A New Method for Quantitative Determination of BHA[1,2]

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A qualitative test developed by Laszlo has been adapted to the quantitative determination of BHA. The determination is based on measurement of the specific and sensitive color resulting from reaction of BHA with diazotized sulfanilic acid in alkaline solution. The red-purple color, which is stable for long periods, has an absorption maximum at 535 mμ. Beer's law is obeyed, and a concentration range of 0.0001% to 0.01% BHA may be measured. The method requires careful adjustment of antioxidant-reagent ratios for rapid and maximum color development. The optimum ratios, the effect of alcohol, and interfering substances are discussed.

The method, as described, also provides for an accurate specific determination of BHA in fats and oils even when other antioxidants are present.

CURRENT METHODS for qualitative and quantitative determination of antioxidants are based on absorption spectra or colorimetric methods. Many of these methods have limitations of nonspecificity, varying degrees of sensitivity and precision, and interference by other antioxidants.

Laszlo (2) has described a new color reaction for BHA (butylated hydroxyanisole is a mixture of 2,t-butyl-4-hydroxyanisole and 3,t-butyl-4-hydroxyanisole) and its application as a rapid qualitative test for BHA in oils and fats. The method, now widely used in Brazil, is sensitive, specific, and easy to perform and only requires readily available reagents such as sodium nitrite, sulfanilic acid, and sodium hydroxide.

The reagents used in the qualitative color test and in the quantitative method to be described are a 0.5% solution of $NaNO_2$ in distilled water and a 0.5% solution of sulfanilic acid in distilled water containing 5% concentrated hydrochloric acid. The nitrite solution should be freshly prepared every three weeks. The solutions are kept refrigerated and are mixed each working day in a ratio of 1:100 of nitrite to sulfanilic acid to preform the reagent (diazobenzenesulphonic acid) known as Ehrlich reagent. The qualitative test is performed in a test tube by shaking 1 ml. of melted fat or oil with 2 ml. of 72% ethanol. The emulsion formed is shaken with 1 ml. of Ehrlich reagent, then immediately with 1 ml. of N NaOH. The development of a red-purple color indicates the presence of BHA.

This report is concerned with the development of a quantitative method for determination of BHA, utilizing this color reaction.

Colors Formed with Antioxidants. The color with BHA was developed by reaction of commercial food-grade BHA with diazotized sulfanilic acid in alkaline solution. The absorption maximum for the colored product was at approximately 535 mμ, and it shifted slightly with concentration changes (Figure 1). The intensity was found to vary with concentration.

Since BHA is used commercially in combination with other antioxidants, the color reaction and absorption maximum formed by a number of these antioxidants with the reagent system was determined. Propyl gallate was found to give a yellow color, which faded rapidly and gave no measurable maximum in the visible range. Other gallates behaved similarly. Nordihydroguaiaretic acid yielded a wine-red color, which rapidly changed to brown and brown-yellow. BHT gave a distinct salmon-pink color with an absorption maximum near 505 mμ. Figure 2 shows the absorption curves for BHA, BHT (butylated hydroxytoluene), propyl gallate, and nordihydroguaiaretic acid. The color produced with BHT developed very slowly under the conditions mentioned above.

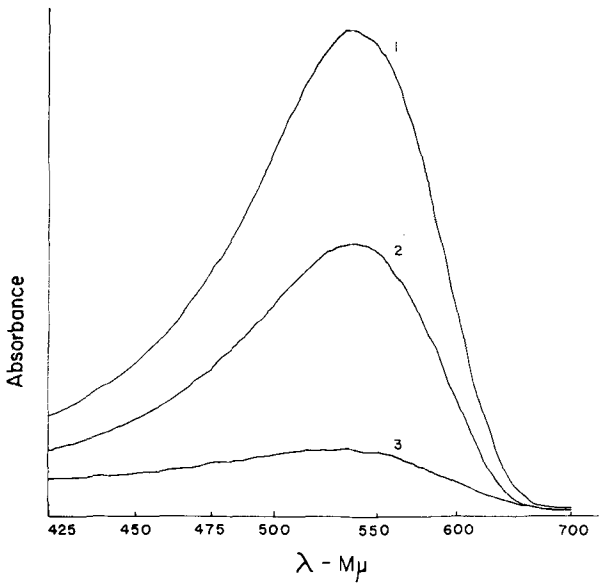

FIG. 1. Absorption curves from reaction of diazobenzenesulphonic acid with: 1, BHA 0.009%; 2, BHA 0.005%; 3, BHA 0.001%.

