Fluorimetric Determination of Butylated Hydroxy Anisole in Food Products and Packaging Material

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A fluorimetric method for the determination of butylated hydroxy anisole in lard, ready-to-eat cereals, and waxed packaging liners is described. Solutions of lard samples are analyzed directly by a standard addition method that corrects for the interference due to propyl gallate or butylated hydroxy toluene. Samples of cereals and packaging materials require prior separation of butylated hydroxy anisole by combination of extraction and thin-layer chromatography. In all procedures the native fluorescence of butylated hydroxy anisole is measured; thus, the problems encountered with colorimetric methods are avoided.

BHA (2- and 3-*tert*-butyl-4-hydroxyanisole) is widely used in many food products and waxed package liners as an antioxidant to preserve freshness. It is used alone, or together with other antioxidants such as BHT (3,5-di-*tert*-butyl-4-hydroxytoluene) and PG (*n*-propyl gallate). Mixtures of the antioxidants are more effective in preserving freshness since no one compound is equally effective in all food products under all conditions. Because of government regulations and the presence of other antioxidants, an analytical method for the determination of BHA in food products must be sensitive in the parts per million range, as well as being specific, rapid, and accurate.

Sahasrabudhe (1964) has successfully utilized thin-layer chromatography to determine quantitatively BHA, BHT, PG, and NDGA in lard. McCaulley *et al.* (1967) have developed a vacuum sublimation method for isolating the antioxidants BHA, BHT, Ionox-100, PG, THBP, and TDPA from lard and quantitatively determining the antioxidants by gas chromatography. Johnson (1967) took advantage of the open *ortho* position in the BHA molecule to form a colored nitroso derivative, and spectrophotometrically determined BHA in vegetable oils. Recently Takahashi (1967) developed a gas chromatographic method using a hydrogen flame ionization detection system to determine BHA and BHT in ready-to-eat corn and rice cereals. In a later publication, Takahashi (1968) gave the results of a collaborative study for the two phenolic antioxidants. Schwecke and Nelson (1964) also used gas chromatography with a flame ionization detector to determine BHA and BHT in ready-to-eat cereals, potato products, and waxed paper.

This paper describes a new approach for the quantitative determination of BHA in lard, ready-to-eat cereals, and waxed package liners. The native fluorescence of BHA is used to determine quantitatively BHA directly in lard either alone or in the presence of BHT and PG. BHA in cereal and in packaging materials is determined by the fluorescence emission of BHA after separation of the antioxidant.

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EXPERIMENTAL

Apparatus and Equipment. All fluorescence and phosphorescence measurements were obtained with an Aminco-Bowman spectrophotofluorometer equipped with a phosphoroscope accessory. The source for the excitation monochromator was a Xenon lamp (American Instrument Co., Catalog No. 416-992). The detector for the emission monochromator was a 1P21 phototube or 1P28 phototube. The latter tube provided greater sensitivity.

The thin-layer chromatographic equipment consisted of a rectangular Kensco developing tank, 10 × 20 cm glass plates, plastic template to apply accurately and locate spots, and a "moving plate" apparatus to coat the glass plates. A Hamilton 50-μl or 10-μl syringe was used to spot samples on thin-layer plates.

Reagents. ABSOLUTE ETHANOL. The ethanol was distilled once using a distilling column similar to the one described by Winefordner and Tin (1964).

LIGROINE (bp 63–75° C). Eastman practical grade treated the same as ethanol.

N,2,6-TRICHLORO-P-BENZOQUINONEIMINE. A 2.0% solution of the Eastman compound was made up in absolute ethanol.

BHA, BHT, AND PG. These food grade antioxidants were used without further purification and were obtained from Eastman Chemical Products, Inc., Kingsport, Tenn., and Universal Oil Products Co., East Rutherford, N.J.

QUININE SULFATE. A stock solution of 0.8 μg per ml of quinine sulfate in 0.1N H_2SO_4 was used daily to check the sensitivity of the spectrophotofluorometer for lard analyses. The excitation monochromator was set at 250 nm and the emission monochromator was set at 450 nm. The sensitivity was then adjusted to the same value as previously obtained using a 1P21 phototube.

