Synthesis and Authentication of Natural Vanillins Prepared by Fermentation

Techniques, regulations, source vartiations and flavor characteristics

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Known since the age of the Aztecs, and by Europeans after 1520, vanilla is and will certainly remain the most appreciated flavor in the world, as shown by the incredible number of ice creams, dessert creams and baked goods containing vanilla flavors. The recent successful launch of new vanilla colas confirms this affection will continue and spread into new applications.

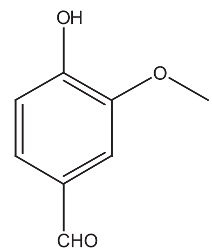
Perhaps one of the most interesting reasons for vanilla's popularity as a flavor is that many infants have been bottle-fed vanilla-flavored milk. Indeed, an independent study conducted in Germany questioned 133 people (71 women and 62 men) to learn if they were bottle or breast-fed as a baby.[1] These people were then asked to taste two similar ketchups, one containing (surprisingly) 0.05 percent vanillin. Among the 38 percent who preferred the ketchup with vanillin, 67% of them were bottle-fed vanilla-flavored milk as infants. This phenomenon of persistent vanilla flavor preference reaches across generations.

Vanillin (or 4-hydroxy-3-methoxybenzaldehyde, F-1) is the major 'olfactive' component of vanilla beans; it is contained at around 2 percent in the pod. The massive demand for vanilla flavorings cannot be met by the limited amount of vanilla beans being grown. This has lead to the development of synthetic vanillin. Today, consumers consider vanillin the 'real' taste of vanilla.

It is not surprising, then, that the worldwide market of synthetic vanillin is estimated to be around 12,000 MT per year compared to an estimated 2,000 MT of vanilla pods in the best growing years — that is to say, only 40 MT in equivalent vanillin.

The major chemical route to manufacturing synthetic vanillin is by far the one starting from catechol (o-dihydroxybenzene), which is transformed into guaiacol, or o-methoxyphenol, and then into vanillin.[2] This is the route used by western companies.

Vanillin structure **F-1**



Considering that 1) the vanilla pod is the only source of natural vanilla flavoring, 2) the demand for more natural foodstuffs is ever increasing and, 3) there has been a recent shortage of vanilla, a tremendous rise in vanilla prices (since 1999) is not surprising. The shortage has spurred the rapid commercial development of natural alternatives. Among these alternatives, Rhodia decided, in 1998, to develop Rhovanil[a] *Natural*, a vanillin obtained by fermentation of natural ferulic acid extracted from rice bran. This fermentation process starts from natural ferulic acid and imitates the natural formation of vanillin in vanilla pods, which occurs by enzymatic degradation of ferulic acid deriva-

[a]Rhovanil is a trademark of Rhodia.

tives during the curing process.

In this article, we will compare the American and European natural flavor legislation and discuss the latest European developments. We will then review the major natural routes to biotechnologic vanillin and their industrial constraints, as well as the essential analytical methods needed to identify the origins of different vanillins and ascertain their natural status. We will conclude with some examples of applications and an organoleptic comparison of vanillins manufactured by different processes.

American and European Natural Flavors Regulations: Common Points and Differences

The two current regulation directives in force are the 88/388 for Europe and the US federal CFR 21. What are the definitions of natural flavor for Europe and the United States? According to EC 88/388, a natural flavoring substance is obtained by appropriate physical processes (including distillation and solvent extraction) or enzymatic or microbiological processes from material of vegetable or animal origin either in the raw state or after processing for human consumption by traditional food-preparation processes (including drying, torrefaction and fermentation).

According to US federal CFR-21 §101-22, the term 'natural flavor' means the essential oils, oleoresin, essence or extractive, protein hydrolysate, distillate or any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit, or fruit juice, vegetable or vegetable juice, edible yeasts, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional.

The European Directive 88/388, approved in June 1988, defines raw materials used for the preparation of flavorings into six categories of flavoring agents:

- Natural flavoring substances
- Nature-identical flavoring substances (synthesis, like vanillin)
- Artificial flavoring substances (like ethyl vanillin)
- Flavoring preparations (plant extracts, EMC…)
- Process flavorings (Maillard products)
- Smoke flavorings (liquid smoke extracts, etc.)

