AUTHENTICITY AND TRACEABILITY OF FOOD AND FOOD FLAVOURINGS USING A STABLE ISOTOPE APPROACH

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Doctoral Dissertation
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PRISTNOST IN SLEDLJIVOST ŽIVIL IN AROM ZA ŽIVILA Z UPORABO STABILNIH IZOTOPOV LAHKIH ELEMENTOV

Doktorska disertacija

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Abstract

Food fraud or economically motivated adulteration has been an issue throughout history. Even today, it remains a significant and growing problem, driven by globalisation, economic opportunity and the low probability and severity of punishment. Fraud is a concern for food producers, consumers, regulatory agencies and scientific organisations, and food fraud prevention is paramount to protecting consumer trust and maintaining fair and sustainable business practices. This doctoral thesis focuses on two fraudulent acts, adulterating natural flavours with cheaper synthetic counterparts and the mislabelling of the country of origin of food crops. In both cases, verification of the authenticity of flavourings and geographical traceability of products from the market can be achieved through the following steps: 1) the development of suitable analytical methods; 2) the establishment of databases; and 3) data processing using chemometric approaches. This thesis addresses all three. Finally, developed methods and databases are used to determine the authenticity of commercial products using fruit, vanilla and truffle flavourings, and selected fruit and vegetable crops: asparagus, garlic, strawberry, cherry, apple and kaki as exemplary commodities, presented in eight interrelated scientific papers/studies.

The first part of the thesis, which is devoted to developing, optimising, and validating robust analytical techniques for flavour authenticity, shows the advantages of coupling the HS-SPME extraction technique with the GC-IRMS method. Accurate and reproducible $\delta^{13}C$ or $\delta^{2}H$ values were achieved for key volatile organic compounds (VOCs) present in various fruits, vanilla and truffle samples, over varying concentrations measured in short analysis time, within the same run and without using solvents. In addition, isotope fractionation was not observed when using optimised measurement parameters. Moreover, the procedure that includes a multiple-point isotopic linear normalisation method with peak size/linearity correction significantly improves the measurement error of small peaks (below 1 nA) from 3 ‰ to 0.5 ‰. For selected fruit and vegetable crops, stable isotope and multi-elemental analysis were used for the geographical origin tracing.

Data interpretation in the selected cases of frauds requires extensive reference data set of authentic food samples, i.e. a database or databank against which a sample under investigation can be compared. This thesis establishes dedicated databases of authentic, sufficiently representative samples that cover the natural variation of isotopic and elemental values. Altogether, ten different extensive databases were established over the period 2017-2021. Four include isotope values of fruit, vanilla and truffle VOCs and the other six include stable isotope (C, N, O and S) values and elemental composition of selected fruits and vegetables. In this thesis, isotopic characterisation of 35 VOCs was performed for the first time.

Data analysis and the interpretation of results are also essential parts of this thesis. Comparing isotope ratios of VOCs collected in the flavour databases allowed the successful discrimination between synthetic and naturally produced VOCs, although the addition of $\delta^{2}H$ data would further improve discrimination. However, a comparative analysis alone could not provide a definitive answer concerning the geographical origin of fruits and vegetables. For this reason, different chemometric approaches were explored. DD-SIMCA was chosen as most suitable method for determining whether the commercial product

complies with its declaration (i.e. Slovenian origin). The most important variables for classification were Sr, Ba Cs, S, Mo, Ni and Fe within elements and δ^{18}O and δ^{13}C within stable isotopes.

The developed approach represents an excellent foundation to verify the authenticity in real-word application. Therefore, the final part of this thesis is dedicated to verifying the naturalness of flavourings and the geographical origin of selected fruits and vegetables from the market. Results of commercial fruit, vanilla and truffle flavourings and their flavoured products imply that the authenticity can be questioned, most often within natural flavoured vanilla and truffle samples. Mislabelling of truffle species has also been identified. Also, 46 of the 124 (37 %) investigated fruit and vegetable samples did not correspond to their stated declaration.

In summary, this thesis shows that the developed analytical techniques used to establish a comprehensive set of databases and selected chemometric models ensure confidence in flavour authenticity studies and provide a sound basis for establishing an adequate traceability system for fruit and vegetables. Although the authenticity and traceability systems cover the needs of the food flavour industry and enforcement agencies in Slovenia, they can be readily transferred to other food commodities and countries. The thesis concludes by highlighting several new research questions and new avenues of research, especially in further developing HS-SPME-GC-IRMS for δ^{2}H determination of VOCs and more robust generalised DD-SIMCA models.

Povzetek

Prevare v živilski industriji oz. potvorbe v zvezi z živili z namenom pridobivanja finančne koristi so bile prisotne skozi vso zgodovino. Globalizacija, ekonomski izplen, nizka verjetnost odkritja in višina kazni se odražajo skozi naraščajoče število potvorb tudi v današnjem času. Te predstavljajo resno skrb za proizvajalce hrane, potrošnike, regulativne agencije in znanstvene organizacije. Zato je preprečevanje goljufij na področju živilstva ključnega pomena za zaščito zaupanja potrošnikov ter ohranjanje poštenih in trajnostnih poslovnih praks.

Doktorsko delo izpostavlja dve različni vrsti goljufij: ponarejanje naravnih arom s cenejšimi sintetičnimi analogi in napačno označevanje države izvora živilskih pridelkov. V obeh primerih je preverjanje mogoče doseči skozi proces, ki ga obravnava to doktorsko delo, in je sestavljen iz več faz: 1) razvoj ustreznih analitskih metod, 2) vzpostavitev baz podatkov in 3) obdelava pridobljenih podatkov s kemometričnimi metodami. Ne nazadnje pa je v delu prikazana uporabna vrednost razvitih metod in baz podatkov pri ugotavljanju pristnosti komercialnih proizvodov na primerih sadnih arom, arome vanilije in tartufov ter na izbranih vrstah sadja in zelenjave: špargljev, česna, jagod, češenj, jabolk in kakija. Rezultati so predstavljeni v osmih medsebojno povezanih znanstvenih prispevkih/študijah.

Prvi del doktorskega dela, ki je namenjen razvoju, optimizaciji in validaciji robustnih analitičnih tehnik, primernih za preverjanje pristnosti arom, prikazuje številne prednosti uporabe ekstrakcijske metode HS-SPME v kombinaciji z GC-IRMS. Izmerjene so bile natančne in ponovljive $\delta^{13}C$ in $\delta^{2}H$ vrednosti pomembnih hlapnih organskih spojin (HOC). Te spojine, prisotne v različnih vzorcih sadja, vanilije ter tartufov v različnih koncentracijah, so bile pomerjene v kratkem času, znotraj posamezne analize ter brez uporabe topil, ob tem pa metoda ob uporabi optimiziranih analitskih pogojev ne povzroča izotopske frakcionacije. Poleg tega postopek, ki vključuje metodo večtočkovne izotopske linearne normalizacije s korekcijo velikosti vrha/linearnosti, znatno izboljša merilno napako majhnih vrhov (pod 1 nA) s 3 ‰ na 0,5 ‰. Nadalje smo za sledenje geografskega izvora izbranih pridelkov sadja in zelenjave uporabili metodologijo določanja razmerij stabilnih izotopov z elementno analizo.

Interpretacija podatkov v izbranih primerih goljufij zahteva obsežen nabor podatkov o avtentičnih vzorcih (zbirke podatkov). Te uporabimo za referenčno primerjavo s preiskovanim vzorcem. Pomemben cilj doktorskega dela tako predstavlja vzpostavitev podatkovnih zbirk, reprezentativnih vzorcev, ki zajemajo naravno variacijo izotopskih in elementnih vrednosti preiskovanih živil. V obdobju 2017–2021 je bilo skupaj vzpostavljenih deset različnih obsežnih zbirk podatkov. Štiri izmed njih so vključevale izotopske vrednosti HOS sadja, vanilije in tartufov, ostalih šest pa izotopske vrednosti lahkih elementov (C, N, O in S) ter elementno sestavo izbranega sadja in zelenjave. V tem doktorskem delu je bilo prvič izotopsko okarakteriziranih 35 HOS.

Bistveni del tega doktorskega dela predstavljata tudi analiza podatkov in interpretacija rezultatov. Primerjava izotopskih razmerij izotopov HOS, zbranih v kreiranih podatkovnih zbirkah, omogoča v veliki meri uspešno ločbo med sintetičnimi in naravno proizvedenimi HOS. Kljub temu bi lahko podatek o izotopski sestavi vodika ($\delta^{2}H$) pri nekaterih spojinah vplival na doseganje boljše ločbe. Vseeno pa primerjalna analiza sama po sebi ne more dati dokončnega odgovora glede geografskega izvora sadja in zelenjave. V

ta namen so bile raziskane različne kemometrične metode. Metoda DD-SIMCA se je izkazala kot najbolj primerna pri določitvi, ali vzorec s tržišča ustreza njegovi označbi porekla (v našem primeru "slovensko poreklo"). Najpomembnejše spremenljivke za razvrstitev so bile Sr, Ba Cs, S, Mo, Ni in Fe med elementi in pa δ^{18}O ter δ^{13}C izmed stabilnih izotopov.

Razvit pristop predstavlja odlično podlago za preverjanje pristnosti v realni uporabi. Zadnji del doktorskega dela je tako namenjen preverjanju naravnosti arom in geografskega izvora izbranega sadja in zelenjave s tržišča. Rezultati analiz komercialnih arom sadja, vanilije in tartufov in njihovih aromatiziranih proizvodov kažejo na vprašljivo pristnost arom, najpogosteje zaznano v izdelkih, ki vsebujejo naravno aromo vanilije ali tartufov. Prepoznano je bilo tudi napačno označevanje vrst tartufov. Pri določanju geografskega izvora sadja in zelenjave pa kar 46 od 124 (37 %) preiskovanih vzorcev ni ustrezalo navedeni slovenski označbi porekla.

Če povzamemo, to doktorsko delo kaže, da razvite analitske metode, uporabljene pri vzpostavitvi obsežnega nabora podatkovnih zbirk, in izbrani kemometrični modeli zagotavljajo zaupanje v študije pristnosti arom in zagotavljajo trdno podlago za vzpostavitev ustreznega sistema sledljivosti sadja in zelenjave. Čeprav sistemi pristnosti in sledljivosti pokrivajo potrebe industrije arom in organov pregona v Sloveniji, jih je mogoče zlahka prenesti na druge živilske proizvode in države. Doktorsko delo na koncu predlaga tudi smernice za nadaljnje raziskovalno delo, predvsem na področju razvoja HS-SPME GC-IRMS metode za določanje δ^2H vrednosti HOS in razvoja robustnih DD-SIMCA modelov.

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Abbreviations

ANN	...	Artificial Neural Network
C3	...	C3 plants, Calvin cycle
C4	...	C4 plants, Hatch-Slack cycle
CRMs	...	Certified Reference Materials
CAGR	...	Compound Annual Growth Rate
CSIA	...	Compound Specific Isotope Analysis
CAM	...	Crassulacean Acid Metabolism
DD-SIMCA	...	Data-Driven Soft Independent Modelling of Class Analogy
δ	...	delta, relative isotopic composition, value given in part per thousand or "permille"
DVB/CAR/PDMS	...	Divinylbenzene/Carboxen/Polydimethylsiloxane
DHS	...	Dynamic Headspace Sampling
EA-IRMS	...	Elemental Analyser - Isotope Ratio Mass Spectrometer
EFSA	...	European Food Safety Authority
FoM	...	Figure of Merit
GC	...	Gas Chromatography
GC-C-IRMS	...	Gas Chromatography-Combustion Isotope Ratio Mass Spectrometry
GC-IRMS	...	Gas Chromatography-Isotope Ratio Mass Spectrometry
GC-MS	...	Gas Chromatography-Mass Spectrometry
GC-P-IRMS	...	Gas Chromatography-Pyrolysis Isotope Ratio Mass Spectrometry
GMOs	...	Genetically Modified Organisms
HS	...	Headspace
HS-SPME	...	Headspace-Solid Phase Microextraction
IRMM	...	Institute for Reference Materials and Measurements
i	...	mass number of the heavier isotope of an element (E)
ISO	...	International Organization for Standardization
IPS	...	International Postgraduate School
IRMS	...	Isotope Ratio Mass Spectrometry
IsoVoc	...	Isotope Volatile organic compounds
δ^{13}C	...	isotopic composition of carbon
δ^{2}H	...	isotopic composition of hydrogen
δ^{15}N	...	isotopic composition of nitrogen
δ^{18}O	...	isotopic composition of oxygen

$\delta^{34}S$	...	isotopic composition of sulphur
j	...	mass number of the lighter isotope of an element (E)
JSI	...	Jožef Stefan Institute
LOD	...	limit of detection
LDA	...	Linear Discriminant Analysis
LLE	...	Liquid–Liquid Extraction
MU	...	Measurement Uncertainty
MW	...	Molecular Weight
nA	...	nano-ampere
NIST MS	...	National Institute of Standards and Technology Mass Spectrometry
NMR	...	Nuclear Magnetic Resonance
No.	...	number(s)
OCC	...	One-Class Classification
OPLS-DA	...	Orthogonal Partial Least Squares Discriminant Analysis
PLS-DA	...	Partial Least Squares Discriminant Analysis
ppb	...	parts per billion
ppm	...	parts per million
PDMS	...	polydimethylsiloxane
PCA	...	Principal Component Analysis
PCs	...	Principal Components
PDO	...	Protected Designation of Origin
R_P	...	the ratio between the heavier and the lighter isotope in the sample
R_{Ref}	...	the ratio between the heavier and the lighter isotope in the reference material
^{1}H NMR	...	Proton Nuclear Magnetic Resonance
$^{13}C/^{12}C$	...	ratio of ^{13}C to ^{12}C
$^{15}N/^{14}N$	...	ratio of ^{15}N to ^{14}N
$^{18}O/^{16}O$	...	ratio of ^{18}O to ^{16}O
$^{2}H/^{1}H$	...	ratio of ^{2}H to ^{1}H
$^{34}S/^{32}S$	...	ratio of ^{34}S to ^{32}S
SDE	...	Simultaneous Distillation Extraction
^{2}H-SNIF NMR	...	site-specific natural isotopic fractionation as measured by nuclear magnetic resonance
SIMCA	...	Soft Independent Modelling of Class Analogy
SPME	...	Solid Phase Microextraction
SPE	...	Solid-Phase Extraction
SFE	...	Supercritical Fluid Extraction
EFFA	...	The European Flavour Association
US FDA	...	United States Food and Drug Administration
VCDT	...	Vienna Cañon Diablo Troilite

VIP	...	Variable Importance in Projection
VOCs	...	Volatile Organic Compounds
VPDB	...	Vienna Pee Dee Belemnite
VSMOW	...	Vienna Standard Mean Ocean Water
wt./vol.	...	Weight/Volume percentage concentration

Chapter 1

Introduction

The role of food in everyday life is essential and multifaceted. Food provides us with the nutrients and energy to develop and grow, be active and healthy, to move, play, work, think and learn. The main driver for eating is unequivocally hunger, but what we choose to eat is not determined solely by our physiological or nutritional needs. Factors that influence food choice include attitudes, beliefs and knowledge about food (EUFIC, 2006). Indeed, consumers have long been concerned about the quality, and particularly the safety, of the foods they eat. In recent years, this concern has taken on additional prominence. Reports about new risks posed by "mad cow" disease and familiar sources of risk, such as food-borne pathogens, pesticides, and hormones, have sharpened consumer focus on food safety. Trends also show increased consumer demand for various fresh and tasty foods available year-round, fostering increased global trade in food (Krissoff, Bohman, & Caswell, 2002).

Globalization has significant benefits, both in terms of access to food that can be grown more efficiently and cheaply elsewhere or those – especially fruit and vegetables – that may be seasonal but are in demand year-round. This kind of production often translates into more intensive production, increased fertilizer usage, and agricultural expansion, such as converting forested lands to fields. Such practices have significant environmental consequences, affecting water and soil quality, greenhouse gas production, and reduced biodiversity (Benton, 2017; FAO, 2018; Ranganathan, Waite, Searchinger, & Hanson, 2018).

Awareness of child labour, low wages, and animal welfare makes many consumers interested in knowing how and where their food is produced. There has also been an increase in interest in foods linked to a specific place or region. For example, consumers increasingly demand local food, food with a traditional character or image, more sustainable food that fulfils cultural identity needs (Pieniak, Verbeke, Vanhonacker, Guerrero, & Hersleth, 2009), and minimally processed food without unnecessary additives such as artificial colours flavours or preservatives. Food produced in this way is often perceived as higher quality, for which people are willing to pay a premium. Unfortunately, higher premiums make such foods vulnerable to food fraud.

Food fraud (Table 1) encompasses a range of deliberate fraudulent acts such as counterfeits and simulations, product tampering, production over-runs, theft, smuggling, document fraud, and diversions, adulterations intended to cause public health harm, economic harm, or terror, i.e., food defence issues (FAO, 2021; USP, 2016).

Table 1: Types of food fraud (FAO, 2021).

Term	**Definition**	**Example**
Adulterate	A component of the finished product is fraudulent	Melamine added to milk
Tampering and mislabelling	Legitimate products and packaging are used in a fraudulent way	Changed expiry information; fraudulent description of production method or origin
Over-run	The legitimate product is made in excess of production agreements	Under-reporting of production
Theft	Legitimate production is stolen and passed off as legitimately procured	Stolen products are mixed with legitimate products
Diversion	The sale or distribution of legitimate products outside of intended markets	Relief food redirected to markets where aid is not required
Simulation	An illegitimate product is designed to look like but not exactly copy the legitimate product	"Knock-offs" of popular foods not produced with the same food safety guarantees
Counterfeit	All aspects of the fraudulent product and packaging are fully replicated	Copies of popular foods not produced with the same food safety guarantees

This thesis addresses a specific type of food fraud, i.e., intentional and economically motivated adulteration. Adulteration is defined either as the fraudulent addition of non-authentic substances or the removal or replacement of authentic substances without the purchaser's knowledge for the economic gain of the seller (USP, 2016). All types of fraud are detrimental to the reputation of the agrifood industry and cause harm to consumers and legitimate businesses. Adulteration can also constitute risks to human and animal health and the environment and ignore moral and ethical principles. Such activities continue to increase. Between 2016 and 2019, the number of suspected food fraud cases in the EU increased by 85 %. The COVID-19 pandemic is also predicted to increase further the presence of substandard goods on the market (European Commission, 2021; Europol, 2020).

Some foods and ingredients are more vulnerable to food fraud than others. Supply and pricing, product attributes resulting in added value, differences in pricing due to regulatory diversity in different countries, the economic health of businesses, level of competition, and the financial strains imposed on suppliers are all economic factors affecting fraud vulnerability. In addition, products with a high value per kilogram, a defined provenance or production system (e.g., organic) or food processed in a specific way (e.g., artisanal products), and food with exceptional qualities (e.g., protected designation of origin, PDO) represent tempting targets. An increase in the complexity of supply chains also increases a

foodstuffs vulnerability to fraud since it decreases the transparency of the network (van Ruth, Huisman, & Luning, 2017). Currently, olive oil, milk, honey, saffron, orange juice, apple juice, grape wine, vanilla extract and fish are the most commonly adulterated foods ("Food Fraud | Knowledge for policy," n.d.). Except for vanilla extract, fraud relating to food flavours is less talked about, but as the demand for natural aromas increases together with the cost of raw materials, so does the potential for economically motivated adulteration (Mordor Intelligence, n.d.).

With increasing fraud, regulatory authorities and food processing industries have pushed for more accurate, standardised, robust analytical tools to confirm food product authenticity (e.g., label compliance) and support law enforcement (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Su, Arvanitoyannis, & Sun, 2018). However, developing such analytical methodologies for food authentication remains a challenge. The interpretation of the results has to be performed in the light of analytical uncertainty, natural variation, and any tolerance permitted by the requirements defining a particular food product. Another challenge is finding a marker (or markers) that characterises a food product, one of its ingredients, the adulterants in question, or its processing, production or geographic origin. A marker must be specific, have a limited natural variation, be well characterised, and there must exist the ability to measure it accurately (Primrose, Woolfe, & Rollinson, 2010).

In this regard, when assessing authenticity, apart from the measurement, data interpretation requires the existence of sufficient authentic food sample reference data, i.e., a database or a databank (Camin et al., 2017) against which an unknown food sample can be compared, usually by using different chemometric approaches (Granato et al., 2018; Medina, Perestrelo, Silva, Pereira, & Câmara, 2019). Since the selection of samples and chemometric approach depends on the problem of interest (Oliveri, 2017), the definition of the specific purpose of a particular database needs to be addressed at the beginning of the study (Donarski, Camin, Fauhl-Hassek, Posey, & Sudnik, 2019). A database must also be developed with the end-user and the intended purpose in mind. A database designed without a specific use in mind is likely to overlook crucial considerations for particular problems (Donarski et al., 2019).

1.1 The Authenticity of Food Flavourings

When food is consumed, the interaction of taste, odour, and textural feeling provides an overall sensation best defined by the term "flavour". Often people refer to a food's 'smell', 'odour' or 'scent' and sometimes about its 'aroma'. What is the difference? Flavour results from compounds divided into two broad classes, those responsible for taste and those responsible for odours. The latter are often designated as aroma substances. Compounds accountable for taste are generally non-volatile at room temperature, whereas aroma substances are volatile compounds perceived by the odour receptor sites of the smell organ, i. e., the olfactory tissue of the nasal cavity. We talk about 'odour' when substances reach the receptors as they are drawn in through the nose (ortho-nasal detection). While an odour is sometimes attributed to something foul, scent is usually associated with pleasant smells. When aroma substances reach the receptors via the throat (Fig. 1), we talk about the 'aroma' of food after being released by chewing, i.e., retro-nasal detection (Belitz, Grosch, & Schieberle, 2009). We, therefore, perceive odour molecules from food twice – once, directly, through the nose and a second time, indirectly, via the mouth (Academy, n.d.).

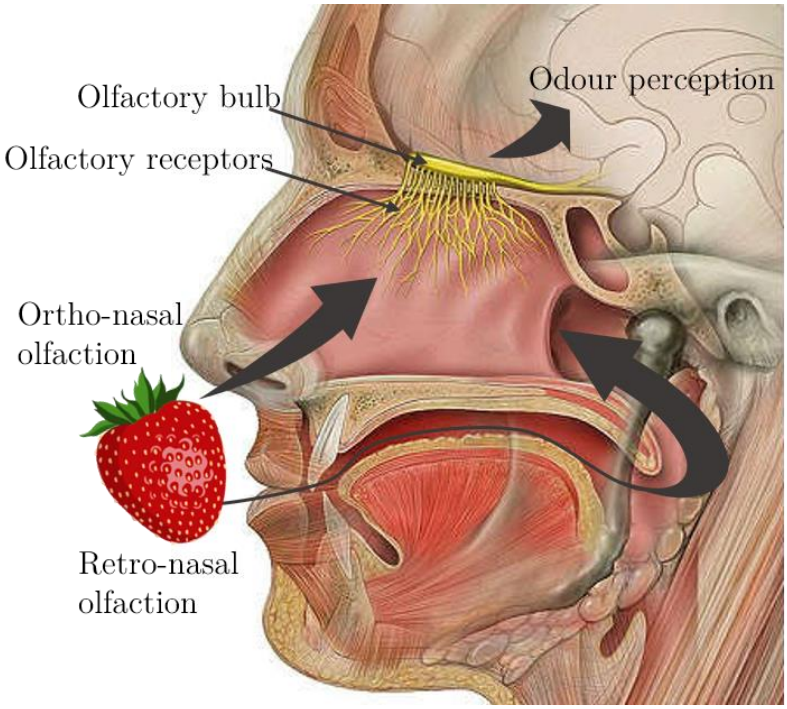

Figure 1: The perception of flavour (Figure adapted from Discover Magazine, (2019)).

Trillions of different odour compounds can be detected by humans (Bushdid, Magnasco, Vosshall, & Keller, 2014), and foods are made up of mixtures of these compounds. The amount of volatile substances present in food is low (ca. 10–15 mg/kg). The characteristic aroma of food products results from a complex mixture, often containing hundreds of compounds, which influence the enjoyment and acceptance of foodstuffs. The chemical structures of aroma compounds also vary and include, for example, acid and alkali compounds, sulphur and nitrogen compounds, alcohols, aldehydes, ketones, hydrocarbons, and esters. There are also significant differences in their volatility, ranging from components with boiling points well below room temperature (hydrogen sulphide, -60 °C) to those that are solid at room temperature (vanillin, 284 °C). In general, the mixture of many volatile compounds forms an aroma (odour). Especially foods made by thermal processes, either alone (e.g., coffee) or in combination with a fermentation process (e.g., bread, beer, cocoa, or tea), can contain more than 800 volatile compounds. A great variety of compounds is often present in fruits and vegetables (Belitz et al., 2009), e.g., apple can have more than 300 aroma compounds making up its olfactory character (Salas et al., 2016). However, of all the volatile compounds present, only a few have a considerable impact on the overall aroma (odour). Only in some cases can a food product's characteristic aroma be narrowed down to a particular compound, e.g., 4-hydroxy-3-methoxy-benzaldehyde (vanillin). Consequently, compounds that provide the characteristic aroma of the food are called key odorants and are essential in the flavouring industry (Belitz et al., 2009).

While flavour should only be used to describe the effects on the senses, flavourings are products added to food to impart, modify, or enhance the flavour of food and have been used since ancient times (International Organization of the Flavor Industry, 2020). After processing and preserving, perishable foods tend to lose their flavour over time, which creates the need to use flavouring substances to help maintain the flavour. The food and beverage industry also require flavourings for different purposes, such as new product

development, changing the taste of existing products, and maintaining consistent product flavour. High demand for new flavours also arises from the food and beverages industry, and continuous innovation drives the growth of the food flavours market. In addition, increasing demand from the fast-food industry is expected to create growth opportunities in the food flavours market. In 2020, the food flavours market was valued at $12.7 billion and is expected to reach $19.2 billion by 2030, registering a CAGR of 3.6 % from 2021 to 2030 (Kumar & Roshan, 2021).

1.1.1 Synthetic and natural flavourings

According to Regulation (EC) No. 1334/2008, flavourings refer to products made or consisting of the following categories: flavouring substances (defined chemical substance, e.g., vanillin), flavouring preparations (undefined chemical substance, e.g., vanilla extract), thermal process flavourings, smoke flavourings, flavour precursors or other flavourings or mixtures thereof. Producing flavours is a complicated task as the original taste and flavour is reduced during the extraction process, making it challenging to retain the original flavour (Kumar & Roshan, 2021). A raw material often contains low concentrations of the desired flavour compounds. Therefore, this natural raw material is rarely used in its native form, and the goal is to produce the densest concentration of aromatic compounds.

The starting point for maximizing an aromatic effect frequently involves using various physical separation methods, including extraction, distillation, or cold pressing of the natural material. Additional purification processes are often used to produce a sufficiently flavourful product, including fractional distillation, topping (removal of volatile parts), solvent extraction, supercritical extraction, thin-film evaporation and molecular distillation. Also, additional re-distillation may be used to remove colour, water, resinous material and unpleasant aroma or taste perceptions. Distillate/oil may then be combined with other sources or chemical constituents of the same distillate/oil to create a suitable flavour. When all the flavour parts have been mixed, the natural flavour complex may contain several hundred chemical constituents. Alternatively, flavourists can make synthetic flavours by combining chemicals made from inedible ingredients, such as paper pulp or petroleum, which smell and taste similar to natural flavourings. These mixtures are often simpler and contain fewer compounds than their natural counterparts, although the chemical structure of individual molecules is often the same (Kumar & Roshan, 2021).

In the 1960s, the flavour industry primarily manufactured more lucrative synthetic flavours, whereas nowadays, with health-conscious consumers preferring natural ingredients, there has been a market switch to natural flavours (Goodman, 2017). Consequently, the word “natural” is increasingly used in marketing food products (Longo & Sanromán, 2006). Currently, no regulatory agency overseeing food labelling defines natural ingredients, except for natural flavours. A search of natural flavours in the Environmental Working Group’s food database containing over 80,000 food products reveals that natural flavours are the fourth most common food ingredient listed on food labels (Goodman, 2017). The US FDA, European Food Safety Authority (EFSA), Japanese Ministry of Health, and other agencies, have precise requirements for labelling flavours as “natural”. However, definitions can vary significantly. Natural flavours are defined in the United States under regulation 21 CFR 101.22 as deriving from natural raw materials that contain no artificial constituents. Artificial within the meaning of this regulation means synthetic or petrochemical in origin. The raw materials that meet the natural definition include all animal products such as meat, egg and dairy. It also includes all botanical and microbiological sources, including fermentation products. Flavours obtained from genetically modified organisms (GMOs), including those modified using synthetic biology, are also considered natural.

In the EU, natural flavours are defined in Regulation (EC) 1334/2008 (2008). This regulation defines three criteria for natural flavours; 1) they must be "obtained by appropriate physical, enzymatic or microbiological processes", 2) they must be "from a material of vegetable, animal or microbiological origin", and 3) they must "correspond to substances that are naturally present and have been identified in nature." The EU definition of natural flavours is stricter than that in the US, and as a result, EU natural flavours meet the US requirement, but the reverse is not necessarily true. In the EU, the term "natural" also depends on the manufacturing method and the origin of the raw material. With so many different definitions of natural flavours, it is essential to know where they will be marketed for correct labelling. Also, a thorough understanding of the raw materials used and the manufacturing method is often needed to determine if the substance meets the local definition of a natural flavour (Grocholl, n.d.).

This thesis refers to current European legislation, which allows four terms for the sales description of natural flavourings. The term "natural flavouring substances" may only be used for flavourings in which the flavouring component contains exclusively natural flavouring substances. In contrast, the term "natural >x< flavouring" may only be used in combination with x representing a reference to food, food category or a vegetable or animal flavouring source if the flavouring component has been obtained exclusively or by at least 95 % by w/w from the source material. The other two terms are "natural >x< flavouring with other natural flavourings" and "natural flavouring". Other categories of flavourings described in EU regulation EC 1334/2008 are flavouring substances, thermal process flavourings, smoke flavourings, flavour precursors and other flavourings (Fig. 2). The category "flavouring substance" comprises all three categories referred to in Directive 88/388/EEC, i.e., natural flavouring substances, nature-identical flavouring substances and artificial flavouring substances. The denominations "nature-identical" and "artificial" no longer exist (European Flavour and Fragrance Association, 2019)

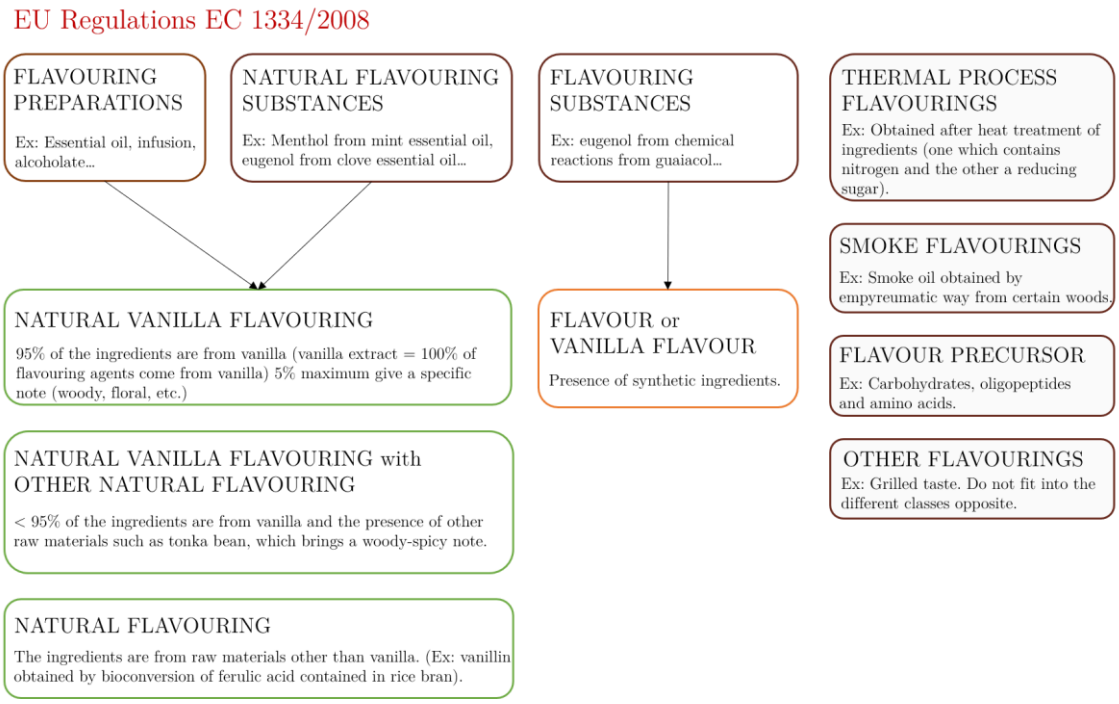

Figure 2: Denomination of different categories of flavourings, with examples of vanillin labelling, EU Regulation EC 1334/2008.

From a food manufacturer's perspective, the difference between a natural flavour and synthetic analogues often comes down to cost and consumer preference. The high costs associated with the extraction of natural flavours and dependence on their supply and other factors that are difficult to control, such as weather conditions and plant diseases, means that the price of natural flavours is often a factor of ten or higher compared to the price of synthetic analogues (Longo & Sanromán, 2006). High costs and the difficulty in differentiating between natural and synthetic flavours means that natural flavourings are substituted by synthetic ones. This substitution can place major food companies at legal and economic risk (G. Martin, Remaud, & Martin, 1993). Non-authentic products could also pose a potential health risk.

Several chemically defined substances are now no longer supported by industry or have even been removed from the "community list" of flavourings and source materials approved for use in and on foods due to safety concerns (Regulation (EU) No 872/2012, 2012). In addition, consumer confidence may be dampened by buying an inferior product sold as a genuine item, and the ability to trace and authenticate food products is becoming a priority in the food industry (van Leeuwen, Prenzler, Ryan, & Camin, 2014). Therefore, establishing analytical criteria to control naturalness is of the utmost importance to ensure fair trade in food flavourings. Quality Assurance managers in the food industry should also be aware of the definition, regulation and analytical methods used to monitor the authenticity of these specific and high-value ingredients (Jamin, & Tomas, 2018).

1.1.2 Analytical techniques for flavour characterization and authentication

Characterization of aroma compounds in natural products and foods remains challenging, despite the sophisticated techniques available to the analyst. Aroma substances consist of highly diversified classes of compounds, some of which are highly reactive and present in food in extremely low concentrations at ppm or even ppb levels (Da Costa & Eri, 2005). In addition, they have a wide range of polarities, solubilities, volatilities, and thermal and pH stabilities. The difficulties usually encountered in qualitative and quantitative analysis of aroma compounds are based on these features. The matrices that contain them may be very complex and interfere with the isolation techniques (Belitz et al., 2009). Therefore, there is no single, simple method for identifying aroma compounds. Instead, analysts must ask themselves what they wish to accomplish with the analysis and then choose the best analytical method or combination of methods. Typical analytical goals include obtaining a complete aroma profile of the sample, identifying specific compounds, or comparing samples (Da Costa & Eri, 2005).

Controlling the conformity of a flavour starts with a series of tests according to the recommendations of certification and regulatory authorities (e.g., ISO, Pharmacopoeia) to ensure identity, quality and safety of the extract. The first step is a sensory analysis typically performed by a sensory-analysis panel or by a group of assessors selected to form the sensory-analysis panel. Sensor analysis has the advantage of avoiding costly investments in analytical instruments but requires time and assiduity of the assessors for training and evaluation. The second step comprises a series of standardised physical and chemical methods, such as determining the ester, acid or carbonyl index, refraction, density, optical rotation, freezing or boiling points, or quantifying ethanol or moisture. However, these simple, effective methods are insufficient for more subtle adulterations (Do, Hadji-Minaglou, Antoniotti, & Fernandez, 2015). It is then necessary to use, optimise and develop more sophisticated analytical techniques (Schieber, 2018). According to the European

Flavour and Fragrance Association (EFFA), the best analytical methodologies to identify adulteration are fingerprint analysis, chiral analysis, site-specific deuterium nuclear magnetic resonance (NMR), ^{14}C radiocarbon determination and isotope ratio mass spectrometry (IRMS) analysis (European Flavour and Fragrance Association, 2019).

1.1.2.1 Fingerprint analysis

A first approach – the fingerprint analysis or flavour profiles applies the gas chromatography-mass spectrometry method (GC-MS). GC-MS chromatograms usually show around one hundred identifiable and quantifiable compounds in full scan mode, most of which can be identified through the NIST MS database. Accurate quantification requires determining the individual response factors for each compound, considering both extraction efficiency and the chromatographic response. For fingerprinting, absolute values are not essential for assessing authenticity. The relative proportions and their orders of magnitude must match the authentic product's. Any significant imbalance could demonstrate the use of specific flavour compounds instead of whole extracts (Jamin & Tomas, 2018). Foreign molecules such as undeclared solvents or atypical compounds could also indicate a synthetic origin.

1.1.2.2 Chiral analysis

Chiral or enantiomeric analysis provide a characterisation of enantiomers of chiral molecules and compounds and has proven to be a reasonably effective method when reviewing the source authenticity of juices and nectars. Many volatile organic compounds (VOC) present in food are chiral, i.e. both enantiomers are found even if one isomer is predominant in the mixture. Any changes in these ratios could indicate illicit manipulation with products, incorrect treatment procedure or addition of synthetically produced chemicals (Špánik, Pažitná, Šiška, & Szolcsányi, 2014). It is infrequent to find flavouring substances with an enantiomeric excess of 100 %. In the same way, very few, if any, aroma compounds are found to be “racemic”. The European Flavor Association (EFFA) published its interpretation for optically active flavouring substances in a guidance document: “mixture of optical isomers shall be allowed in any ratio provided that all the isomers have been identified in nature” (European Flavour and Fragrance Association, 2019). For many years now, the overall content of active components and their relative percentage were accepted criteria in assessing flavourings (Schäfer et al., 2015). However, this information is not definitive evidence of naturalness as not all chiral compounds are found in a stable ratio of both enantiomeric forms. Racemisation may occur during processing or storage, and enantiomers ratios may vary. Since chiral, single component and flavour profile analyses have limited applicability, new methodologies are being developed to detect food fraud (Martin, 1993; Richling et al., 2006; Schipilliti, Bonaccorsi, & Mondello, 2011).

1.1.2.3 ^{14}C Radiocarbon determination

^{14}C radiocarbon determination can be used in the authentication of natural processes. Its measurement indicates whether a material is “of recent biological origin” or whether it contains fossil carbon, in which essentially all of the ^{14}C has decayed (Rowe, 2012). Typically, it is used to assess the natural origin of pure compounds or mixtures containing some major compounds, such as essential oils (Jamin & Tomas, 2018). However, ^{14}C gives little or no information about a compound’s natural status since, in some cases, synthetic compounds produced from natural precursors are not detected by ^{14}C activity measurements (Jamin & Tomas, 2018).

1.1.2.4 Stable isotope analysis

Currently, the most specific and sophisticated methods for determining flavour authenticity based on stable isotopes are site-specific natural isotope fractionation using nuclear magnetic resonance (SNIF-NMR) spectroscopy and gas chromatography-isotope ratio mass spectrometry (GC-IRMS). While IRMS gives a mean value of the isotope concentration studied between all molecule sites, SNIF-NMR makes it possible to go one step further by determining isotopic ratios at different positions within a molecule, thus providing information that is more precise. Initially applied to deuterium, SNIF-NMR has been used to authenticate key-flavour molecules such as vanillin (AOAC Official Method, 2006; Jamin et al., 2007; Remaud, Yves, Gilles, & Gerard, 1997), benzaldehyde, anethole, and raspberry ketone (Jamin, & Tomas, 2018). Although both are powerful techniques, they require databases, an experienced operator and significant investment (Cicchetti et al., 2010; Do et al., 2015; Frey, 2005; Guyader et al., 2019). However, IRMS does not require extensive isolation and purification of compounds, making the analysis more rapid and cost-effective (Rowe, 2012).

The most widely applied stable isotope ratios are for the biogenic elements carbon, nitrogen, oxygen, hydrogen and sulphur (Jamin, & Tomas, 2018; Mihailova, 2012). Generally, the lighter isotope is much more abundant, e.g., approximately 98.89 % of all carbon in nature is ^{12}C, while ^{13}C accounts for only 1.1 % (Rowe, 2012). Since natural isotopic variations are small, the absolute isotopic abundances are less important than the changes in isotopic abundances that have occurred. Therefore, stable isotope abundances are usually expressed as ratios of the rarer isotope to the abundant isotope relative to an internationally accepted standard. Multiplying small absolute abundance ratios by 1000 expresses the fractional differences in parts per thousand using the δ notation expressed in "per mil" (‰). The delta value is defined according to the equation:

$$\delta\left({}^{i/j}E\right) = \delta\ {}^{i/j}E = \frac{{}^{i/j}R_P - {}^{i/j}R_{Ref}}{{}^{i/j}R_{Ref}} \qquad \text{Eq. 1}$$

Here, i and j denote the highest and the lowest atomic mass number of element E, respectively, R_P and R_{Ref} indicate the ratio between the heavier and the lighter isotope ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) in the sample (P) and reference material (Ref), respectively (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014). The δ^2H and $\delta^{18}O$ values are typically reported relative to the VSMOW (Vienna- Standard Mean Ocean Water) standard, $\delta^{13}C$ relative to the VPDB (Vienna-Pee Dee Belemnite) standard and $\delta^{34}S$ to the VCDT (for Vienna Cañon Diablo Troilite) and $\delta^{15}N$ to air (atmospheric nitrogen gas), respectively (Brand et al., 2014). By definition, standards have a δ value of 0 ‰ (Kelly et al., 2002; Mihailova, 2012). A positive δ value means that the ratio of heavy to light isotope is higher in the sample than in the standard, and *vice versa* for a negative δ value (Sharp, 2017).

The chemical behaviour of two isotopes of an element is qualitatively similar. However, differences in the masses of two isotopes of an element result in different reaction rates and bond strengths. This change in the portioning of heavy and light isotopes between a source substrate and the product(s) is termed isotopic fractionation. There are two types of isotopic fractionation: "equilibrium" and "kinetic." An equilibrium isotope effect will cause one isotope to concentrate in one component of a reversible system that is in equilibrium. If the heavier isotope concentrates in the component of interest, then that component is commonly referred to as enriched or heavy, whereas if it is the light isotope that concentrates, then the component is referred to as depleted or light. Reactions that take place are temperature-dependent and occur in closed, well-mixed systems, where substrates and products remain in close contact and reverse reactions can take place. In most

circumstances, the heavy isotope concentrates in the component in which an element is more strongly bound. Thus, equilibrium isotope effects usually reflect relative differences in bond strengths in various system components.

A kinetic isotope effect occurs when one isotope reacts more rapidly in an irreversible system or a system in which the products are removed from the reactants before they have an opportunity to reach equilibrium. Usually, the lighter isotope will react more rapidly than the heavy isotope, and thus the product will be lighter than the reactant. In biological systems, kinetic fractionations are often catalysed by an enzyme that discriminates between the isotopes in the mixture such that the substrate and product become isotopically distinct from one another. All organisms preferentially use lighter isotopic species because "energy costs" are lower, resulting in a significant fractionation between the substrate (heavier) and the biologically mediated product (lighter) (Mihailova, 2012; Sharp, 2017). Differences in isotopic values can also arise due to evaporation and condensation, diffusion, crystallization and melting, absorption and desorption (Mihailova, 2012). Isotope ratio measurement enables us to determine the nature of transformations that have taken place in the production of a specific material. In the context of natural aroma chemicals, this can, in some instances, enable us to differentiate between chemical and enzymatic conversions, and in the latter case, between enzymatic and microbial conversions, and between different types of organism (Rowe, 2012).

The most important process for food fraud detection is the process of photosynthetic fixation by plants. There are three distinct photosynthetic pathways. Most terrestrial plants use the Calvin or C3 pathway, while other plants, mainly tropical grasses, such as maize and sugar cane, use the Hatch-Slack or C4 pathway. A third photosynthetic class of plants uses the CAM (Crassulacean acid metabolism) pathway. Typical CAM plants are succulents. All three pathways discriminate against ^{13}C, but to different degrees (Rowe, 2012). Typical δ^{13}C values are -16 ‰ to -10 ‰ for C4 plants, -32 ‰ to -23 ‰ for C3 plants, and -30 ‰ to -12 for CAM plants (Fig. 3). It is important to note that synthetic compounds, derived from coal and petroleum, which originate from reservoirs of carbon formed from ancient C3 plants, have δ^{13}C values between -30 ‰ to -25 ‰ and are similar to δ^{13}C values in modern C3 plants (van Leeuwen et al., 2014). This similarity can make detecting substitutions difficult.

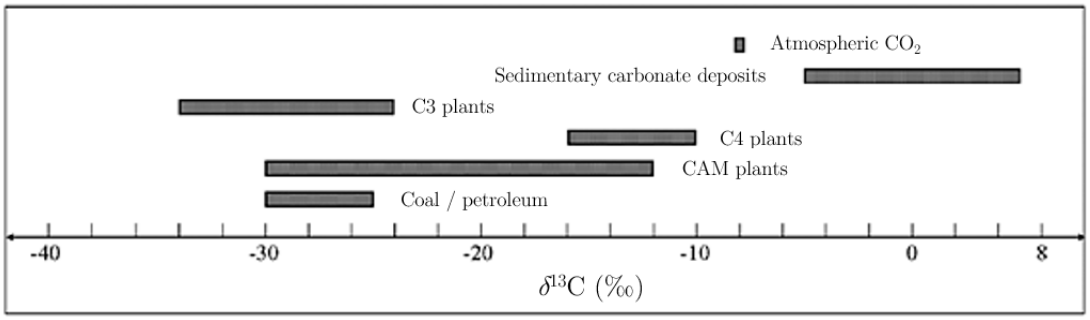

Figure 3: Natural abundances of carbon isotope ratios expressed in δ-notation (Figure adapted from van Leeuwen et al. (2014)).

Stable isotopes ratios are measured using IRMS. The method involves quantitatively converting the samples to a pure gas (usually CO_2, CO, N_2, N_2O, O_2, H_2, or SO_2) which is then introduced into a mass spectrometer. The ratios of the isotopes in the ionised gas can be measured using IRMS either in the entire (bulk) sample or in the individual compounds isolated from the sample before isotopic analyses. Bulk analyses can be performed using an

elemental analyser-IRMS (EA-IRMS) (Mihailova, 2012). Although isotope ratios of flavour compounds have been studied in detail, most research has focused on bulk or global stable carbon isotope ratios ($^{13}C/^{12}C$) (Rowe, 2012).

1.1.2.4.1 Compound-specific isotope analysis

With the introduction of compound-specific isotopic analysis (CSIA) in flavourings, the authentication of flavoured food has advanced even further by allowing the isotopic analysis of individual compounds at the molecular level (Guillou & Reniero, 2001; van Leeuwen et al., 2014). At present, CSIA is perhaps the most specific and sophisticated method for determining food authenticity, and studies have shown that it is possible to distinguish between natural and synthetic aromas based on the isotopic values of individual VOCs (Jochmann & Schmidt, 2012; Martin et al., 1993; Richling et al., 2006; van Leeuwen et al., 2014). CSIA is achieved by coupling a gas chromatograph to an isotope ratio mass spectrometer. After separation on a GC column, the analytes are transferred to either a combustion (GC-C-IRMS) or a pyrolysis (GC-P-IRMS) interface where CO_2 or H_2 is formed for $^{13}C/^{12}C$ or $^{2}H/^{1}H$ measurements, respectively (Zwank, Berg, Schmidt, & Haderlein, 2003) (Fig. 4).

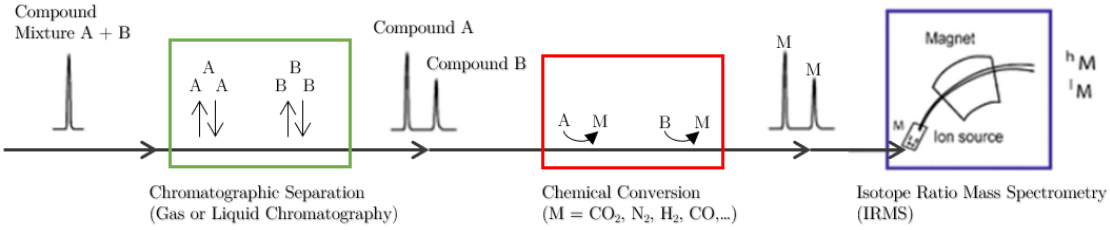

Figure 4: Scheme of compound-specific isotope analysis.

The use of GC-C-IRMS in aroma compounds is the subject of a review by van Leeuwen et al. (2014), in which the authors emphasise the necessary conditions to obtain precise isotope ratios of analytes in complex sample matrices. For example, achieving good reproducibility requires extensive method development aimed at producing optimal instrumental conditions, determining stability and linearity, tuning the injection volume so that the analyte signal is within the linearity range of the instrument, preventing carryover, and choosing appropriate reference materials (Caimi, Houghton, & Brenna, 1994; Carter & Fry, 2012; Mosandl, 1995; Mottram & Evershed, 2003). However, the analytical procedures for organic stable isotope analysis are, in many cases, non-standardised and limited certified reference materials (CRMs) available. Several authors, however, describe appropriate data normalisation methods and the correct use of isotopic reference materials (Jochmann & Schmidt, 2012; Meier-Augenstein & Schimmelmann, 2019; Neves et al., 2015; Meier-Augenstein, 2018). For instance, Jochmann & Schmidt (2012) present possible normalisation pathways for GC-C-IRMS analysis. However, data for VOCs are missing. The lack of reference materials makes method validation challenging since most CRMs are certified using EA-IRMS and generally cover non-volatiles. Only a few reference materials, such as those produced by the University of Indiana (n-alkanes, ethanol, and fatty acid esters), are compatible with GC-C-IRMS. Although they are not internationally agreed-upon reference materials, they are widely used in IRMS measurements. Ethanol from wine produced by the Institute for Reference Materials and Measurements – IRMM (BCR-656) is another example. In this study, we followed

Jochmann & Schmidt (2012) since they found that materials (compounds) calibrated separately using EA-IRMS are suitable for GC-C-IRMS as a "reference material mixture" under identical treatment.

Vital to obtaining repeatable and reproducible results is scale normalisation. The method is based on two simultaneously analysed scale anchors whose isotopic composition bracket that of the samples (Paul, Skrzypek, & Fórizs, 2007; Skrzypek, 2012). Paul et al. (2007) reviewed six commonly used normalisation methods and recommended a two-point calibration. However, multi-point normalisation reduces the random error associated with analysing reference materials to anchor the linear scale (Jochmann & Schmidt, 2012). The measured isotope ratio should ideally be independent of sample size. Still, in practice, the response is often nonlinear, and thus, the isotope ratio depends on the amount of the sample (Ohlsson & Wallmark, 1999). Therefore, only the values within a linear range should be reported. In addition, laboratory-induced irreproducible isotope fractionation that describes variations in the stable isotope ratios of carbon brought about by non-natural causes such as derivatization, poor injection, and incomplete combustion must also be avoided (Blessing, Jochmann, & Schmidt, 2008).

Stable isotope ratio analysis of a single element (e.g. carbon) itself may not provide the discriminatory power required to determine authenticity, which means that an additional element, such as hydrogen, would be beneficial. The primary source of hydrogen in nature is the hydrosphere, also called the "water sphere", as it includes all of the Earths water found in streams, lakes, oceans, ice, groundwater and air. Isotopic fractionation associated with evaporation, condensation and precipitation of meteoric water ultimately results in drinking water having different isotopic compositions depending on geo-location. Several factors such as latitude, altitude, temperature and distance to the open seas can affect the isotopic composition of meteoric water and hence freshwater globally. Also, the underlying fractionation processes associated with evapotranspiration in plant leaves and biosynthetic pathways makes it possible to use the δ^2H of plant material as an indicator of source water, that is, local precipitation and, hence, provenance (Meier-Augenstein, 2018). It is also a reliable tool for elucidating biosynthetic pathways and as an indicator of determining the authenticity of natural compounds (Mosandl, 2007). To date, only a few studies have included hydrogen isotope measurements, although a clear-cut differentiation between synthetic compounds and different natural sources has been shown by correlation between δ^{13}C and δ^2H values (Richling et al., 2006).

1.1.3 Volatiles extraction

The major drawback of GC-IRMS for trace analysis in environmental applications is its relatively low sensitivity. In order to increase method sensitivity, choosing the correct analyte-isolation and pre-concentration technique, as well as a careful optimisation of operational parameters, without compromising accurate and precise isotope ratio determinations are of paramount importance (Augusto, Leite e Lopes, & Zini, 2003; Zwank et al., 2003). Some general techniques like LLE (liquid-liquid extraction) and SPE (solid-phase extraction) are occasionally employed for flavour analysis, although simultaneous distillation extraction (SDE) is the most commonly used method, especially for testing fruit juices and fruit products. Its advantage is that it enables a wide range of compounds to be extracted. It is, however, not suitable for matrices containing a significant amount of alcohol, where LLE offers better compound recoveries (Jamin & Tomas, 2018). Methods involving direct LLE, SPE (solid-phase extraction) or SFE (supercritical fluid extraction) of the samples to isolate odorants are, however, still necessary for species with high odour impact and in extremely reduced concentrations and volatilities that are too low to provide

a suitable concentration in the headspace (Augusto et al., 2003). However, these procedures are time-consuming and use significant amounts of organic solvents (Mottaleb, Meziani, & Islam, 2014). The current trend is to replace them with extraction techniques that are less aggressive and capable of dealing with ultra-low concentrations of analytes. Most of the applications in flavour analytical chemistry are focused on variations of dynamic headspace sampling (DHS) or solid-phase microextraction (SPME) (Augusto et al., 2003).

1.1.3.1 Solid-phase microextraction

In the early 1990s, Pawliszyn and co-workers developed SPME, a solvent-free method designed to extract analytes from gaseous, liquid, and solid matrices and allow easy automation (Arthur & Pawliszyn, 1990). The method utilizes a 1-2 cm fused silica fibre coated with a thin polymeric stationary phase adsorbent such as polydimethylsiloxane (PDMS). The polymer coating acts like a sponge, concentrating the analytes by absorption/adsorption processes (Vas & Vékey, 2004). The fibre is mounted in a syringe-like device (Fig. 5a) and can be exposed to the headspace above the sample (headspace SPME) or directly into the liquid sample (direct immersion SPME) (Lafarge & Cayot, 2019). Several factors can influence extraction efficiency and equilibrium time. The time needed for equilibrium is a function of the analyte and conditions used (Zwank et al., 2003). Fibre selection, namely the fibre coating, has the most impact on the extraction efficiency.

Different fibre materials offer a range of polarities for extracting volatile and semi-volatile compounds. Therefore, different materials have been combined to create fibres able to sample compounds with a broad range of properties (Zwank et al., 2003). In general, volatile extraction is best achieved when the polarity of the fibre matches the polarity of the target molecules, i.e., non-polar fibres for non-polar molecules and polar fibres for polar molecules (Lafarge & Cayot, 2019).

Extractions typically take 15-20 minutes but can be as short as 30 seconds. Headspace extractions are usually faster than immersion, and extraction time will depend on the size of the compounds, fibre coating, type of extraction used and sample concentration (Lafarge & Cayot, 2019). Extraction times can be shorter when analysing small compounds (<150 MW), using thinner, absorbent type fibre coatings, using the headspace technique and working with more concentrated samples (high ppb or ppm range). In specific applications involving non-volatile or high boiling semi-volatile compounds, heating the sample during headspace extractions can help release the analyte, improve sensitivity, and shorten the extraction time. However, too high a temperature can drive the analytes out of the fibre, reducing sensitivity. Adjusting the pH or adding salt can also improve the extraction efficiency by changing the solubility of the analytes in the sample. The addition of 25-30 % (wt. /vol.) of NaCl will increase the ionic strength of the sample and reduce analyte solubility. The addition of salt is beneficial when analysing polar analytes in water. The pH of the sample should be buffered to decrease analyte solubility, improve the volatility of bases and acids, and assure a constant pH between extractions (Supelco, 2004). According to Vas & Vékey (2004), sampling time and other sampling parameters are more important than complete equilibration for high accuracy and precision from SPME. It is also essential to keep the vial size and the sample volume constant. The sample headspace should also be as small as practical (Sigma-Aldrich, 1998). After sampling, desorption of the analyte from a SPME fibre occurs in the split/splitless injector of the gas chromatograph (Zwank et al., 2003) (Fig. 5b) and depends on the boiling point of the analyte, the thickness of the fibre coating, and on the temperature of the injection port (Sigma-Aldrich, 1998).

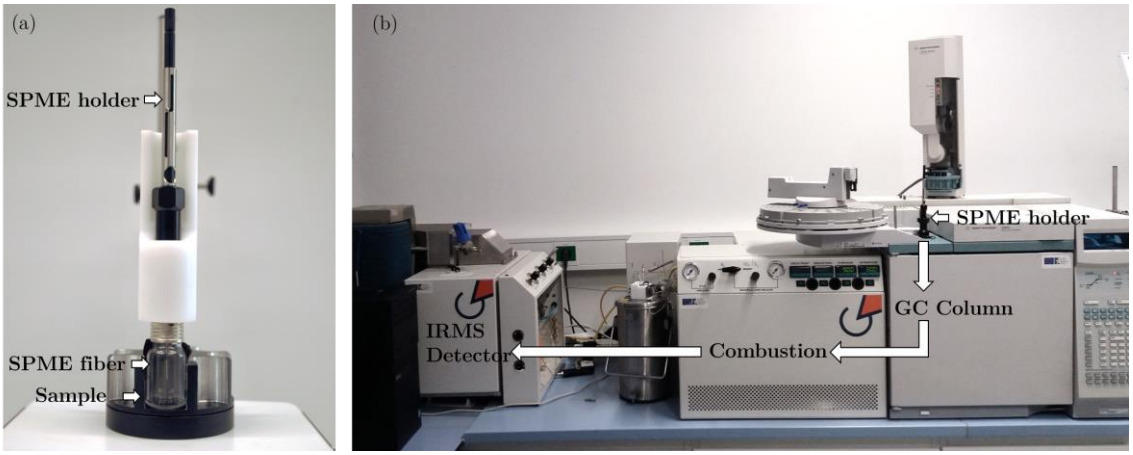

Figure 5: HS-SPME procedure: extraction (a) and thermal desorption on GC injection port of GC-C-IRMS (b).

However, the relative concentration of compounds in the headspace does not accurately reflect the concentration in the sample due to differences in compound volatility. Also, the collected volatiles profile depends on the type, thickness, and length of the fibre used and the sampling time and temperature. Therefore, it is essential to analyse the samples under well-defined and constant conditions (Cajka & Hajslova, 2011) using internal standards. Under such conditions, SPME can be a reliable part of a formal, quantitative analysis (Sigma-Aldrich, 1998). Due to the combination of sampling, extraction, pre-concentration and sample introduction into an analytical instrument in one step, SPME has been used in many food analyses in recent years (Merkle, Kleeberg, & Fritsche, 2015; Souza-Silva, Gionfriddo, & Pawliszyn, 2015).

Despite its numerous advantages, e.g., lower time consumption, an increase of simplicity, lower probability of sample contamination and higher repeatability (Merkle et al., 2015), its combination with GC-C-IRMS has been used in only a few aroma authenticity studies (Schipilliti, Bonaccorsi, Cotroneo, Dugo, & Mondello, 2015; Schipilliti, Bonaccorsi, Occhiuto, Dugo, & Mondello, 2018; Schipilliti et al., 2011). One reason is the limited knowledge concerning SPME and isotopic fractionation. Hattori et al. (2010) reported that SPME could cause isotopic fractionation resulting from fibre immersion or sampling headspace, fibre type, extraction temperature and time, and compound properties.

