Edited by
Barbara Siegmund & Erich Leitner

# FLAVOUR SCIENCE

Proceedings of the
**XV WEURMAN FLAVOUR RESEARCH SYMPOSIUM**

18.-22. September 2017
Graz University of Technology
Austria

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**Barbara SIEGMUND & Erich LEITNER**

Graz University of Technology
Institute of Analytical Chemistry and Food Chemistry
Graz – Austria


**Imprint**

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# Preface

The Weurman Flavour Research Symposium has been a unique platform and probably the most representative conference for flavour scientists to present and discuss recent trends and developments in the field of flavour research. The conference - named after the flavour pioneer Cornelius Weurman - took place in 1975 for its first time and was then held every three years in different European countries. In 2017, it took its turn to Graz, Austria. It was a great honour for us to be the organisers of the XV Weurman Flavour Research Symposium and to welcome 230 flavour scientists from 30 countries at the Old Campus of our university.

The symposium covered six major areas of flavour science: (i) Flavour Generation and Flavour Release, (ii) Flavour Perception and Psychophysics, (iii) Impact of Flavour Compounds on Humans, (iv) Flavour and Off-Flavour of Non-Food Products, (v) Industry-Related Flavour Issues and (vi) Recent Developments in Analytical Techniques. During the conference, we had the chance to follow the presentation of 38 lectures, 14 flash presentations and to discuss an impressive amount of topics and results in front of 130 posters. In the run-up to the event, 65 attendees took the opportunity to attend one of the two satellite symposia and to deepen their knowledge in real-time flavour release analysis or regarding flavour analysis by advanced chromatographic methods. The participation of attendees from industry and academia with different flavour perspectives launched lively discussions in the inspiring atmosphere of our university. We hope that the young colleagues could feel the 'Weurman spirit' and that they are encouraged to continue their work in flavour science.

The present edition of the book 'Flavour Science' follows the structure of the symposium. We are very pleased that many colleagues followed our invitation to publish their results as full contribution in this book. With 108 interesting contributions, we hope that this book will be useful for many flavour scientists, whether or not they attended the symposium. The opportunity to publish the contributions as open access papers will hopefully make flavour science accessible to a large audience.

The organisation of this symposium and the editing of the proceedings could only be achieved with the support and the help of a lot of people. We would like to acknowledge the members of our Scientific Committee for assessing abstracts, selecting contributions and helping us to set up the scientific programme, chairing sessions and reviewing full contributions to be published in this book. We would like to thank the members of the Local Organising Committee for organising this wonderful event at the campus of our university. Thanks to many master- and PhD students who assisted us during the symposium! We are grateful for the generous support by our sponsors. Thanks to their donations we were able to offer reduced student fees to 35 PhD students and, thus, to enable their participation. Finally, we would like to thank Larissa Kolb who worked hard on correcting and formatting of the manuscripts and Gabriele Gross from the Verlag der TU Graz for her general assistance with the book.

The XVI Weurman Flavour Research Symposium will be organised by Elisabeth Guichard and Jean-Luc Le Quéré and will take place in Dijon, France in 2020. We wish Elisabeth and Jean-Luc all success for the organisation and we are looking forward to attending the next Weurman Flavour Research Symposium!

Barbara Siegmund and Erich Leitner

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# Contents

**Preface** **iii**

**FLAVOUR GENERATION AND FLAVOUR RELEASE** **1**

Terpenoid biosynthesis in plants 3
*Arman Beyraghdar Kashkooli, Alexander van der Krol, Harro J. Bouwmeester*

Production of esters and phenylpropenes are linked through *MdAAT1* in apple fruit 13
*Ross G. Atkinson, Edwige J.F. Souleyre, Adam J. Matich, Ye Han, Xiuyin Chen, Mindy Y. Wang, Blue Plunkett, Nadeesha Ileperuma, Andrew P. Dare, Richard V. Espley, Sumathi Tomes, David Chagné, Yar-Khing Yauk*

Shedding light on the modulation of key Riesling wine aroma compounds in a changing climate 19
*Josh Hixson, Yevgeniya Grebneva, Neele Glameyer, Kathrin Vollmer, Cory Black, Mark Krstic, Markus Herderich*

Changes in key aroma compounds during cocoa powder process 25
*Ondrej Novotny, Isabel Pellon, Thierry Dufosse, Tomas Davidek, Sabine Belet*

Flavour release from wine glycosides during tasting 31
*Mango Parker, Alice Barker, Wes Pearson, Yoji Hayasaka, Josh Hixson, Leigh Francis*

A saliva reactor to mimic in-vivo aroma release from flavoured ice-creams 37
*Charfedinne Ayed, Sara I.F.S. Martins, Ann-Marie Williamson, Elisabeth Guichard*

Screening of yeast strains for flavour potential in meat products under reduced concentration of preservative nitrifying agents 43
*Mónica Flores, Daniel Moncunill, José Javier López-Díez, Carmela Belloch*

Identification of odor-active trace compounds in Damask rose (*Rosa damascena*) 47
*Teruhisa Ohashi, Yamato Miyazawa, Tetsuro Shibuya, Susumu Ishizaki, Yoshiko Kurobayashi, Tsukasa Saito*

Characterisation of aroma-active compounds in horseradish (*Armoracia rusticana*) 51
*Eva-Maria Kroener, Andrea Buettner*

Effect of nitrate reduction on the development of oxidized aroma in dry fermented sausages during storage 55
*Laura Perea, Carmela Belloch, Mónica Flores*

Shelf-life model: Useful tool to predict sensory and nutritional quality of infant formulas 61
*Christine Kersch-Counet, Petra Vossenberg, Rudy de Wit, Wim Timmermans, Hans Cruijsen, Jaganmohan Rallapalli, Paul Deckers*

Milk fat globule membrane and its role in flavour development in cheese during ripening 65
*Zahra Haddadian, Graham T. Eyres, Philip J. Bremer*

The effect of sugar type on VOC generation in a model baked system 69
*Patrick Silcock, Julien Brecheteau, Loic Toupin, Sam Heenan*

Understanding the impact of sodium on the structural properties of sweet biscuits 73
*Charfedinne Ayed, Mui Lim, William Macnaughtan, Robert Linforth, Ian D. Fisk*

The impact of plant proteins on vanilla flavour perception 77
*Lizeth Lopez Torrez, Lucian van Belzen, Mathieu Michalet, Odile Janinet, Solange Dalmas*

Decoding the unique peaty aroma of Islay scotch single malt whisky by means of the Sensomics concept 83
*Veronika Mall, Peter Schieberle*

Flavour generation from microalgae in mixotrophic cultivation 87
*Andriéli Borges Santos, Karem Rodrigues Vieira, Pricila Nass Pinheiro, Bruno Nicolau Paulino, Juliano Lemos Bicas, Eduardo Jacob-Lopes, Leila Queiroz Zepka*

Unraveling the complexity of a savory fermented product using a holistic sensory-analytical approach 91
*Arne Glabasnia, Joséphine Charve*

Effects of drying methods on the composition of volatile compounds in fruits and vegetables 95
*Azime Özkan-Karabacak, Gülşah Özcan-Sinir, Ömer Utku Çopur*

Characterization of aroma-active compounds in canned tuna by fractionation and GC/Olfactometry 99
*Fei He, YanPing Qian, Christina Dewitt, Michael C. Qian*

Aroma compounds generation in brown and polished rice during extrusion 103
*Fei He, YanPing Qian, Yan Zhang, MinWei Zhang, Michael C. Qian*

Aroma profile and proximate composition of Roselle seeds: Effects of different origins and different sample preparation methods 107
*Nurul Hansiah Juhari, Mikael Agerlin Petersen*

Effects of sodium chloride, potassium chloride and calcium chloride on flavour formation during heating of a wheat flour-glucose model system 111
*Tolgahan Kocadağli, Dimitris P. Balagiannis, Vural Gökmen, Jane K. Parker*

An assessment of the effect of cinnamon spice on cocoa nibs (Theobroma cacao L.) - An approach to changing flavour in stored roasted cocoa nibs 115
*Matthew Escalante, Gerrit Meerdink, Pathmanathan Umaharan*

Impact of water-soluble precursors leaching from green eans on aroma generation during coffee roasting 119
*Chujiao Lui, Ni Yang, Robert Linforth, Ian D. Fisk*

Revisiting the role of glycosidic aroma precursors on wine aroma: Effects of microorganisms and of slow hydrolytical processes 123
*Inês Oliveira, Vicente Ferreira*

Organoleptic properties of dark chocolates investigated by direct-injection mass spectrometry (PTR-ToF-MS) and GC-Olfactometry 127
*Zoé Deuscher, Isabelle Andriot, Karine Gourrat, Etienne Sémon, Marie Repoux, Elisabeth Guichard, Sébastien Preys, Renaud Boulanger, Hélène Labouré, Jean-Luc Le Quéré*

Influence of salt reduction on flavour release in ready-to-eat meal 131
*Cécile Rannou, Florence Texier, Philippe Courcoux, Véronique Cariou, Carole Prost*

Characterisation of the flavour of the old Austrian apple variety 'Ilzer Rose' 135
*Iris Tauber, Georg Innerhofer, Erich Leitner, Barbara Siegmund*

*Aronia melanocarpa* – the Styrian 'super berry': A flavour characterisation of black chokeberry juice 139
*Susanne Robert, Barbara Siegmund*

On-line coffee flavour formation analysis using PTR-ToF-MS during roasting under different atmospheres 143
*Samo Smrke, Anja Rahn, Alexia N. Gloess,Chahan Yeretzian*

Characterization of key aroma compounds in two types of Keemun tea 147
*Tetsuya Yoshida, Johanna Kreissl, Yoshiko Kurobayashi, Tsukasa Saito, Andreas Dunkel, Thomas Hofmann*

Age-related changes in oral and nasal physiology and their significance in aroma release and perception 151
*Sophie Lester, Taylor M., Corbier C., Cornacchia L., Ian Fisk*

Influence of the brewing process and degree of milling on the taste characteristics of pigmented rice wine 155
*Sanchai Yotmanee, Maria Jose Oruna-Concha, Jane K. Parker*

Characterisation of the aroma developed during fermentation and roasting of jackfruit seeds 159
*Fernanda Papa Spada, Solange Guidolin Canniatti-Brazaca, Jane K. Parker*

Investigating the phytochemical, flavour and sensory attributes of mature and microgreen coriander (*Coriandrum sativum*). 163
*Maria Jose Oruna-Concha, Stella Lignou, Emma L. Feeney, Karen Beegan, Owen Kenny, Niamh Harbourne*

HPLC-ESI(+)MS/MS quantitation of the newly evidenced glutathione S-conjugates in two dual-purpose hop varieties: Citra and Sorachi Ace 167
*Laura Decourriere, Cécile Chenot, Carlos Silva Ferreira, Sonia Collin*

Tracking of hop-derived compounds in beer during fermentation with PTR-ToF-MS 171
*Tobias M. Richter, Alberto Algarra, Patrick Silcock, Graham T. Eyres, Phil J. Bremer, Vittorio Capozzi, Franco Biasioli*

Formation pathways of 2,3-pentanedione in model systems and real foods 175
*Tomas Davidek, Ondrej Novotny, Thierry Dufossé, Luigi Poisson, Imre Blank*

Effect of several food processing methods on volatile composition of strawberry 179
*Gülşah Özcan Sinir, Canan Ece Tamer, Ömer Utku Çopur*

**FLAVOUR PERCEPTION AND PSYCHOPHYSICS** **183**

Influence of genetic variation on flavour perception and food choices 185
*John E. Hayes*

Investigating perception and liking of non-nutritive sweeteners according to taste receptor genotypes 193
*Lisa Methven, Gabriele Kavaliauskaite, Lauren Ellis, Karani S. Vimaleswaran*

Sugar reduction in flavoured beverages: The robustness of aroma-induced sweetness enhancement 199
*Celine Brattinga, Petrus Maria Theresia De Kok, Johannes Hendrikus Franciscus Bult*

Perception of odour mixtures: The next challenge in flavour analysis 207
*Thierry Thomas-Danguin, Charlotte Sinding, Sébastien Romagny, Maiken Thomsen, Elisabeth Guichard, Gérard Coureaud*

Structure-odour relation in homologous series of alkane-1,1-dithiols and dithio(hemi)acetals 213
*Johanna Elisabeth Grimm, Jia-Xiao Li, Martin Steinhaus*

Effect of carbonation level on the perception of sourness in sparkling wine 217
*John C. Castura, Kenneth M. McMahon, Carolyn F. Ross*

Explaining fat sensitivity in cottage cheeses by aroma release and oral physiology parameters 221
*Elisabeth Guichard, Rachel Schoumacker, Sébastien Romagny, Hélène Labouré, Christophe Martin, Eric Neyraud, Jean-Luc Le Quéré, Thierry Thomas-Danguin*

Elucidating the mechanisms of individual variation in fat perception and preference 225
*Xirui Zhou, Jane K. Parker, Orla B. Kennedy, Lisa Methven*

Molecular and cellular mechanisms of the pungent and tingling impression of black pepper (*Piper nigrum* L.) 229
*Corinna Dawid, Leopoldo R. Beltrán, Andrea Henze, Oliver Frank, Madeline Beltrán, Janina Levermann, Sascha Titt, Sini Thomas, Viktoria Puerschel, Miriam Satalik, Günter Gisselmann, Hanns Hatt, Thomas Hofmann*

Temporal processing of odor mixtures in humans 233
*Chloé Albietz, Terry E. Acree*

Real-time percept flavor balance derived from retronasal threshold and *in vivo* measurements of retronasal aroma release with PTR-MS 237
*Shinichiro Ito, Ken Omori, Hideki Miyazaki, Toshihiro Takeda, Kunihide Hoshino*

Perceptive Interactions in Red Wines: How Physico-Chemical Pre-Sensorial Effects May Affect Red Wine Fruity Aromatic Expression? 241
*Margaux Cameleyre, Georgia Lytra, Sophie Tempere, Jean-Christophe Barbe*

Why does this wine smell like apricots? 245
*Tracey E. Siebert, Sheridan R. Barter, Alice Barker, Wes Pearson, Miguel A. de Barros Lopes, Philippe Darriet, Markus J. Herderich, I. Leigh Francis*

Structure-odor relationship study of C-6 unsaturated acyclic monoterpene alcohols: A comparative approach 249
*Shaimaa Awadain Elsharif, Andrea Buettner*

**IMPACT OF FLAVOUR COMPOUNDS ON HUMANS 253**

Taste receptors in respiratory innate immunity 255
*Alan D. Workman, Michael A. Kohanski, Noam A. Cohen*

Bitter taste: prediction, relation to toxicity, and effect on emotions 263
*Ayana Dagan-Wiener, Ido Nissim, Nitzan Dubovski, Masha Y. Niv*

Can flavour enhancement modulate appetite and food intake in women? 269
*Wenting Yin, Ian Fisk, Louise Hewson, Robert Linforth, Moira Taylor*

The TRP channel agonists nonivamide and cinnamaldehyde augment cold-induced mitochondrial biogenesis in white adipocytes 275
*Barbara Lieder, Kerstin Schweiger, Veronika Somoza*

Biotransformation, transmission and excretion processes of garlic odorants in humans: impact on human milk, urine and exhaled breath 281
*Laura Scheffler, Nina Sillner, Yvonne Sauermann, Constanze Sharapa, Jonathan Beauchamp, Andrea Buettner*

The novel umami peptides identified from *Takifugu Obscurus* and *Takifugu Rubripes* 285
*Yuan Liu, Bei Li*

The role of the salivary proteome in salt sensitivity 289
*Theresa Stolle, Freya Grondinger, Andreas Dunkel, Chen Meng, Guillaume Médard, Bernhard Kuster, Thomas Hofmann*

Comparison of the sensory properties of fragrant and non-fragrant rice: The role of the popcorn-like aroma compound 2-acetyl-1-pyrroline 293
*Xuan Wei, Lisa Methven, J. Stephen Elmore*

**INDUSTRY-RELATED FLAVOUR ISSUES 297**

Safety assessment of flavourings in the European Union 299
*Karl-Heinz Engel*

Impact of Nagoya protocol on flavour research 307
*Robin Clery, Klaus Gassenmeier*

Acetals in food flavourings 313
*Ján Pet'ka, Johann Leitner, Jose Maria Bueno, Peter Szolcsányi*

Novel Strategies of addressing increasing complexity in flavour research 319
*Imre Blank, Tomas Davidek, Heiko Oertling*

Effect of muscle, ageing and packaging on marker volatiles for beef flavour 325
*Linda J. Farmer, Terence D.J. Hagan, David Sanderson, Alan W. Gordon, Rod J. Polkinghorne*

Discovering aroma patterns in food products using Latent Dirichlet Allocation and Jensen Shannon divergence 331
*Michael Fitzke, Alicia Olivares*

Searching for naturally generated volatiles from *Tuber Melanosporum* as authenticity markers for black truffle infused vegetable oils 335
*Consol Blanch, Carles Ibáñez, Montse Argelagués, Amparo Tàrrega, Míriam Torres*

Odour qualities and odour thresholds of halogenated, alkylated, alkenylated and methoxylated guaiacol-derived odorants 339
*Katja Lorber, Maria Wagenstaller, Maria Schranz, Florian Juhlke, Katharina Klos, Julia Kerschbaumer, Andrea Buettner*

Characterisation of the key aroma compounds in alcohol- free beer base by gas chromatography-olfactometry 343
*Jose A. Piornos, Dimitris P. Balagiannis, Elisabeth Koussissi, Eric Brouwer, Jane K. Parker*

Studies on off-flavours in lamb 347
*Janeen S. Speers, Aurelie Aubry, Terence D.J. Hagan, Frank Monahan, W. Colin McRoberts, Alison White, Linda J. Farmer*

Key aroma compounds in meat bouillons: Comparison between industrial and traditional preparation processes 351
*Johannes Polster, Maria Monteiro de Araújo Silva, Giuseppe Lubes, David Komarek, Imre Blank*

Malolactic fermentation of sea buckthorn (*Hippophaë rhamnoides* L.) berry juice with *Lactobacillus plantarum*: impact on sugars, sugars alcohols, and organic acids 355
*Niko Markkinen, Sarianna Koivula, Oskar Laaksonen, Baoru Yang*

Impact of enzyme treatment on flavour of aronia juice 359
*Mari Sandell, Anita Vanag, Oskar Laaksonen*

Validity of marker compounds for authenticity control case study: Methyl cinnamate 363
*Michael Backes, Jekaterina Ongouta, Uwe Schäfer, Lars Meier, Birgit Nölting, Nadine Heinemeier, Margit Liebig, Gerhard E. Krammer*

Mapping on the origins of cajuput essential oil for its compatibility as *Cajuputs® Candy* functional flavour 367
*Siska Septiana, Nancy D. Yuliana, Boy M. Bachtiar, C. Hanny Wijaya*

Influence of protective inert gas atmospheres on the aroma stability of orange juice with pulp 371
*Jana Sádecká, Mária Kopuncová, Emil Kolek, Jaroslav Blaško, Martin Polovka, Pavel Farkaš, Ján Durec*

**FLAVOUR & OFF-FLAVOUR OF NON-FOOD PRODUCTS 375**

Odorants in non-food products – Is there more to them than just their smell? 377
*Andrea Buettner, Jonathan Beauchamp, Philipp Denk, Miriam Strangl, Christoph Wiedmer*

The toilet malodor challenge 383
*Christian Starkenmann, Charles Chappuis, Jana Pika*

Key odorants in the artificial leather of car interiors 389
*Klaas Reglitz, Martin Steinhaus*

What does wood smell like? Characterization of odorants in wood 395
*Linda Schreiner, Rahil Ghadiriasli, Patrick Bauer, Andrea Buettner*

Odour-active compounds in an inflatable PVC beach ball 399
*Isabella Märkl, Klaas Reglitz, Martin Steinhaus*

Identification of malodourous emissions of wood pellets during storage 403
*Barbara Pöllinger-Zierler, Irene Sedlmayer, Hermann Hofbauer, Elisabeth Wopienka, Barbara Siegmund*

Describing the smell of wet cat food using a common sensory language: Petscript® 407
*Perrine Delime, Uwe Schaefer, Katja Tiitinen, Chloé Champion, Aurélie De Ratuld*

Comparison of different analytical methods for the quantification of odor-active haloanisoles in food contact materials 411
*Brigitte Jaksa, Thomas Lehner, Dorothea Leis, Sigrid Hager, Erich Leitner*

Emissions of compost bedded pack barn for cattle 415
*Alfred Pöllinger, Barbara Pöllinger-Zierler, Christian Kapp, Nina Haar, Larissa Kolb, Erich Leitner*

**RECENT DEVELOPMENTS IN ANALYTICAL TECHNIQUES 419**

PARADISe - a ground-breaking tool to treat complex GC-MS datasets 421
*Mikael Agerlin Petersen, Rasmus Bro*

Exploring 2-acetyl-1-pyrroline loss by high resolution mass spectrometry and nuclear magnetic resonance 427
*Bethany J. Hausch, Keith R. Cadwallader*

A sophisticated setup for rapid, sensitive and selective food and flavor analysis 433
*Felix Piel, Klaus Winkler, Rene Gutmann, Stefan Haidacher, Jens Herbig, Gregor Mayramhof, Simone Jürschik, Alfons Jordan, Lukas Märk, Philipp Sulzer*

DOLC-NMR: Differential off-line LC-NMR analyses of nutrient-induced metabolome alterations in *S. cerevisiae* and their taste impact 439
*Richard Hammerl, Oliver Frank, Thomas Hofmann*

Process control in flavour generation: NIR-MVA as a tool to monitor key odorants formation 445
*Ana Rita Monforte, Sara I. F. S. Martins, A. C. Silva Ferreira*

Investigation of odour-active compounds in grapefruit (*Citrus paradisi*) 451
*Akihiro Kawaraya, Shingo Chiba, Aki Kurabe, Yuichiro Yamazaki, Yumi Kusano, Kyoko Zaizen, Eri Hiraki, Yoshihiro Yaguchi, Yukihiro Kawakami*

Rapid aroma analysis and data interpretation using on-line mass spectrometry and visualisation software 455
*Jun Hatakeyama, Andrew J. Taylor*

Rapid analysis of important taste active components in chocolate by ultra high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-QToF-MS) 459
*Katherine Filer, Daniel Festring*

Evaluation and optimization of sample preparation techniques towards the regional differentiation of Chinese green teas 463
*Mark Bücking, T. T. Ho, Hongbo Yang, T. Albinus, M. Kotthoff*

On-line high-throughput analysis of the volatilome of microorganisms that have agroindustrial relevance 467
*Iuliia Khomenko, Luca Cappellin, Michele Pedrotti, Patrick Silcock, Franco Biasioli*

Headspace solid phase microextraction (SPME) *vs* dynamic headspace extraction (DHS) to explore breast milk volatile fraction 471
*Sara Le Roy, Angélique Villière, Catherine Fillonneau, Carole Prost*

Continuous collection of volatiles produced by *Streptomyces* grown on oatmeal agar by headspace extraction and GC-MS 475
*Raju Podduturi, Nina Clausen, Nanna Bender Petersen, Niels O. G. Jørgensen, Mikael Agerlin Petersen*

InnOscent chromatographic system: An innovative device to revisit aroma analysis and recombination perspectives 479
*Angélique Villière, Sarah Le Roy, Catherine Fillonneau, Carole Prost*

Comparison of the volatile sulfur compounds in onion oil to those in fresh onion juice 483
*Sytze Haasnoot, Gijs Koopmanschap, Hans Colstee, Cor Niedeveld, Clint Wermes, Robert Cannon, Neil C. Da Costa*

The odour activity value of aroma-active esters - An appropriate means to assess the aroma quality of apple juices 487
*Johanna Kreissl, Katharina Lehmann, Peter Schieberle*

WheelOscent: presentation of an innovative olfactometry-dedicated software using intuitive aroma wheel interface 491
*Angélique Villière, Fabrice Guillet, Sarah Le Roy, Catherine Fillonneau, Carole Prost*

Scope for improvement in the sensomics approach 495
*Lewis L. Jones, Richard Haydock, Kathleen Pinfold, James B. Addison, Neil Desforges*

Rapid ATR-FTIR method for monitoring the release of major components of clove essential oil encapsulated into a complex organic matrix 499
*José Daniel Wicochea Rodriguez, Emanuelle Gastaldi, Thierry Ruiz, Peggy Rigou, Stéphane Peyron, Pascale Chalier*

The impact of pod storage on the formation of different alkylpyrazines from Ghanaian cocoa roasted at four roasting temperatures 503
*Ann de Winne, Michael Hinneh, Julie Haeck, Davy Van de Walle, Jim Van Durme, Emmanuel Ohene Afoakwa, Koen Dewettinck, Luc De Cooman*

Method development for multiple partition coefficients determination to understand headspace aroma distribution of complex mixture. 507
*Margaux Cameleyre, Georgia Lytra, Charlotte Herice, Jean-Christophe Barbe*

Characterisation of wines produced from fungus resistant grape varieties 511
*Dorothea Leis, Wolfgang Renner, Erich Leitner*

**AUTHOR INDEX** **515**

**KEYWORD INDEX** **519**


# FLAVOUR GENERATION AND FLAVOUR RELEASE

# Terpenoid biosynthesis in plants

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## Introduction

Plants produce a wide range of structurally diverse natural products. These natural products play key roles in the interaction of plants with organisms in their environment. They, for example, act as defence compounds against herbivores and pathogens, or as attractants of insects for pollination. They also provide a natural resource for humans and are used as medicine (e.g. artemisinin and parthenolide), pigments (e.g. β-carotene and lycopene), fragrance (e.g. limonene and linalool) and flavours (e.g. vanillin and menthol).

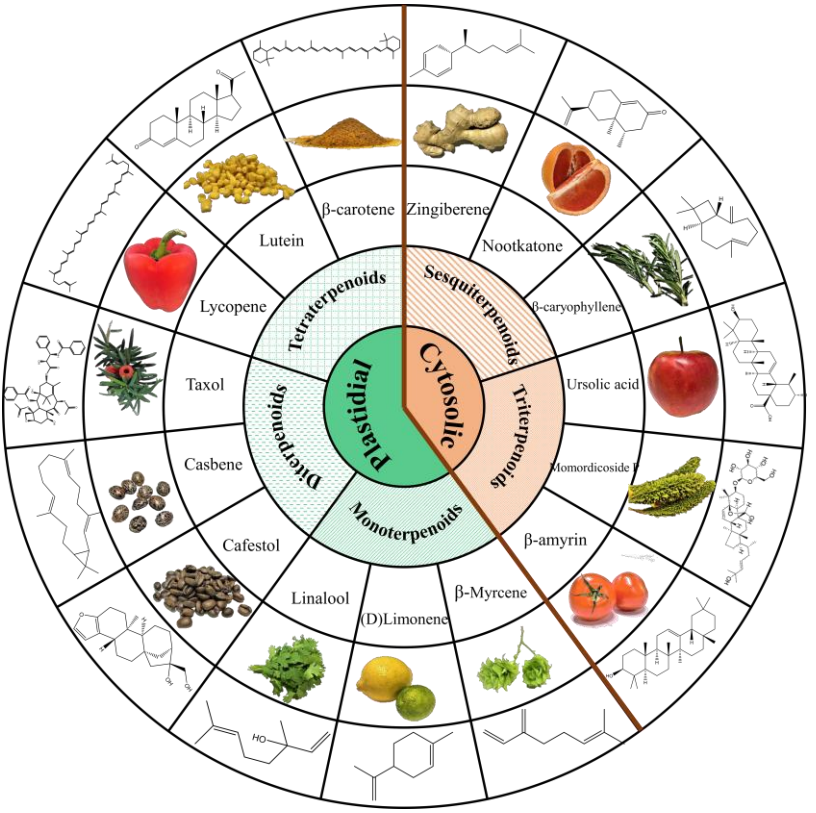


**Figure 1**: Schematic representation of plastidial and cytosolic terpenoids. Examples of different plant derived terpenoids and plant organs (seed, fruit, leaf or root) containing these terpenoids are shown.

Plant natural products can be divided into several classes such as nitrogen-containing (e.g. alkaloids, glucosinolates and cyanogenic glucosides) and nitrogen-free metabolites such as terpenoids, phenolics and flavonoids [1]. Among these, the terpenoids are the

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most diverse class, constituting almost 12% of all known plant metabolites [2] and possessing many different functions in plants. Low molecular weight volatile terpenoids are involved in plant protection mechanisms during biotic and abiotic stresses [3,4] and when emitted from flowers can act as pollinator attracting signals [5]. Terpenoids can be antifeedant compounds that protect the plant against insects, such as for example, geranyllinalool in *Nicotiana obtusifolia* [6] and can be an activator signal for systemic acquired resistance [7]. Several terpenoids, such as gibberellins, abscisic acid and strigolactones are plant hormones and act as signalling molecules in physiological processes. Strigolactones, for example, are a regulator of plant axillary bud outgrowth and thus branching [8]. In addition to their role as plant hormone, strigolactones are also secreted into the soil surrounding the plant's roots where they recruit the symbiotic arbuscular mycorrhizal fungi.

## Biosynthesis of Terpenoids

### *Biosynthesis of the basic building blocks of terpenoids*

Terpenoids are produced from the universal building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are synthesized through two different biosynthetic pathways. One of these occurs in the plastids and supplies mostly the substrates for the production of monoterpenoids (often present in essential oils), diterpenoids and tetraterpenoids (carotenoids) (Figure 2). The other pathway, known as the mevalonate (MVA) pathway takes place in the cytosol. The IPP and DMAPP derived from this pathway are mostly used as substrates in the production of sesquiterpenoids and triterpenoids.

The condensation of the IPP and DMAPP building blocks produced by the MEP and MVA pathways provides the prenyl diphosphate substrates such as C10 (geranyl diphosphate, GPP), formed by condensation of IPP and DMAPP through enzymatic activity of geranyl diphosphate synthase (GPS) or C15 (farnesyl diphosphate, FPP), formed from condensation of GPP and IPP by farnesyl diphosphate synthase (FPS) (Figure 2). GPP and FPP are the universal precursors of monterpenoids (C10) and sesquiterpenoids (C15), respectively. Geranylgeranyl diphosphate synthase (GGPS) catalyses the condensation of FPP with IPP, which results in the formation of the C20 precursor of the diterpenes, geranylgeranyl diphosphate (GGPP), while dimerization of two FPP molecules and removal of the diphosphate groups through the activity of squalene synthase (SQS) results in biosynthesis of squalene (C30) [9]. Squalene monooxygenase or epoxidase adds an oxygen group to the squalene, resulting in production of 2,3-oxidosqualene, the precursor of triterpenoids (C30) as well as sterols and steroids in plants. Dimerization of two GGPP molecules and elimination of the two diphosphate groups by phytoene synthase (PS) results in the formation of a C40 compound, phytoene, the precursor of the tetraterpenoids or carotenoids (Figure 2).

### *Biosynthesis of monoterpenoids (C10)*

Monoterpenoids are C10 compounds derived from GPP. Monoterpenoids are well-known for their biological activity, but also for their strong odour and aromatic properties [10]. These compounds are used for various applications such as fragrances, drinks, food additives, perfumes and cosmetics [11]. Geraniol (isolated from rose flowers) and linalool (from coriander; Figure 1) are two of most important monoterpenoids used in the flavour industry which is reaching to annual consumption of 5000 tons/year [12]. Monoterpenoids in plants are often induced upon biotic and/or abiotic stress conditions and they are supposed to possess properties to enable plants to deal with these stresses

[13]. 1,8-Cineole, for example, is toxic to certain insects and is produced by *Artemisia annua* upon infestation by the root feeding insect, *Diuraphis noxia* [14]. GPP synthase (GPS), the enzyme responsible for synthesis of the monoterpenoid precursor GPP, was first characterised from the essential oil glands of sage [15]. Formation of monoterpenoids takes place through the activity of enzymes called monoterpene synthases (sometimes also called monoterpene cyclase if catalysing the formation of a cyclic monoterpene).

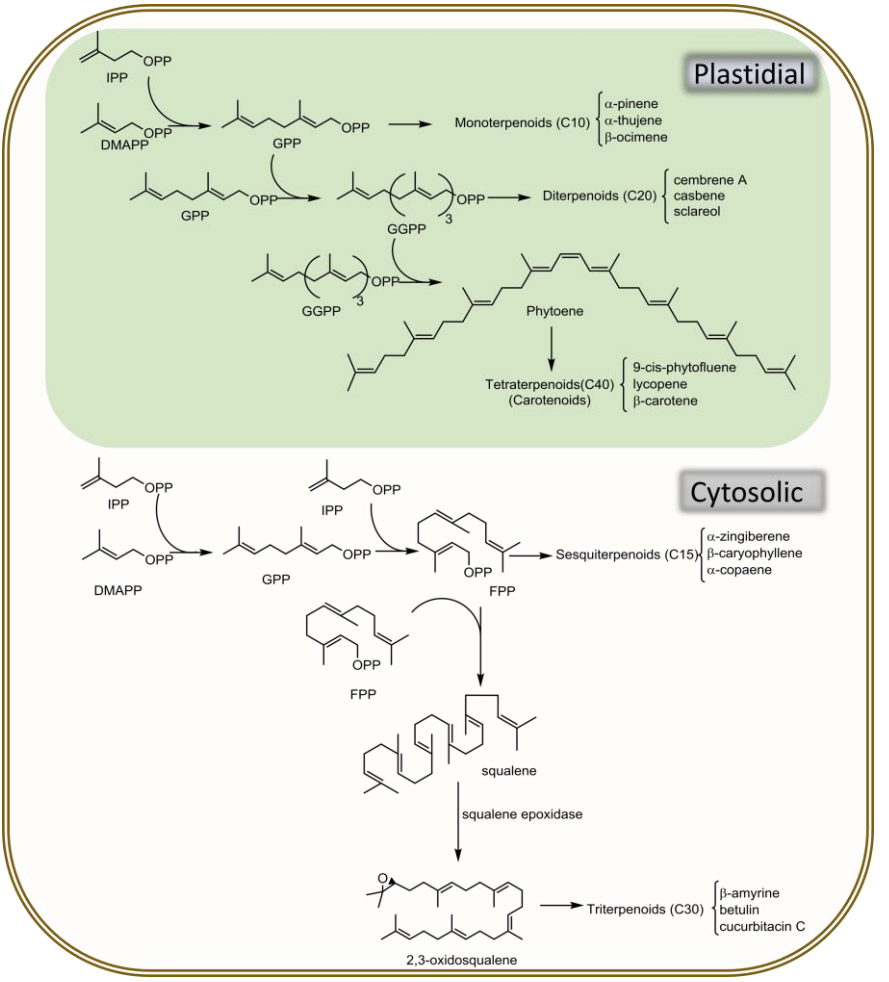


**Figure 2**: Terpenoid biosynthesis pathways. Biosynthesis of monoterpenoids (C10), diterpenoids (C20) and teteraterpenoids (C40) takes place in the plastids (in green) while the biosynthesis of sesquiterpenoids (C15) and triterpenoids (C30) is localized in the cytosol (in white).

The DDxxD motif for $Mg^{2+}$ cation binding is conserved among all terpene synthases, which allows their identification [16]. A single monoterpene synthase often catalyses formation of several monoterpenoids from GPP. For example, a promiscuous monoterpene synthase enzyme, *Cs*TPS2FN, isolated from *Cannabis sativa* encodes the formation of (+)-α-pinene, (+)-β-pinene, myrcene, (-)-limonene and β-phellandrene [17]. As detailed above, it is generally assumed that the plastids are the major organelle for production of monoterpenoids and their substrate, GPP. Intriguingly, however, exchange

of mitochondrial produced GDP to the plastids for production of monoterpenoids has been demonstrated [18].

*Biosynthesis of sesquiterpenoids (C15)*

Sesquiterpenoids are produced from FPP, again through the catalytic activity of terpene synthases, in this case called sesquiterpene synthases. Sesquiterpenoids are often aromatic, and constituents of plant essential oils. The sesquiterpene β-caryophyllene (Figure 1) has been reported to be present in many plant species; it is the major essential oil component of basil (*Ocimum spp*.), oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis*) [19] (Figure 1) and, together with humulene, is the main sesquiterpene obtained from cannabis plants and responsible for its odour [20]. β-caryophyllene is widely used in frozen dairy, chewing gums and beverages [21]. Zingiberene, a sesquiterpene present in ginger (*Zingiber officinale*) is a spider mite repellent (Figure 1) [22]. In chamomile (*Matricaria chamomila*) it was shown that sesquiterpene biosynthesis starts in the plastids with GPP that is exported to the cytosol where IPP is added [23]. Interestingly, results from transient expression of a sesquiterpene synthase from feverfew (*Tancetum parthenium*) show that addition of mitochondrial targeting to a sesquiterpene synthase will result in higher sesquiterpene biosynthesis, presumably because mitochondrial FPP is accessed [24]. Indeed, localization of one of the Arabidopsis FPP synthases in the mitochondria has been demonstrated [25]. Protein localization studies using GFP fusions of *cis*-FPS, and santalene and bergamotene synthase (SBS) suggest that biosynthesis of these unusual sesquiterpenoids take place in the plastids using IPP and DMAPP from the MEP pathway [26].

Sesquiterpene lactones are a sub-class of the sesquiterpenoids with over 4000 different known structures. Sesquiterpene lactones are mainly colourless, bitter, compounds found mainly in plant species in the *Asteracea* [27]. Their biological properties such as antibacterial (e.g. vernolide [28]), antifungal (8α-hydroxy-4-epi-sonchucarpolide [29]), anticancer (e.g. parthenolide [30]) make them of interest for medical use. Sesquiterpene lactones are classified in six bicyclic or tricyclic classes named guianolides, pseudoguaianolides, xanthanolides, eremophilanolides, eudesmanolides and germacranolides [31]. Costunolide may then serve as the precursor of the other germacranolides (e.g parthenolide) and/or guaianolides (e.g. the main constituents of bitter compounds in chicory and endive). Further modification of sesquiterpene lactones is carried out by double bond reductases and glycosyl transferases [32,33].

*Biosynthesis of diterpenoids (C20)*

With more than 10,000 different natural plant derived structures, the diterpenoids are one of the most diverse classes of plant secondary metabolites [34]. They are also part of plant primary metabolism as plant growth regulators such as the gibberellins are diterpenes [35]. Many diterpenoids have medicinal properties, such as taxol (Figure 1), which is isolated from the Pacific yew (*Taxus brevifolia*) [36], and is used for the treatment of ovarian and breast cancer [37]. Cafestol (Figure 1) and the structurally related kahweol are two diterpenes from *Coffea arabica* that induce apoptosis in malignant pleural mesothelioma (MPM) cancer cells [38]. A valuable compound for the fragrance industry is *cis*-abienol which is an aromatic diterpene isolated from fir trees (*Abies balsamea*). *Cis*-abienol is an important oxygen containing diterpenoid serving as the precursor of Ambrox® in perfume formulations [39] and the major labdane type diterpenoid responsible for the fragrance of tobacco leaves. Biosynthesis of *cis*-abienol proceeds in two sequential steps. First a diterpene synthase converts GGPP to 8-hydroxy-copalyl

diphosphate and then a kaurene synthase like enzyme converts the latter into *cis*-abienol by removing the diphosphate group [40].

*Biosynthesis of triterpenoids (C30)*

This class of specialized metabolites constitutes more than 20,000 identified plant compounds so far [41]. Triterpenoids show a lot of diversity in plant families. Saponins, glycosylated triterpenoids, are, for example, found in *Quillaja saponaria* (a native Chilean tree) and *Camellia oleifera*. Saponins are used in detergents, shampoos and emulsifiers due to their foaming properties [42,43]. Many plants produce saponin type triterpenoids during normal growth (e.g. apple fruit peel, producing ursolic acid [44]), however their saponin levels strongly depends on plant species, organs and developmental stage [45]. Butelin, isolated from the bark of *Butela spp*., is another natural triterpenoid which is used in cosmetic products such as hair conditioners [46]. Many triterpenoids are used to cure major diseases such as cancer and HIV. Celastrol, a triterpenoids isolated from *Tripterygium wilfordii* exhibits Tat inhibitory action [47]. 'Tat' is a virus encoded protein which is required for HIV genome transcription. Triterpene synthases convert 2,3-oxidosqualene through a Chair-Boat-Chair (CBC) or the Chair-Chair-Chair (CCC) conformation into the different triterpene skeletons. An example of a triterpene synthase is β-amyrin synthase [48] responsible, for example, for β-amyrin biosynthesis in tomato (Figure 1). P450s and glycosyl transferases play an important role in further decoration of triterpenoids, for example, for the production of the triterpene glycoside glycyrrhizin.

*Biosynthesis of tetraterpenoids (C40)*

The tetraterpenoids contain 750 different reported structures [49]. The carotenoids [50](tetraterpenoids) are the most common natural pigments and also possess antioxidant properties. Carotenoids are industrially used as dyes and colorants, in the food industry (e.g. β-carotene), as nutraceuticals and in the pharmaceutical industry, as well as in cosmetics [51] (Figure 1). They are mostly present in photosynthetic organisms [50] and often are responsible for red, orange and yellow colours [52]. Carotenoids are essential and play a vital role in photosynthesis. Carotenoid biosynthesis starts with the activity of phytoene synthase making pre-phytoene diphosphate [53]. Phytoene synthase then converts pre-phytoene diphosphate to 15-*cis*-phytoene. Several other enzymes namely a desaturase and an isomerase are involved to produce *trans*-lycopene. Cyclisation is the next step; activity of an α-cyclase results in α-carotene biosynthesis, while a β-cyclase can convert *trans*-lycopene to β-carotene. Another class of naturally occurring carotenoid-derived terpenoid type molecules are the strigolactones. Their biosynthesis starts with isomerization of β-carotene by D27 [54]. Then a carotenoid cleavage (CCD7) cleaves the resulting 9-*cis*-β-carotene which then results in production of 9-*cis*-β-apo-10-carotenal and β-ionone [55]. Then, another carotenoid cleavage enzyme, CCD8, converts 9-*cis*-β-apo-10-carotenal into carlactone [54]. This ubiquitous strigolactone precursor will be oxidised by a cytochrome P450, the MAX1 homologs, which results in the formation of carlactonoic acid or *ent*-2-*epi*-5-deoxystrigol [56,57].

**Heterologous production of terpenoids in plants and micro-organisms**

As explained above, the terpenoids are very important compounds from medicinal, nutraceutical and nutritional point of view. However, commercialization of these compounds is often restricted due to their low concentrations in the plant and their high structural complexity which makes chemical synthesis approaches too costly [58]. In addition, some of the plant species that produce attractive molecules grow slowly, may

have a low yield, are threatened by extinction, or are susceptible to environmental conditions. Several approaches have been followed in the last decades to overcome these limitations. In an approach called metabolic engineering, scientists use alternative organisms (expression platforms) to optimize production of these metabolites. Terpenoid production in microbial systems, for example, is an appealing approach. Rapid growth and regeneration (e.g. 1 to 3 days for *Escherichia coli* and *Saccharomyces cerevisiae*, respectively) and well established tools for transformation make them suitable organisms for metabolic engineering purposes. However, ectopic expression of plant derived genes (enzymes) in these microbial platforms comprise some limitations which needs to be solved for a successful engineering strategy. For example, neither *E. coli* nor *S. cerevisiae* contain plastids. Hence in order to prevent possible miss-folding of the enzymes in these platforms, removal of a possible plastid targeting signal is suggested [59]. The subcellular targeting strategy used by plants makes expressing cytochrome P450s in micro-organisms even more challenging. S. cerevisiae, however is a suitable expression platform for cytochrome P450s as it is a eukaryotic microorganism containing endoplasmic reticulum, the maturation and activity site of cytochrome P450s. Another advantage of yeast is the ability of in vivo recombination of DNA fragments, such that several DNA fragments (harbouring homologous flanking regions) can be recombined upon transformation into yeast in a so called transformation associated recombination (TAR) [60]. Almost all required precursors for the biosynthesis of the different terpenoids are produced in yeast. Carotenoid and diterpenoid production in yeast is achieved often by overexpression of a GGPP synthase as yeast produces GGPP in small quantities. Carotenoids biosynthetic pathway genes have been successfully expressed in yeast [61]. Overexpression of genes such as HMGR, the rate limiting enzyme in the MVA pathway, has been shown to enhance the pool of precursor for the biosynthesis of, for example, sesquiterpenoids and triterpenoids. Alternatively, down regulation of competing pathways like sterol biosynthesis through down regulation of *ERG9* (squalene synthase) [62] are molecular strategies which are implemented for successful engineering programs. Successful in-vivo production of artemisinic acid and costunolide in WAT11 yeast (optimal yeast strain for expression of recombinant cytochrome P450s) is reported by introduction of sesquiterpene synthases (amorphadiene synthase and germacrene A synthase) and P450s (amorphadiene oxidase (for artemisininc acid).

Metabolic engineering can also be pursued in the plant species that is already making the attractive product by overexpression of biosynthetic pathway genes or downregulation of competing pathways. However, this homologous engineering - optimization and boosting of metabolic pathways in the original plant species - is sometimes difficult and time consuming. Hence, other *in planta* expression systems have been explored for heterologous expression of genes involved in the biosynthesis of secondary metabolites. Here we discuss a number of examples of such hosts that have been used for metabolic engineering and reconstruction of terpenoid biosynthesis pathways. Overexpression of taxadiene synthase, which converts GGDP to taxadiene - a precursor of the anti-cancer molecule taxol (Figure 1) - has been studied in *Nicotiana benthamiana*. Taxadiene was produced to an astonishing yield of 27 µg/g dry weight [63]. An example of successful reconstruction of a full biosynthetic pathway is the biosynthesis of parthenolide in *N. benthamiana*. The transient co-expression of germacrene A synthase (*GAS*), germacrene A oxidase (*GAO*), costunolide synthase (*COS*) and parthenolide synthase (*PTS*) yielded 1.4 mg/g fresh weight parthenolide in the leaves [30]. Artemisinin was also successfully synthesized in *N. benthamiana*, by transient expression of five biosynthestic pathway genes, amorphadiene synthase (*ADS*), amorphadiene oxidase (*ADO*), alcohol

dehydrogenase 1 (*ALDH1*), artemisinic aldehyde double-bond reductase (*DBR*) and aldehyde dehydrogenase 1 (*ALDH1*). *Physcomytrella patens* is another plant expression platform which recently has raised a lot of interest for metabolic engineering of valuable terpenoids. Novel and relatively easy transformation technology [64] has made this platform a suitable putative heterologous system for bulk production of terpenoids. Successful artemisinin production in *P. patens* was shown recently with a yield of 0.21 mg/g dry weight [65]. This yield was obtained upon co-expression of the same five biosynthesis pathway genes mentioned above, *ADS*, *ADO*, *ALDH1*, *DBR* and *ALDH1*. Yield in these heterologous production platforms is still quite low. A better knowledge of the natural site of biosynthesis and accumulation, the chemical properties of the terpenoids produced, and the mechanisms involved in their transport (from the biosynthesis site to the accumulation site) will provide novel solutions to be implemented in metabolic engineering programs [66,67].

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## References

1. Wink M. (2010) Introduction. Annual Plant Reviews Volume 39: Functions and Biotechnology of Plant Secondary Metabolites: Wiley-Blackwell. pp. 1-20.
2. Springob K., Kutchan T.M. (2009) Introduction to the different classes of natural products. Plant-Derived Natural Products: Springer. pp. 3-50.
3. Loreto F., Dicke M., Schnitzler J.P., and Turlings T.C. (2014) Plant, cell & environment 37: 1905-1908.
4. Bleeker P.M., Diergaarde P.J., Ament K., Schütz S., Johne B., Dijkink J., Hiemstra H., de Gelder R., de Both M.T., and Sabelis M.W. (2011) Phytochemistry 72: 68-73.
5. Byers K.J., Bradshaw H., and Riffell J.A. (2014) Journal of Experimental Biology 217: 614-623.
6. Jassbi A.R., Zamanizadehnajari S., and Baldwin I.T. (2010) Phytochemistry 71: 1115-1121.
7. Chaturvedi R., Venables B., Petros R.A., Nalam V., Li M., Wang X., Takemoto L.J., Shah J. (2012) The Plant Journal 71: 161-172.
8. Cardoso C., Zhang Y., Jamil M., Hepworth J., Charnikhova T., Dimkpa S.O., Meharg C., Wright M.H., Liu J., Meng X. (2014) Proceedings of the National Academy of Sciences 111: 2379-2384.
9. Blagg B.S., Jarstfer M.B., Rogers D.H., Poulter C.D. (2002) J Am Chem Soc 124: 8846-8853.
10. Bakkali F., Averbeck S., Averbeck D., Idaomar M. (2008) Food and Chemical Toxicology 46: 446-475.
11. Aharoni A., Jongsma M.A., Bouwmeester H.J. (2005) Trends in plant science 10: 594-602.
12. Schwab W., Fischer T.C., Giri A., Wüst M. (2015) Applied microbiology and biotechnology 99: 165-174.
13. Holopainen J.K., Gershenzon J. (2010) Trends in Plant Science 15: 176-184.
14. Tripathi A.K., Prajapati V., Aggarwal K.K., Kumar S. (2001) Journal of Economic Entomology 94: 979-983.
15. Croteau R., Purkett P.T. (1989) Archives of biochemistry and biophysics 271: 524-535.
16. Bohlmann J., Meyer-Gauen G., Croteau R. (1998) Proceedings of the National Academy of Sciences 95: 4126-4133.
17. Booth J.K., Page J.E., Bohlmann J. (2017) PLOS ONE 12: e0173911.
18. Dong L., Jongedijk E., Bouwmeester H., Van Der Krol A. (2016) New Phytologist 209: 679-690.

19. Fidyt K., Fiedorowicz A., Strządała L., Szumny A. (2016) Cancer Medicine 5: 3007-3017.
20. Nissen L., Zatta A., Stefanini I., Grandi S., Sgorbati B., Biavati B., Monti A. (2010) Fitoterapia 81: 413-419.
21. Flavoring, Association E.M. (1997) FEMA Database: Beta-Caryophyllene (FEMA No. 2252). Washington, DC: Flavor and Extract Manufacturers Association.
22. Maluf W.R., Campos G.A., das Graças Cardoso M. (2001) Euphytica 121: 73-80.
23. Adam K.-P., Thiel R., Zapp J. (1999) Archives of biochemistry and biophysics 369: 127-132.
24. Liu Q., Majdi M., Cankar K., Goedbloed M., Charnikhova T., Verstappen F.W.A., de Vos R.C.H., Beekwilder J., van der Krol S., Bouwmeester H.J. (2011) PLOS ONE 6: e23255.
25. Cunillera N., Boronat A., Ferrer A. (1997) Journal of Biological Chemistry 272: 15381-15388.
26. Sallaud C., Rontein D., Onillon S., Jabès F., Duffé P., Giacalone C., Thoraval S., Escoffier C., Herbette G., Leonhardt N., Causse M., Tissier A. (2009) The Plant Cell 21: 301-317.
27. Picman A.K. (1986) Biochemical Systematics and Ecology 14: 255-281.
28. Rabe T., Mullholland D., van Staden J. (2002) Journal of Ethnopharmacology 80: 91-94.
29. Skaltsa H., Lazari D., Panagouleas C., Georgiadou E., Garcia B., Sokovic M. (2000) Antifungal activity. Phytochemistry 55: 903-908.
30. Liu Q., Manzano D., Tanic N., Pesic M., Bankovic J., Pateraki I., Ricard L., Ferrer A., de Vos R., van de Krol S., Bouwmeester H. (2014) Metab Eng 23: 145-153.
31. Lepoittevin J.P., Berl V., Giménez-Arnau E. (2009) The Chemical Record 9: 258-270.
32. Zhang Y., Teoh K.H., Reed D.W., Maes L., Goossens A., Olson D.J., Ross A.R., Covello P.S. (2008) Journal of Biological Chemistry 283: 21501-21508.
33. Chadwick M., Trewin H., Gawthrop F., Wagstaff C. (2013) International Journal of Molecular Sciences 14: 12780-12805.
34. Zerbe P., Hamberger B., Yuen M.M.S., Chiang A., Sandhu H.K., Madilao L.L., Nguyen A., Hamberger B., Bach S.S., Bohlmann J. (2013) Plant Physiology 162: 1073-1091.
35. Martínez C., Espinosa-Ruiz A., Prat S. (2016) Annual plant reviews 49: 285-322.
36. Goodman J., Walsh V. (2001) The story of Taxol. Nature and politics in the pursuit of an anti-cancer drug 8.
37. Suffness M. (1995) Taxol: science and applications: CRC press.
38. Lee K.-A., Chae J.-I., Shim J.-H. (2012) Journal of Biomedical Science 19: 60.
39. Zerbe P., Chiang A., Yuen M., Hamberger B., Hamberger B., Draper J.A., Britton R., Bohlmann J. (2012) Journal of Biological Chemistry 287: 12121-12131.
40. Sallaud C., Giacalone C., Töpfer R., Goepfert S., Bakaher N., Rösti S., Tissier A. (2012) The Plant Journal 72: 1-17.
41. Thimmappa R., Geisler K., Louveau T., O'Maille P., Osbourn A. (2014) Annual Review of Plant Biology 65: 225-257.
42. Chen Y.-F., Yang C.-H., Chang M.-S., Ciou Y.-P., Huang Y.-C. (2010) International journal of molecular sciences 11: 4417-4425.
43. Copaja S., Blackburn C., Carmona R. (2003) Wood science and technology 37: 103-108.
44. Yamaguchi H., Noshita T., Kidachi Y., Umetsu H., Hayashi M., Komiyama K., Funayama S., Ryoyama K. (2008) Journal of Health Science 54: 654-660.
45. Moses T., Papadopoulou K.K., Osbourn A. (2014) Critical Reviews in Biochemistry and Molecular Biology 49: 439-462.
46. Patocka J. (2003) J Appl Biomed 1: 7-12.
47. Narayan V., Kodihalli R.C., Chiaro C., Cary D., Aggarwal B.B., Henderson A.J., Prabhu K.S. (2011) Journal of molecular biology 410: 972-983.
48. Brendolise C., Yauk Y.K., Eberhard E.D., Wang M., Chagne D., Andre C., Greenwood D.R., Beuning L.L. (2011) The FEBS journal 278: 2485-2499.
49. Rodriguez-Amaya D.B. (2015) Food Carotenoids: Chemistry, Biology, and Technology: 1-23.
50. Stange C. (2016) Carotenoids in nature: biosynthesis, regulation and function: Springer.
51. Zakynthinos G., Varzakas T. (2016) Current Research in Nutrition and Food Science 4: 38-51.

52. Alcaíno J., Baeza M., Cifuentes V. (2016) Carotenoid Distribution in Nature. Carotenoids in Nature: Springer. pp. 3-33.
53. Dogbo O., Laferriére A., d'Harlingue A., Camara B. (1988) Proceedings of the National Academy of Sciences 85: 7054-7058.
54. Alder A., Jamil M., Marzorati M., Bruno M., Vermathen M., Bigler P., Ghisla S., Bouwmeester H., Beyer P., Al-Babili S. (2012) Science 335: 1348-1351.
55. Bruno M., Hofmann M., Vermathen M., Alder A., Beyer P., Al-Babili S. (2014) FEBS Letters 588: 1802-1807.
56. Abe S., Sado A., Tanaka K., Kisugi T., Asami K., Ota S., Kim H.I., Yoneyama K., Xie X., Ohnishi T. (2014) Proceedings of the National Academy of Sciences 111: 18084-18089.
57. Zhang Y., Van Dijk A.D., Scaffidi A., Flematti G.R., Hofmann M., Charnikhova T., Verstappen F., Hepworth J., Van Der Krol S., Leyser O. (2014) Nature chemical biology 10: 1028-1033.
58. Misawa N. (2011) Current opinion in biotechnology 22: 627-633.
59. Schalk M., Pastore L., Mirata M.A., Khim S., Schouwey M., Deguerry F., Pineda V., Rocci L., Daviet L. (2012) Journal of the American Chemical Society 134: 18900-18903.
60. Larionov V., Kouprina N., Graves J., Chen X., Korenberg J.R., Resnick M.A. (1996) Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. Proceedings of the National Academy of Sciences 93: 491-496.
61. Verwaal R., Wang J., Meijnen J.-P., Visser H., Sandmann G., van den Berg J.A., van Ooyen A.J.J. (2007) Applied and Environmental Microbiology 73: 4342-4350.
62. Ro D.-K., Paradise E.M., Ouellet M., Fisher K.J., Newman K.L., Ndungu J.M., Ho K.A., Eachus R.A., Ham T.S., Kirby J. (2006) Nature 440: 940-943.
63. Hasan M.M., Kim H.-S., Jeon J.-H., Kim S.H., Moon B., Song J.-Y., Shim S.H., Baek K.-H. (2014) Plant cell reports 33: 895-904.
64. King B.C., Vavitsas K., Ikram N.K.B.K., Schrøder J., Scharff L.B., Hamberger B., Jensen P.E., Simonsen H.T. (2016) Scientific reports 6: 25030.
65. Khairul Ikram N.K.B., Beyraghdar Kashkooli A., Peramuna A.V., van der Krol A.R., Bouwmeester H., Simonsen H.T. (2017) Frontiers in Bioengineering and Biotechnology 5.
66. Wang B., Kashkooli A.B., Sallets A., Ting H.-M., de Ruijter N.C.A., Olofsson L., Brodelius P., Pottier M., Boutry M., Bouwmeester H., van der Krol A.R. (2016) Metabolic Engineering 38: 159-169.
67. Fu X., Shi P., He Q., Shen Q., Tang Y., Pan Q., Ma Y., Yan T., Chen M., Hao X., Liu P., Li L., Wang Y., Sun X., Tang K. (2017) Front Plant Sci 8: 723.

# Production of esters and phenylpropenes are linked through *MdAAT1* in apple fruit

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## Abstract

Major QTLs (quantitative trait loci) for the production of two-character impact compounds in apple (*Malus x domestica*), namely 2-methylbutyl acetate (2-MBA, over ripe, fruity, sweet notes) and estragole (anise, licorice notes), both map to the *MdAAT1* (*alcohol acyl transferase 1*) locus in the apple genome. Biochemical analysis shows that *MdAAT1* is required for the production of volatile acetate esters such as 2-MBA, hexyl acetate and butyl acetate and for the production of *p*-hydroxycinnamyl acetates that are substrates for the production of phenylpropenes such as eugenol, chavicol and estragole in ripe apple fruit. The importance of the *MdAAT1* gene in ester and phenylpropene production was validated in transgenic 'Royal Gala' knockdown lines that produced significantly reduced 2-MBA and estragole levels in ripe fruit. Manipulation of flux through the phenylpropanoid pathway in apple using *MdCHS* (chalcone synthase) knockout and *MdMYB10* (transcription factor) over-expression lines increased phenylpropene production. Transient over-expression of alcohol acyl transferases (AATs) from ripe strawberry and tomato fruit showed these enzymes can also produce *p*-hydroxycinnamyl acetates, indicating that ripening-related AATs are likely to link volatile ester and phenylpropene production in many different fruit. These results significantly increase our understanding of volatile synthesis in fruit and provide the basis for breeding new apple varieties with improved flavour profiles by marker assisted selection.

## Introduction

The characteristic taste and aroma of different fruit species and cultivars is derived from non-volatile components such as sugars (providing sweetness), acids (sourness, tartness) and tannins (astringency, bitterness), as well as volatile compounds such as esters, phenylpropenes, alcohols, aldehydes, terpenes and furans. Fruit such as apple, banana, kiwifruit, melon and pear produce high levels of volatile esters which contribute characteristic 'fruity' notes to the aroma. In 'Gala' apples, the major odour-active esters are hexyl acetate (fruity, green, apple notes), butyl acetate (ethereal, solvent, fruity) and 2-methylbutyl acetate (overripe, fruity, sweet) [1]. Volatile esters are synthesised via fatty acid degradation or from amino acid precursors with the final step being catalysed by alcohol acyl transferases (AATs). AATs catalyse the transfer of an acyl group from a CoA donor to an alcohol acceptor.

Phenylpropenes are typically found at low levels in fruit, and impart flavour notes associated with aromatic spices [2]. In apple, estragole (anise, licorice notes) is the most widely described odour-active phenylpropene; with eugenol (sweet, spicy, clove) and chavicol (clove, spicy) also being reported [2]. In tomato, eugenol and guaiacol content

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is correlated with a 'pharmaceutical' aroma [3], whilst in musk strawberry the high content of eugenol, methyleugenol, and methylisoeugenol has been associated with a cinnamon smell [4]. Phenylpropenes are produced as a side branch of the general phenylpropanoid pathway and the initial biosynthetic steps are shared with the lignin biosynthetic pathway up to the production of *p*-coumaryl alcohol and coniferyl alcohol. The first committed step in phenylpropene production involves the conversion of *p*-coumaryl and coniferyl alcohols to *p*-coumaryl and coniferyl acetates (*p*-hydroxycinnamyl acetates). The acetates are then reduced by NADPH-dependent phenylpropene reductases. Methoxylated phenylpropenes such as estragole and anethole are formed by *O*-methyltransferases (OMT) using S-adenosylmethionine (SAM) as the methyl donor.

QTLs (quantitative trait loci) for volatile production have recently been described for aldehydes, esters, alcohols and phenylpropenes in apple (*Malus x domestica*). Forty-six QTLs for ester and alcohol production were reported in a cross between the highly aromatic cultivar 'Royal Gala' (RG) and the low aroma cultivar 'Granny Smith' (GS) [5]. The major QTL for the production of 2-methylbutyl acetate (2-MBA) and 34 other volatiles in this population was located on linkage group 2 (LG2) and co-located with the *Malus x domestica alcohol acyl transferase 1* (*MdAAT1*) gene. Two QTLs for production of the phenylpropene estragole were also identified in the same segregating population. The first QTL was located on LG1 and was responsible for 9.2% of the variation. The *MdoOMT1* (*O-methyltransferase 1*) gene was shown to co-locate with the LG1 QTL and biochemical and molecular analysis showed that this gene was required for estragole production in ripe RG fruit [6].

## Results and discussion

### *QTL analysis*

The major QTL for the production of estragole (accounting for 24% of the variance) in the segregating RG x GS population is located on LG2. The nearest marker to the maximum logarithm of the odds (LOD) peak for estragole production was identical to that previously identified as co-segregating with the production of volatile esters such as 2-MBA, butyl acetate and hexyl acetate in ripe apple fruit [7]. This result suggested that MdAAT1 might be the enzyme responsible for an acylation step in both the ester biosynthetic and phenylpropene biosynthetic pathways.

### *Biochemical characterisation*

The enzymatic activity of MdAAT1 has previously been reported with respect to a wide range of alcohols and acyl CoAs involved in volatile ester production in ripe apple fruit [8]. In Yauk et al., 2017 [7], MdAAT1 was also shown to convert coniferyl and *p*-coumaryl alcohols to *p*-hydroxycinnamyl acetates that serve as substrates for phenylpropene production in apple. The relative activity of recombinant MdAAT1-RGa from RG towards alcohols such as butanol, 2-methylbutanol and hexanol used for volatile ester production was high (64–100%, Table 1). In contrast, relative activity towards *p*-coumaryl alcohol and coniferyl alcohol was much lower (< 2%, Table 1). Kinetic studies indicated that the affinity of MdAAT1-RGa towards *p*-coumaryl alcohol was comparable to that reported for hexanol and butanol, however the $V_{max}$ was much lower [7]. Compared with MdAAT1-RGa, recombinant MdAAT1-GSa from GS showed weak activity (< 3%) towards alcohols used for volatile ester biosynthesis and barely detectable activity (0.11%) towards *p*-coumaryl alcohol (Table 1). This difference in kinetic properties likely explains the QTL in the segregating RG x GS population.

**Table 1**: Activity of recombinant MdAAT1 enzymes from 'Royal Gala' (RG) and 'Granny Smith' (GS). Activity assays contained MdAAT1-RGa (0.5 µg) or MdAAT1-GSa (31.2 µg), 10 mM alcohol, 1 mM CoA, in 50 mM Bis-Tris propane pH 8.0. Activity was set at 100% for acetyl-CoA and hexanol using MdAAT1-RGa. Data are presented as mean ± SE (n=3). Chavicol was synthesised by esterification of *p*-coumaric acid followed by DIBAL reduction to *p*-coumaryl alcohol. Radiolabelled acetyl-CoA was obtained from American Radiolabeled Chemicals and all other chemicals from Sigma-Aldrich. Data derived from Yauk et al., 2017 [7].

| Esters | | | |
|---|---|---|---|
| *Substrate 1* | *Substrate 2* | *MdAAT1-RGa* | *MdAAT1-Gsa* |
| Hexanol | acetyl-CoA | 100±1.4 | 2.94±0.16 |
| Butanol | | 63.8±3.0 | 1.03±0.06 |
| 2-methylbutanol | | 77.6±3.3 | 0.26±0.02 |

| Phenylpropenes | | | |
|---|---|---|---|
| *Substrate 1* | *Substrate 2* | *MdAAT1-RGa* | *MdAAT1-GSa* |
| *p*-coumaryl alcohol | acetyl-CoA | 1.2±0.2 | 0.11±0.01 |
| coniferyl alcohol | | 0.3±0.02 | not detected |

*Analysis of MdAAT1 knockdown lines*

To validate the importance of *MdAAT1* in phenylpropene production, transgenic RG lines containing an RNAi construct of *MdAAT1* were examined. Our hypothesis was that decreasing *MdAAT1* expression (Figure 1A) would reduce the production of *p*-hydroxycinnamyl acetates and the subsequent accumulation of phenylpropenes in ripe fruit. The results from solvent extraction and GC-MS analysis on ripe fruit samples from two transgenic lines and RG controls is presented in Figure 1B. Total ester production in the *MdAAT1* lines was reduced by > 90%, confirming the results previously reported in Souleyre et al., 2014 [5]. Production of the phenylpropenes chavicol, (*E*)-isochavicol, eugenol and estragole were also reduced in the *MdAAT1* knockdown lines.

*Manipulation of flux in the phenylpropanoid pathway*

Two additional sets of transgenic apple plants were investigated to determine what effect manipulating flux through the phenylpropanoid pathway would have on phenylpropene accumulation. The first set of transgenic lines were down-regulated for expression of the *MdCHS* (chalcone synthase) gene, a key biosynthetic gene in the phenylpropanoid pathway. *MdCHS* knockout lines do not accumulate anthocyanins or dihydrochalcones which are normally abundant in apple fruit [9]. Our hypothesis was that redirection of flux in these plants would lead to accumulation of higher levels of phenylpropenes (Figure 2A). The results from solvent extraction and GC-MS analysis on ripe fruit samples from three transgenic lines and RG controls is presented in Figure 2B. Production of the phenylpropenes chavicol and (*E*)-isochavicol increased in all three *MdCHS* lines compared to the control. Production of eugenol and estragole was elevated in lines A2 and A7 respectively. As expected, total ester production in the *MdCHS* knockout lines was similar to the RG control.

Analysis of the glycosides present in two of the *MdCHS* knockout lines compared to the RG control indicated that production of chavicol and eugenol glycosides was elevated in the transgenic lines (Figure 2B). Chavicol glycosides were found at 80–100 fold higher levels in the *MdCHS* lines compared to controls, whilst eugenol glycosides were found at 6–12 fold higher levels. Much of the increased flux towards phenylpropene production in the *MdCHS* knockout lines appeared to be directed towards glycoside sequestration. The total phenylpropene glycoside concentration in the *MdCHS* lines was 35,000–40,000 ng/g

(vs 700 ng/g in controls), whilst the total 'free' phenylpropene concentration was 1,400–4,000 ng/g in the *MdCHS* lines (vs 800 ng/g in controls).

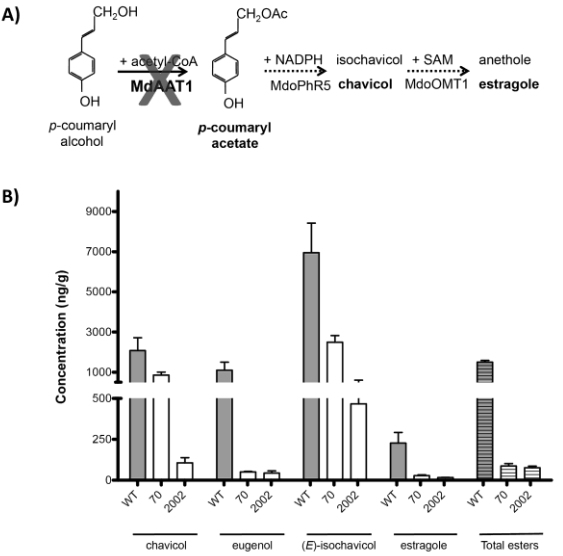


**Figure 1**: Analysis of *MdAAT1* knockdown lines (**A**) In *MdAAT1* knockdown lines the production of *p*-coumaryl acetate and phenylpropene derivatives should be reduced. *MdAAT1 = Malus domestica alcohol acyl transferase 1*, *MdoPhR5 = Malus domestica phenylpropene reductase 5*, *MdoOMT1 = Malus domestica O-methyltransferase 1*. (**B**) Volatile phenylpropene and ester production in 'Royal Gala' wildtype (WT) controls (grey bars) and *MdAAT1* knockdown lines (AS70 and AS2002, white bars). Volatiles were obtained from ripe fruit skin tissue extracted into diethyl ether. GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer [10]. Data are presented as mean ± SE and are derived from Yauk et al., 2017 [7].

The second set of transgenic apple plants in which phenylpropene flux was manipulated constitutively over-expressed a copy of the *MdMYB10* gene, a transcription factor that up-regulates flux through the phenylpropanoid pathway. *MYB10* over-expression lines have red foliage and red-fleshed fruit and accumulate much higher levels of anthocyanins, flavonols and total phenolics due to increased expression of several phenylpropanoid biosynthetic genes [12]. Our hypothesis was that the *MYB10* over-expression lines would also accumulate higher levels of phenylpropenes either as a result of higher flux through the phenylpropanoid pathway or that *MdMYB10* might transcriptionally activate genes involved in phenylpropene biosynthesis. The results of GC-MS analysis indicated that compared to controls, the *MYB10* lines accumulated higher levels of phenylpropenes at all four stages of fruit development tested, but particularly at the two latter time points [6]. No evidence for transcriptional activation of *MdAAT1*, *MdoPhR5 (phenylpropene reductase 5*) or *MdoOMT1* in *MYB10* over-expression lines was observed [7]. Together these results suggested that the increased phenylpropene levels in the *MYB10* fruit was due to some of the increased flux in the phenylpropanoid pathway being diverted into the phenylpropene biosynthetic pathway.

*Do AAT genes link ester and phenylpropene biosynthesis in other fruit?*

Our results in apple clearly demonstrated the importance of *MdAAT1* to ester and phenylpropene production in apple, but is this true for *AAT* genes from other species that accumulate both esters and phenylpropenes such as strawberry and tomato? To test this

hypothesis, AATs from ripe strawberry (*SAAT*) and tomato (*SlAAT1*) fruit were transiently expressed in *Nicotiana benthamiana* in coupled reactions with *MdoPhR5*.

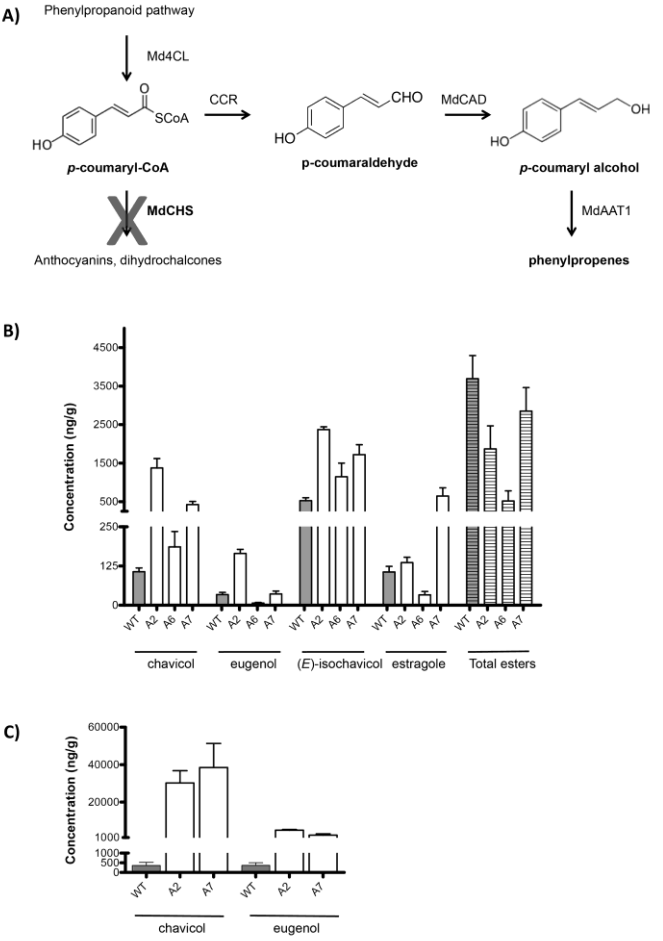


**Figure 2:** Analysis of *MdCHS* knockout lines (**A**) In *MdCHS* knockout lines the phenylpropanoid pathway is blocked leading to the accumulation of *p*-coumaryl-CoA which is then metabolised via *p*-coumaraldehyde to *p*-coumaryl alcohol. *p*-Coumaryl alcohol is the substrate for *MdAAT1* and the entry point for production of phenylpropenes. *Md4CL = Malus domestica 4-coumarate CoA-ligase*, *MdCHS = Malus domestica chalcone* synthase, *MdCCR = Malus domestica cinnamoyl-CoA reductase*, *MdCAD = cinnamyl alcohol dehydrogenase*, *MdAAT1 = Malus domestica alcohol acyl transferase 1*. (**B**) Volatile phenylpropene and ester production in 'Royal Gala' wildtype (WT) controls (grey bars) and *MdCHS* knockout lines (A2, A6 and A7, white bars). Volatiles were extracted from ripe fruit skin tissue into diethyl ether. GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer [10]. Data are presented as mean ± SE and are derived from Yauk et al., 2017 [7]. (**C**) Phenylpropene glycoside production in 'Royal Gala' WT controls (grey bars) and *MdCHS* knockout lines (white bars, A2 and A7). Glycosides were prepared using Amberlite XAD-2 columns as described in Yauk et al., 2014 [11] from ~ 3 g of ripe apple fruit skin tissue. Glycosides were digested with Rapidase AR2000 for 16 h at 37ºC in reactions overlaid with 100 µL diethyl ether/pentane. GC-MS analysis was performed as described in Nieuwenhuizen et al., 2013 [10]. Data are presented as mean ± SE.

Coupled reactions were used as the phenylpropene products are stable and readily detected by GC-MS analysis [7]. The results presented in Figure 3 show that chavicol

was produced by both *SAAT* and *SlAAT1* when leaves were infiltrated with *p*-coumaryl alcohol. Eugenol was also produced by both *SAAT* and *SlAAT1* when leaves were infiltrated with coniferyl alcohol, but at much higher levels with *SAAT* from strawberry. These results suggest that ripening-related AATs may link volatile ester and phenylpropene production in many different fruit and provide a rational basis for breeding new varieties with improved flavour profiles by marker assisted selection or metabolic engineering.

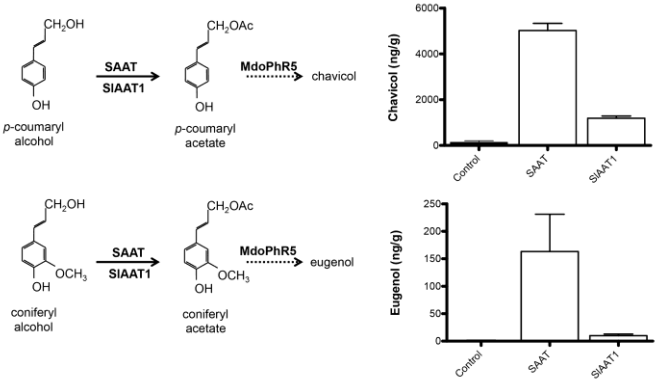


**Figure 3:** Functional characterisation of AATs from ripe strawberry and tomato fruit. The activities of strawberry AAT (*SAAT*) and tomato AAT *(SlAAT1*) were determined by transient expression analysis in *Nicotiana benthamiana*. Leaves were initially infiltrated with *SAAT* or *SlAAT1* coupled with *MdoPhR5* (*Malus domestica phenylpropene reductase 5*). GUS + *MdoPhR5* was used as the control. After seven days, leaves were infiltrated with either *p*-coumaryl or coniferyl alcohol. GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer [10]. Data are presented as mean ± SE and are derived from Yauk et al., 2017 [7].

## References

1. Plotto A., McDaniel M.R., Mattheis, J.P. (2000) J. Am. Soc. Hort. Sci. 125: 714-722.
2. Atkinson R.G. (2017). J. Agric. Food Chem., DOI: 10.1021/acs.jafc.6b04696.
3. Causse M., Saliba-Colombani V., Lecomte L., Duffe P., Rousselle P., Buret M. (2002) J. Exp. Bot. 53: 2089-2098.
4. Pet'ka J., Leitner E., Parameswaran B. (2012) Flavour Fragr. J. 27: 273-279.
5. Souleyre E.J.F., Chagné D., Chen X., Tomes S., Turner R.M., Wang M.Y., Maddumage R., Hunt M.B., Winz R.A., Wiedow C., Hamiaux C., Gardiner S.E., Rowan D.D., Atkinson R.G. (2014) Plant J. 78: 903-915.
6. Yauk Y.-K., Chagné D., Tomes S., Matich A.J., Wang M.Y., Chen X., Maddumage R., Hunt M.B., Rowan D.D., Atkinson R.G. (2015) Plant J. 82: 937-950.
7. Yauk Y.-K., Souleyre E.J.F., Matich A.J., Chen X., Wang M.Y., Plunkett B., Dare A.P., Espley R.V., Tomes S., Chagné D., Atkinson R.G. (2017) Plant J. 91: 292-305.
8. Souleyre E.J., Greenwood D.R., Friel E.N., Karunairetnam S., Newcomb R.D. (2005) FEBS J. 272: 3132-3144.
9. Dare A.P., Yauk Y.-K., Tomes S., McGhie T.K., Rebstock R.S., Cooney J.M., Atkinson R.G. (2017) Plant J. 91: 237-250.
10. Nieuwenhuizen N.J., Green S.A., Chen X., Bailleul E.J.D., Matich A.J., Wang M.Y., Atkinson R.G. (2013) Plant Physiol. 161: 787-804.
11. Yauk Y.-K., Ged C., Wang M.Y., Matich A.J., Tessarotto L., Cooney J.M., Chervin C., Atkinson R.G. (2014). Plant J. 80: 317-330.
12. Espley R.V., Bovy A., Bava C., Jaeger S.R., Tomes S., Norling C., Crawford J., Rowan D., McGhie T.K., Brendolise C., Putterill J., Schouten H.J., Hellens R.P., Allan A.C. (2013). Plant Biotechnol. J. 11: 408-419.

# Shedding light on the modulation of key Riesling wine aroma compounds in a changing climate

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## Abstract

As Riesling ages, there exists a delicate balance between the loss of young fresh and fruity characters, and the formation of aged notes, including 'kerosene' due to 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). Early formation of 'aged' notes in young wine can lead to unbalanced wines that are not necessarily appreciated by consumers. The vineyard drivers associated with earlier evolution of TDN have been examined in this work, along with glycosylated precursors, to aid in better understanding of TDN formation in grapes and wine.

Two commercial vineyards with a difference in temperature of around 2 °C underwent treatments to modulate light exposure to the grape bunches, yielding significant difference in total TDN and providing information into the roles of light and temperature in TDN formation. These treatments allowed for LC-MS/MS studies into glyosidic precursors and tentative identification of several compounds that are expected to contribute to higher amounts of TDN.

## Introduction

Characters of young Riesling wine like estery, citrus and floral result from compounds such as monoterpenes or 2-phenylethyl acetate. As Riesling ages these diminish and the wine develops lime, caramel, and kerosene notes. One of the compounds responsible for aged Riesling character, and the 'kerosene' descriptor, is 1,1,6-trimethyl-1,2-dihydronaphthalene, or TDN [1]. With a sensory threshold of 2 µg/L [2], TDN can be polarising with sensory intensity not always relating to concentration [3]. While considered important to aged Riesling, it can result in an unbalanced wine especially if it starts to emerge in younger wines and dominate delicate floral or fruity notes.

The evolution of TDN in wine is thought to occur via the breakdown of carotenoids and through reaction and rearrangement of norisoprenoids, which are present in grapes as glycosidically bound species. As such, TDN itself is not present in grapes but forms and accumulates as wine ages. Considered a thermodynamic end-point, TDN is very stable, unlike the monoterpenes responsible for young Riesling characters which degrade as wine ages [4]. Due to light-induced changes in carotenoid profiles during grape growing and structural diversity of the carotenoid end group from which the C13-norisoprenoids are formed, elucidation of the exact carotenoid(s) that give rise to TDN, and hence the pathway to formation, has proved difficult so far.

It is well understood that the timing and intensity of light exposure of grapes modulates the amount of TDN produced in wine [5], but recently it has been proposed that as growing seasons get warmer due to climate change, increasing temperatures result in kerosene notes being more prevalent in younger Riesling wines [6]. Currently, there exists evidence that winemaking practices can be useful in managing the amount of TDN present in a wine, including yeast choice [7], wine pH [8], and closure type (Figure 1) [3].

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But the question remains to be answered: is limiting TDN evolution using winemaking interventions is just a case of too little, too late?

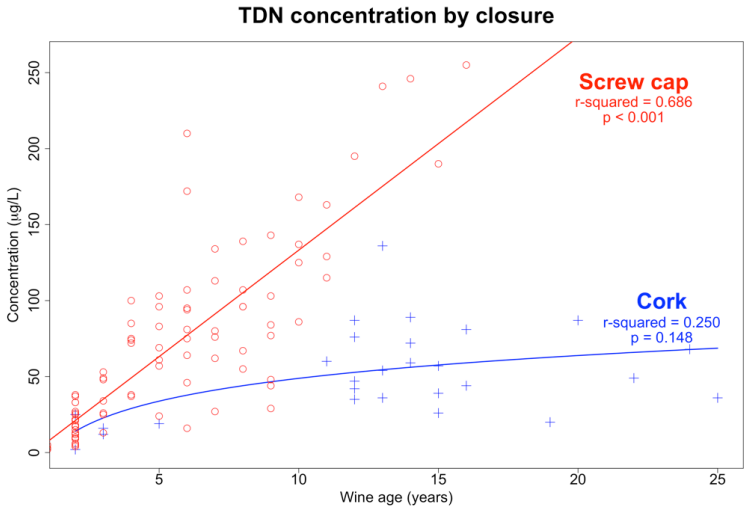


**Figure 1**: The evolution of TDN in 116 commercial Riesling wines, separated into those with screw cap closures (red circles) and cork closures (blue crosses).

The biggest questions that remain surround the key driving forces in the vineyard that determine the speed and extent of TDN production as the wine ages. Is the changing climate a driving force that will result in Riesling obtaining aged characters earlier in future, and do we continue to employ viticultural techniques to modify light exposure of grapes as a means for achieving optimal maturity? This work is the first step in determining practical solutions for managing TDN concentrations in wine, including a better understanding of the pathway(s) by which TDN is formed, identifying markers in grapes that allow us to predict TDN formation in wine, and ascertaining the true vineyard-based driving forces of high TDN wines.

## Experimental

### *Trial sites and grape sampling*

During the 2014/15 growing season, trials were conducted in two commercial vineyards in Barossa Valley (BV) and Eden Valley (EV) of South Australia, separated by 13.7 km, 180 m of altitude, and a mean January temperature differential of 1.94 °C. Treatments were applied 30-days past berry set (11 December for EV, 23 December for BV), where one-third of the bunch zone leaves were removed (leaf-plucked), compared with a control, both replicated 6 times in an alternating manner within two adjacent rows. Within these treatments, light exclusion boxes were applied to single bunches (plucked boxed and control boxed), and temperature was monitored in both canopy and boxes (Tinytag Transit 2 temperature loggers). At commercial harvest (11 February for BV, 19 February for EV) grapes were hand-picked. Grape berries were immediately plucked and randomised. Grapes (300 g) were homogenised (20 s, 8,000 rpm, Retsch Grindomix GM200) and the homogenate stored at -20 °C until further use. When required, the homogenate was thawed, centrifuged and the supernatant used for analyses.

*Analysis of total TDN in grape samples*

The analysis of total TDN was based on the solid-phase extraction (SPE) protocol described by Kwasniewski et al. [5], using $d_8$-nathphalene (Sigma-Aldrich) as the internal standard. Quantification of TDN in the hydrolysed samples using GC-MS was based on the report of Daniel et al. [8], with minor modifications to the oven parameters.

*Preparative HPLC of glycosidic material*

Based on a reported analytical-scale SPE isolation [9], glycosidic material was isolated from 50 mL of Eden Valley grape homogenate supernatant using 25 SPE cartridges (2 mL per cartridge), to increase scale. The pooled material was concentrated to approximately 2 mL and made up to 4 mL using milli-Q water. An aliquot was diluted 1:10 and 500 µL injected on a Dionex UltiMate® 3000 Binary Semi-preparative HPLC-DAD system and separation achieved on a Synergi Hydro-RP column (80 Å, 4 µm, 250 x 21.2 mm) with a C18 guard column (15 x 21.2 mm). A binary gradient with mobile phases consisting of 0.1 % acetic acid in water (A) and 0.1 % acetic acid in acetonitrile (B) with a flow rate of 8 mL/min and elution profile starting at 5% B, increasing linearly to 15% B over 10 mins, then increasing to 30% B over 40 mins, then to 90% B over one minute and held for further 19 mins. The gradient was reduced to 5% B over 1 minute and re-equilibrated for 40 minutes. The column effluent passed through a diode array detector (190-400 nm) and then into a fraction collector. Fractions were collected every 30 seconds, with those representing one peak combined, as were consecutive fractions representing no DAD peak. These were concentrated to dryness and reconstituted in 1 mL of water. Half of this (0.5 mL) was used for LC-MS/MS investigations and half (0.5 mL) analysed for total TDN (as above).

*LC-MS/MS investigation into glycosidic TDN precursors*

Potential glycosidic precursors to TDN were investigated using a Liquid Chromatography Quadrupole Time-of-Flight-system (Agilent 1200 series LC-system). Due to concentration factors 10 µL were injected for the exposed and 5 µL were injected for the control samples, and separation was carried out on a Kinetex PFP column (100Å, 2.7um, 150 x 2.1 mm) using the same mobile phases as above. A flow rate of 0.2 mL/min was used and an elution profile starting at 5% B, increasing linearly to 15% B over 7 mins, then increasing to 30% B over 13 mins, then to 90% B over 12 minutes and held for further 5 mins. The gradient was dropped back to 5% B over 1 minute and then re-equilibrated for 19 minutes. A Bruker micrOTOF-Q II mass spectrometer equipped with an orthogonal ESI source was used for high resolution mass spectrometric analysis. The ionisation was in negative APCI mode with nitrogen curtain, nebulizer and collision gas. The instrument conditions were: capillary voltage (3500 V), end plate offset (-500 V), drying gas (4 L/min, 250 °C), nebulizer gas pressure (0.4 bar); mass scan range (50−1650), and ramped collision energy. External instrument calibration was using sodium formate solution (10 mM NaOH in isopropanol/0.2% formic acid (1:1)).

*Statistics and graphics*

All graphing and statistical analyses were carried out with the open source statistical programming language R, using custom scripts.

**Results and discussion**

The two sites represented a climatic shift of approximately 2 °C (1.94 °C based on our temperature data), and provide a good model for the temperature increase predicted under climate change scenarios [10]. The treatments represented extreme defoliation to

increase light exposure and confirm the previous findings correlating exposure and TDN production, plus boxed treatments to create a negative control, no-light scenario. The changes in total TDN brought about by the light modulating treatments were marked (Figure 2), although no significant inter-site variation was observed.

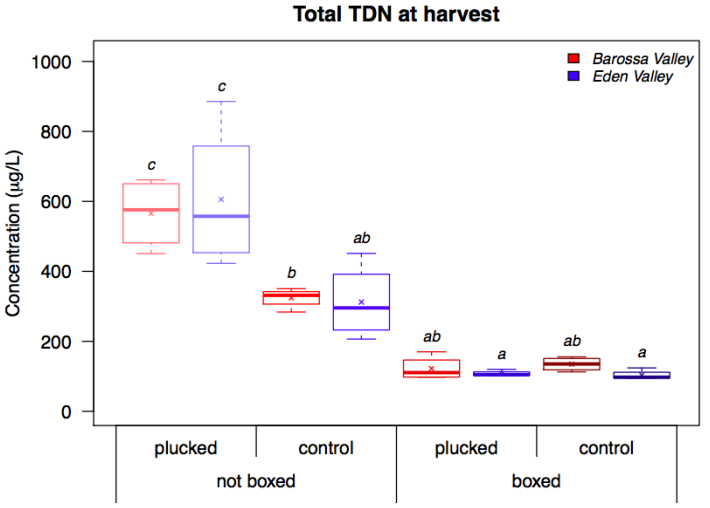


**Figure 2**: Total TDN in grape homogenates from each of the treatments and sites. Different letter denotes significant difference between treatments ($P = 0.05$).

This confirms the previous findings that light is important in modulating the amount of potential TDN that can evolve as a wine ages [5], but also shows no obvious difference between the two sites that differed by approximately 2 °C. This does not necessarily mean that temperature has no effect, as differences between the vineyards (e.g. trellising, soil, humidity) could be offsetting any temperature effect. Although, this result does imply that increases in grape growing temperature does not necessarily have to result in an increase in TDN production in wine.

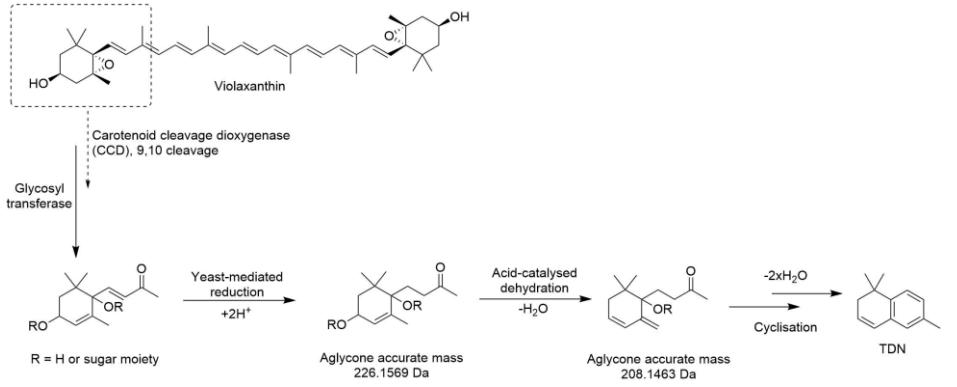


**Figure 3**: Carotenoid cleavage and one proposed pathway for production of TDN via glycosylated precursors, showing accurate masses for protonated intermediates in this proposed pathway [4, 7]

The C13-norisoprenoids that result from carotenoid cleavage are generally bound to sugars in grapes. Hence, the leaf-plucked treatments provided a means to better

understand TDN production as sun-exposed grapes are expected to contain an increased amount of the glycosylated precursors within the TDN formation pathway. As such, glycosidic fractions were isolated from the Eden Valley control and leaf-plucked grapes, then fractionated using preparative HPLC. The fractions that were collected were then divided, and one half used to determine the total TDN content, to ascertain the potential of the compounds present in that fraction to give rise to TDN, and the other half kept for future LC-MS/MS investigation of fractions of interest.

Although several HPLC-separated fractions gave rise to TDN under hydrolysis conditions, some gave significantly higher proportions from leaf-plucked grapes than from control grapes, and hence were targeted for understanding the up-regulation of TDN formation. Although pooling fractions and differences in dilution makes comparison between fractions and with absolute total TDN values obtained in whole grapes hard, the relative amounts between leaf-plucked and control samples could be determined. Fraction 32 showed the highest concentration of total TDN, and a relative ratio of 3.1 between the leaf-plucked and control samples. Fraction 30 gave a 1.5-fold increase in the leaf-plucked samples, and the pooled fractions 37-50 a 2.6-fold increase. These three fractions were taken through to LC-MS/MS analysis to better understand the compounds present that were giving rise to increased TDN when hydrolysed.

For LC-MS/MS investigation, the masses of hydroxylated compounds (possible aglycones) that are present in the proposed TDN formation pathways (Figure 3) [4, 7] were combined with the known masses of the sugars that predominate in grapes [9, 11, 12] (as well as the potential acetate analogues) to produce a table of masses of interest. A number of ions were identified that fit our requirements: they were present in both treatments; more abundant in the leaf-plucked samples; and equivalent to a mass of interest (Table 1).

**Table 1**: Ions observed in LC-MS/MS experiments present in higher abundance in leaf-plucked samples and relating to glycosidically bound masses of interest in the proposed TDN formation pathway.

| *Ion $[M+CH_3OO]^-$ (Da)* | *Fraction* | *Retention time (min)* | | *Matching structure/mass of interest* |
|---|---|---|---|---|
| | | *Control* | *Leaf-plucked* | |
| 447.2230 | 32 | 12.0 | 11.8 | m/z = 226.1569 + hexose |
| 561.2547 | 37-50 | 14.5 | 14.6 | m/z = 208.1463 + hexose + pentose |
| 593.2909 | 30 | 12.7 | 12.6 | m/z = 226.1569 + rutinose |

Figure 4 shows an example fragmentation pattern from precursor ion 593.2809 Da in the leaf-plucked sample. Here, the acetate adduct ion fragments (-60 Da) to yield the mass of the proposed rutinoside (~553.26 Da), composed of an aglycone mass that is commonly observed in the proposed TDN formation pathways (226.1569 Da), and rutinose. This fragments further via a neutral loss of the aglycone (226.1566 Da). These fragments align with product ion spectra previously observed for guaiacol rutinoside in grapes [11].

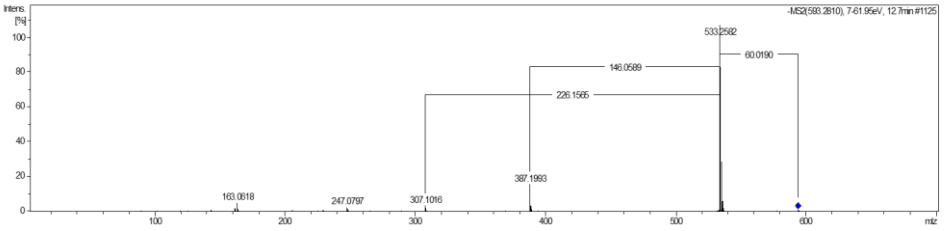


**Figure 4**: Fragmentation pattern for the ion 593.2809 Da in the leaf-plucked sample.

In summary, these viticultural trials have confirmed the importance of light exposure for increasing the total TDN content of grapes, and shown no significant difference in grapes from two vineyards with an approximate 2 °C growing season difference. Preparative HPLC separation allowed for the LC-MS/MS identification of numerous ions that are more abundant in leaf-plucked samples, with tentative elucidation including disaccharide bound norisoprenoids. These structures will provide a starting point for in depth studies into the formation pathway of TDN in wine.

**Acknowledgements**

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## References

1. Simpson, R.F. (1978) Chemistry and Industry. p37.
2. Sacks, G.L., Gates, M.J., Ferry, F.X., Lavin, E.H., Kurtz, A.J. and Acree, T.E. (2012) J. Agric. Food Chem. 6:. 2998-3004.
3. Black, C.A., Francis, I.L., Henschke, P., Capone, D.L., Anderson, S., Day, M., Holt, H., Pearson, W., Herderich, M.J. and Johnson, D. (2012) Wine and Vitic. J. 27: .20-26.
4. Black, C.A., Parker, M., Siebert, T.E., Capone, D.L. and Francis, I.L. (2015) Aust. J. Grape and Wine Res. 21: 582-600.
5. Kwasniewski, M.T., Vanden Heuvel, J.E., Pan, B.S. and Sacks, G.L. (2010) J. Agric. Food Chem. 58: 6841-9.
6. Winterhalter, P. and Gök, R. (2013) in Carotenoid Cleavage Products. ACS Symposium Series 1134, Washington, DC, USA. p. 125-137.
7. Gök, R. (2015) PhD. Thesis. Technical University of Braunschweig.
8. Daniel, M.A., Capone, D.L., Sefton, M.A. and Elsey, G.M. (2009) Aust. J. Grape and Wine Res. 15: 93-96.
9. Hayasaka, Y., Baldock, G.A., Pardon, K.H., Jeffery, D.W. and Herderich, M.J. (2010) J. Agric. Food Chem. 58: 2076-81.
10. IPCC (2014) Climate Change 2014: Synthesis Report [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.
11. Hayasaka, Y., Baldock, G.A., Parker, M., Pardon, K.H., Black, C.A., Herderich, M.J. and Jeffery, D.W. (2010) J. Agric. Food Chem. 58: 10989-10998.
12. Parker, M., Black, C.A., Barker, A., Pearson, W., Hayasaka, Y. and Francis, I.L. (2017) Food Chem. 232: 413-424.

# Changes in key aroma compounds during cocoa powder process

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## Abstract

Changes of selected odorants were followed in entire cocoa powder process. Contribution of the intrinsic aroma content of fermented cocoa and the impact of individual processing steps such as preheating, alkalization, roasting and pressing on the odorant content was evaluated. The intrinsic aroma content of raw cocoa beans together with pre-heating before breaking and winnowing appeared to be the most important source of odorants in cocoa powder. Roasting and alkalization surprisingly showed low impact on *Maillard* derived odorants probably due to the depletion of precursors during preliminary processing. It was observed that the distribution of odorants after cocoa pressing is driven by the polarity of the odorant; polar odorants were predominantly retained in cocoa powder while nonpolar odorants in cocoa butter.

## Introduction

Cocoa powder is the product obtained by grinding the solids remaining after cocoa butter have been pressed out of cocoa liquor. Cocoa powder has gained a significant attention in industry, not only because of its volatile price, but also because of the increasing number of its applications including confectionary, biscuits, powdered beverages, dairy, ice cream, cereals and bakery segments. Approximately half of cocoa bean production is used for manufacturing cocoa powder and cocoa butter.

Flavour character of cocoa powder originates from genotype and origin of cocoa tree, post-harvest treatments (fermentation and drying) and from the manufacturing processes such as alkalization, roasting and pressing. Twenty-four key aroma active compounds were identified in cocoa powder by a sensomics study comprising odorants formed by biosynthesis, during the fermentation and in *Maillard* reaction upon heat treatment [1]. Changes in key aroma compounds during the cocoa bean roasting [2] as well as the impact of alkalization-roasting interaction [3, 4] on the aroma content were described. Yet, there is no comprehensive study clarifying the origin of key cocoa odorants in whole cocoa powder process. Moreover, the majority of reported studies were performed under laboratory conditions that do not exactly match the conditions of the industrial process.

The objective of the study was to evaluate the contribution of the intrinsic aroma content of fermented cocoa and the impact of individual processing steps on the aroma content in cocoa powder.

## Experimental

### *Materials*

Fermented and dried cocoa beans (Ivory Coast origin) and corresponding cocoa nibs (pieces of de-shelled cocoa beans) were obtained from Nestlé La Penilla factory. Potassium carbonate was purchased from Univar (Bradford, United Kingdom). Standards of aroma compounds were purchased from Sigma-Aldrich (Buchs, Switzerland);

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isotopically labelled standards were obtained either from Aroma Lab (Planegg, Germany) or upon customized synthesis from AtlanChim Pharma (Saint Herblain, France).

*Alkalization and roasting*

Alkalization and roasting of cocoa nibs (14 kg batch) were conducted in the pilot plant at Bühler Barth (Germany). Alkalization was conducted with 3% potassium carbonate in CN50 alkalizer under pressure of 1.5 bar (128°C) for 30 min. The nibs were then dried under vacuum for 10 min. The roasting was conducted in RSX Tornado rotating drum roaster with convective heating under a drum pressure of -0.5 bar. The temperature was first set to 90°C and held for 15 min and then raised to 122°C and held for 10 min. At temperature of 110°C, water was injected for the purpose of debacterization. A small scale roasting was performed under ambient pressure with 1.4 kg nibs using a laboratory drum roaster (Probatino S) that operated with the same roasting profile as used during pilot plant trial.

*Production of cocoa powder*

Cocoa powder was produced using laboratory equipment. Cocoa nibs were ground into cocoa liquor a using planetary ball mill (Retsch PM 400 CM) and then pressed in 1/400 GSR cocoa press. The kibble cake was broken using G10S GSR breaker and finally pulverized into powder in ultra-centrifugal mill ZM200 (Retsch).

*Aroma analysis*

The content of fifteen odorants was determined using Head Space Solid Phase Micro Extraction in combination with Gas Chromatography and tandem Mass Spectrometry (HS-SPME-GC/MS/MS). Quantification was accomplished by Stable Isotope Dilution Assay (SIDA). HS-SPME was conducted with 50 mg cocoa sample (original or grinded) in 5 mL water and 100 µL methanol solution of internal standards using DVB-CAR-PDMS fibre of 2cm (Supelco). GC separations were achieved on column DB-624-UI 60 m x 0.25 mm i.d., and film thickness 1.4µm (J&W Scientific).

## Results and discussion

There are several processes for the production of cocoa powder varying mainly in the stage where alkalization (also known as “Dutching”) is applied. Alkalization can be applied either before or after the roasting and can be performed with cocoa nibs, cocoa liquor, cocoa cake or cocoa powder. The process addressed in this study (Figure 1) is the most common one. It starts with cleaning and preheating of cocoa beans followed by breaking and winnowing that result in cocoa nibs. Cocoa nibs are alkalized and then roasted, debacterized and finally ground into cocoa liquor. The liquor is then pressed to obtain cocoa butter and cocoa cake that is further broken and pulverized into cocoa powder. Fifteen key aroma compounds were selected based on literature data [1] and their content was measured in five different stages of the process as indicated by numbers in Figure 1.

A relative contribution of intrinsic aroma of raw nibs and a contribution of two processing steps, alkalization and roasting, to the aroma content of alkalized-roasted nibs is depicted in Figure 2. The contribution of the alkalization and the roasting was assessed based on changes (increase or decrease) of odorant concentrations during these two processes.

Surprisingly, carry over from raw cocoa nibs had the highest impact on the aroma of alkalized-roasted nibs followed by alkalization and roasting. The impact of these two processing steps was rather low.

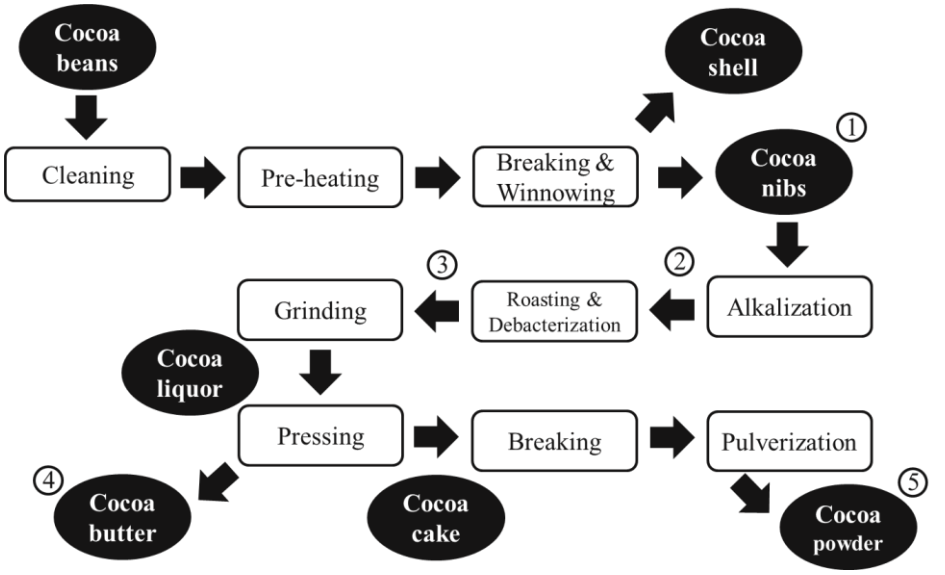


**Figure 1:** Cocoa powder process (numbers indicate sampling points)

*Impact of alkalization on aroma content*

Alkalization has traditionally several purposes: neutralize acidity, decrease bitterness, reduce astringency, modify the colour and improve dispersability of cocoa powder in beverages. Impact of alkalization on aroma compounds is not yet fully understood.

Alkalization revealed a decrease of the majority of the odorants, usually by 30% to 40% as compared to raw nibs. Amounts of 2,3-butanedione (-62%) and dimethyltrisulfide (-81%) were reduced more significantly. Surprisingly, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF) did not change after the alkalization. Only three odorants increased after the alkalization; phenylacetaldehyde that doubled its amounts and guaiacol and 2,3-pentanedione that increased by a factor 6. Yet, the levels of the latter two odorants were very low, thus this increase is less sensory relevant. The decrease of odorants during the alkalization process can be explained either by degradation in basic pH or by stripping of the odorants during vacuum drying applied at the end of alkalization (probably facilitated by water evaporation). It is possible that the generation of certain aroma compounds takes place during alkalisation, however it is outbalanced by the degradation or stripping.

*Impact of roasting on aroma content*

Roasting is considered as an important step for flavour development during cocoa processing. Several studies have shown significant increase of *Maillard* derived odorants upon cocoa roasting [2-5].

In order to evaluate changes of odorants during roasting, aroma content of alkalized nibs before and after the roasting was compared. Surprisingly, roasting of alkalized cocoa nibs had only limited and for majority of the odorants negative impact on the aroma content (Figure 2). More significant changes were detected only for 2,3-pentanedione that decreased by 63% and for dimethyltrisulfide that increased by 77% after the roasting.

Our results are in contradiction with literature data [2-4]. The reason for this could be that published data come exclusively from the trials conducted in a laboratory scale (either with a small coffee roaster or with an oven). In order to understand this phenomenon, a small scale roasting employing only 1.4 kg of alkalized cocoa nibs and a laboratory drum roaster operating with the same roasting profile as used during the pilot plant roasting was conducted. The results were indeed surprising as after small scale roasting a significant increase of many odorants was detected; especially *Maillard* derived odorants such as Strecker aldehydes, HDMF, dimethyltrisulfide and pyrazines increased by 2 to 10 folds.

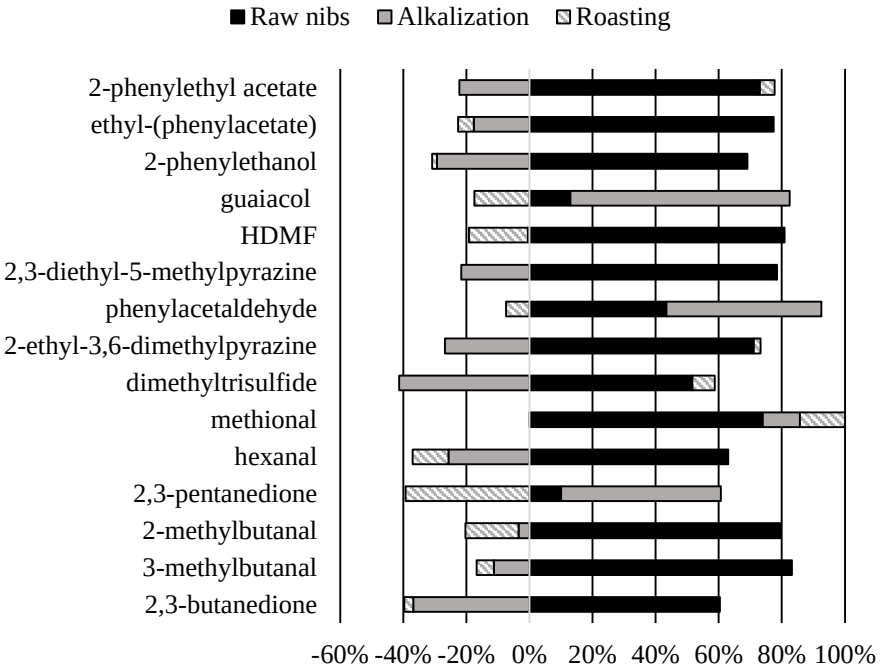


**Figure 2:** Origin of odorants in alkalized-roasted cocoa nibs: relative contribution of intrinsic aroma content of raw nibs and two processes alkalization and roasting

Small scale roasting also resulted in a substantial decrease of esters. Obtained results thus indicate that larger scale roasting influences the flavour development in a much lower extent as compared to small scale roasting (scale-up effect). This is likely linked to heat transfer that depends on batch size and type of heating (conductive heating in a small roaster versus convective heating in a pilot plant roaster).

*Intrinsic aroma content in raw nibs*

Carry over from raw cocoa nibs had by far the highest impact on the aroma of alkalized-roasted nibs (Figure 2). This finding was especially surprising for *Maillard* derived odorants whose formation typically requires higher temperatures. There are theoretically four ways how these odorants can originate in raw cocoa nibs: (i) biosynthesis in cocoa plant, (ii) fermentation, (iii) drying and (iv) preheating before breaking and winnowing. Temperature during fermentation and drying is rather low (between 25°C to 65°C), but both processes can last very long (up to eight days each),

thus the formation of odorants by *Maillard* reaction cannot be excluded. In our study, the focus was put on pre-heating before breaking and winnowing as the most likely process for generation of *Maillard* odorants. During preheating cocoa beans are exposed to temperatures between 90 to 100°C for 20 to 30 min. These thermal conditions are comparable to those applied by Frauendorfer & Schieberle [2] in their roasting study. The authors reported that roasting of cocoa beans in a laboratory coffee roaster (95°C/14min) triggers significant increase of *Maillard* odorants. The highest increase was reported for phenylacetaldehyde (85 folds), HDMF (71 folds) and 3-methylbutanal (21 folds).

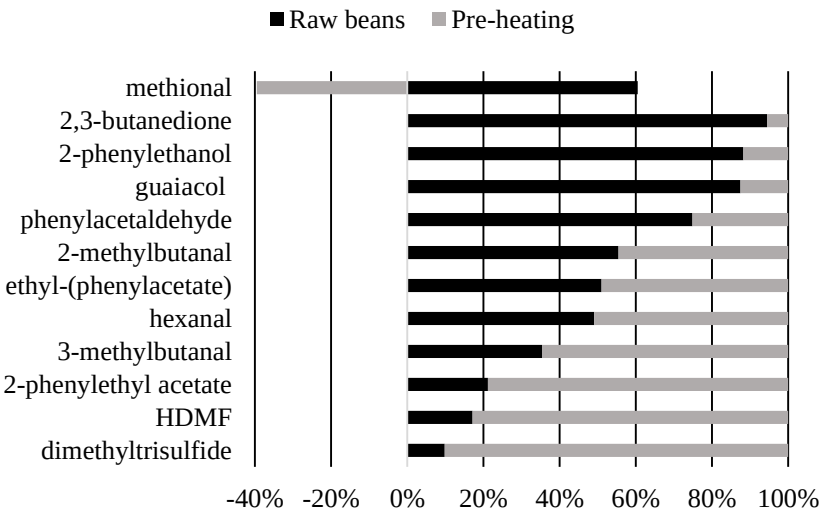


**Figure 3:** Origin of odorants in cocoa nibs: relative contribution of intrinsic aroma content in raw cocoa beans and pre-heating applied during breaking and winnowing

Inspired by this finding the fermented and dried cocoa beans and de-shelled cocoa nibs produced from the same batch were sampled and analysed. Aroma content in both samples was compared in order to evaluate origin of odorants in the nibs (Figure 3). The sniffing of both samples already pointed out the huge difference in aroma quality (non-heated beans possessed beany and earthy notes, while the nibs had already a strong cocoa aroma). The analytical results indeed confirmed that pre-heating applied during breaking and winnowing leads to substantial increase of the odorants, yet intrinsic aroma of raw cocoa still contributes significantly. The odorants in the nibs can be classified into those predominantly carried over from raw cocoa including 2,3-butanedione, 2-phenylethanol, but surprisingly also some Strecker aldehydes like phenylacetaldehyde and 2-methylbutanal and those that are predominantly generated during the preheating including 3-methylbutanal, 2-phenylethylacetate, HDMF and dimethyltrisulfide. Methional was the only odorant whose amount decreased during the preheating.

*Distribution of odorants between cocoa powder and cocoa butter after pressing*

The relative distribution of odorants between cocoa powder and cocoa butter after the pressing of cocoa liquor is depicted in Figure 4. The distribution was established from odorant concentration determined in cocoa powder and cocoa butter and mass ratio between these two products. The distribution was driven mainly by the polarity of the odorants. More polar odorants like Strecker aldehydes, diketones and HDMF were

predominantly retained in the powder, while less polar odorants like esters, pyrazines, dimethyltrisulfide and hexanal were predominantly retained in butter. The distribution of the odorants after the pressing is a final step that determines the concentration ratios between the odorants and consequently flavour signatures of both cocoa powder and cocoa butter.

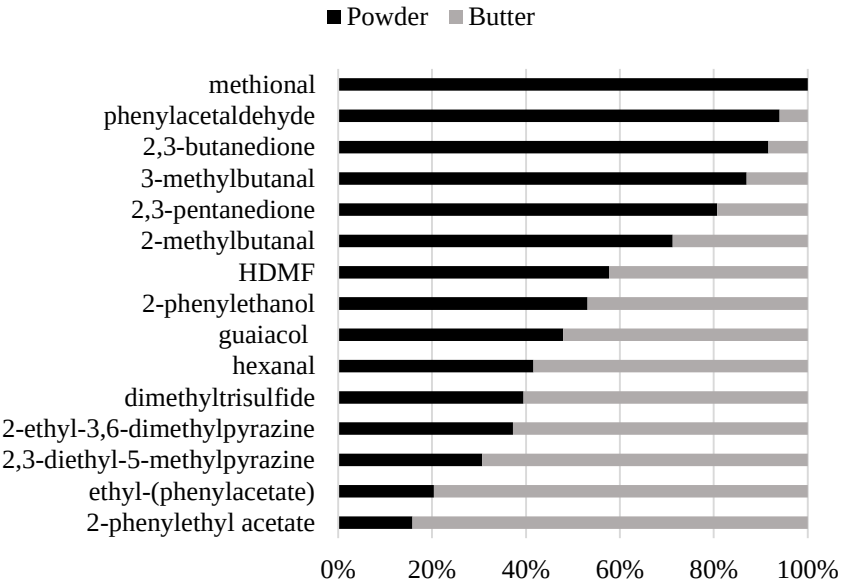


**Figure 4:** Relative distribution of odorants between cocoa powder and cocoa butter after pressing cocoa liquor

In conclusion, the intrinsic aroma content of raw cocoa beans together with preheating before breaking and winnowing appeared to be the most important source of odorants in cocoa powder produced from Ivory Coast cocoa. Roasting and alkalization surprisingly showed low impact on Maillard derived odorants probably due to the depletion of precursors during preliminary processing. Distribution of the odorants after cocoa pressing is driven by the polarity of the odorant. More studies are required to understand the generation of *Maillard* derived odorants during post-harvest processing such as fermentation and drying as well as the impact of cocoa origin.

## References

1. Frauendorfer, F. and Schieberle, P. (2006) J. Agric. Food Chem., 54(15): p. 5521-5529.
2. Frauendorfer, F. an, Schieberle, P. (2008) J. Agric. Food Chem., 56(21): p. 10244-10251.
3. Huang, Y. and Barringer, S.A. (2010) J. Food Sci., 75(1): p.C121-C127.
4. Huang, Y. and Barringer, S.A. (2011) J. Food Sci., 2011. 76(2): p.C279-C286.
5. Afoakwa, E.O., Paterson, A., Fowler, M. and Ryan, A. (2008) Crit. Rev. Food Sci. Nutr., 48(9): p. 840-857.

# Flavour release from wine glycosides during tasting

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## Abstract

Grape-derived glycosides contribute some of the most important aroma characteristics to wine, with volatiles released from glycosides during vinification. Wine can retain high concentrations of these non-volatile flavour precursors. Juice and wine made from aromatic varieties such as Gewürztraminer and Riesling are particularly rich in glycosides of the monoterpenes geraniol, linalool, nerol and α-terpineol. Glycosides from these varieties were extracted and purified to remove phenolics and free volatiles, and extensively characterised. GC/MS analysis following enzyme hydrolysis, hydrolysis by human saliva, and analysis of breath after tasting glycosides showed that monoterpene glycosides can release monoterpenes upon hydrolysis *in vitro* and *in vivo*. The possibility that hydrolysis could contribute to flavour via retronasal odour perception was investigated in a series of sensory experiments. Time-intensity sensory studies showed that *fruity* flavour resulted from assessors tasting glycosides at elevated concentrations. The effect was not significant at wine-like concentrations. There was substantial variability in response to glycosides, and a study of 39 people and several glycosides showed that 77% could detect flavour from at least one glycoside. This study provided evidence that non-volatile glycoconjugates can contribute previously unrecognised flavour during tasting, as well as contributing to aftertaste, a sought-after aspect of wine quality. Following on from these experiments, wines were made with additional glycosides extracted from grape skins. The addition of glycosides increased *floral*, *fruity* and *confectionary* aromas and flavours. Floral aftertaste was especially increased for those panellists who were tested as perceiving flavour from geranyl glucoside.

## Introduction

Austria and Australia are home to some of the world's best regarded Riesling wines [1], notable for their floral, citrus, perfumed and fruity aroma. Wine grapes generally contain low concentrations of free volatiles, and it is accepted that varietal flavour predominantly arises from non-volatile precursors in the grapes, with the main classes being glycosides of volatiles possessing an alcohol functional group, and amino acid conjugates of volatile thiols [2-4]. Free volatiles are present in some grapes, such as monoterpenes in Muscat grapes, rotundone in Shiraz, and methoxypyrazines in Cabernet Sauvignon and Sauvignon Blanc. However, these are exceptions rather than the rule.

Glycosides and other precursors are readily transferred from the grapes into wine, where hydrolysis can occur through the enzyme or acid hydrolysis [5].

Studies have shown that precursors can hydrolyse in the human mouth, including thiol precursors [6], and hexyl glucoside [7]. Glycosides hydrolysed in-mouth have also been shown to be important to the smoky flavour of wines made from grapes exposed to bushfire smoke, although in this case the concentration of smoke-related phenol glycosides is unusually high [8].

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The human oral cavity is a complex system, with many factors influencing sensory perception, especially retronasal perception of odorants from food. Large inter-individual variability has been observed in factors such as oral microbiota, salivary flow rate, saliva composition, breathing and swallowing behaviour [9-12].

For wine glycosides to impart retronasal odour, they must be present in sufficient concentrations, even after swallowing or expectorating the sample, and then be hydrolysed in the mouth cavity. The released odorant must then travel via the retronasal route to the olfactory cleft where it can be perceived by olfaction if the concentration is high enough. And these steps must happen quite quickly to be noticed as a part of the flavour of the wine, in the first 30 seconds to 2 minutes after consuming the wine.

The hypothesis for this research is that grape-derived odourless aroma precursors in wine can be hydrolysed to release odorants in the mouth, leading to perceivable retronasal odour.

## Experimental

*Wines and chemicals*

A Riesling wine from Eden Valley, a Gewürztraminer wine from Goulburn Valley and a Gewürztraminer juice from Adelaide Hills were chosen for the study. Geranyl glucoside, guaiacol glucoside and $d_2$-geranyl glucoside were synthesised in-house. Gewürztraminer marc from Eden Valley and Riesling juice from Adelaide were used for the winemaking study.

*Glycoside extraction, purification and characterisation*

Glycosides were extracted using polymeric resin Amberlite FPX66, and purified to remove phenolic glycosides and volatile impurities. Glycosides were incubated with a commercial enzyme preparation with a wide glycosidase activity, Lallzyme beta, and glycosides were also incubated with whole fresh saliva used within one hour of sampling. The volatiles released were measured using HS-SPME-GC-MS. Glycosides, including monoterpene glucosides, pentosylglucosides and rutinosides were also quantified directly using LC-MS-MS. Experimental details have been previously reported [13].

*Sensory time-intensity studies*

Glycosides from Gewürztraminer juice and wine were assessed by sensory analysis at five times the original concentration in the juice or wine, in model wine with 10.7% v/v aqueous ethanol and pH 3.50. Preliminary sensory assessment of the aroma of the glycosides confirmed the absence of *fruity* or *floral* aroma. Geranyl glucoside was also included in the study, at 3,080 µg/L. A panel (n=11, eight females) was recruited from AWRI staff with at least two years' wine descriptive analysis experience. Details of the panel training and sensory methods have been previously reported [13]. A second study assessed Riesling and Gewürztraminer volatiles and glycosides at wine-like concentrations, using a different sensory panel (n=11, five females), all of whom had previous sensory analysis experience, and none had participated in the first time-intensity experiment

For both studies, *overall fruit* flavour (defined as citrus, floral, stone fruit and confectionary-like) was then rated continuously using FIZZ data acquisition software, over a period of 120 s. Samples were presented monadically, with a forced rest of at least ten minutes between each sample in the formal sessions. All sensory data were obtained in compliance with institutional ethical procedures for sensory evaluation, involving risk

assessment and informed consent, and all samples were expectorated. Fisher's least significant difference (P=0.05) was calculated using analysis of variance of the maximum intensity.

*Inter-individual variability in response to a range of glycosides*

Thirty-nine people (18 males) experienced in wine sensory evaluation were assessed for their ability to perceive flavour from three different glycosides assessed individually in water: geranyl glucoside (3,080 µg/L), glycosides isolated from Gewürztraminer wine (2,930 µg/L), and guaiacyl glucoside (500 µg/L). A water control sample was also evaluated. The participants were instructed to hold the entire sample in the mouth for five seconds, then expectorate and rate *floral/fruity flavour, smoke/medicinal flavour* and, if needed, *other flavour*, rinse with water, and then rest for two minutes before the next sample. Individual judge responses for flavour attributes *fruity/floral* and *smoky* were examined using analysis of variance with P<0.15, compared to the water blank. Those with a significant response to a glycoside were classed as tasters of that glycoside.

*Winemaking with added glycosides from Gewürztraminer marc*

Glycosides were extracted from Gewürztraminer marc (the skin, stem and seed by-product of grape juice production) and purified using a polymeric resin column to remove phenolic compounds. The glycosides were added to Riesling juices at 0.4 g/L ('juice add') and to the wine at 0.4 g/L at bottling ('wine add'). The wines were fermented in 20 L stainless steel containers, in duplicate.

*Sensory descriptive analysis of wines made with added glycosides*

A panel of AWRI staff members with previous wine sensory experience (n=11, five females, six tasters of geranyl glucoside) was convened and a consensus-based descriptive methodology was used as described previously [14]. Nine aroma, twelve flavour and five aftertaste attributes were rated using an unstructured 15 cm line scale. The wines were assessed in duplicate over four days of formal sessions. All samples were expectorated, and there were forced one-minute rests between samples and a ten-minute rest after the fifth sample.

Analysis of variance assessed the effects of wine, judge (random effect), replicate, variety, and the corresponding two-way interactions. The least significant difference (Fisher's, 95% confidence) was calculated using Minitab 18.

## Results and discussion

*Chemical characterisation of extracted glycosides*

Glycosides from Gewürztraminer juice and wine liberated geraniol and other monoterpene alcohols when hydrolysed with enzyme and whole fresh saliva. Direct analysis of the glycosides using LC-MS-MS confirmed the presence of geraniol glucoside as the major glycoside present, with minor components of monoterpene glycosides of diverse structures, including glucosides, pentosylglucosides and rutinosides.

*Sensory time-intensity studies*

Gewürztraminer wine glycosides tasted at five times original concentration gave a fruity flavour with onset approximately 7 s after the sample was taken into the mouth, reaching maximum intensity at 22 s, and lasting until 52 s. Gewürztraminer juice glycosides had a slightly longer delay with flavour onset at 12 s, reaching maximum intensity at 31 s and lasting until 80 s. Pure geranyl glucoside had a similar profile with a slightly

earlier flavour onset. Close examination of the individual panellist data revealed that only six of the eleven panellists were consistently rating the flavour effect.

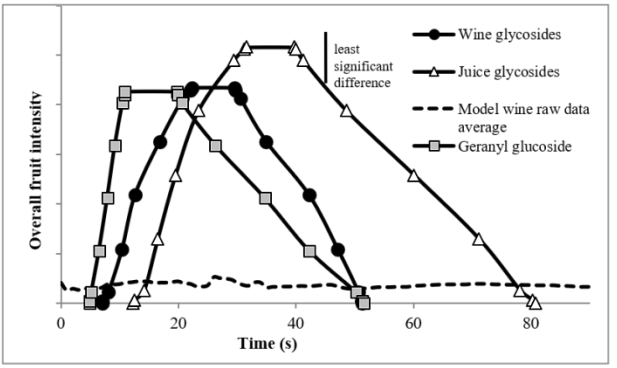


**Figure 1**. Mean time intensity curves for 'overall fruit' flavour intensity, generated by extracting parameters from individual raw data curves from 11 judges x 3 replicates for the three samples with added glycosides assessed in model wine (10% v/v ethanol, pH 3.50). Gewürztraminer wine and juice glycosides were tasted at five times original concentration, geranyl glucoside at 3,080 µg/L. The Fisher's least significant difference value (P=0.05), calculated from the maximum intensity data for the effect of sample, is also shown. (Originally published in AWRI Technical Review issue 214, 2015, with minor modifications.)

There was no significant flavour effect from the Riesling or Gewürztraminer glycosides when tasted at wine-like concentrations in model wine, in the presence or absence of wine volatiles for the sensory panel mean. However, close examination of the individual panellist responses revealed that five panellists out of the twelve responded to some of the glycosides in the study. In the first sensory study six out of eleven panellists perceived flavour. Perhaps only half of the population can perceive flavour from glycosides?

*Inter-individual variability in response to a range of glycosides*

There was large inter-individual variation in response to glycosides (Figure 2), with 77% responding to one or more of the glycosides. Some people responded to all three glycosides, some people responded to two of the glycosides, some responded to one of the glycosides, and some responded to none. Overall, 54% of the panellists rated a significant response to the Gewürztraminer glycosides, 46% rated a significant response to geranyl glucoside, and 64% rated a significant response to guaiacyl glucoside.

*Winemaking with added glycosides from Gewürztraminer marc*

Glycoside additions increased the concentration of geranyl glucoside and free monoterpenes in the resulting wines, regardless of whether the glycosides were added to the juice or to the wine. The concentration of geranyl glycoside increased by more than 2,000 µg/L, and linalool increased by approximately 50 µg/L, presumably due to hydrolysis during the winemaking process, followed by rearrangement of the monoterpene alcohols in the acidic wine matrix.

*Fruity, floral* or *confectionary* aroma and flavour attributes were boosted by the glycosides. Six of the eleven panellists in this study were separately assessed as able to detect flavour from geranyl glucoside, and the tasters rated much higher floral aftertaste in the wines with glycoside additions than the non-tasters. Bitterness was not significantly higher in the glycoside addition wines.

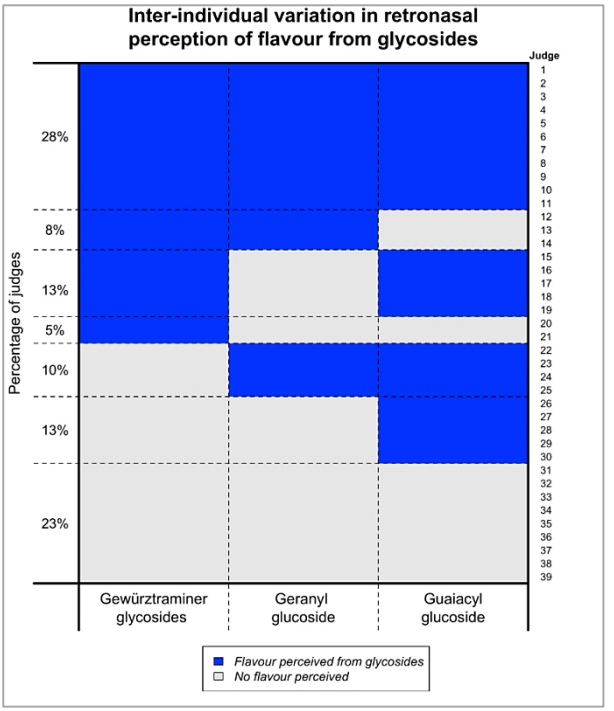


**Figure 2:** Response to various types of glycosides tasted individually in water. Gewürztraminer wine glycosides tasted at five times original concentration, geranyl glucoside at 3080 µg/L, guaiacyl glucoside tasted at 500 µg/L. n=39 people, triplicate presentations, ANOVA 0.15 significance.

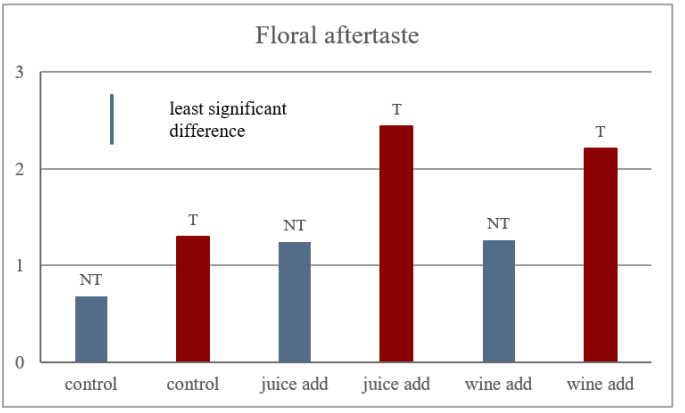


**Figure 3:** Floral aftertaste intensity mean score of Riesling wines made with single additions of glycosides from Gewürztraminer marc, added to the juice before fermentation (juice add), or to the wine at bottling (wine add). The panel was divided into two groups, those who had a significant ($p<0.15$) flavour response to geranyl glucoside in water at 3080 µg/L, who were labelled as tasters (T) (n=6), and nontasters (NT) (n=5) who did not have a significant flavour response to the geranyl glucoside. The Fisher's least significant difference value (P=0.05), calculated for the effect of wine, is also shown.

Overall, monoterpene glycosides were shown to break down in the mouth and contribute to flavour by retronasal perception of the released volatile odorants when tasted

at elevated concentrations. The effect was not significant when tested at wine-like concentrations, and large inter-individual variability was observed. A small survey of 39 individuals and three types of glycosides showed most people (77%) were capable of detecting flavour from some glycosides. Additions of glycosides from Gewürztraminer marc increased *fruity* and *floral* aromas and aftertaste in Riesling wines. The *floral* aftertaste was enhanced for panellists able to taste geranyl glucoside, even in the control wine, providing evidence that release of glycosides in-mouth is an important part of the sensory experience for many people.

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## References

1. Robinson, J., Harding, J. and Vouillamoz, J. (2012). Wine Grapes: A Complete Guide to 1,368 Vine Varieties, including their Origins and Flavours. New York: Harper Collins.
2. Robinson, A.L., Boss, P.K., Solomon, P.S., Trengove, R.D., Heymann, H., and Ebeler, S.E. (2013). American Journal of Enology and Viticulture, 65(1): p. 1-24.
3. Hjelmeland, A.K. and Ebeler, S.E. (2014). American Journal of Enology and Viticulture, 66(1): p. 1-11.
4. Dimitriadis, E. and Williams, P.J. (1984). American Journal of Enology and Viticulture, 35(2): p. 66-71.
5. Loscos, N., Hernández-Orte, P., Cacho, J. and Ferreira, V. (2010). Food Chemistry, 120(1): p. 205-216.
6. Starkenmann, C., Le Calve, B., Niclass, Y., Cayeux, I., Beccucci, S., and Troccaz, M. (2008). Journal of Agricultural and Food Chemistry, 56(20): p. 9575-80.
7. Hemingway, K.M., Alston, M.J., Chappell, C.G., and Taylor, A.J. (1999). Carbohydrate Polymers, 38(3): p. 283-286.
8. Parker, M., Osidacz, P., Baldock, G.A., Hayasaka, Y., Black, C.A., Pardon, K.H., Jeffery, D.W., Geue, J.P., Herderich, M.J. and Francis, I.L. (2012). Journal of Agricultural and Food Chemistry, 60(10): p. 2629-37.
9. Buettner, A. and Beauchamp, J. (2010). Food Quality and Preference, 21(8): p. 915-924.
10. Muñoz-González, C., Feron, G., Brulé, M. and Canon, F. (2018). Food Chemistry, 240: p. 275-285.
11. Muñoz-González, C., Cueva, C., Ángeles Pozo-Bayón, M. and Victoria Moreno-Arribas, M. (2015). Food Chemistry, 187: p. 112-9.
12. Avila, M., Ojcius, D.M. and Yilmaz, O. (2009). DNA Cell Biol, 28(8): p. 405-11.
13. Parker, M., Black, C.A., Barker, A., Pearson, W., Hayasaka and Y., Francis, I.L. (2017). Food Chemistry, 232: p. 413-424.
14. Bindon, K., Holt, H., Williamson, P.O., Varela, C., Herderich, M. and Francis, I.L. (2014). Food Chem, 154: p. 90-101.

# A saliva reactor to mimic *in-vivo* aroma release from flavoured ice-creams

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## Abstract

The aim of the present study was to develop a saliva reactor allowing temperature control and the addition of human saliva in order to follow the release of five aroma compounds from different ice creams. The developed method was a useful tool to mimic the in-mouth process by taking into account sample quantity, mouth volume, temperature, salivary flux, and mastication. The reactor was fit with solid phase micro-extraction for gas chromatography allowing data collection similar to nose-space sampling. Different ice creams were assessed, with varying fat type and level, and protein level. The results showed that the effect of saliva is relatively low and only observed at the higher fat level. Also the effect of the fat type was smaller than that of the fat level. The ice cream with a low fat level released more hydrophobic aroma compounds than the one with a high fat level. The ice creams with both low fat level and low protein level, showed the highest release of aroma compounds. Less added proteins led to less interaction with the aroma compounds and increased their rate of release from the aqueous to the vapour phase. Overall, an innovative tool was provided to guide food industries to reformulate ice creams answering nutritional recommendations in line with consumer demands.

## Introduction

The consumption of ice cream is highly determined by its overall sensory acceptability, mainly flavour perception.

During consumption, ice cream undergoes phase changes from semi-solid to liquid, due to the combined actions of temperature increase and dilution with saliva, before swallowing [1]. In water and oil model systems, the addition of artificial saliva modifies the air/liquid partitioning of aroma compounds [2], inducing either a retention or a salting out effect. This effect has not been explored yet in real food emulsions. Even if some general trends of flavour release from ice cream during eating have already been reviewed [3] there is still a need for a better understanding of the relative impact of fat level, fat type and protein content on aroma release from ice creams, taking into account thermal exchanges occurring in the mouth and the effect of human saliva. A device simulating the retronasal aroma release was developed by Robert and Acree (1995) [4] in order to mimic *in vivo* aroma release of a model wine with artificial saliva. Later in 2001, Deibler *et al.* showed that the ratios of aroma compounds from this device were closely related to those from the subjects' breath [5]. More recently, a saliva reactor has been developed within our research group to mimic the in-mouth breakdown of fat spreads [6], which highlighted the impact of human saliva on aroma release. The aim of the present paper is to adapt the saliva reactor to mimic ice cream consumption in order to determine the effect of fat type, fat level and protein level on aroma release in conditions as close as possible to human

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consumption. The effect of fat type and fat level on either aroma release or sensory perception in ice creams has been the subject of different studies, realised under *in vitro* conditions without addition of human saliva, showing an effect of fat type [7], fat level [8] or protein type and level [9] on aroma release. However, none of these studies combined these effects with that of saliva and they were not realised on the same aroma compounds which renders the comparison of the results difficult even if some general trends are common. An increase in fat level decreases the release of hydrophobic aroma compounds [10]. The nature and amount of protein in the ice cream will change the structure of the emulsion by modifying the interfacial properties and the fat droplet agglomeration in the emulsion [11]. and thus impacting the rate of transfer of aroma compounds from oil to water and then from the emulsion to the gas phase [12].

Our aim was therefore to design an experimental protocol with the saliva reactor to reproduce the thermal exchanges occurring in the mouth during ice cream consumption and worked with a pool of human saliva. The reactor was then used to determine the combined effects of food composition and human saliva on the release of aroma compounds from ice creams. This work will provide innovative tools to guide food industries to reformulate ice creams answering nutritional recommendations such as less fat, more sustainable fat and protein type with a limited effect on aroma release and, thus, on perception.

## Experimental

### *Saliva reactor*

A saliva reactor cell was used to reproduce ice cream breakdown in the mouth as close as possible (Figure 1). This device was specifically designed to evaluate the particular role of saliva during liquid and semi-solid food consumption [6]. It was composed of a water-jacketed glass flask (250 mL), which allowed a temperature control of the sample, equipped with four orifices, one for the temperature sensor, two others to introduce the sample and the SPME fibres and the last one equipped with a 3-blade marine propeller with digital speed control.

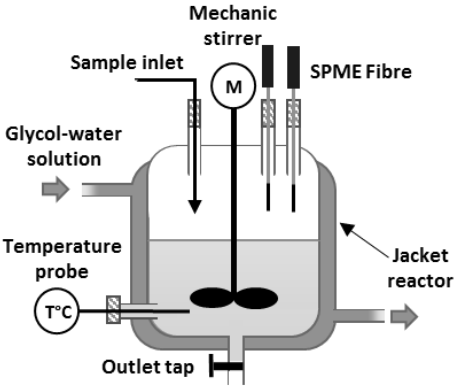


**Figure 1:** Schematic diagram of the saliva reactor

### *Samples composition*

The study was done with different samples of ice creams realised with two fat types (A and B) varying in their solid fat content (SFC). Fat A had 83.3% and 39.6% SFC and fat B, 0.9% and 0.1% SFC at the temperatures of 10°C and 20°C. Each fat type was added

at two different fat levels (L for low = 3 %; H for high = 9 %). The ice creams contained two different levels of skimmed milk powder enriched with whey protein (level 1: standard - SMP: 6.4% Whey: 2.3%; level 2: low - SMP: 3.2% Whey: 1.15%). They were flavoured with a mixture of 5 aroma compounds (acetoin: 450 mg/Kg ice cream; vanillin: 550 mg/Kg; benzaldehyde: 18 mg/Kg; hexanal: 54.9 mg/Kg; ethyl octanoate: 18 mg/Kg). To study the impact of human saliva on aroma release the experiments were realised after diluting the samples in either ultra-pure water (MilliQ®, Bedford, MA) (W) or human saliva (S). Thus, a total of 16 samples were analysed.

*Human saliva collection*

Resting human saliva was collected, centrifuged and stored from 20 volunteers as already described by Poette *et al.* [13]. It should be noted, however, that in a previous study, no effect of saliva storage was observed on the retention of 2-heptanone and ethyl heptanoate by human saliva [14].

*Solid phase micro-extraction – gas chromatography – mass spectrometry (SPME-GC-MS) analysis*

Two fibres were introduced into the reactor (each in one orifice) to follow the aroma release, and were exposed 25 sec. after the introduction of the ice cream, which corresponds to the time at which the mixture reaches the minimum temperature (-22°C). Extraction was then performed for 1 minute. All experiments were realised in triplicate.

SPME fibres were injected in splitless mode (250ºC, 5 min) in a Gas Chromatograph (Agilent 6890N) coupled to a quadrupole Mass Detector (Agilent 5973N). After desorption of the SPME fibre, volatile compounds were separated on a DB-Wax polar capillary column (30 m × 0.25 mm i,d×0.50 µm film thickness) from Agilent (J&W Scientific, Folsom, USA). Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 40ºC, then increased at a rate of 5ºC/min until 240ºC and held for 10 min. The fibres were regenerated 15 min at 240°C before novel use.

For the MS system, the temperatures of the transfer line, quadrupole and ion source were 250ºC, 150ºC and 230ºC, respectively. Electron impact mass spectra were recorded at 70 eV ionization voltage and the ionization current was 10 µA. The acquisitions were performed in Scan mode (from 29 to 350 amu). The semi-quantification was done on the peak areas. However, the linearity of the peak area as a function of aroma concentration was previously verified by doing a calibration curve using 7 concentrations of the 5 aroma compounds diluted in a model emulsion.

*Statistical analysis*

The statistical analyses were done on the GC peak areas for each aroma compound after headspace SPME-GC-MS in the different ice cream samples. Data were subjected to univariate analysis of variance (ANOVA – $\alpha$=0.05) and the [Student]-Newman-Keuls Procedure (SNK) mean comparison test was performed separately in water and saliva, to determine significant differences between the foods matrices for each aroma compound. Microsoft® Excel 2010/XLSTAT©-Pro (2013.4.03, Addinsoft, Inc., Brooklyn, NY, USA); was used for statistical evaluation.

## Results and discussion

*Experimental protocol design in the saliva reactor*

The amount of water/saliva to be added to the reactor and the temperature changes of the ice cream was estimated from preliminary tests with a panel of 10 volunteers. As

an average of 1.6 g of saliva was produced by consuming 8 g of ice cream and considering that 50 g was the minimum amount of ice cream needed in the reactor for a good stirring, 10 mL of water/saliva were transferred into the reactor (250 mL), which was kept at 37ºC, and then 50 g of ice cream (at -22°C) were added and the mixture stirred (400 rpm; maximum available speed in this device). The temperature of the mixture in the reactor decreased from 37ºC to 15ºC after 25 sec. which follows the temperature decrease in the mouth after the introduction of the sample (oral-phase) then the jacket of the reactor was warmed-up in order to increase to 15°C which corresponds to the swallowing temperature of the mixture after 80 sec. (Figure 2).

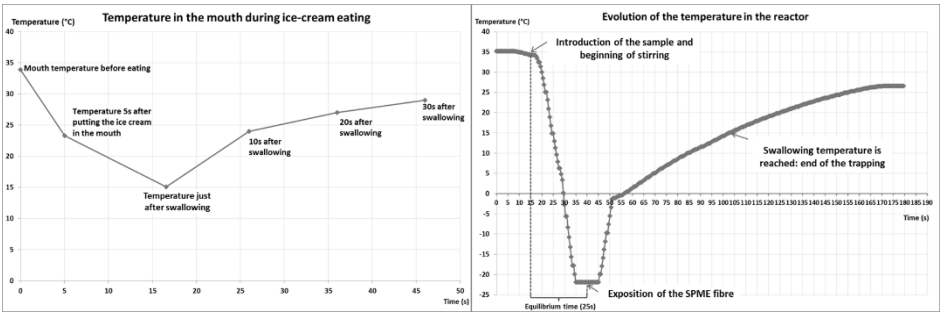


**Figure 2:** Temperature evolution in the mouth (left) and in the reactor (right)

*Effect of ice cream composition and saliva addition on total amount of aroma release*

An analysis of variance was performed (Table 1) with 4 factors (medium, fat type, fat level and protein level).

**Table 1:** ANOVA test on the effect of saliva, fat type fat and protein level on the total amount of release for 5 aroma compounds (univariate tests of Significance - $\alpha$=0.05)

| | | Acetoin | Vanillin | Benz aldehyde | Hexanal | Ethyl octanoate |
|---|---|---|---|---|---|---|
| | logP | -0.66 | 1.21 | 1.5 | 1.78 | 3.8 |
| Medium | F | 0.779 | 1.572 | 0.028 | 1.042 | 0.034 |
| | P-value | 0.390 | 0.228 | 0.870 | 0.323 | 0.856 |
| | Factor effect | - | - | - | - | - |
| Fat type | F | 0.020 | 7.945 | 0.036 | 302.876 | 34.394 |
| | P-value | 0.889 | 0.012 | 0.852 | < 0.0001 | < 0.0001 |
| | Factor effect | - | **B<A** | - | **B<A** | **B<A** |
| Fat level | F | 4.942 | 42.422 | 28.083 | 148.240 | 235.247 |
| | P-value | 0.041 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| | Factor effect | **L<H** | **H<L** | **H<L** | **H<L** | **H<L** |
| Protein level | F | 4.215 | 4.816 | 4.674 | 0.227 | 18.486 |
| | P-value | 0.057 | 0.043 | 0.046 | 0.640 | 0.001 |
| | Factor effect | **1<2** | **1<2** | **1<2** | - | **1<2** |

The 5 aromas are sorted by increasing logP and three parameters are presented: F-test, P-value and factor effect highlighting composition impact on each aroma.

The effect of human saliva seems negligible in comparison to that of the fat type, fat content and protein content. This might be explained by the fact that our work was conducted on clarified saliva and a recent paper showed that the effect of human saliva on the metabolism of aroma compounds, mainly aliphatic aldehydes and di-ketones, was reduced after centrifugation [15]. However, in that study, no such effect was observed for

alcohols, aliphatic ketones and benzaldehyde and the other aroma compounds present in our ice cream, which allows us to conclude that our results are fairly representative of the mechanisms in the mouth. Interactions between salivary proteins and aroma compounds in water was observed in previous studies and it might be modified here in emulsions containing fat and other proteins [2, 14].

Changing the nature of fat modified the release profile. Fat type is significant for the most hydrophobic aroma compounds (logP > 1.7 hexanal and ethyl octanoate; p-value < 0.001). This might be explained by a higher release of hydrophobic compounds from matrices with a greater percentage of SFC at 15°C (cannot solubilise the aroma compounds) [16]. A significant effect of the fat level (p-value < 0.001) was observed for the majority of the aroma compounds. Decreasing fat content led to a higher release for hydrophobic aroma compounds (logP > 1). This is probably due to a high solubility of hydrophobic aroma compounds in fat (more retained) [9b]. A significant effect of protein level (p-value: < 0.05) was observed for 3 volatiles and they are less released from ice creams with a high protein content (protein level 1).

*Effect of ice cream composition and saliva addition on the initial rate of aroma release*

The aim of this part was to determine if the modifications observed on the total amount of aroma release during the eating process were initiated at the beginning of the eating process. This study was conducted on four selected samples (WAH2, SAH1, WAL1, and WAH1 as the reference). Figure 3 represents the percentages of increase/decrease in the rate of release (between 0 and 100 seconds) as a function of WAH1 for the 3 other samples.

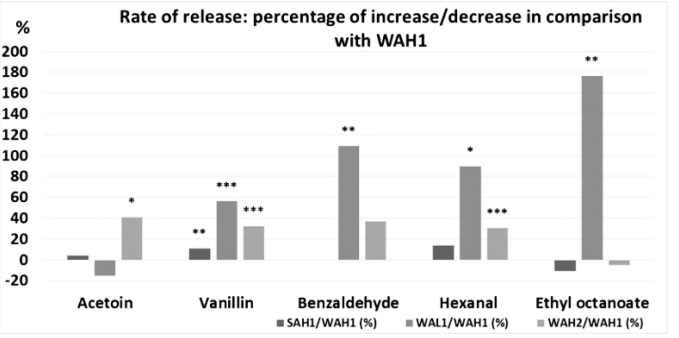


**Figure 3:** Impact of medium, fat type, fat level and protein level on the rate of release (The increase/decrease is significant at: *** p-value < 0.0001 ; ** p-value < 0.001 ; * p-value < 0.05)

A significant impact of saliva was observed for vanillin leading to an increase of the rate of release with saliva (SAH1/WAH1; p-value < 0.001). This compound might be more sensitive to a salting-out effect of salivary proteins [2]. Less fat (WAL1/WAH1) led to a significant better rate of release for hydrophobic (logP > 1) aroma compounds. Confirming that hydrophobic aroma compounds are more retained in fat. A decrease in protein level (WAH2 vs WAH1) induced a significant increase in the rate of release. Less added proteins lead to less interaction with the aromas and increase their rate of release from the aqueous to the vapour phase.

*Discussion and conclusion*

As a conclusion, the saliva reactor was a simple and useful tool to mimic the in-mouth process by taking into account sample quantity, mouth volume, temperature,

salivary flux, and mastication. Connecting the reactor with the use of SPME makes the technique easy to use, and provides data similar to nose-space sampling.

An ANOVA test on the collected data highlighted the different effects of composition on aroma release. The effect of saliva is relatively low and only observed at the higher fat level. The main effect is that of fat level (from 3 to 9%), then the effect of fat type at the higher fat level. The effect of protein level is more significant at the lower fat level. Decreasing fat content in ice cream led to a higher total amount of release for hydrophobic aroma compounds. Changing the nature of fat also modified the release profile, with a higher release of the more hydrophobic compounds from fat with a greater percentage of solid fat at the temperature of eating ($\leq 15°C$). The effect of protein level depends on both fat type and fat level. The level of whey proteins impacted more the aroma release at a low fat level, with a higher amount of aroma released at a low level of protein. However, a small effect was also evidenced at the high level of the fat type with the higher solid fat content. The obtained results showed that the reformulation of ice creams impacts aroma release as a function of fat type, fat level and protein level and also depending on the nature of the aroma compound. The combined effects of fat and protein have also to be taken into consideration.

This *in-vitro* study using a saliva reactor could be easily applied now to study the impact of saliva or reformulation on aroma release. It can potentially provide a big amount of data allowing the computation of a flavour behaviour model in complex liquid or semi-liquid matrices.

## References

1. Salles C., Chagnon M.-C., Feron G., Guichard E., Labouré H., Morzel M., Sémon E., Tarrega A., Yven C. (2011) Crit. Rev. Food Sci. Nutr. 51: 67-90.
2. van Ruth S.M., Grossmann I., Geary M., Delahunty C.M. (2001) J. Agric. Food Chem. 49: 2409-2413.
3. Chung S.J. (2007) Food Science and Biotechnology 16: 8-17.
4. Roberts D.D., Acree T.E. (1995) Chemical Senses 20: 246-246.
5. Deibler K.D., Lavin E.H., Linforth R.S.T., Taylor A.J., Acree T.E. (2001) J. Agric. Food Chem. 49: 1388-1393.
6. Poette J., Lubbers S., Maison B., Andriot I., Pernin K., Guichard E., Cavellec A., Feron G. (2010) In: Advances and challenges in flavor chemistry & biology._Proceedings of the 9th Wartburg Symposium (Hofmann T., Meyerhof W., Schieberle P., ed.), Deutsche Forschungsanstalt für Lebensmittelchemie, Freising (Germany), pp. 386-389.
7. Welty W.M., Marshall R.T., Grün I.U., Ellersieck M.R. (2001) J. of Dairy Sc. 84: 21-30.
8. Miettinen S.M., Piironen V., Tuorila H., Hyvönen L. (2002) J. of Food Sc. 67: 425-430.
9. (a) Friedeck K.G., Aragul-Yuceer Y.K., Drake M.A. (2003) Journal of Food Science 68: 2651-2657; (b) Guichard E. (2002) Food Reviews International 18: 49-70.
10. (a) Doyen K., Carey M., Linforth R.S.T., Marin M., Taylor A.J. (2001) J. Agric. Food Chem. 49: 804-810; (b) van Ruth S.M., King C., Giannouli P. (2002) J. Agric. Food Chem. 50: 2365-2371.
11. Sourdet S., Relkin P., Cesar B. (2003) Colloids and Surfaces B-Biointerfaces 31: 55-64.
12. Druaux C., Voilley A. (1997) Trends in Food Science & Technology 8: 364-368.
13. Poette J., Mekoué J., Neyraud E., Berdeaux O., Renault A., Guichard E., Genot C., Feron G. (2014) Flavour Fragr. J. 29: 39-49.
14. Pagès-Hélary S., Andriot I., Guichard E., Canon F. (2014) Food Res. Int. 64: 424-431.
15. Munoz-Gonzalez C., Feron G., Brulé M., Canon F. (2017) Food Chemistry: in press.
16. Relkin P., Fabre M., Guichard E. (2004) J. Agric. Food Chem. 52: 6257-6263.

# Screening of yeast strains for flavour potential in meat products under reduced concentration of preservative nitrifying agents

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## Abstract

Aroma characteristics of fermented sausages depends on the processing factors such as ingredients and preservatives as well as on the starters used during the fermentative process. Consumers' demand for healthier products is leading to a reduction in the preservative curing agents (nitrate and nitrite) used in the processing of meat products while preventing detriment to sausage aroma. *D. hansenii* yeasts are known contributors to sausage flavour, however little is known about their potential to produce volatiles under reduced concentration of curing agents or its consequences for amino acid metabolism. *D. hansenii* strains isolated from sausages manufactured with different raw materials (meat from pork or llama) were evaluated in a model system resembling the sausage formulation containing free amino acid and additives (salt and glucose) and variable concentrations of nitrite and nitrate. The different ability of the yeast strains to produce volatile compounds from different amino acids and the changes in aroma profile due to nitrifying agents' reduction were evaluated.

## Introduction

The conversion of amino acids, generated through proteolysis during sausage manufacturing [1], into aroma compounds depends largely on microbial metabolism during fermentation where yeasts play an important role [2]. The occurrence of *D. hansenii* as the dominant yeast in a large number of fermentation and ripening processes for production of dry meat products has led to its utilization as starter culture for meat fermentation. Aroma characteristics of the sausages depend not only on the yeast strain used for fermentation but also on the processing factors (raw materials, meat ingredients, preservatives, technological parameters, presence of starter cultures) that can affect the metabolic activity of the yeasts.

Actual trends to reduce the use of preservatives (nitrite and nitrate) in meat products, despite their role in safety and technological properties, has led the industry to look for strategies to maintain safety and quality. However, it is unknown the effect of a reduction in concentration of nitrite and nitrate used as preservatives in fermented sausages on yeast amino acid metabolism and its contribution to generation of volatile compounds. The objective of this study is to search for yeast with flavour production potential in meat products and determine the effect of a reduction in nitrate and nitrite concentration on their amino acid metabolism and volatiles production.

## Experimental

*Yeast strains*

*Debaryomyces hansenii* strains: L1-L9 were isolated from naturally fermented sausages manufactured with pork meat (L1-L6) [3-5] and llama meat (L7-L9) [6].

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*Meat model system*

The meat model system was prepared with a similar composition of dry fermented sausages in terms of additives and amino acid content [7]. The model was prepared using 0.67 % YNB (Yeast Nitrogen Base, Difco Inc.), 30 g/L NaCl, 10 g/L glucose, amino acids in concentration reported by Corral et al. [7] and variable concentrations of $NaNO_2$ and $KNO_3$ as follows: 0.150 g/L each in control medium (C) and 0.128 and 0.113 g/L in media RN15 and RN25, respectively. A total of 11 experiments (50 mL media in 100 mL Erlenmeyer flasks) were carried out using each media C, RN15 and RN25. Nine experiments were inoculated with *D. hansenii* strains and two not inoculated and used as controls before and after incubation [8]. Incubation was at 25°C for 16 d. Experiments were performed in triplicate. After incubations all media were centrifuged and the supernatant recovered for volatile and amino acid content analyses [8].

The free amino acids content was determined by reverse phase HPLC using phenylthiocarbamyl amino acid derivatives according to Aristoy & Toldrá [9] using norleucine (65.6 µg) as internal standard. Quantification of amino acids was done relative to the internal standard and expressed as a percentage of concentration present in the control media before incubation [8].

The volatile analysis was done by SPME-GC-MS using an automatic injector Gerstel MPS2 multipurpose sampler (Gerstel, Germany) and an 85µm CAR/PDMS fibre [8]. Compounds were identified by comparison with mass spectra from the NIST/EPA/NIH Mass Spectral Database, linear retention index and by comparison with authentic standards. Identified volatile compounds were quantified and the abundance expressed as the increase respect to control media after incubation [8].

*Statistical analysis*

Data were analysed using Generalized Linear Model (GML) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). The model included the effect of yeast inoculation as fixed effects and replicates as random effects. Principal component analysis (PCA) was used to evaluate the relationships among aroma compounds, free amino acids and model inoculated media. The inoculated model media represented the difference experiments (L1-L9) carried out using each media C, RN15 and RN25.

## Results and discussion

Nine yeast strains isolated from pork or llama sausages and pertaining to the species *D. hansenii* were screened for their ability to produce volatiles on amino acid rich media containing nitrifying agents used as preservatives in meat products. The most usual nitrite/nitrate concentration used for the elaboration of meat products around Europe were added to the medium (150 ppm) although specific regulations for other traditional European meat products exist [10]. The volatile compounds produced by yeast strains from the degradation of val, ile, leu, met and phe are summarized in Table 1. Statistical analysis revealed a clear difference among yeast strains to produce these volatile compounds from the selected amino acids (Figure 1). Yeasts isolated from llama sausages were characterized by the production of propanoate ester compounds and branched alcohols, while those from pork sausages produced branched aldehydes and acids. Yeast L5, isolated from pork sausages displayed a distinct volatile profile characterized by the presence of ethyl esters derived from methyl branched acids.

**Table 1.** Volatile compounds identified in yeast inoculated model systems after incubation.

| *Volatile Compounds* | *LRI*[1] | *RI*[2] | *Volatile Compounds* | *LRI* | *RI* |
|---|---|---|---|---|---|
| *Valine derived comp* | | | *Leucine derived compounds* | | |
| 2-mehylpropanal | 592 | a | 3-methylbutanal | 689 | a |
| 2-methyl-1-propanol | 682 | a | 3-methylbutanol | 794 | a |
| Ethyl 2-methylpropanoate | 788 | a | Ethyl 3-methylbutanoate | 881 | a |
| 2-methylpropyl acetate | 805 | a | 3-methylbutanol acetate | 906 | a |
| 2-methylpropanoic acid | 862 | a | 3-methylbutanoic acid | 937 | a |
| Propyl 2-methylpropanoate | 896 | a | 3-methylbutanol propanoate | 996 | a |
| *Isoleucine derived compounds* | | | *Phenylalanine derived compounds* | | |
| 2-methylbutanal (58)[3] | 700 | a | Benzaldehyde | 1017 | a |
| 2-methylbutanol | 797 | a | Phenylethyl alcohol | 1194 | a |
| Ethyl 2-methylbutanoate | 878 | a | 2,3-dimethylbenzaldehyde | 1292 | b |
| 2-methylbutanol acetate | 909 | a | 2-Phenylethyl acetate | 1315 | a |
| 2-methylbutanoic acid | 943 | a | 2-Phenylethyl propanoate | 1405 | b |
| 2-methylbutanol propanoate | 999 | a | | | |
| *Methionine derived compounds* | | | | | |
| Dimethyl disulfide | 772 | a | | | |

[1]LRI: Linear Retention Index calculated for DB-624 column. [2]RI: Reliability of identification: (a) mass spectra and LRI in agreement to standard compound, (b) tentatively identified by mass spectra. [3]Target ion in brackets used to quantify the compound when the peak was not completely resolved.

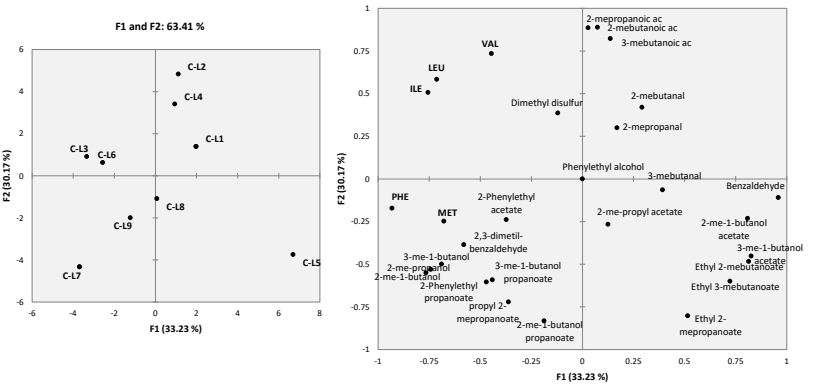


**Figure 1:** Loadings of the first two principal components F1 and F2 of volatile compounds derived from the degradation of amino acids Ile, Leu, Val, Phe and Met in media inoculated with yeast strains (pork strains L1-L6; llama strains L7-L9).

Among the yeast studied, those isolated form llama sausages (L7-L9) and L5 were the highest producers of ester compounds and could be useful to impart specific flavour notes in dry meat products. Ester compounds have been identified in fermented sausages providing fruity aromas and contributing to mask rancid and vegetable cooked odours [11]. In contrast, branched aldehydes, identified in fermented meats as contributors to the overall flavour [12], have been found in sausages inoculated with *D. hansenii* strains [13]. The main branched aldehydes producers were among the pork isolated yeasts (L1-L4) which may also be suitable for fermentative processes.

Regarding the effect of preservative reduction on yeast metabolism, nitrate-nitrite reduction affected the yeast ability to produce volatiles. Particularly, yeasts L1 and L5, isolated from pork sausages, increased the production of branched acids (L1) and ethyl ester compounds (L5), as can be seen in the PCA graph (Figure 2). Until now, there are no reports regarding the effect of nitrate and nitrite reduction on amino acid catabolism of *D. hansenii* strains from meat products. Previous research on other meat starters reported a reduction in leucine catabolism when nitrate and nitrite were added to an experimental model system containing a staphylococcus starter [14]. Under these premises, the current trend to reduce the use of nitrites and adjust their levels in meat

products makes necessary an evaluation of its impact on microbial starters in terms of volatile compounds generation [8].

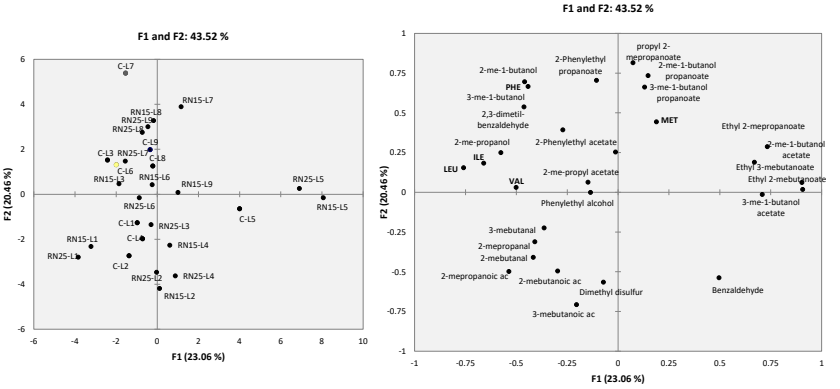


**Figure 2:** Loadings of the first two principal components F1 and F2 of volatile compounds derived from the degradation of amino acids Ile, Leu, Val, Phe and Met in control (C) and nitrifying reduced media RN15 and RN25 media, produced from yeast inoculation (L1-L9).

## Conclusions

Yeast strains isolated from sausages manufactured with different raw materials (meat from pork or llama) have different ability to produce volatile compounds. Yeast amino acid metabolism and production of volatiles are significantly affected by the presence of variable nitrate/nitrite concentrations. The inoculation of selected yeast strains during manufacturing of dry sausages may produce a significant effect on the overall sausage flavour.

### Acknowledgements

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## References

1. Toldrá F and Flores M. (2014) Sausages, types of; Dry and semi-dry. In: Carrick Devine & Michael Dikeman, editors-in-chief. Encyclopedia of Meat Sciences 2e, Vol. 3, Oxford, Elsevier pp 248-255.
2. Flores, M., Corral, S., Cano-García, L., Salvador and A., Belloch, C. 2015 Int J Food Microbiol 212: 16-24.
3. Bolumar, T., Sanz, Y., Flores, M., Aristoy, M. C., Toldrá, F., and Flores, J. Meat Sci., 2006, 72, 457–466.
4. Durá, M. A., Flores, M., and Toldrá, F. Food Chem., 2004, 86, 91–399.
5. Cano-García, L., Flores, M., and Belloch, C. (2013). Food Res Int 52: 42–49.
6. Mendoza, L.M., Padilla, B., Belloch, C. and Vignolo, G. Food Res. Int., 2014, 62, 572-579
7. Corral, S., Leitner, E., Siegmund, B., and Flores, M. (2016). Food Chem 190: 657-664
8. Flores, M., Moncunill, D., Montero, R., López-Díez, J.J. and Belloch, C. (2017) J Agric Food Chem 65: 3900-3909.
9. Aristoy, M. C., and Toldra, F. (1991). J Agric Food Chem 39: 1792-1795.
10. Regulation (EC) no 1333/2008 of the European parliament and of the council of 16 December 2008 on food additives.
11. Stahnke, L. H. (1995). Meat Sci 41: 193-209
12. Montel, M. C., Masson, R., and Talon, R. (1998). Meat Sci 49: S111–S124.
13. Andrade, M. J., Córdoba, J. J., Casado, E. M., Córdoba, M. G., and Rodríguez, M. (2010). Meat Sci 85: 256-264.
14. Olesen, P. T., Meyer, A. S., and Stahnke, L. H. (2004). Meat Sci, 66: 675-687.

# Identification of odor-active trace compounds in Damask rose (*Rosa damascena*)

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## Abstract

The flower scent of Damask rose (*Rosa damascena*) was investigated. Two ultratrace components that exhibited high flavor dilution factors were detected as odor-active compounds by aroma extract dilution analysis (AEDA). One of the trace compounds with a woody note was identified as rotundone by multidimensional gas chromatography–mass spectrometry/olfactometry (MD-GC-MS/O), whereas the other with a citrus note was identified as 4-(4-methyl-3-pentenyl)-2(5*H*)-furanone (MPF) via fractionation of a commercial rose absolute from *R. damascena*. To the best of our knowledge, this is the first study that reflects the organoleptic importance of these two compounds for the rose scent. Sensory analyses were performed to assess the effects of rotundone and MPF. Results revealed that the addition of 50 μg/kg rotundone and 5 μg/kg MPF to the aroma reconstitute of *R. damascena* provided blooming and natural aspects to it. In addition, the presence of rotundone and MPF in five types of fragrant roses was examined. MPF was also detected in fruits (e.g., lemon, orange, grapefruit, apple, and Muscat grape), black tea, and beer.

## Introduction

The rose scent is crucial for flavors and fragrances. In particular, the rose note is essential for floral perfume compositions. Among the large varieties and forms of roses, Damask rose (*Rosa damascena*) is one of the main species of roses that are cultivated in the fragrance industry. The typical aroma concentrates used for fragrance products include rose oil, rose water, rose absolute, and rose concrete. Nevertheless, the aroma of these processed products is different from that of natural rose flowers. Furthermore, the rose aroma reconstitutes with chemical compounds are different from those of natural rose flowers, thus possibly suggesting that the remaining unknown components are the key to the secret of the rose scent. Volatile compounds of natural rose products have been extensively analyzed for many years; however, not many studies have reported the headspace aroma of natural rose flowers. Therefore, this study aims to sensorically characterize and identify the main odorants that are present in the aroma concentrate of the headspace volatiles of *R. damascena* by aroma extract dilution analysis (AEDA), and identify the compounds that differentiate the scent of natural rose from that of artificial rose aroma reconstitutes.

## Experimental

### *Materials*

The petals of *R. damascena* were handpicked from the garden of the T. Hasegawa R&D center in the morning. Absolute from *R. damascena* was purchased from Biorandes Co., (Le Sen, France).

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*Dynamic headspace analysis and AEDA*

First, immediately after picking the petals of *R. damascena* (42.6 g), they were placed into a glass chamber. A constant flow rate of 1.5 L/min was used with air entering the chamber through a charcoal filter and leaving the chamber via passage through 2.0 g of a Tenax TA 60/80 adsorbent (GL Sciences Co., Tokyo, Japan). After collecting the volatiles for 6 h, 20 mL of pentane and 20 mL of diethyl ether were used for elution. The eluent was collected and concentrated to ca. 100 µL via solvent distillation using a Vigreux column at 43 °C. The concentrate was subjected to GC-MS/FID and GC-O analyses equipped with a polar column (InertCap WAX). In addition, MD-GC-MS/O analysis (first column: InertCap WAX, second column: InertCap 1MS) was performed to elucidate the two unknown compounds. For AEDA [1], the concentrated volatile was diluted stepwise with diethyl ether to obtain dilutions of 1:5, 1:25, 1:125, 1:625, etc.

*Elucidation of the citrus-like odor compound*

The absolute from *R. damascena* (330 g) was fractionated by distillation, silica-gel column chromatography, and two-step high-performance liquid chromatography (HPLC). GC-O analysis was performed to confirm the presence of the target citrus-like odor compound, thus finally obtaining the HPLC fraction (2.8 mg). The chemical structure of the target compound was assumed from high-resolution mass and NMR spectra. Identification was further confirmed by matching the analytical data and odor qualities of the isolated citrus-like odor compound with those of the estimated compound synthesized according to a previously reported method [2].

*Threshold measurement of MPF*

The odor threshold of MPF in water was determined according to a previously reported method [3]. Panelists [$n$ = 23 (16 males and 7 females; age range 20–60 years)] were employees of the R&D Center of T. Hasegawa Co., Ltd. Assessments were conducted orthonasally. Panelists also simultaneously evaluated the odor of the sample that they had successfully recognized.

*Evaluation of the effect of rotundone and MPF*

The triangle test was performed to assess the effects of rotundone and MPF on the rose aroma reconstitutes of *R. damascena*. Four aroma reconstitutes (samples A–D) were evaluated. Sample A comprised an aroma reconstitute of *R. damascena* diluted in dipropylene glycol at 5% w/w. Samples B, C, and D comprised fragrance solutions with the same aroma reconstitute with rotundone (50 µg/kg), MPF (5 µg/kg), and rotundone (50 µg/kg) and MPF (5 µg/kg), respectively.

*Identification of rotundone and MPF in various types of roses and foods*

The headspace gases of the living flowers of *Rosa centifolia*, "Neige Perfum," "Pope John Paul II," "Lady Hilingdon," and "Grand Mogul" were pumped to pass through Tenax TA. The adsorbents were eluted with pentane and diethyl ether, followed by concentration. The concentrates were subjected to MD-GC-MS/O analysis to tentatively identify rotundone and MPF.

Cold-pressed oils of lemon, orange, grapefruit, and distilled oil of lime were purchased. The aroma concentrates of apple, Muscat grape, black tea, and beer were prepared by solvent extraction and solvent-assisted flavor evaporation method [4]. The above-mentioned samples were subjected to MD-GC-MS/O analysis to tentatively identify MPF.

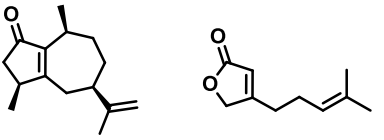
## Results and discussion

*Dynamic headspace analysis and AEDA*

Dynamic headspace sampling was employed to prepare the headspace aroma concentrates of *R. damascena*. From the AEDA results, along with the major compounds such as 2-phenylethanol, geraniol, and citronellol, two ultratrace components with a high flavor dilution factor (FD 625) were detected as odor-active compounds. One trace compound was identified as rotundone, with a woody note (Figure 1) by MD-GC-MS/O analysis with the same retention time, MS spectrum, and odor qualities as those of the authentic synthesized rotundone. However, the other (citrus note) was detected at sufficiently low trace levels such that its structure could not be identified. Rotundone has been identified as an odor-active component in patchouli oil [5], frankincense oil [6], Shiraz wine [7], peppers [7], and several fruits [8]. To the best of our knowledge, rotundone has not been detected in roses.

*Elucidation of the citrus-like odor compound*

The citrus-like odor compound was detected in commercially available rose absolute from *R. damascena*. Therefore, the target citrus-like compound was isolated from rose absolute by distillation, silica-gel column chromatography, and two-step HPLC. As confirmed by GC-FID analysis, the final purity of the isolated compound was 97%. This compound was assumed to be 4-(4-methyl-3-pentenyl)-2(5*H*)-furanone (MPF; Figure 1) from high-resolution mass spectra and NMR spectra. Identification was further confirmed by matching the analytical data and odor qualities of the isolated MPF with those of synthesized MPF. MPF has been identified in rose oil [9] as well as in the secretion of acarid mites [10]. However, to the best of our knowledge, this is the first study that reports on the chemosensory properties of MPF. In the threshold measurement of MPF, panelists have described that MPF emits a citrus-like (lemon, orange, and grapefruit) and floral odor (muguet and jasmine) with a fairly low threshold of 3.6 µg/kg in water.



rotundone MPF

**Figure 1**: Chemical structures of the elucidated odor-active trace compounds from the headspace aroma of rose petals.

*Effects of rotundone and MPF*

The triangle test was performed to examine the effects of rotundone and MPF on the rose aroma reconstitutes of *R. damascena.* Figure 2 shows the results of this test. Sample B and C were not significantly distinguished from sample A. Only sample D was significantly distinguished from sample A. Moreover, panelists who could distinguish sample D from sample A evaluated the aroma of sample D as “more blooming than A” or “more natural than A.” These results suggested that the aroma of the rose aroma reconstitute with the addition of rotundone and MPF was more similar to that of a natural rose flower. Interestingly, the panelists did not differentiate samples A and D as “woody” or “citrus-like.” The effect of these two added compounds is expected to differentiate the aroma of natural rose from that of the artificial rose aroma reconstitutes.

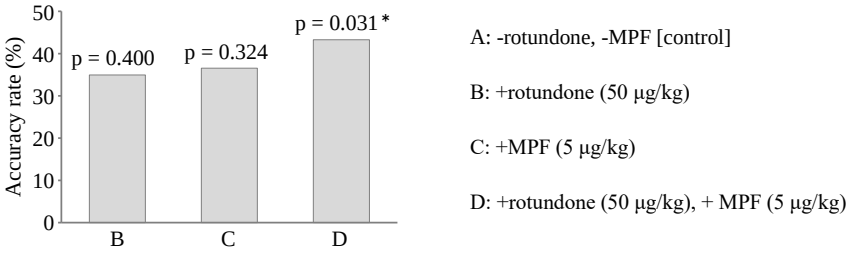



**Figure 2**: Accuracy rate of the triangle test (Identification of sample B, C, or D against A). n = 52, *Binominal test, $p < 0.05$

*Identification of rotundone and MPF in various types of roses and foods*

MD-GC-MS/O analysis was employed to examine the presence of rotundone and MPF in five types of fragrant roses. The former was detected in all roses, while the latter was detected in three roses (i.e., *Rosa centifolia*, "Neige Perfum," and "Pope John Paul II," respectively). Rotundone has been identified in several fruits as a potent odor-active compound [8]; therefore, we examined the presence of MPF in fruits. The results revealed that MPF was detected in lemon, orange, grapefruit, apple, and Muscat grape. In addition, MPF was also detected in black tea and beer. These results indicated that MPF is widely distributed not only in roses but also in various foods. To confirm the contribution of MPF to the aromas of natural resources, quantitative studies need to be performed.

## References

1. Grosch, W. (1994) Flavour Frag. J. 9: 147-158.
2. Poulter, C. D., Wiggins, P. L. and Plummer, T. L. (1981) J. Org. Chem. 46: 1532-1538.
3. Meilgaard, M., Civille, G. V. and Carr, B. T. (2016) Sensory Evaluation Techniques (5th ed) CRC Press: Boca Raton, FL, pp. 153-163.
4. Engel, W., Bahr, W. and Schieberle, P. (1999) Eur. Food Res. Technol. 209: 237-241.
5. Kaiser, R. (2006) In: Meaningful Scents around the World. Olfactory, Chemical, Biological and Cultural Considerations (Kisakürek, M. V., ed.) Verlag Helvetica Chimica Acta/Wiley-VCH, pp 59−64.
6. Niebler, J., Zhuravlova, K., Minceva, M. and Buettner, A. (2016) J. Nat. Prod. 79: 1160-1164.
7. Wood, C., Siebert, T. E., Parker, M., Capone, D. L., Elsey, G. M., Pollnitz, A. P., Eggers, M., Meier, M., Vössing, T., Widder, S., Krammer, G., Sefton, M. A. and Herderich, M. J. (2008) J. Agric. Food Chem. 56: 3738-3744.
8. Nakanishi, A., Fukushima, Y., Miyazawa, N., Yoshikawa, K., Maeda, T. and Kurobayashi, Y. (2017) J. Agric. Food Chem. 65: 4464-4471.
9. Ohloff G. and Demole E. J. (1987) Chromatog. 406: 181-183.
10. Tarui H., Mori N., Nishida R., Okabe K. and Kuwahara Y. (2002) Biosci. Biotechnol. Biochem. 66: 135-140.

# Characterisation of aroma-active compounds in horseradish (*Armoracia rusticana*)

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## Abstract

The aroma composition of freshly ground main roots of horseradish was investigated. Purified extracts of horseradish roots were analysed using the concept of aroma extract dilution analysis (AEDA) and gas chromatography-olfactometry, gas chromatography-mass spectrometry and two-dimensional heart-cut gas chromatography-mass spectrometry/olfactometry. Besides already reported compounds like allyl isothiocyanate and 2-phenylethyl isothiocyanate, a series of odorous substances belonging to different structural classes could be identified, some of them previously unknown or first-time reports in horseradish, and some with high odour potency and potential impact on the overall aroma of horseradish.

## Introduction

Horseradish (*Armoracia rusticana* Gaertn., Mey. et Scherb.) (Figure 1) is a hardy perennial plant belonging to the family of *Brassicaceae* [1] and is distantly related to well-known representatives of this family like cabbage, broccoli, mustard and rapeseed. Horseradish plants possess large leaves [2] and produce white flowers [1], though horseradish is mainly propagated asexually via sets that are obtained from the harvested secondary roots of the previous growing season [3]. It is cultivated in temperate climates in many parts all over the world, but is supposed to have originated from the eastern, temperate regions of Europe [4]. In Europe, the countries with the main growing areas are Austria, Germany, Hungary and Poland. The main reason for cultivation is its white and fleshy root, which is processed to condiments, mainly spicy pastes or sauces; moreover, horseradish is used in traditional phytomedicine, for example as treatment for bronchitis and coughs [5], and has been reported in relation to antimicrobial effects [6, 7].

**Figure 1:** Photograph of harvested horseradish plants

Responsible for the pungent note of the typical horseradish aroma are isothiocyanates (ITCs), which are enzymatically formed from glucosinolates upon cell disruption, when the root is cut or ground [1]. Thiocyanates, nitriles and epithionitriles

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are other possible glucosinolate degradation products that can, besides ITCs, undergo further reactions to various chemical substances [8, 9]. ITCs activate branches of the trigeminal nerve and generate a pain sensation. Numerous studies have dealt with the ITC composition and their content in horseradish roots [10-12], but only few have attempted to explore the substances responsible for the overall aroma impression of horseradish [12, 13]. Accordingly, we applied state-of-the-art methods that cover both sensory and analytical techniques to unravel the composition of horseradish aroma.

## Experimental

*Samples and sample preparation*

Seven horseradish main roots from different varieties were chosen for investigation (n = 7). They were grown on different acreages around the city of Baiersdorf, Germany from April till November 2014. Each main root was peeled and shredded with a Moulinex Moulinette. 1 g ground material was extracted with 30 ml dichloromethane for 30 min, the extract filtrated and dried over anhydrous $Na_2SO_4$. The extract was purified via solvent-assisted flavour evaporation (SAFE) and the aroma fraction thereby recovered. Afterwards the purified sample extracts were gently concentrated up to 100 µl through Vigreux distillation and micro-distillation.

*Sensory evaluation*

Aroma extract dilution analysis (AEDA) was applied to determine the relative contribution of the aroma-active compounds to the overall aroma of horseradish roots. Therefore, the 100 µl root extracts were diluted stepwise in a ratio of 1:2 (v/v). The original extracts (= FD 1) and their dilutions were consecutively analysed by means of gas chromatography-olfactometry, applying the cold on-column application technique, until no odour could be perceived at the sniffing port, and the flavour dilution (FD) factors of each odorous substance were determined. The sniffing analysis was primarily conducted by one trained panellist on two different capillary columns, DB-5 and DB-FFAP (J&W Scientific), and cross-checked by two other trained panellists.

*Mass spectrometric analysis*

The horseradish extracts were analysed via gas chromatography-mass spectrometry and two-dimensional heart-cut gas chromatography-mass spectrometry/olfactometry as has been described previously [14]. EI-mass spectra were created at 70 eV ionisation energy in full scan mode (m/z range 40-250/400).

## Results and discussion

A total of 39 odour-active substances was detected [14]. 30 of these substances could be identified, nine of them tentatively via matching retention indices (RI) and odour impressions with those of authentic reference standards.

21 aroma-active compounds from different structural groups could be detected in all seven samples (cf. Table 1). From the group of organic ITCs and nitriles allyl ITC, *sec*-butyl ITC, isobutyl ITC, 3-butenyl ITC, 4-pentenyl ITC, 2-(methylthio)ethyl ITC, 3-(methylthio)propyl ITC, benzyl ITC, 2-phenylethyl ITC and 1-cyano-2,3-epithiopropane (CETP) could be found. Those substances derive enzymatically from glucosinolates when the horseradish root is ground, and exhibit an overall pungent aroma sensation. As group they were detected in a very wide range of FD factors, starting from FD 1 for isobutyl ITC up to FD 2048 for 2-phenylethyl ITC. Acetic acid, *(Z)*-3-hexenal, 3-(methylthio)propanal, 2-phenylacetaldehyde, 1-octen-3-one, 1-nonen-3-one and 2-

phenylethanol, belonging to the structural group of carbonyl compounds, were detected with low or medium FD factors, for example FD 1 to 4 for acetic acid and FD 16 to 64 for *(Z)*-3-hexenal. As the ITCs, many carbonyl compounds are likely to be primarily formed upon root cutting from fatty acids undergoing lipid oxidation. The pyrazines 3-*sec*-butyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine showed ranges from FD 8 to 256 and FD 512 to 4096 respectively. Those pyrazines are most likely formed within the horseradish root through amidation of α-amino acids, condensation with α,β-dicarbonyls and subsequent methylation [15]. The same FD range as for 3-isopropyl-2-methoxypyrazine with likewise high FD factors was determined for the sweet, smoky, peach- and coconut-like smelling (3*S*,3a*S*,7a*R*)-wine lactone (FD 512-4096). 3-Methylindole with its faecal odour impression was detected with medium FD factors of 16 to 128.

**Table 1:** Aroma-active compounds detected in all horseradish samples (n = 7)

| Odorant [a] | *Odour quality* [b] | *RI value* [c] *on* | | *FD factor range* [d] |
|---|---|---|---|---|
| | | *DB-5* | *DB-FFAP* | |
| Acetic acid [f] | Vinegar-like | 636 | 1447 | 1-4 |
| *(Z)*-3-Hexenal [f] | Grassy, green | 806 | 1145 | 16-64 |
| Allyl ITC [f] | Pungent, mustard-like, horseradish-like, onion-like | 883 | 1353 | 512-1024 |
| 3-(Methylthio)propanal [e] | Cooked potato-like | 910 | 1450 | 4-16 |
| *sec*-Butyl ITC [f] | Pungent, green | 933 | 1263 | 4-32 |
| Isobutyl ITC [f] | Pungent, mustard-like | 954 | 1313 | 1-2 |
| 1-Octen-3-one [e] | Mushroom-like | 979 | 1303 | 4-16 |
| 3-Butenyl ITC [f] | Pungent | 982 | 1447 | 4 |
| 1-Cyano-2,3-epithiopropane [f,g] | Onion-like, pungent | 1000 | 1827 | <1-64 |
| 2-Phenylacetaldehyde [f] | Honey-like, sweet | 1044 | 1636 | 2-4 |
| 1-Nonen-3-one [e] | Mushroom-like, fatty | 1079 | 1397 | 1-8 |
| 4-Pentenyl ITC [f] | Pungent | 1082 | 1524 | 2-4 |
| 3-Isopropyl-2-methoxypyrazine [f] | Pea-like, green pepper-like | 1095 | 1423 | 512-4096 |
| 2-Phenylethanol [f] | Flowery, rose-like | 1119 | 1904 | 1-8 |
| 3-*sec*-Butyl-2-methoxypyrazine [f] | Green pepper-like | 1172 | 1493 | 8-256 |
| 2-(Methylthio)ethyl ITC [f] | Pungent | 1206 | 1892 | 1-4 |
| 3-(Methylthio)propyl ITC [f] | Mushroom-like | 1309 | 1967 | 8-128 |
| Benzyl ITC [f] | Pungent, watercress-like, green | 1363 | 2087 | 8-32 |
| 3-Methylindole (skatole) [f] | Faecal | 1391 | 2480 | 16-128 |
| (3*S*,3a*S*,7a*R*)-Wine lactone [e] | Sweet, peach-like, coconut-like, smoky | 1463 | 2214 | 512-4096 |
| 2-Phenylethyl ITC [f] | Horseradish-like, pungent, watercress-like, green | 1467 | 2205 | 512-2048 |

[a] The compounds were identified by comparing them with the reference odorant based on the given criteria (see below).
[b] Odour quality as perceived at the sniffing port.
[c] Retention indices according to Kovats (1958) [16].
[d] Flavour dilution (FD) factor on the capillary column DB-5.
[e] Identification criteria: RIs on capillaries named in table, odour quality and intensity at the sniffing port.
[f] Identification criteria: same as in ([e]) and MS-EI data.
[g] Detected via GC-MS in all samples, but only in four samples contents above the odour threshold.

Based on the FD factor values for each aroma compounds, the most potent odorants in freshly grated horseradish roots were 3-isopropyl-2-methoxypyrazine, (3*S*,3a*S*,7a*R*)-wine lactone, 2-phenylethyl ITC and allyl ITC. Accordingly, it is conceivable that the general aroma impression of horseradish is mainly defined by those four substances. Nevertheless, other substances, particularly those with medium FD factors like the faecal smelling 3-methylindole and the mushroom-like smelling 3-(methylthio)propyl ITC are also likely to contribute to the overall aroma of horseradish. ITCs with medium or low FD factors like benzyl ITC and isobutyl ITC may further enhance the overall pungency of the horseradish flavour, thereby acting as a group. Likewise, green, vegetable-like notes are likely to be contributed by the green pepper-like smelling 3-*sec*-butyl-2-methoxypyrazine and the grassy, green smelling *(Z)*-3-hexenal.

We further detected four substances with medium or low FD factors, respectively, that were found in six out of the seven samples. Those were the cheesy smelling butanoic acid (FD 2-4) and the pungent 3-methylbutyl ITC (FD 1-2), as well as two unknown odorous substances. One had an onion-, vinegar- and cabbage-like smell (FD 4-32), the other was perceived as earthy, mouldy and dusty (FD 1-16). We assume that the former substance could be a sulphur-containing molecule and the other an alkylated pyrazine, as they show sensory traits typical for these substance groups.

Summarising our findings, we detected substances that covered a wide FD range between the different samples, like CETP with a difference of seven dilution steps, whereas others were surprisingly consistent in the investigated varieties, like allyl ITC with a difference of one dilution step only between samples.

Accordingly, the results of this study make a significant contribution to the general knowledge of the chemical principles of horseradish aroma.

## References

1. Agneta R., Moellers C., Rivelli A.R. (2013). Genet Resour Crop Evol. 60 (7): 1923-1943.
2. Courter J.W., Rhodes A.M. (1969). Economic Botany. 23 (2): 156-164.
3. Walters S.A., Wahle E.A. (2010). HortTechnology. 20 (2): 267-276.
4. De Candolle A. (1885). Origin of cultivated plants. New York: D. Appleton and Co.
5. Bladh K.W., Olsson K.M. (2011). J Herbs, Spices Med Plants. 17 (3): 197-213.
6. Kienholz M., Kemkes B. (1960). Arzneimittelforschung. 10: 917-918.
7. Halbeisen T. (1957). Arzneimittelforschung. 7: 321-323.
8. Hanschen F.S., Lamy E., Schreiner M., Rohn S. (2014). Angew Chem, Int Ed. 53 (43): 11430-11450.
9. Springett M.B., Adams J.B. (1989). Journal of the Science of Food and Agriculture. 46 (2): 211-219.
10. Masuda H., Harada Y., Tanaka K., Nakajima M., Tabeta H. (1996). ACS Symp Ser. 637 (Biotechnology for Improved Foods and Flavors): 67-78.
11. Sultana T., Savage G.P., McNeil D.L., Porter N.G., Clark B. (2003). J Food, Agric Environ. 1 (2): 117-121.
12. Gilbert J., Nursten H.E. (1972). J Sci Food Agr. 23 (4): 527-539.
13. D'Auria M., Mauriello G., Racioppi R. (2004). Ital J Food Sci. 16 (4): 487-490.
14. Kroener E.-M., Buettner A. (2017). Food Chemistry. 232: 455-465.
15. Wuest M. Biosynthesis of Plant-Derived Odorants (2017). In. Springer Handbook of Odor (Buettner A., ed.). Dordrecht Heidelberg London New York: Springer International Publishing. pp. 13-37.
16. Kovats E. (1958). Helv Chim Acta. 41: 1915-1932.

# Effect of nitrate reduction on the development of oxidized aroma in dry fermented sausages during storage

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## Abstract

The effect of nitrate reduction on the development of fermented sausage aroma and its stability during vacuum storage has been studied. Different sausage formulations were manufactured with different nitrate contents as a source of nitrite as preservative. The oxidation of sausages was evaluated by analyzing TBARS compounds and extraction of the volatile compounds using solid phase microextraction (SPME) and gas chromatography mass spectrometry. Aroma compounds related to oxidation processes were identified by olfactometry technique. The study revealed the relation of nitrate reduction and fat content on aroma compounds related to oxidation process and their effect on sausage aroma during vacuum storage

## Introduction

Aroma characteristics of fermented sausage depend on processing factors such as raw material, meat ingredients, preservatives, technological parameters and presence of starter cultures. Despite the role of nitrites and nitrates in meat product safety and technological properties, there is a trend to reduce its use [1]. However, the effect of nitrite on flavor formation in meat products is essential to develop cured aroma. Thomas et al., [2] indicated that cured cooked ham aroma is due to the balance of sulfur compounds and oxidation compounds produced during cooking and in the absence of nitrite, the aroma is disturbed due to the excessive formation of oxidation compounds that mask the sulfur meaty notes. In dry fermented sausage aroma, nitrite plays a fundamental role in developing the typical dry cured aroma [3] although it is not known the effect of nitrite reduction on aroma generation and stability during shelf life. Therefore, our aim is to determine the effect of reduced nitrate concentrations used as preservatives on the development of sausage aroma in dry fermented sausages after storage under vacuum at ambient temperature.

## Experimental

*Dry fermented sausages preparation*

Dry fermented sausages were manufactured using lean pork (50%), pork fat (50%) and the following additives added in g/kg to the sausage formulation: lactose (20); dextrose (20); sodium chloride (20.25); glucose (7); potassium chloride (6.75); sodium ascorbate (0.5); starter culture (0.1) and sodium nitrate (0.25 for control sausage (C) or reduced in 15% (RN15) and 25% (RN25). The starter culture TRADI-302 (Danisco, Cultor, Madrid, Spain) was added containing *Lactobacillus sakei, Pediococcus pentosaceus, Staphylococcus xylosus* and *Staphylococcus carnosus*. The sausages were submitted to a slow fermentation process as described by Corral et al., [4]. At the end of ripening, sausages were vacuum packed and stored at room temperature to study its shelf life at 0, 36, 70 and 100 days.

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*Physicochemical analysis*

The lipid content was determined by organic extraction with $Cl_2CH_2$:$CH_3OH$ (2:1) [5]. Lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) [6] and expressed as μg of malonaldehyde per gram of dry mater (μg MDA/g dm).

*Volatile compound analysis*

The analysis of volatile compounds was carried out by solid phase micro extraction (SPME) with an 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) fiber (Supelco, Bellefonte, PA). 5 g of sausage sample (with BHT to avoid oxidation) was weighed into a headspace vial. The vial was incubated at 37 °C for 30 min. Then, the fibre was exposed into the headspace vial for 120 min while maintaining the sample at 37 °C. The compounds adsorbed by the fibre were desorbed in the injection port of the GC-MS for 5 min at 240 °C in splitless mode. A gas chromatograph (Agilent HP 7890 series II (Hewlett-Packard, Palo Alto, CA) with a mass detector (HP 5975C (Hewlett-Packard) equipped with an autosampler (Gerstel MPS2 multipurpose sampler (Gerstel, Germany) was used [7]. The compounds were identified by comparison with mass spectra from the library database (Nist'05), with linear retention indices [8] and with authentic standards.

*Aroma compound analysis*

A gas chromatograph (Agilent 6890, USA) equipped with a FID detector and sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) was used to analyze aroma compounds [6]. Each assessment was carried out with 5 g of sample using the detection frequency method [9]. Four trained panelists evaluated the odors from the GC-effluent. A total of 12 assessments were carried out. The aroma compounds were identified by comparison with mass spectra, with linear retention indices of authentic standards injected in GC-MS and GC-O and by the coincidence of the assessor's descriptors with those in the Fenaroli's handbook of flavor ingredients [10].

*Statistical analysis*

Analysis of variance (ANOVA) using the statistic software XLSTAT 2011, version 5.0 (Addinsoft, Barcelona, Spain) was performed at each storage time among sausage formulations. Correlation tests (Pearson) among variables were also studied.

**Results and discussion**

Fat content in sausages was analyzed as it is responsible for the generation of lipid oxidation compounds during sausage fermentation [3]. Although all sausages were manufactured with the same lean and fat content, slightly differences among formulations were obtained due to pork back fat variability. Control sausages had a fat content of 33-38%, while nitrate reduced sausages RN15 and RN25 contained between 29-33% and 29-31%, respectively.

The lipid oxidation level (TBARS values) during sausage vacuum storage is shown in Figure 1A. It showed a slight increase during the first month of storage and a decrease during the following months [11]. This behavior may be due to the high reactivity of malonaldehyde with sugars, aminoacids and nitrite [12]. In addition, the absence of oxygen in vacuum storage prevent the sausages for an increase in oxidation. However, differences among formulations were observed ($p<0.001$): the lipid oxidation was the highest in the control formulation. This fact can be due to the highest fat content of this control sausage as a positive ($p<0.05$) relation between lipid oxidation (TBARS values)

and fat content was obtained among sausages analyzed at the end of the ripening process (Figure 2A) [13].

Regarding sausage aroma, GC-O analysis revealed 23 odour active zones (Table 1). The main odorants were Ethyl butanoate, Hexanal, Ethyl 2-hydroxypropanoate, 1-hexanol, 2-acetyl-1-pyrroline, 3-(Methylthio)propanal, 1-Octen-3-ol and 1 unknown compound. Among them, only three aroma compounds were derived from lipid oxidation reactions: Hexanal (Figure 1B), Heptanal (Figure 1C) and 2-Pentylfuran (Figure 1D) which contributed to fresh cut grass, green-unpleasant and garlic-grass odour notes. The concentration of the three volatile compounds showed a general slight increase during vacuum storage of sausages. However, few differences were observed at each storage time among formulations. Hexanal was the most abundant compound and was positively related to fat sausage content at the end of the ripening (Figure 2B).

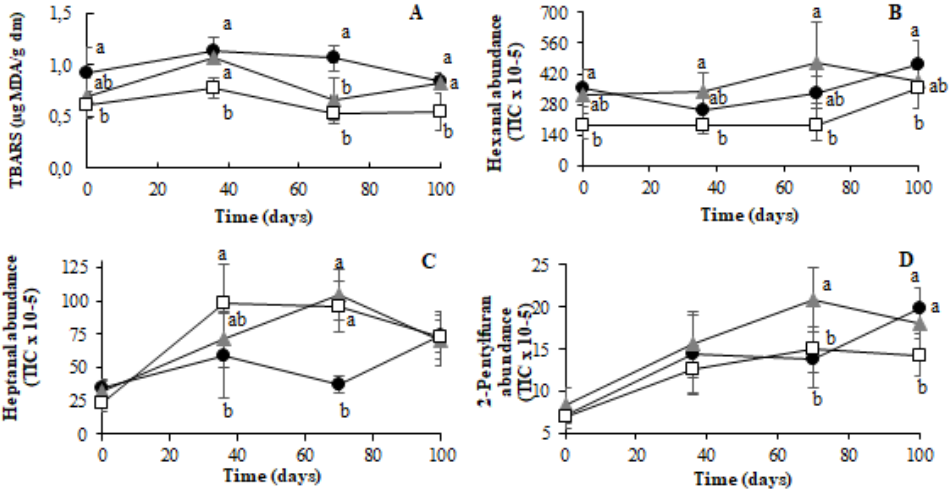


**Figure 1**: Changes in TBARS (A), Hexanal (B), Heptanal (C) and 2-Pentylfuran (D) during vacuum storage of dry fermented sausages: C (control, ●), RN15 (15% reduced nitrate, ▲) and RN25 (25% reduced nitrate, □).

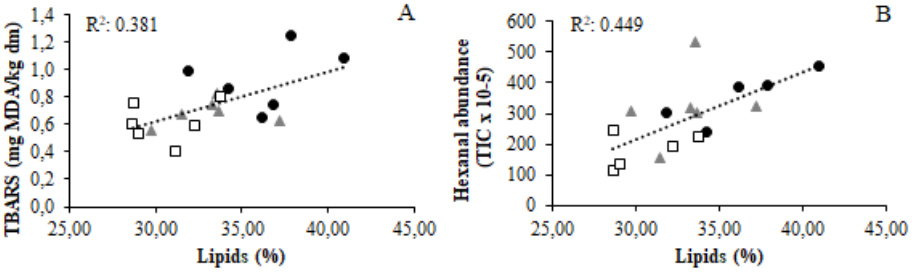


**Figure 2:** Pearson Correlation between fat content and lipid oxidation (A) or Hexanal (B) in dry fermented sausages at the end of the ripening: C (control, ●), RN15 (15% reduced nitrate, ▲) and RN25 (25% reduced nitrate, □).

