A Review of Antioxidant Analysis in Food Products

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Abstract

To permit proper usage of antioxidants by the food processor and permit control under applicable governmental regulations, it is necessary to have adequate analytical methods. Such methods should both positively identify and provide a quantitative assay for the additive. Due to the complex nature of foods, analysis of an antioxidant in the very small quantities used presents a very real problem. Solvent extraction and/or steam distillation techniques are employed to separate the antioxidants from the food. Ultraviolet spectra, gas chromatography and colorimetric techniques are used to identify and determine the quantity of each antioxidant present. Slight variations are necessary for each type of food.

Introduction

THE FOOD PROCESSOR is faced with the problem of using sufficient antioxidant to provide adequate shelf life. He is limited on the amount of antioxidant permitted by various government regulations, by economics, and by the fact that extreme quantities of some antioxidants act as prooxidants. Some antioxidant is often lost or utilized during processing; thus the finished product must be analyzed for antioxidant content rather than depending on the quantity of antioxidant added as a quality control check.

One point should be emphasized. The actual antioxidant content of a food will vary inversely with time lapsed since addition of the antioxidant. Figure 1, taken from Anderson et al. (1), shows this loss of antioxidant during storage of wheat flake cereals.

The complex nature of foods causes a problem in analysis of an antioxidant in the small quantities used. Two general routes are used—one for high-fat foods such as nuts, prepared meat foods, and some baked goods; and another for low fat foods such as potato flakes, rice, and some cereals.

High-Fat Foods

The initial step in the isolation of antioxidants from high-fat foods is Soxhlet extraction with petroleum ether or similar solvents. The extract contains the fats, oils, antioxidants and probably some other products. The high-mol-wt proteins, phospholipids, etc., remain in the Soxhlet cup. Of course, with pure fats such as lard or shortening, extraction is not necessary. Incidentally, extraction is a field which often causes trouble and deserves further investigation.

Anderson et al. (1) have shown a direct correlation between extractable antioxidant and peroxide number of stored samples of wheat flakes, corn flakes and puffed oats. However, experience in the Eastman laboratories shows that antioxidant extraction from high-fat foods often does not correlate with shelf life and particularly peroxide value. The question arises as to whether the extraction removes antioxidant which has migrated to the center of a food particle. In addition, phenolic antioxidants may complex or react with the naturally occurring fats, such as the phospholipids, and are not extracted; thus there is the question as to whether or not these "bound" antioxidants still function as such or whether they no longer provide protection from oxygen attack.

The extract from a high-fat food can be treated in two ways. One procedure is to analyze directly, and this technique will be treated subsequently. By another method, the solvent is evaporated from the extract and the 3-t-butyl-4-hydroxyanisole (BHA) and 3,5-di-t-butyl-4-hydroxytoluene (BHT) removed from the fat by the method of Anglin, Mahon and Chapman (3) which involves steam distillation at about 160C. BHA and BHT steam distill, but propyl gallate (PG) and nordihydroguaiaretic acid (NDGA) either do not steam distill or are destroyed by the high temp. Superheated steam is necessary because, under normal steam distillation conditions, BHA and BHT distill from this fat system too slowly for practical purposes.

Low-Fat Foods

For determination of BHA and BHT in low-fat foods, an initial extraction is not required. The food is steam-distilled directly by the method of Filipic and Ogg (8) which involves a rapid steam distillation using atmospheric steam. As sodium sulfite is used in the processing of potato flakes, sulfur dioxide must be removed by means of a magnesium oxide scrubber in analyzing this product because it interferes in subsequent analysis.

The next step is to analyze the extract and/or steam distillate for antioxidant. The following methods are used.

Ultraviolet Spectra

Each phenolic antioxidant has a characteristic UV spectra, but naturally occurring materials absorbing in the UV range interfere with the measurement. A UV method for BHT in lard has been reported (13). The lard is dissolved in cyclohexane and passed over a silicic acid column. The eluate is analyzed for BHT at 240 and 320 mμ. BHA, PG, tocopherols and nordihydroguaiaretic acid do not interfere.

