THESIS ON NATURAL AND EXACT SCIENCES B131

Identification of Aroma Compounds in Food Using SPME-GC/MS and GC-Olfactometry

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Kristel Kaseleht

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LOODUS - JA TÄPPISTEADUSED B131

Aroomiühendite määramine toidus kasutades SPME-GC/MS ja GC-olfaktomeetriat

KRISTEL KASELEHT

TTÜ KIRJASTUS

CONTENTS

ABSTRACT – KOKKUVÕTE vii
LIST OF PUBLICATIONS xi
LIST OF CONFERENCE PRESENTATIONS xii
ACKNOWLEDGMENTS xiii
ACRONYMS xiv

THESIS 17

1 INTRODUCTION 19
2 LITERATURE REVIEW 21
2.1 Sample Preparation for Volatile Compound Analysis 21
2.2 SPME Technique for Volatile Compound Analysis 23
2.2.1 Partitioning 24
2.2.2 Optimization of the SPME Method 25
2.2.3 Matrix Effect 27
2.2.4 Quantification with SPME 28
2.3 GC-Olfactometry in Food Aroma Analysis 31
2.3.1 Principles of GC-O 31
2.3.2 Humans as a Detector 32
2.3.3 GC-O Methods 36

3 AIMS OF THIS DISSERTATION 39

4 MATERIALS AND METHODS 41
4.1 Materials and Chemicals 41
4.2 Methods 41
4.2.1 SPME-Gas Chromatography 41
4.2.2 GC-Olfactometry 42

5 RESULTS AND DISCUSSION 43
5.1 Applicability of SPME-GC/MS and GC-O for Determining key Aroma Active Compounds 43
5.2 Multiple Headspace Extraction with SPME-GC/MS 44
5.3 Quantifiability with SPME-GC/MS 45
5.4 GC-O Panel Training 45
5.5 Discussion 46

SUMMARY 49

6 CONCLUSIONS 51
6.1 Conclusions from Publication I 52
6.2 Conclusions from Publication II 52
6.3 Conclusions from Publication III 52
6.4 Conclusions from Publication IV 52

BIBLIOGRAPHY 53

CURRICULUM VITAE 62

APPENDICES 67

PUBLICATION I 69
PUBLICATION II 79
PUBLICATION III 89
PUBLICATION IV 101
DISSERTATIONS DEFENDED AT TUT IN NATURAL AND EXACT SCIENCES 119

ABSTRACT

Aroma is an important feature determining the perception of food. Different methods, both sensory and analytical, are combined in aroma studies. The overall aim of this thesis is to study the feasibility of the headspace solid phase micro-extraction (SPME) method to deal with various analytical challenges in the food industry. The main challenge was optimizing the method for identification of food odor-active compounds to study the mechanisms of aroma formation in specific Estonian products: rye sourdough, "Kama" flour and kvass. In addition, the possibility of using stable isotope labeling for determining compounds of interest is studied. An additional aim was to train an olfactometry panel to recognize aroma compounds and provide an approximate quantification. The instrumental methods used in this work include headspace SPME, gas chromatography (GC), gas chromatograph-olfactometry (GC-O), and measurements with electronic ionization time-of-flight mass-spectrometer (TOF-MS), quadrupole mass spectrometry (qMS) and flame ionization detector (FID).

A suitable method based on SPME was introduced and optimized for determining aroma-active compounds in the Estonian national food "Kama" flour using SPME-GC/MS and GC-O. "Kama" flour is a cereal product of Estonian origin that has recently become an article of export to many countries. Aroma is an important sensory characteristic, yet this is the first study to characterize the chemical components forming the aroma of "Kama" flour. The optimized SPME method can be applied for industrial roasting regime development.

Another study within this doctoral work compared volatile organic compound (VOC) profiles in rye sourdough prepared using different species of *Lactobacilli* using multiple headspace extraction. This study was carried out to understand aroma formation during sourdough fermentation and to provide information for the bread industry about their sourdough constituents and characteristics. The multiple headspace extraction method applied in this study is mostly used to avoid matrix effects in quantification, however, to the author's best knowledge, it has not been used for continuous process monitoring, which proved to be a useful and unique output of the technique.

A method to use SPME was developed to quantify acetaldehyde in solid food matrices. This method provides accurate quantification of acetaldehyde in yogurts, purees, curd creams, etc. especially those consumed by children. The accurate quantification of acetaldehyde in food products became important after the International Agency for Research on Cancer changed the cancer risk classification of acetaldehyde from an agent possibly carcinogenic to humans (Group 2B) to an agent carcinogenic to humans (Group 1). The amount of acetaldehyde, however, was found to be acceptable in the products tested.

Last, but not least, a method for GC-O panel training is proposed. GC-O has proven to be a useful tool in aroma analysis and several GC-O instruments are available on market. However, to utilize the full potential of the method a team of trained assessors is required. A lack of a universal method or set of standards for GC-O panel training prompted the development of a potential standard technique in this study. The method developed was

found to be robust and easy to apply and is based on information available in the literature for GC-O assessor training and sensory panel training. Three different GC-O methods were utilized during panel training to detect key aroma-active compounds in kvass. Kvass is a popular soft drink in Eastern Europe and this study is the first attempt to characterize its aroma notes.

The publications resulting from this thesis describe the aroma of traditional Estonian food products like "Kama" flour, kvass and rye sourdough. The systematic use of instrumental analysis in food flavor determination is fairly novel and only occasionally employed in industrial practice. Combining instrumental analysis with food quantitative sensory evaluation and consumer expectation/acceptance studies examined by Kadri Koppel [1] will benefit the Estonian food industry in food category appraisal. The Competence Center of Food and Fermentation Technologies, where these doctoral works were conducted, will maintain the high level food research activities and modern laboratory infrastructure to support product development in the food sector by consulting and developing consumer tailored methods on descriptive sensory analysis, instrumental analysis of flavor components and combined methods for flavor quality and stability control as well as design flavor profiles of new products.

KOKKUVÕTE

Toidu kvaliteeti määratakse peamiselt välimuse, lõhna, tekstuuri ja maitse järgi ent toidu aroom ja selle stabiilsus on üks tarbijat kõige enam mõjutavamatest näitajatest. Stabiilsuse all käsitletakse nii produkti aroomi püsivust ajas kui ka samasust erinevate tootepartiide lõikes. Säilivusaja ja toidu kvaliteedi seisukohalt on väga oluline ka toidu värskuse kao või toidu käitlemisel tehtud vigadega seotud võõrlõhnade instrumentaalanalüütiline tuvastamine.

Uurimistöö peamine eesmärk oli töötada välja tahkefaasiline mikroekstraktsiooni gaaskromatograaf/mass-spektromeetri (SPME-GC/MS) ja gaas-kromatograaf/olfaktomeetria (GC-O) meetodid, mis tagavad mudeltoodete (kamajahu, kali, rukki haputaigen) aroomianalüüsi hea reprodutseeritavuse ning töötada välja vahendid mudeltoodete aroomi moodustumise dünaamika uuringuteks. Üheks ülesandeks oli ka välja töötada treeningmeetod GC-O assessorite treenimiseks ja moodustada paneel, kes suudaks representatiivselt ning reprodutseeritavalt detekteerida ja nimetada toidus leiduvaid lõhnavaid komponente ning määrata nende suhtelist intensiivust.

Uurimistöö praktiline osa viidi läbi AS Toidu- ja Fermentatsioonitehnoloogia Arenduskeskuses TFTAK, kuhu antud doktoritöö raames installeeriti ka toidu aroomi uuringuteks vajalik aparatuur, kuid lisaks käidi stažeerimas ning viidi osaliselt analüüsid läbi Grazi Tehnikaülikoolis.

Esmalt optimeeriti SPME-l põhinev meetod aroomi ühendite määramiseks kamajahust. Kamajahu on Eestile omane teraviljatoode, mida tarvitatakse koos jogurti, keefiri, hapupiima, vms. Kamajahu aroomi ei ole varem keemiliselt kirjeldatud ning arendatud meetodit saab rakendada tööstuses näiteks röstimisrežiimi optimeerimiseks.

Rukkileiva puhul põhjustab suuri maitse ja aroomi erinevusi erinevate piimhappe juuretiste ja pärmitüvede kasutamine. Selleks, et võrrelda erinevat liiki piimhappebakterite lenduvate ühendite profiili rukki haputaignas, kombineeriti SPME-GC/MS automatiseeritud pideva gaasifaasi ekstraktiooni meetodiga (i.k. multiple headspace extraction e. MHE). MHE on algupäraselt välja töötatud kvantitatiivseks analüüsiks vältimaks toidu maatriksi mõju, kuid seda pole siiani kasutatud pidevaks protsessi monitoorimiseks, mis aga tõestas end üsna kasuliku unikaalse meetodi rakendusena. Töö tulemusena mõisteti paremini aroomi ühendite moodustumise dünaamikat rukki haputaigna fermenteerimisel mudelprotsessis, dünaamikat mõjutavaid tegureid ning anti teavet leivatööstustele nende haputaigna koostisest ja omadustest.

Lisaks töötati välja SPME-l baseeruv ning stabiilse isotoopiga rikastatud sisestandardi kasutamisel põhinev meetod, mis võimaldab mingi kindla toidu komponendi kvantifitseerimist ja ei küllasta gaaskromatograafi väga tundlikku detektorit. Meetodit rakendati atseetaldehüüdi määramiseks pool-tahketes toiduainetes, nagu jogurtid, püreed, kohupiimakreemid jne, mis on poplaarsed toidud ka laste menüüs. Atseetaldehüüdi määramine analüütiliselt ilmus huviorbiiti, kui Rahvusvaheline Vähiuuringute Agentuur (i.k. International Agency for Research on Cancer) muutis atseetaldehüüdi ohtlikkuse klassifikatsiooni

gupist 2B (tõenäoliselt kantserogeenne) gruppi 1 (kantserogeenne toimeaine inimestele). Uuritud Eestis müüdavates toodetes jäi atseetaldehüüdi kogus lubatud piiridesse.

Veel on antud doktoritöös käsitletud olfaktomeetria assessorite problemaatikat. Kuigi GC-O on olnud kasutusel juba üle 20 aasta, puuduvad erinevalt sensoorika paneelist meetodid või standardid GC-O paneeli treenimiseks. Käesolevalt on välja töötatud lihtne meetod GC-O paneeli treenimiseks, baseerudes vähesel kättesaadaval kirjandusel ning kandes üle mõned põhimõtted sensoorika paneeli treenimise standarditest. Treenigu käigus uuriti ka kolme enam kasutatud GC-O meetodit võtmekomponentide määramiseks ning rakendati seda kalja aroomi uurimisel. Kali on Kesk- ja Ida-Euroopas tuntud fermenteeritud karastusjook ning ka kalja aroomi põhjustavaid ühendeid ei ole siiani kirjanduses kirjeldatud.

Käesoleva doktoritöö publikatsioonides on kirjeldatud traditsiooniliste Eesti toodete, nagu kamajahu, kali ja rukki haputaigen aroomi. Toiduaroomi süstemaatilist uurimist viiakse läbi enamikes tööstusriikides, küll aga on see võrdlemisi uus ning minimaalselt kasutatud Eesti toidutööstuses. Kombineerides instrumentaalanalüüsi kvantitatiivse kirjeldava sensoorse analüüsi ning tarbijaeelistuste uuringutega, mis on käsitletud Kadri Koppeli doktoritöös [1], antakse toiduteadlastele ning -tööstusele kasulikku lisainformatsiooni. TFTAK, kus mõlemad doktoritööd on teostatud, on saavutanud seeläbi kõrgtasemelised toiduanalüüsi meetodid, taristu ja teadmised toetamaks toiduettevõtete tootearendust.

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation and are reproduced in the appendices with permission from the publishers.

I Kaseleht K, Leitner E, Paalme T. **Determining aroma-active compounds in Kama flour using SPME-GC/MS and GC-Olfactometry**. *Flavour and Fragrance Journal*, 26(2):122-128, (2011)

II Kaseleht K, Paalme T, Mihhalevski A, Sarand I. **Analysis of volatile compounds produced by different species of *Lactobacilli* in rye sourdough using multiple headspace extraction**. *International Journal of Food Science and Technology*, 46(9):1940-6, (2011)

III Kaseleht K, Paalme T, Nisamedtinov I. **Quantitative analysis of acetaldehyde in foods consumed by children using SPME/GC-MS(TOF), On-fiber derivatization and deuterated acetaldehyde as an internal Standard**. *Agronomy Research*, 9(2): 395-401, (2011)

IV Kaseleht K, Leib M, Kadri K, Paalme T, Leitner E. **Method for GC-Olfactometry Panel Training** *Submitted to the Journal of Sensory Studies*, (February, 10th, 2012)

SUMMARY OF AUTHOR'S CONTRIBUTION

I In Publication I, the author performed the experimental work and interpreted the GC/MS and GC-Olfactometry data, wrote the manuscript and is the corresponding author.

II In Publication II, the author carried out the experimental study with an automated SPME-GC/MS method, interpreted the data, wrote the manuscript and is the corresponding author.

III In Publication III, the author performed the experimental work and interpreted the GC/MS data, wrote the manuscript and is the corresponding author.

IV In Publication IV, the author conducted the training of the panel, wrote the manuscript and is the corresponding author.

LIST OF PRESENTATIONS

I Kaseleht K. **Optimization of Solid-Phase Microextraction for the Analysis of Volatile Compounds in Different Food Matrices**. *2nd Baltic Conference on Food Science and Technology*, June 13-14, 2007, Kaunas, Lithuania

II Kaseleht K. **Optimization of SPME Method for the Analysis of Volatile Compounds in Kvass Using GC/MS**. *EFFoST/EHEDG Joint Conference*, November 14-16, 2007, Lisbon, Portugal

III Kaseleht K. **Traditional Estonian Food —- The Analysis of the Aroma-active Compounds**. *Österreichische Lebensmittelchemiker tage*, May 28-30, 2008, Eisenstadt, Austria

IV Kaseleht K, Leitner E. **Optimization of the Automated SPME Method for the Analysis of Volatile Compounds in Kama using GC/MS**. *First European Food Congress*, November 4-9, 2008, Ljubljana, Slovenia

V Kaseleht K, Leitner E. **Determination of Aroma Active Compounds in Kama using SPME-GC/MS and GC-Olfactometry**. *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy*, March 8-13, 2009, Chicago, USA

VI Kaseleht K. **Quantitative Analysis of Acetaldehyde in Bread with SPME- GC/MS**. *4th Baltic Conference on Food Science and Technology*, May 12-13, 2009, Kaunas, Lithuania

VII Kaseleht K, Paalme T, Mihhalevski A, Sarand I. **Comparison of Volatile Organic Compounds (VOC) in Rye Sourdoughs Prepared Using Different Starter Cultures of *Lactobacilli***. *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy*, March 28-5, 2010, Orlando, USA

VIII Kaseleht K, Leib M, Leitner E, Paalme T. **GC-Olfactometry Panel Training**. *9th Pangborn Sensory Science Symposium*, September 4-8, 2011, Toronto, Canada

IX Kaseleht K, Paalme T, Nisamedtinov I. **Acetaldehyde in foods for children**. *Flavor Conference*, October 25-28, 2011, Tallinn, Estonia

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This work is dedicated to my grandfather Vambola Puru – ei ole enam "supikeetja".

ACRONYMS

AEDA	aroma extraction dilution analysis
ASE	accelerated solvent extraction
CHARM	combined hedonic and response measurement
DF	dilution factor
FID	flame ionization detector
GC	gas chromatography
GC-O	gas chromatograph-olfactometry
IDA	isotope dilution assay
ITEX	in-tube extraction
LAB	lactic acid bacteria
LC	liquid chromatography
LLE	liquid-liquid extraction
μ-LLE	micro liquid-liquid extraction
LPME	liquid-phase microextraction
LRI	linear retention index
LTPRI	linear temperature programmed retention index
MASE	microwave assisted solvent extraction
MHE	multiple headspace extraction
MS	mass-spectrometry
NIF	nasal impact frequency
OAV	odor active value
OT	odor threshold
SAFE	solvent assisted flavor evaporator
SBSE	stir bar sorptive extraction
SDE	single drop extraction
SIDA	stable isotope dilution assay
SPDE	solid-phase dynamic extraction
SPE	solid phase extraction
SPME	solid phase micro-extraction
TIC	total ion count
TOF-MS	time-of-flight mass-spectrometer
VOC	volatile organic compound
qMS	quadrupole mass spectrometry

CHEMICALS AND ENZYMES

AdC	Adenylyl cyclase, EC: 4.6.1.1
ATP	adenosine-5'-triphosphate, KEGG: C00002
cAMP	adenosine-3',5'-cyclic phosphate, KEGG: C00575
CAR	Carboxen™: adsorbent very similuar to activated carbon.
CW	Carbowax™: polyethylene glycol with specific molecular weight.
DVB	p-Divinylbenzene, PubChem: 24878089
PA	polyacrylate, PubChem: 24866330
PDMS	polydimethylsiloxane, PubChem: 24888348
PFBAH	o-[(2,3,4,5,6-pentafluorophenyl)methyl]hydroxylamine hydrochloride, PubChem: 114916636
Tenax™	poly(2,6-diphenyl-p-phenylene oxide), Sigma Aldrich™: 181781
PTFE	polytetrafluoroethylene, PubChem: 24870488
vanillin	4-hydroxy-3-methoxy-benzaldehyde, PubChem: 24430806

THESIS

1

INTRODUCTION

The characteristic aroma of food products is a result of complex multisubstance mixtures, containing often hundreds of compounds. These volatile components influence the enjoyment and acceptance of foodstuffs.

Aroma compounds are relatively small (< 400 Da) mostly organic compounds [2] that have an odor when two conditions are met: (I) the compound is volatile, so it can be transported to the olfactory system in the upper part of the nose, and (II) it interacts with one or more of the olfactory receptors. As all aroma compounds are volatile, it must be noted that not all volatile compounds react with human receptors or might be present in an insufficient concentration to trigger a signal. The chemical structures of aroma compounds vary widely; they include acid and alkali compounds, sulfur and nitrogen compounds, alcohols, aldehydes, ketones, hydrocarbons, esters etc. There are also large differences in the volatility of aroma compounds, ranging from components with boiling points well below room temperature (hydrogen sulfide, -60 °C) to compounds that are solid at room temperature (vanillin, 284 °C).

Only in some cases the characteristic aroma of a food product can be narrowed down to one particular compound, for example, 4-hydroxy-3-methoxy-benzaldehyde (vanillin) owing to their association with the aromas of vanilla [3]. More commonly, the mix of volatile compounds forms an aroma. An example is coffee where over 800 volatiles have been found, however, only three dozen have a considerable impact on the overall aroma and none of the individual volatiles really smells like coffee [3]. Accurate identification of each unique volatile compound that contributes to the overall aroma of a particular food is the ultimate goal of aroma research.

When determining the aroma profile of food products, one must consider the aroma extraction procedure. Headspace techniques for extracting volatile compounds from food have several advantages. They are solvent-free, simple and fast sample preparation methods to use with any (food) product and do not contaminate the gas chromatography (GC) with non-volatiles. Ideally, one would inject a known volume of static headspace above a sealed food sample because the untreated headspace reflects the original composition of flavor compounds in the gas phase above a product. However, the concentration of some compounds would be insufficient to detect with some detectors. To concentrate the volatiles, the solid phase micro-extraction (SPME) technique is widely used. Volatiles are adsorbed onto fibers followed by desorption in the GC to achieve a relatively high sensitivity. However, the quantification of volatiles using SPME has a number of issues. Firstly, the analytical data is received only for the headspace of the sample, but the relationship between concentrations in the headspace versus the food can be very complex. Secondly, the SPME fiber

does not adsorb volatiles in equal proportions and thus introduces a bias of the composition of the headspace. In this thesis the applicability of SPME is tested for resolving different food-related problems.

For the past four decades the standard approach to aroma analysis has been to use gas chromatography coupled with mass spectrometry (GC/MS) to establish a chromatographic fingerprint representing the volatile composition or volatile profile of the product. This technique often falls short of accurately defining the true aroma profile of a food because it does not provide direct information regarding odor description and often character-impact aroma components are present at levels below the detection limits of conventional GC/MS instruments. MS-detectors are not as sensitive to odor-active compounds as the human nose [4]. The use of gas chromatograph-olfactometry (GC-O) was proposed as early as 1964 [5] and has shown to be a valuable method for the selection of odor active compounds from complex mixtures. GC-O combines an instrumental method (GC) with human assessors, involving extraction of aroma volatiles from the sample, separation of chemical components as they pass through a GC column, and finally smelling of the individual chemical compounds as they elute from the column. The accuracy of GC-O is very much dependent on the performance of olfactory assessors (sniffers). Individual human assessors have different abilities to detect the odor of each compound that emerges from the GC. To ensure that the panel provides representative and reproducible data a team of assessors needs to be trained to be able to measure the duration of an odor from the start to the end, to describe the odor and to quantify its intensity.

This work was initiated by the practical need of the Estonian food industry to estimate food quality, including food aroma stability, as well as detection of off-flavor and package-related contaminants. In terms of analytical objectives, one may wish to accurately identify and quantify every aroma constituent in a food or only key components of an aroma profile, that is, those components that are responsible for the characteristic food aroma. GC-O could also be used to identify off-note compounds, and determine why they are present in a food product, monitor aroma changes in time, or predict sensory attribute(s) or determine if a food flavoring has been adulterated.

This doctoral work makes use of SPME combined with GC/MS and GC-O to address several aroma-related challenges including determining key aroma compounds, volatile quantification, and studying the production of volatiles by different species of bacteria. This study is the first to characterize a number of key odor-active components of Kama flour, and is the first to apply the multiple headspace extraction (MHE) method for investigating in-vial volatile organic compound production by lactic acid bacteria in sourdough. In addition, a method was developed to accurately quantify acetaldehyde from solid food matrices using SPME. Also, a method for GC-O panel training was developed and critically reviewed.

2

LITERATURE REVIEW

The purpose of modern aroma analysis is to qualitatively and quantitatively decipher the aroma profile defined by nature and, most importantly, to recognize and prioritize organoleptically interesting aroma compounds and distinguish them from other volatile compounds that may have no organoleptic relevance or may merely balance an aroma.

2.1 SAMPLE PREPARATION FOR VOLATILE COMPOUND ANALYSIS

Sample preparation is most often required before aroma analysis to either (I) concentrate the sample, (II) in the case where the sample matrix is not suitable for direct injection into a GC, or (III) removal of interfering compounds. The primary chemical properties used to isolate aroma constituents from food components are volatility and/or solubility. It is problematic that water is, with few exceptions, the most abundant volatile constituent in a food. This creates a problem because isolation methods based on volatility also include water from the food [6]. Additional step needs to be applied to concentrate aroma compounds from water. Most (but not all) aroma compounds have greater solubility in organic solvents than in water, while the bulk of the major food constituents are more hydrophilic [6]. Unfortunately food lipids are also soluble in organic solvents, thus solvent extraction cannot be used if lipids are present or further steps need to be taken to separate the aroma from lipids. The use of molecular distillation, steam distillation, purge-and-trap, or dialysis all further complicate the isolation process and introduce additional biases in isolation.

Various methods exist for extracting the volatile components from foods into a form more easily analyzed. These methods include static and dynamic headspace sampling, solvent extraction, and distillation techniques. Each type of method has distinct advantages and disadvantages and each of these factors must be considered when choosing a method of extraction. In flavor chemistry, for example, high temperature extraction methods (*e.g.* steam distillation) often lead to artifact formation and ultimately inaccurate data [7]. In case of solvent extractions, not all solvents extract organic compounds equally (dichloromethane versus pentane) [8], and not all solvents may be used with GC-O. Also, highly volatile compounds may be masked by the solvent in GC analysis, so for these compounds headspace methods are preferred.

Liquid-liquid extraction (LLE) is one of the oldest, yet most frequently used sample preparation techniques. It is as simple as putting the food sample (*e.g.* apple juice) into a separatory funnel, adding a solvent (*e.g.* dichloromethane) and shaking. The dichloromethane is collected from the separatory funnel, dried with an anhydrous salt and then concentrated

for GC analysis. The disadvantages of this approach include, the time required for analysis, the use of large amounts of harmful solvents, the often toxic waste generated by LLE, the nondiscriminatory nature of this approach, the required application of post-extraction purification, and the loss of volatile components during concentration.

Several strategies have been devised to address these problems. The development of micro liquid-liquid extraction (μ-LLE) drastically reduces the amount of solvent used while simultaneously leading to an increase in sensitivity due to a more favorable phase ratio [9]. Other variants include microwave assisted solvent extraction (MASE) [9] where microwaves are used to enhance extraction efficiency and ultrasonic extraction where ultrasound is used for the same reason. Another technique, termed accelerated solvent extraction (ASE) [10] makes use of temperature and pressure to increase extraction efficiency and reduce extraction time. Recently, single drop extraction (SDE) [11] has been described.

