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Stable Isotope Ratio Mass Spectrometry and Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance applications to discriminate between synthetic and natural analogs: A review

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ABSTRACT

Consumers are nowadays increasingly demanding products containing molecules of natural origin, as they are considered healthier than the synthetic or semi-synthetic counterparts. The higher costs associated with the production of plant raw materials and/or the extraction and purification of these natural substances set the basis for possible counterfeiting, resulting in the addition of cheaper, chemically indistinguishable synthetic form. In this review, we will discuss the effectiveness of stable isotope ratio mass spectrometry and/or Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance for the discrimination between molecules of natural and synthetic origin belonging to the families of flavourings, essential oils, foodstuff, dietary supplements, drugs and steroids.

1. Introduction

From the end of the 19th century and with the development of organic chemistry, many natural-based chemicals started to be produced both using living organisms (biosynthesis) and through chemical fragmentation of molecules (normally of petrochemical origin) into smaller ones (synthesis). Whilst plant metabolism dictates the production of many natural-based chemicals, the biosynthetic and synthetic ones are artificially produced on laboratory/industrial scale. Several biosynthetic and synthetic products are chemically identical as for structure and composition to their natural-based analogs, therefore having the same chemical and/or physical properties. As for the biosynthetics, the compound of interest can be isolated, synthesised or obtained through fermentation from a natural substrate other than the best-known and usually more expensive starting material (e.g. vanillin from rice ferulic acid is much cheaper than the best-known vanillin extracted from *Vanilla* species orchid). As for the synthetics, the compound of interest can be obtained by chemical synthesis from petrochemical origin starting materials (e.g. vanillin obtained from guaiacol) [1].

The demand for natural-based chemicals, sometimes called "botanicals", is constantly growing, based also on erroneous associations like "natural equals healthy" and "synthetic equals toxic". It is worth mentioning that the global market for natural flavours and fragrances has been valued at 9.15 billion dollars in 2021 and it is supposed to expand at a compound annual growth rate (CAGR) of 6.3 % from 2022 to 2030 [2]. This market trend influences the price of both natural and the synthetic forms, making the latter up to ten times cheaper than the former. In this way, the economic motivation for counterfeiting of commercial products, consisting of the substitution or extention of natural-based chemicals with their biosynthetic/synthetic form, is highly promoted. To counter this phenomenon and guarantee products authenticity, various analytical approaches have been developed, such as gas chromatography – mass spectrometry (GC-MS) [3–5], enantiomer testing [6,7], electronic nose [8,9] and carbon-14 analysis [10].

The GC-MS is an analytical technique that merges the principles of gas chromatography (which employs a capillary column, with its separation capacity determined by the column dimensions—length, diameter, and film thickness—and the characteristics of the stationary phase) and mass spectrometry (which ionizes molecules into fragments and identifies them by their mass-to-charge ratio). This method is particularly effective for analyzing complex matrices, such as oils, where the absence of specific secondary components can reveal product adulteration or substitution [5,11]. On the other hand, it is less efficient when the natural or synthetic origin of a specific isolated molecule has to be verified. In this case, quantifying the isomers and verifying their ratio is often effective, unless partial racemization occurred during storage or

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during technological treatment, or unless racemes of natural origin were generated by non-enzymatic reactions as auto-oxidation or photo-oxidation.

The electronic nose can be a valid analytical approach to detect the addition of artificial flavourings in food, and it is widely used to analyse odours and flavours. In this device, the inputs from the samples are collected by a sensor array and are then evaluated through a pattern recognition system [8,9].

Products deriving from plants or animals usually contain the isotope carbon-14. While this isotope can be found in all living organisms, it is not present in petrochemical-derived products. Liquid scintillation counting [12,13] and accelerator mass spectrometry [14,15] were successfully applied for the identification of synthetic and biosynthetic analogues of natural compounds. Liquid scintillation counting is a radiocarbon dating technique frequently used in the 1960s. The liquid sample is added with a scintillator, which produces a flash of light when it interacts with a beta particle. A sample vial is passed between two photomultipliers which record a signal only when both devices register the flash of light. Accelerator mass spectrometry (AMS) is a modern radiocarbon dating method that is considered to be the more efficient way to measure a sample radiocarbon content. In this method, the carbon-14 content is directly measured in relation to the carbon-12 and carbon-13 ones. Unlike the scintillation counting, this method does not count beta particles, but the atoms of carbon in the sample and the isotopic proportions. The carbon-14 quantification analysis is definitely one of the most effective techniques to discriminate between the natural and synthetic forms of a specific compound, but it requires expensive devices, which only a few laboratories in the world are equipped with, and long sample preparation and analysis, resulting in a high-cost approach.

The stable isotope ratio analysis (SIRA) through stable isotope ratio mass spectrometry (IRMS) and Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance (SNIF-NMR) are established analytical techniques applied to distinguish between natural and synthetic chemicals based on the relative abundance of bioelement stable isotopes of carbon ($\delta^{13}C$), hydrogen ($\delta^{2}H/D/H_n$), nitrogen ($\delta^{15}N$) and oxygen ($\delta^{18}O$). Through the isotopic ratio variations of these elements, we can gain insight into determining the origin of chemical compounds. This technique finds application in several fields, from agriculture to pharmacology, and from geology to ecology.

By the end of 2023, approximately 130 scientific articles applying IRMS and SNIF-NMR for the discrimination between molecules of natural and synthetic origin have been published in journals with impact factor (Scopus database source). This review aims to provide a comprehensive exploration of these practical and powerful techniques. We will delve into their fundamental principles, highlighting the effectiveness of the stable isotopes in making clear distinctions between natural and synthetic, even for molecules having identical chemical structures. Furthermore, these techniques can improve our understanding about the sources of important organic compounds in a wide range of scientific disciplines, but it also offers a tool to face counterfeiting of high-value-added products. Nevertheless, its widespread adoption hinges on the access to advanced training and specialised instrumentation, underlining the importance of ongoing professional development in this field.

1.1. Isotopes in the discrimination between natural and synthetic

1.1.1. $\delta^{13}C$ and plant photosynthetic cycle

Approximately 85 % of the earth's plants convert atmospheric carbon dioxide (CO_2) absorbed through leaf stomata into glucose via the Calvin cycle. Plants using only this "standard" carbon fixation mechanism are called C3 plants, due to the three-carbon compound (3-PGA) the reaction produces.

In C4 plants, the light-dependent reactions and the Calvin cycle are physically separated, with the light-dependent reactions occurring in cells of the mesophyll (spongy tissue in the center of the leaf) and the Calvin cycle occurring in special cells around the leaf veins. These cells are called bundle sheath cells. Atmospheric CO_2 is fixed in mesophyll cells to form four-carbon organic acid (oxaloacetate) hence the name C4 cycle. This is then converted into a similar molecule, malate, which can be transported into bundle sheath cells. Inside the bundle sheath, the malate decomposes, releasing a molecule of CO_2 which is then fixed by the RuBisCO enzyme and transformed into sugars through the Calvin cycle.

In CAM plants (Crassulacean Acid Metabolism), photosynthesis is divided in two temporally separated phases. During the night (Nocturnal Phase), when temperatures are low and humidity is high, CAM plants open their stomata and absorb atmospheric CO_2. The CO_2 is converted into malate through the enzyme phosphoenolpyruvate carboxylase (PEPc). Malate is stored in the vacuoles of plant cells. During the day (Diurnal Phase), when temperatures rise and humidity drops, CAM plants close their stomata to reduce water loss through transpiration. During this phase, stored malate is transported to the chloroplasts, where it is degraded to release CO_2. The CO_2 is then used in carbon fixation reactions through C3 photosynthesis.

Compared to atmospheric CO_2, having a $\delta^{13}C$ value of around −8.5 ‰ (see Scripps CO_2 programme: http://scrippsco2.ucsd.edu/), all plants are ^{13}C-depleted. Specifically, C3 plants preferentially select the $^{12}C^{16}O_2$ for sugar production during photosynthesis to a greater extent than C4 and CAM plants [12]. In C3 plants, the preferential selection of ^{12}C during photosynthesis is based on two main factors: the diffusion of CO_2 through the stomata and the preferential selection of ^{12}C by the primary carboxylating enzyme RuBisCO [13,14]. As a consequence, C4 and CAM plants, as well as the metabolites of both, are enriched in ^{13}C compared to their C3 counterparts.