BHT can also be determined in lard by extracting the fat with isooctane and passing the isooctane solution over alumina (14). Absorbance measurement at 276 and 283 mμ will determine BHT concentrations as low as 0.001%.

Berger, Sylvester and Haines (4) have developed a method for separating BHA, BHT and gallate esters from other fat constituents by column chromatography over Silastic 181, a synthetic rubber. BHT is determined in the UV region by measuring the drop in optical density at 306 mμ when the eluate is made basic. BHA and the gallates do not interfere with this change in absorbance, and are determined by colorimetric methods discussed below.

2,4,5-Trihydroxybutyrophenone (THBP) can be determined in fats and oils by dissolving the sample in aqueous isopropyl alcohol and passing the solution over alumina (7). The THBP is retained on the column while interfering substances are eluted. The THBP is removed from the alumina by treating with

hydrochloric acid and is then dissolved in isoamyl alcohol. The amount of THBP is determined by measuring the absorption at 350 mμ.

The methods described above all depend on separation of the antioxidant by column chromatography which involves a fairly tedious elution and the handling of large volumes of solvent. Since other antioxidants are strongly adsorbed on the columns, the method is in general limited to the much less strongly adsorbed BHT. THBP is so firmly adsorbed by the column packing that interfering materials can be eluted without moving the THBP so that separation is achieved. Any other alkyl substituted phenol which is eluted with BHT would give a positive interference with the BHT analysis. Because of the tedious elution and the possibility of interferences, BHT in general is not analyzed in foods by this method in the Eastman laboratories.

However, since the method is extremely sensitive, it can be very valuable for routine analysis of BHT in a given food when it can be shown that no interferences exist by carrying out the analysis on the food with no antioxidant present and where the elution apparatus can be set up on a permanent basis.

Colorimetric Methods

Currently, the most widely used analytical procedures involve colorimetric methods. Ideally, a food extract, steam-distillate or fat solution should be treated directly with reagents to give noninterfering colors specific for each antioxidant. In practice, of course, such is not the case. Generally used methods for the various antioxidants are as follows:

BHA

2,6-Dichloroquinonechloromide Method (3, 8). The steam distillate, from either the Anglin, Mahon and Chapman or the Filipic and Ogg methods, as a 50% ethanol solution is treated with 2,6-Dichloroquinonechloromide [Gibbs reagent (9)] and with borax buffer to give a pH of 9.4. A blue compound is formed, and the amount is analyzed by measurement of absorption at 620 mμ. This reaction is specific for BHA in the presence of BHT because the reactive sites ortho and para to the phenolic hydroxy group in BHT are blocked. In a fat containing only BHA, the BHA can be extracted directly with 70% ethanol and the extract analyzed colorimetrically with ferric chloride-2,2′-bipyridine (11) or Gibbs reagent (12). It has been found in the Eastman laboratories, however, that small amts of naturally occurring material, presumably phenols, present in commercial lards and other pure fats give positive results with these reagents. In our laboratories therefore, the Anglin, Mahon and Chapman steam distillation method is always used for these products.

Sulfanilic Acid Method. Lazlo and Dugan (10) have described the use of the color produced by reaction of BHA and diazotized sulfanilic acid in basic solution as a quantitative analysis method. BHT also reacts with the reagent but so slowly that little interference is encountered if measurements are made within 5–10 minutes after adding the reagent. PG inhibits the color development, so must be removed prior to analysis by extraction with ammonium acetate solution.

BHT

Ferric Chloride—2,2 -Bipyridine Method (3, 8). The steam distillate, from either the Anglin, Mahon and Chapman or Filipic and Ogg methods, as 50% alcohol solutions is treated with ferric chloride—2,2′-bipyridine reagent, and the absorbance determined at 515 mμ. The result is a total of the BHA and the BHT concns. Subtraction of the BHA contribution as determined by Gibbs reagent gives the amount of BHT. The reaction of BHT with the ferric chloride is not complete within the normal analysis time; thus controlling the time of color development is very important. As a further complication, BHA accelerates the BHT reaction rate. However, addition of *n*-butyl alcohol to the reaction mixtures just prior to spectrophotometric measurement inhibits the color reaction and results in reproducible results.

