Detection of Sophisticated Adulterations of Natural Vanilla Flavors and Extracts: Application of the SNIF-NMR Method to Vanillin and *p*-Hydroxybenzaldehyde

Gérald S. Remaud,*,† Yves-Loïc Martin,† Gilles G. Martin,† and Gérard J. Martin‡

Eurofins Laboratories, rue Pierre Adolphe Bobierre, Site de la Géraudière, CP 4001, 44073 Nantes Cedex 03, France, and Laboratoire de RMN et Réactivité Chimique, CNRS URA 472, 2 rue de la Houssinière, 44072 Nantes Cedex 03, France

This paper describes recent progress in the isotopic analysis of the two main aromatic constituents of vanilla flavor (vanillin and *p*-hydroxybenzaldehyde (pHB)). Some improvements concerning the SNIF-NMR analysis of vanillin are presented. They include (i) improvement of the analytical precision by using new software for automatic phasing, baseline correction and curve fitting of the signals of the ^{2}H-NMR spectra; (ii) significant enrichment of the database containing measurements performed on vanillin extracted from vanilla beans harvested in different countries and on synthetic vanillin; (iii) standardization of the purification process to obtain pure vanillin, in order to avoid isotopic fractionation; and (iv) improvement of the statistical tools for both proving and quantifying adulterations. All these improvements were also successfully applied to site specific deuterium NMR analysis of pHB. It is now also possible with the SNIF-NMR method to discriminate between the natural and chemical origins of pHB. New results concerning the δ^{13}C deviation of pHB are also presented. Thus the lowest value of δ^{13}C of pHB extracted from vanilla bean can be set at −19.5‰. We recommend the methodology presented in this paper as a standard procedure for purifying vanillin and pHB, without significant isotopic fractionation, from most matrices (for example from ice cream, yogurts, etc.), in order to perform isotopic analyses (^{13}C IRMS and ^{2}H-NMR).

Keywords: *SNIF-NMR; vanillin; pHB; δ^{13}C; adulteration; vanilla flavor; vanilla extract; ice cream; purification*

I. INTRODUCTION

Being able to distinguish between the natural and synthetic constituents of a flavor is a great advantage in food quality control (Martin et al., 1993). In the case of vanilla flavor, the economic aspects are considerable because of the relatively high price of the natural constituents of the flavor (vanillin, *p*-hydroxybenzaldehyde (pHB), etc.). The possibility of differentiating between vanillin and/or pHB extracted from vanilla beans and synthetic vanillin and/or pHB (i.e., made from guaiacol, lignin, or eugenol) is therefore of particular interest.

Several different approaches have been developed in order to characterize the genuineness of vanilla extracts. Some of these methods are based on compositional analyses, and in particular, potassium, phosphorus, nitrogen, and ash values (Martin et al., 1977; Martin et al., 1981). Other methods rely on isotopic determinations, in which the total deuterium and/or carbon-13 content of vanillin are measured using isotope ratio mass spectrometry (IRMS). Taking into account the difference in total ^{13}C between vanilla (a CAM plant) and vanillin which has been chemically produced from petroleum or lignin, vanillin ex-beans can be distinguished from vanillin ex-lignin and/or ex-guaiacol (Bricout et al., 1974; Derbesy and Touche, 1978; Krueger and Krueger, 1983; Hoffman and Salb, 1979; Schmidt, 1986). Later it became evident that it was possible to enrich synthetic vanillin in ^{13}C on both the methoxy group and the aldehyde group (Krueger and Krueger, 1983, 1985), resulting in the same ^{13}C deviation as vanillin extracted from beans. Consequently, the methods described above were no longer able to detect this type of sophisticated adulteration, and additional analytical tools were needed to identify isotopically-manipulated vanilla extracts.

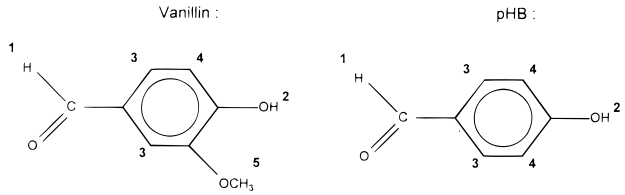

Figure 1. Vanillin and *p*-hydroxybenzaldehyde (pHB) molecules. Deuterium concentrations at all positions numbered can be determined using SNIF-NMR analyses. This numbering is consistent with earlier publications and follows peak order in the ^{2}H-NMR spectra but is not based on IUPAC references.

Interest in ^{2}H-NMR to characterize the origin of vanillin appeared in 1983 (Toulemonde et al., 1983) and in 1988, G. J. Martin and co-workers proposed the SNIF-NMR method (site-specific natural isotopic fractionation studied by nuclear magnetic resonance; registered trademark of Eurofins Laboratories) to determine the origin of vanillin (Maubert et al., 1988). This method had been developed in the early 1980s (Martin and Martin, 1981) and had become the official method of the European Community for detecting the addition of sugar in wines (OIV, 1990). It is also a CEN and an ENV protocol, and recently it became AOAC official method 995.17 for detecting added sugar in fruit juices (Martin et al., 1996a). Since the first experiments (Maubert et al., 1988) carried out on vanilla flavor with

* Author to whom correspondence should be addressed.
† Eurofins Laboratories.
‡ Laboratoire de RMN et Réactivité Chimique.

S0021-8561(96)00518-3 CCC: $14.00

this method, considerable progress has been made both in the software used and in NMR spectrometer technology.

The adulteration of vanilla has become increasingly sophisticated—^{13}C-enriched vanillin, specific ^{13}C enrichment—and since the publication of Maubert et al., (1988), deuterium enrichment. It has therefore become extremely necessary to improve the available techniques able to detect such cleverly adulterated products on the market.

The present paper has two main objectives. On one hand, it describes several improvements in the analysis of the site specific deuterium content of vanillin. These include the following: the repeatability of the analysis, its robustness to various vanillin extraction procedures, and its increased sensitivity. On the other hand, it presents isotopic data (^{13}C and ^{2}H) on the secondary metabolite of the vanilla flavor: pHB. Surprisingly, to the best of our knowledge, there are very few publications dealing with the isotopic analysis of pHB. The only known data concern the limits set by IOFI (letter no. 775, 1989) and by the French government for the ^{13}C content of natural pHB, which have been reported by the Institut de Chimie Analytique et du Contrôle de la Qualité (1984). Currently a minimum limit of −22.5‰ is used by French regulatory authorities for the δ^{13}C deviation of natural pHB (French Directive of June 11, 1987). Through our own data presented in this paper, we show that this value is far too low and has probably led to extracts containing synthetic pHB being accepted as natural vanilla.

