Partenariat Analyse Authenticité Arômes

P3A

Focus
The role of isotopic analysis in checking the regulatory compliance of natural flavourings

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This document has been prepared by the Scientific Committee of the Partenariat Analyse Authenticité Arômes (Flavouring Authenticity Analysis Partnership or P3A). P3A was set up by SNIAA (French Flavour Association) in conjunction with several analytical laboratories with the aim of boosting confidence both in the results produced by analytical laboratories and in the products offered by flavouring manufacturers.

Members of the P3A are indeed frequently called upon to conduct analyses to confirm the compliance of natural flavourings.

The P3A Scientific Committee has decided to draft a serie of documents to provide practical and explanatory information regarding the role of each of these analytical methods available for checking the regulatory compliance of natural flavourings.

1.Isotopes and isotopomers

A chemical element is made up of a nucleus, itself made up of nucleons (protons and neutrons), around which gravitate the electrons. The atomic number Z, which describes both the number of protons and the number of electrons, is used to identify each element. The mass of the proton (m_{proton}=1,67625.10^{-27} kg) and that of the neutron ($m_{neutron}$=1,67495.10^{-27} kg) are very close and far greater than that of the electron ($m_{electron}$=9,10953.10^{-31} kg).

The mass of the atom is consequently virtually guaranteed by that of the nucleus, and is therefore characterised by its mass number, noted A, corresponding to the number of nucleons (Z protons + (A-Z) neutrons).

Atoms that have the same atomic number Z but a different mass number A, i.e. a different number of neutrons, are called *isotopes*. Isotopes have the same atomic number, so belong to the same element and are in the same position in the periodic table.

The isotope with the highest mass number is said to be heavy (e.g. for carbon: ^{13}C) as opposed to the light isotope (e.g. for carbon: ^{12}C) with the lowest mass number.[1]

[1] This concept applies in particular to the C, H, O, N and S atoms most frequently found in organic molecules such as those constituting flavourings.

There are two types of isotopes:

› Stable isotopes: these have a structurally stable nucleus that remains unchanged over time in the absence of external energy input,

› Radioactive isotopes: these have an unstable nucleus that spontaneously transforms into another, emitting radiation or a particle.

Isotopomers are molecules of the same composition and configuration in which one of the atoms has been substituted by one of its less abundant isotopes. Due to the very low natural abundance of heavy isotopes (Table 1), the probability of detecting a molecule containing two or more heavy isotopes of the same element is negligible. Consequently, an isotopomer is defined as a molecule containing only one heavy atom.

Element	Stable Isotopes	Natural Abundance in Average (in %)
Hydrogen	^{1}H	99.9844
	^{2}H (or D)	0.0156
Carbon	^{12}C	98.891
	^{13}C	1.108
Oxygen	^{16}O	99.759
	^{17}O	0.037
	^{18}O	0.204
Nitrogen	^{14}N	99.635
	^{15}N	0.365

Table 1: Stable isotopes of the main constituents of flavouring molecules, and their natural abundances.

2.The different isotopic parameters

The parameters used to describe and measure isotopic distribution phenomena are defined as follows:

The Natural Abundance noted NA and expressed in ppm, indicates the relative proportion of the heavy isotope in question. It is a kind of "isotopic mole fraction":

$$NA = \frac{isotope\ (heavy)}{isotope\ (heavy) + isotope\ (light)} \quad \left(example\ of\ carbon\ NA_{carbon} = \frac{^{13}C}{^{13}C + {}^{12}C}\right)$$

The *isotopic ratio*, noted R and expressed in ppm, is defined as the quotient of the number of heavy isotopes over the number of light isotopes of a given element:

$$R = \frac{isotope\ (heavy)}{isotope\ (light)} \quad \left(example\ of\ carbon\ R_{carbon} = \frac{^{13}C}{^{12}C}\right)$$

These two parameters (R and NA) vary from one compound to another. However, their values can be difficult to handle, as small variations are observed over small quantities. It is therefore better to express this same physical reality using a third relative parameter called *isotopic deviation*, noted δ and expressed in ‰, defined according to the following formula:

$$\delta = 1000 \times \frac{R_{sample} - R_{ref}}{R_{ref}}$$

R_{sample} and R_{ref} being respectively the isotopic ratios of the sample to be measured and the reference sample.

The use of reference materials calibrated against international references (listed in Table 2) enables inter-calibration between measuring instruments and between laboratories.

This isotopic deviation makes it possible to assess small differences in isotopic content.

