Natural Vanillin Obtained by Means of Bioconversion

Authentication of botanic origins.

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Vanilla has traditionally been, and will certainly remain, one of the most widely appreciated flavors in the world, reminiscent of wholesomeness, tenderness and comfort of childhood, while remaining a very trendy flavor.

Vanillin naturally occurs in levels up to 2% of the dry matter of cured vanilla beans (**F-1**); vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main organoleptic characteristic aroma component.

The large demand for vanilla in sweet flavors cannot be met by the available volume of cultivated vanilla beans alone. In order to satisfy increasing global demand, ingredient suppliers have developed ways to produce natural vanillin identical to the one present in vanilla beans.

Natural vanillin[a] obtained via the bioconversion of ferulic acid from rice was first produced more than a decade ago.[1] The present paper describes the authentication tools enabling the differentiation of the botanic origins of natural vanillin: vanillin from vanilla beans and vanillin bioconverted from plants presenting C3 and C4 photosynthetic pathways.

Vanilla Bean Market Situation

Vanilla is farmed in tropical areas around the world. The quantity and quality of yields vary due to changeable weather, diseases such as fusarium, speculative early harvesting and other factors. This affects availability, quality and the stability of market prices. For example, tropical storms in 2003 led to a shortage of vanilla supply from Madagascar and a drastic rise in bean prices. As a result, in 2005, prices increased tenfold. In addition, quality challenges, and therefore profitability complications for producers, is currently being observed in Madagascar. Early indications this year reveal an upward pricing trend after several years of stable prices.

Nonetheless, there is an increasing consumer-led demand for natural flavors consistent with healthy, organic trends in Western markets. Due to the limited volume and varying quality of some vanilla beans, only about 50 metric tons of pure vanillin can be extracted from the total annual production of vanilla beans, representing about 0.3% of worldwide vanillin demand.

Determining Natural Status

Vanillin from ferulic acid is defined as a natural flavor, according to both E.U. and U.S. regulations. As far as flavoring substances are concerned, regulations in force in Europe are EC 1334/2008 and, in the United States, the Food and Drug Administration (FDA) 21CFR101.22 .[2,3]

To allow a flavoring substance to be labeled "natural" under E.U. regulations, three conditions must be strictly met:

- The flavoring substance is naturally present in nature
- The raw material is natural
- The process is traditional

Glossary

AIR: Agro-Industrial Research

Bioconversion: The conversion of one substance to another by biological means. The fermentation of sugars to alcohols, catalyzed by yeasts, is an example of bioconversion.

Catabolic: A metabolic process in which complex molecules are broken down into simple ones by living cells with the release of energy; destructive metabolism. For example: ferulic acid lost carbons and oxygen to be transformed into vanillin

C: Carbon

DGCCRF: Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes

EA-IRMS: Elemental analysis-isotopic ratio mass spectrometry

EC: European Community

EU: European Union

FDA: Food and Drug Administration

GMO: Genetically modified organism

H: Hydrogen

INRA: Institut National de Recherche Agronomique

PCA: Principal component analysis

SNIF-NMR: Site-specific natural isotope fractionation nuclear magnetic resonance

TTB: Alcohol and Tobacco Tax and Trade Bureau

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[a] In this article, the term "natural vanillin" will be used to describe "natural vanillin through the bioconversion of natural ferulic acid."

F-1. Green and cured vanilla beans

Vanilla beans (green pods and cured beans); courtesy of Solvay

Authorized traditional food preparation processes highlighted in Annex II of EC 1334/2008 include extraction, distillation, fermentation, microbiological processes, heating, cooking, baking, frying (up to 240°C at atmospheric pressure) and pressure cooking (up to 120°C).

According to U.S. regulations, to define a flavoring substance as "natural," the raw material and the process have to be natural. The FDA has established that the fermentation process (or bioconversion) for producing vanillin from ferulic acid can be labeled as natural flavor.

Rice Bran: the Historical Source of Ferulic Acid

Due to consumer demand for natural flavors in the 1990s, the flavor industry sought to produce natural vanillin through bioconversion from sources other than the vanilla bean. One of the most intensively studied processes to produce natural vanillin is the bioconversion of ferulic acid, a naturally occurring cinnamic acid.[4]

Ferulic acid from rice has been historically available in Japan and China; it is a byproduct of the production of rice bran oil, a long and costly multi-step extraction process from soap stock leading to many co-products and, often, a low yield.