BHA. A stock solution of 2.0 μg per ml of BHA in ethanol was used daily to check the sensitivity of the spectrophoto-

fluorometer for cereal and packaging analyses. The excitation monochromator was set at 290 nm and the emission monochromator was set at 327 nm. The sensitivity was then adjusted to the same value as previously obtained using a 1P28 phototube.

DETERMINATION OF BHA IN LARD

The cell used to measure the fluorescence intensity of the antioxidants must be cleaned and completely dry before each reading. Also, if PG is present, its concentration must be previously determined. This can be easily accomplished by using the method of Latz and Hurtubise (1969) for determining PG directly in lard without interference from either BHA or BHT.

Separate standard solutions of BHA and PG were prepared containing 10.0 μg per ml of food grade antioxidant in 50% ethanol-ligroine. Between 2.400–2.600 g of lard were weighed in a 100-ml beaker, melted, and dissolved in approximately 50 ml of 50% ethanol-ligroine. The sample was quantitatively transferred to a 100-ml volumetric flask and diluted to volume. Aliquots of 10 ml of lard solution were placed in seven 25-ml volumetric flasks. Samples of 5.0, 2.0, and 1.0 ml of the standard BHA solution were added to three of the volumetric flasks. Then samples of 5.0, 2.0, and 1.0 ml of the standard PG solution were added to three of the remaining four volumetric flasks. All seven flasks were diluted to 25 ml with 50% ethanol-ligroine. The spectrophotofluorometer was adjusted with a standard solution of quinine sulfate using slit arrangement five. The relative fluorescence intensities of the seven lard solutions were measured using a 1P21 phototube with the excitation monochromator at 292 nm and the emission monochromator at 342 nm. The relative fluorescence intensity obtained from the blank lard solution was subtracted from the relative fluorescence intensities of all the other solutions.

The concentration of BHA was determined as follows. The standard addition curve for PG was prepared by plotting the corrected relative fluorescence intensities obtained from the unknown solution and the three solutions spiked with PG against μg per ml of PG added to the lard solutions. The best straight line through these points provides a calibration curve with a slope representative of the fluorescence intensity of PG alone. An intensity value corresponding to the previously determined PG concentration is obtained from this curve and subtraction of the unknown intensity from this value gives the absolute intensity due to PG. The latter value is then subtracted from the intensities obtained from the unknown and the three BHA standard addition solutions and these values are plotted against μg per ml of BHA added to the unknown. Extrapolation of the best straight line through these points to zero relative intensity gives the concentration of BHA in the unknown solution.

DETERMINATION OF BHA IN CEREAL

Extraction of BHA. The method used was similar to that described by Schwecke and Nelson (1964). In this method both BHA and BHT are quantitatively extracted from cereal into ethyl ether. A 10.0-g sample of cereal was ground to a powder with a mortar and pestle and poured into a glass column 49.5 cm in length and 1.0 cm in diameter, which was fitted with a course fritted glass disc. (For spiked samples an aliquot of a 1000 μg per ml or 100 μg per ml solution of BHA in ethanol was added directly to the dry cereal in the column.) Ethyl ether was percolated through the column until 25 ml were collected in a 25-ml Erlenmeyer flask. The 25 ml of eluent were evaporated to dryness with a stream of nitrogen. Two milliliters of ethyl ether were added to the dried residue and added to a 5-ml volumetric flask. The Erlenmeyer flask was rinsed and the rinsings were added to the 5-ml volumetric flask and diluted to 5 ml with ethyl ether.

Thin-Layer Preparation and Sample Spotting. A thin layer of silica gel-G (20 cm × 10 cm × 0.20 mm) was applied to the thin-layer plate. The slurry of silica gel-G used for spreading was prepared and applied as described by the manufacturer. After the plate was coated with silica gel-G, it was dried at room temperature and then activated at 110° C for 30 min. The plate was cooled to room temperature and four aliquots of the extract were applied to the plate. The aliquots were spaced on the plate using a template. A 50-μl aliquot of the extract was applied on the far right of the plate. A similar 50-μl aliquot was applied next to the first. On top of the second aliquot 5 μl of 1000 μg per ml of BHA in absolute ethanol was applied. Next to this spot a 5-μl aliquot of the same BHA solution was applied. Finally, another 50-μl aliquot of the extract was applied on the far left side of the plate.