3,3-Dimethoxybenzidine (Dianisidine) Method. Szalkowski and Garber (17) developed a specific method for determining BHT based on its reaction with dianisidine and nitrous acid. Of antioxidants, only ethoxyquin interfered enough to give an error of greater than 3%. The procedure involves a modified Anglin, Mahon and Chapman steam distillation followed by adjustment of the distillate to give a 50% methanol solution. The dianisidine-nitrous acid is added, the colored product is extracted with chloroform, and the absorbance is measured at 520 mμ.

Sloman, Romagnoli and Cavagnol (16) have combined the Gibbs' reagent method for BHA and the dianisidine method for BHT in an over-all scheme for analysis of these two antioxidants in food products.

Propyl Gallate (PG)

PG does not steam distill; thus its analysis must be conducted on an extract.

Ferrous Ion Method (8). The fat is dissolved in carbon tetrachloride and extracted with 50% aqueous ethyl alcohol. The extract is diluted with water to give 25% ethyl alcohol; the solution is buffered at pH 10 with sodium carbonate-bicarbonate and ferrous sulfate is added. The absorbance of the purple solution is measured at 515 mμ. NDGA interferes with this analysis, but NDGA and PG have similar antioxidant properties and are not normally used together.

In the United Kingdom, propyl, octyl and dodecyl gallates are all used. Vos, Wessels and Six (18) developed a scheme for their analysis using the ferric ion reagent. By this method, PG is selectively extracted with water from a petroleum ether solution of the fat. The amt of PG is determined by adding ferrous tartrate at a pH of 7.0–7.6 and measuring the optical density at 530 mμ. The higher gallates are extracted with methanol. After addition of ferrous

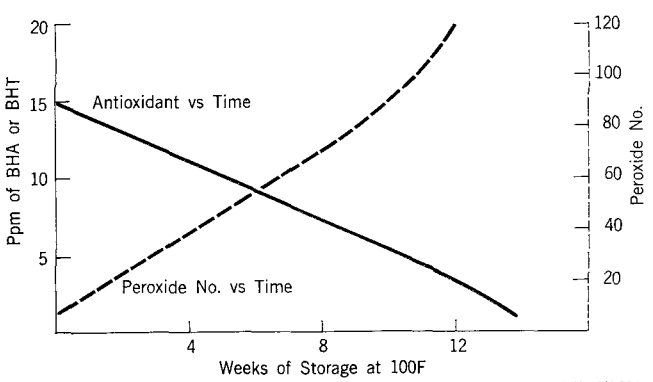

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FIG. 1. Wheat flake cereals to which 15 ppm of each BHA and BHT were added. Stored at 100F in packages with double glassine paper liners.

TABLE I

Chromatographic Columns for BHA/BHT Separation[a]

Column[c]	Approximate Retention Times, Min[b]			Column Temp
	BHA	BHT	Di-BHA	
2 ft 33% Carbowax 20M	10.5	4.5	7	200—280C[d] @ 5C/min.
4 ft 15% Oxiron 2000	9.5	5	7.5	200—260C[d] @ 10C/min.
4 ft 15% Tween 61	14	6.5	11	200—225C[d] @5C/min.
4 ft 5% Tween 61, 25% Silicone SE-30[e]	10.5	8	14	215C
4 ft 20% Silicone-Fluoro-FS-1265-QF-1	7.5	6	9.5	125—180C[d] @ 10C/min.

[a] Instrumental conditions: Sample size 100 μl; Helium flow 60 ml/min; Injection port temp 325C; Detector temp 290C; Detector current 200 ma; Sensitivity XI

[b] The exact retention time will vary somewhat with different preparations of the same column packing.

[c] Support in all cases was untreated Chromosorb W (product of Johns-Manville).

[d] The temp was held at the higher point until all three products eluted.

