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DETERMINATION OF ANTIOXIDANTS IN PALM OIL PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were found to be the common antioxidants used in the palm oil industry. They are added to palm oil products either singly or in combination within the permissible limit of 200 parts per million. A procedure for the determination of the phenolic antioxidants which is based on the AIIBP method using a single step methanolic extraction and reverse phase gradient elution by HPLC was proven to be very simple and reliable.

INTRODUCTION

The widespread use of synthetic antioxidants such as t-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) in oils and fats and oil-containing food products, and the perceived carcinogenic effect of these compounds has stimulated the development of numerous techniques for their determination. Qualitative and quantitative methods are needed for experimental and quality control purposes and to determine whether the level of any of these compounds in a product is within the permissible limit.

There are many methods for the determination of the phenolic antioxidants, but High Performance Liquid Chromatography (HPLC) is now considered the most efficient. The technique is more specific than the traditional spectrometry methods, which often determine the concentration of total reducing substances and give no indication of the nature of the individual antioxidant(s) present. HPLC methods are relatively easier to perform and more precise than Gas Liquid Chromatography (GLC) techniques which require derivatization. From a practical viewpoint, HPLC is the method of choice as it can be used to determine a full range of antioxidants, from the polar PG to the less polar BHT, in a single analysis.

A procedure for extracting 50-100 ppm of

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BHA and BHT from vegetable oils using heptane, dimethyl sulphoxide (DMSO), sodium chloride and petroleum ether has been reported (Phipps, 1973). A reversed phase gradient elution method to quantify BHA, BHT, PG, octyldodecylgallate (OG) and dodecylgallate (DG) in oils and fats and a simultaneous determination of nine phenolic antioxidants in vegetable oils, lard and shortenings has also been reported (Hammond, 1978; Page, 1979). Page's procedure was adopted as the AOCS Ce 6-86, AOAC 983.15 and IUPAC 2.432 standard methods (AOCS, 1990; AOAC, 1992; IUPAC, 1992) since it gave excellent recoveries and low coefficients of variation when tested in a collaborative study (Page, 1983).

In the collaborative study (Page, 1983) , the organizer reported that seven out of eight laboratories that participated showed mean recoveries of 93.2, 95.6, 98.3 and 84.8% and mean coefficients of variation of 5.02, 19.3, 3.75 and 3.45% for PG, TBHQ, BHA and BHT respectively at levels between 20 and 200 ppm. The recovery for BHT was relatively low because it is less polar and difficult to extract from the oil/fat matrices. In 1987, an international interlaboratory test was carried out by the IUPAC (IUPAC, 1992). The organizer reported that good agreements were obtained between most of the eleven participating laboratories in which PG, TBHQ, BHA, BHT were among the seven antioxidants tested.

In evaluating the AOAC/IUPAC/AOCS methods based on the method developed by Page, we obtained comparable results in term of recoveries and precision for four antioxidants – PG, TBHQ, BHA and BHT – when spiked in palm oil at levels between 50 and 200 ppm. The extraction procedure requires the oil to be dissolved in hexane, with subsequent partitioning of the antioxidants into acetonitrile. This is followed by concentration and dilution with isopropanol to give a solution in isopropanol - acetonitrile (1:1) prior to reverse phase gradient eluation by HPLC. The drawback of this method is that it is tedious and time consuming.

In the work reported here, a simpler method (AIIBP, 1994) was used, involving one-step methanol extraction of the antioxidants from the oil/fat matrices prior to reverse-phase gradient eluation by HPLC. The method was used to survey antioxidant(s) in palm oil and palm oil products, and in monitoring the retention of antioxidant(s) in frying oil (palm olein) during deep-frying of potato chips and during handling of palm olein *i.e.* during storage and shipment and at the port of discharge. Marketplace food samples that were known to contain palm oil were also analysed to ascertain the type and level of the synthetic antioxidant(s) used by the manufacturers.