Spot Development. The sample plate was then placed in a developing tank containing mobile solvent (200 ml of ligroine, 20 ml of ethanol, and 3 ml of acetic acid) and developed twice to a solvent front height of 10 cm.

Spot Detection. The first three spots applied were covered while the spot on the far left of the plate was sprayed with N,2,6-trichloro-*p*-benzoquinoneimine reagent. BHA showed up as a pink spot if a small amount was present, or as a blue spot for a larger amount. Three circular areas of 1.7 cm diameter corresponding to the location of BHA on the sprayed area were scraped from the unsprayed area into three 25-ml volumetric flasks fitted with small glass funnels. A blank was obtained by scraping off an area where no sample had migrated. A constant area for each spot was obtained by making an impression on the plate with a metal cylinder of 1.7 cm diameter. Next, 10.00 ml of absolute ethanol were added to the four 25-ml volumetric flasks. Each flask was vigorously hand-shaken for 3 min and decanted into test tubes. After centrifuging, relative fluorescence intensities were obtained from the four clear supernatants using a 1P28 phototube and slit arrangement four. The excitation monochromator was set at 290 nm and the emission monochromator was set at 327 nm. The relative fluorescence intensity obtained from the blank solution was subtracted from the other three.

Calculation of Parts Per Million BHA. The concentration of BHA is obtained using the following expression:

$$\text{BHA} = \text{R.I.U.}/\text{R.I.M.} - \text{R.I.U.} \times 0.500\ \mu\text{g/ml}$$

where R.I.U. = relative intensity of the unknown, R.I.M. = relative intensity of the mixture of standard and unknown, and 0.500 μg/ml = concentration of standard. The relative intensity of the standard by itself is used to monitor the chromatographic procedure and should have a value reasonably close to the difference between the mixture and the unknown.

If 10.00 g of cereal were used in the determination, the μg per ml of BHA was multiplied by 100 to obtain parts per million of BHA.

DETERMINATION OF BHA IN WAXED PACKAGING MATERIAL

Low Fluorescence Background. Some packaging materials are found to have a very low fluorescence background; there-

fore, the following procedure was used. A 0.5000 g sample of waxed paper obtained by punching holes with a paper punch was placed in a 100-ml beaker. Approximately 50 ml of ligroine were added to the beaker and the contents of the beaker were stirred for 30 min on a magnetic stirrer. (For spiked samples, aliquots of a 100 μg per ml BHA solution in ligroine were added directly to the beaker.) The solution was transferred to a 100-ml volumetric flask, and the paper in the beaker was rinsed four times with 10-ml aliquots of ligroine. The rinsings were added to the 100-ml volumetric flask, and the flask was diluted to volume. Ten milliliter aliquots of this solution were added to two 25-ml volumetric flasks. A 2.0-ml aliquot containing 10.0 μg per ml of BHA in 50% ethanol-ligroine was added to one flask and both were then diluted to volume with 50% ethanol-ligroine. Ten milliliters of ligroine were added to two other 25-ml volumetric flasks. One flask was diluted to 25 ml with 50% ethanol-ligroine, and to the other 2.0 ml of 10.0 μg per ml of BHA in 50% ethanol-ligroine were added and then diluted to 25 ml with 50% ethanol-ligroine. Relative fluorescence intensities were then obtained for all solutions with the excitation monochromator at 290 nm and the emission monochromator at 327 nm. The fluorescence intensity was detected with a 1P28 phototube using slit arrangement four. The relative fluorescence intensity from the blank solution was subtracted from the other three solutions.

Calculation of Percent BHA. The calculations were similar to those for cereal, except the ratios of relative intensities were multiplied by 0.800 μg per ml of BHA and the μg per ml of BHA was multiplied by 0.050 to obtain a percentage based on a 0.5000-g sample of packaging material.

