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Gas Chromatographic Determination of Synthetic Antioxidants in Edible Fats and Oils – A Simple Methylation Method

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ABSTRACT

A simple method was developed for determining the antioxidants, BHA, BHT and TBHQ in commercial edible fats and oils. In 2-3 drops (about 60-80 mg) of an oil sample, 10% tetramethylammonium hydroxide (TMAH) methanol solution 1 mL was added and vortex-mixed for 10-minute methylation. The mixture was then extracted with 4-6 mL ether, 0.2% tetradecanol (14C-OH) 50 μL was added and thoroughly mixed. An aliquot of 0.3 μL of the ether layer was then injected to GC for BHA, BHT and TBHQ analyses. The recoveries from commercial oils were in a range of 94-106%, with a coefficient of variation < 9%. Eighteen commercial edible fat and oil samples of olive, sunflower, peanut, soybean, vegetable and blend oils were analyzed using the developed method. The contents of BHA, BHT and TBHQ were found to be 0-50, 0-267 and 0-37 μg/g. Fourteen out of 18 samples were detected to contain antioxidants. The total antioxidant contents in the three blend oils ranged 259-294 μg/g, which exceeded the Chinese National Standards (CNS) control level of 200 μg/g for edible oils.

Key words: synthetic antioxidant, methylation, fats and oils, gas chromatography

INTRODUCTION

Edible fats and oils provide indispensable nutrition in foods and can also help add special flavors for foods. However, foods that contain fats and oils have potential risks, such as the ability to oxidize or acidify. In such cases, the quality of foods will be reduced and human health will be harmed. Therefore, it is essential to block or retard the oxidization of fats and oils. Currently, one of the major methods to stop the oxidization for fats and oils is the addition of an antioxidant[1].

At present, the most commonly used antioxidants are synthetic, such as butylated hydroxyanisole (BHA), butyl hydroxytoluene (BHT) and tertiary-butyl hydroquinone (TBHQ). Many manufacturers prefer to use synthetic antioxidants because they are stable, cheap and easily available. However, these synthetic antioxidants have potential risks[2] raising safety concerns in a growing number of consumers[3-6]. Regarding this aspect of safety and hygiene for foods, it is predictable that there will be stricter limits on the use of synthetic antioxidants in the future.

In order to comply with safety and hygiene controls for foods and also improve concerns of hygiene quality control for food manufacturers, it is very important to establish an analysis method for determining antioxidants in edible fats and oils. Currently, there are official detection methods for BHA and BHT in Taiwan[7], but none for other antioxidants. Because antioxidants work synergistically if more than one antioxidant is combined together[8], using two or more antioxidants at the same time is quite prevalent among manufacturers. Thus, it is even more urgent to establish a determination method for analyzing various antioxidants in edible fats and oils.

The determination methods for analyzing antioxidants in foods include the colorimetric method[9], UV spectrophotometric method[10-12], paper and thin-layer chromatographic method[13-14], gas chromatographic method[15-20] and high performance liquid chromatographic method. However, these methods are either procedure-complicated or only one target antioxidant can be detected in one operation time. Therefore, there are still some difficulties for routine application.

Though there is much literature on analyzing phenolic antioxidants in edible fats and oils by the gas chromatographic method[15-20], these authors have still adopted solvent extraction or steam distillation as the isolation method. After isolating antioxidants, BHA or BHT is injected into GC for analysis directly or derivatively[15]. But TBHQ and propyl gallate (PG) are required to become derivatives prior to GC analysis. Therefore, the sample pretreatment procedures are very complicated. In addition, the derivation condition can become a variable to the recurrence and accuracy for determining antioxidants during analysis processes.

The gas chromatographic method has a high resolution and sensibility, and is one of today's major analysis technologies[27-28]. Therefore, this study adopted a simple method to extract fatty acid methyl ester and phenolic antioxidants such as BHA, BHT and TBHQ from various edible fats and oils by using a small amount of organic solvent after the methylation procedure[29], and then injecting directly into GC for analysis determination. The purpose of this study was to establish a simple, rapid and precise method for analyzing various phe-

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nolic antioxidants, such as BHA, BHT and TBHQ in edible fats and oils.