MATERIALS AND METHODS

Instrumentation

The liquid chromatograph was a Waters Assoc. Model 600-E pump from the USA, equipped with a 20 µl sample loop and a Rheodyne injector valve. A Waters Model 490-E UV detector was used to measure absorbance at 280 nm. Separation was carried out on a Supelco (of USA) stainless steel chromatographic column (150 x 4.6 mm i.d.) packed with RP-18 silica gel (5 µm), bonded with octadecyl dimethyl derivates and fitted with an Alltech (of USA) guard column of RP-18 (5 µm).

Centrifugation was by means of a Sigma Model 3 MK laboratory centrifuge with a rotor of internal diameter 14 cm, allowing up to 15,000 rpm, linked to an NEC Model P-3200 dot-matrix printer. All glassware used was rinsed with chloroform followed by acetone and methanol and dried under nitrogen.

Reagents

All the solvents used, *viz.* methanol, acetonitrile, hexane and acetic acid, were of HPLC grades purchased from Merck (of Germany). Standards of PG, BHA and BHT were purchased from Sigma while TBHQ was from Tokyo Chemical Industry Ltd., Tokyo, Japan.

Mobile Phase Solutions

A solution of acetonitrile containing 1% acetic acid (solvent A) and a solution of distilled water containing 1% acetic acid (solvent B) were prepared.

Stock Solutions

25 mg of PG, TBHQ, BHQ and BHT were weighed accurately into the same 50 mL

volumetric flask and methanol was added to the mark. The flask was shaken until a homogenous and clear solution was formed. This stock solution, containing 500 ppm of the mixed antioxidants, was transferred into an amber bottle and stored in a freezer at -20°C.

Reference Standard Solutions

A reference standard solution containing a mixture of 50 ppm of each of the above antioxidants was prepared daily by pipetting 1 mL of the stock solution into a 10 mL volumetric flask and topping up to the mark with methanol.

Recovery Studies

Three lots of palm olein (with no added antioxidant) were spiked with respectively, a mixture of 50, 100 and 200 ppm each of PG, TBHQ, BHA and BHT. This was done by heating 1 kg of olein at 70°C and adding an appropriate amount of the antioxidants, first as a 10% solution of palm olein and then into the bulk of the heated oil. Using a magnetic stirrer, the mixture was vigorously stirred for 7 hours to achieve complete dispersion of the antioxidants in the oil matrix. The spiked samples were transferred into amber bottles, flushed slowly with N_2 and stored in a freezer (-20°C) before extraction and analysis by HPLC.

Extraction of Antioxidants from Palm Oil Samples

Palm olein samples were heated to 60°C and 2 g of each (in duplicate) were accurately weighed into test tubes and exactly 10 mL of methanol was added. The mixtures were vigorously shaken for 15 min and centrifuged for 30 min at 2000 rpm. The clear upper layer were recovered for HPLC analysis. A double extraction of the same palm olein sample containing 50 ppm of the four antioxidants was also carried out.

Chromatography

A linear gradient was used, with a flow rate of 2 mL per minute from 30% of solvent A in 70% of solvent B to 100% solvent A in seven minutes then holding for five minutes at 100% of A. The elution programme was returned to 30% solvent A over one minute at 2 mL per minute and then allowed to stabilize for 2-3 minutes.

20 µL of the working standards were injected and the elution programme was applied. Similarly, 20 µL of sample solutions was injected and the same elution programme was applied. Dilution with methanol (dilution factor D – see below) was done for samples with any off-scale peak. A reagent blank was performed by injecting 20 µL of the methanolic extract of 2 g hexane in place of oil sample.

CALCULATION

Measurement of Peaks

The average peak area of duplicate injections of the mixed antioxidants reference standard was taken after correcting for the reagent and gradient blank. The average peak areas of duplicate injections of palm oil samples (prepared in duplicate) were also taken after correcting for the reagent and gradient blank.

Expression of Results

The antioxidant content (A), expressed in mg per kg (ppm) can be calculated from the formula:

$$A = \frac{C_{st} \times R_s \times 10 \times D}{R_{st} \times m}$$

Where:

C_{st} is the concentration of the standard solution
D is the dilution factor
R_s is the peak area of the sample antioxidant
R_{st} is the peak area of the standard antioxidant
m is the mass of the sample (g) in the final extract.