1.1.4 Authenticity assessment

Apart from detecting single compounds that may indicate food/beverage fraud, it is also possible to detect the isotopic authenticity range of a particular product. This range is determined by measuring many authentic samples for compounds found within the product of interest, providing a minimum and maximum range (typically 95 % of variability) per compound. For example, verification of aroma authenticity is achieved by measuring the isotope values of the investigated aroma compounds and comparing the obtained isotopic values with those of reference samples in a database. Samples containing one or more compounds outside the “range” established for authentic aroma compounds will be suspected of being adulterated (van Leeuwen et al., 2014). Essential to this work is having a comprehensive database of authentic samples (Donarski et al., 2019; Kelly, Heaton, & Hoogewerff, 2005). The sampling guidelines for building and curating food authenticity databases are given by Donarski et al. (2019). Specifically, the areas of database scope, analytical methodology, sampling, collection and storage of data, validation and curation are discussed (Donarski et al., 2019). Notably, the development of

a particular database should be related to a specific purpose. Since developing and maintaining a food authenticity database is time-consuming, demanding and resource-intensive, current flavour databases tend to contain a minimal number of samples and flavour compounds. Whether the database adequately covers the variability of samples is a common question. The primary purpose of creating a specific flavour database for food flavour authentication should be its applicability and usefulness for either industrial partners, enforcement agencies or even regulatory authorities to determine the authenticity of raw ingredients. Any database must also contain as broad a range of samples as possible to cover the widest variety of flavoured food products. Despite all described shortcomings, the establishment of databases represents a necessary step in the food flavour authentication process (van Leeuwen et al., 2014). This thesis deals with three specific case studies: fruit, vanilla, and truffle flavours.

1.1.5 Case studies

1.1.5.1 Fruit flavourings

Fruits play a significant role in human nutrition. They are consumed not only for their nutritional and health value but mainly for their highly esteemed flavour and taste. Many aromatic characteristics are shared between different fruits. However, each fruit has a distinctive aroma that depends on the VOCs present, their concentration and the perception threshold of each volatile compound (El Hadi, Zhang, Wu, Zhou, & Tao, 2013; Matheis et al., 2007). For instance, in apple flavour (E)-2-hexenal, (E)-2-hexenol, (E)-2-hexenyl acetate, and hexanal are responsible for its fresh, green-fruity basic flavour. Ethyl-2-methyl butyrate, hexyl acetate supports the fruity-estery note, and additional compounds like 3-methyl butyl acetate, hexyl-2-methyl butyrate, damascenone and linalool impart the specific species character. Benzaldehyde intensifies the pip note (Grab, 2007). The basic flavour complex of strawberries is built of 2,5-dimethyl-4-hydroxyfuran- 3(2H)-one and 2,5-dimethyl-4-methoxy-furan-3(2H)-one. Both impart the ripe, fruity, caramel, cooked character, ethyl hexanoate, and fresh fruity, estery note, while (E)-2-hexenal and (E)-2-hexenyl acetate are responsible for the fresh, green impression. 2-Methyl butanoic acid leads to a refreshing fruity acidity and linalool is responsible for the fruity, floral note (Grab, 2007). Fruity notes in flavoured products continue to play well with all consumers, including those associated with health and wellness. The most popular are classic fruit flavours such as apple, berry, and citrus fruits, although flavour preferences are continuously evolving (Graham, 2020).

Fruit juice aroma is an aqueous liquid with a characteristic individual flavour and a chemical composition that reflects the fruit's type and maturity (Matheis et al., 2007). It is recovered and concentrated separately from the juice while producing juices and concentrates in order to add it back at a later time. Depending on the type of fruit, 10-40 % of the first vapours extracted at the beginning contains the highest amount of aroma and thus are suitable for aroma recovery. The aroma is then concentrated (100-200 fold) and the recovered aroma solution is known as fruit juice hydrolate or aromatic water (Dawiec-Liśniewska, Szumny, Podstawczyk, & Witek-Krowiak, 2018; Elss, Preston, Appel, Heckel, & Schreier, 2006; Taylor, 2016). Most water phase/recovery aromas are added to the juice before bottling. However, some can be used as a naturally produced flavouring in dairy, bakery, and cereal products and beverages such as fruity infusions. Flavours typically contribute only a small part to a finished product, both cost and volume. The typical cost contribution of natural flavour to a manufactured soft drink is approximately $0.01 per litre. However, juice manufacture is enormous, with many tons of fruit passing through a single processing plant (Frey, 2005). Despite this, the cost of natural flavours is still a

factor of 10 (or more) higher than the price of synthetic analogues. Frey (1988) gives us an idea of how profitable it is to sell synthetic chemicals as natural by stating how an unscrupulous manufacturer could purchase linalool at $3/lb and sell it at $500/lb.

Papers on determining the authenticity of fruit volatile organic compounds based on GC-IRMS are summarised in Table 2. Authenticity studies include raw fruits, essential oils, fruit products, and flavours obtained synthetically and/or using biotechnological processes. Most research shows that GC-IRMS can distinguish between natural and synthetic aromas. Still, the results are limited to a few common aroma compounds present in different fruits and are based on a small number of samples produced using different extraction procedures (van Leeuwen et al., 2014).

Table 2: Literature data on stable isotope analysis of fruit volatile organic compounds.

Fruit Type	Method of Sample Preparation [a]	Stable Isotopic Value	Samples with Known Origin (No. Samples)	Aroma Compound (No. of Samples Where VOC Was Detected)	Reference
strawberry, peach, plum, apricot	SDE	$\delta^{13}C$	fruit (NS); synthetic (NS); microbial (NS)	γ-decalactone (7; 3; 2)	(Bernreuther et al., 1990)
raspberries	NA	$\delta^{13}C$	fruit (NA); synthetic (NS); biotechnological (NS)	(E)-α-ionone (2; NS; NS), (E)-β-ionone (2; NS; NS)	(Braunsdorf, Hener, Lehmann, & Mosandl, 1991)
bergamot oil	SCF extraction	$\delta^{13}C$	essential oil (2)	linalyl acetate (NS), limonene (NS), linalool (NS), β-pinene (NS), γ-terpinene (NS), β-mycrene (NS), neryl acetate (NS), geranyl acetate (NS)	(Martin, 1993)
raspberries	NA	$\delta^{13}C$	fruit (NS)	α-ionone (NS), β-ionone (NS), δ-decalactone (NS), cis-3-hexen-1-ol (NS)	(Casabianca & Graff, 1994)
strawberry	SDE	$\delta^{13}C$	fruit (NS)	methyl hexanoate (NS), pentyl valerate (NS), butanoic acid (NS), 2-methylbutanoic acid (NS), 4-methylvaleric acid (NS), hexanoic acid (NS), γ-decalactone (NS), γ-uncalactone (NS), γ-dodecalactone (NS)	(Schumacher, Turgeon, & Mosandl, 1995)
raspberry	SCF extraction	$\delta^{13}C$	fruit (NS); synthetic (NS)	(E)-α-ionone (NS), (E)-β-ionone (NS)	(Mosandl, 1995)
banana	LLE	$\delta^{13}C$	fruit (7)	pentan-2-one (NS), isobutyl acetate (NS), isoamyl acetate (NS), pentan-2-ol (NS), isobutyl butyrate (NS), isoamyl butyrate (NS), isoamyl isovalerate (NS), isobutyl acetate (NS), isoamyl acetate (NS), isoamyl alcohol (NS), isoamyl butyrate (NS)	(Salmon, Martin, Remaud, & Fourel, 1996)
bitter almond oils, sweet cherry, sour cherry, peach, nectarine	SDE	$\delta^{2}H$	fruit (NS); essential oil (NS); synthetic (NS); "natural" (NS)	benzaldehyde (NS)	(Ruff, Hör, Weckerle, Schreier, & König, 2000)
cactus pear	SDE	$\delta^{13}C$	fruit (NS)	1-hexanol (NS), E-2-hexenol (NS), E-2-nonenol (NS), E,Z-2,6-nonadienol (NS)	(Weckerle et al., 2001)
different essential oils, orange, apple, nectarine/peach	LLE, SDE	$\delta^{2}H$	fruit/juice/aroma (NS); essential oil (NS); synthetic (NS); "natural" (NS)	linalool (5; 50; 0; 1), linalyl acetate (0; 19; 5; 2), E-2-hexenal (23; 0; 5; 4), E-2-hexenol (35; 0; 5; 0)	(Hör, Ruff, Weckerle, König, & Schreier, 2001)

Fruit Type	Method of Sample Preparation [a]	Stable Isotopic Value	Samples with Known Origin (No. Samples)	Aroma Compound (No. of Samples Where VOC Was Detected)	Reference
pineapple	LLE	$\delta^{13}C$, $\delta^{2}H$	fruit/recovery aroma (16); synthetic (NS); "natural" (NS)	methyl 2-methylbutanate (6; 5; 2), ethyl 2-methylbutanoate (9; 5; 6), methyl hexanoate (17; 5; NA), ethyl hexanoate (14; 0; 0), 2,5-dimethyl-4-methoxy-3-(2H)-furanone (12; 1; 0)	(Preston et al., 2003)
raspberries	SDE	$\delta^{13}C$, $\delta^{2}H$	fruit (NS); nature-identical (NS); "natural" (NS)	(E)-α-ionone (10; 4; 1), (E)-β-ionone (10; 4; 1)	(Sewenig, Bullinger, Hener, & Mosandl, 2005)
peach, apricot, nectarine	SDE	$\delta^{13}C$, $\delta^{2}H$	fruit (18); nature-identical (NS); "natural" (NS)	γ-decalactone (16; 2; 5), δ-decalactone (12; 1; 3)	(Tamura, Appel, Richling, & Schreier, 2005)
pear	SDE, LLE	$\delta^{13}C$, $\delta^{2}H$	fruit (20); synthetic (NS); "natural" (NS)	butyl acetate (14; 3; 3), 1-butanol (18; 3; 2), hexyl acetate (16; 2; 7), 1-hexanol (3; 11; 8), methyl E, Z-2,4-decadienaoate (6; 2; 0), ethyl E,Z-2,4-decadienoate (10; 5; 3), ethyl E,E-2,4-decadienoate (2; 0; 0)	(Kahle, Preston, Richling, Heckel, & Schreier, 2005)
apple	SDE	$\delta^{13}C$, $\delta^{2}H$	juice/recovery aroma (62)	E-2-hexenal (NS), 1-hexanol (NS), E-2-hexenol (NS)	(Elss et al., 2006)
raspberry, litsea cubeba, lemongrass	SDE	$\delta^{13}C$, $\delta^{2}H$	fruit (8); essential oil (NS); synthetic (NS); "natural" (NS)	acetone (NS), citral (NS), α-ionone (NS), β-ionone (NS)	(Caja, Preston, Kempf, & Schreier, 2007)
blackberry	SDE	$\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$	fruit (10); synthetic (3)	2-heptanol (10; 1), trans-linalool oxide (10; 1), cis-linalool oxide (10; 1)	(Greule & Mosandl, 2008)
strawberry, pineapple, peach	HS-SPME	$\delta^{13}C$	fruit (NS)	methyl butanoate (NS), ethyl butanoate (NS), E-2-hexenal (NS), methyl hexanoate (NS), butyl butyrate (NS), ethyl hexanoate (NS), hexyl acetate (NS), linalool (NS), hexyl butyrate (NS), octyl isovalerate (NA), γ-decalactone (NS), octyl hexanoate (NS)	(Schipilliti et al., 2011)
sweet orange	HS-SPME	$\delta^{13}C$	essential oil (20)	myrcene (20), limonene (20), nonanal (20), decanal (20), linalool (20), α-terpineol (20), geranial (20)	(Schipilliti et al., 2015)
wolfberry	HS-SPME	$\delta^{13}C$	fruit (52)	limonene (NS), tetramethylpyrazine (NS), safranal (NS), geranylacetone (NA), β-ionone (NS)	(Meng et al., 2019)

[a] Method of sample preparation: SDE (simultaneous distillation extraction), HS-SPME (headspace solid-phase microextraction), LLE (liquid-liquid extraction), SCF (supercritical fluid extraction). NS: not specified.

Despite numerous already mentioned advantages of SPME (Merkle et al., 2015), its combination with GC-C-IRMS has so far been used only in a few aroma authenticity studies (Schipilliti et al., 2015, 2018, 2011). One reason is the limited knowledge concerning SPME and isotopic fractionation. Hattori et al. (2010) reported that SPME could influence δ-values resulting from fibre immersion or sampling headspace, fibre type, extraction temperature and time, and compound properties. Isotopic fractionation may occur during the volatilisation of the sample and the combustion process and during the chromatographic process itself. Moreover, necessary conditions (injection reproducibility, appropriate data normalisation methods and the correct use of isotopic reference materials, peak size/linearity corrections, method validation) to obtain to obtain reproducible and accurate δ-values for VOCs in complex sample matrices makes the use of GC-IRMS demanding (van Leeuwen et al., 2014).

Since 2010, HS-SPME GC-C-IRMS has been used in three fruit authenticity studies: Meng et al. (2019) and Schipilliti et al. (2011, 2015). Besides fruits, the method has been successfully applied to vanilla (Hansen, Fromberg, & Frandsen, 2014; Schipilliti, Bonaccorsi, & Mondello, 2017), citrus essential oils (Schipilliti et al., 2015, 2018), wine (Jin et al., 2021; Spangenberg, Vogiatzaki, & Zufferey, 2017; Xiaobo & Jiewen, 2008), and truffle oil (Wernig, Buegger, Pritsch, & Splivallo, 2018).

Stable isotope ratio analysis of a single element (e.g. carbon) itself may not provide the discriminatory power required to determine authenticity, which means that the characterisation of an additional element, such as hydrogen, could be beneficial. Previously, however, only a few studies have included hydrogen isotope measurements, and only Hattori et al. (2010) combined SPME with gas-chromatography - high temperature conversion - specific isotope ratio mass spectrometry (GC-P-IRMS) to study acetic acid.

A goal of the current thesis was to develop a single method able to obtain reproducible and accurate δ^{13}C and δ^{2}H values for VOCs based on HS-SPME GC-IRMS during the same analytical run. Such a method would allow us to characterise isotopically compounds with different chemical properties over a wide concentration range, thereby avoiding repeated analysis due to peak intensities falling outside of the isotopic linearity range. It also shows that optimising HS-SPME and chromatographic conditions, appropriate data normalisation and reference material selection, calculation of peak size/linearity corrections and method validation are mandatory steps to obtain meaningful data for use in authenticity studies (Strojnik, Camin, & Ogrinc, 2020).

An extensive stable isotope database based on HS-SPME GC-C-IRMS data is needed to recognise the authenticity of various fruit aromas used in food products, but no such database exists. Also, studies looking at more than five different VOCs are rare (Kahle et al., 2005; Salmon et al., 1996; Schipilliti et al., 2011; Schumacher et al., 1995), and within those, only Schipilliti et al. (2011) address more than one fruit type. However, using a single fruit type, which is the case in several studies, is insufficient for determining authenticity since different fruit types can have different isotopic ratios (Bernreuther et al., 1990; Schipilliti et al., 2011; Tamura et al., 2005). In addition, all properties that might influence isotopic values must be carefully studied and included in the database. For instance, the aroma extraction process must be investigated for evidence of isotopic fractionation, i.e., enrichment of one isotope relative to another. So far, this phenomenon has only been addressed by Elss et al. (2006) when investigating the technological processing of apple aroma. In this instance, the authors did not observe any isotopic fractionation. To build a database, obtaining distillates/extracts of various food types requires extensive resources. It also needs to contain the isotopic values of authentic natural samples since it is impossible to differentiate between natural and synthetic samples;

otherwise, it will likely result in misclassification. The present thesis deals with constructing a stable isotope database (IsoVoc). It includes information about sampling and standard selection, sample preparation, compound identification, $\delta^{13}C$ measurements, data processing and database creation (Strojnik et al., 2021). The constructed database is based on apple and strawberry data. The possibility of using fresh fruits instead of distillates to construct an authentic database of aroma compounds was also investigated with careful consideration of selecting the appropriate volatile organic compounds for assessing the authenticity of fruit aromas used in food products. Based on isotopic mass balance using $\delta^{13}C$ values of individual compounds, the average amount of the synthetic compound that must be added to the natural sample to detect adulteration can be estimated (Strojnik et al., 2019).

1.1.5.2 Vanilla flavourings

The flavour of cured vanilla beans has been appreciated since its discovery in Mexico and is widely used as a flavouring ingredient in food and beverages (Zhang et al., 2014). The rich flavour shows many aspects: the basic creamy, sweet odour is surrounded by warm, woody, slightly phenolic, smoked notes (the vanilla bean character), while rum notes, combined with dried fruit, slightly floral notes round off the whole picture (Grab, 2007). Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the basic key ingredient for the creamy, sweet character obtained from the aromatic black pods of *Vanilla planifolia* and *Vanilla tahitensis* orchids, which are the most commercialized species (Sathuluri & Gokare, 2020). These species are mainly produced in Madagascar (3500 tons in 2020), Indonesia (3400 tons), China (1350 tons), Papua New Guinea (400 tons) and Mexico (390 tons) (Maps of World, 2020). However, the original unprocessed vanilla beans are flavourless until they are processed under a laborious curing process that lasts more than six months to give them their characteristic aroma (Zhang et al., 2014). The isolation of vanillin requires enormous quantities of pods: given that a high-quality pod contains around 2 % of vanillin, 50 kg of pods are needed to produce 1 kg of pure vanillin (Toth, 2012). Periodically the production of *V. planifolia* suffers from poor climatic conditions, resulting in the degradation of both the quality and the quantity of the pods.

In 2019, the price of Madagascar vanilla pods rose to a record of €600 from €53 per kg in 2013. However, by the end of 2019, prices collapsed to as low as €350 per kg (Commodafrica, 2020). Vanilla is sold either as the whole pod, an alcoholic extract or as a powder. The vanilla extract is an aqueous-alcoholic extract containing soluble organics from the vanilla beans. Vanilla flavouring or flavour is similar to vanilla extract, but the amount of ethanol is less than 35 % by volume (Toth, 2012).

An alternative to natural vanillin derived from the vanilla pods can be obtained through fermentation, biotransformation, or bioconversion using three main molecules currently used as precursors: ferulic acid extracted from *Oryza sativa L.* (ex- ferulic acid from rice) and ferulic acid extracted from *Zea mays L.* (ex- ferulic acid from maize), eugenol obtained from *Syzygium aromaticum (L.)*, (ex- eugenol from clove oil), and curcumin extracted from *Curcuma L.* (ex- curcumin from turmeric).

Recently a new process for obtaining natural vanillin was developed using glucose as a precursor (Guyader et al., 2019). Their price is much lower than vanillin from vanilla pods but still cannot compete with synthetic vanillin ($12 per kg) (Schipilliti et al., 2017), the latter being produced from lignin (ex-lignin), a paper derivative, and guaiacol (ex-guaiacol) a petrol derivative (Fig. 6). The development of these cheaper synthetic analogues has resulted in different types of fraud. Examples include the substitution of natural vanillin with synthetic vanillin, but the product is still labelled as natural or a product being sold with an incorrect geographical origin (Guyader et al., 2019).

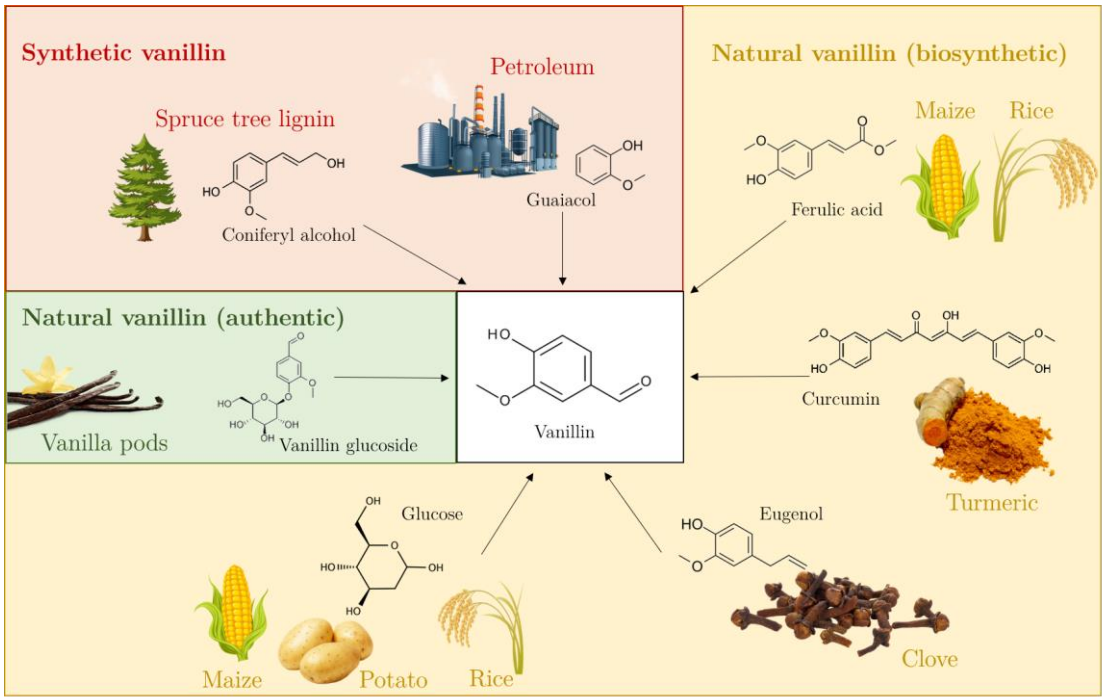

Figure 6: Different production routes of synthetic, biosynthetic and natural vanillin (Figure adapted from Wilde et al. (2019)).

Isotope ratio mass spectrometry is considered an efficient tool for the authentication of vanillin since the δ^{13}C-ranges of vanillin from different precursors (petroleum, C3, C4 and CAM plants) differ from each other (Meier-Augenstein, 1999). Indeed, natural vanillin from tropical orchids is produced via the CAM photosynthetic pathway and has a δ^{13}C value between −22 ‰ and −14 ‰, whereas nature-identical (ex-ferulic acid and turmeric) and synthetic (ex-lignin and guaiacol precursors) vanillin have significantly lower δ^{13}C values, i.e., −38 ‰ to −29 ‰ and −30 ‰ to −27 ‰, respectively (Bensaid, Wietzerbin, & Martin, 2002; John & Jamin, 2004; D. Krueger & H.W. Krueger, 1983, 1985; Lamprecht, Pichlmayer, & Schmid, 2002; van Leeuwen, Prenzler, Ryan, Paolini, & Camin, 2018; Schipilliti et a., 2017, 2016). However, δ^{13}C analysis is sometimes not sufficient to discover vanillin adulteration due to the practice of adding δ^{13}C to the methylic site of synthetic vanillin (D. Krueger & H.W. Krueger, 1985). In order to improve the power of the isotopic approach, δ^{13}C analysis has been combined with analysis of the stable isotope ratio of O (Bensaid et al., 2002; Hener et al., 1998) and H of vanillin (Culp & Noakes, 2002; Greule et al., 2010; Hansen et al., 2014) and vanillin methoxyl groups (Greule et al., 2010), or site-specifically using ^{2}H-SNIF NMR (Remaud et al., 1997). Specifically, vanillin from Vanilla species (Fig. 7) has negative δ^{2}H values ranging from −115 ‰ to −3 ‰ (Culp & Noakes, 2002; Greule et al., 2010; Hansen et al., 2014) but higher than ex-lignin vanillin that ranges from −204 ‰ to −170 ‰ (Culp & Noakes, 2002), whereas synthetic vanillin has positive values from 57 ‰ to 75 ‰ (Greule et al., 2010).

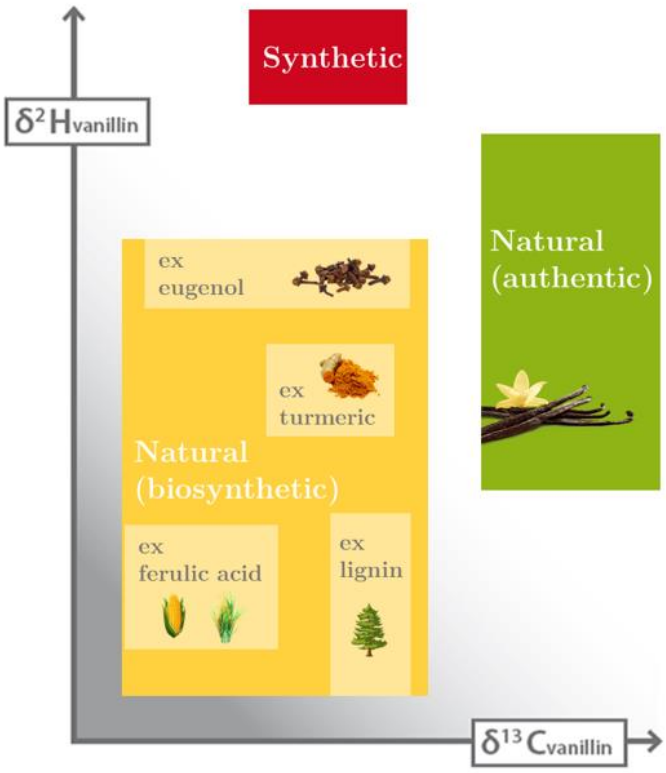

Figure 7: Illustration showing the isotopic fingerprints of vanillin (δ^{13}C and δ^{2}H) depending on the respective source (Figure adapted from Imprint Analytics, n.d.).

Vanillin is a volatile compound, making solvent extraction followed by GC/C (Combustion) or GC/P (Pyrolysis)-IRMS after from the sample the most suitable techniques (Greule et al., 2010; Hansen et al., 2014; John & Jamin, 2004; Kaunzinger, Dieter, & Mosandl, 1997; van Leeuwen et al., 2018). Despite its usefulness, solvent extraction involves considerable amounts of solvent and time (>30 min/sample for solvent extraction and concentration with N_2 stream). Besides that, in more complex matrices (yoghurt, ice-cream and pudding), solvent extraction becomes challenging due to the high-fat content, which requires a long extraction time (>6 hrs) (Bononi, Quaglia, & Tateo, 2015). Recently, headspace solid-phase microextraction (HS-SPME) has been proposed as an alternative rapid method to extract vanillin from different matrices while avoiding isotopic fractionation GC-C-IRMS (Schipilliti et al., 2016). For δ^{2}H, a method based on HS-SPME is not available in the literature and was developed here to achieve a quick, robust and effective method to assess the authenticity of vanillin (Perini, Pianezze, Strojnik, & Camin, 2019).

1.1.5.3 Truffle flavourings

Truffles are the fruiting bodies (ascocarps) of fungi belonging to the genus *Tuber* that grow in the soil or leaf litter in a symbiotic mycorrhizal association with plants (Vita et al., 2015). The unique and intense aroma of truffles and their rarity makes them one of the most expensive foods in the world. Prices range from a few hundred Euros per kg to thousands of Euros per kg (Vahdatzadeh & Splivallo, 2018). The white truffle (*T. magnatum*) is among the most appreciated foods in French and Italian cuisine and is one of the most expensive and rarest commercial truffle species (Costa et al., 2015; Splivallo &

Ebeler, 2015). It grows in spontaneous colonies in certain regions in Italy (Tuscany, Piedmont, Marche, Umbria), Croatia, Slovenia, Hungary, as well as in several Balkan regions (Marjanović, Grebenc, Glišić, Marković, & Milenković, 2010; Mello, Murat, & Bonfante, 2006; Vita et al., 2015). Alba white truffles are among the priciest delicacies, fetching almost $7,000 per kg (Sciarrone et al., 2018). During poor harvests, prices can be even higher (Piltaver & Ratoša, 2006; Reyna & Garcia-Barreda, 2014). Volatile compound bis(methylsulfanyl)methane (also known as 2,4-dithiapentane) is the key compound responsible for white truffle aroma (Splivallo, Ottonello, Mello, & Karlovsky, 2011) and is reminiscent of cheese and garlic (Costa et al., 2015; Splivallo & Ebeler, 2015). The price of black truffle (*T. melanosporum* Vittad.) is two-thirds that of *T. magnatum* (Piltaver & Ratoša, 2006). It is one of the most aromatic species with an aroma similar to a damp forest with hints of radish, chicory and hazelnut (Culleré et al., 2010). *T. brumale* Vittad. has a characteristic musky odour, with accompanying earthy notes (Wang and Marcone, 2011; Katanić et al., 2017) and is one-fifth to one third the price of *T. magnatum* (Piltver & Ratoša, 2006). *T. aestivum* also called the summer truffle, is highly prized and distributed widely across Europe. It is also the most common truffle and matures over a long period of the year (Molinier, Murat, Frochot, Wipf, & Splivallo, 2015), which makes it less expensive than either *T. melanosporum* or *T. magnatum*. Summer truffle (*T. aestivum*) reaches a tenth of the price of T. magnatum and even slightly lower than *T. mesentericum*, which are sometimes not consumed in France due to their sharp odour (Piltaver & Ratoša, 2006). Its aroma has characteristic sulphur, cooked potato, leather and metallic notes (Culleré et al., 2010). Since truffles are highly prized for their distinctive smell, they are also a prime target of food fraud.

Figure 8: Important commercial white and black truffle species (Nourriture, 2015).

The most abundant volatiles commonly present in truffle aroma includes bis(methylthio)methane, dimethylsulfide, hexanal, 2-methylbutanal, and 3-methylbutanal, with quantitative and qualitative variations according to the truffle species and geographical origin. These compounds can be synthesized from petrochemicals and added to foods to deliver a truffle taste (Sciarrone et al., 2018). However, there are significant differences in the market price between the synthetic (100$/Kg) and the biotechnological (5000$/Kg) compounds (Sciarrone et al., 2018).

To date, gas chromatography coupled to combustion-isotope ratio mass spectrometry (GC-C-IRMS) with HS-SPME extraction is a recognized method for evaluating δ^{13}C values in bis(methylthio)methane in different natural truffles and truffle-aromatised samples. It can also be used for authenticity and traceability purposes (Sciarrone et al., 2018) since the authors found that the δ^{13}C values bis(methylthio)methane from genuine Italian white truffles (-42.6 ‰ to -33.9 ‰) were distinguishable from two standards of petrochemical origin (-56.4 ‰ and -77.1 ‰). A third standard a natural flavouring substance obtained by physical, enzymatic or microbiological processes from materials of vegetable, animal or microbiological origin, had the most positive value (-28.5 ‰). Alternatively, Wernig et al. (2018) concluded that it was impossible to differentiate between natural and synthetic flavours based on the δ^{13}C values of bis(methylthio)methane. However, only a limited number of samples were available in both studies. Therefore, a comprehensive database for bis(methylthio)methane and other important truffle volatile compounds is necessary to determine the authenticity of flavoured commercial truffle products.

Besides adding synthetic aroma compounds to food products, which gives them a distinct truffle aroma that is very subtle and is quickly lost during storage and processing, synthetic truffle aroma is sometimes added to fruiting bodies (raw truffles). For instance, truffles from the Far East like *T. indicum*, *T. himalayense* and *T. pseudohimalayense* from China are practically odourless and tasteless, with a value of around € 15 per kilogram. Nevertheless, adding synthetic truffle aroma provides a distinct truffle aroma. Consequently, they can be sold as the prized European black truffle (*T. melanosporum*) for more than a thousand Euros per kg (Culleré, Ferreira, Venturini, Marco, & Blanco, 2013). Counterfeiters also sell other underground fungi as high-quality truffles, like "desert truffle" (*Terfezia sp.)* and "whitish tuber" (*T. oligospermum*), which grows on the shores of the Mediterranean and enters on the black market mainly from Morocco, where it is commonly sold as *T. borchii* or even T. magnatum (Piltaver & Ratoša, 2006). False identification of truffle species means that it is not only economic interests that are at stake, but there are also possible health issues for consumers since not all truffles are fit for consumption. Examples include *T. fulgens*, *T. maculatum*, *T. rufum* and *T. excavatum*; their intense pungent aroma can provoke undesired reactions such as nausea or malaise, a fact well-known by truffle hunters (Piltaver & Ratoša, 2006).

In the Istria region of Slovenia, there is a strong tradition of hunting white and black truffles, and all the necessary conditions exist to develop a truffle culture. Developing such a culture would benefit from having methods that allow objective identification of different species to avoid fraud associated with marketed products or even determine the influence of different growing parameters on the aroma profile (Strojnik, Grebenc, & Ogrinc, 2020).

1.2 Geographical Traceability of Fruits and Vegetables

Various analytical techniques and parameters have been studied to verify the provenance of regional foods, including the use of gas and liquid chromatography to analyse VOCs (i.e., aroma compounds), sugars, phenolic and "fingerprinting" or chemical profiling by ^{1}H

NMR, near Infra-Red and Fluorescence spectroscopy (Kelly et al., 2005). However, determining the origin of a product by analysis of its organic constituents is not always possible since organic components of foods are dependent on various conditions, e.g., fertilisation, botanical origin, history of the field, climatic conditions, location, and soil composition (Katerinopoulou, Kontogeorgos, Salmas, Patakas, & Ladavos, 2020). Despite this, the stable isotopic ratios of light elements (H, C, N, O and S) and the elemental composition of foods are the preferred choice for geographical origin authentication (Katerinopoulou et al., 2020; Kelly et al., 2005).

Stable isotope ratios of light elements allow the relationships between isotope ratios and the fractionation processes associated with local climate data, as well as plant and animal physiology, geology, and pedology, to be analysed. This approach transfers isotopic signals of light elements (H, C, N, O and S) from different natural sources (water, soil, and atmosphere) to plant and animal tissues. For instance, the δ^{2}H and δ^{18}O values of water can provide critical information about the origin of water (e.g., local precipitation, surface water, and groundwater) that depend on latitude, altitude, distance from the sea, amount of precipitation and the degree of evapotranspiration (Gat, 1996). In plants, the δ^{13}C value is mainly regulated by the plants' specific CO_2-fixation pathway (e.g. C3 or C4 plants). However, other factors include atmospheric CO_2 concentration, plant variety, physiology, the cell's nutritional status, growth rate, water-use efficiency, and cultivation practices. While δ^{15}N values have been widely used to distinguish between organic and conventional products, it has also proven valuable in geographical origin studies (Gatzert et al., 2021). The distinctive δ^{15}N values of plants depend on the soil's δ^{15}N values and are influenced by the local climate, general soil conditions, long-term soil treatment, and land use (Camin et al., 2011). The δ^{34}S values reflect geology, volcanism, the influence of sea spray (i.e., distance from the sea), and specific anthropogenic effects, e.g. such as the source of atmospheric SO_2 (Danezis et al., 2016), can also act as local markers (Gatzert et al., 2021).

The verification of regional origin can be even more effective when stable isotopes are combined with elemental composition since a plant's elemental profile is related to soil composition of the location where the plant grows; consequently, bioavailable nutrients can provide direct information about an agricultural products' geographical origin (Drivelos & Georgiou, 2012). However, the availability of elements depends on various factors, including soil pH, moisture, clay and other characteristics (Katerinopoulou et al., 2020), such as water availability and climate (Perini, Giongo, Grisenti, Bontempo, & Camin, 2018) (Fig. 9), while elements such as Sr, Ba, Cs, S, Mo, and Ni are related to geology (Saaltink, Griffioen, Mol, & Birke, 2014; Skordas, Papastergios, & Filippidis, 2013). Strontium is already widely used to trace the geographical origin of agricultural produce (Hiraoka et al., 2016). Equally, Rb and Cs can be quickly mobilised in the soil and taken up by plants, making them suitable source markers (Kelly et al., 2005). Other elements are Na and P. The content of Na is usually related to the distance to the sea, and elevated amounts can be found within several kilometres from the coast due to the steady input of marine aerosols (Saaltink et al., 2014). Phosphorous levels could be related to excessive phosphoric fertiliser and organophosphate agrochemical application. However, it is unlikely that the use of fertilisers affects the elemental profiles in such a way to change the country of origin prediction, as reported by Smith (2005). The high concentrations of some of the remaining elements (e.g. As, Cd, Cu) in the samples could be attributed to the extensive usage of fertilisers, pesticides, fungicides and insecticides (Skordas et al., 2013).

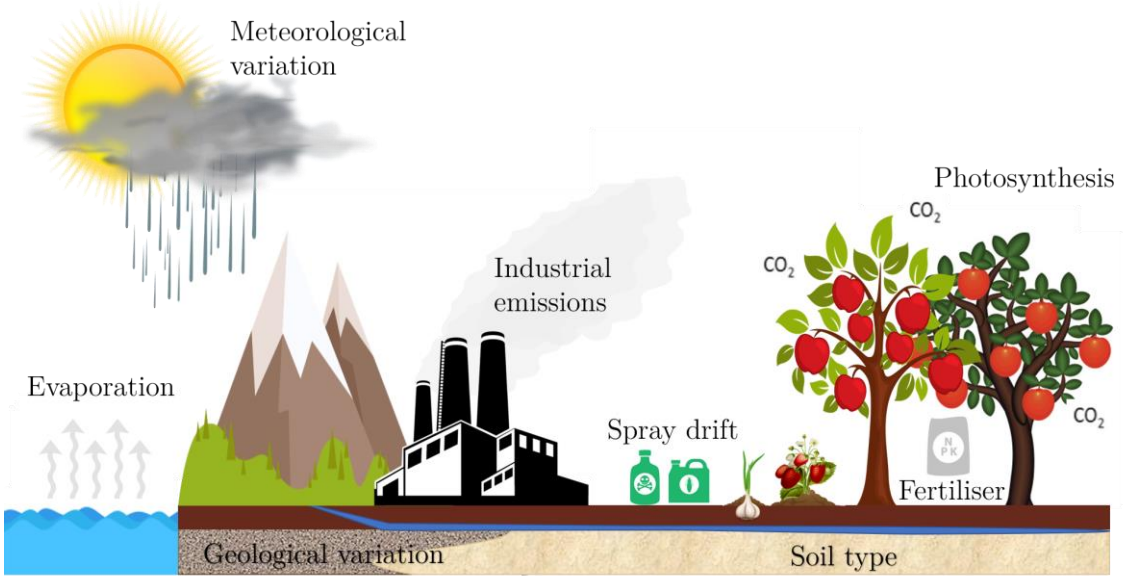

Figure 9: Factors affecting stable isotope value and elemental composition of agricultural products.

1.2.1 Databases

Apart from the measurement, data interpretation requires detailed knowledge of a food commodity in question and sufficient authentic food sample reference data, i.e., a database or databank (Camin et al., 2017). The most important criterion is that the samples contained within the database are authentic. Ideally, samples should be collected from primary producers (i.e., farms and fields) by impartial collectors (i.e., individuals with no economic incentive to corrupt the database) to ensure that traceability and integrity of reference samples are maintained (Donarski et al., 2019). The final sample size depends on several factors, including access to authentic samples, project budget, timeframe, objectives for the completed database, and sample collection logistics (Donarski et al., 2019).

Overall, the sampling design must demonstrate that the samples taken are sufficiently representative and cover natural variation caused, e.g., geographical location, variety, age and health, physical and climate stresses, processing method, temporal or seasonal variation and anthropogenic contamination (Donarski et al., 2019). The most efficient procedure is to create yearly databanks, especially for vegetable and fruit commodities that show a more significant year of harvest/production variability (Camin et al., 2017). When preparing a sampling strategy, it is also necessary to consider the required statistical analysis as this will affect the total number of samples needed (Donarski et al., 2019).

1.2.2 Chemometric approaches

Chemometric methods or multivariate data analysis are used to separate information from noise, uncover hidden correlations, and visually represent them. There are three general chemometric approaches: explorative analysis, classification, and calibration. All three are used in food chemistry and food science, but which method should be chosen depends on the problem and the type of experimental data (Granato et al., 2018).

Principal component analysis (PCA) is often used as an initial step in the multivariate analysis of the data since PCA is an unsupervised technique used for dimensionality reduction, which provides information on the most representative features with a minimum loss of initial information. PCA also reduces the contribution of less significant variables

and generates a new group of variables known as principal components (Covaciu et al., 2016). However, because PCA does not consider group membership, to obtain the best visualisation of group clusters, chemometric methods are typically used for classification and class modelling in situations where the attention is focused on the origin of a product. The term "classification" is often used as a synonym of discriminant methods because they assign objects to predefined classes. The discriminant approach (linear discriminant analysis – LDA, k-nearest neighbours, partial least squares-discriminant analysis – PLS-DA, artificial neural network – ANN) separates samples originating from predefined classes, e.g., two or more different regions. It is also possible to identify the predominant features using this approach that distinguish the two sets of samples (Marini, 2009).

Linear discriminant analysis is one of the simplest classifiers, albeit to compute a centroid for each class and the pooled variance-covariance matrix, a sufficient ratio (≥3) between the number of samples and the number of variables is required. Also, LDA cannot cope with highly collinear data common in chemical problems (Marini, 2009). Other techniques, particularly PLS-DA, have been devised to overcome these limitations. The resulting model is a linear model proven statistically equivalent to the solution obtained by Linear Discriminant Analysis. Due to its being a latent vector-based technique, problems like a minimum sample to variable ratio and the absence of collinearity can be overcome (Marini, 2009). Alternatively, orthogonal partial least squares-discriminant analysis (OPLS-DA) is a modification of the PLS-DA method, which improves interpretability by filtering out any variation not related to the discriminating response by separating that part of the variance used for predictive purposes from the non-predictive variance, which is then made orthogonal (Rongai et al., 2017). The performance of models is then evaluated by their explained variation (R^2X for PCA and R^2Y for OPLS-DA) and predictive ability (Q^2). Internal sevenfold cross-validation is also used to determine the significant components of the models and thus minimise overfitting. The prediction performance of the OPLS-DA model is evaluated in terms of sensitivity (true positives) and specificity (true negatives), calculated as described by Fiamegos et al. (2021). Candidates for discriminant markers are selected by Variable Importance in the Projection (VIP) values of the OPLS-DA models where a value higher than one is considered the threshold.

Studies that have used either stable isotopes or elemental composition or both report the usefulness of discriminant analysis and (orthogonal) partial least squares discriminant analysis for revealing differences between samples of different geographical origins (Bat et al., 2012; Camin et al., 2010; D'Archivio et al., 2019; Fiamegos et al., 2021; Foschi et al., 2020; Magdas et al., 2021; Mimmo et al., 2015; Nie et al., 2021; Opatić et al., 2017; Palacios-Morillo et al., 2014; Perez et al., 2006; Pianezze et al., 2019; Zhang et al., 2019). Confirmation that a specific sample originates from a particular region represents a task where general properties that characterise a set of samples from specific areas independently of other classes and regions need to be identified. The problem is that those discriminant methods are often forcedly used for one-class classification problems. In such cases, although the target class (of compliant samples) is typically well sampled, the non-target class (non-compliant samples) is often not adequately defined and often poorly sampled. Therefore, applications of discriminant classification lead to biased classification rules and biased predictions for new samples (Oliveri, 2017).

Several papers highlight the inappropriateness of discriminant analysis and propose using class-modelling for authentication studies (Oliveri, 2017; Rodionova, Titova, & Pomerantsev, 2016). Methods used for solving authentication problems comprise a separate class among pattern recognition/classification techniques. These methods are called one-class classifiers or class modelling (Rodionova, Titova, et al., 2016). For example, soft

independent modelling of class analogy (SIMCA) is an OCC method widely used in chemometrics.

The original version of SIMCA has undergone numerous modifications, primarily related to constructing the acceptance boundary. This thesis uses the data-driven version of SIMCA (DD-SIMCA) since it can calculate misclassification errors theoretically (Zontov, Rodionova, Kucheryavskiy, & Pomerantsev, 2017).

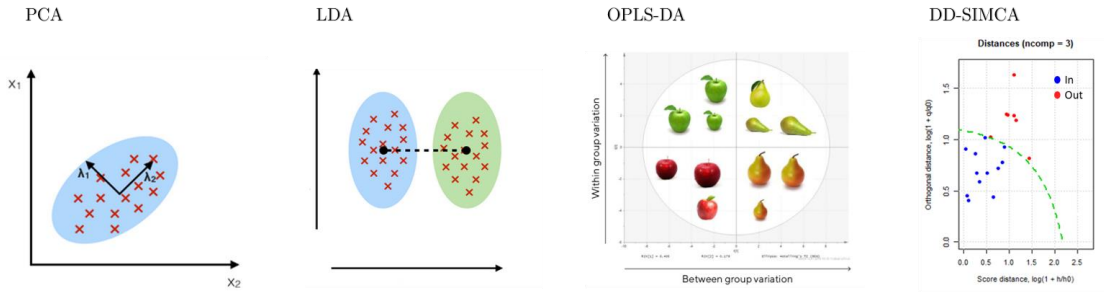

Figure 10: Chemometric methods.

DD-SIMCA consists of two steps. In the first step, principal component analysis (PCA) is applied to the training dataset, and in the second, two distances are calculated for each object from the training set: the score distance (SD) and the orthogonal distance (OD) and corresponding critical limits. The SD represents the position of a sample within the score space, while the OD is the orthogonal Euclidean distance of the sample to the score space. DD-SIMCA adds the possibility of estimating the data-driven distribution parameters, making it possible to develop an acceptance area/decision rule for a given value (Pomerantsev & Rodionova, 2014). The classification results are described as 'sensitivity' and 'specificity' or in standard statistical terms as type I error (a) and type II error (b). The sensitivity denotes a share of correctly identified samples of the target class, while specificity is a portion of objects of an alternative class correctly identified as members of that alternative class. The sensitivity is defined as 100 (1–a)% and specificity as 100 (1–b)% (Fidelis et al., 2017; Rodionova, Titova, et al., 2016). This model represents an excellent approach for verifying geographical origin.

1.2.3 Geographical traceability

The need to protect high quality and locally produced fruit and vegetables is becoming more pronounced. Since most agri-food products only hold a reputation and marketability at the local level, they need to be protected locally, especially from the uncontrolled import of qualitatively inferior products labelled as 'local' or emanating from a specific geographic region. To accomplish this requires geographical authentication, i.e., verifying a product's declared origin.

Recent applications that successfully combine stable isotope ratios with multi-elemental analyses include determining the geographical origin of tomato and lettuce (Mahne Opatić, Nečemer, Lojen, & Vidrih, 2017; Spalla et al., 2009), potato (Mahne Opatić, Nečemer, Budič, & Lojen, 2018; Zampella et al., 2011), sweet pepper (Mahne Opatić et al., 2017), strawberry (Covaciu et al., 2016; Perez et al., 2006), blueberry, pear (Perez et al., 2006), mango (Muñoz-Redondo et al., 2021), apple (Bat et al., 2012) and garlic (Liu, Lin, & Peng, 2018; Opatić et al., 2017). Also, although several studies implemented DD-SIMCA in their research (Arif et al., 2021; de Araújo et al., 2021; de Oliveira Moreira & Braga, 2021; Faqeerzada et al., 2020; Fidelis et al., 2017; Gerônimo et al., 2021; Gomes et al., 2021;

Mazivila et al., 2020; Mohammed & Shuming, 2021; Neves & Poppi, 2020; Rodionova, Oliveri, et al., 2016; Wang et al., 2020), so far none have used DD-SIMCA class-modelling in combination with stable isotopic and multi-elemental compositional data.

Given this specific knowledge gap, this thesis uses a combined stable isotope and elemental approach using a one-class chemometric model to assess its potential as a 'screening' tool to combat the misdeclaration of Slovenian products on the market. The selected vegetables and fruits were selected as a study commodity. Despite its potential, this approach is yet to be applied, and such an approach has yet to be adopted by Slovenian national regulators.

1.2.4 Case study

1.2.4.1 Asparagus, garlic, strawberry, cherry, apple and kaki

This case study focuses on asparagus (*Asparagus officinalis L.*), garlic (*Allium sativum L.*), strawberry (*Fragaria × ananassa*), cherry (*Prunus avium L.*), apple (*Malus domestica Borkh.*), and kaki (*Diospyros kaki L.*) fruits because of their economic and regional relevance in Slovenia. Furthermore, it allows the possibility to promote the production and consumption of high-nutrient sometimes discontinued products. Although Slovenia is a relatively small country (20 273 km^2) with a low level of self-sufficiency, especially regarding fruit (30 %) and vegetables (48 %) ("Podatkovna baza SiStat," n.d.), locally produced varieties have become more appreciated by the Slovenian consumer. In parallel, such fruits and vegetables have become more vulnerable to food fraud. Fruits and vegetables were also selected that differ in climate, soil conditions, fertiliser application, irrigation water, harvest time and storage conditions to test how the variability in isotopic ratios and elemental profiles influences the statistical models for characterising geographical origin.

Seasonal and annual variations in environmental conditions and agricultural practices also alter the isotopic composition of plants. Covering annual differences in the stable isotope ratios and elemental composition of plant materials requires collecting samples over many years. Such an expanded database requires more complex interpretations due to increased natural variation and overlap of the measured parameters in the authentic sample population (Kelly et al., 2005). Nevertheless, a constant expansion of the database should be enabled, and the robustness of all established models should be tested yearly.

Chapter 2

Aims and Hypotheses

The complex nature of our globalized food supply chain and the economic motivation to provide cheaper food products increase the possibility of fraud. Food fraud prevention is paramount to protect our consumers' trust and maintain fair, sustainable business practices. The development of analytical techniques, establishing databases, and chemometric approaches allow us to assess the authenticity of products from the market.

Despite the sophisticated techniques available, food flavours' characterisation and authenticity assessment remain challenging. The introduction of compound-specific isotope analysis, however, means that it is now possible to distinguish natural aromas from synthetic ones based on the isotopic values of VOCs (Jochmann & Schmidt, 2012; Martin et al., 1993; Richling et al., 2006; van Leeuwen et al., 2014). The result is limited to a few common aroma compounds present in different fruits and is based on a small number of samples produced using time-consuming extraction procedures and significant amounts of organic solvents (van Leeuwen et al., 2014). To my knowledge, no study has developed HS-SPME with GC-P-IRMS to determine δ^2H values of food flavourings. Therefore, the first objective of this thesis will be to develop a methodology based on HS-SPME coupled with GC-C-IRMS and GC-P-IRMS to determine δ^{13}C or δ^2H values for several VOCs within the same run with a focus on establishing a procedure for routine laboratory application.

The use of stable isotope ratios with multi-elemental analyses is already well developed and is the method of choice in various food applications for geographical authentication (Katerinopoulou et al., 2020; Kelly et al., 2005). Nevertheless, apart from the measurement, data interpretation requires detailed knowledge of the food commodity in question and sufficient authentic food sample reference data located in a specific database (Camin et al., 2017). Both in the case of flavour authenticity and geographical traceability, there is a lack of comprehensive and representative databases. For this reason, the second objective is to establish such databases.

Besides preparing a sampling strategy, it is also necessary to consider statistical analysis (Donarski et al., 2019). While comparative analysis is used for flavour authentication, this analysis alone cannot provide a definitive answer concerning the geographical origin of an unknown sample (López Vilardell, 2015). Also, despite that discriminant analysis is the most widely used method in authenticity studies (Marini, 2009), several papers highlight the inappropriateness of discriminant analysis for authentication studies and propose using one-class classification methods, like DD-SIMCA (Oliveri, 2017; Rodionova, Titova, et al., 2016). However, no one has applied such a methodology combined with stable isotopic and multi-elemental compositional data for determining geographical origin. Given this knowledge gap, the third objective of the thesis is to create an appropriate DD-SIMCA model for selected fruits and vegetables based on reliable predictions of new unknown samples with the potential to combat misdeclaration.

The interpretation of the results is also of great importance, and the conclusion made must be beyond a reasonable doubt (Primrose et al., 2010). However, the naturalness of the flavours (except vanillin) and the geographical origin of fruits and vegetables are still under investigation. Therefore, the fourth objective of this study is to assess the naturalness of flavourings (fruit, truffle and vanilla) and to verify if selected fruits (strawberries, cherries, apples and kaki) and vegetables (garlic and asparagus) available on the market agree with their declaration.

This thesis tests the following hypotheses:

- HS-SPME extraction procedure can be coupled with GC-C-IRMS and GC-P-IRMS to determine $\delta^{13}C$ or $\delta^{2}H$ values for several VOCs within the same run.
- Established databases are suitable for verifying flavour authenticity or the geographical origin of crops.
- Stable isotopes of light elements and elemental profiles combined with a one-class chemometric model DD-SIMCA can verify the declared origin of selected vegetables (garlic, asparagus) and fruits (cherry, apple, and kaki) on the market.
- Most of the fruit, truffle and vanilla flavours declared as natural, and fruits and vegetables declared as Slovenian are correctly labelled.

Chapter 3

Publications

3.1 Scientific Paper: "Compound-Specific Carbon and Hydrogen Isotope Analysis of Volatile Organic Compounds using Headspace Solid-Phase Microextraction"

This chapter presents the paper entitled "Compound-Specific Carbon and Hydrogen Isotope Analysis of Volatile Organic Compounds using Headspace Solid-Phase Microextraction" by Lidija Strojnik, Federica Camin and Nives Ogrinc. The paper was published in Talanta in 2020. It describes the coupling of HS-SPME with GC-IRMS to determine $\delta^{13}C$ or $\delta^{2}H$ values of VOCs within the same run and to establish a routine laboratory application.

Nine different VOCs (ethyl butanoate, ethyl-2-methylbutanoate, hexanal, 2-methylbutyl acetate, (E)-hex-2-enal, hexyl acetate, [(Z)-hex-3-enyl] acetate, benzaldehyde and ethyl hexanoate), commonly present in apple aroma and important in authenticity studies, were chosen based on their chemical and organoleptic properties. The method was first optimized using a GC-MS to maximise signal intensity (peak area) and factors, such as fibre coating, sample volume, extraction and desorption time, and temperature. The optimised method was then transferred to GC-IRMS to improve the limit of detection (LOD) using HS-SPME as a preconcentration technique. To achieve reproducible and accurate results, a combination of a multiple-point isotopic linear normalization method, and nonlinear correction (calibration of the output of the IRMS instrument in terms of isotope ratio based on different analyte signals – peak heights), was tested. The method gives reproducible and accurate results for different chemical classes of VOCs over varying concentrations in the same analysis. This approach can significantly improve the measurement error of small peaks (below 1 nA) from 3 ‰ to 0.5 ‰. Method validation was also performed, and the average combined measurement uncertainty (MU) was 0.42‰.

The main concern of using SPME in combination with isotope techniques is isotope fractionation caused by fibre immersion or sampling headspace, fibre type, extraction temperature and time, and the physicochemical properties of the compounds. Isotopic fractionation may occur during the volatilisation of the sample, during combustion or during the chromatographic process itself. Therefore, special care was taken to avoid

isotopic fractionation, and the effects of equilibration, adsorption, desorption times and temperatures on $\delta^{13}C$ or $\delta^{2}H$ values were examined. All the $\delta^{13}C$ values were below ± 3*MU, regardless of analytical conditions. In contrast, for $\delta^{2}H$ values, only temperature below 30 °C, with an equilibration time of 15 min and adsorption time between 10 and 20 min, produced a noticeable effect (<10 ‰). Therefore, method optimisation can minimise MU, and data normalisation and validation are essential for obtaining meaningful data in flavour authenticity studies.

In this study, I was responsible for selecting and preparing pure volatile compounds for analysis. I also performed stable all of the carbon and hydrogen isotope measurements, data correction and normalisation, method validation and evaluation of isotope fractionation. I was also responsible for interpreting the data and preparing the manuscript. Results of stable hydrogen isotope analysis were obtained within my two-month training at the Fondazione Edmund Mach Institute in Italy.

The work was presented as a poster presentation at the 2nd Food Chemistry Conference, 17-19 September 2019 in Seville, Spain and at XXII, and during the International Mass Spectrometry Conference, IMSC 2018, August 26-31, 2018, in Florence, Italy.

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Compound-specific carbon and hydrogen isotope analysis of volatile organic compounds using headspace solid-phase microextraction

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ABSTRACT

Natural flavouring materials are in high demand, and a premium price is paid for all-natural flavourings, making them vulnerable to fraud. At present, compound-specific isotope analysis (CSIA) is perhaps the most sophisticated tool for determining flavour authenticity. Despite promising results, the method is not widely used, and the results are limited to the most common volatile organic compounds (VOCs). This paper describes a robust protocol for on-line measurements of $\delta^{13}C$ and $\delta^{2}H$ using HS-SPME coupled with GC-C-IRMS and GC-HTC-IRMS for common fruit VOCs. To achieve reproducible and accurate results, a combination of a peak size/linearity correction with drift correction were used. Finally, the results were normalised by multiple point linear regression using the known and measured values of reference materials. Special care was taken to avoid irreproducible isotopic fractionation and the effects of equilibration, adsorption, desorption times and temperatures on $\delta^{13}C$ or $\delta^{2}H$ values were examined. Method validation was performed, and the average combined measurement uncertainty (MU) was 0.42‰. All the $\delta^{13}C_{VPDB}$ values were below ± 3*MU, regardless of analytical conditions. In contrast, for $\delta^{2}H_{VSMOW\text{-}SLAP}$ values, only low temperature (30 °C) with equilibration time (15 min) and shorter adsorption time (between 10 and 20 min) can produce an isotopic difference of < 10‰. Therefore, method optimisation can minimise MU, and data normalisation and method validation are essential for obtaining meaningful data for use in flavour authenticity studies.

1. Introduction

Consumer demand for natural source products is growing with the primary factors being modern lifestyle and globalization [1]. With increasing pressure to satisfy consumer needs and the high price of natural flavourings compared to synthetic ones, makes naturally flavoured products especially vulnerable to economically motivated adulteration [2]. The EU has legal requirements that every product labelled as containing natural flavours should contain flavours of natural origin. It is, therefore, necessary to develop analytical techniques to assist in enforcing regulations and verify food label claims. At present, compound-specific isotope analysis (CSIA) is perhaps the most specific and sophisticated method for determining food authenticity, and studies have shown that it is possible to distinguish between natural and synthetic aromas based on the isotopic values of VOCs [3–9]. These studies, however, are limited to a few common compounds, stable isotopes ratio of carbon ($^{13}C/^{12}C$, expressed in $\delta^{13}C$), and based on a limited number of samples [8]. In the past, the extensive sample preparation like simultaneous distillation extraction (SDE) and liquid-liquid extraction (LLE) [8], solid phase extraction (SPE), headspace (HS) analysis, striping, and purge and trap methods were required to extract flavour components from fruit and purify the extracts [10]. These procedures are both time-consuming and use significant amounts of organic solvents [10]. In the early 1990s, Pawliszyn and co-workers developed solid-phase microextraction (SPME), which is a solvent-free method designed for the extraction of analytes from gaseous, liquid, and solid matrices, and allows easy automation [11]. Despite its advantages namely, reduced time, simplicity, lower probability of sample contamination and improved repeatability [12], there are only a few studies that combine SPME with gas-chromatography compound-specific isotope ratio mass spectrometry (GC-C-IRMS) [7,13–16]. One reason is the limited knowledge concerning SPME and isotopic fractionation. Isotopic fractionation refers to the fluctuation in the stable isotope ratios as a result of natural biochemical processes as a function of their

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atomic mass. Fractionation also describes variations in the stable isotope ratios of carbon brought about by non-natural causes (laboratory-induced fractionation) [17]. Irreproducible isotopic fractionation can vary depending on fibre placement (immersion or headspace), fibre type, temperature, time, and the physicochemical properties of the analytes [18].

The use of GC-C-IRMS is the subject of a review by van Leeuwen et al. (2014) [8] in which the authors emphasise the necessary conditions to obtain precise isotope ratios of analytes in complex sample matrices. For example, achieving good reproducibility requires extensive method development aimed at producing optimal instrumental conditions, determining stability and linearity, tuning the injection volume so that the analyte signal is within the linearity range of the instrument, preventing carryover, and choosing the appropriate reference materials [19–22]. The measured isotope ratio should ideally be independent on sample size. Still, in practice the response is often non-linear and thus, the isotope ratio depends on the amount of the sample [23]. Therefore, only the values within a linear range could be reported. Possible irreproducible isotope fractionation occurring as a result of derivatization, poor injection, and incomplete combustion must also be avoided [24]. Moreover, an important step to obtain both repeatable and reproducible results is scale normalisation based on two simultaneously analysed scale anchors whose isotopic composition bracket those of the samples [25,26]. Stable isotope ratio analysis of a single element (e.g. carbon) itself may not provide the discriminatory power required to determine authenticity, which means that the characterisation of an additional element, such as hydrogen could be beneficial. Previously, however, only a few studies have included hydrogen isotope measurements, and only Perini et al. (2019) [16] and Hattori et al. (2010) [18] combine SPME with gas-chromatography-high temperature conversion-specific isotope ratio mass spectrometry (GC-HTC-IRMS), to study vanillin and acetic acid, respectively [8].

The objective of this paper was to evaluate the suitability of the coupling of HS-SPME with GC-C-IRMS and GC-HTC-IRMS to determine $\delta^{13}C$ or $\delta^{2}H$ values for several VOCs within the same run with the particular focus on establishing a procedure for routine laboratory application. The specific aims include: 1) evaluation and optimisation of SPME parameters and chromatographic conditions for IRMS measurements; 2) utilising of peak size/linearity corrections and data normalisation by means of multiple-point isotopic linear normalisation; 3) quality control and quality assurance of the measurements by GC-C-IRMS; 4) identification of any irreproducible isotopic fractionation during the measurements. Method performance has been evaluated using the VOCs present in apple aroma, but can be applicable for stable isotope analysis of other volatile organic compounds.

