

The Determination of Butylated Hydroxyanisole (B.H.A.), Butylated Hydroxytoluene (B.H.T.) and Individual Gallate Esters in Fats and Oils by High Performance Liquid Chromatography

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The method describes a simple extraction of fats and oils followed by the simultaneous determination of five antioxidants by high performance liquid chromatography. A separate extraction procedure for the quantitative confirmation of B.H.T. is also proposed.

The "Antioxidants in Food Regulations 1974", SI 1974 No. 1120, define propyl, octyl and dodecyl gallate, B.H.A. and B.H.T., among others, as being permitted antioxidants. In Schedule 3 to these Regulations, the maximum permitted level of antioxidant in anhydrous oils and fats is listed at 100 p.p.m. for propyl gallate or octyl gallate or dodecyl gallate or any mixture thereof and 200 p.p.m. for B.H.A. or B.H.T. or any mixture thereof. There are a considerable number of publications¹⁻²⁰ covering the determination of one or more of the above mentioned antioxidants.

Extraction techniques include steam distillation,¹ solvent distillation,² solvent extraction,³⁻¹⁶ and vacuum sublimation²⁰. Detection has been accomplished by colorimetry,¹ thin layer chromatography (T.L.C.),^{6,10,11,12,15,16} spectrophotometry,^{8,9,18,19} polarography,¹⁷ paper chromatography,^{13,14} gas chromatography (G.C.)^{2,3,4,5,20} and high performance liquid chromatography (H.P.L.C.)⁷.

When the analyses of fats and oils for antioxidants are contemplated, problems may arise with the need for removal of potentially interfering co-extractives. Steam distillation provides a clean extract although time consuming when routine analyses are required and it may give rise to low recoveries⁷. Solvent distillation alone is not suitable for individual determination of gallates² and solvent extraction techniques prior to T.L.C. or G.C. require extensive clean-up.

The proposed method describes a simple extraction of the melted sample with methanol followed by removal of excess fat prior to the simultaneous analysis of the five antioxidants with the aid of an internal standard by gradient elution H.P.L.C.

A modification of a method by Phipps⁷ is employed for the quantitative determination of B.H.T.

Experimental

APPARATUS

Two Waters Associates M 6000A HPLC pumps.

† Now with Waters Associates Limited, 324 Chester Road, Hartford, Northwich, Cheshire CW8 2AH.

Waters Associates U6K sample injection system.

Waters Associates M 660 solvent programmer.

Applied Chromatography Services 750-21 variable wavelength u.v. monitor, operating range 200–600 nm.

Servoscribe RE 571.20 pen recorder.

Water Associates 10 μ m Bondapak C₁₈ 30 cm $\times$ 4 mm i.d. reverse phase column.

Precision Sampling Corporation B-110 pressure lok 25 μ l syringe.

M.S.E. "Multex" centrifuge.

Jobling type 319 vacuum rotary evaporator.

Waters Associates organic clarification kit comprising a 0.5 μ m filter.

REAGENTS

1. *Distilled water*.
2. *Methanol* (Rathburn glass distilled grade).
3. *Acetic acid glacial* (Fisons AR), a molar solution prepared in distilled water.
4. *N-Heptane* (Fisons SLR).
5. *Dimethyl sulphoxide* (DMSO) (BDH AR).
6. *Petroleum ether* (B.P. 30–40°C) (Fisons low in aromatics).
7. *Anhydrous sodium sulphate* (BDH AR) heated at 450°C overnight to remove organics.
8. *2-Ethoxyethanol* (Fisons SLR).
9. *Methanol*: M acetic acid in distilled water (80:20).
10. *3:4-Xylenol* (Fisons SLR).

PREPARATION OF SOLUTIONS

- (1) An internal standard comprising 100 p.p.m. of 3:4-xylenol in methanol.
- (2) A mixture of B.H.A., B.H.T., octyl gallate, dodecyl gallate each at 100 p.p.m. and propyl gallate at 50 p.p.m. in methanol.
- (3) A standard solution made up of equal volumes of (1) and (2) above.
- (4) A 100 p.p.m. B.H.T. standard in methanol is prepared for the quantitative confirmation of B.H.T.