RESULTS AND DISCUSSION

Figure 1A shows a typical chromatogram of the four antioxidants, *i.e.* PG, TBHQ, BHA and BHT. The four antioxidants were well separated in less than eight minutes. Good baselines and sensitivities were obtained for the standards mixtures. PG was the first to be

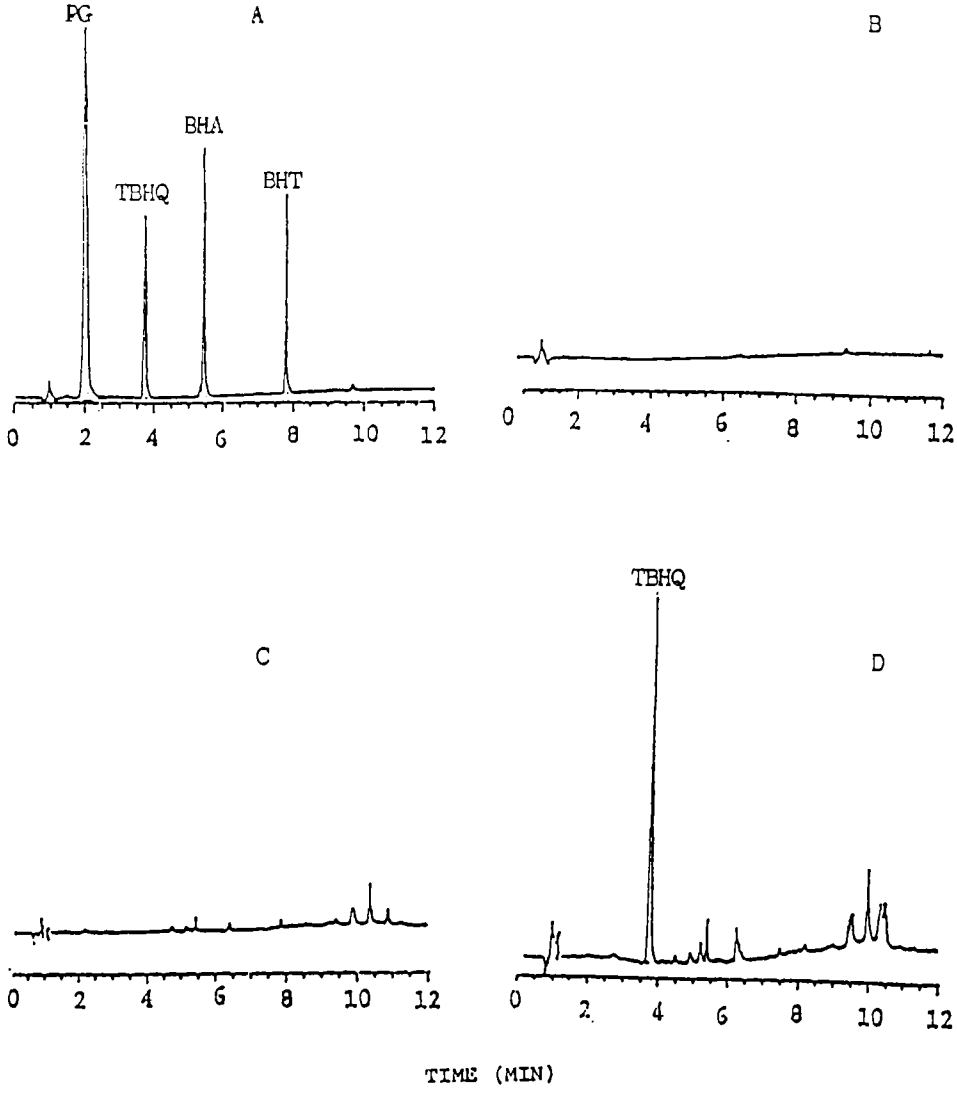

Figure 1. HPLC chromatograms of
A: Antioxidant standards of about 50 µg/ml each of PG, TBHQ, BHA and BHT;
B: Blank gradient;
C: Palm olein (cooking oil) containing no added antioxidant(s);
D: Palm olein containing about 187 ppm TBHQ.

eluted since it is the most polar, while BHT, being the least polar of the four was eluted last: the results of this were consistent with other studies (Page, 1979; Page, 1983; Page and Charbonneau, 1989). In term of individual response, PG was the most sensitive, followed by BHA, BHT and TBHQ.