While C4 plants have $\delta^{13}C$ values ranging between −14 ‰ and −12 ‰, C3 plants show values ranging from −30 ‰ to −23 ‰ [15–20] and CAM plants do not usually have $\delta^{13}C$ lower than −22 ‰ [21]. The mentioned ranges of variability can be also considered typical for products of "natural" origin, obtained through different processes from either C3, C4 or CAM plants. On the other hand, biosynthetic molecules, usually obtained through the fermentation of low-cost sugars (e.g. from cane and corn), have $\delta^{13}C$ values normally falling within the variability range of C4 plants.

The fully synthetic molecules are generally obtained from petrochemical sources, such as fuel oil, which has a wide $\delta^{13}C$ variability from −35 ‰ to −19 ‰ with an average value around −30 ‰ [22]. Thermogenic natural gas from fuel and biogenic gas have an average $\delta^{13}C$ value of −40 ‰ and −65 ‰, respectively [23]. The consistent difference between the $\delta^{13}C$ of natural, biosynthetic and synthetic compounds makes it possible to efficiently distinguish their origin.

1.1.2. Other isotopes ($\delta^{2}H$, D/H_n, $\delta^{18}O$ and $\delta^{15}N$)

Significant differences between natural, biosynthetic and fossil fuel-derived molecules can be easily detected when considering the $\delta^{13}C$, but for the isotope ratios of nitrogen, oxygen and hydrogen, the biosynthetic pathways that lead to these differences are not so defined. The different isotopic parameters were found to be variably effective in their ability to discriminate depending on the molecule investigated. Generally, the $\delta^{2}H$ values tend to be lower in the natural form of the molecule compared to the synthetic homologue. This is likely due to fractionation processes occurring during the synthesis rather than to a direct correlation with the starting fossil source having very negative values (from −181 ‰ to −85 ‰) [24].

1.2. Stable isotope ratio mass spectrometry (IRMS) technique

The analysis of stable isotope ratios (SIRA) of the bioelements through stable isotope ratio mass spectrometry (IRMS) in organic molecules, both of natural and synthetic origin, is a powerful technique to obtain relevant information about their provenance and the steps

involved in their biological or chemical synthesis. Carbon ($^{13}C/^{12}C$), hydrogen ($^{2}H/^{1}H$), oxygen ($^{18}O/^{16}O$), sulfur ($^{34}S/^{32}S$), and nitrogen ($^{15}N/^{14}N$) are usually considered. As they can become enriched or depleted through a variety of kinetic and thermodynamic processes, measurements of the isotope ratios can be used to discriminate among samples that would be otherwise indistinguishable, as they share identical chemical compositions.

Some of the analytical aspects to which particular attention must be paid when applying the IRMS are listed below:

1. Isotopic Fractionation: Prior to analysis, it is essential to take into account isotopic fractionation which may occur during sample preparation. For example, some chemical reactions can bring about variations in the isotopic abundance. In literature, isotopic fractionation pathways are commonly divided into two classes: equilibrium and kinetic ones [27]. Equilibrium fractionation consists of a different partitioning of identical molecules depending on their isotopic composition. The presence of heavy isotopes in the molecular structure results in decreased bond energies, related to lower and therefore thermodynamically favoured energy states. On the other hand, fractionation associated with unidirectional, time-dependent or other non-equilibrium partitioning is classified as kinetic. This phenomenon can be the result of both physical dynamics and chemical transformation, regarding thus all mass-dependent differences between isotopologues (i.e. molecules having the same chemical formula and bonding structure, but differing in their isotopic composition). A simple way to double check for unwanted fractionation is to compare the isotopic values of a pure sample (such as or added to a matrix) before and after the extraction process. If the values match, no fractionation processes are ongoing.
2. Sample preparation and technique selection: The sample can be either analysed without any prior preparation if it is pure, otherwise it must be processed to extract and isolate the compound(s) (single or multiple molecules) of interest. This can represent a critical step, as it must be ensured that the sample isotopic composition does not get altered. The compound(s) can be analyzed as such whether in solid, liquid or gaseous form through elemental analyzer (EA) or pyrolyzer (P). If the target chemical is volatile or can be turned into a volatile analog through derivatization, the most used technique is the gas chromatography coupled to isotope ratio mass spectrometry [25]. If a derivatization step is needed, it will always be necessary to further correct the data considering the isotopic contribution of the derivatizing agent. As for water-soluble molecules, the instrumental coupling of liquid chromatography (LC) to IRMS is now available with various applications illustrated in the review by Perini et al. [26]. As for molecules that are neither volatile nor water-soluble, an additional option could be represented by the separation and purification via preparative - high performance liquid chromatography (HPLC) with organic solvents in the organic phase of a single fraction, followed by rotary evaporation/freeze-drying and stable isotope analysis.
3. Combustion or pyrolysis: The compound(s) of interest, either pure, in a mixture or previously separated from each other (e.g. through GC or LC), are therefore analysed through the elemental analyzer (EA), where they are converted into CO_2, N_2 and SO_2, or through the pyrolyzer (P), where the pyrolysis converts them into CO and H_2. This is also known as thermochemical conversion (TC).
4. Working conditions of the isotope ratio mass spectrometer: The gases obtained from the combustion or pyrolysis are carried to the IRMS, where they are turned into ions, usually by electron ionisation (EI). The charged molecules produced this way are accelerated through an electric field and separated based on their mass-to-charge ratio (m/z) by a magnetic field inside the mass spectrometer. The number of ions having a specific mass is then recorded through dedicated Faraday cup collectors, with suitable amplification of the ion current related to their relative natural abundance. The correct instrumental setting is an essential requirement to guarantee high performances of this device.
5. Expression of the isotopic composition: Isotope ratios are calculated based on the data provided by the device (for example, the value of the ratio $^{12}C/^{13}C$, as for carbon isotopic analysis). The isotopic composition is expressed in delta (e.g. $\delta^{13}C$), according to Brand and Coplen (2012). The results are therefore multiplied and expressed in units "per mil" (‰) or in milliurey (mUr) [28].
6. Equilibration: To measure the $\delta^{2}H$ in organic compounds it is necessary to proceed with a distinction among molecules containing or not exchangeable hydrogens. In fact, only the latter will need to be equilibrated, as reported by Wassenaar et al. (minimum 24 h with atmospheric air hydrogen in a desiccator [29] or by the use of a specific autosampler [30]).
7. Correction, normalisation and standardisation: The collected data must be corrected using known reference standards. These samples have known isotopic composition and are used to establish a correlation between the result given by the device and the certified isotopic values. The recent Skrzypek et al. (2022) publication [31] reports how to proceed to normalise and correctly express the isotopic results. Data normalisation by using two standards (two-points) having isotopic values respectively higher and lower than the sample ones is strongly recommended. However, the availability of certified reference standards resembling the sample under analysis and covering a wide isotopic range is a current issue.
8. Validation and quality control: It is essential to include quality control samples in each analytical batch to ensure the reliability of the results [32]. Quality control and test samples should be identical (e.g. quality control vanillin in vanillin samples analysis) and they must be treated the same way. To validate and estimate measurement uncertainty, the participation in inter-laboratory trials involving the techniques, preparations and matrices under examination is required [32]. Uncertainty can also be calculated from Proficiency test data [33].
9. Statistical processing and interpretation: Data can be subjected to statistical analysis to identify significant trends and to interpret the results according to specific research goals.

By following this approach, it is possible to obtain detailed information about the isotopic composition of inorganic/organic molecules, providing valuable insights into their origin and into the biological/chemical processes influencing their composition.

1.3. Hydrogen and carbon Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance techniques

The techniques based on the Nuclear Magnetic Resonance (NMR) offer powerful tools to discriminate between natural and synthetic compounds based on several factors, including their isotopic composition. In particular, hydrogen and carbon Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance (^{2}H and ^{13}C SNIF-NMRTM) are the main NMR techniques used in this scope in recent years.

The SNIF-NMR is an analytical methodology combining the nuclear magnetic resonance (NMR) spectroscopy with the analysis of isotopes sited at specific positions in the molecular structure (SNIF). It is used to measure $^{2}H/^{1}H$ (^{2}H SNIF-NMR spectroscopy) and $^{13}C/^{12}C$ (^{13}C SNIF-NMR spectroscopy). It can be applied in different sectors, including food, agriculture and environment.