Supercritical CO_2 extraction offers the advantage of a very low boiling point of the solvent leaving no residue to interfere with any subsequent analysis. It penetrates food matrices and its solvent properties can be altered through temperature and pressure changes or the use of chemical modifiers (*e.g.* methanol) [6]. However, application to food products is limited by the high cost of equipment and the non-polar nature of CO_2 (without modifiers) [12]. Another common solvent-extract technique is simultaneous distillation extraction, which simultaneously distills and solvent-extracts a sample [13]. Samples are prepared for simultaneous distillation extraction by making a homogeneous mixture of the sample with water. The sample and solvent are contained in separate flasks. Both flasks are boiled, vapors mix together, condense, and are separated into respective flasks by density. When performed under vacuum, the potential for artifact formation is reduced. A relatively modern approach termed solvent assisted flavor evaporator (SAFE), is a method of separating volatile from nonvolatile food components and is typically applied in conjunction with solvent extraction. It has gained popularity due to the ability to isolate volatiles from numerous food matrices also without solvent extraction [14].

Developed as an alternative to LLE, solid phase extraction (SPE) is a very popular sample preparation technique because of its versatility [15]. A suitable sorbent material is packed into a cartridge, the sample is loaded, and interfering compounds can be rinsed from the cartridge before the analytes of interest are eluted with a strong solvent to remove them from the stationary phase. Flow can be obtained by using a vacuum manifold or by applying positive pressure. The versatility of this technique is a result of the different separation mechanisms it offers such as adsorption, partitioning, affinity or ion exchange. SPE offers several attractive benefits such as high sensitivity, low solvent consumption, high selectivity and the option of automation [15].

One property that an aroma constituent must inherently possess is volatility. Several techniques use this property directly, such as static headspace, dynamic headspace, molecular distillation, steam distillation, and direct injection techniques (where food is placed in the apparatus itself and heated to volatilize aroma constituents). Direct analysis of the equilibrium headspace above a food product would appear to be an ideal method for aroma studies. This method analyzes exactly what the olfactory receptors receive. Also, the method is very simple and gentle – one simply draws a few milliliters of vapor above a food into a gas-tight syringe and makes a direct injection into a GC. The primary limitation of the technique is inadequate sensitivity.

One technique for aroma isolation based on volatility is purge and trap. It is used for the extraction of volatiles from liquid samples, providing high sensitivity and highly purified samples. An inert gas is bubbled through the liquid sample and volatiles are released into the gas phase prior to being trapped on an adsorbent trap (typically Tenax™ or activated charcoal) at low temperature. After sampling, the trapped volatiles are desorped, either in a thermal desorption system, or by using a suitable solvent for elution.

Around 15 years ago, SPME was introduced as a solventless equilibrium microextraction method [16]. Since then, other related microextraction methods such as stir bar sorptive extraction (SBSE), liquid-phase microextraction (LPME) and several in-tube or in-needle extraction techniques were developed to overcome various fiber related drawbacks. Methods such as in-tube extraction (ITEX) offers the advantage that a variety of commercially available sorbent materials and larger amounts of sorbent material can be used to obtain higher extraction yields compared to coated extraction phases [17]. Another in-tube technique is termed solid-phase dynamic extraction (SPDE). In this technique the sorptive layer is coated on the inside of a syringe needle and dynamic sampling is achieved by continuously filling and emptying the syringe with the sample.

This study reviews techniques for measuring the release of aroma compounds in foods using the SPME method.

2.2 SPME TECHNIQUE FOR VOLATILE COMPOUND ANALYSIS

The method of SPME, developed by Pawliszyn [16], utilizes a short, thin, solid rod containing fibers of fused silica, coated with an absorbent polymer. The polymer is attached to the silica fiber without bonding, with bonding, partial cross-linking or high cross-linking [18]. Generally, very small amounts of polymers are used with a coating thickness that is usually between 7 and 100 μm (for PDMS fibers). The total phase volume is less than 1 μL, depending on the coating dimensions. For analysis, the sample is placed in a vial, which is then closed with a septum and a cap. Fiber is then introduced into the headspace of the sample or in a direct extraction mode into the sample. The volatiles then partition between the sample matrix, the air and the immobilized stationary phase. The partitioning of the volatiles between these phases depends on the volatility of each compound, their concentrations, the volume and composition of the phases, as well as the sorption time, which is often diffusion limited.

The amount of the absorbed volatile depends on many SPME parameters such as the type of the fiber, film thickness, sample volume, temperature, extraction time, salting, adjusting pH, mode of extraction (headspace or direct extraction) and derivatization. Each chemical component will behave differently depending on its polarity, volatility, organic/water partition coefficient, the volume of the sample and headspace, the rate of agitation, pH of the solution, and the temperature of the sample.

Following sampling, the concentrated compounds are thermally desorbed in the injector of a gas chromatograph and transferred to the capillary column [19]. The parameters to be optimized in this procedure are desorption time and temperature. Care should be taken that the compounds of interest may be thermally labile and could decompose in the hot GC injector. This problem has been reported for a number of volatile sulfur compounds [20].

The extraction and desorption steps of SPME-GC are amenable to automation and provide the additional capability of analysis in series. In an automated system, the exposure time of the fiber can be precisely controlled. As a result, relative standard deviation values (% RSD) are less than 5% for most target analytes [19].

The solid-phase microextraction technique is independent of the form of the matrix; liquids, solids, and gases all may be sampled readily. Because the SPME technique requires no solvents and can be performed without heating the sample, the formation of chemical artifacts is reduced compared with other methods. Volatile compounds sorb from the sample naturally and chemical interactions are negligible between volatile compounds in the headspace as well as on and within the adsorbent.

2.2.1 *Partitioning*

Solid-phase microextraction equilibrium is a multiphase system, and in the following discussion a three-phase system is considered: (I) a homogeneous sample matrix, (II) the headspace above the sample, and (III) the fiber coating. The equations governing the equilibrium process between the three phases are:

$$K_{fh} = \frac{C_f}{C_h}, \qquad (2.1) \qquad K_{hs} = \frac{C_h}{C_s}, \qquad (2.2) \qquad K_{fs} = \frac{C_f}{C_s}. \qquad (2.3)$$

Where, K_{fh} is the partitioning coefficient of an analyte between the fiber coating and headspace phases, K_{hs} between the headspace and sample phases, and K_{fs} between the fiber coating and sample phases. C_f, C_h, and C_s are the concentrations of the analyte in these phases.

As a result, the amount of analyte absorbed by the fiber coating in the headspace sampling can be expressed as:

$$\eta_f = \frac{K_{fs} \cdot V_f \cdot V_s \cdot C_o}{K_{fs} \cdot V_f + K_{hs} \cdot V_h + V_s}. \qquad (2.4)$$

Where, η_f is the amount extracted by the coating and C_o is the initial concentration of a given analyte in the sample. The three terms in the denominator represent the analyte capacity for each phase: fiber ($K_{fs} \cdot V_f$), headspace ($K_{hs} \cdot V_h$), and the sample itself, V_s.

In Equation 2.4, several parameters can be changed to affect the amount of analyte adsorbed, such as fiber coating volume V_f determined by fiber surface area and layer thickness, the coating/water distribution constant K_{fs}, by changing to a fiber coating that is more selective for the target analytes, and optimizing the sample temperature. Thicker coatings require longer extraction times. Often, shorter equilibration times can be achieved by stirring the sample to increase the mass transfer to the fiber.

Because the adsorption process is limited by diffusion on the fibers, the sample concentration has little effect on the concentration time profile and the equilibration time, and

instead depends on processes that occur on the adsorbed layer [21]; This means that as long as the extraction method and distribution constants between the SPME/sample system remains constant, the system will behave linearly [16].

The distribution constants between the adsorbent and the headspace can be predicted using the gas chromatographic retention time of the target analyte relative to the retention times of n-alkanes [16]. The linear temperature programmed retention index (LTPRI) must be determined using a chromatographic column with the same stationary phase as the SPME coating. These retention indexes are found in tables or can be determined experimentally.

For a given analyte, the LTPRI is calculated as follows:

$$\text{LTPRI} = 100 \cdot \left(N + \frac{t_{R(a)} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}} \right). \tag{2.5}$$

Where N is the number of carbon atoms of the smaller n-alkane; $t_{R(a)}$, the flavor compound retention time in the chromatographic column (in seconds); $t_{R(n)}$ and $t_{R(n+1)}$ are the n-alkane retention times in the chromatographic column, one less and one more than $t_{R(a)}$, respectively.

2.2.2 *Optimization of the SPME Method*

A properly optimized method provides good accuracy and precision together within the detection limits of the instrument. The most important principles which should be considered when optimizing SPME methods are summarized in this section.

The selection of *fiber coating* is based on the chemical nature of the target analyte, primarily on the polarity, hydrophobicity and volatility. At the moment three types are commercially available: special alloy, fused silica and stable flex polymer, the latter being mostly used in food applications. The extraction of the analyte by the polymer film can either be an absorption process, in which analyte molecules are absorbed by the polymer film, or an adsorption process where analyte molecules are adsorbed on the surface of the polymer film. To extract a wider range of flavor compounds several adsorbents are combined in SPME fibers. Also the coating thickness and the distribution constant determine the sensitivity of the method. Thick coatings offer increased sensitivity, but require much longer equilibration times [22]. It is not recommended that SPME fibers be used for more than one hundred extraction-desorption cycles [23]. After several uses, SPME coatings are damaged, exposing the silica fiber. However, using an auto-sampler, the cycle amount is increased. Metal fibers have been demonstrated to be stable over 350 extractions with the CombiPAL automated system [24]. Fiber coating damage can be monitored using a microscope. Also, fiber coatings of one type can differ slightly from fiber to fiber [25] and therefore, if possible, all parallel samples should be analyzed for better reproducibility with one fiber.

The linear dynamic range for the SPME method should also be determined. SPME coatings include polymeric liquids, such as PDMS, which, by definition, exhibit capability for a very broad, linear range. For solid sorbents, such as CW/DVB or PDMS/DVB, the linear range is narrower due to the limited number of sorption sites on the surface, however, it may still span several orders of magnitude for typical analytes in pure matrices [26]. In some rare

cases, when the analyte has an extremely high affinity towards the adsorption surface, saturation can occur at low analyte concentrations. In such cases, the linear range can be expanded by shortening the extraction time.

The fiber must also be *conditioned* prior to first use by desorption in a GC injector for 30 minutes to 2 hours depending on the fiber type. Fiber conditioning has to be repeated also every time before sampling for 10 minutes to remove compounds adsorbed from laboratory air.

In SPME analysis it is ideal if equilibrium is reached between the sample, headspace and the fiber coating, and the adsorption processes collects all the volatiles present in the sample on the fiber. In practice, compromises are required. If the extraction time and mass transfer conditions are strictly controlled, good precision may be achieved. At the steep part of the adsorption curve, the relative error is large even with small variations in the extraction time. Under these conditions auto-samplers should be used.

Agitation facilitates mass transport between the sample, the headspace and the fiber. Magnetic stirring and shaking is most commonly used in SPME experiments, however, care must be taken to avoid splashing of liquid samples onto the fiber. The *volume of the sample* is yet another parameter to optimize. It depends on the estimated distribution constant K_{fs}. This distribution constant can be estimated using literature values for the target analyte or a related compound with the coating selected. K_{fs} can be calculated or determined experimentally by equilibrating the sample with the fiber and determining the amount of analyte extracted by the coating. As the sample volume V_s increases, so does the amount of analyte extracted until the volume of the sample becomes significantly larger than the product of the distribution constant and volume of the coating ($K_{fs} \ll V_s$) [22].

Adjustment of the *pH* of the sample can improve the sensitivity of the method for basic and acidic analytes. Unless ion exchange coatings are used, SPME can extract only neutral (non-ionic) species from water [22]. By properly adjusting the pH, weak acids and bases can be converted to their neutral forms which are more volatile and can be extracted by the SPME fiber [22].

Salt modifies the equilibrium partition of flavor compounds by decreasing the water activity, which leads to an increase in the release of flavor compounds into the vapor phase. This phenomenon is described as a "salting-out" effect. In order to increase the amounts of flavor compounds extracted on adsorbents, salts (NaCl, KCl) are often added to food matrices. Nevertheless, the salting-out effect is limited. Steffen and Pawliszyn [27] reported a saturation effect for a PA fiber with higher salt content. Saturation was noted at 36% salt in aqueous solutions; the amounts of flavor compounds extracted were not higher than with 42% NaCl, except for hexyl alcohol and ethanol.

Conclusively the most important factors affecting the SPME are: agitation conditions, sampling time (if non-equilibrium conditions are used), temperature, sample volume, headspace volume, vial shape, condition of the fiber coating (cracks, adsorption of high molecular weight species), geometry of the fiber (thickness, length), sample matrix components (salt, organic material, humidity, ethanol etc.), time between extraction and analysis, analyte losses (adsorption on the walls, permeation through Teflon, absorption by septa), geometry of the injector, fiber positioning during injection, condition of the injector (pieces of septa), stability of the detector response, and moisture in the needle.

To ensure good reproducibility of the SPME measurement, the experimental parameters listed above should be kept constant or internal standards should be used. The optimization of solid-phase microextraction has been well documented in [28] and [29]. Detailed overviews of theoretical aspects are available in [16], [30] and [22]. Also, the latest edition of [31] includes a complete discussion of these topics.

2.2.3 *Matrix Effect*

The headspace SPME-GC method concentrates odorants in the sample by adsorption onto coated fibers. This does not take into account how a compound is held or released from a food matrix, where only a proportion of the compounds present will be volatile in the gas phase. For headspace SPME, the proportion of flavor compounds extracted in a 100 µm PDMS fiber varied by 0.22% (ethyl hexanoate) and 0.01% (trans-2-hexen-1-ol) of the total amount of each compound in the sample [32]. This variability in extraction is due to the fact that the amount of polymer is very small compared to the amounts of the other sample phases (liquid, solid and vapor) [33] and compounds present in the gas phase depend upon the solubility and binding to nonvolatile components [34].

For example, polysaccharides, such as dextrins and gums, are known to interact with flavor compounds and are used to stabilize flavors in food preparations. Higher concentrations of simple sugars generally result in increased gas/liquid partition coefficients [35, 36]. A sort of "salting-out" effect is likely to be the reason for this phenomenon, probably by changing the activity coefficients in this thermodynamic system [37].

Two types of interactions can occur between flavor compounds and proteins: (I) reversible physical adsorption via non-covalent interaction and (II) chemical reaction via covalent linkages. Flavor compounds bind to proteins only when binding sites are available, and not when the sites are saturated by protein-protein or other interactions [38].

Fats will act as a strong solvent depending upon the relative hydrophobicity of volatile compounds. In the absence of fat, the food matrix retains lipophilic flavors poorly and the resulting headspace concentrations are high. Binding to the water phase tends to reduce the volatility of polar compounds in much the same way that oils bind non-polar flavor compounds. This results in an increase in vapor pressure for a number of components at low concentrations and a marked decrease for others [39].

In addition, ethanol can be considered as a type of matrix effect, it does not bind aroma compounds, but it might saturate the small volume of the fiber coating and affect the binding properties. In this case, it becomes necessary to dilute the solution with water before the analysis.

Although, SPME is particularly sensitive to matrix effects and the matrix is usually not reproducible even for samples from the same origins. Most of these concerns can be alleviated by converting the solid material into a solution or suspension before analysis [40]. It should be taken into account that in case of suspended sample we have already a four phase system.

To overcome the matrix effect and recover the low volatile flavor compounds that are bound to food matrices, a combination of the immersion and headspace extraction methods has been developed. The method consists of inserting a vapor permeable device into the

matrix. A vapor phase in equilibrium with the matrix is created inside the device. The adsorbent is then inserted inside of this vapor phase to extract the flavor compounds. Several applications have been developed for SPME. The SPME fiber was inserted in a porous tube and then placed in the sample. This extraction mode is called in-tube SPME. One disadvantage of extraction in the vapor phase is the fact that the analytes must be volatile enough to reach the headspace above the sample to be extracted on the adsorbent [33].

2.2.4 *Quantification with SPME*

In the ideal case quantitative analysis involves completely extracting an analyte and analyzing the entire sample in the analytical instrument. This approach is only rarely possible with SPME. However, it is not always necessary to extract all of the analyte in a sample. It might be sufficient to be able to consistently measure the relative amount of a component over a period of weeks or months to satisfy the requirements of a shelf-life study, for example.

Another point to consider with SPME is fiber-to-fiber variations, which have been recognized as a problem in quantitative analysis. Considerable differences were observed between the responses of the three CAR-PDMS fibers employed, which corroborates the low reproducibility of this type of fiber. Because of this, the complete set of experiments should be performed with a single fiber or a control sample should be used [25]. The use of a control sample is also recommended to chart the aging of the polymer.

Despite these shortcomings there are a number of commonly applied methods for quantitative analysis with SPME.

2.2.4.1 *External Standard Calibration*

An external standard curve with different known concentrations is made with the compound of interest and is used to calculate the concentration in unknown samples. This method, however, requires a pure reference compound of the analyte to be available and a very reproducible, preferably automated, injection system.

External SPME calibration technique rarely produce acceptable results due to matrix effects that cause considerable differences in the distribution coefficients and release rates for different analytes. This method has thus been suggested to be useful for analyzing less complex matrices such as aqueous solutions [33]. When performing calibration of the extraction method, care should be taken that the fiber is not saturated. This means the amount of flavor compound is lower than that leading to saturation of the relevant isotherm [33]. Otherwise, the internal standard and the other target flavor compounds will compete and displace each other from the adsorption sites. This will modify the efficiency of sorption of each analyte on the adsorbent and lead to an erroneous calibration and subsequent quantification. With SPME, saturation is possible at relatively low flavor concentrations because the adsorption capacity is limited by the relatively low amount of adsorbent used. Several authors, for example Yeung*et al.* [41] who studied the peppermint flavor loss in a taste-masked tablet formulation, as well as Ostroukhova and Zenkevich [42] who compared the accuracies of the external standard and standard addition methods by analyzing model samples

containing known amounts of pesticides in various plant materials, have observed the matrix effect and found that the use of the standard addition method considerably decreases the systematic error during quantification.

2.2.4.2 *Standard Addition*

Standard addition (spiking) involves adding known quantities of the target analyte to the sample matrix, which initially contains an unknown concentration of the analyte, and this mixture is then analyzed [31]. A plot of the responses for the range of target analyte concentrations is then developed, and the extrapolation of the response to zero defines the original concentration in the unspiked sample. To obtain the best precision (2-5%) it is recommended that one analyze at least three standard addition samples at different concentrations in triplicate [31]. The major drawback to this method is the large number of additional samples that must be analyzed for each unknown.

2.2.4.3 *Internal Standard Method*

Using the internal standard method, a pure reference compound is not needed. Instead, an analog of the compound of interest can be used as an internal standard which has similar physical and chemical properties. For example, decanal may be used as an internal standard for determining characteristic flavor compounds (E,Z)-2,6-nonadienal and (E)-2-nonenal in fresh cucumbers [43].

The internal standard must be chosen with great care. The standard must be pure and chemically inert, have a similar retention time and concentration level and be well-resolved from all other chromatographic peaks [44]. With SPME, extraction time profiles must be determined for all compounds, otherwise, if the analyte is extracted at equilibrium, large errors will result if the internal standard is not also extracted at equilibrium [31]. Moreover, if the pK of the analyte of interest is very different from that of the internal standard, and the sample pH is adjusted to favor the analyte of interest, but not the internal standard, the results will be poor [31].

Typically, multiple internal standards are used with each standard representing some subclass of the overall volatile profile. As an example one could use 4-methyl-2-pentanol and 2-ethylbutyric acid as internal standards to analyze volatile compounds in bread crumb [45].

2.2.4.4 *Stable Isotope Dilution Analysis*

The method of choice for exact quantitative analysis of volatile compounds with SPME is currently isotope dilution assay (IDA). To simplify laboratory procedures and also avoid health risks, only stable isotopes are used in food flavor analysis, and one can therefore extend the term to stable isotope dilution assay (SIDA), with S representing stable.

Isotopes are variants of a particular chemical element that have the same number of protons but a different number of neutrons in the nucleus (*i. e.* they have different mass numbers, but display similar chemical and physical behavior). The principle of isotope dilution analysis consists of synthesizing the isotope labeled flavor compounds (for example,

deuterium or ^{13}C) and adding them to the food matrix before sample workup [19]. The flavor compounds that are labeled with stable isotopes differ only slightly from the analyte. Their physical and chemical properties (*e.g.* volatility, reactivity, distribution coefficient and chromatographic behavior—are the same as those of the unlabeled flavor compounds). The mass difference may have a small impact on the diffusivity, however, this effect is observed to be small and is insignificant with regards to the other sources of variability discussed above. The isotope labeled flavor compounds are added to foods as internal standards as early as possible, namely before the first extraction, so that they undergo virtually the same losses as the flavor compounds to be studied during the isolation method and enrichment steps that are employed. For this reason, labeled compounds satisfy nearly all of the requirements for an ideal internal standard and can also tolerate workup methods with very low recovery percentages, provided that the detection sensitivity is not too low [19]. For example, SIDA was applied to quantify linalool enantiomers in beer using synthesized [$^{2}H_2$]R/S-linalool as the internal standard [46].

A fundamental prerequisite to ensure correct quantification is achieving a thoroughly homogeneous distribution of the labeled standard and analyte in the sample. In this regard, slight differences in adsorption characteristics between the analyte and the labeled standard must be tolerated, because the native compounds that are present in the sample matrix are bound to surfaces in a manner that differs from those in the added isotope-labeled standard. With the aid of a known amount of added labeled standard and the mass-spectrometry (MS) response factor—which is determined at defined weight ratios of labeled to unlabeled compounds under identical GC/MS conditions—it is possible to exactly calculate the concentration of flavor compounds in foods [19]. This method has been applied when a limited number of volatiles are of interest, for example, in studies with a small number of "key" aroma compounds [47].

2.2.4.5 *Multiple Headspace Extraction*

The technique of MHE was originally used to quantify analytes in solid samples to avoid matrix effects (implying several consecutive extractions from the same sample until no volatiles are present) [48]. The theoretical principals of MHE were presented by Kolb and Pospisil [49]. It applies stepwise headspace extraction for the quantitative analysis of volatile compounds [50]. The method calculates the total amount of analyte in a solid sample after a few successive extractions [51]. Theoretical aspects of multiple SPME are described by Koster and de Jong [52].

A portion of the headspace is removed in the first extraction and the equilibrium in the vial is disturbed. After re-equilibration, more analyte has migrated from the sample into the headspace; however, the concentrations in the two phases will now be smaller than during the first extraction. The ratio, however, between the analyte concentrations in the two phases will be the same. The second extraction results in a smaller peak and by continuing this procedure it is possible to strip off all the volatiles from the sample. The peak areas are summed up to get the total peak area, which corresponds to the total amount of the analyte in the sample. The influence of the sample matrix is thus eliminated by exhaustive extraction. As the MHE procedure follows a logarithmic function it is not required that the extractions are carried out until all the analyte is removed from the sample matrix. Instead,

the logarithms of the areas under consecutive chromatographic peaks are plotted versus the number of extractions in a linear scale and the total area is obtained by regression from the areas obtained in only a few extraction steps [53]. Multiple SPME has been used for the analysis of solid and liquid samples, for example in studies by Ye *et al.* [54] and Gomez-Ariza and Garcia-Barrera [55]. However, the usefulness of the method is limited for SPME. In some cases the amount of the analyte in the vial almost remains constant, and the area for successive extractions is the same. In addition, the adsorption phenomena and fiber coating saturated by matrix components can invalidate multiple SPME for quantification.

2.3 GC-OLFACTOMETRY IN FOOD AROMA ANALYSIS

It is well documented that only a small fraction of the large number of volatiles occurring in food actually contributes to the aroma or odor of foods. GC detectors provide relevant information on volatile composition; often a large number of them. However, commercial detectors are not as sensitive for odor active compounds as the human nose [4]. Headspace analysis makes an important contribution to aroma analysis when the positions of the aroma substances in the chromatogram are determined by olfactometry [56].

2.3.1 *Principles of GC-O*

GC-Olfactometry refers to the use of human assessors as a sensitive and selective detector for analyzing odor-active compounds. The description of a gas chromatograph modified for the sniffing of its effluent to determine volatile odor activity, was first published in 1964 by Kuehner *et al.* [5]. It was initially described as a screening method to determine whether a compound found in a sample had odor activity or not [57].

GC-O is carried out on a standard GC that has been equipped with an odor port in place of, or in addition to, the conventional detector. When an FID or MS detector is also used to record, the effluent is most often split in equal proportion to both the conventional detector and the odor port [34]. The odor port is usually a nose-cone, to where the eluting volatiles are derived via a heated connection transfer line. The nose-cone is typically positioned ca 30-60 cm away from the instrument so that the assessor is in a comfortable sitting position, considering that the GC-sniff runs may be longer than 30 minutes. It is preferable that the odor port extends from the side of the GC rather than its top [34]. To avoid drying out the assessor's nose, volatiles are carried in a stream of heated and humidified air (50-75% relative humidity). Commercial sniff ports for GC-O are available from DATU (Geneva, NY, USA), SGE (ODO I and ODO II; SGE International B.V., Veldhoven, The Netherlands), ATAS GL (PHASER; Veldhoven, The Netherlands) and Gerstel (ODP; Gerstel; Mühlheim an der Ruhr, Germany) and can be combined with any GC.