Using the GC−C-IRMS technique, a combination of gas chromatography and isotopic mass spectrometry, it is theoretically possible to determine simultaneously the δ^{13}C deviation of both vanillin and pHB of a vanilla flavor. However, when applied in our laboratory, this method did not give satisfactory results for the ^{13}C deviation of pHB whereas it had been successfully used for vanillin (Bréas et al., 1994; Fayet et al., 1995). The main problem was the repeatability and the accuracy of the δ^{13}C measurement of pHB. This therefore led us to develop a new approach based on an efficient purification procedure which was also required for the ^{2}H-NMR analysis. Finally, we show that the SNIF-NMR method can also be used for pHB even when a relatively small amount of material is available.

II. MATERIALS AND METHODS

(a) Nature and Origin of the Products. Most of the samples of vanillin and pHB used as references for natural origins have been extracted in our laboratory from beans grown mainly in Madagascar, Comores, Reunion, and Indonesia. The extraction was performed with dichloromethane as extractive solvent. Some authentic vanilla extracts (ethanol, aqueous, CO_2) were also studied and were used as secondary references. The vanillin and pHB were purified according to the procedure described below. The semisynthetic (ex-lignin) and synthetic (ex-guaiacol) vanillin samples were purchased directly from the manufacturers (Rhône-Poulenc, Eurovanillin, Ontario paper, Monsanto, etc.) or were commercially available through suppliers of laboratory grade chemicals (Aldrich, Lancaster, Fluka, Kodak, Prolabo, Janssen, Panreac, BDH, Carlo Erba, Merck, etc.). Some of these were also synthesized in the laboratory. Commercial samples of pHB were purchased and used as reference for the synthetic origins.

(b) Purification of Vanillin and pHB from Vanilla Extracts. We have developed specific procedures for the purification of vanillin and pHB from several matrices (ethanol extracts, ice creams, preparations with propylene glycol, etc.). Our main goals were a universally applicable method and a simple and efficient procedure. For example, the extraction of vanillin and pHB from an alcohol extract requires several steps which can be summarized by the following procedure.

1. Extraction of Organic Molecules. The aim of this step is to produce a concentrate which is very rich in vanillin and pHB, but contains no gums, sugars, propylene glycol, etc. This concentrate can then be loaded onto the silica column. The extraction is based on the separation properties of the aqueous ethanolic solution at high ionic strength. For example, 1 L of a 50% (vol) ethanol solution gives two phases in the presence of a mixture of salts (120 g of $(NH_4)_2SO_4$ and 85 g of $Na_2HPO_4 \cdot 12H_2O$), i.e., the ethanol no longer mixes with the water. In such a medium, organic molecules will "prefer" the ethanol phase. Indeed, the vanillin and pHB are found in the ethanol, while sugars, gums, and others ingredients are still soluble in the water phase. According to the type of extract and the amount of ethanol, water and/or ethanol has to be added to reach an alcoholic grade of about 50% (vol). The amount of salts is then adjusted depending on the final volume and the solution stirred for 2−3 h. Two layers appear (a better separation is achieved at low temperature, about 5 °C). The alcoholic phase is collected, and the ethanol is evaporated under a light vacuum. In some cases, the organic phase may contain a relatively high amount of residual water, and therefore an extraction with ether is necessary.

2. Purification on Silica Column Chromatography. Separating pHB from vanillin, when the amount of the latter is 10−20 times that of the pHB, represents quite a challenge for the analyst. The separation should be achieved without significant isotope fractionation both for vanillin and pHB. The method used should also be efficient, if a large number of vanilla flavor samples are to be analyzed on a routine basis. With this in mind, we chose to improve the silica column chromatography separation procedure, already used for ^{13}C analysis of vanillin (Fellous et al., 1992). The main modification consists in the choice of silica gel; in this case Merck Kieselgel G silica gel is preferred. However, gravity and atmospheric pressure are not sufficient for the elution, and an air pressure of at most 1 bar (optimum 0.5 bar) is applied on the top of the column (Hunt and Rigby, 1976). A rapid and repeatable elution of the organic molecules is reached when a precolumn treatment is performed. This is achieved by adding classical silica (silica 60 A, 70−200 μm) on top of the already conditioned gel. The product to be separated is then loaded uniformly onto the column. To purify about 2 g of vanillin, 100 g of silica gel G and 100 g of silica gel 60A are necessary, in a 3 cm o.d. glass column equipped with a fritted disk. The elution is initiated by a mixture of 70% pentane and 30% dichloromethane in volume. When all the vanillin has been collected, the gradient is regularly increased in CH_2Cl_2. Each collected fraction (about 300 mL) is controlled by TLC (thin layer chromatography). The main impurities (pigments, oil, etc.) stick on the precolumn. Vanillin is usually collected alone without contamination with pHB. The last fraction of pHB is obtained by an elution of pure dichloromethane. The composition of all the fractions, after concentration, is checked by TLC. If one of them shows a mixture of vanillin and pHB, a short separative column has to be performed to collect the vanillin and pHB fractions which are then added to those obtained previously for each molecule. This should seldom be necessary, particularly if the composition of the initial extract is determined beforehand (using HPLC analysis, for example) in order to avoid overloading the short column. Finally, each fraction of vanillin and pHB is filtered and evaporated to dryness.

3. Recrystallization. This step removes the oily traces which can go through the silica column. The recrystallization can be performed either with pure water or with a mixture of 90% water and 10% ethanol (*caution*: vanillin or pHB should not be allowed to stand in boiling water for too long; see paragraph III). The vanillin or pHB crystals are filtered and dried on P_2O_5 overnight under vacuum (constant weight). The purity of the molecule of interest is usually higher than 99%. For other matrices such as ice cream, the same procedure can be applied but must be adapted to the amount of product to be treated.

(c) Analysis of the Results: Statistical Interpretation. The interpretation in terms of origin (whether a sample is natural, synthetic, etc.; whether it has been adulterated or not, ...) is done by comparison of the isotopic parameters of the sample analyzed with the same isotopic parameters determined on a group of reference molecules of known origin. For this purpose, we have developed isotopic knowledge bases containing reference grids for each source. These databases are regularly updated by collecting and analyzing new authentic samples every year. The diagnosis itself can be performed using a Monte Carlo simulation (Güell and Holcombe, 1990).