Many strains, such as *Streptomyces setonii* ATCC 39116, CNCN I-1555, *Amycolatopsis* DSM 9991 and 9992, *Pseudomonas putida* NCIMB 40988 and *Rhodotorula glutinis* IMI 379896, have been claimed by many companies as catabolics for the bioconversion of ferulic acid into vanillin.[5–10]

Vanillin obtained from ferulic acid isolated from rice is currently the only vanillin recognized as a natural flavor by organizations such as the Directorate General for Competition, Consumer Affairs and Repression of Fraud (DGCCRF) in France and the Alcohol and Tobacco, Trade and Tax Bureau (TTB) in the United States.

In 2003, the French authorities (DGCCRF) issued note no. 2003-61 stating that, "At the present time, the only vanillin corresponding to this definition (the EC 88/388 regulation in force at that time) is the one obtained by fermentation from natural ferulic acid isolated from rice."[11]

In October 2004, natural vanillin from ferulic acid obtained from rice was recognized by the FDA as, "vanillin derived naturally through fermentation." The TTB stipulates that only vanillin from fermentation of ferulic acid can be considered as natural.

In early 2000, a bioconversion process was industrialized on a large scale to produce natural vanillin from ferulic acid present in rice. This natural vanillin is widely used in natural flavors, especially natural fruity flavors, and sweet, creamy and dairy notes. These flavors can be used in all kinds of food and fragrance sectors such as dairy, ice cream, beverages, chocolate, confectionery, bakery, perfumes, fragrance oils and essential oils. Natural vanillin from rice therefore brings into reach a natural alternative to insufficient volumes of vanilla bean extracts.

Ferulic Acid: Naturally Occurring in a Wide Range of Plants

Ferulic acid, a naturally occurring phenolic compound, is the most abundant hydroxycinnamic acid found in the cell walls of

Botanic Sources: C3, C4 and CAM Plants[13]

Photosynthesis, the process by which plants take energy from the sun and store it within the bonds of sugars, is a simple process that needs only light, water and carbon dioxide to take place. Plants can be divided into three different types, depending of their photosynthetic pathway: C3, C4 and CAM plants.

C3 Photosynthesis

C3 plants are the most numerous on the planet (among them, rice and wheat) and profit most from conditions that are cool and moist with normal light. C3 photosynthesis gets its name because the carbon dioxide that is breathed in by the plant in order to convert light into high-energy sugars is incorporated into a 3-carbon compound (3-phosphoglycerate). Photosynthesis takes place in all parts of the leaf in C3 plants.

C4 Photosynthesis

C4 plants include maize and several types of grass. These plants take in carbon dioxide through their stomata (pores on the leaf surface through which carbon dioxide enters and water and oxygen exits) at a faster rate than C3 plants, allowing carbon dioxide to be delivered immediately for photosynthesis in inner cells. The C4 indicates that carbon dioxide is incorporated into a 4-carbon compound (malate). These types of plants have adapted to high-temperatures and bright conditions in order to reduce the damage caused by photorespiration (a process that occurs when carbon dioxide levels are low and the cells burn oxygen, which slows plant growth).

CAM Photosynthesis

The third type of photosynthesis is called CAM (Crassulacean Acid Metabolism). It is an intermediate mechanism between C3 and C4 in which the plant uses the C3 or C4 pathway, depending on the environmental conditions, especially hygrometry. CAM plant examples include vanilla plants, cacti and agaves.

C3, C4 or CAM plants (see **Botanic Sources: C3, C4 and CAM Plants**), where it is ester-linked to polysaccharides.[12] As such, it also occurs in common agricultural residues such as cereal bran. However, it does not exist in a free form, but as a glycosidic bond with carbohydrate chains in the cell wall; therefore, hydrolytic methods, either enzymatic or alkaline, are used to release it.

The three photosynthetic processes from C3, C4 or CAM plants will generate isotopic effects, in particular the ^{13}C isotopic effect, which helps traceability of the botanic origins.

How to Authenticate Vanillin from C3 and C4 Plants

As previously mentioned, natural vanillin obtained via the bioconversion of ferulic acid from rice, a C3 plant, is the only natural vanillin from a non-vanilla source available at present.