As suggested by Delahunty *et al.* [34], GC-O instruments should be located in a dedicated laboratory, the air should be filtered to remove any unwanted odors, and pumped into the room to maintain positive pressure, and the laboratory should also be temperature controlled for assessor comfort as well as to ensure instrument reproducibility. Some investigators ask assessors to listen to music through soundproof headphones to prevent distraction from noise, however most will simply ensure that the laboratory door is closed

during analysis [34]. For better concentration, it is also suggested to sniff with your eyes closed.

GC-O is different from sensory analysis, in which trained panelists taste the food and try to assess the level of taste attributes (salty, sour, vanilla, etc.) usually one attribute at a time and perceived flavor of the sample involves simultaneous tasting of all flavor compounds. In GC-O, odors are first separated from each other and presented to the assessors for a few seconds at undefined intervals over a relatively long period (30 min or more). During sensory evaluation the accurate assessment of the level of a particular flavor compound is difficult. GC/MS instruments in combination with GC-O are able to analyze many samples with hundreds of chemical components.

There are numerous weaknesses in using GC-O methodologies. According to Friedrich and Acree [58], it is often criticized as being a subjective method, yielding inconsistent results, but independent judgments of well-trained subjects should minimize this concern. It must be pointed out that there are no proper universal guidelines for the panel training.

The duration of a routine GC run is often more than 30 minutes. Odor fatigue can set in well before the end of the analysis, leading to incorrect odor descriptions. Thus, one must be conscious of the time a subject is asked to perform this task (it may be limited to 20 minutes or in case of long GC runs it can be split between two assessors). It is ethical that assessors should be told that they could come into contact with hazardous chemicals, especially if they are concentrated on the SPME fiber. Luckily, food samples are comparatively harmless, except perhaps some biological toxins if spoilage has occurred.

Odor characteristics of some favor compounds tend to vary as a function of concentration [6]. Skatole (3-methylindole) has a characteristic fecal odor at high levels but becomes pleasant, sweet and warm at very low levels. Fortunately, there are not many aroma compounds exhibiting such a large concentration-dependent odor character [6]. Furthermore, attempts to indicate the perceived intensity of a GC peak can be in error owing to masking in mixtures [6].

Also, food aromas consist of chemically related and perceptually similar odorants. Therefore, cross-adaptation processes, the decrease in sensitivity to one odorant following exposure to a different odorant, and sequential context effects may influence GC-O results [59].

2.3.2 *Humans as a Detector*

2.3.2.1 *The Sense of Smell*

The immense diversity of flavors associated with foods are primarily derived via the volatile compounds that are released in the oral cavity when foods or liquids are chewed and swallowed. Volatile compounds flow from the mouth via the naso-pharynx passage and access receptors in the nasal cavity (retronasal). The sense of smell is also activated via active sniffing of air where volatile compounds travel to the olfactory receptors through the nostrils (orthonasal).

To demonstrate the influence that the sense of smell has on flavor perception a simple experiment can be made. When tasting grated apple and onion with the nose plugged it is nearly impossible to distinguish between the two, yet with the nose unplugged the task is very easy. When the nose is plugged, there is no airflow over the olfactory epithelium

effectively removing aroma from the overall flavor and distinguishing between the two samples relies on taste attributes alone.

Aroma detection depends on sensory receptors that respond to airborne chemicals. In humans, these chemoreceptors are located in the olfactory epithelium – a patch of tissue about the size of a 2.5 cm^2 located high in the nasal cavity [60]. The olfactory epithelium houses the olfactory receptors on olfactory neuronal cilia within the mucus layer. The mechanism of odor transduction is provided in Figure 1 below.

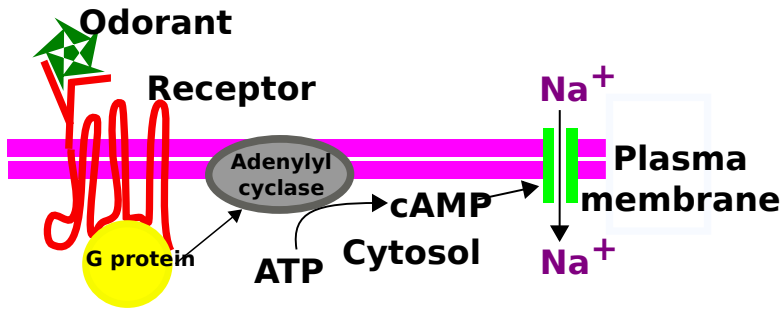

Figure 1 – Human odor receptor adapted from Kimball [60]. Odorant molecules dissolve in the mucus and bind to receptors on the cilia. These are"7-pass" transmembrane proteins. Binding of the odorant activates a G-protein coupled to the receptor on its cytoplasmic side. This activates adenylyl cyclase, an enzyme embedded in the plasma membrane of the cilia. Adenylyl cyclase (AdC) catalyzes the conversion of ATP to the "second messenger" adenosine-3',5'-cyclic phosphate (cAMP) in the cytosol. cAMP opens up ligand-gated sodium channels for the facilitated diffusion of Na^+ into the cell [60]. The influx of Na^+ reduces the potential across the plasma membrane. If this depolarization reaches threshold, it generates an action potential. The action potential is conducted back along the olfactory nerve to the brain. The brain evaluates this and other olfactory signals reaching it as a particular odor.

Interestingly, many odorous substances activate not only the olfactory system but also the somatosensory system – the nerve endings in the noses which are sensitive to temperature, pain etc. [61]. This is why anosmics are often able to detect menthol, for example.

Quantitatively, humans have 6-12 million receptor neurons housed in the olfactory epithelium that are comprised of approximately 360 qualitatively different receptors [62]. Odorants dissolved in the surrounding mucus can access the olfactory receptors, however, the transduction process of smell perception (*e.g.* rose, caramel, cut grass) will only proceed if the volatile compound has the chemical key for the receptor [63]. The system is broadly tuned because a single odorant is often recognized by multiple receptors and multiple odorants are recognized by the same receptor [63]. For example trans-2-hexenal, when activating one receptor, smells green, while activation of another receptor provides the smell of bitter almond. An electrical signal is sent to the olfactory bulb where the neuronal activity pattern responsible for the quality is established.

The perception of smell consists not only of the sensation of the odors themselves but of the experiences and emotions associated with these sensations. Olfactory receptors are directly connected to the limbic system, the most ancient and primitive part of the brain,

which is thought to be the seat of emotions. Smell sensations are relayed to the cortex, where cognitive recognition occurs, only after the deepest parts of our brains have been stimulated. Thus, by the time we correctly name a particular scent as, for example, vanilla, the scent has already activated the limbic system, triggering more deep-seated emotional responses [61].

Many researchers have investigated genetic differences in smell perception and found that people perceive scents differently. Keller and Vosshall [64] reported that each individual has a unique olfactory sense because many have so called olfactory "blind spots", which have been related to the loss of evolutionary pressure allowing the mutations in 400 genes encoding the olfactory receptors. After a number of mutations, some have become pseudogenes, meaning they no longer encode a functioning receptor. Each person has different combination of pseudogenes, hence the dissimilarity in smelling odors resulting in so called "specific anosmias". Specific anosmia is when olfactory function is normal with the exception of a single odorant or small group of closely related odorants that cannot be detected or are poorly detected [65]. Examples of the differing capacity to smell are that one in a thousand cannot smell skunks or that trans-2-hexenal is perceived as green by some people [66] and bitter almond by others [67].

A recent study at the University of Pennsylvania suggests that, contrary to popular belief, blind people do not necessarily have a keener sense of smell than sighted people, trained (sighted) assessors were top performers on most tests, which leads to the conclusion that training is the factor most likely to enhance performance on smell tests [61].

2.3.2.2 *Training of GC-O Assessors*

The accuracy of GC-O is very much dependent on the performance of olfactory assessors. For each of the separated compound that emerges from the GC, a human assessor has the potential to detect this by its odor, to measure its duration from the start to the end, to describe the odor, and to quantify its intensity. The results, however, must be representative and reproducible, which can be complicated due to the variability of humans as pointed out in the previous section. For some GC-O methods, where only detection of odorous compounds is needed, training of assessors is not required if an adequate amount of assessors (8-12) is used. However, to describe the odor and to quantify its intensity requires experience.

The most complicated task for the assessor is odor recognition; therefore the odor memory is what the training should focus on. Fortunately, once an odor has been encoded into memory, it tends to persist there. According to numerous studies long-term odor recognition memory shows relatively little decay over extended periods of time [68].

Lyman and McDaniel [69] suggest that the significance of identifiability in odor recognition memory lies in its providing the opportunity for dual encoding in both a verbal-linguistic and a presumably non-verbal, olfactory imagery system, with veridical identifications allowing for more precise and elaborate processing. However, coming up with accurate verbal labels for odors is extremely difficult, even when the odors are recognized as being highly familiar (so called "tip-of-the-nose state") [70]. On average, subjects are able to name 35–45% of any given set of commonly encountered smells correctly [68]. Desor and Beauchamp [71] were the first to demonstrate that subjects can quickly approach perfect identification of large sets of odors if prompted with their names and given subsequent

corrective feedback. The connection between olfactory perception and verbal labels seems to be one that requires extensive training.

To begin assembling a GC-O panel, potential assessors should be screened for sensitivity, motivation, ability to concentrate, and ability to recall and recognize odor qualities [72]. Also for factors, which include gender; age (general olfactory ability); the presence of respiratory disease, such as asthma, seasonal allergies, or active colds; medication use; smoking; and occupational history [73]. Although smoking does not always affect scores on smell-tests, it is widely believed to reduce sensitivity. Women consistently out-perform men on all tests of smelling ability [61], however, their sensitivity is influenced by menstrual cycles [74].

Once the odor panelists are selected, they are screened for specific anosmia, using a standard test mixture, if no insensitivities are found, the panelists are trained on sniffing different dilutions of standard compounds [75]. Because a similar understanding of the smell of aroma compounds between panelists is crucial, the description is generally based on glossaries of olfactive descriptors with the aim of normalizing the language between panelists (see Figure 2 below). Terminologies are likely to work better on a product-by-product basis (*e.g.* for beer, cheese) than across product categories.

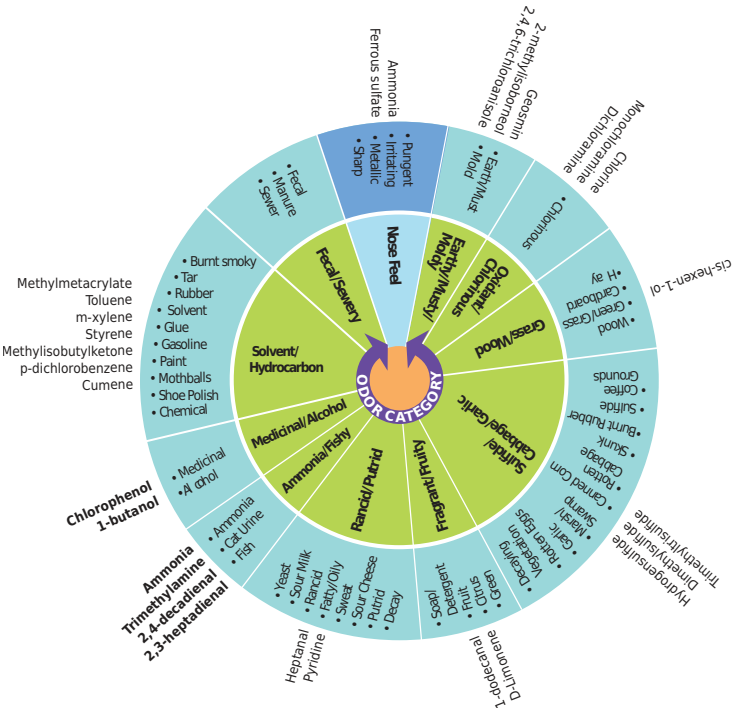

Figure 2 – Odor wheel for taste and odor in drinking water from Suffet *et al.* [76].

In addition, if intensity measurements are to be carried out, the panelists have to learn the scale applied. Because variation between assessors is expected it is common practice to interpret GC-O panel results as a single detector [34].

2.3.3 *GC-O Methods*

To estimate the sensory contribution of a single compound, GC-O can be combined with methods such as dilution, time-intensity, detection frequency and posterior intensity methods.

Dilution analysis, the most applied method, is based on successive dilutions of an aroma extract until no odor is perceived by the panelists. This procedure, usually performed by a reduced number of assessors, is mainly accomplished using the combined hedonic and response measurement (CHARM) and aroma extraction dilution analysis (AEDA). AEDA was developed by Ullrich and Grosch [77]. The samples are evaluated by the panelists in increasing dilution order and the impact of an odor-active compound is given by its dilution factor (DF) value. The DF value is calculated by dividing the largest volume analyzed by the lowest volume in which the respective odor-active compound was still detectable. CHARM was introduced by Acree *et al.* [78]. Dilutions are presented to the panelists in a randomized order, avoiding bias introduced by the knowledge of the dilution being analyzed. Panelists record the start and the end of each detected odor. The detection duration of each individual is then compiled, and an aromagram is generated by plotting the duration of the odor sensation against the dilution value.

Time-intensity methods, such as OSME (from the Greek word meaning smell, first introduced by da Silva *et al.* [79]), are dynamic methods and the odor intensity is recorded along with time during peak elution by moving a variable resistor along a category scale or using finger span method. In OSME, the intensity is attained in a single run, although the results are reliable only when trained assessors are used. A finger span method has been adequately applied for determining odor intensities of wines [80].

Detection frequency uses the number of evaluators detecting an odor-active compound in the GC system's effluent as a measure of its intensity. This GC-O method is performed with a panel of untrained evaluators; 8-10 assessors are a good compromise between low variation of the results and analysis time. It must be added that the results attained are not based on real intensities and are limited by the scale of measurement. Pollien *et al.* [81] developed the nasal impact frequency (NIF) method from the latter method. The NIF method does not require a trained panel, and no intensity scale has to be learned by the evaluators, and therefore no intensity measurement is performed. In this method, eight to ten untrained individuals sniff the GC effluent (one at a time) and simply note when they smell an odor. The aroma isolate used is adjusted to a concentration such that about 30 odorants are perceivable to the sniffers [6]. This adds an element of selection that only the more intense aroma compounds will be evaluated. The number of sniffers detecting an odorant is tabulated and plotted. Those odorants (GC peaks) being detected by the greatest number of individuals are considered the most important. The method has its weaknesses, however. One problem is that, for two compounds, one may be barely over the sensory threshold of all sniffers while another may be a well above its sensory threshold for all sniffers, and yet both these compounds would be viewed as being equal by this methodology [7]. It is also relatively time-consuming method.

The posterior intensity method is a static method, which involves scoring of the perceived intensity on a previously determined scale after a peak has eluted from the GC col-

umn. This might cause considerable variance between assessors. Results may be correlated with the detection frequency method rather than with dilution methods [75].

Another method to characterize aroma compounds is the concept of odor active value (OAV), which takes into account both the thresholds and the actual concentrations of the compound [82].

AEDA and CHARM show a real efficiency to detect odor-active compounds, however, they have two critical disadvantages: they are time-consuming, and base their method on detection thresholds and not on real odor intensities. Researchers now consider the AEDA, OAV, and CHARM methods to be screening in nature. These methods are used to determine the aroma compounds that are most likely to make a contribution to the odor of a food, compounds with the highest DF values are candidates for further study to evaluate their true contribution [6].

Sensory work (*e.g.* omission testing) needs to be done to determine which aroma compounds are truly contributory. To test this, the determined concentrations of the odorants are dissolved in a suitable odorless medium, which is not difficult in case of liquid foods. An ethanol/water mixture, for example, is suitable for wine. In the case of solid foods, however, compromises are required. The aroma profile of the model is then compared to that of the food. To be sure that each compound really contributes to the aroma of the product, one or several aroma substances are omitted in the aroma model and a triangle test is performed. This test is used to examine which of three samples (two complete and one reduced aroma model) offered to the testers in random order differs in aroma from the others. If the panel cannot determine a difference between the complete formulation and the formulation minus one, the omitted component is viewed as not making a contribution to aroma. Prof. Grosch from Technical University of Munich has published papers in this area, particularly on coffee, finding that for example that damascenone had the highest OAV of all compounds in coffee yet sensory testing determined that it makes no contribution to the odor character of coffee [6]. Making matters more complicated, one may find that compounds A and B may individually be omitted from a mixture without perceptibly changing the sensory character of the mixture. However, the deletion of both may result in a change [6].

The number of evaluators which shall be comprised in a panel is a rather controversial matter. Dilution methods are often performed using only 1–3 assessors, while in detection frequency techniques, higher reliability is attained with 8–10 assessors. A large number of trained panelists are also required for intensity evaluations because a high variability may be commonly observed within and between panelists [75].

In the search for the ideal method of aroma isolation, there is no perfect method for selecting key odorants in foods. Each method has weaknesses. The result is that more sensory work is being done to evaluate the analytical data. Very often, researchers are using recombination studies followed by sensory analysis to determine the components that contribute to the aroma of a food and which ones do not. If one is to attempt any re-creation of a flavor, one must have a list of odorants to study and have some reasonable basis for their selection and ranking. The GC-O methods that have been developed have served this purpose with varying levels of success [6].

3

AIMS OF THIS DISSERTATION

The main objective of this doctoral work is to develop and apply methods to study the aroma and volatile composition of food. In addition, the aroma development and stability during food processing is assessed. Four publications resulted from the following investigations:

I Publication I explores aroma-active compound detection in Kama flour.

II Publication II studies volatile compound production by individual species of lactic acid bacteria (LAB) in sourdough using multiple headspace extraction.

III In Publication III, a method was developed to quantify acetaldehyde in food products.

IV In Publication IV, a method was developed to train a GC-Olfactometry panel.

4

MATERIALS AND METHODS

4.1 MATERIALS AND CHEMICALS

Kama flour ("Kamajahu", Pere Leib, Tartu, Estonia), used in Publication I to investigate the aroma-active compounds, was purchased from a local store in Estonia. The same batch was used throughout the study. Rye flour, used in Publication II to compare volatile compound production by individual lactic acid bacteria (LAB) in sourdough, was purchased from Tartu Veski (Tartu, Estonia). The food products for children, used to analyze acetaldehyde content in Publication III, and Kvass (A le Coq, Tartu, Estonia), used for panel training in Publication IV, were purchased from local stores in Estonia. All solvents, reference compounds, and standards of chromatographic grade were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Deuterated acetaldehyde was purchased from Cambridge Isotope Laboratories Inc., MA. Ethanol, whenever used, was acquired from Rakvere Piiritusetehas (Rakvere, Estonia). Water was purified with Millipore (Millipore Corporation, Bedford, MA) whenever samples were prepared. Sniffing strips used in panel training were bought from Orlandi Inc. (Farmingdale, NY, USA).

Silica SPME fibers with commercially available coatings were obtained from Supelco (Bellefonte, PA, USA) (85 μm CAR/PDMS; 65 μm DVB/PDMS; 60 μm CW/PDMS; 50 and 30 μm DVB/CAR/PDMS; 85 μm polyacrylate (PA); 50 and 30 μm DVB/CAR/PDMS 2 cm long). All new fibers were conditioned according to the manufacturer's recommendations prior to first use. Clear glass SPME crimp vials (20 ml) with the polytetrafluoroethylene (PTFE)/silicone septa (20 mm) were purchased from La-Pha-Pack (Langerwehe, Germany), VWR International (West Chester, PA) and Supelco. Glass covered magnetic stirring sticks were purchased from VWR International. Helium, purity 5.0 (Air Liquid, Schwechat, Austria) was used as the carrier gas in the GC system for Kama flour analysis. Helium of purity 5.5 (AGA Eesti AS, Estonia) was used in all other studies.

4.2 METHODS

4.2.1 *SPME-Gas Chromatography*

For analyzing the volatile compounds in Kama flour sourdough, and quantifying the acetaldehyde content in foods, and while training the GC-O panel, the headspace SPME technique was adopted with an auto-sampler CombiPAL (CTC Analytics, Zwingen, Switzerland) and Agilent Technologies Inc. GC (Santa Clara, CA). The detectors used were TOF-MS

(Waters, Manchester, UK), quadrupole mass spectrometry (qMS) (Agilent Technologies Inc., Santa Clara, CA), flame ionization detector (FID) (Agilent Technologies Inc.) or olfactory detection port (Gerstel).

The volatiles adsorbed onto the SPME fibers were thermally desorbed for 10 minutes at 250 - 270 °C (depending on the fiber type) in the injection port of the GC equipped with a DB-5MS column (J&W Scientific, Folsom, CA) with a 0.25 or 1 µm film thickness, 30 m length and 0.25 or 0.32 mm inner diameter (ID).

Helium at a constant of flow ($0.8 - 1.7\ \frac{\text{ml}}{\text{min}}$) was used as the carrier gas and the injector was used in splitless mode for 10 minutes using an inlet liner of 0.75 mm ID specialized for SPME analysis. MS detector temperatures were 200 °C (TOF-MS) and 250 °C (qMS) and for ionization, electron energy of 70 eV was used. The column temperatures mostly started at 35 °C (except for Kama flour at -10 °C and acetaldehyde at 60 °C) and ended at 280 °C at different rates and total run times.

Volatile compounds were tentatively identified by comparing their spectra and retention indices with those present in the NIST05 library (NIST, Gaithersburg, MD) using MassLynx™ and ChromaLynx™ software (Waters) and/or Wiley275 library using ChemStation™ software (Agilent Technologies Inc.). To determine the retention indices, the retention of a series of straight chain alkanes (C8-C20) were compared with Kovats indices [4] and aromatic properties published in Flavornet[1], Pherobase[2] and TU Graz GC laboratory own database (SKAF), together with GC/MS data. For intensity analysis, the peak areas were integrated and compared using MassLynx™ or ChemStation™ software.

4.2.2 *GC-Olfactometry*

Kama flour aroma analysis and GC-O panel training was carried out using a GC-Olfactometer. The column flow was divided by a fixed 1:1 splitter using 100 – 150 µm deactivated fused silica columns between the sniffing port and a flame ionization detector. Aroma active compounds perceived by the assessors were recorded by voice recognition software (Gerstel) and directly imported to the Agilent ChemStation™ software.

For Kama flour aroma analysis (Publication I) three trained panelists (all female, age 24-30 years) were used. In the GC-O panel training study (Publication IV) twenty-nine volunteers (5 male) were trained to become GC-O assessors. Some had previous extensive sensory training, some had a food science background, while others were typical consumers. Panelists with a variety of backgrounds were needed to investigate the influence of previous experience on the results of training. The panelists were between 22 and 50 years old and all were healthy non-smokers.

1 http://www.flavornet.org
2 http://www.pherobase.com

5

RESULTS AND DISCUSSION

Four publications form the basis of this dissertation. The main results from Publication I are presented in Section 5.1, while those from Publication II are presented in Section 5.2. Section 5.3 and Section 5.4 discuss Publication III and Publication IV, respectfully. A unifying general discussion in Section 5.5 is also provided.

5.1 APPLICABILITY OF SPME-GC/MS AND GC-O FOR DETERMINING KEY AROMA ACTIVE COMPOUNDS

An SPME-GC/MS and GC-Olfactometry method for analyzing aroma active compounds in different food matrices was applied for determining key aroma active compounds in Kama flour.

First, the optimization of the SPME method to extract the maximum number of the most aroma active compounds from the sample was carried out. Five different fibers were studied, sample amount, water and salt addition, extraction time and temperature were varied to optimize the extraction conditions. Most volatiles were extracted with CAR/PDMS fiber (Publication I, Figure 1). As expected, the peak intensity increased with the increasing amount of Kama flour in the vial. Water and salt addition decreased the peak intensities and several peaks of polar compounds were not detected probability due to their strong interaction with water (Publication I, Figure 2). Increasing extraction temperature and time increased the chromatographic response. This suggests that equilibrium between the sample and the fiber was not achieved.

Analysis with GC/MS detected 89 volatile compounds. 62 of which were reported in the literature to have odor impression, the rest were either not identified or had no odor according to literature. The peaks in the GC/MS chromatogram (Publication I, Figure 2) were identified and 62 aroma compounds were listed in Publication I, Table 1. In this study, the interest was to identify which of those odor compounds could be detected using GC-Olfactometry at the same extraction and chromatographic conditions as GC/MS. The odor threshold (OT) values in Publication I, Table 1 allow one to predict if the compound could be odor active. In total, 30 compounds (Publication I, Table 2) were detected in Kama flour using GC-O and were used to identify the odor perception. Retention indices were compared to those listed in Publication I, Table 1.

It was found that seven odor active compounds having very high OT (retention indices of 868.4; 901.4; 1007.7; 1032.5; 1051.9; 1173.3 and 1214.8) were not detected by the GC/MS and therefore listed in Publication I, Table 2 as unknown. For the most intense odor active

compounds an aroma extraction dilution analysis (AEDA) was carried out. An AEDA aromagram with concentrations (relative units) calculated relative to internal standards is given in Publication I, Figure 3. According to the aromagram, the highest DF values correspond to for 2,3-butanedione (1), hexanal (7) and 1-octen-3-one (15), meaning that they are detected in 1 mg of Kama flour.