(d) The SNIF-NMR Method. The determinations of the site specific isotope ratio $(D/H)_i$ were performed using ^{2}H-NMR experiments on an AM500 Bruker spectrometer at a temperature of 308 K. The spectra were recorded at 76.77 MHz using a specific 10 mm (o.d.) probe equipped with a ^{19}F locking device. A broad band ^{1}H decoupling was applied continuously. Quantitative data were obtained by using a 90° pulse (13 μs) followed by a relaxation delay of more than 5 T1 (relaxation time). An internal referencing procedure was used (Martin and Martin, 1995). The chemical shifts of ^{2}H signals are referred to TMS, in ppm. A precisely known quantity of a working standard (TMU), 50 μL of C_6F_6 for ^{19}F locking and trifluoroacetic acid (TFA) for OH site displacement (to avoid overlapping with site 3), were introduced in each sample, in addition to pure vanillin or pHB. The mean values of three spectra gives the final $(D/H)_i$. The reference used is certified tetramethylurea (TMU) purchased from the European Union Institute for Reference Materials and Measurements located in Geel, Belgium. The $(D/H)_i$ ratios are expressed in parts per million (ppm). The molar fractions of the monodeuterated isotopomers, f_i, are directly calculated from the signal area, S_i.

The phasing and the base line corrections require great care since any misadjustments can lead to erroneous and nonreproducible $(D/H)_i$. We have recently introduced an advanced global least-squares curve-fitting procedure (Martin, 1994). This procedure is available to other laboratories as the EUROSPEC-LISS Software. With this new algorithm, the base line and the phase corrections are performed together with the calculations of the signal areas. It has been proven that this procedure leads to the best possible results in terms of precision and accuracy (Martin, 1994). It should be noted that the $(D/H)_2$ ratios for site 2 (OH) of vanillin and of pHB are not computed since they are not representative of the origin but rather of the deuterium content of the water used for the recrystallization.

(e) ^{13}C IRMS Determinations. The mass spectrometric determinations of the carbon isotope ratio were carried out by on-line analysis using a Carlo-Erba NA 1500 II elemental analyzer fitted to a Fisons Instruments Optima mass spectrometer. Samples placed in tin containers were submitted to a flash combustion in a stream of helium enriched with pure oxygen. A conventional procedure was used for carbon determinations (Barrie and Lemley, 1989). The results are expressed on the δ ‰ scale with respect to the international standard PDB.

III. RESULTS

(a) Extraction and Purification of Vanillin and pHB from Vanilla Beans and from Extracts. For isotopic determinations, the control of the purity of the product to be analyzed is of importance. A bench extraction and/or purification of vanillin and pHB is/are therefore prerequisite step(s) of paramount importance. Indeed it was shown (Moussa et al., 1990) that where the raw material has undergone physical or chemical treatment, the occurrence of associated isotope effects must be carefully monitored. We have therefore devoted a special paragraph to this aspect.

The purification of vanillin and/or pHB can be performed on alcoholic extracts, powders, sugars, ice creams, etc. It is known that the physical processes applied to vanillin or pHB can lead to isotopic fractionation (separation of heavy molecules from the light molecules) (Hoffman et al., 1979). Such phenomena may slightly change the values obtained from the isotopic measurements and, therefore, if adequate care is not taken, could lead to erroneous interpretation of the results. This point has already been reported (Fellous et al., 1992) for the ^{13}C content of vanillin measured by mass spectrometry. We have therefore conducted a similar study on ^{2}H isotopic fractionation during the different purification steps of vanillin. For pHB, both ^{13}C and ^{2}H fractionations have been studied.

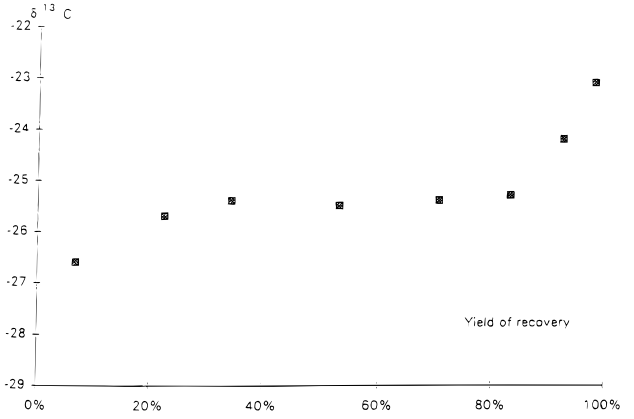

Figure 2. δ^{13}C ‰ of pHB versus the cumulative yield of recovery during a fractionated silica column chromatography. The values plotted correspond to those measured on each fraction and not on the pooled recovered pHB.

The study of isotope fractionation which may occur during each of the above steps was performed systematically. It has already been demonstrated that no isotopic fractionation effect can be detected when liquid−liquid or solid−liquid extractions are performed (Dautraix et al., 1995; Salmon et al., 1996). Therefore it can be safely assumed that the first step of liquid−liquid phase transfer is not creating an isotope disturbance. The liquid chromatographic separation, on the other hand, is known to produce isotope effects (Fellous et al., 1992). The present study on the purification of pHB shows that the carbon-13 content of pHB is affected in a way similar to that of vanillin during fractionated chromatographic separation (Figure 2). It can be concluded from Table 1 that, except for the very tail ends of the eluent, the isotopic fractionation on the site specific $(D/H)_i$ ratio is very small both for vanillin and for pHB. A visible fractionation effect was only observed for the first and last fractions which account for less than 10% of the total. Of course, pooling together all fractions recovered eliminates these effects. It appears that site 1 (aldehyde group) is the most sensitive to noncomplete recovery. A safe way to carry out the purification on a silica column is to obtain a slightly less pure compound but being certain that all the material (vanillin or pHB) has been recovered. The recrystallization will remove the remaining impurities.

