Luminescence Analysis of Food Antioxidants

Determination of Propyl Gallate in Lard

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The luminescence properties of the food antioxidants BHA, BHT, and PG are presented for the first time. A simple, rapid, and accurate fluorometric method is used to determine PG directly in lard in the presence of BHA, BHT, or both. Chloroform, used as a solvent, quenches the fluorescence of BHA and BHT, as well as the background fluorescence of lard, but not PG. As little as 0.0005% PG can be determined in a lard sample by this method without interference from BHA or BHT.

Numerous methods have been reported for the determination of the food antioxidants BHA (mixture of isomers, 2-*tert*-butyl-4-methoxyphenol and 3-*tert*-butyl-4-methoxyphenol), BHT (2,6-di-*tert*-butyl-4-methylphenol), and PG (propyl gallate) (Berger *et al.*, 1960; Johnson, 1967; McCaulley *et al.*, 1967; Mahan and Chapman, 1951; Scheidt and Conroy, 1966; Stuckey and Osborne, 1965; Vos *et al.*, 1957). Many methods lack sensitivity and specificity for measuring accurately parts per million concentrations and are lengthy. In most cases it is necessary to isolate the antioxidants and then determine their concentration by some appropriate method of analysis. Stuckey and Osborne (1965) have recently reviewed ultraviolet absorption, colorimetric, and gas chromatographic methods of determining food antioxidants.

EXPERIMENTAL

Apparatus. Fluorescence and phosphorescence spectra 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. Spectra were recorded with an Aminco-Bowman X-Y recorder.

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

CHLOROFORM. Mallinckrodt analytical reagent or Baker analyzed reagent was distilled once with the same column used for purification of ethanol.

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

QUININE SULFATE. A stock solution of 0.8 μg. per ml. of quinine sulfate in 0.1*N* H_2SO_4 was used daily to check the sensitivity of the spectrophotofluorometer. The excitation monochromator was set at 250 mμ, and the emission monochromator was set at 450 mμ. The sensitivity was then adjusted to the value previously obtained.

General Procedure for Standard Addition Method. The cell used to measure the fluorescence intensity of PG must be clean and completely dry before each reading.

A standard solution was prepared containing 100 μg. per ml. of food grade PG in chloroform. PG does not dissolve readily in chloroform; therefore, it was necessary to stir the solution for about 20 minutes. Between 2.400 and 2.600 grams of lard were weighed in a 100-ml. beaker, melted, and dissolved in approximately 50 ml. of chloroform. The sample was transferred to a 100-ml. volumetric flask, the beaker was rinsed, and the rinsings were added to the 100-ml. volumetric flask and diluted to 100 ml. Aliquots of 10 ml. of lard solution were placed in four 25-ml. volumetric flasks. Samples of 0.5, 0.2, and 0.1 ml. of the standard PG solution were added to three of the volumetric flasks. Then the contents of all four flasks were diluted to 25 ml. with chloroform. The spectrophotofluorometer was adjusted with a standard solution of quinine sulfate, using slit arrangement 5. The relative fluorescence intensity of the four lard solutions was measured with the excitation monochromator at 272 mμ and emission monochromator at 352 mμ. The standard addition curve was prepared by plotting relative fluorescence intensity against micrograms per milliliter of PG added to the lard solutions. The line intersecting the relative intensity axis was extrapolated to zero relative intensity to obtain the concentration in the unknown solution of PG in 25 ml. of chloroform.

General Procedure for Obtaining Fluorescence Spectra of Antioxidants. The antioxidant (BHA, BHT, or PG) was dissolved in absolute ethanol and diluted to a 1 to 5 μg. per ml. solution of the antioxidant. The excitation and emission spectra were obtained using slit arrangement 4.