[e] This column was excellent, but subsequent attempts to duplicate the packing met with only partial success.

tartrate, the methanol solution is diluted with water, extracted with a mixture of isoamyl alcohol and petroleum ether, and the absorbance of the aqueous layer then determined at 550 mμ. Dodecyl and octyl gallates are distinguished by a qualitative color reaction in which ferrous tartrate is added to the methanol solution. This solution is then shaken with a mixture of isoamyl alcohol and petroleum ether. Formation of a violet-blue color in the upper layer indicates the presence of dodecyl gallate since octyl gallate gives no color with this reagent. A similar method for total gallate content based only on extraction with 95% aqueous alcohol has also been developed (6).

Colorimetric reactions in general are excellent for BHA, BHT and PG analyses in food. However, they are not completely specific. 2,6-Dichloroquinonechloromide and diazotized sulfanilic acid will react with any phenol with a vacant ortho or para position, not just with BHA; ferric chloride — 2,2′-bipyridine will give color with any phenol; PG analysis depends on formation of a colored, iron complex and, as already pointed out, NDGA will interfere. In the Eastman laboratories, experience has shown that these objections are not too serious since interfering color has not been encountered in PG analyses, and steam distillation procedures apparently remove most interferences in the BHA and BHT analyses. As with the UV analyses, one is on much firmer ground when the analysis is a routine one where the food containing no antioxidant can be run through the analysis to determine if interferences exist.

Gas Chromatography

Since its development in the 1950's, gas chromatography has become one of the more powerful analytical tools. The fact that excellent commercial instruments can be purchased in the range of only $2000–$4000 has led to its widespread use throughout the chemical and allied industries. One major advantage is that detection of very small quantities of material is possible. Analysis of antioxidants in foods involves quantities in the ppm range, thus it is natural that this tool should be applied in this area.

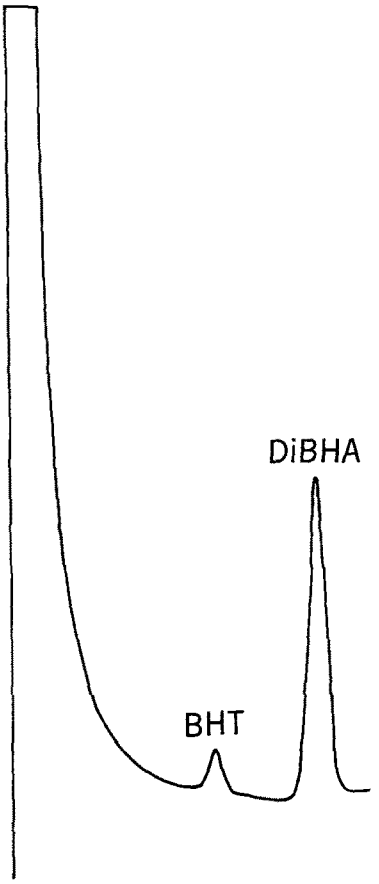

FIG. 2. BHT separation with Carbowax 20M Column.

Buttery and Stuckey (5) described direct extraction of BHA and BHT from potato granules followed by gas chromatography of the eluate on an Apiezon L Column using flame ionization detection. A range of 0.5 ppm to 10 ppm of antioxidant was analyzed with an average error of less than 11%.

Anderson, Schwecke, Nelson and Huntley (1) extended the gas chromatographic work using the direct extraction technique. Schwecke and Nelson (15) introduced the use of 3,5-di-t-butyl-4-hydroxyanisole (DiBHA) as an internal standard. In the internal standard method, the unknown amounts of BHA and BHT are determined by comparison with a known concentration of Di-BHA in the same sample. This procedure obviates having to measure carefully the amt of a standard solution. These workers analyzed in the range of 5–30 ppm antioxidant using a Silicone Gum SE-30-Tween 80 column and flame ionization detection. Anderson et al. (1) used this method in studying the decrease in antioxidant content in cereals with storage time.

