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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-methyl-butyl 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.

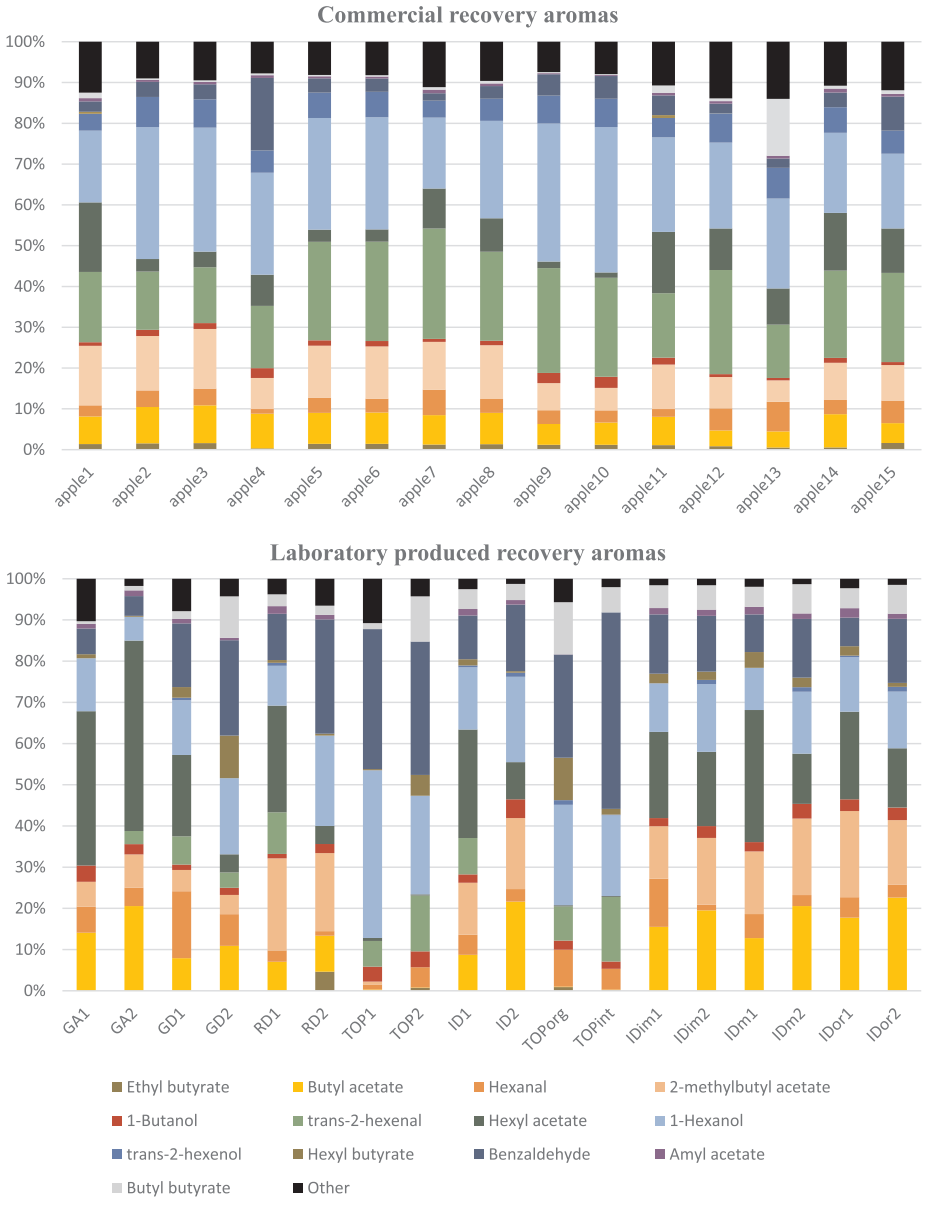

Fig. 1. Relationship between peak areas of aroma compounds in the commercial and laboratory produced recovery aromas obtained by HS-SPME GC–MS (GA = Gala, GD = Golden Delicious, RD = Red Delicious, TOP = Topaz, ID = Idared, 1 = without of storage, 2 = after 2 months of storage, org = organic, int = integrated, 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, &