MATERIALS AND METHODS

I. *Materials*

Eighteen samples of olive oils, sunflower oils, peanut oils, soybean oils, vegetable oils and blend oils, three of each, were obtained from supermarkets in Tainan and Pingtung. Standards included n-octanol, n-decanol, n-dodecanol, n-tetradecanol, BHA, BHT, TBHQ and 8-quinolinol (each has a purity of over 98%) along with 10% TMAH/CH_3OH (tetramethylammonium hydroxide methanol) solution; all were obtained from TCI in Tokyo, Japan. Other reagents and organic solvents included acetone, methanol, acetonitrile, ethanol, ethyl ether, n-hexane and ethyl acetate were all high quality products obtained from ALPS Chemical Co. in Taiwan.

II. *Preparation of Standard Solutions*

0.2 g standards of n-tetradecanol (internal standard 14C-OH), BHA, BHT and TBHQ (each has a purity of over 98%) were weighed individually in 100 mL volumetric flasks with the accuracy of 0.0001 g on scale, dissolved in 20 mL ethyl acetate solution, and then added to volume in n-hexane or ethyl acetate solution, respectively.

III. *Determination of the Relative Response Factor (RRF) of BHA, BHT, and TBHQ to 14C-OH*

0.2% (w/v) 14C-OH was mixed individually with 0.2% (w/v) BHA, BHT and TBHQ in various ratios: BHA, BHT, TBHQ:14C-OH = 1:2, 1:1, 2:1, v/v. The relative response factors were then calculated according to the peak area ratios and concentration ratios of BHA, BHT and TBHQ to 14C-OH in a gas chromatographic device with each sample analysis performed three times:

$$RRF = (A_{BHA\ or\ BHT\ or\ TBHQ})/(W_{BHA\ or\ BHT\ or\ TBHQ}) \div (A_{IS})/(W_{IS}) \quad \text{.....(1)}$$

RRF: the relative response factor of BHA, BHT and TBHQ to the internal standard; $A_{BHA\ or\ BHT\ or\ TBHQ}$: the peak area of BHA, BHT and TBHQ; $W_{BHA\ or\ BHT\ or\ TBHQ}$: weight ($\mu$g) of BHA, BHT and TBHQ; A_{IS}: GC peak area of internal standard 14C-OH; W_{IS}: weight (μg) of internal standard 14C-OH.

IV. *Determination the Contents of BHA, BHT and TBHQ*

(I) *TMAH Methylation Method*

2-3 drops (about 60-80 mg) of various fat and oil samples were dropped into a 7 mL vial, respectively. After adding 1 mL of 10% tetramethylammonium hydroxide (TMAH) methanol solution, the mixture was vortexed for 10 min, adding 0.5 mL distilled water to stop the reaction followed by adding 0.2% (w/v, which was equal to 100 μg) of 50 μL internal standard solution, and then the mixture was extracted with ethyl ether for two to three times. Finally, 0.3 μL of the extraction along with ethyl ether was injected directly into GC for analysis (If the concentrations of BHA, BHT and TBHQ were too low, ethyl ether layer could be condensed to 1 mL prior to GC analysis). The contents of BHA, BHT and TBHQ were then calculated by the following equation (2), each sample was performed in duplicate at least:

$$\text{BHA, BHT, TBHQ } (\mu g/g) = (A_{BHA\ or\ BHT\ or\ TBHQ})/A_{IS} \times W_{IS}/RRF \times 1/W_{sample} \quad \text{.....(2)}$$

W_{sample}: weight (g) of fat and oil samples.

(II) *The AOAC Method*[26]

BHA and BHT were extracted from 5 g of fat and oil samples with 10 mL acetonitrile three times, and the sample solutions were then placed into centrifuges individually with a spin speed at 3000 rpm for 20 min. The upper layer was isolated and pentadecane (C_{15}) was used as an internal standard while the ethyl ether layer was decompressed and condensed to 1-2 mL and analyzed by GC for determination.