2. Materials and methods

2.1. Reagents and materials

Pure aroma compounds (synthetic) of ethyl butanoate, ethyl-2-methylbutanoate, hexanal, 2-methylbutyl acetate, (*E*)-hex-2-enal, hexyl acetate, [(*Z*)-hex-3-enyl] acetate, benzaldehyde and ethyl hexanoate were purchased from Sigma Aldrich. A Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (50/30 μm thickness), 10 mL headspace vials with a PTFE/silicone septum were purchased from Merck (Supelco, USA). Fibres were conditioned at 270 °C for 4 h and at 250 °C for 5 min before each analysis and 250 °C for 20 min after analysis. All vials were cleaned and conditioned at 250 °C.

2.2. Analysis of volatile organic compound by headspace SPME (HS-SPME)

A working standard was prepared by diluting pure VOCs (1 μL) in deionised water (20 mL). The VOCs were extracted from the headspace of a 10 mL SPME vial. Different sample volumes (0.5 mL and 1 mL), temperatures (30 °C, 50 °C, 70 °C), exposure times (10, 20, 30, 40, 50 min), desorption times (60, 120 and 160 s), and injection modes (split and splitless) were tested. Blanks were tested between samples to check for possible analyte carryover.

2.3. Stable isotope ratios of synthetic working reference materials

The $^{13}C/^{12}C$ ratios of synthetic standards were determined using an IsoPrime 100 – Vario PYRO Cube, a Vario liquid sampler, and an OH/CNS Pyrolyser/Elemental Analyzer (IsoPrime, UK) operated using IonVantage for Isoprime Build 1, 6, 1, 0 software. The oxidation and reduction reactor temperatures were 900 °C and 680 °C, respectively. The $^{2}H/^{1}H$ ratios of synthetic standards were measured using a pyrolyzer (TC-EA, Thermo Scientific, Germany) furnished with an autosampler (Finnigan AS 200, Thermo Scientific) and interfaced to a DELTA V IRMS (Thermo Scientific, Germany) via a ConFlo IV dilutor (Thermo Finnigan, Germany). All values are denoted in the delta-notation relative to the international VPDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$ and VSMOW-SLAP (Vienna Standard Mean Ocean Water–Standard Light Antarctic Precipitation) for $\delta^{2}H$ according to $\delta i\,E = \left(\frac{i\,RSA - i\,RREF}{i\,RREF}\right)$, where "i" is the mass number of the heavier isotope of an element E, "RSA" is the respective isotope ratio of the sample and "RREF" is the isotope ratio of a relevant internationally recognised reference materials. Delta values are expressed in "per mil" (‰) [27]. The method was checked using internal laboratory reference materials: absolute ethanol ($\delta^{13}C_{VPDB}$: 27.37 ± 0.09‰, MERCK, Germany), a rum distillate ($\delta^{13}C_{VPDB}$: 13.81 ± 0.09‰) and a wine distillate ($\delta^{13}C_{VPDB}$: 27.77 ± 0.08‰). These reference materials were calibrated against BCR-656 wine alcohol ($\delta^{13}C_{VPDB}$: 26.91 ± 0.07‰, IRMM, Belgium). The combined uncertainty (u_c, k = 1) of measuring $\delta^{13}C_{VPDB}$ in ethanol was ± 0.236‰, while the expended uncertainty (U, k = 2) was ± 0.42‰. The $\delta^{2}H$ values were normalised against a fuel oil NBS-22 ($\delta^{2}H_{VSMOW\text{-}SLAP}$ = −116.9 ± 0.3‰, NIST RM 8539, Sigma-Aldrich), USGS70 ($\delta^{2}H_{VSMOW\text{-}SLAP}$ = −183.9 ± 1.4‰), and USGS 71 ($\delta^{2}H_{VSMOW\text{-}SLAP}$ = −4.9 ± 1.0‰) (C20 icosanoic acid methyl esters, USGS). Reproducibility was tested by injecting freshly prepared working reference materials on at least three different days (for carbon in two different laboratories operated by two different persons), measured at least in duplicate in one day. The reproducibility was < 0.3‰ for the $\delta^{13}C$ and < 3‰ for $\delta^{2}H$.

2.4. Gas chromatography isotope ratio mass spectrometry (GC-IRMS)

The carbon isotopic compositions of VOCs in the working reference materials were obtained using a GC-C-IRMS (GC: Agilent 6890 N, CT-PolaPLOT Q 30 m 30 μm (Agilent Technologies, USA), Combustion: CuO treatment at 900 °C to convert the CO_2 gas (IsoPrime, GV Instruments, UK), IRMS (IsoPrime, GV Instruments, UK). Water vapour was removed with a Nafion water trap. The faraday collector was setup to detect CO_2 ion contributions at *m/z* 44, 45 and 46. The hydrogen isotopic composition was obtained using a GC-HTC-IRMS: TRACE GC Ultra gas chromatograph, equipped with a TriPlus autosampler, hyphenated to a Delta V Advantage IRMS, (Thermo Scientific, Italy) and in parallel, with a single-quadrupole GC-MS (ISQ Thermo Scientific, Italy). Data were collected using Isodat 2.5 software (Thermo Scientific, Italy).

For $\delta^{2}H$, each compound was passed through a high-temperature reactor (1400 °C). The $[H_3]^+$ factor, quoted before starting each analytical run, was < 8, generally around 5, with a maximum daily variation of 0.05. The separation was achieved using an Agilent J&W VF-WAXms capillary column (30 m × 0.25 × 0.25) (Agilent Technologies, USA). The temperature program was as follows: 40 °C (held 1 min) to 60 °C at 5 °C min^{-1} (held 1 min), 60–100 °C at 7 °C min^{-1}, 100–180 °C at 10 °C min^{-1}, finally 180–200 °C at 15 °C min^{-1} (held 1 min). The carrier gas was He at 1.5 mL min^{-1} for carbon and 2.0 mL min^{-1} for hydrogen measurements. Injections were performed at 250 °C in split

Table 1
Comparison of the $\delta^{13}C_{VPDB}$ values (‰) obtained by GC-C-IRMS to the 'reference values' obtained by EA-IRMS.

	ethyl butanoate	ethyl 2-methylbutanoate	butyl acetate	hexanal	2-methylbutyl acetate	ethyl hexanoate	hexyl acetate	[(Z)-hex-3-enyl] acetate	hexan-1-ol	(E)-hex-2-en-1-ol	benzaldehyde
EA											
Average	−25.72	−24.68	−28.57	−25.48	−32.86	−32.82	−26.99	−28.70	−24.37	−27.51	−25.99
Stdev	0.01	0.01	0.06	0.02	0.04	0.05	0.01	0.03	0.05	0.04	0.01
Liquid injection/splitless/different concentration											
2.5 µL/mL	−25.72	−24.80	−28.28	−26.16	−32.61	−33.01	−26.33	−28.22	−23.41	−27.54	−25.45
5 µL/mL	−25.33	−24.85	−27.80	−26.08	−32.80	−33.10	−26.47	−28.30	−23.70	−27.57	−25.92
10 µL/mL	−25.96	−24.91	−27.99	−26.19	−32.90	−32.94	−26.46	−28.42	−23.75	−27.54	−25.78
15 µL/mL	−25.36	−24.72	−28.00	−26.25	−32.86	−33.04	−26.48	−28.58	−24.04	−27.87	−25.66
Average	−25.59	−24.82	−28.02	−26.17	−32.79	−33.03	−26.43	−28.38	−23.72	−27.63	−25.70
Stdev	0.26	0.07	0.17	0.06	0.11	0.06	0.06	0.14	0.22	0.14	0.17
Difference from EA	0.13	−0.14	0.55	−0.69	0.07	−0.21	0.56	0.32	0.65	−0.12	0.29
Liquid injection/split											
Split 2	−26.31	−25.36	−28.76	−26.72	−33.23	−33.07	−26.61	−28.42	−23.94	−27.68	−25.54
Split 5	−26.45	−25.24	−28.66	−26.23	−33.38	−33.00	−26.63	−28.49	−23.87	−27.78	−25.65
Average	−26.38	−25.30	−28.71	−26.47	−33.30	−33.03	−26.62	−28.46	−23.90	−27.73	−25.60
Stdev	0.11	0.09	0.07	0.35	0.11	0.05	0.01	0.05	0.05	0.07	0.08
Difference from EA	−0.66	−0.62	−0.14	−0.99	−0.44	−0.21	0.37	0.24	0.47	−0.22	0.39
SPME/split/different sample volume											
1 mL	−26.07	−24.98	−28.66	−25.45	−33.47	−32.50	−26.19	−27.79	−23.98	−27.92	−25.85
0.5 mL	−26.30	−24.81	−28.68	−25.54	−33.36	−33.00	−26.31	−27.88	−24.50	−27.87	−25.74
Average	−26.19	−24.90	−28.67	−25.50	−33.41	−32.75	−26.25	−27.84	−24.24	−27.89	−25.80
Stdev	0.16	0.12	0.01	0.06	0.07	0.35	0.09	0.07	0.36	0.03	0.08
Difference from EA	−0.47	−0.22	−0.10	−0.02	−0.55	0.07	0.74	0.86	0.13	−0.38	0.19
SPME/splitless											
Splitless	−26.03	−24.79		−25.53	−32.99	−32.72	−26.46	−28.52		−26.55	−26.26
Stdev	0.14	0.09		0.05	0.11	0.08	0.01	0.16		0.14	0.17
Difference from EA	−0.31	−0.11		−0.05	−0.13	0.10	0.53	0.18		0.96	−0.27

(split ratio 5:1 or 2:1) and splitless mode.

Data analysis was performed firstly by applying a correction for peak size (peak size/linearity correction). Difference between the known and measured values of carbon/hydrogen isotope ratios was evaluated using linear regression and was compensated for measurement error due to variations in peak heights relative to the concentration of the analyte in the sample. For analysis of samples with small intensity peaks (< 1.5 nA), usually logarithmic function was used. Next a correction for drift was applied and finally the results were normalised by multiple point linear regression using the known and measured values for working reference materials. The isotopic composition of C and H in the working reference materials were determined using EA/HTC-IRMS.

Reproducibility was tested by injecting freshly prepared working reference materials on at least three different days (for carbon in two different laboratories), measured at least in duplicate in one day. The reproducibility was < 0.6‰ for the $\delta^{13}C$ and < 8‰ for $\delta^{2}H$. The limit of detection (LOD) was defined as the minimum amount of carbon dioxide obtained after combustion of individual VOC to achieve a precision of 0.3‰ and as the minimum amount of hydrogen obtained after pyrolysis of individual VOC to achieve a precision of 5‰. For the minimum acceptable signal size (0.6 nA), approximately 6 nmol of CO_2 is required, while for the minimum acceptable signal size (1000 mV), for hydrogen approximately 20 nmol of H_2 is required.

3. Results and discussion

Nine different volatile organic compounds (VOCs) were tested in this study based on their chemical and organoleptic properties (in detail described in Supporting Information). Their different chemical characteristics allow the capability of HS-SPME with GC-C-IRMS and GC-HTC-IRMS to be tested in order to obtain information about $\delta^{13}C$ or $\delta^{2}H$ values. In food, VOCs are usually present in different concentrations, and even minor VOCs can significantly add to the overall aroma. The compounds selected for this study are common VOCs present in apple aroma and important in authenticity studies.

3.1. Optimisation of HS-SPME GC-MS method

The HS-SPME method was optimised using GC-MS to maximise signal intensity (peak area) while avoiding fibre saturation [28]. In IRMS, the detector is less sensitive than in a standard GC mass spectrometer (ng L^{-1}) [9]. Therefore, the emphasis was on improving LOD using SPME as a preconcentration technique and optimizing factors, such as fibre coating, sample volume, extraction and desorption time, and temperature. The effect of these variables was studied on the extraction of fruit VOCs. The sample vial volume was fixed at 10 mL. Two commercially available SPME fibres: a 100 µm PDMS and 50/30 µm DVB/CAR/PDMS, were tested. The DVB/CAR/PDMS fibre had a better extraction efficiency overall and was used in all further optimisation steps. The effects of sample volume (0.1 mL, 0.5 mL, 1 mL and 5 mL) on absorptivity and equilibrium were also investigated. A 1 mL sample volume gave the best absorptivity response, while for terpenoids, a larger sample volume was preferable. Temperatures (30 °C, 45 °C, 60 °C) and exposure time (5, 10, 15, 20, 40, 60, 80 and 100 min) were also studied. All target compounds reached adsorption capacity after 20 min at 30 °C. Optimal desorption time was 60 s at 250 °C. Regarding chromatographic conditions, peak overlap/shape had a detrimental effect on trueness and precision. For $^{2}H/^{1}H$ ratio measurements, co-elution and peak tailing significantly influenced δ-values similar to that observed by Vetter et al. (2007) [29].

3.2. Coupling of HS-SPME to GC-C-IRMS

The aim of this study was to investigate if HS-SPME can cause irreproducible isotope fractionation. Hattori et al. (2010) [18] reported

Table 2
Calculated repeatability, reproducibility, combined and expanded uncertainty for carbon isotope analysis of aroma compounds.

In-house standard	Reference values[1] (‰)	Repeatability (r)	Reproducibility (R)	Combined uncertainty (u_c, k = 1)	Expanded uncertainty (U = $2u_c$, k = 2)
ethyl butanoate	−25.72	0.08	0.31	0.47	0.93
hexanal	−25.48	0.17	0.58	0.32	0.64
(E)-hex-2-enal	−27.51	0.21	0.43	0.76	1.52
hexyl acetate	−26.99	0.20	0.32	0.64	1.27
benzaldehyde	−25.99	0.28	0.55	0.75	1.49
ethyl 2-methylbutanoate	−24.68	0.04	0.29	0.14	0.28
2-methylbutyl acetate	−32.86	0.14	0.38	0.25	0.50
ethyl hexanoate	−32.82	0.25	0.33	0.20	0.40
[(Z)-hex-3-enyl] acetate	−28.70	0.16	0.58	0.23	0.46
All compounds		0.17	0.42	0.42	0.84

[1]Reference values were previously defined using EA-IRMS.

that SPME could cause isotopic fractionation resulting from fibre immersion or sampling headspace, fibre type, extraction temperature and time, and compound properties and influence δ-values. Isotopic fractionation may occur during the volatilisation of the sample and the combustion process, as well as during the chromatographic process itself. Injection reproducibility is also crucial in GC-C-IRMS, whereas these effects are less pronounced in bulk stable isotope analysis (BSIA) using EA-IRMS. The EA-IRMS instrument is more robust, precise and measurements are traceable to the VPDB via the NBS22 reference materials as reported by Neves et al. (2015) [30] which makes it more suitable for obtaining 'reference values' of pure VOCs.

After EA-IRMS analysis, the samples were analysed by GC-C-IRMS using classical liquid injection. A comparison of $\delta^{13}C_{VPDB}$ values obtained by EA-IRMS and GC-C-IRMS is presented in Table 1. The differences were between −0.69 and 0.65‰. Different concentrations levels of VOCs (2.5 μL, 5 μL, 10 μL and 15 μL) were also tested, and the results were repeatable (± 0.26‰). Jochmann and Schmidt (2012) [9] also suggested that split injection could be a source of fractionation. In response, we compared injections in split (2:1 and 5:1) and splitless modes with the 'reference values'. Although there were no differences in isotope values at split ratios of 2:1 and 5:1, slightly lower $\delta^{13}C_{VPDB}$ values were observed for ethylbutanoate, ethyl 2-methylbutanoate, hexanal and 2-methylbutyl acetate compared to splitless injection. Finally, we performed HS-SPME with different sample volumes (1 mL and 0.5 mL) in split mode. Except for butyl acetate and hexan-1-ol the best results, were obtained using splitless injection mode (Table 1).

3.3. The normalisation of stable isotope ratios data

Analytical procedures in the field of organic stable isotope analyses and characterisation are, in many cases, non-standardised and certified reference materials (CRMs) are unavailable. Several authors, however, describe appropriate data normalisation methods and the correct use of isotopic reference materials [9,30–32]. For instance, Jochmann and Schmidt (2012) [9] present possible normalisation pathways for GC-C-IRMS analysis. However, data for VOCs are missing. The lack of reference materials also makes method validation challenging since the majority of CRMs are certified using EA-IRMS and generally cover non-volatiles. However, some reference materials, such as those produced by the University of Indiana are compatible with GC-C-IRMS systems and can be used for calibration and data normalisation, namely *n*-alkanes, ethanol, and fatty acids esters. Although they are not internationally agreed-upon reference materials, they are widely used in IRMS measurements. Ethanol from wine produced by the Institute for Reference Materials and Measurements – IRMM (BCR-656) is another example. In this study, we followed Jochmann and Schmidt (2012) [9] who suggest that materials (compounds) calibrated separately using EA-IRMS are suitable for GC-C-IRMS as a "reference material mixture" under identical treatment.

Paul et al. (2007) [26] reviewed six commonly used normalisation methods and recommended a two-point calibration. In this study, a multi-point normalisation is used in order to reduce the random error associated with the analysis of a reference materials used to anchor the linear scale [9]. Multiple-point isotopic linear normalisation method with peak size/linearity correction is considered the most appropriate method for data normalisation. It gives reproducible and accurate results for different chemical classes of VOCs over varying concentrations in the same analysis. The linear range in terms of peak size of the instrument is narrow (3–10 nA of peak height), and since many compounds fall out of this range, peak size/linearity correction (calibration of the output of an IRMS instrument in terms of isotope ratio based on different analyte signals – peak heights) was developed as described previously in *Materials and methods*. This approach can significantly improve measurement error of small peaks (below 1 nA) from 3‰ to 0.5‰. Since many compounds were measured simultaneously, their normalised values should be compared to the 'reference values', and the difference between the two represents the method error.

3.4. Quality control and quality assurance

The HS-SPME GC-C-IRMS method was validated for ethyl butanoate, ethyl-2-methylbutanoate, hexanal, 2-methylbutyl acetate, (E)-hex-2-enal, hexyl acetate, [(Z)-hex-3-enyl] acetate, benzaldehyde and ethyl hexanoate. Validation was performed according to the EURACHEM/CITAC Guide CG 4 [33]. The criteria for repeatability and reproducibility was as follows: limit of repeatability r = 0.30‰ and limit of reproducibility R = 0.60‰. Repeatability was obtained by performing seven analyses over a short period, while long-term reproducibility and accuracy were tested by injecting freshly prepared working reference materials on three different days. The results are given in Table 2. The maximum repeatability was 0.28‰ for benzaldehyde and 0.17‰ for the method in general (for all compounds). Reproducibility of the $\delta^{13}C$ values, calculated by repeated analysis (n = 16) of the laboratory reference material, was good with an overall R < 0.60‰ (Table 2). Calculated combined and expanded uncertainty for all selected aroma compounds are presented in Table 2. The calculated combined uncertainty was for the method 0.42‰, multiplied by a k-factor (k = 2) so that an expanded uncertainty was 0.84‰ at the 95% confidence level. The results are comparable or better than the reported data, e.g., repeatability (1σ) for acetic acid measurements in vinegar for carbon was 0.4‰ [18] while for vanillin measurements were between 0.8 and 1.0‰ [16,34,35].

The results show that it is possible to obtain reproducible and accurate *δ*-values for VOCs and the methodology has been successfully applied to differentiate between natural and synthetic apple VOCs [15]. The methodology can be also applicable for stable isotope analysis of other VOCs.

3.5. Evaluation of the isotopic fractionation using HS-SPME GC-C/HTC-IRMS method

To see how isotopic fractionation was influencing the $\delta^{13}C$ values,

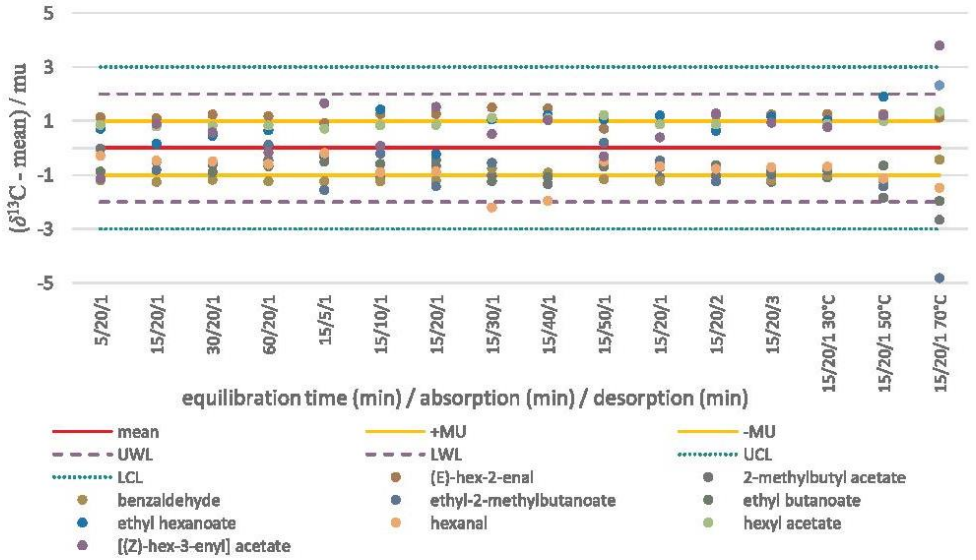

Fig. 1. Evaluation of SPME parameters based on measurement uncertainty calculated for each of the selected compounds. On the x-axis equilibration time (min)/ extraction time (min)/desorption time (min) are reported.

four experiments were performed using a working reference material mixture of nine characteristic apple aroma compounds: 1) varying equilibration time (5, 15, 20 and 60 min) with constant adsorption (20 min) and desorption (1 min) times and temperature (30 °C); 2) varying absorption time (10, 20, 30, 40 and 50 min) with constant equilibration (15 min) and desorption (1 min) times at 30 °C; 3) varying desorption times (1, 2 and 3 min) after 15 min of equilibration, and 20 min of absorption at 30 °C, and 4) testing three different equilibration and absorption temperatures (30, 50 and 70 °C).

The $\delta^{13}C_{VPDB}$ values were evaluated using QA/QC charts where the limits are based on measurement uncertainty of working reference materials. The results are expressed as value $Y = \frac{(x-X)}{u_c}$; where x is the measured value obtained using GC-C-IRMS, X is the assigned or 'reference values' obtained using EA-IRMS, and u_c is the measured combined uncertainty (k = 1) for each compound. This way, the deviation of the measured value from the accepted value (mean) for all VOCs can be presented in one figure. Also, line of measured uncertainty (k = 1) (± MU), upper (UWL) and lower (LWL) warning limit (± 2*MU) and upper (UCL) and lower (LCL) control limit (± 3*MU) are presented (Fig. 1). The standard deviation for any measurement points is below 0.18‰.

The results show that equilibration time, absorption time and desorption time do not affect the $\delta^{13}C_{VPDB}$ values. The temperature of equilibration and absorption is on the other hand critical since high temperatures (≥ 70 °C) are inappropriate for individual VOCs (e.g. [(Z)-hex-3-enyl] acetate and ethyl 2-methylbutanoate) since the measured values deviate from the accepted values by > ± 3*MU.

When different conditions were tested to look at hydrogen isotopic fractionation, out of the nine VOCs selected, only five were used for further testing. The $\delta^{2}H_{VSMOW\text{-}SLAP}$ value of benzaldehyde is approx. + 470‰ and outside of the values of normalisation reference materials used for calibration. Ethyl butanoate, hexanal, and (E)-hex-2-en-1-ol were also excluded because they were outside of the region of isotopic linearity. The results reveal differences in δ-value between experimental conditions only for certain VOCs. The $\delta^{2}H_{VSMOW\text{-}SLAP}$ values obtained for 2-methylbutyl acetate varied the most concerning equilibration time and temperature. Besides high temperature (50 and 70 °C) and long absorption time (40 and 50 min), a short equilibration time (5 min) can lead to differences in δ-value of > 10‰. The best approximation to the 'reference values' (MU < 10‰) was obtained with the following conditions: 15/20/1 (equilibration time (min)/extraction time (min)/desorption time (min)) or 15/10/1 (Fig. 2). Similarly, Hatorri et al. (2010) [18] reported measurement repeatability (1σ) of for acetic acid using HS-SPME GC-IRMS of ± 5.0‰ for hydrogen, while Perini et al. (2019) [16] using HS-SPME GC-HTC-IRMS reported a measurement reproducibility of < 7‰ for vanillin.

The method error can be minimised by optimizing measurement conditions while emphasising that for hydrogen, this is only a preliminary study.

4. Conclusions

This study shows that it is possible to obtain reproducible and accurate $\delta^{13}C$ and $\delta^{2}H$ values for VOCs measured during the same analytical run. These robust methods allow us to isotopically characterise a set of compounds with different chemical properties present over a wide concentration range and overcome issues such as loss of compounds and need for repeated analysis due to their low peak intensities falling outside of the isotopic linearity range. It also shows that the optimisation of HS-SPME and chromatographic conditions, appropriate data normalisation and reference material selection, calculation of peak size/linearity corrections and method validation are mandatory steps to obtain meaningful data for the use in authenticity studies.

Credit author statement

Lidija Strojnik: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing-Original Draft; Federica Camin: Investigation, Validation, Writing-Review & Editing; Nives Ogrinc: Resources, Writing-Review & Editing, Supervision.

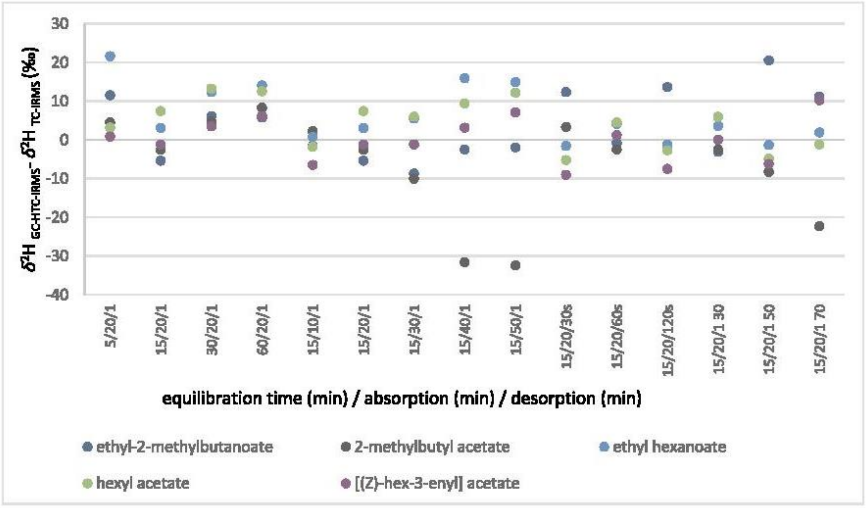

Fig. 2. Calculated reproducibility of materials (compounds), which were calibrated separately on the TC-system, and used as the "working reference material mixture" on the GC-HTC-IRMS. On the x-axis equilibration time (min)/extraction time (min)/desorption time (min) are reported.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121264.

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3.2 Scientific Paper: "Authentication of Key Aroma Compounds in Apple Using Stable Isotope Approach"

In this chapter, I present my paper entitled "Authentication of Key Aroma Compounds in Apple Using Stable Isotope Approach" by Lidija Strojnik, Matej Stopar, Emil Zlatič, Doris Kokalj, Mateja Naglič Gril, Bernard Ženko, Martin Žnidaršič, Marko Bohanec, Biljana Mileva Boshkovska, Mitja Luštrek, Anton Gradišek, Doris Potočnik and Nives Ogrinc. The paper was published in Food Chemistry in 2019. It discusses the potential of combining HS-SPME with GC-C-IRMS to obtain δ^{13}C values of volatile apple compounds.

The present research characterises 18 laboratory-produced and 15 commercial apple recovery aromas, establishes a database of δ^{13}C values of 16 aroma compounds regarding their origin (synthetic and natural) and assesses the authenticity of commercially available aroma compounds. The first step in creating a δ^{13}C database involved obtaining robust data by optimising the extraction procedure, identifying and minimising sources of contamination and isotopic fractionation. Importantly, isotopic fractionation did not occur, and the optimized method was appropriate for all the studied aroma compounds. This study also found that the accurate determination of δ^{13}C values depends on good chromatographic separation and the integration parameters. Since many compounds in varying concentrations are present in a single sample, selecting reference material and appropriate processing and interpretation of the results obtained was crucial. Finally, a database of δ^{13}C values was established, containing an isotopic authenticity range of synthetic standards and many authentic samples. Most of the δ^{13}C values for the aroma compounds were reported for the first time. Data analysis revealed some overlap in the δ^{13}C values between natural and synthetic compounds showing butyl acetate and 1-butanol could not be used in authenticity studies. Analysis of commercial recovery aromas labelled as natural revealed that the δ^{13}C value of most of the compounds present was within the expected authentic range. The data also revealed possible falsifications. The sensitivity of the method was evaluated through simple isotope mass balance calculation. Notably, the study showed that identifying falsifications is possible for most aromatic substances when the fraction of added synthetic compound is in the order of a few 10 %.

In this study, I was responsible for selecting and preparing synthetic volatile compounds and distillates (recovery aromas) before analysis. I performed GC-MS analysis, stable carbon isotope measurement on EA-IRMS and GC-C-IRMS, data processing, database creation and evaluation of the results. I also prepare the manuscript.

The work was presented as an oral presentation at the 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019), November 5-8, 2019 in Prague, Czech Republic, at 2nd Isotope Ratio MS Day, June 27-29, 2018, in Messina, Italy, at the MASSTWIN Workshop on Mass spectrometry in support of the environment, food, and health interaction and disease, April 18-20, 2018 in Antwerp, Belgium. It was also presented at the 10th Jožef Stefan International Postgraduate School Students' Conference and 12th Young Researchers' Day 10th and 11th May 2018 in Piran, Slovenia, and at the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, 15th and 16th May 2019 in Planica, Slovenia.

The work was also presented as a poster presentation at 5th MS Food day, October 11-13, 2017 in Bologna, Italy, at 8th International Symposium in Recent Advances in Food Analysis, (RAFA), November 7-10, 2017 in Prague, Czech Republic, at ASSET 2018, Belfast Summit on Global Food Integrity, May 28-31, 2018 in Belfast, Ireland and during the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, April 1-3, 2019 in Portorož, Slovenia.

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Authentication of key aroma compounds in apple using stable isotope approach

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ABSTRACT

Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) for the analysis of key volatile compounds sampled using headspace solid phase microextraction (HS-SPME) is an appropriate tool for authenticity assessment of apple aromas. The current research characterises 18 laboratory produced and 15 commercial apple recovery aroma samples, establishes a database of $\delta^{13}C$ values of 16 aroma compounds with respect to their origin (synthetic and natural), and assesses the authenticity of commercially available aroma compounds. Analysis of so-called natural aroma products, revealed $\delta^{13}C$ values that were within the expected authentic range although the data did reveal possible falsifications. The sensitivity of the method was evaluated through simple isotope mass balance calculation. Falsification identification is possible for most aromatic substances when the amount of added synthetic compound is in tens of percent.

1. Introduction

Apples are a highly flavoured fruit with unique flavour characteristics, and apple juice is one of the most popular juices in the world (Elss, Preston, Appel, Heckel, & Schreier, 2006). In industrial juice production, several 100 kgs of mashed apples per hour are processed into apple juice. This juice is then sold as commercial single strength juice or further processed to apple juice concentrate and water phase where volatile aroma compounds are recovered and concentrated usually by means of distillation or steam distillation. Recovered aroma solution is also known as fruit juice hydrolate or aromatic water (Dawiec-Liśniewska, Szumny, Podstawczyk, & Witek-Krowiak, 2018; Elss et al., 2006; Taylor, 2016). Water phase/recovery aromas can then be used as naturally produced flavouring in many different dairy, bakery, and cereal products and also in beverages such as a fruity infusion.

The demand for flavourings is increasing, and apple aroma is no exception. The primary factors leading to this increase are globalisation and modernisation. In 2016 the global flavour market was about USD 9.2 billion and is set to increase at a compound annual growth rate (CAGR) of 3.8% and reach nearly USD 12.8 billion by 2023 (Modor Intelligence, 2018). Nowadays, most flavouring compounds are produced by chemical synthesis or by extraction from natural materials. Today's consumers more than ever are demanding naturally flavoured products, and the word "natural" is increasingly used in the marketing of food products (Longo & Sanromán, 2006). Current European legislation allows 4 terms for the sales description of natural flavourings. The term "natural flavouring substances" may only be used for flavourings in which the flavouring component contains exclusively natural substances while the term "natural < x > flavouring" may only be

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used in combination with reference to a food, food category or a vegetable or animal flavouring source if the flavouring component has been obtained exclusively or by at least 95% by w/w from the source material. The other two terms used in regulation EC No. 1334/2008 are "natural < x > flavouring with other natural flavouring" and "natural flavouring" (European Commission, 2008). However, a raw material often contains low concentrations of the desired flavour compounds, making their extraction costly. Moreover, their supply depends on factors that are difficult to control such as weather conditions and plant diseases (Longo & Sanromán, 2006). The cost of natural flavours is often a factor of 10 or more higher than the price of synthetic analogues. Owing to this premium price and the difficulty in differentiating between natural and synthetic flavours, synthetic flavours are sometimes sold as natural ones. This can place major food companies, who think in good faith, that they are purchasing natural flavours and who are typically paying a premium for them, at legal and economic risk (Martin, Remaud, & Martin, 1993), and non-authentic products could also pose a potential health risk. Several chemically defined substances are no longer supported by the industry or have been removed from the "community list" of flavourings and source materials approved for use in and on foods due to safety concerns. Approved flavourings are listed in regulation EU No 872/2012 (European Commission, 2012). In addition, consumer confidence may be dampened by buying an inferior product, sold as the genuine item (van Leeuwen, Prenzler, Ryan, & Camin, 2014). Therefore the ability to trace and authenticate food products/ingredients is of major concern in the food industry.

The most widely adopted analytical techniques used in flavour authentication are based on the analysis of single components, total aroma spectra and chiral separation of enantiomers (Martin et al., 1993; Richling et al., 2006; Schipilliti, Dugo, Bonaccorsi, & Mondello, 2011). New methodologies are also being studied in order to solve current food fraud issues where classical methods fail to detect them. For example, adulteration of natural fruit aroma with synthetic aroma cannot be easily detected by well-established techniques due to their identical chemical characteristics. At this point in time, gas chromatography isotope ratio mass spectrometry (GC-IRMS) is perhaps the most specific and sophisticated method for determining food authenticity (Elss et al., 2006; Kahle, Preston, Richling, Heckel, & Schreier, 2005; Martin et al., 1993; Richling et al., 2006; Schipilliti et al., 2011; van Leeuwen et al., 2014). To provide more information about specific compounds in food and beverages, isotope ratio mass spectromete (IRMS) may be coupled to a gas chromatograph (GC) *via* either a combustion (GC-C-IRMS) or a pyrolysis (GC-P-IRMS) chamber to obtain information about C/N or H/O isotopes, respectively. The use of GC-C-IRMS is the subject of a review by van Leeuwen et al. (2014). Interestingly, although previous studies have investigated the authenticity of the aromatic components in many types of fruits, non have applied GC-C-IRMS for determining the authenticity of apple aromas. One study did investigate apples in order to determine whether or not processing modifies the isotopic ratios in aromatic components in apple juice aroma (Elss et al., 2006). So far, studies include raw fruits (such as pear, pineapple, raspberry, strawberry, cactus pear, blackberry, lemongrass and banana), essential oils, fruit products, and flavours obtained synthetically and/or using biotechnological processes. Most of the research shows that GC-C-IRMS can distinguish between natural and synthetic aromas, but the results are limited to a few of the most common aroma compounds and are based on a small number of samples (van Leeuwen et al., 2014).

Sample preparation methods typically involve techniques like simultaneous distillation extraction (SDE) and liquid-liquid extraction (LLE) (van Leeuwen et al., 2014), Solid Phase Extraction (SPE), headspace (HS) analysis, striping, and purge and trap methods (Mottaleb, Meziani, & Islam, 2014). These procedures take a long time and/or use relatively large amounts of organic solvents (Mottaleb et al., 2014). In the early 1990s, Pawliszyn and co-workers (Arthur & Pawliszyn, 1990) developed solid-phase microextraction (SPME), which is solvent-free method that can be used for the extraction of analytes from gaseous, liquid, and solid matrices, and is also easy to automate. Despite the numerous advantages of SPME, e.g., reduced time, simplicity, lower probability of sample contamination and improved repeatability (Merkle, Kleeberg, & Fritsche, 2015), its combination with GC-C-IRMS has so far been used only in a few aroma authenticity studies (Schipilliti, Bonaccorsi, Cotroneo, Dugo, & Mondello, 2015; Schipilliti, Bonaccorsi, Occhiuto, Dugo, & Mondello, 2018; Schipilliti et al., 2011). Due to a combination of sampling, extraction, pre-concentration and sample introduction into the instrument in a single step, SPME has been used in many food analyses in recent years (Merkle et al., 2015; Souza-Silva, Gionfriddo, & Pawliszyn, 2015). Although numerous studies provide aroma profiles of apples, apple juices and other apple products, no study has investigated the authenticity of the apple aroma compounds. About 15–20 compounds have been identified as the principal contributors to apple aroma in different cultivars (Fructuoso & Cortada, 2010), which makes them ideal candidates for falsification.

The present study deals with the development of a procedure including sampling and standard selection, sample preparation, compound identification, $\delta^{13}C$ measurements, data processing and database creation to detect possible frauds of apple aroma compounds. The overall objectives are: (i) to characterise the aroma of laboratory produced and commercial apple recovery aroma samples by dynamic headspace solid-phase microextraction (SPME) methodology used with GC–MS and GC-C-IRMS analysis; (ii) to establish a database of $\delta^{13}C$ values of synthetic and natural aroma compounds; (iii) assess the authenticity of commercially available aroma compounds.

Hypothesis of our study is "authenticity assessment of commercial apple recovery aromas is possible, by SPME methodology used with GC-C-IRMS analysis".

2. Materials and methods

2.1. Samples

Samples of aroma volatiles, recovered in the water phase in apples (n = 18), were produced by steam distillation at the Biotechnical Faculty, University of Ljubljana. Apple fruits of 5 different varieties (Gala, Idared, Golden Delicious, Red Delicious, Topaz), at 3 different stages of maturity (Idared variety – immature, mature, overripe), and 2 different production types (Topaz variety – organic and integrated) harvested in 2016 were provided by the Agricultural Institute of Slovenia. Commercial samples (n = 15) labelled as natural apple recovery aromas were also analysed.

Samples of 16 pure synthetically derived aroma compounds were purchased from Sigma Aldrich: **1**, ethyl acetate; **2**, ethyl butyrate; **3**, ethyl-2-methyl butyrate; **4**, butyl acetate; **5**, 1-hexenal; **6**, 2-methylbutyl acetate; **7**, 1-butanol; **8**, amyl acetate; **9**, butyl butyrate; **10**, trans-2-hexenal; **11**, hexyl acetate; **12**, 2-hexen-1-ol, acetate; **13**, 1-hexenol; **14**, trans-2-hexenol; **15**, benzaldehyde; **16**, 1-octanol.

2.2. Sample preparation

The volatile components from both laboratory and commercial recovery aromas were extracted using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (50/30 µm thickness) purchased from Sigma-Aldrich (Supelco, Bellefonte, USA) initially conditioned at 270 °C for 4 h. Before each analysis, the fibre was again conditioned at 250 °C for 5 min and after analysis for 20 min at the same temperature. Volatile compounds were extracted from the headspace of a 10 mL SPME vial (with silicone/PTFE septa) filled with 1 mL of sample. Equilibration time was 10 min at 30 °C, and the extraction time was 20 min at 30 °C. Volatile compounds were desorbed from the fibre at 250 °C for 1 min. A working standard solution was prepared by diluting 1 µL of each synthetically derived aroma compounds (**1 to 15**) in 20 mL of water.

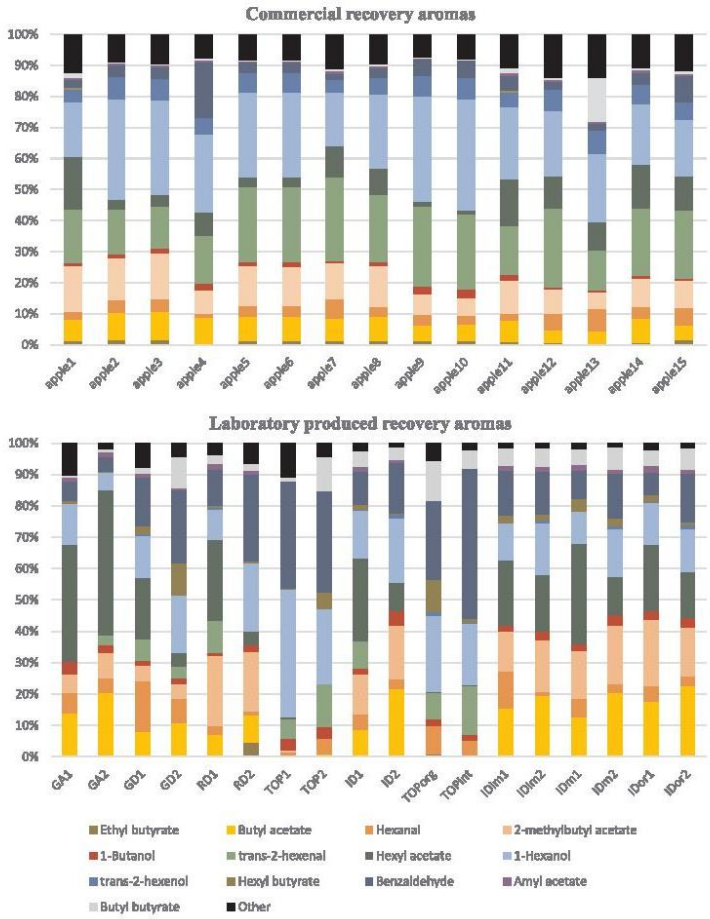

Fig. 1. Relationship between peak areas of aroma compounds in the commercial and laboratory produced recovery aromas obtained by HS-SPME GC–MS (GA = Gala, GD = Golden Delicious, RD = Red Delicious, TOP = Topaz, ID = Idared, 1 = without of storage, 2 = after 2 months of storage, org = organic, int = integrated, im = immature, m = mature, or = overripe). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Gas chromatography-mass spectrometry (GC–MS)

GC–MS analyses were performed using a 7890B GC & 5977A Series GC/MSD (Agilent Technologies, USA). Separation was achieved on a VF-WAXms capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J&W, USA). The temperature program was as follows: 40 °C (held 1 min) to 60 °C at 5 °C/min (held 1 min), then to 100 °C at 7 °C/min, then to 180 °C at 10 °C/min, then to 200 °C at 15 °C/min (held 1 min). Helium was used as a carrier gas with a constant flow of 1.5 mL/min. A Straight Ultra Inert Liner for SPME (Sigma-Aldrich/Supelco, USA) was used, and the injection was performed at 250 °C in the split mode (1:10). In the MS the ion source was set to 230 °C, the interface temperature to 250 °C, and the scan range to 30–400 *m/z*. GC–MS data were acquired using ChemStation software (Agilent, USA). Identification was performed using spectral similarity with the NIST14 library (Agilent, USA).

2.4. Elemental analysis-isotope ratio mass spectrometry (EA-IRMS)

The $^{13}C/^{12}C$ ratios of synthetic standards were determined using IsoPrime 100 – Vario PYRO Cube combined with Vario LS sampler (Liquid sampler for "cube" analyzer line) (OH/CNS Pyrolyser/Elemental Analyzer) (IsoPrime, Cheadle, Hulme, UK) and the IonVantage for Isoprime Build 1, 6, 1, 0 software. The oxidation and reduction reactors were set at 900 °C and 680 °C, respectively. To assure the accuracy of IRMS measurements two internal working standards: absolute ethanol MERCK (Germany) with $\delta^{13}C = -27.38 \pm 0.09‰$ and a distillate of rum with a $\delta^{13}C = -13.81 \pm 0.09‰$ were used. Working standards were previously calibrated against the certified reference material BCR-656 wine alcohol ($\delta^{13}C$ value of $-26.91 \pm 0.07‰$) available from the Institute for Reference Materials and Measurements (IRMM, Belgium).

The carbon isotope data are expressed with the conventional δ-notation using the general formula (Brand, Coplen, Vogl, Rosner, &

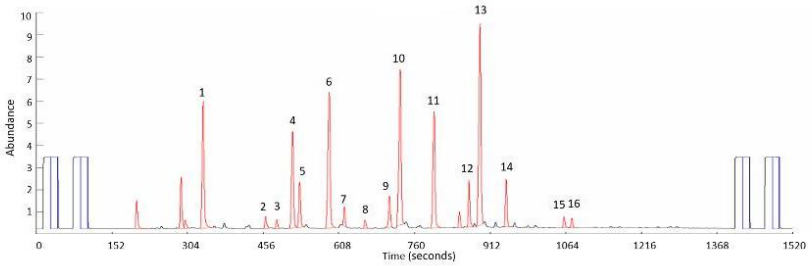

Fig. 2. GC-C-IRMS chromatogram related to natural apple recovery aromas on a VF-WAXms column. Peak identification of 16 pure synthetically derived aroma compounds purchased from Sigma Aldrich: 1, ethyl acetate; 2, ethyl butyrate; 3, ethyl-2-methyl butyrate; 4, butyl acetate; 5, 1-hexenal; 6, 2-methylbutyl acetate; 7, 1-butanol; 8, amyl acetate; 9, butyl butyrate; 10, trans-2-hexenal; 11, hexyl acetate; 12, 2-hexen-1-ol, acetate; 13, 1-hexenol; 14, trans-2-hexenol; 15, benzaldehyde; 16, 1-octanol.

Prohaska, 2014):

$$\delta^{i}E = \left(\frac{R(^{i}E/^{j}E)_{sample}}{R(^{i}E/^{j}E)_{standard}}\right) - 1$$

where E is carbon (C), R is the isotope ratio between the heavier "*i*" and the lighter "*j*" isotope ($^{13}C/^{12}C$) in the sample and relevant internationally recognised reference standard. The delta values are multiplied by 1000 and expressed in units "per mil" (‰). For carbon the Vienna Pee Dee Belemnite (VPDB) is used as a reference standard. The reproducibility of measurements for $\delta^{13}C$ was ± 0.1‰.

2.5. Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)

The isotopic compositions of aroma components were obtained using an Agilent 6890 N GC-C system coupled to an IsoPrime GV IRMS. Separation was achieved using an Agilent J&W VF-WAXms capillary column (30 m × 0.25 × 0.25). The temperature program was as follows: 40 °C (held 1 min) to 60 °C at 5 °C/min (held 1 min), then to 100 °C at 7 °C/min, then to 180 °C at 10 °C/min, then to 200 °C at 15 °C/min (held 1 min). Helium was used as a carrier gas with a constant flow of 1.5 mL/min. The injection was performed at 250 °C in the split mode (1:5). The oxidation reactor (Cu/O) in the 6890 N GC/C system was set to 900 °C.

Before each measurement sequence, stability and linearity were checked. Acceptable values were < 0.03‰. Reproducibility and accuracy were evaluated routinely using the working standard. The carbon isotope ratio of each compound in the recovery aroma sample was compared to the reference solution determined by EA-IRMS and then analysed with GC-C-IRMS. For data normalisation, the multiple-point linear normalisation method was used (Paul, Skrzypek, & Fórizs, 2007). The reproducibility of the GC-C-IRMS measurements based on duplicate analysis ranged from ± 0.1 to ± 0.5‰. Peak recognition was performed using retention times of reference compounds and by comparison of chromatograms obtained from GC–MS.

3. Results and discussion

3.1. Aroma screening/identification

Aroma profiles of laboratory and commercial apple recovery aroma samples were first characterised by GC–MS. Numerous studies provide aroma profiles of apples, apple juices and other apple products and more than 300 volatile molecules have been reported in fresh apples (Dixon & Hewett, 2001). The aroma profile also changes as apple fruits progress through maturation, harvest, and subsequent storage and especially during technological processing of apples (Dixon & Hewett, 2000; El Hadi, Zhang, Wu, Zhou, & Tao, 2013; Espino-Díaz, Sepúlveda, González-Aguilar, & Olivas, 2016; Fructuoso & Cortada, 2010). Aroma profiles obtained by GC–MS of laboratory and commercial apple distillates show a difference in regards to the presence or absence of certain aromatic components and the relationships between them (Fig. 1). Commercial samples have more uniform composition compared to the laboratory samples. The quality of the commercial samples is probably due to the special care taken during preparation by aroma experts. Regardless of environmental and technological impacts, consumers demand product consistency. In contrast, laboratory samples vary greatly. The most varied and odorous compounds (summarized by Mehinagic, Royer, Symoneaux, Jourjon, & Prost, 2006) in the laboratory samples detected by olfactometry with their sensory descriptions are butyl acetate (fruity, sweets), hexanal (green), 2-methylbutyl acetate (fruity, sweets, apple), trans-2-hexenal (green, apple), hexyl acetate (sweets, pear, apple), 1-hexanol (fresh, green), hexyl butyrate, benzaldehyde and butyl butyrate (rotten fruits). Variability is also a result of variety (GA = Gala, GD = Golden Delicious, RD = Red Delicious, TOP = Topaz, ID = Idared). Within variety, variability was also observed due to different storage treatment of the sample (1 = without of storage, 2 = after 2 months of storage). Different types of production (org = organic, int = integrated) affected mainly the presence of hexyl butyrate, which occurs primarily in the organically produced sample. Interestingly, only small differences between the immature (im), mature (m) and overripe (or) apples were observed, which is inconsistent with the literature data where aldehydes are reported to be the dominant volatiles detectable in immature apple fruit, whereas maturing and ripening fruits produce primarily esters and alcohols (Dixon & Hewett, 2000; El Hadi et al., 2013; Espino-Díaz et al., 2016; Mehinagic et al., 2006). The difference between our data and the literature data probably emerges due to different types of material that have been used. All studies focus on apple fruit, and not on apple recovery aroma like in our case. It is also well known that the processing of raw material significantly changes the aroma (Espino-Díaz et al., 2016).

A statistical difference between laboratory produced and commercial samples are observed for trans-2-hexenal, and its ratio to 1-hexanol, which is approximately 1:1 in commercial samples, while in laboratory-produced samples, levels of trans-2-hexenal are much lower. Further, the presence of hexyl acetate and its ratio to trans-2-hexenal is not greater than 1:1. Next, to 2-ethylbutyl acetate and butyl acetate, hexyl acetate was identified as one of the key odorant volatiles. Trans-2-hexenal is the main compound responsible for the freshness of apple-juice flavour and can be used as an additive to give flavours a greener apple-like aroma (Mehinagic et al., 2006). A large difference between laboratory produced and commercial aromas is in the level of benzaldehyde. Laboratory produced samples contain a higher amount of benzaldehyde compared to commercial samples, but its contribution to the overall apple aroma is yet to be evaluated.

All volatile compounds are important for characterising the aroma

Table 1
$\delta^{13}C$ (‰) values of different aroma compounds in natural apple samples, synthetic standards and literature data.

	Ethyl acetate	Ethyl butyrate	Ethyl-2-methyl butyrate	Butyl acetate	1-Hexanal	2-methylbutyl acetate	1-butanol	Amyl acetate	Butyl butyrate	Trans-2-hexenal	Hexyl acetate	2-hexen-1-ol, acetate	1-Hexanol	trans-2-hexenol	Benzaldehyde	1-octanol
Synthetic standards measured																
median	−27.1	−25.7	−24.7	−28.6	−25.5	−34.8	−31.5	−32.5	−26.8	−27.5	−27.0	−37.0	−24.4	−28.0	−26.0	−28.8
Synthetic standards literature																
No. of samples		1	5	3			3				2		11			
median		−24.7	−25.0	−30.5			−28.9				−26.5		−26.0			
min			−27.2	−31.3			−30.0				−26.7		−27.0			
max			−22.8	−29.7			−27.7				−26.2		−25.0			
Natural apple samples measured																
No. of samples	1	1	1	6	12	10	15	8	8	12	8	1	15	2	15	8
median	−33.0	−28.5	−27.9	−34.2	−36.4	−32.4	−42.3	−33.5	−37.8	−35.1	−32.5	−33.9	−40.6	−43.7	−31.5	-39.9
min				−36.0	−38.9	−34.4	−44.5	−37.0	−41.4	−39.4	−35.7		−41.8	−43.8	−34.1	−41.9
max				−32.7	−34.9	−30.5	−40.8	−30.0	−33.6	−34.1	−31.0		−39.4	−43.7	−29.7	−38.3
Natural samples literature																
No. of samples	ND	ND		32			31		ND	41	24		67	41		
median	−25.3	−31.3		−31.3			−37.5		−32.4	−31.9	−30.1		−38.6	−37.1		
min	−27.5	−32.6		−39.8			−41.8		−33.0	−39.1	−38.3		−42.7	−43.3		
max	−23.1	−30.3		−27.2			−28.2		−31.8	−27.3	−28.0		−34.30	−36.8		

ND not defined.

profile of apples, but only a few of them contribute significantly to the fruit aroma. To investigate this, 16 key active aroma components, which could be analysed by GC-C-IRMS, were selected (Fig. 2). This selection agrees with the study of Elss et al. (2006), who found that the major constituents of the apple recovery aroma were: 1-hexanol, 1-butanol, trans-2-hexenal, trans-2-hexenol, butyl acetate, 2-methylpropanol, hexanal, ethyl butyrate, cis-3-hexenol, ethyl butyrate, 1-propanol and hexyl acetate. The presence or absence of certain aroma compounds and the ratio between different aroma compounds appears to be an important indicator of quality in the sensory evaluation of recovery aromas and may be helpful in authenticity studies. In the present study, a similar approach is not sufficient to assess the authenticity of aroma due to the diversity of the samples and that the analysis is based only on the aroma profile.

3.2. GC-C-IRMS measurements/database creation

At the moment, GC-C-IRMS is one of the most powerful techniques available for detecting fraudulent practices in the food and beverages industry. Many studies, mainly of aroma compounds in different essential oils and in different fruits have used the stable isotope approach for differentiating between synthetic and natural compounds (van Leeuwen et al., 2014). Since synthetic compounds, derived from coal and petroleum, which originate from reservoirs of carbon formed from ancient C3 plants, have $\delta^{13}C$ values between −30‰ and −25‰ and are similar to $\delta^{13}C$ values in modern C3 plants (van Leeuwen et al., 2014) makes detecting substitutions difficult. Research shows that GC-C/P-IRMS is capable of distinguishing between natural and synthetic aromas, but the results are limited to a few common aroma compounds present in different types of fruits and are based on a small number of samples produced using different extraction procedures (van Leeuwen et al., 2014). The $\delta^{13}C$ values obtained from the literature for different natural and synthetic samples with a number of analysed samples are reported in Table 1. Literature data without information about a number of analysed samples are marked in Table 1 as not defined (ND).

The first steps in developing a $\delta^{13}C$ database are extraction optimisation, identification and minimisation of sources of contamination and isotopic fractionation since both processes will lead to bias in the isotopic values. Since the method involves HS-SPME, it is necessary to optimise all those parameters affecting SPME, such as fibre coating, sample volume, extraction and desorption time and temperature. In this case, the optimised methodology was appropriate for all the studied apple aroma compounds. Importantly, isotopic fractionation did not occur. The accurate determination of $\delta^{13}C$ values mainly depends on good chromatographic separation and on the integration parameters. Since many compounds in varying concentrations are present in a single sample, the selection of reference material and appropriate processing and interpretation of the results obtained is crucial. Hence, samples of pure synthetic aroma compounds were used. The $\delta^{13}C$ value for each of the compounds was first determined using EA-IRMS and then measured with GC-C-IRMS. The $\delta^{13}C$ values are presented in Table 1 and agree with the literature data. Most of the measured synthetic standards were then used as an internal standard during analysis by GC-C-IRMS.

A database of $\delta^{13}C$ values for the most common aroma compounds present in apple recovery aromas was then established. Table 1 presents the $\delta^{13}C$ (‰) values of synthetic standards and of a large number of authentic samples from which an isotopic authenticity range of a particular product together with the minimum, maximum and median $\delta^{13}C$ values was obtained. Most of the $\delta^{13}C$ values for the aroma compounds extracted from apple aroma are reported for the first time. Elss et al. (2006) also reported $\delta^{13}C$ values for trans-2-hexenal (from −39.1‰ to −31.5‰), 1-hexanol (from −42.5‰ to −38.4‰) and trans-2-hexenol (from −42.2‰ to −36.8‰) in apple aroma. Their results agree with the results of this study. A certain amount of overlap in the $\delta^{13}C$ values between natural and synthetic aroma compounds is reported in the literature data, while no overlap was observed in laboratory-derived

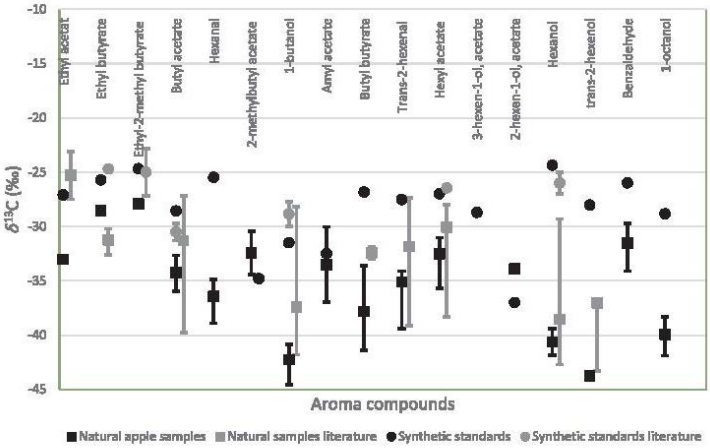

Fig. 3. δ^{13}C values of natural and synthetic samples (this study) and literature values.

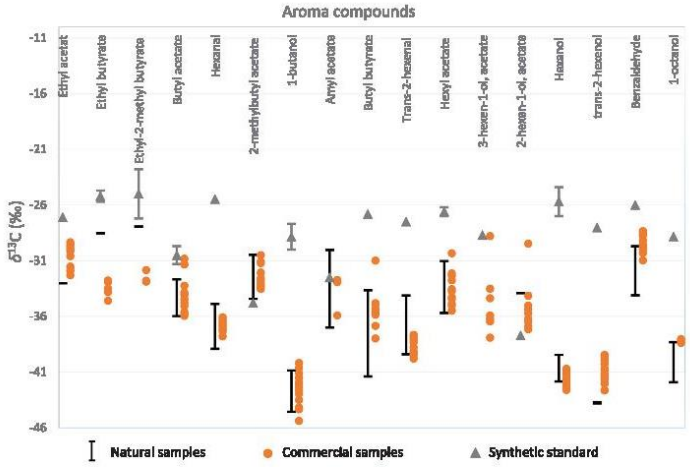

Fig. 4. δ^{13}C values of commercial apple recovery aromas with δ^{13}C values from the established database for natural samples and synthetic standard.

samples with synthetic values, with exception of 2-methylbutyl acetate and amyl acetate (Fig. 3). Differences in δ^{13}C values between the natural samples (this study) and values from literature is a likely consequence of the different types of fruit analysed (apple/pear/peach/strawberry/orange/passion fruit) and samples (fruit/juice/aroma/brandy) (Byrne, Wengenroth, & Kruger, 1986; Elss et al., 2006; Kahle et al., 2005; Parker, Kelly, Sharman, Dennis, & Howie, 1998; Preston et al., 2003; Schipilliti et al., 2011; Swift, 2002). However, the isotopic composition of individual compounds in the natural samples is independent of variety, stage of maturity, and the type of production, and therefore represents an ideal tool for determining the authenticity of aromas.

3.3. Authenticity assessment

To verify the authenticity of commercial samples, δ^{13}C values of different aroma compounds were determined and compared to the isotopic authenticity range. Any sample with one or more compounds outside this range was suspected of being adulterated. Analysis of commercial recovery aromas, labelled as natural, revealed that the majority of compounds in the samples were within this range (Fig. 4). Nevertheless, several compounds had δ^{13}C values that fell within the range of synthetic samples (butyl acetate in two samples with δ^{13}C values −30.8‰ and −31.3‰). In addition, two samples, one for butyl butyrate and another for 2-hexenol acetate, have different δ^{13}C values than the natural samples. Results confirms our working hypothesis that authenticity assessment of commercial apple recovery aromas is possible, by SPME methodology used with GC-C-IRMS analysis. To gain greater confidence in the interpretation of the obtained results, an extensive database, also for synthetic samples, is required.

Based on the obtained data one can estimate the quantity of the synthetic compounds that must be added to the natural sample to detect adulteration. As seen in Fig. 4, synthetic compounds show a significant shift in isotope values from natural aroma compounds. From the span of

Table 2
The estimated minimum added fractions of synthetic aroma compounds that are likely to be detected in a commercial samples using isotope model calculation.

