Authenticity of aroma components

Enantiomeric separation and compound specific stable isotope analysis

Anne-Mette Sølvbjerg Hansen
PhD Thesis
2015

DTU Food
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Preface

The research presented in this thesis is a result of a PhD study at the Technical University of Denmark. The work for this PhD study was conducted at the Division of Food Chemistry, National Food Institute, DTU in the period 15th of February 2012 to 14th of February 2015. The project was financed by Ejnar Willumsen A/S and The Technical University of Denmark.

Many people have contributed during the work of this PhD for which I am very grateful. First and foremost I would like to thank my supervisors Henrik L. Frandsen and Arvid Fromberg for their guidance, encouragement, and valuable suggestions during this research work.

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Great thanks to all my colleagues at the department of food chemistry for providing a nice working environment and making me feel welcome. It has always been a pleasure to discuss both scientific and not so scientific subjects with you. A special thanks to Liljana and Maud for your invaluable help in the laboratory.

I would like to thank my friends and family for support and understanding during the project and for taking my mind somewhere else when needed.

Finally, I would like to thank my parents for always being there for me when I needed it. Without your love and support I would not have been able to do this.

Odense, August 2015

Anne-Mette Sølvbjerg Hansen
Ph.d.-student

Abbreviations

ANOVA: Analysis of Variances
CAM: Crassulacean Acid Metabolism
CAR: Carboxen
CSIA: Compound Specific Isotope Analysis
DVB: Divinylbenzene
EA: Elemental Analyzer
GC: Gas Chromatography
GC-C-IRMS: Gas Chromatography Combustion Isotope Ratio Mass Spectrometry
GC-P-IRMS: Gas Chromatography Pyrolysis Isotope Ratio Mass Spectrometry
GC-Q-TOF: Gas Chromatrograph-Quadropole-Time of Flight
HTC: High Temperature Conversion
IAEA: International Atomic and Energy Agency
IRMS: Isotope Ratio Mass Spectrometry
MDGC: Multi-Dimensional Gas Chromatography
NBS: National Bureau of Standards
NMR: Nuclear Magnetic Resonance
PDMS: Polydimethylsiloxane
PEP: Phosphoenolpyruvate carboxylase
RCWIP: Regionalized Cluster-based Water Isotope Prediction
Rubisco: Ribulose biphosphate carboxylase/oxygenase
SBSE: Stir Bar Sorptive Extraction
SNIF: Site-specific Natural Isotope Fractionation
SPME: Solid Phase Micro Extraction
TBDMS: *tert*-butyldimethylsilyl
V-PDB: Vienna-Pee Dee Belemnite
V-SMOW: Vienna-Standard Mean Ocean Water

List of publications

Papers

Paper 1:

Authenticity of raspberry flavor in food products using SPME-chiral-GC-MS

Anne-Mette Sølvbjerg Hansen, Henrik Lauritz Frandsen, and Arvid Fromberg.

Submitted to Food Science & Nutrition.

Paper 2:

Authenticity and traceability of vanilla flavours by analysis of stable isotopes of carbon and hydrogen

Anne-Mette Sølvbjerg Hansen, Arvid Fromberg, and Henrik Lauritz Frandsen.

Published in Journal of Agricultural and Food Chemistry 2014.

Paper 3:

Authenticity of vanillin in food products determined by SPME-GC-IRMS

Anne-Mette Sølvbjerg Hansen, Henrik Lauritz Frandsen and Arvid Fromberg.

Submitted to Food Chemistry.

Poster

Conference Poster 1:

Optimization of GC injection for δ^2D analysis of vanillin

Anne-Mette Sølvbjerg Hansen, Arvid Fromberg, and Henrik Lauritz Frandsen.

Contribution to ”Food integrity and traceability conference” 2014, Queens University, Belfast.

Summary

The word “authenticity” is increasingly used in the marketing of food products. A product can be marketed claiming its authenticity such as containing only natural ingredients or originating from a special location produced using local traditional production methods. Within the area of food ingredients a problem with authenticity of aroma compounds has occurred, because natural aromas are wholly or partly replaced with synthetic ones. This is a large economic problem, since natural aromas are often more expensive than artificial ones. Furthermore, the European Union has legal requirements that every product marketed as containing natural flavours should be able to document that the flavours are in fact of natural origin. Such parameters are very difficult to verify because similar aromas often have the same chemical composition regardless of the production method or origin. It is therefore necessary to develop new methods able to verify whether the authenticity of the food products is in agreement with the labelling.

Some aroma compounds are chiral, meaning that they can exist as two enantiomers with different spatial orientation. Synthetic aroma compounds will consist of almost equal amounts of both enantiomers contrary to natural aromas where often only one of the enantiomers will be in excess. Consequently, if equal amounts of enantiomers are detected in a food product labelled “Natural” it could be an indication of adulteration.

Artificial aroma compounds often have very different ratios of stable carbon isotopes compared to compounds synthesized in plants. Furthermore there can be large variations in isotopic compositions in plants synthesized compounds, since different plants utilize carbon dioxide through various cycles which will be reflected in different fractionations of carbon isotopes. This fact can be used for authenticating aroma compounds. Weather and climate conditions will affect the ratios of stable isotopes of hydrogen in the plants and consequently, differences of stable hydrogen isotopes can be used for tracing the geographical origin of the plant from which a flavour is extracted.

The purpose of this PhD project was to establish an analytical platform based on gas chromatographic enantiomeric separation and compound specific isotope analysis.

Aromas of known authenticity were then analysed in order to create a database of reference values. Based on this database the authenticity of aromas from food products was evaluated.

Before gas chromatographic enantiomeric separation could be performed, it was necessary to extract and concentrate the aroma components. The use of solid phase micro extraction was investigated for an extraction of aroma components, especially the chiral α-ionone, from raspberry aroma. A mixed fiber coating consisting of divinylbenzene, carboxen, and polydimethylsiloxane was found suitable for the purpose. The addition of sodium chloride was found to inhibit the extraction of α-ionone. The two enantiomers of α-ionone could be separated using a chiral column consisting of cyclodextrins. Aroma components from raspberries and samples labelled to contain raspberry aroma were extracted using the above method. 13 of the 27 samples analysed contained almost racemic mixtures of α-ionone. Hereof was 4 products falsely indicated to contain raspberries or raspberry juice.

Vanilla aroma made by chemical synthesis, microbiological processes, and extracted from natural vanilla pods were analyzed using Gas Chromatography-Isotope Ratio Mass Spectrometry. Based on measurements of ratios of stable isotopes of carbon it was possible to differentiate between the vanilla aroma extracted from vanilla pods and vanilla aroma made otherwise. Furthermore, the species of the vanilla pods was identifiable by measurements of carbon stable isotopes. By combining measurements of stable isotopes of carbon and hydrogen for natural vanilla, it was seen that adjacent geographic origins of growth had a tendency to cluster. Compound specific isotope analysis can therefore be a valuable tool in authenticating and tracing a vanilla aroma.

Methods for extracting vanilla aroma from food products were investigated and it was found that vanilla aroma could be extracted and concentrated using solid phase micro extraction with a polyacrylate fiber coating. The isotopic composition of the aroma extracted from vanilla custard powder, vanilla sugar, and cookies were determined and their authenticity evaluated accordingly. Extraction of vanilla aroma from ice cream was more complicated and a preliminary extraction was investigated. A liquid-liquid extraction was chosen in combination with solid phase micro extraction. With this method it was possible to analyze vanillin from 7 different ice creams and to evaluate their authenticity based on the measured carbon isotopic composition.

Resumé (in danish)

Ordet ”Autenticitet” benyttes ofte indenfor markedsføring af fødevarer. Et produkt kan markedsføres på, at det er autentisk - for eksempel i den forstand at produktet udelukkende indeholder naturlige ingredienser, men det kan også være i form af en bestemt geografisk oprindelse, som er forbundet med en traditionel fremstillingsmetode eller en særlig lokal delikatesse. Indenfor fødevareingrediensområdet, opleves der problemer med autenticiteten af aromastoffer, idet naturlige aromaer helt eller delvist erstattes af tilsvarende syntetiske aromaer. Dette er problematisk, da syntetiske aromaer ofte er langt billigere end naturlige. Desuden er det i Europa ulovligt at mærke fødevarer som indeholdende naturlige aromaer, hvis dette ikke er sandt. Det er imidlertid svært at bestemme autenticiteten af aromaer, da disse ofte er ens på molekylært niveau uafhængig af produktionsmetode eller oprindelsessted. Det er derfor nødvendigt at udvikle nye analytiske metoder, der er i stand til at verificere autenticiteten af aromaer.

Nogle aromastoffer har en molekylær opbygning som gør, at de kan forefindes som spejlbilledeisomerer af hinanden, også kaldet enantiomerer. Sådanne stoffer kaldes kirale og vil som oftest forefindes i lige mængder i en syntetiskfremstillet aroma, hvorimod en plantesyntetiseret aroma vil have en overvægt af den ene enantiomer. Dette kan udnyttes ved autenticitetsbestemmelser, da tilstedeværelse af lige mængder af begge enantiomerer vil indikere, at der er tale om en ikke-naturlig aroma.

Syntetisk fremstillede aromaer adskiller sig fra naturlige plantesyntetiserede aromaer ved at have forskelligt indhold af stabile karbonisotoper. Yderligere varierer indholdet af stabile karbonisotoper også imellem planter, på grund af planternes forskellige måder at optage og inkorporere karbondioxid. Dette faktum kan benyttes ved autenticitets-bestemmelse af aromaer. Forskellige klimaforhold påvirker fordelingen af stabile brintisotoper i planter kloden over og indholdet af brintisotoper kan derfor benyttes til at spore det geografiske voksested for den plante hvorfra en aroma stammer.

Formålet med dette Ph.d. projekt var at etablere en analytisk platform baseret på kiral separation samt komponent specifik stabil isotop analyse. Dernæst blev der udarbejdet en række referenceværdier for aromaer af kendt autenticitet. Disse referenceværdier

kan efterfølgende danne grundlag for verifikation af ukendte aromaer ekstraheret fra fødevarer.

Før der kan udføres gas kromatografisk enantiomerisk separation er det nødvendigt at ekstrahere og koncentrere aromastofferne. Til ekstraktion af aromastoffer fra hindbær, i særdeleshed det kirale molekyle α-ionone, blev forskellige parametre af metoden Fastfase-mikroekstraktion undersøgt. En fiber belagt med en blanding af divinylbenzen, carboxen og polydimethylsiloxan blev fundet egnet til formålet. Desuden blev det vist, at tilsætning af natrium klorid hæmmede ekstraktion af analytter til fiberen. De to enantiomerer af α-ionone fra hindbær blev adskilt ved hjælp af gas kromatografi med en kiral kolonne med en fase af cyclodextrin. Samme metode blev dernæst anvendt til analyse af hindbær samt produkter med deklareret indhold af hindbæraroma. 13 af de 27 produkter som blev analyseret, viste sig at indeholde en racemisk blanding af α-ionone. Af disse 13 produkter var 4 deklareret til at indeholde naturlig hindbær eller hindbærjuice, hvilket er i modstrid med fordelingen enantiomerer for α-ionone.

Vaniljearoma kan fremstilles syntetisk, ved mikrobielle processer eller ekstraheres fra naturlige vaniljestænger. Indholdet af stabile karbon- og brintisotoper for disse tre typer af vanilje blev bestemt ved Isotop Ratio Massespektrometri. Ud fra indholdet af stabile karbon isotoper for en vanilje aroma var det muligt, at bestemme om denne stammede fra en vaniljestang eller om aromaen var produceret ved en anden metode. Yderligere var det muligt at adskille to typer af vaniljeplanter baseret på målinger af stabile karbonisotoper. Et plot af resultater for stabile karbonisotoper mod stabile brintisotoper viste en tendens til at tætliggende geografiske områder grupperede sig. Isotop ratio massespektrometri kan derfor være en mulighed i autenticitetsbestemmelse og sporbarhed af vaniljearoma.

Det er nødvendigt med ekstraktion og koncentrering af vaniljearoma fra en fødevare før aromaen kan analyseres ved isotop ratio massespektrometri. Metoder til dette formål blev derfor undersøgt. Til ekstraktion af aroma fra vaniljecremepulver blev fastfase mikroekstraktion med en fiber af polyacrylat fundet egnet. Indholdet af stabile karbonisotoper for vanilje creme pulver, vaniljesukker og småkager blev målt og autenticiteten af den tilsatte vanilje blev herudfra vurderet. Ekstraktion af vanillin fra vaniljeis var mere vanskelig og en passende prøveforbehandling blev derfor undersøgt. Væske-væske ekstraktion i kombination med SPME blev valgt og med denne metode

blev 7 typer vaniljeis analyseret. Autenticiteten af den tilsatte vanilje blev dernæst vurderet ud fra det målte karbon isotop forhold.

Table of contents

1. Introduction

Authenticity is increasingly used in the marketing of food products. Buzzwords such as “authentic”, “natural”, “homemade” and “local” are examples used on food packaging appealing to consumer’s emotions in order to make them buy a product. The choice of the consumer is based not only on safety and hygiene concerns, but also on the story behind the product. This is especially true for high quality food products where authenticity plays an important role and for which consumers are willing to pay extra.

In this context, “authenticity” means that a food product actually contains what is labelled on the packaging. Aromas are often added to food products in order to obtain or enhance a specific flavor. In the case of aroma components, the issue of authenticity is often the naturalness of an aroma, but also the geographic indication and/or the plant species from where the aroma originates are important parameters. For example, a vanilla ice cream found in a local supermarket was labelled to contain bourbon vanilla originating from Madagascar. Such parameters are often not possible to see, taste, or smell. This makes verification of authenticity impossible for the consumer and replacements with cheaper aroma components possible for forgers. There is a huge economic gain in replacing the natural bourbon vanilla with a cheaper vanilla aroma. The fact that the cost of natural vanilla can be up to 350 times the price of synthetic vanilla aroma makes fraud very tempting (Korthou & Verpoorte, 2007).

European flavor regulations state that the aroma of food products may only be labeled “natural” if the flavor contains exclusively natural flavoring substances. A natural flavor should be obtained by appropriate physical, enzymatic or microbiological processes from the material of vegetable, animal or microbiological origin (European Parliament, 2008). For the case of vanilla, it means that a vanilla aroma can be labeled as “natural” if it originates from a vanilla pod but also “biovanillin” obtained from fermentation processes can be labelled as “natural”.

Fraudulent business within aroma components has been known for years and methods for detecting fraud have been developed accordingly. However, forgers become more

advanced and able to cheat traditionally analytical methods, which is why new and more sophisticated methods for detecting fraud within aroma components are needed.

Authenticity of raspberry aroma can be investigated using measurements of enantiomers. The raspberry aroma is very complex and consists of many different aroma components all contributing to the characteristic aroma of raspberry. One of the main aroma contributing molecules is α-ionone. α-ionone is chiral, meaning that it exists as two enantiomers (Robertson, Griffiths, Woodford, & Birch, 1995). A natural raspberry aroma will be dominated by one of the enantiomers while a synthetic produced raspberry flavour will have an almost equal distribution of both enantiomers (Werkhoff et al., 1993; Werkhoff, Bretschneider, Güintert, Hopp, & Surburg, 1991). Consequently, authenticity of raspberry aroma can be evaluated by separating these enantiomers and measuring the distribution. Enantiomers can be separated by Gas Chromatography using a stationary phase of derivatized cyclodextrins. This separation was used by Werkhoff et al.in 1991, who found natural ripe raspberries to contain 99.9% (R)-α-ionone (Werkhoff et al., 1991).

The method of enantiomeric separation of aroma components for authenticity purposes has its limitations. First of all, it is only applicable for chiral aroma compounds. Secondly, it is possible to separate a racemic mixture of enantiomers using chromatographic techniques, so that only the enantiomer found in natural products are added as aroma. Thirdly, it is possible to make an aroma compound by chemical synthesis using a natural precursor extracted from plants. The formed aroma compound can be labelled as “natural” but will contain a racemic mixture. Analyzing such an aroma by chiral-Gas Chromatography-Mass Spectrometry (chiral-GC-MS) would lead to a find of both enantiomers and could thereby be falsely characterized as an aroma of non-natural origin. Therefore, it is necessary to develop new methods in order to overcome such problems.

Another method for authenticating aroma components is by comparing the ratios of stable isotopes. Plants discriminate against the heavy stable isotope of carbon (^{13}C) and thus a natural aroma component synthesized from a plant will have different amounts of ^{13}C than an aroma made by chemical synthesis. Furthermore, stable hydrogen isotopes (^{1}H and ^{2}H) are not distributed evenly around the world. This is

reflected in the meteoric water worldwide and thereby also in plants and consequently, can be used for tracing an aroma.

Authenticity of vanilla aroma can be investigated using stable isotope ratios. Vanillin is generally the most abundant compound in vanilla aroma and present in all types of vanilla, which is why isotope ratios of vanillin were chosen for authenticity purposes. Stable isotope ratios can be measured using Gas Chromatography-Isotope Ratio Mass Spectrometry (GC-IRMS). The GC makes it possible to separate the components of an aroma and measure the isotopic ratio of each individual component also called Compound Specific Isotope Analysis (CSIA).

This project focuses on two methods for the authentication of aroma components: separation of enantiomers and measurements of stable isotope ratios. Stable isotope analysis is a relatively new technique capable to determine the ratio of heavy to light stable isotopes. Previous studies have used measurements of stable isotopes of vanillin for authentication: Natural vanilla, vanilla made by fermentation (biovanillin), and synthetic vanilla aroma were differentiated by measurements of stable carbon isotope ratios (Greule et al., 2010; Scharrer & Mosandl, 2002). However, little attention has been on the combined use of stable carbon and hydrogen isotopes for authentication and traceability. In this thesis both synthetic, biovanillin, and a large number of natural vanilla pods originating from different places around the world, have been investigated using compound specific isotope analysis of hydrogen and carbon.

Sample preparation is needed for both CSIA and enantiomeric separation for which purpose Solid Phase Micro Extraction (SPME) has been investigated. The use of SPME for the extraction of α-ionone from natural red raspberries were investigated including tests of different SPME fibers and the addition of salt for the optimization of extraction. Then 27 samples labelled to contain raspberry aroma was analyzed using SPME-enantio-GC-MS. Likewise SPME was used for extraction of vanillin from food products and extracts hereof for authenticating vanilla aroma.

The aim of this project was to test the hypothesis that authenticity of aroma components in food can be verified by:

- Measurements of enantiomeric composition of chiral aroma constituents

- Measurements of stable isotope ratios of hydrogen and carbon of selected aroma constituents
- Developments of sample purification procedures that enables the analysis of aroma compounds from food products by chiral-GC-MS or GC-IRMS

The first part of this PhD project was to implement methods for the verification of authenticity using IRMS and chiral separation. Hereafter, an analytical platform was established based on measurements of both ratios of stable isotopes and measurements of enantiomers for aroma components of known authenticity. This platform was then used for authenticating aroma compounds extracted from food products.

The first part of this thesis describes authentication using enantiomeric separation. Chapter 2.1 provide background information of chiral compounds and how to separate them using gas chromatography. Sample preparation is necessary before separation of enantiomers. For this purpose Solid Phase Micro Extraction has been used and the background for the method described in 2.1.3. In chapter 2.2 existing literature within enantiomeric separation of aroma compounds are reviewed.

In chapter 2.3 the studies of Paper 1 is presented. In this study, extraction of aroma components where optimized before separation on a chiral capillary GC column. The enantiomeric distribution of α-ionone of authentic raspberries are presented and compared to results for raspberry aroma extracted from 27 different food products to establish the authenticity of the aroma added.

Chapter 3.1 describes the theory of stable isotopes, their natural fractionation, and theory behind the Isotope Ratio Mass Spectrometer. In chapter 3.2 relevant literature within compound specific isotope analysis is reviewed.

Chapter 3.3.1 describes the preliminary studies necessary to carry out the work of Paper 2. In chapter 3.3.2 and 3.3.3 the studies which form the basis of Paper 2 is presented. In Paper 2 the CSIA of synthetic vanillin, biovanillin, and vanillin extracted from vanilla pods are presented. Authenticity studies of vanilla aroma from food products are described in chapter 3.4 and in Paper 3.

Finally concluding remarks and future perspectives are presented in chapter 4 and 5. Description of materials and methods for the studies described in this thesis are attached in appendix 1 and publications are found in appendix 2.

2. Authenticity of aroma compounds using enantiomeric separation

Aroma compounds are small organic molecules with a certain degree of volatility. Due to the volatile nature of aroma compounds, they can be carried as vapours into the human main olfactory epithelium where they can interact with the odorant receptors and thereby be detected by the human brain as a characteristic smell. Even low concentrations of aroma compounds can be detected by olfaction (Berg, Tymoczko, & Stryer, 2006).

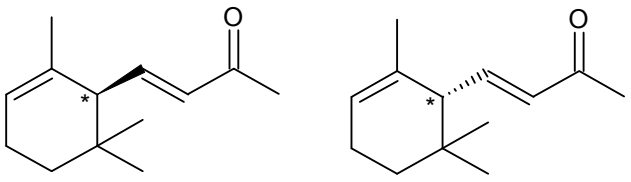
The volatile nature of aroma compounds makes them suitable for analysis by gas chromatography, which is the basic chromatographic method of separation for the research presented in this project.

Natural aromas are often complex mixtures of many different compounds, which each contribute more or less to the human perception of the aroma in question. Some aroma compounds exist as different kinds of isomers; for this chapter, the focus is on chiral compounds also called enantiomers. The extraction and separation of enantiomers are investigated in order to evaluate the authenticity of aroma components.

2.1 Background

2.1.1 Chirality

Chiral separation of aroma compounds can be used for the determination of naturalness (Werkhoff et al., 1991). A chiral molecule is defined by having a non-superimposable mirror image (Berthod, 2010). (E)-α-ionone known from raspberry flavour is an example of a chiral molecule which has two enantiomers, Figure 1 (Werkhoff et al., 1991). The two enantiomers are differentiated in R or S configurations using the Cahn-Ingold-Prelog nomenclature (Berthod, 2010).



(R)-(E)-α-ionone (S)-(E)-α-ionone

Figure 1: The two enantiomers of (E)-α-ionone

A chemical synthesis often results in a racemic mixture containing almost equal amounts of both enantiomers. However, in plants the biosynthesis of aroma components is often catalyzed by stereospecific enzymes leading to enantiomeric purity, where one of the enantiomers is dominating. This is also the case for raspberry flavour where (R)-(E)-α-ionone is dominating. Consequently, the presence of (S)-(E)-α-ionone could be an indicator of adulteration with artificial aroma components (Aprea, Biasioli, Carlin, Endrizzi, & Gasperi, 2009; Taylor & Linforth, 2010).

2.1.2 Separation of chiral compounds

The first chromatographic method for analysis of enantiomers was the “indirect” method where the enantiomers are converted into diastereomeric derivatives by letting them react with an enantiomeric pure chiral reagent. The diastereomeric derivatives could then be separated on a traditional achiral GC column. In contrast, the “direct” method for separation of enantiomers uses a column with a chiral stationary phase. This stationary phase can consist of e.g. chiral diamides, methal chelates, or cyclodextrins. Especially derivatisation of different types of cyclodextrins have made this type of stationary phases very versatile and capable of separating a wide range of aroma components (Werkhoff et al., 1993). Separation of enantiomers using cyclodextrins have been used for this project and will be the only method of enantiomeric separation described.

Chiral compounds can be separated on a GC column containing a chiral stationary phase made of cyclodextrins derivatized with appropriate functional groups. Cyclodextrins (CD) are cyclic polysaccharides consisting of 6 (α-CD), 7 (β-CD), or 8 (γ-CD) D-glucopyranose units bonded through 1,4-linkages, Figure 2. The glucopyranose units form a toroid with chiral hydroxyl groups pointing outwards making the exterior toroid hydrophilic. These chiral hydroxyl groups are the main reason for the capabilities to separate enantiomers (Beesley, 2010). The inside of the toroid is hydrophobic due to the oxygen from the glucosidic linkage and hydrogen atoms (Song, Bai, Xu, He, & Pan, 2009). The outside of the CD toroid can be derivatized with different functional groups in order to optimize separation of specific compounds (Beesley, 2010; Schurig, 2001).

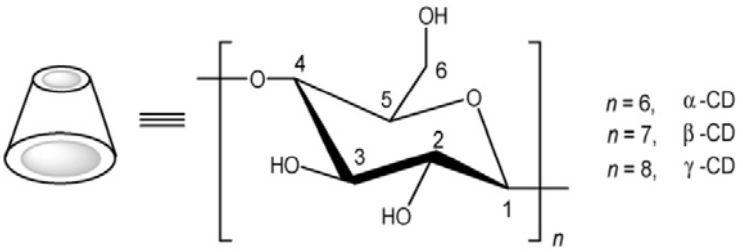


Figure 2: Conformation of a cyclodextrin toroid and a cyclodextrin monomer (Song et al., 2009)

An example of a chiral GC column is 25 % 2,3-di-O-acetyl-6-0-*tert*-butyldimethylsiloxane-β-cyclodextrin embedded in 20 % phenyl/80 % dimethylsiloxane. *Tert*-butyldimethylsilyl (TBDMS) is attached to the C6 hydroxyl where it blocks smaller cavities in the CD affecting enantioselectivity (Schurig, 2001). The CD is diluted in polysiloxanes to make them suitable for gas chromatography. This particular column is suitable for separating smaller enantiomeric molecules (Sigma-Aldrich, 2015).

2.1.3 Extraction of aroma compounds – Solid Phase Micro Extraction

Preparation of a sample is often necessary before it can be analyzed on GC. Solid Phase Micro Extraction (SPME) is concentrating analytes and can be selected to match a certain group of compounds. This preconcentration have been used in combination with both GC-MS and GC-IRMS throughout this project.

In general, SPME consists of a thin solid fiber of fused silica which is coated with a relatively thin film of stationary phase that acts as an organic “solvent” where to analytes can absorb/adsorb. The fiber can be coated with pure or mixed stationary phases, which is chosen based on the type of analyte (Marsili, 2002; Wercinski, 1999).

For extraction of aroma components the SPME fiber is placed in the headspace of a vial containing the sample. It is also possible to immerse the fiber into an aqueous sample, but for this project only headspace extraction has been used. When a SPME fiber is placed in the headspace, analytes from the headspace will partition between the sample matrix, the headspace, and the fiber coating. This partitioning can be facilitated by an agitation of the sample, heating the sample matrix or/and adding salt. Next, the fiber is placed in the GC injector liner where the analytes are desorbed directly onto the GC column under high temperatures. The partition between sample

matrix and SPME fiber, in the case of headspace SPME extraction, is an equilibrium between three phases:

1) fiber coating (f) and headspace (h): $K_{fh} = \frac{C_f}{C_h}$ equation 1

2) headspace (h) and sample solution (s): $K_{hs} = \frac{C_h}{C_s}$ equation 2

3) fiber coating (f) and sample solution (s): $K_{fs} = \frac{C_f}{C_s}$ equation 3

The amount of analyte extracted by the fiber can be expressed by the following equation: $n = \frac{K_{fs}V_fV_sC_0}{K_{fs}V_f+K_{hs}V_h+V_s}$ (equation 4) (Pawliszyn, 2012a; Wercinski & Pawliszyn, 1999). Here, n is the concentration of analytes on the fiber, V is the volume of the fiber (f), sample (s) and headspace (h) respectively and C_0 is the initial concentration of analyte in the sample. SPME is an equilibrium sampling method and thus does not exhaustively extract analytes from the sample matrix which is why it is important to keep sampling parameters constant for each analysis.

2.2 Description of existing research within chiral separation of aroma components

A natural aroma is a complex mixture of many different aroma compounds, each contributing more or less to the human perception of the specific aroma. To imitate a specific natural aroma, only some of the most characteristic aroma compounds present in the natural extract are needed. This makes the profile for a synthetic aroma much less complex than its natural counterpart. This fact can be useful for authenticity purposes. However, often the aroma is added to a food matrix with lots of interfering compounds, which makes it impossible to establish which compounds originates from the added aroma. In such a case the separation of enantiomers can be useful for authentication.

Some of the aroma compounds exist as enantiomers as described in section 2.1.1. For aroma compounds produced by biosynthesis in a plant, the distribution of R and S enantiomers are fixed, because these biosynthesis´ are catalyzed by stereospecific enzymes. Often one of the enantiomers is dominating also called enantiomeric excess or enantiomeric purity. In contrary, a chemical synthesis of a chiral compound will result in a racemic mixture containing almost equal amounts of both enantiomers.

Accordingly, the ratio between the two enantiomers of an aroma compound can be used to determine how this aroma was produced. Enantioselective gas chromatography has been used extensively for the authenticity control of essential oils but also fresh fruit and some few products have been investigated. In the following chapters some examples of previous studies regarding analysis of aroma compounds and enantiomeric separation is reviewed.

2.2.1.1.1 Bergamot (*Citrus bergamia)* and other fruits containing linalool

Bergamot aroma is used mainly in perfumes and tea. The main components of bergamot aroma are limonene, linalool, and linalyl acetate. Casabianca et al used a stationary phase consisting of heptakis(tri-*O*-methyl)-β-cyclodextrin for separation of the enantiomers, while Ravid et al. used heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin and octakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-γ-cyclodextrin phase. Limonene and linalool have been found almost exclusively as the R-enantiomer in natural bergamot oil aroma (oil and peel), while only a small portion (≤ 2%) of S-limonene was present (Casabianca & Graff, 1994; Ravid et al., 2010). Accordingly, these compounds can be used for authenticity control of bergamot aroma.