V. *Detection Limits of BHA, BHT and TBHQ on GC-FID*

The standard solutions of BHA, BHT and TBHQ were diluted to concentrations of 10, 5.0, 2.5 and 1.0 μg/mL, respectively. 0.3 μL of each dilution was injected into GC equipped with the flame ionization detector (FID) (range = 1, attenuation = 1), and the detection limits of BHA, BHT and TBHQ on GC-FID were then evaluated (peak/noise ratio > 3).

VI. *Fortification Recovery Test*

0.2% (w/v) of BHA, BHT and TBHQ were diluted to concentrations of 1× and 2×, respectively. 50 μL of the above standard solutions (which were equal to 25, 50 μL of BHA, BHT and TBHQ) were individually placed in a 7 mL vial containing 0.1g blend oil. After each mixture was methylated according to method IV-(I), 50 μL of 0.2% (w/v, 100 μg) internal standard solution 14C-OH was added followed by extracting with 2 mL ether three times and then 0.3 μL of the ether layer along with ethyl ether extraction was injected directly into GC for analysis. Each addition was performed in triplicate while a blank test was operated in the same time. The recoveries of BHA, BHT and TBHQ were then evaluated.

VII. *The GC Conditions*

This study used a GC (GL Science 390B, Tokyo, Japan) equipped with a flame ionization detector (FID) with H_2 flow

rate at 30 mL/min and air flow rate at 300 mL/min. The analytical column was a CP-SIL 8CB megapore capillary column (0.53 mm × 30 m, 1.5 μm. Chrompack, Netherland). The flow rate of carrier gas (N_2) was set at 4 mL/min. The temperatures of injection port and detector were set at 270˚C and 300˚C, respectively. The oven temperature was programmed to initiate at 145˚C and held for 1 min. The temperature was raised to 170˚C at a rate of 6˚C/min followed by increasing to 240˚C at a rate of 10˚C/min, and finally increased to 300˚C at a rate of 40˚C/min and held for 5 min. The injection volume was 0.3 μL with a splittless injection mode.

RESULTS AND DISCUSSION

I. *Sample Pretreatment – Methylation Procedure*

Fats and oils are not suitable for analyzing by GC directly because they have a high volatilily point. Normally, they are transformed into fatty acid methyl ester derivatives prior to GC analysis[30]. The most commonly used methylation methods include the boron tirfluoride method, sulfuric acid-reflux method, sodium methoxide method and tetramethylammonium hydroxide method.

This study developed a simple method for determining antioxidants in fats and oils (such as BHA, BHT and TBHQ), by transforming fats and oils into fatty acid methyl ester after a methylation procedure, followed by extracting with a small amount of organic solvent prior to GC analysis. With respect to the selection of methylation methods, because heating processes are needed for the boron trifluoride method, sulfuric acid-reflux method and sodium methoxide method, which has certain effects on the stability of antioxidants, thus the TMAH method was selected in our study. This method has a more moderate reaction condition, as the reaction temperature is at room temperature around 25-30˚C. Furthermore, the procedures of the TMAH method are more simple and rapid (only 10 min needed), and the methylation extent is more complete[30]. Our results also showed that the sample pretreatment in the TMAH method had less effect on the stability of antioxidants (BHA, BHT and TBHQ).

II. *About the GC Conditions*

This study showed that after fats and oils were methylated according to the TMAH method followed by extracting with a proper amount of ethyl ether, using a middle-polar CP-SIL 8CB (30 m × 0.53 m, 1.5 μm) as the analytic column was more suitable when analytical materials were analyzed by the splittless injection mode. The temperature increasing procedures for analysis were: the initial temperature was held at 145˚C for 1 min, then programmed to 240˚C at 10˚C/min to isolate analytical materials (BHA, BHT and TBHQ), followed by rapidly increasing to 300˚C at 40˚C/min to elute fatty acid methyl ester. According to the above analysis conditions, the retention times of BHA, BHT and TBHQ were 8.13, 8.63 and 8.98 min, respectively. The GC chromatograms of standards and commercial blend oils were shown as (A) and (B) in Figure 1.

With respect to the selection of internal standards, a small amount of octanol, decanol, dodecanol, tetradecanol and 8-quinolinol were individually added into methyled oil samples and then analyzed according to the above GC conditions. Results showed that the GC retention times of the above samples were 2.96, 5.86, 7.83, 10.62 and 6.73 min, respectively. The GC peaks of tetradecanol (14C-OH) were the only ones that did not overlap with other ingredient peaks of methyled oil samples. Thus, this study found that 14C-OH is suitable to be used as an internal standard for determining BHA, BHT and TBHQ. According to the above GC conditions, only 20 min was needed for each oil sample analysis.