Aroma compound	Mean of a natural compound $\delta^{13}C$ (‰)	Standard deviation σ	Mean of synthetic compound $\delta^{13}C$ (‰)	Detectable synthetic fraction x	
				50% threshold	95% threshold
Butyl acetate	−34.3	1.4	−28.6	0.49	0.66
Hexanal	−36.7	1.3	−25.5	0.23	0.37
1-butanol	−42.4	1.1	−28.9	0.16	0.27
Butyl butyrate	−37.8	2.4	−26.8	0.43	0.60
Trans-2-hexenal	−35.9	1.6	−27.5	0.39	0.56
Hexyl acetate	−32.8	1.6	−27.0	0.55	0.71
Hexanol	−40.5	0.7	−24.4	0.09	0.17
Benzaldehyde	−31.5	1.1	−26.0	0.38	0.55
1-octanol	−40.0	1.2	−28.8	0.21	0.35

$\delta^{13}C$ values of individual compounds obtained for the different aromas (Fig. 4), it is possible to estimate an average amount of the synthetic compound that needs to be added to the natural aroma to cause a detectable shift in the $\delta^{13}C$ values. In this case, a reasonable interval for $\delta^{13}C$ values is considered to be twice the standard deviation (2σ) around the average value of the natural compounds. In a mixture of the natural and synthetic compound, the $\delta^{13}C$ value is proportional to the isotope mass balance of the two fractions as:

$$\delta^{13}C_{mix} = (1 - x) * \delta^{13}C_{nat} + x * \delta^{13}C_{syn}$$

where *nat* and *syn* denote the $\delta^{13}C$ value of natural and synthetic compounds, and x relates to the fraction of the added synthetic compound in the mixture ($x = 0.5$ corresponds to both components being present in equal amounts). Taking 2σ as a maximum acceptable deviation from the average value a for the natural compounds, the limit value of x that can be detected can be expressed as the following:

$$x = \frac{a + 2*\sigma - \delta^{13}C_{nat}}{\delta^{13}C_{syn} - \delta^{13}C_{nat}}$$

As an example, hexanal with a mean $\delta^{13}C_{nat}$ value of −36.7‰ and σ = 1.3‰ is chosen, the $\delta^{13}C_{syn}$ value is −25.5‰ resulting in $x = 0.23$, meaning that if a mixed sample contains more than 23% of a synthetic fraction, it is likely (with 50% chance) that the falsification will be suspected since the shift in the $\delta^{13}C$ values will be significant. If the calculation is made with $\delta^{13}C_{nat}$ = a − 2σ value of −39.3‰, the resulting fraction is $x = 0.37$, meaning that if a mixed sample contains more than 37% of a synthetic fraction, it is almost certain (with 95% chance) that falsification will be suspected.

The calculated minimum fractions of synthetic aromas that are likely to be detected, for different compounds, is presented in Table 2. Model estimations are only presented for compounds where the database is composed of at least 5 samples of the same aroma compound so that standard deviation (σ) of natural compounds can be evaluated.

The necessary synthetic fraction of selected aroma compounds lies between 9% and 55% for 50% detection threshold, and between 17% and 71% for 95% detection threshold. The method is the most sensitive for falsification of hexanol and least sensitive for adulteration of hexyl acetate. 2-methylbutyl acetate and amyl acetate are not included in the Table 2 because these two samples have overlapping $\delta^{13}C$ values between natural and synthetic aroma, and thus, it is not possible to discriminate between them.

4. Conclusions

While the demand for natural aromas continues to grow and natural raw materials are becoming more expensive, there is increasing pressure on prices and quality. This study has shown that GC-C-IRMS analysis of key volatile compounds is an appropriate tool for determining the authenticity of aromas. Measurements can be performed using headspace solid phase microextraction (HS-SPME), which offers many advantages such as low-concentration sample measurements, short analysis time, solvent-free analysis, and importantly, it does not cause isotopic fractionation. However, since many different compounds with different concentration ranges are present in the sample, the selection of reference materials, appropriate data processing and interpretation of the results is crucial. When assessing authenticity, the most important thing is having a suitable database composed of authentic natural and synthetic aroma compounds that are present in the sample and are the ones most likely to be falsified. The sensitivity of the method was estimated, and it should permit detection of added synthetic compounds with sensitivity threshold between 9% and 71% depending on the aromatic substance. When analysing commercial distillates, labelled natural, $\delta^{13}C$ values of most aroma compounds were within an authentic range. Possible falsifications were, however, identified. An extensive database is currently under construction. An accurate determination of authenticity is feasible when multiple parameters are studied. In this regard, a multi-analysis approach such as GC-C/P-IRMS ($\delta^{13}C$ and $\delta^{2}H$ measurements) combined with metabolomics (fatty acids, amino acids analysis) and chemometrics (machine learning approach) could discriminate between, for example, not only apple aroma but also other types of aromas according to their source.

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Declarations of interest

None.

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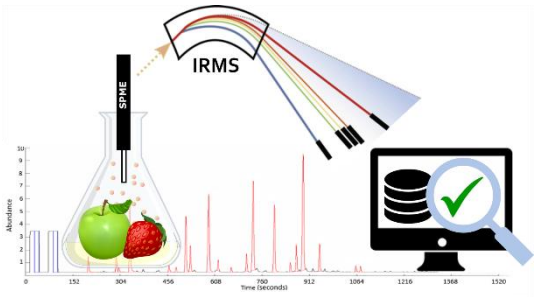
3.3 Scientific Paper: "Construction of IsoVoc Database for the Authentication of Natural Flavours"



This chapter presents the paper entitled "Construction of IsoVoc Database for the Authentication of Natural Flavours" by Lidija Strojnik, Jože Hladnik, Nika Cvelbar Weber Darinka Koron, Matej Stopar, Emil Zlatič, Doris Kokalj, Martin Strojnik and Nives Ogrinc. The paper describes establishing a database for determining flavour authenticity. It was published in Foods in 2021. It deals with constructing a stable isotope database (IsoVoc) based on HS-SPME GC-C-IRMS data to recognise the authenticity of fruit aroma. The primary purpose of creating the IsoVoc stable isotope database is its applicability and usefulness for industrial partners to determine the authenticity of raw ingredients. Therefore, in establishing the IsoVoc database, the following steps were included: selecting authentic reference samples, database creation and authenticity assessment of commercial samples.

The paper also investigates using fresh fruit instead of distillates to construct an authentic database of aroma compounds. The data revealed a difference in the δ^{13}C values of seven VOCs in apples and three in strawberries. It is known that different types of fruit processing can cause isotope fractionation resulting in differences in the isotopic ratios between distillates and fruit juice samples. Besides fruit processing, fractionation of compounds could be a consequence of flavour changes occurring through maturation, harvest, and subsequent storage. The paper found that δ^{13}C values of certain VOCs can only be obtained from fresh fruits, and a database should consist of both distillates and fruit samples. Further, apple and strawberry data were compared with raspberries, blueberries, peaches, pears, and sour cherries to determine which aroma compounds are common to all fruit types and the most frequent. It was found that apples and strawberries account for the most variability in the natural range of δ^{13}C values and contain the highest number of VOCs, making them the most appropriate fruits for database creation.

Finally, an extensive stable isotope database (IsoVoc) was established consisting of 39 authentic flavour compounds with well-defined origin: apple (148), strawberry (33), raspberry (12), pear (9), blueberry (7), and sour cherry (4) samples. The database also consists of 31 VOCs of synthetic origin. The data are comparable with literature data, and despite some overlap between the natural and synthetic range of values, the method allows the successful separation of 25 of the 33 target VOCs. When tested on 33 commercial fruit flavourings, including natural banana, blueberry, peach, grape, pear, apple, strawberry, kiwi, raspberry, blackberry, plum, and sour cherry, possible falsification for several fruit aroma compounds were identified.

In this study, I was responsible for selecting and preparing synthetic volatile compounds and distillates (recovery aromas) for analysis. My contribution involved performing GC-MS analysis, EA-IRMS and GC-C-IRMS analysis, analysing and processing the data using RStudio, establishing the IsoVoc database. I also wrote and prepared the manuscript for publication.

The work was presented as an oral presentation at the 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019), November 5-8, 2019 in Prague, Czech Republic, at 2nd Isotope Ratio MS Day, June 27-29, 2018, in Messina, Italy, and at

MASSTWIN Workshop on Mass spectrometry in support of the environment, food, and health interaction and disease, April 18-20, 2018 in Antwerp, Belgium, at the 10th Jožef Stefan International Postgraduate School Students' Conference and 12th Young Researchers' Day 10th and 11th May 2018 in Piran, Slovenia, and at the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, 15th and 16th May 2019 in Planica, Slovenia.

It was also presented as a poster presentation at XXII. International Mass Spectrometry Conference, IMSC 2018, August 26-31, 2018 in Florence, Italy, at 8th International Symposium in Recent Advances in Food Analysis, (RAFA), November 7-10, 2017 in Prague, Czech Republic, at ASSET 2018, Belfast Summit on Global Food Integrity, May 28-31, 2018 in Belfast, Ireland and at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, April 1-3, 2019 in Portorož, Slovenia.

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Construction of IsoVoc Database for the Authentication of Natural Flavours

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Abstract: Flavour is an important quality trait of food and beverages. As the demand for natural aromas increases and the cost of raw materials go up, so does the potential for economically motivated adulteration. In this study, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis of volatile fruit compounds, sampled using headspace-solid phase microextraction (HS-SPME), is used as a tool to differentiate between synthetic and naturally produced volatile aroma compounds (VOCs). The result is an extensive stable isotope database (IsoVoc—Isotope Volatile organic compounds) consisting of 39 authentic flavour compounds with well-defined origin: apple (148), strawberry (33), raspberry (12), pear (9), blueberry (7), and sour cherry (4) samples. Synthetically derived VOCs (48) were also characterised. Comparing isotope ratios of volatile compounds between distillates and fresh apples and strawberries proved the suitability of using fresh samples to create a database covering the natural variability in $\delta^{13}C$ values and range of VOCs. In total, 25 aroma compounds were identified and used to test 33 flavoured commercial products to evaluate the usefulness of the IsoVoc database for fruit flavour authenticity studies. The results revealed the possible falsification for several fruit aroma compounds.

Keywords: IsoVoc; database; volatile aroma compounds; fruits; headspace-solid phase microextraction (HS-SPME); gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS); authenticity

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1. Introduction

Fruits play a significant role in human nutrition. While their consumption depends on several factors, including colour, texture, appearance, and nutritional value, the flavour, which is a combination of aroma and taste, remains the primary selection criteria [1]. The same is valid for fruit-flavoured products where fruity notes continue to play well with all consumers, including those who associate them with health and wellness. The most popular are classic fruit flavours such as apple, berry, and citrus fruits, although flavour preferences are continuously evolving [2]. Most flavourings are produced either by chemical synthesis or by extracting natural materials, although today's consumers increasingly demand naturally flavoured products [3]. The problem is that the pressure to satisfy consumers and the higher market value of natural flavourings makes naturally flavoured products vulnerable to economically motivated adulteration [4].

New methodologies are being developed to detect food fraud since the analysis of chiral separation of enantiomers, single components, and total aroma spectra have limited applicability [5–7]. One such method involves using isotope ratios of light elements such

as hydrogen and carbon ($^{2}H/^{1}H$, $^{13}C/^{12}C$), which is increasingly being applied in food quality control and in determining the authenticity of natural flavourings. Much effort has also gone into developing isotopic methods to detect adulteration of natural products with synthetic compounds. Among them, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) seems to be the most specific and sophisticated method that can discriminate between natural and synthetic aromas based on the isotopic values of selected volatile organic compounds, VOCs [7–25]. The use of GC-C-IRMS is the subject of a review by van Leeuwen et al. [8] and a paper by Strojnik et al. [26] in which the authors emphasise the importance of the relevant analytical conditions to obtain precise isotopic ratios.

Articles on determining the authenticity of fruit volatile organic compounds based on GC-C-IRMS are summarised in Appendix A (Table A1). These studies cover, for example, raspberry [10,13,18,19,27], peach [7,11,23,25], strawberry [7,8,20], apple [16,25,28], nectarine [11,23,25], pineapple [7,9], and orange [15,25]. Many aromatic characteristics are shared between different fruits. However, each fruit is characterised by a distinctive aroma that depends on the VOCs present, concentration and perception threshold of individual volatile compounds [29]. Among these aromatic substances, special attention is paid to those compounds that create a fruit's characteristic aroma and are, therefore, likely to be falsified [29,30]. Studies looking at more than five different VOCs are scarce [7,12,16,20,22], and within those, only two address more than one fruit type [7,16]. In both studies, headspace solid-phase microextraction (HS-SPME) is used to extract the VOCs. The method offers many advantages such as simplicity, reduced time, improved repeatability, lower sample contamination probability [31], and finally, it does not cause isotopic fractionation [26]. HS-SPME-GC-C-IRMS has been used in all four fruit authenticity studies after 2010, proving its suitability as a tool for authenticity verification [7,15,16,32]. Besides fruits, the method has been successfully applied for determining the authenticity of other aromas such as vanilla [31,33,34], citrus essential oils [15,35], wine [36–38], and truffle oil [39].

When assessing authenticity, it is essential to have a comprehensive database of authentic samples [16,40–42]. The guidelines on the structure and data collection of such a database are presented by Donarski et al. [41]. For example, verification of aroma authenticity is achieved by measuring the isotope values of investigated aroma compounds and comparing the obtained isotopic values with those of reference samples present in the database. Samples containing one or more compounds outside the range established for authentic aroma compounds will be suspected of being adulterated [18]. A limitation of such a database is that it represents only a snapshot in time and requires continuous updating, evaluation, and processing. In addition, using a single fruit type, which is the case in several studies, is insufficient for determining authenticity since different fruit types can have different isotopic ratios [7,8,11]. In addition, all properties that might influence isotopic values must be carefully studied and included in the database. For example, the aroma extraction process must be investigated for evidence of isotopic fractionation, i.e., enrichment of one isotope relative to another. So far, this phenomena has only been addressed by Elss et al. [28] when investigating the technological processing of apple aroma. In this instance, the authors did not observe any isotopic fractionation.

Obtaining all of the necessary distillates of various fruit types to build a database requires extensive resources. However, this task could be significantly reduced by directly analysing the aroma of fresh fruits. Therefore, the present study deals with constructing a stable isotope database (IsoVoc) based on HS-SPME-GC-C-IRMS data to recognise the authenticity of fruit aroma used in food products. Further, the possibility to use fresh fruits instead of distillates for constructing the authentic database on aroma compounds was also investigated. Thus, the specific objectives were to (1) compare $\delta^{13}C$ values of multiple VOCs between distillates and fresh fruit samples (apples and strawberries), (2) compare $\delta^{13}C$ values of VOCs between different fruit types to define the natural variability in $\delta^{13}C$ values of VOCs, (3) establish a database of $\delta^{13}C$ values of authentic natural and synthetic aroma compounds, and (4) identify aroma compounds as markers of fruit flavour authenticity.

2. Materials and Methods

2.1. Samples

Fresh apples (n = 86), strawberries (n = 17), raspberries (n = 4), blueberries (n = 3) and pears (n = 2) harvested in Slovenia were obtained from the Agricultural institute of Slovenia and from local food producers, between 2016 and 2019. Additional fresh apples were obtained from the Czech Republic (n = 4), Italy (n = 2), Japan (n = 2), Poland (n = 2), and Slovakia (n = 3). Samples of aroma volatiles, recovered in the water phase (used in text as distillates) in apples (n = 43) and strawberries (n = 17), were prepared by steam distillation at the Biotechnical Faculty, University of Ljubljana. Additional distillates of apples (n = 6), strawberries (n = 2), blueberries (n = 4), raspberries (n = 8), sour cherries (n = 4) and pears (n = 7) with known origin were obtained from a commercial flavour supplier.

Pure synthetically derived aroma compounds were obtained from Sigma Aldrich and from a commercial flavour supplier and include (number in the brackets present the number of obtained samples): **1**, ethyl acetate (n = 2); **2**, ethyl butyrate (n = 2); **3**, ethyl 2-methyl butyrate (n = 2); **4**, butyl acetate (n = 1); **5**, hexanal (n = 3); **6**, 2-methyl butyl acetate (n = 2); **7**, 1-butanol (n = 1); **8**, amyl acetate (n = 1); **9**, butyl butyrate (n = 1); **10**, (E)-2-hexenal (n = 3); **11**, hexyl acetate (n = 1); **12**, (E)-2-hexen-1-ol, acetate (n = 2); **13**, 1-hexanol (n = 1); **14**, (E)-2-hexen-1-ol, (n = 3); **15**, benzaldehyde (n = 2); **16**, hexyl 2-methyl butyrate (n = 1); **17**, methyl butyrate (n = 1); **18**, hexyl butyrate (n = 1); **19**, propyl acetate (n = 1); **20**, methyl acetate (n = 1); **21**, 2-heptanone (n = 1); **22**, 1-octanol (n = 2); **23**, linalool (n = 1); **24**, isopropyl butyrate (n = 1); **25**, propyl propionate (n = 1); **26**, hexanoic acid (n = 1); **27**, isoamyl acetate (n = 3); **28**, methyl hexanoate (n = 2); **29**, acetic acid (n = 1); **30**, E-nerolidol (n = 1) and **31**, ethyl hexanoate (n = 2).

2.2. Sample Preparation

Fruit samples were mashed and filtered to obtain fresh juice immediately before analysis. Volatile organic components were extracted from both juice, and the distillates using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (50/30 µm thickness) purchased from Sigma-Aldrich (Supelco, Bellefonte, PA, USA). The SPME fibre was previously conditioned according to the supplier recommendation. The analysis of the VOCs was performed by sampling the headspace of a 10 mL SPME vial (with silicone/PTFE septa) containing 1 mL of sample. The equilibration time was 10 min at 30 °C, followed by extraction for 20 min at the same temperature. The analytes were then desorbed at 250 °C for 1 min. A working standard solution was prepared by diluting 1 µL of each synthetically derived aroma compound (samples 2 to 15) in 20 mL of water, which was then stored at 4 °C before use. All analyses were performed within three months.

2.3. Elemental Analysis-Isotope Ratio Mass Spectrometry (EA-IRMS)

The $^{13}C/^{12}C$ ratios were determined using a Vario PYRO Cube analyser (OH/CNS Pyrolyser/Elemental Analyzer) couple to an IsoPrime 100 isotope ratio mass spectrometer, IRMS (IsoPrime, Cheadle, Hulme, UK). The accuracy and precision of measurements were controlled using internal working standards: absolute ethanol ($\delta^{13}C = -27.38 \pm 0.09‰$) and a rum distillate ($\delta^{13}C = -13.81 \pm 0.09‰$) previously calibrated against the certified reference material BCR-656 wine alcohol ($\delta^{13}C$ value $= -26.91 \pm 0.07‰$) from the Institute for Reference Materials and Measurements—IRMM, Belgium).

Carbon isotope ratios are expressed in the δ-notation in per mil (‰) relative to the VPDB international standard as follows:

$$\delta^{13}C_{VPDB}\ (‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 1000, \quad (1)$$

where R refers to the $^{13}C/^{12}C$ ratios in the sample and standard, respectively. The precision of the measurements was $\pm$ 0.1‰.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS)

VOCs were identified using a 7890B & 5977A Series GC/MSD (Agilent Technologies, Santa Clara, CA, USA), where chromatographic separation was achieved on a VF-WAXms capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). The overall temperature program was as follows: 40 °C (1 min) to 60 °C at 5 °C min^{-1} (held 1 min), then to 100 °C at 7 °C min^{-1}, to 180 °C at 10 °C min^{-1} and finally to 200 °C at 15 °C min^{-1} (held 1 min). The carrier gas was helium at a constant flow of 1.5 mL min^{-1}. The injection was performed at 250 °C in the split mode (1:10), using a Straight Ultra Inert Liner for SPME (Agilent Technologies, Santa Clara, CA, USA). The ion source was set to 230 °C and the interface temperature to 250 °C, with a scan range of 30 to 400 m/z. The system was controlled using the ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The identification procedure was performed by comparing retention times and mass spectra with the NIST 14 Mass Spectral Library (Agilent Technologies, Santa Clara, CA, USA) and pure standards.

2.5. Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS)

Method optimisation and validation are described in detail in Strojnik et al. [26]. Briefly, GC-C-IRMS analysis was performed on Agilent 6890N GC-C system coupled to an IsoPrime GV IRMS. Separation was achieved using an Agilent J&W VF-WAXms capillary column (30 m × 0.25 × 0.25). The overall temperature program was the same as for GC-MS analysis. The carrier gas was also helium at a constant flow of 1.5 mL min^{-1}. Injections were performed at 250 °C in splitless mode. The oxidation reactor (Cu/O) in the 6890N GC/C system was set to 900 °C.

Before each measurement, stability and linearity were checked. Acceptable values were < 0.03‰. Reproducibility and accuracy were determined routinely using the working standard. The $^{13}C/^{12}C$ ratio was first determined in pure standards and compared with the values obtained by EA-IRMS. Next, the $^{13}C/^{12}C$ ratios were determined in the distillates and fruit samples. The multiple-point linear method was used for data normalisation [26]. The reproducibility of the measurements based on duplicate analysis ranged from ±0.1 to ±0.5‰. Finally, the identification of aroma compounds was performed by matching the retention times of pure standards and comparing the chromatograms obtained by GC-MS.

2.6. Data Analysis

Metadata (fruit type, fruit variety, processing method, geographical location, and data type, i.e., training or test data) and isotopic values were imported into Excel. Then, data visualisation and data analysis were performed in RStudio. A Mann-Whitney U test for non-normally distributed data was then used to reveal any statistically significant differences. In this case, principal component analysis (PCA) could not be applied since the dataset included many missing values of individual variables.

3. Results and Discussion

The primary purpose of creating the IsoVoc stable isotope database for food flavour authentication within the present study is its applicability and usefulness for industrial partners to determine the authenticity of raw ingredients. For this reason, the database must be as broad as possible to cover the widest variety of flavoured fruit products. Therefore, in establishing the IsoVoc database, the following steps were included: selecting authentic reference samples, database creation and authenticity assessment of commercial samples.

3.1. Selection of Authentic Reference Sample

To address the usefulness of fresh fruits instead of distillates for database creation, we compared the $\delta^{13}C$ values of specific VOCs present in distillates and fresh fruit samples of apple and strawberry. Only aroma compounds that occur in at least five samples per group (fruit samples and distillates), wherein the compound must be present in both groups,

were accepted for further data analysis. In this way, we identified 15 characteristic aroma compounds for apple and 11 aroma compounds for strawberry.

We included 49 recovery aromas and 99 samples of fresh apple juice (Figure 1). For the 15 aroma compounds, δ^{13}C values ranged between −43.7‰ (acetone in fruit) and −24.1‰ (methyl hexanoate in fruit). A Mann-Whitney U test revealed statistically significant differences in median isotope values for (E)-2-hexanal (−42.2‰; −35.1‰), amyl acetate (−36.5‰; −31.4‰), butyl acetate (−36.2‰; −33.6‰), butyl propionate (−36.8‰; −30.5‰), hexanal (−40.1‰; −35.9‰), hexyl 2-methyl butyrate (−33.9‰; −37.6‰) and hexyl acetate (−36.7‰; −32.3‰) between fruit samples and distillates respectively.

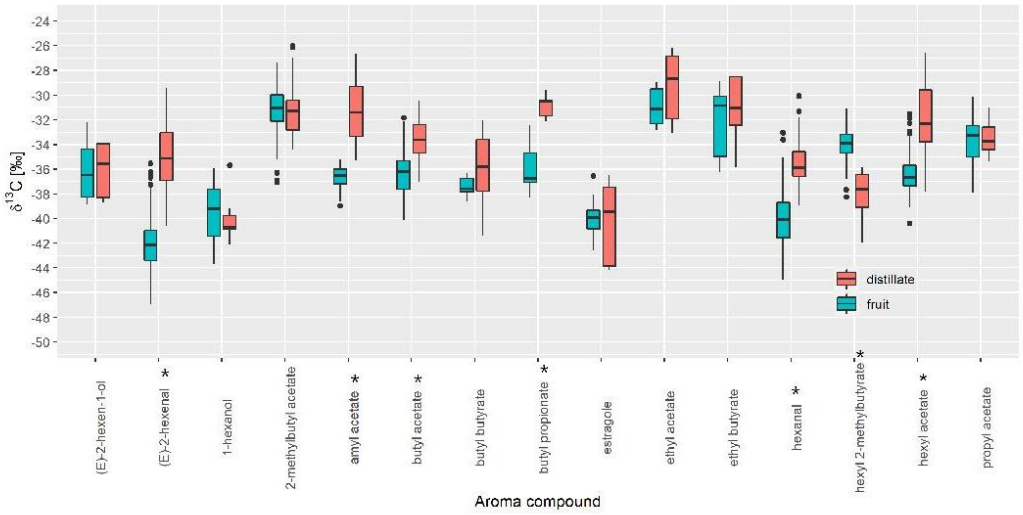

Figure 1. Comparison of δ^{13}C values of volatile organic compounds (VOCs) present in apple fruit and apple distillates. * $p < 0.05$ (Mann-Whitney test, statistically significant).

For strawberry, 17 distillates and 17 fresh strawberry samples were analysed (Figure 2). For the selected 11 aroma compounds, δ^{13}C values ranged between −47.0‰ ((E)-2-hexenal in fruit) and −26.0‰ (2-methylbutyl acetate in distillate). In this case, the Mann-Whitney U test revealed statistically significant differences for (E)-2-hexanal, (−30.5‰; −33.6‰), acetone (−42.1‰; −37.7‰) and ethyl butyrate (−31.4‰; −29.7‰) between fruit samples and distillates, respectively.

Data analysis also revealed several outliers in apple and strawberry that were not a result of analytical problems or random errors but are likely due to differences in variety, stage of maturity, and geographical origin, although further investigation is required. The outliers were not removed from the database. It is also necessary to consider natural isotopic variation caused by, e.g., geographical location, variety, temporal and seasonal variation, and processing to make the database more robust. Further, before data analysis it is important to understand if various technological processes such as distillation cause any isotope fractionation in isotopic values of aroma compounds in specific sample type. When comparing distillates with the freshly prepared fruit juice, we observed a difference in δ^{13}C values for seven investigated VOCs in apple and three in strawberry. Although Ells et al. [28] did not find a significant isotopic effect caused by technological treatment for (E)-2-hexanal, (E)-2-hexanol, and hexanal, in this study, we found that for (E)-2-hexanal and hexanal, preparing the fresh juice can modify the isotopic data. In apple, the isotopic values for (E)-2-hexanal are lower for fruit juice than distillates. In the case of strawberry aroma, the opposite is true. Except for hexyl-2-methyl butyrate, the median isotope values

are lower in the fruit samples. Isotope fractionation that we observed in many volatile compounds is most likely compound dependent. For volatiles that are produced as a result of tissue disruption (secondary compounds, like aldehydes (E)-2-hexanal and hexanal), it seems that different types of fruit processing can cause isotope fractionation in different ways resulting in the difference in isotopic ratios between distillates and fruit juice samples. Beside fruit processing, different fractionation of compounds could be a consequence of flavour changes occurring through maturation, harvest, and subsequent storage. We also observed some differences in isotope values between varieties (such as in red delicious for apples) and samples from different geographical locations (samples from Poland and Japan differentiate from Slovenian ones) that could be important when understanding isotope fractionation rates. However, we cannot draw any general conclusion on this subject since we are dealing with a limited number of samples, and further investigation in this field is required.

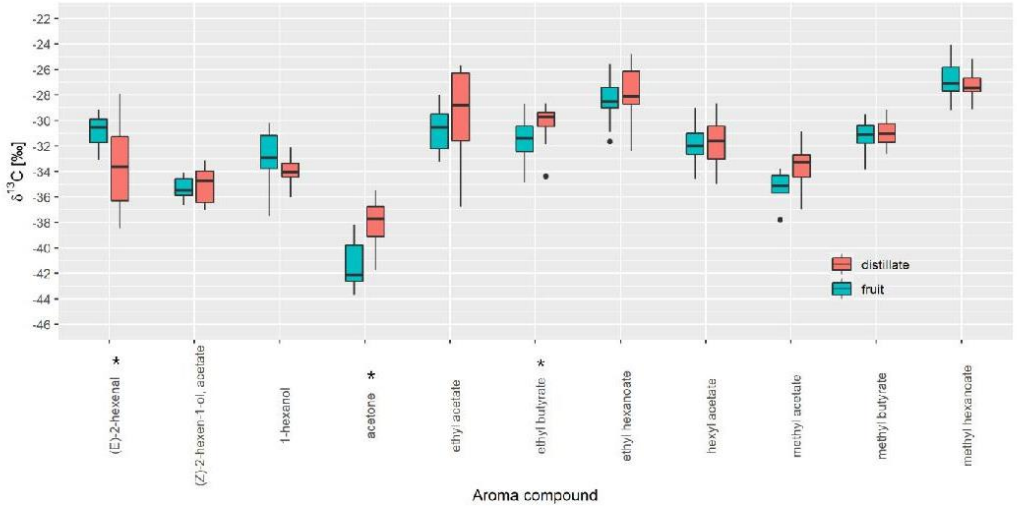

Figure 2. Comparison of δ^{13}C values of VOCs present in strawberry fruit and distillate. * $p < 0.05$ (Mann-Whitney test).

Importantly, the following question arises: can fresh fruit samples substitute distillates in the database? For all aroma compounds where the δ^{13}C values are within the min and max value of the distillates, this is possible, as in the case of 1-hexanol, (E)-2-hexen-1-ol, 2-methylbutyl acetate, ethyl butyrate, and propyl acetate for apples and 1-hexanol, ethyl butyrate, hexyl acetate, methyl butyrate, and methyl hexanoate for strawberries. That means that an authenticity assessment will not be influenced by this effect. However, if we want to cover more fruit VOCs, we need to include both distillates and fruit samples in the database.

Next, for apple and strawberry, distillates and fruits were first combined into a single group (Figure 3). Finally, we compared the data with that for raspberries, blueberries, peaches, pears, and sour cherries to find out which aroma compounds are common to the selected fruit types and which occur most frequently. We then evaluated the δ^{13}C values of 1-hexanol, (E)-2-hexen-1-ol, (E)-2-hexenal, benzaldehyde, butyl acetate, ethyl acetate, ethyl butyrate, ethyl hexanoate, hexanal, and hexyl acetate. We also found that apples and strawberries account for most of the most variability in the natural range of δ^{13}C values and contain the highest number of VOCs, which makes them the most appropriate fruits for database creation.

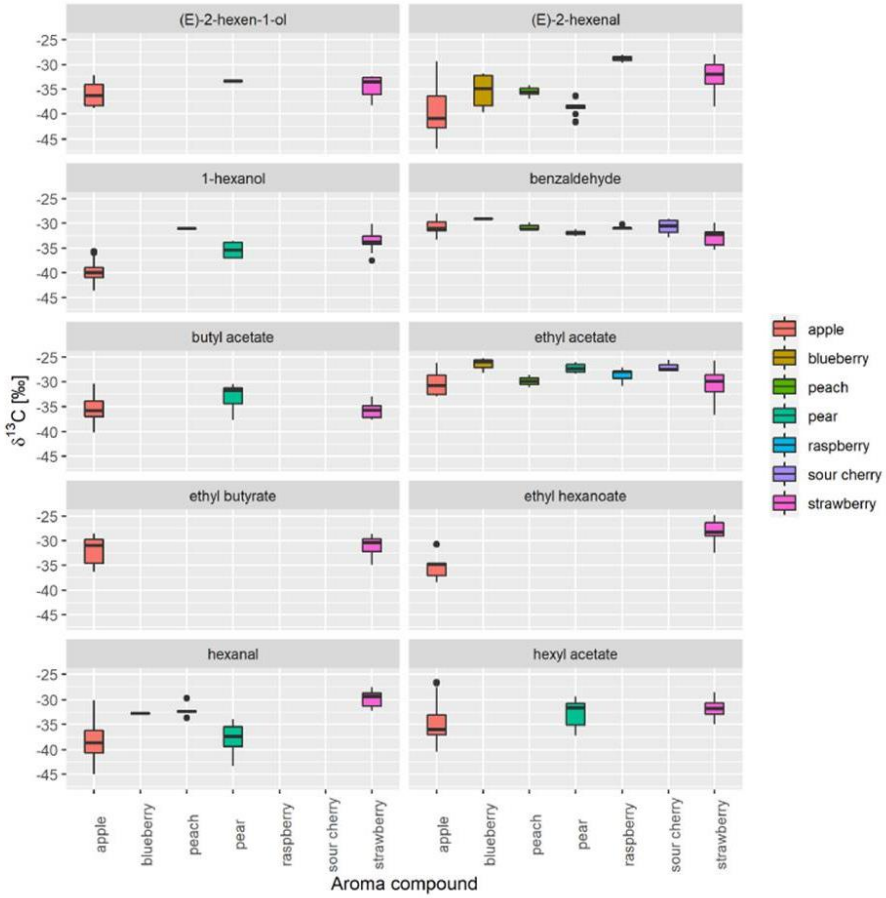

Figure 3. Comparison of δ^{13}C values of VOCs in different fruit samples. Only aroma compounds present in at least five samples are shown.

3.2. Database Creation

The number of unique VOCs present in at least five samples was 39 (Table 1). Not all variables were present in all samples. Since the dataset included many missing values for individual variables and contained only δ^{13}C values for authentic natural samples and not for the synthetic samples, we used the Mann-Whitney U test rather than principal component analysis, PCA. We also excluded butyl propionate, isobutyl acetate, estragole, acetone, (Z)-2-hexen-1-ol, acetate, and isopropyl butyrate from Table 1, since there are no data on δ^{13}C values reported in the literature. However, the δ^{13}C values for authentic compounds determined in our study are presented, since they could be of a general interest to other researchers and are as follows:: butyl propionate (n = 18; δ^{13}C from −38.3 to −29.6‰), isobutyl acetate (n = 8; δ^{13}C from −34.6 to −27.7‰), estragole (n = 34; δ^{13}C from −44.1 to −36.5‰) in apple, and acetone (n = 23; δ^{13}C from −43.7 to −35.5‰), (Z)-2-hexen-1-ol, acetate (n = 14; δ^{13}C from −37.1 to −33.2‰) and isopropyl butyrate (n = 9; δ^{13}C from −52.9 to −45.1) in strawberry.

Table 1. $\delta^{13}C$ (‰) values of volatile organic compounds in synthetic standards, fruits obtained experimentally and from the literature.

	(E)-2-hexen-1-ol	(E)-2-hexen-1-ol, acetate	(E)-2-hexenal	1-butanol	1-hexanol	1-octanol	2-heptanone	2-methylbutyl Acetate	Acetic Acid	Amyl Acetate	Benzaldehyde	Butyl Acetate	Butyl Butyrate	E-nerolidol	ethyl 2-methylbutyrate	Ethyl Acetate	Ethyl Butyrate	Ethyl Hexanoate	Hexanal	Hexanoic Acid	hexyl 2-methylbutyrate	Hexyl Acetate	Hexyl Butyrate	Isoamyl Acetate	Linalool	Methyl Acetate	Methyl Butyrate	Methyl Hexanoate	Propyl Acetate	Propyl Propionate	α-ionone	β-ionone	γ-decalactone
Synthetic																																
No.	3	2	3	1	1	2	1	2	1	1	2	1	1	1	2	2	2	2	3	1	1	1	1	3	1	1	1	2	1	1		
min	−30.2	−37.0	−33.9	−31.4	−24.4	−29.2	−29.4	−34.8	−42.0	−32.5	−26.0	−28.6	−26.8	−32.0	−27.0	−27.1	−25.7	−32.8	−26.2	−35.6	−27.6	−27.0	−33.5	−28.5	−25.6	−26.7	−26.7	−35.9	−27.2	−25.5		
max	−28.0	−33.2	−33.4	−31.4	−24.4	−28.8	−29.4	−32.9	−42.0	−32.5	−25.3	−28.6	−26.8	−32.0	−24.7	−27.0	−16.5	−26.2	−25.3	−35.6	−27.6	−27.0	−33.5	−18.6	−25.6	−26.7	−26.7	−35.1	−27.2	−25.5		
Apple																																
No.	19	1	127	20	59	4		100		60	33	100	33		15	13	18	6	121		35	102	24	2					25	9		
min	−38.8	−31.9	−47.0	−44.5	−43.7	−33.3		−37.1		−39.0	−33.3	−40.1	−41.4		−34.3	−33.0	−36.2	−38.4	−45.0		−41.9	−40.4	−40.0	−36.9					−37.9	−35.7		
max	−32.2	−31.9	−29.4	−40.8	−35.7	−31.5		−26.0		−26.7	−28.0	−30.4	−32.1		−27.0	−26.2	−28.5	−30.8	−30.1		−31.0	−26.6	−34.8	−36.4					−30.2	−29.6		
Strawberry																																
No.	12	10	22		13	7	13	1	5		16	10		5	3	24	29	26	7	6		27	1	3	8	20	29	28				7
min	−38.2	−32.4	−38.5		−37.5	−36.5	−27.6	−33.4	−31.3		−35.4	−37.6		−33.8	−29.1	−36.7	−34.8	−32.4	−32.2	−29.9		−35.0	−35.0	−33.7	−26.9	−37.8	−33.8	−29.2				−35.6
max	−32.4	−29.5	−27.9		−30.2	−34.4	−20.7	−33.4	−28.1		−29.9	−33.0		−27.8	−27.4	−25.7	−28.6	−24.8	−27.5	−20.2		−28.6	−35.0	−30.2	−24.5	−30.9	−29.2	−24.1				−28.7
Blueberry																																
No.			5						1		1					3			1						1	4						
min			−39.6						−28.4		−29.1					−28.2			−32.7						−28.5	−38.2						
max			−31.8						−28.4		−29.1					−25.2			−32.7						−28.5	−32.5						
Pear																																
No.	1		9		4			3		4	5	7				6			9			6		1								
min	−33.4		−41.6		−37.0			−30.8		−31.9	−32.6	−37.7				−28.4			−43.4			−37.2		−30.9								
max	−33.4		−36.3		−33.5			−27.3		−27.5	−31.2	−30.5				−26.0			−33.9			−29.3		−30.9								
Raspberry																																
No.			2					2			5					6									5						8	6	
min			−29.6					−32.7			−31.2					−30.9									−32.0						−34.2	−33.4	

Table 1. *Cont.*

	(E)-2-hexen-1-ol	(E)-2-hexen-1-ol, acetate	(E)-2-hexenal	1-butanol	1-hexanol	1-octanol	2-heptanone	2-methylbutyl Acetate	Acetic Acid	Amyl Acetate	Benzaldehyde	Butyl Acetate	Butyl Butyrate	E-nerolidol	ethyl 2-methylbutyrate	Ethyl Acetate	Ethyl Butyrate	Ethyl Hexanoate	Hexanal	Hexanoic Acid	hexyl 2-methylbutyrate	Hexyl Acetate	Hexyl Butyrate	Isoamyl Acetate	Linalool	Methyl Acetate	Methyl Butyrate	Methyl Hexanoate	Propyl Acetate	Propyl Propionate	α-ionone	β-ionone	γ-decalactone
Raspberry																																
max			−28.0					−31.4			−30.2				−27.1									−30.1							−32.6	−32.4	
Peach																																
No.			5		1						3				3				5													2
min			−37.0		−31.0						−31.2				−31.1				−33.7													−37.7
max			−34.2		−31.0						−29.8				−28.6				−29.7													−37.5
Sour cherry																																
No.											4.0				3.0									1.0								
min											−32.8				−27.8									−25.5								
max											−29.1				−25.5									−25.5								
Synthetic literature																																
min	−28.0			−31.5	−27.0	−28.8		−34.8			−32.5	−26.0	−31.3	−26.8	−27.2	−27.1	−25.7		−25.5		−27.0						−36.1					
max	−28.0			−27.7	−24.4	−28.8		−34.8			−32.5	−26.0	−28.6	−26.8	−22.8	−27.1	−25.7		−25.5		−26.2						−36.1					
Natural literature																																
min	−43.8			−39.4	−44.5	−42.5	−41.9	−34.4			−37.0	−34.1	−39.8	−41.4	−27.9	−33.0	−32.6	−31.2	−38.9	−33.2	−38.3	−32.9		−36.8		−33.7	−28.2					
max	−36.8			−27.3	−37.0	−36.9	−38.3	−30.5			−30.0	−29.7	−31.3	−33.6	−27.9	−33.0	−28.5	−30.0	−34.9	−32.1	−29.7	−30.3		−28.0		−29.3	−26.5					

All authentic samples were combined in one range (shown in dark green in Figure 4). What is clear is the overlap between individual ranges in $\delta^{13}C$ values. Most data are in good agreement with literature data. For example, Elss et al. [28] reported $\delta^{13}C$ values for trans-2-hexenal ranging from −39.1 to −31.5‰, 1-hexanol ranging from −42.5 to −38.4‰ and trans-2-hexenol ranging from −42.2 to −36.8‰ in apple aroma.

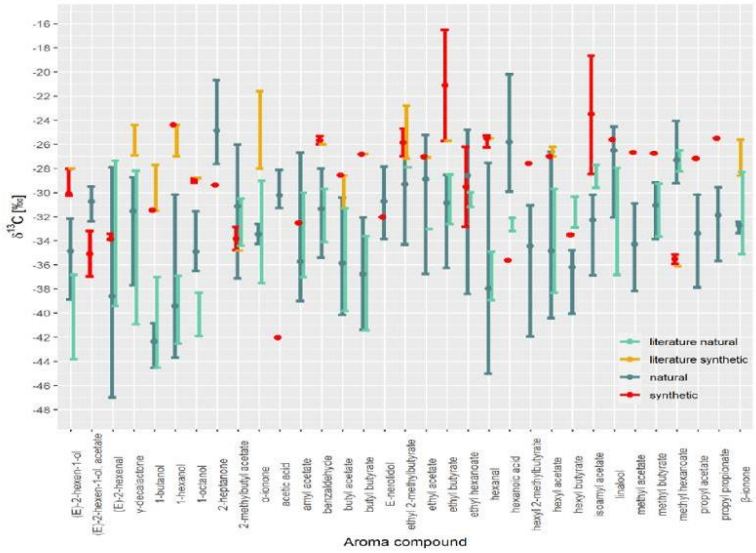

Figure 4. $\delta^{13}C$ values of VOCs of natural and synthetic samples (this study) and literature values.

However, for certain VOCs, the authentic range of $\delta^{13}C$ values observed in the literature is slightly shifted (e.g., 1-octanol, (E)-2-hexen-1-ol, hexanoic acid, hexyl butyrate, isobutyl acetate and linalool) compared with this study. For example, isoamyl acetate $\delta^{13}C$ values for bananas are higher, ranging from −29.6 to −27.2‰ [22] compared to our study. This difference likely results from the different preparation method used or technological production, or unique fruit characteristics. In addition, the broad range of $\delta^{13}C$ values in the literature data for γ-decalactone, α-ionone, β-ionone is most likely due to the low number of samples and, consequently, the smaller variation in $\delta^{13}C$ values in samples used in our study. The $\delta^{13}C$ values for γ-decalactone and δ-decalactone in *prunus* fruits such as peaches, apricots, and nectarines ranged from −38.4 to −34.0‰ [11] and were lower compared to strawberries, where the $\delta^{13}C$ values of γ-decalactone ranged from −31 to −28‰ [7]. In this study, the $\delta^{13}C$ values for γ-decalactone in organically produced strawberries range from −29.7 to −28.2‰ and cannot be distinguished from other natural strawberry samples. Raspberry has also been subjected to isotope analysis [13]. The $\delta^{13}C$ values for α-ionone, β-ionone, and α-ionol in these samples ranged from −36.6 to −30.3‰, which shows that it is possible to differentiate between natural and synthetic derived compounds. Although compounds appearing in peach and raspberry are well researched, many more samples are needed to represent better the actual variation in $\delta^{13}C$ values for those compounds.

A database should not contain only the isotopic values of authentic natural samples. For example, we observed several overlaps in the isotopic values of synthetic samples, which meant that it was not possible to differentiate between natural and synthetic samples, mainly (E)-2-hexanal, 2-methylbutyl acetate, amyl acetate, E-nerolidol, ethyl acetate, ethyl hexanoate, hexyl acetate, and linalool $\delta^{13}C$ values. In addition, having only natural aroma compounds in the database is likely to result in samples being misclassified. Different

market brands should also be analysed to increase the range of $\delta^{13}C$ values observed in synthetic samples.

Despite this overlap, the method could successfully separate 25 of the 33 VOCs for which we have data for the synthetic compounds (γ-decalactone, α-ionone, β-ionone from the literature).

3.3. Authenticity Assessment of Commercial Samples

Based on isotopic mass balance using $\delta^{13}C$ values of individual compounds, estimating the average amount of synthetic compound in the sample is possible, as shown by Strojnik et al. [16]. Here we adapted the method to work for any distribution, not necessarily a normal one, where $\delta^{13}C$ distribution is described by the min, max, and median value. Thus, the $\delta^{13}C$ value of a mixture (*mix*) of two compounds, both natural (*nat*) and synthetic (*syn*), can be expressed with the following relationship:

$$\delta^{13}C_{mix} = (1 - x) * \delta^{13}C_{nat} + x * \delta^{13}C_{syn}, \quad (2)$$

where fraction x corresponds to the added synthetic compound in the mixture, and in the case where $x = 0.5$, both components are present in equal amounts. Assuming an extensive dataset of samples that is representative of the population, we can use $\delta^{13}C_{nat,max}$ *and* $\delta^{13}C_{nat,min}$ as the range of $\delta^{13}C$ values in natural compounds (with a median value M) to estimate the lowest value x that can be detected for compounds in those regions where natural and synthetic samples are separated.

When the range of synthetic $\delta^{13}C$ values is higher than the natural range, we use the following expression:

$$x = \frac{\delta^{13}C_{nat\ max} - \delta^{13}C_{nat}}{\delta^{13}C_{syn} - \delta^{13}C_{nat}}. \quad (3)$$

For example, we chose benzaldehyde with a median $\delta^{13}C_{nat}$ value of −31.3‰ and max value $\delta^{13}C_{nat\ max}$ of −28.0‰. If the median $\delta^{13}C_{syn}$ value is −25.7‰, then $x = 0.59$, meaning that if a mixed sample contains more than 59% of a synthetic fraction, it is likely (with 50% chance) that adulteration will be suspected since the shift in the $\delta^{13}C$ values will be significant (i.e., out of range of natural compounds). If the calculation is made using the minimum measured values of $\delta^{13}C_{nat} = \delta^{13}C_{nat\ min}$ and $\delta^{13}C_{syn} = \delta^{13}C_{syn\ min}$, then $x = 0.79$, meaning that if a mixed sample contains more than 79% of a synthetic fraction, the measured $\delta^{13}C$ value will fall out of natural sample range.

If the range of synthetic $\delta^{13}C$ values is lower than natural ones, the corresponding fraction x of a synthetic sample is calculated by replacing the maximum values of the measured ranges with the minimum values and vice versa. The detectable fractions of synthetic compounds for different VOCs are presented in Table 2.

The necessary synthetic fraction of selected aroma compounds lies between 5% and 83% for a 50% detection threshold and between 19% and 100% for a 100% detection threshold. Therefore, the method is the most sensitive for detecting adulteration with β-ionone, acetic acid, α-ionone, and 1-butanol and less sensitive at detecting adulteration of ethyl 2-methyl butyrate and hexanal. However, evaluating their usefulness will require a study on how adding different amounts of synthetic aroma compounds to a sample with a weak intrinsic aroma can affect the odour.

Table 2. The calculated fractions of added synthetic volatile organic compounds in commercial samples based on the isotope mass balance model.

Aroma Compound	Natural Compound			Synthetic Compound			Detectable Synthetic Fraction	
	Min	Max	Median	Min	Max	Median	x	x
	δ^{13}C (‰)			δ^{13}C (‰)			50% Threshold	100% Threshold
γ-decalactone	−37.7	−28.7	−31.5	−26.9	−24.4	−26.1	0.52	0.83
1-butanol	−44.5	−40.8	−42.3	−31.4	−31.4	−31.4	0.14	0.28
1-hexanol	−43.7	−30.2	−39.4	−24.4	−24.4	−24.4	0.61	0.70
1-octanol	−36.5	−31.5	−34.9	−29.2	−28.8	−29.0	0.57	0.68
2-heptanone	−27.6	−20.7	−24.8	−29.4	−29.4	−29.4	0.61	0.80
(E)-2-hexen-1-ol	−38.8	−32.2	−34.9	−30.2	−28.0	−30.1	0.56	0.78
(E)-2-hexen-1-ol, acetate,	−32.4	−29.5	−30.8	−37.0	−33.2	−35.1	0.37	0.78
acetic acid	−31.3	−28.1	−30.2	−42.0	−42.0	−42.0	0.09	0.23
benzaldehyde	−35.4	−28.0	−31.3	−26.0	−25.3	−25.7	0.59	0.79
butyl acetate	−40.1	−30.4	−35.8	−28.6	−28.6	−28.6	0.75	0.84
butyl butyrate	−41.4	−32.1	−36.8	−26.8	−26.8	−26.8	0.47	0.64
ethyl 2-methyl butyrate	−34.3	−27.0	−29.3	−27.0	−24.7	−25.8	0.66	1.00
ethyl butyrate	−36.2	−28.5	−30.9	−25.7	−16.5	−21.1	0.24	0.73
hexanal	−45.0	−27.5	−38.0	−26.2	−25.3	−25.5	0.83	0.93
hexanoic acid	−29.9	−20.2	−25.8	−35.6	−35.6	−35.6	0.42	0.63
hexyl 2-methyl butyrate	−41.9	−31.0	−34.4	−27.6	−27.6	−27.6	0.49	0.76
hexyl butyrate	−40.0	−34.8	−36.2	−33.5	−33.5	−33.5	0.52	0.80
isoamyl acetate	−36.9	−30.2	−32.3	−28.5	−18.6	−23.5	0.24	0.80
methyl acetate	−38.2	−30.9	−34.3	−26.7	−26.7	−26.7	0.45	0.63
methyl butyrate	−33.8	−29.2	−31.1	−26.7	−26.7	−26.7	0.44	0.66
methyl hexanoate	−29.2	−24.1	−27.3	−35.9	−35.1	−35.5	0.23	0.47
propyl acetate	−37.9	−30.2	−33.4	−27.2	−27.2	−27.2	0.52	0.72
propyl propionate	−35.7	−29.6	−31.9	−25.5	−25.5	−25.5	0.36	0.60
α-ionone	−34.2	−32.6	−33.4	−28.0	−21.6	−25.9	0.11	0.26
β-ionone	−33.4	−32.4	−32.7	−28.6	−25.6	−27.7	0.05	0.19

Finally, we assessed the authenticity of 33 commercial fruit flavourings, including natural banana, blueberry, peach, grape, pear, apple, strawberry, kiwi, raspberry, blackberry, plum, and sour cherry aroma. A shift in the δ^{13}C values towards the synthetic range for 2-heptanone, benzaldehyde, butyl acetate, ethyl butyrate, hexanal, hexanoic acid, isoamyl acetate, methyl acetate, methyl hexanoate, and propyl acetate suggest the possible falsification of specific fruit VOCs despite being labelled as natural fruit extracts (Figure 5). Results imply that the authenticity of flavoured products on the market can be questioned, and extensive testing is necessary.

We can also estimate the amount of synthetic fraction x present in the commercial fruit distillates by using the measured $\delta^{13}C_{mix}$ values and the minimum, maximum, or average synthetic fraction from the variability of data defining the natural and synthetic database range. By using the median values of VOCs for natural and synthetic samples ($\delta^{13}C_{nat}$ and $\delta^{13}C_{syn}$) we can estimate the median amount of the added synthetic fraction. In the same way, we can also determine the maximum and minimum range of synthetic fraction by using limit database values (minima or maxima) of natural and synthetic samples. Table 3 shows examples for several measured commercial samples that fall outside of the natural range. For example, in the exemplary case of benzaldehyde where the commercial sample has a δ^{13}C = −26.4‰, using the max value of the synthetic range (−25.3‰) and natural range (−28.0‰), we can estimate that the minimum amount of synthetic fraction is 59%, but it can range up to 95%.

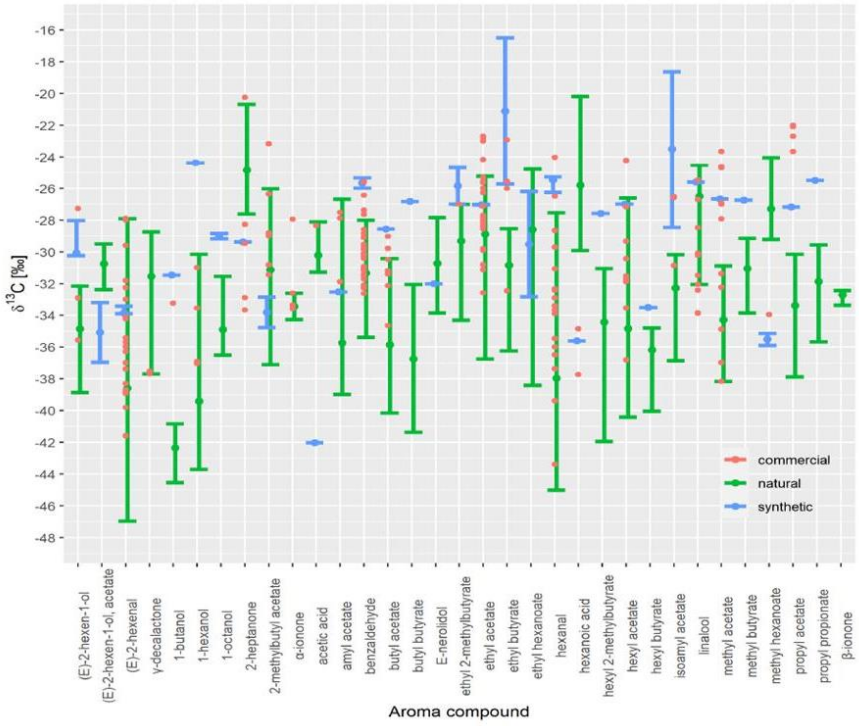

Figure 5. Comparison of $\delta^{13}C$ values of commercial fruit distillates with $\delta^{13}C$ values from the database of natural samples and synthetic standards.

Table 3. Estimation of the amount of synthetic fraction in commercial fruit distillate.

Aroma Compound	Commercial Fruit Distillate Isotope Value	Min Estimated Synthetic Fraction	Median Estimated Synthetic Fraction	Max Estimated Synthetic Fraction
	$\delta^{13}C$ (‰)	x	x	x
hexanal	−26.5	0.46	0.92	0.99
butyl acetate	−29.8	0.34	0.83	0.89
benzaldehyde	−26.4	0.59	0.86	0.95
ethyl butyrate	−26.0	0.21	0.50	0.97
methyl hexanoate	−34.0	0.71	0.81	0.89
methyl acetate	−27.0	0.92	0.96	0.97
2-heptanone	−28.3	0.38	0.76	0.87
hexanoic acid	−34.8	0.86	0.92	0.95

It is impossible to distinguish certain synthetic (petroleum-based) VOCs from natural ones since they can have a $\delta^{13}C$ signature similar to that of a plant [42], which was also observed in our study for (E)-2-hexanal, 2-methylbutyl acetate, amyl acetate, E-nerolidol, ethyl acetate, ethyl hexanoate, hexyl acetate, and linalool. Therefore, developing an HS-SPME-GC-IRMS method for 2D-isotope fingerprinting (^{13}C and ^{2}H) of VOCs would be needed to prove their natural authenticity.

4. Conclusions

The falsification of natural flavours by either dilution, mixing with synthetic compounds, or false declaration of origin of natural ingredients creates a demand to control their authenticity. HS-SPME-GC-C-IRMS analysis of volatile fruit compounds is a valuable tool for differentiating between synthetic and naturally occurring aromas and, together with a database containing flavour compounds with well-defined origins, can be used for authenticity assessment. However, selecting suitable reference samples that need to be analysed to create such a database is critical. When comparing distillates with freshly prepared fruit juice, differences in isotope values in some of the investigated VOCs in apple and strawberry samples is observed. We needed to include both distillates and raw fruit samples to obtain maximal natural stable isotope variability of selected VOCs. A database of $\delta^{13}C$ values for 39 aroma compounds present in fruit and distillate samples (apples, strawberries, blueberries, pears, raspberries, peaches, and sour cherries) and 31 VOC of synthetic origin were established. This study proves that samples of apple and strawberry are sufficient for database construction regarding the variability in $\delta^{13}C$ values. The data are comparable with literature data, and despite some overlap between the natural and synthetic range of values, the method allows the successful separation of 25 of the 33 target VOCs. When testing commercial fruit distillates, the results show possible falsification for several fruit aroma compounds. However, extensive testing of flavoured products on the market is necessary to determine the extent of adulteration and evaluate the usefulness of IsoVoc for different flavoured products. Moreover, the development of an HS-SPME-GC-IRMS method for $^{2}H/^{1}H$ ratio determination of fruit VOCs is required to upgrade the existing database, which could be more successfully used in fruit flavour authenticity studies.

Author Contributions: Conceptualisation, L.S.; methodology, L.S., E.Z., D.K.; software, L.S. and M.S. (Martin Strojnik); validation, L.S.; formal analysis, L.S.; investigation, L.S.; resources, N.O., J.H., N.C.W., D.K., M.S. (Matej Stopar); data curation, L.S.; writing—original draft preparation, L.S.; writing—review and editing, N.O.; visualisation, L.S., M.S. (Martin Strojnik); supervision, N.O.; project administration, N.O.; funding acquisition, N.O. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Supplementary data to this article can be found in Table A1.

Table A1. Literature data on stable isotope analysis of fruit volatile organic compounds.