TABLE II

Sensitivity of a 33% Carbowax 20M Column

Sample	Determinations	BHA, ppm				BHT, ppm			
		Actual	Anal. Av.	Range	Std. Dev.	Actual	Anal. Av.	Range	Std. Dev.
40B	8	10	10.2	9.5–11.0	0.55	10	9.95	9.0–11.0	0.80
40D	8	5	5.70	5.5– 6.0	0.28	5	5.45	5.0– 6.0	0.42
40C	9	2.5	2.52	2.3– 2.7	0.16	2.5	3.02	2.8– 3.6	0.27
40D	8	1	1.00	0.8– 1.2	0.13	1	0.91	0.85– 1.0	0.05

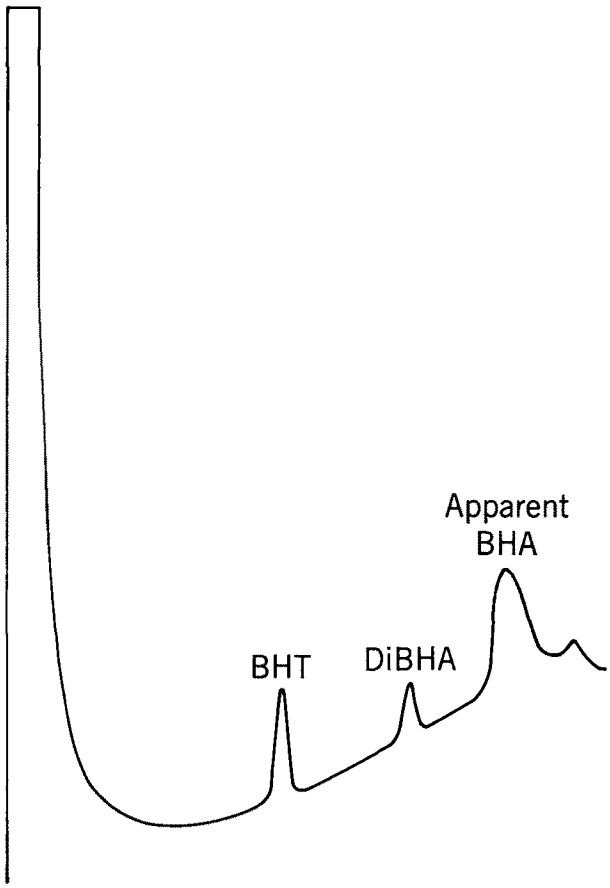

FIG. 3. BHA, BHT separation with Carbowax 20M Column.

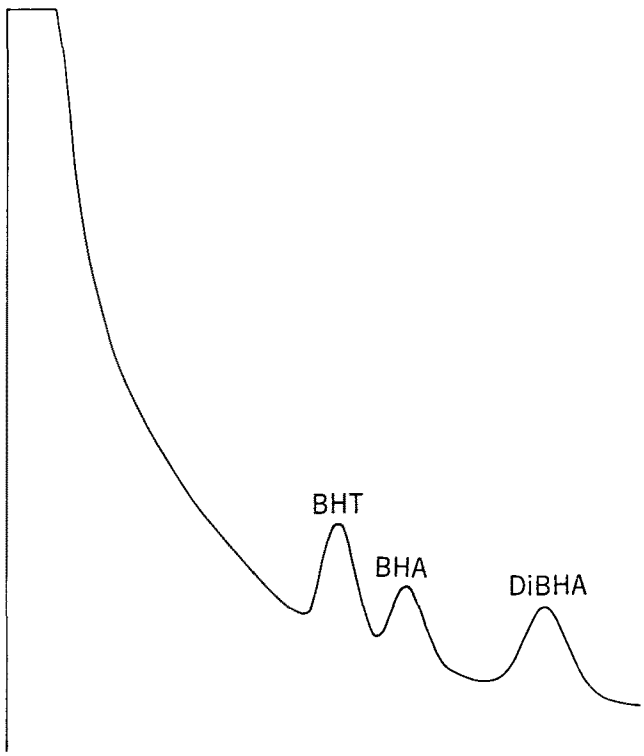

FIG. 4. BHA, BHT separation with Silicone-Tween 61 Column.

