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Isolation and Stable Isotope Ratio Analysis of Vanillin

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A method has been developed for the routine determination of the origin of vanillin found in vanilla extracts and products. This method uses gas chromatographic isolation coupled with C-13/C-12 stable isotope ratio analysis to differentiate between vanillin from vanilla beans and that produced synthetically from lignin, eugenol, and guaiacol.

One of the world's most important and desirable flavors is vanilla, formed by the extraction of the cured fruit of an orchid plant. In 1976, 2.23 million pounds of vanilla beans were imported into the United States at an approximate value of fifteen million dollars. A large majority of the beans came from the Malagasy Republic (Madagascar). With the high demand and limited supply of quality beans and the continuing increase in their cost, the use of inexpensive vanillin from lignin in vanilla extracts and products has become attractive. To insure the quality of vanilla extracts and flavors, it is important to develop techniques to verify their authenticity.

Several methods have been developed for this purpose. The most commonly used methods are found in the Association of Official Analytical Chemists' "Methods of Analysis" (1975). However, the composition of an altered sample can be adjusted to mimic the authentic sample, reducing the usefulness of these methods. Furthermore, changes in the source of vanilla beans in the past few years requires additional experimentation to use some of these techniques. For instance, Indonesia (Java) has become the second largest exporter of vanilla beans and was not included in developing these methods. Recently, Martin et al. (1975, 1977) developed a method which uses identification ratios determined from the vanillin, potassium, nitrogen, and inorganic phosphate content of each type of vanilla extract. This method is applicable but requires sophisticated equipment, a significant input of time, and regular determination of the ratios for authentic standards; e.g., extracts of Java beans in 1975 had an undetectable vanillin content whereas the 1976 crop has about 500 ppm. Therefore, a procedure dependent upon an intrinsic property of the compound vanillin (the major flavor constituent of vanilla) and one which could also determine the origin of the vanillin would be more advantageous and consistent. This procedure would, in addition, be applicable to vanilla products as well as extracts.

It is known that photosynthesis is accompanied by isotopic fractionation of carbon in favor of carbon-12. Stable isotope ratio analysis (SIRA) of carbon, used earlier for correcting radiocarbon dating, was developed by Bender (1971) and Smith and Epstein (1971) into a method for determining biosynthetic pathways since it was found that plants discriminate differently against car-

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bon-13. Those which discriminated the most against carbon-13 were found to follow the Calvin cycle, while those which discriminated the least follow the Hatch-Slack cycle. Between these two are the plants following the crassulacean acid metabolism (CAM) which is a combination of the previous biosynthetic pathways. Therefore, the ratio of the carbon-13 to carbon-12 of the sample determined in relationship to the ratio of a standard, a fossil Beleminite found at Pee Dee, South Carolina (PDB), is an intrinsic property of a compound due to its biogenesis. Bricout et al. (1974) and Bricout and Koziet (1975) used this technique to determine the origin of citral and vanillin, while White and Doner (1978) used it to detect the adulteration of honey with high fructose corn sugar (HFCS). Hillaire-Marcel et al. (1977) detected the adulteration of maple syrup with sugar at the 10% level.

Because of McCormick's interest in vanilla, it was decided to expand on Bricout's work by including Javan and Tahitian vanilla bean extracts and synthetic vanillin from eugenol and guaiacol along with a larger number of Madagascar and Mexican bean extracts and vanillins from lignin. Utilizing his suggestion of gas chromatographic isolation of vanillin, a quick and simple method was developed, which can be used routinely for detecting adulteration of vanilla products.

EXPERIMENTAL SECTION

Vanilla Extracts. Three Mexican, six Javan, and three Tahitian extracts, conforming to the Food and Drug Administration's definition of a standard onefold extract, were prepared from authentic beans in our laboratory. Each variety was obtained from several importers. The authentic Madagascar extracts were obtained from McCormick & Co. These included three onefold, five 3.33-fold, one tenfold, and one 20-fold extracts, each made from different lots of vanilla beans.

Vanillins. *Lignin.* Eight samples of vanillin from lignin were obtained from various suppliers and were representative of the major manufacturers in the United States, Canada, and Japan.

Eugenol. Four samples of vanillin were prepared from several sources of clove oil eugenol using the manufacturing procedure described by Bedoukian (1967) but on a laboratory scale, using nitrobenzene as the oxidizer and azobenzene as the solvent and also by the method of Lampman et al. (1977).

Guaiacol. Three samples of vanillin were prepared from guaiacol by a scaled-down manufacturing procedure described by Bedoukian (1967) using dimethylaniline, nitrous acid, and formaldehyde. It was also prepared by a method developed by Russell and Luther (1955) using sodium hydroxide and chloroform. Here also, several sources of guaiacol were used.