Linalool is present in a wide range of fruits and aromas and thus can be used as an indicator of adulteration as long as the enantiomeric distribution is known. Bernreuther and Schreier investigated the enantioselective distribution of linalool in different fruits. They found the R enantiomer of linalool to be dominating in guava, plum, and peach. The S enantiomer of linalool was dominating in arctic bramble, mango, yellow passion fruit, raspberries, and strawberries (Bernreuther & Schreier, 1991). Likewise, Ravid et al and Werkhoff et al found the enantiomers of linalool to differ depending on the type of flavor; in coriander oil, orange, and papaya the S-enantiomers were dominating, while the R-enantiomer was the main constituent of basil oil. Almost racemic linalool was found in yellow passion fruit flavor (Ravid et al., 2010; Werkhoff et al., 1993).

2.2.1.1.2 Rosemary (*Rosmarinus officinalis)*

Zawirska-Wojtasiak et al. quantified 15 volatile compounds from rosemary leafs and oils by the use of SPME-GC-MS. 8 of these compounds were separated in their enantiomers using a capillary column consisting of β-cyclodextrin embedded into 14% cyanopropylphenyl/86% dimethyl polysiloxane. The 8 compounds were α-pinene,

camphene, β-pinene, limonene, linalool, camphor, borneol, and α-terpineol. Of the analysed enantiomers only the content of (S)-borneol was detected in all samples analysed and found in high enantiomeric purity (>85%) (Zawirska-Wojtasiak & Wąsowicz, 2009). König et al also reports the S-enantiomer to be the most abundant in rosemary oil, however they find the enantiomeric purity of borneol to vary according to origin. Also the content and enantiomeric purity of 1,8-cineol and camphor in rosemary have been found to vary depending on the origin (König, Fricke, Saritas, Momeni, & Hohenfeld, 1997).

2.2.1.1.3 Tea tree oil (*Melaleuca alternifolia)*

There are many varieties of tea tree and accordingly a large variation in the aroma profile. α-pinene, β-pinene, α-phellandrene, limonene, sabinene, linalool, terpinen-4-ol, and α-terpineol are some of the compounds found in tea tree oil. (R)-α-pinene has been found with the highest enantiomeric purity (86-91%), while the other chiral compounds measured was closer to a racemic mixture. Additionally, eucalyptus oil contain α-pinene, β-pinene, limonene, and α-terpineol in almost the same enatiomeric range as found in tea tree oil, making it difficult to separate the two type of oils. However, the concentration and enantiomeric purity of terpinen-4-ol differs for the two type of oils, and can be used for authenticity determinations (Kreck, Scharrer, Bilke, & Mosandl, 2002; Mosandl, 2007).

2.2.1.1.4 Raspberries (*Rubus ideaus)*

Aprea et al identified 28 volatile compounds and further tentatively 18 compounds in two cultivars of raspberries[1] using SPME-GC-MS. Aprea et al found both quantitatively and qualitatively differences between the two cultivars. Especially aldehydes, alcohols and hexenal differed for the two cultivars (Aprea et al., 2009). Malowicki et al quantified 29 compounds from five different raspberry cultivars[2] using stir bar sorptive extraction-GC-MS. They found especially α-ionone, β-ionone, geraniol, linalool, and (Z)-3-hexenol to vary between the analysed cultivars. Additionally, Malowicki et al found the aroma profile of raspberries of same cultivar but grown in different location to differ. The enantiomeric ratio of the following chiral compounds where determined: α-ionone, α-

[1] Raspberry cultivars analysed by Aprea et al: “Tullameen” and “Polka”

[2] Raspberry cultivars analysed by Malowicki et al.: “Tullameen”, “Meeker”, “Yellow Meeker”, “Chiliwack”, and “Willamette”

pinene, linalool, terpinen-4-ol, δ-octalactone, δ-decalactone, 6-methyl-5-hepten-2-ol. The one enantiomer was found predominating with more than 96% of (R)-α-ionone, (R)-α-pinene, (S)-δ-octalactone, and (S)-δ-decalactone. Enantiomers of terpinen-4-ol and linalool was found in more eaqual amounts, the ratio of linalool even varied 37-51% due to cultivar and growing location (Malowicki, Martin, & Qian, 2008).

2.2.1.1.5 Authenticity of aroma extracted from food and food products

Ravid et al investigated commercial bergamot tea samples using SPME-GC-MS and found all of the samples to contain almost racemic mixtures of the R and S enantiomers of linalool and linalyl acetate. Since natural bergamot has been found to be enantiomerically pure for these compounds, the findings of Ravid et al indicate that the tea samples have been adulterated (Ravid et al., 2010).

In 1991 Werkhoff et al extracted natural α-damascone from black tea and tobacco by the use of multidimensional preparative chromatographic techniques and further separated the enantiomers of α-damascone using a capillary column consisting of heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin in polysiloxane. They found extracts of both black tea and tobacco to be almost racemic with a slight excess of the R-enantiomer. Werkhoff et al assumed that α-damascone was formed during the production of black tea/tobacco and therefore did not represent the enantiomeric distribution of the original ingedients (Werkhoff et al., 1991).

Werkhoff et al also separated the enantiomers of α-ionone from natural products such as *Boronius megastigma* Nees, *Osmanthus fragrance* Lour,*Costus* root oil, *Viola odorata*, carrots, and vanilla pods and found α-ionone to occur mainly as the R enantiomer. Investigation of three raspberry concentrates revealed two of them to contain 38 and 46% S-α-ionone respectively, indicating prescence of α-ionone of non-natural origin (Werkhoff et al., 1991).

The enantiomeric purity of 7 chiral compounds found in 6 different strawberry varieties were determined by SBSE-enantio-Multi Dimensional GC-MS. (S)-2-methylbutanoate, (S)-ethyl-2-methylbutanoate, (R)-4-octanolide, (R)-α-ionone, (R)-4-decanolide, and (R)-4-dodecanolide were found in high enantiomeric purity of more than 96.5%.
The percentage of enantiomers found, were then compared with the findings from strawberry products in order to establish the authenticity of these products. The

concentration of the compounds differed for the 6 strawberry varieties analyzed. This will however, not affect the enantiomeric distribution. The products analyzed were strawberry jams, sweet dessert, milk, syrup, yoghurt, and aroma. For some of the analytes the concentration was under the limit of detection, but in that case the authenticity could be judged based on other of the measured chiral compounds, and the method proved valid for authenticity purposes (Kreck, Scharrer, Bilke, & Mosandl, 2001).

Ravid et al. investigated the authenticity of products containing raspberry flavor using HS-SPME-chiral-GC-MS. The products analyzed was syrup, yoghurt, jam, tea, and candy. The samples were added NaCl and adsorbed on a SPME fiber coated with a 65µm polydimethylsiloxane-divinylbenzene phase for 30 or 90 min (depending on the type of sample) at 25 °C. The column used for separation of enantiomers was a heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin 30m x 0.25mm i.d., 0.25µm. 12 of the 20 products analyzed was found to contain almost racemic mixtures of both enantiomers indicating presence of non-natural raspberry flavor (Ravid et al., 2010).

Preconcentration of aroma components before GC analysis is necessary. Werkhoff et al used headspace stripping in vacuum for concentration of aroma components of raspberries (Werkhoff et al., 1991). However, in the following years new methods for sample extractions and concentrations were developed: Malowicki et al. used Stir Bar Sorptive Extraction (SBSE) to quantify volatiles from different raspberry cultivars while Aprea et al. and, Ravid et al. investigated the use of Solid Phase Micro Extraction (SPME) for the extraction of volatiles from raspberries (Aprea et al., 2009; Malowicki et al., 2008; Ravid et al., 2010).

2.3 Paper 1 - Authenticity of raspberry flavor in food products using Solid Phase Micro Extraction-chiral-GC-MS

Naturalness of aroma components can be evaluated based on the content of enantiomers. The distribution of enantiomers in natural aroma components are determined by stereospecific enzymes involved in the biosynthesis. Ratios of enantiomers can vary for different types of plants but often one enantiomer is dominating for natural aroma components. On the contrary a chemical synthesis of an aroma compound is most likely to result in a racemic mixture. Consequently, separation and measurements of enantiomers of an aroma component can be used for the verification of authenticity.
Until recent most of the research using enantioslective-GC-MS has been on essentials oils or samples for which the aroma components can easily be extracted, i.e. tea.

Paper 1 describes an optimization study for extraction of raspberry aroma from raspberries and samples containing raspberry aroma by the use of headspace solid phase micro extraction (SPME). SPME was chosen because it has the advantage that it extracts and concentrates volatile analytes from a food matrix without the use of solvents. The main focus is to achieve the best extraction of the chiral compound α-ionone, but the extraction of other volatiles can also contribute to the authenticity of raspberry aroma, since the natural aroma is often complex and contains more aroma compounds than synthetic ones. Ravid et al have demonstrated that SPME successfully can be used for the extraction of raspberry flavours using a polydimethylsiloxane-divinylbenzene fiber and addition of NaCl (Ravid et al., 2010). In this paper we have investigated different parameters for optimization of the SPME extraction of raspberry aroma. Furthermore, 27 samples of food labelled to contain raspberry aroma was analyzed. This is, to the author's knowledge, the largest dataset obtained for the enantiomeric purity of raspberry samples.

2.3.1. Paper 1 – Solid Phase Micro Extraction, optimization for extraction of raspberry flavor

Previous studies have established that natural raspberries contain over 99 % of R-(E)-α-ionone (Ravid et al., 2010; Werkhoff et al., 1993). Therefore, it is not necessarry to analyze a range of natural raspberries of different cultivars to establish reference

intervals for enantiomers in raspberries. However, it is necessary with a sample preparation that extracts and concentrates raspberry aroma compounds before analysis.

Based on equation 4, different parameters can be changed in order to optimize the extraction of analytes to the SPME fiber. The focus of this study was to increase K_{fs} by choice of fiber and by the addition of salt.

2.3.1.1. Selection of fiber coating

By choosing a fiber coating that is more selective towards the analytes, the partition between the fiber coating and the sample will be shifted in favor of the fiber thus increasing the term K_{fh} and thereby increasing the concentration of analytes on the fiber. Three bipolar fiber coatings were tested for the extraction of volatiles from raspberries. The coatings on the tested fibers are combinations of polydimethylsiloxane (PDMS), divinylbenzene (DVB) and carboxen (CAR). See fiber description in Table 1.

Table 1: Description of SPME fibers used for extraction of volatiles from raspberries (Sigma-Aldrich, 2015)

Name	Description	Primary extraction	Application
DVB/PDMS	65 µm PDMS/DVB coating Partially cross linked phase	Adsorption	M_w: 50-300 g/mol Semi volatiles, aromatic hydrocarbons, aromatic amines, VOC´s
CAR/PDMS	75 µm CAR/PDMS coating. Partially cross linked phase	Adsorption	M_w: 30-225 g/mol VOC´s, hydrocarbons
DVB/CAR/PDMS	50 µm DVB and 30 µm CAR on PDMS coating Highly cross linked	Adsorption	C3-C20 M_w: 40-275 g/mol

The SPME fibers were used for extracting aroma compounds from raspberries followed by GC-MS analysis. Materials and methods used are described in Appendix 1. Figure 3 shows peak areas for 8 compounds extracted from raspberries. For this study, only the ability to extract analytes from raspberries was of interest and not the concentration of the analytes. The varying concentrations for the different analytes were not caused by inefficient extractions, but rather due to the different concentrations of analytes in raspberries.

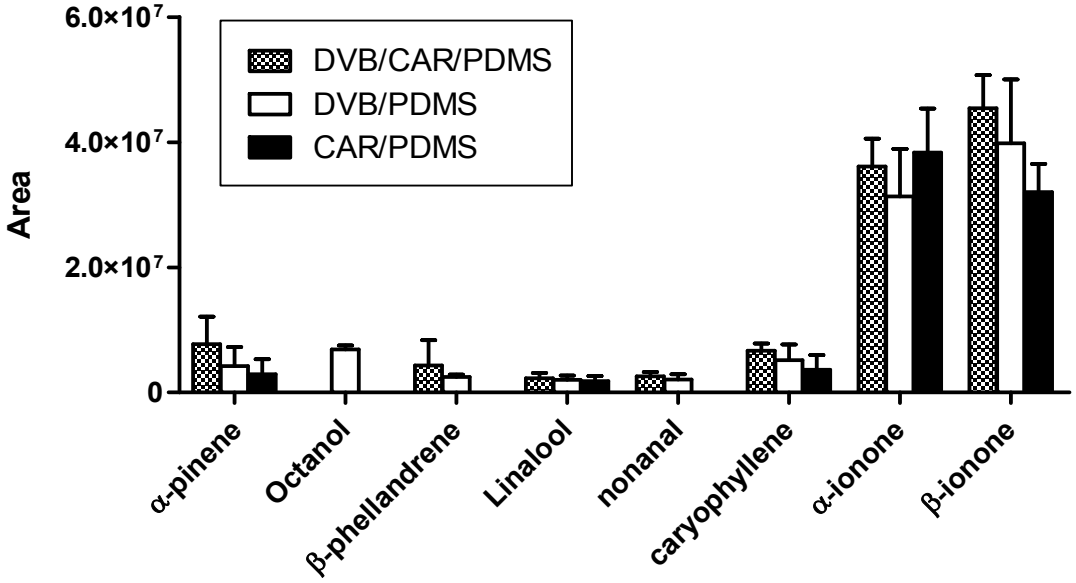


Figure 3: Peak areas for 8 analytes extracted from raspberries with DVB/CAR/PDMS, DVB/PDMS, and CAR/PDMS SPME fibers. n=4

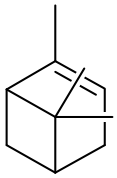
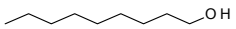
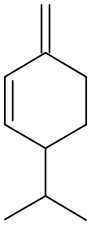
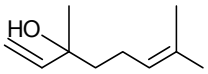
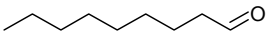
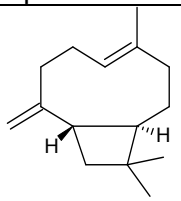
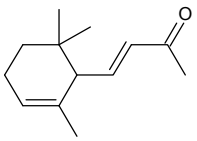
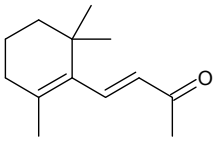
 α-pinene M_w: 136.234 bp: 156.3°C	 Octanol M_w: 130.228 bp: 194.7°C	 β-phellandrene M_w: 136.234 bp: 177°C	 Linalool M_w: 154.249 bp: 198°C
 Nonanal M_w: 142.238 bp: 195 °C	 Caryophyllene M_w: 204.352 bp: 122°C[a]	 α-ionone M_w: 192.297 bp: 145°C[b]	 β-ionone M_w: 192.297 bp: 124°C[c]

Table 2: Name, structure, molar mass [g/mol], and boiling point (760mmHg) of the analytes extracted from raspberries. Superscript letters indicate boiling points at reduced pressure: a=13.5mmHg, b=28mmHg, c=10mmHg (Haynes, 2015).

The three SPME fibers showed approximately similar abilities to extract analytes from raspberry headspace considering the rather large standard deviations. Octanol was the only analyte not found by DVB/CAR/PDMS and CAR/PDMS.

DVB is a porous polymer and carboxen a carbon molecular sieve. Both these coatings extract analytes via adsorption into their porous material. Pore size determines the size of the analytes adsorbed, given that a pore can contain a molecule about half the size of the pore diameter (Shirey, 2012). DVB has an average pore size with a diameter of 16Å and carboxen 12Å (Shirey, 2012). This means that in general carboxen is better suitable for smaller volatile analytes than DVB. However, the porous material is not only composed of a single pore size, but is a combination of macro-, meso-, and micro-pores. I.e. carboxen contains tapered pores going all the way through the sphere, also enabling it to extract analytes of a larger size. This could mean that a higher desorption temperature could be needed in order to desorb all analytes from the CAR/PDMS fiber. For this study, all types of fibers were desorbed at 230°C, which might have been too low for all analytes to desorb from carboxen, leading to undetected or lower concentrations of some analytes.

Caryophyllene, α-pinene, and both ionones were found using CAR/PDMS fiber coating. Except for caryophyllene, these molecules are rather small and able to fit into the pores of carboxen. The smallest diameter of caryophyllene is 8Å (Chemicalize.org, 2015) which in theory makes it too large to be adsorbed by carboxen. Instead, caryophyllene must be assumed to be absorbed by PDMS.

Only octanol was not found using either DVB/CAR/PDMS fiber coating or CAR/PDMS fiber coating, while all three fibers extracted linalool. None of the fibers have a high degree of polarity and the extraction mechanism for alcohols on the fibers must be assumed to be of more adsorptive character. It is therefore strange that octanol is not seen for CAR/PDMS and DVB/CAR/PDMS. Since the DVB/CAR/PDMS fiber consists of an outer layer of DVB embedded in PDMS, it should be able to extract the same compounds as DVB/PDMS. However, the alcohols were not the main focus for an analysis of raspberries; otherwise, a more polar fiber like Polyacrylate (PA) should have been chosen.

The three fiber coatings tested had very similar extraction capabilities and each of them could be used for studies of α-ionone from raspberries. The SPME fiber coated with DVB/CAR/PDMS was found suitable especially for an extraction of α-ionone and β-ionone and was chosen for further analysis.

2.3.1.2. Addition of sodium chloride

The effect of adding sodium chloride to the matrix was investigated. The general theory of NaCl addition is that when the ionic strength of the matrix is increased, the solubility of the analytes decreases and thereby K_{fs} could be increased (Kudlejova, Risticevic, & Vuckovic, 2012). In this study sodium chloride was added to the sample matrices to a concentration of 25 % and analytes extracted with a SPME fiber coated with DVB/CAR/PDMS. Extraction, desorption and GC parameters were as described in Appendix 1.

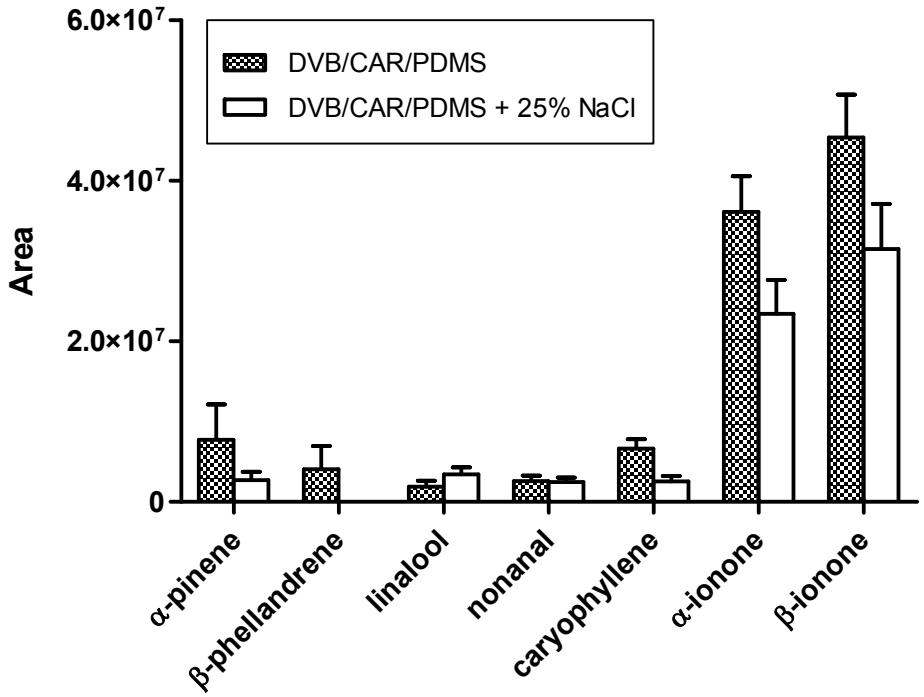


Figure 4: Effect of added sodium chloride upon the extraction of aroma compounds from raspberries. Extraction performed with a DVB/PDMS/CAR fiber

From Figure 4 it is clear that an addition of sodium chloride does not have an effect upon the extraction of the majority of the analytes extracted from raspberries. On the contrary, it looks like the salt has a negative effect especially for α-pinene, caryophyllene, α- and β-ionone. For the other compounds analyzed the effect was less pronounced. Only the extraction of linalool was higher when sodium chloride was added. Pawliszyn 2012 reported that the positive effect of salt increases with increased polarity of the analytes, which is in agreement with the results found in this study (Pawliszyn, 2012b).

The negative effect by salt addition is possibly caused by a decrease in activity coefficients for the analytes which will decrease K_{fs} and thereby the extraction efficiency (Kudlejova et al., 2012).

The results for β-ionone are somewhat in agreement with the results of Yang and Peppard, who found that the extraction of β-ionone decreases with higher sodium chloride concentrations when extracted with a SPME fiber, coated with 100 µm PDMS (Yang & Peppard, 1994).

2.3.1.3. Further improvement of extraction with SPME fibers

Besides changing fiber coating and adding salt there are other parameters that can be changed to facilitate solid phase micro extraction.

The SPME needle is hollow and has a large diameter compared to needles used for liquid injections. Therefore SPME needles can easily damage the septum during injections and septum and liner needs to be changed more often (Penton, 1999). This can be optimized by using a special optimized septum-less injector seal, which was not used for this study. Also liner dimensions should be kept small, but still large enough so there would be enough space for the SPME fiber.

Increasing the volume of the fiber (V_f) can also increase the amount of analytes extracted. The fiber volume can be increased by making a thicker fiber, but this will often result in longer extraction times. Instead, the length of the fiber is normally increased. This will not require longer time for adsorption but since the fiber is longer it is also more fragile and inclined to break. Another parameter that is more easily changed is the decreasing of the headspace volume (V_h). This will increase the concentration of non-polar analytes on the fiber (Penton, 1999). For this experiment the headspace above the sample was kept at a minimum, so there was just enough space for exposing the 1cm fiber.

An increased desorption temperature for CAR/PDMS could enhance desorption of especially larger analytes. Also, extraction time and temperature could be optimized. An increased extraction temperature would facilitate evaporation of semi volatile analytes and thereby increase the adsorption to the fiber. Conversely a high

temperature could also result in desorption of small volatiles from the fiber and/or thermal decomposition of analytes. Analytes were extracted at 60°C in this study. Increasing the extraction temperature can increase K_{fs} by facilitating the transfer of analytes from the sample to the headspace and thereby also the fiber. But excessive temperature increase can cause some of the analytes to desorb while the fiber is still immersed in the head space, which will result in a loss of analytes (Balasubramanian & Panigrahi, 2011).

Also extraction time can be increased so more sites of the fiber get occupied by analytes. The disadvantage by long absorbtion/adsorbtion time is that analytes can start to desorb and analytes with higher affinity for the fiber can replace analytes with less affinity.

2.3.2. Paper 1 - Authenticity of raspberry flavour from food products

Before analysis of food samples, preliminary studies were performed on natural raspberries, synthethic made α-ionone, and raspberry juice to test the method and establish reference data.

The naturalness of raspberry flavours was evaluated using SPME-chiral-GC-MS. SPME was used for the extraction and concentration of analytes from the matrix. Hereafter analytes were separated on a GC column able to separate enantiomers. The presence of only R-(E)-α-ionone would indicate a flavour of natural origin.
The coating of the SPME fiber was made of DVB/CAR/PDMS, which was found suitable for extracting α-ionone. The GC column used was a β-DEX 225 consisting of non-bonded 25% 2,3-di-O-acetyl-6-O-TBDMS-β-cyclodextrin in a SPB-20 phase (Sigma Aldrich, Denmark), see description in chapter 2.1.2 and appendix 1 for materials and methods.

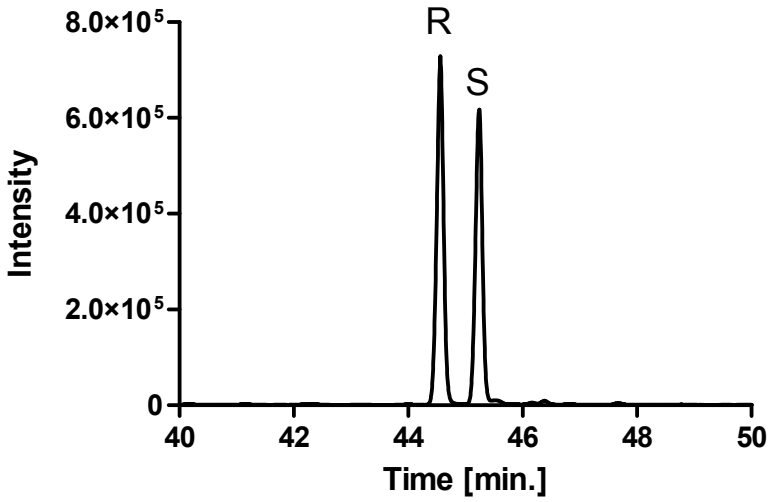


Figure 5: Chromatogram of synthetic made α-ionone obtained by selective ion monitoring

The chiral column used was found suitable for separating enantiomers of synthetic made α-ionone, Figure 5. Figure 6 shows total ion chromatograms (TIC) of analytes extracted from blended natural raspberries (red) and raspberrry juice (grey). The raspberry juice was kindly provided by A/S Ejnar Willumsen. At first sight, the two chromatograms look quite different. The raspberry flavour of unknown origin contains many of the same compounds as natural raspberries, but in much different concentrations, some higher and some lower. This does not discard the flavour as being natural since aroma compounds might have evaporated during the processing, especially if it has been heated, and/or compounds might have decomposed or has been converted into other compounds. Furthermore, the concentration of analytes will vary for raspberries depending on type and ripeness. The raspberry juice can be made from another type of raspberries than used in this study, which would give different aroma profiles.

For authenticity purposes, the most interesting compound is α-ionone. Figure 7 shows a zoom on the chromatogram in Figure 6. It is seen from Figure 7 that (S)-α-ionone is barely detectable in natural raspberries (red) compared to the peak of (R)-α-ionone. The same is true for the raspberry juice (grey); in fact the (S)-α-ionone peak has almost the same intensity as the baseline before the peak of (R)-α-ionone. The missing (S)-α-ionone indicates that this aroma is not made by chemical synthesis. In the case of synthetic raspberry aroma both enantiomers would be present in almost equal amounts. Synthetic α-ionone can be added to natural raspberry aroma i.e. in order to

enhance flavour and/or reduce price. In that case the enantiomeric ratio will not be 50:50, but it would still be possible to detect (S)-α-ionone indicating a non pure natural aroma.

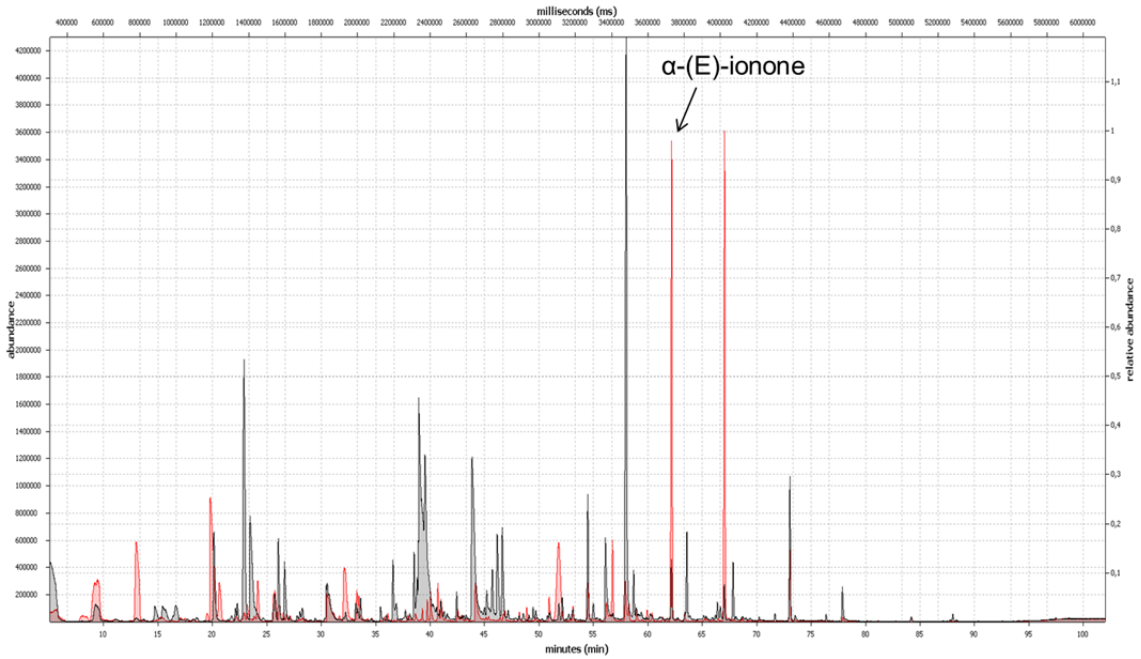


Figure 6: Total ion chromatogram of analytes extraced with SPME from natural raspberries (red) and a raspberry juice (grey)

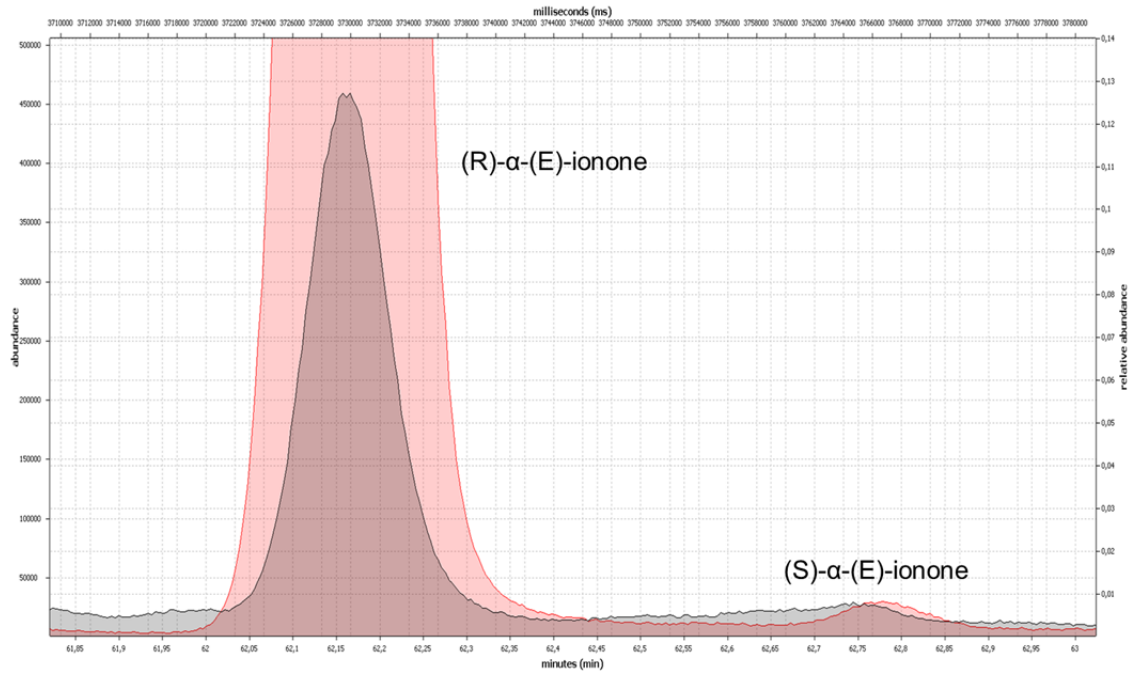
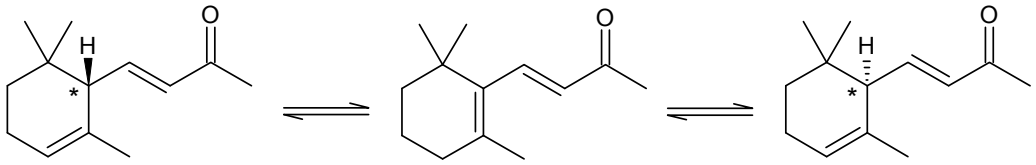


Figure 7: Zoom of α-(E)-ionone from chromatogram in Figure 6. The peak at the left is the R-enantiomer while the hardly detectable peak at the right is where the S-enantiomer would elute

Even though natural α-ionone is biosynthesized primarily in the R form, it is possible to find small amounts of the S form present. It is possible for the R form to be converted into the S form: a hydrogen atom can be removed from the asymmetric center of ionone as a result of shift in the double bond resulting in either β-ionone or the isomeric ketone, Figure 8. When the hydrogen atom returns to the center there is an equal probability that it will return to the original position or to its mirror image (Sobotka, Bloch, Cahnmann, Feldbau, & Rosen, 1943).