High Fluorescence Background. For packaging material with high fluorescence background, the initial extract was evaporated to less than 2 ml with a stream of nitrogen. The extract was then added to a 10-ml volumetric flask. The beaker was rinsed with ligroine and the rinsings were added to the 10-ml volumetric flask.

The BHA was then separated by the thin-layer chromatographic procedure described above, except for the following modifications. Only 2 μl of 1000 μg per ml of BHA standard were spotted, the plate was developed only once, and 5 ml of ethanol were used to elute the spots.

Calculation of Percent BHA in Packaging Material. The calculations were similar to those for "low fluorescence background" except the ratios of relative intensities were multiplied by 0.400 μg per ml of BHA and the μg per ml of BHA was multiplied by 0.200.

RESULTS AND DISCUSSION

Determination of BHA. LARD. The advantages of using fluorescence for the determination of antioxidants in lard has been demonstrated by Latz and Hurtubise (1969). They used chloroform as a solvent to quench the fluorescence of BHA and BHT, which enabled them to determine PG in lard without prior separation. The determination of BHA in lard has also been accomplished without separation by using 50% ethanol-ligroine as a solvent in which lard and all of the antioxidants are soluble. Also, the fluorescence intensity of BHA is increased by a factor of three in 50% ethanol-ligroine, as compared to BHA in ligroine. In 50% ethanol-ligroine, the fluorescence intensity of BHT is very low, and the maximum emission wavelengths of BHA and PG are separated by 30 nm. Because of these factors, BHA can be determined with BHT and PG present in solution. Problems with colorimetric methods such as differences in reaction rates of the isomers of BHA with a color reagent and differences in molar absorptivities of the final color complex do not occur with fluorimetric methods (Johnson, 1967; Mahon and Chapman, 1951, 1952). These problems are eliminated because a solution of the isomers gives only one maximum emission wavelength. The relative intensity at the maximum emission wavelength is used to obtain an accurate determination of BHA.

Table I. Determination of BHA in Lard Samples by Standard Addition

No. of Determinations	Added, ppm			BHA found, ppm	Error, %
	BHA	BHT	PG		
4	120	...	40	128	+ 6.67
3	24.0	...	12.0	27	+12.5
2	10.0	...	30.0	14	+40.0
2	120	240	40.0	126	+ 5.00
2	40.0	40.0	...	40.0	0.00
2	80.0	240	...	84.5	+ 5.62
3	120	120	40.0	125	+ 4.17
3	80	...	...	81.7	+ 2.12

Table I shows the results for a number of determinations of BHA in spiked lard samples. The concentration of PG was assumed to be the same as added to the lard solutions. For all solutions measured, a blank relative fluorescence intensity value was subtracted. The blank value was obtained from an antioxidant-free lard solution. The background emission from four different antioxidant-free lard solutions was found to be similar. The average value of the relative fluorescence intensity of the lard solutions was 0.35. The relative fluorescence intensity of a typical unknown solution was 6.40; therefore, the background emission would contribute about 5.47% to the total fluorescence intensity. If a blank value is not subtracted, an error of about 10.0% or greater can be introduced in the determination of BHA. The results in Table I indicate that with other antioxidants present and when a blank value is subtracted, the percentage error is not very large.

Standard addition has several advantages. These advantages have been discussed by Latz and Hurtubise (1969). One disadvantage of the standard addition method in this work was that the maximum excitation and emission wavelengths of PG are not used to obtain the standard addition plot for PG. Because of this any small error in relative fluorescence intensity will be magnified, since the slope of the standard addition curve does not have a maximum value.

Cereal. Two separation steps were necessary for the determination of BHA in cereal because of interfering fluorescent and phosphorescent components. The initial extraction of antioxidant-free cereals yielded solutions that contained components whose fluorescence and phosphorescence emission wavelengths were similar to BHA and thus would interfere with the determination of BHA. Therefore, luminescence analysis of the eluent from the extraction column could not be used to determine BHA. Fluorescence was used to determine BHA after scraping from a thin-layer plate rather than phosphorescence because the fluorescence intensity of BHA is much greater than its phosphorescence intensity (Latz and Hurtubise, 1969).

