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ScienceDirect
Food Chemistry 105 (2007) 389–394
Food Chemistry
www.elsevier.com/locate/foodchem

Determination of synthetic phenolic antioxidants in food items using reversed-phase HPLC

Bahruddin Saad [a,*], Yong Yek Sing [a], Mohd Asri Nawi [a], NoorHasani Hashim [a], Abdussalam Salhin Mohamed Ali [a], Muhammad Idiris Saleh [a], Shaida Fariza Sulaiman [b], Khairuddin Md Talib [c], Kamarudzaman Ahmad [c]

[a] School of Chemical Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
[b] School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
[c] Food Quality Control Laboratory, Ministry of Health Malaysia, Bukit Kayu Hitam, Kedah, Malaysia

Received 9 September 2006; received in revised form 9 November 2006; accepted 16 December 2006

Abstract

A HPLC with gradient elution method for the determination of the synthetic phenolic antioxidants (SPAs) propyl gallate (PG), tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) in food items is described. A C18 column served as the stationary phase; the gradient elution was formed by acetonitrile and water:acetic acid (1%). The UV detector was set at 280 nm. Under the recommended conditions, separation of the four SPAs was achieved in less than 8 min. Analytical characteristics of the HPLC method such as limit of detection, linear range, and reproducibility were evaluated. Extraction parameters were optimized for the recoveries of the SPAs in different types of food items (cooking oil, margarine and butter, and cheese). Before the HPLC separation, the SPAs were extracted with methanol/acetonitrile (1:1, v/v) and were subjected to vortex/ultrasonic treatment. The extracts were next kept in a freezer (~2 h) to precipitate co-extracted components. Recoveries of the SPAs when spiked to cooking oil, margarine, butter and cheese at 50 and 200 mg l^{-1} were in the ranges 93.3–108.3% for PG, 85.3–108.3% for TBHQ, 96.7–101.2% for BHA and 73.9–94.6% for BHT. The method was applied to the determination of SPAs in 38 food items (16 cooking oils, ten margarine, six butter and six cheese samples). The levels of SPAs in positive samples are all below the legal limits of Malaysia.

Keywords: Synthetic phenolic antioxidants; HPLC; Extraction; Food items

1. Introduction

Antioxidants (natural and synthetic) play a significant role in retarding the lipid oxidation reactions in food products. The detrimental effects of excessive lipid oxidation such as the formation of off-flavours and undesirable chemical compounds (aldehydes, ketones and organic acids, etc.), are well-known. Since natural antioxidants are usually of poor stability, food manufacturers prefer to use synthetic phenolic antioxidants (SPA) such as propyl gallate (PG), tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) (Fig. 1). These SPAs are deliberately added to prevent or delay the lipid oxidation during processing and storage of fats, oils and lipid-containing foods and have been used by the food industries for over 50 years (Formanek et al., 2001; McCarthy, Kerry, Kerry, Lynch, & Buckley, 2001). The use of these SPAs, however, is not without their problems. BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis in laboratory animals (Grice, 1986; Wichi, 1988). Thus, the use of SPAs in foodstuffs is strictly regulated by governments but regulations of permitted levels often vary from country to country. For example, TBHQ is

* Corresponding author. Tel.: +60 4 65777888; fax: +60 4 6574854.
E-mail address: bahrud@usm.my (B. Saad).

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doi:10.1016/j.foodchem.2006.12.025

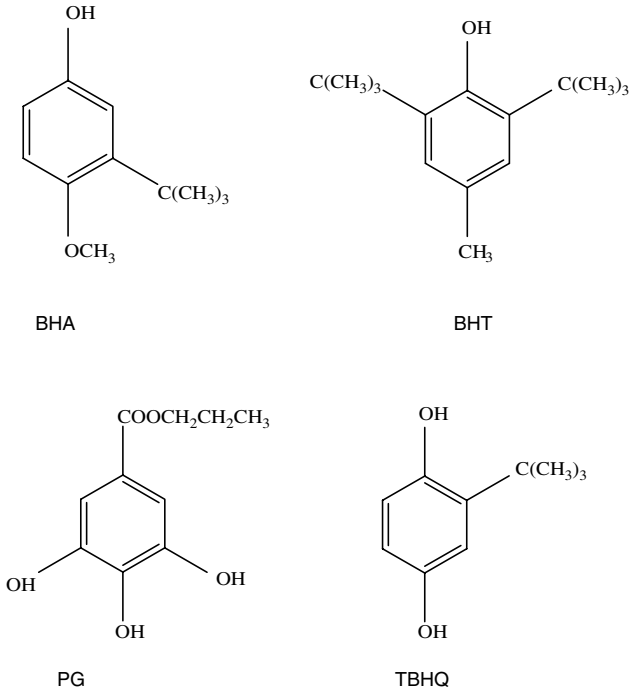

Fig. 1. Chemical structures of SPAs studied.