Most of the odor active compounds found by SPME-AEDA belong to pyrazines (14 of the identified 23 compounds) and quite likely most of the unidentified compounds that elicit a roasted odor also belong to pyrazines. However, the aroma compounds detected from lowest sample size (1 mg) were 2,3-butanedione which elicits a buttery odor, hexanal responsible for a green note and 1-octen-3-one for mushroom.

Furthermore, the GC/MS peak area was clearly not proportional to odor intensity. For example furfural, detected by GC/MS and having according to literature a nutty almond-like odor impression [83], was not detected while sniffing using GC-O. Seven compounds were not obtained with the GC/MS or FID chromatogram, which shows they are present in quantities too low to detect, however, have a very low odor threshold so they are detectable only with the human nose. This illustrates the importance of GC-Olfactometry while detecting low concentration aroma compounds.

The determination of the absolute concentration of different aroma compounds in Kama flour is more complicated because it requires external calibration involving adding different concentrations of pure Kama flour aroma compounds and the addition of internal standards to the Kama flour. However, the relatively small standard deviations of the parallel samples using GC/MS demonstrate that the use of internal standard makes it possible to quantitatively compare the aroma analysis in different batches of Kama flour. Although the behavior of the analog internal standards are not exactly the same as the aroma compounds in the matrix, they still reveal the relative ratio between pyrazines and provide the possibility to compare the intensities of aroma profiles of different Kama flour preparations (batches).

5.2 MULTIPLE HEADSPACE EXTRACTION WITH SPME-GC/MS

The multiple headspace extraction (MHE) method was developed for quantification purposes, however, exploiting the idea for in-vial process investigation has not been published before. In this study, MHE was conducted with automated SPME-GC/MS and used to characterize volatile profiles produced by different species of lactic acid bacteria in their natural environment. Results demonstrate that multiple extractions from the same sample vial using SPME fibers is a very simple and convenient method, particularly due to the automation that also minimizes the influence of human variability by allowing precise sampling and less fiber damage. It enables one to study the dynamics of volatile formation (Publication II, Figure 2) and analyze several different samples in parallel.

Comparing SPME chromatograms of dough prepared from sterile rye flour demonstrate that chromatographic patterns change during incubation significantly (Publication II, Figure 1 A and B). Although the compounds were repeatedly taken out with the fiber during incubation, the concentration of some compounds increased in the vials due to the activity of the indigenous flour enzymes or chemical reactions in the dough. Compounds that de-

crease are either consumed in chemical reactions or transferred out with the fiber during repeated sampling.

Inoculation of dough significantly affects the volatile profiles in fermentation vials, for example 24-hour *Lactobacillus sakei* (Publication II, Figure 1 C) versus the 24-hour blank (Publication II, Figure 1 B). A comparison was made between the dynamics of peak intensities (total ion count (TIC)) during 24 h fermentation of nine different sourdough samples and a blank sample containing only sterile flour and water (1:1). All of the compounds tentatively identified in different dough are listed in Publication II, Table 1. The behavior of a certain compounds in a sourdough associated with the growth of different starter *Lactobacilli* is described in the table by either minus (decreasing), plus (increasing) or 0, when the compound was not found in the sample. Results indicate that changes take place in the sample vial during 4 hours after the start of intensive growth.

5.3 QUANTIFIABILITY WITH SPME-GC/MS

The development of a SPME-GC/MS method was initiated by the practical need to quantify acetaldehyde in food products after the International Agency for Research on Cancer (IARC) changed the cancer risk classification of acetaldehyde from an agent possibly carcinogenic to humans (Group 2B) to an agent carcinogenic to humans (Group 1) (IARC Press release №196). Importantly, a quantitative method for determining the concentration of acetaldehyde in solid food matrices was not available.

Considering the low evaporation temperature and high reactivity of acetaldehyde, the method contains as few sample pretreatment steps as possible in order to avoid losses. For both the LC/MS- and enzyme-based quantification, insoluble particles in the sample needed to be removed by centrifugation and/or filtration, which caused losses via evaporation and thus made the precise quantification extremely difficult. In this study, a quantification method based on SPME-GC/MS with stable isotope addition was developed. To increase the sensitivity and selectivity of the method, derivatization of acetaldehyde with PFBAH was used.

The exact amount of acetaldehyde in food products consumed by babies and small children was determined. The highest concentration of acetaldehyde was found in yoghurts (31.5 ± 0.05 $\frac{\text{mg}}{\text{kg}}$), which exceeded the limit set by the Council of Europe by 30-40% (23 $\frac{\text{mg}}{\text{kg}}$ in beverages and 20 $\frac{\text{mg}}{\text{kg}}$ in food). Nevertheless, we did not find proof that the concentration of acetaldehyde is considerably higher than the mutagenity level in foods for children.

5.4 GC-O PANEL TRAINING

The gas chromatograph-olfactometry (GC-O) approach has been used extensively in aroma research for the selection of odor active compounds from the whole range of volatile compounds in food products. In this type of analysis, the human nose is used as a detector and the accuracy of GC-O is very much dependent on the performance of olfactory assessors. This is particularly true when one requires that the assessors rate the intensity of perceived odors, rather than simply indicate that they are above the detection limit. Objective training methods and assessor performance benchmarks are desirable to ensure high quality

data. Surprisingly, to the author's best knowledge, guidelines for training panelists with the technique have not been published.

In the current study a robust training method that applies a four step approach is proposed:

A. Introduction of the method (2-days theory lecture presenting the method to the panelists).

B. Vocabulary training using standard compounds and learning the use of the scale. Assessors are subjected to the "Sniffing Strips" of standard aroma compounds at different concentrations to study the vocabulary and learn the scale.

C. Training with a standard mixture. A standard mixture, with the same compounds used for training the vocabulary, is sniffed using GC-O by each assessor three times. Two parallels are used to detect and identify the smell, and a third is used to determine the intensity.

D. Training with the product of interest (kvass, a fermented non-alcoholic soft drink was used in Publication IV). Assessors are divided randomly into smaller groups that apply different olfactometric methods. In Publication IV, ten people were used in detection frequency, ten used AEDA, and nine used posterior intensity to describe key aroma-active compounds.

Twenty-nine volunteers were trained to detect and identify the odor of gas chromatograph effluents and, in addition, rate the intensity. Three GC-O methods were compared (aroma extraction dilution analysis, detection frequency, and the posterior intensity method) to evaluate key compounds of kvass aroma. The posterior intensity method was found to provide a sufficient amount of data for key compound analysis, enabled easy data handling and was chosen as the method for further training and panel monitoring.

Following the steps in this study, most panelists learned to perform a GC-O task, however, large variances among subjects were observed. A group of twelve assessors was chosen based on their performance and interest in becoming a professional GC-O assessor. To function efficiently, this group is assembled monthly to train their odor memory, learn new odor-active compounds, and sniff different standard mixes using GC-O.

5.5 DISCUSSION

In this thesis, SPME-GC/MS and GC-Olfactometry were applied to solve several flavor related issues, however, the results raise some points to discuss. Utilizing the SPME method for key aroma-active compound analysis is very convenient and numerous articles have been published using this method, however, it is extremely biased regarding which volatiles it extracts and therefore needs to be carefully applied. Some compounds of interest might not be extracted when using an inappropriate fiber. When researchers set out to determine the most important volatiles contributing to the aroma of a food, several aroma isolation methods should be used. No single method of isolation yields an isolate truly representing the food aroma. Other modern methods such as solvent assisted flavor evaporator (SAFE) and/or high-pressure extraction with supercritical CO_2 could be applied in parallel to SPME in future studies.

Furthermore, while optimizing the SPME method for Kama flour aroma analysis (Publication I) and choosing the best fiber, the abundance of the volatiles extracted that was used in the article, is not an adequate indicator of aroma compounds due to biased extraction. To ensure that the aroma isolate adsorbed on SPME fiber is truly representative of the aroma of a food product, it needs to be conditioned to sensory analysis. Obtaining an aroma isolate after fiber desorption that smells like the food is probably the only reliable way to select the best fiber to study the aroma compounds that contribute to the flavor of a food.

Another drawback of SPME analysis is the partial extraction of volatile compounds, due to the fact that the amount of polymer on the SPME fiber is very small compared to the amounts of the other sample phases (liquid, solid and vapor). By increasing the amount and surface area of the polymer coating, it is then possible to extract more flavor compounds. Other sorption-based methods can be considered, for example, the stir bar sorptive extraction (SBSE) or solid-phase dynamic extraction (SPDE). SPDE can be automated with CTC CombiPAL, but SBSE needs an additional thermal desorption instrumentation mounted on the GC. An additional drawback for both is that there is still only one type of sorbent (polydimethylsiloxane) commercially available, which inevitably leads to low recoveries of highly polar molecules [44].

GC-O methods, especially dilution methods (including AEDA used in Publication I) for key aroma active compound analysis are considered to be screening methods and compounds with the highest values are candidates for further study. Sensory work (*e.g.* recombination studies) needs to be performed to determine which aroma compounds are truly contributory. Dilution methods falsely assume that compounds present at the greatest multiple of their threshold are the most important to aroma. There is not a power function relationship between concentration and sensory intensity and the relationship is different from one aroma compound to another [6], thus, one cannot unequivocally rank compound intensity based on OAV, CHARM or AEDA values. This weakness is recognized and compounds with the highest values are "candidates for further study" to evaluate their true contribution. For that, each of the volatiles suggested in Publication I to be important based on AEDA data must be quantified, then added to a deodorized food base at an appropriate concentration and evaluated using olfaction if the recombined food product has the same aroma as original product. Moreover, omission testing should be carried out subsequently to evaluate the contribution of each compound to the overall aroma. If a compound can be omitted from the recombined aroma mix without affecting the aroma, it is not a key compound for this product, as for example, the damascenone in coffee [84].

Identification of compounds is another controversial matter. Several MS detectors such as quadrupole, the ion trap, time of flight, and magnetic sector instruments may be used to gain structural information. In this study two MS detectors were used, the first was a quadrupole (qMS) detector and the second a time of flight (TOF-MS) detector. Additionally, other detectors could have been used to help in identification, for example an electron capture detector, which is specific for electronegative species (*e.g.* halogenated compounds), a nitrogen phosphorus detector for nitrogen and phosphorus containing molecules and a flame photometric detector for sulphur or phosphorus containing compounds. Some aroma researchers claim that MS data and LRI data (comparing published LRI with observed LRI) is enough to ensure a compound is identified, however, some insist confirming with standard

compounds to assure the identification. The author agrees with the latter opinion that at least some standards should be used to confirm the identification.

Column selection also makes compound identification easier. In this study, only one column (DB-5, nonpolar) was used, which best separates nonpolar compounds (*e.g.* lactones, esters, ketones, and aldehydes). Highly polar compounds, such as free fatty acids, are well separated by polar columns, like DB-WAX [6]. Because most foods contain some quantity of both polar and nonpolar compounds, samples should be analyzed on at least one type of each column [85]. An additional driver for multiple column analysis is to increase the certainty of identification of an unknown compound. Although coelution of compounds may be common on a single column, the likelihood that the same compounds will coelute across multiple columns is much less likely [85]. Therefore, analyzing a sample with multiple columns helps nullify the effects of coelution and to positively identify unknown compounds.

In this study two methods to measure the concentration of flavor compounds were used: internal standard addition (Publication I) and stable isotope dilution assay (Publication III). 1,2,3-trichloropropane was used as an internal standard. This compound does not appear in nature, however, it does have similar physical and chemical properties of all the aroma compounds of interest in Kama flour. Multiple internal standards, with each standard representing some subclass of the overall volatile profile, would have improved the precision of the results. Furthermore, considering that a very small amount of internal standard was added to Kama flour, an even distribution of this compound in the matrix might not have not been achieved. Possibly more important is the competition among flavor compounds for the adsorption sites. These interactions modify the extraction of flavor compounds where some analytes reduce the extraction of others. This is due to the higher affinity of one compound for the fiber, which could displace others from the fiber and therefore lead to erroneous interpretations of quantification data.

For the quantification of one specific compound (an off-flavor or a toxic volatile, such as acetaldehyde), the use of SIDA can be applied. Complications did occur, however, while using the TOF-MS. It is a very sensitive instrument and allows for good detection of compounds in trace levels as well as precise mass measurement for better identification, however, one drawback is a rather low dynamic range of the detector. This causes the so called "dead time" (in the short time after registering an ion the system is not able to record another ion). Therefore, the detector can be used for quantification only if it is not saturated by the compound. This can be checked by analyzing several dilutions of each sample. To overcome this effect an FID detector can be used for quantification. On the other hand, in case of stable isotope addition, the use of exact mass extraction from the total ion count might be crucial if the isotopes are not separated in a GC column and are overlapping with the compound of interest.

Another complication with aroma analysis (both quantification and identification of aroma compound) is the fact that character-impact aroma components are often present at levels below the detection limits of GC/MS instruments. For example, some of the most potent thiols can be detected in concentrations by olfactometry as low as $6 \times 10^7 \frac{\text{molecules}}{\text{mL}}$ air (2-propene-1-thiol) [86], which is too low for detection with conventional MS.

Most of these issues are recognised by aroma researchers and better methods, instruments and appliances are being developed.

SUMMARY

6

CONCLUSIONS

It is known that research into new flavor compounds is a growing field that is continuously influenced by consumer demands. For this reason, the investigation and identification of odor-active compounds, especially key-odor notes, in food samples, as well as the determination of their relevance and release from the matrix, are of extreme importance for the characterization of a food. The investigations preformed in this doctoral work represent a very small part, and in some cases is only the beginning of method development for application in commercial food flavor development.

In this study SPME-GC/MS and GC-Olfactometry methods were applied to analyze the aroma active compounds in Kama flour. It was found that most of the aroma-active compounds detected were pyrazines; additionally, 2,3-butanedione, hexanal and 1-octen-3-one contribute to the aroma. This method, with small modifications, could be used for food aroma or off-flavor analysis of other similar products.

Additionally, quantitative analysis using an on-fiber derivatization method was applied for accurate measurement of the concentration of acetaldehyde in products for children. The method developed was shown to be adequate to analyze acetaldehyde (or other aldehydes) in solid and half-solid food matrices.

A multiple headspace extraction method was applied to investigate the dynamics of volatile organic compound production of different lactic acid bacteria. It proved to be a perspective high throughput method for this purpose and gives good qualitative information about volatile formation and conversion processes taking place in the headspace vial during dough incubation and enables one to determine the differences between different strains of *Lactobacilli*.

As a result of this study an aroma laboratory with the following equipment was set up: GC/TOF-MS; auto-sampler CombiPAL with SPME, headspace and liquid sampling option; GC-O with flame ionization detector and voice recognition software. For the latter instrument, an olfactory panel was trained and a professional panel of 12 assessors was put together from the best sniffers and is being monitored and further trained monthly to solve current and future food aroma challenges.

The key to resolving some flavor problems is to match the appropriate tool (difference sensory testing, descriptive analysis, GC/MS, olfactometry, etc.) to the specific problem being studied. If a food technologist has attempted to duplicate a competitor product and has created three prototypes to compare, then sensory analysis is likely the preferred approach, with chemical analysis supplying a secondary supporting role. On the other hand, if the sample has developed an unfamiliar off-flavor after production or during shelf-life, then chemical analysis will likely give more information and help sensory scientists get to

the solution of the problem more quickly. Overall, it can be stated that flavor chemistry research (*e.g.* instrumental analysis) has little relevance to flavor characterization without sensory analysis.

This dissertation focuses on applying SPME-GC/MS and GC-Olfactometry methods to solve different food aroma related issues. The main conclusions from this work are listed below, grouped according to the publication they originate from.

6.1 CONCLUSIONS FROM PUBLICATION I

It was found that the majority of aroma-active compounds detected in Kama flour were pyrazines; additionally, 2,3- butanedione, hexanal and 1-octen-3-one contribute to the aroma, and have the highest flavor dilution factor.

6.2 CONCLUSIONS FROM PUBLICATION II

This work demonstrates that multiple extractions from the same sample vial using automated SPME is a perspective high throughput method for the characterization of volatile formation profiles of microorganisms.

Publication II provides good qualitative information about volatile formation and conversion processes taking place in the headspace vial during dough incubation and enables one to see differences between strains of *Lactobacilli*.

6.3 CONCLUSIONS FROM PUBLICATION III

A method was developed and shown to be appropriate for the precise and accurate quantification of acetaldehyde in solid and half-solid food matrices. The concentration of acetaldehyde in the various food products tested is below the threshold of considerable health risk to the children who consume these products.

6.4 CONCLUSIONS FROM PUBLICATION IV

The training strategy presented in Publication IV is suitable for training a panel of assessors to perform a GC-O task. It was found that female assessors performed the GC-O task better than male assessors, however, no significant difference was found between assessors with different backgrounds. A posterior intensity method was found to be feasible for the newly trained GC-O panel.

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APPENDICES

PUBLICATION I

Kaseleht K, Leitner E, Paalme T.

Determining aroma-active compounds in Kama flour using SPME-GC/MS and GC-Olfactometry.

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Determining aroma-active compounds in Kama flour using SPME-GC/MS and GC–olfactometry

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ABSTRACT: Kama flour is a traditional Estonian food, consisting of a newly milled powder mixture of roasted barley, rye, wheat and pea flour. For consumption it is normally mixed with sour milk and sweetened with sugar. In this study the aroma profile of Kama flour was studied. For the analysis, the solid-phase microextraction (SPME) method was optimized and used in combination with gas–chromatography/mass spectrometry (GC/MS) and GC–olfactometry (GCO), where the human nose is used as a sensitive and specific detector for odour-active compounds. For a positive identification of the aroma-active compounds, calculation of retention indices combined with the odour impression of pure reference compounds in a comparable concentration, and the mass spectrum under the same conditions as GC-O were used. The analysis of Kama flour headspace by GC/MS led to the detection of 89 compounds, of which 62 were found to have odour impression according to the literature. However, in total, 30 odour-active compounds were detected with GCO from which seven could not be detected under the same chromatographic conditions either by GC/MS or GC with FID. Most of the identified aroma compounds belonged to pyrazines coming from roasting process of Kama flour due to the Maillard reaction. Additionally, 2,3-butadedione, hexanal and 1-octen-3-one contributed to the aroma of Kama flour. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: Kama flour; aroma-active compounds; aroma analysis; GC-olfactometry; SPME; pyrazines

Introduction

Kama is a traditional Estonian food originating from 19th century, where it was made from stewed or boiled grains and legumes, which were dried in an oven, roasted, milled and mixed with sour milk. It was mostly enjoyed for breakfast, as a refreshing drink on hot summer days, or as a snack between main courses.[1] Nowadays, this finely milled powder mixture of roasted rye (30%), pea (20%), wheat (30%) and barley (20%) flour is mixed by the consumer with sour milk, yogurt or kefir, sweetened with sugar, fruits or honey, or served unsweetened, and is also used in numerous recipes.[2] The consumption of Kama has several health benefits, such as the intake of dietary fibre, vitamins and minerals from grains, calcium and beneficial lactic acid bacteria from sour milk and essential amino acids from all ingredients.

Recently, Kama flour has become an export item to other Baltic States, Finland, Germany, Russia and Austria, and therefore the quality, including the stability of Kama flour aroma, needs more attention. To the best of the authors' knowledge the aroma of Kama flour has not been characterized until now. Aroma characteristics of various cereals such as corn, rye, triticale, wheat, roasted barley, malted barley or rice have been investigated from the standpoint of volatile-compound composition, using solvent extraction techniques.[3] Taking into consideration the analysis of volatile compounds in food, solid-phase microextraction (SPME) has become a widely popular technique.[4–7] Some studies that use SPME for analysing cereal volatile composition, like barley[3] and oat volatiles have recently been published.[8,9] SPME is a fast, solvent-free technique that extracts aroma compounds from headspace. It combines sensitivity and selectivity due to the concentration of the fibre and the availability of different coatings. Optimization of SPME prior to GC–olfactometry (GCO) analysis is an important step for reaching the balanced extraction of volatiles and sufficient analytical sensitivity over the whole range of aroma compounds. The theoretical aspects of SPME have been well documented by Pawliszyn,[10] Scheppers-Wercinski,[11] and Pawliszyn,[12] and reviews dedicated to SPME in food analysis have been published by Kataoka *et al.*[13] and Wardencki *et al.*[14] Also, an in-depth critical review has been published by Nongonierma *et al.*,[15] but still the advantages of the method seem to outweigh the disadvantages, particularly when the aroma (the 'headspace') rather than the content of the product is analysed.

In this study the aroma profile of Kama flour was studied combining analytical instrumentation and the human nose. The work was carried out in order to optimize an automated SPME-GC/MS method and develop a GCO method for investigating the effect of roasting conditions and the stability of different batches of Kama flour in the future.

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Experimental

Chemicals and Materials

Kama flour ('Kamajahu'; Pere Leib, Tartu, Estonia) was purchased from a local store in Estonia. The optimization and detection of aroma-active compounds were determined from one batch of the product. Sodium chloride (99.5%) was purchased from Fluka (Buchs, Switzerland) and the water used was purified with Millipore (Millipore Corporation, Billerica, MA, USA). Helium, purity 5.0 (Air Liquid, Schwechat, Austria) was used as the carrier gas in the GC system. Silica SPME fibres with commercially available coatings (85 μm Car/PDMS; 65 μm DVB/PDMS; 60 μm CW/PDMS; 50/30 μm DVB/Carboxen/PDMS; 85 μm polyacrylate) were obtained from Supelco (Bellefonte, PA, USA). All new fibres were conditioned according to the manufacturer's recommendations prior to their first use. Clear glass SPME crimp vials (20 ml) with polytetrafluoroethylene (PTFE)/silicone septa (20 mm) were purchased from La-Pha-Pack (Langerwehe, Germany) and VWR International (West Chester, PA, USA). Magnetic sticks covered with glass where purchased from VWR International. The reference compounds 2,3-butanedione, acetic acid, 2-butanone, 3-methylbutanal, 2-methylbutanal, 1-penten-3-ol, 2,3-pentanedione, pentanal, 1-octen-3-one, 2 methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, ethylpyrazine, hexanal, 5-methylfurfural, 2-heptanone, furfural, trimethylpyrazine, acetylpyrazine, 2-ethyl-6-methylpyrazine, acetylpyrrole, 2,3-diethyl-5-methylpyrazine, 2-ethyl-3,5-dimethylpyrazine and the internal standard 1,2,3-trichloropropane were purchased from Sigma-Aldrich (St. Louis, MO, USA).

GC-MS Analysis

To identify and quantify volatile compounds of Kama flour the headspace SPME technique was adopted with an autosampler CombiPAL (CTC Analytics, Zwingen, Switzerland) and GC/MS (Agilent Technologies Inc., Santa Clara, CA, USA). The volatiles adsorbed onto the SPME fibre were thermally desorbed in the injection port of an Agilent 5890 GC equipped with a HP-5 column (J&W Scientific, Folsom, CA, USA) of 1 μm thickness, 30 m length and 0.25 mm inner diameter (i.d.). Helium (0.8 ml/min) was used as the carrier gas and the injector was in splitless mode for 10 min using an inlet liner of 0.75 mm i.d. The injector and detector temperatures were 270°C and 250°C, respectively. The column temperature was initially maintained at −10°C for 1 min before increasing to 280°C at a rate of 12°C/min and held for 1 min (total run time of 26.32 min). An oven starting temperature of −10°C was used for gaining sharper peaks and a thicker column for increasing adsorption efficiency. Volatile compounds were identified by comparing their spectra and retention indices with those present in the Wiley275 library using ChemStation software (Agilent Technologies) and the NIST Library (NIST, Gaithersburg, MD, USA). All samples were analysed in five parallels and an empty vial was analysed prior to every sample list in order to detect any contaminants from the environment.

Different fibres (CW/DVB, Car/PDMS/DVB, DVB/PDMS, Car/PDMS, polyacrylate) were studied to find the most efficient fibre towards the most volatiles in Kama flour. Also, the sample amount, water and salt addition were investigated. Kama flour was added in amounts of 0.5, 1 and 1.5 g into the 20 ml SPME vials. To improve the transport of compounds from the sample to the gaseous phase 5 ml of HPLC water or 5 ml of HPLC water with 0.5–2 g of sodium chloride was added to the vial with the Kama flour. The samples containing either only flour, or flour with water, or flour with water and sodium chloride were pre-incubated for 5 min at 40°C. Fibre was exposed to the headspace of the Kama flour or the slurry for 20 min at 40°C at 250 rpm. Additionally, the fibre exposure time and temperatures were investigated. Kama flour (1.5 g) was extracted for 20 min at 40, 50, 60 and 65°C. Fibre exposure time (5, 10, 20, 30 and 40 min) was investigated at 65°C.

For relative quantification of odour-active compounds 1,2,3-trichloropropane was used as an internal standard. One hundred microlitres of internal standard solution (5.00 mg/l in methanol) was added to 20 ml vial containing 1 g of pure Kama flour (final concentration 500 μg/kg) and mixed thoroughly in a magnetic mixer.

Analysis by GC–Olfactometry

The aroma of Kama flour was analysed with GCO with a HP-5 column (30 m × 0.25 mm × 1 μm), under the chromatographic conditions of 35°C held for 1 min, 8°C/min to 280°C, held for 1 min. The column flow was divided by a fixed 1:1 splitter using 100 μm deactivated fused silica columns between the sniffing port (ODP; Gerstel, Muelheim an der Ruhr, Germany) and a flame ionization detector (FID) (Agilent Technologies).