In order to assess the isotopic fractionation which may occur during the recrystallization step, a sample of vanillin ex-guaiacol has been recrystallized four times consecutively in solvent constituted of 10% ethanol and of 90% water. After the sample was dried overnight on P_2O_5 the recovery yield and the ^{2}H-NMR parameters (the molar fraction) were calculated (Table 2). The yield corresponds to the recovery ratio of vanillin after each recrystallization, while the cumulated yield refers to the

Table 1. Specific Deuterium and Carbon-13 Content of Vanillin F and pHB[b] Collected during a Silica Column Chromatography According to the Methodolodgy Described in the Text

	$(D/H)_1$ (ppm)	$(D/H)_3$ (ppm)	$(D/H)_4$ (ppm)	$(D/H)_5$ (ppm)	cumulativ yield (%)	δ^{13C} (‰)
			Vanillin			
fraction 1	111.0	122.5	159.6	103.0	21.0	−27.4
fraction 2	122.4	126.2	158.6	103.3	38.5	−26.8
fraction 3	124.2	128.7	157.6	104.0	57.4	−26.8
fraction 4	129.1	127.1	157.5	103.0	86.1	−26.5
fraction 5	146.1	132.0	165.0	104.4	94.6	−25.9
reference	126.4	128.7	161.9	104.5	100.0	−26.8
			pHB			
fraction 1					6.9	−26.6
fraction 2	253.1	166.2	161.1		22.5	−25.7
fraction 3					34.4	−25.4
fraction 4	249	156.3	151.8		53.1	−25.5
fraction 5	nm	nm	nm		70.6	−25.4
fraction 6	266.5	165.4	154.8		83.2	−25.3
fraction 7					92.5	−24.2
fraction 8	290.1	156.8	156.1		98.1	−23.1
reference	254.2	164.7	158.5		100	−25.3

[a] The values given for the reference were measured on the pure synthetic vanillin used for the experiment. [b] The values given for the reference were measured on the pure synthetic pHB used for the experiment. The SNIF-NMR results given for the fractions 2 and 8 correspond to the results of analyses performed on fraction 1 pooled with fraction 2 and fraction 7 pooled with fraction 8 respectively. [c] nm = not measured.

Table 2. Study of Isotope Fractionation during the Recrystallization Process of Vanillin in a Mixture of 90% Water and 10% Ethanol[a]

sample no.	f_1	f_3	f_4	f_5	recovery yield for each experiment (%)	cumulated recovery yield (%)	^{13}C (‰)
reference	0.293	0.226	0.122	0.358			−28.9
exp no. 1	0.293	0.229	0.123	0.355	92.7	92.7	−29.0
exp no. 2	0.293	0.228	0.122	0.357	92.5	69.0	−28.9
exp no. 3	0.294	0.228	0.118	0.360	84.4	44.4	−29.0
exp no 4	0.292	0.225	0.119	0.365	66.2	18.4	−28.8

[a] The ^{2}H-NMR parameters are the molar fractions f_i. Results given for the reference were measured on a sample of pure vanillin (ex-guaiacol). The cumulated yield corresponds to the effective vanillin obtained after each recrystallization compared to the starting amount of vanillin. The amount collected after each experiment is used as starting material in the next recrystallization.

initial amount of vanillin. One can notice that even when about 80% of material is lost, there is no significant change in the deuterium distribution. The same conclusion can be drawn for the ^{13}C content. However, caution should be taken when dealing with recrystallization of vanillin (and pHB) in aqueous media. We have observed in extreme conditions (i.e., in boiling water during several days) that exchange between hydrogen of site 4 and water may sometimes occur. The acidic properties of this hydrogen are supported by its enolic feature.

(b) Site Specific Deuterium Content $(D/H)_i$ of Vanillin. The internal reproducibility (ISO standard 5725 1987) of the SNIF-NMR analysis is shown in Table 3. These values were obtained by measuring 17 times the same vanillin sample over one year. This reproducibility is in fact the combination of the reproducibility of the NMR sample preparation step and of the NMR measurement itself. Such good results are due to the technical improvements presented in this paper. On the

Table 3. Standard Deviation (SD) and Internal Reproducibility (R_i) of the SNIF-NMR Method Applied to Vanillin, According to the ISO Standards (Norme ISO 5725, 1987)

site *i*	SD (%)	R_i (%)
1	1.0	2.8
3	1.3	3.8
4	1.3	3.8
5	0.5	1.4

average, this is a 4-fold improvement over previously published results (Maubert et al., 1988).

It appears that all the origins of vanillin are well discriminated using ^{2}H-NMR data (Figure 3). There is no overlap origins. Particularly, vanillin ex-bean is well separated from the other sources: semisynthetic (ex-lignin) and synthetic (ex-guaiacol). The formation of the aldehyde function is the main source of variation within the groups ex-guaiacol and ex-lignin. For the guaiacol origin, two subgroups are clearly identified. We believe that they correspond to two types of chemical processes used for creating an aldehyde function. The guaiacol core remains consistent with the starting material. The lignin origin is also spread mainly because of the aldehyde function. The oxidation mode is therefore important, but the lignin core is somehow also different.

However, the internal variations within a group which were discussed above are small enough so that, in a first approximation, each origin (beans, lignin, guaiacol) can be considered as only one homogeneous group. The mean values for these three main origins are presented in Table 4. The standard deviation around these mean values are relatively low, but are still larger than the reproducibility of the method. Each group is well separated from the other, allowing studies of mixtures (Figure 3). The discriminating power of the different isotope ratios $(D/H)_i$ is not identical. Therefore a global approach using the $(D/H)_i$ values all together is necessary. By using not only one site but all discriminating sites, the minimum threshold of detection is significantly lowered. Generally speaking, the present method enables the detection of about 5% of vanillin ex-guaiacol in vanillin ex-bean and about 10% of vanillin ex-lignin in vanillin ex-bean. This can be observed visually in Figure 3 or by observing that the addition of 5% vanillin from guaiacol increases $(D/H)_1$ of about 10 ppm, thus pushing it out of the range for natural vanillin. The multidimensional Monte Carlo interpretation discussed below is even more sensitive as it takes into account simultaneously the effect on all four sites. These thresholds decrease when comparison is made with more precise reference groups. The quantification of each type of vanillin present in a mixture is not straightforward. We applied a Monte Carlo approach (Güell and Holcombe, 1990). To illustrate this, various mixtures of vanillin ex-lignin, vanillin ex-guaiacol, and vanillin ex-beans (Madagascar) were made up. Table 5 summarizes the results. The experimental values agree almost perfectly with the actual composition of the mixtures (especially the natural vanillin portion). Using this approach, the composition of specific mixtures such as the French product type "Arôme renforcé", which is a mixture of 60% vanillin ex-bean and 40% synthetic vanillin, can be easily verified.