## Conclusions

Aroma compounds derived from lipid oxidation reactions contribute to the aroma of fermented sausages. The increase in shelf life by vacuum storage produced variation in

**Table 1**: List of aroma compounds detected in GC-FID/Olfatometry

| Compound | *LRI std*[a] *GC-O* | *LRI*[b] *GC-O* | *Descriptor* | *DF*[c] |
|---|---|---|---|---|
| Methanethiol | 471 | 472 | Rotten, unpleasant | 8 |
| 2-Methylfuran | 619 | 615 | Green, garlic, toasted, yeast, malt | 4 |
| 2,3-Butanedione | 632 | 629 | Fruit, cheese, butter, floral, fresh, broth | 4 |
| 2-Butanone | 638 | 636 | Cheese, butter, dairy, strawberries, fruity, sweet, flower | 8 |
| Acetic acid | 700 | 699 | Vinegar, acid, unpleasant, sweet | 8 |
| 2,3-Pentanedione | 739 | 740 | Sweet, candy, fruit, glue, meat | 4 |
| 3-hydroxy-2-butanone | 777 | 782 | Strawberry, sweet, fruity, apple, orange, acid, fresh, green | 9 |
| Ethyl butanoate | 825 | 824 | Sweet, apple, banana, orange, fruit, strawberry, floral | 10 |
| **Hexanal** | **836** | **834** | **Fresh cut grass, vegetable, lemon, aromatic herbs, fresh** | **10** |
| Ethyl 2-hydroxy propanoato | 859 | 865 | Cheese, fruit, strawberry, sweet, rancid, acid | 11 |
| Ethyl 3-methyl butanoato | 876 | 874 | Strawberry, fruit, glue, sweet | 9 |
| 1-hexanol | 919 | 920 | Cheese, oxidized fat, humidity | 11 |
| 2-Heptanone | 931 | 931 | Cheese, rancid, burnt, irritating, garlic, vinegar, strawberry | 4 |
| **Heptanal** | **937** | **938** | **Green, unpleasant, toasted** | **5** |
| 2-acetyl-1-pyrroline | 960 | 960 | Toasted, fried corn, bread, citrus, floral | 12 |
| 3-(methylthio)propanal | 969 | 965 | Cooked potato, roast meat | 10 |
| **2-Pentylfuran** | **1011** | **1008** | **Garlic, onion, fried, unpleasant, cured, grass** | **8** |
| 1-Octen-3-ol | 1028 | 1023 | Mushrooms, humidity, spicy | 11 |
| Unknown | - | 1031 | Burnt, mushrooms, garlic, unpleasant, humidity, closed, herbs | 8 |
| Unknown | - | 1037 | Green, grass, earth, burnt, spicy, aromatic herbs | 6 |
| Unknown | - | 1162 | Spices, garlic, spicy, fried corn, unpleasant | 5 |
| Unknown | - | 1178 | Cooked potato, fried corn, toasted, dried fruit | 10 |
| Ethyl octanoate | 1226 | 1223 | Cured sausages, onion, fruit, cooked potato | 7 |

[a]LRI std: Linear retention index of standard compounds in the GC-FID-O. [b]Linear retention index of the compounds eluted from the GC-FID-O. [c]Detection frequency value.

these aroma compounds that are affected not only by the presence of preservatives (curing agents) but also by the matrix composition (fat content).

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## References

1. Bolger, Z., Brunton, N.P., Lyng, J.G. and Monahan, F.K. (2017). Food Reviews International 33, 143-166.
2. Thomas, C., Mercier, F., Tournayre, P., Martin, J-L. and Berdagué, J-L. (2013). Food Chemistry 139, 432-438.
3. Flores, M. and Olivares, A. (2015). In: Handbook of Fermented Meat and Poultry (2nd ed.). (Toldra, F., ed.). West Susex, UK: John Wiley & Sons,Ltd., pp. 217-225.
4. Corral, S., Salvador, A., Belloch, C. and Flores, M. (2015). Food Control 47, 526-535.
5. Folch, J., Lees, M. and Stanley, G.H.S. (1957). Journal of Biological Chemistry 226, 497-509.
6. Olivares, A., Navarro, J.L. and Flores, M. (2011). Meat Science 87, 264-273.
7. Corral, S., Salvador, A. and Flores, M. (2013). Meat Science 93, 776-785.
8. Kovats, E. S. (1965). In: Advances in chromatography (Giddings, J.C., & Keller, R.A., ed.), New York: Marcel Dekker, Inc. pp. 229–247.
9. Pollien, P., Ott, A., Montigon, F., Baumgartner, M., Muñoz-Box, R. and Chaintreau, A. (1997). Journal of Agricultural and Food Chemistry 45, 2630−2637.
10. Burdock, G. A. (2002). Fenaroli's handbook of flavor ingredients (6th ed.). Boca Raton, Florida: CRC Press Inc.
11. Ansorena, D. and Astiasarán, I. (2004). Meat Science 67, 237-244.
12. Rubio, B., Martinez, B., Garcia-Cachan, M.D., Rovira, J. and Jaime, I. (2008). Meat Science 80, 1182-1187.
13. Olivares, A., Navarro, J.L. and Flores, M. (2015). Food Science and Technology International 21, 110-123.

# Shelf-life model: Useful tool to predict sensory and nutritional quality of infant formulas

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## Abstract

Shelf life prediction gains an increasing interest in food industries. It is especially relevant for long shelf-life products where degradations have more time to occur. In this study, we show that it is possible to build a reliable shelf-life kinetics model for infant formula (powder) packed in metal cans. The model varies the following parameters: storage temperature (5-40°C), storage time (0-2 years), and oxygen level in the pack (protected or unprotected atmosphere). The effects of light and moisture were discarded as they cannot penetrate through the metal can. A model was build based on chemical kinetics. The model is able to predict the taste, the level of vitamin C, and the aromas concentrations based on the chemical reactions occurring in the infant formula. The kinetic reactions were fitted based on data of aroma concentrations and oxygen level in the package. Several examples of accelerated shelf life tests simulating a normal shelf life at 2 years are illustrated. The results are compared to the most common practice in shelf-life: using a fixed Q10 temperature coefficient. It is advised to use multiple accelerated shelf life tests to mimic the normal shelf life of the relevant sensory or nutritional aspects of the product.

## Introduction

In this study, we show that it is possible to build a reliable shelf-life kinetics model for infant formula (powder) packed in metal cans.

## Experimental

*Shelf-life conditions*

In order to build the model several conditions were varied in the infant formula:

- Storage temperature: 5°C, 20°C, 30°C, and 40°C,
- Storage time: 0 to 2 years,
- Oxygen level (O2) in the pack: protected (N2 flushed) or unprotected atmosphere (21 % O2).

The effects of light and moisture were discarded in this study as they cannot penetrate through the metal can [1, 2, 3].

*Analysis*

Several selected parameters have been measured in the infant formula (powders):

- Sensory attributes (Quantitative Descriptive Analysis - QDA, scale 0-100) were evaluated by a trained panel of 16 persons,
- Aromas concentration was determined by GC-MS (most relevant aromas selected based on literature [4] and internal check; method adapted from [5]),
- Oxygen content in the package (metal can) and vitamin C content.

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*Model building*

The relevant correlations between the parameters mentioned above were identified using multivariate analysis methods (Unscrambler). The gPROMS model builder was used to calculate the kinetics of the chemical reactions (aromas, vitamin; [6]). A user-friendly shelf-life model was finally created in Excel and linked to the gPROMS model builder interface. The predictive power of the model was validated with real data. Utilizing this model, sensory attributes scores or vitamin C level can be predicted based on aroma compounds or/and oxygen evolution in the package during storage.

## Results and discussion

*General trends*

During shelf-life, infant formula powders were very sensitive to oxygen exposure. This effect is even more prevalent if the temperature increased during storage. As an example (Figure 1a), an infant formula packed in a metal can without protected atmosphere (high level of oxygen) developed higher oxidation flavour and showed high losses of vitamin C during storage. These results were expected as vitamin C is known to be one of the most unstable vitamins to oxygen and heat [7]. Similar results were obtained for liquid dairy products where other vitamins (B1, B2, D or A) always showed less degradation than vitamin C during storage with oxygen (data not shown).

In contrast to unprotected atmosphere, infant formula powders packed in the metal can with protected atmosphere (low oxygen level) were extremely well protected. Vitamin C was stable at any temperature tested (30-60°C) and only a slight increase (not significant) of oxidation flavour occurred after 2 years storage for the common temperatures of 30-40°C (end of shelf-life; Figure 1b). At 60°C, the oxidation reactions with the residual $O_2$ content were increased.

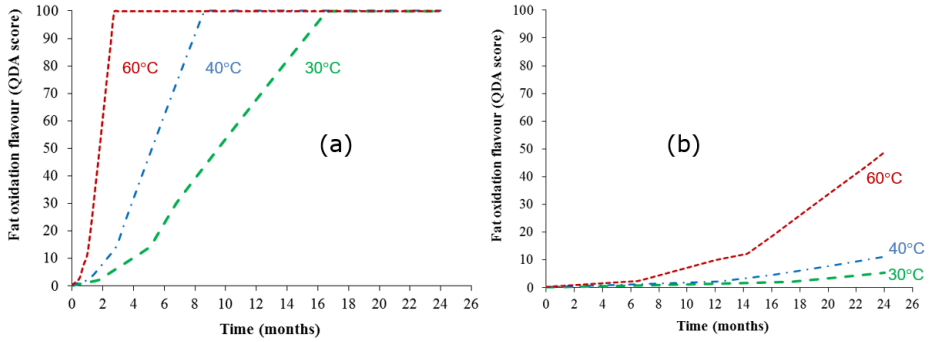


**Figure 1**: Increase of fat oxidation flavour with storage time (months) for (a) an infant formula in a metal can containing 21% $O_2$; (b) an infant formula in a metal can flushed with inert gasses (protected atmosphere; 1,5% $O_2$ residual).

*Fitting with aroma compounds*

A very good fitting was identified between the fat oxidation flavour and several aromas for the infant formula (correlation >0.6, good fitting in the model): hexanal (impact ~60%), pentanal (impact ~ 25%), 2,4,-tr, tr-decadienal (impact <5%), 4-cis-heptenal (impact < 5%), penten-3-one (impact < 5%). Furthermore, furfural fitted with the burned odour observed during storage in the dried infant formula powder. Such a result is logical as furfural is produced through Maillard reactions [8]. Those reactions

trend to happen during storage at high temperature and can increase the caramelized, sweet or burned notes of the product. During storage, production of furfural in infant formula was only linked to heat state and not to the oxygen level. Though it is mainly recognized that Maillard reactions increase in the presence of oxygen [8] some authors however underlined that those reactions can also occur in anaerobic conditions [9]. The latter statement is supported by the finding that browning reactions were also observed during storage in dairy drinks in anaerobic conditions (data not shown).

*Q10 method*

A tool commonly used in accelerated shelf-life studies is the Q10 method. Q10 is the factor that indicates the increase in the rate of the reactions when the temperature is increased by 10°C. It is unit less and can be calculated with the following equation for 2 reactions 10°C apart: Q10 = k (T+10°C) / k (T°C), where k= reaction rate constant. For most products, the Q10 value is 2.0, which means for every increase of 10°C, the rate of a chemical reaction will double. As an example, if a food has a stability of 20 weeks at 20°C and 10 weeks at 30°C, then the Q10 will be 20/10 or 2.

*Accelerated shelf-life tests simulating a normal shelf-life at 2 years*

As it can be seen from Tables 1-2, the model was used to predict the accelerated shelf life test to mimic a normal storage of 2 years at 30°C of infant formula packed in a metal can. The results were compared with the common approach, i.e. the Q10 method (see description above).

**Table 1:** Accelerated shelf life of infant formula mimicking the values obtained after 2 years at 30°C in a metal can flushed with inert gasses (1.5% $O_2$ residual in the headspace).

| | *Value after 2 years at 30°C** | *Corresponding months at 40°C* | *Corresponding months at 60°C#* | *Equivalent Q10 30°C vs 40°C* |
|---|---|---|---|---|
| Vitamin C (mg/kg) | 873 | 12.3 | 3.3 | 2.0 |
| Fat oxidation flavour | 5 | 16.5 | 8.3 | 1.5 |
| Hexanal (ppb) - A | 254 | 15.8 | 7.3 | 1.5 |
| Furfural (ppb) - B | 60 | 5.0 | 0.3 | 4.8 |

***** Initial value before storage: vitamin C: 890 mg/kg; fat oxidation flavour:1; hexanal: 10 ppb; furfural: 20 ppb. Indicator of oxidation reactions (A) or of Maillard reactions (B). # Values at 60°C were generated by the shelf-life prediction model.

**Table 2:** Accelerated shelf life of infant formula mimicking the values obtained after 2 years at 30°C for a metal can in unprotected atmosphere conditions (21% $O_2$ in the headspace).

| | *Value after 2 years at 30°C** | *Corresponding months at 40°C* | *Corresponding months at 60°C#* | *Equivalent Q10 30°C vs 40°C* |
|---|---|---|---|---|
| Vitamin C (mg/kg) | 738 | 10.3 | 2.2 | 2.3 |
| Fat oxidation flavour | 100 | 8.8 | 3.0 | 2.7 |
| Hexanal (ppb) - A | 14992 | 12.5 | 4.0 | 1.9 |
| Furfural (ppb) - B | 60 | 5.0 | 0.3 | 4.8 |

***** Initial value before storage: vitamin C: 890 mg/kg; fat oxidation flavour:1; hexanal: 10 ppb; furfural: 20 ppb. Indicator of oxidation reactions (A) or of Maillard reactions (B). # Values at 60°C were generated by the shelf-life prediction model.

The shelf-life parameters of the infant formula (vitamin C, fat oxidation, hexanal, furfural) showed different kinetics and therefore, they should be tested using different accelerated shelf-life (Table 1). For example, the furfural (indicator of Maillard reactions) needed an accelerated shelf-life at 40°C of 5 months to mimic the normal shelf life of 2

years at 30°C while the fat oxidation needs 16.5 months. This also means that the Q10 coefficient of the different parameters varied from 1.5 to 4.8. Only one factor has a Q10 coefficient of 2 in a metal can with protected atmosphere: the vitamin C. The Q10 factor can vary in function of the conditions, for example a Q10 of 1.5 was observed for fat oxidation in protected atmosphere (Table 1) while it was closer to 3 in unprotected atmosphere at 40°C (Table 2). The accelerated shelf-life of the infant formula at 60°C showed that all reactions can be accelerated but that for several parameters still some months were required to reach the same value found in normal shelf-life (2 years at 30°C). As an example, an accelerated shelf-life of 7.3 months and 2.8 months, both at 60°C were needed for fat oxidation and vitamin C, respectively (Table 1). This is logical since metal cans with protected atmosphere are extremely good protective packaging [2, 3].

The same observations were seen in unprotected atmosphere conditions: the accelerated shelf-life conditions (Table 2) as well as the Q10 coefficient depended on the parameter types (vitamin C, fat oxidation). The fat oxidation flavour as well as oxidation reactions indicator, hexanal increased sharply in unprotected conditions (see values of normal shelf life at 30°C for 2 years Table 1 vs Table 2). The Maillard reactions indicator (furfural) was similar at 30°C for protected and unprotected atmosphere. This is because those reactions were dependent on the applied temperature and not on the oxygen level present in the headspace of the packaging.

The results indicate that using only one accelerated shelf-life test (one time/temperature) to mimic the normal shelf-life is not optimal. The best approach would be to use one accelerated shelf-life test for each parameter of interest. In other words, a multiple shelf-life approaches should be used, respecting the reaction kinetic of each parameter. In the near future, shelf-life model will help to better predict the behaviour of the key parameters of infant formula powders and to correlate the results to normal shelf life. With this model, the duration of accelerated shelf life study is expected to reduce while still guarantying a good prediction of the normal shelf-life.

## References

1. Robertson, G. L. (2009). In Food packaging and shelf-life: a practical guide (Robertson, G. L., ed). CRC Press, USA.pp 1-16.
2. Gargouri, B., Zribi, A., Bouaziz, M. (2015). J Food Sci Technol, 52: 1948–1959.
3. Properties of packaging material: http://www.fao.org/Wairdocs/X5434E/x5434e0g.htm.
4. Venkateshwarlu, G., Let, M. B., Meyer. A. S., Jacobsen, C. (2004). J. Agric. Food Chem, 52: 311-317.
5. Jeleñ, H.H., Dabrowska, A., Klensporf, D., Nawrocki, J., Wasowicz, E. (2004). Chem. Anal, 49: 869.
6. Van Boekel, T. (2010). In Analysis in Controlling Maillard Pathways to Generate Flavors (Mottram and Taylor, eds), ACS Symposium: Washington, DC: pp. 1-11.
7. Paulus, K. (1989). Bibliotheca Nutritio et Dieta, 43 : 173–187.
8. Mottram, D. S. (2007). In Flavours and Fragrances (Berger, R.G., ed.), Springer Verlag Berlin Heidelberg, pp. 269-283.
9. Hayase, F., Shibuya, T., Sato, J., Yamamoto, M. (1996). Biosci Biotechnol Biochem. 60: 1820-5.

# Milk fat globule membrane and its role in flavour development in cheese during ripening

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## Abstract

Despite the well-recognised role of milk fat in flavour development in cheese, research investigating the importance of the milk fat globule membrane (MFGM) on flavour in cheese is scarce. This study investigated the impact of MFGM composition and structure on the volatile profile of ripened Cheddar cheese samples. Three types of MFGM recombined cheeses were manufactured using MFGM fractions isolated from dairy by-products and were compared to two reference cheeses. After 6 months' maturation the MFGM recombined cheeses had a higher concentration of volatile compounds (short chain fatty acids, alcohols, methyl ketones and sulfide compounds) compared to the reference-cheeses. These results demonstrate that the MFGM composition as well as structural rearrangement at the fat globule interface had a significant effect on the development of volatile compounds in cheese during maturation.

## Introduction

Milk fat plays a vital role in determining the texture, flavour, and physico-chemical properties of cheese [1]. The milk fat globule (MFG) consists of a lipid core surrounded by a three-layer membrane termed the MFGM. The MFGM contains a complex mixture of glycoproteins, enzymes, and phospholipids. Phospholipids within the membrane possess a high water-holding capacity and the moisture captured by them can serve as a reservoir where enzymes can act and enhance flavour development [2]. MFGM components can act as substrates for both lactic acid bacteria (LAB) and non-starter lactic acid bacteria (NSLAB) [3] during the later stages of cheese ripening. Furthermore, the MFGM contains redox enzymes such as xanthine oxidase (XO), which are capable of catalysing the oxidation of a broad range of substrates, and therefore may play a role in determining the flavour of cheese.

Buttermilk is a by-product of butter manufacturing, produced during churning; whereas α-serum (αS) and β-serum (βS) are by-products of anhydrous milk fat manufacturing. Buttermilk, αS and βS were once considered to be waste material, but are now recognised as good sources of MFGM. The procedures used to produce buttermilk powder (BMP), αS, and βS affect the protein and lipid moiety of the isolated MFGM fractions, and the content and activity of XO and other enzymes [4]. As such, BMP, αS, and βS were used in this study as a source of MFGM fractions with different protein and lipid composition and XO enzymatic activities. The isolated fractions were then used to investigate the importance of the MFGM structure and composition on development of volatile compounds in model Cheddar cheese samples during six months of ripening.

## Experimental

### *Materials*

Raw milk was collected from Jersey cows at late lactation from a local dairy farm (Outram, New Zealand) on the day of milking. Freeze-dried αS and βS and spray dried BMP were obtained from a dairy factory in New Zealand. AMF was obtained from New

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Zealand Milk Products (Auckland, New Zealand). Freeze-dried mesophilic starter culture containing *Lactococcus lactis ssp. lactis* plus *Lactococcus lactis ssp. cremoris* (R704) was obtained from Hansen A/S, Horsholm (Denmark).

*MFGM isolation and model cheese production*

Freeze-dried αS and βS and BMP were used for MFGM isolation as outlined in Haddadian et al. [4]. Three types of 5% milkfat emulsion were prepared using the three MFGM isolates (2%) as the emulsifier, as previously described [4]. Three separate batches of model Cheddar cheese were manufactured for each treatment using the 5% milk fat emulsions, according to a standard Cheddar cheese making procedure [5]. Two reference cheeses were also manufactured as comparison samples: (1) Native-cheese containing cream and skim milk to evaluate the role of MFGM structure in flavour development; and (2) Tween-cheese containing recombined MFGs emulsified by Tween 80 to evaluate the effect of the MFGM composition in the flavour development process (Table 1). Cheeses were sampled after 1, 90, and 180 d of ripening. Ripened samples taken at each sampling date were frozen at -20°C until the end of the trial so that the complete sample set could be analysed together.

**Table 1**: Compositional properties of cheese milk samples for model cheese production

| Cheese samples | Cheese milk composition (mL) | | | | Emulsifier | PFR* |
|---|---|---|---|---|---|---|
| | Cream# | Emulsion (mL) | Water (mL) | Skim milk (mL)† | | |
| Native | 30 | - | 200 | 170 | - | 1.02 |
| Tween | - | 230 | - | 170 | Tween80 | 1.02 |
| α-cheese | - | 230 | - | 170 | α-MFGM | 1.02 |
| β-cheese | - | 230 | - | 170 | β-MFGM | 1.02 |
| BMP-cheese | - | 230 | - | 170 | BMP-MFGM | 1.02 |

* Protein to fat ratio
# Pasteurised cream; 36% fat and 2.2% protein

*Determination of volatile compounds by SPME-GC/MS*

The analysis of volatile compounds in miniature model cheeses was carried out using solid phase micro extraction (SPME) with a fibre coated with a film of DVB/CAR/PDMS (Supelco, Bellefonte, PA, USA) and analysed by gas chromatography–mass spectrometry (GC-MS). Volatile compounds were separated on a polyethylene glycol capillary column (Zebron ZB-Wax 60m x 0.32mm x 0.50 µm, Phenomenex, Torrance, CA, USA). A complete randomised design, blocked by replicate, was used for the volatile analysis. Vacuum-packed frozen cheese samples were ground with liquid nitrogen and $Na_2SO_4$ (1.5 g/g) to give fine particles. A subsample (5 g) of each powdered sample was mixed with 2 µL of an aqueous solution of 12.5 mg $L^{-1}$ fenchol in a 20 mL sealed GC vial. Vials were placed on autosampler tray (PAL3 RSI 85, Agilent Technologies) for analysis.

## Results and discussion

*Effect of MFGM on development of volatile compounds during cheese ripening*

A total of 28 significantly different compounds were detected among the model cheese samples for the three time points during the six-month ripening period. The differences between samples over ripening were visualised using PCA on normalised peak areas (Figure 1).

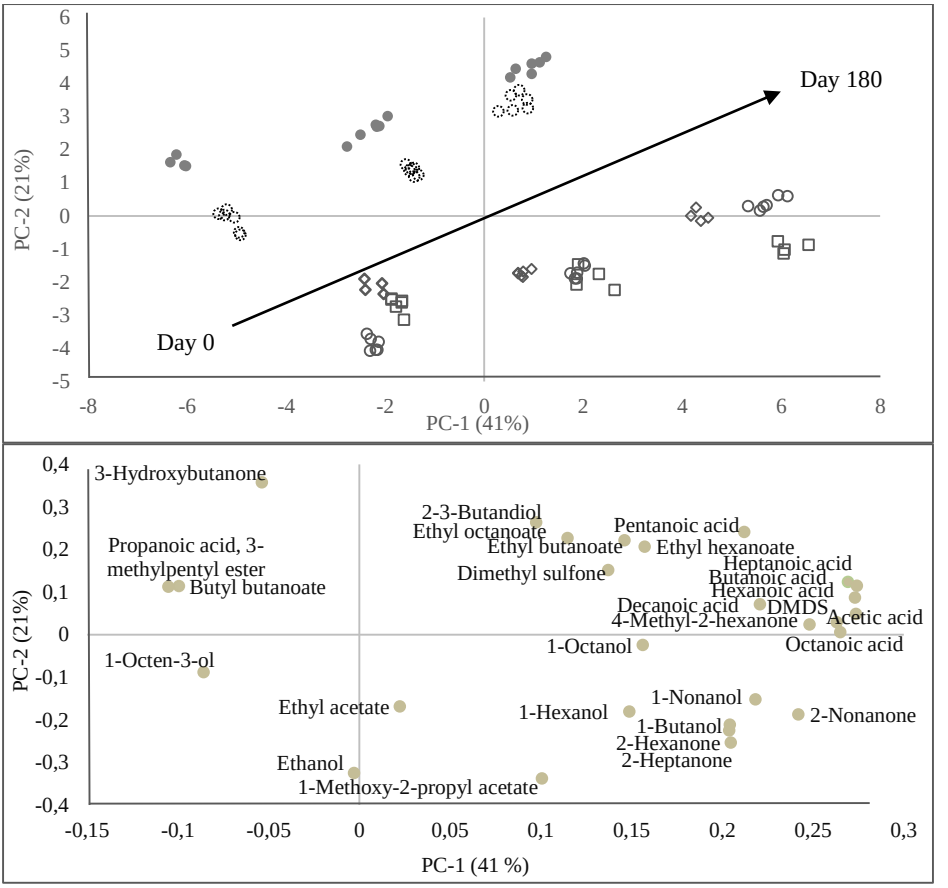


**Figure 1**: Principal component analysis of significantly different volatile compounds detected in model cheese samples at three time points during six months ripening. Scores plot (top) of cheese samples; α (○), β (□), BMP (◊), Native (●), and Tween (○); Loadings plot (bottom) of significantly different volatile compounds identified by GC-MS. Points represent 3 batches x 2 analytical replicates per treatment per time point.

The first two principal components accounted for 62% of the total variability (PC1 41%, and PC2 21%) and clearly separated the model cheese samples into three groups consisting of Native-cheese, Tween-cheese, and MFGM-recombined cheeses of α, β, and BMP (Figure 1, top). Ripening time proceeded from left to right on PC1. The number and concentration of most compounds increased as ripening proceeded, except for a few compounds, such as ethanol, and octen-3-ol, which decreased significantly over the ripening in all model cheese samples. The volatile profile of the MFGM-recombined cheeses developed the most, while the Tween cheese, followed by the Native-cheese showed the least volatile development during the ripening period.

At six months of ripening, MFGM-recombined cheeses containing αS, βS, and BMP had a higher concentration of short chain fatty acids (SCFAs), alcohols, methyl ketones and sulfide compounds (Table 2). In MFGM recombined cheeses, the higher XO activity in β-serum (7.2 ± 0.8 mmol/L uric acid/min) and α-serum (7.6 ± 0.5 mmol/L uric acid/min) compared to BMP (no detected activity) was correlated to higher concentrations of SCFAs. Native and Tween cheeses had higher concentrations of 2,3-butanediol and 3-hydroxybutanone compared to the recombined cheeses of αS, βS, and

BMP. Tween cheese was uniquely associated with higher levels of three esters; ethyl butanoate, butyl butanoate, and ethyl hexanoate, but overall had the lowest concentration of volatiles, thus showing the importance of MFGM to the flavour development in cheese.

**Table 2**: Concentrations of significantly different volatile compounds detected in experimental cheese samples after six months ripening.

| | AS-cheese | BS-cheese | BMP-cheese | Tween-cheese | Native-cheese |
|---|---|---|---|---|---|
| Acetic acid | 26.4 ± 1.6 $^{a}$ | 22.1 ± 1.0 $^{b}$ | 12.1 ± 0.6 $^{c}$ | 6.7 ± 0.2 $^{e}$ | 9.8 ± 0.3 $^{d}$ |
| Butanoic acid | 89.3 ± 5.5 $^{a}$ | 88.3 ± 2.7 $^{a}$ | 66.8 ± 1.8 $^{b}$ | 34.3± 3.0 $^{c}$ | 63.7 ± 2.6 $^{b}$ |
| Hexanoic acid | 93.8 ± 2.4 $^{a}$ | 99.3 ± 3.4 $^{a}$ | 72.2 ± 2.6 $^{b}$ | 45.0 ± 5.5 $^{d}$ | 61.5± 7.8 $^{c}$ |
| Ethanol | 322.8 ± 5.0 $^{a}$ | 285.0 ± 2.9 $^{b}$ | 187.1 ± 13.6 $^{c}$ | 248.1 ± 13.4 $^{e}$ | 129.8 ± 8.2$^{d}$ |
| 1-Butanol | 7.8 ± 0.4 $^{b}$ | 9.0 ± 0.2 $^{a}$ | 6.8 ± 0.3 $^{c}$ | 5.9 ± 0.5 $^{d}$ | 2.7 ± 0.3 $^{e}$ |
| 1-Nonanol | 1.1 ± 0.05 $^{b}$ | 1.0 ± 0.06 $^{d}$ | 1.1 ± 0.03 $^{b}$ | 0.8 ± 0.03 $^{c}$ | 0.6 ± 0.04 $^{a}$ |
| 1-Hexanol | 4.3 ± 0.1$^{c}$ | 4.9 ± 0.3 $^{ab}$ | 4.4 ± 0.2 $^{c}$ | 4.8 ± 0.1$^{bc}$ | 2.6 ± 0.4 $^{d}$ |
| Dimethyldisulfide | 0.7 ± 0.06 $^{a}$ | 0.7 ± 0.02 $^{a}$ | 0.6 ± 0.07 $^{b}$ | 0.04 ± 0.05 $^{c}$ | 0.5 ± 0.04 $^{b}$ |
| Dimethyltrisulfide | 0.5 ± 0.04 $^{b}$ | 0.6 ± 0.03 $^{a}$ | 0.4 ± 0.03 $^{c}$ | 0.07 ± 0.01 $^{d}$ | 0.1 ± 0.04 $^{c}$ |
| 2-Hexanone | 40.1 ± 0.1 $^{a}$ | 40.7 ± 1.9 $^{a}$ | 40.3 ± 1.4 $^{a}$ | 28.8 ± 0.3 $^{b}$ | 8.6 ± 0.1 $^{c}$ |
| 2-Heptanone | 13.2 ± 0.4 $^{b}$ | 15.0 ± 0.6 $^{a}$ | 13.8 ± 0.4 $^{b}$ | 9.8 ± 0.3 $^{c}$ | 4.0 ± 0.7 $^{d}$ |
| 2-Nonanone | 4.9 ± 0.3 $^{a}$ | 5.4 ± 0.2 $^{a}$ | 5.0 ± 0.2 $^{a}$ | 3.7 ± 0.1 $^{b}$ | 2.4 ± 0.3 $^{c}$ |
| 4-Methyl-2-hexanone | 5.9 ± 0.6$^{ab}$ | 6.3 ± 0.4 $^{b}$ | 5.4 ± 0.2 $^{a}$ | 3.5 ± 0.4 $^{c}$ | 2.0 ± 0.2 $^{d}$ |
| 2-3-Butanediol | 0.9 ± 0.1 $^{e}$ | 1.4 ± 0.1 $^{d}$ | 2.0 ± 0.2 $^{c}$ | 5.5 ± 0.07 $^{a}$ | 3.1 ± 0.3 $^{b}$ |
| 3-Hydroxybutanone | 4.6 ± 0.4 $^{d}$ | 4.0 ± 0.2 $^{d}$ | 5.7 ± 0.2 $^{c}$ | 10.1 ± 0.2 $^{a}$ | 8.8 ± 0.3 $^{b}$ |
| Ethyl butanoate | 1.4 ± 0.05 $^{a}$ | 1.2 ± 0.06 $^{a}$ | 1.0 ± 0.07 $^{a}$ | 1.5 ± 0.08 $^{a}$ | 1.0 ± 0.1 $^{a}$ |
| Ethyl hexanoate | | | | 0.7 ± 0.2 | |
| Butyl butanoate | 3.9 ± 0.2 $^{b}$ | 4.8 ± 0.6 $^{b}$ | 4.3 ± 0.1 $^{b}$ | 11.9 ± 0.6 $^{a}$ | 4.7 ± 0.6 $^{b}$ |

Letters denote significant differences ($p < 0.05$) between samples according to Tukey HSD post-hoc test.

## Conclusion

By comparing the volatile profile of the MFGM-recombined cheeses with Tween-cheese, and Native-cheese, new insights were revealed into the role of MFGM and its composition and structure on flavour development in cheese. Rearrangement of the MFGM structure and the higher activity of the MFGM-enzymes such as XO, favoured the production of volatile compounds during ripening. These results demonstrate the potential of using MFGM components from commercial by-products as a functional ingredient to enhance the flavour development of cheese.

## References

1. Fox, P. F., Guinee, T. P., Cogan, T. M., and McSweeney, P. L. H. (2000). Fundamentals of Cheese Science. New York: Springer US.
2. Goudédranche, H., Fauquant, J., and Maubois, J. L. (2000). Fractionation of globular milk fat by membrane microfiltration. Le Lait, 80(1), 93–98.
3. Moe, K. M., Porcellato, D., and Skeie, S. (2013). Metabolism of milk fat globule membrane components by nonstarter lactic acid bacteria isolated from cheese. Journal of Dairy Science, 96(2), 727–39.
4. Haddadian, Z., Eyres, G. T., Carne, A., Everett, D. W., and Bremer, P. (2017). Impact of different milk fat globule membrane preparations on protein composition, xanthine oxidase activity, and redox potential. International Dairy Journal, 64, 14-21.
5. Kosikowski, F. V., and Mistry, V. V. (1997). Cheese and fermented milk foods. In Vol. 2: Procedures and Analysis, 3rd edition (Ed. FV Kosikowski). Westport: LLC.

# The effect of sugar type on VOC generation in a model baked system

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## Abstract

Due to the multiple functions of sugar in foods, in particular the contribution of sugar to the desirable "fresh baked" aroma, efforts to improve the nutritional profile of baked products by reducing sugar is problematic. As such to produce consumer accepted reduced sugar products it is necessary to understand how removing and/or modifying sugar composition influences the final product flavour. Model baked products (muffins) were produced containing variable amounts of sucrose, fructose, glucose and lactose and the volatile organic compound (VOC) composition isolated by solvent assisted flavor extraction (SAFE) and measured by gas chromatography mass spectrometry (GC-MS). Overall changing the sugar composition changed the VOC composition with lactose containing systems producing a VOC composition that was most different from the uncooked and sucrose containing muffins. In comparison to the lactose containing muffins the glucose and fructose containing muffin produced VOC compositions more similar to sucrose containing muffins. Not all compounds increased with increasing levels of sugar.

## Introduction

Replacing sugar in baked products is a major challenge. Sugar not only imparts sweetness, but contributes to the fresh flavour quality of baked foods during thermal processing and acts as a tenderiser by retarding and restricting gluten formation [1]. Reducing sugars have a direct influence on the Maillard reaction, which can either promote or reduce Strecker degradation, resulting in the formation of important compounds such as pyrazines that are character impact odorants of freshly baked foods [2]. Sucrose, a non-reducing sugar, can degrade during baking forming the reducing sugars fructose and glucose. Therefore, it is necessary to understand how removing and/or modifying sugar composition influences the final product flavour.

The objective of the study was to investigate the effect of sugar type (sucrose, glucose, fructose and lactose) at two sugar levels (3.7%, 14.7% of batter recipe) on volatile organic compound (VOC) generation in a model baked system (muffins).

## Experimental

Model baked systems (muffins) were produced using the generic formulation in Table 1 and sugar composition in Table 2. Dry ingredients (flour, sugar, baking powder, salt, polydextrose and sugar mixture) were mixed with the liquid ingredients (egg white, water and oil) and baked at 200 °C for 18.5 min. Muffin cooked weight was 55 +/- 0.5 g. Muffins were immediately frozen after baking using liquid nitrogen.

Ground frozen muffins (200g) were added to 150mL distilled water and 200mL diethyl ether (*99.7%, Merck KGaA, Germany*). This mixture was shaken for 40min then filtered and 30ppm carvone added.

SAFE (*Glasbläserei, Bahr, Manching, Germany*) distillation was carried out at about $10^{-6}$ mbar over two hours (including sample addition time of one hour). The 500mL

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sample flask was maintained at 35°C. The 500mL receiver flask was cooled using liquid nitrogen. Circulating Water was held at 42°C. Diethyl ether (25mL) was used to rinse the sample bottle and dropping funnel.

**Table 1:** Generic formulation of the muffins

| *Ingredient* | *% (weight/weight)* |
|---|---|
| Flour | 31.3 |
| Baking powder | 1.8 |
| Salt | 0.5 |
| Polydextrose | 2.7 |
| Egg white | 11.2 |
| Canola oil | 11.1 |
| Water | 26.7 |
| Sugar mixture | 14.7 |

**Table 2:** Composition of the sugar mixture used in each muffin variant

| *Variant number* | *Sugar composition* |
|---|---|
| 1 | 100% sucrose |
| 2 | 100% fructose |
| 3 | 100% glucose |
| 4 | 100% lactose |
| 5 | 25% sucrose, 75% polydextrose |
| 6 | 25% fructose, 75% polydextrose |
| 7 | 25% glucose, 75% polydextrose |
| 8 | 25% lactose, 75% polydextrose |
| 9 | 100% sucrose - uncooked |
| 10 | 100% polydextrose |

Distillates were dehydrated with anhydrous sodium sulphate, filtered through celite then concentrated to 1 mL in a Kuderna Danish apparatus under oxygen-free nitrogen. All the extracts were stored in a freezer (-20°C) until GC-MS analysis. Distillates were analysed using an Agilent 6890 GC coupled with Agilent 5973 Quadrapole MS fitted with a BPX5 column (30m x 0.25mm id, 0.25um film thickness).

Data Analysis: Peak alignment and peak area extraction were performed using XCMS [3]. Principal component analysis (PCA) was used to investigate the relationships between samples and peak areas.

## Results and discussion

The use of different sugar formulations impacted on the extent of browning upon baking (most browning, 100% fructose; least browning, polydextrose). Differences in the total amount of volatile organic compounds (VOCs) produced, as measured by summed normalised peak areas, were also observed. Summed normalised peak areas were highest

for muffins containing 100% lactose followed by 25% lactose, 100% fructose, 100% glucose, 25% glucose, 25% fructose, 100% sucrose, 25% sucrose and polydextrose, respectively.

The effect of sugar type on VOC composition was examined by normalising the peak areas to the sum of the peak areas then assessed by principal component analysis (PCA). The PCA plot explained 78% of the variation on the 1st and 2nd PCs (PC1 60%; PC 2 18%) (Figure 1). Along PC 1 the VOC composition of muffins containing lactose were most different from the uncooked muffin batter.

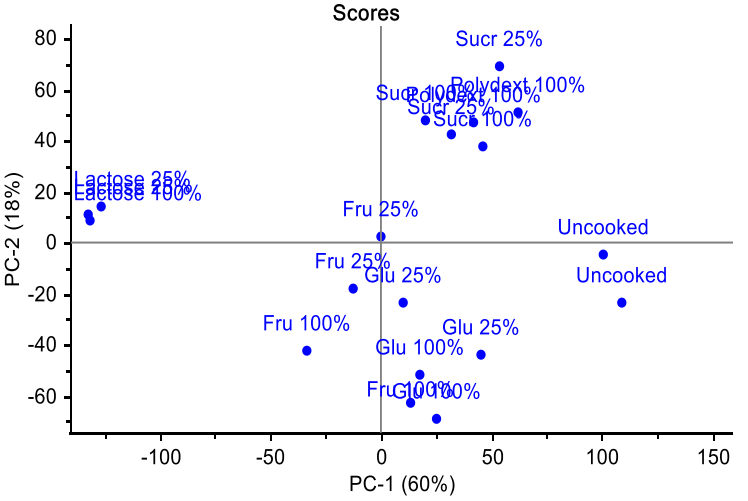


**Figure 1:** Principal component analysis scores plots of muffins containing different sugar compositions

The separation of the cooked muffins from the uncooked muffin batter on the PCA appeared to be related to a combination of number of compounds detected and higher proportions of common compounds. Separation towards the lactose containing muffins was due the presence of higher proportions of 2-furanmethanol, maltol, γ-butyrolactone, 2(5H)-furanone, and lower proportions of acetic acid, hexanoic acid and three unknown compounds. PC2 separated the muffins containing glucose/fructose from muffins containing sucrose/ polydextrose due to higher proportions of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, 5-(hydroxymethyl)-2-furancarboxaldehyde, 5-methyl 2-furanmethanol, 5-hydroxymethylfurfural, furfural, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, hexanoic acid and 5-hydroxymethylfurfural; and higher proportions of methyl pyrazine, benzeneacetaldehyde, nonanal and two anhydro-glucopyranose compounds, respectively.

Relative peak areas of the main VOC's responsible for the separation of muffins on the PCA plot are shown in Figure 2. Furan methanol and maltol are highest for lactose 100% (Figure 2A and 2B). Acetic acid and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one are highest in the fructose and glucose containing muffins (2C and 2F). For these VOC's the muffins containing 25% lactose, 100% sucrose, 100% polydextrose and uncooked muffin all contained similar relative peak areas. Their contribution to the separation on PC 1 was probably due to lower total peak areas for sucrose, polydextrose and uncooked muffins compared to lactose containing muffins. Methyl pyrazine and

benzeneacetaldehyde show a similar trend with the sucrose containing muffins containing the highest peak areas (2D and 2E).

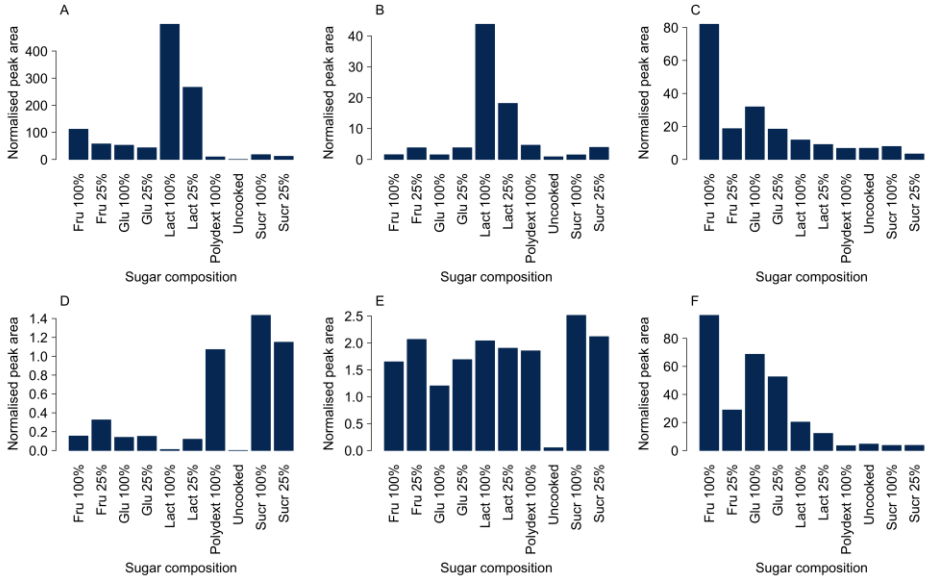


**Figure 2:** Relative peak areas for compounds responsible for descrimination based on sugar composition (relative peak area of internal standard =30); A. 2-furanmethanol ; B. maltol ; C. acetic acid ; D. methyl pyrazine; E. benzeneacetaldehyde; F. 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one

Overall changing the sugar composition changed the VOC composition with lactose containing systems producing a VOC composition that was most different from the uncooked and sucrose containing muffins. In comparison to the lactose containing muffins the glucose and fructose containing muffin produced VOC compositions more similar to sucrose containing muffins. In some instances higher relative peak areas were obtained for some compounds (e.g. benzeneacetaldehyde) in the 25% level of muffins containing fructose and glucose compared to the 100% levels. This may reflect some compounds present are intermediates and react to form other compounds.

## References

1. Gelinas, P., Roy, G., and Guillet, M. (1999). Journal of Food Science, 64 (5), 937–940.
2. Pozo-Bayon, M. A., Guichard, E., and Cayot, V. (2006). Food Reviews International, 22(4), 335–379.
3. Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R., and Siuzdak G. (2006). Analytical Chemistry, 78, pp. 779–787.

# Understanding the impact of sodium on the structural properties of sweet biscuits

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## Abstract

The impact of sodium inclusion on the structural properties of sweet biscuits was investigated. Mass loss behaviour of four biscuit doughs (four levels of added salt) during baking (rate of loss, mass loss) was monitored using TGA, and texture properties of the baked biscuits were established with a texture analyser. Reducing the amount of added salt significantly increased the rate of mass loss at the baking phase, and hence, impacted biscuit hardness. Furthermore, less sodium chloride in the dough decreased the intrinsic break strength of the biscuits. This could be explained at a molecular level by changes in the glutenin gliadin cross-linking leading to changes in the gluten network. In contrast, when high levels of sodium chloride were added to the dough, an increased intrinsic biscuit break strength was observed. The present study demonstrates the significant impact of sodium on gluten polymerization during biscuit baking and confirms that sodium inclusion led to a retention of free water necessary for the gluten formation.

## Introduction

Although sodium is required for normal body functions, it is often consumed in excess, this has led to a major global health problem for both adults and children. A high consumption of salt causes an increase in blood pressure and therefore increased risks of cardiovascular disease, stroke and coronary heart disease. The World Health Organization (WHO) recommends that adults consume less than 5g of salt daily. However, the average global intake significantly exceeds this level (e.g. 10g/day in the UK) [1]. Salt is used for 3 principal applications: processing, sensory (enhancement properties of others ingredients) and preservation [2]. More precisely, sweet biscuits have been highlighted because they often contain significant amounts of hidden salt. In 2013, a survey found that biscuits are in the top ten contributors of salt intake in the UK diet [3]. To ensure that biscuits with a lower sodium content remain appealing to consumers, sodium reduction in food products must not modify quality such as texture, or preservation and taste properties. Several studies have investigated the impact of sugar and fat in sweet biscuits, but to the best of the authors' knowledge, the impact of sodium reduction was exclusively performed on bread and salty snacks [4]. Sweet biscuits are a complex food matrix composed of various ingredients such as wheat flour (containing gluten and starch), fat (butter), sugar (sucrose), salt, and a low amount of water (< 5 %). During dough making, high sugar and fat levels and low water levels result in poor gluten hydration [5], leading to a non-elastic dough with a low gluten development [6]. During dough heating, fat, sugar, and gluten react. Starch granules could potentially swell but in short dough, this phenomenon might be very limited. A degradation of starch particles could also be observed but the high sucrose and low water levels prevent complete gelatinization [7]. Gaines (1990) stated that gluten proteins remain functional during the baking phase in this kind of matrix. Chevallier *et al.* (2000) and Pareyt *et al.* (2009) observed that the level of extractable proteins after baking decreased significantly, suggesting the formation of a gluten network in the dough during baking [8]. Moreover, Chevallier *et al*. suggest that

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the structure of the matrix after baking could be attributed to the sucrose [8a]. As the biscuit could be defined as a complex matrix made of sugars, lipids, starch granules and protein aggregates, they suggested that the structure cohesiveness might be mainly achieved by sugars that become glassy after the baking phase during the cooling step. It still appears that the quality of the gluten network is the most important factor that affects the structural properties of the biscuits [9]. The aim of this study was to observe and understand the impact of sodium on gluten polymerization in sweet biscuits and the impact on the structure after baking.

## Experimental

### *Materials*

Reference dough (L3) was prepared from the ingredients listed below: (1) Unsalted Butter 23.2 g/100 g; (2) Caster sugar 18.6 g/ 100 g; (3) Semi-skimmed long life milk 11.1g/100 g; (4) Salt, 0.6 g/100 g; (5) Flour containing self-raising agent 46.5 g/100 g. Unsalted butter, caster sugar, semi-skimmed long life milk and sodium chloride were sourced from Sainsburys (Supermarket company, UK), and flour was sourced from Morrisons (Supermarket company, UK).

### *Biscuit dough making and baking*

The ingredients from (1) to (4) were weighed and blended manually then (5) was added and the dough mixed by a Food processor blender (Multipro Home, Kenwood, UK). A homogeneous dough was then formed, rolled to 40 mm thickness using an industrial laminator (Fritsch, Rollfix, Germany), and shaped by a model cutter (24 mm diameter, round with a smooth edge). The biscuits were placed on the same tray, placed in a Deck oven (Tom Chandley Compacta, UK) and baked at 180°C for 12 min. Subsequently, the biscuits were cooled to room temperature (20°C). The biscuit dimensions and weight were (average): height: 0.6mm; diameter: 32mm, and weight 3g. The biscuits were carefully packed and stored in sealed aluminium bags with a minimum headspace within the bag to reduce the effect on moisture content. Four doughs, from L0 to L3, were formulated and each contained different quantities of sodium chloride (respectively: 0.53; 0.75; 0.96; 1.20g of salt/100g of dough). L3 was the reference, comparable to the higher quantity of salt in commercial biscuits available in supermarkets (i.e. 1.3g per 100g of biscuit).

### *Thermogravimetry (TGA)*

The weight loss of samples was measured with a Mettler-Toledo TGA/SDTA 851 thermal gravimetric analyser, using a nitrogen atmosphere (3 replicates). TGA is an analytical technique used to determine a material's thermal stability by monitoring the weight change that occurs as a specimen is heated; the weight is recorded as a function of the increasing temperature. In dynamic measurements, 10.0 ± 0.2mg of sample were placed in the aluminium pans and heated from 30 to 200°C at a heating rate of 10°C/min.

### *Moisture content*

Moisture content of all biscuits was assessed by drying the biscuit using an OHAUS MB25 moisture balance. 2 g of sample were ground using a pestle and mortar and then placed on the moisture balance pan. The balance was programmed to run at 120°C for 12 min. 12 replicates of each type of biscuit were run.

### *Three-point bend*

A Texture Analyser (TAXT Texture Analyser, Stable Micro Systems) was used to measure fracture force (Newton, N) of biscuits in compression mode, in a 3-point bending

test using a 3-point bending rig (HDP/3PB), a heavy-duty platform (HDP/90) and a load cell of 5 kg. The inner gap distance between 2 plates was 18 mm and the upper blade linked to the probe moved vertically with 5.5 mm either side of the plate.

*Statistical analysis*

The data obtained from colour, moisture content, water activity, texture analyser and aroma release experiments were statistically evaluated using the software Microsoft® Excel 2010/XLSTAT©-Pro (2013.4.03, Addinsoft, Inc., Brooklyn, NY, USA). Data were subjected to univariate analysis of variance (ANOVA). The significance level was set at p-value<0.05. Significant differences among means of treatments were evaluated by the post-hoc multiple comparisons Fisher test.

## Results and discussion

Reducing the salt content resulted in more rapid weight loss probably due to a lower water retention during baking. Rates for L0 and L3 were -4.12 and -3.28 µg/s respectively. Fessas & Schiraldi (2001) suggested that water in the dough would mainly be in two states, namely, i) free to diffuse through a medium, whose viscosity increases with increasing temperature because of the drying and transformations affecting starch and gluten, and ii) tightly bound to the gluten network and thus able to flash off only at higher temperatures [10]. The observed phenomena here could be due to release of "free water" in L0 (high rate of release; low temperature) and the added sodium in L3 might lead to an increase of the amount of "bound water". This could also explain why we need a higher temperature to release water in L3 (maximum rate of loss for L3=106°C while for L0=104°C). Moisture content analysis of biscuits showed a significant difference between the samples L0 to L3 with respectively 3.13 and 3.60 %.

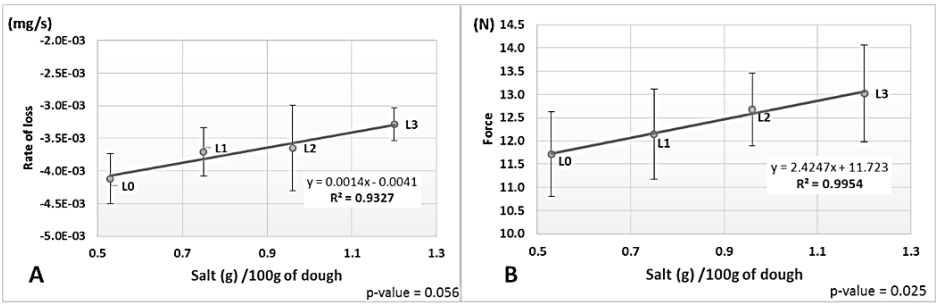


**Figure 1:** Maximum rate of mass loss (mg/s) between 104 and 106°C (**A**) and force (Newton) required to fracture (**B**) biscuits L0 to L3

However, there is no difference between L1 and L2 but the global trend shows a decrease of the moisture content when the quantity of added sodium chloride decreases. This observation tends to confirm our hypothesis that reducing the amount of added salt led to a matrix which retains less water leading to a smaller moisture content of L0 than L3. The force required to bend and fracture the samples was measured and a significant decrease in the force needed to reach the point of break was observed (p-value < 0.05) in biscuits without added salt, meaning less resistance to fracture and lower elastic response in L0 (11.70 N) than L3 (12.75 N). Decreasing values from L3 to L0 could be here related to the development of a less elastic structure in biscuits during baking. However, it must be stressed that due to the low moisture content in these doughs before baking (≈17%), and a high fat level (23.4%) and sugar level (18.6%), gluten proteins may not be properly hydrated and may form a non-continuous network [11]. Lynch *et al.* showed that sodium

chloride increased the strength of the gluten network in bread doughs by enhancing the orientation uniformity [12]. The impact of the sodium on the gluten network strength was established by McCann & Day as added sodium chloride reduced the proteins charge leading to less repulsive forces (enhanced non-covalent hydrophobic interactions), and they observed higher interactions between them, leading to an increase in the gluten network strength [13]. Therefore, the added sodium chloride increased the force required to break the biscuits due to the formation of the gluten network being more resistant after baking. This is hypothesised to be due to the sodium chloride retaining more water in the matrix and decreasing the quantity of free water. So, the increase of the force required to break the salted biscuits could be due to the strengthening of the gluten network mixed with sugar in a glassy state (forming a more elastic matrix – lowering Young modulus).

## Conclusion

The objective of this study was to understand the impact of sodium on the physico-chemical characteristics (colour, aroma release, texture) and sensory properties of sweet biscuit by baking biscuits with less added salt. When sodium chloride was added up to 1.20% (L3 as reference), the biscuit required more force to be broken, and had a higher moisture content than the biscuits with no added sodium chloride. Salt reduction may reduce the formation/strength of the gluten network [13]. It was suggested that there is an increase of "free water" in L0 (high rate of release at a lower temperature) and that the added sodium chloride in L3 might lead to an increased amount of "bound water" due to a more developed/strengthened gluten network (lower rate of release; higher temperature). A good gluten network might retain more water in the matrix (L3) and that more "bound water" will lead to a more resistant and elastic matrix which could potentially retain more aroma compounds in the matrix during the baking step.

## References

1. World Health Organization, Sodium intake for adults and children (2012) http://www.who.int/nutrition/publications/guidelines/sodium_intake/en/
2. Hutton T. (2002) Br Food J 104: 126-152.
3. Consensus Action on Salt and Health (CASH) (2013) http://www.actiononsalt.org.uk/news/surveys/2013/Biscuits/100901.html
4. (a) Israr T., Rakha A., Sohail M., Rashid S. and Shehzad A. (2016) Trends in Food Science & Technology 51: 98-105; (b) Silow C., Axel C., Zannini E. and Arendt E. K. (2016) Journal of Cereal Science 72: 135-145.
5. Gaines C. S. (1990) Cereal Chemistry 67: 73-77.
6. Wade P., Biscuits, cookies and crackers. Vol. 1 (1988), Elsevier Applied Science, London.
7. Chevallier S., Della Valle G., Colonna P., Broyart B. and Trystram G. (2002) Journal of Cereal Science 35: 1-10.
8. (a) Chevallier S., Colonna P., Buleon A., and Della Valle G. (2000) Journal of Agricultural and Food Chemistry 48: 1322-1326; (b) Pareyt B., Brijs K., and Delcour J. A. (2009) Journal of Agricultural and Food Chemistry 57: 7814-7818.
9. (a) Pareyt B., Delcour J. A. (2008) Critical Reviews in Food Science and Nutrition 48: 824-839; (b) Pareyt B., Bruneel C., Brijs K., Goesaert H., Delcour J. A. (2010) Journal of Agricultural and Food Chemistry 58: 353-360.
10. Fessas D., and Schiraldi A. (2001) Food Chemistry 72: 237-244.
11. Manley D., Pareyt B., and Delcour J. A. (2011) In: Manley's Technology of Biscuits, Crackers and Cookies (Fourth edition) (Duncan M., ed.), Woodhead Publishing, pp. 331-346.
12. Lynch E. J., Dal Bello F., Sheehan E. M., Cashman K. D., and Arendt E. K. (2009) Food Research International 42: 885-891.
13. McCann T. H., and Day L. (2013) Journal of Cereal Science 57: 444-452.

# The impact of plant proteins on vanilla flavour perception

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## Abstract

Interactions between proteins and flavours have been reported to produce flavour retention and to decrease flavour perception in food products. Protein/flavour interactions, a type of flavour retention, can either be reversible such as hydrophobic, hydrogen, and electrostatic interactions or irreversible such as covalent binding. Proteins can also transmit undesirable off-flavours to food products affecting their organoleptic properties and thus also altering flavour perception. It has been previously confirmed that vanilla flavour intensity was reduced due to interactions between vanillin and milk proteins. However, less is known about plant protein/flavour interactions. Therefore, the aim of this study was to investigate interactions between vanillin and plant proteins (wheat, soy, lupin, pea, and potato) in aqueous systems and their impact on flavour perception. Results showed that interactions were dependant on the protein source. Vanillin was bound mainly by pea protein, followed by wheat protein. The final sensory profiles of model beverages were influenced by both, protein/vanillin interactions and off-flavour related to each protein.

## Introduction

Multiple studies have shown that proteins can interact with various flavour components resulting in flavour retention and affecting flavour perception [1]–[3]. Protein/flavour interactions differ according to the amino acid composition of proteins and the chemical structure of flavour components. Retention of flavour by physico-chemical interactions can be either reversible such as hydrophobic, hydrogen, and electrostatic interactions or irreversible such as covalent binding. Protein/flavour interactions have been confirmed for vanillin (4-hydroxy-3-methoxybenzaldehyde), the main compound of vanilla flavour which is widely applied in food products [2], [4]–[9]. Vanillin binding affinity and flavour perception has been largely investigated for milk proteins [1], [2], [4]–[8]. Studies showed that sodium caseinate or whey proteins interact with vanillin, and that the binding affinity increases with protein concentration [2], [4]–[6]. Reversible interactions can even occur quickly and influence the flavour perception of food immediately [2], [9], [10]. On the other hand, fewer studies have focussed on interactions between plant proteins and flavours, although the plant protein usage is predicted to increase in the future [11]. Plant protein/flavour interactions have been previously investigated for soy protein [6], [12]–[15], in lesser extent for pea [16], [17] and wheat proteins [18], and no studies have focused on lupin or potato proteins. The usage of proteins may not only cause flavour retention but also transmit unwanted off-flavours, which represent the main limitation for their use in food [2], [19]. This sensory dimension is less taken into account in studies that focussed on protein/flavour interactions. Therefore, the aim of this study was to investigate both, flavour retention and flavour perception when vanillin is mixed with plant proteins (wheat, soy, pea, lupin, and potato), as well as the contribution of protein off-flavours in the final sensory profile of model beverages.

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## Experimental

Vanillin (Mane, France), wheat protein concentrate (Tereos, France), soy protein concentrate (ADM, USA), pea protein isolate (Roquette, France), lupin protein-rich powder (Terrena, France), and potato protein isolate (Avebe, The Netherlands) were used to investigate protein/vanillin interactions and sensory flavour perception. The protein content in dry base were 80 %, 69 %, 83 %, 42 %, and 90 % for wheat, soy, pea, lupin, and potato proteins, respectively. Solutions were prepared in demineralised water by adding proteins and sugar at 3 % w/w and at 2.5 % w/w concentrations, respectively. The pH of wheat, soy, pea, lupin, and potato protein solutions was not adjusted and was around 5.8, 7.4, 7.2, 7.5, and 6.0, respectively. When vanillin was added to samples the final concentration was 100 ppm.

*Sensory evaluation*

Descriptive sensory analyses were performed by an internal panel composed between 10 and 15 panellists using the rank-rating evaluation method [20]. Per session, panellists tested protein and protein/vanillin solutions and evaluated the vanillin flavour and the off-flavours: cereal/wheat, herbal/vegetal, and bitterness, on a 0-10 scale. These three protein off-flavour descriptors were selected by their frequency from a separate sensory session testing protein solutions. Changes in the perceived intensity of each descriptor were determined by the difference between pure vanillin and protein/vanillin solutions. Data obtained was treated using an analysis of variance (ANOVA).

*Determination of protein/vanillin interaction*

Physico-chemical interactions between vanillin and plant proteins were determined by equilibrium dialysis experiments and High Performance Liquid Chromatography (HPLC) analysis for quantification of vanillin. In equilibrium dialysis experiments proteins were kept separated by using semi-permeable membranes (Spectra/Por1 MWMO: 6-8 kDa). Protein solutions were first dialysed overnight against demineralised water to purify samples prior to vanillin addition. After the equilibrium was reached (~72 h), samples were taken from the side of the membrane without proteins and centrifuged at 4500xg for 30 min. HPLC analysis was done using a UPLC HSS C18 column (150 mm x 2.1 mm with 1.8 µm particle size) (Waters, France) coupled to a UV spectrophotometric detector set at 280 nm. The mobile phase consisted of a mixture of demineralised water, acetic acid, and acetonitrile (83:2:15). 1 µl sample was injected at 0.4 $mL.min^{-1}$ of flow rate and 40°C of temperature. The loss of vanillin by interaction with proteins was calculated by the following relationship: *% Loss of vanillin = (concentration of vanillin in the control - concentration of vanillin in the sample)*100 /concentration of vanillin in the control*. Experiments were performed in triplicate and control samples did not contain proteins. Results were normalised by the protein content in solutions.

## Results and discussion

To understand the impact of protein addition on flavour perception, the sensory profile of plant protein solutions containing vanillin or not were evaluated by a panel. The off-flavours of pure wheat, soy, pea, lupin, and potato protein solutions were mainly described as bitter, herbal, vegetal, cereal, wheat, astringent, flour, metallic, yeast, earthy, metallic, hay, fatty, soapy, and paper cardboard. Among these terms, the most frequents off-flavour descriptors generated for all proteins were: bitter, cereal/wheat, and herbal/vegetal which were later used for sensory evaluations. The off-flavour intensity scores in pure wheat, soy, pea, lupin, or potato protein solutions are presented in Table 1.

**Table 1:** Off-flavours intensity scores of wheat, soy, pea, lupin, and potato protein sweet solutions without vanillin. Analysis of differences between categories (a, ab, b) with a confidence level of 95%.

| *Protein* | *Cereal/Wheat* | *Herbal/Vegetal* | *Bitter* |
|---|---|---|---|
| Wheat | 5,6 [a] | 3,8 [a] | 2,2 [b] |
| Soy | 6,3 [a] | 3,5 [a] | 4,1 [ab] |
| Lupin | 5,8 [a] | 4,9 [a] | 6,0 [a] |
| Pea | 6,6 [a] | 3,4 [a] | 4,2 [ab] |

Results showed that the cereal/wheat flavour was characteristic for most of protein solutions, except for potato protein. Herbal/vegetal flavours were perceived at different degrees among all proteins. Bitterness was mainly pronounced in solutions containing lupin and potato proteins, while it was the least present in wheat protein solutions. Similarly, other studies on soy, pea, and lupin proteins described beany, green, bitter, grassy, metallic, and astringent off-flavours [16], [21], [22]. Especially, green and beany off-flavours in pulse and legume ingredients were explained by the presence of unsaturated lipids susceptible to oxidative deterioration by endogenous lipoxygenases [19], [22]. Changes on the perceived intensity of vanillin flavour and off-flavours (cereal/wheat, herbal/vegetal, and bitterness) of wheat, soy, pea, lupin, and potato protein solutions after addition of vanillin are shown in Figure 1. As expected, the perception of vanillin increased in most of protein solutions after addition of vanillin. However, the perceived intensity of vanillin was different for each protein. The vanillin flavour was best perceived in solutions containing lupin protein, producing an intensity increase of 2.4 significantly higher than the other proteins. In contrast, the vanillin flavour was least perceived in potato protein solutions. Off-flavours seemed to decrease after addition of vanillin in most of protein solutions, expect for potato protein.

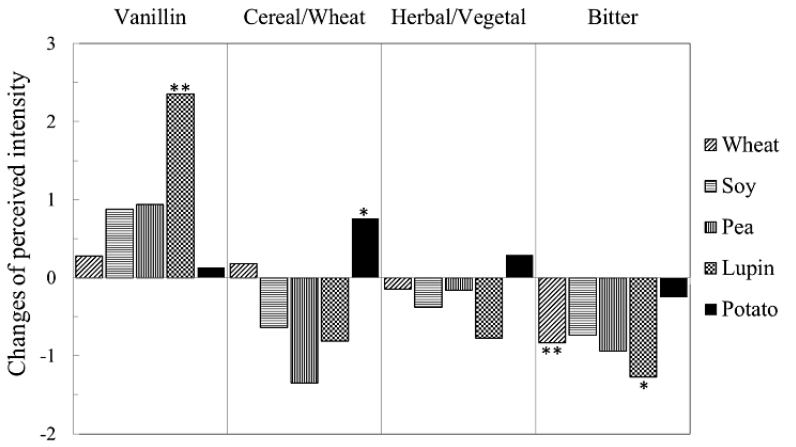


**Figure 1:** Changes in the perceived intensity of bitterness, herbal/vegetal, cereal/wheat, and vanillin flavours in wheat, soy, pea, lupin, and potato protein sweet solutions after addition of vanillin. Significant difference between categories with 90% (*) and 95% (**) of confidence level.

Protein/vanillin interactions were quantified in terms of vanillin loss for wheat, soy, pea, lupin, and potato protein solutions (figure 2). The loss of free vanillin varied

depending on the protein source. The strongest interaction with vanillin was observed for pea protein followed by wheat protein, with a vanillin loss of around 50 % and 22 %, respectively, compared to the control. In contrast, soy, lupin, and potato proteins slightly interacted with vanillin under the tested conditions. Different degrees of flavour retention by plant proteins were expected since there are many factors that can play a role on protein/flavour interactions, and there is no universal mechanism. Protein/flavours interactions have been reported to be mainly of reversible nature in aqueous system [2], [9], [14].

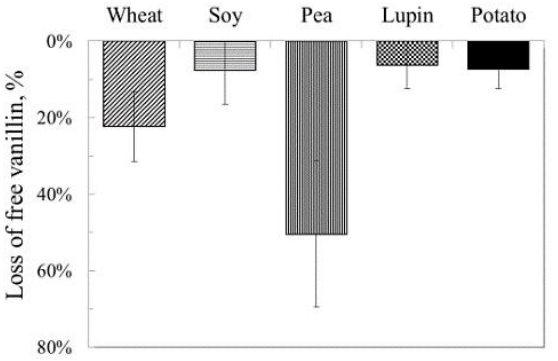


**Figure 2:** Loss of free vanillin (%) by interactions with wheat, soy, pea, lupin, or potato proteins in sweet aqueous systems with respect to the control without vanillin. Values were normalised by the protein content in dry base and error bars represent one standard deviation.

This study suggested that lupin was the most suitable source of plant protein to be used with vanillin, and thus vanilla flavour. Lupin protein had moderated off-flavours, and vanillin was almost not retained by the protein. Therefore, vanillin stayed free and enhanced the vanilla flavour profile of model beverages. In line with this statement, other studies showed that lupin ingredients had cheese-like, milky, fruity, and fatty off-flavours [21]. This creamy-like sensory profile certainly contributed to a better vanillin perception and, simultaneously, to the decrease of off-flavours such as bitterness in lupin protein solutions. On the other hand, potato protein also displayed low interaction with vanillin but did not produce an increase in vanillin perception after its addition. Contrary to lupin, solutions containing potato protein and vanillin displayed a slight increase of cereal/wheat and herbal/vegetal flavours. This was likely due to the strong and characteristic off-flavours related to this protein (i.e. earthy, paper cardboard, algae). So, for masking potato protein off-flavours, we may suggest to use other warm flavours rather than vanilla (e.g. chocolate). Controversially, soy protein did not have strong affinity for vanillin but displayed relatively low vanillin perception. Soy proteins are known to interact reversibly by hydrophobic binding with carbonyl compounds, such as vanillin [6], [13]. Soy protein/flavour interactions were mainly entropy driven, which means that conformational changes of soy protein may be important in binding of vanillin [6], [14], [23]. The traditional extraction of our commercial soy protein could tentatively explain the low interaction with vanillin. Due to thermal treatment and/or acid precipitation, the protein may have aggregated irreversibly and reduced its flavour binding capacity. Anyhow, further research is necessary to evaluate protein denaturation. Finally, in this study, pea and wheat proteins primarily interacted with vanillin. Similar to our findings, previous studies showed that pea globulins had more flavour binding capacity than wheat

gluten [18]. Pea protein/flavour interactions were mainly of hydrophobic nature [16], [17], while for wheat gluten also inter- and intra-molecular disulphide linkages can participate in flavour binding [18]. Interestingly, even if pea protein retained almost twice more vanillin than wheat protein, the later protein obtained lower scores in vanillin perception. Intuitively, we can think that larger retention produces lower flavour perception. However, the type and strength of interactions could also influence the loss of flavour perception. Since our commercial wheat protein was hydrolysed for better solubility, we can think that as a result, gluten peptides increased the number of binding sites and had better access to primary structures, including sulphur-containing residues [1], [3], [6]. Therefore, if disulphide bridges were somehow involved in wheat protein/vanillin interactions, they were probably stronger and more stable as compared to hydrophobic ones, producing larger impact on the flavour perception.

In conclusion, the impact of plant protein (wheat, soy, pea, lupin, and potato) on flavour perception was studied and tentatively correlated to the protein off-flavours and physico-chemical interactions with vanillin in aqueous systems. Understanding these protein/flavour implications is allowing the flavour industry to have better control on the flavour release and the reduction of off-flavours in plant protein based products.

## References


1. Guichard, E. (2002) Food Rev. Int. 18, 49–70.
2. Kuhn J, Condisine, T. and Singh, H. (2006) J. Food Sci. 71, 72–82.
3. Guichard, E. (2006) Biotechnol. Adv. 24, 226–229 (2006).
4. Chobpattana, W., Jeon, I., Smith, J. and Loughin, T. (2002) Food Chem. Toxicol. 67, 973–977.
5. Li, Z., Grun, I. and Fernando, L. (2000) Food Eng. Phys. Prop. 65, 997–1001 (2000).
6. Hansen, A. and Booker, D. (1996) in Flavour-Food Interactions 75–89.
7. Mcneill, V. and Schmidt, K. Vanillin (1993) J. Food Sci. 58, 1142–1147 (1993).
8. Landy, P., Druaux, C. and Voilley (1995) Food Chem. 54, 387–392.
9. Reiners, J., Nicklaus, S. and Guichard, E. (2000) Lait 80. 347–360.
10. Reineccius, G. Flavor chemistry and Technology. (CRC Press Taylor and Francis Group, 2006).
11. Williams, L. A. Top Ten Trends for 2017 by Innova Market Insights Innova Market Insights ' Top 10 Trends for 2017. 28 (2017).
12. O'Keefe, S., Wilson, L., Resurreccion, A. and Murphy, P. (1991) J. Agric. Food Chem. 39, 1022–1028.
13. O'Neill, T. and Kinsella, J. (1987) J. Food Sci. 51, 98–101.
14. Damodaran, S. and Kinsella, J. (1981) J. Agric. Food Chem. 29, 1253–1257.
15. O'Keefe, S., Resurreccion, A., Wilson, L. and Murphy, P. (1991) J. Food Sci. 56, 802–806.
16. Heng, L. et al. (2004) Food Sci. Technol. 15, 217–224.
17. Dumont, J. and Land, D. (1986) J. Agric. Food Chem. 34, 1041–1045.
18. Wang, K. and Arntfield, S. (2014) Food Chem. 157, 364–372.
19. Rackis, J., Sessa, D. and Honig, D. (1979) J. Am. Oil Chem. Soc. 56, 262–271.
20. Kim, K. and O'Mahony, (1998) M. J. Sens. Stud. 13, 241–249.
21. Roland, W., Pouvreau, L., Curran, J., van de Velde, F. and de Kok, P. (2017) Cereal Chem. 94, 58–65.
22. Sessa, D. and Rackis, J. (1977) J. Am. Oil Chem. Soc. 54, 468–473.
23. Damodaran, S. and Kinsella, J. (1981) J. Agric. Food Chem. 29, 1249–1253.

# Decoding the unique peaty aroma of Islay scotch single malt whisky by means of the sensomics concept

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## Abstract

After application of an Aroma Extract Dilution Analysis and Stable Isotope Dilution Assays, 39 odorants with Odour Activity Values ≥ 1 were mixed in their natural concentrations in 40 % ABV (alcohol by volume) ethanol for a recombination experiment to verify the correct characterisation of all aroma impact compounds. The smoky, clove-like and phenolic character of the peaty whisky was caused by a set of 14 phenol derivatives, such as 3-ethylphenol with an Odour Activity Value up to 940. Comparing the concentrations of phenol derivatives in the raw whisky with the matured ready to drink product, it seems that the maturation process also contributes to the smoky aroma by increasing the concentrations of 4-allyl-2-methoxyphenol and 2-ethylphenol, while the other phenol derivatives mainly originated from the special kilning process with peat reek.

## Introduction

Whisky making has a long tradition in Scotland and its islands. After mashing barley malt with yeast, a double-batch distillation yields the raw spirit, which is then aged for at least 3 years in second hand oak casks before bottling as single malt whisky. Especially, whiskies from the island Islay are particularly known to elicit a peaty odour. The malting process on Islay contains the traditional step of kilning with so-called peat reek (peat smoke) which is responsible for the typical smoky and phenolic aroma of the spirit. It is already suggested that this "peatiness" is caused by a spectrum of phenolic compounds including phenol, methylphenol and dimethylphenol derivatives and 2-methoxyphenol with a total amount up to 80 ppm [1-5]. Early studies could correlate the cumulated concentrations of all phenolic compounds to the degree of peatiness [1,2] or identified some phenol derivatives, such as 4-ethyl-2-methoxyphenol and 2-, 3-, and 4-methylphenol in Scotch and Japanese whiskies as aroma impact compounds based odour activity values (OAV) calculated, however, using threshold data in 10 to 20 % ABV ethanol [3-5]. Poisson and Schieberle were the first to fully characterise an American Bourbon whiskey by means of the Sensomics concept. Their investigations resulted in a set of 26 impact aroma compounds, including ethyl (*S*)-2-methylbutanoate, 3-methylbutanal, 4-hydroxy-3-methoxybenzaldehyde and (*E*)-*β*-damascenone with highest OAV [6,7]. They also investigated a peaty whisky from Islay and could trace back the distinctive smoky aroma to the high OAVs of several phenol derivatives, such as 2-methoxyphenol, 4-allyl-2-methoxyphenol and 5-methyl-2-methoxyphenol. However, their recombination experiments did not lead to a satisfying outcome [8] as not all phenol derivatives could be identified. In order to decode the unique aroma with focus on the peatiness of Scotch Single Malt whiskies from Islay on a molecular basis, a whisky from the Ardbeg distillery was investigated by means of the Sensomics concept [9]. Additionally, selected aroma compounds were quantitated in a sample of the corresponding raw whisky to investigate the impact of the maturation process to the smoky aroma of the whisky.

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## Experimental

*Samples.* The whisky "Uigeadail" from the distillery Ardbeg was purchased at a local spirit shop. The shop owner also kindly provided a sample of the raw whisky, intended to be matured into an "Uigeadail" whisky.

*Workup.* After solvent extraction and SAFE (solvent assisted flavour evaporation) distillation and Vigreux column distillation, the concentrated distillate was subjected to aroma extract dilution analysis (AEDA), which was carried out by two panellists to assure the detection of the whole set of important odorants. Impact aroma compounds with high FD factors were quantitated by means of stable isotope dilution assays (SIDA), using $^{13}C$ or $^{2}H$-labelled analogues. OAVs were then calculated by using the respective odour threshold concentration in 40 % ABV ethanol from the literature [7,8,10].

*Sensory trials.* Unavailable odour threshold concentrations were newly determined in 40 % ABV ethanol by a sensory trained panel according to the method reported previously [10]. For a descriptive analysis of the recombinate and the original whisky, the sensory panel was asked to rate the intensities of nine aroma attributes from 0 (no perception) to 10 (very strong intensity) on an unscaled line.

## Results and discussion

AEDA and identification experiments resulted in 36 aroma active compounds with FD factors ranging from 32 to 4096. Next to (*E*)-*β*-damascenone, *cis*-whisky lactone and 4-hydroxy-3-methoxybenzaldehyde with high FD factors, a group of phenol derivatives with FD factors ranging from 4 to 4096 with smoky, phenolic or clove-like odour attributes were identified (data not shown). Based on these data quantitations followed by the determination of OAVs of 44 aroma compounds were carried out. Highest OAVs were found for 3-ethylphenol (940), followed by 3-methybutanal (640), (*S*)-ethyl 2-methylbutanoate (410), ethanol (390), 2-methoxy-5-methylphenol (590) und 2-methoxyphenol (280). Altogether, 39 aroma compounds with an OAV ≥ 1 (Table 1), including 14 phenol and 2-methoxyphenol derivatives, contributed to the complex aroma of the peaty single malt whisky. A recombination experiment with all 39 impact aroma compounds in their natural concentration could mimic the original whisky very well (Figure 1) confirming their correct characterisation as impact aroma compounds. The typical smoky and phenolic aroma of the whisky was generated by the set of phenol and 2-methoxyphenol derivatives with high to very high OAVs, such as 3-ethylphenol, 2-methoxy-5-methylphenol, 4-ethyl-2-methoxyphenol, 4-methylphenol, 2-methoxy-4-propylphenol, 2-methylphenol und more.

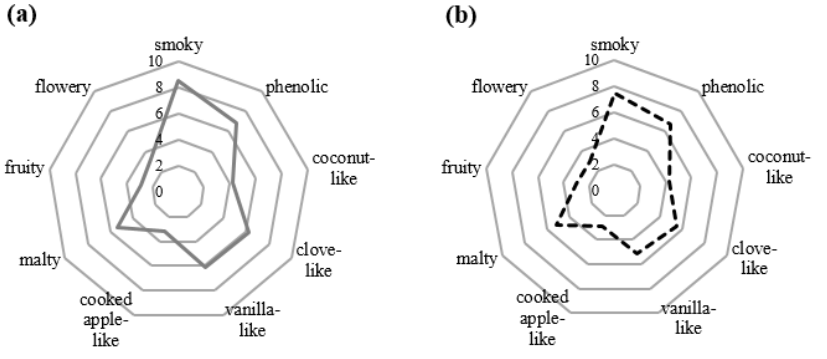


**Figure 1:** Aroma profiles of the original whisky (a) and the corresponding aroma recombinate (b)

**Table 1:** Impact aroma compounds with OAV ≥ 1 of the peaty single malt whisky from Islay.

| odorant | *OAV*[1] | odorant | *OAV*[1] |
|---|---|---|---|
| 3-ethylphenol | 940 | 2-phenylethyl acetate | 29 |
| 3-methylbutanal | 640 | (*E*)-2-nonenal | 29 |
| (*S*)-ethyl 2-methylbutanoate | 410 | 3-methylbutyl acetate | 25 |
| ethanol | 390 | 4-allyl-2-methoxyphenol | 20 |
| 2-methoxy-5-methylphenol | 380 | ethyl cinnamate | 18 |
| 2-methoxyphenol | 280 | 4-ethylphenol | 16 |
| ethyl octanoate | 250 | 3-methyl-1-butanol | 15 |
| (*E*)-*β*-damascenone | 220 | 2-phenylethanol | 14 |
| 4-ethyl-2-methoxyphenol | 200 | 3-methylphenol | 12 |
| ethyl methylpropanoate | 160 | 2-ethylphenol | 10 |
| vanillin | 140 | decanoic acid | 9 |
| ethyl 3-methylbutanoate | 120 | ethyl 3-phenylpropanoate | 9 |
| 4-methylphenol | 97 | 2-methoxy-4-methylphenol | 7 |
| ethyl hexanoate | 80 | methyl-1-propanol | 5 |
| 1,1-diethoxyethane | 68 | *γ*-nonalactone | 5 |
| ethyl butanoate | 67 | phenol | 4 |
| 2-methoxy-4-propylphenol | 52 | acetaldehyde | 3 |
| 2-methylphenol | 46 | 2,3-dimethylphenol | 3 |
| 2-methylbutanal | 43 | 3,5-dimethylphenol | 1 |
| *cis*-whisky lactone | 30 | | |

[1] OAV; odour activity value using odour threshold concentrations in 40 % ABV ethanol.

In order to investigate the impact of the maturation process on the smoky aroma of the whisky, a sample of the raw spirit intended for the production of "Uigeadail" with 69 % ABV ethanol was investigated focussing on known maturation derived compounds, such as *cis*-whisky lactone and 4-hydroxy-3-methoxybenzaldehyde, as well as on the previously identified phenol derivatives. Since the investigated whisky had cask strength (59 % ABV) meaning the spirit did not undergo dilution after the maturation process, the concentrations of the selected compounds in the raw spirit and final whisky were directly compared without conversion.

Next to the typical maturation derived compounds, only two phenol derivatives, such as 2-ethylphenol and 4-allyl-2-methoxyphenol showed noteworthy concentration increases after oak cask maturation. The remaining phenol derivatives were already present in the raw whisky, thus confirming their origin from the peat smoke used for kilning the malt. Minor concentration differences could be explained by the use of different starting material and vintage.

**Table 2:** Concentrations of impact aroma compounds in the raw and matured whisky.

| odorant | *concentration [µg/L]* raw whisky | matured whisky | increase/ decrease [%] |
|---|---|---|---|
| *maturation compounds* | | | |
| *cis*-whisky lactone | < 1.3 | 2000 | +150000 |
| vanillin | 23.1 | 3140 | + 13500 |
| *phenol derivatives* | | | |
| 2-ethyphenol | 411 | 870 | + 112 |
| 4-allyl-2-methoxyphenol | 89.2 | 139 | + 56 |
| 3-ethylphenol | 444 | 537 | + 21 |
| 2-methoxy-4-propylphenol | 88.6 | 97.6 | + 10 |
| 4-ethyl-2-methoxyphenol | 1380 | 1370 | - 1 |
| 2-methylphenol | 4290 | 4120 | - 4 |
| 2-methoxy-4-methylphenol | 2010 | 1790 | - 11 |
| 4-methylphenol | 3260 | 2900 | - 11 |
| 4-ethylphenol | 3330 | 2740 | - 18 |
| 3-methylphenol | 1770 | 1400 | - 21 |
| 2-methoxyphenol | 3480 | 2600 | - 25 |
| 2-methoxy-5-methylphenol | 236 | 122 | - 48 |

## Conclusions

By applying the Sensomics concept to the Single Malt Scotch whisky from Islay, its aroma could be successfully characterised. The typical smoky and phenolic aroma was traced back to the multiplicity of phenol and 2-methoxyphenol derivatives with high OAVs. The additional investigation of the raw spirit confirmed their origin mainly from the peat reek used for malt kilning in the making process of these especially peaty whiskies.

## References

1. Bathgate, G.N. and Cook, R. (1989), In: The science and technologies of whiskies. Piggott, J.R.; Sharp, R.; Duncan, R.E.B. (Eds.), Longman Scientific & Technical, Harlow Text.
2. Bathgate, G.N. and Taylor, A.G. (1997) J. Inst. Brew, 83(3), 163–168.
3. Nishimura, K. and Masuda, M., (1971) J. Food Sci. 36, 819–822.
4. Swan, J.S. and Burtles, (1978), S.M. Chem. Soc. Rev., 7, 201–223.
5. Jounela-Eriksson, P.; Lehtonen, M., (1981), Quality of Food and Beverages: Chemistry and Technology. 1, 167–181.
6. Poisson, L. and Schieberle, P. (2008), J. Agric. Food Chem. 56, 5813–5819.
7. Poisson, L. and Schieberle, P. (2008), J. Agric. Food Chem. 56, 5820–5826.
8. Poisson, L. Dissertation, Technical University of Munich (Prof. Dr. P. Schieberle), (2003).
9. Schieberle, P. and Hofmann, T. (2011), In: Food Flavors - Chemical, Sensory and Technological Properties (Jelen, H.; Ed.); CRC Press, Taylor and Francis Group, 411-437.
10. Czerny, M.; Christlbauer, M.; Christlbauer, M.; Fischer, A.; Granvogl, M.; Hammer, M.; Hartl, C.; Hernandez, N. and Schieberle, P. (2008), Eur. Food Res. Technol., 228, 265–273.

# Flavour generation from microalgae in mixotrophic cultivation

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## Abstract

Microalgae are known to produce several volatile organic compounds that can be obtained from the biomass or released extracellularly into the medium. The aim of this study was to evaluate the generation of volatile organic compounds with flavour potential from the microalga *Phormidium autumnale* in mixotrophic cultivation. The experiment was conducted in a New Brunswick Scientific BioFlo®310 bioreactor operating under a batch system, with a 1.5 L working volume. The experimental conditions were as follows: initial inoculum concentration 100 mg $L^{-1}$, temperature 25°C, pH adjusted to 7.6 and aeration of 1.0 volume air per culture volume per minute, supplemented with 5 $g.L^{-1}$ of sucrose and constant light intensity of 4 klux. The volatile compounds were isolated by solid phase micro-extraction applied in headspace of residence time (144 hours), separated by gas chromatography and identified by mass spectrometry (HS-SPME-GC/MS), co-injection of standards and Kovats index. The major products in the bioreactor were 2,4-decadienal (46.03%), 3-methyl-1-butanol (12.39%), hexanol (4.17%) and 2-ethyl-1-hexanol (3,51%). The descriptor flavour of the compounds detected in experiments was mainly classified as fried food, fruity, spice, and floral compounds. In conclusion, the results have shown that the mixotrophic cultivation of the *Phormidium autumnale* could be a potential biotechnological to produce natural flavours.

## Introduction

Microalgae are a group of photosynthetic microorganisms typically unicellular and eukaryotic. Although cyanobacteria belong to the domain of bacteria, and are photosynthetic prokaryotes, they are often considered microalgae [1]. Microalgae and cyanobacteria are considered some of the most promising feedstocks for the supply of food and nonfood industries [2; 3]. Because they present a high content of macronutrients (proteins, carbohydrates, and lipids), microalgae have the potential to enhance the nutritional value of foods [4]. They may also be used as a feed source for many aquatic organisms and livestock [5]. Microalgae-based systems for chemicals production are an emergent area, representing a great promise for industrial application.

The growing interest in natural products guides the development of the technologies that employ microorganisms, including microalgae, which are able to synthesize specific volatile organic compounds. Therefore, the selection of a mode of cultivation of microalgae is of vital importance. Four major modes of microalgae cultivation can be adopted, namely photo-autotrophic, heterotrophic, photo-heterotrophic, and mixotrophic [6]. Mixotrophic microalgae use different energy and carbon sources so that they may use organic or inorganic sources and light in different combinations. Mixotrophy makes microalgae more flexible because it may gather both the carbon and energy demand from organic or inorganic sources and light simultaneously [7].

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The occurrence of volatile organic compounds in microalgae is a consequence of their versatile metabolism. The compounds produced may belong to different classes of compounds such as esters, alcohols, hydrocarbons, ketones, terpenes, carboxylic acids and sulphur compounds [8, 9]. Many of these volatiles present odour descriptors such as floral, fruity, spice, sweet, roasted, and can, therefore, be used as a flavouring agent in the food industry and others used in the pharmaceutical and fine chemicals industries.

Thus, the objective of this study was to evaluate the generation of volatile organic compounds with flavour potential from the microalga *Phormidium autumnale* in mixotrophic cultivation.

## Experimental

*Microorganism and culture conditions*

Axenic cultures of *Phormidium autumnale* were originally isolated from the Cuatro Cienegas desert (26°59′ N, 102°03′, W. Mexico). Stock cultures were propagated and maintained in solidified agar-agar (20 g $L^{-1}$) containing BG11 medium [10]. The cultures were illuminated with 20 W fluorescent day light-type tubes (Osram Sylvania, Brazil), located in a photo period chamber at a photon flux density of 15 μmol photons $m^{-2}s^{-1}$ and a photoperiod of 12/12 h light/dark at 25°C. The photon flux density was adjusted and controlled by using a digital photometer (Spectronics, model XRP3000). To obtain the inoculum in liquid form, 1 mL of sterile medium was transferred to slants, and the colonies were scraped off and then homogenized with the aid of mixer tubes. The entire procedure was performed aseptically.

The experiment was conducted in a New Brunswick Scientific BioFlo®310 bioreactor operating under a batch system, with a 1.5 L working volume. The bioreactor including filtration units was sterilized by autoclaving at 121ºC for 20 min. The experimental conditions were as follows: initial concentration of inoculum of 100 mg $L^{-1}$, temperature of 26°C, pH adjusted to 7.6, aeration of 1.0 VVM (volume of air per volume of culture per minute per minute). The culture medium consisted of a BG11 synthetic medium supplemented with 5g $L^{-1}$ of sucrose and a constant light intensity of 4 klux.

*Isolation of the volatile organic compounds*

The volatile organic compounds were analysed at 144 h of the residence time using headspace solid-phase micro-extraction (HS-SPME) with a 50/30μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, USA). Sample preparation was performed using 20 mL of culture medium, equally separated into two portions. Each of these portions was analysed by HS-SPME coupled with GC/MS for the quantitative determination of the volatile compounds. The aliquot was placed in a headspace septum vial containing 3 g of NaCl. The SPME fiber was inserted into the headspace of the vial containing the sample (previously kept at 40ºC for equilibration temperature) for 45 min at 40°C, with agitation provided by a magnetic stir bar. After this period, the fiber was removed from the vial and immediately desorbed into the injector of the GC. The analytical procedure was performed twice and in duplicate. Therefore, the data refer to the mean value of two repetitions.

*GC/MS analysis*

The volatile organic compounds were analysed in a GC system (Agilent 7890A) coupled to a mass spectrometer detector (Agilent 5975) using a DB-Wax fused silica

capillary column (60 m in length, 0.25 mm id and 0.25 μm film thickness). The initial oven temperature was held at 35°C for 5 min., followed by a linear increase at 5°C/min to 220°C, and held at this temperature for 5 min. For the identification of the compounds was based on GC-MS, electron-impact ionization voltage of 70 eV was applied, and helium was used as the carrier gas. The volatile compounds were identified by a comparison of their MS spectra with those provided by the computerized library (NIST MS Search). In addition, to assist with identification, each volatile linear retention index (LRI) was calculated using the retention times of a standard mixture of paraffin homologues prepared in hexane and compared with the LRI values published in the literature for columns with the same polarity (www.flavornet.net). Co-injection of the sample and the standard mixture provided experimental LRIs for the compounds, which were compared with those of standards analysed under similar conditions.

## Results and discussion

The volatile organic compounds produced by *Phormidium autumnale* cultivated in mixotrophic conditions are presented in Table 1. A total of 16 compounds (aldehydes, alcohols, ketones, and hydrocarbons) with different odour descriptors were found. Among the chemical classes identified, 2,4-decadienal (46.03%), 3-methyl-1-butanol (12.39%) and 1-hexanol (4.17%) were the major compounds identified.

**Table 1:** Volatile organic compounds produced by *Phormidium autumnale* cultivated in a mixotrophic microalgal reactor. The odour description presented was extracted from the literature in comparison to the compound name, chromatographic column and Kovats index (www.flavornet.org).

| Compound | *Kovats Index* | *Description of odour* | *Relative peak area (%)* |
|---|---|---|---|
| acetaldehyde | 714 | pungent, ether | 2.37 |
| hexanal | 1084 | grass, tallow, fat | 1.96 |
| 2-methyl-1-propanol | 1099 | wine, solvent, bitter | 0.73 |
| 3-methyl-1-butanol | 1205 | whiskey, malt, burned | 12.39 |
| 1-pentanol | 1255 | balsamic | 0.75 |
| 1-hexanol | 1360 | resin, flower, green | 4.17 |
| 2-octenal (E) | 1408 | green | 1.62 |
| (E,E)-2,4-heptadienal | 1463 | nut, fat | 3.02 |
| 2-ethyl-1-hexanol | 1487 | rose, green | 3.51 |
| benzaldehyde | 1495 | almond, burnt sugar | 0.57 |
| hexadecane | 1600 | alkane | 3.28 |
| 2-octen-1-ol (E) | 1608 | soap, plastic | 0.72 |
| acetophenone | 1645 | must, flower, almond | 1.43 |
| 2,4-decadienal (E,E) | 1710 | fried, wax, fat | 46.03 |
| trans-geranylacetone | 1840 | green | 1.83 |
| β-ionone | 1912 | seaweed, flower, raspberry | 0.82 |
| Other Compounds | | | 14.80 |
| *Total* | | | *100* |

Mixotrophic cultivation occurs when the microalga uses photosynthesis and oxidation of organic compounds concomitantly: the oxygen produced in the photosynthesis is consumed in the heterotrophic route. At the same time, the carbonic gas generated in the oxidation of the organic compound is exploited in photosynthesis. This cultivation is already widely exploited in terms of biomass production [6, 7]. The volatile organic compounds biosynthesis mainly depends on the availability of carbon and

nitrogen as well as energy provided by primary metabolism. The formation of volatile organic compounds can occur during both primary and secondary metabolism of microorganisms as secondary products, thereby we can suggest that the presence of these compounds is due to the secondary metabolism of these microorganisms.

According to Santos [8], aldehydes proved to be the most prevalent volatile organic compounds and, due to their low odour threshold values, might be important headspace volatiles compounds contributing to desirable aromas as well as rancid odours and flavours. Saturated aldehydes have a green-like, hay-like, paper-like odour, whereas unsaturated aldehydes have a fatty, oily and frying odour. Whereas the shorter chain linear aldehydes are often derived from chemical lipid oxidation, branched and aromatic aldehydes are typically formed due to enzymatic lipid and protein oxidation.

Microalgae can produce a variety of industrially relevant volatile compounds that can represent an improvement in the supply of a large volume of inputs for different types of industry (odour, flavours, energy).

In conclusion, the results show that the mixotrophic cultivation of the *Phormidium autumnal* could be an alternative to obtain flavours by this biotechnological route. More knowledge about the biochemical routes should be taken into account, thereby increasing the production of compounds of interest and the use of all the products generated during the bioprocess.

## References

1. Bueno, S.; Langellotti, A.L.; Martello, A.; Rinna, F. and Fogliano, V. (2014). Food & Function 5:1669-1685.
2. Borowitzka M.A (2013) J Appl Phycol 25:743–756.
3. Santos, A. B.; Fernandes, A. S.; Wagner, R.; Jacob-Lopes, E. and Zepka, L. Q (2016) J Appl Phycol, 60:32-42.
4. Robertson, R. C., Mateo, M. R. G., O'Grady, M. N., Guiheneuf, F., Stengel, D. B., Ross, R. P., Fitzgerald, G. F., Kerry, J. P., and Stanton, C. (2016). Innovative Food Science and Emerging Technologies, 237-246.
5. Hosoglu, M. I. (2017) Food Chem, Accepted Manuscript.
6. Wang J, Yang H, and Wang F (2014) Appl Biochem Biotechnol 172:3307–3329.
7. Perez-Garcia, O. and Bashan, Y. (2015). Algal Biorefineries. 61-131.
8. Santos, A. B.; Vieira, K. R.; Nogara, G. P.; Wagner, R.; Jacob-Lopes, e. and Zepka, L. Q. (2016) In: Julian Patrick Moore. (Org.). Volatile Organic Compounds: Occurrence, Behavior and Ecological Implications. 1ed.: Nova Science Publishers, pp. 1-18.
9. Papaleo, M.C; Romoli, R.; Bartolucci, G. and Maida, I. (2013). New Biotechnology 30:824-838.
10. Rippka, R., Derueles, J., Waterbury, J.B., Herdman, M., and Stanier, R.Y. (1979) J Gen Microbiol 111:1–61.

# Unravelling the complexity of a savoury fermented product using a holistic sensory-analytical approach

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## Abstract

The fermented corn starch hydrolysate reveals more complexity in savoury flavour that goes beyond the umami-taste of MSG, despite the presence of glutamic acid amongst other amino acids. Glutamyl-dipeptides were identified as major class of known taste compounds present in the savoury paste but, at the found concentrations, their impact is not sufficient to match the product's taste. Fru-Glu was found as the major Amadori compound. However, its individual taste impact could not be proven. Additionally, sensory-guided fractionation has revealed a class of N-acyl derivatives of amino acids as possible taste-actives in that fermented product. Taste activity for some derivatives has already been described elsewhere but their final impact on the taste profile of the current product is currently under investigation.

## Introduction

In culinary food products, umami compounds like monosodium glutamate (MSG) and 5′-nucleotides are often used to impart savoury taste. As consumers get increasingly more sensitive to the addition of such pure ingredients, which are classified as flavour enhancers, alternative natural sources have gained interest in the past years such as products obtained through the fermentation of different raw materials (e.g., wheat gluten, soybean). In the past years, several studies have been conducted to determine the presence of taste-active or modifying compounds in such products, mainly in soy sauce [1,2]. However, the link to the sensory characteristics of the products was studied less intensely and the role of the individual taste compounds on the overall flavour remains in many cases questionable [3]. The fermented savoury product herein investigated (Savoury Base 100) is produced by fermentation of hydrolysed corn starch using *C. glutamicum*, a Gram-positive bacterium. *C. glutamicum* has been widely used for industrial production of amino acids, such as L-glutamic acid, and fermented cereals. The aim of the study was to unravel the complex savoury flavour by combination of analytical and sensory approaches.

## Experimental

The investigated product (Savoury Base (SB) 100) is produced by fermentation of hydrolysed corn starch using non genetically-modified proprietary strains of *Corynebacterium* sp. (*Corynebacterium glutamicum* ATCC 13032).

Sensory assessment was performed with a trained panel using nose-clips for descriptive and comparative profiling either in water or model broth.

Glutamyl-peptides and Amadori compounds were quantified by LC/MS in MRM mode on a BEH amide (Waters) using isotopically labelled standards. Nucleotides were quantified by LC-UV using a PBr column (Cosmosil) and external calibration. Determination of basic composition was performed by ion chromatography for sugars, amino acids, organic acids and minerals with external calibration.

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Ultrafiltration was performed using a stirring cell and membranes of 1 kDA cut-off. Further fractionation of the low molecular weight fraction was performed by 1D preparative HPLC and 2D-fractionation with a Sepbox (Sepiatec) using the polar set-up.

HR-mass spectroscopy of individual fractions was performed using a BEH amide column and MS detection was done on a Q Exactive Focus (Thermo) in full scan mode and auto MS/MS of the 3 most abundant ions at three different collision energies.

For structure elucidation, NMR of isolated peaks was performed on a 600 Mz NMR from Bruker using 1D/2D experiments.

## Results and discussion

The savoury powder used in this study contains a specific intrinsic mix of various compounds, including amino acids, organic acids, and minerals. The considerable amounts of glutamic acid raised the question how the sensory profile of this novel ingredient is compared to a pure MSG solution. Sensory evaluation (Fig. 1) performed with a trained panel (n=6) wearing nose-clips comparing MSG and the savoury base at same glutamic acid level, revealed that the umami taste of the fermented savoury product is higher than a pure MSG solution. In addition, a simple recombinant including basic tastants (NaCl, glutamic acid, acetic acid, pH adjusted) did not match the initial product's taste either. Panellists described the taste of SB100 as being more complex and round.

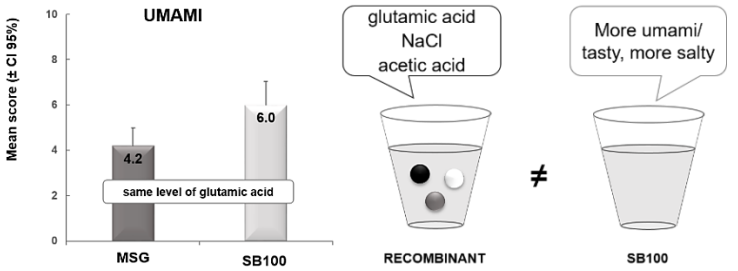


**Figure 1**: Sensory evaluation of MSG and savoury base providing same amount of glutamic acid (left) and comparison of simple recombinant with entire product SB 100 (right)

Based on the gap identified by the sensory panel, known taste-active molecules were quantified in the corresponding product and their individual contribution to overall taste was evaluated by calculating dose-over-threshold (DoT) values (Table 1). Glutamic acid was found to be the dominant amino acid with a DoT value of 6.4 followed by alanine and proline as second most abundant amino acids. Amongst the well-known γ-glutamyl-dipeptides, Glu-Glu and Glu-Gln were found as the most abundant members of that family, beside small amounts of other derivatives. The DoT values of these di-peptides were below their reported taste thresholds. However, it is known that these peptides have taste-modulating properties rather than showing taste-activity on their own. In addition, Amadori compounds were identified, with N-(1-deoxy-D-fructos-1-yl)-L-glutamic acid (Fru-Glu) as the main compound beside traces of other Amadori compounds. These Amadori compounds were also below individual threshold but might be of importance due to modifying properties. Other compounds that were identified were minerals and organic acids with NaCl and acetic acid, respectively, being the dominating ones, as well as sugars and ribonucleotides found in trace levels in SB100. Among those, most individual compounds were found well below their individual taste thresholds except for

NaCl, and acetic acid, which showed DoT-factors of 0.6 and 0.3, respectively. A recombinant sample, including compounds with DoT >0.1, still showed a gap in taste profile indicating the presence of other compounds contributing to the taste of the savoury powder.

**Table 1:** Concentrations and Dose-over-threshold factors of taste compounds found in SB 100

| *Taste* | *Compound* | *Av. Conc. (% dm)* | *DoT at 0.2% solution* |
|---|---|---|---|
| *umami* | Glutamic acid | 52.1 | 6.4 |
| | Glutamyl-peptides | 1.8 | < 0.01 |
| | Fru-Glu | 0.7 | 0.03 |
| | Ribonucleotides | 0.1 | < 0.01 |
| | Other Amadori | 0.2 | n.c |
| *salty* | NaCl | 4.9 | 0.6 |
| | Other minerals | 0.9 | < 0.01 |
| *sour* | Acetic acid | 1.7 | 0.3 |
| | Other acids | 0.3 | < 0.01 |
| *other* | Alanine | 1.8 | 0.05 |
| | Proline | 1.1 | 0.01 |
| | Sugars | < 0.1 | < 0.01 |
| | Other free amino acids | 0.9 | < 0.01 |
| | Nucleosides | 0.2 | < 0.01 |

As the recombinant of the known taste-active compounds showed no taste match with the initial powder, the product was submitted to a sensory-guide fractionation approach, using ultrafiltration (1 kDa) followed by preparative HPLC of the LMW fraction. Each fraction was then tested in water or model broth (MSG, NaCl, sucrose) for any taste activity (Fig. 2). Sensory evaluation of fractions revealed two fractions showing low taste activity when tasted alone in water, but having umami (F3 and F4) and salt modulating effects (F4), when tasted in a model broth.

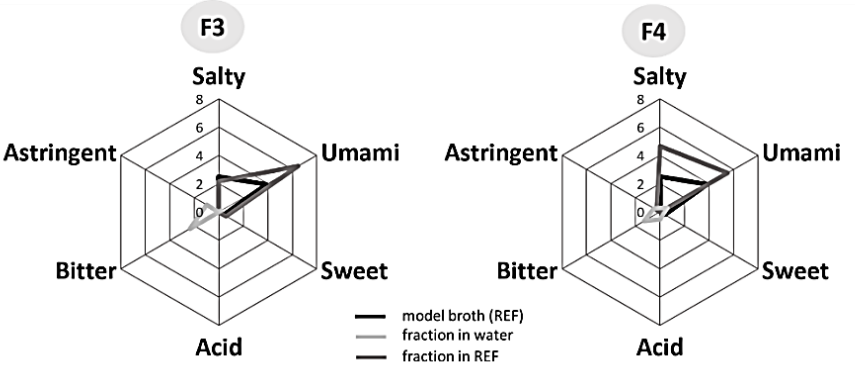


**Figure 2**: Sensory evaluation of most taste-active fractions F3 and F4

Taste-active fractions were then submitted to LC-HR-MS analysis and a library search was performed for first identification of peaks. This confirmed the presence of the already quantified glutamyl-peptides in fractions F3 and F4. Recombination of these peptides was nevertheless not sufficient to mimic the taste properties of fractions F3 and F4. Thus, the most prominent unknown peaks were isolated by preparative LC and structure elucidation was performed by means of NMR and HR-MS/MS. This led e.g. to the identification of N-acetyl-glutamine in fraction F4. By using molecular networks of the MS data several more of N-acyl derivatives of amino acids could be tentatively identified. Confirmation by reference molecules and sensory evaluation is currently ongoing and structure elucidation of further unknown compounds is also in progress. The results so far show that the fermented savoury base is a complex mixture of several taste-active and taste-modifying molecules, which probably contribute even in sub-threshold concentrations to the complex taste of the product by additive, synergistic and modulating effects.

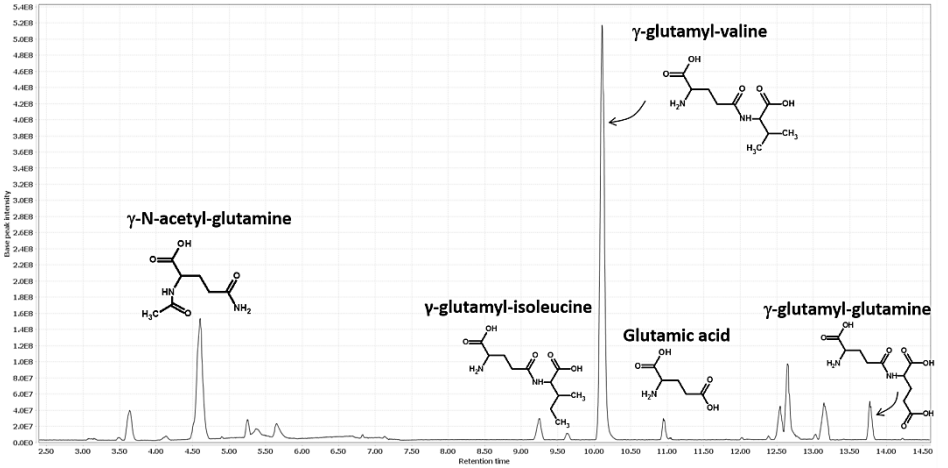


**Figure 3**: HR-MS chromatogram of fraction F4 containing taste-active/modifying molecules

## References

1. H. Katayama, Y. Tatemichi, and A. Nakajima, (2017), Food Chemistry, 228, 279-286
2. M. Zhuang, L. Lin, M. Zhao, Y. Dong, D. Sun-Waterhouse, H. Chen, C. Qiu, and G. Su, (2016) Food Chemistry, 206, 174-181
3. S. Yamamoto, K. Shiga, Y. Kodama, M. Imamura, R. Uchida, A. Obata, T. Bamba, and E. Fukusaki, (2014) Journal of Bioscience and Bioengineering, 118, 56-63
4. S. Tafazoli, T. Vo, A. Petersen, A. Constable, M. Coulet, P. Phothirath, J. Lang, and N. Baldwin, (2017) Regulatory Toxicology and Pharmacology, 87, 71-87

# Effects of drying methods on the composition of volatile compounds in fruits and vegetables

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## Abstract

The mixtures of different volatile and non-volatile compounds create the unique aroma and taste of foods. Aroma, derived from combinations of volatile components, is essential for determining the quality of foods. Aroma profile of dried foods may be affected by loss, destruction, change or improvement of unexpected flavours during drying processes. The Maillard reaction and autoxidation are the main chemical reactions responsible for the formation of new compounds during drying. These reactions have considerable effects on the flavour of dried fruit and vegetables. The Maillard reaction derived compounds are classified in three groups which are sugar dehydration/fragmentation products (furans, pyrones, cyclopentenes, carbonyl compounds and acids), amino acid degradation products (aldehydes, sulphur compounds and nitrogen compounds) and volatiles produced by further interactions (pyrroles, pyridines, pyrazines, imidazoles, oxazoles, thiazoles and thiophene). Some of the flavour compounds (aldehydes and esters) might be formed through lipid oxidation or biosynthesis of alcohols and acids. The concentration of volatile compounds and activity of volatile forming enzymes are affected by drying methods and conditions. Besides that, loss of the precursors may also cause the loss of volatile compounds after drying. Conventional drying techniques adversely affect colour, aroma and flavour due to increased temperature and long exposure to heat and oxygen. On account of the negative effects of conventional drying processes, freeze drying and vacuum drying have been alternatively used in recent years. These technologies are expensive and time consuming; even they preserve flavour better than conventional drying. This review highlights the effects of drying methods on the volatile compounds of fruits and vegetables.

## Introduction

Fruits and vegetables are readily perishable foods because of their high moisture content [1]. Drying of fruits and vegetables are important in preserving food quality, forming suitable option for economic postharvest management, increasing food safety and shelf-life. In the drying process, water is removed to slow down or stop the existent chemical reactions, in addition to inhibit growth of spoilage microorganisms [2]. However, drying leads to loss and change in volatiles (e.g. stripping process, oxidation and thermal degradation), formation of new volatile compounds (e.g. enzymatic reactions, Maillard reaction and lipid oxidation), and negative impact on colour, texture and nutritional value [3, 4, 5].

Different drying methods are commercially utilized to remove moisture from fruits and vegetables. These methods are basically divided into three subgroups; solar drying, atmospheric drying (e.g., tunnel, cabinet, fluidized bed, spray and microwave drying) and sub atmospheric drying (e.g. vacuum and freeze drying) [6]. In order to maintain the characteristic aroma of fruits and vegetables during drying, novel or improved drying methods have been developed [3]. Therefore, this study is aimed to collect recent information on volatile flavour compounds of dried fruits and vegetables.

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## Drying methods

*Sun and solar drying:* In sun drying, sunshine is used to dehydrate fruits and vegetables which are spread out under the sun and dried. It is widely used in tropical and semitropical countries due to its low cost by using free renewable energy source, whereas there are adverse effects of this method such as inapplicability in all seasons and hygiene problems [6]. The different solar drying methods use equipment to gather sunrays in a unit. Compared to sun drying, the temperature in solar unit is usually 20-30°C higher. The handicap of these methods is that fruits and vegetables that are dried outdoors must be covered during cool nights because air condenses and can moisturize foods back [2].

*Conventional drying*: Drying times in conventional driers change remarkably, depending on room temperature, humidity, the amount of food and its moisture content. Air temperature and circulation are important aspects which should be controlled during drying. When the temperature is too low, the food will dry slowly and microbial growth may occur, but if the temperature is too high, a hard shell can develop and the inside of product remains wet [2].

*Tunnel dryers:* The tunnel driers consist of fans, heaters and wagons in which products are carried. During drying the wagons are moved in the tunnel. Tunnel dryers decrease the drying time and enable closer control of moisture content [7, 8, 9].

*Drum dryers:* Drum dryers consist of a cylinder which is heated on the inside and turns continuously. During drying, the product is carried out in a thin film on the outside of the drum and dries quickly. After every rotation, the dry solid is scraped off the roll, which is revolving slowly. This method is convenient for highly or low viscous foods [10].

*Spray drying:* Spray dryers are used to remove moisture from foods especially those in puree or liquid forms. In this method, atomization and evaporation of water are carried out when the dispersed / sprayed material passes through the drying chamber. Higher drying rates, low energy consumption, preservation of food quality and prevention of oxidation are the main advantages of spray drying [2, 10].

*Freeze drying:* Freeze drying technique uses extreme cold temperatures as low as -50°C in a wide variety of products [11]. In regards to low processing temperatures applied in this method, thermal degradation reactions are excluded, high aroma retention and high quality product is attainable with excellent rehydration properties [12].

*Microwave drying:* Microwave drying is an another alternative method with various advantages like providing higher drying rate, shorter drying time, homogeneous energy delivery on the material and better process control [13, 14, 15, 16].

*Vacuum drying:* Vacuum drying is used under reduced pressure, which enables food to be dried at lower temperatures. With this method, oxidation reactions are inhibited due to the absence of air while the flavour, colour and texture of the dried foods are maintained [10, 17].

## The volatile flavour compounds of dried fruits and vegetables

More than a few hundred volatile compounds are present in fruits and vegetables. Many vegetables contain aroma compounds such as allicin in garlic [18] terpenes, sesquiterpenes, styrene, alkanes and a few alcohols in carrots [1], sulphur compounds, alcohols and esters in shiitake mushrooms [19] and sesquiterpene lactones in chicory and lettuce [20, 21]. Moreover, citrus fruits such as lemon and orange are abundant in terpenoids, while aroma compounds of the other non-citrus fruits such as banana, apple,

apricot and cranberry are described by esters and aldehydes [22]. These volatile compounds may change, be lost or form new compounds during drying with some reactions such as stripping process, oxidation, thermal degradation, enzymatic and non-enzymatic reactions.

Nunes et al. [23] reported that among thirty-one volatile compounds of fresh guava fruit, terpenes were predominant even after oven (55°C, 22 h) and freeze drying (50°C, 0.025 mbar, 48 h) processes. However, aldehydes and esters were other main compounds diminished by dehydration of guava fruit.

Allicin, which is the principal volatile of organosulfur compound in garlic, was affected by drying time and temperature when dried convectively at 50 and 60°C, respectively, with airflow of 1.5 m/s. Allicin retention after drying was significantly affected by temperature and variance in the structural properties of garlic. Researchers reported that drying at 60° C lowered loss of allicin content [18].

Rajkumar et al. [1] showed that freeze drying is an extremely useful technique for higher aroma retention in carrots. They also indicated that terpenes had a greater effect in giving aroma to the samples. The key flavour components of fresh carrots were mostly kept during drying.

Narain et al. [24] evaluated the retention of volatile compounds in tomato juice and its products (A: prepared with 5% maltodextrin, B: prepared with 5% tapioca flour) dehydrated by a forced air circulation dryer (temperature: 60°C, relative humidity: 25%, air velocity: 5 m/min). The volatiles, mostly sulphur compounds, were more retained in product A than product B. The concentration of dimethyl sulphide, hydroxymethyl furfural, acetaldehyde, 2-ethyl furan and $\alpha$-terpineol in tomato powder rose with drying, whereas ethanol and geranyl butanoate decreased during dehydration.

In another study reported by Huang et al. [25], aroma composition of apple slices dried by a combination of freeze drying and microwave-vacuum drying (A) was evaluated and compared with only freeze dried (B) samples. They also indicated that volatile compounds in apple slices were classified as esters (principal compounds in apple), aldehydes, alcohols and acids. From the results of aroma retention between drying methods applied, researchers observed that dried apple slices by B application were retained aroma better than A application.

Shiga et al. [26] studied the influences of spray drying on powdery encapsulation of shiitake flavours. It was reported that flavour retention increased with the rise of drying air temperature and solid content and decreased with the rise of dextrose equivalents of maltodextrin. Lenthionine concentration was increased with heat treatment but other flavours were not affected by heat treatment.

The study of Jeyaprakash et al. [27] was attempted to identify the effects of heat pump dehumidifier dryer on flavour retention of tomato samples and compared with fresh, freeze dried and commercial spray dried samples. The quality parameters were determined as volatile, non-volatile and odour intensity. Heat pump dried tomato showed better retention with regard to volatile and sensory profiles of tomatoes than freeze drying. However, loss of the fresh aroma compounds (*E*)-2-hexenal, 1-penten-3-one, 1-hexanol) and the availability of heat induced compounds (dimethyl sulphide, furfural, pyrrole) were identified in spray dried tomato samples.

## Conclusion

Consumers demand processed products, which retain their original properties. During drying, important flavour components could degrade and be lost due to high

temperatures and long drying times. Among the drying technologies, freeze drying, vacuum drying and heat pump drying offer great scope for the dried products retaining aroma components.

## References


1. Rajkumar, G., Shanmugam, S., Galvao, M.S., Neta, M.T.S.L., Sandes, R.D.D., Mujumdar, A.S., and Narain, N. (2017) Drying Technology 35 (6): 699-708.
2. Nabais, R. (2010) In: Handbook of fruit and vegetable flavors (Hui, Y.H., ed.), John Wiley & Sons, Inc., pp.487-514.
3. Nijhuis, H.H., Torringa, H.M., Muresan, S., Yuksel, D., Leguljt, C., and Kloek, W. (1998) Trends in Food Science & Technology 9:13-20.
4. Phoungchandang, S., and Saentaweesuk, S. (2010) Food and Bioproducts Processing 89 (4): 429-437.
5. Ding, S.H., An, K.J., Zhao, C.P., Li, Y., Guo, Y.H., and Wang, Z.F. (2012) Food and Bioproducts Processing 90: 515-524.
6. Komes, D., and Ganic, K.K. (2010) In: Handbook of fruit and vegetable flavors (Hui, Y.H., ed.), John Wiley & Sons, Inc., pp.487-514.
7. Wrolstad, R.E., Lombard, P.B., and Richardson, D.G. (1991) In quality and preservation of fruits (Eskin, N.A. ed.), Boca Raton, FL: CRC Press, pp. 67-95.
8. Singh, R.P., Heldman, D.R. (2015) Introduction to food engineering. Nobel Publication, Ankara, pp.859.
9. Kutlu, N., İşçi, A., and Şakıyan Demirkol, Ö. (2015) Gıda, 40(1): 39-46.
10. Geankoplis, C.J. (2011) Transport Processes and Unit Operations, Pearson, Turkey, pp. 1052.
11. Nawirska, A., Figiel, A., Kucharska, A. Z., Sokol-Letowska, A., and Biesiada, A. (2009) Journal of Food Engineering 94 (1): 14-20.
12. Coumans, W.J., Kerkhof, P.J.A., Bruin, S. (1994) Drying Technology 12 (1-2): 99-149.
13. Maskan, M. (2000) Journal of Food Engineering 44: 71-78.
14. Zhang, M., Tang, C.J., Mujumdar, A.S., and Wang, S. (2006) Trends Food Sci Technol 17(10): 524-534.
15. Arslan, D., and Ozcan, M.M. (2010) LWT-Food Science and Technology 43: 1121-1127.
16. Incedayı, B., Tamer, C.E., Özcan Sinir, G., Suna, S., and Çopur, Ö.U. (2016) Food Science and Technology: Campinas 36(1): 171-178.
17. Zielinska, M., Zapotoczny, P., Alves-Filho, O., Eikevik, T.M., and Blaszczak, W. (2013) Journal of Food Engineering 115: 347-356.
18. Mendez-Lagunas, L., Rodriguez-Raminez, J., Reyes-Vasquez, D., and Lopez-Ortiz, A. (2017) Food Measure 11: 1127-1232.
19. Tian, Y., Zhao, Y., Huang, J., Zeng, H., and Zheng, B. (2016) Food Chemistry 197: 714-722.
20. Peters, A.M., and Amerongen, A.V. (1998) J Amer Soc Hort Sci 123 (2): 326 – 329.
21. Sessa, R.A., Bennett, M.H., Lewis, M.J., Mansfield, J.W., and Beale, M.H. (2000) The Journal of Biological Chemistry 275: 26877-26884.
22. Venir, E. (2010) In: Handbook of fruit and vegetable flavors (Hui, Y.H., ed.), John Wiley & Sons, Inc., pp.515-529.
23. Nunes, J.C., Lago, M.G., Castelo-Branco, N., Oliveira, F.R., Torres, A.G., Perrone, D., and Monteiro, M. (2016) Food Chemistry 197:881-890.
24. Narain, N., Sousa Galvao, M., Santana, K.L., and Silveira Moreira, J.J. (2010) Drying Technology 28: 232-239.
25. Huang, L., Zhang, M., Wang, L., Mujumdar, A.S., and Sun, D. (2012) LWT-Food Science and Technology 47: 183-188.
26. Shiga, H., Yoshii, H., Ohe, H. Yasuda, M., Furuta, T., Kuwahara, H., Ohkawara, M., and Linko, P. (2004) Bioscience, Biotechnology and Biochemistry 68 (1): 66-71.
27. Jeyaprakash, S., Frank, D.C., and Driscoll, R.H. (2016) Drying Technology 34 (14): 1709-1718.

# Characterization of aroma-active compounds in canned tuna by fractionation and GC/Olfactometry

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## Abstract

Odour-active compounds from two canned tunas (albacore and skipjack) were isolated using solvent extraction followed by solvent-assisted flavour evaporation and normal phase chromatography separation. Aroma-active compounds were identified by gas chromatography/olfactometry-mass spectrometry (GC/O-MS). Many sulfur-containing compounds (2-methylthiophene, 2-methyl-3-furanthiol, 1-acetyl-1-pyrroline, dimethyl sulfide, dimethyl trisulfide, 2-furfurylthiol, methional) were identified contributing to meaty, chicken-like aroma. The majority of the odour-active compounds, however, were saturated and unsaturated aldehydes such as hexanal, nonanal, (*Z*)-4-heptenal, (*E,Z*)-2,6-nonadienal, (*E,E*)-2,4-decadienal, and acids. Most of these compounds were identified in both skipjack and albacore species, but their aroma intensities were different. Results demonstrated that normal phase chromatography is a useful tool to help compound identification in complex mixture.

## Introduction

Many factors can affect the aroma profile of canned tuna, including processing conditions [1], fish species [2,3], and storage conditions [4,5]. Oxidation of unsaturated fatty acids in fish generates saturated and unsaturated aldehydes [6], and some of these compounds have been reported as useful markers for fishy off-flavours in fish and fish products [7]. Besides lipid oxidation, off-flavours may originate from environmental pollutants, microbial spoilage, or endogenous enzymatic decomposition [8,9]. The objectives of this study were to identify the odour-active compounds responsible for the canned tuna aroma.

## Experimental

### *Materials*

Two types of commercially canned tuna (skipjack and albacore species) were provided by an industrial collaborator (Bumble Bee Foods, San Diego, CA). Each of the species was procured from five different fish suppliers/regions, including Europe, Asia, and America. All samples were stored at 4 °C until use.

### *Tuna aroma isolation with Solvent-Assisted Flavour Evaporation (SAFE)*

For each tuna species, one can of tuna sample (125 g) from each supplier/batch was blended with liquid nitrogen into fine powders and all five samples were mixed together (625 g totally). The tuna powder was mixed with 200 mL of saturated salt water and then extracted with 200 mL of freshly distilled diethyl ether. The mixture was shaking vigorously for 1 hour at room temperature in a Teflon centrifuge bottle. The organic phases were separated by centrifuge at 5500 rpm for 10 min at 5 °C. The organic phase was saved and the sample was extracted two more times. The organic phases from three extractions were combined and distilled using solvent assisted flavour evaporation (SAFE) (Glasblaserei Bahr, Manching, Germany) at 50 °C under vacuum. The distillates

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were dried over anhydrous sodium sulfate and concentrated to 1mL at 40 °C using a Vigreux column, then concentrated to 0.5 mL using a gentle nitrogen.

*Normal Phase Chromatography and Gas Chromatography/Olfactometry-Mass Spectrometry (GC/O-MS)*

To facilitate the GC/O analysis, aroma extracts were separated by fractionation prior to GC-O analysis. A column packed with 5 g of silica gel was washed with 100 ml methanol, then 100 ml diethyl ether, and then with 100 ml pentane. After sample loading, pentane (fraction 1), 50 ml pentane: diethyl ether (98:2, fraction 2), pentane:diethyl ether (95:5, fraction 3), pentane:diethyl ether (90:10, fraction 4) and diethyl ether (fraction 5) were sequentially applied to elute the aroma compounds from the column at a flow rate of 3 ml/min. All elutes were slowly concentrated to 10 ml and then to 100 µL with a stream of nitrogen for GC–O and GC–MS analysis.

The GC-O and GC-MS analysis were performed using an Agilent 6890 GC-MS (5973N, Agilent, Willmington, DE), and a Gerstel olfactory detection port (Gerstel, Baltimore, MD). All the samples were analysed on a DB-Wax column (30 m, 0.25mm ID, 0.5 µm film thickness). One microliter of fractionated aroma extract was injected into the GC in splitless mode. The oven temperature was programmed initially at 40 °C for 1 min, then increased to 70 °C at a rate of 8 °C/min, then increased to 200 °C at a rate of 3 °C/min and increased to 230 °C at a rate of 8 °C/min with 15 min holding. The column carrier gas was helium at a flow rate of 2 mL/min. The flow was split between MS and ODP at 1:1 ratio to provide one stream for MS identification and another stream to the sniffing port for odour detection simultaneously. The olfactometry analysis was achieved by five experienced panellists for all samples. The odour intensities were evaluated on a five-point intensity scale, where 1 meant a volatile has a slight sensory impact, 3 was for moderate, and 5 was for extreme impact. The intensity was the average from all panellists. Compounds' identification was achieved by comparing mass spectral data from the MS spectra database and confirmed by comparing Kovats retention indices of standards under the same conditions or those reported in the literature, in addition to odour description.

## Results and discussion

Normal phase chromatography separates the tuna extract into five fractions. The number in each fraction represented the odour intensity ranging from 1 to 5, where 5 was the strongest odour, and 1 was the weakest.

**Table 1.** Odour-active compounds in canned tuna detected by GC-O and Normal Phase Fractionation.

| Compounds | *RI (DB-wax)* | *ID* | *Odour* | *Skipjack* | | | | | | *Albacore* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | F1 | | F3 | F4 | F5 | All | F1 | F2 | F3 | F4 | F5 | All |
| Dimethyl sulfide | 842 | 1,2 | cabbage | 3 | | | | | 3 | | | | | | 0 |
| 2,3-Butanedione | 982 | 1,2 | buttery | | 2 | 2 | 2 | 3 | 9 | | | | 1 | 4 | 5 |
| Methyl thioacetate | 1014 | 1,2 | roasted | 1 | 1 | | 2 | | 4 | 2 | | | | | 2 |
| Dimethyl disulfide | 1029 | 1,2,3 | fishy | | | | | | 0 | | | 2 | | | 2 |
| 2,3-Pentanedione | 1046 | 1,2,3 | buttery | | 2 | | | | 2 | 3 | | | 1 | 2 | 6 |
| Hexanal | 1067 | 1,2,3 | grassy | | 3 | | | | 3 | | 2 | | | | 2 |
| 2/3-Methylthiophene | 1079 | 1,2,3 | roasty | 2 | | | | | 2 | 3 | | | | | 3 |
| Heptanal | 1169 | 1,2,3 | oily | | 3 | 1 | | | 4 | | | | | | 0 |

**Table 1 (continued)**

| Compounds | *RI (DB-wax)* | *ID* | *Odour* | *Skipjack* | | | | | | *Albacore* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (*Z*)-4-Heptenal | 1228 | 1,2,3 | meaty | 4 | | 3 | 4 | 3 | 14 | 3 | 3 | 3 | 3 | | 12 |
| Octanal | 1287 | 1,2,3 | soapy | 2 | 2 | 2 | 2 | | 8 | 2 | | | | | 2 |
| 1-Octen-3-one | 1301 | 1,2 | mushroom | 2 | 2 | 2 | | 2 | 8 | 3 | 3 | 3 | | 2 | 11 |
| 2-Methyl-3-furanthiol | 1309 | 1,2 | meaty | 3 | 2 | 2 | | | 7 | 3 | 4 | | | 2 | 9 |
| 2-Acetyl-1-pyrroline | 1340 | 1,2 | popcorn | | | | | | 0 | | 3 | | | 1 | 4 |
| Ethyl thioacetate | 1360 | 1,2 | sulfury | | 2 | 1 | | 2 | 5 | 4 | 3 | | | | 7 |
| Dimethyl trisulfide | 1380 | 1,2 | onion | 4 | | | | | 4 | 5 | | | | | 5 |
| Nonanal | 1389 | 1,2,3 | fruity | | | | | | 0 | | | 1 | | | 1 |
| (*E*)-2-Octenal | 1426 | 1,2,3 | oily | | 2 | | | | 2 | | | | | | 0 |
| 2-Furfurylthiol | 1430 | 1,2,3 | coffee | | 4 | | 3 | | 7 | 4 | 5 | 4 | 2 | 4 | 19 |
| Acetic acid | 1445 | 1,2,3 | vinegar | | | | 4 | | 4 | | | | | 3 | 3 |
| 1-Octen-3-ol | 1450 | 1,2,3 | mushroom | | | 4 | | | 4 | | | | 3 | | 3 |
| Methional | 1454 | 1,2 | nutty | | | | 3 | | 3 | | 4 | | | | 4 |
| (*Z*)-1,5-octadien-3-ol | 1486 | 1,2,3 | earthy | | | | | | 0 | | 1 | 1 | | | 2 |
| (E, E)-2,4-Heptadienal | 1497 | 1,2,3 | earthy | 2 | | | | | 2 | | | | | | 0 |
| (*Z*)-2-Nonenal | 1501 | 1,2,3 | oily | | 3 | | | 2 | 5 | | | | | | 0 |
| Benzaldehyde | 1520 | 1,2,3 | nutty | | 4 | | | | 4 | | | | | | 0 |
| Isobutyric acid | 1564 | 1,2,3 | sweaty | | | | 3 | | 3 | | | | 2 | | 2 |
| (*E,Z*)-2,6-Nonadienal | 1581 | 1,2,3 | cucumber | | | 5 | | | 5 | | | | | | 0 |
| 2-Undecanone | 1592 | 1,2,3 | oily | | | 3 | | | 3 | | | 2 | | | 2 |
| 2-Ethylthiophene | 1597 | 1,2,3 | fishy | | | | 3 | | 3 | | | 2 | | | 2 |
| (*E,E*)-2,4-Octadienal | 1619 | 1,2,3 | mushroom | | 2 | | | | 2 | | | | | | 0 |
| Butanoic acid | 1628 | 1,2,3 | sour | | | 5 | 5 | | 10 | | | 4 | 3 | 3 | 10 |
| (*E*)-2-Decenal | 1639 | 1,2,3 | oily | | | | 1 | | 1 | | | | | | 0 |
| 2-Acetylthiazole | 1663 | 1,2,3 | popcorn | | | | | | 0 | 3 | | | | | 3 |
| Isovaleric acid | 1670 | 1,2,3 | sweaty | | | 5 | | 3 | 8 | | | 4 | 4 | | 8 |
| Valeric acid | 1744 | 1,2,3 | sour | | | 2 | | | 2 | | | | | | 0 |
| (*E*)-2-Undecenal | 1756 | 1,2,3 | green | | | | | | 0 | | | | | | 0 |
| β-Damascenone | 1816 | 1,2,3 | sweet | | | | | | 0 | | | | | 2 | 2 |
| (*E,E*)-2,4-Decadienal | 1819 | 1,2,3 | oily | | | 4 | | | 4 | | | | | | 0 |
| Hexanoic acid | 1856 | 1,2,3 | sour | | | 4 | 4 | | 8 | | | 3 | | | 3 |
| Heptanoic acid | 1965 | 1,2,3 | sour | | | | | | 0 | | 2 | | | | 2 |
| Furaneol | 2049 | 1,2,3 | candy | | | | | | 0 | | | | 3 | 2 | 5 |
| Octanoic acid | 2070 | 1,2,3 | sour | | 2 | | | | 2 | | | 3 | | | 3 |
| *p*-Cresol | 2097 | 1,2,3 | horse | | | 4 | 4 | | 8 | | | 4 | 3 | | 7 |
| Sotolon | 2229 | 1,2,3 | sweet | | | | 3 | | 3 | | | | | | 0 |
| Vanillin | 2572 | 1,2,3 | vanilla | | | | | | 0 | | | | | 3 | 3 |

*1: compounds were identified by the aroma descriptors; 2: compounds were identified by retention indices compared with pure compound standard;3: compounds were identified by the MS spectra.*

The major odour compounds identified in canned tuna were sulfur-containing compounds, aldehydes, ketones, alcohols and short-chained fatty acids. The sulfur-containing compounds are generated via Maillard reactions during cooking and generally contribute to meaty, chicken-like aroma. The aldehydes, ketones as well as some alcohols are generated via lipid oxidation, and they contribute to fishy, oily off-flavour in the products. This research provided directions for future research and actionable steps to improve flavour quality of canned tuna fish.

## References

1. Aubourg, S., Gallardo, J. M., and Medina, I. (1997) International journal of food science and technology 32(5): 427-431
2. Medina, I., Aubourg, S. P., and Martín, R. P. (1995) Lipids 30(12): 1127-1135.
3. Saito, H., Ishihara, K., and Murase, T. (1996) Bioscience, biotechnology, and biochemistry, 60(6): 962-965.
4. Medina, I., Sacchi, R., Biondi, L., Aubourg, S. P., and Paolillo, L. (1998) Journal of Agricultural and Food Chemistry, 46(3): 1150-1157.
5. Purnomo, H., Surjoseputro, S., and Mali, T. (2003) Indonesian Food and Nutrition Progress, 10(1): 13-18.
6. Iglesias, J., Medina, I., Bianchi, F., Careri, M., Mangia, A., and Musci, M. (2009) Food Chemistry 115(4): 1473-1478.
7. Venkateshwarlu, G., Let, M. B., Meyer, A. S., and Jacobsen, C. (2004) Journal of Agricultural and Food Chemistry 52(6): 1635-1641.
8. Wilkes, J. G., Conte, E. D., Kim, Y., Holcomb, M., Sutherland, J. B., and Miller, D. W. (2000) Journal of Chromatography A 880(1): 3-33.
9. Ghaly, A. E., Dave, D., Budge, S., and Brooks, M. (2010) American Journal of Applied Sciences 7(7): 859.

# Aroma compounds generation in brown and polished rice during extrusion

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## Abstract

The effect of mechanic extrusion on aroma compounds in brown and polished rice was studied by gas chromatography-olfactometry (GC-O). Aroma compounds were isolated using solvent extraction followed by solvent-assisted flavor evaporation. Aroma extract dilution analysis (AEDA) was performed on both brown rice and polished rice before and after the extrusion process. A total of 71 odorants were identified. On the basis of flavor dilution (FD) factors, the most important aroma compounds in extruded rice could be hexanal, heptanal, 2-acetylpyrroline, 1-octen-3-ol, octanal, (*E*)-2-octenal, nonanal, decanal and (*E, E*),2,4-nonadienal. The aroma compounds were similar in all rice samples but FD factors were different. The FD factors of 2-acetylpyrroline, 1-octen-3-ol in brown rice were much higher than in polished rice. The extrusion process greatly increased the FD factors of most aroma compounds, particularly aldehydes in brown rice.

## Introduction

The aroma and volatile profile of cooked rice can be affected by postharvest processes (harvesting, drying, milling and storage) and cooking processes (boiling, puffing or extrusion) [1]. Extrusion is a high-temperature/short-time cooking process, producing breakfast cereals and other snack food products [2]. Extrusion conditions such as temperature and screw speed can affect product quality such as expansion, bulk density, and texture. Those conditions are also critical for the development, retention, and degradation of flavor components in the finished products [3].

In brown rice, the bran and germ are present while in milled rice, they are partly or totally removed[1]. Rice bran contains amino acid, lipids, minerals and antioxidants. Milled rice has a different chemical composition according to the degree of milling, and therefore could lead to differences on the formation of rice aroma during cooking [4][5].

In this work, the aroma compounds in brown and polished rice powder were analyzed by gas chromatography-mass spectrometry/olfactometry (GC-MS/O). Aroma extract dilution analysis (AEDA) was used to study the generation of aroma compounds in brown and polished rice before and after the extrusion process.

## Experimental

### *Materials*

'Huanghuazhan' rice cultivar was used in this study because it is one of the main commercial cultivars in South China. The rice was grown in the Experimental Station of the Rice Research Institute of Guangdong Academy of Agricultural Sciences on a sandy loam soil in 2016. They were sown in late March and harvested in mid-July. The rice grains were then air-dried to a moisture content of approximately 13% and stored at room temperature for 3 months. The rice samples were milled to separate the husk from the brown rice. The brown rice was then polished using a rice milling machine (Satake Co. Hiroshima, Japan) to obtain approximately 90% (w/w) polished rice. The brown rice and

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polished rice samples were sieved by passing through a 60-mesh sieve using a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO, U.S.A.) for further process.

*Extrusion*

Extrusion was performed using a twin-screw extruder (Continua 37, Werner and Pfleiderer, Stuttgart, Germany) system with co-rotating. The screw diameter was 37 mm, overall L/D ratio was 27, and the diameter of extrusion die was 6 mm. The feed rate (25 kg/h) and screw speed (200 rpm) were kept constant. The extrusion was carried out at 120℃ with the temperature of different barrel zones set at 60, 100 and 120℃. The feed moisture was conditioned to 12–17%. The extrudates were cooled to room temperature, packed in polyethylene bags and milled later to flour using a grinder (Sujata, India) to a particle size < 250 μm and stored at -20℃ until further analysis. All samples, including raw polished rice (RPR); extruded polished rice (EPR), raw brown rice (RBR), and extruded brown rice (EBR), were kept in a refrigerator at 4 ℃ until analysis.

*Rice aroma isolation with Solvent-Assisted Flavor Evaporation (SAFE)*

The aroma compounds from four rice samples were extracted using organic solvent. For each variety, 200 g of sample was mixed with amylase (0.2% w/w) and Milli-Q water (1:1, v/v) and shaked for 1 hour. Then 100 mL of pentane/diethyl ether mix (2:1, v/v) was added to the rice mixture. The mixture was shaken vigorously for 1 hour at room temperature in a Teflon centrifuge bottle. The organic phases were separated by centrifugation at 5000 rpm for 15 min at 5 °C. The organic phase was saved and the sample was extracted two more times. The organic phases from three extractions were combined and distilled using solvent assisted flavor evaporation (SAFE) (Glasblaserei Bahr, Manching, Germany) technique to remove the nonvolatile constituents at 50 °C under high vacuum. After distillation, the receiving part of SAFE in the system was carefully rinsed with 5 mL of pentane/diethyl ether mix, and combined with the distillates in the volatile-receiving flask. The final distillates were dried over anhydrous sodium sulfate overnight and concentrated to about 1 mL at 40 °C using a Vigreux column, then concentrated to 0.1 mL using a stream of gentle nitrogen flow for further analysis.

*Gas Chromatography/Olfactometry-Mass Spectrometry (GC/O-MS)*

The GC-O and GC-MS analysis were performed using an Agilent 6890 GC with an Agilent 5973N mass selective detector (MSD, Willmington, DE, U.S.A.), and a Gerstel olfactory detection port (ODP series 2, Baltimore, MD, U.S.A.). All samples were analyzed on a DB-Wax column (30 m, 0.25 mm ID, 0.5 μm film thickness). One microliter of sample was injected into the GC in splitless mode. The oven temperature was programmed initially at 40 °C for 4 min, then increased to 230 °C at a rate of 4 °C/min with 20 min holding. The column carrier gas was helium at a flow rate of 2.5 mL/min. The flow was split between MS and ODP to provide one stream for MS identification and another stream the sniffing port for odor detection simultaneously. Six experienced panelists (2 males and 4 females) performed the GC-O analysis on the original extracts. Each sample was sniffed by each panelist in duplicates. Compounds' identification was achieved by comparing mass spectral data from the database and confirmed by comparing Kovats retention indices (RI) of standards obtained under the same conditions in the lab, in addition to odor description.

*Aroma Extract Dilution Analysis (AEDA)*

The aroma extracts were diluted stepwise with 1:1 (v/v) distilled pentane/ ether mix (1:1, v/v). Analyses were performed on the same instrument as described previously on a DB-5 column (30 m, 0.25 mm ID, 0.5 µm film thickness). One microliter of sample was injected into the GC in splitless mode. Determination of the flavor dilution (FD) factors was then done by two panelists, and each dilution was evaluated by each panelist in duplicates.

## Results and discussion

GC/Olfactometry analysis of the four rice extract revealed 71 odor-active areas in the gas chromatogram (*data not shown*). AEDA revealed 28 compounds with FD factors ranging from 1 to 2048 (Table 1). Although the aroma-active compounds identified were similar among all the samples, their FD factors varied in different samples, demonstrating the flavor differences among the products.

**Table 1:** Aroma -active compounds in polished and brown rice, before and after extrusion

| Compounds | *Odor* | *RI* | *ID* | *FD factor* | | | |
|---|---|---|---|---|---|---|---|
| | | | | *RPR* | *EPR* | *RBR* | *EBR* |
| Dimethyl sulfide | cabbage | 723 | RI, A | 8 | 8 | 8 | 8 |
| Butan-2,3-dione | buttery | 736 | RI, A | 2 | 8 | 8 | 8 |
| 3-Methylbutanal | malty | 761 | MS,RI,A | Na | na | 2 | 1 |
| Hexanal | green | 819 | MS,RI,A | 32 | 64 | 32 | 256 |
| Methional | potato | 898 | MS,RI,A | 16 | 64 | 8 | 256 |
| 4-Mercapto-4-methylpentan-2-one (4MMP) | Grapefruit | 912 | RI, A | 8 | 32 | 16 | 8 |
| 2-Acetylpyrroline | popcorn | 917 | MS,RI,A | 16 | 64 | 512 | 2048 |
| Pentanoic acid | sweaty | 941 | MS,RI,A | 8 | 8 | 8 | 16 |
| 1-Octen-3-ol | mushroom | 972 | MS,RI,A | 32 | 64 | 1024 | 1024 |
| Octanal | oily | 996 | MS,RI,A | 1 | 16 | 32 | 1024 |
| Hexanoic acid | sour | 1032 | MS,RI,A | 16 | 2 | 2 | 8 |
| (*E*)-2-Octenal | oily | 1057 | MS,RI,A | 16 | 32 | 32 | 64 |
| Linalool oxide | floral | 1080 | MS,RI,A | Na | na | 2 | 16 |
| Nonanal | oily | 1098 | MS,RI,A | 16 | 64 | 2 | 128 |
| Ethyl hexanoate | fruity | 1127 | MS,RI,A | Na | na | 8 | 8 |
| (*E*)-2-Nonenal | oily, green | 1130 | MS,RI,A | 2 | na | 2 | 32 |
| Decanal | waxy | 1195 | MS,RI,A | 2 | 64 | 8 | 128 |
| (*E,E*)-2,4-Nonadienal | oily | 1207 | MS,RI,A | 4 | 8 | 16 | 64 |
| 4-Vinylphenol | woody | 1227 | MS,RI,A | 2 | na | 32 | 64 |
| Octanoic acid | sour | 1281 | MS, | na | na | 16 | 16 |
| 4-Vinylguaiacol | woody | 1312 | MS,RI,A | 2 | 8 | 2 | 64 |
| Vanillin | vanilla | 1376 | MS,RI,A | 2 | na | 8 | na |

*ID representes identification method. RI: compounds were identified by retention indices compared with pure compound standard; A: compounds were identified by the aroma descriptors; MS: compounds were identified by the MS spectra.*

Among all the compounds identified, 2-acetylpyrroline, 1-octen-3-ol, hexanal, octanal, nonanal and decanal had relatively high FD factors, suggested their potentially higher aroma contribution. 2-Acetylpyrroline is a well-known character compound for rice products, whereas 1-octen-3-ol, hexanal, octanal, nonanal and decanal are generated from lipid oxidation of unsaturated fatty acids. 4-Mercapto-4-methylpentan-2-one (4-MMP) was also identified as a key odor-active compound.

Compared with the raw polished rice, the raw brown rice had higher FD factors for 2-acetylpyrroline, 1-octen-3-ol, octanal, and 4-vinylphenol, suggesting these compounds were associated with the bran and germ of the rice. The reason that the brown rice had higher FD factors for 1-octen-3-ol and octanal could be due to the fact that brown rice is more susceptible to off-flavor development, mainly due to oxidation of rice oil catalyzed by enzymes such as lipase and lipoxygenase and autooxidation. It is interesting to notice that the brown rice also showed a higher FD factor for 2-acetylpyrroline.

Extrusion changed the FD factors of many compounds. Extrusion increased the FD factors of 4-mercapto-4-methylpentan-2-one, 2-acetylpyrroline, and some lipid derived compounds (i.e. octanal, nonanal) in polished rice, and the increases were much more pronounced for brown rice, especially for lipid derived compounds including hexanal, heptanal, octanal, nonanal, decanal. During the extrusion process, thermal processing of the raw ingredients occurs under high temperature and shear, with limited moisture conditions. This process causes decomposition, degradation, denaturation, cross-linking, and various chemical reactions such as oxidation, polymerization, hydrolysis and other reactions in the extruded material. Thermal oxidation will generate straight-chained aldehydes. Linalool oxide was only detected in brown rice, and the extrusion process greatly increased its FD factor. 3-Methylbutanal, ethyl hexanoate and octanoic acid were also detected only in brown rice, however their FD factors were not greatly influenced by extrusion process.

In conclusion, brown rice had higher FD factors than polished rice for most of aroma-active compounds. The extrusion process greatly increased the FD factors of most aroma compounds, particularly aldehydes in brown rice.

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## References

1. Handoko, D.D., L. Methven, J.S. Elmore, and D.S. Mottram. (2013) In: Flavour Science: Proceedings from XIII Weurman Flavour Research Symposium (p. 83). Academic Press.
2. Norajit, K., B.-J. Gu, and G.-H. Ryu. (2011) Food Chemistry, 129 (4):1919-1925
3. Yuliani, S., P.J. Torley, B. D'Arcy, T. Nicholson, and B. Bhandari. (2006) Food Research International 39 (3): 318-331.
4. Tsugita, T., T. Kurata, and H. Kato. (1980) Agricultural and Biological Chemistry 44 (4): 835-840.
5. Shobana, S., N. Malleshi, V. Sudha, D. Spiegelman, B. Hong, F. Hu, W. Willett, K. Krishnaswamy, and V. Mohan. (2011) International journal of food sciences and nutrition 62 (8): 800-810.
6. Burbank, H.M. and M.C. Qian. (2005) Journal of Chromatography A 1066 (1): 149-157.

# Aroma profile and proximate composition of Roselle seeds: Effects of different origins and different sample preparation methods

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## Abstract

The influence of different origin on proximate composition of Roselle seeds and different sample preparation methods on the aroma profiles of Roselle seeds were studied. It was seen that sample origin affected the proximate composition and volatile profiles. Ground dry (GD) was chosen as the preparation method because it was an efficient method with less chemical changes of the samples whereas, Roselle seeds of Malaysian origin was selected as potential food ingredient because it has high lipid, protein, and total dietary fiber content.

## Introduction

Roselle (*Hibiscus sabdariffa* L.) is an important food and medicinal plant, among other things due to its high content of antioxidants, for example anthocyanins and vitamin C. It is also used as a natural food colorant. Normally, in food industry only Roselle calyces are used to produce various food products; the seeds are removed and disposed as a by-product. However, Roselle seeds are also edible [1]. To our knowledge, the study of Roselle seeds is limited and there are no aroma profiles of Roselle seeds being reported. Therefore, this study addresses the influence of different origin on proximate composition of Roselle seeds and tests different sample preparation methods to determine the aroma profiles of Roselle seeds.

## Experimental

### *Materials*

Two types of sun dried Roselle (*Hibiscus sabdariffa* L.) seeds commercially available were obtained to study aroma profiles and proximate analysis: 1) Roselle seeds of the UMKL cultivar (obtained from HERBagus Sdn. Bhd., Penang, Malaysia) 2) Roselle seeds of Chinese origin (obtained from Sichuan Keren Imp & Exp Trading Co. Ltd, Sichuan, China).

### *Sample preparation*

For aroma analysis, samples were prepared by two different procedures and analyzed in triplicate: Ground, dry (GD): Whole Roselle seeds were ground for 90 sec using a laboratory blender Model 38BL41 (Waring, USA). Internal standard (1 mL of a 5 ppm 4-methyl-1-pentanol solution) was added to 25 g of Roselle seeds and volatiles were sampled by Dynamic Headspace Sampling (DHS).

Ground, mixed with water (GMW): Whole Roselle seeds were ground as mentioned above and then 25 g of ground Roselle seeds were mixed with 100 mL of tap water, ratio (1:4). Again, 1 mL of a 5 ppm 4-methyl-1-pentanol solution was added and DHS was carried out.

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*Dynamic Headspace Sampling (DHS) and Gas Chromatography-Mass Spectrometry (GC-MS)*

The DHS method was adopted and modified from Starr et al. [2]. Each sample was placed in a 500 mL glass flask. A trap containing Tenax-TA (200 mg) was attached to the sealed flask. The flasks containing the samples were immersed in a water bath held at 40 °C. Under magnetic stirring (200 rpm), the sample was tempered for 10 min and then purged with nitrogen (100 mL $min^{-1}$) for 40 min. The traps were purged with a flow of nitrogen (100 mL $min^{-1}$) for an extra 10 min to remove water.

In GC-MS analysis, the collected volatiles were determined as previously described by Starr et al. [2]. Volatile compounds were identified by probability based matching of their spectra with those of a commercial database (Wiley275.L, HP product no. G1035A). The software program, MSDChemstation (Version E.02.00, Agilent Technologies, Palo Alto, California), was used for data analysis. Amounts are presented as peak areas. Volatile compound identification was confirmed by comparison with retention indices (RI) of authentic reference compounds or retention indices reported in the literature.

*Proximate composition*

In proximate analysis, samples were treated according to the AOAC standard methods [3]. Moisture content (hot-air oven method), ash (dry ashing method), lipid (Soxhlet extraction), protein (Micro-Kjeldahl method) and total dietary fiber [4] were analyzed and calculated. All measurements were conducted in triplicate. The results were expressed as a percentage (wet weight).

*Data analysis*

Multivariate data analysis (principal component analysis (PCA)) using the Latentix software (LatentiXTM 2.0 Latent5, Copenhagen, Denmark, www.latentix.com) was applied to GC-MS data to evaluate the variation between the different samples from different countries and one-way analysis of variance (ANOVA) was performed using the software JMP (version 12.0, SAS Institute Inc.) to test for differences in proximate composition.

## Results and discussion

A total of 61 volatile compounds were identified including alcohols (18), terpenes (15), aldehydes (13), ketones (9), furans (2), phenols (2), ester (1), and lactone (1). An equal number of aroma compounds was recovered in GD and GMW. Roselle seeds from China and Malaysia had different volatile profiles (chromatogram not shown). But both volatile profiles were dominated by alcohols, terpenes and aldehydes and had phenols, an ester and a lactone present in traces. A Principal Component Analysis was carried out using the peak areas obtained, in order to provide an overview of the influences of different sample preparation methods and different origins of Roselle seeds (Figure 1). The first principal component (PC1) explained 58 % of the variance while PC2 explained 22 % of the variance. The samples were clearly separated according to country and also by different sample preparation methods. The differences are probably due to different harvesting time, harvesting place, climate zone and varieties.

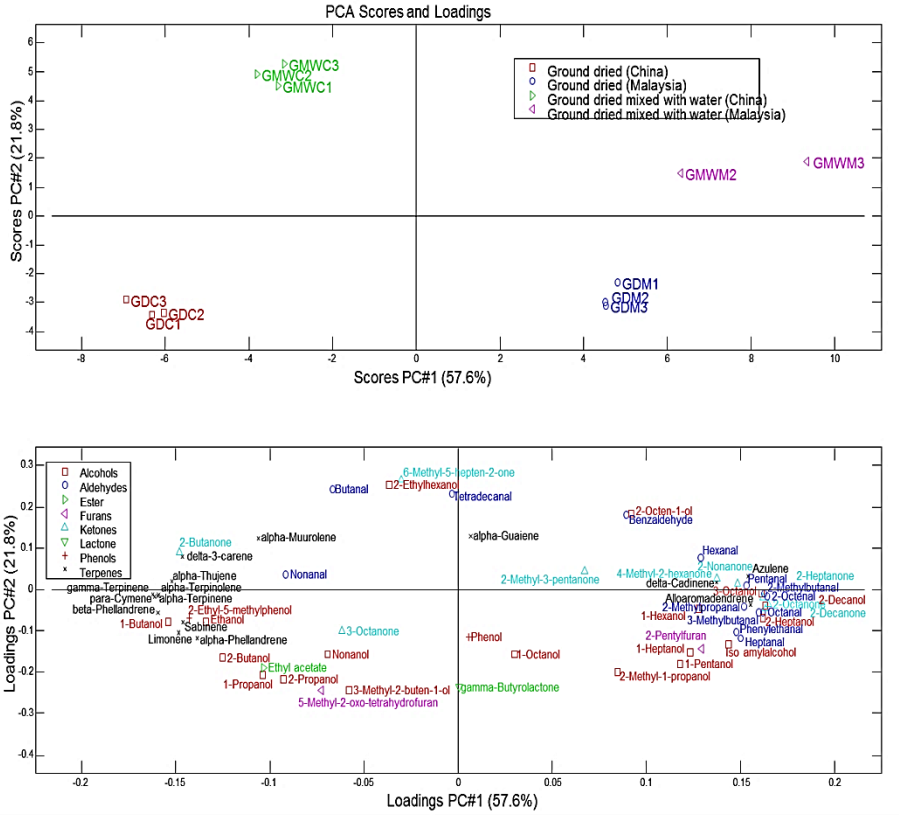


**Figure 1:** PCA scores and loadings plot of volatile compounds for Roselle seeds

For most volatiles the largest peak areas were obtained using the GD sampling technique: only some ketones had higher peak areas in the GMW samples. GD would therefore be the preferred sample preparation technique compared to GMW. It seems that addition of water during sample preparation does not improve the efficiency of sampling of Roselle seeds. Nevertheless, care must be taken to avoid sampling procedures which may alter the substances being studied. GD was assumed to cause less chemical changes of the samples, it was simple, and it was easy to handle. Therefore, it was decided to use the GD preparation method in further experiments.

The loadings plot shows that Roselle seeds from Malaysia were found to have a larger amount of many alcohols, aldehydes, and ketones, whereas samples from China were high in most terpenes (Figure 5). The major volatile compounds (by peak size; 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, α-phellandrene, hexanal, 2-methyl-1-propanol, sabinene, 4-methyl-2-hexanone, β-phellandrene, 3-methylbutanol, 2-pentylfuran, 1-pentanol, ρ-cymene, 1-hexanol, and 2-ethyl-5-methylphenol) were found in all samples in varying levels.

The composition of Roselle seeds is rarely studied compared to the calyces and studies on proximate composition of Roselle seeds are limited compared to studies on other seeds such as black cumin seed (*Nigella sativa* L.) and jojoba seed (*Simmondsia chinensis*) [5]. The results of proximate compositions of Roselle seeds are presented in

Table 1. The total dietary fiber, protein and lipid of Roselle seeds ranged between 47.1 and 47.3 %, 21.3 and 23.6 %, 11 and 16.2 %, respectively. The differences in proximate composition may be attributed to the different origins, agricultural practices, and varieties. Previous studies have also shown that Roselle seeds contain high protein, dietary fiber, and minerals such as phosphorus, magnesium and calcium. This contributes to the strength of the seed compared to other common sources of dietary fiber such as wheat and rice bran, oat, and fiber from fruits [5]. Furthermore, El-Adawy and Khalil [6] reported that the lipid from Roselle seeds contained more than 70 % of polyunsaturated fatty acids and Dhar et. al. [7] found high content of γ-tocopherol.

In conclusion, it was found that Roselle seeds of Malaysian origin had more volatile compounds recovered in both types of sample preparation, GD and GMW, and had higher lipid content. The effect of the volatile profile on the sensory quality remains to be elucidated, but a high lipid content in the seeds is considered an advantage due to its richness in polyunsaturated fatty acids (PUFAs) and γ-tocopherol which possess potential health benefits. Thus, Roselle seeds of Malaysian origin were selected instead of Roselle seeds of Chinese origin as a potential food ingredient for further exploration in development of bakery products using Roselle seeds.

**Table 1:** Proximate composition of Roselle (*Hibiscus sabdariffa* L.) seeds

| *Type of analysis* |  |  | *Significance* |
|---|---|---|---|
| | *Malaysia* | *China* | |
| Moisture content (%) | 8.4[a] | 7.9[b] | *** |
| Ash (%) | 6.5[a] | 4.8[b] | *** |
| Lipid (%) | 16.2[a] | 11[b] | *** |
| Protein (%) | 21.3 | 23.6 | ns |
| Total dietary fiber (%) | 47.3 | 47.1 | ns |

Values in a column not marked with the same letters are significantly different, Student t-test ($p<0.05$).
*** Indicates significant at $p<0.001$; ns, no significant difference between the samples.

## References

1. Wilson, F. D.; and Menzel, M. Y. (1964) Econ. Bot. 18: 80–91.
2. Starr, G.; Petersen, M.; Jespersen, B.; and Hansen, A. (2015) Food Chem. 174: 527–537.
3. A.O.A.C (2000) In: Official Methods of Analysis, Association of Official Analytical Chemists International (17th ed), Gaithersburg, Maryland, USA
4. Prosky, L.; Asp, N.; Schweizer, T.; DeVeries, J.; Furda, and I. Prosky. (1988) J. Assoc. Off. Anal. Chem. 71: 1017–1023.
5. Ismail, A.; Ikram, E. H. K.; and Nazri, H. S. M. (2008) Food 2: 1–16.
6. El-Adawy, T. A.; and Khalil, A. H. (1994) J. Agric. Food Chem. 42: 1896–1900.
7. Dhar, P.; Kar, C.; Ojha, D.; Pandey, S.; and Mitra, J. (2015). Ind. Crops Prod. 77, 323–332.

# Effects of sodium chloride, potassium chloride and calcium chloride on flavour formation during heating of a wheat flour-glucose model system

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## Abstract

The presence of metal salts in a food system can change the quantitative distribution of the reaction products formed during Maillard and caramelisation reactions. In this respect, dough formulae containing NaCl, KCl and $CaCl_2$ were prepared and a set of heating experiments was performed at 180 °C. To determine the volatile compounds formed during the Maillard reaction, headspace analyses were carreid out using solid-phase microextraction (SPME) coupled with gas chromatography mass spectrometry (GC-MS). It was found that the quantitative distribution of aroma compounds, such as Strecker aldehydes, pyrazines, pyrroles and furan derivatives, changed in the presence of salts.

## Introduction

Metal cations can interact with sugars and amino acids in a food system, especially during thermal processing when they can alter the kinetics of rate-limiting pathways that control the Maillard and caramelisation reactions. Degradation of sugars is accelerated in the presence of metal cations, and 5-hydroxymethyl-2-furfural and 2-furfural are formed in higher concentrations as a result [1,2]. On the contrary, the reactions of amino acids may be inhibited in the presence of metal cations, as evidenced by the mitigation of acrylamide formation from asparagine in the presence of calcium salts during the Maillard reaction [1,3].

Metal cations directly interact with the nucleophilic oxygens of sugars, which are key in dehydration, and isomerisation reactions [4]. It has also been suggested that metal cations coordinate with the ring oxygen, facilitating ring-opening reactions [5]. Alkali metal cations change the quantitative distribution of the products in two different ways: (*i*) by increasing the rate of isomerisation and (*ii*) changing the rates of different dehydration and fragmentation reactions [6]. It has been shown that under pyrolytic conditions, the sodium ion changes the reaction rate constants of glucose degradation by catalysing most of the reactions but also inhibiting others [7]. The effect of sodium cations is related to how the particular stereochemistry of the transition state interacts with the ions, therefore changing the reaction rate constants [7]. In contrast to the metal cations, chloride anions interact with the partially positively charged hydrogen atoms by locating farther from reaction centres [4].

Although the effect of sodium, potassium and calcium on the formation of acrylamide and furfurals is well established, their effect on the formation of flavor compounds during the Maillard reaction is not entirely known. The aim of this study was to investigate changes in the flavour profile generated in Maillard reaction model systems composed of wheat flour and glucose in the presence of NaCl, KCl, $CaCl_2$.

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## Experimental

Wheat flour (100 g) was mixed with 50 mmol glucose, 5 mmol of either NaCl, KCl or $CaCl_2$, and 50 mL water to form a dough. The dough was freeze-dried and ground prior to dry heating at elevated temperatures. The ground dried mixtures (0.5 g) were transferred to tubes with PTFE sealed screw caps and heated in duplicate at 180 °C for 1, 3, and 5 min in an oil bath.

Heated mixtures were analysed by headspace SPME-GC/MS after adding 1 mL saturated NaCl solution containing the internal standards of 2-methylpentanal (0.5 mg/L), isopropylpyrazine (0.05 mg/L) and 3-furfural (0.05 mg/L). Saturated NaCl solution was used for adjusting the ionic strength in all the formulae, to standardise the flavour release. A Supelco 50/30 μm DVB/CAR/PDMS SPME fibre was used. Volatile analyses were carried out on an Agilent 7890A GC system coupled to an Agilent 5975C mass spectrometer. A ZB-WAX column (30 m × 0.25 mm i.d., 1 μm film thickness; Phenomenex, UK) was used for chromatographic separation. The data were approximately quantified as ng of volatile compounds in 0.5 g of heated mixture by comparing the area of the analytes to the response of the internal standards.

## Results and discussion

A model system, simulating the drying conditions on the surface of bakery products during thermal treatment, was created to monitor the effect of salts on the Maillard reaction. Although sucrose is the major sugar source in bakery products, glucose was chosen as a reducing sugar. It is known that sucrose hydrolysis and degradation increase in the presence of metal salts, producing glucose and fructose [8]. Therefore, the aim was to observe the effects with a single reducing sugar by keeping the model system simpler at first.

The concentration of volatile compounds was found to increase during 5 min heating at 180 °C. NaCl and KCl, at concentrations of 0.3 and 0.4 g/100 g flour respectively, had minor effects on aroma formation compared to the control (Figure 1). Slight increases were observed in the Strecker aldehydes, 2-methylbutanal and 3-methylbutanal, in the presence of KCl. $CaCl_2$, at a concentration of 0.6 g/100 g flour, had no effect on Strecker aldehyde formation during heating.

Remarkable changes were observed for pyrazines and furan derivatives in the presence of $CaCl_2$. Pyrazines were found to decrease in the presence of $CaCl_2$ whereas furan derivatives increased dramatically. Pyrrole and pyridine derivatives showed an increment in case of prolonged heating.

In conclusion, the quantitative distribution of flavour compounds changed in the presence of salts to varying degrees. The effect of salts on the Maillard reaction and caramelisation needs detailed investigation to be able to control flavour development during processing when considering sodium reduction and use of calcium salts to reduce acrylamide formation.

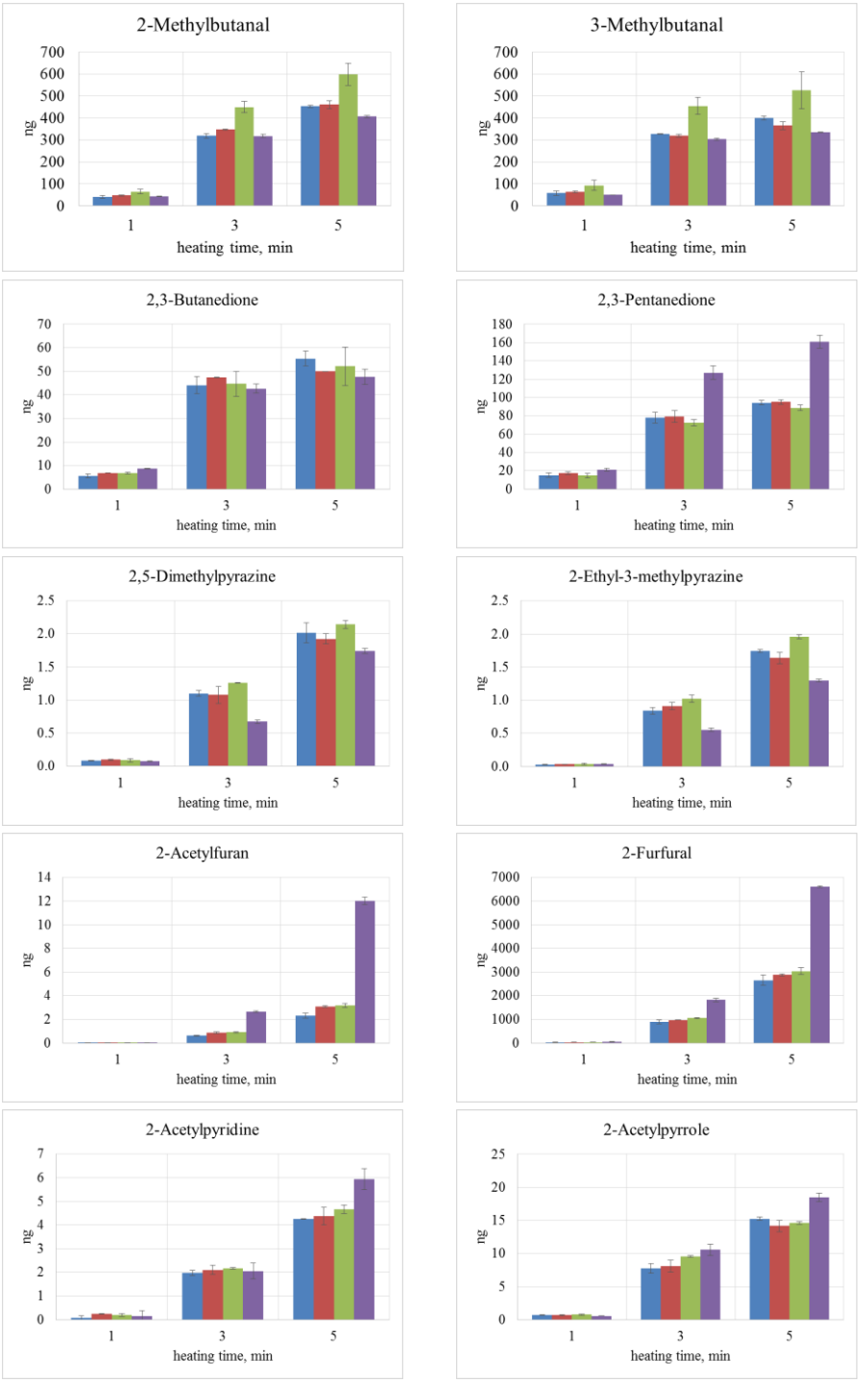


**Figure 1:** Formation of certain volatile compound during heating wheat flour-glucose mixture in the absence of salts (control, ■) and presence of NaCl (■), KCl (■) and $CaCl_2$ (■).

## References

1. Gökmen, V., and Şenyuva, H. Z. (2007) Eur. Food Res. Technol. 225(5-6): 815-820.
2. Açar, Ö. Ç., Pollio, M., Di Monaco, R., Fogliano, V., and Gokmen, V. (2012) Food Bioprocess. Technol. 5(2): 519-526.
3. Gökmen, V., and Şenyuva, H. Z. (2007) Food Chem. 103(1): 196-203.
4. Mayes, H. B., Tian, J. H., Nolte, M. W., Shanks, B. H., Beckham, G. T., Gnanakaran, S., and Broadbelt, L. J. (2014) J. Phys. Chem. B, 118(8): 1990-2000.
5. Zhao, H., Holladay, J. E., Brown, H., and Zhang, Z. C. (2007). Science, 316(5831): 1597-1600.
6. Kocadağlı, T., and Gökmen, V. (2016) J. Agric. Food Chem. 64(32): 6333-6342.
7. Mayes, H. B., Nolte, M. W., Beckham, G. T., Shanks, B. H., and Broadbelt, L. J. (2015) ACS Catal. 5(1): 192-202.
8. Fiore, A., Troise, A. D., Mogol, B. A., Roullier, V., Gourdon, A., Jian, S. E., Hamzalioglu, B. A., Gokmen, V., and Fogliano, V. (2012) J. Agric. Food Chem. 60: 10808−10814.

# An assessment of the effect of cinnamon spice on cocoa nibs (*Theobroma cacao L.*) - An approach to change flavour in stored roasted cocoa nibs

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**Abstract**

Fine flavours in chocolate are influenced by factors such as cacao variety, growing environment, post-harvest processing and chocolate manufacturing processes. The objective of this experiment was to develop a method to enhance the flavour of cocoa nibs without affecting their inherent fine flavours. Concentration of cinnamon, method of packaging, and exposure time were manipulated to obtain a unique ancillary spice note as detected via descriptive sensory evaluation of cocoa liquor processed from the cinnamon infused cocoa nibs. Increased concentration enhanced the detection of cinnamon flavour, more so under vacuum packaging, while simultaneously reducing the perception of an undesirable note. This study shows that addition of ancillary spices to a mass of nibs can enhance the taste experience, allowing for novel favourable and unique liquor products. This method has particular potential for small to medium size chocolate makers and chefs.

## Introduction

Cocoa beans are categorized globally into two groups, "fine or flavour" cocoa and bulk cocoa. Fine or flavour cocoa beans are generally obtained from cocoa trees of Criollo or Trinitario ancestry. They are characterized by desirable ancillary flavour notes such as floral and fruity, with a robust chocolate flavour. In contrast, bulk cocoa has a robust chocolate note with no significant ancillary notes. Cocoa nibs are fermented, dried, roasted and crushed cocoa beans [1]. Currently, there is an increase in demand for "fine or flavour" cocoa beans and nibs largely due to the buoyant craft chocolate and culinary industry that uses it to produce exclusive chocolates and chocolate products.

This craft industry uses innovations through the transference of novel flavours into cocoa and therefore can provide a competitive advantage in an increasingly competitive marketplace. Cocoa or chocolate flavours can be enhanced through the addition of direct spices and flavouring during conching but this may give negative mouthfeel properties.

Desirable flavours can result in unique flavour notes and thus further enhance the quality of cocoa and the potential price it can fetch in the market. Bio-generated atmospheres or vacuum storage demonstrated a positive impact on stored cocoa beans [2]. Little work has been done to show the significance of vacuum storage or its influence on flavour infusion on "fine or flavour" roasted cocoa nibs. Aromas could potentially be transferred onto cocoa by a process known as mass transfer [3]. By manipulating concentration and distance one should be able to affect a flavour change in stored roasted cocoa nibs validated through descriptive sensory evaluation.

Descriptive sensory evaluation of cocoa liquor has been used as a tool to judge cocoa bean quality. A major strength of descriptive sensory evaluation is that it can link instrumental measurements of quality with consumer acceptance [4]. An optimised

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protocol for descriptive sensory evaluation was developed to quantify different flavour attributes of cocoa liquors in Trinidad and Tobago [1].

The overall objective of this work is, therefore, to develop a standardised method for cocoa nib infusion with cinnamon spice aroma to be used by small to medium size chocolate makers and chefs.

## Experimental

### *Nib and cocoa liquor preparation*

Cocoa beans from the International Cocoa Gene Bank Trinidad (ICGT) were selected and used for the experiment. Roasting of beans was done according to Sakha et al. [1]. After roasting, beans were cooled to room temperature on cooling racks for further processing. Cocoa beans were broken using a cocoa breaker to an average size of 0.2-0.5 cm and collected into neutral and non-odorous plastic containers. The broken beans were then winnowed and winnowed nibs were manually fine cleaned using stainless steel forceps. Cocoa nibs were packaged based on the experiment design below and stored at 22°C room temperature.

**Table 1:** Overview of the experimental set-up and design.

| *Experiment Replication* | *Treatments of ingredient infusion* | *Storage method* | *Levels of infused ingredient in 100 grams of nibs* | *Sampling days* |
|---|---|---|---|---|
| Two replicates (0 grams cinnamon was not repeated in experiment) | Cinnamon sticks (placed in the bags with nibs) | Vacuum (1) sealable bags<br><br>Aerated (2) sealable bags | • 2 grams<br>• 10 grams<br>• 25 grams<br>• 0 grams | 3, 7, 14, 28, 56 |

Each vacuum sealed and aerated sample bag was opened and 50 g of nibs were removed and used to make liquor. Bags with the remainder of the nibs were resealed. Nibs were broken down using a Magic Bullet®USA blender. The blender was pulsed 9 times for one second and 3 times more for 3 seconds. The ground sample was placed in a Cocoa Town® USA mini 500g bowl to be milled for one and a half hours in a Cocoa Town® ECGC-12SLTA Melanger. The cocoa paste or liquor was then transferred to a sterile plastic cup, which was labelled with the date and sample information. The cups were then sealed with tape and placed in a freezer at -4 °C.

### *Sensory evaluation*

Frozen samples were thawed and one ounce of each liquor sample was placed in one-ounce cups. Each cup was given a random code [1]. A sensory panel, consisting of 6 people, was trained for four days to taste cocoa liquors prior to the actual sensory evaluation of the infused nibs. Each person was trained to taste 46 different descriptors and to use the extended sensory sheet called “Cocoa and Chocolate Flavour Evaluation” [5]. Panellists were also asked to provide comments, an overall score and assessment of ‘uniqueness’. Sensory evaluation was done in an air conditioned room at 23°C. Each panellist was given samples in random order. Seventy-four coded liquors were tasted over a period of 10 days; 70 from the experiment and four samples of Ghana liquor as additional controls. The latter was used to test consistency of the panellists in scoring. Liquors were placed on a VWE Analog 2 block heater (USA) prior to tasting. Each panellist was given a jar of warm water, a jar of room temperature water and Carr's table water crackers to clear their palate. A tasting spoon and a small plate were also provided.

The method for cleaning the palate and tasting liquor were as described by "ESSeguine-DASukha Cocoa and Chocolate Flavour Evaluation method 2017" [5].

Panellist scored each flavour note perceived on a 10 point hedonic scale. The data was subjected to analysis of variance (ANOVA) to determine the significance of the major effects and interactions. Data were subsequently used in statistical analysis to determine the effects of cinnamon concentration, storage methods (aerated or vacuum stored), and storage time.

## Results and discussion

The average mean scores for "Spice other", "Wood resin" and "overripe fruit" were significantly affected by the infusion of cinnamon. The average mean scores are calculated based on all samples tested for time, concentrations and aerated and vacuum packaging. Not all sensory evaluation results are recorded on this paper.

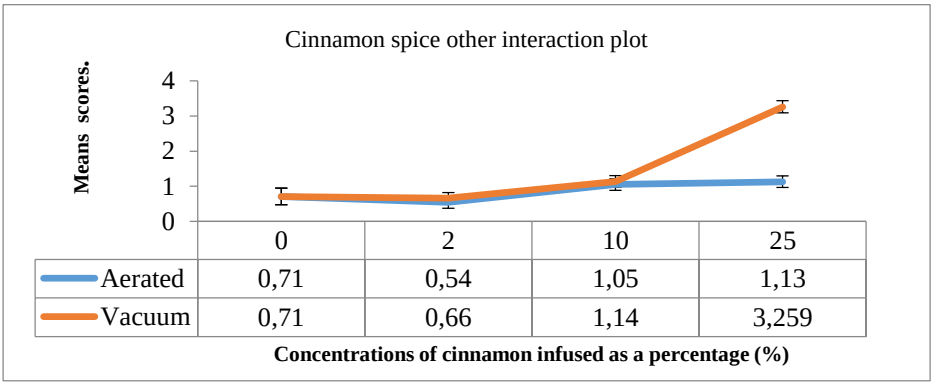


**Figure 1:** The effect of increasing concentration of cinnamon spice on vacuum and aerated packaging for the infusion of "spice other" flavour note on cocoa nibs.

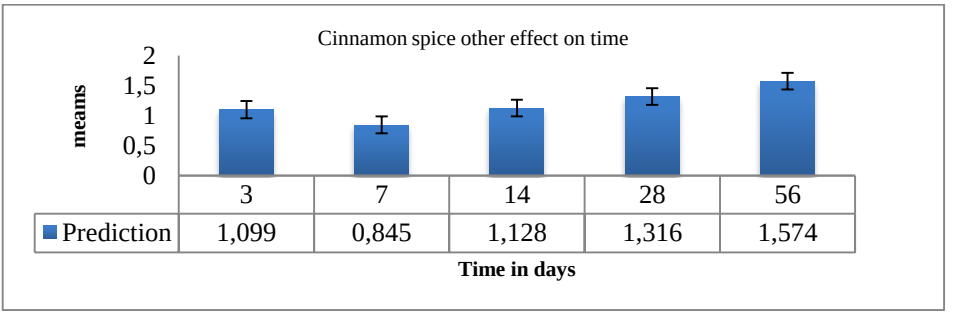


**Figure 2:** Evaluation of the spice other flavour note of cocoa nibs after selected days of infusion.

*"Spice other"*: "Spice other" flavour was the note that the sensory panellists determined as the cinnamon flavour in the liquors. Interaction between concentration and packaging method was significant ($P < 0.001$) and least significant difference (LSD) 0.3846 indicating that the effect of vacuum packaging was more evident at the 25% cinnamon concentration than at the lower concentrations. In general, the intensity of the spice note increased with increasing time of exposure to the spice ($P < 0.05$).

*"Cinnamon spice"* aroma is perceived by two main volatile compounds: cinnamon aldehyde (cinnamon), and 2-propenoic acid, 3-phenyl ester (cinnamon) [7]. This experiment showed that with increased percentages of cinnamon bark, and increased storage time, especially under vacuum storage, a spice note can be effectively transferred to cacao nibs.

*Overall score:* This is a score that rates how good or bad the cocoa liquor samples are. The panellists significantly ($P < 0.001$) preferred vacuum over aerated storage. The overall score was significantly ($P < 0.05$) higher for nibs stored with 25% cinnamon. Thus, infusing the nibs with 25% cinnamon under vacuum storage, improve the general quality of the nibs.

## Conclusion

The objective of this experiment was to develop a novel method to change cocoa nib flavour. Here this method was tested using cinnamon. Vacuum storage and higher cinnamon concentration allowed for superior intensity of cinnamon flavour. Length of exposure also enhanced the spice flavour in cocoa nibs. Given the positive results of this study, this method can be recommended for the infusion of flavour to nibs.

Nonetheless more research using other spices remains necessary to show the general usefulness of the methods. Moreover, quantifying the mass transfer of volatiles from spice to nibs and the inclusion of analytical data such as solid phase micro extraction (SPME)-gas chromatography (GC)-and mass spectroscopy (MS), will help to identify relationships or linkages between the panel's sensory analysis and instrumental quantitative data [7].

## References

1. Sukha D.A., Butler D.R., Umaharan P. and Boult E. (2008) European Food Research and Technology 226(3): 405-413.
2. Navarro S., de Bruin T., Montemayor A.R., Finkelman S., Rindner M. and Dias R. (2007) IOBC Bulletin Volume 30: 20-23.
3. Fair J. R. (2009) In: Chap 8 Mass transfer. Albright's Chemical Engineering Handbook (Lyle, F., ed.) Albright CRC Press.
4. Murray J. M., Delahunty C. M., and Baxter I. A. (2001) In: Descriptive sensory analysis: past, present and future. Food research international 34(6): 461-471.
5. ESSeguine-DASuhka Cocoa and Chocolate Flavour Evaluation, by ESSeguine and DA Sukha (2017) Licensed under a Creative Commons Attribution-ShareAlike 4.0 International License.
6. VSN International (2011) GenStat for Windows 14th Edition. VSN International, Hemel Hempstead, UK. Web page: GenStat.co.uk
7. Albak F. and Tekin A.R. (2015) Journal of Food Science and Technology 52(4): 1960.

# Impact of water-soluble precursors leaching from green beans on aroma generation during coffee roasting

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## Abstract

In this investigation green Robusta coffee beans were pre-soaked with different time-temperature profiles before roasting in normal conditions and grinding to a standardized particle size. Aroma profile of roasted coffee beans and water soluble precursors such as sucrose and total protein content from soaking water were examined by using Solid Phase Micro Extraction -Gas Chromatograph Mass Spectrometry and Liquid chromatography–mass spectrometry and BCA Protein Assay Kit respectively. A significant impact of soaking time-temperature profile was observed on the yield of water-soluble precursors in the soaking water. The loss of these precursors significantly de-creased aroma formation during roasting. The results also suggested that water-soluble precursors could modify the quality of Robusta coffee.

## Introduction

Coffee species such as Arabica and Robusta are most common coffee varieties in the world, which account for 61% and 38% of the coffee production worldwide. Arabica, perceived as a smooth, and rich flavour is usually more desirable than Robusta, which is often described as having a muddy odour. Robusta coffee beans are often blended with Arabica coffee beans to create specific aroma profiles, enhance cream formation or reduce cost, but the maximum that can be included is often limited due to the loss of aroma quality [5].

Aroma formation in coffee is directly related to the chemical composition of the green coffee beans and typical coffee aromas are developed during the roasting pro-cess due to complex reactions such as, Maillard reactions, Strecker degradation, thermal degradation and oxidation [2]. A number of studies have improved the quality of Robusta coffee by passing the green Robusta beans through steam to remove sub-stances such as 2-methylisoborneol, which is responsible for the muddy odour [1]. However, during this process important water soluble precursors such as sucrose and protein are leached into water, hence compromising the flavour generation potential of the roasted coffee. The amino acids and sugar are considered to be the main precursors in the aroma generation and colour formation during coffee roasting [3]. Therefore, the objective of this study was to investigate how much water-soluble precursors are lost during pre-soaking of green coffee beans and its impact on aroma generation during coffee roasting.

## Experimental

### *Coffee preparation*

Coffee beans were purchased from Edgehill coffee, Warwick, United Kingdom, where both Robusta beans (Vietnam) and Arabica beans (Kenya) are single-origin washed beans. Robusta green beans were soaked in water solution at different time (2, 4, 6, 8, 10 and 12 h) and temperatures (20, 40, 60, 80 and 100 ˚C), four replicates each. Soaked Robusta green beans and non-treated Robusta green were placed into a desiccator with saturated sodium nitrate solution (relative humidity 65.5%) at room temperature

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(20±2 °C) for 20 d to control the moisture content (around 11.5%). Determination of the water changes during soaking and coffee roasting was carried out by weighting the coffee sample at every step. Soaked Robusta green beans and non-treated Robusta green beans were roasted in a convection oven (Mono Equipment, Swansea, UK) at 200 °C for 20 min. Roasted samples were ground with an electronic coffee grinder (KG 49, Delonghi, Australia) then passed through a metal sieve size 710 um (Endecotts, Essex, UK) and stored in the freezer at -80°C prior to analysis.

*Gas Chromatograph Mass Spectrometry (GC-MS)*

The ground coffee (1.5 g) was transferred into glass vials (20 ml), four replicates for GC-MS analysis. An internal standard was prepared by adding 10 µL 3-heptanone (Sigma, Saint Louis, USA) into 10 ml methanol (Laboratory reagent grade, Fisher Scientific, UK). 2 µL of internal standard was added into each coffee sample and kept for 1 h equilibrium prior to GC analysis. All analytical samples were randomised for GC-MS analysis. A trace 1300 series Gas Chromatograph coupled with the Single-Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Hemel Hemptead, UK) was used for analysis of volatile compounds. Samples were incubated at 40 °C for 5 min with shaking. A 50/30 µm DVB/CAR/PDMS SPME Fibre (Supelco, Sigma Aldrich, UK) was used to extract volatile compounds from the sample headspace (extraction for 5 min then desorption for 2 min). The injector temperature was set at 200 °C in splitless mode (constant carrier pressure was at 18 psi). Separation was carried out on a ZB-WAX Capillary GC Column (length 30 m, inner diameter 0.25 mm, film thickness 1 µm; Phenomenex Inc., Macclesfield, UK). Column temperature was held initially at 40 °C for 5 min, increased by 3 °C/min to 180 °C, then 8 °C/min to 240 °C and held for 2 min. Full scan mode was used to detect the volatile compounds (mass range from m/z 20 to 300).

*BCA protein assay kit and Liquid Chromatography-Mass Spectrometry (LC-MS)*

Pierce TM BCA protein assay kit (23225/23227, Thermo Scientific) was used to measure the total protein content for both green beans and soaking water. Liquid Chromatography-Mass Spectrometry (LC-MS) was used to measure the sucrose content for both green beans and soaking water. The LCMS analysis was performed following standard protocol described in Perrone et al, 2008. All results were analysed by Design-Expert version 7.0.0 and Microsoft excel 2010 using samples as the fixed effect and a Tukey's HSD post-hoc test. Principal Component Analysis (PCA) was performed by Excel XLSTAT Version 2015.5.01.23373.

## Results and discussion

*Water-soluble precursors*

In figure 1, protein content showed a significant decrease with increased soaking temperature ($p < 0.05$). Similarly, a significant decrease in the sucrose content was observed in soaked Robusta green beans at 20 ˚C for 12 h when compared with non-soaked Robusta green beans. However, there were no significant differences between the soaked green beans at 20 ˚C and 40 ˚C for 12 h. Significant decrease in the sucrose content showed in the soaking temperature at 60 C˚, 80 C˚ and 100 C˚ for 12 h. A significant decrease in the sucrose content was observed with at 60, 80 and 100 ˚C soaking temperature for 12 h.

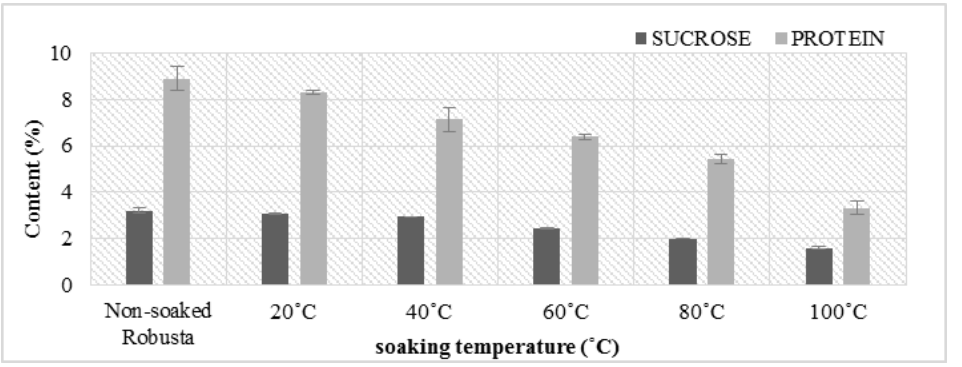


**Figure 1**: Sucrose and protein content in the non-soaked and soaked Robusta green beans at different soaking temperature at constant soaking time (12 h). The error bars are standard derivation.

Figure 2 showed that the soaking time also play an important role on the sucrose and protein content. In summary, a significant impact of soaking temperature and time on the water soluble precursors from green coffee beans was observed, this can significantly impact the aroma profile of roasted coffee beans ($p < 0.001$). In addition, in-crease soaking temperature results in higher loss in protein (from 8.3% to 3.3%) and sucrose (from 3.1% to 1.6%) content (Figure 1) when compare with increase soaking time the protein loss from 7.04% to 3.3% and sucrose from 2.6% to 1.6% (Figure 2).

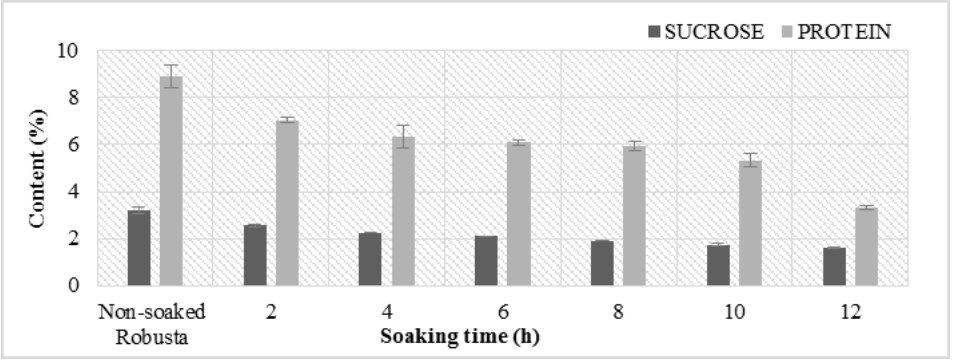


**Figure 2**: Sucrose and protein content in the non-soaked and soaked Robusta green beans at constant soaking temperature 100˚C for different soaking time. The error bars are standard derivation

*Aroma*

A range of volatile compounds were observed with roasted Robusta coffee beans with different functional groups such as 2 organic acids, 1 alcohol, 2 aldehydes, 3 key-tones, 2 furans, and 5 heterocyclic compounds (N containing). All aroma com-pounds showed a significant decrease in their content with increasing soaking time and temperature ($p < 0.05$). These volatile compounds are associated with sensory odour description such as malty, nutty, grassy, sour, burnt, and smoky [3].

Principal component analysis (PCA) was used to illustrate the variation between the 15 aroma compounds across the 10 soaked Robusta samples (including different time and temperature) and 1 non-soaked Robusta sample (Figure 3). PCA results indicated that both soaking time and temperature have a significant effect on aroma generation during the coffee roasting. The first principal component (PC1) accounted for 75.51% of the

variance in the whole dataset and showed separation between the soaked Robusta (left) and non-soaked Robusta samples (right). The second principal component (PC2) accounted for 17.37% of the variance in the dataset and discriminated the difference between increasing soaking time (top) and soaking temperature (bottom). Sample soaked at 20 ˚C and 40 ˚C for 12 h, showed more closed to the furfural, acetic acid, 2-methylfuran, 2, 3-butanedione, 2-furanmethanol and 2, 3-pentanedione content as compared to the samples soaked at a higher temperature (60 ˚C, 80 ˚C and 100 ˚C). Non-soaked beans have a significantly higher concentration of all these volatile compounds ($p < 0.001$) as compared to soaked green beans. This change can be explained by the leaching of sucrose during soaking process at higher temperature as shown in Figure 1. Volatiles such as furfural, acetic acid, 2-methylfuran, 2, 3-butanedione, 2-furanmethanol and 2, 3-pentanedione have been reported as sugar degradation products [3]. Therefore, in conclusion the reduction sucrose content in the soaked green beans has significantly affected aroma formation during the roasting process.

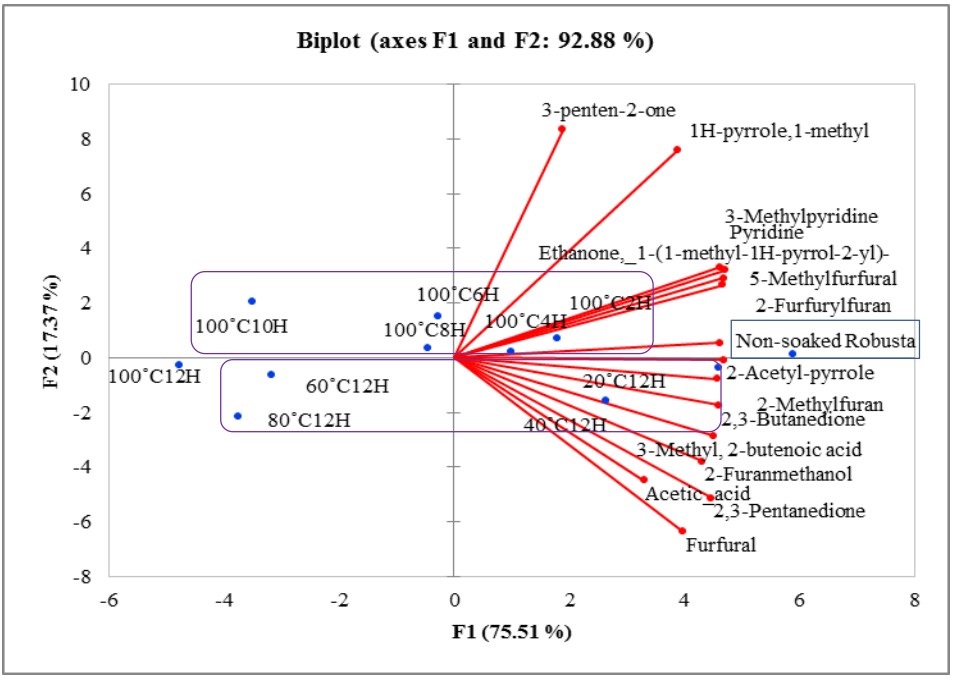


**Figure 3:** Principle component analysis (Bi-plot) of the volatiles compounds associated with soaked and non-soaked Robusta coffee analysed by GC-MS.

## References

1. Becker, R., Schlabs, B., and Weisemann, C. (1991). Process for improving the quality of Robusta coffee. In): Google Patents.
2. Fisk, I. D., Kettle, A., Hofmeister, S., Virdie, A., and Kenny, J. S. (2012) Flavour, 1(1), 14.
3. Flament, I. (2002). Coffee flavour chemistry. West Sussex.
4. Perrone, D., Donangelo, C. M., and Farah, A. (2008) Food Chem, 110(4), 1030-1035.
5. Robertson, C. (2010) The little book of coffee law: American Bar Association, 50-52.

# Revisiting the role of glycosidic aroma precursors on wine aroma: Effects of microorganisms and of slow hydrolytical processes

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## Abstract

Grapes are determinant for the quality of the final wine since they not only provide a specific profile of nutrients which strongly determines the production of secondary metabolites by yeast, but they provide precursors of key aroma components. Glycosidic precursors were the first category of aroma precursors discovered and for long it has been known that they constitute the main source of relevant wine aroma molecules such as linalool and β-damascenone, but also main sources of some potential off-flavours such as 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN).

In spite of their known importance, their role in wine aroma formation is not completely understood due to the complexity of their genesis. Difficulties arise because many aglycones undergo different chemical rearrangements to produce the aroma molecule.

In order to better understand the role of yeast and of aging two large fractions of precursors from highest quality Garnacha grapes were obtained by SPE, and used to prepare model musts. The musts have been fermented by different yeasts and resulting wines have been subjected to accelerated aging under strict anoxic conditions. Analytical controls have been included all throughout the process in order to ensure an efficient control of the mass balance.

Results show that aromas formed from different grape varieties and in combination with different microorganisms lead to high aroma diversity. Besides, this study has allowed to differentiate the aroma formation influenced by enzymatic or hydrolytic activity, as well as their evolution during bottle aging, enlightening the principal formation mechanism and the fate of these aromas during the shelf-life of wines.

## Introduction

Most wine grapes are aromatically neutral, nonetheless, they are important providers of aroma precursors that can be released during wine making and wine aging. Different families of odorants can be formed from precursors such as polyols by chemical rearrangements, glycosides by enzymatic or acidic hydrolysis, or cysteinyl derivatives by the action of yeast β-lyases. Glycosides are related with the genesis of important aroma volatiles, some of them are considered as varietal wine aroma compounds [1][2]. Since different strains of *Saccharomyces* contain different types and activities of glycosidases, some specific strains have been proposed as having abilities to enhance the varietal aroma of wines made of grapes from a single variety. Moreover, different *genera* of yeast can lead to very different fermentative outcomes due to their diverse genetic pool and that is why the usage of non-*Saccharomyces* yeast strains as enhancers of the organoleptic properties of wine is gaining more and more attention [3][4].

The processes that lead to the release of aroma from non-floral grape precursors by yeast is not yet fully understood, since large amount of reactions and interconversions

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take place during this stage of winemaking. Particularly, the bound fraction of grape glycosides can be source of several families of odorants, such as monoterpenes, volatile phenols, $C_{13}$-norisoprenoids or vanillin derivates. These compounds are very important to establish the varietal character of wines and some can be very powerful odorants, even at small concentrations. While the release of some compounds can occur by direct enzymatic or acidic hydrolysis of the glycosylated bound between the glycone (sugar moieties) and the aglycone of volatile aroma compounds, others require more complex reactions or even chemical rearrangements. In addition, wine aging can also be determinant to the appearance of certain odours, positive or negative, which can further contribute to the development of the varietal character of wine [1][2].

The present research intends to further investigate the effects of yeast (*S. cerevisiae* and non-*Saccharomyce*) on the formation of aroma compounds derived from glycosidic precursors taking into account aging time.

## Experimental

Glycosidic precursors fraction was extracted from 23 kg of Garnacha grapes from Spain. After grape crushing and addition of $SO_2$ (5 mg/kg) and pectolitic enzymes (Lafazym, 127 mg/kg), cold maceration took place during 48 hours inside a closed recipient. Grapes were then pressed and the liquid obtained was sulfited (90 mg/L) and let to sediment at 4˚C for 24hours, after which the clean must was further filtered, divided into two 5L-batches and sulfited again (90 mg/L). Five grams of conditioned LiChrolut-EN resins were added to each batch and kept under magnetic stirring for 48 hours at 10˚C. Resins were further recovered using paper filter, washed with water and re-packed into beds. Free aroma compounds were washed out with 45ml of DCM and the glycosidic fraction was eluted with 90ml of Ethyl acetate-methanol (95:5, v/v). The extracted must was further sulfited (50 mg/L) and re-extracted with a second 5g-batch of clean resins, which were similarly processed to obtain the glycosidic fraction which was collected with the previous one and evaporated to dryness under Nitrogen.

A complex synthetic grape must with pH 3.5 containing oligoelements, vitamins, glucose, fructose, Tween, and amino acids imitating Garnacha grapes was prepared under aseptic conditions. Three-hundred and fifty mL volumes of synthetic must, containing or not glycosidic precursors were inoculated with non-*Saccharomyces* yeast strains at day 0 and with *S. cerevisiae* after 4 days. Control samples fermented only with *S. cerevisiae* were also prepared. The strains used were *Pichia kluyveri* (Frootzen), *Lachancea thermotolerans* (Concerto), *Torulaspora delbruekii* (Prelude), all from Chr. Hansen. Air locks were used to seal the fermenters and fermentation was carried out at 21˚C.

Once the_fermentation was over, the wines were centrifuged, introduced into an anoxic chamber, aliquoted into three air tight tubes and further bagged in high density plastic bags containing oxygen scavengers. The tubes were subjected to anoxic accelerated aging at 50˚C for 1, 2 and 5 weeks.

Resulting wine samples were characterized according to their general enological parameters; major volatiles were analysed by liquid-liquid microextraction followed by a GC-FID analysis [5], and minor volatiles were isolated by SPE and determined by GC-MS [6].

## Results and discussion

The effects of the presence of glycosidic precursors on the aroma profile were assessed by comparing the odorant profiles (paired t-test) of corresponding ferments with

or without precursor fractions. Overall, the presence of glycosidic precursors caused significant increases in the levels of 28 compounds, 9 of which were above or close to the odour threshold. Eight out of the 28 compounds were in fact fermentative compounds, which suggest that the precursor fraction –in synthetic must- exert a general effect on the secondary metabolism of yeast.

