PHENOLIC ANTIOXIDANTS DETERMINATION IN FOOD ITEMS USING REVERSED-PHASE HPLC

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Abstract: The determination of synthetic phenolic antioxidants (SPAs) including propyl gallate (PG), tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food items is reported using high performance liquid chromatography (HPLC). A C_{18} column is used as the stationary phase, acetonitrile and water:Acetic acid (1%) is used as the mobile phase of gradient elution and the UV detector is set at 280 nm. Under the above conditions, four antioxidents is completely separated within 8 min. The limit of detection, linear range, and reproducibility of HPLC are evaluated. Isolation parameters of SPAs from different types of food items (cooking oil, margarine and butter, and cheese) are optimized. SPAs are extracted from food items through extraction with methanol/acetonitrile (1 : 1, in volume), vortex, ultrasonic treatment and precipitation in a freezer (2 h). For cooking oil margarine, butter and cheese at 50 and 200 mg/L, recoveries of SPAs are 93.3%—108.3% (PG), 85.3%—108.3% (TBHQ), 96.7%—101.2% (BHA), and 73.9%—94.6% (BHT). The method is applied to the determination of SPAs in 38 food items (16 cooking oils, 8 margarine, 6 butter and 6 cheese samples). The levels of SPAs in positive samples are all below the legal limits of China.

Key words: phenolic antioxidants (PAs); high performance liquid chromatography (HPLC); gradient elution; food

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INTRODUCTION

Natural and artificial antioxidants play important roles in inhibiting oil oxidation reaction of food. It is well known that too much oxidation reaction will cause adverse influence, producing unpleasant odors and harmful compounds including aldehydes, ketones and organic acids. Since natural antioxidants are generally unstable, food producers prefer artificial antioxidants including propyl gallate (PG), tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Their structures are shown in Fig. 1. Adding synthetic phenolic antioxidants (SPAs) to fat, cooking oil can inhibit oxidation reaction in production and deposit process, thus there have been more than 50 years for the use of SPAs in food industry[1-2]. However, there exist problems in the use of SPAs that BHA and BHT are revealed to be harmful to liver and cause cancer[3-4]. So, the use of SPAs in food is strictly regulated, though different countries have their different permitted limits. For example, the use of TBHQ is allowed in USA, but not in some European countries. Generally, SPA concentration of 100—200 ug/g in oil and fat is allowed, and this is the direct reason for the determination of SPAs in the food industry quality control process[5].

For the analysis of antioxidants, several different techniques have been used, including thin-layer chromatography (TCL)[6], gas chromatog-

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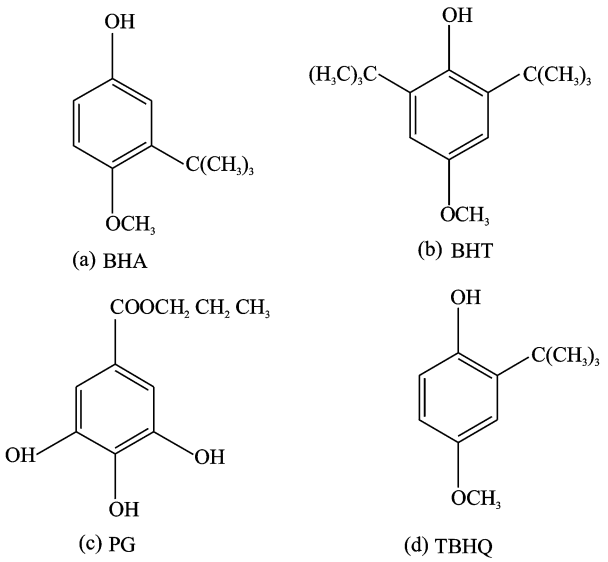

Fig. 1 Chemical structures of studied SPAs

raphy (GC)[7], capillary electrophoresis (CE)[8] and stripping voltammetry[9], yet all of the method above may be interfered by the samples. With its advantages of high precision and sensitivity, the high performance liquid chromatography (HPLC) became the main technique for the analysis of SPAs in meat juice, dehydrated soups, meat gravy, dehydrated meat, dehydrated pet food, baked food, palm oil, potatoes and corn chips, popcorn, cheese, breakfast cereals, drink powder mixture and livers[10]. In the related analysis, the recoveries of spiked PG, TBHQ and BHA are above 90%, though the recovery of BHT is only 75%—90%, which does not meet the analysis requirements.