Influence of Temperature and Mixing Time of the Color-Forming Reagent on the Color Development. Since the color-forming reagent is a solution of diazotized sulfanilic acid and the formation and stability of diazo-compounds are heat-sensitive, the effect of temperature on the Ehrlich reagent was examined.

Tests were made by using different conditions of temperature before and after mixing the nitrite solution and sulfanilic acid solution as well as by allowing the preformed Ehrlich reagent to stand for different periods of time. These showed that cold, freshly-mixed reagents should be used for maximum color production and stability. It was also established that, when the Ehrlich reagent was prepared by mixing cold solutions and storing at refrigerator temperatures 8–10°C., it was necessary to prepare the reagent only once each working day.

Optimum Reagent Ratios. Phenols form colored products with the Ehrlich reagent in alkaline solution. However the rate of color development and its intensity will vary with the amount of sodium hydroxide used. Approximately 0.6 ml. N NaOH was

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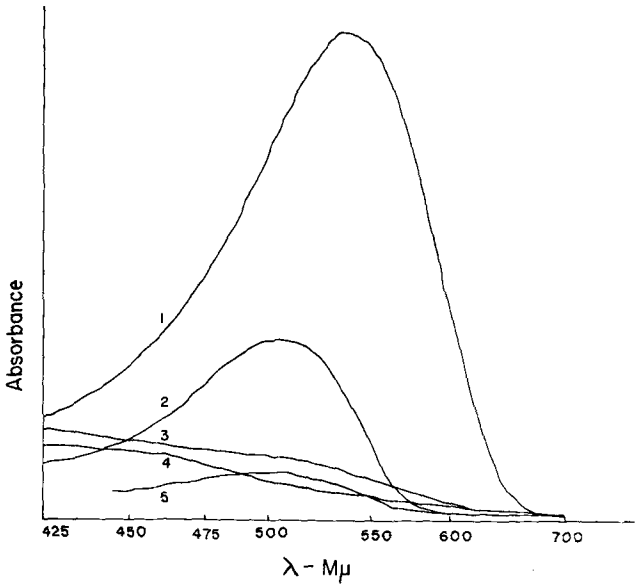

FIG. 2. Absorption curves from reaction of diazobenzenesulphonic acid with: 1, BHA 0.009%; 2, BHT 0.02% after 24 hrs.; 3, nordihydroguaiaretic acid 0.01% after 10 min.; 4, propyl gallate 0.01% after 15 min.; 5, BHT 0.01% after 10 min.

required for neutralization of 1 ml. of Ehrlich reagent (1:100). Colors produced by using a constant quantity of BHA in 72% ethanol, 1 ml. of Ehrlich reagent, and varying amounts of N NaOH in 10-ml. final volume were orange with 0.6 ml., purple-orange with 0.7 ml., and red-purple with 0.8 ml. or more of N NaOH. Excessive amounts of either the Ehrlich reagent or sodium hydroxide solution caused rapid fading of the color.

Conditions were established for immediate development of maximum color, which was stable for periods of longer than 30 min. This involved the use of 1 ml. of BHA solution in 72% ethanol with 2 ml. of Ehrlich reagent and 7 ml. of N NaOH to provide a final volume of 10 ml. The color so developed followed Beer's law.

A series of tests was run to determine the optimum ratio of sodium nitrite to sulfanilic acid in the Ehrlich reagent used. The ratio 1:100 was found to be critical since 0.5:100 gave less color and 1.5:100 gave color which started to fade more rapidly.

Procedure for Determination of BHA. A 1-ml. aliquot of a solution of BHA in 72% ethanol (quantity of BHA in the range from 0.01 to 0.1 mg.) was pipetted into a 50-ml. Erlenmeyer flask. To this were added 2 ml. of ice-cold Ehrlich reagent and 7 ml. of N NaOH immediately with shaking to mix well (local excesses of NaOH may produce light discoloration). The colored solution was read at 535 mμ in a spectrophotometer against a blank prepared in the same manner, using 1 ml. of 72% ethanol instead of the BHA solution.