Isolation of Vanillin from Extracts. Sufficient extract containing approximately 0.1–0.2 g of vanillin was extracted three times with ether. The combined ether extracts were concentrated, and the residue was taken up in methylene chloride removing any water and dried (Na_2SO_4). This solution was concentrated to a gum or solid from which approximately a 10% solution in ether was prepared. Pure vanillin was then isolated by preparative gas chromatography of the solution.

Preparative Gas Chromatography (GC). The isolated crude vanillin or synthetic vanillin dissolved in ether was chromatographed on a Hewlett Packard 5712 gas chromatograph equipped with a thermal conductivity (TC) detector. The injection port temperature was 250 °C and the detector temperatue 250 °C. The column was a 5 ft × 0.25 in. i.d. glass column packed with 5% Carbowax 20M on Chromosorb WAW. (Later evaluation indicated that a 6 ft × 0.25 in. i.d. glass column packed with the above material commercially prepared by Supelco worked equally as well.) The pressure at the head of the column was 40 psi and the column oven temperature was programmed from 70–230 °C at 8 °C/min. Approximately 50 μL was injected on the GC to help saturate the column and remove previous contamination. With this injection the peak height was adjusted so that similar quantities of vanillin were collected from run to run and sample to sample (approximately 50–80% pen deflection). The collection was made from a horizontal line approximately 5–10% of the peak height above the base line (Figure 1, point A to A′). This gave consistent and reproducible results. The vanillin eluant was collected from the detector port in glass tubes, 1.6–1.8 × 100 mm, available from Kimax. The tubes had been cleaned by extraction with CCl_4 in a Soxlet and oven-dried.

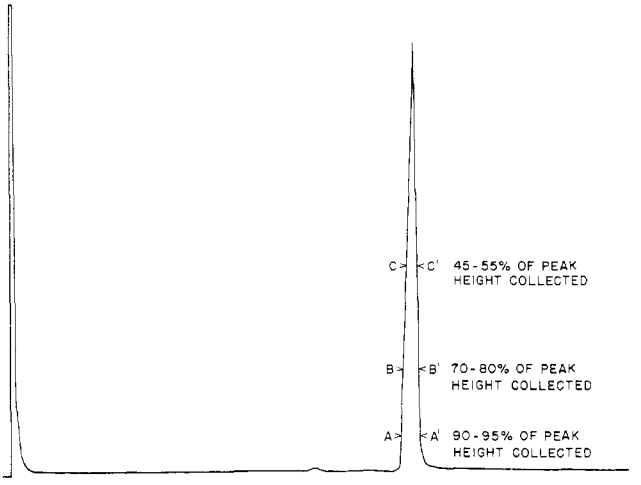

Figure 1. Gas chromatograph of vanillin, indicating collection points.

Consultation with Mr. Reeseman of Geochron and later analysis determined that 3–5 mg of sample was adequate for a determination. In most cases two collections were necessary to assure this quantity. In some cases where the vanillin content was low (i.e., Javan and Tahitian), requiring an inordinately large GC injection, three or four collections were necessary. The tubes were carefully sealed, avoiding charring and reducing the size for convenience, and sent to Geochron for analysis.

Stable Isotope Ratio Analysis (SIRA). The analyses were done by Geochron Laboratories Division, Krueger Enterprises, Inc., Cambridge, MA, using a Micromass 602D spectrometer. Results are reported in δ (δ_{PDB} ^{13}C) units expressed in parts per thousand (ppt) compared with the reference Standard Pee Dee Beleminite (PDB) calculated using the following equation:

$$\delta\ ^{13}C\ (\text{ppt}) = \left[\frac{(^{13}C/^{12}C)\ \text{sample}}{(^{13}C/^{12}C)\ \text{standard}} - 1 \right] \times 1000$$

The analytical labs overall reproducibility is better than ±0.5 ppt. Details of this procedure are adequately described elsewhere (White and Doner, 1978)

RESULTS AND DISCUSSIONS

The isolation of vanillin as crystals and subsequent purification for analysis by standard techniques was quickly abandoned because these methods were both time-consuming and inconsistent. Therefore, directed by a comment of Bricout and Koziet (1975), a preparative gas chromatographic method to isolate vanillin was developed.