The SNIF-NMR analysis is now in widespread use, and some instances of sophisticated isotopic manipulations to get around the method have been noticed. For example, synthetic vanillin samples, enriched in deu-

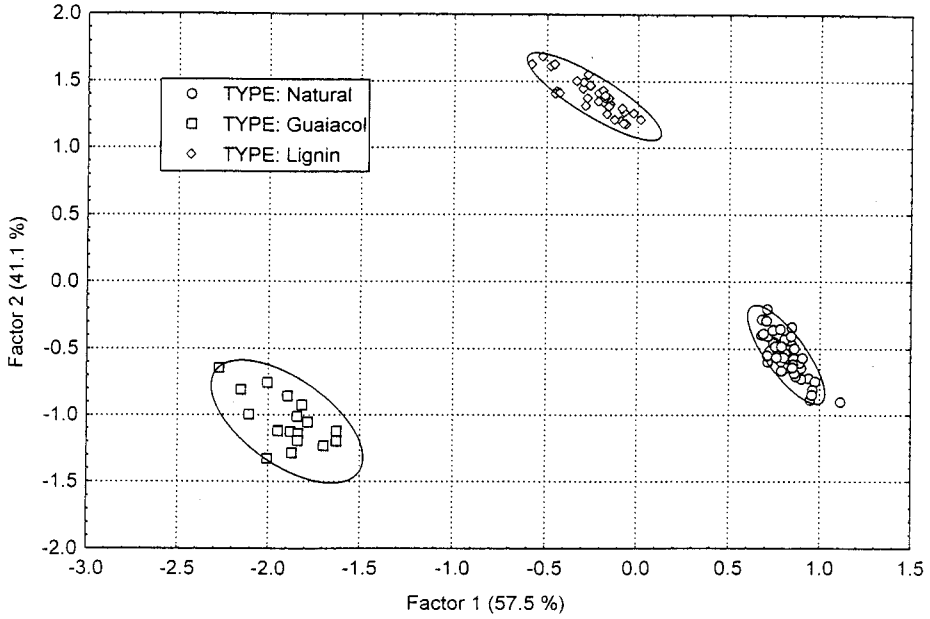

Figure 3. Representation of the reference groups of vanillin (ex-beans, ex-lignin, and ex-guaiacol) projected in the plane of the canonical variables. $(D/H)_1$, $(D/H)_3$, $(D/H)_4$, and $(D/H)_5$ were the initial parameters. The ellipses drawn correspond to the 95% confidence interval.

Table 4. Mean Values and Standard Deviations (SD) of $(D/H)_i$ of Vanillin from the Main Origins: Beans, Guaiacol, and Lignin[a]

origin	$(D/H)_1$ (ppm)	$(D/H)_3$ (ppm)	$(D/H)_4$ (ppm)	$(D/H)_5$ (ppm)
ex-beans (49)	130.8	157.3	196.4	126.6
SD (ppm)	3.1	3.0	2.5	1.7
ex-lignin (64)	119.9	132.1	168.8	105.9
SD (ppm)	6.4	2.6	5.9	1.4
ex-guaiacol (30)	315.2	138.8	143.8	139.1
SD (ppm)	56.9	6.7	5.3	8.4

[a] The numbers in brackets following the origin of vanillin correspond to the number of samples used.

Table 5. Determination of Mixtures of Vanillin from Several Origins, Based on SNIF-NMR Experiments[a]

	actual values[b]			experimental values[b]		
sample no.	ex-L (%)	ex-G (%)	ex-B (%)	ex-L (%)	ex-G (%)	ex-B (%)
mixture A	25.4		74.6	24.5		75.4
mixture B	50.0		50.0	47.0		53.0
mixture C	11.8	9.8	78.4	17.2	9.6	73.3
mixture D		15.1	84.9		14.6	85.4
mixture E	15.5		84.5	15.3		84.7
mixture F		90.4	9.6		89.3	10.6

[a] The actual values are the calculated percentages, while the experimental values correspond to the percentages obtained from the SNIF-NMR experiment after statistical calculations. [b] L = lignin, G = guaiacol, B = beans.

terium on several sites, have been detected for some time—an adulteration which shows up particularly on site 5. Indeed, the adjustment of site 5 (OCH_3) leads to the appearance of an additional peak on the ^{2}H-NMR spectrum whose intensity is proportional to its abundance (see, for example, Figure 4). This peak occurs at the right side of peak number 5 and corresponds to vanillin containing a deuterated methoxy group. This type of molecule does not exist at that abundance in natural vanilla (there is 2000 times less). Its occurrence in the ^{2}H-NMR spectrum points to an isotopic manipulation. This seems to indicate that one of the methodologies used to adjust the deuterium content of the

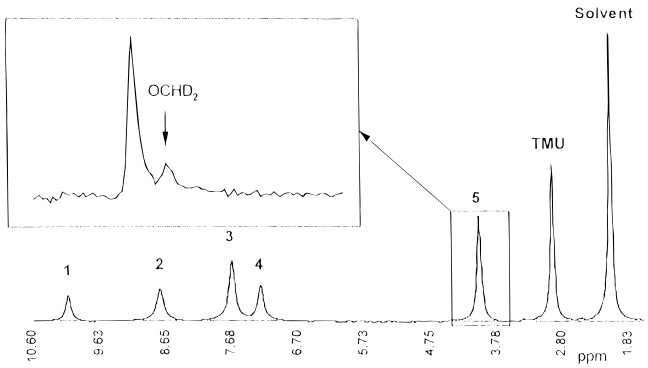

Figure 4. Zoom of ^{2}H-NMR spectrum showing the presence on the right side of the peak corresponding to the methoxy group (site 5) of a small peak due to molecules containing probably $OCHD_2$. This is an indication that an isotopic manipulation of vanillin was performed on that sample.

methoxy group is a methylation with deuterated methyl iodide.