III. *Determination of the Relative Response Factors (RRF) of BHA, BHT and TBHQ to the Internal Standard (14C-OH)*

In order to determine the contents of oil samples including BHA, BHT and TBHQ accurately, it is necessary to determine the RRF of BHA, BHT and TBHQ to the internal standard 14C-OH. The contents of BHA, BHT and TBHQ in oil samples could be calculated according to RRFs by equation (2). The coefficients of linearity (R^2) plotted by the peak area ratios (axis Y) of BHA, BHT and TBHQ to 14C-OH versus concentrations (axis X) of BHA, BHT and TBHQ to 14C-OH were all above 0.97. The RRFs of BHA, BHT and TBHQ were then calculated to be 1.04, 1.42 and 0.65, respectively.

IV. *The Detection Limit of GC-FID for BHA, BHT and TBHQ*

The gas chromatographic device equipped with FID detector (FID range = 1, attenuation = 1) and the splittless injection mode were adopted in this study to determine BHA,

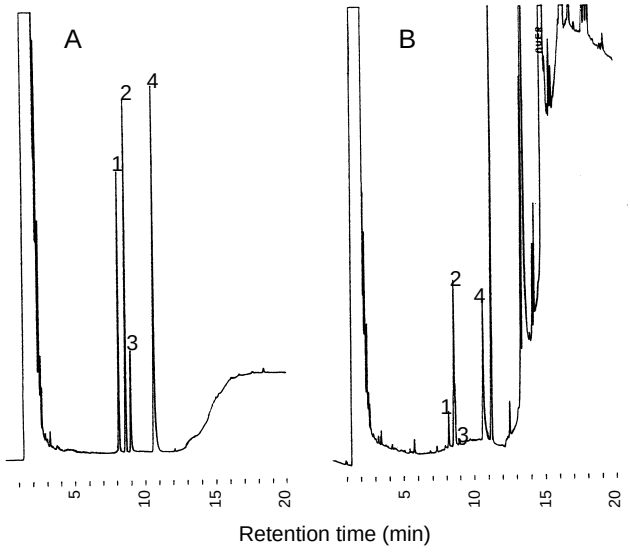

Figure 1. Gas chromatograms of synthetic antioxidants by splittless injection GC method.
(A) n-tetradecanol (14C-OH), BHA, BHT and TBHQ authentic compounds.
(B) 10% tetramethylammonium hydroxide (TMAH) methylated blend oil.
Peaks : 1 = BHA, 2 = BHT, 3 = TBHQ, 4 = 14C-OH (IS).

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BHT and TBHQ. Results showed that the detection limit of GC-FID for BHA, BHT and TBHQ was around 1 μg/mL.

V. *The Effects of Extracting with Different Solvents on the Recoveries of BHA, BHT and TBHQ after TMAH Methylation Procedure*

This study managed to establish a simple methylation method for determining BHA, BHT and TBHQ by extracting with a small amount of organic solvent from edible fats and oils. Therefore, it was necessary to select an appropriate solvent that has a best extraction effect on BHA, BHT and TBHQ. Three kinds of solvents including ethyl ether, ethyl acetate and n-hexane were selected for the test. Results showed that ethyl ether had a best extraction effect on BHA, BHT and TBHQ among those three solvents as shown in Table 1. The recoveries of BHA, BHT and TBHQ were found to be 91.4, 88.4 and 70.2%, respectively, while ethyl acetate came second with the recoveries of 73.9, 78.0 and 66.1%, and n-hexane came last with the recoveries of 50.3, 68.7 and 49.8%, respectively.