Comparing the existing, or even the new, European natural flavoring definition with the FDA's CFR-21, one notes no significant differences. Indeed, both directives consider as natural every substance obtained by physical and biotech processes, starting from natural materials of vegetable or animal origin (either in their original state or obtained after a traditional food-preparation).

The notions of 'physical' and 'heating' processes can lead to a wide range of interpretations. A typical example is the vanillin ex-curcumin when obtained by a chemical process at around 300°C under a pressure of around 100 bar.[3] Another illustration is vanillin ex-eugenol when chemical processes are used. These types of interpretations will be clarified by the new European flavor regulation expected in 2004.

This European directive proposes the following definition of what a natural flavoring substance is: "Chemically defined flavoring substances obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable or animal origin either in the raw state or after processing for human consumption by traditional food preparation processes…"

In order to have a strict application of this definition, a list of traditional food preparation processes and appropriate physical processes are mentioned: crystallization, precipitation, distillation, drying, solvent extraction, filtration, roasting and heating up to 150°C. The following processes are excluded: singlet oxygen, ozone, inorganic catalysts, metal or organometallic reagents and UV radiation.

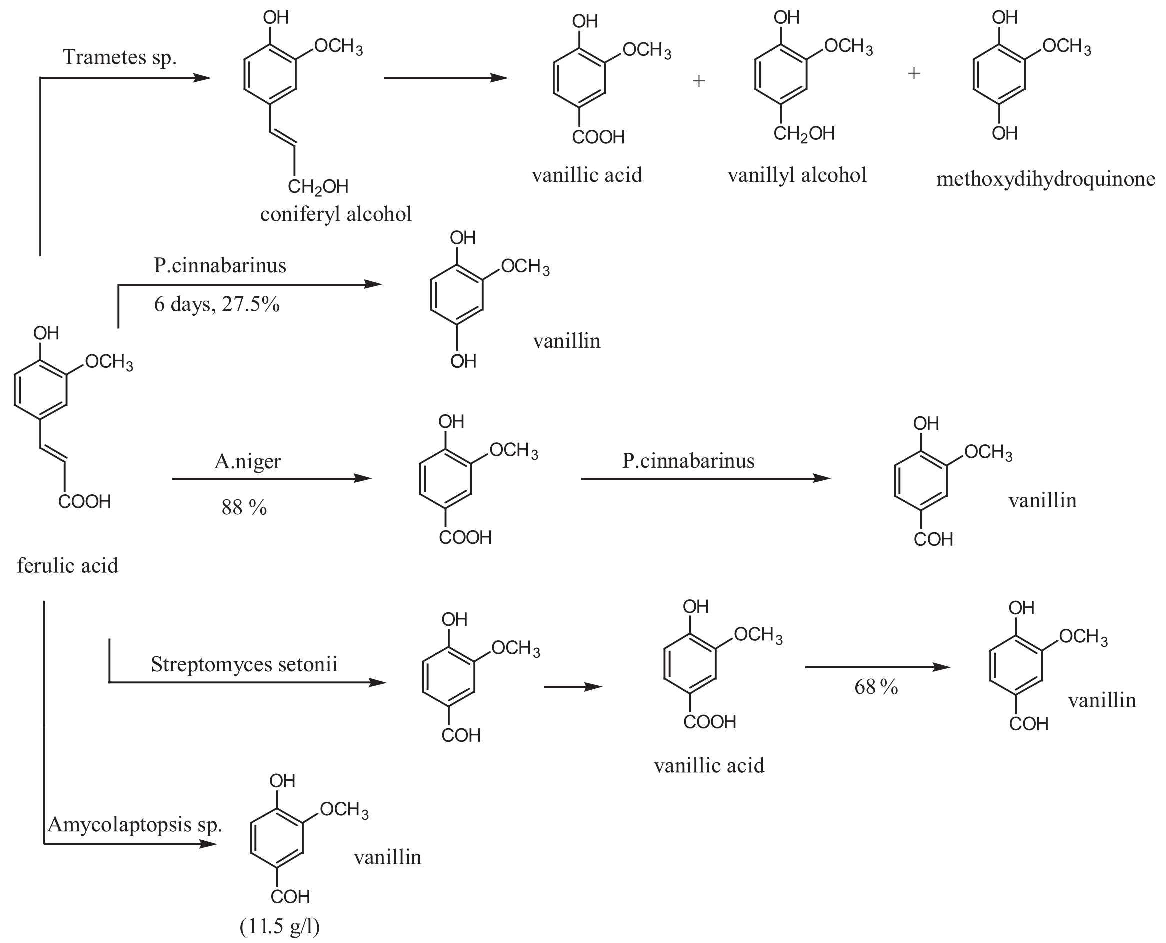
In addition, the French Authorities, among the leaders on vanilla flavoring regulation, have recently stated, in their latest Information Note N° 2003-61 (June 16th, 2003), that according to their best knowledge, the vanillin obtained by fermentation process from natural ferulic acid from rice bran is the only current commercial natural vanillin available on the market.