Natural vanillin obtained via the bioconversion of ferulic acid from maize, a C4 plant, has been the subject of studies in recent years. Initial investigations were carried out in 1990 within the framework of the FAIR Consortium, involving the INRA (Institut National Recherche Agronomique) and, among

Isotopes and Isotopic Effects

Isotopes are species of the same element differing by the number of neutrons. The natural average proportion of common isotopes is given in **F-2**.

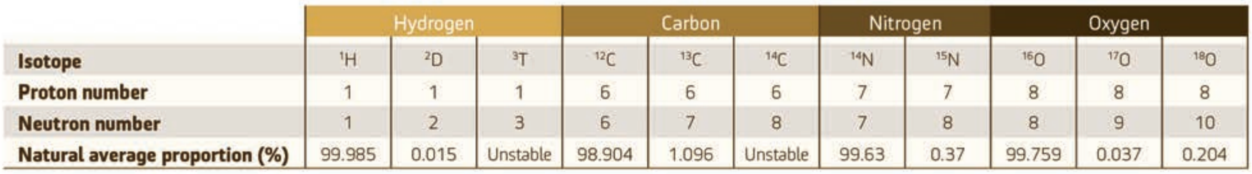
The mass difference between isotopes of the same element may be sufficient to induce differences in physical and chemical properties. This is why the natural abundance of isotopes is not uniform around the world in the biosphere and living organisms.

Natural isotopic variations observed in chlorophyll tissues versus atmosphere are due to fractionation phenomena, also called isotopic discrimination, occurring between "light" molecules, such as $^{12}CO_2$ and "heavier ones," such as $^{13}CO_2$ and $^{14}CO_2$, during carbon assimilation at light. As a matter of fact, the isotopic mass difference between different types of CO_2 molecules leads to differences in behavior such as lowering of gaseous diffusion through the stomatic pores of leaves ($^{13}CO_2$, being slightly heavier, will diffuse less rapidly than $^{12}CO_2$), lowering of the reaction rate constant of enzymatic reactions. Thus, photosynthesis discriminates the light isotope ^{12}C to the detriment of the heavy isotopes such as ^{13}C and ^{14}C in leaves. The consequence is a decrease in ^{13}C (and ^{14}C) of the plant organic matter, compared to the carbon of atmospheric CO_2.

Away from industrial activity, atmospheric carbon dioxide displays a mean isotopic deviation of about $\delta^{13}C$ = -8‰ all over the world. The effect of CO_2 integration by the plant leads to a decrease of ^{13}C isotopic ratio in plants of about -20‰ for plants with a C3 photosynthetic pathway. The C3 photosynthetic pathway is very discriminative toward ^{13}C, whereas C4 plant discrimination toward ^{13}C is lower. As a result, the $^{13}C/^{12}C$ isotopic deviation is only lowered by about -3-4‰.[13]

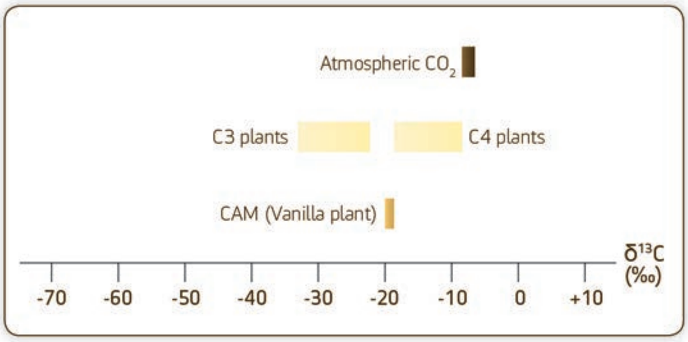
As a consequence, $\delta^{13}C$ isotopic deviation of plants will vary depending of the photosynthetic mechanism. Plants with a photosynthetic metabolism of the C3 type, such as rice and wheat, display a mean isotopic deviation $\delta^{13}C$ of about -28‰. Meanwhile, plants with a C4 photosynthetic mechanism, such as maize, will display a mean isotopic deviation of about - $\delta^{13}C$ = 14‰, as shown in **F-3**.