VI. *The Effects of Ethyl-ether Extraction Times on the Recoveries of BHA, BHT and TBHQ after TMAH Methylation Procedure*

50 μL of 0.2% internal standards were added individually into oil samples after the TMAH methylation procedure and 2 mL of ethyl ether, which had the best extraction effect as shown in the previous test, was used to extract for three times as shown in Table 2. The recoveries of BHA, BHT and TBHQ reached as high as 90% when using ethyl ether to extract for two times. The above results indicated that methylated oil samples could be injected into GC for analysis after extracting with 2 mL ethyl ether for two to three times (samples were to be condensed to increase the concentrations if the GC peaks were too small). At last, good determination results for BHA, BHT and TBHQ could be achieved.

Table 1. Effect of different solvents on the recovery of BHA, BHT and TBHQ from TMAH methylated oil

Solvent[a]	Recovery (%)[b]		
	BHA	BHT	TBHQ
Ether	71.4	88.4	70.2
Ethyl acetate	73.9	78.0	66.1
n-Hexane	50.3	68.7	49.8

[a] After TMAH methylation, 2 mL solvent was added.

[b] The sample oil was spiked 104, 102 and 104 μg/g BHA, BHT and TBHQ, respectively.

Table 2. Effect of ethyl ether extraction times on the recovery of BHA, BHT and TBHQ from TMAH methylated oil

Extraction times[a]	Reecovey (%)[b]		
	BHA	BHT	TBHQ
1 st	71.4	88.4	70.2
2 nd	89.3	93.5	90.1
3 rd	92.8	96.8	93.6

[a] After TMAH methylation, 2 mL solvent was added.

[b] The sample oil was spiked 104, 102 and 104 μg/g BHA, BHT and TBHQ, respectively.

VII. *Fortification Recovery Test*

26, 52 μg of BHA, BHT and TBHQ were added individually into 0.1 g blend oils. After the methylation procedure, 50 μL of 0.2% internal standards was added and extracted with ethyl ether three times according to method IV (I) prior to GC analysis. Results showed that the recoveries of BHA, BHT and TBHQ were at the ranges of 97-106%, 93-98% and 94-95%, respectively, with the coefficient of variation (CV%) less than 8.7% as shown in Table 3. This indicated that the method adopted by this study for determining BHA, BHT and TBHQ was very precise. One single sample, which needs only a small quantity (about 0.1 g), could be analyzed within 20 min. Only 4-6 mL of solvent was required in this study. While the AOAC method[(26)] needs a larger amount of sample (about 5-10 g) and solvent (more than 30-60 mL), and it takes more than 60 min for each sample analysis. The solid phase-HPLC method adopted by Yang, *et al*. needs about 2 g of oil sample and 13 mL of solvent and takes 35 min for each sample analysis. By comparing the above results, the method developed in this study was more superior.

VIII. *Comparisons of this Developed Method and the AOAC Method*

Comparisons of the TMAH methylation-ethly ether extraction method and the AOAC method are shown in Table 4. By adopting the AOAC method, fats and oils antioxidants (BHA and BHT) were extracted with a polar organic solvent followed by the decompression and condensation processes and then analyzed by GC. Results showed that 5.9 μg/g BHA, 218.3 μg/g BHT and 16.2 μg/g TBHQ were detected respectively by using this developed method for analyzing commercial blend oils, while only 167.6 μg/g BHT and none of BHA and TBHQ were determined by using the AOAC method. This indicated that the polar organic solvent (acetonitrile) used in the AOAC method had a lower extraction effect on BHA, BHT and TBHQ, while in this study methods the extraction effect could exceed 90% by using low polar ethyl ether to extract BHA, BHT and TBHQ after the TMAH

Table 3. Recoveries of the spiked antioxidants from TMAH methylated commercial blended oil by direct injection gas chromatographic method

Antioxidants	Blank (μg) (A)[a]	Spiked amount (μg) (B)	Amount determined (μg) (C)[b]	Recovery (%)[c]	CV (%)[d]
BHA	0.0	52.0	50.4	96.9	8.4
	0.0	26.0	27.6	106.2	5.8
BHT	236.4	51.0	283.6	92.5	3.9
	236.4	25.5	261.3	97.6	6.6
TBHQ	0.0	52.0	49.1	94.4	8.7
	0.0	26.0	24.7	95.0	8.2

[a] The amount of BHA, BHT and TBHQ in 0.1 g of blend oil, average of 5 tests.

[b] Average of triplicates analyses.