These reports show that gas chromatography is an excellent tool for BHA and BHT analyses. In the Eastman laboratories, however, some problems have occurred in use of the flame ionization detection system, the major problem being column "bleed." In the published reports, the techniques followed used less than 1-μl samples and a very high detector sensitivity. This procedure results in detection of any small amount of column packing which bleeds off and confuses the analysis. Under these conditions, the column must be conditioned for an extended period of time to drive off column packing which is volatile at the column temp employed. In addition, flame ionization detection does not give an equal response for equal amts of different compounds, so calibration curves must be prepared. Anderson and Nelson (2) described the use of the much less sensitive thermal conductivity detection system using 23% Silicone Gum SE-30 column packing. A 100-μl sample must be used to obtain sufficient detector response. The response is essentially the same for BHA, BHT and the internal standard, Di-BHA. Similar work in the Eastman laboratories confirms the utility of this method using an F and M Scientific Company Model 720 Gas Chromatograph. A discussion of some of this work follows:

A 10- to 15-g sample of food was packed in a 13 × 20-mm glass column with a 55 × 30-mm solvent reservoir at the top. The sample was washed with one 10-ml portion and four 5-ml portions of ethyl ether. About 1 lb nitrogen pressure was used to obtain reasonable flow. Concurrently, a gentle nitrogen stream was used to evaporate the emerging eluate. Toward the end of the elution, 500 μg of Di-BHA in ether solution was added to the conc. The final volume was about 1 ml.

Instrument conditions for the F and M Scientific Company Model 720 are found in Table I. A number of columns have been devised which adequately separate BHA, BHT and Di-BHA. In our laboratories, the separation of BHA and BHT using the 23% Silicone Gum SE-30 of Anderson and Nelson (2) was not as good as is desired. We have developed the columns listed in Table I which give adequate separation and reasonable retention times.

In no case was column bleeding a problem and only about 30 min was required to obtain a steady baseline. However, it should be pointed out that the Model 720 is a dual column instrument where a reference column is in parallel with the analysis column which results in compensation of bleeding. We have also found that the response of the BHA, BHT and Di-BHA is equal for equal amts, within experimental error, so that no calibration need be made and direct peak area comparisons are valid. Table II shows the sensitivity of the 33% Carbowax 20M Column. These data were carried out with solutions prepared to correspond to the indicated concns of antioxidant.

This column was used to analyze rice samples for BHT in the 3–30 ppm range; the chromatogram contained no peaks other than those of the BHT and Di-BHA. Figure 2 illustrates the chromatogram obtained. A problem involving BHA and BHT in the 3–30 ppm range in potato flakes was also investigated using this column. However, it was found that a constituent from the potatoes came off the column in the BHA region, completely obscuring the BHA peak. Figure 3 shows this interference. Changing to a 4 ft 5% Tween 61, 25% Silicone SE-30 column eliminated this problem; clean, sharp peaks with no interference were obtained. These are shown in Figure 4. In the potato flake analysis, the concentrated extract contained a good deal of insoluble material. However, allowing it to settle as much as possible minimized the amount of solid taken with the sample, and even

when a relatively large amount of solid was occluded in the sample shot on the column, there was no interference in the BHA, BHT, Di-BHA peaks. With the variety of columns developed here, it should be possible to eliminate such interference in other problems by changing columns. Another positive advantage in having various columns available is that BHA and BHT can be identified positively since not only can peak times be measured, but order of elution can be varied from BHT, BHA, Di-BHA to BHT, Di-BHA, BHA.

The problem encountered in this potato flake analysis illustrates quite clearly a general point which is indicative of our experience in antioxidant analyses. Each type of food presents a slightly different problem and while general methods can be discussed, variations may be necessary for individual cases.

The gas chromatographic technique is valuable in eliminating interferences since, as in the case of the potatoes, interference is immediately obvious and methods can be varied to eliminate it. Interference in colorimetric work would be far harder to detect in individual cases, especially where a single wavelength reading is used with no check of the rest of the spectrum for other peaks.

Gas chromatographic analysis needs further investigation, particularly in high fat food where the direct extraction technique removes large amounts of various fats and oils along with the antioxidants. These interfering materials not only result in a multiplicity of peaks, but also completely plug up the column on occasion.

The use of the gas chromatographic technique to analyze BHA and/or BHT in a single food product is relatively simple and can be handled by a technician with little supervision. However when substrates are varied or when instrumentation difficulties arise, an operator with a thorough knowledge of gas chromatographic techniques and instrumentation experience is a must.

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