One of the most important applications of SNIF-NMR is the geographical traceability and authenticity of food and flavours [34]. For example, this technique can determine the geographic origin of wine, honey, or olive oil whose site-specific $^{2}H/^{1}H$ ratio varies by latitude, altitude and distance from the sea. The SNIF-NMR can be also used to study chemical, biological or synthetic processes, providing detailed information on isotopic variations in different production or transformation steps [35]. Since the isotopic composition is affected by

geographic, climatic and process factors, SNIF-NMR can be used to detect food fraud or manipulation in products. The SNIF-NMR represents a particular application of NMR spectroscopy, which is mostly used in organic chemistry for structure elucidation based on ^{1}H and ^{13}C measurements.

Here are some of the aspects to which particular attention must be paid when carrying out the NMR analyses considered in this review:

1. Sample preparation: During extraction, purification and analysis the possibility of fractionation must be double checked. Moreover, the sample should be weighed with ±0.1 g precision and ±0.1 g accuracy (see for example method OIV-MA-AS311-05). The same should be applied to solid samples which have to be dissolved in appropriate solvents for NMR analysis (e.g., deuterated acetone for ^{13}C NMR). When working with this method, an internal calibration standard (e.g. tetramethylurea for ^{2}H–SNIF-NMR or sodium acetate for ^{13}C–SNIF-NMR) and a field stabiliser, or lock-compound (e.g. hexafluorobenzene), are always required.
2. Measurement of $^{2}H/^{1}H$ or $^{13}C/^{12}C$: After carefully setting the instrument, a minimum of 150 scans per measurement of the same sample must be repeated during time (min 8 times). Controlled experimental conditions (i.e. temperature, relaxation delays, and acquisition times for the best signal-to-noise ratio) should be used when acquiring spectra with a high-field NMR spectrometer. This would help minimise variations and artefacts. For an accurate quantification, the use of ^{13}C decoupling is usually recommended to remove satellite peaks and prevent their overlap with other interfering peaks.
3. Expression of the isotopic composition and comparison with other isotopic approaches: The $^{2}H/^{1}H$ and $^{13}C/^{12}C$ values can be measured site-specifically in the different molecular sites. They are expressed in parts per million (ppm) and can be converted in ‰ or milliUrey, which can be useful when comparing SNIF-NMR and IRMS results. As previously mentioned, while SNIF-NMR performs site-specific measurements, the IRMS is based on bulk analyses in the whole molecule. Therefore, to compare the results given by the two techniques, the isotopic values determined by SNIF-NMR have to be considered according to their contribution. In particular, it is necessary to sum the isotopes contributions from each site, considering both relative abundance and site distribution.
4. Validation, quality control, statistical processing and interpretation: see Chapter 1.2.

1.4. Advantages and disadvantages of the IRMS and SNIF-NMR techniques

Listed below are some advantages and disadvantages of applying these isotopic techniques

1. High performances in the discrimination between molecules of natural and synthetic origin: as will be shown in this review, both techniques allow for a clear discrimination.
2. Cost and Accessibility: Both SNIF-NMR and IRMS are costly and require specialised equipment and expertise. However, SNIF-NMR tends to be even more specialised and less widely available than IRMS.
3. Detail and Specificity: SNIF-NMR offers molecular site-specific information, resulting in more detailed insights compared to the broader isotopic information which IRMS provides.
4. Precision and Sensitivity: The IRMS technique offers high precision in isotopic measurement, allowing for accurate and reliable results. Reference values for repeatability, reproducibility, sensitivity and/or precision for different matrices are reported in several studies [36–38].
5. Application Versatility: While both techniques are versatile, IRMS has broader applications across various scientific fields. SNIF-NMR is particularly powerful in food and beverage authentication.
6. Sample Preparation and Handling: Both techniques require careful sample preparation and handling to avoid contamination, but SNIF-NMR can be more complex and time-consuming in this regard. The analysis is not usually conducted on complex matrices (e.g. wine) but on the target analyte which must be previously separated and purified (e.g. ethanol obtained from wine distillation).
7. Complex Interpretation: Interpreting isotope data can be challenging and requires a deep understanding of both isotopic fractionation and study context.
8. Limited Reference Materials: In IRMS analysis there may be a lack of standardised reference materials for certain isotopes or samples, complicating calibration and comparison.

In summary, while both techniques provide valuable isotopic information, SNIF-NMR is more specialised and detailed in its molecular-specific analysis, whereas IRMS offers broader applicability and is slightly more accessible.

1.5. Regulation on the naturalness of compounds

The use of natural compounds is regulated in several fields, which are described as follows and are partially the subject of this review.

1.5.1. Food and beverages

Regulation (EC) No 1334/2008 [39] on flavourings and specific food ingredients with flavouring properties to be added in and on foodstuffs defines what natural flavourings are and establishes criteria for their use and labelling. Regulation (EU) 2018/848 [40] establishes standard references for what can be labelled as organic, including requirements for the use of natural substances and methods in agricultural production.

1.5.2. Cosmetics

Regulation (EC) no. 1223/2009 [41] includes provisions on the safety and labelling of cosmetics, ensuring that any claims about natural or organic ingredients are not misleading and are supported by evidence. ISO 16128 [42] provides guidance on definitions and criteria for natural and cosmetic ingredients and organic products.

1.5.3. Pharmaceuticals

Directive 2001/83/EC [43] concerns medicinal products for human consumption and includes provisions on the quality and safety of active pharmaceutical ingredients (APIs), whether natural or synthetic.

1.5.4. Environmental and sustainability standards

EU Ecolabel Regulation (EC) n. 66/2010 [44] establishes the EU Ecolabel scheme, which includes criteria for the naturalness and sustainability of products. Products carrying the EU Ecolabel must meet strict environmental and natural ingredient standards.

2. Applications

2.1. Flavourings

2.1.1. Vanillin

Vanillin is one of the volatile organic compounds (VOCs) that has been most widely studied through isotopic analysis. It can be obtained through three processes: by extraction from the best-known and expensive orchid belonging to the genus *Vanilla* (mainly *Planifolia* or *Tahitensis*), being therefore classified as “natural from *Vanilla*”; by biosynthesis starting from natural sources such as curcumin from turmeric, ferulic acid form rice or corn, lignin from wood pulping, eugenol and iso-eugenol from clove, glucose from wheat or corn, processed through fermentation technologies (solid-state fermentation),

microorganisms bioengineering, enzymatic production, biosynthetic systems, bioconversion of agro-industrial wastes and production by microorganisms, being classified as "natural" vanillin; by chemical synthesis starting from petrol-based intermediates, specifically guaiacol, being classified as "synthetic" vanillin [45]. The combination of $\delta^{13}C$ and $\delta^{2}H$ has been shown to be effective in the discrimination among the three sources of vanillin [46–56].

Vanilla orchid is a CAM (*Crassulacean Acid Metabolism)* plant. Unlike C3 plants, CAM ones follow a photosynthetic pathway that allows them to better face water stress and to adapt to arid environments. The fractionation phenomena occurring in the different phases of the CAM photosynthetic cycle determine specific values of $\delta^{13}C$ that are completely different from those characterising plants following the C3 photosynthetic cycle. On the other hand, due to a certain variability related to local climatic conditions, they can instead overlap with the values found in C4 plants, e.g. cane sugar/pineapple.

Several authors have already tried to set the limit values for $\delta^{13}C$ of vanillin based on numerous studies available in the literature [46–56]. An overview of the reference values for natural and synthetic vanillin is given in Table 1. For the natural form obtained from *Vanilla planifolia,* it is possible to identify a $\delta^{13}C$ range of variability between −22.2 ‰ and −17.8 ‰, while for the *Vanilla tahitensis* the $\delta^{13}C$ values range from −19.7 ‰ to −14.6 ‰ [46–56]. Greule et al. has recently studied *Vanilla pompona*, reporting a $\delta^{13}C$ range of variability going from −18.0 ‰ to −17.1 ‰ [50].