All determinations were accomplished with a Beckman DU spectrophotometer, using 1-cm. cells with lids. Readings were made at 10-, 15-, and 20-min. intervals, after the addition of the color-forming agent, to confirm that maximum color development had been achieved in 10 min. and remained stable for at least 20 min.

Interference of Alcohol Concentration on Development of Color. When extraction of BHA from fat results in solutions lower in concentration than 0.001%, it is necessary to use larger aliquots of BHA solution and smaller volumes of more concentrated base in order to maintain the final volume at 10 ml. This can be accomplished by using 7 ml. of BHA solution and 1 ml. of 7 N NaOH. The amount of alcohol in the final solution affects the slope of the standard curve (Figure 3). Line A was obtained with 1 ml. of BHA solution and 7 ml. of N NaOH. Lines B, C, D, and E were obtained by using the 7 N NaOH and BHA solutions with an ethanol concentration of approximately 10%, 40%, 70%, and almost 100%, respectively. The differing slopes indicated that standard curves must be prepared in accordance with the alcohol concentration of the BHA solution to be used for the determination. If methanol is used, the slope of the standard curve will also vary with the concentration of methanol but will be different from the slope produced by an identical ethanol concentration.

Interference by Other Antioxidants. Although propyl gallate has no appreciable absorption at 535 mμ, it has not been possible to determine BHA in its presence since the color developed is less than for BHA alone. This is undoubtedly due to reaction with the Ehrlich reagent and base to upset the balance required for optimum color development. It is necessary to remove the propyl gallate by extraction with ammonium acetate solution, followed by a water-wash before the appropriate value for BHA concentration can be demonstrated.

BHT develops measurable color under the test conditions, but the rate of color development is quite slow. When measurements are made within 5 to 10 min. from the time of the addition of reagents, there is no appreciable effect on the BHA values even when the two antioxidants are present in equal quantities. For example, a mixture of 0.005% BHA and 0.005% BHT in alcohol showed 0.0052% BHA in 5 min. and 0.0053% BHA in 10 min. At 25 min. a content of 0.0056% BHA was indicated so that corrections may be required if longer color development times are

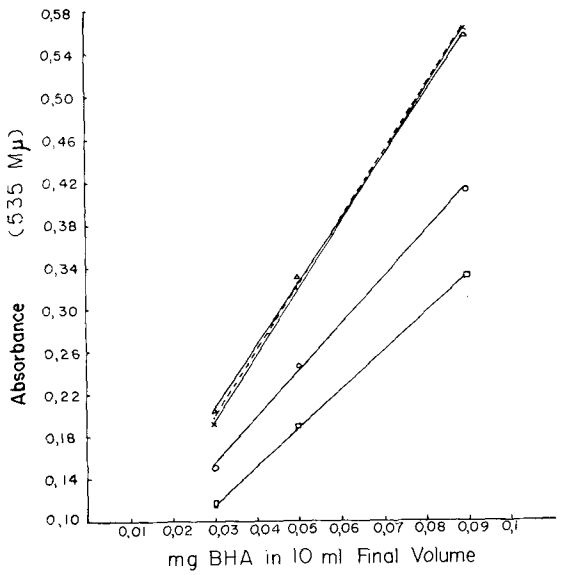

FIG. 3. Effect of varying concentration of ethanol on the slope of the standard curve: x–x, 10% ethanol (1 N NaOH) (A); – – –, 10% ethanol (7 N NaOH) (B); △–△, 40% ethanol (C); ○–○, 70% ethanol (D); □–□, almost 100% ethanol (E).

used. Corrections may also be necessary if the BHT/BHA ratio is high.

Extraction and Recovery of BHA from Fats. Solvent extraction was the preferred method for recovery of BHA. It permits the separation of BHA from BHT (if present) and allows a higher BHA concentration than other methods.

