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## Detection of Fraudulent Vanillin Labeled with $^{13}\text{C}$ in the Carbonyl Carbon

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Vanillin is oxidized with sodium chlorite to vanillic acid, which is then decarboxylated with bromine. The  $\text{CO}_2$ , derived from the carbonyl carbon, is analyzed mass spectrometrically to determine  $^{13}\text{C}/^{12}\text{C}$ . Vanillin from *Vanilla planifolia* gives carbonyl values near to  $-25\%$ ; vanillin from lignin yields carbonyl values near to  $-35\%$ . Lignin vanillin, labeled with  $^{13}\text{C}$  from [carbonyl- $^{13}\text{C}$ ]-vanillin to resemble that from authentic vanilla, gives carbonyl values near  $+20\%$ . The implications for vanilla quality control are discussed.

### INTRODUCTION

Adulteration of vanilla extracts with synthetic, lignin-derived vanillin is a major problem in the commercial market for this product. Low vanillin content Indonesian extracts can be made to simulate premium Bourbon extracts by addition of synthetic vanillin. Adulteration of this type can often frustrate the conventional quality assurance tests of lead number, organic acid profile, and vanillin/potassium ratio.

This type of adulteration may be readily detected by carbon Stable Isotope Ratio Analysis (SIRA) (Hoffman and Salb, 1979). It was found that the  $^{13}\text{C}/^{12}\text{C}$  ratio of the carbon in vanillin from vanilla differed from that in synthetic vanillin derived from lignin and other sources (Table I). SIRA of vanillin was found to readily distinguish natural from synthetic vanillin and has since become a widely used test for vanilla quality assurance.

Recently, a means of circumventing SIRA of vanillin was discovered. Since natural vanillin contains slightly more  $^{13}\text{C}$  than does lignin vanillin, addition of  $^{13}\text{C}$ -enriched vanillin to lignin vanillin can make the lignin vanillin appear natural to routine SIRA.

The simplest and cheapest means of evading the SIRA test in this manner involves the use of [methyl- $^{13}\text{C}$ ]-vanillin as the source of excess  $^{13}\text{C}$ . Two new tests have been developed to detect the addition of [methyl- $^{13}\text{C}$ ]-vanillin (Bricout et al., 1981; Krueger and Krueger, 1983) (Figure 1, Table II). In one, the methyl carbon of vanillin is

Table I. Carbon SIRA of Vanillin from Various Sources

source of vanillin	$\delta^{13}\text{C}_{\text{total}}$
vanilla (Madagascar)	-20.4
vanilla (Java)	-18.7
lignin	-27.0
eugenol (clove oil)	-30.8
guaiaicol	-32.7

Table II. Analysis of [methyl- $^{13}\text{C}$ ]-Vanillin

sample	$\delta^{13}\text{C}_{\text{total}}$	$\delta^{13}\text{C}_{\text{DHB}}$	$\delta^{13}\text{C}_{\text{methyl}}$
lignin vanillin	-27.0	-26.7	
altered lignin vanillin	-20.0	-26.0	
lignin vanillin	-27.3		-28.4
altered lignin vanillin	-20.6		+25.8

Table III. Methyl Analysis of Boston Retail Vanilla Extracts

sample	$\delta^{13}\text{C}_{\text{total}}$	$\delta^{13}\text{C}_{\text{methyl}}$
1	-23.3	+8.1
2	-20.6	+13.6
3	-20.9	+16.8
4	-22.9	-22.7
5	-23.5	-26.7
6	-20.7	+17.4
7	-21.5	-12.8
8	-20.6	+10.3
9	-27.3	-23.2
10	-21.5	-25.4
11	-20.4	-24.7

removed with  $\text{BBr}_2(\text{CH}_3)_2\text{S}$ , and SIRA is performed on the resulting dihydroxybenzaldehyde.  $^{13}\text{C}$ -Altered lignin gives typical lignin results by this method. In the other, the methyl carbon is removed as  $\text{CH}_3\text{I}$  in refluxing  $\text{HI}$ .

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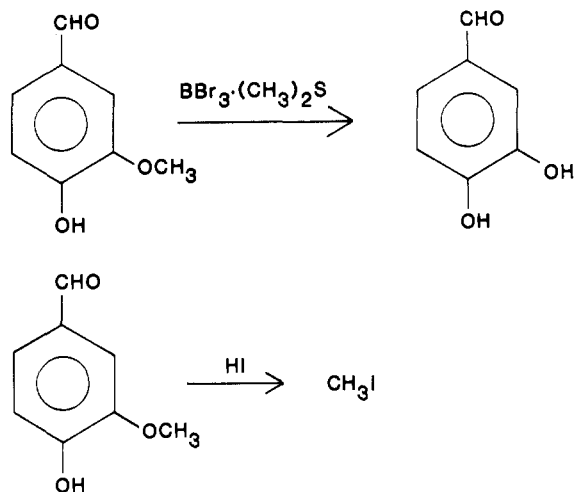


Figure 1.