**Table 1:** Aroma compounds whose levels significantly increase in the presence of glycosidic precursors. Compounds marked with * are at levels above or close to the odour threshold.

| | *Varietal odorants* |
|---|---|
| *Monoterpenols* | Linalool, α-Terpineol, Geraniol* |
| *Norisoprenoids* | β-Damascenone* |
| *Volatile Phenols* | 4-Vinylphenol, E-Isoeugenol*, Eugenol, 4-Vinylguaiacol*, Guaiacol*, 2,6-Dimethoxyphenol, 4-Ethylguaiacol, 4-Allyl-2,6-dimethoxyphenol |
| *Lactones* | γ-Nonalactone, γ-Butyrolactone |
| *Vanillin derivates* | Methyl vanillate, Ethyl vanillate, Acetovanillone, Syringaldehyde, Vanillin |
| *Cinnamates* | Ethyl dihydrocinnamate* |
| | *Fermentative odorants* |
| *Higher alcohols* | 1-Hexanol, Benzyl alcohol, Methionol* |
| *Ethyl esters* | Ethyl hexanoate*, Ethyl octanoate*, Ethyl decanoate* |
| *Acetate esters* | Butyl acetate |
| *Carbonyl compounds* | Acetoine |

In order to further investigate the role of the different strains of yeasts and the effects of accelerated wine aging on the formation of these aroma compounds a two-way ANOVA was carried out. As summarized in Figure 1 for the particular case of 4-vinylguaiacol, the effects of the precursor fraction increased with aging time and were just slightly dependent on the strain of yeast which conducted the fermentation, in apparent disagreement with previous results [3][4].

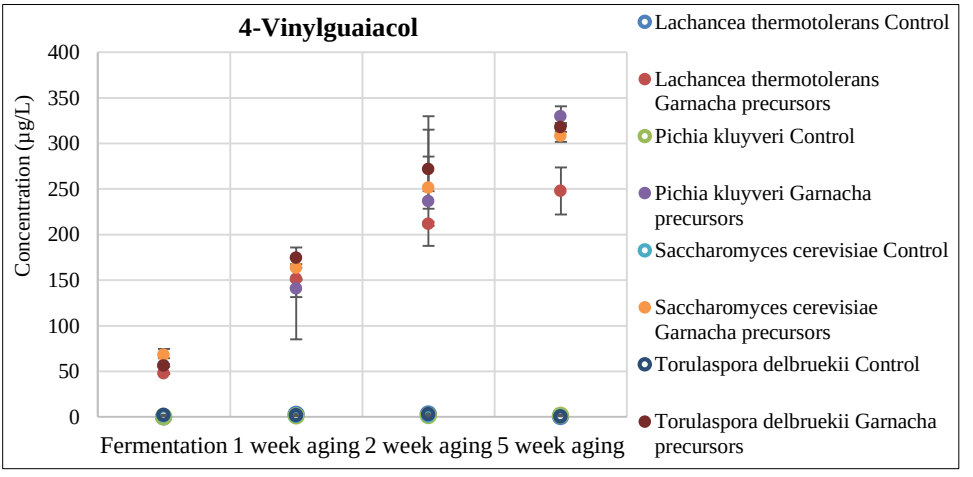


**Figure 1:** 4-Vinylguaiacol content in fermentations carried out by *L. thermotolerans*, *P. kluyveri* and *T. delbruekii*, sequentially inoculated with *S. cerevisiae* and *S. cerevisiae*, individually inoculated (yeast control) in control must and in musts spiked with glycosidic precursors fraction. Samples were analysed at the end of fermentation and after 1, 2 and 5 weeks of accelerated wine aging under strict anoxia conditions.

Two relevant exceptions to this general pattern of low yeast dependence were the cases of ethyl dihydrocinnamte and of geraniol, for which levels were significantly influenced by the strain of yeast. In the cases of ethyl dihydrocinnamate, *T. delbruekii* seems to be able to produce this compound at relatively large levels regardless of the presence of glycosidic precursors.

By contrast, wines made with the other yeasts contained low levels of this important aroma compound, just slightly higher in samples fermented in the presence of glycosidic precursors.

The case of geraniol was still more challenging. In wines obtained from *L. thermotolerans* and *T. delbruekii*, highest levels of this compound were observed by the end of fermentation in those samples containing glycosidic precursors. Levels of this unstable compound later decreased throughout aging. Contrarily, wines fermented with *P. kluyveri* and *S. cerevisiae* did not contain any geraniol by the end of fermentation, but its levels later increased during aging, regardless of the presence of precursors. Again, this suggests that both yeasts are able to form de novo a precursor of this odorant.

In conclusion, while this research confirms that the glycosidic aroma precursor fraction has an important effect on the levels of many wine aroma components, it also revealed that the effects of the yeast carrying out the first step of fermentation on the levels of most aromatic aglycones were surprisingly low. In contrast, some yeasts showed a specific activity to form *de novo* aroma molecules or aroma precursors. All this suggests that the different role played by yeast are more related to their specific secondary metabolism and not to their differential glycosidase activities.

**References**

1. Ferreira V.G., and San Juan F. (2011) In: Flavor of Wine, Food Flavours: Chemical, Sensory, and Technological Properties, pp. 269-299.
2. Parker M., Capone D.L., Francis, I.L. and Herderich M.J. (2017) Journal of Agricultural and Food Chemistry.
3. Hernández-Orte P., Cersosimo M., Loscos N., Cacho J., Garcia-Moruno E., and Ferreira V. (2008) Food chemistry, pp.1064-1077.
4. Mendes Ferreira A., Clímaco M.C., and Mendes Faia A. (2001) Journal of Applied Microbiology, 91: 69-71.
5. Ortega C., López, R., Cacho J., and Ferreira V. (2001) Journal of Chromatography A, 923:.205–214.
6. Lopez R., Aznar M., Cacho J., and Ferreira V. (2002) Journal of Chromatography A, 966:167–177.

# Organoleptic properties of dark chocolates investigated by direct-injection mass spectrometry (PTR-ToF-MS) and GC-olfactometry

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## Abstract

A preliminary sensory study conducted on a set of 187 dark chocolates varying in terms of cocoa origin and variety allowed their classification into four distinct sensory categories. Fingerprints in volatile organic compounds (VOCs) of these chocolates were obtained by a direct-injection mass spectrometry headspace method using Proton Transfer Reaction Mass Spectrometry (PTR-MS). This chemical analysis allowed discriminating the four sensory poles, so the sensory discrimination seemed to be mainly based on volatile compounds. Then, the key odorants responsible for chocolates differentiation were determined through identification of targeted aroma compounds by GC-MS after GC-O analyses of extracts representative of each subset of chocolates. Twelve dark chocolates were studied using the detection frequency method. The odour events generated by a panel of 12 assessors were grouped into 124 odorant areas (OAs). Correspondence analyses allowed distinguishing the samples while identifying 34 OAs that appear relevant to discriminate the chocolates sensory poles. Among these characteristic OAs, five were identified unambiguously with GC-MS and the remaining need to be resolved from numerous coeluted peaks.

## Introduction

Dark chocolates develop several organoleptic characteristics depending on cocoa origin, cocoa variety and fabrication process. These parameters influence the chemical composition of the chocolates, and particularly their qualitative and quantitative content in volatile organic compounds (VOCs) responsible for their aroma [1]. A set of 187 dark chocolates varying in terms of cocoa origin and variety, obtained with exactly the same fabrication process, was submitted to sensory evaluation based on 36 descriptors (32 aromas and 4 tastes). Four distinct sensory poles (SPs) were subsequently clearly established. As their sensory differentiation was essentially based on aroma descriptors, we hypothesized that the sensory classification of the chocolates should be mainly based on their composition in VOCs. VOCs investigation can be carried out by headspace analysis using direct-injection mass spectrometry such as Proton-Transfer Reaction Mass Spectrometry (PTR-MS), an untargeted approach that leads to aroma profiles (fingerprints). Identification of targeted aroma compounds is possible using gas chromatography combined with olfactometry (GC-O) and GC-MS. GC-O has been commonly used to investigate key aroma compounds in several products, including cocoa and chocolate [2-4]. The aim of this study was to identify key aroma compounds of the four sensory poles. To achieve this goal, we first checked that the sensorial differentiation

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was mainly based on VOCs composition by studying the chemical fingerprints of the 187 chocolates. Then we identified the key odorants responsible for chocolates differentiation by GC-MS analyses of targeted aroma compounds selected after GC-O analyses of extracts representative of each subset of chocolates.

## Experimental

### *Samples*

Dark chocolates were provided by the Valrhona company. All the samples originating from different cocoa varieties and sources were produced using the same transformation process with the same mass of cocoa, sugar, soy lecithin and vanillin.

### *Headspace analysis using PTR-ToF-MS*

Samples of chocolate (1 g) mixed with 1 mL of artificial saliva were transferred to 20 mL vials that were maintained under stirring at 36.2°C for 2 hours equilibration time. Headspace measurements of 187 samples were performed in triplicates using a Proton Transfer Reaction - Time of Flight - Mass Spectrometry (PTR-ToF-MS) instrument (PTR-ToF 8000, Ionicon Analytik GmbH, Innsbruck, Austria) with $H_3O^+$ as reagent ion. The instrument drift-tube was set to a pressure of 2.30 mbar, a temperature of 80°C and a voltage of 480 V, which resulted in E/N ratio (electric field strength to gas number density) of 111 Townsend (Td, 1 Td=$10^{-17}$ V.cm$^2$). Total inlet flux was adjusted to 65 ml/min and the transfer line maintained at 110°C. To assure a constant flux into the PTR and avoid drift-tube depression, a flux of 100 ml/min of zero-air was used with a leak allowing the flux excess to escape. The designed experimental setup allowed analysing successively background air, the sample and the molecule used for mass calibration of the instrument (headspace of aqueous ethyl decanoate (Sigma-Aldrich)) just by twisting four three-way valves. A sample analysis lasted 5 minutes and was followed by cleaning the tubing by flushing the transfer line with zero-air until baseline recovery. This protocol allowed the analysis of successive samples every 10 min. The measurement order was randomized using a Latin square design to avoid possible systematic memory effects. The average areas under the curves obtained for the 2 min release of 314 significant ions present in the mass spectra were used to perform unsupervised (PCA) and supervised (PLS-DA) multivariate data analyses.

### *Extraction of the volatiles of 12 samples*

30 g of chocolate were mixed with 100 mL ultra-pure water and 300 µl 2-methylheptan-3-one (93 ng/µl in water) as internal standard. This mixture was vacuum distilled under stirring for 1h45 using a SAFE apparatus [5] in a thermostated bath at 37°C. The aqueous distillate was extracted with dichloromethane (3 x 15ml). Finally, the extract was concentrated to 400µl with a Kuderna-Danish apparatus in a 70°C water bath.

### *Identification of odorous compounds with GC-O and GC-MS*

Twelve assessors evaluated the extracts using detection frequency methodology. Samples were analysed using a 6890A gas chromatograph (Agilent Technologies, Massy, France) equipped with a flame ionization detector (FID) using a DB-FFAP column (30 m x 0.32 mm x 0.5 µm; J&W Scientific, Folsom, CA, USA). The effluent was split into two equal parts to the FID and the sniffing port via Y-type seal glass and two deactivated capillaries. The assessors generated sensorial attributes at the same time they detected an odour events. These were grouped into olfactive areas (OAs) on the basis of the closeness of their linear retention indices (LRIs). A detection filter of 30% was set to finally retain 124 significant OAs. A correspondence analysis (CA) was performed on the detection

frequencies of the discriminant OAs found in the 12 samples. Identification of the compounds responsible for OAs was done by gas chromatography-mass spectrometry (GC-MS) by injection on the same column as in the GC-O study. Reliability of compounds identification was assured by comparison of mass spectra to databases (NIST 08 and an in-house database, INRAMass) and by comparison of LRIs to LRIs on DB-FFAP cited in literature.

## Results and discussion

A Principal Component Analysis (PCA) conducted on the PTR-MS data revealed partial separation of the four sensory poles (SPs) (data not shown). To go further a Partial Least Squares Discriminant Analysis (PLS-DA) was conducted on the 314 ions obtained in the PTR-MS study (X variables) to try to better distinguish the four sensory poles (Y variables) and identify the most explanatory ions used for the classification. PLS-DA revealed 7 significant latent variables with $R^2 = 0.847$.

Figure 1 displays the plane defined by the two first latent variables that carried out significant explained variance (28% for X and 26% for Y on the first factor and 9% for X and 20% for Y on the second). The robustness of the model was obtained using leave-one-out cross validation. The groups formed by samples of each SPs were differentiated, especially those from the SP 1 and 2, found in the positive side of the first factor while SP4 were find on the opposite side. The groups formed by samples affected to the SP3 and the SP4 are better distinguished on the plan defined by the factors 1 and 3. Explanatory ions could be inferred from the model and could be considered as molecular markers of SPs and could be used to predict to which SP an unknown sample belongs. This classification could be compared to the one obtained with the sensory data and globally revealed the same features (data not shown).

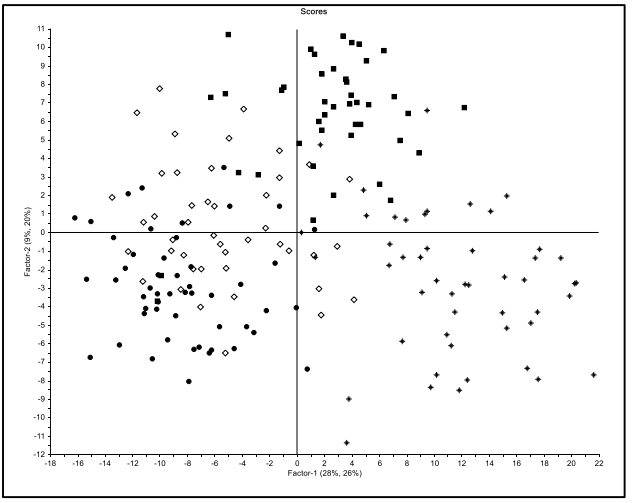


**Figure 1:** PLS-DA with chemical data (factors 1 and 2) 187 samples distributed in 4 sensory poles (Y variables) / 314 ions (X variables) (star: pole 1; box pole 2; dot: pole 3; open diamond: pole 4)

The GC-O experiment revealed 124 OAs after application of a 30 % threshold on the detection frequencies. Among them, 34 showed significant detection frequencies differences between samples and were included in a correspondence analysis in the aim to discriminate the samples and associate corresponding OAs. The samples were clearly

discriminated along factor 1 of the CA (Figure 2) and characteristic OAs were found for each SP. Factor 3 discriminated samples belonging to the poles 1 and 2 (data not shown). Furthermore 90 OAs exhibited no real changes in detection between samples and therefore may represent the background of the overall chocolate aroma. Only five OAs have been positively identified so far by comparing their experimental data to the literature data (retention indices, mass spectra and aroma descriptors).

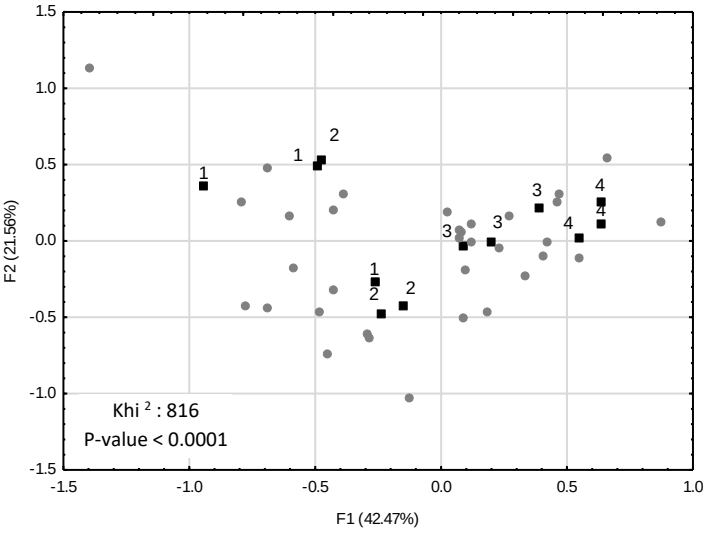


**Figure 2:** Correspondence analysis (factors 1 and 2): detection frequencies of 34 OAs (grey dots) within 12 samples (black diamonds). The different numbers (1, 2, 3 and 4) indicate the sensory poles.

To conclude, the "chemical map" obtained in the PTR-MS analyses of the chocolates headspace allowed retrieving the classification of the 187 samples into the four sensory categories previously determined. Thus, it could be deduced that the composition of chocolates in VOCs explained in a large part the sensory classification. Using GC-Olfactometry, discriminant OAs for each pole were identified thanks to a correspondence analysis. Some discriminant OAs have been positively identified using GC-MS. The remaining unidentified OAs required additional analyses for their identification (a different GC column, chemical ionization, 2DGC-MS-O…).

## References

1. Aprotosoaie, A.C., Luca, S.V., and Miron, A., (2016) Comprehensive Reviews in Food Science and Food Safety, 15: 73-91.
2. Counet, C., Callemien, D., Ouwerx, C., and Collin, S., (2002) Journal of Agriculture and Food Chemistry, 50: 2385- 2391.
3. Liu, J., Liu, M., He, C., Song, H., Guo, J., Wang, Y., Yang, H., and Su, X., (2015) Journal of the Science of Food and Agriculture, 95: 1362-1372.
4. Schnermann, P., and Schieberle, P., (1997) Journal of Agriculture and Food Chemistry, 45: 867-872.
5. Engel, W., Bahr, W., and Schieberle, P., (1999) European Food Research and Technology, 209: 237-241.

# Influence of salt reduction on flavour release in ready-to-eat meals

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## Abstract

Salt reduction in food is becoming a major concern for public authorities since a high sodium diet is associated with an increased risk of hypertension and obesity [1,2,3,4]. As convenience products and ready-to-eat meals are one of the main sources of dietary sodium, the food industry is encouraged to produce low-sodium formulations. However, salt is a well-known flavour enhancer and its reduction could modify the release of volatile compounds, thereby affecting flavour perception. In this study, a salt reduction of 40% in a meal composed of chicken, pasta and cheese sauce significantly impacts its flavour perception evaluated in sensory analysis compared to the reference meal with no salt reduction. The decrease of flavour intensity could be related to the decreased amount of terpenes as these compounds are known to be highly odour-active.

## Introduction

Sodium chloride, usually referred to as salt, provides about 90% of people's dietary sodium intake [1]. The World Health Organization recommends a maximum salt intake of 5 g/day for adults. However, in the industrial countries, the mean sodium intake is generally higher [2]. Dietary sodium intake mainly originates from processed foods (75-80%), from non-processed foods (5-10%), and from the salt added during the preparation of meals or at the table (10-15%) [1]. A salt consumption higher than the physiological needs is known to increase blood pressure, leading to the development of noncommunicable diseases, such as hypertension, cardiovascular diseases or coronary heart disease [1,2,4]. Lowering the salt intake of individuals is one of the main challenges for authorities to prevent health diseases [5].

During industrial food processing, salt is largely used as a flavour enhancer. A reduction of its amount in foods may modify their organoleptic properties, especially taste. However, taste has been pointed out to be one of the main drivers of liking, which motivates consumers to purchase a product [6]. Considering the pressure of the public authorities on the food industry to reduce salt in their products, the main challenge is to formulate food with lower sodium content while maintaining satisfying organoleptic qualities [6]. Processed foods are particularly rich sources of sodium. In Europe, the main sources of sodium are bread and cereal products, delicatessen, sauces and condiment, ready-to-eat meals, cheese, soups, pasta dishes and pizzas [1,3,7]. The consumption of convenience foods and ready-made meals is steadily increasing, as is the development of obesity and other diseases related to high sodium intake. This phenomenon is related to our modern life style which involves less time spent for meal cooking [8].

Many solutions have been tested to reformulate foods with lower sodium content while maintaining an acceptable organoleptic quality. Each solution must be adapted to the type of food, as salt may also have a technological role, especially for microbial safety [1,9]. For ready-to-eat meals, the solutions tested involve direct salt reduction, use of substitutes such as potassium chloride and flavour enhancers such as yeast extract and

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addition of natural flavour enhancers such as aromatic herbs [1,10]. In fact, the study of odour-taste interactions could compensate salt reduction with the use of aromas congruent with salt perception [10,11].

Many studies were conducted to test solutions to compensate salt reduction and check their acceptability by consumers, however only a few were performed to characterize the impact of salt reduction on the flavour of food, especially ready-meals, with both instrumental and sensorial methods. As salt is a well-known flavour enhancer, a reduction of its amount could modify the release of the volatile compounds and thus flavour perception. In this study, a ready-to-eat meal composed of chicken with pasta and cheese sauce with various levels of salt content was chosen to study the impact of salt reduction on sensory perception as well as on volatile compounds release. The aim was to characterize the modifications in the aroma profile due to salt reduction of ready-to-eat meals by means of a descriptive profile to determine the effect of salt reduction on the sensory properties, and, secondly, to identify if modifications in the volatile compounds can be observed due to salt reduction.

## Experimental

### *Materials*

Ready-to-eat meals were produced containing pasta (38%), chicken (24%) and cheese sauce (38%). Various salt levels were tested: 100% salt (0.80g salt/100g food), 80% salt (0.64g salt/100g food), 70% salt (0.56g salt/100g food) and 60% salt (0.48g food/100g food).

### *Sensory analysis*

A panel consisting of 21 trained assessors (23-55 years) was recruited. Sensory analysis took place in a sensory analysis room equipped with sensory booths. The 4 samples were conditioned in isotherm boxes and delivered at 63°C to the panellists. The samples were presented in a randomized order and identified with a three-digit code. A ranking test on 11 attributes was performed. The attributes were chosen to describe odour (O), texture (T) and flavour (F). A Friedman test ($\alpha$=5%) was applied on these results.

### *Chromatographic analysis*

Volatile compounds were extracted using HS-SPME (Headspace Solid Phase Micro Extraction). The fibre used was 1cm Car/PDMS 85µm. Samples were weighed (5 g) in a 20mL vial. The equilibrium phase lasts 15min at 49°C. The extraction phase lasts 50min at 49°C. After extraction, the volatile compounds were injected in the GC-MS (column DB-WAX). Injector was maintained at 260°C. The program temperature ranged from 40°C (5min) to 230°C (10min) at 5°C/min. Identification of the volatile compounds was performed with comparison of the Kovats linear retention indices (LRI) with the literature, comparison of the mass spectra with a database and standard injection. Relative areas were used for semi-quantification. Comparison of the amount of each volatile compounds in each sample was performed with one-way analysis of variance ($\alpha$=5%) followed by Least Significant Difference test.

## Results and discussion

### *Sensory properties of pasta/chicken/cheese sauce meal*

A sensory characterization of the 4 samples of pasta/chicken/cheese sauce meals was performed (figure 1).

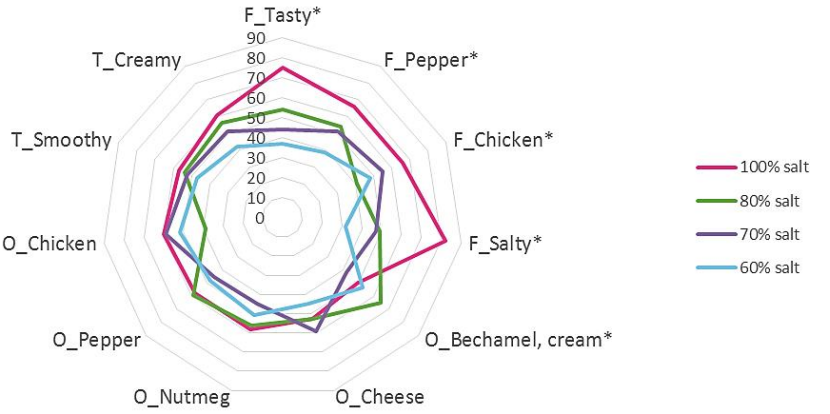


**Figure 1**: Sum of ranks computed for each sample and each attribute. Significant differences between samples are indicated by an asterisk (α=5%). Different letters mean significant differences.

Differences between the samples were mainly observed for the flavour in mouth. The control 100% salt was always perceived more aromatic than the salt-reduced samples. As expected, the salty taste obtained significant different scores between samples. Moreover, the 3 others flavour attributes (F_Pepper, F_Chicken, F_Tasty) were also impacted by salt reduction, emphasizing its role on flavour release. However, no differences were perceived for texture and odour, except for the odour of béchamel. Such interactions between taste and aroma may be explained by physico-chemical, physiological and psychological interrelationships [12].

A characterization of the aroma volatile compounds was performed to explain the results observed in sensory analysis. Chromatographic analysis revealed that the samples had the same total number of volatile compounds (82) varying only in quantity. Volatile compounds belong to various chemical classes. The most significant differences between samples occurred with respect to terpenes, with a decrease of their concentration associated with the salt reduction (Table 1).

**Table 1**: Sample means of the quantity of terpenes identified in the 4 ready meals with various salt content (expressed in area $x10^5$/g of product). Superscripts refer to results from post-hoc LSD tests associated with each volatile com-pound (α=5%). When identical, means are not significantly different.

| Volatile compounds | *LRI* | *100% salt* | *80% salt* | *70% salt* | *60% salt* |
|---|---|---|---|---|---|
| α-pinene | 1026 | 5,3 (7)$^{a}$ | 5,3 (7)$^{a}$ | 4,5 (3)$^{b}$ | 4,4 (5)$^{b}$ |
| β-pinene | 1111 | 6,5 (9)$^{a}$ | 4,7 (10)$^{b}$ | 5,0 (4)$^{b}$ | 5,2 (8)$^{b}$ |
| Sabinene | 1125 | 5,2 (7)$^{a}$ | 3,1 (10)$^{ab}$ | 3,4 (4)$^{b}$ | 2,9 (9)$^{c}$ |
| δ-3-carene | 1154 | 5,8 (6)$^{a}$ | 4,6 (13)$^{b}$ | 4,7 (8)$^{b}$ | 4,9 (3)$^{b}$ |
| β-myrcene + l-phellandrene | 1171 | 10,3 (3)$^{a}$ | 9,5 (9)$^{ab}$ | 8,7 (2)$^{b}$ | 9,2 (7)$^{b}$ |
| α-terpinene | 1186 | 7,0 (8)$^{a}$ | 5,9 (11)$^{b}$ | 5,4 (4)$^{b}$ | 5,8 (4)$^{b}$ |
| d-limonene | 1205 | 30,1 (3)$^{a}$ | 23,5 (8)$^{c}$ | 26,8 (7)$^{b}$ | 28,9 (4)$^{ab}$ |
| β-phellandrene | 1209 | 8,4 (11)$^{a}$ | 6,1 (10)$^{b}$ | 5,9 (5)$^{b}$ | 5,3 (5)$^{b}$ |
| γ-terpinene | 1238 | 12,4 (5)$^{a}$ | 10,4 (10)$^{b}$ | 9,6 (3)$^{b}$ | 10,8 (4)$^{b}$ |
| p-cymene | 1268 | 13,2 (10)$^{a}$ | 10,4 (9)$^{b}$ | 9,8 (7)$^{b}$ | 10,6 (5)$^{b}$ |
| α-terpinolene | 1279 | 4,5 (5)$^{a}$ | 4,1 (12)$^{ab}$ | 3,8 (4)$^{b}$ | 3,1 (6)$^{c}$ |
| 4-terpineol | 1601 | 17,5 (3)$^{a}$ | 16,1 (4)$^{b}$ | 16,0 (2)$^{b}$ | 16,4 (5)$^{ab}$ |

The presence of terpenes, known as highly odour-active compounds originating from natural products, may be explained by the use of pepper and nutmeg in the sauce. Among the 15 volatile compounds with significant higher concentration in the 100% salt sample 12 are terpenes. Indeed, the impact of salt on the release of the volatile compounds, known as the "salting out" effect, is particularly noticeable for terpenes. This latter aspect may explain the increase of the intensity of the flavour attributes F_Pepper and F_Tasty perceived by assessors in the sensory analysis for 100% salt sample. Similar results were obtained with tomato soups rich in vegetables [6]. Our results show that terpenes are particularly sensitive to salt reduction even when natural products are present in very small quantity in a complex matrix, and these modifications are perceived by consumers.

To improve the nutritional properties of processed foods, salt reduction is strongly advised. However, such a salt reduction might impair the organoleptic quality of food, resulting in a loss of aroma. With regard to cheese sauce-topped chicken and pasta dishes produced within this study, sensory analysis indicated that a salt reduction beyond 20% is perceived by assessors. A characterisation of the volatile compounds revealed that the aroma loss is mainly due to the decrease of the amount of terpenes which was associated with salt reduction ($r^2$=82% without d-limonene). These compounds, generated by plants, are highly odour-active, and play a significant role in the global aroma of the dish. The complementary use of sensory and instrumental analyses allows us to identify those volatile compounds responsible for aroma loss and permits to consider solutions to compensate it. Indeed, the increase of pepper or nutmeg in the recipe may be an efficient solution to increase the content of terpenes, as well as the use of other herbs and spices or salt-associated flavours [11]. Further sensory analysis performed on the various formulations tested may be necessary to determine the most efficient solution to compensate salt reduction and to produce dishes with satisfying organoleptic qualities.

## References

1. Kloss L., Dawn Meyer J., Graeve L. and Vetter W. (2015) NFS Journal, 1: 9-19.
2. World Health Organization (WHO) (2012) In: WHO Press, Geneva, Switzerland.
3. AFSSA (2009) In: Rapport de la commission AFSSA
4. He F.J. and MacGregor G.A. (2010) Progress in Cardiovascular Diseases, 52: 363-382.
5. World Health Organization (WHO) (2013) In: WHO Press, Geneva, Switzerland.
6. Michell M., Brunton N.P. and Wilkinson M.G. (2012) Food Res Int, 44: 1036-1043.
7. Kanzler S., Hartmann C., Gruber A., Lammer G. and Wagner K.-H. (2014) Public Health Nutrition 17(11): 2459-2466.
8. Alkerwi A., Crichton G.E. and Hébert J.R. (2015) British J Nutr 113: 270-277.
9. Durack E., Alonso-Gomez M. and Wilkinson M.G. (2013) J Food Safety 33(3):302-312.
10. Liem D.G., Miremadi F. and Keast R.S.J. (2011) Nutrients 3(6): 694-711.
11. Lawrence G., Salles C., Septier C., Busch J. and Thomas-Danguin T. (2009) Food Quality and Preference 20: 241-248.
12. Arvisenet G., Guichard E. and Ballester J. (2016), Food Quality and Preference, 52, 211-221

# Characterisation of the flavour of the old Austrian apple variety 'Ilzer Rose'

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## Abstract

The old apple variety 'Ilzer Rose', coming from the region near the village Ilz (Austria), is an old variety that has been described since approximately 1900. The rather small, intense-red apples with white flesh have a very pleasant, intense fruity and rose-like flavour. The aim of this study was to characterize the flavour of the old apple variety 'Ilzer Rose' but also to identify differences in distribution of volatiles between the skin and the flesh of the apples. The use of comprehensive GC x GC-MS resulted in the detection of more than 600 volatile compounds and offers a completely new picture of the apple volatilome.

## Introduction

Styria is Austrian's apple cultivation hot spot. About 80% of the annual yield (corresponding to about 130.000 tons) is harvested in this region. The majority of apples – mainly new apple varieties as Golden Delicious, Gala or Idared – are cultivated in plantations. However, about 25% of the apples are grown in so-called meadow orchards. The traditional meadow orchards have been part of a specific type of landscape for hundreds of years and have hosted an enormous number of old apple varieties since then. Even though these varieties have been cultivated in this region for many decades, their flavour properties have not been characterised so far. Most varieties lack a molecular characterisation of flavour compounds.

In general, the flavour of apples is composed by several hundred different volatile compounds such as alcohols, aldehydes, esters, etc. The composition of the apple volatiles depends on variety, climate, maturity/ripening level and storage conditions [1]. Primary flavour compounds are formed via the enzymatic and biological processes in the intact fruit during growth, maturation and ripening, whereas secondary flavour compounds develop as results of tissue disruption. Apple flavour compounds are produced by several biosynthetic pathways, such as the β-oxidation of fatty acids, which is the primary biosynthetic pathway for ester formation. After cell disruption, the lipoxygenase (LOX) pathway is active and is mainly responsible for the formation of straight chain C6 and C9 aldehydes whereas amino acid degradation reactions lead for example to methyl branched aldehydes and alcohols. It is generally assumed that terpene biosynthesis plays a minor role for apple flavour. However, terpenes are formed via the mevalonic (MVA) pathway or the 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway. In general, compounds such as (*E*)-2-hexenal, hexanal, ethyl-2-methylbutanoate, ethyl butanoate and propyl butanoate are regarded to play a significant role for the apple flavour.

The formation of flavour compounds depends on the presence of precursor compounds and enzyme activities of the fruits, for processed fruits also on the conditions used during fruit processing. In this study we aimed to investigate primary flavour compounds in different parts of 'Ilzer Rose' apples. To reach this aim we applied 1-dimensional GC-MS as well as comprehensive GC x GC-MS for the identification of

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'Ilzer Rose' volatiles after enrichment by Headspace Solid Phase Microextraction (HS-SPME). The enormous capacity regarding separation as well as sensitivity of comprehensive GC x GC-MS allows deep insight into the flavour composition of this old apple variety. In addition, sensory methods were used to characterize the overall flavour properties.

## Experimental

*Apple samples*

Apples were harvested in 2016 from traditionally grown trees from meadow orchards in Styria. Apple skin was carefully separated from the flesh. To inactivate apple enzymes as far as possible, apple flesh and skin were prepared separately according to Aprea et al [2] prior to GC analysis.

*Gas chromatographic analysis*

Aliquots of the homogenised samples (250 mg each for 1-dim GC-MS and 50 mg for comprehensive GC x GC-MS) were transferred into headspace vials, 2-octanol was used as internal standard (50 ng absolute). Four replicates of each sample were prepared and analysed. After enrichment of the volatiles by HS-SPME (30°C, 20 min, 50/30 µm DVB/CAR/PDMS fibre, 2 cm stable flex fibre) analyses were performed with 1-dimensional GC-MS (Agilent GC 7890, MS 5975c VL MSD, Santa Clara, CA, USA; HP5 30 m*0.25 mm*1 µm, EI (70eV)) and comprehensive GC x GC-MS (Shimadzu GC-2010 Plus coupled with Shimadzu GCMS-QP2010 Ultra, , Shimadzu Europa Gmbh; 1$^{st}$ dim.: ZB-5MS 30 m *0.25 mm*0.25 µm and 2$^{nd}$ dim.: BPX50 2.5 m *0.15 mm*0.15 µm, Zoex cryo modulator, 5s modulation frequency, Hot Jet 280°C, 350 msec pulse time; EI (70 eV)). Identification of the compounds was based on the comparison of the obtained mass spectra to those from MS libraries or authentic reference compounds as well as on retention indices (RI). Linear-temperature programmed RI were calculated using n-alkanes ($C_5$-$C_{26}$) and compared to data from authentic reference compounds and data from literature. For comprehensive GC x GC-MS retention indices were calculated for the 1$^{st}$ dimension.

*Sensory evaluation*

For sensory evaluation, the fruits were cut into cylinders and treated with an antioxidant solution according to Corollaro et al. [3] to avoid (i) browning of the apple pieces and (ii) excessive formation of secondary flavour compounds. Sensory evaluation was performed by 14 well-trained panellists under standardised conditions using quantitative descriptive analysis (QDA®). All panellists had vast experience in evaluating fruits and had undergone apple-specific training prior to this study. Data acquisition was performed by the use of Compusense Sensory Software (Compusense Inc., Guelph, Canada).

## Results and discussion

It was the aim of this study to characterize the flavour of the old apple variety 'Ilzer Rose', but also to investigate the distribution of the volatile compounds between the skin and the flesh of the apples.

Sensory evaluation was performed from standardised 'Ilzer Rose' apple pieces after inactivation of apple enzymes at the sample surface. Nine different odour/flavour attributes were chosen by the panel to describe the sensory characteristics of 'Ilzer Rose'.

Results from QDA® demonstrate the pronounced rose-like/floral and fruity properties of ‘Ilzer Rose’ apples (Figure 1).

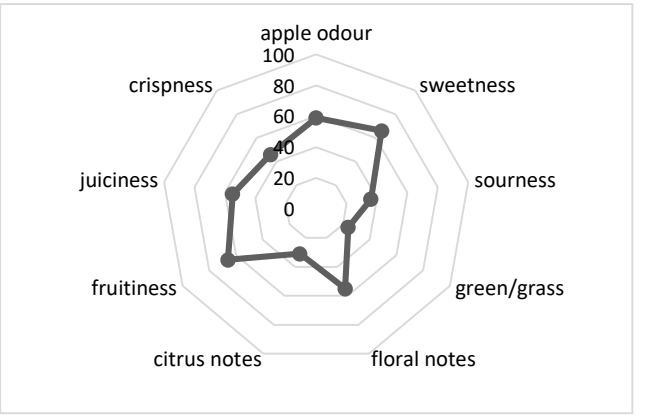


**Figure 1**: Results from QDA® of ‘Ilzer Rose’ after inactivation of fruit enzymes at the sample surface

A total of 82 volatile compounds was identified from the skin of the ‘Ilzer Rose’ by 1-dimensional GC-MS, in contrast to only 55 volatiles in the flesh alone**.** Significantly higher concentrations of most volatile compounds were found in the skin than in the flesh of the Ilzer Rose apples. Table 1 gives a comparison of the relative concentrations of selected volatiles in the skin and the flesh, respectively. Interestingly, not only the carotinoid cleavage product 6-methyl-5-hepten-2-one and the sesquiterpene α-farnesene – that had already been described in apple coating decades ago [5] – are significantly higher in concentration in the skin, but also esters like hexyl butanoate, hexyl 2-methyl butanoate and hexyl hexanoate (Table 1).

**Table 1**: Selected volatile compounds semi-quantified in the headspace of the apple skin and flesh samples by 1-dim GC-MS. Concentrations are expressed as relative concentrations to the internal standard 2-octanol

| Compound | *RI (HP5) exp* | *RI (HP5) lit* | *Skin (**mg** kg$^{-1}$)* | *Flesh (**µg** kg$^{-1}$)* |
|---|---|---|---|---|
| 6-Methyl-5-hepten-2-one | 986 | 987[a] | 1.6 | n.d. |
| Hexyl acetate | 1008 | 1014[b] | 6.6 | 7 |
| Hexyl butanoate | 1188 | 1193[a] | 3.5 | 3 |
| Hexyl-2-methyl butanoate | 1236 | 1236[c] | 2.5 | 3 |
| Hexylhexanoate | 1384 | 1386[c] | 5.0 | n.d. |
| α-Farnesene | 1516 | 1508[d] | 24.6 | 25 |

[a] RI obtained from authentic reference compounds and collected in the SKAF Flavor database for Food Research Institute, Slovakia, © 2001−2002

[b] RI obtained from www.flavornet.org

[c] RI obtained from http://webbook.nist.gov/

[d] RI obtained from literature [4]

Chromatograms obtained from comprehensive GC x GC-MS analysis clearly demonstrate the differences between flesh and skin (Figure 2). More than 600 volatile compounds were (tentatively) identified in ‘Ilzer Rose’ apples, many of them seen in the apples for the first time. These results are in accordance with recently published data on the volatilome of strawberries – nearly 600 volatiles were described from strawberries after analysis by comprehensive GC x GC-MS [6]. The identified compounds include

well-known apple volatiles like esters, alcohols, aldehydes and ketones, but also a large number of mono- and sesquiterpenes. The presence of high numbers of terpenes predominantly in the skin of 'Ilzer Rose' is of special interest as, so far, terpenes have not been regarded to be important contributors to apple flavour. However, they might be the reason for the expressed floral/rose-like notes that are known from 'Ilzer Rose' apples.

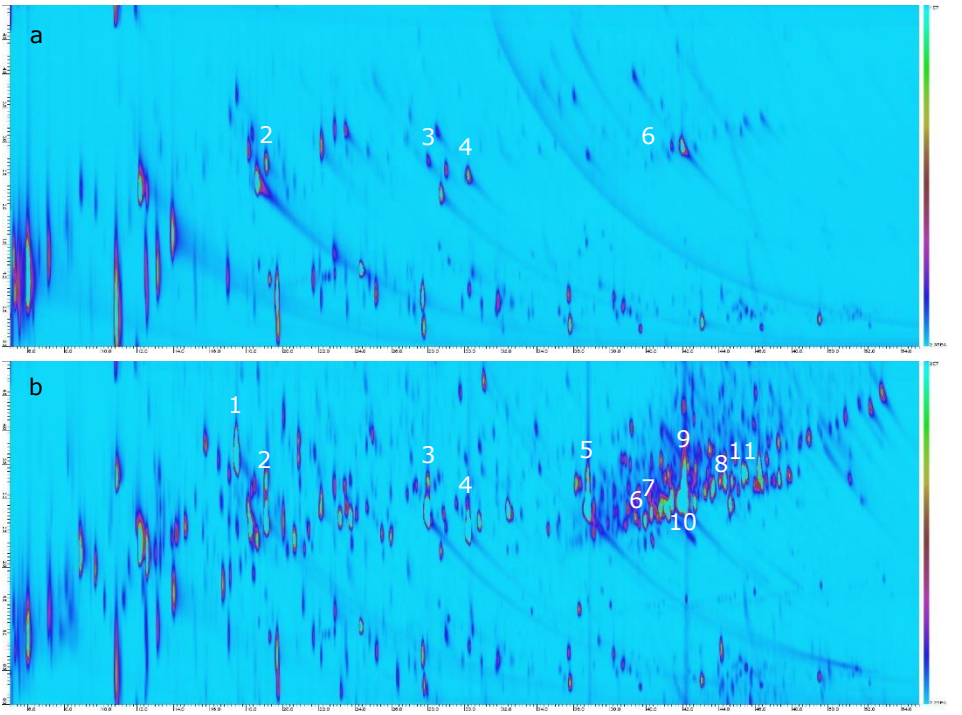


**Figure 2**: Chromatograms obtained from comprehensive GC x GC-MS; analysis of the (a) flesh and (b) skin of Ilzer Rose apple. Retention times in the first (x-axis) are given in minutes, retention times in the second dimension (y-axis) are given in seconds. (1) 6-methyl-5-hepten-2-one, (2) hexyl acetate, (3) hexyl butanoate, (4) hexyl-2-methylbutantoate, (5) hexyl hexanoate, (6) α-farnesene, (7) cis-β-farnesene$^t$, (8) cis-thujopsene$^t$, (9) β-longipinene$^t$, (10) β-vatirenene$^t$, (11) cis- α-santalol$^t$; $^t$tentatively identified by probability-based matching of the obtained mass spectra with the mass spectra from the NIST library

The results obtained from this study demonstrate that the use of comprehensive GC x GC-MS offers a completely new insight into the apple volatilome. The preliminary results from this study serve as a basis for future investigations of volatiles in different parts of apples in general and of the floral, rose-like odour of 'Ilzer Rose' in particular.

## References

1. Dixon, J. and Hewett, E.W. (2000) New Zeal. J. Crop Hortic. Sci., 28:3, 155-173.
2. Aprea, E., Gika, H., Carlin, S., Theodoridis, G., Vrhovsek, U. and Mattivi, F. (2011) J. Chromatogr. A 1218, 4517–4524.
3. Corollaro, M. L., Endrizzi, I., Bertolini, A., Aprea, E., Demattè, L. M., Costa, F., Biasioli, F. and Gasperi, F., (2013) Postharvest Biol. Technol. 77, 111–120.
4. Fuhrmann, E. and Grosch, W. (2002) Nahrung - Food 46, 187–193.
5. Huelin, F.E. and Murray, K.E. (1966) Nature, 5042, 1260-1261.
6. Cannon, R.J., Agyemang, D., Curto, N.L., Yusuf, A., Chem, M.Z. and Janczuk, A.J. (2015) Flavour Fragr. J., 30, 302-319.

# *Aronia melanocarpa* – the Styrian 'super berry': A flavour characterisation of black chokeberry juice

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## Abstract

The juice of black chokeberries (*Aronia melanocarpa*) was in the focus of this investigation. Whereas there are several studies available about the health beneficial effects of aronia products, which are mainly based on the extraordinary high polyphenol concentrations, little is known about the flavour properties of aronia products. The volatile compounds of aronia juices were investigated by one- and two-dimensional gas chromatographic methods. In addition, gas chromatography-olfactometry as well as sensory evaluation was applied to explore the sensory properties of compounds and juices, respectively. The results show an interesting composition of the flavour compounds that is dominated by alcohols, aldehydes, free fatty acids, terpenes, norisoprenoids and cinnamic acid metabolites. Most striking is the lack of fruit esters in aronia juices when compared with volatiles from other fruit and berry juices, leading to very weak fruity notes in the aronia products. These results serve as a basis for future investigations on the technological impact on flavour formation during the production of aronia juices.

## Introduction

The black chokeberry (*Aronia melanocarpa*) is a shrub that has traditionally been cultivated in Eastern European countries as well as in North America where it has also been used as domestic remedy. Recently, the black chokeberry has been included into the group of 'superfoods' which made this berry type popular. In Southern Austrian regions, the crop area for the cultivation of aronia has increased drastically within the last few years with the aim to produce a domestic superfood. Its superfood status is mainly based on the very high antioxidative capacities due to exceptionally high concentrations of polyphenols (i.e. anthocyanins and proanthocyanins, flavonols as well as phenolic acids) [1]. Furthermore, the black chokeberry is rich in minerals and trace elements as well as some vitamins [2]. Several studies proved the health benefits of aronia showing positive impact on blood pressure values, cholesterol- and trigylceride concentrations, anti-inflammatory effects, anti-tumor activity as well as the exhibition of immunomodulatory activity in breast cancer patients [2, 3].

Due to the high concentrations of anthocyanins, and as a consequence the extraordinary colour intensity, aronia products (e.g. extracts, concentrates or dried products) have been of interest for food industry as a natural food colourant. Only recently, the consumption of aronia products as health promoting food has become popular. Austrian farmers founded a consortium named 'Aronia Austria' to promote NFC (not from concentrate) aronia juice as a domestic superfood. However, in contrast to other juices and nectars from domestic fruits, the flavour characteristics of high quality aronia juice are not well described. As a consequence, we investigated aronia juice produced from Austrian aronia berries with emphasis on volatile compounds and sensory properties.

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## Experimental

*Material*

Aronia juices were prepared from Styrian aronia berries (variety Nero) from the harvest 2015 by a small local fruit processing company. All investigated juices were NFC juices. The juices were prepared after enzymatic treatment and were pressed using a belt press. All juices were stored in glass bottles in the dark at 5°C until further use. Only juices that were awarded with at least 18 out of 20 points at a local juice tasting competition prior to this study were included in these investigations.

*Sensory evaluation*

Sensory evaluation of the juices was performed by an expert panel (14 well-trained panellists) under standardised conditions. All panellists had vast sensory experience with fruit products and achieved specific training on aronia products prior to this study. Descriptive analyses to select appropriate attributes for the products as well as quantitative descriptive analyses (QDA®) were applied. Sensory data were recorded using Compusense sensory software (Compusense Inc., Guelph, Canada).

*Analysis of the volatile compounds*

Enrichment of the volatile compounds was performed by headspace solid phase microextraction (HS-SPME; 60°C, 20 min, 50/30 µm DVB/CAR/PDMS fibre, 2 cm stable flex fibre) for all types of GC-analyses. 200 µL of aronia juice with the addition of 50 mg NaCl were transferred into 20 mL headspace vials. 2-Octanol (100 ng absolute) was added as internal standard. Four replicates of each sample were prepared and analysed. 1-dimensional GC-MS analysis was performed on Agilent GC 7890, MS 5975c VL MSD, Santa Clara, CA, USA; HP5 30 m*0.25 mm*1 µm, EI (70eV), scan range 35-350 amu. Comprehensive GC x GC-MS was carried out on Shimadzu GC-2010 Plus coupled with Shimadzu GCMS-QP2010 Ultra, Shimadzu Europa GmbH; 1st dim.: ZB-5MS 30 m *0.25 mm*0.25 µm and 2nd dim.: BPX50 2.5 m *0.15 mm*0.15 µm, Zoex cryo modulator, 5s modulation frequency, Hot Jet 280°C, 350 msec pulse time; EI (70 eV). Identification of the compounds was based on the comparison of the obtained mass spectra to those from MS libraries or authentic reference compounds as well as on retention indices (RI). Linear-temperature programmed RI were calculated using n-alkanes ($C_5$-$C_{26}$) and compared to data from authentic reference compounds and data from literature. For comprehensive GC x GC-MS, RI were calculated for the 1st dimension.

For GC olfactometry, 1 mL of aronia juice with the addition of 500 mg NaCl was used. GCO/GC-FID analysis was performed on a non-polar column (Hewlett Packard 5890 series II equipped with an FID and a Gerstel Olfactory Detection Port; Split ratio FID:ODP 1:1; analytical column DB5, 30 m*0,32 mm*0.25 µm; splitless injection). Detection frequency (DF) with the use of 5 trained panellists was performed to determine the odour active compounds with the potentially highest sensory impact. Each GCO run was performed in duplicate resulting in a total of 10 GCO runs for DF analysis. Identification of the odour active compounds was performed by the determination of linear temperature programmed RI and the odour descriptors given for the odour impressions from authentic reference compounds or literature.

*Statistical evaluation of the results*

Principal component analysis (PCA) using a Pearson correlation matrix was performed to correlate concentrations of volatile compounds from 15 different aronia

juices with results from QDA®. PCA was performed with XLSTAT Sensory by Addinsoft (France).

## Results and discussion

Due to its pronounced antioxidative properties, the consumption of aronia juice has gained increasing popularity on the (local) market. However, little is known about the sensory properties and the composition of flavour compounds of aronia juice. In this study, we therefore aimed for a basic characterisation of aronia juice volatiles.

In comparison to other juices, aronia juice is somehow different as the products do not show dominant fruitiness. Depending on the juice, adstringency and bitterness on the one hand, and woody, balsamic, green and sweaty notes on the other hand significantly influence aronia juice flavour. Figure 1 shows a typical chromatogram (comprehensive GC x GC-MS) of a high quality aronia juice. Several hundred volatile compounds could be detected, 50 thereof were identified. These results show that the volatiles count to the chemical classes of alcohols, aldehydes and ketones, (methyl-branched) short-chain fatty acids, terpenoid compounds and norisoprenoids. In addition, several aromatic compounds as polyphenol degradation products (formed most likely via the shikimic acid pathway and degradation of cinnamic acid, respectively) were identified. The enormous concentrations of 5,6-dihydro-2H-pyran-2-one (up to 1.250 µg/L) have to be pointed out. However, with an odour threshold of higher than 100 mg/L (in water), this compound is not considered to be of relevance for aronia juice flavour. Noticeable is the lack of the typical fruit esters which is most likely the reason for the lack of the fruity notes in the juice. Ethyl-2 (3)-methyl butanoate was identified in the GCO experiment as the only pronounced fruity odour with medium impact and is thus supposed to be responsible for the moderate fruity attributes of the products.

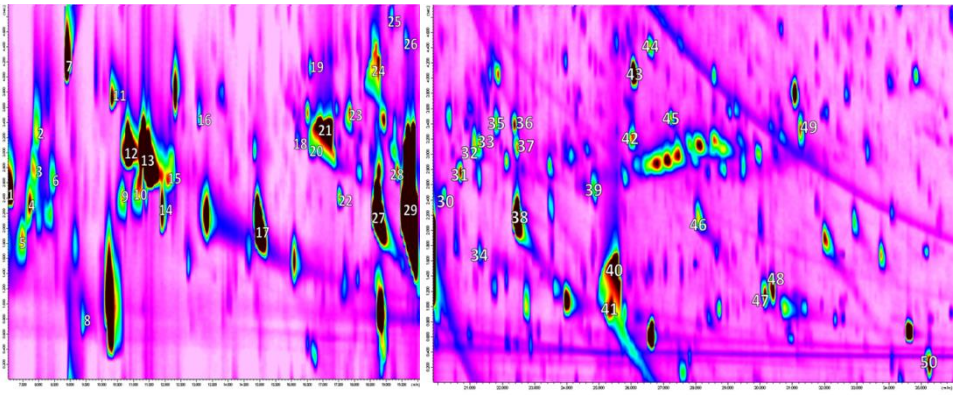

**Figure 1**: Chromatogram from comprehensive GC x GC-MS of a selected aronia juice (divided into 2 parts); retentions times in x-axis are given in minutes and in y-axis in seconds; **1**: (*E*)-3-penten-2-one, **2**: 2,3-butandiol, **3**: acetylacetone, **4**: (*Z*)-2-penten-1-ol, **5**: 2-methylpropanoic acid, **6**: hexanal, **7**: 4-hydroxy-2-pentanone, **8**: furfural, **9**: 2-methylbutanoic acid, **10**: 3-methylbutanoic acid, **11**: (*E*)-2-hexenal, **12**: (*Z*)-3-hexen-1-ol, **13**: (*E*)-2-hexen-1-ol, **14**: γ-butyrolactone, **15**: 1-hexanol, **16**: heptanal, **17**: benzaldehyde, **18**: 1-heptanol, **19**: 6-methyl-5-hepten-2-one, **20**: 1-octen-3-ol, **21**: hexanoic acid, **22**: β-myrcene, **23**: (*E*)-3-hexenoic acid, **24**: 2-hexenoic acid, **25**: sorbic acid, **26**: hexyl-2-methyl-2-propenoate, **27**: benzyl alcohol, **28**: limonene, **29**: 5,6-dihydro-2H-pyran-2-one, **30**: β-ocimene, **31**: γ-terpinene, **32**: cis-linalool oxide (furanoid), **33**: heptanoic acid, **34**: guaiacol, **35**: trans-linalool oxide (furanoid), **36**: nonanal, **37**: linalool, **38**: 2-phenylethanol, **39**: 1-phenyl-1,2-propanedione, **40**: benzoic acid, **41**: ethyl benzoate, **42**: octanoic acid, **43**: terpinen-4-ol, **44**: α-terpineol, **45**: decanal, **46**: 3-phenylpropanol, **47**: 4-ethylguaiacol, **48**: 2,3,6-trimethylphenol, **49**: acetovanillone, **50**: β-damascenone

Figure 2 shows the results from PCA of 20 aronia volatiles of 15 investigated aronia juices. The selection of the compounds was based on the results from GCO. Products that can be found in quadrants I and IV are described to possess well-balanced odour with slight fruity notes. Unfortunately, the concentrations of ethyl-2(3)-methyl butanoate were too low for quantification and could, thus, not be included in the PCA. Interestingly, volatiles like benzaldehyde, benzyl alcohol or 2-phenylethanol or ethylbenzoate are important for these products and obviously contribute positively to the overall flavour with their balsamic, woody, slighty floral attributes. These volatile also showed high impact in the GCO experiments. Juices with high concentrations of hexanal, (*Z*)-3-hexen-1-ol and hexanoic acid (quadrant II) were perceived as mainly imbalanced and dominated by green notes, lacking any fruity and berry like odour.

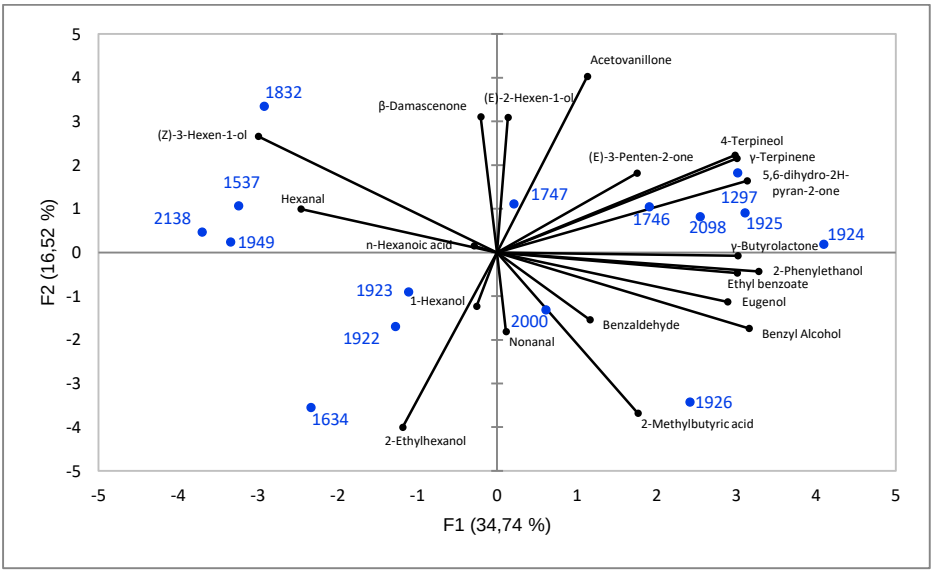


**Figure 2**: PCA based on the relative concentrations to the internal standard of 20 volatile compounds [µg/L] for 15 investigated aronia juices; four digit numbers are sample codes.

The results of this study demonstrate that the flavour of black chokeberry (*Aronia melanocarpa*) juice differs significantly from the juices of other fruits and berries, mainly due to the lack of esters with fruity notes. However, these results serve as a good basis for future investigations of the technological impact on the flavour formation in aronia juices.

**Acknowledgment**: The authors would like to thank the members of the TUG expert panel for evaluating the samples. Georg Thünauer (Styrian Chamber of Agriculture) and *Aronia Austria* are acknowledged for providing sample material.

## References

1. L. Jakobek, M. Drenjancevi, V. Juki, M. Seruga (2012), Scientia Horticulturae, 147: 56-63.
2. S.E. Kulling, H.M. Rawel (2008), Planta Medica 74: 1625-1634.
3. B. Olas (2014), in V. Breedy (Ed.) Cancer: Oxidative Stress and Dietary Antioxidants, Elsevier, pp.151-157.

# On-line coffee flavour formation analysis using PTR-ToF-MS during roasting under different atmospheres

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## Abstract

The impact of the atmosphere on the flavour formation of coffee aroma during roasting was investigated by means of on-line proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS). Roasting under inert atmosphere (nitrogen) was compared to roasting under oxidative conditions (air). Roasting under air has resulted in overall higher intensities of PTR-ToF-MS time-intensity profiles for seven mass peaks, which were significantly higher in intensity for ≥30 % of the roasting duration. Conversely, to the coffee roasted in air, coffee roasted under nitrogen had an unpleasant smell and lacked the distinctive coffee aroma. The results show clear differences between the flavour formation during coffee roasting in different atmospheres and provide evidence that a certain degree of oxidation during roasting is essential to formation of coffee aroma.

## Introduction

Coffee roasting contributes most significantly to coffee aroma by transforming the green coffee beans both physically and chemically into its characteristic end-product. Thermally induced pathways, including the Maillard reaction, generate a plethora of volatile organic compounds (VOCs) that contribute to the coffee's characteristic aroma. During coffee roasting, these reactions occur throughout the bean, resulting in a portion of the volatiles being released into the roaster exhaust while the rest remains trapped within the bean matrix. Roaster exhaust is primarily composed of water vapour, driven off the beans through air convection. This study aims to investigate the influence of oxidative flushing of the roasting chamber during roasting on the VOC composition of the roaster exhaust gas. Previous EPR studies suggest that oxygen does not contribute to radically driven pathways during roasting [1]; however, this does not exclude it from being essential in non-radical pathways.

Traditionally studied using static measurement techniques, such as gas chromatography mass spectroscopy (GC/MS), coffee aroma analysis has recently adopted more dynamic approaches, for example PTR-ToF-MS. Several groups have used PTR-ToF-MS to monitor the exhaust gas of coffee roasters. Wieland and associates [2] used this highly sensitive, time resolved technique to predict the coffee roast degree based on the evolution of the exhaust gas composition. Whereas Gloess and colleagues [3] found that the exhaust gas composition was coffee origin dependent, providing evidence that different VOC pathways were occurring. The sensitivity of PTR-ToF-MS was a key feature in the present study to investigate the impact of anaerobic and oxidative conditions on the roaster exhaust gas VOC composition.

## Experimental

### *Coffee roasting*

Arabica coffee beans from Guatemala were used for roasting experiments. The coffee was conditioned before experiments for 20 min at 105 °C and roasted in 10 g batches. A modified pilot plant type 4E Reactor vessel (Büchi, Uster, Switzerland) was

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used for roasting. The reactor was set horizontally and consisted of an internal fan, rotating sample basket and two heaters (internal and in the vessel jacket). The inlet to the reactor vessel was connected to air or nitrogen supply for purging the reactor at approximately 20 $L_N$/min (normal litre per minute). The outlet of the reactor was left open. Coffee was roasted 20 min to reach a set point of 180 °C at the sensor in the reaction vessel.

*PTR-ToF-MS*

The PTR-ToF-MS was interfaced directly to the inside gas of the reaction vessel using a custom built dilution system. The experimental setup is shown schematically in Figure 1. The outlet of the dilution system was actively pumped and nitrogen was introduced to the dilution stream at 3.9 $L_N$/min. The sampling flow rate was set to 24.0 ± 0.4 $mL_N$/min (mean ± SD) from the roasting chamber, to achieve dilution of about 160-fold. The gas lines were heated to 90 °C and the dilution system was heated to 120 °C. A PTR-ToF-MS 8000 mass spectrometer (Ionicon) was used. The PTR drift tube was operated at 80 °C and 140 Td. The mass axis calibration was performed on $[H_3{}^{18}O]^+$, acetone ($[C_3H_7O]^+$) and caffeine ($[C_8H_{11}N_4O_2]^+$).

Gas chromatography

Headspace GC/MS (system with cryogenic $CO_2$ oven cooling) was performed on roasted and ground coffee beans. Two grams of coffee powder were transferred into vials and analysed with HS GC/MS based on a previously published method [4]. Peak identification was based on comparing the mass spectra with the NIST08 database. In total, 58 peaks were evaluated.

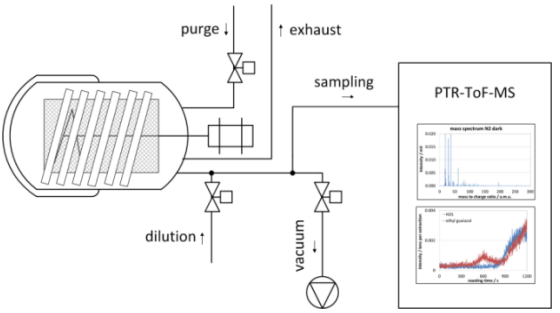


**Figure 1**: Schematics of the coupling of the PTR-ToF-MS to the reaction vessel for on-line PTR-ToF-MS analysis of the exhaust gas during roasting under controlled atmosphere.

## Results and Discussion

The aerobic conditions realised by flushing the roasting chamber with air were found to have a significant influence on the coffee aroma. Sensory observations parallel to those made by Tai and Ho's [4] for cysteine model systems, suggesting that cysteine's oxidative state plays an essential role in the development of coffee's aroma profile. These observations are further supported by differences observed, using PTR-ToF-MS, in the exhaust gas composition.

Roasting under inert atmosphere increased the intensity of m/z 34.996, tentatively assigned to dihydrogen sulphide ($H_2S$). The increased intensity of $H_2S$ (Figure 2a) was accompanied with an unpleasant aroma, a similar unpleasant aroma was observed by Tai and Ho [4]. These authors observed that when cysteine's sulphur group was reduced sulphur containing molecules were dominant within the product profile [4].

Roasting under oxidative conditions generated characteristic differences within the PTR-ToF-MS profile and restored the characteristic coffee aroma. Tai and Ho [4] observed an absence in sulphur containing compounds as well as, “a strong coffee note” when the sulphur side chain of cysteine was oxidized to cysteinesulfinic acid.

Aerobic roasting increased the intensities and shape of several VOC PTR-ToF-MS profiles (Table 1) demonstrating the influence of oxygen on the evolution of coffee aroma formation pathways. Amongst the most prominent differences was the higher intensity observed for m/z 153.0910, tentatively assigned to 4-ethylguaiacol (Figure 2b).

**Table 1:** Compounds of significantly higher intensities generated during coffee roasting in either air or inert (nitrogen) atmosphere.

| Atmosphere | *On-line PTR-ToF-MS*[a] | *Headspace GC/MS*[b] |
|---|---|---|
| Nitrogen | m/z 34.996 ($H_2S$) | Methanethiol, dimethyl sulfide |
| Air | m/z 31.0178 ($CH_2O$, formaldehyde), m/z 44.0174 ($CH_2NO^+$), m/z 55.0542 ($C_4H_6$, butadiene), m/z 67.0542 ($C_5H_6$), m/z 107.0491 ($C_7H_6O$, benzaldehyde), m/z 135.1 (unresolved), m/z 153.0910 ($C_9H_{12}O_2$, 4-ethylguaiacol) | Dimethyl disulphide |

[a] PTR-ToF-MS: mean intensities (n=4) differ for at least 2 SD for >30% of roasting time, compounds were tentatively assigned based on molecular mass.

[b] HS GC/MS: t-test, $P < 0.1$

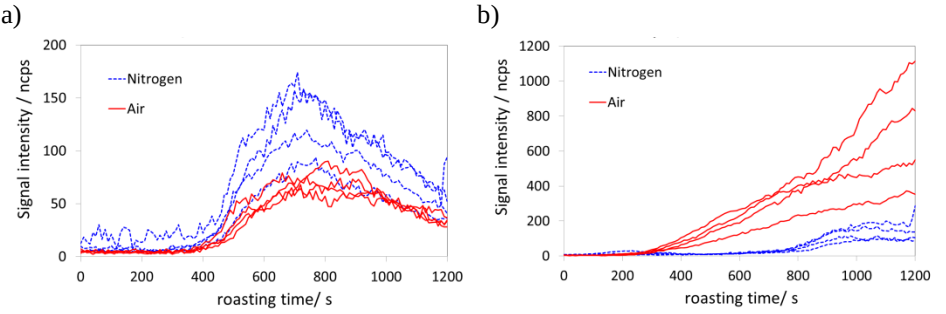


**Figure 2:** Time-intensity profiles of (two samples: air and nitrogen, each four repetitions) (a) a VOC of mass-to-charge-ratio (m/z) 34.996 (tentatively assigned to protonated $H_2S$) and (b) m/z 153.091 (tentatively assigned to protonated 4-ethyl guaiacol) of roasting in air and nitrogen.

PTR-ToF-MS time-intensity profile of m/z 107.049 (tentatively assigned to protonated benzaldehyde) was observed at higher intensity when roasting in air (Figure 3a), but no difference in the shape of the profile was seen. Despite no obvious difference in profile, the larger amount of benzaldehyde formed during aerobic roasting is consistent with studies on model systems [5], where oxidative degradation of phenylalanine at high temperatures was studied. Phenylacetaldehyde, the Strecker aldehyde of phenylalanine has been suggested as an intermediate for benzaldehyde formation, but it does not show a significant difference between air and nitrogen roasting (Figure 3b). This could be caused by less reproducible signal for phenylacetaldehyde, or alternatively that oxidative

formation of benzaldehyde in coffee matrix does not go through a phenylacetaldehyde intermediate.

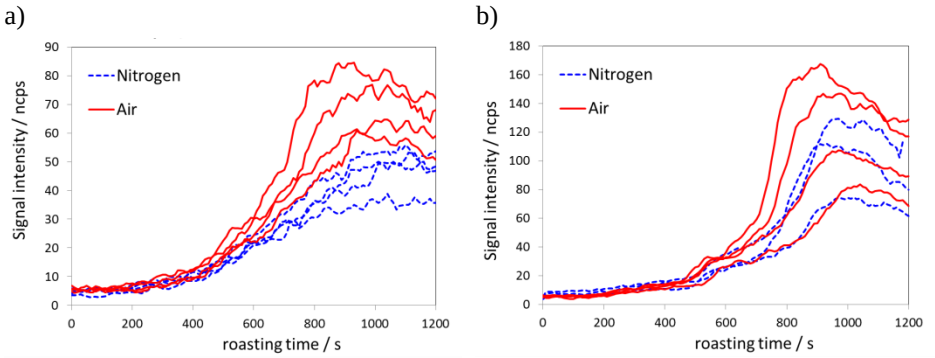


**Figure 3**: Time-intensity profiles of (two samples: air and nitrogen, each four repetitions) (a) m/z 107.049 (tentatively assigned to protonated benzaldehyde) and of (b) m/z 121.067 (tentatively assigned to phenylacetaldehyde) of roasting in *air* and *nitrogen*.

The GC/MS analysis was performed seven days after coffee roasting. The higher intensity of methanethiol in samples roasted in nitrogen indicated that there is less oxidative degradation during storage. This is consistent with higher amounts of dimethyl disulphide in the samples roasted in air. Dimethyl disulphide is a product of methanethiol oxidation and is used as an indicator of coffee freshness in its ratio against methanethiol [6].

The aerobic conditions in the roaster play an essential role in the development of characteristic coffee aroma. Under anaerobic conditions dihydrogen sulphide ($H_2S$) serves as the dominant nucleophile leading to high concentrations of primarily sulphur containing VOCs, which unbalances coffees aroma profile leading to an undesirable sensory experience. Oxidative conditions suppress the formation of $H_2S$ by oxidizing coffee's sulphur groups. Suppression of $H_2S$ allows ammonia to become the dominant nucleophile allowing for the development of more desirable aroma profiles.

## References

1. Goodman B.A., Pascual E.C., Yeretzian C. (2011) Food Chem 125: 248-254.
2. Wieland F., Gloess, A.N., Keller M., Wetzel A., Schenker S., Yeretzian C. (2012) Anal. Bioanal. Chem. 402: 2531-2543.
3. Gloess A.N., Vietri A., Wieland F., Smrke S., Schonbachler B., Lopez J.A.S., Petrozzi S., Bongers S., Koziorowski T., Yeretzian C. (2014) Int. J. Mass Spectrom 365: 324-337.
4. Tai C.-Y., Ho C.-T. (1997) J. Agric. Food Chem. 43: 3586-3589.
5. Chu F.L., Yaylayan V.A. (2008) J. Agric. Food Chem. 56: 10697-10704.
6. Gloess A.N., Schonbachler B., Rast M., Deuber L., Yeretzian C. (2014) Chimia 68: 179-182.

# Characterization of key aroma compounds in two types of Keemun tea

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## Abstract

Keemun tea is one of the most popular Chinese black teas, and it is highly appreciated by consumers because of its sweet, floral, and slightly smoky odor. In this study, two types of Keemun tea that differ in terms of raw material and manufacturing process, namely "*gōngfū*" and "*míngyōu*" type Keemun tea, respectively, were investigated by aroma extract dilution analysis (AEDA).

From the AEDA results, 34 odorants with flavor dilution (FD) factors ranging from 64 to 1024 were detected from the volatiles obtained from the isolated fractions of Keemun tea infusions. In particular, geraniol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, and coumarin exhibited the highest FD factor, which was followed by methional, 2-phenylethanol, phenylacetic acid, and 3-methyl-2,4-nonanedione. These odorants were detected in both Keemun teas. Stable isotope dilution assays (SIDA) were performed, and odor activity values (OAVs) were calculated for the quantitative evaluation of 38 odorants: 27 key odorants with an OAV $\geq 1$ were identified. The obtained quantitative data permitted the preparation of aroma recombinates from both types of Keemun tea. Comparative aroma profile analyses between the recombinates and their respective Keemun tea indicated excellent similarity in terms of the overall aroma, thus validating these volatiles as the key components that contribute to the unique odor profile of Keemun tea.

## Introduction

Tea (*Camellia sinensis*), which is one of the most popularly consumed beverages in the world, is mainly cultivated in tropical, subtropical, and temperate climates. India, Sri Lanka, and China are the main tea-producing countries. The following two principal varieties are grown in the tea-producing areas: small-leaved Chinese plant (*Camellia sinensis V. sinensis*) and a large-leaved Assamese plant (*Camellia sinensis V. assamica*). Darjeeling and Keemun tea are classified into the former group, while Assamese and Ceylon tea are classified into the latter group. Among Chinese plants, numerous studies have reported on the Darjeeling tea aroma [1]. Although Keemun tea exhibits unique flowery, sweet, and slightly smoky notes [2,3], few studies have reported on the Keemun tea aroma.

Black tea production generally comprises the following four steps: withering, rolling, fermentation, and firing. In particular, several biochemical reactions occur in tea leaves during fermentation. Keemun tea is categorized into two types mainly based on the manufacturing method. One is "*gōngfū*"-type Keemun tea (GK) that is used for exports, while the other is "*míngyōu*"-type Keemun tea (MK) that is used for domestic

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consumption. The rolling process (i.e., rubbing and twisting processes) is different for GK and MK. GK is tightly rolled using a machine, while MK is softly rolled by hand.

This study aimed to clarify the key aroma compounds in Keemun black tea by aroma extract dilution analysis (AEDA) and stable isotope dilution assays (SIDA) as well as to determine whether the difference in the rolling process considerably affects the volatile profiles of tea infusions.

## Experimental

First, tea leaves (6 g) were soaked in hot water (95°C, 300 mL). After 5 min, tea leaves were separated by filtration, and the infusion was cooled to 15°C using an ice bath. Second, the beverage (50 mL) was repeatedly extracted with dichloromethane (2 × 50 mL), and the volatile compounds were isolated by solvent-assisted flavor evaporation [1].

GC-O was employed to analyze the aroma extract, and the most important aroma-active compounds were determined by AEDA. After identification (RI on two capillary columns, odor quality, and mass spectra), the aroma compounds with the highest FD factors were quantified by SIDA. Finally, odor activity values (OAVs) of the key odorants were calculated from the concentrations of the aroma compounds and their odor thresholds.

Aroma reconstitution models were prepared by utilizing natural concentrations of the key odorants with an OAV greater than or equal to one dissolved in water. Sensory analysis was performed in a sensory room with single booths. The sensory panel comprised 15–21 trained assessors.

## Results and discussion

*Screening of aroma-active compounds by AEDA*

The elucidation of the aroma-active compounds by AEDA revealed 34 odorants in the two types of Keemun tea with FD factors ranging from 64 to 1024. The highest FD factors in both Keemun tea were observed in case of odorants such as flowery-smelling geraniol and sweet-smelling 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (FD 1024). However, in GK, oat-like smelling (*E*,*E*,*Z*)-nonatrienal (FD 1024) was followed by the cooked potato-like smelling methional, flowery-smelling 2-phenylethanol, and sweet-smelling coumarin (FD 512). Meanwhile, in MK, coumarin (FD 1024) was followed by hay-like-smelling 3-methyl-2,4-nonanedione (FD 512).

*Quantitation of the key odorants by SIDAs*

Aroma-active compounds that exhibited high FD factors from AEDA in addition to four compounds (i.e., $\alpha$-ionone, (*Z*)-4-heptenal, (*E*,*E*)-2,4-decadienal, and (*E*,*Z*)-2,6-nonatrienal, respectively) were quantified. All compounds concentrations were determined by SIDA, and OAVs were calculated on the basis of these concentration and odor thresholds [4] in water revealed 27 key odorants in each Keemun tea (Table 1). Geraniol, 2-phenylethanol, and linalool were determined as the key floral odorants, and 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, coumarin, and (*E*)-$\beta$-damascenone were determined as the key sweet odorants; finally, 4-vinylguaiacol, 4-vinylphenol, and guaiacol were determined as the key smoky odorants in the Keemun tea.

*Sensory profile analysis*

To validate the results obtained from these investigations, 27 key odorants were recombined in their natural concentrations, and each aroma model was compared to the

original Keemun tea infusions by aroma profiling (Figure 1). Both mixtures considerably matched the original Keemun tea infusions in terms of all attributes; these investigations demonstrated that the aroma of the two types of Keemun tea can be simulated by 27 compounds.

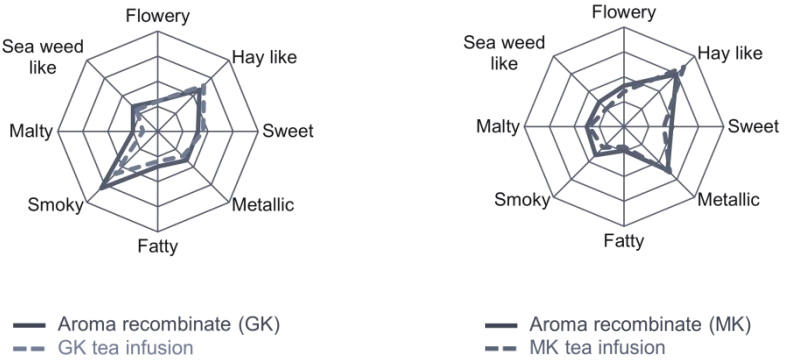


**Figure 1:** Comparative aroma profiles of aroma recombinate and original Keemun tea infusions

The comparison of the aroma profiles of the original Keemun tea revealed clear differences among metallic, malty, and smoky attributes. Odorants responsible for these attributes were *trans*-4,5-epoxy-(*E*)-2-decenal (metallic), 4-vinylguaiacol (smoky), 2-methylbutanal, and 3-methylbutanal (malty), and their OAVs were clearly different in the two types of Keemun tea (Table 1).

**Table 1:** FD factors and OAVs of aroma-active compounds (OAV ≥ 1) in Keemun tea (GK and MK)

| Odorant | *Odor quality* | *FD factor* | | *OAV* | |
|---|---|---|---|---|---|
| | | *GK* | *MK* | *GK* | *MK* |
| geraniol | flowery, fruity | 1024 | 1024 | 290 | 530 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | caramel, sweet | 1024 | 1024 | 3 | 5 |
| (*E*,*E*,*Z*)-2,4,6-nonatrienal | oat | 1024 | 64 | 36 | 19 |
| methional | cooked potato | 512 | 32 | 7 | 7 |
| 2-phenylethanol | flowery, honey | 512 | 256 | 3 | 4 |
| coumarin | woodruff, sweet | 512 | 1024 | 1 | 2 |
| linalool | flowery | 256 | 32 | 530 | 610 |
| (*E*)-*β*-damascenone | cooked apple, sweet | 256 | 256 | 23 | 25 |
| *trans*-4,5-epoxy-(*E*)-2-decenal | metallic | 256 | 256 | 8 | 15 |
| eugenol | clove | 256 | 32 | 1 | 1 |
| 3-ethylphenol | phenol | 256 | 256 | 2 | 3 |
| 4-vinylguaiacol | clove, smoky | 256 | 256 | 2 | <1 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | celery, seasoning | 256 | 256 | 2 | 2 |
| 4-vinylphenol | phenol, smoky | 256 | 16 | 3 | 2 |
| phenylacetaldehyde | honey, bees wax | 128 | 64 | 76 | 54 |
| guaiacol | smoky, sweet | 128 | 256 | 9 | 9 |

**Table 1:** continued

| Odorant | *Odor quality* | *FD factor* | | *OAV* | |
|---|---|---|---|---|---|
| | | *GK* | *MK* | *GK* | *MK* |
| 3-methyl-2,4-nonanedione | hay, fishy | 64 | 512 | 63 | 67 |
| 2,3-butanedione | buttery | 64 | 32 | 11 | 15 |
| 3-methylbutanal | malty | 32 | 64 | 421 | 618 |
| 2-methylbutanal | malty | 32 | 64 | 155 | 220 |
| hexanal | green, grassy | 32 | 64 | 24 | 43 |
| 2-acetyl-1-pyrroline | roasted, popcorn | 16 | 64 | 1 | 2 |
| 3-methylindole (skatole) | fecal, mothball | 16 | 64 | 2 | 3 |
| 1-octen-3-one | mushroom | 4 | 64 | 5 | 8 |
| (*Z*)-4-heptenal | fishy, fish oil | 4 | 8 | 15 | 10 |
| (*E*,*E*)-2,4-decadienal | fatty, fried | - | - | 12 | 11 |
| (*E*,*Z*)-2,6-nonadienal | cucumber | 8 | 8 | 8 | 8 |

In conclusion, a majority of the key odorants in Table 1 had been previously identified as the major contributors to the aroma of Darjeeling tea [1]; meanwhile, some smoky-smelling compounds such as 4-vinylphenol and guaiacol and sweet-smelling compounds such as coumarin have been reported to be crucial contributors to the aroma of Keemun tea. Our study has revealed the key aroma compounds that can characterize the overall aroma of Keemun tea and the potent aroma compounds that differentiate between the two types of Keemun tea; however, further investigation is necessary to clarify the presence of a high number of odorants that contribute to smoky attributes in the Keemun tea rolled by a machine and a high number of odorants contributing to the metallic and malty attributes in the Keemun tea rolled softly by hand.

## References

1. Schuh, C.; Schieberle, P. J. Agric. Food Chem. 2006, 54(3), 916-924.
2. Japan Perfumery and Flavoring Association. The Koryo 2003, 217, 145-150.
3. Kawakami, M.; Granguly, N. S.; Banerjee, J.; Kobayashi, A. J. Agric. Food Chem. 1995, 43(1), 200-207
4. Czerny, M.; Christlbauer, M.; Christlbauer, M.; Fischer, A.; Granvogl, M.; Hammer, M.; Hartl, C.; Hernandez, M. N.; Schieberle, P. Eur Food Res Technol. 2008, 228, 265–273.

# Age-related changes in oral and nasal physiology and their significance in aroma release and perception

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## Abstract

With aging comes many physiological changes, including those in the oral and nasal cavity, such as impaired olfactory function, reduced salivary flow and compromised dental status and function [1-3]. These changes may impact on aroma release and flavour perception, and subsequently the enjoyment of foods, leading a risk of undernutrition. This review aimed to summarise current literature on how olfaction is affected by aging, the major physiological parameter in flavour perception. With the worldwide projected increase in the older population, the economic and social burden of undernutrition is expected to be severe. Tackling this issue is of interest to both clinical practice and industry, as there is potential for new products to be developed which meet the needs and sensory preferences of this specific, increasing population.

## Introduction

Due to socioeconomic development people are living longer than ever [4]. For example, somebody born in Japan in 2015 was projected to live until 90 years old, this is compared to only 83 years if born in 1985 [4]. Although extra years are added to life, unavoidable changes in sensory capacity may reduce functionality and life quality. It is well documented that aging is associated with a decline in both vision and hearing[4]; however, a lesser acknowledged sensory impairment, is the sense of olfaction.

## Discussion

### *Importance of olfaction*

Olfactory impairments have been proposed to be a key contributor in the aetiology of "anorexia of aging" [5, 6] a term which alludes to the high prevalence of undernutrition within the older adult population. The "anorexia of aging" leads to multifaceted clinical conditions, such as frailty and sarcopenia, which are common among frail older persons, and are related to many comorbidities and ultimately an increased risk of mortality [5].

Olfaction is a key contributor to the anorexia of aging due to the impact it may have on hunger and appetite [7] and on reducing nutritional quality and altering dietary habits [8]. For example, Duffy *et al.* (1995) [9] found that older women (aged 65 to 93 years) with olfactory dysfunction had lower interest in food-related activities (i.e. cooking) and Aschenbrenner *et al.* (2008) [10] found that more than one-third of patients with olfactory loss reported changes in their social food-related activities. In terms of altered nutritional quality, Duffy *et al.* (1995) [9] found that olfactory impairments led to a lower preference for foods with predominant sour/bitter taste such as fruits and vegetables, and higher intake of sweets. Such evidence is supported by the work of Griep *et al.* (1996) [11] who found that older individuals with olfactory impairments had lower nutrient intake levels than older individuals with good odour perception. One recent study has suggested that community dwelling older adults with impairments in sensory perception, including olfaction, are at a greater risk of frailty [12] due to decreased appetite and food intake. It

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has been suggested that weight loss is more frequent in individuals with olfactory impairments [8, 10, 13, 14]; Gopinath *et al.* (2012) [14] found that BMI was significantly lower in participants with, than without, olfactory impairment. Weight loss is a significant problem in the population of older adults as it can lead to muscle wasting, decreased immunocompetence and increased rate of complications, along with being highly predictive of morbidity and mortality [15]. Thus olfactory impairments have been proposed to be predictive of overall mortality, over a 5 year period [14]. Schiffman and Graham (2000) [16] also drew attention to how undernutrition itself may be a risk factor for olfactory impairments as deficiencies in the B Vitamins Niacin and Vitamin B12 and Zinc impair olfactory function [14], so it is easy to understand how undernutrition may become exacerbated and maintained.

*Prevalence of age-related olfactory impairment*

The prevalence of impaired olfactory function in the older adult population is high. Using a smell identification test, Doty *et al.* (1984) [17] conducted a cross-sectional study in 1955 individuals and found that 60% of those aged 65-80 were experiencing major olfactory impairments. More recently, Murphy *et al*, (2002) [18] conducted a population-based study with 2491 individuals aged 57-93 years. They found the prevalence of olfactory impairment to be 24.9% and also that the prevalence increases with age; within the population of 80- to 97-year-olds, 62.5% were experiencing olfactory impairments.

*Causes of age-related olfactory impairment*

The cause of olfactory impairments is likely to be multi-factorial, involving age-related alterations within the nose, olfactory epithelium, olfactory bulb and higher levels of the brain that receive olfactory input [1, 19, 20]. The complex causes of age-related olfactory impairment are discussed in detail in the review by Doty and Kamath, (2014) who summarised potential contributing factors to be: altered nasal engorgement and airflow, increased propensity for nasal disease, cumulative damage to the olfactory epithelium from viral and other environmental insults, decrements in mucosal metabolizing enzymes, ossification of cribriform plate foramina, loss of selectivity of receptor cells to odorants and changes in neurotransmitter and neuromodulator systems. A potential genetic contribution to odour identification ability has also been identified [21, 22].

There is a strong association between olfactory impairment and age-related neurodegenerative disease, such as Alzheimer's and Parkinson's disease. Olfactory impairments can be an early symptom of these diseases, which Doty and Kamath, (2014) [1] proposed to be due to expression of aberrant proteins. The significance of these proteins was shown by Wilson *et al*, (2007) [23], who found inverse correlations between Brief Smell Identification Test (B-SIT) scores obtained before death and the post-mortem density of neurofibrillary tangles. In another study, Wilson *et al*, (2011) [24] found an inverse relationship between B-SIT scores and post-mortem measures of Lewy bodies in limbic and cortical brain regions. This evidence suggests that olfactory impairment in older adults in not confined to structural changes within the nose, but its aetiology is likely to involve higher brain structures.

Lastly, the influence of medications should be taken into account. Many drugs used to treat age-related conditions, such as antihypertensive medications and statins, are known to affect both taste and smell [20]. A comprehensive discussion of these medications and diseases can be found in Schiffman and Zervakis (2002) [20] who states

that older adults experience an exaggerated burden of chemosensory disorders from these medications, compared to younger individuals.

*Aroma- specificity of age-related olfactory impairment*

While older adults experience an impairment in their olfactory function, only a few studies have investigated how their perception of single aroma compounds changes with aging. In a large survey involving 1.2 million National Geographic readers, Wysocki and Gilbert (1989) [25] reported differences in the rate of age-related olfactory loss to six odorants. More recently, Seow *et al,* (2016) [26] conducted a study using The Specific Sensitivity test involving 281 participants of various age groups. They tested the identification rates and detection thresholds of 10 odorants, with various chemical and sensory properties, and found large differences in detection thresholds for some odorants, between age groups. For example, participants in their 70s had a detection threshold 179 times higher than the young for the rose-like aroma compound phenylethyl alcohol, whereas for the onion-like aroma compound 2-methyloxolane-3-thiol, the threshold was only 3 times higher. Interestingly, they also found that the older subjects had higher identification rates if they rated the odorants as pleasant. This is supported by Wysocki and Gilbert (1989) [25] who found no age effect for the intensity rating of galaxolide (which may be considered a pleasant aroma), whereas a 26% age decline was observed for methanethiol (which could be considered an unpleasant aroma). These findings are is in contrast to Konstantindis *et al,* (2006) [27] who found that, unlike pleasant odours, unpleasant odours were not sensitive to age-related olfactory loss.

In an effort to explain the physiological phenomenon of aroma-specific age-related loss, Sinding *et al,* (2014) [28] investigated if there was a difference in age-related odour perception between aroma molecules with heavy and light molecular weights, based on the idea that the molecules would bind differently to olfactory receptors. They found that older adults experience olfactory loss more specific to heavier molecules, suggesting that aroma-specific age-related loss bears connection to the molecular structure of individual aroma molecules.

Considering these findings on age-related aroma-specific sensory loss, it is reasonable to conclude that it is not simply the case that older adults perceive flavour at a weaker intensity, it is likely that their overall flavour perception becomes distorted as the contribution made by individual aroma compounds to a flavour mixture is altered.

*Previous food-based aroma strategies to counteract olfactory changes*

To endeavour to combat the effects of olfactory impairments in older adults, a reasonable response undertaken is to modify the aroma in food in an effort to counteract impairments, and ultimately improve food liking and intake. Many studies have investigated this, however, results have not been consistently successful. For example, Koskinen, *et al,* (2003) [29] heightened the aroma in a yogurt-like fermented oat bran *product and found that older adults liking and intake of the product was lower, when* compared with the regular product. Considering that olfactory loss is aroma-specific [25-27], heightening of aroma may have distorted flavour perception [26], and may explain why some panellists reported an “artificial flavour”.

*Future approaches and conclusion*

In order to combat age-related olfactory impairments, more tailored aroma-modification strategies are needed. Seow *et al,* (2016) [26] stated that, in order to design targeted remedies for the effects of chemosensory losses (including olfaction), it is imperative to first gain insight on the extent of olfactory loss to specific single aroma

compounds. Developing foods which meet the sensory needs of the aging population is a challenging and complex task, but considering the social and economic burden of undernutrition, it is a vital challenge to overcome.

**References**


1. Doty RL, Kamath V (2014) Applied Olfactory Cognition 5:213-232
2. Martin-Harris B, Brodsky MB, Michel Y, Ford CL, Walters B, Heffner J (2005) Archives of Otolaryngology–Head & Neck Surgery 131:762-770
3. Vandenberghe-Descamps M, Labouré H, Prot A, Septier C, Tournier C, Feron G, Sulmont-Rossé C (2016) Journal of Texture Studies 47(4):353-360
4. WHO (2015) World report on ageing and health. World Health Organization
5. Landi F, Calvani R, Tosato M, Martone AM, Ortolani E, Savera G, Sisto A, Marzetti E (2016) Nutrients 8:69
6. Wysokiński A, Sobów T, Kłoszewska I, Kostka T (2015) Age 37:81
7. de Jong N, Mulder I, de Graaf C, van Staveren WA (1999) Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences 54:B324-B331
8. Mattes RD, Cowart BJ, Schiavo MA, Arnold C, Garrison B, Kare MR, Lowry LD (1990) The American journal of clinical nutrition 51:233-240
9. Duffy VB, Backstrand JR, Ferris AM (1995) Journal of the American Dietetic Association 95:879-884
10. Aschenbrenner K, Hummel C, Teszmer K, Krone F, Ishimaru T, Seo H-S, Hummel T (2008) The Laryngoscope 118:135-144
11. Griep M, Verleye G, Franck A, Collys K, Mets T, Massart D (1996) European journal of clinical nutrition 50:816-825
12. Somekawa S, Mine T, Ono K, Hayashi N, Obuchi S, Yoshida H, Kawai H, Fujiwara Y, Hirano H, Kojima M (2017) The journal of nutrition, health & aging 21:710-714
13. Frasnelli J, Landis B, Heilmann S, Hauswald B, Hüttenbrink K, Lacroix J, Leopold D, Hummel T (2004) European Archives of Oto-Rhino-Laryngology and Head & Neck 261:411-415
14. Gopinath B, Anstey KJ, Kifley A, Mitchell P (2012) Maturitas 72:50-55
15. Alibhai SM, Greenwood C, Payette H (2005) Canadian Medical Association Journal 172:773-780
16. Schiffman S, Graham B (2000) European Journal of Clinical Nutrition 54:S54
17. Doty RL, Shaman P, Applebaum SL, Giberson R, Siksorski L, Rosenberg L (1984) Science 226:1441-1443
18. Murphy C, Schubert CR, Cruickshanks KJ, Klein BE, Klein R, Nondahl DM (2002) Jama 288:2307-2312
19. Attems J, Walker L, Jellinger KA (2015) Gerontology 61:485-490
20. Schiffman SS, Zervakis J (2002) Advances in food and nutrition research 44:247-346
21. Calhoun-Haney R, Murphy C (2005) Brain and cognition 58:178-182
22. Hedner M, Nilsson L-G, Olofsson JK, Bergman O, Eriksson E, Nyberg L, Larsson M (2010) Frontiers in aging neuroscience 2
23. Wilson RS, Arnold SE, Schneider JA, Tang Y, Bennett DA (2007) Journal of Neurology, Neurosurgery & Psychiatry 78:30-35
24. Wilson RS, Yu L, Schneider JA, Arnold SE, Buchman AS, Bennett DA (2011) Chemical senses 36:367-373
25. Wysocki CJ, Gilbert AN (1989) Annals of the New York Academy of Sciences 561:12-28
26. Seow Y-X, Ong PKC, Huang D (2016) Chemical Senses 41:487-495
27. Konstantinidis I, Hummel T, Larsson M (2006) Archives of Clinical Neuropsychology 21:615-621
28. Sinding C, Puschmann L, Hummel T (2014) Chemical senses:bju004
29. Koskinen S, Kälviäinen N, Tuorila H (2003) Appetite 41:87-96

# Influence of the brewing process and degree of milling on the taste characteristics of pigmented rice wine

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## Abstract

The taste characteristics of pigmented rice wine were investigated with respect to brewing conditions, and the extent to which the rice had been milled to remove the bran. Both the saccharification and the subsequent alcoholic fermentation processes were monitored over time at 25 ºC and 30 ºC. The following conditions were selected based on maximising ethanol content and minimizing acetic acid: saccharification for two days at 30 ºC and alcoholic fermentation for nine days at 30 ºC. This brewing process was applied to pigmented rice which had been milled to various degrees (0%, 30%, 50% and 65%) and the wine was analysed for taste compounds (sugars, organic acids, amino acids and cyclic dipeptides (2,5-diketopiperazines)). The results showed that the higher degree of milling significantly increased the glucose in the wine, however there was a concomitant loss of glutamic acid ($p<0.05$). Cyclo(Pro-Val), cyclo(Pro-Ile), cyclo(Pro-Leu) and cyclo(Pro-Pro) were detected in pigmented rice wine for the first time. They can impart bitter and metallic tastes, but they were present at concentrations below their reported taste thresholds. Their formation increased as the degree of milling increased and the pH decreased. Based on reported taste thresholds, the compounds most likely to contribute to the taste of pigmented rice wine are acetic acid and glutamic acid.

## Introduction

Rice wine or *Sake* is a traditional fermented alcoholic beverage which is becoming increasingly popular in some Asian countries [1]. The production of sake is well-documented. Chinese or Japanese rice wine is prepared using high quality polished glutinous rice, wheat and *koji*, a starter culture which contains both the fungi for the saccharification step, and the yeast for the subsequent fermentation step. During these processes, the brewing temperature is important as it affects the cell growth, cell density, starch hydrolysis and the production of ethanol and organic acids [1]. The taste of rice wine has been described as having sweet, sour, harmonious, mellow, and fresh characteristics [2], which are mainly generated during fermentation when proteins present in the rice are converted to small peptides and amino acids by proteases from the microorganisms [3]. Other non-volatile metabolites, including organic acids and sugars can contribute to the taste of rice wine.

Polished glutinous rice is not the only rice used for wine manufacture. Unpolished pigmented rice, where the bran is retained, is also used to produce pigmented rice wine, especially in countries such as Thailand and the Philippines. However, contrary to *Sake*, it has a unique savoury flavour, and much less is known about the compounds contributing to the characteristic taste and aroma of the pigmented rice wine. Therefore, this study investigated the influence of (i) the brewing process and (ii) degree of milling on the generation of the taste characteristics of pigmented rice wine.

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## Experimental

### *Materials*

Glutinous pigmented rice (Double Elephant, Thailand) was purchased from a local supplier at Reading, UK. Brewing microorganisms were *Aspergillus oryzae* ATCC 22787 and *Saccharomyces cerevisiae* NCYC 478 obtained from LGC Standards (UK) and The National Collection of Yeast Cultures (UK) respectively, and cyclic dipeptide standards (2,5-diketopiperazines) were purchased from Bachem AG (Switzerland).

### *Selection of brewing process*

#### *Saccharification*

Pigmented rice was steamed for 60 min at 100 ºC and inoculated with the fungi *A. oryzae*, followed by incubation at 25 ºC or 30 ºC for 8 days. Sugars and organic acids were analysed every 24 h. The optimum saccharification process was determined by the conditions (time and temperature) that produced the highest concentration of glucose.

#### *Alcoholic fermentation*

The optimum saccharification process was applied to the steamed pigmented rice, which was subsequently inoculated with *S. cerevisiae* and left to ferment for 10 days at either 25 ºC or 30 ºC. Samples were collected every day to determine the levels of sugars, organic acids and the ethanol content. The optimum fermentation conditions were selected on the basics of high ethanol content and reduced levels of acetic acid. Samples were pasteurized at 70 ºC for 10 min.

### *The brewing of rice wine from pigmented rice with different degree of milling*

Pigmented rice was milled in a Twinbird rice polishing machine (Japan) to (partially) remove bran and produce rice of various degrees of milling (DM0% (whole grain), DM30%, DM50% and DM65% (fully polished grain)). The grains were used for brewing under the selected brewing conditions.

### *Analysis of compounds responsible for taste*

#### *Sugars, organic acids and ethanol content*

The analysis of sugars, organic acids and ethanol was performed as described by Zeppa et al. [4]. Separation was carried out on an Aminex HPX-87H column (300 x 7.8 mm, 9µm) from Bio-Rad (UK) with 5 mM sulfuric acid as the mobile phase for the separation of the compounds of interest. The selected wavelength for the organic acids was 210 nm, whereas an RI detector was used for the analysis of sugars and ethanol.

#### *Free amino acids*

Free amino acids were analysed using the EZfaast™ amino acid derivatization technique (Phenomenex, Torrance, CA), followed by GC-MS (Agilent, Germany) as described by Elmore et al. [5].

#### *Cyclic dipeptides*

Analysis of cyclic dipeptides was carried out as described by Oruna-Concha et al. [6]. Briefly, pigmented rice wine (15 mL) was mixed with 50 µL of 3-chlorophenol (100 mg/L) as internal standard, and then passed through the SPE cartridge (Strata-X 33 µm polymeric reversed phase giga tube, Phenomenex). HPLC water and methyl acetate were used for washing and elution, respectively. The eluent was concentrated by flushing with $N_2$, and then injected into the GC-MS equipped with a ZB-Wax column.

## Results and discussion

*Selection of brewing conditions for the production of pigmented rice wine*

Saccharification is one of the important steps during brewing, as the starch present in cooked rice is converted to simple sugars, thus acting as nutrients for the subsequent fermentation stage and contributing to the taste and flavour of the rice wine [1]. Sugars including maltotriose, maltose and glucose were monitored throughout the saccharification process (Figure 1). Sugar levels were low on day 1 regardless of the temperature, however the concentration of maltotriose and maltose significantly increased by day 2 as the rice starch was degraded to maltotriose and maltose by the fungi. From day 2, an increase in glucose levels was observed as both maltotriose and maltose were converted to glucose. The highest levels of glucose were observed at day 6. After that, the sugars levels decreased as their rate of formation was less than their rate of consumption by *A. oryzae*. Slightly higher levels of sugars were observed at 30 ºC and therefore the optimum saccharification process for this study was set at 30 ºC for 2 days.

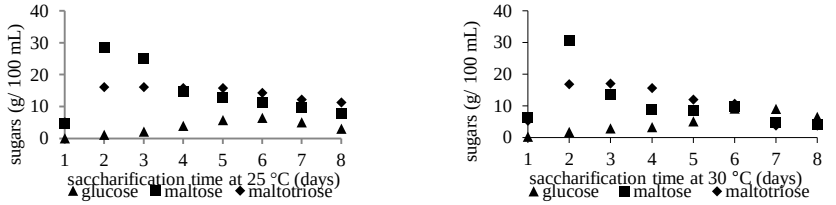


**Figure 1**: Glucose, maltose and maltotriose generated from pigmented rice during saccharification by *A. oryzae*; n=3 brews. The standard deviation was generally <2%, and <5% in all cases.

Following saccharification, the rice was inoculated with *S. cerevisiae* for alcoholic fermentation. During this step, and regardless of temperature, the levels of maltose and glucose decreased, whereas an increase in ethanol was observed, particularly on day 9 (Figure 2). Malic, lactic, succinic and acetic acid were also formed by yeast metabolism which used glucose as a substrate [1]. Moreover, fermentation at 30 ºC produced more ethanol and lower levels of acetic acid ($p<0.05$). Although acetic acid is the most abundant volatile acid in wine, its excessive concentration (>0.9 g/L) affects negatively the quality of wine because it can contribute a bitter or sour aftertaste [7]. Therefore, the optimum fermentation conditions for this study were set at 30°C for 9 days.

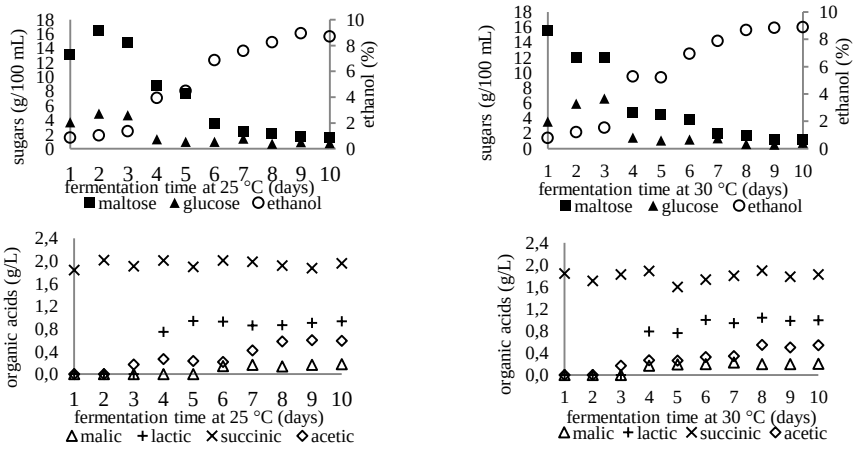


**Figure 2**: Sugars, ethanol and organic acids produced during fermentation from pigmented rice; n=3 brews. The standard deviation was generally <2%, and <5% in all cases.

*Effect of degree of milling on the characteristic taste compounds of pigmented rice wine*

Four proline-based cyclic dipeptides, namely cyclo(Pro-Val), cyclo(Pro-Ile), cyclo(Pro-Leu) and cyclo(Pro-Pro) were identified in pigmented rice wine (Table 1) however their concentrations were lower than the reported threshold [8] and they are therefore unlikely to contribute to the taste of rice wine. It is likely that these cyclic dipeptides are generated by the action of the yeast [6] during the brewing process. A Pearson correlation ($p=0.01$) showed a correlation between pH and the formation of cyclic dipeptides, the lower pH being more favourable for formation of cyclic dipeptides [8].

Table 1 shows that glucose significantly increased (0.79-1.78 g/L, $p<0.05$) as the DM increased, however no significant differences were observed in the ethanol content. The predominant acid was acetic acid (0.36-0.65 g/L) and glutamic acid was the predominant amino acid (0.62-1.17 g/L), all present at concentrations higher than their reported thresholds [9], thus contributing respectively to the unique sour and umami taste characteristics of pigmented rice wine. Moreover, this study has shown that retaining the bran increases the glutamic acid in pigmented rice wine ($p<0.05$).

**Table 1**: pH, ethanol and taste compounds found in pigmented rice wines

| taste compounds | | *degree of milling* | | | | *threshold* |
|---|---|---|---|---|---|---|
| | | *0%* | *30%* | *50%* | *65%* | |
| pH | | $4.76^{d}$ | $4.24^{c}$ | $3.94^{b}$ | $3.69^{a}$ | - |
| ethanol (%) | | $11.9^{a}$ | $12.2^{a}$ | $11.6^{a}$ | $11.8^{a}$ | - |
| glucose (g/L) | | $0.79^{a}$ | $1.20^{b}$ | $1.19^{b}$ | $1.78^{c}$ | 3.24 [9] |
| glutamic acid (g/L) | | $1.17^{c}$ | $0.81^{b}$ | $0.62^{a}$ | $0.62^{a}$ | 0.18 [9] |
| organic acids (g/L) | *lactic acid* | $0.73^{a}$ | $0.90^{a}$ | $1.04^{a}$ | $0.76^{a}$ | 1.39 [9] |
| | *acetic acid* | $0.65^{c}$ | $0.39^{a}$ | $0.36^{a}$ | $0.48^{b}$ | 0.12 [9] |
| cyclic peptides (mg/L) | *cyclo(Pro-Val)* | $1.07^{a}$ | $1.88^{b}$ | $1.79^{b}$ | $2.06^{b}$ | 251 [8] |
| | *cyclo(Pro-Ile)* | $5.04^{a}$ | $14.2^{b}$ | $16.3^{b}$ | $17.2^{b}$ | 101 [8] |
| | *cyclo(Pro-Leu)* | $8.96^{a}$ | $9.74^{a}$ | $9.34^{a}$ | $9.81^{a}$ | 250 [8] |
| | *cyclo(Pro-Pro)* | $2.98^{a}$ | $4.14^{b}$ | $4.27^{b}$ | $4.94^{c}$ | 501 [8] |

Values are the mean of three replicates. Means with different letters are significantly different at $p=0.05$.

## References


1. Liu, D., Zhang, H.T., Xiong, W., Hu, J., Xu, B., Lin, C., Xu, L., Jiang, L. (2014). Biomed. Res. Int. 1-8.
2. Yu, H., Zhao, J., Li, F., Tian, H., Ma, X. (2015). J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 129-135.
3. Iizuka-Furukawa, S., Isogai, A., Kusaka, K., Fujii, T.,Wakai, Y. (2017). J. Biosci. Bioeng. 123(2): 209-215.
4. Zeppa, G., Conterno, L. and Gerbi, V. (2001). J. Agric. Food Chem. 49(6): 2722-2726.
5. Elmore, J.S., Koutsidis, G., Dodson, A.T., Mottram, D.S., Wedzicha, B.L. (2005). J. Agric. Food Chem. 53(4): 1286-1293.
6. Oruna-Concha, M., Mottram, D.S., Blumenthal, H. (2014). In: Flavour Science, Proceeding of 14th Weurman Flavour Research Symposium (Taylor, A.J. and Mottram, D.S., ed.), Context Products, pp. 127-130.
7. Shang, Y.H., Zeng, Y.J., Zhu, P., Zhong, Q.P. (2016). Biotechnol. Biotechnol. Equip. 30(3): 512-520.
8. Borthwick, A.D. and Da Costa, N.C. (2017). Crit. Rev. Food Sci. Nutr. 57(4): 718-742.
9. Hufnagel, J.C. and Hofmann, T. (2008). J. Agric. Food Chem. 56(19): 9190-9199.

# Characterisation of the aroma developed during fermentation and roasting of jackfruit seeds

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## Abstract

When jack fruit seeds are fermented with banana leaves, and dried and roasted in a process similar to that used in the production of roasted cocoa nibs, a chocolate aroma develops. By using SPME GC-olfactometry, we have shown that compounds such as 2- and 3-methylbutanal, trimethylpyrazine and phenylacetaldehyde, which are key components of cocoa aroma, are present in the headspace of roasted fermented jack fruit (FJS) seeds at similar levels to that of a typical Brazilian cocoa powder. However, a series of less desirable higher molecular weight pyrazines with branched chain substituents was also found in the headspace of the FJS, but not in the cocoa. The valine-derived substituents imparted carbolic and cardboard aromas typical of the jackfruit seeds. Minimisation of these is important for improving the flavour of this sustainable and inexpensive cocoa substitute.

## Introduction

The flesh of the jackfruit (*Artocarpus heterophyllus* Lam.) is popular in tropical countries where it grows in abundance in the wild. The seeds, which are usually discarded, account for 15-18% of the weight of the fruit and they are an under-utilised waste stream which could be exploited by local communities in Brazil. Recently, Spada et al. [1] showed that when jackfruit seeds are fermented and roasted using a process similar to that used for cocoa beans, a distinctive chocolate aroma develops. Various novel applications are currently being developed for the use of ground and roasted jackfruit seeds as a partial substitute for cocoa powder in cakes, cappuccino and cosmetics.

The development of the desirable chocolate aroma is very dependent on the post-harvest treatment, and the subsequent drying and roasting processes. Response surface methodology was used to compare fermentation and acidification steps prior to roasting, and to identify optimum roasting conditions to maximise the chocolate aroma of the ground roasted seeds. Twenty-seven different roasted jackfruit seed powders were assessed for "chocolate aroma" by a sensory panel (n=162) using ranking tests [1]. Optimum roasting conditions from each of the three processes (dried, acidified and fermented) were selected and the corresponding powders analysed by GC-MS and GC-olfactometry and compared to a standard Brazilian cocoa powder. In this paper, we focus on the fermented product (FJS) which had the highest ranking score for chocolate aroma.

The aim of the work was to confirm the presence of key chocolate aroma compounds in the FJS powder and to identify those compounds responsible for the less desirable "jackfruit seed" aromas.

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## Experimental

*Materials*

The jackfruit were collected from the local countryside, the flesh discarded and the seeds, pulp and banana leaves were placed in a closed container for 3 days to encourage anaerobic fermentation and production of alcohol. Over 5 days, the container was opened daily and the fermentating mass was turned over manually to encourage oxidation and production of acetic acid. After 8 days the pulp and banana leaves were removed and the fermented seeds dried at 60 ºC for 24 h. The seeds were then roasted at 154 ºC for 35 min and ground to a powder.

The cocoa powder was obtained from Cargill, Brazil. It was of Brazilian origin and the cocoa beans had been fermented and roasted. All reference standards were obtained from Sigma Aldrich, Gillingham, UK.

*Model reactions*

Equimolar amounts (0.1 mM) of glucose, glycine and another amino acid (valine, leucine or isoleucine) were adjusted to pH 7 and heated in an autoclave at 125 °C for 30 min. They were diluted 10 times prior to GC-MS analysis on two columns under identical conditions to the analysis of the powders.

*Analysis of the volatile compounds by SPME and GC-MS*

FJS powder (3 g) or Brazilian cocoa powder (3 g) were mixed with HPLC grade water (3 ml) in an SPME vial and vortexed for 2 min. After equilibrating the sample at 45 °C for 15 min, the triple phase fibre (65 µm PDMS/DVB/Carboxen from Supelco) was exposed to the headspace for 55 min as previously described [1]. SPME extracts were analysed by GC-MS on an Agilent HP5890 Series II GC, coupled to a 5975 MSD. The GC was equipped with either a Zebron ZB-wax column or a Zebron DB5 column (both Phenomenex® 30 m x 0.25 mm x 0.25 µm film thickness) and a standard 5 °C/min temperature ramp programme was used.

*Analysis of the volatile compounds by SPME and GC-Olfactometry*

SPME extracts were also analysed on the same two columns using an Agilent HP5890 Series II GC-FID system coupled to an ODO 2 odourport (SGE). The outlet was split between a flame ionisation detector and a sniffing port, each with a flow of 1 ml/min. The contents of the SPME fibre were desorbed for 3 min in a split/splitless injection port, in splitless mode, onto five small loops (5 cm diameter) of the column in a coil, which were cooled in solid carbon dioxide, contained within a 250 mL beaker. After 3 min the beaker was removed and a standard 5 °C/min temperature ramp programme employed. The eluting aroma regions were described and scored by two assessors in duplicate on a scale of 0 (none) to 7 (strong). Mean values are reported in Table 1.

## Results and discussion

*GC-MS*

The extract contained ~200 volatile compounds, most of which were identified and at least 70 of the identities were confirmed by comparison with the appropriate standard reference compound. Of the 200 volatiles, we believe that >60 are pyrazines, although reference standards were only available for 10 of these. For that reason, model reactions were prepared in order to distinguish the many pyrazines that were generated during the roasting process. By using either valine, isoleucine or leucine in a simple glucose/-glycine

Maillard reaction, it was possible, in conjunction with mass spectra and LRI data on two columns, to confirm the identity of the substituents on many pyrazines as 2-methylpropyl, 2-methylbutyl or 3-methylbutyl respectively. However, the position of the substituents could not be determined unless there were authentic standards available for some of the isomers. This information was vital in attributing the aroma regions in the GC-Olfactometry to particular compounds.

*GC-Olfactometry*

Over 50 aroma regions were detected by the assessors on the DBWax column. Of these, 40 were assigned to compounds, most of which were also found in the GC-MS. The identities of these 40 compounds were further confirmed by carrying out GC-O on a DB5 column. Most of the aromas detected on the DBWax were found at the correct LRI on the DB5 column. Many of these were generic aroma compounds found in most foods as described by Dunkel et al. [2].

In this paper, the main focus is on the aroma regions which obtained high scores from the GC-O assessors, particularly those which are known to be important in the aroma of chocolate [3] or cocoa powder [4], or those which resembled the less desirable character of the jackfruit seeds which dominated the aroma of some of the earlier trial samples. These are summarised in Table 1.

**Table 1**: Comparison of GC-Olfactometry scores for selected compounds for roasted fermented jackfruit seeds (FJS) and Brazilian cocoa powder, mean score of 2 assessors in duplicate where 0 = none and 7 = strong

| LRI on DBWax | | | | LRI on DB5 | | | GC-O Score | |
|---|---|---|---|---|---|---|---|---|
| GC-O expt | GC-MS expt | GC-MS au | Identity of compound | GC-MS au | GC-O expt | GC-MS expt | FJS | Cocoa |
| *i)Compounds typically found to be important in chocolate or cocoa aroma, detected in both FJS and cocoa* | | | | | | | | |
| 909 | *911* | *925/ 928* | 2/3-methylbutanal | 656/ 665 | <600 | 651/ 662 | 7 | 6 |
| 1390 | *1394* | *1394* | trimethylpyrazine | 1008 | coelute | 1003 | 7 | 6 |
| 1628 | *1624* | *1624* | phenylacetaldehyde | 1058 | 1053 | 1049 | 6 | 5 |
| *ii) Compounds only found in roasted jackfruit seeds, typically with jackfruit, carbolic, cardboard aroma* | | | | | | | | |
| 1487 | *1489* | *1489** | methyl-2-methyl-propylpyrazine 1 | 1134* | 1133 | 1140 | 5 | 0 |
| 1494 | *1495* | *1495** | methyl-2-methyl-propylpyrazine 2 | 1134* | 1145 | 1149 | 5 | 0 |
| 1553 | *1553* | *1555** | a dimethyl 2-methyl-propylpyrazine | 1206* | 1205 | 1206 | 3 | 0 |

**found in corresponding reaction mixture*

The upper half of Table 1 shows compounds which are typically associated with chocolate or cocoa aroma and have been reported by GC-Olfactometry, and deemed to be important, in many cocoa based products including milk chocolate [3] and cocoa powder [4]. These compounds were detected in both the Brazilian cocoa and the FJS,

suggesting that these too might be important in the chocolate component of the FJS aroma.

The lower half of the table shows the compounds which had relatively high GC-O scores and were detected in FJS, but not in the cocoa powder. These were 2-methylpropyl substituted pyrazines which were far more abundant in the FJS chromatograms compared to the cocoa, and were described by the GC-O assessors with less desirable terms such carbolic, cardboard and "roasted jackfruit seed flour". All three isomers of methyl-2-methylpropylpyrazine were detected in the FJS by GC-MS, and in the valine model system, two of which corresponded to the very characteristic jackfruit seed aromas which were detected in the GC-O at the corresponding LRIs. Similarly, all three isomers of dimethyl-2-methylpropylpyrazine were detected by GCMS in both FJS and the valine model system, but only the most abundant isomer was detected by GC-O. The LRI of this isomer on a DB5 column matches the LRI of one of the two isomers (2,5- and 2,6-dimethyl-3-(2-methylpropyl)pyrazine) synthesised in our lab and reported previously [5].

Further development of the flavour of this potential cocoa substitute needs to focus on removing or decreasing the contribution from the branched chain substituted pyrazines, particularly those which are likely to be derived from valine during the roasting process.

## References

1. Spada, F.P., Masson Zerbeto, L., Cabreira Ragazi, G., Roel Gutierrez, E., Coelho Souza, M., Parker, J. K., Canniatti-Brazaca, S. (2017) J. Ag. Food Chem. 65: 1196-1208.
2. Dunkel, A., Steinhaus, M., Kotthoff, M., Nowak, B., Krautwurst, D., Schieberle, P., Hofmann, T. (2014) Angew. Chem. Int. Ed Engl. 53(28):7124-43.
3. Schnermann, P., Schieberle, P. (1997) J. Agric. Food Chem. 45: 867–872.
4. Frauendorfer, F., Schieberle, P. (2006) J. Agric. Food Chem. 54: 5521- 529.
5. Elmore, J.S., Dodson, A.T., Muttucumaru, N., Halford N.G., Parry, M.A.J., Mottram, D.S. (2010) Food Chem. 122:753-760.

# Investigating the phytochemical, flavour and sensory attributes of mature and microgreen coriander (*Coriandrum sativum*)

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## Abstract

Microgreens, young stem and leaves of growing plants, have recently been the subject of much interest due to their higher concentrations of nutritive and purported bioactive compounds in comparison to their mature plant counterparts. However, there is currently limited information available in relation to the flavour and sensory attributes of microgreen species, which may ultimately prove important in determining consumer acceptance. This paper reports the total phenolic, carotenoid and chlorophyll contents as well as the aroma volatile profile and sensory attributes of both mature and microgreen coriander. Microgreen coriander was shown to contain significantly higher levels of phenolic compounds, elevated concentrations of terpenes as the main aromatic compounds and a more intense bitter/sweet taste characteristics compared to the mature coriander.

## Introduction

The term 'microgreen' is generally used to describe young (7 – 21 days) stem and leaves of growing plants [1]. In recent years, microgreens have become a growing trend in the food industry due to their nutritional density and ease of growth. These small but powerful greens have been shown to contain higher concentrations of vitamins, minerals, and phytonutrients than their mature counterparts [2,3] and continue to increase in popularity due to their appealing appearance and use as a flavourful, edible garnish.

Microgreens are considered a novel crop and therefore not much scientific information is available. Previous research on microgreens has shown that the chemical composition has a major impact on its acceptability. As such, it has been shown that sugars, phenolics and other non-volatile compounds (such as ascorbic acid) are important in microgreens as per their direct correlations to consumer preference and overall eating quality [4]. However, there is very little published research on the flavour profile of plants specifically on their microgreen stage.

## Experimental

### *Materials*

Mature coriander (MC) and microgreen coriander (MGC) were obtained from McCormack Farms Ltd (Co. Meath, Ireland). Sensory evaluation was carried out in fresh samples. Coriander leaves were plucked from the stem, washed and air-dried before presenting them to the panellists. Micro coriander leaves were prepared in the same way. For the remaining analysis, the herbs were harvested and immediately freeze-dried. Solvents and authentic compounds were purchased from established laboratory chemical suppliers.

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*Analysis of volatile compounds*

The extraction of volatile compounds was performed using a headspace solid-phase microextraction system (HS-SPME). A 50/30 µm divinylbenzene (DVB)/polydimethylsiloxane (PDMS) fibre (Supelco, Bellefonte, Pennsylvania, USA). Freeze-dried herb (0.5g) reconstituted in 4.5mL of water containing 5000 ng of IS propyl propanoate were placed in a SMPE vial of 15 mL fitted with a screw cap. After equilibration at 40°C for 10 min, the fibre was exposed to the headspace above the sample for 30 min. The sample was kept under stirring at 40°C and desorpted for 20 min in the GC injector at 230°C and analysed by GC-MS as described by Morales-Soto et al. [5].

*Analysis of free amino acids*

Free amino acids were analysed using the EZ-Faast amino acid derivatisation technique (Phenomenex, Torrance, CA) followed by GC-MS analysis, as described by Elmore et al. [6]. For each plant sample, 0.2 g of freeze-dried powder was weighed in glass vials and suspended in 10 mL of 0.01 M HCl. The suspensions were stirred for 15 minutes with a magnetic stir bar and plate. After standing for 15 minutes, 2 mL of the supernatant was removed and placed into Eppendorfs that were centrifuged for 15 minutes at 12,100g in a MiniSpin Eppendorf centrifuge.

*Analysis of total phenolics*

The extraction of phenolic compounds was carried as described by Sun et al. [7]. Freeze-dried herb (0.1g) was extracted with 5 mL of methanol/water (60:40, v/v) using sonication for 60 min at 21°C. The sample was centrifuged at 1000g for 15 minutes and supernatant used for analysis. Total phenolic determination was carried as described by Singleton & Rossi [8].

*Analysis of total carotenoids & chlorophyll*

The carotenoids & chlorophyll were extracted as described by Giallourou et al. [9] with slight modifications. Methanol (4 ml) was added to 25 mg of powder and the samples were shaken for 15 min at 8000 rpm. Following centrifugation at 4000 rpm for 5 min, the supernatant was transferred to a clean tube and the process was repeated until a colourless supernatant was obtained. The absorbance of the combined supernatants was measured at 470, 645 and 662 nm. The total amount of carotenoids & chlorophyll was calculated according to the equations by Lichtenthaler & Buschmann [10].

*Sensory analysis*

Sensory evaluation was carried out using Quantitative Descriptive Analysis (QDA) on micro and mature coriander fresh leaves via a trained panel (n=11) on a gLMS scale [11,12].

## Results and discussion

Microgreen coriander had significantly higher ($p<0.05$) levels of total phenols in comparison to mature plants (24.1mg GAE/g and 16.4 mg GAE/g (d.w.), respectively), however there was no significant difference in the content of total carotenoids (1.6 vs 1.6 mg/g d.w.) or chlorophylls (8.5 vs 8.3 mg/g d.w.) between MGC and MC.

In general, higher levels of amino acids (more than 2 fold) were found in the MGC compared to the mature counterpart (24.5 mg/g and 11.0 mg/g (d.w), respectively). Of sixteen amino acids identified, the predominant one was asparagine (15.82 vs 5.01 mg/g (d.w) in MGC and MC, respectively) followed by glutamine (1.99 vs 1.05 mg/g (d.w)), aspartic acid (1.75 vs 1.48 mg/g (d.w)) and glutamic acid (1.44 vs 1.09 mg/g (d.w))

although differences for these three amino acids were not significant. Free amino acids may contribute to the flavour quality of the herbs by their own taste characteristics including sweet, sour and bitter taste. Significant differences ($p<0.05$) were found in the levels of glycine and tryptophan, thus potentially contributing to the sweet and bitter taste of the MGC.

Thirty-six compounds were identified in the headspace of the coriander herbs and the significant ones are listed in Table 1. Terpenes were the major compounds identified in the MGC comprising 62% of the total volatile compounds collected from the headspace whereas aldehydes, particularly hexanal, together with alkanes and alkenes represented 87% of the total volatile compounds collected from the headspace of the MC. The most abundant compound present in the MGC was linalool (more than 30 fold higher in microgreen coriander compared to mature coriander). Previous research on the chemical profile of coriander essential oil has also indicated that it is a rich source of oxygenated monoterpenes, with linalool as the principal constituent [11]. Additionally, α-pinene, γ-terpinene, limonene and p-cymene were also detected as the main compounds in the MGC samples.

**Table 1:** Volatile compounds in the headspace of microgreen (MGC) and mature (MC) coriander.

| | *LRI*[A] | *MGC*[B] | *MC*[B] | *P** |
|---|---|---|---|---|
| Methyl 2-methylbutanoate | 777 | 238 | 113 | * |
| Hexanal | 799 | 804 | 1613 | * |
| Methyl 2-methyl-2-butenoate | 825 | 335 | 70 | ** |
| α-Pinene | 940 | 4539 | nd | *** |
| Camphene | 956 | 643 | nd | ** |
| cis-Sabinene | 979 | 259 | nd | ** |
| β-Pinene | 984 | 208 | nd | ** |
| β-Myrcene | 994 | 676 | nd | ** |
| Linalool | 1102 | 11636 | 370 | *** |
| Nonanal | 1105 | 703 | 439 | ** |
| p-Cymene | 1030 | 1587 | nd | ** |
| Limonene | 1035 | 1727 | 550 | ** |
| (Z)-β-Ocimene | 1050 | 136 | nd | ** |
| γ-Terpinene | 1064 | 2374 | nd | ** |
| Terpinolene | 1095 | 248 | nd | ** |
| Camphor | 1158 | 774 | 18 | *** |
| Borneol | 1178 | 414 | 1 | ** |
| Dodecane | 1200 | 579 | 264 | * |

[A] Linear retention index on DB-5 column, calculated from a linear equation between each pair of straight chain alkanes C6–C20.

[B] Estimate quantities (ng) of compound in the headspace of 0.5g of herb calculated by comparison with 5000ng of propyl propanoate used as internal standard.

*Significant at the 5% level;

**Significant at the 1% level;

***Significant at 0.1% level. Means of three replicate samples; nd, not detected

Results from the sensory analysis are show in Figure 1. MGC was rated as more intense for both bitterness and sweetness which could be associated with significantly higher levels of phenolic compounds as well as bitter and sweet tasting amino acids in MGC (Figure 1A). However, no significant differences in umami were observed between the microgreen and mature coriander thus confirming the amino acid results where similar levels of aspartic acid and glutamic acid, responsible for umami taste, were found in both samples.

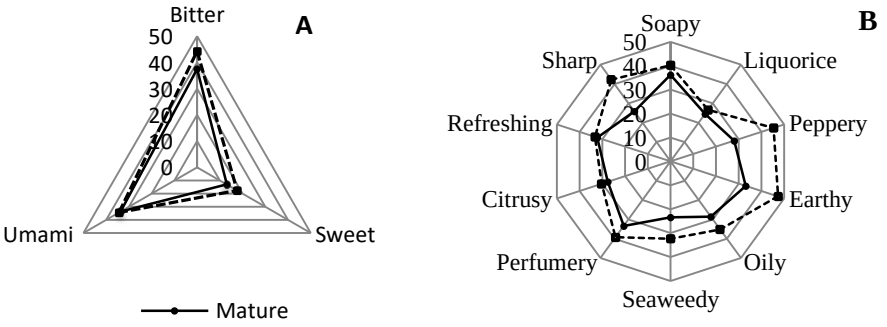


**Figure 1:** Radar plot and cobweb representing the taste (A) and flavour (B) profiles of microgreen (MGC) and mature (MC) coriander. Intensity of each attribute was marked on a gLMS scale (n=11) ($p< 0.05$).

Flavour characteristics (Figure 1B), on the other hand, showed significant differences between MGC and MC in the attributes "peppery", "earthy" and "sharp", commonly used to describe the flavour of coriander [12], on the gLMS scale with the MGC scoring higher than MC, which could be associated with higher levels of β-myrcene (peppery) and α-pinene (earthy). Furthermore, higher "perfumery" and "citrusy" notes were also associated with MGC. Linalool which was the major compound in the MGC generally contributes to the floral and pleasant notes. Several other terpenes such as limonene, γ-terpinene and terpinolene, present at higher level in MGC, could be responsible for the citrus notes described by the panellists.

Results of the current study suggest that microgreen coriander could potentially be used as novel culinary ingredients whose widespread popularity may be dependent on familiarization of consumers with their particular sensory attributes.

## References

1. J.S. Lee, W.G. Pill, B.B. Cobb and M. Olszewski (2004) J. Hort. Sci. Biotech. 79:565–570.
2. E. Pinto, A.A. Almeida, A.A. Aguiar and I.M.P.L.V.O. Ferreira (2015) J. Food Compost. Anal. 37, 38-43.
3. Z. Xiao, G.E. Lester, Y. Luo and Q. Wang (2012) J. Agric. Food Chem. 60, 7644-7651.
4. Z. Xiao, G.E. Lester, E. Park, R.A. Saftner, Y. Luo, and Q. Wang (2015) Postharvest Bio. Tech. 110:140–148.
5. Morales-Soto, M.J. Oruna-Concha, J.S. Elmore, E. Barrajón-Catalán, V. Micol, C. Roldán, and A. Segura-Carretero (2015) Ind. Crops and Prod. 74, 425-433.
6. J.S. Elmore, G. Koutsidis, A.T. Dodson, D.S. Mottram, and B.L. Wedzicha (2005) J. Agric. Food Chem. 53, 4, 1286-1293
7. J. Sun, Z. Xiao, L. Lin, G.E. Lester, Q. Wang, J.M. Harnly, and P. Chen (2013). J. Agric. Food Chem. 61(46): 10960-10970.
8. V.L. Singleton, and J.A. Rossi (1965) J. Enol. Vitic. 16, 144-158
9. N. Giallourou, M.J. Oruna-Concha, and N. Harbourne (2016) Food Chem. 212 :411-419
10. H. K. Lichtenthaler, and C. Buschmann (2001). In CPFAC. John Wiley & Sons, Inc.
11. H.T. Lawless, and H. Heymann (2010) Springer Science & Business Media.
12. L.M. Bartoshuk, V.B. Duffy, B.G. Green, H.J. Hoffman, C.-W. Ko, L.A. Lucchina, L.E. Marks, D.J. Snyder, and J.M. Weiffenbach (2004) Physiol & Behav., 82, 109-114.
13. K. Msaada, K. Hosni, M.B. Taarit, T. Chahed, M.E. Kchouk, and B. Marzouk (2007) Food Chem. 102, 1131-1134.
14. L. Mauer, and A. El-Sohemy (2012) Flavour, 1, 1-5.

# HPLC-ESI(+)MS/MS quantitation of the newly evidenced glutathione S-conjugates in two dual-purpose hop varieties: Citra and Sorachi Ace

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## Abstract

After the evidence in hop of the cysteinylated precursors of 3-sulfanyl-4-methylpentan-1-ol (3S4MPol) and 3-sulfanylhexan-1-ol (3SHol), S-glutathione precursors were recently investigated in Amarillo, Hallertau Blanc and Mosaic. The aim of the present work was to assess the linked-potential in two other dual-purpose hop cultivars, namely Citra and Sorachi Ace. The occurrence of S-3-(1-hydroxyhexyl)glutathione (G-3SHol) was confirmed in all cultivars, at levels well above those reported for the cysteinylated counterpart, while S-3-(4-methyl-1-hydroxypentyl)glutathione (G 3S4MPol) revealed more specific of the Hallertau Blanc variety.

## Introduction

Dual-purpose hop cultivars are characterized by high contents of both bitter acids (>7% humulones) and essential oils. Among the essential oils, odorant polyfunctional thiols, present in much lower amounts (1-150 µg/kg) than terpenols (10−100 mg/kg), are viewed as key contributors to hop flavour in beer, especially when dry hopping or bottle refermentation are applied. Most of them have a 3-carbon distance between the SH group and the other chemical function (alcohol, ester, carbonyl, etc.). [1-5] 41 Volatile polyfunctional thiols have been found in hop, and each cultivar exhibits a unique thiol profile. [5-7] Among them are found 3S4MPol with its very nice grapefruit/rhubarb-like flavours (odour perception threshold = 70 ng/L in beer), and 3SHol, also with a grapefruit-like flavour (odour perception threshold = 55 ng/L in beer). [6,7] Hoppy flavours can be enhanced in beer by applying either late hopping (addition of hop at the end of wort boiling or in the whirlpool) or dry hopping (addition of hop during beer fermentation or maturation). As described by Gros *et al*., the thiol content of the final beer reaches higher values than might be expected on the basis of hopping rate and hop free thiol contents, due to the presence of heavy precursors including cysteine adducts (levels 20-120 times higher than the free forms). [5] In plants, cysteine-S-conjugates usually arise through the glutathione detoxification pathway, where the tripeptide is added to an α,β-unsaturated carbonyl in the presence of glutathione-S-transferase. The resulting glutathione-S-conjugate is further converted to the corresponding S-cysteine conjugate after successive enzymatic cleavages of glycine and glutamate residues. [8,9] The occurrence in hop of glutathione S-conjugates was evidenced for the first time in 2016. [10] Very high concentrations of G-3SHol were quantitated in Amarillo, Hallertau Blanc and Mosaic cultivars (up to 32 mg/kg). The aim of the present work was to investigate G-3SHol and G-3S4MPol in two other dual-purpose hop varieties: Citra and Sorachi Ace.

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## Experimental

*Extraction of cysteine and glutathione S-conjugates*

Thiols S-conjugates were extracted (according to Kankolongo *et al.* [10]) from the Citra and Sorachi Ace hop varieties. S-Benzylcysteine (Cys-IST) was used as an internal standard at 8 mg/kg of hop. Milled pellets (100 g) were stirred with 1000 mL of a 1% (v/v) formic acid aqueous solution for 2 h at 45 °C. After centrifugation for 30 min, the supernatants were collected and loaded on a column of IR-120 cation exchange resin (100 g preconditioned with 100 mL of aqueous 2M HCl followed by 1 L of water). The column was then washed with 500 mL of water and the thiol precursors were recovered by elution with aqueous ammonia solutions from 0 to 3.3 mol/L (increment of 0.3 mol/L). Glutathione adducts are eluted in the 1.2−2.4 mol/L fractions (also containing the cysteine S-conjugates). Those fractions were pooled and concentrated under reduced pressure. The obtained extract was dissolved in a formic acid aqueous solution for analysis by HPLC-ESI(+)-MS/MS with the Cyclobond I 2000 RSP chiral column. The elution solvents were water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). An isocratic elution with 95% solvent A and 5% solvent B was applied, with a flow rate of 300 µL/min. 5 µL of sample was injected onto the column at room temperature. The mass spectra were acquired with a BrukerDaltonics Esquire 3000 ion trap mass spectrometer equipped with an electrospray ion source (Bruker) operated in positive mode (ESI+). The ESI inlet conditions were as follows: source voltage, 4.5 kV; capillary temperature, 360 °C; nebulizer, nitrogen, 12 Psi. Nitrogen was also used as drying gas, at a flow rate of 8 mL/min. For identification, collision-induced dissociation MS/MS spectra were recorded at a relative collision energy of 0.2 V. For quantitation, the MRM mode was applied (relative collision energy of only 0.05 V to maximize the [M + $H^+$] ions). Calibration curves of G-T relative to Cys-IST were determined and the following equation was used: concentration of G-T (in µg/kg) = concentration of Cys-IST (in µg/kg) × (peak area of G-T/peak area of Cys-IST) × (mass response coefficient of Cys-IST/mass response coefficient of G-T). All analyses were carried out in duplicate.

## Results and discussion

As depicted in Figure 1, HPLC-ESI(+)MRM analyses enabled us to evidence both diastereomers of G-3SHol in Citra and Sorachi Ace hop cultivars, at concentrations similar to those reported by Kankolongo *et al.* for the Amarillo, Hallertau Blanc and Mosaic hops. On the other hand, no bound 3S4MPol was found. [10]

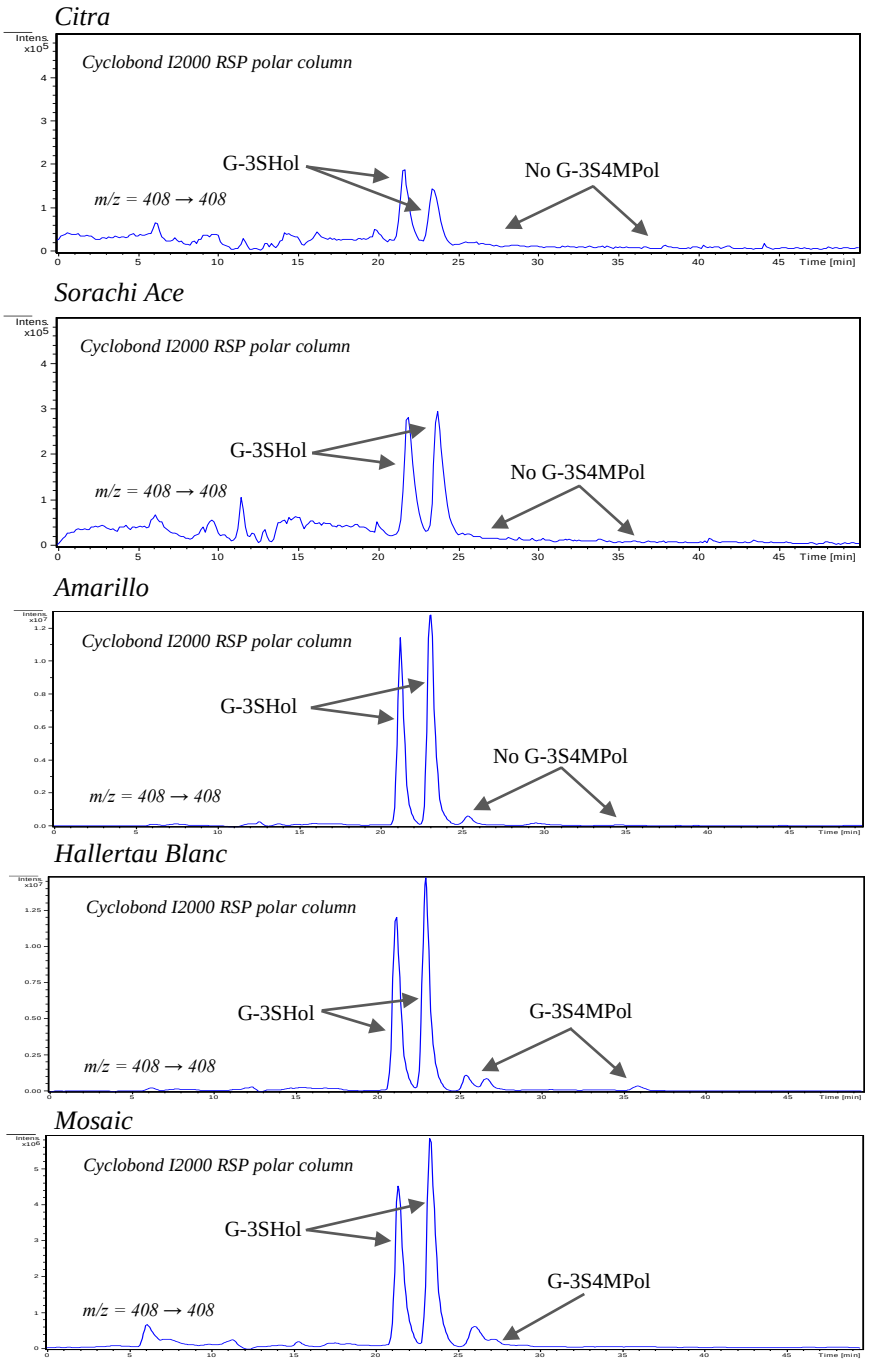


**Figure 1:** HPLC-ESI(+)MRM analysis of G-3SHol and G-3S4MPol diastereomers in Citra and Sorachi Ace hops on the Cyclobond I 2000 RSP column. Comparison with three previously investigated cultivars. [10]

Given in free thiol equivalents (Figure 2), the glutathionylated 3SHol emerged as the key fraction in all cultivars while for 3S4MPol, cysteinylated and free fractions remain important to be considered.

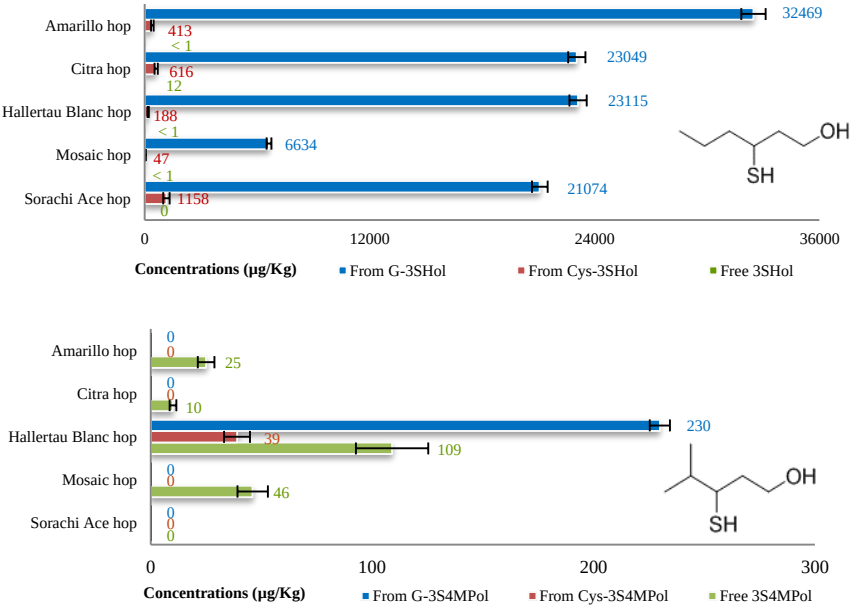


**Figure 2:** Concentration (µg/kg) of free and bound potentials (given in free thiol equivalents) of 3SHol and 3S4MPol in Citra and Sorachi Ace hop varieties. Comparison to Amarillo, Hallertau Blanc and Mosaic hops. [10]

In conclusion, 3SHol seems relatively ubiquitous in free, cysteinylated, and glutathionylated forms while the glutathione adduct of 3S4MPol was found only in the Hallertau Blanc variety. Further research is needed to understand how more thiols could be released from glutathione forms through the brewing process.

## References

1. Gros, J., Nizet, S., Collin, S. J. Agric. Food Chem., 2011, 59, 8853-8865.
2. Kankolongo Cibaka, M. L., Gros, J., Nizet, S., Collin, S. J. Agric. Food Chem. 2015, 63, 3022−3030.
3. Collin, S., Nizet, S., Gros, J. Cerevisia, 2012, 36, 119-124.
4. Nizet, S., Gros, J., Peeters, F., Chaumont, S., Robiette, R., Collin, S. J. Am. Soc. Brew. Chem., 2013, 71, 15-22.
5. Gros, J., Peeters, F., Collin, S. J. Agric. Food Chem. 2012, 60(3), 7807−7816.
6. Kishimoto, T.; Wanikawa, A.; Kono, K.; Shibata, K. J. Agric. Food Chem. 2006, 54, 8855−8861.
7. Takoi, K., Degueil, M., Shinkaruk, S., Thibon, C., Maeda, K., Ito, K., Bennetau, B., Dubourdieu, D., Tominaga, T. J. Agric. Food Chem. 2009, 57, 2493−2502.
8. Marrs, K. Annu. Rev. Plant Biol. 1996, 47, 127–158.
9. Starkenmann, C., Troccaz, M., Howell, K. Flavour Fragr. J. 2008, 23, 369–381.
10. Kankolongo-Cibaka, M-L., Decourrière, L., Lorenzo-Alonso, C-J., Bodart, E., Robiette, R., Collin, S. J. Agric. Food Chem., 2016, 64, 8572-8582.

# Tracking of hop-derived compounds in beer during fermentation with PTR-ToF-MS

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## Abstract

Proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS) was used as a novel, direct and real-time analytical method to monitor small-scale fermentations carried out in 20 mL vials (3 mL sample volume) at 20 °C with repeated measurements of the headspace volatile organic compounds (VOCs) for four days. A design matrix of two yeast biotypes (California Ale and Edinburgh Scottish Ale) and two New Zealand aroma hop cultivars (Motueka and Nelson Sauvin), together with their respective no addition controls, were used to investigate yeast-hop interactions. The results highlighted the advantages of using online analytical measurements, such as PTR-ToF-MS, to understand temporal changes that occur in VOCs during fermentation. Distinct differences were observed in the VOCs profile of the different beers based on combinations of yeast biotype and hop cultivar; e.g. samples with Motueka and Scottish Ale had higher concentrations of *m/z* 89.057 (3-methyl-1-butanol). Complex dynamics were observed for VOC development during the fermentation; e.g. production maxima for masses such as *m/z* 145.121 (2-nonanol or ethyl hexanoate) and *m/z* 173.153 (isoamyl isovalerate or ethyl octanoate).

## Introduction

The craft beer market is experiencing a rapid increase in growth. To help brewers optimise hop character, and to make beer with distinctive hop profiles, a better understanding of the role that yeast play in the development of hop character is required. Anecdotally, brewers report that some yeasts "promote" hop flavour or accentuate one hop character over another, while other yeast biotypes are considered to be "hop neutral" or known to reduce hop intensity. However, to date very little scientific research has been published on the impact of different yeast biotypes and fermentation parameters on hop flavour in beer or on the mechanisms responsible for differing aroma and flavour development [1].

Traditionally, hop flavour development has been assessed using GC-MS based approaches, where hop derived compounds in the final beer are identified and measured. The drawback with this approach is that it is time consuming and provides little information on changes that occur during brewing and fermentation. Furthermore, the sensitivity of GC-MS to detect volatile organic compounds (VOCs) is dependent on the extraction method employed and the volatility and polarity of the target analytes and their affinities towards the chosen solvent or solid phase material [2,3].

In this paper PTR-ToF-MS was used to measure and compare the dynamic changes in VOCs during the production of beer containing one of two aroma hops (Motueka and Nelson Sauvin) in combination with one of two yeast biotypes (California Ale and Scottish Ale) and their respective no addition controls.

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## Experimental

Laboratory scale beer samples were produced with wort standardised to 10.2˚P [degree Plato] (1044 OG [Original Gravity]) and 20 IBU [International Bittering Units]. Aroma hops (5 g/L) (Table 1) were added at 90˚C for 5 min before being cooled to 20˚C. Wort was inoculated with yeast (Table 1) (pitching rate: ~$1.0\times10^7$ cells/mL) and divided into 6 aliquots (3 mL) of each treatment. Fermentation was carried out in HS-vials at 20˚C with consecutive headspace sampling every 6 hours using PTR-ToF-MS. Compounds were identified through an elemental composition calculator and preceding GC-MS measurements [4]. Microfermentations (3 mL) may be regarded to not closely represent industrial fermentations due to differences in convection and pressure in large scale ferments, which might impact on the magnitude of concentrations of some VOCs (e.g. ester formation might be altered in micro ferments). However, the production pathways and the sequence of changed in the volatile profile are expected to stay the same.

**Table 1:** Experimental design with yeast and hop combinations (treatments)

| *Treatment* | *No yeast (NY)* | *California Ale yeast (CA)* | *Scottish Ale yeast (SA)* |
|---|---|---|---|
| No aroma hop (NH) | Blank | California Ale control | Scottish Ale control |
| Motueka hop (MT) | Motueka control | California Ale with Motueka | Scottish Ale with Motueka |
| Nelson Sauvin hop (NS) | Nelson Sauvin control | California Ale with Nelson Sauvin | Scottish Ale with Nelson Sauvin |

## Results and discussion

The fermentation was monitored for four days and information from 672 mass ions (*m/z*) over 14 time points were collected. Two-way ANOVA was carried out to select *m/z* with a significant change during the fermentation; overall, 182 *m/z* were found to have a significant ($p < 0.01$) change during the fermentation. A principal component analysis (PCA) was carried out on all significant ($p < 0.01$) ions for all treatments (except controls). A score plot coded to highlight treatment effects of hop cultivar is shown in Figure 1, where each point represents the VOC profile of the selected *m/z* of each sample at each time point. Reproducibility of the replicates (n=6) was found to be very good. Separation along PC-1 (39% explained variance) and PC-2 (10% explained variance, data not shown) were mainly due to changes during fermentation. As illustrated on PC-3 (4% explained variance), hop cultivar had a major impact on the VOC profile at the beginning of the fermentation (black circles), with this impact disappearing over time due to either modification by the yeast cells or stripping due to $CO_2$ production during the fermentation. Towards the end of fermentation yeast biotypes dominated the VOC profile differentiation owing to differences in the metabolites they were producing (red circles).

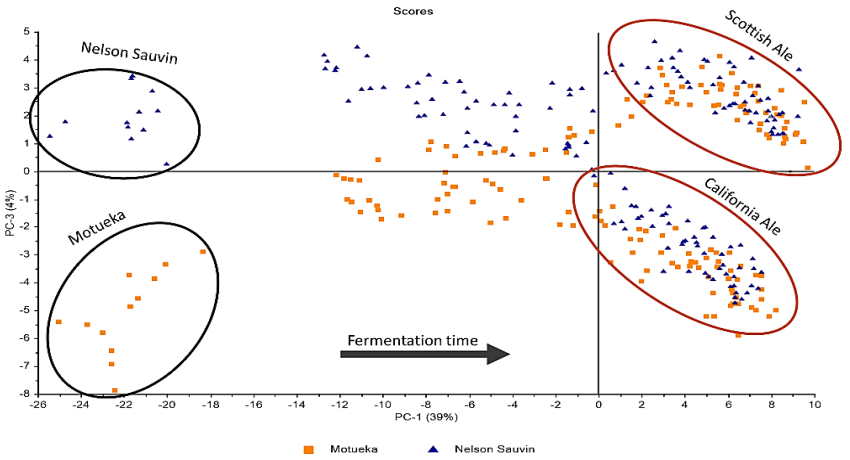


**Figure 1:** PCA of the samples with the significant *m/z* VOC profile over the fermentation time for the treatments

Selected *m/z* were tracked over time to observe VOC development throughout fermentation. Three different dynamics were observed during the fermentation: reduction (through stripping, yeast uptake, or metabolic conversion), production, and increase with a subsequent decrease. In some cases, multiple VOCs shared the same *m/z*.

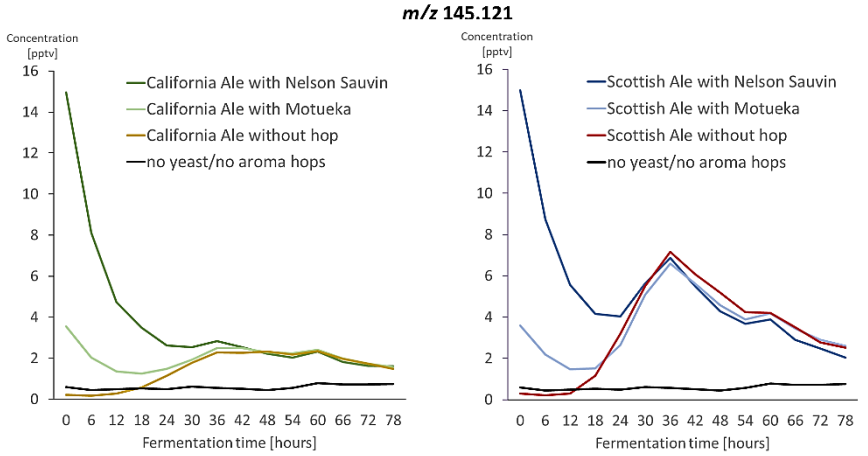


**Figure 2:** Changes in generation of *m/z* 145.121 during the fermentation of each treatment

An example of the varying dynamics present during the fermentation is illustrated by *m/z* 145.121 (Figure 2). This compound was tentatively identified at the beginning of the fermentation as octanoic acid and/or methyl heptanoate, which were determined to be hop-derived compounds. Towards the end of the fermentation *m/z* 145.121 was more likely to be either ethyl hexanoate and/or 2-nonanol, which were fermentation-derived compounds. Scottish Ale yeast with Nelson Sauvin hop demonstrated a greater reduction in *m/z* 145.121 towards the end of fermentation indicating an interaction between yeast biotype and hop cultivar. It is believed that differences observed in fermentation-derived VOC production could be due to differential gene expression.

The impact of the hop cultivar is evident for some fermentation-derived compounds, e.g. *m/z* 89.057 (Figure 3A), which was tentatively identified as ethyl acetate and/or 3-methyl-1-butanol, and *m/z* 173.153 (Figure 3B) was tentatively identified as isoamyl isovalerate, pentyl pentanoate, 2-methylbutyl-2-methylbutyrate, and/or ethyl octanoate. For both masses, Motueka hops resulted in higher production compared to Nelson Sauvin. Two production maxima (~34 hours and ~60 hours) were congruent with the rate of change in ethanol production during the fermentation, possibly related to yeast metabolism and ester production.

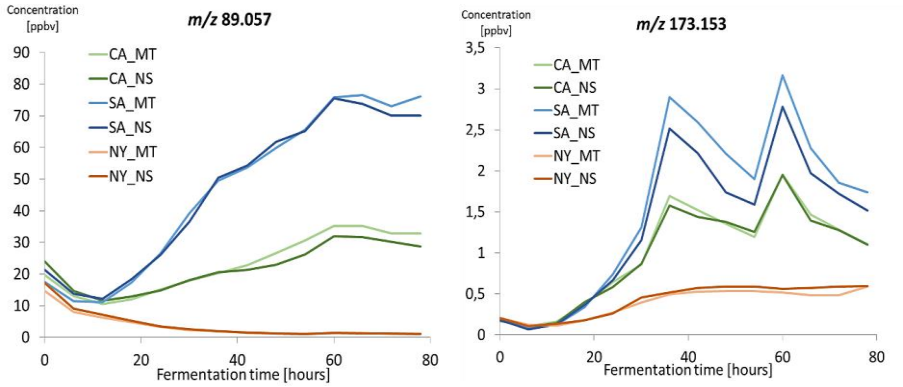


**Figure 3:** Changes in generation of *m/z* 89.057 (A) and *m/z* 173.153 (B) during the fermentation of each treatment

In conclusion, PTR-ToF-MS can successfully differentiate and monitor the change in VOCs during fermentation in real time and demonstrate how interactions between hop cultivars and yeast biotypes result in unique VOC profiles. Dynamic monitoring has the capability to enhance understanding of how metabolic pathways and stress factors influence the production of VOCs and this knowledge will facilitate a better understanding of beer flavour. A better understanding of how yeast biotypes influence hop-derived compounds during fermentation will improve our understanding of hop aroma generation in beer and will give insight on how to accentuate a desired hop character by selecting yeast biotypes and modifying fermentation parameters.

## References

1. King, A. J., & Dickinson, J. R. (2003). FEMS Yeast Research, 3, 53-62.
2. Rodrigues, F.; Caldeira, M.; Câmara, J. S. (2008). Analytica Chimica Acta, 609 (1), 82-104.
3. Richter, T. M., Eyres, G. T., Silcock, P., Bremer, P. J. (2017). Journal of Separation Science, DOI:10.1002/jssc.201700676
4. Cappellin, L., Biasioli, F., Granitto, P. M., Schuhfried, E., Soukoulis, C., Costa, F., Märk, T. D., & Gasperi, F. (2011). Sensors and Actuators B: Chemical, 155(1), 183-190.

# Formation pathways of 2,3-pentanedione in model systems and real foods

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## Abstract

The formation of the buttery smelling 2,3-pentanedione was studied in glucose/glycine and glucose/proline reaction systems under different conditions and compared with the formation of 2,3-pentanedione upon extrusion cooking. The CAMOLA approach was applied to determine the relative importance of different reaction pathways. The results indicate a strong impact of moisture on the formation of 2,3-pentanedione. Under dry heating conditions, the majority of 2,3-pentanedione (70% to 82%) was formed from the intact glucose backbone, irrespectively of the pH and the type of amino acid. On the other hand, under aqueous conditions, both pH and the type of amino acid played an important role. At pH 5 the majority of 2,3-pentanedione was formed from the intact sugar backbone (60% in the presence of proline and 86% in the presence of glycine) while at pH 9 this diketone was almost exclusively formed by recombination of $C_3/C_2$ and $C_4/C_1$ sugar fragments. Upon extrusion cooking the major part of the 2,3-pentanedione (83%) was formed via the intact glucose backbone.

## Introduction

Numerous studies were conducted up to date to better understand the generation of the buttery smelling 2,3-butanedione from reducing sugars. The use of labelled precursors and the introduction of the so-called Carbon Module Labeling (CAMOLA) technique have allowed to propose several formation pathways, but also to determine their relative importance [1]. The formation mechanisms were shown to be strongly affected by reaction conditions such as moisture, temperature, pH and type of amino acid [1-4]. For example, under aqueous conditions at pH 7 and 135°C, the glucose/proline model system generated 2,3-butanedione exclusively by recombination of sugar fragments, whereas at pH 5 the same precursor system generated 2,3-butanedione both from the intact glucose skeleton (about 30%) and by recombination of sugar fragments (70%). Similar to 2,3-butandione, the generation of 2,3-pentanedione was shown to proceed via several mechanisms, e.g. from intact skeleton, recombination of sugar fragments (both of $C_4/C_1$ and $C_3/C_2$) or by alanine-mediated chain elongation of methylglyoxal [3-6]. Nevertheless, the impact of reaction conditions on the importance of the individual pathways is much less understood as compared to 2,3-butanedione.

The aim of this study was to better understand the impact of reaction conditions on the formation of 2,3-pentandione in model systems containing glucose and glycine or proline and to compare the results with those obtained for extruded cereals.

## Experimental

### *Materials*

The following chemicals were commercially available: D-glucose, glycine, L-proline, 2,3-butanedione, 2,3-pentanedione, monosodium dihydrogenphosphate anhydrous, disodium hydrogenphosphate dihydrate, trisodium phosphate, sodium

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sulphate anhydrous (Sigma-Aldrich, Buchs, Switzerland); [U-$^{13}C_6$]-glucose (Cambridge Isotope Laboratories, Inc., Andover, USA); [$^{13}C_4$]-2,3-butanedione, [$^{13}C_2$]-2,3-pentanedione, (Aroma Lab, Planegg, Germany).

*Aqueous systems*

Amino acid (either glycine or proline; 0.1 mmol each) and a 1:1 mixture of [$^{12}C_6$]-glucose (0.15 mmol) and [U-$^{13}C_6$]-glucose (0.15 mmol) were placed in a 20 mL headspace vial and dissolved in phosphate buffer (1 mL; 0.5 M; pH 5, 7 or 9). Vials were sealed with a crimp cap and heated in a silicon oil bath at 135 °C for 20 min. After cooling down with ice water, anhydrous sodium sulphate (2 g) was added, the vials were vortexed, and directly analysed by HS-SPME GCxGC-TOFMS.

*Dry systems*

Mixtures were prepared as described for aqueous systems, however the samples were freeze dried prior to heating (135 °C for 20 min). After cooling down with ice, the mixtures were dissolved in water (1g), anhydrous sodium sulphate (2 g) was added, the vials were vortexed, and directly analysed by HS-SPME GCxGC-TOFMS.

*Extrusion trials*

The extrusion trials were performed on a twin-screw extruder BC-21 (Clextral, France) using a model rice recipe. Rice flour was spiked with glycine (0.05 mol/kg) and a 1:1 mixture of [$^{12}C_6$]-glucose (0.075 mol/kg) and [U-$^{13}C_6$]-glucose (0.075 mol/kg) and extruded under moderate extrusion conditions (135 °C, 20% moisture, 400 rpm). The extruded products were dried in an Aerotherm oven (Wiesheu, Germany) at 120 °C for 6 min.

*Gas-Chromatography-Mass spectrometry*

The samples were analysed by HeadSpace Solid Phase Micro-Extraction in combination with 2D Gas Chromatography-Time-of-Flight-Mass Spectrometry (HS-SPME-GCxGC-TOFMS) as described previously [2]. The contribution of individual reaction pathways to the formation of 2,3-pentanedione was calculated from the relative distribution of the isotopologues. All results were corrected for the $^{13}C$ content of the natural isotope. The obtained percentage after correction <0.5% was set to 0% by definition.

## Results and discussion

The formation of 2,3-pentanedione from hexoses has been shown to proceed via several pathways including recombination of fragments as well as formation from the intact sugar skeleton [3-6]. The impact of reaction conditions was studied in model systems containing equimolar mixtures of unlabelled and $^{13}C_6$-labelled glucose (CAMOLA approach) in the presence of glycine or proline. The relative importance of the individual pathways generating 2,3-pentanedione in glucose/proline systems under different reaction conditions is shown on Figure 1.

Under aqueous conditions, the importance of individual pathways depended on the pH of the reaction mixture. While the formation from the intact glucose skeleton was the major pathway contributing to 2,3-pentanedione at pH 5 (60%), this pathway was not active at pH 7 and pH 9. Under neutral and alkaline aqueous conditions, 2,3-pentanedione was exclusively formed by recombination of glucose fragments. The recombination of $C_3/C_2$ fragments (e.g. 1-hydroxypropanone and acetaldehyde as proposed by Hofmann [5]) was the major pathway (72 % to 74%) while the recombination of $C_4/C_1$ fragments (e.g. 2,3-butanedione and formaldehyde as proposed by Weenen [4]) contributed to about

one quarter of the 2,3-pentanedione formed (24% to 28%). Contrary to aqueous conditions, under dry heating the formation of 2,3-pentanedione was almost independent of the pH value. The majority of the compound was formed from the intact sugar skeleton (72% to 82%) followed by recombination of $C_3/C_2$ fragments (12% to 18%). The recombination of the $C_4/C_1$ fragments contributed only marginally under dry heating conditions (6% to 8%).

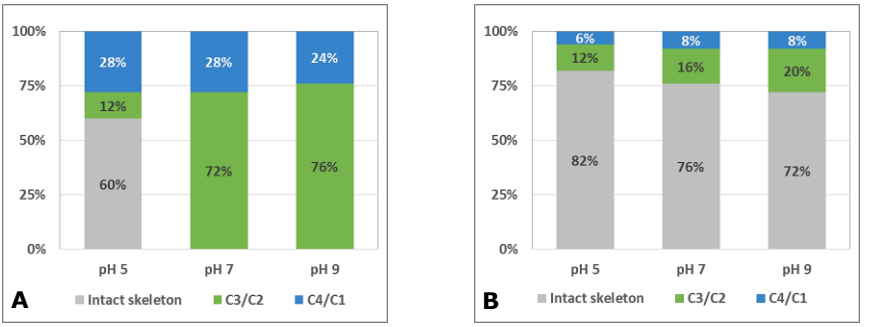


**Figure 1:** Relative contribution of different pathways generating 2,3-pentanedione in glucose/proline model systems under aqueous (A) and dry (B) heating conditions as calculated from the isotopologue distribution of CAMOLA experiments

In the presence of glycine, the contribution of the individual pathways to 2,3-pentanedione was different as compared to the system containing proline (Figure 2). In general, the contribution of the intact skeleton was more pronounced in the system containing glycine. The formation from the intact sugar skeleton was the major pathway generating 2,3-pentanedione at pH 5 (86%). The formation through recombination of sugar fragments was very limited at pH 5, however the importance of these pathways strongly increased with pH. At pH 9 the majority of 2,3-pentanedione was formed by recombination of $C_3/C_2$ fragments (62%), followed by recombination of $C_4/C_1$ fragments (24%). The presence of glycine, as compared to proline, triggered also limited formation of 2,3-pentanedione by recombination of $C_4$ sugar fragment and $C_1$ glycine fragment (most probably formaldehyde, the Strecker aldehyde of glycine). The importance of the latter pathway slightly increased with the pH of the aqueous system, but remained marginal.

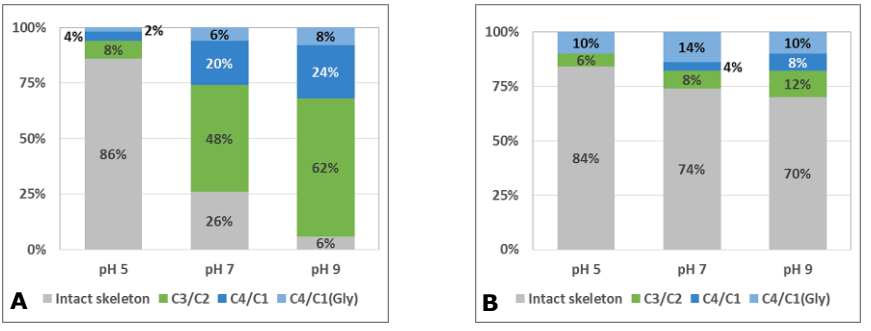


**Figure 2:** Relative contribution of different pathways generating 2,3-pentanedione in glucose/glycine model systems under aqueous (A) and dry (B) heating conditions as calculated from the isotopologue distribution of CAMOLA experiments

Under dry heating condition, the results obtained in the glucose/glycine system were quite similar to that obtained in the glucose/proline system. Irrespectively of the pH, the

majority of the 2,3-pentanedione has been formed from intact glucose backbone. The generation of 2,3-pentanedione through recombination of $C_3/C_2$ and $C_4/C_1$ sugar fragments was slightly less important in the systems containing glycine as compared to those containing proline, in favour of generation via recombination of the $C_4$ sugar fragment and $C_1$ glycine fragment. Indeed, at pH 5 and pH 7, the dry heating system seems to produce more easily the $C_1$ fragment (formaldehyde) from glycine than from glucose, which is not the case for pH 9 where generation of the $C_1$ fragment from both precursors was comparable. In contrast, under aqueous conditions the generation of the $C_1$ fragment from glucose was favoured over the generation from glycine, irrespectively to pH value.

The relative contribution of different pathways generating 2,3-pentanedione from glucose in the presence of glycine under extrusion cooking is shown in Figure 3. The results indicate that upon extrusion cooking, the major part of the 2,3-pentanedione originates from the added precursors and only a small part (about 7%) is formed from the inherent precursors of rice flour. The majority of 2,3-pentanedione (83%) that originated from added precursors was generated from the intact glucose backbone. The recombination of $C_3/C_2$ sugar fragments contributed to only about 11% and recombination of $C_4$ sugar fragment and $C_1$ glycine fragment to the remaining 6% of 2,3-pentanedione. Under extrusion conditions, the generation of the $C_1$ fragment from glycine is favoured over the generation from glucose, indicating that extruded systems seem to behave more like dry systems than aqueous systems.

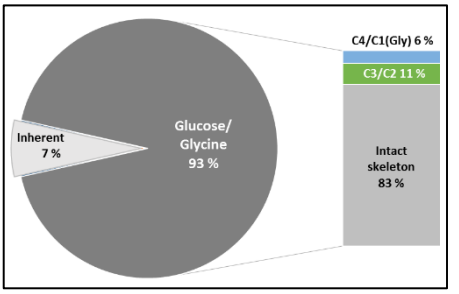


**Figure 3:** Relative contribution of different pathways generating 2,3-pentanedione upon extrusion cooking from added precursors (glucose/glycine) and inherent precursors of rice as calculated from the isotopologue distribution of CAMOLA experiments

In conclusion, the generation of 2,3-pentanedione from glucose strongly depends on the reaction conditions as well as on the type of co-reacting amino acids. Therefore, extrapolation of the results from models systems to food systems must be done with caution and should be validated by experiments using authentic food systems.

## References

1. Schieberle, P. Ann N.Y. Acad. Sci., 2005, 1043, 236-248
2. Blank, I.; Novotny, O.; Davidek, T., In Advances and Challenges in Flavor Chemistry & Biology; Hofmann, T., Meyerhof, W., Schieberle, P., Eds.; Deutsche Forschungsanstalt für Lebensmittelchemie: Freising, Germany, 2011; pp 161−166.
3. Yaylayan, V. A.; Keyhani, A. J. Agric. Food Chem. 1999, 47, 3280–3284.
4. Weenen, H. Food Chem. 1998, 62, 393−401.
5. Hofmann, T. Dissertation, Technical University, Munich, Germany, 1995; pp 118-119.
6. Poisson, L. ; Auzanneau, N. ; Mestdagh, F. ; Blank, I. Davidek, T. J. Agric. Food Chem. DOI: 10.1021/acs.jafc.6b04849.

# Effect of several food processing methods on volatile composition of strawberry

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## Abstract

Strawberry is one of the most economically important fruit in the food industry, because it is used as an ingredient in jam, jelly, yogurt, several milk based products, ice cream, syrup, fruit juice, tea, and other processed foods. Strawberry has a unique fresh and fruity flavour with the contribution of more than 360 volatile compounds, which were widely studied by many researchers. Strawberry aroma is a complex mixture of furanones, esters, aldehydes, acids, alcohols and sulphur compounds. Besides, it is highly changed during processing. Jam making and fruit juice processing are important methods to preserve strawberry, which require heat treatment. Actually, fresh characteristic of strawberry volatiles is mostly replaced in processed strawberry by certain heat-induced volatile compounds, such as isobutyraldehyde, furan, furfural and dimethyl sulphide. In this review, change of volatile compounds of strawberry after several processes (heating, blanching, osmotic dehydration, high hydrostatic pressure, pulsed electric field etc.) is summarized.

## Introduction

Strawberry (*Fragaria* x *ananassa* Duch) has a specific, enticing aroma and is one of the most popular fruits. Strawberry is the 6$^{th}$ most eaten fresh fruit after banana, apple, orange, grape and watermelon in the world. The United States is the world's largest producer of strawberry. Turkey, Spain, Egypt, Korea, Mexico, and Poland are the next highest producing countries. Consumers prefer to purchase strawberry and its derived products for their unique sensory characteristics and nutritional value. It is commonly used as an ingredient in several food products such as jam, jelly, yogurt, several milk based products, ice cream, syrup, fruit juice, tea and other processed foods.

Strawberry is rich in phytochemicals such as phenolic acid, ellagic acid, anthocyanins, catechins, quercetin and kaempferol (and their glucosides) which make a positive impact to human health. Studies made by several researchers determined that total phenolic content of strawberry ranges from 43 to 273 mg/100 g fresh weight (FW). Total anthocyanin content was identified between 6 to 102 mg/100 g FW and total ellagic acid content was less than 84 mg/100 g FW [1]. The predominant anthocyanin in strawberries is pelargonidin 3-glucoside and it defines the red colour of the fruit [2, 3]. Some researchers determine that quercetin 3-rutinoside is the major flavonol while others indicate quercetin 3-glucoside and quercetin 3-glucuronide [4].

Strawberry has unique fresh and fruity flavour with contribution of more than 360 volatile compounds [5]. Furanones, esters, aldehydes, acids, alcohols, and sulphur compounds are the main groups, which form desired strawberry aroma [6]. Several processes such as heat treatments and storage can impact the concentrations of aroma characteristics [7, 8]. In this review, differentiation in aroma profile of strawberry after several processes and storage is summarized.

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DOI: 10.3217/978-3-85125-593-5-40, CC BY-NC-ND 4.0

**Thermal Processes**

Andujar-Ortiz et al. [9] investigated the cooked and fermented flavour in strawberry juices. They stated that, heating of juice to 60ºC and 90ºC generates cooked flavour, similar to the report of Schieberle [8]. Some of the volatile compounds are present in strawberries, such as mesifurane, methyl butanoate, hexyl acetate, and butyl butanoate [10, 11], while some of them have not previously been identified in strawberries such as α-bisabolol, (*Z, E*)-farnesol and nerol. These alcohols and γ-undecalactone found in strawberry jams [12]. Andujar-Ortiz et al. [9] also found that some chemical compounds such as α-bisabolol oxide, epoxy-linalool oxide and 1-octen-3-one were contributing to fermented flavour of strawberry mainly came from fresh fruit, while others could be formed during processing.

Heat has an important effect on aroma profile of strawberry. Sterilization treatment at 120°C for 20 min caused a significant increase in the concentration of butyl acetate, hexanal, linalool, heptan-2-one, hexen-2-al, 2-methyl propanoic acid, butanoic acid, hexanoic acid, benzene methanol, furaneol, nerolidol, octanoic acid and γ-decalatone when compared to raw strawberry [13]. Sterilization also increases the concentration of acetaldehyde [14]. However, the flower-scented strawberry flavour is lost due to heat treatment, with a significant decrease in nerolidol and furaneol concentrations. Sterilization (120°C, 20 min) also formed geraniol and vanillin [13]. Also dimethyl sulphide and isobutyl aldehyde, which are not present in raw strawberry puree, were formed. Also isobutyraldehyde may be formed by Strecker degradation of valine. 2-Furaldehyde, 2-acetyl furan and ethyl furoate were the heat generated compounds found in strawberry jam [14]. Sloan et al. [14] identified heat induced dimethyl sulfide in strawberry puree which is heated at 120°C for 30 min. It was not determined in freshly prepared puree but was noticed higher than its threshold level after heating for 10 min.

Heating affect the odour characteristics of strawberries. Sweet caramel like odour turns into a dominant odour in heated strawberries while green and fruity odours are the most desirable odour in the fresh strawberry [8]. Short thermal treatments retain volatiles which contain fruity and fresh flavours better than long thermal treatments [15]. Thermal treatments with long time and high temperature lead to caramelization as well as Maillard reaction, which cause undesired burnt and caramel flavours during strawberry jam production [16, 17].

Ozcan and Barringer [11] studied the concentration change of volatile compounds in the headspace of whole undamaged, whole punctured, and whole bruised strawberries under refrigerated storage conditions for 8 days. (*E*)-2-hexenal, (Z)-3-hexenal, hexanal, and hexanoic acid level increased in undamaged strawberries due to the continuing activity of enzymes during storage. The concentration of (*E*)-2-hexenal and hexanoic acid was significantly higher in bruised strawberries, which have severe damage, than undamaged strawberries.

Also some processes such as freezing and thawing did not change the furaneol and mesifurane level of strawberries while esters changed [18].

**Non-thermal Processes**

The traditional preservation methods require high temperatures which can destroy several food components [19]. In contrast to this, novel or non-thermal processes preserve the colour, flavour, nutritious and bioactive components of food.

Lambert et al. [13] investigated the differences in aromatic volatile composition of strawberry after high pressure treatment. Researchers applied 200 and 500 MPa for 20 min to strawberry puree and found no major difference in aromatic profile compared to untreated fruit. When they used 800 MPa for 20 min, 3,4- dimethoxy 2-methyl furan and γ-decalactone were detected as new compounds and concentration of many volatiles contributing to fresh strawberry flavour, such as nerolidol, furaneol, linalool and some esters were importantly lower in the strawberry puree compared to unprocessed sample. Also pressure processed samples did not have geraniol and vanillin which are typical volatiles originated from sterilization (120°C, 20 min.).

Esters are the most important flavour compounds, which give fruity note to strawberry. According to Lambert et al. [13], many esters remain after high pressure treatment, while other researchers did not determine any ester compounds after high pressure treatment (200, 400, 600 or 800 MPa/18-22°C/15 min) [20].

Cao et al. [21] studied the effects of high hydrostatic pressure (HHP) combined with blanching on volatile profile of cloudy and clear strawberry juices. In comparison with cloudy and clear juices, the concentration of total volatile constituents in HHP-treated (600 MPa for 0, 2, 4, and 6 min) cloudy juice increased by 13.21% while HHP-treated clear juice declined by 6.92%. The acid esters such as butanoic acid methyl ester, butanoic acid ethyl ester, and acetic acid hexyl ester decreased and the content of (*E*)-2-hexenal increased in both HHP-treated cloudy and clear juices. They also determine rise in one of the key aroma compounds of strawberry, 2,5-dimethyl-4-methoxy-3(2H)-mesifurane, 19.76 and 3.80% in HHP-treated cloudy and clear juices, respectively.

High pressure processing is also used in jam making process. Gimenez et al. [22] declared that application of 400 or 800 MPa pressure at 22°C for 5 min caused soured and lower fruity smell than conventional processed strawberry jam.

Bermejo-Prada et al. [23] investigated the effect of hyperbaric storage (0.1, 50, and 200 MPa for 15 days) at 20°C on the aroma profile of strawberry juice. They found that volatile content of samples stored under pressure were similar to beginning day. Even no changes were observed in important aroma compounds after hyperbaric storage. The study showed that hyperbaric storage was more effective than refrigeration in retaining the volatile profile of strawberry juices unchanged for 15 days.

Combined osmotic-blanching treatments were applied to strawberries for increasing shelf life and retain fresh flavour during that time [24]. When blanching step is implemented before osmotic dehydration process, volatile profile of strawberries remained like the original. Whereas, esters and furaneol promoted when blanching is performed after the osmotic process.

Geveke et al. [25] determined the effect of pilot plant pulsed electric field process (field strengths: 24.0–33.6 kV/cm, outlet temperatures: 45.0–57.5ºC and flow rate: 100L/hr) on flavour of strawberry puree. Researchers did not measure specific compound concentrations but noticed that colour and flavour of a strawberry beverage containing pulsed electric field processed purée was bright red and fresh.

Beyond the free flavour compounds, high amount of flavour compounds are aggregated as non-volatile and flavourless glycol conjugates [26]. Application of enzyme hydrolysis for clarification of juices allows freeing the volatile constituents from the attached fraction by hydrolyzing glycosides, and also reduces the consistency of the mixture by degrading pectin. This process makes possible the volatile components to deliver from the complex structure of food more easily [27].

## Conclusion

The results obtained by several studies showed that heating affects the aroma profile of strawberries while non-thermal processes preserve flavour of fruit better. If blanching process applies before osmotic dehydration process, volatile compounds of strawberries remained like fresh. Pulsed electric field process, hyperbaric storage and refrigerated storage also had minimal negative effect on fresh strawberry flavour.

## References

1. Zhao Y. (2007) Berry Fruit edited by CRC Press Taylor & Francis Group
2. Wang S.Y., Zheng W., Galletta G.J. (2002) J. Agric. Food Chem. 50: 6534-6542.
3. Aaby K., Skrede G., Wrolstad R.E. (2005) J. Agric. Food Chem. 53: 4032-4040.
4. Seeram N.P., Lee R., Scheuller H.S., Heber, D. (2006) Food Chem. 97: 1-11.
5. Latrasse A. (1991) In: Volatile compounds in foods and beverages (Maarse, H., ed.) New York, CRC Press pp. 329-410.
6. Perez A.G., Sanz C., Olias R., Rios J.J., Olias J.M. (1997) Acta Hortic. 1: 337-340.
7. Shamaila M., Powrie W.D., Skura B.J. (1992) J. Food Sci. 5: 1168–1184.
8. Schieberle P. (1994) In: Trends in Flavour Research (Marrse, H., Van der Heij, D. G., ed.) Elsevier Sciences, pp. 345−351.
9. Andujar-Ortiz I., Peppard T.L., Reineccius G. (2015) In: The Chemical Sensory Informatics of Food: Measurement, Analysis, Integration (Guthrie, B., Lavine, B.K., Beauchamp, J., Buettner, A., ed.) American Chemical Society., pp.293-312.
10. Forney C.F., Kalt W., Jordan M.A. (2000) Hort. Science 6: 1022–1026.
11. Ozcan G., Barringer S. (2011) J. Food Sci. 2: 324–333.
12. Barron D., Etievant P.X. (1990) Z. Lebansm.-Unters Forsch 191: 279–285.
13. Lambert Y., Demazeau G., Largeteau A., Bouvier, J.M. (1999) Food Chem. 67: 7-16.
14. Sloan J.L., Bills D.D., Libbey L.M. (1969) J. Agric Food Chem. 17:1370–1372.
15. Sun-Pan B., Kuo J.M., Wu C.M. (2007) In: Chemical and Functional Properties of Food Components (Sikorski, Z.E., ed.) CRC Press, pp. 295-328.
16. Viberg U. (1998) Studies of the effects of Industrial Processing of fruit, Lund: The department of Food Engineering, Lund University, 47-52
17. Coultate T.P. (2009) Food – The chemistry of its components, 5th edition, Cambridge, RSC Publishing, 2009, 30-41
18. Deuel C.L., Plotto A. (2005) In: Processing fruits (Barrett D.M., Somogyi L.P., Ramaswamy H.S. ed.) CRC Press, pp. 550-581.
19. Hayashi R. (1996). Use of high pressure in bioscience and in bio-technology. In R. Hayashi, C. Balney, (Eds.), High pressure bioscience and biotechnology (Vol. 13, pp.1±7 Amsterdam: Elsevier Applied Science.
20. Zabetakis I., Koulentianos A., Orruno, E., Boyes, I. (2000) Food Chem. 71: 51-55.
21. Cao X., Liu F., Wu J., Liao X., Hu X. (2014) Int. J. Food Prop. 17: 1900–1920.
22. Gimenez J., Kajda P., Margomenou L., Piggott J.R., Zabetakis I. (2001) J. Sci. Food Agr. 81: 1228-1234.
23. Bermejo-Prada A., Vega E., Perez-Mateos M., Otero L. (2015) LWT - Food Sci. Technol. 62: 906-914.
24. Escriche, I., Chiralt, A., Moreno, J., Serra, J.A. (2000) J. Food Sci. 65 (7): 1107-1111.
25. Geveke D.J., Aubuchon I., Zhang H.Q., Boyd G., Sites J.E., Bigley A.B.W. (2015) J. Food Eng. 166: 384–389.
26. Pogorzelski E., Wilkowska A. (2007) Flavour and Fragrance Journal 22, 251–254.
27. Li C., Hao J., Zhong H., Dang M., Xie B. (2009) J. Sci. Food Agr. 89 (14): 2405–2414.


# FLAVOUR PERCEPTION AND PSYCHOPHYSICS

# Influence of genetic variation on flavour perception and food choices

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## Abstract

In 1932, Blakeslee and Fox published detailed reports on the inability of some people to taste phenylthiocarbamide (PTC), calling this heritable trait 'tasteblindness'. In 2003, the molecular mechanism underlying this trait was finally elucidated. Over the last 15 years, molecular genetics and modern psychophysics have made it clear that this heritable dimorphism is only one of many, some of which are directly relevant to the food supply. Nor are these differences restricted to taste, as other sensory modalities involved in flavour perception also show genetic variability. Here I review some mechanisms involved in systematic variation in chemosensation across individuals, and highlight a few examples that are relevant to ingestive behavior, food choice, and consumer behavior. Other complications are also discussed.

## Introduction

Each year, the food industry spends millions of dollars formulating new products and reformulating existing products. For existing products, these efforts typically focus on either product improvement or margin improvement (a euphemism for cost cutting). In both cases, there is an implicit assumption that formulation influences the sensations arising from the food. That is, a classical psychophysical relationship is assumed: if I add more sucrose, my product will get sweeter. In turn, sensations are then assumed to affect the hedonic responses for the food. Finally, it has long and widely been accepted liking drives intake [1, 2], although more precisely, this relationship is heteroskedastic and disliking drives non-use [3, 4]. Whether implicitly or explicitly, extensive resources are deployed in research and development efforts under the assumption that a causal chain linking formulation to sensation to pleasure to use exists [5].

However, at each step along this chain, the relationship between pairs is clearly not perfect (i.e., the correlation is certainly less than one). Accordingly, the final relationship between formulation and use is highly attenuated. As a rough estimate, reasonable values of the individual correlations can be drawn from existing literature (e.g., [2, 6]). As shown in Figure 1, when these values are multiplied together, the potential correlation between formulation and use is between .47 and .12, suggesting total variance in use that can be explained by formulation is depressingly low, somewhere between 2 and 22%. Of course, this is not entirely surprising given the myriad other factors which influence food choices and use, including availability, cost, context, health concerns, prior experience, physiological state, personality, parental modelling, culture, etc. [7-9]. Yet despite this relatively weak relationship, the continued expenditure of substantial resources on formulation and reformulation suggest that despite all these other factors that influence use, the presumed chain outlined here must still have some influence on purchase and use, or the food industry would have abandoned this approach years ago.

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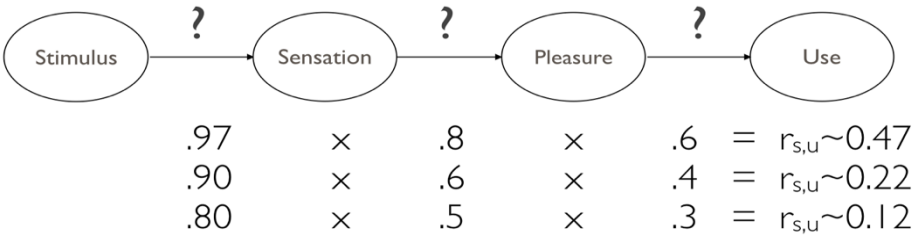


**Figure 1:** Putative causal chain linking stimulus to use, with estimated correlations between each step

At risk of oversimplification, for many decades, the food industry focused much of their work on formulation and reformulation toward producing foods that please the largest possible number of consumers. Acceptance tests are performed with demographi ically appropriate consumers and sample sizes [10] that allow for interferential statistics to be used, under the assumption that means estimated under controlled laboratory conditions generalize back to the broader population in the real world. Besides the issues inherent to simple measurement error (see discussion in [5]), this also leads to what I call *the paradox of the modern product development process*.

Specifically, sensory and market research studies typically use mean liking or acceptability to predict liking in some population, while decisions about what to eat are typically made at the level of the individual, and critically, individual vary. This is similar to the dilemma currently faced by the pharmaceutical industry, wherein clinical trials for new drugs are based on average responses while individuals differ in their responses to these drugs. There, one proposed solution is to use genetics to understand this variation (i.e., pharmacogenetics) towards a goal of personalized medicine [11]. Similarly, genetics can be used to systematically understand differences in chemosensation that may influence food choices and dietary behaviour [12]. That is, in addition the influence of formulation shown above, genetics also has the potential to influence the sensations from foods, with downstream implications for liking and use of various foods.

**Sources of genetic variation with the potential to influence chemosensation**

By some estimates, humans share about 99.9% of their genetic information. That is, given a total of ~3 billion base pairs in our DNA, on average, two randomly selected individuals will differ by about 3 million basepairs (i.e., 1 in 1000 basepairs). This variation can be broadly grouped into three categories: substitutions of individual basepairs, insertion or deletion of a string of basepairs, or structural variation. Here, I will focus on the first and third, as there is more evidence of meaningful variation in relation to flavour, with the caveat this may change in the future, as this is an active area of research.

When an individual nucleotide basepair is changed (e.g., Thymine for Cytosine), this is called a single nucleotide polymorphism, abbreviated SNP, and pronounced “snip”. Within sections of DNA known as coding regions, base pair triplets encode which specific amino acid is transcribed, so a SNP may or may not alter the resulting amino acid sequence. If the nucleotide substitution does not change which amino acid is transcribed, the SNP is termed a “synonymous SNP”, as it typically assumed that such variation does not meaningfully influence the protein structure. Conversely, a “non-synonymous SNP” results in a different amino acid being transcribed, with the potential to alter the secondary

or tertiary structure of the protein, depending in the chemical properties of the amino acid. In the case of taste or smell receptor proteins, this can affect the binding pocket, resulting in altered receptor function. Separately, SNPs also occur in so-called non-coding regions of DNA. Despite being outside the gene per se, these SNPs can also influence protein expression, as SNPs in the promoter region of a gene can influence regulatory mechanisms that control when a gene is turned on or off.

Small groups of SNPs are inherited together, meaning variation at one point in the genome may not be statistically independent from variation at another spot. Known as linkage disequilibrium (LD), this results in haplotypes, where a set of SNPs cluster within or even across genes. Critically, the existence of haplotypes can explain why robust statistical associations between SNPs and specific outcomes may still be false positives, mechanistically speaking. An example of this will be given below.

A separate source of variation with the potential to influence flavour perception comes from a type of structural variation known as a copy number variant (CNV). In a CNV, a large section of DNA, typically in excess of a kilobase (1000 basepairs), is repeated one or more times. Higher CNVs influence the level of protein that is expressed, with the downstream potential to influence flavour perception.

### *TAS2R* polymorphisms, perception and behaviour

The best known and studied example of taste variation is the ability or inability to taste a small class of structurally similar compounds that contain a thiourea moiety [13, 14]. 'Tasteblindness' to PTC was briefly described in April 1931, followed by a more detailed formal report by Fox in 1932 [13]. Almost immediately, Snyder [15] and Blakeslee [16] each replicated Fox's initial finding, and more critically, showed that this dimorphism was heritable. In 1932, Blakeslee and Fox conducted a 'Taste Exhibit' at the American Association for the Advancement of Science meeting held in New Orleans in December 1931 and January 1932 [17], where they noted that "*Thomas Jefferson said all men are created equal, but he had not tried [phenylthiocarbamide] crystals. Taste tests show people are different. Our world is what our senses tell us. Each [of us] lives in a different world*." However, PTC is synthetic, so why would we have evolved the ability to taste it? In 1950, Boyd [18] concluded this ability must have evolved to protect us from natural anti-thyroid toxins found in plants, like 5-vinyloxazolidine-2-thione. (Interested readers should see [19] and [20] for more on early work in this area).

The ability to taste PTC and related compounds, like 6-n-propylthiouracil (PROP), is due to SNPs in the *TAS2R38* bitter receptor gene (HGCN: 9584). Three SNPs result in amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) that alter receptor function [21, 22]. In Americans of European ancestry, the minor allele frequency of the Pro49Ala SNP is high (~.43), and the linkage disequilibrium (LD) with the other SNPs is strong, resulting in 2 common (PAV and AVI) and 4 (AAI, AAV, PAI, PVI) rare haplotypes. Diplotypes are roughly balanced between the common haplotype homozygotes (25% AVI/AVI, and 23% PAV/PAV), with proportionally more AVI/PAV heterozygotes (43%). The balance (~9%) have rare diplotypes. Of the 2 common haplotypes, the PAV variant associates with greater suprathreshold bitterness and lower (more sensitive) thresholds, while the AVI variant associates with less bitterness and higher thresholds (e.g., [23, 24]); the rare haplotypes show intermediate phenotypes [25].

Despite PTC and PROP being synthetic compounds not found in nature or the food supply, tasteblindness is not merely an academic curiosity. As presaged by Boyd's speculation, responses to 5-vinyloxazolidine-2-thione (i.e., goitrin), show the same (but

weaker) patterns of responses as PTC and PROP for PAV homozygotes, heterozygotes and AVI homozygotes [26]. Indeed, recent work on *TAS2R38* variation and vegetable intake is highly consistent with earlier work associating PROP phenotype with vegetable intake (e.g., [27, 28]). Specifically, PAV carriers report more bitterness from vegetables [29, 30], lower liking [30], and thus less intake [31], in general agreement with the model in Figure 1. Work by Duffy et al. [31] suggests these effects are not small: AVI/AVI homozygotes (i.e., those who experience the least bitterness) reported eating vegetables much more frequently than heterozygotes or PAV homozygotes (roughly 700 versus 400 times per year). Also, these findings seem to be robust, as similar effects on intake have also been reported in Italians [32], Brazilians [33], and Finns [34].

The influence of *TAS2R38* variants on diet is not limited to vegetables, as multiple studies show an association with alcohol use. Using a quantity-frequency measure in non-dependent European-Americans, Duffy et al. found PAV homozygotes drank less than heterozygotes, who drank less than AVI homozygotes [35]. Hayes et al. replicated this for both drinking occasions and total intake [36]. In older white mostly male cancer patients, *TAS2R38* SNPs associated with drinking frequency and heavy drinking frequency, but not drinks per drinking day [37]. In Mexicans, drinker status associated with the *TAS2R38* Pro49Ala and Ala262Val SNPs [38]. In older Australians undergoing colonoscopy, intake associated with *TAS2R38* Pro49Ala SNP (although effects varied by gender and beverage type) [39]. Again, consistent with the model in Figure 1, these associations appear to be mediated via differences in bitterness [40] and liking [41].

Critically, *TAS2R38* is only one of 25 different bitter receptor genes in humans, and several others also appear to have functional polymorphisms that potentially influence ingestive behavior and food choices. For example, *TAS2R31* (formerly *TAS2R44* before being renamed; HGNC: 19113) is activated by numerous ligands including the plant derived compound aloin and the sweeteners saccharin and acesulfame potassium (aceK). SNPs in *TAS2R31* alter receptor function and associate with differences in the bitterness from saccharin and aceK [42, 43]. As would be expected, this SNP also associates with differential liking of aceK [44]; whether it also predicts differences in use of aceK or saccharin containing products is currently unknown, at least in the open literature. Separately, yet another variant, the Val96Leu SNP in *TAS2R4* (HGNC: 14911), associates with differential bitterness of the non-nutritive sweetener stevioside [45].

Finally, it should be noted that all these SNPs and haplotypes are unrelated and independent of each other. That is, the bitterness of PROP is unrelated to the bitterness of saccharin or aceK [43, 46], and the bitterness of the sweetener aceK does not predict the bitterness of stevia derived sweeteners like rebaudioside A [47]. This highlights that being sensitive to bitterness is not a monolithic trait where an individual is universally a sensitive or insensitive responder. Indeed, back in 1932, Blakeslee and Fox [17] noted “a person may be an acute taster for one kind of bitter but a poor taster for another.”

**Odorant receptor variation, food sensations, and affective responses**

Like taste receptors, odor receptors are G-protein coupled receptors (GPCRs) that bind ligands, initiating the signal cascade we eventually perceive as a sensation. And like taste receptor genes, genetic variants have the ability to alter sensation. The observation that individuals are smell blind to specific odorants is not new, as Amoore first described what he called specific anosmia a half century ago [48]. However, unlike taste, direct evidence of the influence on food liking and intake is much more sparse. The best example to date is the meat defect known as boar taint. Androstenone is a hormone

produced in the testicles of male pigs, and this steroid can be found in adipose tissue. Notably, not all humans can smell this compound, but those who do describe it as having a sweaty / urine-like character. In humans, the *OR7D4* gene contains multiple SNPs, two of which (R88W and T113M) are in very strong LD, resulting two common haplotypes: RT and WM. When the RT/RT homozygotes are compared to RT/WM heterozygotes, or the WM/WM homoyzygotes, they report more intense as well as less pleasant sensations from pure androstenone sniffed in a laboratory setting [49]. Notably, these effects also generalize to cooked meat samples spiked to contain varying levels of androstenone: the RT/RT individuals dislike the androstenone samples significantly more [50]. To date, there is no published data showing this variant influences food intake, but this seems highly likely, as can be attested to by anyone who has ever been served tainted pork. Other examples of genetic variants in odor receptors that may potentially influence food choice include β-ionone [51], guaiacol [52], and cilantro [53].

**Influence of genetics on texture perception**

There is some evidence that texture perception differs across people due to genetic variability, at least with respect to starch. Salivary amylase is encoded by the gene *AMY1*, and humans have between 2 and 15 copies [54]. As with other CNV (see above), this has the potential to influence the amount of protein produced. In the case of salivary amylase, those with higher copy numbers have both higher amounts of amylase and higher amylase activity [55, 56]. Because salivary amylase begins breaking down starch while it is still in the mouth, this has the potential to influence texture perception. Indeed, those with great amylase activity experience faster breakdown and greater overall changes in perceived viscosity [55]. There is no evidence that *AMY1* CNV influences food liking or intake to date, but this may change as this is an active area of research.

**Further complications: perceptual interactions and false positives**

Despite the model given above, it is not sufficient to know the stimulus concentration in the food and the genetic makeup of the consumer. Even if they could each be measured perfectly, that ignores the key role of interactions that occur centrally [57]. Mixture suppression describes the phenomenon that occurs when two qualitatively distinct stimuli are mixed: in a mixture, the intensity of each quality is lower than the intensity would have been had the same stimulus been given in isolation. For example, sweetness from sucrose suppresses the bitterness from caffeine; the reverse is also true, although the effect is smaller [58]. This asymmetry is consistent across studies [59, 60], meaning that sweetness reduces bitterness more than bitterness reduces sweetness. Critically, such interactions can influence liking in non-intuitive ways: bitterness is normally aversive, but adding small amounts of quinine to concentrated sucrose can actually increase pleasantness ratings, due to mixture suppression [59]. Nor are such effects limited to model systems. Grapefruit juice is both sweet and bitter. Accordingly, when *TAS2R* variants cause some individuals to experience more bitterness from grapefruit juice, they also tend to experience less sweetness, presumably due to mixture suppression; as expected, more bitterness and less sweetness lead to lower liking [36].

Additional complications come from false positives that can arise from haplotypes within and across genes. For example, multiple studies have consistently suggested the Arg299Cys SNP in *TAS2R19* (neé *TAS2R48;* HGNC: 19108) predicts the bitterness of quinine and grapefruit juice [36, 61] and liking of grapefruit juice [3, 36]. Critically however, newer data show the *TAS2R19* Arg299Cys SNP is in strong LD with *TAS2R31*

SNPs, which also predict grapefruit liking and quinine bitterness [62]. As the major bitter constituents from grapefruit juice fail to activate hT2R19 receptors in vitro, this suggests prior findings for *TAS2R19* were false positives, at least mechanistically.

## Conclusions

Flavour is, ultimately, a perceptual construct that occurs within a human, so it must be studied interdisciplinarily using multiple levels of analysis. There is a causal chain from stimulus to food intake, via sensation and affect, even if we only focus on one narrow part of this chain within our own research. Biologically driven differences in perception are very common, and exist for taste, smell, and texture. This ubiquity also implies that past work with very low numbers of observers need to be interpreted cautiously. Further work is needed to better understand how flavour drives food choices.