allowed in the USA while it is banned in the European Union countries (Noguera-Ortí, Villanueva-Camañas, & Ramis-Ramos, 1999). Usually, up to 100–200 μg g^{-1} of SPA in oils or fats, either singly or in combination, is allowed. Thus, the determination of SPAs in foods is necessary to ensure the fulfillment of the legal requirements as well as quality control procedures in the food industries.

At present, reversed-phase HPLC is the method of choice for the analysis of SPA because of its versatility, precision and adequate sensitivity (Karovičová & Šimko, 2000) although other analytical methods such as thin-layer chromatography (TLC) (Ragazzi & Veronese, 1973), gas chromatography (GC) (González, Gallego, & Valcárcel, 1999), capillary electrophoresis (CE) (Boyce & Spickett, 1999) and stripping voltammetry (Guanghan, Yu, Leiming, & Shuanglong, 1994) have also been reported. UV-spectrophotometric methods, due to its simplicity, used to be popular analytical technique. However, these methods are subjected to severe interferences from the sample matrix (González et al., 1999).

HPLC procedures for the quantitative determination of SPAs in diverse food products (Page & Charbonneau, 1989; Perrin & Meyer, 2002; Pinho, Ferreira, Oliveira, & Ferreira, 2000; Razali, Norhaya, & Norasimah, 1997; Rafecas, Guardiola, Illera, Codony, & Boatella, 1998) including gravies and dehydrated soups, bouillons, dehydrated meat and dry pet food, bakery products, palm oil products, potato and corn chips, popcorn and cheese snacks, breakfast cereals, dry beverage mixes and liver pates have been reported. In general, good recoveries (over 90%) of added PG, TBHQ and BHA were observed. However, the recovery for BHT was less satisfactory (75–90%) in most of the food items.

Another important issue in the analysis of the SPAs is the sample preparations that are involved prior to the HPLC separation. Solid-phase extraction, due to its environment-friendly characteristics, has been used for the preconcentration and isolation of antioxidants (Chen, Zuo, & Deng, 2001; Shui & Leong, 2004). However, the classical liquid–liquid extraction continued to be used widely and often mixtures of solvents that require multisteps are involved. Thus, the objective of this work is to develop and evaluate simplified procedure that can be applied for the routine determinations of SPAs in common food items such as cooking oils, bread spreads (margarines and butters) and cheese samples as reports on these items are scarce.

2. Experimental

2.1. Food samples

Thirty-eight food samples were analyzed, comprising 16 vegetable oils, 16 bread spread (six butters and ten margarines), and six cheese samples. The samples were purchased from local supermarkets around Penang Island. All samples, with the exception of cooking oils, were kept refrigerated before use.

2.2. Chemicals and SPA standards

Standards of PG (97%), TBHQ (97%), BHA (98%), and BHT (99%) were purchased from Aldrich (WI, USA). Methanol (MeOH) and acetonitrile (ACN) (both HPLC grade) were obtained from Merck (Darmstadt, Germany). Glacial acetic acid was supplied by J.T. Baker (Philipsburg, USA) and isopropyl alcohol (analytical grade) was from Systerm (Kuala Lumpur, Malaysia). Nanopure water (18 milliQ) obtained using NANOpure Diamond™ was from Barnstead.

2.3. Preparation and storage of SPA standards

A stock standard solution containing 500 mg ml^{-1} of each of the SPA (PG, TBHQ, BHA, and BHT) were prepared in MeOH/ACN (1:1, v/v). The flask was shaken until a homogenous and clear solution was formed. The stock solution was covered with aluminum foil and stored in a freezer (4 °C) and away from light for a maximum of one month. Before use, standard working solutions were prepared by diluting appropriate amounts of the stock solution in MeOH/ACN (1:1, v/v).