(c) Carbon-13 and Site Specific Deuterium Content $(D/H)_i$ Data on pHB. *(i) Carbon-13 Content of pHB.* Since there was very little data available for pHB, we decided to perform a full isotopic analysis of pHB from several sources. The mean value for the ^{13}C content of natural pHB extracted from vanilla beans is -18.2 ± 0.6‰ (30 samples). The purification method used is the one described in this paper. This method is safe enough and repeatable (see Table 6). Therefore a mean value of -18.3‰ for pHB present in vanilla beans can be validated. This is a very important conclusion since, until now, it was commonly assumed (French Directive of June 11, 1987) that the normal δ^{13}C for natural pHB from vanilla beans could be as negative as -22.5‰. We have observed no authentic vanilla extracts where the δ^{13}C of pHB is more negative than -19.3‰. In fact, only one sample was found with a value more negative than -19‰ (at -19.3‰), and its origin was uncertain. Thus -19.5‰ can be proposed as a safe lower limit (statistically it also corresponds to the mean value (-18.3) minus 2 standard deviations). In our opinion this limit is conservative enough and

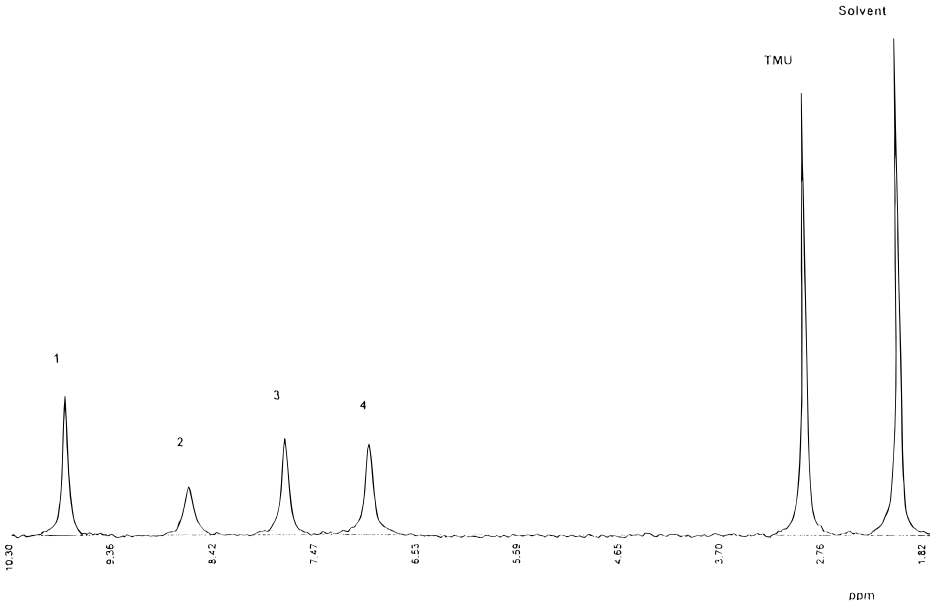

Figure 5. Typical ^{2}H-NMR spectrum of pHB obtained on a 500 MHz Bruker spectrometer. Officially calibrated TMU (tetramethylurea) is the internal reference. The solvent used to dissolve pHB is CH_3CN.

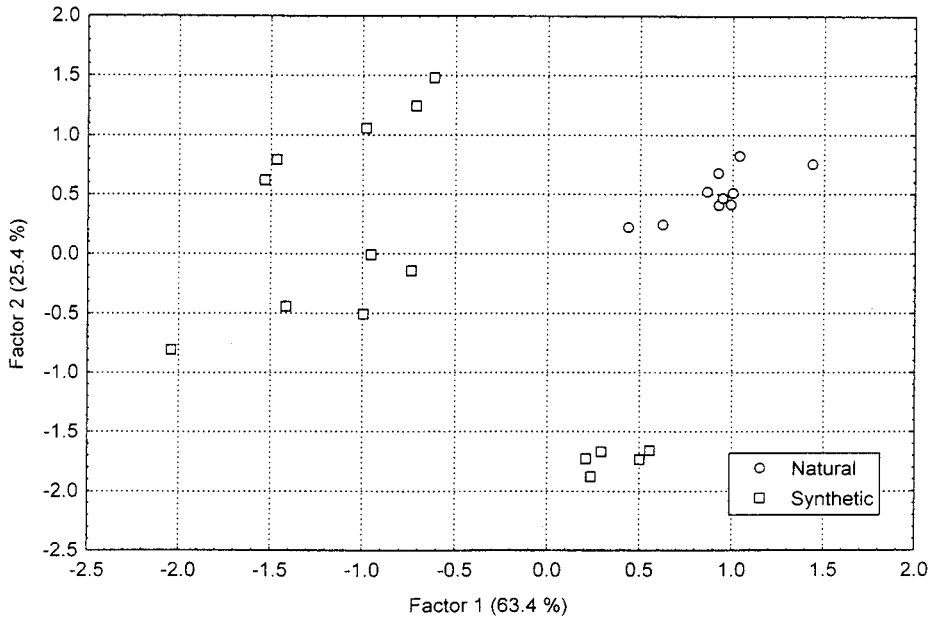

Figure 6. Representation of the reference groups of pHB (ex-beans and synthetic) projected in the plan of the canonical variables. $(D/H)_1$, $(D/H)_3$, and $(D/H)_4$ were the initial parameters.

should not cause authentic products to be classified as adulterated as in industrial products, which are produced in large volumes, the standard deviations are smaller than in small laboratory samples (Martin et al., 1996b). This result shows that in vanilla beans the pHB molecule is indeed enriched in ^{13}C with respect to the vanillin molecule. This kind of difference was already observed in some cases (Gensler et al., 1995) when comparing the main molecule to secondary metabolites. The mean value of ^{13}C content of synthetic pHB is −28.3 ± 2.8‰ (14 samples). Addition of synthetic pHB into natural pHB (extracted from vanilla beans) is detectable on the basis of ^{13}C measurements.

(ii) SNIF-NMR Analysis of pHB. Figure 5 shows a typical ^{2}H-NMR spectrum of a pHB sample. With 250−500 mg of pure pHB, good spectra can be recorded with an acceptable spectrometer accumulation time. The signal-to-noise ratio is not as high as that obtained for vanillin, where, when possible, we use about 1 g of pure vanillin. However, after about 15 h three ^{2}H-NMR spectra can be recorded (for 300 mg of pHB) and can be mathematically processed (Martin, 1994) to yield reliable site specific $(D/H)_i$ isotope ratios. Table 7 summarizes the $(D/H)_i$ ratios of pHB ex-vanilla beans and synthetic pHB. The natural pHB ex-vanilla beans is well discriminated as shown in Figure 6. Further data are currently being collected to increase the database of authentic pHB samples.