Values falling outside these ranges indicate vanillin samples obtained from natural sources other than the orchid, such as ex eugenol, ex ferulic acid, ex lignin, ex turmeric, ex tannin or ex guaiacol (see Table 1). For Wilde et al. [57] "*if the $\delta^{13}C$ V-PDB < −22.2 ‰, it is below the range for natural vanillin from vanilla pods and the vanillin originates from C3 or petrochemical precursor material (can be synthetic vanillin or biovanillin). If the sample ranges between -22.2 ‰ and −14.6 ‰, it is in the range for natural vanillin from vanilla pods and the vanillin originates from vanilla pods or the vanillin originates from corn (*via *ferulic acid). If the value $\delta^{13}C$ V-PDB > −14.6 ‰, it is above the range for natural vanillin from vanilla pods and the vanillin originates from glucose, most likely from C4 precursor material, for example corn*".

In order to improve the power of this discrimination, the $\delta^{13}C$ of bulk vanillin has been recently combined with its $\delta^{2}H$ [49,50,53] and $\delta^{18}O$ [58,59], with $\delta^{2}H$ and $\delta^{13}C$ of vanillin methoxyl groups [50] and with site-specific analysis using both ^{2}H SNIF-NMR [60] and ^{13}C SNIF-NMR [61,62]. Vanillin from *Vanilla* species showed negative $\delta^{2}H$ (−115 ‰ to −3‰ [49,53]), higher than the values reported for vanillin obtained through biosynthesis from natural sources (−204 ‰ to −36 ‰ [53]), whereas synthetic vanillin from fossil showed positive values (+38 ‰ to +104 ‰ [50,53]) (see Table 1). ^{13}C Site-specific stable isotope analysis was also measured on vanillin by low temperature pyrolysis EA-IRMS [63] showing that the produced CO consisted of an average of methoxy, aldehyde and ring carbon attached to hydroxyl oxygen through analysis of isotopically enriched vanillin.

Hener et al. reported that samples of natural vanillin presented higher values of $\delta^{18}O$ (from −2.19 ‰ to +0.33 ‰) compared to the synthetic one (−6.17 ‰) [59]. Bensaid et al. confirmed this statistical difference, but reported other ranges of variability for vanillin samples from natural ($\delta^{18}O$ from +11.8 ‰ to +16.7 ‰) and synthetic ($\delta^{18}O$ from +8 ‰ to +10.1 ‰) sources and for the corresponding chemical degradation-derived guaiacol (natural $\delta^{18}O$ from +7.5 ‰ to +13.1 ‰ and synthetic $\delta^{18}O$ from +5.0 ‰ to +8.9 ‰) [58]. The cause of this discrepancy could be due to the use of different non-certified standards for the isotopic analysis of oxygen, and to the fact that the $\delta^{18}O$, although figuring as a promising parameter, is still essentially unexplored.

Position-Specific Isotope Analysis (PSIA) reported by Greule et al. consists in the chemical cut of the methoxy group from vanillin using Zeisel reaction, making it possible to measure hydrogen and carbon isotopic composition of this functional group [50]. This procedure only requires a few milligrams of pure vanillin and the comparison between the isotopic data measured on the bulk and on the methoxy fraction allows the vanillin samples to be identified with higher accuracy. The $\delta^{13}C_{Methoxyl}$ of vanillin from orchids varies from −27.4 ‰ to −7.1 ‰. The synthetic analog has very low $\delta^{13}C_{Methoxyl}$ (−52.2 ‰ to −29.7 ‰) compared to the natural vanillin. As for $\delta^{2}H_{Methoxyl}$, no significant differences among natural and synthetic values are reported, except for the spruce wood-derived synthetic vanillin (−313.2 ‰).

Regarding the ^{2}H SNIF-NMR, Remaud et al. reported the D/H_n measured in the five different sites of natural vanillin molecule (Fig. 1). They resulted on average 130.8 ± 3.1 ppm in $(D/H)_I$ site, 157.3 ± 3.0 ppm in $(D/H)_{III}$, 196.4 ± 2.5 ppm in $(D/H)_{IV}$ and 126.6 ± 1.7 ppm in $(D/H)_V$. These values were statistically different from those reported for synthetic vanillin (ex guaiacol) and allow the identification of minimum 5 % additions to the natural one [60,64].

Tenailleau et al. [61] and Pironti et al. [62] evaluated the applicability of ^{13}C SNIF-NMR spectroscopy in discriminating the carbon isotopic composition of different vanillin samples (see Table 1).

As reported by Guyader et al., the SNIF-NMR effectiveness in verifying the authenticity of vanillin can be improved by combining the information provided by the ^{2}H and ^{13}C SNIF-NMR profiles [65]. Furthermore, the ^{13}C NMR based on pulse sequences such as INEPT (polarisation transfer enhanced insensitive nuclei) offers the possibility to reduce the amount of product required (from 1 g to less than 50 mg) and the analysis time (from 15 h to less than 8 h) compared to the ^{2}H NMR method [39].

Table 1
Variability range for the $\delta^{13}C_{bulk}$, $\delta^{13}C$ SNIF-NMR-$\delta^{13}C$, $\delta^{13}C_{methoxyl}$, $\delta^{2}H_{bulk}$, $\delta^{2}H_{methoxyl}$ parameters of vanillin from natural and synthetic sources (Krueger and Krueger 1983; Bricout, Fontes, and Merlivat 2020; Hoffman and Salb 1979; Hansen, Fromberg, and Frandsen 2014; Greule et al., 2010; Geiβler et al., 2017; Kaunzinger, Juchelka, and Mosandl 1997; Matteo Perini, Pianezze et al., 2019; Schipilliti, Bonaccorsi, and Mondello 2017; Lamprecht, Pichlmayer, Schmid 1994, Tenailleau 2004 and van Leeuwen 2017).

		$\delta^{13}C_{bulk}$ (‰, vs V-PDB)		$\delta^{13}C$ SNIF-NMR (‰, vs V-PDB)		$\delta^{13}C_{methoxyl}$ (‰, vs V-PDB)		$\delta^{2}H_{bulk}$ (‰, vs V-SMOW)		$\delta^{2}H_{methoxyl}$ (‰, vs V-SMOW)	
		min	max	min	max	min	max	min	max	min	max
Natural from *Vanilla*	Vanilla from orchid	−22.2	−14.6	−21.0		−27.4	−7.1	−115	−3	−207	−85
	Vanilla planifolia	−22.2	−17.8								
	Vanilla tahitensi	−19.7	−14.6								
	Vanilla pompona	−18.0	−17.1								
Natural from other sources	Ex eugenol	−36.9	−29.9					−91	−75		
	Ex ferulic acid (rice)	−37.9	−35.1	−46.4	−36.4			−176	−168		
	Ex ferulic acid (corn)	−19.9	−19.0								
	Ex lignin	−29.5	−26.5	−27.3		−28.8	−19.6	−204	−169	−236	
	Ex turmeric	−30.4	−27.8					−112			
	Ex wood tannin	−28.8	−26.9					−142	−36		
Synthetic	Ex guaiacol	−36.2	−27.4	−38.0	−25.0	−52.2	−29.7	38	104	−182	−42

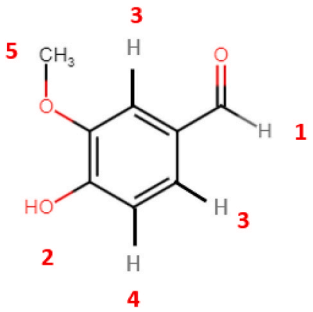

Fig. 1. Vanillin structure. The sites where the $(D/H)_n$ isotope ratio can be measured are numbered in red.

2.1.2. Truffle flavour

White truffle (*Tuber magnatum*) is the best-known species belonging to the genus *Tuber*. This species is appreciated for the presence of specific compounds giving their peculiar aroma. According to EU Regulation 1334/2008, "if the term natural is used to describe a flavour, the flavouring components used should be entirely of natural origin, the source of the flavourings should be labelled, and if a source is mentioned, at least 95 % of the flavouring component should be obtained from the material referred to". It is not even possible to use the term "natural white truffle flavour ", "white truffle flavour" or "natural flavouring substance'". In case the flavoring compound bis(methyl-thio)methane (BMTM) does not originate for more than 95% from the *Tuber magnatum*, only the term "flavoring substance" can be used. Since the synthetic form of the aromatic molecule is commercially available, it is possible to hypothesise its use in order to correct or enhance the aroma naturally present in the truffle. For Bononi et al., the $\delta^{13}C$ of synthetic BMTM ranged between −42.24 ‰ (σ 0.35) and −43.40 ‰ (σ 0.34) [66], while for Sciarrone et al., genuine white truffles harvested in Italy showed $\delta^{13}C$ values between −42.6 ‰ and −33.9 ‰ [67].

