Determination of Synthetic Phenolic Antioxidants in Cake by HPLC/DAD after Mixed Micelle–Mediated Cloud Point Extraction

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Summary: A mixed micelle-mediated cloud point extraction (MMCPE) system was developed for the extraction and preconcentration of four synthetic phenolic antioxidants (SPAs) (propyl gallate (PG), tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and octyl gallate (OG)) in cake. The mixture of two kinds of non-ionic surfactants polyoxy ethrlene nonyl phinyl ether (NP-7) and polyoxy ethrlene nonyl phinyl ether (NP-9) was utilized as a suitable micellar medium for preconcentration and extraction of SPAs. The surfactant-rich phase was then analyzed by high performance liquid chromatography-diode array detection (HPLC–DAD). The effect of different parameters such as concentration of surfactants, proportion of NP-7 and NP-9, equilibration time and temperature on the cloud point extraction (CPE) was carefully optimized. Under the studied conditions, four SPAs were successfully separated within 12 min. The relative standard deviations (RSD, n=6) were 1.2–2.0% and the limits of detection (LOD) were 1.5 ng mL^{-1} for PG, 3.6 ng mL^{-1} for TBHQ, 2.9 ng mL^{-1} for BHA, and 0.8 ng mL^{-1} for OG, respectively. Recoveries of the SPAs in spiked cake samples were in the range of 92% to 99%. The MMCPE method showed potential advantage for the preconcentration of the SPAs, with enrichment factor of 25. Moreover, the method is simple, has high sensitivity, consumes much less solvent, and has significant advantage in extraction efficiency compared to traditional CPE methods.

Keywords: Cloud-point extraction, Mixed micelle, Cake, High-performance liquid chromatography, preconcentration, Synthetic phenolic antioxidants

Introduction

Antioxidants are a large group of phenolic compounds which are widely used in food industry to prevent deterioration caused by oxidation during the storage of some food containing unsaturated fatty acids, such as fats, oils and cakes. They are roughly classified as natural and synthetic antioxidants. The mechanism of action of antioxidants is to interfere with the formation of free radicals that propagate oxidation [1]. Natural antioxidants components mainly include vitamins, flavonoids and carotenoids from fruits, vegetables and medicinal plants. Synthetic phenolic antioxidants (SPAs) permitted for use in food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (DG), usually at concentrations up to 100–200μg g^{-1} in oils or fats, either singly or in combination [2].

Natural antioxidants have become highly in demand because of their health benefits. The regular intake of natural antioxidants can decrease the risks of cancer, cardiovascular disease, diabetes and other ageing-related diseases by reducing oxidative stress [3] However, natural antioxidants are not stable. Industries often carry out freezing and drying processes or use decontaminant chemical agents systems to achieve the objective of long-term storage of foods containing natural antioxidants [4, 5]. Therefore, synthetic antioxidants have been used by the food industry for over 50y instead of natural antioxidants due to their high stability, low cost, and wide availability [6]. However, people have also found that excessive use of these artificial antioxidants may cause a loss of nourishment and even produce toxic substances to harm the public health [7–9]. Therefore, establishing a method to determinate SPAs is necessary to ensure public's safety.

Various analytical methods have been reported for the determination of SPAs in oil, fat, cake and other food stuffs. High-performance liquid chromatography (HPLC) is the most widely used for its high selectivity for the separation of SPAs under chromatographic conditions [7, 8]. Other methods including GC–MS [9, 10], thin–layer chromatography (TLC) [11], micellar electrokinetic capillary chromatography [12], spectrophotometry [13] have also been used for determinating SPAs.

An appropriate extraction and preconcentration procedure prior to the HPLC separation of the sample is another important factor

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in the analysis of the SPAs. Dispersive liquid–liquid microextraction (DLLME) [14], liquid-liquid extraction (LLE) [15], solid phase extraction (SPE) [16], solid-phase microextraction (SPME) [17] are used. Among these methods, DLLME and LLE are particularly unfriendly to the environment for the use of organic solvents, while the SPE and SPME are time-consuming and the equipments can be quite expensive. Thus, an effective, inexpensive, environment-friendly method for the extraction of SPAs is still demanding.