F-2. Natural average proportion of common isotopes; source: Eurofins Analytics France



	Hydrogen			Carbon			Nitrogen		Oxygen		
Isotope	^{1}H	^{2}D	^{3}T	^{12}C	^{13}C	^{14}C	^{14}N	^{15}N	^{16}O	^{17}O	^{18}O
Proton number	1	1	1	6	6	6	7	7	8	8	8
Neutron number	1	2	3	6	7	8	7	8	8	9	10
Natural average proportion (%)	99.985	0.015	Unstable	98.904	1.096	Unstable	99.63	0.37	99.759	0.037	0.204

F-3. Mean $\delta^{13}C$ isotopic deviation (in ‰) found in nature; source: "L'isotope: traceur d'origine. Distribution isotopique dans les composés naturels," G Remaud and S Akoka



Isotopic Deviations Analysis as Traditional Method

The natural abundance of isotopes (see **Isotopes and Isotopic Effects**) in the biosphere and living organisms is not uniform around the world. The isotopic ratio depends on several parameters such as natural presence (vs synthetic origin) and, for natural raw materials extracted from biomass, on the photosynthetic path of the plant (C4 vs C3 plants), and even on geographical production zones.

other partners, Pernod Ricard, a key beverage industry player.[14] The objectives of this project were to evaluate the potential for the development of natural flavors by microbial and enzymatic bioconversion of specific components (such as ferulic acid) of plant cell-wall residues, including brans derived from wheat or maize.

The vanillin obtained displayed a ^{13}C/^{12}C IRMS isotopic ratio of -18.3‰ vs PDB, which was expected due to the isotopic deviation of the raw material (see **Isotopic Deviations Analysis as Traditional Method**) a natural ferulic acid from maize, a C4 plant, and the fact that bioconversion is conservative towards ^{13}C isotopic ratio.[14,15]

It should be noted that ^{13}C/^{12}C IRMS cannot discriminate vanillin from maize and vanillin from the bean.

Isotopic deviation analyses such as ^{13}C IRMS and ^{2}H-SNIF-NMR[b] (site-specific natural isotopic fractionation-nuclear magnetic resonance) are traditional methods for the assessment of the authenticity of vanilla, and are commonly used to unequivocally discriminate vanillin from vanilla bean from all other known sources of vanillin or mixtures thereof.

It has been demonstrated that ^{13}C IRMS and ^{2}H-SNIF-NMR isotopic deviation methods have perfectly discriminated vanillin obtained by bioconversion of ferulic acid from rice bran from vanillin coming from the vanilla bean, thus guaranteeing genuineness of the source.[1]

As seen in **Isotopes and Isotopic Effects**, natural vanillin obtained by bioconversion of ferulic acid from C4 plants, such as maize, is expected to display a ^{13}C isotopic signature distinct from that of natural vanillin from ferulic acid from C3 plants, such as rice.

For each new source of vanillin previously unknown in the isotopic database, such as natural vanillin from ferulic acid from maize, the demonstration of discrimination must be carried out.

How to Discriminate the Botanic Origin of Natural Vanillin

Traditional authentication methods exist to guarantee the genuineness of the source of natural vanillin from vanilla beans and C3/C4 plants, as illustrated in **F-4**.

Vanillin obtained by bioconversion of ferulic acid from C3 plants (such as rice) is easily differentiated from C4 plant origins (such as maize) by ^{13}C IRMS analysis.

Thanks to the ^{2}H-SNIF-NMR, method, natural vanillin from C4 plants (such as maize) can be discriminated from the vanilla bean (CAM plant).

^{13}C/^{12}C Isotopic Ratio by EA-IRMS—the Method to Discriminate Botanic Origin

Since ferulic acid and vanillin are pure, no prior separation analysis is required. Therefore, EA-IRMS (elemental analysis-isotopic ratio mass spectrometry) is carried out.

13C/12C isotopic deviation is determined by IRMS consisting of combustion in the first step (**F-5**).