Fruit Type	Method of Sample Preparation [a]	Stable Isotopic Value	Samples with Known Origin (No. Samples)	Aroma Compound (No. of Samples Where VOC Was Detected)	Reference
strawberry, peach, plum, apricot	SDE	$\delta^{13}C$	fruit (NS); synthetic (NS); microbial (NS)	γ-decalactone (7; 3; 2)	[8]
raspberries	NA	$\delta^{13}C$	fruit (NA); synthetic (NS);biotechnological (NS)	(E)-α-ionone (2; NS; NS), (E)-β-ionone (2; NS; NS)	[18]
bergamot oil	SCF extraction	$\delta^{13}C$	essential oil (2)	linalyl acetate (NS), limonene (NS), linalool (NS), β-pinene (NS), γ-terpinene (NS), β-mycrene (NS), neryl acetate (NS), geranyl acetate (NS)	[5]
raspberries	NA	$\delta^{13}C$	fruit (NS)	α-ionone (NS), β-ionone (NS), δ-decalactone (NS), cis-3-hexen-1-ol (NS)	[19]
strawberry	SDE	$\delta^{13}C$	fruit (NS)	methyl hexanoate (NS), pentyl valerate (NS), butanoic acid (NS), 2-methylbutanoic acid (NS), 4-methylvaleric acid (NS), hexanoic acid (NS), γ-decalactone (NS), γ-uncalactone (NS), γ-dodecalactone (NS)	[20]
raspberry	SCF extraction	$\delta^{13}C$	fruit (NS); synthetic (NS)	(E)-α-ionone (NS), (E)-β-ionone (NS)	[27]
banana	LLE	$\delta^{13}C$	fruit (7)	pentan-2-one (NS), isobutyl acetate (NS), isoamyl acetate (NS), pentan-2-ol (NS), isobutyl butyrate (NS), isoamyl butyrate (NS), isoamyl isovalerate (NS), isobutyl acetate (NS), isoamyl acetate (NS), isoamyl alcohol (NS), isoamyl butyrate (NS)	[22]
bitter almond oils, sweet cherry, sour cherry, peach, nectarine	SDE	$\delta^{2}H$	fruit (NS); essential oil (NS); synthetic (NS); "natural" (NS)	benzaldehyde (NS)	[23]
cactus pear	SDE	$\delta^{13}C$	fruit (NS)	1-hexanol (NS), E-2-hexenol (NS), E-2-nonenol (NS), E,Z-2,6-nonadienol (NS)	[24]
different essential oils, orange, apple, nectarine/peach	LLE, SDE	$\delta^{2}H$	fruit/juice/aroma (NS); essential oil (NS); synthetic (NS); "natural" (NS)	linalool (5; 50; 0; 1), linalyl acetate (0; 19; 5; 2), E-2-hexenal (23; 0; 5; 4), E-2-hexenol (35; 0; 5; 0)	[25]
pineapple	LLE	$\delta^{13}C$, $\delta^{2}H$	fruit/recovery aroma (16);synthetic (NS); "natural" (NS)	methyl 2-methylbutanate (6; 5; 2), ethyl 2-methylbutanoate (9; 5; 6), methyl hexanoate (17; 5; NA), ethyl hexanoate (14; 0; 0), 2,5-dimethyl-4-methoxy-3-(2H)-furanone (12; 1; 0)	[9]

Table A1. *Cont.*

Fruit Type	Method of Sample Preparation [a]	Stable Isotopic Value	Samples with Known Origin (No. Samples)	Aroma Compound (No. of Samples Where VOC Was Detected)	Reference
raspberries	SDE	$\delta^{13}C$, $\delta^{2}H$	fruit (NS); nature-identical (NS); "natural" (NS)	(E)-α-ionone (10; 4; 1), (E)-β-ionone (10; 4; 1)	[10]
peach, apricot, nectarine	SDE	$\delta^{13}C$, $\delta^{2}H$	fruit (18); nature-identical (NS); "natural" (NS)	γ-decalactone (16; 2; 5), δ-decalactone (12; 1; 3)	[11]
pear	SDE, LLE	$\delta^{13}C$, $\delta^{2}H$	fruit (20); synthetic (NS); "natural" (NS)	butyl acetate (14; 3; 3), 1-butanol (18; 3; 2), hexyl acetate (16; 2; 7), 1-hexanol (3; 11; 8), methyl E, Z-2,4-decadienaoate (6; 2; 0), ethyl E,Z-2,4-decadienoate (10; 5; 3), ethyl E,E-2,4-decadienoate (2; 0; 0)	[12]
apple	SDE	$\delta^{13}C$, $\delta^{2}H$	juice/recovery aroma (62)	E-2-hexenal (NS), 1-hexanol (NS), E-2-hexenol (NS)	[28]
raspberry, litsea cubeba, lemongrass	SDE	$\delta^{13}C$, $\delta^{2}H$	fruit (8); essential oil (NS); synthetic (NS); "natural" (NS)	acetone (NS), citral (NS), α-ionone (NS), β-ionone (NS)	[13]
blackberry	SDE	$\delta^{13}C$, $\delta^{2}H$, $\delta^{18}O$	fruit (10); synthetic (3)	2-heptanol (10; 1), trans-linalool oxide (10; 1), cis-linalool oxide (10; 1)	[14]
strawberry, pineapple, peach	HS-SPME	$\delta^{13}C$	fruit (NS)	methyl butanoate (NS), ethyl butanoate (NS), E-2-hexenal (NS), methyl hexanoate (NS), butyl butyrate (NS), ethyl hexanoate (NS), hexyl acetate (NS), linalool (NS), hexyl butyrate (NS), octyl isovalerate (NA), γ-decalactone (NS), octyl hexanoate (NS)	[7]
sweet orange	HS-SPME	$\delta^{13}C$	essential oil (20)	myrcene (20), limonene (20), nonanal (20), decanal (20), linalool (20), α-terpineol (20), geranial (20)	[15]
wolfberry	HS-SPME	$\delta^{13}C$	fruit (52)	limonene (NS), tetramethylpyrazine (NS), safranal (NS), geranylacetone (NA), β-ionone (NS)	[32]
apple	HS-SPME	$\delta^{13}C$	recovery aroma (18); synthetic (16)	ethyl acetate (1; 1), ethyl butyrate (1; 1), ethyl-2-methyll butyrate (1; 1), butyl acetate (6; 1), 1-hexanal (12; 1), 2-methylbutyl acetate (10; 1), 1-butanol (15; 1), amyl acetate (8; 1), butyl butyrate (8; 1), trans-2-hexenal (12; 1), hexyl acetate (8; 1), 2-hexen-1-ol, acetate (1, 1), 1-hexanol (15; 1), trans-2-hexenol (2; 1), benzaldehyde (15; 1), 1-octanol (8; 1)	[16]

[a] Method of sample preparation: SDE (simultaneous distillation extraction), HS-SPME (headspace solid-phase microextraction), LLE (liquid-liquid extraction), SCF (supercritical fluid extraction). NS: not specified.

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3.4 Scientific Paper: "C and H Stable Isotope Ratio Analysis Using Solid-Phase Microextraction and Gas Chromatography-Isotope Ratio Mass Spectrometry for Vanillin Authentication"

This chapter presents the paper entitled "C and H Stable Isotope Ratio Analysis Using Solid-Phase Microextraction and Gas Chromatography-Isotope Ratio Mass Spectrometry for Vanillin Authentication" by Matteo Perini, Silvia Pianezze, Lidija Strojnik and Federica Camin. The paper was published in the Journal of Chromatography A in 2019. This paper describes the development of a novel method to analyse δ^2H in vanillin based on SPME-GC-IRMS. The aim was to develop a quick, robust and effective method to measure δ^2H and δ^{13}C in vanillin to gauge its authenticity. Fifty authentic samples from vanilla pods, nature-identical (ex) and synthetic vanillin, and four commercial food products were analysed.

For δ^2H, the method based on HS-SPME was first optimised in fibre selection, extraction temperature, and NaCl addition. The PDMS/CAR/DVB fibre provides a much higher uptake and affinity toward the volatile components, showing a 7-fold higher extraction yield than the PA fibre. The minimum detectable concentration of vanillin was also found to be 0.13 mg/mL. The addition of NaCl was found to induce isotopic fractionation or react with vanillin, modifying the ratio between deuterium and hydrogen in the headspace randomly. Based on these results, a protocol was proposed based on PDMS/CAR/DVB fibre and conditioning of samples at 80° C in water solution without NaCl addition. The method avoids isotopic fractionation and provides repeatability SD and internal reproducibility SD of less than 7‰, similar to solvent extraction. Moreover, the HS-SPME method guarantees savings in time (from 30 min/sample to 5 min/sample) and solvent (e.g., ethanol or diethyl ether).

The protocol was also tested to analyse the δ^{13}C and δ^2H of vanillin in more complex matrices such as tea, yoghurt, ice cream and pudding. In order to demonstrate the absence of isotopic fractionation or matrix effect, white yoghurt and a chocolate pudding without vanillin were tested and compared with vanillin samples with known isotopic values. The obtained δ^{13}C and δ^2H values were similar ($p < 0.01$) to the "reference" values, confirming the absence of isotopic fractionation.

The proposed SPME method was finally applied to a selection of commercial food products stated to be flavoured with natural vanillin (yoghurt, ice cream, pudding and tea). Results show that all the samples labelled as containing natural vanillin from Vanilla beans contained synthetic vanillin. In this study, I developed the HS-SPME IRMS (δ^2H) method, jointly analysed the data and co-wrote the manuscript.

The findings from this study were presented as an oral presentation at the 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019), November 5-8, 2019, in Prague, Czech Republic. As an oral and a poster presentation at the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, 15th and 16th May 2019 in Planica, Slovenia. It was also presented as a poster presentation at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, April 1-3, 2019, in Portorož, Slovenia.

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C and H stable isotope ratio analysis using solid-phase microextraction and gas chromatography-isotope ratio mass spectrometry for vanillin authentication☆

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ABSTRACT

It is possible to distinguish precious vanillin from *Vanilla* species (*planifolia* or *tahitensis*) from much less expensive synthetic and nature-identical vanillin on the basis of the stable isotope ratios of H and C ($^2H/^1H$, $^{13}C/^{12}C$). Analysis is usually performed using GC-IRMS (Gas Chromatography - Isotope Ratio Mass Spectrometry) after solvent extraction of vanillin from the sample. Recently, head-space solid-phase microextraction (HS-SPME) has been proposed as an alternative for determining $^{13}C/^{12}C$.

The aim of this study was to develop a method to analyse $^2H/^1H$ in vanillin using SPME-GC-IRMS for the first time, by testing different operating conditions and comparing the results with those obtained after solvent extraction. The ultimate scope was to develop a quick, robust and effective method to measure $^2H/^1H$ and $^{13}C/^{12}C$ in vanillin to assess the authenticity of labelling.

Almost 50 authentic samples from vanilla pods, nature-identical (ex) and synthetic vanillin and 4 commercial food products were taken into account. All the samples were subjected to HS-SPME-GC-IRMS analysis and most of them to GC-IRMS analysis after solvent extraction of vanillin.

The SPME method developed for $^2H/^1H$ analysis guarantees the absence of isotopic fractionation, repeatability and reproducibility standard deviation of below 7‰ and savings in terms of time (from 30 to 5 min) and solvent.

HS-SPME GC-IRMS analysis of δ^2H and $\delta^{13}C$ can be proposed as a rapid and robust method to discriminate different types of vanillin and assess the authenticity of natural vanillin, also contained in food matrices.

1. Introduction

Vanilla extract is widely used as a flavouring ingredient in food and beverages. It is extracted from different species of tropical orchids belonging to the *Genus Vanilla*, of which *Vanilla planifolia* and *Vanilla tahitensis* are the most widely used [1], mainly produced in Indonesia (3200 tons in 2017), Madagascar (3100 tons), Mexico (463 tons), Papua New Guinea (433 tons) and China (335 tons) [2].

Vanilla from *Vanilla* species is the 2nd most expensive spice in the globe (4,000$/Kg [3]) second only to saffron. The increased demand for *Vanilla* and the limited supply of extract from orchids have encouraged the development of new sources of vanillin, which are synthetically or biotechnologically produced from natural precursors, e.g. lignin, eugenol, ferulic acid and curcumin. The price of these types of vanillin is much lower compared to that of products from *Vanilla* species, being around 12 $/kg for synthetic vanillin [3], and this leads to possible adulteration of the natural product from the orchid.

Authenticity assessment of vanilla extract is thus an important and challenging issue and there is increasing interest in the development of authentication criteria. One of the most commonly used methods is stable isotope ratio analysis of $^{13}C/^{12}C$ (expressed as

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$\delta^{13}C$). Indeed, natural vanillin from tropical orchids is produced via the CAM photosynthetic pathway and has a $\delta^{13}C$ value between -22‰ and -14‰, whereas nature-identical (ex-ferulic acid and turmeric) and synthetic (ex-lignin and guaiacol precursors) vanillin have significantly lower $\delta^{13}C$ values (from -38‰ to -29‰ and from -30‰ to -27‰ respectively) [3–9].

Analysis of the $\delta^{13}C$ of vanillin, once extracted from different matrices and in some cases purified using HPLC, has been performed directly using Isotope Ratio Mass Spectrometry (IRMS), after demethylation and conversion to methyl iodide [5] or oxidation to vanillic acid [4], or by using an EA (elemental Analyser)-IRMS directly in vanillin [8,10,11] or after its conversion to guaiacol [7]. More sophisticated approaches involve quantitative isotopic ^{13}C-Nuclear Magnetic Resonance (NMR) [12]. As vanillin is a volatile compound, the most suitable technique is GC/C (Combustion)-IRMS after solvent extraction from the sample [6,9,13]. Recently, head-space solid-phase microextraction (HS-SPME) has been proposed as an alternative and more rapid method to extract vanillin from different matrices without encountering isotopic fractionation [3].

Nowadays, $\delta^{13}C$ analysis is sometimes not enough to discover vanillin adulteration, due to the practice of adding ^{13}C to the methylic site of synthetic vanillin [4]. In order to improve the power of the isotopic approach, $\delta^{13}C$ analysis has been combined with analysis of the stable isotope ratio of O [7,14] and H of vanillin [15–17] and vanillin methoxyl groups [16], or site-specifically using ^{2}H-SNIF NMR [10]. Specifically, vanillin from *Vanilla* species has negative values for the $^{2}H/^{1}H$ ratio, expressed as $\delta^{2}H$ (from -115‰ to -3‰ [15–17] but higher than ex-lignin vanillin (from -204 to -170‰ [18]), whereas synthetic vanillin has positive values (from +57 to +75‰ [16]). Analysis is carried out after solvent extraction or dilution, using IRMS after off line conversion of vanillin to H_2 [15] or GC/P (Pyrolysis)-IRMS [16,17].

The aim of this study was to develop and validate a method to analyse $\delta^{2}H$ in vanillin using SPME-GC/P (Pyroliser)-IRMS for the first time, by testing different operating conditions and comparing the results with those obtained by GC-IRMS after solvent extraction.

Almost 50 authentic samples from vanilla pods, nature-identical and synthetic vanillin and 6 commercial food products were taken into account.

The ultimate scope was to develop a quick, robust and effective method to measure $\delta^{2}H$ and $\delta^{13}C$ in vanillin in order to assess the authenticity of labelling.

2. Materials and methods

2.1. Samples and chemicals

Samples were provided by different suppliers, who guaranteed their authenticity. 33 vanilla extracts (from *Vanilla* plants) of unknown geographical origin, 10 tannins as a natural source of ex-lignin vanillin (flavourings and food ingredients (EC No 1334/2008, EU Regulation No. 872/12) and clarification agents for protein stabilisation in must and wine), 1 ex lignin, 2 ex eugenol, 2 ex ferulic acid, 1 ex turmeric and 6 synthetic vanillins were considered. Moreover, a selection of commercial food products stated to be flavoured with natural vanilla from *Vanilla* pods were taken into account (1 ice-cream, 1 tea, 1 pudding and 1 yoghurt).

To demonstrate the absence of isotopic fractionation and matrix effect, a sample of white yoghurt and chocolate pudding, both without vanillin, were added to a synthetic and a natural vanillin from *Vanilla* in the laboratory. 3 g of a white yoghurt and a chocolate pudding without vanillin content were mixed separately in two 20 mL SPME vials with 1 mL of natural vanillin extract from *Vanilla* having a known $\delta^{13}C$ value of -20.3‰ and $\delta^{2}H$ value of -35‰. The experiment was repeated using 1 mg of synthetic vanillin with a known $\delta^{13}C$ value of -29.3‰ and $\delta^{2}H$ value of +80‰.

Ethanol (>99.8‰), sodium chloride ≥99.5% and diethyl ether were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Sample preparation

The solid samples (ex-lignin, ex-eugenol, ex-ferulic acid, ex-turmeric and synthetic vanillin) were diluted with ethanol (0.3 mg/mL) and injected.

2.2.1. Extraction of vanillin from vanilla extracts

Vanillin was extracted from the vanilla extracts and purified by adjusting the method reported by Fayet et al. (1999) [19]. Briefly, 3 ml of extract was mixed with 3 ml of diethyl ether and shaken for 1 min with a vortex device. The diethyl ether layer containing vanillin was removed and concentrated to dryness under N_2 stream at room temperature. The concentrate was dissolved in ethanol (1 mL) and then vortexed. The sample was diluted eightfold and then injected into the GC-IRMS instrument.

2.2.2. Extraction of vanillin from tannins

Vanillin was extracted from tannins following the method reported by van Leeuwen et al. (2018) [9]. Briefly, ethanol (0.9 mL) was added to tannin (100 mg) and vortexed. Deionised water (2.1 mL) and diethyl ether (3 mL) were added, the sample was shaken by hand and the layers were allowed to separate. The diethyl ether layer was removed and retained. The water layer was re-extracted with diethyl ether (1 mL) and the ether extracts were combined and then concentrated to dryness under N_2 stream at 30 °C. The sample was dissolved in ethanol (0.3 mL), vortexed and transferred to an insert in a 2-mL vial before analysis.

2.2.3. Vanillin head-space solid-phase microextraction (HS-SPME)

2.2.3.1. $\delta^{13}C$ analysis. 0.3 mg of synthetic or nature-identical pure vanillin, 1 mL of vanillin extracted from vanilla extracts or tannins, 6 g of ice-cream, tea, pudding or yoghurt were individually mixed with 5 mL of a saturated NaCl water solution in a 20 mL SPME vial with a silicone/PTFE septum [3].

PDMS/Car/DVB (2 cm, 50/30 μm) SPME fibres (Sigma-Aldrich/Supelco, Bellefonte, USA) were exposed to the headspace (HS) of the samples for 30 min at 80 °C and then they were desorbed in the GC injector port for 2 min at 260 °C.

2.2.3.2. $\delta^{2}H$ analysis. 1 mg of pure synthetic vanillin was first mixed with 3 mL of saturated NaCl water solution (experiment A) or 3 mL of deionized water without NaCl (experiment B) in a 20 mL SPME vial with a silicone/PTFE septum. Two SPME fibres (Sigma-Aldrich/Supelco, Bellefonte, USA) PDMS/Car/DVB (2 cm, 50/30 μm) and polyacrylate (PA) (1 cm, 85 μm) were evaluated. The fibres were exposed to the HS of the samples for 30 min at different temperatures (40 and 80 °C) and then desorbed in the GC injector port for 2 min at 260 °C.

Then, after having identified the best conditions, 2 mL of liquid vanilla extract, 1 mg of vanillin powder or 10 g of ice-cream, tea, pudding or yoghurt were mixed with 3 mL of deionized water in a 20 mL SPME vial with a silicone/PTFE septum and analysed using PDMS/Car/DVB (2 cm, 50/30 μm) SPME fibre (Sigma-Aldrich/Supelco, Bellefonte, USA).

2.3. Elemental analyser (EA) and pyrolizer (TC) analysis of standard synthetic and ex-lignin vanillin

The $\delta^{13}C$ and $\delta^{2}H$ values of a synthetic vanillin ("standard 1") and ex-lignin vanillin ("standard 2") were measured using an elemental

analyser (Flash EA 1112, Thermo Scientific, Bremen, Germany) and a pyrolyzer (TC-EA, Thermo Scientific, Bremen, Germany) furnished with an autosampler (Finnigan AS 200, Thermo Scientific) and interfaced to a DELTA V isotope ratio mass spectrometer (Thermo Scientific) through a ConFlo IV dilutor (Thermo Finnigan, Bremen, Germany).

According to the IUPAC protocol, the values are denoted in delta in relation to the international V-PDB (Vienna-Pee Dee Belemnite) for $\delta^{13}C$ and V-SMOW (Vienna-Standard Mean Ocean Water) for $\delta^{2}H$, according to the following general equation:

$$\delta i\,E = \frac{(i\,RSA\text{-}i\,RREF)}{i\,RREF}$$

where i is the mass number of the heavier isotope of element E, RSA is the respective isotope ratio of the sample and RREF is the relevant internationally recognised reference material.

The delta values are multiplied by 1000 and expressed in units "per mil" (‰).

For $\delta^{13}C$, the isotopic ratios were calculated against two in-house standards covering the range between −10 and −30‰ and calibrated against the following international reference materials: fuel oil NBS-22 ($\delta^{13}C$= -30.03‰), sucrose IAEA−CH-6 ($\delta^{13}C$= -10.45‰) obtained from IAEA-International Atomic Energy Agency, (Vienna, Austria) and L-glutamic acid USGS 40 ($\delta^{13}C$= -26.39‰, $\delta^{15}N$= -4.52‰) from U.S. Geological Survey, (Reston, VA, USA).

The $\delta^{2}H$ values were calculated against fuel oil NBS-22 ($\delta^{2}H$ = -120.0 ± 1‰), USGS 70 ($\delta^{2}H$ = -183.9 ± 1‰) and USGS 71 ($\delta^{2}H$ = -4.9 ± 1‰) through the creation of a linear equation.

The uncertainty (2 s) of measurements was <0.3‰ for $\delta^{13}C$ and <3‰ for $\delta^{2}H$.

2.4. GC-IRMS analysis

The system consisted of a GC oven, Trace GC Ultra, equipped with a TriPlus autosampler, retrofitted to the combustion interface GC/IsoLink, hyphenated to the isotope ratio mass spectrometer, Delta V Advantage (Thermo Scientific, Milan, Italy) and in parallel, with a single-quadrupole GC–MS (ISQ Thermo Scientific, Milan, Italy) to clearly identify vanillin. Data were collected in triplicate using Isodat 2.5 software (Thermo Fisher Scientific). The capillary column was a VF-WAXms 30m × 0.25 mm i.d., 0.25 μm film thickness (Agilent, Santa Clara, CA, United States). The carrier gas was He at a constant flow of 2.0 mL/min. The programmed operating temperature for analysis after solvent extraction was as follows: 65 °C (held for 3 min) to 250 °C at 20 °C/min, then to 260 °C for 8 min. Injection was performed at 260 °C in splitless mode (2 min). The programmed operating temperature for HS-SPME analysis was as follows: 50 °C (held for 3 min) to 250 °C at 7 °C/min, then to 260 °C for 15 min. Injection was performed at 260 °C in splitless mode (2 min).

To determine $\delta^{13}C$, the eluted compounds were combusted into CO_2 and H_2O in a combustion furnace reactor (GC/Isolink), operating at 1030 °C and consisting of a non-porous alumina tube (320 mm long) containing three wires (Ni/Cu/Pt, 0.125 mm diameter, all 240 mm long) braided and centred end-to-end within the tube. Water vapour was removed with a water-removing trap consisting of a Nafion dryer. The He carrier gas and CO_2 reference gas were set respectively at 1 and 0.6 bar. The Faraday collector was set to detect CO_2 ion contributions at *m/z* 44, 45 and 46.

Vanillin was identified using GC/MS with selected ions and compared with the NIST library (NIST Standard Reference Database 1A NIST/EPA/NIH Mass Spectral Library (NIST 08) and NIST Mass Spectral Search Program (Version 2.0f)).

For $\delta^{2}H$, each compound passed through a high temperature reactor, operating at 1400 °C, where it was subjected to high temperature pyrolysis with development of H_2 gas.

Before measuring the $^{2}H/^{1}H$ ratio, the $[H3]^{+}$ factor was verified to be lower than 8, the limit suggested by Thermo Scientific in the Trace GC Ultra instrumental manual. The "[H3]+ factor" or "H3+correction" describes an algorithm which corrects the measured δ2H data for the contribution of H3+species formed by ion/molecule reactions in the ion source at increasing gas pressures. It is determined by measuring the intensity of m/z 3 of working gas as a linear function of m/z 2. The $\delta^{13}C$ and $\delta^{2}H$ values were calculated against two in-house standard vanillins in powder form, one synthetic ("standard 1") ($\delta^{13}C$= -31.4‰, $\delta^{2}H$= +72‰) and one ex lignin ("standard 2") ($\delta^{13}C$= -28.9‰, $\delta^{2}H$= -169‰), calibrated against international reference materials as reported in Section 2.3 by using EA and TC-IRMS.

For $\delta^{13}C$ and $\delta^{2}H$ analysis of samples after solvent extraction, the uncertainty (2 s) of measurements was <0.6‰ for $\delta^{13}C$ and <6‰ for $\delta^{2}H$. The results are comparable to those reported in Greule et al. (2010) [16].

2.5. Statistical analysis

The data were statistically evaluated using Statistica v 13.1 (Dell Inc.). Parametric tests (Tukey's HSD for an unequal number of samples test, t Student test, ANOVA Analysis of Variance) were applied to the data because they were normally distributed and fulfilled the assumptions for the application of these tests.

3. Results and discussion

3.1. $\delta^{13}C$ and $\delta^{2}H$ of vanillin in samples after solvent extraction

Table 1 and Fig. 1 show the results for samples extracted from vanilla extracts and tannins, and for ex-lignin, ex-eugenol, ex-ferulic acid, ex-turmeric and synthetic vanillin diluted with ethanol.

Vanillin from *Vanilla* species has $\delta^{13}C$ ranging between -22‰ to -17.9‰ and $\delta^{2}H$ from -110‰ to -6‰. Ex-eugenol and ex-ferulic acid showed the lowest $\delta^{13}C$ values, ranging from -36.9‰ to -31.1‰, while ex-lignin (including that from tannin) and ex-turmeric vanillin have $\delta^{13}C$ between -29.7 to -26.9‰. Synthetic vanillin covers a range of $\delta^{13}C$ values between -31.4‰ and -29.4‰, outside the range of natural vanillin. Stable isotope carbon analysis

Table 1
$\delta^{13}C$ and $\delta^{2}H$ values of vanillin analysed after solvent extraction (*from vanilla extract* and *tannin*) or dilution in ethanol (*ex eugenol, ex ferulic acid, ex lignin, ex turmeric* and *synthetic* vanillin).

		$\delta^{13}C$ (‰, vs V-PDB)				$\delta^{2}H$ (‰, vs V-SMOW)			
	n	Mean	SD	Min	Max	Mean	SD	Min	Max
Ex *Vanilla* plants	33	−20.1	1.0	−22.0	−17.9	−32	25	−110	−6
Ex eugenol	2	−34.0		−36.9	−31.1	−83		−91	−75
Ex ferulic acid	2	−36.6		−37.4	−35.7	−172		−176	−168
Ex lignin	1	−27.9				−169			
Ex turmeric	1	−29.7				−112			
Ex wood tannin	10	−27.7	0.6	−28.8	−26.9	−104	32	−142	−36
Synthetic commercial	6	−30.5	0.8	−31.4–31.4	−29.4	63	23	38	104

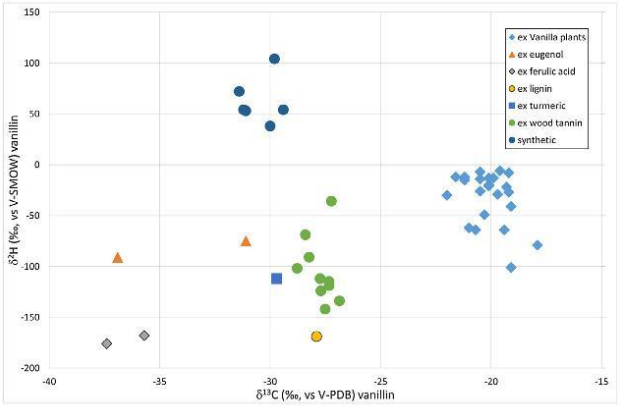

Fig. 1. Plot of $\delta^{13}C$ vs $\delta^{2}H$ of vanillins from natural or synthetic source.

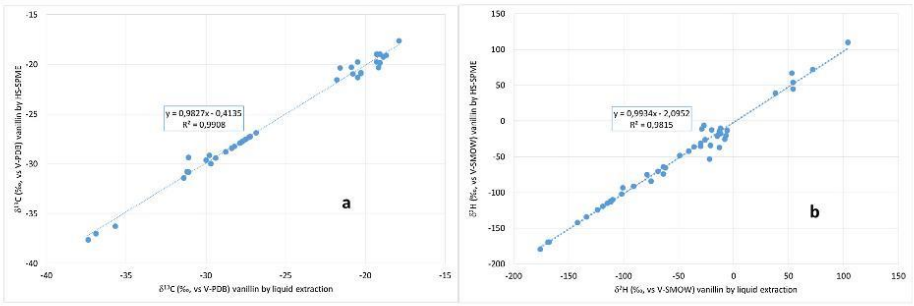

Fig. 2. Correlation between the $\delta^{13}C$ (a) and $\delta^{2}H$ (b) of vanillin analysed using the GC-IRMS method after solvent extraction or dilution and HS-SPME-GC-IRMS.

is thus confirmed as a powerful method for discriminating between natural from *Vanilla* species, nature-identical and synthetic vanillin.

With the exception of ex-eugenol vanillin, ranging between -91‰ and -75‰, all the nature-identical vanillin samples had $\delta^{2}H$ lower than natural vanillin (from *Vanilla* plants): ex-ferulic acid (ranging between -176‰ and -168‰), ex lignin (-169‰) and ex turmeric (-112‰). In contrast, synthetic vanillin was characterised by positive values (ranging between +38‰ and +104‰).

The $\delta^{13}C$ and $\delta^{2}H$ values reported here fall within the ranges cited in the literature [16,17] and confirm the usefulness of $\delta^{2}H$ analysis in terms of detecting the addition of synthetic to natural vanillin, also when it has been added with ^{13}C at the methylic site [4]. The addition of ex-ferulic, ex lignin and ex turmeric vanillin can also be detected on the basis of $\delta^{2}H$ values.

Despite the usefulness of the analysis, the solvent extraction method involves considerable use of solvent and is quite time-consuming (more than 30 min/sample for solvent extraction and concentration with N_2 stream). This has stimulated the development of more rapid analytical protocols, and an HS-SPME protocol was developed.

3.2. $\delta^{13}C$ analysis of vanillin using HS-SPME-GC -IRMS

Following the method reported by Schipilliti et al. (2016) [3], the $\delta^{13}C$ of all the samples (natural extract from *Vanilla*, synthetic and nature-identical vanillin) was analysed using HS-SPME. Data are reported in Table 1S (Supplementary Materials). As shown in Fig. 2a, there was a high correlation between the $\delta^{13}C$ values obtained using solvent extraction and HS-SPME methods ($R^2 = 0.99$). The difference between the values obtained with the two methods was on average less than 0.8‰.

This value is similar or lower than the repeatability limit estimated by Schipilliti et al. (2016) (1.0‰) [3] and by van Leeuwen et al. (2018) (0.8‰) [9] and calculated as $2^* \sqrt{2}\ ^*SD_{repeatability}$.

3.3. $\delta^{2}H$ analysis of vanillin using HS-SPME-GC -IRMS: method development

For $\delta^{2}H$, the method based on HS-SPME is not available in the literature and was developed here with the scope of achieving a quick, robust and effective method.

In the tests, we took into account a pure vanillin sample in powder form ("standard 1"), previously analysed using pyrolysis (see section 2.4), having a "reference" $\delta^{2}H$ value of +72‰.

Two types of fibre (PA and PDMS/Car/DVB) and different operating conditions were tested, and the results are shown in Table 2. PA and PDMS/Car/DVB fibres were chosen due to the high affinity with vanillin already reported by Sostaric et al. (2000) [20] and Schipilliti et al. (2016) [3].

The performance of PA and PDMS/Car/DVB fibres were compared by analysing standard 1 in SPME vials without water (Table 2, Experiment 1) and in a saturated NaCl water solution (Table 2, Experiment 2) at 40 °C and 80 °C.

Table 2
Evaluation of the performance of two different fibres (PA and PDMS/Car/DVB) for δ^2H analysis of vanillin at different temperatures (40 °C and 80 °C) and in different operating conditions (without NaCl and water, with NaCl and water, and with water but without NaCl).

δ^2H (‰)	Experiment 1 **PA fibre**		Experiment 2		Experiment 3
	Without NaCl + water		With NaCl + water		With water and without NaCl
	40 °C	80 °C	40 °C	80 °C	80 °C
Mean (n)	72 (11)	nd	34 (8)	3 (30)	38 (10)
SD	8	nd	37	18	35
δ^2H (‰)	**PDMS/Car/DVB fibre**				
	Without NaCl + water		With NaCl+ water		With water and without NaCl
	40 °C	80 °C	40 °C	80 °C	80 °C
Mean (n)	94 (10)	nd	66 (9)	61 (12)	67 (12)
SD	9	nd	19	37	7

Table 3
δ^{13}C and δ^2H values obtained using HS-SPME-GC-IRMS of vanillin added in the laboratory to a white yoghurt and a chocolate pudding for evaluating isotopic fractionation.

	δ^{13}C (‰)vs V-PDB	SD	δ^2H (‰)vs V-SMOW	SD
Natural vanillin extract (NVE)	−20,3	0,2	−35	7,0
White yogurt + NVE	−20,6	0,2	−31	5,0
Chocolate pudding + NVE	−20,5	0,2	−41	9,0
Synthetic vanillin (SV)	−29,3	0,2	80	5,0
White yogurt + SV	−29,4	0,2	75	4,0
Chocolate pudding + SV	−29,0	0,2	78	6,0

In Experiment 1 the samples were only conditioned at 40 °C, because at 80 °C vanillin powder melts and degrades. As reported in Table 2, in these conditions, both PA and PDMS/Car/DVB fibres provided an acceptable repeatability SD, below 9‰, but PA guaranteed a more accurate isotopic value. In any case, the applicability of the method to samples in powder form is very limited, as vanillin is normally in the form of an alcoholic extract.

For this reason, in Experiment 2 we diluted the sample in water by adding NaCl up to saturation. The addition of NaCl or another salt to the sample matrix makes it possible to enhance the tendency of volatiles to go in the headspace (salting out effect) [21–23] and hence to enhance the partition with SPME fibre (or to achieve higher enrichment factors). The data reported in Table 2 show that the presence of NaCl had a negative effect on analysis, both at 40 °C and 80 °C. The repeatability SD was very high (over 18‰) and the average values were significantly lower than the reference value, despite the linear correction adopted. It is hard to find an explanation for it. Possible reasons can be that NaCl, accelerating the salting out of vanillin, would induce isotopic fractionation or that the salt would react with vanillin, modifying the ratio between deuterium and hydrogen in the HS space randomly.

We therefore decided to set up another trial (Table 2, Experiment 3), avoiding NaCl addition. The performance of both fibres (PA and PDMS/Car/DVB) was only explored at 80 °C, because at 40 °C vanillin, in water solution without NaCl, is not sufficiently volatile to go in the headspace.

At 80 °C the absence of the salt also forced us to increase the operating concentration of the samples in the vial (0.33 mg/ml instead of 0.06 mg/ml).

As reported in Table 2, we obtained the best results with PDMS/Car/DVB fibre, with a repeatability SD of below 7‰ and an average value not significantly different ($p < 0.01$) from the reference value (+67‰ vs +72‰). In these analytical conditions, differently from PDMS/Car/DVB fibre, the use of PA fibre provides isotopic fractionation.

As reported by Schipilliti et al. [3] PA and PDMS/Car/DVB fibre differ in sensitivity and selectivity. The PDMS/Car/DVB fibre performs better, because it provides a much higher uptake and a higher affinity toward the volatile components, showing an about 7-fold higher extraction yield compared to the PA fibre.

In the reported operating conditions (PDMS/Car/DVB fibre, 30 min exposure time at 80 °C, GC injector port for 2 min at 260 °C), by increasing the concentration of vanillin from 0.05 to 0.33 mg/ml, we found that the minimum detectable concentration of vanillin was 0.13 mg/mL.

On the basis of these results, we propose a protocol based on the use of PDMS/Car/DVB fibre and conditioning of samples at 80 °C in water solution without NaCl addition. This method avoids isotopic fractionation and is applicable when the vanillin concentration is higher than 0.13 mg/mL.

3.4. δ^2H analysis of vanillin using HS-SPME-GC -IRMS: validation

With the scope of verifying if isotopic fractionation occurs and of calculating the repeatability limit of the developed protocol, two samples of pure vanillin (one synthetic vanillin, "standard 1" and a nature-identical vanillin, "standard 2") were analysed several times. Both were previously analysed using pyrolysis, and "reference" values (δ^2H = +72‰ and -169‰ respectively) were assigned. As shown in Table 2S (Supplementary Materials), the method avoids isotopic fractionation (mean values of standard 1 and 2: +67‰ and -164‰, respectively) and provides repeatability SD of less than 7‰, therefore not different from the protocol based on solvent extraction. Moreover, the HS-SPME method guarantees savings in terms of time (from more than 30 min/sample to 5 min/sample) and solvent (e.g. ethanol or diethyl ether).

Table 2S (Supplementary Materials) also shows the results of δ^2H analysis of the same nature-identical vanillin (standard 2) carried out on different days. The average value (-166‰) is again not different from the 'reference' value, thus confirming absence of isotopic fractionation and the internal reproducibility SD is again estimated to be lower than 7‰.

Fig. 2b shows that the δ^2H values obtained with the SPME method were comparable with those obtained using traditional solvent extraction. The correlation was highly significant ($R^2 = 0.98$) and the difference between the two δ^2H values was always below 10‰, therefore below the repeatability limit.

This means that the HS-SPME GC-IRMS protocol is a promising alternative to a GC-IRMS method based on solvent extraction for analysis of both δ^{13}C and δ^2H in vanillin, guaranteeing absence of isotopic fractionation, comparable repeatability and reproducibility and savings in terms of time and solvent use.

Table 4
$\delta^{13}C$ and $\delta^{2}H$ of vanillin contained in commercial food products.

	$\delta^{13}C$ (‰)vs V-PDB	$\delta^{2}H$ (‰)vs V-SMOW
Ice cream	−29,8	89
Tea	−30,2	112
Pudding	−27,4	36
Yoghurt	−28,6	39

3.5. $\delta^{13}C$ and $\delta^{2}H$ analysis of vanillin in food products using HS-SPME-GC-IRMS: method development and validation

The protocol was also tested for analysis of the $\delta^{13}C$ and $\delta^{2}H$ of vanillin contained in more complex matrices such as tea, yoghurt, ice-cream and pudding. With these matrices, a method based on solvent extraction is very challenging due to the high fat content, which requires a long extraction time (over 6 h) [24].

To demonstrate the absence of isotopic fractionation or matrix effect, a white yoghurt and a chocolate pudding without vanillin were added in a SPME vial with vanillin samples having known isotopic values: a natural vanillin from *Vanilla* ($\delta^{13}C$ -20.3‰ and $\delta^{2}H$ -35‰) and a synthetic one (-29.3‰ and +80‰) and the spiked yoghurt and chocolate were analysed using the developed protocol. As reported in Table 3, the $\delta^{13}C$ and $\delta^{2}H$ data were not significantly different ($p < 0.01$) compared to the "reference" values, confirming absence of isotopic fractionation. It follows that the proposed method can also be used for the analysis of complex food products, without isotopic fractionation.

The proposed SPME method was thus applied to a selection of commercial food products stated to be flavoured with natural vanillin (yoghurt, ice-cream, pudding and tea). The results reported in Table 4 shows that all the samples considered and stated to be flavoured with natural vanillin from *Vanilla* beans actually contained synthetic vanillin, because both the $\delta^{13}C$ values (ranging from -30.2‰ to 27.4‰) and the $\delta^{2}H$ values (ranging from +36‰ to +112‰) fell into the range of variability of the synthetic vanillin.

With the scope of also calculating the repeatability limit of the method developed for food products, the sample of ice-cream ($\delta^{13}C$ -29.8‰ and $\delta^{2}H$+89‰) was analysed several times. As shown in Table 2 Supplementary, the method had a repeatability SD of 7‰, comparable with that obtained for vanillin extracts.

4. Conclusion

The HS-SPME GC-IRMS method was shown to be a rapid, effective and promising alternative to the GC-IRMS approach for the analysis of both $\delta^{13}C$ and $\delta^{2}H$ in vanillin, guaranteeing absence of isotopic fractionation, comparable repeatability and reproducibility and savings in terms of time and solvent use.

For the first time, an SPME method for $\delta^{2}H$ analysis was developed, and performance in terms of accuracy, repeatability and reproducibility standard deviation was demonstrated.

The HS-SPME GC-IRMS method can also be used to verify the natural or synthetic origin of vanillin contained in commercial food products.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.02.032.

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3.5 Scientific Paper: "Species and Geographic Variability in Truffle Aromas"

This chapter presents the paper entitled "Species and Geographic Variability in Truffle Aromas" by Lidija Strojnik, Tine Grebenc and Nives Ogrinc. The paper was published in Food and Chemical Toxicology in 2020. It included the classification of truffle species based on their aroma profile, an analysis of the differences in the volatile organic composition of truffle species over a geographical area, and, in more detail, a study of T. aestivum from four natural truffle-growing sites in Slovenia. HS-SPME GC-MS was used to characterise the VOC profiles of fresh samples.

This study provides an extended exploration of the impact of different VOCs on the aroma quality of 460 fresh ascocarps of nine truffle species: *T. aestivum, T. magnatum, T. melanosporum, T. brumale, T. mesentericum, T. excavatum, T. macrosporum*, and *T. rufum*, from 10 different European countries (Slovenia, Croatia, Bosnia and Herzegovina, Macedonia, Italy, Spain, France, United Kingdom, Germany and Poland) and *T. indicum* obtained from China. Samples were harvested in the 2018/19 and 2019/2020 seasons and provided by local truffle hunters.

Before analysis, each sample's odour perception and quality were recorded to identify possible quality markers. Samples of *T. aestivum* with aroma profiles dominated by 3-octanone (sometimes also in combination with 1-octen-3-ol) have a characteristic rotten odour. The presence of dimethyl sulphide (> 60 %) and 1-propene-1-methylthio-, (E)- ($>$ 25 %) also contribute to its unpalatable bouquet. In all of the samples, the level of 2-butanone was low (< 15 %), which means that 2- butanone could act as a quality marker for *T. aestivum*. Despite the variability in their aromatic profile, truffles of a given species share common VOCs that can act as species-specific fingerprints. The statistical model developed in this study revealed that all of the investigated truffle species produced an overall correct classification rate of 97 %. This study also looked at the differences in VOC profiles of truffles spread over a geographical area. A detailed study of *T. aestivum* from four geographically related areas in Slovenia achieved only a 50.5 % correct classification rate. It revealed that the variability in VOCs could be attributable to many factors, including genotypic variability, maturation, microbial community, and geographical origin.

As part of this work, my responsibilities included preparing fresh truffle samples for analysis. I also developed and optimized the HS-SPME GC-MS method for characterising the truffle VOC profiles. I was also responsible for experimental design, data analysis, and writing and preparing the manuscript for publication.

This work was presented as an oral presentation at the 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019), November 5-8, 2019, in Prague, Czech Republic, and at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, April 1-3, 2019 in Portorož, Slovenia, and at the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, 15th and 16th May 2019 in Planica, Slovenia.

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Species and geographic variability in truffle aromas

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ABSTRACT

The gastronomic relevance and price of truffles are related mainly to its unique aroma. In this study, we explore the impact that different volatile compounds have on the aroma quality of fresh truffles using gas chromatography-mass spectrometry (GC-MS). Four hundred sixty fresh ascocarps of nine truffle species (*Tuber aestivum, Tuber magnatum, Tuber melanosporum, Tuber mesentericum, Tuber brumale, Tuber excavatum, Tuber rufum, Tuber indicum and Tuber macrosporum*) harvested in 2018/19 and 2019/2020 from 11 different countries (Slovenia, Croatia, Bosnia in Herzegovina, Macedonia, Italy, Spain, France, United Kingdom, Germany, Poland and China) were collected. Our investigation included the classification of species based on their aroma profile, a study of the differences in the volatile organic composition of truffle species over a geographical area, and, in more detail, a study of *T. aestivum* from four natural truffle growing sites in Slovenia. Our models can distinguish between groups, with small classification error. These models could form the basis of a predictive framework to detect fraud concerning truffle products and to determine the influence of different growing parameters on the aroma profile of truffles.

1. Introduction

Truffles are the fruiting bodies (ascocarps) of fungi belonging to the genus *Tuber* that are produced in the soil or leaf litter in a symbiotic mycorrhizal association with plants. Bacteria represent the third component of these associations and produce volatile organic compounds (VOCs) that contribute significantly to truffle aroma together with other *Tuber*-associated microbes (yeast and fungi). It is thought that uses these volatile compounds enable the truffle to communicate with plants (symbiotic hosts), animals (vectors) and other microorganisms (decomposers). Besides their biological role, VOCs create the unique truffle aroma sensed by humans, which in turn determines their economic value (Vita et al., 2015).

Molecular based data suggests the existence of 180 truffle species in various regions of the world (Bonito et al., 2013). At present, approximately 30 species of truffle are commercially traded, and because of their rarity and unique aroma, are one the most expensive foods in the world, and prices range from a few hundred Euros per kg to thousands of Euros per kg (Vahdatzadeh and Splivallo, 2018). The price of truffles is highly dependent on supply and demand, and this changes daily. For example, an intact, perfectly shaped, healthy, fragrant, firm and fresh (not older than three days) specimen of white truffle (*T. magnatum*), can fetch 3,500 Euros per kg, while a second- or third-class specimen can fetch anything from one-third to two-thirds of that price. During poor harvests, prices can be even higher (Piltaver and Ratoša, 2006; Reyna and Garcia-Barreda, 2014). *Tuber magnatum* has limited geographical distribution and grows in spontaneous colonies in certain regions in Italy (Tuscany, Piedmont, Marche, Umbria), Croatia, Slovenia, Hungary, as well as in several Balkan regions (Marjanović et al., 2010; Mello et al., 2006; Vita et al., 2015). The price of *T. melanosporum* is two-thirds that of *T. magnatum*, and *T. brumale* is one-fifth to one-third the price (Piltaver and Ratoša, 2006). *Tuber aestivum,* also called the summer truffle, is highly prized and broadly distributed across Europe. It also the most common truffle and matures over a long period of the year (Molinier et al., 2015), which makes it less expensive than either *T. melanosporum* or *T. magnatum*. Summer (*T. aestivum*) reach a tenth of the price of *T. magnatum*, and even slightly lower than *T. mesentericum*, which are sometimes not consumed in France due to their sharp odour (Piltaver and Ratoša, 2006).

The VOCs responsible for the distinctive scent of truffles are a blend of alcohols, ketones, aldehydes, aromatic and sulphur compounds, albeit only a small fraction of all the VOCs emitted by truffles ("aroma-active compounds") are responsible for what humans perceive as truffle aroma. In terms of composition, specific odorants are common to many truffle species, while other odour compounds are species-specific or limited to only a few species. For example, 2-methylbutanal, 3-

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methylbutanal, 2- methylbutan-1-ol, 3-methylbutanol and oct-1-en-3-ol are common to most truffle species, while 2,4-dithiapentane is exclusive to the fruiting bodies of *T. magnatum* (Vahdatzadeh and Splivallo, 2018). The concentration of a single aroma compound can also vary significantly in truffles of the same species and even in truffles collected from the same orchard (Molinier et al., 2015). These differences arise because of biotic factors (e.g. fungi, yeasts, bacteria, mesofauna, and plant host) and abiotic factors (e.g. rainfall and temperature, mycelial connectivity, soil properties, and microclimatology) that often co-vary in truffle orchards (Büntgen et al., 2017; Mello et al., 2006). Aroma variability in truffles has been attributed to maturation stage (*T. borchii*) and to environmental (*T. magnatum*) and genetic factors (Splivallo et al., 2012; Vahdatzadeh and Splivallo, 2018). Scientists have also documented a significant variability in the concentration of four (2-butanone and 2-butanol), and eight carbon-containing volatiles (1-octen-3-one, 1-octen-3-ol and trans-2-octenal) in *T. aestivum* ascocarps collected only a few centimetres apart in the same truffle orchards (Molinier et al., 2015; Vahdatzadeh and Splivallo, 2018). Other factors such as hydration and storage conditions can affect the aroma profile of truffles (Boyce, Mary C., James White, Danielle Hudson, Nick Malajczuk, 2018; Pennazza et al., 2013; Torregiani et al., 2017). Sample preparation may also influence the aroma profile, for instance, freezing can lead to the *de novo* formation of volatiles by disrupting tissues and allowing enzymes to work on specific substrates (Molinier et al., 2015). Culleré et al. (2013a,b) report that frozen samples are more abundant in compounds such as diacetyl, 1-octen-3-one, and 1-octen-3-ol. Splivallo et al. (2012) found the effect of storing truffles at 4 °C for up to 6 days was negligible, whereas the freezing process significantly influenced the aroma profile. Also, Culleré et al. (2013a,b) reported that after only 24 h of freezing, there was a significant reduction in their "characteristic" aroma. Washing truffles will also affect the microbial population within the truffle-fruiting bodies, which has a role in the synthesis of truffle odorants (Molinier et al., 2015). There is even indirect evidence that bacteria might be exclusively responsible for the emission of 2,4-dithiapentane in *T. magnatum* (Vahdatzadeh et al., 2015).

Truffle hunters identify truffles of different species not just by their odour, but also by the size and shape of their spores and asci, spore wall ornamentation, structure of the peridium and gleba, but despite this, their identification remains unreliable (Mello et al., 2006). Misidentification is especially problematic for species with similar morphological features, and therefore less valuable truffles (or even false truffles) can be (un)intentionally traded as high-quality species. Frauds are also common, for instance, truffles from the Far East, with a value of around € 15 per kilogram, are sold as the prized European black truffle (*T. melanosporum*) for more than a thousand Euros per kg (Cullere et al., 2013a). *Tuber indicum*, *T. himalayense* and *T. pseudohimalayense* from China are practically odourless and tasteless but by adding synthetic truffle aroma, which can be purchased in the form of an oil concentrate, gives them a distinct truffle aroma. These fakes, however, have no real taste. Counterfeiters also sell other underground mushrooms as high-quality truffles, like "desert truffle" (*Terfezia sp.*) and "whitish tuber" (*T. oligospermum*), which grows on the shores of the Mediterranean and comes to the black market mainly from Morocco, and is commonly sold as *T. borchii* or even *T. magnatum* (Piltaver and Ratoša, 2006).

False identification of truffle species means that it is not only economic interests which are at stake, but there are also possible health issues for consumers since not all truffles are fit for consumption. Examples include *T. fulgens*, *T. maculatum*, *T. rufum* and *T. excavatum*, and their intense pungent aroma that can provoke undesired reactions such as nausea or malaise, which are symptoms well known among truffle hunters (Piltaver and Ratoša, 2006). The freshness of the truffle is also of concern as only fresh truffles have the best organoleptic value, and like many other vegetable commodities, they are highly perishable. Their perishability is mainly due to bacteria and mould growth and dehydration, which results in the rapid loss of organoleptic properties such as texture, aroma, and taste (Culleré et al., 2013b). The ageing of truffles implies that several biochemical reactions are taking place, which changes their flavour with time (Caboni et al., 2020; Falasconi et al., 2005).

In Slovenia, there is a strong tradition of hunting white and black truffles in Slovenian Istria, and all the necessary conditions exist for the development of a truffle culture. This economic interest in truffles means that it is essential to develop methods that allow objective identification of different species to avoid the fraud associated with marketed products or even to determine the influence of different growing parameters on the aroma profile. This study provides an extended exploration of the impact of different VOCs on the aroma quality of fresh *T. aestivum, T. magnatum, T. melanosporum, T. brumale, T. mesentericum, T. excavatum, T. macrosporum, and T. rufum,* which are common in Europe and *T. indicum* obtained from China. Since not all the investigated truffle species provide pleasing odour and some of them are also considered inedible, we investigated the classification of species based on aroma profiles. This study also looked at the differences in VOC profiles of truffles spread over a geographical area; in this case, *T. aestivum* from the four main natural truffle growing regions in Slovenia. The overall study objectives were to (i) characterise the VOC profiles of fresh truffle species using headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS); (ii) identify potential markers of truffle quality; (iii) build a statistical model for classifying species based on VOC profiles; (iv) build a statistical model for classifying geographical origin based on VOC profiles, and (v) characterise the aroma variability between the main truffle growing sites of *T. aestivum* in Slovenia. Overall, this work represents a contribution to our understanding of the aroma profile of fresh truffles, especially of those hunted in Slovenia.

2. Materials and methods

2.1. Samples

Fresh ascocarps (n = 460) of *T. aestivum (n = 240), T. magnatum (n = 49), T. melanosporum (n = 36), T. mesentericum (n = 22), T. brumale (n = 69), T. excavatum (n = 23), T. rufum (n = 8), T. indicum (n = 3) and T. macrosporum (n = 10)* were collected in 2018/19 and 2019/2020 in 11 different countries: Slovenia (n = 242), Croatia (n = 20), Bosnia in Herzegovina (n = 6), Northern Macedonia (n = 87), Italy (n = 15), Spain (n = 3), France (n = 25), United Kingdom (n = 4), Germany (n = 15), Poland (n = 3) and China (n = 3). The truffles were collected from the wild with the help of local truffle hunters and from the market (n = 37). Regardless the country of origin, truffle samples were collected with a specialised truffle hunting dogs and in accordance with national regulatives. Truffles were transported to the laboratory as soon as possible but no later than in five days. Each sample was placed in either a glass jar or a plastic ziplock bag wrapped in a fresh piece of unperfumed paper towel to absorb any moisture, which was changed daily. The containers were placed and transported in a cooling box (4–8 °C). Slovenian samples were collected from four main collecting sites: Sežana (n = 15), Bloke (n = 20), Žlebič (n = 57) and Spodnje Blato (n = 19). The samples were transported separately in paper bags to the laboratory where the ascocarps were brushed with a soft wet brush and rinsed with tap water. The truffles were then identified based on the morphology of spores and asci, spore wall ornamentation, and the structure of the peridium and gleba. Each species was wrapped separately in paper and stored in glass jars at 4 °C. All samples were analysed within 24–48 h.

2.2. Sample preparation

Immediately before analysis, the peridium was removed, and 0.3 g of the truffle gleba was cut into thin slices using a sharp ceramic knife and placed in a 10 mL amber glass SPME vial. Deionised water was added (1 ml) and the vial immediately capped with a silicone/PTFE

Table 1
Analysed truffle samples per species and country of origin. The number within the brackets indicates the number of excluded samples from the original sample set.

Row Labels	Slovenia	North Macedonia	France	Italy	Croatia	Bosnia in Herzegovina	United Kingdom	China	Spain	Unknown	Grand Total
TUBAES	139 (15)	13 (15)		4	2	2	4			21 (7)	**185**
TUBBRU	31 (3)	19 (2)	2 (2)		5 (3)	(1)				1	**58**
TUBMAG	29 (4)	2		3	(5)	3				3	**40**
TUBMEL	5		17 (4)	7					3		**32**
TUBMES	11	8		1						2	**22**
TUBEXC	3 (1)	13 (3)			1					2	**19**
TUBMAC		7			3						**10**
TUBRUF	1	4 (1)			1					1	**7**
TUBIND								3			**3**
Grand Total	**219**	**66**	**19**	**15**	**12**	**5**	**4**	**3**	**3**	**30**	**376**

The number within the brackets indicates the number of excluded samples from the original sample set (See 2.1).

septa. The fibres used for VOCs extraction were divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibres (50/30 μm thickness) purchased from Sigma-Aldrich (Supelco, Bellefonte, USA) and conditioned at 270 °C for 4 h when new. Before each extraction, the fibre was conditioned at 250 °C for 5 min, and again for a further 20 min after analysis. Sample equilibration time was 10 min at 30 °C, and the extraction time was 10 min at 30 °C. Volatile compounds were desorbed from the fibre in the GC inlet at 250 °C for 1 min.

2.3. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analyses were performed using a 7890B GC & 5977A Series GC/MSD (Agilent Technologies, USA). The separation was achieved on a VF-WAXms capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W, USA). The temperature program was as follows: 30 °C (held 1 min) to 40 °C at 1 °C min^{-1} (held 2 min), from 40 °C to 60 °C at 7 °C min^{-1} and finally from 60 °C to 200 °C at 20 °C min^{-1} (held 5 min). The carrier gas was helium maintained under constant flow at 1.5 mL min^{-1}. The injector was fitted with a straight Ultra Inert SPME liner (Sigma-Aldrich/Supelco, USA) and operated in the split mode (1:5). The temperatures for the injection port, transfer line, ion source and quadrupole were 250 °C, 250 °C, 230 °C and 150 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV within a mass range m/z 35–300 at scan rate 1.9 scans s^{-1} (Full scan mode). Data were acquired using ChemStation software (Agilent, USA). Identification was performed using spectral similarity with the NIST14 library (Agilent, USA).

2.4. Statistical experimental design and data analysis

Multivariate linear discriminant analysis (LDA) was used to classify samples based on the aroma profiles. The analysis was performed using the XLSTAT software package (Addinsoft, New York, USA).

3. Results and discussion

The HS-SPME/GC-MS analysis was performed on 460 fresh truffles representing *T. aestivum*, *T. magnatum*, *T. melanosporum*, *T. mesentericum*, *T. brumale*, *T. excavatum*, *T. rufum*, *T. indicum* and *T. macrosporum*. Although SPME adsorbs VOCs to varying degrees, a comparison across samples is permissible because the influence of differential absorption is equivalent for all the samples as long as the analysis conditions are kept constant. Peak areas were summed and expressed as a percentage of the total VOCs for each sample. Compounds present at < 1% were classified in the group named "Other". In this way, the number of variables (VOCs) was reduced from 458 to 81.

Before analysis, the odour perception and quality of each sample were also recorded to identify possible quality markers. Initial results revealed that several species to be wrongly identified, i.e., their odour and aromatic profile more closely matched that of other species. Individual samples were also identified as being of poor quality, which was also reflected in their odour and aromatic profiles. Samples of *T. aestivum* with aroma profiles dominated by 3-octanone (sometimes also in combination with 1-octen-3-ol) have a characteristic rotten odour. The presence of dimethyl sulfide (> 60%) and 1-propene-1-methylthio, E− (> 25%) also contribute to its unpalatable bouquet. In all of the samples, the level of 2-butanone was low (< 15%), which means that 2-butanone could act as a quality marker for *T. aestivum*. 2-Butanone is also present in low levels in immature truffles (Molinier et al., 2015), but in this study, samples with a low percentage of 2-butanone are at a different stage of maturity. In total, 84 samples were excluded from further consideration, including three samples from Poland, which in addition to having a repulsive odour, contained a high proportion of toluene. It also includes all the German samples (n = 15), which appear to have been frozen given their soft texture, atypical odour (sweet baked cookies), and the presence of large amounts of 3-octanone (20–50%). Since this study required fresh samples, these samples were also excluded from further research, although the presence 3-octanone may be a specific feature based on geographical location. Culleré et al. (2013a) found that frozen samples are more abundant in compounds such as 1-octen-3-ol and 1-octen 3-one. Thirteen samples that have significantly different aroma profiles from other *T. aestivum* samples in terms of their high levels of 1-octen-3-ol were excluded from the aroma profile of *T. aestivum* but otherwise included in further data treatment since eight-carbon-containing volatiles are dependent on clones/genets (Molinier et al., 2015). In the other 30 samples, there was also no reliable information on the country of origin since they were purchased from the market.

The removal of these samples meant that 376 samples *T. aestivum* (TUBAES), *T. brumale* (TUBBRU), *T. magnatum* (TUBMAG), Tuber melanosporum (TUBMAC), *T. mesentericum* (TUBMES), *T. excavatum* (TUBEXC), *T. macrosporum* (TUBMAC), *T. rufum* (TUBRUF) and *T. indicum* (TUBIND) from 9 different countries (Slovenia, North Macedonia, France, Italy, Croatia, Bosnia and Herzegovina, United Kingdom, China and Spain) were included in the data analysis (Table 1). Chromatograms for each species are shown in Fig. 1.