Cloud-point extraction (CPE) or micelle-mediated extraction (MME) has a lot of advantages including lower toxicity, lower cost, more environmentally friendly as an alternative to traditional extraction. When the temperature is above cloud-point temperature of the surfactant, the solution becomes turbid, then the extraction occurs, analytes are successfully extracted and preconcentrated into the surfactant-rich phase (SRP) which can be diluted with a minimum volume organic solvent to reduce viscosity before detected. In this process, a preconcentration factor of more than 10 was obtained. This procedure is based on the surfactant-mediated phase separation.

In order to obtain highest efficiency in separation and preconcentration of the targets, mixed micelle-mediated extraction (mixed-MME) system has also been used in CPE. Some physical characteristics of the mixed micelle have been investigated, such as light scattering and electrophoretic [18]. Publications on CPE using mixed micelle applications for the determination of penicillin antibiotics [19], ginsenoside [20] and trace amounts of metals [21–24] have been reported. The mixed micelles used in these reporting were all made up of nonionic and anionic surfactant. However, to our knowledge, CPE of SPAs using two kinds of non-ionic surfactants has not been reported yet.

In this research, a CPE using mixed micelle made up of two kinds of non-ionic surfactants NP-7 and NP-9 was investigated for extracting SPAs (PG, TBHQ, BHA and OG; Fig. 1) in cake. Compared with Triton X-114, Tergitol TMN-6, a much lower volume of surfactant and higher extraction efficiency were obtained by this CPE system. Different experimental conditions were studied to determine the optimal condition for the analysis of SPAs.

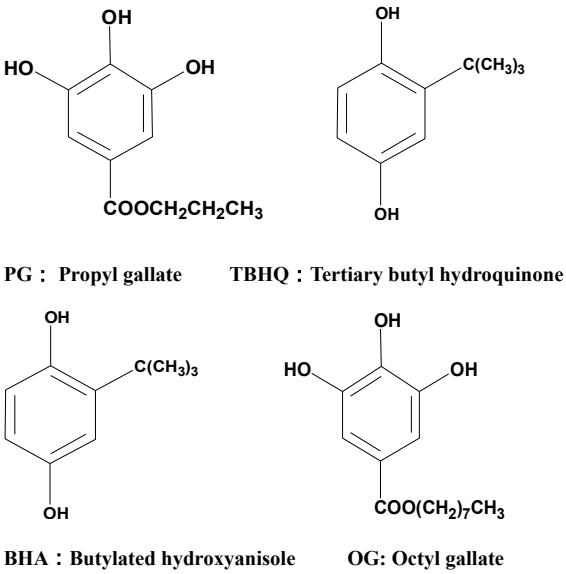

Fig. 1: Molecular structures of PG, TBHQ, BHA and OG.

Results and Discussion

For the extraction and preconcentration of SPAs, lots of CPE were recorded before, but cloud point extraction using mixed micelles made up of two kinds of non-ionic surfactants NP-7 and NP-9 has not been reported yet. Our group made a comparison of the proposed method with the most commonly used non-ionic surfactants TX-114 CPE and TMN-6 CPE. From the results (Fig. 2) we can see that the MMCPE is more effective in extraction efficiency evidently without using centrifugation and salt which present a threat to the chromatogram column.

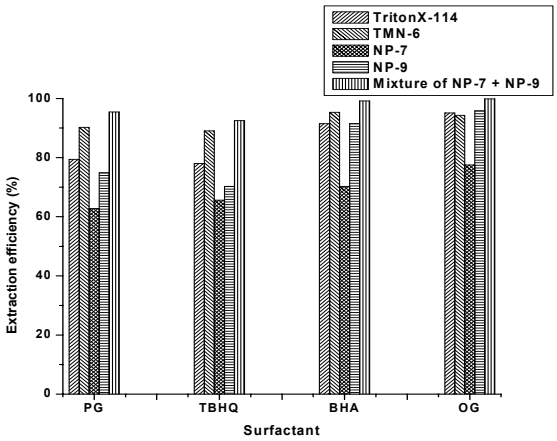

Fig. 2: Effect of different kinds of surfactants on the extraction efficiency of the SPAs spiked in cake: concentrations of Triton X-114, Tergitol TMN-6, NP-7 or NP-9 are 2.0% (v/v), respectively

Fig. 3 shows typical HPLC chromatograms of extracted and preconcentrated SPAs, Fig. 3a is the chromatogram of blank cake. Fig. 3b is the chromatogram of standard SPAs without MMCPE and Fig. 3c is the chromatogram of SPAs after MMCPE from cake spiked with it. The preconcentration effect of MMCPE is clearly demonstrated in Fig. 3b and 3c. Although the mixture of NP-7 and NP-9 has high UV absorbance at the wavelength of 280 nm as shown in Fig. 3c, it does not interfere with the determination of SPAs. For achieving the highest efficiency and sensitivity in MMCPE of SPAs several different parameters that can influence the extraction efficiency were investigated and optimum conditions were obtained.