As reported by Wernig et al., the $\delta^{13}C$ values were not effective in the discrimination between natural and synthetic 2,4-dithiapentane, a characteristic truffle odorant detected in most flavoured oil samples [68]. Based on the results obtained, only truffle-flavoured oils having $\delta^{13}C$ lower than −45 ‰ can be classified as synthetic.

2.1.3. Flavours in essential oils of flowers and fruits

In the fragrances sector, flowers and fruits essential oils play a leading role thanks to their characteristic flavouring substances. They are used not only by the perfume industry but also as additives in the food sector to give a different or more intense flavour and/or smell. Their high production cost makes them particularly exposed to adulteration issues, consisting in the replacement of one or more natural compounds with the corresponding synthetic ones in commercial products.

For the analysis of $\delta^{13}C$ and $\delta^{2}H$ of essential oil components, the normally used technique is the Compound Specific Isotope Analysis (CSIA), via Gas Chromatography Combustion (GC-*c*-IRMS) and/or Gas Chromatography Pyrolysis (GC-*p*-IRMS) both coupled with the Isotope Ratio Mass Spectrometry. Braunsdorf et al. defined the $\delta^{13}C$ natural variability for the most abundant compounds in lemon (*Citrus limon (L.) Burm.*) essential oil, namely neral and geranial [69]. Braunsdorf et al. and Schipilliti et al. included additional compounds in their investigation: α-pinene, β-pinene, limonene, α-terpineol, neryl acetate, geranyl acetate, caryophyllene, trans-α-bergamotene, β-bisabolene, 2, 3-dimethyl- 3-(4-methyl-3-pentenyl)-2-norbornanol, campherenol, α-bisabolol [70,71]. Faulhaber et al. and Schipilliti et al. reported the ranges of $\delta^{13}C$ natural variability of the main compounds in mandarin (*Citrus reticulata* L.) essential oil: α-thujene, α-pinene, β-pinene, myrcene, limonene, γ-terpinene, terpinolene, terpinen-4-ol, α-terpineol, decanal, thymol, methyl N-methylanthranilate, α-farnesene and α-sinensal [72,73]. The adulteration can therefore be detected when $\delta^{13}C$ values fall outside the reported limits.

Lemon balm (*Melissa officinalis* L.) was investigated by Wagner et al. based on the $\delta^{13}C$ of its main compounds (citral and its enantiomers geranial and neral, citronellal, β-caryophyllene, germacrene D and caryophyllene oxide). In particular, the analysis of citronellal lead to the unequivocal identification of adulterations consisting in the replacement of lemon balm oils (deriving from C3 plants) with citronella ones (deriving from C4 plants) [74]. As reported by Kumar et al., the sophistication of Lemongrass (*Cymbopogon flexuosus*) and Palmarosa (*Cymbopogon martinii*) essential oils, both C4 plants, is easily detected through the isotopic analysis [75]. The synthetic adulterants geraniol and citral have $\delta^{13}C$ values significantly lower than their natural counterparts. On the other hand, Pellati et al. reported that unexpectedly high $\delta^{13}C$ values found in commercial *Rosa damascena Mill.* samples can be explained by the addition of the cheaper natural Palmarosa (*Cymbopogon martinii*) oil, figuring in this case as an adulterant [76].

In addition to the peculiar molecules associated with genus *Citrus* flavour, VOCs related to the aroma of other fruits were also investigated through IRMS, to guarantee their natural origin. Some authors tried to define the $\delta^{13}C$ authenticity limits for natural aromatic compounds of apple (*Malus domestica* L.) [77,78], pear (*Pyrus communis* L.) [78,79], strawberries (*Fragaria ananassa* L.), peach (*Prunus persica* L.) [78,80], raspberry (*Rubus idaeus* L.), sour cherry (*Prunus cerasus* L.) and blueberry (*Vaccinium corymbosum* L.) [78]. Bernreuther et al. reported significantly different ranges of variability for natural and synthetic γ-decalactone, while Strojnik et al. showed it is not always possible to distinguish between synthetic (petroleum-based) and natural VOCs, since they may have similar $\delta^{13}C$ values [78,80].

To improve the identification of synthetic adulterants or substituents, the $\delta^{13}C$ parameter was considered together with the $\delta^{2}H$, measured through GC-*p*-IRMS. As reported by Mao et al., natural (E)-cinnamic acid, vanillin, and benzoic acid extracted from Sumatra Benzoin Balsam (a natural balsamic resin exuded from *Styrax benzoin* tree), had $\delta^{13}C$ values ranging from −41.6 ‰ to −29.8 ‰ and $\delta^{2}H$ values ranging from −156 ‰ to −19 ‰. On the other hand, their synthetic analogs had $\delta^{13}C$ values ranging from −31.1 ‰ to −26.9 ‰ and $\delta^{2}H$ values ranging from +42 ‰ to +83 ‰ [81]. For Fink et al., the $\delta^{13}C$ and $\delta^{2}H$ of (E)-methyl cinnamate synthetic form, a common substance used in the flavour industry, ranged between −31.4 ‰ and −29.5 ‰ and +328 ‰ to +360 ‰, respectively. The esters produced from natural products such as galgant rhizomes, Peru balsam, strawberries, carambola (*A. carambola*), and cinnamon extracts and oils, revealed $\delta^{13}C$ between −30.1 ‰ and −25.6 ‰ and $\delta^{2}H$ between −169 ‰ and −162 ‰ [82].

Cinnamon oil derives from the bark or leaves of several types of trees, including ceylon (*Cinnamomum ceylanicum Breyn*), cassia (*C. cassia*) and cassia vera (*C. burmanii*). Culp et al. and Sewenig et al. investigated $\delta^{2}H$ and $\delta^{13}C$ values of cinnamaldehyde, its main aromatic component, deducing characteristic ranges of authenticity of the natural compound compared to the synthetic one [83,84]. The $\delta^{13}C$ and $\delta^{2}H$ values of natural (+/−)-Dihydroactinidiolide from tea (*Camellia sinensis*) and rooibos tea (*Aspalathus linearis*) ranged from −34.1 ‰ to −24.4 ‰ and from −274 ‰ to −153 ‰, respectively, whereas the values for the synthetic analog ranged from −28.4 ‰ to −27.0 ‰ and from −169 ‰ to −28 ‰, respectively [85].

The naturalness of wintergreen (*Gaultheria genus*) essential oils can be verified by GC-*c*-IRMS and GC-*p*-IRMS. While the $\delta^{13}C$, $\delta^{2}H$ and $\delta^{18}O$ values of natural methyl salicylate, its principal component, ranged between −38.9 ‰ and −34.2 ‰, −149 ‰ and −119 ‰ and −8.0 ‰ and +5.3 ‰ respectively, the synthetic ones ranged between −34.3 ‰ and −28.3 ‰, −97 ‰ and −13 ‰ and +18.0 ‰ and +27.8 ‰, respectively [86–88].

Estragole and methyl eugenol [89], trans anethol [90] from anise (*Pimpinella anisum*) and fennel (*Foeniculum vulgare*) seed and α-inone, β-ionone, α-ionol [91] from raspberry (*Rubus idaeus*) fruits and may chang (*Litsea cubeba*) and lemongrass (*Cymbopogon citratus*) oil are

organic compounds in which carbon, deuterium and, if available, oxygen isotopic compositions differ significantly between the synthetic and the natural form, which can derive from different sources.

The $\delta^{13}C$ and $\delta^{2}H$ values of the natural and synthetic form of other organic compounds deriving from different fruits have also been reported: 2-methylbutanoate, ethyl 2-methylbutanoate, methyl hexanoate, ethyl hexanoate, and 2,5-dimethyl-4-methoxy-3 [2H]-furanone from pineapple (*Ananas comosus*) [92]; butyl acetate, 1-butanol, hexyl acetate, 1-hexanol, methyl *E,Z*-2,4-decadienoate, ethyl *E,Z*-2,4-decadienaote, and ethyl *E,E*−2,4-decadienoate, from pear (*Pyrus communis*) [79]; γ-decalactone and δ-decalactone from peach (*Prunus persica* var. *persica*), apricot (*Prunus armeniaca*), and nectarine (*Prunus persica* var. *nectarina*) [93].