General Procedure for Obtaining Phosphorescence Spectra and Lifetimes of Antioxidants. The antioxidant (BHA, BHT, or PG) was dissolved in absolute ethanol and diluted to a 1 to 5 μg. per ml. solution of the antioxidant. The sample tube containing the antioxidant solution was placed in a Dewar flask which contained liquid nitrogen, and the phosphoroscope was set at

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Table I. Luminescence Properties of Antioxidants

Compound	Solvent	Excitation Max., Mμ	Emission Max., Mμ		Phos. Mean Lifetime, Sec.	Relative Intensity[a]		Concn., μg./Ml.	
			Fluor.	Phos.		Fluor.	Phos.	Fluor.	Phos.
BHA	Ethanol	293	342	420	2.9	11.7	1.56	5.0	4.0
BHT	Ethanol	287	350	439	0.5	0.069	0.027	5.0	100.0
PG	Ethanol	286	372	454	2.7	4.40	2.70	3.5	4.0
BHA	Chloroform	290	345	...	...	0.009	.....	4.0	...
BHT	Chloroform	290	330	...	...	0.004	.....	4.0	...
PG	Chloroform	272	352	...	...	1.50	.....	4.0	...

[a] Compared to fluorescence of 0.8 μg. per ml. quinine sulfate solution in 0.1N H_2SO_4, relative intensity 12.0.

maximum speed. The phosphorescence spectrum was then recorded.

The phosphorescence decay times (the time for the relative intensity to drop from a given value to 1/e of that value) were obtained using the recorder, and a blank slit inserted in the slit holder facing the excitation source was manually moved to block the light from the source. The slit program was as follows: 4-4-4-4-4.

RESULTS AND DISCUSSION

Fluorescence and Phosphorescence of Antioxidants in Ethanol. Table I gives the first reported luminescence data on the antioxidants BHA, BHT, and PG. The data and spectra presented have not been corrected for instrumental artifacts. The purpose of this work was to investigate the luminescence properties of the antioxidants and use these properties to develop analytical methods for the quantitative determination of the antioxidants used in foods. The investigation of the luminescence properties indicates that there are many possible approaches utilizing both fluorescence and phosphorescence. For example, Table I indicates that the emission wavelengths of BHA and BHT are close to each other, and an analysis of a mixture of the two might appear to be impossible. However, BHT gives a relative intensity of 0.07 at 350 mμ; while BHA gives a relative intensity of 11.1 at 350 mμ with both antioxidants at the same concentration. Since the fluorescence of BHT is so weak, BHA could be determined quantitatively in a mixture of the two at low concentrations of BHT.

The fluorescence and phosphorescence emission intensities of both BHA and PG are strong compared to BHT and could be used easily for analytical purposes. The fact that their emission maxima are 30 mμ apart suggests further that either fluorescence or phosphorescence could be used for the determination of both antioxidants in a mixture without the need for separation (Figures 1 and 2). A study of phosphorescence decay of the three antioxidants has shown that the mean lifetime of BHT is short compared to BHA and PG (Table I). It should therefore be possible to determine either BHA or PG in the presence of BHT by the use of decay curves (Winefordner, 1966). The above suggestions do not exhaust all possible methods of luminescence analysis for the antioxidants.

Fluorescence of Antioxidants in Chloroform. As can be seen from Figure 3 and Table I, the fluorescence of both BHA and BHT in chloroform is extremely weak. In fact, their fluorescence is almost identical with the background emission of chloroform, and therefore it was not possible to determine accurately a maximum excitation and emission wavelength for BHA and BHT in chloroform. The weak fluorescence of BHA might be due partially to the heavy atom effect (Wehry, 1967), whereby there is an increase in the rate of intersystem crossing from the singlet to the triplet state of the molecule. Since phosphorescence usually occurs from the lowest triplet state of a molecule, the relative phosphorescence intensity of BHA in chloroform should be greater than for fluorescence, if the rate of intersystem crossing is increasing. Even though chloroform forms a snow at liquid nitrogen temperature, which would decrease the measured phosphorescence intensity,