(R)-α-ionone β-ionone (S)-α-ionone

Figure 8: Racemization of α-ionone

Ravid et al. 2010 reported racemization of α-ionone when exposed to UV light. However, it was found in Paper 1 (see Table 3) that even the treatment the raspberry aroma undergoes for production of jam, does not alter the enantiomeric distribution to an extent, where it is not useful for authentication.

The optimized SPME method was also used to determine the naturalness of α-ionone from raspberry flavor extracted from 27 different food products, Table 3. Raspberry jam, dried raspberries, and sodas declared to contain natural aroma all contained almost only the R-enantiomer of α-ionone, confirming the declaration. Eight types of sweets were analyzed of which six did not declare a content of natural raspberry aroma on the labelling. This was in agreement with the almost racemic mixture found. Two of the sweets analyzed were declared to contain raspberry juice. However, these samples had an almost racemic mixture of enantiomers indicating presence of only synthetic raspberry flavor. Likewise, a fruit bar and yoghurt was declared to contain natural aroma, but showed almost equal amounts of both enantiomers, indicating that no natural raspberry aroma was present.

Table 3: Measured percentages of R-α-ionone and S-α-ionone from food samples obtained from the Danish retail market

Sample	R-α-ionone [%]	S-α-ionone [%]	Natural aroma declared	Aroma declared	Fruit content declared	Compliance with EU legislation
Jam #1	97	3			Raspberry, 35%	Yes
Jam #2	97	3			Raspberry, 50%	Yes
Jam #3	97	3			Raspberry, 50%	Yes
Jam #4	96	4			Raspberry, 45%	Yes
Jam #5	98	2			Raspberry, 40%	Yes
Jam #6	97	3			Raspberry, 45%	Yes
Soda #1	50	50		X		Yes
Soda #2	50	50		X		Yes
Soda #3	100	0	X			Yes
Soda #4	100	0	X			Yes
Soda #5	51	49		X		Yes
Dried berries #1	100	0			Dried fruits	Yes
Sweet #1	50	50		X		Yes
Sweet #2	50	50		X		Yes
Sweet #3	50	50		X		Yes
Sweet #4	50	50		X	Raspberry juice	No
Sweet #5	51	49		X	Raspberry juice	No
Sweet #6	50	50		X		Yes
Sweet #7	50	50		X		Yes
Sweet #8	50	50		X		Yes
Fruit bar #1	49	51	X		Raspberry 1.3%	No
Yoghurt #1	100	0			Raspberry 14%	Yes
Yoghurt #2	100	0			Raspberry 7.5%	Yes
Yoghurt #3	49	51	X		Raspberry 1.7%	No
Yoghurt #4	100	0	X		Raspberry 7%	Yes
Yoghurt #5	100	0	X		Raspberry 8%	Yes
Yoghurt #6	100	0	X		Raspberry 6%	Yes

3. Authentication of aroma components using Compound Specific Isotope Analysis (CSIA)

3.1 Background

Separation of enantiomers is a valuable tool in the authenticity control of aroma, especially to determine if an aroma is of natural or synthetic origin. However, this method has certain limitations, i.e. not all aroma compounds are chiral and even for those, which possess chirality, the distribution of R and S enantiomers are not always of enantiomeric purity. Furthermore, racemization during processing/storage of food products have been observed, which will lead to false measurements of the enantiomeric distribution. Compound Specific Isotope Analysis (CSIA) can be used in such cases or/and in combination with enantiomeric separation. Authenticity control also involves the geographic origin of an aroma, for which enantiomeric distribution is insufficient. The use of Compound Specific Isotope Analysis (CSIA) for authenticity and traceability studies are here investigated.

3.1.1. Stable isotopes

Isotopes are atoms of the same element but with different numbers of neutrons. The different numbers of neutrons lead to different atomic masses for isotopes of the same element, although the atomic number is the same (Housecroft & Constable, 2006), see Table 4. The term “stable” means that the isotopes do not undergo radioactive decay. For this project it is the stable isotopes of hydrogen and carbon that are of interest. Hydrogen has two stable isotopes with the mass number 1 and 2 which are denoted ^{1}H and ^{2}H. Likewise carbon has two stable isotopes denoted ^{12}C and ^{13}C. The number in the superscript is the mass number. The atomic number can be noted as a number below the atomic mass (Brand & Coplen, 2012). However, this notation is not used throughout this thesis, since the atomic number is constant for the same element and because only carbon and hydrogen are of primary interests in this project.

Table 4: Masses and natural abundances of isotopes of selected elements (Harris, 2012)

Element	Stable isotopes	Mass [Da]	Abundance [%]
Hydrogen	^{1}H	1.007825	99.988
	^{2}H	2.01410	0.012
Carbon	^{12}C	12	98.93
	^{13}C	13.00335	1.07
Nitrogen	^{14}N	14.00307	99.632
	^{15}N	15.00011	0.368
Oxygen	^{16}O	15.99491	99.757
	^{17}O	16.99913	0.038
	^{18}O	17.99916	0.205
Sulphur	^{32}S	31.97207	94.93
	^{33}S	32.97146	0.76
	^{34}S	33.96787	4.29
	^{36}S	35.96708	0.02

The delta notation is traditionally used when measuring stable isotopes, defined by: $\delta = \frac{(R_{sample} - R_{ref})}{R_{ref}} \cdot 1000‰$ (equation 5).

The ratio of the heavy to light isotopes of a sample (R_{sample}) are measured and correlated to the ratio of an internationally predetermined reference (R_{ref}) also called primary standard. Since the isotopic ratios are very small, the delta values are expressed in the unit “per mill” in order to reduce the number of decimals. The reference materials used are normally enriched in the heavy isotope which is why most measured delta values are negative (Groot, 2004; Sessions, 2006).

Stable isotope ratios of carbon are expressed relatively to the primary standard Pee Dee Belemnite (PDB). This material consist of $CaCO_3$ from the rostrum of an extinct order of cephalopods (Belemnitella Americana) found in the Pee Dee formation in South Carolina, USA (Groot, 2004). PDB is now exhausted, but a new standard called NBS-19 has been developed by the National Bureau of Standards (NBS). NBS-19 is also called TS-limestone and is a carbonate material derived from white marble of unknown origin (Groot, 2004). A new scale was established for measurements of carbon stable isotopes based on NBS-19, this new scale was called Vienna-Pee Dee Belemnite and is nowadays used as basis for measurements of carbon stable isotopes (Carter & Barwick, 2011; Groot, 2004).

Stable isotope ratios of hydrogen are expressed relatively to the primary standard: Standard Mean Ocean Water (SMOW). This material has never physically existed but is defined according to the water standard NBS-1. Meanwhile, another standard was made by blending different types of water so that the isotopic composition became close to SMOW; this standard was called Vienna-Standard Mean Ocean Water (V-SMOW). V-SMOW is now replaced by V-SMOW2 (Carter & Barwick, 2011).

3.1.2. Natural isotopic fractionation

In general, the lighter isotope is the most abundant, i.e. there is a natural mean occurrence of 99.988% of ^{1}H while only 0.012% is ^{2}H. For carbon the natural mean occurrence of ^{12}C is 98.93% and 1.07% of ^{13}C, Table 4. However, this distribution is not uniform. Due to fractionation the isotopic ratio will change.

3.1.2.1. Fractionation of isotopes

Even though isotopes of the same element behave almost identical, the difference in mass can cause fractionation. In Figure 9 the potential energy of a diatomic molecule are shown as a function of the interatomic distance. To the right in Figure 9 the interatomic distance is so large that the two atoms move independently. When the distance between the atoms decreases attractive forces begin to form and when the interatomic distance is small enough bonds are formed between the two atoms. The vibrational frequency of a molecule depends inversely on the mass so that two isotopologous will have different vibration frequencies. The lighter molecule will have a higher vibrational frequency and thereby a higher potential energy than the heavier molecule. The zero point energy of the molecules where the bonds are most stable is shown as the two horizontal lines in Figure 9. Because the lighter molecule has a higher zero point energy than the heavier molecule, less energy is needed to break the bond in the lighter molecule. This means that for a given amount of energy applied more bonds in lighter molecules will break than bonds in heavier molecules and consequently fractionation takes place (Bigeleisen, 1965; Fry, 2008a).

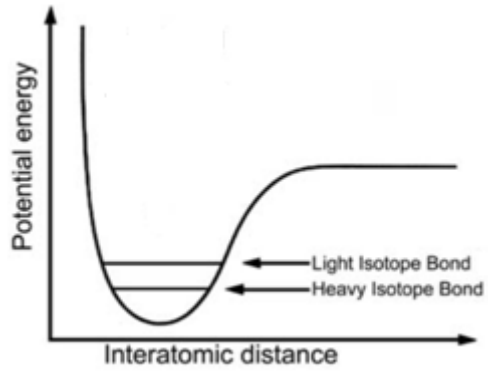


Figure 9: Illustration of the energy of a diatomic molecule as a function of the distance between atoms (Fry, 2008a)**.**

Fractionation also occurs during equilibrium exchange reactions. As described above, the bonds in molecules containing heavier isotopes are stronger and need more activation energy to break. Heavy isotopes tend to concentrate where bonds are strongest and thereby create fractionation. Likewise bond formation of heavy isotopes requires higher activation energy and is therefore formed slower than for lighter isotopes (Bigeleisen, 1965; Fry, 2008a, 2008b).

3.1.2.2. Natural fractionation of hydrogen

Hydrogen mainly cycles around the atmosphere in the form of water. The mass difference between the two hydrogen isotopes are large, and consequently, large fractionation occurs leading to a non-uniform distribution of stable hydrogen isotopes around the world (Hoefs, 2009). Fractionation of hydrogen isotopes can be divided into 4 main effects: continental, altitude, latitude, seasonal, and amount effect. However, the most significant effects are the temperature, which is most pronounced near the poles, and the amount of rainfall mainly affecting tropical areas (Dansgaard, 1964).

The continental effect occurs when water evaporates from the ocean. The first fractionation happens when ocean water evaporates: the formed water vapor will be depleted in the heavy isotopes compared to the ocean. The cloud of water vapor can be considered a closed system where the condensed water immediately is removed. This rainout causes the fraction of the remaining water vapor to decrease, a phenomenon called Rayleigh distillation. The continental effect implies that the clouds moving inland from the coast will become more and more depleted in the heavy isotope (Bowen, 2010; Dansgaard, 1964).

The temperature affects the fractionation during the condensation of rain. In winter times when the temperature is low, the fractionation of the heavy isotopes increases leading to precipitation depleted in ^{2}H, also called seasonal effect.
The isotopic composition of water is also affected by altitude. An increase in altitude means that the atmospheric pressure decreases necessitating larger temperature drops in order for rain to form. Consequently values of $\delta^{2}H$ will decrease with increasing altitude (Bowen, 2010; Dansgaard, 1964).

Values of $\delta^{2}H$ at the equator are high and decreases with increasing latitude. This effect is due to more rainout and lower temperatures of condensation moving away from equator. Observations have shown that rain becomes more depleted in the heavy isotopes with heavy rainfall (Dansgaard, 1964). This is called the amount effect and is mostly pronounced in the tropics but has also been observed in Northern Europe for precipitation caused by thunderstorms (Dansgaard, 1964).

All of these effects lead to different distributions of $\delta^{2}H$ in precipitation around the world. The International Atomic and Energy Agency (IAEA) has developed a world map showing the predicted annual $\delta^{2}H$ in precipitation (Figure 10) via a Regionalized Cluster-based Water Isotope Prediction (RCWIP) model (IAEA/WMO 2014. Global Network of Isotopes in Precipitation., 2014; International Atomic Energy Agency, Vienna, 2014; Terzer, Wassenaar, Araguás-Araguás, & Aggarwal, 2013)

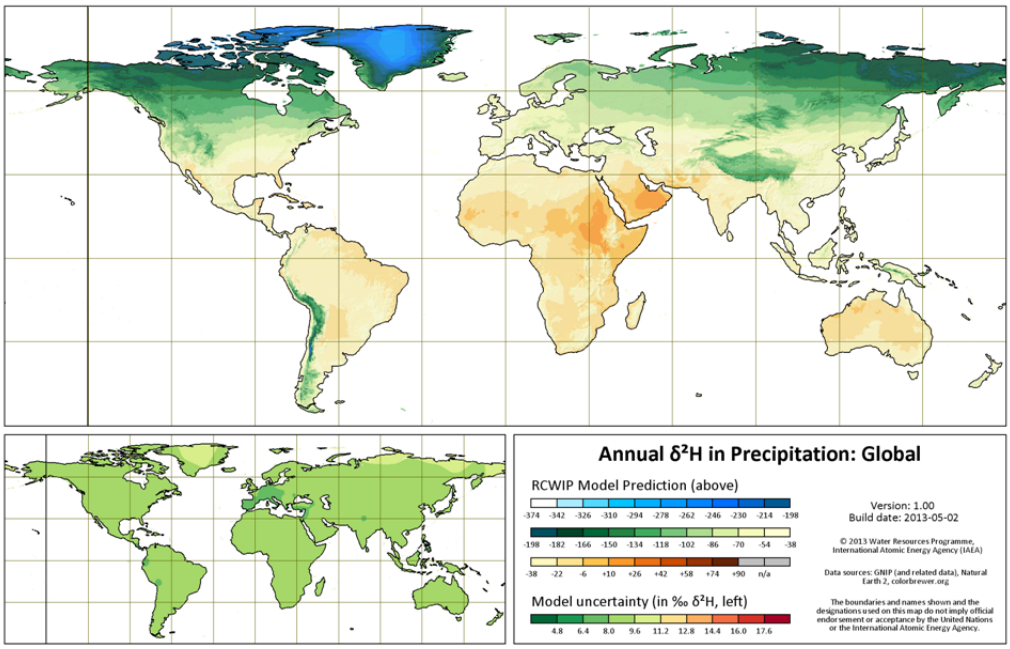


Figure 10: World distribution of δ^2H in precipitation. Developed by IAEA using the model of Regionalized Cluster-based Water Isotope Prediction

The ratio of stable hydrogen isotopes in compounds biosynthetsized by the plant, is reflected by the isotopic composition of the source water taken up by the plant and by diffusion/evaporation of water by the plant (Barbour, 2007). No isotopic fractionation of hydrogen stable isotopes are observed during the uptake of source water (Ehleringer & Dawson, 1992). Consequently, plants growing in different parts of the world will exihibit differences in ratios of stable hydrogen isoptopes. This isotopic composition would be difficult/impossible to estimate, which is why empirical data are needed.

3.1.2.3. Natural fractionation of carbon

Atmospheric CO_2 is a mixture of approximately 98.93% $^{12}CO_2$ and 1.07% $^{13}CO_2$ (Housecroft & Constable, 2006). Plants take up CO_2 from the air via photosynthesis and use it as building blocks for the formation of primary and secondary metabolites. However, plants discriminate against ^{13}C during photosynthesis so that plants are more depleted in the heavy carbon isotopes than atmospheric air (O'Leary, 1988).

For C3 plants the first step where discrimination takes place is during the uptake of external CO_2 by tiny pores, called stomata, in the plant. This process is reversible because there is some diffusion of CO_2 from the stomata back to the atmosphere. From the stomata CO_2 is dissolved in water and enters the outer layer of the mesophyll

cells where photosynthesis takes place. The fixation of CO_2 is catalyzed by the enzyme ribulose biphosphate carboxylase/oxygenase (Rubisco). This process is called the Calvin cycle and results in a formation of 3 carbon molecules which is why plants fixating CO_2 by this pathway is called C3 plants. Rubisco discriminates against ^{13}C resulting in the formation of molecules with a lower value of $\delta^{13}C$ than the surrounding atmosphere (Fry, 2008a; O'Leary, 1988).

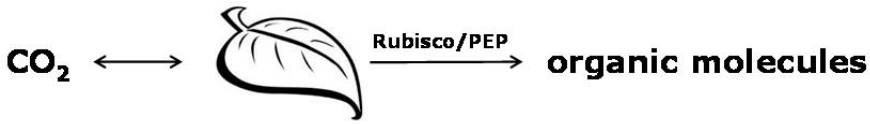


Figure 11: Illustration of plant uptake and fixation of CO_2

The degree of discrimination depends to a large extent on Rubisco, but may also be influenced by the diffusion of CO_2 from the plant. Plants will close their stomata when the temperature is high in order to prevent loss of water and vice versa (Hopkins & Hüner, 2004). If the plant stomata are open, the diffusion rate of CO_2 is increased compared to closed stomata. The higher the diffusion rate of CO_2 from the plant, the more the discrimination depends on diffusion, even though it is still the enzymatic discrimination that dominates. Consequently, the predicted values of $\delta^{13}C$ for C3 plants may vary between -33 and -24‰ (Fry, 2008a; Hoefs, 2009; O'Leary, 1988).

High diffusion of CO_2 from the stomata in C3 plants leads to deficiency of CO_2 causing Rubisco to use O_2 instead, also called photorespiration, which is unfavorable. This problem is overcome in C4 plants where external CO_2 firstly is converted into the 4 carbon molecule, oxaloacetate, by the enzyme phosphoenolpyruvate carboxylase (PEP), called the Hatch-Slack pathway. Oxaloacetate is then transferred from the mesophyll cells to the inner layer of the bundle sheath cells. Here, oxaloacetate is converted via Rubisco in the same way as for C3 plants. Diffusion from the mesophyll cells is larger than for the inner bundle sheath cell causing a higher diffusion of CO_2 for C3 plants than for C4 plants. This means that C4 plants are slightly more enriched in the heavy carbon isotope than C3 plants, leading to values of $\delta^{13}C$ between -16 and -10‰ (O'Leary, 1988).

A third type of plants uses the Crassulacean acid metabolism (CAM) for fixation of CO_2. CAM plants open stomata during night to absorb CO_2 which are converted and stored as C4 molecules by processes resembling the ones taking place in C4 plants. During the day stomata closes and the stored CO_2 are converted by Rubisco. CAM plants are able to open their stomata during the afternoon and directly convert CO_2 via the Calvin Cycle. Accordingly, CAM plants have different values of $\delta^{13}C$ during night and day leading to an average $\delta^{13}C$ value between -20 and -10‰ (O'Leary, 1988).

Consequently, measurements of stable carbon isotopes can be used to differentiate compounds originating from C3, C4, and CAM plants, respectively. The ranges of $\delta^{13}C$ for C3, C4, and CAM plants are summarized in Table 5.

Table 5: Values of $\delta^{13}C$ vs V-PDB for C3, C4, and CAM plants

C3 plants	CAM plants	C4 plants
-33 to -24‰	-20 to -10‰	-16 to -10‰

3.1.3. Gas Chromatography Isotope Ratio Mass Spectrometry

An Isotope Ratio Mass Spectrometer is capable of precise measurements of the natural abundance of stable isotopes. The high presicion of $\delta^{13}C$ measurements makes it able to detect changes of 1‰. Actually, the instrument is measuring the ratio between two isotopes, hence the name (Meier-Augenstein, 1999). For this project the IRMS are coupled to a Gas Chromatograph, making it suitable for volatile and semi volatile compounds, but other peripherals are available such as elemental analyzer and gas bench. Gas bench has not been used for this project and will not be described.

The structure of the instrument used for this study is shown in Figure 12. The main focus is on the IRMS coupled to a GC (thicker horizontal arrows). The Single Quadropole has been used as a secondary tool for the verification of the identity of compounds measured by the IRMS. The Elemental Analyzer has been used to compare results for known standards. This chapter describes Isotope Ratio Mass Spectrometry based on the setup shown in Figure 12.

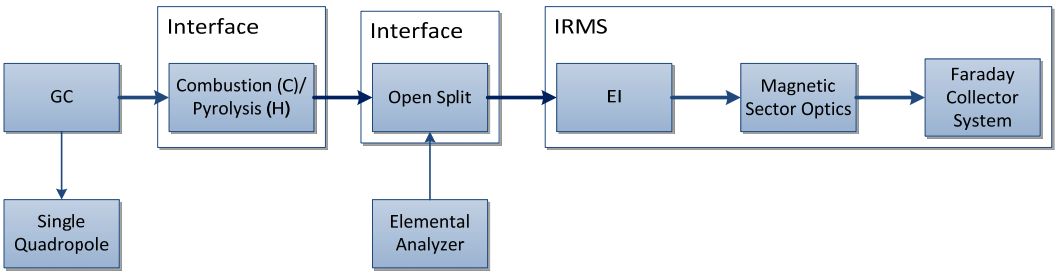


Figure 12: Schematic overview of the instrument used for this project

When using the instrument shown in Figure 12, analytes are separated by GC. Hereafter the flow is split so that 1/10 is led to a single quadropole and the rest of the flow is passed through interfaces and into the IRMS. The first interface consists of combustion and pyrolysis reactors where analytes are quantitatively converted to gasses: CO_2 for measurements of carbon stable isotopes and H_2 for measurements of hydrogen stable isotopes. Only one reactor is used at a time depending on which element is being analyzed. For analyzing carbon (and nitrogen) stable isotopes the analytes are combusted in a combustion reactor at 1000°C. The combustion reactor consists of a ceramic capillary containing copper and nickel wires. Any NO_x formed in the reactor will be reduced due to a surplus of Ni/NiO in the capillary reactor (Sessions, 2006). NO_x is unwanted because of its mass 46, the same as $C^{12}O^{18}O^{16}$. Since NO_x and $C^{12}O^{18}O^{16}$ are isobaric they would not be separated before detection leading to a measured signal intensity with contribution from both compounds (Sessions, 2006). The flow from the reactor is passed through a nafion membrane which has a counter flow of helium for the removal of H_2O (Thermo, 2012). Removing H_2O is crucial because H_2O could protonate CO_2 in the ion source which will lead to the formation of $^{12}CO_2H^+$ with m/z 45. Since this is also the mass of $^{13}C^{16}O_2$, the protonated CO_2 will interfere with the detection of m/z 45 and result in incorrect isotope ratio measurements (Groot, 2004).

For measurements of ratios of stable isotopes of hydrogen, the analytes eluting from the GC are converted to H_2 by pyrolysis in a High Temperature Conversion reactor (HTC reactor or pyrolysis reactor). This reactor consists of an empty ceramic tube where analytes are converted at a temperature of 1420°C. The hot zone of this reactor is limited, which is why the flow from the GC has to be lower than for the combustion reactor, so there is time for a quantitative conversion (van Leeuwen, Prenzler, Ryan, & Camin, 2014).

The gaseous analytes are then introduced into the ion source of the IRMS via a moveable open split. The open split ensures a constant gas flow rate to the IRMS (Sessions, 2006). The ion source is an Electron Ionization (EI) type and will not be explained in details this thesis. In the EI analytes are ionized by electrons with an energy of 70eV produced by a heated filament. The ionized analytes are drawn out of the ionization chamber and through a series of electrostatic lenses designed to focus and define the ion beam plus get rid of excess electrons. The high potential voltage difference between the ionization chamber and the ion exit slit of the source, results in acceleration of the ionized analytes of energies up to 3keV (Sessions, 2006).

The ion beam enters a magnetic sector field where it is deflected. The deflection can be described by the following equation: $\frac{m}{z} = \frac{r^2}{2U} \cdot H^2$ (equation 6). Here m/z is the mass to charge ratio, H is the magnetic field strength, U the accelerating voltage and, r the nominal radius of the ion path which is constant. The magnetic field strength and the accelerating voltage of the positive ions can be varied in order to analyze for different species (Groot, 2004; Thermo, 2012).

The magnetic sector field is generated by an electromagnet which has a maximum field strength of 0.75T corresponding to a mass range of up to 80 amu at 3keV. The ionized analytes are deflected so that each mass hits into a specific faraday cup. The applied magnetic field is kept constant for a specific element, while the accelerating voltage is adjusted resulting in a majority of the ion beam hitting the cups. This is called peak centering and is performed before each analysis in order to optimize the signal.
The collector system used for this project consists of a total of five faraday cups (Figure 13). For ratio measurements of stable carbon isotopes three faraday cups are used for measuring the isotopologous of CO_2, see Table 6 (Groot, 2004; Thermo, 2012).

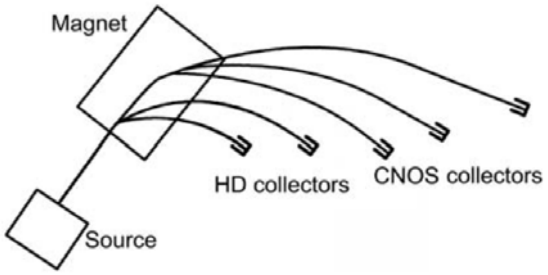


Figure 13: Schematic overview of IRMS. The gaseous analytes are ionized in the ion source and accelerated into a magnetic field. The accelerating voltage and the strength of the magnetic field are optimized for each element. The small mass difference of the ions determines the reflection path into the collection system consisting of faraday cups (Fry, 2008c)**.**

These faraday cups can also be used for ratio measurements of other elements i.e. nitrogen and sulphur. On each side of the three cups, two cups are placed for measurements of mass 2 and 3. When an ionized analyte hits a faraday cup the charge of the ion is passed to the faraday cup. The current obtained by the charge of the ion is amplified and monitored (Groot, 2004; Sessions, 2006). The current of two or three ions are measured simultaneously in order to obtain a more stable output. The reason for this is that a current from one ion can vary while the difference between the currents from two ions are constant. Furthermore the contribution from ^{17}O has to be considered when determining the $^{13}C/^{12}C$. As seen in Table 6 the cup measuring isotopologous of mass 45 has contributions from both ^{13}C and ^{17}O. The software corrects for the ^{17}O contribution using an algorithm based on measurements of ^{18}O (Carter & Barwick, 2011; Groot, 2004). For this thesis the Santrock, Studley, and Hayes (SSH) algorithm has been used (Santrock, Studley, & Hayes, 1985).

Table 6: The most abundant isotopologous of CO_2

$^{12}C^{18}O^{16}O$	46
$^{13}C^{16}O^{16}O$	45
$^{12}C^{17}O^{16}O$	45
$^{12}C^{16}O^{16}O$	44

Corrections of the measurements are also necessary for determination of hydrogen stable isotopes. In the ion source H_3^+ is formed due to the following ion-molecule reaction: $H_2^+ + H_2 \rightarrow H_3^+ + H$ (equation 7).

H_3^+ is isobaric with DH^+ and will contribute to the ion current measured at m/z 3 and therefore needs to be corrected for. The contribution of H_3^+ to the m/z 3 signal is determined as the H_3 factor by the following:

$$\frac{I(3)}{I(2)} = \frac{DH^+ + H_3^+}{H_2^+} = \frac{DH^+}{H_2^+} + \frac{k(H_2^+)^2}{H_2^+} => \frac{DH^+}{H_2^+} = \frac{I(3)}{I(2)} - k \cdot I(2) \quad \text{(equation 8)}.$$

In equation 8 I(2) is the measured ion current on m/z 2, I(3) is the measured ion current at m/z 3 and k is the H_3 factor. The H_3 factor is determined by introducing a series of reference gas peaks with varying amplitude but with identical isotopic composition. The H_3 factor is the factor which minimizes the differences in the measured isotopic composition of the reference peaks (Sessions, 2006; Thermo, 2012).

3.1.1 Elemental Analyzer

The elemental analyzer is peripheral for IRMS which can be used to introduce bulk samples of solid organic and inorganic material as an alternative to the GC described in the previous section. Furthermore, the elemental analyzer can be used for introduction of reference compounds and thereby analyzing the same pure standard on both GC-IRMS and EA-IRMS.