The measurement of the $\delta^{13}C$ has been associated with that of the site-specific hydrogen ratio via SNIF-NMR in a study on linalool and linalyl acetate isolated from essential oils of various plants including lavender (*Lavandula angustifolia*) [94]. This gave the possibility to calculate the percentage of addition of the synthetic form, evaluating the ten different ratios (D/H) (Fig. 2) [94]. The two mentioned lavender flavourings had higher $\delta^{2}H$ values in the synthetic form (linalool −209 ‰ to −185 ‰; linalyl acetate −197 ‰ to −173 ‰) compared to the natural one (linalool −307 ‰ to −265 ‰; linalyl acetate −276 ‰ to −238 ‰) [95]. These results confirmed what previously reported by Hor et al., who considered decanal, E2-exanal and E2-exanol, besides linalool and linalyl acetate from lavender, bergamot (*Citrus aurantium* var. *bergamia Loisel*), coriander (*Coriandrum sativum* L.) and thyme (*Thymus vulgaris*) [96].

For some matrices, the IRMS was sufficient for the authentication of natural compounds such as benzaldehyde from bitter almond oils, fruits, kernels and leaves [89]; citral (neral and geranial) in essential oils from *Cymbopogon flexuosus* and *C. citratus*, *Litsea cubeba*, *Lippia citriodora*, *Melissa officinalis* and the Citrus species *C. aurantium*, *C. limon*, *C. sinensis*, *C. paradisi* and *C. aurantifolia* [97]; thymol, carvacrol, gamma-terpinene and *p*-cymene in thyme (*Thymus vulgaris*), savory (*Satureja montana* subsp. *montana*) and oregano essential oils (*Origanum heracleoticum* L., *Coridothymus capitatus* L. *and Origanum compactum* L.) [98].

The coffee alkyl pyrazines contribute to its flavour and the synthetic analogs could be added to fraudulently correct it. The $\delta^{2}H$ showed an excellent ability to discriminate between natural and synthetic products in most of the forms investigated, while the $\delta^{15}N$ was not as effective [99].

2.2. Foods

Food represents an important application field for the SIRA to discriminate between molecules of natural and synthetic origin. The added value of many of these products is directly correlated with the presence and/or a certain concentration of characteristic molecules. Adulterations may consist in selling synthetic food additives instead of the more expensive natural analogs (e.g. caffeine) and/or in producing counterfeit foodstuff (e.g. vinegar obtained from fermentation of raw materials such as cane sugar).

Caffeine is a natural alkaloid which can be found in coffee, cocoa, tea, cola, guarana and mate plants as well as in their drinks, and represents the most widespread and consumed stimulating psychoactive substance in the world, being used both for recreational and medical purposes.

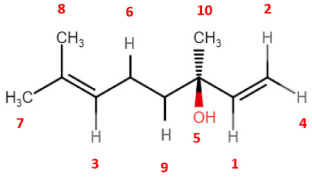

Fig. 2. Linalool structure. The sites where the $(D/H)_n$ isotope ratio can be measured are numbered in red.

The $\delta^{13}C$ ranges for natural (−32 ‰ to −25 ‰) and synthetic (−40 ‰ to −33 ‰) caffeine have been reported [100–103]. Additionally, $\delta^{15}N$ and $\delta^{18}O$ seem to allow for a significant discrimination between the natural and synthetic form. As reported by Weilacher et al., the $\delta^{15}N$ varies from −2‰ to +5 ‰ in the product obtained from coffee and/or tea and between −33 ‰ and −2‰ in the synthetic one [103]. As for the $\delta^{18}O$, it is possible to define a variability range from +12 ‰ to +17.1 ‰ for the synthetic product and from −12 ‰ to +11 ‰ for caffeine from coffee (*Coffea arabica*) and tea (*Camellia sinensis*) [101–103] or guarana (*Paullinia cupana*) [104].

The isotopic analysis of carbon via IRMS and of hydrogen via SNIF-NMR in ethanol has been reported since the 1990s as an official method for the identification of the addition of synthetic alcohol to must and wine (OIV-MA-AS311-05 R2011 and OIV-MA-AS312-06 R2009). Since the 2000s, the method has been adapted, validated, and then extended to wine vinegar. The isotopic ratios of the acetic acid extracted from this product were reported [105–107]. As previously mentioned, the $^{13}C/^{12}C$ ratio of the different sugars reflects the photosynthetic pathways the plant follows for CO_2 fixation. In turn, the amount of deuterium in the methyl site of ethanol, which formed during alcoholic fermentation, derives 85 % from the ^{2}H contained in fermentable carbohydrates [108]. Typical $(D/H)_I$ and $\delta^{13}C$ values for ethanol derived from C3 (as grape) and C4 plants (as cane or maize) and from synthetic sources are summarised in Table 2 [109–112]. These values can also be used for the evaluation of isotope ratios measured in the acetic acid extracted from wine vinegar.

Besides the grape-derived distillates, many other products can be analysed through IRMS: cane, tapioca and wheat ethanol [108]; pear, apple, sweet cherry, sour cherry, plum and apricot brandy [113]; beet, cane and maize sugar [110]; grain and potato ethanol [110], blue agave spirit tequila (*A. tequilana Weber* var. *Azul*) [114]; spirit vinegars [115]; canned vegetables containing vinegar pickle [116].

Fang et al. developed an innovative approach to identify the addition of synthetic acetic acid to brewed rice vinegar, based on the difference between the $\delta^{13}C$ of the extracted acetic acid and the precipitated proteins, or based on the $\delta^{13}C$ measurement of acetoin proteins, which represents one of the major flavours formed during vinegar fermentation. They established a 95 % confidence interval difference of 0.27–2.10 ‰ for the $\Delta\delta^{13}C_{protein\text{-}acetic\ acid}$ [117] and of 0.61–2.27 ‰ for the $\Delta\delta^{13}C_{acetoin\text{-}acetic\ acid}$ [118]. As reported by Hsieh et al., the ^{2}H SNIF-NMR analysis gave $(D/H)_{CH3}$ values of 98.50, 108.46 and 131.58 ppm for rice vinegar, molasses spirit vinegar, and synthetic vinegar, respectively [119].

As demonstrated by Perini et al., synthetic ethanol has $\delta^{18}O$ values

Table 2
Ranges of variability of $(D/H)_I$ and $\delta^{13}C$ of ethanol samples obtained by synthesis and by fermenting grapes and fruits.

		$(D/H)_{I\ ethanol}$ (ppm)		$\delta^{13}C_{ethanol}$ (‰, vs V-PDB)	
		min	max	min	max
Ethanol from C3 plants (Calvin Cycle)	Grape	99	106	−30	−24
	Fruits	97	105	−27	−25
	Grain	96	99	−26	−24
	Patato	93	97	−28	−25
Ethanol from C4 plants (Hatch-Slack Pathway)	Cane, Maize	108	113	−14	−11
Ethanol from CAM plants (Crassulacean Acid Metabolism)	Pineapple, Agave	100	113	−30	−12
Synthetic		123	133	−38	−25

(−2.6 ‰ to +12.4 ‰) significantly different from grape-derived (+24 ‰ to +36 ‰) and fruit- and tuber-derived (+13.9 ‰ to +28.8 ‰) one. This parameter can be therefore used to identify synthetic ethanol addition [120].

Safranal, the main VOC of saffron (*Crocus sativus*), guarantees the organoleptic quality of this expensive spice, but the synthetic analog can be fraudulently added in small quantities to powdered stigmas. As demonstrated by Moras et al., the addition can be identified via δ^2H analysis, discriminating between natural (mean $\delta^2H = +36 \pm 40$ ‰) and synthetic safranal (mean $\delta^2H = -210 \pm 35$ ‰) [121].

Since L-ascorbic acid is very unstable, it represents a suitable quality and freshness indicator for fruit juices. To correct its deficiency, L-ascorbic acid obtained from glucose through microbiological processes can be fraudulently added to different products. Since commercial glucose is usually obtained from C4 plants, the biosynthetic L-ascorbic acid has typical values around −11.3 ‰, different from the fruit-derived one, having values around −20.7 ‰ [122].