French definition of natural flavor: According to the French Information Note N° 2003-61 (June 16, 2003): flavor substances obtained by biotechnologic process are considered, according to the European flavor regulation, as natural substances. Consequently, to be presented as a natural substance, a vanillin not coming from vanilla beans must be obtained by an enzymatic or microbiologic or physical process from a natural raw material.

Right now, the only known vanillin to the French regulation authorities (DGCCRF) corresponding to this definition is the one obtained (by fermentation) from natural ferulic acid isolated from rice. Its isotopic deviation of C^{13} is between -37 percent and -36 percent.

We can also mention that Fenaroli's Handbook of flavor has clearly stated in the last edition that:

Biotransformation of ferulic acid by different microorganisms F-2



Natural vanillin refers to vanillin produced from natural precursors consistent with processes described in 21 CFR 101.22. For example, fermentation or enzymolysis of curcumin, eugenol or ferulic acid derived from natural sources can produce natural vanillin. Natural vanillin has odor and taste characteristics that are closer to vanilla than synthetic vanillin … Microbial biotransformation (also referred to as fermentation) appears to be the most promising at producing large quantities of natural vanillin at high quality.[4]

Processes for the Preparation of Natural Vanillin

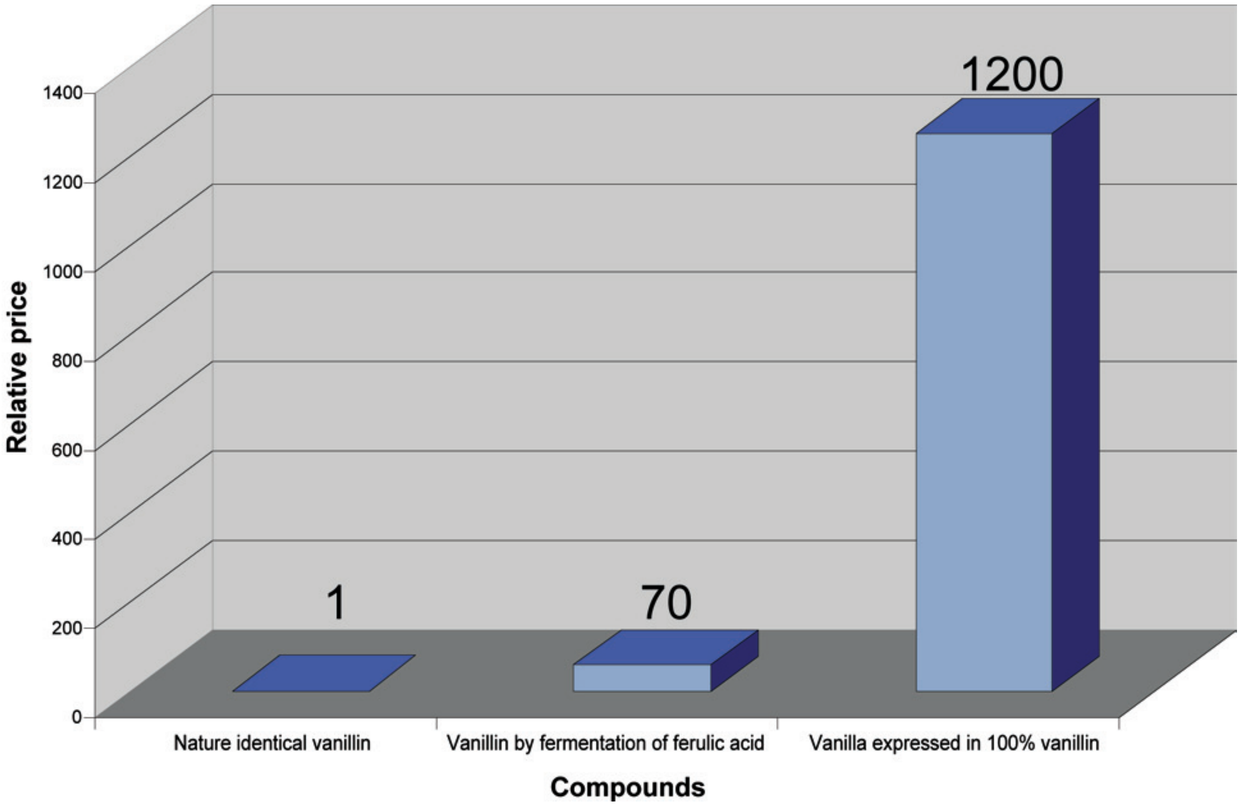
During the last 10 years, much work has been published on the manufacturing of vanillin from natural raw materials.[5-6] The main potential natural raw materials for the bio preparation of natural vanillin are ferulic acid, eugenol and lignin.