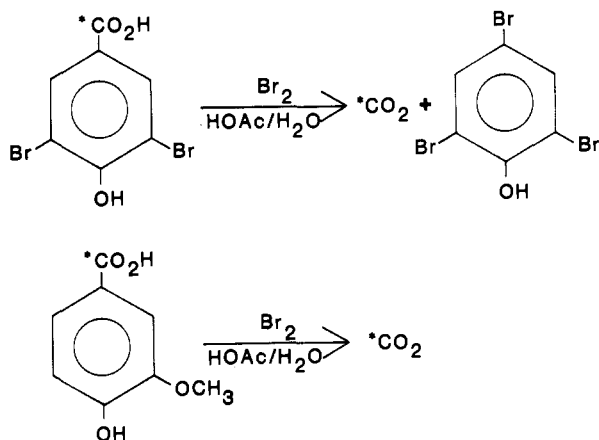


Figure 2.

SIRA is performed on the  $CH_3I$ .  $^{13}C$ -Altered lignin gives values approximately 50 ‰ positive than those from natural vanillin. While the latter is more convenient and more precise, both can unambiguously determine adulteration of this type. Recent work has shown that this means of evading SIRA of vanillin has become widespread (Table III) (Krueger, 1983).

In principle, there are seven other molecular positions in which a  $^{13}C$  label could be placed in the vanillin molecule, none of which will be efficiently detected by the above methods. There is concern that an economically feasible means of producing one of these, [*carbonyl- $^{13}C$* ]-vanillin, may be found, thus further confusing the interpretation of SIRA on vanillin. To alleviate this concern, a method for the detection of [*carbonyl- $^{13}C$* ]-vanillin addition to vanillin is desirable.

An examination of the isotope literature revealed a procedure for the quantitative decarboxylation of 3,5-dibromo-4-hydroxybenzoic acid with bromine (Figure 2), and the subsequent SIRA of the evolved  $CO_2$  (Grovenstein and Henderson, 1956; Grovenstein and Ropp, 1956). It was hoped that an analogous reaction of vanillic acid might prove the key to SIRA of the carbonyl carbon of vanillin. A method for detecting [*carbonyl- $^{13}C$* ]-vanillin employing this chemistry is described below.

#### EXPERIMENTAL SECTION

**Samples.** Lignin vanillin and six natural Bourbon vanilla extracts were supplied by McCormick & Co., Inc. Four vanilla extracts were obtained from wholesale commercial suppliers. [*carbonyl- $^{13}C$* ]-Vanillin was obtained from Cambridge Isotope Laboratories, Inc. The 90 atom % material was mixed with an appropriate amount of lignin

vanillin, dissolved completely in ethyl ether, and evaporated to ensure homogeneous mixing.

**Isolation of Vanillin.** The following procedure was suggested by Dr. Warren Wong of Givaudan Corporation. A 100-mL portion of 1-fold extract (or an equivalent amount of multi-fold extract diluted to 100 ml with 35% alcohol) is placed in a 400-mL beaker. Methylene chloride (200 mL) is added and the solution is stirred magnetically overnight. (It was found that extraction in a separatory funnel yielded intractable emulsions.) The aqueous layer is then decanted and discarded. The methylene chloride layer is dried over  $Na_2SO_4$  and evaporated in vacuo to dryness. The residue in the flask is extracted with 50 mL of boiling pentane, and vanillin crystallizes from the pentane on cooling in an ice bath. The crystalline vanillin is vacuum filtered, washed with 10 mL of cold pentane, and air-dried overnight.

**SIRA.** Carbon SIRA are performed on  $CO_2$  with an isotope ratio mass spectrometer in the usual manner (AOAC, 1980). Results are reported relative to the PDB standard according to the formula:

$$\delta^{13}C_{\text{sample}} = \left[ \frac{(^{13}C/^{12}C)_{\text{sample}}}{(^{13}C/^{12}C)_{\text{PDB}}} - 1 \right] 1000$$

**Preparation of  $CO_2$  from Vanillin and Vanillic Acid.** The sample (5 mg) is combusted with oxygen over  $CuO$  at 850 °C in the usual manner (AOAC, 1980).