3.1. Aroma profile

Table 2 (shown graphically in Fig. 2) presents the average area per cent of aroma compounds of each truffle species. Thirteen samples that have significantly different aroma profiles from *T. aestivum* samples in terms of their high levels of 1-octen-3-ol and were excluded from its average aroma profile and presented separately as the group TUBAES Other. Several compounds appear to be species-specific. For instance, 2,4-dithiapentane was detected only in *T. magnatum*, and 2,4-diathiapentane (bis(methylthio) methane) was the major contributor to the aroma, a finding that agrees with previous studies (Splivallo et al., 2011; Talou et al., 2007). Dimethyl disulphide was mainly present in the aroma of *T. magnatum*, while butanenitrile, 2-methyl-, pentane, 2-

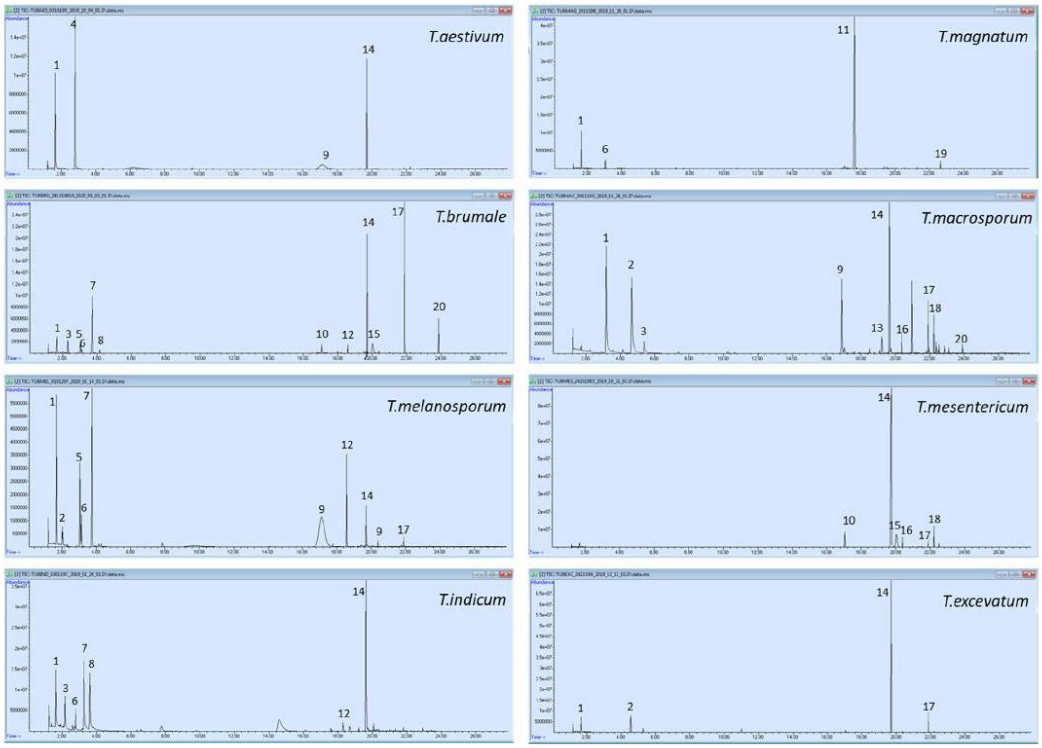

Fig. 1. Gas chromatographic profile of the headspace components sampled by solid-phase microextraction from the fresh truffle species: *T.aestivum, T. magnatum, T. brumale, T. macrosporum, T. melanosporum, T. mesentericum, T.indicum* and *T. excevatum*. Peak identification: 1 = Dimethyl sulfide, 2 = Acetone, 3 = Formic acid, 1-methylethyl ester, 4 = 2-Butanone, 5 = Butanal, 2-methyl, 6 = Butanal, 3-methyl, 7 = Formic acid, 1-methylpropyl ester, 8 = Formic acid, 2-methyl propyl ester, 9 = 1-Butanol, 2-methyl, 10 = 3-Octanone, 11 = 2,4-dithiapentane, 12 = Anisole, 13 = 3-Octanol, 14 = Benzene, 1-methoxy-3-methyl-, 15 = 1-Octen-3-ol, 16 = 2,3-Dimethylanisole, 17 = Benzene, 1,4-dimethoxy-, 18 = Benzene, 1,4-dimethoxy-2-methyl-, 19 = 2-Methylthioacetic acid, 20 = 1,2,4-Trimethoxybenzene.

nitro- and butane, 2-bromo-2-methyl- were unique to *T. rufum,* which has a distinctive ethereal, fruity odour (Talou et al., 2007). The source and its role, however, in truffles remain unknown. 2-Butanone and 2-butanol were detected mainly in *T. aestivum* samples, and although 2-butanone is linked to truffle maturation (Molinier et al., 2015), there was no correlation with maturity. It does appear, however, to be a marker of quality since all of the samples containing low amounts of 2-butanone had an unpleasant rotten odour, except for samples with a high amount of 1-octen-3-ol (also up to 80%), where the aroma was acceptable. The presence of 1-octen-3-ol in place of 2-butanone could be explained by gene/clone testing. Molinier et al. (2015) reported that the concentrations of C_4–VOCs: 2-butanone and 2-butanol or of C_8–VOCs: 1-octen-3-ol, 1-octen-3-one and 3-octanol correlate with and are dependent on clones/genets and not on genetic clusters. In an earlier study of *T. aestivum,* Splivallo et al. (2012) found a strong link between the concentration of eight-carbon-containing volatiles and the genotype. 1-Octen-3-ol, arising from the oxidation of linoleic acid, has been identified in mature fruit bodies. Zeppa et al. (2004) found that 1-octen-3-ol was a component of a mixture of alcohols responsible for truffle aroma and an extremely potent olfactory attractant for many insect species. Propane, 1-(methylthio)- is another compound present only in *T. macrosporum* has an alliaceous, creamy green, leek odour. Two compounds typical for *T. macrosporum* are 1-propene, 1-(methylthio)- (E)- and 1-propene, 1-(methylthio)- (Z)-, which were also present in *T. excavatum*. *Tuber excavatum* and *T. macrosporum* possess similar aroma compounds just in different ratios. Similarly to that reported by Talou et al. (2007), our study show that benzene-1-methoxy-3-methyl- (3-Methylanisole) was typical for *T. mesentericum, T. brumale, T. indicum* and *T. excavatum* and has a strong unpleasant spicy odour reminiscent of car paint. In the literature, it is described as having musky-mouldy odour notes, however the odour contribution of anisole cannot be excluded (Talou et al., 2007). We detected anisole mostly in *T. brumale* and *T. melanosporum*. Fresh *T.melanosporum* had the highest amount of 1-butanol, 2-methyl (21.9%), formic acid, 1-methylpropyl ester (15.7%), dimethyl sulphide (11.7%), butanal, 2-methyl (8.4%) and butanal, 3-methyl- (6.5%); however, this may be a result of extended storage (up to 7 days) at 4 °C during transport. Boyce et al. (2018) found that after seven days of storage, the VOC profile did not change significantly except for dimethyl sulphide, which was no longer detectable, the detection of 3-methylbutanol, and the formation of esters. 1-Butanol, 2-methyl and butanal, 2-methyl- are typical for *T. melanosporum* and are responsible for its sulfurous, animal odour (Talou et al., 2007). In the 80s, a mixture of 2-methylbutanal and dimethyl sulphide was patented to mimic the smell of *T. melanosporum* (Vahdatzadeh et al., 2015). The most abundant aroma compounds in all 9-truffle species are dimethyl sulphide, benzene,1-methoxy-3-methyl-, 1-octen-3-ol, 3-octanone and butanal, 3-methyl-. According to the literature 3-methyl-1-butanol, 1-octen-3-ol, 3-methylbutanal, 3-octanone, hexanal,

Table 2
Volatile organic compounds identified in *T. aestivum* (TUBAES), *T. brumale* (TUBBRU), *T. excavatum* (TUBEXC), *T. indicum* (TUBIND), *T. macrosporum* (TUBMAC), *T. magnatum* (TUBMAG), *T. melanosporum* (TUBMEL), *T. mesentericum* (TUBMES) and *T. rufum* (TUBRUF) and corresponding average area per cent.

	TUBAES	TUBAES other	TUBBRU	TUBEXC	TUBIND	TUBMAC	TUBMAG	TUBMEL	TUBMES	TUBRUF
1-methoxy-3-methylbenzene	3.7	5.7	28.3	37.9	44.5	10.6	0.3	4.2	69.5	17.4
bis(methylsulfanyl)methane	nd	nd	nd	nd	nd	nd	67.8	nd	nd	nd
butan-2-one	53.3	10.4	0.6	0.4	0.3	0.2	nd	1.3	0.3	0.8
(E)-1-methylsulfanylprop-1-ene	0.2	nd	nd	29.6	nd	27.0	nd	nd	0.3	nd
2-methylbutanenitrile	nd	nd	nd	nd	nd	0.1	nd	nd	nd	21.9
1-methylsulfanylpropane	0.1	nd	nd	0.4	nd	22.1	nd	nd	nd	nd
2-methylbutan-1-ol	4.7	1.7	0.4	nd	nd	0.6	1.2	21.9	nd	3.2
methylsulfanylmethane	18.9	3.8	4.5	4.9	4.3	1.3	14.6	11.7	1.2	8.2
butan-2-yl formate	nd	nd	7.0	nd	11.4	0.2	nd	15.7	nd	2.2
oct-1-en-3-ol	0.9	47.4	11.2	2.4	0.2	4.7	nd	2.0	9.6	3.1
3-methylbutan-1-ol	nd	nd	nd	1.8	6.5	nd	0.2	nd	nd	5.4
octan-3-one	2.6	16.1	8.8	2.0	0.2	7.3	0.3	4.2	4.2	5.3
1,4-dimethoxybenzene	0.1	nd	12.1	2.8	nd	2.7	0.1	0.1	0.9	0.7
butan-2-ol	6.3	1.9	0.1	nd	nd	nd	nd	nd	nd	0.3
2-methylpropyl formate	nd	nd	0.9	nd	nd	nd	nd	1.0	nd	1.4
2-methylbutanal	0.1	nd	0.8	nd	nd	nd	0.1	8.4	nd	0.4
undec-1-ene	0.1	nd	2.9	1.8	nd	0.4	1.8	nd	nd	nd
(Z)-1-methylsulfanylprop-1-ene	nd	nd	nd	6.7	nd	8.0	nd	nd	0.2	nd
1,4-dimethoxy-2-methylbenzene	nd	nd	1.4	0.1	1.2	1.6	nd	0.1	7.8	0.1
anisole	nd	nd	3.4	0.7	1.4	0.8	nd	7.1	nd	0.2
3-methylbutanal	1.2	1.6	0.6	0.8	2.8	nd	1.5	6.5	nd	1.6
2-nitropentane	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.9
2-bromo-2-methylbutane	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.3
(methyldisulfanyl)methane	nd	nd	0.1	0.1	nd	0.2	2.8	0.2	nd	nd
Other	7.7	11.5	17.0	7.6	27.2	12.3	9.4	15.6	6.0	21.7

'nd' = not detected.

acetaldehyde occur in more than 50% of all truffle species. Axenic cultures of truffle mycelium, as well as numerous fungal and bacterial phyla, are known to produce these compounds (Vahdatzadeh et al., 2015).

3.2. Species separation by aromatic profile

Despite the variability in their aromatic profile, truffles of a given species share common VOCs that can act as a species-specific fingerprint. These VOCs also contribute to the specific aroma of a particular species (Splivallo et al., 2011). For this reason, a statistical model was constructed based on the aroma profiles of different truffle species. The model was built using multivariate linear discriminant analysis (LDA). Linear discriminant analysis maximises the ratio between-class variance and minimises the ratio of within-class variance (Drivelos and Georgiou, 2012). The statistical method was used to check the two- dimensional charts to test if the groups to which the observations belong are distinct and to reveal the properties of these

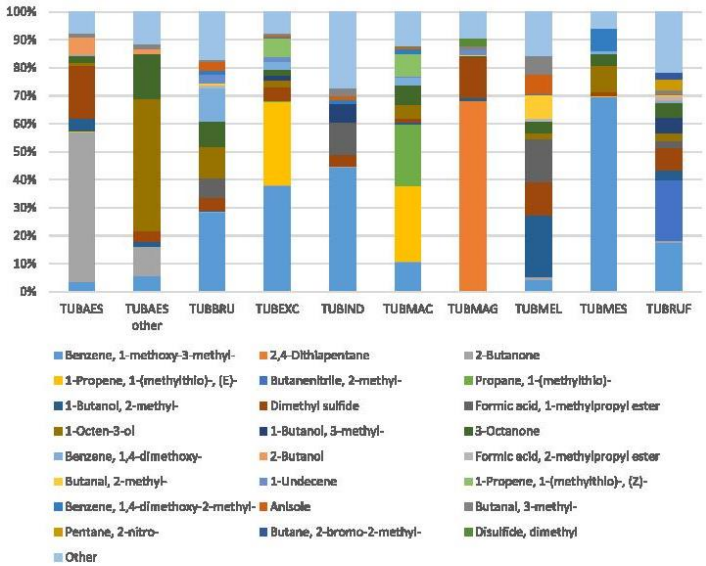

Fig. 2. Relative percentages of volatile compounds in truffle species identified by HS-SPME/GC-MS analysis (mean values).

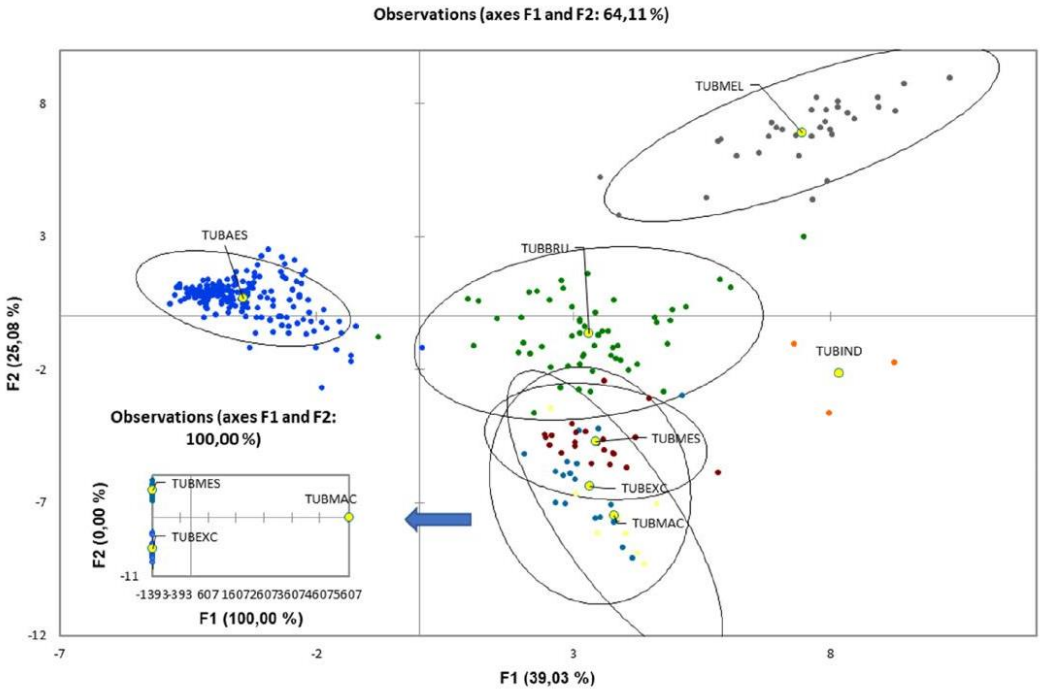

Fig. 3. Scatter plot of linear discriminant analysis (LDA) of truffle samples different species (*T. aestivum*– TUBAES, *T. brumale* – TUBBRU, *T. melanosporum* – TUBMEL, *T. indicum* – TUBIND, *T. mesentericum* – TUBMES, *T. excavatum* – TUBEXC and *T. macrosporum* – TUBMAC).

Table 3
Confusion matrix for the validation sample of LDA of different truffle species.

Confusion matrix for the validation sample:

from \ to	TUBAES	TUBBRU	TUBEXC	TUBIND	TUBMAC	TUBMEL	TUBMES	Total	correct (%)
TUBAES	57	0	0	0	0	0	1	58	98.3
TUBBRU	0	14	0	0	0	0	1	15	93.3
TUBEXC	0	0	5	0	0	0	0	5	100
TUBIND	0	0	0	1	0	0	0	1	100
TUBMAC	0	0	0	0	1	0	0	1	100
TUBMEL	0	1	0	0	0	10	0	11	90.9
TUBMES	0	0	0	0	0	0	7	7	100
Total	**57**	**15**	**5**	**1**	**1**	**10**	**9**	**98**	**96.9**

groups using explanatory variables. A confusion matrix was then used to evaluate the classification accuracy. The method can also be used to create a predictive framework. The analysis was performed on 81 different aroma compounds belonging to the origin classes (TUBAES, TUBBRU, TUBMAG, TUBMEL, TUBMES, TUBMAC, TUBEXC, TUBIND and TUBRUF). Fig. 3 shows a two-dimensional chart with centroids and confidence circles at a significance level of 5%. Since *T. magnatum* and *T. rufum* are well separated and plot far from the other groups (not reported), a second LDA was performed, excluding TUBMAG and TUBRUF (Fig. 3). The model was built using a calibration set (70% of data) and validated using a random validation set (30% of data) to avoid over-optimistic and misleading results. Several misclassifications were identified in the data, which resulted in a 97% correct classification (Table 3). In Fig. 3, an overlap is observed between *T. mesentericum*, *T. excavatum* and *T. macrosporum,* which is not surprising since these groups possess similar aroma compounds, for example, benzene,1-methoxy-3-methyl present in high amounts in *T. mesentericum* and *T. excavatum*, and 1-propene, 1-(methylthio)- (E)- and 1-propene, 1-(methylthio)- (Z)-, that are present in *T. macrosporum* and *T. excavatum* in varying amounts. However, the LDA analysis of only these groups gave a 100% correct classification rate (Fig. 3). Table 4 lists the variables (aroma compounds) that contribute to the separation of specific species together with their odour description ("Food safety and quality: details," n.d., "Perflavory Search," n.d.). The obtained results are consistent with the key odorous notes of *T. magnatum, T. rufum, T. aestivum, T. indicum, T. mesentericum, T. excevatum* and *T. macrosporum* as described by Talou et al. (2007).

3.3. Geographical origin separation by aromatic profile

Similar to building a model for the classification of different truffle species, an additional model was built to identify, within species,

Table 4
Variables (aroma compounds) that contribute to the separation of species.

Aroma compound	Odour description[a]	TUBMAG	TUBRUF	TUBAES	TUBMEL	TUBIND	TUBMES	TUBEXC	TUBMAC
2,4-Dithiapentane	alliaceous sulfurous fresh onion garlic vegetable cabbage spicy mustard horseradish	X							
Butanenitrile, 2-methyl-	–		X						
Pentane, 2-nitro-	–		X						
Acetone	solvent ethereal apple pear		X		X				
2-Butanone	acetone ethereal fruity camphoraceous			X					
Dimethyl sulfide	sulfurous onion sweet corn vegetable cabbage tomato green radish			X				X	
Butanal, 2-methyl-	musty cocoa coffee nutty				X				
1-Butanol, 2-methyl-	roasted winey onion fruity fusel alcoholic whiskey				X				
Formic acid, 1-methylpropyl ester	–				X	X			
Anisole	phenolic gasoline ethereal anise aromatic odour				X				
1-hexanol	ethereal fusel oily fruity alcoholic sweet green				X				
Propanal, 2-methyl	fresh aldehydic floral pungent				X				
Butanal, 3-methyl-	ethereal aldehydic chocolate peach fatty				X				
Butanoic acid, 2-methyl-	fruity sweet				X				
Formic acid, 2-methylpropyl ester	–					X			
Benzene, 1-methoxy-3-methyl-	narcissus						X		
Benzene, 1,4-dimethoxy-2-methyl-	–						X		
1-Propene, 1-(methylthio)-, (E)-	acrid strong garlic-like odour							X	
1-Propene, 1-(methylthio)-, (Z)-	acrid strong garlic-like odour							X	
Benzene, 1,4-dimethoxy-	sweet green hay newly mown hay fennel							X	
Propane, 1-(methylthio)-	alliaceous creamy green leek								X
3-Octanone	fresh herbal lavender sweet mushroom								X
3-Octanol	earthy mushroom herbal melon citrus woody spicy minty								X
	Key odorous notes (Talou et al., 2007)	alliaceous	ethereous, fruity	fruity, green	sulfurous, animal	sulfurous, earthy	musky, mouldy	fungus, alliaceous	sulfurous, alliaceous

[a] Data from "The Good Scents Company Information System".

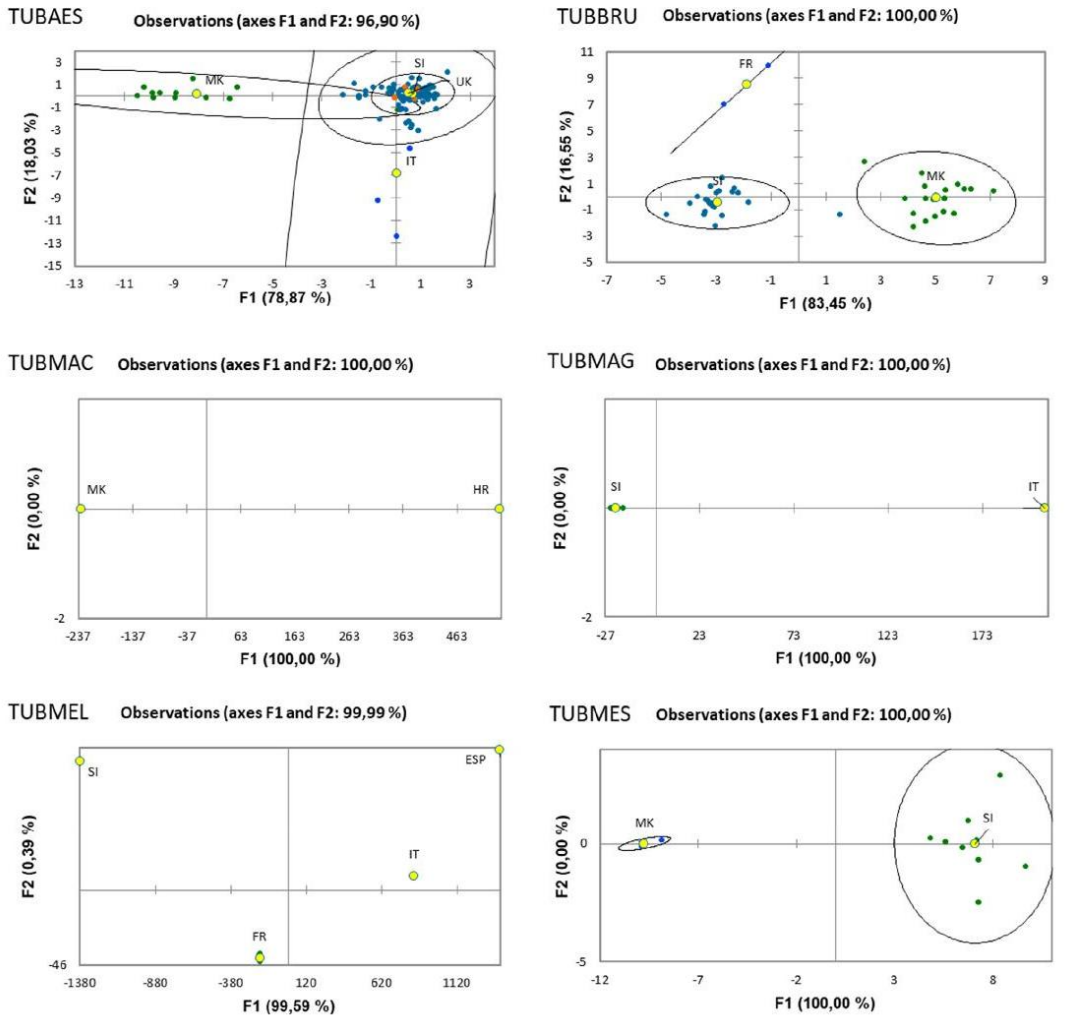

Fig. 4. Scatter plots of linear discriminant analysis (LDA) of different species of truffle (*T. aestivum* – TUBAES, *T. brumale* – TUBBRU, *T. melanosporum* – TUBMEL, *T. indicum* – TUBIND, *T. mesentericum* – TUBMES, *T. excavatum* – TUBEXC and *T. macrosporum* – TUBMAC) regarding their geographical origin (France – FR, Croatia – HR, North Macedonia – MK, Slovenia – SI, Spain – ESP, Italy – IT, Bosnia and Herzegovina – BIH, United Kingdom – UK).

possible markers of geographical origin. Namely a model for *T. aestivum* (collected from SI, BIH, HR, IT, MK and UK), *T. brumale* (collected from SI, HR, MK, FR), *T. macrosporum* (collected from HR and MK), *T. magnatum* (collected from SI, BIH, IT and MK), *T. melanosporum* (collected from SI, ESP, FR and IT) and *T. mesentericum* (collected from SI and MK). Despite the low quantity of samples from certain countries, a good separation of truffle species from different countries was obtained with a 93.7% (TUBMAG), 87.4% (TUBAES), 80.0% (TUBMAC), 68.4% (TUBMES), 63.5% (TUBBRU) and 37.5% (TUBMEL) correct classification rate (Fig. 4). However, truffles from certain countries, namely *T. brumale* from Croatia, *T. aestivum* from Croatia and Bosnia and Herzegovina, and *T. magnatum*: Bosnia and Herzegovina and North Macedonia should be excluded since they plot far from the other groups. Table 5 lists the most important variables that separate truffle aromas from different countries. The results of geographical origin are; however, preliminary, and in order to use such a mode, as an accurate predictive framework, will require a more extensive sample database. Also, since many parameters can influence the aroma profile of truffles from different geographical regions, observed differences may be better explained by combining VOC profiling with other methods such as multi-elemental, stable-isotope, genome and microbiome using appropriate chemometric tools.

3.4. Slovenian truffles

The tradition of truffles collecting in Slovenia is first reported in the 18th-century (Piltaver and Ratoša, 2006), and *T. magnatum* (white truffles) and *T. melanosporum* (black truffle) were well known in

Table 5
The variables (aroma compounds) that contribute to the separation of countries (Slovenia – SI, Croatia – HR, Bosnia and Herzegovina – BIH, Italy – IT, Northern Macedonia – MK, United Kingdom – UK, France – FR and Spain - ESP) for specific truffle species.

Aroma compound	Odour type[a]	SI	HR	BIH	IT	MK	UK	FR	ESP
Sulfide, allyl methyl-	sulfurous		AES, BRU						
Propane, 1-(methylthio)-	alliaceous		AES, BRU						
Anisole	phenolic			AES	MAG				
Butanoic acid, 2-methyl-	fruity			AES	MEL				
Acetic acid	acidic			AES	MEL				
Ethanol	alcoholic	MAG		AES					MEL
3-Octanol	earthy		BRU		MEL	MAC			
2-Nonanone	fruity		BRU						
Benzene, 1,4-dimethoxy-	green	MES			MAG	BRU			
Benzene, 1,3-dimethoxy-	medicinal	MEL				MES			
Benzene, 1-methoxy-3-methyl-	floral	MEL			MAG	BRU, MAC, MES			
Benzene, 1,4-dimethoxy-2-methyl-	–					BRU			
3-Ethylphenol, methyl ether	–					BRU			
Formic acid, 1-methylpropyl ester	–							BRU	
Formic acid, 1-methylethyl ester	sweet-ethereal	BRU							
1-Octen-3-ol	earthy	BRU, MES			MAG	AES			
3-Octanone	herbal	BRU			MAG	AES			
2-Butanone	ethereal	AES					AES		MEL
1-Propene, 1-(methylthio)-, (E)-	sulfurous					MAC			
Butanal, 2-methyl-	chocolate				MAG			MEL	
Benzaldehyde	fruity	MAG							
1-Butanol, 2-methyl-	roasted	MAG							MEL
Dimethyl sulfide	sulfurous	AES, MAG, BRU					AES		
Butanal, 3-methyl-	aldehydic							MEL	
1-Hexanol	herbal								MEL
1-Propanol, 2-methyl-	ethereal								MEL
Acetone	solvent				MEL				
1-butanol, 2-methyl acetate	fruity				MEL				
Propanal, 2-methyl	aldehydic				AES				

[a] Data from "The Good Scents Company Information System". The abbreviations are as follows: AES - *T. aestivum*; BRU - *T. brumale*; MAG - *T. magnatum;* MEL - *T. melanosprum;* MAC - *T. macrosporum;* MES - *T. mesentericum.*

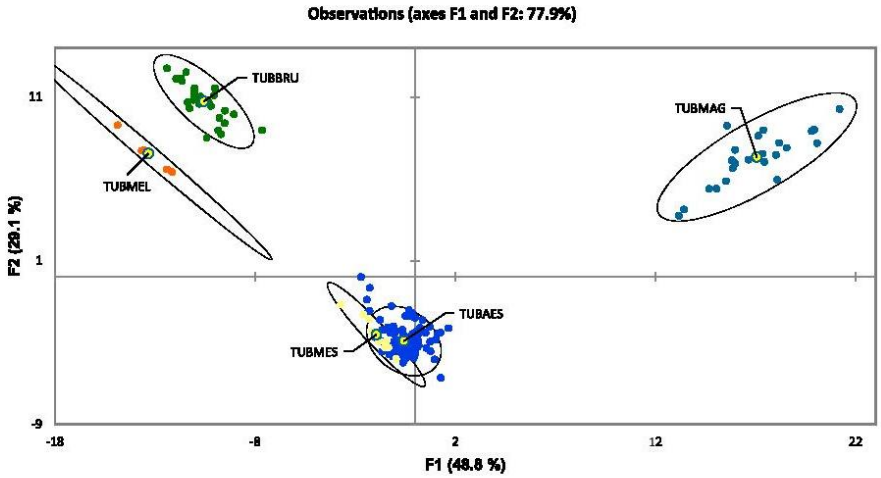

Fig. 5. Scatter plot of linear discriminant analysis (LDA) of different truffle species (*T. aestivum*– TUBAES, *T. brumale* – TUBBRU, *T. melanosporum* – TUBMEL, *T. indicum* – TUBIND, *T. mesentericum* – TUBMES) collected in Slovenia.

Slovenian Istria a region in southwest Slovenia. Piltaver and Ratoša (2006) report 16 known taxa in Slovenia, including *T. aestivum, T. brumale, T. excavatum, T. magnatum, T. melanosporum, T. mesentericum* and *T. rufum,* which are the focus of this study. The results show that except for *T. aestivum* and *T. mesentericum,* which overlap, the remaining species form distinct clusters in the LDA space (Fig. 5). The observed overlap is an indication that either not all the samples of *T. aestivum* were correctly identified or that within a species specificity exists. Indeed, specific samples of *T. aestivum* - similar to *T. mesentericum,* contain large amounts of benzene-1-methoxy-3-methyl-, which like other eight-carbon organic volatile compounds, is under genetic control (Molinier et al., 2015; Splivallo et al., 2012). However, LDA analysis of the five most common groups (*T. rufum* and *T. excavatum* were excluded due to an insufficient number of samples) produced a

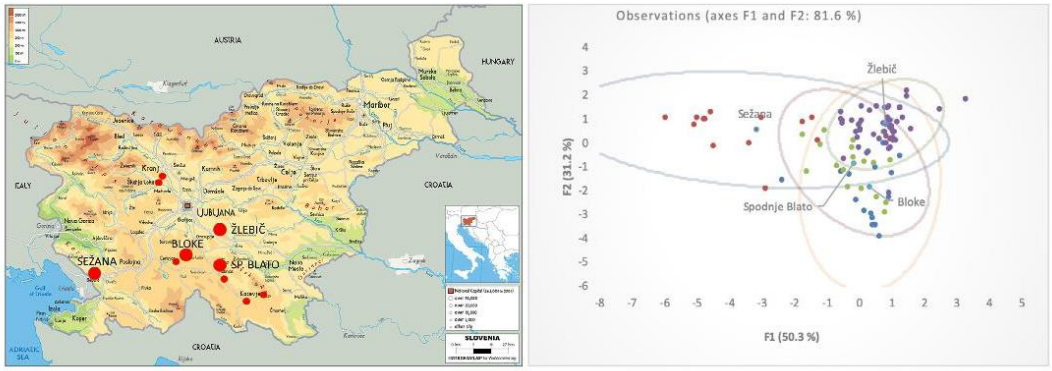

Fig. 6. Geographical location of *T. aestivum* samples collected in Slovenia (left) and scatter plot of linear discriminant analysis (LDA) discriminating *T. aestivum* according to the growing areas: Bloke, Sežana, Žlebič and Spodnje Blato (right).

95.8% correct classification rate.

It is known that the concentration of a single aroma compound can vary significantly in truffles collected from the same orchard (Splivallo et al., 2012). Therefore, a more detail study was performed of the variability of VOCs in *T. aestivum* — a species broadly distributed across Slovenia. The study involved analysing the VOC profile of 137 ascocarps of *T. aestivum* from ten closely related geographically areas (Fig. 6). Discriminant analysis achieved only a 39.4% correct classification rate, which is likely due to an insufficient number of samples from specific very closely related areas. When only the four main truffle growing areas: Sežana (n = 15), Bloke (n = 20), Žlebič (n = 57), and Spodnje Blato (n = 19), were taken into account, the classification rate increased to 50.5% (Fig. 6). The variables that contribute to this separation were for Spodnje Blato: benzene, 1,4-dimethoxy, benzene, 1,4-dimethoxy-2-methyl-, benzene, 1,4-dimethoxy-3-methyl and 3-octanone; Bloke: butanal, 2-methyl- and 1-propene, 1-methoxythio-(E)-; Sežana: 1-butanol, 2-methyl-, acetate, anisole, 1-octen-3-ol, 2-octen-1-ol, butanol, 2-methyl- and Žlebič: sec-butyl acetate, ethyl acetate, n-hexane and butanoic acid, 3-methyl.

The reason for this large variability in aroma profile according to the areas is uncertain. Splivallo et al. (2012) reported that truffles producing a different concentration of C8–VOCs clustered around distinct host trees. This clustering was not associated with maturity, but with fungal genotype, and as mentioned previously, the variation in C8–VOCs is most likely under genetic control. Other possible factors that can explain the intraspecific aromatic variability are the maturity of the ascocarp (Zeppa et al., 2004), the host plant and the microbial flora (Splivallo et al., 2011). Acetic acid, ethanol, 2-methyl-1-butanol, 2-methyl-1-propanol, acetaldehyde, 3-methylbutanal, 2-methylbutanal, propanal, hexanal, 2-methyl-2-butenal formed by truffle fermentation are possible markers of product spoilage (Costa et al., 2015; Falasconi et al., 2005). As mentioned earlier, it is expected that multiple approaches would better explain variability in aroma profile.

4. Conclusions

This study shows that SPME is a useful tool for determining VOCs in truffle aromas. It also proves that aroma profiling, in combination with odour determination, can be a useful method when evaluating aroma quality. In the case of *T. aestivum,* the VOCs are dominated by 3-octanone and is characterised by having a rotten odour. 2-Butanone does appear to be a marker of quality since all samples with low amounts ($< 15\%$) had an unpleasant rotten odour, except for samples with high amounts of 1-octen-3-ol ($\leq 80\%$) had a pleasant odour. Interestingly, 1-octen-3-ol seems to be dependent on clones/genets.

Despite the variability in aromatic profile, truffles of a given species share common volatiles that can serve as a fingerprint for different species. The statistical model developed in this study revealed that all of the investigated truffle species *T. aestivum, T. brumale, T. magnatum, T. melanosporum, T. macrosporum, T. mesentericum, T. excavatum, T. rufum* and *T. indicum* produced an overall correct classification rate of 97%, which means that the model could be used to identify samples of unknown or suspicious origin. A similar model constructed within species showed good discrimination of samples based on their geographical origin. However, to build an accurate predictive framework would require a more extensive sample database. A detailed study of *T. aestivum* from four geographically related areas in Slovenia revealed that the variability in VOCs could be attributable to many factors, including genotypic variability, maturation, microbial community as well as geographical origin. In future studies, the influence of the microbiome and genome beside and other abiotic factors will be addressed. Additionally, odour perception will be correlated with species-specific markers and markers of freshness/spoilage in order to determine quality parameters.

CRediT authorship contribution statement

Lidija Strojnik: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Writing - original draft. **Tine Grebenc:** Investigation, Writing - review & editing. **Nives Ogrinc:** Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.6 Scientific Paper: "The potential of stable isotope technique in the determination of truffle aroma formation and in the authentication of truffles and products containing truffles"

In this chapter, the paper entitled "The potential of stable isotope technique in the determination of truffle aroma formation and in the authentication of truffles and products containing truffles." by Lidija Strojnik, Tine Grebenc and Nives Ogrinc is presented. The paper is currently under review in the Journal of Fungi. The study describes the development of a procedure including $\delta^{13}C$ measurements using HS-SPME GC-C-IRMS methodology, data processing and database creation of authentic truffle VOCs.

The work involved determining $\delta^{13}C$ values of 21 flavour compounds present in 87 fresh ascocarps of six truffle species (*Tuber aestivum*, *Tuber magnatum*, *Tuber mesentericum*, *Tuber brumale*, *Tuber excavatum*, and *Tuber macrosporum*) harvested in 2018/19 in 11 countries (Slovenia, Croatia, Bosnia in Herzegovina, North Macedonia, Italy, and Poland). The $\delta^{13}C$ values of the 20 truffle aroma compounds were reported for the first time, and 13 synthetically derived VOCs were also characterized. Only one compound, benzene, 1-methoxy-3-methyl-, has been identified in all investigated truffle species with minimal variability between them. Compounds such as 1-butanol, 2-methyl, butanal, 2-methyl-, 3-octanone, and dimethyl sulphide were also present in almost all truffle species and show the most natural isotope variation between truffle species. Besides truffle species, harvest location and the quality of the sample was also found to influence $\delta^{13}C$ values. The role of microbes in aroma formation was also observed for certain VOCs and their impact on $\delta^{13}C$ values. Compounds with more negative ^{13}C values, 1-hexanol, 2-hexen-1-ol, (E)-, ethyl acetate, benzene, 1,4-dimethoxy- and 2,4-dithiapentane, also likely derived from microbial activity.

Moreover, aroma compounds might be derived from truffles and microbes, resulting in a broad authentic range found in 1-butanol, 2-methyl-, 3-octanone, butanal, 2-methyl-, butanal, 3-methyl- and dimethyl sulphide. Therefore, it is not surprising to observe overlapping in $\delta^{13}C$ values between natural and synthetic compounds. The method could still successfully separate nine of the 15 VOCs for which there are values for synthetic samples. Eleven aromatised commercial truffle products were also analysed to evaluate the database's usefulness. The results revealed possible falsifications, but further development of an HS-SPME GC-IRMS method for determining the $\delta^{2}H$ values of truffle VOCs is required to upgrade the existing database, which could be more successfully used in authenticity studies.

In this study, I prepared fresh truffle samples and synthetic volatile compounds for analysis, GC-MS analyses and stable carbon isotope measurement using EA-IRMS and GC-C-IRMS. I was responsible for data processing, interpretation and using RStudio. I collected the data, established the database, and wrote and prepared the manuscript for publication.

The potential of stable isotope technique in the determination of truffle aroma formation and in the authentication of truffles and products containing truffles.

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Abstract

Volatile organic compounds released by truffles enable the truffle to communicate with plants (symbiotic hosts), animals (vectors) and other microorganisms (decomposers). Besides their biological role, VOCs create the unique truffle aroma hugely treasured by humans, of great gastronomic relevance and highly vulnerable to food frauds. In this study, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) analysis of volatile truffle compounds, sampled using headspace-solid phase microextraction (HS-SPME), is used as a tool to differentiate between synthetic and naturally produced volatile aroma compounds (VOCs). The result is an extensive stable isotope database consisting of 21 authentic flavour compounds with well-defined origin: *Tuber aestivum* (n=58), *Tuber magnatum* (n=14), *Tuber mesentericum* (n=3), *Tuber brumale* (n=5), *Tuber excavatum* (n=3), and *Tuber macrosporum* (n=4). Synthetically derived VOCs (13) were also characterised. To evaluate the usefulness of the database, 11 aromatised commercial truffle products were analysed. The results revealed possible falsifications. Moreover, in this study, we also explore the potential of stable isotope technique in the determination of role of microbes in truffle aroma formation and influence of different factors on $\delta^{13}C$ value. Compounds with more negative $\delta^{13}C$ values, 1-hexanol, 2-hexen-1-ol (E)-, ethyl acetate, benzene, 1,4-dimethoxy- and 2,4-dithiapentane are supposed to be derived only by microbes.

Keywords: authenticity, aroma, truffle, HS-SPME, GC-C-IRMS, database

1. Introduction

Since time immemorial, truffles have been of interest to people. Their unique aroma, unusual appearance, special hunting and cultivation make them globally valued in the culinary world (Allen & Bennett, 2021). Among consumers, the *Tuber magnatum*, the white truffle, is considered the most famous and appreciated of all edible fungi. Its price can fetch 3,500 Euros per kg (Piltaver & Ratoša, 2006; Reyna & Garcia-Barreda, 2014). Its characteristic odour is reminiscent of the smell of cheese and garlic and is carried by the volatile compound 2,4-dithiapentane (Costa et al., 2015; Splivallo & Ebeler, 2015). However, fresh truffles are highly perishable. Their shelf life is 7-10 days, and this is also a time when they exhibit their maximum sensorial properties (Campo, Marco, Oria, Blanco, & Venturini, 2017). Losses of volatile organic compounds (VOCs), oxidation and enzymatic reactions are considerable problems during storage (Palacios, Guillamón, García-Lafuente, & Villares, 2014). Cheaper, synthetically derived 2,4-dithiapentane ($ 100/kg) can be easily added to commercial products to enhance truffle aroma. Its use as a flavouring preparation is permitted by the law. However, the correctness of labelling is often in question (Bononi, Tateo, Benevelli, Pennetta, & Benedetto, 2018; Sciarrone et al., 2018). An analytical approach that can discriminate between natural and synthetic origin of VOCs utilises the use of gas chromatography coupled to combustion-isotope ratio mass spectrometry (GC-C-IRMS) (van Leeuwen,

Prenzler, Ryan, & Camin, 2014). Despite that synthetic compounds, derived from coal and petroleum have $\delta^{13}C$ values between -30‰ to -25‰ and are similar to $\delta^{13}C$ values in modern C3 plants (van Leeuwen et al., 2014) research shows that GC-C-IRMS is capable of distinguishing between natural and synthetic aromas compounds (Strojnik et al., 2021; van Leeuwen et al., 2014). Three independent studies from 2018 started to transfer this approach to truffles (Bononi et al., 2018; Sciarrone et al., 2018; Wernig, Buegger, Pritsch, & Splivallo, 2018). Due to the economic relevance of *T.magnatum* is therefore not surprising that all three studies focus on 2,4-dithiapentane compound. Bononi et al., 2018 characterised the synthetic standards with $\delta^{13}C$ values between -43.40‰ to -42.24‰. Similarly found Wernig et al., 2018 who found that only samples with a $\delta^{13}C$ value lower than -45‰ can be classified as synthetic. The third research characterised two synthetic standards with very low $\delta^{13}C$ values (-56.4‰ and -77.1‰) and one synthetic standard with more positive value (-28.5‰). The $\delta^{13}C$ values of genuine white truffles collected from Italy range from -42.6% to -33.9%. Therefore, literature data suggest possible differentiation between synthetic and natural (from truffle) origin of truffle VOCs. However, the results are limited to a 2,4-dithiapentane aroma compound present only in *T.magnatum* and are based on a small number of samples. In addition to *T. magnatum*, there are quite a few culinary interesting species from the genus *Tuber*. Black truffle (*T. melanosporum*) is considered one of the most aromatic species. Its aroma is described as rotten, radish, chicory and hazelnut (Katanić, Marina et al., 2017; Wang & Marcone, 2011). Other species such as *T.brumale*, *T. aestivum*, *T.mesentericum* possess a lower market value, although they can have a good aroma quality (Piltaver & Ratoša, 2006). Despite the variability in their aromatic profile, truffles of a given species share common VOCs. According to the literature, 3-methyl-1-butanol, 1-octen-3-ol, 3-methylbutanal, 3-octanone, hexanal, acetaldehyde, dimethyl sulphide, dimethyl disulphide, ethyl butyrate, benzene,1-methoxy-3-methyl- are the most abundant aroma compounds in truffles (Vahdatzadeh, Deveau, & Splivallo, 2015). However, there are no data about the stable isotope composition of those VOCs. Those data may be helpful in the detection of the unlabeled addition of synthetic compounds to raw truffles or their processed products.

However, the biological role of volatile compounds is to enable the truffle to communicate with plants (symbiotic hosts), animals (vectors) and other microorganisms (decomposers) (Vita et al., 2015). Indeed, truffle fruiting bodies are colonised by a diverse microbial community of bacteria, yeast, guest filamentous fungi, and viruses. The exact origin of truffle volatiles is unclear. It has been speculated that truffle aroma might result from the intimate interactions of truffles and their microbiomes. Some volatiles might be produced by truffles and microbes, while others might be derived from a single player (i.e., yeasts, bacteria, or truffles). Literature data on the ability of organisms to produce volatiles suggests that truffles and microbes might produce common truffle odorants, whereas more specific compounds might be of microbial origin only (Vahdatzadeh et al., 2015). At the same time, many authors cite the great variability of the aromatic profile within a particular species, even in samples from the same location or even within the same mycelium (Virginie Molinier, Murat, Frochot, Wipf, & Splivallo, 2015). These differences arise because of biotic factors (e.g. fungi, yeasts, bacteria, mesofauna, and plant host) and abiotic factors (e.g. rainfall and temperature, mycelial connectivity, soil properties, and microclimatology) that often co-vary in truffle orchards (Büntgen et al., 2017; Mello, Murat, & Bonfante, 2006). Aroma variability in truffles has been attributed to the maturation stage (*T. borchii*) and to environmental (*T. magnatum*) and genetic factors (Splivallo et al., 2012; Vahdatzadeh & Splivallo, 2018). Despite researches, the factors influencing the development of aromatic compounds are still unexplained or only predicted. However, VOCs' stable isotope composition may help explain factors that influence aroma formation.

The present study deals with developing a procedure including $\delta^{13}C$ measurements, data processing and database creation to detect possible frauds of truffle aroma compounds. The $\delta^{13}C$ values for all investigated aroma compounds,

except for 2,4-dithiapentane, were, to our knowledge, identified for the first time. Moreover, the role of microbes in aroma formation has been investigated using stable isotopes for the first time. Therefore, the overall objectives are: (i) to establish a database of $\delta^{13}C$ values of synthetic and natural truffle aroma compounds; (ii) to assess the authenticity of commercially available truffle products (iii) assess the role of microbes in the formation of truffle aroma compounds.

2. Materials and Methods

2.1. Samples

Sampling of fruiting bodies. Fresh ascocarps (n=87) representing six truffle species (*Tuber aestivum* (n=58), *Tuber magnatum* (n=14), *Tuber mesentericum* (n=3), *Tuber brumale* (n=5), *Tuber excavatum* (n=3), and *Tuber macrosporum* (n=4)) harvested in 2018/19 in 11 different countries (Slovenia (n=61), Croatia (n=8), Bosnia in Herzegovina (n=1), North Macedonia (n=11), Italy (n=3), and Poland (n=3) were collected with the help of local truffle hunters. Each truffle species were transported separately in paper bags to the laboratory, where they were brushed with a soft wet brush and rinsed with tap water. The truffles were re-identified based on the length and shape of spores and asci, spore wall ornamentation, and the peridium and gleba structure. Each sample was then wrapped in paper, and depending on the species of truffle, stored in glass jars. All samples were kept at 4°C until the analysis, usually done within 24-48 h.

Commercial samples. A total of 11 commercial products aromatised with truffles were purchased from stores and, namely, four olive oils, four sauces, one fish spread, one summer truffle and one white truffle.

Synthetic standards. Samples of 13 pure synthetically derived aroma compounds of 1-butanol, 2-methyl; 1-octen-3-ol; 1-octen-3-one, 2-butanol; 2-butanone; 2-hexen-1-ol (E)-; 2,4-dithiapentane; 3-octanone; acetaldehyde; benzene, 1-methoxy-3-methyl-; dimethyl disulphide; dimethyl sulphide; and ethyl acetate were purchased from Sigma Aldrich.

2.2. Sample preparation

Sample preparation was performed immediately before analysis, and volatile components were extracted from the headspace (HS) using a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (50/30 µm thickness) according to the procedure described by Strojnik, Grebenc, & Ogrinc, 2020.

2.3. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analyses were performed using a 7890B GC & 5977A Series GC/MSD (Agilent Technologies, USA). The separation was achieved on a VF-WAXms capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J&W, USA). The analytical conditions and procedure are in detail described by Strojnik et al., 2020.

2.4. Elemental Analysis-Isotope Ratio Mass Spectrometry (EA-IRMS)

The $^{13}C/^{12}C$ ratios of synthetic standards were determined using IsoPrime 100 – Vario PYRO Cube combined with Vario LS sampler (Liquid sampler for "cube" analyser line) (OH/CNS Pyrolyser/Elemental Analyzer) (IsoPrime, Cheadle, Hulme, UK) and the IonVantage for Isoprime Build 1, 6, 1, 0 software. The analysis follows the analytical procedure described by Strojnik et al., 2019. To assure the accuracy of IRMS measurements, two internal working standards: absolute ethanol MERCK (Germany) with $\delta^{13}C = -27.38 \pm 0.09‰$, a distillate of rum with $\delta^{13}C = -13.81 \pm 0.09‰$ were used. Working standards were previously calibrated against the certified reference material BCR-656 wine alcohol ($\delta^{13}C$ value of -26.91 ± 0.07‰) obtained from the Institute for Reference Materials and Measurements (IRMM, Belgium).

The carbon isotope ratios were expressed in the δ-notation in per mil (‰) deviation relative to the VPDB international standard as follows:

$$\delta^{13}C_{VPDB}\ (‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \times 1000,$$

where R is the isotope ratio $^{13}C/^{12}C$ in the sample and standard, respectively. The reproducibility of measurements for $\delta^{13}C$ was ±0.1‰.

2.5. Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS)

The isotopic compositions of aroma components were obtained using an Agilent 6890N GC-C system coupled to an IsoPrime GV IRMS. Separation was achieved using an Agilent J&W VF-WAXms capillary column (30 m × 0.25 × 0.25). The temperature program was as follows: 30 °C (held 1 min) to 40° C at 1 °C min^{-1} (held 2 min), from 40 °C to 60 °C at 7 °C min^{-1} and finally from 60 °C to 200 °C at 20 °C min^{-1} (held 5 min). Helium was used as a carrier gas with a 1.5 mL/min constant flow. The injection was performed at 250 °C in the split (1:5) / splitless mode. The oxidation reactor (Cu/O) in the 6890N GC/C system was set to 900 °C.

Before each measurement sequence, stability and linearity were checked. Acceptable values were <0.03‰. Reproducibility and accuracy were evaluated routinely using the working standard. The carbon isotope ratio of each compound in the reference solution, previously determined by EA-IRMS, was then obtained by GC-C-IRMS. For data normalisation, the multiple-point linear normalisation method was used (Paul, Skrzypek, & Fórizs, 2007). The reproducibility of the GC-C-IRMS measurements based on duplicate analysis ranged from ±0.1 to ±0.8‰. Peak recognition was performed using retention times of reference compounds and by comparison of chromatograms obtained from GC-MS.

3. Results and discussion

3.1. GC-C-IRMS measurements / Database creation

One of the first steps when developing a $\delta^{13}C$ database of truffle VOCs was a selection of authentic reference samples of natural origin (truffles). We decided to analyse only raw truffle samples, freshly prepared immediately before analysis to avoid introduced isotope fractionation, i.e., enrichment of one isotope relative to another. Wernig et al., 2018 reported significant difference $\delta^{13}C$ values between fresh and frozen fruiting bodies and between fresh samples analysed in time series over two days. On the contrary, Sciarrone et al., 2018 did not find any considerable variation of the $\delta^{13}C$ value after reanalysis a sample after two months of storage. Similarly, our study did not find a difference in the $\delta^{13}C$ value of the sample analysed within 15 days of storage at 4 °C. Our research focused mainly on *T. aestivum*, the most common species found in Europe and on *T.magnatum*, the most expensive and rarest of all commercial species. To investigate whether truffle species influence $\delta^{13}C$ value, we include other *Tuber* species, namely *T. brumale*, *T. excevatum*, *T. mesentericum*, and *T. macrosporum*. A database of $\delta^{13}C$ values of the most common aroma compounds present in truffles was then established. Table 1 presents the $\delta^{13}C$ (‰) values of synthetic standards and many fresh truffle samples from which an isotopic authenticity range together with the minimum, maximum and median $\delta^{13}C$ values of 21 truffle aroma compounds was obtained.

149 Table 1: $\delta^{13}C$ (‰) values of different aroma compounds in fresh truffle samples, synthetic standards and literature data.

	2-butanone	2-butanol	3-octanone	dimethyl sulphide	1-butanol, 2-methyl-	1-octen-3-ol	2-octen-1-ol, (E)-	acetaldehyde	1-hexanol	benzene, 1-methoxy-3-methyl-	ethyl acetate	1-octen-3-one	1-butanol, 3-methyl-	dimethyl disulphide	2,4-dithiapentane	butanal, 2-methyl-	benzene, 1,4-dimethoxy-	butanal, 3-methyl-	1-propene, 1-(methylthio)-, (E)-	1-propene, 1-(methylthio)-, (Z)-	propane, 1-(methylthio)-
											Synthetic										
No.	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
min	-27,8	-27,1	-33,5	-31,3	-29,0	-30,1	-28,5	-35,4	-24,4	-33,9	-27,0	-27,7	-23,1	-29,5	-34,3						
max	-27,8	-27,1	-33,5	-31,3	-29,0	-30,1	-28,5	-35,4	-24,4	-33,9	-27,0	-27,7	-23,1	-29,5	-34,3						
											Tuber aestivum										
No.	36	16	14	46	42	26	4	13	2	10	6	7	3	1		1					
min	-29,8	-25,8	-32,3	-40,1	-30,7	-29,2	-36,0	-30,4	-34,8	-37,1	-35,1	-26,5	-27,8	-27,9		-20,7					
max	-21,8	-17,6	-24,5	-21,5	-24,4	-20,1	-29,2	-20,4	-31,1	-32,1	-30,0	-23,2	-25,8	-27,9		-20,7					
											Tuber magnatum										
No.			4	14	13					6				11	12	14	10	6			
min			-35,1	-28,8	-34,2					-35,5				-32,3	-39,1	-32,2	-30,6	-30,3			
max			-24,1	-24,9	-22,2					-29,4				-27,1	-35,7	-19,4	-24,2	-24,4			
											Tuber excevatum										
No.			1	1					1	3	1		1			1			2	2	1
min			-24,4	-26,2					-33,0	-34,5	-32,8		-28,5			-32,5			-28,2	-28,2	-28,9
max			-24,4	-26,2					-33,0	-30,6	-32,8		-28,5			-32,5			-26,7	-27,5	-28,9
											Tuber brumale										
No.	1		4	5	5					3						4		5	3	1	2
min	-26,1		-37,6	-30,5	-34,6					-34,8						-30,3		-33,8	-25,3	-20,4	-32,0
max	-26,1		-31,0	-27,3	-26,8					-33,4						-18,6		-15,0	-20,0	-20,4	-26,9
											Tuber mesentericum										
No.			1							3											

	2-butanone	2-butanol	3-octanone	dimethyl sulphide	1-butanol, 2-methyl-	1-octen-3-ol	2-octen-1-ol, (E)-	acetaldehyde	1-hexanol	benzene, 1-methoxy-3-methyl-	ethyl acetate	1-octen-3-one	1-butanol, 3-methyl-	dimethyl disulphide	2,4-dithiapentane	butanal, 2-methyl-	benzene, 1,4-dimethoxy-	butanal, 3-methyl-	1-propene, 1-(methylthio)-, (E)-	1-propene, 1-(methylthio)-, (Z)-	propane, 1-(methylthio)-
min			-24,5							-33,7											
max			-24,5							-31,6											
										Tuber macrosporum											
No.			2	3	2					4						1			4	4	4
min			-37,2	-28,8	-28,6					-34,4						-28,3			-24,7	-24,4	-31,0
max			-32,9	-24,1	-28,4					-31,3						-28,3			-21,6	-19,9	-26,2
										Synthetic literature											
min															-77,1						
max															-42,3						
										Natural literature											
min															-33,9						
max															-42,6						

150

151

152 Isotopic authenticity ranges of 21 most common truffle VOCs were also presented graphically in Figure 1. We
153 identified benzene, 1-methoxy-3-methyl- in all investigated truffle species with minimal natural isotope variability.
154 Similarly, 1-butanol, 2-methyl, butanal, 2-methyl-, 3-octanone and dimethyl sulphide were present in almost all truffle
155 species. But they show the most extensive natural isotope variation between truffle species. Numerous factors have been
156 suggested to influence truffle aroma, including fruiting body maturity (Zeppa et al., 2004), possibly the association with
157 the host trees (Gioacchini et al., 2008; Vita et al., 2018), genetics (V Molinier, Murat, Frochot, Wipf, & Splivallo, 2015;
158 Splivallo, Ottonello, Mello, & Karlovsky, 2011; Vahdatzadeh & Splivallo, 2018) microbes colonising truffle fruiting
159 bodies (Splivallo et al., 2015; Vahdatzadeh et al., 2015), and other environmental factors (i.e. soil, climate). We observed
160 some differences in the $\delta^{13}C$ value of truffle VOCs between different truffle species for 1-butanol, 2-methyl, butanal,
161 2-methyl- and 3-octanone. Interestingly all three compounds have the same pattern, with the similar lowest values in *T.*
162 *brumale* and *T. macrosporum*, and more positive values, similar in *T. aestivum* and *T. magnatum*. However, we observed
163 some differences within certain species, e.g. for dimethyl sulphide in T. aestivum that seems to be related to harvest
164 location or some other biotic factors (e.g. microbes or plant host) specific for that location. Geographic distances from
165 the sea could not be the reason, since all sites are just a few kilometres apart from each other and all located in the south-
166 eastern part of Slovenia. Also, all samples are of good quality, proven with aroma profile and sensory analysis. The low
167 quality of samples is interestingly reflected in more positive $\delta^{13}C$ values (higher than -25‰) of butanal, 2-methyl- and
168 1-butanol, 2-methyl-. The odour of those samples is reminiscent of mould or earth. More positive values of butanal, 2-
169 methyl- was also typical for samples with a low intense aroma. Therefore, isotope values of this compound may be

attributed to the maturation stage of truffles since more mature samples synthesise higher concentrations of butanal, 2-methyl- (Splivallo et al., 2011).

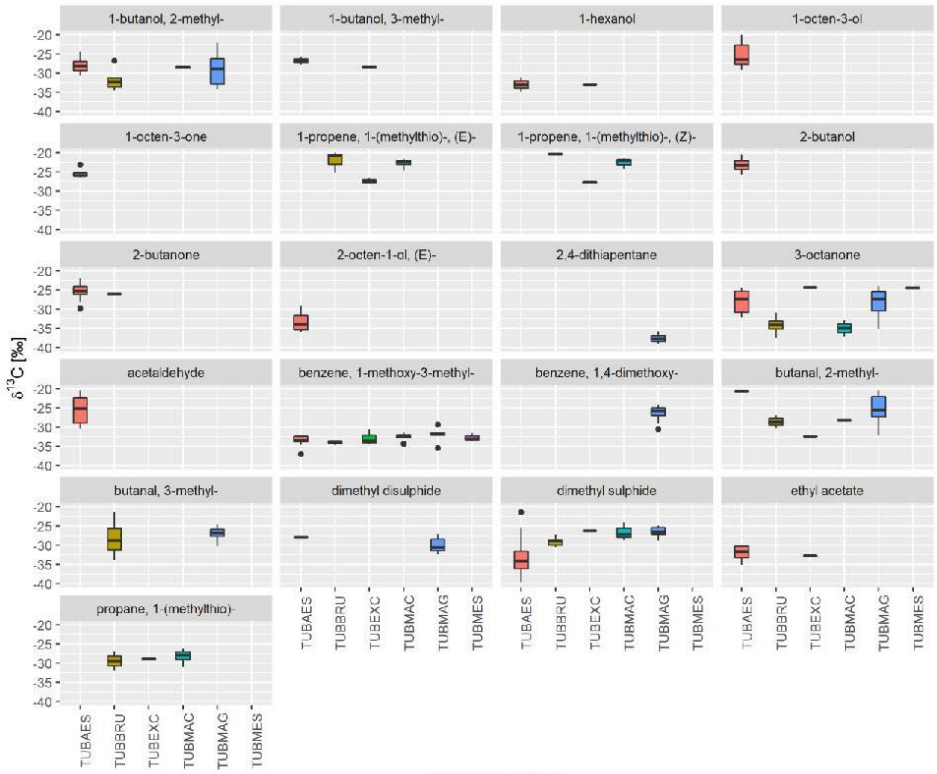

Figure 1: Comparison of isotope authenticity ranges of 21 most common truffle VOCs between six different truffle species, *T. æstivum* (TUBAES), *T. brumale* (TUBBRU), *T. excevatum* (TUBEXC), *T. macrosporum* (TUBMAC), T. magnatum (TUBMAG) and *T. mesentericum* (TUBMES).

To investigate whether the isotope values of *T. magnatum* VOCs reflect the geographical origin of samples, we select them from Slovenia, Italy, Croatia, and North Macedonia. The results are presented in Figure 2. We can observe several differences between countries. However, it must be emphasised that a detailed study with more samples per country is required to confirm differences. Anyway, we can observe similar distribution between countries for 1-butanol, 2-methyl-, 2,4-dithiapentane, benzene, 1,4-dimethoxy-, dimethyl disulphide and dimethyl sulphide. Italy (IT) with the most

negative values, followed by slightly more positive values in Slovenia (SI) and the most positive in Croatia (HR). More positive values of 1-butanol, 2-methyl in Croatian samples are related to the low quality of those samples.

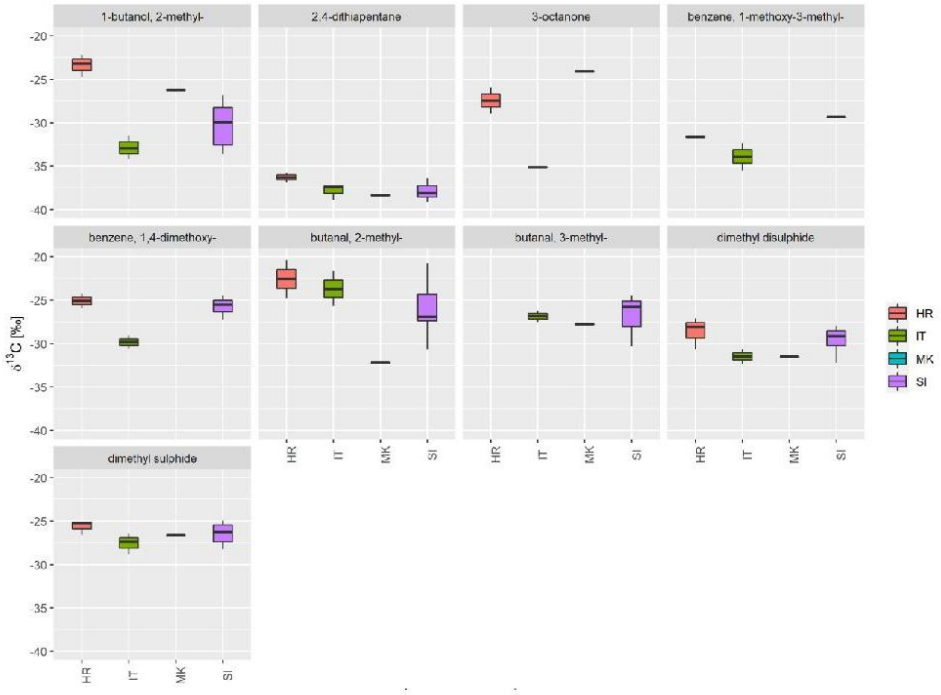

Figure 2: Comparison of isotope authenticity ranges of 9 most common truffle VOCs identified in T. magnatum collected from four different countries, Croatia (HR), Italy (IT), North Macedonia (MK) and Slovenia (SI).