## References

1. Schutz, H.G., Preference ratings as predictors of food consumption. Am. Psychologist, 1957. 12(7): p. 412.
2. Tuorila, H., et al., Comparison of affective rating scales and their relationship to variables reflecting food consumption,. Food Quality and Preference,, 2008.
3. Duffy, V.B., et al., Surveying Food/Beverage Liking: A Tool for Epidemiological Studies to Connect Chemosensation with Health Outcomes. Ann NY Acad Sci, 2009. 1170: p. 558-568.
4. Randall, E. and D. Sanjur, Food preferences – their conceptualization and relationship to consumption. Ecol Food Nutr, 1981. 11: p. 151–161.
5. Hayes, J.E., Measuring sensory perception in relation to consumer behavior, in Rapid Sensory Profiling Techniques, J. Delarue, J.B. Lawlor, and M. Rogeaux, Editors. 2015, Woodhead Publishing. p. 53-69.
6. Li, B., J.E. Hayes, and G.R. Ziegler, Interpreting consumer preferences: Physicohedonic and psychohedonic models yield different information in a coffee-flavored dairy beverage. Food Quality and Preference, 2014. 36(0): p. 27-32.
7. Gidding, S.S., et al., Implementing American Heart Association pediatric and adult nutrition guidelines: a scientific statement from the American Heart Association Nutrition Committee of the Council on Nutrition, Physical Activity and Metabolism, Council on Cardiovascular Disease in the Young, Council on Arteriosclerosis, Thrombosis and Vascular Biology, Council on Cardiovascular Nursing, Council on Epidemiology and Prevention, and Council for High Blood Pressure Research. Circulation, 2009. 119(8): p. 1161-75.
8. Rozin, P., The socio-cultural context of eating and food choice, in Food choice, acceptance and consumption. 1996, Springer. p. 83-104.
9. Cardello, A., The role of the human senses in food acceptance, in Food choice, acceptance, and consumption, H.L. Meiselman and H.J.H. MacFie, Editors. 1996, Blackie Academic & Professional: London ; New York.
10. Hough, G., et al., Number of consumers necessary for sensory acceptability tests. Food Quality and Preference, 2006. 17(6): p. 522-526.
11. Drew, L., Pharmacogenetics: The right drug for you. Nature, 2016. 537: p. S60.
12. Hayes, J.E., E.L. Feeney, and A.L. Allen, Do polymorphisms in chemosensory genes matter for human ingestive behavior? Food Quality and Preference, 2013. 30(2): p. 202-216.
13. Fox, A.L., The Relationship between Chemical Constitution and Taste. PNAS, 1932. 18(1): p. 115-120.
14. Barnicot, N.A., H. Harris, and H. Kalmus, Taste thresholds of further eighteen compounds and their correlation with P.T.C thresholds. Ann Eugen, 1951. 16(2): p. 119-28.
15. Snyder, L.H., Inherited Taste Deficiency. Science, 1931. 74(1910): p. 151.
16. Blakeslee, A.F., Genetics of sensory thresholds: Taste for phenyl thio carbamide. Proceedings of the National Academy of Sciences, 1932. 18: p. 120-130.

17. Blakeslee, A.F. and A.L. Fox, Our different taste worlds PTC as a demonstration of genetic differences in taste. Journal of Heredity, 1932. 23(3): p. 97-107.
18. Boyd, W.C., Taste reactions to antithyroid substances. Science, 1950. 112(2901): p. 153.
19. Guo, S.W. and D.R. Reed, The genetics of phenylthiocarbamide perception. Ann Hum Biol, 2001. 28(2): p. 111-42.
20. Wooding, S., Phenylthiocarbamide: a 75-year adventure in genetics and natural selection. Genetics, 2006. 172(4): p. 2015-23.
21. Kim, U.K., et al., Positional cloning of the human quantitative trait locus underlying taste sensitivity to phenylthiocarbamide. Science, 2003. 299(5610): p. 1221-5.
22. Bufe, B., et al., The molecular basis of individual differences in phenylthiocarbamide and propylthiouracil bitterness perception. Curr Biol, 2005. 15(4): p. 322-7.
23. Hayes, J.E., et al., Supertasting and PROP Bitterness Depends on More Than the TAS2R38 Gene. Chem Senses, 2008. 33(3): p. 255-265. Epub 21 January 2008, doi:10.1093/chemse/bjm084.
24. Garneau, N.L., et al., Crowdsourcing taste research: genetic and phenotypic predictors of bitter taste perception as a model. Frontiers in Integrative Neuroscience, 2014. 8: p. 33.
25. Boxer, E.E. and N.L. Garneau, Rare haplotypes of the gene TAS2R38 confer bitter taste sensitivity in humans. Springerplus, 2015. 4: p. 505.
26. Wooding, S., et al., Genetics and Bitter Taste Responses to Goitrin, a Plant Toxin Found in Vegetables. Chem Senses, 2010.
27. Keller, K.L., et al., Genetic taste sensitivity to 6-n-propylthiouracil influences food preference and reported intake in preschool children. Appetite, 2002. 38(1): p. 3-12.
28. Dinehart, M.E., et al., Bitter taste markers explain variability in vegetable sweetness, bitterness, and intake. Physiol Behav, 2006. 87(2): p. 304-13.
29. Sandell, M.A. and P.A. Breslin, Variability in a taste-receptor gene determines whether we taste toxins in food. Curr Biol, 2006. 16(18): p. R792-4.
30. Shen, Y., O.B. Kennedy, and L. Methven, Exploring the effects of genotypical and phenotypical variations in bitter taste sensitivity on perception, liking and intake of brassica vegetables in the UK. Food Quality and Preference, 2016. In press.
31. Duffy, V.B., et al., Vegetable Intake in College-Aged Adults Is Explained by Oral Sensory Phenotypes and TAS2R38 Genotype. Chemosensory Perception, 2010. 3(3): p. 137-148.
32. Sacerdote, C., et al., Lactase persistence and bitter taste response: instrumental variables and mendelian randomization in epidemiologic studies of dietary factors and cancer risk. Am J Epidemiol, 2007. 166(5): p. 576-81.
33. Colares-Bento, F.C., et al., Implication of the G145C polymorphism (rs713598) of the TAS2r38 gene on food consumption by Brazilian older women. Arch Gerontol Geriatr, 2012. 54(2): p. e13-8.
34. Sandell, M., et al., Genetic variation in the hTAS2R38 taste receptor and food consumption among Finnish adults. Genes Nutr, 2014. 9(6): p. 433.
35. Duffy, V.B., et al., Bitter receptor gene (TAS2R38), 6-n-propylthiouracil (PROP) bitterness and alcohol intake. Alcohol Clin Exp Res, 2004. 28(11): p. 1629-37.
36. Hayes, J.E., et al., Allelic Variation in TAS2R Bitter Receptor Genes Associates with Variation in Sensations from and Ingestive Behaviors toward Common Bitter Beverages in Adults. Chem Senses, 2011. 36(3): p. 311-9.
37. Dotson, C.D., et al., Variation in the Gene TAS2R13 is Associated with Differences in Alcohol Consumption in Patients with Head and Neck Cancer. Chem Senses, 2012. 37(8): p. 737-44.
38. Ramos-Lopez, O., et al., Association of a novel TAS2R38 haplotype with alcohol intake among Mexican-Mestizo population. Ann Hepatol, 2015. 14(5): p. 729-34.
39. Beckett, E.L., et al., A potential sex dimorphism in the relationship between bitter taste and alcohol consumption. Food & Function, 2017.
40. Nolden, A.A., J.E. McGeary, and J.E. Hayes, Differential bitterness in capsaicin, piperine, and ethanol associates with polymorphisms in multiple bitter taste receptor genes. Physiol Behav, 2016. 156: p. 117-27.

41. Hayes, J.E. and A.A. Nolden. Biologically driven differences in sensation: implications for the wine industry. in Proceedings of the 16th Australian Wine Industry Technical Conference: Adelaide, South Australia 24-28 July 2016,. 2017. Adelaide, SA: Australian Wine Industry Technical Conference Inc.
42. Roudnitzky, N., et al., Genomic, genetic and functional dissection of bitter taste responses to artificial sweeteners. Hum Mol Genet, 2011. 20(17): p. 3437-49.
43. Allen, A.L., et al., Bitterness of the Non-nutritive Sweetener Acesulfame Potassium Varies With Polymorphisms in TAS2R9 and TAS2R31. Chemical Senses, 2013. 38(5): p. 379-89.
44. Bobowski, N., D.R. Reed, and J.A. Mennella, Variation in the TAS2R31 bitter taste receptor gene relates to liking for the nonnutritive sweetener Acesulfame-K among children and adults. Sci Rep, 2016. 6: p. 39135.
45. Risso, D., et al., Genetic signature of differential sensitivity to stevioside in the Italian population. Genes Nutr, 2014. 9(3): p. 401.
46. Horne, J., et al., Bitter taste of saccharin and acesulfame-k. Chem Senses, 2002. 27(1): p. 31-8.
47. Allen, A.L., J.E. McGeary, and J.E. Hayes, Rebaudioside A and Rebaudioside D bitterness do not covary with Acesulfame K bitterness or polymorphisms in TAS2R9 and TAS2R31. Chemosens Percept, 2013. 6(3).
48. Whissell-Buechy, D. and J.E. Amoore, Odour-blindness to musk: simple recessive inheritance. Nature, 1973. 242(5395): p. 271-3.
49. Keller, A., et al., Genetic variation in a human odorant receptor alters odour perception. Nature, 2007. 449(7161): p. 468-72.
50. Lunde, K., et al., Genetic variation of an odorant receptor OR7D4 and sensory perception of cooked meat containing androstenone. PLoS One, 2012. 7(5): p. e35259.
51. Jaeger, S.R., et al., A mendelian trait for olfactory sensitivity affects odor experience and food selection. Curr Biol, 2013. 23(16): p. 1601-5.
52. Mainland, J.D., et al., The missense of smell: functional variability in the human odorant receptor repertoire. Nat Neurosci, 2014. 17(1): p. 114-20.
53. Eriksson, N., et al., A genetic variant near olfactory receptor genes influences cilantro preference. Flavour, 2012. 1(1): p. 22.
54. Perry, G.H., et al., Diet and the evolution of human amylase gene copy number variation. Nat Genet, 2007. 39(10): p. 1256-60.
55. Mandel, A.L., et al., Individual differences in AMY1 gene copy number, salivary alpha-amylase levels, and the perception of oral starch. PLoS One, 2010. 5(10): p. e13352.
56. Yang, Z.-M., et al., The roles of AMY1 copies and protein expression in human salivary α-amylase activity. Physiology & Behavior, 2015. 138: p. 173-178.
57. Lawless, H.T., Evidence for neural inhibition in bittersweet taste mixtures. Journal of Comparative & Physiological Psychology, 1979. 93(3): p. 538-547.
58. Kamen, J.M., et al., Interactions of suprathreshold taste stimuli. Journal of Experimental Psychology, 1961. 62(4): p. 348-356.
59. Lawless, H.T., The pleasantness of mixtures in taste and olfaction. Sensory processes, 1977. 1(3): p. 227.
60. Green, B.G., et al., Taste mixture interactions: suppression, additivity, and the predominance of sweetness. Physiol Behav, 2010. 101(5): p. 731-7.
61. Reed, D.R., et al., The perception of quinine taste intensity is associated with common genetic variants in a bitter receptor cluster on chromosome 12. Hum Mol Genet, 2010.
62. Hayes, J.E., et al., Quinine Bitterness and Grapefruit Liking Associate with Allelic Variants in TAS2R31. Chem Senses, 2015. 40(6): p. 437-43.

# Investigating perception and liking of non-nutritive sweeteners in individuals representing different taste receptor genotypes

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## Abstract

This study investigates whether variations in taste receptor genotypes account for differences in perception and liking of the non-nutritive sweeteners sucralose and Rebaudioside A (RebA). Single nucleotide polymorphisms (SNPs) of sweet taste receptor subunits *TAS1R2* and *TAS1R3* (8 SNPs), bitter taste receptors *TAS2R4* and *TAS2R14* (2 SNPs), and carbonic anhydrase 6 (*CA6*, GUSTIN) were studied. Consumer liking and perception of apple beverages varying in sucralose or RebA concentration were measured. Of the sweet receptor SNPs, *TAS1R2 rs12137730* had a significant effect on sweet perception of sucralose beverages. No sweet taste receptor SNPs had any significant effect on liking. The bitter taste receptor SNP *TAS2R4 rs2234001*, however, significantly affected bitter perception of stevia beverages; the more bitter sensitive consumers, homozygous for the GG allele, liked the RebA-sweetened drinks substantially but not significantly less than the homozygous CC group.

## Introduction

Individual differences exist in liking and perception of sucrose sweetness [1]. Consumers can be classified into 'sweet likers' and 'sweet dislikers' (SLs, SDs) [2]. SDs like sweet taste at relatively high levels; their liking of sucrose solutions decreased at around 12 % (w/v), whereas SLs continued to like sucrose at 36 % (w/v) [2]. Whether hedonic phenotypes for non-nutritive sweeteners correlate with distinct genotypes is less clear. G-protein coupled receptors responding to sweet stimuli, are T1R2 and T1R3. Several SNPs in *TAS1R2* and *TAS1R3* genes have been investigated, focusing on either sweet perception or carbohydrate intake. One study of *TAS1R2* SNPs found *rs12033832* was significantly associated with sucrose taste thresholds and sugar intake, yet cofounded by the body mass index (BMI) [3]. A study of *TAS1R3* found correlations between sucrose sensitivity and two SNPs, *rs307355* and *rs35744813*, where in both cases individuals carrying the T allele were less sensitive to sucrose [4]. *TAS1R3 rs35744813* has been reported to impact on a preference for sucrose concentrations [5]. Regarding diet, *TAS1R2 rs35874116* has been shown to influence carbohydrate intake [6]. Two dental studies found that *TAS1R2 rs3935570, rs35874116*, and *rs307355* are related to dental caries risk [7, 8]. Finally, the CA6 gene is linked to taste cell proliferation; SNP rs2274333 A allele carriers have been shown to have produced more taste cells [9]. There is a lack of research into genotype/phenotype associations and non-nutritive sweeteners. Sucralose is a widely used artificial sweetener, whereas steviol glycosides (SGs), such as RebA, are natural non-nutritive sweeteners obtained from the leaves of the Stevia rebaudiana shrub. However, SGs are also bitter due to their affinity for *TAS2R4* and *TAS2R14* receptors. SNPs *rs2234001* and *rs3741843*, of *TAS2R4* and *TAS2R14*, respectively, have been proposed to account for individual differences in bitter perception from SGs [10, 11]. This study investigates associations between receptor genotype and differences in individual liking and perception of the non-nutritive sweeteners, sucralose and stevia (RebA).

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## Experimental

*Subjects, study design and stimuli*

Participants (n=62; 11 male, 51 female, ages 18-62, non-smoking) were recruited (study number 34/16). Each participant attended two 30 min visits. In visit 1 they rated liking of beverages, had buccal samples collected and answered demographic questions. On visit 2 they rated perception of the same beverages. An Apple cordial beverage was developed containing an apple flavouring (0.017% w/v, International Flavours and Fragrances), malic acid (0.2% w/v, Sigma-Aldrich), potassium sorbate (0.02% w/v, Young's Group), sucrose (2% w/v, Silver Spoon), water (Harrogate Spa), plus the non-nutritive sweetener (sucralose, Tate and Lyle; RebA, Cargill) at varying levels (Table 1). To calculate equivalent sweetness (ES), it was estimated that sucralose and RebA were 600 and 250 times sweeter than sucrose, respectively.

**Table 1:** Concentration of sweetener added to apple cordial beverage models

| *Equivalent sweetness to sucrose (% w/v)* | *Equivalent sweetness to sucrose required from sweetener* | *Sucralose (g/L)* | *Equivalent sweetness to sucrose (% w/v)* | *Equivalent sweetness to sucrose required from sweetener* | *Reb A (g/L)* |
|---|---|---|---|---|---|
| 3 | 1 | 0.017 | 4 | 2 | 0.08 |
| 11 | 9 | 0.15 | 6 | 4 | 0.16 |
| 20 | 18 | 0.30 | 8 | 6 | 0.24 |
| 28 | 26 | 0.43 | 16 | 14 | 0.56 |
| 36 | 34 | 0.57 | 32 | 30 | 1.2 |

*Sensory methods*

The liking of samples was rated on a 9-point hedonic scale. The five sucralose-sweetened samples were presented first (monadic sequential presentation, balanced order, random code labelling and allocation), with a 30 s time delay to cleanse the palate (water, crackers) between samples. Following a 5 min break, the five RebA-sweetened samples were presented in the same manner. Perceived sweetness (all samples) and bitterness (RebA samples) were rated using the general Labelled Magnitude Scales (gLMS). Prior to sample rating, a gLMS practice session was performed where four food items ("salty crisps", "black coffee", "lemon", and "honey") were rated for their respective tastes (by recall). Testing was carried out in individual booths with artificial daylight at 23°C. Data were collected using Compusense at-hand software (Canada).

*Genotyping*

Two replicate buccal swab samples were collected per participant by rubbing a sterile swab along the inside of the cheek for 1 min. Swab heads were placed into individual tubes with Isohelix Dri-capsules and stored in a dry place at ambient temperature. Samples were sent to iDNA Genetics Ltd (Norwich, UK) for genotyping.

*Statistical analysis*

Analysis was carried out within the individual sweetener sample set. To avoid scale bias, sweet and bitter perception data were normalised using the gLMS practice data. ANOVA was used to investigate liking and taste perception depending on the sweetener concentration. Agglomerative Hierarchical Clustering (AHC) of liking data used dissimilarity (Euclidean distance) and agglomeration by Ward's method. A chi-squared test of independence determined associations between receptor genotypes. Due to the high number of significant associations, subsequent analysis by ANOVA was performed

for each SNP independently. Liking and taste perception were reanalysed fitting sample, genotype and interaction. Using Bonferroni correction for multiple testing, a significance of the main effect was assumed at $p<0.001$. Trends at $p<0.05$ were discussed due to the small sample size. Multiple pairwise comparisons used Tukey HSD ($p<0.05$). XLSTAT software (Paris, France) was used for all statistical data analysis. Error bars on all figures represent standard error of the mean.

## Results and discussion

### *Population genotype*

Genotypes for the receptor SNPs examined are given in Table 2. Proportion of participants with the minor allele types was similar to those reported in the literature, except for CA6 *rs227433,* where the proportion of the GG genotype was much lower (5%) as compared to previous literature (21%) [9].

**Table 2**: Distribution of receptor genotypes within the study population

| *Cat.* | *Receptor Gene* | *SNP* | *Allele Frequency* | *Homozy. wild type n (%)* | *Heterozy. type n (%)* | *Homozy. polymorphic type n (%)* |
|---|---|---|---|---|---|---|
| s | *TAS1R2* | *rs35874116* | T > C | 36 (58) | 23 (37) | 3 (5) |
| s | *TAS1R2* | *rs12033832* | G > A | 27 (43) | 29 (47) | 6 (10) |
| s | *TAS1R2* | *rs12137730* | A > C | 30 (48) | 25 (40) | 7 (12) |
| s | *TAS1R2* | *rs4920566* | G > A | 16 (26) | 32 (52) | 14 (22) |
| s | *TAS1R2* | *rs3935570* | G > T | 31 (50) | 30 (48) | 1 (2) |
| s | *TAS1R2* | *rs4920564* | G > T | 32 (52) | 24 (39) | 6 (9) |
| s | *TAS1R3* | *rs307355* | C > T | 42 (68) | 14 (23) | 6 (9) |
| s | *TAS1R3* | *rs35744813* | C > T | 46 (74) | 10 (16) | 6 (10) |
| g | *CA6* | *rs2274333* | A > G | 33 (53) | 26 (42) | 3 (5) |
| b | *TAS2R4* | *rs2234001* | C > G | 23 (37) | 25 (40) | 14 (23) |
| b | *TAS2R14* | *rs3741843* | A > G | 45 (72) | 11 (18) | 6 (10) |

**Cat. = category; s = sweet, g = gustin, b = bitter*

### *Influence of sweet stimuli and genotype on the sweet perception*

Sweetness increased with increasing sweetener concentration as expected. Fig.1a demonstrates psychophysical relationship between perceived sweetness against stimuli concentration (log-log plot). As samples contained two different types of sweetener, and in all cases 2 % sucrose was included, the stimulus concentration is represented as ES. In the case of sucralose, the relationship for sweetness approximated a decelerating relationship (exponent 0.7), whereas for stevia the relationship is close to proportional (exponent 0.9). There was no effect of CA6 *rs2274333* on sweetness perception (data not shown). Of the 8 type-1 receptor SNPs investigated, there was only one significant association between sweetness perception of sucralose which was for *TAS1R2* SNP *rs12137730* ($p=0.0001$) (Fig. 1b), with a tendency for an effect of *rs35874116* ($p=0.011$) (data not shown). Of these two SNPs, *TAS1R2* SNP *rs12137730* also had a tendency for association with sweetness perception of stevia ($p=0.005$) (Fig. 1c). However, there was no clear link to the wild or minor allele (Fig 1b-c); consumers with the AC genotype rated sweetness higher than either homozygous group for both sweetener types. This result should be treated with caution as the CC group size was small (n=7). In the case of

*TAS1R2 rs35874116*, there was a tendency for the TT homozygotes to rate sweetness from sucralose higher than the CC homozygotes, but this effect was not replicated for stevia, and the CC group was extremely small (n=3) (data not shown). Neither of these two SNPs influenced liking.

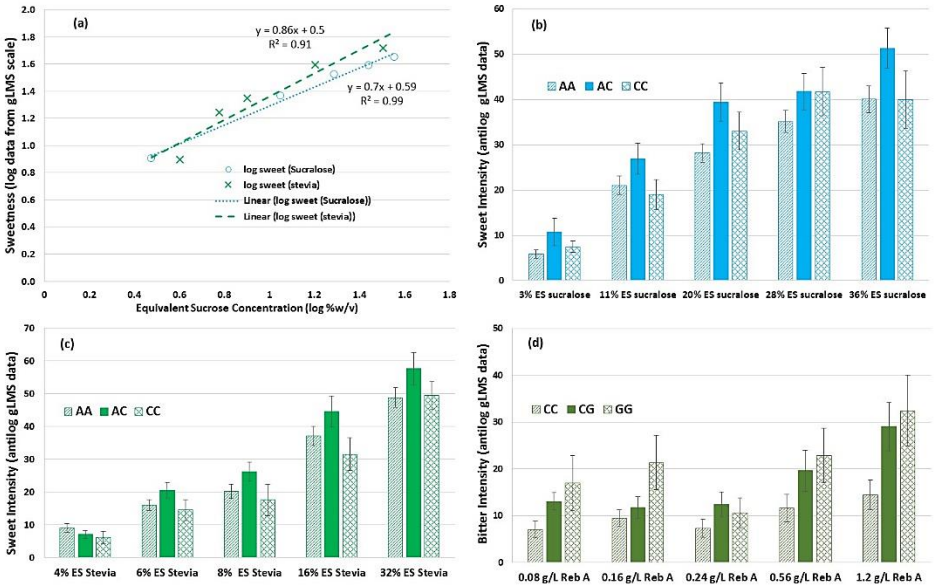


**Figure 1**: (a) Psychophysical relationship between perceived sweetness (log gLMS data) and equivalent sucrose concentration (log %w/v), (b) Sweet perception of sucralose sweetness according to *TAS1R2 r12137730* genotype, (c) Sweet perception of stevia sweetness according to *TAS1R2 r12137730* genotype, (d) Bitter perception of stevia beverages according to *TAS2R4 rs2234001* genotype. (ES = equivalent sweetness)

*Influence on genotype on bitter perception of Stevia*

In addition to sweet taste, RebA imparts bitter taste and liquorice flavour [10]. Previous studies have shown that bitterness becomes noticeable above 1000µM [10], which is equivalent to 0.97g/L, between samples 4 and 5 in the present study. The relationship between bitterness and Reb A concentration was far less than proportional (exponent 0.28 on log-log plot, data not shown), and indeed the bitterness perceived was very low until 0.56 g/L. In the present study, there was no relationship between *TAS2R14 rs3741843* and RebA bitter perception; however the influence of *TAS2R4 rs2234001* was significant ($p<0.0001$), where the homozygous GG group (n=14) rated bitterness significantly higher than the CC group ($p<0.0001$%) (Fig. 1d), as expected from previous literature [10]. In addition, the CA6 SNP *rs2274333* demonstrated a relationship that was close to significance ($p=0.003$; data not shown); the homozygous GG group tended to rate bitterness lower than the other two groups, however, there were extremely few GG consumers (n=3). Although *TAS1R3 rs307355* and *rs35744813* did not influence sweet perception, there was a trend for an effect on bitter perception ($p=0.004$ and $p=0.003$, respectively; data not shown). For both SNPs the homozygous polymorphic type (TT) rated bitterness lower than the wild type CC groups ($p=0.005$; data not shown) however, there were only 6 TT participants for each of these SNPs. These SNPs were not associated with the type 2 bitter receptor genotypes tested, therefore, there is no clear hypothesis for this trend.

*Influence on sweet stimuli and genotype on liking of Apple Cordial Samples*

Table 3 demonstrates liking of the apple beverages across the study population. With both sweetener types the mean liking increased from the first to second concentration, plateaued from the second to fourth sample, and decreased at the highest sweetener concentration. The sweetness varied from an ES of 3 to 36 % (w/v) sucrose.

**Table 3**: Mean liking of apple beverages sweetened with varying levels of sucralose or rebaudioside A (with 2% sucrose w/v). (S1 to S5 = samples 1 to 5). [abc]Values without the same letter significantly different ($p<0.05$)

| | *S1* | *S2* | *S3* | *S4* | *S5* | |
|---|---|---|---|---|---|---|
| Sucralose (g/L) | 0.017 | 0.15 | 0.3 | 0.43 | 0.57 | |
| Reb A (g/L) | 0.08 | 0.16 | 0.24 | 0.56 | 1.2 | Significance (p) |
| Sucralose | $4.3^{a}$ | $6.3^{c}$ | $6.2^{c}$ | $6.1^{bc}$ | $5.3^{b}$ | <0.0001 |
| RebA | $4.4^{ab}$ | $5.6^{c}$ | $5.6^{c}$ | $5.0^{bc}$ | $3.7^{a}$ | <0.0001 |

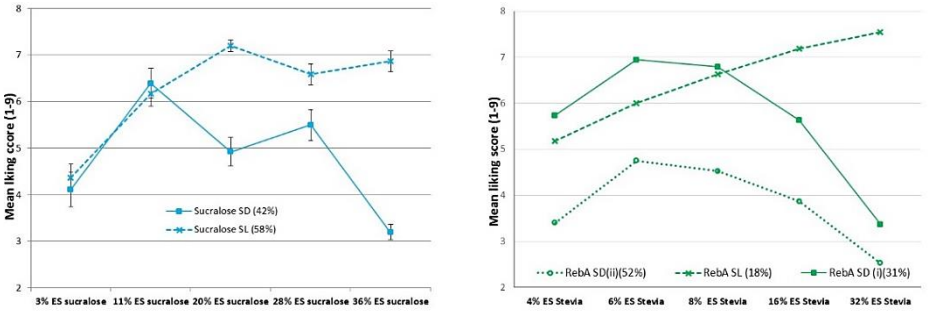


**Figure 2**: (a) Liking of sucralose beverages by consumers clustered into two distinct liking groups, (b) Liking of RebA beverages by consumers clustered into three distinct liking groups. (ES = equivalent sweetness, SD = sweet liker, SL = sweet liker)

AHC revealed two liking clusters for sucralose beverages (Fig. 2a): for the larger cluster (58%), sucralose SLs, liking reached a maximum at an ES of 19.5%, which was then maintained. The sucralose SDs reached maximum liking at 11% ES, above which liking decreased. For RebA there were 3 clusters (Fig. 2b): there was an outright RebA SL group (18%), where liking increased with increasing RebA, however, there were two SD groups. The first SDs(i) (31 %) showed a similar pattern of liking to the SLs up to an ES of 6-8%, above which their liking ratings decreased. The second SD(ii) group (52%) rated their liking at all levels of RebA lower than the other 2 groups, and again their liking for the RebA sweetened beverages decreased when ES above 8%. Considering the sweet perception of the sweet liking groups, there was no significant difference between the sucralose sweet perception between the 2 clusters ($p=0.07$). However, there was a significant difference in sweet perception for the stevia sweet liking clusters ($p=0.006$) where the SLs had lower sweet perception than both the SD(i) and SD(ii) groups ($p=0.002$ and 0.006, respectively). In addition, there was a difference in bitterness perception between these groups, where the participants that particularly disliked RebA beverages (SD(ii)) had significantly higher bitter ratings than the SD(i) group, that only disliked the higher RebA levels ($p=0.008$).

None of the sweet receptor SNPs, nor the CA6 SNP, had any significant effect on liking of either sucralose or RebA sweetened beverages at $p<0.001$. However, for RebA there were trends for two *TAS1R2* SNPs (*rs4920566* $p=0.01$; *rs12033832* $p=0.04$), the 2

*TAS1R3* SNPs (*rs307355* p=0.01, *rs35744813* p=0.008) and the *TAS2R4 rs2234001* (p=0.004). The more bitter sensitive *TAS2R4 rs2234001* GG group liked the RebA sweetened drinks substantially less than the homozygous CC group (p=0.003). However, for *TAS1R3 rs307355* and *rs35744813*, the CC groups which rated bitterness higher had a tendency to give higher mean liking scores which cannot readily be explained. For *TAS1R2 rs4920566* the trend in liking was attributed clearly to the minor allele, as the heterozygotes rated liking higher than either homozygous group. For *TAS1R2 rs12033832* the homozygotes with the minor allele (AA) rated liking higher for stevia beverages. Although they did not differ here in sweet perception, a previous study [3] found the AA group of normal BMI to have higher taste thresholds for sucrose.

In conclusion, consumers varied in their liking for sweetness of sucralose and RebA, as previously shown for sucrose. Such differences in liking were not associated with differences in their perception of sucralose. However, for stevia-sweetened beverages our study revealed that those participants with a higher liking had a lower sweet perception, and those that particularly disliked these beverages found them to be more bitter. There were a number of trends for the receptor genotypes tested to influence perception and liking of the apple beverages, however, there were only two significant differences at p<0.001: *TAS1R2 rs12137730* had a significant effect on the sweet perception of the sucralose beverages, and *TAS2R4 rs2234001* had a significant effect on the bitter perception of the stevia beverages. To reduce free sugar intake, beverage manufacturers are replacing sugar with non-nutritive sweeteners. The findings of this study may help to explain why consumers differ in their sensorial appreciation of non-nutritive sweeteners. 23% of our study sample were of the *TAS2R4 rs2234001* GG genotype, suggesting that a substantial proportion of the population may find RebA to be too bitter, which may influence their beverage choice.

## References

1. Kim, J.-Y., Prescott, J., and Kim, K.-O. (2014) Food Quality & Pref. 36:96-103.
2. Methven, L., Xiao, C., Cai, M., and Prescott, J. (2016) Food Quality & Pref. 52:74-80.
3. Dias, A.G., Eny, K.M., Cockburn, M., Chiu, W., Nielsen, D.E., Duizer, L., and El-Sohemy, A. (2015) J Nutrigenetics & Nutrigenomics. 8(2):81-90.
4. Fushan, A.A., Simons, C.T., Slack, J.P., Manichaikul, A., and Drayna, D. (2009) Current Biology. 19(15)1288-1293.
5. Mennella, J.A., Pepino, Y., and Reed, D.R. (2005) Pediatrics. 115(2):E216-E222.
6. Ramos-Lopez, O., Panduro, A., Martinez-Lopez, E., and Roman, S. (2016) Nutrients. 8(2).
7. Robino, A., Bevilacqua, L., Piratsu, N., Situlin, R., Di Lenarda, R., Gasparini, and P., Navarra, C.O., (2015) Genes & Nutrition. 10(5).
8. Haznedaroglu, E., Koldemir-Gunduz, M., Bakir-Coskun, N., Bozkus, H.M., Cagatay, P., Susleyici-Duman, B., and Metnes, A. (2015) Caries Research. 49(3):275-281.
9. Melis, M., Atzori., E., Cabras, S., Zonza, A., Calo, C., Muroni, P., Nieddu, M., Padiglia, A., Sogos, V., Tepper, B.J., and Barbarossa, I.T. (2013) Plos One. 8(9):15.
10. Hellfritsch, C., Brockhoff, A., Staehler, F., Meyerhof., W., and Hofmann, T. (2012). J Agric Food Chem. 60(27):6782-6793.
11. Risso, D., Morini, G., Pagani, L., Quagliariello, A., Giuliani, C., De Fanti, S., Sazzini, M., Luiselli. D., and Tofanelli, S. (2014) Genes & Nutrition. 9(3).

# Sugar reduction in flavoured beverages: The robustness of aroma-induced sweetness enhancement

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## Abstract

Aroma-induced sweetness enhancement (AISE) is a cross-modal perceptual interaction repeatedly demonstrated in model foods but rarely in real foods. Previously, we hypothesized that the taste of flavoured foods can be enhanced by aroma components associated with naturally occurring taste-intense versions of that food. This was proven for apple juice sweetness by adding ethyl hexanoate (HEX), an odourant synthesized in apples during ripening. Here, we investigated whether AISE persists after repeated exposure or whether humans eventually learn to discriminate between sugar- and aroma contributions to sweetness. A case series study was performed to assess the effects of sucrose feedback on the perceived sweetness of apple juice. Feedback effects were assessed by pre-test/post-test evaluations by 21 subjects of the sweetness of HEX-containing (0, 1, 2, 5 ppm) apple juices with 0% or 2% sugar added. Juices were evaluated naively and after 4 and 8 intermittent sucrose feedback sessions. Finally, subjects rated sweetness after a 35-day washout period in which no further feedback was given.

Significant enhancement of sweetness by HEX confirmed previous findings that AISE occurs with naïve subjects (HEX effect: $p < 0.001$) and is most profound at low sucrose concentrations (HEX x Sucrose effect: $p < 0.05$). Furthermore, AISE was suppressed to an extent proportional to the amount of feedback received (HEX x Feedback effect: $p < 0.001$), but recovered significantly after washout for all but the highest HEX concentration (HEX x Washout effect: $p < 0.05$). Results contradict that subjects acquired perceptual skills to distinguish between sucrose- and odour-induced sweetness. Instead, we conclude that subjects temporarily adopted the response strategy to reduce sweetness ratings with a factor proportional to the perceived intensity of the HEX odour. Results indicate the long-term applicability of AISE to reduce sugar in naturally flavoured beverages.

## Introduction

Overweight and obesity are increasing health threats in the western society [1]. In part, their incidence is attributed to a global shift in diet towards increased intake of energy-dense foods that are high in fats and sugars [2, 3]. Sweetened beverages appear to play an exceptional role in this dietary shift as drinking beverages results in higher ad-lib calorie intakes than spooning beverages [4, 5]. A recent investigation under Dutch primary school pupils substantiated the effect of liquid calorie intake on weight gain. It showed that the sugar content of fruit-based juices, consumed as single 250-mL servings during daily lunch, significantly contributes to weight gain over a period of one year [6]. Hence, lowering the sugar contents of beverages that are drunk on a daily basis may reduce overweight incidence.

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In order to reduce sugar in beverages without affecting taste, synthesized intense sweeteners are being applied extensively in spite of resistance from consumer organizations against the use of synthesized sweeteners [7, 8]. Yet, alternative sugar reduction strategies are available that do not require substitute sweeteners. First of all, consumers may adapt to a gradual reduction of sugar from all beverages. This could challenge consumer loyalty for beverages and the successful gradual reduction of sugar would therefore require broad commitment in the industry. More elegantly, compensation of sweet taste may be compensated for by aromas, as was shown for simple taste solutions [9, 10].

Reports on such aroma induced sweetness enhancement (AISE) of real foods are few. This may be due to the fact that foods have pronounced aromas already, which makes improvement more challenging than in simple aqueous taste solutions. To deal with the complexity of adjusting existing food aromas for taste enhancement, we introduced a modified approach to the AISE paradigm [11]. This approach was born from the hypothesis that, by mere exposure to many instances of foods, humans learn to associate food aromas with the taste (intensity) that it usually accompanies. If so, mimicking the aroma of sugar-rich versions of a food would raise perceived sweetness by mere suggestion through the aroma. This was confirmed by the demonstration that ethyl hexanoate (HEX), an aroma component that is synthesized simultaneously with sugars in apples during ripening [12], raises the sweetness of apple juice. Although demonstrated repeatedly [11], it is yet unclear whether this effect is robust over long-term repeated presentations to the same subjects.

In the present study, we tested the robustness of the previously demonstrated enhancement of sweetness by mimicking the aroma of sweeter versions of the same food. To that end, we monitored AISE of apple juice by HEX over repeated exposures during a 6-month period. To maximize the opportunity of panellists to learn to distinguish between the contributions of sucrose and aroma to sweet taste, explicit feedback on sucrose-calorie content was provided intermittently in dedicated sessions. To prevent that sweetness differences could only be attributed to aroma differences, two concentrations of sucrose were used in the apple juices. This was expected to aid subjects in distinguishing between AISE-induced and sweetener-induced sweetness. After repeated sucrose-feedback sessions, a wash-out period was observed during which no sucrose feedback was given. AISE is still to be considered robust if it recovers from initial suppression by sucrose feedback during this wash-out period.

## Experimental

### *Subjects*

Forty-five naïve subjects enrolled in the experiment. Of these, 26 passed tests for normogeusia and normosmia. In line with ISO guidelines on the selection of panellists (ISO 8586-1:1993) normogeusia entailed the correct identification of 9 out of 12 duplicate presentations of water and aqueous tastant solutions of sucrose, sodium chloride, citric acid, caffeine and mono-sodium glutamate. Smell acuity was assessed by the Dutch odour identification test GITU; [13], an odour recognition test using 36 common odours varying in familiarity. Test results of 18 or more correct identifications were considered normosmic. Of the 26 selected subjects, 21 completed the study (mean age 40.7 years, 7 male). Their acuity scores were 10.0 and 22.6, respectively. This panel size was considered sufficient to replicate the AISE studies by Knoop [11], involving 17 or 18 subjects.

Under the Dutch regulations, a medical-ethical evaluation was not indicated at the time of execution of this behavioural study (2009-2010). However, the application of stimulus materials in naïve panellists was medically-ethically approved by the Wageningen University medical ethical board (NL25364.081.08). The study was conducted in compliance with the Declaration of Helsinki on Medical Research Involving Human Subjects. Subjects gave written informed consent and were paid for their participation.

*Methods*

Stimuli: Stimuli were apple juices with varying contents of sucrose and additional aromas. Apple juices were prepared by diluting a commercial apple juice concentrate, low in aroma content ('medium acid', FrieslandCampina, the Netherlands) in water (Evian, Danone, France) at a concentration of 130 g/L. To this dilute, 10 ppm (vol/vol) of a commercial food-grade apple aroma (Aroma Type 'apple'; IFF, Hilversum, the Netherlands) was added. Crystalline sucrose (0 or 2 % w/w) obtained from the local supermarket and food-grade ethyl hexanoate (0, 1, 2 or 5 ppm (vol/vol); Sigma-Aldrich, Zwijndrecht, the Netherlands) were added to the apple juice in a full-factorial manner to obtain eight different apple juices.

Procedure: Thirty-ml medicups (King, Tiel, the Netherlands) were filled with 25-ml aliquots of apple juice and closed by a lid with a straw (8.0 mm o.d.) stuck through a hole of the same diameter. In this way, retronasal aroma stimulus presentation was favoured as it is assumed that AISE is optimal for retronasal aroma presentation [14]. The apple juice without added HEX or sucrose was presented as reference stimulus in transparent plastic cups of 100 ml (King, the Netherlands). These cups were sealed with Parafilm® (Pechiney Plastic Packaging, US) through which a straw was inserted. Stimuli were labelled with 3-digit codes for identification without revealing their composition.

Subjects started each session by first evaluating the reference stimulus. They could taste this reference stimulus at will throughout the experiment. Subjects then rated the sweetness of the apple juices on visual-analogue scales anchored 0 at the left extreme ('not sweet at all') and 100 at the right extreme ('very sweet'). To prevent that perceived stimulus differences due to aroma manipulation would, for lack of response alternatives, be dumped into the sweetness ratings [15], three additional attributes were evaluated in parallel to sweetness on identical scales. These attributes, 'sourness', 'apple-like aroma' and 'flowery-like aroma', were previously identified as important quality descriptors of the apple juices in the Knoop studies [11]. Reference scores for these attributes were also derived from the Knoop studies. The stimulus presentation order was individually randomized. Attribute ratings were collected with EyeQuestion software (Logic8, Elst, the Netherlands).

*Experimental design*

Subjects evaluated apple juices that varied systematically in HEX content (0, 1, 2, 5 ppm) and added sucrose content (0, 2% w/w), combined in a full-factorial manner. In addition, the amount of sucrose-content feedback given was manipulated systematically. For this, the experiment consisted of 7 phases (Figure 1) during each of which all 8 apple juices were evaluated. The first, naïve, evaluation phase (I) was in essence a replication of the study by Knoop [11] performed in two consecutive sessions. During the subsequent sucrose feedback phase (4 sessions), sucrose content information was presented to subjects during stimulus evaluation. In a subsequent informed evaluation phase (II; 2 sessions), subjects evaluated stimuli in an identical fashion as during the first stimulus

evaluation phase. Because subjects received feedback prior to this evaluation phase, they were not naïve regarding possible discrepancies between the perceived sweetness and the actual sucrose contents. This procedure was repeated in a second sucrose feedback phase (4 sessions) and a further informed evaluation phase (III; 2 sessions). After a subsequent wash-out period of 3 weeks, subjects performed another evaluation (IV; 2 sessions), which took place 5 weeks after they received the last sucrose feedback.

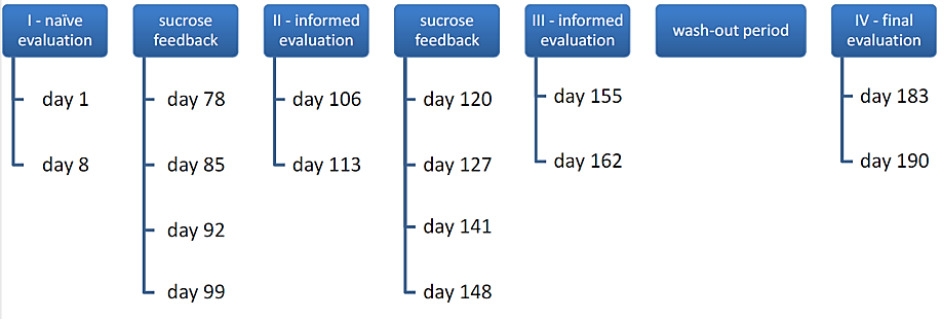


**Figure 1**: Scheduled experimental sessions in days after start. Subjects rated the sweetness of apple juices in duplicates on fixed week days under four information conditions: (I) 'naïve' regarding the actual sucrose contents, (II) after 4 evaluation sessions in which sucrose content feedback was provided, (III) after further 4 sessions in which sucrose content feedback was provided, and (IV) 5 weeks after the last exposure to sucrose content feedback.

*Data analysis*

Only sweetness ratings are evaluated since aroma and sourness attributes were merely included in the experiment to prevent dumping. The statistical evaluation of sweetness results is divided in three relevant sub-tests:

Naïve evaluation. First, to test whether the AISE results of Knoop [11] were reproduced, sweetness ratings from the (naïve) evaluation phase I were subjected to repeated-measures ANOVA, testing for main effects of HEX concentration (HEX; 0, 1, 2, 5 ppm; within-subject), added sucrose (Sucrose; 0, 2% w/w; within-subject) and Replicates (within-subject), and for the respective 2-way and 3-way interactions.

Sucrose content feedback. Second, the effect of sucrose content feedback on sweetness ratings was tested by comparing sweetness ratings for the three conditions of increasing sucrose content feedback (Feedback; 'naïve evaluation I', 'informed evaluation II' and 'informed evaluation III'). Between these evaluation conditions, the total exposure of subjects to sucrose content information was the distinguishing variable. Hence, the factors tested were Feedback (naïve, 4x feedback, 8x feedback), HEX (0, 1, 2, 5 ppm), Sucrose (0, 2% w/w) and Replicates and their mutual 2-way and 3-way interactions (all within-subject).

Recovery. Third, to test whether recovery of AISE occurred after the wash-out period, sweetness ratings collected after the wash-out period in evaluation phase IV were compared to ratings collected during evaluation phase III. The factors thus tested by ANOVA were Recovery (most-informed [III] vs. after washout [IV]), HEX, Sucrose and Replicates, along with their mutual 2-way and 3-way interactions (all within-subject).

All statistical tests were performed with Statistica (version 10, 2011; Statsoft, Inc, Tulsa, OK).

## Results and discussion

*Naïve evaluations*

For the naïve evaluations of apple juice, significant effects were observed for Sucrose [$F(1,20) = 49.1$; $p < 0.001$], HEX [$F(3,60) = 8.91$, $p < 0.001$] and the Sucrose x HEX interaction [$F(3,60) = 3.06$, $p < 0.05$]. Observed sweetness ratings (Figure 2) reflect the observation by Knoop [11] that both sucrose and HEX enhance sweetness, and that the contribution of HEX to sweetness is more pronounced at lower sucrose concentrations. No significant effects were observed for replicates or for any of the interactions involving replicates.

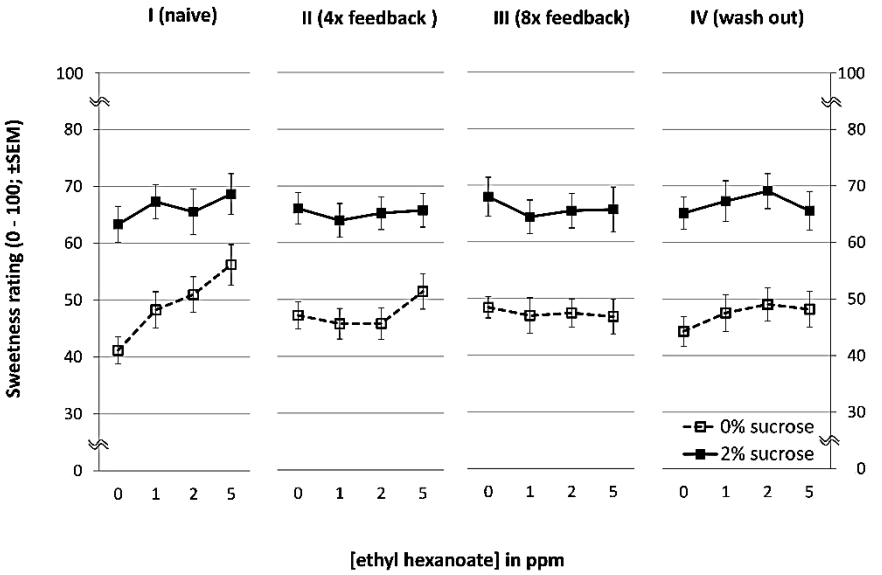


**Figure 2:** Rated sweetness as a function of ethyl hexanoate- and sucrose concentrations in apple juice under different sucrose content feedback conditions: I = no information provided, II = after 4 sucrose content feedback sessions, III = after 8 sucrose content feedback sessions and IV = after a subsequent 'wash-out' period of 5 weeks during which no sucrose content information was communicated.

*Sucrose content feedback*

Sweetness ratings were affected by Sucrose [$F(1,20) = 85.4$, $p < 0.001$], HEX [$F(3,60) = 3.81$, $p < 0.05$] and the HEX x Feedback interaction [$F(6,120) = 5.85$, $p < 0.001$]. No main effects of Feedback or Replicate or other interactions were observed. Inspection of sweetness ratings for the first three evaluation phases in Figure 2 shows that the main effect of Sucrose is due to an overall sweetness enhancement upon addition of 2% w/w sucrose. The addition of increasing concentrations HEX results in increasing sweetness ratings in the naïve evaluation setting, and to a lesser extend also in the evaluation after the first sucrose-content feedback phase, explaining the main HEX effect. The observed HEX x Feedback interaction is reflected in a gradual decrease of HEX-induced sweetness enhancement for increasing amounts of feedback on sucrose content. In fact, after two feedback blocks, no HEX-induced sweetness enhancement is observed at all (Figure 2, plot III).

*AISE recovery effects after wash-out*

Analysis of the effects of a wash-out period on AISE (evaluation IV versus evaluation III) resulted in significant effects of Sucrose [$F(1,20) = 70.7$, $p < 0.001$] and a non-significant trend for the HEX x Recovery interaction [$F(3,60) = 2.56$, $p = 0.063$]. Inspection of the interaction effect of HEX x Recovery on sweetness learns that AISE appears to be restored for the lower HEX concentrations (0, 1 and 2 ppm) but not for the highest HEX concentration (5 ppm). For the 3 lowest HEX concentrations, this comparison results in a significant HEX x Recovery interaction [$F(2,40) = 3.62$, $p = 0.036$]. No main effects of Replicate or HEX were observed, nor for any of the remaining interactions.

*Discussion*

Aroma-induced taste enhancement is a cross-modal perceptual interaction that received a fair amount of attention in the scientific literature. This interest could originate from the prospect of exploiting cross-modal effects to reduce sugars and sodium in foods. Nonetheless, only few reported on the successful application of single odourants to enhance the taste of model systems reminiscent of real foods [11, 16, 17], possibly because of the challenge to modify the existing aromas of these foods without ill effects on their quality. The aroma-induced taste enhancement paradigm proposed by Knoop [11] differs from classical cross-modal interaction approaches because it entails the balancing of selected aroma components in line with their natural occurrence in sweeter versions of the food, rather than the classical combination of singular aroma components or entire aroma mixtures with a mere taste solution. Consequently, literature reports on AISE in simple taste solutions may still be a poor indicator of the reliability and robustness of the few observations of aroma-induced taste enhancement by Knoop. Therefore, the repeated observation of AISE in apple juice by an odourant that is synthesized in sugar-rich apples provides further support for the hypothesis that AISE exists in real foods and that it relies on previously learned aroma-taste associations.

In the present study, explicit feedback on sucrose contents of stimuli elicited a profound suppression of AISE. After four sucrose feedback sessions, HEX contributions to sweetness ratings nearly disappeared and after eight feedback sessions HEX did not contribute to sweetness ratings at all. As the contributions of sucrose to sweetness remain unchanged over feedback conditions, it is expected that the feedback-induced sweetness reductions are entirely due to a changed processing of aroma information, and not to a changed processing of sweetness in general. We therefore conclude that subjects have learned to use aroma information to adjust their sweetness ratings on basis of feedback regarding the stimulus sucrose contents. The conscious exposure to calorie feedback therefore appears to mimic acceleration of the effects of repeated exposure to stimuli on the reduction of sweetness ratings.

Central to the interpretation of these results is the question whether the observed suppression of AISE after sucrose content feedback reflects a limited robustness of AISE for repeated exposure. We think that the answer lies in the mechanism involved in the observed AISE suppression. Either, subjects became unresponsive to HEX after sucrose feedback because (i) they successfully acquired the skill to perceptually isolate the aroma contribution to sweetness from the sucrose contribution, or (ii) they learned to apply the response strategy that if HEX is perceived, the perceived sweetness should be diminished with a corresponding amount. The former explanation reflects a genuine refinement of perceptual skills whereas the latter implies that only response behaviour is affected by feedback. In the latter case, a reduction of AISE after sucrose content feedback does not

unequivocally demonstrate a poor robustness of AISE because perception is overruled by the application of a response rule. Given the general long-term persistence of acquired perceptual skills and perceptual odour associations [18, 19] and the more transient nature of non-reinforced cognitive strategies in perceptual tasks [20], it is expected that response strategies will decay after discontinued feedback, whereas perceptual associations will not. If a response strategy invokes AISE suppression, AISE is expected to recover after a wash-out period during which no further feedback is given on sucrose content. Therefore, the observed AISE recovery after the final wash-out period is indicative of robust sweetness enhancement by HEX and of a response rule in decay. Further support for this interpretation is that, after the wash-out period, sweetness enhancement was restored for the two intermediate HEX concentrations (1ppm and 2ppm), but not for the highest (5ppm) HEX concentration. This suggest that AISE recovered, although partially compensated for by a remainder of the response strategy that only kicks in when the enhancing aroma becomes too apparent, i.e. at its highest concentration.

The partial recovery of AISE after a wash-out period favours an explanation in terms of robust AISE and a decaying application of a response strategy. Would AISE have recovered even more after longer wash-out periods? As the present results do not rule out a persistent partial suppression of AISE, we invited the panel for a repeated evaluation of the used stimuli, one year after the start of the experiment, to verify whether further recovery of AISE occurred. Unfortunately, panellist drop out had increased by then and the sweetness ratings collected for the remaining subgroup of panellists showed a decrease of general task performance (higher intrinsic response variation). This may reflect a general problem in longitudinal studies of multimodal perception in which a panel should be kept naïve and untrained during wash-out periods.

In general, studies on taste enhancement by factors other than the tastant concentration show that enhancement is most pronounced at lowered tastant concentrations. This is, for instance, observed for aroma-induced taste enhancement of sweet tasting stimuli [11, 21], salty stimuli [22] and the effects of salt distribution on the salty taste of bread [23]. The present study confirms this dependency, which further supports the applicability of AISE for enhancing the sweetness of beverages with reduced sugar contents.

This study is premised on the idea that by informing panellists on the amounts of sucrose in the apple juice, the worst case scenario is created for the extinction of AISE. If explicit sucrose feedback cannot forestall AISE in the long run, does this therefore really imply that AISE is robust for long-term repeated exposure? Or may, alternatively, the chosen feedback regime in the present study not have challenged AISE robustness enough? For instance, one may argue that after repeated exposure to low-sugar juices with the enriched aroma, subjects may cease to associate the aroma with sweetness and the sweetness-enhancing effect of the aroma would disappear. This scenario is improbable if perceived sweetness were the main driver for the learned aroma-taste association because a full compensation of sugar reduction by AISE would not reduce the perceived sweetness. However, sugar is not only a sweetener but also an important energy source. Pairing a particular aroma with low-energy foods may then, in the long run, invoke consumers to associate aromas with caloric content as was previously shown for rodents [24]. Attempts to replicate such controlled intake experiments in humans failed, as foods in which aromas signalled different calorie contents [25, 26] invoked no behavioural changes due to metabolic impact. With this in mind, we consider the present study a greater challenge to the robustness of AISE than mere repeated consumption. Regarding the comparison with explicit sucrose feedback in real-life, we argue that even if

consumers would spell out ingredient lists, the discrepancy between sucrose contents and the perceived taste intensity would not be as explicit as it was in this study. Nonetheless, only a longitudinal consumer study that monitors daily intake under natural conditions without revealing the focus on taste-aroma interactions, taste intensity and preference may provide a conclusive answer.

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## References

1. de Onis M., Blössner M., Borghi E. (2010) American Journal of Clinical Nutrition, 92: 1257-1264.
2. van Dam R.M., Seidell J.C. (2007) European Journal of Clinical Nutrition, 61 (1): 75-99.
3. Rosenheck R. (2008) Obesity Reviews, 9: 535-547.
4. Mattes R.D. (2005) Physiology & Behavior, 83 (5): 739-747.
5. Mattes R.D. (2006) Physiology & Behavior, 89 (1): 66-70.
6. Ruyter J.C., Olthof M.R., Seidell J.C., Katan M.B. (2012) The new England Journal of Medicine, 367: 1397-1406.
7. Marinovich M., Gallj C.L., Bosetti C., Gallus S., La Vecchia C. (2013) Food Chemistry Toxicology, 60C: 109-115.
8. Nettleton J.A., Lutsey P.L., Wang Y., Lima J.A., Michos E.D., Jacobs D.R. (2009) Diabetes Care, 32 (4): 688-694.
9. Frank R.A., Byram J. (1988) Chemical Senses, 13 (3): 445-455.
10. Frank R.A., Ducheny K., Mize S.J.S. (1989) Chemical Senses, 14 (3): 371-377.
11. Knoop J.E. (2011) Cross-modal interactions in complex food matrices. PhD thesis, Wageningen University and Research Center, Wageningen, The Netherlands, .
12. Poll L. (1985) Lebensmittel Wissenschaft und Technologie, 18 (4): 205-211.
13. Hendriks A.P. (1988) Rhinology, 26 (4): 229-251.
14. Small D.M., Gerber J.C., Mak Y.E., Hummel T. (2005) Neuron, 47: 593-605.
15. Frank R.A., Van der Klaauw N.J., Schifferstein H.N.J. (1993) Perception & Psychophysics, 54 (3): 343-354.
16. Lawrence G., Salles C., Palicki O., Septier C., Busch J.L.H.C., Thomas-Danguin T. (2011) International Dairy Journal, 21 (2): 103-110.
17. Busch J.L.H.C., Knoop J.E., Bult J.H.F., Smit G. (2007) In: Proceedings of the 7th Rosemary Pangborn Symposium, August 12-16, Minneapolis, MA, (P2.34), Amsterdam, Elsevier.
18. Stevenson R.J., Boakes R.A., Wilson J.P. (2000) Journal of Experimental Psychology: Learning, Memory and Cognition, 26 (2): 423-440.
19. Stevenson R.J., Boakes R.A., Wilson J.P. (2000) Learning and Motivation, 31: 114-127.
20. Miltenberger R.G. (2012) In: Behavior modification, principles and procedures, 5 edn., Wadsworth Belmont CA, pp. 88-99.
21. Schifferstein H.N.J., Verlegh P.W.J. (1995) Acta Psychologica, 94: 87-105.
22. Nasri N., Beno N., Septier C., Salles C., Thomas-Danguin T. (2011) Food Quality and Preference, 22 (7): 678-682.
23. Noort M.W.J., Bult J.H.F., Stieger M.A. (2012) Journal of Cereal Science, 55 (2): 218-225.
24. Sclafani A., Glendinning J.I. (2003) Physiology & Behavior, 79: 783-788.
25. Hogenkamp P.S., Mars M., Stafleu A., De Graaf C. (2010) American Journal of Clinical Nutrition, 91 (4): 841-847.
26. van Wymelbeke V., Béridot-Thérond M.E., de La Guéronnière V., Fantino M. (2004) European Journal of Clinical Nutrition, 58: 154-161.

# Perception of odour mixtures: The next challenge in flavour analysis

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## Abstract

The olfactory dimension of food flavour is critical to the food identity and typicality. Food odour and aroma result from the processing of complex mixtures of volatile compounds activating the sense of smell. The perceptual properties of odour mixtures have been explored from both the aroma analysis point of view and the psychophysical point of view, thus revealing perceptual effects such as masking, synergy, or perceptual blending. However, considering odorants separately, the classical aroma analysis approach misses the central role of perceptual integration in odour mixture processing. Therefore, the challenge of food flavour analysis is now to integrate the mechanisms of complex odorant mixtures perception. Here, we briefly review recombination strategies and tools that are already available to go one step forward and consider not only key-odorants but also key-associations involved in overall flavour perception.

## Introduction

The olfactory dimension of food flavour is critical to the food identity and typicality. This has been nicely showed in a basic experiment by Mozell et al. [1], in which a group of subjects had to identify real food flavours. In order to minimize identification by nonchemical cues, 20 food samples were prepared to be presented as liquids of about the same apparent viscosity. Subjects were allowed to swirl the liquid around their mouth before being asked to identify the flavour by a food name (e.g. "chocolate, "coffee," "onion,"). All the samples were presented twice to each subject but following two experimental procedures. In a first condition, the olfactory dimension was removed since subjects were equipped with an air stream apparatus, connected to their nostrils, which blew odourless air in the direction opposite to the movement of volatile molecules from the mouth to the nose via the nasopharynx. In the second condition, without the air stream apparatus, the nose remained normally accessible to the molecules. When deprived of the olfactory and the nasal trigeminal inputs, subjects were poorly able to identify the samples and were even unable to identify the flavour of coffee or chocolate.

Food odour and aroma are both percepts, namely cerebral representations, constructed on the basis of the olfactory processing of complex mixtures of volatile compounds able to activate olfactory receptors [2]. Following the aroma analysis classical methodology, GC/MS-O (Gas Chromatography/Mass Spectrometry-Olfactometry) is used to separate and identify those odorants that contribute to the odour of a given food sample headspace extract. The analysis process requires around half an hour to detect and identify the main odorants of the mixture that constitutes the headspace [3]. In contrast, the human nose, when confronted to the same mixture of odorants, analyses simultaneously all the chemicals to provide a pattern that is integrated by the brain to produce, in less than one second, a mental representation of the food sample. The result will be the rapid categorisation and likely recognition of the odour as an odour object [4].

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Therefore, we always have to keep in mind that there is a critical difference between the chemical analysis strategy and the perceptual strategy when focusing on complex odour mixtures responsible for the flavour of food.

## The perception of odour mixtures, the case of perceptual blending

The processes underlying the perception of complex mixture of odorants as patterns and the elaboration of odour object representation in the brain are based on odour coding and perceptual interactions that take place along the olfactory pathway [4]. The simultaneous interplay of several odorants with the olfactory system induces various interactions at all the levels of integration, from the very periphery where competition at the olfactory receptors level takes place [5,6] to high order integrative processes involving cognitive and top-down modulations [7]. From a theoretical point of view, it is possible to consider several cases of perceptual interactions in odour mixtures (Figure 1).

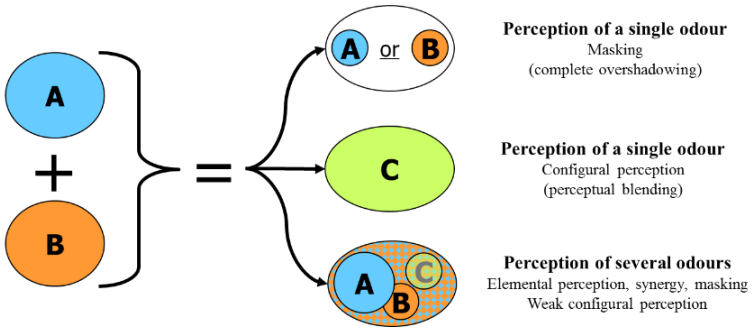


**Figure 1**: Theoretical outcomes on odour quality when two odorants are processed in mixture by the olfactory system. One odorant has an odour noted A and the other B, while odour C is specific to the mixture and results from configural processing of the so-called blending mixture (adapted from [4]).

In one case, the mixture carries a specific odour, which is not the superposition of the odorants' odour. Such a perceptual outcome is the result of the configural processing of certain mixtures called blending mixtures [8], which may be the chemical signature of odour objects [4]. Another processing strategy of odour mixture, namely elemental processing, leads to the recognition of the odorants' quality within the mixture (Figure 1). These two processing strategies, likely concurrent, can be influenced by individual-related factors such as physiological or cognitive state but also by the stimulus features, especially the odour quality of each of the odorants and their relative concentrations.

In a recent study, we investigated the configural and elemental perception of two 6-odorants mixtures in two mammal species, human adults and newborn rabbits, which have both assets with regard to the study of odour mixtures [9]. Using free-sorting tasks in humans, we evaluated the perception of a blending mixture (RC), which evoked the specific odour of Red Cordial and another mixture ($RC^{mod}$), made of the 6 same odorants but in different proportions, in comparison to the perception of the single odorants. In newborn rabbits, the perception of the same mixtures was assessed by measuring the orocephalic sucking response to the mixtures or their components after conditioning to one of these stimuli. The results revealed that the blending mixture (RC) was indeed configurally processed both in humans and rabbits. In contrast, the other mixture ($RC^{mod}$), containing the same odorants but in different concentration ratio, was elementally processed. These results demonstrate that configural perception is specific not only to the

odorants included in a blending mixture but also to their respective proportion [10]. Interestingly, rabbit neonates also responded to each odorant after conditioning to the red cordial mixture, which demonstrated their ability to perceive elements in addition to the configuration in the mixture [11] and, in turn, supports the hypothesis that both elemental and configural processing are concurrent.

**Key odorants and key associations in odour mixtures**

Within the aroma chemical analysis framework, it is usually considered that if the omission of an odorant from a recombined mixture changes the overall perception, then this odorant is a key aroma compound [12]. However, key odorants may have a different status depending on whether the mixture has blending properties or not, which may also explain why key odorants reported in the literature for a lot of food sometimes carry an odour similar to the overall food odour and sometimes not. We tested this hypothesis through the study of the perceptual roles of the odorants that are included in mixtures elementally or configurally perceived. We examined, in humans, the perceptual impact of the nature and concentration ratio of the odorants included in two 6-components mixtures and their sub-mixtures containing 2 to 5 components [13]. The 6-odorants mixtures were RC and $RC^{mod}$ used in the previous study [9]. Mixture processing was explored through a similarity rating task, in which 61 subjects rated the similarity of odour samples containing 1 to 6 components to either the RC or the $RC^{mod}$ reference mixtures.

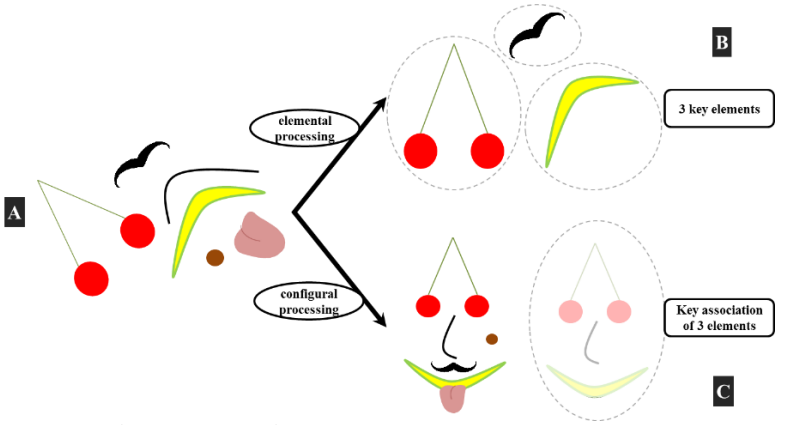


**Figure 2**: Illustration of the perception of key elements and key association in a 6-components mixture (A). Some of the elements carry a specific object identity (boomerang, bird and cherry); at a specific ratio (represented here by the spatial arrangement of the elements), their perception as individual elements can be still salient (B); they are *key elements*; the perception is *elemental*. In contrast, at another ratio (another spatial arrangement), the same elements may lead to a *key association* (C), in which the elements lose their object's identity but create another feature (a basic face); these elements are contributors to the *key association*. Adding other elements, which do not necessarily refer to specific objects (point, curved line), "polishes" the key association and provides an identity for the whole mixture; the perception is *configural*. (adapted from [13]).

The results highlighted that elemental perception depended primarily on the odour quality and concentration ratio of many of the mixed odorants, whereas configural perception depended on specific associations of odorants in strict concentration ratios. These findings led us to reconsider the impact of key elements in odour mixtures within the framework of a perceptual model, illustrated in Figure 2 owing to a visual analogy. In mixtures, some odorants may preserve their perceptual features such that the individual odour they carry as single molecules is still identifiable within the mixture. In that case,

the mixture is elementally processed. It is perceived as a collection of a few individual odours carried by some of the odorants, which can be qualified as *key odorants*. Still, several odorants may lose some of their perceptual features [14] and create meaningful associations that strongly contribute to the mixture odour quality. These associations can be considered as *key associations*.

**New developments in aroma analysis**

If the aroma analysis methodology, relying on GC/MS-O, has been repeatedly shown to be efficient to identify impact odorants in complex food flavour, it appears that it can only point those molecules that are key odorants. Indeed, key associations can only be identified through the study of mixtures. Nevertheless, some odorants, likely contributors to key associations, may have been spotted during confirmatory recombination approaches, in which odorants, which odour is not similar to the odour of the overall food flavour, can appear as impacting compounds (e.g. [15,16]). The need for new tools to rapidly evaluate the perceptual importance of odorants in complex mixtures have led to the development of several methods based on dynamic reconstitution of mixtures online during GC-O analysis [17–20]. The Olfactoscan system couples two devices: a GC-O apparatus and a multi-channel dynamic dilution olfactometer [21]. The humidified air stream at the outlet of the olfactometer is connected to the GC-O sniffing port so that controlled mixtures of odorants, provided by the olfactometer, can be mixed with the odorants coming from the GC-O. Therefore, the olfactoscan system enables the screening of the olfactory active compounds delivered during a GC-O run, while mixed with a well-controlled background odour generated with the olfactometer.

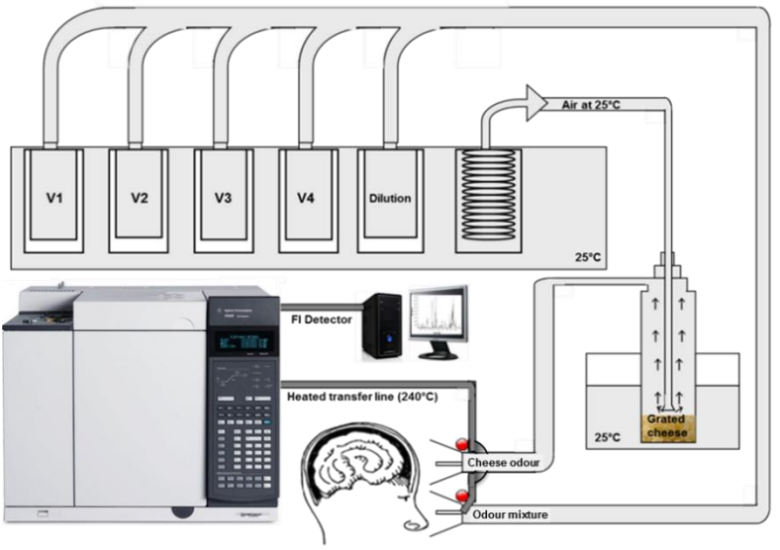


**Figure 3**: Olfactoscan setup including an olfactometer that delivered a precise mixture of odorants from the four vessels (V1, V2, V3, V4) to be combined with odorants eluted from a gas-chromatograph coupled to the outlet of the olfactometer; this formed the *Odour mixture* olfactory port. The olfactometer was also used to control the delivery of real cheese odour at the *Cheese odour* olfactory port, which served as the reference odour for the direct similarity rating task (adapted from [22]).

We used the Olfactoscan system to screen for specific associations of odorants responsible for the odour specificity of 3 non-processed semi-hard cheeses (setup

presented on Figure 3) [22]. Eight odorants, identified as contributors to the basic odour of the cheeses, were dispatched into the four vessels of the olfactometer to form an optimal basic composition, specific to each of the three cheeses. Eight odorants, among which four were also present in mixture in one of the olfactometer vessels, were individually added to the basic composition owing to the GC-O system. All the combinations formed complex odour mixtures that were systematically compared to the real odour of each cheese, by 16 trained subjects through a direct similarity rating.

The results highlighted that the relative concentrations of the same few odorants in a mixture can be adjusted via a recombination approach to reach an optimum of similarity with the odours of different non-processed semi-hard cheeses. More precisely, when combined with acetic acid, butan-2,3-dione and methional, the odorant dimethyl trisulphide contributed to one cheese odour, whereas butanoic acid contributed to another cheese odour. Still, for the third cheese odour, the combination of dimethyl trisulphide, butanoic acid, 3-methylbutanoic acid and 3-methylbutan-1-ol is required.

## Conclusion

Odour mixture processing, which constitutes the basic rule when perceiving the flavour of a food, induces several perceptual effects that contribute to the olfactory system striking efficiency in coding complex odour objects. The concept of configural-elemental dual olfactory processing has led to consider a new perspective in the identification of key components of odour sources, namely the importance of key odorants but also of key associations. In the framework of food flavour analysis, online recombination strategies and specifically relevant tools have been developed and are now available to go one step forward and take up the challenge of integrating odour mixture processing specificity into the aroma analysis path. Beyond the expected impact in terms of food flavour analysis, the study of odour mixtures is an original window allowing the investigation of olfaction-specific mechanisms certainly crucial to interpret -and provide an efficient representation of- our food and more broadly our environment.

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## References


1. Mozell, M.M., Smith, B.P., Smith, P.E., Sullivan, R.L., and Swender, P. (1969). Arch. Otolaryngol. 90, 367–73.
2. Dunkel, A., Steinhaus, M., Kotthoff, M., Nowak, B., Krautwurst, D., Schieberle, P., and Hofmann, T. (2014). Angew. Chem. Int. Ed. Engl. 53, 7124–43.
3. d'Acampora Zellner, B., Dugo, P., Dugo, G., and Mondello, L. (2008). J. Chromatogr. A 1186, 123–143.
4. Thomas-Danguin, T., Sinding, C., Romagny, S., El Mountassir, F., Atanasova, B., Le Berre, E., Le Bon, A.-M., and Coureaud, G. (2014). Front. Psychol. 5, 504.
5. El Mountassir, F., Belloir, C., Briand, L., Thomas-Danguin, T., and Le Bon, A.-M. (2016). Flavour Fragr. J. 31, 400–407.
6. Chaput, M., El Mountassir, F., Atanasova, B., Thomas-Danguin, T., Le Bon, A.-M., Perrut, A., Ferry, B., and Duchamp-Viret, P. (2012). Eur. J. Neurosci. 35, 584–97.
7. Gottfried, J.A. (2010). Nat. Rev. Neurosci. 11, 628–41.
8. Sinding, C., Coureaud, G., Chabanet, C., Chambault, A., Béno, N., Dosne, T., Schaal, B., and Thomas-Danguin, T. (2014). Chapter 5 - Perceptual Interactions in Complex Odor Mixtures: The Blending Effect. In Flavour Science: Proceedings from XIII Weurman Flavour Research Symposium, V. Ferreira and R. B. T.-F. S. Lopez, eds. (San Diego: Academic Press), pp. 27–31.
9. Sinding, C., Thomas-Danguin, T., Chambault, A., Béno, N., Dosne, T., Chabanet, C., Schaal, B., and Coureaud, G. (2013) PLoS One 8, e53534.
10. Le Berre, E., Béno, N., Ishii, A., Chabanet, C., Etiévant, P., and Thomas-Danguin, T. (2008). Chem. Senses 33, 389–95.
11. Coureaud, G., Thomas-Danguin, T., Wilson, D.A., and Ferreira, G. (2014). Proc. R. Soc. B Biol. Sci. 281, 20133319–20133319.
12. Grosch, W. (2001). Senses 26, 533–545.
13. Romagny, S., Coureaud, G., and Thomas-Danguin, T. (2017). Flavour Fragr. J., 1–9.
14. Jinks, A., and Laing, D.G. (2001). Physiol. Behav. 72, 51–63.
15. Escudero, A., Gogorza, B., Melus, M.A., Ortin, N., Cacho, J., and Ferreira, V. (2004). J. Agric. Food Chem. 52, 3516–3524.
16. Paravisini, L., Septier, C., Moretton, C., Nigay, H., Arvisenet, G., Guichard, E., and Dacremont, C. (2014). Food Res. Int. 57, 79–88.
17. Hallier, A., Courcoux, P., Sérot, T., and Prost, C. (2004). J. Chromatogr. A 1056, 201–208.
18. Johnson, A.J., Hirson, G.D., and Ebeler, S.E. (2012). PLoS One 7, e42693.
19. Hattori, S., Takagaki, H., and Fujimori, T. (2005). Food Sci. Technol. Res. 11, 171–174.
20. Williams, R.C., Sartre, E., Parisot, F., Kurtz, A.J., and Acree, T.E. (2009). Chemosens. Percept. 2, 173–179.
21. Burseg, K., and de Jong, C. (2009). J. Agric. Food Chem. 57, 9086–9090.
22. Thomsen, M., Dosne, T., Beno, N., Chabanet, C., Guichard, E., and Thomas-Danguin, T. (2017). Flavour Fragr. J. 32, 196–206.

# Structure-odour relation in homologous series of alkane-1,1-dithiols and dithio(hemi)acetals

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## Abstract

Twenty 1,1-dithioalkane derivatives were synthesized including ten compounds structurally derived from acetaldehyde and the ten corresponding compounds structurally derived from propanal. Compounds included ethane-1,1-dithiol and propane-1,1-dithiol as basic structures and their methyl, ethyl, and propyl derivatives 1-(methylsulfanyl)ethane-1-thiol, 1-(ethylsulfanyl)ethane-1-thiol, 1-(propylsulfanyl)-ethane-1-thiol, 1,1-bis(methylsulfanyl)ethane, 1-(ethylsulfanyl)-1-(methylsulfanyl) ethane, 1-(methylsulfanyl)-1-(propylsulfanyl)ethane, 1,1-bis(ethylsulfanyl)ethane, 1-(ethylsulfanyl)-1-(propylsulfanyl)ethane, 1,1-bis(propylsulfanyl)ethane, 1-(methyl-sulfanyl) propane-1-thiol, 1-(ethylsulfanyl)propane-1-thiol, 1-(propylsulfanyl)propane-1-thiol, 1,1-bis(methylsulfanyl)propane, 1-(methylsulfanyl)-1-(ethylsulfanyl)propane, 1-(methylsulfanyl)-1-(propylsulfanyl)propane, 1,1-bis(ethylsulfanyl)propane, 1-(ethyl-sulfanyl)-1-(propylsulfanyl)propane, and 1,1-bis(propylsulfanyl)propane. GC-O analyses revealed onion-like odour qualities for the majority of the compounds and additional fruity notes for some higher homologues. Thresholds showed a clear tendency towards higher values with increasing alkyl chain length, particularly the dithiohemiacetals showed consistently lower thresholds than the dithioacetals.

## Introduction

1,1-Dithioalkane derivatives such as alkane-1,1-dithiols, dithiohemiacetals, and dithioacetals (Figure 1) have scarcely been reported in food so far. One of the rare exceptions is durian, the fruit of the Southeast Asian tropical rainforest tree *Durio zibethinus.* Durian is famous for its strong and penetrating odour which combines fruity notes with a strong sulfury, oniony smell. The latter was recently assigned to a series of 1,1-dithio compounds and some short-chain alkanethiols [1,2]. The odour-active 1,1-dithio compounds in durian were all structurally related to acetaldehyde and propanal as carbonyl component and hydrogen sulfide, methanethiol, ethanethiol, and propane-1-thiol as thio component. This prompted us to synthesize all possible 1,1-dithio compounds available from these building blocks and have a closer look at the relation of their structure to their odour properties.

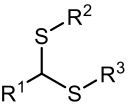


**Figure 1:** General structure of alkane-1,1-dithiols ($R^1$ = alkyl; $R^2$, $R^3$ = H), dithiohemiacetals ($R^2$ = H; $R^1$, $R^3$ = alkyl), and dithioacetals ($R^1$, $R^2$, $R^3$ = alkyl)

## Experimental

*Syntheses*

Compounds **1-6**, **8**, **12**, and **13** were synthesized as detailed in [1].

Synthesis of 1-(methylsulfanyl)-1-(propylsulfanyl)ethane (**7**) was accomplished by adding $CsCO_3$ (820 mg, 25 mmol) and $Bu_4NI$ (930 mg, 2.5 mmol) in anhydrous DMF

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(13 ml) under Ar to **2** (270 mg, 2.5 mmol). After stirring (1 h, RT), PrBr (338 mg, 2.75 mmol) was added dropwise at 0 °C. After further stirring (1 h, RT), water (30 ml) was added and the mixture was extracted with DCM (3 × 30 ml). Combined solvent extracts were washed with water (3 × 30 ml) and brine (30 ml) and dried. The solvent was evaporated and the product was purified by flash chromatography [1] to give 145 mg of **7** in 97% purity (GC-FID) and with 37% yield.

Synthesis of 1-(ethylsulfanyl)-1-(propylsulfanyl)ethane (**9**) was done by adding **4** (272 mg, 2 mmol) to a mixture of aqueous NaOH (10 M, 0.2 ml) and MeOH (5 ml) at 0 °C. After stirring (5 min), EtI (2.4 mmol, 0.2 ml) was added and stirring was continued (2 h, RT). MeOH was removed (Vigreux column), water (20 ml) was added, and the mixture was extracted with $Et_2O$ (3 × 20 ml). Combined extracts were washed with brine (60 ml) and dried. The solvent was evaporated and the product was purified by flash chromatography [1] to afford 155 mg of **9** (85% purity, 40% yield).

Synthesis of 1,1-bis(propylsulfanyl)ethane (**10**), 1,1-bis(ethylsulfanyl)propane (**18**), and 1,1-bis(propylsulfanyl)propane (**20**) was achieved from the corresponding aldehydes and alkanethiols by applying the approach detailed for **8** in [1]. Yields were 1.31 g (73%) (**10**), 1.31 g (79%) (**18**), and 1.29 g (67%) (**20**), all in 99% purity.

Propane-1,1-dithiol (**11**) and 1-(propylsulfanyl)propane-1-thiol (**14**) were synthesized by adding propanal (2.9 g, 50 mmol), PrSH (3.8 g, 50 mmol), and acetate buffer (5.4 M, pH 5, 40 ml) to a mixture of $Na_2S \cdot 9\ H_2O$ (12 g, 50 mmol) and DCM (20 ml) at -60 °C under Ar. After stirring (3 h at -60 °C, then 3 d at RT), the organic layer was separated and the aqueous phase was extracted with DCM (50 ml). Combined extracts were washed with water (100 ml), dried and the solvent was evaporated. Vacuum distillation (60 °C, 5 kPa) afforded 230 mg of **11** (90% purity, 3.8% yield). DCM (50 mL) was added to the residue, the mixture was washed with aqueous $Na_2CO_3$ (5%, 50 ml), dried, and the solvent was evaporated in vacuo. The residue was purified by flash chromatography. Elution with pentane afforded 330 mg of **14** in 99 % purity (GC-FID).

1,1-bis(Methylsulfanyl)propane (**15**) was synthesized from $Me_2S_2$ (1.32 g, 21.3 mmol) and propanal (1.24 g, 21.3 mmol) by the approach detailed for **5** [1], resulting in 500 mg product (97% purity, 17% yield).

The approach detailed above for **9** was also used to synthesize 1-(ethylsulfanyl)-1-(methylsulfanyl)propane (**16**) from **13** (180 mg, 1.2 mmol), 10 M NaOH solution (0.1 ml, 1 mmol) and MeI (0.1 ml, 1.6 mmol), 1-(methylsulfanyl)-1-(propylsulfanyl)propane (**17**) from **14** (150 mg, 1 mmol), 10 M sodium hydroxide solution (0.1 ml, 1 mmol) and MeI (0.1 ml, 1.6 mmol), as well as 1-(ethylsulfanyl)-1-(propylsulfanyl)propane (**19**) from **14** (150 mg, 1 mmol), 10 M sodium hydroxide solution (0.1 ml, 1 mmol) and EtI (0.1 ml, 1.6 mmol). Yields were 42 mg (22%) in 93% purity (**16**), 50 mg (30%) in 95% purity (**17**), and 62 mg (34%) in 98% purity (**19**).

*Odour threshold values (OTVs) in air*

These were determined by aroma extract dilution analysis using (2*E*)-dec-2-enal as internal standard [3,4]. Results of two panellists were averaged by calculating the geometrical mean of the individual thresholds.

## Results and discussion

In total, 1,1-dithio compounds were synthesized. Ten compounds were derived from acetaldehyde as carbonyl component (Figure 2) and ten compounds were derived from propanal as carbonyl component (Figure 3). Each series started from the 1,1-dithiol, i.e.

ethane-1,1-dithiol (**1**) and propane-1,1-dithiol (**11**). Then the structure was modified by adding alkyl groups of increasing length, namely methyl, ethyl, and propyl to both of the two sulfur atoms. In doing so, for each of the two carbonyl compounds, four homologues series resulted, each including four members.

For the acetaldehyde derivatives (Figure 2), the first homologues series consisted of ethane-1,1-dithiol (**1**) and the three 1-(alkylsulfanyl)ethane-1-thiols 1-(methylsulfanyl)ethane-1-thiol (**2**), 1-(ethylsulfanyl)ethane-1-thiol (**3**), and 1-(propylsulfanyl)ethane-1-thiol (**4**). In this series, all compounds exhibited an onion-like smell and rather low odour thresholds. Thresholds decreased from **1** to **3** and increased from **3** to **4**, with **4** showing the highest threshold value in the series.

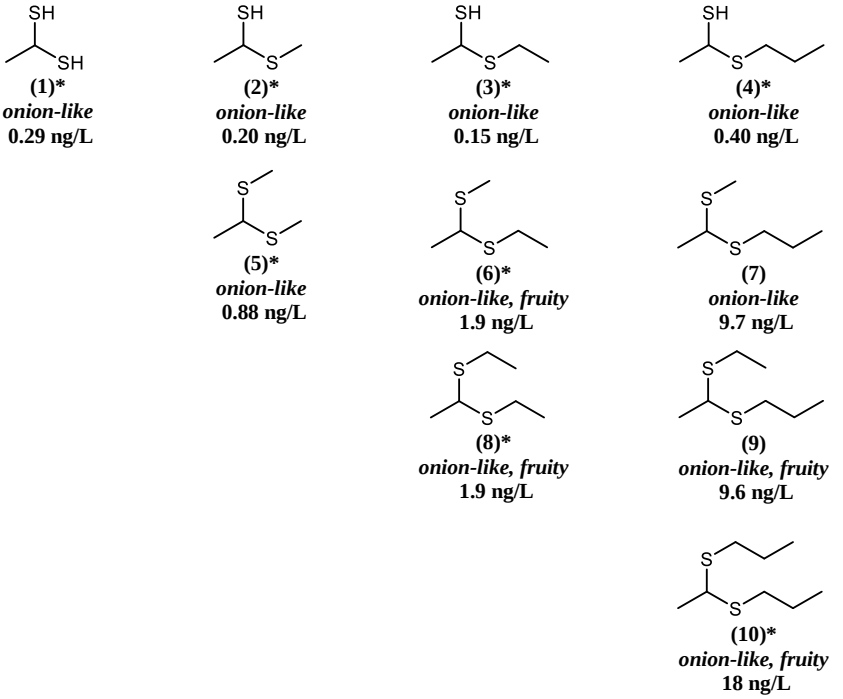


**Figure 2:** Structures, odour qualities as perceived during GC-O, and OTVs of 1,1-dithioethane derivatives; asterisks indicate compounds found among the odour-active compounds in durian [1]

The second homologues series of acetaldehyde derivatives consisted of 1-(methylsulfanyl)ethane-1-thiol (**2**) and the three 1-(methylsulfanyl)-1-(alkylsulfanyl)ethanes 1,1-bis(methylsulfanyl)ethane (**5**), 1-(ethylsulfanyl)-1-(methylsulfanyl)ethane (**6**), and 1-(methylsulfanyl)-1-(propylsulfanyl)ethane (**7**). Thresholds increased in this series, odour qualities were described as onion-like, but **6** showed an additional fruity note.

In the third homologues series of acetaldehyde derivatives, including 1-(ethylsulfanyl)ethane-1-thiol (**3**) and the three 1-(ethylsulfanyl)-1-(alkylsulfanyl)ethanes 1-(ethylsulfanyl)-1-(methylsulfanyl)ethane (**6**), 1,1-bis(ethylsulfanyl)ethane (**8**), and 1-(ethylsulfanyl)-1-(propylsulfanyl)ethane (**9**), thresholds again increased with increasing carbon number and fruity notes were prevalent, though the onion-like note dominated.

The fourth homologues series of acetaldehyde derivatives consisted of 1-(propylsulfanyl)ethane-1-thiol (**4**) and the three 1-(propylsulfanyl)-1-(alkylsulfanyl)ethanes

1-(methylsulfanyl)-1-(propylsulfanyl)ethane (**7**), 1-(ethylsulfanyl)-1-(propylsulfanyl)-ethane (**9**), and 1,1-bis(propylsulfanyl)ethane (**10**). All compounds showed onion-like odours, but the higher homologues **9** and **10** additionally exhibited fruity notes and thresholds increased by trend.

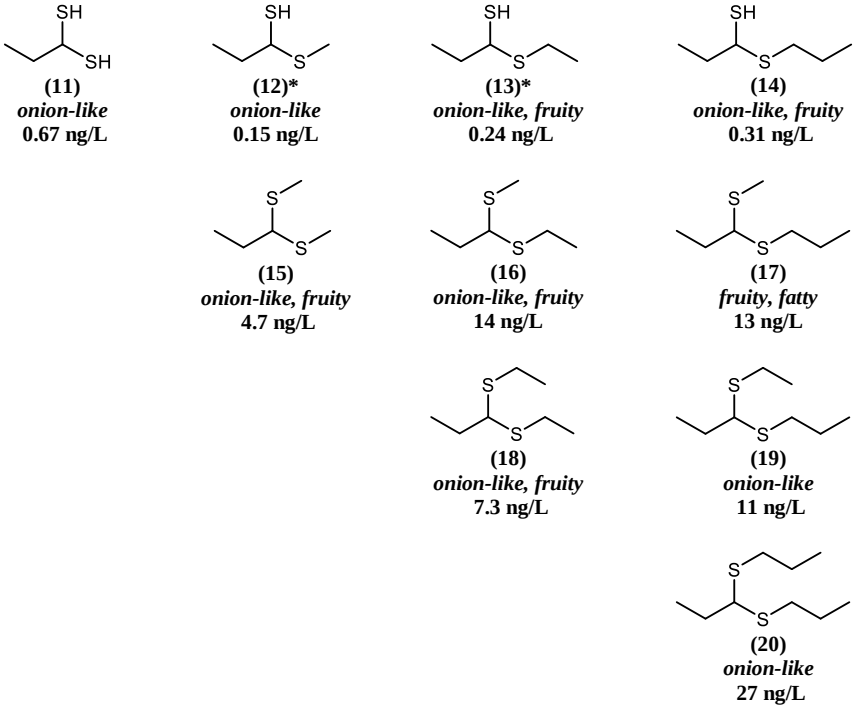


**Figure 3:** Structures, odour qualities as perceived during GC-O, and OTVs of 1,1-dithiopropane derivatives; asterisks indicate compounds found among the odour-active compounds in durian [1]

In summary, all analysed acetaldehyde derivatives showed an onion-like odour, but an additional fruity note was prevalent in the higher homologues. Thresholds showed a tendency towards higher values with increasing chain length. Similar observations were made with the propanal derivatives (Figure 3). The comparison of the propanal derivatives with the respective acetaldehyde derivatives showed a tendency towards slightly higher thresholds in the propanal derivatives. For both, acetaldehyde and propanal derivatives, the dithiohemiacetals (**1**-**4** and **11**-**14**) showed consistently lower thresholds than the dithioacetals (**5**-**10** and **15**-**20**).

## References

1. Li J.-X., Schieberle P., Steinhaus M. (2012) J Agric Food Chem 60: 11253-11262.
2. Li J.-X., Schieberle P., Steinhaus M. (2017) J Agric Food Chem 65: 639-647.
3. Ullrich F., Grosch W. (1987) Z Lebensm Unters Forsch 184: 277-282.
4. Teranishi R., Buttery R.G., Guadagni D.G. (1974) Ann N Y Acad Sci 237: 209-216.

# Effect of carbonation level on the perception of sourness in sparkling wine

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## Abstract

The relationship between instrumental and sensory measurements were investigated in 11 wines varying in their carbonation level. Although sourness intensities of the wines were not significantly different, increased carbonation concentration affected the dynamics of sourness perception. Both the onset and extinction of the sourness perception were delayed with increased carbonation. Amongst potential explanations are that dynamic effects of carbonation draw attention away from sensations that arise in other sensory modalities, including gustation, and that carbonation has an anaesthetizing effect which partially reduces the ability to perceive sourness. Findings suggest potential for further research for systematically investigating how carbonation level affect how products are perceived in mouth.

## Introduction

From a sensory perspective, sparkling wines are highly complex products. Carbonation increases surface area and kinetic energy. It also imparts characteristic mouthfeel effects that include tingling and other sensations, and may trigger gustatory, olfactory, trigeminal, and auditory perceptions as well [1]. A mechanism for carbonation perception as sourness has been proposed [2]. The effect of carbonation on the perception of sourness intensity (as determined using static sensory measurements) has been investigated in various beverages but reported results are inconsistent [3-8]. Although effects on sourness intensity are often reported to be slight, overall impact of carbonation may have a more pronounced effect on taste quality perceptions, e.g. on sweet and salty perception [9].

In a previous study, eleven wines with different carbonation levels were created [10] then evaluated according to a replicated experimental design by trained assessors using (i) sensory descriptive analysis (which provides static data on attribute intensities), and (ii) temporal check-all-that-apply (TCATA) [11] in which attribute applicability is determined dynamically over time.

Specifically, a trained descriptive sensory panel (n=11) conducted a replicated evaluation of the eleven wines according to the intensities of 20 sensory attributes. The panel discriminated wines based on 12 of the attributes (9 mouthfeel, 1 aroma, 1 flavour, and 1 taste), but the wines were not discriminated according to their sourness intensities [1]. TCATA data from trained assessors (n=12) indicated that the duration during which sensations are elicited is elongated with increasing carbonation concentration, yet the average citation rates for sourness (proportional to the area under the curve) were not significantly different across carbonation levels.

In this study, we further investigate these data to determine potential relationships between carbonation level in sparkling wine and the dynamic perception of sourness.

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## Experimental

### *Materials*

Eleven wines were made starting from the same base cuvee, resulting in one (still) base wine and ten sparkling wines, each at a different carbonation level (1.2-7.5 g $CO_2$/L), using materials and winemaking techniques described in [10]. Wine chemistry analysis confirmed differences amongst samples with respect to carbonation, as well as similarity in terms of sensory threshold levels in recorded concentrations of total sugars, titratable acid, pH, and ethanol [10].

### *Sensory evaluation*

Twelve assessors evaluated the 11 wines in triplicate via TCATA using eight attributes: six mouthfeel attributes (Bite/Burn, Carbonation/Bubble pain, Foamy, Numbing, Prickly/Pressure, Tingy) and two taste attributes (Bitter, Sour). The evaluation period was 120 s. Details related to attribute definitions, training, sample evaluation protocols, and other experimental parameters are found in [1].

### *Statistical analysis*

TCATA curves were obtained for sourness citation rates per $CO_2$ level using the R package tempR [12]. Cumulative citation rates leading up to 15, 30, 45, 60, and 75 s were obatined per $CO_2$ level and stacked, such that rows indicated unique combinations of $CO_2$ level and time for both the predictor matrix (with variables ethanol, $CO_2$ concentration, titratable acidity) and response variables (TCATA citation rate for the eight TCATA attributes). These X and Y matrices were then submitted to multivariate PLS regression using the plsreg2 function in the R package plsdepot [13].

The predictor variable $CO_2$ level and response variable sourness citation rates per 15-s interval were submitted to least squares regression to investigate how sourness characterization changes with $CO_2$ level and time.

## Results and discussion

TCATA curves for sourness citation rates per $CO_2$ level are presented in Figure 1. In this figure, we are looking at aggregated raw panel data in which the sourness curves are right-shifted and damped as the $CO_2$ concentration increases. Increased carbonation delayed the perceptual onset and extinction of sourness.

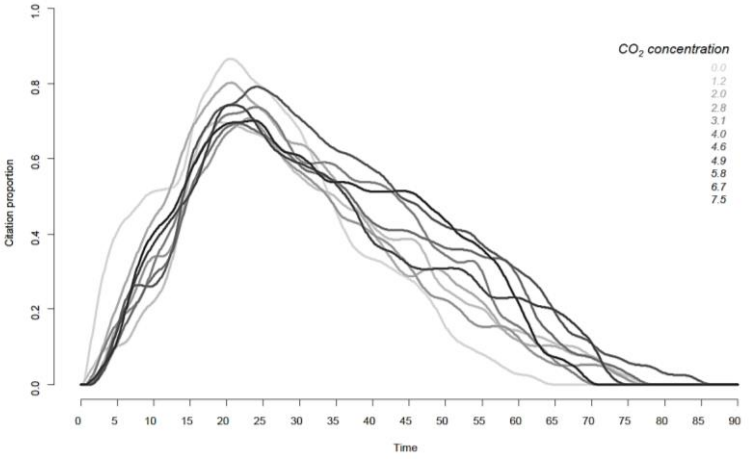


**Figure 1:** TCATA curves for the attribute Sour for the eleven samples which varied in $CO_2$ concentration.

Multivariate PLS-regression analysis gives correlations between the predictor and response variables; the relationships in the first two latent vectors are given in Figure 2, and show a temporal relationship between $CO_2$ concentration and time for sourness characterization.

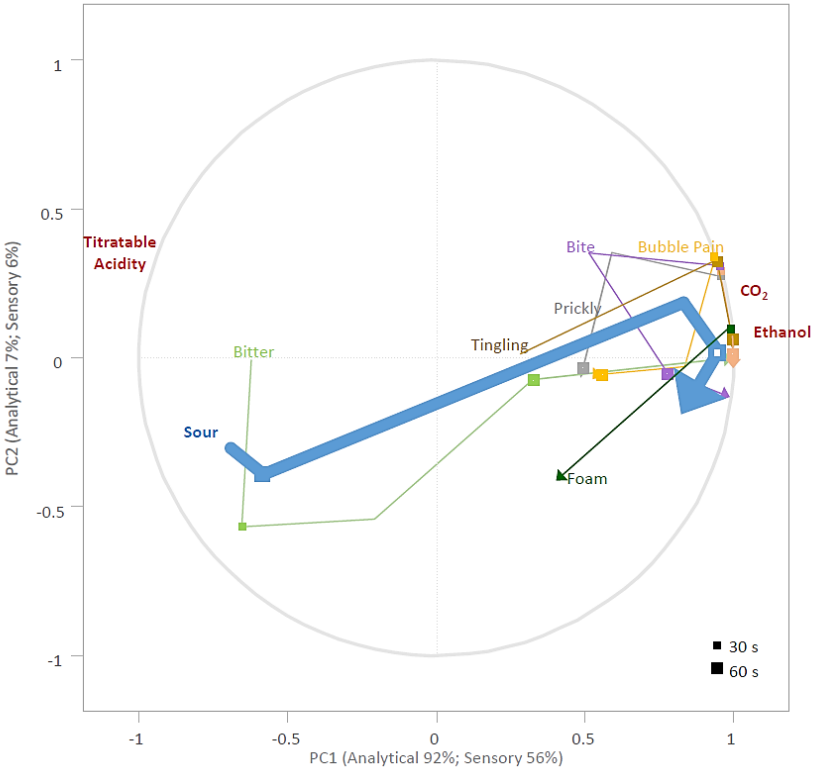


**Figure 2:** Partial least squares regression analysis of analytical measurements (Titratable Acidity, $CO_2$, and Ethanol) vs. TCATA Citation Proportion for 15-s intervals leading up to 15, 30, 45, 60, and 75 s. For each attribute shown these five time intervals are joined. The line that starts at 15 s and kinks at each time interval, with the closed square indicating 30 s, the open square 60 s, and the cumulating arrow (which indicate the progression of time) 75 s. Sour, which was the focus of this paper, is shown as a thick blue line.

Results from least squares regression indicate a strong relationship between sourness citation proportion and time. The proportion of assessors describing the wine as sour is highest in the 15-s interval leading up to 30 s across all $CO_2$ concentrations. There is significant interaction between time and $CO_2$ concentration (which is visualized here as differences in slopes). Leading up to 30 s, assessors describe low-$CO_2$ wines as sour more often than high-$CO_2$ wines; thereafter, the low-$CO_2$ wines are described as sour less often than high-$CO_2$ wines. Thus, the sourness citation proportion depends on both time and $CO_2$ concentration.

The wines described herein are similar in pH [10] and perceived intensity of sourness [1], yet differ in dynamic perception of sourness. Why might low-$CO_2$ wines be characterized as sour early, more often, and for a shorter duration, and high-$CO_2$ wines be characterized as sour later, less often, but for a longer duration? Potential explanations include the possibility that $CO_2$ has a masking or distracting effect (e.g. dynamic effects

of carbonation draw attention away from sensations that arise in other sensory modalities) or an anaesthetizing effect (e.g. carbonation partially reduces the ability to perceive sourness). Additionally, the right-shifted curves in Figure 1 may indicate adaptation, with perceived sourness attenuating after initial perception. Findings are relevant to product developers working on carbonated products, and suggest potential for further research for systematically investigating how carbonation level interacts with other wine components at different concentrations to affect how products are perceived in mouth.

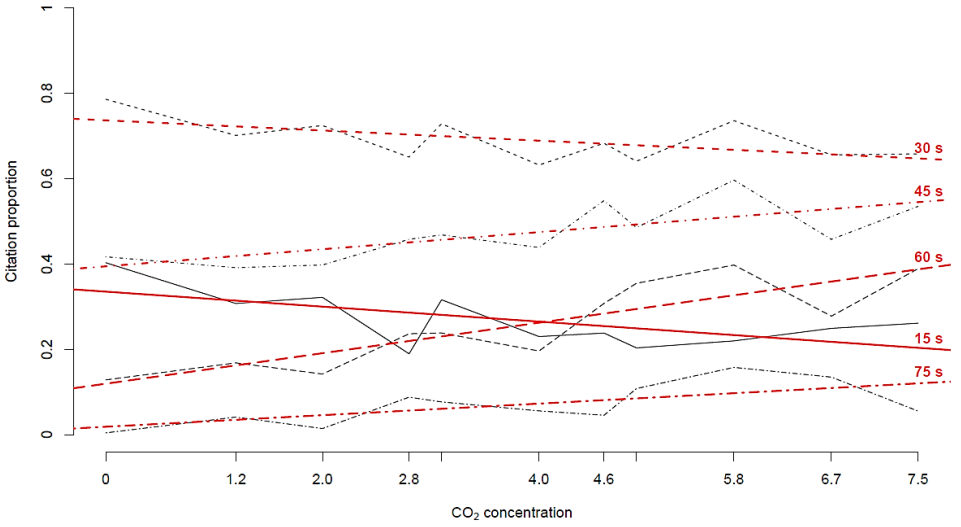


**Figure 3:** Interaction plot showing cumulative TCATA Citation Proportion for the 15-s intervals leading up to 15, 30, 45, 60, and 75 s vs. $CO_2$ concentration. The observed citation proportions are shown in black, and slopes are presented in red.

## References

1. McMahon K.M., Culver C., Castura J.C., Ross C.F. (2017b) Food Qual. Pref., 59, 14-26.
2. Chandrashekar J., Yarmolinsky D., von Buchholtz L., Oka Y., Sly W., Ryba N.J.P., Zuker C.S. (2009). Science, 326: 455–458.
3. McLellan M.R., Barnard J., Queale D.T. (1984) J. Food Sci., 49 (6): 1595-1597.
4. Cometto-Muñiz J.E., Garcia-Medina M.R., Calviño A.M., Noriega G. (1987) Perception, 16: 629-640.
5. Lederer C.L., Bodyfelt F.W., McDaniel M.R. (1991) J. Dairy Sci., 74 (7): 2100-2108.
6. Yau N.J.N., McDaniel M.R. (1992) J. Food Sci., 57: 1412-1416.
7. Hewson L., Hollowood T., Chandra S., Hort J. (2009) Chem. Percept., 2: 94–107.
8. Cowart B. (1998) Chem. Senses 23: 397–402.
9. Saint-Eve A., Déléris I., Feron G., Ibarra D., Guichard E., Souchon, I. (2010) Food Qual. Pref., 21, 1026–1033.
10. McMahon K.M., Culver C., Ross C.F. (2017a) J. Wine Res., 28(2), 123-134.
11. Castura J.C., Antúnez L., Giménez A., Ares G. (2016) Food Qual. Pref., 47, 79-90.
12. Castura J.C. (2016) tempR version 0.9.9.10. http://CRAN.R-project.org/package=tempR
13. Sanchez G. (2012) plsdepot version 0.1.17. https://CRAN.R-project.org/package=plsdepot

# Explaining fat sensitivity in cottage cheeses by aroma release and oral physiology parameters

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## Abstract

Great inter-individual differences exist in fat perception. Forty subjects were grouped according their global fat perception in cottage cheeses. The more sensitive subjects were also more sensitive to fatty odorants, they had a higher respiratory flow and thus a higher rate of release of aroma compounds in the nasal cavity, which could explain the role of the olfactory modality in fat perception. Fat sensitive subjects had a lower saliva flow, less viscous saliva, and less amount of product remaining in the mouth after swallowing, which could explain, why they were more sensitive to taste and textural modalities of fat perception.

## Introduction

In the aim to increase sensory acceptability of low fat content foods, a better understanding of the physiological mechanisms involved in fat perception is needed. Fat perception is considered as a multimodal sensation in itself involving smell, taste and texture perception [1, 2]. In a previous experiment conducted on 40 subjects and focusing on fat perception in cottage cheese, great inter-individual differences in fat sensitivity were observed in both absolute and difference detection thresholds [3]. An Ascending Hierarchical Classification evidenced three subsets of subjects with contrasted sensitivity profiles: high, medium and low absolute and difference thresholds. For each group of subjects, thresholds were always lower when the subjects did not wear nose clips, suggesting a strong impact of the olfactory modality in fat perception. The aim of the present paper was to determine, on the same well-characterized 40 subjects, the physiological parameters related to aroma release and/or aroma sensitivity that better explain the differences of fat perception in cottage cheeses.

## Material & Methods

Odour detection and recognition thresholds were determined for 3 aroma compounds (pentane-2,3-dione; hexane-3,4-dione and 3-hydroxy-2-butanone) using the AS'SCENT International Olfactometer (St. Croix Sensory, Stillwater, MN). Detection thresholds were estimated using a 3-Alternative Forced Choice (AFC) procedure based on 14 dilution steps. Recognition threshold were estimated using a 4-AFC method in which subjects has to choose among 4 odour labels at each detection trial. Thresholds were expressed as the absolute value of the logarithm of threshold dilution level; the threshold could range from 0 for the less sensitive to 5 for the most sensitive.

General olfactory capabilities were estimated using the European Test of Olfactory Capabilities [4]. The overall score to the test is usually expressed as a percentage, here as a value between 0 and 1.

*In vivo* release of 2 aroma compounds imparting fatty notes (pentane-2,3-dione and hexane-3,4-dione) was followed by a Proton Transfer Reaction-Mass Spectrometer

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equipped with a Time-of-flight analyser (PTR-ToF 8000, Ionicon Analytik, Innsbruck, Austria), while consuming 1% fat content cottage cheese. Sampling was performed at a total flow rate of 60 mL/min with the transfer line maintained at 80°C. All the release data were calculated from the breath concentration ncps data, using Microsoft Excel 2010. Ten parameters were extracted from the smoothed release curves as described in Figure 1.

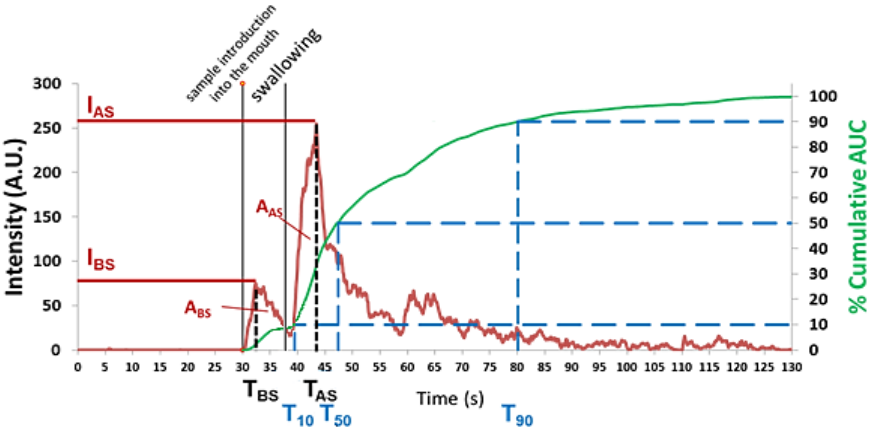


**Figure 1:** Parameters extracted from in vivo release curves**.** Maximal intensity before swallowing (**$I_{BS}$**) and after swallowing (**$I_{AS}$**), Time to reach maximal intensity before swallowing (**$T_{BS}$**) and after swallowing (**$T_{AS}$**), area under the curve before swallowing (**$A_{BS}$**) and after swallowing (**$A_{AS}$**), and time to reach 10% (**$T_{10}$**), 50% (**$T_{50}$**) and 90% (**$T_{90}$**) of the total area (**AUC**).

Mouth coating, defined as the residual food that sticks to the oral surface after food ingestion, was quantified by the "mouth rinse" method [5]. Dry matter of residual food (DM) was measured after lyophilisation. The lipids of residual food were quantified in the lyophilisate after extraction with chloroform/methanol (2:1) [6].

Resting saliva was collected as previously described [7] by instructing the subjects to spit out the saliva whenever they felt like into a pre-weighed cup over a period of 10 minutes. The cups were weighed and the salivary flow rates were expressed in mL/min. Saliva viscosity at rest (mPa.s) was measured with a Vibro – viscosimeter type SV-A (A&D Compagny Limited Japan).

An Eccovision® acoustic pharyngometer (Hood Laboratories, USA) was used to measure the oral volumes [8].

Respiratory flow was measured at rest using a spirometer (Pulmo System II, MSR, Rungis, France). Subjects were asked to breathe normally by the nose for three minutes. Respiratory frequency represents the number of respiratory cycles per minute and current volume, the volume of air used during each respiratory cycle.

Analyses of variance (ANOVA) were performed using XLSTAT® Software (Excel 97, version 8.0, Paris, France).

## Results and discussion

The 40 subjects included in the panel pertained to 3 groups of sensitivity for fat perception in cottage cheese: 7 high sensitive subjects (S+), 24 medium sensitive (S0) and 9 low sensitive (S-) [3]. Among the different physiological parameters measured in

the study, only those presenting significant differences between the three groups of sensitivity are reported (Figure 2).

Subjects less sensitive to fat (S-) had lower overall olfactory capabilities reflected by lower scores to the ETOC (Figure 2a). They were less sensitive to aroma compounds imparting fatty notes (Figure 2a). They had especially a higher recognition threshold for 2,3-hexanedione and a higher detection threshold for acetoin. These results confirmed our previous hypothesis that olfaction is important for global fat perception.

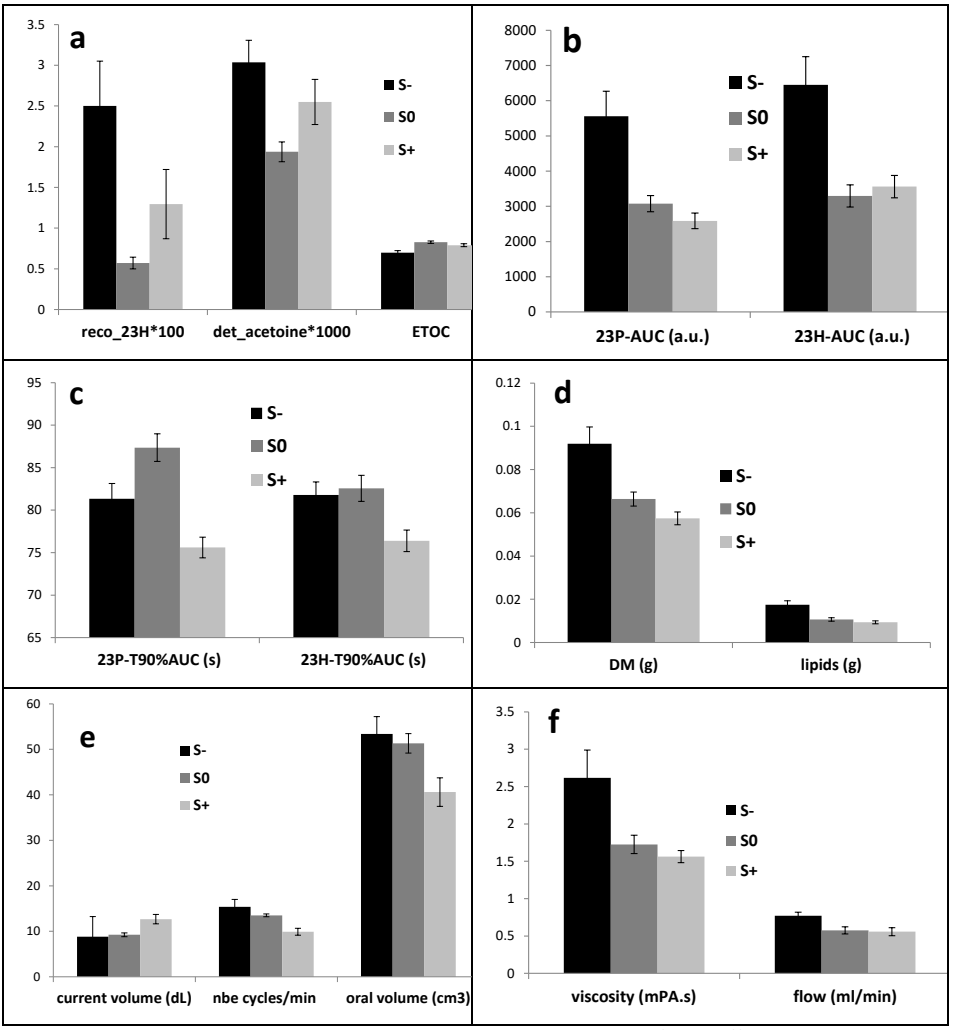


**Figure 2:** Physiological parameters (mean and standard error) showing significant differences between groups of fat sensitivity threshold (S+: high sensitive, S0: medium sensitive, S-: low sensitive) a) recognition threshold for 2,3-hexanedione, detection thresholds for acetoin, ETOC test; b) area under the curve (AUC) for 2,3-pentanedione (23P) and 2,3-hexandione (23H); c) time to reach 90% AUC for 23P and 23H; d) amount of dry matter (DM) and lipids remaining in the mouth after swallowing; e) respiratory parameters: current volume, number of cycles and oral volume; f) saliva viscosity and saliva flow.

Subjects less sensitive (S-) to fat significantly released a higher total amount of aroma than medium (S0) and high (S+) sensitive (AUC, Figure 2b), which cannot explain

their greater sensitivity. They needed a longer time to reach 90% AUC (Figure 2c), which means that their rate of release was slower. It has previously been suggested that the rate of release impacts more aroma perception than the total amount of aroma compounds release [9]. Moreover, subjects S- had a higher amount of product remaining in the mouth (DM and lipids, Figure 2d), which explains their higher amount of total aroma in the nasal cavity [8].

Subjects less (S-) and medium (S0) sensitive to fat had a higher respiratory frequency (nbe cycles/min) and a higher oral volume than high sensitive (S+) and subject's medium (S0) sensitive to fat had a significant lower respiratory flow (current volume) than high sensitive (S+) (Figure 2e). All these parameters could explain that the S- subjects had a higher rate of aroma release and thus aroma compounds will reach their olfactory receptors in a longer time.

Subjects less sensitive to fat (S-) also presented a higher salivary flow and saliva viscosity than medium (S0) and high sensitive (S+) (Figure 2f). These parameters, in addition to a higher mouth coating, could decrease the accessibility to taste and chemesthesic receptors and thus decrease textural and taste modalities of fat perception [3]. A high amount of lipid remaining in the mouth will form a fat barrier, which could limit the access to the receptors. A high viscous saliva will limit the diffusion of stimuli.

## Conclusion

Fat perception in cottage cheese is multimodal and involves smell, taste and texture perception, with great interindividual differences. Subjects more sensitive to fat have higher olfactory capabilities, a lower respiratory frequency and a higher rate of aroma release in the nasal cavity; all these physiological features converge to increase aroma perception. Subjects less sensitive to fat have a higher saliva viscosity, a higher amount of product remaining in the mouth after swallowing, which could limit the access of the fat stimulus to the taste and chemesthesic receptors in the mouth.

## References

1. Mattes R. D. (2009). Annual Review of Nutrition; Vol. 29, 305.
2. Martin C., Schoumacker R., Bourjade D., Thomas-Danguin T., Guichard E., Le Quéré J.-L., Laboure H. (2016) Dairy Science and Technology. 96(5): 735-746.
3. Schoumacker R., Martin, C., Thomas-Danguin, T., Guichard, E., Le Quéré J.L. and Labouré H. 2017. Food Quality and Preferences 56: 241-246.
4. Thomas-Danguin T., Rouby C., Sicard G., et al. Development of the ETOC: A European test of olfactory capabilities. 2003. Rhinology 41(3): 142-151.
5. Repoux M, Septier C, Palicki O, Guichard E, Feron G, et al. (2012) Archives of Oral Biology 57: 81-86.
6. Folch, J., M. Lees, and G. H. S. Stanley. (1957). Journal of Biological Chemistry, 226(1), 497-509.
7. Neyraud E, Cabaret S, Brignot H, Chabanet C, Labouré H, Guichard E, Berdeaux O. 2017. Scientific Reports 7: 5969.
8. Feron, G., Ayed, C., Qannari, E.M., Courcoux, P., Labouré, H., Guichard, E. (2014) PlosOne 9(4), e93113.
9. Gierczynski I, Laboure H., Guichard E. (2008) Journal of Agricultural and Food Chemistry, 56: 1697-1703.

# Elucidating the mechanisms of individual variation in fat perception and preference

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## Abstract

Fat is perceived through three modalities; mouthfeel, odour and, also taste where free fatty acids are the stimuli generating this sensation. Individuals showed variation in oral fat taste perception which significantly influenced fat intake and preference. The mechanisms causing differences in individual oral fat taste perception could be explained by genetic variation of the fatty acid translocase CD36 and lipase activity. Individuals had different lipase activities in saliva to release free fatty acids from dietary fat, which influenced oral fat perception. In addition, CD36 genotypes influenced oral fat perception and the influence of CD36 genotype on oral fat perception differed between subjects with high salivary lipase activity and those with low lipase activity.

## Introduction

Taste is one of the influential determinants driving individual food preference and consumption. Fat contributes to the unique texture and odour of foods, and recent studies proposed that fat can also be perceived through taste. Fatty acids are proposed as the effective stimuli in generating fat taste sensation due to the discovery of fatty acid receptors and activation of transduction pathways in mouth [1]. Individuals present diverse oral fat taste perception and these variations could contribute to individual differences in food liking and consumption [2]. As triglyceride is the major component in dietary fat and free fatty acids are only present in foods at very low levels, recent study report that salivary lipase can hydrolysis triglyceride in the mouth into free fatty acids [3]. This may cause an increase in free fatty acid in the mouth and hence result in a greater oral taste sensation. However, it is still unclear whether differences in individual salivary lipase activity may be a potential cause of individual differences in oral fat perception. CD36, as the fatty acid translocase, has been regarded as a putative candidate for the oral fat sensor. Genetic variations in CD36 have been proposed as another potential factor that could influence individual differences to oral fat taste perception.

This study aims to understand how fat is perceived in mouth. It also aims to understand inter-subject variability in fatty acid sensitivity, fat perception, fat preference and choice of high fat foods, and to elucidate the impact of CD36 genotype and salivary lipase activity on individual variation in fat perception.

## Materials and methods

### *Participant*

Ninety participants of age range 18-55 years were recruited and self-reported to be healthy. Three participants dropped out before they had completed the sessions, and two participants were excluded from the data analysis due to the incomplete questionnaires.

### *Fat intensity rating*

The samples and method for the intensity test were developed as described in the study of Zhou et al., 2016 [4]. Non-fat skimmed milk, single cream and double cream (Tesco, UK) were used to generate seven samples of different fat levels: 0%, 2.5%, 5%,

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7.5%, 10%, 15% and 20%. Mouthfeel masked samples (containing thickener (Nestlé Nutrition Resource ThickenUp Clear, UK) and liquid paraffin (Care, Thornton & Ross, UK)) and mouthfeel non-masked samples were prepared. Participants were asked to rate the perceived fat intensity on a generalised labelled magnitude scale (gLMS). Nose-clips were provided for mouthfeel-masked samples to obtain intensity ratings under the "taste" modality in isolation from odour and mouthfeel.

*Fatty acid sensitivity test*

Samples and methods for threshold sensitivity were developed as described by Zhou et al. (2016). Food-grade oleic acid (Sigma, UK) was chosen and the concentration of oleic acid ranged from 0.098 to 55.9 mM (0.0028 to 1.58% w/v), with dilution differing by 0.25 log units. The rapid 3 alternative forced choice (3AFC) approach was used to measure fatty acid sensitivity. During the whole process, participants were asked to wear nose-clips to avoid any olfactory effects.

*Dietary intake and preference*

A Food Frequency Questionnaire (FFQ) and a Food Preference Questionnaire (FPQ, adapted from Deglaire et al., 2012 [5]) were used to collect recalled food intake and food preference data.

*Salivary lipase activity measurement*

The lipase activity in saliva was reflected by the free oleic acid generation in expectorated almond samples. Participants were asked to chew one almond (15s) without swallowing and the expectorated almond was collected. The free oleic acid in expectorated almond samples was analysed using Folch extraction of fat from expectorated almond samples, separation of free oleic acids from fat (using solid phase extraction with aminopropyl cartridges (ISOLUTE® NH2, Biotage)), derivatization of free oleic acids to oleic acid methyl ester at room temperature for 5 min (by using 1.5% $H_2SO_4$ in methanol) and gas chromatography analysis (using gas chromatography flame ionization detector (Hewlett Packard 5890 Series II) with non-polar capillary Agilent J&W DB-5 column (60 m × 0.25 mm × 0.25 μm)). The total fat in the expectorated almond sample was measured by using Folch extraction to calculate the free oleic acid in total fat (g/ml).

*CD36 genotype measurement*

Participants were asked to swab the inside of their cheek 7 times to collect buccal cells using sterile Omni swabs (Whatman, UK). The buccal swab samples were collected in duplicate. Genotyping of three CD36 SNPs (*rs1761667, rs1527483, rs3840546*) was carried out at iDNA genetics Ltd (Norwich, UK).

*Statistical analysis*

Fat intensity ratings were collected by Compusense at-hand (Canada). Data was analysed by XLSTAT (version 2016.8, Addinsoft). Latent cluster analysis was conducted to classify participants into different liking groups based on their recalled liking ratings of foods. Multivariate ANOVA with Bonferroni pairwise comparison was conducted to examine the difference in perceived fat intensity between groups (e.g. liking groups, sensitivity groups or genotypes). Significance was set at 0.05.

## Results and Discussion

### *Taste modality on oral fat perception*

Significant differences were found between fat levels under the "taste" condition ($p<0.05$), which implies that fat can be distinguished by taste. The perceived fat intensity rated in the "taste" modality was significantly higher than in the "overall" modality ($p<0.0001$), which was due to the addition of paraffin and thickener in the mouthfeel-masked samples. This confirms that mouthfeel, such as thickness and lubrication, is an important indicator of oral fat perception.

### *Food liking, food intake and oral fat perception*

Based on liking results collected by FPQ, high fat likers (HFLs) and high fat dislikers (HFDs) were established. HFLs (n=34) displayed higher liking scores in most food items (42 out of 46) which was significant for 25 items ($p<0.05$). HFLs showed higher fat intake (as % total energy) ($p=0.004$), which implies that high liking to foods rich in fat could stimulate the consumption of these foods. In addition, HFLs showed significantly lower perceived fat intensity under the "taste" modality compared to HFDs (n=51, Figure 1A). Fat taste generated by fatty acids has been reported to be an unpleasant taste sensation [6], so this might explain why the HFDs do not like foods rich in fat.

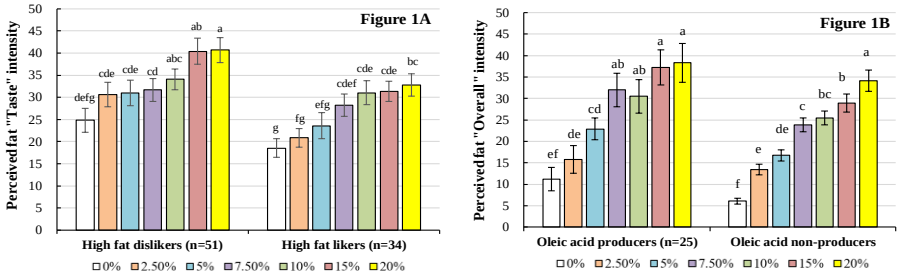


**Figure 1:** Perceived fat "taste" intensity between HFLs and HFDs (A, left) and perceived fat "overall" intensity between oleic acid producers/non-producers (B, right). Bars not sharing a common letter differ significantly ($p<0.05$) between fat levels and between groups. Error bars represent standard error of the mean.

### *Fatty acid sensitivity and oral fat perception*

Individual threshold sensitivity to oleic acid varied, and participants were divided into high (n=47) / medium (n=19) / low (n=21) sensitivity groups (grouping approach was developed by Zhou et al. (2016)). No significant difference in the perceived fat intensity rating was found between fatty acid sensitivity groups ($p=0.46$). However, the high sensitivity group could discriminate more pairs of fat levels in the fat intensity rating, which implies that individual sensitivity to oleic acid influenced the ability to distinguish fat levels in the real food model.

### *Salivary lipase activity and oral fat perception*

Free oleic acid as a percentage of total fat in expectorated almond samples ranged from 0.024% to 3.75%w/w. Compared with free oleic acid (as % of total fat) in whole ground almond (0.027% to 0.26%), participants with expectorated free oleic acid (%) above 0.26% (n=20) were grouped as "oleic acid producers", and those below 0.26% (n=65) were "oleic acid non-producers". No significant difference in fat "taste" intensity ratings was found between producers and non-producers ($p=0.39$), however, under the "overall" condition, oleic acid producers rated oral fat perception higher than non-

producers (p<0.0001, Figure 1B). Therefore, to some extent, subjects who could produce free oleic acid in the mouth perceived a stronger oral sensation of fat in the cream model.

*CD36 genotype and oral fat perception*

CD36 *rs1761667* genotype influenced perceived fat "taste" intensity rating (p=0.003), where A/A carriers (n=27) had significantly higher perceived fat "taste" intensity than G/G (n=21) and A/G (n=35). No significant effect of *rs1527483* nor *rs3840546* was found on perceived fat intensity (p=0.22, p=0.14, respectively).

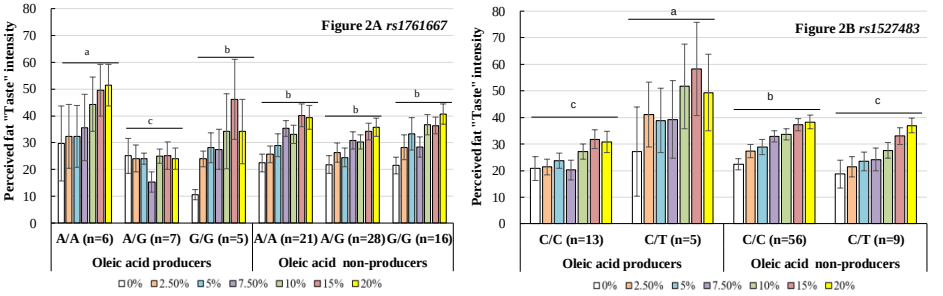


**Figure 2:** Perceived fat "taste" intensity between oleic acid producers and non-producers with different CD36 genotypes at *rs1761667* (A) and at *rs1527483* (B). Bars not sharing a common letter differ significantly (p<0.05) between groups. Error bars represent standard error of the mean.

A significant interaction between *rs1761667* genotypes and oleic acid producers/non-producers, and between *rs1527483* genotypes and producers/non-producers on perceived fat "taste" intensity was observed (p=0.007, p<0.0001, respectively). In oleic acid producers, *rs1761667* A/A carriers (n=6) showed higher perceived fat "taste" intensity than G/G carriers (n=5, p=0.014) and A/G carriers (n=7, p<0.0001, Figure 2A). In addition, *rs1527483* C/T carriers (n=5) presented higher perceived fat "taste" intensity than C/C carriers (n=13) (p<0.0001, Figure 2B). Regarding non-producers, no significant difference in perceived fat "taste" intensity was found between *rs1761667* A/A (n=21), G/G (n=28) and A/G (n=16); however, *rs1527483* C/C carriers (n=56) showed higher perceived fat "taste" intensity than C/T carriers (n=9) (p=0.035, Figure 2B). This implies that the influence of CD36 genotypes on oral fat perception varied between subjects according to their ability to generate free fatty acids.

## Conclusion

Fat can be perceived through taste. Individual preference to high-fat foods varies. High-fat likers had significantly lower fat taste perception in dairy model. Both lipase activity and CD36 genotype influence oral fat perception. The influence of CD36 genotypes on oral fat perception varies between subjects with high or low lipase activity.

## References

1. Godinot N., Yasumatsu, K., Barcos M. E., Pineau N., Ledda M., Viton F., Ninomiya Y., Le Coutre J., Damak S. (2013). Neuroscience 250: 20-30.
2. Stewart J.E., Feinle-Bisset C., Golding M., Delahunty C., Clifton P.M., Keast R.S. (2010). Br J Nutr 104: 145-152.
3. Voigt N., Stein J., Galindo M.M., Dunkel A., Raguse J.D., Meyerhof W., Hofmann T., Behrens M. (2014). J Lipid Res 55: 870-882.
4. Zhou X., Parker J.P., Kennedy O.B., Methven, L. (2016). Chemosens Percept 9: 105-119.
5. Deglaire A., Mejean C., Castetbon K., Kesse-Guyot E., Urbano C., Hercberg S., Schlich P. (2012). Food Qual Prefer 23:110-124.
6. Running C.A., Craig B.A., Mattes D. (2015). Chemical Senses 40: 507–516

# Molecular and cellular mechanisms of the pungent and tingling impression of black pepper (*Piper nigrum* L.)

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## Abstract

Sensory-directed fractionation of an ethanol extract prepared from black pepper corns (*Piper nigrum* L.) followed by LC-TOF-MS, LC-MS/MS, and 1D/2D-NMR experiments as well as synthesis revealed in sum 25 key tingling and/or pungent stimuli, which belong to two chemical classes of non-volatiles: the piperine-type analoges and unsaturated, long-chain fatty acid amides. While sensory evaluation of human recognition threshold concentrations by means of a modified half tongue test demonstrated the structural features causing the tingling and/or pungent impression of these pepper amides, the data obtained did not correlate with those reported for heterologously expressed TRP channels TRPV1 and TRPA1 in literature. Screening experiments with two-pore domain (KCNK, $K_{2P}$) $K^+$ channels suggest that pungent/tingling chemosensates from pepper exhibit a marked effect on three KCNK channels, namely TASK-1 (KCNK3, $K_{2P}$ 3.1), TASK-3 (KCNK9, $K_{2P}$ 9.1) and TRESK (KCNK18, $K_{2P}$ 18.1), respectively, which are likely to play a complementary role to TRP channels in the complex orosensory impression elicited by black pepper corns.

## Introduction

Due to the fact that nearly every cuisine all around the world appreciates black pepper (*Piper nigrum* L.) for its characteristic pungent and tingling orosensory impression, its corns remain the world's most important spice today. Although multiple research groups isolated next to piperine (**1a**), several other piperine-type analoges, like piperylin (**1b**), piperettine (**2b**) or retrofractamide (**3c**), all of which share a piperonal moiety, and identified them as the key players imparting the pungent impression of pepper [1], reliable data published on their human taste threshold concentrations are still lacking (Figure 1). In addition, next to its pungency black pepper corns exhibit a long-lasting tingling impression, which has never been characterized before. Therefore, the first objective of the present investigation was to target the sensory active key molecules in black pepper corns by application of a Sensomics approach. Thereby, we identified 25 key pungent and tingling chemosensates, among which, interestingly, exclusively piperine analogues exhibited a clear pungent orosensory impression while a group of 2,4-dienoic acid amides with an additional *cis*-configurated double bond exhibited both, a pungent and a long-lasting tingling sensation at higher concentrations [2]. However, the data obtained did not correlate with those from heterologously expressed TRP channels, like TRPV1 and TRPA1, which are known to be activated by several pungent sensing amides from black pepper [2-4]. For decades, the pungency perception has rested almost exclusively on polymodal TRP channels. However, there are publications suggesting additional targets for pungent substances [3, 5-9] and we found that even in the presence

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of TRP-antagonists, piperine (**1a**) was still able to activate a fraction of trigeminal neurones. Two-pore domain $K^+$ channels (KCNK) channels are among the most plausible candidates for a complementary role in the chemoperception of pungent stimuli [8,9]. Three of those channels have been previously shown in mice to be molecular targets of the tingling active hydroxy-α-sanshool [10].

Therefore, the aim of the present investigation was, on the one hand to target the sensory active key molecules in black pepper corns by means of a Sensomics approach and on the other hand to investigate whether two-pore domain $K^+$ channels (KCNK) channels could play a physiologically relevant role in their perception.

## Experimental

### *Materials*

Black peppercorn samples were purchased from the German retail market. Prior to the cell-culture assays and the psychophysical experiments, spectroscopic data and the purity (>98%) of each individual pepper amides **1a**−**11c** were checked by means of HPLC-UV, $^{1}H/^{13}C$ NMR, LC-MS/MS, and LC-TOF-MS experiments. Thereby, spectroscopic data were in good agreement with those published in the literature [2]. All experimental procedures including isolation, identification and psychophysical experiments of pepper amides as well as all cell experiments have been described in detail previously [2,8,9].

## Results and discussion

Aimed at characterizing the pungent and tingling orosensory impression of black pepper corns data from human psychophysical experiments, collected by means of a Sensomics approach, were combined with their effect on background $K^+$ currents.

### *Molecular definition of black pepper taste by means of a Sensomics approach*

A Sensomics approach, including taste dilution analysis, followed by UHPLC-TOF-MS, LC-MS/MS and 1D/2D NMR experiments as well as synthesis, led to the structure identification of 12 piperine analogues (**1a-6c**) and 13 2,4-dienoic acid amides (**7a-11c**) [2] (Figure 1). Depending on the chemical structure of the amides, sensory studies by means of a modified half-tongue test revealed human orosensory recognition thresholds of these phytochemicals to range from 3.0 to 1150.2 nmol/cm$^2$ for pungency and from 520.6 to 2162.1 nmol/cm$^2$ for tingling [2]. Interestingly, while the piperine analogues **1a-6c** exclusively exhibited a clear pungent orosensory impression, 2,4-dienoic acid amides with an additional *cis*-configurated double bond in the fatty acid chain (**7a-8c**,**10a-11c**) were found to exhibit both a pungent (at lower concentrations) and a tingling impression (at higher concentrations) [2].

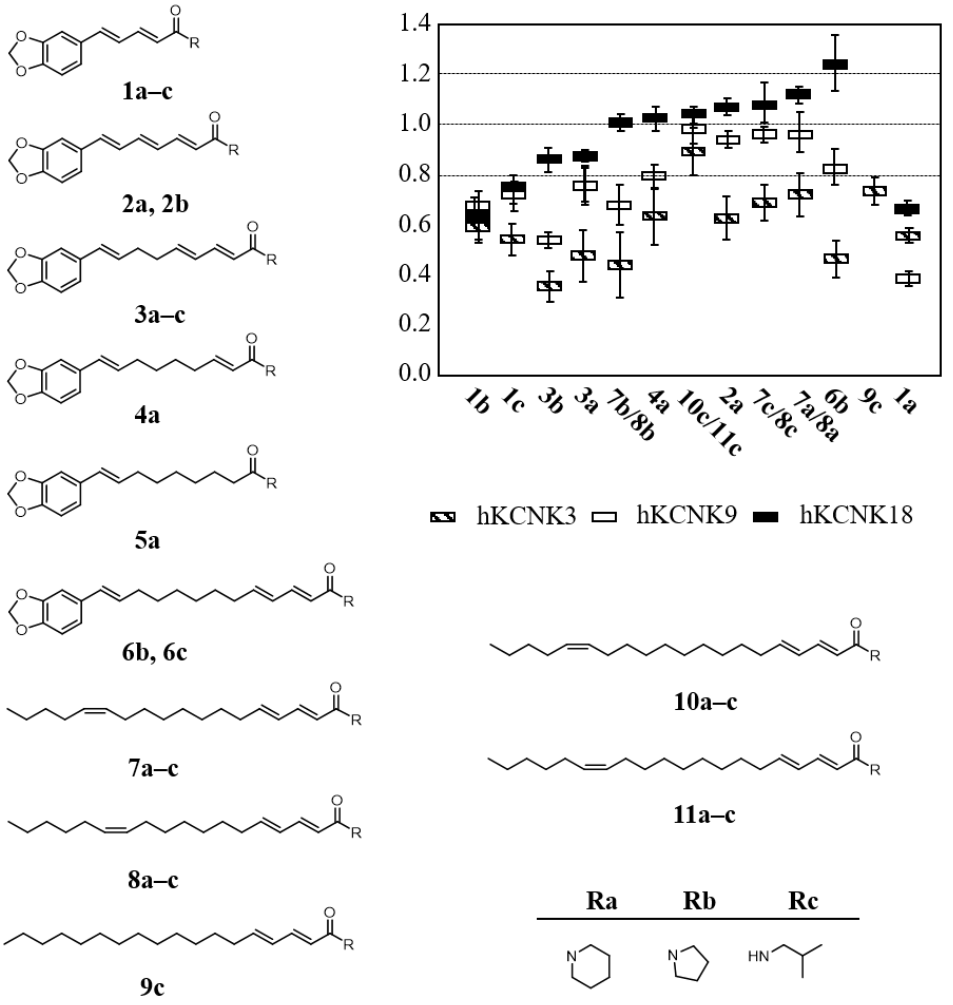


**Figure 1**: The chemical structures of pungent and tingling compounds **1a**-**11c** in black pepper corns and its pungent[p] and tingling[t] recognition threshold concentrations in nmol/ $cm^2$ as reported ealier [8]: piperine (**1a**) [3.0[p]], piperyline (**1b**) [5.1[p]], piperlonguminine (**1c**) [10.4[p]], piperettine (**2a**) [5.2[p]], piperoleine (**2b**) [10.3[p]], dehydropipernonaline (**3a**) [152.1 [p]], 1-[1-oxo-9(3,4-methylenedioxyphenyl)-2E,4E,8E-nonatrienyl]-pyrrolidine (**3b**), retrofractamide A (**3c**) [25.3[p]], pipernonaline (**4a**), piperroleine B (**5a**) [1150.2[p]], brachyamide A (**6b**), guineensine (**6c**) [810.1[p]], 1-(octadeca-2E,4E,13Z-trienyl)piperidine (**7a**), 1-(octadeca-2E,4E,13Z-trienyl)pyrrolidine (**7b**), (2E,4E,13Z)-N-isobutyl-octadeca-2,4,13-trienamide (**7c**) [540.5[p], 2162.1[t]], 1-(octadeca-2E,4E,12Z-trienyl)piperidine (**8a**), 1-(octadeca-2E,4E,12Z-trienyl)pyrrolidine (**8b**), (2E,4E,12Z)-N-isobutyl-octadeca-2,4,12-trienamide (**8c**), (2E,4E)-N-isobutyl-octadeca-2,4-dienamide (**9c**) [763.0[p]], 1-(eicosa-2E,4E,15Z-trienyl) piperidine (**10a**), 1-(eicosa-2E,4E,15Z-trienyl)pyrrolidine (**10b**), isobutyl-eicosa-2,4,15-trienamide (**10c**), 1-(eicosa-2E,4E,14Z-trienyl) piperidine (**11a**), 1-(eicosa-2E,4E,14Z-trienyl)pyrrolidine (**11b**), (2E,4E,14Z)-N-isobutyl-eicosa-2,4,14-trienamide (**11c**) [741.2[p], 1482.3[t]]. The compounds **7c**/**8c** [540.5[p], 2162.1[t]], **10a**/**11a** [260.2[p], 520.6[t]], **10b**/**11b** [405.8[p], 811.6[t]] and **10c**/**11c** [741.2[p], 1482.3[t]] were tasted as binary mixtures. Graphic top right: Normalized currents showing the effect of 12 chemosensates at 1 mM on *Xenopus laevis* oocytes expressing hKCNK3, hKCNK9 and hKCNK18. The currents were normalized to the current measured prior to the application of each tastant (middle line). If a pepper amide induces a change greater than 20% of the basal activity (upper and lower lines), a relevant effect is suggested. Figure adapted by [9].

*Pungent and tingling substances inhibit the human two-pore domain potassium channels TASK-1, TASK-3 and TRESK*

For a long time, the focus of pungent trigeminal chemoperception has rested almost exclusively on two members of the TRP family, TRPV1 and TRPA1 [3,4,8,9]. However, we found that the human recognition threshold concentrations for many pepper amides did not correlate with the data obtained from heterologously expressed TRP channels [2,3,9]. In addition to this, we observed that even in the presence of TRP-antagonists, piperine (**1a**) was still able to activate a large fraction of trigeminal neurones [8]. Therefore, we assumed that additional receptors, like two-pore domain ($K_{2P}$) potassium channels, which have been shown by Noël et al. [11] to "fine-tune" the cellular response to stimuli that activate TRP channels [8, 9], possibly interact with our taste stimuli.

Next to piperine, 6-gingerol and capsaicin 12 other pungent/tingling amides from black pepper corns, which were additionally screened, exhibited a marked effect on two-pore domain (KCNK, $K_{2P}$) $K^+$ channels, namely TASK-1 ($K_{2P}$ 3.1), TRESK ($K_{2P}$ 18.1) and especially TASK-3 ($K_{2P}$ 9.1) (Figure 1) [8,9]. Although tingling compounds from Szechuan pepper have been shown to induce neuronal excitation by inhibiting KCNK channels before [10], our results demonstrate, for the first time, that next to tingling and pungent stimuli, exclusively pungent tasting compounds from *Piper nigrum*, like **1a-c**, **2a**, **3a** or **6c**, possess an inhibitory effect on two-pore domain $K^+$ channels. This inhibitory effect was dose-dependent and fully, although slowly, reversible. Thereby, 1-(octadeca-2*E*,4*E*,13/12*Z*-trienoyl)-pyrrolidine (**7b/8b**) was found to be the most potent naturally occurring inhibitor of *h*KCNK3. In addition, we observed when His98, the amino acid which is thought to be the main proton sensor in TASK-1 and TASK-3 [12,13], is mutated to Glu, the piperine-induced inhibition is significantly reduced [8].

In conclusion, a Sensomics approach led to the structure determination of 25 key phytochemicals, which elicit the typical pungent/tingling flavour of black pepper corns. In addition, our results suggest that pungent/tingling tasting pepper compounds, possesses a marked effect on KCNK channels, especially on KCNK3, which are likely to play a complementary role to TRP channels in the complex orosensory impression elicited by black pepper corns.

## References

1. Friedman M., Levin C.E., et al. (2008) J. Agric. Food Chem., 56, pp. 3028-3036
2. Dawid C., Henze A, et al. (2012) J. Agric. Food Chem., 60 (11), pp. 2884-2895
3. Okumura Y., Narukawa M., et al. (2010) Biosci. Biotechnol. Biochem. 74, pp. 1068-1072
4. McNamara F.N., Randall A., Gunthorp M.J. (2005) Br. J. Pharmacol. 144, pp. 781-790
5. Green B.G. (1996) Pain 68, pp. 245-253
6. Green B.G. (1998) Physiol. Behav. 65, pp. 517-523
7. Caterina M.J., Schumacher M.A., et al. (1997) Nature 23, 816-824
8. Beltrán L.R., Dawid C., et al. (2017) Front. Pharm., 8 (408), pp. 1-14.
9. Beltrán L.R., Dawid C., et al. (2013) Front. Pharm., 4 (141), pp. 1-11.
10. Bautista D.M., Sigal Y.M., et al. (2008) Nat. Neurosci. 11, pp. 772-779.
11. Noël J., Zimmermann K., et. al. (2009) EMBO J. 28, pp. 1308-1318
12. Rajan S., Wischmeyer, E., et al. (2000) J. Biol. Chem. 257, pp. 16650-16657
13. Lopes C.M., Zilberger N., Goldstein S.A. (2001) J. Biol. Chem. 276, pp. 24449-24452

# Temporal processing of odor mixtures in humans

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## Abstract

There is evidence that odorants in a mixture produce their sensations at different times. If true, then temporal processing may be an important component of olfactory decoding. Starting with binary odorant mixtures at which subjects had equal probability to detect one compound or the other, Equal Odd Ratio (EOR), we prepared solutions of each component separately and puffed these separated solutions at different times between 0 to 800 ms (latency times) and different concentration ratios. The results indicate a linear relationship between latency and concentration ratios confounding the meaning of the temporal delays reported in earlier psychophysical experiments.

## Introduction

Human perceptions of odorant mixtures are created from olfactory receptor output combined with information from many brain functions, i.e. memory, emotion, other sensory input, etc. and each of these can operate at different speeds. Using four different odorant pairs Laing in 1994 observed a latency ranging from 92 ms (Carvone-Limonene) to 580ms (Carvone-Benzaldehyde). [1] Twelve years later, Rinberg studied the speed-accuracy tradeoff in mice and observed that the time required to reach the maximal accuracy can be up to 600 ms (harder tasks). [2] In 2015, Resulaj demonstrate that mice process odor information in 70-90ms after odor inhalation indicating that mice can make decisions surprisingly fast. [3] This evidence of temporal differences in human and murine response to different odorants led us to use a sniff olfactometer (SO) to study the effect of stimulus onset time for 3 odorant-pairs and compare these differences between mixtures of the same odorants at different concentration ratios. [1,3,4]

## Experimental

### *Materials*

The three odorants tested had thresholds that ranged over 10,000 fold: benzaldehyde (threshold 350 ppb), R(-)-carvone (threshold 2 ppb) and 2,4,6,-trichloroanisole (threshold 0.027ppb). They were tested starting at 5 times their threshold in binary mixtures and at concentration ratios above and below their equal odds ratio (EOR) in Experiment 1 and as pairs of single component solutions puffed simultaneously in Experiment 2. They were dissolved in ethanol and aliquots diluted to a target concentration with 7% ethanol and water to yield 7% ethanol for all samples tested.

### *Psychophysics*

Four subjects participated in this study; 3 females and 1 male ranging from 25 to 32 years old. None of them were smokers and reported any olfactory dysfunction. They were students and employees of Cornell University's Department of Food Science and did not have any prior experience with this type of psychophysical testing. [5] Figure 1 shows a cartoon of the Sniff Olfactometry (SO) used. The SO delivers a 15ml puff of headspace gas from above 50 ml solutions of odorants with a duration of 70ms. The puffs were presented 500ms after a visual cue directing the subjects to inhale was shown on the monitor. After an additional 750 ms the subjects were asked to answer a question using

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the mouse. Shown on the right in Figure 1 is Binomial Generalized Linear Model (B-GLM) plot of the responses to the cue, "which is stronger: the 'mint' or the 'almond' smell?" replicated 9 times for each of 4 sessions covering a range of responses from 100% "mint" to 100% "almond". The dotted lines indicate the outer limits of the 95% prediction interval for the data. In Experiment 1 they were asked, "which odor was the strongest" but in Experiment 2 they were asked "which odor came first". They answered either "mint" or "almond".

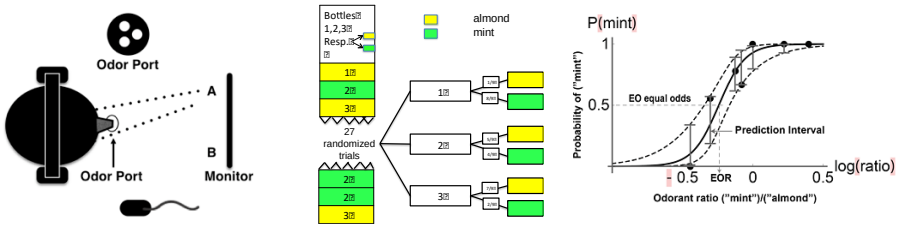


**Figure 1:** The Sniff Olfactometer, DATU, Inc., Geneva, NY, shown in the cartoon on the left shows a subject from above waring noise-canceling headphones, the mouse used for input, the shape and location of the odor port and the monitor used to provide cues to the subject. [4] In the center is shown the script for the 27 randomized trials each replicated 9 times. When testing mixtures, each bottle contains a different concentration ratio. [6] On the right is Binary-GLM fit of the carvone-benzaldyde data showing the probability of "mint", the EOR and the prediction interval (between the dotted lines).

*Experiment (1):*

As outlined on the left in Figure 2 the odorant pairs were tested as a mixed head space above a solution containing both. Iterative tests (n=9) of a range of odor ratios yielded response probabilities for 3 odorant pairs and 4 subjects.

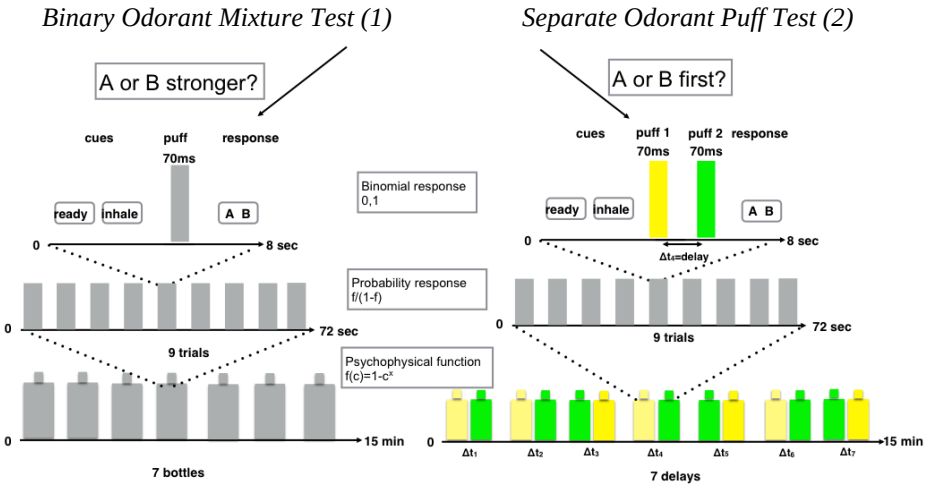


**Figure 2:** Shows the two experimental protocols: 1) used to determine the response probability at different concentration ratios of a binary mixture and 2) used to determine the response probability to the binary odorants puffed separately at various times.

*Experiment (2):*

To test the temporal effect on binary odorant detection, separate solutions were prepared at their EOR concentrations determined in Test (1). These solutions were placed in separate bottles and instead of puffing a mixture two bottles with separate odorants

were puffed simultaneously. Then, they were puffed with different latencies between the puffs. The right side of Figure 2 is a cartoon of this experiment showing the randomly interlaced puffs of single odorants at different delay but all at same concentration ratio, in contrast to Experiment 1 where the samples were presented as single puffs of mixture at different ratios.

**Results and discussion**

Figure 3. summarizes the results of Experiment 1 (a. and b.) and Experiment 2 (c. and d.). The plots are generalized linear model fits to the binomial data produced from the SO. Dotted lines indicate the extent of the 95% prediction intervals and the 3 colors in a. and c. indicate different odorant pairs of the three odorants tested. The 4 colors shown in b. and d. indicate the four different subjects used in the study.

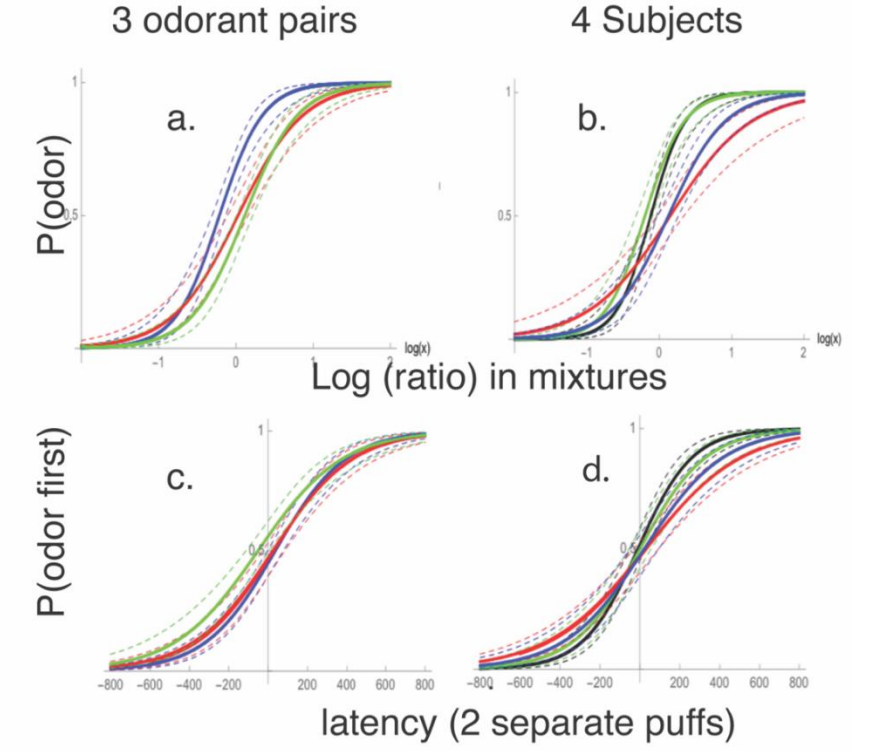


**Figure 3:** Summarizes the resutls of Experiment 1. and 2. Plots a. and b. show the combined Binomial-GLM plots for responses to mixtures of odorant. In a., blue, red and green, are the data from the 3 odorant pairs and b. shows the compined data GLM plots for the 4 different subjects. The EOR concentrations produced by subject "black" was used (arbitrarerly) to define the concentration ratio used in Experiment 2.

It is well documented that odorants differ greatly in their odor potency therefore we would expect the response probabilities for the 3 odorant pairs plotted in Fig.1(a.) to have different plots and indeed they do. Exactly how these differences affect odorant mixture perception remains to be determined but that behavior is compositionally determined is clear. Furthermore, Figure 1 (b.) shows an even greater difference in the perceptions for different subjects to the same odorant pair, a result also well documented in the literature. Far from being a confounding factor it implies that SO studies may be an excellent way to investigate individual differences. Speed is the main advantage SO tests have over

more traditional sensory testing but SO studies require knowledge of the key odorants in a mixture.

In Experiment 2 the subjects were presented with single puffs separated in time by a range of latencies from -800ms to +800ms. All experiments began with odorants in separate bottles at their EOR determined in Experiment 1 for each odorant pair. For every pair and each subject, the response probability was 0.5 when separate puffs of single odorants were presented to the subject as it was when single puffs of mixtures were presented. At the EOR concentrations all the models were within the prediction interval at a 95% probability. In this study, no difference in temporal response different odorants or by different subjects. It is as though the brain does not distinguish between the sensations produced by a puff of air containing a uniform mixture of two different odorants and the sensations produced by two puffs of air each containing uniform sample of a single odorant. Whatever the mechanism that translates odorant composition from a sniff into a perception the intensity of each odorant is concentration dependent and independent of the delivery mechanism, i.e. individually or in a mixture. The receptor system evaluates each odorant separately and the two puffs in the SO do not dilute each other. Such a mechanism would allow organisms to perceive turbulent mixing of odorant sources as undiluted by the turbulence and indicative of the source composition. At least until diffusion completely dilutes the odorants and the gas is uniform.

As both figures c and d indicate the puffing of odorant pairs with different latencies has a marked effect on which odorant is perceived most frequently as first to be detected. All 3 pairs of odorants and all 4 subjects showed the same relationship between probability of detection and latency within a 95% prediction interval and reaching 100% detection of one odorant first with a 600-800ms separation. In light of these results from 4 subjects and 3 odorants it is not clear if the 580ms carvone-benzaldehyde latency measured by Laing was caused by differences in concentration ratios or differences in temporal processing. A better psychophysical experiment may be one that measures the effects of odorant composition on reaction time. [1]

## References

1. Laing, D. G.; Eddy, A.; Francis, G. W.; Stephens, L. (1994) . Brain Research 1994, 651, 317-328.
2. Rinberg, D.; Koulakov, A.; Gelperin, A. (2006) Neuron 2006, 51, 351-8.
3. Resulaj, A.; Rinberg, D. (2015) J Neurosci 2015, 35, 11667-73.
4. Wyckoff, S. G.; Acree, T. E. Multi-Modal Olfactometer - US Patent 9,642,570. May 9, 2017, 2017.
5. Albietz, C. Temporal Processing of Odor Mixtures in Humans. Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland, 2017.
6. Rochelle, M. M.; Prevost, G. J.; Acree, T. E. (2017). J Agric Food Chem 2017.

# Real-time percept flavor balance derived from retronasal threshold and *in vivo* measurements of retronasal aroma release with PTR-MS

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## Abstract

We devised to convert the contributions of retronasal aroma fluctuating with time during consumption into numerical values. Retronasally perceived aroma compounds were directly analyzed with proton transfer reaction mass spectrometry (PTR-MS) while subjects drank several samples of aqueous solutions of aroma compounds. The behavior of aroma compounds released from the nostrils was detected with breath-by-breath AUC (area under curve in a plot concentrations vs time) and was approximated as a power law function. Separately, subjects determined the flavor threshold for each aroma compound by drinking its aqueous solution. AUC of each compound at the 1st breath released from the nostrils was newly defined as retronasal threshold when subjects drunk an aqueous solution at its threshold concentration. Then, the value which was calculated from the AUC of retronasal aroma and retronasal threshold was defined as the contribution value of retronasal aroma (CRA). A contribution of an aroma compound at an arbitrary time after swallowing foods and drinks can be indicated with CRA. Consequently, our study enables the visualization of a perceived flavor balance and calculation of its time changes during consumption.

## Introduction

Retronasal aroma is one of the most important factors for palatability of foods and drinks during consumption. It is different from orthonasal aroma inhaled from the nostrils. It is thought that nosespace analysis of aroma compounds released from the nostrils and olfactory sensation during ingestion of foods and drinks have a high correlation [1]. Aroma extract dilution analysis (AEDA) and odor activity value (OAV) are widely known as methods to determine the contribution value for aroma compounds in a food by sniffing via the orthonasal route; both are very useful to indicate the aroma contribution [2]. Even though several studies reported calculation methods of retronasal OAV by using odor thresholds determined retronasally [3, 4], they did not indicate time changes of the aroma contribution. An aroma contribution on AEDA or OAV is just derived from an aroma compound's concentration in a target sample so that it does not indicate flavor perception nor time changes of aroma during consumption. Real-time measurement of volatile compounds using atmospheric pressure chemical ionization mass spectrometry (APCI-MS) or PTR-MS has been possible since the 1990s, and has been used to measure aroma release from foods or *in vivo* aroma release from nostrils [5, 6]. Few reports, however, referred to time changes of the contribution value of retronasal aroma. In this study, we developed a method which shows time changes of aroma contribution during the ingestion of foods and drinks and predicts the time changes of the perceived flavor balance.

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## Experimental

*Sample preparation for nosespace analysis and odor profile creation*

Nineteen aroma compounds were selected from aroma compounds which are known to be present in a coffee drink, and were separated into several groups in order to analyze them with PTR-MS. Each mixture of compounds was dissolved in water. The concentration of each compound was adjusted to be detected reliably with PTR-MS until the measurement was finished.

Separately, for odor profile verification, a coffee model flavor was prepared with the nineteen aroma compounds mentioned above at an appropriate composition ratio as coffee flavor. The model flavor was added to water purified by ion-exchange at 0.1% w/w, and it was used as a model coffee drink.

*Nosespace analysis with PTR-MS*

A commercial PTR-MS instrument (Ionicon Analytic GmbH, Innsbruck, Austria) was used for nosespace analysis. Two subjects sucked each aqueous aroma solution (10 ml) through a straw and swallowed at once. Then, expiration from each subject's nostrils was measured for one minute under controlling their breathing pattern (once per 3 sec). Each compound released from the nostrils was introduced with a flow of 100 sccm into the drift tube (2.0 mbar, 105 °C, 480 V drift voltage). The E/N ratio was 136 Td. The mass ion counts were normalized to $H_3O^+$ ion counts. The behavior of each retronasal aroma compound analyzed with PTR-MS was changed over to breath-by-breath AUC [7]. The behavior of AUC was approximated as a power law function [8]. The results of two subjects' measurement were averaged.

*Determination of retronasal thresholds*

Nineteen aroma compounds were dissolved in water separately, and were stepwise diluted with dilution factor 10 (0.1ppt to 1000ppm). Two assessors drank aqueous aroma solutions in ascending order of concentrations. The lowest concentration of each aqueous aroma solution that the assessors perceived on average was determined as flavor threshold of the aroma compound, respectively. When subjects drink an aqueous aroma solution, there is a proportional relationship between an aroma concentration in water and the aroma concentration released from a nostril [8]. AUC of each compound at the 1$^{st}$ breath released from nostrils was defined as retronasal threshold when subjects drink an aqueous aroma solution at its threshold concentration.

*Calculation of the contribution value of retronasal aroma (CRA)*

The behavior of AUC of each aroma compound is shown as a power law function by nosespase analysis with PTR-MS. In the case of any aroma concentration in water, a power law function of each compound can be used, because previous reports demonstrated an approximate linear relationship between each aroma concentration in water and breath concentration of each compound [8]. Therefore, AUC at arbitrary breath can be calculated in proportion to the aroma concentration in water. The contribution value of retronasal aroma (CRA) was calculated to divide AUC at arbitrary breath by retronasal threshold.

*Sensory evaluation of odor intensity*

Seven trained panelists assessed the odor intensity of aroma attributes according to seven descriptors (malty, butter, nutty, roast, green coffee bean, brown sugar, smoky/medicine) on a seven-point scale from 0 (not perceivable) to 6 (strongly perceivable) in order to visualize the sensory profile during the ingestion of the model

coffee drink. Each odor intensity was evaluated immediately after swallowing the drink. In addition, each odor intensity was evaluated after about 30 seconds after swallowing the drink.

## Results and discussion

*Prediction of CRAs*

First of all, AUC of the 1st breath of each compound was calculated from each concentration of 19 aroma compounds in water during the ingestion of the model coffee drink. AUC of the 10$^{th}$ breath of each compound was calculated using the individual power law function. The 1$^{st}$ breath is assumed to be right after swallowing the drink and 10$^{th}$ breath is assumed to be after about 30 seconds from swallowing the drink. Then, CRA of each aroma compound was calculated by dividing AUC of 1$^{st}$ and 10$^{th}$ breath, respectively by retronasal threshold, and the results showed that CRA of 11 compounds exceeded 1.0 at 1$^{st}$ breath CRA (Table 1). CRA of other compounds was smaller than 1.0. In other words, this shows that we are not able to recognize these compounds at the concentration level in the model coffee drink, because their AUC of 1$^{st}$ breath was considered smaller than retronasal threshold.

**Table 1:** Contribution values of retronasal aroma (CRA) of 11 compounds in model coffee drink

| Aroma compound | *Aroma Attributes* | *CRA (1$^{st}$ Breath)* | *CRA (10$^{th}$ Breath)* |
|---|---|---|---|
| 2-Methylpropanal | Malty | 10 | 0.1 |
| 2-Methylbutanal | Malty | 36 | 0.1 |
| 3-Methylbutanal | Malty | 40 | 0.2 |
| Diacetyl | Butter | 10 | 1.6 |
| 2-Ethyl-3-methylpyrazine | Nutty | 4 | 1.3 |
| 2-Ethyl-3,5-dimethylpyrazine | Nutty | 20 | 6.7 |
| Furfuryl mercaptan | Roast | 50 | 0.1 |
| Furfuryl alcohol | Roast | 10 | 2.6 |
| 2-Methoxy-3-isobutyl pyrazine | green coffee bean | 50 | 3.9 |
| Methyl cyclopentenolone | brown sugar | 20 | 6.8 |
| Guaiacol | smoky, medicine | 10 | 2.3 |

*Comparison of the sensory profile and the predicted profile*

In order to verify the advantage of our procedure, we compared the CRAs and the results obtained from sensory evaluation. It is difficult to linearly compare the CRAs of aroma compounds with the sensory evaluation because the CRAs were derived from quantitative values and it would be predominated by Fechner law when converted to a predicted flavor profile. So, CRAs of the same aroma attribute were first summed up. Next, the total CRA's value of each aroma attribute was taken as a logarithmic value. In addition, the calculated logarithmic values at 1$^{st}$ breath were multiplied by 2.1 so that the "roast" values in Figure 1A overlapped. And the values at 10$^{th}$ breath were multiplied by 3.7 so that the "brown sugar" values in Figure 1B overlapped. Therefore, the predicted profile and the sensory profile can be easily compared visually.

Figure 1A shows a comparison of the sensory intensity at right after swallowing and the predicted profile at the 1st breath. Profiles resulted in almost overlapping profiles. Figure 1B shows a comparison of the sensory intensity at after about 30 seconds and the predicted value at the 10th breath. Both of them changed over time from Figure 1A. The predicted profile deviated partly from the sensory profile recorded after about 30 seconds. However, we think that the profiles are still similar at several aroma attributes like nutty, green coffee bean, brown sugar, and smoky/medicine.

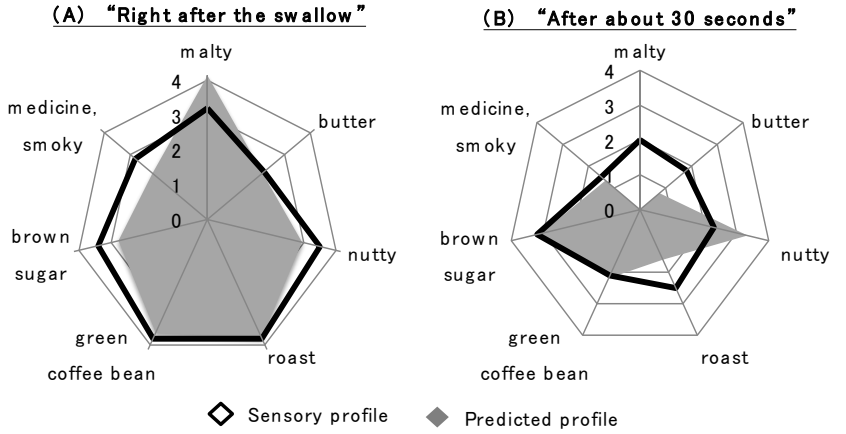


**Figure 1:** Comparison of sensory profile and predicted profile

## Conclusion

We devised a method to predict aroma contributions and the time changes that we perceive after swallowing in the following sequence: *in vivo* measurements of retronasal aroma with PTR-MS, relationship between aroma concentration in water at swallowing and aroma concentration released from nostrils, then newly defined retronasal threshold derived from the relationship. The results of verification using the model coffee drink showed a good correlation between the predicted profile and the sensory evaluation. Therefore, it was suggested that this study is useful to indicate the real-time perceived flavor balance. On the other hand, there is an inevitable deviation between our prediction system and sensory evaluation because cross- or multi-modal sensory integration of olfaction and gustation has occurred during consumption [9]. Individual or genetic differences among subjects should be considered as well in future investigations.

## References

1. M. Mestres, R. Kieffer, A. Buettner (2006) J. Agric. Food Chem., 54, 1814-1821
2. W. Grosch (1994) Flavour and Fragrance Journal, 9, 147-158
3. J. Reiners, W. Grosch (1998) J. Agric. Food Chem., 46, 2754-2763
4. A. Stephan, H. Steinhart (1999) J. Agric. Food Chem., 47, 4357-4364
5. A. J. Taylor, R.S.T. Linforth (1996) Trends in Food Science & Technology, 7, 444-448
6. M. L. Mateus, C. Lindinger, J. C. Gumy, R. Liardon (2007) J. Agric. Food Chem., 55, 10117-10128
7. E. Szymanska, P. A. Brown, A. Ziere, S. Martins, M. Batenburg, F. J. Harren, L. M. C. Buydens (2015) Anal. Chem., 87, 10338-10345
8. M. D. Hodgson, J. P. Langridge, R. S. T. Linforth, A. J. Taylor (2005) J. Agric. Food Chem., 53, 1700-1706
9. J. H. F. Bult, T. Hummel (2007) Neuroscience Letters, 411, 6-10

# Perceptive interactions in red wines: How physico-chemical pre-sensorial effects may affect red wine fruity aromatic expression?

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## Introduction

More than thousand volatiles have been identified in wine. To be perceived, these aroma compounds need to be first volatilized from the matrix to the headspace in order to reach the olfactory epithelium of the taster. From a physico-chemical point of view, compounds' release may be explained by their partition coefficient, which represents the ratio of aroma concentration between gas and liquid phase.

In red wine, a part of the fruity aroma is the consequence of perceptive interactions between various aromatic compounds, thanks to synergistic effects (Lytra et al., 2014, 2015), as well as masking effects, thus modulating the overall fruity expression (Cameleyre et al., 2015; Lytra et al., 2012). Even if these effects have been clearly described, the levels where they occur have been poorly investigated (Tempere et al., 2016).

This work proposes to explore the pre-sensorial level, where the release of flavour-active compounds from the matrix take place, using multiple partition coefficients determination.

## Experimental

*Sensory analysis*

Sensory profiles were established for previously highlighted perceptive interactions. Impact on fruity perception of diacetyl, acetoin, acetic acid and γ-butyrolactone, but also dimethyl sulphide (DMS) at various concentrations and hydroxylated esters was studied.

Typical fruity aroma found in red wine was represented in the fruity aromatic reconstitution by 13 esters at concentrations listed in Table 1.

The panel consisted of 21 judges, 9 males and 11 females aged 28.7 ± 5.3 (mean ± SD) years. All panellists were research laboratory staff at ISVV, Bordeaux University, selected for their experience.

*Partition coefficients calculation*

Partition coefficient ($k_{g/m}$) represents, at the thermodynamic equilibrium, the ratio of concentrations between the gas phase ($C_{gas}$) and the liquid matrix ($C_{liq}$) of a volatile compound:

$$k_{g/m} = \frac{C_{gas}}{C_{liq}}$$

Partition coefficient determination was realized using Phase Ratio Variation (PRV) method developed by Ettre et al (1993), who established the following equation, where the concentration of volatiles in the headspace is proportional to the sample volume in the vial:

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$$\frac{1}{A} = \frac{1}{f_i \times C_i^{liq} \times k_{g/m}} + \frac{1}{f_i \times C_i^{liq}} \times \beta$$

$k_{g/m}$ is the partition coefficient between the gas and the matrix, where A is the chromatographic peak area at the thermodynamic equilibrium, $f_i$ is the detector response factor, $C_i^{liq}$ is the initial compound concentration in the vial and β is the ratio between the headspace ($V_g$) and the liquid ($V_l$) volume (Ettre et al., 1993).

By plotting as 1/A against β, Equation 2 can be written as a linear relationship between 1/A and β, as follows:

$$\frac{1}{A} = a + b\beta$$

Where $a = \frac{1}{f_i \times C_i^{liq} \times k_{g/m}}$ and $b = \frac{1}{f_i \times C_i^{liq}}$.

The value of the partition coefficient $k_{g/m}$ is thus equal to the ratio between a and b, with $k_{g/m} = {}^{b}/_{a}$, expressed as a concentration ratio.

Partition coefficients were determined by plotting the inverse of the chromatographic areas against the phase ratio β, in order to obtain values for a and b. Glass vials (22.8 mL, Chromoptic, France) were filled with 6 amounts of volatiles solutions in dilute alcohol solution (0.05, 0.1, 0.5, 1, 1.5 and 2 mL), with phase ratios from 227 to 10.4 (according to the liquid samples volumes).

**Table 1:** Ethyl ester concentrations used for sensory analysis

| *Ethyl Esters and Acetates (µg/L)* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $C_3C_2$ | $C_4C_2$ | $C_6C_2$ | $C_8C_2$ | $2MeC_3C_2$ | (2S)-$2MeC_4C_2$ | (2S)- and (2R)-$2OH4MeC_5C_2$ | $C_2C_4$ | $C_2C_6$ | $C_2iC_4$ | $C_2iC_5$ | $3OHC_4C_2$ | $3MeC_4C_2$ |
| 150 | 200 | 200 | 200 | 250 | 50 | 400 | 10 | 2 | 50 | 250 | 300 | 50 |

$C_3C_2$, ethyl propanoate; $C_4C_2$, ethyl butanoate; $C_6C_2$, ethyl hexanoate; $C_8C_2$, ethyl octanoate; $2MeC_3C_2$, ethyl 2-methylpropanoate; S-$2MeC_4C_2$, S-ethyl 2-methylbutanoate; $2OH4MeC_5C_2$, ethyl 2-hydroxy-4-methylpentanoate; $C_2C_4$, butyl acetate; $C_2C_6$, hexyl acetate; $C_2iC_4$, 2-methylpropyl acetate; $C_2iC_5$, 3-methylbutyl acetate; $3OHC_4C_2$, ethyl 3-hydroxybutanoate; $3MeC_4C_2$, ethyl 3-methylbutanoate; 2MB, 2-methylbutan-1-ol; 3MB, 3-methylbutan-1-ol; 2MP, 2-methylpropan-1-ol; P, propan-1-ol; B, butan-1-ol.

## Results and discussion

### *Sensory profiles establishment*

Sensory analysis, and more precisely sensory profiles in dilute alcohol solution, showed modulation of fruity aroma perception in the presence of diacetyl, acetoin, acetic acid and γ-butyrolactone, but also with addition of dimethyl sulfide (DMS) at various concentrations (5, 10, 50 and 70 µg/L) or hydroxylated esters. Presence of these molecules led to masking and synergistic effects of fruity aromatic reconstitution, *via* some remarkable sensory interactions (results not shown).

*Esters partition coefficients determination*

SHS-LP-GC/MS method was applied, in order to research potential modifications of equilibrium release in headspace when compounds were mixed together.

Significant decrease in esters partition coefficients was observed demonstrating a masking effect when diacetyl, acetoin, acetic acid and γ-butyrolactone were added. This indicated a reduction in esters' presence in the headspace (Figure 1). This fact may explain, at least partially, that the taster was stimulated differently and was therefore possibly related to the masking effect observed for fruity notes, with an existence of pre-sensorial effects.

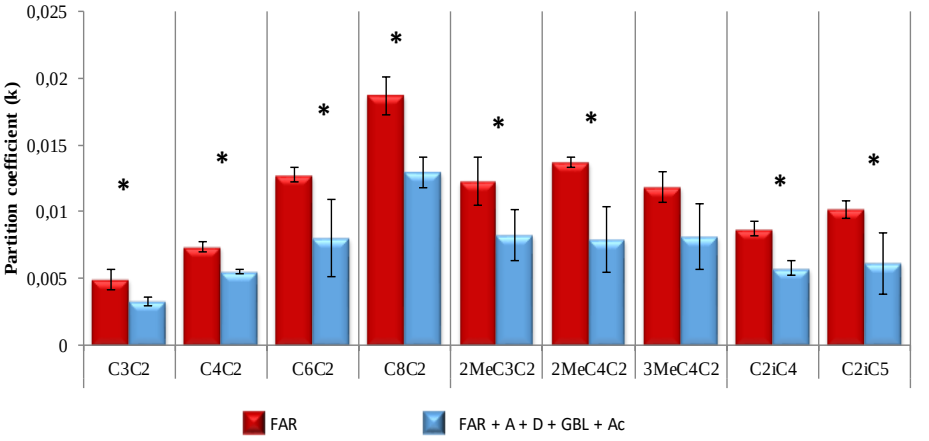


**Figure 1:** Impact of diacetyl, acetoin, acetic acid and γ-butyrolactone on partition coefficient of ethyl esters and acetates in dilute alcohol solution (12% v/v.). *, 5% significant level; error bars represent standard deviation; D, diacetyl; A, acetoin; Ac, acetic acid; GBL, γ-butyrolactone; FAR, fruity aromatic reconstitution.

DMS addition led to an increase in esters partition coefficients, especially as DMS concentration increased. Moreover, adding the 13 esters also led to an intensification of DMS partition coefficients (Figure 2). These observations suggested an intensification of these compounds release in the headspace when they were mixed together in dilute alcohol solution. These results may be correlated with the ones obtained using sensory analysis. Indeed, addition in fruity aromatic reconstitution of increasing concentrations of DMS led to a significant synergistic perception of black-fruit and blackcurrant notes.

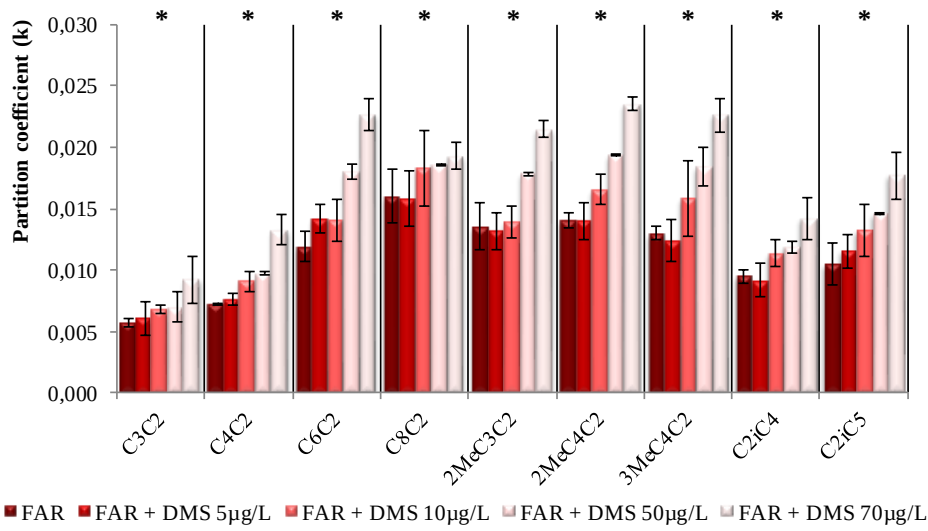


**Figure 2:** Impact of DMS at various levels on partition coefficient of esters in dilute alcohol solution (12% v/v.). *, 5% significant level; error bars represent standard deviation; FAR, fruity aromatic reconstitution.

The analytical tool application for hydroxylated esters highlighted that omission of ethyl 2-hydroxy-4-methylpentanoate or ethyl 3-hydroxybutanoate from fruity aromatic reconstitution did not change the partition coefficients of the other esters (results not shown). This fact was surprising, because omitting ethyl 2-hydroxy-4-methylpentanoate or ethyl 3-hydroxybutanoate from fruity aromatic reconstitution led to decrease of black- and fresh-fruit or red-, fresh- and jammy-fruit perception, respectively, even if these compounds were present at level below their olfactory thresholds. These data suggested that these synergistic effects related to hydroxylated esters were not the result of pre-sensorial interactions, but the consequence of interactions at sensorial level.

In general, our work highlighted the complexity of the mechanisms involved in perceptual interaction phenomena, whose origins can take place on several levels. They also contribute to the understanding of new perception modifications, especially between fruity and non-fruity compounds found in red wines.

## References

1. Cameleyre, M., Lytra, G., Tempere, S., and Barbe, J.-C. (2015). J. Agric. Food Chem. *63*, 9777–9788.
2. Ettre, L.S., Welter, C., and Kolb, B. (1993). Chromatographia. *35*, 73–84.
3. Lytra, G., Tempere, S., Revel, G. de, and Barbe, J.-C. (2012). J. Agric. Food Chem. *60*, 12260–12269.
4. Lytra, G., Tempere, S., Zhang, S., Marchand, S., De Revel, G., and Barbe, J.-C. (2014). J. Int. Sci. Vigne Vin *48*, 75–85.
5. Lytra, G., Cameleyre, M., Tempere, S., and Barbe, J.-C. (2015). J. Agric. Food Chem.
6. Tempere, S., Schaaper, M.H., Cuzange, E., de Lescar, R., de Revel, G., and Sicard, G. (2016). Food Qual. Prefer. *50*, 135–144.

# Why does this wine smell like apricots?

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## Abstract

The compounds in white wine that give rise to varietal 'stone fruit' aroma characters are not well understood. Relating the compositional differences among wines to their sensory attributes can help uncover the cause of specific sensory differences. Viognier and Chardonnay wines with differing levels of 'stone fruit' character, ranging from low to high intensity, were characterised by sensory descriptive analysis and comprehensive quantitative chemical analysis. Several aroma compounds were positively associated with the 'apricot' aroma attribute notable in some of the Viognier wines, including γ-lactones, monoterpenes and aldehydes. Sensory reconstitution experiments verified that a mixture of three monoterpenes, linalool, geraniol and nerol, was the most important group for the model to be perceived as having an 'apricot' attribute.

## Introduction

'Stone fruit' aroma attributes are important to many varieties of white wine, such as Chardonnay and Viognier. Some γ-lactones, monoterpenes and aldehydes have been reported as important aroma compounds in fresh stone fruits [1]. However, very little is known about the chemical basis of 'stone fruit' aromas in wine. Previous Chardonnay wine sensory studies have included a 'stone fruit' descriptor but these studies did not focus on wines with clear 'stone fruit' aroma attributes. Little has been reported about aroma compounds in Viognier wine. Multivariate statistical techniques can be used to find relationships between compositional differences among wines to their sensory attributes [2]. Using an appropriate set of wines, highlighting the sensory attribute of interest, is more likely to provide well modelled predictions of the aroma compounds responsible for that attribute and to help determine if sensory difference is due to variation in concentrations of aroma compounds, an absence/presence of certain aroma compounds or an additive effect of several aroma compounds. To confirm or dismiss these predictions, reconstitution, addition and omission experiments in a realistic model are required [3].

## Experimental

### *Materials*

A set of 18 commercially available wines (six wines each: Australian Chardonnay; Australian Viognier; and French Viognier) were selected by a small group of experienced wine tasters from 75 potential wines to encompass a wide range of 'stone fruit' intensities.

### *Sensory evaluation*

Descriptive sensory analysis was carried out on the wines using similar methodology to Mayr et al. 2014 [4]. A sensory panel, consisting of 10 trained panellists, rated the intensity of sensory attributes of the wines in triplicate under controlled conditions.

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*Analysis of the volatile compounds*

Wines were characterised by comprehensive quantitative chemical analysis, targeting over 100 volatile aroma compounds using previously published methods [5-13] with stable isotope dilution analysis (SIDA-MS) and basic wine chemical composition.

*Statistical analysis*

Sensory panel performance was evaluated using Fizz, Senstools and PanelCheck software. Analysis of variance (ANOVA) was carried out using Minitab 17.1.0. Following ANOVA, Fisher's least significant difference (LSD) value was calculated ($P = 0.05$). The sensory attribute ratings were related to chemical composition by partial least squares regression (PLSR) using The Unscrambler X software.

*Aroma reconstitution*

Preliminary model reconstitutions were promising for 'apricot' but not for 'peach'. Therefore, only apricot was further investigated.

Addition and omission descriptive sensory studies were conducted in a wine-like base model wine: ethanol (13.3 %v/v), tartrate (2 g/L), glucose/fructose (2 g/L), glycerol (4.6 g/L), citric acid (0.4 g/L), malic acid (2.6 g/L), succinic acid (0.6 g/L), $SO_2$ (20 mg/L), pH 3.33 and food colouring. All models contained a mixture of 55 aroma compounds (mean concentrations of the 18 wines from the wine sensory study, Table 1). The aroma compounds predicted from the PLSR to be important to apricot were added as groups at the mean concentrations measured in the three wines with the highest intensity rating of apricot (Table 1). Sensory assessments were performed by a panel of eight in the same manner as for the wines, but only aroma attributes were assessed not palate.

**Table 1:** Volatile compounds included in Control model reconstitution and concentrations of compounds that were included in the reconstitution study for 'apricot' aroma

| *Compounds in Control model* | *Addition to Control model* | | *µg/L* |
|---|---|---|---|
| 14 ethyl and acetate esters | Lactones | γ-nonalactone | 5.9 |
| 5 alcohols | | γ-decalactone | 1.2 |
| 8 fatty acids | | (Z)-6-dodeceno-γ-lactone | 0.05 |
| 3 sulphur compounds | Monoterpenes | linalool | 83 |
| 3-mercaptohexanol | | geraniol | 27 |
| 8 oak-derived volatiles | | nerol | 5.3 |
| 10 oxidation-related volatiles | Aldehydes | benzaldehyde | 206 |
| 2,3-butanediol | | (*E*)-2-hexenal | 0.37 |
| α-terpineol, β-damascenone | | (*E*)-2-nonenal | 1.8 |
| γ-octa and γ-decalactone | | (*E*)-2-hexenol | 3.2 |
| 6-amyl-α-pyrone | | | |

## Results and discussion

*Relationships between the sensory and chemical data*

The sensory descriptive analysis data established that the selected 18 wines had distinct descriptors of stone fruit aroma attributes, 'apricot' (tinned apricots) and 'peach' (fresh white peach), with widely differing intensity ratings. Notably, the two stone fruit attributes were not closely correlated.

Of the 104 targeted wine aroma compounds, 79 were detectable and quantified in the 18 wines. The odour activity values (OAVs) of many of the aroma compounds were below 0.5. However, OAVs do not account for additive, synergic, antagonistic or perceptual interactions that might be occurring. Hence, all compositional measurements were used in the multivariate analysis.

From the PLSR analysis, several aroma compounds were positively associated with the 'apricot' aroma attribute notable in some of the Viognier wines (Figure 1). γ-Nonalactone, γ-decalactone and the previously little studied and potent aroma compound (Z)-6-dodeceno-γ-lactone were associated with the apricot sensory attribute, together with the monoterpenes linalool, geraniol and nerol, several aldehydes and (E)-2-hexenol. 3-Mercaptohexyl acetate and *trans*-ethyl cinnamate were negatively correlated. In contrast, other wine aroma studies have generally reported monoterpenes to imbue 'floral', 'citrus' and 'pine-like' characters [14]. The 'peach' aroma attribute was associated with a range of fermentation-derived ethyl and acetate esters, fatty acids and monoterpenes (Figure 1). However, the association with monoterpenes was strongly influenced by a few Viognier wines rated highly in both 'apricot' and 'peach', thus confounding the 'peach' reconstitution model.

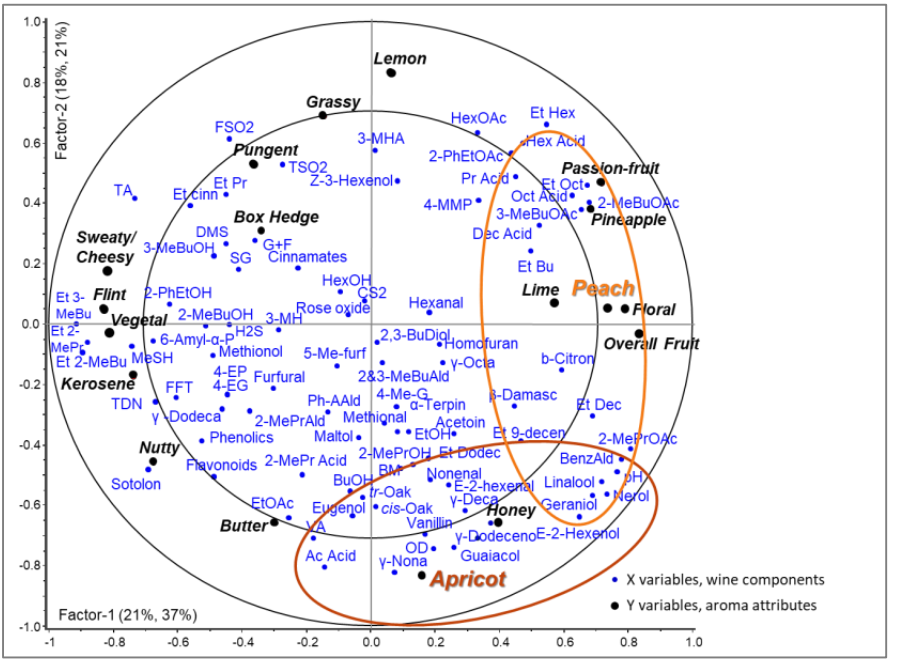


**Figure 1:** PLSR of chemical composition and sensory data loadings plot: volatile aroma compounds that explain apricot and peach aroma attributes.

## *Aroma reconstitution*

All models containing the monoterpenes were rated higher in 'apricot' than the Control model (Figure 2). However, the lactones did not increase the intensity of 'apricot'. Aldehyde additions did not enhance 'apricot' scores (data not shown). 'Peach' aroma attribute intensities were not significantly different across the models.

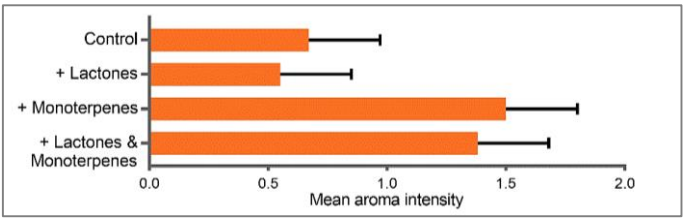


**Figure 2:** Mean ratings of 'apricot' aroma attribute in white wine reconstitution models. Compounds in the reconstitution models are listed in Table 1. Error bars are plus half LSD (P = 0.05).

'Tropical', 'confection/floral', and 'cardboard' aroma attribute intensities were significantly different across the models ($p < 0.05$) and the effects of the additions and omissions for these attributes were varied (data not shown). To our knowledge, this is the first time that this comprehensive approach has been used to identify the aroma compound(s) responsible for a particular wine aroma attribute from a set of wines specifically selected with that attribute. Subsequently, reconstitution studies confirmed that the mixture of the three monoterpenes linalool, geraniol and nerol, in the presence of ubiquitous wine compounds, was the most important group for the model to be perceived as having an 'apricot' attribute.

**Acknowledgements**

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## References

1. Greger, V., Schieberle, P. (2007) J. Agric. Food Chem. 55, 5221-5228.
2. Noble, A.C.; Ebeler, S.E. (2002) Food Rev. Int. 18, 1-20.
3. Polášková, P., Herszage, J., Ebeler, S.E. (2008) Chem. Soc. Rev. 37, 2478-2489.
4. Mayr, C.M., Geue, J.P., Holt, H. E., Pearson, W.P., Jeffery, D.W., Francis, I.L. (2014) J. Agric. Food Chem. 62 (20), 4528-36.
5. Siebert, T.E., Smyth, H.E., Capone, D.L., Neuwohner, C., Pardon, K.H., Skouroumounis, G., Herderich, M.J., Sefton, M.A., Pollnitz, A.P. (2005) Anal. Bioanal. Chem. 381, 937-947.
6. Siebert, T.E., Solomon, M.R., Pollnitz, A. P., Jeffery, D.W. (2010) J. Agric. Food Chem. 58, 9454-9462.
7. Capone, D.L., Ristic, R., Pardon, K.H., Jeffery, D.W. (2015) Anal. Chem. 87, 1226-1231.
8. Pollnitz, A.P. Pardon, K.H., Sykes, M., Sefton, M.A. (2004) J. Agric. Food Chem. 52, 3244-3252.
9. Mayr, C.M., Capone, D.L., Pardon, K.H., Black, C.A., Pomeroy, D., Francis, I.L. (2015) J. Agric. Food Chem. 63, 3394-3401.
10. Varela, C.A., Kutyna, D. R., Solomon, M.R., Black, C.A., Borneman, A., Henschke, P.A., Pretorius, I.S., Chambers, P.J. (2012) Appl. Environ. Microbiol. 78, 6068-6077.
11. Pedersen, D.S. Capone, D.L., Skouroumounis, G.K., Pollnitz, A.P., Sefton, M.A. (2003) Anal. Bioanal. Chem. 375, 517-522.
12. Cooke, R.C. Capone, D.L., Van Leeuwen, K.A., Elsey, G.M., Sefton, M.A. (2009) J. Agric. Food Chem. 57, 348-352.
13. Siebert, T.E., Barker, A., Barter, S.R., De Barros Lopes, M.A., Herderich, M.J., Francis, I.L. (2017) Food Chem. submitted for publication.
14. Black, C.A., Parker, M., Siebert, T.E., Capone, D.L., Francis, I. L. (2015) Aust.J. Grape Wine Res. 21, 582-600.

# Structure-odor relationship study of C-6 unsaturated acyclic monoterpene alcohols: A comparative approach

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## Abstract

Acyclic monoterpenes are a valuable class of compounds useful for the flavor and fragrance industries [1]. Among them are the C-6 unsaturated monoterpene alcohols, namely linalool, geraniol, nerol and β-citronellol. These substances exhibit pleasant smell properties, are prevalent in the essential oils of many plants and are pharmacologically and physiologically active. Thereby, it is interesting to note that linalool and geraniol, specifically, do not only activate olfactory receptors, but have also other physiological activities, e.g. acting as anti-cancerogens [2, 3]. Systematic elucidation of the sensory characteristics of metabolic derivatives of this substance group, however, is very limited as most work, until today, focused on the basic acyclic monoterpene compounds. Our studies demonstrated that a series of these metabolites are odor active compounds, at times exhibiting exceptionally pleasant smells [4, 5]. In the course of our studies, we started from the respective monoterpene alcohols and their corresponding acetates, yielding a total of 24 oxygenated derivatives via diverse synthetic strategies, and characterized their olfactory properties. Specifically, these compounds were tested with regards to their odor qualities, relative odor thresholds (OTs) in air, and potential inter-individual variations in human sensory perception for each single substance. Finally, a comprehensive substance library was established comprising the respective retention index data (RI values) as well as mass spectrometric and nuclear magnetic resonance data, to aid in future analytical studies on this sensorially fascinating substance class.

## Introduction

Apart from being fragrant compounds, linalool, geraniol, nerol and citronellol are characterized by several pharmacological and physiological properties. Linalool, found in lavender plant, potentiates $GABA_A$ receptor modulatory activity in the central nervous system; this mechanism is supposed to be the underlying principle for sleep-inducing and balancing effects in humans [6]. Similarly, nerol, present in lemon balm, showed to exhibit an anxiolytic effect in mice [7]. Geraniol, found in palmarosa, is a plant-based insect repellent especially active against mosquitos [8]. β-citronellol, a main component of lemon grass leaves, has showed a vasodilatory effect and therefore is claimed to be a hypotensive agent [9]. These monoterpenes and their acetate esters have previously been studied in view of their odor characters, without comprehensively correlating these smell properties with their chemical structure. In addition, the metabolic derivatives of these compounds in plants and animals have been studied [10, 11]. The main metabolic pathway includes C-8 hydroxylation of these monoterpenes yielding 8-hydroxy compounds which are further oxidized to the corresponding 8-carboxy derivatives. Due to the lack of commercial availability of these metabolites, the present work aimed at the synthesis of a total of 24 C-8 oxygenated compounds, and the determination of their odor qualities and odor thresholds (OT) in air using gas chromatography-olfactometry (GC-O). It was found that most of these derivatives elicited distinct smells [4, 5, 12]. Therefore,

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a structure-odor relationship study was established in a comparative approach comprising all the aforementioned monoterpenes, their acetates and their derivatives, highlighting the main structural features and functional groups that impact the odor quality and potency of this substance class.

## Experimental

### *Syntheses*

General synthetic pathways are shown in Figure 1. Chemicals required for synthesis were purchased from Sigma-Aldrich or Fischer Scientific. Data comprising nuclear magnetic resonance spectra ($^{1}H$ and $^{13}C$), mass spectra as well as retention indices were recorded and are described in Elsharif, Banerjee [4], Elsharif and Buettner [5], Elsharif and Buettner [12].

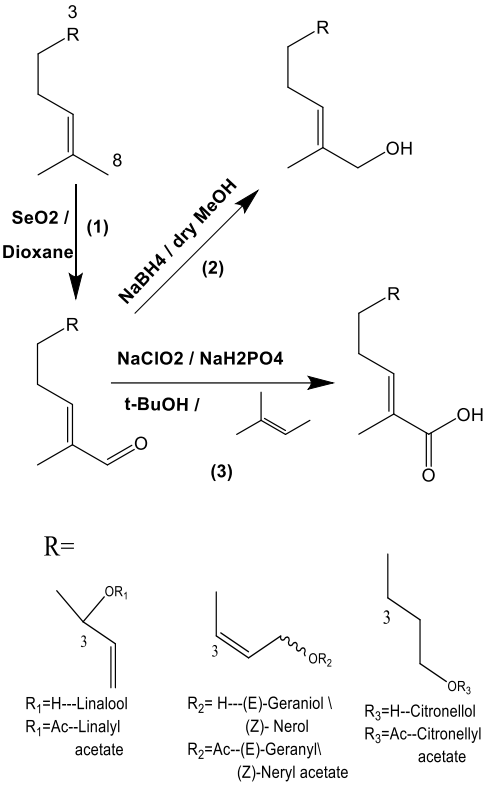


**Figure 1:** General synthetic pathways leading to the oxygenated derivatives.

### *Evaluation of odor quality and odor threshold*

Odor qualities and thresholds in air were determined according to the procedure of Czerny, Brueckner [13] using GC-O involving five panelists who are trained volunteers from the University of Erlangen. Compounds were evaluated by each panelist repeatedly on different days on different capillary columns (DB-FFAP and DB-5). Panelists were asked to relate their sensory impression to an in-house developed flavor language.

## Results and discussion

Tables 1 (monoterpene alcohols and their oxygenated derivatives) and 2 (monoterpene acetates and their oxygenated derivatives) show a comparison of the odor attributes perceived by at least 60% of the panel and group odor thresholds calculated as a geometric mean of the individual thresholds of panelists.

We found that parent monoterpene alcohols and their 8-hydroxy derivatives elicited citrus-like, fresh odor attributes (Table 1). Only two 8-oxo derivatives, 8-oxolinalool and 8-oxocitronellol showed similar odor attributes, i.e. citrus-like and fresh. On the other hand, 8-oxogeraniol and 8-oxonerol exhibited a fatty, musty odor. All 8-carboxy derivatives of this group were odorless with the sole exception of the 8-carboxynerol which elicits a fatty, waxy odor. Odor potencies of the parent monoterpene alcohols were much higher than that of their corresponding oxygenated derivatives. Although the additional OH group at C-8 preserved the citrus-like odor of the parent monoterpene alcohols, it tremendously decreased their potency. In case of 8-oxogeraniol and 8-oxonerol, the aldehyde group turned the odor to musty. A C-8 carboxy group added to linalool, geraniol or citronellol yielded odorless substances. To sum up: 1) the OH-group at C-1 or C-3 is responsible for the citrus-like odor and the low OT of the parent monoterpenes, 2) an additional OH at C-8 only retains the odor quality but not the potency, 3) oxidation of the OH at C-8 to the corresponding aldehyde group commonly turns the odor to musty and fatty, and 4) further oxidation of the aldehyde to the respective acid leads to odorless compounds.

**Table 1:** Odor qualities and thresholds for the acyclic monoterpene alcohols and their synthesized oxygenated derivatives

| Name | *Odor quality*[a] | *% of panelists* | *Odor threshold*[b,c] *ng/L$_{air}$* |
|---|---|---|---|
| Linalool | Citrus | 80 | 3.2 |
| Geraniol | Citrus, fresh, fatty | 80, 60 | 11.5 |
| Nerol | Citrus, fresh | 60 | 68 |
| β-Citronellol | Citrus, floral, fresh | 100, 40 | 11 |
| 8-Oxolinalool | Citrus, fatty | 80, 60 | 50 |
| 8-Oxogeraniol | Fatty, musty | 60 | 139 |
| 8-Oxonerol | Fatty, musty | 80, 60 | 534.4 |
| 8-Oxocitronellol | Citrus, fresh | 80, 60 | 879 |
| 8-Hydroxylinalool | Citrus, sweet | 80, 40 | 123.6 |
| 8-Hydroxygeraniol | Citrus, fatty | 60 | 310.2 |
| 8-Hydroxynerol | Citrus, sweet, vanilla | 40, 60 | 451 |
| 8-Hydroxycitronellol | Citrus, fresh | 100, 80 | 233 |
| 8-Carboxynerol[d] | Fatty, waxy | 40 | 297 |

The parent monoterpene acetates elicited similar odor characters closely related to their monoterpene alcohols (citrus-like) with the sole exception of neryl acetate which smells sweet and phenolic (Table 2). Similarly, the 8-hydroxy acetates provoked citrus-like, soapy smell. 8-Oxogeranyl and 8-oxocitronellyl acetates were perceived as fatty, musty and rotten, musty. Interestingly, all 8-carboxy acetates were found to be odor active compounds with the sole exception of 8-carboxycitronellyl acetate. The panel described their smells as fatty for 8-carboxylinalyl acetate, sweet and coconut-like for 8-carboxygeranyl acetate, and green for 8-carboxyneryl acetate.