Sample preparation is the experiment step before the HPLC separation, and it is a key point for the experiment. The solid phase extraction method is mainly used in antioxidant concentration and separation which is based on solubility principle. The liquid-liquid extraction method is widely used in multi-component solution separation of SPAs in food. So, the experiment is to set up a simple method for the analysis of SPAs.

1 MATERIALS AND METHODS

There are 38 food samples including 16 kinds of cooking oil, 16 kinds of bread spread and 6 kinds of bread cream. The 16 kinds of bread spread include 6 kinds of butter and 10 kinds of cheese.

PG (97%), TBHQ (97%), BHA (98%) and BHT (99%) are purchased from Aldrich Co. (WI, USA), and MeOH and ACN (HPLC grade) are purchased from Merck (Darmstadt, Germany). Glacial acetic acid and isopropanol are A. R. grade. Water is two times distilled water.

SPAs stock standard solution are prepared in MeOH/ACN (1 ∶ 1, in volume) with 500 mg/ml as the concentration, after being shakened to a clear solution, sealed by aluminum foil, and stored at 4 ℃ for one month. Before used in the HPLC analysis, the stock standard solutions are diluted with 1 ∶ 1 (in volume) MeOH/ACN to be suitable concentrations.

The instrumentation used in the sample preparation process includes ultrasound bath (up 5200H) and vortex vibrator (VORTEX-6, Beijing Chuangbo Bio-Tech Co. Ltd.). The UV absorption spectra are performed using UV-visible spectrometer (Varian, Cary 50).

A sample (10.0 g) is extracted with MeOH/ACN in a corked flask (100.0 ml) for 15 min by shaking under high spread, and then is centrifuged at 3 500 r/min for 10 min. The supernatant is collected and cooled in the refrigerator for 1 h. The clear liquid obtained is injected directly into the HPLC system. The extraction process is shown in Fig 2.

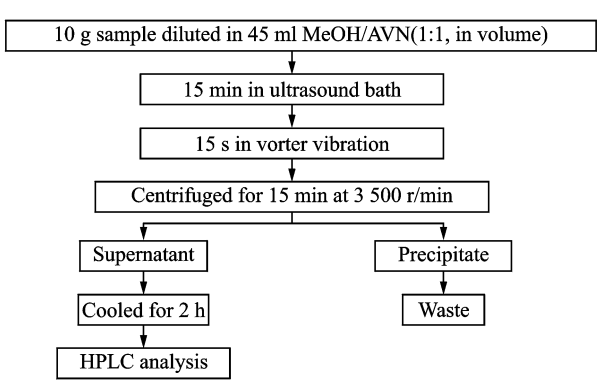

Fig. 2 Scheme for determination of SPAs

The HPLC analyses are carried out on a Varian HPLC system (Agilent HPLC 120), consisting of an autosampler, a 240 Pro Star pump, a 320 Pro Star UV detector operated by CP-SCAN-

VIEW version 6 software. HPLC separation is carried out on a particle size 250 mm × 4.0 mm and Lichrospher column 5 um. The mobile phase is composed of water (1% HAC) as mobile phase A and acetonitrile (1% ACN) as mobile phase B. The analytical separation is performed using gradient elution. A segmented gradient of mobile phase B is increased from 30% to 90% in 5 min, followed by ramping of mobile phase B to 100% in 4 min and is held constantly for 1—2 min. Then the mobile phase is filtered and degassed. The UV detection wavelength is set at 280 nm. The flow rate is maintained at 1.5 ml/min. After each experiment, the system is eluted with water/MeOH (20 : 80, in volume) for 30 min.