2.4. Apparatus

The following apparatus were used during the course of sample preparation: ultra sonic bath, Ultrasonic 28×, (Ney Dental, Yucaipa, CA, USA); vortex mixer, (IKA MS1 Minishaker, IKA Works Labortechnik, Staufen, Germany); centrifuge, Kubota 2100, (Kubota Corporation,

Tokyo); filtration and pump, Waters model DDA-V130-BN, (Millipore, Milford, USA); membrane filters, Whatman PTFE (0.5 μm × 45 mm) and Supelco Nylon 66 (0.45 μm × 47 mm) membrane filters. The absorption spectrum of the SPAs (10 mg l^{-1} dissolved in MeOH/ACN (1:1, v/v)) were obtained using a Varian, Cary 50, UV–Vis spectrophotometer equipped with CaryWin UV program. MeOH/ACN (1:1, v/v) was used as the reference solution.

2.5. Extraction procedure

Oil samples (10 g) were weighed into a 100 ml stoppered conical flask. MeOH/ACN (45 ml) was added, and placed in an ultrasonic bath for 15 min. The mixture was next vortexed and centrifuged at 3500 rpm for 10 min. The upper organic layer was kept in a deep-freezer for at least an hour and the clear portion was injected into the HPLC unit. The procedure is summarized in Fig. 2.

2.6. HPLC analysis

Chromatographic analysis was conducted using a Varian HPLC unit equipped with a Model 240 Pro Star solvent delivery system, a Rheodyne injector with a 20 μl loop, a Model 320 Pro Star UV detector, and CP-SCANVIEW version 6.0 Software. A LiChrospher® reversed-phase column (250 mm × 4.0 mm length, 5 μm particle size) served as the stationary phase. A gradient mode comprising ACN and water with 1% acetic acid that was varied between 30% and 95% over 5 min, and continued to 100% in another 4 min. Next, the system was allowed to stabilize for 1–2 min under the initial conditions. The prepared mobile phase was filtered and degassed using ultrasonic agitation. UV detection was performed at 280 nm and a flow rate of 1.5 ml min^{-1} was used throughout this study. At the end of each working day, the chromatographic system was rinsed with water/MeOH, (20:80, v/v) for at least 30 min.

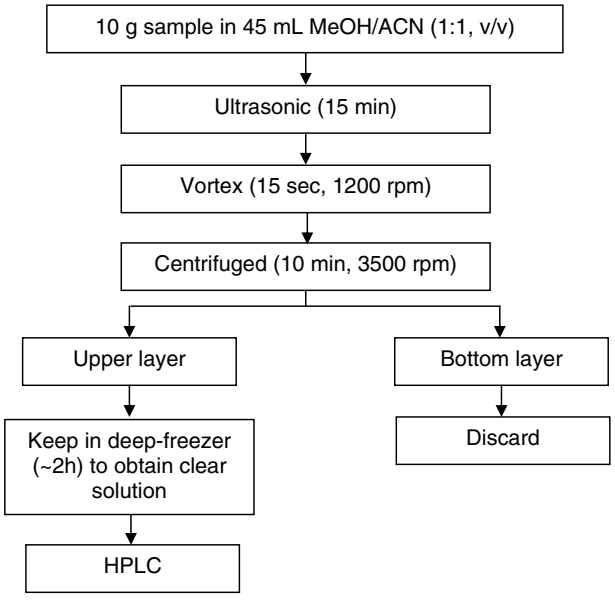

Fig. 2. Scheme for the determination of SPAs.

3. Results and discussion

3.1. UV spectrum of SPAs

The UV spectrum of the SPA standards exhibited maximum absorption at 275, 295, 290, and 280 nm for PG, TBHQ, BHA and BHT, respectively. For the HPLC analysis, the detector was fixed at 280 nm throughout the work.

3.2. HPLC conditions

Initially, the more straightforward isocratic elution condition was investigated. The elution involving the use of MeOH/ACN (1:1, v/v) at flow rate of 1.0 ml min^{-1} was tested. It was found that PG and TBHQ were not separated, although there was good separation between BHA and BHT. Another mobile phase consisting of MeOH/ACN (1:1, v/v): H_2O/acetic acid (99:1, v/v) (pH 2.95) at (98:2, v/v) with flow rate of 0.5 ml min^{-1} was also tried. Again, the separation of PG and TBHQ was not satisfactory.