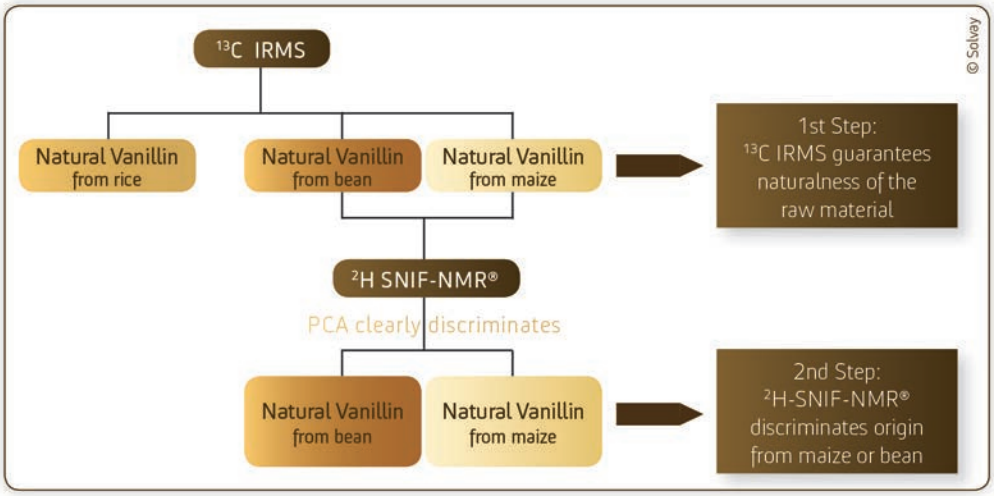
Next, $^{13}CO_2$ and $^{12}CO_2$ are separated by gas chromatography; $^{13}CO_2$ and $^{12}CO_2$ are respectively quantified by mass spectrometry at MW=45 and MW=44. Isotopic deviation is expressed as δ^{13}C with about 0.3% margin of error (**F-6**).

It should be noted that ^{13}C isotopic deviation obtained by this method is a mean value of all the carbon atoms in the sample.

^{13}C EA-IRMS Measurement of Natural Vanillin

δ^{13}C isotopic deviations of natural vanillin produced via bioconversion of ferulic acid from maize have been measured by EA-IRMS.

F-4. Decisional logigram: natural vanillin discrimination of botanic origin; source: Solvay Vanil'Expert Center, Lyon France



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[b] ^{2}H-SNIF-NMR is a trade name of Eurofins

F-5. $^{13}C/^{12}C$ isotopic deviation is determined by IRMS, consisting of combustion in the first step

$$\text{Produit} \xrightarrow{\text{Comb}} N_2 + CO_2 + H_2O + SO_2$$

F-6. Isotopic deviation is expressed as $\delta^{13}C$ with about 0.3% margin of error

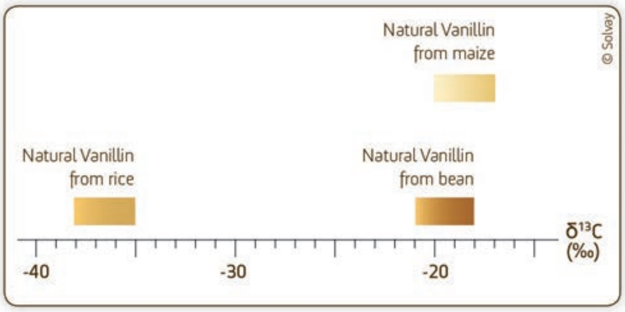
$$\delta^{13}C\ (°/oo) = \left(\frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{ref\ PDB}} - 1 \right) \times 1000$$

F-7. $\delta^{13}C$ EA-IRMS isotopic deviation of natural vanillin from maize; sources: Solvay Vanil'Expert Center; measurements carried out by Eurofins Analytics France

Ferulic acid from maize $\delta^{13}C$ (in ‰ vs PDB*)	Natural vanillin from maize	
	Sample number	^{13}C IRMS $\delta^{13}C$ in ‰ vs PDB
-19 to -16‰	Sample 1	-19.3 +/- 0,3
	Sample 2	-19.6 +/- 0,3
	Sample 3	-18.2 +/- 0,3
	Sample 4	-18.2 +/- 0,3
	Sample 5	-19.6 +/- 0,3
	Sample 6	-19.7 +/- 0,3

*PDB= Pee Dee Belemnite

F-8. $\delta^{13}C$ IRMS data for natural vanillin; source: Solvay Vanil'Expert Center; measurements carried out by Eurofins Analytics France



F-9. ^2H SNIF-NMR values of natural vanillin from maize vs from rice; source: Solvay Vanil'Expert Center/measurements carried out by Eurofins Analytics France