Element	Molecule	Name	Origin	NA (ppm)	R (ppm)
Carbon	$CaCo_3$	PDB (Pee Dee Belemnite)	South carolina limestone	11112.33	11237.2 (±2.9)
Hydrogen	H_2O	V.SMOW (Vienna Standard Mean Ocean Water)	Blend of ocean waters	155.74	155.76 (±0.05)
Oxygen	H_2O	V.SMOW	Blend of ocean waters	2001.2	2005.2 (±0.43)
Nitrogen	N_2	Air	Atmospheric nitrogen	3663	3676.5 (±2.9)

Table 2: International references used for isotopic analysis

3.Stable isotope enrichment or isotopic distribution

3.1. Carbon-13

During photosynthesis (Figure 1), plants assimilate atmospheric carbon dioxide by means of different metabolisms. Only this part of photosynthesis, which has a significant isotopic effect on plants, will be explained here.

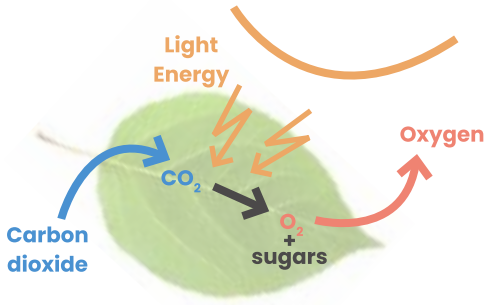

Figure 1: Simplified representation of photosynthesis

Three main types of primary metabolism are possible, with different isotopic fractionations leading to different isotopic distributions:

› C3 metabolism (3-carbon intermediate), known as the Calvin cycle, is by far the most widespread, with 98% of green plants subject to it, including vines, orange trees, wheats, sunflowers and beetroots.

› C4 metabolism (4-carbons intermediate), known as Hatch and Slack, represented by sugar cane and maize, among others.

› CAM metabolism (Crassulacean Acid Metabolism), comparable to C4 metabolism, but delayed in time (the phases of C4 metabolism are not continuous but alternate day and night). It is less widely observed because it only affects so-called 'fat' plants such as cacti, pineapples, vanillas and agaves.

It is known that natural molecules derived from these plants retain an isotopic signature specific to the environment and to these modes of photosynthesis. For example, products from C4 plants, such as sugars and alcohol derived by fermentation, have higher carbon-13 contents than their counterparts from C3 plants. Measuring the carbon-13 content therefore makes it possible to detect and quantify sugar of C4 origin (cane sugar or maize isoglucose) added to products derived from C3 type plants, such as wine or honey, in order to conclude whether the product has been adulterated (particularly in the case of a claim of botanical origin).

Regarding typical values, C3 plants have a $\delta^{13}C$ deviation between - 33‰ and -20‰ on average. For C4 plants, this value is between -14‰ and -9‰. Thus, each natural substance has a characteristic isotopic deviation (Figure 2). Consequently, being able to measure the carbon isotopic ratios ($^{13}C/^{12}C$) of a molecule with sufficient accuracy enables us to trace it back to the primary metabolism of the plant that produced it. Using ad hoc databases, isotopes can be used as a genuine tracer of botanical or geographical origins and can also be used to differentiate between molecules according to the natural or synthetic origin of the carbon atoms they contain.