Panelists were trained on the aroma qualities prior to GCO analysis by using aqueous solutions of approximately 20 odorants to become familiar with an aroma lexicon; for example, hexanal for a 'green' odour or 3-methylbutanal for a malty odour. The sniffing of 1.5 g of Kama flour was carried out in duplicates by three trained assessors (female, age 24–30 years) and aroma-active compounds perceived by the assessors were recorded by voice recognition software (Gerstel) and directly imported to the Agilent Chemstation software.

For identification the retention indices were calculated using a series of straight chain alkanes (C8–C20) and compared to Kovats indices[16] and aromatic properties published in Flavornet (www.flavornet.org), Pherobase (www.pherobase.com) and the database of TU Graz GC laboratory (SKAF), together with GC/MS data. In addition, pure reference compounds were sniffed to confirm the results.

In order to find the most important odour-active compounds, which have the lowest odour thresholds, the amount of sample was reduced from 1.5 g to 1 g, 100 mg, 10 mg and 1 mg according to an aroma extraction dilution analysis (AEDA),[17] where samples are evaluated by the panelists in increasing dilution order and the impact of an odour-active compound is given by its dilution factor (FD) value.[18]

Results and Discussion

Optimization of the SPME Method

Prior to GCO analysis for identifying the key aroma-active compounds, optimization of the SPME method to extract the maximum number of the most aroma-active compounds from the sample was carried out. Adsorption and absorption (depending on the fibre) of aroma compounds on different fibres (CW/DVB, Car/PDMS/DVB, DVB/PDMS, Car/PDMS, polyacrylate) were studied to find the most efficient fibre towards the most volatiles in Kama flour. Also, the amount of sample, the amount of water and salt added, and the extraction time and temperature were varied to optimize the extraction conditions.

The GC/MS chromatograms (Figure 1) show that Car/PDMS fibre was able to adsorb volatiles from Kama flour more efficiently than the others. The intensities were higher, except for isopropyl dodecanoate and some other high boiling compounds. Compounds like 2-oxo-propanal, 2-butenal, 2-pentanone and carylene were observed only with this fibre. One fibre, however, might not give the complete list of volatiles present in Kama flour as the extraction depends on the hydrophobicity of the fibre and the competition among flavour compounds for the adsorption sites.[15] However, Car/PDMS extracted the widest range of flavour compounds compared to other fibres and was therefore chosen for further optimization of extraction conditions and GCO analyses.

The amount of sample, and effect of adding water and salt to Kama flour were investigated next. As expected, the higher intensities were obtained with 1.5 g of Kama flour compared to 0.5 and 1.0 g. The addition of water and salt decreased the peak intensities and several peaks of polar compounds

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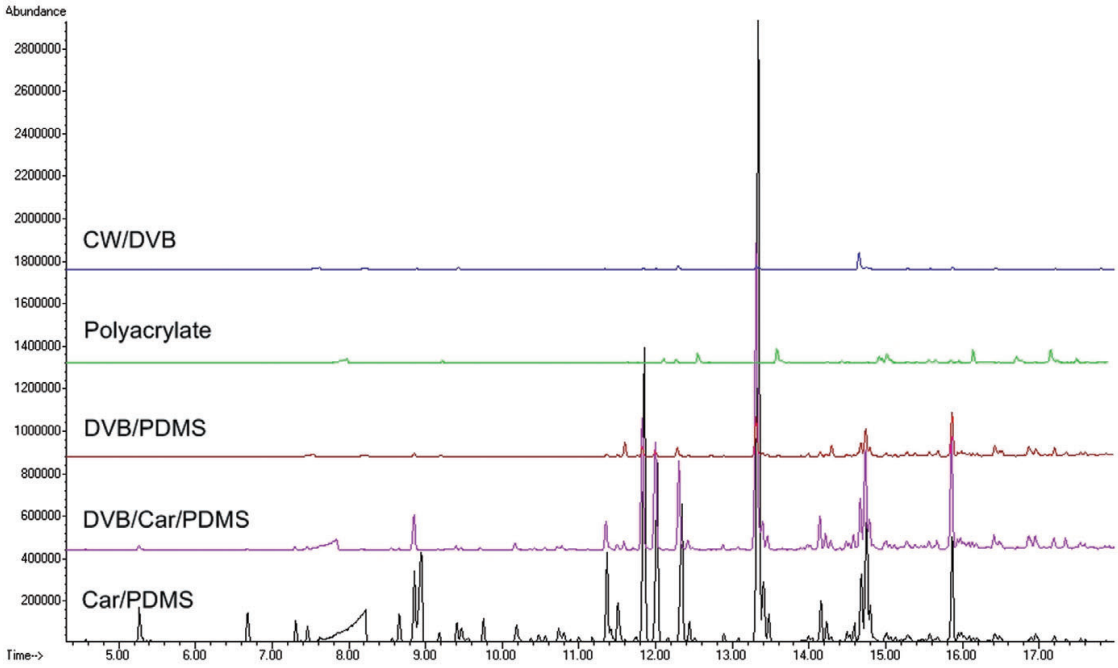

Figure 1. A section of overlaid GC/MS chromatograms of four different fibres. Extraction conditions: 1.5 g Kama flour, 65°C, 30 min

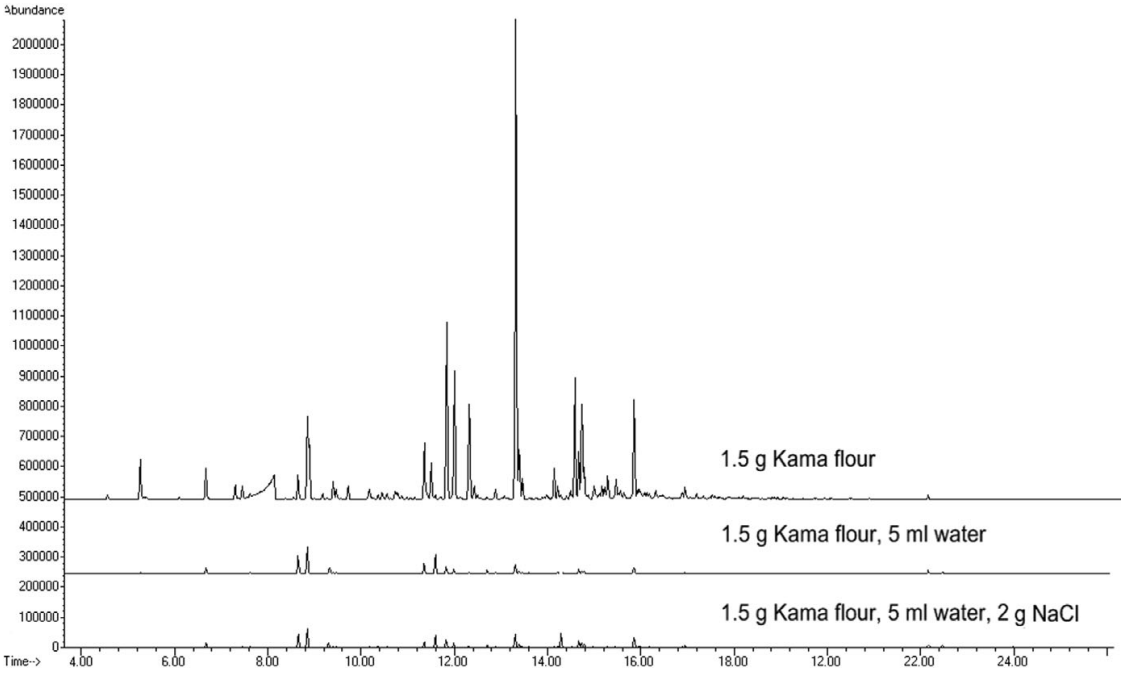

Figure 2. GC/MS chromatograms with 1.5 g Kama flour alone, compared to 1.5 g Kama flour in 5 ml water, and 1.5 g Kama flour in 5 ml water to which 2 g NaCl had been added. Extraction conditions: 1.5 g Kama flour, 40°C, 20 min

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Table 1. Compounds identified with GC/MS, corresponding odour perceptions, Kovats retention indices (DB5 column), observed retention indices and odor thresholds Gemert[26]

Compound name	Kovats RI[a]	Observed LRI	Threshold (mg/m^3 in air)	Odour perception[a,b]
Ethanol	668	—	2–988	Sweet, alcohol
2-Propanone	477	—	1–936	Acetone
iso-Butyraldehyde	662	—	0.015–0.41	Pungent, malt, green
2,3-Butanedione	593	—	0.000007–0.02	Butter
2-butanone	597	—	0.21–250	Ether, sweet
2-Methylfuran	603	—	—	Chocolate, sweet, solvent, burnt
Acetic acid	600	—	0.25–500	Sour
2-Butenal	648	—	0.18–0.57	Flower
3-Methylbutanal	650	—	0.0016–0.004	Malt
2-Methylbutanal	641	—	0.001–0.1	Cocoa, almond
1-Penten-3-ol	686	—	0.66–4.3	Butter, pungent
2-Pentanone	711	—	6.7–48	Sweet, ethereal, fruity, wine, acetone
2,3-Pentanedione	700	—	0.01–0.063	Cream, butter
Pentanal	732	—	0.009–40	Almond, malt, pungent
2-Ethylfuran	700	—	—	Sweet, ethereal, burnt, coffee, chocolate
3-Hydroxy-2-butanone	718	—	—	Butter, cream
Pyrazine	732	—	—	Pungent, sweet, corn, roasted hazelnuts
Dimethyldisulfide	785	—	0.0011–3.5	Cabbage, vegetable, onion, putrid
1-Pentanol	766	—	0.02–225	Strong, sweet, balsamic
1-Hydroxy-2-butanone	—	—	—	Sweet, coffee, musty, grain
Methylbenzene	[773]	—	0.4–260	Paint
2,3-Butanediol	806	803.6	—	Fruit, onion
Hexanal	801	807	0.02–0.33	Grass, tallow, fat
Pentan-3-ol	[759]	809.6	—	Fruit
2-Methylpyrazine	[828]	820	1.9	Popcorn, nut, cocoa, green, roasted
Furfural	829	829.5	0.008–1	Bread, almond, sweet
Furan methanol	[851]	852	32	Burnt
1,3-Dimethylbenzene	[802]	863	0.052–86	Plastic
2-Heptanone	895	889.8	0.045–3.3	Sulfur, pungent, green
Heptanal	901	901.4	0.006–9.5	Nut-like, fatty, fruity
Styrene	[893]	903	0.012–8	Balsamic, gasoline, pungent, roasted
2,5-Dimethylpyrazine	[905]	905.3	0.17–1.82	Cocoa, roasted nut, roasted beef, medicine
2,6-Dimethylpyrazine	913	909.2	0.25–1.72	Roasted
Ethylpyrazine	[907]	916.9	0.25–2	Peanut butter, wood
2,3-Dimethylpyrazine	892	920.2	0.88	Nut, peanut butter, cocoa, meat
trans-2-Heptenal	957	954.9	13–80	Pungent, green, vegetable, fatty, almond
Benzaldehyde	960	958.1	0.014–13	Almond, burnt sugar
5-Methylfurfural	978	961.5	0.005–6	Almond, caramel, burnt sugar
1-Octen-3-one	982	978.1	0.00003–0.0022	Mushroom
6-Methyl-5-hepten-2-one	974	986.8	—	Herb, oily, pungent, pear, pepper, mushroom
2-Pentylfuran	993	991.7	0.27	Green bean, butter
(*E,E*)-2,4-Heptadienal	1000	999.5	0.057	Fried
2-Ethyl-6-methylpyrazine	1003	1001.5	—	Roasted, nutty, cocoa
2-Ethyl-5-methylpyrazine	[993]	1004.1	0.036	Fruity, sweet, pungent
Trimethylpyrazine	1000	1005.5	0.05–0.19	Roast, potato, must
2-Ethyl-3-methylpyrazine	[1047]	1013.1	0.035–0.15	Roasted
2-Methyl-6-vinyl pyrazine	—	1019.2	—	Hazelnut, roasted
2-Ethyl-1-hexanol	1032	1028.8	0.4–0.74	Mild, oily, sweet, slight rose, green
Acetylpyrazine	1023	1032	0.0004	Roast
Benzene acetaldehyde	1049	1043.1	0.0006–0.0017	Harsh, green, honey
Acetylpyrrole	1045	1058.9	2	Bread, walnut, licorice
3,5-Octadien-2-one	1068	1072	—	Fresh, sweet, woody, mushroom
3-Ethyl-2,5-dimethylpyrazine	1082	1078.7	0.00245–0.02	Roast
2,3-Dimethyl-5-ethylpyrazine	1084	1081	—	Burnt, popcorn
2-Ethyl-3,5-dimethylpyrazine	1083	1084	0.000007–0.000011	Potato
2-Methoxyphenol	1089	1090.8	0.0001–0.64	Smokey, sweet, medicine
Nonanal	1104	1104.5	0.0003–1.7	Fat, citrus, green
2,3-Diethyl-5-methylpyrazine	1158	1155.5	0.000009–0.023	Potato, meat, roast
3,5-Diethyl-2-methylpyrazine	1160	1162	—	Baked
2,5-Dimethyl-3-isobutylpyrazine	1184	1176	—	Hazelnut
Dodecane	1200	1200.5	11.8–50	Alkane
4-Vinyl-2-methoxyphenol	1323	1327	0.0004–0.0008	Clove, curry

[a] www.flavornet.org.
[b] www.pherobase.com.

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Table 2. Odour-active compounds in Kama flour and concentrations (GC/MS signal) relative to IS in the headspace of Kama flour

No.	Compound name	Calculated RI	Odour detected	Concentration (relative units)	Standard deviation
1	2,3-Butanedione	—	Butter	23.6	0.93
2	Acetic acid	—	Sour	338.6	45.02
3	3-Methylbutanal	—	Green	21.9	1.84
4	2-Methylbutanal	—	Roasty	62.1	6.25
5	2,3-Pentanedione	—	Butter	48.3	5.48
6	Pentanal	—	Grass	53.0	6.41
7	Hexanal	807	Grass	181.0	21.76
8	2-methylpyrazine	826	Rancid, weak smell	864.9	77.20
9	Unknown	868.4	Roast	—	—
10	Unknown	901.4	Algae	—	—
11	2,6-Dimethylpyrazine	905.3	Roasted potato	2183.2	214.74
12	2,5-Dimethylpyrazine	909.2	Roast	—	—
13	ethylpyrazine	916.9	Roast	261.2	23.21
14	2,3-Dimethylpyrazine	920.2	Roasted meat	84.2	7.56
15	1-Octen-3-one	978.8	Mushroom	traces	—
16	2-Ethyl-5-methylpyrazine	996.6	Sweet	233.1	21.64
17	Trimethylpyrazine	1000.0	Ether-like roasty	—	—
18	2-Ethyl-6-methylpyrazine	1002.9	Roast, hazelnut	576.2	60.72
19	Unknown	1007.7	Rubber	—	—
20	Acetylpyrazine	1020.8	Roast, nut	26.3	7.79
21	Unknown	1032.5	Rubber	—	—
22	Benzene acetaldehyde	1044.6	Honey, sweet	—	—
23	Unknown	1051.9	Roast, grain	—	—
24	3-Ethyl-2,5-dimethylpyrazine	1077.7	Sweet, roast	38.0	5.00
25	2-Ethyl-3,5-dimethylpyrazine	1081.1	Roast	441.5	58.32
26	2,5-Diethylpyrazine	1089.3	Roast, green	0.9	1.29
27	2,3-Diethyl-5-methylpyrazine	1157.0	Grass, roast	48.1	4.95
28	3,5-Diethyl-2-methylpyrazine	1158.5	Roast	52.6	8.86
29	Unknown	1173.3	Roast	—	—
30	Unknown	1214.8	Roast	—	—

(ethanol, acetic acid, 1-penten-3-ol, propanoic acid, pyrazine and dihydro-2-methyl-3(2*H*)-furanone) were not detected, probably because of their strong interaction with water (Figure 2). Also, better reproducibility was obtained with Kama flour than with a flour–water suspension, probably because of a smaller matrix effect and higher signal intensity. Furthermore, the addition of water and salt to the flour, as used in many studies,[3,19,20] changes the ratio of the volatile compounds in the headspace and using just pure flour would reflect the aroma of Kama flour more accurately.

Additionally, the effect of extraction temperature and time was optimized. Kama flour (1.5 g) was extracted for 20 min at 40, 50, 60 and 65°C with exposure times of 5, 10, 20, 30 and 40 min. The results demonstrated that increasing temperature increased the chromatographic response. As expected, aldehydes, acids, pyrazines and compounds with higher molecular mass increased with higher temperatures, because the volatilization is favoured by the temperature increase, while smaller molecules, like ethanol, isobutyraldehyde, 2-propanone, 2-butanone and 2,3-butanedione were temperature independent. It was also seen that, generally, lower boiling compounds, like isobutyraldehyde, 3-methylbutanal, 2-propanone, 2,3-butanedione etc. were not affected by extraction time. This suggests that a distribution equilibrium of compounds with low boiling points was reached between food matrix, headspace and fibre. For pyrazines and other higher-boiling compounds the chromatographic response increased over 40 min. Eventually, 1.5 g of Kama flour after an extraction time of 30 min at 65°C with the Car/PDMS fibre was chosen as the optimum for aroma analysis.

Analyses with GC/MS and GC–Olfactometry

The total number of compounds detected with GC/MS was 89, and according to the literature, 62 of these were found to have an odour impression. The remainder were either not identified or, according to the literature, had no odour (e.g. isopropyl dodecanoate). The peaks in the GC/MS chromatogram (Figure 2) were identified and 62 aroma compounds were listed in Table 1. In this study, the aim was to identify which of those odour compounds might be detected by GCO under the same extraction and chromatographic conditions as in GC/MS. Odour threshold (OT) values in Table 1 allow us to predict whether a compound would be odour active. For example, 2,3-butanedione and 2-ethyl-3,5-dimethylpyrazine, which have an OT value as low as 0.00007 mg/m^3 in air, were both detected by the GCO. In total, 30 compounds (Figure 2) were detected in Kama flour using GCO and for identification the odour perception and retention indices were compared to those listed in Table 1. It was found that seven odour-active compounds having very high OT values (retention indices of 868.4, 901.4, 1007.7, 1032.5, 1051.9, 1173.3 and 1214.8)

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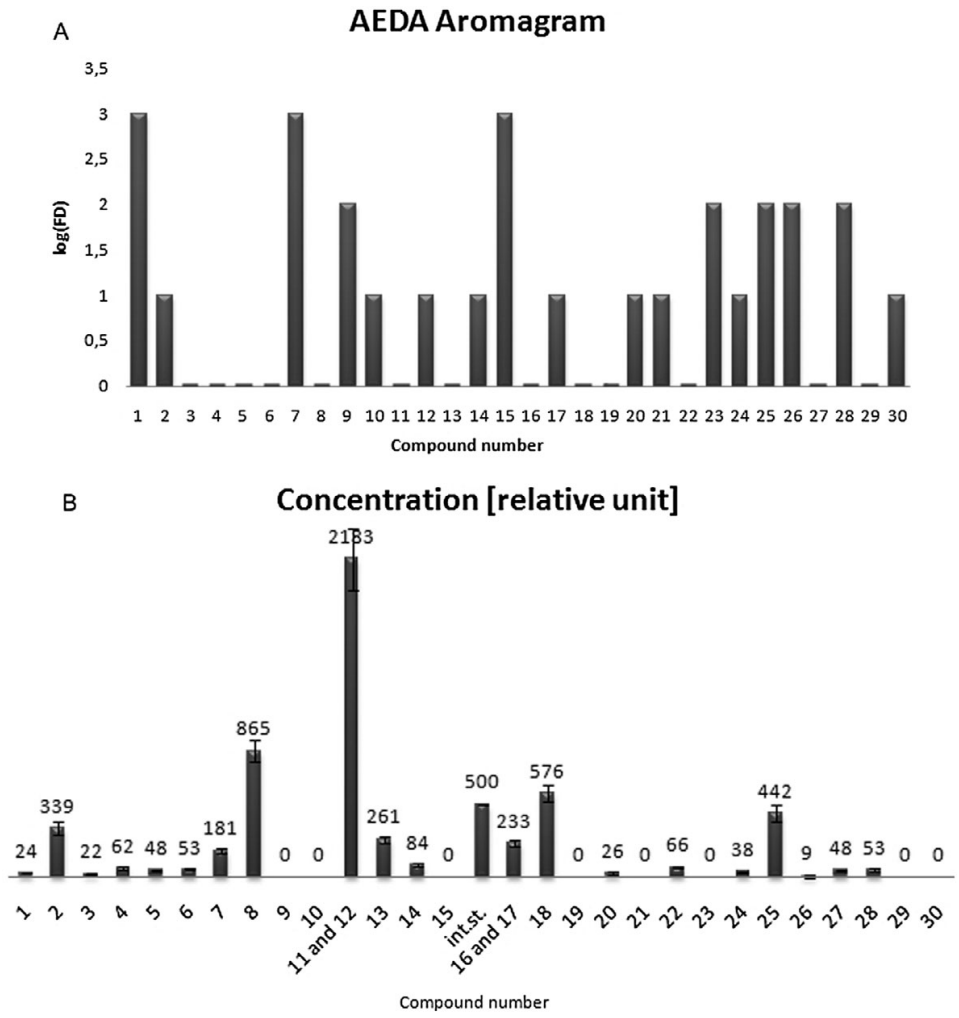

Figure 3. (A) AEDA results attained from the GCO analysis of volatiles in Kama flour; and (B) concentrations (GC/MS signal) of odour-active compounds relative to 1,2,3-trichloropropane (internal standard). The compound numbers are as given in Table 2

were not detected by the GC/MS and are therefore listed in Table 2 as unknown. For the most intense odour-active compounds, the amount of sample was reduced from 1.5 g to 1 g, 100 mg, 10 mg, 1 mg until no odour was detected and an AEDA was carried out. An AEDA aromagram with concentrations (relative units) calculated relative to internal standard is given in Figure 3 (the numbers correlate to Table 2). According to aromagram, the highest FD factors were found for 2,3-butanedione (compound 1), hexanal (7) and 1 octen-3-one (15), meaning they were detected in 1 mg of Kama flour. The AEDA method is, however, considered to be a screening method and compounds with the highest values are candidates for further study to evaluate their true contribution to the odour of a food.[21] Therefore these compounds should be considered as 'most likely' the most important compounds of the Kama flour. In relative concentration chart, compounds 9, 10, 15, 19, 21, 23, 29 and 30 were either not detected or only traces of the compound was detected by the GC/MS, like for 1-octen-3-one (15). 2,5-Dimethylpyrazine (11), 2,6-dimethylpyrazine (12) and 2-ethyl-5-methylpyrazine (16) and trimethylpyrazine (17) could not be separated by GC/MS and are given as one peak.

Most of the odour-active compounds found by SPME-AEDA belong to the pyrazines (14 of the identified 23 compounds) and quite likely most of the unidentified compounds eliciting a roasty odour (compounds 9, 23, 29 and 30) also belong to the pyrazines. However, the aroma compounds detected from the lowest sample size (1 mg) were 2,3-butanedione, eliciting a buttery odour, hexanal responsible for a green note, and 1-octen-3-one for mushroom. 2,3-Butanedione is formed during fermentation and the Maillard reaction,[22] hexanal is a result of lipid oxidation[22] and 1 octen-3-one is derived from flour fatty acids.[23] Pyrazines are formed during the roasting process via the Maillard

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reaction. Additionally, pentanal, benzene acetaldehyde, 2- and 3-methylbutanal (common lipid oxidation products),[3] acetic acid (a fermentation product) and 2,3-pentanedione (fermentation and lipid oxidation products) were found to give Kama flour aroma, but compounds 10, 19 and 21 still remain unknown.

Concerning the origin of the detected compounds, it has been noted that different cereals or grain varieties commonly have similar volatile compounds but in different concentrations.[24] However, according to Cramer *et al*.,[3] 2- and 3-methylbutanal, pentanal and hexanal have not been found in wheat flour , but were present in barley flour and 2- and 3-methylbutanal were determined as the most odour-active compounds in roasted barley. Additional information on the volatiles of dried pea was not found in the literature.

Further, the GC/MS peak area was clearly not proportional to odour intensity. For example, furfural detected by GC/MS has a nutty almond-like odour impression, according to the literature,[25] but was not detected while sniffing using GCO. Seven compounds that are marked as unknown in Table 2 were not obtained with the GC/MS or FID chromatogram, which shows they were present in too low quantities to detect, but had a very low odour threshold so they were detectable only with the human nose. It indicates the importance of GCO when detecting aroma compounds at low concentrations.

The determination of absolute concentration of different aroma compounds in Kama flour is more complicated as it requires external calibration involving adding different concentrations of a standard mixture of Kama flour aroma compounds and internal standard to Kama flour. However, a relatively small standard deviation of the parallel samples using GC/MS demonstrates that by using an internal standard the comparative quantitative aroma analysis of different batches of Kama flour would be possible. Although the behaviour of the internal standard is not the same as that of each compound in the matrix, it still reveals a relative ratio between pyrazines and makes it possible to compare the intensities of aroma profiles of different Kama flour preparations (batches), which is the aim for the future.