In the elemental analyzer a sample is converted to simple gasses that can be analyzed by IRMS, just like as in the reactors following the GC. The measured isotopic ratio is therefore a result of the bulk isotopic ratio for all compounds present in the sample. In EA-IRMS two techniques are used depending on which element is analyzed. High temperature conversion (TC-EA) is used for analysis of hydrogen and oxygen and combustion EA for analysis of carbon, nitrogen, and sulphur.

For TC-EA the sample is weighed in a silver capsule which is then dropped into the reaction tube. The tube consists of an outer tube of alumina and an inner tube of glassy carbon filled with glassy carbon particles. Here the sample is converted to H_2 and CO gasses at temperatures above 1400°C. The formed gasses are then isothermally separated on a GC column and enter the IRMS via the open split. The ionization of gasses, separation, and detection of isotopes in the IRMS is the same as described in the previous section (Benson, 2012; Carter & Barwick, 2011; Thermo, 2015b).

For analysis of C, N, and S the flash combustion EA is used. The samples are weighed in tin capsules and then dropped into the reaction tube. Here a pulse of pure O_2 is introduced in order to combust the sample material and convert it into simple gasses. The temperature in the reactor tube reaches 1800°C because of an exothermic reaction with the tin capsule. Excess oxygen is removed and NO_x reduced to N_2 by Cu. H_2O is removed and the gaseous analytes separated on a GC column before reaching the IRMS (Benson, 2012; Carter & Barwick, 2011; Thermo, 2015a).

3.2 Description of existing research within Compound Specific Isotope Analysis

3.2.1 Authentication of naturalness

Isotope Ratio Mass Spectrometry has been used especially for detecting addition of sugar to wine and fruit juices. Sugar is added to obtain a sweeter tasting product or in the case of wine, a higher content of alcohol (chaptalization). If the sugar added originates from a C4 plant i.e. corn syrup from maize, it would affect the values of $\delta^{13}C$ for glucose/ethanol. Wine and fruit juices are made from C3 plants which in general have lower values of $\delta^{13}C$ than C4 plants. Addition of corn syrup to wine/juice would therefore increase the measured values of $\delta^{13}C$ (Meier-Augenstein, 1999; Andreas Rossmann, 2001). Also measurements of $\delta^{18}O$ were used to differentiate freshly pressed juices and juices diluted with tap water. $\delta^{18}O$ of water from authentic juices are higher than $\delta^{18}O$ of tap water. However, the values of $\delta^{18}O$ vary depending on geographic origin, weather conditions, and season. Hence a large dataset of reference samples are needed for verification of authenticity (Andreas Rossmann, 2001).

CSIA have the advantage that it is able to measure the isotopic ratios for several compounds in a sample. This can give a unique fingerprint for the specific aroma and is a valuable tool for authenticity control. Braunsdorff et al measured $\delta^{13}C$ values of 9 compounds in authentic lemon oils. Here, one of the compounds were chosen as an internal isotopic standard. This standard is set to the same value for all authentic samples and the values of $\delta^{13}C$ for the other components are then correlated based on the internal isotopic standard. By using the internal isotopic standard the fingerprint becomes unique for this type of plant and is only dependent on the enzymatic reactions during formation of secondary metabolites. The fingerprint obtained can then be used for authenticating lemon oils (Braunsdorf et al., 1993).

Bonaccorsi et al conducted a similar study on lime oils. They measured values of $\delta^{13}C$ for 9 aroma compounds from two types of lime oils. An internal isotopic standard was used to correlate the $\delta^{13}C$ values found for the two lime types and found them to be within the same range except for two compounds. This range together with enantiomeric purities could then be used for authenticating lime oils of unknown origin (Bonaccorsi et al., 2012). CSIA have also been reported used for coriander oil (Frank, Dietrich, Kremer, & Mosandl, 1995), lavender oil (Hanneguelle, Thibault, Naulet, & Martin, 1992), black Ceylon, Assam, and Darjeeling tea (Weinert, Ulrich, & Mosandl, 1999). CSIA have previously mostly been used for the above described types of products.

3.2.2 Authentication of geographic origin

In recent years CSIA has found use in tracing the geographical origin of aroma and food products. As part of the Food traceability project "TRACE" Schellenberg et al measured stable isotope ratios of carbon, hydrogen, sulphur, and nitrogen for honey originating from 20 different regions around Europe. They found that the hydrogen isotopes of honey protein were determined by the groundwater in the geographical region from where the honey originated. Also the sulphur isotopes were correlated to the region, while carbon isotopes were affected by the climate. Together the four elements constitute a strong correlation to the geographic origin and can be used for traceability studies (Schellenberg et al., 2010).

Multielement CSIA has been used for authenticating (tracing) a number of products i.e. lavender oil (Jung, Sewenig, Hener, & Mosandl, 2005), clementines (Benabdelkamel et al., 2012), glycerol in wine (A Rossmann, Schmidt, Hermann, & Ristow, 1998), and blueberries (Camin, Perini, Bontempo, & Giongo, 2009).

Stable isotope ratios of hydrogen are good indicators of geographical origin because they are strongly correlated to meteoric water. Hydrogen also has the advantage that it is more widespread than sulphur and easier to measure. The isotopic ratios of oxygen is correlated to the ratios of hydrogen and can therefor also be used as a tracer for geographic origin (Dansgaard, 1964; Krivachy (Tanz), Rossmann, & Schmidt, 2014; Terzer et al., 2013).

3.2.3 Analysis of isotopic ratios for authenticating vanilla aroma

Vanilla is a valuable aroma and consequently susceptible to fraud. Measurements of stable isotope ratios have therefore been investigated for authentication of vanilla aroma. In the early days of IRMS it was necessary to extract and isolate vanillin from vanilla beans before isotopic analysis. Bricout et al. measured $\delta^{13}C$ and D/H for vanillin from *V. planifolia* and synthetic vanillin and found the isotopic values of the two types of vanillin to differ (Bricout, Fontes, & Merlivat, 1974).

In 1979 Hoffmann and Salb also isolated vanillin from vanilla extracts and measured values of $\delta^{13}C$ using EA-IRMS. The isolation of vanillin was obtained by extraction with ether followed by preparative gas chromatography. This is a laborious extraction method and care has to be taken using preparative GC, because the heavier isotopes will elute immediately before the lighter isotopes. This was considered in their study and they found that vanillin with a $\delta^{13}C$ value lower than -21.0 ‰ would originate from another source than vanilla beans (Hoffmann & Salb, 1979). Lamprecht et al. used preparative HPLC for isolation of vanillin and measured $\delta^{13}C$ of vanillin extracted from vanilla pods and synthetic vanillin (Günther Lamprecht, Pichlmayer, & Schmid, 1994).

Authenticity based on measurements of $\delta^{13}C$ could be circumvented by synthetic vanillin enriched in ^{13}C. Krueger and Krueger therefore developed a method were $\delta^{13}C$ are measured only for specific groups of the vanillin molecule. By converting the methyl group to CH_3I, the values of $\delta^{13}C$ for this specific group could be determined (Krueger & Krueger, 1983). Likewise vanillin was oxidized to vanillic acid and decarboxylated in order to measure values of $\delta^{13}C$ for the carbonyl group of the vanillin molecule (Krueger & Krueger, 1985).

Compound specific isotope analysis was greatly facilitated after the online coupling of GC and IRMS via a combustion/pyrolysis interface. This setup has reduced sample preparation since most of the compounds in a sample could be separated directly at the GC and the isotopic ratio measured immediately. Mosandl et al. used this method for analysis of $\delta^{13}C$ values for vanillin and minor compounds present in vanilla extracts in order to obtain an authenticity range. The compounds analyzed were vanillin, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, anisic acid, and anisic alcohol. They found that the concentration of the components in vanilla extract could vary from year

to year, but the values of $\delta^{13}C$ remained more or less constant. Furthermore, their studies showed that the $\delta^{13}C$ values of vanillin differed for different species of vanilla, while values of $\delta^{13}C$ for 4-hydroxybenzaldehyde were more alike (Kaunzinger, Juchelka, & Mosandl, 1997; Scharrer & Mosandl, 2002). A similar study on Indian vanilla beans was carried out by John and Jamin. They measured $\delta^{13}C$ for vanillin, 4-hydrobenzaldehyde, vanillic acid, and 4-hydrobenzic acid and found values to be in accordance with previous findings (John & Jamin, 2004).

Authenticity of vanillin produced by biotechnology (fermentation of natural precursors such as eugenol or ferulic acid) constitutes another analytical challenge. Bensaid et al. degraded vanillin into guaiacol and measured $\delta^{13}C$ and $\delta^{18}O$ for guaiacol for authenticity purposes (Figure 14). By this method the exchange of oxygen in the formyl group becomes insignificant (Bensaid, Wietzerbin, & Martin, 2002).

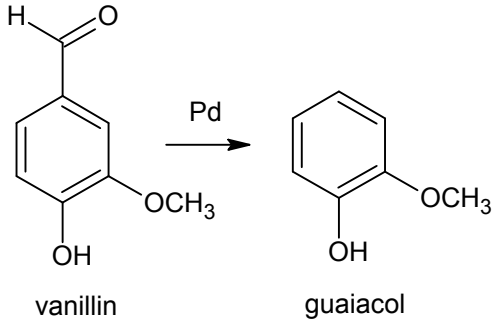


Figure 14: Degradation of vanillin to guaiacol

Greule et al. measured values of $\delta^{13}C$ and $\delta^{2}H$ for both vanillin and the methoxyl group of the vanillin molecule. The latter was done by using the “Zeisel method” where the methoxyl group are transformed to methyl iodide and then analysed by IRMS. The samples analyzed were synthetic vanillin, semisynthetic vanillin, vanillin extracted from vanilla pods of different species, and vanilla of unknown origins. They found that values of $\delta^{13}C$ and $\delta^{2}H$ for vanillin alone was sufficient to distinguish between vanillin from vanilla pods and vanillin produced otherwise. Additionally, they concluded that values of $\delta^{13}C$ and $\delta^{2}H$ for the methoxyl group of vanillin would be a useful tool for authentication of vanillin that have been enriched in ^{13}C or ^{2}H (Greule et al., 2010).

In general the values of $\delta^{13}C$ measured in different studies for the different types of vanillin are in good agreement. However, not much attention has been on the use of $\delta^{2}H$ or the utilization of a combination of $\delta^{13}C$ and $\delta^{2}H$ for authenticity and traceability

studies. In 1998 Hener et al. measured $\delta^{13}C$ and $\delta^{18}O$ for vanilla from Comores, Mexico, Tahiti, and for vanillin produced by chemical synthesis and by biotechnology. However, the focus of their study was the successful simultaneous analysis of $\delta^{13}C$ and $\delta^{18}O$ by a pyrolysis interface and not traceability. They found one sample of vanilla from Tahiti to have values of $\delta^{18}O$ close to one vanilla sample from the Comores. A sample of Mexican vanilla differentiated from the other two by almost -2‰ (Hener et al., 1998).

3.3 Paper 2 – Authenticity and traceability of vanilla flavors by analysis of stable isotopes of carbon and hydrogen

The aim of the second part of this project was to develop IRMS methods able to authenticate aroma compounds extracted from food products. But before doing so, it was necessary to establish an analytical platform based on measurements of stable isotope ratios of carbon and hydrogen for aromas of known authenticity. Chapter 3.3.1 focuses on the preliminary precautions important for correct stable isotope ratio measurements of vanilla aroma. Next, a dataset was established consisting of measurements of ratios of stable isotopes of carbon and hydrogen for vanilla flavours of known authenticity. These results are presented in chapter 3.3.2 and in Paper 2 (appendix 2).

The IRMS used for analysis was a Thermo Delta V Advantage coupled to a GC Trace and a Single Quadropole (ISQ). The interface between the GC and IRMS is called GC IsoLink and consists of a combustion reactor and a high temperature reactor (HTC). The interface between all peripherals and the IRMS was a ConFlo IV. A schematic overview of the instrument is shown in Figure 12, chapter 3.1.3.

3.3.1 Preliminary studies

3.3.1.1. Standards

All measurements of stable isotope ratios are given as deviations from an international predetermined standard material – also called a primary standard, see Table 7. Stable isotope ratios of carbon are expressed relatively to the primary standard Pee Dee Belemnite (PDB), while stable isotope ratios of hydrogen are expressed relatively to the primary standard Standard Mean Ocean Water (SMOW). Both of these materials are now exhausted and supplementary primary standards have been made. To make sure

that the new primary standards are not exhausted, secondary standards have been developed, see Table 7.

Standards are used for all measurements of stable isotope ratios in order to express results relative to the international determined standard. During each isotope analysis, pulses of CO_2/H_2 from an internal laboratory tank are introduced[3]. The ISODAT software uses the delta value of this reference CO_2/ H_2 gas to calculate delta values for the following measurements of analytes. The reference gas pulses also serves as an indicator of stability in the sense that large deviations in delta values of the reference pulses indicate stability problems. This reference gas cannot directly be used for calibrating the measurements according to the internationally accepted scale, which is why standards of known isotopic compositions need to be analyzed accordingly.

Many of the primary and secondary standards available are not suitable for GC analysis. Instead, the internal laboratory reference CO_2/ H_2 gas can be assigned with a delta value based on Elemental Analyzer-IRMS measurements of a secondary standard. Future measurements can then be calibrated against the CO_2/ H_2 reference gas. This would mean that the delta value calculated by the software would be the correct value correlated to the V-PDB/V-SMOW scale. However, experiments have shown that the same standard analyzed on EA-IRMS and GC-IRMS deviated more than 1‰ (study performed by the author but not published). An IAEA caffeine standard was found to be more depleted in the heavy isotope when analyzed on EA-IRMS compared to GC-IRMS. The described measurements were done at the same instrument using the same CO_2/ H_2 as reference gas, and are therefore comparable. These results indicate that it would be wrong to assign the internal laboratory reference CO_2/ H_2 gas with a value based on measurements of EA-IRMS and to use this reference gas for calibrating results found by GC-IRMS. Instead, reference standards must be analyzed on GC-IRMS along with the samples.
Another reason for not assigning the internal laboratory reference CO_2/ H_2 gas a delta value based on EA-IRMS measurements is that the isotopic composition of the reference gas can change over time (i.e. reduced tank volume or change of tank) which will lead to incorrect calculations of sample delta values.

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[3] Pulses of CO_2 are used for measurements of carbon stable isotopes ratios, while H_2 are used for measurements of hydrogen stable isotope ratios.

Instead, it was decided to analyze tertiary standards in each sequence. A mixture of three alkanes (see also Table 7) with known delta values correlated to the V-PDB scale was used as tertiary standards for measurements of carbon stable isotope ratios. The delta values of the three alkanes had a range covering the expected delta values of the measured aroma compounds. For measurements of hydrogen stable isotope ratios two caffeine samples (see Table 7) with defined and different values of δ^2H were used as tertiary standards.

The selection of in-house standards was chosen based on the following:

- Should be able to analyze on GC.
- Isotopically homogenous (Carter & Barwick, 2011). This was assured by grinding a portion of the solid standard material in a mortar before dissolution. A large batch was made and distributed into small vials/glass ampoules which were sealed and stored at -18°C. Storing the in-house standard in small portions could be critical due to the fact that the risk of evaporation is larger for small samples than for larger samples. However, it was judged that storage at low temperatures would significantly decrease evaporation. On the contrary, small portions are advantageous at a daily basis where only one portion of the in-house standard is needed. If the standard was stored in larger portions, it would be necessary to thaw all of it in order to take out a smaller portion for analysis. Thawing and exposure to surroundings each time an aliquot was needed would increase the risk of evaporation. Delta values have to be measured for each new batch produced.
- The standard should be stable and not change isotopic composition over time (Carter & Barwick, 2011).
- The delta values of the in-house standards should be in the range of expected delta values for the samples measured and preferably cover a range in where the sample delta values are found (Carter & Barwick, 2011).
- For measurements of hydrogen, it is important that the in-house standards does not contain exchangeable hydrogens (Carter & Barwick, 2011).

In-house standards were analyzed regularly. The in-house standards were meant to eventually replace the more expensive tertiary standards in the daily isotopic measurements.

	Primary	Secondary	Tertiary	Quaternary (in-house)
$\delta^{13}C$	V-PDB NBS-19	NBS-22 (oil) USGS-24 (graphite) IAEA-CH-7 (PET) IAEA-600 (caffeine)	Alkanemix: C_{11},C_{15},C_{20}[(a)]: C_{11}: $\delta^{13}C$ = -26.11‰ C_{15}: $\delta^{13}C$ = -30.102‰ C_{20}: $\delta^{13}C$ = -33.06‰	Caffeine (Sigma) γ-decalactone (Sigma) γ-octalactone (Sigma)
$\delta^{2}H$	V-SMOW V-SMOW2	NBS-22 (oil) IAEA-CH-7 (PET)	Caffeine #1[(b)]: $\delta^{2}H$ = 91.4‰ Caffeine #2[(b)]: $\delta^{2}H$ = -152.8‰	Caffeine (Sigma) γ-decalactone (Sigma) γ-octalactone (Sigma)

Table 7: Four types of standard reference materials. [(a)]characterized by Chiron, Trondheim, Norway, [(b)]characterized by A. Schimmelmann, Inidana University, Bloomington, USA

3.2.1.2. Isotopic fractionation during GC-injection

A critical parameter in measurements of stable isotope ratios is fractionation. Fractionation should as far as possible be avoided because this would result in incorrect delta values. Fractionation during split and splitless injections has been investigated during preliminary studies for Paper 2 and presented as Poster 1. See materials and methods in appendix 1 and Poster in appendix 2.

About 30ng of hydrogen on column are required when measuring stable isotope ratios of hydrogen compared to only 10ng of carbon for measurements of carbon stable isotope ratios (Thermo, 2012). The high amount of hydrogen required is due to the low natural abundance of deuterium and low ionization efficiency of H_2. Extracts of vanilla pods contains about 40ng/µL of hydrogen from vanillin which will overload a DB-5ms column with a film thickness of 0.25µm when injecting 1µL using splitless injections. Overloading of the column appears as peaks which gradually increase to a maximum and then incline steeply to baseline, also called “shark-finned” peaks. To overcome this problem, injection with a split ratio of 1:20 was performed. This resulted in better chromatography with more “Gaussian-shaped” peaks but the amount of hydrogen on the column was now in the low range for precise δD measurements, see Figure 15.

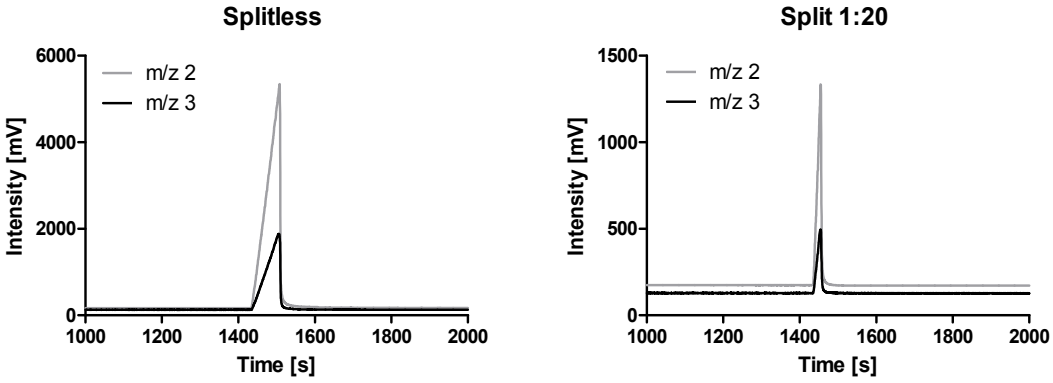

Figure 15: m/z 2 and 3 for standards of vanillin analyzed using GC-P-IRMS with splitless injection (left) and split injection 1:20 (right).

Standards of vanillin with concentrations ranging from 40 to 3000ng/µL of hydrogen were analyzed using both splitless and split injections, however the δ^2H values were not standardized against V-SMOW because this would require that standards were analyzed with the exact same conditions as the samples (Figure 16).

Figure 16 shows that low concentrations of analytes (hydrogen on column) result in lower values of δ^2H corresponding to a depletion of deuterium compared to the true δ^2H value. This amount dependency is valid for both injection techniques; however, the decline in delta values starts at higher concentrations when using split injections with a ratio of 1:20. For split injections the decline seems to start around 150ng hydrogen on column and is evident around 100ng H on column. For splitless injections the decline starts at concentrations around 40ng H on column.

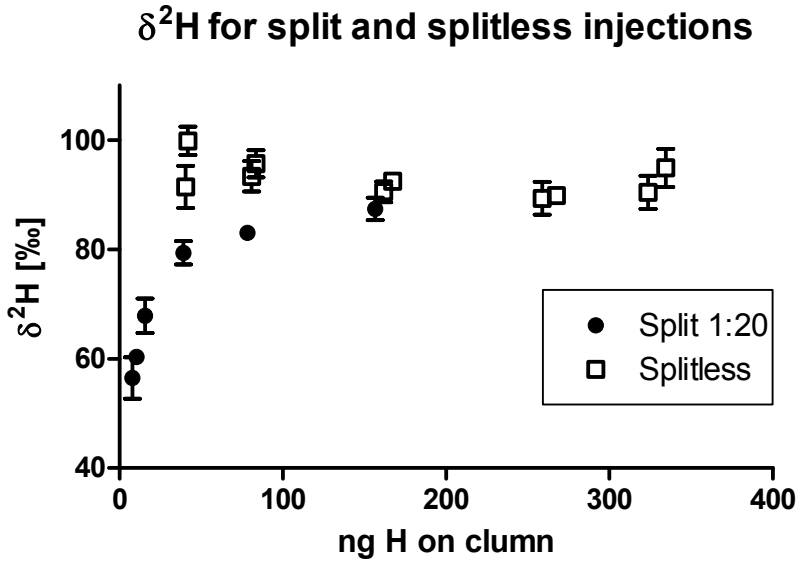

Figure 16: GC-P-IRMS measurements of different concentrations of a vanillin standard solution using split 1:20 and splitless injection

Vanilla extracts have concentrations of vanillin corresponding to approximate 40ng hydrogen per µL. This means that measurements of δ^2H for vanillin using split injections would lead to a too low δ^2H value unless this deviation is corrected. The loss of deuterium during split injections must be due to the sample lost through the split valve. A possible explanation is that the lighter isotopes will evaporate before the heavy isotopes and therefore a larger portion of the lighter isotope will reach the column, while the rest of the sample carried away in the split will be slightly enriched in the heavy isotope. It must be assumed that this effect is more pronounced for semi volatiles, like vanillin, than for more volatile analytes.

Schmitt et al. 2003 found that the length of the injecting needle affected the amount dependency of stable carbon isotope ratio measurements (Schmitt, Glaser, & Zech, 2003). An injection close to the end of the liner will make sure that the sample vapor reaches the column rapid and therefore has less time for fractionation. The needle used for this experiment had a length of 8mm, which is longer than normally used, and the amount dependency can therefore not be optimized by increasing the needle length. When using splitless injections at concentrations above 16ng of hydrogen on column, the δ^2H standard deviations for the vanillin standards measured were lower than 4‰ and very close to the expected precision of the instrument at 3‰.

Studies have shown that measurements of carbon stable isotope ratios are also amount dependent. A decrease in carbon concentrations on column leads to higher measured values of $\delta^{13}C$, contrary to what was seen for $\delta^{2}H$. These measurements are in accordance to the experiments made by Schmitt et al. 2003.

For carbon the problem with amount dependency is less pronounced when analyzing vanilla extracts. About 63% of the weight of the vanillin molecule is composed of carbon, contrary to only 2.5% for hydrogen. Furthermore, the amount of carbon needed on column is only 10ng. Accordingly, extracts of vanilla pods contain sufficient amounts of vanillin for carbon isotopic measurements.

Figure 16 shows that the measured delta values of hydrogen decreases when the concentration of hydrogen on column is low and that this effect is most pronounced when using split injections of 1:20. For vanilla extracts with concentrations above 40ng hydrogen per µL splitless injections give the most correct results.

3.3.2 Paper 2 – Aroma profile of vanilla pods of known authenticity

Natural vanilla extracts contain lots of different compounds contributing to the characteristic vanilla flavour, whereof vanillin is the most abundant. The presence of other compounds found in natural vanilla extracts can be used for authentication of an unknown vanilla aroma. A synthetic made vanilla flavour can be made solely of vanillin, which would result in a simpler aroma profile than for natural vanilla. Furthermore, aroma profiles can be used for the determination of the vanilla specie. To illustrate the different aroma profiles, pods of *V. planifolia* and *V. tahitensis* were extracted as described in Paper 1 and analyzed using a Gas Chromatograph-Quadropole-Time of Flight-Mass Spectrometer. Examples of the obtained chromatograms are shown in Figure 17.

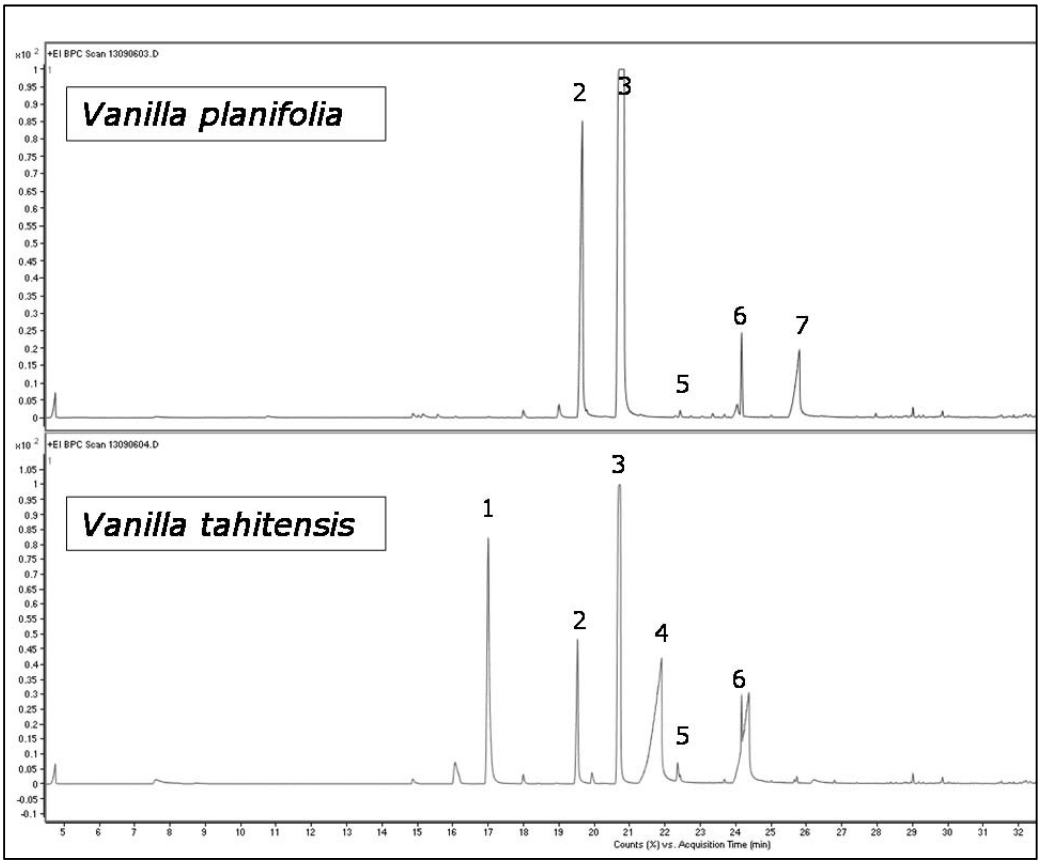

Figure 17: Chromatograms of extracts of *Vanilla planifolia* and *Vanilla tahitensis*. 1=anis alcohol, 2=4-hydroxybenzaldehyde, 3=vanillin, 4=*p*-anisic acid, 5=vanillyl akcohol, 6=4-hydroxy benzoic acid, 7=vanillic acid

As seen from Figure 17 *V. planifolia* contains higher amounts of vanillin and 4-hydroxybenzaldehyde than *V. tahitensis*. *V. tahitensis* contains anis alcohol and *p*-anisic acid, which were not found in *V. planifolia*. Consequently, the different aroma profile could be used for identification of species for an unknown vanilla flavour (not the scope of this thesis). However, the concentration of these compounds in a food matrix is often very low and it is more likely that vanillin and 4-hydroxybenzaldehyde is the only compounds found for extracts of *V. planifolia*, while anis alcohol could be an indicator for extracts from *V. tahitensis*.

3.3.3 Paper 2 – CSIA of vanilla pods of known authenticity

Vanilla is a popular aroma and used in a wide range of products such as bakery, ice cream, chocolate, beverages, and perfumes. The main compound contributing to the vanilla aroma is vanillin (Figure 18). The annual demand for vanillin is about 12.000 tons of which only 20 tons originates from pods of the vanilla orchid (Rao &

Ravishankar, 2000). Synthetic vanillin accounts for the majority of the vanillin consumed and only a minor part is made by microbiological processes. The price of natural vanilla is high due to troublesome cultivation and preprocessing of the vanilla pods. The price of natural vanillin is between $1200 and $4000 per kg. while the price of synthetic vanillin was $11-15 pr. kg (Korthou & Verpoorte, 2007). The price difference between natural and synthetic vanillin makes fraudulent business appealing.

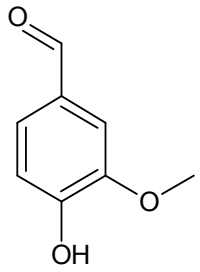

Figure 18: 4-hydroxy-3-methoxybenzaldehyde (vanillin)

In Paper 2 $\delta^{13}C$ and $\delta^{2}H$ were measured for vanilla extracts of known authenticity. The aim of this study was to investigate if there were any similarities in stable isotopic ratios for similar parameters, such as production method, origin, and geographical origin. The large dataset obtained in Paper 2 would afterwards function as a reference dataset used for evaluating vanilla aroma of unknown authenticity.