3.2. Authenticity assessment

To evaluate the appropriateness of the established database in authenticity studies, all authentic samples were combined in one range (shown in dark green in Figure 3) and compared with $\delta^{13}C$ values of synthetic samples. What is clear is the overlap between several ranges in $\delta^{13}C$ values. Anyway, the method could successfully separate 9 of the 15 VOCs for which we have values for synthetic samples. Data show that synthetic samples are necessary for the database. It seems that different market brands should be analysed to increase the range of $\delta^{13}C$ values observed in synthetic samples and avoid misclassification of samples. When we compare our data with literature, we can find that synthetic 2,4-dithiapentane could be more positive, like in our study (-34.3‰). Or more negative, Sciarrone et al., 2018 reported very low $\delta^{13}C$ values (-56.4‰ and -77.1‰) of two standards of petrochemical synthetic origin. Wernig et al., 2018 reported $\delta^{13}C$ values of four synthetic standards between -50‰ and -40‰. Since they obtain overlapping between natural and synthetic range, they conclude that only samples with a $\delta^{13}C$ value lower than -45‰ can be classified as synthetic. Therefore, when we analyse commercial samples, some of their $\delta^{13}C$ values fall within the range of natural even they were labelled "contain truffle flavour", which usually means synthetic origin. On the contrary, some of them labelled "contain natural truffle flavour" fall outside but closely to the natural range. Therefore, we cannot be very sure

about their origin. They may be of mixed origin of natural flavouring substance obtained by physical, enzymatic, or microbiological processes from a material of vegetable, animal, or microbiological origin (-28.5‰, Sciarrone et al., 2018) and flavouring substance of petrochemical synthetic origin. We found one black summer truffle sauce (*T. aestivum*), probably of natural origin. Still, the producers add to the product also 2,4-dithiapentane, the compound of *T. magnatum*, also probably of natural origin (-30.8‰). When product odour reminiscent to something else (even this is other species) to what product is advertised, the question of appropriate labelling is here in question. Moreover, commercial samples (one white truffle oil, one raw *T. magnatum* and one raw *T. aestivum*) with $\delta^{13}C$ values of 2,4-dithiapentane around -58‰ indicate synthetic origin. Also, we were surprised by the addition of 2,4-dithiapentane to the fresh *T. aestivum* fruiting body. The compound was added to the fresh *T. magnatum* fruiting body, probably to enhance its weak aroma. Beside 2,4-dithiapentane, we identify 6 other truffle VOCs, namely 1-butanol, 2-methy-, 1-octen-3-ol, 2-butanone, benzene,1-methoxy-3-methyl-, butanal, 2-methyl- and dimethyl sulphide. From them, we can discriminate just between the synthetic and natural origin of 1-octen-3-ol. Samples of two black truffle sauce labelled "contain natural truffle flavour" fall outside the natural range with similar $\delta^{13}C$ values as olive oil that contain truffle flavour of synthetic origin.

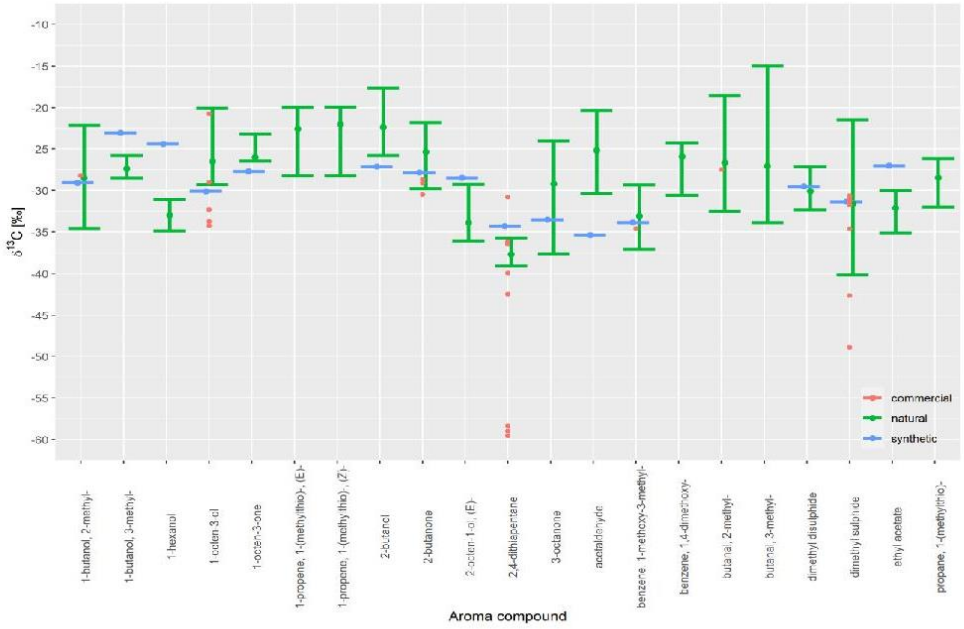

Figure 3: Comparison of $\delta^{13}C$ values of commercial fresh truffles or their products with $\delta^{13}C$ values from the database of natural samples and synthetic standards.

3.3. Role of microbes in aroma formation

Microbes colonise truffles at all stages of their life cycle (Vahdatzadeh et al., 2015). The bacteria are selectively assimilated from the surrounding soil microbiome into the fruiting body during development and maturation. Therefore, it has been speculated that soil microorganisms affect their formation (Perlińska-Lenart et al., 2020) and can produce VOCs that contribute significantly to truffle aroma (Mustafa et al., 2020). Despite researches on the truffle microbiome that try to explain truffle aroma formation, the exact origin of truffle volatiles is still unclear (Vahdatzadeh et al., 2015). However, the analysis of stable isotope values of truffle VOCs may analyse microbial function.

The very first isotopic imprint on the carbon source for the production of plant volatiles derives from isotopic discrimination during photosynthetic carbon fixation by either RuBisCO (primary metabolism of C_3-plants) or PEP-carboxylase (primary metabolism of C_4-plants), resulting in different $\delta^{13}C$ values of the plant material (Blaser & Conrad, 2016). Truffles are mycorrhizal fungi that form symbioses with plants in which plant-supplied sugars are exchanged for nutrients obtained by fungi from the soil (Hobbie, 2005). Symbiosis between truffles and plants alters the bacterial community structure of plant roots. These community shifts then promote the growth and activity of bacteria with metabolisms associated with sugar and protein metabolism, thereby promoting plant growth and development (Yang et al., 2019). Belowground litter and root exudates of the canopy-forming plant species with typical $\delta^{13}C$ values of –29 to –26‰. Direct feeding on plant tissue results in enrichment in ^{13}C of herbivores by 0.5–1‰ (Potapov, Tiunov, & Scheu, 2019). In comparison, mycorrhizal fungi are enriched by 1 to 5‰ in ^{13}C compared to their host trees (Hobbie, 2005). Typically, primary decomposers feeding on litter and saprotrophic microorganisms are enriched in ^{13}C by 3–4‰ as compared to litter. In turn, some decomposers and predators were strongly enriched in ^{13}C (up to 6–7‰ compared to leaf

litter), suggesting that microorganisms largely processed dead plant material. Animal species with low $\delta^{13}C$ values suggesting feeding on mycorrhizal fungi. Hence, soil animals feeding on mycorrhizal fungi are presumably depleted in ^{13}C compared to animals feeding on saprotrophic microorganisms (Potapov et al., 2019).

Yet, the carbon isotope composition can be different between compound classes. Carbon isotope discrimination occurs during reactions required to produce the mixture of nucleic acids, proteins, carbohydrates, and lipids that form biomass (Hayes, 2001). Further carbon isotope discrimination occurs during the different metabolic processes associated with the chemical breakdown of macromolecules (carbohydrates, proteins, and lipids) and the synthesis of micromolecules (e.g. volatile metabolites). Biochemical reactions involving carbon often display kinetic isotope effects, which usually discriminates against the heavy ^{13}C isotope so that the $\delta^{13}C$ value of the product is always lower than that of the substrate. Biochemical pathways typically consist of a sequence of reactions that may influence the overall fractionation factor to various extents (Blaser & Conrad, 2016). Moreover, even when starting points and downstream processes are closely similar, the separation of pathways between compartments can mean that carbon flow divisions at branch points differ significantly and, therefore, that final isotopic composition differ sharply (Hayes, 2001).

Based on $\delta^{13}C$ values of several truffle VOCs obtained in this study, we observed a broad authentic range of more than 15‰ difference between min and max value, found in 1-butanol, 2-methyl-, 3-octanone, butanal, 2-methyl-, butanal, 3-methyl- and dimethyl sulphide. Those compounds may be of mixed origin, produced by truffle mycelia and microbes. The result agrees with Vahdatzadeh et al., 2015, who found that compounds like 3-methylbutanal, 3-methyl-1-butanol, hexanal and acetaldehyde may be produced by specific bacterial classes as well as by truffle mycelia. Moreover, the Ehrlich pathway, which consists of the catabolism of specific amino acids leading to odorants dimethyl sulphide, 2-methylethanol, 2- and 3-methylbutanol, and others common to microbes and truffles (Vahdatzadeh et al., 2015). On the contrary, compounds with a span in an authentic range smaller than 15‰ seem to be derived from truffles or microbes only. Compound related with truffle origin seem to be 1-butanol, 3-methyl, 1-octen-3-ol, 1-octen-3-one, 1-propene, 1-(methylthio)-,(E)-, 1-propene, 1-(methylthio)-,(Z)-, 2-butanol, 2-butanone, acetaldehyde, benzene, 1,4-dimethoxy-, butanal, 3-methyl-, dimethyl disulphide and propane, 1-(methylthio)-. Their $\delta^{13}C$ values are comparable with isotope values determined in bulk truffle samples ranging from -28.5‰ to -24.5‰ reported by Gregorčič et al., 2020. Eight-carbon containing volatiles (i.e., octan-3-one, 1-octen-3-ol) were believed to be of strict fungal origin (Vahdatzadeh et al., 2015). Compounds with more negative $\delta^{13}C$ values, 1-hexanol, 2-hexen-1-ol (E)-, ethyl acetate, benzene, 1,4-dimethoxy- and 2,4-dithiapentane are supposed to be derived only by microbes. Splivallo et al., 2015 found that sulphur-containing volatiles such as thiophene derivatives, volatiles unique to *T. borchii*, are derived from bacteria and resulted from the biotransformation of non-volatile precursor(s) into volatile compounds. Non-volatile precursors of thiophene compounds might be produced by the intimate interactions of truffles, yeasts and bacteria. However, the biosynthetic pathway leading to thiophene derivatives as well as for 2,4-dithiapentane remains unknown (Splivallo & Ebeler, 2015). Anyway, there is indirect evidence that bacteria might be exclusively responsible for the emission of 2,4-dithiapentane in *T. magnatum*. However, more specific sulphur volatiles might be derived from truffles or microbes only (Vahdatzadeh et al., 2015). At the same time that microorganisms play an important role in the biodegradation of organic molecules (Fester, Giebler, Wick, Schlosser, & Kästner, 2014). Our study observed more positive $\delta^{13}C$ values (> -24‰) of 1-

butanol, 2-methyl, butanal, 2-methyl and butanal, 3-methyl in low quality samples. Vahdatzadeh, Deveau, & Splivallo, 2019 also reported 2-methylbutanal and 3-methyl-1-butanol as markers of spoilage.

4. Conclusions

The present work demonstrates that HS-SPME-GC-C-IRMS analysis of volatile compounds is a valuable tool for differentiating between synthetic and natural truffle flavourings. The development of an authenticity database is of particular importance when detecting fraudulent practices. However, we observed great variability in $\delta^{13}C$ value of several aroma compounds. We observed some differences in the $\delta^{13}C$ value of truffle VOCs between different truffle species. Also, the truffles' location and quality seem to influence $\delta^{13}C$ value. Moreover, aroma compounds might be derived from truffles and microbes, resulting in a broad authentic range found in 1-butanol, 2-methyl-, 3-octanone, butanal, 2-methyl-, butanal, 3-methyl- and dimethyl sulphide. Therefore is not surprising that we observed several overlaps between a natural and synthetic range of values. The method could still successfully separate 9 of the 15 VOCs for which we have values for synthetic samples. However, when analysing commercial samples, we obtain $\delta^{13}C$ values only for 1-butanol, 2-methyl-, 1-octen-3-ol, 2-butanone, 2,4-dithiapentane, benzene, 1-methoxy-3-methyl-, butanal, 2-methyl- and dimethyl sulphide. From those compounds, we can differentiate between synthetic and natural origin based on data from the established database only for 1-octen-3-ol and 2,4-dithiapentane. Therefore we can conclude that an established database of $\delta^{13}C$ values of the 21 most common aroma compounds represents an important contribution in this not well-researched field. $\delta^{13}C$ values of the 20 truffle aroma compounds were reported for the first time. However, the development of an HS-SPMEGC-IRMS method for 2H/1H ratio determination of truffle VOCs is required to upgrade the existing database, which could be more successfully used in authenticity studies.

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Conflicts of Interest: The authors declare no conflict of interest.

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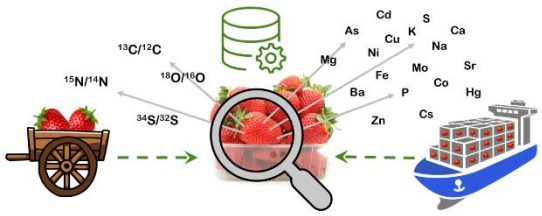
3.7 Scientific Paper: "Geographical Identification of Strawberries Based on Stable Isotope Ratio and Multi-Elemental Analysis Coupled with Multivariate Statistical Analysis: A Slovenian Case Study



This chapter presents the paper entitled "Geographical Identification of Strawberries Based on Stable Isotope Ratio and Multi-Elemental Analysis Coupled with Multivariate Statistical Analysis: A Slovenian Case Study" by Lidija Strojnik, Doris Potočnik, Marta Jagodic Hudobivnik, Darja Mazej, Katja Babič, Boštjan Japelj, Andrija Ćirić, Nadja Škrk, Suzana Marolt, David Heath and Nives Ogrinc. The paper was published in Food Chemistry in 2022. This study demonstrates the appropriateness and usefulness of explorative analysis, classification and class modelling chemometric approaches in determining the geographical origin of strawberries using stable isotope ratio and multielement analysis. The overall objective was to assess their potential in building a classification model that provides reliable predictions of unknown samples with the potential to combat food misdeclaration. The work involved creating a database of 92 authentic Slovenian strawberry samples, 32 imported samples (Italy (18), Croatia (6), Spain (3), France (3), Greece (1) and Serbia (1)), and 43 commercially available samples (test samples) with declared Slovenian origin. All samples were harvested between 2018 and 2020.

The work involved determining stable isotope ratios of light elements ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$) and the elemental composition of 25 elements (Na, Mg, Al, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, Cs, Ba, Hg and Pb). Different statistical procedures were used, including PCA, LDA, OPLS-DA and DD-SIMCA. The PCA method was used to explore the whole dataset and allowed the grouping of samples corresponding to geographical origin. The results also show that separating specific Croatian and Italian samples from Slovenian samples is impossible despite their geographical proximity. Two supervised methods were also applied: LDA and OPLS-DA to obtain the best visualization of group clusters. Both conventional discriminant strategies allowed the strawberry samples to be classified according to their origin with good accuracy (95 %). Also, the most important variables for projection were P, Na, K, As, Ba, Sr, Mo, Cr and Zn. The study also shows that distinguishing between production years is also possible. However, discriminant analysis can lead to biased classification rules and predictions for new samples when confirming that a specific sample originates from a particular region. In order to overcome this, the study applied DD-SIMCA, a one-class classification technique, to build a classification model that could provide reliable predictions of new unknown samples with the potential to combat food misdeclaration. This approach, to our knowledge, is the first application of its kind to strawberries. The classification model was built and validated first for each year separately and finally for all years together. Excellent sensitivity was obtained (96.2 % to 97.0 %) and good specificity (81 % to 90.9 %) for each year separately. When all years were included in a single model, sensitivity slightly reduced (94.3 %); however, specificity was only 62.5 %. The study revealed that all variables are essential in modelling and that strawberry production depends on soil composition and is susceptible

to environmental conditions. Significant year-to-year variations meant that DD-SIMCA could not provide a robust model when all years were combined. Finally, a model based on production year was used to investigate the origin of commercial samples with declared Slovenian origin. Overall, 39 % of samples were found to be potentially mislabelled.

My responsibility included designing the sampling strategy, sample handling and documentation management. I was also responsible for sample preparation (obtaining water from samples, pulp isolation and freeze-drying) and performing stable isotope analysis (determination of $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$) and normalization of results. In addition, I pre-processed all of the isotopic and elemental data, and with the help of Dr. Japelj, performed data analysis using DD-SIMCA in RStudio. Finally, I interpreted the data, wrote and prepared the manuscript for publication.

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Geographical identification of strawberries based on stable isotope ratio and multi-elemental analysis coupled with multivariate statistical analysis: A Slovenian case study

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ABSTRACT

The geographical classification and authentication of strawberries were attempted using discriminant and class-modelling methods applied to stable isotopes of light elements and elemental composition. The work involved creating a database of 92 authentic Slovenian strawberry samples and 32 imported samples. All samples were harvested between 2018 and 2020. A good geographical classification of Slovenian and non-Slovenian strawberries was obtained despite different production years using discriminant approaches. However, for verifying compliance with a given specification (geographical indications), a class-modelling approach was used to build an unbiased verification model. Class models generated by data-driven soft independent modelling of class analogy (DD-SIMCA) had high sensitivity (96% to 97%) and good specificity (81% to 91%) on a yearly basis, while a more generalised model combining total yearly data gave a lower specificity (63%). Of the 33 commercially available samples (test samples) with declared Slovenian origin, 39% were from outside of Slovenia.

1. Introduction

Seasonal locally produced fruits are becoming more and more popular. Local (seasonal) fruit and vegetables are typically considered fresher, tastier, and higher quality than similar imported commodities or those produced out of season. Such attributes mean that consumers will pay a premium for local and regional products, with seasonal strawberries being a good example. Unfortunately, this increase in demand and higher value makes commodities such as locally grown strawberries vulnerable to origin fraud. There are also many other benefits of purchasing local produce, i.e., it supports regional economies, local agriculture, self-sufficiency, the environment (reducing CO_2 emissions by reducing food miles, maintaining farmland and green and open spaces) while ensuring that small community farms exist.

Protecting local produce is not easy because determining the geographical origin of agricultural produce is challenging and depends on various conditions, e.g., fertilisation, botanical origin, history of the field, climatic conditions during cultivation, location, and soil composition (Katerinopoulou, Kontogeorgos, Salmas, Patakas, & Ladavos, 2020). Therefore, the objective in food authentication is to develop appropriate analytical methods and tools to identify unique markers, or groups of markers, capable of establishing the origin of a food or identifying potential exogenous compounds, which can resolve issues around authenticity. Various analytical techniques and parameters have been studied to verify the provenance of regional foods, including the use of gas and liquid chromatography to analyses characteristic volatile organic compounds (i.e., aroma compounds), sugars, phenolic compounds and "fingerprinting" or chemical profiling by 1H NMR, near Infra-Red and Fluorescence spectroscopy (Armenta & de la Guardia, 2016; Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Downey,

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2016; Katerinopoulou et al., 2020; Oliveira, Cruz-Tirado, & Barbin, n.d.; Sotiropoulou et al., 2021; Su, Arvanitoyannis, & Sun, 2018). However, the stable isotopic approach using light elements (H, C, N, O and S) (Camin et al., 2017) and elemental profiling are the two most powerful tools for determining the geographical source (Drivelos & Georgiou, 2012; Gonzalvez, Armenta, & de la Guardia, 2009; Kelly, Heaton, & Hoogewerff, 2005; Nakano & Zhao, 2018).

Apart from the measurement, data interpretation requires detailed knowledge of the food in question and an appropriate database or databank of authentic food samples (Camin et al., 2017) against which an unknown food sample can be compared. The most important criteria is that the samples contained within the database are authentic, sufficiently representative and cover natural variation caused by many factors, including geographical location, variety, age and health, physical and climate stresses, processing method, temporal or seasonal variation and anthropogenic contamination (Donarski, Camin, Faulhl-Hassek, Posey, & Sudnik, 2019). The most efficient way is to create yearly databanks, especially for vegetable and fruit commodities that show a more significant year of harvest/production variability (Camin et al., 2017).

When preparing a sampling strategy, it is necessary to consider the appropriate statistical analysis to make sense of the significant amounts of data produced (Donarski et al., 2019). Overall, there are three general chemometric approaches: explorative analysis, classification, and class modelling (Marini, 2009). The term "classification" is often used as a synonym of discriminant methods because they assign objects to predefined classes. Statistical (e.g., linear discriminant analysis – LDA, partial least squares-discriminant analysis – PLS-DA) and machine learning discriminant approaches (e.g. k-nearest neighbours, artificial neural network – ANN) separate samples according to predefined classes, e.g., two or more different regions. However, it is possible to identify the predominant features using an approach that distinguishes the two sets of samples. Studies that have used either stable isotopes or elemental composition or both report the usefulness of discriminant analysis and (orthogonal) partial least squares discriminant analysis for revealing differences between samples of different geographical origins (Bat et al., 2012; Camin et al., 2010; D'Archivio et al., 2019; Fiamegos et al., 2021; Foschi et al., 2020; Magdas et al., 2021; Mimmo et al., 2015; Nie et al., 2021; Opatić et al., 2017; Palacios-Morillo et al., 2014; Perez et al., 2006; Pianezze et al., 2019; Zhang et al., 2019). Interestingly, despite strawberries being such an important crop, only two studies, one by Perini et al. (2018) and the other by Pérez-Silva et al. (2006), have used isotopes and elemental composition to determine their geographical origin. Perini et al. (2018) used the $\delta^{13}C$ and $\delta^{15}N$ in pulp and $\delta^{18}O$ in water to identify the different geographical origins of the strawberry fruit cultivated in Italy, Poland and Romania. However, the authors found that covering the crop and fertilisation practices (organic or mineral fertiliser) affects their isotopic values. Perez et al. (2006) differentiated the geographic origin (Oregon vs Mexico) of strawberry samples using stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and trace element profiling (Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, V, and Zn) combined with principal component analysis and canonical discriminant analysis for data visualisation achieving a correct classification rate of 70 to 100%.

Confirmation that a specific sample originates from a particular region represents a task where those features that distinguish a set of samples from a specific area independently of other classes and regions are identified. However, discriminant methods are often forcedly used for one-class classification problems. In such cases, although the target class (of compliant samples) is typically well sampled, the non-target class (non-compliant samples) is often not adequately sampled and is poorly defined. As a result, discriminant classification can lead to biased classification rules and biased predictions for new samples (Oliveri, 2017).

Several papers highlight the inappropriateness of discriminant analysis and propose using class-modelling for authentication studies instead (Oliveri, 2017; Rodionova, Titova, & Pomerantsev, 2016). Methods used for solving authentication problems comprise a separate class among pattern recognition/classification techniques called one-class classifiers or class modelling. For example, soft independent modelling of class analogy (SIMCA) is an OCC method widely used in chemometrics (Zontov, Rodionova, Kucheryavskiy, & Pomerantsev, 2017). The original version of SIMCA has undergone numerous modifications, primarily related to constructing the acceptance boundary. In SIMCA, the number of samples can be as low as ten samples per class, and there is no limitation on the number of variables. Also, as Pirhadi, Shiri, & Ghasemi (2015) write, because the number of variables in a data set may be greater than the number of samples, many standard discrimination approaches would be violated in such situations due to issues relating to collinearity and chance classification.

This study uses the data-driven version of SIMCA (DD-SIMCA) since it can calculate misclassification errors theoretically (Zontov et al., 2017). Also, although several studies have implemented DD-SIMCA in their research so far, none have used DD-SIMCA in combination with stable isotopic and multi-elemental data for determining the geographical origin of long grain rice (Arif et al., 2021), coffee (de Araújo et al., 2021), copaiba oil (de Oliveira Moreira & Braga, 2021), almond powder (Faqeerzada et al., 2020), juices (Fidelis et al., 2017), bar soaps (Gerônimo, Oliveira, Soares, Peralta-Zamora, & Nagata, 2021), Tokaj wines (Gomes et al., 2021), milk powder (Mazivila, Páscoa, Castro, Ribeiro, & Santos, 2020), cow milk (Mohammed & Shuming, 2021), coconut oil (Neves & Poppi, 2020), olives (Rodionova, Oliveri, & Pomerantsev, 2016), lamb meat (Wang et al., 2020). Given this specific knowledge gap, this study explored different chemometric approaches using stable isotopic and elemental data to determine possible discriminant markers for Slovenian and non-Slovenian strawberries. The overall objective was to assess their potential in building a classification model that provides reliable predictions of new unknown samples with the potential to combat food misdeclaration.

What is clear is that there is a growing demand for reliable and robust analytical tools among enforcement agencies to verify food product compliance with its label description (Danezis et al., 2016). The approach developed in our study where DD-SIMCA has been used in combination with stable isotopic and multi-elemental data for verifying geographical origin has been for the first time applied to strawberries. Moreover, the presented methodology can be easily transferred to other food commodities and used in other countries to help companies and official control policies tackle fraudulent practices. In this paper, we set out to (1) explore the dataset of strawberries using PCA, (2) investigate the possible differences between years of production using LDA, (3) determine discriminant markers of Slovenian strawberries using OPLS-DA, and (4) establish a DD-SIMCA one-class classifier model to identify the possible misdeclaration of Slovenian strawberries.

2. Materials and methods

2.1. Samples

In total, 157 fresh strawberry samples were included in the study. To establish an appropriate database of authentic samples, 92 samples harvested in 2018 (35), 2019 (26) and 2020 (31) were obtained from the regional units of the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection from various geographical production fields within Slovenia. In aDDition, the database also included 32 foreign samples: Italy (18), Croatia (6), Spain (3), France (3), Greece (1) and Serbia (1), as well as 33 commercially available samples with declared Slovenian origin: 2018 (6), 2019 (12), and 2020 (15). Foreign and commercial samples were obtained from the market by government food inspectors, and their country of origin was obtained from the traceability documentation.

2.2. Sample preparation

A known weight of sample (250 g) was homogenised in a blender fitted with a titanium knife and afterwards divided into three parts. The first part was used for water extraction, where the sample was filtered through a GF/PVDF 45/25 filter (Macherey-Nagel, Chromofil, Germany) and stored at 4 °C until analysis. The second part obtained pulp extract by diluting approximately 35 mL of homogenised sample to 50 mL with Milli-Q water (Merck, Millipore, Watertown, MA, USA). The sample was then centrifuged at 3200 rpm for 10 min (Type Centric 322A, TEHTNICA, Slovenia). The supernatant was discarded, and the pulp was washed five times with Milli-Q water (40 mL, Merck, Millipore, Watertown, MA, USA) and three times with acetone (25 mL, Carlo Erba, Italy) before being freeze-dried (Liof Gamma 1–16 LSC plus, Christ, Germany). Finally, the third portion of the homogenised sample intended for multi-element analysis was transferred into a plastic container and freeze-dried before analysis.

2.3. Stable isotope and multi-element analysis

Determination of $\delta^{18}O$ in water from samples: The analytical protocol is based on the exchange of oxygen isotopes between water and CO_2 (Epstein & Mayeda, 1953). The method involves purging 200 µL of the sample with CO_2. Once equilibrated, the oxygen isotopic composition was measured using an isotope ratio mass spectrometer (IRMS, IsoPrime 100, IsoPrime, Cheadle, UK), connected to a Multi-flow Bio preparation system (Multi-Flow; IsoPrime, Cheadle, UK). Results were normalised against two laboratory reference materials seawater ($\delta^{18}O = +0.36 \pm 0.04$‰) and snow ($\delta^{18}O = -19.73 \pm 0.02$‰) and one international reference material, USGS53 ($\delta^{18}O = +5.47 \pm 0.03$‰). A laboratory reference material, Milli-Q water ($\delta^{18}O = -9.12 \pm 0.04$‰), was used for independent control.

Determination of $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ values in the pulp: Ratios of stable isotopes of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$) and sulphur ($^{34}S/^{32}S$) were determined in lyophilised pulp samples. Approximately 5 mg of each sample and 5 mg of WO_3 was transferred to a tin capsule. The capsule was then closed and placed in the sampler of elemental analyser. Determination of stable isotope ratios of light elements, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$ was performed simultaneously for each sample using an IRMS equipped with a preparative module for solid samples (IsoPrime100 - Vario PYRO Cube EA/CNS Pyrolyser/Elemental Analyser). All analyses were normalised against the following reference materials: USGS61 ($\delta^{13}C = -35.05 \pm 0.04$‰ and $\delta^{15}N = -2.87 \pm 0.04$‰); USGS89 ($\delta^{13}C = -18.07 \pm 0.07$‰, $\delta^{15}N = 6.25 \pm 0.10$ ‰ and $\delta^{34}S = 3.86 \pm 0.40$‰); USGS43 ($\delta^{13}C = -21.28 \pm 0.10$‰, $\delta^{15}N = 8.44 \pm 0.10$‰ and $\delta^{34}S = 10.46 \pm 0.22$ ‰); USGS91 ($\delta^{13}C = -28.27 \pm 0.07$‰, $\delta^{15}N = 1.78 \pm 0.10$‰ and $\delta^{34}S = -20.85 \pm 0.40$‰). Other reference materials included CRP-CASEIN ($\delta^{13}C = -20.3 \pm 0.09$‰, $\delta^{15}N = 5.62 \pm 0.19$‰, $\delta^{34}S = 4.18 \pm 0.74$‰), B2155 Protein Sercon ($\delta^{13}C = -26.98 \pm 0.13$‰, $\delta^{15}N = 5.94 \pm 0.08$‰, $\delta^{34}S = 6.32 \pm 0.80$‰), and IAEA-600 caffeine ($\delta^{13}C = -27.73 \pm 0.04$‰, $\delta^{15}N = 1.02 \pm 0.05$‰). Mean values were calculated from triplicate analyses. The reproducibility for $\delta^{13}C$ and $\delta^{15}N$ was ± 0.2‰, and ± 0.3‰ $\delta^{34}S$.

Elemental composition of strawberries: A known weight of the sample (0.15 g) was weighed into a Teflon digestion vial along with 1 mL of Suprapur 65% HNO_3. The sample was then subjected to microwave digestion (ULTRAWAVE, Single Reaction Chamber Microwave Digestion System, MILESTONE, Italy). The program was as follows: heating to 240 °C in 20 min and held for 15 min at max 100 bar at a max power of 1500 W. The digested solution was transferred into a polyethylene-graduated tube and diluted to 10 mL using Milli-Q water (Merck, Millipore, Watertown, MA, USA). Before analysis, samples were diluted with 5% HNO_3 in a ratio 1:5 and 1:10. Determination of selected elements (Na, Al, Mg, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Cd, Mo, Cs, Ba, Hg and Pb) were performed on an inductively coupled plasma triple quadrupole mass spectrometer (ICP-QQQ, Agilent 8800, CA, USA). Concentrations were referred to the dry samples. Quantification was achieved using external calibration, and every tenth sample was prepared in duplicate. Blanks and selected reference materials (Spinach Leaves NIST 1570, Tomato Leaves NIST 1573a, and Peach Leaves NIST 1547) were also prepared in the same way as the samples to ensure the quality of the results. Results of the accuracy of selected RM were collected in Appendix A – Table A.1. Detection limits were calculated based on three standard deviations of the blank measurements and were: for Na 2.4 µg/g, Mg 0.002 mg/g, Al 3 µg/g, P 0.0005 mg/g, S 0.003 mg/g, K 0.01 mg/g, Ca 0.04 mg/g, V 3.5 ng/g, Cr 15 ng/g, Mn 0.02 µg/g, Fe 0.5 µg/g, Co 1.2 ng/g, Ni 40 ng/g, Cu 0.2 µg/g, Zn 1 µg/g, As 3 ng/g, Se 3 ng/g, Rb 0.2 µg/g, Sr 0.2 µg/g, Mo 10 ng/g, Cd 1 ng/g, Cs 1 ng/g, Ba 0.2 µg/g, Hg 0.4 ng/g, and Pb 15 ng/g, respectively. A more detailed description of the optimisation of the method for elemental analysis can be found in (Potočnik, Jagodic Hudobivnik, Mazej, & Ogrinc, 2021).

2.4. Statistical analysis

The final elemental data were prepared in Microsoft Excel. Lead (Pb) was removed from the dataset since 72% of its concentrations were < LOD. Each value was then divided by the LOD and converted to its logarithm (log10) to compare concentrations over several orders of magnitude. The whole isotope and element dataset was further normalised to mean = 0 and standard deviation = 1 (auto-scaling) according to Richter et al. (2019).

For multivariate statistical analyses, different statistical procedures were used, including principal component analysis (PCA), linear discriminant analysis (LDA), orthogonal partial least squares discriminant analysis (OPLS-DA) and data-driven soft independent modelling of class analogy (DD-SIMCA).

PCA was used as an initial step in the multivariate analysis of the data since PCA is an unsupervised technique used for dimensionality reduction and provides information on the most representative features with a minimum loss of information. Also, PCA reduces the contribution of less significant variables and generates a new group of variables known as principal components/factors (Covaciu et al., 2016). Because PCA does not consider group membership, two supervised methods were used to obtain the best visualisation of group clusters: linear discriminant analysis (LDA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The methods were used to assign samples to predefined classes to classify new unknown samples. Thus, supervised methods rely on *priori* assumptions regarding sample groupings. LDA is one of the simplest classifiers, albeit to compute a centroid for each class, and the pooled variance–covariance matrix requires a sufficient ratio (≥3) between the number of samples and the number of variables. Also, LDA cannot cope with highly collinear data common in chemical problems (Marini, 2009).

Other techniques – in particular, PLS-DA have been devised to overcome these limitations. The resulting model is a linear model proven statistically equivalent to the solution obtained by LDA. Due to its being a latent vector-based technique, problems like a minimum sample to variable ratio and the absence of collinearity can be overcome (Marini, 2009). Alternatively, OPLS-DA is a modification of the PLS-DA method, which improves interpretability by filtering out any variation not related to the discriminating response by separating that part of the variance used for predictive purposes from the non-predictive variance, which is then made orthogonal (Rongai et al., 2017). This study evaluated performance using the explained variation (R^2X for PCA and R^2Y for OPLS-DA) and predictive ability (Q^2). Internal sevenfold cross-validation was then used to determine the significant components of the models and thus minimise overfitting.

The prediction performance of the OPLS-DA model was evaluated via sensitivity (true positives) and specificity (true negatives), calculated as described by Fiamegos et al. (2021). Accuracy (TP + TN)/(TP + FP + FN + TN) and F1 Score (F1 Score = 2* (Recall * Precision) / (Recall +

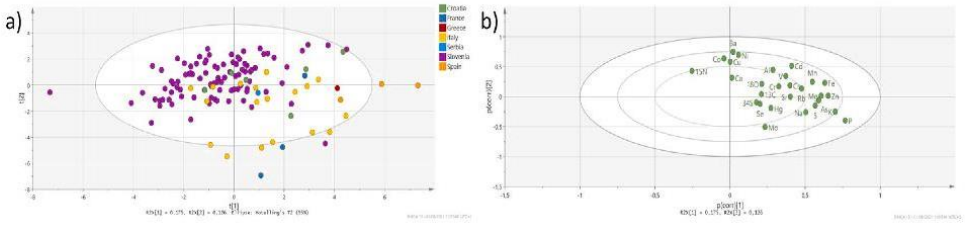

Fig. 1. a) PCA score plot for the 124 strawberry samples coloured based on their country of origin, b) Loading plot for the PCA model.

Precision)) of the model were calculated: Recall = TP/(TP + FN) and Precision = TP/(TP + FP) where TP is a true positive, TN a true negative, FP a false positive, and FN a false negative. Accuracy is the ratio of the correctly labelled subjects (samples) to the whole pool of subjects (samples) and the F1 Score is the harmonic mean (average) of the precision and recall. The best values for F1 Score are obtained if there is a balance between precision and recall in the system.

Candidates for discriminant markers were selected based on a variable's importance in the projection (VIP) values of the OPLS-DA models where a value higher than one was considered the threshold. PCA and OPLS-DA were obtained using SIMCA-P (version 15, Sartorius Stedim Biotech, Umeå, Sweden). LDA and the Kruskal Wallis test was carried out using the XLSTAT software package (ADDinsoft, New York, USA). Following PCA and OPLS-DA, a modification of the SIMCA method called data-driven SIMCA or DD-SIMCA (Pomerantsev & Rodionova, 2014a) was then used as a one-class classifier (OCC) technique.

DD-SIMCA consists of two steps: (1) principal component analysis (PCA) is applied to the training dataset, and (2) for each object from the training set, two distances are calculated: the score distance (SD) and the orthogonal distance (OD), and the corresponding critical limits. The SD represents the position of a sample within the score space, while the OD is the orthogonal Euclidean distance of the sample to the score space. DD-SIMCA adds the possibility of estimating the data-driven distribution parameters. Thus, it is possible to develop an acceptance area/decision rule for a given value (Pomerantsev & Rodionova, 2014a). The classification results are described as 'sensitivity' and 'specificity' or in standard statistical terms such as type I error (α) and type II error (β). When alternative classes are presented, the β-error can be calculated for a given α-error as described by Pomerantsev and Rodionova (2014b). The sensitivity denotes a share of correctly identified samples of the target class, while specificity is a portion of objects of an alternative class correctly identified as members of that alternative class. The sensitivity can be defined as 100 (1– α)% and specificity as 100 (1– β)% (Fidelis et al., 2017; Rodionova et al., 2016). DD-SIMCA calculations were performed using the DD-SIMCA MATLAB GUI tool (Zontov et al., 2017) and the mdatools package for R 4.0.3 (Kucheryavskiy, 2020).

3. Results and discussion

3.1. Principal component analysis (PCA)

The PCA method was applied to explore the whole dataset and reveal hidden data patterns. The first step was to use the distance to the model (DModX) method to detect outliers. In this case, a single outlier was detected but was not removed from the model. As a result, a model with three PCs was established, explaining 38.9% of the total variance. The PCA (Fig. 1 a) allowed the grouping of samples that correspond to the different geographical origins of the samples. It also suggests that separating certain Croatian and Italian samples from Slovenian samples may be impossible and is unsurprising given their geographical proximity.

The loading plot (Fig. 1 b) provides information about the elements that contribute most to the grouping of the samples in the PCA models. For example, Ba, Co, Ni and Cu play an essential role (higher levels) in grouping Slovenian samples. Also, δ^{15}N and Ca seem to be typical for Slovenian samples. Alternatively, the remaining variables were higher in the non-Slovenian samples and, in particular, P, K, Mo, Na, S, As, Zn and Mg are important in differentiating the non-Slovene samples.

3.2. Discriminant techniques

Linear Discriminant Analysis (LDA)

Since the dataset includes samples from three harvest years, linear discriminant analysis was applied to reveal possible differences (Fig. 2).

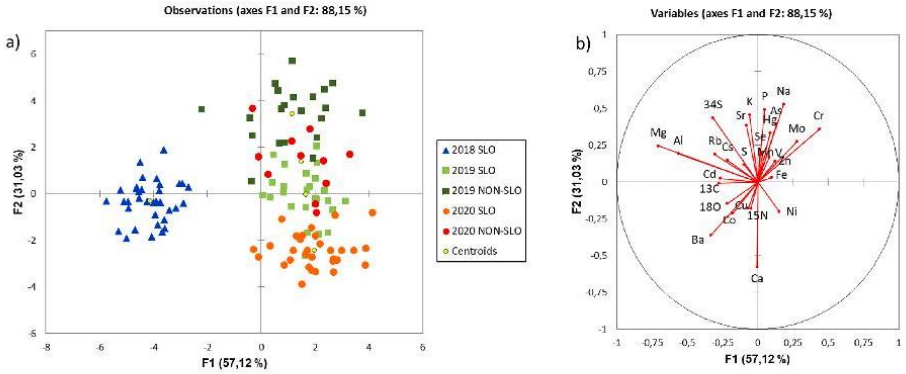

Fig. 2. Discriminant function score plot (a) and a discriminant loadings plot (b) for strawberry samples from Slovenia and other countries harvested in 2018 to 2020 (2018 SLO (n = 35); 2019 SLO (n = 26); 2020 SLO (n = 31); 2019 NON-SLO (n = 21); 2020 NON-SLO (n = 11)).

Table 1
Summary of OPLS-DA models for discerning Slovenian strawberry samples from non-Slovenian samples and production year.

Classification based on	Year	Number of components	R2Y	Q2(cum)	F1 Score (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
SLO vs NON-SLO	2018, 2019, 2020	1 + 3 + 0	0.72	0.57	96.8	98.9	84.4	95.2
SLO vs NON-SLO	2019	1 + 1 + 0	0.65	0.46	92.6	96.2	85.7	91.5
SLO vs NON-SLO	2020	1 + 1 + 0	0.75	0.37	96.9	100	81.8	95.2

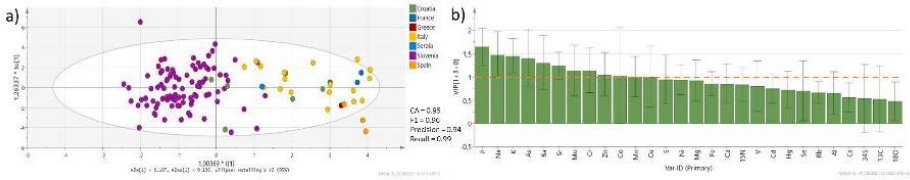

Fig. 3. OPLS-DA score plots (a) and VIP values (b) in the pairwise comparison between Slovenian and non-Slovenian strawberry samples. Samples are coloured based on their country of origin. The ellipse on the score plot represents the 95% confidence interval. A red-dashed line indicates the criteria used to identify the most important variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The first two discriminant components account for 88.1%, and five groups are visible in the LDA plots. The Slovenian samples from 2018 form the most distinct group. The most critical variables for classification were Mg, Al, $\delta^{13}C$, Rb and Ba, which were higher in the 2018 samples, while non-Slovene samples were higher in Na, Cr, P, As, Mo and Hg. The 2020 non-Slovene and 2019 Slovene groups were poorly separated (36.4%). The highest prediction ability (81.5%) was obtained for the 2018 Slovene group (100%). The third discriminant component explains 10.31% of the total variability and mostly contributes to discrimination between Slovenian and non-Slovenian samples. According to the Kruskal-Wallis test, the variables significant for separation between production years were $\delta^{18}O$, $\delta^{13}C$, $\delta^{34}S$, Na, Mg, Al, Ca, Cr, Rb, Mo, Cd, Ba and Hg.

4. Orthogonal partial least squares discriminant analysis (OPLS-DA)

Despite yearly differences, OPLS-DA was applied to analyse the observed differences between Slovene and non-Slovene samples in the PCA analysis and investigate the goodness of fit (R^2X) and prediction (Q^2) for the model. In the case of Slovenian vs non-Slovenian samples, a good OPLS-DA model was obtained, resulting in one predictive and three orthogonal components (1 + 3) producing an $R^2X = 0.40$, $R^2Y = 0.72$, and $Q^2 = 0.57$. The F1 Score rates, obtained by internal cross-validation, were between 92.6% and 96.9% for the 2018 and 2020 samples, respectively (Table 1).

The separation between classes in the OPLS-DA score plots is evident (Fig. 3), although specific Croatian and Italian samples are misclassified. The separation of these two classes (Fig. 3 b) is governed by P, Na, K, As, Ba, Sr, Mo, Cr and Zn (VIP > 1). According to the Kruskal-Wallis test, Mn, S, Fe, Ca, $\delta^{15}N$, Hg, V and Se are significant for separating these two groups, besides already mentioned elements. The medians and standard deviations of measured variables are given in Appendix B – Table B.1.

From the literature, Perez et al. (2006) found that P, Cu, Zn and Mg are important in classifying Oregon and Mexican strawberries, and increased levels of Cu in Mexican strawberries likely derive from atmospheric deposition, irrigation water, fertiliser, and soil amendments. Their study also showed that varietal and sub-regional differences did not affect geographic origin modelling when combined with elemental profiling. However, neither Cu nor Mg was influential in classifying Slovenian and non-Slovenian strawberries.

Perini et al. (2018) found that growing strawberries in high tunnels also affected $\delta^{18}O$ values and that production year had little effect on isotopic variability due to similar meteorological and climatic conditions in the studied years. Overall, however, they found that by comparing $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values, they could distinguish the different geographical origins of the samples. In the present study, $\delta^{15}N$ values were significant for distinguishing Slovenian from non-Slovenian strawberries, probably due to the different fertilisation procedures used. Also, although the production year influenced $\delta^{13}C$ and $\delta^{18}O$ variability, these two variables were not important in the OPLS-DA classification model with the reason being that all foreign countries are combined into a single non-Slovenian group, thereby eliminating any differences in $\delta^{13}C$ and $\delta^{18}O$ values between individual countries. This grouping was made because of the small number of non-Slovenian samples. Despite this, clustering was observed for Slovenian and non-Slovenian strawberries.

4.1. Modelling techniques

Data-Driven Soft Independent Modelling of Class Analogies (DD-SIMCA)

The drawback of discriminant methods is the necessity of the exhaustive representation of all classes. Unlike discriminant methods, DD-SIMCA distinguishes objects of a particular class, i.e., the target class (authentic SLO samples), from all other objects and classes, referred to as alternative sets (non-Slovenian samples). Information from the non-target samples, in this case, was used only for evaluating the model's performance. The target set was randomly divided into a training set (70% of data from the primary dataset) and a test set (30% of data from the primary dataset) to assess the model's validity. This roughly corresponds to a 4–5-fold cross-validation and represents a good compromise between having sufficient data to build a good model from the test dataset and the test dataset's error estimate (Gholamy, Kreinovich, & Kosheleva, 2018). Also, the Procrustes cross-validation (Kucheryavskiy, Zhilin, Rodionova, & Pomerantsev, 2020) was used for model optimisation since it is a better alternative to regular cross-validation for short datasets. Overfitting is where the model performs well on training data but poorly on data not seen during training. In our case, the extreme plot, which is sensitive to overfitting, was used to estimate model complexity regarding the test set and the optimal number of principal components. However, since validation of the model based on the extreme plot did not reveal any overfitting, modelling was performed first for each year separately and finally for all years together since the goal was to develop a robust DD-SIMCA model independent of the production year.

The exploratory data analysis was performed using the entire dataset with selected variables to explore the data structure and analyse the

Table 2
Summary of DD-SIMCA models used for distinguishing Slovenian from non-Slovenian strawberry samples and production year based on selected variables.

Year	Removed variables	Number of components	TP	FN	TN	FP	Sensitivity (%)	Specificity (%)	Accuracy (%)
2018	Pb	1	32	/	/	1	97.0	/	/
	$\delta^{18}O$, $\delta^{13}C$, Cr, Ni, Se, Rb, Hg, Pb	3	32	/	/	1	97.0	/	/
2019	Pb	2	25	4	17	1	96.2	81.0	89.4
	$\delta^{18}O$, $\delta^{13}C$, Cr, Ni, Se, Rb, Hg, Pb	2	26	6	15	0	100	71.4	87.2
2020	Pb	2	27	1	10	1	96.4	90.9	94.9
	$\delta^{18}O$, $\delta^{13}C$, Cr, Ni, Se, Rb, Hg, Pb	2	27	2	9	1	96.4	81.8	92.3
All	Pb	3	83	12	20	5	94.3	62.5	85.8
	$\delta^{18}O$, $\delta^{13}C$, Cr, Ni, Se, Rb, Hg, Pb	3	85	11	21	4	95.5	65.6	87.6

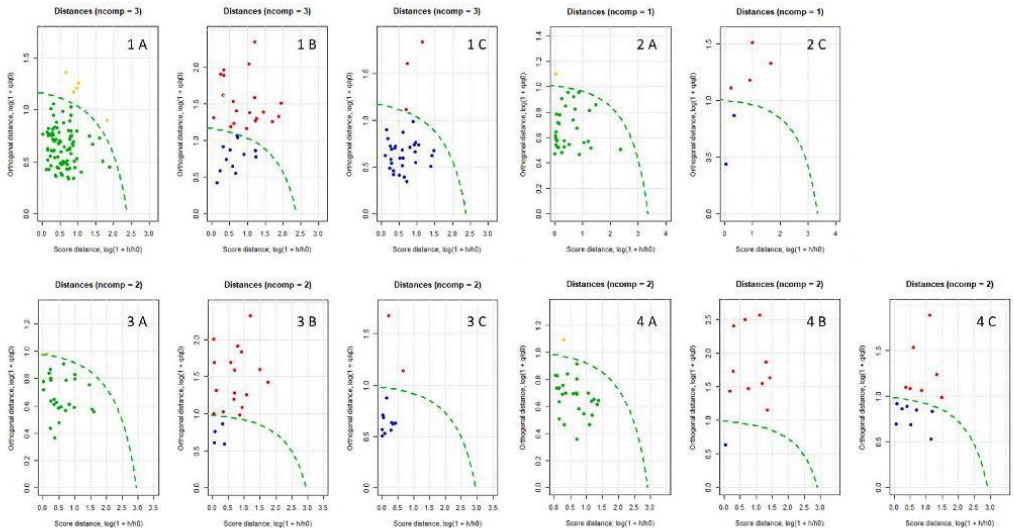

Fig. 4. DD-SIMCA classification model of a) authentic strawberry samples (green dots; 1A, three year model, n = 92; 2A, 2018 model, n = 35; 3A, 2019 model, n = 26; 4A, 2020 model, n = 31) cultivated in Slovenia, b) strawberry samples cultivated outside Slovenia (red dots; 1B, two year model, n = 32; 3B, 2019 model, n = 21; 4B, 2020 model, n = 11), c) test samples of strawberry from the market labelled as Slovenian (1C, three year model, n = 33; 2C, 2018 model, n = 6 ; 3C, 2019 model, n = 12; 4C, 2020 model, n = 15). The green dashed line represents the 100(1-α)% acceptance area, i.e. decision boundary whether the sample belongs to the target class or not. The green points in Fig. 4A represent the correctly identified samples of the target class. The orange points are wrongly rejected samples based on the α value. The red points in Fig. 4B represent the true positive values based on the β value while the blue points represent the wrong acceptance of foreign samples. Blue points in Fig. 4C represent correctly identified samples from the test set and red points represent the wrongly rejected samples based at the α level of 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

figure of merit (FoM) results concerning the number of PCs. A classification model was then built using the target set, the alternative set (samples from abroad) and the PCA model with a selected number of PCs. Sensitivity and specificity were used as figures of merit obtained from the target and alternative set (Table 2).

Excellent sensitivity was obtained for each year separately (96.2% to 97.0%) and good specificity (81% to 90.9%). However, since there were no foreign samples for 2018, specificity could not be obtained. When all years were included in a single model, sensitivity slightly reduced (94.3%); however, specificity was only 62.5%. Certain variables were removed to improve specificity following Arif et al. (2021). This method involves sequentially removing variables from the dataset (in this case, $\delta^{18}O$, $\delta^{13}C$, Cr, Se, Rb, Cs and Hg) and calculating a new figure of merit (FoM), i.e., if the new FoM is greater than the original FoM, the variable was removed from the model. The result was a slight improvement in sensitivity (94.3 to 95.5%) and specificity (62.5 to 65.6%).

Modelling revealed how important all variables are and how strawberry production depends on soil composition and is susceptible to environmental conditions. Significant year-to-year variations and overlapping between groups, observed in the LDA model, including many variables and a high number of samples, does not provide a robust model when all years are included. This result is exemplified when using the model to reveal possible mislabelling.

In response, a classification model was built using the target set (authentic Slovenian samples), a test set of samples from the market declared as Slovenian and the PCA model with selected PCs. When an object is similar to the other objects in a class, it will be located near them in the PC map. The benefit of SIMCA is that the test sample only belongs to the class with a high probability. Also, while the residual variance of an observation exceeds the upper limit for any modelled class in the data set, the sample is not assigned to any of the classes since it is either an outlier or belongs to a class that is not represented in the data set (Pirhadi et al., 2015).

The results show three strawberry samples fall outside the three-year model's acceptance area and are classified as non-Slovenian (Fig. 4 (1 C)). However, more samples with possible misclassification are uncovered when a year-to-year model is used. For example, in 2018, 4 out of 6 samples were potentially mislabelled a Slovenian (Fig. 4 (2 C)), in 2019,

2 out of 12 samples (Fig. 4 (3 C)) and in 2020, 7 out of 15 samples (Fig. 4 (4 C)). Overall, 39% of 33 samples in this data do not correspond to the declaration.

5. Conclusions

A new approach was successfully proposed and tested for the geographical classification and authentication of strawberries using discriminant analysis and class modelling of stable isotopes ($\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) and multi-elemental data. Conventional discriminant strategies, LDA and OPLS-DA, allowed the strawberry samples to be classified according to their origin, achieved good accuracy (95%). Distinguishing between production years was also possible. It also identified those variables important for separating Slovenian and non-Slovenian classes, i.e., P, Na, K, As, Ba, Sr, Mo, Cr and Zn (VIP > 1), and based on a Kruskal-Wallis test, additionally, Mn, S, Fe, Ca, $\delta^{15}N$, Hg, V and Se.

In addition, class modelling, using DD-SIMCA, was applied to individually model each strawberry harvest year and create a generalised model for all production years. The method was highly sensitive when applied to specific years, but unfortunately, the generalised model resulted in poor specificity (62.5%), which meant that it could not predict the origin of the samples. Also, significant year-to-year variations and overlapping between groups meant that DD-SIMCA could not provide a robust model when all years were combined.

Since a reduction in variables did not significantly improve the model specificity, each yearly model was used to investigate the origin of commercial samples with declared Slovenian origin. Overall, 39% of samples were potentially mislabelled. DD-SIMCA class modelling seems to be the recommended statistical treatment for the origin verification of Slovenian strawberries based on their isotopic composition and elemental profiles. Compared to standard discrimination approaches, DD-SIMCA is indifferent to the number of samples, the number of variables and importantly test sample only belongs to the class for which there is a high probability. In addition, discriminant approaches can have problem with classification of new samples which do not belong to any of the predefined classes. The first results are promising, but further investigation involving more production years should be addressed to establish a more robust general model with variables independent of the harvest year. Significantly, such a model could reduce the number of authentic samples required in the dataset. Equally, these methods will ensure confidence and trust in the local food supply chain's integrity and could be used in other authenticity studies.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132204.

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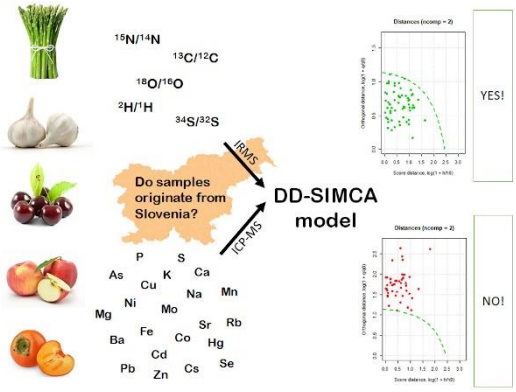
3.8 Scientific Paper: "Verifying the Origin of Slovenian Fruit and Vegetables Based on Isotopic and Elemental Profiles Using a One-Class Chemometric Model"



In this chapter, a paper entitled "Verifying the Origin of Slovenian Fruit and Vegetables Based on Isotopic and Elemental Profiles Using a One-Class Chemometric Model" by Lidija Strojnik, Doris Potočnik, Marta Jagodic Hudobivnik, Darja Mazej, Katja Babič, Boštjan Japelj, Andrija Ćirić, Nadja Škrk, Suzana Marolt, David Heath and Nives Ogrinc is presented. The paper is currently under review in the Journal of Agricultural and Food Chemistry. The study demonstrates how stable isotopes of light elements and elemental profiles combined with a one-class chemometric model DD-SIMCA can be used to verify the declared origin of selected vegetables (garlic, asparagus) and fruits (cherry, apple, kaki). Samples were obtained from the regional units of the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection from different geographical production areas in Slovenia between 2018 and 2020. Overall, the study includes 222 authentic samples collected directly from the field, 128 imported samples and 91 test samples from the market with Slovenian declaration.

Stable isotope ratios of light elements ($\delta\,^{13}$C, $\delta\,^{15}$N, $\delta\,^{18}$O, $\delta\,^{34}$S) and elemental composition of 25 elements (Na, Mg, Al, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, Cs, Ba, Hg and Pb) were first pre-processed for each fruit and vegetable separately and then used in DD-SIMCA one-class classifier model. The dataset was first divided into the target set (authentic Slovenian samples), alternative set (samples from abroad) and set of samples whose origin is was to be verified. A uniform sampling algorithm using a 70:30 split ratio was used to divide each fruit or vegetable's primary dataset (target set) into two subsets – a training set used for building the model and a test set used to assess its validity.

In the first step, exploratory data analysis was performed using the whole dataset with selected important variables to explore the data structure and analyse the figure of merit (FoM) results regarding the number of PCs. The sensitivity and specificity values were obtained from the training and test set validation. Based on FoM values, the optimal number of PCs was selected at which specificity was sufficiently large with satisfactory sensitivity. The second step classification model was created using the target set (of compliant samples) and the PCA model with a selected number of PCs. The alternative set (samples from abroad or non-compliant samples) was used for external validation. Although dependent on the number and diversity of samples in the dataset, it was possible to achieve good sensitivity and specificity except for cherry, where a lower specificity (66.7 %) was obtained. The isotopes and elements, which were the most important for classification, were also determined. The study found that Sr, Ba Cs, S, Mo, Ni, and Fe were the most important variables within the selected crops. These elements reflect soil composition and are most likely related to geology. Unlike trace elements, stable light isotopes create patterns that are more predictable since they are a product of geographically dependent processes. This study shows that δ^{18}O and δ^{13}C are important factors in all five crops.

Finally, the model was used to reveal the possible mislabelling of products on the Slovenian market. A classification model was built using the target set (authentic Slovenian samples), a test set of samples from the market declared as Slovenian and the PCA model with selected PCs. Twenty-five per cent of garlic, 37 % of asparagus, 30 % of cherry, 50 % of apple and 36 % of kaki samples were outside the acceptance area and were classified as non-Slovenian

In this study, I was responsible for developing the sampling strategy, sample handling, documentation, sample preparation (obtaining water from samples, pulp isolation and freeze-drying, stable isotope analysis (determination of $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$), and normalization of results. I also collected and pre-processed all of the stable isotope and elemental data. With the help of Dr. Japelj, I performed data analysis, interpreted the results, wrote and prepared the manuscript.

Article

Verifying the Origin of Slovenian Fruit and Vegetables Based on Isotopic and Elemental Profiles Using a One-Class Chemometric Model

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Abstract

This study demonstrates how stable isotopes of light elements and elemental profiles combined with a one-class chemometric model DD-SIMCA (Data-Driven Soft Independent Modelling of Class analogy) can be used to verify the declared origin of selected vegetables (garlic, asparagus) and fruits (cherry, apple, kaki) on the market. Samples were obtained from different geographical production areas in Slovenia from 2018 to 2020 for garlic, 2019 to 2020 for cherry and asparagus, while apples and kaki were collected in 2020. A database of 222 authentic Slovenian samples was used to develop the verification models, while 128 imported samples were used to assess their reliability. The models were then used to check the validity of 91 test samples from the market with declared Slovenian origin. The method proved highly sensitive and specific, ranging from 94.1% to 97.6% and 66.7 to 100%, respectively. The main important discriminating variables were $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, Na, S, Fe, Ni, Sr, Mo, Cd, Cs and Ba. Of the commercial samples with declared Slovenian origin, approximately 35% were classified as non-Slovenian. Overall, the presented methodology provides a firm basis on which an appropriate traceability system could be established.