A comparison of the ^{2}H isotopic ratios of pHB and vanillin from beans at the same position clearly shows that the aromatic ring is generated from an identical intermediate: the deuterium contents of the two aromatic sites measured by ^{2}H-NMR are very similar both in natural pHB and vanillin. Interestingly the aldehyde group is very different. At the moment no good explanation can be made for this fact; it is supposed that a common aromatic intermediate exists for pHB and vanillin, but the metabolism of each molecule is then

Table 6. Repeatability of the Separation of Vanillin from pHB on Short Silica Chromatography Column, Using the Method Described in This Paper, for the Determination of Carbon-13 Content of both Vanillin and pHB

vanillin sample no.	δ^{13C} (‰)	experiment[a]	pHB sample no.	δ^{13C} (‰)
9A060427	−26.7	a	9B060431	−25.5
9A060428	−26.7	a	9B060432	−25.4
9A060429	−26.8	a	9B060433	−25.6
9A060430	−27.0	a	9B060434	−25.5
9A060435	−26.8	b	9B060439	−25.6
9A060436	−26.6	b	9B060440	−25.5
9A060437	−26.7	b	9B060441	−25.4
9A060438	−26.9	b	9B060442	−25.2
mean	−26.78		mean	−25.46
SD	0.13		SD	0.13
reference	−26.8		reference	−25.3

[a] Experiment a is a separation of 200 mg of vanillin from 200 mg of pHB. Experiment b corresponds to the separation of 6 g of vanillin from 1 g of pHB. The values given after reference correspond to the pure pHB and vanillin used, analyzed before being mixed together.

Table 7. Results of SNIF-NMR Analyses of pHB Samples (Ex-beans and Synthetic) as $(D/H)_1$, $(D/H)_3$, and $(D/H)_4$ Parameters[a]

origin of pHB	$(D/H)_1$ (ppm)	$(D/H)_3$ (ppm)	$(D/H)_4$ (ppm)
ex-beans (10)	273.7	159.0	197.4
SD (ppm)	8.0	5.6	3.1
synthetic (16)	248.5	146.6	146.3
SD (ppm)	102.7	9.0	8.5

[a] Mean values and standard deviations (SD) of $(D/H)_i$ of pHB extracted from beans (ex-beans) and chemically produced (synthetic). The numbers in parentheses following the origin of pHB correspond to the number of samples used.

very different, leading to a creation of the aldehyde function *via* a different mode.

IV. CONCLUSION

The robustness of the overall method enables us to propose a general approach for characterizing the authenticity of vanilla flavors and extracts as well as of finished products (ice cream, desserts, etc.) containing this flavor.

(a) SNIF-NMR Analysis of Vanillin. The SNIF-NMR method now makes it possible not only to prove that wholly synthetic vanillin has been substituted for natural vanillin but also to detect and quantify additions of as little as 5% of synthetic vanillin ex-guaiacol and 10% of semisynthetic vanillin ex-lignin in natural vanillin (ex-beans). In the same experiment, a careful inspection of the ^{2}H-NMR spectrum may reveal an isotopic enrichment in deuterium, aimed at circumventing this analysis.

(b) ^{13}C Content of pHB. ^{13}C IRMS analysis on pHB is an easy additional step to control the authenticity of the whole vanilla flavor or extract. A conservative lower limit of carbon-13 deviation of pHB ex-beans can be set at −19.5‰. On the basis of our results, any sample of vanilla extract or flavor where pHB shows a δ^{13}C deviation lower than this value cannot be considered as originating only from vanilla beans.

(c) SNIF-NMR Analysis of pHB. A confirmation of the naturality of the flavor under investigation can now also be achieved by performing a SNIF-NMR analysis of pHB.

The above analyses can be carried out on all vanillin containing products. We have already tested the present methodology on vanilla ice cream, yogurt, dessert cream. In order to be able to perform steps a and b, 15 kg of ice cream has to be extracted.

ABBREVIATIONS USED

AOAC, Association of Official Analytical Chemists; CEN, Comité Européen de Normalisation; ENV, provisional European standard; GC−C−IRMS, Gas chromatography ^{13}C isotope ratio, mass spectroscopy; HPLC, high-performance liquid chromatography; IOFI, international organization of the flavor industry; IRMS, isotope ratio by mass spectroscopy; NMR, nuclear magnetic resonance; OIV, Office International de la Vigne et du Vin; PDB, Pee Dee Belemnite; pHB, *p*-hydroxybenzaldehyde (4-hydroxybenzaldehyde); SNIF-NMR, registered trademark; TMS, tetramethylsilane; TMU, tetramethylurea.

ACKNOWLEDGMENT

We thank Miss Laurence Adam for her contribution to the study of pHB and vanillin isotope fractionation.

LITERATURE CITED

Barrie, A.; Lemley, M. Automated ^{15}N/^{13}C analysis of biological materials. *Int. Lab.* **1989**, *19* (7), 82−91.

Bréas, O.; Fourel, F.; Martin, G. J. ^{13}C analysis of aromas and perfumes by a coupled GC-IRMS technique. The case of vanillin and leaf alcohol extracts. *Analusis* **1994**, *22*, 268−272.

Bricout, J.; Fontes, J. C.; Merlivat, L. Flavors and nonalcoholic beverages. Detection of synthetic vanillin in vanilla extracts by isotopic analysis. *J. Assoc. Off. Anal. Chem.* **1974**, *57* (3), 713−715.

Dautraix, S.; Gérola, K.; Guilluy, R.; Brasier, J. L.; Château, A.; Guichard, E.; Etiévant, P. Test of isotopic fractionation during liquid-liquid extraction of volatile components from fruits. *J. Agric. Food Chem.* **1995**, *43*, 981−983.

Derbesy, M.; Touche, J. Techniques d'isolement de la vanille à partir de gousses et d'extraits naturels de vanille en vue de l'analyse isotopique. (Techniques for the isolation of vanillin from pods and from natural vanilla extracts for the purposes of isotope analysis.) *Parfums, Cosmét., Arômes* **1978**, *21*, 97−101.