**Preparation of  $CO_2$  from the Carbonyl Position of Vanillic Acid.** Vanillic acid (0.2 mM) is placed in one arm of a two-armed vacuum flask, and 5 mL of a solution of 1% bromine in 50% acetic acid is placed in the other arm. The flask is sealed and the bromine solution is rapidly frozen in crushed dry ice. The flask is then evacuated and placed in a 50 °C water bath. When the bromine solution has melted, it is mixed with the vanillic acid until the latter has dissolved. The flask is replaced in the 50 °C bath for 1 h. It is then opened to vacuum, and the contents are passed through a dry ice/acetone trap and a pentane ice trap and are frozen in a liquid nitrogen trap. The liquid nitrogen trap is then isolated, and the  $CO_2$  is distilled into a sample flask for SIRA.

**Preparation of Vanillic Acid.** Vanillin (1 mM) and 2 mM sulfamic acid are dissolved with stirring in 20 mL of  $H_2O$ .  $NaClO_2$  (1.5 mM, 80%) is added, and the reaction is stirred for 1 h, during which time crystalline vanillic acid precipitates. The suspension is then centrifuged, the supernatant is decanted, and the vanillic acid is washed with 2 mL of 0 °C water. The suspension is again centrifuged, the supernatant is decanted, and the vanillic acid is dried at 70 °C overnight. The consumption of vanillin is monitored by GC: 6 ft  $\times$  1/4 in. 5% Carbowax 20M on Chromasorb WAW column at 230 °C.

**Preparation of  $CO_2$  from the Carbonyl Position of Vanillin.** Vanillin (10.0 mg), 12.8 mg of sulfamic acid, and 11.1 mg of 80%  $NaClO_2$  are added to one arm of a two-armed vacuum flask. To these is added 5 mL of  $H_2O$ . The mixture is agitated on a vortex mixer until the vanillin is completely dissolved. The mixture is allowed to react for 1 h, at which time 5 mL of 1% bromine in 50% acetic acid are added to the other arm. The flask is sealed and the solutions are rapidly frozen in crushed dry ice. The flask is evacuated and placed in a 50 °C bath. When the solutions have melted, they are thoroughly mixed and replaced in the 50 °C bath for 1 h. The flask is then opened to vacuum, and the contents are passed through a dry ice/acetone trap and a pentane ice trap and then frozen in a liquid  $N_2$  trap. The liquid  $N_2$  trap is isolated and the  $CO_2$

Table IV. Vanillin Carbonyl SIRA

sample	$\delta^{13}\text{C}_{\text{total}}$	$\delta^{13}\text{C}_{\text{carbonyl}}$
Bourbon	-21.4	-25.7
lignin	-27.22 $\pm$ 0.02 (8)	-37.3
altered lignin	-19.94 $\pm$ 0.03 (10)	+17.1

is frozen into a sample flask for SIRA.

**CO<sub>2</sub> Yield Measurement.** All CO<sub>2</sub> yields are measured by condensing the sample in a manometer and recording the mercury displacement on warming to room temperature. The manometer is calibrated with the CO<sub>2</sub> generated by 1 N HCl decomposition of weighted amounts of CaCO<sub>3</sub>.

## RESULTS AND DISCUSSION

The goal of this study was to develop a method for removing the carbonyl carbon of vanillin in the form of CO<sub>2</sub> for SIRA. This had to be done with negligible isotopic fractionation, or at least with a consistent fractionation. The reported decarboxylation of 3,5-dibromo-4-hydroxybenzoic acid with bromine appeared to be a good model reaction to try, since vanillic acid, a simple oxidation product of vanillin, is functionally very similar to the former compound.

We reacted vanillic acid with 1% bromine in 50% acetic acid and obtained CO<sub>2</sub> in 105% of the theoretical yield. Blank runs yielded a CO<sub>2</sub> reagent blank of about 3–5%. Thus the reaction appeared to proceed quantitatively. The blank had a  $\delta^{13}\text{C}$  value of -30.8 ‰, so a small correction could easily be made on the vanillic acid carbonyl  $\delta^{13}\text{C}$  value. Since the reaction is quantitative, we assume no isotopic fractionation of the generated CO<sub>2</sub>.

Since the decarboxylation proceeded smoothly, we needed only to develop an efficient means of oxidizing vanillin to vanillic acid. This proved more troublesome. Our first choice of silver oxide proved difficult to scale down to the small sample sizes ( $\leq$ 50 mg) that we wanted to use. After a number of unsuccessful attempts with other oxidants, we settled upon sodium chlorite. This was reported to oxidize vanillin to vanillic acid in good yield under simple conditions. It further seemed suited to scaling down, and possibly suited to a one-pot decarboxylation of vanillin.