Conclusion

This study was carried out to characterize the aroma of Kama flour. It was found that most of the aroma-active compounds detected in Kama flour were pyrazines; additionally, 2,3-butanedione, hexanal and 1-octen-3-one contribute to the aroma, and have the highest flavour dilution factor. The results obtained comparatively by using GC/MS and GCO demonstrated that not all aroma-active compounds in Kama flour detected by sniffing could be detected by MS or FID detectors. One of the future aims, comparing the aroma profiles of different Kama flour batches, was also gained as the optimized automated SPME technique proved to be successfully applicable to analyse the volatile compounds of Kama flour. This technique will be used for comparative analysis of aroma profile and intensity of different batches of Kama flour and aroma stability during storage.

Acknowledgements

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PUBLICATION II

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Original article

Analysis of volatile compounds produced by different species of lactobacilli in rye sourdough using multiple headspace extraction

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Summary Profiles of volatile organic compound (VOC) produced by nine individual lactic acid bacteria (LAB) during rye sourdough fermentation were compared by automated SPME and GC/MS-Tof. The dough samples were inoculated with individual strains, placed inside the headspace vials and incubated during next 24 h. The production or loss of VOC-s was followed by adsorbing volatiles onto 85-m Car/PDMS fibre in every 4 h. Volatile profiles differed among LAB species and divided LAB into two main groups – hetero- and homofermentative. Hetrofermentative LAB (*Lactobacillus brevis*; *Leuconostoc citreum*; *Lactobacillus vaginalis*, *Lactobacillus panis*) showed high production of acetic acid, CO_2, ethanol, ethylacetate, producing also hexyl acetate, ethyl hexanoate and isopentyl acetate. Whereas homofermentative LAB species (*Lactobacillus helveticus*; *Lactobacillus casei; Lactobacillus sakei*; *Lactobacillus curvatus*) produced a considerable amount of 2,3-butanedione. Production of L-leucine methyl ester was unique for *Lb. sakei*, *Lb. casei* and *Lb. curvatus* strains. *Lb. helveticus* was the only LAB that produced benzaldehyde.

Keywords Lactic acid bacteria, multiple headspace extraction, rye sourdough, solid-phase microextraction, volatile compounds.

Introduction

Sourdough is essential to rye bread by improving bread quality in terms of delaying staling, improving taste, texture, aroma and generally increasing shelf life (Plessas *et al*., 2008). As rye does not contain enough gluten, the structure of rye bread depends on the starch in the rye flour, as well as other carbohydrates known as pentosans (Wing & Scott, 1999).

Rye bread flavour is influenced by the recipe, processing conditions and its starter culture composition. Lactic acid bacteria (LAB) are the main part of sourdough starter. Although LAB are mostly responsible for the sourdough acidification, they also liberate aroma precursors. It is, however, not fully clear to what extent different LAB contribute to flavour formation.

The generation of odorants occures in sourdough mainly because of enzymatic and microbial processes during sourdough fermentation; however, some compounds are already present in the rye flour (Kirchoff & Schieberle, 2002).

Homofermentative LAB present in sourdough are able to convert hexoses almost completely into lactic acid (> 85%), whereas heterofermentative LAB degrade hexoses into lactic acid, acetic acid, ethanol and CO_2 (Hansen & Schieberle, 2005). In addition, LAB liberate aroma precursors, such as free amino acids, which concentrations increase significantly during flour hydration (Mihhalevski *et al*., 2010) and sourdough fermentation (Hansen & Schieberle, 2005). The key degradation reaction of amino acids during dough fermentation is the *Ehrlich* pathway leading to formation of aldehydes or the corresponding alcohols (Hansen & Schieberle, 2005). The composition of volatile fraction of rye sourdough has been found to consist of acetic acid, ethanol and volatile compounds. In addition, the increased amounts of esters, such as acetates, propionates, hexanoates, lactates and octanoates, have been reported; also, (*E*)-2-butenal, (*Z*)-3-hexenal, nonanal and heptadienal were characterised as sourdough constituents (Kirchoff & Schieberle, 2002). The number of aldehydes was lower in sourdoughs fermented by heterofermentative LAB (Kirchoff & Schieberle, 2002). Whereas the content of ethyl acetate as well as hexyl acetate has been shown to be higher in rye sourdoughs fermented with heterofermentative LAB compared with sourdoughs fermented with homofermentative LAB (Lund *et al*., 1989).

The aim of this work was to study the impact of different LAB strains on volatile organic compounds

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formation in sourdough and its significance on rye bread aroma development using multiple headspace extraction (MHE). MHE is originally a technique for quantifying analytes in solid samples, which avoids matrix effect (implying several consecutive extractions from the same sample until no volatiles are present) (Ezquerro *et al*., 2003), but is used for monitoring fermentation dynamics in this study.

Although volatile compounds in rye sourdough have been studied previously (Kirchoff & Schieberle, 2002; Hansen & Schieberle, 2005), no data are available on the dynamics of volatile compound patterns during dough fermentation with single LAB strains. The 10 kGy irradiated flour was used to prevent the influence of indigenous flour LAB on aroma formation. A 24-h fermentation at 24 °C without addition of yeasts was used to simulate rye sourdough fermentation conditions used in the local bakery.

Materials and methods

Microorganisms

The lactobacilli strains *Lactobacillus helveticus* N92, *Lactobacillus helveticus* E96, *Lactobacillus casei* N726, *Lactobacillus vaginalis* N1113, *Lactobacillus panis* N915 were isolated from industrial sourdough. *Lactobacillus curvatus* 0E12-11, *Lactobacillus sakei* 0E12-10, *Leuconostoc citreum* 3N18-10, *Lactobacillus brevis* 0E12-37 were isolated from spontaneously fermented rye flour. The bacteria were isolated and identified based on 16S r-RNA sequence (Viiard *et al*., unpublished) essentially as described by Van der Meulen *et al*. (2007). The strains were maintained in stock as 25% glycerol cultures at −80 °C and re-cultivated anaerobically in De Man, Rogos and Sharpe (MRS) broth (Lab M, Bury, UK) 48 h at 30 °C for the dough fermentation experiments (Mihhalevski *et al*., 2010).

Chemicals and materials

Rye flour was purchased from Tartu Veski (Tartu, Estonia) and irradiated at 10 kGy using dosimetric system GEX WinDose (Gex Corporation, Centennial, CO, USA). Helium, purity 5.5 (AGA Eesti AS, Tallinn Estonia), was used as the carrier gas in the GC system. Silica SPME fibre with 85-μm Car/PDMS coating (chosen according to Kaseleht *et al*., 2010) was obtained from Supelco (Bellefonte, PA, USA). Fibre was conditioned according to the manufacturer's recommendations prior to its first use. Clear glass SPME crimp vials (20 mL) with the polytetrauoroethylene (PTFE)/silicone septa (20 mm) were purchased from Supelco. MilliQ water (Millipore Corp., Molsheim, France) was used whenever samples were prepared.

Sample preparation

Bacterial suspension ($1–5 \times 10^7$ cfu mL^{-1}) in 0.5% NaCl was mixed with sterile rye flour (1:1) and mixed for 15 min using a model 400 circulator stomacher (Seward Ltd., Worthing, UK). For VOC analysis, 1 g of sourdough was weighed in two parallels into 20 mL headspace vial and sealed with the crimp cap. Noninoculated sterile dough was used as control. First, VOC analysis of dough was performed immediately after sealing the vial and placing it in the autosampler tray (about 15 min after flour hydratation and inoculation), after which the sample vials were held sealed and incubated at 24 °C on the sample tray for MHE. The VOC was extracted by SPME technique from the vials at 4, 9, 14, 20 and 24 h to monitor the changes in sourdough VOCs during growth of nine different LAB and a noninoculated control.

GC/MS analysis

To analyse sourdough volatile compounds in the sample vials, the SPME technique was adopted with an autosampler CombiPAL (CTC Analytics, Zwingen, Switzerland) and GC/MS-Tof (Agilent Technologies Inc., Santa Clara, CA, USA and Waters, Manchester, UK). The volatiles adsorbed onto the SPME fibre during 20-min extraction were thermally desorbed 10 min at 260 °C in the injection port of an Agilent 6890 gas chromatograph equipped with a DB-5MS column (J&W Scientific, Folsom, CA, USA) with a 0.25 μm film thickness, 30 m length and 0.32 mm inner diameter (i.d.). Helium (1.7 mL min^{-1}) was used as the carrier gas, and the injector was in splitless mode for 10 min using an inlet liner of 0.75 mm i.d. The detector temperature was 200 °C, and for ionisation, electron energy of 70 eV was used. The column temperature was initially maintained at 35 °C for 3 min after injection before increasing to 155 °C at a rate of 7 °C min^{-1} and to 280 °C at a rate of 45 °C min^{-1} with a total run time of 22.92 min. Volatile compounds were tentatively identified by comparing their spectra and retention indices with those present in the NIST05 library (NIST, Gaithersburg, MD, USA) using MassLynx and ChromaLynx software (Waters). For intensity analysis, the peak areas were integrated and compared using MassLynx software.

Results and discussion

Volatile formation in noninoculated rye sourdough

Comparison of SPME chromatograms of the noninoculated dough prepared from 10 kGy rye flour demonstrated that chromatographic pattern changed during 24-h incubation (Fig. 1a, *peaks 1–34*, and b, *peaks 1–34*

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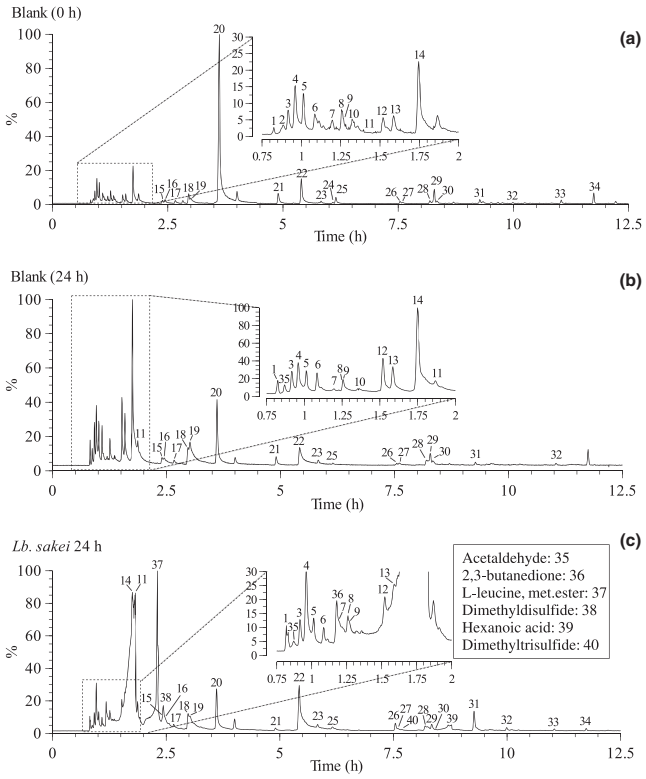

Figure 1 Chromatograms of 10 kGy irradiated dough at 0 and 24 hour (a and b). Chromatogram of 10 kGy irradiated dough after inoculation with Lb. sakei after 24 hours (c). Peak numbers correspond to the compounds in Table 1.

and peak 35). The change was the result of biological and chemical processes in sourdough and removal of volatiles by SPME fibre. Although the compounds were repeatedly taken out with the fibre, the concentration of several compounds like 3-methylbutanal (*peak 12*), 2-methylbutanal (*13*), carbon dioxide (*1*), acetaldehyde, ethanol (*3*), propanal (*4*), 2-methylpropanol (*5*), 2-butanone (*7*), ethyl acetate (*9*), acetic acid (*11*), 1-penten-3-ol (*14*), (Z)-2-penten-1-ol (*19*) increased in the vials during incubation. Compounds that decreased during 24-h incubation were 2-propenal (*2*), 2(3)-methylfuran (*8*), trichloromethane (*10*), 3-methylbutanol (*15*), 2-methylbutanol (*16*), (E)-2-pentenal (*17*), 1-pentanol (*18*), hexanal (*20*), 2-hexenal (*21*), 1- hexanol (*22*), 2-heptanone (*23*), (Z)-4-heptenal (*24*), heptanal (*25*), (E)-2-heptenal (*26*), benzaldehyde (*27*), 1-octen-3-ol (*28*), 2-pentylfuran (*29*), 2-octanone (*30*), 2-octenal (*32*), nonanal (*33*), 2-nonenal (*34*). The production of those compounds was less than the amount transferred out with the fibre during repeated sampling.

Volatile compound formation in dough inoculated by individual LAB strains

Analysis of volatile peak intensities (Total Ion Count, TIC) at different time points of SPME extraction during 24-h fermentation of rye dough inoculated with nine different LAB was carried out. Both homofermentative (*Lb. sakei, Lb. casei, Lb. curvatus, Lb. helveticus E96 and Lb. helveticus N92*) and heterofermentative (*Lb. brevis, Ln. citreum, Lb. panis, Lb. vaginalis*) LAB strains were used for inoculation. To determine the volatile compound production profiles of studied LAB, the 10 kGy noninoculated dough incubation profiles were subtracted from the profiles obtained for correspondent inoculated dough samples. All the compounds tentatively identified in dough are listed in Table 1.

Inoculation of dough significantly affected the SPME volatile profiles in fermentation vials, for example 24-h *Lb.sakei* (Fig. 1c, *peaks 1–34* and *peaks 35–40*) vs. 24-h blank (Fig. 1b). Appearance of hexanoic acid, by-product of fatty acid biosynthesis, on chromatogram

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Table 1 Profiles of volatile compounds formation during growth of individual bacteria in dough. 'Peak' presents the peak number in Fig. 1, 'Control' is noninoculated control prepared from 10 kGy irradiated rye flour. Compounds were either not detected (0), decreasing (−) or increasing (+) during growth phase of corresponding lactic acid bacteria

			Heterofermentative				Homefermentative				
Compound	**Peak**	**Control**	***Lb. brevis***	***Ln. citreum***	***Lb. panis***	***Lb. vaginalis***	***Lb. sakei***	***Lb.curvatus***	***Lb.casei***	***Lb.helv.E96***	***Lb.helv.N92***
Carbon dioxide	1	+	++	++	++	++	++	++	+	+	+
2-propenal	2	–	0	0	–	–	0	0	0	–	–
2-propen-1-ol		0	–	0	–	+	0	0	0	0	0
Acetaldehyde	35	+	– –	– –	– –	– –	+	+	–	+	+
Ethanol	3	+	+++	+++	+++	+++	+	+	+	+	+
Acetic acid	11	+	++++	++++	+++	+++	+++	+++	+	++	+
Ethyl acetate	9	+	+++	+++	+++	++	–	–	–	–	–
Propanal	4	+	–	– –	– –	– –	+	+	+	+	+
2-methylpropanal	6	+	–	– –	– –	–	+	+	–	+	+
2-methylpropanol	5	+	+	+	+	+	+	+	+	+	+
3-methylbutanal	12	++	– –	– –	– –	– –	+	–	–	–	+
3-methylbutanol	15	– –	+	+	+	+	+	+	+	+	+
2-butanone	7	+	–	–	–	–	+	+	+	+	+
2-methylbutanal	13	++	– –	– –	– –	– –	–	–	–	–	+
2-methylbutanol	16	– –	+	+	+	+	+	+	+	+	+
2-pentenal (E)	17	–	0	–	–	–	+	+	+	+	+
1-pentanol	18	–	+	+	+	+	+	+	+	+	+
1-penten-3-ol	14	+	–	+	+	–	+	+	+	+	+
2-penten-1-ol (Z)	19	+	+	+	+	+	+	+	+	+	+
Isopentyl acetate		0	+	+	+	+	0	0	0	0	0
Hexanal	20	– – –	– – –	– – –	– – –	– – –	–	– –	– – –	– –	– –
1-hexanol	22	– – –	++	++	++	++	+	+	+	+	–
2-hexenal	21	– –	– –	– –	0	– –	–	–	–	+	–
Hexanoic acid	39	0	++	+++	++	++	++	+++	++	++	++
Hexyl acetate		0	++	++	+	+	0	0	0	0	0
Ethyl hexanoate		0	+	+	+	0	0	0	0	0	0
Heptanal	25	– – –	– – –	– – –	0	– – –	–	–	–	–	–
4-heptenal (Z)	24	– – –	– – –	– – –	0	– – –	– – –	–	– – –	– – –	– – –
2-heptenal (E)	26	– –	+	–	+	–	++	+	+	+	+
2-heptanone	23	–	–	–	+	+	+	+	+	+	+
2-octanone	30	– – –	–	–	–	–	–	–	–	–	–
2-octenal	32	– – –	0	– – –	0	– – –	+	+	– – –	+	– – –
1-octen-3-ol	28	–	+	+	+	+	+	+	+	–	– –
Nonanal	33	– –	–	–	–	– –	+	+	+	+	+
2-nonenal	34	– – –	– – –	– – –	0	– – –	–	–	– – –	– – –	–
2(3)-methylfuran	8	–	–	–	–	–	–	–	–	–	–
2-pentylfuran	29	– – –	+	+	+	+	+	+	+	+	+
Trichloromethane	10	–	0	0	0	–	0	–	0	0	–
Benzaldehyde	27	– –	– –	– –	0	– –	–	–	– –	++	++
Dimethyldisulfide	38	0	++	+	+	+	++	++	+	+	+
Dimethyltrisulfide	40	0	+	0	0	0	+	+	0	0	0
L-leucine,met.ester	37	0	0	0	+	0	+++	+++	++	0	0
2,3-butanedione	36	0	0	0	0	0	+	++	++++	+	+
Unknown 9.27	31	– –	+	++	–	–	++	++	++	++	+

was used as an indicator of growth start of LAB. As growth rates of the inoculated LAB strains differ, appearing of hexanoic acid on chromatogram was used as starting point for comparative characterisation of volatile compound formation during growth of individual species, for example during 14–19 h after inoculation for *Lb. panis* and 19–26 h for *Lb. helveticus N92* (Fig. 2).

The results obtained show that hexanoic acid, 1-hexanol, 1-pentanol, 2- and 3-methylbutanol,

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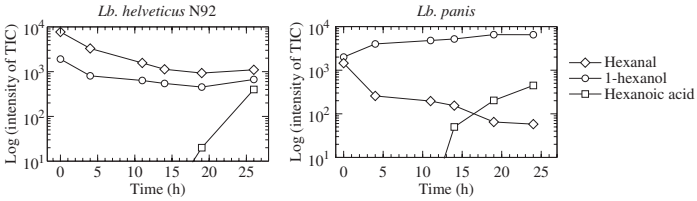

Figure 2 Intensities (total ion count) of hexanal, 1-hexanol and hexanoic acid during 24 hours in homofermentative *Lb. helveticus* N92 and heterofermentative *Lb. panis*.

2-pentylfuran and dimethyldisulfide were produced and 2- and 3- methylbutanal consumed during growth of all LAB species in sourdough (Table 1).

The production of alchohols suggests that so-called *Ehrlich* aldehydes, including 2- and 3-methylbutanal, originally present in the flour and additionally forming during dough incubation were reduced by LAB into corresponding alcohols: 2- and 3-methylbutanol (peaks *15* and *16*), respectively. Taking into account also the formation of hexanol and heptanol, it can be suggested that dough environment with growth of LAB becomes reducing (Vermeulen *et al.* 2007). Most probably, LAB use the aldehydes present in dough as electron acceptors in alcohol dehydrogenase reaction (EC 1.1.1.1) to oxidise NADH produced in excess during the growth of LAB. The ability to reduce aldehydes was more pronounced for heterofermentative species than for homofermentative.

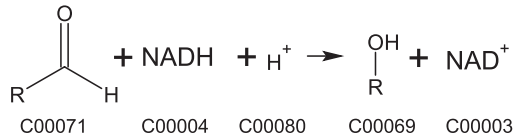

Figure 3 Reaction pathway of alcohols derived from aldehydes.

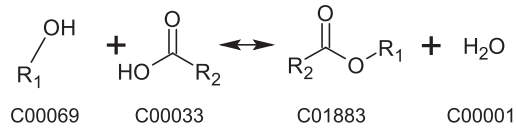

Figure 4 Reaction pathway for esters from alcohol and organic acid.

Heterofermentive species

The aroma profiles of different species in sourdough correspond well to division of LAB to homo- and heterofermentative species. As expected, the inoculation of dough with heterofermentative species using phosphoketolase pathway resulted in remarkable CO_2, ethanol and acetic acid production. Those compounds were produced also by homofermentative species but at much lower rate.

While reduction in aldehydes (2- and 3-methylbutanal, 2-hexenal, (E)-2-heptenal, hexanal, heptanal and 2-octenal) was more strongly expressed in hetero- than homofermentative species, the reduction in acetaldehyde, propanal, 2-methylpropanal, 2-butanone into corresponding alcohols was observed only in case of heterofermentative species. The differences were probably owing to the higher alcohol dehydrogenase activity and/or higher surplus of reductive power (NADH) during the growth of heterofermentative species (Fig. 3).

Homofermentative species, instead of degrading, produced (E)-2-pentenal and nonanal and also produced less 1-hexanol than heterofermentative species.

Production of esters like ethyl acetate, isopentyl acetate, hexyl acetate, ethyl hexanoate was also specific only to heterofermentative species. That is because of the higher production of ethanol and acetate by heterofermentative species (Fig. 4).

Our results show that there were no significant differences in volatile compound formation profiles within heterofermentative species studied.

Homofermentive species

All homofermentative lactobacilli produced 2,3-butanedione (particularly *Lb. casei*), which could not be seen in case of heterofermentative species. This was also observed by Hansen & Schieberle, 2005; who stated that the content of diacetyl was higher in sourdoughs manufactured with homofermentative compared with heterofermentative cultures and was also higher in the corresponding breads. Probably, the heterofermetative bacteria lack pathway for diacetyl synthesis, or/and the redox potential is too high to allow oxidation of acetoin (Fig. 5).

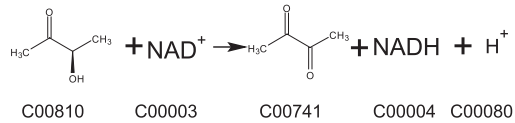

Figure 5 Reaction pathway of diacetyl from acetoin.

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Lb. sakei, *Lb. curvatus* and *Lb. casei* were producing L-leucine methyl ester; however, a very small peak was also found in heterofermentative *Lb. panis*.

L. sakei and *L. curvatus* strains were rather similar. They were isolated from the first stages of spontaneous fermentation of sourdough. These species had high maximum specific growth rate in rye dough (0.8–0.9 h^{-1}) (Mihhalevski *et al.*, 2010) and were unique in producing considerable amount of L-leucine methyl ester. The compound is probably derived by esterification of leucine and methanol. The reasons for the formation of L-leucine methyl ester are not clear. Also, significant acetate production was observed in those species compared with other homofermentative LAB.

Lb. helveticus E96 and *Lb. helveticus* N92 were dominating strains in repeated cycle of industrial rye sourdough in local bakery. Compared to other species studied the *Lb. helveticus* was unique in producing benzaldehyde. Benzaldehyde has been reported to be produced from phenylalanine in *Lb. plantarum* (Groot & de Bont, 1998). In addition, different from all the other dough studied, 1-hexanol decreased in dough inoculated with *Lb. helveticus* N92 strain. This might be explained by the low extent of reduction in 1-hexanal to 1-hexanol compared with other strains.

Significant formation of L-leucine methyl ester was observed for dough inoculated with the *Lb. casei* strain. Also, the amount of synthesised 2,3-butanedione was the largest among homofermentative LAB strains studied.

Multiple headspace extraction of volatiles

The MHE/SPME method presents certain drawbacks, which limits its use for quantitative purposes. In case of sourdough volatile analysis, MHE technique could be used for quantification after the fermentation is stopped or samples are fixed. Doing that we can loose the main advantages of the technique: minimal processing of samples and high throughput as well as ability to follow dynamics of volatile formation during fermentation in single vial.

During MHE of dough samples, the amount VOC detected is the result of two processes: volatile formation and headspace extraction. If the amount of extracted volatile (GC peak area) remains constant in two consequent measurements, it can be expected that production rate between those two points is equal to amount of extracted compound divided by time interval between extractions. For different fermentations and compounds, this point in different growth phases at different biomass concentrations might be different. It makes thus quantitative comparison of production rates of different volatiles in different species difficult, and process quantification using SPME method would be possible using labelled internal standards.

In this study, the method conducted with automated SPME-GC/MS was used for semi-quantitative purposes and to identify the volatile production profiles during growth of different species of sourdough bacteria in their natural environment and relate them to corresponding chemical and biochemical transformations in sourdough. The culture conditions during sourdough fermentations in headspace vials were similar as possible to those used in the bakery. The temperature used was 24 °C, although higher fermentation temperatures are used in many bakeries. All those temperatures remain probably in range of Arrhenius plot, which affect mainly the specific growth rate but not other physiological properties of the culture (Adamberg *et al.*, 2003). To follow the dynamics of the process, the fermentation was started from lower cell densities and higher pH than in industry. Low pH can cause the acid stress and affect the both volatile production rate and extraction coefficient. Next subject to study would be aroma formation in mixed cultures to answer the question if the aroma profiles in mixed culture differ from the sum of individual cultures.