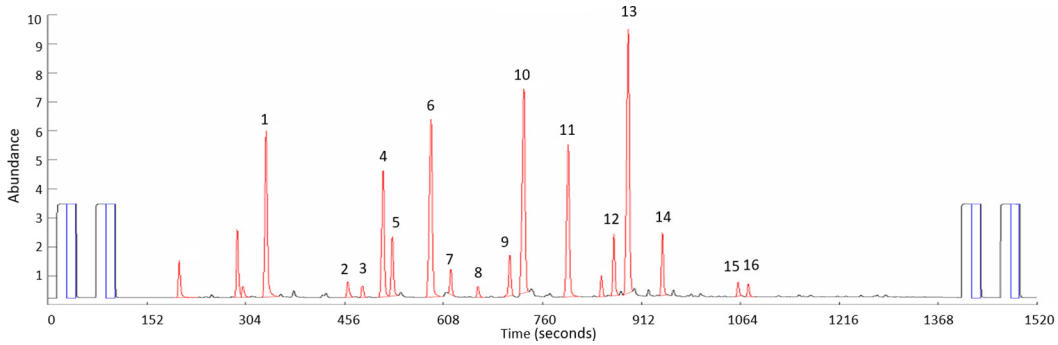

Fig. 2. GC-C-IRMS chromatogram related to natural apple recovery aromas on a VF-WAXms column. Peak identification of 16 pure synthetically derived aroma compounds purchased from Sigma Aldrich: 1, ethyl acetate; 2, ethyl butyrate; 3, ethyl-2-methyl butyrate; 4, butyl acetate; 5, 1-hexenal; 6, 2-methylbutyl 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

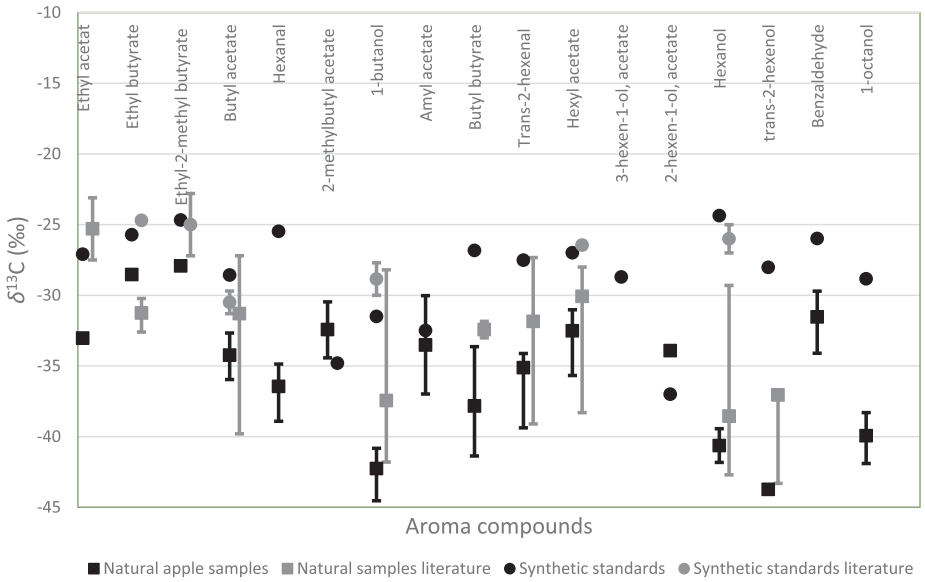

Fig. 3. δ^{13}C values of natural and synthetic samples (this study) and literature values.

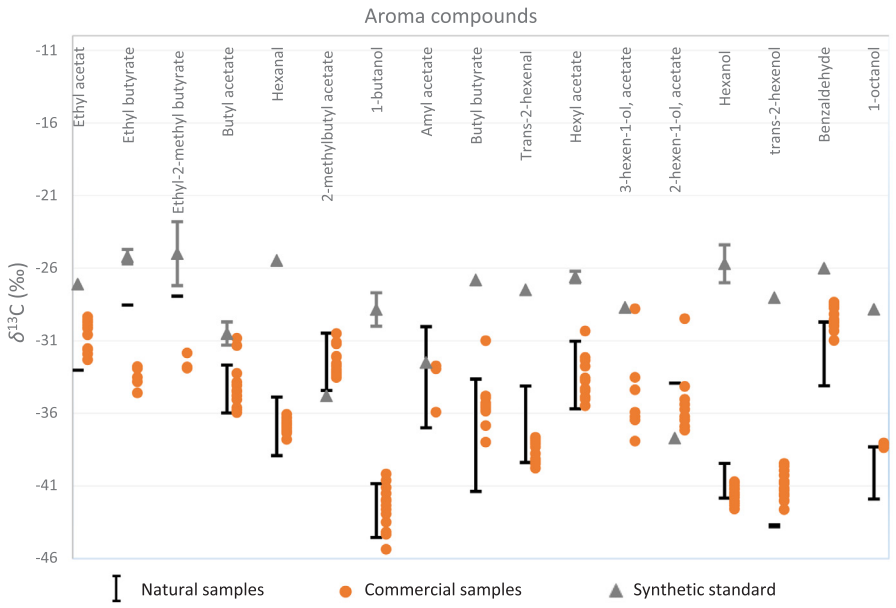

Fig. 4. δ^{13}C values of commercial apple recovery aromas with δ^{13}C values from the established database for natural samples and synthetic standard.

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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