From ferulic acid: Ferulic acid is the most common raw material. 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 for biotransformation of ferulic acid into vanillin.[7-12]

The transformation of vanillin into vanillic acid has also been described using *Pycnoporus cinnabarius* CNCN DEG1-937 or CNCN DEG 1-938, *Aspergillus niger*, *Pseudomonas putida* NCIMB 40988 or *Rhodotorula glutinis* IMI 379896.[12-14] This approach needs a second biochemical step for the reduction of vanillic acid into vanillin. F-2 summarizes main biotransformation of ferulic acid by different microorganisms.

However, today, a limited number of processes have been developed on an industrial scale. The main producers using

F-3 Relative price of nature-identical vanillin, 'natural' vanillin prepared from ferulic acid and natural vanillin ex beans



ferulic acid are Symrise and Rhodia.

From eugenol: Because eugenol is an antiseptic compound, the biotransformation of eugenol is difficult. In addition, the concentrations in eugenol are usually low. The transformation of eugenol into vanillin is patented with Serratia, Klebsiella, Enterobacter, Lipoxygenase, Lipoxisase Sigma L8383 and Pseudomonas TK2101.[15-18] In all cases, the performances of biochemical transformation starting from eugenol are lower than those using ferulic acid as raw materials. Many publications describe the use of genetically modified microorganisms to improve the transformation of eugenol into vanillin.[19]

From lignin: Theoretically, the biodepolymerisation of lignin is a source of vanillin, vanillic alcohol, vanillic acid, ferulic acid, coniferyl aldehyd, etc. In the publications describing the biodegradation of lignin, the yields of vanillin are very low.

Conclusion: Today, ferulic acid seems to be the best raw material for the bio production of vanillin. In terms of productivity, Symrise and Rhodia claim to produce vanillin at a concentration greater than 10 g/l.

Industrial Constraints

As already mentioned in the above paragraph, although the concentration of vanilla produced by bioconversion of ferulic acid is acceptable on a lab scale, industrial vanillin production needs to be competitive to allow medium and long term development.

The competitiveness of vanillin produced by bioconversion mainly depends on two factors: 1) the fermentor's size, to minimize the production cost, and 2) the facilities for the separation and treatment of vanillin.