**Table 2:** Odor qualities and thresholds for the acyclic monoterpene acetates and their synthesized oxygenated derivatives

| Name | *Odor quality*[a] | *% of panel* | *Odor threshold*[b,c] *ng/L*$_{air}$ |
|---|---|---|---|
| Linalyl acetate | Citrus, fatty | 60 | 134 |
| Geranyl acetate | Citrus | 60 | 57.1 |
| Neryl acetate[d] | Phenolic, sweet | 40 | 108 |
| Citronellyl acetate | Citrus, soapy | 60 | 665 |
| 8-Hydroxylinalyl acetate | Citrus, fresh | 80, 60 | 120.3 |
| 8-Hydroxygeranyl acetate | Citrus, soapy | 80, 60 | 62 |
| 8-Hydroxyneryl acetate[d] | Citrus | 80 | 92 |
| 8- Hydroxycitronellyl acetate | Citrus, soapy | 100, 80 | 1261 |
| 8-Oxolinalyl acetate | Citrus, fatty | 60 | 6 |
| 8-Oxogeranyl acetate | Fatty, musty | 60 | 20.5 |
| 8-Oxoneryl acetate | Citrus, fatty | 80 | 26.1 |
| 8-Oxocitronellyl acetate | Musty, rotten | 80, 60 | 346 |
| 8-Carboxylinalyl acetate | Fatty | 100 | 7 |
| 8-Carboxygeranyl acetate | Sweet, coconut | 60, 40 | 37.1 |
| 8-Carboxyneryl acetate | Green | 40 | 24 |

[a]Common odor attributes given by the panel as perceived at the sniffing port. [b]Odor thresholds in air were determined as described by Ullrich and Grosch [14]. [c]Odor threshold was calculated as a geometric mean of the individual thresholds of panelists. [d]Anosmia observed.

The findings can be summarized as follows: 1) the acetate group at C-1 or C-3 decreases the odor potency at least by a factor of 5, but preserves the citrusy odor of the parent monoterpene alcohols with the sole exception of neryl acetate, 2) addition of an OH- group at C-8 enhances the citrus odor with an increase in potency, 3) the C-8 aldehyde group leads to the appearance of a musty odor for 8-oxogeranyl and 8-oxocitronellyl acetates, and 4) an acid moiety at C-8 of the acetates induces odor attributes other than citrusy, but with a further increase in odor potency. It is important to note that single cases of anosmia were observed for individuals with the following compounds: 8-hydroxynerol, neryl acetate and 8-hydroxyneryl acetate.

## References

1. King, A.J. and J.R. Dickinson, (2003) FEMS Yeast Research, 3(1): p. 53-62.
2. Sun, X.-B., Wang, S.M., Li, T., and Yang Y.Q., (2015) Trop. J. Pharm. Res., 2015. 14(4): p. 619-625.
3. Madan, K.A. and T. Devaki, (2015) Int. J. Pharm. Pharm. Sci., 7(4): p. 67-70.
4. Elsharif, S.A., A. Banerjee, and A. Buettner, (2015) Frontiers in chemistry, 3: p. 57.
5. Elsharif, S.A. and A. Buettner, (2016) Journal of Agricultural and Food Chemistry,
6. Kessler, A., H. Sahin-Nadeem, S.C.R. Lummis, I. Weigel, M. Pischetsrieder, A. Buettner, and C. Villmann (2014) Mol Nutr Food Res, 58(4): p. 851-62.
7. Marques, T.H.C.,et.al., (2013) World Journal of Neuroscience, 2013. 03(01): p. 32-38.
8. Omolo, M.O., M.O.Omolo, D. Okinyo, I.O. Ndiege, W. Lwande, and A. Hassanali, (2004) Phytochemistry, 65(20): p. 2797-802.
9. Ribeiro-Filho, H.V., de Souza Silva C.M., de Siqueira R.J., Lahlou S., dos Santos A.A., and Magalhães P.J., (2016) European Journal of Pharmacology, 775: p. 96-105.
10. Luan, F., A. Mosandl, A. Degenhardt, M. Gubesch, and M.Wüst, (2006) Analytica Chimica Acta, 563(1-2): p. 353-364.
11. Chadha, A. and K.M. Madyastha, (1984) Xenobiotica, 14(5): p. 365-74.
12. Elsharif, S.A. and A. Buettner, (2017) Food Chemistry, 232: p. 704-711.
13. Czerny, M., R. Brueckner, E. Kirchhoff, R. Schmitt, and A., (2011) Chem Senses, 36(6): p. 539-53.
14. Ullrich, F. and W. Grosch, (1987) Zeitschrift für Lebensmittel-Untersuchung und Forschung, 184(4): p. 277-282.

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# IMPACT OF FLAVOUR COMPOUNDS ON HUMANS

# Taste receptors in respiratory innate immunity

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## Abstract

Over the past several years, taste receptors have emerged as key players in the regulation of innate defenses in the mammalian respiratory tract. Several cell types in the airway, including ciliated epithelial cells, solitary chemosensory cells, and bronchial smooth muscle cells all display chemoresponsive properties that utilize taste receptors. A variety of bitter products secreted by microbes are detected with resultant downstream inflammation, increased mucous clearance, antimicrobial peptide secretion, and direct bacterial killing. Genetic variation of bitter taste receptors also appears to play a role in susceptibility to infection in respiratory disease states, including chronic rhinosinusitis. Ongoing taste receptor research may yield new therapeutics that harness innate defenses in the respiratory tract and offer alternatives to antibiotic treatment.

## Introduction

Canonically, taste has been thought of as an adaptive sense for organisms that feed on matter in the environment: food that nourishes is considered to have a pleasant taste, while poisons and inedible material tend to be far less palatable. Specifically, bitter taste receptors are often tuned to respond to toxic chemicals or products that compromise digestive health. Over the past several years, a growing body of literature supports a broader role for taste receptors throughout the body, with functions extending far beyond the sensory capacity of the tongue [1-6]. Both bitter and sweet taste receptors are expressed in the airway, where they appear to play several important roles in innate defenses [7, 8].

## Taste receptor mechanisms

Bitter and sweet taste receptors are G-Protein Coupled Receptors (GPCR's) that were first identified in taste bud type II cells [9, 10]. Those from Taste Receptor Family 1 subtype 2 and 3 (TAS1R2/TAS1R3) respond to sugars [5, 11] such as glucose, fructose, and sucrose [12]. Bitter taste receptors, from Taste Receptor Family 2 (TAS2R's), have a much wider diversity of subtypes, with each tuned to specific bitter compounds [13]. These compounds include the plant sesquiterpene lactones, strychnine, and denatonium [14]. Humans are known to have at least 25 TAS2R subtypes [11, 15], and there are many others that have been discovered in mammalian species [16]. The type II taste cells of the tongue most often express only one taste modality, but some cells do express multiple unique receptors [17].

The pre-synaptic mechanisms for taste receptor stimulation and signal transduction are relatively conserved in the tongue and the airway. Briefly, a bitter or sweet ligand binds its respective GPCR, triggering activation of phospholipase C isoform β2 (PLCB2). PLCβ2 then causes inositol 1,4,5-trisphosphate (IP3) production, activating the IP3 receptor on the endoplasmic reticulum (ER) with release of calcium (Ca2+) [18]. While this process occurs, the GPCR stimulation also activates phosphodiesterases (PDE's) that cause the reduction of cAMP levels and corresponding protein kinase A (PKA) activity.

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PKA acts as an inhibitor of the type III IP3R through phosphorylation, so removal of this inhibitory pathway further enhances calcium release from the ER [19]. The released calcium activates the TRPM5 channel [20], which depolarizes the cell membrane, activates voltage-gated sodium (Na+) channels generating an action potential that causes ATP release through the CALHM1 ion channel [5, 19, 21, 22]. In the tongue, this ATP release activates purinergic receptors on presynaptic taste cells and sensory fibers, transmitting the sensation of taste to the central nervous system [5, 22, 23].

## Taste receptors and airway immunity

GPCR taste receptors are expressed in a number of organ systems, including the brain, pancreas, testicles, bladder, respiratory and GI tracts [1-4, 24] The present review will focus on taste receptors expressed in the airways.

### *Overview of innate airway immunity*

Several respiratory immune mechanisms work in concert to achieve a relatively low microbial biomass in the lower airway, in spite of the vast number of bacteria, fungi, and viruses that are inhaled into the upper respiratory tract with each breath. During infection or debris inhalation, ciliary beat frequency (CBF) increases to speed up mucociliary clearance (MCC) [25]. In addition to transporting the mucus to the pharynx where it is cleared by swallowing, innate immune products are disseminated on the airway surface [26]. These immune products include direct anti-microbial compounds such as defensins, lactoferrin, cathelicidins, and lysozyme, in addition to reactive oxygen species (ROS) and nitric oxide (NO) that also display potent antimicrobial activity [27].

In order to activate all of these defense mechanisms, recognition of foreign organisms or toxins both immediately and throughout bacterial colonization is paramount. Toll-like receptors (TLR's) are expressed by airway epithelial cells and recognize pathogen-associated molecular patterns (PAMP's), which are bacterial cell wall components or bacterial products. TLR signaling and downstream immune effect takes up to 12 hours and works through gene expression, creating a sustained immune response [28]. However, a portion of antimicrobial peptide secretion and changes in MCC in response to pathogens occurs almost immediately [29], suggesting the existence of a molecular pathway that rapidly detects foreign compounds and effects timely responses. Bitter taste receptors may provide a missing link in this pathway as initiators of these rapid defenses.

### *Airway bitter taste receptors*

A wide variety of bitter taste receptors are expressed in various parts of both the human and rodent airway [8, 29-32]. While some bitter taste receptors in the airway are upstream of a nervous system signaling cascade [33], others act in a cell-autonomous fashion without any nervous innervation with the bitter products detected an entirely local phenomenon. In 2009, bronchial epithelial cells were shown to have $Ca^{2+}$ increases following bitter compound stimulation, thus increasing CBF thereby accelerating clearance of the noxious compound [32]. These TAS2R receptors are located on the motile cilia themselves. In response to phenythiocarbamide (PTC) stimulation of sinonasal epithelial cell TAS2R's, an increase in NO production is also observed, with potent bactericidal consequences [8]. NO diffuses rapidly into bacteria such as *P. aeruginosa*, where it causes cellular destruction and death [34]. However, recent *in vitro* experiments demonstrated differential bactericidal activity of NO depending on the specific organism in question [35]. In addition to this direct antimicrobial activity, NO

acts as a second messenger to activate protein kinase G (PKG) and guanylyl cyclase to phosphorylate proteins within the cilia and speed up CBF [36]. Other experiments have further investigated this NO pathway and found that both the TRPM5 channel and PLCB2, two of the components in canonical taste transduction, are necessary for NO production but not the canonical taste G-protein gustducin [8].

Lactones are bitter chemicals that can stimulate TAS2R's in the airway [8, 37], and acyl-homoserine lactones (AHL's) are a subclass of lactones that are produced by many gram-negative bacteria [38, 39]. AHL's serve as biofilm "quorum-sensing molecules" for the bacteria. Once a sufficient concentration of AHL's are produced in a localized environment, bacteria will form a biofilm, which confers increased protection for the bacteria from host immune defenses [40]. It is proposed that detection of these AHL's before bacteria reach a density adequate for biofilm formation is an adaptive mechanism, allowing for an increased immune response before microbial protection occurs in the biofilm formation [7].

*Solitary chemosensory cells*

Ciliated epithelial cells are not the only cells to express bitter taste receptors in the airway. Over a decade ago, a class of cells that is sparsely scattered in rodent respiratory epithelium was shown to be immunoreactive with alpha-gustducin (a component of taste signaling) [41]. These cells were named "solitary chemosensory cells" (SCC's), and they share many similarities with cells found in the taste buds of the tongue [30]. Approximately one out of every hundred cells in the sinonasal cavity is a SCC [33]. The function of these airway taste-like cells were explored further, and it was discovered that they express sweet and bitter taste receptors [29, 42], and in the mouse capable of responding to AHL's and other bitter agonists [7, 43, 44]. These murine SCC's show intracellular calcium responses in the presence of AHL's [33], but they do not appear to activate downstream NO production. Instead, when mouse sinonasal SCC's are stimulated with AHL's or denatonium, the calcium response results in acetylcholine (ACh) release that stimulates trigeminal nerve peptidergic nociceptors, with downstream effects of breath holding and inflammatory mediator release [7, 33, 43]. The inflammatory response is intuitively antimicrobial, while the breath holding response may also represent an adaptive reflex to limit toxin or organism aspiration in the host.

SCC's have been identified in human upper airway tissue as well [29, 45], along with additional physiological function beyond what has been elucidated in the rodent system. TAS1R1 and 2, and TAS2R4, 10, and 47 are all expressed on SCC's in the human nasal cavity [31, 45]. Denatonium, a bitter compound that shows activity in mouse SCC signaling [30], also stimulates a $Ca^{2+}$ response in human SCC's that spreads to neighboring cells via gap junctions [31]. Just as in the NO response seen in ciliated cells, the calcium signaling requires canonical taste signaling pathways, including gustducin, PLCβ2, the IP3 receptor, and TRPM5 [31]. Gap junction spread of the signal causes immediate release of antimicrobial peptides (AMP's) from the adjacent ciliated cells [29]. These AMP's include beta defensin 1 (DEFB1) and beta defensin 2 (DEFB2), and the secreted products have potent activity in killing of gram-positive and gram-negative organisms [46], including methicillin-resistant *S. aureus* and *P. aeruginosa*. This rapid secretion of antimicrobial products contrasts directly with the TLR mechanism of AMP messenger RNA upregulation, causing a sustained response that does not appear until several hours after bacterial stimulation [28]. Pre-formed stores of AMP's are released in the TAS2R response, rather than *de-novo* synthesis [46].

*T2R38*

TAS2R's are very genetically diverse, a phenomenon that helps to explain the wide variety of taste preference both within and between cultures [47, 48]. Many individuals find bitter foods such coffee or herbs to be detestable, while others do not have an aversive response. This genetic variation of TAS2R's is not exclusively found in the tongue; TAS2R receptor variation in the airway appears to also play a key role in respiratory defense. TAS2R38, a receptor that is localized to motile cilia in humans, responds to at least three AHL's produced by *P. aeruginosa*, N-butyrl-L-homoserine lactone, N-hexanoyl-L-homoserine lactone and N-3-oxo-dodecanoyl-L-homoserine lactone [8]. Additionally, PTC and propylthiouricil (PROP) are bitter compounds that also agonize TAS2R38 in a similar fashion [49]. When TAS238 in nasal cells is stimulated by AHL's, PTC, or PROP, NO is produced to speed up MCC and directly kill pathogens in the human upper airway [31]. However, the genetic locus for TAS2R38, has three common polymorphisms that tend to segregate together, yielding a functional receptor (PAV) and a non-functional receptor (AVI) [48]. Individuals who have an AVI/AVI genotype do not taste the bitter compounds PTC or PROP [50], and epithelial cells from these patients grown at an air-liquid interface (ALI) show significantly lower NO production in response to AHL's when compared to epithelial cells from a PAV/PAV individual. The consequent reductions in MCC and bacterial killing are also significant in the AVI/AVI group [51].

The implications of these differences are broad. Patients with chronic rhinosinusitis (CRS) have pathological mucociliary stasis, which harbors bacteria and allows infection to perpetuate [52]. This creates a very stagnant and favorable environment for bacteria to proliferate, and for bacterial toxins to continually cause destruction of both cells and cilia [53]. It was previously shown that sinonasal epithelial explants from patients with CRS show an attenuated response to a variety of compounds that stimulate CBF in normal controls [54]. Additionally, further studies demonstrated that there were differences in NO levels in patients with CRS or other airway diseases [55]. However, a review of the nasal NO literature was unable to demonstrate any trends in rhinopathologies with regard to nasal NO measurements [56]. The pathophysiology behind this disparity is not entirely clear, but the TAS2R38 genotype (or not controlling for TAS2R38 genotype) may help to explain the conflicted literature. Individuals who have the PAV/PAV genotype are less likely to need surgical intervention for their CRS symptoms than those with the AVI/AVI genotype [50, 57]. PAV/PAV patients are additionally less prone to developing gram-negative infection, such as that of *P. aeruginosa* [50, 57, 58]. In light of this data, it appears that variation in bitter taste receptor function in humans has a phenotypic effect on upper respiratory disease. In the near future, bitter taste testing with PTC or PROP could potentially help to stratify CRS patients who are more likely to benefit from standard sinus procedures as well as those who should receive alternative or more aggressive management [8]. Further, the bitter compounds themselves could even serve as therapeutic agents, in speeding up MCC and strengthening host responses to counter bacterial proliferation in CRS [59].

**Table 1:** Overview of bitter and sweet receptors and their functions in airway immune defense.

| Cell Type | *Receptor(s) Expressed* | *Animal* | *Function* |
|---|---|---|---|
| Solitary Chemosensory Cells (sinuses) | TAS2R bitter receptors | Mouse<br>Human | Breath holding, inflammation<br>Antimicrobial peptide release |
| | TAS1R sweet receptors | Mouse<br>Human | Silence TAS2R stimulation<br>Unknown |
| Ciliated cells (sinuses) | TAS2R38 | Human | NO production (MCC stimulation and direct killing) |
| Ciliated cells (bronchi) | TAS2R bitter receptors | Human | MCC stimulation |
| Brush cells (trachea) | TAS2R bitter receptors | Mouse | Breath holding |
| Smooth muscle cells (bronchi) | TAS2R bitter receptors | Mouse<br>Human | Bronchodilation |

Sources: [7, 8, 30-32, 43, 45, 51, 60-64]

*Sweet taste receptors*

The TAS1R receptors (dimer of isoform 2 and 3) detect sweet compounds and are also found in the respiratory mucosa [30]. They have been isolated in the human vomeronasal duct [30] as well as in SCC's [29]. In the sinuses, the sweet receptors respond to concentrations of glucose and other sugars that are far lower than those detected on the tongue [65]. Normally, individuals have a glucose concentration of approximately 0.5 mM in the airway surface liquid (ASL), and there is a constant leak and reuptake of glucose from the serum that maintains this constant concentration [31]. The T1R2/3 sweet receptors are tonically activated by this low level of glucose, and appear to function in an antagonistic role to that of the bitter taste receptors. Depletion of ASL glucose is a harbinger of bacterial infection, as the bacteria consume the sugar rapidly. It is hypothesized that this reduction in glucose deactivates the sweet receptors, which then release their inhibition on the action of the TAS2R receptors to bitter compounds [31]. While low-level colonization by bacteria is expected in the sinonasal tract, any perturbation in this homeostasis towards glucose depletion (i.e., more than colonization) causes a balance in favor of TAS2R activation with subsequent mobilization of local defenses against the pathogen, resulting in decreased microbial numbers and restoration of physiologic airway surface glucose concentrations. Paradoxically, a recent study correlated in vitro SCC hyper-activation to disease recurrence for patients with chronic rhinosinustis [66].

This hypothesis has been supported by several experiments. The addition of glucose and sucrose (both TAS1R2/3 agonists) to the ASL of an ALI culture blocked the $Ca^{2+}$ response of bitter taste receptors to denatonium, while mice that did not express these sweet receptors [67] showed a normal response to the compound [31]. Antagonists of the TAS1R2/3 receptors, such as lactisole [68] and amiloride [31], also could release the inhibition of the denatonium response. D-amino acids produced by bacteria in the airway also could activate TAS1R2/3 sinonasal taste receptors [69]. Work by Lee and colleagues demonstrated that *S. aureus* produced at least two TAS1R2/3-activating D-amino acids, and these D-amino acids could suppress sinonasal SCC innate immune responses with resultant decreased secretion of antimicrobial peptides. These D-amino acids may be produced by the bacteria for protection from host innate defenses and may allow for increased colonization and potential opportunistic infection. Just as is the case with bitter receptors, there is genetic variation in TAS1R genes that manifests as individual preference in sweet taste [70]. While no single locus has yet been identified, there are allele variations among the TAS1R genes that show frequency differences of >10% in 16 loci between patients with CRS and controls [58]. TAS1R2/3 antagonists such as lactisole

may prove useful in the future in augmentation of host airway bitter taste receptor responses.

*Additional functions of taste receptors in the airway*

The previous experiments discussed focused on SCC's and ciliated cells that populate the upper airway, and SCC cells are unique to that location of the respiratory tract. Bronchial tissue, which contains an abundance of smooth muscle cells, do not demonstrate SCC responses or secretion of AMP's following stimulation [31]. However, the smooth muscle cells do express several TAS2R's, and activation of these receptors causes bronchodilation [30, 51]. This phenomenon potentially occurs due to an increase in $Ca^{2+}$ that modifies potassium currents within the muscle cells that causes them to become hyperpolarized and relax [63]. These cells lack innervation, so this response is similar to that of the NO production within ciliated cells, in that it is a local defense. Interestingly, asthmatics have an upregulation in TAS2R gene expression [71].

Allele expression studies in patients with CRS showed that TAS2R38 is not the only genetic determinant of disease severity. Several other loci, such as that of TAS2R14 and TAS2R49 show an allele frequency difference of >10% between CRS patients and controls [58]. It will be important for future research to determine the full expression pattern of taste receptors throughout the length of the respiratory tract, as well as explore the full complement of bitter products that are secreted by organisms.

## Conclusions

Airway taste receptors play an important role in innate respiratory defense, and they function in regulating inflammation and antimicrobial activity within the respiratory tract. These responses are quick in onset and are complementary to traditional antimicrobial pathways, such as those involving TLR's. Dysfunction or genetic variation of bitter or sweet taste receptors appears to play a key role in respiratory disease, including CRS and increased susceptibility to infection in diabetes. Conventional management of respiratory diseases often involves antibiotics, but strengthening endogenous defense mechanisms may be possible by using TAS1R and TAS2R receptors as novel therapeutic targets.

## References

1. Laffitte, A., F. Neiers, and L. Briand. (2014) Curr Opin Clin Nutr Metab Care, 17: 379-85.
2. Clark, A.A., S.B. Liggett, and S.D. Munger; (2012) FASEB J,. 26: 4827-4831.
3. Depoortere, I., Gut, (2014) 63: 179-190.
4. Behrens, M. and W. Meyerhof, (2010) Results Probl Cell Differ, 52: 87-99.
5. Kinnamon, S.C., (2012) Acta Physiol (Oxf), 204: 158-168.
6. Sternini, C., L. Anselmi, and E. Rozengurt, (2008) Curr Opin Endocrinol Diabetes Obes, 15: 73-78.
7. Tizzano, M., Gulbransen BD, Vandenbeuch A, Clapp TR, Herman JP, Sibhatu HM, Churchill ME, Silver WL, Kinnamon SC and Finger TE., (2010) Proc Natl Acad Sci U S A, 107: 3210-3215.
8. Lee, R.J., Xiong G, Kofonow JM, Chen B, Lysenko A, Jiang P, Abraham V, Doghramji L, Adappa ND, Palmer JN, Kennedy DW, Beauchamp GK, Doulias PT, Ischiropoulos H, Kreindler JL, Reed DR and Cohen NA., (2012) J Clin Invest, 122: 4145-4159.
9. Zhang, Y., Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS and Ryba NJ, (2003) Cell, 112: 293-301.
10. Iwata, S., R. Yoshida, and Y. Ninomiya, Curr Pharm Des, 2014. 20: 2684-2692.
11. Margolskee, R.F., (2002) J Biol Chem. 277: 1-4.
12. Treesukosol, Y., K.R. Smith, and A.C. Spector, (2011) Physiol Behav. 105: 14-26.
13. Adler, E., James N. Palmer, Nithin D. Adappa and Noam A. Cohen, (2000) Cell, 100: 693-702.

14. Brockhoff, A., Behrens M, Massarotti A, Appendino G, Meyerhof W., (2007) J Agric Food Chem, 55: 6236-6243.
15. Chandrashekar, J., Mueller K.L., Hoon M.A., Adler E., Feng L., Guo W., Zuker C.S., and Ryba N.J., (2000) Cell,. 100: 703-711.
16. Wu, S.V., M.C. Chen, and E. Rozengurt, (2005) Physiol Genomics, 22: 139-149.
17. Liman, E.R., Y.V. Zhang, and C. Montell, (2014) Neuron., 81: 984-1000.
18. Giovannucci, D.R., Groblewski G.E., Sneyd J. andYule D.I., (2000) J Biol Chem, 275: 33704-33711.
19. Taruno, A., Matsumoto I., Ma Z., Marambaud P. and Foskett J.K., (2013) Bioessays, 35: 1111-1118.
20. Zhang, Z., Zhao Z., Margolskee R. and Liman E., (2007) J Neurosci, 27: 5777-5786.
21. Miyoshi, M.A., K. Abe, and Y. Emori, Chem Senses, 2001. 26: 259-265.
22. Taruno, A., Vingtdeux V., Ohmoto M., Ma Z., Dvoryanchikov G., Li A., Adrien L., Zhao H., Leung S., Abernethy M., Koppel J., Davies P., Civan M.M., Chaudhari N., Matsumoto I., Hellekant G., Tordoff M.G., Marambaud P. and Foskett J.K., (2013) Nature, 495: 223-226.
23. Behrens, M., S. Born, U. Redel, N. Voigt, V. Schuh, J.D. Raguse, and W. Meyerhof, (2012) PLoS One, 7: e40304.
24. Malki, A., J. Fiedler, K. Fricke, I. Ballweg, M.W. Pfaffl and D. Krautwurst, (2015) J Leukoc Biol, 97: 533-545.
25. Shaari, J., Palmer J.N., Chiu A.G., Judy K.D., Cohen A.S., Kennedy D.W. and Cohen N.A., (2006) Am J Rhinol, 20: 150-154.
26. Sleigh, M.A., J.R. Blake, and N. Liron, (1988) Am Rev Respir Dis, 137: 726-741.
27. Parker, D. and A. Prince, (2011) Am J Respir Cell Mol Biol, 45: 189-201.
28. Hume, D.A., D.M. Underhill, M.J. Sweet, A.O. Ozinsky, F.Y. Liew and A. Aderem, (2001) BMC Immunol, 2: 11.
29. Barham, H.P., Cooper S.E., Anderson C.B., Tizzano M., Kingdom T.T., Finger T.E., Kinnamon S.C. and Ramakrishnan V.R., (2013) Int Forum Allergy Rhinol, 3: 450-457.
30. Tizzano, M., M. Cristofoletti, A. Sbarbati and T.E. Finger, (2011) BMC Pulm Med, 11: 3.
31. Lee, R.J., Kofonow J.M., Rosen P.L., Siebert A.P., Chen B., Doghramji L., Xiong G., Adappa N.D., Palmer J.N., Kennedy D.W., Kreindler J.L., Margolskee R.F. and Cohen N.A., (2014) J Clin Invest, 124: 1393-1405.
32. Shah, A.S., Ben-Shahar Y., Moninger T.O., Kline J.N., Welsh M..J, (2009) Science, 325: 1131-1134.
33. Saunders, C.J., Christensen M., Finger T.E. and Tizzano M. , *(2014)* Proc Natl Acad Sci U S A, 111: 6075-6080.
34. Barraud, N., Hassett D.J., Hwang S.H., Rice S.A., Kjelleberg S. and Webb J.S.*(2006)* J Bacteriol, 188: 7344-7353.
35. Workman, A.D., R.M.Carey, M.A. Kohanski, D.W. Kennedy, J.N. Palmer, N.D. Adappa and N.A. Cohen, (2017) Int Forum Allergy Rhinol, 7: 770-776.
36. Salathe, M., (2007) Annu Rev Physiol, 69: 401-422.
37. Chadwick, M. H. Trewin, F. Gawthrop and C. Wagstaff, (2013) Int J Mol Sci, 14: 12780-12805.
38. Jimenez, P.N., Koch G., Thompson J.A., Xavier K.B., Cool R.H. and Quax W.J, (2012) Microbiol Mol Biol Rev, 76: 46-65.
39. Li, Z. and S.K. Nair, *(2012)* Protein Sci, 21: 1403-1417.
40. Parsek, M.R. and E.P. Greenberg, *(2000)* Proc Natl Acad Sci U S A, 97: 8789-8793.
41. Zancanaro, C., Caretta C.M., Merigo F., Cavaggioni A. and Osculati F., *(1999)* Eur J Neurosci, 11: 4473-4475.
42. Osculati, F., M. Bentivoglio, M. Castellucci, S. Cinti, C. Zancanaro and A. Sbarbati, *(2007)* Eur J Histochem, 51: 65-72.
43. Gulbransen, B., W. Silver, and T.E. Finger, *(2008)* J Comp Neurol, 508: 62-71.
44. Lin, W., E.A.D. Ezekwe Jr., Z. Zhao, E.R. Liman and D. Restrepo, (2008) BMC Neurosci, 9: 114.
45. Braun, T., B. Mack, and M.F. Kramer, *(2011)* Rhinology, 2011. 49: 507-512.

46. Mosimann, B.L. White M.V., Hohman R.J., Goldrich M.S., Kaulbach H.C. and Kaliner M.A., (1993) J Allergy Clin Immunol, 92: 95-104.
47. Hayes, J.E., Wallace M.R., Knopik V.S., Herbstman D.M., Bartoshuk L.M. and Duffy V.B. (2011) Chem Senses, 36: 311-319.
48. Bufe, B., P.A.S. Breslin, C. Kuhn, D.R. Reed, C.D. Tharp, J.P. Slack, U.-K. Kim, D. Drayna and W. Meyerhof1, (2005) Curr Biol, 2005. 15: 322-327.
49. Kim, U.K. and D. Drayna, (2005) Clin Genet, 67: 275-80.
50. Adappa, N.D., Zhang Z., Palmer J.N., Kennedy D.W., Doghramji L., Lysenko A., Reed D.R., Scott T., Zhao N.W., Owens D., Lee R.J. and Cohen N.A., (2014) Int Forum Allergy Rhinol, 4:3-7.
51. An, S.S., Wang W.C., Koziol-White C.J., Ahn K., Lee D.Y., Kurten R.C., Panettieri R.A. Jr. and Liggett S.B., (2012) Am J Physiol Lung Cell Mol Physiol, 303: L304-L311.
52. Rom, D.I., Christensen J.M., Alvarado R., Sacks R. and Harvey R.J., (2017) Rhinology, 55: 90-94.
53. Min, Y.G., Seung Jun Oh, Tae-Bin Won, Yong Min Kim, Woo Sub Shim, Chae-Seo Rhee, Jin-Young Min and Hun-Jong Dhong, *(2006)* Acta Otolaryngol, 126: 941-947.
54. Chen, B., Shaari J., Claire S.E., Palmer J.N., Chiu A..G, Kennedy D.W. and Cohen N.A., (2006) Am J Rhinol, 20: 325-329.
55. Naraghi, M., Deroee A.F., Ebrahimkhani M., Kiani S. and Dehpour A., *(2007)* Am J Otolaryngol, 28: 334-337.
56. Phillips, P.S., Sacks R., Marcells G.N., Cohen N.A. and Harvey R.J., (2011) Otolaryngol Head Neck Surg, 144: 159-169.
57. Adappa, N.D., Howland T.J., Palmer J.N., Kennedy D.W., Doghramji L., Lysenko A., Reed D.R., Lee R.J. and Cohen N.A., (2013) Int Forum Allergy Rhinol, 2013. 3: 184-187.
58. Mfuna Endam, L., Filali-Mouhim A., Boisvert P., Boulet L.P., Bossé Y. and Desrosiers M., (2014) Int Forum Allergy Rhinol, 2014. 4: 200-206.
59. Lee, R.J. and N.A. Cohen, (2013) Am J Rhinol Allergy, 27: 283-286.
60. Finger, T.E., Böttger B., Hansen A., Anderson K.T., Alimohammadi H. and Silver W.L., (2003) Proc Natl Acad Sci U S A, 100: 8981-8986.
61. Krasteva, G., Canning B.J., Hartmann P., Veres T.Z., Papadakis T., Mühlfeld C., Schliecker K., Tallini Y.N., Braun A., Hackstein H., Baal N., Weihe E., Schütz B., Kotlikoff M., Ibanez-Tallon I. and Kummer W., (2011) Proc Natl Acad Sci U S A, 108: 9478-9483.
62. Saunders, C.J., S.D. Reynolds, and T.E. Finger, (2013) Am J Respir Cell Mol Biol, 49: 190-196.
63. Deshpande, D.A., Wang W.C., McIlmoyle E.L., Robinett K.S., Schillinger R.M., An S.S., Sham J.S. and Liggett S.B., (2010) Nat Med,. 16: 1299-1304.
64. Robinett, K.S., Koziol-White C.J., Akoluk A., An S.S., Panettieri R.A. Jr. and Liggett S.B., (2014) Am J Respir Cell Mol Biol, 50: 678-683.
65. Kalsi, K.K., Baker E.H., Fraser O., Chung Y.L., Mace O.J., Tarelli E., Philips B.J. and Baines D.L., (2009) Pflugers Arch, 457: 1061-1070.
66. Carey, R.M., Workman A,D., Hatten K.M., Siebert A.P., Brooks S.G., Chen B., Adappa N.D., Palmer J.N., Kennedy D.W., Lee R.J. and Cohen N.A., (2017) Int Forum Allergy Rhinol, 7: 699-704.
67. Lemon, C.H. and R.F. Margolskee, (2009) J Neurophysiol, 101: 2459-2471.
68. Jiang, P., Cui M., Zhao B., Liu Z., Snyder L.A., Benard L..M, Osman R., Margolskee R.F. and Max M., (2005) J Biol Chem, 280: 15238-15246.
69. Lee, R.J., Hariri B.M., McMahon D.B., Chen B., Doghramji L., Adappa N.D., Palmer J.N., Kennedy D.W., Jiang P., Margolskee R.F. and Cohen N.A., (2017) Sci Signal, 10: eaam7703
70. Bachmanov, A.A., N.P. Bosak, C. Lin, I. Matsumoto, M. Ohmoto, D.R. Reed, and T.M. Nelson, (2014) Curr Pharm Des, 20: 2669-2683.
71. Orsmark-Pietras, C., James A., Konradsen J.R., Nordlund B., Söderhäll C., Pulkkinen V., Pedroletti C., Daham K., Kupczyk M., Dahlén B., Kere J., Dahlén S.E., Hedlin G. and Melén E., (2013) Eur Respir J, 42: 65-78.

# Bitter taste: prediction, relation to toxicity, and effect on emotions

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## Abstract

Bitter taste sensation is considered a signal of toxicity and is elicited by molecules of widely varying chemical structures, as summarized in BitterDB (http://bitterdb.agri.huji.ac.il). We developed a machine-learning (decision trees-based) tool BitterPredict, and showed that only 60% of the toxic compounds are known or predicted to be bitter, similar to the predicted bitter abundance in FDA-approved drugs and lower than in natural compounds. This suggests that there are many non-bitter toxic compounds. Interestingly, bitter mouth-rinse leads to lower mood scores and the effect depends on perceiving the solution as bitter.

## Introduction

Bitter taste is one of the basic taste modalities and is typically considered a sentinel of toxicity. Yet, several examples of tasteless poisons or bitter non-toxic molecules are known. Intriguingly, the molecules that elicit taste sensation are numerous and chemically diverse [1] (Figure 1).

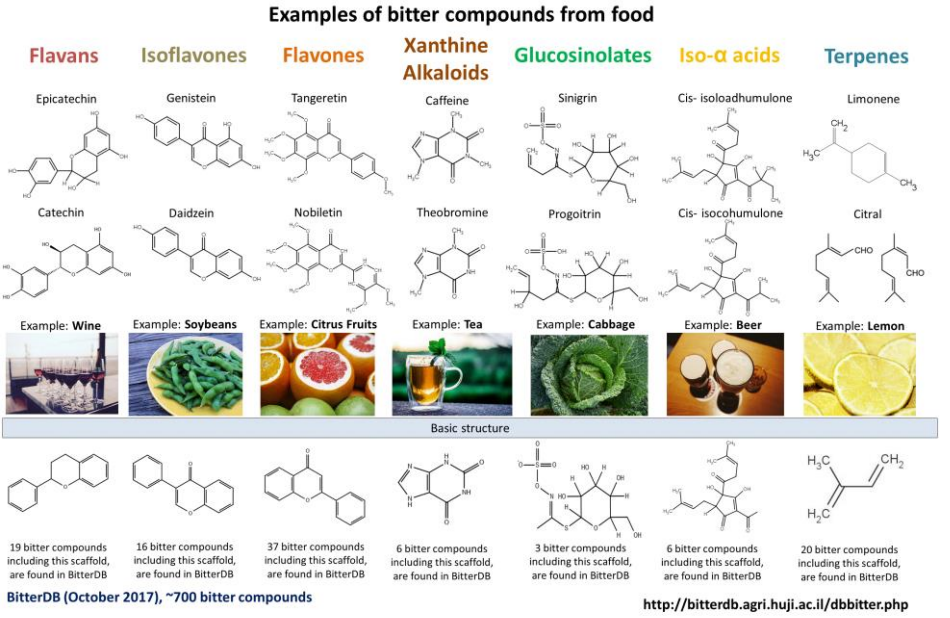


**Figure 1:** Examples of bitter compounds from food

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To facilitate the study of bitter taste, we have established the BitterDB (http://bitterdb.agri.huji.ac.il) [2], which has served over 30,000 users so far. The BitterDB contains data on molecules that were reported as bitter or were shown to activate at least one bitter taste receptor (T2R). Close to 700 bitter molecules have been gathered in the BitterDB, but clearly many additional bitter compounds exist. Some additional bitter molecules can be unraveled using a combination of computational, cell-based and sensory techniques [3]. Machine-learning approaches are proving to be extremely powerful in many areas of research and engineering, including sensory science [4]. Here we describe BitterPredict [5], a machine learning adaptive boosting program to classify molecules as bitter or non-bitter, and apply it to datasets of toxic and other compounds [6]. Finally, complementing the vast literature on emotional effects of odors, we present the effects of bitter (quinine or 6-n-propylthiouracil (PROP) dissolved in water) and sweet (sucrose in water) mouth rinse on mood [7].

## Experimental methods

*I. BitterPredict [5]*

14 physicochemical properties and 47 Absorption, Distribution, Metabolism, Excretion and Toxicity (ADME/T) descriptors from the QikProp package (Schrödinger, LLC) were calculated for the molecules. BitterPredict is an AdaBoost model constructed from 200 decision trees. AdaBoost is an ensemble method, where the final prediction uses the weighted average of the predictions given by each of the decision trees in the ensemble. The data was divided into a training set (70%) and a test set (30%). The model was trained only on the training set. Additionally, 3 external validation sets (molecules which were not used for training or testing) were collected and used to assess the performance of BitterPredict.

*II. Toxicity datasets [6]*

Two datasets were created to represent toxicity: FocTox is a focused, relatively small dataset aptly named FocTox, comprised of ~40 compounds from the *FAO/WHO food contaminants list* and ~350 compounds from the *List of extremely hazardous substances* defined in *Section 302* of the *U.S. Emergency Planning and Community Right-to-Know Act*. CombiTox is a broad dataset, combining two publicly available datasets: T3DB (The Toxin and Toxin-Target Database, contains ~3000 compounds) and DSSTox (Distributed Structure-Searchable Toxicity Database, contains ~140,000 compounds).

*III. Sensory panel testing [7]*

Participants tasted a solution without swallowing, and then had to: identify the taste they perceive (by choosing either sweet, sour, salty or bitter as the main taste modality), rank the taste intensity, perform seemingly unrelated behavioral tasks, and fill a standard *Positive and Negative Affect Schedule* (PANAS) [8] mood questionnaire. The questionnaire consists of 10 positive and 10 negative affect items. Each item was rated on a Likert scale of 1 (not at all) to 5 (very much). The total score was calculated by subtracting the sum of the 10 negative items, from the sum of the 10 positive items. T2R38 genotype was determined by collecting saliva samples from the participants, using OG-500 Saliva collection kits (Pronto Diagnostics Ltd). Nucleotides and amino acid codons for the two alleles of each panelist were carried out in The Monell Chemical Senses Center.

## Results and discussion

Bitter compounds are very diverse in their chemical structures. Various chemical classes (terpenes, flavones, glucosinolates and more) are found among bitter compounds in food (Figure 1). So what are the chemical properties of bitter compounds, and can these be used for bitterness prediction?

A principle component analysis (PCA) of the bitter set, non-bitter set and 2000 random molecules, using physicochemical properties suggests that the non-bitter sub-sets are capturing different narrow chemical spaces (Figure 2). Most of the bitter molecules (97%) have molecular weight below 700 and range in -3 < AlogP < 7. This range was defined as the applicability domain for BitterPredict, which was then trained using the physicochemical and the ADME/T descriptors of the bitter and non-bitter set.

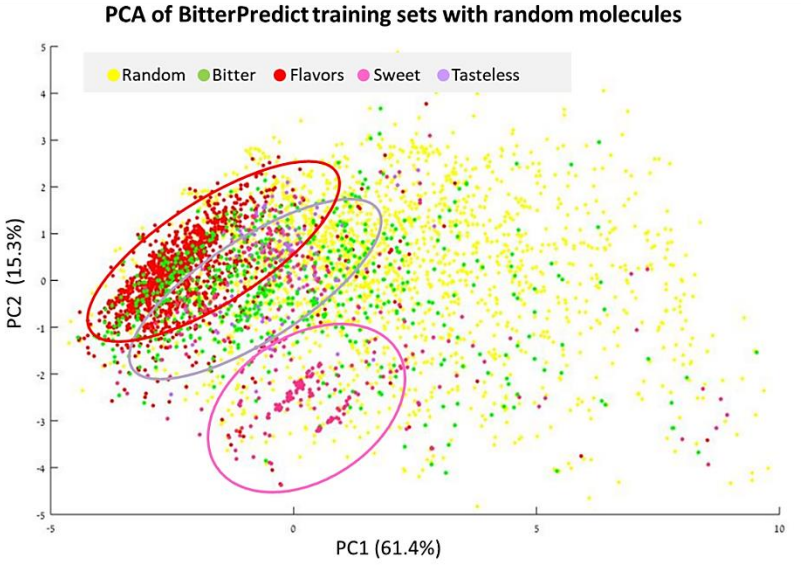


**Figure 2:** PCA of the negative sets (flavors, sweet and tasteless molecules), positive set (bitter) and random molecules within the Bitter Domain. The bitter molecules (green) spread widely inside the Bitter Domain. Each non-bitter sub set covers distinct sub-space (red, pink and purple); however, the combined non-bitter set covers almost all the domain, though not uniformly distributed. Principle components (PC) PC1 and PC2 explain ~61% and ~15% of the variation, respectively.

BitterPredict outputs a numerical score, positive for bitter and negative for non-bitter. Higher absolute values indicate higher confidence scores. Score > 0.6 can selected as a high confidence bitter score (leading to a false positive rate lower than 0.05) and < -0.7 can be selected as a high confidence non-bitter score (leading to a false negative rate lower than 0.1). BitterPredict separates well between the bitter and non-bitter molecules, with sensitivity (true positive/true positive+false negative) of 0.77 and specificity (true negative /true negative+false positive) of 0.85 on the test set (Table 1).

**Table 1:** BitterPredict performance on train set, test set and validation sets
Sensitivity = (true positive) / (true positive + false negative)
Specificity = (true negative) / (true negative + false positive)

| | | *Sensitivity* | *Specificity* | *Accuracy* |
|---|---|---|---|---|
| | train set | 0.9 | 0.94 | 0.93 |
| | Test set | 0.77 | 0.85 | 0.83 |
| Negative subsets | Non-Bitter flavors | | 0.83 | |
| | sweet | | 0.82 | |
| | tasteless | | 0.86 | |
| Validation Sets | BitterNew | 0.75 | | 0.75 |
| | Phyto | 0.98 | 0.69 | 0.88 |
| | UNIMI | 0.78 | 0.85 | 0.82 |

The high performance of BitterPredict was confirmed via external sets, sensory evaluation, and datamining of prospective predictions [5] and enabled us to estimate the abundance of bitter compounds in toxic, random, natural and other datasets (Table 2). Interestingly, only 60% of the toxic compounds were predicted as bitter. This prediction is higher than in food compounds, but lower than in natural products and in approved drugs, suggesting existence of many toxic compounds that are not bitter. All in all, the number of predicted bitter compounds in the entire chemical space may be higher than initially thought.

**Table 2:** Approximate percentage of molecules predicted by BitterPredict as bitter/non-bitter with different confidence levels, in datasets with defined orientation

| | FooDB | DrugBank Approved | DrugBank Experimental | Natural Products ZINC15 | ChEBI | CombiTox | FocTox |
|---|---|---|---|---|---|---|---|
| | FooDB | DRUGBANK | DRUGBANK | ZINC15 | ChEBI | EPA T3DB | World Health Organization |
| **% Molecules predicted as bitter** | **38.36%** (7,926 / 20,661) | **65.94%** (1,024 / 1,553) | **49.93%** 2,506/5,019 | **77.21%** (21,786 / 28,217) | **43.71%** (16,188 / 37,033) | **55.84%** (77,712 / 139,165) | **60.18%** (192 / 319) |
| **% Molecules predicted as bitter with 0.6 cutoff** | **29.05%** (6,001 / 20,661) | **52.54%** (816/ 1,553) | **33.35%** 1,674/5,019 | **62.39%** (17,604 / 28,217) | **30.01%** (11,115 / 37,033) | **37.33%** (51,960 / 139,165) | **37.30%** (119 / 319) |
| **% Molecules out of Bitter Domain (considered non bitter)** | **34.23%** 7,703 / 20,661) | **11.46%** 178/1,553 | **14.05%** 705/5,019 | **2.88%** (813 / 28,217) | **19.84%** (6,688 / 37,033) | **7.95%** (11,050 / 139,165) | **15.99%** (51 / 319) |
| **% Molecules predicted as non-bitter in bitter domain** | **27.40%** 5,662/ 20,661 | **22.60%** 351/1,553 | **36.02%** 1,808/5,019 | **20.16%** (5,688 / 28,217) | **29.24%** (10,827 / 37,033) | **36.22%** (50,403 / 139,165) | **23.82%** (76 / 319) |
| **% Molecules predicted as non-bitter with -0.7 cutoff** | **14.99%** 3,098 / 20,661 | **10.56%** 164/1,553 | **17.99%** 903/5,019 | **9.24%** (2,606 / 28,217) | **19.84%** (13,53/ 37,033) | **17.20%** (23,932 / 139,165) | **8.46%** (27 / 319) |

Bitter taste may be generally associated with unpleasant and difficult situations [9], and thus may evoke negative emotions. A direct negative change in PANAS score compared to water baseline score, was induced by oral exposure to quinine or to PROP, but not to sucrose. PROP taster/non-taster status was determined by the participants' genotype of T2R38, which underlies the ability to taste PROP as bitter-. The negative affect caused by exposure to PROP depends on the taster/non-taster status of the participants (Figure 3A). Furthermore, the mean PANAS score for a group of participants

that tasted quinine solution was significantly lower than for groups of participants that tasted water, sweet or bitter-sweet mixture solutions (Figure 3B). However, the reverse effect – positive mood changes as a result of sweet solution – was not observed.

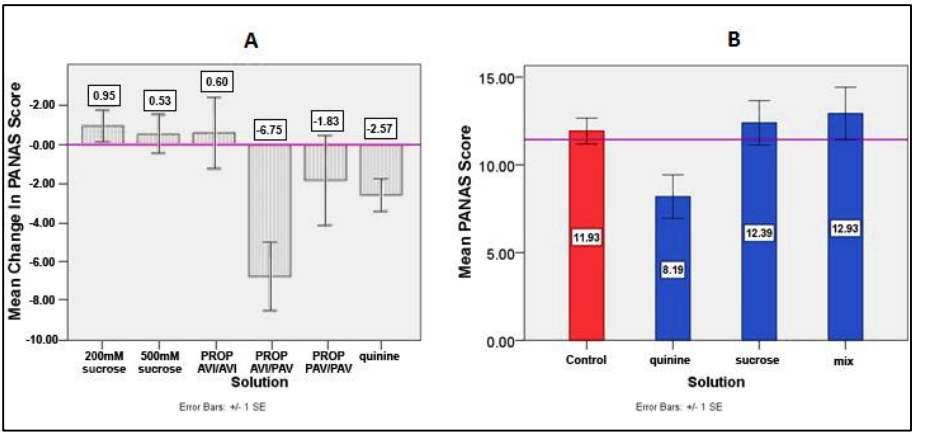


**Figure 3: (A)** Mean change in PANAS score, after exposure to the examined solutions, compared to water baseline. Bars indicate standard error. The horizontal line represents 0 (no change). PAV and AVI represent PROP tasters and non-tasters, respectively. PAV/PAV homozygotes have high sensitivity to PROP compound, PAV/AVI heterozygotes have intermediate sensitivity, while AVI/AVI homozygotes are PROP non-tasters. **(B)** Mean PANAS score of the examined solutions (blue), compared to the water group (red). Bars indicate standard error. The horizontal line represents the mean for all participants.

## Summary

Bitter ligands can be accessed via BitterDB (http://bitterdb.agri.huji.ac.il). The BitterPredict bitter/non-bitter classifier works well despite tremendous chemical diversity of bitter compounds and can be applied to drug repurposing and bitterness prediction. Many random compounds may be bitter and only 60% of toxic compounds are predicted to be bitter. The high percentage of predicted bitter compounds in the datasets tested – including food-derived compounds – suggests that bitter taste may not be a strong marker for toxicity [6]. Mood scores were decreased by quinine solution that was perceived as bitter. PROP mouth rinse lead to negative mood change among in PROP tasters only. Conversely, while sweet mouth rinse ranked higher hedonic scores, it did not positively affect mood scores.

## References

1. Meyerhof, W., Batram C., Kuhn C., Brockhoff A., Chudoba E., Bufe B., Appendino G. and Behrens M., (2010) Chem Senses 35: 157-170
2. Wiener, A., Shudler, M., Levit, A. and Niv, M. Y. (2012) Nucleic Acids Res 40: D413-419
3. Bahia, M. S., Nissim, I. and Niv, M. Y., (2017) Int J Pharm, 2017.03.076
4. Keller, A. R.C. Gerkin, Y. Guan, A. Dhurandhar, G. Turu, B. Szalai, J.D. Mainland, Y. Ihara, C. Wen Yu, R. Wolfinger, C. Vens, L. Schietgat, K. De Grave, R. Norel, G. Stolovitzky, G.A. Cecchi, L.B. Vosshall and P. Meyr, (2017) Science 355: 820-826
5. Dagan-Wiener A., I. Nissim, N.B. Abu, G. Borgonovo, A. Bassoli and M.Y. Niv. (2017) Sci Rep 7: 12074
6. Nissim, I., Dagan-Wiener, A. and Niv, M. Y. (2017) IUBMB life 69: 938-946,
7. Dubovski, N., Ert, E. and Niv, M. Y. (2017) Food Quality and Preference 60. 154-164
8. Watson, D., Clark, L. A. and Tellegen, A. (1988) Journal of personality and social psychology 54: 1063-1070
9. Reed, D. R. and Knaapila, (2010) Prog Mol Biol Transl Sci 94: 213-240

# Can flavour enhancement modulate appetite and food intake in women?

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## Abstract

Food flavour might be helpful for weight management by regulating appetite sensation and food intake. This chapter investigated whether the enhancement of flavour intensity or flavour complexity can modulate appetite sensation and food intake within a female sample. A single flavour modality, aroma or taste, seems to have no actual effect on appetite sensation and food intake. Sensory-specific satiety was developed for a flavour modality (sweet taste) after the consumption of a drink containing that flavour modality (sweet taste). An enhancement of flavour complexity due to multimodal interaction of two flavour modalities (aroma + taste) suppressed hunger sensation significantly but did not affect subsequent food intake.

## Introduction

Excessive food energy intake has promoted the global overweight and obesity pandemic which accounts for approximately 9.1% of a country's total healthcare expenditure [1]. Food intake and appetite are mediated by successive satiation and satiety signals [2, 3]. "Satiation" is the process that results in the termination of a meal when appetite for food has been satisfied; "Satiety" describes the lingering feeling of satisfaction during the inter-prandial period before hunger returns [4]. To date, limited attention has been paid on the effect of food flavour which may act as a pre-ingestive satiation or satiety signal on the regulation of appetite and food intake [2].

Taste alone has been shown to contribute to the improvement of food palatability, resulting in an increase in hunger sensation and food intake [5-8]. On the contrary, a single flavour modality per se, aroma or taste, has been reported to enhance fullness sensation, suppress hunger sensation and reduce subsequent food intake [9, 10]. The preliminary findings from the current literature suggests that flavour can be appetizing due to its positive influence on food palatability, at the same time, a prolonged exposure to food flavour may also induce satiation or satiety signals over the time course of an eating event [9, 11]. A decrease in the palatability of a food after the food is consumed to satiation has been repeatedly observed, resulting in a reduction in subsequent consumption of foods with a similar flavour profile [12]. This phenomenon is described as sensory-specific satiety (SSS), which may partly contribute to the satiating effect of food flavour [12].

Aroma and taste modalities are two primary drivers in food flavour perception, but flavour perception is not a mere sum of the two modalities [13, 14]. Flavour is the synergistic combination of multisensory modalities; however, no study has been focused on the interactive effect of two flavour modalities on appetite and food intake. [13, 14]. This chapter investigated whether flavour enhancement can modulate appetite sensation and food intake. Flavour was enhanced in two dimensions: 1) a simple increase in the intensity of a flavour modality; 2) an increase in flavour complexity because of multimodal interaction. Specifically, two behaviour experiments were conducted to answer several unresolved questions in this field.

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Experiment 1: Whether enhancing the intensity of a single flavour modality (sweet taste) affected the ad libitum food intake and sensory-specific satiety;

Experiment 2: Whether two combined flavour modalities (aroma + taste) affected appetite sensation and subsequent food intake because of their multimodal interaction on flavour enhancement.

## Experimental

### Experiment 1

#### *Protocol*

The objective of this experiment was to study the effect of sweetness intensity of a milkshake on ad libitum intake of the milkshake and on SSS. Based on a cross-over experimental design, 24 female participants consumed one of the three pre-selected milkshakes with high sweetness (HS), ideal sweetness (IS) or low sweetness intensity (LS) over 3 sessions on 3 separate visits.

Having fasted from food and drink except water overnight (from 21.00 one evening before a visit), participants arrived at the laboratory at 8.45 in the next morning. Participants consumed 50 g porridge (190 kcal) ("So Simple Original Porridge Original Pot", Quaker Oat, UK) dissolved in 100 ml hot water as a standard breakfast between 9.00 and 9.20. From 9.20 to 10.25, participants fasted from any food and drink while sitting quietly in a waiting room. From 10.30 to 10.40, participants were served one of the milkshake (HS, IS or LS) with a serving portion of 800 ml to consume until they felt comfortably full. Participants were encouraged to ask for another portion of the milkshake if they needed. The volume of the milkshake consumed were recorded. The total energy intake of the milkshake was calculated by multiplying the volume (ml) consumed by its energy density (kcal/ml). Subjective appetite sensations were rated immediately before breakfast at 9.00 (baseline appetite), immediately before and after the consumption of milkshake using a 100-mm visual analogue scales (VAS) [15]. SSS was characterised by the changes (Δ) in the pleasantness of the milkshake after consumption of the milkshake, compared to the initial pleasantness of the milkshake. Immediately before and after intake of the milkshake, participants tasted and rated 5 ml of each of the HS, IS and LS milkshakes on the pleasantness of the milkshakes on a 100 mm VAS [11].

#### *Participants and materials*

24 recruited female participants had normal BMI with a mean of 20.2 ± 1.6 kg/m2. They were aged from 19 to 27 years (mean age: 22 ± 2 years), non-restraint eaters and not clinical depressed [16, 17].

The milkshakes, in every 100 ml, consisted of 50 ml mineral water (Evian, Danone Group, France), 50 ml of a commercial milkshake drink (Yazoo banana, Friesland Campina, Belgium), and varying concentrations of a low-caloric sweetener (Canderel Spoonful artificial sweetener, Merisant, UK). The HS, IS and LS milkshake samples were selected on an individual basis. Each participant tasted 10 milkshake samples with varying Canderel sweetener concentrations at 480, 576, 691, 829, 995, 1194, 1433, 1720, 2064 and 2477 mg. They rated their perceived intensities of sweetness, relative-to-ideal sweetness, pleasantness, and desire to drink the milkshake on 100-mm VAS scales. The geometric distance of the sweetener concentrations between each pair of HS and LS samples was similar between participants, which was 3-4 folds of the common ratio 1.2.

Experiment 2

*Protocol*

Using a "preloading paradigm" design, the impact of aroma and taste, in combination and independently, on appetite sensation and subsequent food intake were investigated. 26 female participants visited the laboratory 4 times to consume 4 different sample drinks as preloads, followed by ad libitum past meal. They were asked to fast from 21.00 at one evening before their visit until arriving at the laboratory next morning. During each visit, they arrived at 08.45 and baseline subjective appetite sensation was rated at 09.00 on a 100-mm visual analogue scales [15]. The appetite sensation include hunger, fullness, satisfaction, desire to eat and prospective consumption. Participants consumed a standardised breakfast containing cereal and milk between 09.00 and 09.20. At 11.00, they consumed one of the four sample drinks over 15 minutes using a fine straw (diameter: 0.625 mm, Altec Ltd., UK). VAS ratings were collected immediately at several time intervals before, during the 15 minutes' sample drink consumption, and until 65 minutes after the sample drink consumption. At 12.20, participants were provided an ad libitum pasta meal. They were given a 530 g (878 kcal) portion of pasta and instructed to terminate the meal only when they felt comfortably full. They were instructed to ask for another portion once the previous one was finished. The weight of the pasta consumed was recorded. Pasta energy intake was calculated by multiplying the weight (g) of pasta consumed by the energy density of pasta lunch (kcal/g). Participants seated in a quiet waiting room within the laboratory centre when not asked to conduct any experimental activity.

*Participants and materials*

The recruited 26 female participants had normal BMI with a mean of 20.9 ± 1.9 kg·m-2. They were aged from 18 to 45 years (mean age of 22 ± 4 years), non-restraint eaters and not clinical depressed [16, 17].

The 4 sample drinks were: water (S1, 150 ml, 0 kcal), water with 0.05% strawberry aroma (S2, 150 ml, 0 kcal), water with 8% sucrose and 0.1% citric acid (S3, 150 ml, 48 kcal) and water with 0.5% strawberry aroma, 8% sucrose and 0.1% citric acid (S4, 150 ml, 48 kcal). The volatile compounds in strawberry aroma (Mane Co. Ltd., Derby, UK) were ethyl butyrate, ethyl 2-methyl butyrate, and ethyl hexanoate, diluted in propylene glycol.

The breakfast consisted of Rice Krispies (Kellogg's UK Limited, Manchester, UK) and semi-skimmed milk (Sainsbury's Supermarkets Ltd., London, UK). It was equivalent to 10% of the participant's estimated total daily energy requirement and containing 14%, 14%, and 72% energy from fat, protein, and carbohydrate, respectively [18].

The pasta meal consisted of penne pasta, olive oil, cheddar cheese (Sainsbury's Supermarkets Ltd., London), and Dolmio Garden Vegetable pasta sauce (MARS Food, USA). Its energy density was 1.66 kcal/g, of which 14%, 52%, and 34% provided by protein, carbohydrate and fat, respectively.

## Results and discussion

Experiment 1

*Effect of sweetness intensity on ad libitum intake of milkshake*

The hunger sensation reduced significantly after the consumption of milkshakes ($p < 0.05$), but appetite sensation was not affected by the sweetness intensity of the milkshake. The consumption amount (ml or kcal) of a high sweetness milkshake (HS)

was not different from the intake of a less sweet milkshake (LS or IS), even when the two milkshakes shared a similar palatability (Figure 1 and Figure 2). It suggests that the *ad libitum* intake of the milkshake (satiation) was not affected by the sweetness intensity of the milkshake.

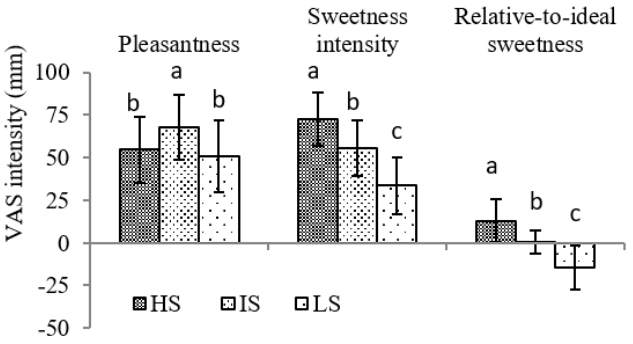


**Figure 1:** Mean (n=24) of the initial pleasantness, sweetness and relative-to-ideal sweetness for HS, IS and LS milkshakes. For each measurement, the samples without a same small letter were significantly different. Error bars represent the standard deviations

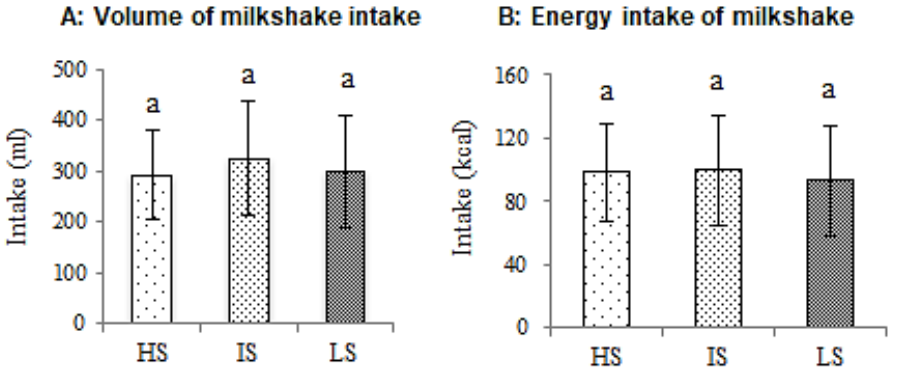


**Figure 2:** Mean values of *ad libitum* intake of HS, IS and LS milkshake samples measured as total volume consumed (A) and energy (B) consumed. Error bars represent the standard deviations. Values within a column without a same letter superscript are significantly different ($p < 0.05$)

*Effect of sweetness intensity on sensory-specific satiety*

The pleasantness of the HS milkshake was reduced significantly ($p < 0.05$), following the intake of the HS, IS or LS milkshake. This suggests that SSS for the HS milkshake was developed following *ad libitum* intake of any sweet milkshake. However, the reduction in the pleasantness of the HS milkshake was not affected by the sweetness intensity of the milkshake. This indicated that the extent of SSS for the milkshake was not affected by the sweetness intensity of the milkshake.

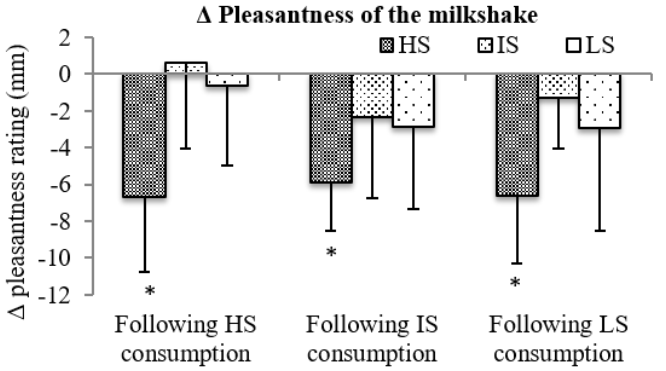


**Figure 3:** Change (Δ) in pleasantness of HS, IS and LS milkshakes. Δ pleasantness were calculated by subtracting the initial pleasantness ratings of the milkshake from the pleasantness ratings after consumption of the milkshake. Error bars are standard errors. '*' represents a significant reduction in the pleasantness. $p < 0.05$.

Experiment 2

*Effect of combined aroma and taste on appetite sensation*

The consumption of the sample drinks containing only aroma (S2) or only taste (S3) did not affect appetite sensation differently from the water control (S1) (Figure 4) [19]. But the combined aroma and taste (S4) suppressed hunger sensation more than the water control (S1) or the sample drink with only aroma (S2) or only taste (S3), during the 15-min sample drink consumption. The drink condition with combined aroma and taste (S4) suppressed hunger sensation more than the water control (S1), the aroma condition (S2) and the taste condition (S3) for a further 30 minutes, 20 min and 5 minutes after the sample drink was consumed, respectively ($p < 0.05$). This suggests that the combined aroma and taste induced a greater satiation and a greater short-term satiety than aroma, taste and the water control. This was potentially due to an increase on the perceived intensity of flavour and an enhancement of the complexity of the perceived flavour because of aroma-taste cross-modal interaction [19].

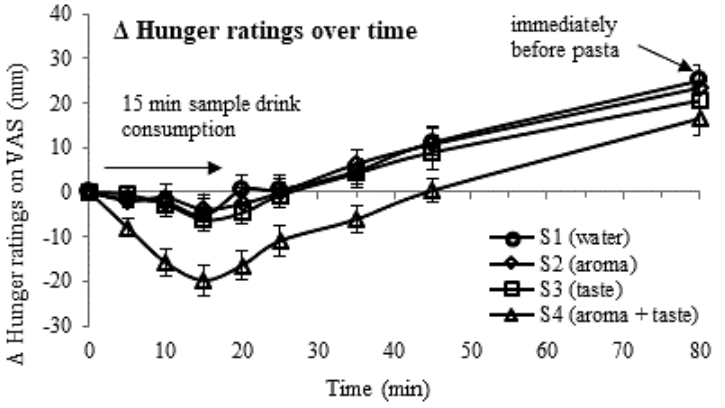


**Figure 4:** Mean Δ hunger over 5, 10, 15, 20, 25, 35, 45, and 80 min after starting to consume sample drink of S1 (water), S2 (aroma), S3 (taste) or S4 (aroma + taste), n = 25 participants. Error bars represent standard errors [19].

*Effect of combined aroma and taste on subsequent food intake*

The subsequent energy intake of the pasta meal, provided 65 min following the sample drink consumption, was not different by the different sample drink preloads (Table 1) [19]. The accumulative energy intake did not differ between the sample drink conditions.

**Table 1:** Mean ± standard deviation (n=26) of energy intakes from pasta meal and accumulative energy intake of pasta and sample drink in the 4 sample drink conditions [19].

| Sample drink conditions | *Pasta meal energy intake (kcal)* | *Accumulative (sample + pasta) energy intake (kcal)* |
|---|---|---|
| S1 | 776 ± 96 [a] | 776 ± 96 [a] |
| S2 | 781 ± 75 [a] | 781 ± 75 [a] |
| S3 | 759 ± 82 [a] | 807 ± 82 [a] |
| S4 | 757 ± 89 [a] | 806 ± 89 [a] |

Values within a column without a same letter superscript are significantly different ($p < 0.05$)

## Conclusion

A single flavour modality, aroma or taste, had no actual effect on appetite and food intake. The consumption of a drink containing one flavour modality (sweet taste) induced sensory-specific satiety for that flavour modality (sweet taste). However, increasing the intensity of a single flavour modality did not affect appetite sensation, food intake and sensory-specific satiety. An enhancement on the flavour complexity due to the multimodal interaction of two flavour modalities (aroma + taste) suppressed hunger sensation significantly but did not affect subsequent food intake.

## References

1. Withrow, D., and Alter, D.A., (2011) Obes Rev, 12: 131-141.
2. Blundell, J.E. and Bellisle, F., (2013) In: Woodhead publ food s; Woodhead Publishing: Cambridge: p. 8-9.
3. Blundell, J.E., Rogers, P.J. and Hill, A.J., (1987) Food Acceptance and Nutrition, 205-220.
4. Benelam, B., (2009) Nutrition Bulletin, 34: 126-173.
5. Yeomans, M.R., (1996) Appetite, 27: 119-133.
6. Spiegel, T.A., Shrager, E.E. and Stellar, E., (1989) Appetite, 13: 45-69.
7. Bobroff, E.M. and Kissileff, H.R., (1986) Appetite, 7: 85-96.
8. Hill, A.J., Magson, L.D. and Blundell, J.E., (1984) Appetite, 5: 361-371.
9. Bolhuis, D.P., Lakemond, C.M.M., de Wijk, R.A., Luning, P.A. and de Graaf, C., (2011) J Nutr, 141: 2242-2248.
10. Ramaekers, M.G., Luning, P.A., Ruijschop, R.M.A.J., Lakemond, C.M. and Van Boekel, M.A.J.S., (2011) Appetite, 57.
11. Yeomans, M.R., (1998) P Nutr Soc, 57: 609-615.
12. Rolls, B.J., (1986) Nutr Rev, 44: 93-101.
13. Auvray, M. and Spence, C., (2008) Conscious Cogn, 17: 1016-1031.
14. Wallace, M.T., (2015) Curr Biol, 25: R986-R988.
15. Flint, A., Raben, A., Blundell, J.E. and Astrup, A., (2000) Int J Obesity, 24: 38-48.
16. Beck, A.T., Steer, R.A. and Garbin, M.G., (1988) Clin Psychol Rev, 8: 77-100.
17. Stunkard, A.J. and Messick, S., (1985) Journal of Psychosomatic Research, 29: 71-83.
18. Astbury, N.M., Taylor, M.A. and Macdonald, I.A., (2011) J Nutr, 141: 1381-1389.
19. Yin, W., Hewson, L., Linforth, R., Taylor, M. and Fisk, I.D., (2017) Appetite, 114: 265-274.

# The TRP channel agonists nonivamide and cinnamaldehyde augment cold-induced mitochondrial biogenesis in white adipocytes

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## Abstract

An increased conversion of white adipocytes to a "brite" adipocyte phenotype, characterized by increased number of mitochondria and increased expression of uncoupling protein 1 (UPC-1), represents a promising anti-obesity approach. Beside an activation of beta-adrenergic signalling by a cold ambient temperature, also the activation of several temperature-sensing transient receptor potential (TRP) ion channels has been associated with browning of white adipocytes. In the present study, we hypothesized that the two food-derived aroma compounds and TRP-channel agonists nonivamide and cinnamaldehyde augment the thermogenic response of 3T3-L1 white adipocytes to a cold stimulus. We found that upon incubation at 29°C, 3T3-L1 adipocytes show an increased expression of UCP-1 at the levels of gene transcripts and protein level as well as increased mitochondrial biogenesis in comparison to 37°C, confirming the validity of the cellular model. In addition, we demonstrate here that treatment of 3T3-L1 cells with 0.1 µM nonivamide or 10 µM cinnamaldehyde for 48 h increased mitochondrial biogenesis when incubated at 29°C, but not at 37°C. These data hint towards beneficial effects of the two aroma compounds when applied as a chronic treatment in addition to a cold stimulus, which has to be validated in future studies.

## Introduction

For the first time, the current generation of humans may have a shorter life span than the previous [1]. This is mostly due to an inactive lifestyle combined with the consumption of energy-dense food, resulting in an expanding population of chronically ill people with obesity-associated diseases [2]. This underlines the urgent need for new weight loss-supporting measures.

Weight loss is achieved by a negative energy balance, which can be accomplished by an increased energy expenditure, determined by the total metabolic rate. The total metabolic rate is divided into basal metabolic rate, physical activity, and thermogenesis [3]. Recent calculations demonstrate that the adaptive thermogenesis, which is mediated by brown adipose tissue, is accounting for ~5% of the total thermogenesis and can be a relevant target to achieve a difference in body weight [4]. The brown adipocytes are, in contrast to white adipocytes, densely packed with mitochondria that express UCP-1. Upon activation, this protein shortcuts the circuits of the electrochemical gradient of the respiratory chain, which is the driving force in ATP production. Thus, UCP-1 activates heat production from available substrates, leading to an increased energy expenditure [5]. More recently, upon stimulation, clusters of UCP-1 expressing adipocytes with an increased number of mitochondria have been identified in white adipose tissue. This specific phenotype of cells is often referred to as "brite" (brown in white) adipocytes [6, 7].

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The probably most widely studied inducer of a brite adipocyte phenotype is cold temperature, which stimulates sympathetic nerve activity by beta-adrenergic receptor activation, followed by increased cyclic adenosine monophosphate (cAMP) and protein kinase A levels, finally leading to activation of UCP-1 [8].

Beside a cold ambient temperature, also activation of the warm-temperature-sensing transient receptor potential (TRP) ion channels TRPV1 [9] and TRPV4 [10] has been associated with browning of white adipocytes. In addition, also the food-derived TRPA1 agonist cinnamaldehyde has been demonstrated to unfold thermogenic effects in primary white adipocytes [11]. However, the response of TRP channel agonists in addition to cold ambient temperatures has not been studied so far. Thus, in the present study, we hypothesized that the food-derived aroma compounds nonivamide and cinnamaldehyde, which are agonists for the TRPV1 or TRPA1 channel, respectively, may augment cold-induced browning-responses in 3T3-L1 white adipocytes as a model. The first part of this study investigated the suitability of the 3T3-L1 cells to demonstrate browning of white adipocytes using UCP-1 gene expression and protein level, and mitochondrial biogenesis as novel marker for compound screening. The second part of the study assessed the effects of the TRPV1 agonist nonivamide and the TRPA1 agonist cinnamaldehyde on mitochondrial biogenesis with or without an additional cold stimulus.

## Experimental

### *Materials*

3T3-L1 (mouse fibroblasts) cells were purchased at ATCC. Nonivamid was kindly provided by Symrise AG, Holzminden, Germany. All other used reagents were obtained from Sigma Aldrich, Austria, unless stated otherwise.

### *Cell culture*

3T3-L1 pre-adipocytes were cultured and differentiated to mature adipocytes as described before [12]. Adipocytes were used for experiments on day 10 after initiation of differentiation. Incubation of the cells was carried out in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 4.5 [g/L] glucose and 2 mM pyruvate in the absence of phenol red and bicarbonate.

### *Cell viability*

Effect of applied concentrations of cinnamaldehyde (CA, 10-100µM) and nonivamide (NV, 0.1-1µM) on cellular proliferation as a measure for the viability of the cells was analysed via standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Carl Roth, Germany) assays as described before [12].

### *Quantitative real time-polymerase chain reaction*

*UCP-1* gene expression was analysed in mature 3T3-L1 cells by using quantitative Real-Time PCR. The total RNA was isolated using MasterPure™ RNA Purification Kit (Epicentre®) according to manufacturer's protocol. Quality and concentration of isolated RNA was analysed using NanoQuant Plate on an Infinite M200 Plate reader (both Tecan). RNA was subsequently reversely transcribed into cDNA using the high capacity RNA to cDNA Kit (ABI, Thermo Fisher) and the PCR was performed on a StepOnePlus device (ABI, Thermo Fisher) using SYBR Green Fast Master Mix (ABI, Thermo Fisher). Gene expression of *UCP-1* was measured in triplicates and normalized to the reference genes *HPRT* and *ACTB* as endogenous controls. The hypothetical respective starting mRNA levels were calculated using LinReg v 12.8 software and presented as fold change relative to control cells (37°C, set to 1). Detailed Primer information can be obtained in Table 1.

**Table 1:** Sequences of the primers used during PCR reaction.

| Gene | *Sequence (3'-5')* | *Product length [bp]* | *Reference* |
|---|---|---|---|
| *HPRT* | FW: GAGAGCGTTGGGCTTACCTC<br>RV: ATCGCTAATCACGACGCTGG | 136 | [12] |
| *ACTB* | FW: TCTTTGCAGCTCCTTCGTTG<br>RV: CATTCCCACCATCACACCCT | 188 | [13] |
| *UCP-1* | FW: AGGCTTCCAGTACCATTAGGT<br>RV: CTGAGTGAGGCAAAGCTGATTT | 133 | [14] |

*Mitochondrial biogenesis*

For assessing mitochondrial biogenesis, mitochondria were stained using MitoTracker® Deep Red (Molecular Probes, Thermo Fisher) after incubation of 3T3-L1 adipocytes at 37°C or 29°C for 48 h in a 96-well format with or without addition of the test substances based on methods described by Chowanadisai et al. [15] and Huang et al. [16]. In brief, cells were incubated with 50 nM MitoTracker ® dissolved in phenol red-free DMEM for 30 min, and washed three times with phenol red-free DMEM before fluorescence was measured at 640 nm excitation and 665 nm emission by means of a Tecan Infinite M200 (Tecan) plate reader. Results were calculated in percent relative to untreated control cells.

*Immunocytochemistry*

For intracellular UCP-1 staining, 3T3-L1 pre-adipocytes were differentiated on round glass slides (12mm, Carl Roth) in 24-well plates (Sarstedt) before incubation at 37°C or 29°C for 48 h. After fixation with 4 % formalin and blocking for 60 min with 5% FBS (Gibco) and 0.5% Trition-X100 (Carl Roth), cells were incubated under gentle agitation with a specific UCP-1 primary antibody (1:200; Abcam) for further 60 min. Detection was carried out using Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Molecular Probes). The corrected total cell fluorescence (CTFC) was analysed using Image J 2.0.0. As a control, background fluorescence intensity after incubation of the cells with the secondary antibody alone was analysed (data not shown).

*Statistical analysis*

Data are presented as fold change or percent of control ± SEM, calculated from at least three different experiments with multiple technical replicates each after excluding outliers identified using Nalimov outlier test. Normal distribution and equal variance of the data were tested using Shapiro-Wilk or Brown-Forsythe test, respectively. Significant differences between two groups were assessed using T-Test, or Mann-Whitney-U test, in case of not normally distributed data. Likewise, significant differences between multiple treatments were tested using One-Way ANOVA with Holm-Sidak post hoc test, or One-Way ANOVA on Ranks with Dunn's post-hoc test, respectively. Differences between treatment groups are marked with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. SigmaPlot 13.0 was used for statistical analysis.

## Results and discussion

Browning of white adipocytes is, amongst others, characterized by an increased number of mitochondria combined with an upregulation of *UCP-1* expression [17]. The

process of browning can not only be mediated by cold-stimuli via beta-adrenergic signalling, but also by activation of TRP channels like TRPV1 and TRPV4 [9, 10]. In the present study, we hypothesized that the food-derived aroma compounds nonivamide (NV) and cinnamaldehyde (CA), which are agonists for TRPV1 or TRPA1 cation channels, respectively, may augment cold-induced browning responses in 3T3-L1 white adipocytes.

In a first set of experiments, 3T3-L1 adipocytes were tested for their browning response when exposed to a cold ambient temperature. Since pyruvate serves as an easily catabolized substrate for mitochondrial futile cycles, and pyruvate metabolism partly regulates lipogenesis during cold exposure [18], the impact of pyruvate on *UCP-1* expression was addressed as well. *UCP-1* gene expression was analysed as a marker of the induction of a beige phenotype in 3T3-L1 cells after exposure to 29°C for 6 h (Figure 1A), and revealed an 8.98 ± 3.61 fold increased expression. When no pyruvate was added to the incubation media, the *UCP-1* gene expression was significantly reduced to a 2.06 ± 0.25 fold change increase (data not shown). This result strengthens the assumption that the increased *UCP-1* gene expression indicates a thermogenic response in 3T3-L1 adipocytes.

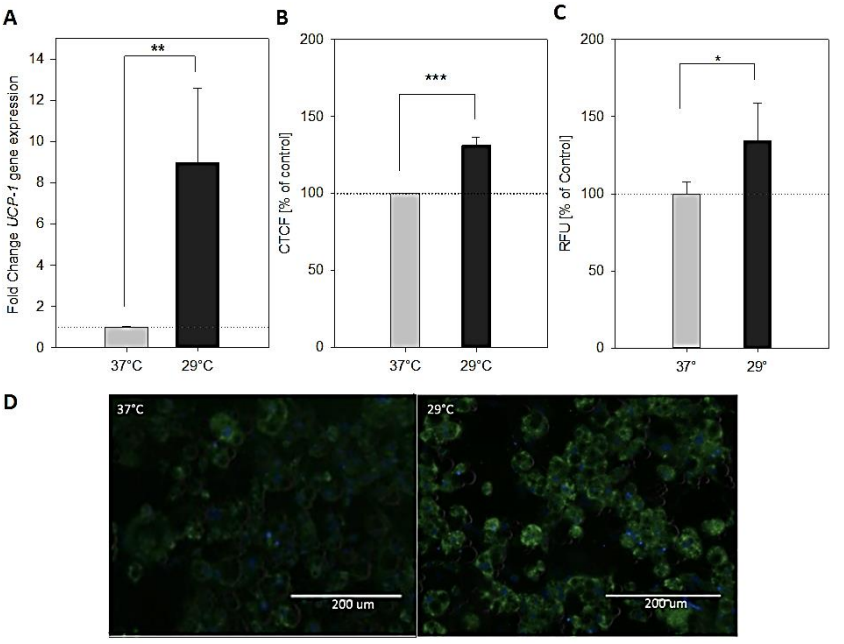


**Figure 1:** Analysis of *UCP-1* gene expression (**A**, n=10), UCP-1 immunostaining (**B**,**D**, analysis of the corrected total cellular fluorescence (CTCF) in a total of 50-100 cells) and mitochondrial biogenesis (**C**, n=4) in 3T3-L1 adipocytes after incubation for 6 h (**A**) or 48 h (**B**-**D**) at 37°C or 29°C. Statistics: Student's T-test, **P*<0.05, ***P*<0.01, ****P*<0.001.

Moreover, the increased *UCP-1* gene expression was confirmed by immunocytostaining on protein level after incubation for 48 h at 29°C, demonstrating 31.3 ± 5.22% increased UCP-1 levels in cells kept at 29°C compared to cells incubated at 37°C (Figure 1B & 1D). In addition, staining of the mitochondria using a specific mitochondric dye demonstrated that the number of mitochondria was increased by 36.6 ± 24.1% after 48 h at 29°C in comparison to 37°C (Figure 1C). These data point to an

increased mitochondrial biogenesis following exposure to cold temperatures and confirm the validity of the 3T3-L1 cells as a model for browning.

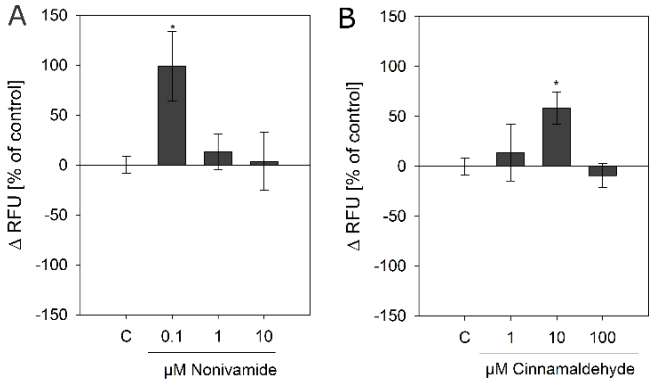


**Figure 2:** Differences in the mitochondrial biogenesis after treatment with 0.1-10 µM nonivamide (A) or 1 to 100 µM cinnamaldehyde (B) for 48 h at 29°C. n=3-4. Statistics: One-Way ANOVA *vs.* control with Holm-Sidak post hoc test (A), One-Way ANOVA on Ranks *vs.* control with Dunn's post hoc test (B). * $P<0.05$

Beside cold temperatures, also activation of thermosensitive TRP channels like TRPV4 and TRPV1 has been shown to induce a brite adipocyte phenotype [9, 10]. In addition, the thermosensitive TRPV1 and, more recently, also TRPA1 has been shown to be involved in the differentiation and maturation process of 3T3-L1 adipocytes [12]. Moreover, the TRPV1 agonist NV has been associated with a decreased body fat content following oral administration of 0.15 mg per day in a 12-week human intervention trial with overweight test subjects [19]. Likewise, also addition of 1% of the TRPA1 agonist CA to a high-fat diet has been associated with anti-obesity effects accompanied by increased mitochondria protein levels in mice [20]. However, it has not been clarified yet if thermogenic responses of adipocytes to a cold-stimulus can be enhanced by a treatment with NV or CA. Thus, in the present study, we investigated whether NV or CA induce mitochondrial biogenesis in 3T3-L1 adipocytes at ambient temperatures of 37°C or 29°C as a marker for potential browning effects. The range of test concentrations of the two compounds was chosen based on the $EC_{50}$ values for activation of TRPV1 (1.4 µM, NV [21]) or TRPA1 (63 µM, CA [22]), respectively. Negative effects of NV or CA in the applied concentrations on cellular proliferation as a measure for cell viability were excluded using standard MTT assays (data not shown). Neither incubation with 0.1-10 µM NV nor with 1-100 µM CA for 48 h at 37°C led to increased mitochondrial biogenesis (data not shown). However, when incubated at 29°C for 48 h, 0.1 µM NV as well as 10 µM CA increased the mitochondrial biogenesis by 99.2 ± 16.2% or 58.5 ± 16.2%, respectively. A very recent report on increased markers of thermogenesis at 37°C after treatment with CA was carried out with higher test concentrations of 400 µM CA. Induction of thermogenesis markers was analysed after short-term incubation up to 4 h [11], although longer-lasting effects have not been investigated yet on the mitochondrial level. Thus, it cannot be excluded that higher test concentrations of CA will lead to an increased number of brite adipocytes at 37°C as studied here. Data on the effect of NV on markers of thermogenesis in adipocytes are not available so far, however, the more pungent structural analogue of NV, capsaicin treatment has been shown to increase expression of genes associated with a brite adipocyte phenotype by Baboota *et al.*[23],

however, without addressing mitochondrial biogenesis directly. Overall the current data emphasize the need for more studies to evaluate the efficacy of food-derived TRP agonists like NV and CA to stimulate mitochondrial biogenesis at support of body weight loss.

To summarize, the presented data hint towards a long-term beneficial effect of the TRP channel agonists NV and CA on thermogenesis in addition to a cold-stimulus. Future studies are needed to confirm browning effects *in vitro* and *in vivo* and to clarify an involvement of TRPV1 and TRPA1.

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## References

1. S. J. Olshansky, D. J. Passaro, R. C. Hershow, J. Layden, B. A. Carnes, J. Brody and D. S. Ludwig, N Engl J Med, 2005, 352: 1138-1145.
2. T. Pischon, H. Boeing, K. Hoffmann, M. Bergmann, M. B. Schulze, K. Overvad and E. Riboli, N Engl J Med, 2008, 359, 2105-2120.
3. P. Stiegler and A. Cunliffe, Sports Med, 2006, 36, 239-262.
4. S. Enerback, Cell Metab, 2010, 11, 248-252.
5. D. Ricquier, Front Endocrinol (Lausanne), 2011, 2, 85.
6. M. Ghorbani and J. Himms-Hagen, Int J Obes Relat Metab Disord, 1997, 21, 465-475.
7. A. Vitali, I. Murano, M. C. Zingaretti, A. Frontini, D. Ricquier and S. Cinti, J Lipid Res, 2012, 53, 619-629.
8. M. L. Bonet, P. Oliver and A. Palou, Biochim Biophys Acta, 2013, 1831, 969-985.
9. P. Baskaran, V. Krishnan, J. Ren and B. Thyagarajan, Br J Pharmacol, 2016, 173, 2369-2389.
10. L. Ye, S. Kleiner, J. Wu, R. Sah, R. K. Gupta, A. S. Banks and B. M. Spiegelman, Cell, 2012, 151, 96-110.
11. J. Jiang, M. P. Emont, H. Jun, X. Qiao, J. Liao, D. I. Kim and J. Wu, Metabolism, 2017.
12. B. Lieder, M. Zaunschirm, A. K. Holik, J. P. Ley, J. Hans, G. E. Krammer and V. Somoza, Front Pharmacol, 2017, 8, 316.
13. J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen and T. L. Madden, BMC Bioinformatics, 2012, 13, 134.
14. X. Wang, A. Spandidos, H. Wang and B. Seed, Nucleic Acids Res, 2012, 40, D1144-1149.
15. W. Chowanadisai, K. A. Bauerly, E. Tchaparian, A. Wong, G. A. Cortopassi and R. B. Rucker, J Biol Chem, 2010, 285, 142-152.
16. C. Huang, D. Chen, Q. Xie, Y. Yang and W. Shen, Biochem Biophys Res Commun, 2013, 438, 211-217.
17. M. Harms and P. Seale, Nat Med, 2013, 19, 1252-1263.
18. K. S. McCommis and B. N. Finck, Biochem J, 2015, 466, 443-454.
19. C. M. Hochkogler, B. Lieder, P. Rust, D. Berry, S. M. Meier, M. Pignitter and V. Somoza, Mol Nutr Food Res, 2017, 61.
20. Y. Tamura, Y. Iwasaki, M. Narukawa and T. Watanabe, J Nutr Sci Vitaminol (Tokyo), 2012, 58, 9-13.
21. K. C. Thomas, M. Ethirajan, K. Shahrokh, H. Sun, J. Lee and T. E. Cheatham, 3rd, C. A. Reilly, J Pharmacol Exp Ther, 2011, 337, 400-410.
22. M. Bandell, G. M. Story, S. W. Hwang, V. Viswanath, S. R. Eid, M. J. Petrus and A. Patapoutian, Neuron, 2004, 41, 849-857.
23. R. K. Baboota, D. P. Singh, S. M. Sarma, J. Kaur, R. Sandhir, R. K. Boparai and M. Bishnoi, PLoS One, 2014, 9, e103093.

# Biotransformation, transmission and excretion processes of garlic odorants in humans: impact on human milk, urine and exhaled breath

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## Abstract

The impact of raw garlic consumption (3 g) on the composition of human milk (n=18), urine (n=19) and exhaled breath (n=11) was explored. Urine and milk samples gathered prior to and after garlic ingestion (up to 24 h and 4 h, respectively) were analyzed by GC-MS and GC-GC-MS. Milk samples were additionally assessed by aroma profile analyses. Exhaled breath analysis was performed by PTR-MS. The compound allyl methyl sulfide was identified as garlic-derived metabolite in all three excreta. Furthermore, allyl methyl sulfoxide and allyl methyl sulfone were identified as metabolites in both urine and milk. These garlic-derived metabolites were quantified by means of stable isotope dilution assays. Two garlic-derived metabolites were identified in breath, namely allyl methyl sulfide and methanethiol. The measurements revealed inter-individual differences in metabolite concentrations and removal kinetics after consumption of 3 g of raw garlic.

## Introduction

Garlic is savoured for its characteristic aroma and has been associated with beneficial health effects [1-3]. The active principles of garlic are not fully understood, although allicin and its metabolic derivatives have been proposed as active components [4]. Constituents ingested with food can be strongly modified in the body and evoke physiological effects [5]. In previous studies we identified three garlic-derived metabolites in human milk and urine, namely allyl methyl sulfide (AMS), allyl methyl sulfoxide (AMSO) and allyl methyl sulfone ($AMSO_2$) [6, 7] .These compounds have been identified as the dominant metabolites in rat liver, plasma and urine after treating rats with diallyl disulfide, a constituent compound in garlic [8]. To further characterize their biotransformation processes, the present study aimed to quantify these metabolites in human milk and urine and additionally explore the influence of raw garlic consumption on the odorant and metabolite composition of exhaled breath.

## Experimental

### *Samples*

This study was conducted in agreement with the Declaration of Helsinki. Written, informed consent was given by all volunteers prior to participation. Withdrawal from the study was possible at any time. The study (registration number 49_13B) was approved by the Ethical Committee of the Medical Faculty, Friedrich-Alexander Universität Erlangen-Nürnberg.

Milk samples were collected using mechanical or electrical breast pumps according to the mothers' preferences. Sampling took place within the 9 to 36 weeks postpartum

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lactation period. Milk samples (n=18) were collected at the times indicated in *Figure 1*. Sampling intervals reflected the normal lactation period of individual mothers.

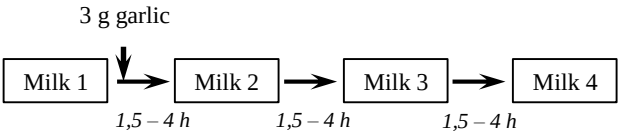


**Figure 1:** Times of milk sample taking

Urine samples (n=19) were collected in brown glass bottles. Breath analysis (n=11) was performed by having individuals exhale deeply into a buffered-end-tidal (BET) sampling tube that was connected to a proton-transfer-reaction mass spectrometer (PTR-MS) for on-line analysis. Sampling times of urine and breath samples are indicated in Figure 2.

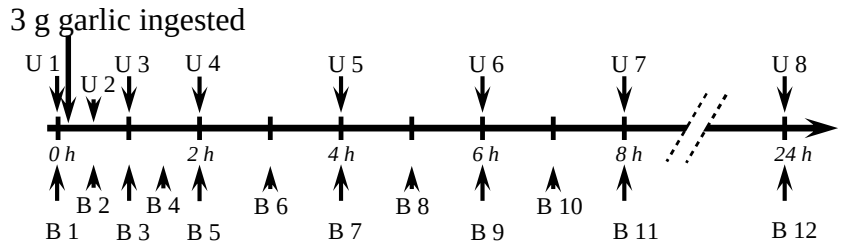


**Figure 2:** Urine and breath sampling. U: urine sample B: breath sample

*Stable isotope dilution assays (SIDA)*

Defined amounts of $^{2}H_3$-isotopically-labeled reference compounds were added to each milk and urine sample. Dichloromethane (DCM) was then added at a ratio of 1:2 (DCM:sample, v/v) to the sample. The mixture was stirred (30 min, room temperature) and then distilled via solvent-assisted flavour evaporation (SAFE; 50 °C) and subsequently concentrated to a volume of 100 µL. Analyses of AMSO and $AMSO_2$ were performed using GC-MS in selected ion monitoring (SIM) mode. The analysis of AMS was performed with GC-GC-MS. The metabolite concentrations in milk and urine samples were calculated from the intensity ratios of labeled to unlabelled compounds in the extracts.

*Creatinine content*

The creatinine content of each urine sample was determined using a creatinine kit (Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany). The concentrations of the metabolites in urine samples were normalized to the creatinine content of the respective urine sample.

*Sensory analyses*

All milk samples underwent sensory analyses. These analyses took place prior to the work-up described above and were performed by a trained panel that evaluated the samples orthonasally.

*Breath gas analyses*

On-line PTR- time-of-flight-MS (PTR-TOFMS) was used for monitoring the presence of garlic metabolites in exhaled breath gas [9]. Use of a BET sampling apparatus extended the analysis time of end-tidal breath and reduced the likelihood of interferences of garlic-constituents emanating from the stomach.

## Results and discussion

The analyses revealed three garlic-derived metabolites in human milk and urine, namely AMS, AMSO and $AMSO_2$. Of these three, only AMS had a garlic-/cabbage-like door whereas the other two metabolites were odourless. AMS had an impact on the aroma profile of the milk samples, whereby milk samples collected prior to garlic consumption did not exhibit the garlic-/cabbage-like door whereas samples collected after garlic intervention clearly did. Furthermore, the quantitative analyses revealed differences in concentrations and excretion kinetics of metabolites between individuals. Three examples of milk and urine sample sets are shown in *Figure 3* and *4*, respectively.

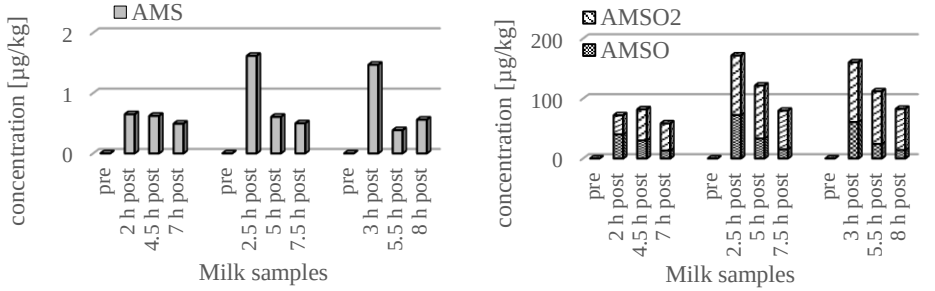


**Figure 3:** Concentrations of garlic metabolites (left: AMS; right: AMSO and $AMSO_2$) in three different milk sets. pre: sample prior garlic ingestion. x h post: samples after garlic ingestion.

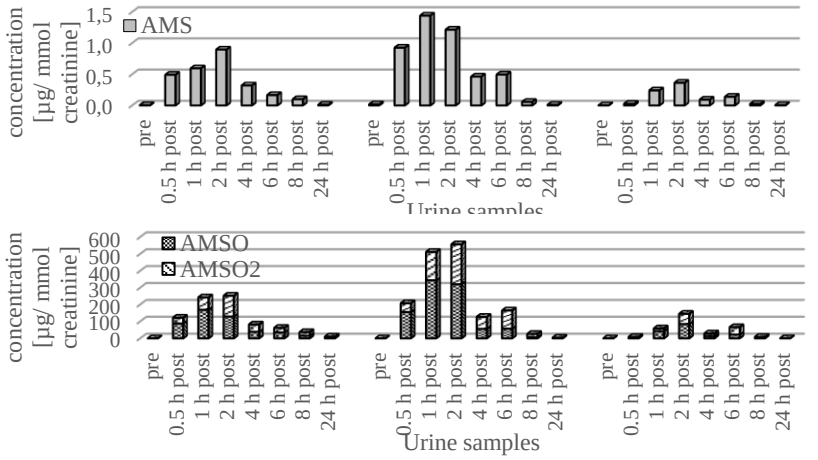


**Figure 4**: Concentrations of garlic metabolites (left: AMS; right: AMSO and $AMSO_2$) in three different urine sets. pre: sample prior garlic ingestion. x h post: samples after garlic ingestion.

The highest concentrations of AMS and AMSO in the investigated milk samples were detected in the first samples taken after garlic consumption. In contrast, $AMSO_2$ maxima were only detected in the second milk sample after garlic consumption for some

individuals. Similar results were observed in urine samples, with the highest concentrations of the garlic metabolite being detected 1-2 h after garlic intervention. By comparison, the $AMSO_2$ maxima often appeared slightly later than AMSO. In breath, the highest AMS concentration was observed between 0.5-4 h after garlic consumption (see *Figure 5*). Methanethiol displayed similar excretion kinetics to AMS.

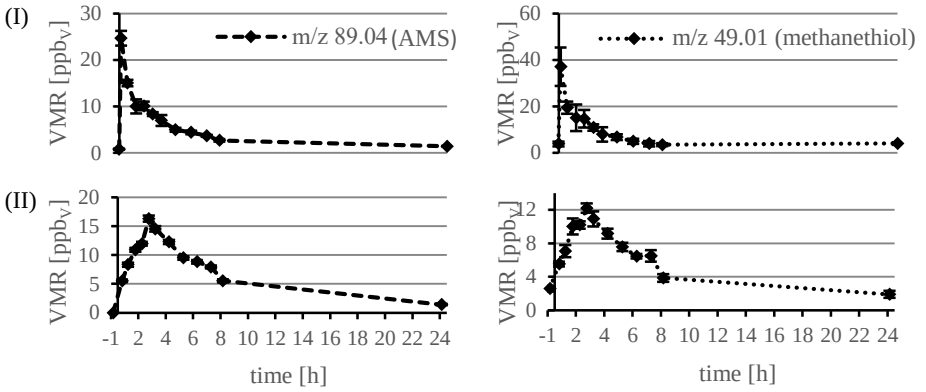


**Figure 5:** Breath profiles of AMS (left; m/z 89.04) and methanethiol (right; m/z 49.01) for two individuals after consumption of 3 g raw garlic. t=0: time of garlic ingestion.

Overall, the duration and concentration of excretion of the metabolites exhibited large inter-individual variations, despite all individuals consuming identical quantities of raw garlic. The concentrations observed in urine samples were approximately twice or three times higher than those observed in the milk samples. Additional possible elimination routes such as via feces were not considered in this study. Nevertheless, these findings suggest that different garlic-derived metabolites are excreted via different metabolism pathways.

**Acknowledgement**

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## References

1. Adler, A.J. and B.J. Holub, (1997) The American Journal of Clinical Nutrition, 65(2): p. 445-50.
2. Sobenin, I.A., et al., Lipids in Health and Disease, 2010. 9(1): p. 1-6.
3. Ried, K., C. Toben, and P. Fakler, (2013) Nutrition Reviews, 71(5): p. 282-299.
4. Lawson, L.D., (1998) Phytomedicines of Europe 691: p. 176-209.
5. Kirsch, F., Horst K., Röhrig W. et al., (2012) Metabolomics, 9(2): p. 483-496.
6. Scheffler, L., Sauermann Y., Zeh G., Hauf K., Heinlein A., Sharapa C. and Buettner A., (2016) Metabolites, 6(2): p. 18.
7. Scheffler, L., Sauermann Y., Zeh G., Heinlein A., Sharapa C. and Buettner A., (2016) Metabolites, 6(4).
8. Germain, E., Auger J., Ginies C., Siess M.H., and Teyssier C., (2002)Xenobiotica, 32(12): p. 1127-1138.
9. Herbig, J., Titzmann T., Beauchamp J., Kohl I., and Hansel A., (2008) Journal of Breath Research, 2(3).

# The novel umami peptides identified from *Takifugu obscurus* and *Takifugu rubripes*

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## Abstract

Consuming Takifugu is very popular in China because of its delicious taste. In this study, Takifugu obscurus (*T. obscurus*) and Takifugu rubripes (*T. rubripes*), edible puffer fishes, were used as the raw materials to isolate and identify the umami peptides. Different fractions were separated and purified using some isolation technologies such as membrane separation. Then sensory evaluation was performed of theses fractions to distinguish the peptides with the umami taste. The separated umami peptides were synthesized to verify their umami taste using the sensory evaluation. Finally, 12 novel umami peptides were identified and validated. This study could provide theoretical basis and technical support to understand the flavour of Takifugu and develop the new flavouring using the umami peptides.

## Introduction

Flavour perception involves in the olfactory and gustatory interaction, which results from the volatile and non-volatile compounds releasing from the food. It also has a decisive influence on the eating quality, preference and acceptability of foods, especially for meat products [1]. And this is the reason why the number of research on understanding the chemistry of meat flavour, and determining the dominant influencing factors on flavour quality during the production and processing of meat has increased over the years. Inorganic salts, peptides, amino acids, organic acids, ATP breakdown products (ATP derivatives) and sugars contribute to generating the taste of meat and meat products in their water soluble substances [2]. Among these contributors, flavour peptides possess unique taste properties owing to their primary structure and amino acid sequence, which participates in the formation of the flavour and improve the overall flavour of food [3].

The pioneer study of the flavour peptide can be traced back to 1987. Japanese scientist firstly isolated the beefy meaty peptide (BMP) from beef meat, which was identified as a savoury taste similar to monosodium glutamate (MSG) [4]. After that, many researchers are gradually focusing on isolating the novel flavour peptides using gel filtration chromatography, reverse phase high performance liquid chromatography, tandem mass spectrometry, sensory evaluation and the electronic tongue.

Puffer fish, known as blowfish, bubble fish, fugu and porcupine fish, is a popular and edible fish species in both China and eastern Asian countries, owing to its unique umami taste. Peptides and free amino acids are the key components to release the delicious taste of puffer fish [5]. The relationship between the formation of flavour and free amino acids, ATP in puffer fish has been well studied. However, the isolation and identification of the novel umami peptides in puffer fish is a neglected topic.

Therefore, our present study is to identify the novel umami peptides from *Takifugu obscurus* and *Takifugu rubripes.* Different treatments including water soluble, heating, and enzymatic hydrolysate were used on *Takifugu* to extract and separate the umami peptides. The meaning of the study is not only to identify the novel umami peptides, but also to provide information on developing the new umami flavours or seasonings.

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## Experimental

*Materials*

*Takifugu obscurus* and *Takifugu rubripes* fish were both purchased from Jiangsu Zhongyang Group Co., Ltd (Nantong City, Jiangsu Province, China). And they were killed by a puffer fish licensed chef. Muscle filets were removed and three different treatments, including water soluble, heating, and enzymatic hydrolysate were used to extract the fractions from fresh cultured muscle filets. The supernatants were collected freeze-dried and stored at -80℃.

*Isolation and purification* [6]

The T. *obscurus* and T. *rubripes* extractions with a molecular weight of less than 3KDa were fractionated by ultra-filtration using membranes with a MW cut off size of 3KDa. The condition of ultrafiltration was at 5℃ under 2.5-3.0 Psi $N_2$ pressure. All the ultrafiltration fractions were collected for freeze-drying and stored at -80℃. Sephadex G-15 gel filtration chromatography (column 1.6*60cm) was used to elute the extracted fractions with deionized water as the eluting solvent and the flow rating being 0.75ml/min. The UV absorbance of the eluent was monitored at 220nm. Each filtration fraction from successive runs were pooled and lyophilized for the sensory evaluation (described in the following).

The most intense umami taste fraction, obtained from the gel filtration chromatography, was separated using a Waters 2695 Allicance® HPLC system under the condition of a Kromasil C18 column at 30℃ to get several sub-fractions. The elution condition was an isocratic elution with eluting solvent 90% A (Milli-Q water) and 10% B (ACN HPLC grade) for 20 minutes at a flow rate of 1ml/min. And the elution peaks were monitored at 215nm.

*Identification of the umami peptides by MALDI-TOF/TOF MS/MS* [6]

Freeze-dried RP-HPLC fraction with the most intense umami taste was first re-dissolved in 50% ACN, 0.1% TFA containing 4mg/ml a-cyano-4-hydroxycinnamic acid (HCCA) and filtered. Myoglobin digested with trypsin was used to calibrate the mass instrument with internal calibration mode. The MALDI-TOF/TOF MS/MS (mass range scanning from 450 to 2000Da) was run in the positive refractor mode. MS/MS spectra were acquired from 2000 shots by adjusting the laser intensity above the threshold for generation of molecular ions for each umami peptide.

*Synthesis of the umami peptides*

The sequenced umami peptides by *MALDI-TOF/TOF MS/MS* were synthesised using the Solid phase peptide synthesis technique. The purity of synthetic peptides was higher than 97%.

*Sensory evaluation* [6]

The sensory panel consisted of 3 males and 5 females, who were screened by recognising the basic tastes. The sensory evaluation was conducted in the sensory lab. The sensory attributes, including sweetness, sourness, bitterness, umami, and kokumi, were evaluated to describe the taste perception of sample fractions, including the ultrafiltration, Sephadex G-15 gel filtration, RP-HPLC fractions, and the synthetic umami peptides.

## Results and discussion

After ultrafiltration and gel chromatography, the fractions of *T. obscurus* and *T. rubripes* were separated and subjected to the sensory evaluation in order to screen out the most intense fraction with the umami taste. RP-HPLC was conducted on this fraction to get several individual sub-fractions. The descriptive analysis was used to describe and assess the sensory properties of the sub-fractions. The sub-fractions with the most intense umami taste were considered as the umami peptides and their sequences were identified. In order to validate the umami taste with these identified peptides, the synthetic peptides were subjected to the sensory evaluation. The umami taste was also found and described by the synthetic peptides, which indicates the validation of these identified peptides as the umami peptides.

Table 1 lists the sensory description of the synthetic umami peptides. The result shows that totally, 12 novel umami peptides were identified and validated from *T. obscurus* and *T. rubripes* using the different treating methods. Additionally, regardless of the different treatments, another attribute kokumi, is one of the main sensory properties of these umami peptides, which is more likely to contributing the delicious taste of puffer fish.

**Table 1:** The sequences, sources and sensory description of the synthetic umami peptides [7]

| *The sequences of identified umami peptides* | *Sources* | *Sensory Properties* |
|---|---|---|
| Leu-Tyr-Glu-Arg | Takifugu Obscurus enzymatic hydrolysate | Sweetness, Umami, Kokumi |
| Val-Arg-Ser-Tyr | Takifugu Obscurus enzymatic hydrolysate | Sweetness, Umami, Kokumi |
| Cys-Ala-Leu-Thr-Pro | Takifugu Obscurus (100℃) | Umami, Kokumi |
| Arg-Pro-Leu-Gly-Asn-Cys | Takifugu Obscurus (100℃) | Umami, Kokumi |
| Glu-His-Ala-Met-Leu-Asn | Takifugu Rubripes (4℃) | Umami, Kokumi |
| Lys-Gly-Arg-Tyr-Glu-Arg | Takifugu Obscurus enzymatic hydrolysate | Sweetness, Umami, Kokumi |
| Thr-Leu-Arg-Arg-Cys-Met* | Takifugu Obscurus (4℃) | Umami, Kokumi |
| Pro-Gly-Gly-Val-Arg-Asn-Gly | Takifugu Rubripes (4℃) | Umami, Kokumi,Sourness |
| Pro-Val-Ala-Arg-Met*-Cys-Arg | Takifugu Obscurus (4℃) | Umami, Kokumi |
| Tyr-Gly-Gly-Thr-Pro-Pro-Phe-Val | Takifugu Obscurus (100℃) | Umami, Sweetness |
| Tyr-Lys-Cys-Lys-Asp-Gly-Asp-Leu-Arg | Takifugu Obscurus enzymatic hydrolysate | Umami, Kokumi, Fish flavour |
| Glu-Phe-Lys-Glu-Tyr-Asn | Takifugu Rubripes (4℃) | Umami, Kokumi, Sourness |

## References

1. Mottram, D. S. (1998). Food Chem., 62(4), 415-424.
2. Mateo, J., Dimínguez, M. C., Aguirrezábal, M. M. and Zumalacárregui, J. (1996). Meat Sci., 44(4). 245-254.
3. Lu, C.Y. (2006). Peptides as flavour precursors in Maillard reaction. New Brunswick Rutgers New Jersey.
4. Yamasaki, Y. and Maekawa, K. (1978). Agric. Biol. Chem., 42(9), 1761-1765.
5. Deng, J. C. (2009). Research on the differences of mouthfeel and flavour between Fugu obscurus and Fugu rubripes. Shanghai: Shanghai Ocean University.
6. Zhang, M. X., Wang, X. C., Liu, Y., Xu, X. L., Zhou, G.H. (2012). Food Chem., 135, 1463-1470.
7. Yu, X.Y., Zhang L. J., Miao, X. D., Li, Y.Y. and Liu, Y. (2017). Food Chem., 221, 599-605.

# The role of the salivary proteome in salt sensitivity

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## Abstract

Understanding individual subjects' taste sensitivity and the mechanisms involved in peri-receptor events taking place in the oral cavity would open new avenues for the reformulation of food products. Salivary proteins are believed to interact with key food taste molecules like sodium chloride and, by doing so, seem to impact taste receptor activation. Therefore, the present study set a particular focus on the salivary proteome level before and upon chemosensory stimulation using a combination of liquid chromatography mass spectrometry and sensory experiments. Interestingly, dynamics upon stimulation and differential proteome pattern between sensitivity groups seemed to be two largely independent conditions. Gene ontology enrichment analysis of key proteins with regard to sodium chloride sensitivity revealed augmented endopeptidase activity for sensitive subjects. Non-sensitive subjects, in contrast, were high abundant in proteins showing endopeptidase inhibitor activity. In the context, increased sensitivity could be demonstrated to arise from enriched serine-type endopeptidase activity and an *in-vivo* generation of salt-modulating peptides. Decreased sensitivity, in contrast, could be correlated to increased abundances of lipocalin-1 and lysozyme C and furthermore, predicted at an individual subject's level.

## Introduction

Dietary salt intake is challenging. On the one hand, salt is essential for homeostatic regulation and nerve conductance [1]. On the other hand, excess sodium chloride has been correlated to cardiovascular diseases [2]. Understanding mechanisms involved in salivary peri-receptor events and sodium-induced ion channels, pharmacology would open new avenues to reformulate low-sodium food products without compromising on salt taste quality. Several mechanisms have been reported in terms of salt taste transduction involving the amiloride sensitive epithelial sodium chloride channel (ENaC). However, the response of the ENaC in its external environment also plays an important role. In doing so, the activity of the ENaC has been described to be modulated by serine proteases, e. g. kallikrein and trypsin, and thus, leading to facilitated signal transduction [3, 4]. Ion channels and taste receptors have further been considered to be activated by saliva during oral food processing. Alternatively, salivary constituents such as proteins or peptides may interact with sensory stimulants and therefore, influence the concentration which is available at a receptor stage [5, 6]. The wide-ranging influence of saliva on chemosensory perception raised the question as to whether the salivary composition and dynamic changes upon tastant stimulation affect salt taste perception and, drive salt taste sensitivity at an individual panellist's level. Therefore, the objective of the present study is to classify panellists according to sodium chloride sensitivity, collect saliva before and upon salty tastant stimulation and investigate time-dependent dynamic changes in the salivary proteome by means of tryptic in-solution digestion followed by protein quantitation using isobaric tags for relative and absolute quantitation (iTRAQ). The second part of this study

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focuses on sensitivity at individual panellist's level and consequently, a targeted quantitation of identified key proteins within each subject's saliva. To investigate as to whether a salt taste enhancing effect might be correlated to endoprotease-catalysed ion channel modulation or an *in-vivo* release of salt enhancing peptides, panellists were further challenged with a serine-type endoprotease and degradation products identified using sequential windowed acquisition of all theoretical mass spectra (SWATH-MS), followed by sensory evaluation.

## Experimental

*Study subjects and classification according to sodium chloride sensitivity*

31 panellists were screened in their full sodium chloride detection range by means of threshold determination using 3-alternative forced-choice tests (ISO 13301:2002) and individually adapted concentration ranges [7]. Thereupon, psychometric functions were calculated for each panellist using logistic regression models and a 95 % confidence interval and subjects classified accordingly.

*Collection of saliva and analyses of proteome pattern as well as degradation products*

The collection of unstimulated and stimulated saliva was conducted according to literature [7, 8]. The four most NaCl-sensitive ($S^+$) and four most NaCl-insensitive panellists ($S^-$) were challenged with aqueous salt solution and saliva before, upon and after chemosensory stimulation taken for tryptic in-solution digestion and shotgun proteomics as described in literature [7]. Unstimulated saliva was collected from 20 subjects classified according to NaCl-mediated salt taste sensitivity and saliva samples analysed using targeted proteomics [9]. A subset of four panellists was further challenged with trypsin (0.1 mg/mL in bottled water, 2 mL) and saliva samples before and upon trypsin challenge were analysed using SWATH-MS as described in literature [9].

## Results and Discussion

*Classification according to NaCl-mediated salt taste sensitivity*

31 healthy panellists performed 3-alternative forced-choice tests (3-AFC) with individually adapted concentration ranges to determine NaCl detection threshold concentrations. However, since detection thresholds may be subject to day-to-day variability, each panellist's reproducibility in identifying threshold level sodium chloride samples had to be evaluated prior to classification. To achieve this, logistic regression models were calculated on the basis of each subject's daily performance at respective NaCl sample concentrations. The resulting psychometric functions revealed that four panellists consistently detected NaCl concentrations of less than 1.2 mmol/L and consequently, were classified most sensitive ($S^+$). The four panellists consistently detecting NaCl concentrations above 8.1 mmol/L were classified most insensitive ($S^-$). The classification of 20 panellists into sensitive, medium sensitive non-sensitive subjects was carried out as described in literature [9].

*Salivary proteome patterns affecting salt taste sensitivity*

To investigate salt taste sensitivity in the context of the salivary proteome and dynamic changes upon salt taste stimulation, collected saliva samples were analysed using shotgun proteomics and results illustrated in figure 1.

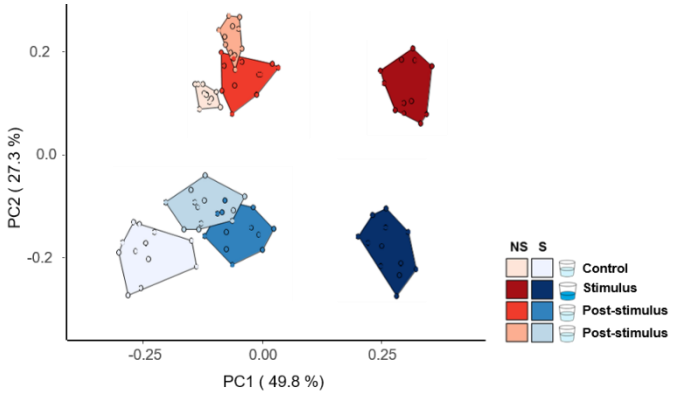


**Figure 1:** Principal component analysis of saliva samples upon salty tastant challenge

Principal component analysis demonstrated that dynamics upon stimulation (PC1) and sensitivity (PC2) seemed to be largely independent and further suggested that sodium chloride sensitivity may depend on the proteome pattern in resting saliva only. Subsequently, t-tests with a 5 % FDR cut-off revealed that lipocalin-1, lysozyme C, cystatin-D, cystatin-S and cystatin-SN are highly abundant within the $S^-$-group. Immunoglobulin heavy constant y1, cathepsin G, haptoglobin, kallikrein and myeloblastin, in contrast, were found to be key proteins for the $S^+$-group. When taken for gene ontology enrichment analysis, identified marker proteins showed significant enrichment in contrasting biological functions: The $S^+$-group demonstrated an augmented serine-type endopeptidase activity (*p-value* = $8.52 \cdot 10^{-8}$) whereas the $S^-$-group exhibited a significantly enriched cysteine-type endopeptidase inhibitor activity (*p-value* = $8.74 \cdot 10^{-9}$) and thus, suggesting that proteolytic events in the oral cavity may play a role in salt taste perception.

*Key proteins affecting individual subject's salt taste sensitivity*

To identify key proteins which may be predictive for individual panellist's sensitivity, saliva of 20 subjects classified according to NaCl-mediated salt taste was analysed by using targeted proteomics with stable isotope incorporation. In doing so, a pseudo-inverse logarithmic response between salt taste sensitivity and the abundance of lipocalin-1 and lysozyme C was found as illustrated in figure 2.

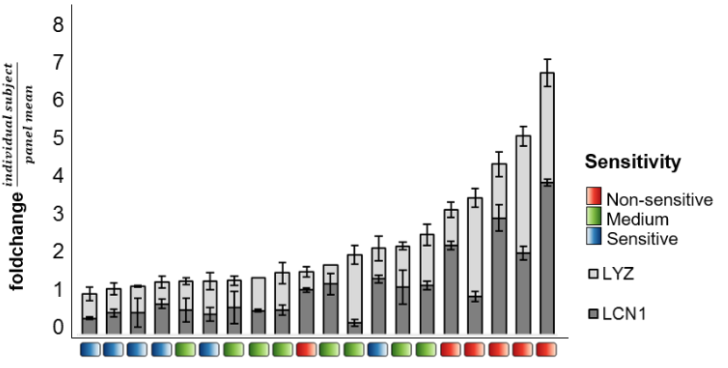


**Figure 2:** Individual panellists' sodium chloride sensitivity in correlation with abundances of lipocalin-1 and lysozyme C

Interestingly, both proteins have been demonstrated to be co-localised in salivary von Ebner glands and interact to form a thin film layer [10] which may lead to a decreased accessibility of the ENaC.

*Serine-type endopeptidases and in-vivo generation of salt taste enhancing peptides*

To answer to question as to whether enriched endopeptidase activity facilitates salt taste perception by activation of the ENaC [4] or an *in-vivo* release of salt taste enhancing peptides, a subset of four panellists was challenged with trypsin and saliva samples before and after stimulation analysed by using sequential window acquisition of all theoretical mass spectra (SWATH-MS). Interestingly, an unknown compound with a mass of 570.327 Da was observed to be significantly (*p-value* = 0.01) upregulated upon trypsin challenge demonstrating a fold change of 29. A targeted data extraction of SWATH-MS data resulted in the identification of tetrapeptide PLWR which could be confirmed by using a reference standard. A sensory evaluation of PLWR in model broth using a triangle test design [9] further revealed a salt taste enhancing effect at concentrations of 6.5 µmol/L and above. To the best of our knowledge, this is the first time that salt taste sensitivity could be correlated to an *in-vivo* endoprotease-catalysed release of salt taste modulating peptides and consequently, a facilitated salt taste perception.

## References

1. Denton D.: The hunger for salt. An anthropological, physiological and medical analysis. Springer, Berlin, 1982
2. Wardener, H. de and MacGregor, G. A. (2002) J. Hum. Hypertens. 16, 213–223.
3. Kleyman, T. R.; Carattino, M. D.; and Hughey, R. P., (2009) J. Biol. Chem. 284 (31).
4. Hughey, R. P.; Carattino, M. D.; and Kleyman, T. R., (2007) Curr. Opin. Nephrol. Hypertens. 16, 444–450.
5. Poette, J.; Mekoue, J.; Neyraud, E.; Berdeaux, O.; Renault, A.; Guichard, E.; Genot, C. and Ferron, G., (2014) Flavour Frag. J. 29, 39-49.
6. Ganfornina, M. D.; Gutiérrez, G.; Bastiani, M.; and Sanchez, D., (2000) Mol. Biol. Evol., 17 (1), 114–126.
7. Stolle, T.; Grondinger, F.; Dunkel, A.; Meng, Ch.; Médard, G.; Kuester, B.; and Hofmann, T., (2017) J. Agric. Food Chem. 65 (42), 9275-9286
8. Lorenz, K.; Bader, M.; Klaus, A.; Weiss, W.; Görg, A.; and Hofmann, T., (2011) J. Agric. Food Chem. 59 (18), 10219–10231.
9. Stolle, T.; Grondinger, F.; Dunkel, A.; and Hofmann, T., (2017) Food Chem. in press.
10. Glasgow, B. J., (1995) Graefes Arch. Clin. Exp. Ophthalmol. 233 (8), 513–522.

# Comparison of the sensory properties of fragrant and non-fragrant rice: The role of the popcorn-like aroma compound 2-acetyl-1-pyrroline

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## Abstract

2-Acetyl-1-pyrroline (2-AP) has been widely reported as being a key contributor to the popcorn-like aroma of fragrant rice. To understand more about the contribution of 2-AP to the aroma of fragrant rice and to highlight the sensory differences between fragrant and non-fragrant rice, quantitative descriptive analysis was conducted, to examine the sensory properties of six boiled rice samples (three fragrant rice and three non-fragrant rice) by 11 panellists, with emphasis on popcorn-like odour and flavour. The results showed perceived intensity of popcorn odour and flavour in fragrant rice were higher than in non-fragrant rice ($p = 0.016$, $p = 0.026$, respectively). However, the panellists could not differentiate between fragrant boiled rice varieties based on popcorn odour or flavour. 2-AP was extracted from the six boiled rice samples by headspace solid-phase microextraction and quantified by gas chromatography-mass spectrometry. 2-AP was found in fragrant rice samples (146 µg/kg in Jasmine, 113 µg/kg in Basmati and 80 µg/kg in Sintanur) but could not be quantified in non-fragrant varieties (below 5 µg/kg). These results suggested that although 2-AP is a key contributor to popcorn-like notes in fragrant rice, the differences in level of 2-AP content between different boiled fragrant rice samples may be too small to cause perceptual discrimination. In addition, popcorn-like notes were perceived in non-fragrant rice samples, despite levels of 2-AP being below detection limits.

## Introduction

2-Acetyl-1-pyrroline (2-AP) is a volatile compound with a popcorn-like odour and a low detection threshold (0.1 µg/kg in water). It was firstly identified in boiled fragrant rice [1]. When popcorn odour intensities in several fragrant rice varieties were ranked, 2-AP was considered as the most important contributor to this odour [2]. However, Yang et al. [3] reported that popcorn-like note may not be the only important characteristic in boiled fragrant rice odour. In addition, Limpawattana et al. [4] reported no correlation between popcorn flavour and 2-AP. Moreover, 2-AP has been reported to be generated only during fragrant rice growth and not during other postharvest procedures or cooking [5].

Lexicons of rice descriptors have been established in several studies, especially for fragrant rice [6-8]. The selection of descriptors depends on the panellists' culture and familiarity with the samples [9]. However, no rice lexicon has previously been reported using a UK sensory panel. In this study, different boiled rice varieties were evaluated using quantitative descriptive analysis (QDA). A lexicon was developed for both boiled fragrant and non-fragrant rice varieties using a UK-based panel. Differences in flavour and odour between fragrant and non-fragrant rice were evaluated. In addition, the relationship between popcorn flavour/odour and 2-AP content in boiled fragrant and non-fragrant rice was examined.

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## Experimental

### *Materials*

Six varieties of white rice were obtained in summer 2016, including three fragrant rice varieties (Basmati and Thai Jasmine from ASDA supermarket; Sintanur from Indonesia Centre of Rice Research) and three non-fragrant rice varieties (American long grain from ASDA supermarket; Arirang from Korea Foods Company Limited; and Ciherang from Indonesia Centre of Rice Research). 2-AP and deuterated 2-AP (2-AP-d2) standards were used for 2-AP quantification (both 30,000 ppm in dichloromethane (DCM), Aroma Lab, Germany).

### *Quantitative Descriptive Analysis (QDA) in boiled rice*

Milled rice (200 ± 1 g) was weighed and then boiled using 300 mL mineral water in a rice cooker (0.8 L, Lloytron, UK). Cooking proceeded for 20 min before the rice cooker automatically turned to warm mode. The samples were kept warm (65 °C) for 20 min before serving to panellists for evaluation.

Quantitative descriptive analysis (QDA) was conducted for six rice samples, using 11 trained UK panellists. A vocabulary was developed for appearance, odour, taste, flavour, mouthfeel and after-effect. A pre-heated ceramic cup (50 mL) filled with boiled rice (20 g) covered by foil was served to panellists for developing odour attributes and another 20-g sample was then served in the same manner for developing all the other attributes. The scoring for each attribute of sample was conducted in individual booths in duplicate on separate days, and samples labelled with three-digit codes were presented randomly in a balanced order. Data were collected using Compusense at-hand (Canada).

### *2-Acetyl-1-pyrroline quantification in boiled rice*

Rice samples (1 g ± 0.001 g) and 1.5 mL HPLC-grade water were added to 20-mL SPME glass vials with metal screw-caps and PTFE-faced silicone septa. Vials were then heated in a GC oven at 100 °C for 20 min. A 1.5-mL aliquot of 2-AP-$d_2$ aqueous solution (approximately 100 µg/kg, prepared by replacing DCM with HPLC-grade water) was added into the vials after they were cooled to room temperature. 2-AP in boiled rice was extracted from these samples by automated SPME (GC Sampler 120, Agilent). Samples were incubated with magnetic shaking for 10 min at 40 °C, and then extracted with a Supelco DVB/CAR/PDMS SPME fibre for 1 hour at 40 °C. After extraction, the extracts were analysed by gas chromatography-mass spectrometry (GC-MS) using a 7890 GC with 5975C MS (both Agilent). The SPME fibre was desorbed in the GC injector at 250 °C for 20 min, in splitless mode, onto the front of a Zebron ZB-Wax column (30 m × 0.25 mm; 1 µm film thickness; Phenomenex). The carrier gas was helium at a constant column flow rate of 0.9 mL/min. The initial GC oven temperature was 40 °C and held for 2 min, then increased to 60 °C at the rate of 2 °C/min; then the rate increased to 6 °C/min until the temperature reached 250 °C. Electron ionisation (EI) mode was used at 70 eV. Full scan mode was used for analysis from m/z 30 to 280. Simultaneous selective ion monitoring was also applied: ions *m/z* 68, *m/z* 83 and *m/z* 111 were monitored for 2-AP; *m/z* 86 and *m/z* 114 were monitored for 2-AP-$d_2$. The dwell time of monitored ions was set at 100 ms/ion.

## Results and discussion

### *Quantitative Descriptive Analysis (QDA) in boiled rice*

Thirty-seven attributes (covering appearance, odour, taste, flavour, mouthfeel and after-effect) were found in six boiled rice samples by 11 trained UK panellists. Significant differences between samples were found in all appearance attributes ($p < 0.0001$), popcorn odour ($p = 0.028$) and cohesive mouthfeel ($p < 0.0001$). Popcorn-like attributes were not only found in fragrant rice, but also in non-fragrant rice. When the six samples were grouped into fragrant (Jasmine, Basmati and Sintanur) and non-fragrant rice (long grain, Arirang and Ciherang), the perceived intensities of popcorn odour and flavour in fragrant rice were found significantly higher than in non-fragrant rice ($p = 0.016$, $p = 0.026$, respectively; Figure 1a). Although a significant difference in perceived popcorn odour was observed by ANOVA between different rice varieties (Figure 1b, $p = 0.028$), this difference was caused by a difference between Jasmine and Ciherang ($p < 0.05$); differences between other rice varieties were not observed. Jasmine and Sintanur tended to show higher perceived popcorn flavour than other samples, but no significant differences in popcorn flavour were found between rice varieties (Figure 1b, $p = 0.134$). These results indicated that although the panellists could not detect a difference in popcorn odour and flavour between individual boiled rice varieties, fragrant and non-fragrant rice could be categorised based on popcorn odour or flavour.

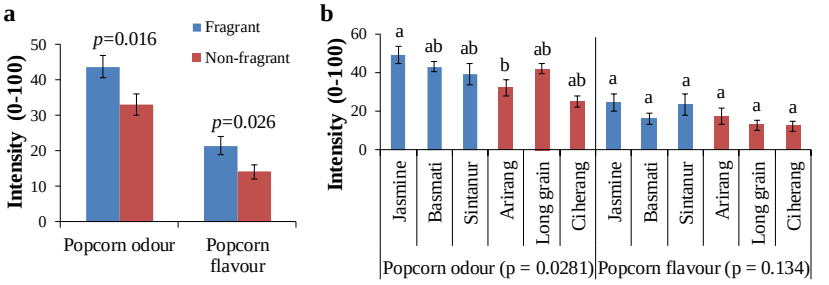


**Figure 1**: popcorn odour and flavour in fragrant and non-fragrant rice (a); perceived popcorn odour and flavour among six rice samples (b). Bars not sharing a common letter are significant different ($p<0.05$). Error bar represents standard error. Blue bars represent fragrant rice; red bars represent non-fragrant rice.

### *Quantification of 2-acetyl-1-pyrroline in boiled rice*

Concentrations of 2-AP in six boiled rice samples are shown in Figure 2. Significant difference in 2-AP concentrations was observed between the three boiled fragrant rice samples ($p = 0.028$). The concentrations of references (four levels of 2-AP standards) used for popcorn odour in QDA training were 5 or 10-fold in difference, and the trained panellists could rank these samples in order of intensity with no difficulty. However, the two-fold difference in 2-AP (Jasmine *vs* Sintanur) was not great enough to be noticed by panellists, which might explain why there was no significant difference in popcorn odour or flavour between fragrant rice samples. Limpawattana et al. [4] reported that although 2-AP was the only contributor to popcorn-like note in boiled rice, this compound did not correlate with popcorn flavour. Therefore, as rice contains numerous volatile compounds, the interaction of other compounds with 2-AP might affect the perception of popcorn odour and flavour. In three non-fragrant rice varieties, although traces of 2-AP were detected in the GC-MS chromatograms, the concentration in these samples was too low to be quantified (Figure 2). The lowest concentration of 2-AP that could be quantified in

this study was 5 µg/kg. However, these concentrations may be still about 50-fold higher than the 2-AP detection threshold (0.1 µg/kg in water), which could be the reason that popcorn-like attributes in non-fragrant rice were also detected by panellists.

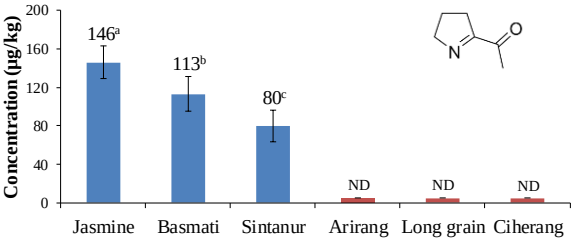


**Figure 2**: 2-acetyl-1-pyrroline concentrations in six boiled rice samples. Bars not sharing a common letter are significantly different ($p < 0.05$). Error bar represents standard deviation. ND: not detected, concentration lower than 5 µg/kg. Blue bars represent fragrant rice; red bars represent non-fragrant rice.

Yang et al. [3] analysed odour-active compounds in five boiled fragrant rice and one boiled non-fragrant rice samples. They found that 2-AP was detected in all six rice varieties, and popcorn-like odour was also detected in the non-fragrant rice variety. However, no other compounds that contributed popcorn-like odour were detected in their study. As no other compounds known to possess popcorn aroma were found in the current study, this suggests that trace levels of 2-AP in the non-fragrant varieties may be responsible for their popcorn-like aroma.

## Conclusion

A lexicon was developed by a trained UK panel to describe six boiled rice varieties (three fragrant and three non-fragrant rice types). Popcorn odour and flavour were found in both fragrant and non-fragrant rice, but it was difficult to differentiate all six boiled rice varieties based on these attributes. However, significant differences were observed in both popcorn odour and popcorn flavour when fragrant and non-fragrant rice were compared by t-test. Significant differences in 2-AP concentration were found between the three fragrant rice varieties, although such differences were too small to cause a significant perceptual difference. Much higher levels of 2-AP were found in fragrant rice than non-fragrant rice. However, trace levels of 2-AP may contribute to popcorn attributes in non-fragrant rice varieties.

## References

1. Buttery, R. G. and Ling L.C. (1982). Chemistry and Industry, 12, 958-959.
2. Buttery, R. G., Ling L.C., Juliano B.O., and Turnbaugh J.G. (1983). Journal of Agricultural and Food Chemistry, 31, 823-826.
3. Yang, D. S., Shewfelt R.L., Lee K.-S. and Kays S.J. (2008). Journal of Agricultural and Food Chemistry, 56(8), 2780-2787.
4. Limpawattana, M., Yang, D., Kays, S. and Shewfelt, R. (2008). Journal of Food Science, 73(9), 456-461.
5. Yoshihashi, T. (2002). Journal of Food Science, 67(2), 619-622.
6. Goodwin, J., Koop, L. A., Rister, M. E., Miller, R. K., Maca, J. V., Chambers, E., Hollingsworth, M., Bert, K., Webb, B. D., and McClung A. (1996). TAMRC Consumer Product Market Research CP2-96. Texas A&M: College Station.
7. Piggott, J. R., Morrison W.R., and Clyne J. (1991). International Journal of Food Science and Technology, 26, 615-628.
8. Yau, N. J. N., and Liu, T. T. (1999). Journal of Sensory Studies, 14(2), 209-233.
9. Paule, C. M., and Powers, J. J. (1989). Journal of Food Science, 54, 1515-1520.


# INDUSTRY-RELATED FLAVOUR ISSUES

# Safety assessment of flavourings in the European Union

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**Abstract**

In the European Union (EU) the regulatory framework for the use of flavourings in and on foods is provided by Regulation (EC) No 1334/2008. It contains as Annex the so-called Union list, i.e. a list of flavouring substances authorized for use in and on foods to the exclusion of all others. The principles underlying a group-based approach applied for the safety evaluation of flavouring substances prior to their entry into the Union list are outlined. The application of a decision-tree that takes into consideration structure-activity relationships, metabolism, intake and toxicity is described. Examples with particular emphasis on testing for genotoxic potential are given, and criteria for future safety evaluations of chemically defined substances and of flavourings other than flavouring substances are presented.

## Regulatory framework

In the European Union (EU) the regulatory framework for the use of flavourings in and on foods is provided by Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 [1]. The Regulation applies to flavourings, food ingredients with flavouring properties, to food containing flavourings and/or food ingredients with flavouring properties, and to source materials for flavourings and/or source materials for food ingredients with flavouring properties. Flavourings to be used in or on food must meet the following conditions: (i) They do not, on the basis of the scientific evidence available, pose a safety risk to the health of the consumer, and (ii) their use does not mislead the consumer.

Regulation (EC) No 1331/2008 of 16 December 2008 [2] laid down a common procedure for the assessment and the authorization of so-called food improvement agents, i.e. food additives, food enzymes and food flavourings. A Union list, i.e. a list of flavourings and source materials for use in and on foods that are authorized to the exclusion of all others, is included as Annex to Regulation (EC) No 1334/2008.

## Principles of the safety assessment

The procedure to establish the Union list had been laid down in Regulation (EC) No 2232/96 of the European Parliament and of the Council [3]. Member States were requested to notify to the Commission a list of flavouring substances which at that time were legally accepted on their territory. The resulting register of about 2800 substances was adopted by Commission Decision (1999/217/EC) [4]. The measures for the evaluation programme were laid down by Commission Regulation (EC) No 1565/2000 [5]. Considering the large number of substances, it was decided to make use of already existing safety assessments. Flavouring substances that had been considered as being not of safety concern at the current levels of intake either by the Scientific Committee on Food of the European Commission (SCF), the Experts on Flavouring Substances of the Council of Europe (CEFS) or by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) before 2000 did not need to be re-evaluated within the evaluation programme. Flavouring substances classified after 2000 by JEFC as to present no safety concern at the current level of intake had to be considered by the European Food Safety

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Authority (EFSA), in order to decide whether no further evaluation is necessary. The remaining flavouring substances had to be evaluated by EFSA.

In order to make the evaluation process as efficient as possible, a group-based approach was followed. The flavouring substances contained in the register were divided into 34 structurally related chemical groups; substances within a group are considered to have some common metabolic and biological behaviours. An additional important feature is that data may be provided either for a candidate substance as such or for supporting representatives showing sufficient structural and metabolic similarity.

The evaluation procedure is based on a stepwise decision-tree approach that considers information on structure-activity relationships, metabolism, intake and toxicity (Figure 1). This corresponds to a procedure developed by JECFA [6] and subsequently applied in an adjusted version to the evaluation of various flavouring substances [7-9]. The only differences are that the option to accept flavouring substances with the only argument that their estimated intake is lower than the threshold of concern of 1.5 µg/person/day was not adopted and that flavouring substances should be particularly examined for structural alerts of potential genotoxicity [10].

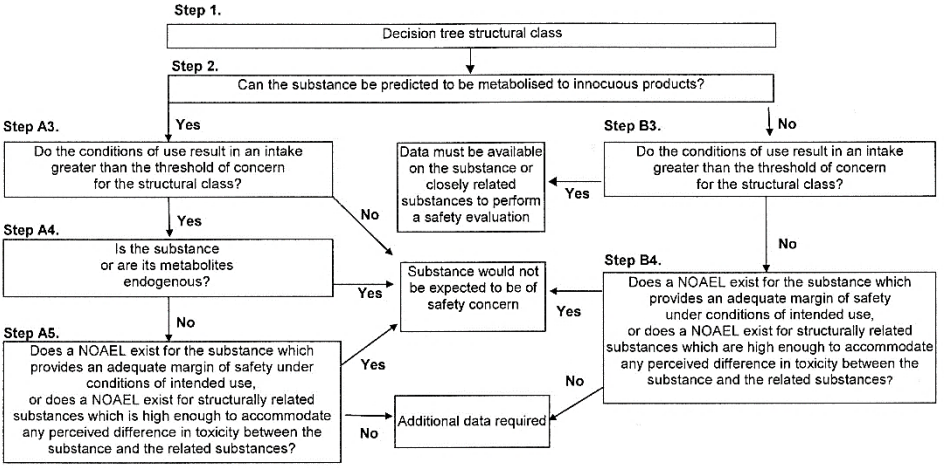


**Figure 1**: Procedure for the safety evaluation of chemically defined flavouring substances

The first step of the decision tree is the assignment of a flavouring substance to one of three classes for which thresholds of concern (human exposure thresholds) have been specified. Class I contains flavouring substances with simple chemical structures and efficient modes of metabolism, suggesting a low order of oral toxicity. Class II contains substances with structural features that are less innocuous, but are not suggestive of toxicity. Class III includes flavouring substances with structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity [11]. The thresholds of concern for these structural classes (1800, 540 and 90 µg/person/day, respectively) have been derived from a large dataset of subchronic and chronic animal studies [6,12].

In the following step, the answer to the question whether the flavouring substance can be predicted to be metabolized to innocuous products determines whether the evaluation proceeds via the A- or the B-side of the decision tree. Another decisive

question is whether the intended conditions of use of the flavouring substance result in an intake greater than the threshold of toxicological concern for the structural class. The answer determines whether the substance is not expected to be of safety concern or whether information is required on a no-observed-adverse-effect level (NOAEL) for the flavouring substance as such or structurally related substances, which provides an adequate margin of safety under the intended conditions of use.

The intake assessment plays an important role in the application of the Procedure. As a default, the so-called "Maximised Survey-derived Daily Intakes" (MSDI) approach, which is based on annual production volumes, was used [13]. However, the MSDI approach in a number of cases grossly underestimates the intake by regular consumers of products flavoured at the use levels reported by Industry. Therefore, the intakes were also estimated using the "modified Theoretical Added Maximum Daily Intake" (mTAMDI) approach, which is based on normal use levels reported by industry and consumption data for certain food categories [13]. The mTAMDI value was not considered in the Procedure but was only used as tool to prioritise the flavouring substances according to the need for a refined intake screen and the request for more precise data. Accordingly, the following types of conclusions can be found in the scientific opinions, the so-called Flavouring Group Evaluations (FGEs): (i) Based on the default MSDI approach, the candidate substance, which was evaluated through the Procedure, would not give rise to safety concern at the estimated level of intake arising from the use as flavouring substance. (ii) Based on the mTAMDI approach, the estimated intake of a flavouring substance is above the threshold of concern for the respective structural class. In this case, more reliable exposure data are required. On the basis of such additional data, the flavouring substance should be re-evaluated using the Procedure; subsequently, additional toxicological data might become necessary.

**Implementation of the Union list**

The Union list of flavouring substances has been adopted by Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 [14]. It contains information on the identities and the purities (at least 95%; otherwise composition is given) of the flavouring substances. It may also contain restrictions of use, e.g. that a substance may only be added to the listed food categories and under the specified conditions of use. The scientific body that has carried out the evaluation is given and finally, footnotes indicate for which flavouring substances the evaluation is to be completed, and the time limits for applicants to comply with EFSA´s requests expressed in published opinions.

**Requests for additional genotoxicity data**

In the pending requests for additional information, particular attention is paid to the provision of genotoxicity data. According to the guidance expressed in the opinion of the EFSA Scientific Committee [15], genotoxicity testing should start with a basic battery of *in vitro* tests, i.e. a bacterial reverse mutation assay and an *in vitro* micronucleus test. If all *in vitro* endpoints are negative, there is no genotoxic potential. If one or two tests are positive, the following *in vivo* tests should be considered: (i) an *in vivo* mammalian erythrocyte micronucleus test, (ii) a transgenic rodent cell gene mutation assay, and (iii) an *in vivo* Comet assay. The *in vivo* test selected should relate to the genotoxic endpoint(s) identified as positive in the *in vitro* tests. If any of the *in vivo* tests is positive, there is a genotoxic potential and the flavouring substance is considered to be of safety concern.

The α,β-unsaturated aldehyde and ketone structures are considered as structural alerts for genotoxicity. FGE.19 contains 360 α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation. These substances were divided into structurally related subgroups, representative substances were selected, and the Flavouring Industry had to provide additional genotoxicity data [16]. If on the basis of these data a genotoxic potential can be ruled out, the substances are merged with structurally related substances in other FGEs and evaluated using the Procedure.

An example of such a subgroup of FGE.19 are the three alicyclic aldehydes with α,$\beta$-unsaturation in the ring/side chain and the seven precursors for such aldehydes shown in Figure 2.

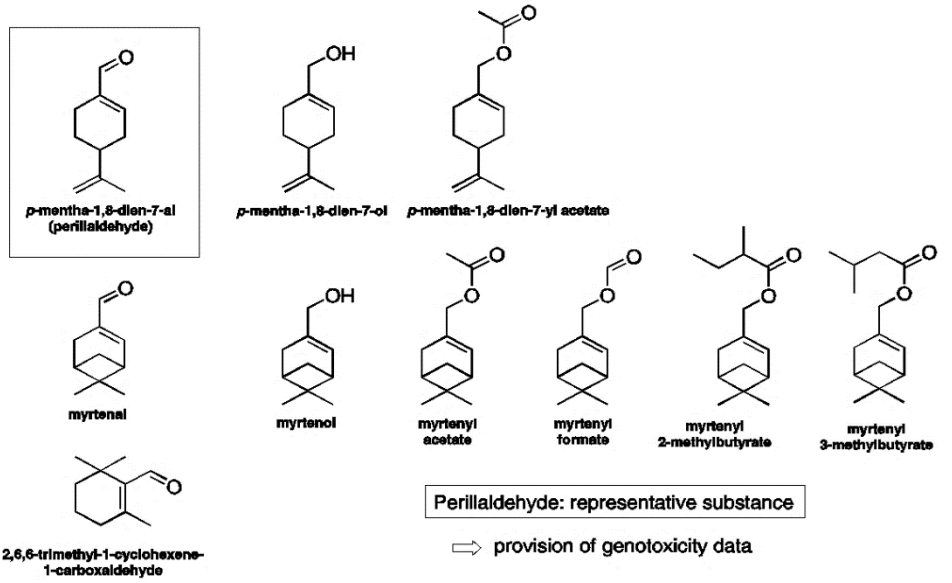


**Figure 2**: Examples of α,$\beta$-unsaturated carbonyls and their precursors (subgroup 2.2 of FGE.19)

*p*-Mentha-1,8-dien-7-al [FL-no: 05.117] was selected as representative substance for which genotoxicity data were requested. According to the data submitted, the EFSA Panel concluded that *p*-mentha-1,8-dien-7-al is genotoxic *in vivo* [17], and the flavouring substance was removed from the Union list [18]. This, however, meant that were also concerns regarding potential genotoxicity for the other flavouring substances in this subgroup represented by *p*-mentha-1,8-dien-7-al. Subsequently, the flavor industry withdrew the support for 2,6,6-trimethyl-1-cyclohexen-1-carbox-aldehyde [FL-no: 05.121], myrtenyl formate [FL-no: 09.272], myrtenyl 2-methylbutyrate [FL-no: 08.899] and myrtenyl 3-methylbutyrate [FL-no: 09.900] which then were also removed from the Union list. For myrtenol [FL-no: 02.091], myrtenyl acetate [FL-no: 09.302] and p-mentha-1,8-dien-7-yl acetate [FL-no: 09.278] new genotoxicity data were provided; they allowed to rule out the concerns regarding genotoxicity for these substances. Only for myrtenal [FL-no: 05.106] the genotoxicity data submitted were considered equivocal and therefore this flavouring substance presently cannot be evaulated through the Procedure [19].

In contrast to this group-based approach involving a representative substance, the rather unique structure of 4,5-epoxydec-2(trans)-enal [FL-no: 16.071] resulted in an evaluation as stand-alone substance. The genotoxic effect observed *in vitro* was confirmed in an *in vivo* Comet assay in the liver of rats. Accordingly, the EFSA Panel concluded that 4,5-epoxydec-2(trans)-enal raises a safety concern with regard to genotoxicity [20], and consequentially this flavouring substance was removed from the Union list [21].

**Evaluation of newly submitted flavouring substances**

The established Union list is open and can be amended in the light of scientific and technical developments. EFSA has elaborated a guidance document for the risk assessment of flavourings newly submitted after the adoption of the Union list [22]. As a starting point of the assessment genotoxicity testing is required. Flavourings which can be assigned to one of the existing FGEs on the basis of structural and metabolic similarities can be evaluated according to the scientific principles and to the group-based approach underlying the former evaluation programme. For flavouring substances which cannot be assigned to one of the existing FGEs individual evaluations via the tiered approach shown in Figure 2 have to be performed. The type of data required depends on (i) whether there are experimental data available for the substance to demonstrate that the metabolites can be considered innocuous, and (ii) whether the chronic dietary exposure, based on added use levels, is below or above the threshold of concern of the structural class to which the flavouring substance belongs.

For the assessment of dietary exposure, a new approach called “Added Portions Exposure Technique” (APET) has been introduced [22]. The APET is calculated based on the occurrence levels provided by the applicant in a defined list of food categories by summing the highest potential dietary exposure within each of the two groups of “Beverages” and “Solid foods”. Such an estimate, based on daily consumption of one single standard portion of beverage and one single portion of solid food, is considered to provide a conservative assessment of long-term average dietary exposure for consumers of flavoured products. A case study on the use of the APET technique to estimate total dietary exposure to flavouring substances has been provided [23].

The applicant needs to provide: (i) Normal and maximum occurrence levels as added flavouring substance; (ii) normal and maximum occurrence levels of the substance from other sources, e.g. as natural constituent, as substance developed through the processing of foods, as carry-over originating from the use in animal feed or as residues of packaging; (iii) normal and maximum combined occurrence levels of the substance, taking into account all sources. In addition, the applicant needs to indicate the non-food uses of the flavouring substance.

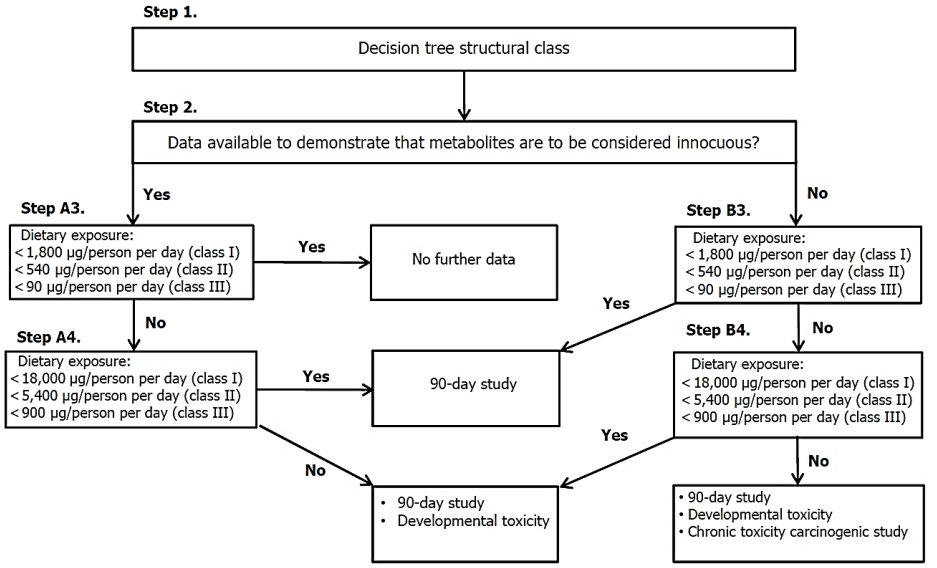


**Figure 3**: Individual evaluation of flavouring substances

A recent example for the application of the approach outlined in Figure 2 is the assessment of 3-(1-((3,5-dimethylisoxazol-4-yl)methyl)-1H-pyrazol-4-yl)-1-(3-hydroxybenzyl)-imidazolidine-2,4-dione [FL-no: 16.127], a substance intended to be used as flavour modifier [24]. Data provided for the substance demonstrated that there is no concern regarding genotoxicity. It was assigned to Cramer class III; potential metabolites could not be considered to be innocuous. The cumulative dietary exposure using the APET technique was 850 µg/person/day for an adult (60 kg) and 536 µg/person/day for a 3.year-old child (15 kg). Considering that this intake is higher than the threshold of concern of substances belonging to Cramer class III, i.e. 90 µg/person/day, but lower than 10 times this threshold, i.e. 900 µg/person/day, a 90-day feeding study and a developmental study were required. In a developmental toxicity study with rats no differences between treated and control groups up to 100 mg/kg bw/day were observed. In a 90-day feeding study with rats an NOAEL of 100 mg/kg bw/day could be derived. The comparison of this NOAEL with the estimated intakes resulted in margins of safety of > 7,000 for adults and > 2,000 for a 3-year-old child.

## Evaluation of flavourings other than flavouring substances

In addition to flavouring substances, Article 9 of Regulation (EC) No 1334/2008 of the European Parliament specifies the following categories of flavourings for which an evaluation and approval is required: (i) Flavouring preparations obtained from material of vegetable, animal or microbiological origin, other than food. (ii) Thermal process flavourings for which ingredients for their production are source materials other than food and/or for which the conditions of their production and/or the maximum levels of undesirable substances set out in Annex V of Regulation 1334/2008 (EU, 2008) are not met. (iii) Flavour precursors obtained from source material other than food. (iv) Other flavourings. The information requested for a safety evaluation of these categories of flavourings is described in a guidance document [22].

For the categories (i) – (iii) no applications have been submitted so far. Examples of recently assessed "Other flavourings" are two "grill flavours", i.e. high oleic sunflower oils subjected to short-time heating at high temperatures [24, 25], and "rum ether", a complex mixture of volatiles obtained by pyrolysis of wood (oak, beech, hickory) and esterification of the resulting pyroligneous acid with ethanol, under oxidative conditions in the presence of sulfuric acid and manganese oxide [26].

## Conclusion

The establishment of the Union list of flavourings substances constitutes a basis change in paradigm in the regulatory oversight on flavourings in the EU. On the one hand, this creates economically relevant hurdles for applicants, on the other hand such a list increases transparency, it can serve as reliable platform for involved stakeholders, and it may finally help to increase the acceptance of flavourings by consumers.

## References

1. Regulation (EC) No 1334/2008 of the European Parliament and of the Council on 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. Official Journal of the European Union, L354/34, 31.12.2008
2. Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorization procedure for food additives, food enzymes and food flavourings. Official Journal of the European Union, L354/1, 31.12.2008
3. Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996 laying down a Community procedure for flavouring substances used or intended for use in or on foodstuffs. Official Journal of the European Communities, L 299/1, 23.11.1996
4. Commission Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996 (1999/217/EEC). Official Journal of the European Communities, L84/1, 27.03.1999
5. Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council. Official Journal of the European Union, L180/8, 19.7.2000
6. Evaluation of certain food additives and contaminants. 44th Report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Reports Series 856, Geneva 1995
7. Toxicological evaluation of certain additives and contaminants in foods. Prepared by the Joint FAO/WHO Expert Committee on Food Additives, WHO Food Additives Series 35, Geneva 1996
8. Evaluation of certain food additives and contaminants. 46th Report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Reports Series 868, Geneva 1997
9. Evaluation of certain food additives and contaminants. 49th Report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Reports Series 884, Geneva 1999
10. Opinion on a programme for the evaluation of flavouring substances. Scientific Committee on Food. SCF/CS/FLAV/TASKF/11 Final 6/12/1999. Annex I of minutes of the 119th Plenary meeting. European Commission Health and Consumer Protection Directorate – General
11. Cramer G.M., Ford R.A., Hall R.L. (1978) Estimation of toxic hazard – a decision tree approach. Food Cosmet. Toxicol. 16, 255-276
12. Munro I.C., Ford R.A., Kennepohl E., Sprenger J.G. Correlation of Structural Class with No-Observed-Effect Levels: A Proposal for Establishing a Threshold of Concern. Food and Chemical Toxicology *34*, 829-867 (1996)

13. Bergsten C., Andersen N.L., Gry J. Methods for calculating the intake of flavourings. TemaNord 2002:550; Nordic Council of Ministers, Copenhagen
14. Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. Official Journal of the European Union, L 2676/1, 2.10.2012
15. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Scientific Committee. EFSA Journal 2011; 9(9):2379
16. EFSA CEF Panel. List of alpha, beta-Unsaturated Aldehydes and Ketones representative of FGE.19 substances for genotoxicity testing. EFSA Journal 2008, 910; 1-7
17. EFSA CEF Panel. Scientific Opinion on the Flavouring Group Evaluation 208 Revision 1 (FGE.208Rev1): Consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the a,ß-unsaturation in ring/side chain and precursors from chemical subgroup 2.2 of FGE.19. EFSA Journal 2015, 13(7):4173
18. Commission Regulation (EU) 2015/1760 of 1 October 2015 amending Annex I to regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards removal from the Union list of the flavouring substance *p*-mentha-1,8-dien-7-al. Official Journal of the European Union. L257/27, 2.10.2015
19. EFSA CEF Panel. Scientific Opinion on Flavouring Group Evaluation 208 Revision 2 (FGE.208Rev2): Consideration of genotoxicity data on alicyclic aldehydes with a,ß-unsaturation in the ring/side chain and precursors from chemical subgroup 2.2 of FGE.19. EFSA Journal 2027, 15(5):4766
20. EFSA CEF Panel. Scientific Opinion on Flavouring Group Evaluation 226 Revision 2 (FGE.226Rev1): consideration of genotoxicity data on one a,ß-unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19. EFSA Journal 2017, 15(5):4847
21. Commission Regulation (EU) 2017/1250 of 11 July 2017 amending Annex I to Regulation No 1334/2008 of the European Parliament and of the Council as regards removal from the Union list of the flavouring substance 4,5-epoxydec-2(trans)enal. Official Journal of the European Union L179/3, 12.7.2017
22. Guidance on the data required for the risk assessment of flavourings to be used in or on foods. EFSA Journal 8(6): 1623 (2010)
23. Piccinelli, R., Mistura L., Raffo, A., Sette S., Le Donne C., Charrondiere R., Leclercq C. (2013) A case study on the use of the "Added Portions Exposure Technique – APET" to estimate total dietary exposure to flavouring substances. Trends in Food Science & Technology *32*, 51-70
24. EFSA CEF Panel. Scientific Opinion of Flavouring group Evaluation 502 (FGE.502): grill flavor "Grillin´5078". EFSA Journal 2017, 15(9):4973
25. EFSA CEF Panel. Scientific opinion of Flavouring Group Evaluation 503 (FGE.503): grill flavor "Grillin`CB-200SF". EFSA Journal 2017, 15(9): 4963
26. EFSA CEF Panel. Scientific Opinion of Flavouring Group Evaluation 500 (FGE.500): rum ether. EFSA Journal 2017, 15(8): 4897

# Impact of Nagoya protocol on flavour research

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## Abstract

On 12th October 2014 the *Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization* came into effect on being ratified by the 50th party. So, three years after ratification, what has happened and what impact can be expected on commercially orientated flavour research?

The overall aims and intentions of the Nagoya Protocol are relatively clear in principle; researchers who obtain biological materials from a Nagoya country with ABS legislation in place, and develop and launch a new ingredient based on that research, now have an obligation to establish a benefit sharing agreement with the provider.

However, the specific national laws and regulations are often complex and unclear. On the provider side, where national access legislation exists it is variable in its scope and application while the official processes and documentation are still evolving in many countries. On the user side, the EU has enacted the first compliance legislation and is currently working on the guidance documentation and the processes for making the declarations as required by the legislation.

The main impact for those involved in flavour research appears to be more paperwork, a need to carry out additional due diligence concerning the origin of natural materials, and in some cases additional agreements or contracts when obtaining biological materials for use in research programs. At present there are many more questions than answers and most of the activity is in the realm of industry associations and corporate legal departments but as the obligations under the EU legislation become clearer it is now beginning to impact at the research laboratory level.

## Introduction

For most of the long history during which mankind has harvested the wealth of nature, the natural resources of the planet could be claimed and used by those who invested the time and effort to obtain and develop them. Throughout the 17$^{th}$ to 19$^{th}$ century, as colonialization and international trade developed, botanic gardens and agricultural experimental stations were established around the world to assist the transfer of valuable species to alternative locations where they could be developed and traded. The benefit of this trade was mostly gained by those doing the trading such as the French, British, Portuguese and Dutch through their respective East India companies. Rubber trees were relocated from Brazil to Malaysia, vanilla relocated from Mexico to Madagascar, tea, coffee and cocoa redistributed to plantations worldwide, and this was encouraged by the governments of the time. An early example is that of Pierre Poivre, who in 1770 as Governor of Mauritius established the botanic garden there and obtained clove, nutmeg, pepper and other plants from the Spice Islands, now Maluku islands in Indonesia to be grown in Mauritius for the benefit of France. When plants were relocated, whether purchased or plundered, there was often little benefit for the local communities, although some colonial enterprises did establish local plantations and trading posts that enhanced the wealth of at least some of the local population.

Things changed significantly in 1992 with the “Rio Earth Summit”, a landmark United Nations conference covering many topics related to sustainable development and

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the economic development of natural resources. At the Rio conference the Convention on Biological Diversity (CBD) was opened for signature and it came into force in 1993[1]. Among many other things, this established that countries could assert sovereign rights over the '*genetic resources'* found in their territory. Thus biological resources now belong to the country in which they are found and, following the principles of Access and Benefit Sharing (ABS) established by the CBD, anyone wishing to develop those biological resources for commercial gain should negotiate a benefit sharing deal with the country of origin. Although the principles had been established, it has taken many years of discussion and negotiation for further treaties to evolve such as the Bonn Guidelines (2002) and the Nagoya Protocol (2010) which define the principles of Access and Benefit Sharing in more detail. These are international treaties and, as such, have no legal bearing on individuals, companies or institutions. It is up to the countries that are parties to these conventions to establish their own policy measures and enact their own legislation to address these principles, thus the application of the Nagoya Protocol can be very different in different countries.

**The Nagoya protocol**

To give it its full title, '*The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity*' was adopted at the Conference of the Parties in 2010 in Japan and finally came into force on 12th October 2014 with the 50th signatory [2]. The full text is available online [3].

From the perspective of a '*User'*, the principle of the Nagoya protocol can be summarised as;

*"If genetic resources or traditional knowledge associated with genetic resources are obtained from a country that is a party to the Nagoya protocol for the purposes of research and development, then some of the benefits from its subsequent commercialisation should be shared with the provider."*

The protocol sets out the regulatory, administrative and policy measures to be undertaken by the parties at national level, and also establishes the concept of national focal points and an international clearing-house mechanism [5] for the exchange of information relating to access and benefit sharing in each country.

One of the challenges in understanding the scope and application of the Nagoya protocol comes in the interpretation of the limited definitions in Article 2 of the CBD and Article 2 of the Nagoya protocol [6,3]. The following is a simplified interpretation of the words used in the title of the protocol:

**Access:** Obtaining genetic resources or traditional knowledge *in situ*, or from *ex situ* collections or through trade. Requires Prior Informed Consent (PIC)

**Genetic resources:** Plants, animals, microbes, their DNA/RNA, and extracts made from them.

**Equitable sharing:** Mutually Agreed Terms (MAT), benefit sharing agreement.

**Benefits:** Monetary or non-monetary; payments, shared results and IP, community projects, etc.

**Utilization:** Carrying out Research & Development.

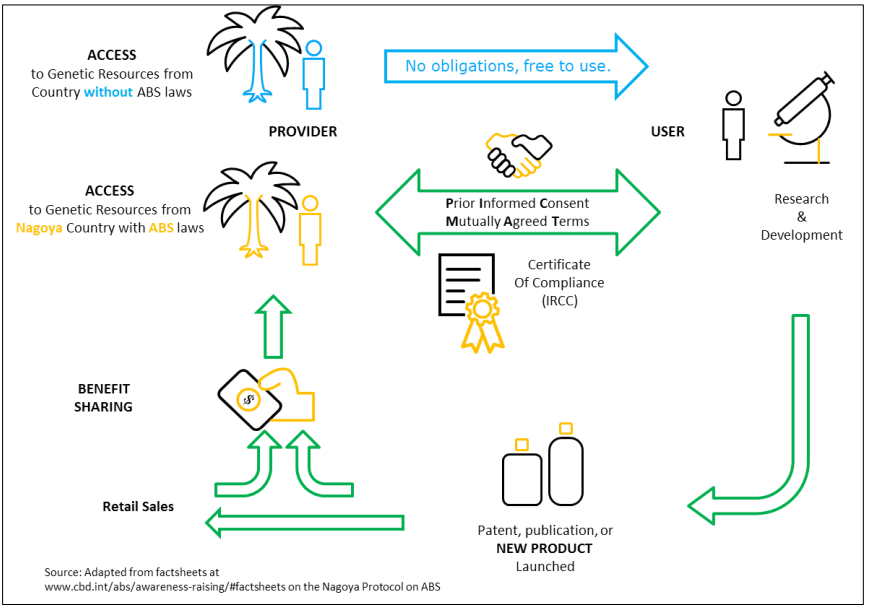


**Figure 1:** Principles of access and benefit sharing [4]

There are some specific exceptions, notably the exclusion of human genetic resources, certain pathogenic organisms, and genetic resources covered by other instruments such as the ITPGRFA for crop plants [7].

Currently, 32 '*Provider'* countries have registered some form of legislative, administrative or policy measures on the clearing-house website [5] and others are in the process of developing their legislation. So far, only the European Union has enacted compliance legislation as a '*User'* of genetic resources and it in turn transfers the obligations for administering the measures to its member states. The registration to date of over 100 internationally recognised certificates of compliance (IRCC) on the clearing house website is evidence that the system is beginning to function, and there are certainly many more ABS agreements that have been successfully concluded in some form or other.

## The EU Regulation EU-ABS 511 / 2014

Since the EU represents countries that are mainly '*Users'* rather than '*Providers'* of genetic resources it has enacted compliance legislation but not access legislation. The regulation: *EU-ABS 511 / 2014 on compliance measures for users from the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization in the Union* [8] came into force in 2014 and applies, along with the Nagoya Protocol from 12th October 2014. The text contains several cumulative requirements, and is also based on definitions that are open to interpretation, but which can be summarised as:

*If genetic resources or traditional knowledge associated with genetic resources are obtained …by a user carrying out R&D in the EU*

*…from a country that is party to the Nagoya Protocol with ABS laws in place*

*…after 12th Oct 2014,*

*then the user is required to comply with the legislation in the country of origin.*

The user should carry out due diligence to determine if the EU ABS regulations and any laws in the country of origin apply. If the regulations apply, a declaration of the due diligence should be submitted to the EU via the 'DECLARE' system which is currently in development. The regulations identify two trigger points at which the due diligence should be carried out and a declaration made; on receiving funding for the work, and on launching a new product on the market in the EU. And by default there is a trigger point on '*Access'* to the genetic resources or traditional knowledge.

The EU has published a general Guidance Document [9] to assist the interpretation of the regulations and is in the process of preparing Sectorial Guidance Documents for each of 7 sectors; Cosmetics, Animal Breeding, Plant Breeding, Biocontrol, Pharmaceuticals, Food and Feed, Biotechnologies, and also for Upstream Actors including collections and research institutions. These guidance documents have been prepared in consultation with relevant industries through their associations which, for the purposes of the flavour industry, includes IOFI, IFRA, EFFA, and EU SpecialityFoodIngredients. The guidance documents will provide more specific interpretations of the scope and application of the regulations but they are not themselves legally binding. There are an increasing number of law firms and lawyers specialising in Biodiversity Law and a variety of NGOs that champion the cases of the providers as well as facilitating benefit sharing agreements between providers and users. The Union for Ethical Biotrade (UEBT) is one such organisation that is well established in the flavour, fragrance and cosmetics area.

There are several unresolved issues which are still subject to on-going discussions at various levels right up to the UN. Notably the topic of Digital Sequence Information (DSI) which is currently understood to be outside the scope of Nagoya, but certain countries cover this in their national legislation. It is being discussed and reviewed by many interested parties including UN, ITPGR, WHO, ICC [10] etc., and is on the agenda for the CBD conference of the parties in 2018.

An unresolved topic with direct impact on researchers is the definition of research and development itself. This is of critical importance, since it is the act of carrying out R&D on a genetic resource than triggers the need to carry out due diligence. However, it is not clear which activities fall within the definition. The proposed definitions are based on the Frascati Manual of the OECD [11] and the activities under discussion include, among others, routine QC tests, screening to de-select material from further study, and toxicological tests for regulatory purposes.

The concept and definition of "derivatives" has been a point of much discussion since the outset. It is defined in article 2e) of Nagoya as "*…a naturally occurring biochemical compound resulting from the genetic expression or metabolism of biological or genetic resources…"*, and referred to in the definition of utilization, but not mentioned elsewhere. The EU guidance document [8] interprets this to mean that a derivative is in scope when accessed in combination with the genetic resource from which it is derived. So it may be inferred that "isolated derivatives" such as many food and flavour ingredients purified from plants or animals such as proteins, fats & oils, essential oils and flavour extracts would be out of scope when accessed without any associated access to their original genetic resources.

As it stands in 2017, the guidance from the EU and the processes and systems of the competent authorities are still very much in development, nonetheless, it is slowly becoming clearer which research activities involving genetic resources fall in scope and what, if any, legal obligations apply.

## Implications for flavour research

Biodiversity and genetic resources are important for flavour research and it is our responsibility as scientists to use them wisely, and as an industry to carry out commercial developments ethically.

The landscape around the use of natural biological materials as a starting point for research projects in the EU is changing. With the gradual introduction of both access legislations in provider countries and compliance legislation in user countries the act of obtaining biological material for an R&D project may now carry certain obligations.

For anyone involved in obtaining biological materials for a research project, this means ensuring that the relevant checks concerning ABS legislation in the country of origin are carried out and documented, and that any corresponding requirements are met. For research institutions or collections of biological resources this may relate to the transfer of relevant information to subsequent users. For commercial and applied research activities this may involve some form of benefit sharing with the original provider, or the transfer of relevant information to subsequent retailers of the new product developed from their research.

For flavour and fragrance houses with their own R&D departments and for traders obtaining new products from abroad, this will mean more checks and more paperwork for everyone along the supply chain, but not necessarily more constraints on the scope or quality of flavour research that can be successfully carried out in the EU.

## References

1. History of The Convention on Biological Diversity. https://www.cbd.int/history accessed Sept.2017
2. The Nagoya Protocol on Access and Benefit Sharing, www.cbd.int/abs accessed Sept.2017
3. Full Text of Nagoya Protocol. https://www.cbd.int/abs/doc/protocol/nagoya-protocol-en.pdf
4. ABS Information Kit. www.cbd.int/abs/awareness-raising/#factsheets on the Nagoya Protocol on ABS. accessed Sept.2017
5. The ABS Clearing House. https://absch.cbd.int/ accessed Sept.2017
6. The Text of the CBD. https://www.cbd.int/doc/legal/cbd-en.pdf
7. International Treaty on Plant Genetic Resources for Food and Agriculture. http://www.fao.org/plant-treaty/en/
8. EU ABS Regulation 511/2014. http://ec.europa.eu/environment/nature/biodiversity/international/abs/legislation_en.htm
9. EU Guidance Document for regulation no. 511/2014. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.C_.2016.313.01.0001.01.ENG
10. ICC Position Paper on DSI. https://cdn.iccwbo.org/content/uploads/sites/3/2017/05/ICC-IP-position-paper-on-digital-sequence-information.pdf
11. The Frascati Manual 2015. OECD. http://www.oecd.org/innovation/inno/frascati-manual.htm

# Acetals in food flavourings

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## Abstract

Matching of competitor flavours is one of the most common tasks for the majority of flavour chemists working for a flavour house. It is obvious to find various artefacts in those flavourings, where carbonyl compounds, alcohols and acids are together part of the recipe. Unfortunately, mass spectra of many of these compounds are not available in the commercial mass spectra databases. We decided to systematically investigate the reaction products of carbonyl compounds with the most common solvents used in the flavour industry – ethanol and propylene glycol, by means of gas chromatography– mass spectrometry. In a second study, we investigated the formation of acetals of selected alcohols and aldehydes, naturally occurring in concentrated apple condensates. Mass spectra of unpublished acetals are presented, together with results of a complementary storage study at different pH levels of the base.

## Introduction

Aldehydes are essential constituents of the flavour of various foods, such as citrus fruits or apples [1]. Aldehydes are quite reactive because of the electronegativity of oxygen on the carbonyl group, what in the presence of alcohols leads to the formation of labile hemiacetals. This process can be both acid and base catalysed. In the acid catalysed reaction, protonation of carbonyl group occurs and a carbocation intermediate is produced. Acidic conditions and excess of alcohol cause transformation of the carbocation to acetal and water [2]. Diols and triols are capable of reacting intra-molecularly to form cyclic acetals. These reactions generally proceed rapidly due to their low activation energies. Acetals are stable at neutral pH, because the equilibrium reaction needs the protons, which are at pH 7 not available. Up to four isomers can be formed in the case of propylene glycol due to its chirality and diastereomerism [3].

In current business world, the matching of competitor's flavours is one of the main tasks for the creators of food flavours – the flavourists. Acetal formation makes this task much more complicated, because mass spectra of acetals of many aldehydes are still not available in the commercial mass spectra databases, such as NIST [4] or Wiley [5].

The aim of this study was to systematically synthetize acetals of all aldehydes we had on our shelf with the most common solvents used in flavour industry: ethanol and propylene glycol. In this work, we present the mass spectra of acetals not found in literature. We additionally synthetized the acetals of the most common aldehydes and alcohols as found in apple condensate (FTNF), because we hypothesized that some of them could be among the unknown peaks we found in the FTNFs concentrated to high degree with the Spinning Cone Column (Flavourtech, Griffith, AU) technique. We performed a storage study as well in following media: soft drink base, mineral water, water and yoghurt to prove the stability of some acetals.

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## Experimental

*Materials*

All compounds used in this study were of food grade, purchased from Sigma-Aldrich (Munich, D).

*Aldehydes to be reacted with ethanol and propylene glycol (PG)*:

2-methyl-propanal, 2-phenyl-2-butenal, 5-methylfurfural, acetaldehyde, α-amylcinnamaldehyde, anisaldehyde, benzaldehyde, β-homocyclocitral, butyraldehyde, cinnamaldehyde, citral, citronellal, decanal, dodecanal, ethylvanillin, furfural, heptanal, hexanal, isovaleraldehyde, melonal, nonanal, octanal, perillaldehyde, phenylacetaldehyde, piperonal, p-tolualdehyde, trans-2-decenal, trans-2-hexenal, trans-2-methyl-2-butenal, undecanal, valeraldehyde, vanillin.

*Flavouring compounds for the FTNF study*:

acetaldehyde, benzaldehyde, hexanal; propanol, 1-butanol, 2-methylbutanol, isobutanol, isopropanol, 3-methylbutanol, n-hexanol, n-pentanol.

*Preparation of acetals*:

100 µL of carbonyl compound, 890 µL of solvent, 10 µL of acetic or formic acid, 2 days at 37°C.

*Gas Chromatography-Mass spectrometry*:

Gas chromatograph Agilent 7890B equipped with autosampler Gerstel Robotic and mass detector Agilent 5977B, operated at 70 eV (all Gerstel, Mühlheim a.d. Ruhr, Germany), S/SL injector, injection volume 0.5 µL, split ratio 200:1, injector temperature: 230ºC, column: Restek Vms 20 m x 0.8 mm x 1 µm (Restek GmbH, Bad Homburg, Germany), carrier gas: helium, constant flow = 1 mL/min, acquisition mode: Scan, 26-250 amu, oven program: 50ºC (3min), 10ºC/min, 250ºC (6min), retention indices: $C_7$-$C_{20}$.

*Headspace analysis*:

Alpha M.O.S. Heracles II (Toulouse, France), PAL autosampler, S/SL injector, injection volume: 2mL from 1g/L solution of the corresponding acetals, splitless mode, injector temperature: 200ºC, columns: Restek MXT-5 and MXT-1701, both 10 m x 0,18 mm x 0,4 µm, carrier gas: hydrogen, oven program: 40ºC (5s), 0.6ºC/s, 250ºC (60s), detector: 2x flame ionization detector.

## Results and discussion

Acetals of aldehydes with propylene glycol and / or ethanol were subject of various studies [3, 6-8]. In table 1 we show the spectra of those acetals, which have not been published in any mass spectra database yet [9].

We were not able to find the following acetals at our reaction conditions:

- With ethanol were not reacting: 2-phenyl-2-butenal, 5-methylfuraldehyde, α-amylcinnamaldehyde, anisaldehyde, citral, ethylvanillin, perillaldehyde, piperonal, trans-2-decenal, trans-2-hexenal, trans-2-methyl-2-butenal and vanillin
- With isopropanol were not reacting benzaldehyde and trans-2-hexenal
- With propylene glycol we observed only sluggish reaction of: 2-phenyl-2-butenal, 5-methylfurfural and perillaaldehyde

**Table 1:** The first 10 most abundant mass fragments of acetals, which have not been published yet

| Aldehyde | *Alcohol* | *RI* | *m/z (Abundance)* |
|---|---|---|---|
| β-Homo-cyclocitral | PG | 1575 | 87(1000), 59(235), 41(72), 88(45), 91(36), 79(32), 95(30), 107(30), 31(29), 77(24) |
| 5-Methylfurfural | PG | 1311 | 168(1000), 108(984), 95(977), 94(704), 153(673), 167(480), 79(472), 82(471), 81(168), 111(149) |
| α-Amylcinnam-aldehyde | PG | 1971 | 189(1000), 131(308), 190(139), 117(121), 115(105), 91(83), 87(74), 129(67), 59(58), 128(53) |
| Citronellal | PG | 1479 | 87(1000), 127(915), 121(750), 59(474), 41(368), 69(349), 95(284), 136(218), 81(169), 109(142) |
| p-Tolualdehyde | PG | 1496 | 177(1000), 119(967), 163(514), 91(420), 92(368), 178(312), 105(225), 104(132), 133(130), 103(118) |
| trans-2-Decenal | PG | 1578 | 113(1000), 127(444), 69(273), 55(262), 169(251), 41(189), 211(115), 83(107), 114(107), 59(79) |
| trans-2-Methyl-2-butenal | PG | 1075 | 127(1000), 69(420), 83(301), 87(243), 59(211), 41(159), 55(151), 97(126), 67(92), 39(72) |
| β-Homo-cyclocitral | Ethanol | 954 | 103(1000), 75(462), 47(200), 195(75), 149(74), 107(66), 104(57), 93(48), 81(47), 91(45), 123(45) |
| Melonal | Ethanol | 1343 | 103(1000), 86(883), 75(558), 82(385), 47(349), 123(241), 69(226), 41(207), 125(171), 81(161) |
| p-Tolualdehyde | Ethanol | 1394 | 149(1000), 121(511), 93(248), 91(211), 119(144), 150(120), 77(80), 65(46), 122(45), 29(31) |
| Undecanal | Ethanol | 1602 | 103(1000), 199(264), 75(182), 47(107), 97(87), 83(72), 57(69), 104(56), 69(56), 55(55), 85(54), 200(38) |
| Benzaldehyde | Propanol | 1457 | 149(1000), 107(949), 79(230), 77(135), 105(127), 150(113), 43(86), 108(76), 41(50), 27(29) |
| Hexanal | Propanol | 1281 | 131(1000), 143(981), 89(806), 43(757), 83(647), 101(364), 55(255), 41(228), 144(96), 57(95) |
| Benzaldehyde | Butanol | 1639 | 163(1000), 107(962), 79(169), 164(124), 105(106), 77(93), 108(78), 41(67), 29(50), 51(16) |
| Hexanal | Butanol | 1454 | 57(1000), 157(798), 159(630), 83(508), 83(647), 103(402), 101(351), 41(244), 55(191), 29(144) |

**Table 1:** continued

| Aldehyde | *Alcohol* | *RI* | *m/z (Abundance)* |
|---|---|---|---|
| Benzaldehyde | Isobutanol | 1535 | 107(1000), 163(798), 77(135), 105(127), 150(113), 43(86), 108(76), 41(50), 27(29) |
| Hexanal | Isobutanol | 1281 | 57(1000), 157(462), 83(200), 159(197), 103(163), 101(162), 41(155), 55(91), 29(74), 43(50) |
| Acetaldehyde | Pentanol | 1307 | 115(1000), 71(961), 43(420), 187(229), 45(170), 41(118), 29(72), 55(61), 42(64), 97(56) |
| Benzaldehyde | Pentanol | 1826 | 177(1000), 107(926), 79(230), 77(135), 105(127), 150(113), 43(86), 108(76), 41(50), 27(29) |
| Hexanal | Pentanol | 1634 | 71(1000), 171(766), 181(477), 43(473), 83(388), 101(360), 117(296), 55(161), 41(134), 42(65) |
| Benzaldehyde | 2-Methylbutanol | 1740 | 107(1000), 177(821), 71(178), 43(131), 178(109), 79(99), 108(84), 105(61), 77(56), 41(50), |
| Hexanal | 2-Methylbutanol | 1545 | 71(1000), 171(385), 43(271), 101(167), 83(148), 187(109), 117(106), 77(56), 41(76), 29(48) |
| Acetaldehyde | 3.Methylbutanol | 1232 | 71(1000), 115(723), 43(373), 41(90), 55(70), 72(67), 116(55), 45(40), 29(36), 39(26) |
| Hexanal | 3-Methylbutanol | 1556 | 71(1000), 171(384), 43(303), 187(248), 55(76), 41(70), 72(56), 117(48), 172(46), 29(31), 188(30) |
| Benzaldehyde | Hexanol | 2014 | 191(1000), 192(145), 43(129), 79(89), 105(79), 108(72), 41(49), 85(48), 55(26), 29(22) |
| Hexanal | Hexanol | 1815 | 85(1000), 185(634), 43(463), 215(335), 101(306), 83(277), 131(175), 57(138), 55(128), 41(121) |

Due to the diminished electrophilicity of C=O group in conjugated aldehydes (via positive mesomeric effects), these carbonyl derivatives are less susceptible to AdN (nucleophilic addition) reactions of O-nucleophiles (e.g. alcohols in hemi/acetalization) in comparison to more reactive aldehydes. This is due to the low reactivity of such compounds with ethanol, and thus, no formation of acyclic acetals occurs.

On the other hand, the use of propylene glycol as bis-O-nucleophile leads to the formation (though sluggish) of the corresponding cyclic acetals of the same carbonyls because the generation of 5-membered rings is thermodynamically favourable.

Unfortunately, we were not able to find any of the aldehydes we synthetized from apple alcohols and aldehydes in apple FTNF itself. The unknown peaks might possibly be rather combined acetals of aldehydes with two alcohols, such as acetals of acetaldehyde with ethanol and some other alcohol (ethyl-methyl, ethyl-butyl, etc.) This will be the subject of our further study.

In the storage study, we observed complete elimination of acetals in the low pH environment (soft drink base, yoghurt) within 2 hours after mixing (Figure 1). In media with higher pH, such as near-water drinks or mineral water, the decomposition of acetals was reduced considerably (Figure 2), which may raise the need for testing of these compounds by the food safety bodies, as many of acetals are still not in the Union list of flavourings and source materials [10].

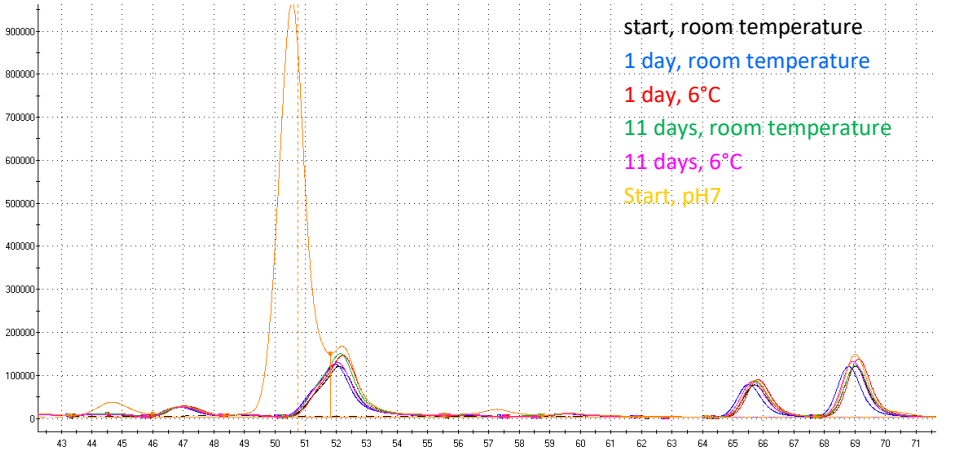


**Figure 1:** Headspace chromatograms showing complete decomposition of acetaldehyde propylene glycol acetalat at pH 3

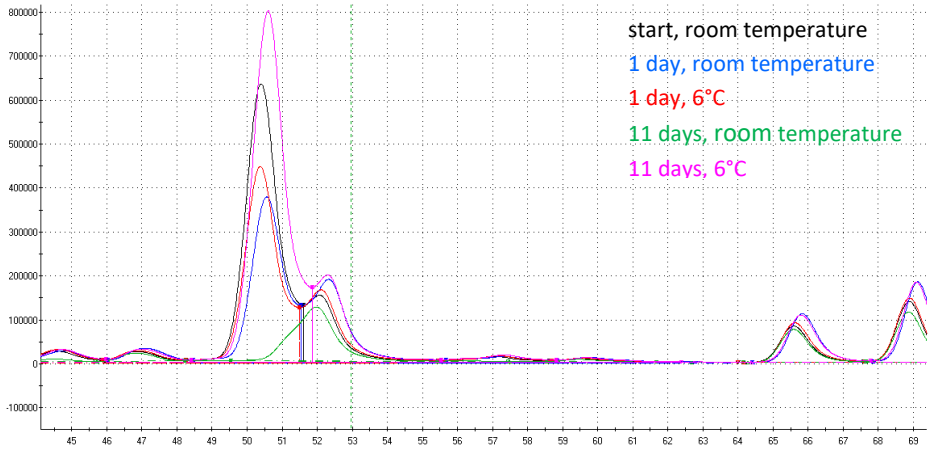


**Figure 2:** Headspace chromatograms showing increased stability of acetaldehyde propylene glycol acetal stored at higher pH and at lower temperature

## References

1. Maarse, H. Volatile compounds in foods and beverages. (1991). New York: Marcel Dekker. 764 p.
2. Dewick, P.M. (2006) In: Essentials of organic chemistry : for students of pharmacy, medicinal chemistry and biological chemistry. J. Wiley: Chichester, West Sussex, England ; Hoboken, N.J. pp. 221-282.
3. Coleman, W.M. (2006) J Chrom Sci, 44(3): 167-173.
4. NIST Mass Spectral Database, (2014). National Institute of Standards and Technology.
5. Wiley Mass Spectral Database, (2017). John Wiley & Sons, Inc.
6. Woelfel, K., Hartman, T.G. (1998) In: Flavor analysis : developments in isolation and characterization, (Mussinan, C.J. and Morello, M.J., eds.). American Chemical Society: Washington, DC. pp. 193-210.
7. Heydanek, M.G., Min, D.B.S. (1976) J Food Sci, 41: 145-147.
8. Zhang, J., Reineccius, G.A. (2017) Perf Flav, 42(9): 32-49.
9. Search NIST 17 and Wiley MS Databases. (2017) [cited 2017 October]; Available from: http://www.sisweb.com/software/ms/nistsearch.htm.
10. Authorisation of Food Flavourings. (2017) [cited 2017; Available from: https://webgate.ec.europa.eu/foods_system/main/?sector=FFL&auth=SANCAS.

# Novel strategies of addressing increasing complexity in flavour research

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## Abstract

Foods and beverages are highly complex systems in terms of composition and chemical and material changes during processing. The expected quality and benefits represent a delicate balance between sensory properties (aroma, taste, mouthfeel, texture), nutrition, health, and safety. This calls for a holistic and system-type approach to obtain the best product quality. Therefore, it is important to consider chemical and physical interactions, to study the formation kinetics of both desired and undesired compounds, and to know more about the release of bioactive compounds from the food and beverage matrix including the consumption event as well as during digestion and resorption. This requires sophisticated experimental setups, the use of non-targeted ("omics"-type) analytical methods and advanced data processing, working at the interface of scientific disciplines and establishing correlations between product quality and consumer benefits.

## Introduction

Flavour research has been a key activity in academia as well as in flavour and food industry. Many key odorants and taste compounds have been identified, their sensory characteristics described, their formation mechanisms studied using thermal and/or bio-assisted approaches, and ways for their formulation and controlled release developed. For a long time, the discovery of new molecules has been the primary focus, using targeted analytical methods as well as synthetic chemistry. More recently, high-throughput receptor-based assays have been designed for the screening of taste-active components. While identifying new sensorially relevant molecules will remain an active area of interest, generating and delivering the desired, complex, and well-balanced flavour profile by natural means and mild processing has become a major focus. This paper briefly describes new approaches of dealing with increasing complexity in flavour research and options to transform challenges into opportunities.

## Results and discussion

*Flavour Chemistry*. Our understanding of complex phenomena in food chemistry is largely based on the attempt to simplify intricate systems and to study individual phenomena in model systems, i.e. typically lipid oxidation and Maillard-type reactions. This approach has led to major breakthroughs highlighting the reaction mechanisms and relevant parameters of control. However, in food matrices these reactions cannot be seen in isolation, as food is composed of many different chemical entities such as lipids, carbohydrates, amino acids, peptides and proteins, but also polyphenols, alkaloids, vitamins, terpenoids, minerals, etc. They interact at various stages of different reaction cascades and influence and shape food properties and quality attributes such as aroma, taste, colour, texture and the nutritional profile. In this respect, chemical transformations taking place in foods can be regarded as a subset of "systems chemistry". In the following, complex food chemistry will be depicted from different perspectives, such as *i)* chemical

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interactions, *ii)* food as a complex system, *iii)* formation of defined molecules from various precursors, and *iv)* flavour generation in self-assembly systems.

As examples, amino acid degradation products characteristic for Maillard-type reactions (e.g. Strecker aldehydes, thermogenic amines, vinylogous compounds) can be produced in the presence of lipid oxidation products such as α,β-unsaturated aldehydes [1]. Lipid-derived reactive aldehydes can also be replaced by polyphenols as shown for the Strecker degradation of phenylalanine in the presence of *o*- and *p*-diphenols [2]. Epicatechin reactions have been shown to influence the mechanism of Maillard product formation in low moisture systems [3]. Hydroxyhydroquinone, a degradation product of chlorogenic acids, is trapping 2-furfurylthiol (FFT), a character-impact odorant of coffee aroma, in the presence of transition metals, thus changing the overall coffee aroma from fresh to stale [4]. The triple role of polyphenols has recently been discussed, resulting in a multitude of chemical interactions based on their chelating, free radical-scavenging, and carbonyl-trapping regions [5].

Looking at food as a complex system, it is mandatory to perform studies not only in simplified model systems but in real food matrices. Coffee constitutes one of those examples. The coffee bean can be seen as a mini-reactor. Consequently, the most appropriate approach studying chemical transformations upon roasting is using the coffee bean itself as a reaction system. Therefore, it is not surprising that the formation of FFT in coffee is different from what we learned from the respective model systems. It has been shown that FFT is generated in arabinose/cysteine model systems *via* 3-deoxypentosone and furfural maintaining the intact carbon chain [6]. However, 'in-bean' experiments using fully $^{13}$C-labelled arabinose resulted in only 1% fully labelled FFT upon coffee roasting while almost 90% of the FFT formed was not labelled at all [7]. This strongly suggests alternative formation pathways of FFT in coffee, which are still not well understood.

A specific molecule can derive from one individual source material or, on the other side, from many different precursors. As an example and depending on the food composition, furan might be formed from various sugars, amino acids, polyunsaturated fatty acids (PUFAs), carotenoids, and ascorbic acid [8, 9]. Therefore, it is mandatory to screen for all potential sources to mitigate the formation of this undesirable compound during food preparation. Contrastingly, acrylamide is primarily formed from asparagine as a well-defined precursor [10] while 2,4-decadienal is known as a lipid degradation product of PUFAs. As recently shown [11], the choice of the oil in combination with heat treatment has a strong impact on the level of acrylamide and flavour active components (2,4-decadienals) exhibiting deep fried notes (Table 1). Therefore, it is recommended to study the formation of undesirable and desirable compounds in parallel in order to enable mitigation while delivering desired sensory properties.

**Table 1:** Concentrations (µg/kg) of acrylamide and 2,4-decadienal (sum of the (*E*,*E*)- and (*E*,*Z*)-isomers) in potato chips after deep-frying at 180 °C and 140 °C for 2.5 min

| Odorant | *Safflower oil 180 °C* | *Safflower oil 140 °C* | *Linseed oil 180 °C* | *Linseed oil 140 °C* |
|---|---|---|---|---|
| Acrylamide | 160 | 94 | 1690 | 1240 |
| 2,4-Decadienal | 4697 | 468 | 321 | 46 |

A characteristic feature of systems chemistry is the formation of self-assembled structures, also referred to as mesophases, which can be observed in many food products. Molecular organisation of flavour precursors can play an important role in food systems

containing ingredients that tend to form self-assembly structures, as for example in reversed microemulsions. This may lead to increased yields in flavour formation due to favouring certain formation pathways and increased flavour stability by protecting labile flavour compounds in compartments of the structured medium. As shown in Figure 1, the yield of FFT generated from xylose (Xyl) in the presence of cysteine (Cys) increased continuously during the entire heating period in both reaction media. However, highest FFT yields were obtained in the mesophasic system as compared to phosphate buffer [12].

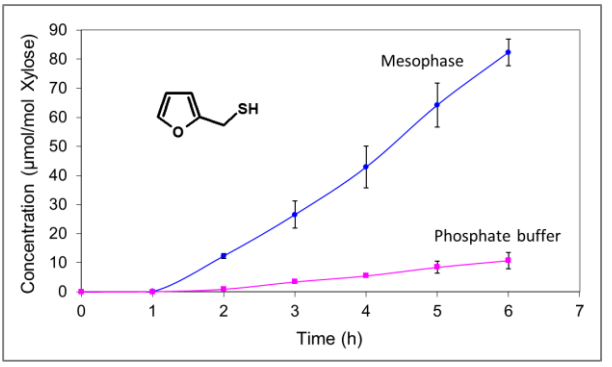


**Figure 1:** Formation of 2-furfurylthiol (FFT) from Xyl/Cys in phosphate buffer and in self-assembled structures (mesophase: reversed microemulsion) at 95 °C and as a function of time.

In such structures, three domains of submicrometre size are present, i.e. aqueous, amphiphilic, and lipophilic. A given molecule is preferably dissolved in one particular domain and may display a specific spatial orientation. When two molecules are located in the same domain (e.g. amphiphilic domain), their concentration is higher, thus increasing the probability of reaction. On the other hand, a molecule in the aqueous domain is unlikely to react with a molecule in the lipid domain. As a result, this domain fragmentation (compartmentalization) may favour certain reactions while inhibiting others. For Maillard-type reactions involving cysteine and xylose, both reactants are hydrophilic whereas the reaction products are more lipophilic, such as FFT for example. Thus, this type of reactions in mesophases may lead to high yields considering that the product concentration in water will remain low, as formed FFT will migrate into the lipophilic domain once generated. Furthermore, isolation of FFT in the lipid domain may protect it against reactants present in the aqueous media.

*Advanced Analytics*. Novel insights and the data quality obtained usually correlate with the advancement in analytical techniques applied. New key odorants and tastants have been identified thanks to sensory-guided chemical analyses, i.e. GC-Olfactometry [13, 14] and LC-Taste [15]. Quantitative results can be obtained using the Stable Isotope Dilution Assay (SIDA) method [16]. Reaction mechanisms can be elucidated using labelling experiments and the relative importance of concurrent pathways estimated by the carbon module labelling (CAMOLA) technique [17]. These techniques, e.g. primarily targeted methods, have contributed to major new discoveries and our current understanding of flavours.

We have applied the CAMOLA technique in kinetic studies to study the formation of 2,3-butanedione (diacetyl) from various precursors [18]. Figure 2 shows the formation of diacetyl from sucrose and other sources, e.g. bound carbohydrates. While the total amount of diacetyl is constantly increasing over time and with roasting degree (up to seven minutes and in particular after three minutes), sucrose is progressively losing

importance as a source of diacetyl in favour of other precursors (e.g. bound carbohydrates). Furthermore, the contribution of the intact carbohydrate skeleton decreases with increased roasting level (data not shown) due to fragmentation favoured at higher temperatures. These data give a new insight into the relative role of various formation pathways, which are the base for adapting process conditions and selecting raw materials. Understanding the relative importance of various alternative reaction pathways helps to single out the relevant formation patterns and to identify how they could potentially be influenced *via* adapted processing conditions.

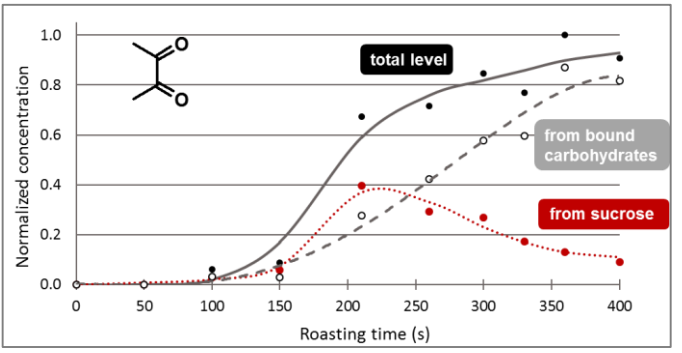


**Figure 2:** Formation of 2,3-butanedione (diacetyl) upon coffee roasting obtained in a CAMOLA study using $^{13}$C-labelled and unlabelled sucrose in a 1:1 ratio.

The techniques mentioned above are suitable to study known compounds and relationships in a targeted manner. However, they show some limitation when it comes to unknown molecules and intricate formation pathways. Data independent acquisition (DIA) of mass spectrometry (MS) data has been proven a very effective tool in Life Sciences to unravel complex correlations and, thus, identify new molecular targets and mechanistic relationships. In the food context, they may correlate with phenomena of interest such as aroma, taste, and health benefits. The sequential window acquisition of total high-resolution mass spectra (SWATH-MS) method is measuring all signals in one run. SWATH-MS is a DIA method that generates, in a single measurement, a complete recording of the fragment ion spectra of all analytes in a sample for which the precursor ions are within a predetermined *m/z* versus a retention time window [19]. SWATH-MS results in a digital fingerprint of the sample (digital twin) allowing retrospective data interpretation. It can be used as a new method in food and flavour research to compare differences between samples and changes upon processing. Targeted analysis can subsequently be performed with a focus on significant chemical differences. This untargeted method is considerably gaining importance in food research as a complementary approach to targeted molecular characterization. It is frequently associated with the term “foodomics” as shown at the recent RAFA symposium [20].