The results of this study demonstrated that multiple extractions from the same sample vial using SPME fibre is a perspective high throughput method for characterisation of volatile formation profiles of microorganisms, in particular during fermentation in solid phase, like dough. That is particularly because of the automatisation that allows analysing several different samples in parallel and minimises human factor by allowing precise sampling and less fibre damage.

Present work gives good qualitative information about volatile formation and conversion processes taking place in the headspace vial during dough incubation and enables to see the differences between different strains of lactobacilli. How the aroma profile of sourdough bacteria affects the final sensory properties of bread is the goal for another study. Multiple headspace extraction in combination with sensory analysis is certainly perspective approach in such studies.

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PUBLICATION III

Kaseleht K, Paalme T, Nisamedtinov I.

Quantitative analysis of acetaldehyde in foods consumed by children using SPME/GC-MS(TOF), On-fiber derivatization and deuterated acetaldehyde as an internal Standard.

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Quantitative Analysis of Acetaldehyde in Foods Consumed by Children using SPME/GC-MS(Tof), On-fiber Derivatization and Deuterated Acetaldehyde as an Internal Standard

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Abstract. The aim of this study was to develop a precise quantitative method for acetaldehyde determination in solid food matrixes as, to the authors' best knowledge, no such method was available. The method was applied for quantification of acetaldehyde in various foods consumed by children such as yoghurt, purees, curd creams etc. On-fiber derivatization of acetaldehyde with PFBHA was used to increase the method sensitivity and deuterated acetaldehyde was used as an internal standard for exact quantification. The article is mostly focused on method development, including sample preparation. The amount of acetaldehyde in foods was found to be rather negligible, with the highest concentration (up to 31.5 ± 0.05 mg kg^{-1}) detected in yoghurts.

Key words: acetaldehyde, food products, PFBHA, SPME/GC-MS

INTRODUCTION

Acetaldehyde (AA) is a widely occurring compound in nature and is also industrially produced for various applications, including food processing. In nature, acetaldehyde is commonly found in fruits as an intermediate product in the respiration of higher plants and in alcoholic fermentation (Miyake & Shibamoto, 1993). AA is added to foods as a flavour enhancer (fruit flavour) but also as a preservative of fruits and fish products (IARC, 1985).

Exact quantification of acetaldehyde in food products has become of increased interest as the International Agency for Research on Cancer (IARC) has changed the cancer risk classification of AA from an agent possibly carcinogenic to humans (Group 2B) to an agent carcinogenic to humans (Group 1) (IARC Press release Nº196). The European Flavour and Fragrance Association (EFFA, 2010) has reported that the concerns raised for AA are mainly linked to the risk of potential cancer formation in the upper digestive tract after alcohol abuse and the combined effect of alcohol and smoking. Notably, there are no known safety concerns raised by EFFA so far related to the use of AA as a flavouring substance under normal conditions of use in food.

On the other hand, food products are the primary source of AA exposure to non-smokers and non-drinkers. The greatest concern regarding AA is focused on children and neonates, whose weight-adjusted AA exposure may be the highest. The Committee

of Experts on Flavouring substances from the Council of Europe (CoE) has approved AA concentration in beverages 23 mg kg^{-1} and in food 20 mg kg^{-1}. The FDA has reported in the Code of Federal Regulations, Title 21 (revised April 1, 2010) that AA is generally recognized as safe (GRAS), but that it should be used in the minimum quantity required to produce its intended effect, and otherwise in accordance with all the principles of good manufacturing practice. The Joint FAO/WHO Committee on Food Additives (JECFA) has reported no safety concern at the current level of intake (Burdock, 2001). Nevertheless, studies conducted in Finland show that the average AA concentration in yoghurts sold in Finland exceeds the level of mutagenicity eight-fold (Salaspuro, 2010). Therefore, foods typically consumed by small children in Estonia are under focus in this study. According to scientific literature, the dairy industry has engaged in an active years-long attempt to develop yeasts whose acetaldehyde-producing capacity is as high as possible (Salaspuro, 2010) to improve product quality by enhancing its flavour. Additionally, it is known that AA is lethal to yeasts and moulds (Barkai-Golan & Aharoni, 1976), and its use as a microbial inhibitor in foods is intriguing. Furthermore, Salaspuro (2009) has reported that AA appears to act as a cumulative carcinogen in the upper digestive tract of humans. This strongly suggests the importance of world-wide screening of AA levels in many beverages and foodstuffs, as well as an urgent need for regulatory measures and consumer guidance.

Even though AA has been found in many foods, the number of reports describing its quantification in food samples is rather scarce. Quantitative determination of AA is complicated because it is very volatile (T_{evap} = 20ºC), reactive and is present in very small concentrations. Thus, proper and sensitive methods need to be used in order to get reliable results. Basic methods relying on concentration of AA by distillation and following determination either by liquid chromatography (LC) or enzymatic methods are tedious and often difficult to apply on solid food matrices. To increase the sensitivity and selectivity of the method, derivatization of AA in combination with subsequent detection and quantification by mass spectrometry (MS) has been used. For example, solid phase micro extraction (SPME) combined with on-fiber derivatisation using O-2,2,4,5,6- (pentafluorobenzyl)-hydroxylamine hydrochloride (PFHBA) has been previously used to extract aldehydes from water (Tsai & Chang, 2003) and in exhaled breath (Poli et al., 2010). In light of these studies, but also those of others (Martos & Pawliszyn, 1998; Koziel et al. 2001; Wang et al. 2005), the method was modified in the present work. Solid-phase micro extraction (SPME) combined with GC/MS-Tof analysis and stable isotope dilution assay was used for quantification of AA in different food products.

MATERIALS AND METHODS

Materials

Food products used in the study were as follows: i) purees: Hipp vegetable puree with rice and Hipp fruit puree (Pfaffenhofen, Germany), Milupa apple-pumpkin puree and Milupa chicken stew with potatoes and carrots (Dublin, Ireland), Põnn peach-yoghurt dessert (Salvest, Estonia), Gerber pear puree (Vevey, Switzerland), Bebivita apple puree with banana (München, Germany); ii) curd creams and desserts: Tere blueberry curd cream and Tere cherry dessert (Tallinn, Estonia), Valio Alma yoghurt

dessert with strawberries and blueberries (Laeva, Estonia); iii) yoghurts: Valio Alma wild-strawberry yoghurt (Laeva, Estonia), Tere Mumuu yoghurt with rye bread and hazelnuts and Tere Hellus wild berry yoghurt (Tallinn, Estonia), Valio Gefilus blueberry yoghurt (Laeva, Estonia), Farmi rhubarb yoghurt (Rakvere, Estonia), Nopri farm yoghurt with sea buckthorn (Võrumaa, Estonia); iv) puddings: Tere vanilla pudding (Tallinn, Estonia); v) yoghurt drinks: Activia raspberry and Actimel raspberry-cranberry semi-solid yoghurt (Paris, France), Valio Gefilus peach yoghurt drink (Laeva, Estonia), Tere Hellus tropic fruit yoghurt drink; vi) ready-to-drink baby milks: Semper baby milk (Sundbyberg, Sweden).

AA and O-2,2,4,5,6- (pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA, 98%) was purchased from Sigma-Aldrich, deuterated acetaldehyde (D_4-AA) from CIL (≥ 98%, M = 48.08 Da, Cambridge Isotope Laboratories Inc., MA), ethanol was from Rakvere Piiritusetehas (Rakvere, Estonia). Clear glass SPME crimp vials (20 ml) with the polytetrauoroethylene (PTFE)/silicone septa (20 mm) were purchased from Supelco and glass-covered magnetic stirrers (1 cm) from VWR Int. (PA, USA). MilliQ water (Millipore Corp., Molsheim, France) was used whenever samples were prepared.

Principle of the method

The PFBHA loaded on SPME fiber was used to absorb and derivatize AA from the headspace of the water extract of the samples. The reaction of derivatisation with PFBHA reagent gives two PFBHA-oxime isomers (cis and trans), which are desorbed and analysed by time-of-flight gas chromatography–mass spectrometry (Tof-GC/MS). D_4-AA, added before the extraction of the sample, was used as an internal standard for precise AA quantification in the sample extract. The exact amount of AA was calculated against internal standard integrating the peak area of the ions m/z = 209.08 (AA) and m/z = 213.08 (D_4-AA).

Stock solution preparation and determination of linear range of AA

All solutions and sample preparation were prepared in a cooling chamber at +4ºC. First, the D_4-AA stock solution was prepared by transferring 117 µl D_4-AA and 132 µl ethanol into a 100 ml volumetric flask and filled with cold oxygen-free water (MilliQ water treated with gaseous N_2), total concentration for both 1,000 µg ml^{-1}. An equal amount of ethanol was added to the stock in order to determine the exact concentration of D_4-AA by HPLC and the refractive index (RI) detection (considering that the RI values for ethanol and AA are very similar - 1.36 and 1.33, respectively). The concentration of internal standard was checked every time the standard was used.

Secondly, the calibration curve was prepared for determination of the linear range of AA extracted with SPME. Shortly thereafter, 100 µl of AA (99.9%, $\rho_{25ºC}$ = 0.785) was transferred into the 100 ml volumetric flask filled with cold oxygen-free water to produce an unlabeled AA stock solution with a concentration of 785 µg ml^{-1}. 6.37 ml of this unlabeled AA stock solution and 5 ml of D_4-AA stock solution (prepared previously) were transferred intoa 50 ml volumetric flask and filled with cold oxygen-free water to produce a solution with a concentration of 100 µg ml^{-1} for both standards. Then the dilutions (100, 80, 50, 20, 5 and 1 µg ml^{-1}) were transferred directly into the headspace vials, 4 ml in each vial. The linear range was determined with Targetlynx software (Waters Inc., Manchester, UK).

Sample preparation

For analysis of AA in food samples, 100 ± 2 g of baby puree, yoghurt, milk or curd cream was weighed into a Stomacher® bag with a filter containing a cooled (4ºC) mixture of MilliQ water (99 ml) and D_4-AA stock (1 ml, with conc. 1,000 μg ml^{-1}). The bag was quickly sealed with tape and placed into a Stomacher® blender. Homogenization was carried out at 4 ºC at 300 rpm for 1 min. Then 2 ml of the mixture was quickly transferred to a 20 ml headspace vial and capped (magnetic cap with PTFE septum). The vial with a sample was kept at +4ºC before sampling and brought to room temperature for 30 min using 250 rpm stirring the prior sampling with PFBHA-loaded SPME fiber.

Sample analysis

All analyses were carried out in triplicates. On-fiber derivatization with PFBHA reagent was carried out using polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (65 μm) from Supelco. Before each use, the fiber was cleaned in the CombiPAL (CTC Analytics, Zwingen, Switzerland) fiber conditioning station for 10 min at 250ºC in order to release any contaminants, and then exposed to the headspace of a PTFE-capped 20 ml vial containing 1 ml aqueous solution of PFBHA (17 mg ml^{-1}) for 10 seconds at room temperature under stirring conditions (250 rpm). After loading with PFBHA the fiber was placed directly into the headspace of the sample vial for 2 min at 22ºC (the on-fiber derivatisation phase) under stirring conditions (250 rpm). The sample had been stirred previously for 30 minutes at 22ºC for equilibrium. The fiber was then thermally desorbed in the GC injection port for 10 min at 250ºC and the absorbed compounds were separated using GC/MS-Tof (Agilent Technologies Inc., Santa Clara, CA and Waters, Manchester, UK).

GC/MS-Tof conditions and data analysis

Agilent 6890 gas chromatograph was equipped with a DB-5MS column (J&W Scientific, Folsom, CA) with a 1.0 μm film thickness, 30 m length and 0.25 mm inner diameter (i.d.). Helium (purity 5.5, 0.9 ml min^{-1}) obtained from AGA Eesti AS (Estonia) was used as the carrier gas and the injector was in splitless mode for 10 minutes using an inlet liner of 0.75 mm i.d. The detector temperature was 200ºC and for ionization, electron energy of 70 eV was used. The column temperature was initially maintained at 60ºC and instantly increased to 95ºC at a rate of 10ºC min^{-1} and held for 25.50 min, then increased to 280ºC at a rate of 40ºC min^{-1} and held for 1.38 min with a total run time of 35.01 minutes. Data were analysed using Targetlynx software (Waters Inc., Manchester, UK). Concentrations of AA (C_{AA}) in the samples were calculated based on the ratio of the integrated area of the Tof chromatogram of D_4-AA internal standard (m/z=213.08) and that of unlabeled AA (m/z=209.08) containing the sample (Eq 1).

$$\frac{(Area\ ion\ 209.08\ (cis) + area\ ion\ 209.08\ (trans)) \times conc\ D_4\text{-}AA\ in\ stock \times RF}{(Area\ ion\ 213.08\ (cis) + area\ ion\ 213.08\ (trans)) \times 100} \quad (1)$$

RESULTS AND DISCUSSION

The calibration curve prepared with 1, 5, 20, 50, 80 and 100 μg ml^{-1} of AA and D_4-AA demonstrated that under described conditions the linear relation of AA concentration and the respective MS response was up to a concentration of at least 20 μg ml^{-1}. At concentrations higher than 50 μg ml^{-1} the standard curve flattened, which suggests the saturation of the PFBHA-loaded fiber or the TOF detector. Thus, if the determined concentration of AA in the sample exceeded 20 μg ml^{-1}, dilutions of the extract were made in order to remain in the linear range of MS response. The response factor (RF) calculated for unlabeled and D_4-AA was 1.32 ± 0.06 (Fig. 1).

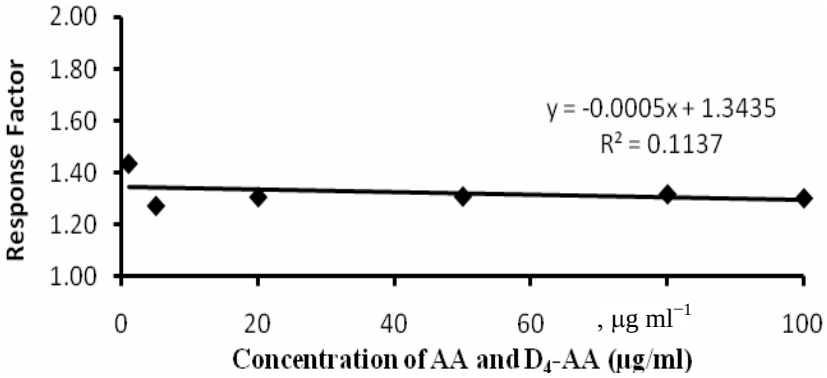

Figure 1. Response factor for unlabeled and D_4-AA is 1.32 ± 0.06.

Quantification of AA in food products

Results of AA concentration determined in different food products are shown in Fig. 2. The highest concentration of AA was determined in yoghurts. This is not surprising because AA is formed during lactic acid fermentation. Nevertheless, the concentration of AA in all studied yoghurts was in the range of the limits set by the Council of Europe (23 mg kg^{-1} in beverages and 20 mg kg^{-1} in food). Therefore, the concern that AA is considerably higher than the mutagenicity level in foods for children could not be shown in this study. The concentration of AA in all baby purees was remarkably lower (up to 6.4 ± 0.02 mg kg^{-1}) than the limits suggested by the Council of Europe.

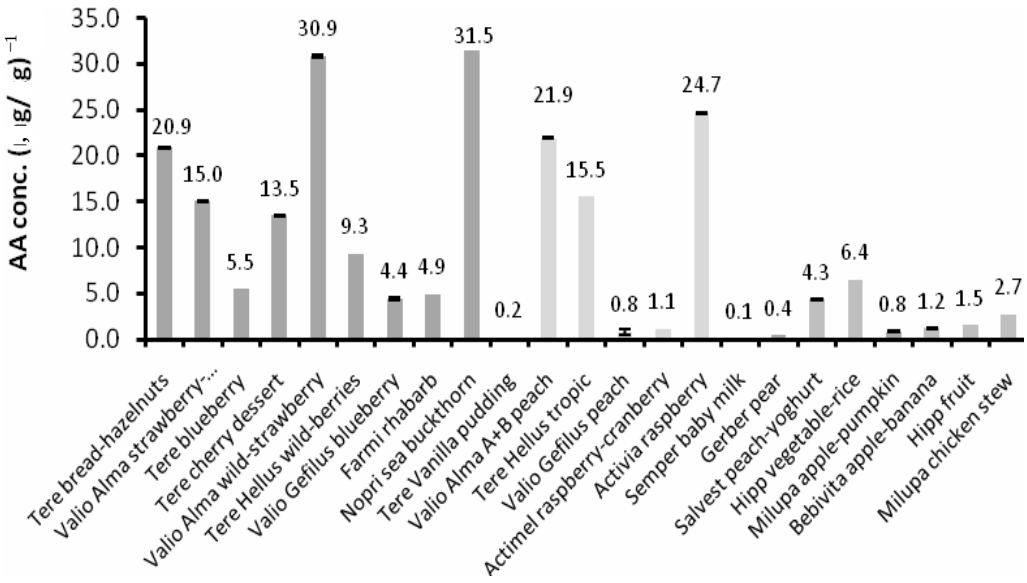

Figure 2. AA concentration in yoghurts and other milk desserts (dark gray), in yoghurt and milk drinks (light gray) and in baby purees (middle gray).

CONCLUSIONS

The method developed in the present study was shown to be adequate to analyse acetaldehyde (or other aldehydes) in solid and half-solid food matrixes. The concentration of AA in different food products, mostly consumed by small children, is not a considerable health risk.

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PUBLICATION IV

Kaseleht K, Leib M, Kadri K, Paalme T, Leitner E.

Method for GC-Olfactometry Panel Training

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A Method for GC-Olfactometry Panel Training

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Running title: Method for GC-Olfactometry panel training

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Abstract

Odor active compounds are commonly analyzed using gas-chromatography/olfactometer (GC-O). However, the guidelines available for panelist training with this technique are limited. In the current study 29 volunteers were trained to detect, identify, and rate the intensity of odors. In addition, three GC-O methods, i.e., aroma extraction dilution method, detection frequency, and posterior intensity were used to evaluate the newly trained panelists' aptness to analyze key compounds of kvass aroma. A four step approach is proposed for training: (1) introduction of the method; (2) vocabulary training using standard compounds and learning the use of the scale; (3) training with the reference mixture; (4) training with the real product of interest – kvass (fermented non-alcoholic drink). Following these steps, all panelists learned how to perform GC-O identification. Some variances among subjects were observed, however, the background of the trainees was insignificant. Assessors for the professional group were chosen from people with a sensory and food science background, but also from consumers without knowledge in sensory analysis and/or food chemistry. Posterior intensity method was found to provide sufficient amount of data for key compound analysis, enabled easy data handling and was subjected for further training and panel monitoring.

Keywords: GC-Olfactometry, panel training, kvass aroma

1. Introduction

Gas-chromatography/olfactometry (GC-O) refers to the use of human olfaction as a sensitive and selective detector for odor-active compounds separated using GC. The accuracy of GC-O is very much dependent on the performance of olfactory assessors (sniffers). For each of the separated compounds that emerge from the GC, a human assessor has the potential to identify and describe each compound by its odor, determine its retention time, and quantify its intensity. However, without prior training and practice this is difficult or even impossible. Debonneville (2002) analyzed a flavor model analysis with three different panelists and found large deviations in both sensitivity and the ability to recognize different compounds. It is clear that assessors have different potentials to detect each compound, however, a panel must be representative and reproducible, which is heavily compromised if there is a lack of consistency in the way panelists have been trained. At present, GC-O methods that quantify the potency or intensity of an odor can be classified in the following categories: dilution analysis (AEDA and Charm), detection frequency (DF), posterior intensity (PI) and time intensity methods (Van Ruth, 2001). Pollien *et al.*, (1997) reported that DF, where only odor detection is conducted, does not require training. However, Van Ruth and O'Connor (2001) show that training is still beneficial, as it increases the sensitivity of the method by reducing the signal-to-noise level of the group of assessors.

Training guidelines or even standards have been published for sensory detection methods. For example, Chambers *et al.*, (1981) compared a trained and a semi-trained panel and proved that the use of a small highly trained panel is justified in descriptive sensory analysis. More recently Del Castillo *et al.*, (2008) describe methods of training, validation, and maintenance of a sensory panel with an objective of being able to discriminate between dry bean texture properties. Another example presents a case study of a panel trained for the sensory evaluation of carrots (Kreutzmann *et al.*, 2007). This case study shows that for some attributes the learning process can be longer and training has a significant effect.

A limited number of guidelines have been proposed for GC-O methods. Delahunty *et al.*, (2006) reviewed the use of GC-O identification methods and discuss the importance of panel training which has an influence on the quality of the results. Hulin-Bertaud *et al.*, (2001) trained 8 panelists and evaluated their ability to describe blue cheese extracts using a time-intensity method. Another study by van Ruth and O'Connor (2001) trained panelists over a 4-month period and observed an improvement in precision. Panelist training for the olfaction analysis of aroma extracts was described by Bianchi *et al.*, (2009). While GC was not used in this study, the training process they describe may still be applicable to a GC-O panel as well.

Due to the inherent differences between GC-O identification and sensory analysis methods, each requires different training procedures. Sensory techniques are usually based on trained panelists, who taste the food and try to assess the intensity of flavor attributes (salty, sour, vanilla, etc.), but it is possible to assess one attribute several times before scoring, if necessary. In GC-O identification, odors are presented to the assessors for a few seconds at undefined intervals over a relatively long period (up to 30 min or more), requiring the panelist to react quickly. During sensory evaluation the perceived flavor of the sample involves simultaneous tasting of all flavor chemicals and therefore accurate assessment of the level of a particular flavor chemical may not be possible, and often is not the objective. Odor analysis using a GC/MS instrument in combination with GC-O is able to separate the components of a

sample composed of hundreds of chemicals while determining the odor potential as well as the quantity of each chemical.

The objective of this study is to introduce a method for training GC-O assessors, and determine the most suitable GC-O identification method (AEDA, DF, PI) to train the aroma analysis skills of an inexperienced panel. This panel is trained to identify the key aroma-active compounds in kvass. Kvass is a refreshing non-alcoholic drink (less than 1% ethanol) that is well known in Central and Eastern Europe and made by simultaneous acid and alcoholic fermentation of rye bread, rye/barley malt or rye flour with addition of sugar, containing living lactic acid bacteria and yeast.

2. Materials and Methods

2.1 Materials

2,3-butanedione, 3-methylbutanol, hexanal, dimethylsulfide, isoamylacetate, methional, benzaldehyde, 1-octen-3-ol, damascenone were acquired from Sigma-Aldrich (St. Louis, MO, USA) (Tab. 1). All nine odor-active compounds used as standards were diluted with ethanol from Rakvere Piiritustehas, Estonia. Kvass (A. Le Coq AS, Tartu, Estonia) was purchased from a local store. Sniffing strips were bought from Orlandi Inc. (Farmingdale, NY, USA). MilliQ water (Millipore SAS, Molsheim, France) was used for making kvass solutions.

2.2 Selection of the Panelists

Twenty-nine volunteers (5 male) are trained to become GC-O assessors. Some have had previous extensive sensory training, some have a food science background while others are typical consumers. Panelists with a variety of backgrounds are needed to investigate the influence of previous experience on the results of training. The panelists are between 22 and 50 years old and are all healthy non-smokers.

2.3 Training of the GC-O Assessors

Training was carried out in four steps: (1) introduction of GC-O identification methods to the panelists during two days of theory lectures; (2) vocabulary training using standard compounds and learning the use of the assessment scale; (3) training with the reference mixture by detecting and measuring the intensity of the components in the mixture with the GC-O; (4) training with the product of interest. To investigate the effect of training, we compare the results with a standard olfaction analysis of kvass.

2.3.1 GC-O Analysis of Kvass Using Untrained Assessors

To investigate the effect of training a GC-O analysis of kvass was carried out before training, where only limited instructions were given to the assessors. Assessors were asked to detect an odor and describe it with his/her choice of words. Pre-test samples were prepared as detection frequency and posterior intensity samples.

2.3.2 Introducing the Method

The GC-O identification method was presented to the panelists over two days in a lecture format. An overview was given on human senses, odor-activity of chemical compounds, and odor sensation and threshold. Panelists were introduced to sample preparation procedures,

gas-chromatography, including comprehensive and two-dimensional GC, mass-spectrometry and GC-O identification. In addition, they were introduced to the following GC-O methods: (1) aroma extraction dilution analysis (AEDA), combined hedonic response measurement (CHARM), (2) detection frequency (DF), (3) time intensity (TI), and (4) posterior intensity methods (PI).
Panelists were familiarized with the test protocol which included avoiding alcoholic drinks, very spicy meals, and coffee before sniffing. During the session disturbance from the outside was minimized and no communication was permitted during the session. Assessors were not allowed to rush to the session. The panel leader was supposed to announce the time of each session early enough and use regular times/dates for the sessions. The best times for sessions is in the morning between 9:30-10:30 and in the afternoon between 14:00-14:30. Assessors should not sniff over 6 samples a day and not over 15 min at a time to avoid fatigue. In case of longer GC runs it is suggested to split the run between two assessors, however, in this case at least two parallels with different switching times must be carried out.