TABLE I
Recovery of BHA from Lard

Lot and sample No.	Weight of sample g.	Antioxidant added to 200 g. lard, mg.		BHA found in 100 g. lard, mg.
A_1	10	10.0	BHA	9.6
A_2	10	10.0	BHA	9.6
A_3	10	10.0	BHA	9.6
A_4	10	10.0	BHA	9.8
		3.0	PG [a]	
A_5	10	10.0	BHA	9.8
		3.0	PG	
B_1	10	10.0	BHA	10.1
B_2	10	10.0	BHA	10.1
B_3	15	10.0	BHA	10.7
C_1	10	5.0	BHA	4.6
C_2	15	5.0	BHA	5.0
C_3	15	5.0	BHA	5.0
D_1	15	10.0	BHA	9.7
		10.0	BHT	

[a] Propyl gallate.

Various difficulties involving turbidity, emulsion formation, or interference with maximum color development were encountered. These ruled out extraction with 72% ethanol from a solution of fat in cyclohexane (5), extraction with methanol from a chloroform solution of fat (1), or direct extraction with 72% ethanol stirred through fat with a Blendor.

Best results were obtained with the modification of the method of Mahon and Chapman (4), which involved extraction from a solution of a larger sample (15 g.) of lard dissolved in petroleum ether since this resulted in better recovery of BHA. When propyl gallate was present, it was removed prior to extraction of the BHA (3).

Freshly rendered lard with a peroxide value of 1.5 m.e./kg. was used for the recovery studies. The BHA was extracted immediately after incorporation because repeated warming and cooling of a fat containing BHA resulted in a significant lowering of BHA concentration in a very short time. Whether this is due to loss of antioxidant by volatilization or conversion to forms which no longer give the color reaction has not been determined.

Determination of BHA in Fats and Oils. Transfer 15 g. of melted fat or oil to a 500-ml. separatory funnel with the aid of 50 ml. of petroleum ether (b.p. 30–60°C.). Extract with three 25-ml. portions of 72% ethanol by continuously inverting the funnel for 3 min. Follow with a 1-min. extraction, using 60 ml. of 72% ethanol. Let the phases separate well between each extraction. Filter the combined extracts through 2 Whatman #54 filter papers and make up to a final volume of 150 ml. with 72% ethanol.

If propyl gallate is present, it should be extracted from the dissolved fat by the methods of Mahon and Chapman (3) prior to the extraction with ethanol.

Add 2 ml. of cold Ehrlich reagent (1:100) to 7 ml. of the extract and then, drop by drop, add 1 ml. of 7 N NaOH with constant shaking. Read the optical density after 10 min. in a spectrophotometer at 535 mμ, using a blank of 7 ml. 72% ethanol, 2 ml. of Ehrlich reagent, and 1 ml. of 7 N NaOH. (Blanks made of extracts of the same lard containing no BHA differ from the 72% ethanol blank only by the range of error of the method.)

Calculate the quantity of BHA in the fat in percentage from the following equation:

$$\% \text{ BHA in fat} = (1000/7)c$$

c = concentration of BHA in the 10-ml. final volume of the determination. Determine "c" from the standard curve.

Precision of the Method. The precision of the method has been checked by using different aliquots of a 72% ethanolic extract from fat containing BHA and making them up to 7 ml. with 72% ethanol prior to color development. The BHA concentration also was measured in 6 ml. of the extract plus 1 ml. of a solution of known concentration of BHA in 72% ethanol. The deviations ranged from 0.0001 to less than 0.0003% of the BHA in the fat or 1 to less than 3% of the antioxidant added (0.01%).

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Report of the Spectroscopy Committee, 1959-60

AT A MEETING held at the Roosevelt hotel, New Orleans, La., during the 50th Annual Meeting of the Society, April 20–22, 1959, the Spectroscopy Committee decided that collaborative tests to extend the scope of the infrared absorption method for isolated *trans* ethylenic bonds[1] to the analysis of long-chain fatty acids directly should be the next activity. It was also decided that efforts to make the secondary standards, required by users of this method, readily available should be expedited. Accordingly during the past year collaborative investigation of an analysis of fatty acids directly for their *trans* content, *i.e.*, without prior conversion to the methyl esters, has been undertaken. In addition, fatty acid methyl esters and triglycerides of high and low *trans* isomer content have been analyzed by the entire committee and established as secondary standards for the method as published (1).

A single meeting was held during the year, in connection with the Annual Meeting in Dallas, April 4–6, 1960.

[1] Hereinafter in this report referred to as "*trans* content" or "*trans*-isomers."