Gradient elution based on the earlier report (Razali et al., 1997), with slight modification either using (i) MeOH/ACN (1:1 v/v) and water/acetic acid (99:1 v/v) or (ii) ACN and MeOH/ACN (1:1 v/v) was next carried out. Acidified water was used as eluent component to prevent ionization of the hydroxyl groups of the phenolic compounds (López, Martínez, Del Valle, Orte, & Miró, 2001).

Both gradient conditions produce baseline separation of the SPAs, but the second system was preferred as it gave faster analysis time (Fig. 3).

Using the gradient elution program, the four SPAs were eluted within 8 min. PG was eluted first followed by TBHQ, BHA and BHT. The order of elution correlates well with the polarity of these SPAs that decreased from PG to BHT.

3.3. Linearity and detection limits

The sensitivity of the UV detector was tested by injecting standard mixtures of these SPAs (0.1–1.0 mg l^{-1}) into the HPLC unit. The limit of detection (LOD) was taken when the signal: noise was 3. The LOD for PG was 0.3 mg l^{-1} and was 0.5 mg l^{-1} for TBHQ, BHA and BHT. Linear calibration curves obtained by plotting the peak area against the concentration of the respective compounds were found to be linear over the range 1.0–300.0 mg l^{-1} for all the SPAs.

3.4. Reproducibility studies

The repeatability of the peak areas and retention times were studied by repeatedly injecting mixtures containing

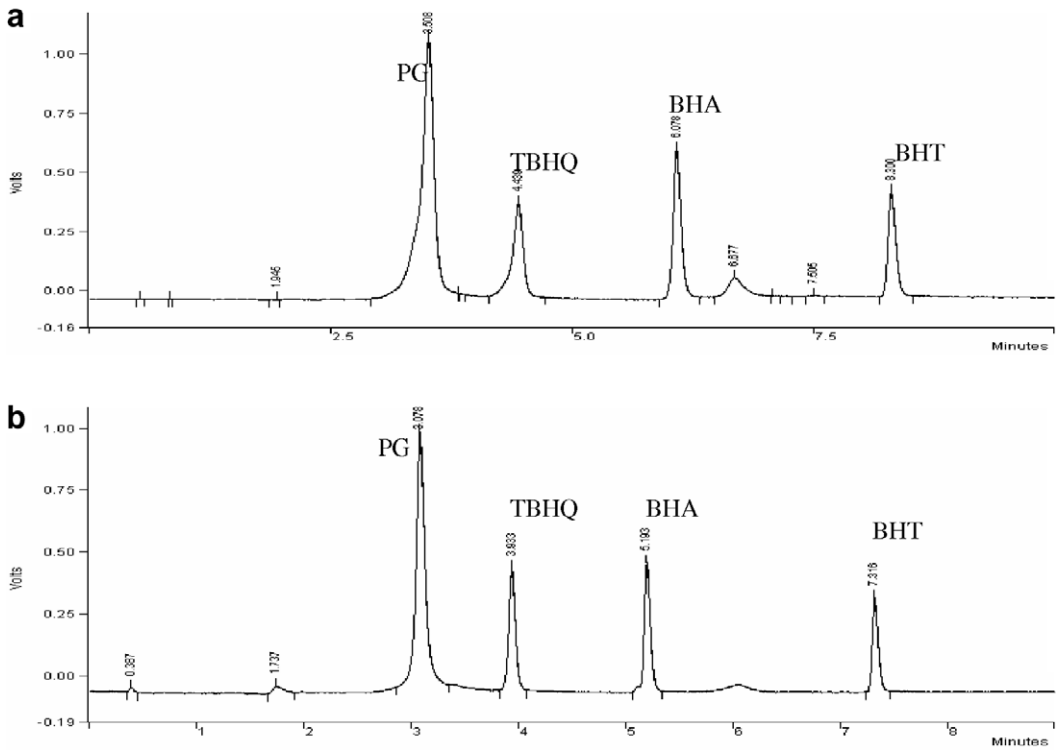

Fig. 3. Chromatograms for the gradient elution of SPA-free palm olein sample that had been spiked with 50 mg l^{-1} each SPA at flow rate 1.5 ml min^{-1}. (a) MeOH/ACN (1:1, v/v) and water/acetic acid (99:1, v/v) (pH 2.95). (b) Acetonitrile, ACN and water/acetic acid (99:1, v/v) (pH 2.95).