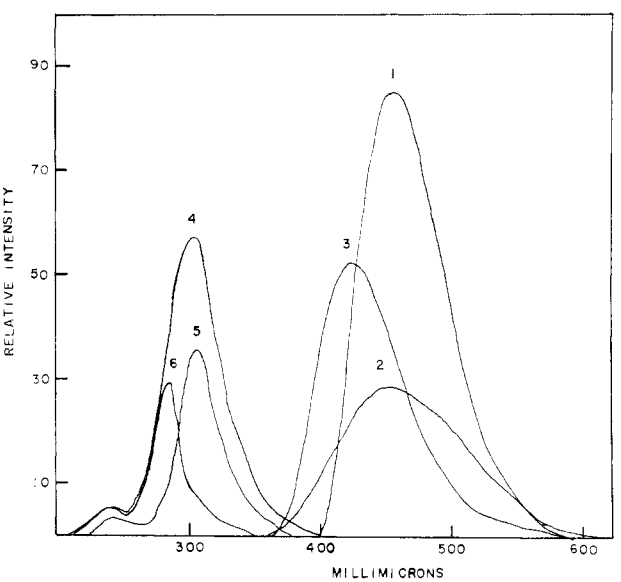

Figure 1. Fluorescence and excitation spectra of antioxidants in ethanol

1. Fluorescence spectrum of PG
2. Fluorescence spectrum of BHA
3. Fluorescence spectrum of BHT
4. Excitation spectrum of PG
5. Excitation spectrum of BHT
6. Excitation spectrum of BHA

No intended correlation from plot to plot of relative intensities

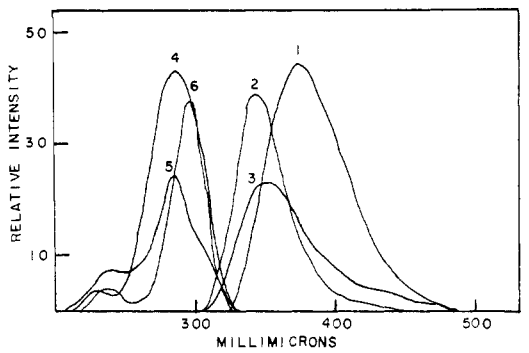

Figure 2. Phosphorescence and excitation spectra of antioxidants in ethanol

1. Phosphorescence spectrum of PG
2. Phosphorescence spectrum of BHT
3. Phosphorescence spectrum of BHA
4. Excitation spectrum of PG
5. Excitation spectrum of BHA
6. Excitation spectrum of BHT

No intended correlation from plot to plot of relative intensities

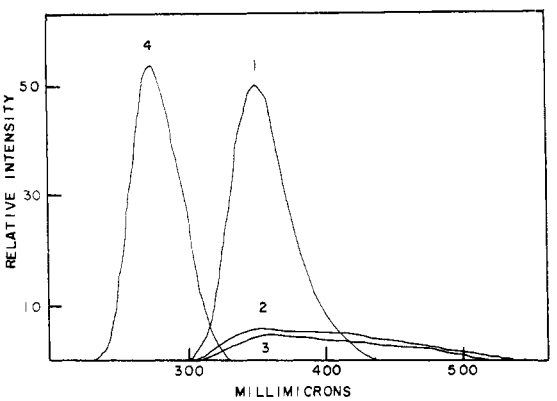

Figure 3. Fluorescence spectra of antioxidants in chloroform
All antioxidants at 4 μg./ml. and excited at 272 mμ

1. Fluorescence spectrum of PG
2. Fluorescence spectrum of BHA (sensitivity × 27 compared to 1)
3. Fluorescence spectrum of BHT (sensitivity × 27 compared to 1)
4. Excitation spectrum of PG

the intensity of BHA was about 10 times greater than the fluorescence intensity of BHA in chloroform at room temperature. The fact that the emission intensity is still low indicates that the absorbed energy is being lost by other processes. No definite conclusions could be drawn for BHT, because the phosphorescence intensity in chloroform was very low.

Determination of PG Directly in Lard in Presence of BHA and BHT. Since BHA and BHT essentially do not fluoresce in chloroform, and the background emission from antioxidant-free lard samples is very low (Table II), it was felt that the fluorescence emission of PG could be used for its qualitative and quantitative determination in lard. Table III gives the results of a number of analyses where the antioxidants were added to uninhibited lard samples. Some of the concentrations that were added were within the range of governmental regulations. The lard samples were checked for the antioxidants by a thin-layer chromatography procedure similar to the one used by Scheidt and Conroy (1966). However, the excitation and emission spectra of PG in a lard sample in chloroform can be used for qualitative identification of PG directly in lard in the presence of BHA, BHT, or both. By comparing the relative intensities of the blank solutions with the solutions containing PG in Table II, it can be seen that as little as 0.12 μg. per ml. and less of PG can be detected. In general Table III indicates that the percentage of PG in a sample will be high if a blank value is not subtracted and can be high or low if a blank value is subtracted. The error involved in both cases, however, is not serious at such low concentrations. Therefore, PG can be determined accurately with or without subtracting a blank value. Samples 7 and 8 contained five times as much BHA and BHT as PG and there was virtually no interference from BHA and BHT in the determination of PG.