If BHT is present in a cereal sample, it will not interfere in the determination of BHA because BHT moves with the solvent front on the thin-layer plate. The isomers of BHA which migrated at the same rate appeared as one pink or blue spot and had an average R_f value of 0.42. Thin-layer plates were

Table II. Recoveries of BHA Added to Cereals

Sample	BHA added, ppm	BHA found, ppm	Recovery, %
Wheat	50.0[a]	47.7	95.6
	50.0[a]	55.0	110
	10.0	7.00	70.0
	10.0	9.56	95.6
Corn	50.0	51.8	103
	50.0	50.0	100
	10.0	11.6	116
	10.0	8.66	86.6
Rice	50.0	47.8	95.8
	50.0	48.8	96.4
	10.0	8.85	88.5
	10.0	8.59	85.9

[a] 20 μl of extract spotted.

Table III. Determination of BHA in Spiked Extracts from Waxed Paper with Low Fluorescence Background

BHA, added, %	BHA found, % blank not subtracted[a]	BHA found, % blank subtracted
0.100	0.110	0.105
0.100	0.114	0.111
0.050	0.051	0.048
0.050	0.056	0.051

[a] Blank solution, 10 ml of antioxidant free extract in ligroine diluted to 25 ml with 50% ethanol-ligroine.

developed twice with mobile solvent because with one development some cereal extracts showed a blue spot on the plate corresponding to almost the same position as BHA. However, two developments allowed BHA to be distinguished, and BHA always appeared above the unknown spot.

When the experimental procedure was carried out on BHA-free extracts of corn, rice, and wheat cereals, there was no background interference in almost all samples. With some samples a very small background fluorescence was observed, but it was not intense enough to cause error.

Table II shows that the method for determining BHA is sensitive and reasonably accurate in the part per million range. Also the method is generally applicable to corn, rice, and wheat cereals. The lower percent recovery at the 10 ppm level most likely results from the use of two separation steps for such a small amount of material. Recovery studies indicate that the initial cereal extraction is efficient using the procedure described, so it is suspected that the second extraction from silica gel is the primary source of error at the lower concentration levels. Repetitive determinations of BHA in three commercial samples of cereals containing this antioxidant produced results of 3.8 ± 0.4, 0.98 ± 0.6, and 2.0 ± 0.2 ppm. It was necessary to use 100 μl of concentrated extract for thin-layer separation to obtain these results and recovery experience suggests that the low values are most probably due to decomposition or volatilization during storage (Stuckey and Osborne, 1965).

The method of spotting the different aliquots on thin-layer plates was chosen because this allowed the detection of errors such as uneven migration on the thin-layer plate or inefficient scraping of BHA from the plate. For example, if there was a large difference between the relative fluorescence intensity of the standard sample and the difference between the relative intensity of the unknown and standard addition sample, then it was assumed that some error, such as uneven migration on the thin-layer plate, had occurred. The method used to calculate the amount of BHA was employed because it corrected for any increase or decrease in the fluorescence emission of BHA that might result from some component present in the cereal extract or a component eluted from the silica gel.

Packaging Material. Table III gives the results for the determination of BHA from extracts of waxed paper that had a low fluorescence background. The results show that BHA can be accurately determined by a simple extraction procedure. However, with other antioxidant-free packaging materials, high fluorescence background was obtained and an accurate determination of BHA by simple extraction was impossible. Therefore, it is necessary to know beforehand the magnitude of the background fluorescence. Also, the glue used to seal the packaging material may be highly fluorescent; therefore, the sample of waxed paper used to determine BHA should not contain any traces of glue. In some cases the extracts of waxed paper were slightly cloudy, but this did not interfere with the determination.

Because some of the extracts from waxed paper had a high fluorescence background, it was again necessary to use thin-layer chromatography to separate BHA following extraction. Duplicate determinations of BHA in commercial waxed paper were performed using extraction alone and extraction combined with a thin-layer separation. The two approaches produced results of 230 and 60 ppm, respectively, which provides an indication of the extent of interference. The latter result is lower than might be expected, and although poor recovery might be partially responsible, it is felt that volatilization during storage may have occurred. Samples of freshly prepared wax paper were not available for verification.

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