50 mg l^{-1} of the SPAs ($n = 5$). The relative standard deviation (RSD) for the retention time and peak area ranged from 0.5 –1.6% to 1.1–3.8%, respectively.

The reproducibility over different days was checked by injecting the same standard solution (50 mg l^{-1}) over five days. It was observed that both retention times and peak areas were reproducible and all the RSD values obtained were satisfactory (<4.0%).

4. Optimization of the extraction procedures

4.1. Recovery studies for oil samples

Sample preparation is an important procedure in chromatographic applications. One of its main aims is to remove interfering matrix components and particulates as well as to concentrate analytes of interest to enhance the sensitivity.

Refined palm olein oil samples (10 g) of Malaysian origin that were free from SPA were spiked with standard solution of PG, TBHQ, BHA, and BHT which had been prepared in isopropyl alcohol so that the final concentrations were 50 and 200 mg l^{-1}. The mixtures were mixed well to ensure complete dissolution of the antioxidants in the oil matrix.

The extraction method using methanol as described by Razali et al. (1997) was chosen as it involved the same matrix (palm oil) and furthermore used apparatus that can be commonly found in analytical laboratories. As results of the methanol extraction procedure was not satisfactory especially for BHT, thus the effect of using extraction solvent of MeOH/ACN (1:1, v/v) was investigated. A disadvantage of methanol extraction was that the recoveries for PG and TBHQ are high probably due to the co-extraction of interfering compounds such as lipids and proteins (Page & Charbonneau, 1989) while for BHT is less due to its lower polarity. It had been reported that ACN was a suitable solvent for extracting SPAs from fats (Karovičová & Šimko, 2000). Thus, the combination of MeOH/ACN as extracting solvent was applied in this study. After the extraction and ultrasonic step, the extracts were all kept in the freezer for about 2 h as carried out by Karovičová and Šimko (2000) to aid the solidification of any excess fat from the extraction solvent before injecting into the HPLC unit.

The use of MeOH/ACN (1:1, v/v) was found to significantly improve the recovery of BHT in cooking oil. The ultrasonic/vortexing procedure further improve the recoveries of the extraction, especially for BHT. Overall results indicate that the recoveries of the four SPAs were in the range of 94.6–108.3%.

4.2. Recovery studies for bread spread and cheese samples

Recovery results when the same condition for oil samples were applied to SPA-free bread spread (margarine, Australian origin) and cheese (New Zealand origin) samples indicated that BHT in breadspread was poorly

Table 1
Comparison of the recoveries of SPAs in food products ($n = 3$)

SPAs	Average recovery, % ($n = 3$)					
	50 mg l^{-1}			200 mg l^{-1}		
	Oil	Bread spread	Cheese	Oil	Bread spread	Cheese
PG	108.3	107.3	99.5	103.5	103.6	93.3
TBHQ	104.0	108.3	85.3	103.9	95.0	93.1
BHA	97.6	97.5	101.2	96.7	97.7	97.4
BHT	94.6	80.2	89.5	88.2	73.9	91.4

Table 2
Levels of SPAs found in SPA-positive food items

Sample type	Manufacturing country	SPAs (mg kg^{-1})				
		PG	TBHQ	BHA	BHT	Total
Refined palm olein	Malaysia	n.d.	n.d.	n.d.	88.9	88.9
Refined palm olein	Malaysia	n.d.	20.2	n.d.	n.d.	20.2
Butter	Malaysia	n.d.	n.d.	n.d.	36.2	36.2
Margerine	Malaysia	n.d.	n.d.	103.9	71.1	175.0
Butter	Malaysia	n.d.	n.d.	25.7	38.6	64.3
Butter	Malaysia	n.d.	n.d.	n.d.	14.4	14.4
Butter	Malaysia	n.d.	n.d.	45.8	42.0	87.8
Margerine	Malaysia	n.d.	n.d.	n.d.	143.1	143.1
Margerine	Malaysia	n.d.	n.d.	66.7	54.9	121.6
Margerine	Malaysia	n.d.	n.d.	72.6	53.3	125.9
Margerine	Malaysia	n.d.	n.d.	64.0	55.9	119.9
Margerine	Malaysia	n.d.	n.d.	44.8	72.7	117.5
Margerine	Malaysia	n.d.	n.d.	5.2	154.2	159.4