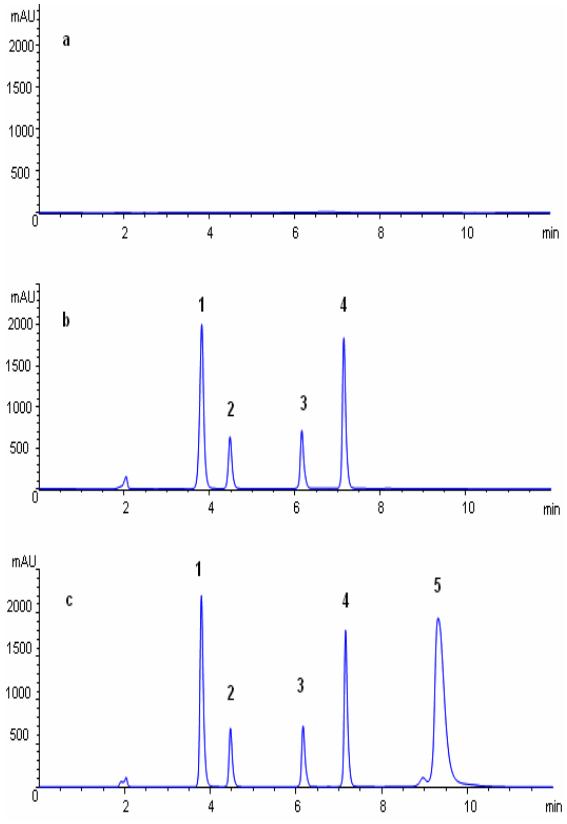

Fig. 3: HPLC–UV chromatograms: a blank cake, b standard (600 µg mL^{-1}) and c cake spiked with SPAs (600 µg mL^{-1}, peaks were obtained by dilute the surfactant-rich phase from 0.3mL to 0.6mL). Peak nr: (1) PG, (2) TBHQ, (3) BHA, (4) OG, (5) NP-7 + NP-9. HPLC conditions-gradient separation using methanol and 0.1 % (v/v) acetic acid; injection volume 10 µl; flow rate 1 mL min^{-1}; wavelength 280 nm

Effect of NP-7 and NP-9

It was found that cloud point extraction of SPAs is more efficient in the presence of both NP–7 and NP–9 simultaneously. Therefore the effects of both the mixing proportion (NP-7 and NP-9) and mixed surfactant concentration were investigated.

Firstly, aqueous solutions containing a mixture of standard SPAs (10 μg mL^{-1} each) were extracted by MMCPE with different mixing proportion of surfactant in the range of 1:0.5–1:10 (NP-7:NP-9; v/v), while the other conditions were as follows: mixed surfactant concentration is 2.0%, 55 °C equilibration for 40 min. Fig. 4 shows that the extraction efficiency of SPAs is the best at a proportion of 1:4 (NP-7:NP-9; v/v). As the mixing proportion was obtained, the concentration of the mixed surfactant was investigated in the range of 0.5% to 4.0% (v/v). The results can be seen in Fig. 5, extraction efficiency increased gradually by increasing mixed surfactant concentration up to 2.0% (v/v) and decreased sharply at higher concentrations. Therefore, a concentration of 2.0% (v/v) of the mixed surfactant was selected as optimum. Based on these results, a mixing proportion of 1:4 (NP-7:NP-9; v/v) and a mixed concentration of 2.0% (v/v) were adopted to achieve the best analytical signals and highest extraction efficiency.