Fayet, B.; Fraysse, C.; Tisse, C.; Pouliquen, I.; Guerere, M.; Lesgards, G. Analyse isotopique par GC-SMRI du carbone 13 de la vanille dans des crèmes glacées. (Isotopic analysis by GC-IRMS of carbon 13 of vanillin in ice creams.) *Analusis* **1995**, *23*, 451−453.

Fellous, R.; George, G.; Schippa, C. Vanilline : Anomalies dans le protocole d'identification par ^{13}C. (Vanillin: anomalies in the carbon-13 identification protocol.) *Parfums, Cosmét., Arômes* **1992,** *106*, 95−98.

French Directive of June 11, 1987. Méthode Officielle pour la détermination de la déviation isotopique du carbone 13 de la vanilline. In *Journal Officiel de la République Française*, 22/07/87.

Gensler, M.; Rossmann, A.; Schmidt, H.-L. Detection of added L-ascorbic acid in fruit juices by isotope ratio mass spectrometry. *J. Agric. Food Chem.* **1995**, *43*, 2662−2666.

Güell, O. A.; Holcombe, J. A. Analytical applications of Monte Carlo Techniques. *J. Anal. Chem.* **1990**, *62*, 529−542.

Guinamant, J. L. L'extraction en phase supercritique. (Supercritical phase extraction.) *Analusis* **1992**, *20* (1), 36−38.

Hoffman, P.; Salb, M. Isolation and stable isotope ratio analysis of vanillin. *J. Agric. Food Chem.* **1979**, *27*, 352−355.

Hunt, B. J.; Rigby, W. Short column chromatography. *Chem. Ind.* (*London*) **1976**, 1868−1869.

Institut de Chimie Analytique et du Contrôle de la Qualité (ICACQ). Préparations naturelles vanillées. *Option Qualité* **1984**, *18*, 12−15.

IOFI Information letter no 775, 1989.

Krueger, D. A.; Krueger, H. W. Carbon isotopes in vanillin and the detection of falsified "natural" vanillin. *J. Agric. Food Chem*. **1983**, *31*, 1265−1268.

Krueger, D. A.; Krueger H. W. Detection of fraudulent vanillin labeled with ^{13}C in the Carbonyl carbon. *J. Agric. Food Chem*. **1985**, *33*, 323−325.

Martin, G. E.; Ethridge, M. W.; Kaiser, F. E. Determining the authenticity of vanilla extracts. *J. Food Sci.* **1977**, *42* (6), 1580−1586.

Martin, G. E.; Alfonso, F. C.; Figert, D. M.; Burggraff, J. M. Stable isotope ratio determination of the origin of vanillin in vanilla extracts and its relationship to vanillin / potassium ratios. *J. Assoc. Off. Anal. Chem*. **1981**, *64*, (5), 1149−1153.

Martin, G. G.; Remaud, G.; Martin, G. J. Isotopic methods for control of natural flavours authenticity. *Flavour Fragrance J.* **1993**, *8*, 97−107.

Martin, G. G.; Wood, R.; Martin, G. J. Detection of added beet sugar in concentrated and single strength fruit juices by Nuclear Magnetic Resonance (SNIF-NMR method): Collaborative Study. *J. AOAC Int.* **1996a**, *79*, 917−928.

Martin, G. G.; Hanote, V.; Lees, M.; Martin, Y. L Interpretation of combined ^{2}H SNIF-NMR® and ^{13}C SIRA/MS analyses of fruit juices to detect added sugar. *J. AOAC Int.* **1996b**, *79*, 62−72.

Martin, G. J.; Martin, M. L. Deuterium labelling at the natural abundance level as studied by high field quantitative ^{2}H NMR. *Tetrahedron* **1981**, *22* (36), 3525−3528.

Martin, G. J.; Martin, M. L. Stable isotope analysis of food and beverages by NMR spectroscopy. *Annu. Rep. NMR Spectrosc.* **1995**, *31*, 91−104.

Martin, G. J.; Koziet, J.; Rossmann, A.; Dennis, J. Site-specific natural isotope fractionation in fruit juices determined by deuterium NMR, a European inter-laboratory comparison study. *Anal. Chim. Acta* **1996**, *321*, 137−146.

Martin, Y. L. A global approach to accurate and automatic quantitative analysis of NMR spectra by complex least-squares curve fitting. *J. Magn. Reson*. **1994**, Series A111, 1−10.

Maubert, C.; Guérin, C.; Mabon, F.; Martin, G. J. Détermination de l'origine de la vanilline par analyse multidimensionnelle du fractionnement isotopique naturel spécifique de l'hydrogène. (Determination of the origin of vanillin by multivariate analysis of the site-specific natural isotope fractionation factors of hydrogen.) *Analusis* **1988,** *16*, 434−439.

Moussa, I.; Naulet, N.; Martin, M.-L.; Martin G. J. A site-specific and multielement approach to the determination of liquid-vapor isotope fractionation parameters. The case of alcohols. *J. Phys. Chem*. **1990**, *94*, 8302−8309.

Norme ISO 5725 : 1986, Fidélité des méthodes d'essai - Détermination de la répétabilité et de la reproductibilité d'une méthode d'essai normalisée par essais interlaboratoires. In *Méthodes statistiques - recueil de normes ISO 3*, Geneva, **1989**, 413−463.

Office International de la Vigne et du Vin. Détection de l'enrichissement des moûts, des moûts concentrés, du sucre de raisin et des vins par application de la résonance magnétique nucléaire du deutérium (RMN-FINS/SNIF-NMR®). In *Recueil des méthodes internationales d'analyse des vins et des moûts*; OIV: Paris, France, 1990p pp 107−118.

Salmon, B.; Martin, G. J.; Remaud, G.; Fourel, F. Compositional and isotopic studies of fruits flavors. Part 1: the banana aroma. *Flavor Fragrance J*. **1996**, *11*, 353−359.

Schmidt, H. L. Food quality control and studies on human nutrition by mass spectrometric and nuclear magnetic resonance isotope ratio determination. *Fresenius Z. Anal. Chem*. **1986**, *324*, 760−766.

Toulemonde, B.; Horman, I.; Egli, H.; Derbesy, M. Applications of high resolution NMR. Part II. Differentiation between natural and synthetic vanillin samples using ^{2}H-NMR. *Helv. Chim. Acta* **1983**, *66*, 2342−2345.

Received for review July 11, 1996. Accepted December 9, 1996.[⊗]

JF960518F

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.