extracted. Thus, ultrasonic time was increased from 15 to 25 min and vortex time from 15 s (1200 rpm) to 5 min (1400 rpm for 4 min and 1600 rpm for 1 min). It is evident that increasing the ultrasonic time and vortex time as well as vortex speed improve the recoveries for the four compounds in bread spread and cheese samples.

The optimum extraction procedures were also carried out for oil, bread spread and cheese samples that are spiked with 200 mg l^{-1} SPAs. The average recoveries for the three types of samples are summarized in Table 1. It can be seen that the recovery for BHT is the lowest among the four SPAs but these are typical levels found by other researchers (e.g. ~87% when extracted with ACN/isopropanol 1:1 (v/v) in bakery products (Rafecas et al., 1998); ~74% from margarines when extracted with ACN (Karovičová & Šimko, 2000). The present study showed that the use of MeOH/ACN (1:1, v/v) in combination with vortexing/ultrasonic steps further improves the extraction compared to the use of MeOH alone. Keeping the extracts in a freezer helps to precipitate any fatty materials that might be co-extracted. This minimizes the complexity of the injected samples and thus helps to extend the lifetime of the HPLC column.

4.3. Analysis of food items

Details of the food items that were found to contain SPAs are shown in Table 2. The listed manufacturing country was as listed on these products although a few of them (e.g., olive, sesame, soya bean, canola and sunflower oils) are not native of Malaysia. All the samples were analyzed according to the optimized extraction procedure described earlier. Peak identification of the SPAs was based on the comparison between the retention times of standard compounds and was confirmed by spiking known standards to the sample. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis.

Sixteen cooking oil samples were analysed and it was observed that the four SPAs were not found in most of the samples, except two samples, which contained 88.9 mg kg^{-1} BHT and 20.2 mg kg^{-1} TBHQ, respectively (Table 2). Of the 16 bread spread samples that were analysed, eleven samples were found to contain BHT (range, 14.4–175.0 mg kg^{-1}) and eight were found to contain BHA (range, 5.2–103.9 mg kg^{-1}) (Table 2). It is interesting to note that all the samples that contained SPA are of Malaysian origin that had been added either with BHA and BHT either singly or their combination. On the other hand, no SPAs were detected in imported bread spread products. PG and TBHQ, were not found in all the bread spread samples analysed. No SPAs were found in all the six cheese samples that were analysed. None of the positive samples violates the legal limit of 200 mg kg^{-1} for total SPAs as specified in the Food Act and Regulations of Malaysia. For all the SPA-positive samples, the manufacturers declared the presence of synthetic antioxidants, but neither the type nor its level was stated.

It was also found that some bread spread and except one sample, all the cheese samples, showed a big unknown peak at about 3.5 min (chromatograms not shown). It was suspected that this was due to preservatives such as sorbic acid or benzoic acid. To verify this, standard sorbic acid and benzoic acid (100 mg l^{-1}) prepared in MeOH/ACN (1:1, v/v) were injected into the HPLC unit. Peaks at retention times of 3.5 min were found for both sorbic and benzoic acids, suggesting the use of these preservatives in some of these samples.

5. Conclusion

The analytical determination of SPAs in foods is a continuous activity for law enforcement agencies to ensure the safety of food consumed. A single step liquid–liquid extraction procedure using MeOH/ACN (1:1, v/v) offer good recoveries to the extraction of SPAs in cooking oils, bread spreads and cheese samples studied. The extraction was further enhanced by incorporating a vortexing/ultrasonic step. Being a single step extraction procedure, the method uses less organic solvents as compared to the previous reports (Razali et al., 1997; Rafecas et al., 1998). 12.5% cooking oil samples were found to contain SPA while none was found in cheese samples. 68.7% bread spread samples (range, 14.4–75.0 mg kg^{-1}) contain SPA. However, even among the SPA-positive samples, the levels of SPA do not exceed the legal limit of 200 mg kg^{-1}.