A serious valley-to-valley baseline drift during elution of a mixture containing seven antioxidant standards (including PG, TBHQ, BHA and BHT) was observed by some participants in the collaborative work organized by Page (1983). In our study repeated calibration with the mixture of four antioxidant standards did not give this problem, which otherwise would result in underestimating the standard peak area/ height. *Figure 1B* shows a chromatogram of the blank gradient with a somewhat sloping baseline. A slopping baseline and/or interference from the BHT peak were also observed by others (Page, 1979; Page, 1983; Page and Charbonneau, 1989) and the problems were attributed to impurities in the acetic acid and/ or water used. In this study, it was observed that the size of the sloping baseline and any peaks eluting with it was very small and they usually appeared after eight minutes.

Chromatograms of methanolic extracts of palm oil products such as shown in *Figures 1C* (palm olein or cooking oil containing no antioxidants) and *1D* (palm olein containing about 187 ppm TBHQ) showed larger sloping baselines accompanied by a number of peaks, however. This is expected and similar findings have been reported for other oils and fats (Page, 1979; Page 1983; Page and Charbonneau, 1989). This phenomenon was attributed to some lipids and other non-polar materials being co-extracted and accumulated at the head of the column. These artefacts then appeared during the course of elution. In any case the slopping baseline and peaks eluted with it disappeared during the stabilization programme used prior to the injection of the next palm oil sample. The elution profiles of some samples of palm oil-based vanaspati (ghee), margarine, dough fat (baking fat) and shortening containing different types and levels of the antioxidant(s) are shown in *Figures 2A, 2B, 2C* and *2D* respectively.

A study on recovery of the four antioxidants was also carried out. Palm olein was spiked with a mixture of the four antioxidants from 50-200 ppm (w/w) to reflect the levels normally added by palm oil refiners or processors. The results of the recovery studies at the 50 and 100 ppm levels are shown in *Table 1.* The recovery rates, standard deviations and coefficients of variation were found to be comparable with those reported for other oils and fats (Page, 1979; Page, 1983; IUPAC, 1992). As usual, BHT, being less polar, was quite difficult to extract completely from the oil matrix, even with centrifuging times extended to 30 minutes, and this accounted for

TABLE 1. RECOVERY OF ANTIOXIDANTS ADDED TO PALM OLEIN (AFTER A SINGLE EXTRACTION)

	50 ppm added				100 ppm added			
Antioxidant	**Found[a] (ppm)**	**Recovery (%)**	**S.D. (ppm)**	**C.V. (%)**	**Found[a] (ppm)**	**Recovery (%)**	**S.D. (ppm)**	**C.V. (%)**
PG	47.7	95.4	1.9	4.0	96.7	96.7	3.1	3.2
TBHQ	47.0	94.0	2.5	5.3	97.3	97.3	3.0	3.1
BHA	51.5	103.0	2.1	4.1	101	101	2.6	2.6
BHT	44.5	89.0	3.7	8.3	90.7	90.7	5.4	5.9

[a]Mean of 5 determinations.