Keywords: agricultural products; geographical origin; authenticity; stable isotope; element composition; DD-SIMCA

1 Introduction

The need to protect high-quality and locally produced fruit and vegetables is becoming more pronounced. Since most agri-food products only hold a reputation and marketability at the local level, they need to be protected locally, especially from the uncontrolled import of qualitatively inferior products labelled as 'local' or emanating from a specific geographic region. To accomplish this requires geographical authentication, which is the process of verifying a products declaration of origin. To date, several analytical techniques and various parameters have been studied in an attempt to verify the provenance of regional foods of which methods based on the stable isotope ratios of light elements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) and elemental composition are the preferred choice for geographical origin authentication (Katerinopoulou et al., 2020; Kelly et al., 2005).

Stable isotope ratios of light elements allow the relationships between isotope ratios and the fractionation processes associated with local climate data, as well as plant and animal physiology, geology, and pedology, to be analysed. This approach is based on transferring isotopic signals of light elements (H, C, N, O and S) from different natural sources (water, soil, atmosphere) to plant or animal tissues. For instance, the ratio of stable water isotopes ($^{2}H/^{1}H$ and $^{18}O/^{16}O$) can provide key information on water origins (e.g., local precipitation, surface water, groundwater) that depend on latitude, altitude, distance from the sea, amount of precipitation and the degree of evapotranspiration (Gat, 2003). In plants, the carbon isotope ratio ($^{13}C/^{12}C$) is mainly regulated by the plants' specific CO_2-fixation pathway (e.g. C3 or C4 plants). However, other factors include atmospheric CO_2 concentration, plant variety, plant physiology, nutritional status of the cell, plant growth rate, water-use efficiency, and cultivation practices. While $^{15}N/^{14}N$ isotope ratios have been widely used to distinguish between organic and conventional products, it has also proven valuable in geographical origin studies (Gatzert et al., 2021). The distinctive $^{15}N/^{14}N$ isotope ratios of plants depend on the soil's $^{15}N/^{14}N$ isotope ratios, which is influenced by the local climate, general soil conditions, long-term soil treatment, and land use (Camin et al., 2011). The $^{34}S/^{32}S$ isotope ratios reflect geology, volcanism, the influence of sea spray (i.e., distance from the sea), and specific anthropogenic effects, e.g. such as the source of atmospheric SO_2 (Danezis et al., 2016), can also act as local markers (Gatzert et al., 2021). The verification of regional origin can be even more effective when stable isotopes are combined with elemental composition since a plant's elemental profile is related to soil characteristics (pH, moisture, elemental composition), water availability and climate (Perini et al., 2018).

Using an isotope and elemental approach to determine the geographic origin of food requires establishing a traceability system based on a database of authentic products with known elemental and isotopic compositions against which an unknown food sample can be compared. However, a comparative analysis alone cannot provide a definitive answer concerning the geographical origin of an unknown sample, only that its isotopic ratios and elemental composition are consistent (or not) with authentic samples from the same geographical location. In most cases, verifying compliance with a given specification could be provided by applying appropriate classification strategies. For instance, given the possibility to sample meaningfully two or more classes, discriminant classification methods (e.g., linear discriminant analysis – LDA, k-nearest neighbours, partial least squares-discriminant analysis – PLS-DA, artificial neural network – ANN) are the most appropriate solution. Conversely, when the focus is on whether or not a specific product agrees with its

declaration, a class-modelling approach, such as SIMCA (soft independent modelling of class analogies) or UNEQ (unequal class modelling) techniques are more appropriate (Marini, 2009). Despite this, in more than half of food authentication studies, discriminant methods are used. The problem is that when discriminant methods are forcedly used for one class problems, the target class (of compliant samples) is usually representatively sampled. In contrast, the non-target class (non-compliant samples) is often not adequately defined and is poorly sampled, introducing a bias in the outcomes (Oliveri, 2017). Unlike discriminant methods, class-modelling methods or one-class classifiers (OCC) are meant to distinguish one particular class of objects, i.e., the target class, from the other objects and classes. This process is similar to a non-targeted analysis of adulterants since the acceptance boundaries are developed around the authentic samples without the need for an authentic adulterant training sample. However, access to authentic samples could be sometimes limited due to environmental or economic conditions. Nevertheless, one-class approaches are considered robust even when dealing with highly unbalanced datasets composed of high-dimensional noisy feature space (Zheng et al., 2013).

SIMCA is one of the OCC methods widely used in chemometrics. The original version of SIMCA has undergone numerous modifications, primarily related to constructing the acceptance boundary. Since discriminant analysis is inappropriate for authentication studies (Oliveri, 2017; Rodionova, Titova, et al., 2016), many studies have begun implementing the data-driven version of SIMCA (DD-SIMCA) as a class-modelling approach (Arif et al., 2021; de Araújo et al., 2021; de Oliveira Moreira & Braga, 2021; Faqeerzada et al., 2020; Fidelis et al., 2017; Gomes et al., 2021; Maciel Gerônimo et al., 2021; Mazivila et al., 2020; Mohammed & Shuming, 2021; Neves & Poppi, 2020; Rodionova, Oliveri, et al., 2016; Wang et al., 2020). Part of the reason is that the misclassification errors can be calculated theoretically (Zontov et al., 2017).

Recent applications that combine stable isotope ratios with multi-elemental analyses include the geographical origin determination of tomato and lettuce (Mahne Opatić et al., 2017; Spalla et al., 2009), potato (Mahne Opatić et al., 2018; Zampella et al., 2011), sweet pepper (Mahne Opatić et al., 2017), strawberry (Covaciu et al., 2016; Perez et al., 2006), blueberry, pear (Perez et al., 2006), mango (Muñoz-Redondo et al., 2021), apple (Bat et al., 2012) and garlic (Liu et al., 2018; Opatić et al., 2017). However, such studies have yet to apply DD-SIMCA, and limited data exist in its use in this field. For this reason, we decided to investigate whether stable isotopes combined with multi-elemental composition are appropriate for building a classification model capable of providing reliable predictions of new unknown samples, i.e., if examples of popular Slovenian fruit and vegetables correspond to their declaration of geographical origin.

In this study, we selected asparagus (*Asparagus officinalis L.*), garlic (*Allium sativum L.*), cherry (*Prunus avium L.*), apple (*Malus domestica Borkh.*), and kaki (*Diospyros kaki L.*) fruits because of their economic and regional relevance in Slovenia. Furthermore, it allows the possibility to promote the production and consumption of high-nutrient – sometimes discontinued – products. Although Slovenia is a relatively small country (20 273 km^2) with a low level of self-sufficiency, especially regarding fruit (30%) and vegetables (48%) (*Podatkovna Baza SiStat*, n.d.), locally produced varieties have become more appreciated by the Slovenian consumer. In parallel, such fruits and vegetables have become more vulnerable to food fraud.

We also deliberately selected fruits and vegetables, which differ in climate, soil conditions, fertiliser application, irrigation water, harvest time and storage conditions, to test how the variability in isotopic ratios

and elemental profiles influence our statistical models for characterising geographical origin. Garlic, for example, is a vegetable that grows underground year-round, and its elemental profile is closely connected to the soil type and quality and the geology of the location where it is grown (D'Archivio et al., 2019; Smith, 2005; Vadalà et al., 2016). Pianezze et al. used stable isotope ratios to determine the geographical origin of Italian red garlic (Pianezze et al., 2019). The authors found that garlic from nearby sources could be differentiated according to their oxygen and hydrogen isotopic composition. In Slovenia, Mahne Opatić et al. were the first to establish an isotopic and elemental database of garlic (Opatić et al., 2017). Using discriminant analysis, they used their database to discriminate between distinct growing regions in Slovenia and obtained an overall classification rate of 77%. However, the authors did not question the provenance of garlic on the market labelled as Slovenian and the study includes only organically produced garlic. Liu et al. also used stable isotopes and trace elements to discriminate between garlic from different Asian countries (Liu et al., 2018). They concluded that despite no single trace element that could act as a specific country marker, trace-element concentrations are "*fundamental to PCA being able to discriminate garlic's country of origin*".

Asparagus was included in this study because of its limited harvesting period, which lasts only 7-8 weeks during spring. Its production also requires a specific soil composition and is grown in the same spot for up to 20 years (Pegiou et al., 2020). Despite the popularity of asparagus, authenticity data are scarce. Richter et al. used elemental profiling and machine learning to discriminate between samples of white asparagus from Germany, Poland, the Netherlands, Greece, Spain, China and Peru (Richter et al., 2019). Their approach resulted in a 91.2% prediction accuracy (98% on subsets of samples with high support vector machine (SVM) scores).

Apple trees are cultivated worldwide with more than 7,500 known cultivars (cultivated varieties). Its economic importance has meant that apple fruit, apple juice and pomace have been investigated more frequently in authenticity studies, including stable isotope approach, and in some cases, combined with elemental composition (Bat et al., 2012; Bizjak Bat et al., 2016; Ogrinc et al., 2009). For example, Mimmo et al., 2015 who built a multivariate classification model based on linear discriminant analysis (LDA) of the $\delta^{13}C$ and $\delta^{15}N$ values of the fruit and sub-fractions, i.e., peel, seed and pulp, were capable of discriminating (99% classification rate) between apples grown in orchards just a few hundred kilometres apart in northern Italy.

In Slovenia, cherries are produced all over the country, but the majority, and the most appreciated ones, are produced in the Brda region located close to the border with Italy. The problem is that Italy is also a known cherry producer, and there is a high risk of mislabelling. Unfortunately, the discrimination of cherries from both origins has not been attempted. However, in the only paper to investigate the geographical origin of cherry, Longobardi et al., 2015 describe how cherries from two regions of Italy: Emilia Romania and Apulia, could be successfully discerned (92.3% classification rate) using a combination of isotopic fingerprinting and an electronic nose.

The last selected crop is kaki, a common and increasingly popular fruit among consumers in the Mediterranean region and represents an important local crop. Unlike many other widely cultivated fruit trees, it requires minimal care and intervention (Bellini et al., 2008). Kaki fruit matures in late autumn and can stay on the tree until winter. To our knowledge, only one study on determining the geographic origin of kaki was performed by Mir-Marqués et al., 2015. By analysing the mineral profiles of 167 samples of kaki from across

153 Spain, the authors concluded that differences between samples from different Spanish regions (using ANOVA)
154 could offer a way to authenticate the origin of PDO samples.
155 In the present study, we use a stable isotope and elemental approach combined with DD-SIMCA as a
156 'screening' tool to combat mislabelling of selected Slovenian fruits and vegetable products. Our aims were to
157 (i) create an appropriate DD-SIMCA one-class classifier model for regionally important fruits and vegetables
158 based on authentic samples; (ii) identify the most important isotope and elemental variables for classification,
159 and (iii) use the model to verify the origin of garlic, asparagus, cherries, apples and kaki declared as Slovenian.
160 Although the paper deals with selected fruits and vegetables, the methods and approaches can be readily
161 adapted to newly identified priority food products and transferable to other countries of interest.
162

163 **2 Materials and Methods**

164 *2.1 Samples and sample preparations*

165 Samples of selected vegetables (garlic, asparagus) and fruits (cherry, apple, kaki) were obtained
166 from the regional units of the Administration of the Republic of Slovenia for Food Safety, Veterinary
167 and Plant Protection, from different geographical production areas in Slovenia between 2018 to 2020
168 for garlic, 2019 to 2020 for cherry and asparagus, while apples and kaki were collected in 2020. While
169 Slovenia is a small country (20 273 km2), 20 to 30 samples taken from all production areas have been
170 sufficient to populate the database. The number of non-compliant samples (samples from abroad)
171 was limited with the market availability. Overall, the study includes 222 authentic samples collected
172 directly from the field, 128 imported samples and 91 test samples from the market with Slovenian
173 declaration (Table 1). Due to the high validity of the data, particularly the guaranteed authenticity of
174 the samples - all samples have been collected from primary producers, i.e., directly from the plant by
175 impartial collectors (inspectors), the established database is considered to be of outstanding quality.
176

177 **Table 1.** Number of authentic Slovenian samples (Authentic SLO), samples from foreign countries (Abroad) and samples taken from the market for verification of declared Slovenian
178 origin for selected vegetables (garlic, asparagus) and fruits (cherry, apple, kaki) per harvest year.

179

Garlic	2018	2019	2020	Total	Asparagus	2019	2020	Total	Cherry	2019	2020	Total	Apple	2020	Total	Kaki	2020	Total
Authentic SLO	**29**	**24**	**10**	**63**	**Authentic SLO**	**22**	**30**	**52**	**Authentic SLO**	**24**	**30**	**54**	**Authentic SLO**	**27**	**27**	**Authentic SLO**	**26**	**26**
Test SLO	**1**	**18**	**1**	**20**	**Test SLO**	**5**	**14**	**19**	**Test SLO**	**7**	**16**	**23**	**Test SLO**	**18**	**18**	**Test SLO**	**11**	**11**
Abroad	**8**	**32**	**9**	**49**	**Abroad**	**17**	**12**	**29**	**Abroad**	**17**	**10**	**27**	**Abroad**	**9**	**9**	**Abroad**	**14**	**14**
Argentina		1		1	Croatia	1		1	Greece	6	3	9	Chile	1	1	Italy	2	2
Egypt	1	4	1	6	Italy	11	12	23	Croatia	1	1	2	France	2	2	South Africa	1	1
France		5		5	Peru	1		1	Italy	5	5	10	Germany	1	1	Spain	11	11
Croatia	1	2		3	Spain	4		4	Spain	4		4	Italy	2	2			
Italy		3		3					Serbia		1	1	New Zealand	1	1			
China	1		1	2					Turkey	1		1	Serbia	2	2			
Mexico		1		1														
Spain	5	16	7	28														
Total	**38**	**74**	**20**	**132**	**Total**	**44**	**56**	**100**	**Total**	**48**	**56**	**104**	**Total**	**54**	**54**	**Total**	**51**	**51**

180

Sample preparation: At least 250 g of each fruit and vegetable were used for analysis. For elemental analysis, the samples were first freeze-dried, while for water extraction, samples were homogenised in a blender with a titanium knife and the juice filtered through GF/PVDF 45/25 filters (Chromofil) and stored in refrigeration until analysis. For pulp separation, approximately 35 mL of homogenised sample was diluted (50 ml) with pure water (Millipore). The sample was then centrifuged at 3200 rpm for 10 min (Type Centric 322A). Following the procedure of Rossmann et al. [45], the supernatant was discarded. The pulp was additionally washed five times with deionised water (40 mL, Millipore) to remove free sugars and three times with acetone (25 mL, Carlo Erba) to remove lipids (according to the *SIST ENV 13070:1999 - Fruit and Vegetable Juices - Determination of the Stable Carbon Isotope Ratio*, n.d.) and freeze-dried. The pulp was selected based on the experience from the previous study performed by Jamin et al., where the authors show that the isotopic signature of the pulp is related to the provenience of the fruit (Jamin et al., 1998).

2.2 Stable isotope and multi-element analysis

Stable isotope composition is reported as δ-values, i.e., as deviations from an international standard using the following equation:

$$\delta^i E = \left(\frac{R\left({}^{i}E / {}^{j}E\right)_{sample}}{R\left({}^{i}E / {}^{j}E\right)_{standard}} \right)$$

where superscripts i and j are the higher and lower atomic masses of analysed element E ($^2H/^1H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$, $^{34}S/^{32}S$), while subscripts' sample' and 'standard' refers to analysed sample and the reported reference material, respectively. The data are reported as per mil (‰) versus the following standards: 'Vienna Standard Mean Ocean Water' for H and O; 'Vienna PeeDee Belemnite' for C; atmospheric N2 (air) for N and 'Vienna Canyon Diablo Troilite' for S.

Determination of $\delta^{18}O$ in water samples: The analytical protocol is based on the exchange of oxygen isotopes between water and CO_2 (Bat et al., 2012). For this, 200 µL of the sample was placed in a vial and purged with CO_2. After equilibration, the isotopic composition of oxygen was measured using an isotope ratio mass spectrometer, IRMS (IsoPrime 100, IsoPrime, Cheadle, UK), connected to a Multi-flow Bio preparation system (Multi-Flow; IsoPrime, Cheadle, UK). Results were normalised against seawater ($\delta^{18}O$ = +0.36 ± 0.04‰), snow ($\delta^{18}O$ = −19.73 ± 0.02‰) and USGS53 ($\delta^{18}O$ = +5.47 ± 0.03‰). A laboratory reference material, Milli-Q water ($\delta^{18}O$ = −9.12 ± 0.04‰), was used as an independent control.

Determination of $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ values: $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ values were determined in lyophilised pulp samples (cherry, apple and kaki) or in the lyophilized sample (asparagus, garlic). A known amount of pulp (5 mg) and 5 mg of WO_3 was transferred to a tin capsule. Measurements were then performed simultaneously using an isotope ratio mass spectrometer (IRMS) equipped with a preparative module for solid samples (IsoPrime100 - Vario PYRO Cube EA/CNS Pyrolyser/Elemental Analyser). All analyses were normalised against the following reference materials: USGS61 ($\delta^{13}C$ = -35.05 ± 0.04‰ and $\delta^{15}N$ = -2.87 ± 0.04‰); USGS89 ($\delta^{13}C$ = -18.07 ± 0.07‰, $\delta^{15}N$ = 6.25 ± 0.10 ‰ and $\delta^{34}S$ = 3.86 ± 0.40‰); USGS43 ($\delta^{13}C$ = -21.28 ± 0.10‰, $\delta^{15}N$ = 8.44 ± 0.10‰ and $\delta^{34}S$ = 10.46 ± 0.22 ‰); USGS91 ($\delta^{13}C$ = -28.27 ± 0.07‰, $\delta^{15}N$ = 1.78 ± 0.10‰ and $\delta^{34}S$ = -20.85

± 0.40‰). Other reference materials included CRP-CASEIN ($\delta^{13}C$ = -20.3 ± 0.09‰, $\delta^{15}N$ = 5.62 ± 0.19‰, $\delta^{34}S$ = 4.18 ± 0.74‰), B2155 Protein Sercon ($\delta^{13}C$ = −26.98 ± 0.13‰, $\delta^{15}N$ = 5.94 ± 0.08‰, $\delta^{34}S$ = 6.32 ± 0.80‰), and IAEA-600 caffeine ($\delta^{13}C$ = -27.73 ± 0.04‰, $\delta^{15}N$ = 1.02 ± 0.05‰). Each sample was analysed in triplicate, and the mean value calculated. The reproducibility for $\delta^{13}C$ was ±0.2‰, and ±0.3‰ for $\delta^{15}N$ and $\delta^{34}S$.

Elemental composition of fruits and vegetables: A known weight of the freeze-dried sample (0.15 g) was weighed into Teflon tubes along with 1.5 mL Suprapur 65% HNO_3. Samples were subjected to microwave digestion (ULTRAWAVE, Single Reaction Chamber Microwave Digestion System, MILESTONE). The solution was quantitatively transferred into 10 mL polyethylene graduated tubes and filled to the mark with Milli-Q water. Before measurement by ICP-MS, samples were additionally diluted with 5% HNO_3 if needed. Quantification was done by external calibration, and every tenth sample was prepared in duplicate. Blanks and reference materials were prepared in the same way as the samples. Detection limits were calculated based on three standard deviations of the blank measurements, and accuracy was checked using Spinach Leaves NIST 1570, Tomato Leaves NIST 1573a, and Peach Leaves NIST 1547 certified reference materials.

2.3 Statistical analysis

For statistical analysis, we used a modified version of the well-known soft independent modelling of class analogy (SIMCA) method called data-driven SIMCA or DD-SIMCA (Pomerantsev & Rodionova, 2014a) as a one-class classifier (OCC) technique. DD-SIMCA consists of two steps (1) principal component analysis (PCA) is applied to the training dataset, and (2) for each object from the training set, two distances are calculated: the score distance (SD) and the orthogonal distance (OD) and corresponding critical limits. The SD represents the position of a sample within the score space, and the OD characterises the orthogonal Euclidean distance of the sample to the score space. DD-SIMCA adds the possibility of estimating the data-driven distribution parameters. In this way, it is possible to develop an acceptance area/decision rule (Pomerantsev & Rodionova, 2014b). The classification results are described in terms of 'sensitivity' and 'specificity' or using terms such as type I error (a) and type II error (b). Sensitivity represents the proportion of correctly identified samples in the target class, whereas specificity is the portion of objects from the alternative class correctly identified as members of that alternative class, i.e., sensitivity is 100 (1–a)% and specificity as 100 (1–b)% (Fidelis et al., 2017; Oliveri, 2017; Rodionova, Titova, et al., 2016). All calculations were performed using DD-SIMCA – A MATLAB GUI tool (Zontov et al., 2017) and the mdatools library in the R 4.0.3 programming language (Kucheryavskiy, 2020).

3 Results and discussion

Data pre-processing: elemental concentrations were prepared in Microsoft Excel. Elements with > 30% of their concentrations were < LOD were removed. This included Al (52%), V (71%), Cr (70%), Hg (78%) and Pb (92%) for garlic; Hg (54%) and Pb (73%) for asparagus; Na (36%), Al (62%), V (38%), Cr (74%), As (74%), Se (72%), Cd (48%), Hg (78%) and Pb (92%) for cherry; Al (31%), As (75%), Se (87%), Cd (38%), Hg (65%) and Pb (89%) for apple, and Al (75%), V (79%), Cr (33%), As (69%), Se (71%), Cd (69%), Hg (31%) and Pb (94%) for kaki. Unfortunately, $\delta^{34}S$ could not be analysed due to too low concentrations in apple and kaki samples. Each value

was divided by LOD and converted to its logarithm (log10) to compare concentrations over several orders of magnitude. The data were further normalised to mean=0 and standard deviation=1 for each element (auto-scaling) (Richter et al., 2019)

3.1 One-class classification (DD-SIMCA): exploratory data analysis

For DD-SIMCA, the dataset was first divided into the target set (authentic Slovenian samples) and alternative set (samples from abroad). Moreover, a uniform sampling algorithm using a 70:30 split ratio was used to divide each fruit or vegetable's primary dataset (target set) into two subsets – a training set used for building the model and a test set used to assess its validity. This would roughly correspond to 4-5-fold cross-validation and makes a sensible compromise between having sufficient data to build a good model from the test dataset and estimating the accuracy of the test dataset's (Gholamy et al., 2018). Information from the non-compliant samples (from abroad) was used only to evaluate the model's performance and not for building the model.

The exploratory data analysis was carried out using the whole dataset with selected important variables to explore the data structure and analyse the figure of merit (FoM) results concerning the number of PCs. In this step, possible outliers and extreme samples were detected and were removed from the model. The sensitivity and specificity values were obtained from the training and test set validation, and based on FoM values (Figure 1), the optimal number of PCs was selected as two for garlic, three for asparagus and one for kaki samples. In the case of cherry and apple, we found that six and five were the optimal numbers of PCs for the final DD-SIMCA, respectively. Our goal was to select the number of PCs at which specificity is large enough and sensitivity is satisfactory. It has been reported that the selection of too many PCs could lead to an overfitted model. Overfitting is a scenario where the model performs well on training data but performs poorly on data not seen during training. The extreme plot that is sensitive to overfitting has been used to estimate the model complexity regarding the test set and selection of an optimal number of principal components to explain a sufficient % of the total variance. Validation of the model based on the extreme plot showed that the models were not overfitted.

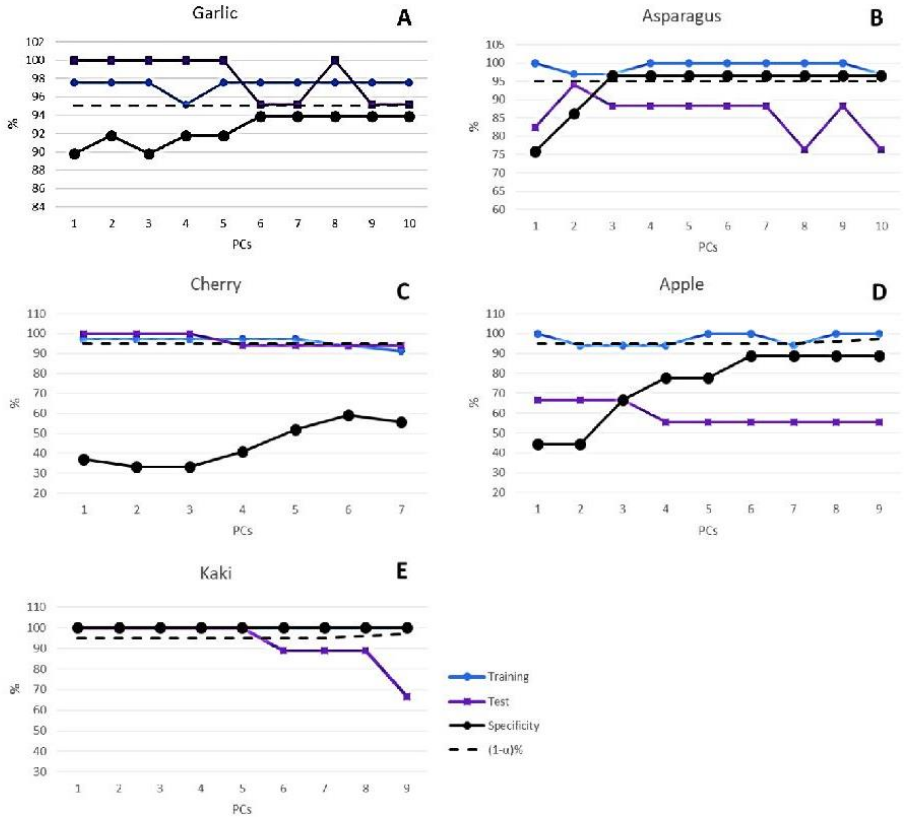


Figure 1. Plots showing the figures of merit (FoM) for each validation test set: garlic (A), asparagus (B), cherry (C), apple (D) and kaki (E) samples.

3.2 One-class classification (DD-SIMCA): establishment of models and selection of important variables

In this step, the classification model was created using the target set (of compliant samples), the alternative set (samples from abroad or non-compliant samples) and the PCA model with a selected number of PCs (Table 2). The acceptance area α was 0.05, which meant that one out of every 20 samples is likely to be classified as extreme. All objects within this area are considered members of the target class. Table 2 shows the archived sensitivity and specificity values derived from the target and alternative sets and described in separate subsections below. For the means and standard deviations of the measured variables, see Appendix A.

297 **Table 2.** Sensitivity and specificity values obtained by DD-SIMCA for α = 0.05 and selected No of PCs.

DD-SIMCA	Model parameters	Sensitivity	Specificity
	α = 0.05		
Garlic	2 PCs	97.6	91.8
Asparagus	3 PCs	95.9	96.6
Cherry	6 PCs	94.1	66.7
Apple	5 PCs	96.2	88.9
Kaki	1 PCs	96.3	100

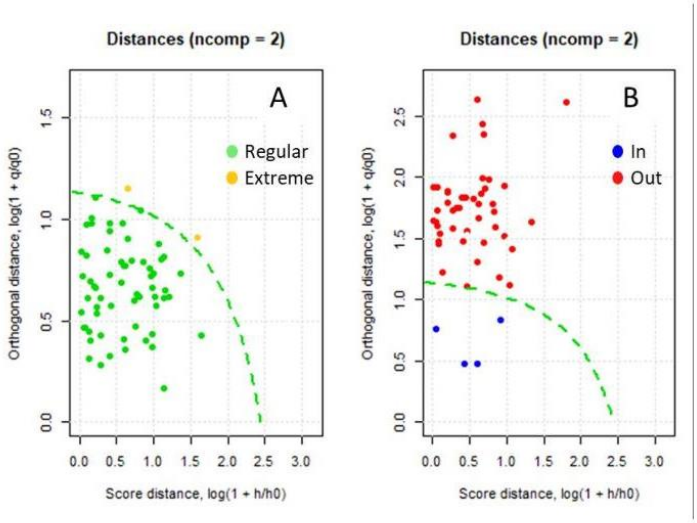
298

299 To determine which isotopes and elements are the most important in the classification model, the authors
300 followed the method Arif et al., 2021. First, the PCA loading plot was analysed using the entire dataset. The
301 variables important for classification (VIC) were identified as follows. Variables were successively removed
302 from the dataset, and a new figure of merit (FoM) calculated. This new FoM was then compared with the
303 original FoM. If the new FoM was lower than the original (VIC<0), the variable is considered important for the
304 model, whereas if FoM remained unaltered (VIC = 0), the variable is considered not influential but kept in the
305 model. However, if the new FoM is greater than the original FoM (VIC>0), the variable is removed from the
306 model.

307

308 3.2.1. GARLIC

309 Sixty-one of the Slovenian garlic samples were correctly classified, and two were extreme (Figure 2a). The
310 sensitivity achieved is 97.6%. The results of non-Slovenian garlic samples are shown in Figure 2b. Out of the
311 49 samples, 45 are outside the acceptance area and are correctly classified as non-Slovenian (specificity =
312 91.8%).



313
314 **Figure 2.** DD-SIMCA classification model of a) authentic garlic samples (green dots; *n* = 63) cultivated in Slovenia, b) garlic
315 samples (red dots; *n* = 49) cultivated outside Slovenia. The green line signifies the acceptance area at α = 0.05.

316 The most significant variables for discriminating between Slovenian and non-Slovenian garlic were $\delta^{18}O$,
317 $\delta^{34}S$, Na, S, K, Sr, Cd and Ba (VIC = -1). Those variables that were not influential (VIC = 0) but were kept in the
318 model were $\delta^{13}C$, P, Ni, while those removed from the model (VIC = 1) included $\delta^{15}N$, Mg, Ca, Mn, Fe, Cu, Zn,
319 Rb, Co, As, Mo, Se, and Cs.

320 Opatić et al., 2017 who investigated the use of stable isotope ($\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) and multi-element (P,
321 S, Cl, K, Ca, Zn, Br, Rb, Sr) in Slovenian garlic found that the main parameters that differentiate the garlic
322 samples' origin were $\delta^{13}C$, $\delta^{34}S$, Zn, P and Cl. In Vadala et al.'s study of garlic from Spain, Italy (Sicily), and
323 Tunisia, the authors found that Ni and Fe concentrations could serve as a biomarker for geographical origin.
324 Nickel was not an influential variable in our study, and the model's specificity was reduced when Fe was
325 included. Vadalà et al., 2016 also suggest that the high content of Se in the Nubia red garlic variety from Sicily
326 acts as a potential marker for discriminating the geographical origin of this cultivar, but again, Se was not
327 important in our model. D'Archivio et al., 2019 and Pianezze et al., 2019 also studied the geographical
328 discrimination of red garlic coming from Italy. However, whereas they found that Ba, Ca, Fe, Mg, Mn, Na and
329 Sr were important in class modelling when using SIMCA (D'Archivio et al., 2019), only Ba, Na, and Sr were
330 important in our study. Pianezze et al., 2019)found that the most influential variables $\delta^{34}S$, $\delta^{2}H$ and $\delta^{18}O$.
331 Although $\delta^{13}C$ was a non-significant parameter in our case, it was included in the model, while $\delta^{15}N$ was not
332 important. Similar results were obtained by Nie et al., 2021, who studied isotopes and chemometrics to
333 discriminate PGI Chinese garlic from others. Their results are similar to this study, although $\delta^{2}H$ was not
334 analysed. Since garlic's elemental profile is strongly dependent on the soil's composition, it is not surprising
335 that each study identified different important variables since each study looked at a specific growing region;
336 however, stable isotopes are more robust and, therefore, important in class modelling.
337
338 3.2.2. ASPARAGUS

339 Forty-seven Slovenian asparagus samples were correctly classified, and two were extreme (Figure 3a). The
340 sensitivity was 95.9%. The results of non-Slovenian asparagus samples are shown in Figure 3b. Out of 29
341 samples, 28 are outside the acceptance area and are correctly classified as non-Slovenian (specificity of
342 96.6%). The most discriminating variables were $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, Na, Mg, S, Ca, Mn, Fe, Co, Ni, Cu, As, Se,
343 Rb, Sr, Mo, Cd, Cs, Ba. The selection of variables is quite extensive; however, all the important elements for
344 discriminating garlic were also important for asparagus, except for K, Al, Zn, V and Cr, which were removed
345 from the model. Phosphorous was not influential and kept in the model. Besides stable isotopes, we expect
346 mainly those elements related to soil composition, given that asparagus grows only for a specific time
347 underground and at the same location for many years. Richter et al. also observed a similar elemental profile
348 to this study (Richter et al., 2019).

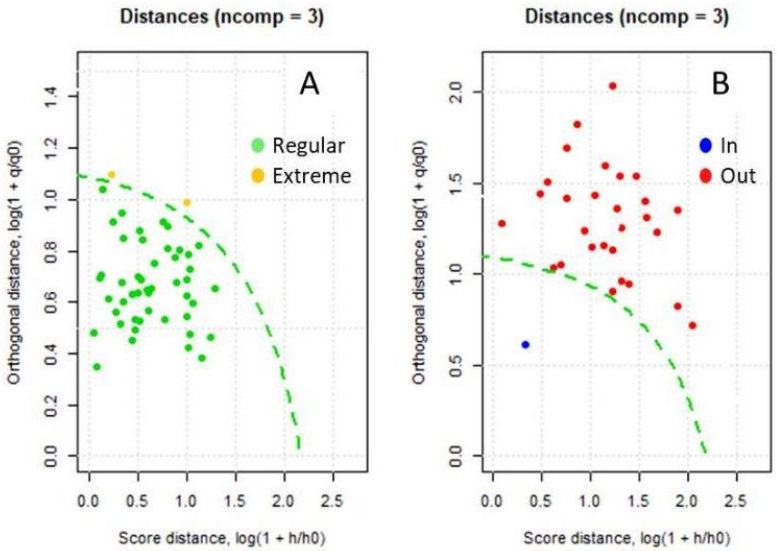


Figure 3. DD-SIMCA classification model: a) authentic asparagus (green dots, *n* = 49) cultivated in Slovenia, b) asparagus samples (red dots, *n* = 29) from outside Slovenia. The green line indicates the acceptance area (α = 0.05).

3.2.3. CHERRY

Forty-eight Slovenian cherry samples were correctly classified, and three were extreme (Figure 4a). The sensitivity was 94.1%. The results of non-Slovenian cherry samples are shown in Figure 4b. Out of 27 samples, 18 are outside the acceptance area and were correctly classified as non-Slovenian with a specificity of 66.7%.

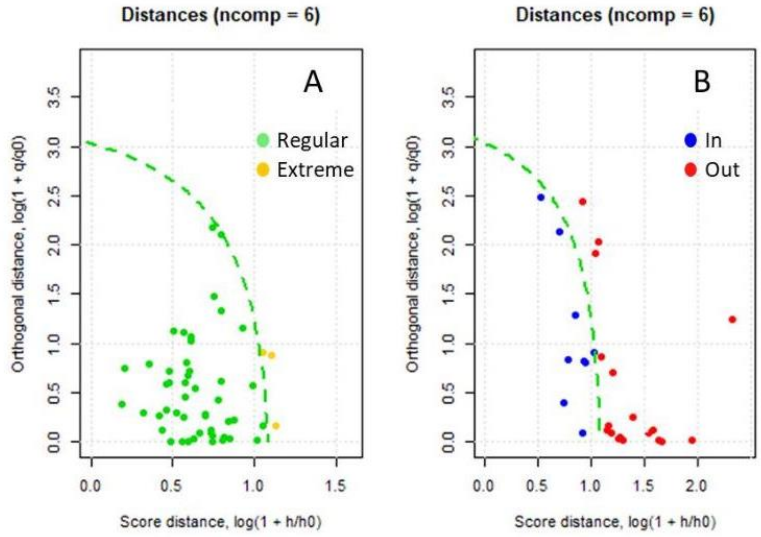


Figure 4. DD-SIMCA classification model: a) authentic cherries samples (green dots, n = 51) from Slovenia, b) cherries samples (red dots, n = 27) from outside Slovenia. The green line signifies the acceptance area (α = 0.05).

The important variables for cherries were $\delta^{18}O$, $\delta^{13}C$, Rb; non-influential variables were Mn, Fe, Sr, Cs while variables that were removed from the model were $\delta^{15}N$, $\delta^{34}S$, Mg, P, S, K, Ca, Cu, Zn, Ba, Co, Ni and Mo. To our knowledge, only Longobardi et al., 2015 have used stable isotopes to determine the geographical origin of Italian cherries. They confirmed that $\delta^{18}O$ and $\delta^{2}H$ (both measured in solid samples) help differentiate their geographic origin and that $\delta^{13}C$ is a useful parameter when using multivariate analysis, a finding which is comparable with the results of this study.

3.2.4. APPLE

Twenty-six apple samples were correctly classified as Slovenian and one extreme (Figure 5a). The sensitivity achieved is 96.2%. The results of non-Slovenian apple samples are shown in Figure 5b. Out of the nine apple samples, eight are outside the acceptance area and are thus correctly classified as non-Slovenian (specificity of 88.9%).

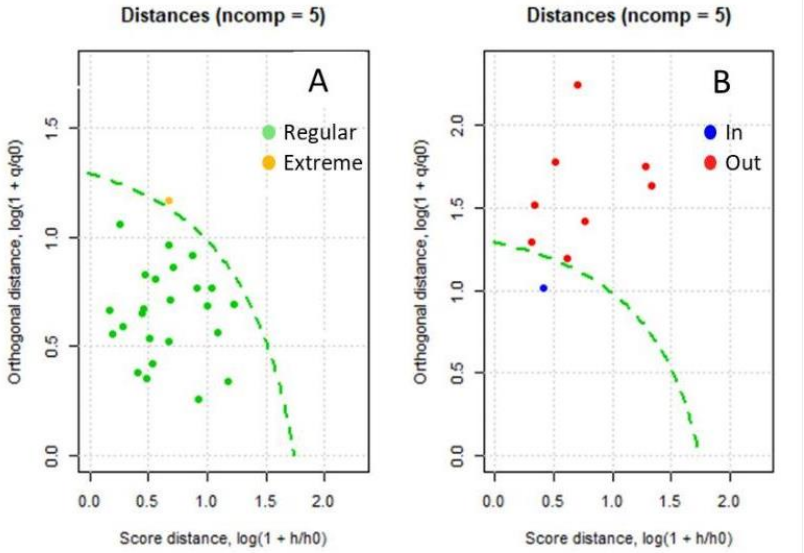


Figure 5. DD-SIMCA classification model of a) authentic apple samples (green dots; n = 27) cultivated in Slovenia, b) apple samples (red dots; n = 9) cultivated outside Slovenia. The green line signifies the acceptance area at $\alpha = 0.05$.

The variables that discriminate the most between Slovenian and non-Slovenian apples (VIC = -1) were $\delta^{15}N$, S, Fe, Cu, Zn, Sr, Mo and Ba. Variables where VIC=0 are $\delta^{18}O$, $\delta^{13}C$, P, K, Mn, Ni, Cs, while Mg, Ca, Na, Rb, Cr and Co had VIC = 1 and were removed to improve the overall classification rate. The stable isotope data is also comparable with the previous studies by Bat et al., who found that the most important variables for Slovenian apples were $\delta^{18}O$ and $\delta^{2}H$ in water, $\delta^{13}C$ in sugar, S, Ca and Rb (Bat et al., 2012; Bizjak Bat et al., 2016). They found that the level of S was the major variable in the discriminant function. Other important elements were Cl, Fe, Cu, Zn and Sr (Bizjak Bat et al., 2016). Further, Mimmo et al. prove that $\delta^{13}C$ and $\delta^{15}N$ values could be used to discriminate the geographical origin of apples produced only a few hundred kilometres apart (Mimmo

et al., 2015). However, based on our experience, the influence of harvest year must be studied to fully understand the isotopic differences and patterns observed among different varieties and geographical origins.

3.2.5. KAKI

Twenty-six kaki samples were correctly classified as Slovenian (sensitivity of 96.3%), with one classed as extreme (Figure 6a). The results of non-Slovenian apple samples are shown in Figure 6b. Out of 14 samples, all are outside the acceptance area and correctly classified as non-Slovenian (specificity of 100%).

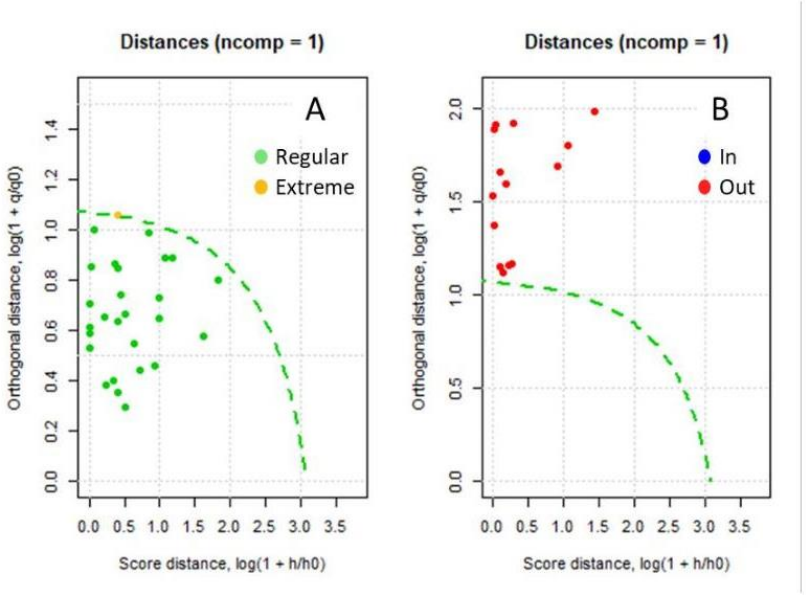


Figure 6. DD-SIMCA classification model: a) authentic kaki samples (green dots, n = 26) from Slovenia, b) kaki samples (red dots, n = 14) from outside Slovenia. The green line signifies the acceptance area at $\alpha = 0.05$.

The most important discriminating variables were $\delta^{13}C$, $\delta^{15}N$, Mg, S, Ca, Na, Fe, Sr, Mo, Cs. In their absence, the model's specificity is significantly reduced. All variables that were not influential but were kept in the model were $\delta^{18}O$, Cu, Zn, Rb, Ba, Co, Ni. The variables removed from the model since their exclusion improve the value of FoM were P, K, Mn. When comparing mineral composition (Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Ni, Sr, Zn), the data were comparable with data reported by Mir-Marqués et al., 2015 who studied kaki fruit from different production areas in Spain.

3.3. Overall discussion

We observed that the FoM – sensitivity and specificity could be improved by optimising variable selection. In this way, the country of origin or the variability in the data in the alternative class was important. Within selected crops, the most important variables for classification within elements were Sr, Ba Cs, S, Mo, Ni and Fe. Those elements seem to reflect soil composition and could be related to geology (Saaltink et al., 2014; Skordas et al., 2013). Strontium is already widely used to trace the geographical origin of agricultural produce

405 (Hiraoka et al., 2016). Alkaline elements, especially Rb and Cs, can be easily mobilised in the soil and taken up
406 by plants, making them good source markers (Kelly et al., 2005). Other important elements are Na and P. The
407 content of Na is usually related to the distance to the sea. Elevated amounts can be found within several
408 kilometres from the coast due to the steady input of marine aerosols (Saaltink et al., 2014). It is interesting to
409 note that Na, and Mg and Sr, are essential for classifying kaki samples grown near the coast in Slovenia.
410 Phosphorous levels could be related to excessive applications of phosphoric fertilisers or/and
411 organophosphate agrochemicals. However, it is unlikely that the use of fertilisers affects the elemental profiles
412 in such a way to change the country of origin prediction, as reported by Smith (Smith, 2005).

413 The high concentrations of some of the remaining elements (e.g. As, Cd, Cu) in the samples could be
414 attributed to the extensive usage of fertilisers, pesticides, fungicides and insecticides (Skordas et al., 2013).
415 We found that As is an important discriminating variable for asparagus, Cd for garlic and asparagus, and Cu
416 for asparagus and kaki samples. Further, we also found the highest Mg, K, Ca, Mn, Fe, Cu, Zn, and Co levels in
417 asparagus samples irrespective of the country of origin, suggesting that these elements are likely more related
418 to variety than geographical origin.

419 Unlike trace elements, stable light isotopes create more predictable patterns and are the product of
420 geographically dependent processes, i.e., the water cycle. Our study shows that $\delta^{18}O$ and $\delta^{13}C$ are important
421 factors in all five crops. However, the influence of storage conditions on the $\delta^{18}O$ stable isotope composition
422 of fruits and vegetables has not been investigated. Still, it could be important for garlic and apples that are
423 stored for a long time and for asparagus, which is prone to rapid water loss. Seasonal and annual variations in
424 environmental conditions and agricultural practices can also alter the isotopic composition of plants. To cover
425 annual differences in the stable isotope ratios and elemental composition of plant materials would require
426 collecting samples over many years. Such an expanded database will require more complex interpretations
427 due to increased natural variation and overlap in measured parameters from the authentic sample population
428 (Kelly et al., 2005). Nevertheless, a constant expansion of the database should be enabled every year, and at
429 the same time robustness of all established models should be tested.

430

431 *3.4. One-class classification (DD-SIMCA): origin assessment of garlic, asparagus, cherries, apples and kaki with*
432 *declared Slovenian origin*

433 Finally, the model was used to classify samples from the Slovenian market for revealing possible
434 adulteration. A classification model was built using the target set (authentic Slovenian samples), a test set of
435 samples from the market declared as Slovenian and the PCA model with selected PCs. The acceptance area is
436 delineated by the green line corresponding to $\alpha = 0.05$ (Figure 7a for garlic, 7b for asparagus, 7c for cherries,
437 7d for apples and 7e for kaki).

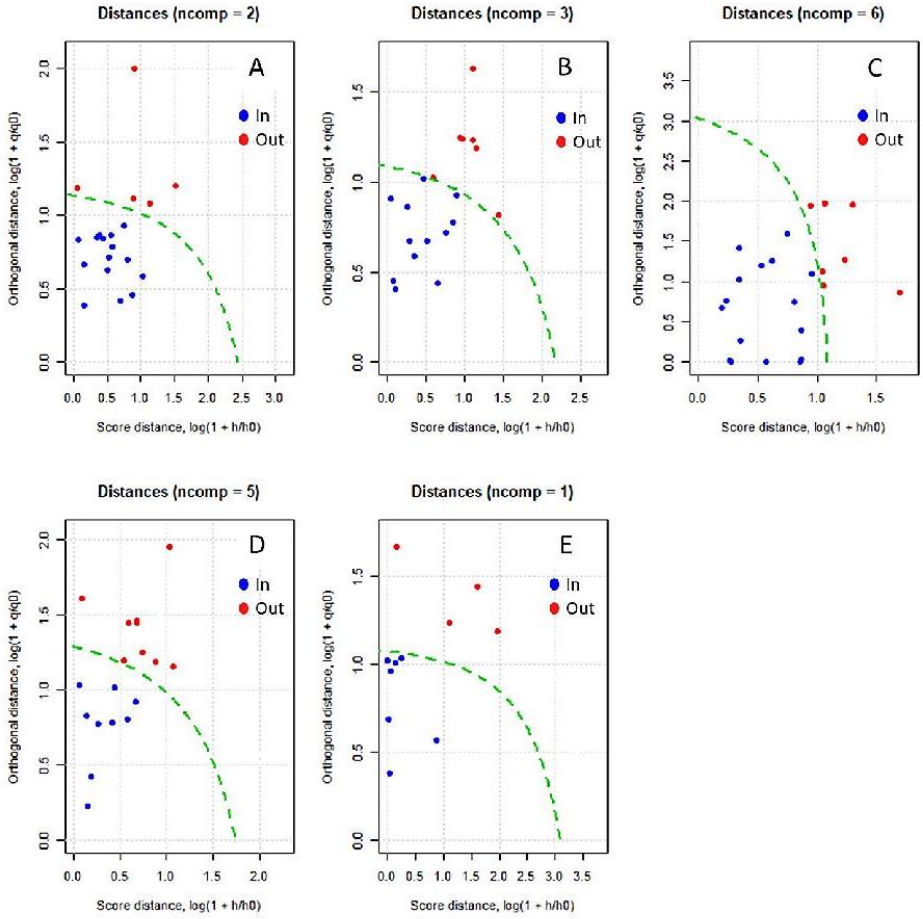


Figure 7. DD-SIMCA classification model of test samples of garlic (**A**), asparagus (**B**), cherries (**C**), apples (**D**) and kaki (**E**) from the market labelled as Slovenian. The green line indicates the acceptance area at α = 0.05.

Twenty-five per cent of garlic, 37% of asparagus, 30% of cherry, 50% of apple and 36% of kaki samples fall outside the acceptance area and are classified as non-Slovenian (Table 3). The PCA model for cherry was built with six PCs, and based on only seven variables and is probably too sensitive. Data covering two harvests with this number of samples is insufficient for a reliable, robust classification model.

Table 3. Results of classification of samples from the market declared as Slovenian (test class).

DD-SIMCA	Model parameters	No of samples in test class	No samples classified as non-Slovenian	% samples classified as non-Slovenian
	α = 0.05			
Garlic	2 PCs	20	5	25
Asparagus	3 PCs	19	7	37

Cherries	6 PCs	23	7	30
Apples	5 PCs	18	9	50
Kaki	1 PCs	11	4	36

4. Conclusions

The methodology reported herein provides a sound basis for establishing an adequate traceability system for fruit and vegetables. Although dependent on the number and diversity of samples in the dataset, we achieved good model sensitivity and specificity except for cherry, where we obtained lower specificity (66.7%). We can conclude that for building a generalised cherry model, samples from only two harvest years are insufficient to identify the general properties that characterise the Slovenian samples independently of other countries. The variability of the selected parameters is probably too big and monitoring over an extended period of more than one year is advisable to capture seasonal effects and changes, including agricultural practices. In apples and kaki, where we develop only a one-year model, the robustness of the model must be confirmed in further years. With several models (like garlic and asparagus), the number of samples per year required for the database may be reduced. The variables important for classification were the $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and elements Sr, Ba, Cs, Ni, S, Mo, Fe, Na and P. Importantly of the samples with a declared Slovenian origin, 35% were potentially mislabelled.

Overall, this study demonstrated that the isotope composition and elemental profiles combined with DD-SIMCA represents a promising tool for determining the declared origin of Slovenian fruits and vegetables. Such methods and approaches can also ensure confidence and trust in the food supply chain's integrity and can be adapted to newly identified priority food products and transferable to other countries of interest.

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Chapter 4

Conclusions

This thesis has contributed to science by improving how we tackle and deter fraudulent practices in the agro-food chain. It does this by developing and validating robust routine analytical procedures to establish the authenticity of flavourings and the geographical origin of fruits and vegetables with applicability to other crops. It also shows the advantages of coupling HS-SPME with GC-IRMS to obtain $\delta^{13}C$ or $\delta^{2}H$ values for several VOCs within a single run, which, when combined with multiple-point isotopic linear normalisation and a nonlinear correction, opens up new possibilities for its application through a series of case studies. Moreover, the thesis covers important aspects of establishing a database, such as access to authentic samples and the number of representative samples needed to cover natural isotopic variation. The result is ten databases that are of significant importance for producers or enforcement agencies for determining the authenticity or geographical traceability of raw ingredients.

The work also delivers the most appropriate chemometric approaches. For example, the utilisation of DD-SIMCA in this field is the first application of its kind in Slovenia (for selected crops: strawberries, cherries, apples, kaki, garlic and asparagus also globally). Notably, this approach has already been used as a part of official control in Slovenia. The methods, databases and models developed were used to assess the authenticity of flavourings and geographical traceability of food commodities, allowing insight into potential mislabelling and adulteration, benefiting producers and enhancing consumer trust. This work has resulted in eight papers (six published and two under review) in SCI international journal and presented at 11 international conferences.

The main conclusions of this thesis can be summarised into four interrelated research topics as follows:

1. **Developing suitable analytical techniques and protocols:** A robust protocol was developed for measuring $\delta^{13}C$ using HS-SPME coupled with GC-C-IRMS which can be used for routine laboratory application. Optimising HS-SPME parameters is critical to avoid isotopic fractionation and minimise method error. Also, the use of multiple-point isotopic linear normalisation enabled simultaneous evaluation of multiple compounds, which, together with peak size/linearity correction, significantly improved the measurement error of small peaks (below 1 nA) from 3 ‰ to 0.5 ‰. Its application to actual samples demonstrated that it could be an essential tool for determining the authenticity of apple and strawberry aroma and is easily transferred, not only to various fruit aromas such as raspberry, blueberry, peach, pear, and sour cherry but also to other aromas such as truffle and vanilla.

In this study, HS-SPME was also combined with GC-P-IRMS for the first time to determine the δ^2H value of vanillin. HS-SPME is an appropriate alternative to solvent extraction, and using this method meant that it was possible to overcome issues of solvent use, large sample volumes, need for concentrated samples, long analysis times, isotope fractionation and irreproducible results. It also avoids matrix effects, making it suitable for analysing complex food products. Overall, the method makes a significant contribution to vanillin authentication.

2. **Establishing databases:** This thesis exposed the importance of having a comprehensive database of authentic samples when assessing flavour authenticity and geographical traceability. Ten different databases were established: a database of δ^{13}C values of apple VOCs; a database of δ^{13}C values of various fruit VOCs (IsoVoc); a database of δ^{13}C and δ^2H values of vanillin; a database of truffle aroma profiles with corresponding δ^{13}C values; and stable isotope (C, N, O and S) and elemental composition databases of selected fruits and vegetables (asparagus, garlic, strawberries, cherries, apples and kaki). These databases cover the period 2017-2021 and contain representative samples covering the natural variation of isotopic and elemental values. Despite several limiting factors, such as access to authentic samples and sample collection logistics, the databases are extensive and can be used for statistical analysis. The δ^{13}C values of 61 aroma compounds with natural origin from the source material were determined in total within 350 samples. δ^{13}C values of 35 VOCs were reported for the first time, and 474 fruit and vegetable samples were included in establishing databases for geographical traceability. The databases themselves can be considered high-quality since the samples were collected from the field by impartial collectors. Overall, all databases were applicable and have proven useful for industrial partners or enforcement agencies to determine raw ingredients' authenticity or geographical traceability.

3. **Data analysis and model development:** Flavour authentication and geographical traceability require appropriate data analysis, but the choice of statistical method depends on the study goal and the characteristics of the objects and variables included. The presence of outliers and missing values must also be considered. In the case of flavour authentication, not all investigated VOCs were present in each sample, and values were missing in the flavour databases, which meant that it was only possible to make a comparative analysis of natural and synthetic isotope ranges. Although it allowed an assessment of the naturalness of the VOCs, this was only possible in those cases where the isotopic ranges between natural and synthetic samples did not overlap. In the case of overlap, additional information, such as δ^2H values, would be beneficial. The thesis also compares the obtained ranges with the literature and exposes the influence of sample characteristics on data variability. For example, apples and strawberries account for the most natural variability in δ^{13}C values and contain the highest number of VOCs, making them the most appropriate fruits for database creation.

   This thesis also found that different types of fruit processing cause isotopic fractionation, as evidenced by the differences in the isotopic ratios between distillates and fruit juice samples. This observation means that distillates and fruit samples should be included in a database. Data collected in the IsoVoc

and vanilla database allowed the successful discrimination between natural and synthetic VOCs, thereby increasing the chance of detecting falsifications. Within truffles, truffle species, harvest location sample quality and the presence of microbes influence the $\delta^{13}C$ value of truffle VOCs creates a broad range of authentic $\delta^{13}C$ values. However, the established $\delta^{13}C$ database did allow the successful discrimination between synthetic and natural VOCs for which values for synthetic samples exist. The addition of 2,4-dithiapentane, a compound typical for "white" T. magnatum, to "black" truffle samples could also be detected based on aroma profiling. It was also possible based on the aroma profile and linear discriminant analysis (LDA), to discriminate between investigated truffle species *T. aestivum*, *T. brumale*, *T. magnatum*, *T. melanosporum*, *T. macrosporum*, *T. mesentericum*, *T. excavatum*, *T. rufum,* and *T. indicum* with 97 % correct classification rate. A similar model constructed within species showed good discrimination of samples based on their geographical origin with a 93.7 % (*T. magnatum* collected from SI, BIH, IT and MK), 87.4 % (*T. aestivum* collected from SI, BIH, HR, IT, MK and UK) correct classification rate.

While comparative analysis is successfully used for flavour authentication, it alone can not provide a definitive answer concerning the geographical origin of fruits and vegetables. Instead, multivariate data analysis should be applied. This thesis identifies DD-SIMCA as most appropriate to inspect whether the sample from the market complies with its declaration (i.e., Slovenian origin). Within the fruit species studied, excellent sensitivity and good specificity were only obtained for each production year separately, while for vegetable species, robust models were created, where all harvest years were combined. The variables important for classification within the selected crops were Sr, Ba Cs, S, Mo, Ni, Fe and $\delta^{18}O$ and $\delta^{13}C$.

4. **Authenticity and geographical traceability of commercial samples:** The developed methods, databases, and models were finally used to control the naturalness of fruit flavours and vanillin and to verify if the commercially available fruit (strawberries, cherries, apples and kaki) and vegetables (garlic and asparagus) comply with their Slovenian declaration. The analysis of commercial fruit flavours, including natural banana, blueberry, peach, grape, pear, apple, strawberry, kiwi, raspberry, blackberry, plum, and sour cherry aromas, suggest possible falsification of specific fruit VOCs despite being labelled as natural fruit extracts. Results imply that the authenticity of flavoured products on the market can be questioned. Moreover, all investigated samples claimed to be flavoured with natural vanillin from Vanilla beans containing synthetic vanillin. Further, eleven aromatised commercial truffle products were analysed, from which three are labelled as "contain natural flavour". In most cases, the results were not conclusive, except for the addition of 2,4-dithiapentane to fresh *T. aestivum* and *T. magnatum* fruiting bodies, which produced $\delta^{13}C$ values indicative of a synthetic origin. Regarding the geographical origin of investigated fruit and vegetable crops, over a third of the samples did not correspond to their given declaration.

Overall, this thesis demonstrates that an established methodology can ensure confidence in flavour authenticity studies and provide a sound basis for establishing an adequate traceability system for fruit and vegetables. Although the authenticity and traceability systems cover the needs of the food flavour industry and enforcement

agencies in Slovenia, they can be easily transferred to any other food commodity or country.

Despite that this thesis answers many questions, it also raises new scientific questions and identifies needs that should be addressed in the future. For example, this would mean developing an HS-SPME GC-IRMS method for δ^2H determination of fruit and truffle VOCs to upgrade the existing database. Another is the need to establish a database of natural flavourings of biotechnological origin and where the source is from CAM and C4 plants. It is also necessary to understand better the influence of parameters on the isotope values of fruit and vegetable flavours, such as the effects of different distillation and concentration techniques, storage conditions, storage time, geographical origin, freshness and ripeness. Further investigations are also needed to study the effect of production year to establish a robust general DD-SIMCA model with variables independent of the year of harvesting, seasonal effects and changes, including agricultural practices. Equally important is the further validation of these models for flavour authenticity and traceability testing using laboratory adulterated samples with different amounts of adulterant. Estimation of the sensitivity of the models should also need to be done. Finally, in the case of truffles, the influence of the microbiome, genome and other abiotic factors on truffle aroma should be studied.

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Other Publications (optional)

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Biography

Lidija Strojnik began her studies at the Biotechnical Faculty University of Ljubljana, Slovenia, in 2007. In 2010, she was awarded a BSc degree in food science and nutrition and then, in 2014, a Master of food science (MSc.) for the thesis entitled "Diversity of moulds population on red grapes and in musts". In 2016, she enrolled as a doctoral student in the Ecotechnology program at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Prof. Dr. Nives Ogrinc at the Department of Environmental Sciences. Her research focuses on developing analytical methods, establishing databases, data processing using chemometric approaches and evaluation of the authenticity of commercial fruit, vanilla and truffle flavourings and origin traceability of selected fruits and vegetables. During this time, she has become proficient in different isotopic techniques for determining stable isotope ratios of hydrogen, carbon, nitrogen, oxygen and sulphur and multielement analysis and chromatographic methods. In her specialist field, she has paid particular attention to determining the isotopic composition of volatile organic compounds using the headspace solid-phase microextraction (HS-SPME) technique coupled with gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). Her work was also dedicated to data processing and interpretation. She has also attended several workshops on isotopic measurement techniques and data processing in SIMCA and RStudio and presented her work at international conferences. During her PhD study, she was also involved in an accreditation scheme at the Department of Environmental Sciences at Jožef Stefan Institute, where she is currently employed. This involvement allowed her to be part of expert studies on verifying the geographical origin of selected fruits and vegetables in cooperation with the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection.