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Oxidation of Vanillin to Vanillic Acid

Significance to Determination of Vanillin

D. T. ENGLIS AND MERLE MANCHESTER¹

University of Illinois, Urbana, Ill.

By means of ultraviolet absorption studies, it is shown that a dilute solution of vanillin in air is slowly oxidized to vanillic acid. Ethyl vanillin shows the same type of change. This change is responsible for low results when either of the vanillins is determined by a direct ultraviolet examination. Confirmation is also given for the high results by the official Folin-Denis colorimetric method following

IN 1944 Englis and Hanahan (3) described a method for de-termining vanillin (4-hydroxy-3-methoxybenzaldehyde) in flavoring extracts, which was based upon the absorption characteristics of vanillin in the ultraviolet region. Since that time it has been found that samples which have been allowed to stand for some time yield low results by this method. Under similar conditions, the official colorimetric method (4) based upon the reduction of the Folin-Denis phosphomolybdate-phosphotungstate reagent gives high results. These high values have been attributed to the formation of 4-hydroxy-3-methoxybenzoic acid (vanillic acid), which is reported to have a greater reducing power for the reagent than the corresponding aldehyde, vanillin. Therefore, it seemed important to study the effect of standing and exposure to air, to determine the nature and rate of change which is characteristic of vanillin, and to learn whether coumarin would undergo any adverse change under the same conditions.

There has long been a discussion as to the reaction that takes place when a dilute solution of vanillin is allowed to stand. Tiemann (?) in 1875 stated that he had obtained traces of vanillic acid by subjecting the vanillin to the action of moist air for a long period of time. Hubbard (5) said that it was likely that the vanillin was slowly oxidized to pyrocatechuic acid. Ciamician and Silber (1) obtained vanillic acid by exposing vanillin to sunlight in the presence of nitrobenzene. Pearl (θ) vanillin to sunlight in the presence of nitrobenzene. worked out a direct method for the preparation of vanillic acid from vanillin based on the classical oxidation of aldehyde by means of silver oxide. He found that with an excess of alkali and 0.5 mole of silver oxide with 1.0 mole of vanillin the reaction gave quantitative results.

In the colorimetric procedures (4, 5) for the quantitative de-termination of vanillin Hubbard (5) and Curl and Nelson (2) noticed that the standard vanillin solution tended to increase in apparent strength on standing in a partially filled bottle. Curl and Nelson (2) believed that this was due to the oxidation of vanillin, as a full bottle of standard solution kept in the refrigerator checked with a freshly prepared solution. A partially filled bottle kept several months at room temperature gave a color increase of 19% over a freshly prepared standard solution. They also compared the color formed by standard solutions of vanillin

¹ Present address, Firestone Tire & Rubber Co., Pottstown, Pa.

atmospheric oxidation. Fairly complete oxidation of the vanillin compounds by air requires several weeks for the very dilute aqueous solution at room temperature. Neither coumarin nor vanillic acid shows appreciable change under the same conditions. Prompt examination is necessary to avoid serious errors in the quantitative estimation of vanillin in flavoring extracts.

and vanillic acid. Vanillic acid gave about 50% more color than did the vanillin solution of the same concentration. The colorimetric results obtained were higher than those from the gravimetric analysis. A colorimetric determination made on the residue from the extraction of the vanillin in the gravimetric method showed the substance was definitely not vanillin.

The official colorimetric method (4) for the determination of vanillin has been modified slightly by Wilson (8) in regard to the quantity of the sodium carbonate reagent and the instrument employed to measure the color value.

The ultraviolet method (3) was designed for quantitative determination of vanillin and coumarin when present together and depends upon their differences in chemical structure. Vanillin has a maximum absorption where coumarin shows only slight absorption. This greatest difference occurs at 2313 Å., whereas at 2875 Å, the extinction value for equal weights of either constituent is the same. Thus, by determining the extinction value at 2875 Å, the total concentration for the mixture of the two may be determined, and the amount of each individual constituent may be calculated by a simple equation from the extinction value at 2313 Å. When the measurements are made immediately after separation of the constituents or dilution of the sample, the results are good; if the solutions are allowed to stand, there is a marked reduction in the absorption at 2313 Å., leading to low results for apparent vanillin.

EXPERIMENTAL

A Beckman Model D.U. spectrophotometer Equipment. employing 1-cm. cells was used in the absorption studies.

Procedure. Solutions of vanillin, coumarin, and vanillic acid were prepared in concentrations of 10 mg, per liter of water containing about 10% of ethyl alcohol. A mixture containing 2.5 mg, per liter each of vanillin and coumarin was prepared by taking 250 ml. of each of the primary solutions and diluting to 1 liter. The solutions were sampled after 5 months and the absorption of each was determined with the spectrophotometer.

A marked change took place in the vanillin curve as a result of allowing the solution to stand. There was a pronounced decrease



Figure 1. Ultraviolet Absorption Curves







Figure 2. Changes in Ultraviolet Absorption Spectrum of Vanillin on Standing

1.	l week
II.	2 weeks
III-	3 weeks
IV.	4 weeks

in the peaks for vanillin near 2300 and 2800 Å. (curve I, Figure 1) for the freshly prepared solution when compared to curve II for the solution allowed to stand. Such a change causes a tremendous error in the apparent vanillin by the ultraviolet method. In curve II a peak has developed near 2540 Å. and its whole nature conforms closely to that shown for vanillic acid (curve III).

The vanillic acid alone and solution of coumarin showed no measurable change upon standing. The absorption curve for the latter is available in a previous article (3). The change in absorption characteristics of the vanillin-coumarin mixture upon standing appeared to be solely due to a change in the vanillin.

Inasmuch as 10 mg. of vanillin should yield about 11 mg. of vanillic acid, one would anticipate the peak at 2540 in curve II of the exposed vanillin to exceed that of curve III. Because it is slightly lower one must conclude that the oxidation of the vanillin was not complete even after 5 months.

Stronger solutions of vanillin in 95% alcohol appeared to change less rapidly than did the dilute aqueous solution. In regular extracts the solvents and extraneous materials present may exert some protective action.

The vanillin solution of Figure 1 had been allowed to stand in a container which had a considerable air space above the surface of the solution and was opened frequently to the air.

In order to determine whether the conditions had accelerated the oxidation, a second solution containing 10 mg. of vanillin per liter was prepared. No special treatment was given the distilled water to expel dissolved oxygen. The absorption data were determined immediately on the fresh solution; then The absorption part of the remaining solution was placed in glass-stoppered bottles which were filled so that there was no air space remaining above the solution, then sealed with paraffin wax. These solutions were allowed to stand at room temperature in the laboratory. Once a week, one of the solutions was examined with the spectrophotometer.

There was a marked change in the shape of the curve over a period of a few weeks (Figure 2). At the end of a month (curve IV) the general characteristics of the vanillic acid curve are evident. It is apparent that the additional precautions to exclude oxygen, after the solutions were once prepared, had little benefit for this concentration of vanillin.

For the oxidation of vanillin to vanillic acid, 152 parts of the former would require 16 parts of oxygen. Assuming the water used in the preparation of the solutions was about half-saturated with oxygen and contained about 4 parts per million, this would be sufficient for the oxidation of 38 mg. per liter of vanillin-a quantity much greater than the 10 mg. actually present.

In ordinary flavoring extracts the concentration of vanillin is about 2000 mg. per liter. Although a certain amount of oxidation may take place in the stored extract, it is not likely under ordinary conditions to be a significant amount in proportion to the total vanillin present. However, when a tremendous dilution has been made for the purpose of the colorimetric determinations and the solutions are allowed to stand, the effect upon the analytical results may be very serious.

Study of Ethyl Vanillin. Ethyl vanillin (ethyl protocatechuic aldehyde) has a higher flavoring ability than regular vanillin. It has come into wide use as a constituent of imitation vanilla extracts and sometimes as an adulterant of extracts represented to be genuine. Therefore, it was desirable to subject this malerial to the same type of study as had been accorded the vaniltin. A solution containing 10 mg. of ethyl vanillin in approximately 10% ethyl alcohol was prepared and its absorption curve evaluated. Curve I, Figure 3, is very similar to the vanil-





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lin curve. Substitution of the ethoxy for the methoxy group produces little change in the absorption. The effect of standing for 5 months (curve II) is of the same nature as for vanillin, There is a conversion to the corresponding acid. The extent of conversion appears to be a little less than that for vanillin in the same period of time. It appears safe to conclude that errors in its estimation would be affected to about the same extent.

COLORIMETRIC ANALYSIS WITH FOLIN-DENIS REAGENT

While the work on the ultraviolet method was in progress, it seemed advantageous to include experiments in which the official method, as modified by Wilson (8), was utilized.

The procedure followed was essentially as Wilson has outlined, except that the solutions were examined at the specified wave lengths (6100 and 6500 Å.) with the Beckman instrument in 1-cm. cells in place of the wedge photometer.

The solutions were prepared in concentrations such that 10 ml. at the stage of analysis contained the quantity of material shown in Table I. To each portion analyzed, 5 ml. of the Folin-Denis reagent and 10 ml. of 20% sodium carbonate were added. The solutions were thoroughly mixed, allowed to stand 10 to 15 minutes, and made up to 100 ml. Then the color values were read.

The data of Table I supplement the findings of Curl and Nelson (2). Vanillic acid has a much stronger reducing action than freshly prepared vanillin, and the samples of vanillin and ethyl vanillin which have been allowed to stand have greatly increased color values, approaching those of the vanillic acid.

If one plots the E values against concentration for the freshly prepared vanillin and vanillic acid (series A), Beer's law is observed up to a concentration of 1 mg. per 10 ml. of solution analyzed. Beyond this point, it is apparent that the concentration of the oxidant (Folin-Denis reagent) is not sufficient to give

Table I. Color Values with Folin-Denis Reagent

	Mg. per 10 Ml. Portion	E at 6100 Å.		<i>E</i> at 6500 Å.	
	of Solution Analyzed	Vanillin	Vanillic acid	Vanillin	Vanillic acid
Series A	0.2 0.4 0.6	$0.057 \\ 0.098 \\ \dots$	$\begin{array}{c} 0.147 \\ 0.264 \\ 0.353 \end{array}$	$0.062 \\ 0.105 \\ \dots$	$\begin{array}{c} 0.159 \\ 0.276 \\ 0.376 \end{array}$
Freshly prepared solutions	$1.0 \\ 1.4 \\ 2.0$	$\substack{0.213\\0.227\\0.248}$	0.574	$\begin{array}{c} 0.225 \\ 0.237 \\ 0.260 \end{array}$	0.593
			Ethyl vanillin		Ethyl vanilli n
Series B	0.2	0.114^a 0.117b 0.129^c	0.118	${0.122^a \atop 0.125^b \atop 0.137^c}$	0.122
Solutions allowed to stand several weeks	0.5	0.212° 0.247b 0.285°	0.247	0.219ª 0.254b 0.295°	0.257
4 b 6 Slightly	different times o	fstanding	and differ	ent solution	ns.

a linear response. It makes little difference which wave length is selected; both ranges give linear response if the quantity of material is in the specified range.

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Vitamin A in Mixed Feeds

Chromatographic Separation and Estimation

MAXWELL L. COOLEY, Larrowe Division, Rossford, Ohio, JAMES B. CHRISTIANSEN, Larro Research Farm, Detroit, Mich., AND RAY C. KOEHN, General Mills, Inc., Minneapolis, Minn.

A method for determining vitamin A in mixed feeds is improved by extracting the sample with a hot solvent and passing the extract through a chromatographic column which serves to remove plant pigments. The column is packed with equal parts of magnesia and diatomaceous earth. Vitamin A esters are eluted free from significant amounts of carotene and carotenoids. Carotene is, however, eluted together with the vitamin A alcohol from saponification mixtures.

N A previous publication, Cooley, Christiansen, and Schroeder (2) advocated the determination of vitamin A in mixed feeds by extraction, partial purification of the extract by chromatographic adsorption on sodium carbonate, and measurement of total antimony trichloride color. Vitamin A content was obtained by correcting total color for that produced by carotene also present in the eluate, following a separate determination of the latter pigment. Although this method has been used successfully several years, certain improvements have been developed.

When the sodium carbonate adsorbent is used, some pigments such as lycopene are difficult to separate, the application of the carotene correction is cumbersome and a source of error, and the sodium carbonate must be a particular type and usually must be dried and ground before use.

More recently, Brew and Scott (1) have proposed a somewhat different technique for vitamin A determination in mixed feeds, which also involves partial chromatographic purification of feed extracts and correction of the total antimony trichloride color for

that formed by nonvitamin substances present. This method has some of the same drawbacks as that of Cooley, Christiansen, and Schroeder.

Objectionable features of the earlier methods can be overcome by modification of the adsorption step in the determination. The activated magnesia-Hyflo Super Cel mixture suggested by Strain (6) and by Wall and Kelley (7) for chromatographic isolation of carotene is an effective agent in vitamin A determination and incidentally is more convenient to use than the special sodium carbonate recommended by Coolev et al.

The Waring Blendor originally suggested for extraction is effective, particularly when large samples of feeds are used. However, when it is employed with the magnesia-Super Cel mixture, an additional filtration is required. The hot solvent extraction technique suggested here has been found more convenient.

The proposed method affords quantitative separation a