According to European legislation it is permitted to label an aroma as “Natural” when it is obtained by appropriate physical, enzymatic or microbiological processes from materials of vegetable, animal or microbiological origin (European Parliament, 2008). Biovanillin is obtained by the fermentation of natural precursors e.g. eugenol and can therefore be labelled as a “natural” aroma exactly as vanillin extracted from vanilla pods. To distinguish the two and emphasize that a vanilla aroma originates from natural vanilla pods, the aroma can be labelled with the vanilla specie or geographic origin. This information is, however, very difficult to verify and easy to falsify. This is why methods able to authenticate vanilla aroma are needed.

The samples analyzed in Paper 2 consisted of synthetic vanillin (n=2), biovanillin (n=1) and vanillin extracted from natural vanilla pods (n=79). The vanilla pods used were of the type *V. planifolia* (n=54) and *V. tahitensis* (n=15) (10 of the vanilla pods were of unknown species).The geographic origins were known for 71 of the vanilla pods.

Values of $\delta^{13}C$ vs V-PDB for vanillin extracted from vanilla pods were expected to be between -20 and -10‰, since the vanilla orchid is a CAM plant (see chapter 3.1.2). The biovanillin analyzed was made from natural eugenol. The specific origin of the eugenol used for biovanillin was unknown, but natural eugenol is traditionally derived from clove oil. Clove is a C3 plant with values of $\delta^{13}C$ between -33 and -24‰. It is assumed that the $\delta^{13}C$ value of eugenol is largely maintained during fermentation, since most of the carbon skeleton is retained and no extra carbon atoms are inserted. There are of course many unknowns during the fermentation process, but if all of the substrate is converted to vanillin the possibility of fractionation decreases. Consequently, biovanillin ex. eugenol is expected to have values of $\delta^{13}C$ between -33 and -24‰.

Result of $\delta^{13}C$ for synthetic vanillin was -29.6 $\delta \pm$ 3.0‰, while biovanillin was found to -30.9‰. Mean values of $\delta^{13}C$ for *V.planifolia* was -20 ± 1.2 ‰ and -16.6 ± 1.1 ‰ for *V. tahitensis*, see Figure 19. Figure 19 shows that values of $\delta^{13}C$ for natural and biovanillin falls within the expected intervals. Consequently, vanillin from vanilla pods can be differentiated from biovanillin based on values of $\delta^{13}C$. However, more samples of biovanillin are desirable and would highly strengthen this conclusion.

$\delta^{13}C$ values of synthetic vanillin were close to the values of biovanillin, accordingly measurements of $\delta^{13}C$ cannot be used to differentiate between the two. In contrast, values of $\delta^{13}C$ proved to differ significantly[4] for vanillin extracted from *V. planifolia* and vanillin extracted from *V. tahitensis*.

[4] t-test; $p < 0.001$

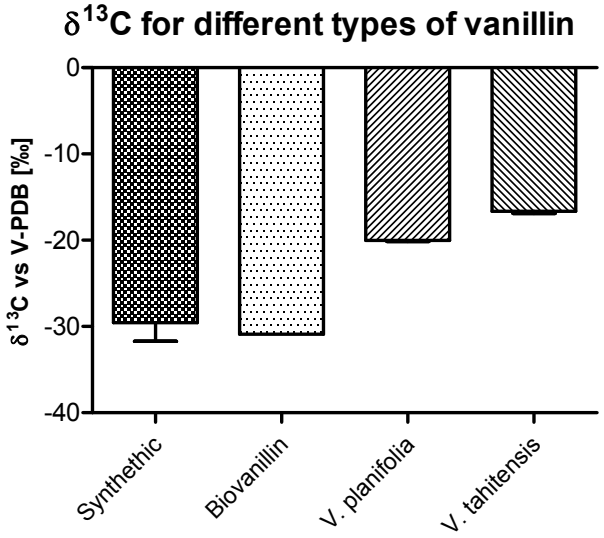

Figure 19: $\delta^{13}C$ vs V-PDB for vanillin of known authenticity: obtained from chemical synthesis (n=2), fermentation of eugenol (n=1), and vanilla pods of the type *V. planifolia* (n=54) and *V. tahitensis* (n=15).

Brand et al. 1998 and Scharrer et al. 2002 measured $\delta^{13}C$ values of vanillin made by fermentations and both found them to be -37‰ vs V-PDB. However, the precursor for the fermentation was not given. Bensaid et al. 1998 found values of $\delta^{13}C$ for biovanillin ex. ferulic acid to be -36.1‰ vs. V-PDB. The ferulic acid originated from rice, which is a C3 plant and therefore compounds derived from rice would be expected to have values of $\delta^{13}C$ between -33 and -24‰. Even though eugenol and ferulic acid are both obtained from C3 plants, a large difference in carbon isotopic composition would not be surprising. Even within the same plant genus the isotopic composition can vary, as seen for *V. planifolia* and *V. tahitensis.* Unfortunately, the isotopic value of biovanillin ex. eugenol makes it impossible to differentiate it from synthetic vanillin using the described method. However, the method is assumed to be able to distinguish biovanillin ex. ferulic acid from synthetic vanillin. It was not possible to provide biovanillin ex. ferulic acid, so this has not been tested.

Furthermore, sophisticated methods have now made it possible to enrich the methoxy and aldehyde group (Figure 18) of vanillin in the heavy carbon isotope (Remaud, Martin, Martin, & Martin, 1997). This makes it possible to increase values of $\delta^{13}C$ for synthetic vanillin. Since IRMS is measuring the isotope ratio of the bulk vanillin molecule, such enrichment would not be detected by IRMS. In that case compound

specific isotope analysis of other compounds in the vanilla flavour could be useful or another method should be considered i.e. Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance (SNIF-NMR). SNIF-NMR measures the isotopic ratio for each carbon atom in the vanillin molecule.

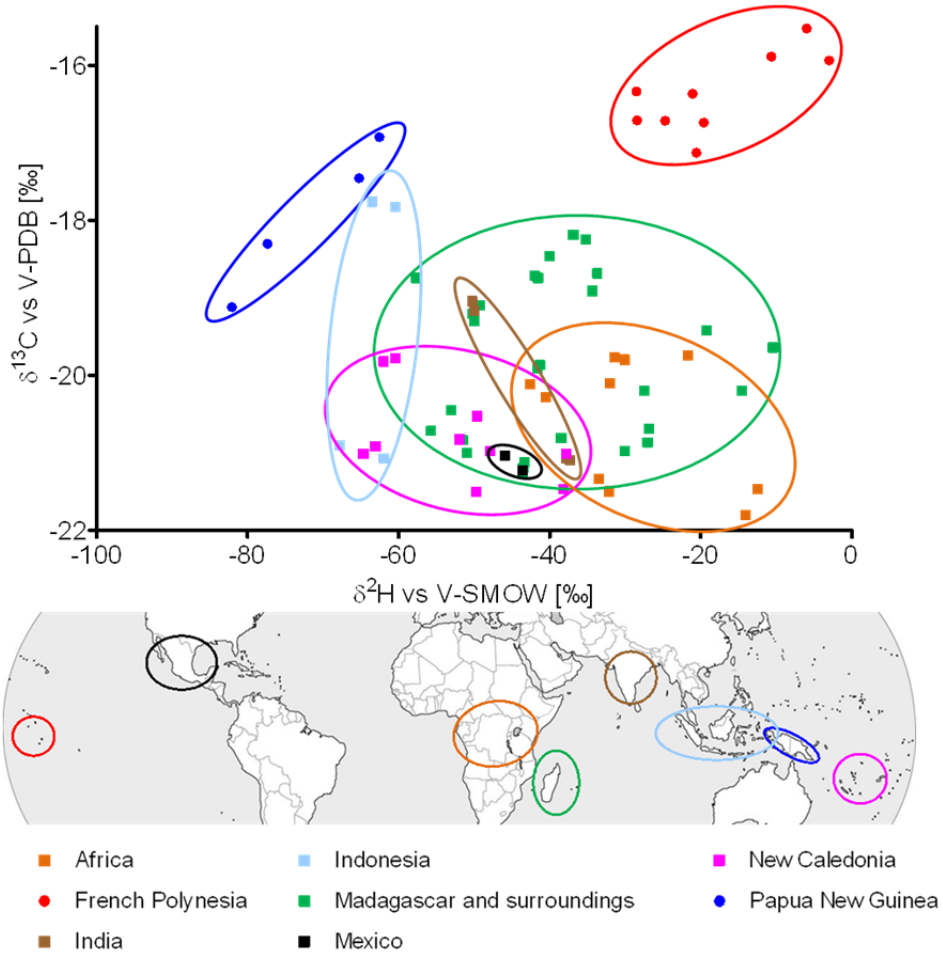

Figure 20: Scatterplot of δ^2H vs V-SMOW and $\delta^{13}C$ vs V-PDB for vanillin extracted from vanilla pods. Points are labelled with geographic origin. The geographical origins are separated by colors. The circles in the scatterplot are color-correlated to the circles in the world map, showing the regions from where the samples originates

Paper 2 also describes measurements of δ^2H of vanillin in vanilla extracts. In Figure 20 results of $\delta^{13}C$ are depicted against δ^2H in a scatter plot. As seen in Figure 20 there is a tendency for δ^2H values of vanillin from vanilla pods from adjacent geographic locations to group together. The trend in isotopic composition appears to be in accordance with the Regionalized Cluster-based Water Isotope Prediction shown in Figure 10. Analysis by one-way analysis of variances (ANOVA) followed by Tukey´s

test showed that values of δ^2H for vanillin originating from Indonesia can be separated from vanillin originating from Africa and Madagascar. Vanillin from New Caledonia can be separated from vanillin originating from Africa and Madagascar. Furthermore, δ^2H of vanillin from French Polynesia was found to be significantly different from vanillin originating from New Guinea.

Vanillin was chosen as the only analyte for stable isotope ratio measurements in this study. This was because vanillin was the only component occurring in all vanilla extracts examined at measureable concentrations.

Concentration of analytes by evaporation of the solvent could be necessary. A small study was carried out investigating the effect of δ^2H values of vanillin after evaporation of vanilla extracts (ethylacetate/cyclohexane 1:1) from approximately 1mL to 200µL. The evaporated samples showed no significant difference in δ^2H from extracts with none evaporation. The measurements of this study only focused on vanillin. Other more volatile flavours from the vanilla extract might behave differently. To make sure that no fractionation takes place, it would be necessary to investigate each component before using evaporation for concentrating samples.

For GC-P-IRMS measurements of δ^2H it is important to be aware of exchangeable hydrogens. For the vanillin molecule the hydrogen of the hydroxyl group can readily exchange with hydrogen/deuterium from the solvent (ethanol/water). In theory, this exchange can alter the measured δ^2H. Yet, this alteration was considered insignificant, because in vanillin there is only one exchangeable hydrogen out of eight. Also, the water used for extraction probably does not change the isotopic composition considerably and the conditions/exchange for the extracts is therefore identical. As long as all samples are analyzed in the same laboratory this hydrogen exchange will be no problem.

From the study described in chapter 3.3.3 and in Paper 2 it can be concluded that the combination of $\delta^{13}C$ and δ^2H of vanillin can be a helpful tool in the authentication of vanilla flavours. Results from this study were used as basis for the verification of authenticity of vanilla flavours from food products, described in chapter 3.4.

3.4 Paper 3 - Authenticity of vanillin in food products determined by SPME-GC-IRMS

Vanilla aroma added to food products can originate from extracts of natural vanilla pods, chemical synthesis, or from vanillin made by fermentations. As shown in chapter 3.3.3 stable isotope ratios of carbon and hydrogen can be used for authenticating vanilla aromas. When analyzing vanilla aroma from food products there are several parameters to consider. First, the constituents of the vanilla aroma get diluted when added to a food matrix and thus become more difficult to measure. Furthermore, there is the food matrix to consider. Ideally, the aroma components should be extracted and concentrated from the matrix before analysis without any isotopic fractionation taking place. Until recently most authenticity studies using CSIA have been made for pure vanillin or extracts of vanilla, which have a less complex matrix than food products.

In the first part of Paper 3 vanilla aroma from vanilla custard powder, vanilla sugar, cookies, and vanilla ice creams were measured by GC-C-IRMS. A polyacrylate (PA) SPME fiber was used for extracting and concentrating vanillin from the food matrix. The polyacrylate coating is of moderate polarity and the best choice for extracting polar semi volatiles like vanillin (Sigma-Aldrich, 2014). SPME of vanillin using a PA fiber has been carried out successfully by Sostaric et al. for GC-MS analysis, but not in combination with IRMS as is the case in this study (Sostaric, Boyce, & Spickett, 2000).

Lamprecht et al. analyzed vanillin from food products such as ice creams, desserts, and yoghurt using precipitation of milk proteins, liquid-liquid extraction of vanillin, purification by preparative HPLC followed by EA-IRMS analysis. A similar extraction method was used by Fayet et al. who found that the extraction did not lead to isotopic fractionation (Fayet et al., 1995; Guenther Lamprecht & Blochberger, 2009). In the second part of Paper 3 and in chapter 3.3.3 an optimization of the extraction of vanillin from ice creams followed by GC-C-IRMS analysis is investigated.

3.4.1 Paper 3 - SPME extraction of vanillin from food products

Vanilla custard powder, vanilla sugar, and cookies were analyzed using head space SPME-GC-C-IRMS. Extraction was carried out at 60°C for 30 minutes for approximately 4 g of sample. Hereafter, the fiber was desorbed in the GC inlet at 230°C for 5 minutes. These desorption conditions were sufficient to prevent carry over. The GC oven was initially at 50°C and held for 1 min, then raised at 10°C/min to

120°C, then raised at 2°C/min to 150°C, raised at 20°C/min to 260°C and held at this temperature for 5 min. The helium flow was 1mL/min.

Table 8: Results from SPME-GC-C-IRMS measurements of vanillin extracted from vanilla custard powder, vanilla sugar, and cookies containing vanilla aroma

Food product	Aroma declaration	$\delta^{13}C$ vs. V-PDB [‰]
Custard powder #1	Natural bourbon vanilla, vanillin	-27.2
Custard powder #2	Aroma	-29.3
Custard powder #3	Natural vanilla extract	-20.9
Custard powder #4	Bourbon vanilla	-27.4
Vanilla sugar #1	Vanilla powder	-20.9
Vanilla sugar #2	Natural bourbon vanilla aroma, aroma	-30.5
Cookies #1	Bourbon vanilla grains, natural aroma	-33.3

Measured values of $\delta^{13}C$ for vanillin extracted from food products are given in Table 8. Vanilla custard powders #1 had too low values of $\delta^{13}C$ to be of purely natural origin. Vanilla custard powder #1 was on the front side of the packaging declared by its content of natural Bourbon vanilla, while on the backside it declared to also contain “vanillin”. It is assumed that a synthetic origin is implicit when only using the word “vanillin” or “aroma”. The blend of natural and synthetic vanillin explains the $\delta^{13}C$ found for custard powder #1 very well. This blend will result in a mixture of the $\delta^{13}C$, where the natural vanillin will increase the delta values compared to the values of synthetic vanillin. The $\delta^{13}C$ measured for Custard powder #1 is still quite low compared to what has been measured for vanillin from *V. planifolia* which is why the amount of natural vanillin is assumed to be rather low compared to synthetic vanillin. Vanilla custard powder #2 is declared to contain “aroma” which implies a not natural aroma. This is in accordance with the isotopic value measured.

Vanilla custard #1 and #2 were produced by the same manufacturer, and it is therefore expected that the same type of synthetic vanillin has been used for both custard powders. In that case the values of $\delta^{13}C$ would be expected to be the same for synthetic vanillin in both powders. An increase in $\delta^{13}C$ can only be due to the addition

of vanillin with higher values of $\delta^{13}C$. If this speculation is correct it would support the conclusion that custard powder #1 also contains natural vanilla, because addition of natural vanillin would increase the value of $\delta^{13}C$.

It would be an advantage if a correlation could be made between values of $\delta^{13}C$ for synthetic and natural vanillin. In that case the content of synthetic and natural vanillin in an unknown sample could be estimated. Unfortunately, $\delta^{13}C$ intervals for synthetic and natural vanillin are too large to make a sufficient correlation. Such a correlation would at least require that the same type of synthetic and natural vanillin were used, which is unrealistic for different types and producers of food products. Although, if the assumption that the synthetic vanillin used in both custard powders are of the same type, then custard powder #1 would contain about 20% natural vanillin.

Custard powder #4 is declared to contain bourbon vanilla, which refers to the specific species of the vanilla orchid, *Vanilla planifolia*. However, the isotopic value of -27.4 ‰ is too low for the vanillin to be a pure extract of vanillin from this type of vanilla orchid. The isotopic value of -20.9 ‰ for vanilla custard powder #3 is rather high, and within the interval found for extracts of *Vanilla planifolia*. The labelling of Vanilla custard powder #3 is therefore in agreement with the measured isotopic values.

The measured isotopic value of -20.9‰ for vanilla sugar #1 is in agreement with the declared content of vanilla powder, whereas the measured value of -30.5‰ for vanilla sugar #2 cannot confirm the presence of natural Bourbon vanilla aroma.

The one cookie sample analysed is declared to contain Bourbon vanilla grains and natural aroma and have a measured isotopic value of -33.3 ‰ Biovanillin made from rice bran and ferulic acid have been reported to have δ values ranging from -37‰ and -33‰ (Hilmer, Hammerschmidt, & Losing, 2010), and according to EU legislation it is allowed to label biovanillin as “Natural” (European Parliament, 2008). The cookies were also labelled to contain Bourbon vanilla, which is a type of vanilla flavour that can only be extracted from the vanilla pod. The addition of both these flavours to the cookies might very well results in the measured isotopic value of -33.3 ‰.

The extraction of vanillin by SPME fibers is performed from the headspace of a sample. SPME has successfully been used in combination with IRMS: Schipilliti et al. 2011

measured values of carbon stable isotopes using SPME for the extraction of volatiles from strawberries and Winterová et al. 2008 measured stable isotope ratios of hydrogen for fruit spirits using SPME. Isotopic fractionation using SPME fiber was investigated by Dias et al. and SPME was found suitable for water-soluble organics, even though a small fractionation during the adsorption was found. The effect differed for hydrophobic compounds and organic acids (Dias & Freeman, 1997; Sessions, 2006). However, a possible fractionation will not be large enough to significantly influence the evaluation of naturalness, since the difference between ratios of carbon stable isotopes of synthetic and natural vanillin is large.

3.4.2 Paper 3 – Extraction of vanillin from vanilla ice cream

For vanilla ice cream the concentration of vanillin was low compared to the other food products tested and SPME alone was not sufficient. Sostaric et al. 2000 described the SPME-GC method for ice cream, but it was not applicable for GC-IRMS, since a high amount of analytes are required to measure the isotopic ratios. Optimization of the SPME procedure was unsuccessfully carried out; instead a preliminary extraction of vanillin was investigated.

3.4.2.1. Extraction method A

Lamprecht & Blochberger 2009 and Fayet et al. 1995 both used liquid-liquid extraction methods for extracting and concentrating vanillin from vanilla ice cream. In this study a method for extracting vanillin from vanilla ice cream was developed inspired by the work of Lamprecht 2009 and Fayet 1995. After investigating different steps of sample cleaning the following method was decided:

100g of ice cream was mixed with 200mL 20V/V% ethanol/H_2O and 10mL 15W/V% Na_2CO_3 and mixed for 1 hour. Then centrifuged at 3500g for 10 minutes and kept in the freezer overnight at -18°C. Next day, the samples where thawed and extracted with 50mL heptane. The remaining aqueous phase was acidified with 10mL 6M HCl. The acidified aqueous phase was then extracted twice with 50mL methyl isobutyl ketone. Centrifuging at 3500g for 10 min. between extractions was necessary. The organic phases were pooled and vanillin transferred to an aqueous phase by adding 10mL 0.1M NaOH. The alkaline aqueous phase was removed and acidified by adding 200µL glacial acetic acid. Vanillin was extracted from the headspace by a PA SPME fiber for 30 minutes at 60°C. Desorption and GC-C-IRMS analysis was identical to the method

described for vanilla custard powder, see also appendix 1 for materials and methods. Results are presented in Table 9.

Table 9: Results from SPME-GC-C-IRMS measurements of vanillin extracted from vanilla ice cream

Food product	Aroma declaration	$\delta^{13}C$ vs. V-PDB [‰]
Vanilla ice cream #1	Natural vanilla extract, aroma (vanillin)	-24.9
Vanilla ice cream #2	Natural bourbon vanilla, vanilla aroma	-31.9

It was only possible to extract enough vanillin for measurements of $\delta^{13}C$ from two of the ice cream samples analyzed. Ice cream #1 has an isotopic composition too high to exclusively originate from synthetic vanillin and too low to be solely of natural origin. Like for vanilla custard powder #1, the measured value of $\delta^{13}C$ is probably due to a blend of synthetic and natural vanillin. This is also in agreement with the declaration. Ice cream #2 has a very low $\delta^{13}C$ value. The label declared this ice cream to contain natural Bourbon vanilla. Judging from the isotopic measurements the portion of natural vanilla must be very small/non existing in order to obtain such a low value of $\delta^{13}C$. The result would be better explained if the vanilla flavour originated from synthetic vanillin and biovanillin.

The method used for extraction of vanillin from ice cream was not suitable for all ice creams tested. For other ice cream samples extracted the concentrations of vanillin were too low to be measured at GC-C-IRMS. Interestingly, the two ice creams, from where vanillin extraction was adequate, both had a low fat content. It is possible that vanillin binds to the content of fat in ice cream, so increasing fat content would lead to less extracted vanillin. This theory is supported by the findings of (Li, Marshall, Heymann, & Fernando, 1997). They found that the content of free vanillin was significantly lower for ice creams containing between 6-10% of fat and non-fat solids than for ice creams with 0.5-4% fat and solids. The average percentage of free vanillin in the group of ice creams with low fat was 59%, while ice creams with a high fat content were found to have an average of 52% free vanillin. The difference in free vanillin from ice creams containing high fat and low fat are fairly low. If it is assumed that this difference would be the same for the high and low fat ice creams analyzed in this study, it would have been expected also to detect vanillin in ice creams of high fat

content. The intensity of mass 44 for vanillin in the successful measurements was above 1000mV (about 500mV is needed for obtaining good precision). If this intensity equals about 59% free vanillin, then 52% free vanillin from the high fat ice creams would also be measurable at IRMS.

The content of vanillin in the ice creams analyzed was not given. Douglas et al. 2013 reports that higher concentrations of vanillin in ice creams are needed when the amount of fat and non-fat solids increase (Douglas & Hartel, 2013). If this is the case, it would have been expected to detect vanillin at IRMS for ice creams of high fat content even though some of the vanillin was bound.

In the first step of the extraction method for ice creams, the pH is increased in order to ionize the vanillin molecule. If vanillin is bound to fat and non-fat solids in the ice cream, the ionization was apparently not enough to release vanillin. Ice cream is a complex matrix consisting of fat globules, air, water, sugar, proteins, and salt. The interface between fat and water are stabilized by milk proteins, casein micelles and monoglycerides (Moonen & Bas, 2004). The emulsifying characteristics of these compounds can facilitate binding of vanillin, and therefore making it difficult to extract using the described liquid-liquid extraction method.

Since it was possible to extract vanillin from two extracts of ice cream, it is assumed that the SPME method was suitable for extraction of vanillin. However, care should be taken not to reduce the extraction capabilities of the fiber. During this study it was noticed that even though the ice cream extracts were aqueous, they still contained small amounts of methyl isobutyl ketone. This solvent has a lower boiling point than vanillin and there is the risk that methyl isobutyl ketone will compete with vanillin, so that less vanillin is adsorbed by the fiber. This effect could be avoided by getting rid of all solvents in the aqueous extract before SPME extraction.

3.4.2.2. Extraction method B

Ice cream is a complex matrix consisting of air, water, lipid globules, proteins, and sugar. Emulsifiers are added in order to obtain a stable suspension of the ice cream. Egg yolk is traditionally used as emulsifier in ice creams because of its high content of phospholipids and lecithin. These emulsifiers can bind to the surface of the lipid globules present in the milk and to the surrounding water, and hereby stabilize the

lipid/water interface. Lamprecht et al and Fayet et al precipitated proteins in ice cream before extraction with methyl isobutyl ketone. They used reagent Carrez 1 and 2 for precipitation (Fayet et al., 1995; Guenther Lamprecht & Blochberger, 2009). Phospholipids and proteins were reported to precipitate by addition of acetone (Maximiano et al., 2008). A new method for extraction of vanillin from ice cream was developed using acetone for precipitation of phospholipids and proteins followed by extraction with ethyl acetate: method B:

Approximately 25g of ice-cream was weighed in a beaker glass and allowed to thaw. 50ml of acetone was added under stirring with a magnetic stirring bar. The suspension was maintained at 4˚C for 30min to precipitate phospholipids and proteins. The precipitate was removed by filtration and the filtrate was concentrated on a rotary evaporator at 40˚C to a final volume of approximately 10ml. The residue was extracted twice with 5ml of ethyl acetate and the combined organic phase was dried with $MgSO_4$ and evaporated to dryness in a 10ml head space vial at 40˚C under a gentle stream of nitrogen.

Method B gave sufficiently intense peaks of vanillin in the analyses for the rest of the ice-cream samples. Results are presented in Table 10.

Table 10: Measured values of $\delta^{13}C$ vs. V-PDB [‰] for ice cream samples #3 - #7

Food product	Aroma declaration	$\delta^{13}C$ vs. V-PDB [‰]
Vanilla ice cream #3	Vanilla	-22.9
Vanilla ice cream #4	Vanilla	-21.1
Vanilla ice cream #5	Vanilla	-21.7
Vanilla ice cream #6	Natural vanilla extract, aroma	-27.2
Vanilla ice cream #7	Aroma, vanilla extract	-31.8

The measured isotopic values for ice cream #3, #4, and #5 rather high and indicate a content of vanillin extracted from the vanilla orchid, presumably of the type *V. planifolia.* Ice creams #6 and #7 have low isotopic values indicating presence of synthetic vanillin. Both are declared to contain vanilla extract and aroma, but based on the isotopic measurements the content of natural vanilla extract must be fairly low.

The SPME-GC-IRMS was found suitable for vanilla custard powder, vanilla sugar, and one type of cookies. Because of the high content of vanillin, only a short extraction time at ambient temperatures was necessary. It is therefore assumed that this method also would be applicable for other products containing rather high amounts of vanillin such as vanilla extracts, powder for various vanilla desserts etc.

The liquid-liquid extraction of vanillin using methyl isobutyl ether (Method A) and subsequent SPME-GC-C-IRMS analysis was only found suitable for two ice creams. For all other vanilla ice creams tested, the method did not extract enough vanillin to be able to measure the ratios of carbon stable isotopes. On the contrary, addition of acetone and extraction with ethyl acetate worked successfully for extraction of vanillin from ice cream samples of high fat content.

4. Conclusion

The aim of this thesis was to investigate methods for authentication of aroma components. An analytical platform was built based on enantiomeric separation of chiral aroma components and compound specific isotope analysis. Afterwards the authenticity of aroma compounds from food matrices was evaluated.

In order to use enantiomeric separation for authentication of raspberry aroma SPME was at first used for extraction and concentration of aroma compounds. A DVB/CAR/PDMS fiber coating was found suitable for extraction of aroma components from raspberries, especially the chiral compound α-(E)-ionone. Furthermore, it was found that addition of salt had a negative effect on the fiber adsorption of α-(E)-ionone. 27 food products containing raspberry aroma was analysed using the optimized SPME method and subsequently analyzed by enantio-GC-MS. Based on the enantiomeric distribution of α-(E)-ionone the naturalness of the aromas were evaluated.

Vanilla extracts of two different species were analyzed using GC-Q-TOF in order to obtain an aroma profile. The two aroma profiles where differentiated in that *V. tahitensis* contained anis alcohol and *p*-anisic acid which was not seen in extracts of *V. planifolia*. On the contrary, *V. planifolia* contained higher amounts of vanillin and 4-hydroxbenzaldehyde than *V. tahitensis*.

Vanillin of synthetic origin, biovanillin and vanillin from *V. planifolia* and *V. tahitensis* were measured for ratios of stable isotopes of carbon and hydrogen. Based on measurements of ratios of stable isotopes of carbon it was possible to differentiate between vanillin extracted from vanilla pods and vanillin made otherwise. Also the species of the vanilla pods was identifiable by this method. A combination of ratios of stable isotopes of carbon and hydrogen showed tendencies to cluster due to the geographical origin of natural vanilla pods. Compound specific isotope analysis can therefore be a valuable tool in determination of authenticity and traceability of vanilla aroma.

The authenticity of vanillin in different food products was evaluated based on measurements of stable isotopes of carbon. Solid phase micro extraction combined

with GC-IRMS was sufficiently for analysis of vanilla custard powder, vanilla sugar, and one type of cookies. A preliminary extraction step was needed for analysis of vanillin in ice cream. Two methods were described: Method A: variations of pH to get rid of lipids and sugars followed by extraction with methyl isobutyl ketone and Method B: precipitation of phospholipids and proteins using acetone followed by extraction of vanillin with ethyl acetate. Method A was found useful only for ice cream with low fat content, while Method B extracted sufficiently vanillin from ice creams for isotopic analysis.

5. Future perspectives

IRMS measurements in this study focused solely on vanillin, because this is the main constituent of vanilla aroma and it is the only compound found in all types of vanilla aroma. It could, however, be interesting to measure stable isotope ratios for other compounds. Natural vanilla extracts contain lots of other compounds beside vanillin, which all contribute to the unique flavour of vanilla. Analysis of minor components from vanilla extracts would require a preliminary concentration of the extracts. This could be done by evaporating some of the solvents under a flow of nitrogen. Although, it must be assured that that no isotopic fractionation occur during sample preparation.