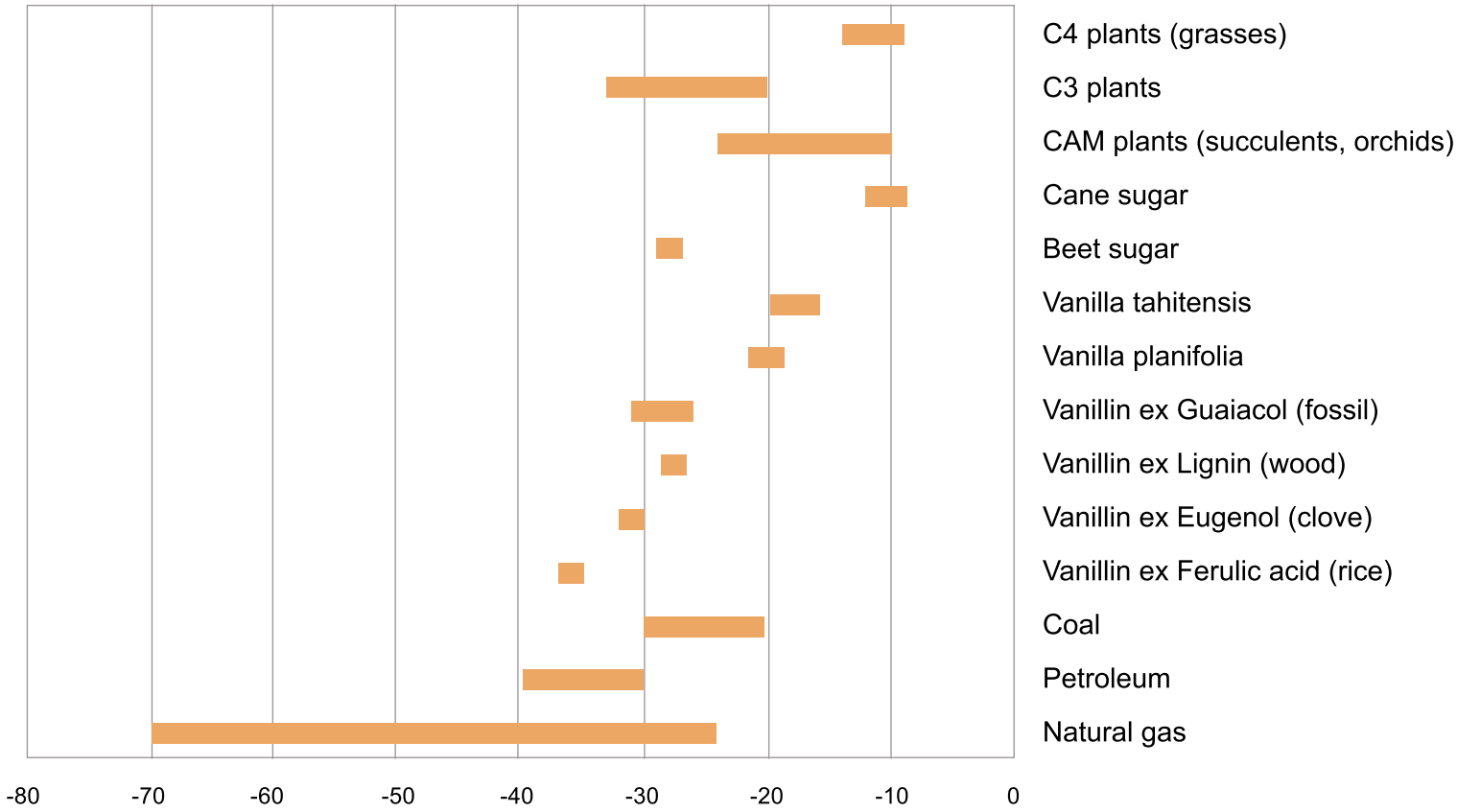

Figure 2: Average $\delta^{13}C$ isotopic deviations (in ‰) of different natural products.

3.2. Deuterium (^{2}H) and Oxygen-18

Fresh water (rain, irrigation, etc.) is the only source of hydrogen (^{1}H) for photosynthesis. Unlike carbon-13 isotopic deviation values, which remain broadly the same regardless of the geographical area from which the plant originates, deuterium (^{2}H) levels in plants can vary widely. The levels of the heavy isotopes ^{2}H and ^{18}O in irrigation water depend on a number of factors, including geography, climate, latitude, altitude, etc. All these factors lead to a wide disparity in deuterium isotopic ratios in the water, which influences deuterium isotopic abundance in the biomass.

Plant evapotranspiration is also an important factor in isotopic fractionation. For example, above-ground plants such as vines have a higher evapotranspiration rate than below-ground plants such as beetroot. This will result in greater deuterium enrichment in the sugars from grapes than in those from beets.

Measuring the isotopic deviation of deuterium is most often used to verify the natural or synthetic origin of a substance. In the first instance, this measurement identifies the use of a chemical synthesis process (not natural) to obtain a substance.

More generally, the value of the isotopic deviation of deuterium is used in combination with the value of the isotopic deviation of carbon-13 when the analysis of the carbon isotopic deviation is not sufficiently discriminating to assess the naturalness or the origin of a substance (Figure 3).