COLUMN OPERATING CONDITIONS A

For simultaneous antioxidant analysis:

<i>Mode</i>	Gradient elution.
<i>Mobile phases</i>	M acetic acid in distilled water in pump A, methanol in pump B.
<i>Gradient</i>	The programme consists of a linear gradient from 55 per cent. methanol to 85 per cent. methanol in 15 minutes and a 10 minute hold at 85 per cent. methanol. To return to initial conditions a reverse programme of 10 minutes is used.
<i>Flow rate</i>	1.5 ml/min: this produces a back pressure of approximately 3500 lb/in ² at 55 per cent. methanol.

Detector U.v. at 280 nm, 0.1 absorbance units full scale.
Figure 1 shows a chromatogram obtained from a 25 μ l injection of the standard solution.

Chart speed 300 cm/hour.

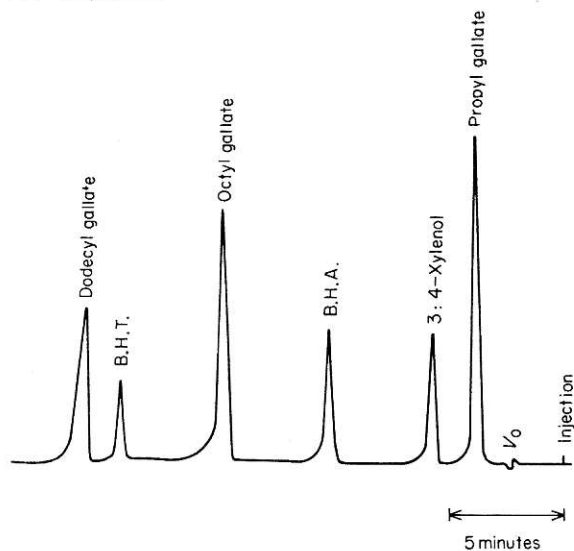


Fig. 1. HPLC trace from a 25 μ l injection of standard solution.

COLUMN OPERATING CONDITIONS B

For quantitative confirmation of B.H.T.:

Mode Isocratic elution.

Mobile phase 80 per cent. methanol/20 per cent. M acetic acid.

Flow rate 1.5 ml/min: this produces a back pressure of approximately 2750 lb/in².

Detector U.v. at 280 nm, 0.1 absorbance units full scale.
Figure 2 shows a chromatogram obtained from a 25 μ l injection of the B.H.T. standard.

Chart speed 300 cm/hour.

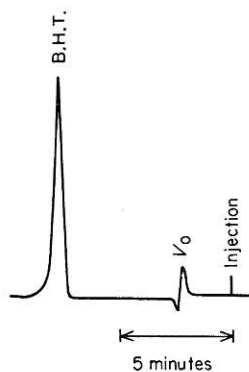


Fig. 2. HPLC trace from a 25 μ l injection of standard solution of B.H.T..

METHOD

Weigh approximately 20 gm of sample into a stoppered centrifuge tube of 70 ml capacity and warm in a water bath at 40–50°C until the sample has melted. Add 20 ml of methanol by pipette and shake vigorously for 2 minutes, allow to settle and centrifuge for 10 minutes at 3000 rev./min. Transfer to a deep freeze for a few hours to aid the solidification of any excess fat from the methanol layer, then decant and filter the methanol extract through an organic clarification kit or suitable alternative. Dilute a portion of this filtrate with an equal volume of internal standard solution and inject 25 μ l on to the column using operating condition A. Following the reverse programme and re-establishment of equilibrium at the initial conditions (shown by the return to a steady back pressure) make a 25 μ l injection of the standard solution for evaluation purposes. Retention times are shown in Table I.

TABLE I
RETENTION DATA FOR THE FIVE ANTIOXIDANTS UNDER COLUMN OPERATING CONDITION A

Compound	Retention time, minutes	Retention time relative to 3:4-xylene
Non-retained peak (V_0)	2.3	0.39
Propyl gallate	4.0	0.68
3:4-xylene	5.9	1.00
B.H.A.	10.3	1.75
Octyl gallate	14.9	2.53
B.H.T.	19.5	3.31
Dodecyl gallate	21.1	3.56

If B.H.T. is identified from its retention time and quantitative confirmation is required, the following method is proposed.