Perini et al. proposed the analysis of scyllo-inositol carbon isotopes as a method to identify the adulteration of concentrated grape must, due to its replacement with sugar syrups or fruit musts. This polyalcohol must be found in grape products, while its biosynthetic analog, having values around −11.8 ‰, could be fraudulently added [123].

As demonstrated by Camin et al., the analysis of δ^{15}N protein fraction allows for the discrimination between the animal rennet (min $\delta^{15}N = +5.7$ ‰) used for the production of high quality traditional PDO, PGI and TSG cheeses and the low-cost chymosin (genetic chymosin), produced through fermentation carried by the host microorganism *Aspergillus niger* var *awamori* (δ^{15}N from −5.3 ‰ to +2.2 ‰) [123,124].

2.3. Dietary products and drugs

The food supplements market significantly grew in recent years. These products contain molecules carrying health benefits and, as they claim to be "natural", they are often erroneously considered safer than traditional drugs. From a commercial point of view, the economic value of the ingredients used in the formulation of the food supplement is linked to the concentration of the molecule(s) of interest (e.g. the monacolin K content in fermented red rice or the curcuminoids concentration in turmeric extracts) and to its origin (higher value for natural ones compared to synthetic ones). The content of substances with beneficial properties, declared exclusively of natural origin, could actually derive from the addition of synthetic or biosynthetic substances to a natural base, resulting in a commercial offence. Begg et al. reported cases of dietary supplement frauds and demonstrated how this represents a formidable regulatory challenge [125].

Due to their role as efficient antioxidants, carotenoids are of nutritional relevance. Synthetic β-carotene, lycopene, lutein and canthaxanthin exhibit higher δ^{13}C and δ^2H compared to the natural analogs obtained from different sources [126].

Much lower δ^2H values for, the natural compared to the synthetic form were also recorded for curcuminoids, which are characterised by antioxidant, anti-inflammatory, antimutagenic, antimicrobial, and anticancer properties (mean $\delta^2H = -93$ ‰ ± 11 ‰ in natural and +51 ± 1.8 ‰ in biosynthetic samples) [127]. The same was also reported for cannabidiol (CBD), a non-psychoactive cannabinoid of *Cannabis sativa* exhibiting several beneficial pharmacological properties, including anti-inflammatory and antioxidant effects (mean $\delta^2H = -244$ ‰ ± 15 ‰ in natural and −171 ± 25 ‰ in biosynthetic samples) [128]. In the case of CBD, the δ^{18}O parameter represented an important contribution for the discrimination between the two types of molecules. In the biosynthetic CBD, the δ^{18}O averages +26.9 ± 0.6 ‰, while in the natural form it averages +20.1 ± 1.7 ‰ [128].

The δ^{13}C is not always sufficient by itself to discriminate between natural and synthetic products, as reported by Chen et al. in the case of melatonin [129]. Perini et al. reported that the δ^{18}O of the bulk sample, unlike the δ^{13}C, is a key parameter to guarantee the authenticity of the *Serenoa Repens* (Saw Palmetto) extract, used in the treatment of benign prostatic hypertrophy. The extract is a mixture of different fatty acids (FAs), therefore the commercial product can be falsified by formulating a blend of FAs having the concentrations prescribed by the United States Pharmacopeia (USP) monograph. The FAs used to mimic the vegetal extract usually derive from animals (processing waste) and have therefore characteristic δ^{18}O values (mean $\delta^{18}O = +16.2 \pm 2.0$ ‰), lower than the authentic ones, deriving from the *Serenoa* plant (mean $\delta^{18}O = +34.3 \pm 3.7$ ‰) [130]. The site-specific isotopic analysis of individual FAs is certainly more in-depth and useful, especially in the case of mixtures and it can also include the measurement of other parameters like hydrogen.

The molecules biosynthetically obtained usually derive from fermentation processes involving yeasts growing on a substrate. The carbon source is usually cane (C4 plant) sugar or glucose, which is commercially produced via enzymatic hydrolysis of starch [131]. In turn, starch mainly derives from corn (C4 plant) crops [132]. As reported by O'Leary, the normal range of C4 plants, like cane or maize, ranges between −10 ‰ and −16 ‰ [133]. Most of the plant sources of dietary supplements, and therefore of the natural molecules that characterise their composition, belong to C3 plant group, whose range of variability lays between −30 and −23 ‰ [134]. Thanks to this significant difference in the δ^{13}C isotope ratio, Perini et al. assessed the possibility to discriminate between natural monacolin K of red yeast rice (mean $\delta^{13}C = -29.6$ ‰) and the chemically indistinguishable cholesterol-lowering drug lovastatin (mean $\delta^{13}C = -16.7$ ‰), biosynthetically produced and fraudulently added to red coloured rice products [135,136]. L-theanine obtained from the tea plant (*Camelia Sinensis*) and used for its health properties, can be distinguished from the biosynthetic one thanks to both δ^{13}C (mean $\delta^{13}C = -24.4 \pm 1.3$ ‰ in natural and −14.3 ± 1.5 ‰ in biosynthetic products) and δ^{15}N (mean $\delta^{15}N = +7.4 \pm 1.6$ ‰ in natural and +2.0 ± 1.5 ‰ in biosynthetic products) [137]. In this matrix, the relatively low δ^{15}N values are likely due to the addition of ammonium sulphate (δ^{15}N - 3.1 ± 0.66 ‰) as a source of nitrogen to the yeast broth during biosynthesis.

The characterization of the illicit drug methylamphetamine represents an important and extensively studied field of application for the IRMS. The identification of the starting material of this psychostimulant, whether ephedrine/pseudoephedrine or phenyl-2-propanone (P2P), is performed using a combination of δ^{13}C, δ^{15}N and δ^2H. Moreover, as reported by Collins and Salouros, "*using the stable isotope ratios of methylamphetamine it is possible to determine which of the 3 common industrial processes* (Fig. 3) *was used to make the ephedrine/pseudoephedrine: (1) natural – extracted from the Ephedra plant, (2) semi-synthetic or bio-synthetic – a procedure involving benzaldehyde and sugar, or (3) fully-synthetic – a method of synthesis starting with the precursor propiophenone*" [138].

Grzechnik et al. defined the δ^{13}C and δ^{15}N variability ranges of the three forms [139]. As displayed in Fig. 4, they obtained a good discrimination with significantly lower δ^{13}C and higher δ^{15}N values in the natural form compared to the semi-synthetic and synthetic ones.

In some studies, only the synthetic form of specific molecules has been investigated. These data could be useful for future scientific works aiming to characterise the natural analogs. The IRMS of pharmacologically active synthetic molecules and their precursors, whether drugs or dietary supplements, was reported for molecules like creatine-monohydrate [140], benzylpiperazine [141], ibuprofen [35,142], naproxen [35] and 1-octacosanol [143]. These studies aimed to trace the chemicals producer (authorised vs clandestine labs) and the raw materials that have been used in the productive chain, or to determine whether a synthetic process was chosen for their production.

Silvestre et al. (2009) speculated on the origin of the acetic acid employed to build the side chain of acetylsalicylic acid and paracetamol, establishing its provenance according to the site specific δ^{13}C value. Some samples having very negative values ($\delta^{13}C \simeq -50$ ‰) seemed to have a petrochemical origin, whereas for other samples with less negative values ($\delta^{13}C \simeq -17$ ‰) a natural origin was suggested. This

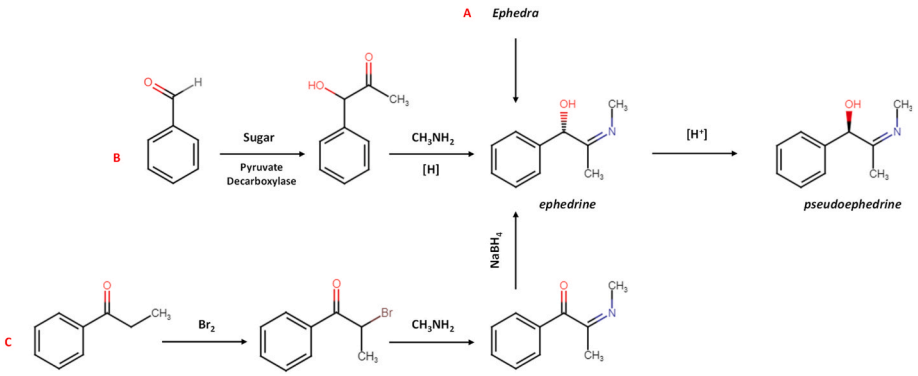

Fig. 3. The three major industrial methods for production of ephedrine (pseudoephedrine): (A) extraction of ephedra (natural route), (B) the semi-synthetic preparation by fermentation of a sugar source with benzaldehyde, and (C) the fully synthetic preparation commencing with bromination of propiophenone.

approach was proposed for the batch-to-batch traceability [144].