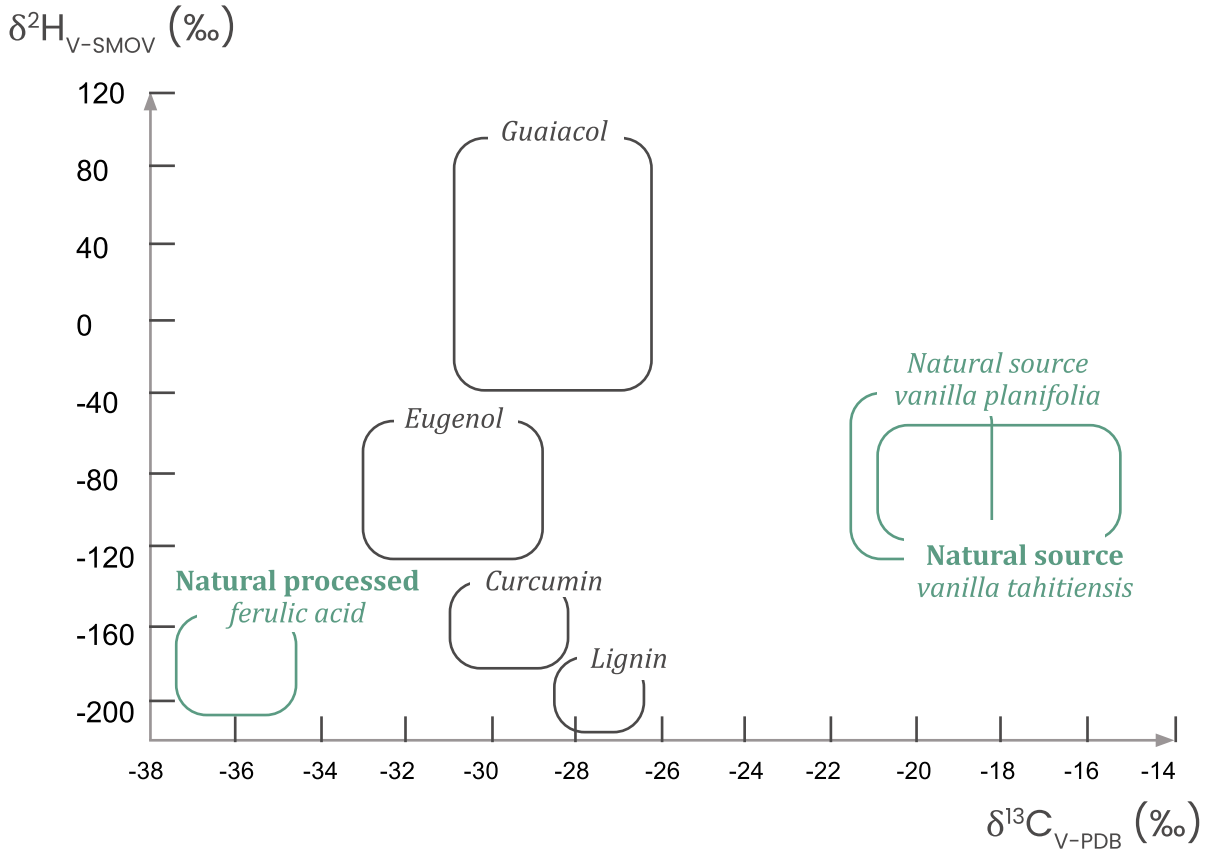

Figure 3: Average isotopic deviations $\delta^{13}C$/ $\delta^{2}H$ (in ‰) of vanillin.[2]

[2] Christoph N., Schellenberg A., Zander W., Krammer G. Stable isotope ratio analysis for authenticity control. Handbook of Odour, Springer 2016, 439-458

3.3. Nitrogen 15

The isotopic deviation of nitrogen gives information on the trophic level of the compound: in nature, a mineral or fossil origin will have a deviation close to 0‰ (vs AIR), a plant origin around 3‰ and an animal origin around 6‰.

Furthermore, in the case of chemical reactions attacking nitrogen-containing chemical functions/groups, the parameter $\delta^{15}N$ will become a true tracer of the product's origin.

For example, natural caffeine shows deviations between -1‰ and +6‰ whereas synthetic caffeine has much more negative values, between -10‰ and -30‰.

The $\delta^{15}N$ parameter is also useful for checking the naturalness of amino acids (leucine, cysteine, etc.), and for verifying the organic or conventional nature of certain products. It is also a relevant parameter for origin control (cereals, meat, honey, milk and dairy products).

3.4. Sulphur 34

The ratio of sulphur isotopes $^{34}S/^{32}S$ is also a parameter that can be used to trace the origin of the product. It is affected by proximity to the sea (marine deposits) and is also highly dependent on anthropogenic activities. It will therefore be an informative parameter for controlling the origin of products (particularly animals, which contain sulphur more than plants).

Also during organic synthesis, the use of sulphur groups will lead to an increase in the sulphur isotope content. It is measured against the international standard CDT (Canyon Diablo Troilite). More recent in its routine use in laboratories due to its constraints (dangerousness of the reference gas SO_2, risk of clogging, etc.), it has been well mastered since the early 2010s due to technological advances in instrumentation and is included in the packages for controlling origin because it is very informative.

4.Isotopic fractionation linked to manufacturing processes of flavourings

The difference in mass between isotopes gives atoms and molecules significantly different physical and chemical properties. During a manufacturing process involving transformation, *isotopic fractionation* occurs when the ratio of two isotopes of the same element is different in the initial molecules and in the molecules produced. This fractionation is the consequence of two types of isotopic effects, kinetic and thermodynamic, and often accompanies the transformation of chemical species.

This isotopic fractionation can vary according to:

› The botanical specie/variety used and its geographical origin: this is referred to as isotopic distribution (see part 3.),

› The physical, chemical or biochemical transformations involved in making a product.

As explained above, measuring the isotopic deviation in stable isotopes makes it possible not only to trace the geographical and/or botanical origin of a substance, but also to obtain clues as to how it was manufactured (natural or synthetic process).

To measure the isotopic deviation of a compound present in a complex matrix, it is important to ensure beforehand that the purification or extraction step used do not lead to isotopic segregation, which would affect the analytical result.