Table I. Confirmation of Fractionation

amount collected, mg (portion)	δ_{PDB} ^{13}C	weighed average
Natural Vanillin		
4.05 (front)	−19.4	−8.6
5.04 (back)	−21.5	−11.9
	calcd	−20.6
	found	−20.7
Mixture of Purified Natural and Purified Synthetic		
3.85 (front)	−22.4	−9.2
5.48 (back)	−26.2	−15.3
	calcd	−24.6
	found	−24.8

Table II. Dependence of δ_{PDB} ^{13}C on Portion of Peak Collected

GC collection	δ_{PDB} ^{13}C
Synthetic Lignin Vanillin	
base line to base line	−26.9
A to A′ (see Figure 1)	−27.0
recrystallized vanillin	−27.3
Natural Extract	
A to A′ (see Figure 1)	−20.6
B to B′	−20.7
C to C′	−21.0
isolated and purified vanillin	−20.7

Isotopic fractionation of vanillin by GC was subsequently found to occur such that the heavier isotope was concentrated in the earlier portion of the GC peak. Similar GC fractionation has been reported for carbon dioxide by Gunter and Gleason (1971) and for sulfur hexafluoride and carbon tetrafluoride by Bayer and Nicholson (1970). Also, Baertschi et al. (1953) showed that distillation of $CHCl_3$, CCl_4, CH_3OH, and C_6H_6, using a special column, resulted in concentration of the carbon-13 in the distillate. This indicated that care in the isolation of vanillin would be necessary.

The fractionation of vanillin was confirmed using a method similar to that used by Gunter and Gleason (1971). The front and back portions of the GC peak were collected in tared tubes, analyzed, and weighted averages of the individual δ values were used to calculate the experimentally determined δ value for the entire sample. This was done on a natural vanillin and a mixture of a purified natural and a purified synthetic (see Table I).

Because of this fractionation, and the presence of closely eluting minor constituents found in the natural isolates, it was necessary to determine the relationship of the δ (δ_{PDB} ^{13}C) value to the portion of the peak collected. These interfering peaks could then be eliminated by collecting further up the gas chromatographic peak as long as the loss in vanillin had little effect on the final δ (δ_{PDB} ^{13}C) value.

A commercial synthetic vanillin void of any interfering components was totally collected (i.e., from base line to base line) and from a position which would be convenient for routine collections (see Figure 1, point A to A′). The analyses on these two collections compared favorably with analysis on the recrystalized solid (see Table II). A natural isolate was collected three times starting and ending at horizontal lines at successively greater distances from the base line (see Figure 1, point A to A′, B to B′, and C to C′) and these were analyzed. From these δ values and analysis on the vanillin isolated from this same extract but purified by preparative thin-layer chromatography, followed by crystalization, it was found that if greater than 80% of the peak height was collected, consistency was maintained (see Table II). Normally greater than 90% of the peak height was isolated without interference from minor constituents.

Table III. Confirmation of Reproducibility

replicate analysis on one isolate over one month		duplicate analysis on different isolates of extracts		
			extract	
δ_{PDB} ^{13}C			A	B
	−20.7	isolate 1	−20.4	−20.8
	−20.6	isolate 2	−20.2	−20.6
	−20.2			
	−20.3			
mean	−20.4			

Table IV. Vanillin Carbon SIRA

vanilla extracts				
Madagascar	(fold)	Javan	Mexican	Tahitian
−20.2	(1×)	−19.2	−20.3	−16.7
−20.2	(1×)	−18.2	−20.2	−17.1
−20.7	(1×)	−18.7	−20.4	−16.7
−20.2	(3.33×)	−18.6	−20.3[a]	−16.8[a]
−20.2	(3.33×)	−18.3		
−20.7	(3.33×)	−19.0		
−20.5	(3.33×)	−18.7[a]		
−20.3	(3.33×)			
−20.2	(10×)			
−20.4	(20×)			
−20.4[a]				
0.2 SD				

synthetic vanillins		
lignin	clove oil eugenol	guaiacol
−26.8	−29.9	−36.2
−27.0	−29.9	−29.0
−27.0	−31.7	−32.8
−26.9	−31.5	−32.7[a]
−27.3	−30.8[a]	
−27.1		
−26.9		
−27.4		
−27.0[a]		
0.2 SD		

[a] Mean value.

The consistency and reproducibility of the gas chromatographic isolation was demonstrated by four replicative analyses on one isolate over a 1-month period and also duplicate analyses on two extracts isolating the vanillin separately for each determination (see Table III).