*Release Phenomena*. Aroma and taste components present in foods and beverages need to reach the respective receptors in order to elicit the desired aroma note or taste response. One critical step is the release of those aroma- or taste-active molecules during mastication in the mouth and their transport in the saliva. In-mouth release phenomena are studied with the aim of maximizing the inherent flavour potential of sensory-active components before they are being swallowed [21, 22]. During this in-mouth process, flavour compounds are progressively released from the food matrix. This phenomenon is mainly dependent on food texture, composition, in-mouth breakdown, and on

impregnation with saliva. The saliva composition and its activity may represent another opportunity of influencing flavour perception. As all these factors will affect release kinetics, this could potentially be an option to reduce the amount of ingested sodium and sugar while maintaining the desired taste characteristics.

Tailored design of materials in the solid state, for instance as a co-crystal, constitutes a novel concept to modulate taste perception. Co-crystals are little known in the food industry [23], however, co-crystallization as a concept has been broadly applied in the pharmaceutical industry to improve solubility and bioavailability of the respective active compound. In a food context, modulating dissolution kinetics could be of interest for delivering salt and sweet taste through the use of co-crystalline salt and carbohydrate materials. Co-crystals of glucose and NaCl are well known in the literature and easy to obtain *via* direct crystallization from aqueous solution [23]. Synthetic protocols to obtain co-crystals of sucrose and NaCl have not been described previously. This material is preferably accessible *via* isomorphous seeding with the co-crystalline NaBr heterologue: their synthesis and physico-chemical characterisation have recently been reported [24].

The dissolution kinetics in saliva are key for the sensory perception of water-soluble tastants consumed in the solid state, e.g. salt and sugar. This concept has been explored in the past *via* micronization, e.g. using powdered sugar or dusted salt. Interestingly, co-crystalline formulations can display faster dissolution properties, possibly giving rise to a stronger taste impact. Figure 3 shows the dissolution kinetics of pure NaCl, anhydrous glucose, glucose monohydrate and the respective co-crystal $(Glucose)_2 \cdot NaCl \cdot H_2O$, the structure of which is presented in the bottom right of Figure 3.

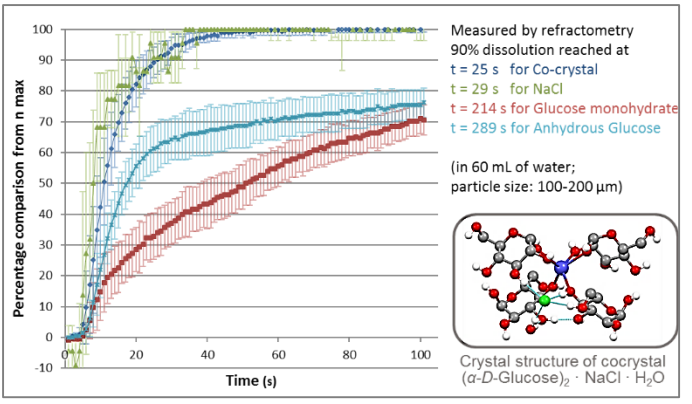


**Figure 3:** Dissolution kinetics of co-crystalline glucose sodium chloride *vs.* its individual pure ingredients indicating that the co-crystal dissolves faster than glucose or glucose monohydrate alone.

This co-crystalline material dissolves faster compared to pure glucose or pure glucose monohydrate alone, taking into account parameters like crystal size, crystal size distribution, concentration and molar composition. It dissolves comparably to NaCl with respect to kinetics. From a sensory perspective, the salt taste perception is much stronger than the simultaneously perceived faint sweetness of glucose. However, the co-crystal also dissolves faster than a simple dry-mix of glucose with NaCl. Therefore, such co-crystalline forms of NaCl could potentially offer a boost of saltiness, as carbohydrates are omnipresent in food products [25].

In conclusion, flavour research is facing an increasing complexity. Product quality is not only depending on one individual attribute, e.g. aroma, but on a multitude of

features (e.g. taste, mouthfeel, texture) which need to be well balanced. In addition, it is equally important to ensure nutrition and to maintain or develop health benefits (e.g. appropriate amount of carbohydrates, minerals and lipids), as well as mitigation of process contaminants. While the concurrent study of all these phenomena represented a clear challenge in the past, we have got emerging analytical techniques from Life Sciences using extensively non-targeted methods ("omics"). Their transfer and application to food science ("foodomics") has become a trend and it is an excellent opportunity to connect flavour research with other disciplines delivering additional benefits. Another recent development of equal importance stems from Material Sciences allowing the use of tailored solid-state structures to better master flavour formation and release.

## References


1. Hidalgo, F.J.; Zamora, R. Crit. Rev Food Sci. Nutr. 2016, 56, 1242-1252.
2. Delgado, R.M.; Zamora, R.; Hidalgo, F.J. J. Agric. Food Chem. 2015, 63, 312-318.
3. Totlani, V.M.; Peterson, D.G. J. Agric. Food Chem. 2007, 55, 414-420.
4. Müller, C.; Hofmann, T. J. Agric. Food Chem. 2007, 55, 4095-4102.
5. Zamora, R.; Hidalgo, F.J. Trends Food Sci. Technol. 2016, 54, 165-174.
6. Tressl, R.; Helak B.; Kersten, E.; Nittka, C. In Recent Developments in Flavor and Fragrance Chemistry, Hopp R., Mori, K. (eds.), VCH, Weinheim, Germany, 1993, pp. 167-181.
7. Poisson, L.; Schmalzried, F.; Davidek, T.; Blank, I.; Kerler, J. J. Agric. Food Chem. 2009, 57, 9923-9931.
8. Perez, L.C.; Yaylayan, V.A. J. Agric. Food Chem. 2004, 52, 6830-6836.
9. Blank, I. In Bioactive Compounds in Foods; Gilbert J., Senyuva, H. Z., Eds.; Blackwell Publishing: Oxford, 2008; pp. 291-322.
10. Stadler, R.H.; Blank, I.; Varga, N.; Robert, F.; Hau, J.; Guy, Ph. A.; Robert, M.-C.; Riediker, S. Nature 2002, 419, 449-450.
11. Thürer, A.; Granvogl, M. J. Agric. Food Chem. 2016, 64, 9107–9115.
12. Sagalowicz, L.; Moccand, C.; Davidek, T.; Ghanbari, R.; Martiel, I.; Negrini, R.; Mezzenga, R.; Leser, M. E.; Blank, I.; Michel, M. Philos. Trans. R. Soc. A: Math. Phys. Eng. Sci. 2016, 374, 20150136.
13. Acree, T. E.; Barnard, J.; Cunningham, D. G. Food Chem. 1984, 14, 273-286.
14. Grosch, W. Chem. Senses 2001, 26, 533-545.
15. Frank, O.; Ottinger, H.; Hofmann, T. J. Agric. Food Chem. 2001, 49, 231-238.
16. Schieberle, P.; Grosch W. J. Agric. FoodChem. 1987, 35, 252-257.
17. Schieberle, P. Ann N.Y. Acad. Sci. 2005, 1043, 236-248.
18. Poisson, L.; Auzanneau, N.; Mestdagh, F.; Blank, I.; Davidek, T. J. Agric. Food Chem., 2017, DOI: 10.1021/acs.jafc.6b04849.
19. Liu, Y.; Hüttenhain, R.; Surinova, S.; Gillet, L.C.J.; Mouritsen, J.; Brunner, R.; Navarro, P.; Aebersold, R. Proteomics 2013, 13, 1247–1256.
20. 8th Recent Advances in Food Analysis, RAFA symposium, Prague, November 7-10, 2017, http://www.rafa2017.eu/
21. Salles, C; Chagnon, M.C.; Feron, G.; Guichard, E.; Laboure, H.; Morzel, M.; Semon, E.; Tarrega, A.; Yven, C. Crit Rev Food Sci Nutr. 2011, 51, 67-90.
22. 4th International Conference on Food Oral Processing, Lausanne, July 3-6, 2016, https://meeting.artegis.com/lz/LoginPage?T=1&custom=1451&navid=12720&event=11650
23. Oertling, H. Cryst. Eng. Comm. 2016, 18, 1676-1692.
24. Oertling, H.; Besnard, C.; Alzieu, T.; Wissenmeyer, M.; Vinay, C.; Mahieux, J.; Fumeaux, R. *Cryst. Growth Des.* 2017, *17*, 262–270.
25. Oertling, H.; Alzieu, T.; Fumeaux, R.; Le Reverend, B.; Dupas-Langlet, M.; Wissenmeyer, M.; Mahieux, J. WO 2017016907 (A1).

# Effect of muscle, ageing and packaging on marker volatiles for beef flavour

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## Abstract

Many of the key flavour impact compounds for cooked beef are present at very low concentrations and are challenging to analyse. Marker compounds for desirable flavour have been identified and may be used to monitor flavour-forming reactions. In this paper, this approach is used to follow the impact of muscle, ageing and packaging on grilled beef flavour.

Different muscles and ageing periods show some alterations in the profile of marker volatile compounds that may reflect changes in consumer perception. Some significant and consistent differences are observed between muscles and ageing periods, while grilled beef that has previously been modified atmosphere packed, vacuum packed and over-wrapped show differences in numerous volatile flavour compounds, especially in the products of lipid oxidation.

This approach is yielding a new understanding of the factors affecting the formation of flavour compounds in cooked beef, which could enable new processing methods to be proposed to manage flavour formation in commercial beef products.

## Introduction

Consumer assessments of beef from across Europe have shown that palatability is not as consistent as might be expected from a high value product [1]. The proportion of beef judged to be "unsatisfactory" ranges from 19.5% for grilled sirloin and 25% grilled rump to 54% of roasted topside. An inconsistency in quality delivered to the consumer was one of the catalysts for the development of "Meat Standards Australia" (MSA), a cuts-based quality assurance grading scheme developed by Australian scientists [2,3]. This system is now widely used in Australia, and has been tested and found effective in other countries including South Korea, Northern Ireland, Ireland, USA, New Zealand, France and Poland [4].

Despite its effectiveness at predicting eating quality and tenderness, there is some evidence that the MSA prediction of flavour for some consumers could be improved [5]. Flavour can be as important as tenderness for consumers [5,6]. For this reason, studies have been conducted to determine the relationship between volatile flavour compounds in beef and consumer-perceived quality.

Many of the key flavour impact compounds for cooked beef are present at very low concentrations and are challenging to analyse. Therefore, marker compounds for desirable flavour have been proposed [7] to provide a cost-effective and accessible method of monitoring flavour-forming reactions. In this study, this approach is used to follow the impact of muscle, ageing and packaging on grilled beef flavour.

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DOI: 10.3217/978-3-85125-593-5-67, CC BY-NC-ND 4.0

## Experimental

### *Materials*

Beef was obtained from an experiment conducted in Australia, which investigated the impact of muscle, packaging and ageing on sensory quality. Samples were blast frozen after the designated ageing period and selected samples were transported frozen to Northern Ireland by commercial courier. Samples from three muscles (striploin, fillet and rump), three packaging methods (modified atmosphere packaging (MAP with 80% $O_2$:20% $CO_2$), overwrapped (OWP) and vacuum skin packaging (VSP)) and three ageing periods (14, 21 and 49 days) were selected for analysis. Table 1 summarises the treatments evaluated and the numbers of samples analysed for volatile compounds.

**Table 1:** Experimental design

| Cut | Muscle | *Abbreviation* | *Ageing* | *MAP** | *OWP* | *VSP* | *Total* |
|---|---|---|---|---|---|---|---|
| Striploin | *Longissimus thoracis/ lumborum* | STR045 | 14 | 4 | 5 | 5 | 14 |
| | | | 21 | 4 | 5 | 4 | 13 |
| | | | 49 | 2 | 4 | 5 | 11 |
| Fillet | *Psoas major* | TDR062 | 14 | 5 | 5 | 5 | 15 |
| | | | 21 | 5 | 4 | 5 | 14 |
| | | | 49 | 4 | 5 | 4 | 13 |
| Rump | *Gluteus medius* | RMP131/ RMP231[#] | 14 | 4 | 5 | 5 | 14 |
| | | | 21 | 5 | 4 | 4 | 13 |
| | | | 49 | 4 | 5 | 6 | 15 |
| | | | | 37 | 42 | 43 | 122 |

** MAP = modified atmosphere packaging; OWP = overwrapped; VSP = vacuum skin packaging.*
*[#] RMP131 and 231 are two parts of the same muscle; similar numbers of samples were taken from each: 22 from RMP131 and 20 from RMP231.*

### *Analysis*

Beef was grilled according to the standard MSA protocol for "medium" cooked beef [8] and the volatiles were collected using Solid Phase Micro Extraction, prior to analysis by electron impact GC-MS, as described previously [9]. The results were statistically analysed using linear mixed methodology, using restricted maximum likelihood (REML) estimation.

## Results and discussion

Differences between muscles and ageing periods are significant for some compounds but generally small, while those caused by packaging are more extensive.

### *Effect of muscle*

Comparison of the volatile compounds from different muscles (Figure 1) shows the quantities of selected compound classes (Strecker aldehydes and ketones) from the grilled muscles, relative to that obtained from striploin, which was common to both trials. Of the Strecker aldehydes, only benzaldehyde showed a significant difference between muscles (P=0.018), with striploin producing less than the other muscles. Other Strecker aldehydes showed a non-significant trend also towards lower quantities in striploin. This agrees with previous findings [9] that benzaldehyde (but not the other Strecker aldehydes) were lower in striploin than tenderloin, rump or topside. Three ketones showed significantly higher

levels in tenderloin than the other muscles, with 2-butanone showing a similar non-significant pattern.

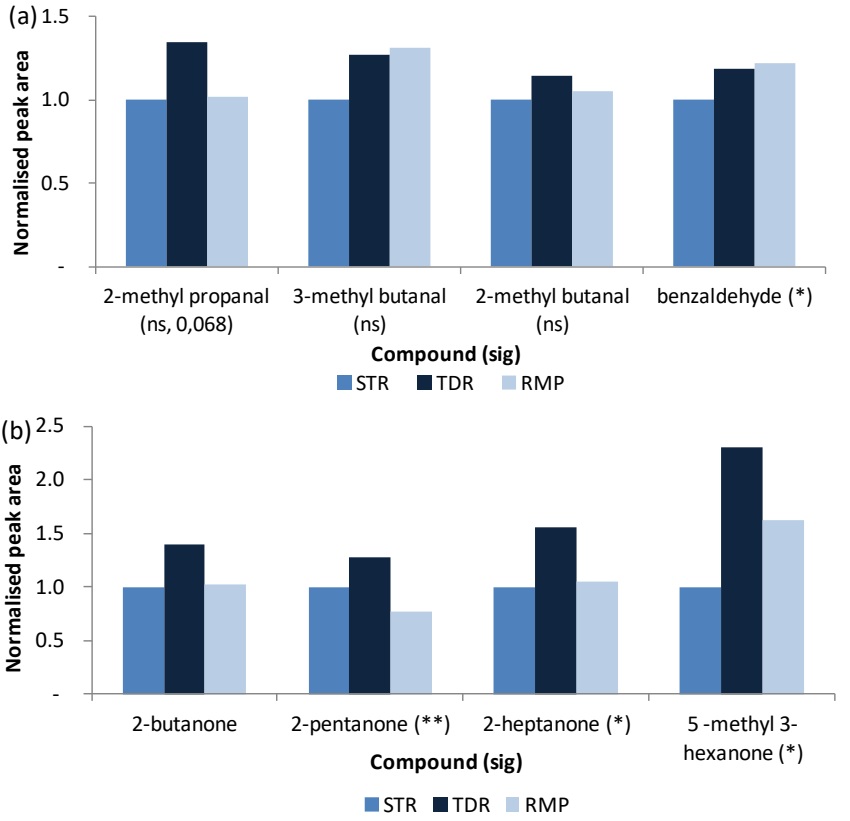


**Figure 1** (a – b). Relative qualities of Strecker aldehydes (a) and ketones (b) from grilled beef, different muscles, shown relative to striploin, STR045 = 1. Abbreviations and replication may be found in Table 1.

Previous research [9] showed a similar pattern for 2-propanone ($P<0.001$) and 2-butanone (ns), but did not report findings for the remaining ketones. Most of the remaining volatile compounds were not significantly different between muscles. These results indicate that different muscles produce a similar balance of volatile compounds on grilling, but with some significant and consistent differences. The changes in flavour formation pathways reflected by these differences may contribute to variations in flavour between muscles.

*Effect of ageing*

Figure 2 shows the effect of ageing from 14 and 21 to 49 days on selected volatiles. While there were few significant differences, there were some trends, with the C7 to C9 n-aldehydes showing an apparent increase at 21 days that was not replicated at 49 days (Figure 2). The large variation within treatments for these compounds meant that these results were generally not statistically significant and further analyses are ongoing to clarify these effects. The Strecker aldehydes, heterocyclic compounds and C4 ketones formed by the Maillard reaction showed no significant effects of ageing and nor were there significant ageing x muscle interactions (results not shown). Research has shown

that the concentrations of sugars, amino acids and ribonucleotides increase with age [10, Farrell, unpublished data], and it might have been expected that the volatile products would follow a similar pattern. Only 3-methylbutanal and 2-methylbutanal showed a non-significant trend correlating with ageing (results not shown).

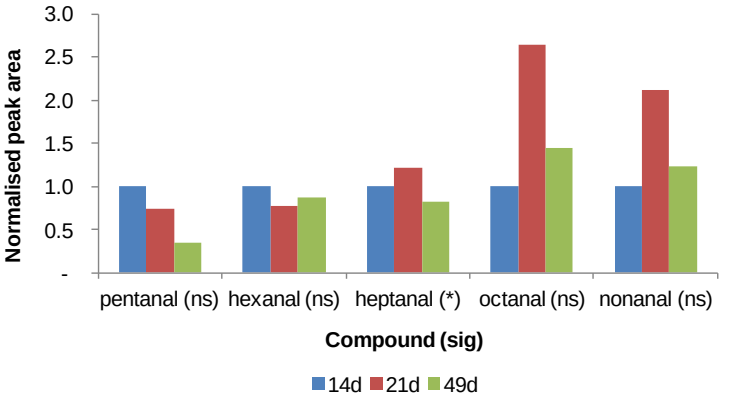


**Figure 2:** Relative qualities of n-aldehydes from grilled beef from different muscles, shown relative to 14 days = 1. Abbreviations and replication may be found in Table 1.

*Effect of packaging*

Changes in packaging caused significant differences in the generation of a number of different volatile flavour compounds (Figure 3). Benzaldehyde ($P < 0.001$) was lowest in modified atmosphere packed (MAP) beef and highest in vacuum packed beef. Other Strecker aldehydes followed the same pattern (though non-significantly), as did dimethyltrisulphide ($P < 0.01$). MAP is reported to cause oxidation of proteins and it is possible that this could affect the concentrations of free amino acids available for the formation of these compounds [11,12]. Strecker aldehydes have been closely associated with desirable flavour of beef for consumers [7,13], so changes in these compounds could contribute to differences in consumer preference between packaging treatments.

Amongst the n-aldehydes, only pentanal shows a significant difference with at least five times more in MAP-packed beef than the other two packaging treatments. The remaining n-aldehydes follow the same pattern as hexanal (shown in Figure 3). Vacuum-packed beef has significantly lower concentrations than overwrapped beef of 5-methyl-3-hexanone and 2-pentyl furan while 3-heptanone and 2-ethyl-1-hexanol are lower in both VSP and MAP beef. These compounds can be formed by oxidation pathways [14-16] and it is possible that the reduced oxygen in vacuum-packed beef and higher oxygen permeability of overwrapped beef has caused this effect. Further studies are ongoing to elucidate these effects.

While products of the Maillard and lipid oxidation reactions often follow a similar pattern, a number of products show different effects due to treatment. In some cases, a significant effect is mirrored by a non-significant trend in related compounds, but in others, there are widely different effects within a compound class. Thus, care will be required when identifying marker compounds for desirable flavour [7] that these apply in all cases.

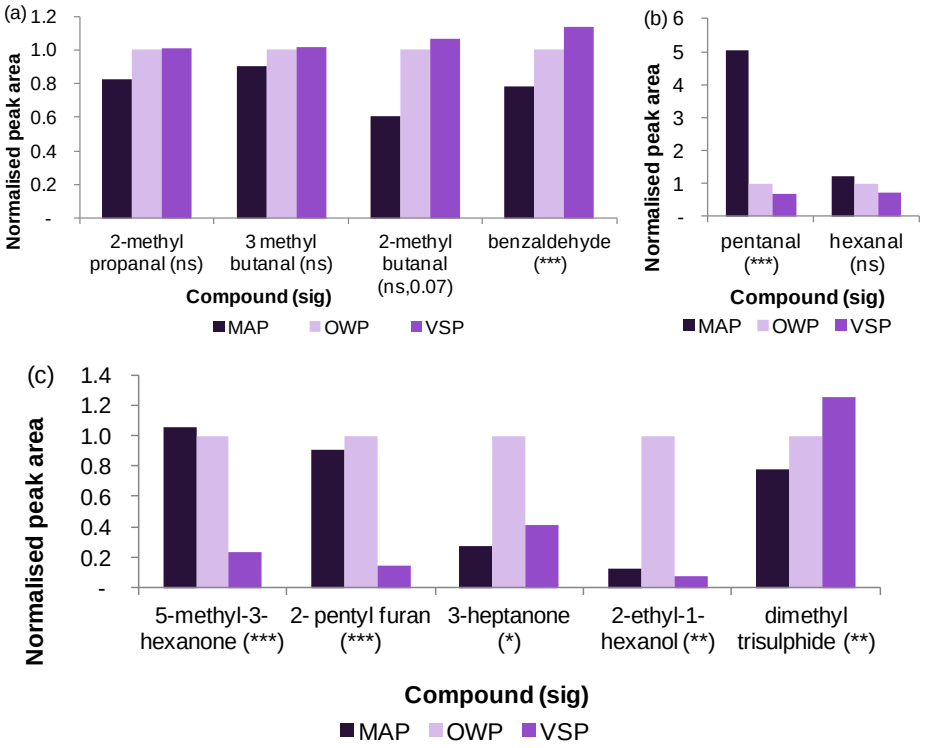





**Figure 3:** Relative qualities of selected volatile compounds of grilled beef from different packaging methods, shown relative to overwrap (OWP) = 1: (a) Strecker aldehydes; (b) n-aldehydes from Trial 2. Abbreviations and replication may be found in Table 2.

## Conclusions

Differences in volatile odour compounds are observed due to muscle, ageing and packaging method. These changes are most extensive due to packaging. The resulting changes in the balance of flavour compounds are likely to alter the flavour profile perceived by consumers.

While products of the Maillard and lipid oxidation reactions often follow a similar pattern, some demonstrate different effects due to treatment. Thus, care will be required when identifying marker compounds for desirable flavour. Further analyses are ongoing to clarify further these effects.

## References


1. Farmer, L. J., Bowe, R., Troy, D. T., Bonny, S. P. F., Birnie, J., Dell'Orto, V., Polkinghorne, R. J., Wierzbicki, J., de Roest, K., Scollan, N. D., Henchion, M., Morrison, S. J., Legrand, I., Roehe, R., Hocquette, J. F., and Duhem, K. (2016). Viandes & Produits Carnés 32, VPC-2016-2032-2011-2016
2. Egan, A. F., Ferguson, D. M., and Thompson, J. M. (2001). Australian Journal of Experimental Agriculture 41, 855-859
3. Ferguson, D., Thompson, J., and Polkinghorne, R. (1999). In: 45th Internatinal Congress of Meat Science and Technology, Yokohama, Japan
4. Polkinghorne, R. J., and Thompson, J. M. (2010) Meat standards and grading A world view. Meat Science 86, 227-235
5. Polkinghorne, R. (2007). In: 60th Reciprocal Meat Conference, Brookings, USA
6. Oliver, M. A., Nute, G. R., Furnols, M. F. I., San Julian, R., Campo, M. M., Sanudo, C., Caneque, V., Guerrero, L., Alvarez, I., Diaz, M. T., Branscheid, W., Wicke, M., and Montossi, F. (2006). Meat Science 74, 435-442
7. Farmer, L. J., Hagan, T. D. J., Devlin, Y., Gordon, A. W., and Oltra, O. R. (2012). In: International Congress on Meat Science and Technology Montreal
8. Watson, R., Gee, A., Polkinghorne, R., and Porter, M. (2008). Australian Journal of Experimental Agriculture 48, 1360-1367
9. Legako, J. F., Brooks, J. C., O'Quinn, T. G., Hagan, T. D. J., Polkinghorne, R., Farmer, L. J., and Miller, M. F. (2015). Meat Science 100, 291-300
10. Koutsidis, G., Elmore, J. S., Oruna-Concha, M. J., Campo, M. M., Wood, J. D., and Mottram, D. S. (2008). Meat Science 79, 270-277
11. Frank, D. C., Geesink, G., Alyarenga, T., Polkinghorne, R., Stark, J., Lee, M., and Warner, R. (2017). Meat Science 123, 126-133
12. Fu, Q., Liu, R., Zhang, W., Li, Y., Wang, J., and Zhou, G. (2015). Food and Bioprocess Technology 8, 580-588
13. Farmer, L. J., Hagan, T. D. J., Oltra, O. R., Devlin, Y., and Gordon, A. W. (2013). In: Wartburg Flavour Symposium (T., H., and Schieberle, P. eds.), Eisenach, Germany
14. Larick, D. K., Turner, B. E., Schoenherr, W. D., Coffey, M. T., and Pilkington, D. H. (1992). J. Anim. Sci. 70, 1397-1403
15. Gasser, U., and Grosch, W. (1988). Z. Lebensm. unters. Forsch. 186, 489-494
16. Mottram, D. S. (1991). In: Volatile Compounds in Foods and Beverages (Maarse, H. ed.), Marcel Dekker, New York. pp 107-177

# Discovering aroma patterns in food products using Latent Dirichlet Allocation and Jensen Shannon divergence

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## Abstract

Aroma Extract Dilution Analysis (AEDA) evaluates volatile compounds most likely contributing to the overall aroma of a food sample by means of flavour dilution (FD) factors. In the food industry, this can be useful to compare aroma-active profiles of raw materials or finished products and to select those that are statistically similar. When multiple samples are analysed, the high number of variables makes it difficult to take conclusions. Principal Component Analysis (PCA) should not be applied to FD values as they are discrete numbers. To our knowledge, there are no appropriate methods available to interpret AEDA results from multiple samples. In this study, a new rapid methodology to interpret AEDA results was developed. Latent Dirichlet Allocation (LDA) was developed in the context of text analysis as a mean of dimensionality reduction and has been successfully applied for the analysis of AEDA outcomes. Furthermore, Jensen Shannon divergence measure was a useful tool to compare the distribution of volatile compounds with similar descriptions ("berries", "cheese" or "fruits") among different samples.

## Introduction

Gas chromatography-olfactometry (GC-O) is used to judge the sensory relevance of the volatiles present in foods. In particular, AEDA evaluates the odour activities of the volatiles by sniffing the effluent of a series of dilutions of the original aroma extract. The result is expressed as the flavour dilution (FD) factor that corresponds to the maximum dilution value detected. Compounds with the highest FD are assumed to be most likely contributing to the overall aroma of a food product. AEDA is a time-consuming technique and generally research articles report the analysis of 1-3 samples where it is fairly easy to see differences. However, when multiple samples are analysed, the interpretation of AEDA results becomes challenging. This is because AEDA data set is fairly high-dimensional but sparse and it is difficult to conclude similarity among samples. A common approach in situations like this is to map the data into an adequate lower dimensional sub space where the comparison and clustering is done. When the data is normally distributed, PCA is often used. However, PCA should not be applied to AEDA because the data are discrete. This may be the reason why in other works, the statistical interpretation of AEDA has been claimed to be controversial or even not applicable [1, 2] although the authors did specify the reasons.

Latent Dirichlet Allocation (LDA) was developed in the context of text analysis as a means of dimensionality reduction [3]. For example, LDA can be used to cluster documents where instead of cluster them word by word, they can be clustered by topic (a topic would be described by a distribution over words). In probability theory and statistics, the Jensen–Shannon divergence is a method of measuring the similarity between two probability distributions [4].

The aim of this work was to develop a rapid methodology using LDA and Jensen-Shannon divergence to interpret AEDA results from multiple samples. In particular, the method was used to investigate the similarities in the aroma profile of pet foods.

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## Materials and methods

*Samples*

8 pet foods samples from different brands and varieties were used in this study. 20 g of each sample were suspended with 20 mL $H_2O$ and extracted with 100 mL diethyl ether (distilled before use). The organic layer was separated from the residue and the volatiles were isolated via Solvent Assisted Flavour Evaporation. The distillate was dried over sodium sulphate and concentrated to 200 µL using a Vigreux column.

*GC-O analysis*

High resolution gas chromatography was performed by means of a Trace GC (Finnigan, Bremen) and a column FFAP (30 m x 0.25 mm x 0.25 µm, J&W Scientific). The samples (1 µl) were injected using "on column" injection technique at 40 °C. After 1 min, the temperature was raised 6 °C/min until 240 °C were reached. The flow rate of the carrier gas (helium) was set on 1.5 mL/min. At the end of the capillary, the effluent was split 1:1 into a flame ionization detector (FID) and a sniffing port by using two deactivated, uncoated fused silica capillaries (20 cm × 0.25 mm). The FID and sniffing port were held at 250 °C. Linear retention indices (LRI) were calculated by the equation given by Kovats. The volatile fraction was diluted stepwise 1+1 with solvent and each dilution step was sniffed until no odourant in the effluent was perceived. The odour extract dilution analysis was performed by two trained panellists. FD factors were expressed in logarithmic scale units.

*Statistical analysis*

LDA was used to model aroma profiles as random mixtures over latent topics, each topic was characterized as a distribution over aroma compounds and was interpreted as a basic aroma profile.

The following generative process was assumed for each product aroma profile $I_n$:

1. Choose $N$ ~ *Poisson* ($\xi$) as the sum of all logarithmized FD-factors. in $I_n$
2. Choose $\Theta$ ~ *Dirichlet* ($\alpha$)
3. For each of the $N$:
   (a) Choose topic $Z_n$ ~*Multinomial* ($\Theta$)
   (b) Choose a DF from the aroma compounds from $p(I_n|Z_n,\beta)$, a multinomial probability conditioned on the topic $Z_n$

Model fitting and inference based on this process was done by Variational Bayes.

To determine the similarity of the aroma profiles of two products, to use information-theoretically motivated measure of distance of two probability distributions $\boldsymbol{P}$ and $\boldsymbol{Q}$ like the Kullback-Leibler divergence $\boldsymbol{D_{KL}(P||Q) = \sum_i P(i) \cdot \log \frac{P(i)}{Q(i)}}$ is appropriate.

Jensen-Shannon Divergence is the symmetric version of Kullback-Leibler divergence and was used a distance metric to describe distances between products, as follows:

$$\boldsymbol{JSD(P||Q) = \frac{1}{2} D_{KL}(P||M) + \frac{1}{2} D_{KL}(Q||M)}$$
$$\textit{Where } \boldsymbol{M = \frac{1}{2}(P + Q)}.$$

## Results and discussion

A total of 77 odour-active compounds was detected in the samples although 10 of them could not be identified (Table 1). The 67 identified compounds include 11 alcohols, 10 aldehydes, 10 acids, 8 ketones, 7 sulphur compounds, 4 esters, 4 pyrazines, 4 lactones,

3 hydrocarbons, 2 pyrrolines, 2 furans and 2 nitrogen compounds. Not all of the flavour active compounds were present in all the samples and for those present in all the samples, the FD values were different in many cases. From the FD factors it was not obvious if samples were statistically different to each other (Figure 1).

**Table 1**: Volatile compounds in the pet food samples and their odour description.

| Compound/chemical class | Odour descriptor | LRI FFAP | Compound/chemical class | Odour descriptor | LRI FFAP |
|---|---|---|---|---|---|
| *Ketones* | | | *Alcohols* | | |
| 2,3-butanedione | butter | 967 | linalool | floral 1 | 1529 |
| 3-mercapto-2-butanone | catty, blackcurrant | 1267 | geraniol | rose | 1839 |
| 1-octen-3-one | mushroom | 1294 | 2-methoxyphenol | smoky | 1857 |
| 3-mercapto-2-pentanone | catty | 1356 | 2-phenylethanol | honey 1 | 1900 |
| (Z)-1,5-octadien-3-one | geranium | 1367 | maltol | caramel 2 | 1957 |
| 3-methyl-2,4-nonandione | minty 2 | 1706 | 4-ethyl-2-methoxyphenol | clove 1 | 2014 |
| β-damascenone | apple | 1807 | 4-methylphenol | barnyard | 2083 |
| β-ionone | violet | 1920 | eugenol | clove 2 | 2162 |
| *Aldehydes* | | | 3-/4-ethylphenol | leather | 2169 |
| 2-/3-methylbutanal | malty | 911 | 2,6-dimethoxyphenol | smoky, clove | 2258 |
| hexanal | grassy | 1077 | isoeugenol | clove 3 | 2333 |
| (Z)-4-heptenal | fishy | 1233 | *Pyrrolines* | | |
| octanal | citrus | 1289 | 2-acetyl-1-pyrroline | roasty 1 | 1328 |
| (E,Z)-2,6-nonadienal | cucumber | 1582 | 2-propionyl-1-pyrroline | roasty 2 | 1406 |
| phenylacetaldehyde | floral 2 | 1625 | *Terpenes and hydrocarbons* | | |
| (E,E)-2,4-nonadienal | fatty 1 | 1688 | á-pinene | resinous | 1007 |
| (E,E)-2,4-decadienal | fatty 2 | 1800 | (E,Z)-1,3,5-undecatriene | pineapple | 1378 |
| (E,E,Z)-2,4-6-nonatrienal | oatflakes 1 | 1860 | vanillin | vanilla | 2560 |
| tr.-4,5-epoxy-(E)-2-decenal | metallic | 1986 | *Esters* | | |
| *Acids* | | | ethyl-2-methylbutanoate | fruity 1 | 1038 |
| acetic acid | vinegar | 1433 | methylhexanoate | fruity 2 | 1174 |
| propanoic acid | cheese 1 | 1511 | ethyl-3-phenylpropanoate | cinnamon 1 | 1867 |
| 2-methylpropanoic acid | cheese 2 | 1553 | ethylcinnamate | cinnamon 2 | 2113 |
| butanoic acid | cheese 3 | 1606 | *Nitrogen compounds* | | |
| 2-/3-methylbutanoic acid | cheese 4 | 1656 | indol | mothballs 1 | 2440 |
| pentanoic acid | cheese 5 | 1724 | 3-methylindol | mothballs 2 | 2480 |
| 3-/4-methylpentanoic acid | cheese 6 | 1781 | *Terpenes and hydrocarbons* | | |
| hexanoic acid | goat 1 | 1833 | á-pinene | resinous | 1007 |
| phenylacetic acid | honey 2 | 2530 | (E,Z)-1,3,5-undecatriene | pineapple | 1378 |
| phenylpropionic acid | goat 2 | >2600 | vanillin | vanilla | 2560 |
| *Sulfur compounds* | | | *Lactones* | | |
| 3-methyl-2-buten-1-thiol | beer | 1107 | γ-octalactone | coconut | 1906 |
| dimethyltrisulfide | cabbage 1 | 1370 | sotolon | seasoning 1 | 2185 |
| 2-fufurylthiol | burnt | 1418 | δ-dodecalactone | peach | 2383 |
| methional | cooked potato | 1444 | 3-hydroxy-2(2H)-pyranone | meaty | 1953 |
| benzenemethanthiol | cress, burnt | 1616 | *Unknowns* | | |
| dimethyltetrasulfide | cabbage 2 | 1713 | unknown 1 | sulphurous | 1150 |
| 2-acetyl-2-thiazolin | roasty 3 | 1744 | unknown 2 | caramel 1 | 1415 |
| *Pyrazines* | | | unknown 3 | minty 1 | 1555 |
| 2,3,5-trimethylpyrazine | earthy 1 | 1400 | unknown 4 | catty, rhubarb | 1933 |
| 2-ethyl-3,5-dimethylpyrazine | earthy 2 | 1450 | unknown 5 | oatflakes 2 | 1975 |
| 2,3-diethyl-5-methylpyrazine | earthy 3 | 1478 | unknown 6 | sour | 2029 |
| 2-vinyl-3,5-dimethylpyrazine | earthy 4 | 1542 | unknown 7 | minty 3 | 2079 |
| *Furans* | | | unknown 8 | fatty 3 | 2150 |
| furaneol | caramel 3 | 2017 | unknown 9 | foxy | 2208 |
| abhexone | seasoning 2 | 2246 | unknown 10 | chemical | 2300 |

LDA was used to reduce the dimensions by clustering the odour descriptors into “aroma topics”. The 77 odour-active compounds were narrowed down to 3 aroma topics, each aroma topic being a distribution of odour-active compounds as shown in Figure 2. Aroma topic 1 was mainly defined by compounds having sweet, roasted notes, Aroma Topic 2 by spicy, fruity floral notes and Aroma Topic 3 by stable, fatty and cheese notes.

In Figure 3, the aroma topics per sample are shown. As it can be seen the aroma topic 1 was common to all the samples. It could be argued that it contains the basic flavour active compounds for pet foods. The presence of aroma topics 2 and 3 varied among the samples contributing to the specific notes. It was observed that products 2, 4 and 6 had similar flavour active profiles, as well as products 7 and 8.

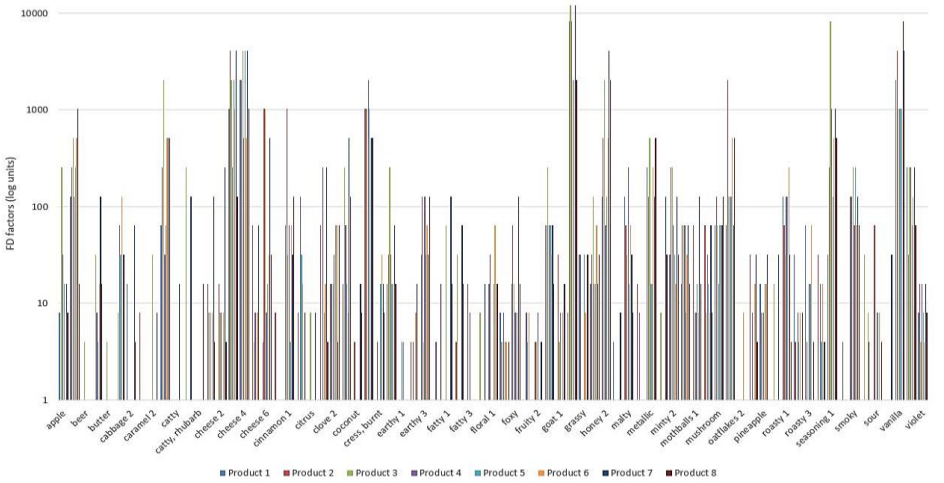


**Figure 1**: FD factors for the 8 samples analysed and the corresponding descriptors identified for each of the flavour-active compounds.

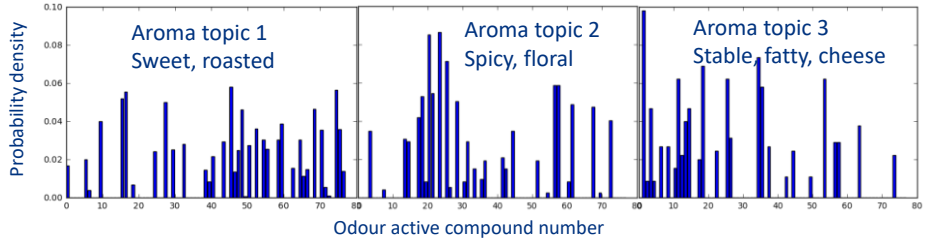


**Figure 2**: Aroma topics obtained by LDA. Bars represent the distribution of each odour-active compound.

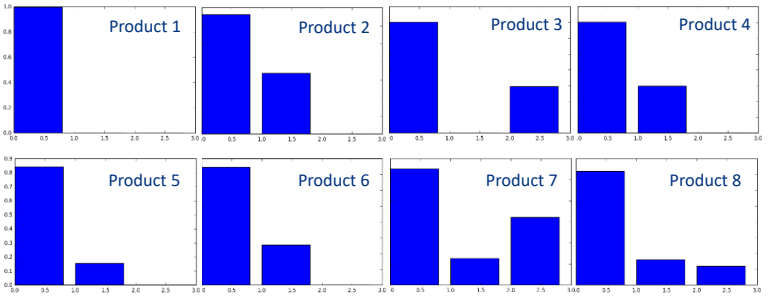


**Figure 3**: Aroma topics 1, 2 and 3 in the samples (Left, centre and right columns respectively).

The developed method was successfully applied to pet food and could be a useful tool for the food and flavour industry to select raw materials with similar aroma profiles. The correlation between this method and the traditional quantification of compounds could be explored in the future.

## References

1. Zellner B., Dugo P., Dugo G., Mondello L. (2008). J.Chromatogr. A, 1186, 123–143.
2. Chin S., Marriott P. J. (2015). Anal. Chim. Acta 854 1–12.
3. Blei D.M., Ng A.Y., Jordan M. I. (2003). Latent dirichlet allocation. J. Mach. Learn. Res. 3, 993-1022.
4. Lin J. (1991). Divergence measures based on the Shannon entropy. IEEE Trans. Inf. theory 37, 1, 145-151.

# Searching for naturally generated volatiles from *Tuber Melanosporum* as authenticity markers for black truffle infused vegetable oils

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## Abstract

The secret of the great popularity of truffles and its derivatives resides mainly in its volatile aromatic fraction, which contributes to their unique aroma. Some culinary preparations are made with this fungus, such as truffle-infused oils. The adulteration of these products must be controlled and prevented due to the high economic cost of natural black truffles.

In this preliminary work the volatile composition of truffles and some commercial truffle-infused oil samples were determined by headspace solid-phase micro-extraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) in order to confirm the authenticity of the infused oils. Complementary, a descriptive sensory analysis was also performed with the same purpose.

Principal component analysis (PCA) was applied to the data obtained and different groups were established according to the sensory profiles and the variation among samples.

## Introduction

Truffles are hypogenous fungi that live in symbiosis with the roots of several host trees. These fungi are widely appreciated for their organoleptic properties. As it is well-known, the culinary and commercial value of truffles is mainly due to their sensorial properties such as their aroma [1, 2] the quality of which clearly provides the economic value of this edible fungus.

The aim of this preliminary study was to characterize sensory and analytically the organic volatile compound composition of commercial truffle oils by headspace-solid phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) coming from the truffles and not from the oil in order to characterize its natural origin or to detect the presence of added flavourings.

## Experimental

### *Materials*

Two fresh *Tuber melanosporum* black truffle samples in its optimum maturation level purchased in a local market were analysed as a reference for the generic volatile compound profile. Six samples from different geographical origins of commercially available vegetable oils infused and/or aromatized with truffle were evaluated. Samples

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were purchased in local (Spain: S1, S2, S3) and foreign markets (France: F1, Italy: I1, I2) August- November 2013. All samples were labelled as artificially aromatized except S3. S1 and S2 were a mixture of naturally infused and aromatized oil. One more sample (ES) was studied: a truffle-infused oil sample prepared in our laboratory with 5 g of minced *Tuber melanosporum* in 250 ml of olive oil in order to have a positive authenticity control sample. The infusion was made at ambient temperature and darkness during 2 weeks. The oil infused samples were stored at 4ºC and the analysis was done maximum 24-48h after each bottle was opened.

*Sensory evaluation*

A group of thirteen trained panellists (7 women and 6 men, between 25 and 65 years old) participated in the evaluation of the aroma of truffle oils. First, the descriptors or sensory terms for describing the odour sensations perceived from truffle were established. After that, the panellists were trained in the identification of descriptors and the use of continuous scales for evaluating the intensity of each descriptor. Finally, the trained panellists evaluated the seven truffle oil samples and the aroma profile of each sample was obtained. For each sample, panellists scored the perceived intensity in duplicate using an unstructured 10 cm line with anchors "weak" and "strong". Panel performance was studied using Panelcheck software. For each sensory attribute, a 2-way ANOVA (sample and panellist) with interaction was applied to the data obtained. To study the significance of the sample effect a mixed model ANOVA, considering panellists as random factor and the sample as fixed factor was performed for each attribute. In order to study the sensory differences among samples, taking into account all sensory attributes, a principal component analysis (PCA) was performed. A one-factor analysis of variance (ANOVA) was used in order to study differences between samples on aroma compounds of truffle-infused oil samples. Significance of differences among means was established using Tukey's Test ($\alpha \leq 0.05$). Principal component analysis was used to evaluate relationships among selected aroma volatile compounds obtained by GC-MS data and samples. A one-factor analysis of variance (ANOVA) was used to study differences between samples on aroma sensory attributes. Principal component analysis was used to evaluate relationships among selected aroma attributes and samples. Partial Least Squares Regression (PLSR) was applied to model the relation among the variance of sensory attributes among samples (Y variables) and the variance in volatile compounds obtained by GC (X variables). All the analysis were carried out with XLSTAT Pro software version 2013 (Addinsoft, France).

*Analysis of the volatile compounds*

Extraction of organic volatile compounds was performed with static headspace solid phase microextraction (HS-SPME) using 2 g of sample. At least two replicates of each sample were prepared and analysed and the final results are the average of all samples analysed. For fresh *Tuber Melanosporum* black truffle samples two fibres from Supelco were used: 50/30 µm DVB-CAR-PDMS and 100 µm PDMS Truffle samples were sliced, incubated for 5 min at 50ºC and extracted for 10 min at the same temperature. For truffle-infused oil samples only the triple phase fibre was used due to its better results, obtained in previous studies. A direct 30 min extraction of 2 g sample at 50ºC was made to avoid oil oxidation. After extraction, the volatiles were thermally desorbed for 10 minutes at 250ºC in splitless mode. Volatiles were separated on two different columns: a polar column and an apolar one. Detection was carried out in a single quadrupole mass spectrometer.

## Results and discussion

107 volatile components were identified in fresh black truffles. Only 43 of those components were also found in an olive oil sample infused with the same type of truffles in our laboratory. From those 43 products some alcohols like ethanol, isobutanol, 2-methyl-1-butanol, 2-butanol, 2-pentanol, 2,3-butanediol, 2-methylthioethanol and 3-methylthiopropanol, were higher in the authentic infused sample than in the flavoured samples. The high quantity of ethanol found could be due to truffle fermentation processes in the oil at ambient temperature and in this case it cannot be considered a true marker. On the other hand, 1-octen-3-ol, a typical mushroom component and 2,4-dithiapentane, a typical white truffle component, were only present in trace quantities in our analysis made to fresh black truffles. Both have been found in huge quantities in flavoured samples.

Looking to the sensory analysis a total of eleven odour attributes were found to be useful for describing the odour of truffle oils: fungus, fermented, cockle, moist soil, rancid, hazelnut, faecal, boiled cabbage, garlicky gas, potato and carob. Fifteen panellists initially evaluated the intensity of the odour attributes of the seven oil samples. Data from two panellists that showed low concordance with the rest of panel were not considered in further analysis. The results of a mixed model ANOVA showed significant differences ($\alpha$= 0.05) among samples for all attributes, even in those (faecal, garlicky gas and potato) for which the effect of panellists' x sample interaction had been found significant. The mean values of the perceived intensity for each attribute in the oil samples were obtained and the sensory profile of each sample is presented in spider web plots (Figure 4)

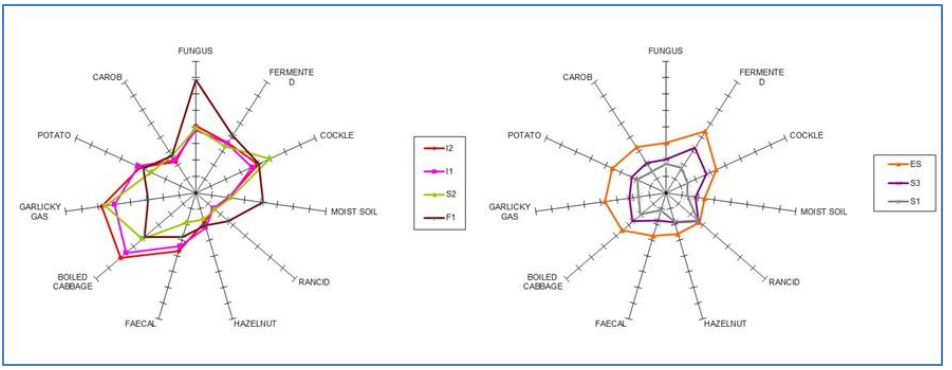


**Figure 1:** Mean values of the intensity perceived for each attribute in the oil samples

For samples infused with truffle the odour intensity was low (S1 and S3) or high (ES) but it was equilibrated among the different attributes. However, oil samples aromatized with truffle flavours presented high intensity of only certain attributes, such a: fungus, cockle, garlicky gas and boiled cabbage.

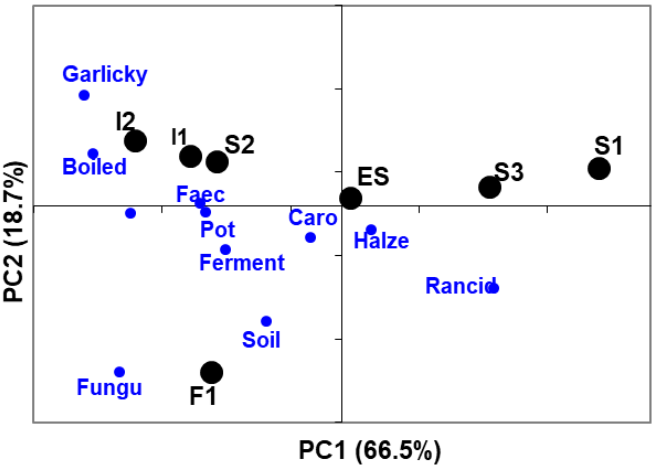


**Figure 2:** Sensory attributes PCA Analysis of truffle infused oils

The PCA (principal component analysis) of the data (Figure 5) showed that the first two dimensions accounted for 85.2% of the variability in the odour of truffle oil. The first dimension clearly separated on the right side the oil samples infused naturally with truffle and on the left side the oil samples aromatized with truffle flavourings. The second dimension separated the aromatized oil samples S2, I1 and I2 (upper side) with more intense garlicky gas and boiled cabbage odours and aromatized oil sample F1 (bottom side) with more intense fungus and soil odours.

## Conclusions

Analytical and sensory differences were clearly seen between oil samples naturally infused with *Tuber Melanosporum* fresh black truffles and artificially aromatized oil samples. Some volatile components frequently present in other mushroom flavours but not present in black truffles were found in aromatized samples. Some alcohols present in the flavour of fresh black truffles and also present in naturally infused oils were found in higher quantities in those samples than in artificially flavoured oil samples.

## References

1. T. Talou, Flavour and Fragrance Journal, 4 (1989 )109.
2. G. Mauriello, J. Chromatogr. Sci. 42 (2004) 299.
3. A.M. Gioacchini, Rapid Communications in Mass Spectrometry, 19(2005)2365.
4. R.E. March, Int. J. Mass Spectrometry, 249 (2006) 60.
5. Mello, C. Murat, P. Bonfante, 2006, FEMS Microbiology Letters, 260 (2006) 1
6. R. Splivallo, S. Bossi, M. Maffei, P. Bonfante, Phytochemistry, 68(20) 2007 2584
7. P. Díaz, LWT- Food Science and Technology, 42 (2009) 1253.
8. L. Culleré, Food Chemistry,122 (2010) 300.
9. M. Kiss, Journal of Applied Botany and Food Quality, 84 (2011) 102.
10. R. Splivallo, New Phytologist, 189 (2011) 688.
11. G. Pacioni, Food Chemistry, 146, (2014) 30.

# Odour qualities and odour thresholds of halogenated, alkylated, alkenylated and methoxylated guaiacol-derived odorants

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## Abstract

In the present study, we compare the odour qualities and odour thresholds of guaiacols with different structural moieties with special focus on the impact of halogenation on their sensory properties. Thereby, a series of substances, which were not commercially available, was synthesized. All compounds were systematically analysed regarding their retention indices, odour qualities and odour thresholds.

Odour qualities of alkylated, alkenylated and methoxylated guaiacols were mainly smoky, clove-like and vanilla-like. Halogenated derivatives also exhibited smoky, sweet and vanilla-like odours, but also medicinal and plaster-like smells. Odour thresholds in air were very low, namely between 0.00018 and 111 ng/L for all compounds. Huge inter-individual differences were found for odour thresholds, whereas the perceived odour qualities were quite comparable between different individuals.

The analytical and sensory data library created in this study will aid future analytical discovery of this interesting substance class. Parts of this work are also published in [1] and [2].

## Introduction

Guaiacol-derived odorants are commonly found in nature. Guaiacols are produced by various plants as well as by animals, and widely used in food and perfume industry. They are employed inter alia as antiseptic and anesthetic agents [3, 4]. Guaiacol derivatives have been found in smoked foods like smoked ham [5], in wheat beers [6] and brandy amongst a row of other foods. Halogenated guaiacols are, however, up to now mainly found in nature due to human intervention. Halogenated guaiacols are for example present in waste water of pulp mills and therefore responsible for some off-odours in fish [7, 8]. However, comprehensive data on sensory characteristics of guaiacol derivatives and the impact of halogenation have not been reported until now.

## Experimental

*Gas chromatography*

GC-FID and GC-olfactometry (GC-O) were carried out with a Trace CT Ultra using a DB-5 and FFAP capillary. Helium at a flow rate of 2.5 mL/min was used as carrier gas. Samples were injected at 40 °C, 40 °C was kept for 2 minutes, then the oven temperature was raised at 10 °C/min to 200 °C or at 6 °C/min to 250 °C, then raised at 20 °C/min or 40 °C/min to 300 °C (DB-5), or at 8 °C/min to 240 °C (FFAP), respectively, and held for 5 or 10 minutes. GC-MS analyses were performed with an Agilent MSD 5975C using the same temperature programs and types of capillaries as described above. Mass spectra

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were generated in the electron impact mode (EI) at 70 eV. Retention indices were determined according to the method of Van den Dool and Kratz [9].

*Odour thresholds and odour qualities*

Panellists were trained assessors form the University of Erlangen. Odour thresholds in air were determined according to the method described by Ulrich & Grosch [10] using *(E)*-2-decenal as internal standard. 2 µL were injected of every dilution. Odour thresholds were determined by 8 assessors for all compounds. Odour qualities were determined during GC-O and panellists were asked to freely choose odour quality descriptors.

*Syntheses*

Compounds, which were not commercially available, were synthesized according to the literature procedures named in [1] and [2].

## Results and discussion

Figure 1 gives an overview of odour thresholds of all investigated compounds.

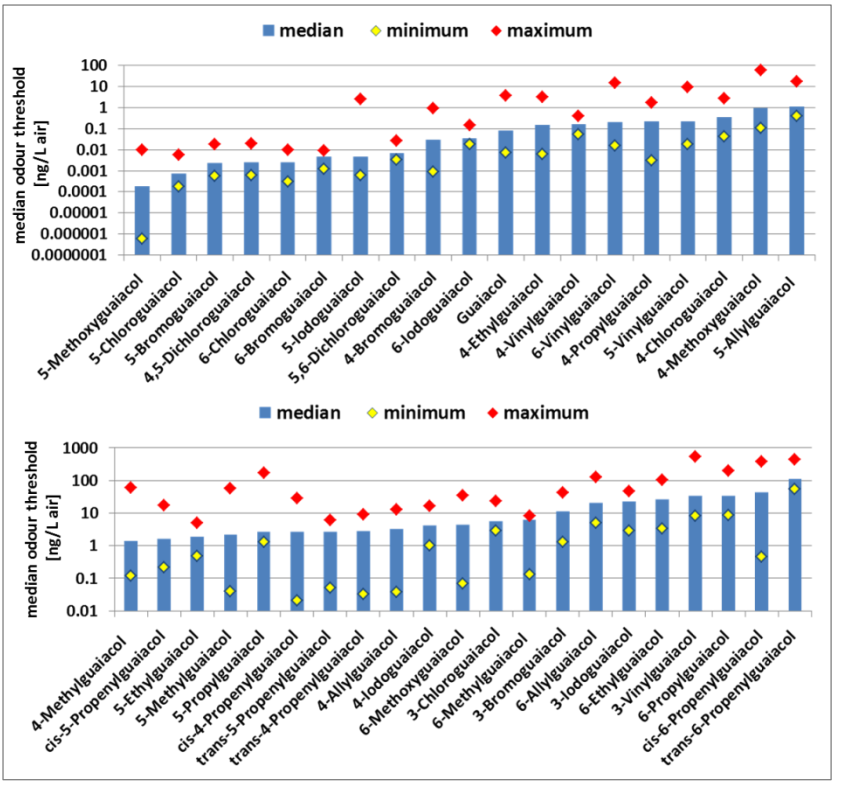


**Figure 1:** Odour thresholds of guaiacol derivatives

With the exception of 5-methoxyguaiacol, all compounds with lower odour thresholds than guaiacol itself were halogenated derivatives. The compound with the lowest odour threshold was 5-methoxyguaiacol with a median odour threshold about 500-times lower than that of guaiacol, namely 0.00018 ng/L. Halogenated compounds with an odour threshold lower than guaiacol were some chloro-, bromo-, and iodoguaiacols with the halogen in positon 4, 5, or 6. Additionally, two dichloroguaiacols were tested. Both had a lower odour threshold than guaiacol. Compounds with odour thresholds higher than

guaiacol were alkylated guaiacols as well as alkenylated guaiacols. Additionally, some halogenated compounds also exhibited odour thresholds higher than guaiacol. These were derivatives with halogens in position 3 or 4. The compounds with the highest odour thresholds of the investigated substances were cis- and trans- 6-propenylguaiacol with thresholds of 44 and 111 ng/L air, respectively. Of the halogenated compounds, the substances with halogens in position 3 showed the highest odour thresholds. These were 3-chloro-, 3-bromo- and 3-iodoguaiacol.

Inter-individual differences in odour thresholds were quite pronounced. The most prominent inter-individual differences were found for 5-methoxyguaiacol with a factor of about 17000 between the lowest and the highest individual odour threshold. Other compounds with high inter-individual differences in odour thresholds were 5-iodo-, 5-methyl-, cis-4-propenyl-, and 4-bromoguaiacol, all with factors over 1000 between highest and lowest individual odour thresholds. On the other hand, there were also compounds with small inter-individual variations, like 6-iodo-, 3-chloro-, 4,5-dichloro-, 4-vinyl-, 6-bromo-, and trans-6-propenylguaiacol, all with a factor of 8 between highest and lowest individual threshold.

Odour qualities were mainly smoky, clove-like and vanilla-like for alkylated, alkenylated and methoxylated guaiacols. Halogenated guaiacols also exhibited smoky, sweet and vanilla-like odour qualities. However, none of the halogenated compounds exhibited a clove-like odour. Conversely, some of the halogenated derivatives also showed medicinal and patch-like smells. Table 1 provides an overview of the odour qualities of the investigated compounds. The most frequently named attributes were smoky, vanilla-like and sweet. Several substances also exhibited a ham-like odour, but only one of them was a halogenated substance, namely 5-chloroguaiacol. All in all, odour impressions were quite consistent between individuals. Additionally, Table 1 shows odour thresholds in [pmol/$L_{air}$]. By giving odour thresholds in [pmol/$L_{air}$] (cf. Table 1) in addition to the values in [ng/L] (cf. Figure 1), one can also see the impact of the molecular weight on the odour threshold values.

These results form a basis for future analytical discovery of this substance class.

**Table 1:** Odour qualities and odour thresholds (OT) of all investigated guaiacol derivatives

| Odourant[a,b] | *Odour qualities* | *OT [pmol/$L_{air}$] range*[c] |
|---|---|---|
| 5-Methoxyguaiacol | sweet, clove, vanilla | 0.000004 - 0.065 |
| 5-Chloroguaiacol | smoked, smoky, ham-like | 0.0011 - 0.037 |
| 6-Chloroguaiacol | smoky, sweet | 0.0020 - 0.063 |
| 5-Iodoguaiacol | sweet, smoked | 0.0024 - 10 |
| 5-Bromoguaiacol | smoky, sweet | 0.0028 - 0.089 |
| 4,5-Dichloroguaiacol | smoky, sweet, vanilla-like | 0.0032 - 0.10 |
| 4-Bromoguaiacol | vanilla-like, sweet, smoky | 0.0045 - 4.6 |
| 6-Bromoguaiacol | medical, smoky, patch-, plastic-like | 0.0059 - 0.045 |
| 5,6-Dichloroguaiacol | smoky, medical, patch-like | 0.018 - 0.14 |
| 4-Propylguaiacol | smoky, clove, sweet | 0.018 - 10 |
| 4-Ethylguaiacol | clove, smoky | 0.039 - 21 |
| Guaiacol | smoky, vanilla, ham | 0.056 - 30 |
| 6-Iodoguaiacol | medical | 0.072 - 0.60 |
| 6-Vinylguaiacol | smoky, ham | 0.11 - 100 |

**Table 1.** continued

| Odourant[a,b] | *Odour qualities* | *OT [pmol/$L_{air}$] range*[c] |
|---|---|---|
| 5-Vinylguaiacol | smoky, ham, clove, sweet, vanilla | 0.12 - 63 |
| cis-4-Propenylguaiacol | clove | 0.13 - 177 |
| trans-4-Propenylguaiacol | clove | 0.20 - 55 |
| 4-Allylguaiacol | clove | 0.23 - 79 |
| 4-Chloroguaiacol | sweet, vanilla-like | 0.27 - 18 |
| 5-Methylguaiacol | vanilla, sweet, smoky | 0.29 - 413 |
| trans-5-Propenylguaiacol | vanilla, sweet | 0.30 - 38 |
| 4-Vinylguaiacol | clove, smoky | 0.35 - 2.7 |
| 6-Methoxyguaiacol | smoky, sweet | 0.45 - 227 |
| 4-Methoxyguaiacol | clove, sweet, smoky, vanilla, ham | 0.71 - 383 |
| 4-Methylguaiacol | vanilla, sweet, ham, smoky | 0.87 - 441 |
| 6-Methylguaiacol | smoky, plastic, sweet, bacon | 0.94 - 60 |
| cis-5-Propenylguaiacol | smoky, clove, ham | 1.3 - 104 |
| 5-Allylguaiacol | smoky, ham, clove, sweet | 2.4 - 104 |
| cis-6-Propenylguaiacol | smoky, ethereal, clove | 2.8 - 2259 |
| 5-Ethylguaiacol | smoky, sweet, ham | 3.1 - 33 |
| 4-Iodoguaiacol | vanilla-like, smoky, sweet | 4.0 - 64 |
| 3-Bromoguaiacol | musty, old | 6.4 - 212 |
| 5-Propylguaiacol | clove, vanilla | 7.8 - 999 |
| 3-Iodoguaiacol | musty, moldy | 12 - 184 |
| 3-Chloroguaiacol | smoky, medical | 18 - 145 |
| 6-Ethylguaiacol | smoky | 22 - 683 |
| 6-Allylguaiacol | plastic, clove, smoky | 30 - 773 |
| 6-Propylguaiacol | plastic, sweet | 51 - 1203 |
| 3-Vinylguaiacol | smoky, clove | 55 - 3509 |
| trans-6-Propenylguaiacol | ham, smoky | 335 - 2698 |

[a] Odorants are displayed in the order of their minimum odour threshold.
[b] Retention indices of all compounds on DB-5 as well as on FFAP can be found in [1, 2].
[c] Odour thresholds were established according to the method described by Ullrich & Grosch [10].

## References

1. Schranz, Lorber, Klos, Kerschbaumer, Buettner (2017). Food Chem., 232, 808-819.
2. Juhlke, Lorber, Wagenstaller, Buettner, (2017), Front. Chem., 5 (120), doi: 10.3389/fchem.2017.00120.
3. Hunnuis (1997). Hunnius pharmazeutisches Wörterbuch: De Gruyter.
4. Jadhav, Khandelwal, Ketkar, Pisal (2004). Drug Dev. Ind. Pharm., 30(2), 195-203.
5. Baloga, Reineccius, Miller (1990). J. Agr. Food Chem., 38(11), 2021-2026.
6. Langos, Schieberle (2013). J. Agr. Food Chem., 61 (47), 11303–11311.
7. Couillard, Nellis (1999). Environ. Toxicol. Chem., 18(11), 2545-2556.
8. Owens, Swanson, Birkholz (1994). Chemosphere, 29(1), 89-109.
9. Van den Dool, Kratz (1963). J. Chromatogr. A, 11, 463-471.
10. Ullrich, Grosch (1987), Z. Lebensm. Unters. For., 184(4), 277-282.

# Characterisation of the key aroma compounds in alcohol-free beer base by gas chromatography-olfactometry

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## Abstract

The pleasant fruity flavour of lager beers is one of the most appreciated features of these beverages, whereas alcohol-free beers (AFB) also exhibit a flavour reminiscent of wort. Even though several studies have been carried out to characterise the key odorants in different alcoholic beers, there are no similar works for AFB. Hence, the aim of this research is to identify the compounds contributing to the characteristic aroma of AFB. In this work, the volatile fraction of an AFB-base (without added flavourings) was isolated using solvent assisted flavour evaporation (SAFE) and analysed by GC-MS and GC-Olfactometry. Twenty-three odour regions showed odour activity in GC-O experiments, amongst which the most potent were methional, phenylacetaldehyde, 2-methoxyphenol, β-damascenone, 2-phenylacetic acid, 2-phenylethanol, and 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone. The presence of these compounds plays a crucial role in AFB aroma.

## Introduction

AFB consumption has increased over the last few years, mainly in response to strict drink driving legislation, medical recommendation or religious grounds, but also due to a growth in health awareness. According to current UK legislation, the description "alcohol-free" may be applied to products containing "an alcoholic strength by volume of not more than 0.05 per cent".

These beers usually exhibit a flavour reminiscent of wort. Recent literature shows that Strecker aldehydes, particularly 2-methylbutanal, 3-methylbutanal and methional, are responsible for the negative attributes associated with AFB flavour [1], and these compounds are also present in barley malt [2]. These aldehydes have exceptionally low odour thresholds (1.25 μg/L, 0.6 μg/L and 0.25 μg/L for 2-methylbutanal, 3-methyl-butanal and methional, respectively [3]) and impart potent worty, malty aromas even at very low concentrations.

Although worty aroma of AFB has been related to Strecker aldehydes [1], there is no information in literature about the possible contribution of other odour-active compounds to the overall aroma of AFB. Sensomic methodology has been employed to identify the key odorants in different beers, such as pale lager [4] and wheat beers [5]. In the latter example, the authors found more than 30 odorants contributing to the characteristic aroma of wheat beer, Strecker aldehydes being amongst them. The aim of this study was to identify a more complete set of odour-active volatile compounds present in AFB by means of Sensomic methodology.

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## Experimental

*Materials*

An alcohol-free beer-base (AFB-base), without any external flavour added, was brewed, bottled and pasteurised in Heineken's pilot brewery (Zoeterwoude, The Netherlands) in January 2016 following a standard cold-contact fermentation procedure (brewing conditions not specified). Diethyl ether and saturated alkane standards were purchased from Sigma (Dorset, UK).

*Isolation of the volatile fraction*

For the isolation of volatiles from the AFB-base, the procedure described by Langos et al. was employed with slight modifications [5]. Briefly, 1 kg of sample was extracted with redistilled diethyl ether (250 mL × 4). The organic phase was dried over anhydrous $Na_2SO_4$ and filtered before concentration using a Vigreux distillation column (60 cm, 1 cm i.d.) at 40 °C until a final volume of approximately 100 mL was reached. To separate the non-volatile materials from the extract, this was submitted to a high-vacuum distillation process known as solvent assisted flavour evaporation (SAFE) technique (evaporation at 25 °C and $10^{-5}$ Pa). The distillate was fractioned into an acidic and a basic/neutral fraction using $NaHCO_3$ 0.5 M solution (60 mL × 3). After washing with 30 mL of a saturated NaCl solution three times, the organic layer was kept for further treatment (basic organic extract). In parallel, the basic aqueous phase was acidified to pH 2.25±0.10 by adding HCl solution (10 M or 1 M) and extracted using redistilled diethyl ether (60 mL × 3) and the extracts combined (acidic organic extract). Both basic and acidic organic extracts were concentrated using a Kuderna-Danish concentrator at 45 °C (final volume ~400 µL for each extract). The concentrated aroma extracts were kept at -80 °C until use.

*Gas chromatography analyses of concentrated aroma extracts*

In order to identify odour-active compounds in the concentrated aroma extracts, these were analysed by GC-Olfactometry (GC-O) using a 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) provided with an FID detector held at 250 °C. A sample (2 µL) was injected and two capillaries with different polarities were employed: Rxi®-5 Sil MS capillary (30 m, 0.25 mm i.d., 1.0 µm df) non-polar column and a Stabilwax®-DA (30 m, 0.25 mm i.d., 0.25 µm df) polar column, both from Restek (Bellefonte, Pennsylvania, USA). The temperature gradients were set as follows: 40 °C for 2 min, then a rise of 5 °C/min up to 200°C and 15 °C/min from 200 °C to 300 °C, and then held for 19 min for the non-polar column; 40 °C for 2 min, then rise of 4 °C/min up to 200 °C, then from 200 °C up to 250 °C at 15 °C/min, and then held for 15 min for the polar column. Helium was used as a carrier gas (2 mL/min). The sample was split 1:1 at the end of the column, followed by two untreated silica-fused capillaries of the same dimensions (1 m, 0.32 mm i.d.). An ODO II sniffing port (SGE, Ringwood, Victoria, Australia), where the flow was diluted with a moist make up gas, was utilised. Every sample was analysed by at least 3 assessors in duplicate. The assessors scored the intensity of the aromas perceived on a scale from 1 ("very weak") to 10 ("very strong"). These results were reported as the modified frequency, defined as $MF(\%)=[F(\%)\cdot I(\%)]^{1/2}$, where F(%) is the detection frequency and I(%) is the average intensity expressed as the percentage of the maximum intensity [6].

The concentrated aroma extracts were also analysed by GC-MS using equivalent capillaries and chromatographic conditions as used for the GC-O analyses. The instrument employed for these analyses was a gas chromatograph model 7890A coupled

to a 5975C inert XL EI/CI MSD triple axis mass spectroscopy detector and a 7683B Series autosampler (Agilent Technologies, Santa Clara, CA, USA). The carrier gas was helium at a flow rate of 1mL/min. Mass spectra were recorded in the electron-impact mode at an ionisation voltage of 70 eV and source temperature of 200 °C.

## Results and discussion

The sensomic approach was applied for the identification of key odorants in alcohol-free beer. Recently, this methodology has been applied to identify key flavour compounds in a wide variety of foodstuff and beverages, such as hazelnuts [7] and rapeseed oil [8]. For this reason, concentrated aroma extracts (basic and acidic fractions) were prepared from AFB-base using the methodology described previously [5].

**Table 1:** Odour regions and attributed compounds found by GC-Olfactometry (n=3 in duplicate) in acidic and/or basic fractions of a SAFE extract of an alcohol-free beer-base

| *LRI* | | | | | |
|---|---|---|---|---|---|
| *Rxi-5* | *StabW* | *Odour quality*[a] | *Odorant*[b] | *Fn*[c] | *%MF*[d] |
| 579 | 1000 | cream, butter | butanedione | b | 80 |
| 648 | 950 | malty, cocoa | 3-methylbutanal | a | 65 |
| 664 | 1429 | vinegar | acetic acid | a | 76 |
| 680 | 950 | cocoa | 2-methylbutanal | b | 60 |
| 725 | 1225 | banana, alcoholic | 3-methyl-1-butanol | b,a | 44 |
| 845 | 1609 | cheese | butanoic acid | a | 76 |
| 886 | 1646 | cheese, rancid | 3-methylbutanoic acid | a | 83 |
| 917 | 1470 | boiled potato | methional | b,a | 91 |
| 992 | 1354 | cooked rice | 2-acetyl-1-pyrroline[e] | b | 31 |
| 1059 | 1649 | rose, honey | phenylacetaldehyde | b,a | 95 |
| 1103 | 1872 | smoky | 2-methoxyphenol | b,a | 92 |
| 1109 | 2188 | smoky, spicy | 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | a | 56 |
| 1125 | 2074 | candy floss | 5-ethyl-4-hydroxy-2-methyl-3(2*H*)-furanone | a | 48 |
| 1127 | 1930 | rose, honey | 2-phenylethanol | b,a | 86 |
| 1130 | 2223 | cloves, woody | 2-methoxy-4-vinylphenol | b | 67 |
| 1154 | 2223 | curry, spicy | 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone | b,a | 89 |
| 1180 | 1983 | spicy, smoky | 2-methoxy-4-methylphenol | b,a | 55 |
| 1206 | 2380 | leather | 4-vinylphenol | b,a | 68 |
| 1293 | 2540 | honey, floral | 2-phenylacetic acid | a | 87 |
| 1382 | 2022 | honey, rubber | 2'-methoxyacetophenone | b | 73 |
| 1389 | 1835 | apple, apricot | β-damascenone | b | 87 |
| 1400 | - | hospital, phenolic | unknown. | a | 56 |
| 1472 | 2556 | vanilla | vanillin | a | 73 |

[a]Odour perceived at the sniffing port of the GC-O.
[b]Compounds were identified by comparison of their mass spectrum and LRI on two columns with those of authentic standards, and confirmed by detection in the extract by GC-MS
[c]Fraction where the compound was found: basic/neutral (b) or acidic (a).
[d]$MF(\%)=[F(\%)\cdot I(\%)]^{1/2}$, where F(%) is the detection frequency and I(%) is the average intensity expressed as the percentage of the maximum intensity
[e]Tentative identification based on odour description and LRI.

These extracts from the AFB-base were sniffed by GC-O on columns of different polarity and mass spectra were obtained from GC-MS analyses. Twenty-three odour regions were found in total in both basic and acidic fractions from the AFB-base. Table 1 shows the most active odour regions found in the SAFE extracts. Amongst them, the highest MF values corresponded to 2-methoxyphenol, β-damascenone, 5-ethyl-3-hydroxy-4-methyl-2(*5H*)-furanone, 2-phenylacetic acid, 2-phenylethanol and the Strecker aldehydes methional and phenylacetaldehyde. The presence of these compounds might explain the honey-like, worty aroma of alcohol-free beers brewed by cold contact fermentation. Moreover, two Strecker aldehydes were found to be important: 2-methylbutanal and 3-methylbutanal. These two, along with methional, have been previously reported as contributors to malty and worty aromas in alcohol-free beers [1, 2].

Similar work has been carried out in other beers, such as wheat beer [5] and pale lager beer [4], where higher alcohols and esters were found to be main contributors to the overall aroma. Examples of these are ethyl hexanoate, ethyl butanoate, 3-methylbutyl acetate, and 3-methyl-1-butanol. In our case, no fruity esters were detected by GC-O. This was associated with the mild conditions for cold contact fermentation process, where yeast was not active enough to synthesise esters throughout the Ehrlich pathway [9]. Butanedione, also found in this study, has been reported as an off-flavour in lager beers [10].

Note, however, that in this study we used an alcohol-free beer "base" which was prepared without the addition of external flavours which provide the desirable fruity note which is not generated during cold contact fermentation. The addition of external flavours to commercial alcohol-free beers is common practice of brewers to improve the flavour of AFB.

We conclude that the information generated from this study will help in the identification of the less desirable worty notes in alcohol-free beers. Further quantitative and sensory analysis will elucidate the actual role of the key odorants in the overall aroma of these beverages.

## References

1. Perpète P., Collin, S. (1999) J. Agric. Food Chem. 47(6): 2374-2378.
2. Beal A.B., Mottram D.S. (1994) J. Agric. Food Chem. 42(12): 2880-2884.
3. Meilgaard M.C. (1975) Tech. Q. Master Brew. Assoc. Am. 12(3): 151-168.
4. Schieberle P. (1991) Z. Lebensm. Unters. For. 193: 558-565.
5. Langos D., Granvogl M., Schieberle P. (2013) J. Agric. Food Chem. 61: 11303-11311.
6. Stewart A., Grandison A.S., Ryan A., Festring, D., Methven L., Parker J.K. (2017) J. Agric. Food Chem. 65(6): 1186-1195.
7. Kiefl J., Pollner G., Schieberle P. (2013) J. Agric. Food Chem. 61(22): 5226-5235.
8. Matheis K., Granvogl M. (2016) Eur. Food Res. Technol. 242(9): 1565-1575.
9. Pires E.J., Teixeira J.A., Brányik T., Vicente A.A. (2014) Appl. Microbiol. Biotechnol. 98: 1937-1949.
10. Dresel, M. (2017) In: Springer Handbook of Odor (Buettner, A., ed.). Springer International Publishing, pp. 129-142.

# Studies on off-flavours in lamb

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## Abstract

The trend across Europe is towards using rams in production due to welfare reasons, and because they reach slaughter weight faster. Two trials were conducted to determine the role of gender, breed and diet on the incidence and cause of off-flavour in lambs. Lambs were slaughtered and the loin subjected to sensory profiling and analysis of branched chain fatty acids was also conducted.

Results showed effects of breed and diet on flavour, but fewer effects of gender. Close examination of the data indicated that some animals from all treatments had elevated scores for off-flavour. These occurrences were apparent in rams and castrates across both trials. Analysis of branched chain fatty acids showed that for the three compounds studied, there was no clear link between diet or gender and a higher level of branched chain fatty acids.

## Introduction

Across Europe, there is a move to produce lamb meat from entire males rather than castrates for welfare reasons and because they are more efficient [1]. They reach slaughter weight faster and produce a leaner carcass; the value of a lamb's carcass is determined mainly by the yield of lean meat [2]. Some studies found that meat from ram lambs is of inferior quality [2], whilst others conclude there are no differences in sensory quality [3] or that meat from ram lambs was actually of a superior quality than castrates or ewes [4, 5].

Research on off-flavour in sheep meat has identified a number of compounds that may contribute to the characteristic flavour of lamb and/or off-flavour. These include medium-chain branched fatty acids, namely 4-methyloctanoic acid, 4-ethyloctanoic acid and 4-methylnonanoic acid [3, 7], carboxylic acids, aldehydes, ketones, indoles (such as skatole) and sulphur-containing compounds [8]. It was proposed that off-odours and other sensory attributes may be associated with ram lambs as a result of them reaching sexual maturity [5], or that bacteria in the rumen may produce skatole, which may lead to a farmyard, slurry-like taint [6].

It is believed within the industry that there is a potential issue concerning the quality of ram lamb meat over castrated lambs and ewes. However, there appears to be little evidence to support this. Where there are claims that meat quality is lower in rams, it generally refers to colour, texture analysed by Warner Bratzler shear force, and higher ultimate pH, but it is accepted that if differences are apparent, they are small [2]. Lamb meat quality is influenced by breed, slaughter weight and sex according to Teixera et al. [9], and the sensory characteristics of tenderness, juiciness and flavour [10] as well as aroma and taste [11] are most important, as these are what the consumer experiences.

This study compares the sensory evaluation of the meat from rams & castrates, from two breeds, fed on a variety of diets. It also investigates individual incidences of off-odour/off-flavour, as assessed by the trained panellists. Analysis of the branched chain fatty acids (BCFA) is also reported.

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## Experimental

*Materials*

Trial A (144 lambs), and Trial B (132 lambs) of two genders (entire male or castrate), two breeds (Suffolk cross (Trial A), Suffolk-Texel cross (Trial B), and Blackface-Swaledale) were reared on six diet housing regimes as follows: **Trial A** – concentrate C, grass silage, clover silage (indoors), grazed grass, rape and stubble turnip (outdoors), **Trial B** – concentrate A, concentrate B, grass silage (indoors), grazed grass, rape and stubble turnip (outdoors). There were 6 animals per treatment for Trial A, and the experimental design included 50 % rams and 50% castrates. Trial B used the same experimental design as Trial A but for the indoor lambs there was just one breed with 11 animals per treatment. Lambs were slaughtered between 8-10 months old (November to January) and the *longissimus dorsi* removed, aged for 9 days at 4$^{o}$C after boning, before being cut into 25 mm steaks, blast frozen and stored for future analyses.

*Sensory evaluation*

The left loin from each animal was subjected to sensory profiling by eight trained assessors using quantitative descriptive analysis to evaluate the samples over an unstructured line scale from 0 - 100. Assessors developed a common vocabulary to describe the characteristics of the fat and lean meat (presented separately) during training and they agreed upon definitions for each descriptor. Sensory evaluation was carried out over 12 sessions according to a latin square design.

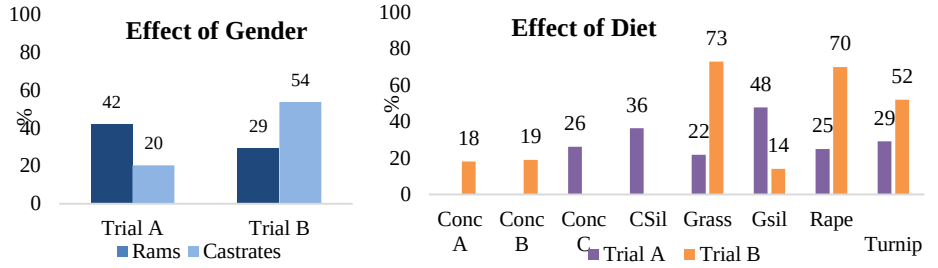
Samples were grilled to an internal temperature of 75$^{o}$C with fat attached. Prior to serving, the fat was removed and served in a ceramic dish with a lid. Panellists assessed the aroma of the fat before assessing the lean meat for a number of attributes including aroma, texture, flavour and aftertaste. The results were statistically analysed using linear mixed methodology, using restricted maximum likelihood (REML) estimation. The incidences of off-flavours and off-odours in individual animals was also assessed by determining samples that were scored highly for particular attributes by assessors. Samples were defined as scoring highly if the score was greater than the mean + 2 x S.D for each individual panellist.

*Branched chain fatty acids*

Branched chain fatty acids were determined in adipose tissue attached according to an adaptation of the method of O'Fallon et al [12]. The limit of detection was 0.6 μg/g using mass spectrometry as the detector.

## Results and discussion

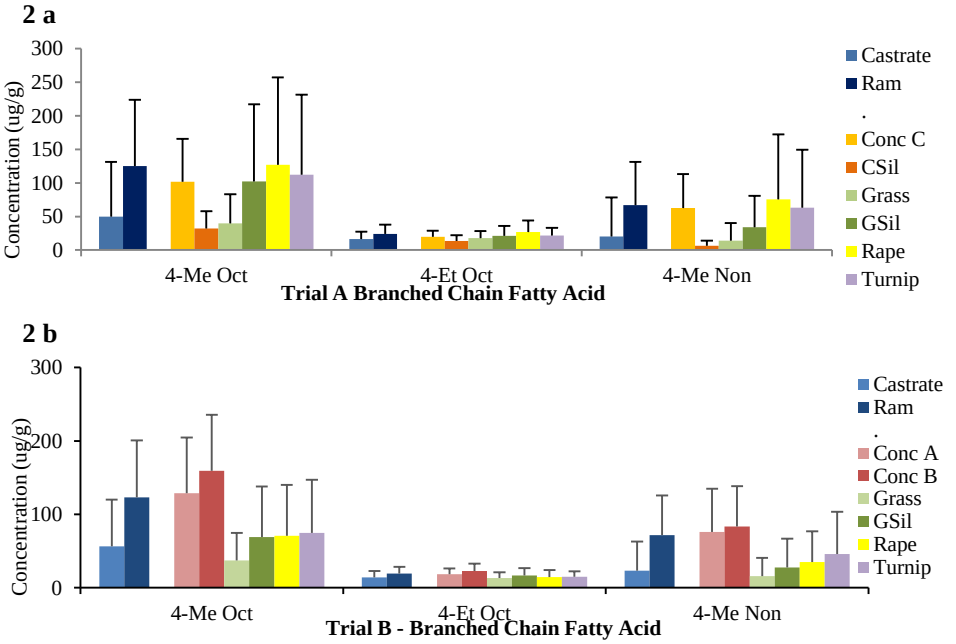
Sensory profiling results (not shown here) did not show significant effects due to gender or breed that would suggest increased off-flavour or off-odour. There were some significant gender x breed interactions but they were small. Therefore, this paper focuses on the occurrence of sporadic incidences of off-flavour and off-odour in individual animals. Sensory evaluation showed that not all panellists were equally sensitive to off-flavours and that they used the scoring system differently. Therefore, detection of off-flavours in meat from an animal was defined as when an individual panellist scored more than two standard deviations above their mean score.




**1 a** **1 b**

**Figures 1 a & 1 b:** Incidences of off-odour and off-flavour exceeding mean + 2*S.D for a minimum of 2 assessors. There were 2 genders (Ram and Castrate) and 8 diets (Conc A (Concentrate A), Conc B (Concentrate B), Conc C (Concentrate), CSil (Clover Silage), Grass, GSil (Grass Silage), Rape and Turnip).

Figure 1a illustrates that in Trial A the incidences of off-flavour and off-odour was higher for rams than castrates but, in Trial B, the opposite was true. Figure 1b shows that each diet had at least 14% of lambs which demonstrated off-odour/off-flavour, rising to more than 70% for some diets in Trial 2. However, there is no consistent effect of diet on sporadic off-flavours between trials, which may indicate a seasonal effect, or that off-flavours are caused by some other factor. Work is ongoing to establish if there is a link with sire line and the farms where the lambs were born.




**Figures 2 a & b:** Branched Chain Fatty Acids from 2 genders; Ram and Castrate, and 6 diets; Conc (Concentrate), CSil (Clover Silage), Grass, GSil (Grass Silage), Rape and Turnip.

Figure 2 shows the mean concentrations of the three main BCFAs of interest. For all three compounds, loin from rams contained higher levels, although the relative difference was smaller for 4-ethyloctanoic acid than the other two. Quantities of 4-methyloctanoic acid were higher overall which is consistent with the findings of Young et al. [3]. These results were in keeping with other work which found that 4-ethyloctanoic

acid is present in much smaller amounts when compared to the more abundant 4-methyloctanoic acid. All BCFAs studied are present in quantities greater than the odour threshold values according to Brennand et al. [13] and Wong et al. [14]. The lambs in this study were slaughtered between late November and early January. It is possible that the BCFAs were highest in the rams due to them reaching sexual maturity by October when they were approximately 30 weeks old [15]. Previous studies indicated that lambs should ideally be slaughtered before October to avoid potential issues with off-flavour in the meat [5]. The incidence of high levels of BCFAs for both trials (Figure 2) did not correspond with high scores for off-flavours and off-odours (Figure 1). This suggests that, although BCFAs are detectable at above the threshold, they do not have a consistent effect on sensory quality. Therefore, there may be another cause of the sporadic off-odours/flavours in these lambs and research is ongoing.

## Conclusion

There is no evidence from this study of any consistent off-flavour problem with ram lamb meat, and only small differences between meat from ram lambs and castrates. Therefore, there appears to be little argument from this data for castrating rams to improve meat quality. Incidences of off-odour/off-flavour occur in individual lambs, both rams and castrates and the influence of diet is variable. The evidence suggests that BCFAs alone are not responsible and other compounds may contribute.

## References

1. Seideman, S. C., Cross, H. R., Oltjen, R. R., and Schanbacher, B. D. (1982). Journal of Animal Science 55: 826-840.
2. Johnson, P. L., Purchas, R. W., McEwan, J. C., and Blair, H. T. (2005). Meat Science 71: 383-391.
3. Young, O. A., Lane, G. A., Podmore, C., Fraser, K., Agnew, M. J., Cummings, T. L., and Cox, N. R. (2006). New Zealand Journal of Agricultural Research 49: 419-430.
4. Dransfield, E., Nute, G. R., Hogg, B. W., and Walters, B. R. (1990) Animal Science 50: 291-299.
5. Mushi, D. E., Eik, L. O., and Sørheim, Ådnøy, T. (2008). Acta Agriculturae Scandinavica, Section A – Animal Science 58: 23-30.
6. Schreurs, N. M. (2013). New Zealand Society of Animal Production 73: 68-70.
7. Young, O. A, Berdagué, J-L., Viallon, C., Rousset-Akrim, and S., Theriez, M. (1997). Meat Science 45: 183-200.
8. Mottram, D. S. (1998). Food Chemistry 62: 415-424.
9. Teixera. A., Batista, S., Delfa, R., and Cadavez, V. (2005). Meat Science 71: 530-536.
10. Safari, E., Fogarty, N. M., Ferrier, G. R., Hopkins, L. D., Gilmour, A. (2001). Meat Science 57: 153-159.
11. Resconi, V. C., Escudero, A., and Campo, M. M. (2013) Molecules 18: 6748-6781.
12. O'Fallon, J. V., Busboom, J. R., Nelson, M. L., and Gaskins, C. T. (2007). Journal of Animal Science 85: 1511-1521.
13. Brennand, C. P., and Lindsay, R. C. (1992). Meat Science 31: 411-421.
14. Wong, E., Johnson, C. B., and Nixon, L. N. (1975). New Zealand Journal of Agricultural Research 18: 261-266.
15. Sutherland, M. M., and Ames, J. M. (1996). Journal of Agriculture and Food Chemistry 44: 3113-3116.)

# Key aroma compounds in meat bouillons: Comparison between industrial and traditional preparation processes

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## Abstract

Sensory evaluation of traditionally prepared and industrially manufactured meat bouillons showed a striking difference in their flavour profiles. Notably, the latter were less intense in chicken or beef signature flavours. In order to gain an insight into the molecular basis responsible for these aroma differences, traditionally prepared and commercially available products were screened for aroma compounds by applying comparative aroma extract dilution analysis (cAEDA). In general, traditionally prepared samples showed much higher FD-factors for α,β-unsaturated aldehydes, e.g., (*E*)-2-nonenal and (*E*,*E*)-2,4-decadienal, resulting in boiled, fatty aroma notes, whereas commercial samples revealed high FD-factors for organic acids, leading to sour, sweaty odours.

## Introduction

The increasing consumer demand for organic, natural and authentic culinary products, free from taste enhancers or artificial antioxidants, has led to a surge in "all-natural" meat bouillons in the markets.

Sensory evaluation (Figure 1) of meat bouillons prepared at industrial-scale showed different flavour profiles when compared to bouillons prepared in a traditional manner.

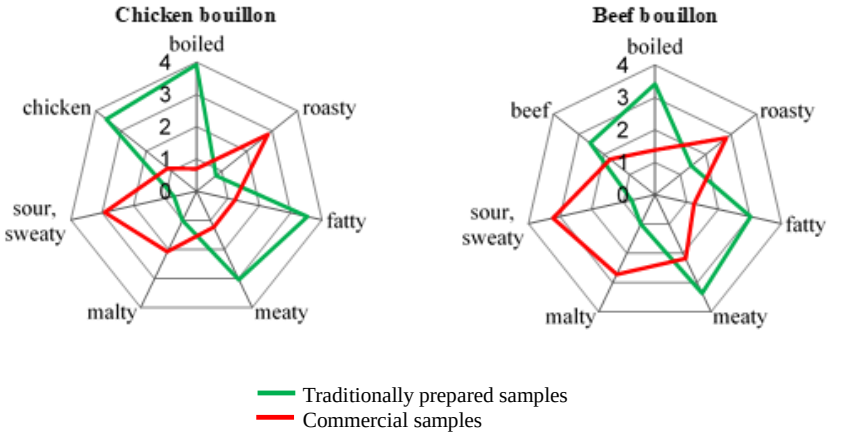


**Figure 1:** Aroma profiles of beef and chicken bouillons. Commercial samples (C) versus traditionally prepared samples (TP). Scale: 0 = aroma not detectable; 4 = strong aroma.

Traditionally prepared chicken and beef bouillons showed much stronger meaty, fatty and boiled aroma notes whereas commercial samples revealed a more sour, malty and roasted aroma. In general, traditionally prepared samples were much more intense in

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chicken or beef signature flavours. These differences might be caused by aroma losses, degradation upon processing or low quality of the raw materials.

While aroma compounds of traditionally prepared chicken [1, 2] and beef bouillons [1, 2, 3] have been well studied, no data were available regarding the difference in aroma compared to commercial products. In order to gain more insight into the molecular composition responsible for this aroma difference, the aim of the present study was to identify the key aroma compounds by applying aroma extract dilution analysis (AEDA).

## Experimental

Commercial (C) meat extracts were prepared according to instructions on the packet. Traditionally prepared (TP) standards were produced by experienced kitchen chefs using beef or chicken meat and water.

Volatile compounds were isolated using high vacuum distillation (SAFE) after liquid extraction (diethyl ether). The distillate was concentrated (200 µl) and odour-active compounds were located by AEDA. Structural identification of aroma compounds was achieved by comparison of their mass spectra (EI), retention indices and odour characteristics with data of reference compounds analysed in parallel. Differences between TP and C samples were evaluated using comparative AEDA.

## Results and discussion

*Aroma-active compounds in traditionally prepared samples*

The results of the identification experiments in combination with the FD factors revealed 2-acetyl-1-pyrroline, 2-furfurylthiol, methional and (*E*)-2-nonenal as important aroma contributors in traditionally prepared boiled chicken and beef (Tables 1 and 2). The highest FD factor in chicken bouillon was found for (*E,E*)-2,4-decadienal (FD 1024), whereas this compound was of minor importance (FD < 4) in beef. For beef, FD-factors for furaneol (sweet, caramel-like aroma) were much higher, whereas for chicken more fatty aroma notes, e.g., (*E*)-2-decenal, (*E,E*)-2,4-nonadienal, were identified. These results are in good accordance with literature data [1, 2, 4]. Interestingly, 2-methyl-3-furanthiol and bis(2-methyl-3-furyl)disulphide were not identified by AEDA in beef or chicken. These sulphur compounds were evaluated as important contributors for beef and chicken aroma by Gasser, 1990 [1], whereas in other studies [4, 5] their influence was rated rather low. In contrast to some literature studies on beef aroma [2, 6], 12-methyltridecanal, which was identified as an important species-specific odorant was not detected in this study.

*Comparison of commercial and traditionally prepared samples*

Comparative AEDA showed significant differences between traditionally prepared and commercial samples, in good accordance with sensory results. Commercial chicken and beef samples presented higher FD factors for organic acids, e.g., acetic acid, butanoic acid, 2-methylbutanoic acid (Tables 1 and 2), resulting in significantly increased sour and sweaty aroma notes (Figure 1).

In contrast, FD factors of α,β-unsaturated aldehydes, e.g., (*E*)-2-octenal, (*E*)-2-nonenal, (*E,E*)-2,4-nonadienal and (E,E)-2,4-decadienal for chicken and (*E*)-2-octenal, (*E*)-2-nonenal and (*E,Z*)-2,6-nonadienal for beef were considerably lower for the commercial samples. The highest differences were found for (*E*)-2-nonenal (FD 256 compared to FD 32 in chicken, FD 128 compared to FD 16 in beef) and (*E,E*)-2,4-decadienal (FD 1024 compared to FD 32 in chicken). These aldehydes are well known to

contribute to the characteristic boiled, fatty aroma of meat and they are responsible for the typified aroma notes, in particular for boiled chicken [1]. Therefore, these aroma qualities were clearly lower in the aroma profiles of the commercial samples (Figure 1). Lower FD factors for pyrazines and 2-acetylthiazole were observed in the traditionally prepared sample. This may be a reason for significantly lower roasted aroma notes, whereas increased FD factors for 2-/3-methylbutanal may be correlated with increased malty odour notes in the commercial samples.

**Table 1**: AEDA of chicken bouillons: traditionally prepared samples (TP) compared to commercial samples (C) (selected results).

| Compound | *Odour quality* | *TP* | *C* |
|---|---|---|---|
| 2-/3-methylbutanal | malty | 8 | 64 |
| 1-octen-3-one | mushroom-like | 32 | 8 |
| 2-acetyl-1-pyrroline | roasty | 128 | 128 |
| 2,3,5-trimethylpyrazine | roasty, earthy | 16 | 64 |
| (*E*)-2-octenal | fatty | 32 | 8 |
| 2-furfurylthiol | roasty, coffee-like | 128 | 16 |
| 3-ethyl-2,5-dimethylpyrazine | earthy, roasty | 32 | 128 |
| methional | cooked potato-like | 128 | 256 |
| acetic acid | vinegar-like | 8 | 32 |
| (*E*)-2-nonenal | fatty | 256 | 32 |
| (*E*)-2-decenal | fatty | 16 | n.d. |
| butanoic acid | sweaty, sour | 8 | 128 |
| 2-methylbutanoic acid | sweaty, sour | n.d. | 32 |
| 2-acetylthiazole | roasty | 32 | 64 |
| (*E,E*)-2,4-nonadienal | fatty, fried | 64 | 16 |
| (*E,Z*)-2,4-decadienal | fatty | 16 | n.d. |
| (*E,E*)-2,4-decadienal | fatty, fried | 1024 | 32 |
| hexanoic acid | sour, sweaty | 8 | 32 |
| furaneol | sweet, caramel-like | 32 | 64 |
| phenylacetaldehyde | flowery | 8 | 16 |

**Table 2**: AEDA of (selected results) for beef bouillons: Traditionally prepared samples (TP) compared to commercial samples (C).

| Compound | *Odour quality* | *TP* | *C* |
|---|---|---|---|
| 2-/3-methylbutanal | malty | 16 | 64 |
| 2,3-pentanedione | butter-like | 8 | n.d. |
| 1-octen-3-one | mushroom-like | 32 | 4 |
| 2-acetyl-1-pyrroline | roasty | 256 | 256 |
| (*E*)-2-octenal | fatty | 16 | 8 |
| 2-furfurylthiol | roasty, coffee-like | 128 | 16 |
| 3-ethyl-2,5-dimethylpyrazine | earthy, roasty | 64 | 128 |
| acetic acid | vinegar | 16 | 64 |
| methional | cooked potato-like | 128 | 256 |
| 2,3-diethyl-5-methylpyrazine | earthy, roasty | 32 | 64 |
| (*E*)-2-nonenal | fatty | 128 | 16 |
| (*E*,*Z*)-2,6-nonadienal | cucumber-like | 16 | n.d. |
| butanoic acid | sour, sweaty | 32 | 128 |
| 2-methylbutanoic acid | sweaty, sour | n.d. | 32 |
| 2-acetylthiazole | roasty | 64 | 128 |
| β-ionone | violet-like | 16 | 8 |
| furaneol | sweet, caramel-like | 256 | 512 |

In conclusion, key aroma compounds responsible for the differences in flavour profiles of industrially manufactured and traditionally prepared meat bouillons were identified by comparative AEDA. During industrial processing, on the one hand, a loss of α,β-unsaturated aldehydes, responsible for characteristic boiled, fatty aroma notes was observed, e.g., (*E*,*E*)-2,4-decadienal, whereas on the other hand organic acids, e.g., butanoic acid, responsible for sour, sweaty odours were increasing. Preliminary results (data not show) indicate that the concentration process is a critical step for aroma development. To obtain a closer insight into specific processing parameters, different model studies will be performed. The study shows the importance of identifying and monitoring character impact compounds. Additionally, manufacturing steps should be adapted in the best possible manner to obtain an authentic meat bouillon character.

## References

1. Gasser, U. and Grosch, W. (1990) Z. Lebensm. Unters. Forsch. 190: 3-8.
2. Kerscher, R. and Grosch, W. (2000) In: Frontiers of Flavour Science, Proceedings of the 9. Weurman Flavour Research Symposium (Schieberle, P.; Engel, K. H., ed.) Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, pp. 17-20.
3. Brinkman, H. W., Copier, H., De Leuw, J. J. and M., Tjan, S. B. (1972) J. Agric. Food Chem. 20: 177-181.
4. Kerler, J., and Grosch, W. (1997) Z. Lebensm. Unters. Forsch. A 205: 232-238.
5. Cerny, C., and Grosch, W. (1992) Z. Lebensm. -Unters. Forsch. 194: 322-325.
6. Guth, H., and Grosch, W. (1994) J. Agric. Food Chem. 42: 2862-2866.

# Malolactic fermentation of sea buckthorn (*Hippophaë rhamnoides* L.) berry juice with *Lactobacillus plantarum*: impact on sugars, sugars alcohols, and organic acids

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## Abstract

Potential to decrease sourness, and thus increase sensory value of sea buckthorn berries, by using malolactic fermentation with *Lactobacillus plantarum* was investigated. Sea buckthorn juice samples were fermented with four different *L. plantarum* strains, and chemical changes related to fermentation were determined by analysing sugars, sugar alcohols and organic acids as trimethylsilyl-derivates from fermented sea buckthorn juices with GC-FID. There was a clear difference in fermentation efficiency between studied strains, strain 10492 being the most effective, resulting in total conversion of malic acid into lactic acid. Additionally, levels of total sugars maintained comparable to the non-treated juice with all strains, and thus sweetness was maintained. Therefore, *L. plantarum* with selected strains is potential candidate for malolactic fermentation of sea buckthorn.

## Introduction

Sea buckthorn (*Hippophaë rhamnoides* L.) berries contain a versatile combination of chemical compounds having health promoting features such water-soluble vitamins (C, B1, and B2), fat-soluble vitamins (A, K, and E), fatty acids, flavonoids, and plant sterols [1]. However, the sour, bitter and astringent taste characteristics limit its regular consumption. The main chemical factors related to the sourness of sea buckthorn are the high concentrations of malic and quinic acids. Additionally, strong sourness intensifies the perception of astringency [2].

One potential treatment to increase the value of sea buckthorn would be to use malolactic fermentation, a method currently used for decreasing acidity of sour wines. In this process, certain lactic acid bacteria convert malic acid into lactic acid. In the wine industry, *Oenococcus oeni* is the most commonly used lactic acid bacteria [3]. However, while being effective in wines, *O. oeni* has specific nutrient requirements and a relatively slow growth rate [4], and thus other candidates for malolactic fermentation of atypical materials (such as sea buckthorn) are worth investigating. One potential candidate is *Lactobacillus plantarum*, a bacterial species commonly found in and responsible of the fermentation of plant-based lactic acid fermented foods such as sauerkraut and table olives. Potential benefit of *L. plantarum* is in its robustness: it has a relatively fast growth rate, tolerance of low pH, and low nutrient requirements [5]. However, studies related using *L. plantarum* as malolactic organism are currently limited. Here we evaluate the potential to use *Lactobacillus plantarum* to decrease acidity of sea buckthorn juice, and thus increase its sensory value.

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## Experimental

*Materials*

Frozen sea buckthorn berries (*Hippophaë rhamnoides subp. mongolica*) were obtained from Asterpajutooted OÜ (Tõrva, Estonia). Berries were originated from South Estonia, collected from multiple producers by the distributor. Berries were stored at –20 °C until use.

Four strains of *Lactobacillus plantarum* (DSM 16365, DSM 20174, DSM 10492, DSM 100813) were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz, Germany) as freeze-dried cultures. Cultures were revived as instructed by the manufacturer, and stored in 10 % (v/v) glycerol in food-grade medium (FGM) [6] at –20 °C until use.

*Sample preparation and fermentation set-up*

In order to prepare the juice, frozen berries were thawed in a microwave at 650 W for 5 minutes with intermittent mixing. Berries were made into a mash with an immersion blender. Juice was extracted from the mash with mechanical pressing. Prior to pasteurization, the juice was diluted 1:1 with active-carbon filtered water. Juices were pasteurized in an autoclave (Systec D-150, Linden, Germany) at 85 °C for 5 minutes. After pasteurization, juice samples were cooled down on an ice bath and stored at +4 °C for 24 hours before inoculation.

To produce the starter cultures, each strains was inoculated into 250 ml of FGM by a scrape from the glycerol stock with a sterile inoculation loop. Cells were grown at +30 °C for 24 hours. From each culture, cells were collected with centrifugation at 3410 × *g* for 10 minutes from 200 ml of o/n growth. Cells were washed twice with sterile saline solution and concentrated into a volume of 4,5 ml. Each juice sample of 100 ml was inoculated with 1 ml of respective cell concentrate. Fermentation was performed for +30 °C for 72 h in iCinac equipment (Unity Scientific, Milford, USA) equipped with TW8 water bath (Julabo, Seelbach, Germany). Each fermentation was prepared in duplicates. After fermentation, samples were collected in sterile 2 ml tubes and stored at –80 °C until analysis.

*Analysis of sugars, sugars alcohols, and organic acids*

First, the juice samples were diluted with reverse-osmosis water to achieve an appropriate concentration for the analyses. Aliquots of the diluted samples were dried under nitrogen flow, followed by derivatization of the sugars, sugars alcohol and organic acids with chlorotrimethylsilane reagent with pyridine and hexamethylsilazane (Tri-Sil HTP, Thermo Scientific, Bellefonte, PA, USA). Each sample was prepared in triplicate.

TMS-derivated samples were analysed by using a Shimadzu 2010Plus gas chromatograph (Kyoto, Japan) equipped with flame ionization detector and Shimadzu AOC-20i autosampler. Analyses were performed on SPB-1 column (30 m x 0,25 mm ID, liquid film 0,25 μm, Supelco, Bellefonte, PA, USA). Internal standards were used for quantification, xylitol for sugars and sugar alcohols, and tartaric acid for organic acids. External standards were used for the calculation of correction factors and for the identification of the analytes.

## Results and discussion

The purpose of this work was to investigate the potential to utilize malolactic fermentation with *Lactobacillus plantarum* to decrease the acidity in sea buckthorn juice. The content of malic acid in the control juice was 11,5 ± 0,08 mg/ml (Figure 1). The level of malolactic fermentation varied greatly among the studied *L. plantarum* strains. Strain DSM 10492 was the most effective, with all malic acid converted into lactic acid. Strains DSM 20174 and 100813 had moderate conversions, and malic acid contents were reduced to 8,73 ± 0,19 and 8,34 ± 0.14 mg/ml, respectively. No conversion was detected with the strain 16365.

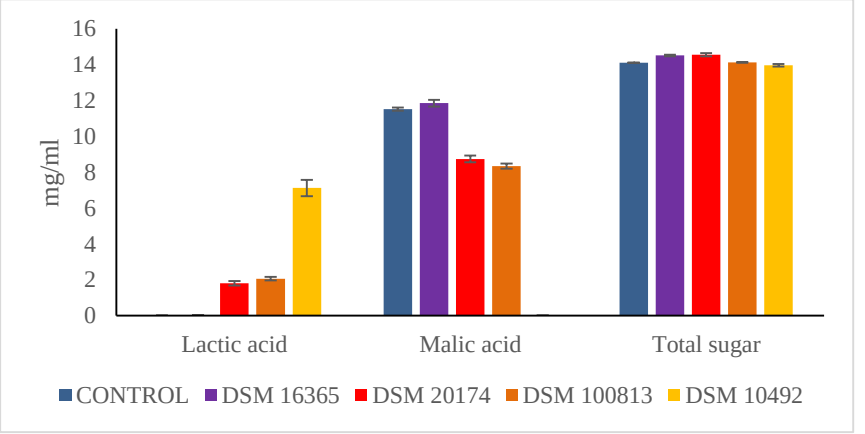


**Figure 1:** Lactic acid, malic acid and total sugars (sum of fructose, glucose, myo-inositol and methyl-myo-inositol) in control sea buckthorn juice and juices fermented with *L. plantarum*. Error bars present standard deviation (N = 6).

As malic acid is converted into lactic acid, acidity is reduced due to decarboxylation, as was observed in the increase in pH of the fermented juices (Figure 2). Although lactic acid bacteria can use a variety of carbon sources, including monocarbohydrates such as glucose and fructose, the content of total sugars remained similar in the fermented juices compared to the control. A similar phenomenon was observed when sea buckthorn juice was fermented with other malolactic bacteria, *Oenococcus oeni* [7]. This is most likely due to the high acidity of the material. At low pH, *L. plantarum* seems to prefer malic acid as an energy source over sugars, possibly due to passive diffusion, as the acids are predominately present in the protonated form [3].

Other identified and quantified compounds were the organic acids citric acid, quinic acid, and ascorbic acid, and the sugars ethyl-glucose, myo-inositol, and methyl-myo-inositol. Additionally, the sugar alcohol L-quebrachitol was identified. Compared to the control, samples fermented with strain 10492 had a small but significant ($P<0,05$) decrease in levels of citric acid, fructose, and quinic acid. Comparing the same samples, significantly higher levels ($P<0,05$) of ascorbic acid and ethyl-glucose were measured in the fermented samples compared to the control.

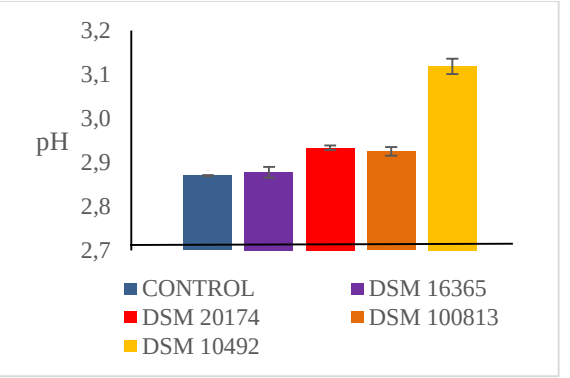


**Figure 2:** Measured pH in sea buckthorn control juice and in juices fermented with *L. plantarum*. Error bars present standard deviation (N = 4).

All in all, our results indicate that *L. plantarum* could be used for malolactic fermentation of sea buckthorn juice without additional nutrients or by increasing the pH. Additionally, other acids, sugars and sugar alcohols remained mostly unfermented. Therefore, this method also maintains the sweetness of the berry juice. However, the effectiveness of the fermentation is highly affected by the strain. Thus, prior investigation of the suitable strains is important. On the other hand, prolonged malolactic fermentation can also produce unwanted off-flavours on strain-dependent basis [7], possibly due to the production of alcohols [8]. On the other hand, malolactic fermentation with *O. oeni* was shown to increase fruity notes in sea buckthorn juice by releasing more ethyl esters or acetate esters of fatty acids [8]. Our work should therefore in the future be combined with aroma analysis and sensory evaluations to confirm how the sensory value and the flavour of sea buckthorn are affected by lactic acid fermentation with *L. plantarum*.

## References

1. Bal L.M., Meda V., Naik S.N. and Satya, S. (2011) Food Res Int 44: 1718–1727.
2. Sowalsky R. A. and Noble A. C. (1998) Chem Senses 23: 343–349.
3. Cibrario A., Peanne C., Lailheugue M., Campbell-Sills H. and Dols-Lafargue, M. (2016) BMC Genomics 17: 984.
4. Theobald S., Pfeiffer P. and König H. (2005) J Wine Res, 16:171–178.
5. Rodríguez, H., Curiel J.A, Landete J.M., de las Rivas B., de Felipe F.L., Gómez-Cordovés C., Mancheño J.M. and Rosario Muñoz R. (2009) Int J Food Microbiol 132: 79–90.
6. Saarela, M., Rantala, M., Hallamaa, K., Nohynek, L., Virkajärvi, I. and Matto, J. (2004). J Appl Microbiol 96: 1205–1214.
7. Tiitinen, K.M., Vahvaselkä, M., Hakala, M., Laakso, S., and Kallio, H. (2005). Eur Food Res Technol 222, 686–691.
8. Tiitinen, K., Vahvaselkä, M., Laakso, S., and Kallio, H. (2007). Eur Food Res Technol 224: 725–732.

# Impact of enzyme treatment on flavour of aronia juice

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## Abstract

Aronia (*Aronia mitschurinii*) is rich in different polyphenolic compounds and sorbitol. Pressed aronia juice has a beautiful colour. The aim of this work was to analyse smell and taste of aronia juices prepared from berries grown in South-West of Finland. Sensory properties were studied using projective mapping with consumers and applying qualitative profiling with experienced sensory panel. Sugar and acid composition was analysed with GC-FID. Based on our results, pectinase treatment had a negative impact on both odour and taste of aronia juice.

## Introduction

Berries, in general, are rich in polyphenols and various other bioactive components and their possible health inducing properties are intensively studied. However, some eatible berries such as bilberry (*Vaccinium myrtillus*), lingonberry (*Vaccinium vitis-idaea)*, sea buckthorn (*Hippophae rhamnoides*), black currant (*Ribes nigrum), red currant (Ribes rubrum)* have challenging flavour and taste properties which may limit their further utilization despite their healthiness [1-2]. Aronia (*Aronia mitschurinii***)**, also called chokeberry, is a shrub that originates from the eastern part of North America. Aronia is a popular garden decoration also in Europe and in Finland, mainly consumed by birds instead of human consumers. The colour of aronia juice is deep violet and stable and it has high contents of various polyphenols. However, due to the strong and mostly unfamiliar orosensory properties of the berry [3], chokeberries are usually used in blended juices.

The aims of this work were to 1) analyse smell and taste of aronia juices prepared from berries grown in South-West of Finland, 2) study the effect of enzymatic pectinase treatment on odour, taste and flavour, 3) study the effect of sucrose or citric acid addition on the sensory properties of juice.

## Experimental

Samples are shown in Table 1 and the protocol is shown in Figure 1. Aronia berries were grown in Turku (Finland) and harvested in 2016. Juices (n = 6) were pressed from crushed berries without (I) and with (II) pectinolytic enzyme (Pectinex Ultra SP-L, Novozymes) treatment applying incubation for 5h at 50°C. Also sucrose (1 %) or citric acid (0.15 %) was added to some samples.

**Table 1:** Sample set included 6 different juice samples.

| Juice samples | *w/o pectinase* | *w pectinase* |
|---|---|---|
| Juice | No enzyme | Enzyme |
| Juice + Sucrose 1 % | No enzyme / added sugar | Enzyme / added sugar |
| Juice + Citric acid 0.15 % | No enzyme / added acid | Enzyme / added acid |

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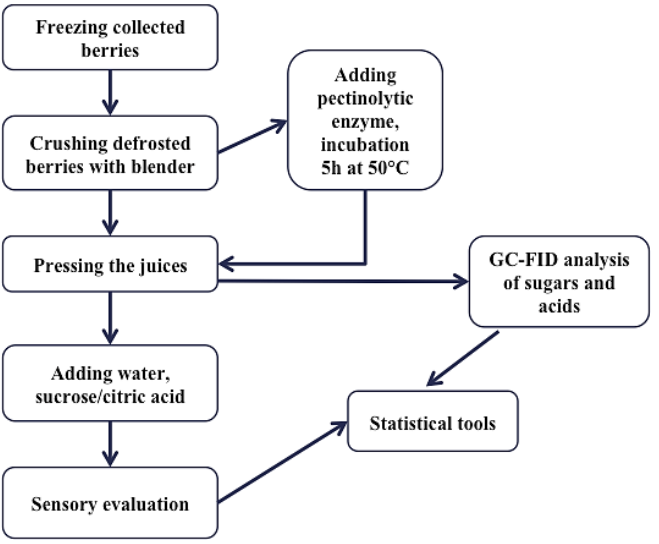


**Figure 1:** Study protocol for flavour research moving from aronia berries to pressed aronia juices applying enzymatic treatment.

Differences and similarities of all the samples were studied using projective mapping (PM) with volunteer participants (n = 32) in controlled sensory laboratory environment (ISO 8589). Pure juices were diluted with water (carbon-filtrated) 1/2 before the sensory evaluation. Moreover, qualitative descriptive analysis was applied to describe the sensory properties of juices in our sensory laboratory by experienced sensory panellists (n = 7).

In addition, gas chromatography (GC) with flame ionization detection (FID) was applied to determine the sugar and acid contents in juices without any sugar or acid addition. Sugars and acids were measured by GC as trimethylsilyl derivatives [4]. GC-FID was Shimadzu with a column (SPB-1, 30 m x 0.25 mm x 0.25 um, Supelco), temperature of injector: 210 °C, temperature of detector: 290 °C. GC-analysis was 150 °C (hold 2 min), rate 4 °C/min => 210 °C/min, rate 40 °C/min 0 => 275 °C (hold 5 min) with a total time 28.6 minutes).

Results of projective mapping were processed with Principal Component Regression (PCR) and full cross validation using Unscrambler X (Camo, Norway). In PCR-model X-variables were sample coordinates from project mapping and sensory descriptors were defined as Y-variables.

## Results and discussion

Our results showed a clear impact of enzyme treatment on smell and taste of aronia juices (Figure 2). Two separate PCR models were created based on odour and flavour evaluations (Figure 2A and 2B, respectively) with sample coordinates by 32 participants as X-data explaining the variances in sensory descriptors data (Y-data) with three validated components in both models. Differences between juices produced with or without enzymes are shown on the first PCs in both models whereas notably less significant components 2 and 3 show the impact of added sugar or acid on the sensory quality. The key odour and flavour descriptors describing the differences between the two juice types are shown in Table 2. In the PM test based on flavour and taste (Figure 2B), juice with added acid locate on lower section of plot on PC-2 together with astringent

descriptors as opposed to (loadings plots not shown). In the PM test based on odour, addition either sucrose or citric acid to the juice samples had little impact on the sensory descriptors.

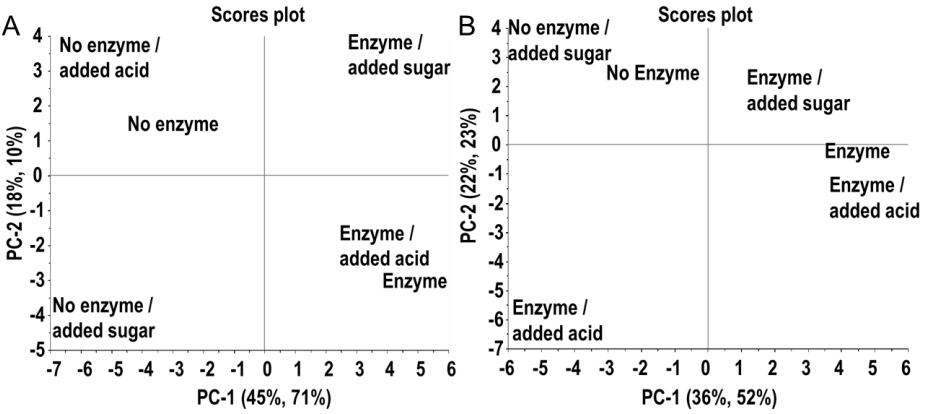


**Figure 2:** PCR Score plots showing the distributions of six aronia juice samples by the participants in PM test based on odour (A) or flavour and taste characteristics (B).

Most common sensory descriptors for aronia juice samples were “sour”, “sweet” and “astringent”. Juices produced with pectinase were described as “unpleasant” with descriptions such as “almond”, “nutty” and “oat-like” or “grainy” odour and their flavours were “stale” and with various astringent descriptors (Table 2). Somewhat expected notable increase in astringent properties was due to the release of polyphenolic compounds from berry skins by the enzymes [5]. Juices without enzymatic assistance were described as more pleasant with odour attributes such as “forest”, “aronia” and “sweet” and flavours “berry-like” and “sweet”. Addition of low concentration of sucrose or citric acid did not result in notable new odour or flavour descriptors in comparison to descriptor differences between juice treatments.

**Table 2:** Odour and flavour or taste properties for diluted juice samples.

| *Odour w/o pectinase* | *Odour w/ pectinase* | *Flavour and taste w/o pectinase* | *Flavour and taste w/ pectinase* |
|---|---|---|---|
| Aronia | Earthy | Berry-like | Almond |
| Forest | Fermented | Fermented | Astringent |
| Fresh | Nutty | Leaf-like | Mouth-drying |
| Pleasant | Stale | Sweet | Puckering |
| Sour | Unpleasant | Bitter | Stale |
| Sweet | Oat/grain | | Watery |
| Feed | | | Berry |

Based on GC-analyses main sugars were glucose, sorbitol and fructose, and main acids were malic and quinic acid (Table 3) in both juices (w/ and w/o pectinase). Also there were no differences in concentrations of sugars and acids. Although in some berries and berry products the ratio between sugars and acids is a critical predictor of flavour [1-

2,6], we may conclude that flavour differences between pectinase treated and natural juice could be better explained by other compounds than original sugars and acids.

**Table 3:** Sugar and acid composition (mg/100 ml) of juice samples

| *mg/100ml* | *Juice w/o pectinase* | *Juice w/ pectinase* |
|---|---|---|
| *Succinic acid* | 1.39±0.3 | 1.57±0.3 |
| *Malic acid* | 421±15 | 416±10 |
| *Isocitric acid* | 20.8±4.8 | 22.2±4.0 |
| *Citric acid* | 3.0±2.6 | 2.9±3.6 |
| *Quinic acid* | 142±7.2 | 147±7.4 |
| *Fructose* | 1150±130 | 1120±160 |
| *Glucose* | 10900±760 | 10000±1200 |
| *Sorbitol* | 2550±52 | 2490±85 |
| *Sucrose* | 358±270 | 404±370 |
| *Sugar-acid ratio* | 25.4 | 23.8 |

Inclusion of pectinolytic enzyme to the juice pressing process gave the aronia juices in this study very strong odour and flavour characteristics different from juices without enzymatic assistance. Enzyme treatments used in food industry will typically contribute to yields of pressed juice instead of focusing on flavour. However, in the case of aronia, they may also create flavours, which may be considered as undesired and unpleasant by consumers.

## References

1. Sandell M., Tiitinen K., Pohjanheimo T., Kallio H., and Breslin P.A.S. (2007) In ACS Symposium Series No988. Food Flavor: Chemistry, Sensory Evaluation and Biological Activity. Eds. Takeoka, G.; Ebeler, S.; Kubota, K.; Tamura, H. American Chemical Society, Oxford University Press, pp. 219–228.
2. Sandell M., Laaksonen O., and Lundén S. (2012) In: Berries: Properties, Consumption and Nutrition (ed. Carlo Tuberoso), Nova Science Publishers, Inc., pp 105–126.
3. Laaksonen O., Knaapila A., Niva T., Deegan K., and Sandell M. (2016). Food Qual Pref 53:117–126.
4. Kallio H, Hakala (Sandell) M, Pelkkikangas AM, and Lapveteläinen A (2000) Sugars and Acids of Strawberry Varieties, Eur Food Res Tech 212:81-85.
5. Laaksonen O., Sandell M., Nordlund E., Heiniö R.-L., Malinen H.-L., Jaakkola M., and Kallio, H. (2012) Food Chem, 130(1):31–41.
6. Kårlund A., Hanhineva K., Lehtonen M., Karjalainen R., and Sandell M. (2015). J Agric Food Chem 63(3):1010–1019.

# Validity of marker compounds for authenticity control case study: Methyl cinnamate

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## Abstract

Methyl 3-methoxy-3-phenyl-propanoate (3-MMPP) is seen as a potential marker, which is used by analytical laboratories and NGO´s to conclude on the correct production of natural methyl cinnamate (MC) qualities according to the EU flavour directive 1334/2008. However, there have been questions about its validity, as a marker for adulteration is only valid, if it can unmistakably be associated with non- permitted starting materials and/or process conditions. There are two obvious routes to get access to natural methyl cinnamate, which would be in full compliance with the requirements for natural flavouring substances according EU flavour regulation 1334/2008. As potential starting materials natural cinnamic acid or *Alpinia malaccensis* roots can be used. Within this study we shed light on the potential formation of 3-MMPP during processing and proved that this trace compound is not appropriate as a marker for adulteration.

## Introduction

There is a global trend towards natural food solutions. Hence the industry is looking for ways to increase their portfolio of natural aroma compounds. Good examples are the esters of cinnamic acid, which are widely used for fragrance compositions as well as for the creation of flavours. Especially the methyl ester of cinnamic acid is widely used due to its sweet, aromatic and balsamic notes combined with a fruity odour. Methyl cinnamate occurs naturally in a variety of plants, including fruits like strawberry and some culinary spices, such as thyme and basil. However, the isolation from these species is not economically feasible. Alternative sources are essential oils of rhizomes of various *Alpinia* species, e.g. *Alpinia malaccensis* [1] containing approximately 78% methyl cinnamate (Figure1). [2,3]

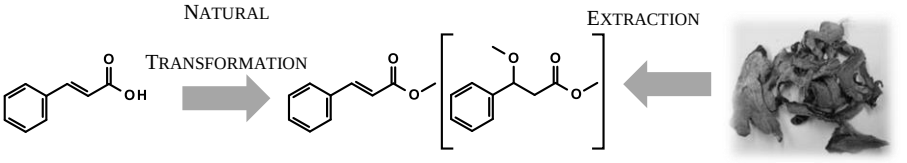


**Figure 1:** Potential ways to generate methyl cinnamate from different sources.

Another alternative would be the esterification of widely available natural cinnamic acid with methanol form natural sources under conditions permitted by corresponding national or international laws. Natural aroma compounds are mostly more expensive than their synthetic equivalents and are therefore prone to adulteration. Several sophisticated methods, e.g. isotopic ratio mass spectrometry [4] or SNIF-NMR, have been developed to identify adulterated material. As these methods are not always conclusive, the presence of trace components is often considered as an additional marker. The presence of 3-

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MMPP is often referenced as an indicator for potential adulteration, when found in natural methyl cinnamate samples. At the same time there is no published evidence supporting the quality of this potential indicator. Within this study we attempted to check the suitability of 3-MMPP by answering the question whether or not 3-MMPP can be formed during processing of a suitable raw material. In this study it was also investigated, whether the formation of 3-MMPP could serve as a potential indicator for the use of a mineral acid during the esterification of cinnamic acid.

## Experimental

### *Materials and methods*

All chemicals were purchased from commercial suppliers. Dried roots of *A. malaccensis* were purchased from Indonesia *via* a German distributor.

GC/MS-Analysis was conducted using a HP 6890N (Agilent, Santa Clara, CA, USA), fitted with a DB-WAXms capillary column (Phenomenex, Torrance, CA, USA) (30m*0.25mm i.d., df 0.25µm), coupled with an MSD 5975C (Agilent, Santa Clara, CA, USA). The GC conditions for the GC/MS-analysis were: split injection (split ratio 20:1), injector temperature 230°C; initial oven temperature at 60°C for 1min, ramp at 4°C/min to 230°C for 20min. Helium was used as the carrier gas and the flow rate was 2.0mL/min. Quantities of 3-MMPP were determined via semi-quantification against the internal standard 2-Nonanol using the GC-FID signal.

All stable isotope measurements were performed using a gas chromatography-combustion/ high temperature conversion-isotope ratio mass spectrometry (GC-C/HTC-IRMS) system consisting of an HP 7890B gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a robotic autosampler (CTC Analytics, Zwingen, Switzerland), coupled to a BiovisION isotope ratio mass spectrometer (Elementar, Langenselbold, Germany) via an oxidation reactor ($\delta^{13}C$) or via a pyrolysis reactor ($\delta^{2}H$) in a GC5 interface (Elementar, Langenselbold, Germany). All $\delta^{13}C$ and $\delta^{2}H$ values were normalized relative to V-PDB or V-SMOW by a two-point calibration using two vanillin working standard with distinct isotope signatures.

## Results and Discussion

### *Isolation of methyl cinnamate from Alpinia malaccensis*

The processing of the dried rhizomes of *Alpinia malaccensis* can either be done by extraction with a suitable solvent (e.g. methanol) or hydrodistillation with subsequent rectification to obtain sensorially acceptable products. Dried *Alpinia malaccensis* rhizomes from Indonesia were extracted by simultaneous distillation extraction (SDE) employing water and a mixture of diethylether/pentane to mimic hydrodistillation conditions. In addition, the rhizomes were extracted with methanol and the solvent removed with and without the addition of $Na_2CO_3$ to prevent the decomposition by acidic byproducts during rectification. As expected, it was not possible to distinguish the different processing conditions *via* IRMS data (Table 1).

**Table 1:** IRMS data and formation of 3-MMPP during different extraction conditions.

| *work-up procedure* | *Methyl Cinnamate* | | *3-MMPP* |
|---|---|---|---|
| | delta $^{13}C$ | delta $^{2}H$ | |
| SDE | − 26.5 ± 1.0 ‰ | − 150 ± 15 ‰ | ./. |
| methanolic extract w/o addition of $Na_2CO_3$ | − 26.6 ± 1.0 ‰ | − 151 ± 15 ‰ | ./. |
| methanolic extract with addition of $Na_2CO_3$ | − 27.2 ± 1.0 ‰ | − 146 ± 15 ‰ | 720 ppm |

However, the addition of $Na_2CO_3$ led to the formation of significant amounts of 3-MMPP which is in line with related reactions described in chemical literature. [5] So even during processing of a widely accepted raw material, traces of 3-MMPP can be formed and good communication with the corresponding supplier is mandatory.

*Generation of methyl cinnamate via esterification*

Our studies showed that the esterification of cinnamic acid and methanol is possible under various conditions. Surprisingly, simple heating of a mixture of both starting materials led to significant formation of methyl cinnamate without the need of any additional additives (Table 2). Moreover, there is no significant difference regarding the formation of 3-MMPP neither between the use of a mineral or organic acid nor without the use of any additive.

**Table 2:** Generation of methyl cinnmate starting from cinnamic acid and methanol.

| *Cinnamic acid [mmol]* | *Methanol [mmol]* | *Additive [mmol]* | *Conditions* | *Conversion [%]* | *MC : 3-MMPP* |
|---|---|---|---|---|---|
| 34 | 244 | Sulfuric acid 16.9 | 6h reflux | > 90 | 50:1 |
| 27.0 | 244 | Tartaric acid 2.7 | 9h, 120 °C/ 4.2 bar | 15 | 67:1 |
| 67 | 280 | none | 26h, 120 °C/ 4.2 bar | 19 | 63:1 |

In many regulations heating of methanol and cinnamic acid under slightly elevated temperature and pressure is a process which is allowed for the generation of natural aroma compounds. Hence the use of 3-MMPP as a potential marker for authenticity is highly questionable, as there is no reliable evidence for unequivocal differentiation between the use of permitted and non-permitted process conditions according to the EU flavour regulation 1334/2008 during the generation of natural methyl cinnamate.

## Summary

Available natural methyl cinnamate (European legislation) can at least be generated *via* two different processes. Firstly, an extraction/distillation of *Alpinia malaccensis* roots should be feasible. Here the generation of 3-MMPP could be traced back to the potential use of processing aids (e.g. $Na_2CO_3$) which are widely used and comply with current legislation for the generation of natural aroma chemicals. Secondly esterification of methanol and cinnamic acid delivers a suitable product, providing the used raw materials are sourced in a natural quality. Here we could show that methyl cinnamate could be formed easily as assumed under the addition of a mineral acid. However, it could also be formed just by addition of an organic acid or even without any additives. In all cases 3-MMPP could be detected. Taking this into account, the use of 3-MMP as potential marker for adulteration should be challenged.

## References

1. Lim, T. K. (2016) Alpinia malaccensis. Edible Medicinal and Non-Medicinal Plants: Volume 12 Modified Stems, Roots, Bulbs. Cham, Springer International Publishing: 172-177.
2. Nor Azah, M. A., Sam, Y. Y., Mailina, J., and Chua, L. S. L. (2005) Journal of Tropical Forest Science 17(4): 631-633
3. Sirat, H. M., Basar, N., andJani, N. A. (2011) Natural Product Research 25(10): 982-986
4. Fink, K., Richling, E., Heckel, F., and Schreier, P. J. (2004) Agric. Food Chem. 52(10): 3065-3068
5. Guo, S.-H., Xing, S.-Z., Shuai Mao, S., Ya-Ru Gao, Y.-R., Wen-Liang Chen, W.-L., and Yong-Qiang Wang, Y.-Q. (2014) Tetrahedron Letters 55(49): 6718-6720

# Mapping on the origins of cajuput essential oil for its compatibility as *Cajuputs® candy* functional flavour

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## Abstract

Cajuput essential oils (CEO) were utilized as a functional flavour in *Cajuputs® candy*. CEO are produced in several areas of Indonesia such as Buru Island, Bupolo, Namlea, Belu, Gelaran, Sendang mole, Tanjung enim, Indramayu, Ponorogo, Mojokerto, Gundih, Kuningan, and Pasuruan. The quality of these CEO, however, might be influenced by its geographical origin. The current product is utilizing CEO originated from Buru Island as its flavouring. The aim of this study was to obtain compatible CEO as alternative functional flavour for *Cajuputs® candy*. The physicochemical properties such as density, refractive index, optical rotation, solubility in alcohol 70% and cineol content were examined. Hedonic test and different from control test were also conducted to evaluate their sensory characteristics. Principal Component Analysis (PCA) was applied to observe the mapping of CEO from different origin based on their physicochemical and sensory properties. The results showed that CEO from Bupolo, Ponorogo and Mojokerto showed good compatibility in terms of the best acceptance rate of taste, aroma and overall attributes, and having the least difference to the reference characteristics.

## Introduction

Cajuput essential oils (CEO) derived from *Melaleuca cajuputi* species are known to have antibacterial and antifungal activity related to their bioactive compounds [1,2]. CEO has been long used for tropical medicine, yet it was developed as functional flavour in *Cajuputs® candy*, an Indonesian herbal-based lozenge which was also proved to maintain the oral health from pathogenic microbial infections. Since 1997 this functional candy has been produced by using CEO from Buru island as its main flavouring. However, the availability of this CEO was gradually decreasing since most of their producers had become gold miners. Therefore, it is necessary to explore alternative CEO with compatible flavour characteristics for *Cajuputs® candy*.

Indonesia has many sources of CEO due to the excellent adaptability of the plants to grow both in dry and wetlands or even in swamp areas. However, the quality of CEO seems different depending on the origin. The aim of this study was to obtain compatible CEO, in terms of their physicochemical and sensory properties, as alternative functional flavouring for cajuputs *Cajuputs® candy.*

## Experimental

### *Materials*

CEO were obtained from steam distillation of leaves and twigs of cajuput plants (*Melaleuca cajuputi*) collected from 13 different locations, with Buru island CEO used as the reference. Others CEO samples were obtained from private suppliers (Bupolo, Namlea and Belu), Dinas Kehutanan dan Perkebunan Daerah Istimewa Yogyakarta (Gelaran and Sendang mole), Bukit Asam Company (Tanjung enim), and Kesatuan Bisnis

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Mandiri IMKP Surabaya (Indramayu, Ponorogo, Mojokerto, Gundih, Kuningan, and Pasuruan). Peppermint oil was purchased at PT. Brataco Chemika.

*Preparation of candy*

The original *Cajuputs*® *candy* non sucrose was made based on the procedures conducted by Wijaya [3].

*Physicochemical analysis*

The physicochemical properties of CEO were examined based on Indonesian national standard for CEO (SNI 06-3954-2006).

*Sensory evaluation*

The hedonic rating test and different from control test of *Cajuputs*® *candy* were performed for the CEO sensory analysis. They were done according to Meilgaard *et al.* [4]. In case of the hedonic test, the samples were given to the panellists in the block system according to the Balanced Incompleted Block Design [5].

*Statistics analysis*

Analysis of variance (ANOVA) was conducted to assess significant differences between samples *Cajuputs*® *candy* using the SPSS version 22.0 program (SPSS Inc., Chicago, IL, USA). Dunnet test was performed for the different from control test data while the hedonic test data was analysed by Duncan's test. P value < 0.05 was considered statistically significant. Principal component analysis was performed to map 13 CEO towards their physicochemical properties and hedonic scores using Minitab 16 (Minitab Inc., USA).

## Results and discussion

The CEO had different colour, ranging from colourless to greenish. They also had strong cajuput-like odour, except for Belu CEO which was showing off-flavour. As shown in table 1, none of these 12 CEO showed significant differences among the CEO. In addition, they also had a high similarity to the reference except for the cineol content and optical rotation. Most of the CEO samples had a good quality based on SNI standard. Tanjung enim, Gelaran, Sendang mole and Namlea were out of the range due to their lower cineol content.

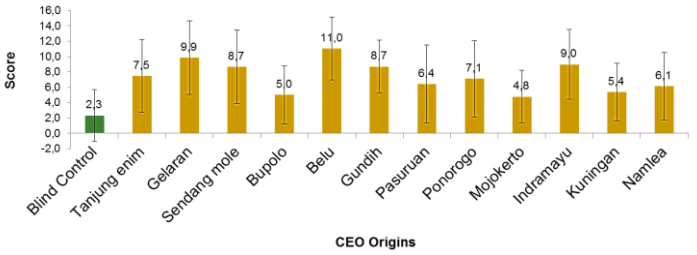



**Figure 1:** Different from control test of *Cajuputs*® *candy* derived from several CEO in Indonesia (Blind Control = Reference)

Panellists' given response showed that there were significant differences between the samples and the reference ($p<0.05$) on the overall attributes (Figure 1). *Cajuputs*® *candy* with CEO from Belu had the most different sensory characteristic from the reference. It might be due to its strong metallic odour and off-flavour. Similar phenomenon was found on the CEO from Indramayu. On the other hand, Bupolo and

Namlea CEO had a huge similarity with the reference. This could be due to their similar geographical origin. However, the most similar sensory characteristic of CEO in terms of flavour was shown by CEO from Mojokerto. Interestingly, Mojokerto is located on a different island, however, it showed better similarity. It indicated that the compatibility of the CEO is not only due to the geographical impact, but also due some other factors. There should be specific chemical compounds on CEO which could affect their similarity. Similar tendencies were also found on Kuningan, Pasuruan and Ponorogo CEO.

**Table 1:** Physicochemical properties of CEO from different origins

| *No* | *CEO Origins* | *Analysis parameters* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Smell test* | *Density* | *Refractive index* | *Optical rotation* | *Solubility in alcohol 70%* | *Cineol content (%)* |
| | | *cajuput-like* | *0,900 – 0,930* | *1,450 – 1,470* | *(-) 4,00 – 0,00* | *1:1 – 1:10* | *50 – 65* |
| 1 | Buru Island (Reference) | cajuput-like | 0,915 | 1,463 | (-) 0,10 | 1:3 | 50,10 |
| 2 | Tanjung enim | slightly metallic | 0,924 | 1,470 | (-) 0,30 | 1:3 | 43,16 |
| 3 | Gelaran | cajuput-like | 0,911 | 1,468 | (-) 7,60 | 1:3 | 41,51 |
| 4 | Sendang mole | cajuput-like | 0,913 | 1,468 | (-) 0,40 | 1:3 | 46,29 |
| 5 | Bupolo | cajuput-like | 0,917 | 1,467 | (-) 0,35 | 1:3 | 54,62 |
| 6 | Belu | Off-flavour | 0,917 | 1,465 | (-) 3,40 | 1:3 | 52,78 |
| 7 | Gundih | cajuput-like | 0,910 | 1,467 | (-) 6,10 | 1:3 | 51,44 |
| 8 | Pasuruan | cajuput-like | 0,910 | 1,467 | (-) 4,10 | 1:3 | 64,79 |
| 9 | Ponorogo | cajuput-like | 0,916 | 1,465 | (-) 5,40 | 1:3 | 53,79 |
| 10 | Mojokerto | cajuput-like | 0,913 | 1,468 | (-) 3,40 | 1:3 | 56,71 |
| 11 | Indramayu | Slightly burned | 0,914 | 1,461 | (-) 0,50 | 1:3 | 57,76 |
| 12 | Kuningan | cajuput-like | 0,915 | 1,468 | (-) 0,90 | 1:3 | 60,50 |
| 13 | Namlea | cajuput-like | 0,915 | 1,466 | (-) 2,00 | 1:3 | 45,86 |

The results from the hedonic test results (Figure 2) showed that the level of panellists' preference was influenced also by the origin of the CEO. The results showed that the preferences upon *candy* which was added with CEO from Belu showed the lowest score. CEO from Ponorogo, Mojokerto, Kuningan, Bupolo, and Namlea had a similar preference level, equal to the reference. These results supported the previous finding that the CEO with high similar sensory characteristic to the reference tends to obtain high score of preference level approach to the reference.

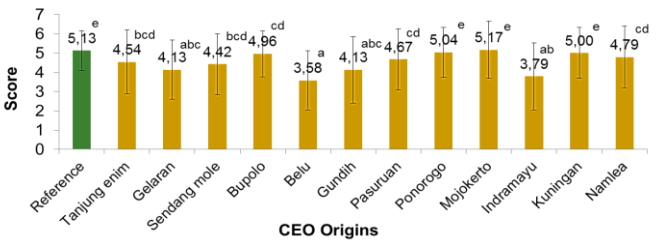
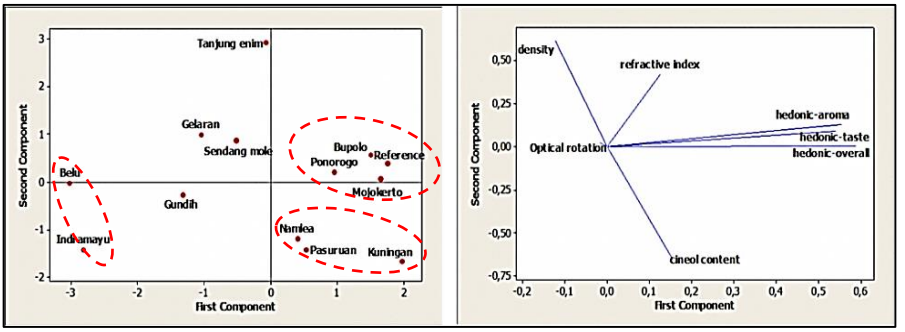



**Figure 2:** Hedonic test of *Cajuputs® candy* derived from several CEO in Indonesia ($p < 0,05$)

The mapping of the physicochemical properties into the sensory characteristics showed that the CEO from Bupolo, Mojokerto, and Ponorogo were the most potential CEO to be developed as compatible functional flavour ingredients in *Cajuputs® candy*

due to their similarity with the reference (Figure 3). This grouping tends to be dominated by the panellists' preferences in terms of taste, aroma (data not shown) and the overall attributes. Cineol content also gives a significant contribution. The second option comes to the group of CEO from Pasuruan, Kuningan and Namlea.




(a) (b)

**Figure 3:** Score plot (a) and loading plot (b) PCA of different origins CEO towards its physicochemical properties and sensory characteristics

Moreover, the data proved that although the physicochemical characteristic of the CEOs (Table 1) looks similar to the reference, the sensory characteristics toward the *Cajuputs®candy* showed diversity. Similar with the previous phenomena, Mojokerto, Ponorogo, Kuningan, and Pasuruan CEO which were located in a different island as the CEO reference origin, having similar sensory characteristic as the reference (Figure 1). Surprisingly, not all of the CEOs which met the requirements of SNI standard obtained a good sensory preference level (Figure 2). Otherwise, some CEO those had high level of preferences and high similarity on sensory characteristic actually did not met the requirements of SNI (Pasuruan, Ponorogo and Namlea). Therefore, further studies are necessary to be done in order to investigate the correlation of the CEO origin with their chemicals composition, particularly for the volatiles which contributed to the flavour perception.

**Acknowledgments**

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## References

1. Siddique S., Parveen Z., Firdaus-e-Bareen, and Mazhar S. (2017). Chemical composition, antibacterial and antioxidant activities of essential oils from leaves of three Melaleuca species of Pakistani flora. Arab. J. Chem.
2. Pujiarti R., Yoshito O., and Hideaki I. (2012). J. Wood Sci., 58:429–436.
3. Wijaya C.H. (2015). Indonesia Patent Submission No. P00201406482: Komposisi dan pembuatan permen cajuputs non sukrosa sebagai perawatan kesehatan mulut, Departemen Kehakiman dan Hak Asasi Manusia Republik Indonesia.
4. Meilgaard M.C., Civille G.V., and Carr B.T. (2007). In: Sensory evaluation techniques (4th ed). CRC Press, New York, pp. 92-100.
5. Cochran W.G., and Cox G.M. (1957). In: Experimental Designs (2nd ed). Wiley, New York, pp. 376-380.

# Influence of protective inert gas atmospheres on the aroma stability of orange juice with pulp

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## Abstract

The efficacy of three divers processing atmospheres: nitrogen ($N_2$), carbon dioxide ($CO_2$), and conventional "air" ($O_2$) (as a control), for the protection of the volatile aroma compounds of commercially produced orange juice during its guaranteed four-month shelf life was investigated over two successive production years. Headspace-solid phase microextraction (HS-SPME), gas chromatography-mass spectrometry (GC-MS), and in parallel gas chromatography coupled to the flame ionization detector and with olfactometry (GC-FID/O) were used for the isolation, and subsequently for investigation of the volatiles with the emphasis on the key odour-active constituents of the aroma. Gained results showed that inert processing atmospheres can partly preserve the aroma profile of the orange juice. The best results were obtained with $N_2$ application. Concerning the outcomes of $CO_2$ application on orange juices, the results were comparable with $N_2$, but acceptability of juices treated by $CO_2$ have to be considered by consumers because of the sparkling character of final products evoking more sour taste. Nevertheless, none of two investigated inert gases was able to avoid all changes in the composition of volatiles during the storage time. However, from a sensory point of view, GC-FID/O analyses proved that these changes are not significant to that extent to lead to deterioration in the overall flavour of juices. On the contrary, certain negative sensory changes were observed for juices processed in conventional "air" ($O_2$) atmosphere as early as in second month of four-month shelf life, and they were getting worse gradually over the storage time. GC-FID/O revealed that the generation of some aldehydes, mainly hexanal, nonanal and perillaldehyde, as a consequence of oxidative changes could be responsible for this off-flavour phenomenon. In these juices obvious increase in bitter and waxy odour and taste was noticed, as well as the appearance of considerable astringent taste, a certain loss of freshness and fruity sweetness, and undesirable colour changes.

## Introduction

Generally, the flavour of fresh hand-squeezed orange juice is considered to be the most attractive one, and it is used as a reference etalon against which all other types of orange juices are judged. Nevertheless, sensory perception evoked by commercial produced orange juice can be quite different because individual stages of industrial processing (freezing/thawing, depulping, deaeration, pasteurisation of raw juice) [1-9], influence of used packaging materials, as well as long-term storage in retail chain (impact of temperature, time, oxygen content, light exposure) result in some alterations in original fresh juice aroma [10-15]. It is obvious that a lot of effort has been devoted to the research of commercial orange juice up to now, so one potential way how to reduce degradation of fruit juices during storage can be their production under inert atmosphere.

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## Experimental

### *Materials*

Raw, unconcentrated orange juice imported from Costa Rica in frozen state was obtained, and afterwards technologically processed by McCarter a.s., Bratislava, Slovakia. After unfreezing, juice was enriched with pulp, mixed, pasteurised at up to 95 °C during 20 s and filled aseptically into the 200 ml polyethylene terephthalate (PET) bottles with oxygen scavengers. The first year, one series of samples from the same batch of raw juice was processed under $N_2$ atmosphere, and the second one by the traditional technology in conventional "air" ($O_2$) atmosphere. In the second year, one series of samples was produced under $N_2$, and the second one under $CO_2$ atmosphere. Bottled samples were stored in lab at 7±1 °C in the showcase refrigerator under conditions simulating the daylight exposure, i.e. typical conditions in a retail chain, within 4-month shelf life period. Analyses were performed in 24 h after delivery of samples to the lab, and then on a monthly basis.

### *Head-space solid phase microextraction (HS-SPME)*

Each sample of orange juice (5.0 ml) was incubated statically in a 40 ml glass vial in a metallic block thermostat at 35 °C for 30 min, with a SPME fibre with 50/30 µm DVB/Carboxen/PDMS film (2 cm stable flex) placed in the headspace of sample. HS-SPME isolates were desorbed at 250 °C in GC injector during the entire GC runs.

### *Gas chromatography-mass spectrometry (GC-MS)*

Obtained complex mixtures of the volatiles were analysed by GC-MS using the gas chromatograph Agilent 6890N coupled to the mass spectrometric detector 5973 inert equipped with fused silica capillary GC column Ultra 1 (50 m × 0.32 mm × 0.52 µm) operating with a temperature programme 35 °C (2 min), 4 °C.min$^{-1}$, 200 °C. The linear velocity of carrier gas helium was 33 cm.s$^{-1}$ (measured at 143 °C). Splitless injection mode was used at an injector temperature of 250 °C. Ionization voltage (EI) was set to 70 eV. Identification of compounds was performed by comparison of measured mass spectra with available mass spectral libraries Wiley and NIST MS. Relative proportions of individual volatiles as semi-quantitative parameters were calculated by the method of internal normalization and expressed as a percentage; the values were the averages of triplicates (data not shown).

### *Gas chromatography-olfactometry (GC-FID/O)*

GC-FID/O was involved using the detection frequency concept of posterior evaluation of odour quality and odour intensity of individual odorants. A sniffing panel was formed from 5 sniffers who were chosen from 11 well-trained assessors in sensory analysis. Results of these analyses were expressed as the average values of estimated odour intensities in a scale from 0 to 3 with increments of 0.5, obtained from 5 independent measurements. Each sensory perception was based on at least 4 citations. The value ±0.5 was considered as measurement deviation. For the performance of these analyses, as well as for the determination of linear retention indices the gas chromatograph Agilent 7890A was coupled to FID and an olfactory detection port (ODP3, Gerstel). GC column Ultra 1 (50 m × 0.32 mm × 0.52 µm) operated with the temperature programme 35 °C (2 min), 4 °C.min$^{-1}$, 200 °C. Hydrogen was used as a carrier gas at the linear velocity of 44.6 cm.s$^{-1}$ (measured at 143 °C). Splitless injection mode was used at injector temperature of 250 °C. The linear retention indices ($LRI^{U1}$) were calculated according to the equation of Van den Dool and Kratz [16], using n-alkanes $C_6$–$C_{14}$ as reference compounds. For GC-FID/O experiments the effluent of the

column was splitted with a ratio of 1:1 to the FID and ODP, which operated at the temperature of 180 °C, interface temperature was 230 °C, the flow of added $N_2$ in ODP humidifier 12 ml.min$^{-1}$. The sniffing time of each judge did not exceed 30 min.

## Results and discussion

*GC-FID/O study of juices produced under $N_2$ atmosphere vs "air"($O_2$) atmosphere*

GC-FID/O technique was used in order to detect and identify volatiles which can be responsible for the sensory differences observed between juices processed in inert and conventional "air" atmosphere during storage, as well as to reveal potential off-flavour compounds causing negative changes in the aroma of juices. In general, 24 odour-active compounds were detected in the orange juice irrespective of used processing atmosphere (Fig. 1, Tab. 1), however, only 23 olfactory responses were recorded, due to the overlap between odours of octanal + β-myrcene. Odorants D-limonene, (Z)-β-ocimene, δ-3-carene, α-terpinolene$^t$, linalool, L-limonene$^t$ and decanal were principal in the volatile fraction of orange juice. They contributed with their high odour intensities (from 2 to 3) to the overall odour of orange juice to a decisive degree and thus, they were the most characteristic components of its odour. With regard to odorants such as (E)-2-hexenal, D-limonene, (Z)-β-ocimene, α-terpinolene$^t$, linalool, perillaldehyde and unknown compound No. 23, their odour intensities remained unchanged during the entire storage period in both processing atmospheres.

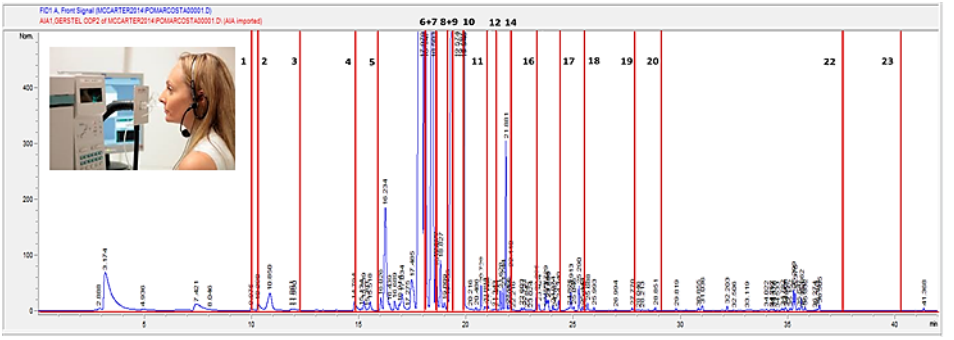


**Figure 1:** Gas chromatogram + olfactogram of orange juice volatiles (production without inert gas ($O_2$), 0. month of storage) obtained by HS-SPME coupled to GC-FID/O. The numbering verticals, marking olfactory responses corresponds to Tab. 1.

On the contrary, hexanal (green, grassy, leafy and bitterish odour), and also nonanal (soapy, waxy, tallow-like odour) were detected only in samples produced in conventional $(O)_2$ atmosphere (Tab.1). Perillaldehyde (smoked, cumin, spicy odour) was noticed in both atmospheres, but in conventional one showed higher odour intensity. Intensity of decanal (orange peel-like, waxy odour) dropped in $N_2$ atmosphere, whereas in conventional one was stable. Only undecanal (fatty, citrus, aldehydic, waxy odour) showed increasing trend in both atmospheres. Concerning the observed changes in odour intensities of some aldehydes, they can explain deterioration of the organoleptic properties of juice processed in conventional atmosphere that occurred during the second, but especially the third month of storage. Mainly, it was increased bitter and astringent taste of juice, it was registered a certain loss of freshness and fruity sweetness, accompanied by undesirable colour changes. In contrast, juice processed in $N_2$ atmosphere had standard organoleptic quality comparable to the fresh product during the entire storage period.

**Table 1:** Principal odorants of industrially processed orange juice with application of $N_2$ or without inert gas ($0_2$), revealed by the method HS-SPME coupled to GC-FID/Olfactometry

| No. | LRI U1 | Compound | Odour intensity during storage | | | | | | | | | | Odour description | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 month | | 1 month | | 2 months | | 3 months | | 4 months | | | |
| | | | $O_2$ | $N_2$ | $O_2$ | $N_2$ | $O_2$ | $N_2$ | $O_2$ | $N_2$ | $O_2$ | $N_2$ | | |
| 1 | 772.4 | hexanal | 0.5 | – | 0.5 | – | 0.5 | – | 0.5 | – | 0.5 | – | grassy, leafy, green, slight fruity | LRI, MS, ST, OD, LIT |
| 2 | 782.9 | ethyl butanoate | 1 | 1 | 1 | 1 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 2 | fruity, apple-like, sweet | LRI, MS, ST, OD, LIT |
| 3 | 822.8 | (E)-2-hexenal | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | green, leafy, apple pip-like, slightly bitter | LRI, MS, ST, OD, LIT |
| 4 | 926.6 | α-pinene | 1 | 1 | – | – | 0.5 | 0.5 | – | – | 0.5 | – | sharp, pine, terpenic | LRI, MS, ST, OD, LIT |
| 5 | 979.1<br>981.8 | octanal<br>β-myrcene | 1 | – | 1 | – | 1 | 0.5 | 1 | 0.5 | 1 | 1 | herbaceous, bitterish, terpenic, hop oil-like | LRI, MS, ST, OD, LIT |
| 6 | 1001.3 | δ-3-carene | 2 | 2 | 2 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | turpentine-like, sweet citrus, sharp | LRI, MS, ST, OD, LIT |
| 7 | 1005.9 | α-terpinene | 0.5 | – | 0.5 | – | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | balsamic, herbaceous, marjoram-like | LRI, MS, ST, OD, LIT |
| 8 | 1008.6 | p-cymene | 1 | – | 1 | – | 1 | 1 | 1 | 1 | 1 | 1 | citrus-peel, fresh, weak fuel-like | LRI, MS, ST, OD, LIT |
| 9 | 1018.1 | D-limonene | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | citrus, terpenic, intensive citrus-peel odour | LRI, MS, ST, OD, LIT |
| 10 | 1018.5 | (Z)-β-ocimene | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | lime, green, sweet, lemon, orange | LRI, MS, ST, OD, LIT |
| 11 | 1054.3 | 1-octanol | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1.5 | 1 | 1.5 | herbaceous, earthy, waxy | LRI, MS, ST, OD, LIT |
| 12 | – | α-terpinolene[t] | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | mushroom-like, plastic | MS, OD, LIT |
| 13 | 1081.4 | nonanal | – | – | 1 | – | 2 | – | 2 | – | 2 | – | soapy-fruity, waxy, tallowy | LRI, MS, ST, OD, LIT |
| 14 | 1083 | linalool | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | refreshing, floral, fragrant | LRI, MS, ST, OD, LIT |
| 15 | 1102.2 | ethyl 3-hydroxyhexanoate | – | – | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | smoky, leather, tobacco | LRI, MS, ST, OD, LIT |
| 16 | – | L-limonene[t] | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | intensive fresh floral, rose, sweet orange | OD, LIT |
| 17 | 1157.1 | terpinen-4-ol | 1 | – | 1 | 1.5 | 2 | 1.5 | 1 | 1 | 1 | 1 | earthy, woody, musty, waxy | LRI, MS, ST, OD, LIT |
| 18 | 1180.3 | decanal | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 1 | 2 | 1 | orange peel-like, waxy | LRI, MS, ST, OD, LIT |
| 19 | – | perillaldehyde[t] | 1 | 0.5 | 1 | 0.5 | 1 | 0.5 | 1 | 0.5 | 1 | 0.5 | fresh, herbal, cumin, spicy | MS, OD, LIT |
| 20 | 1285.3 | undecanal | 0.5 | 0.5 | 0.5 | 0.5 | 1 | 1 | 1 | 1 | 1.5 | 1.5 | fatty with orange and rose undertone, waxy | LRI, MS, ST, OD, LIT |
| 21 | 1360 | geranyl acetate | – | 0.5 | – | 0.5 | – | 0.5 | – | 0.5 | – | 0.5 | fresh, green, lavender | LRI, MS, ST, OD, LIT |
| 22 | – | δ-cadinene[t] | 1 | – | 1 | – | 1 | – | 1 | – | 1 | – | thyme, slightly sweet, herbal, woody | MS, OD, LIT |
| 23 | – | unknown[o] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | pleasant, floral, slight fruity, conditioner-like | – |

Compounds identified on the basis of following criteria: LRI [U1]–linear retention index measured on GC column Ultra 1; MS(EI)-mass spectrum; ST-comparison with the reference compound; OD-odour quality; LIT-literature reference. [t]- tentative identification (only on the basis of mass spectra); [o]- compound detected only by GC-O

One of the principal findings of the study is that production of juices under inert atmosphere $N_2$ or $CO_2$ can protect their standard organoleptic quality from undesirable changes caused by oxidative load or acid-catalysed reactions during the guaranteed storage period.

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## References

1. Seideneck R., Schieberle P. (2011) Eur Food Res Technol 232: 995-1005.
2. Brat P., Rega B., Alter P., Reynes M., Brillouet J.M. (2003) J Agric Food Chem 51: 3442-3447.
3. Rega B., Fournier N., Nicklaus S., Guichard E. (2004) J Agric Food Chem 52: 4204-42012.
4. Averbeck M., Schieberle P. (2009) Eur Food Res Technol 229: 611-622.
5. Jordán M.J., Goodner K.L., Laencina J. (2003) Lebensm Wiss Technol 36: 391-396.
6. Jordán M.J., Goodner K.L., Castillo M., Laencina J., Peyron S., Guillard V., Chalier, P. (2005) J Sci Food Agric 85: 1065-1071.
7. Sádecká J., Polovka M., Kolek E., Belajová E., Tobolková B., Daško Ľ., Durec (2014) J Food Nutr Res 67: 113-121.
8. Farnworth E.R., Lagacé M., Couture R., Yaylayn V., Stewart B. (2001) Food Res Int 34: 25-30.
9. Vervoort L., Grauwet T., Kebede B.T., Van der Plancken I., Timmermans R., Hendrickx M., Van Loey, A. (2012) Food Chem 134: 2303-2312.
10. Petersen M.A., Tønder D., Pool L. (1998) Food Qual Prefer 9: 43-51.
11. Moshonas M.G., Shaw P.E. (1989) J Agric Food Chem 37: 157-161.
12. Averbeck M., Schieberle P. (2011) Eur Food Res Technol 32: 129-142.
13. Moshonas M.G., Shaw P.E. (2000) J Food Qual 23: 61-71.
14. Berlinet C., Ducruet V., Brillouet J.M., Reynes M., Brat P. (2005) Food Addit Contam 22: 185-195.
15. Berlinet C., Brat P., Ducruet V. (2008) Packag Technol Sci (2008) 86: 2206-2212.
16. Van den Dool H., Kratz P. (1963) J Chromatogr A 11: 463-471.


# FLAVOUR & OFF-FLAVOUR OF NON-FOOD PRODUCTS

# Odorants in non-food products – Is there more to them than just their smell?

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## Abstract

Odours associated with contemporary products are encountered frequently on a daily basis, often with intense potency, yet their chemical nature has been barely investigated, to date. Several studies were performed to identify the substances that cause sensory defects in products that included children's toys, adhesives, and post-consumer plastic waste and their corresponding recycled polymers, which were analysed using odorant analytical techniques derived from the methods used in flavour research. A wide range of odorants were identified in these diverse matrices, with noticeable clustering of certain functional groups or substance classes. This chapter summarises previous findings according to the substance classes of the corresponding odorants and describes the analytical procedures employed for the targeted identification of odorants in polymer matrices.

## Introduction

Contemporary products often emanate unusual or unpleasant smells that people are repeatedly confronted with in their everyday lives [1-6]. As with many undesirable smells, consumers report concerns over associated health risks, yet equally, an increasing number of consumers no longer react to such olfactory (warning) signals due to their (mis)belief that some smells, for example, 'plastic', are entirely normal and should not be a cause for concern. In many cases, such smells indeed might be simply harmless by-products of production processes that dissipate quickly after purchase, yet smells of other products can be caused by hazardous compounds. Conversely, however, odourless is not synonymous with harmless.

The continual development and global ubiquity of contemporary materials and associated products presents an increasingly pressing need to monitor and control their quality. In view of this it is interesting to note that the general issue of non-intentionally added substances (NIAS) has received increasing attention in product quality control screenings [7,8].

Recently, we published several studies that explored the odorous artefacts associated with modern materials and products, including those derived from woods, plastics, polymers, consumer waste regrinds, colouring agents, glues, adhesives and binders, and reported on how products of potential concern can be 'sniffed out' analytically. Knowledge of the underlying chemical structures of smells related to these products provides an essential basis to elucidate their formation pathways and is the main premise for developing targeted avoidance strategies and adapted sensor technologies for controlling for such substances. Furthermore, the analytical results provide the basis for risk assessment strategies for those who are exposed to such odorous emissions, not only consumers, but also people that regularly handle such products vocationally.

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## Experimental

*Sensory evaluation*

The descriptive analyses of the non-food samples under investigation were carried out by a trained sensory panel. The samples were presented individually in covered glass-vessels. The panel members were asked to open the lid of the vessel and note their perceived odour impressions. After a consensus decision of the odour attributes by the panel, the selected impressions were rated on a scale from 0 (not perceived) to 10 (very strongly perceived). Additionally, the overall intensities were evaluated together with the hedonic ratings. The intensity assessment was performed according to an in-house method based on the industrial standard EN ISO 13299:2016.

*Isolation of the volatiles*

The identification of odour-active compounds in the samples utilised a non-selective extraction method. Samples were dissolved in high purity dichloromethane and stirred vigorously at room temperature for 30 min. After filtration, the resulting solvent extract was subjected to distillation under high vacuum using solvent-assisted flavour evaporation (SAFE) [9]. This technique ensures a careful isolation of the volatile compounds due to the mild distillation conditions, whereby the temperature of the water bath is held at 50°C and the apparatus at 55°C and under high vacuum. The distillate was then concentrated by Vigreux distillation and micro-distillation [10].

*Gas chromatography-olfactometry*

The presence of odour-active compounds in the sample distillates was screened using gas-chromatography olfactometry (GC-O). An aliquot of each distillate was applied to the GC-system by the cold on-column technique. This injection technique avoids the formation of breakdown products and the generation of new odorants. At the end of the GC capillary, the effluent was split (1:1; v/v) to a flame ionisation detector (FID) and an odour detection port (ODP), at which a trained panellist determined the odour quality of the odorous regions in the eluent gas. A linear retention index (RI) for each odour-active region was calculated according to van den Dool and Kratz [11]. Experimental details are given in the corresponding publications [1-6].

*Odour extract dilution analysis*

Odour extract dilution analysis (OEDA) is used to screen for the most odorous components in a sample distillate and thereby provides a measure of the impact of individual odorants to the overall odour impression of the sample [12]. For this purpose, each distillate was volumetrically diluted stepwise with dichloromethane (1+2; v/v) resulting in different solutions corresponding to odour-dilution (OD) factors. An aliquot of each dilution step was analysed by GC-O.

*Two-dimensional gas chromatography-mass spectrometry/olfactometry*

Unambiguous identification of the constituent odorants of a sample extract was carried out by comparing the mass spectra (electron ionisation (EI) mode at 70 eV), RI, and odour quality with those of corresponding reference substances. This procedure was conducted using two-dimensional gas chromatography-mass spectrometry/olfactometry (2D-GC-MS/O). This system allows odorous portions that elute after separation in the first capillary to be cryo-trapped and subsequently transferred onto a second capillary column with different polarity to that of the first. This provides further separation of volatiles that co-elute from the first capillary column.

## Results and discussion

*Mono- and polyunsaturated carbonyl compounds*

Carbonyl compounds are well known as aroma compounds in food, as are their mechanism of formation, such as for the unsaturated aldehydes (*E*)-non-2-enal or (*E*,*E*)-deca-2,4-dienal whereby the autoxidation of unsaturated fatty acids forms these fatty, cucumber or cardboard-like smelling compounds [13]. Diverse carbonyl compounds were identified in our recent investigations of non-food materials like toys, post-consumer packaging waste, and adhesives. (*E*)-Non-2-enal (fatty), (Z)-non-2-enal (fatty, green, musty), (*E*,*E*)-nona-2,4-dienal (fatty, peanut-like) and (*E*,*E*)-deca-2,4-dienal (fatty) were all found to be present in packaging waste and selected toys [1,2,4]. Other aldehydes included (*E*)-oct-2-enal (fatty, musty, peanut-like) in post-consumer packaging waste and toys [2,4], (*E*,*Z*)-nona-2,4-dienal (fatty) in diverse toys [1,2], and (*Z*)-dec-2-enal and (*E*)-dec-2-enal (fatty, metallic, and fatty, respectively) in packaging waste. Beside mono- and polyunsaturated aldehydes, some unsaturated ketones like the mushroom-like smelling oct-1-en-3-one [1,2,4] and hex-1-en-3-one with a glue-like odour [1,2] were additionally identified. Also, some epoxidised derivatives with metallic odorous attributes like *trans*-4,5-epoxy-(*E*)-non-2-enal [1] and *trans*-4,5-epoxy-(*E*)-dec-2-enal [1,2,4,5] were detected. All of these substances are likely to be formed by the degradation of fatty acids from trace contaminants, such as residual food matter in packaging waste or fatty acid-derived constituents such as fatty lubricants [14], which are used for the production of such materials.

*Phenol, guaiacol and alkylated or halogenated derivatives*

Phenol and guaiacol derivatives are classes of odorants that are ubiquitous in flora and fauna and are widely used as aromatising agents in the food and perfume industries [15,16], but they were also found to be present in modern plastic products. Phenol (typical phenolic odour), for example, was successfully identified in diverse aquatic toys and swimming aids [1], as well as in a plastic toy sword [6], and its derivative *p*-cresol (horse stable-like) was detected in a fancy-dress handbag for children [2], a toy sword [6], but also in adhesives, where the isomer *o*-cresol was also found to be present [5]. Another phenol derivative that was detected in several products was the leather-like smelling 3-ethylphenol [2,5,6], although its isomer 2-ethylphenol (phenolic odour) was only detectable in the toy sword sample [6]. The latter sample was found to additionally contain traces of 2-isopropylphenol (phenolic), 2-propylphenol (smoky), 3,5-dimethylphenol (phenolic), and 3- and 4-propylphenol (both leather-like, phenolic) [6]. In contrast, guaiacol (smoky) and the halogenated phenol derivative 2-bromophenol (medicinal, plaster-like) were only detected in acrylic adhesives.

The formation of these phenolic compounds in polymer matrices is not fully resolved, although their presence might relate to the use of phenolic antioxidants during the production of such products [17] and their formation during degradation. Nevertheless, other pathways, such as contaminated raw materials (e.g., pigments), might equally contribute to their presence.

*Naphthalene and derivatives*

Naphthalene is the smallest member of the polycyclic aromatic hydrocarbons (PAHs), a substance class that is commonly associated with health hazards due to the carcinogenic potential of these chemicals. In general, PAHs can be transferred into plastic products by contaminated raw materials such as extender oils or the pigment Carbon black [18].

Naphthalene contaminations in products are not only critical due to potential health hazards, but a secondary issue is the characteristic faecal or mothball-like odour that it can impart to the corresponding products. Polyvinyl chloride products, for instance, have been found to contain this compound, for example, with 74 mg/kg naphthalene detected in an inflatable plastic toy that had been investigated – and subsequently withdrawn from the market – due to its potent smell. It is worthy of note that the article in question was only sent to a specialised laboratory and analysed because its strange odour was noticed by a television crew, leading to media coverage on the issue [19]; as such, it is unlikely that this serendipitous finding is an isolated incident.

Methylnaphthalenes have also previously been responsible for product recalls, with a prominent case in 2010 when the Kellogg Company issued a voluntary recall of 28 million boxes of breakfast cereals in response to consumer reports of off-flavour and off-odour. This sensory defect could be traced back to hydrocarbon contaminations, including methylnaphthalene, in the wax paper liners of the cereal boxes. Unsurprisingly, neither the specific isomer nor the levels of 1- or 2-methylnaphthalene found in the tainted cereals have been made public [20].

These anecdotal examples give us an impression of the range of products in which contaminations of PAHs can occur, thus the detection of naphthalene and a variety of its derivatives in malodorous toys is unsurprising. Two of the products tested in our analyses – namely a fancy dress accessory handbag and a children's toy sword – were found to contain naphthalene as well as both 1- and 2-methylnaphthalene [2,6]. In addition, several dimethylnaphthalene isomers, namely 1,2- and 1,7-dimethylnaphthalene, were detected in both products [2,6]. The handbag additionally contained the isomers 1,4-, 1,5-, 1,6- and 2,6-/2,7-dimethylnaphthalene, as well as 2,3,5-trimethylnaphthalene, whereas 1,2-dihydronaphthalene and 2,6-diethylnaphthalene were present in the sword, and 3-methylisoquinoline was detected in both toys [2,6].

As mentioned above, naphthalene and both 1- and 2-methylnaphthalene exhibit faecal or mothball-like odours, and most of the aforementioned dimethylnaphthalene isomers exhibit similar odours. Surprisingly, the isomers 2,6- and 2,7-dimethylnaphthalene, as well as 2,6-diethylnaphthalene, exhibit anise-like odours, thus these results additionally provide new insights into structure-odour relations of these PAH compounds.

In addition to comprehensive molecular elucidation using classical and enhanced GC-MS approaches, often the absolute concentrations of such compounds within the sample matrix are not indicative of their emissions into the gas-phase, and consequently their exposure potential. GC-MS analyses can partially address this issue by the use of headspace gas sampling, but such methods are intermittent and provide only snapshots of emission profiles. In order to characterise the kinetics of release with higher resolution and accuracy, on-line chemical ionisation mass spectrometry in the form of proton-transfer-reaction mass spectrometry (PTR-MS) can be applied to follow the emissions of PAHs from such products [21]. Preliminary results (unpublished) from such PAH analyses on selected children's products revealed different release kinetics depending on the initial concentration in the material and nature of the material itself. Such insights assist in estimating risk assessment for exposure to affected products.

*Terpenes and their oxidation products*

Terpenes occur naturally in a large group of plants, bacteria, and in some insects, often as signalling molecules. Two biosynthetic pathways are involved in their formation,

the mevalonate pathway and the deoxylulose pathway, yet the formation of the building block isopentenylpyrophosphate (IPP) is common and fundamental in both. By coupling with another IPP unit, linear terpenes are formed and several changes by reactions such as cyclisation and hydrolysis lead to the large variety of terpenes [22, 23].

Terpenes have been used throughout the ages for a range of purposes, including perfumery and medical treatments such as aromatherapy. Resolving the underlying odorants of plants and resins such as frankincense is essential in this context in order to better understand the potential physiological benefits of their use. In the case of frankincense, several mono- and sesquiterpenes, often oxygenated, were found to be natural constituents of different varieties of the resin, specifically 1,8-cineole (eucalyptus odour), linalool (flowery, fresh, balsamic), verbenone (spicy, soup, bread), *trans*-carveol (mint, eucalyptus, green), carvone (mint, caraway, spicy), and thymoquinone (flatbread, black cumin) [24-26]. Furthermore, the hydrocarbon monoterpenes $\alpha$-pinene (rosiny, pine), $\beta$-myrcene (geranium), *p*-cymene (solvent-like, fruity), limonene (citrus, soapy, fresh), the sesquiterpene hydrocarbons germacrene (fruity, woody, cherry), $\alpha$-copaene (spicy, broth, woody) and the two oxygenated sesquiterpenes rotundone and mustakone were detected in frankincense samples.

As terpenes often exhibit flowery or fruity notes, they find application as fragrances, deodorants or masking ingredients in a variety of products including cosmetics, washing and cleaning agents. As the biosynthesis of these compounds are rare, terpenes used for such applications are typically produced by chemical synthesis. For example, $\beta$-ionone is routinely produced from citral and acetone via aldol condensation and cyclisation [27]. Besides toiletries and detergents, foods are often also rich in terpenes such as linalool and 1,8-cineol, especially spices [28]. This presents an issue for packaged foods, whose volatile compounds can migrate from the filling good into the packaging material, and indeed several terpenes have been identified to be present in plastic packaging waste and associated recycled materials [4], including, for example, β-ionone (violet-like) and $\alpha$-isomethylionone (rosy). In the case of $\beta$-ionone, this compound is not only a common perfuming ingredient, but may also be formed by oxidative degradation in food containing carotenes [29].

## Summary

Our analyses demonstrate that comprehensive state-of-the-art odorant analytical techniques – as routinely used in food science – are also a powerful tool to identify the odorants responsible for the intense smells of contemporary products. The odours of the analysed products were found to arise from diverse substances. Many of them, however, belong to the substance class of (poly-)unsaturated carbonyl compounds or derivatives of either phenol, guaiacol or naphthalene. Several odorants were also found to be terpenes or their derivatives. Depending on the substance, odorants might stem from contaminated raw materials or can be formed (as by-products) from contaminants or additives or via oxidation processes during production or storage.

## References


1. C. Wiedmer, C. Velasco-Schön and A. Buettner, (2017), Anal. Bioanal. Chem. 409: 3905–3916.
2. C. Wiedmer, C. Velasco-Schön and A. Buettner, (2017) Sci. Rep. 7: 1807.
3. L. Schreiner, H. Loos and A. Buettner (2017) Anal. Bioanal. Chem. 409: 3719-3729
4. M. Strangl, T. Fell, M. Schlummer, A. Maeurer and A. Buettner, (2017) J. Sep. Sci. 40: 1500-1507
5. P. Denk and A. Buettner, (2017) Int. J. Adhes. 78: 182-188.
6. P. Denk, C. Velasco-Schön and A. Buettner (2017) Anal. Bioanal. Chem. 409: 5249-5258.
7. J. Félix, F. Isella, O. Bosetti and C. Nerín, (2012) Anal. Bioanal. Chem. 403(10): 2869-2882.
8. C. Nerin, P. Alfaro, M. Aznar and C. Domeño (2013) Anal. Chim. Acta 775: 14-24.
9. W. Engel, W. Bahr and P. Schieberle (1999) Eur Food Res Technol. 209(3): 237-241.
10. J.M.H. Bemelmans. Land D.G. and Nustern H.E. (1979) Applied Science Publ. London Progress in flavour research, 79–98.
11. H. van Den Dool and Dec. P. Kratz. (1963) J Chromatogr A., 11: 463-471.
12. W. Grosch. Chem. Senses. 2001, 26(5): 533-545
13. W. Grosch and Chan H.W.-S. (ed.) Autoxidation of Unsaturated Lipids, London, Academic Press, 95–139. (1987)
14. F. Mayer and K. Breuer, (2006), Indoor air. 16(5): 373-82.
15. M. Schranz, K. Lorber, K. Klos, J. Kerschbaumer and A. Buettner, (2017) Food Chem. 232: 808-819.
16. J.A. Maga and I. Katz. (1978) Crit Rev Food Sci Nutr. 10: 323-37.
17. E. Baur, S. Brinkmann, T.A. Osswald, N. Rudolph, E. Schmachtenberg and H. Saechtling. Saechtling Kunststoff Taschenbuch. Carl Hanser Verlag; (2013).
18. P. Lassen, L. Hoffmann and M. Thomsen. PAHs in toys and childcare products. http://www2.mst.dk/udgiv/publications/2012/01/978-87-92779-49-6.pdf, 2012, Date of access: 05.10.2017.
19. M. Jost and M. Fuchs. Der Tchibo-Check. http://www.daserste.de/information/ratgeber-service/markencheck/sendung/der-tchibo-check-folge-2-102.html, Das Erste: 2016; aired on 2.5.2016.
20. S. M. Snedeker. In: Toxicants in Food Packaging and Household Plastics - Exposure and Health Risks to Consumers (ed. S. M. Snedeker), 2014, chapter 10, pp. 245 – 264.
21. J. Beauchamp and E. Zardin. In: Springer Handbook of Odor (ed. A. Buettner), 2017, chapter 18, pp. 355 – 408.
22. J. Chappell, (1995) Annu. Rev. Plant Biol., 46: 521-547.
23. H.K. Lichtenthaler, (1999) Annu. Rev. Plant Biol., 50: 47-65.
24. J. Niebler and A. Buettner, (2015) Phytochemistry, 109: 66-75.
25. J. Niebler, K. Zhuravlova, M. Minceva and A. Buettner, (2016) J. Nat. Prod., 79: 1160-1164.
26. J. Niebler and A. Buettner, (2016) A. Chemistry & biodiversity, 13: 613-629.
27. C. Noda, G. Alt, R. Werneck, C. Henriques and J. Monteiro, (1998) Braz. J. Chem. Eng., 15(2)
28. M.C. Díaz-Maroto, M.S. Perez-Coello and M.D. Cabezudo. (2002) J Chromatogr A. 94:23-29.
29. P. Kanasawud and J.C. Crouzet., (1990) J. Agr. Food Chem. 38: 237-243.

# The toilet malodor challenge

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## Abstract

Globally, 2.5 billion people lack access to adequate sanitation. To help address this issue, Firmenich partnered with the Bill & Melinda Gates Foundation initiative: Reinvent the Toilet Challenge.

A receptor-based discovery program was developed to identify malodor antagonists and to bring affordable, novel and effective toilet cleaning and freshening products to global markets. When integrated into cleaning products, and used as part of a regular hygiene and maintenance regime, our malodor counteracting technologies aim to promote sanitary environments.

This presentation will focus on human waste and toilet malodor analysis. We will explain how pit latrine headspace analyses were performed in crowded slums in Africa and India. Based on the diverse volatiles found, rigorous sensory analysis allowed us to develop fecal reconstitutions using only four molecules. Hedonic appreciation of odors (like or dislike) is driven by diverse factors including cultural heritage. We therefore validated the authenticity of our reconstitutions via sensory surveys of more than 400 subjects in Switzerland, Africa and India. In the meantime, the four key malodorant molecules were used to identify odor receptors which were expressed and screened using a library of volatile organic compounds to identify potential antagonists. We recreated the exact toilet conditions in terms of temperature, humidity and ventilation in model latrine cabins. These cabins were equipped with devices that delivered malodors, including $H_2S$ and methyl mercaptan. We concurrently monitored perfume release by solid supported delivery systems, analyzed the concentrations of antagonists in the air and conducted sensory analyses.

## Introduction

Offending toilet malodors resulting from the action of environmental microbiota on human waste to produce volatile organic chemicals (VOCs) makes toilet/latrine use undesirable to populations more accustomed to defecating in the open.

The compounds responsible for fecal malodor have been well-known since 1878 when a Swiss doctor distilled 50 kg of human fecal material to yield a crystalline compound he named skatole [1]. The first analysis of odorant compounds from feces was published in 1987 [2]. Subsequently, Sato et al reported the first exhaustive analysis of odorant compounds from human waste sludge [3] and the analysis of fresh fecal odorants in Western style toilets [4]. Unfortunately, these studies were insufficient to help us answer key questions related to the toilet malodor challenge, specifically, what are the odor differences between conventional and urine diverted toilets and how different is the smell of an African pit latrine compared with an Indian toilet. Additionally, we couldn't find any documented quantitative headspace analysis of key latrine odorants.

The project was based on two approaches to the control of malodor. The first was a traditional fragrance engineering approach, where perfumers used psychophysical data and their experience to build a fragrance which combined with the malodor to produce an acceptable or even pleasant smell. The second approach relied on a new breakthrough

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technology based on the identification of molecules that antagonize temporarily but very specifically the odor receptors (OR) involved in the perception of the malodorous chemicals. Humans express an estimated 360 ORs in their noses, and according to the experiments performed within this project, only a small fraction of these receptors are activated by fecal malodors. This second approach was riskier, because it required the development of new technologies, but we anticipated that the final perfume including malodor antagonists would be considerably more performant. For the second approach it was critical to find the key malodorant compounds to identify which ORs were activated. It was also critical to simplify the screening protocols and for this reason we focused on four key malodorant molecules.

This presentation reviews the odors associated with different toilet systems in India and Africa. The analytical challenges of working with potentially pathogenic materials, in crowded informal settlements, is explained. The development of new tools to evaluate our new malodor counteractant technologies and their validation process are also discussed.

## Experimental

The qualitative and quantitative analyses of latrine sludge VOCs by SPME and SPE was described in J. Environ. Sci. Technol. 2013 [5]. The quantification of $H_2S$, $CH_3SH$ and other selected VOCs in toilet headspace was described in the same journal [6]. The sensory survey of reconstituted latrine malodors was described in Flavour Fragr. J. [7].

## Results and discussion

*1. Challenges to the analysis of human excrement and sludge in informal settlements*

Analyzing urine is easier compared to feces because it is generally sterile, and, if not, can be easily sterilized using membrane filters [8]. Conversely, feces contain gut bacteria remains, proteoglycans, cellulosic fibers debris and can be contaminated by pathogenic species. While fresh stools can vary in consistency, they can usually be suspended in water and sterilized by ultra-filtration. Waste sludge, however, was a colloid and the membrane fouling made processing for analysis almost impossible. Consequently, SPME was gauged to be the most suitable analytical technique. Human waste, urine and feces were collected in a bucket which was covered with a lid into which a small hole had been drilled to allow insertion of a SPME fiber. The draw back was that the composition of VOCs was evolving. The action of ureases caused an increase in sample pH resulting in deprotonation of the organic acids which could consequently not be detected in the headspace. When fresh stools were analyzed without urine the VOC profile changed. Dynamic headspace was performed using Tenax cartridges but this technique had two drawbacks: we could not smell the extract, the rapid breakthrough of highly volatile compounds and finally we noticed an important diffusion of volatiles over time, even in closed tubes, which makes this technique not suitable for shipping Tenax tubes from Africa or India. Static headspace analysis using Porapak resin was better but the absorption process was slow, and it was not convenient to leave a device containing the resin hanging in the toilet without attendance overnight. In fact, many were stolen from the sampling sites. Finally, the analysis of the exposed Porapak resin, extracted with $Et_2O$ was very instructive. It was possible to smell the headspace extract back in the lab with perfumers and to perform GC-olfaction to determine that four classes of compounds were key sludge odorants, namely sulfur-containing compounds, short chain fatty acids, phenols, indole and skatole (Figure 1).

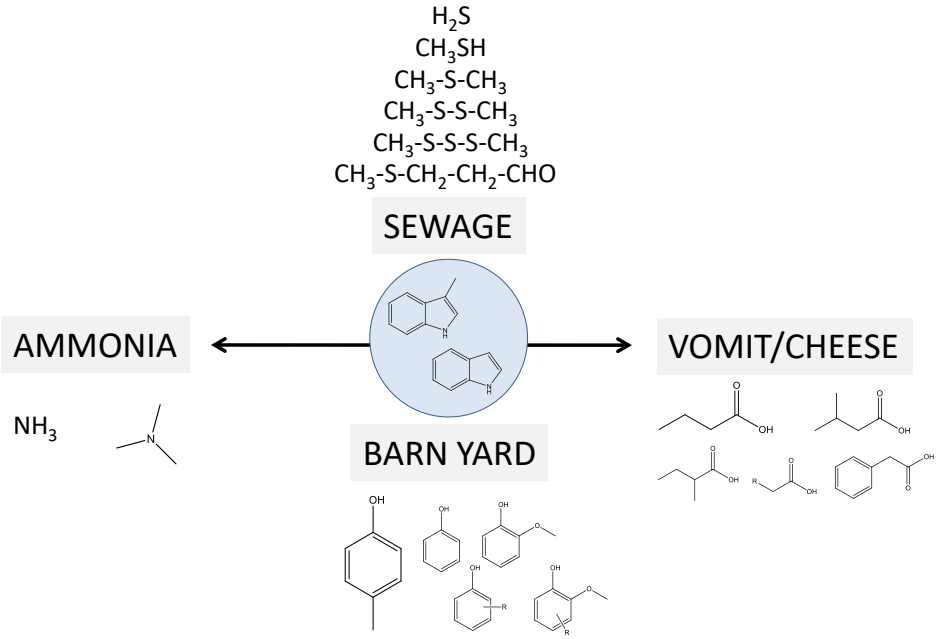


**Figure 1:** Schematic summary of VOC impairing toilet smell

Finally, sludge samples spiked with deuterated standards were analyzed in closed vials using SPME. The SPME fibers were thermally desorbed and analyzed by GC-MS in our labs in the USA.

*2. Learning from the first analytical campaign*

Latrine odors can be classified as resulting from anaerobic and aerobic microbial degradation. Typically, anaerobic latrines produced a strong $H_2S$, $CH_3SH$ sewage odor. This was mainly the case for toilets in India connected to sewage pipes or in South Africa when the sludge was covered by rain or infiltrated water. Latrines equipped with efficient ventilation ports didn't smell much. Urine-diverted toilets systems smelled more barn-yard and stale urine.

Based on the VOCs detected during our study, the malodor was reconstituted using pure chemicals. It was possible to simplify the formula to four chemicals while retaining its authenticity. Dimethyltrisulfide was used as a proxy for methylmercaptan, which was difficult to handle, and indole was used instead of skatole which was declared carcinogenic [9].

The logical next step was to confirm that the reconstitutions were representative of toilet malodors. To achieve this goal, sensory surveys were conducted in Africa, India and Switzerland. Three bad smells, one complex fecal odor reconstitution, one fecal odor containing only the four compounds and one urine odor reconstitution were submitted to panelists along with three pleasant smells: banana, citrus, lavender.

From the sensory surveys the conclusion was that the fecal reconstitutions were both evaluated as latrine malodor and unpleasant. From these results we were confident that identifying ORs activated by the four key malodorant compounds, and subsequently

antagonists to the activation, would help us to design the first malodor counteractant prototypes based on antagonists.

*3. Toilet headspace analysis*

For the sensory analyses, we used smelling sticks that had been impregnated with the reconstituted latrine malodor. The real latrine smell was quite different from what we could smell from the smelling sticks. The reason was that $H_2S$ and $CH_3SH$ are very important contributors to the real odor and the partition coefficients of short chain fatty acids is related to sludge pH and matrix interactions. During our second campaign we attempted to precisely quantify key malodor molecules in Indian and African latrines.

This was achieved by bubbling 350 L of latrine air through water in order to trap the VOCs. $H_2S$ and $CH_3SH$ were derivatized using N-ethylmaleimide (NEM). The aqueous extract was loaded on a SPE-Oasis cartridge which was shipped to Switzerland. To abide by flight regulations, the water eluted from the first SPE-Oasis cartridge was not acidified with HCl but with acidic sulfonic resin and reloaded onto a fresh SPE-Osasis cartridge to quantify butyric acid. Recovery factors, reproducibility, limits of detection and limits of quantification were established and verified using olfactometers and certified diluted $H_2S$ and $CH_3SH$ standards (Figure 2).

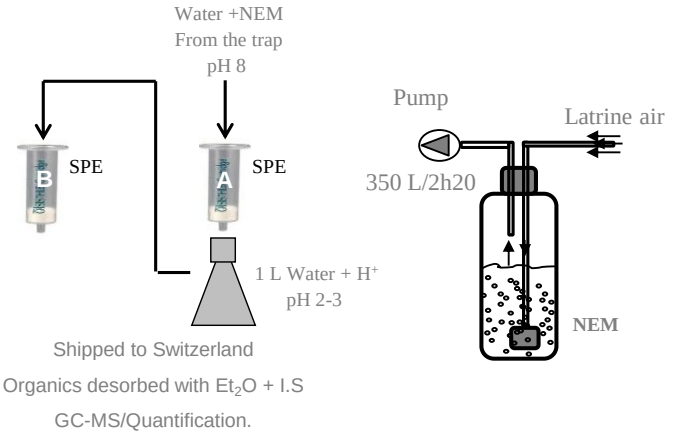


**Figure 2:** Schematic analysis of toilet headspace and quantification of $H_2S$ and $CH_3SH$ in addition to p-cresol, indole and butyric acid.

*4. Recreating latrine conditions in the laboratory to assess perfume performance*

Delivery systems can be used for extended release of and enhanced fragrance performance. When we obtained the first generation of malodor control prototype fragrances, we committed to evaluate their performance under realistic conditions. In the field many problems emerged. When cellulosic pads were used as delivery systems they were stolen and the citrus fragrance-containing pads were partially eaten. The air flows were totally different for different latrines, even those in the same block. When we conducted a sensory survey with local subjects we experienced all kinds of problems. For example, the toilet cleaning person cooked a curry in the ablution block, therefore the hedonic results were confounded, not due to the perfume but due to his cuisine.

For these reasons it was decided to build model toilet system using climate-controlled chambers where exact concentrations of malodors, at constant humidity and

temperature, were injected. Under these conditions the perfume prototype-containing delivery system performance was precisely evaluated by sensory analyses or by quantification of the VOCs in the headspace (Figure 3).

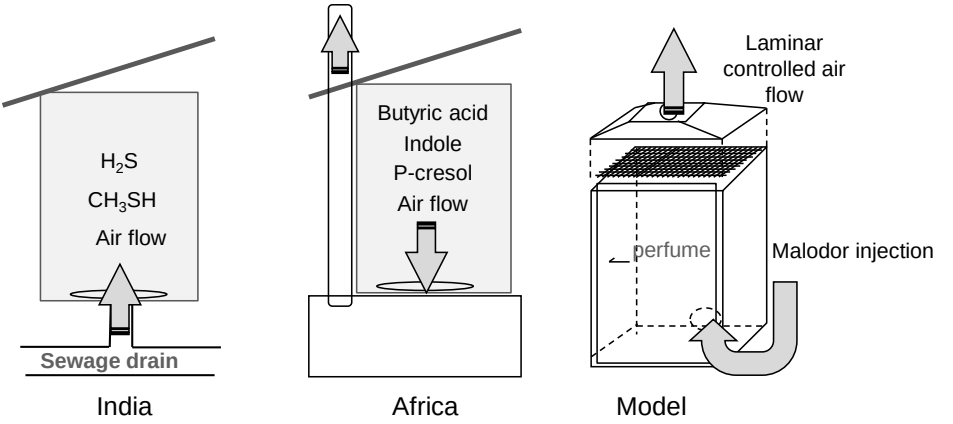


**Figure 3:** Trends in toilet systems visited. Arrows explain air flows.

## References

1. Brieger, L. (1878) J. Prakt. Chem., 179, 124−138.
2. Moore, J. G.; Jessop, L. D.; and Osborne, D. N. (1987) Gastroenterology, 93, 1321−1329
3. Sato, H.; Morimatsu, H.; Kimura, T.; Moriyama, Y.; Yamashita, T.; and Nakashima, Y. (2002) J. Health Sci., 48, 179−185
4. Sato, H.; Hirose, T.; Kimura, T.; Moriyama, Y.; and Nakashima, Y. (2001) J. Health Sci., 47, 483−490
5. Lin, J.; Aoll, J.; Niclass, Y.; Velazco, M. I.; Wünsche, L.; Pika, and J.; Starkenmann, C. (2013) Environ. Sci. Technol., 47, 7876−7882.
6. C.J. Chappuis, Y. Niclass, C. Vuilleumier, and C. Starkenmann. (2015) Environ. Sci. Technol. 49, 6134−6140
7. C.J. Chappuis, Y. Niclass, I. Cayeux and C. Starkenmann. (2016) Flavour Fragr. J., 31, 95–100
8. M. Troccaz, Y. Niclass, P. Anziani and C. Starkenmann, (2013) Flavour Fragr. J., 28, 200–211
9. J.M. Weems and G.S. Yost (2010) Chem. Res. Toxicol.; 23: 696–704.

# Key odorants in the artificial leather of car interiors

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## Abstract

Application of an aroma extract dilution analysis (AEDA) to the volatiles isolated from a PVC based automotive artificial leather with representative odour characteristics by solvent extraction and solvent-assisted flavour evaporation (SAFE) revealed 22 odour-active compounds in the flavour dilution (FD) factor range of 4 to 64. The three compounds with the highest FD factor of 64 were plastic-like, pungent smelling 1-hexen-3-one, mushroom-like smelling 1-octen-3-one, and plastic-like smelling acetophenone. Quantitation of these three compounds was accomplished by stable isotope dilution assays (SIDAs). On the basis of the obtained concentrations and oil as matrix, an odour reconstitution model was prepared that clearly reflected the typical odour of the artificial leather. Omission tests in combination with quantitative descriptive analyses (QDAs) showed that 1-hexen-3-one contributed most to the overall odour, but 1-octen-3-one and acetophenone modified the sensory impression of the mixture. Quantitation of 1-hexen-3-one in a variety of other PVC materials, including baby toys, inflatable beach toys, and a flexible PVC tubing for beverage industry applications finally showed that the problem of odour-active amounts of 1-hexen-3-one is not limited to artificial leather.

## Introduction

The interior of new cars often exhibits a more or less strong odour. Many modern consumers consider this "new car odour" as unpleasant and it has been shown that it may have a huge influence on the purchase decision [1]. As a consequence, the automotive industry currently tries hard to reduce the interior odour towards an almost odourless new car. A targeted minimization approach, however, requires knowledge of the causal compounds and their source materials. So far, little data has been published on this topic. Polyurethane foams included in headliners and floor carpets have been identified as source of fishy smelling compounds such as benzyldimethylamine (BDMA) and pentamethyldipropylenetetramine (PMDPTA) [2, 3]. The role of PVC materials, however, was yet unclear.

The aim of the present study was to get an insight into the major compounds contributing to the odour of PVC based artificial leathers. Such materials are widely used in the automotive industry for upholstery and interior covers. On the one hand, artificial leathers are very versatile, allowing for a wide range of optical (colour, gloss) and haptic adjustments. On the other hand, they are very resistant against scratching and aging [4]. However, PVC materials are known to be highly odorous and numerous volatiles emitted from PVC have been characterized including plasticizers, solvent residues, unreacted monomers, and secondary degradation products [5]. However, little information is available on the odour activity of volatiles emanated from PVC materials [6, 7].

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## Experimental

### *Materials*

The automotive artificial leather was provided by a German car manufacturer. It was ~1 mm thick and consisted of a main layer of expanded PVC, which was glued to a polyester fabric on the back side and covered by a layer of dense PVC and a transparent lacquer film on the front side. A flexible PVC tubing for industrial beverage handling was obtained from ESSKA.de (Hamburg, Germany). All other PVC products were purchased from local shops in Freising, Germany. For analyses, the materials were cut into small pieces by scissors or knives and further crushed by use of a cryomill (Retsch, Haan, Germany) at −196 °C.

### *Reference odorants*

1-Hexen-3-one and acetophenone were purchased from Sigma-Aldrich (Taufkirchen, Germany). 1-Octen-3-one was from Alfa Aesar (Karlsruhe, Germany).

### *Workups*

Solvent extraction was accomplished by using dichloromethane. Extraction time was 72 h. Light was excluded during extractions. Non-volatile material was removed by solvent-assisted flavour evaporation (SAFE) [8]. SAFE distillates were concentrated by using a Vigreux column (50 cm × 2 cm). For the volatile isolate used for gas chromatography-olfactometry and aroma extract dilution analysis (AEDA), 150 g material and 1 L solvent were used and the distillate was concentrated to 1 mL.

### *Gas chromatography-olfactometry (GC-O)*

GC-O was performed by using a gas chromatograph equipped with a cold-on-column injector and a DB-FFAP column, 30 m × 0.32 mm i.d. × 0.2 µm film thickness, or a DB-5 column, 30 m × 0.32 mm i.d. × 0.2 µm film thickness (both J&W, Agilent Technologies, Waldbronn, Germany). The eluate of the column was split 1:1 using a glass splitter and the volatiles were simultaneously transferred to an FID and a tailor-made sniffing port [9]. AEDA was done as detailed in [10].

### *Quantitations*

These were accomplished by stable isotope dilution assays using the following stable isotopically substituted analogues of the target compounds as internal standards: ($^2H_2$)-1-hexen-3-one [11], ($^2H_4$)-1-octen-3-one [12], and ($^2H_5$)acetophenone (Sigma-Aldrich). Work-up was done as detailed above. Internal standards were added at the beginning of the extraction period. Selective recording of analytes and standards was done by GC-MS analysis using a GC×GC-TOFMS system [13] and concentrations were calculated as detailed in [13].