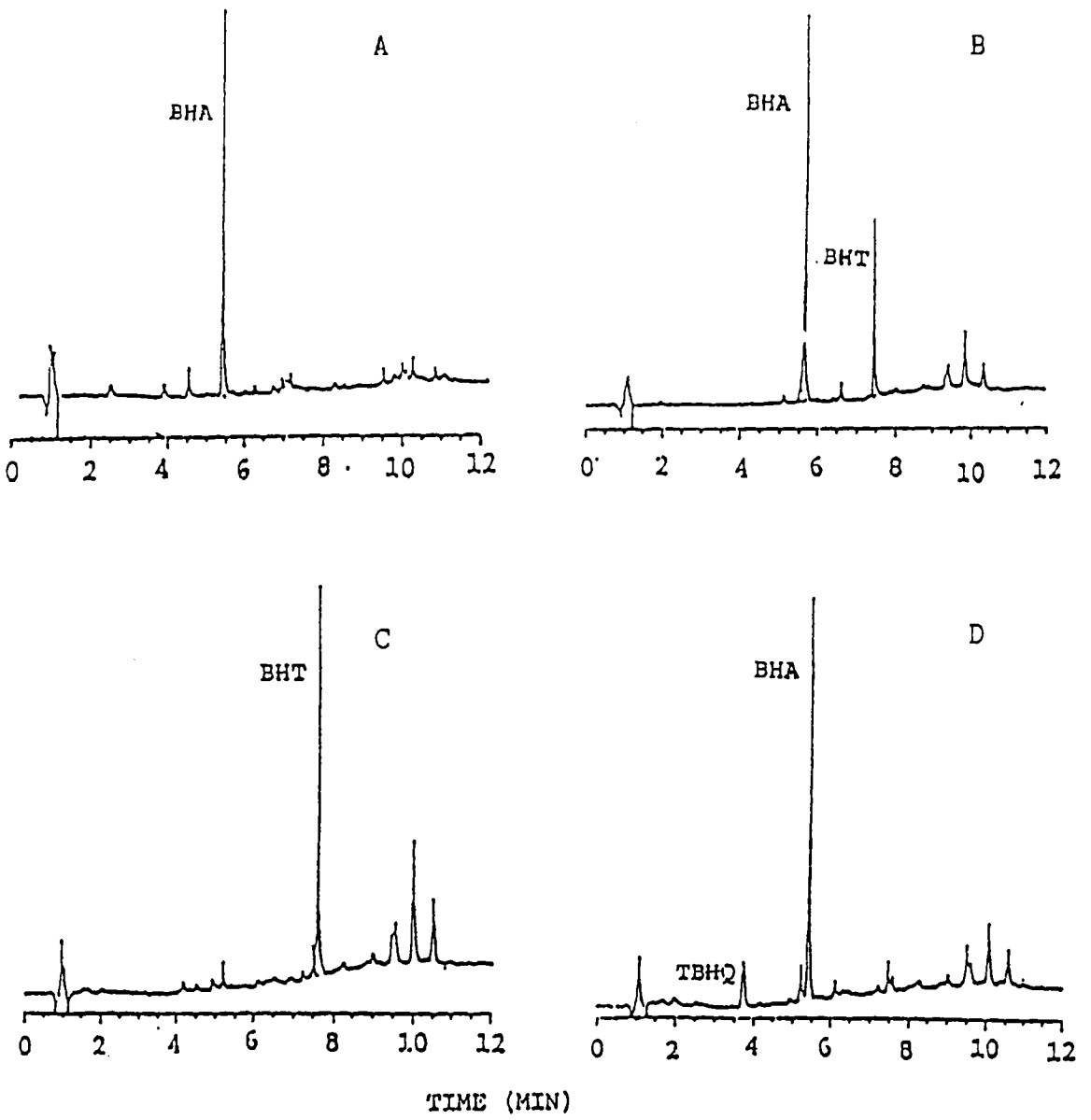

Figure 2. HPLC chromatograms of
A: Vanaspati containing 101 ppm BHA,
B: Margarine containing 110 ppm BHA and 81 ppm BHT,
C: Dough fat containing 150 ppm BHT, and
D: Shortening containing 50 ppm TBHQ and 152 ppm BHA.

TABLE 2. RECOVERY OF ANTIOXIDANTS (50 PPM) ADDED TO PALM OLEIN (AFTER A DOUBLE EXTRACTION)

Antioxidants	Found[a] (ppm)	Recovery (%)	S.D. (ppm)	C.V. (%)
PG	51.5	103.0	2.2	4.3
TBHQ	50.3	100.5	2.8	5.7
BHA	52.4	104.7	2.0	3.8
BHT	47.2	94.4	4.0	8.5

[a]Mean of 5 determinations.

the lower recovery values observed. A double extraction at the 50 ppm level was found to improve the recoveries of PG, TBHQ and BHA to about 100% and BHT to about 94% *(Table 2)*.