Acknowledgement

One of us (Y.Y. Sing) is indebted to the National Science Foundation, Malaysia, for providing a scholarship to pursue her MSc degree.

References

Boyce, M. C., & Spickett, E. E. (1999). Separation of food grade antioxidants (synthetic and natural) using mixed micellar electrokinetic capillary chromatography. *Journal of Agricultural and Food Chemistry, 47*, 1970–1975.

Chen, H., Zuo, Y., & Deng, Y. (2001). Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *Journal of Chromatography A, 913*, 387–395.

Page, B. D., & Charbonneau, C. F. (1989). Liquid chromatographic determination of seven antioxidants in dry food. *Journal of the Association of Official Analytical Chemists, 72*, 259–265.

Formanek, Z., Kerry, J. P., Higgins, F. M., Buckley, D. J., Morrissey, P. A., & Farkas, J. (2001). Addition of synthetic and natural antioxidants to a-tocopheryl acetate supplemented beef patties: effects of antioxidants and packaging on lipid oxidation. *Meat Science, 58*, 337–341.

González, M., Gallego, M., & Valcárcel, M. (1999). Gas chromatographic flow method for the preconcentration and simultaneous determination of antioxidant and preservative additives in fatty foods. *Journal of Chromatography A, 848*, 529–536.

Grice, H. C. (1986). Safety evaluation of butylated hydrotoluene (BHT) in the liver, lung and gastrointestinal tract. *Food and Chemical Toxicology, 24*, 1127–1130.

Guanghan, L., Yu, W., Leiming, Y., & Shuanglong, H. (1994). Determination of ascorbic acid in fruits and vegetables by stripping voltammetry on a glassy carbon electrode. *Food Chemistry, 51*, 237–239.

Karovičová, J., & Šimko, P. (2000). Review: determination of synthetic antioxidants in food by high-performance liquid chromatography. *Journal of Chromatography A, 882*, 271–281.

López, M., Martínez, F., Del Valle, C., Orte, C., & Miró, M. (2001). Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography. *Journal of Chromatography A, 922*, 359–363.

McCarthy, T. L., Kerry, J. P., Kerry, J. F., Lynch, P. B., & Buckley, D. J. (2001). Evaluation of the antioxidant potential of natural food/plant extracts as compared with synthetic antioxidants and vitamin E in raw and cooked pork patties. *Meat Science, 57*, 45–52.

Noguera-Ortí, J. F., Villanueva-Camañas, R. M., & Ramis-Ramos, G. (1999). Direct injection of edible oils as microemulsions in a micellar mobile phase applied to the liquid chromatographic determination of synthetic antioxidants. *Analytica Chimica Acta, 387*, 127–134.

Perrin, C., & Meyer, L. (2002). Quantification of synthetic phenolic antioxidants in dry foods by reversed-phase HPLC with photodiode array detection. *Food Chemistry, 77*, 93–100.

Pinho, O., Ferreira, I. M. P. L. V. O., Oliveira, M. B. P. P., & Ferreira, M. A. (2000). Quantification of synthetic phenolic antioxidants in liver pâtés. *Food Chemistry, 68*, 353–357.

Rafecas, M., Guardiola, F., Illera, M., Codony, R., & Boatella, J. (1998). Liquid chromatographic determination of phenolic antioxidants in bakery products. *Journal of Chromatography A, 822*, 305–309.

Ragazzi, E., & Veronese, G. (1973). Quantitative analysis of phenolic compounds after thin-layer chromatography separation. *Journal of Chromatography, 77*, 369–375.

Razali, I., Norhaya, H., & Norasimah, A. S. (1997). Determination of antioxidants in palm oil products by high performance liquid chromatography. *Elaeis, 9*, 25–33.

Shui, G., & Leong, L. P. (2004). Analysis of polyphenolic antioxidants in star fruit using liquid chromatography and mass spectrometry. *Journal of Chromatography A, 1022*, 67–75.

Wichi, H. P. (1988). Enchanced tumpr development by butylated hydroxyanisole (BHA) from the perspective of effect on forestomach and oesophageal squamous epithelium. *Food and Chemical Toxicology, 26*, 717–723.