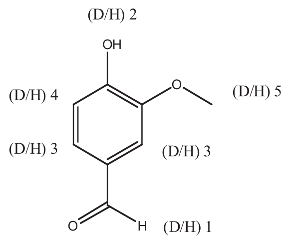
Economic Overview

The current shortage of vanilla beans has led to a significant price increase. Therefore, the development of biotechnologic vanillin is of high interest to many because it will provide flavorists a cost effective alternative in the formulation of natural flavorings.

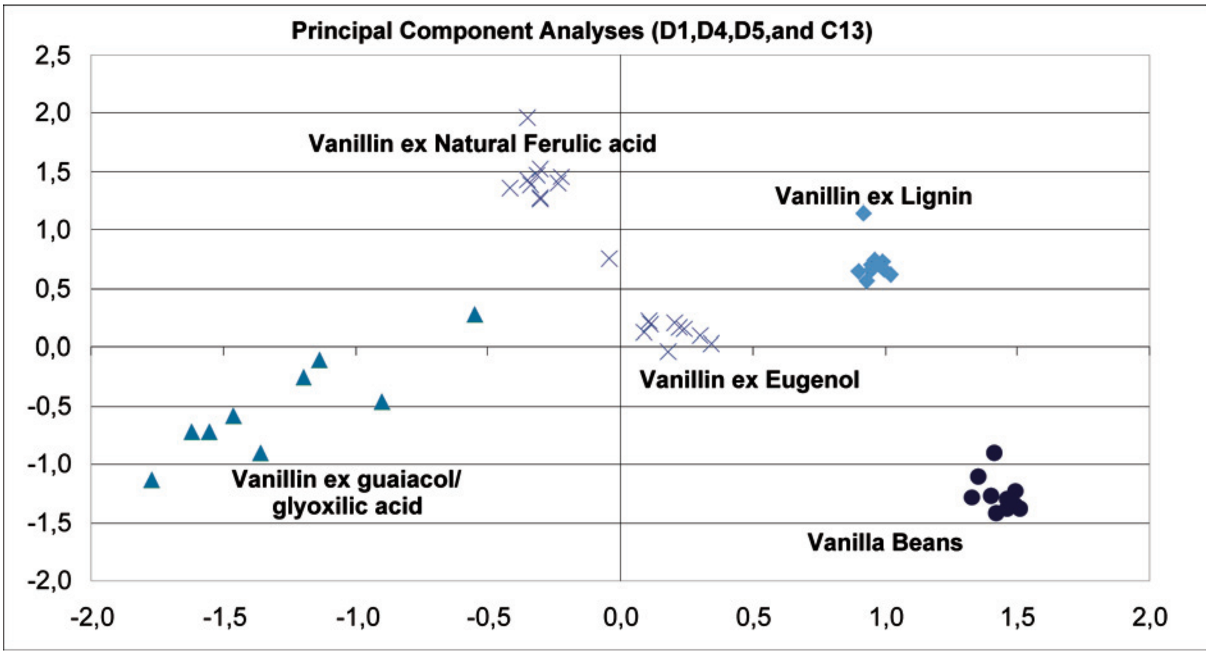
IRMS is employed for the determination of isotopic deviation δ ¹³C given by the formula; δ ¹³C is a function of the raw material origin **F-4**

$$\delta\,^{13}C = \left[\frac{13C/12C\text{ (compound) - }13C/12C\text{ (standart)}}{13C/12C\text{ (standart)}}\right] \text{X } 1000$$

D1 through D5 **F-5**



Authentication of vanillins by ²H-NMR after principal component analysis based on D1, D4, D5 and δ ¹³C **F-6**



Hereafter, we compare the relative cost of nature-identical vanillin to natural biotech vanillin and to vanilla beans from Madagascar, for instance (F-3). It is clear, from the presented evidence, that biotechnologic vanillin can bring to flavorists an interesting cost effective alternative for natural flavorings.