Table IV illustrates the determination of PG in inhibited commercial lard samples, all of which contained either BHA or BHT. A blank value was not subtracted in the analysis of these samples. The presence of PG in one supposedly antioxidant-free lard sample was detected by obtaining the fluorescence excitation and emission spectra of PG in lard and then checked by thin-layer chromatography. With another sample it was necessary to spot four times the amount

Table II. Comparison of Relative Fluorescence Intensity of Uninhibited and Inhibited Lard Samples in Chloroform

Sample	Relative Intensity, Uninhibited Lard[a]	Sample	Relative Intensity, Inhibited Lard[b]	PG Concn., μg./Ml.
1	0.06	6	2.37	2.00
2	0.05[c]	7	0.55	0.40
3	0.06	8	0.17	0.12
4	0.06	9	1.04	0.80
5	0.07	10	0.52	0.40

[a] Blank solutions, 0.010 g./ml. of lard.
[b] 0.010 g./ml. of lard, blank value not subtracted.
[c] Contained BHT.

Table III. Analysis of Lard Samples of Known PG Content by Standard Addition Method

Sample	Added, % BHA	Added, % BHT	Added, % PG	PG Found, %, Blank Not Subtracted[a]	Error, %	PG Found, %, Blank Subtracted	Error, %
1	0.0120	.....	0.0040	0.0040	0.0	0.0037	−7.5
2	0.0120	.....	0.0040	0.0036	−10.0	0.0032	−20.0
3	0.0400	.....	0.0200	0.0215	+7.5	0.0205	+2.5
4	0.0400	.....	0.0200	0.0205	+2.5	0.0190	−5.0
5	0.0080	0.0080	0.0040	0.0042	+5.0	0.0040	0.0
6	0.0080	0.0080	0.0040	0.0050	+25.0	0.0045	+12.5
7	0.0400	0.0400	0.0080	0.0087	+8.8	0.0085	+6.3
8	0.0400	0.0400	0.0080	0.0085	+7.5	0.0080	0.0
9	.....	.....	0.0012	0.0016	+33.4	0.0010	−16.7
10	.....	.....	0.0012	0.0019	+41.6	0.0013	+8.5

[a] Blank solution 0.010 g./ml. of uninhibited lard in chloroform.

of solution normally specified for TLC analysis (Scheidt and Conroy, 1966). However, by obtaining the excitation and emission spectra of PG in a chloroform lard solution PG was quickly identified.

A standard addition method was used for a number of reasons. First, variations in source intensity and phototube aging can change a calibration curve, even though the instrument sensitivity is adjusted with a standard. By using a standard addition method these problems are practically eliminated, since the relative intensity data for one analysis can be obtained within 30 minutes. More importantly, slight variations in the composition of different lard samples could possibly alter the relative fluorescence intensity of PG from one lard sample to another, but by using a standard addition method this source of error should be eliminated. Also, in the standard addition method the concentration of PG increases with each reading and the background emission from the lard remains constant. Therefore, as the concentration of PG increases the background emission from the lard is less important. For example, one of the antioxidant-free samples in Table IV gives a relative intensity of 0.06. The final relative intensity reading in a typical analysis would be 2.54. Therefore, the background emission does not contribute greatly to the total emission.

The limit of detection by the standard addition method is near 0.0005% PG in a solid lard sample, which would yield a concentration of 0.05 μg. per ml. in the final analysis solution using the method described. At lower concentrations it would be necessary to use larger samples of lard and subtract a blank value from the relative intensity values. By doing this the lower limit of detection can be extended even more. The maximum amount of PG that can be detected in a solution of chloroform is 4 μg. per ml., because concentration quenching begins to occur near this concentration. Therefore, if any of the solutions used in the analysis contained more than 4 μg. per ml. it would be necessary to dilute the solutions to eliminate concentration quenching.

Table IV. Determination of Propyl Gallate in Commercial Lard Samples

Sample	% PG
1	0.0025
	0.0026
2	0.0021
	0.0020
3	0.0042
	0.0039

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