The IRMS used for this study is also able to measure stable isotope ratios of nitrogen, oxygen, and sulphur besides carbon and hydrogen. This is an opportunity that should be exploited within authenticity of aroma components. In theory, all five elements could be measured for all constituents of an aroma. This would cause a large dataset where multivariate data analysis could be used for identifying the variables useful for authentication.

Furthermore $\delta^{18}O$ has the possibility to be used for traceability studies. There is a strong correlation between the $\delta^{18}O$ and $\delta^{2}H$ of meteoric water such as precipitation and atmospheric water vapour. Values of $\delta^{18}O$ could therefore also be used as marker for geographical habitat of a plant. Hener et al. measured $\delta^{18}O$ of vanillin extracted from pods originating from Mexico, Tahiti and Comores.(Hener et al., 1998) Hener et al. found vanillin from Mexico to be the most depleted in the heavy oxygen isotope, followed by Comores, and Tahiti. This is in agreement with the results of $\delta^{2}H$ found in Paper 2. Nevertheless, ratios of stable oxygen isotope are not as used for traceability studies as hydrogen, primarily due to instrumental challenges. If these challenges could be overcome, the combination of ratios of stable isotope of hydrogen and oxygen could be a powerful tool for traceability studies.

Indeed Isotope Ratio Mass Spectrometry has a large future potential within traceability. Interest in geographic traceability and local specialties are increasing and labels such as PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication)

are more commonly used. Methods for traceability will therefore be crucial for authorities to verify PDO and PGI labelled products.

Combining enantiomeric separation with IRMS could further improve the versatility of food authenticity, i.e. in the case where an aroma is chemically synthesized using a natural precursor extracted from a C3 plant. Such a synthesis would result in a racemic mixture of the formed aroma compound. A subsequent enantiomeric separation will then show a presence of both enantiomers indicating a flavour of non-natural origin. By analyzing this flavour with enantio-GC-IRMS it would be possible to obtain delta values for each enantiomer. This delta value will be reflected by the isotopic values of the natural precursor and be the same for both enantiomers indicating a common origin.

Compound Specific Isotope Analysis is a valuable tool for evaluation of authenticity and traceability. The use of multielement CSIA combined with multivariate data analysis possesses great future opportunities within authenticity studies.

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7. Appendix 1

Materials and methods

Paper 1 – Selection of fiber coating

Frozen raspberries of unknown type from Poland were purchased in the local supermarket and stored at -18°C. The three SPME fibers tested: 65 µm PDMS/DVB, 75 µm CAR/PDMS, and 50/30 µm DVB/CAR/PDMS where purchased from Sigma Aldrich (Brøndby, Denmark).

The analysis of volatiles extracted from raspberries were carried out using an Agilent 6890 gas chromatograph equipped with a 5979 Mass Selective Detector (Agilent Technologies, Inc., Wilmington, Germany) and a Combi Pal autosampler (CTC Analytics, Zwingen, Switzerland).

The SPME fiber was heated at 250°C for 10min before each analysis. Samples were preincubated at 60°C for 15 min with agitation. Afterwards, the SPME fiber was exposed to the head space of the sample and extraction carried out for 30 min. at 60°C at agitation. Desorption in the GC inlet was carried out for 10 min. at 230°C. The initial temperature of the GC oven was 50°C for 1 min., then increased 1.5°C/min to 125°C, increased 5°C/min to 200°C, increased 9°C/min to 280°C and held for 15 min. The helium flow was 0.8mL/min. Conditions during extraction, desorption, and GC run were kept constant for all fibers. Analytes were separated on a DB-5MS (30m x 0.250mm, d_f 0.25µm). Standard EI mode was used at 70eV. Total ion chromatograms were obtained from 50 to 300 amu.

Paper 1 – Addition of sodium chloride

Frozen raspberries of unknown type from Poland were purchased in the local supermarket (different batch from first part of Paper 1) and stored at -18°C. SPME fibers of the type 50/30 µm DVB/CAR/PDMS were purchased from Sigma Aldrich (Brøndby, Denmark). Sodium chloride was purchased from Merck (Darmstadt, Germany).

Raspberries were thawed and blended. For each sample 4 g of the blended raspberries was placed in a 10mL vial and sodium chloride was added to concentrations of 10, 20, 25, and 30 (w/w)% (duplicate samples of each concentration). SPME and GC conditions were the same as described for Study 1 – Selection of fiber coating

Paper 1 – Authenticity of raspberry flavour from food products

Frozen raspberries of the brand “Minirisk organic” were purchased from the local supermarket. Raspberry juice was kindly provided by A/S Ejnar Willumsen. Raspberry jam was purchased in the local supermarket and was declared to only contain natural flavours. Synthetic α-ionone was purchased from Sigma-Aldrich (Brøndby, Denmark). Sample preparation and SPME extraction was carried out as described in Paper 1 – Selection of fiber coating. Analytes were separated on a β-DEX 225 GC column (30m x 0.250mm, d_f 0.25µm). The initial temperature of the GC oven was 50°C and held for 7 min., then increased 1.5°C/min to 180°C, increased 6°C/min to 200°C and held for 5 min. (GC runtime: 102min.). The helium flow was 0.8mL/min. The transfer line temperature was 250°C, MS source 230°C, MS Quad 150°C The mass spectrometer was used in Electron Ionization (EI) mode using Scan mode (m/z 40-400) for the method optimizing part and for samples Single Ion Monitoring (SIM); m/z 121 as quantifier ion and 192, 136 and 93 as qualifiers to ensure the identity and purity of the peaks.

Samples of jam, sodas, sweets, dried raspberries, fruit bars and yoghurts from the Danish retail market were purchased at different retailers, all declared to contain raspberries, natural flavor and/or flavor. No sample preparation was performed on jam, soda and dried raspberry samples. Sweets and the fruit bar were cut in 5 mm pieces prior to transfer to the sample vials. Approximately 4g of lightly homogenized sample was placed in a 10mL headspace vial. The vial was heated to 60°C before the SPME fiber was exposed to the headspace. Extraction was carried out at 60°C for 30 min. using agitation. Hereafter analytes were thermally desorbed into the GC inlet at 230°C for 5 minutes in splitless mode. The GC was used with the following temperature program: 50°C for 5 min. and then raised at 30°C/min. to 100°C and raised again at 2°C/min. to 145°C and finally 30°C/min. to 200°C for 4 min. (GC runtime: 35 min.). Carrier gas was helium at 0.8 ml/min, 33 cm/sec. Samples were analyzed as single determinations, which were deemed sufficient because percentages of R-α-ionone

close to 100 in the samples would indicate natural flavor whereas values close to 50 would indicate synthetic flavor.

Paper 2 - Preliminary studies, Isotopic fractionation during GC-injection

Vanillin was purchased from Sigma Aldrich (Brøndby, Denmark). Ethyl acetate and cyclohexane were purchased from Rathburn Chemicals, Ltd. (Walkerburn, U.K.).

Standards of vanillin dissolved in ethyl acetate/cyclohexane (1:1) were made in the following concentrations: 6000, 4800, 3000, 1500, and 750ng/µL for splitless injections and concentrations of 60,000, 30,000, 15,000, 6000, 4000, and 3000ng/µL for split injections. Standards were placed in vials and each vial analyzed five times.

Vanillin was analyzed on a GC Ultra (Thermo Scientific, Bremen, Germany) fitted with a DB-5 capillary column (Agilent Technologies, Böblingen, Germany) (30m x 0.250mm, d_f 0.25µm). The initial temperature of the GC was 50°C, then increased at 4°C/min to 150°C, increased at 7°C/min to 260°C and held for 1 min. The inlet temperature was 230°C. A split ratio of 20 was used. The helium flow was 1.2ml/min.
The GC was coupled to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). The interface between the GC and the IRMS was a ConFlo IV and a GC-IsoLink with a combustion reactor and a high temperature reactor (Thermo Scientific, Bremen, Germany). For this study only the high temperature reactor was used. The temperature of the reactor was 1420°C.

Paper 3 - Authenticating vanilla flavour from food products

A polyacrylate SPME fiber with a length of 1 cm was purchased from Sigma Aldrich (Brøndby, Denmark). Vanilla custard powder, vanilla sugar, cookies, and vanilla ice cream were purchased in local supermarkets.

Custard powder and vanilla sugar were analyzed without any sample preparation and cookies were crushed into a fine powder prior to analysis. Approximately 4g of sample was placed in a 10 mL headspace vial.

Method A: 100g of ice cream was mixed with 200mL 20V/V% ethanol/H_2O and 10mL 15W/V% Na_2CO_3 (Merck, Darmstadt, Germany) and mixed for 1 hour. Ethanol was purchased from Merck (Darmstadt, Germany). Then centrifuged at 3500g for 10 minutes and kept in the freezer overnight at -18°C. Next day the samples where thawed and the supernatant extracted with 50mL heptane (Merck, Darmstadt, Germany). The remaining aqueous phase was acidified with 10mL 6M HCl (SCP Science, Quebec, Canada). The acidified aqueous phase was then extracted twice with 50mL methyl isobutyl ketone (Sigma-Aldrich Steinheim, Germany). Centrifuging at 3500g for 10 min. between extractions was necessary. The organic phases were pooled and vanillin transferred to an aqueous phase by adding 10mL 0.1M NaOH (Sigma Aldrich, Brøndby, Denmark). The alkaline aqueous phase is removed and acidified by adding 200µL glacial acetic acid (Merck, Darmstadt, Germany). Vanillin is extracted from the headspace by a PA SPME fiber for 40 minutes at 60°C. Vanillin was purchased from (Sigma Aldrich, Brøndby, Denmark).

Method B: Approximately 25g of ice-cream in a beaker glass was allowed to thaw and added 50ml of acetone (Sigma-Aldrich, Steinheim, Germany) under stirring with a magnetic stirring bar. The suspension was maintained at 4˚C for 30min to precipitate phospholipids and proteins. The precipitate was removed by filtration and the filtrate was concentrated on a rotary evaporator at 40˚C to a final volume of approximately 10ml (Büchi, Buchs, Switzerland). The residue was extracted twice with 5ml of ethyl acetate and the combined organic phase was dried with $MgSO_4$ and evaporated to dryness in a 10ml head space vial at 40˚C under a gentle stream of nitrogen.

For SPME extraction of vanillin the polyacrylate fiber was conditioned for 1h at 280°C according to manufacture recommendation. A 10mL vial is filled 2/3 with sample. The head space vials were heated to 60°C before the SPME fiber was exposed to the headspace. Extraction was carried out at 60°C for 30 min. using agitation. Hereafter analytes were thermally desorbed into the GC inlet at 230°C for 5 minutes in splitless mode. Analytes were separated on a DB-5 capillary column (Agilent Technologies, Böblingen, Germany) (30m x 0.250mm inner diameter, with d_f of 0.25µm). The initial temperature of the GC oven was 50°C for 1 min., and then increased 10°C/min to 120°C, increased 2°C/min to 150°C, increased 20°C/min to 260°C and held for 5 min. The helium flow was 1.0mL/min. The helium flow was 1ml/min. The Trace GC Ultra (Thermo Scientific, Bremen, Germany) was coupled to a Delta V Advantage Isotope

Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). The interface between the GC and the IRMS was a ConFlo IV and a GC-IsoLink with a combustion reactor and a high temperature reactor (Thermo Scientific, Bremen, Germany). Analytes were converted to gasses in the combustion reactor operated at 1000°C.

8. Appendix 2

Publications

Paper 1:

Authenticity of raspberry flavor in food products using SPME-chiral-GC-MS

Anne-Mette Sølvbjerg Hansen, Henrik Lauritz Frandsen, and Arvid Fromberg.

Submitted to Food Science & Nutrition.

Paper 2:

Authenticity and traceability of vanilla flavours by analysis of stable isotopes of carbon and hydrogen

Anne-Mette Sølvbjerg Hansen, Arvid Fromberg, and Henrik Lauritz Frandsen.

Published in Journal of Agricultural and Food Chemistry 2014.

Paper 3:

Authenticity of vanillin in food products determined by SPME-GC-IRMS

Anne-Mette Sølvbjerg Hansen, Henrik Lauritz Frandsen and Arvid Fromberg.

Submitted to Food Chemistry.

Poster

Conference Poster 1:

Optimization of GC injection for δ^2D analysis of vanillin

Anne-Mette Sølvbjerg Hansen, Arvid Fromberg, and Henrik Lauritz Frandsen.

Contribution to ”Food integrity and traceability conference” 2014, Queens University, Belfast.

Title: Authenticity of raspberry flavor in food products using SPME-chiral-GC-MS

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Running head: Authenticity of raspberry flavor SPME-chiral-GC-MS

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Abstract

A fast and simple method for authenticating raspberry flavors from food products was developed. The two enantiomers of the compound (E)-α-ionone from raspberry flavor were separated on a chiral gas chromatographic column. Based on the ratio of these two enantiomers the naturalness of a raspberry flavor can be evaluated due to the fact that a natural flavor will consist almost exclusively of the R enantiomer, while a chemical synthesis of the same compound will result in a racemic mixture.

27 food products containing raspberry flavors where investigated using SPME-chiral-GC-MS. We found raspberry jam, dried raspberries and sodas declared to contain natural aroma all contained almost only R-(E)-α-ionone supporting the content of natural raspberry aroma. Six out of eight sweets tested did not indicate a content of natural aroma on the labelling which was in agreement with the almost equal distribution of the R and S isomer. Two products were labelled to contain natural raspberry flavors but were found to contain almost equal amounts of both enantiomers indicating a presence of synthetic raspberry flavors only. Additionally, two products labelled to contain both raspberry juice and flavor showed equal amounts of both enantiomers, indicating the presence of synthetic flavor.

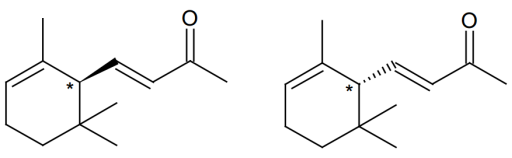
Keywords: Authenticity, SPME, chiral-GC-MS, raspberry flavor, α-ionone, enantioselective GC

Introduction

Authenticity of food products is often used as a parameter of quality in marketing. Food products containing only natural ingredients are preferred by many consumers who are willing to pay a higher price for such products. Natural aroma components are often more expensive than their synthetic equivalent making counterfeit an economical benefit. Counterfeit of aroma components is misleading of the consumers and is illegal according to European legislation (European Parliament 2008) where the term "Natural" can be used only for flavors containing exclusively natural flavoring substances. A natural flavor should be obtained by appropriate physical, enzymatic or microbiological processes from the material of vegetable, animal or microbiological origin (European Parliament 2008).

The flavor of raspberries consist of many different aroma compounds all contributing more or less to the characteristic perception of raspberries. Malowicki *et al*. investigated the volatile composition of raspberries following stir bar sorptive extraction and identified some 30 compounds including (Z)-hexenol, hexanal, (E)-2-hexenal, 2-heptanone, δ-octalactone, δ-decalactone, geraniol, α-ionone, β-ionone and terpinen-4-ol as the major constituents (Malowicki et al. 2008a and b). Some of the flavoring substances are chiral and the enantiomeric composition in raspberry extracts was characterized by chiral GC-MS, α-ionone occurs mainly in the R-form (97-100%) whereas δ-octalactone, δ-decalactone and terpinen-4-ol occurs mainly in the S-form (80-100%), see Figure 1 (Malowicki et al 2008a and b). Also, Werkhoff et al. found an enantiomeric composition of α-ionone in raspberry extract sampled by head space of 99.9% R-form and 0.1% S-form (Werkhoff et al 1991). The enantiomeric composition of α-ionone and δ-decalactone in raspberry extract was by chiral GC determined to 98-100% R-form for ionone and to 98-100% S-form for decalactone (Casabianca and Graff 1994). The enantiomeric composition of a number of chiral 4-alkylated-γ-lactones from C_5 to C_{12} were determined in extracts from apricot, mango, passion fruit, peach, raspberry and strawberry (Bernreuther et al 1989. Guichard et al 1990). For all fruits there seemed to be a preponderance of the R-form of the longer chained γ-lactones > C_8 whereas for the shorter chained γ-lactones some fruits had preponderance of the R-form, some of the S-form and some hardly showed enantiomeric excess.

Figure 1: The two enantiomers of (E)-α-ionone



(R)-(E)-α-ionone (S)-(E)-α-ionone

A previous study (Ravid et al. 2010) on the assessment of the authenticity of natural food fruit compounds in food and beverages used head space SPME chiral GC-MS for the characterization of the enantiomeric composition of: linalool, linalyl acetate and limonene in bergamot oil, γ-decalactone and γ-undecalactone in peach and nectarine products, γ-lactones in passion fruit products and α-ionone in raspberry products. The study showed that (E)-α-ionone in raspberry is efficiently adsorbed on an SPME fiber and occur almost exclusively as the R enantiomer, whereas (E)-α-ionone originating from chemical synthesis is a racemic mixture containing almost equal amounts of both enantiomers. In raspberries the biosynthesis of (E)-α-ionone is catalyzed by stereospecific enzymes leading to a preponderance of (R)-(E)-α-ionone of more than 99% (Ravid et al. 2010). Consequently presence of (S)-(E)-α-ionone could be an indicator of adulteration with artificial aroma components (Aprea et al. 2009; Taylor & Linforth 2010).

Head space SPME is an attractive method to isolate and concentrate volatile compounds from complex samples such as foods because only little sample pretreatment is required. In this study we investigated three different fiber coatings: divinylbenzene/polydimethylsiloxane (DVB/PDMS), carboxen/polydimethylsiloxane (CAR/PDMS), and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) for the capabilities to extract volatile compounds, from raspberry aroma and in particular (E)-α-ionone, as this compound is the major enantiomeric compound bound to the extraction fiber. Furthermore, addition of sodium chloride during head space SPME was investigated for potential improvement of extraction efficiency. Finally, 27 raspberry containing and/or flavored samples of foods, beverages and sweets from the Danish market were analyzed for enantiomer composition of (E)-α-ionone to investigate whether the labeling were in compliance with the EU regulation. The samples set included sodas and sweets, products which have not previously been investigated for authenticity.

Materials and methods

Samples: Samples of jam, sodas, sweets, dried raspberries, fruit bars and yoghurts from the Danish retail market were purchased at different retailers, all declared to contain raspberries, natural flavor and/or flavor, see Table 1. Samples of fresh raspberries were obtained from the local retail market.

Table 1: Samples collected from the Danish marked.

Sample	Fruit content declared	Natural aroma declared	Aroma declared
Jam #1	Raspberry, 35%		
Jam #2	Raspberry, 50%		
Jam #3	Raspberry, 50%		
Jam #4	Raspberry, 45%		
Jam #5	Raspberry, 40%		
Jam #6	Raspberry, 45%		
Soda #1			x
Soda #2			x
Soda #3		x	
Soda #4		x	
Soda #5			x
Dried raspberries #1	Dried fruits		
Sweet #1, fruit gum			x
Sweet #2, fruit gum			x
Sweet #3, fruit gum			x
Sweet #4,fruit gum	Raspberry juice		x
Sweet #5,fruit gum	Raspberry juice		x
Sweet #6, lollipops			x
Sweet #7, fruit gum			x
Sweet #8, fruit gum			x
Fruit bar #1	Raspberry 1.3%	x	
Yoghurt #1	Raspberry 14%		
Yoghurt #2	Raspberry 7.5%		
Yoghurt #3	Raspberry 1.7%	x	
Yoghurt #4	Raspberry 7%	x	
Yoghurt #5	Raspberry 8%	x	
Yoghurt #6	Raspberry 6%		

77 Chemicals: Pure standard of α-ionone was purchased from Sigma-Aldrich (Steinheim, Germany). Sodium chloride was
78 purchased from Merck (Darmstadt, Germany).

79 SPME fibers: SPME fibers were purchased from Supelco, (Supelco, USA), all coated on a fused silica fiber and with a
80 length of 1cm; divinylbenzene (DVB)/polydimethylsiloxane (PDMS) 65µm coating, carboxen (CAR)/(PDMS) 75µm
81 coating and DVB/CAR/PDMS 50µm DVB and 30µm CAR on PDMS coating.

82 SPME-chiral-GC-MS method: The SPME fibers were used for extracting aroma compounds from raspberries and
83 subsequent analysis using automated headspace solid phase micro extraction (HS-SPME) using a GC-Combi PAL
84 (CTC Analytical, Zwingen, Switzerland) on an Agilent 6890 gas chromatograph (GC) (Agilent Technologies, Inc.,
85 Wilmington, Germany) equipped with an Agilent 5979 Mass Selective Detector.

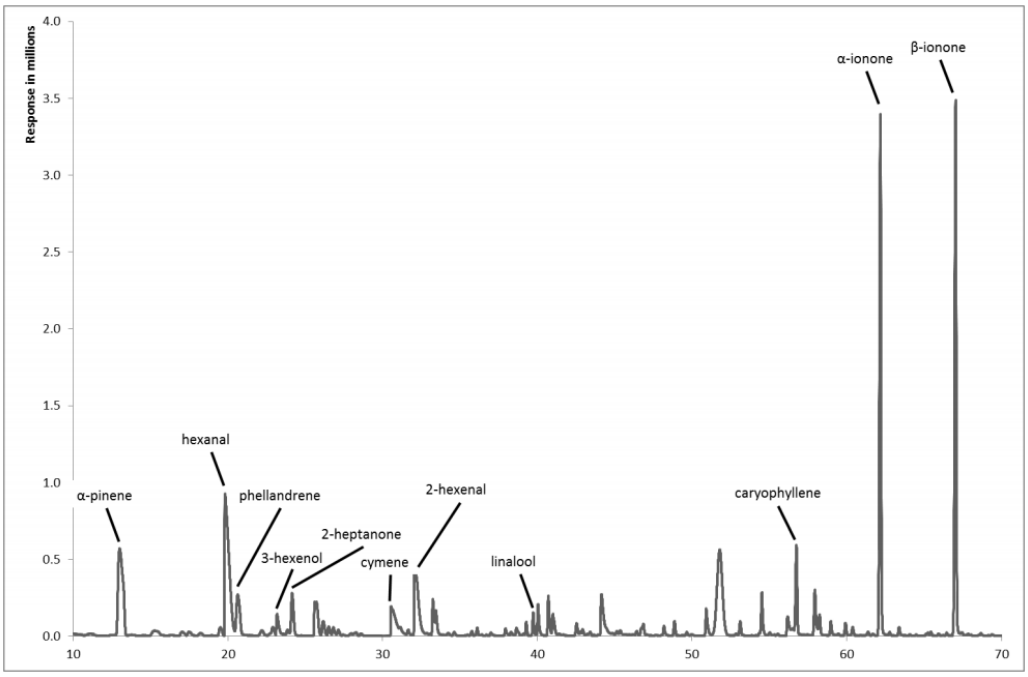
86 Samples of fresh raspberries were macerated with a fork prior to transfer to the sample vials. No sample preparation
87 was performed on jam, soda, youghurt and dried raspberry samples. Sweets and the fruit bar were cut in 5 mm pieces
88 prior to transfer to the sample vials. Approximately 4g of lightly homogenized sample was placed in a 10mL headspace
89 vial. The vial was heated to 60°C before the SPME fiber was exposed to the headspace. Extraction was carried out at
90 60°C for 30 min. using agitation. Hereafter analytes were thermally desorbed into the GC inlet at 230°C for 5 minutes
91 in splitless mode. The GC was used with the following temperature program: 50°C for 5 min. and then raised at
92 30°C/min. to 100°C and raised again at 2°C/min. to 145°C and finally 30°C/min. to 200°C for 4 min. (GC runtime: 35
93 min.). For the method optimizing part, the following temperature program was used: 50°C for 7 min. and then raised at
94 1.5°C/min. to 180°C and raised again at 6°C/min. to a final temperature of 200°C/min. for 5 min. (GC runtime: 102
95 min.). The column was a β-DEX 225 (Supelco, USA) consisting of non-bonded 25% 2,3-di-O-acetyl-6-O-TBDMS-β-
96 cyclodextrin in a SPB-20 phase 0.25mm x 30m, 0.25µm film thickness. Carrier gas was helium at 0.8 ml/min, 33
97 cm/sec. The transfer line temperature was 250°C, MS source 230°C, MS Quad 150°C The mass spectrometer was used
98 in Electron Ionization (EI) mode using Scan mode (m/z 40-400) for the method optimizing part and for samples Single
99 Ion Monitoring (SIM); m/z 121 as quantifier ion and 192, 136 and 93 as qualifiers to ensure the identity and purity of
100 the peaks. Quality ratio control of the qualifiers were ±20% and quality control of the retention time was ±0.2 min..
101 Samples were analyzed as single determinations, which were deemed sufficient because percentages of R-α-ionone
102 close to 100 in the samples would indicate natural flavor whereas values close to 50 would indicate synthetic flavor.

103

Results and discussion

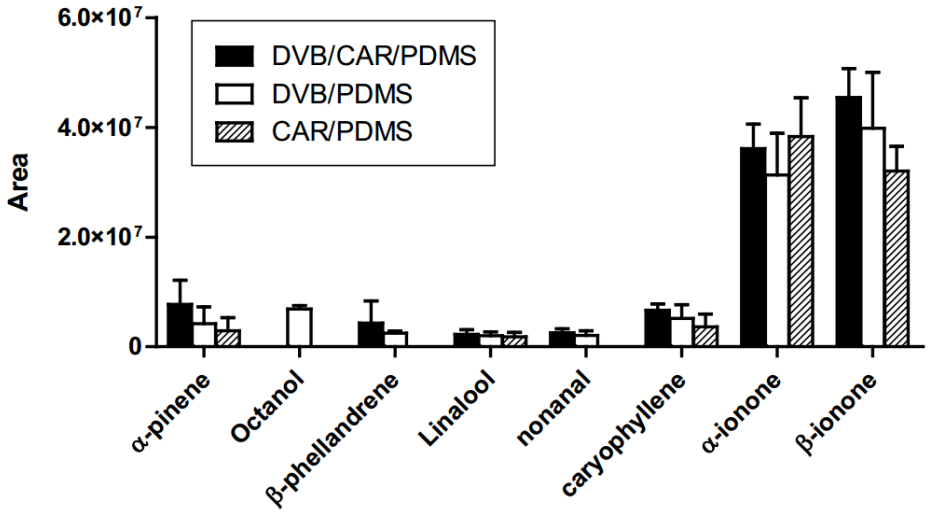
Before analyzing the samples the analytical method were optimized in order to obtain a fast and reliable method for the documentation of the authenticity of raspberry flavor in food products. The optimization was performed using macerated raspberries as samples and included SPME fiber coating selection and the influence of addition of sodium chloride to the samples. A full scan SPME GC-MS chromatogram of macerated raspberries, using a DVB/CAR/PDMS fiber is shown in figure 2, where it appears that α-ionone and β-ionone are the highest peaks.

Figure 2: Full scan head space SPME GC-MS chromatogram of macerated raspberries, GC runtime 102 min.



To study the influence of fiber coating on the SPME the following fiber types were selected, all coated on a fused silica fiber and with a length of 1cm; divinylbenzene (DVB)/polydimethylsiloxane (PDMS) 65µm coating, carboxen (CAR)/(PDMS) 75µm coating and DVB/CAR/PDMS with 50µm DVB coating / 30µm CAR on PDMS coating. Macerated raspberries were used as a basis model for the experiments. For this method optimizing study, only the ability to extract analytes from raspberries was investigated and the levels where therefore not quantified but based on comparison of peak areas and presented in Figure 3.

Figure 3: Peak areas for 8 analytes extracted from raspberries with DVB/CAR/PDMS, DVB/PDMS, and CAR/PDMS SPME fibers

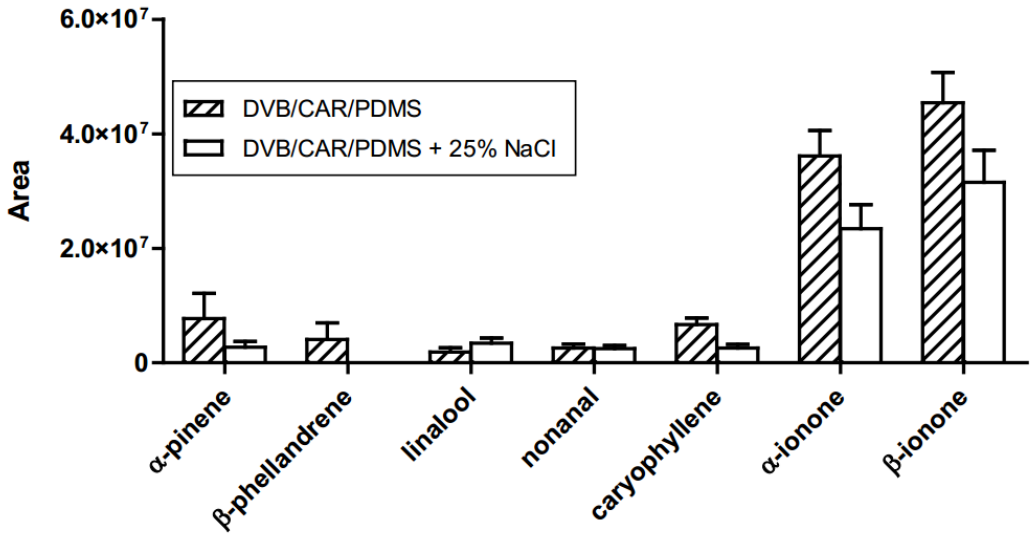


The three SPME fibers showed approximately similar abilities to extract the seven major chemical constituents from raspberry headspace. With the exception that octanol was not extracted by DVB/CAR/PDMS and CAR/PDMS. In addition β-phellandrene and nonanal were not extracted by CAR/PDMS. The DVB/CAR/PDMS fiber showed slightly better extraction efficiency for ionone and was therefore selected for the further studies.