We reacted vanillin with NaClO<sub>2</sub> and sulfamic acid (as a Cl<sub>2</sub> scavenger) in water and observed results similar to those reported (Lindgren and Nilsson, 1973). Vanillic acid was recovered in 50–60% yield in a 50-mg scale reaction. SIRA of the product vanillic acid (-27.07  $\pm$  0.02) and of the starting vanillin (-27.22  $\pm$  0.02) showed minimal fractionation. If all of the difference is attributed to fractionation of the carbonyl carbon, then that fractionation is +1.2  $\pm$  0.2 ‰ (0.15 ‰ change from  $\delta^{13}\text{C}_{\text{total}}$  times 8 carbon atoms per vanillin molecule). Gas chromatographic monitoring of the reaction showed quantitative consumption of vanillin. Only 2–3% of CO<sub>2</sub> was obtained from the reaction, suggesting that overoxidation of the carbonyl carbon was negligible.

With both steps of the decarboxylation procedure in place, we ran decarboxylations of a known natural Bourbon vanillin, a known wood pulp lignin vanillin, and the same lignin vanillin which had added to it sufficient [*carbonyl*-<sup>13</sup>C]-vanillin to change the SIRA results from -27.22 ‰ to -19.94 ‰. The results are listed in Table IV.

The carbonyl carbon of natural vanillin yielded a SIRA value of -25.7, near the value of the total carbon SIRA. The lignin sample yielded a carbonyl SIRA value of -37.3,

Table V. Vanillin Carbonyl SIRA on Various Vanilla Extracts

sample	$\delta^{13}\text{C}_{\text{carbonyl}}$	remarks
lignin	-37.7 $\pm$ 1.4	known lignin vanillin
altered lignin	+22.0 $\pm$ 0.6	lignin vanillin with added [ <i>carbonyl</i> - <sup>13</sup> C]-vanillin
Bourbon	-27.7	known natural vanillin
Bourbon	-23.2	known natural vanillin
Bourbon	-29.9	known natural vanillin
Bourbon	-24.7	known natural vanillin
Bourbon	-24.8	known natural vanillin
commercial	-42.8	$\delta^{13}\text{C}_{\text{methyl}} = +16.0$ ; adulterated methyl- <sup>13</sup> C
commercial	-24.5	probably natural
commercial	-43.1	probably adulterated methyl- <sup>13</sup> C
commercial	+67.1	$\delta^{13}\text{C}_{\text{total}} = -15.6$ adulterated carbonyl- <sup>13</sup> C

again similar to, but somewhat more negative than, the total carbon SIRA. The carbonyl altered lignin vanillin, however, yielded a carbonyl value of +17.1, a value much more positive than the total carbon SIRA. This result both confirms that the carbonyl carbon of vanillic acid is in fact the carbon that is removed by bromine and established that labeling of lignin vanillin with [*carbonyl*-<sup>13</sup>C]-vanillin can be detected.

A mass balance equation (eq 1) shows that the observed result for the altered lignin carbonyl SIRA is close to the predicted value based on the amount of <sup>13</sup>C added.

$$\delta^{13}\text{C}_{\text{carbonyl altered lignin}} = \delta^{13}\text{C}_{\text{carbonyl lignin}} + 8(\delta^{13}\text{C}_{\text{total altered lignin}} - \delta^{13}\text{C}_{\text{total lignin}})$$

$$\delta^{13}\text{C}_{\text{carbonyl altered lignin}} = +21.1$$

We next tried to develop a one-pot decarboxylation procedure for work on a very small scale ( $\sim$ 10 mg). We performed the NaClO<sub>2</sub> oxidation of vanillin in one arm of a two-armed flask, then added the bromine reagent directly to the reaction mixture. The reaction proceeded only to about 70% of theoretical, and the small scale of the reaction resulted in a blank of about 30% of the total CO<sub>2</sub>. The results (corrected for 30% blank of -30.8 ‰) are presented in Table V. While the precision and accuracy are diminished somewhat, particularly at the positive end of the scale, this much simpler procedure will also reliably detect [*carbonyl*-<sup>13</sup>C]-vanillin additions. Also included in Table V are results for several known natural vanilla extracts and commercial formulations.

**Registry No.** Vanillin, 121-33-5; lignin, 9005-53-2; carbon-12, 7440-44-0; carbon-13, 14762-74-4.

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