Authentification of Natural Vanillin to Guarantee Its Origin

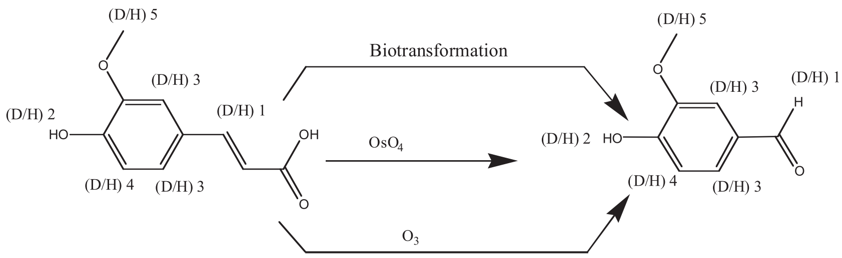
To develop and market a new natural vanillin, one must develop analytical methods in order to insure naturalness of the raw material while controlling the processes used (according to natural flavoring regulations in force).

The classical analytical methods used for the determination of vanillin origin are IRMS (isotope ratio mass spectrometry) and SNIF-NMR (site-specific natural isotope fractionation studied by nuclear magnetic resonance).[20-21] IRMS is employed for the determination of isotopic deviation δ ¹³C given by the formula. δ ¹³C is a function of the raw material origin (F-4).

In SNIF-NMR, the site-specific isotopic ratio $(D/H)_i$ depends on the product origin and the process used for

It is a challenge to detect if natural vanillin is really made by biotransformation and not by chemistry

F-7



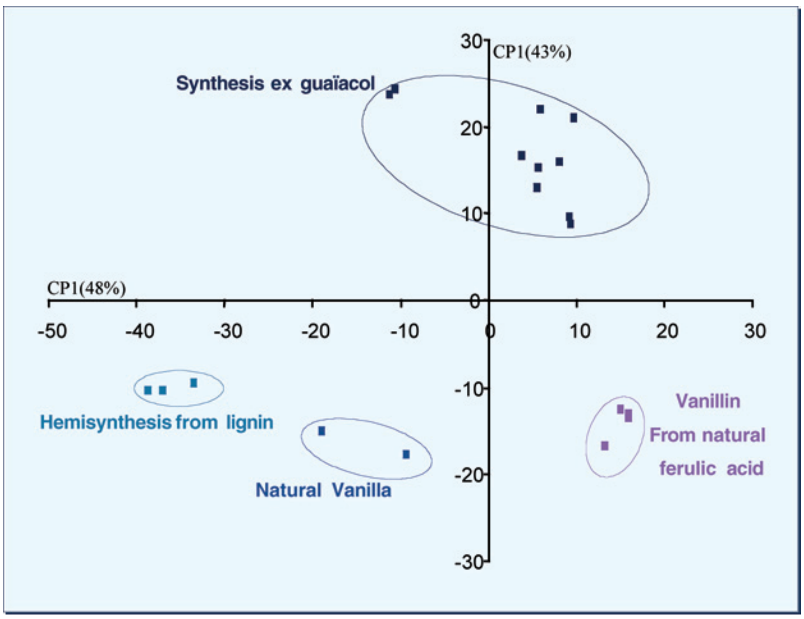
Isotopic ratio $(D/H)_i$ and isotopic deviation δ ^{13}C of ferulic acid ex rice and vanillins prepared from ferulic acid ex rice

T-1

Compound	Transformation	(D/H) 1ppm	(D/H) 3ppm	(D/H) 4ppm	(D/H) 5ppm	δ ^{13}C‰
Ferulic acid	Esterification CH2N2	126.4	135.8	136.7	128.8	-35.3
Vanillin	Biotransformation	125.2	126.7	147.0	130.1	-36.6
Vanillin	OsO_4	118.8	122.8	145.7	127.6	-37.91
Vanillin	O_3	117.6	123.3	143.6	127.7	-36.71

Authentication of vanillins by δ ^{13}C NMR after principal component analysis based on each carbon atoms

F-8



the vanillin synthesis.