Reference	Description	Isotopic analysis								
		^{13}C IRMS	$^2D/^1H$ SNIF-NMR®							
		$\delta^{13}C$	(D/H) 1	(D/H) 3	(D/H) 4	(D/H) 5	F1	F2	F4	F5
		in ‰ vs PDB	ppm	ppm	ppm	ppm	molar fraction	molar fraction	molar fraction	molar fraction
Sample R	Natural vanillin from rice	-37,4	120	117	142	126	0,1123	0,2192	0,1333	0,3552
Sample M	Natural vanillin from maize	-19,6	134	149	125	136	0,1152	0,2561	0,1072	0,3504

Ferulic acid from C4 plants such as maize displays ^{13}C isotopic deviation ranging from -19‰ to -16‰. The bioconversion appears to be conservative on ^{13}C isotopic deviation of ferulic acid; the natural vanillin obtained by bioconversion of natural ferulic acid extracted from maize displays ^{13}C isotopic deviation ranging from -20‰ to -17‰, as shown in **F-7**.[15]

Discrimination of Botanic Origin by ^{13}C IRMS

Vanillin from ferulic acid from C3 plants such as rice displays ^{13}C isotopic deviation ranging from -38‰ to -35‰, which clearly differentiates it from vanillin from ferulic acid of a C4 plant such as maize or vanillin from beans (CAM plants), as illustrated in **F-8**.

Thus, natural vanillin from maize displaying $\delta^{13}C$ of approximately -19‰ is perfectly differentiated by ^{13}C IRMS, guaranteeing the genuineness of natural sourcing.

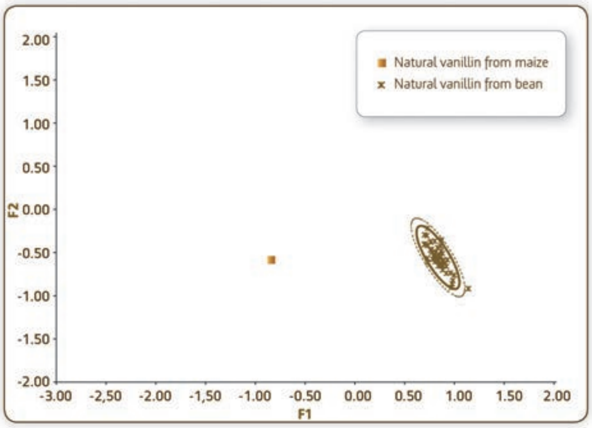
However, ^{13}C IRMS isotopic deviation is not sufficient to differentiate natural vanillin from beans and natural vanillin from maize. Therefore, it can be deduced that ^{13}C IRMS provides a first step of discrimination for naturalness as well as differentiation of ferulic acid from C3 plant origin.

Isotopic Fingerprint by ^2H SNIF-NMR

^2H SNIF-NMR is a tool for acquiring site-specific 2D/1H ratio via NMR, providing a fingerprint allowing multivariate analysis such as principal component analysis (PCA). Typical ^2H SNIF-NMR values for natural vanillin from ferulic acid obtained from rice and maize are shown in **F-9**.

The ^2H SNIF-NMR allows the natural vanillin from maize, a C4 plant, to be differentiated from natural vanillin from rice,

F-10. Principal components analysis (PCA) on vanillin based on ^{2}H SNIF-NMR; source: courtesy of Eurofins Analytics France



a C3 plant. The natural vanillin from maize is differentiated by ^{2}H SNIF-NMR from the vanilla bean, as illustrated by the PCA shown in **F-10**.

Conclusions

Natural vanillin is an interesting case study as it is widely used in natural flavors, and because it can be derived from various plants. The flavor, fragrance and ingredient industry is aware of an increased demand from food manufacturers and final consumers for authenticity and traceability. Therefore, more advanced analytical methods have been developed to discriminate the botanic origins and are now used on a regular basis to comply with regulations for naturalness.

Botanic origins of natural vanillin can be distinguished by isotopic differentiation methods such as ^{13}C EA-IRMS and ^{2}H SNIF-NMR, ensuring full traceability and guaranteeing its botanic authenticity.

Moreover, the elaborated ^{2}H SNIF-NMR method can distinguish natural vanillin from C4 plants and vanillin extracted from vanilla beans, thereby guaranteeing genuineness.

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