5.Isotopic analysis techniques

5.1. Isotopic Ratio Mass Spectrometry (IRMS)

Isotopic Ratio Mass Spectrometry (IRMS) is an essential tool in isotopic analysis. The usefulness of this method has been demonstrated in the field of authenticity, as it makes it possible to determine the exact isotopic ratio of an element (H, C, O, N or S) within a molecule.

This ratio can be used to differentiate between two molecules with identical structures but different origins. The determination can be made by combining the IRMS technique with chromatographic separation methods, elementary analysers, pyrolysis or equilibration systems, and these combinations can be then used to analyse all kinds of samples: solid, liquid or gaseous, including complex mixtures.

The speed, specificity and sensitivity of IRMS detection make it easy to assay targeted elements and compounds present in very small quantities, while providing information on isotopic content in natural abundance.

The principle of IRMS is to measure the isotopic ratios of an analyte transformed into single gases, which are representative of the initial sample. The system can measure the D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ or $^{34}S/^{32}S$ isotopic ratios present in the structure of the molecule.

5.1.1. Elemental analyser (EA-IRMS)

The elemental analyser (EA) is used to obtain the percentage of carbon, hydrogen, nitrogen, oxygen and sulphur in an organic sample and convert the sample into elemental gases that can be analysed by isotopic mass spectrometer (Figure 4). The absence of a molecular separation step limits the use of this coupling to high-purity compounds. This coupling is also of interest for analysing inorganic compounds, polymers or other thermolabile compounds that cannot be analysed by gas chromatography, such as ferulic acid (a precursor of vanillin obtained by biotechnology).

Another advantage of this coupling is that it allows the use of a large proportion of the IAEA (International Atomic Energy Agency) international standards that mostly cannot be analysed by gas chromatography, such as NBS22 (oil), SLAP (water), GISP (water), IAEA-CH-3 (cellulose), IAEA-CH-6 (sucrose), etc., which are required for reference gas calibration.

The samples to be analysed may be in solid or liquid form, depending on the type of autosampler installed on the instrument.

Figure 4: Example of EA/IRMS coupling

5.1.2. Coupling with gas chromatography (GC-c/p-IRMS)

Coupling an isotopic ratio mass spectrometer with a gas chromatograph makes it possible to carry out isotopic analyses of molecules present in complex mixtures, with or without prior purification steps.

The separative power of gas chromatography makes it possible to isolate a target molecule and transform it into an elemental gas using a combustion or pyrolysis furnace. The different isotopomers (ions of different isotopic combinations) making up the formed elemental gas are then analysed by the IRMS on the basis of their mass differences in order to determine the isotopic signature of the molecule of interest.

[3] Elemental gases are gases resulting from the combustion or pyrolysis of the analysed substance such as CO_2, H_2, O_2, N_2...

5.2. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectroscopy is a non-destructive technique for characterising molecules, exploiting the magnetic properties of the nuclei (Figure 5). In particular, it can be used to determine the developed structure of a molecule and its conformation in space. It can be used for samples in the liquid, solid or liquid crystal state. Widely used in organic synthesis on hydrogen and carbon-13 nuclei to identify synthesised compounds, NMR is also intrinsically quantitative. Commonly marketed at high field strength (minimum 400MHz), it can be used to quantify the distribution of isotopes at each site of the molecule.

Figure 5: Example of an NMR spectrometer

The SNIF-NMR® technique (Site-specific Natural-Isotope Fractionation by Nuclear Magnetic Resonance) can be used to quantify deuterium or ^{13}C enrichment at different sites of a molecule, providing additional discrimination possibilities compared with IRMS. The distribution of deuterium and carbon-13 at the different sites of a molecule is not organised statistically but depends directly on the origin of the molecule and its biosynthesis. This measurement therefore provides a very precise 'fingerprint' of the analysed compound. The isotopic ratios for the different sites are calculated from the areas of the different peaks in the NMR spectrum, converted into isotopic ratios or deviations by internal calibration using a reference compound whose isotopic composition is precisely known, or by measuring the overall ratio using IRMS.

The SNIF-NMR method was originally developed in response to a government call for tenders by Professor Gérard Martin and his team at the University of Nantes, with the aim of detecting the addition of sugar to wines (chaptalisation) and the enrichment of grape musts (G. Martin et al, 1981[4]). It was then extended to many natural substances with high added value, including flavouring substances.

One of the limitations of deuterium NMR is its lack of sensitivity, which is directly linked to the low natural abundance of this isotope. This means that the technique requires a large sample size (of the order of a gram) or a relatively long analysis time (several hours) and a high degree of purity of the target molecule, which can be limiting factors for compounds that are often present in small quantities in flavourings.