2 RESULTS AND DISCUSSION

2.1 SPAs UV absorption spectra

The maximum absorption appears at 275, 295, 290 and 280 nm for PG, TBHQ, BHA and BHT respectively. So the HPLC detection wavelength is set at 280 nm.

2.2 HPLC condition

When the mobile phase is chosen as MeOH/ACN(1 : 1, in volume) with flow rate of 1.0 ml/min, BHA and BHT are separated well, but not for PG and TBHQ. With mobile phase A as MeOH/ACN (1 : 1, in volume), B as water/acetic acid (99 : 1, in volume) of ratio A : B = 98 : 2 (in volume) and flowrate of 0.5 ml/min, PG and TBHQ still could not be separated. So, the gradient elution reported by Razali et al[11] is used with little adjustment, by using MeOH/ACN (1 : 1, in volume) and water/HAC (99 : 1, in volume) as mobile phase; or ACN and MeOH/ACN (1 : 1, in volume) as mobile phase. The acidified water is used to prohibit ionization of hydroxyl in phenol compounds[12].

The two mobile phases both can give well separation. The second one is faster, so it is chosen in the experiment. Within only 8 min, PG, TBHQ, BHA and BHT are separated in sequence, which is in accordance with their polarity, as shown in Fig. 3.

2.3 Linearity and detection limits

The SPA standard mixture solution (0.1—1.0 mg/l) are injected into HPLC to obtain its sensitivity. The detection limits are obtained with

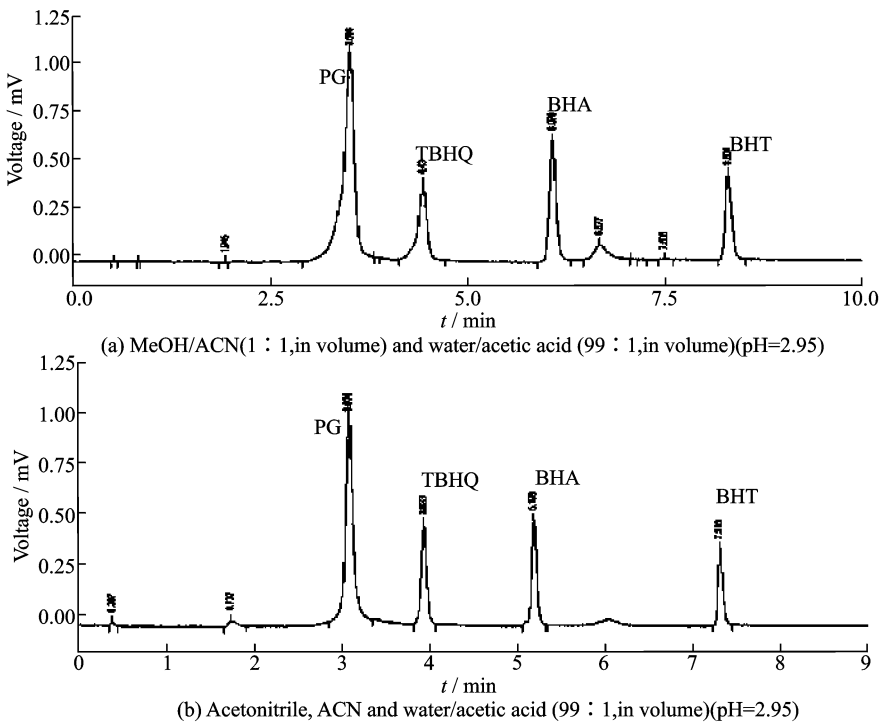

Fig. 3 Chromatograms for gradient elution of SPA-free palm olein sample spiked with 50 mg · l^{-1} each SPA at flow rate of 1.5 ml · min^{-1}

signal noise ratio 3. The detection limit for PG is 0.3 mg/l. The calibration curres are linear over the range of 1.0—300 mg/l for all the four SPAs.