Influence of addition of sodium chloride to samples

The effect of addition of sodium chloride to the matrix was investigated, as increased ionic strength usually improves the extraction efficiency of hydrophilic compounds (Kudlejova et al. 2012). Sodium chloride was added to the sample matrices to a concentration of 25 % and analytes extracted with a SPME fiber coated with DVB/CAR/PDMS and presented in figure 4.

Figure 4: Peak area for 7 analytes extracted from raspberries with DVB/CAR/PDMS SPME fiber with and without addition of NaCl.



From Figure 4 it is clear that addition of sodium chloride does not have a positive effect upon extraction of the majority of the analytes from raspberries. On the contrary it looks like the salt has a negative effect on the extraction especially for α-pinene, caryophyllene, α- and β-ionone. For the other compounds analyzed the effect was less pronounced. Only the extraction of linalool was a little higher when sodium chloride was added. Pawliszyn 2012 reported that the positive effect of salt increases with increased polarity of the analytes, which is in agreement with the results found in this study (Pawliszyn 2012).

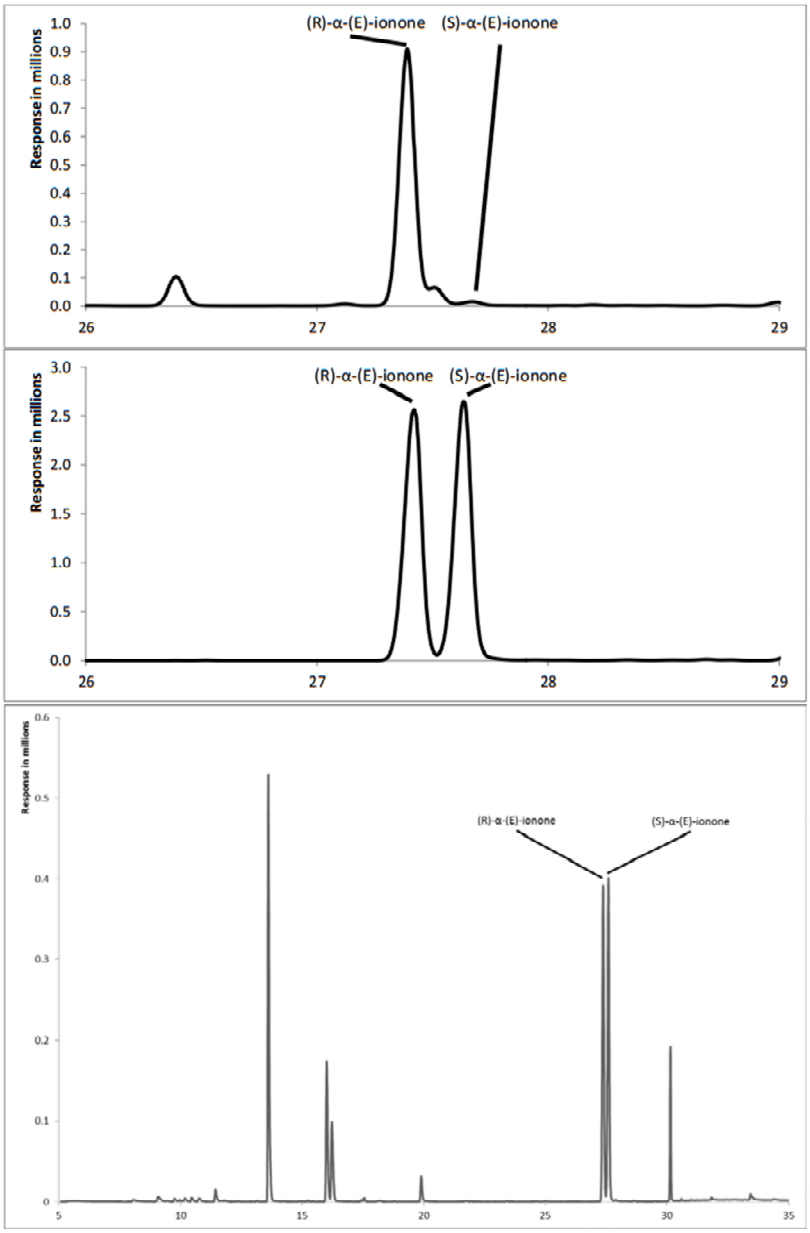
The result for β-ionone is in agreement with the results of Yang and Peppard, who found that the extraction of β-ionone decreases with higher sodium chloride concentrations when extracted with a SPME fiber, coated with 100 µm PDMS (Yang & Peppard 1994).

Content of R-(E)-α-ionone and S-(E)-α-ionone in foods, beverages and sweets from the Danish market

For authenticity investigation of raspberry flavored foods using head space SPME chiral GC-MS the most important compound is α-ionone. From the chromatogram in Figure 5 it can be seen that (S)-α-ionone is barely detectable in macerated raspberry (top) compared to the peak of (R)-α-ionone. This is in accordance with previously published results on the analyses of enantiomer ratios of α-ionone in raspberries showing that (R)-α-ionone constitutes more than

97%. (Sewing et al, 2005; Ravid et al 2010) On the contrary synthetic raspberry aroma (Figure 5 middle) shows the presence of both enantiomers in almost equal amounts. Synthetic α-ionone can be added to natural raspberry flavor i.e. in order to fortify the flavor and/or reduce price. In that case the enantiomer ratio will not be 50:50, but reflect the percentage of synthetic aroma added to the natural raspberry aroma and it would still be possible to detect (S)-α-ionone indicating a not purely natural flavor.

Figure 5: Chromatograms for α-ionone from macerated raspberry containing primary R-(E)-α-ionone (top) and a sample of synthetic raspberry aroma containing both R-(E)-α-ionone and S-(E)-α-ionone (middle). At the bottom a full scale chromatogram of yoghurt sample #3.



Twenty seven samples of food beverages and sweets from the Danish retail market were analyzed for enantiomer ratio of (E)-α-ionone using the described head space solid phase micro extraction method including chiral-gas chromatography combined with mass spectrometric detection using SIM mode. Table 2 presents the results calculated as the percentage of R-(E)-α-ionone and S-(E)-α-ionone in all samples.

Table 2: Results for R-(E)-α-ionone and S-(E)-α-ionone analyses in food and sweet samples from the Danish market.

Sample	R-α-ionone %	S-α-ionone %	Compliance with EU legislation
Jam #1	97.1	3.0	Yes
Jam #2	97.4	2.6	Yes
Jam #3	96.8	3.2	Yes
Jam #4	96.3	3.7	Yes
Jam #5	98.5	1.5	Yes
Jam #6	97.3	2.7	Yes
Soda #1	49.9	50.1	Yes
Soda #2	49.8	50.2	Yes
Soda #3	100	0	Yes
Soda #4	100	0	Yes
Soda #5	50.6	49.4	Yes
Dried raspberries #1	100	0	Yes
Sweet #1	49.9	50.1	Yes
Sweet #2	49.9	50.1	Yes
Sweet #3	49.7	50.3	Yes
Sweet #4	50.4	49.6	?
Sweet #5	51.0	49.0	?
Sweet #6	50.4	49.6	Yes
Sweet #7	50.2	49.8	Yes
Sweet #8	50.4	49.6	Yes
Fruit bar #1	49.2	50.8	No
Yoghurt #1	100	0	Yes
Yoghurt #2	100	0	Yes
Yoghurt #3	49.5	50.5	No
Yoghurt #4	100	0	Yes
Yoghurt #5	100	0	Yes
Yoghurt #6	100	0	yes

Six samples of raspberry jam declared to contain between 35 and 50% fruit, all had a huge preponderance of the (R) enantiomer of α-ionone with the (S) enantiomer being barely detectable. These jams were all declared not to have been added any aroma which the analyses confirmed.

Analyses of two out of five soda´s declared to contain natural aroma only, showed that these contained only (R)-α-ionone which is in accordance with the declared content. The three soda´s declared to contain aroma showed an

enantiomer ratio of 50%, which is, also, in accordance with the declaration and confirm that synthetic α-ionone had been added to the products.

The dried raspberries only contained the (R) enantiomer of α-ionone, so no synthetic α-ionone was added to this product. Six of the sweets samples had a 50:50 enantiomer ratio which is in accordance with the declared use of aroma in these samples. Two of the sweets were declared to contain raspberry juice concentrate, 5% as well as aroma. However, the content of ionone from this source must be very low as addition of raspberry juice containing exclusively the (R) enantiomer would have been expected to change the enantiomer ratio from the observed 50:50%. The fruit bar was declared to contain both 1.3% raspberry and natural aroma, however, the measured enantiomer ratio of 50:50% suggest that synthetic aroma was added to this product and accordingly the declaration is not compliant with legislation.

Finally, six yoghurt samples, declared to contain between 1.3% and 14% raspberries, were analyzed. Three of the samples were declared, in addition to raspberries, also to contain natural aroma. For five of the raspberry yoghurt samples only the (R) enantiomer of α-ionone were found in the products indicating natural raspberries were used in the product. However, for yoghurt #3 declared to contain 1.7% raspberry and natural aroma, a 50:50 enantiomer ratio for R-α-ionone and S-α-ionone was observed indicating that synthetic α-ionone was added as aroma to this product contrary to the declared use of natural aroma.

Conclusions

A fast and simple headspace SPME-chiral-GC-MS method for analyses of authenticity of l raspberry flavor in foods has been developed and used to analyze samples of jams, sodas, sweets, fruits bars, dried raspberries and yoghurts. Raspberry jam, dried raspberries and sodas declared to contain natural aroma all contained almost only R-α-ionone supporting the content of only naturally raspberry or naturally raspberry aroma used in the products. All sweets tested had an almost equal distribution between R-α-ionone and S-α-ionone indication that synthetic aroma was added to these products in agreement with the information from the declaration. For two out of the 27 products tested, the fruit bar and one of the raspberry yoghurts, both R-α-ionone and S-α-ionone was detected at an enantiomer ratio of 50% indicating the use of synthetic aroma in the products, which is in disagreement with the declared information on the products.

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241

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Article

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Authenticity and Traceability of Vanilla Flavors by Analysis of Stable Isotopes of Carbon and Hydrogen

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ABSTRACT: Authenticity and traceability of vanilla flavors were investigated using gas chromatography−isotope ratio mass spectrometry (GC−IRMS). Vanilla flavors produced by chemical synthesis ($n = 2$), fermentation ($n = 1$), and extracted from two different species of the vanilla orchid ($n = 79$) were analyzed. The authenticity of the flavor compound vanillin was evaluated on the basis of measurements of ratios of carbon stable isotopes ($\delta^{13}C$). It was found that results of $\delta^{13}C$ for vanillin extracted from *Vanilla planifolia* and *Vanilla tahitensis* were significantly different (t test) and that it was possible to differentiate these two groups of natural vanillin from vanillin produced otherwise. Vanilla flavors were also analyzed for ratios of hydrogen stable isotopes ($\delta^{2}H$). A graphic representation of $\delta^{13}C$ versus $\delta^{2}H$ revealed that vanillin extracted from pods grown in adjacent geographic origins grouped together. Accordingly, values of $\delta^{13}C$ and $\delta^{2}H$ can be used for studies of authenticity and traceability of vanilla flavors.

KEYWORDS: *isotope ratio mass spectrometry, stable isotope ratios, adulteration, vanillin, geographic origin, GC−IRMS*

INTRODUCTION

Consumers are increasingly interested in the authenticity and traceability of food products. The trend is not only toward more natural products and ingredients, but geographic origin and method of production can also affect the consumer's choice of products.[1] Tracing the geographic origin of a product is important because the origin can imply a characteristic flavor, certain traditional production method, or specific species of plants that only grows in a particular climate.[2] Such parameters are often reflected in a higher price of a product, which is why there could be a financial benefit from falsification. It is illegal in the European Union (EU) to label a product with false information;[3] however, falsification can be difficult to verify using traditional analytical methods. Therefore, new methods are needed to protect consumers and local producers from such fraudulent practices.

Vanilla is commonly used in a wide range of foodstuffs, beverages, and cosmetics. However, the current world production of natural vanilla is not nearly enough to meet the demand.[4] In addition, vanilla pods are difficult to produce,[5] leading to high prices of natural vanilla and, as a consequence, the risk of falsification. Falsification can include partly or wholly substituting vanilla flavor extracted from vanilla beans with a cheaper vanilla flavor and labeling with the incorrect botanical species or the incorrect geographical origin of growth.

Vanilla flavor is traditionally extracted from the tropical orchid of the genus *Vanilla*, which includes over 100 different species of which *Vanilla planifolia* and *Vanilla tahitensis* are the most widely used.[4] Natural vanilla contains a number of compounds contributing to the characteristic vanilla flavor. The main flavor-contributing molecule, 4-hydroxy-3-methoxybenzaldehyde, also called vanillin, is often solely used for the production of artificial vanilla flavor. Besides extraction from vanilla pods, vanillin can be made by chemical synthesis and fermentation. Vanillin produced by fermentation, also called biovanillin, is more expensive to produce than synthetic vanillin but cheaper than vanillin extracted from vanilla pods and, therefore, a potential substitute for this.[4] Biovanillin can advantageously be labeled a "natural flavoring substance", because it is obtained from a natural precursor,[6] namely, plant material, i.e., eugenol derived from clove oil or ferulic acid derived from lignin obtained from agricultural residues.[7]

Vanilla orchids primarily use the crassulacean acid metabolism (CAM) for fixing carbon dioxide, while the substrate mostly used for production of vanillin by fermentation is based on plants fixing carbon dioxide via the C_3 metabolic pathway. The two ways of carbon fixation cause differences in the ratio of the heavy to light stable carbon isotope ($\delta^{13}C$) that is later incorporated into the vanillin molecule, and therefore, it can be used to differentiate natural vanillin from biovanillin. Synthetic vanillin made from petrochemicals are depleted in the heavy carbon isotopes and expected to show the lowest values of $\delta^{13}C$, while vanillin from the vanilla orchid would have the highest values. Vanillin produced by C_3 plants is expected to have values of $\delta^{13}C$ in the same range as vanillin made from petrochemicals.[8,9]

Natural vanilla is a native of Mexico but is today grown over a large area along the equator.[10] The geographical origin of growth can be considered a parameter of quality; consequently, certain vanilla pods are being marketed on the basis of their origin.

The ratios of the heavy/light stable hydrogen isotope ($\delta^{2}H$) in precipitation differ over the world primarily because of longitudinal, altitudinal, and continental effects (Figure 1). As a result, the $\delta^{2}H$ value of water taken up by a plant depends upon

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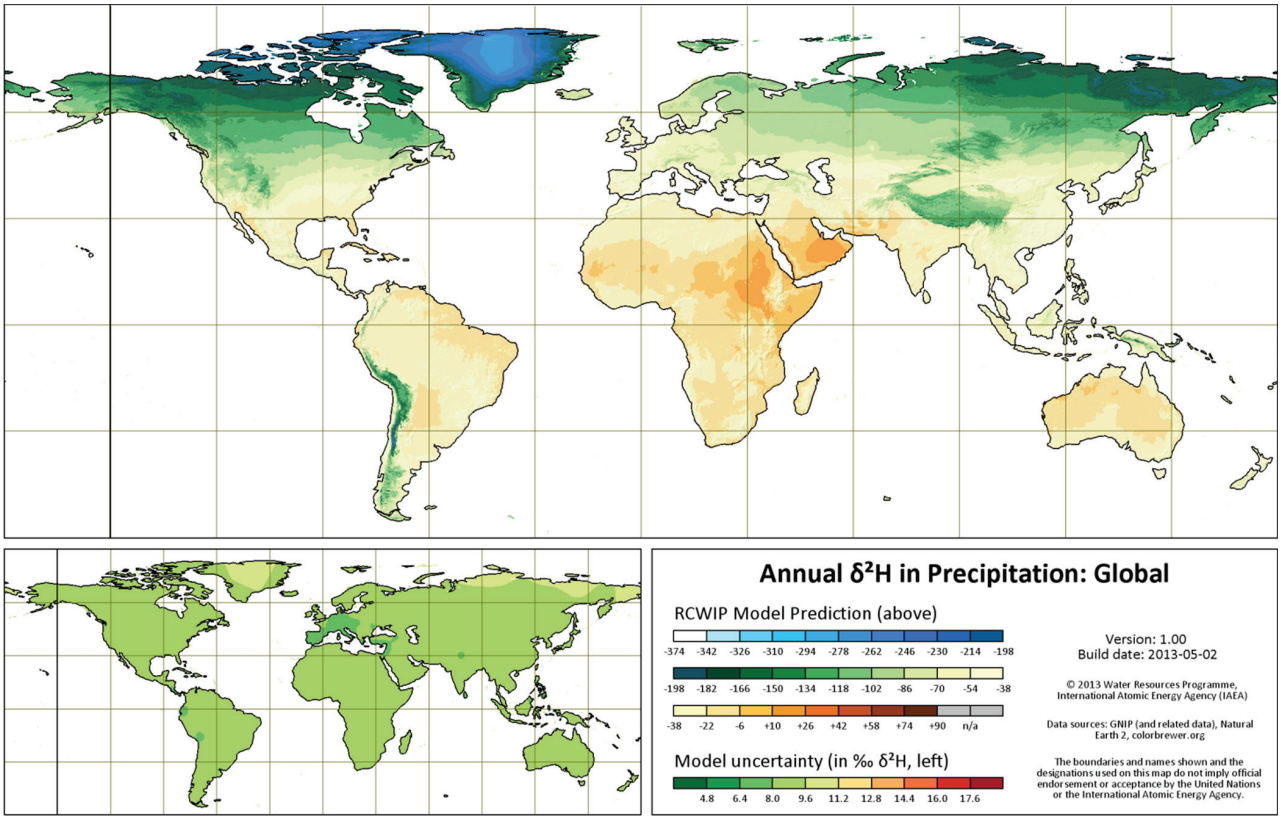

Figure 1. Regionalized cluster-based water isotope prediction model of δ^2H in precipitation, developed by the International Atomic Energy Agency (IAEA).[22−24]

the location of its habitat. Because the plant absorbs water and uses it for biosynthesis of metabolites, the values of δ^2H in the local precipitation will be reflected in these secondary metabolites, for example, vanillin from the vanilla orchid.[11−13]

The values of δ^{13}C and δ^2H can be measured by isotope ratio mass spectrometry (IRMS). IRMS is capable of measuring the natural abundance of stable isotopes.[14] Previous studies have investigated the authenticity of vanilla flavor using either elemental analyzer−IRMS (EA−IRMS) or gas chromatography−IRMS (GC−IRMS).[15−17] In these studies, it was shown that GC can be used to separate vanillin from the other components of a vanilla extract and that compound-specific data can be measured.

In this study, three types of vanillin were analyzed by gas chromatography−combustion−isotope ratio mass spectrometry (GC−C−IRMS) to investigate if the δ^{13}C values differed enough to assign each group with a specific δ^{13}C interval. In this way, an unknown sample of vanillin can be categorized by measuring its δ^{13}C value. Additionally, gas chromatography pyrolysis isotope ratio mass spectrometry (GC−P−IRMS) was used for determining δ^2H of vanillin from vanilla pods from various locations to test if it is possible to assign a δ^2H interval to a specific geographic area. To the knowledge of the authors, this is the first time measurements of δ^2H of vanilla pods from more than three different geographical origins have been characterized.

MATERIALS AND METHODS

Samples. A total of 82 vanilla flavors of different origin were analyzed: 2 produced synthetically by Sigma-Aldrich (Brondby, Denmark), 1 sample produced by fermentation of natural eugenol by Rhodia (www.rhodia.com), and 79 natural vanilla pods of different types and geographical origin: 54 pods of the type *V. planifolia*, 15 pods of *V. tahitensis*, and 10 pods of unknown species. The geographical origin was known for 71 of the vanilla pods. The vanilla pods analyzed, originated from Madagascar, French Polynesia, India, New Caledonia, Tonga, Vanuatu, Mexico, Africa (Congo and Uganda), Indonesia, and New Guinea (including Papua New Guinea).

Chemicals. Ethanol used for extraction was of 96% purity and purchased from CCS Healthcare AB (Borlänge, Sweden). Ethyl acetate and cyclohexane used for extraction were purchased from Rathburn Chemicals, Ltd. (Walkerburn, U.K.), and anhydrous sodium sulfate was purchased from Merck (Darmstadt, Germany). A mixture of C_{11}, C_{15}, and C_{20} alkanes (Chiron, Trondheim, Norway) with known values of δ^{13}C (−26.11, −30.02, and −33.06‰) were used for sample calibration against the reference standard: Vienna-Pee Dee Belemnite (V-PDB). Two samples of caffeine (1 and 2) with different δ values (91.4 and −152.8‰), purchased from Arndt Schimmelmann (Indiana University, Bloomington, IN), were used for δ^2H calibration against the reference standard: Vienna-Standard Mean Ocean Water (V-SMOW).

Sample Preparation. Vanilla pods were cut lengthwise; the seeds were scraped out; and the rest of the pod was cut in pieces less than 5 mm. Vanilla seeds and pods were placed in a closed flask, and for every 15 g of vanilla, 46 g of water and 54 g of 96% ethanol were added and macerated at ambient temperature for approximately 72 h during constant stirring with a stir bar (500 rpm). The flask was protected against sunlight. Afterward, the mixture was filtered, and the filter cake was pressed. This method extracted most of the vanillin because a second extraction resulted in less than 10% vanillin content compared to the first extraction. A total of 3 mL of the ethanol/water extract was further extracted with 3 mL of ethyl acetate/cyclohexane (1:1) and dried with anhydrous sodium sulfate. Samples were diluted 4 times

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with ethyl acetate/cyclohexane (1:1) prior to GC−C−IRMS analyses, while no dilution was used before GC−P−IRMS analyses. One extraction was made for each vanilla pod. Synthetic vanillin and vanillin from eugenol were dissolved in ethyl acetate/cyclohexane (1:1). Each sample was analyzed 3 times by GC−C−IRMS and GC−P−IRMS, and results are given as a mean of the 3 determinations.

GC Analyses. Separation of the samples was performed on a Trace GC Ultra (Thermo Scientific, Bremen, Germany) fitted with a DB-5 capillary column (Agilent Technologies, Böblingen, Germany) (30 m × 0.250 mm inner diameter, with d_f of 0.25 μm). The following GC conditions were employed: splitless injection, injector temperature of 230 °C, initial oven temperature of 40 °C, ramp at 4 °C/min to 150 °C, and ramp at 7 °C/min to 260 °C for 1 min. Helium was used as the carrier gas at 1.4 mL/min for GC−C−IRMS analysis and 1.2 mL/min for GC−P−IRMS; otherwise, the GC parameters were the same for the determination of δ^{13}C and δ^{2}H.

IRMS Analysis. For the determination of δ^{13}C, compounds eluting from GC were combusted in an oxidation reactor (NiO tube with NiO/CuO/Pt) operated at 1000 °C, purchased from Thermo Scientific (Bremen, Germany). For δ^{2}H determinations, compounds eluting from GC were converted in a high-temperature pyrolysis reactor (HTC reactor), consisting of a ceramic tube with no catalyst and operated at 1420 °C. The HTC reactor was purchased from Thermo Scientific (Bremen, Germany). The converted compounds from either the combustion reactor or the HTC reactor were analyzed on a Delta V Advantage isotope ratio mass spectrometer from Thermo Scientific (Bremen, Germany).

Standardization against Primary Standards. The standards of known isotopic values were analyzed in every sequence together with vanilla samples. The measured isotopic values for the standards were used to create an isotopic calibration of the samples, to express the final results in delta notation with the unit "per mille" versus V-SMOW and V-PDB, separately: $\delta = (R_{sample} - R_{standard})/R_{standard} \times 1000‰$, where R is the ratio of the heavy/light isotopes.

Statistical Analysis. t tests have been used for comparison of means between two groups. Variance homogeneity have been established preliminary using F tests. One-way analysis of variation (ANOVA) has been used when more than two groups are compared. Afterward, Tukey's test is used to establish the correlation between the groups.

RESULTS AND DISCUSSION

Vanillin is the main contributing compound to the vanilla flavor. The molecule is identical in its elemental composition regardless of the production method: natural, synthetic, or biovanillin. We determined the ratios of stable carbon isotopes by GC−C−IRMS of 82 vanillin samples to test if these results could be used to differentiate between the three production methods. A total of 2 samples of synthetic vanillin, 1 sample of biovanillin, and vanillin extracted from 79 vanilla pods were analyzed. Figure 2 shows a partial GC−C−IRMS chromatogram of mass 44 for a vanilla extract.

Authenticity of Vanillin: Plant Species. There was a significant difference in the δ^{13}C between vanillin extracted from *V. planifolia* compared to vanillin from *V. tahitensis* (Figure 3) (t test; $p < 0.001$). The range of δ^{13}C values was from −21.8 to −17.8‰ for *V. planifolia* (n = 54) and from −19.1 to −15.5‰ for *V. tahitensis* (n = 15). The results show that *V. tahitensis* is more enriched in the heavy carbon isotope than *V. planifolia*. These findings are consistent with the results obtained by Scharrer et al. and Greule et al.[16,18]

Authenticity of Vanillin: Production Method. Synthetic vanillin from two different productions was found to have values of δ^{13}C at −27.4 and −31.7‰, while biovanillin was measured to −30.9‰ (Figure 3). Because the δ^{13}C value of biovanillin is within the range found for synthetic vanillin, the stable isotope analysis of carbon is not sufficient to differentiate

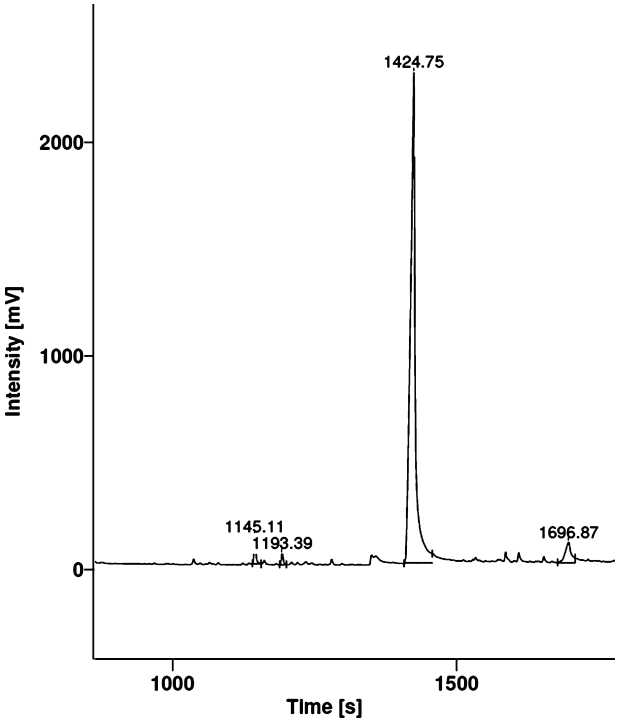

Figure 2. Partial GC−C−IRMS chromatogram showing the ion current of mass 44. The large peak with the retention time at 1424 is vanillin.

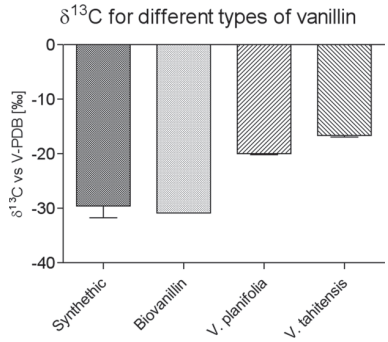

Figure 3. δ^{13}C versus V-PDB for vanillin of known authenticity: obtained from chemical synthesis (n = 2), fermentation of eugenol (n = 1), and vanilla pods of the types *V. planifolia* (n = 54) and *V. tahitensis* (n = 15). Error bars equal standard errors of the means within the sample group.

the two groups and other methods have to be considered for authentification, i.e., measurements of site-specific isotopic ratios.[19] The δ^{13}C value of the sample of biovanillin was −30.9‰, which is within the interval for C_3 plant material. Stable isotope analyses of synthetic and natural eugenol showed values of δ^{13}C similar to biovanillin from eugenol (results not shown) and indicate that the isotope ratio of carbon does not change significantly when converted from eugenol to vanillin during fermentation.

Precursors used for the production of synthetic vanillin are generally derived from petrochemicals, with a δ^{13}C similar or lower than C_3 plants.[20] In the case of biovanillin made by the fermentation of eugenol, the δ^{13}C values found are similar to the values of synthetic vanillin in this study. However, Scharrer

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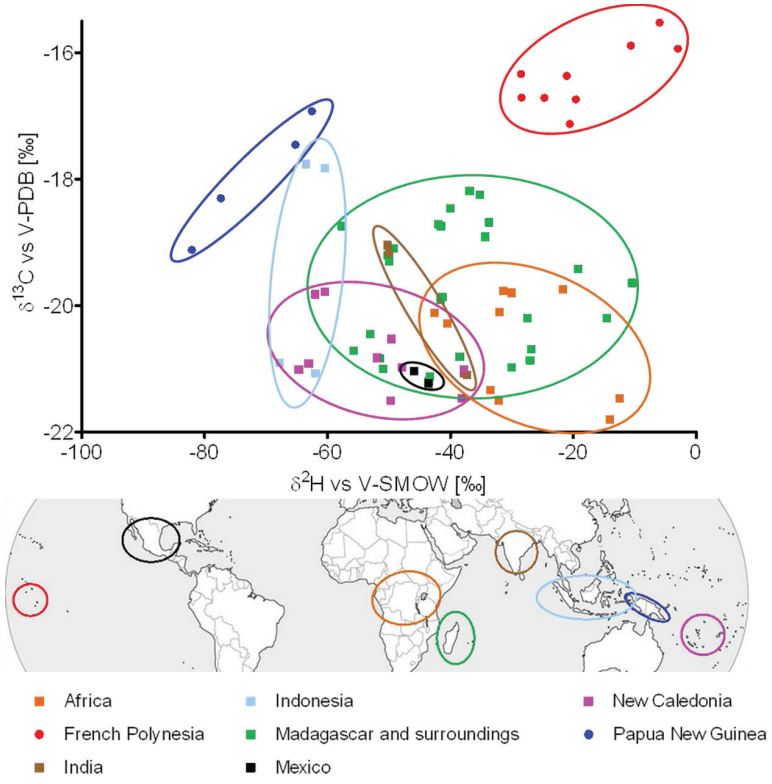

Figure 4. Results of δ^2H versus V-SMOW and δ^{13}C versus V-PDB for vanillin extracted from vanilla pods. Points are labeled with geographic origin. The geographical origins are separated by colors, with *V. tahitensis* represented by circles and *V. planifolia* represented by squares. The large circles in the scatter plot are color-correlated to the circles in the world map, showing the regions from where the samples originate.