The isotopic analysis of an NMR spectrum can be expressed in terms of specific isotopic ratios at each site of the molecule. This isotopic ratio measurement (defined in part 2.) is determined relative to a reference. This reference has a unique and known isotopic ratio.

[4] Martin GJ, Martin ML. Tetrahedron Lett;22:3525-8 (1981)

Let's take the case of a molecule analysed using deuterium isotope NMR. For this analysis, the reference is an internal reference, the tetramethylurea molecule (TMU). For each signal of the molecule to be analysed, an isotopic ratio will be determined based on this internal reference using the formula:

$$(D/H)i = \text{Intensity (site i)} \times C$$

This equation shows that the isotopic ratio specific to site i, (D/H)i - the ratio of the number of deuterium D to the number of hydrogen H - is then a function of the intensity of the signal attributed to site i on the spectrum and of a factor C, all the parameters of which are known when the sample is prepared.

This C factor comprises multiple parameters: the known isotopic ratio of the reference, the intensity of the reference peak, the masses weighed in the tube for the sample and the reference, the molar masses of the molecule under study and the reference molecule, the purity of the reference, the purity of the sample, the numbers of equivalent atoms considered under the respective signal of the site of the molecule and the reference. The intensity of the signal of interest for which we wish to calculate the isotopic ratio has no unit. The only unit used in the constant formula is the isotopic ratio of the reference, expressed in ppm. Consequently, the site-specific isotopic ratio will be expressed in ppm.

SNIF-NMR analyses have also been developed for carbon-13 isotope measurements. In the case of carbon-13 analysis, the methods were developed without an internal reference. Site-specific isotopic measurements are then compared with a value that will be considered as a reference for SNIF-NMR and obtained by another analytical technique, Isotope Ratio Mass Spectrometry (IRMS).

6. Application of isotope analysis to vanillin

IRMS analysis can be carried out either by EA-IRMS on the pure molecule or by GC-c/p-IRMS after an extraction stage on a complex product (vanilla extract, natural vanilla flavouring, vanilla flavoured product, etc.).
The $\delta^{13}C$ value will provide a first general information which will allow an initial discrimination, however partial (Figure 6). For example, vanillin from vanilla beans will have a $\delta^{13}C$ value of about -20‰.

Vanillin derived from rice ferulic acid will have a $\delta^{13}C$ value of around -37‰ and finally vanillin derived from guaiacol, eugenol or curcumin around -30‰. However, as illustrated in Figure 6, a 50/50 mixture of vanillin from vanilla beans and vanillin from rice ferulic acid will also show a value of around -30‰. Thus, the value of $\delta^{13}C$ alone does not provide complete assurance of the origin of the vanillin analysed.

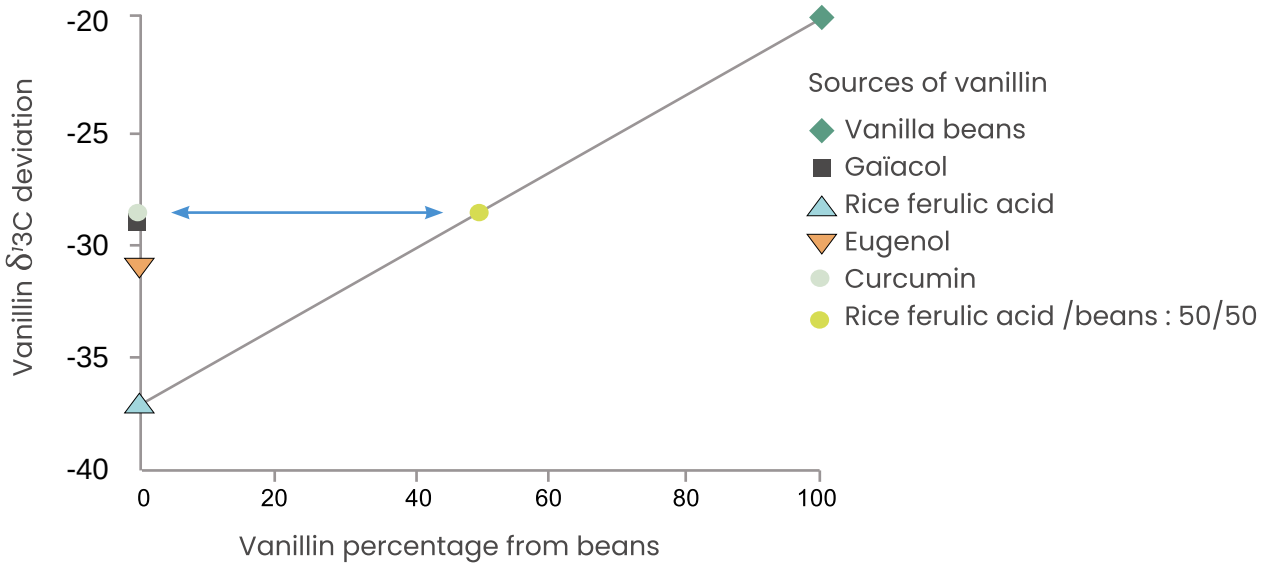

Figure 6: $\delta^{13}C$ of vanillin (AE-SMRI or CG-C-SMRI) - a partial precursor indicator

Using isotopic NMR, vanillin isotopomers can be observed with 5 sites for hydrogen and 8 sites for carbon. The hydrogen in position 2 is too labile and will tend to be exchanged in solution, so should not be considered when interpreting the results (Figure 7).