Weigh accurately approximately 4 gm of sample, dissolve in 20 ml of *n*-heptane and extract four times with 25 ml portions of D.M.S.O. Add 200 ml of distilled water to the combined D.M.S.O. extracts, allow to cool and extract with two 75 ml portions of 30–40°C petroleum ether. Filter the combined petroleum ether through anhydrous sodium sulphate, add 0.5 ml of 2-ethoxyethanol and rotary-evaporate until all the petroleum ether has been removed. Transfer the remaining liquid to a 5 ml volumetric flask and make up to the mark with methanol, rinsing the rotary evaporator flask in the process. Filter as in the initial extraction procedure and inject 25 μ l on to the column using operating condition B. After a suitable time has elapsed (see Table II) inject 25 μ l of the B.H.T. standard onto the column.

TABLE II
RETENTION TIME OF B.H.T. UNDER COLUMN OPERATING CONDITION B

Compound	Retention time, minutes
Non-retained peak (V_0)	2.3
B.H.T.	7.6

Results and Discussion

The chromatogram of the standard solution is shown in Figure 1 whilst the retention data are given in Table I. Quantitative assessment of any antioxidant found in a sample is determined from the ratio of the peak heights of the antioxidant and internal standard for both the standard solution and the sample.

Calibration graphs prepared by plotting the amount of antioxidant against the ratio of the peak height for the antioxidant to the peak height for the internal standard are linear over the range of 25–250 p.p.m. for all the antioxidants. The concentration of antioxidant in a sample is thus determined from the following equation:

$$\text{concentration of antioxidant in sample in p.p.m.} = \frac{20}{S} \times \frac{A_{\text{sam}}}{I_{\text{sam}}} \div \frac{A_{\text{std}}}{I_{\text{std}}} \times X,$$

where S = weight of sample taken in gm,

A_{sam} = peak height measurement of antioxidant in the sample,

I_{sam} = peak height measurement of internal standard in the sample,

A_{std} = peak height measurement of antioxidant in the standard,

I_{std} = peak height measurement of internal standard in the standard,

X = concentration of antioxidant in the standard in p.p.m. before dilution with the internal standard.

The reproducibility of retention data and peak height ratios under gradient elution conditions have been found to be within ± 1 per cent. during a working day. Day to day variations can, however, exceed this figure probably because of changes in ambient temperature affecting the efficiency of the column.

Recoveries from standard additions of antioxidants to fats and oils are listed in Table III.

TABLE III
RECOVERY OF ANTIOXIDANTS ADDED TO FATS AND OILS

Antioxidant	Level of addition, p.p.m.	Sample type and recovery, per cent.				Mean recovery with standard deviation, per cent.
		Lard	Fat	Dripping	Oil	
Propyl gallate	50	108	107	104	104	106 $\pm$ 3
	100	102	105	102	106	104 $\pm$ 2
	200	106	106	106	105	106 $\pm$ 1
Octyl gallate	50	102	101	95.5	94	98 $\pm$ 4
	100	99	104	103	103	102 $\pm$ 3
	200	96	98	95	96	96 $\pm$ 1
Dodecyl gallate	50	91	93	85	96	91 $\pm$ 5
	100	91	92	90	92	91 $\pm$ 3
	200	90	88	89	91	89 $\pm$ 1
B.H.A.	50	72	68	70	63	68 $\pm$ 5
	100	68	70	71	68	69 $\pm$ 1
	200	72	71	70	67	70 $\pm$ 2
B.H.T.	50	40	35.5	34	28	34 $\pm$ 4
	100	39	33	38	33	36 $\pm$ 3
	200	37	34	37	32	35 $\pm$ 2

The low recoveries for B.H.T. necessitate the adoption of an extraction procedure specifically for B.H.T. when an accurate quantitative figure is needed. Using this procedure, Figure 3 shows the chromatogram relevant to a sample of

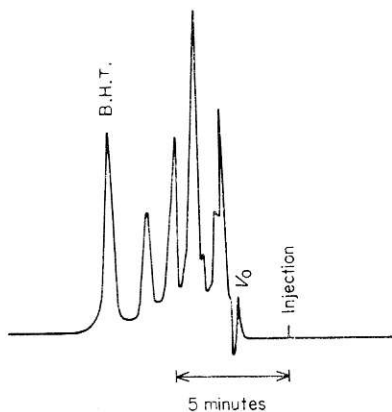


Fig. 3. Trace from a 25 μ l injection of a lard extract spiked with 100 p.p.m. of B.H.T.