[c] Recovery (%) = (C – A) / B × 100%. [d] Coefficient of variation.

Table 4. Comparison of AOAC method and proposed method for determining of BHA, BHT and TBHQ in commercial edible oils

Methods[a]	Content[b] μg/g (cv %)[c]		
	BHA	BHT	TBHQ
Proposed method	5.9	218.3	16.2
	(7.2)	(3.6)	(5.9)
AOAC method	ND	167.6	ND
		(7.8)	

[a] Methylation and then determined by GC = proposed method in this study.
AOAC method = acetonitrile extraction and then determined by GC[(28)].
[b] Average of triplicates analyses. [c] Coefficient of variation.

Table 5. BHA, BHT and TBHQ content in some commercial edible fats and oils

Samples	Content[a] (μg/g)		
	BHA	BHT	TBHQ
Soybean-A	ND	38.6	25.3
Soybean-B	50.4	18.1	ND
Soybean-C	ND	35.7	ND
Olive -D	29.1	ND	ND
Olive -E	34.5	13.0	ND
Olive -F	43.8	13.4	ND
Vegetable-G	40.0	12.7	ND
Vegetable-H	ND	30.9	ND
Vegetable-I	ND	51.4	ND
Blend oil-J	24.8	218.3	16.0
Blend oil-K	15.7	267.1	10.7
Blend oil-L	23.8	236.4	5.9
Sunflower-M	ND	58.5	36.9
Sunflower-N	ND	ND	ND
Sunflower-O	ND	ND	ND
Peanut oil-P	ND	38.8	ND
Peanut oil-Q	ND	ND	ND
Peanut oil-R	ND	ND	ND

[a] Average of duplicates analyses.
ND = not detected.

methylation procedure (Table 2). For the determination of BHT, 218 μg/g was detected with CV% of 3.6% by using this developed method, while 167.8 μg/g was determined with CV% of 7.8% using the AOAC method, indicating that this developed method not only had a higher extraction effect, but also had a higher accuracy than the AOAC method.

IX. *The Contents of BHA, BHT and TBHQ in Commercial Edible Fats and Oils*

Eighteen samples of commercial soybean oils, olive oils, vegetable oils, blend oils, sunflower oils, peanut oils, three of each, were purchased in Taiwan supermarkets. In this study, we developed a simple method for determining the contents of BHA, BHT and TBHQ in these samples. Results showed that the contents of BHA, BHT and TBHQ among these eighteen samples were at the ranges of 0-50.4, 0-267.1 and 0-36.9 μg/g, respectively. Only merchandises of blend oil samples have been labeled "contain antioxidants", while the rest fifteen out of eighteen samples haven't been labeled anything though eleven of them were BHA or/and BHT or/and TBHQ found.

Blend oils that have been labeled "contain antioxidants" were BHA, BHT and TBHQ detected. Besides, the total contents of antioxidants reached as high as 259-294 μg/g exceeding the regulation limit (200 μg/g) according to CNS as shown in Table 5.

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簡易甲酯化處理－氣相層析法分析食用油脂中之合成抗氧化劑

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摘　要

本研究建立了食用油脂中合成抗氧化劑BHA、BHT及TBHQ之簡便氣相層析分析定量法。將2-3滴（60-80 mg）之油脂樣品以10%之tetramethylammonium hydroxide（TMAH）甲醇溶液1 mL甲酯化後，再以4-6 mL乙醚萃取其中之抗氧化劑，加入0.2%（w/v）之tetradecanol（14C-OH）內標準品溶液50 μL，即可直接注入氣相層析儀分析之。添加回收實驗中，BHA、BHT及TBHQ之回收率為94-106%，變異係數均小於9%。以本方法測定18件市售動、植物油脂，包括橄欖油、葵花油、花生油、大豆沙拉油、蔬菜油及動植物混合油等之BHA、BHT及TBHQ含量，分別為：0-50、0-267、及0-37 μg/g，18件樣品中檢出含抗氧化劑者達14件，其中3件動植物混合油之總抗氧化劑含量達259－294 μg/g，超過CNS之標準添加量200 μg/g之規定。

關鍵詞：合成抗氧化劑，甲酯化，食用油脂，氣相層析