Table 1. Measured Values of δ^{13}C versus V-PDB and δ^2H versus V-SMOW for Vanilla Pods Originating from New Caledonia, French Polynesia, Mexico, Africa, Madagascar, India, Indonesia, and New Guinea

		δ^{13}C versus V-PDB (‰)				δ^2H versus V-SMOW (‰)			
	n	$\bar{x}$	*s*	minimum	maximum	$\bar{x}$	*s*	minimum	maximum
New Caledonia, Tonga, and Vanuatu	10	−20.8	0.6	−21.5	−19.8	−52.5	9.8	−64.6	−37.8
French Polynesia	9	−16.4	0.5	−17.1	−15.5	−18.0	9.4	−28.5	−3.0
Mexico	2	−21.1	0.1	−21.2	−21.0	−44.7	1.6	−45.9	−43.6
Africa (Congo and Uganda)	10	−20.6	0.8	−21.8	−19.7	−29.0	10.1	−42.6	−12.4
Madagascar	28	−19.8	1.0	−21.3	−18.2	−38.9	12.1	−57.7	−10.4
India	4	−20.1	1.2	−21.1	−19.0	−43.8	7.3	−50.2	−37.3
Indonesia	4	−19.4	1.9	−21.1	−17.8	−63.4	3.2	−67.8	−60.4
New Guinea	4	−18.0	1.0	−19.1	−16.9	−71.8	9.4	−82.1	−62.5

et al. found that biovanillin could be differentiated from synthetic vanillin using GC−C−IRMS. The precursor for the biovanillin used by Scharrer et al. was not specified.[18] Therefore, it might be possible to authenticate biovanillin made from C_3 plant material other than eugenol using the measurement of δ^{13}C.

Values of δ^{13}C for natural vanillin extracted from vanilla pods were in the range from −21.8 to −15.5‰, while vanillin from other sources had values of δ^{13}C between −31.7 and −27.4‰. Thus, natural vanillin is more enriched in the heavy carbon isotope compared to vanillin originating from fermentations or chemical synthesis. This difference was expected because natural vanillin is extracted from CAM plants, which have higher values of δ^{13}C than C_3 plant materials used for the production of biovanillin by fermentations. O'Leary found that CAM plants often have values of δ^{13}C between −20 and −10‰, while δ^{13}C for molecules from C_3 plants have values between −33 and −24‰.[9] The results from this study are consistent with the findings by O'Leary.

To differentiate between vanillin from vanilla pods and vanillin made otherwise, the values of synthetic vanillin and biovanillin are tested against values of δ^{13}C for vanillin extracted from vanilla pods. Because the two species of vanilla pods are found to have significant different means, the non-natural vanillin has to be tested against both species separately. This results in three groups: one containing synthetic and biovanillin, a second containing all vanillin results for *V. planifolia*, and a third group with vanillin results for *V. tahitensis*. A one-way ANOVA followed by a Tukey's test showed significant difference between the three groups ($p < 0.001$). Values of δ^{13}C versus V-PDB can therefore be used to determine if vanillin originates from a vanilla pod or is produced otherwise.

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Traceability of Vanillin. Ratios of the heavy/light stable isotopes of hydrogen differ around the world primarily as a result of longitudinal, altitudinal, and continental effects. Because plants take up water from their surroundings, the ratio of stable hydrogen isotopes in plants will resemble the ratio of their specific geographic location.[13] In this study, natural vanillin was analyzed for δ^2H by GC−P−IRMS to investigate whether the differences in δ^2H values are large enough to differentiate between the geographic origins of growth.

Figure 4 shows results of δ^{13}C plotted against results of δ^2H for vanillin extracted from vanilla pods. The average, standard deviation, maximum, and minimum values of δ^{13}C and δ^2H for each geographic origin are shown in Table 1. Samples from French Polynesia and New Guinea are all of the species *V. tahitensis*, while the rest of the vanilla samples are *V. planifolia*. Samples of *V. tahitensis* from New Guinea are more depleted in the heavy carbon isotope than samples of *V. tahitensis* from French Polynesia. Growth conditions may be a possible explanation for the differences in values of δ^{13}C between these two countries. West et al. describes how marijuana grown in an indoor environment has significant lower values of δ^{13}C than plants grown outdoors. The respired CO_2 has lower values of δ^{13}C than atmospheric CO_2. Therefore, in a closed environment, with poor ventilation, the respired CO_2 will not mix well with the atmosphere and, thereby, decrease the values of δ^{13}C.[21] In places with intensive sunlight, it is necessary to protect the vanilla orchid, either in the form of support trees or by covering the plants.[5] In the last-mentioned case, the cover could cause poor ventilation and, consequently, lower the values of δ^{13}C in the vanilla plants.

Figure 4 shows results of δ^{13}C versus δ^2H for natural vanillin, and it is seen that adjacent geographic locations are grouped together. Vanillin extracted from vanilla pods originating from French Polynesia differ from the other samples. Because vanilla pods from French Polynesia are solely of the species *V. tahitensis*, which are more enriched in the heavy carbon atom than *V. planifolia*, they can be separated from other geographic origins growing *V. planifolia* based on their values of δ^{13}C. Even within samples of *V. tahitensis*, the two different locations, French Polynesia and New Guinea, are easily separated ($p < 0.001$) because their values of δ^2H differ by more than 30‰.

The lowest δ^2H values were found for vanillin extracted from vanilla pods from New Guinea and Indonesia, with mean values of −71.8 ± 9.4 and −63.4 ± 3.2‰, respectively. India and New Caledonia samples are more enriched in deuterium than New Guinea and Indonesia. δ^2H mean values are −43.8 ± 7.3‰ for India and −52.5 ± 9.8‰ for New Caledonia. Values of δ^2H for vanillin from Madagascar and surrounding islands show more variation than the other origins analyzed (Figure 4). The mean δ^2H value of vanillin samples from Madagascar is −38.9 ± 12.1‰. The highest values of δ^2H are found for French Polynesia and Africa, with a mean value of −18.0 ± 9.4 and −29.0 ± 10.1‰, respectively.

One of the highest values of δ^2H is found for vanilla grown in Africa (Uganda and Congo). From Africa, there is a tendency toward decreasing values of δ^2H moving east. This trend can also be seen in the regionalized cluster-based water isotope prediction (RCWIP) model developed by IAEA,[22−24] which shows the predicted annual δ^2H in precipitation (Figure 1). The agreement between the measured δ^2H values of vanillin and the RCWIP model supports that the plants incorporate water from its surroundings without significant isotopic fractionation.[13]

Tests showed that values of δ^{13}C versus V-PDB for *V. planifolia* are significantly different from those of *V. tahitensis*, To use this knowledge about vanilla species, the geographical origins are separated into two groups: one group for origins growing only *V. planifolia* (Madagascar, Mexico, India, Africa, Indonesia, and New Caledonia) and a second group growing only *V. tahitensis* (French Polynesia and New Guinea). Values of δ^2H versus V-SMOW for the geographical origins in the group of *V. planifolia* are tested using one-way ANOVA followed by a Tukey's post-test. This test shows that values of δ^2H for vanillin originating from Indonesia can be separated from vanillin from Africa ($p < 0.001$) and Madagascar ($p < 0.01$). Furthermore, vanillin from New Caledonia can be separated from vanillin originating from Africa ($p < 0.001$) and Madagascar ($p < 0.05$). Values of δ^2H versus V-SMOW for vanilla pods originating from French Polynesia and New Guinea are tested using a *t* test and found to be significantly different ($p < 0.001$).

The combined use of results of δ^{13}C and δ^2H analyses for vanillin, extracted from vanilla pods, shows a relatively close clustering pending upon species as well as geographic location of growth. Analysis of δ values of carbon and hydrogen by compound-specific IRMS is a useful tool in the verification of the authenticity and traceability of vanilla pods.

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The authors declare no competing financial interest.

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Authenticity of vanillin in food products determined by SPME-GC-IRMS

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Chemical compounds studied in this article:

Vanillin, (PubChem CID: 1183)

Abstract

An analytical method for authentication of vanillin in real food samples by stable isotope ratio analysis is described. Dry and low fat samples, such as custard powder, vanilla sugar and cookies, were analysed by head space SPME prior to GC-IRMS analyses. Ice cream samples were treated with acetone to break the emulsion and to precipitate proteins and phospholipids. After filtration the residue was concentrated on a rotary evaporator to reduce the content of acetone prior to extraction of the vanillin with ethyl acetate. The organic phase was transferred to a head space vial and evaporated to dryness followed by SPME-GC-IRMS analysis. Fourteen vanilla flavoured foods from the Danish retail marked was analysed and for 9 of the food samples the measured $\delta^{13}C$ values for vanillin were in accordance with the declared content of vanilla/aroma and for 5 food samples this was not the case.

1. Introduction

One of the most commonly used flavourings is vanillin which is used in a wide range of food products such as bakery, desserts, sweets, and beverages (Korthou & Verpoorte, 2007). Vanilla flavour is traditionally extracted from fermented and dried vanilla pods from the vanilla orchid. The characteristic flavour of vanilla are composed by many different flavour compounds where of 4-hydroxy-3-methoxybenaldehyde, also called vanillin is the main flavour-contributing compound (Figure 1). The world production of vanilla flavours from vanilla pods are far from large enough to cover the demand, which is why vanilla flavours are also made synthetically. The major part of synthetic vanillin is produced from raw materials such as lignin and guaiacol (Hilmer, Hammerschmidt, & Losing, 2010). Counterfeit of natural produced flavours is a huge problem within the flavour industry. Many consumers prefer the label "natural" and are willing to pay extra for such, which is why natural flavours are often used in food products. There is however, an economical gain in partly or wholly replacing natural flavours with synthetic made flavours. In the case of vanilla flavours, the price of natural vanillin can be more than 350 times the price of its synthetic equivalent (Korthou & Verpoorte, 2007).

The vanillin molecule has the same elemental composition whether it is made synthetically or extracted from vanilla pods, but the amount of stable carbon isotopes differs. Determination of the stable isotope ratio of carbon by GC-IRMS can be used to discriminate between natural and synthetic origin of vanillin in order to verify the authenticity. GC-IRMS has previously been used to assess the authenticity of γ- and δ-decalactones from peach, apricot and nectarine extracts (Tamura, Appel, Richling, & Schreier, 2005). Faulhaber et al. 1997 reported the $\delta^{13}C$ values of characteristic flavour compounds from mandarin essential oils, including: limonene, myrcene, linalool and octanal (Faulhaber, Hener, & Mosandl, 1997). Weinhart et al. 1999 used $\delta^{13}C$ values of methyl

55 salicylate versus $\delta^{13}C$ values of linalool, linalool oxide, trans-2-hexenal and cis-3-hexenol extracted
56 from samples of Ceylon, Assam and Darjeeling teas for the assessment of adulteration with methyl
57 salicylate (Weinert, Ulrich, & Mosandl, 1999). The authenticity of coriander essential oils were
58 assessed by the analyses of $\delta^{13}C$ values for limonene, γ-terpinene and geraniol (Frank, Dietrich,
59 Kremer, & Mosandl, 1995). Also, the analysis of $\delta^{13}C$ values of vanillin by GC-IRMS was used for
60 the assessment of the authenticity of vanillin from extracts from vanilla pods or vanillin from
61 various origins (Greule et al., 2010; Kaunzinger, Juchelka, & Mosandl, 1997) The compounds
62 specific isotope analyses referred above were either conducted on fruit/tea extracts or on essential
63 oils. However, the compound specific isotope analysis of flavouring compounds following the
64 addition to food products usually requires a much more complex sample preparation due to the
65 complexity of the food matrix. Reports on the compound specific isotope analysis of flavour
66 compounds in foods are therefore scarce. Lamprecht et al. developed a protocol for isolation and
67 EA-IRMS analysis of vanillin from ice cream and yoghurt samples which included laborious semi
68 preparative HPLC for isolation of vanillin from the samples (Guenther Lamprecht & Blochberger,
69 2009).

70 Here we report on the authenticity of vanillin in various food samples including ice-cream. The
71 $\delta^{13}C$ values of vanillin were measured by GC-IRMS following solid phase micro extraction
72 (SPME). SPME was used as a final extraction for all samples; however, for ice cream a
73 concentration and purification procedure was needed and a new and relatively simple sample work
74 up procedure was developed.

75

76

77 # 2. Materials and Methods

78 *2.1 Samples and chemicals*

79 Samples of vanilla pods, custard powder, vanilla sugar, cookies and ice-cream were purchased at
80 different retailers, all declared to contain either vanilla pods, natural flavor and/or flavor. Synthetic
81 vanillin, methylisobutylketone and acetone were purchased from Sigma-Aldrich (Steinheim,
82 Germany). SPME fibers were purchased from Supelco, (Supelco, USA), all coated with
83 polyacrylate on a fused silica fiber and with a length of 1cm.

84 *2.2 Sample preparation*

85 Custard powder and vanilla sugar were analyzed without any sample preparation and cookies were
86 crushed into a fine powder prior to analysis. Approximately 4g of sample was placed in a 10mL
87 headspace vial.

88 Two different sample preparation procedures were tested for work-up of ice-cream samples: A;
89 extraction with methylisobutylketone and B; precipitation and extraction with acetone.

90 Method A: approximately 100g of ice cream was mixed with 200mL 20% (V/V) ethanol and 10mL
91 15 % (W/V) Na_2CO_3 and mixed for 1 hour. Then centrifuged at 3500*g* for 10min. and kept in the
92 freezer overnight at -18°C. The next day the fat layer in the top of the tube was removed and the
93 samples thawed. The samples were extracted with 50 mL of heptane. The remaining aqueous phase
94 was acidified with 10mL of 6M HCl. The acidified aqueous phase was then extracted twice with
95 50mL methyl isobutyl ketone (MIBK). Centrifugation at 3500*g* for 10min. between extractions was
96 necessary. The organic phases were pooled and vanillin transferred to an aqueous phase by adding
97 10mL 0.1M NaOH. The alkaline aqueous phase was transferred to a 10ml head space vial and
98 acidified with 200µL glacial acetic acid.

Method B: approximately 25g of ice-cream in a beaker glass was allowed to thaw and added 50ml of acetone under stirring with a magnetic stirring bar. The suspension was maintained at 4°C for 30 min to precipitate phospholipids and proteins. The precipitate was removed by filtration and the filtrate was concentrated on a rotary evaporator at 40°C to a final volume of approximately 10ml (Büchi, Buchs, Switzerland). The residue was extracted twice with 5ml of ethyl acetate and the combined organic phase was dried with $MgSO_4$ and evaporated to dryness in a 10ml head space vial at 40°C under a gentle stream of nitrogen.

2.3 GC-IRMS analysis

The head space vials were heated to 60°C before the SPME fiber was exposed to the headspace. Extraction was carried out at 60°C for 30 min. using agitation. Hereafter analytes were thermally desorbed into the GC inlet at 230°C for 5min. in splitless mode. A Trace GC Ultra (Thermo Scientific, Bremen, Germany) fitted with a DB-5 capillary column (Agilent Technologies, Böblingen, Germany) (30m x 0.250mm inner diameter, with d_f of 0.25µm) was used for the separation of analytes and a Delta V Advantage IRMS (Thermo Scientific, Bremen, Germany) for determinations of $\delta^{13}C$ values.

The GC oven was initially set at 50°C and held at this temperature for 1min. Then the temperature was raised at 10°C/min to 120°C, raised at 2°C/min to 150°C, raised at 20°C/min to 260°C and held at this temperature for 5min. The helium flow was 1.0mL/min.

Samples were analyzed as single determinations, which were deemed sufficient as the result of the analyses is a ratio between ^{13}C and ^{12}C. However, in order to verify the precision of the analyses some samples were analyzed in replicate.

3. Results and discussion

The isolation of vanillin from whole foods is a difficult task due to the complexity of the matrix which may contain various substances such as fat, proteins, carbohydrates, water and emulsifiers. Vanillin is a semi volatile compound and therefore we investigated whether head space SPME was a useful method for isolation of vanillin from whole foods. The procedure worked well for most of the dry and low fat samples such as custard powder, vanilla sugar and a single sample of cookies. Fig 2. shows a GC-IRMS chromatogram. The ion current of the masses 44, 45, and 46 is shown. In other samples the vanillin content was too low to result in a sufficiently intense peak in the GC-IRMS analyses. However, for ice-cream the attempt was not successful and we therefore tested and compared two different extraction methods. In the first method (A), which was based on Lamprecht et al. 2009, vanillin was first extracted into alkaline 20% ethanol/water. Vanillin was ionized by the high pH in the suspension and therefore stays behind in the aqueous phase. After removal of fat on the surface of the suspension, heptane was added to remove any remaining lipids from the samples. In the next step pH was lowered so it was possible to extract vanillin with methyl isobutyl ketone. This step removes unwanted sugar from the matrix. Vanillin was then transferred to 0.1M NaOH solution and finally acidified before extraction using SPME. The first method was useful for the extraction and partial purification of vanillin from two low fat ice-cream samples prior to GC-IRMS

137 analyses. For the rest of the ice-cream samples the purification was insufficient to provide GC-
138 IRMS peaks from vanillin of sufficient intensity. Instead, a second extraction method (B) was
139 attempted. Egg lecithin, a phospholipid, is often used as emulsifier in production of ice-cream and
140 acetone was reported to precipitate phospholipids and proteins (Maximiano et al., 2008). Treatment
141 of ice-cream with two volumes of acetone under magnetic stirring resulted in efficient breaking of
142 the emulsion and in precipitation of phospholipids and proteins. Following filtration and
143 evaporation of most of the acetone, vanillin was extracted with ethyl acetate and transferred to head
144 space vials and evaporated to dryness prior to GC-IRMS analysis. This procedure gave sufficiently
145 intense peaks of vanillin in the analyses for the rest of the ice-cream samples.

146 The analysed food products were all declared to contain either vanilla, vanilla extracts and/or
147 aroma, see Table 1. Several products, in addition, showed pictures of vanilla pods on the package,
148 which might lead the consumer to expect that the product contains authentic vanilla.

149 Reported values for $\delta^{13}C$ are in the range of -15.5‰ to -21.8‰ for vanillin extracted from vanilla
150 pods, in the range of -26.9‰ and -31.7‰ for synthetic vanillin and about - 31‰ for biovanillin
151 made from fermentation using eugenol as precursor (Hansen, Fromberg, & Frandsen, 2014;
152 Lamprecht, Pichlmayer, & Schmid, 1994). Other studies report values of $\delta^{13}C$ for biovanillin
153 ranging from -33‰ to -37‰, but then the substrate used for fermentation was ferulic acid and rice
154 bran (Hilmer et al., 2010).

155 The results of the stable isotope ratio analyses of vanillin extracted from the food samples are
156 presented in Table 1. Custard powder no. 1 is declared to contain natural bourbon vanilla and
157 vanillin, however the δ value of -27.2‰ indicates the presence of only synthetic or biovanillin. For
158 custard powder no. 2 and 3 there seems to be agreement between the declared content and the
159 measured δ values. Custard powder no. 4 is declared only to contain Bourbon vanilla, however the

160 measured δ value of – 27.4‰ cannot confirm this. The measured δ value of – 20.9‰ for vanilla
161 sugar no. 1 is in agreement with the declared content of vanilla powder, whereas the measured δ
162 value of – 30.5‰ for vanilla sugar no.2 cannot confirm the presence of natural Bourbon vanilla
163 aroma. The one cookie sample is declared to contain Bourbon vanilla grains and natural aroma and
164 have a measured δ value of -33.3‰. Biovanillin made from rice bran and ferulic acid have been
165 reported to have δ values ranging from -37‰ and -33‰ (Hilmer et al., 2010), and according to EU
166 legislation it is allowed to label biovanillin as "Natural". The cookies were also labelled to contain
167 Bourbon vanilla, which is a type of vanilla flavour that can only be extracted from the vanilla pod.
168 The addition of both these flavours to the cookies might very well results in the measured δ value of
169 -33.3 ‰.

170 The measured δ value of -24.9‰ for ice-cream no.1 is in agreement with the declared content of
171 both natural vanilla extract and aroma. Also, for ice-cream no. 2 -5 the measured δ values are in
172 agreement with the declared content, although the δ value for ice-cream no. 3 is a little outside the
173 range for natural vanillin. Ice-cream no. 6 and 7 are both declared to contain both vanilla extract
174 and aroma, however the measured δ values of -27.2‰ and -31.8‰, respectively, indicates the
175 presence of synthetic vanillin only or only very low content of natural vanillin.

176

177 4. Conclusion.

178 The authenticity of vanillin in 14 vanilla flavoured foods was investigated by measurement of
179 compound specific isotope ratio analysis. Dry and low fat samples were analysed by head space
180 SPME prior to GC-IRMS analyses. For fat containing samples like ice cream a new purification
181 procedure was developed and applied to the samples prior to analyses. For 9 of the food samples the

measured $\delta^{13}C$ values for vanillin were in accordance with the declared content of vanilla/aroma and for 5 food samples this was not the case.

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Table 1: Values of δ^{13}C-vs VPDB for four types of custard powder, two types of vanilla sugar, one cookie, and seven different types of vanilla ice cream. The numbers of analyses are stated by the number in superscript after each result. The standard deviation for each sample was less or equal to 1.5 ‰.

Table 1:

Food product	Aroma declaration	δ^{13}C-vs VPDB [‰]	Extraction method
Custard powder no.			
1	Natural bourbon vanilla, vanillin	-27.2[1]	SPME
2	Aroma	-29.3[1]	SPME
3	Natural vanilla extract	-20.9[1]	SPME
4	Bourbon vanilla	-27.4[2]	SPME
Vanilla sugar no.			

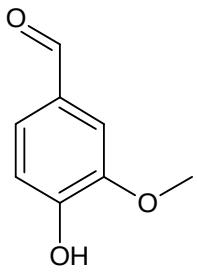
1	Vanilla powder	-20.9[1]	SPME
2	Natural bourbon vanilla aroma, aroma	-30.5[4]	SPME
Cookies no.			
1	Bourbon vanilla grains, natural aroma	-33.3[1]	SPME
Ice cream no.			
1	Natural vanilla extract, aroma (vanillin)	-24.9[1]	MIBK extract/SPME
2	Aroma	-31.9[1]	MIBK extract/SPME
3	Vanilla	-22.8[2]	Acetone extraction/SPME
4	Vanilla	-21.1[1]	Acetone extraction/SPME
5	Vanilla	-21.7[2]	Acetone extraction/SPME
6	Natural vanilla extract, aroma	-27.2[1]	Acetone extraction/SPME
7	Aroma,vanilla extract	-31,8[1]	Acetone extraction/SPME

Figure Captions:

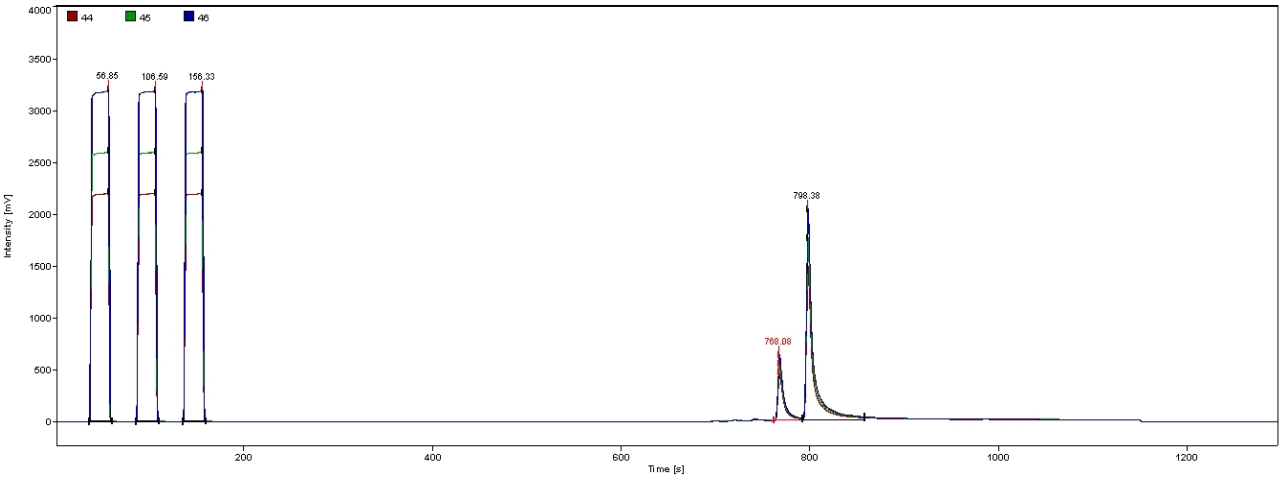
Figure 1: 4-hydroxy-3-methoxybenzaldehyde (vanillin)

Figure 2: GC-C-IRMS chromatogram. The ion currents of the masses 44, 45, and 46 are shown. Vanillin has a retention time at 798s.

Figure 1



241 Figure 2



242

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Optimizing GC Injections when Analyzing δ^2H of Vanillin for Traceability Studies

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Column overloading is a problem when analyzing δ^2H, due to the low natural abundance of deuterium and poor ionization efficiency of H_2. This problem can be overcome by using split injections instead of splitless. In this study we have compared the influence upon the measured isotopic ratios when using the two injection methods.

Introduction

In traceability studies of vanilla, the main aroma constituent 4-hydroxy-3-methoxybenzaldehyde (vanillin) was analyzed for its δ^2H values by Gas Chromatography Pyrolysis Isotope Ratio Mass Spectrometry (GC-P-IRMS). To obtain good precision, it is normally required to have at least 30ng of hydrogen on column depending on the instrument. However, the amount of vanillin in extracts of vanilla pods will overload a DB-5ms column with a film thickness of 0.25µm when injecting 1µL using splitless injection – as seen in figure 1. Chromatography can be improved by using split injections with a split ratio of 1:20. δ^2H should in theory not be affected by sample amount and therefore be the same independently of injection method.

Figure 1: Standards of vanillin containing 1578ng/µL analyzed using GC-P-IRMS with splitless injection (top) and split injection of 20:1 (bottom).

Results

In this study we compared values of δ^2H for vanillin using split and splitless injection. Splitless injections maintains a constant level of δ^2H (see figure 2) with standard deviation around 3‰ corresponding to the expected precision of the instrument. At low concentrations of vanillin the δ^2H values decreases when using split at 1:20.

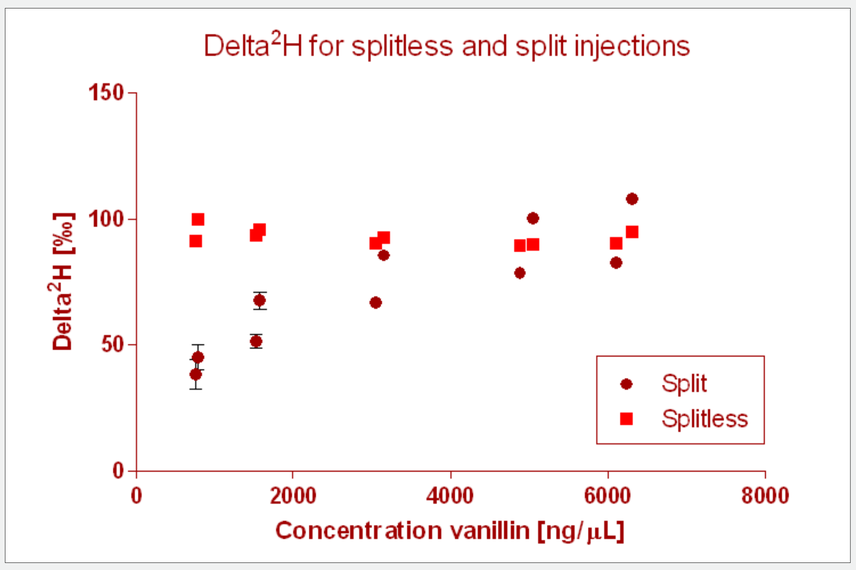

Figure 2: δ^2H for different concentrations of vanillin analyzed with GC-P-IRMS with splitless injection and split injection of 20:1. All other parameter was kept the same for the two experiments. Results are not standardized against V-SMOW because this would require that standards was run at the exact same conditions as the samples.

Accordingly, using split injections at low concentration will lead to discrimination against ^2H compared to splitless injections. It is therefore recommended to use splitless injections for analysis of δ^2H when analyzing extracts of vanilla pods for traceability studies.

When injecting 40ng vanillin on column for both methods, a deviation above 10‰ was found (data not shown). This indicates differences in the obtained values is not solely due to amount-dependent isotopic fractionation, but partly also due to fractionation during split injection.

Materials and methods

GC-IRMS: Trace GC Ultra fitted with a DB-5 capillary column (Agilent Technologies, Böblingen, Germany) (30m x 0.250mm i.d., d_f 0.25 µm) coupled to Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). High Temperature Conversion Reactor consisting of a ceramic tube with no catalyst was operated at 1420 C. Helium was used as carrier gas at 1.2mL/min. Injector temp. 230 C.

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