The panelists were advised to breathe normally, preferably quicker (Hanaoka *et al.*, 2000) and not to forget to breathe out or dizziness may occur. During sniffing the panelists were asked to focus on stronger odors. They were not shown the chromatogram while sniffing as they may imagine a smell as the GC-peak appears.

2.3.3 Training Using Standard Compounds

The second step introduced odor vocabulary and assessment scale usage to the panelists. Nine commercially available aroma compounds were used to train the assessors (Tab. 1). During training it is recommended to use compounds that are present in the product of interest – kvass in this study. The concentrations, odor thresholds (Gemert, 2003), and perceptions (according to www.flavornet.com and www.pherobase.com) of the standard compounds are also given in Tab. 1. Approximately 1 cm of the sniffing strip was dipped into the stock solution, and sealed into screw-cap test tubes after evaporation of ethanol for sniffing (approximately 10 sec at room temperature). Sniffing strips of one compound at a time were used to learn the vocabulary (each assessor's native language was used, mostly Estonian, but also Russian). Assessors memorized the odors and their descriptions and were subsequently tested with the same compounds. The panelists were required to describe the odor after sniffing the paper strip once. Solutions with standard compounds were stored for a maximum of five months, as aldehydes are not stable in ethanol and form acetals.

The assessors rated the perceived intensities of the compound on a five-point intensity interval scale (1 = very weak, not identifiable; 2 = weak, but identifiable; 3 = moderate, easily recognizable, but not strong; 4 = strong; 5 = extremely strong, that you wish to distract your nose) during a group discussion session as suggested by Ferreira *et al.*, (2003) and Berdague *et al.*, (2007). For approximate and tentative intensity measurements new sniffing strips were prepared. Benzaldehyde solution (Tab. 1) was diluted 100 times for intensity 1 (0.0122 g/L); isoamylacetate solution was diluted 10 times for intensity 2 (0.098 g/L); hexanal was diluted 2 times for intensity 3 (1.4 g/L); β-damascenone solution (1.21 g/L) was used for intensity 4 and 3-methylbutanol (100 g/L) was used for intensity 5. Sniffing strips were dipped into the corresponding reference solution, excess ethanol evaporated and the strips were placed into the screw-cap tubes.

2.3.4 GC-O Training with the Reference Mixture

The third step included reference mixture analysis. The reference mixture was composed of 0.1 ml of each solution (Tab. 1), diluted by ethanol 1:1 with a total concentration of 0.06 to 0.16 mg/mL (Tab. 1, last column). It must be noted that after mixing the 9 solutions, the reference mixture consisted of approximately 20 odorous compounds due to impurities. Since some impurities had a similar odor description as the standard compounds, like dimethyl disulfide (rotten cabbage) and 1-octen-3-one, octanol (mushroom), cinnamic acid (kvass) they were also counted as a signal (13 compounds in total), other odorous impurities were ignored in data analysis.

Table . Standard compounds used for GC-Olfactometry panel training.

Compound	CAS number	Odor perception	Odor threshold (air, mg/m^3)	Odor threshold (water, mg/kg)	Conc. of stock solutions in EtOH (g/L)	Conc. in reference mix in EtOH (g/L)
2,3-butanedione	431-03-8	Butter	0.00001-10.2	0.0003-2.3	1.2	0.07
3-Methylbutanol	123-51-3	Whiskey, malty, burnt, pungent, balsamic, alcohol, fruity, ripe, bitter	0.019-6.3	0.7-70	2.8	0.16
Hexanal	66-25-1	Grass, tallow, fat, fruity, fishy, herbal	0.025-0.098	0.0091-0.75	2.8	0.16
β-Damascenone	23726-93-4	Apple, rose, honey	0.000002-0.00004	0.0000075-0.01	1.21	0.7
Methional	3268-49-3	Cooked potato	0.000063-0.06	0.0002-0.0018	1.02	0.6
Dimethylsulfide	324-92-0	Cabbage, sulfur, gasoline	0.003-3.5	0.00016-0.09	1.07	0.06
1-octen-3-ol	3391-86-4	Mushroom	0.012-0.028	0.000005-0.025	1.1	0.06
Isoamylacetate	123-92-2	Banana	0.004-8	0.002-2.5	0.98	0.06
Benzaldehyde	100-52-7	Almond, burnt sugar	0.014-4.3	0.072-111	1.22	0.07

The reference mixture was sniffed three times by each assessor: two times to detect and identify the compounds, and the third time to determine the intensity of a compound. Correctly detected peaks are counted when the assessor detects it at least two times. Correctly recognized and named peaks are counted, when the assessor recognized it at least one time. Detection level is the number of all detections (every time the button was pressed).

Assessors were given individual feedback on their performance, by comparing individual aromagram data to the average group aromagram, including information on intensity scaling, the number of peaks detected, and number of and suitability of descriptors given. It was possible for the panelists to use the sniffing strips in between the sniffing sessions to refresh their memory.

2.3.5 GC-O Training with the Real Product

The fourth step was to train the panelists with the real product, kvass, which is more complex and includes odors unfamiliar to the panelists. Panelists were divided randomly into three groups that each applied a different olfactometric method: detection frequency (*n=10*), AEDA (*n=10*), and posterior intensity (*n=9*). The result from one group was treated as one signal and the signal-to noise level was chosen to be two, as suggested by Van Ruth and O'Connor (2001).

Group 1, which used detection frequency method analyzed kvass (0.5 ml in 20 ml vials, described in Sample Preparation and Instrumental Analysis section) in duplicates and recorded the beginning and the end of an odor. Kvass was not diluted and was adjusted in strength such that about 30 odor compounds were perceivable to the sniffers (Reineccius, 2010). The number of sniffers (*n=10*) detecting an odorant in kvass was either tabulated and plotted in NIF (Nasal Impact Frequency) values using MS Excel (% of assessors detecting the odor) or combined for a SNIF (Surface of Nasal Impact Frequency) chromatogram representing OID values (% frequency × duration (s)) using the Gerstel SNIFF program as described by Plutowska and Wardencki (2008). In MS Excel, if an assessor failed to register a gap between two closely eluting compounds, it was counted as a separate signal.

Group 2 used an AEDA method to analyze kvass in 4 dilutions: 1) 3 ml kvass and 1 ml of water in 20 ml vial (dilution factor (DF) = 1.33), 2) 1 ml of kvass and 3 ml of water in 20 ml vial (DF = 4), 3) 0.1 ml kvass and 490 ml water in 20 ml vial (DF = 40), 4) 0.01 ml kvass and 499 ml water in 20 ml vial (DF = 400). This group detected aroma-active compounds and also recorded the odor description. Results are given in log(DF) value, which is calculated by taking the logarithm of DF of the last dilution at which the signal was above noise level, alternatively, using the Gerstel SNIFF program, in OID values taking into account % frequency, duration and DF.

Group 3, used a posterior intensity method to analyze kvass (0.5 ml in 20 ml vial) in duplicates and recorded the maximum odor intensity as well as description once the compound had eluted. A remote control was used to express the intensity of a compound by pressing buttons from 1 to 5 (1 = very weak, not identifiable; 5 = extremely strong, that you wish to distract your nose). Both the SNIFF software and MS Excel were used to analyze the results. Using MS Excel, the parallel results of each assessor were summed in a table (retention time; odor impression and intensity). All the compounds were sorted in order of retention time. If a compound was detected in both parallels, it was highlighted and not repeated in the list. Afterwards, the results of all assessors were listed together and grouped according to retention time and odor impression. Intensity values were averaged.

2.3.6 Further Training of the Panel

The goal was to train and finally choose 8 assessors for the professional GCO panel to be further trained and monitored. It is suggested to train at least twice as many panelists than are required. In this study, three times more assessors were trained. A good panel should provide results that are accurate, discriminating and precise. Criteria for choosing an assessor for the professional panel were mostly taken form sensory standards DIN 10961 part 1, ISO 8586-2:2008 and Meilgaard et al. (2007). Additional criteria, such as willingness to cooperate and to work in the panel for a longer period and availability of the person, but also sensitivity, motivation, ability to concentrate, and ability to recall and recognize odor qualities as suggested by Marin *et al.* (1988) were taken into account. Factors such as gender, age (general olfactory ability), presence of a respiratory disease (asthma, seasonal allergies, active colds), medication use, smoking and occupational history (Dalton and Smeets, 2004) were noted, but were not discriminative.

Regular examination of the panel is carried out a minimum of twice per month to train odor memory, and to evaluate and keep the olfactory skills. Results from regular training sessions are documented and the performance profile of each assessor is composed. The univariate tool collection is based on sequential ANOVA tests to assess sensitivity and assessor

reproducibility (Kermit and Lengard, 2005). The panel may be called professional after 1.5 years of monitoring.

2.4 Sample Preparation and Instrumental Analysis

For the analysis of the standard mixture, 0.01 ml of the mix was injected into a 20 ml headspace (HS) vial that contained a 1 cm glass covered magnetic stir bar. In case of kvass samples, 0.5 ml or 4 ml of the sample was measured into a 20 ml HS vial that also contained a 1 cm stir bar.

For sample preparation and injection, a CTC CombiPAL auto sampler (Chromtech, Germany) with SPME option was used. The incubation time was 5 minutes at 60 °C, after which a 2 cm SPME fiber (50/30 µm DVB/Car/PDMS Stableflex, Supelco, Bellefonte, PA) was injected into the vial for 20 minutes at 60 °C for extraction. Samples were mixed in a Single Magnetic Mixer (SMM, Chromtech) at 250 rpm. Volatiles were desorbed in an Agilent 7890 gas chromatograph equipped with a flame ionization detector (FID) and a sniffing port ODP-3 (Gerstel, Germany). Column effluent was split 1:1 between the FID and the sniffing port using deactivated fused silica capillaries (1 m length, 0.15 mm ID). The sniffing port was supplied with humidified air at 30 ml/min. Transfer line temperature was 300 °C. A capillary column DB-5MS (30 m length, 0.25 mm i.d. and 1 µl film thickness; J & W Scientific, Folsom, CA) was used in the GC. Helium gas (purity 5.0, AGA Eesti AS, Estonia) was used as a carrier at a constant flow of 2 ml/min. Splitless mode was used in a split/splitless injector at 250 °C. The initial oven temperature was 35 °C. For the standard mixture and AEDA sniffing, a temperature program of 25 °C/min to 280 °C, holding time 6 min was used (total run time 15.8 min). For detection frequency, posterior intensity method and preliminary analysis, initial oven temperatures were followed by a rate of 45 °C/min to 85 °C, then by 9 °C/min to 200 °C and then by 45 °C/min to 280 °C and held for 1 min (total run time 16.6 min).

FID responses confirmed consistency of the injections and sample preparation for replicates and dilutions. Intensity measurement was performed with the Gerstel ODP recorder program. If a panelist recognizes an odor he/she activates a microphone by pushing the specific remote control button for intensities 1-5 and describes the odor by quality. If intensities were not of interest, then button 3 with intensity 3 was chosen in all samples for registering the detection of an odor.

2.5 Statistical Evaluation

Panel average scores were calculated for standard compound sniffing and GC-O analysis of the standard mixture, scores were subjected to Single Factor ANOVA (α=0.05).

3. Results

3.1 Preliminary Analysis of Kvass

All assessors taking part in the training were subjected to pre-testing of kvass. Only 18 assessors were able to describe an odor at least one time, others just detected. Vocabulary learned during the training was used only 15 times (n=29) (Table 2) compared to 137 times after training (n=20, since only AEDA and Posterior intensity groups described the odor, detection frequency group did not).

Table . Using descriptions of aroma-active compouncs in kvass before and after training

Odor impression	Pre-training (N=29)	After training (N=20)
Cabbage	-	12
Butter	-	11
Grass	3	13
Whiskey	-	20
Banana	-	15
Potato	1	24
Mushroom	6	22
Almond	1	7
Kvass	4	13

3.2 Results of the Training Stage with Standard Compounds and Reference Mixture

Nine previously memorized pure compounds were presented to the panelists and the test results are given in Table 3. Results were subjected to ANOVA and no significant difference between assessors with different backgrounds is observed. Data representing the sniffing of the reference mixture is also given in Table 3. According to ANOVA there are no significant differences between assessors with different backgrounds in any category; however there is a significant difference in correctly detected and recognized compounds between men and women (no difference again in detection level). The most difficult compounds for the assessors to detect and recognize with GCO were rotten cabbage (dimethylsulfide), grass (hexanal) and mushroom (1-octen-3-ol and octanol), mostly due to higher odor thresholds (hexanal and 1-octen-3-ol) and low concentration in the mix (impurities like dimethylsulfide and octanol).

Table . Correct average answers (± stdev) during training for nine pure compounds and the reference mixture using GC-O broken down into the three types of panelists (consumers, food technologists, and sensory assessors) as well as the professional group's average.

	Sensory assessors	Food technologists	Consumers	Prof. GCO group
No. assessors in a group	10	10	8	8
Average correct answers (max 9)	7.0 ± 1.29	8.1 ± 1.50	7.6 ± 1.55	8.7 ± 0.43
Average correctly detected compounds (max 13)	9.0 ± 2.18	9.5 ± 2.12	9.3 ± 2.06	11.0 ± 1.73
Average correctly recognized (max 13)	6.1 ± 1.96	8.0 ± 2.65	7.7 ± 1.98	9.5 ± 1.94
Average detection level	15.6 ± 5.59	19.6 ± 6.35	13.7 ± 3.99	17.4 ± 4.09

3.3 GC-O Results of Kvass Samples

For sniffing the real product (kvass) three common olfaction methods were chosen: 1) detection frequency, 2) AEDA, and 3) posterior intensity.

3.3.1 Detection Frequency

In detection frequency (Group 1), the number of sniffers (*n=10*) detecting an odor compound in kvass was manually tabulated and plotted in NIF values (Fig. 1a) and OID values (Fig 1b). In Fig 1a 18 key compounds exceeding threshold value of 60% are numbered. The same compounds are numbered in Fig 1b. Although the peak heights do not exactly coincide, the results are comparable with both manual and automatic data analysis (perhaps only compound 18, which was not distinguished using the SNIFF aromagram). Therefore, in order to make data analysis easier and faster, a SNIFF program can be used.

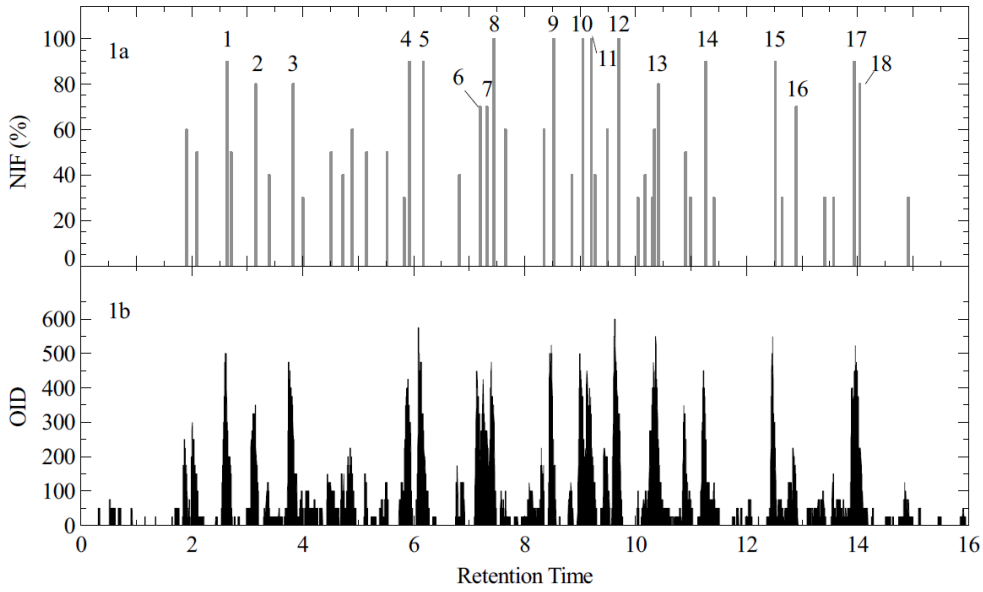

FIG. 1A AND 1B. DETECTION FREQUENCY RESULTS (MANUAL INTERPRETATION IN MS EXCEL) AND OLFACTOGRAM OF DETECTION FREQUENCY RESULTS BY THE SNIFF PROGRAM IN CHEMSTATION (RET.TIME VS OID VALUE). 18 KEY COMPOUNDS OF KVASS ARE NUMBERED.

3.3.2 Aroma Extraction Dilution Analysis

In AEDA, used by Group 2 (*n=10*), samples were evaluated by the panelists in increasing dilution order. The impact of an odor-active compound is given by its dilution factor (DF) in Fig 2a and OID value in Fig 2b. Interpretation of AEDA data was challenging as gaps existed during sniffing of the dilution series. The error caused by the gaps should be minimized by using the equation suggested by Debonneville *et al.*, (2002) and used in the SNIFF program. However, this is only possible in the case of a highly trained panel. For example, three peaks in Fig 2b - butter (ret. time 9.17), unknown (ret. time 9.47) and butter (ret. time 9.75) - were all detected once by one assessor in the most diluted sample. This resulted in high peaks in the aromagram and referred to an important compound, however this error was caused by a beginner assessor. With the SNIFF program, excluding the errors and using a noise level of two was impossible and therefore results were not very comparable with manual data handling.

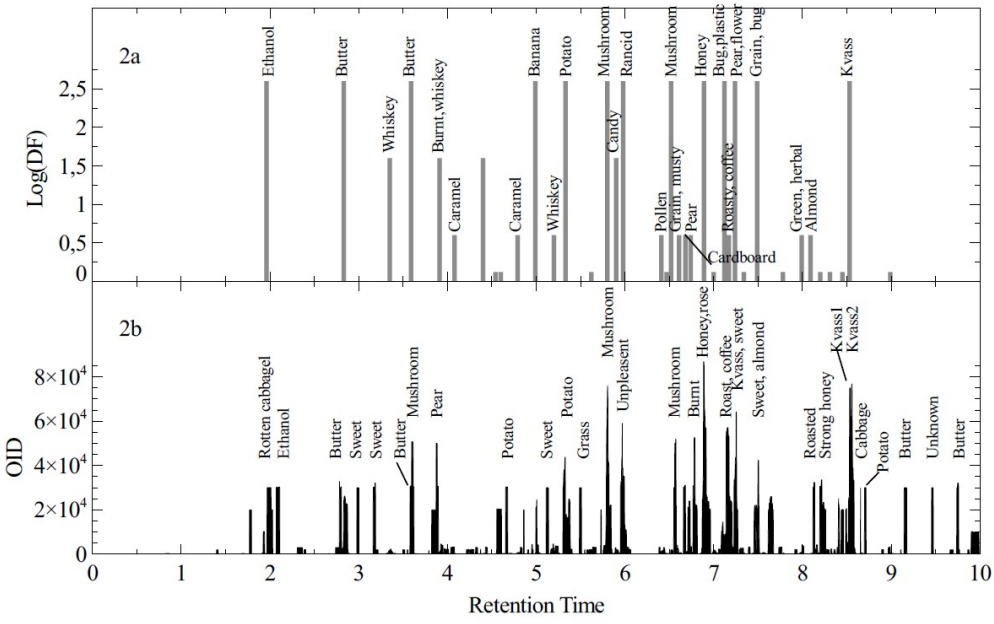

FIG. 2A AND 2B. AEDA RESULTS (MANUAL INTERPRETATION IN MS EXCEL) AND OLFACTOGRAM OF AEDA RESULTS BY THE SNIFF PROGRAM IN CHEMSTATION (RET.TIME VS OID VALUE).

3.3.3 Posterior Intensity Method Results

Posterior intensity group (Group 3) (*n=9*) evaluated the odor impression and rated the intensity of a compound. Results are shown in Fig 3a, using MS Excel and Fig. 3b using the SNIFF software. In total, 115 different compounds were detected by the panelists. However, using a noise level of two, the amount of compounds was 48. Since MS Excel data coincide well with SNIFF data, the program has the benefit of automating data analysis.

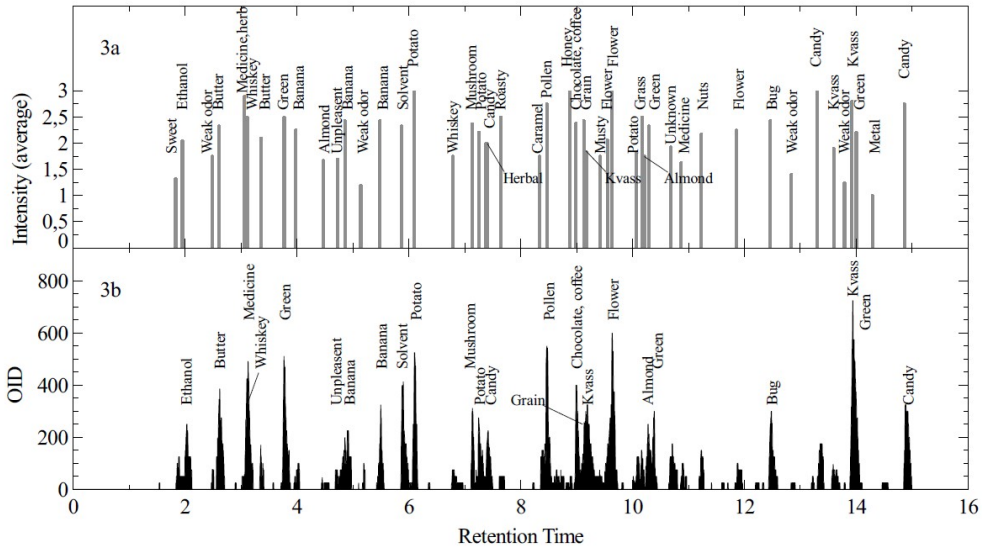

FIG. 3A AND 3B. POSTERIOR INTENSITY RESULTS MANUAL INTERPRETATION IN MS EXCEL (AVERAGED) AND OLFACTOGRAM OF POSTERIOR INTENSITY RESULTS BY THE SNIFF PROGRAM IN CHEMSTATION (RET. TIME VS OID VALUE).

4 Discussion

4.1 GC-O Method Comparison

In this study, different GCO methods were tested to assess their applicability for odor analysis with an inexperienced panel and to study the data handling complexity of each method. There are advantages and disadvantages of each method. Detection frequency did not provide any data on odor description. Some authors (Pollien *et al*., 1997) suggest the use of detection frequency so that each assessor records the duration of the peak as well as the odor description. In this study, panelists did not judge the odor impression, since recording of the exact ending of the odor (for SNIF data) would have been compromised while saying something as the assessor is compelled to breathe out. However, if SNIF data is not an objective, this complementary method can be easily used. Additionally, while using the NIF method for data analysis, two compounds, from which one may be barely over the sensory threshold for all sniffers while another may be a great distance above its sensory threshold for all sniffers, both of these compounds would be viewed as being equal by this method (Reineccius and Vickers, 2004). This can be avoided if a SNIFF program is used. For example, peaks 8, 9, 10, 11, 12 were detected by all sniffers (Fig 1a), but using a SNIFF program, intensities were distinguished more clearly. On the other hand, while using SNIF data analysis (where peak areas, not heights are used for key compound estimation) partially co-eluting compounds may be overestimated due to broader peak width and early eluting compounds with narrow peak may be underestimated (Delahunty *et al.* 2006).
AEDA is criticized for its incorrect assumption that intensity increases with concentration equally for all compounds (Audouin *et al.*, 2001) and is not recommended to use while

determining key odor-active compounds. Also, incompatibility with the conventional sensory measurements of odor intensity has been reported for dilution methods (Audouin *et al.*, 2001). As a result of this study, the professional panel will continue training with the posterior intensity method as it provides enough information on the odorous compounds and proved to be feasible for a newly trained panel.

4.2 Selecting a Professional GC-O Panel and Effect of Training

After training, about 80% of assessors were interested in becoming a professional GCO assessor, however only 8 was chosen according to their good detection and recognition skills. The average results of the professional panel are given in Tab. 2. The number of female assessors was 7 and 1 was male.
The effect of training was also evaluated by the trainees themselves. The theoretical part was found to be helpful for some; all the other stages were either important or very important. It was agreed that steps 2 and 4, learning the vocabulary and sniffing kvass, respectively, were most effective. Assessors found to be often affected by their state of health, mostly common cold due to winter time. All agreed on the importance of the training, but suggested that it should be planned over a shorter period (less than 4 months) and could be more intensive. Also, more standard compounds could be used to enlarge the vocabulary for kvass analysis. In this case more training with reference mixtures and more feedback would be required.

5. Conclusions

A posterior intensity method was found to be feasible for the newly trained GCO panel. The SNIFF program allows for easy data handling, and additionally a voice recognition program (from Gerstel) can be used, where each assessor teaches the machine to recognize his/her words. It was found that female assessors performed the GC-O task better than male, but no significant difference between assessors with different backgrounds was found. The developed method is suitable for training assessors to perform a GC-O task.

6. Acknowledgments

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