A majority of the analyses were done on extracts of Madagascar beans of various folds since they are the higher quality and represent a large portion of the imported beans. Because the Javan beans are increasing in importance and quality, a significant selection of these were analyzed. Although Comoro is the next most significant source of vanilla beans (12% of the total U.S. import), samples of the past crop were unavailable at the time this research was performed, but attempts are being made to acquire samples of the new crop for analysis. However, earlier work by Bricout et al. (1974) and Bricout and Koziet (1975) had shown that these Comoron extracts fell within the same range as the Madagascar and waiting would unnecessarily delay reporting this data. Tahitian and Mexican vanilla extracts were also analyzed, the former since it is a different species (*Vanilla tahitensis* Moore vs. *Vanilla planifolia* Andrews) and the latter since it was once the main source of vanilla and is the origin of all bourbon beans (Madagascar, Java, and Comoro). The synthetic samples were obtained from various producers and suppliers of vanillin. To cover other minor and future sources of synthetic vanillin, it was synthesized from both eugenol (clove oil) and guaiacol (a coal tar distillate). While

Table V. Analysis of Mixture of Known Composition of Purified Natural and Purified Synthetic Vanillins of Known δ_{PDB} ^{13}C

	δ_{PDB} ^{13}C	% composition in mixture	weighted average
natural	−20.6	33.6	−6.9
synthetic	−26.8	66.4	−17.8
		calcd	−24.7
		found	−24.6

other syntheses of vanillin are possible, the cost would not be competitive at this time.

From this data (see Table IV), it is clear that an isolated vanillin sample more negative than δ_{PDB} ^{13}C −21.0 would be considered to contain vanillin from a source other than vanilla beans. Since the mean for Madagascar vanillin is δ_{PDB} ^{13}C −20.4 (standard deviation, 0.2), this can be stated with a confidence of 99.9%. The remainder of the naturals are the same as or less negative than the Madagascar vanillin; Mexican mean δ_{PDB} ^{13}C −20.3, Javan mean δ_{PDB} ^{13}C −18.7, and Tahitian mean δ_{PDB} ^{13}C −16.8. The commercially available synthetics are found to have a mean δ_{PDB} ^{13}C −27.0. The vanillins synthesized from clove oil eugenol and guaiacol were found to be more negative than this at means of δ_{PDB} ^{13}C −30.8 and −32.7, respectively.

The application of this technique was confirmed by mixing isolated and purified crystals of natural Madagascar vanillin of known δ_{PDB} ^{13}C with synthetic vanillin of known δ_{PDB} ^{13}C and experimentally obtaining a value which can be predicted by calculation (see Table V). Thus, this ratio is an intrinsic property dependent on the vanillin composition. Furthermore, evaluation of an altered extract fell in the correct range for predicting sophistication. Vanillin has also been extracted from a liquor and the addition of synthetic vanillin confirmed, indicating the techniques' application to vanilla products.

In addition, geographic origin of the beans or extracts can be determined at the 97% confidence level, differentiating between natural Tahitian, Javan, and Mexican–Madagascar.

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Nonacidic Constituents of Volatiles from Cooked Mutton

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The nonacidic volatiles from cooking mutton have been analyzed by gas chromatography–mass spectrometry, both as ether extracts and by adsorption onto porous polymer traps. Of the 93 compounds identified (some tentatively), 56 have not previously been reported in volatiles from cooked ovine tissues and 15, including the new compound 3,6-dimethyl-1,2,4,5-tetrathiane, have not been previously identified in cooked meats.

Sheep meats find low consumer acceptance in many countries. This has been attributed to the distinctive cooking odor and to the flavor of the cooked meat (Batcher et al., 1969; Weidenhamer et al., 1969; Wasserman and Talley, 1968). Previous studies in this laboratory established the contribution of 4-methyloctanoic acid to mutton flavor (Wong et al., 1975b) and examined the novel constituents of the acidic portion of the steam distillate from cooking mutton (Wong et al., 1975a). It was also suggested that additional contributors to mutton odor are present in the nonacidic fraction of the distillate.

Recently reports have appeared concerning the basic (Buttery et al., 1977) and neutral (Caporaso et al., 1977) portions of the volatiles from cooked ovine adipose tissue. This paper reports the results of our studies of the nonacidic volatiles from cooking mutton mince.

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EXPERIMENTAL SECTION

Details of materials, combined gas chromatography–mass spectrometry, and sensory evaluation procedures have been described (Wong et al., 1975a). Gas chromatography was carried out using a Hewlett Packard 7620A gas chromatograph (all glass system). After cooking and distillation, components of extracts were separated on 2.5 m × 2 mm i.d. glass columns of 10% (w/w) stabilized polyethylene glycol adipate (EGA, Analabs Ltd.) on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories) and 10% (w/w) methyl silicone OV-101 (Applied Science Laboratories) on the same support. The more volatile contents of the porous polymer traps were separated on a 2.5 m × 3.2 mm o.d. stainless steel column of Tenax (Applied Science Laboratories). In addition, an

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