The method was used to survey 22 commercial samples of palm oil products containing up to 80% palm oil (*e.g.* margarines) and 100% palm olein (*e.g.* cooking oil). The results *(Table 3)* indicate that PG is rarely

TABLE 3. ANTIOXIDANTS FOUND IN PALM OIL PRODUCTS

No.	Sample[a]	Antioxidant (ppm)			
		PG	TBHQ	BHA	BHT
1.	Shortening A	–	–	202	–
2.	Shortening B	–	104	–	–
3.	Shortening C	–	–	47	43
4.	Shortening D	–	50	152	–
5.	Vanaspati/Ghee A	–	–	101	–
6.	Vanaspati/Ghee B	–	–	95	89
7.	Vanaspati/Ghee C	–	97	–	–
8.	Pourable Shortening A	–	35	–	–
9.	Pourable Shortening B	–	47	–	–
10.	Dough Fat A	–	–	–	150
11.	Dough Fat B	–	–	–	93
12.	Dough Fat C	–	–	–	99
13.	Margarine A	–	–	84	73
14.	Margarine B	–	45	48	–
15.	Margarine C	–	–	110	81
16.	Cooking Oil A (100% Palm Olein)	–	–	–	–
17.	Cooking Oil B (100% Palm Olein)	–	–	–	–
18.	Cooking Oil C (100% Palm Olein)	–	–	–	–
19.	Cooking Oil D (Blend of Palm Olein)	–	–	–	–
20.	RBD Olein A (Bulk Storage)	–	190	–	–
21.	RBD Olein B (Bulk Storage)	–	–	56	127
22.	RBD Olein C (Bulk Storage)	–	187	–	–

[a]Each sample was analysed in duplicate and the figures were rounded up.

used and that TBHQ, BHA and BHT are the popular antioxidants. These three phenolic antioxidants were added to palm oil products either singly or in combination, but their levels were below the 200 ppm permitted maximum.

The retention of TBHQ in palm olein during deep-fat frying of potato chips or crisps was followed using the same analytical method. The data in *Table 4* show that after 8 hours of frying, over 60% of TBHQ was lost owing to steam volatilization and absorption by the fried food. On the third day, *i.e.* after 24 hours of frying, only a very small amount of TBHQ was left in the oil. TBHQ like PG, BHA and BHT, is known to be practically ineffective in term of protecting oil against deterioration during heating or frying.

The method was also applied to monitor the retention of TBHQ in palm olein starting from the stage of deodorization in a refinery, up to discharge in a port in the USA, which took about two months. The results in *Table 5* indicate that about 90% of the TBHQ was still present in the oil after the various handling stages. TBHQ, either alone or better still in combination with citric acid, has been shown to be very effective in preserving the qualities of palm oil and palm olein during storage and shipping (Fritsch, *et al.*, 1975; Berger, 1985).

TABLE 4. RETENTION OF TBHQ IN PALM OLEIN DURING DEEP-FAT FRYING[a] OF POTATO CHIPS

Time	Level of TBHQ Found
Before frying (fresh oil)	194 ± 3.8 ppm
After 1st day of frying	76 ± 3.0 ppm
After 3rd day of frying	13 ± 2.2 ppm
After 5th day of frying	Not detected

[a]Frying conditions:
A fresh batch of chips was fried at 180°C for 3.5 minutes at half-hourly intervals during 5 consecutive working days, each of 8 hours. The oil level in the fryer was restored daily, using fresh palm olein containing 200 ppm TBHQ. The level of TBHQ originally added to the palm olein was 200 ppm, analyses were done in duplicate.

TABLE 5. RETENTION OF TBHQ IN PALM OLEIN DURING HANDLING

Sampling Point	Level of TBHQ[a] Found
After deodorization (Malaysia)	196 ± 3.7 ppm
At bulking storage tank (Malaysia)	191 ± 4.1 ppm
At ship's tank (Malaysia)	193 ± 3.2 ppm
At port of discharge (USA)[b]	178 ± 2.4 ppm

[a] Level originally added was 200 ppm. Analyses were done in duplicate.
[b]Time taken for the consignment to arrive in USA was about 55 days.

CONCLUSION

A one-step methanol extraction of phenolic antioxidants based on the AIIBP draft method with minor modification, was found to be simple and practical for application to palm oil products. The results obtained are shown to be comparable to those from the AOCS/AOAC/IUPAC methods and the procedure also has the advantage of being rapid.

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