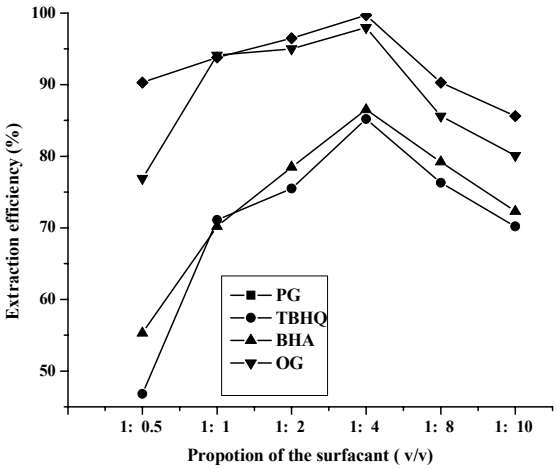

Fig. 4: Eeffect of mixing proportion of NP-7 and NP-9 on the extraction efficiency
Other experimental conditions are concentration of surfacant: 2.0% (v/v); equilibrium temperature: 55 °C; equilibrium time: 40 min

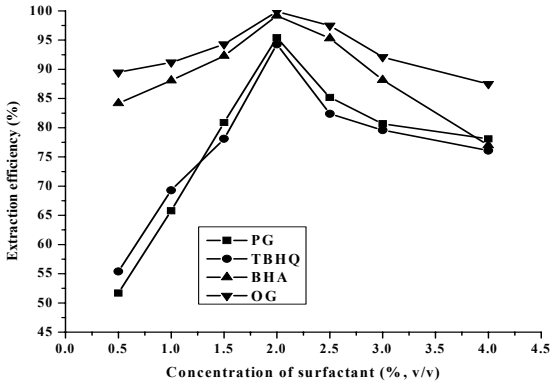

Fig. 5: Eeffect of mixed surfactant concentration of NP-7 + NP-9 on the extraction efficiency

Effect of the Equilibrium Temperature

The effects of temperature on the efficiency of SPAs extraction are illustrated in Fig. 6 at 2.0% (v/v) of mixed surfactant at a proportion of 1:4 (NP-7:NP-9). Equilibrium temperature is necessary to complete the extraction. Easy phase separation and the best extraction results can be achieved when the MMCPE was performed at equilibrium temperature. Although the cloud-point temperature of NP-7 and NP-9 are 20 °C and 54 °C respectively, the CP of the mixed surfactant occured at 45 °C. The optimal equilibrium temperature of the CPE is 15–20 °C higher than the cloud-point temperature of the surfactant [25]. Therefore, to employ the lowest possible equilibrium temperature for efficient separation of phases, the equilibrium temperature in the range of 45 to 65 °C was studied.

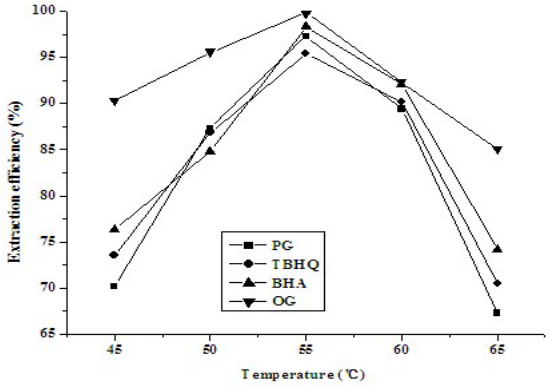

Fig. 6: Eeffect of the equilibrium temperature on the extraction efficiency.
Other experimental conditions are concentration of surfacant: 2.0% (v/v); proportion of NP-7 and NP-9: 1:4; equilibrium time: 40 min.

It is evident from the results that the extraction efficiency of the SPAs increase from 84% to 99% when temperature is raised from 45 to 55 °C, and beyond 55 °C, the extraction efficiency decreased sharply. Taking the previously mentioned findings into account, 55 °C was selected as the equilibrium temperature.

Effect of the Equilibrium Time

In order to determine the optimum equilibrium time of MMCPE, different time in the range of 20 to 50 min was studied using 2.0% (v/v) mixed surfactant at a proportion of 1 : 4 (NP-7 : NP-9) at 55 °C. The results are shown in Fig. 7 and 40 min was chosen as optimal time for equilibration.