Vanillins produced from ferulic acid ex rice, which is the most widely available natural ferulic acid, have been analyzed with IRMS and SNIF-NMR, and compared with vanilla and synthetic vanillins. F-5 and F-6, which display a plane representation of two $(D/H)_i$ functions, shows different areas for all kinds of vanillin.[22]

Natural vanillin produced from natural ferulic acid is easy to detect. In fact, the main challenge is to detect if the natural vanillin is really made by biotransformation and not by chemistry (F-7). If we consider the three following transformations — biotransformation, ozonolisys, oxidation with OsO4) (F-5) — no isotopic effect is observed by IRMS and SNIF-NMR, as is shown by the results in the T-1.[23]

Consequently, IRMS and SNIF-NR can be used for the origin control of natural vanillin, but they do not determine the process used for the vanillin preparation. The main question is to be sure that natural vanillin has been prepared by a biochemical process, and not by a chemical process using a natural ferulic acid.

For these reasons, Rhodia has developed new analytical methods to guarantee that natural vanillin was produced from a natural product and by a natural process. The first method is an SNIF-NMR method based on a quantitative natural abundance carbon-13 determination site by site.[24-25] The graph presented in F-8 was obtained after principal component analysis showed a specific area for vanillins prepared from natural ferulic acid.

Chemical oxydating reagents, such as OsO_4 and ozone, react with all kinds of double bonds, whereas strains or enzymes are usually specific to one substrate. Consequently, the impurities profile will be different following the technology used. In using a special HPLC developed by Rhodia, the analysis of the vanillin ex-ferulic obtained by biotransformation, ozonolyzis and OsO_4 shows very different chromatograms (F-9). Impurities are not the same.

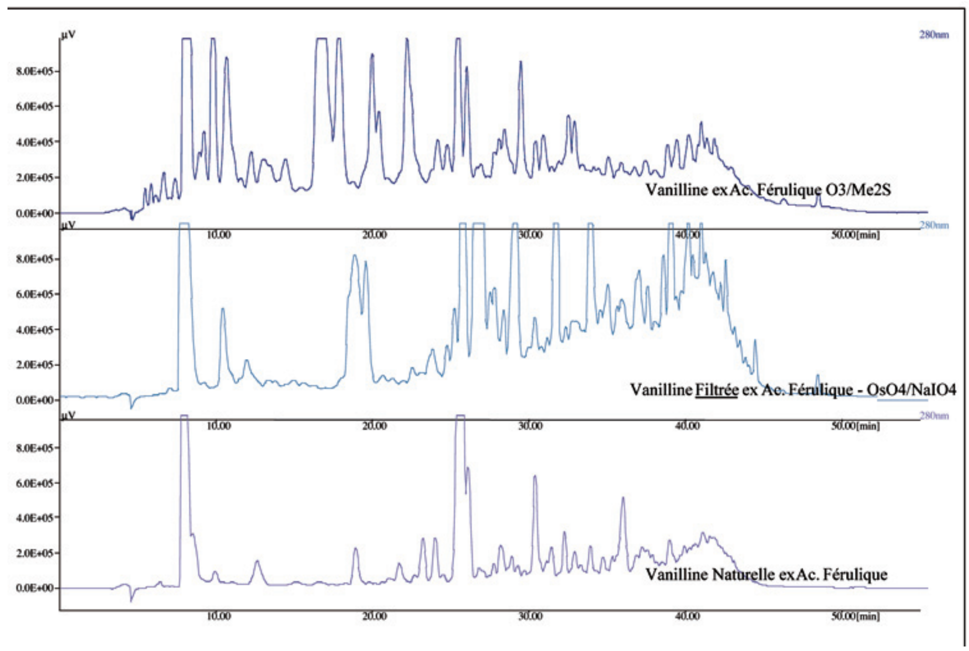
In conclusion, the combination of isotopic analysis methods and HPLC chromatography allows one to guarantee the vanillin label; that is to say, to certify the raw material origin and the process used to make vanillin.

End-Use Value: Comparison of Different Vanillins by a Panel of Experts

We asked a panel of 15 different experts well trained in flavor tasting, including memorization of fragrances and flavoring materials and using sensory analysis techniques, to compare different pure vanillins using chemical referents as descriptors according to Jaubert's method.[27] All the vanillins were first dissolved in ethanol and then diluted in water for a more comfortable tasting.