2.4 Reproducibility study

The intra-day repeatability of the peak area is examined by injecting 50 mg/l SPAs mixture solution 5 times into the HPLC system. Relative standard deviation (RSD) for retention times and peak areas are 0.5%—1.6% and 1.1%—3.8%, respectively. The same method is used to obtain the reproducibility after 5 days, and it is found that RSD for retention times and peak areas are all below 4%.

2.5 Optimization of extraction condition

2.5.1 Recovery of cooking oil sample

Sample preparation is a very important process before HPLC analysis. The aim of the sample preparation process is to increase the analysis sensitivity by removing interfering matrix components and particulates and concentrating the analyses.

PG, TBHQ, BHA and BHT dissolved in isopropanol are added in Malaysia refined palm oil which does not contain SPAs. The final concentrations for these SPAs are 50 and 200 mg/l for each. The solutions are mixed well to have all the SPAs dissolved.

Razali et al[11] used extraction with methanol method to analyze the same palm oil. But extaction with methanol does not suit well with the extraction of SPAs, especially for the extraction of BHT. This is why the paper studies extraction with MeOH/ACN (1∶1, in volume). The shortcoming of extraction with methanol is that the recoveries of PG and TBHQ are high, as the extraction of fat and protein are achieved simultaneously, but the recovery of BHT is low because of its low polarity[13]. According to report, ACN is a suitable extractant. Thus, in the experiment, MeOH/ACN is used. After extraction and ultrasound bath, the extracts are saved in refrigerator for 2 h.

The recoveries are obviously increased by using MeOH/ACN(1∶1, in volume) as extractant. The same effect is achieved by using ultrasound/vortex technique, especially for the extraction of BHT. The final results show that the recoveries for the four SPAs are 94.6%—108.3%.

2.5.2 Recoveries of bread spread and cheese samples

The recovery experiment of bread spread and cheese is done with the same recovery method described above, and the recovery of BHT from the bread spread is found to be very low. So, increase the time for ultrasound bath from 15 min to 25 min, vortex time from 15 s (1 200 r/min) to 5 min 4 min(1 400 r/min) or 1 min (1 600 r/min), thus the recoveries of the four SPAs from bread and cheese can be increased.

The recoveries of bread spread and cheese samples spiked with SPAs 200 mg/l are listed in Table 1, found with the optimized extraction condition. Table 1 shows that the recovery of BHT recovery is similar with the values found by other research groups (for example, Rafecas et al[14] found the value to be 87% with ACN/propanol as extractant, and Karovičová & Šimko[10] found the value to be 74% with ACN as extractant). Our experiment shows that with MeOH/ACN (1∶1, in volume) as extratant and after ultrasound bath and vortex vibration, the extraction rate can be increased. Also, refrigerator storing can help to precipitate fat material, and all of these techniques can simplify the samples which can increase the HPLC column life term.

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

SPAs	50 mg/l			200 mg/l		
	Oil	Cheese	Bread spread	Oil	Cheese	Bread spread
PG	107.9	99.7	108.1	104.2	93.2	104.1
TBHQ	104.1	86.1	107.4	103.6	92.8	94.8
BHA	98.4	102.1	97.6	96.2	97.1	96.8
BHT	95.3	88.9	80.1	89.4	90.9	74.1

2.5.3 Sample analysis

The details of the foods containing SPAs are listed in Table 2. The samples are analyzed with the optimized extraction condition. The SPA peaks are identified by retention time, and this method is proved by spiked sample. The determination is achieved by external standard method using linear regression.