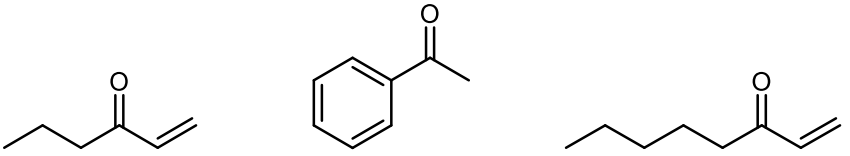
### *Sensory tests*

These were performed by a trained panel of males and females, aged 23-36. Odour threshold values were determined according to the ASTM method [14]. Odour reconstitution models were prepared in odourless sunflower oil. Descriptors used in the QDAs were collected by a preceding free choice profiling test. During QDAs, descriptors were defined by reference solutions of the following odorants: 1-hexen-3-one (pungent, plastic), 1-octen-3-one (mushroom), (*E*, *Z*)-2,4-decadienal (fatty), nonanal (citrusy, soapy), and ethyl 2-methylbutanoate (fruity). For each descriptor the intensity was rated on a seven-point scale using 0.5 increments and a range from 0 to 3 with 0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong.

## Results and discussion

*Odorant screening by GC-O and AEDA*

Automotive artificial leather with a characteristic odour was obtained from a car manufacturer. The volatiles were isolated using solvent extraction and SAFE. GC-O of the concentrated volatile fraction using an FFAP column resulted in 22 odour-active zones. Application of an aroma extract dilution analysis revealed flavour dilution (FD) factors in the range of 4 to 64 (data not shown). Ten odorants showed an FD factor of 4, four showed an FD factor of 8, and five showed an FD factor of 16. The remaining three compounds exhibited an FD factor of 64, thus were the most potent odorants in the extract. Among them, two compounds showed an odour reminiscent of plastic and the third compound smelled like mushrooms. Comparison of their odour qualities and their retention indices on the FFAP column with published data suggested them to be 1-hexen-3-one, acetophenone, and 1-octen-3-one. These structure assignments were confirmed by GC-O analysis of reference compounds in parallel to the artificial leather extract using the FFAP column as well as a DB-5 column and by GC-MS analysis (Figure 1).



**1-hexen-3-one**
odour: plastic-like, pungent
$RI_{DB\text{-}5}$: 775; $RI_{FFAP}$: 1093

**acetophenone**
odour: plastic-like
$RI_{DB\text{-}5}$: 1312; $RI_{FFAP}$: 1654

**1-octen-3-one**
odour: plastic-like
$RI_{DB\text{-}5}$: 979; $RI_{FFAP}$: 1293

**Figure 1:** Most potent odorants (FD 64) in the automotive artificial leather

*Quantitation of 1-hexen-3-one, 1-octen-3-one, and acetophenone*

Quantitation of 1-hexen-3-one, 1-octen-3-one, and acetophenone in the automotive artificial leather was accomplished by stable isotope dilution assays using ($^2H_2$)-1-hexen-3-one, ($^2H_4$)-1-octen-3-one, and ($^2H_5$)acetophenone as internal standards. Results revealed concentrations of 0.40 µg/kg for 1-hexen-3-one, 41.0 µg/kg for 1-octen-3-one, and 5600 µg/kg for acetophenone (Table 2). Comparison of these data with the odour threshold values of the three compounds in water, which were determined to be 0.00069 µg/kg (1-hexen-3-one), 0.016 µg/kg (1-octen-3-one), and 26 µg/kg (acetophenone), confirmed their high odour potency.

**Table 1:** Concentrations of the major odorants in the automotive artificial leather

| *Odorant* | *Odour* | *Concentration (µg/kg)*[a] |
|---|---|---|
| 1-hexen-3-one | plastic-like, pungent | 0.40 ± 0.01 |
| acetophenone | plastic-like | 5600 ± 26 |
| 1-octen-3-one | mushroom-like | 41.0 ± 1.0 |

[a] mean of triplicates ± standard deviation

*Odour reconstitution and omission experiments*

On the basis of the quantitative data detailed in Table 1, a reconstitution model was prepared from the pure compounds and oil as matrix. The model was compared

orthonasally with the artificial leather in a QDA. Results showed a good agreement of the odour profiles of the original material and the model (Figure 2).

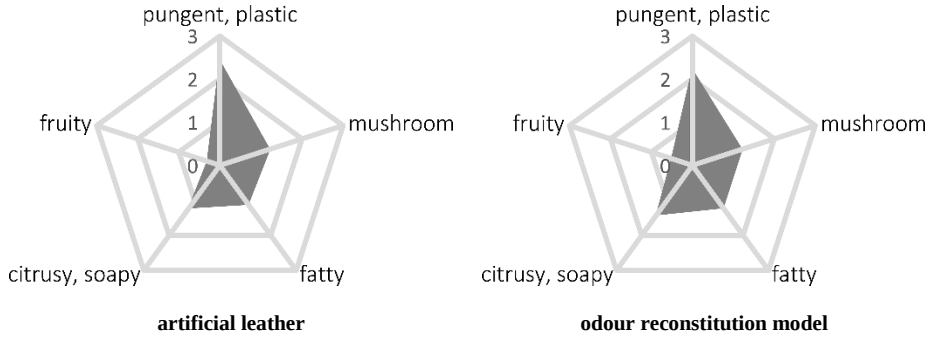



**Figure 2:** Orthonasal odour profiles of the artificial leather (left) and the odour reconstitution model (right) as obtained by QDA

To get a deeper insight into the individual odour contribution of the three odorous components, omission tests were performed. Omission of one component resulted in three binary mixtures that were compared to the complete model in triangle tests. The three tests revealed significant differences, indicating that all three compounds contributed to the odour of the mixture (Table 2). However, omission of 1-hexen-3-one was detected with higher significance ($p$ = 0.00004) than omission of 1-octen-3-one ($p$ = 0.01) and acetophenone ($p$ = 0.005). Thus, 1-hexen-3-one obviously played a key role for the odour of the artificial leather.

**Table 2:** Results of omission tests applied to the artificial leather odour reconstitution model

| *Odorant omitted* | *Correct answers* | *p value* | *Significance* |
|---|---|---|---|
| 1-hexen-3-one | 26/40 | 0.00004 | *** |
| acetophenone | 26/50 | 0.005 | ** |
| 1-octen-3-one | 25/50 | 0.01 | ** |

Results of the triangle tests were confirmed by QDA of the three binary mixtures and by QDA of models containing only one of the three compounds (Figure 3). Omission of 1-hexen-3-one clearly reduced the plastic-like note. On the other hand, the model containing only 1-hexen-3-one (Figure 3, upper left) showed a profile that was already quite close to the profile of the tertiary mixture (Figure 2, right).

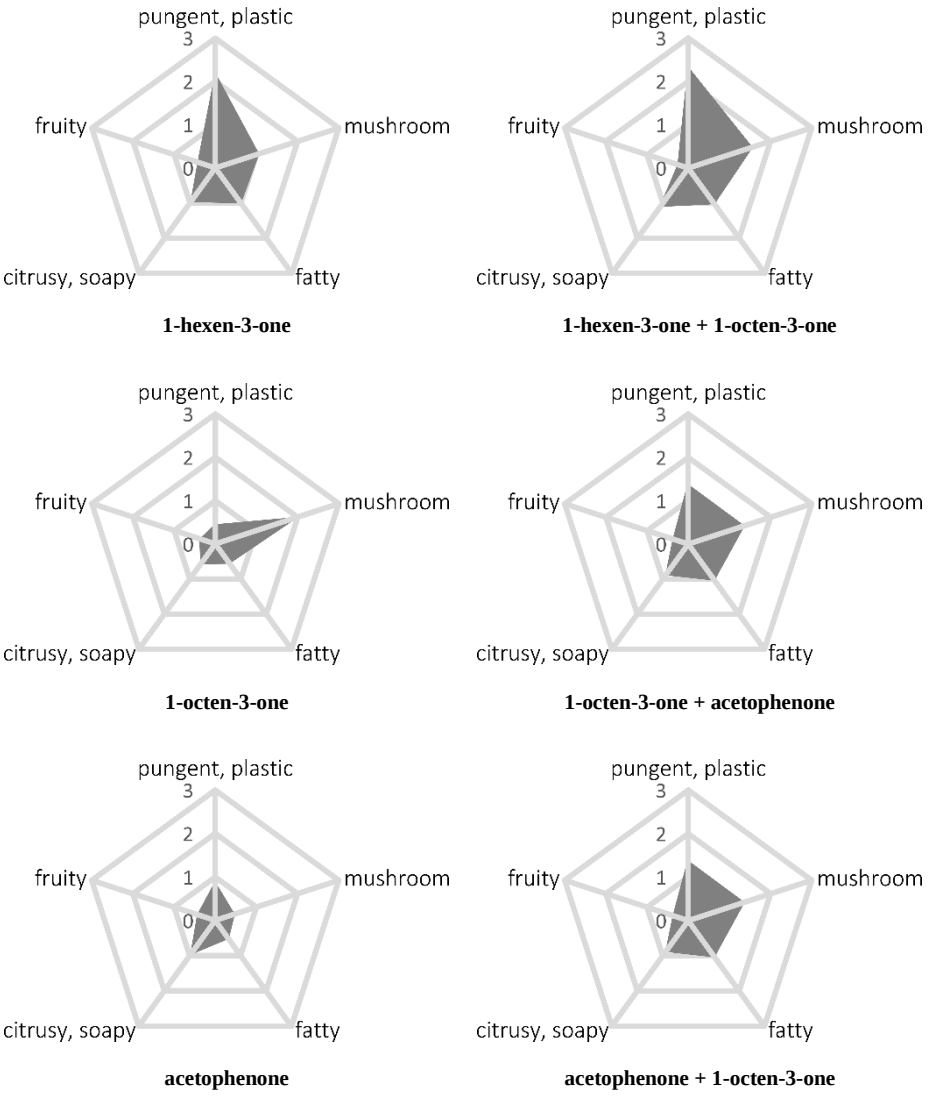



**Figure 3**: Orthonasal odour profiles of single compound models (left) and binary mixtures (right) obtained by omitting one or two compounds from the complete artificial leather odour reconstitution model

*Concentrations of 1-hexen-3-one in other PVC products*

To put the 1-hexen-3-one concentration found in the automotive artificial leather into perspective, we quantitated the compound in various other PVC products. Among them was a small water fun toy in the form of a dolphin, a baby toy in the form of a dinosaur, two inflatable beach balls, swimming aids in the form of inflatable armbands, and a PVC tubing intended for industrial beverage handling. Results (Table 3) showed concentrations in the range of 0.621 to 11.8 µg/kg. Thus, the 1-hexen-3-one concentration in all analysed materials was higher than the concentration previously determined in the

automotive artificial leather. The highest concentration was found in an inflatable beach ball, but high concentrations were also determined for the baby toy in the form of a dinosaur (10.1 µg/kg), another inflatable beach ball of different brand (7.34 µg/kg), and the beverage tubing material (6.93 µg/kg). Clearly lower 1-hexen-3-one concentrations were found in the inflatable armbands (1.81 µg/kg) of a well-known brand and the rather highly priced water fun toy in the form of a dolphin (0.621 µg/kg).

**Table 3:** Concentrations of 1-hexen-3-one in various PVC products

| *PVC material* | *Concentration of 1-hexen-3-one (µg/kg)*[a] |
|---|---|
| water fun toy dolphin | 0.621 ± 0.033 |
| baby toy dinosaur | 10.1 ± 0.8 |
| inflatable beach ball I | 7.34 ± 0.25 |
| inflatable beach ball II | 11.8 ± 1.1 |
| inflatable armbands (swimming aids) | 1.81 ± 0.08 |
| beverage tubing | 6.93 ± 0.34 |

[a] mean of triplicates ± standard deviation

**Acknowledgements**

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## References

1. Knoblich H., Scharf A., Schubert B. (2003) Marketing mit Duft. Oldenbourg, München. pp. 77-78.
2. Steinhaus M., Schieberle P. (2011) In: Advances and Challenges in Flavor Chemistry & Biology. Proceedings of the 9th Wartburg Symposium (Hofmann T., Meyerhof W., Schieberle P., eds.) Verlag Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, pp. 337-340.
3. Rampfl M., Mayer F., Breuer K., Holtkamp D. (2006) In: Proceedings of the 12th International Conference on Indoor Air Quality and Climate, Austin, TX, USA, University of Texas at Austin, paper 993.
4. Schlott S. (2011) ATZ - Automobiltechnische Zeitschrift 113: 884-887.
5. Järnström H., Saarela K., Kalliokoski P., Pasanen A.-L. (2007) Environ. Int. 34: 420-427.
6. Kong P., Lin X., Wang M. (2011) Suliao Keji 39: 95-98.
7. Wypych G. (2013) Handbook of odors in plastic materials, ChemTec Publishing, Toronto, Canada.
8. Engel W., Bahr W., Schieberle P. (1999) Eur Food Res Technol 209: 237-241
9. Steinhaus M. (2015) J Agric Food Chem 63: 4060-4067.
10. Schieberle P., Grosch W. (1987) Z Lebensm Unters Forsch 185: 111-113.
11. Thorkildsen J (2014) Characterization of key aroma compounds formed during peroxidation of bulk fish oil and insights into oxidation mechanisms. Verlag Deutsche Forschungsanstalt für Lebensmittelchemie, Freising.
12. Kubickova J., Grosch W. (1998) Int Dairy J 8: 17-23.
13. Reglitz K., Steinhaus M. (2017) J Agric Food Chem 65: 2364-2372.
14. American Society of Testing and Materials (2005). In: ASTM Book of Standards, Vol. 15.08, standard E679-04, American Society of Testing and Materials, West Conshohocken, PA, USA, pp. 38-44.

# What does wood smell like? Characterization of odorants in wood

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## Abstract

Wood is a material humans come into contact with every day, e.g., in the form of furniture and building materials, products of daily use such as pencils and toys, or secondary products that are derived from wood such as paper and cardboard. Whereas general emissions of volatile organic compounds from wood are well known, only limited information is available on the odour-active substances. The present study therefore aimed at specifically elucidating the odorous constituents of wood. To gain an overview of the odorants emitted by wood, two different wood species were investigated.

Targeted odorant analysis requires specialized techniques combining modern odorant analytical tools with human-sensory evaluation. Following this concept, the wood samples were first evaluated by human sensory analysis. The odorants were then characterized by gas chromatography-olfactometry (GC-O) and ranked according to their odour potency via aroma extract dilution analysis (AEDA). Using this approach, more than 60 odorous substances were detected and the most potent odorants were identified by gas chromatography-mass spectrometry/olfactometry (GC-MS) and two-dimensional gas chromatography-mass spectrometry/olfactometry (2D-GC-MS/O).

## Introduction

Previous investigations have predominantly focused on odorants in wood from wooden barrels that are used for wines and spirits, and their impact on the filling goods. Thus, mainly wood types with a potential usage in the alcoholic beverage production have previously been investigated and the samples were, according to their usage, commonly toasted. Following this conception, different oak woods as well as extracts from chestnut, acacia, cherry, and ash woods have already been analysed regarding their odorants.[1,2] In contrast to that, information about odorous substances in untreated wood, especially in softwoods, is rare. To close this gap, we focused on the elucidation of odorants in natural wood samples. Therefore, wood samples of incense cedar, which is commonly used for a range of products like pencils or furniture, and Scots pine, one of the most common trees in Germany, were investigated.

## Experimental

Wood samples of incense cedar (*Calocedrus decurrens* (Torr.) Florin) and Scots pine (*Pinus sylvestris* L.) were supplied by Staedtler Mars GmbH & Co KG (Nuremberg, Germany). The samples were delivered in form of cuttings which were planed into wood shavings and were then directly used for analysis without any further treatment. The samples were analysed by a trained sensory panel prior to extraction to elaborate the respective odour profiles. For the isolation of the volatiles, 2.5 g of the wood shavings were mixed with 100 ml dichloromethane. The solution was stirred at room temperature for 30 min and thereafter immediately applied for solvent assisted flavour evaporation

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(SAFE) [3]. Aroma extract dilution analysis (AEDA) [4] was performed using GC-O [5]. The most potent odour-active compounds were identified using GC-MS/O and 2D-GC-MS/O by comparing the odour quality, linear retention index [6], and mass spectrum with the properties of the respective reference compounds. Experimental details for the sensory evaluation as well as the instrumental analysis were as described in Schreiner et al. 2017 [5].

## Results and discussion

The odour profile analyses (cf. figure 1) showed that the smell of the incense cedar wood sample was dominated by a pencil-like note showing the highest intensity (5.2) followed by a sawdust-like odour impression (2.9). In contrast to that, the Scots pine wood sample smelled strongly resin-like with an intensity of 7.1 followed by sawdust-like (2.7) and frankincense-like notes (2.6). AEDA showed 16 substances to be the most potent odorants in Scots pine wood or incense cedar wood, respectively, with flavour dilution (FD) factors of ≥729 (cf. table 1). 14 of these substances were successfully identified. Most of the odour-active compounds are commonly known fatty acid degradation products like unsaturated alkenals and dialkenals with fatty smells, or acids like butanoic and heptanoic acid. Moreover, a group of terpenoic substances was found, *inter alia* the woody, resinous smelling α-pinene or α-bisabolol (balsamic, peppery). Another prominent group of odour-active constituents in both wood types were phenyl compounds such as vanillin or p-cresol, occurring due to the degradation of lignin. Two substances with a sweaty, perfume-like, androstenone-like smell remained unknown in the cedar samples, but could be tentatively identified in the pine wood samples as androst-2,16-diene and (5β)-androst-2-en-17-one.

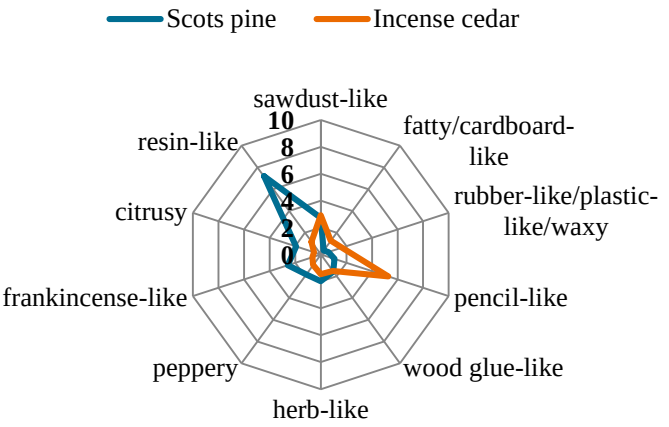


**Figure 1:** Odour profiles of Incense cedar and Scots pine wood

The results of the sensory evaluation show a close agreement with the odour qualities of the identified odorants. The most potent attribute for the Scots pine wood was resin-like which could be correlated with α-pinene, whereas the main attribute chosen to describe the smell of the incense cedar was pencil-like. This smell impression appears to

result from the pronounced smell impact associated with thymoquinone. Thymoquinone as naturally occurring molecule with a pencil-like smell was a new finding [5]. Thymoquinone is known to be the major compound in black cumin seed (*Nigella sativa* L.) [7] and contributes with its bioactivity for example to the antioxidant [8] and anti-inflammatory [9] activities of black cumin seed essential oil.

**Table 1:** Odorant compounds, their retention indices, flavour dilution (FD) factors, and odour qualities as identified in incense cedar and Scots pine wood

| Substance | *Odour quality* | *RI DB-FFAP* | | *FD-factor* | |
|---|---|---|---|---|---|
| | | *Incense cedar* | *Scots pine* | *Incense cedar* | *Scots pine* |
| α-Pinene | woody, resinous | 1029 | 1032 | ≥ 729 | ≥ 729 |
| (*E*)-Non-2-enal | fatty | 1533 | 1526 | ≥ 729 | 243 |
| 3-Methylbutanoic acid | cheesy | 1664 | 1677 | ≥ 729 | 9 |
| (*E,E*)-Nona-2,4-dienal | fatty | 1700 | 1700 | ≥ 729 | ≥ 729 |
| (*E,E*)-Deca-2,4-dienal | fatty | 1810 | 1810 | 81 | ≥ 729 |
| Heptanoic acid | red pepper-like, plastic-like | n.d. | 1942 | n.d. | ≥ 729 |
| δ-Octalactone | coconut-like | 1920 | 1984 | | ≥ 729 |
| p-Cresol | horse-like | 2100 | 2089 | ≥ 729 | 27 |
| Sotolone | savoury | 2222 | 2212 | ≥ 729 | 243 |
| α-Bisabolol | blasamic, peppery | 2250 | 2255 | ≥ 729 | 9 |
| Phenylacetic acid | honey-like | 2563 | 2567 | 243 | ≥ 729 |
| Vanillin | vanilla-like | 2588 | 2594 | ≥ 729 | ≥ 729 |
| 3-Phenylpropanoic acid | metallic, fruity, vomit-like | 2625 | 2640 | ≥ 729 | ≥ 729 |
| (5β)-Androst-2-en-17-one[1] | sweaty, perfume-like, androstenone-like | 2875 | 2878 | ≥ 729 | 81 |
| Androst-2,16-diene[1] | sweaty, perfume-like, androstenone-like | 2986 | 2927 | ≥ 729 | 27 |
| Thymoquinone | pencil-like | 3100 | 3100 | 243 | ≥ 729 |

[1] tentatively identified

Additionally, it was the first time that 3-phenylpropanoic acid, hexanoic acid, α-bisabolol, and thymoquinone are reported to be odour-active substances in wood. α-Bisabolol is a sesquiterpene which was first found in German chamomile (*Matricaria chamomilla*) [10]. It has already been discovered as ingredient in the oil from Candeia wood (*Eremanthus erythropappus*) [11], but its appearance as wood odorant is a new finding. Whereas thymoquinone and α-bisabolol are naturally occurring molecules,

hexanoic acid and 3-phenylpropanoic acid are likely to result from degradation of common wood components such as fatty acids and lignin.

Quantification trials will be a future challenge to trace back the differences in smell between the respective wood samples, as the respective profiles indicate that most likely the sensory differences result from quantitative rather than general qualitative differences in odorant composition. Moreover, more comprehensive investigations will be required targeting the impact of wood smell on wellbeing in humans.

## Conclusions

The found odorants belong to a variety of substance classes and exhibit a great diversity in odour character. Some of the substances are known constituents in wood whereas others were identified for the first time in wood or even for the first time as being odour-active. The successful elucidation of potent odorants in wood is a first important step towards the understanding of the molecular basis of the odour profile of a commonly-used material in daily life.

## References

1. L. Culleré, B. Fernández de Simón, E. Cadahía, V. Ferreira, P. Hernández-Orte and J. Cacho, LWT - Food Science and Technology, 2013, 53, 240-248.
2. M. C. Díaz-Maroto, E. Guchu, L. Castro-Vázquez, C. de Torres and M. S. Pérez-Coello, Flavour and Fragrance Journal, 2008, 23, 93-98.
3. W. Engel, W. Bahr and P. Schieberle, European Food Research and Technology, 1999, 209, 237-241.
4. W. Grosch, Chemical Senses, 2001, 26, 533-545.
5. L. Schreiner, H. M. Loos and A. Buettner, Analytical and Bioanalytical Chemistry, 2017, 409, 3719-3729.
6. H. van Den Dool and P. Dec. Kratz, Journal of Chromatography A, 1963, 11, 463-471.
7. O. R. Johnson-Ajinwo and W.-W. Li, Journal of Agricultural and Food Chemistry, 2014, 62, 5466-5471.
8. M. Burits and F. Bucar, Phytotherapy research, 2000, 14, 323-328.
9. V. Hajhashemi, A. Ghannadi and H. Jafarabadi, Phytotherapy Research, 2004, 18, 195-199.
10. G. P. Kamatou and A. M. Viljoen, Journal of the American Oil Chemists' Society, 2010, 87, 1-7.
11. K. A. Santos, E. J. Klein, Z. C. Gazim, J. E. Gonçalves, L. Cardozo-Filho, M. L. Corazza and E. A. da Silva, The Journal of Supercritical Fluids, 2016, 114, 1-8.

# Odour-active compounds in an inflatable PVC beach ball

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## Abstract

Application of an aroma extract dilution analysis to an inflatable beach ball made of PVC revealed 38 odour-active compounds with flavour dilution (FD) factors of 1 to 4096 among which 13 compounds showed FD factors >128. The most potent odorants were sweet, plastic-like smelling 2-ethylhexanal (FD 4096), fatty smelling (2*E*,4*E*)-nona-2,4-dienal (FD 4096), mushroom-like smelling non-1-en-3-one (FD 2048), plastic-like smelling 2-ethylhexyl 4-methylbenzoate (FD 2048), fatty smelling (2*Z*)-non-2-enal (FD 1024), solvent-like smelling γ-butyrolactone (FD 1024), plastic-like smelling hexan-3-ol (FD 512), green, fatty smelling (2*E*)-hept-2-enal (FD 512), and fruity smelling hexyl hexanoate (FD 512). (2*E*,4*E*)-Nona-2,4-dienal, 2-ethylhexyl 4-methylbenzoate, and (2*E*)-hept-2-enal were previously unknown in PVC material. Structures suggested that autoxidation of unsaturated fatty acids and degradation of di(2-ethylhexyl) terephthalate (DEHT) used as plasticizer were the most important sources of odour-active compounds.

## Introduction

Inflatable beach toys such as beach balls and pool floats are typically manufactured from polyvinyl chloride (PVC). Particularly when new, these PVC toys often exhibit an intense and characteristic "plastic-like" odour. To date, little is known on the molecular background of this odour [1,2]. The aim of the current study was to extract the volatiles from a typical PVC beach toy with a characteristic smell and screen them for odour-active compounds by gas chromatography-olfactometry (GC-O) and aroma extract dilution analysis (AEDA) [3].

## Experimental

### *Materials*

Numerous PVC beach toys including pool floats in different sizes and shapes as well as beach balls were obtained from local shops in Freising, Germany and from German e-tailers. All materials were orthonasally evaluated by a sensory panel using free choice profiling. In open discussion, a beach ball was finally selected for the further investigations, because it showed an intense and highly characteristic smell.

### *Reference odorants*

2-Ethylhexanal (**1**), (2*E*,4*E*)-nona-2,4-dienal (**2**), γ-butyrolactone (**6**), hexan-3-ol (**7**), and (2*E*)-hept-2-enal (**8**) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Oct-1-en-3-one (**10**) was obtained from Alfa Aesar (Karlsruhe, Germany). Non-1-en-3-one (**3**) was synthesized by oxidation of non-1-en-3-ol (Alfa Aesar) with Dess-Martin periodinane (Sigma-Aldrich) [4]. Hexyl hexanoate (**9**) was obtained from hexan-1-ol and hexanoic acid (both Sigma-Aldrich) using the general approach detailed in [5].

The same approach was used to synthesize 2-ethylhexyl 4-methylbenzoate (**4**) from 2-ethylhexan-1-ol and 4-methylbenzoic acid (both Sigma-Aldrich). **4** was obtained as a colourless oil. RI (FFAP) 2300. MS (EI, 70 eV), m/z (%) 41 (35), 55 (26), 65 (20), 70 (90), 83 (20), 91 (45), 112 (30), 119 (100), 137 (20). $^{1}$H-NMR ($CDCl_3$, 400.13 MHz, 298

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K), δ (ppm) 0.90-0.95 (m, 3H), 0.97 (t, 3H), 1.29-1.39 (m, 2H), 1.39-1.46 (m, 4H), 1.46-1.54 (m, 2H), 1.69-1.77 (m, 1H), 2.43 (s, 3H), 4.21-4.29 (m, 2H), 7.25-7.27 (m, 2H), 7.94-7.97 (m, 2H). $^{13}$C-NMR ($CDCl_3$, 100.62 MHz, 298 K): δ (ppm) 11.1 ($CH_3$), 14.0 ($CH_3$), 21.6 ($CH_3$), 23.0 ($CH_2$), 24.0 ($CH_2$), 29.0 ($CH_2$), 30.6 ($CH_2$), 38.9 (CH), 67.1 ($CH_2$), 127.8 (C), 129.0 (CH), 129.5 (CH), 143.4 (C), 166.8 (C).

*Isolation of the volatile compounds*

The PVC beach ball skin was cut into small pieces (1 cm × 1 cm). The mouthpiece was discarded. The pieces (500 g) were stirred with dichloromethane (1 L, 20 h). The extract was filtered and non-volatiles were removed by solvent-assisted flavour evaporation (SAFE) [6] at 40 °C in high vacuum.

*Odorant screening*

The SAFE distillate was concentrated (1 mL) using a Vigreux column (60 cm × 1 cm). An aliquot of the concentrate (1 µL) was analysed by GC-O using an FFAP column (30 m × 0.32 mm i.d. × 0.2 µm film thickness). The GC eluate was split 1:1 between an FID and a heated exit serving as sniffing port [7]. Following the concept of an AEDA [3], the initial concentrate was stepwise diluted 1:2 and each diluted sample was also analysed by GC-O. Each odour-active compound was assigned an FD factor representing the dilution factor of the highest diluted sample in which the odorant was detected by any of three trained panellists.

*Structure assignment of odorants*

Preliminary structure assignments were achieved by comparing odour and retention indices of the PVC beach ball odorants as obtained by GC-O using the FFAP column detailed above and a DB-5 column (30 m × 0.32 mm i.d. × 0.2 µm film thickness) as well as mass spectra as obtained by GC-MS with data compiled in databases. Preliminary structure assignments were then confirmed by GC-O and GC-MS analysis of authentic reference substances analysed in parallel to the PVC ball volatile isolates. To avoid coelution problems during MS analysis, the PVC ball volatiles were previously separated into a fraction containing the acidic volatiles and a fraction containing the neutral and basic volatiles. The latter was further fractionated into five fractions of different polarity by silica gel chromatography as detailed in [7]. Before the fractions were subjected to GC-MS, the PVC beach ball odorants were localized in the fractions by GC-O.

## Results and discussion

The AEDA resulted in a total of 38 odour-active compounds covering an FD factor range of 1 to 4096 (data not shown). Thirteen compounds exhibited FD factors >128 (Figure 1). Among them, sweet, plastic-like smelling 2-ethylhexanal (**1**) and fatty smelling (2*E*,4*E*)-nona-2,4-dienal (**2**) were the most potent (FD 4096), followed by mushroom-like smelling non-1-en-3-one (**3**, FD 2048), plastic-like smelling 2-ethylhexyl 4-methylbenzoate (**4**, FD 2048), fatty smelling (2*Z*)-non-2-enal (**5**, FD 1024), solvent-like smelling γ-butyrolactone (**6**, FD 1024), plastic-like smelling hexan-3-ol (**7**, FD 512), green, fatty smelling (2*E*)-hept-2-enal (**8**, FD 512), fruity smelling hexyl hexanoate (**9**, FD 512), mushroom-like smelling oct-1-en-3-one (**10**, FD 256), plastic-like smelling hex-1-en-3-one (**11**, FD 128), solvent-like smelling cyclohexanone (**12**, FD 128), and plastic-like smelling 2-ethylhexan-1-ol (**13**, FD 128).

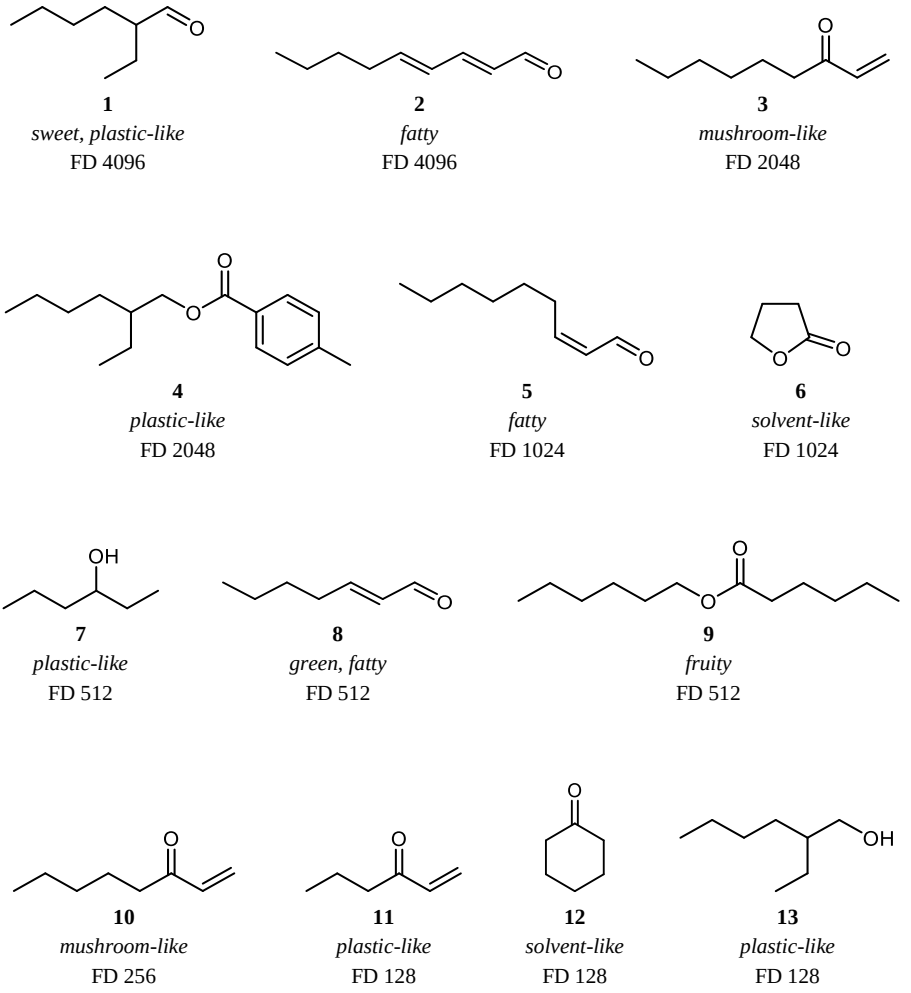


**Figure 1**: Structures, odour qualities, and FD factors of the most potent odorants identified in the beach ball

(2*E*,4*E*)-Nona-2,4-dienal (**2**), 2-ethylhexyl 4-methylbenzoate (**4**), (2*E*)-hept-2-enal (**8**), and γ-butyrolactone (**6**) were found for the first time in a PVC material.

Among the 13 most potent odorants in the PVC beach ball depicted in Figure 1 six were typical autoxidation products of unsaturated fatty acids, namely (2*E*,4*E*)-nona-2,4-dienal, non-1-en-3-one, (2*Z*)-non-2-enal, (2*E*)-hept-2-enal, oct-1-en-3-one, and hex-1-en-3-one [8]. Hex-1-en-3-one was previously identified as key odorant in a PVC-based automotive artificial leather [9].

Three compounds, namely 2-ethylhexanal (**1**), 2-ethylhexyl 4-methylbenzoate (**4**), and 2-ethylhexan-1-ol (**13**) were structurally related to common PVC plasticizers such as di(2-ethylhexyl) phthalate (DEHP) and di(2-ethylhexyl) terephthalate (DEHT) (Figure 2). It may therefore be assumed that **1**, **4**, and **13** are potential decomposition products and/or impurities of such plasticizers. GC-MS analysis of authentic reference

compounds of DEHP and DEHT in comparison with the PVC beach ball volatile isolate showed the presence of DEHT in the beach ball, whereas DEHP was absent. This was in particular also in agreement with the para-structure of **4**. For a long time, DEHP was the standard plasticizer in PVC materials, but today its use is restricted in most parts of the world and DEHT is used as a common substitute. DEHT shows similar plasticizing properties as DEHP, but is considered to be less toxic [10].

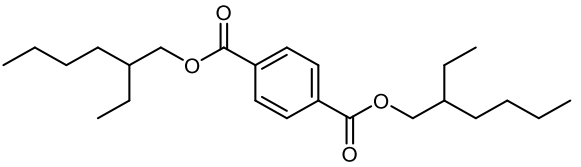


**Figure 2**: Structure of di(2-ethylhexyl) terephthalate (DEHT) used as plasticizer in the beach ball

## References

1. Kong P., Lin X., Wang M. (2011) Suliao Keji 39: 95-98 (in Chinese).
2. Wypych, G. (2013) Handbook of odors in plastic materials, ChemTec Publishing, Toronto, Canada.
3. Schieberle P., Grosch W. (1987) Z Lebensm Unters Forsch 185: 111-113.
4. Dess D.B., Martin J.C. (1983) J Org Chem 48: 4155-4156.
5. Li J.-X., Schieberle P., Steinhaus M. (2017) J Agric Food Chem 65: 639-647.
6. Engel W., Bahr W., Schieberle P. (1999) Eur Food Res Technol 209: 237-241.
7. Steinhaus M. (2015) J Agric Food Chem 63: 4060-4067.
8. Grosch W. (1982) In: Food Flavours (Morton I.D., MacLeod A.J., eds.). Elsevier, pp. 325-397.
9. Reglitz K., Steinhaus M., this book.
10. Kwon B., Ji K. (2016) Hangug Hwangyeong Bogeon Haghoeji 42: 169-188 (in Korean).

# Identification of malodourous emissions of wood pellets during storage

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## Abstract

During storage of pellets, various compounds like e.g. carbon monoxide (CO), carbon dioxide ($CO_2$) and partly malodourous volatile organic compounds (VOCs) can be formed. Research on this so-called off-gassing phenomenon is focusing on the one hand on the identification and characterization of the unpleasant odour-active VOCs and on the toxic atmosphere caused by formation of CO and depletion of oxygen ($O_2$). On the other hand, the mechanism behind the off-gassing phenomenon is a further topic in research. However, both topics have in common that they are necessary research questions in order to prevent insecurity due to the formation and enrichment of harmful CO and complaints by the end-user because of malodourous smell in pellet storage places.

Thus, the aim of this study was to identify and quantify the malodorous VOCs and find correlations with the released amounts of CO. To identify odour-active components in wood pellets, sensory and analytical methods were applied. A trained sensory panel established olfactory descriptors for the wood pellet samples. By means of instrumental methods like GC-MS, volatile and potentially odour-active components were identified. The total amount of VOCs was determined using a flame ionization detector and CO was analysed with a gasanalyzer. The results showed significant differences concerning both types of emission - VOCs and CO - between sensory unremarkable wood pellets and pellets with a pronounced off-flavour. Terpenes, short chain fatty acids and saturated aldehydes were identified to have major impact on the aroma profile of wood pellets.

## Introduction

In recent years, a significant increase in the demand of wood pellets has been observed. Besides beneficial characteristics for the combustion process (e.g. wood pellets exhibit a high energy density) the customer also expects a natural wood pellet flavour of the product [1]. During storage of wood pellets, various compounds like carbon monoxide (CO), carbon dioxide ($CO_2$) and volatile organic compounds (VOCs) are formed, and the oxygen ($O_2$) in the surrounding air decreases [2]. Simultaneously, partly malodourous compounds may be formed leading to unpleasant and disturbing smell in the pellet storage facilities. The formation pathways of the unpleasant odour-active VOCs and CO are not completely clear, but the degradation of natural wood components like resins or fatty acids seem to be one reason for the release of the emissions. In addition, secondary metabolites of microbial growth on the raw material can lead to off-flavour formation during storage [3].

The aim of this study was the identification of odour-active components in wood pellets. To reach this aim, sensory and analytical/gas chromatographic methods were applied on wood pellets (with or without off-odour) made of spruce or/and pine in various ratios. Different sensory unremarkable and malodourous wood pellets were analysed.

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## Experimental

For the analysis of the volatile compounds the pellets were crushed using a laboratory scale mill. For the extraction and enrichment of the volatile compounds from the pellets prior to the GC analysis, headspace solid phase micro extraction (HS-SPME) was used. 100 mg of ground pellet samples were analysed and 50/30 µm DVB/Carboxen/PDMS fibres (2 cm stable flex, Supelco) were used for the enrichment of the volatile compounds. The fibre was exposed into the headspace of the samples for 20 minutes at 40°C while stirring the samples. The separation and the identification of the volatile compounds were performed on an Agilent GC-MS system (GC 7890 with MS 5975c VL MSD, electron impact ionisation 70 eV) using a nonpolar analytical column (HP5MS, 30m*250µm*1 µm) and on a Shimadzu GC-MS system (GC2010 with GCMS-QP 2010 Plus, Shimadzu Europa GmbH, electron impact ionisation 70 EV) system with a polar analytical column (ZB-Wax plus, 20m*180µm* 0.18µm). The identification of the compounds was based on the comparison of the obtained mass spectra with mass spectra from literature or from MS databases as well as the calculation of linear temperature programmed retention indices and comparison with retention indices from authentic reference compounds or data from literature.

Moreover, for the determination of emitted amounts of CO and total VOCs (TVOC, i.e. the sum of all volatile compounds that can be emitted from the wooden material) the pellets samples were stored in closed glass bottles for five days at 22°C. Thus, this method is called storage experiment. For the measurement of CO concentration, a gasanalyzer (Emerson, NGA 2000) and for the determination of total VOCs concentration a flame ionization detector (Thermo-FID) were used [4]. The release of emissions is explained as release of gas per kg pellets on dry basis per day enabling the comparison of different pellet samples.

A sensory panel consisting of 15 well trained panellists performed descriptive analysis of sensory unremarkable and malodourous wood pellets and established corresponding olfactory descriptors.

## Results and discussion

The results of the study show that terpenes (e.g. α-pinene, β-pinene or camphene), short chain fatty acids and saturated aldehydes have major impact on the composition of the wood pellet volatiles. The composition of the volatile compounds of pellets with and without detected off-odour differs significantly. Figure 1 shows the comparison of the aldehyde and free fatty acid distribution, receptively, of a reference sample produced from spruce and pine (50/50) and a rejected, malodourous pellet sample. On the one hand, shifts of the compound ratios were discovered which could be one reason for the detected off-flavours in pellets. Increasing concentrations of compounds like hexanoic acid and aldehydes like octanal or nonanal are supposed to negatively influence the pellet odour. On the other hand, some compounds were detected which are most likely produced by microorganisms as secondary metabolites during microbial growth on sawdust (e.g. 3-methyl-butanoic acid). These VOCs present in higher concentrations in the pellets with malodour most probably influence the off-odour formation.

Concerning the acid composition, a drastic decrease of acetic acid but also of butanoic and pentanoic acid was observed from the ‘good’ to the ‘bad’ sample, as well as a simultaneous increase of hexanoic and heptanoic acid were observed. The methyl branched fatty acid 3-methyl-butanoic acid shows slightly higher concentration in the malodorous pellet than in the ‘good’ one. Due to its low odour threshold (OT;

0,0018mg/m³; [5]) 3-methyl-butanoic acid is considered to be of importance for the pellet off-odour. Regarding aldehydes, hexanal concentrations were reduced while pentanal, octanal and nonanal showed higher concentrations in the malodourous sample. These compounds shift results in pellets with a very strong lacquer-like flavour, whereas the typical woody odour mainly caused by terpenes was no longer detectable. The total concentration of terpenes decreased in the malodourous samples as well as the total concentration of VOCs. The loss of hexanal might be also responsible for the loss of fresh notes.

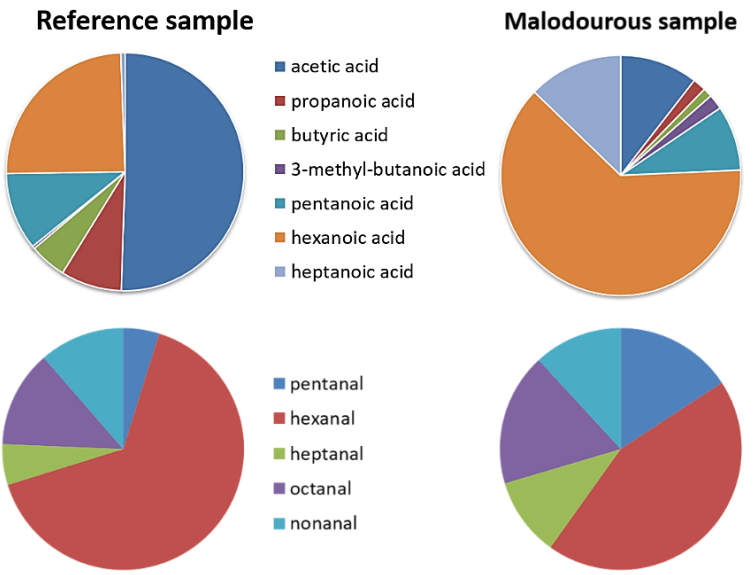


**Figure 1:** Odour-active components in pellets samples

Figure 2 shows the results from CO and TVOC analysis. Interestingly, the so-called reference sample (i.e. good sample) shows a significantly higher emission of CO and total VOCs, respectively, than the malodourous sample.

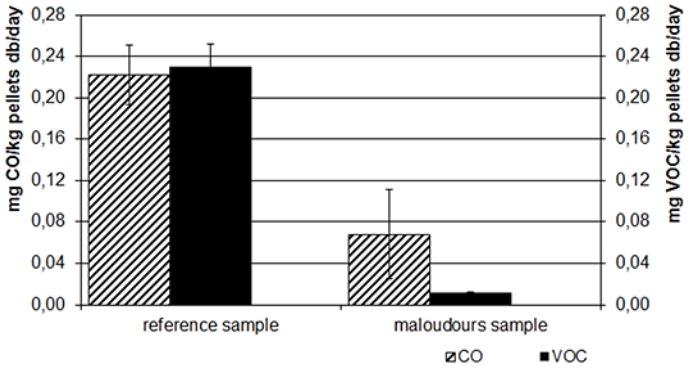


**Figure 2:** Release of CO and total VOCs

The formation pathways for odour-active VOCs, total VOCs and CO have not been explained yet. The identities of the detected compounds indicate that the (oxidative) degradation of natural wood components like resins or fatty acids is one reason for the release of emissions from the pellets.

Results from previous studies show (data not shown), that the microbial spoilage of raw material during storage could be an additional way for the formation of VOCs (especially for aldehydes and acids).

Moreover, interestingly the pellet sample with pronounced off-odour did not emit more CO and total VOCs than an odour unobtrusive pellet sample. Although the pellet sample was rejected due to a distinct off-odour, a decrease of total VOCs could be detected in GC-MS analysis. The results of the study show a shift of different odour-active substances as a reason for the existing off-odour. Thus, the total amount of VOCs is not the decisive parameter alone for the evaluation of a malodourous smelling pellet sample. Since odour of pellets is dependent on the concentration in combination with the odour thresholds (OT) of the respective component (e.g. OT of hexanal 58mg/m³, OT of 3-methylbutanoic acid 0,0018mg/m³) [5], quantification is inevitable to be able to judge the sensory relevance of the compounds of interest.

The results of this study show that the determination of CO emission and the measurement of total VOC release from the pellets are not sufficient to evaluate off-odour formation in wooden pellets. The detailed investigation of the sensory properties in combination with the GC-MS analysis of the potentially (mal)-odorous volatiles is necessary to obtain a comprehensive picture of pellet (off)-flavour. The results serve as a basis for future investigations to elucidate off-flavour formation pathways in wood pellets.

**Acknowledgment**

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## References

1. Calderón C., Gauthier G. and Jossart J.M., (2017) Aebiom Statistical Report 2017, Full Report, AEBIOM Brussels, pp. 149-212.
2. Svedberg U., Petrini C. and Johanson G., (2009) Ann. Occup. Hyg. 53 (8): 779-787.
3. Pöllinger-Zierler B., Sedlmayer I., Reinisch C., Siegmund B., Wopienka E. and Pointner C., Haslinger W., (2015) Proceedings - WSED 2015
4. Emhofer W., (2015) Emission from Wood Pellets during Storage, Doctoral Thesis, Vienna University of Technology, pp. 19-22.
5. Van Gemert L.J., (2011) Odour Thresholds – compilations of odour thresholds in air, water and other media, second edition, ISBN 978-90-810894-0-1

# Describing the smell of wet cat food using a common sensory language: Petscript®

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## Abstract

Meal time is increasingly considered by pet owners as a privileged moment to create emotional bonds with their animal. To make meal time a shared enjoyable moment, pet food manufacturers not only need to satisfy pets' appetite, they also need to satisfy pet owner expectations towards food. For instance, pet food cosmetic and sensory properties such as appearance and smell highly contribute to its acceptance by the pet owner. These factors play an important role in the act of repurchase of the pet food product. Only a few research studies have used human sensory analysis to describe the smell of wet pet food. The purpose of this study was to characterize the smell of different wet cat foods available on the European market using Petscript®, a sensory language specifically developed for this type of product. First, a full set of olfactory descriptors was generated by a human expert panel in order to have a common sensory language to describe the odor profile of wet pet food. Then, several products were selected to offer a representative picture of the market. All these products were assessed via a Rate All That Apply (RATA) method in association with a free description to have an exhaustive odor characterization. The products were then positioned on a map according to their olfactory profile. The Petscript® sensory language was successfully used to build the olfactory space for wet cat food. The results highlight the existence of clusters of products showing similar odor profile. The Petscript® language can be used to support pet fooders' strategy by positioning their products in the olfactory landscape, and helping them to reach a specific smell target and then differentiate from the other products on the market. The ongoing challenge is now to couple these sensory results with human and animal preferences to identify the smell profiles appreciated by both pet owners and their beloved animal.

## Introduction

In the past 10 years, human sensory panels have been used to characterize different pet foods [1, 2]. However, there is a lack of a common sensory language to describe the smell of pet food. Furthermore, it has been observed that prior experience and cultural environment can affect the way people describe the smell of food products [3]. The purpose of this study was to utilize a unique and universal sensory language - the Petscript® - to characterize the smell of different wet cat foods available on the European market.

## Experimental

Market products: 32 premium and super premium wet cat foods were bought in France, UK and Germany (Figure 1). All the products were chicken based recipes in different matrices: chunks in jelly, chunks in gravy, loaf or mousse. They were packed in pouches, cans or aluminum trays.

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**Figure 1:** 32 European wet cat foods used in the study

Sensory sessions: 25 judges were trained on the Petscript® language. Figure 2 presents the Petscript® olfactory descriptors used to characterize wet pet food. The whole study was divided into 9 sensory sessions. Up to 4 products were presented during each sensory session. 11 to 21 trained judges participated to each sensory session. The products were randomized between judges over each session. The sessions were conducted under red light to avoid any sensory bias.


**Figure 2:** The 25 odor terms of Petscript® sensory language for wet pet food

RATA method [4] with a 4 points scale was used to describe all the cat food products, using the 25 odor terms of the Petscript® language. The order of the odor terms was randomized between judges, over each sensory session. The judges could also add up to 5 odor terms of their choice to describe the smell of the products.

Data analysis: a mixed model was used with "judge" as a random effect and "product" as a fixed effect for each descriptor (STATGRAPHICS Centurion XVI.I). A PCA was conducted on adjusted means (SPAD8). An AHC (Ward criteria) was then applied to the factorial coordinates of the products in the spaces defined by PCA (SPAD8).

## Results and discussion

An olfactory space with 6 distinct clusters was obtained from PCA (Figure 3).

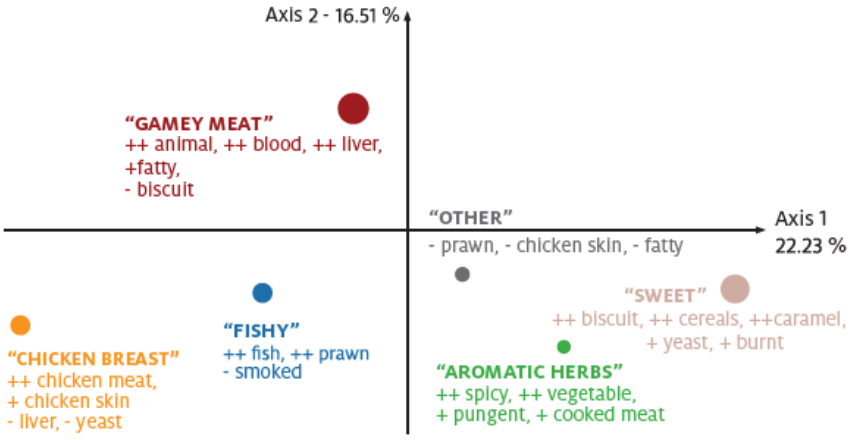


**Figure 3:** Representation of the 6 clusters of products in the 1st and 2nd dimensions of the PCA

Two clusters are opposed on axis 1, the "CHICKEN BREAST" cluster and the "SWEET" cluster. The "CHICKEN BREAST" cluster gathers together products characterized by 'chicken meat' and 'chicken skin' olfactory notes. These hyper premium products were all bought from the UK market. They contain above 45% of chicken meat in a clear white jelly or gravy. On the other hand, the "SWEET" cluster was described with 'biscuit', 'cereals', 'caramel' and 'grilled' terms that are typical of products from the Maillard reaction. The darker color of these products and the mention of 'sugar' in the ingredient list also point towards the Maillard reaction.

Two clusters are opposed on axis 2: the "GAMEY MEAT" cluster, characterized by 'animal', 'blood', 'liver', and 'fatty' olfactory notes; and the "AROMATIC HERBS" cluster, characterized by 'rosemary', 'thyme' and 'basil' olfactory notes. The "GAMEY MEAT" cluster is the most represented on the EU market and could target animal satisfaction [5]. The cluster "AROMATIC HERBS" contains 3 products bought respectively from UK, France and Germany. The use of herbs ingredients in these recipes underlines the trend of 'humanization', with pet food products not only design to attract pets, but also to improve pet owner satisfaction towards smell.

The 2 last clusters "FISHY" and "OTHER" are better represented in the 3rd, 4th and 5th dimensions. The "FISHY" cluster is characterized by 'fishy' and 'prawn' olfactory notes. The "OTHER" cluster contains products with particular olfactory profiles and weaker smell intensity. One product from this cluster was for instance described as 'peanut like'. Two products out of the 5 products in the "FISHY" cluster declare containing at least one fish ingredient (fish oil or fish extract).

In this study, the Petscript® sensory language was successfully used to build the olfactory space for European wet cat food. Petscript® allowed differentiating selected products based on their odor profiles. Odor similarities and differences between products could be due to recipes and raw material origin, process, and manufacturing place. They can also highlight different pet food manufacturers' strategies. Indeed, one pet food

manufacturer has all his products in the cluster “GAMEY MEAT” while another pet food manufacturer has 3 out of 4 products in the cluster “SWEET”. These could traduce pet food manufacturers’ will to create a brand olfactory signature. On the other hand, other brands use smell differentiation according to product positioning. One famous brand of pet food has their products spread in 4 out of the 6 different olfactory clusters.

Petscript® can be used to support pet fooders’ strategy by positioning their products in the olfactory landscape, and helping them to reach a specific smell target or to differentiate from the other products on the market. The ongoing challenge is now to couple these sensory results with human and animal preferences to identify the smell profiles appreciated by both pet owners and their beloved animal.

## References

1. Koppel K. 2014. Sensory analysis of pet foods. J Sci Food Agric; 94: 2148-2153 –
2. Pickering G. J. 2008. Optimizing the sensory characteristics and acceptance of canned cat food: use of a human taste panel. Journal of Animal Physiology and Animal Nutrition.
3. Keller A. and Vosshall L.B. 2016. Olfactory perception of chemically diverse molecules. BMC Neuroscience.
4. Meyners M., Jaeger S.R. and Ares G. 2015. On the analysis of Rate-All-That-Apply (RATA) data. Food quality and preference.
5. Nilsson S. et al. 2014. Behavioral Responses to Mammalian Blood Odor and a Blood Odor Component in Four Species of Large Carnivores. PLoS ONE

# Comparison of different analytical methods for the quantification of odour-active haloanisoles in food contact materials

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## Abstract

2,4,6-trichloroanisol (2,4,6-TCA), the major compound responsible for cork taint in wine, belongs to haloanisoles which are formed by microorganisms, primarily fungi [1]. These substances can contribute to off-flavour in food and food contact material at remarkably low concentrations. Odour threshold concentrations (OTCs) of haloanisoles were determined by our trained expert panel in different matrices. The fact that 2,4,6-TCA and 2,4,6-tribromoansiol (2,4,6-TBA) exhibit extremely low OTCs concludes in a challenge for analytical detection methods. Different analytical methods were developed and compared, highly sensitive and selective systems. It is shown that gas chromatography mass spectrometry (GC-MS) using negative chemical ionization (NCI) in selected ion monitoring (SIM), comprehensive GCxGC-MS using electron impact ionization (EI) in SIM and GC-MS/MS using EI in multiple reaction monitoring (MRM) mode fulfil the desired requirements.

## Introduction

Haloanisoles with their musty, mouldy and earthy odour are known contaminants in recycled paper and paperboard. Using these materials for packaging food, these substances can migrate into the packed good and cause unpleasant off-flavour. Based on sensory evaluation a threshold of 10 ng/kg paperboard, which correlates with the sensitivity of the human nose and covers the OTCs of the most sensitive individuals, was chosen as reference for the required sensitivity of the analytical methods. Since the measurement with EI-GC-MS neither in scan nor in SIM mode reaches the desired sensitivity, a MRM method with increased sensitivity and selectivity was developed. With comprehensive GCxGC-MS using EI in SIM the requested sensitivity was achieved due to the process of solute focusing and reinjection in the modulator, which results in a narrow peak with higher amplitude [2]. Due to its high selectivity for electron capturing species, NCI-GC-MS operated in SIM can be used to analyse haloanisoles at very low concentrations. External calibrations of the 4 most potent haloanisoles were performed to prove sensitivity and linearity. For quantification standard addition and single point internal standard method (where applicable) were conducted. The aim of this study was to compare different gas chromatographic methods that can be used for analysis and quantification of haloanisoles in paperboard samples.

## Experimental

*Sensory analysis*

Determinations of OTCs were performed in duplicate by Graz University of Technology sensory expert panel using a 3 alternative forced choice sample presentation method in an ascending concentration series [3]. The panel consisted of 14 persons (9 women and 5 men in the age between 27 and 50) who were trained in the standard sensory

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evaluation methods and fulfilled all psychological and physical demands required for a sensory panellist. Samples were prepared the day before the trial in the specific matrix and served the panel in covered odour-free plastic cups immediately before testing. OTCs were calculated as the geometric means of the individual thresholds and were evaluated in three different matrices, water, Mygliol®812 (fat matrix) and cellulose.

*Sample preparation*

The sample preparation was performed by headspace solid phase microextraction (HS-SPME) with agitation by magnetic stir bars. Two different fibers, SPME fiber 50/30 µm Carboxen®/DVB/PDMS (2 cm stable flex) and PAL SPME Arrow fiber Carbon Wide Range/PDMS (CTC-ARR11-C-WR-120/20-P3), were compared to prove an alleged increase in sensitivity due to the larger stationary phase volume in the Arrow fiber.

*GC-MS and GC-MS/MS*

The GC-MS system consisted of a Shimadzu GCMS-TQ8050 combined with Shimadzu AOC-6000 multifunctional auto sampler, using a quadrupole mass filter and EI. The headspace exposure of the SPME fiber was performed at 100°C for 20 minutes. A 29.5 m ZB-5MSi Column with 0.25 mm id and 0.25 µm film thickness was used for GC separation. Carrier gas was helium (51.4 kPa, linear velocity, 35.0 cm/sec). Column oven temperature program was 70°C (1 min) @ 10°C/min until 200°C @ 35°C/min until 320°C (1 min). Measurements were conducted in Q3 scan (m/z 50-400, 5000 scans/sec), Q3 SIM and MRM. The m/z filtered in SIM are shown in Table 1, the transitions for the GC-MS/MS MRM method are shown in Table 2.

**Table 1:** GC-MS measurement of haloanisoles in SIM mode, selecting following m/z

| Compound | *m/z* | *m/z* | *m/z* |
|---|---|---|---|
| Internal Standard 2,4,6-dTCA | 217 | 215 | |
| 2,4,6-TCA | 212 | 210 | |
| 2,3,4,6-tetrachloroanisol (2,3,4,6-TeCA) | 246 | 231 | 229 |
| Internal Standard 2,4,6-dTBA | 351 | 349 | |
| 2,4,6-TBA | 346 | 344 | |
| 2,3,4,5,6-pentachloroanisol (2,3,4,5,6-PCA) | 282 | 280 | 265 |

**Table 2:** GC-MS/MS method; transitions (collision energy)

| Compound | *transitions* | *transitions* | *transitions* |
|---|---|---|---|
| 2,4,6-dTCA | 217.00>199.00 (15) | 215.00>197.00 (15) | 217.00>171.00 (30) |
| 2,4,6-TCA | 211.90>197.00 (15) | 210.00>195.00 (15) | 212.00>169.00 (30) |
| 2,3,4,6-TeCA | 230.90>202.90 (12) | 228.90>200.90 (18) | 243.90>201.90 (27) |
| 2,4,6-dTBA | 350.80>332.70 (18) | 348.70>330.70 (15) | 348.70>302.70 (33) |
| 2,4,6-TBA | 345.80>330.80 (18) | 343.70>328.70 (15) | 343.70>300.70 (27) |
| 2,3,4,5,6-PCA | 264.80>236.80 (15) | 279.80>236.80 (27) | 236.90>142.90 (24) |

*Comprehensive GCxGC-MS*

The comprehensive GCxGC-MS system consisted of a Shimadzu QP2010 Ultra instrument combined with an Optic-4 injector and a Shimadzu AOC-5000 Plus auto sampler using EI and was run in SIM mode. The headspace exposure of the SPME fiber was performed at 100°C for 20 minutes. A 30 m HT1 column with 0.25 mm id and 0.25 µm film thickness was combined with a 2.5 m BPX5 column both with 0.15 mm id and

0.25 µm of film thickness. Modulation time was set with 5 seconds. Carrier gas was helium (131.1 kPa, average linear velocity 33.7 cm/sec on both columns). Column oven temperature program was 65°C (1 min) @ 5°C until 230°C. Ion source temperature was 200°C, detector voltage was 1.2 kV. M/z selected in SIM mode are the same as used in GC-MS SIM measurements (Tab.1).

*Negative Chemical Ionisation (NCI)*

The GC-MS system consisted of a Shimadzu QP2010 Plus system combined with AOC-5000 Plus auto sampler. The headspace-exposure of the SPME-fiber was performed at 100°C for 20 minutes. A 30 m ZB-5 MS column with 0.25 mm id and 0.25 µm film thickness was used for separation. Column oven temperature program was 50°C (1 min) @ 10°C/min until 300°C (5 min). Carrier gas was helium (45.6 kPa, linear velocity, 35.0 cm/sec). Isobutane was used for NCI, the ion source temperature was 160°C, the detector voltage 1.4 kV. In SIM mode m/z 35 and m/z 37 were filtered for compounds containing chlorine and m/z 79 and m/z 81 for bromine.

## Results and discussion

*Sensory analysis*

OTCs of 9 different haloanisoles were determined in water. It was shown that the haloanisoles with the positions 2,4 and 6 on the benzene molecule filled with the halogen (2,4,6-TCA and 2,4,6-TBA) show the lowest OTCs. As soon as one position is exchanged or one halogen is added, the OTC increases. However, in general the matrix has great influence on the sensory threshold. To investigate this effect, OTC evaluations of the 4 most odorous compounds were conducted in Miglyol®812, a fat matrix used in the food sector as it is odour neutral due to its oxidation resistant properties. An increase in the sensory threshold of approximately 4 decades compared to the values received in water was observed. Due to the fact that haloanisoles are known to cause off-flavour in paperboard with unpleasant musty, earthy odour, the OTCs of the two most potent substances (TCA and 2,4,6-TBA) were investigated in cellulose as matrix, too. It was shown that the sensory thresholds were 3 decimal powers higher in cellulose than in water. A comparison of the OTCs in the different matrices is given in Table 3. At this point the great variations in the sensory thresholds between the individual panellists should be noticed. Values for 2,4,6-TBA for example were ranging from 0.001 ng/l to 1 ng/l (water).

**Table 3:** BET concentrations [ng/L] / [ng/kg] in different matrices; (n.d.: not determined)

| Compound | *BET [ng/L] in water* | *BET [ng/L] in Miglyol®812* | *BET [ng/kg] in cellulose* |
|---|---|---|---|
| 2,4,6-trichloroanisol | 0.1 | 800 | 360 |
| 2,4,6-tribromoanisol | 0.1 | 5000 | 480 |
| 2,3,4,6-tetrachloroanisol | 2.5 | 11000 | n.d. |
| 2,3,4,5,6-pentachloroanisol | 60 | 284000 | n.d. |
| 2,3,5,6-tetrachloroanisol | 250 | n.d. | n.d. |
| 2,3,4,5-tetrachloroanisol | 1000 | n.d. | n.d. |
| 2,4-dibromoanisol | 1500 | n.d. | n.d. |
| 2,3,4-trichloroanisol | 36000 | n.d. | n.d. |
| 3,5-dibromoanisol | 54000 | n.d. | n.d. |

*Calibrations*

In the progress of method development external calibrations (10 ng/kg; 100 ng/kg; 500 ng/kg and 1000 ng/kg) were performed. With GC-MS (SIM) the desired sensitivity of 10 ng/kg could not be achieved, but was realised with GC-MS/MS in MRM (Figure 1). An improvement of sensitivity of the Arrow fiber compared to the traditional SPME fiber in the MRM measurements could not be shown. Linearity of the calibrations curves of the 4 haloanisoles was shown using the correlation coefficients, where values of > 0.998 (SPME-fiber) and > 0.995 (arrow-fiber) were received.

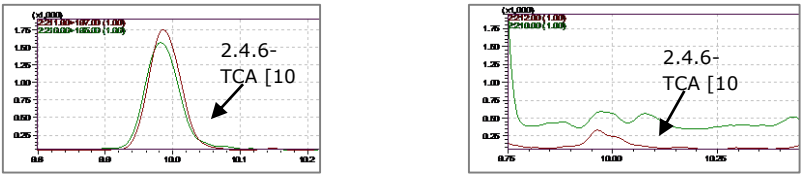


**Figure 1**: 2,4,6-TCA [10 ng/kg]; left: EI-GC-MS/MS in MRM; right: EI-GC-MS in SIM

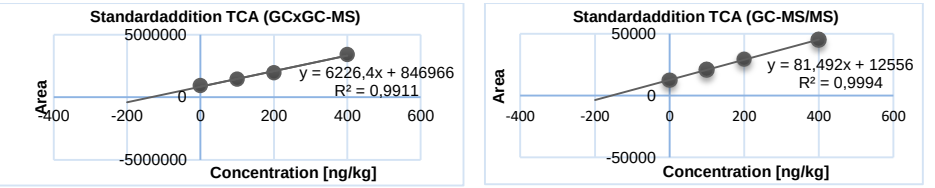


**Figure 2**: Calibration curves showing linear ranges for 2,4,6-TCA using comprehensive GCxGC-MS (left) and GC-MS/MS (right)

*Quantifications*

To investigate if the methods are applicable for the quantification of haloanisoles in real life samples, recycling paperboard samples (100 mg) were analysed. As expected, haloanisoles could not be detected with EI-GC-MS, but with comprehensive GCxGC-MS using EI in SIM, EI-GC-MS/MS in MRM and NCI-GC-MS in SIM (Table 4). Standard addition was conducted to investigate the influence of the matrix. Due to its better applicability single point internal standard method is going to be conducted in routine analysis except for the NCI-GC-MS measurements, where chlorine/bromine ions are selected in SIM and consequently the deuterated standard cannot be separated from the analyte.

**Table 4:** Quantification of the two most odorous compounds, 2,4,6-TCA and 2,4,6-TBA in an example paperboard sample using standard addition and * single point internal standard method

| Compound | *GCxGC-MS* | *GC-MS/MS* | *GC-MS/MS** | *GC-MS/NCI* |
|---|---|---|---|---|
| 2,4,6-TCA [ng/kg] | 172 ± 9 | 154 ± 17 | 177 ± 13 | 137 |
| 2,4,6-TBA [ng/kg] | 1536 | 1104 ± 102 | 1449 ± 234 | 987 |

## References

1. Buser H.R., Zanier, C., Tanner, H. (1982) J. Agric. Food Chem. 30 (2). 359–362.
2. Berscht W. (2000) J. High Resol. Chromatogr. 23 (3) 167-181.
3. ASTM E679; Standard Practice for Determination of Odor Concentration Series Method of Limits

# Emissions of compost bedded pack barn for cattle

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## Abstract

The compost dairy bedded pack barn is an animal-friendly housing system for cattle. It consists of a large, open resting area, usually bedded with sawdust. The most critical success factor for managing a compost dairy bedded pack barn (CDB) is providing a comfortable, dry resting surface for cows at all times.

Within the project "Assessment of compost dairy bedded pack barns with regard of compost quality, odour and ammonia emissions" emission measurements ($NH_3$) of 23 CDB were executed during three different seasons (summer – autumn – winter). Samples for the analysis of odour-active volatile organic compounds (VOCs) and chemical parameters (e.g. pH value, dry matter, C/N ratio) were taken. In addition, an extensive microbiological screening with special attention to harmful bacteria was carried out. The samples were picked from the compost manure mattress. In CDB, mainly sawdust is used as bedding material despite its increasing cost.

Concerning the assessment of emissions in CDB no correlations between ammonia and odour-active VOCs could be determined, but the majority of the analysed CDBs shows emission concentrations below or in the range of cubicle housing systems.

## Introduction

The compost dairy bedded pack barn (CDB) is a new and an animal-friendly housing system for cattle, which is also positively assessed from the perspective of claw health [1, 2]. The most critical success factor for managing a CDB is providing a comfortable, dry resting surface for cows at all times [3]. A CDB consists of a large, open resting area usually bedded with sawdust. Due to its good absorptivity, structural stability and good decomposition under aerobic conditions, sawdust is a very well-suited bedding material. The major drawback of sawdust is the constantly increasing costs, which makes the use of cheaper alternatives like spelt husk, wood chips or hay from nature conservation areas more attractive.

However, until now only scattered investigations about ammonia and odour-active volatile organic compounds (VOCs ) were carried out [4–6]. Knowledge about VOC emission of CBDs is of utmost importance, as potentially odorous emissions will not only effect the animals' well-being. Emission of malodourous compounds would also lead to complaints by neighbouring residents.

Another important parameter in a CDB is the large diversity of microorganisms [7]. In Friesland (The Netherlands), for instance, the group of extremely thermophilic aerobic spore-formers (XTAS) was the reason for a ban on the delivery of milk from composting plants to dairies [8]. Because of this ban, intense investigations and characterization of the compost mattress especially with respect to the microbial load is of increasing importance. Nevertheless, a total screening and characterization of the different

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microorganisms in the compost mattress (e.g. harmful bacteria, like *Klebsiella spp*.) has not yet been conducted.

## Experimental

### *Sampling*

Within this project, emission measurements ($NH_3$) of 23 CDB were performed and samples for the analysis of odour-active VOCs and chemical parameters were taken. The samples were drawn from the compost manure mattress during three different seasons (summer – autumn – winter) and on six different locations of the mattress.

### *Analysis of volatile compounds*

The gaseous $NH_3$ emissions were measured by an open dynamic chamber and analysed by a Multigasmonitor INNOVA 1412. For the analysis of potentially odorous compounds, headspace solid phase micro extraction was carried out prior to GC analysis to enrich the VOCs from the mattress samples (50/30 µm DVB/Carboxen/PDMS 2 cm stable flex fibre, Supelco; enrichment at 40°C for 20 minutes). Gas chromatography mass spectrometry (Agilent 7890A GC Systems and Agilent 5975C VL MSP, electron impact ionisation, 70eV, scan mode) equipped with a nonpolar analytical column (HP5MS, 30m x 250µm x 1µm) was used for the analysis of the VOCs. Quantitation was performed using deuterated dodecane as internal standard. Identification of the compounds was based on the comparison of the obtained mass spectra with mass spectra from MS databases or literature and the linear temperature programmed retention indices and comparison to data from authentic reference compounds and data from literature.

### *Microbial screening*

To receive information about the microbial load of CBD compost mattresses, an extensive microbial screening was carried out. Special attention was paid to the characterization of harmful bacteria, like *Klebsiella ssp*. and XTAS (extreme thermophilic aerobic spore-formers). To determine the bacterial count (colony forming units, CFU) samples were plated on plate-count agar (PCA) and on selective culture media (e.g. Baird Parker RPF Agar or SGC2).

## Results and discussion

Results from the CDB study show, that ammonia emissions are generally lower from CBD than in cubicle housing systems (Figure 1), except for the use of spelt husk and CBD with reduced lying area availability per cow. Concerning the assessment of emissions in CDB, no correlations between ammonia and odour-active VOCs formation were found.

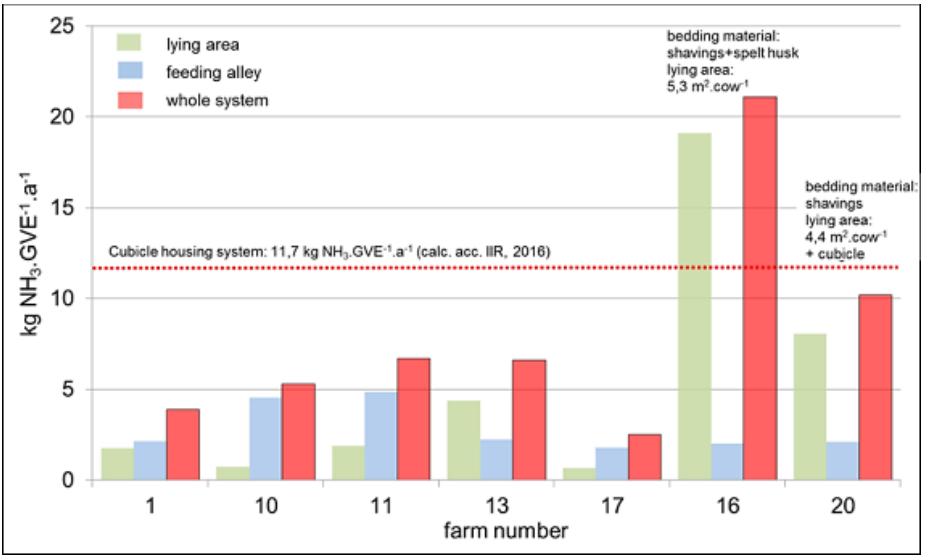


**Figure 1:** Ammonia emissions in CDB summer and autumn 2015 in comparison to a cubicle housing system

Concerning the potentially odour-active VOCs, the same conclusion was drawn as for ammonia emissions. The VOCs have a high dependency regarding seasonal changes and on the bedding material (Figure 2). Especially phenolic and sulphur compounds (e.g. dimethyl disulphide) as well as aldehydes and terpenes (e.g. 3-methylbutanal, camphene, α- and β-pinene) were identified as the major compounds responsible for the odour of the compost manure mattress. Phenols and sulphur substances show constant concentrations, whereas aldehydes and terpenes range from low to high concentrations depending on the bedding material or on the composting state of the resting surface. Furthermore, in summer, the total odour-active VOCs are - as expected – significantly higher than in winter, which shows that the annual temperature fluctuations are one of the major external factors of influence on odour formation of the CDB. VOC measurements in a comparable cubicle housing system showed 10 to 100 times higher VOC values than in CDB (depending on the compound).

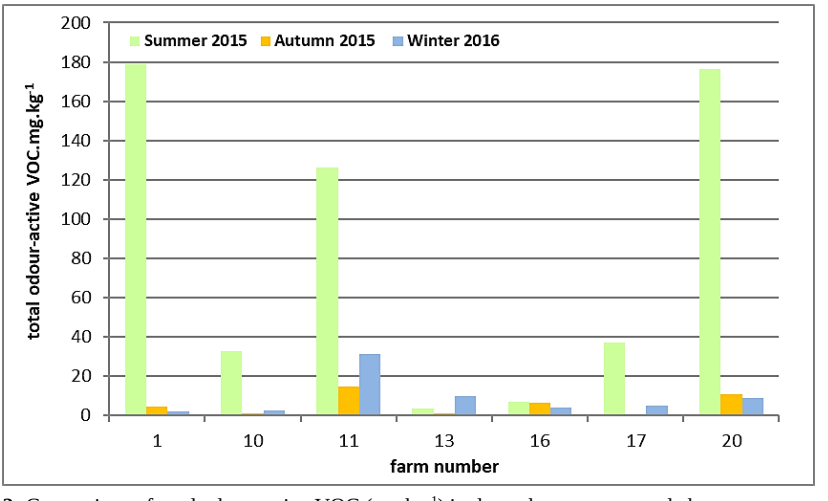


**Figure 2:** Comparison of total odour-active VOC (mg $kg^{-1}$) in dependency to seasonal changes

Microbial analysis of the compost mattresses showed no significant variation neither depending on the individual season nor on the farm (Figure 3). In general, farms using spelt husk showed the highest bacterial counts, whereas the lowest microbial concentrations were found for bedding with sawdust. Extreme thermophilic spore-formers (XTAS) were analysed in a very low concentration range. So far, no limit value exists for the presence of XTAS. Based on the results obtained from the 23 investigated CDBs, it can be assumed that CDBs do not present any hazard potential.

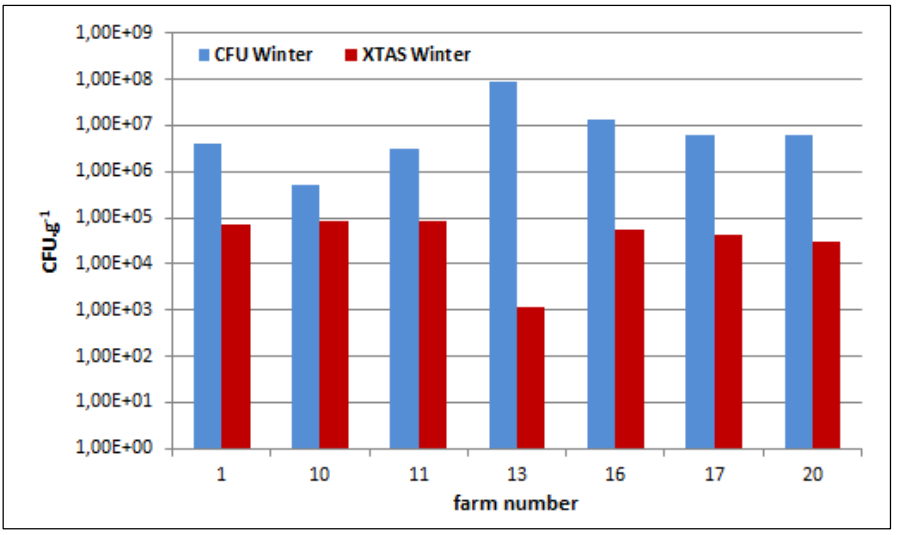


**Figure 3:** Microbiological Screening (total count vs. XTAS count) winter 2015/16

The results of this study show that a well-managed CDB is not only animal-friendly but it is also a farming option with less emissions compared to a commercial used cubicle housing system. Consequently, CDBs are said to be a very recommendable and suitable cattle farming option.

## References

1. J. Burgstaller, J. Raith, S. Kuchling, V. Mandl, A. Hund, and J. Kofler, (2016) Vet. J., vol. 216, pp. 81–86
2. E. Ofner-Schrock, M. Zahner, G. Huber, K. Guldimann, T. Guggenberger, and J. Gasteiner, (2015) Open J. Anim. Sci., vol. 5, no. 2, pp. 124–131
3. A. Pöllinger, B. Pöllinger-Zierler, C. Kapp and M. Konrad, (2016) Österreichische Fachtagung für Biol. Landwirtschaft, pp. 1–6
4. E. M. Shane, M. I. Endres, and K. A. Janni, (2010) Appl. Eng. Agric., vol. 26, no. 3, pp. 465–473
5. B. Bjerg and I. Klaas, (2014), Proc. ASABE and CSBE/SCGAB, pp. 1-7.
6. P. Galama, (2014) Report 707, WUR Livestock Research, Lelystad, p 22
7. K. Schwarzkopf, (2014) "Hygienisch-mikrobiologische Untersuchungen von Kompost-ställen und Einstreumaterialien aus Gärresten und Kompost.," Masterarbeit am Inst. für Umwelt- und Tierhyg.; Tiermed. & Tierklinik, agrarwiss. Fak., Universität Hohenheim
8. P. J. Galama, H. C. De Boer, H. J. C. Van Dooren, W. Ouweltjes, and F. Driehuis, (2015) Wageningen UR Livestock Research Report 873, p 80


# RECENT DEVELOPMENTS
# IN
# ANALYTICAL TECHNIQUES

# PARADISe -
# A ground-breaking tool to treat complex GC-MS datasets

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## Abstract

A new approach to treatment of complex GC-MS datasets is introduced. The approach is based on PARAFAC2 modelling but does not require extensive coding and in-depth mathematical knowledge due to the new 'PARAFAC2 based Deconvolution and Identification System' (PARADISe). PARADISe can, in a user-friendly way, perform all the necessary steps in treatment of GC-MS data. It is demonstrated how PARADISe can efficiently quantify peaks, resolve co-elution, improve identification and save significant amounts of time.

## Introduction

Modern GC-MS systems combined with efficient sampling techniques produce chromatograms with a large number of peaks of which many are not well-resolved. Well-designed experiments and screening investigations include many samples and replicates. The result is unavoidably heavy workload on the investigator to treat this data and extract the chemical information. Many approaches have been used from simple analysis of total ion chromatograms over single-ion techniques to different kinds of deconvolution techniques. They all have significant draw-backs: most are very time-consuming, results can be user-dependent to different degrees, and for almost all techniques, chromatograms are treated independently of each other. Furthermore, many approaches can only handle moderately overlapping peaks and often experience problems with low signal-to-noise peaks. Non-detects remain an issue as well.

Here, a completely different approach using the so-called PARAFAC2 modelling (PARAllel FACtor analysis 2) is demonstrated. Until now, PARAFAC2 modelling has only been available for mathematical users and has required extensive coding for efficient use [1]. An integrated approach called PARAFAC2 based Deconvolution and Identification System (PARADISe) has, however, become available. The solution is user-friendly, extremely time-saving, and produces reliable results that are less user-dependent. It is developed by a group of chemometricians around the 'Chemometrics and Analytical Technology' group at Department of Food Science, University of Copenhagen, and is freely available.

PARADISe benefits from the ability of PARAFAC2 to resolve co-eluting chromatographic peaks for all investigated chromatograms simultaneously [2]. It overcomes the limitation of PARAFAC2 which only works on time intervals, by assisting the user in defining appropriate intervals in the chromatograms, and it can thus perform all the necessary steps from visualization of data to generation of a final table of identified compounds for an entire set of chromatograms.

The steps in an analysis of a set of chromatograms by PARADISe are:

- Conversion of datafiles to AIA format
- Open/import files in PARADISe
- Inspect raw data (zoom/pan, search in NIST, exclude samples…)
- Define intervals

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- Calculate PARAFAC2 models
- Evaluate models (decide number of components)
- Tag relevant compounds
- Make report

In the following, examples are given to compare data treatment of real datasets done with a commonly used vendor software (Agilent ChemStation) and with PARADISe. It will be demonstrated how the techniques perform with regard to integration/baseline-modelling, deconvolution, peak identification, and user's time-consumption.

## Experimental

Chromatograms from datasets exhibiting typical challenges were selected from recent projects carried out in our lab. The chromatograms were from different food products and were all obtained using dynamic headspace sampling in combination with thermal desorption (Perkin Elmer Turbomatrix ATD 650) gas chromatography mass spectrometry (7890A GC-system interfaced with a 5975C VL MSD with Triple-Axis detector from Agilent Technologies, Palo Alto, California) as described by Fjaeldstad et al. [3]. The chromatograms were treated using Agilent's software ChemStation (MSD ChemStation E.02.02.1431) and using PARADISe, a software package developed by Johnsen et al. [4] and available from *http://models.life.ku.dk/paradise* (PARADISe version 1.1.6).

## Results and discussion

*Example 1*

This is a simple case to demonstrate the basic features in PARAFAC2 modelling as carried out in PARADISe. The raw data is the time interval from 3.71 to 3.99 minutes taken from 40 chromatograms. Part of the task in using PARADISe is to determine how many components need to be used. There are several utilities for this in the software and some are explained in Example 3. Figure 1 shows how a PARAFAC2 model with 2 components can separate the raw data into two 'phenomena' or components: Component 1 which includes mass fragments of typical background noise (air, water a.o.) and component 2 which mainly includes the mass fragments 43 and 86 (see Figure 2).

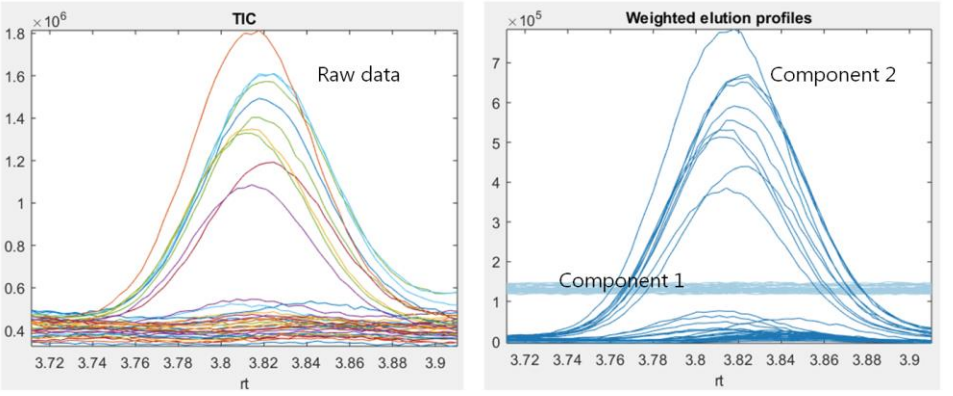


**Figure 1:** Total Ion Chromatograms (TIC) from the interval 3.71 - 3.99 min from 40 chromatograms and weighted elution profiles from a PARAFAC2 model with two components

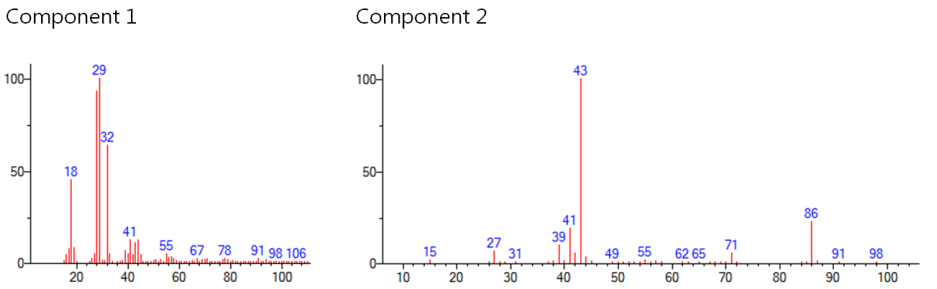


**Figure 2:** Patterns of mass fragments (=mass spectra) constituting component 1 and 2 in Figure 1

The mass fragments of component 2 do actually make up a mass spectrum, and when searched in the NIST database, it was identified as 3-methyl-2-butanone. It is seen that the PARAFAC2 model eliminates the need for integration of peaks. Instead the background is modelled and separated into its own component(s), in this case component 1, so component 2 exclusively represents 3-methyl-2-butanone. Even background noise that changes in intensity and in composition throughout the interval can be modelled, but may then require more than one component.

The PARAFAC2 model extracts one mass spectrum for each component by combining information from all chromatograms. This results in a mass spectrum of higher quality and better match factors are most often experienced. Finally, the PARAFAC2 model creates a concentration profile which is a list of the peak areas in all the chromatograms included. It should be noted that minor retention time shifts (for example as those seen most clearly in the weighted elution profiles in Figure 1) are handled by the model without problems. It is also worth noting that the PARAFAC2 model does not assume any particular shape (e.g. Gaussian or Lorentzian) of the elution profiles. The shape is solely determined by the data.

*Example 2*

This example demonstrates a more complex situation, see Figure 3. The figure shows two coeluting peaks which were expected to be 2- and 3-methylbutanal. PARAFAC2 modelling did, however, reveal that 6 ‘phenomena’ or components could be found in the interval, see Figure 4.

The first component is representing ethyl acetate, but it is only a small remain (or ‘tail’) not belonging to this time interval. Component 2 and 3 represent rather small peaks that were hidden behind 2- and 3-methylbutanal in the TIC, but could still be identified with high match factors as vinyl isopentylether and 2-methyl-2-propanol. Component 4 models background noise. So, in addition to performing a near perfect separation, and thus quantification and identification, of 2- and 3-methylbutanal, two hidden peaks were identified and quantified with high reliability.

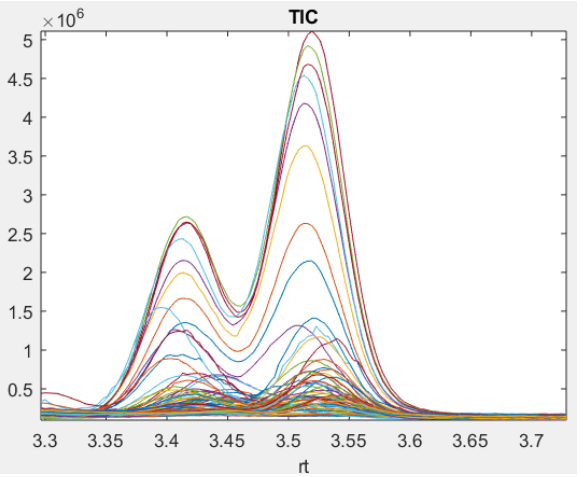


**Figure 3:** Total Ion Chromatograms (TIC) from the interval 3.30 - 3.75 min from 80 chromatograms

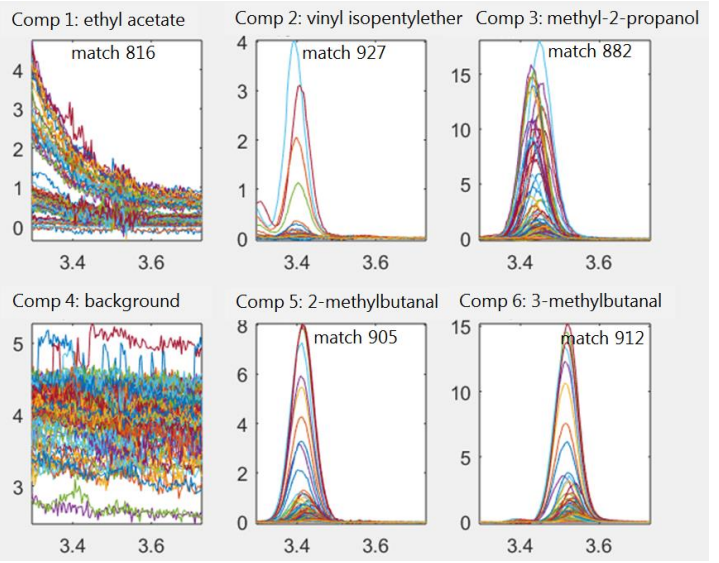


**Figure 4:** Weighted elution profiles (not overlaid) from a 6 component PARAFAC2 model applied to the data shown in Figure 3. Identifications and match factors from search in the NIST database are also shown.

*Example 3*

This example shows how the appropriate number of components is determined and how decisions on number of components affect the data obtained. The same 40 chromatograms and the same retention time interval as in example 1 are used, supplemented by data from the interval 3.73 - 3.92 min which include the compound 2-butanone. Figure 5 and 6 show peak areas of the two compounds from five selected samples. The peak areas were calculated from the TIC's using standard integration settings in ChemStation and by applying 1, 2, 3 and 4 component PARAFAC2 models in PARADISe.

To determine the appropriate number of components in the models, PARADISe includes two diagnostics: Fit and core consistency. Fit will normally increase with increasing number of components while core consistency tends to decrease. Both values should be as high as possible (range: 0-100). Fit and core consistency are included in the figures. The numbers indicate that 2 and 3 components could both be reasonable. When several models are appropriate it is often useful to select the one with most components in order to extract as many chemical pieces of information as possible.

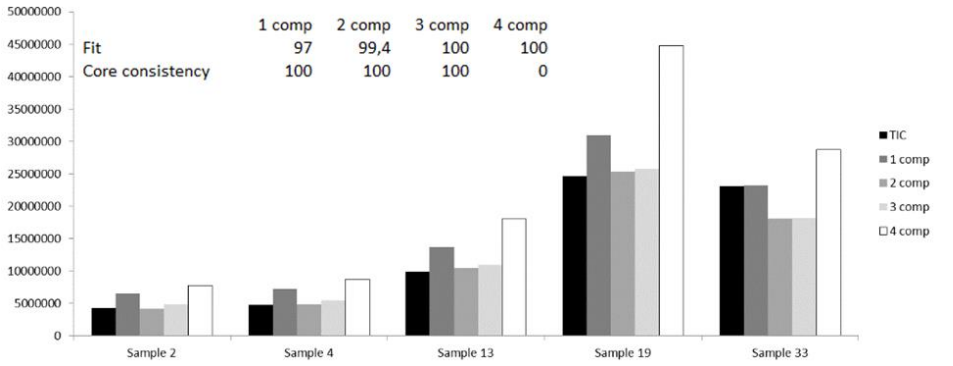


**Figure 5:** Peak areas of 2-butanone in five selected samples. The peak areas were calculated from TIC's using standard integration settings in ChemStation and by applying 1, 2, 3 and 4 component PARAFAC2 models.

2-Butanone (Figure 5) is a medium sized peak. A 3 component model would be the choice since it has fit and core consistency values of 100. The 2 component model works almost equally well, but the 4 component model is obviously wrong, having a core consistency of 0. The 1 component model gives too high peak areas because the background noise is not modelled by a separate component but is included in component 1. The TIC data from ChemStation fits the 2 and 3 component models well. The reason for the discrepancy in sample 33 is a coelution which is not resolved by ChemStation (and neither by the 1 component model).

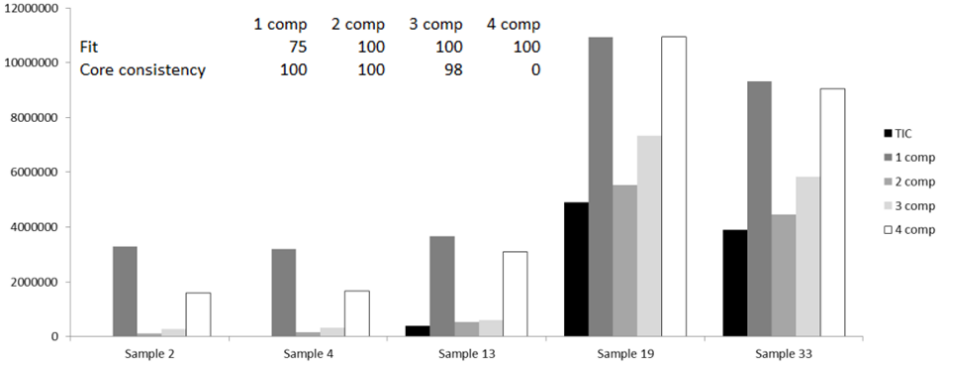


**Figure 6:** Peak areas of 3-methyl-2-butanone in five selected samples. The peak areas were calculated from TIC's using standard integration settings in ChemStation and by applying 1, 2, 3 and 4 component PARAFAC2 models.

3-Methyl-2-butanone (Figure 6) has very small peaks in some of the samples. A 2 component model would be the choice since it has fit and core consistency values of 100.

The 3 and especially the 4 component models have lower core consistency and are therefore less appropriate. The TIC data from ChemStation fits the 2 component model well except in sample 2 and 4 where the peak is too small to be integrated by the ChemStation software.

This example shows that the diagnostics fit and core consistency give good guidance in determining the correct number of components. Even when the guidance is not clear (as for 2-butanone) the two possible selections (2 or 3 components) result in almost equal peak areas. Furthermore, it is demonstrated that PARADISe does not depend on integration settings, but gives areas of all peaks independent of their size, and that the peak areas reported by PARADISe are practically equal to those obtained when well separated TIC peaks are integrated in ChemStation. Note, that even in samples without a certain chemical present, it will still be quantified. All chemicals are quantified in all samples and hence, there is no issue with below limit of detection.

## Time consumption

To go through the steps mentioned in the introduction, a user of PARADISe will typically spend a few minutes to convert and import files. Time used for inspecting raw data depends mostly on the data. Defining intervals can be done within 30 min for an experienced user. The calculation of PARAFAC2 models is very time consuming (few hours to more than a day) but will be carried out by the computer unattended. Evaluating the models and tagging compounds may take up to a couple of hours depending on the complexity of the chromatograms, and finally the report is created within few minutes. In total, the typical time consumption will be 2-3 hours for an experienced user – almost independent on the number of chromatograms included.

## Conclusion

It is concluded that treatment of large datasets with PARADISe results in extraction of more information, the information is more reliable, and user's time-consumption when treating datasets with numerous complex samples/chromatograms is dramatically reduced.

## References

1. Kiers H.A.L., ten Berge J..MF. and Bro R. (1999), Journal of Chemometrics, 13, 275-294.
2. Amigo J.M., Popielarz M.J., Callejón R.M., Morales M.L., Troncoso A.M., Petersen M.A. and Toldam-Andersen T.B. (2010), Journal of Chromatography A, 1217, 4422–4429
3. Fjaeldstad A., Petersen M.A. and Ovesen T. (2017), Chem. Percept., 10, 42–48
4. Johnsen L.G., Skou P.B., Khakimov B. and Bro R. (2017), Journal of Chromatography A, 1503, 57–64

# Exploring 2-acetyl-1-pyrroline loss by high resolution mass spectrometry and nuclear magnetic resonance

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## Abstract

2-Acetyl-1-pyrroline (2AP) is the characterizing odorant in foods such as aromatic rice and popcorn. 2AP is a potent odorant with a detection threshold of 0.1 ppb and contributes a roasted, cracker-like or popcorn-like aroma character. As such, 2AP would be appealing as an added flavoring but this poses a challenge due to the molecule's unstable nature. When 2AP is neat or in a concentrated aqueous solution, the color will rapidly change from colorless to red as 2AP reacts. Mass spectral data from our lab show a decline in a 25 mg/mL aqueous solution of 2AP in only 5 minutes. Buttery et al. postulated in 1983 that this molecule undergoes a polymerization process. Yet, little information is available in the literature to support this hypothesis. Our research has probed 2AP loss in water by high resolution mass spectrometry (HR-MS) and NMR (1D and 2D) and confirmed that a polymerization process does occur. We have observed that 2AP polymerization is a complex process, generating many unstable intermediates. The intermediates are highly unsaturated molecules which contain an increasing number of 2AP moieties, accompanied by the loss of water. Increasing dehydration occurs as molecular weight increases. NMR shows the increase of other small molecules in 2-6 hours of reaction. We have assembled a list of structural features of the polymeric species via 2D NMR and $MS^2$. The research presented will focus on the insights gained about 2AP's reactions products. Stabilization strategies for 2AP will be briefly mentioned.

## Introduction

2-Acetyl-1-pyrroline (2AP) is the characterizing aroma compound in aromatic rice, popcorn and the Pandan leaf with a low odor threshold (0.1 ppb) [1]. 2AP can be formed biochemically, such as in rice, the Pandan leaf and the bread flower. Additionally, it is a Maillard reaction compound; and therefore, 2AP is present in a huge variety of cooked foods. Unfortunately, 2AP is very unstable when isolated in its neat form. 2AP rapidly undergoes a color change from a pale yellow oil to a viscous red material in minutes after being concentrated.

2AP was discovered in 1982 by Ron Buttery's group [2]. In the following year, this research group offered the hypothesis that 2AP undergoes a polymerization process between the carbonyl group and the five position of other molecules [1]. During the 35 years since 2AP's discovery, there has been a wealth of research done on 2AP that has included the development of new synthetic routes, the identification and quantification of 2AP in various foods, and a variety of chemical and physical methods developed to increase the compound's storage stability. Figure 1 shows some highlights from 2AP's history. Yet, 2AP's fate has been accepted as polymerization without analytical characterization.

The current work provides strong data to explain the fate of 2AP. This research is motivated by the clear interest of the scientific community in this molecule and the potential of 2AP as a flavoring molecule in the food industry. A deeper understanding of

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2AP's reaction fate can offer insight into stabilization attempts and provide knowledge about the chemistry of the structurally similar family of cracker-like aroma compounds.

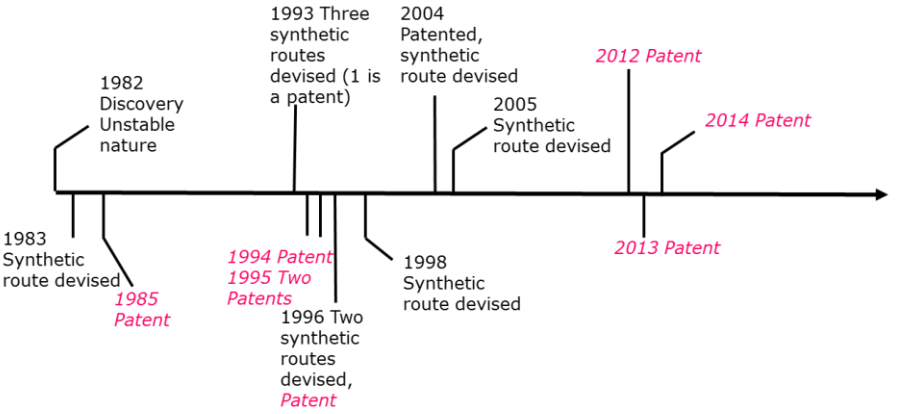


**Figure 1:** Timeline of 2AP's discovery and synthesis and patent history. The italicized work is focused on 2AP stabilization. Compiled from references 1-13.

## Experimental

2AP was synthesized according to known methods [5]. The product was stored as a dilute solution in dry ether (sodium sulfate present) in the freezer. Immediately prior to an experiment, neat 2AP was obtained by filtering away the sodium sulfate and removing the ether under a stream of nitrogen gas. Initial experiments explored 2AP degradation in methanol. However, 2AP was relatively stable (days) and deuterium exchange proved to be a problem when working with deuterated solvents. The acetyl group can undergo keto-enol tautomerization. This is observed in NMR by the loss of a singlet corresponding to 2AP's methyl group, accompanied by the formation a triplet and pentet just upfield of the methyl signal. This is consistent with the incorporation of one or two deuterium atoms, respectively, based on the deuterium 2n+1 rule.

Further studies used water as the dilution solvent for 2AP. This is an appropriate solvent choice since water is a major component of many foods. Normal (protonated) water was used for NMR studies as well to overcome problems with exchange. Time course data was collected with high resolution mass spectrometry (HR-MS) and NMR for times within a few minutes of the reaction start and up to seven hours. MS2 data was used to obtain additional structural insights. 2D NMR experiments (COSY, TOCSY, HSQC, HMBC) were also conducted to gain connectivity data. Isotopically labeled d3-2AP was synthesized from d3-iodomethane. HR-MS studies in deuterium oxide were conducted on the labeled molecule.

## Results and discussion

From the time course data collected with HR-MS (not shown) it is notable that 2AP's peak area declines in only 5 minutes [14]. Table 1 demonstrates the formation of a series of products with increasing molecular weight [14]. These masses are a multiple of 2AP's mass plus a proton. Alternatively, the masses correspond to a multiple of 2AP's mass, accompanied by the loss of water. For example, the mass 205.1338 is the equivalent mass of 2 molecules of 2AP, the loss of 18 Daltons from water and the addition of a proton to

create a positive ion, caused by the ionization process. Another feature observed from the data is that the abundance of the dimer (223.1444) increases at early time points and then declines (data not shown) [14]. This would also be consistent with a polymerization process, where products continue to react and increase in size.

From Table 1, the mass accuracies are all within 3 ppm, which gives confidence that the proposed chemical formulas are correct. The same time course experiment was repeated with d3-2AP. In order to get useful data, the experiment must be conducted in deuterium oxide, since the deuteriums on the acetyl group exchange with the deuteriums of the solvent. Table 2 shows some very interesting results, which indicate that exchange must occur between the solvent and a proton on the pyrroline ring, indicating that a ring opening mechanism is involved [14]. The rationale for this conclusion is based on the fact that the observed masses have one more deuterium that the predicted formulas for the isotopic species. For example, the dimer has six deuterium atoms from the two molecules coming together and one deuterium from the ionization process, giving a total of seven deuterium atoms. Since eight deuterium atoms are actually present on the dimer, this strongly points to a ring opening mechanism. Further, this observation gives further confidence that a chemical reaction is occurring, as opposed to aggregates forming in the mass spectrometer.

**Table 1:** 2AP product masses with chemical formula assignments and qualitative descriptions

| General name | *Chemical formula (protonated)* | *Exact mass* | *Mass accuracy, ppm* |
|---|---|---|---|
| 2AP | $C_6H_{10}NO$ | 112.0760 | 2.762 |
| dehydrated dimer | $C_{12}H_{17}ON_2$ | 205.1341 | 2.731 |
| dimer | $C_{12}H_{19}O_2N_2$ | 223.1447 | 2.669 |
| dehydrated trimer | $C_{18}H_{26}O_2N_3$ | 316.2026 | 2.044 |
| trimer | $C_{18}H_{28}O_3N_3$ | 334.2131 | 1.741 |
| doubly dehydrated tetramer | $C_{24}H_{33}O_2N_4$ | 409.2605 | 1.704 |
| dehydrated tetramer | $C_{24}H_{35}O_3N_4$ | 427.2710 | 1.480 |
| triply dehydrated pentamer | $C_{30}H_{40}O_2N_5$ | 502.3179 | 0.494 |
| doubly dehydrated pentamer | $C_{30}H_{42}O_3N_5$ | 520.3287 | 0.929 |
| triply dehydrated hexamer | $C_{36}H_{49}O_3N_6$ | 613.3874 | 2.175 |
| doubly dehydrated hexamer | $C_{36}H_{51}O_4N_6$ | 631.3980 | 2.169 |

**Table 2:** Products observed from the loss of $d_3$-(Me)-2-acetyl-1-pyrroline in deuterium oxide

| General name | *Chemical formula (protonated)* | *Predicted $d_3$-analogue (in $D_2O$)* | *Actual $d_3$- analogue (in $D_2O$)* | *Exact mass* | *Mass accuracy, ppm* | *Complications* |
|---|---|---|---|---|---|---|
| 2AP | $C_6H_{10}ON$ | $C_6H_6{}^2H_4NO$ | $C_6H_6{}^2H_4NO$ | 116.10081 | 0.108 | none |
| dehydrated dimer | $C_{12}H_{17}ON_2$ | $C_{12}H_{12}{}^2H_5ON_2$ | $C_{12}H_{11}{}^2H_6ON_2$ | 211.17116 | -0.190 | 1 D exchanged for H |
| dimer | $C_{12}H_{19}O_2N_2$ | $C_{12}H_{12}{}^2H_7O_2N_2$ | $C_{12}H_{11}{}^2H_8O_2N_2$ | 231.1942 | -0.512 | 1 D exchanged for H |
| dehydrated trimer | $C_{18}H_{26}O_2N_3$ | $C_{18}H_{18}{}^2H_8O_2N_3$ | $C_{18}H_{15}{}^2H_{10}O_2N_3Na$ | 348.24626 | -1.165 | 2 D exchanged for 2 H |
| trimer | $C_{18}H_{28}O_3N_3$ | $C_{18}H_{18}{}^2H_{10}O_3N_3$ | $C_{18}H_{16}{}^2H_{12}O_3N_3$ | 346.28784 | -1.413 | 2 D exchanged for 2 H |
| trimer | | | $C_{18}H_{16}{}^2H_{11}O_3N_3Na$ | 367.26306 | -1.218 | 2 D exchanged for 2 H |
| dehydrated tetramer | $C_{24}H_{35}O_3N_4$ | $C_{24}H_{24}{}^2H_{11}O_3N_4$ | $C_{24}H_{21}{}^2H_{14}O_3N_4$ | 441.35799 | -0.571 | 3 D exchanged for 3 H |

The NMR data was collected in an array. There was no delay between each step in the array, allowing the maximum sensitivity by continuous acquisition of spectra. The major signals in the time zero data are all associated with 2AP and it structural dimer (data not shown) [14], the latter of which is formed during 2AP's chemical synthesis and is difficult to separate from 2AP using silica gel chromatography. After 2 hours of reaction, it is clear that much of the 2AP has been lost and new species have formed. The challenge of NMR in general is that milligram quantities of product are needed. In this reaction system, numerous products are being generated in trace quantities. Another particular challenge is the system's dynamic nature. NMR sensitivity doubles as the number of scans is quadrupled; however, this system does not give the option of long acquisition times. The problem is being overcome through the use of a quick sample preparation procedure using solid phase extraction. We have successfully isolated the dimer in excess of 90% purity. With the low quantity of 2AP, the dimer was stable for at least a 24-hour time period, quite adequate for acquiring quality 2D data. NMR spectra was collected using protonated solvents (regular water and methanol) due to the exchange problem. Presaturation of the methyl signal from methanol interferes with dimer cross peaks in the 2D data. Studies are currently underway with d3-methanol (CD3OH) to overcome this problem.

The data given here provide strong evidence that 2AP undergoes a polymerization process. Dilution is a simple strategy to extend 2AP's shelf life, also suggested by Buttery when he hypothesized that 2AP undergoes polymerization [1]. Various stabilization strategies have been developed for 2AP. Physical methods have included entrapment in cyclodextrin [9] and spray drying [15]. Unfortunately, these methods have suffered from poor loading and a limited increase in shelf life. The most promising strategy to date has been 2AP complexation with zinc halides [13]. The zinc forms a covalent bond to both the nitrogen and oxygen groups of 2AP. This complex is stable for months if stored under moisture free conditions. Further, since zinc, chloride and 2AP are all GRAS molecules, the complex should not pose a problem for obtaining GRAS status. This concept of holding the reactive groups on 2AP captive is a key idea from both the current work and the zinc halide complexation. Future stabilization work should continue to utilize this concept.

## Summary and Conclusions

From this research we have provided mass spectral data to show that 2AP undergoes polymerization. We see that 2AP forms up to at least a hexamer in dehydrated form. $MS^2$ data indicates that the 2AP ring opens during formation of the dimer. Further, NMR suggests that the dimer contains carbonyl and carbon-carbon and/or carbon-nitrogen double bonds. We are continuing efforts to identify the dimer structure. Holding one or more of the reactive groups on 2AP captive is a promising approach to stabilizing this potent flavor molecule.

## References


1. Buttery R.G., Ling L.C., Juliano B.O. and Turnbaugh J.G. (1983) J. Agric. Food Chem., 31: 823–826.
2. Buttery R.G., Ling L.C. and Juliano B.O. (1982) Chem. Ind., 23: 958–959.
3. Adams A., and De Kimpe N. (2006) Chem. Rev., 106: 2299–2319.
4. Buttery R.G., Ling L.C., Juliano B.O. 2-Acetyl-1-Pyrroline and Its Use for Flavoring Foods. (June 11, 1985) US Patent 4,522,838.
5. De Kimpe N.G., Stevens C.V., and Keppens, M.A. (1993) J. Agric. Food Chem., 41: 1458-1461.
6. Duby P., Huynh-Ba T. Pyrroline-Based Food Flavoring Compounds. (January 18, 1994) U.S. Patent 5,280,127.
7. Duby P., Huynh-Ba T. Preparation of 2-Acetyl-1-Pyrroline. (August 29, 1995) U.S. Patent
8. Duby P., Huynh-Ba T. Preparation of 2-Acetyl-1-Pyrroline Compositions. (March 28, 1995) U.S. Patent 5,401,521.
9. Duby P., Huynh-Ba T. Compositions Containing 2-Acetyl-1-Pyrroline. (April 30, 1996) U.S. Patent 5,512,290.
10. Srinivas P., Gurudutt K.N. Process for the Preparation of 2-Acetyl-1-Pyrroline, the Basmati Rice Flavorant. (April 20, 2004) U.S. Patent 6,723,856 B1.
11. Degenhardt A., Krammer G., Koch J., Tai M.Y. Novel Pandan Extract Articles in Powder Form and Process for the Production Thereof. (August 23, 2012) U.S. Patent 2012/0213904 A1.
12. Morere A., Menut C., Gunata Y.Z., Agrebi A. Method for Synthesizing 2-Acetyl-1-Pyrroline, Stable Ketal Precursors Thereof, and Isotopically Marked Analogs. (December 3, 2013) U.S. Patent 8,597,706 B2.
13. Cadwallader K.R., Fang M.-C. Stabilized Compositions and Methods of Manufacture. (September 18, 2014) U.S. Patent 2014/0275543 A1.
14. Hausch B.J., Cadwallader K.R. Exploring 2-Acetyl-1-Pyrroline Loss by High Resolution Mass Spectrometry and Nuclear Magnetic Resonance. Manuscript in preparation.
15. Apintanapong M., and Noomhorm A (2003) Int. J. Food Sci. Technol., 38: 95–102.

# A sophisticated setup for rapid, sensitive and selective food and flavor analysis

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## Abstract

A novel Proton Transfer Reaction – Mass Spectrometry (PTR-MS) setup for rapid, sensitive and selective food and flavor analysis is introduced and proof-of-concept measurements are presented. The setup enables rapid and sensitive analysis because of the utilization of ion transmission improving technologies, namely an ion funnel and a hexapole ion guide between the PTR drift tube and the mass spectrometer and the interfacing with an autosampler. Furthermore, the setup is highly selective because of the implementation of an advanced fastGC inlet system. Using a certified gas standard, the performance of the PTR-MS instrument is evaluated and compared to conventional devices. Finally, the combination of all instrumental components is tested in real-life conditions by analyzing nine different red wines.

## Introduction

For decades Gas Chromatography – Mass Spectrometry (GC-MS) has been the gold standard for sensitive and selective analysis in food and flavor science. However, soon after its introduction in the 1990s PTR-MS has proven its potential in this field and has rapidly become an established method for real-time monitoring [1]. Early PTR-MS instruments were equipped with quadrupole mass filters and lacked selectivity because of unit mass resolution. They provided real-time quantification capability only for monitoring of selected compounds, as acquiring full mass spectra with quadrupole MS can only be done in mass scanning mode and thus is time-consuming. These drawbacks have been overcome by the introduction of high resolution Time-Of-Flight (TOF) mass spectrometers in the late 2000s [1], [2]. Nowadays PTR-TOFMS instruments can be considered as state-of-the-art, because of their ample advantages over quadrupole based PTR-MS devices and development work of various commercial manufacturers and universities is mainly focused on the further improvement of selectivity and sensitivity.

The importance of high selectivity in food and flavor science is obvious, as often a large number of compounds needs to be analyzed in complex matrices (e.g. coffee [3], wine [4], etc.), with many of them being isobars or even isomers. Whereas high mass resolution of TOF mass spectrometers enables the separation of isobaric compounds, isomers cannot be distinguished regardless of the resolution and require additional means of selectivity improving measures. Early attempts of coupling GC to PTR-MS were successful in considerably improving selectivity (separation of isomers, unambiguous compound identification, etc.) but disabled one of the most important advantages of PTR-MS, namely the real-time capability [5]. Much more rapid methods, which have subsequently evolved, include switching of reagent ions and changing of the reduced electric field strength in the PTR drift tube (E/N) [6], but have not met the gold standard GC in terms of selectivity so far. Eventually, a presumably ideal compromise between separation power and response time has been published in 2014: a rapid GC system consisting of a multi-capillary-column coupled to PTR-TOFMS [7].

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The main advantage of improved sensitivity is not only a better Limit-of-Detection (LoD), but increased measurement speed and better quality of data. If one assumes a typical sensitivity of 25 cps/ppbv of first generation PTR-TOFMS instruments [2], the relative statistical error for measuring a compound at 1 ppbv for 1 s is 20% (square root of count rate divided by count rate). Exactly the same concentration measured for 1 s with an improved instrument with 1000 cps/ppbv would lead to an error of only 3%. Even if the measurement time would be reduced by one order of magnitude to 100 ms, the error would still be only 10%. Thus, sensitivity is of utmost importance for time-critical applications, such as flavor analysis in mouth- and nosespace or rapid aroma releasing processes. Furthermore, sample throughput can be considerably increased if the measurement time per sample can be reduced.

Here we present a sophisticated setup combining a novel high-sensitivity PTR-TOFMS instrument with an advanced fastGC inlet system, which allows for switching between direct injection and fastGC mode, and an autosampler.

## Experimental

The working principle of PTR-TOFMS has been described in detail e.g. in the book by Ellis and Mayhew [8]. In short, $H_3O^+$, $NO^+$, $O_2^+$ [9] and $Kr^+$ [10], respectively, reagent ions are generated in a hollow cathode ion source and injected into a drift tube, where chemical ionization of the analytes takes place. The reagent and product ions are then separated according to their *m/z* in a TOF analyzer and detected with a microchannel plate detector.

The transfer region between the drift tube and the TOF mass spectrometer is what distinguishes the novel instrument (called "PTR-TOF 6000 X2") from conventional designs. Recently, it has been found that this area, traditionally consisting of a series of electrostatic lenses, is the cause for a high amount of ion losses and thus is the main limiting factor for the overall sensitivity. As a countermeasure we developed an ion funnel for being installed at the end of the drift tube. Ion funnels have been invented in the late 1990s [11] and consist of a series of lenses with successively smaller apertures to which an alternating voltage is applied. This setup effectively focuses ions to the exit aperture and has first been applied to PTR-TOFMS by Barber et al. [12], where the ion funnel constituted about 50% of the drift tube and thus formed a major part of the reaction region. We, however, developed a compact and modular funnel design, which i) is primarily for focusing the ions and with only about 1/3 of the drift tube length, not forming an integral part of the reaction region and ii) can easily be installed in existing PTR-TOFMS instruments by replacing the drift tube exit lens with the ion funnel.

In 2014 we introduced a PTR-TOFMS instrument equipped with a quadrupole ion guide instead of a conventional transfer lens system [13]. Now we considerably improved this design by developing a hexapole instead of a quadrupole ion guide, as hexapoles are known to have a better transmission and to be more beneficial for focusing ions of a broad *m/z* range. Besides improving the transmission, i.e. the sensitivity of the instrument, multipole ion guides additionally cause cooling of the ions and thus improve injection conditions into the TOF mass spectrometer, which results in an increased mass resolution. In the results section we present the effects this combination of ion funnel and hexapole ion guide, which is displayed in the schematic view in Figure 1, has on the instrument's sensitivity by analyzing a certified gas standard (TO-14A aromatics mix).

For selectivity improvement we essentially revised the GC design from [7] to an efficient fastGC setup. The present setup consists of an electronically switchable pressure

controlled multiport valve, which enables switching between direct injection and fastGC mode. In direct injection mode the air from the sampling line is split to two lines, with one line leading directly to the PTR drift tube and the other feeding a sample loop. That is, while the instrument is measuring in real-time, as it is common for PTR-MS, the sample loop is continuously flushed with sample air. By switching to the fastGC mode, the content of the sample loop is injected into a 10 m nonpolar MXT-1 column, which can be heated to 400°C with a heating rate of up to 1200°C/min. After being separated according to their retention times the compounds are injected into the drift tube. Typical spectral runs take between 30 to 150 s depending on the temperature profile. This means that by switching between direct injection and fastGC mode the advantages of PTR-MS and GC can be combined, namely real-time analysis and the highest level of selectivity.

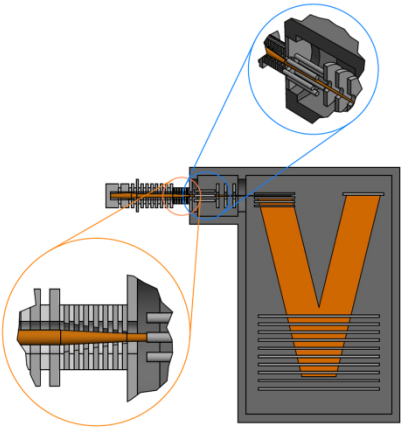

**Figure 1**: Schematic view of the novel PTR-TOF 6000 X2 equipped with an ion funnel (insert bottom left) and hexapole ion guide (insert top right).

The "sophisticated setup for rapid, sensitive and selective food and flavor analysis", as referred to in the title of this contribution, is completed by a commercial autosampler (PAL RSI, CTC Analytics AG, CH) for which we developed a dedicated interface to connect it to the PTR-TOFMS instrument. This interface consists of a heated cell, which is constantly flushed with $N_2$ at a controlled flow rate. Via a septum the content of the autosampler syringe is injected into the cell. Finally, the mixture of headspace and $N_2$ is introduced into the PTR-TOFMS instrument via a common inlet line and excess air is ejected via an overflow port. This design is necessary so that the static headspace of sample vials can be analyzed, because the PTR-TOFMS instrument requires a continuous sample gas flow.

As a proof-of-concept test of the performance of the novel setup we investigated nine different red wines purchased at a local supermarket.

## Results and discussion

In order to evaluate the sensitivity improvement of installing an ion funnel and a hexapole ion guide we analyzed a certified TO-14A aromatics mix with three different PTR-TOFMS instruments. In the left diagram of Figure 2 the results are shown. The PTR-TOF 1000 is a conventional instrument with a system of electrostatic lenses in the transfer region between the drift tube and the TOF mass spectrometer. The PTR-TOF 1000 *ultra* has been upgraded by installing a modular ion funnel, as described in the experimental

section. Finally, the PTR-TOF 6000 X2 is equipped with a hexapole ion guide in addition to the ion funnel and thus combines both transmission improving technologies. However, it should be noted that the latter instrument has a TOF with a longer flight-path, compared to the PTR-TOF 1000, which improves the mass resolution, but somewhat lowers the sensitivity, because a lower pulse rate has to be used. All instruments show increasing sensitivities with increasing *m/z*, which is a well-known effect observed in TOF analyzers. For the conventional PTR-TOF 1000 the resulting sensitivity is in the range of 60 – 130 cps/ppbv. After installing the modular ion funnel the sensitivity is boosted by nearly one order of magnitude to 600 – 1000 cps/ppbv for the PTR-TOF 1000 *ultra*. Eventually, the combination of further improved transmission due to the hexapole ion guide and a lower pulse frequency leads to a boost by another factor of 2 for the PTR-TOF 6000 X2, with values between 1200 – 1800 cps/ppbv.

In the right diagram of Figure 2 the minimum integration times for an arbitrary compound with a low concentration of 100 pptv in order to get a relative statistical error of 5 and 10% (compare introduction), respectively, have been calculated using the measured sensitivities. Assuming one wants to quantify 100 pptv with 5% error e.g. in exhaled nosespace air, the importance of high sensitivity gets immediately obvious. The 30 s of the PTR-TOF 1000 well exceed the duration of a breath cycle and disqualify the instrument for the given task. With the PTR-TOF 6000 X2, however, 100 pptv can be detected with 5% error within about 2 s and if an error of 10% is acceptable, the breath cycles can be monitored with a high time resolution of 600 ms.

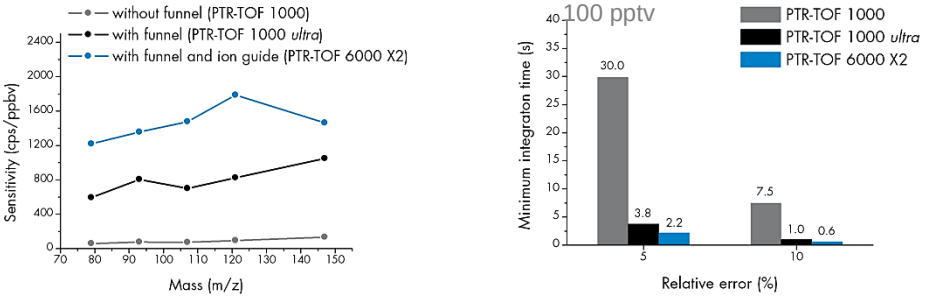


**Figure 2**: Comparison of measured sensitivities for different instrument types (left) and the calculated minimum integration times to reach a relative error of 5 and 10%, respectively (right).

For the LoDs, calculated via the maximum sensitivities and by using the 3σ method, we determined 70, 10 and 10 pptv for 1 s and 10 pptv, 750 ppqv and 550 ppqv for 1 min integration time for the PTR-TOF 1000, *ultra* and 6000 X2, respectively. The mass resolutions (full width at half maximum) at *m/z* 181 were 1700 m/Δm for the PTR-TOF 1000 (*ultra*) and 6000 m/Δm for the PTR-TOF 6000 X2 (data not shown).

Figure 3 shows the instrumental response during the autosampler injection of wine headspace while switching between direct injection and fastGC mode. The various lines in this diagram represent the ion yields for different *m/z* versus time. *Note*: As data evaluation of the wine study is still ongoing and this contribution should only serve as a proof-of-concept, here we do not attempt to identify compounds or go into detail about the different wines. As soon as the autosampler injects the wine headspace into the interface an immediate response can be seen for all *m/z*. In conventional PTR-TOFMS these signal intensities would be attributed to distinct compounds matching the exact *m/z*.

However, switching to fastGC mode unveils that for nearly all *m/z* more than one ion is contributing to the total ion yield, which can be due to the presence of isomers, non-resolved isobars or fragment ions.

The advantage of high selectivity gets even more obvious in Figure 4, where the nine different wines are compared. At *m/z* 131.11 two ions can be separated in fastGC mode (left diagram). Importantly, these two ions, which are detected as one sum signal in direct injection mode, have considerably different ratios. That is, even if the ion yields at a particular *m/z* are similar in intensity for several wines in direct injection mode, it is still possible that the compounds contributing to this ion yield are present in completely different concentrations. This can also nicely be seen in the right diagram of Figure 4, where even three ions can be seen at *m/z* 117.09. For some wines the abundance of all three ions is comparable in intensity, whereas for other wines over 50% originates from the ion at 38 s retention time.

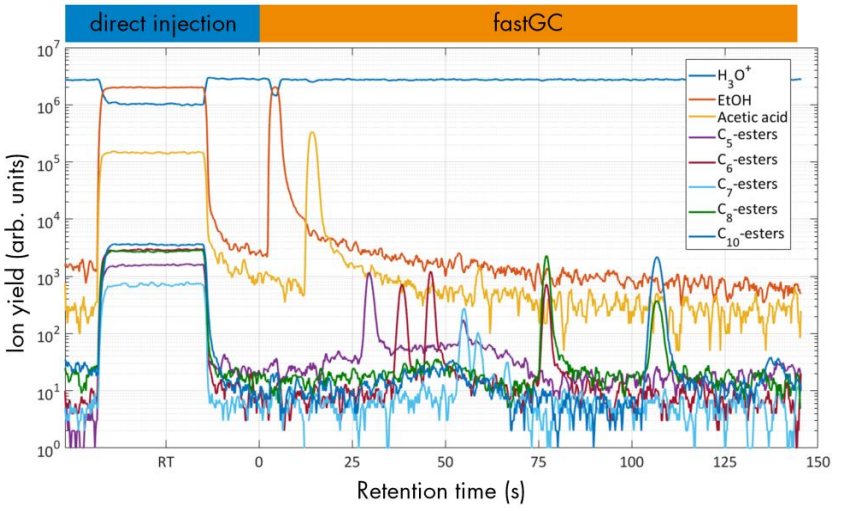


**Figure 3**: Exemplary ion yields during the autosampler injection of wine headspace in direct injection and fastGC mode.

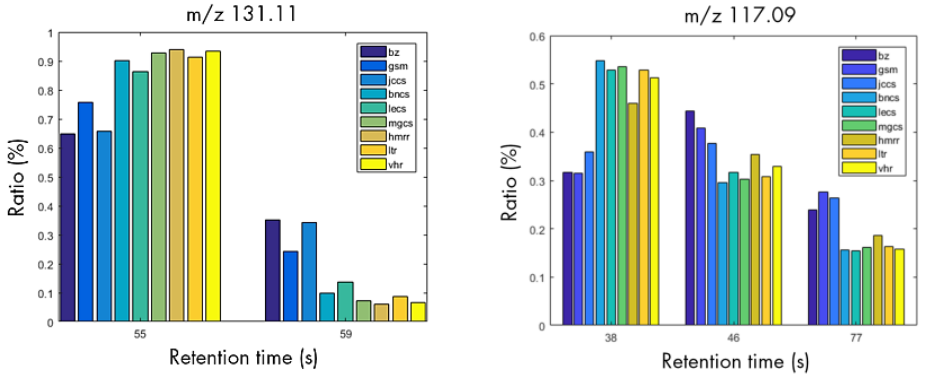


**Figure 4**: Relative intensity distributions for different ions sharing the same exact *m/z*: 131.11 (left) and 117.09 (right); the abbreviations stand for the different wines.

We conclude that the combination of an ion funnel and hexapole ion guide in a high resolution PTR-TOFMS instrument with additionally a fastGC and autosampler inlet system is a powerful setup for rapid, sensitive and selective food and flavor analysis, which produces considerably more high quality data at a higher sample-throughput than established methods. As a next step following this proof-of-concept we will perform statistical analysis on the acquired data in order to distinguish between different brands/vintages of wine.

**Acknowledgement**

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## References

1. F. Biasioli, C. Yeretzian, F. Gasperi and T.D. Märk, Trends in Analytical Chemistry 30/7 (2011) 968-977.
2. Jordan, S. Haidacher, G. Hanel, E. Hartungen, L. Märk, H. Seehauser, R. Schottkowsky, P. Sulzer and T.D. Märk, International Journal of Mass Spectrometry 286 (2009) 122-128.
3. Yeretzian, A. Jordan and W. Lindinger, International Journal of Mass Spectrometry 223-224 (2003) 115-139.
4. E. Boscaini, T. Mikoviny, A. Wisthaler, E. Hartungen and T.D. Märk, International Journal of Mass Spectrometry 239 (2004) 215-219.
5. C. Lindinger, P. Pollien, S. Ali, C. Yeretzian, I. Blank and T. Märk, Analytical Chemistry 77 (2005) 4117-4124.
6. D. Materić, M. Lanza, P. Sulzer, J. Herbig, D. Bruhn, V. Gauci, N. Mason and C. Turner, International Journal of Mass Spectrometry (2017) in press.
7. Romano, L. Fischer, J. Herbig, H. Campbell-Sills, J. Coulon, P. Lucas, L. Cappellin and F. Biasioli, International Journal of Mass Spectrometry 369 (2014) 81-86.
8. A.M. Ellis and C.A. Mayhew, Proton Transfer Reaction Mass Spectrometry: Principles and Applications. John Wiley & Sons, Ltd, Chichester, UK (2014).
9. Jordan, S. Haidacher, G. Hanel, E. Hartungen, J. Herbig, L. Märk, R. Schottkowsky, H. Seehauser, P. Sulzer and T.D. Märk, International Journal of Mass Spectrometry 286 (2009) 32-38.
10. P. Sulzer, A. Edtbauer, E. Hartungen, S. Jürschik, A. Jordan, G. Hanel, S. Feil, S. Jaksch, L. Märk and T.D. Märk, International Journal of Mass Spectrometry 321-322 (2012) 66-70.
11. R.D. Smith and S.A. Shaffer, US patent 6107628 (1998).
12. S. Barber, R.S. Blake, I.R. White, P.S. Monks, F. Reich, S. Mullock and A.M. Ellis, Analytical Chemistry 84 (2012) 5387-5391.
13. P. Sulzer, E. Hartungen, G. Hanel, S. Feil, K. Winkler, P. Mutschlechner, S. Haidacher, R. Schottkowsky, D. Gunsch, H. Seehauser, M. Striednig, S. Jürschik, K. Breiev, M. Lanza, J. Herbig, L. Märk and T.D. Märk, A. Jordan, International Journal of Mass Spectrometry 368 (2014) 1-5.

# DOLC-NMR:
# Differential off-line LC-NMR analyses of nutrient-induced metabolome alterations in *S. cerevisiae* and their taste impact

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## Abstract

Metabolome investigations by means of mass spectrometry are often limited in structure elucidation of unknown and new metabolites. A novel Differential Off-Line LC-NMR approach (DOLC-NMR) was developed to record and quantify nutrient-induced metabolome adjustments in *Saccharomyces cerevisiae*. Off-line coupling of preparative high performance liquid chromatography separation and $^{1}$H-NMR spectroscopy supported by automated comparative NMR bucket analyses, followed by quantitative $^{1}$H-NMR using ERETIC II has been successfully utilized to monitor significant quantitative changes in the metabolome of *S. cerevisiae* upon intervention with the aromatic amino acid *L*-tyrosine. Among the 33 metabolites identified by means of exact mass and 1D/2D-NMR experiments, glyceryl succinate, tyrosol acetate, tyrosol lactate, tyrosol succinate, and *N*-(1-oxoacyl)-*L*-tyrosine derivatives like *N*-(1-oxooctyl)-*L*-tyrosine have not been earlier reported as yeast metabolites. Depending on the chain length of the fatty acid, *N*-(1-oxooctyl)-, *N*-(1-oxodecanyl)-, *N*-(1-oxododecanyl)-, *N*-(1-oxomyristinyl)-, *N*-(1-oxopalmityl)-, and *N*-(1-oxooleoyl)-*L*-tyrosine imparted a kokumi taste enhancement above their human recognition thresholds ranging between 145 and 1432 µmol/L in a savoury model broth. Based on Carbon Modul Labelling (CAMOLA) and Carbon Bond Labelling (CABOLA) experiments using $^{13}C_6$-glucose as carbon source, biosynthesis pathways of the identified key metabolites could be described unequivocally. The aliphatic side chain of *N*-(1-oxooctyl)-*L*-tyrosine could be shown to be generated via de novo fatty acid biosynthesis from four $C_2$-carbon modules (acetyl-CoA) originating from *D*-glucose.

## Introduction

Due to the wide application and its importance in foods production such as bread, wine, and beer, *Saccharomyces cerevisiae* has been one of the most investigated micro-organisms in the past 60 years. Various imaging techniques based on mass spectrometry and NMR spectroscopy were used to monitor stress-induced metabolome alterations and enabled the identification of stress markers. Salt stress, for example, has been reported to be responsible for the increase of trehalose levels to counteract osmotic pressure, whereas concentration levels of mono- and disaccharides depleted upon ethanol stress. Furthermore, different aroma and taste characteristics of fermented beverages are determined by utilizing different amino acids. Aliphatic branched-chain amino acids like *L*-leucine, *L*-isoleucine, and *L*-valine were found to be responsible for the typical aroma of *S. cerevisiae* fermented beverages. Upon entering the Ehrlich pathway, various fusel alcohols were provided by the yeast upon fermentation of different amino acids. [1]

Metabolomic studies on yeast were performed by analysing the metabolite profile emitted by the microorganism into the supernatant during the fermentation process.

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Although LC-MS has been often used for observing metabolite profiles because to its high resolution and rather low limit of detection, the unambiguous identification of important key metabolites undergoing an alternation in their concentration level upon an intervention becomes often a big challenge. Analyses of the whole extract by means of $^{1}$H NMR spectroscopy seems to be promising as it can supply direct structure information as well as quantitative data of unknown metabolites. However, the low resolution and signal overlapping in $^{1}$H-NMR spectroscopy limits the comprehensive analyses of complex natural extracts. Therefore, liquid chromatographic or SPE pre-separation has been described to simplify complex crude mixtures, such as faeces and urine, and to raise the resolution and decrease the complexity of one-dimensional $^{1}$H NMR spectroscopy in metabolome research. [2]

To investigate secondary metabolites from *L*-tyrosine in yeast, a novel differential off-line HPLC-NMR approach (DOLC-NMR) was established to monitor metabolite alterations in *S. cerevisiae*. [3] Before and after an intervention with the aromatic amino acid *L*-tyrosine, yeast supernatants were pre-separated using preparative HPLC prior to a comparative NMR buckets analyses to record relative concentration changes of key metabolites, followed by absolute quantitation via qHNMR using the ERETIC II protocol. [3] In addition, the biosynthesis pathways of newly characterized key metabolites have been monitored by means of $^{13}$C-labeling experiments, namely, carbon module labelling (CAMOLA) and carbon bond labelling (CABOLA) with LC-TOF/MS- and $^{13}$C-NMR-based analyses of isotopologue patterns. [4,5]

## Experimental

*S. cerevisiae fermentation with/without L-tyrosine (Tyr1/Tyr0)*

Dry yeast (*S. cerevisiae*, 460 mg of dried pellets; RUF, Quakenbrück, Germany) was mixed with water (200 mL), *D*-glucose (194 mmol/L) and *L*-tyrosine (12 mmol/L) were added, and the suspension was incubated for 96 h at 36°C under anaerobic conditions ($Tyr^{1}$). In addition, a control experiment ($Tyr^{0}$) was performed without the presence of *L*-tyrosine. Thereafter, the supernatants of $Tyr^{0/1}$ were separated from the yeast cells by filtration (0.45 μm, Sartorius Stedium Biotech GmbH; Göttingen, Germany) and freeze-dried and the residue obtained was used for chromatographic MPLC separation using a Spot Prep II (Gilson, Limburg, Germany) equipped with a preparative 250 × 21.2 mm, 5 μm, PhenylHexyl Luna column (Phenomenex, Aschaffenburg, Germany) to collect a total of 34 fractions in 1 min intervals. [3] After concentration of each fraction in vacuum by means of a HT-12 evaporation system (Genevac Limited, Ipswich, UK), the individual fractions collected from $Tyr^{1}$ and $Tyr^{0}$, respectively, were dissolved in $D_2O$ or MeOD-$d_4$ (Euriso-Top, Gif-sur-Yvette, France) for NMR analyses.

*Stable isotope labelling experiments*

To perform a CAMOLA experiment, a mixture of dry yeast (460 mg of dried pellets), *D*-glucose (97 mmol/L), $^{13}C_6$-glucose (97 mmol/L; Cambridge Isotope Laboratories, Inc., Andover, MA, USA), and *L*-tyrosine (12 mmol/L) in water (200 mL) was incubated for 96 h at 36 °C under anaerobic conditions. After fermentation, the supernatant was obtained by filtration and, then, directly used for UPLC-ESI-TOF/MS (Waters Synapt G2S HDMS; Waters, Manchester, UK, coupled to an Acquity UPLC core system; Waters, Milford, MA, USA) analyses.

For the CABOLA experiment, a mixture of dry yeast (460 mg of dried pellets), *D*-glucose (184.3 mmol/L), $^{13}C_6$-glucose (9.7 mmol/L), and *L*-tyrosine (12 mmol/L) in water (200 mL) was incubated for 96 h at 36 °C under anaerobic conditions. Purified

metabolites from the supernatants were taken up in $D_2O$ or MeOD-$d_4$ and analysed by means of $^{13}$C-NMR spectroscopy to analyse $^{n}J_{13C\text{-}13C}$ coupling patterns of the isolated target fermentation products.

*Nuclear Magnetic Resonance spectroscopy (NMR)*

$^{1}$H/$^{13}$C-NMR experiments were performed on a Bruker AVANCE III 500 MHz system equipped with a cryo-TCI Probe at 300 K (Bruker, Rheinstetten, Germany). The collected HPLC fractions were dissolved in $D_2O$ (1 mL), and aliquots (540 µL) were then mixed with an aliquot (60 µL) of the NMR buffer (phosphate buffer, pH = 7) solution prior to the measurement. The more hydrophobic fractions 20−34 were taken up in MeOD-$d_4$ (1 mL) and aliquots (600 µL) used for NMR analyses. $^{1}$H NMR spectra were acquired using the Bruker standard water suppression pulse sequence (noesygppr1d). The 90° pulse length (P1), PL9, and O1 were adjusted individually on each sample and spectra were acquired using 16 scans (NS) and 4 prior dummy scans (DS) and collected into 64K data points using a spectral width of 10273.97 Hz. The relaxation time (T1) was set to 20 s based on the longest relaxation time of a signal of interest.

The NMR buckets were calculated with Amix Viewer V3.9.13 software (Bruker, Rheinstetten, Germany). Each spectrum was referenced to TMSP (0.0 ppm). After checking the baseline offset and using the underground removal tool, the spectra were used to determine the buckets. Covering the chemical shift region from −1 to 11 ppm, the range of each bucket was set to 0.1 ppm. The area between 4.5 and 5 ppm was excluded from bucketing due to the water signal in the spectra. The calculation of the absolute integral value for each of the 115 buckets was performed successfully when the signal-to-noise ratio was >10. The noise was calculated in the region from 10 to 11 ppm, where no signals appeared. From the yeast fermentation with tyrosine ($Tyr^1$) and the control ($Tyr^0$), the corresponding buckets showing an integral ratio ($Tyr^1/Tyr^0$) of >2 or <0.5 were used for further analyses. Quantitative $^{1}$H-NMR was performed using ERETIC 2 (Electronic REference To access In vivo Concentrations) based on the PULCON (PULse length based CONcentration determination) methodology. [6]

*Sensory analyses for determination of taste threshold concentrations*

Intrinsic taste threshold concentrations of the purified compounds were determined in Evian water using a duo test in triplicate analyses by a well-trained sensory panel (n = 14). Threshold concentrations for taste modulation effects were determined in a savoury model broth (pH 5.9, adjusted with 0.1% FA) consisting of sodium chloride (2.9 g/L), maltodextrin (6.4 g/L), monosodium *L*-glutamate (1.9 g/L) and an amino acid mix (0.38 g/L). The geometric mean of the last correct tasted and the first incorrect tasted concentration was calculated and taken as the individual threshold of each panellist. The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in two independent sessions.

## Results and discussion

*Differential off-line HPLC-NMR analyses*

To overcome the challenge of overlaying signal, the fermentation batches $Tyr^1$ and $Tyr^0$ were separated by means of RP-HPLC into 1 min subfractions prior to $^{1}$H-NMR analyses to increase spectral resolution of the key metabolites. The obtained NMR buckets from fraction 3-34 were used for statistical analyses to visualize relative concentration ratios in the metabolite signature, expressed as the ratio ($Tyr^1/Tyr^0$) of the signal integrals of the corresponding metabolites (Figure 1).

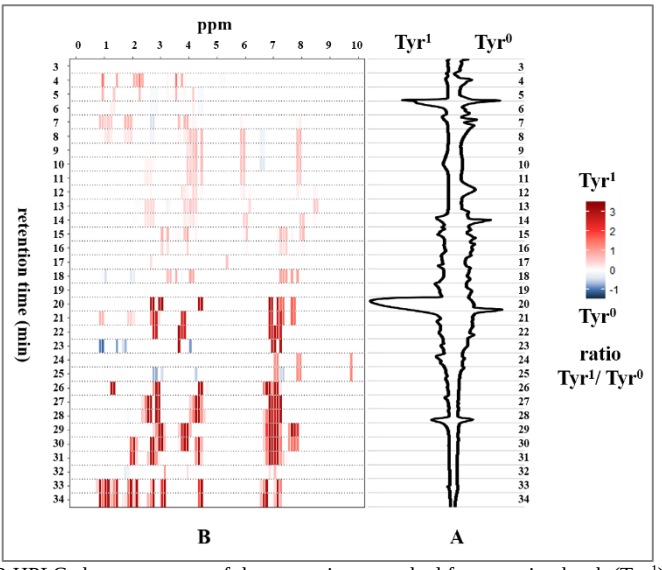


**Figure 1**: (A) RP-HPLC chromatograms of the *L*-tyrosine perturbed fermentation broth ($Tyr^1$) and the control approach without *L*-tyrosine ($Tyr^0$). (B) Differential NMR bucket analyses of fractions 3−34 showing the relative integral ratios ($Tyr^1/Tyr^0$) and chemical shifts of influenced metabolites.

The first-eluting fractions 3−15 showed only marginal differences in relative metabolite concentrations between the broths $Tyr^0$ and $Tyr^1$. Among the identified metabolites, many primary metabolites playing a key role in energy management and growth of the microorganism like intermediates of the citrate cycle such as succinic acid (fractions 8−10; **2**, Figure 3), malic acid (fraction 5), fumaric acid (fractions 7−9) were to be found. Furthermore, amino acids such as leucine (fraction 6/7), isoleucine (fraction 6/7), phenylalanine (fraction 15/16), degradation products of *D*-glucose like glycerol (fraction 4/5; **3**) and lactic acid (fractions 4−6; **4**), and the nucleotides adenosine (fractions 11−15), guanosine (fractions 14−16) and uridine (fractions 8−12) were significantly influenced by the nitrogen source. Additionally, the ester glycerol succinate (**5**), identified in fractions 12−14, has been found for the first time as a metabolite of *S. cerevisiae*.

In comparison to the first eluting polar metabolites, higher differences in metabolite concentrations between $Tyr^0$ and $Tyr^1$ were monitored in fractions 16−34 containing semi- and nonpolar compounds. The aromatic fusel alcohol tyrosol (fraction 21/22; **1**), degradation product of *L*-tyrosine via the Ehrlich pathway, was found in a 100-fold higher concentration in $Tyr^1$. In the further analyses three tyrosol esters, varying in the organic acid side chain, could be clearly identified, namely, tyrosol acetate (**6**) in fraction 30/31, tyrosol succinate (**7**) in fraction 27/28, and tyrosol lactate (**8**) in fraction 26. Neither Tyrosol succinate (**7**) nor tyrosol lactate (**8**) have been reported as a metabolite of *S. cerevisiae*. Moreover, p-hydroxyphenyl lactic acid (fraction 20) and p-hydroxybenzaldehyde (fraction 24/25) were identified as nitrogen-free *L*-tyrosine degradation metabolites. Finally, the amino acid amide *N*-(1-oxooctyl)-*L*-tyrosine (**9**) could be isolated from the $Tyr^1$ fractions 33/34. This amide is already known from *Escherichia coli* but has not yet been reported as a metabolite of *S. cerevisiae*. Furthermore, LC-MS/MS analyses revealed the presence of *N*-(1-oxoacyl)-*L*-tyrosine derivatives with acyl chain lengths of $C_4$, $C_6$, $C_{10}$, and $C_{12}$ along with the predominating *N*-(1-oxooctyl)-*L*-tyrosine (**9**) homologue.

*Absolute metabolite quantitation by $^1$H NMR spectroscopy*

A validated PULCON methodology using ERETIC II was applied to the NMR buckets of fractions 3−34 collected from Tyr$^0$ and Tyr$^1$. Using fraction 14 as an example (Figure 2), glycerol succinate (**5**), uridine and adenosine were quantitated using selected resonance signals that were baseline separated without showing no signal overlap, namely, 2.64 ppm (dd, 2H, H-(3′)) for glycerol succinate (**5**), 7.88 ppm (d, 1H, H-(6)) for uridine and 8.35 ppm (s, 1H, H-(2)) for adenosine, respectively.

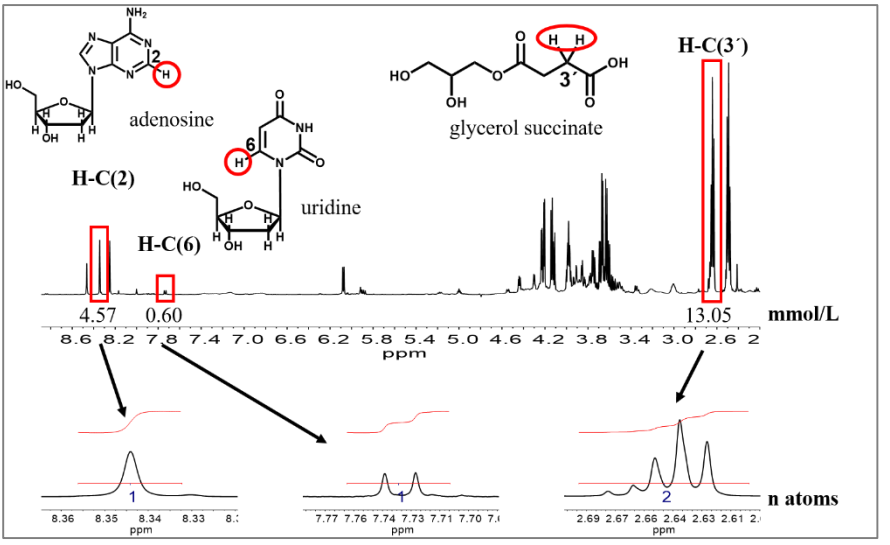


**Figure 2**: Quantitative $^1$H NMR spectroscopy (noesygppr1d, 500 MHz, D2O, 300 K) of fraction 14 containing adenosine (H-(2), 8.34 ppm, 1H), uridine (H-(6), 7,75 ppm, 1H), and glycerol succinate (**5**; H-(3′), 2.64 ppm, 2H). Labelled signals were used for metabolite quantitation.

*Sensory activity of metabolites*

All detected *N*-(1-oxoacyl)-*L*-tyrosine derivatives showed a bitter intrinsic taste within a narrow threshold range from 343 to 647 µmol/L in Evian water (Table 1). The evaluation of the compounds in a spicy model broth demonstrated a clear kokumi enhancing activity of *N*-(1-oxooctyl)-*L*-tyrosine (9) above 1432 µmol/L (Table 1).

**Table 1**: Human taste recognition thresholds in µmol/L of *N*-(1-oxoacyl)-*L*-tyrosine derivatives in Evian water (intrinsic bitter taste) and savoury model broth (kokumi enhancement) with a significance level $\alpha \leq 0.05$

| Compound | *Bitter taste* | *Kokumi enhancement* |
|---|---|---|
| *N*-(1-Oxobutyl)-*L*-tyrosine ($C_4$) | 647 | n.d. |
| *N*-(1-Oxohexyl)-*L*-tyrosine ($C_6$) | 343 | n.d. |
| *N*-(1-Oxooctyl)-*L*-tyrosine ($C_8$) | 631 | 1432 |
| *N*-(1-Oxodecanyl)-*L*-tyrosine ($C_{10}$) | 627 | 537 |
| *N*-(1-Oxododecanyl)-*L*-tyrosine ($C_{12}$) | 480 | 145 |
| *N*-(1-Oxomyristyl)-*L*-tyrosine ($C_{14}$) | 672 | 160 |
| *N*-(1-Oxopalmitylyl)-*L*-tyrosine ($C_{16}$) | 627 | 183 |
| *N*-(1-Oxooleoyl)-*L*-tyrosine ($C_{18:1}$) | 446 | 217 |

A prolongation of the length of the fatty acid side chain reduced the kokumi recognition thresholds concentrations to 145−217 µmol/L as for the $C_{12}$ to $C_{18:1}$ homologues. A shortening of the chain length ($C_4$ and $C_6$) eliminated the kokumi activity.

*Biosynthetic pathway analyses using 13C-labelling experiments*

The analyses of the biosynthetic pathways (Figure 3) of identified metabolites were performed by means of two different $^{13}$C-labelling experiments. The added glucose was diluted with 5% (CABOLA) and 50% (CAMOLA) $^{13}C_6$-glucose, respectively, prior to yeast fermentation in the presence and absence of *L*-tyrosine to monitor the joint transfer of several $^{13}$C atoms *en bloc* into the target metabolite. Supported by these experiments it could be clearly shown that the aliphatic side chain of *N*-(1-oxooctyl)-*L*-tyrosine (**9**) is generated via *de novo* fatty acid biosynthesis from four $C_2$-carbon modules (acetyl-CoA). In addition, the $^{13}$C signatures of the secondary metabolites tyrosol acetate (**6**), tyrosol lactate (**8**), tyrosol succinate (**7**), and glyceryl succinate (**5**) beside the primary intermediates glycerol (**3**), succinic acid (**2**) and lactic acid (**4**) were investigated. The labelling experiments showed the ability of *S. cerevisiae* to form tyrosol (**1**) via the Ehrlich pathway from *L*-tyrosine or *de novo* via the Shikimi pathway from *D*-glucose.

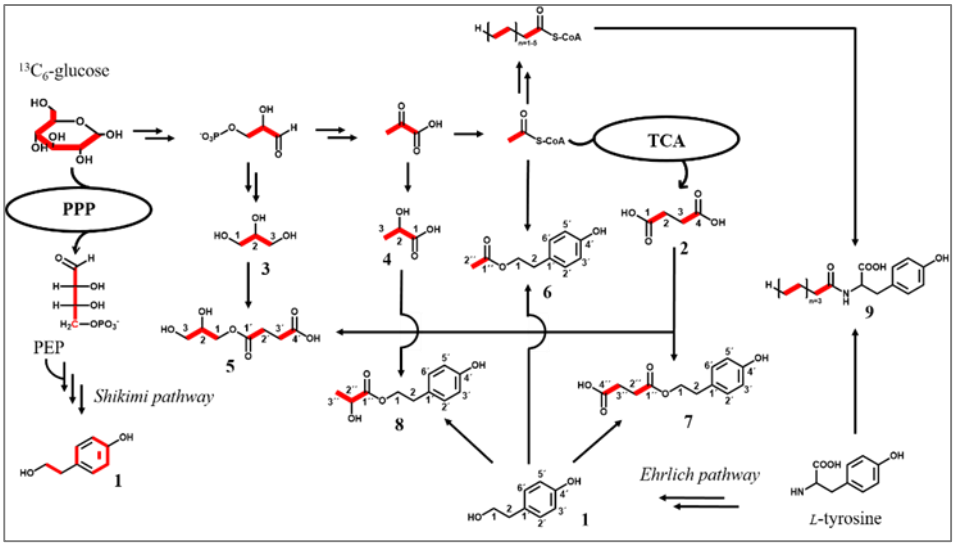


**Figure 3**: Biosynthesis pathways of key metabolites originating from *D*-glucose and *L*-tyrosine (TCA: tricarboxylic acid cycle; PPP: pentose phosphate pathway; PEP: phospho enol pyruvate). Bold lines indicate intact carbon bonds originating from $^{13}$C-glucose as observed by the CABOLA and CAMOLA experiment.

## References

1. Hazelwood L. A., Daran J.-M., van Maris A. J. A., Pronk J. T., Dickinson J. R. (2008) Appl. Environ. Microbiol. 74, 2259−2266
2. Jacobs D. M., Spiesser L., Garnier, M., de Roo N., van Dorsten F., Hollebrands B., van Velzen E., Draijer R., van Duynhoven J. (2012) Anal. Bioanal. Chem. 404, 2349-2361
3. Hammerl R., Frank O., Hofmann T. (2017) J. Agric. Food Chem. 65, 3230-3241
4. Schieberle P. (2005) Ann. N. Y. Acad. Sci. 1043, 236-248
5. Bacher A., Rieder C., Eichinger D., Arigoni D., Fuchs G., Eisenreich W. (1999) FEMS Microbiol. Rev 22, 567-598
6. Frank O., Kreissl J. K., Daschner A., Hofmann T. (2014) J. Agric. Food Chem. 62, 2506−2515

# Process control in flavour generation: NIR-MVA as a tool to monitor the formation of key odorants

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## Abstract

Sensors that are suitable to monitor chemical reactions leading to the formation of potent odorants in foods, and consequently, enabling process control, are of increasing demand. In the present work, real time kinetic analyses were made by developing new methodologies combining near infrared spectroscopy (NIR) with rich information detectors such as GC and LC-MS. These were applied to study the dynamics in phenylacetaldehyde formation through a number of reactions, namely (i) glucose and phenylalanine, (ii) gallic acid and phenylalanine and iii) gallic acid, phenylalanine and glucose. Phenylacetaldehyde as well as other reaction intermediaries were monitored during 60 min with a frequency for data acquisition of 3 spectra/min. Samples were collected in 10 minutes reaction intervals, and target analysis was performed using mass spectroscopy (GC-ITMS and LC-ESI-QqTOF-HRMS). For comparison, the spectral data were analysed in a conventional way fitting kinetics for specific wavelengths. Multivariate alternative least squares (MCR-ALS) method was applied to model the spectral data with the quantification of the reaction compounds, and to perform deconvolution of spectral data. Different reaction rates were observed according to the perturbation, i.e. metals addition, temperature increase and substrate class. The obtained results were in line with those obtained by LC-ESI-QqTOF-HRMS. The developed NIR spectroscopy method showed to be a good alternative for real time, high-throughput and low-cost analysis process monitoring, unlocking chemical information related to specific compounds such as, phenylacetaldehyde, benzaldehyde, quinones and dicarbonyls, and the impact on phenylacetaldehyde formation. Also, it showed that the information captured by NIR spectroscopy can accurately predict the phenylacetaldehyde concentration under real time conditions.

## Introduction

Several factors affect the formation of Strecker aldehydes in wines, in particular the formation of phenylacetaldehyde. In particular compounds such as antioxidants [1], metals [2] and glucose [3] are key compounds in mechanisms responsible for aldehydes formation such as Maillard reaction and oxidation of phenolic compounds. Commonly the kinetic studies are performed by taking points at specific times during an experiment The disadvantage of this time point approach is the loss of information between the intervals.

Process analytical technology (PAT) involves the combined use of in-process monitoring, allowing a dynamic measurement of reaction kinetics in short periods of time. Monitoring is at the core of PAT and is done in situ or at-line on whole samples by multi-parametric methods. Many spectroscopic techniques are multi-parametric methods. Near-

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infrared (NIR) spectroscopy is one of the techniques to be suitable for a variety of PAT applications. Major advantages of NIR spectroscopy regard its non-destructive nature and its immediate (real-time) delivery of results. However, this technique is based on indirect measurements, resulting in convoluted and broad spectra, that are very difficult to interpret with the unaided eye. It requires calibration and the use of a reference technique, such as GC-MS or HPLC with mathematical and statistical tools (chemometrics) to extract analytical information from the corresponding spectra [4]. This represents a major limitation for its usage, nevertheless, in recent studies it has been successfully applied in different food systems such as meat, fruits, vegetables, grains, dairy products, oils and beverages mainly for quality control [5]. In the present study, NIR spectroscopy was applied to investigate its add-value, as tool to monitor key odorants formation, such as phenylacetaldehyde formation in wine related model systems, as well as to verify its potential to unlock chemical information related to specific compounds (phenylacetaldehyde, benzaldehyde, quinones and dicarbonyls) involved in the reaction mechanism of phenylacetaldehyde formation.

## Experimental

*Sample preparation*

Three equimolar model systems (2.4 mM) comprising i) glucose and phenylalanine (M) and ii) gallic acid and phenylalanine (O) and iii) gallic acid, glucose and phenylalanine (MO) were prepared in 12% (v/v) aqueous ethanol and tartaric acid (0.03 M) buffered to pH 3.4 with NaOH. The metal ions, copper(II) and iron(II), were added to the model system at concentrations of 6.3 μM and 0.1 mM, respectively, in the form of Cu(II)sulfate·5H2O and Fe(II)sulfate·7H2O. In the solutions with no metal addition, a 50 μM EDTA solution was also added. The solutions were prepared in 20 mL vials. The vials were closed with an internal silicone septum and an external screw cap.

*Offline measurements*

A design of experiments was performed consisting of storing two model solutions (O & MO) at three different temperatures (T=40, 60 and 90ºC). Phenylacetaldehyde was identified and quantified by GC-MS and the reaction intermediaries such as the gallic acid, quinone and the hydroxysulphonic acid of phenylacetaldehyde (HASA) by LC-ESI-QqTOF-HRMS, respectively [3].

*Online measurements - NIR acquisition and data analysis*

On-line and offline acquisition of spectra was performed with the use of a portable DLP NIRscan Nano Evaluation Module (EVM) (Texas Instruments, Dallas, USA). Absorbance spectra were measured in the wavelength range from 900 to 1700 nm, at intervals of 3.51 nm. Diffuse reflectance NIR spectra were continuously collected in-line and non-invasive during the process, a spectrum has been acquired each 1 minute for 160 minutes.

Data manipulation with NIR spectra was performed using PLS Toolbox version 8,0 (Eigenvector Research, Inc., USA) for MatlabR2014a for Mac (Mathworks, USA). It is common in NIR spectroscopy to apply pre-treatments designed to avoid a mix-up between the relevant information and the spectral noise. After the acquisition either multiplicative scatter correction (MSC) or a 7- point second order Savitzky-Golay filter (second order derivative pre-processing) were performed.

Principal component analysis (PCA) was used for sample classification to obtain process trajectories/perturbations from the spectral data. Partial least squares-regression (PLS-R) was applied to develop a model to predict phenylacetaldehyde content in online measurements.

## Results and discussion

The two major questions addressed in this study were: (1) how much information can NIR spectroscopy capture from Strecker aldehyde formation in wine related model systems and (2) how accurate is the predicted phenylacetaldehyde concentration.

The approach used focused not only on the multi-parametric assessment of NIR spectra but also on the ability to define process kinetics (i.e. process-signatures or fingerprints) related to several perturbations such as the addition of metals and antioxidants during the reaction time taken from consecutive spectra acquisitions.

A representative scheme of NIR spectra pre-processing is shown in Figure 1. The raw spectrum is characterized by broad and unresolved bands; pre-processing techniques (MSC + SG + Mean Centre) clearly allowed a better enhancement of spectral information.

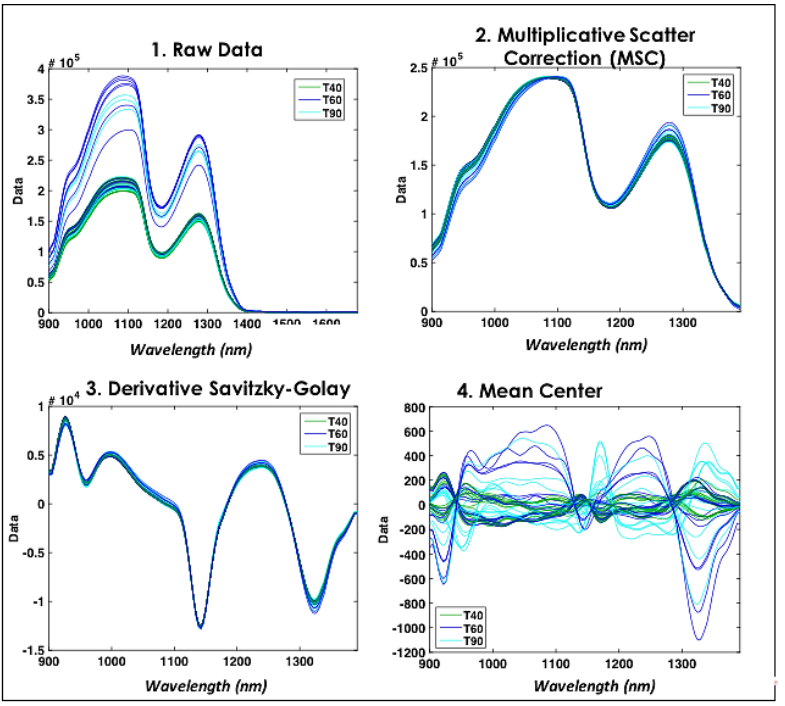


**Figure 1:** A) Pre-processing of spectras obtained in offline measurements (coloured according temperature) B) Scores plots from PCA analysis of PC1 vs. PC2 and PC1 vs. PC3

### *Offline measurements - results*

The effect of temperature was well captured by the score plot of the three first principal components analysis (Figure 2). The PCA was carried out on the offline measurements to investigate the variance in the spectral data and it clearly shows that temperature 90ºC (light blue) was well separated from 60 and 40ºC along PC2. Along

PC3 a clear separation between samples with and without metals, represented in green and red respectively, was also observed. These results show that the developed NIR spectroscopy method was sensible to both temperature and metals presence which is a good indication that the sensor can be used for offline process control.

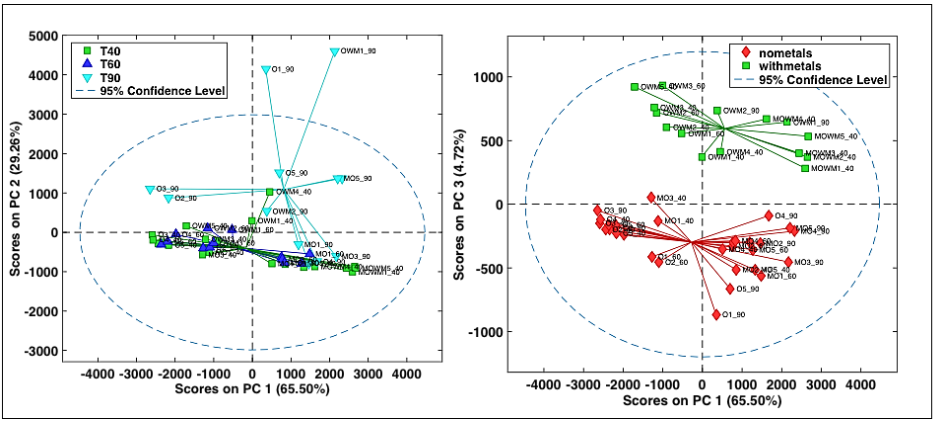


**Figure 2:** PCA results for the NIR data obtained in DOE offline measurements: scores plots from PCA analysis of PC1 vs. PC2 and PC1 vs. PC3

*Online measurements - results*

To better understand the impact of single factors in real time, sequential additions of metals and sulphur dioxide were done to the MO model system. NIR was applied to continuously collect spectra during the process, and a large amount of complex data was obtained per monitored process. Useful information from these data was extracted using suitable chemometric tools.

To further complement the obtained NIR information every ten minutes an aliquot was taken from the reactor and analysed by LC-ESI-QqTOF-HRMS. Phenylacetaldehyde content and reaction intermediates, such as: hydroxysulphonic acid (HASA) of phenylacetaldehyde were quantified.

The NIR data were again modelled using PCA and 87% of the variation was explained by the first two components (results not shown). Since this study was performed in dynamic mode as a function of time, the correlation of score plot expression can be correlated with the time of the experiment. For this purpose, the scores for the first component were plotted versus reaction time and indicated that the Strecker degradation started after 12 minutes and ended at 117 minutes (Figure 3). This endpoint also corresponded to the addition of sulphur dioxide, which was expected to stop the formation of phenylacetaldehyde due to the binding power of bisulphite to phenylacetaldehyde to form the HASA of the aldehyde. In parallel, target quantification of phenylacetaldehyde by GC-MS was done every 10 minutes during the total experiment time. Figure 3 shows that phenylacetaldehyde concentration increased during time, and the addition of $SO_2$ decreased its formation dramatically, confirming the results obtained with NIR Spectroscopy.

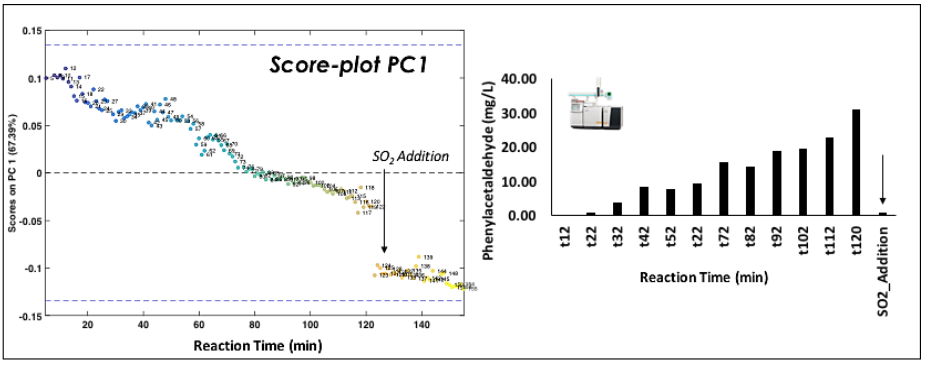


**Figure 3:** PCA results for the NIR data obtained during the reaction time. A) Scores of PC1 versus process time plot. B) Phenylacetaldehyde concentration measured by GC-MS during reaction time (each 10 minutes)

Furthermore, for online measurements of phenylacetaldehyde using NIR spectra a correlation coefficient of r=0,935 was obtained and a Root Mean Square Error of Calibration of 1.8 mg/L was calculated as depicted in Figure 4. An indication is given that the information captured by NIR spectroscopy can rather accurately predict phenylacetaldehyde concentration under real time conditions.

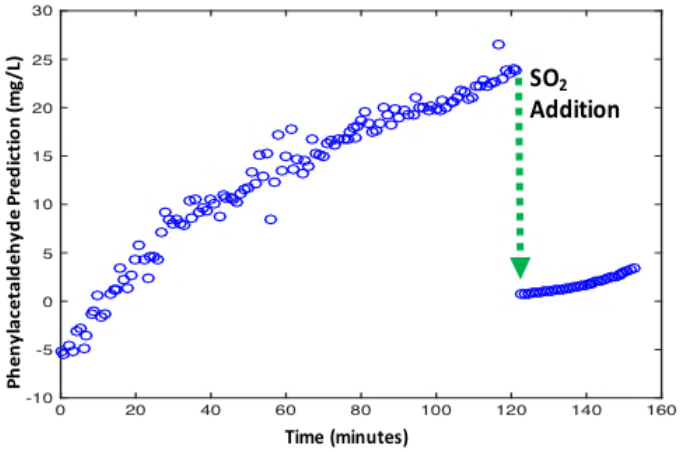


**Figure 4:** Phenylacetaldehyde real time prediction based on the PLS-R regression vector during the reaction time

In summary, this study evaluated the feasibility of NIR spectroscopy as a process analyser (PAT tool) for the non-invasive, on-line and real-time monitoring of Strecker aldehydes in wine related model systems. It clearly showed that it can capture the impact of the addition of metals and temperature variation on the phenylacetaldehyde formation and rather accurately predicted its concentration. The developed NIR spectroscopy method showed potential as a real time, high-throughput and low-cost analysis for process monitoring, unlocking chemical information related with specific compounds.

## References

1. Nikolantonaki, M. and A.L. Waterhouse, (2012) Journal of Agricultural and Food Chemistry, 60(34): p. 8484-8491.
2. Danilewicz, J.C., (2003) American Journal of Enology and Viticulture, 54(2): p. 73-85.
3. Monforte, A.R., S. Martins, and A.C. Silva Ferreira, (2017) Journal of Agricultural and Food Chemistry
4. Porep, J.U., D.R. Kammerer, and R. Carle, (2015) Trends in Food Science & Technology, 46(2, Part A): p. 211-230.
5. Huang, H., Yu, H., Xu, H. and Ying Y., (2008) Journal of Food Engineering, 87(3): p. 303-313.

# Investigation of odour-active compounds in grapefruit (*Citrus paradisi*)

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## Abstract

Odour-active components of grapefruit essence oil obtained by distillation of grapefruit juice were investigated by aroma extract dilution analysis (AEDA). The results showed a total of 15 components exhibited high flavour dilution (FD) factors in the range of 64-1024. By application of a GC omission test, it was clarified that a woody odorant contributed to the juicy aroma of grapefruit. Subsequently, a multi-dimensional GCMS-Olfactometry (MDGC/MS-O) analysis revealed the woody odorant was mustakone. Enantiomeric distribution of mustakone in grapefruit showed (−)-mustakone was predominant over (+)-mustakone. A GC omission test and a reconstitution test with a mixture of both synthesized enantiomers indicated that mustakone enriched the natural juicy aroma note like original essence oil.

## Introduction

Grapefruit (*Citrus paradisi*) has a characteristic odour and its volatile components have been investigated for several decades. So far, over 320 volatile compounds have been identified including intense odorants such as 1-*p*-menthene-8-thiol and 4-mercapto-4-methylpentan-2-one [1]. However, there still remain some unknown odour-active components because the preparation of high quality reconstituted grapefruit flavour like original natural grapefruit aroma is extremely difficult even by blending reported compounds.

Essence oils, obtained by distillation during the concentrated fruit juice production process, are highly concentrated juice aroma. Therefore, they are used as flavour ingredients to enrich the juicy aroma note of foodstuffs and/or beverages. Among them, grapefruit essence oil is one of the most expensive ingredients because the production for processing gradually decreases year by year. Hence, clarification of the remaining odour-active components in grapefruit is very important for us to cope with the essence oil supply shortage and the increase in price.

Omission experiments are enormously effective to evaluate the actual contribution of certain compounds to the overall aroma of the original sample. Recently, we have developed a novel sample preparation method for omission tests using preparative capillary GC [2]. The procedure of our method is: 1) determination of target odorants by GC-O, 2) preparation of original and omitted samples by preparative GC, 3) evaluation of differences of the aroma between the samples by sensory analysis. More details regarding sample preparation, the original sample is a collection of all components of the aroma eluted from preparative GC. The omitted samples are the same collections expect they do not include each target odorant. The preparation is accomplished by preparative GC based only on the retention time range of target odour-active regions. Therefore, this method requires no qualification and quantification. Furthermore, there is little change in the ratio of all components during the experiment.

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The aim of this study is to reveal novel odour contributors in grapefruit essence oil and their effect through omission experiments without complex processes.

## Experimental

### *Materials*

Grapefruit essence oil was purchased from Peace River Citrus Products Inc. (Florida, US). Essence oils are obtained by distillation during concentrated juice production processing.

### *GC-Olfactometry (GC-O) and Aroma Extract Dilution Analysis (AEDA)*

GC-O was conducted on a 7890A GC (Agilent, Santa Clara, USA) equipped with a sniffing port (GL Sciences, Inc., Tokyo, Japan) and a BC-Wax column (50 m × 0.25 mm, 0.15 µm, GL Sciences, Inc.). At the end of the separation column, the effluent was split between an FID and a sniffing port (1:10). For AEDA, the sample was diluted stepwise (1:4) with ethanol and each dilution was investigated by GC-O. All analyses were repeated in triplicate by three trained panellists.

### *Preparative-gas chromatography*

Preparative-GC was carried out on a GC-2010 plus equipped with an FID (Shimadzu Corp., Kyoto, Japan) and a BC-Wax column (30 m × 0.53 mm, 1.0 µm, GL Sciences Inc.) as a separation column. The end of column was connected to both a VPS2800 GC fraction collector (GL Sciences, Inc.) and the FID via a splitter. GC conditions: Helium was used as carrier gas at a head pressure of 103 kPa (flow rate, 6.8 mL /min). The splitter make-up pressure set at 80 kPa. The oven temperature was set at 70 °C, ramped at 4 °C/min to 230 °C and held for 20 min. The temperature of the injector and detector were set at 230 °C. The target compounds were collected using the GC fraction collector equipped with traps cooled at −30 °C.

### *GC omission test*

Grapefruit essence oil was divided into 3 fractions using preparative-GC. The odour-active region of the target odorant by GC-O was 32.9 to 33.0 min, therefore, each fraction was collected as below. Fr.1: 0 min to 32.9 min, Fr.2: 32.9 to 33.0 min, Fr.3: 33.0 to 60 min. The recombination with these fractions provided the original sample A (Fr.1 + Fr.2 + Fr.3) and the omitted sample B (Fr.1 + Fr.3). Subsequently, sensory evaluation was applied in order to compare the overall aroma of these samples [2].

### *Multi-Dimensional GC/MS-O (MDGC/MS-O)*

MDGC/MS-O analysis was performed on a MDGC/GCMS-2010 (Shimadzu Corp.). The first GC column was a BC-Wax (30 m × 0.25 mm, 0.15 µm, GL Sciences, Inc.) and the second GC column was an Rxi-5ms (30 m × 0.25 mm, 0.25 µm, Restek Corp., Bellefonte, USA). For chiral analysis, the second GC column was a CP-Chirasil-DEX CB (25 m × 0.25 mm, 0.25 µm, Agilent).

### *Synthesis of chiral mustakone*

(+)- and (−)-mustakone were obtained by oxidation of (+)- and (−)-alpha-copaene, respectively [3].

### *Preparation of reconstituted sample*

The original grapefruit essence oil was subjected to preparative-GC to give Fr.1, Fr.2 and Fr.3. A mixture of Fr.1 and Fr.3 was used as the omitted sample B. The mustakone solution D was a mixture of both authentic enantiomers in the resulted enantiomeric ratio.

A reconstituted sample C was prepared by the addition of a mustakone solution D to an omitted sample B in a concentration of 100 ppm. All samples were diluted to 1% (w/w) with ethanol and then the solutions were diluted to 0.1% (w/w) with water. The aqueous solutions were subjected to sensory evaluation.

Sample A: Fr.1 + Fr.2 + Fr.3 (recombined original sample)

Sample B: Fr.1 + Fr.3 (without the target woody odorant)

Sample C: sample B + sample D (replaced Fr.2 with mustakone)

Sample D: synthetic mustakone solution

*Sensory evaluation*

All samples were evaluated by trained panellists. The omitted sample B and the reconstituted sample C were evaluated by 10 trained panellists. The panellists were asked to rate given descriptors for each sample on a scale from 0 to 5 in 1 increments, where 0 = very weak and 5 = very strong.

## Results and discussion

*Investigation of a potent odorant contributing to grapefruit juicy aroma*

Odour-active components of grapefruit essence oil were investigated by AEDA. The results showed a total of 14 components exhibited high FD factors in the range of 64-1024 (Table 1). Among them, a woody odorant **13** was focused because the structure could not be identified by conventional GCMS analysis. The GC omission test was carried out to confirm the effect of **13** on overall aroma of grapefruit. By application of the GC omission test, the original sample A and the omitted sample B were prepared using preparative-GC and then a sensory evaluation of overall aroma was performed. The result showed that the typical grapefruit juicy aroma note of the omitted sample B was clearly less intense than that of the original sample A. Thus, the woody odorant **13** should contribute to grapefruit juiciness.

**Table 1**: Flavour Dilution factors (FD factors ≥ 64) of potent odorants in grapefruit essence oil

| | | | LRI [1] | | |
|---|---|---|---|---|---|
| No. | odorant | FD factor | exp. | ref [2] | odour description |
| 1 | alpha-pinene | 256 | 1045 | 1043 | piney |
| 2 | *cis*-3-hexenal | 256 | 1138 | 1128 | green, leafy |
| 3 | limonene | 256 | 1218 | 1207 | citrus |
| 4 | octanal | 1024 | 1284 | 1282 | peely, citrus |
| 5 | nonanal | 64 | 1381 | 1383 | peely, waxy |
| 6 | linalool | 64 | 1529 | 1531 | floral, citrus |
| 7 | (*E*, *E*)-2,4-decadienal | 64 | 1785 | 1787 | oily, fried |
| 8 | *cis*-calamenene | 64 | 1809 | 1813 | spicy, phenolic |
| 9 | geraniol | 64 | 1824 | 1828 | floral, citrus |
| 10 | *trans*-4,5-epoxy-(*E*)-2-decenal | 256 | 1977 | 1977 | metallic, green |
| 11 | *trans*-4,5-epoxy-(E, Z)-2,7-decadienal | 256 | 2032 | 2034 | metallic, green |
| 12 | eugenol | 64 | 2140 | 2146 | spicy, clove-like |
| 13 | unknown | 64 | 2231 | - | woody, peppery, powdery |
| 14 | rotundone | 256 | 2244 | 2248 | woody, peppery |

[1] Linear Retention indices on BC-Wax calibrated by *n*-alkanes

[2] According to internal database

*Identification of the potent odorant*

MDGC/MS-O was conducted to determine the structure of **13**. As a result of mass spectrum analysis, the woody odorant **13** was estimated as mustakone. Its mass spectrum, retention time and odour characteristics were in good agreement with those of an authentic mustakone standard. Therefore, the woody odorant **13** was determined to be mustakone. To the best of our knowledge, mustakone was found for the first time in grapefruit. Mustakone has a pair of enantiomers as shown in Figure 1. As a result of chiral analysis by MDGC/MS, the enantiomeric ratio (+) : (−)-mustakone in the grapefruit essence oil was 8.5 : 91.5 (Fig. 1).

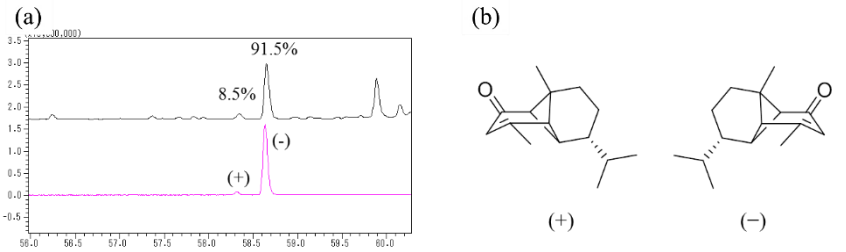


**Figure 1:** (a) MDGC second dimension chromatograms of grapefruit essence oil (top) and mixture of synthesized mustakone ((+) : (−) = 1 : 9) (bottom). (b) Structures of two enantiomers of mustakone.

*Evaluation of the effect of Mustakone on overall aroma*

To confirm the effect of mustakone on grapefruit odour, the omitted sample B and the reconstituted sample C were prepared and then evaluated by sensory analysis. The result indicated that “impact”, “voluminous”, “woody” and “sweet” were rated slightly more intense in reconstituted sample C (Fig. 2). For the overall aroma, all sensory panellists judged that mustakone enriched natural juicy aroma note like original essence oil. It was concluded that mustakone was responsible for the juicy aroma of grapefruit.

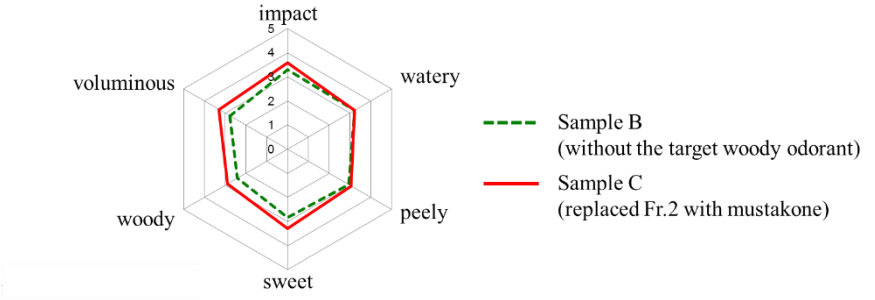


**Figure 2**: Aroma profiles of grapefruit essence oils omitted the woody odorant and added mustakone to omitted sample.

## References

1. Buettner, A. and Schieberle, P. (1999) J. Agric. Food Chem. 47: 5189-5193.
2. Kasuga, H. and Murata, A. (2017) Japan Patent P2017-40536A.
3. Kapadia, V. H., Nagasampagi, B. A., Naik, V. G. and Dev, S. (1965) Tetrahedron 21: 607-618.

# Rapid aroma analysis and data interpretation using on-line mass spectrometry and visualisation software

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## Abstract

Before starting detailed flavour analyses of food or beverage samples, it is often worthwhile carrying out a pre-screen to indicate what kinds of differences are present. Typically, the analyst wants to know: are there different compounds present / missing between samples or are the flavour profiles similar, but just differ in the amounts present and to what extent? With this knowledge, they can then design suitable analyses to investigate these differences more thoroughly. Here we present an example of a set of coffee samples, with different sensory aroma profiles, which were subjected to rapid headspace analyses using on-line APCI-mass spectrometry and the data imported into a visualization package to carry out data quality checks as well as some preliminary data interrogation. This approach yielded useful information in a few days, so that the next analytical steps could be planned, based on the pre-screen evidence.

## Introduction

Modern analytical instruments can analyse complex flavour profiles effectively and provide qualitative and quantitative data on the compounds present (identity and amount). Techniques like GC-MS and LC-MS can deliver detailed information on the odour and tastant profiles, although the process can be time-consuming if full identification and quantification are carried out using LRI, spectral matching and authentic standards. Flavour scientists have recognised this issue already and have introduced techniques like GC-olfactometry [1] to filter the data so that, in this case, identification and quantification will focus only on the odorous compounds and therefore reduce the workload. Another approach is to compare the odour or taste profiles of samples under investigation to determine the nature of the differences and then choose which detailed analyses would be most suitable to characterise the samples. For example, if the analytical odour profiles of the samples are similar, then an obvious step would be to compare the taste profiles to determine what differences lay there. If the same odour compounds were present, but in quite different amounts, then it would be useful to consider the odour threshold values and determine, first whether the compound was present above the odour threshold value, and then, whether the change in amount could cause a significantly different sensory response using techniques like AEDA or Odour Activity Values (OAV) [2]. Pre-screening can provide evidence on which to base an experimental plan and, when coupled with data visualisation, it provides a way to handle all the data, carry out data quality checks, investigate the nature of the differences and produce results in 2D and 3D formats to clearly visualise the differences found in the data set. When full analyses become available, (compound identification, GC-O, odour threshold or sensory data) they can be added to the data table and the multi-factorial data can be subjected to interrogation using various statistical tests or by considering the relationships between chemical compounds. This latter approach can give an insight into the chemistry that occurred in the processes used to manufacture the food or the beverage and might be useful in understanding why the differences occurred.

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## Experimental

*Samples*

Five coffee samples from Brazil (3), Ethiopia (1) and Vietnam (1) were provided by a coffee manufacturer. Samples were characterised by their lightness (L value) and origin only. Brewed coffee was prepared using 11 g of ground coffee and 240 mL of boiled water in a glass bottle fitted with a screw-top lid, providing a sampling port from which headspace was sampled at 65˚C.

*Headspace analysis*

A heat-jacketed, fused-silica transfer line (0.53 mm diameter, Agilent) was maintained at 150˚C and connected to the glass bottle containing the brewed coffee. The APCI-MS venturi inlet introduced a flow of headspace (10 mL/min) into the APCI source for 0.5 min. Triplicate samples were analysed.

*APCI-MS conditions*

A Micromass ZQ mass spectrometer fitted with a gas phase APCI interface (Waters, Manchester, U.K.) was operated as described previously [3]. Raw ion abundance and *m/z* values were recorded in a table, with no data processing.

*Data wrangling*

APCI data were imported into Spotfire (v7.9, Tibco, Palo Alto, USA) and extra identity tags (Sample) and calculated values (mean, standard deviation and %CV) were added. Data were un-pivoted to make columns that were appropriate for data filtering (*m/z*, abundance, sample, replicate, country of origin, lightness, mean, SD and %CV). JMP (v13, SAS Institute Inc., Cary, USA) was used for PCA plots.

## Results and discussion

*Data quality checks*

The first step in any data analysis is to check and clean the data. The filter on the data visualisation screen (Fig 1a) shows that the APCI ion abundance values ranged from 4 to 40 x $10^6$. In all analyses, there is a minimum signal to noise ratio and with knowledge of the analytical procedure being used, the minimum signal can be reset to remove spurious signals. In this case, the minimum acceptable APCI signal is >1000 so the slider was set to 1000, which excluded all values <1000 (Fig. 1b).

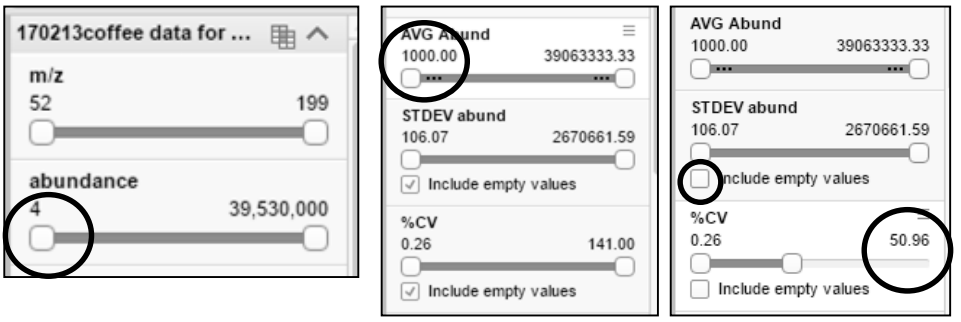


**Figure 1:** Screenshots of the filters in Spotfire and their adjustment to achieve rapid data cleaning. Circles indicate the changes made in steps 1a to 1c (left to right)

The Standard Deviation (Ion Abundance) filter (Figure 1b) also showed that there were some empty values in the data table; these occur when there are insufficient replicates to calculate a value, i.e. only one or two of the replicates showed the presence of that particular ion. These data can be removed by unclicking the "Show empty values" box to further clean the data. Finally, the %CV values show how robust the data are. Again, with knowledge of the samples and the typical analytical variation, the analyst can make informed choices about the degree of data variability they are willing to include in the data table and a final data cleaning step can be applied by adjusting the slider, in Fig 1c it is set at 50.96% but 24% was used to clean the data for PCA analysis. Using this feature of the visualisation software, the data can be cleaned by changing the filter box values to produce a robust data table which can then be interrogated with confidence.

*Qualitative differences in coffee aroma profiles*

A bar chart plot was constructed using Samples and *m/z* values on the X axis and Average Abundance on the Y axis. (Fig 2). Qualitative differences in the profile of the coffees were identified visually and, by hovering the cursor over each bar in Spotfire, the information about that point was displayed and assessed. Visual inspection led to two main conclusions. One was that the ion profiles of the samples in Fig. 2 were very similar, it was the amounts that were different. The other was that all the coffee profiles contained ions from *m/z* 50 to 200, so chromatographic conditions should be chosen to resolve compounds across this molecular weight range and, for quantification, internal standards would need to be added across the molecular weight range.

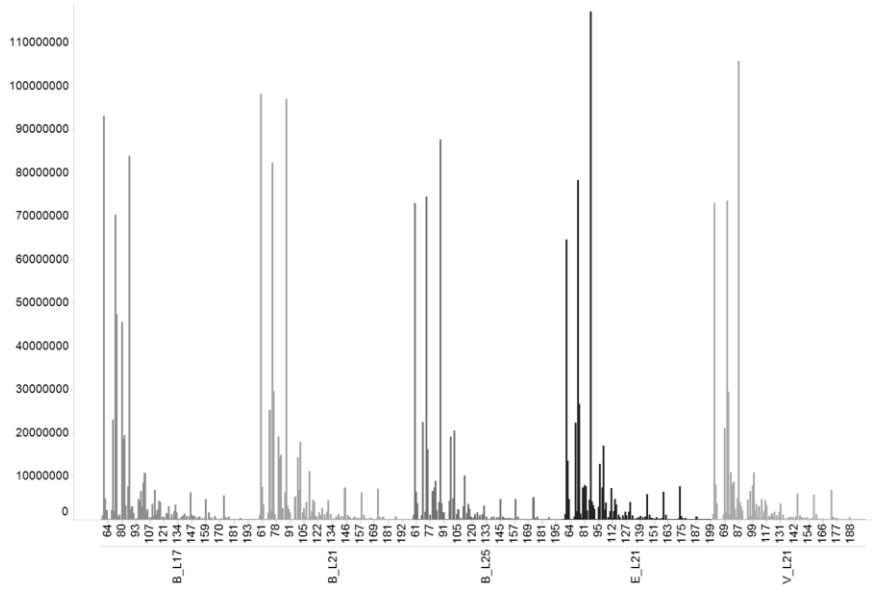


**Figure 2:** Ion profiles for each of the *m/z* values in the five coffee samples (Ion abundance are aggregated values for the three replicates)

Principal component analysis of the data using correlation and covariance matrices indicated that covariance, using the absolute data, not the normalised data, gave the best discrimination of the samples and explained around 85% of the variance (Figure 3). This showed that ions with high abundance contributed strongly to the discrimination of the samples by the PCA.

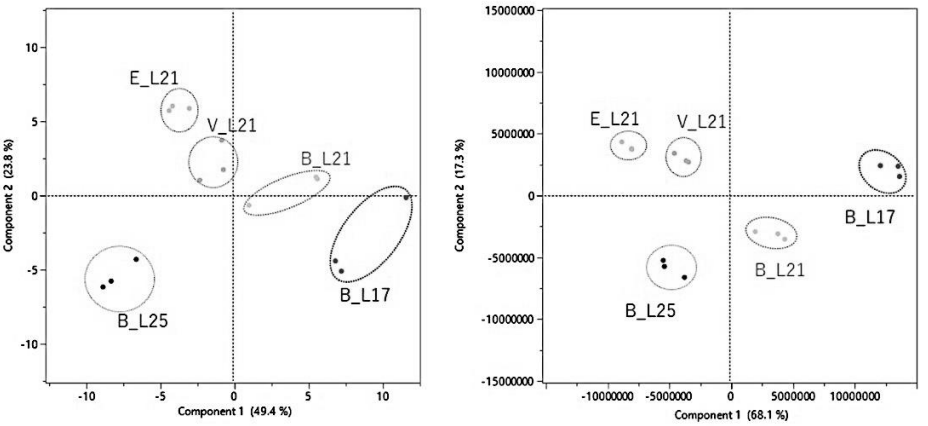


**Figure 3:** PCA plots of normalised data (left) and absolute data (right) using correlation and covariance matrices

Further analysis indicated that the same discrimination could be achieved with 19 high abundance ($>10^6$) ions and these represented the key drivers of the differences in the coffee headspace samples. Therefore, the pre-screen defined the next stage of analysis, which was to identify and quantify the nineteen compounds associated with the ions monitored in APCI using the GC-EI-APCI-MS technique [4].

## Conclusion

Pre-screening of the coffee samples using APCI-MS and visualisation of the results, provided a rapid way to assess the differences between the samples. The information gained could be used to design subsequent analyses, in terms of chromatographic conditions and the type of internal standards needed. The concept is to get the GC- and/or LC-MS analyses right first time to avoid time delays due to having to re-run the analyses. APCI-MS provides an untargeted snapshot of the volatile profiles of the samples and, while not providing a comprehensive analysis, does cover off the main chemical classes involved (esters, acids, aldehydes, pyrazines etc.). Assessing the APCI-MS data in a visualisation package allows rapid data cleaning and checking, followed by easy-to-interpret plots that inform the design of the chromatographic procedures. The advantage of visualisation software is that any ion can be added to this chart using drag and drop, so the analyst can quickly test out ideas and hypotheses about the differences in the coffee profiles. Both JMP and Spotfire offer more sophisticated statistical analyses to interrogate the data. While statistical analysis is obviously key in identifying quantitative differences between samples, our experience is that data visualisation can complement the basic statistical analyses (e.g. ANOVA) and provide a focus for further chemical and statistical analyses.

## References

1. Acree, T.E., Butts, R.M., Nelson, R.R., Lee, C.Y., (1976) Anal. Chem., 48: 1821-1822.
2. Grosch, W., (1993) Trends Food Sci. Technol., 4: 68-73.
3. Hatakeyama, J., Davidson, J.M., Kant, A., Koizumi, T., Hayakawa, F., Taylor, A.J., (2014) Food Chem., 157: 229-239.
4. Taylor, A.J., Sivasundaram, L.R., Linforth, R.S.T., Surawang, S., (2003) In: Handbook of flavor characterization. (Deibler, K.D., Delwiche, J., Eds.); Marcel Dekker: New York: p. 411-422.

# Rapid analysis of important taste active components in chocolate by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-QToF-MS)

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## Abstract

This study illustrates a rapid approach to qualify and quantify taste active compounds in chocolate. Eleven commercially available chocolate samples were extracted with aqueous, acidified methanol and analysed by UHPLC-QToF-MS. This allowed the (semi)-quantification of a series of taste actives within a single injection, while the high resolution of the mass analyser allowed a high confidence in their identification. Dose-over-Threshold (DoT) values were calculated for key chocolate taste compounds allowing a deeper understanding of the orosensory properties of chocolate and creating a link with human-sensory investigations. The outcomes of this study illustrate the benefit of UHPLC in combination with high resolution mass spectrometry, while requiring minimal sample preparation for the extraction of key tastants from chocolate.

## Introduction

Chocolate is appreciated by consumers around the globe due to its unique organoleptic properties. The combination of desirable textural features and a boost of flavours when melting in the mouth made chocolate become one of the most beloved treats in the human diet. Several decades of research have focussed on deciphering the flavour active components in cocoa and chocolate and deepening the understanding of their origin [1, 2, 3, 4]. In particular, the role of cocoa derived aroma active components and their fate during roasting has been studied in detail [2]. Fewer studies are available for the taste active components, but it is generally well understood that besides acidity and astringency, bitterness is a characteristic sensory attribute for cocoa taste [5].

Research applying sensory-guided techniques identified a range of chemically diverse molecules contributing to the bitter taste of roasted cocoa nibs (Figure 1) [5].

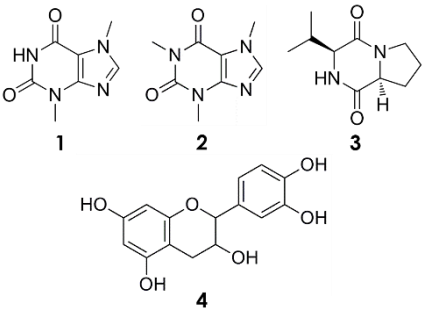


**Figure 1:** Structure of the bitter tastants theobromine (**1**), caffeine (**2**), cyclo(Pro-Val) (**3**) and epi-/catechin (**4a/b**)

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In particular, the alkaloids theobromine and caffeine, the diketopiperazine cyclo(Pro-Val) and the flavan-3-ol epicatechin were attributed with high Dose-over-Threshold (DoT) values [5]. Besides their intrinsic taste, selected diketopiperazines were found to modulate the taste of theobromine solutions resulting in increased bitterness intensity, as well as changing bitterness qualities [6, 7].

The aim of this work was to develop a fast method to identify and quantify key bitter compounds in chocolate and create a link with bitter sensory scores.

## Experimental

### *Materials*

Eleven chocolates from seven markets (with claimed cocoa content from 39 to 70%) were used. Methanol for LC-MS, acetonitrile for LC-MS, water for LC-MS and formic acid were from VWR (Lutterworth, United Kingdom). Caffeine, (+)-catechin, (-)-epicatechin, theobromine, and tyrosine methyl ester hydrochloride were from Sigma-Aldrich (Dorset, United Kingdom). cyclo(Pro-Val) was from Bachem (Bubendorf, Switzerland).

### *Extraction of key tastants and analysis by UHPLC-QToF-MS*

Details of the experimental procedures on the isolation and quantification will be published elsewhere. Briefly, finely ground chocolate was spiked with the internal standard tyrosine methyl ester and extracted with a mixture of methanol/water/formic acid (80/20/0.1, v/v/v). The samples were filtered, diluted and analysed on an ekspert™ ultraLC 100 (AB Sciex, Warrington, United Kingdom) coupled to a Triple TOF 5600$^+$ Mass Spectrometer (AB Sciex, Warrington, United Kingdom). Chromatographic separation was achieved on an ACQUITY® BEH C18 column (2.1 x 150 mm, 1.7 µm) (Waters, Elstree, United Kingdom) equipped with corresponding pre-column using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phases. Calibration curves were prepared by dilution of the commercially available reference substances. The peak areas of the target analytes were extracted using the *m/z* values calculated for $[M+H]^+$ with an extraction window of ± 5 ppm.