Each member of the panel described the mouth-taste by using chemical descriptors for which the

Chromatogram of vanillins ex ferulic acid prepared by ozonolyzis, oxidation and biotransformation **F-9**



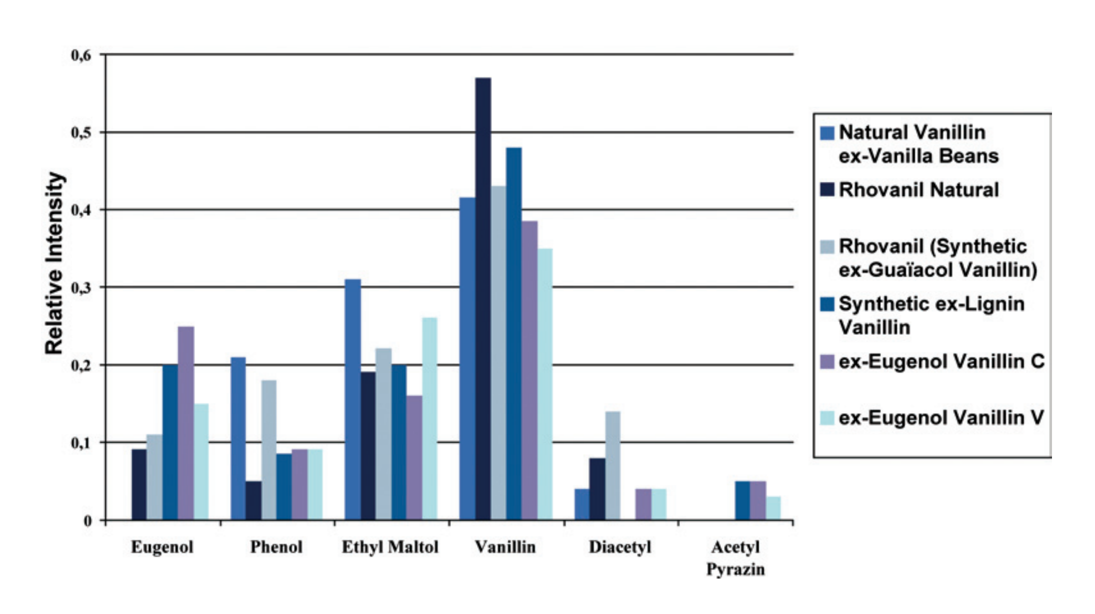
flavor is perfectly identified (eugenol, phenol, ethyl maltol, vanillin, etc.). Only the major notes were represented in the following graph (F-10). The data given by the panel are then treated in order to compare the relative intensity of these different vanillins.

The vanillin obtained by fermentation appears to be quite similar to the vanillin from guaiacol. However, it differentiates itself by its strong natural vanillin character, with a soft (less phenolic), sweet and powdery note, completed by a pleasant caramel note. In Europe, the final labeling rules are under discussion, but the labeling on the final foodstuff of biotechnologic vanillin is 'natural flavor.'

Biotechnologic vanillin is typically used alone or in combination with vanilla extracts in the following food products:

- Ice creams or dairy products like vanilla dessert creams or yogurts, to reinforce the typical, sweet, full bodied and creamy characteristic vanillin note
- Chocolate, caramel, and malt flavors to round off single flavor chemicals and cocoa extracts, burnt sugar
- All kinds of fruit flavors: natural strawberry, raspberry, banana and fruit blends to create sweet, creamy, cotton candy-like profiles appreciated by children
- Confectionery, baking

Comparison of the relative intensity of different vanillins as evaluated by a panel **F-10**



Conclusion

Today, natural vanillin is a reality: the commercial flavor is already available worldwide. This product has found its own market positioning, free of the shortages affecting current vanilla pods production, thus fitting flavorists' needs. Among the options available to overcome economic, regulatory and taste hurdles, vanillin obtained by bioconversion of natural ferulic acid appears to be a promising alternative. While the volume increase will lead to the development of new and more cost-effective industrial processes, consumers will expect a guarantee and a clear indication of the natural origin of flavors. In order to meet this demand, analytical tools must be developed industry-wide to ensure the reliable natural authentication of obtained vanillin.

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