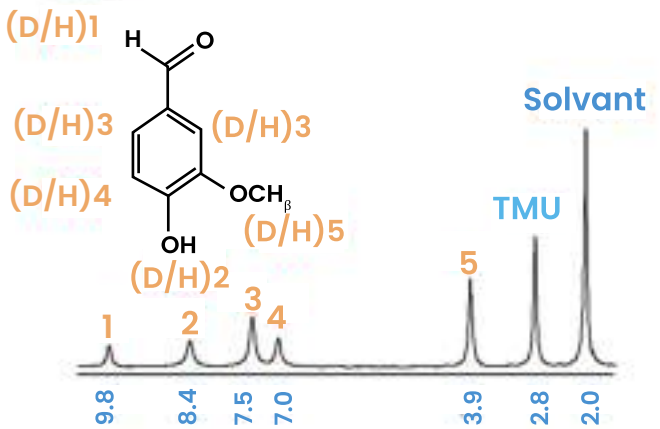

Figure 7: Vanillin molecule and SNIF-NMR spectrum of deuterium

Here Solvent = acetonitrile and TMU = tetramethylurea, calibrated internal reference (international standard)

To determine the vanillin precursors, a principal component analysis[5] (PCA) was carried out on the values of the specific isotope ratios (D/H)1, (D/H)3, (D/H)4, (D/H)5 (Figure 8).

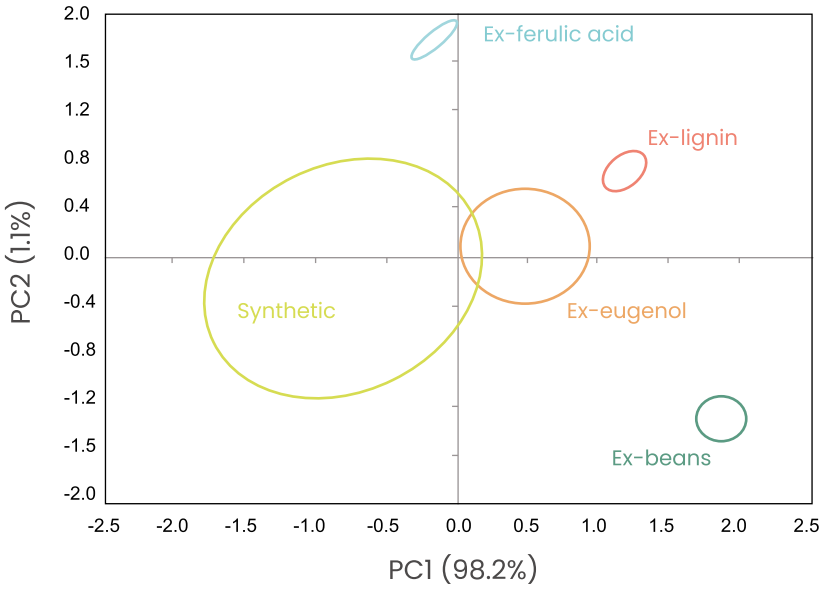

Figure 8: Principal Component Analysis (PCA) used to discriminate the main precursors by SNIF-NMR of vanillin deuterium.

[5] Principal Component Analysis (PCA) is a data analysis method that transforms a 3-dimensional data set into 2 dimensions by grouping the information. In this way, information can be summarised by reducing the number of variables, and a complex representation can be transformed into a simple graphical representation.

However, as mentioned in paragraph 5.2, the quantity of vanillin required is large (~1g) and the acquisition time for the spectra is long (~15 hours).

SNIF-NMR ^{13}C reduces the quantity of vanillin to be analysed (~250mg) and the time required to acquire spectra (~1 hour per spectrum). It also provides better discrimination between the different groups (Figures 9 and 10).