2.4. Steroids

Since 2008, a series of studies demonstrated the potential of the isotopic technique for identifying the illicit intake of molecules belonging to the anabolic androgenic steroids family aiming to improve sports performance (doping practice) [145,146].

Because of the low concentration of anabolic androgenic steroids, and due to the complexity of human urine as a matrix, the preconcentration of steroids followed by their separation via a multi-step extraction is required. It involves a preliminary extraction of analytes by solid phase extraction, the addition of a buffer to regulate the pH, the enzymatic hydrolysis and a final extraction by liquid–liquid extraction [147]. Once the steroid fraction has been isolated, the isotopic composition of the components is analysed using compound specific GC-*c*-IRMS analysis. Endogenous steroids naturally produced by the animal organism showed typical $\delta^{13}C$ ranging from −25.8 ‰ to −16.7 ‰, as animals can be fed both on a C3 and a C4 diet. On the other hand, exogenous synthetic steroids derives from plant sterols such as stigmasterol and sitosterol, obtained from C3 plants, except for progestins, containing an ethyl group at position C-13, which are entirely synthesised from fossil precursors [148]. As reported by Ueki et al. and Griffith et al., these C3 derivatives $\delta^{13}C$ ranges from −36.4 ‰ to −26.1 ‰ [149,150].

Unfortunately, as reported by Brailsford et al., commercially available synthetic nandrolone preparations sold in the UK could display $\delta^{13}C$ values overlapping the endogenous analogs ones ($\delta^{13}C$‰ from −26 to −16 ‰) [151]. Hülsemann et al. demonstrated how athletes should avoid consumption of wild boar, which may give rise to atypical analytical results in sports drugs testing [152].

Different authors have focused their studies on optimising the GC-*c*-IRMS or purification methods to avoid possible fractionation processes and to reduce the quantity of the starting urine sample [153–157]. For instance, Janssens et al. developed and reported a method for the detection of 17β-estradiol given to cattle and breeding animals and for the measurement in urine of 5- androstene-3β,17α-diol as ERC and 17α-estradiol as a metabolite [158,159].

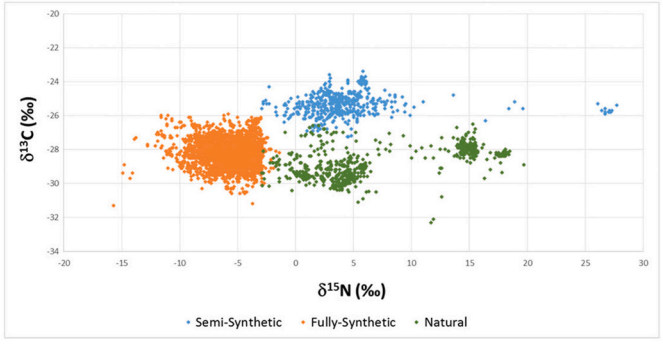

Fig. 4. $\delta^{13}C$ vs $\delta^{15}N$ of ephedrine/pseudoephedrine-derived methylamphetamine samples seized at the Australian Border between 2010 and 2017 (reprinted with permission from Grzechnik et al., 2018).

2.5. Miscellaneous

Several studies on the applicability of the IRMS for the discrimination between natural and synthetic molecules of products not belonging to the previously mentioned categories are reported in the literature.

Surfactants are amphiphilic molecules produced mainly from fossil sources, that represent up to 40 % of laundry detergents. To reduce their environmental impact, derived "bio-based" surfactants, totally or partially synthesised by renewable resources, are now commercially available. Gaubert et al. and Pironti et al. investigated the $\delta^{18}O$, $\delta^{13}C$ and $\delta^{2}H$ of synthetic and bio-sourced surfactants and mixtures. Gaubert et al. found $\delta^{18}O$ to be the most discriminating parameter, with values ranging from −4.8 ‰ to +11.7 ‰ in the synthetic product and from +14.6 ‰ to +28.6 ‰ in the bio-based one. The $\delta^{13}C$, however, provides less clear information, as demonstrated by the different results reported by the two authors [160,161].

Llana-Ruíz-Cabello et al. reported the $\delta^{13}C$ variability of polylactic acid, an aliphatic polyester obtained mainly from renewable agricultural sources (corn, a C4 plant), and demonstrated that the addition of natural additives from C3 plants could influence it [162]. Additionally, González-Pérez et al. isotopically characterised (through the measurement of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$) a plastic molecule of 100% fossil origin, a dye (*o*-chloroaniline) and the polyethylene which contained it [163].

Edible bird's nests (EBNs), also known as swallow nests (Chinese: 燕窝; pinyin: Yàn Wō), are made up of solidified saliva by edible-nest swiftlets, Indian swiftlets and other swiftlets belonging to the genera *Aerodramus, Hydrochous, Schoutedenapus* and *Collocalia*, which are harvested for human consumption. This traditional product was investigated through IRMS in two separated studies [164,165]. Presenting some similarities to EBNs texture, various cheap materials such as pig skin, tremella, agar, food gums, egg white and glucose could be used to adulterate this product by increasing its net weight. Nevertheless, establishing a data processing formula to improve cohesion of the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$ isotope ratios, a discriminant formula to quickly assess the authenticity of EBN was achieved with an accuracy ranging from 88 % to 99 % [165]. The adulteration of EBNs with exogenous sialic acid to improve their quality is easily detectable as the synthetic adulterant has mean $\delta^{13}C$ (−16.26 ± 3.91 ‰) lower than the natural analog ones (−29.90 ± 0.42 ‰) [164].

3. Conclusions

This review highlights that both SNIF-NMR and IRMS can be techniques of choice for the discrimination of chemically identical molecules derived from different sources (natural from plant sources, biosynthetic or synthetic from fossil/petrochemical sources). While both methods are costly and require specialised equipment, SNIF-NMR is less accessible and more complex than IRMS, but it provides more detailed, molecular site-specific information. Despite the high precision and sensitivity of SNIF-NMR, IRMS has broader scientific applications and faces challenges like complex data interpretation and limited reference materials.

The δ^2H by IRMS or $(D/H)_n$ by SNIF-NMR demonstrated the greatest discrimination ability among the isotopic parameters, generally showing lower values in the natural than in the synthetic molecules. The δ^{13}C, despite being the most studied parameter, did not always perform a significant discrimination between natural and synthetic fossil products. It is certainly the parameter of choice in the differentiation between natural molecules extracted from a specific plant and their biosynthetic analogs, synthesised from C4 substrates such as cane and corn (e.g., red yeast rice or L-theanine). In some specific cases, the δ^{15}N (for caffeine) and δ^{18}O (for *Serenoa Repens* extract) allowed excellent characterization results to be obtained.

The top techniques for the compound specific analysis are represented by the GC-*c*-IRMS and the GC-*p*-IRMS when the investigated substance is volatile (e.g., flavour/aroma) and by the LC-IRMS when it is soluble in water (e.g., organic acids). In all other cases, the use of preparative- HPLC carrying organic solvents as mobile phase is still the most advisable choice.

The analysis of δ^{13}C and δ^2H, both in bulk samples (through EA-IRMS or TC-IRMS) and in specific compounds (via GC-*c*-IRMS, LC-IRMS or GC-*p*-IRMS), is of widespread applicability, whereas the analysis of δ^{15}N and δ^{18}O, is still difficult to perform. The causes lie mainly in the technical setup required for the analytical execution, which involves a dedicated column for δ^{18}O measurements in the GC-IRMS and cryogenic traps for the analysis of δ^{15}N to remove CO_2 which can crack in the ion-source to give CO isobaric interference with N_2.

CRediT authorship contribution statement

M. Perini: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Data curation, Conceptualization. **S. Pianezze:** Writing – original draft. **L. Bontempo:** Writing – review & editing, Supervision, Investigation, Conceptualization.

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.

Data availability

Data will be made available on request.

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