### *Quantification of theobromine by HPLC-UV*

The quantitative determination of theobromine in chocolate was performed as described in literature [8].

### *Sensory evaluation*

The bitter taste of the chocolates was scored by twelve trained sensory panellists on a scale ranging from 0 (not bitter) to 10 (very bitter), in duplicate repetition.

## Results and discussion

### *Identification of the analytes*

The developed UHPLC-QToF-MS method enabled analysis of a wide range of taste active components of different functional groups found in the chocolates. The tastants were identified based on their retention times and accurate masses in comparison to references. Figure 2 shows extracted ion chromatograms (EICs) for selected bitter compounds (Figure 1) in a dark chocolate.

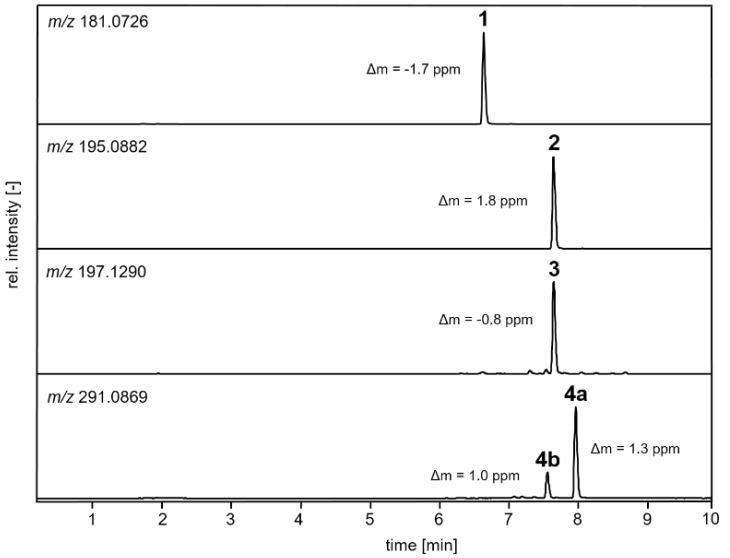


**Figure 2:** Extracted ion chromatograms (EICs) for the $[M+H]^+$ (±5 ppm) for theobromine (**1**), caffeine (**2**), cyclo(Pro-Val) (**3**), catechin (**4b**) and epicatechin (**4a**) from a dark chocolate sample

*Quantification of the analytes*

Following identification, the concentrations of the analytes were quantified using tyrosine methyl ester as internal standard. The values obtained for theobromine using the developed method were compared to that of a conventional HPLC-UV method (Figure 3). A good correlation of the results was observed ($r^2$=0.9171).

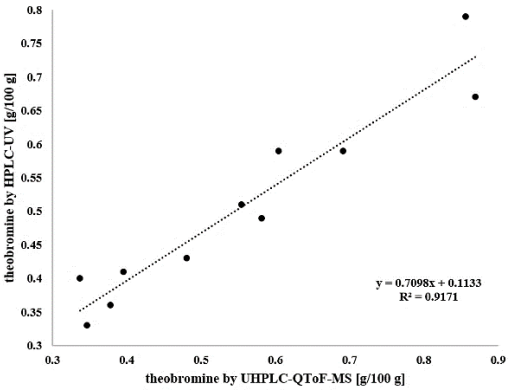


**Figure 3:** Correlation of theobromine values obtained by the developed UHPLC-QToF-MS method and the HPLC-UV reference method

*Calculation of Dose over Threshold (DoT) values and correlation to sensory analysis*

The quantification of key taste actives (theobromine, caffeine, epicatechin, catechin and cyclo(Pro-Val)) allowed the calculation of DoT values based on taste threshold values obtained in literature [5]. A link of the sum of the DoT values for compounds **1-4** (Figure 1) with values obtained by human-sensory assessment of bitter taste was then established (Figure 4). Figure 4 shows that in particular sample **I** is deviating from the

predicted values. This was attributed to the higher concentration of DKPs, shown for cyclo(Pro-Val) in Table 1. This agreed with literature on the taste modifying properties of DKPs [5, 6].

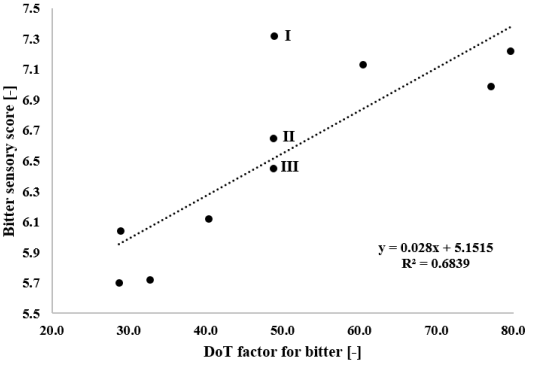


**Figure 4:** Correlation of the sum of the DoT values for bitter for the key tastants (**Figure 1**) and the observed sensory bitter scores

Overall a good correlation was observed between the sum of the DoT factors for the five taste compounds (Figure 1) and the bitter sensory scores. The results confirm in particular that **1** is the most important contributor to the bitterness of commercial dark chocolates (Table 1). Further investigation on the synergism of DKPs and theobromine (**1)** and the relevance of DKPs for the sensory perception of dark chocolates are on-going.

**Table 1:** DoT values for the key tastants and sensory scores for bitter in selected dark chocolate samples. Numbering of components refers to Figure 1.

| *Sample* | *Dose over threshold factor (DoT) [-]* | | | | | *Sensory score bitter* |
|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4a* | *4b* | |
| I | 40.6 | 4.6 | 2.9 | <0.5 | 2.9 | 7.3 |
| II | 38.7 | 4.9 | 0.8 | 0.9 | 0.8 | 6.7 |
| III | 42.2 | 4.4 | 1.9 | <0.5 | 1.9 | 6.5 |

## References

1. Afoakwa, E. O., Paterson, A., Fowler, M. and Ryan, A. (2008) Crit. Rev. Food Sci. Nutr. 48:840-857.
2. Frauendorfer, F. and Schieberle, P. (2008) J. Agric. Food Chem. 56:10244-10251.
3. Schnermann, P. and Schieberle, P. (1997) J. Agric. Food Chem. 45: 867-872.
4. Liu, J., Liu, M., He, C., Song, H., Guo, J., Wang, Y., Yang, H. and Su, X.(2015) J. Sci. Food Agric. 95: 1362-1372.
5. Stark, T., Bareuther and S., Hofmann, T. (2006) J. Agric. Food Chem. 54:5530-5539.
6. Pickenhagen, W., Dietrich, W., Dietrich, P., Keil, B., Polonsky, J., Nouaille, F. and Lederer, E. (1975) Helv. Chim. Acta 58:1078-1086.
7. Stark, T. (2005) Charakterisierung der wertgebenden Geschmacksstofe von geröstetem Kakao (Theobroma cacao) mittels Dekompositions- und Rekonstruktions-Analyse (in German). PhD Thesis, Technische Universität München.
8. Schweizerisches Lebensmittelbuch (1999) Bestimmung des Theobromins und Coffeins mittels HPLC.

# Evaluation and optimization of sample preparation techniques towards the regional differentiation of Chinese green teas

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## Abstract

Aroma profiles of 18 green tea samples from Guizhou province were investigated and compared to teas from other Chinese regions.

Several preparation techniques like Static (SHS) and Dynamic Headspace (DHS), Solid-Phase Microextraction (SPME), Stir-Bar Sorptive Extraction (SBSE) and Solvent Assisted Flavour Evaporation (SAFE) were performed to compare their efficiency and applicability. All methods were measured with gas chromatography coupled to mass spectrometry (GC-MS).

HS-SPME proved to be the most efficient method regarding enrichment of the analytes, robustness, flexibility, ease of handling, and in economic regards. The optimized method identified 43 volatile organic compounds (VOC). Subsequently, all green teas were classified on the basis of these VOC into their provinces. Here, Partial Least Squares Discriminant Analysis (PLS-DA) achieved a separation of teas of the provinces Anhui and Guizhou, whereas the VOC profiles of teas from the remaining provinces overlap with others.

## Introduction

Tea contains substantial amounts of polyphenols, caffeine, volatile oils, vitamins, aroma-forming substances and other compounds that have unique biological activities and health benefits [1].

The flavour of green tea is characterized by up to 300 volatile compounds, of which only eight are responsible for the formation of the distinct green tea aroma [2].

Green tea is mainly consumed in Asia. The overall market in 2016 for Chinese tea was about 37.5 billion Euro. Especially high-quality green teas from special regions like the Guizhou province are of interest. The price per kilogram tea can vary from ten to several thousand euros. The increasing demand for those teas, however, misleads certain producers to sell their low-quality products as high-quality ones from specific regions. As a result, a large number of adulterations have already been registered on the markets. In order to protect consumers from adulteration and food fraud, instrumental analysis is necessarily required to clearly indicate distinctions between the tea aroma profiles of specialty goods and inferior ones. Due to its popularity, the characterization of green tea aroma compounds is widely spread, but does not address the situation mentioned above. Essentially, a method investigating the regional origin may also consider VOC other than key food odorants.

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## Experimental

All green teas were high quality teas from special Chinese regions. 18 green teas derive from Guizhou, six from Anhui, two of each from Henan and Zhejiang and one from Jiangsu.

GC settings: Column: Rxi-5ms and Rtx-624 (Restek GmbH, Bad Homburg, Germany) 60 m x 250 µm x 0,25 µm; oven: initial 40°C, hold 4 min; 5°C/min to 100°C; 2°C/min to 138°C; 5°C/min to 210°C, hold 2 min; 15°C/min to 230°C, hold 16 min; carrier gas: Helium, constant flow = 1 mL/min; aquisition mode: Scan mode, 35-500 amu; GC system: Agilent 7890 (Waldbronn, Germany); MS/MS system: MS Triple Quad 7000 C Agilent.

For the following analyses, an amount of 0.3 g green tea was ground with liquid nitrogen and poured with 8 mL distilled water at 80°C. For direct SPME and SBSE a filtration step was included. At SHS analysis the sample was incubated for one minute at 80°C in the incubator oven of the autosampler (MultiPurposeSampler MPS2, Gerstel, Mülheim a.d. Ruhr, Germany). After that an aliquot of 600 µL was injected into the GC injector.

In the course of DHS (Automated Dynamic Headspace, Gerstel), the sample was incubated for one minute at 45°C. Soon afterwards the headspace was flushed with 100 mL nitrogen (flow of 40 mL/min) and subsequently injected onto a cryogenic trap cooled with nitrogen at –120°C (KAS 4, Gerstel), heated and injected onto a Rtx-624 in an Agilent 6890 GC.

In SBSE (coupled to a Thermal Desorption Unit TDU, Gerstel) and SPME (automated SPME using the Gerstel MPS2) analysis the analytes were incubated for 1 min at 80°C and enriched on a PDMS-based Twister® SBSE respectively a 50/30 µm DVB/Carboxen/PDMS fibre and analysed on a Rtx-624 as well.

SAFE was performed at $10^{-5}$ mbar. Before analysis, the apparatus was set thermostatically at 40°C. 2 g with liquid nitrogen grounded tea were poured with 50 mL of 80°C hot distilled water and stirred for 15 min. 40 mL of the sample was then added to the SAFE apparatus by decantation. After 30 min distillation time the distillate was extracted twice with 15 mL diethyl ether and the ether phases were combined. 1 µL of the ether extract was injected directly for GC-MS analysis.

0.4 g cryo-ground green tea were analysed without infusion. The volatiles were enriched in 20 mL SPME vials by HS-SPME after which GC-MS was performed. For SPME analysis a 50/30 µm DVB/Carboxene/PDMS fibre (2 cm) was used and the analysis was performed with the GERSTEL-Multi Purpose Sampler MPS2. The incubation of the samples took place at 70°C for 40 min. After that the SPME fibre was exposed into the sample's headspace for 40 min extraction at 70°C. A temperature programmed transfer of the analytes was performed in the GC-injector. To include very volatile compounds as well, the initial GC oven temperature was set on 40°C.

## Results and discussion

HS-SPME analysis was preferred over the tested methods direct SPME, direct SBSE, DHS, SHS and SAFE. In a direct comparison, higher signal intensities and a wider range of analytes have been achieved by HS-SPME [5]. SHS merely turns out as a screening method for the analysis of very volatile compounds (e. g. 2-methybutanal or 3-methylbutanal). Later eluting compounds as well as typical terpenoid components of green tea like linalool or geraniol were not covered by this method. DHS, on the other hand, should be able to analyse a wider range of substances and be able to achieve higher

signal intensities as it includes concentration steps like purging and trapping. Due to this the equilibrium between liquid and gas phase is constantly resetting. This ensures that even substances with low volatility and low concentrations can be detected. This method, however, turns out as not suitable for the characterisation of green tea under the given sample preparation conditions. Aqueous samples like tea infusions were rather problematic for DHS analysis. The difficulty is that condensed water may block the GC injector by freezing out. To minimize this effect, DHS analysis was performed at a low incubation temperature of 45°C, which results in a small spectrum of VOC extracted from green tea. According to the results, DHS performance was limited in the case of aqueous samples and was thus not suitable for tea analysis. Additionally, DHS was not preferred over HS-SPME due to economic aspects.

When comparing SPME and SBSE, HS-SPME proves to be preferred over direct SPME and direct SBSE. Compared to the first, HS-SPME is the more robust method, because in direct analysis, the tea infusion had to be filtered before starting the analysis in order to protect the fibre from contamination. In consequence, very volatile compounds escaped during the filtration process. On the contrary, HS-SPME analysis allowed a direct investigation in the vial immediately after tea infusion, so that these losses did not occur. Similar to direct SPME, the deficit of very volatile substances appeared in direct SBSE as well.

Another advantage of HS-SPME is the triple coated fibre that guarantees a wider range of aroma compounds of different polarities. PDMS-Twister® are selective for non-polar compounds, while polar compounds are less effectively analysed. In total, only 8 green tea components were determined by direct SBSE analysis, while 43 substances were analysed by the HS-SPME method [5]. Polar compounds such as volatile esters and alcohols can be determined more efficient by magnetic stirrers coated with ethylene glycol and polyacrylate. On the other hand, extraction deficits would result due to polar materials regarding non-polar substances, so that there should be no advantage over the triple-coated SPME fibre. Additionally, the analysis of several sorption phases with different polarities are connected with extra efforts and a longer analysis time which lead to economic disadvantages.

SAFE was also carried out in comparison with the automated methods. This application, however, was not suitable due to the low analyte concentrations in those green teas.

Using HS-SPME-GC-MS analysis and PLS-DA, it can be stated that round 83% investigated green teas are well classified. Especially samples from Guizhou and Anhui 100% separated from each other. The two samples from Zhejjang have similar aroma profiles like samples from Anhui and Guizhou. In the same way, the aroma profile of green teas from Jiangsu and Henan overlap with these from Guizhou (cp. figure 1).

In general, this data analysis shows that a classification of the green teas by means of their volatiles profile into their provinces is possible. General conclusions for specific provinces can hardly be stated. A broader analysis with equal sample numbers of each province are required to draw general and valid statements. The statistical power of this study is limited, since only 29 teas with varying shares of regions were analysed. For example, Jiangsu province was only represented by one green tea sample (T19).

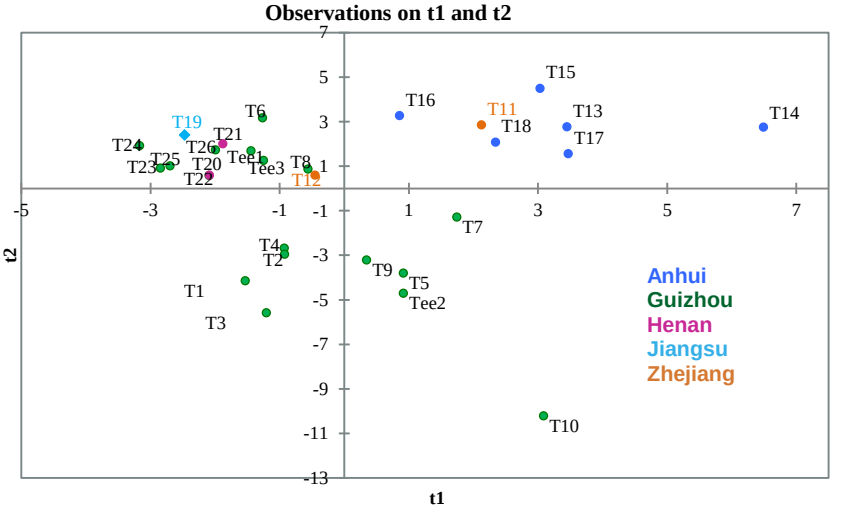


**Figure 1**: PLS-DA plot of 29 green teas of five different provinces; based on 43 identified VOC

Future studies, should therefore contain a variety of green teas from different regions. It would be advantageous if the same numbers of green tea were examined in each province, to assure even weighting of the samples. In parallel, a trained sensory panel should accompany the study and establish relations to influencing factors.

Further work should be carried out in the context of organic teas. By statistical analysis the organic tea of this study (Tea 10) was significantly distinguished from all other 28 teas (s. figure 1). To confirm whether such teas and why are different from conventional teas, a larger number of organic teas should be included in the statistics.

Finally, the analysis of low-volatility components can as well be performed by liquid chromatography coupled to mass spectrometry, since e.g. flavonoids are important constituents with varying and potentially fingerprint concentrations in green tea.

## References

1. Sereshti H., Samadi S., and Jalali-Heravi M. (2013) J. of Chromatogr. A, 1280 pp. 1-8
2. Dunkel A., Steinhaus M., Kotthoff M., Nowak B., Krautwurst D., Schieberle P., and Hofmann T. (2014) Angewandte Chemie. International edition 53, pp. 7124-7143
3. Huo D., Wu Y., Yang M., Fa H., Luo X., and Hou C. (2014) Food Chem 145, pp. 639-45
4. Ye N., Zhang L., and Gu X. (2011) Anal Sci. 27, pp. 765-769
5. Ho T. T. (2017) Master Thesis: Evaluierung von Probenvorbereitungsmethoden und Optimierung der Aromaanalytik mit Hilfe von GC-MS zur Herkunftsbestimmung von chinesischem Grüntee, Lebensmittelchemie, University Wuppertal, Germany (in German).

# On-line high-throughput analysis of the volatilome of microorganisms that have agroindustrial relevance

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## Abstract

Yeast and bacterial fermentations play a key role in producing not only important technological functions in fermented foods but also the characteristic sensory attributes. While key flavor compounds are generally characterized, the kinetics of formation are poorly understood and poorly controlled. Multipurpose head-space automated sampling (MHSA) together with Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) were investigated as a tool to understand volatile organic compound (VOC) changes during fermentation. Automation of the analytical process as provided by MHSA guaranteed reproducibility over the whole microorganism life cycle, the accurate control of process parameters (temperature and sampling times) while maintaining the rapid sampling rates that PTR-ToF-MS enables. Multivariate data analysis techniques are required to identify important trends in the data.

## Introduction

Yeast and bacterial species are widely used for leavening, brewing, wine making or dairy fermentations and play a key role in producing the characteristic sensory profiles and perceived quality of these products through the VOCs they generate [1-3]. These VOCs synthetized by microorganisms as secondary metabolites, not only impart important sensory notes but also have important technological functions [3]. As such, an on-line and non-invasive screening of the microorganism volatilome is of high importance to better understand and control these processes and support innovation in this traditional sector by unlocking the flavor generation process.

Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) coupled with multipurpose head-space automated sampling (MHSA) was investigated to enable the efficient monitoring of agroindustry-relevant microbiological processes: dough leavening, lactic acid fermentation and wine making.

## Experimental

The following three experimental datasets are used to illustrate the usefulness of the coupled PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Austria) and multipurpose headspace automated sampler (Gerstel GmbH & Co. Germany):

1. Lactic acid bacteria in low fat milk [4]; 3 cultures from Chr Hansen (A: FD-DVS YF-L812 Yo-Flex; B: FD-DVS YC-380 Yo-Flex; and C: FD-DVS YC-X11 Yo-Flex) and 1 from Danisco (D: YO-MIX 883 D) x 3 replicates x 12 time points (0 – 240 min)
2. Yeast in bread dough [5]; 4 commercial yeast (Y1: Lessaffre, Parma, Italy; Y2: Lessaffre, Parma, Italy; Y3; Pakmaya, Istanbul, Turkey; and Y4: Italmill, Cologne, Italia) x 5 replicates x 3 time points (0 – 2.7 h); headspace dilution of 2:1 inert gas: sample flow

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3. *Sacchromyces cerevisiae* on agar [6]; 6 strains x 12 replicates x 66 time points (0 – 11 d); dilution of 1:3 sample flow to argon was used to overcome the deleterious effect of ethanol on the acquired spectra.

PTR-ToF-MS drift tube temperature and pressure were 110°C and 2.3 mbar, respectively and the drift tube voltage was about 550 V, which resulted in an E/N ratio of about 140 Td (1 Td = 10−17 cm2 V−1 s−1). The inlet flow was 40 sccm for the yogurt and wine yeast fermentations and 120 sccm for the dough.

Data processing of PTR-ToF-MS included dead time correction, external calibration, peak extraction, peak fitting and baseline extraction [4,5] and concentration was calculated as per Lindinger et al. [6].

**Results and discussion**

The coupling of PTR-ToF-MS with multipurpose headspace automated sampling allows the on-line noninvasive high-throughput screening of microorganism volatilome; and the identification of strain specific features and new metabolic pathways over time frames that are industrially relevant.

PTR-ToF-MS analysis of the VOCs generated during fermentation from doughs fermented with four different commercial yeasts produced complex spectra (Figure 1). After filtering to remove m/z that were unchanged, clusters (water and/or ethanol) and isotopologues ($^{13}C$ and $^{18}O$) 46 m/z discriminated the bread dough with respect to time or yeast type. Yeast types were discriminated by 16 VOC. The high mass resolution was advantageous in allowing the discriminate between separate masses within one nominal mass, e.g. m/z 87 where variation in the signal between yeast could be assigned to variation in an aldehyde/ketone rather than diacetyl (Figure 1 inset).

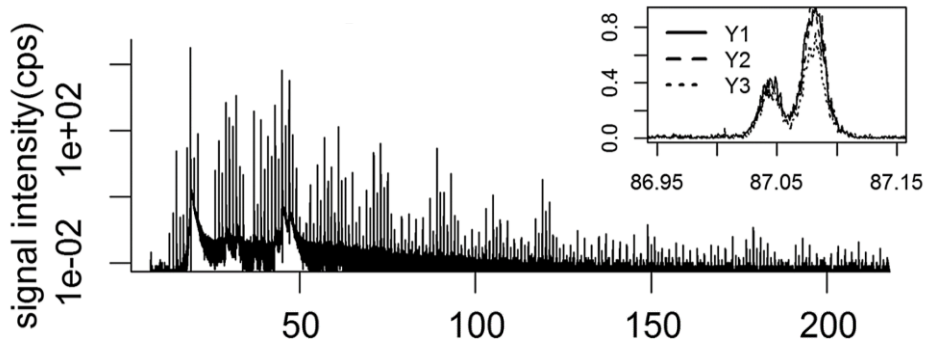


**Figure 1:** Average PTR-ToF-MS spectrum of fermenting dough; inset shows a double peak for the nominal mass 87 [2] *(Copyright © 2014 John Wiley & Sons, Ltd)*

The four cultures that fermented yogurt were distinguished by 13 m/z including 2 isotopologues. During the conversion of milk into yogurt the MHSA coupled with PTR-ToF-MS allowed sampling times with sufficient temporal resolution to allow depletion/consumption of methanethiol and synthesis/appearance of acetoin to be followed (Figure 2).

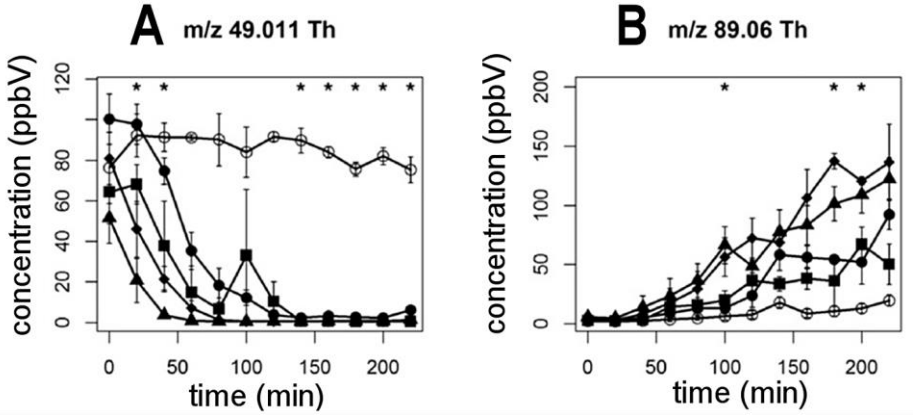


**Figure 2:** Fermentation kinetics of methanethiol (**A**) and acetoin (**B**) (means of three replicates ± standard deviation). Open circle (○), uninoculated milk; filled square (■), starter A; filled circle (●), starter B; filled triangle (▲), starter C; filled rhombi (♦), starter D. Asterisks indicate statistically significant differences (ANOVA, p b 0.05) among commercial starters. [1] *(Copyright © 2015 Elsevier Ltd*)

The VOC generated by the wine yeast grown on agar were significantly discriminated by 70 m/z based on yeast type and time. Of these 50 could be assigned a chemical formula and 37 were tentatively identified. The principal component analysis (PCA) explained 76.1% of the data variation on 2 principal components (Figure 3). The PCA plot shows from left to right the open circles, which represent the times points for each yeast during the lag phase and growth phase, an increase in fermentation time. Separation is largely due to increases in esters and ethanol. In contrast, during the stationary phase ester and ethanol synthesis cease and due to the sampling conditions stripping occurs, i.e., ester and ethanol concentrations decrease.

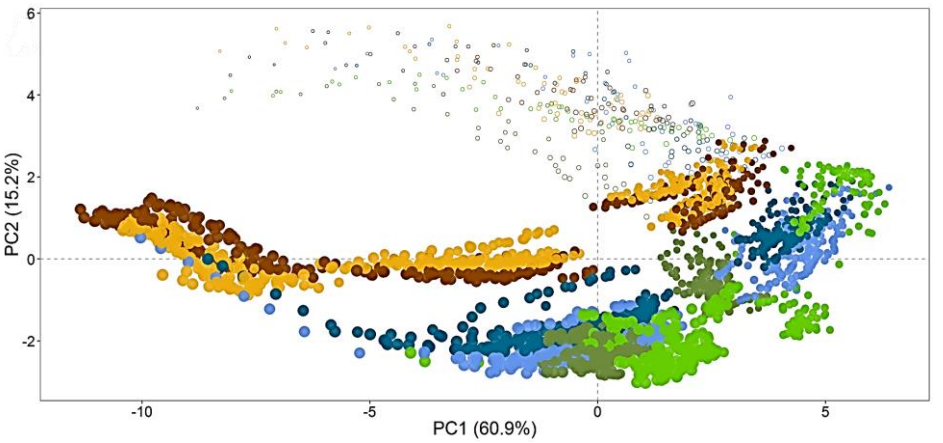


**Figure 3:** Score plot of principal component analysis showing fermenting wine differentiation due to yeast volatilome evolution during 11 days. Data are logarithmically transformed and centered. Different colors indicate different yeast strains, medium and blank samples. Length of fermentation time is represented by an increase in point size; Open circles represent the lag and growth phase; and closed circles represent the stationary phase [3].

Automation of the analytical process as provided by MHSA guaranteed reproducibility over the whole microorganism life cycle, the accurate control of process parameters (temperature and sampling times). Analysis could be completed as frequently as every second but typically the headspace of each sample was measured for one minute while displacing the headspace with zero air or pure nitrogen. In addition, the fermentation processes can automatically be monitored for several hours in the case of dough leavening and lactic acid fermentations or days for alcoholic fermentations or yeast colonies grown on a solid medium. The set-up allows the monitoring of up to 128 samples at each time point.

To deal with data matrices containing several hundreds of mass peaks for each measurement multivariate data analysis is needed to provide the general overview of biological processes and phenotypic variability among different microbial strains. Observations of single VOC emission curves allow the opportunity to study known metabolic pathways and unravel unknown ones.

## References

1. Pretorius, I.S., (2000) Yeast, 16: 675-729.
2. Ur-Salim, R., Paterson, A., and Piggott, J.R., (2006) Trends in Food Science & Technology, 17: 557-566.
3. Cheng, H., (2010) Critical Reviews in Food Science and Nutrition, 50: 938-950.
4. Cappellin, L., Biasioli, F., Fabris, A., Schuhfried, E., Soukoulis, C., Märk, T.D., and Gasperi, F., (2010) International Journal of Mass Spectrometry, 290: 60-63.
5. Cappellin, L., Karl, T., Probst, M., Ismailova, O., Winkler, P.M., Soukoulis, C., Aprea, E., Märk, T.D., Gasperi, F., and Biasioli, F., (2012) Environmental Science & Technology, 46: 2283-2290.
6. Lindinger, W., Hansel, A., and Jordan, A., (1998) International Journal of Mass Spectrometry and Ion Processes, 173: 191-241.
7. Benozzi, E., Romano, A., Capozzi, V., Makhoul, S., Cappellin, L., Khomenko, I., Aprea, E., Scampicchio, M., Spano, G., Märk, T.D., Gasperi, F., and Biasioli, F., (2015) Food Research International, 76: 682-688.
8. Makhoul, S., Romano, A., Cappellin, L., Spano, G., Capozzi, V., Benozzi, E., Märk, T.D., Aprea, E., Gasperi, F., El-Nakat, H., Guzzo, J., and Biasioli, F., (2014) Journal of Mass Spectrometry, 49: 850-859.
9. Khomenko, I., Stefanini, I., Cappellin, L., Cappelletti, V., Franceschi, P., Cavalieri, D., Märk, T.D., and Biasioli, F., (2017) Metabolomics, 13: 118.

# Headspace solid phase microextration *vs.* dynamic headspace extraction to explore breast milk volatile fraction

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## Abstract

Biological matrices of mammals contain many volatile compounds which can act as specific chemical cues for congeners modifying their behavior or their physiological parameters. Gas-chromatography (GC) can be used to assess such matrices, however, this is a challenging technique because of low concentrations of highly volatile analytes. Thus, an extraction technique that (i) preserves the original profile of the volatile compounds, and (ii) concentrates the analytes is required. Headspace extraction methods, such as headspace solid phase micro-extraction (HS-SPME) or dynamic headspace (DHS), show many advantages. Therefore, they are promising methods for the analyses of biological matrices.

In this study, DHS was compared by using two sorbent cartridges, and HS-SPME was performed for the exploration of human milk composition. Volatile compounds of pooled breast milk samples were extracted by HS-SPME using a Car/PDMS fiber, by DHS associated to Tenax® or Bio-monitoring sorbent tubes. Extracts were analyzed by GC coupled to a mass spectrometer and a flame ionization detector. Extraction yields were compared on the basis of qualitative and semi-quantitative chromatograms.

As a result, HS-SPME with Car/PDMS fiber extractions enabled the recovery of a large diversity of compounds from breast milk and displayed a great reproducibility, whereas DHS enabled to recover a larger number of compounds with an about 10-fold higher yield. The DHS method allowed four compounds to be newly identified in breast milk: cyclohexanone, 6-methyl-5-hepten-2-one, pyridine and phenol. Thus, despite a challenging implementation, DHS is a better option than HS-SPME with Car/PDMS fiber for the investigation of volatile fractions of small-scale or low concentrated samples like breast milk. Since the two sorbents used in DHS, Tenax® and Bio-monitoring, demonstrated equivalent extraction capabilities, Tenax®, which is widely used, is the preferred option for a better comparison of the results with the current literature.

## Introduction

Biological matrices of mammals contain many volatile compounds which can act as specific chemical cues for congeners modifying their behavior or their physiological parameters[1]. Extraction of volatile compounds and their subsequent analysis by means of gas-chromatography (GC) could enable key components from biological matrices to be identified. However, since volatile compounds of biological matrices were present in very-small amounts[2] their exploration is particularly challenging. Indeed, analytical methods require the extraction technique to have a strong concentration capability without distorting the original volatile profile. Regarding this issue, the headspace extraction techniques could be of interest since they involve solvent-free procedures and respect matrix integrity[3]. Among them, headspace solid phase micro-extraction (HS-SPME) is widely employed because it is relatively easy to apply and low in costs. However, the low amount of sorbent on fibers could limit its application for samples with low-concentrated headspace. Dynamic headspace (DHS) methods demonstrate enriched

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capabilities through the constant displacement of the headspace equilibrium and the large quantity of sorbent involved. However, the implementation of this technique can be complex, notably due to leaking during desorption of the target compounds.

The objective of this study was to evaluate the capability of DHS associated with the commonly used sorbent, Tenax® or a new combination of sorbent Bio-monitoring and HS-SPME for the exploration of a low-concentrated biological fluid: the breast milk.

## Experimental

*Samples*

Four samples of human milk (50mL) were collected into a pre-cleaned 120mL wide-mouth bottle. The bottle was capped and the sample stored at -20°C until all samples were collected. Once the four samples were collected, they were defrosted and pooled together. The pooled sample was divided into 5mL aliquots, placed into 22,5 mL amber screw cap *vials, sealed with a PTFE septum and stored at -80°C prior to analysis. Before extraction*, samples were defrosted during 10 minutes at ambient temperature.

*Extraction methods*

DHS extraction. A nitrogen flow was bubbled into 5mL sample of human milk with a purge flow of 20mL.min$^{-1}$ during 2 hours adapted from conditions previously published[4]. The compounds were trapped either in a Tenax® (Markes international Ltd, Llantrisant, UK) or a Bio-monitoring cartridge (composed of Tenax® TA and Carbograph 5 TD, Markes).

HS-SPME extraction. Volatile compounds from a 5mL sample of human milk were extracted by HS-SPME with a Car/PDMS fiber (10mm length, 85µm film thickness) placed in the headspace of the vial for 2 hours at 34°C. SPME extraction time was set in order to have comparable conditions with DHS.

*Desorption and chromatographic conditions*

Analyses were carried out using a GC (7890A System Agilent, Wilmington, DE, USA) equipped with a mass spectrometer (5975 inert MSD with Triple Axis Detector MS, Agilent) and a Flame Ionization Detector (FID, Agilent). Triplicates of HS-SPME and DHS extracts were analyzed in a random sequence. Compounds on the SPME fiber were desorbed into the injection port of the chromatograph (T=260°C, splitless) whereas DHS cartridges were desorbed on a thermal desorption system (Unity 2 thermal desorption, Markes) for 20 minutes at 240°C. The thermal desorption system uses a two-stage procedure, where the first stage is a desorption of the cartridge followed by a re-trapping on a Peltier-cooled (10°C) trap. Compounds were then transferred to the GC column during the rapid heating of the Peltier trap from 10°C to 320°C with a rate of 100°C.s$^{-1}$. After desorption, volatile compounds were separated on a DB-WAX column (30m x 0.25mm x 0.5µm film thickness, Agilent). Hydrogen was used as a carrier gas at constant flow (1mL.min$^{-1}$). The oven temperature was programmed from 50°C (0min) to 80°C at 5°C.min$^{-1}$, then from 80°C to 200°C (0min) at 10°C.min$^{-1}$, and finally from 200°C to 240°C (4min) at 20°C.min$^{-1}$. Peak areas were integrated using the MSD Chemstation software (Agilent). Mass spectra were recorded in electron impact mode (70eV) between a mass range of 33 and 300 m/z at a scan rate of 2.7 scan.s$^{-1}$. Compounds were identified by comparing their mass spectra and their linear retention indices (LRI) with those of reference databases (Nist and internal database) and with relevant literature.

*Data processing and statistical analyses.*

One-way analysis of variance (ANOVA) and Least Significant Difference (LSD) tests were performed on FID peak areas obtained from the analysis of HS-SPME or DHS (Tenax® or Bio-monitoring cartridges) milk extracts with a 95 % confidence level. A normalized principal component analysis (PCA) and Ascendant Hierarchical Classification (AHC) were conducted on the data. The Xlstat software (Addinsoft) was used to perform statistical analyses.

## Results and discussion

Chromatograms obtained from the three extraction methods are presented in Figure 1.

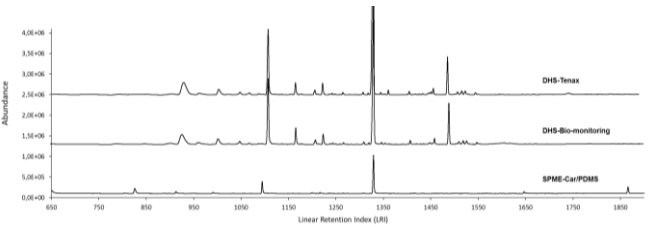


**Figure 1:** FID chromatograms of breast milk extracts obtained with DHS with Tenax® or Bio-monitoring cartridges and HS-SPME with Car/PDMS fiber

Fifty-five peaks were detected in at least one of the three extracts, mostly carbonyl compounds, alcohols, terpenes and carboxylic acids (Fig.2).

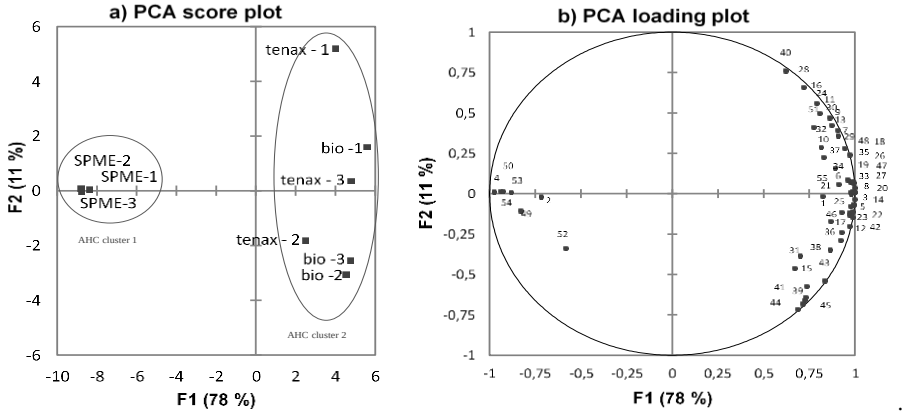


**Figure 2:** Principal component analysis performed on the peak areas of the volatile compounds detected in human milk extracted by HS-SPME, DHS (Tenax®) and DHS (Bio-monitoring) and projection of clusters (AHC). a- Score plot from human milk different extracts. b- Loading plot for volatile compounds 1: Hexane; 2:Unidentified-1; 3: Octane; 4 :2-propanone; 5: Butanal; 6: 2-Butanone; 7: Heptane, 2,2,4,6,6-pentamethyl-; 8: 2-Pentanone; 9: α-Pinene; 10: Toluene; 11: Camphene; 12: Hexanal; 13: β-Pinène; 14: Ethylbenzene; 15: 1-Butanol; 16: 3-Carene; 17: Unidentified-2; 18: 2-Heptanone + Heptanal; 19: Pyridine; 20: dl-Limonène; 21: (E)-2-Hexenal; 22: 2-Pentylfuran; 23: 1-Pentanol + γ-Terpinene; 25: p-Cymene; 26: Octanal; 27: 1-Hexanol; 28: Cyclohexanone; 29: (E)-2-Heptenal; 30: 6-Methyl-5-hepten-2-one; 31: 1-Hexanol; 32: Unidentified-3; 33: 2-Nonanone; 34: Nonanal; 35: Unidentified-4; 36: Unidentified-5; 37: (E)-2-Octenal; 38: Acetic acid; 39: Unidentified-6; 40: Unidentified-7; 41: 2-Ethyl-1-hexanol; 42: Unidentified-8; 43: Unidentified-9; 44: Unidentified-10; 45: Unidentified-11; 46: 3,5-Octadien-2-one; 47: (E)-2-Nonenal + Benzaldehyde; 48: Camphor; 49: Butanoic acid; 50: Unidentified-12; 51: Acetophenone; 52: Pentanoic acid; 53: Hexanoic acid; 54: Heptanoic acid; 55: Phenol

Among detected compounds, 43 were associated with at least one identified compound. All of them have already been identified in human milk except cyclohexanone, 6-methyl-5-hepten-2-one, pyridine and phenol which were tentatively identified. These compounds have already been found in other human materials[5]. The analysis of chromatographic profiles of breast milk samples showed that respectively 38 and 53 compounds were identified in HS-SPME and DHS extracts. The following 20

compounds were only detected in the DHS extracts: butanal, (E)-2-hexenal, 3,5-octadien-2-one, acetophenone, camphene, 3-carene, camphor, 1-hexanol, 2-ethyl-1-hexanol, octane, heptane,2,2,4,6,6-pentamethyl, ethylbenzene, styrene and 7 unidentified compounds (LRIs: 1372, 1418, 1441, 1456, 1493, 1503, 1510) while four compounds: 2-propanone, an unidentified compound (LRI: 1651), hexanoic acid and heptanoic acid where only detected in the HS-SPME extracts. The ANOVA and LSD tests performed on individual peak areas (data not shown) demonstrated that among the 34 compounds common to HS-SPME and DHS extracts, all of them, except butanoic acid, were found at a larger extent in the DHS extracts with a 10-fold higher yield. However, no specific trends were observed for any chemical family. The ratio of five compounds, two mono-terpenes (α-pinene and p-cymene) and three other components (1-butanol, pyridine and 6-methyl-5-hepten-2-one) even exceeded a ratio of 20. Some of these compounds, could have originated from the mother's diet, like terpenes (vegetables) or pyridins (roasted food), and could be flavor cues present in the mother's milk influencing the early and future newborn's feeding behavior[6]. Moreover, among the 53 compounds detected in DHS extracts, none was statistically different between the Tenax® and Bio-monitoring cartridges, except hexane and acetic acid, which were significantly more abundant when using the Bio-monitoring cartridge, although these could also have been artifacts of sorbents.

A PCA performed on the peak areas of each compound (Fig. 1) allowed visualization of these previous observations. Indeed, 89% of the variance was recovered in the PCA map. In the PCA score plot, DHS extracts were positively correlated to the first axis, which represents 78% of the variance, while the HS-SPME extract was negatively correlated to this axis. HS-SPME extraction exhibited a greater repeatability than DHS. However, the loading plot showed that the majority of peaks (48) was positively correlated to DHS extracts, whereas only 7 peaks were correlated to the HS-SPME extract. The AHC shows that HS-SPME and DHS extracts were discriminated, however Tenax® and Bio-monitoring cartridges were not.

In conclusion, even if HS-SPME with the Car/PDMS fiber extraction enables the recovery of a large diversity of compounds from milk and displays a great reproducibility, DHS enables the recovery of a larger number of compounds and in a greater extent than HS-SPME. Thus, despite a challenging implementation, DHS is a better option than HS-SPME to investigate volatile fraction of small-scale or little concentrated samples like breast milk. Tenax® and Bio-monitoring sorbents, tested in DHS, exhibited equivalent extraction capabilities. Thus, Tenax®, which is widely used, should be preferred to Bio-monitoring sorbent to enhance comparison of results with the current literature.

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## References

1. Buettner, A., Wagenstaller, M. and Beauchamp, J. (2015) In: Flavour Development, Analysis and Perception in Food and Beverages (Parker, J.K. ed.), Woodhead Publishing, pp. 387-407.
2. Spitzer, J., Klos, K. and Buettner, A. (2013) Clin. Nutr. 32: 1036–1042.
3. Soria, A. C., García-Sarrió, M. J., Ruiz-Matute, A. I. and Sanz, M. L. (2017) In: Comprehensive Analytical Chemistry (Ibáñez, E. & Cifuentes, A., ed.) Elsevier, pp. 255–278.
4. Schaal, B., Coureaud, G., Langlois, D., Giniès, C., Sémon, E., and Perrier, G et al. (2003) Nature 424: 68–72.
5. De Lacy Costello, B., Amann, A., Al-Kateb, H., Flynn, C., Filipiak, W., Khalid, T., Osborne, D., and Ratcliffe, N.M. (2014) J. Breath Res. 8: 14001-14030.
6. Mennella, J. A. and Beauchamp, G. K. (1993) Pediatr. Res. 34: 805–808.

# Continuous collection of volatiles produced by *Streptomyces* grown on oatmeal agar by headspace extraction and GC-MS

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## Abstract

Volatile organic compounds (VOCs) produced by bacteria are known to play a significant role in interactions among many organisms, but VOCs also impart aroma to many food products. *Streptomyces* bacteria include a large group of organisms that produce a wide range of secondary volatile metabolites with potential for clinical and industrial applications. VOC profiles of bacteria are typically analyzed in liquid media. Yet, many bacteria also grow well on solid media and may here produce different VOCs than in liquid media. However, it is challenging to capture and measure VOCs from bacteria grown on solid media, and such limitations could bias measured VOCs profiles from bacteria. In this study, a special approach was applied to examine VOC production by *Streptomyces* when grown on oatmeal agar in a large gas-washing flask with Tenax-TA traps attached 96 hours of incubation. The obtained VOC profiles of two *Streptomyces* species show presence of geosmin and 2-MIB along with a total of 110 compounds, including 51 terpenes.

## Introduction

Bacterial volatile organic compounds (VOCs) may play a significant role in inter- and intraspecies relations in ecosystems [1], but they also add flavor to many food products [2]. Bacteria belonging to the genus *Streptomyces* are known for their production of the off-flavors geosmin and 2-methylisoborneol (2-MIB), but they produce a wide range of other volatile metabolites with potential clinical and industrial applications [3]. Much attention has been given to geosmin and 2-MIB because these two VOCs are commonly occurring in many aquatic environments. Geosmin and 2-MIB are reported to spoil the quality of fish in aquaculture systems due accumulation in the flesh, and to add unattractive flavor to drinking water that is produced from surface water reservoirs.

The production of volatiles by bacteria is influenced by growth conditions and the metabolism of the organisms [4, 5]. Typically, in studies of VOCs production by microorganisms, bacteria have been grown in liquid media [4], probably because the determination of VOC production from cells grown on solid media is challenging. Liquid media may not provide optimum growth conditions for all microorganisms and may also underestimate the production of volatiles. Thus, in some bacterial species like *Streptomyces* and fungi, the metabolite production is stimulated by growth on surfaces, as compared to liquid media [5].

The objective of the present study was to establish a method for measuring volatiles produced by bacteria when cultured on solid media. Two species of *Streptomyces* were grown on oatmeal agar in gas-washing flasks attached with Tenax traps to facilitate continuous adsorption of volatiles upon equilibration in headspace. Geosmin, 2-MIB and other volatiles produced by *Streptomyces* were monitored for four days by collection of

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VOCs in the headspace onto the Tenax traps by flushing with $N_2$ every 24 hours. The volatiles were quantified by GC-MS analysis.

## Experimental

The two species included in the study are *Streptomyces* 2R (isolated from a Danish aquaculture pond) and *Streptomyces diastatochromogenes* (SD) (from DSMZ, Germany). Thirty ml of oatmeal agar was added to 500 ml sterile gas-washing flasks and inoculated with conidia (spores) to a number of $2\times10^7$ per flask of strain 2R or SD. The flasks were sealed with a purge-head attached to a Tenax-TA trap and incubated at 29°C. Volatiles in the headspace were collected by purging with $N_2$ at 200 ml/min for 15 min. Headspace was collected every 24 h until 96 h and analyzed by GC-MS settings as described in Podduturi et al. [2]. In brief, volatiles from the Tenax traps were analyzed by an automatic thermal desorption unit (ATD 400, PerkinElmer, Norwalk, USA) in combination with gas chromatograph mass spectrometer (GC-MS, 7890A GC-system interfaced with a 5975C VL MSD and a Triple-Axis detector from Agilent Technologies, Palo Alto, California). Separation of the volatiles was carried out on a DB-Wax capillary column (30 m length × 0.25 mm internal diameter and 0.5 µm film thickness) using $H_2$ as carrier gas with an initial flow rate of 1.0 mL/min. The column temperature program was held at 40°C for 10 min, then raised to 240°C at the rate of 8°C/min and finally at 240°C for 5 min. The mass spectrometer was operated in electron ionization mode at 70 eV. Mass-to-charge (m/z) ratios between 15 and 300 were scanned. Tenax-TA traps were changed every 24 h to avoid overloading of the traps. Parallel setups were used to collect cell biomass without interrupting the VOC production. Biomass of the bacteria was estimated using a DNA-based assay after staining of the cells with SYBR green I.

## Results and discussion

The obtained VOC profiles from cultivation of *Streptomyces* showed the presence of geosmin and 2-MIB along with several other terpenes.

### *Geosmin and 2-MIB production*

Production of geosmin and 2-MIB was detected by both strains throughout the 96 h growth period. *Streptomyces* 2R produced rather similar amounts of both compounds, while *Streptomyces* SD produced a higher amount of 2-MIB (800 ng per flask) as compared to geosmin (70 ng per flask) after 96 h (Figure 1). A large increase in the production of geosmin and 2-MIB occurred from 24 to 48 h for both strains. After 48 h, no major changes in production of the metabolites were found, but the biomass of both strains still increased after 48 h. In strain 2R, the geosmin and 2-MIB production rate increased 2- to 3-fold between 24 and 48 h and then declined to the initial 0 to 24 h rate. In strain SD, a high production of 2-MIB ($4 \times 10^{-18}$ g/cell) occurred from 24 to 48 h, while the geosmin production was lower. From 24 to 48 h, the geosmin and 2-MIB production rate increased almost 200-fold, followed by decline in the production rate, as also observed for 2R. *Streptomyces* 2R's geosmin production rate ($0.28 \times 10^{-18}$ g/cell) observed in this study is in similar range as previously reported, *S. albidoflavus* ($0.3 \times 10^{-18}$ g/cell) grown on solid media [6] and *S. citreus* ($0.28 \times 10^{-18}$ g/cell) grown in submerged culture during active growth stage [7].

Species of *Streptomyces* are known to produce several secondary metabolites during transition from compartmentalized mycelium to aerial mycelium, simultaneous with initiation of the sporulation [8]. Among metabolites produced in this phase are geosmin [9]. According to Yagüe et al. [8], *Streptomyces* grown on agar media begin forming

aerial mycelium after about 24 h. This agrees with the observed formation of a dense layer of white spores on the agar surface at bottom of the present culture flasks. The spore formation coincided with the increased geosmin and 2-MIB production by 48 h, relative to 24 h, and resulted in the highest metabolite production rate during 24-48 h.

**Table 1:** Terpenoid compounds found in headspace extracts of *Streptomyces* 2R and *Streptomyces diastatochromogenes*

| | *Calculated RI(DB-Wax)* | *Suggested Compound* | | *Calculated RI* | *Suggested Compound* |
|---|---|---|---|---|---|
| 1 | 1064 | 2-Methylenebornane[a] | 18 | 1688 | Humulene [b] |
| 2 | 1105 | β-Pinene[b] | 19 | 1705 | γ-Muurolene[a] |
| 3 | 1176 | β-Myrcene[b] | 20 | 1721 | γ-Gurjunene[a] |
| 4 | 1197 | D-Limonene[b] | 21 | 1728 | Germacrene D[a] |
| 5 | 1467 | α-Cubebene[a] | 22 | 1739 | Eremophilene[a] |
| 6 | 1503 | α-Copaene[a] | 23 | 1741 | α-Muurolene[a] |
| 7 | 1531 | β-Bourbonene[a] | 24 | 1752 | β-Dihydroagarofuran[a] |
| 8 | 1552 | β-Cubebene[a] | 25 | 1763 | β-Bisabolene[a] |
| 9 | 1584 | (-)-Aristolene[a] | 26 | 1773 | δ-Cadinene[a] |
| 10 | 1589 | β-Copaene[a] | 27 | 1778 | γ-Cadinene[a] |
| 11 | 1602 | β-Elemene[a] | 28 | 1800 | Cadina-1(2),4-diene[a] |
| 12 | 1614 | 2-Methylisoborneol [b] | 29 | 1853 | Calamenene[a] |
| 13 | 1637 | Allo-Aromadendrene[a] | 30 | 1854 | Geosmin [b] |
| 14 | 1642 | α-Himachalene[a] | 31 | 2084 | Caryophyllenyl-alcohol[a] |
| 15 | 1649 | cis-β-Guaiene[a] | 32 | 2094 | Cubenol[a] |
| 16 | 1658 | α-Guaiene[a] | 33 | 2177 | γ-Eudesmol[a] |
| 17 | 1683 | Viridiflorene[a] | | | |

[a] Compounds identified by NIST MS library and RI from literature
[b] Compounds identified by NIST MS library and RI of pure compounds

*Total VOC profile*

A total of 110 different VOCs were found in headspace of strain 2R, while 108 different compounds were identified for strain SD. The VOC composition of both strains was dominated by 51 terpenoids (mono and sesquiterpenes and their derivatives) and 23 hydrocarbon compounds. The list of terpenoids identified with standards and tentatively identified with NIST library similarity and RI values from literature are shown in Table 1. The identity of 12 of the detected 51 terpenoids was unknown. The total number compounds found in this study are considerably higher than the *Streptomyces* grown in liquid media [7].

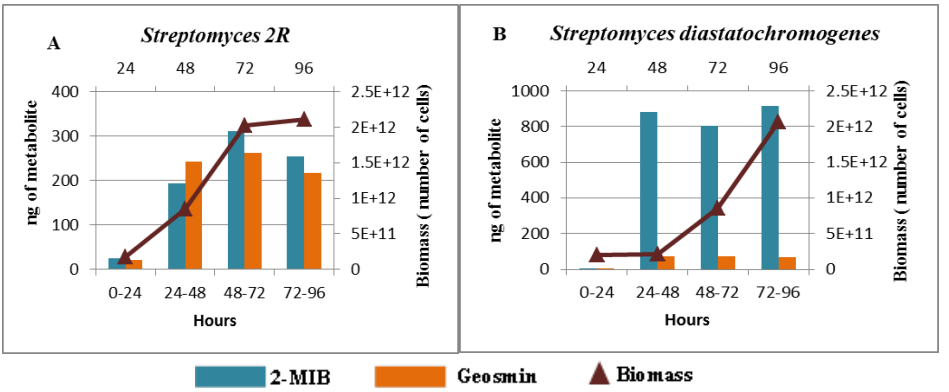


**Figure 1**: Production of geosmin and 2-MIB as well as biomass by *Streptomyces 2R* (A) and *Streptomyces diastatchromogenes* (B) over 96 hours

The headspace composition of various volatiles produced by the *Streptomyces* isolates in this study (dominance of mono and sesquiterpenes) resemblances the volatile profiles by other geosmin-producing *Streptomyces* [3, 10]. Most of the compounds listed in Table 1, including 1, 2, 4, 5, 6, 8, 11, 12, 18, 21, 23, 24, 25, 30 and 32, have previously reported to be produced by various *Streptomyces* strains [3, 10].

## Conclusion

The experimental setup demonstrates that volatile metabolites produced by *Streptomyces*, such as geosmin and 2-MIB, can be detected and identified by cells growing on solid media. The current study also shows that *Streptomyces* grown on solid media produces higher number of metabolites compared to submerged culture with almost similar rate of production. The present approach can be applied for detection and quantification of VOCs produced by bacteria, but the experimental setup could also be useful in studying dynamics and kinetics of volatiles and metabolites produced by bacteria.

## References

1. Wheatley R. E. (2002) A. Van. Leeuw J. Microb., 81: 357-364.
2. Podduturi R., Petersen M. A., Mahmud S., Rahman M. M. and Jørgensen N. O. G. (2017) J. Agr. Food Chem., 65: 3730-3736.
3. Rabe P., Citron C. A. and Dickschat, J. S. (2013). ChemBioChem, 14(17): 2345–2354.
4. Tai E., Perry J. D., Stanforth S. P. and Dean, J. R. (2014) J. Chromatogr. Sci., 52: 363–373.
5. Van der Molen K. M. Raja, H. A., El-Elimat T. and Oberlies, N. H. (2013) AMB Express, 3: 1–7.
6. Schöller C. E. G., Gürtler H., Pedersen R., Molin S. and Wilkins K. (2002) J. Agr. Food Chem., 50(9): 2615–2621.
7. Pollak F. C. and Berger R. G. (1996). Appl. Env. Microbiol., 62(4): 1295–1299.
8. Yagüe P., Rodríguez-García A., López-García M. T., Martín J. F., Rioseras B., Sánchez J. and Manteca, A. (2013) PLoS ONE, 8(3): e60665.
9. Dionigi C. P., Millie D. F., Spanier A. M. and Johnsen P. B. (1992) J. Agr. Food Chem., 40(1): 122–125.
10. Yamada Y., Kuzuyama T., Komatsu M., Shin-Ya K., Omura S., Cane D. E. and Ikeda, H. (2015) P. Natl. Acad. Sci. USA., 112(3): 857–62.

# InnOscent chromatographic system: An innovative device to revisit aroma analysis and recombination perspectives

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## Abstract

Aroma results of a complex mixture of volatile compounds in which the contribution of each volatile depends on its concentration, detection threshold, interaction with the matrix and emergence of complex aroma-aroma interactions. [1] Gas chromatography (GC) coupled to olfactometry is useful to identify and estimate the individual potential of odour-active compounds, but it is irrelevant to predict the effective contribution of odorants to food aromas. Therefore, models reconstituted with chemicals are used to evaluate these contributions in a mixture context. [2] This approach requires time and above all, needs every compound to be identified, quantified, and commercially available. The InnOscent chromatographic device was designed to overcome these constraints and was configured to realize both conventional GC analysis coupled with multiple detectors, and innovative fraction collection experiments including recombination possibilities. This study aims at presenting this system through a wine aroma analysis.

An olfactometric analysis was first performed on a wine made of Cabernet Franc grape variety, with an 8 judges-panel and the InnOscent system configured to operate as a conventional GC coupled to a mass spectrometer (MS) and a dual olfactometric port. On the basis of the olfactometric results, recombined fractions of selected compounds were directly recovered from the system and were submitted to the panel in order to evaluate the contribution of these odour-active compounds to the wine aroma. 32 odorants were detected by at least 3 out of 8 judges in the wine aroma. Evaluation of the recombined extracts demonstrated that the mixture of all these odorants mimics the original wine aroma. Moreover, the mixture of the 14 most intense odorants was demonstrated to be representative of this aroma. The findings of this study illustrate the relevance of the system to realize a comprehensive aroma exploration using a single disposal. While freeing from chemicals, InnOscent system makes it possible to evaluate the contribution of any compound or group of compounds to an overall aroma, and thus go further in aroma analysis.

## Introduction

Food aroma is a major criterion in consumers' appreciation prompting food producers and processors to regard it as a perennial issue. However, comprehension of aroma is still an ongoing scientific challenge since aroma results from a complex mixture of volatile compounds. All do not contribute equally to the aroma mainly due to their respective concentrations, detection thresholds, interactions with the matrix and emergence of complex aroma-aroma interactions. Gas chromatography (GC) coupled to olfactometry is a both analytical and sensory technique used to identify odour-active compounds and estimate their individual odorant potential. However, there is a gap between this individual characterization of compounds and their effective contribution to a food aroma. Thus, investigation of many product aromas turns to models that are reconstituted with chemicals, generally based on odour activity values. While functioning, this approach is time-consuming and requires every compound to be

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identified, quantified, and commercially synthesized. In this context, the InnOscent chromatographic system was designed to overcome these constraints and ease aroma comprehension. This device was configured to perform both (1) conventional analyses by GC coupled with a mass spectrometer, a single or dual olfactometric port, or a flame ionization detector, and (2) innovative fraction collection experiments including recombination possibilities. As a demonstration of the capabilities of this system, the aroma analysis of a wine made of Cabernet Franc grape variety was performed and the contribution of most potent odorants was investigated.

## Experimental

### *Material*

Wine used for the study was a red wine, 13% alcohol, from Bourgueil appellation, elaborated in 2010 from a Cabernet Franc vineyard. Chemical standards and n-alkanes were purchased from Sigma Aldrich (St Quentin Fallavier, France) with purity > 97%.

### *Wine aroma extraction*

Volatiles from a 5mL wine sample were extracted by solid phase micro-extraction with a Car/PDMS fibre (10 mm length, 85µm film thickness; Supelco, Bellefonte, PA, USA) placed in the headspace of the vial for 10 minutes at 34°C after 1hour of incubation. Compounds were directly desorbed from the fibre in the injection port of the GC (T=260 °C).

### *Chromatographic device and conditions*

Analyses were carried out with the InnOscent laboratory-designed system (Figure1) using an Agilent 7890A gas chromatograph combined with a 5975 mass spectrometer (MS, electron impact mode 70 eV, scan m/z 33-300, 2.7 scan.$s^{-1}$, Agilent Technologies, Wilmington, DE, USA). The column was a DB-Wax (Agilent, 30m length × 0.25 mm internal diameter × 0.5 µm film thickness), hydrogen was used as the carrier gas and oven temperature was programmed as follows: 50°C (0 min) to 80 °C at 5 °C·$min^{-1}$, then 80 to 200 °C at 10 °C·$min^{-1}$, and 200°C to 240 °C (4 min) at 20°C·$min^{-1}$.

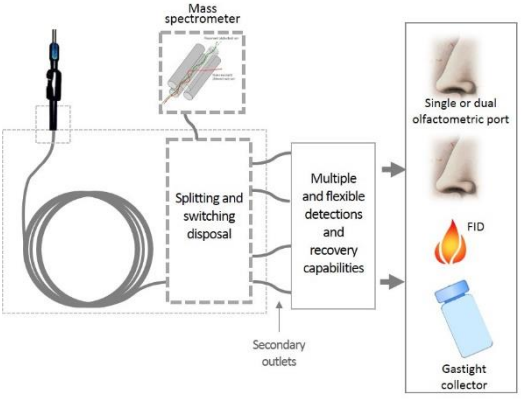


**Figure 1:** Schematic configuration of the InnOscent-GC device (patent pending) [3]

The end of the column was connected to a splitting and switching disposal allowing the eluate to be split and directed for one part to the MS and for the rest of the eluate towards secondary outlets. Throughout the run, the flow can be selectively transferred to one or another secondary outlet, *via* the events control module of Chemstation software (Agilent). Depending on the purpose of the experiments, secondary outlets can be

connected to an olfactometric port (transfer line T=200°C, Gerstel ODP 3, Mülheim an der Ruhr, Germany), a flame ionization detector (FID, T=260°C, Agilent) or a collecting device.

*Olfactometric analysis*

The InnOscent system was first scheduled to get a conventional GC-MS coupled to a dual olfactometric port, connecting two olfactometric ports to secondary outlets. The eluate was analysed by an 8 trained judges-panel, throughout successive runs. Judges' perceptions (time, intensity and description) were recorded *via* the WheelOscent olfactometric software presenting an aroma wheel interface designed for wine analysis. [4] Results are displayed as an aromagram, directly obtained from the software, presenting the number of detections *vs* retention time. Identification of odorants was performed by comparing their linear retention index and mass spectra to those of databases (Wiley, Nist and internal databases) and by injection of standard compounds.

*Recombination experiments and extracts evaluation*

A collector was connected to a secondary outlet of the InnOscent GC-MS system and an FID was also connected to the system allowing to control the recovered extract. The total extracts were first recovered in the collector throughout successive runs. Then the recombined extracts were recovered. Switching events were programmed so that only selected compounds were directed to the collector and the FID, whereas other fractions of the eluate were eliminated through other secondary outlets. Extracts containing odorants perceived by at least, 3, 4, 6 and 8 judges on the basis of the olfactometric results were this way successively collected in different collectors. Timing of the switching events was set according to the GC-MS-O results. Collectors containing the recombined and total extracts were coded with a three-digit random number and submitted to the panel in a randomized order. The judges were asked to smell the content of the collectors and score the similarity of the odour with that of a total extract named reference. Anova was performed on similarity scores obtained for each extract with a 95% confidence level.

**Results and discussion**

The aromagram and chromatogram of the wine aroma obtained from the InnOscent device present different patterns, underlining that compounds with the most abundant peak areas are not necessarily the most frequently perceived, and highlighting that instrumental and human detections are complementary. Thirty-three compounds were perceived by at least 3 out of the 8 judges (Figure) and 25 compounds were identified. Among them a majority of ethyl esters and acetates are found, as well as alcohols, acids, carbonyl and sulfur compounds, phenols and pyrazines. These observations are consistent with extensive literature dealing with wine aroma analysis. [5]

On the basis of these olfactometric results, recombined extract were directly recovered from the InnOscent device and were submitted to the panel to evaluate their contribution to the aroma. The extract that contained the 33 compounds detected by at least 3 out 8 judges, was perceived as representative of the wine aroma with a similarity score of 7.7 out of 10 (Table 1). This is particularly true considering the similarity score (8.2 out of 10) given to the total extract compared to the identical reference, explained by the natural reluctance of the judges to use the ends of the scale. This result illustrates the capability of the olfactometry analysis to point out the compounds involved in the wine aroma.

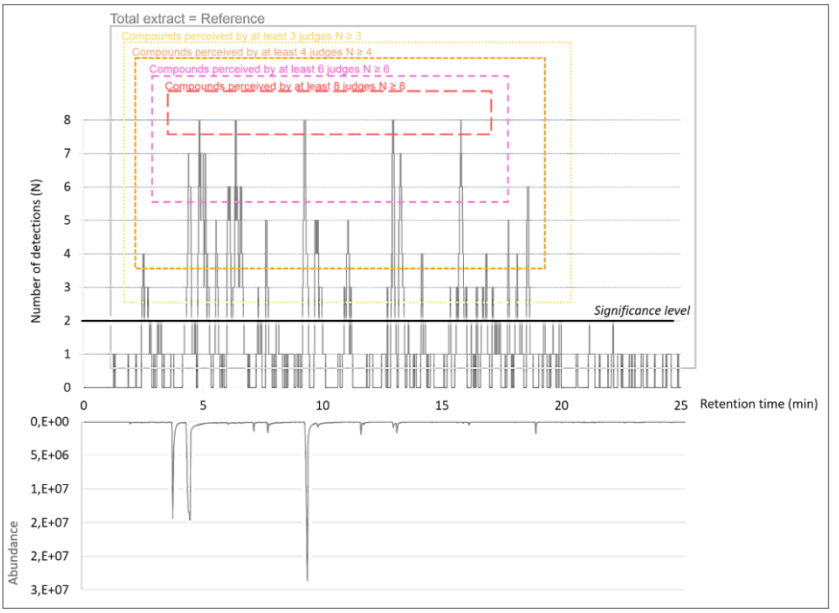


**Figure 2:** Aromagram and MS chromatogram of the wine aroma.

**Table 1:** Similarity scores obtained from the 8 judges-panel for the different extracts recovered directly from the InnOscent GC device compared to a total extract.

| | *Recombined extract with x compounds perceived by at least :* | | | | *Reference* |
|---|---|---|---|---|---|
| | *8 judges* *x= 5* | *6 judges* *x= 13* | *4 judges* *x= 24* | *3 judges* *x= 33* | *= total extract* |
| *Similarity score* | 2.2[b] | 7.2[a] | 7.4[a] | 7.7[a] | 8.2[a] |

Furthermore, if the mixture of the 5 more perceived compounds did not allow to reconstitute the wine aroma, the mixture of the 13 more frequently perceived compounds demonstrated to be sufficient to produce an extract perceived as representative for the wine aroma. This study illustrates the possibilities given by the InnOscent device combining conventional analysis and innovative omission/recombination capabilities. The system provides solutions to directly evaluate the aroma of mixture of compounds or estimate the contribution of any target compound to a global aroma, overcoming constraints of current approaches. This approach will deliver valuable information to understand complex aroma-aroma interactions and to go further into aroma comprehension.

## References

1. Guichard, E. (2002) Food Rev. Int. 18: 49-70.
2. Ferreira V. Ortin N., Escuerdo A., Ricardo L., and Cacho J. (2002) J. Agric. Food Chem. 50: 4048–4054.
3. Villière A., Lethuaut L., Prost C. and Ecole Nationale Vétérinaire, (2013) WO 2013140057 A1 also published as EP 2828652 A1 and EP 2828652 B1.
4. Villière A., Le Roy S., Fillonneau C., Guillet F., Falquerho H., Boussely S. and Prost C. (2015) Flavour 4: 24.
5. Fischer U. (2007) In Flavours and Fragrances (Berger, P., ed.) Springer B.H., pp.241-267.

# Comparison of the volatile sulfur compounds in onion oil to those in fresh onion juice

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## Abstract

The characteristic aroma of fresh onion (*Allium cepa* L.) is formed enzymatically. [1] When the onions are chopped, the cells release enzymes (alliinases), which transform isoalliin into the highly reactive 1-propenyl sulfenic acid. [2,3] The latter compound can condense with itself to form thiosulfinate (pathway I). Thiosulfinates can react with free thiols to form relatively stable compounds such as disulfides. Alternatively, the enzyme lachrymatory factor synthase (LFS) can convert 1-propenyl sulfenic acid into *syn*-propanethial-S-oxide, the lachrymatory compound in onions (pathway II). [4] It is known that, in water, *syn*-propanethial-S-oxide can decompose to form propanal and hydrogen sulfide. These decomposition products can further react with other volatiles present in onion juice, to form new molecules that contribute to the aroma of fresh onion. [5] Propanal and hydrogen sulfide can readily generate a number of high impact flavor compounds. These molecules possess a powerful fresh onion character, but their analytical characterization is often challenging. This is due to their reactivity towards other molecules present in onion juice, their thermal lability in water and their low concentration in the matrix. A pathway was proposed for the formation of the major sulfur compounds in onion juice.

## Objective

The goal of this work was to compare the different types of volatile sulfur compounds in fresh onion juice to those in onion oil. Onion oil can be prepared simply by steam distillation of ground onions in water. The obtained oil contains the volatiles sulfur compounds and can be analyzed directly by GC-MS. The identification of the volatile sulfur compounds in onion juice, however, required a special approach. In *Allium* analysis by GC, cool injection techniques are preferred, because hot injection may result in artifacts. [1,6,7,8] On-column injections have limited sample load ability and may bring non-volatile plant material in the extract onto the column. In this research, it was attempted to overcome these limitations by applying a thiol enrichment step, followed by analysis under mild conditions using an Agilent multimode inlet.

## Approach and results

*Onion oil.* Yellow onions were peeled, chopped and added to water. The mixture was steam distilled under atmospheric pressure. The oil layer was separated from the distillate, dried over magnesium sulfate, filtered and injected directly on an Agilent 5977 single-quadrupole GC/MS system in electron ionization mode. Figure 1 depicts the gas chromatogram of the onion oil. Mass spectral identification was achieved using in-house and commercial libraries. Structure assignment and relative percentages of the seven major signals in the matrix are presented in Table 1.

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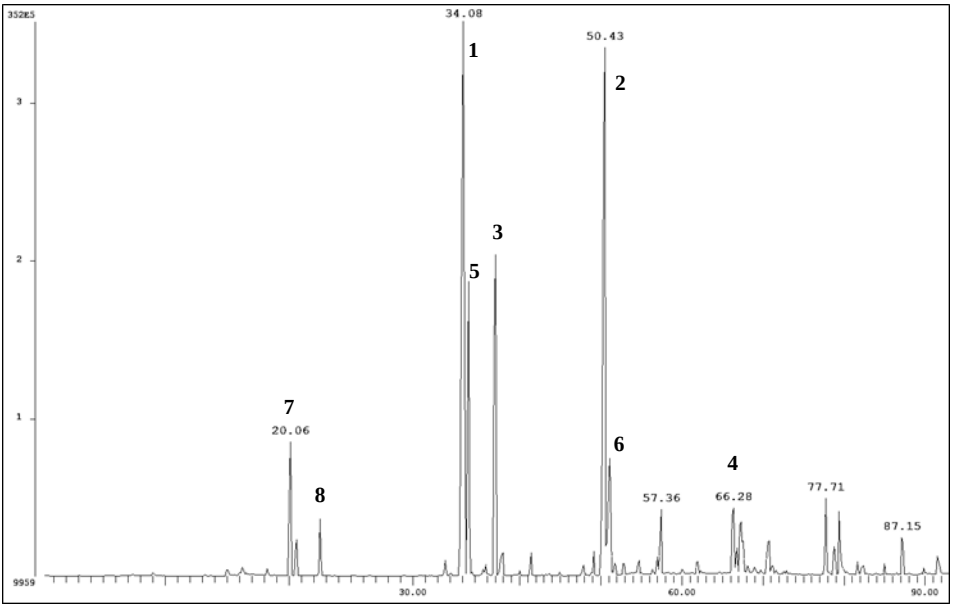


**Figure 1:** Gas chromatogram of onion oil.

**Table 1:** Predominant Sulfur Compounds in Onion Oil

| # | *Compound* | *Relative percentage* |
|---|---|---|
| 1 | dipropyl disulfane | 21.8 % |
| 2 | dipropyl trisulfane | 17.1 % |
| 3 | methyl propyl trisulfane | 10.1 % |
| 4 | dipropyl tetrasulfane | 5.8 % |
| 5 | *trans*-1-propenyl propyl disulfane | 5.8 % |
| 6 | 1-propenyl propyl trisulfane | 4.8 % |
| 7 | *cis*-1-propenyl methyl disulfane | 3.0 % |
| 8 | dimethyl trisulfane | 1.2 % |

Numbers match the signals in the chromatogram.

*Onion juice.* Yellow onions were peeled, cut and processed with a commercial juicer. The juice was collected and allowed to stand for 30 minutes prior to extraction. During this period, enzymatic reactions and subsequent chemical reactions could take place, by which the compounds of interest were formed. The juice was extracted with methylene chloride, followed by partial evaporation of the solvent. The residue was passed through a mercuric agarose gel which was eluted with methylene chloride / dithiothreitol. [9,10] Prior to the actual analysis, a multimode inlet, operating in the vent mode, was used to evaporate the solvent to further concentrate the sample in the GC inlet liner at low temperature. Only the (semi-)volatile compounds were swept onto the column under mild thermal conditions (150 °C). Analysis was done on an Agilent 5977 single-quadrupole GC/MS system in electron ionization mode. Figure 2 depicts the gas chromatogram of the thiol enriched onion juice extract. Mass spectral identification was achieved using in-house and commercial libraries, as well as synthesized or purchased reference standards. Structure assignment and relative percentages of the seven major signals in the matrix are presented in Table 2.

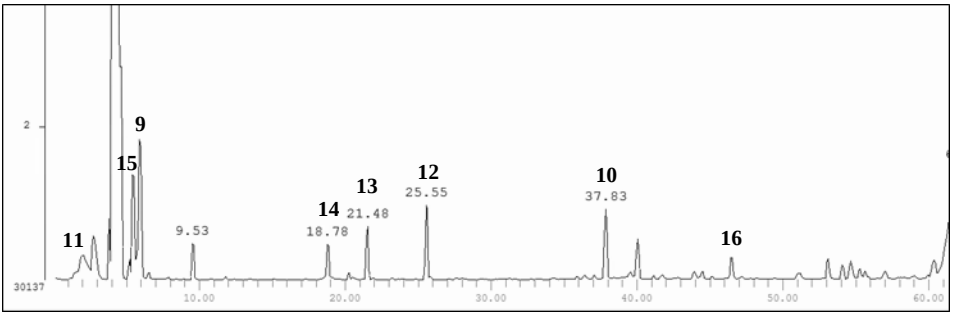


**Figure 2:** Gas chromatogram of the thiol enriched onion juice extract

**Table 2:** Predominant Sulfur Compounds in Raw Onion Juice [11]

| # | *Compound* | *Relative percentage* |
|---|---|---|
| 9 | Propanethiol | 21.5 % |
| 10 | 1-propylsulfanyl-1-propanethiol | 15.7 % |
| 11 | Methanethiol | 15.3 % |
| 12 | 1-methylsulfanyl-1-propanethiol | 12.0 % |
| 13 | 1,2-propanedithiol | 10.1 % |
| 14 | 1,1-propanedithiol | 7.1 % |
| 15 | Allylthiol | 3.6 % |
| 16 | 3-sulfanyl-2-methylpentan-1-ol | 2.2 % |

Numbers match the signals in the chromatogram.

## Discussion and conclusion

The sulfur compounds that are found in onion juice are rather different from the sulfur compounds that are found in onion oil. Onion oil has a 'boiled onion' aroma and its composition analysis has well been described in literature. It consists mainly of disulfanes, trisulfanes and tetrasulfanes, which are relatively stable end products. These components are formed by reactions of thiosulfinates during boiling in water. Reaction of a thiosulfinate with a free thiol will result in the formation of a disulfane. If a thiosulfinate reacts with hydrogen sulfide, an alkyl hydropersulfide will be formed as an instable intermediate. Hydropersulfides can again react with a thiosulfinate to form trisulfane. From the sulfur compounds found in the oil, it can be concluded that by heating in water, 1-propenyl sulfenic acid follows reaction pathway I in Figure 3. Raw onion juice has a 'fresh onion' aroma and contains rather different classes of sulfur compounds; disulfanes and trisulfanes are not the major components in the thiol enriched extract of the juice. The major sulfur compounds found in onion juice can be classified in groups: free thiols, thiohemiacetals and aldol condensation products. Figure 3 proposes a potential pathway for their formation. From the sulfur compounds found by analysis of the juice, it can be concluded that in the absence of a boiling step in water, 1-propenyl sulfenic acid follows reaction pathway II in Figure 3.

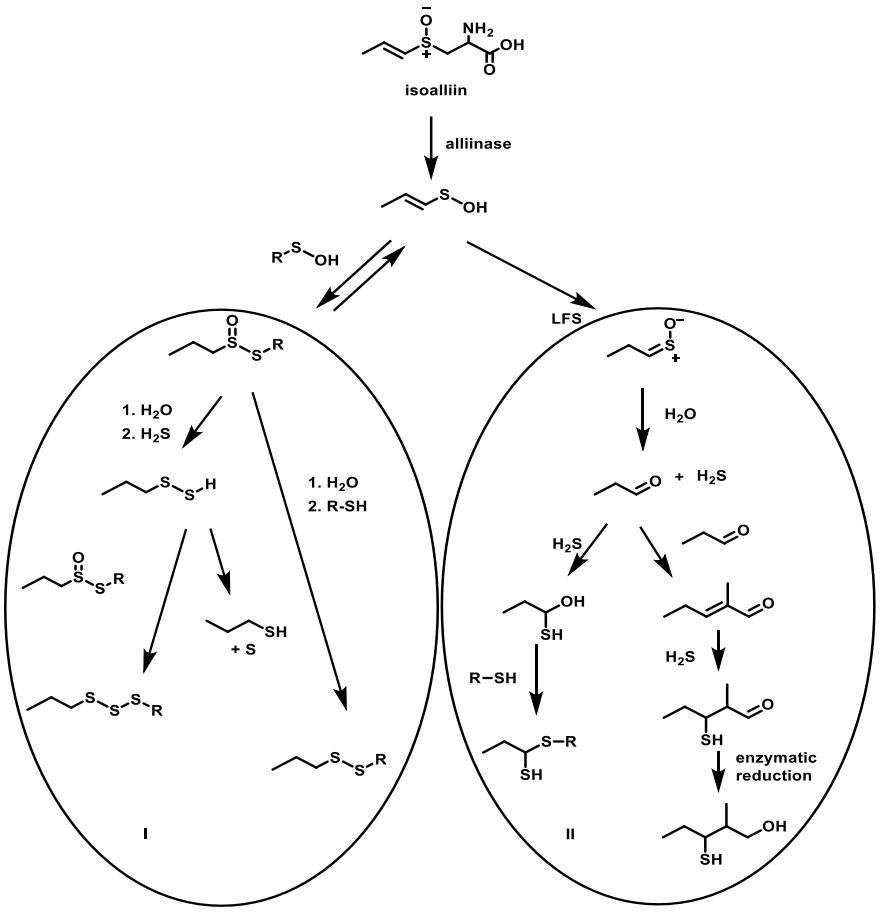


**Figure 3:** Proposed pathways of 1-propenyl sulfenic acid for the formation of the thiol compounds in onion oil (I) and in fresh onion juice (II)

## References

1. Block E, and Calvey EM, (1994) Sulfur Comp. in Foods, Washington DC, Am. Chem. Soc. 63-79.
2. Feilberg A, Lokke MM, Edelenbos M, and Larsen E, Sensors, (2012) 12, 16060-16076.
3. Poll L, Nielsen G, and Larsen L, J. Agric. Food Chem. (2003), 51, 1970-1976.
4. Storsberg J, Schulz H, Keusgen M, Tannous F, Dehmer K, Keller E-R, (2004) J. Agric. Food Chem. 2004, 52, 5499-5505.
5. Boelens M, et al., J. (1974) Agric. Food Chem. 22, 1071-1076.
6. Block E, Putman D, and Zhao S-H, (1992) J. Agric. Food. Chem., 40, 2431-2438.
7. Block E, Naganathan S, Putnam D, and Zhao S-H, (1993) Pure & Appl. Chem., 65, 625-632.
8. Iranshahi M, (2012) J. Ess. Oil. Res., 24, 393-434.
9. Pavez C, Agosin E, and Steinhaus M, (2016) J. Food Chem. 64, 3417-21.
10. Steinhaus M, Munafo JP, Didzbalis J, Schnell RJ, and Schieberle P, (2014) J. Agric. Food Chem. 2014, 62, 4544-4551.
11. Wermes, C. et al, (2017) Journal of Food Science , 82(11), 2606-2613.

# The odour activity value of aroma-active esters – An appropriate means to assess the aroma quality of apple juices

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## Abstract

Aroma quality of apple juice from concentrate depends on an appropriate rearomatisation. Nevertheless, until now, available concepts to analytically evaluate the aroma quality of apple juices are non-satisfying. Most concepts focused only on the concentration of esters to rate the aroma quality. However, without consideration of the different odour thresholds of the esters no successful results were obtained. To address this challenge, odour-active compounds were characterized in apple juices by means of the Sensomics concept including gas chromatography-olfactometry and GC×GC-MS(TOF) and a set of 16 esters was selected for quantitation. The concentrations and OAVs of these esters were determined in 23 defined apple juices using a fast, multicomponent stable isotope dilution assay (SIDA), based on headspace-solid phase micro extraction (HS-SPME) in combination with GC×GC-MS(TOF). Thus, for each ester limits in terms of OAV ranges were determined representing a correct re-aromatisation and a good aroma quality of apple juice. The new method enabled a high and fast throughput of samples due to the absence of any sample work-up.

## Introduction

Beside orange, multivitamin, and grape juices, apple juice is one of the most favoured juices in Germany [1]. About 700 million litres of apple juice are consumed per year in Germany. However, its consumption dropped from more than 12 litres in 2005 to 8 litres per person and year in 2010. Beside juice not from concentrate (NFC), mainly juice from concentrate (FC) is produced. For its manufacturing, the fresh juice is concentrated, while recovering the valuable apple aroma. After storage and/or transportation, the reconstitution of the juice concentrate, the apple aroma and water takes place, followed by a pasteurisation step. However, thermal stress and an inappropriate re-aromatisation may affect the aroma quality. This has also been criticised by consumer protection organisations [2]. However, until now, there is no appropriate concept available to evaluate the aroma quality respectively an adequate re-aromatisation of apple juices by means of analytical parameters.

Esters are known as important odorants in apple juice. Former proposed concepts (sum of esters, aroma index) included the concentration of esters to rate the aroma quality of apple juices, but these concepts did not consider the (big) difference in odour thresholds of individual odorants. To evaluate the aroma of apple juice not only the amount of an aroma compound is important, the potential (odour threshold) of an odorant has to be regarded, as well. For example, while hexyl acetate has an odour threshold of 15 µg/L (water), ethyl 2-methylbutanoate has a threshold of 0.013 µg/L. This is the reason why, when considering the sensory potential, of the 72 esters already found in apple juices,

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only a few (~7) are sensory relevant [3-6], because not all esters reach or exceed their individual odour threshold.

The aim of the study was to develop a reliable and fast, multicomponent quantitation method, taking the sensory potential of single esters into consideration for the evaluation of the aroma quality of apple juices.

## Experimental

### *Materials*

23 apple juices (freshly pressed, NFC, and FC) and in cased of FC, correctly re-aromatized, were obtained from defined processes directly provided by the manufacturers. The apples were from different varieties (not known) from South Tyrol, Germany, and Poland and harvested in the years 2010 to 2013. In addition, 17 commercially available apple juices of different origin and appearance were purchased form several supermarkets: 10 from Germany, 2 from United Kingdom, and 1 each from Poland, Hungary, France, Belgium, and Netherlands. Among them 4 cloudy NFC juices, 1 clear NFC juice, 2 cloudy FC juices, and 10 clear FC juices.

### *Hedonic, ranking order*

Hedonic evaluation was performed using an incomplete balanced block plan with 18 tests and 4 juices per test. 40 panellists were asked to rank the juices on a scale from 1 (most wanted) to 4 (most unwanted).

### *Screening for esters*

For the screening of esters volatiles were extracted with dichloromethane, distilled in high vacuum (SAFE) at 40 °C, concentrated, and analysed by GC-olfactometry (GC/O) on a GC 8000 (Fisons Instruments, Mainz, Germany). After the separation on an FFAP column (30 m×0.25 mm, 0.25 µm film thickness, J&W, Köln) using the following temperatures: 40 °C (2 min), 6 °C/min, 190 °C (0 min), 12 °C/min, 230 °C (5 min) the effluent was split 1:1 via an Y-splitter and transferred to an FID and a sniffing port.

### *Quantitation of esters*

For the quantitation of the esters, stable isotope dilution assays (SIDA) were applied. Measurements were done by HS-SPME/GC×GC-MS(TOF) analysis on a Pegasus 4D instrument (Leco, St. Joseph, MI) consisting of a 7890A GC (Agilent), a dual-stage quad-jet thermal modulator and a secondary oven coupled to the mass spectrometer. Apple juice samples (0.5 mL) and stable isotope internal standards were mixed in a SPME vial (20 mL), equilibrated for 30 min and afterwards, the enrichment of the esters was done by exposing a Stable Flex fibre (65 µm, PDMS/DVB, Supelco, Sigma-Aldrich, Taufkirchen) for 30 min at 40 °C in the headspace of the sample. Desorption was performed in the GC Multimode inlet system at 250 °C. In the first dimension an FFAP column (30 m×0.25 mm, 0.25 µm film thickness, J&W, Köln) was installed running the following temperatures: 40 °C (3 min), 6 °C/min, 230 °C (7 min). In the second dimension a VF-5 column (1.5 m×0.15 mm, 0.30 µm film thickness, Agilent, Böblingen) was installed running the following temperature program: 80 °C (3 min), 6 °C/min, 250 °C (10 min).

## Results and discussion

*Screening for esters and additive effects*

First, aroma-active esters in the solvent extract of an NFC apple juice were screened by GC/O to detect esters in trace amounts but with a high aroma potential. In addition, less odour-active esters were identified by GC×GC/MS(TOF). In total 29 esters were found whereof 17, with low odour thresholds and/or present in huge amounts, were selected for quantitation. These compounds were quantitated in the NFC apple juice by HS-SPME/GC×GC/MS(TOF) using stable isotope dilution assays. Finally, odour activity values (OAV, ratio of the concentration of an odorant to its odour threshold) were calculated (Table 1).

**Table 1:** Odour activity values of 17 esters in NFC apple juice

| No. | *Compound/variety* | *Odour activity value (OAV)** |
|---|---|---|
| 1 | ethyl 2-methylbutanoate | 1700 |
| 2 | ethyl butanoate | 100 |
| 3 | 2-ethylbutyl acetate | 64 |
| 4 | methyl 2-methylbutanoate | 27 |
| 5 | ethyl 2-methylpropanoate | 20 |
| 6 | hexyl acetate | 11 |
| 7 | butyl acetate | 9.6 |
| 8 | propyl 2-methylbutanoate | 4.4 |
| 9 | ethyl hexanoate | 3.7 |
| 10 | ethyl propanoate | 1.9 |
| 11 | pentyl acetate | < 1 (0.77) |
| 12 | 2-methylpropyl acetate | < 1 (0.31) |
| 13 | butyl 2-methylbutanoate | < 1 (0.17) |
| 14 | butyl butanoate | < 1 (0.09) |
| 15 | hexyl 2-methylbutanoate | < 1 (0.07) |
| 16 | hexyl butanoate | < 1 (0.03) |
| 17 | butyl propanoate | < 1 (0.03) |

** Ratio of the concentration of an odorant to its odour threshold.*

The calculation of OAVs served as basis for the investigation of additive effects of esters in sensory tests. For this purpose, a mixture of all esters (no. 1-17) was compared in a triangle test to a mixture of esters containing only esters with an OAV ≥ 1 (no. 1-10). The sensory evaluation clearly showed that the esters with OAVs < 1 did not contribute to the overall aroma. Due to this fact, further investigations were done with ethyl 2-methylbutanoate, ethyl butanoate, 2-methylbutyl acetate, ethyl 2-methylpropanoate, methyl 2-methylbutanoate, butyl acetate, propyl 2-methylbutanoate, hexyl acetate, ethyl propanoate, and ethyl hexanoate (ester no. 1 to 10).

*Determination of OAV ranges and application to commercial juices*

Esters 1 to 10 were quantitated in all 23 defined juice samples (freshly pressed, NFC, and FC) and OAV were calculated. Thus, OAV ranges for a well-balanced apple juice aroma were established (Table 2).

These OAV ranges were applied to 17 commercially available apples juices. A sensory trained panel ranked these juices concerning their hedonic preference. In addition, esters 1 to 10 were quantitated and OAVs were calculated. The OAVs of the (significantly) most appreciated (best) and the most unpopular (worst) juice were applied

to the established OAV ranges and highlighted a clear trend: while all OAVs were within these OAV ranges for the best, only the OAVs of 2-methylbutyl acetate and hexyl acetate were within its range for the most unpopular juice (Table 2).

**Table 2:** OAV Ranges of 10 Selected Esters and OAVs of Juice 6 and 13

| Compound/variety | *OAV range* * | *OAV best juice* | *OAV worst juice* |
|---|---|---|---|
| ethyl 2-methylbutanoate | 900 – 12000 | **12000** | 280 |
| ethyl butanoate | 55 – 390 | **270** | 17 |
| 2-methylbutyl acetate | 11 – 110 | **31** | **11** |
| ethyl 2-methylpropanoate | 8 – 110 | **95** | 3 |
| methyl 2-methylbutanoate | 12 – 80 | **75** | 3 |
| butyl acetate | 5 – 45 | **10** | 4 |
| propyl 2-methylbutanoate | 4 – 37 | **23** | < 1 |
| hexyl acetate | 1 – 26 | **4** | **2** |
| ethyl propanoate | 1 – 12 | **11** | < 1 |
| ethyl hexanoate | 1 – 11 | **4** | < 1 |

** Values resulted from the investigation of 23 sensory proper juices (freshly pressed, NFC, FC) from defined processes directly provided by different apple juice manufacturers.*

The results of these investigations showed that OAV ranges of only 10 aroma-active esters may serve as markers for the fruity aroma quality of apple juices. Using HS-SPME/GC×GC/MS a suitable method was established for the simultaneous quantitation of all esters of interest. By considering the aroma perception of a sensory panel (consumer) and the analysis of the aroma quality of apple juices, expressed in OAV ranges, the production and also a correct re-aromatisation of apple juices can be lifted to a reliable basis.

## References

1. Unknown (2014) VdF (Verband der deutschen Fruchtsaft-Industrie e.V.), Bonn, brochure.
2. Unknown (2009) Stiftung Warentest, Test 9:20-26. (www.test.de/Apfelsaft-Drei-sind-durchgefallen-1801978-0/, last access 01.2018)
3. Flath R.A., Black D.R., Guadagni D.G., McFadden D.G., Schultz T.H. (1967) J Argic Food Chem 1:29-35.
4. Poll (1988) LWT Food Sci Technol 21:87-91.
5. Young H., Gilbert J.M., Murray S.H., Ball R.D. (1996) J Agric Food Chem 71:329-336.
6. Bogen J. (2010) Ph.D. Thesis, Verlag Deutsche Forschungsanstalt für Lebensmittelchemie Freising

# WheelOscent: Presentation of an innovative GC-Olfactometry-dedicated software using intuitive aroma wheel interface

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## Abstract

GC-Olfactometry is a valuable methodology commonly used to investigate odor active compounds in complex food aroma profiles. Considering the number of studies using this technique, little is done to improve olfactometric data acquisition although it is essential for quality results. Efforts were mainly done to automate recording of moments of perception but intensity and description of perception are still often communicated orally, which disrupts the judge's breathing rhythm during analysis. Solutions that integrate all recording parameters result in a multiple steps acquisition procedure, scarcely compatible with the transience of the perceptions evaluated during olfactometry. The objective of this work was to develop a new olfactometric software that include olfactometric data acquisition and processing capabilities. The WheelOscent software was designed to improve the users' task and overcome constraints and bias of existing systems. More specifically the software, coded with Java technologies, implements innovative components:

- a data acquisition interface based on intuitive aroma wheels, adaptable to each product studied, which enables judges to characterize all parameters related to odors perceived in a simple and intuitive move,
- a data store, for collected data,
- a data analysis interface, which provides easy and direct analysis of data displayed into interactive and graphical visualization.

Providing good usability, this software enables a precise characterization that allows to point out special features of products even with close and complicated aroma profiles. This disposal is now used for wine analysis, where judges take advantage of the wheel aroma presentation, currently used for wine sensory characterization.

## Introduction

GC-Olfactometry is a valuable methodology commonly used to investigate odor active compounds in the aroma of food products. Considering the number of studies using this technique, small number of papers deals with the improvement of olfactometric data acquisition although it is essential for the quality of the results. Despite many drawbacks, some olfactometric studies are still conducted with an oral transmission of judges' sensory impressions. This practice leads to perturbation of breathing rhythm, breakdown of sensorial perceptions and complicates the recording of the judge's perception. Besides, it mobilizes an operator to capture judges' comments and restricts olfactometry sessions to a single judge. To avoid these bias, instrumental devices were developed to automate the acquisition of judges' perceptions. A pushing button was first employed to record time and duration of odor events and finger-span system was developed to record the intensity perceived by using the distance between the thumb and the major finger of the judge to represent the odor intensity score. [1,2] However, when recorded, descriptors

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were independently captured. Tape and digital recorders could be used to overcome the presence of an operator and devices can merge vocal information into numerical data through a voice recognition system, but these appliances don't prevent perturbations associated with speaking. Besides, when descriptors are freely chosen by judges, a consensus can be complicated to obtain for a same odor event. Even if training of judges strongly reduces these difficulties linked to individualities, this lack of consensus can persist due to the difficulty for human brain to link the olfactory and semantic memories and thus, to clearly associate a word to an odor. [3] To come through this problem, an acquisition software proposed to constrain judges to first choose an odor category and then a more precise term. [4] Despite the intuitiveness of this software using pictures, the odor description is made in several steps which delayed data recording and could fail to characterize closely eluted odorants. Currently, and according to literature, there was no device that, all at once, prevents judges from speaking, enables to record simultaneously all odor event parameters, and permits data processing.

The objective of this work was to introduce an innovative olfactometric software based on an intuitive wheel interface that allows a simultaneous and automatic recording of moment, duration, intensity and description of the perceived sensations (patented). [5,6] This approach was conceived to respect the breathing rhythm and the continuity of sensorial judge's perceptions while offering direct data processing possibilities. This original integrative system named WheelOscent is herein presented through a wine aroma analysis.

## Experimental

### *Material*

The wine used for the study was a red wine, 13% alcohol, from appellation Bourgueil, elaborated in 2010 from a Cabernet Franc vineyard. Chemical standards and n-alkanes were purchased from Sigma Aldrich (St Quentin Fallavier, France) with purity > 97%.

### *Wine aroma extraction and chromatographic conditions*

Volatiles from a 5 mL sample of wine were extracted by solid phase micro-extraction with a Car/PDMS fiber (10mm length, 85µm film thickness; Supelco, Bellefonte, PA, USA) placed in the headspace of the vial for 10 minutes at 34°C after 1 hour of incubation. The fiber was then directly introduced into the injection port of the gas chromatograph (T=260 °C). Besides the analyses of the samples, a solution of C5 to C32 n-alkanes was injected under the same chromatographic conditions. Analyses were carried out with a gas chromatograph (GC 7890A, Agilent Wilmington, DE, USA) equipped with a DB-Wax column (Agilent, 30m length, 0.25mm internal diameter, 0.5µm film thickness). Hydrogen was used as the carrier gas and the oven temperature was programmed as follows: 50°C (0min) to 80 °C at 5 °C·min$^{-1}$, then 80 to 200 °C at 10 °C·min$^{-1}$, and 200°C to 240 °C (4min) at 20 °C·min$^{-1}$. GC was coupled to a mass spectrometer (MS 5975, electron impact mode 70 eV, scan m/z 33-300, 2.7 scan.s$^{-1}$, Agilent) and a dual olfactometric port (transfer line T= 200°C, Gerstel ODP 3, Mülheim an der Ruhr, Germany). The olfactometric ports were equipped with nose glass funnels and supplied with humidified air to prevent dehydration of the nasal mucosa.

### *Olfactometric analysis*

The eluate was analyzed by 8 judges throughout successive runs. Judges were trained to aroma recognition and the use of an intensity scale. Judges' perceptions (time, intensity

and description) were recorded in real time *via* the WheelOscent software coded with Java technologies, described above (Figure ). Descriptors were presented on a dedicated aroma wheel especially designed for wine aroma. The wheel is structured in 23 poles associated to general odor families written in capital letters. These poles can be divided into sections associated to more precise descriptors.

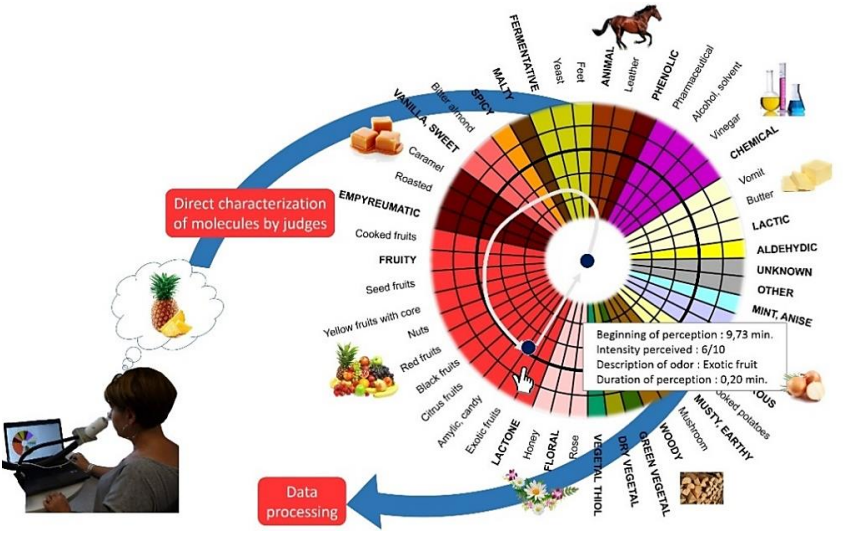


**Figure 1:** Schematic functioning of the WheelOscent olfactometric software presenting the aroma wheel interface (patent pending) [5,6]

Colors were also associated to poles to help the judges to rapidly find terms corresponding to the perceived odors. During the GC-Olfactometric run, judges were asked to signal the perception of an odor by directing the mouse pointer outside the central zone and then to direct it to the section of the wheel corresponding to the adequate odor term. They were also asked to score the intensity of the odor by clicking on the 0-10 intensity scale represented by the radius of the wheel (center of the wheel= 0, edge of the wheel=10). When an odor was no longer perceived, judges were asked to direct the pointer of the mouse back to the center of the wheel. Judges were encouraged to describe each odor perceived using terms proposed on the wheel. If the odor perceived did not correspond to any descriptor, judges were invited to describe the odor by the name of the pole corresponding to the general odor family or failing that, by the "Unknown" or "Other" sections.

**Results and discussion**

The results are displayed directly from the software. Concatenated aromagrams can be obtained presenting either the number of detections or the mean intensity of odors perceived *vs* retention time or linear retention index (LRI, Figure). For the investigated wine, 33 odorant zones were perceived by at least 3 judges. Individual aromagrams are also available for each judge with their associated descriptors. Moreover, a table of results that summarize the recorded data for each odorant zone is accessible from the software.

Identification of odorants was performed by comparing their LRI and mass spectra to those of databases (Wiley, Nist and internal databases) and by injection of standard compounds. Descriptors given for each detected compound were also compared with those found in the literature. Identification of compounds associated to each odorant zone and LRI is systematically recorded in the software database, so that a list of plausible

odorants with related descriptors is available for exploration of later samples.

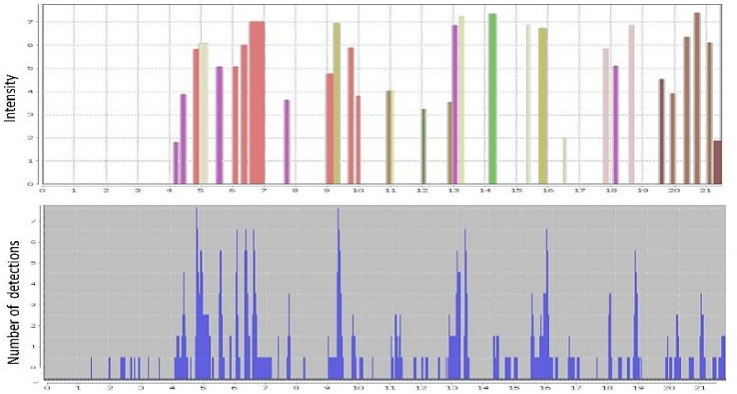


**Figure 2:** Individual and concatenated aromagrams directly obtained from the wheelOscent software after the GCO analysis of the investigated wine

As proved by the excerpt of the table of results obtained after the analysis of the wine sample (Table ), this tool enables to clearly discriminate odorants closely eluted like the 2-ethyl-2-methylpropanoate (4,82min) and the 2,3-butanedione (4,94 min).

**Table 1:** Excerpt of the table of results obtained from the software after olfactometric analysis of the wine

| *LRI apex* | *Retention time* | *Start time* | *End time* | *Judge1* | *Judge2* | *Judge3* | *Judge4* | *Judge5* | *Judge5* | *Judge6* | *Judge7* | *Judge8* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 940 | 4,40 | 4,37 | 4,45 | CHIMIQUE | alcool | CHIMIQUE | | | CHIMIQUE | LACTONE | | |
| 970 | 4,82 | 4,78 | 4,92 | FRUITE | amylique | FRUITE | | VANILLE, DOUX | fruits rouges | amylique | amylique | |
| 978 | 4,94 | 4,92 | 5,18 | INCONNU | beurre | caramel | beurre | beurre | beurre | amylique | beurre | |
| 1018 | 5,57 | 5,52 | 5,62 | INCONNU | alcool | INCONNU | VEGETAL VERT | | CHIMIQUE | | caramel | |

As expected, this software allows a rapid, precise and efficient recording of GC-Olfactometric data, associated with an excellent usability for judges through the intuitive aroma wheel interface. This approach solves bias found in current GC-O data acquisition methods and notably disruption of breathing rhythm inherent to the oral transmission of judges' perceptions. It provides a complete characterization of odor events, and includes data treatment capabilities. The accuracy of the approach makes it a valuable tool to shed light from whatever complex aroma product or compare those with very close aroma profile and point out their significant characteristics. Besides, the wheel presentation of descriptors, consistent with those found in numerous sensory analysis, can facilitate chemometric approaches that attempt to understand the contribution of compounds to a global aroma.

## References

1. Pollien, P. A. Ott, F. Montigon, M. Baumgartner, R. Muñoz-Box, and A. Chaintreau, (1997) J. Agric. Food Chem. 45, 2630–2637.
2. Guichard, H., E. Guichard, D. Langlois, S. Issanchou, N. Abbott, (1995) Z. Für Lebensm.-Unters. Forsch. 201, 344–350
3. Rouby, C. T. Thomas-Danguin a,b, G. Sicard, M. Vigouroux, T. Jiang, J. Poitevineau, and S. Issanchou, (2005). Psychol. Fr. 50, 225–239
4. Cotte, V. M. E., S.K. Prasad, P.H.W. Wan, R.S.T. Linforth, and A.J. Taylor (2008) Expression of Multidisciplinary Flavour Science 498–501
5. Prost, C. and Bertrand, D. Procédé d'analyse d'une impression sensorielle. FR2933788 (2010).
6. Villière, A., S. Le Roy, C. Fillonneau, F. Guillet, H. Falquerho, Sabine Boussely and C. Prost (2015) Flavour 4, 24

# Scope for improvement in the sensomics approach

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## Abstract

Sensomics is a stepwise approach for determining the compounds responsible for food odour. In this work, meta-analysis was carried out on a selection of sensomics publications and previously unpublished research, identifying two steps with scope for improvement. Firstly, it was found that Flavour Dilution (FD) factors, as calculated for odourants using Gas Chromatography – Olfactometry (GC-O), are very poor predictors of an odorants' Odour Activity Value (OAV). FD factors are used to prioritize odorants for quantitation and, following this work, it is recommended that all odorants are quantitated, regardless of FD factor, or other measures are considered in odorant prioritization. Secondly, from the statistical information available, it was found that Flavor Profiling®, used to compare the odour simulation to original material (OM), is able to test for significant difference in specific odour attributes but not overall odour. It is therefore recommended that simulations are assessed by additional methods such as Napping®. All sensory methods should be powered to meet the criteria of the statistical testing to be performed.

## Introduction

Sensomics is an accepted approach to identify the key odorants in food with more than 100 publications determining odorants in over 200 different foods [1]. Sensomics is an established technique, however there is limited literature available for its validation. The sensomics approach to odour analysis is stepwise, combining: (1) The bioactivity guided detection of key odorants using GC-O, where the method of Aroma Extract Dilution Analysis generates FD factors, which are used to prioritise odorants for quantitation. (2) Accurate quantitation, which is used to calculate an odorants' Odour Activity Value (OAV), the ratio of the concentration of an odorant in the food and its odour detection threshold in a suitable matrix. OAV is assumed to relate to an odorants' overall importance in a food. (3) Accurate reconstitution, using odorants with OAV > 1, to create an odour simulation. This simulation is then validated by comparison to the original material (OM) using the human sensory method of Flavour Profiling®. (4) Sensory omission studies to reduce the simulation to the smallest number of odorants.

There is a wealth of literature validating methods for the quantitation of odorants [2] and odour detection thresholds [3]. There are previous publications on the applicability of sensory omission studies [4]. However, there is limited information on the ability of FD factors to determine and prioritise importance of odorants and the ability of Flavour Profiling® to validate odour simulations. Here, a meta-analysis is conducted on results from a sample of sensomics publications focusing on the prediction of an odorant's OAV from the FD factor, and the use of Flavour Profiling® to compare odour simulations to the OM.

## Experimental

Meta-analysis was conducted on a selection of sensomics publications [5-19]. Covering a period from 1993 to 2017, the selection included analysis of fish, meat, coffee,

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nuts and fruit, using the sample preparation techniques of Solvent Assisted Flavour Evaporation, High Vacuum Distillation and Static Headspace. For each publication the odorant data was tabulated with other available information on the odorant and food product [20-22]. Statistical analysis was carried out using R version 3.3.3 and the *ranger* library.

Two statistical modelling methods were used to assess the ability of FD factors to predict OAV. The first was a simple linear model with OAV as response and FD factor as fixed effect, both on the $\log_{10}$ scale. The second was a random forest approach, with 200 trees and 4 variables selected per tree. Additional predictors (variables) were used for the random forest model, including odorants' vapour pressure (VP), air/water partition coefficient ($K_{aw}$) and odour detection threshold. The models were fitted to 70% of the data. The remaining data was then predicted, and used to calculate root mean square error (RMSE), as a measure of fit. Within the reviewed publications there was little statistical information (e.g. variance) available for the comparison of the odour simulation to the OM. Therefore, the data used to first assess Flavour Profiling® and then investigate an alternative approach, Napping®, is from unpublished work on liver and tuna.

## Results and discussion

Results for the prediction of OAV from FD factor using a simple linear model are displayed in Fig. 1A. The figure shows that prediction of OAV from FD factor alone is very poor, RMSE 1.14. It is therefore recommended that FD factors alone are not used to select or prioritise odorants for quantitation. Previous publications have noted that there are differences between FD factors and OAVs. The reasoning was two-fold. Firstly, that FD factors are not corrected for losses in sample preparation [23]. Secondly, that in GC-O the whole aroma extract is vaporised, whereas OAVs are calculated using odour thresholds in a matrix, i.e. considering only the amount of an odorant in the headspace. The example given is that polar compounds are often overestimated by AEDA, because they are quite soluble in water, and thus their vapour pressure is comparatively low [11].

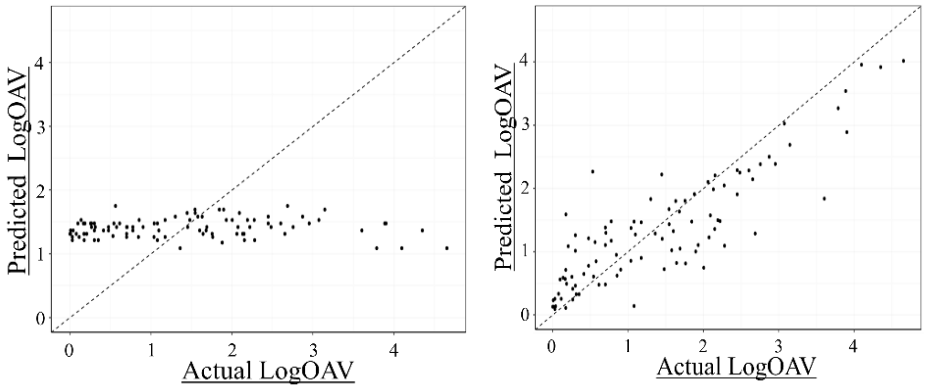


**Figure 1:** A, left, prediction of OAV from FD factor alone, using a simple linear model. B, right, prediction of OAV from FD factor along with additional measures

Results for the prediction of OAV from FD factor and additional variables, using the machine learning model, is shown in Fig. 1B. The figure shows a great improvement in prediction, RMSE 0.58, showing that by incorporating other variables odorants can be selected or prioritised for quantitation. The variables that have highest importance in the

model are displayed in Table 1. Additional variables with their importance were: the amount of water (19.7), protein (15.8), fat (14.8) in the food matrix; the physical chemical constants, VP (23.0), LogP (14.1), exact mass (12.1); Linear Retention Index (LRI) on a standard non-polar column (21.0); the percentage abundance of the odorant in studied food (12.8) as defined by Dunkel et al [1]; FD factor (23.3). The results show that within the model, FD factor is not the best predictor of OAV, even when normalised by taking into account the amount of food used for sample preparation. In fact, LRI on a normal polar column is the best single predictor of OAV. A possible explanation is that LRI is a good correlator of odour release from foods. Whereas, VP and $K_{aw}$ are calculated within systems at equilibria, LRI is calculated within a dynamic system, as is odour release. GC-O can identify odorants, but multiple additional measures are required to predict an odorant's importance.

**Table 1:** Highest importance score of variables used with the machine learnt random forest model.

| *Variable* | *Importance* |
|---|---|
| LRI Standard Polar Column | 52.3 |
| $K_{aw}$ | 39.8 |
| Carbohydrate (% wt.) | 37.6 |
| Normalised FD factor ($g^{-1}$) | 34.17 |
| Odour Detection Threshold (mg /L ) | 33.05 |
| LRI Semi-Standard Non-Polar Column | 24.57 |

In the absence of statistical data from published work using Flavour Profiling®, the data reviewed is from previously unpublished work on liver (Fig. 2a). In statistical testing, by analysis of variance (ANOVA), there was no significant difference between simulation and OM for each odour attribute. Power analysis of the data showed that, for each attribute, a difference of 0.5 would be detected 80% of the time (if present), at a significance level of 5% with 25 assessors. The results therefore show that Flavour Profiling® is able to test odour attribute differences of 0.5 between the simulation and OM. Within Flavour Profiling® this is equivalent to half way between a moderate to strong odour attribute. But what about differences in overall odour? Using a sensory discrimination test, Triangle testing, a significant difference in overall odour was observed ($p<0.01$) with 60 participants. In sensomics publications simulations are described as characteristic (and not similar) to the overall odour of the OM. Indeed, a previous review commented that there are difficulties in producing flavour simulations for solid foods as it is not possible to recreate the composition and distribution on the non-volatiles components in a suitable odourless matrix [23]. In effect, the simulation matrix causes a difference. Since Flavour Profiling® does not assess overall odour the assessment of the overall odour of a canned skipjack tuna odour simulation was carried out by the human sensory method of Napping®. The odour simulation was compared to the OM (Skipjack 1) and other tuna samples of different species (Albacore), manufacturer (Skipjack 2) and samples that had been opened and left in a fridge for 24 h (Aged). The results show that the overall odour of the simulation clusters with tuna samples from the same manufacturer and tuna species. The overall odour is characteristic of a specific manufacturer's canned skipjack tuna product. However, the overall odour is not similar as Triangle testing showed a significant difference ($p<0.01$). In addition to the previous explanation on why simulations are not similar, the Napping® shows that the overall odour of canned tuna changes over time from opening. Food does not have a constant

stable odour. Reviewing the results, it is recommended that for odour simulation assessment Flavour Profiling®, with additional methods such as Napping®, are used. All sensory methods should be powered to meet the criteria of the statistical testing to be performed.

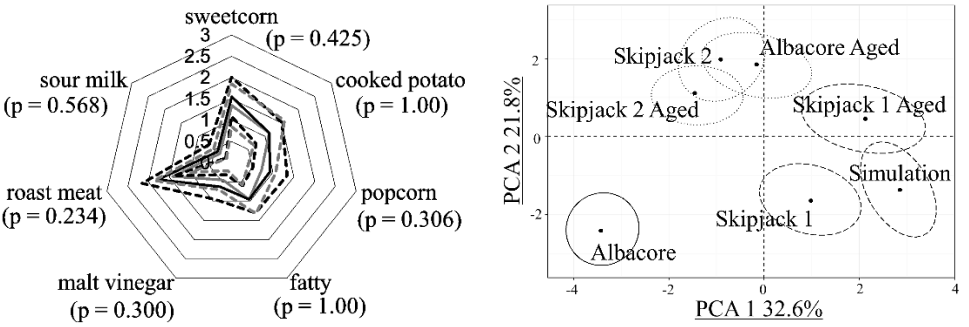


**Figure 2:** A, left, radar plot showing Flavour Profiling® comparison of odour attributes for liver (black) to liver odour simulation (grey). Solid line represent mean, dashed lines 95% confidence limits, n = 20, p-value from testing significant difference between each odour attribute for liver and liver odour simulation. B, right, PCA of Napping® result for the comparison of a canned skipjack tuna odour simulation to skipjack tuna from different manufacturer and species (albacore). All tuna samples were analysed freshly opened and after aging in a fridge for 24 hours. n= 10, ellipsoids represent 95% confidence intervals, line style represents clustering determined by hierarchical cluster analysis: solid black cluster 1, dotted grey cluster 2, dashed cluster 3.

## References

1. Dunkel A., Steinhaus M, Kotthoff M, Nowak B, Krautwurst D, Schieberle P, Hofmann T. (2014) Angew Chem. Int. Ed. 53:7124-7143.
2. Analysis IWGoMo. (2011) Flavour Fragr. J. 26: 297-299.
3. Lawless H.T., Heymann H (1998) Sensory Evaluation of Food, Kluwer Academic / Plenum Publishers London, pp. 173-207.
4. Delime P., Desforges N., Hort J. (2015) In: Flavour Science (Taylor, A.J., Mottram, D.S., eds), Context Products., pp. 291-296.
5. Milo C.; Grosch W. (1993) J. Agric. Food Chem. 41:2076-2081.
6. Heiler C., Schieberle P. Int. Dairy J. (1997), 7:659-666.
7. Kerler J.; Grosch W. (1997) Eur. Food Res. & Technol., 205:232-238.
8. Czerny M.; Grosch W. J. (2000) J. Agric. Food Chem., 48:868-872.
9. Buettner A.; Schieberle P. (2001) J. Agric. Food Chem., 49:2387-2394.
10. Buettner A.; Schieberle P. (2001) J. Agric. and Food Chem., 49:1358-1363.
11. Greger V., Schieberle, P. (2007) J. Agric. Food Chem., 55:5221-5228.
12. Chetschik I., Granvogl M., Schieberle, P. (2008) J. Agric. Food Chem., 56:10237-10243.
13. Averbeck M., Schieberle P. (2009) Eur. Food Res. Technol 229:611-622.
14. Christlbauer M., Schieberle P. (2009) J. Agric. Food Chem.,57:9114-9122.
15. Christlbauer M., Schieberle P. (2009) J. Agric. Food Chem., 59:13122-13130.
16. Li J-X., Schieberle P., Steinhaus M., (2012) J. Agric. Food Chem., 60:11253-11262.
17. Conde-Martínez N., Jiménez A., Steinhaus M., Schieberle P, Sinuco D, Osorio. (2013) Eur. Food Res. Technol. 236:1085-1091.
18. Kiefl J., Schieberle P. (2013) J. Agric Food Chem. 61:5236-5244.
19. Li J.-X., Schieberle P, Steinhaus M. (2013) J. Agric. Food Chem. 65:639-647.
20. PubChem https://pubchem.ncbi.nlm.nih.gov (accessed August 2017).
21. Chemspider https://www.chemspider.com (accessed August 2017).
22. Holland B., Welch A.A., Unwin I.D., Buss D.H., Paul A.A, Southgate, D.A.T, (1991) The Composition of Food, Royal Society of Chemistry.
23. Grosch W. (2001) Chem. Sense 26:533-545.

# Rapid ATR-FTIR method for monitoring the release of major components of clove essential oil encapsulated into a complex organic matrix

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## Abstract

Fourier transform infrared (FTIR) attenuated total reflection (ATR) spectroscopy is a rapid and non-destructive technique that provides an overall infrared fingerprint of a matrix and/or sample. It can prevent time consuming analysis for monitoring changes in an aroma profile and/or aroma compounds transfer (1).

In this study, clove essential oil (EO), previously identified by GC-MS, was encapsulated into an organic solid matrix mainly composed of proteins and polysaccharides. The controlled release of major aroma compounds of the essential oil was followed using a FT-IR method and compared to conventional gas chromatography methodology. The quantification by FTIR was performed after a calibration procedure carried out by depositing pure clove oil and its major components at different concentrations on a constant mass of matrix without essential oil. Several Partial Least Squares (PLS) regression calibration models were optimized on the specific bands of aroma compounds to determine the best correlation ($R^2$>0.90) between the predicted and reference values of clove essential oil major compounds. The limit of detection (LOD) and limit of quantification (LOQ) were determined and the release of major compounds of clove EO proved to be similar as the results obtained using GC-FID. Such ATR-FTIR method can be used as an alternative rapid method for the identification and quantification of major aroma compounds in complex organic samples.

## Introduction

The common method for essential oils (EOs) quantification is extraction followed by a gas chromatography analysis which is expensive and time consuming. Fourier transform infrared (FTIR) attenuated total reflection (ATR) spectroscopy is a rapid and non-destructive technique which provides an overall fingerprint of a sample and can be considered as an interesting alternative way for the rapid quantification of EOs or monitoring their evolution in a complex medium (1). ATR-FTIR has already been successfully used for EOs characterization but to our knowledge there are no previous investigations that have been conducted on the quantification of EO encapsulated in an organic complex matrix. Indeed, the presence of complex matrices can compromise the analysis by affecting the sensitivity and the specificity of method due to spectra superposition of the targeted compounds with the matrix fingerprint.

The aim of this study was to assess the potential of ATR-FTIR method applying a partial least square (PLS) model and a cross validation with a GC quantitative analysis to monitor the release of clove EO encapsulated in a complex matrix.

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## Experimental

*Matrices elaboration and GC-MS analysis of Clove EO*

An organic powder with high content in polysaccharides and proteins was mixed with distilled water and clove EO (Golgemma, Esperza, France). The mixture was processed and dried at a low temperature to obtain a matrix with the ability to retain and release aroma compounds. Clove EO was characterized by a GC-MS (ISQ, ThermoScientific, Austin, Texas, USA) equipped with a DB-WAX polar capillary column (30 m, 0.25 mm i.d. x 0.25 µm of thickness). Helium was used as carrier gas with a flow rate of 1.2 ml/min. The GC-MS oven temperature was kept at 40 °C for 5 min and programed to 260 °C at a rate of 2 °C/min. One µL of diluted sample (dilution 1/100) was injected at constant temperature of 250 °C via split injector (1:20). Spectra were obtained in the electron impact mode (70ev) in full scan mode with a range between 40-500 amu. Identification of components was based on the calculated Kovats indexes estimated by simultaneous injection of alkanes and comparison of spectra with mass spectra libraries (NIST 2.0/Wiley/INRA). Quantification of the identified compounds was expressed as percentage by directly calculating from peak areas.

*ATR-FTIR method*

A FTIR Nicolet 6700 spectrometer (Thermo Scientific) in Attenuated Resonance mode has been used. Clove EO major aroma compounds used as standards were purchased from Sigma-Aldrich and analyzed directly by deposing around 13 mg of aroma compound over the diamond crystal of a Smart DuraSamplIR accessory (Thermo Scientific). Spectral data were recorded from 64 scans with a resolution of 2 $cm^{-1}$ in the range of 800-4000 $cm^{-1}$ wavenumbers.

For establishment of standard curves, organic matrices free of clove EO were elaborated. Different known amounts of clove essential oil were deposited into the several matrices by using a precision pipette and stored in closed glass jars at room temperature until analysis. The matrices were grinded using a laboratory mortar and analyzed directly by the ATR-FTIR spectrometer. In parallel, the EO was extracted from the matrix (18h at constant stir and room temperature) using hexane in the presence of 100 µL of 2-heptanol at 3 g/L as internal standard. The organic extracts were analyzed and the major components previously identified using standard components were quantified by a GC-FID (Varian CP-3800 GC, Les Ulis, France).

*Controlled release of EO from matrices and validation of FTIR method*

The organic matrices loaded with clove EO were put into a Memmert oven HPP IPP plus (Buchenbach, Germany) maintained at controlled temperature of 25 °C and relative humidity at 72 % during a period of 34 days. At interval times, samples were removed and analyzed by the ATR-FTIR method and the GC-FID method for comparison purpose.

*Chemometric analysis*

Spectra treatments were performed using Omnic v7.3 and TQ Analyst v7.3 softwares (Thermo electron). The most prominent spectral band specific of clove EO was selected for the PLS analysis. Calibration models were validated by a cross validation (leaving 40% out) using a regression PLS calibration algorithm. Limit of detection (LOD) and limit of quantification (LOD) was calculated with the guidelines of the International Conference on Harmonisation (ICH) (2).

## Results and discussion

*Essential oil characterization*

The major components of clove essential oil and their relative proportion were identified by GC-MS. Eugenol represents 62.52 % ± 0.35 of total oil, acetyleugenol 23.62 % ± 0.42, β-caryophyllene 9.77 % ± 0.18, α-caryophyllene 1.39% ± 0.02, caryophyllene oxide 0.98% ± 0.01, methyl salicylate 0.91% ± 0.014 and allylphenol 0.47% ± 0.01. These components are the most outstanding major compounds in the EO composition as generally described in literature (3).

*ATR-FTIR method.*

First, the FTIR spectral bands of clove EO were compared with the spectra of an uncharged matrix. As no overlapping with the pure matrix spectra are observed for the ranges of the spectra from the 1250 to 1600 $cm^{-1}$ wavenumbers, a band was selected in this region allowing a good sensitivity of the analysis. For quantification, the 1514 $cm^{-1}$ stretching aromatic C=C vibration (4) was preferentially selected. This band was found for three of the clove EO major compounds (eugenol, acetyleugenol and 4-allyphenol) according to the aroma compounds characterization by ATR-FTIR. These 3 phenolic compounds represent 86% of the essential oil, which explains the proportional increase of the 1514 $cm^{-1}$ peak to 5 concentrations included in the standard curve (Figure 1). This stretching band was chosen to elaborate the PLS regression, a normalization was carried out according to the peak height of $CH_2$ stretching band at 2853 $cm^{-1}$ that corresponds to the organic pure matrix.

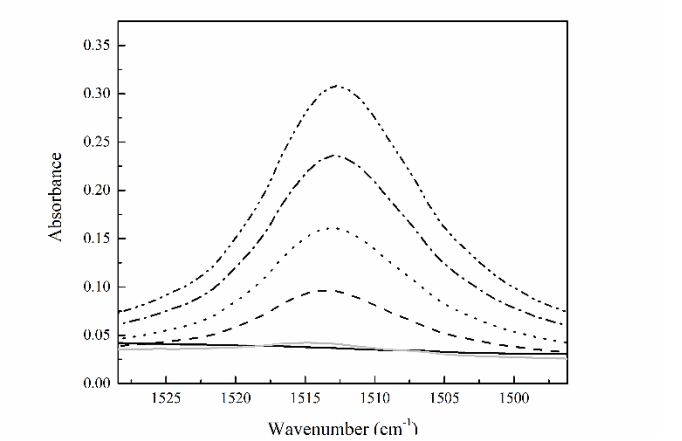


**Figure 1**: Increase of the spectroscopic selected band (1514 $cm^{-1}$) proportionally to concentration.

*Quantitative analysis of trapped EO by ATR-FTIR.*

For the development of the quantification model, averaged spectra issued from 35 samples were correlated with a GC-FID standard curve for which average values were obtained from 5 samples (Figure 2). Although the different amounts of analyzed samples between the two methods (13 mg of matrix for ATR against 300 mg for GC) the correlation between the ATR-FTIR and GC-FID analysis was satisfactory since PLS regression model produced a high coefficient of determination ($R^2$) and RMSECV and RMSEP values were 0.0120 and 0.0162 g/g respectively, indicating that the residuals of the calibration data are low and the model gives a good estimation on how it built the data for unknown samples. LOD (0.003 g of clove EO/g matrix) and LOQ (0.010 g of clove

EO/g matrix) were determined by multiplying the standard deviation measured on 15 blank samples by 3 and 10 times respectively, and dividing the result value by the slope of the ATR-FTIR standard curve.

*Controlled release of EO from matrices and validation of the ATR-FTIR method*

The release of clove EO at controlled relative humidity and temperature was followed both GC-FID and ATR-FTIR methods. It was found that the chemometric method could be applied to predict the release of major compounds of clove essential oil, including eugenol, acetyl eugenol and 4-allylphenol. The fact that the quantification does not cover the totality of the compounds and that small changes in aromatic profile occurred as observed by GC analysis could result from variability within samples.

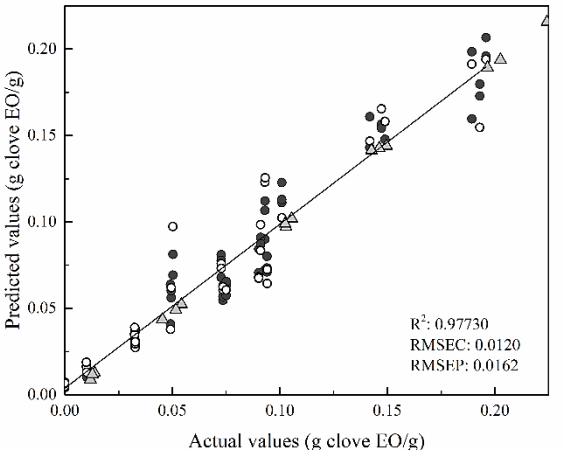


**Figure 2**: Results of PLS cross validation essay: ●PLS calculated value, ○ PLS validated value, ▲ GC-FID validation.

The proposed ATR-FTIR protocol demonstrated to be a reliable and robust quantitative method compared to the GC despite the complexity of the organic matrix studied. The ATR-FTIR method can be used to survey the release of the selected aroma compounds of essential oils trapped in an organic matrix or study the influence of specific external factors over the matrix.

## References

1. Gómez-caravaca, A. M., Maggio, R. M., Verardo, V., Cichelli, A., and Cerretani, L. (2013). LWT - Food Science and Technology 50(1), 153–159.
2. Ermer, J. and Miller, J. H. McB. (2005). Method Validation in Pharmaceutical Analysis (Ed. Wiley-VCH), Wiley-VCH, Germany. pp.. 251 - 252 :
3. Hossain, M. A., Al Harbi, S. R., Weli, A. M., Al-Riyami, A., and Al-Sabahi, J. N. (2014). Asian Pacific Journal of Tropical Disease. 4(4), 262–268.
4. Dhoot, G., Auras, R., Rubino, M., Dolan, K., and Soto-Valdez, H. (2009). Polymer. 50(6), 1470–1482.

# The impact of pod storage on the formation of different alkylpyrazines from Ghanaian cocoa roasted at four roasting temperatures

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## Abstract

The typical flavour characteristics of cocoa are related to the cocoa bean genotype and the growing environmental conditions. However, the flavour does not exist in freshly harvested beans. Flavour is generated by a series of procedures that begins with occasional pod storage (PS) after harvesting, followed by fermentation of the beans, and roasting. PS implies storing harvested cocoa pods for a period of time before opening. The effect of PS is believed to be beneficial for the subsequent development of cocoa flavour in the cocoa beans [1]. During roasting, several volatile heterocyclic compounds are formed, among them alkylpyrazines. These newly formed compounds are considered to be key odour components. Among alkylpyrazines, tetramethylpyrazine and trimethylpyrazine, are the most abundant ones. Other alkylpyrazines with different substituents also contribute to the aroma profile. Hence, monitoring pyrazines can be helpful in optimizing roasting conditions of cocoa beans for attaining the desired aroma of the cocoa liquors. In several studies, cocoa volatiles have been measured using gas chromatography mass spectrometry (GC-MS), frequently using headspace solid-phase microextraction (HS-SPME) to concentrate the volatiles.

The purpose of this study was to investigate the effect of PS (0, 3, 7 days PS) and roasting temperature (100°C, 120°C, 140°C and 160°C) on the formation of alkylpyrazines in Ghanaian cocoa liquors. HS-SPME extraction of the alkylpyrazines was carried out with a DVB/CAR/PDMS fibre. The identified compounds were measured semi-quantitatively and the results were statistically processed by multivariate analysis. In total, 18 different alkylpyrazines were determined. Higher roasting temperatures resulted in the formation of more complex alkylpyrazine profiles, compared to lower roasting temperatures. Moreover, an extended PS time of 7 days leads to highest formation of alkylpyrazines.

## Introduction

During roasting, the typical roasty and chocolate flavours are developed and undesired flavours are eliminated, at least to some extent. Flavour precursors (free amino acids, oligopeptides and reducing sugars) participate in non-enzymatic browning (Maillard) reactions. An important route is the Strecker degradation, which leads to volatile aldehydes, pyrazines and other heterocyclic compounds. Pyrazines are the main class of nitrogenous heterocyclic volatiles and they are also key odour components in cocoa aroma. Several pyrazines contribute to the overall cocoa flavour, especially the alkylpyrazines with different substituents, of which tetramethylpyrazine and trimethylpyrazine are the most abundant ones. Roasting temperature is a critical factor

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that influences the concentration of pyrazines. The aim of this study was to investigate the effect of pod storage (0, 3, 7 days of PS) of Ghanaian cocoa liquor, followed by roasting at 4 different temperatures (100°C, 120°C, 140°C and 160°C), on the formation of alkylpyrazines.

## Experimental

Ghanaian cocoa beans from 31-year old trees (hybrid type *Forastero)* were obtained from a farm in Jachere farming community (Brong Ahafo region). The beans were harvested in September-October; the cocoa beans have different times of PS: 0, 3 and 7 days. After PS, the beans were fermented for 6 days, and sun-dried for two weeks. 1 kg of cocoa beans was weighed and roasted in a conventional oven (Termarks, Lien 79, N-5057 Bergen, Norway) at 100°C, 120°C, 140°C and 160°C for 35 min. After cooling to room temperature, the cocoa beans were wrapped in aluminium foil and stored in odourless buckets. Prior to sample preparation, the beans were manually deshelled. For grinding the de-shelled cocoa beans to liquor an ECGC-12SLTA Cocoa T melanger (CocoaTown, Roswell, USA) was used.

The volatile aroma profiles were recorded using a Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany) equipped with a HS-SPME unit. Cocoa liquor (2 g) was weighed in a 20 ml vial and 0.792µg of 1-octen-3-ol was added as internal standard. Afterwards, the vials were sealed airtight with a magnetic cap equipped with a PTFE septum. Prior to extraction, each vial was heated at 60°C for 10 min for headspace equilibration in a thermostatic agitator. Next, the volatiles compounds were extracted for 25 min at 60°C using a 50/30µm DVB/CAR/PDMS fiber (1 cm) (Supelco, Sigma-Aldrich N.V., Bornem). Volatile components were desorbed (5 min) into the splitless injector (250°C) of an Agilent Technologies 6890-5793 GC-MS system (Agilent Technologies, Santa Clara, CA, USA) and separated on a Phenomenex 30m ZB-Wax plus capillary column (0.25 mm i.d.; 0.25 µm film thickness). The temperature program was 5 min at 35°C; heating at 4°C/min to 182°C and heating at 7°C/min to 240°C. Compounds were fragmented using electron-impact ionization (70eV), with a source temperature of 230°C, a scan range of 40-230 amu and a scan rate of $2s^{-1}$. After injection, each time the fiber was baked out for 7 min at 270°C. Components were identified based on comparison of mass spectra with those of spectral libraries of Wiley 7N Registry of GC Mass spectral Data (John Wiley, NY, USA). Additionally, confirmation of identified compounds was done by determination of Kovats indices (KI), determined after injection of series of n-alkane homologues using the analytical configuration as described above. The calculated values were compared to KI values found in literature obtained on polar columns and are inserted in Table 1.

Statistical analysis was performed using SPSS 22 (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) was used to investigate any significant differences between the samples (significance level at 0.05). Significant differences were identified with the Tukey's multiple range test. Principal Component Analysis (PCA) was performed (using Unscrambler 6.1., Camo, Norway) to visualize complex data matrix and the relationship between the different cocoa beans on their volatile composition.

## Results and discussion

*Formation of different pyrazines at different roasting temperatures*

In total, 18 different pyrazines, derived from Maillard reactions, were identified in the cocoa liquors. In Table 1, all semi-quantitative concentrations (ng/g liquor) of the

**Table 2:** Semi-quantitative data (expressed as ng/g liquor) of different alkylpyrazines at 3 pod storage periods (0PS, 3PS, 7PS) as a function of roasting temperature (100°C, 120°C, 140°C, 160°C) (data are expressed as mean values of 3 replicates ± standard deviation, $^{A-H}$ different letters are significantly different at a significance level of $P < 0.05$ according to Tukey's test)(n.d. not detected).

| *RT* | *Pyrazine* | *KI* | *KI(lit)* | *0PS100* | *0PS120* | *0PS140* | *0PS160* | *3PS100* | *3PS120* | *3PS140* | *3PS160* | *7PS100* | *7PS120* | *7PS140* | *7PS160* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15.25 | **methyl-pyrazine** | 1257 | 1251 | 18.7 ± 5.1$^{D}$ | 181.0 ± 9.1$^{CD}$ | 573.1 ± 69.3$^{B}$ | 1259.9 ± 143.3$^{A}$ | 51.1 ± 2.6$^{D}$ | 287.9 ± 30.7$^{C}$ | 629.0 ± 55.2$^{B}$ | 1243.1 ± 42.5$^{A}$ | 35.0 ± 5.9$^{D}$ | 182.5 ± 15.5$^{CD}$ | 510.4 ± 55.8$^{B}$ | 1235.8 ± 136.5$^{A}$ |
| 17.34 | **2,5-dimethyl-pyrazine** | 1315 | 1290-1358 | 25.9 ± 7.4$^{F}$ | 307.6 ± 42.4$^{F}$ | 1360.8 ± 144.6$^{DE}$ | 2486.2 ± 234.9$^{B}$ | 58.4 ± 12.0$^{F}$ | 450.5 ± 42.0$^{F}$ | 1175.9 ± 68.7$^{E}$ | 2048.4 ± 145.3$^{BC}$ | 46.8 ± 9.9$^{F}$ | 444.9 ± 73.1$^{F}$ | 1719.1 ± 220.4$^{CD}$ | 3681.4 ± 343.0$^{A}$ |
| 17.56 | **2,6-dimethyl-pyrazine** | 1321 | 1296-1358 | 39.7 ± 6.2$^{G}$ | 192.7 ± 23.4$^{EFG}$ | 593.6 ± 66.7$^{D}$ | 1024.9 ± 107.9$^{B}$ | 67.6 ± 4.7$^{FG}$ | 259.5 ± 22.8$^{E}$ | 546.3 ± 48.5$^{D}$ | 862.3 ± 67.7$^{BC}$ | 63.7 ± 3.5$^{FG}$ | 251.4 ± 44.5$^{EF}$ | 687.0 ± 83.6$^{CD}$ | 1392.2 ± 129.6$^{A}$ |
| 17.71 | **ethyl-pyrazine** | 1325 | 1323-1343 | n.d. | 58.1 ± 2.5$^{CD}$ | 183.6 ± 36.6$^{B}$ | 385.7 ± 33.8$^{A}$ | n.d. | 69.0 ± 7.9$^{C}$ | 205.9 ± 31.1$^{B}$ | 330.8 ± 44.7$^{A}$ | n.d. | 55.6 ± 8.3$^{CD}$ | 156.3 ± 13.8$^{B}$ | 376.4 ± 5.4$^{A}$ |
| 18.17 | **2,3-dimethyl-pyrazine** | 1337 | 1315-1344 | 94.1 ± 12.7$^{F}$ | 272.9 ± 16.2$^{EF}$ | 727.4 ± 84.6$^{D}$ | 1194.3 ± 161.0$^{C}$ | 123.8 ± 13.2$^{F}$ | 226.4 ± 22.9$^{EF}$ | 379.9 ± 35.5$^{EF}$ | 539.6 ± 71.3$^{DE}$ | 237.0 ± 10.7$^{EF}$ | 546.2 ± 78.0$^{DE}$ | 1655.9 ± 243.1$^{B}$ | 2541.4 ± 231.4$^{A}$ |
| 19.56 | **2-ethyl-6-methyl-pyrazine** | 1376 | 1381-1415 | n.d. | 204.6 ± 30.5$^{D}$ | 678.9 ± 59.8$^{B}$ | 1295.1 ± 119.5$^{A}$ | n.d. | 125.7 ± 11.2$^{DE}$ | 427.3 ± 31.4$^{C}$ | 735.0 ± 75.7$^{B}$ | n.d. | 141.2 ± 41.0$^{DE}$ | 629.2 ± 76.7$^{B}$ | 1320.8 ± 110.2$^{A}$ |
| 19.76 | **2-ethyl-5-methyl-pyrazine** | 1381 | 1386-1453 | n.d. | 115.4 ± 22.3$^{E}$ | 1198.1 ± 95.0$^{C}$ | 1741.0 ± 141.4$^{B}$ | n.d. | 239.7 ± 86.1$^{E}$ | 874.1 ± 41.5$^{D}$ | 1212.6 ± 129.5$^{C}$ | n.d. | 177.7 ± 28.3$^{E}$ | 1517.0 ± 219.8$^{BC}$ | 2337.1 ± 191.8$^{A}$ |
| 20.34 | **trimethyl-pyrazine** | 1397 | 1381-1413 | 264.7 ± 28.3$^{G}$ | 1168.9 ± 90.7$^{FG}$ | 3658.5 ± 329.8$^{CD}$ | 5417.4 ± 389.7$^{C}$ | 282.2 ± 50.6$^{G}$ | 778.1 ± 58.8$^{FG}$ | 1782.1 ± 72.9$^{EFG}$ | 2393.8 ± 207.6$^{DEF}$ | 884.5 ± 28.1$^{FG}$ | 3039.1 ± 329.4$^{DE}$ | 9230.8 ± 1166.8$^{B}$ | 15353.8 ± 1606.7$^{A}$ |
| 21.59 | **2,5-dimethyl-3-ethyl-pyrazine** | 1431 | 1435 | n.d. | 703.2 ± 240.1$^{DE}$ | 1513.7 ± 152.9$^{C}$ | 2478.3 ± 164.7$^{B}$ | n.d. | n.d. | 783.4 ± 58.3$^{DE}$ | 1062.4 ± 67.2$^{D}$ | n.d. | 470.5 ± 84.2$^{E}$ | 1503.6 ± 232.3$^{C}$ | 3055.7 ± 281.8$^{A}$ |
| 22.08 | **2,3-dimethyl-5-ethyl-pyrazine** | 1445 | 1493 | 87.3 ± 16.7$^{E}$ | 385.5 ± 42.1$^{DE}$ | 1001.1 ± 117.3$^{C}$ | 1920.4 ± 162.8$^{B}$ | 83.8 ± 13.1$^{E}$ | 170.2 ± 3.5$^{E}$ | 429.7 ± 45.2$^{DE}$ | 766.6 ± 29.2$^{CD}$ | 138.9 ± 16.5$^{E}$ | 572.8 ± 45.4$^{CDE}$ | 2095.8 ± 259.2$^{B}$ | 4090.6 ± 574.0$^{A}$ |
| 22.53 | **tetramethyl-pyrazine** | 1457 | 1438-1474 | 1116.8 ± 94.5$^{E}$ | 1971.7 ± 164.2$^{E}$ | 2467.5 ± 269.7$^{E}$ | 2956.2 ± 293.3$^{E}$ | 730.6 ± 74.9$^{E}$ | 674.9 ± 63.0$^{E}$ | 777.2 ± 27.8$^{E}$ | 758.5 ± 57.8$^{E}$ | 7215.8 ± 131.0$^{D}$ | 10141.9 ± 1097.3$^{C}$ | 14325.2 ± 1970,2$^{B}$ | 18542.3 ± 2260.2$^{A}$ |
| 22.81 | **2-methyl-6-vinyl-pyrazine** | 1465 | 1521 | n.d. | 80.5 ± 14.9$^{C}$ | 128.0 ± 19.1$^{ABC}$ | 119.8 ± 13.1$^{ABC}$ | n.d. | 96.6 ± 13.4$^{BC}$ | 101.5 ± 9.2$^{BC}$ | 81.5 ± 8.6$^{C}$ | n.d. | 101.5 ± 6.7$^{BC}$ | 143.4 ± 41.6$^{AB}$ | 152.8 ± 16.9$^{A}$ |
| 23.16 | **3,5-diethyl-2-methyl-pyrazine** | 1474 | 1524 | n.d. | 123.3 ± 14.7$^{DEF}$ | 407.6 ± 49.1$^{B}$ | 653.5 ± 67.9$^{A}$ | n.d. | 55.9 ± 7.7$^{EF}$ | 144.9 ± 11.8$^{DE}$ | 221.0 ± 21.2$^{CD}$ | n.d. | n.d. | 273.9 ± 61.1$^{C}$ | 687.9 ± 99.5$^{A}$ |
| 23.78 | **2,3,5-trimethyl-6-ethylpyrazine** | 1491 | - | 85.4 ± 11.4$^{E}$ | 476.9 ± 22.1DE | 876.9 ± 106.5$^{D}$ | 1325.8 ± 105.4C | 76.1 ± 10.5$^{E}$ | 138.4 ± 22.3$^{E}$ | 271.8 ± 9.8$^{E}$ | 328.7 ± 12.5$^{E}$ | 193.0 ± 10.6$^{E}$ | 826.0 ± 107.6$^{D}$ | 1950.7 ± 271.1$^{B}$ | 3250.0 ± 395.4$^{A}$ |
| 27.03 | **2-isoamyl-6-methyl-pyrazine** | 1581 | - | n.d. | n.d. | 39.0 ± 5.9$^{D}$ | 74.0 ± 15.1$^{B}$ | n.d. | n.d. | 40.6 ± 3.0$^{D}$ | 43.9 ± 2.2$^{CD}$ | n.d. | n.d. | 64.0 ± 12.8$^{BC}$ | 144.6 ± 15.2$^{A}$ |
| 29.49 | **2-methyl-6,7-dihydro-5H-cyclopentapyrazine** | 1648 | - | n.d. | n.d. | 92.2 ± 9.0$^{C}$ | 225.9 ± 14.6$^{B}$ | n.d. | n.d. | 83.0 ± 17.4$^{C}$ | 176.1 ± 35.9$^{B}$ | n.d. | n.d. | 196.2 ± 43.6$^{B}$ | 518.5 ± 55.5$^{A}$ |
| 29.99 | **2,3,5-trimethyl-6-propylpyrazine** | 1662 | - | n.d. | n.d. | 33.5 ± 3.5$^{CD}$ | 60.4 ± 10.9$^{C}$ | n.d. | n.d. | 24.2 ± 6.3$^{CD}$ | 32.4 ± 6.8$^{CD}$ | n.d. | 56.2 ± 5.9$^{C}$ | 116.2 ± 19.9$^{B}$ | 218.9 ± 39.5$^{A}$ |
| 31.89 | **2,3-dimethyl-6,7-dihydro 5H-cyclopentapyrazine** | 1714 | - | n.d. | n.d. | n.d. | 113.3 ± 29.6$^{B}$ | n.d. | n.d. | n.d. | 56.3 ± 13.7$^{BC}$ | n.d. | n.d. | 130.1 ± 26.0$^{B}$ | 545.7 ± 101.6$^{A}$ |
| | **Total** | | | 1732.5 ± 174.8$^{H}$ | 6242.2 ± 689.6$^{FG}$ | 15533.5 ± 1549.2$^{D}$ | 24732.1 ± 2023.3$^{C}$ | 1473.5 ± 174.3$^{H}$ | 3573.0 ± 371.0$^{GH}$ | 8676.6 ± 514.0$^{EF}$ | 12892.9 ± 966.2$^{DE}$ | 8814.6 ± 150.0$^{EF}$ | 17007.3 ± 1895.8$^{D}$ | 36904.7 ± 4841.5$^{B}$ | 59446.0 ± 6453.8$^{A}$ |

alkylpyrazines are presented. The most abundant compound was tetramethylpyrazine (> 18 µg/g), followed by trimethylpyrazine (> 15 µg/g) after 7PS at 160°C. Higher roasting temperatures resulted in the formation of more alkylpyrazines compared to lower roasting temperatures. In addition, at roasting temperatures of at least 140°C, four extra pyrazines were formed: 2-isoamyl-6-methylpyrazine, 2-methyl-6,7-dihydro-5H-cyclopentapyrazine, 2,3,5-trimethyl-6-propylpyrazine and 2,3-dimethyl-6,7-dihydro-5H-cyclopentapyrazine; the latter was only formed at a temperature of 160°C.

At 100°C only 8 pyrazines were detected. At 120°C, already 14 pyrazines were formed and the total amount of pyrazines increased by a factor 3.6 for 0PS; 2.4 for 3PS and 1.9 for 7PS compared to a roasting temperature of 100°C. Starting from 140 °C, for all PS, an increase in the level of pyrazines was observed by a factor greater than 2, compared to a roasting temperature of 120°C. At a temperature of 160 °C, only a rise by a factor 1.5-1.6 for all PS was reached, compared to a roasting temperature of 140°C. In summary, increasing the roasting temperature gives rise to more complex alkylpyrazine profiles in cocoa, and quantitatively higher levels in alkylpyrazines.

*Influence of pod storage on formation of pyrazines*

Figure 1 represents the biplot from PCA on all samples and all detected alkylpyrazines. The amount of variance explained by the two factors in the biplot was 94% (PC1:84%, PC2:10%). From the PCA, it became clear that there is an influence of both roasting temperature and PS. Samples of 7PS were clearly distinct from all other samples. This was mainly due to an exponential increase in tetramethylpyrazine and trimethylpyrazine (Table 1). Samples of 0PS and 3PS migrated in another, but similar way as a function of roasting temperature. At 3 days PS, the total amount of formed pyrazines was always lower than after 0 days PS. Both the data shown in Table 1 and the PCA biplot in Figure 1, point to a pronounced impact of extended PS (7 days) on the formation of alkylpyrazines, independent of the roasting temperature. Even at the lowest roasting temperature (100°C), PS for 7 days gives rise to highly increased alkylpyrazine formation, compared to 0 days PS (difference by a factor 5.1).

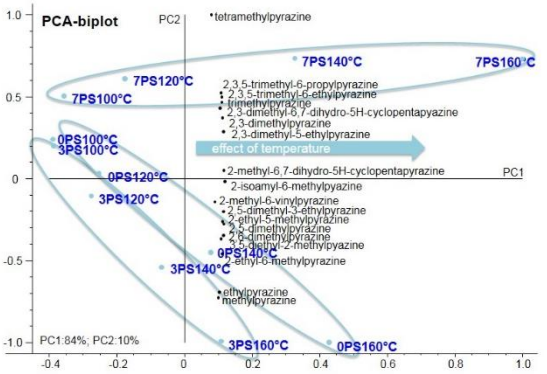


**Figure 1:** PCA-biplot of different alkylpyazines in Ghanaian liquor samples as a function of pod storage and roasting temperatures

## References

1. Afoakwa E.O., Paterson A., Fowler M. and Ryan A. (2009) Food Chem 113: 208-215

# Method development for multiple partition coefficients determination to understand headspace aroma distribution of complex mixture

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## Introduction

Sensory perception is directly related to compounds volatilization from the matrix to the atmosphere. From a physico-chemical point of view, this release may be described by partition coefficients, which correspond to the volatile concentration ratio between the liquid and gaseous phase. Partition coefficients may be determined thanks to Phase Ratio Variation (PRV) method which is based on the fact that, in a closed system, the headspace volatile concentration changes as a function of liquid phase volume (Ettre et al., 1993). This method is generally applied using HS-GC-FID.

For the analysis of various heavy compounds, some authors proposed a design for low-pressure gas chromatography coupled to mass spectrometry (LP-GC-MS), consisting in combining a micro-bore column to a mega-bore one, resulting in faster analysis and a better chromatographic resolution and sensitivity (de Zeeuw et al., 2000).

The goal of this work is to develop and optimize a new approach using multiple partition coefficients calculation in order to study potential modifications of headspace aroma distribution.

## Experimental

The impact of various operating conditions on partition coefficients of esters and higher alcohols in headspace analysis was evaluated (Table 1). Five parameters were considered: (i) the time to achieve thermodynamic equilibrium in the gas phase (from 0 to 2880 minutes); (ii) the filling rate of the syringe (250, 500 and 750 µL/s); (iii) the gas injection rate (250, 500 and 750 µL/s); (iv) the volume ratio between the gas and liquid phase, from 227 and 10.4, corresponding to a liquid volume from 50 µL to 5 mL; and (v) the type of analytical column used (micro-bore BP21 capillary column (50 m x 0.32 mm ID, film thickness, 0.25 µm, SGE) or a mega-bore BP21 capillary column with low-pressure (30 m × 0.53 mm, film thickness, 0.5 µm, SGE, connected with a Siltite µ-union (SGE) to a 7 m x 0.25 mm ID deactivated column (SGE) at the inlet end). Each of the above mentioned parameters was optimized one at a time.

For esters and higher alcohols, the equilibrium time was evaluated in diluted alcohol solutions containing the mix of esters and higher alcohols, all at the average concentrations found in red wine (Table 1). All the solutions were prepared at room temperature (20°C); the vials were filled with 1 mL of each solution, and loaded on a tray cooler at 20°C. The headspace was analyzed at 15 different times, from 0 to 2880 minutes, and the surface area of each compound of interest was evaluated for each time.

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For the syringe filling rate, gas injection rate and the type of analytical column, vials were filled with 1mL of a solution containing the higher alcohols and the esters prepared at room temperature (20°C).

**Table 1:** Ethyl ester, acetate, and higher alcohol concentrations used for method development.

| *Ethyl Esters and Acetates (µg/L)* | | | | | | | | | | | | *Higher Alcohols (mg/L)* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $C_3C_2$ | $C_4C_2$ | $C_6C_2$ | $C_8C_2$ | $2MeC_3C_2$ | (2S)-$2MeC_4C_2$ | (2S)- and (2R)-$2OH4MeC_5C_2$ | $C_2C_4$ | $C_2C_6$ | $C_2iC_4$ | $C_2iC_5$ | $3OHC_4C_2$ | $3MeC_4C_2$ | 2MB | 3MB | 2MP | P | B |
| 150 | 200 | 200 | 200 | 250 | 50 | 400 | 10 | 2 | 50 | 250 | 300 | 50 | 50 | 200 | 100 | 30 | 4 |

$C_3C_2$, ethyl propanoate; $C_4C_2$, ethyl butanoate; $C_6C_2$, ethyl hexanoate; $C_8C_2$, ethyl octanoate; $2MeC_3C_2$, ethyl 2-methylpropanoate; S-$2MeC_4C_2$, S-ethyl 2-methylbutanoate; $2OH4MeC_5C_2$, ethyl 2-hydroxy-4-methylpentanoate; $C_2C_4$, butyl acetate; $C_2C_6$, hexyl acetate; $C_2iC_4$, 2-methylpropyl acetate; $C_2iC_5$, 3-methylbutyl acetate; $3OHC_4C_2$, ethyl 3-hydroxybutanoate; $3MeC_4C_2$, ethyl 3-methylbutanoate; 2MB, 2-methylbutan-1-ol; 3MB, 3-methylbutan-1-ol; 2MP, 2-methylpropan-1-ol; P, propan-1-ol; B, butan-1-ol.

Partition coefficients were determined according to the "Phase Ratio Variation" method developed by Ettre et al. (1993), by plotting the inverse of the chromatographic areas against the phase ratio β, in order to obtain values for a and b. Glass vials (22.8 mL, Chromoptic, France) were filled with 6 amounts of volatiles solutions in diluted alcohol solution or in dearomatised red wine (0.05, 0.1, 0.5, 1, 1.5 and 2 mL), with phase ratios from 227 to 10.4 (according to the liquid samples volumes).

## Results and discussion

### *Method development*

All the conditions tested and optimized did not allow the detection of all molecules involved in this study, as hexyl acetate and ethyl 3-hydroxybutanoate were not detected. For this last one, in view of its Log P value (0.31), this compound could have a high affinity to the matrix (diluted alcohol solution) and thus be retained in this one. Moreover, it could be hypothesized that these compounds were added at concentrations lower than their limits of detection. For hexyl acetate, this last hypothesis could also be related to its concentration in the matrix (2 µg/L). Chromatographic conditions, and more precisely the phase of the column used (BP21 capillary column, SGE, Nitroterephthalic acid modified polyethylene glycol) did not allow the separation of the 2- and 3-methylbutan-1-ol. The use of other types of columns, such as the CP-Wax 57 (50 m × 0.32 mm i.d.; film thickness, 0.25 µm; Agilent) allowed the separation of these two molecules, but not the detection of most esters used for the aromatic reconstitutions, with only 5 esters being detected. Consequently, the 2- and 3-methylbutan-1-ol were studied as a single peak area for the optimization and the validation of the method. In conclusion, except for ethyl 3-hydroxybutanoate and hexyl acetate, the optimization and validation of the method were realized for the 12 other ethyl esters and acetate, and for the 5 higher alcohols (with the 2- and 3-methylbutan-1-ol as the same peak area).

Optimization of the parameters showed that the thermodynamic equilibrium was achieved after 300 min in diluted alcohol solution for all tested compounds. The optimum syringe filling rate was determined at 750 µL/s and the gas injection rate at 500 µL/s. The investigation of the volume ratios between the gas and liquid phase highlighted that at a volume phase higher than 2 mL, no variation in the peak areas of esters and alcohols in headspace was observed. In conclusion, the volumes used for the partition coefficient determination ranged from 0.05 to 2 mL of liquid phase for ethyl esters and acetates and for higher alcohols. The use of a mega-bore column connected to a micro-bore column at the inlet end allowed to find more esters in the headspace, but also a better resolution for the chromatographic peaks. This type of chromatographic column assembly was called "low-pressure chromatography". In view of these results, but also because low-pressure mega-bore capillary column gave greater sample loadability and ruggedness, all the chromatographic analysis were performed using this column coupled to low-pressure injection (static headspace low pressure gas chromatography coupled to mass spectrometry).

*Application of the new SHS-LP-GC/MS method*

The new SHS-LP-GC/MS method developed and optimized in this work was used to calculate partition coefficients of various ethyl esters and acetates but also higher alcohols. Partition coefficients for 9 esters were calculated in diluted alcohol solution alone or supplemented with average concentrations of 5 higher alcohols.

As shown in Figure 1, in diluted alcohol solution, the addition of higher alcohols led to a significant decrease of the partition coefficients for esters ($p = 0.05$), except for ethyl propanoate ($p > 0.05$). Partition coefficients for higher alcohols were also calculated at average concentrations found in red wines in dilute alcohol solution alone or supplemented with a pool of 13 esters at average levels. Unlike the effects observed on esters, partition coefficients of higher alcohols were not impacted by the addition of these last ones ($p > 0.05$) (results not shown).

As the partition coefficient represents the distribution of molecules between gas and liquid phases, a decrease of this parameter indicates a decrease of the volatilization in the gas phase. These results therefore indicated that the addition of higher alcohols led to a decrease of esters concentrations in the gas phase. These observations could be explained by the fact that the addition of these 5 higher alcohols was added in the dilute alcohol solution at concentrations not included in the infinite dilution region. In the present study, the 5 higher alcohols were added at molar fractions from $4.7.10^{-3}$ to $5.7.10^{-1}$. Alessi et al. (1991) have introduced the concept of "infinite dilution" which correspond to the conditions "were the addition of an infinitesimal amount of the component 1 does not modified the thermodynamic behavior of the mixture, that is like the component 2 does not notice the addition of the component 1". It was also defined that the range of infinite dilution in mixture started at a mole fraction less than $10^{-4}$ (Alessi et al., 1991). These data could explain why in our context the addition of higher alcohols, at concentrations higher than the infinite dilution area, modified esters volatility.

Previous sensory analyses have demonstrated that the addition of higher alcohols led to an increase of the olfactory thresholds of the pool of 13 esters, as well as a decrease of the perception of fruity notes and increase the perception of butyric and solvent notes (Cameleyre et al., 2015). The diminution of the volatility of esters (responsible of the fruity character in red wines) in the presence of higher alcohols is a physicochemical fact, which is totally in agreement with the olfactory decrease of fruity perception as a consequence of the addition of higher alcohols.

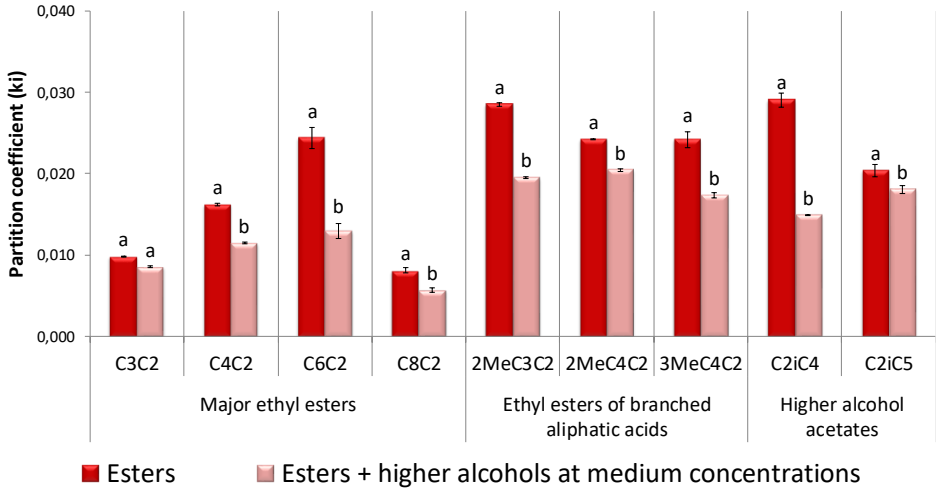


**Figure 1:** Impact of 5 higher alcohols found in red wines on partition coefficient of ethyl esters and acetates in dilute alcohol solution (12% v/v.)

A new approach to calculate multiple partition coefficients in complex mixture has been developed and particularly validated regarding esters and higher alcohols. This approach used a combination of different methods usually found for headspace analysis and characterization on the one hand (static headspace) and for pesticide analysis on the other hand (low-pressure gas chromatography). This methodology consisted in analyzing vial headspace at the thermodynamic equilibrium using a short guard capillary column connected to an analytical mega-bore column finishing at the MS detector. The association of these two techniques led to an increase of the injection volume and to the detection of more compounds compared to a simple micro-bore column, and additionally to a decrease of the run time.

Thanks to this method, it was possible to calculate partition coefficients for a multi-components mixture, and it was highlighted that the addition of higher alcohols in different matrices led to a decrease of the release of esters in the headspace.

## References

1. Alessi, P., Fermeglia, M., Kikic, I. (1991). Fluid Phase Equilibria. 70, 239-250.
2. Cameleyre, M., Lytra, G., Tempere, S., and Barbe, J.-C. (2015). J. Agric. Food Chem. 63, 9777–9788.
3. Ettre, L.S., Welter, C., and Kolb, B. (1993). Chromatographia 35, 73–84.
4. de Zeeuw, J., Peene, J., Jansen, H.-G., and Lou, X. (2000). J. High Resolut. Chromatogr. 23, 677–680.

# Characterisation of wines produced from fungus resistant grape varieties

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## Abstract

To deal with possible consequences of climate change, new varieties of grapes are needed that are resistant to some of the most common diseases that can arise from the changing growing conditions. One possibility is fungus resistant grape (FRG) varieties. Wines produced from these varieties might have different sensory features than wines from conventional cultivars. To characterize FRG wines we used different analytical techniques to quantify odorants in various concentrations. Additionally, a sensory study was done comparing the new wines to common Styrian wines. Using both analytical and sensory tools, a description could be achieved that might reduce consumer bias towards new products.

## Introduction

Wine as an agricultural product is subject to the climatic conditions in its growing area allowing winemakers to cultivate similar grape varieties but still achieve very different wines. However, the changing climate (in Styria a rise of the annual average temperature of +1.5 °C until 2050 is predicted [1]) affects the growing conditions, influencing the vegetation period and might increase the risk of losses because of various plant diseases leading to a larger use of plant protection agents. One possibility to reduce these problems are fungus resistant grape varieties. FRGs are a cross between American and European wine cultivars that are resistant to some of the most common fungal diseases. Earlier attempts to establish FRGs have mostly failed due to unfavourable sensory properties like a ‘foxy note’ (methyl anthranilate; sweet, candy-like strawberry smell). Newer attempts eliminated this and several other problems and the new cultivars are better suited for the production of wines. Nevertheless, a comprehensive characterization of wine can help to find wines that reach the consumers’ expectations and fulfil the winemakers’ needs. Volatile organic compounds contribute to the aroma of a wine depending on their odour thresholds and their concentrations. This means compounds with very low odour thresholds can have an impact even at very low concentrations resulting in the need of analytical techniques with different selectivities and sensitivities to identify and quantify these compounds.

## Experimental

*Wine samples*

Grapes of the FRG varieties Blütenmuskateller, Bronner, Cabernet Blanc, CAL 6-04, Chardonel, Muscaris, Solaris, Souvignier gris and VB 32-7 were grown in an experimental vineyard in the southern Styrian wine region. Out of these, wines were produced at the Fruit Research Station in Haidegg in microvinification using single strain yeasts. This was done to minimize the influence of parameters other than grape variety.

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*GC analysis of the volatile compounds*

Based on the large differences in sensory thresholds for the relevant aroma compounds, the respective instrumental methods had to be adjusted to the required sensitivity. In the following, a few of these techniques are described shortly. In general, volatiles were enriched using headspace solid phase microextraction (HS-SPME) with volumes of 0.1-1 mL of wine. Different methods were applied for some interesting volatile compounds that occur in different quantities (Table 1).

**Table1**: Instrument setting

| | *GC-MS Scan* | *GC-MS SIM* | *GC-MS/MS* |
|---|---|---|---|
| | *Agilent GC 7890* | | *Shimadzu TQ8040* |
| Compound | Aromaprofiles<br>Fatty Acid Ethyl Esters | Linalool | IBMP<br>IPMP |
| Sample | 1 mL wine (Aroma profiles)<br>0.1 mL Ester quantification | 0.1 mL wine + Standard | 1 mL wine + IS Mix |
| SPME | Stable Flex fibre 50/30 µm DVB/Carboxen/PDMS<br>40°C for 30 min | Stable Flex fibre 50/30 µm DVB/Carboxen/PDMS<br>40°C for 30 min | Carboxen Wide Range Arrow fibre<br>60° C for 20 min |
| Column | HP5-MS UI (30 m x 0.25 mm x 1 µm) | HP5-MS UI (27 m x 0.25 mm x 0.25 µm) | ZB5-MS Si (30 m x 0.25 mm x 0.25 µm) |
| Carrier Gas | Helium | Helium | Helium |
| Temperature program | 30 °C for 1 min to 240 °C at 5 °C/min and to 290 °C at 20 °C/min | -10°C (1 min) at 20 °C/min to 100°C to 160 °C at 6 °C/min and to 260 °C at 20 °C/min | 40 °C for 1 min to 200 °C at 40 °C/min and to 310 °C at 25 °C/min for 1 min |
| GC settings | 151 kPa, constant flow 35 cm/s; Injector Temp. 270°C | 8.7 kPa, constant flow 33.25 cm/s; Injector Temp. 270°C | 66 kPa, constant flow 40 cm/s; Injector Temp. 270°C |
| MS settings | Electron ionization<br>Detector voltage relative to tune (2.0 kV)<br>m/z: 35-300<br>5.19 scans/sec | Electron ionization<br>Detector voltage: 1.4 kV<br>Ion used for quantification of Linalool: 93 (dwell time 20 msec) | Electron ionization<br>Detector voltage: 2.5 kV<br>Transitions (Collision Energy):<br>IBMP (RT 10.0-10.8 min):<br>124.10>94.10 (11);<br>124.10>81.10 (7);<br>124.10>79.10 (23)<br>IPMP (RT 7.5-9.5 min):<br>152.10>137.10 (7)<br>137.10>109.10 (7)<br>152.10>124.10 (7) |

*Principal component analysis (PCA)*

With the raw data from the aroma profiles were created using the MASstat software in the 3.02u version. m/z ratios excluded from the calculations were: 28, 32, 77, 133, 151, 207 and 281.

*Sensory evaluation*

To characterize the sensory properties of the FRG wines a tasting with a panel of 11 trained experts (10 men, 1 woman; 24-56 years) was conducted at the Agricultural Research Center using comparative descriptive analysis. The wines were compared to typical regional varieties like Muskateller, Pinot Blanc, Welschriesling and Sauvignon Blanc. In addition, a collection of characteristic descriptors was provided.

## Results and discussion

For the analysis of the wine volatiles, three different techniques were used to get an overview about some of the most abundant volatiles in the upper µg/L range, like short chain fatty acid ethyl esters, as well as to quantify substances down to the low ng/L range like 3-isobutyl-2-methoxy pyrazine (IBMP). IBMP has a sensory threshold of 2 ng/L and is responsible for a characteristic green bell pepper aroma in Sauvignon Blanc wines and other varieties [4]. Higher concentrations of that compound have been associated with unripeness. In the same method, 2-isopropyl-3-methoxypyrazine (IPMP), which has a threshold of 0.32 to 2.29 ng/L [5], was quantified. Additionally, linalool, which is a varietal compound of Muscat wines that has a flowery aroma and a threshold of 15 µg/L [3], was quantified using GC-MS in SIM mode using standard addition.

Looking at Figure 1, three of the wines (Muscaris (with grape skin contact for 2 and 8 h) and Blütenmuskateller) form a separate cluster. Wines from these two cultivars usually have a higher concentration of several terpene compounds. Looking at the data from Table 2, a significantly higher linalool concentration in the wines from the two cultivars corresponds with the PCA results. In addition, the wines of Solaris and the Souvignier gris cultivars show some deviations which can be explained by significantly higher (Souvignier gris) and lower concentrations (Solaris) of short chain fatty acid ethyl esters.

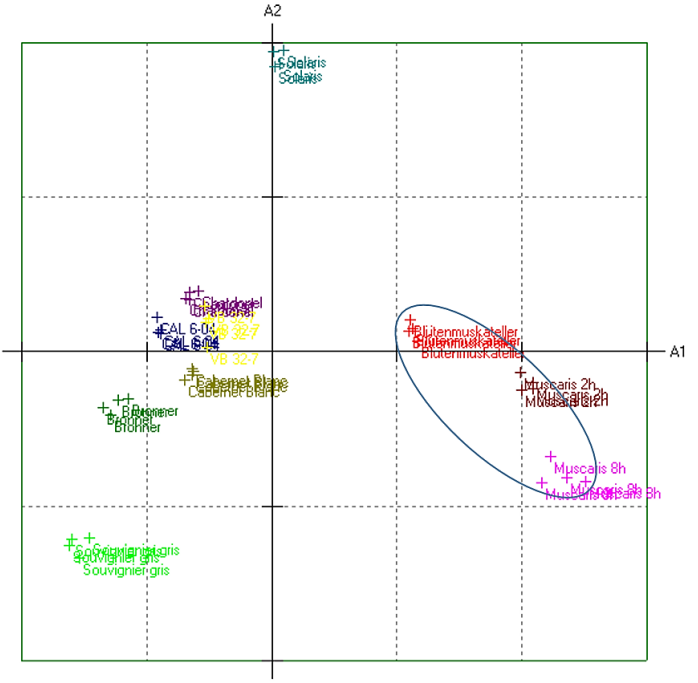


**Figure 1**: PCA on the basis of relative concentrations of different FRG wines (vintage 2016)

Table 2 shows the concentrations of the investigated compounds. Some of them help to explain the results of the PCA given in Figure 1 As this PCA is based on the relative concentrations of the investigated wines, the concentration of IBMP, which cannot be detected in the aroma profiles due to its low concentrations, does most probably not affect these results.

**Table 2:** Concentrations of selected compounds with Odour Activity Values (OAV)

| | *Sum Ester C4-C10 [µg/L]* | *Sum Ester OAV* | *Linalool [µg/L]* | *OAV* | *IBMP [µg/L]* | *OAV* | *IPMP OAV [µg/L]* | |
|---|---|---|---|---|---|---|---|---|
| Blütenmuskateller | 4555 | 1253 | 230 | 15 | <0.001 | <1 | 0.007 | 3 |
| Bronner | 5831 | 1582 | <15 | <1 | <0.001 | <1 | <0.001 | <1 |
| Cabernet Blanc | 5211 | 1476 | <15 | <1 | 0.007 | 4 | <0.001 | <1 |
| CAL 6-04 | 6232 | 1737 | <15 | <1 | 0.006 | 3 | <0.001 | <1 |
| Chardonel | 4692 | 1392 | <15 | <1 | <0.001 | <1 | <0.001 | <1 |
| Muscaris | 3668 | 1032 | 560 | 37 | <0.001 | <1 | <0.001 | <1 |
| Solaris | 2261 | 584 | <15 | <1 | <0.001 | <1 | <0.001 | <1 |
| Souvignier gris | 9735 | 3144 | <15 | <1 | <0.001 | <1 | <0.001 | <1 |
| VB 32-7 | 5904 | 1661 | <15 | <1 | 0.020 | 10 | <0.001 | <1 |
| Analytical Method | GC-MS Scan | | GC-MS SIM | | GC-MS/MS MRM | | | |

**Table 3:** Sensory description of the wines

| *Grape variety* | *Sensory description* |
|---|---|
| Blütenmuskateller | Instantly fragrant, elderberry flower, Muscat-type |
| Bronner | Slightly fruity, neutral, medium bodied, Burgundy-type |
| Cabernet Blanc | Green, spicy, pomaceous fruit, well balanced, Sauvignon-type |
| Chardonel | Apple, banana, neutral, tender, lean, Burgundy-type |
| Muscaris | Flowery, citrus, stone fruits, complex, full bodied, Muscat-type |
| Souvignier gris | Slightly fruity, spicy, full bodied, Burgundy-type |
| VB 32-7 | Green bell pepper, green apple, spicy, full bodied, Sauvignon-type |
| CAL 6-04 | Apricot, apple, lime, black currant |

The results of the sensory evaluations showed a good correlation with the instrumental data. Wines that had higher concentrations of terpenes like linalool were classified as Muscat-type, which usually show higher concentrations of these compounds. Wines with higher IBMP concentrations were the ones that ranked highest in the Sauvignon-type descriptor.

Different instrumental techniques are necessary for interference free quantification of relevant aroma compounds at different concentration levels. The analytical result can provide tools and methods for better understanding of the sensory properties of wine. Knowing the concentration and the impact of some of the key aroma compounds can help to classify new wines in terms that help the communication between winemakers and consumers.

## References

1. APCC (2014): Österreichischer Sachstandsbericht Klimawandel 2014. Verlag der Österreichischen Akademie der Wissenschaften. ISBN 978-3-7001-7699-2.
2. Ferreira V., Lopez R., Cacho J.F. (2000) J. Sci. Food Agric. 11: 1659-1667.
3. Guth H. (1997) J. Agr. Food Chem. 8: 3027-3032.
4. Maga J.A. (1990) In: Flavors and off-flavors. (Charalambous G. ed) Elsevier Science Publishers B.V., pp 61-70.
5. Pickering G.J., (2007) J. Food Science 7: 468-472.
6. http://www.piwi-international.de

# AUTHOR INDEX


**A**

Acree Terry E. 233
Addison James B. 495
Afoakwa Emmanuel Ohene 503
Albietz Chloé 233
Albinus T.T. 463
Algarra Alberto 171
Andriot Isabelle 127
Argelagués Montse 335
Atkinson Ross G. 13
Aubry Aurelie 347
Ayed Charfedinne 37, 73

**B**

Bachtiar Boy M. 367
Backes Michael 363
Balagiannis Dimitris P. 111, 343
Barbe Jean-Christophe 241, 507
Barker Alice 31, 245
Barter Sheridan R. 245
Bauer Patrick 395
Beauchamp Jonathan 281, 377
Beegan Karen 163
Belet Sabine 25
Belloch Carmela 43, 55
Beltrán Leopoldo R. 229
Beltrán Madeline 229
Biasioli Franco 171, 467
Bicas Juliano Lemos 87
Black Cory 19
Blanch Consol 335
Blank Imre 175, 319, 351
Blaško Jaroslav 371
Boulanger Renaud 127
Bouwmeester Harro J. 3
Brattinga Celine 199
Brecheteau Julien 69
Bremer Philip J. 65, 171
Bro Rasmus 421
Brouwer Eric 343
Bücking Mark 463
Bueno Jose Maria 313
Bult Johannes H. F. 199
Buettner Andrea 51, 249, 281, 339, 377, 395,

**C**

Cadwallader Keith R. 427
Cameleyre Margaux 241, 507
Canniatti-Brazaca Solange G. 159
Cannon Robert 483
Cappellin Luca 467
Cariou Véronique 131
Castura John C. 217
Chagné David 13
Chalier Pascale 499
Champion Chloé 407
Capozzi Vittorio 171
Chappuis Charles 383
Charve Joséphine 91
Chen Xiuyin 13
Chenot Cécile 167
Chiba Shingo 451
Clausen Nina 475
Clery Robin 307
Cohen Noam A. 255
Collin Sonia 167
Colstee Hans 483
Çopur Ömer Utku 95, 179
Corbier C. 151
Cornacchia L. 151
Courcoux Philippe 131
Coureaud Gérard 207
Cruijsen Hans 61

**D**

Da Costa Neil C. 483
Dagan-Wiener Ayana 263
Dalmas Solange 77
Dare Andrew P. 13
Darriet Philippe 245
Davidek Tomas 25, 175, 319
Dawid Corinna 229
de Araújo Silva Maria M. 351
de Barros Lopes Miguel A. 245
De Cooman Luc 503
de Kok Peter Maria T. 199
De Ratuld Aurélie 407
de Winne Ann 503
de Wit Rudy 61
Deckers Paul 61
Decourriere Laura 167
Delime Perrine 407
Denk Philipp 377
Desforges Neil 495
Deuscher Zoé 127
Dewettinck Koen 503
Dewitt Christina 99
Dubovski Nitzan 263
Dufosse Thierry 25, 175
Dunkel Andreas 147, 289
Durec Ján 371

**E**

Ellis Lauren 193
Elmore J. Stephen 293
Elsharif Shaimaa Awadain 249
Engel Karl-Heinz 299
Escalante Matthew 115

Espley Richard V. 13
Eyres Graham T. 65, 171

**F**

Farkaš Pavel 371
Farmer Linda J. 325, 347
Feeney Emma L. 163
Ferreira Vicente 123
Festring Daniel 459
Filer Katherine 459
Fillonneau Catherine 471, 479, 491
Fisk Ian D. 73, 119, 151, 269
Fitzke Michael 331
Flores Monica 43, 55
Francis Leigh I. 31, 245
Frank Oliver 229, 439

**G**

Gassenmeier Klaus 307
Gastaldi Emanuelle 499
Ghadiriasli Rahil 395
Gisselmann Günter 229
Glabasnia Arne 91
Glameyer Neele 19
Gloess Alexia N. 143
Gökmen Vural 111
Gordon Alan W. 325
Gourrat Karine 127
Grebneva Yevgeniya 19
Grimm Johanna Elisabeth 213
Grondinger Freya 289
Guichard Elisabeth 37, 127, 207, 221
Guillet Fabrice 491
Gutmann Rene 433

**H**

Haar Nina 415
Haasnoot Sytze 483
Haddadian Zahra 65
Haeck Julie 503
Hagan Terence D.J. 325, 347
Hager Sigrid 411
Haidacher Stefan 433
Hammerl Richard 439
Han Ye 13
Harbourne Niamh 163
Hatakeyama Jun 455
Hatt Hanns 229
Hausch Bethany J. 427
Hayasaka Yoji 31
Haydock Richard 495
Hayes John E. 185
He Fei 99, 103
Heenan Sam 69
Heinemeier Nadine 363
Henze Andrea 229
Herbig Jens 433
Herderich Markus J. 19, 245
Herice Charlotte 507
Hewson Louise 269
Hinneh Michael 503
Hiraki Eri 451
Hixson Josh 19, 31
Ho T.T. 463
Hofbauer Hermann 403
Hofmann Thomas 147, 229, 289, 439
Hoshino Kunihide 237

**I**

Ibáñez Carles 335
Ileperuma Nadeesha 13
Innerhofer Georg 135
Ishizaki Susumu 47
Ito Shinichiro 237

**J**

Jacob-Lopes Eduardo 87
Jaksa Brigitte 411
Janinet Odile 77
Jones Lewis L. 495
Jordan Alfons 433
Jørgensen Niels O.G. 475
Juhari Nurul H. 107
Juhlke Florian 339
Jürschik Simone 433

**K**

Kapp Christian 415
Kashkooli Arman Beyraghdar 3
Kavaliauskaite Gabriele 193
Kawakami Yukihiro 451
Kawaraya Akihiro 451
Kennedy Orla B. 225
Kenny Owen 163
Kerschbaumer Julia 339
Kersch-Counet Christine 61
Khomenko Iuliia 467
Klos Katharina 339
Kocadağli Tolgahan 111
Kohanski Michael A. 255
Koivula Sarianna 355
Kolb Larissa 415
Kolek Emil 371
Komarek David 351
Koopmanschap Gijs 483
Kopuncová Mária 371
Kotthoff M. 463
Koussissi Elisabeth 343
Krammer Gerhard E. 363
Kreissl Johanna 147,487
Kroener Eva-Maria 51
Krstic Mark 19
Kurabe Aki 451
Kurobayashi Yoshiko 47, 147
Kusano Yumi 451
Kuster Bernhard 289

**L**

Laaksonen Oskar 355, 359
Labouré Hélène 127, 221
Le Quéré Jean-Luc 127, 221
Le Roy Sarah 471, 479, 491
Lehmann Katharina 487
Lehner Thomas 411
Leis Dorothea 411, 511
Leitner Erich 135, 411, 415, 511
Leitner Johann 313
Lester Sophie 151
Levermann Janina 229
Li Jia-Xiao 213
Li Bei 285
Liebig Margit 363
Lieder Barbara 275
Lignou Stella 163
Lim Mui 73
Linforth Robert 73, 119, 269
Liu Chujiao 119
Liu Yuan R. 285
Lopez Torres Lizeth 77
López-Diez José Javier 43
Lorber Katja 339
Lubes Giuseppe 351
Lytra Georgia 241, 507

**M**

Macnaughtan William 73
Mall Veronika 83
Märk Lukas 433
Markkinen Niko 355
Märkl Isabella 399
Martin Christophe 221
Martins Sara I. F. S. 37, 445
Matich Adam J. 13
Mayramhof Gregor 433
McMahon Kenneth M. 217
McRoberts W. Colin 347
Médard Guillaume 289
Meerdink Gerrit 115
Meier Lars 363
Meng Chen 289
Methven Lisa 193, 225, 293
Michalet Mathieu 77
Miyazaki Hideki 237
Miyazawa Yamato 47
Monahan Frank 347
Moncunill Daniel 43
Monforte Ana Rita 445

**N**

Neyraud Eric 221
Niedeveld Cor 483
Nissim Ido 263
Niv Masha Y. 263
Nölting Birgit 363
Novotny Ondrej 25, 175

**O**

Oertling Heiko 319
Ohashi Teruhisa 47
Olivares Alicia 331
Oliveira Ines 123
Omori Ken 237
Ongouta Jekaterina 363
Oruna-Choncha Maria José 155, 163
Özcan-Sinir Gülşah 95, 179
Özkan-Karabacak Azime 95
Parker Mango 31
Parker Jane K. 111, 155, 159, 225, 343
Paulino Bruno Nicolau 87
Pearson Wes 31, 245
Pedrotti Michele 467
Pellon Isabel 25
Perea Laura 55
Petersen Nanna Bender 475
Petersen Mikael Agerlin 107, 421, 475
Pet'ka Ján 313
Peyron Stéphane 499
Piel Felix 433
Pika Jana 383
Pinfold Kathleen 495
Pinheiro Pricila Nass 87
Piornos Jose A. 343
Plunkett Blue 13
Podduturi Raju 475
Poisson Luigi 175
Polkinghorne Rod J. 325
Pöllinger Alfred 415
Pöllinger-Zierler Barbara 403, 415
Polovka Martin 371
Polster Johannes 351
Preys Sébastien 127
Prost Carole 131,471, 479, 491
Puerschel Viktoria 229

**Q**

Qian Michael C. 99, 103
Qian YanPing 99, 103

**R**

Rahn Anja 143
Rallapalli Jaganmohan 61
Rannou Cécile 131
Reglitz Klaas 389, 399
Renner Wolfgang 511
Repoux Marie 127
Richter Tobias M. 171
Rigou Peggy 499
Robert Susanne 139
Romagny Sébastien 207, 221
Ross Carolyn F. 217
Ruiz Thierry 499

**S**

Sádecká Jana 371

Saito Tsukasa 47, 147
Sandell Mari 359
Sanderson David 325
Santos Andriéli Borgas 87
Satalik Miriam 229
Sauermann Yvonne 281
Schaefer Uwe 363, 407
Scheffler Laura 281
Schieberle Peter 83, 487
Schoumacker Rachel 221
Schranz Maria 339
Schreiner Linda 395
Schweiger Kerstin 275
Sedlmayer Irene 403
Sémon Etienne 127
Septiana Siska 367
Sharapa Constanze 281
Shibuya Tetsuro 47
Siebert Tracey E. 245
Siegmund Barbara 135, 139, 403
Silcock Patrick 69, 171, 467
Sillner Nina 281
Silva Ferreira Antonio C. 445
Silva Ferreira Carlos 167
Sinding Charlotte 207
Smrke Samo 143
Somoza Veronika 275
Souleyre Edwige J.F. 13
Spada Fernanda Papa 159
Speers Janeen S. 347
Starkenmann Christian 383
Steinhaus Martin 213, 389, 399
Stolle Theresa 289
Strangl Miriam 377
Sulzer Philipp 433
Szolcsányi Peter 313

**T**

Takeda Toshihiro 237
Tamer Canan Ece 179
Tàrrega Amaparo 335
Tauber Iris 135
Taylor Moira 151, 269
Taylor Andrew J. 455
Tempere Sophie 241
Texier Florence 131
Thomas Sini 229
Thomas-Danguin Thierry 207, 221
Thomsen Maiken 207
Tiitinen Katja 407
Timmermans Wim 61
Titt Sascha 229
Tomes Sumathi 13
Torres Míriam 335
Toupin Loic 69

**U**

Umaharan Pathmanathan 115

**V**

van Belzen Lucian 77
Van de Walle Davy 503
van der Krol Alexander 3
Van Durme Jim 503
Vanag Anita 359
Vieira Karem Rodrigues 87
Villière Angélique 471, 479, 491
Vimaleswaran Karani S. 193
Vollmer Kathrin 19
Vossenberg Petra 61

**W**

Wagenstaller Maria 339
Wang Mindy Y. 13
Wei Xuan 293
Wermes Clint 483
White Alison 347
Wicochea Rodriguez José D. 499
Wiedmer Christoph 377
Wijaya Hanny Christofora 367
Williamson Ann-Marie 37
Winkler Klaus 433
Wopienka Elisabeth 403
Workman Alan D. 255

**Y**

Yaguchi Yoshihiro 451
Yamazaki Yuichiro 451
Yang Baoru 355
Yang Hongbo 463
Yang Ni 119
Yauk Yar-Khing 13
Yeretzian Chahan 143
Yin Wenting 269
Yoshida Tetsuya 147
Yotmanee Sanchai 155
Yuliana Nancy D. 367

**Z**

Zaizen Kyoko 451
Zepka Leila Queiroz 87
Zhang Yan 103
Zhang MinWei 103
Zhou Xirui 225

# KEYWORD INDEX


**A**

acetal 213, 313
2-acetyl-1-pyrroline 293, 427
addressing increasing complexity 319
ageing 325
age-related 151
*alcohol acyl transferase 1* (MdAAT1) 13
alkane-1,1-dithiol 213
alkenylated 339
alkylated 339
analytical method 171, 319, 403, 411, 471
appetite 151, 269, 407
apple fruit 13
apple juice 199, 487
apple variety 135
apricot 245
aroma generation 55, 119
aroma pattern 331
aroma precursor 32, 123
aroma quality 29, 119, 487
aroma release 37, 151, 221, 237
aroma stability 371
aroma-active 51, 99, 148, 331, 352, 487
aroma-induced sweetness enhancement 199
aronia 139, 359
*Aronia melanocarpa* 139
*Aronia mitschurinii* 359
artificial leather 389
atmosphere 61, 143, 325, 371, 403
authenticity control case study 363

**B**

beef 285, 325, 351
beer 47, 167, 171, 343, 439
biotransformation 281
bitter taste 151, 193, 255, 263, 459
black chokeberry juice 139
black pepper 229
black truffle 335
breast milk 471
brewing process 155
brown and polished rice 103

**C**

C-6 unsaturated acyclic monoterpene alcohols 249
Cajuputs® candy 367
calcium chloride 111
canned tuna 99
car interior 389
carbonation level 217
cattle 415
cellular mechanism 229
characterisation 51, 83, 135, 159, 343, 511
cheese 65, 131, 221, 331
chocolate 115, 127, 159, 207, 459, 503
cinnamaldehyde augment 275
cinnamon spice 115
Citra and Sorachi Ace 167
clove essential oil 499
cocoa bean 25, 115, 159, 503
cocoa nibs 25, 115, 159, 459
cocoa powder process 25
coffee flavour 143
coffee roasting 119, 143
cold-induced mitochondrial biogenesis 275
complex organic matrix 499
complexity 91, 123, 269, 319, 323
compost bedded pack barn 415
cottage cheese 221

**D**

Damask rose 47
dark chocolate 127
degree of milling 103, 155
development 55, 65, 73, 87, 103, 131, 151, 159, 171, 185, 307, 310, 325, 427, 433, 503, 507
dynamic headspace sampling (DHS) 107, 463, 471
direct-injection mass spectrometry 127
dithio(hemi)acetals 213
dry fermented sausages 55
drying methods/process 95
dual-purpose hop varieties 167

**E**

edible puffer fish 285
emission(s) 377, 395, 403, 415
enzyme treatment 359
esters 13, 95, 125, 135, 139, 179, 241, 249, 363, 487, 507
European Union 299
excretion process 281
exhaled breath 281
extrusion 103, 175

**F**

fabrication process 127
fat 37, 55, 65, 73, 75, 199, 221, 467

fat perception and preference 225
fat sensitivity 221
fatty acids 13, 65, 99, 135, 139, 225, 347, 355, 399, 403, 439
fermentation 25, 43, 55, 91, 147, 155, 159, 167, 171, 355, 439, 467, 503,
flavour analysis 207, 433,
flavour development 27, 65, 171
flavour enhancement 269
flavour generation 87,119, 445, 467
flavour perception 37, 77, 131, 151, 185, 207, 237, 269, 285
flavour potential 43, 87
flavour release 31, 37, 131
flavour research 307, 319, 377
flavoured beverages 199
flavourings 299, 313, 335, 343
food contact material 411
food processing 179, 289
food product(s) 73, 77, 91, 103, 107, 179, 289, 331, 377, 407, 475, 491
formation pathway 175, 327, 377, 403
formation mechanism(s) 123, 175, 319
fractionation 47, 91, 99, 229
fragrant and non-fragrant rice 293
fruit(s) 3, 13, 47, 95, 135, 139, 151, 159, 167, 179, 199, 213, 313, 331, 363, 371, 374
fruity aromatic expression 241
fruity note(s) 19, 139, 213
functional flavour 367
fungus 335, 511
fungus resistant grape (FRG) 511

**G**

garlic odorant 281
gas chromatography-mass spectrometry (GC-MS) 421, 475
gas chromatography-olfactometry (GC-O) 51, 103, 127, 139, 159, 213, 249, 331, 339, 343, 395, 399, 451, 455, 487, 491, 495
GC-Olfactometry-dedicated software 491
genetic variation 185, 225, 255
Ghanaian cocoa 503
glutathione S-conjugates 167
glycosidic aroma 123
grape varieties 123, 511
grapefruit 47, 167, 451
green bean 119
guaiacol-derived odorant 339

**H**

haloanisole 411
halogenated 339
high resolution mass spectrometry 427, 459
high-performance liquid chromatography (HPLC) 167, 285, 439
holistic 91, 319
homologous serie 213
hop 167, 171
hop-derived compound 171
horseradish 51
HPLC-ESI(+)MS/MS 167
Head space solid phase micro extraction (HS-SPME) 87, 176, 335, 371, 463, 471, 478, 503
human milk 281, 283
human nose 207, 411
human recognition 229, 439
human saliva 31, 37

human(s) 3, 31, 37, 179, 199, 229, 233, 249, 253, 255, 275, 281, 339, 359, 383, 395, 407
hydrolytical process 123

**I**

ice-cream 37
Ilzer Rose 135
industrial 25, 87, 91, 99, 131, 351, 371, 475
industrial production 91
infant formula 61
InnOscent chromatographic system 479
intuitive aroma wheel interface 491
*in-vivo* aroma release 37
Islay scotch single malt whisky 83

**J**

jackfruit seeds 159
Jensen Shannon divergence 331

**K**

Keemun tea 147
key aroma compound 25, 127, 147, 343, 351

**L**

*Lactobacillus plantarum* 355
lamb 347
Latent Dirichlet Allocation 331

**M**

malodourous emission 403
malolactic fermentation 355
manufacturing process(es) 25, 115, 147
marker(s) 13, 99, 325, 335, 363, 364, 439
marker volatile 325
mass spectrometry 127, 427, 455, 459
maturation process 83
mature and microgreen coriander 163
meat bouillon 351
meat product(s) 43, 55, 285
mechanism(s) 171, 185, 207, 217, 225, 229, 249, 255, 289, 403, 445
metabolome alterations 439
method development 414

methoxylated 339
methyl cinnamate 363
microalgae in mixotrophic cultivation 87
microorganisms 87, 95, 123, 155, 411, 415, 439, 467, 475
milk fat globule membrane 65
mitochondrial biogenesis 275
model baked system 69
model system(s) 37, 43, 111, 175
modulate appetite 269
molecular 73, 83, 135, 159, 185, 229, 351, 399, 427
multiple partition coefficients determination 241, 507
muscle 255, 325

**N**

Nagoya protocol 307
nasal 151, 207, 221
naturally generated volatile 335
NIR-MVA 445
nitrate reduction 55
nuclear magnetic resonance (NMR) 229, 249, 363, 427, 439
non-food product 377
nonivamide 275
non-nutritive sweetener 193
novel strategies 319
nutrient-induced 439

**O**

odour activity value (OAV) 83, 147, 237, 455, 479, 487, 495
odor-active compound(s) 47, 99, 343, 389, 399, 451, 479, 487
odour mixture 207, 233
odour qualities 213, 339, 340, 349
odour threshold 237, 249, 339, 343, 411, 427, 455, 487, 511,
odour-active 13, 131, 213, 371, 395, 403, 411, 415
off-flavour 77, 99, 102, 123, 347, 371, 403, 411, 475
old Austrian apple variety 135
olfactometry 55, 233, 479, 491
onion juice 483
onion oil 483
on-line 143, 455, 467
On-line high-throughput analysis 467
on-line mass spectrometry 455
optimization 463
oral 225, 289, 367, 491
oral and nasal physiology 151
oral physiology parameter 221
orange juice with pulp 371
organic acids 91, 155, 285, 351, 355
organoleptic properties 77, 123, 127, 131, 335, 459
oxidation processes 55
oxidized aroma 55

**P**

packaging 115, 325, 371, 411
PARADISe 421
peaty aroma 83
2,3-pentanedione 175
Perceptive interactions 241
Petscript® 407
phenylpropene 13
physico-chemical 65, 77, 507
physico-chemical pre-sensorial effects 241
physiological 131, 151, 185, 221, 249, 281, 471
phytochemical 163, 179
pigmented rice wine 155,
plant proteins 77
plants 3, 51, 147, 163, 249, 307, 339, 363, 367, 415
pod storage 503
popcorn-like aroma 293, 427
potassium chloride 55, 111, 131
preparation processes 351
preservative nitrifying agent 43
process control 445
production of esters 13
protective inert gas atmosphere 371
proton transfer reaction – mass spectrometry (PTR-MS) 127, 237, 281, 380, 433
proton-transfer-reaction time-of-flight mass spectrometry (PTR-ToF-MS) 127, 143, 171, 467,
pungent and tingling impression 229
PVC beach ball 399

**R**

rapid 171, 207, 331, 433, 434, 455, 459, 467, 499
ready-to-eat meals 131
real food(s) 37, 175, 199, 207
red wine(s) 241, 433, 507
regional differentiation 463
relation to toxicity 263
respiratory innate immunity 255
retronasal aroma release 37, 237
retronasal threshold 237
rice 427
rice wine 155
Riesling wine aroma 19
ripening 13, 43, 55, 65, 135, 199
roasted cocoa nib 115, 159, 459
roasting 25, 119, 143, 459
roasting of jackfruit seeds 159
roasting temperatures 503
robustness 199, 355, 463
Roselle seed 107

**S**

*S. cerevisiae* 439
safety assessment 299
saliva reactor 37
salivary proteome 289
salt reduction 131
sample preparation method(s) 107, 451
savoury fermented product 91
sea buckthorn (*Hippophaë rhamnoides L.*)
berry juice 355
selective 411, 433
sensitive 91, 143, 193, 221, 289, 411, 433
Sensomics approach 229, 495
Sensomics concept 83, 487
Sensory attributes 61, 163, 217, 245, 347, 467
sensory and nutritional quality 61
sensory properties 139, 293, 319, 339, 359, 367, 407
shelf-life model 61
smell 245, 377, 395, 407
sodium 73, 131,
sodium chloride 73, 111, 131, 289
sourness 13, 217, 355
sparkling wine 217
stability 55, 313, 371, 415,
storage 61, 103, 179, 313, 371, 403, 487
storage conditions 99, 135
storage stability 427
strawberry 13, 179, 363
structural properties 73
structure-odor relationship study 249
structure-odour 213, 249
Styrian ‘super berry’ 139
sugar(s) 13, 95, 111, 119, 155, 163 175, 193, 259, 285, 359, 503
sugar reduction 199
sugar type 69
sweet biscuit 73, 76

**T**

*Takifugu Obscurus* and *Takifugu Rubripes* 287
Taste active compounds 459
taste characteristics 155, 163, 355, 439
taste impact 91, 439
taste receptor 255, 289
taste receptor genotypes 193
taste receptor mechanisms 255
temporal processing 233
terpenoid biosynthesis 3
toilet malodor challenge 383
traditional preparation process 351
transient receptor potential (TRP) channel agonists nonivamide 275
transmission and excretion process 281
*Tuber Melanosporum* 335

**U**

urine 281, 383
ultra-high performance liquid chromatography high resolution mass spectrometry (UHPLC-QToF-MS) 459

**V**

vacuum storage 55, 115
vanilla flavour 77
vegetables 95, 151, 304
vegetable oils 335
volatile organic compound(s) (VOC) 69, 87, 127, 143, 171, 383, 395, 403, 415, 463, 467, 475, 511
volatile composition 179, 335
volatile fraction 343, 471
volatile sulfur compounds 483
volatilome 135, 467

**W**

water-soluble precursors 119
wet cat food 407
wheat flour-glucose model system 111
WheelOscent 491
white adipocytes 275
wine 37, 355, 395, 411, 439, 491, 511
wine aroma 123, 479
wine glycosides 31
wine making 123, 467
wood(s) 377, 395, 415
woody 47, 451
wood pellets 403

**Y**

yeast strain 43, 123