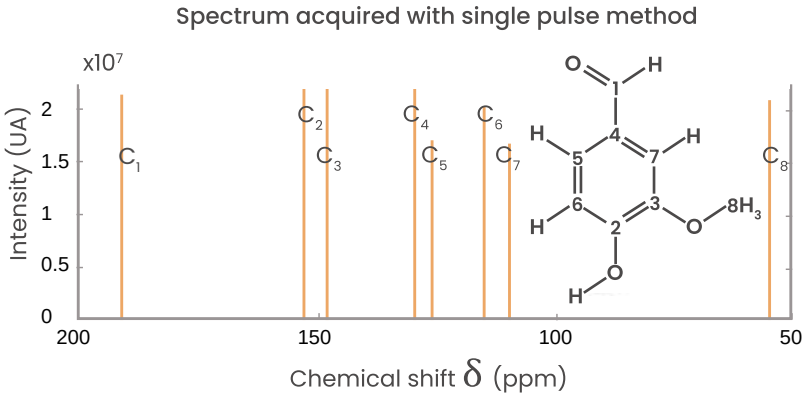

Figure 9: Vanillin molecule and SNIF-NMR spectrum of carbon-13 [6]

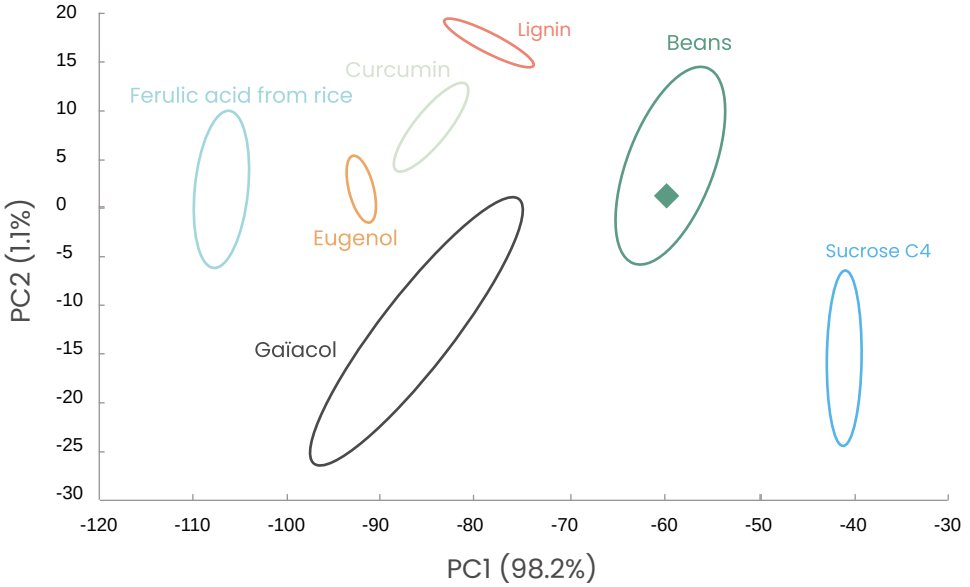

Figure 10: Principal Component Analysis (PCA) used to discriminate the main precursors by SNIF-NMR ^{13}C of vanillin[6]

It is also possible to use the two dimensions ^{2}D and ^{13}C for optimum separation. However, it is not often that the analysis is taken this far.

[6] Vanillin isotopic intramolecular 13C profile through polarization transfer NMR pulse sequence and statistical modelling V. Portaluri, F. Thomas, E. Jamin, B. Lorandel, V. Silvestre, S. Akoka, G. Remaud. Food Control,108345 (2021)

7. Isotopy and regulatory compliance

At European level, Regulation (EC) N° 1334/2008 sets out the regulatory requirements for flavourings and certain food ingredients with flavouring properties. The regulatory compliance of natural-status flavourings is multifactorial, as detailed in the document entitled "Analytical controls and regulatory compliance of natural flavourings" written by the P3A platform.

Determining the relative abundance of certain stable isotopes provides relevant clues about the origin of flavouring ingredients. In particular, it can be used to distinguish a natural molecule from a synthetic molecule (produced by the petrochemical industry). In the case of natural molecules, isotopic analysis can be used to trace the botanical and geographical origin of a compound.

For flavouring ingredients, isotopic analysis of ^{13}C and ^{2}H are the most widely used. The D/H ratio is more difficult to interpret but can reveal the use of production/synthesis processes that do not comply with Regulation (EC) No 1334/2008. Isotopic analyses can sometimes reveal inconsistencies with the manufacturing process indicated by the supplier, while concluding that a molecule is of natural origin. It is essential to have a solid database, as this is essentially a comparative analysis.

Isotopic analysis can therefore be used to raise doubts and identify inconsistencies in flavouring designations, particularly when the isotopic deviation measured indicates a different origin from the one claimed (natural/synthetic, from source/biotechnology).

That said, isotopic analysis alone is not always enough to conclude positively or negatively about the naturalness of a flavouring. This analysis should be combined with:

› Other techniques for identifying molecules (chiral analysis, ^{14}C analysis, detection of the presence of adulterants not detectable by gas chromatography or synthetic impurities, etc.).

› An assessment of the guarantees of compliance established by the flavouring producer in terms of processes, formulation, etc.

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