lard spiked with B.H.T. at the 100 p.p.m. level with peaks for co-extracted material well separated from the B.H.T. peak. Calculation of the amount of B.H.T. in a sample is by comparison with a known standard of B.H.T.

$$\text{Concentration of B.H.T. in sample in p.p.m.} = \frac{H_{\text{sam}}}{H_{\text{std}}} \times \frac{5}{S} \times X,$$

where H_{sam} = peak height measurement of B.H.T. in sample,

H_{std} = peak height measurement of B.H.T. in standard,

S = weight of sample taken in gm,

X = concentration of B.H.T. standard in p.p.m.

The use of isocratic conditions in the determination of B.H.T. provides greater precision than is afforded by the use of gradient elution and an internal standard was not found to be necessary with the U6K injector. Recoveries from this procedure listed in Table IV are in agreement with the recoveries found by

TABLE IV
RECOVERY OF B.H.T. ADDED TO FATS AND OILS

Level of addition, p.p.m.	Recovery, per cent.	
	Phipps method ⁷	Proposed method
100	97 average	100
200		98

Phipps⁷. The limits of detection for the antioxidants in fats and oils using column operating condition A are 10 p.p.m. for the gallates and 20 p.p.m. for B.H.A. and B.H.T. These are by no means the ultimate limits of detection but are set to minimise interference from organic contamination present in the

distilled water and baseline drift caused by the change in solvent composition during the gradient programme. Figure 4 shows a typical sample chromatogram using these operating conditions.

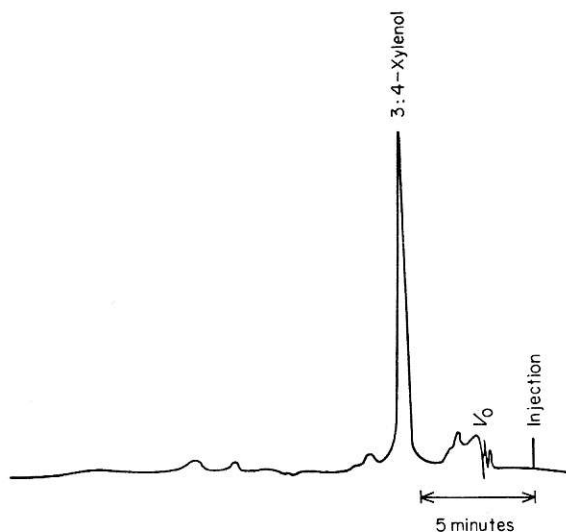


Fig. 4. Trace from a 25 μ l injection of an extract of dripping.

To obtain greater sensitivity, organic contaminants may be removed from the distilled water by passing it through the column and collecting as it elutes. The contaminants are retained on the column and are removed by flushing the column with approximately 30 ml of methanol at a flow rate of 1.5 ml/min.

Excessive baseline drift at high detector sensitivities can give rise to difficulties of measurement. To overcome this, isocratic elution at a suitable solvent composition may prove successful where measurement of an individual peak is

TABLE V
ANTIOXIDANT FOUND IN SUBMITTED SAMPLES

Sample type	Antioxidant found, <i>p.p.m.</i>		
	B.H.T.	B.H.A.	Dodecyl gallate
American lard	35		
American lard	77		
American lard	44		
American lard	41		
English lard	39		
Refined lard	42		19
Lard	52		13
Lard	105		
Lard	32		
Lard	35		
Lard	100		
Lard			18
Refined dripping	20	62	

All the above samples bore labels declaring the presence of permitted antioxidants.

required. Contamination from repeated sample injections is not a problem, as any build-up of fat on the column is simply removed at the end of a working day by flushing with several column volumes of methanol.

Forty-three samples comprising lards, fats, drippings and vegetable oils have so far been analysed. Thirteen of those were found to contain antioxidant. The results are listed in Table V.

Conclusion

The use of HPLC permits the rapid, simultaneous determination of antioxidants in fats and oils without the need for extensive sample clean-up so characteristic of many other techniques. Any accumulation of fat on the column is easily removed, enabling routine assays to be carried out without any deterioration in column performance.

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