Sixteen cooking oils are analyzed, and the results show that most samples do not contain SPAs except for two samples containing 88.9 mg/kg BHT and 20.2 ng/kg TBHQ, as shown in Table 2. Among 16 bread spread samples, 7 samples are found to contain BHT (14.4—175.0 mg/kg) and 8 samples are found to contain BHA (5.2—103.9 mg/kg), as shown in Table 2. All the food items containing SPAs are from Malaysia, which contain BHA or BHT, or both. No SPA is found in imported bread spread samples. No PG or TBHQ is found in any bread spread samples. No SPA is found in 6 cheese samples. The values of SPA amount found are below 200 mg/kg which is the Malaysia maximum permitted levels. The food produer marks the related SPAs containing food about the antioxidant, but not about the kinds and amounts.

The results also show that there is a high absorption at 3.5(R.T.) in each the chromatograms of the cheese samples except for 1 cheese sample, and the peak may correspond to preservatives, such as sorbic acid or benzoic acid. This is proved by injection of sorbic acid and benzoic acid samples dissolved in MeOH/ACN (1∶1, in volume) into the HPLC system under the same condition, and the results show that the peaks of sorbic acid and benzoic acid are at 3.5 min (R.T.), which means that most cheese samples contain preservatives.

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

Sample	PG	BHA	BHT	TBHQ	Total
Refined palm olein	n.d.	n.d.	n.d.	20.6	20.6
	n.d.	n.d.	89.2	n.d.	89.2
Butter	n.d.	n.d.	36.8	n.d.	36.8
	n.d.	25.8	37.4	n.d.	63.2
	n.d.	n.d.	15.4	n.d.	15.4
	n.d.	45.7	40.6	n.d.	86.3
Artificial butter	n.d.	103.8	70.2	n.d.	174
	n.d.	n.d.	142.8	n.d.	142.8
	n.d.	66.5	54.3	n.d.	120.8
	n.d.	71.5	52.1	n.d.	123.6
	n.d.	63.8	54.8	n.d.	118.6
	n.d.	44.6	72.5	n.d.	117.1
	n.d.	5.3	151.7	n.d.	157

3 CONCLUSION

Analysis of SPAs is an important task in executing food safety laws. Using liquid-liquid extraction with MeOH/ACN as extractant can increase the recoveries of SPAs from oil, bread spread and cheese samples. Ultrasound bath and vortex vibrator can increase recoveries. Our extraction method uses less organic solvent compared with previous studies[7, 14]. 12.5% of the oil samples contained SPAs, while no cheese samples contain SPAs. 68.7% bread spread samples contain SPAs (14.4—75.0 ng/kg) though the amount is bellow the permitted level of 200 mg/kg.

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反相高效液相色谱测定食品中酚类抗氧化剂

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摘要:描述了利用高效液相色谱 (HPLC)测定食品中没食子酸丙酯(PG),叔丁基对苯二酚(TBHQ),叔丁基羟基茴香醚(BHA)和二丁基羟基甲苯(BHT)4 种合成酚类抗氧化剂(SPAs)的方法。采用C_{18}色谱柱作为固定相,乙腈-1%乙酸水溶液作为流动相进行梯度洗脱,紫外检测波长设定为 280 nm,在不到8 min 时间内可将4 种合成酚类抗氧化剂分离。研究评价了HPLC 的分析特性,即检测限,线性范围和重现性等,优化了在不同类型食品(食用油,人造黄油,奶油和奶酪)中获取 SPAs 工艺参数。样品在高效液相分离之前,用甲醇/乙腈(1∶1,体积比)提取SPAs,并进行漩涡/超声处理,提取物冷冻2 h,制得沉复合提取物。浓度为50 mg/l 和 200 mg/l 的食用油,人造黄油,奶油和奶酪中,PG 的回收率为93.3%—108.3%,TBHQ 的回收率为85.3%—108.3%,BHA 的回收率为 96.7%—101.2%,BHT 的回收率为 73.9%—94.6%。应用反相HPLC 测定 38 种食品(16 种食用油,10 种人造黄油,6 种奶油和 6 种奶酪样品)中的 SPAs,样品中 SPAs 的含量均低于中国规定的含量。

关键词:酚类抗氧化剂;高效液相色谱(HPLC);梯度洗脱;食品

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