Experimental

Apparatus

Chromatographic separation and evaluation were performed on an HPLC system (containing a vacuum degasser, an auto sampler, a quatpump, and a diode-array detector; Agilent 1200 Series, Agilent Technologies, Calif., U.S.A.) equipped with a reversed phase C18 analytical column of 150 × 4.6 mm (Agilent TC-C18). The empower software was used for data acquisition. A thermostatic water bath (Jintan crystal glass experimental instrument, SHY-2A, Jin Tan, China) was used to implement MMCPE.

Chemicals and Stock Solutions

Standards of PG (≥98.0%), TBHQ (≥99.0%), BHA (≥98.5%) and OG (≥99.0%) were obtained from Sigma (St. Louis, Mo.,U.S.A.). The structures of the studied SPAs are shown in Fig. 7. A stock standard solution (1 mg mL^{-1}) containing each SPA (PG, TBHQ, BHA and OG) was prepared by dissolving an appropriate amount in methanol and stored in a freezer (4 °C). More diluted solutions were prepared using this stock solution. Non-ionic surfactants NP-7 and NP-9 were purchased from Aladdin Chemistry (Co., Ltd. Shanghai, China) and used without further purification. A stock solution of mixed NP-7 and NP-9 was prepared in doubly distilled water. Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). Acetic acid was purchased from Kedi (Tian Jin, China).

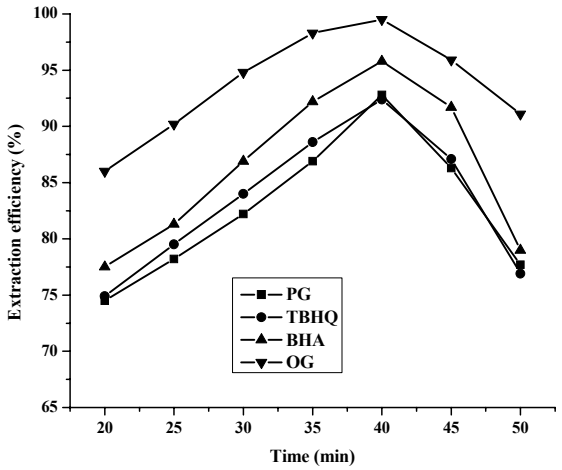

Fig. 7: Eeffect of the equilibrium time on the extraction efficiency
Other experimental conditions are concentration of surfacant: 2.0% (v/v); proportion of NP-7 and NP-9: 1:4; equilibrium temperature: 55 °C

All reagents were of analytical reagent grade.

MMCPE Procedure

Aliquots of 5 mL of sample solution containing different concentrations of SPAs (PG, TBHQ, BHA and OG) with 0.4% (v/v) of NP-7 and 1.6% (v/v) of NP-9 were placed in centrifugal vials. The centrifugal vials were left in a thermostatic water bath at 55 °C for 40 min. In this process, surfactant-rich phase (SRP) stuck to the bottom of centrifugal vials gradually. The aqueous phase (AQ) was removed and the SRP was diluted with methanol to 0.6 mL. Then 10.0 µl of the solution was directly injected into the HPLC system for analysis.

Preparation of Cake Samples

A 1± 0.05 g sample was vortexed in the presence of 2 mL of acetonitrile at room temperature for 5 min until the mixture became a homogenate. A portion of the upper liquid phase was transferred into a 10 mL centrifugal vial through a paper filter. The residue was reextracted with another 2 mL of acetonitrile, and the supernatant was isolated and collected with the 1st extraction fraction and diluted with doubly distilled water to the mark. The extract aliquots of cake samples were subjected to the MMCPE procedure.

HPLC Conditions

The separations were performed on an Agilent TC-C18 column (150 mm×4.6 mm, i.d, 5 µm). An Agilent Chemstation for LC system was utilized to control the system and for the acquisition and analysis of the chromatographic data. Quantification was done by the evaluation of peak areas. Methanol (B) and water with 0.1% acetic acid (A) were used as mobile phase with the gradient program as follows: 50–85% B, 0–4.5 min; 85–90% B, 4.5–6.5 min; 90–50% B, 6.5–9.0 min; Next, the system was allowed to stabilize for 1 to 2 min. The flow rate was maintained at 1 mL min^{-1}. The column temperature was maintained at 40 °C and the injection volume was 10 μL. SPAs were recorded at the wavelength of 280 nm.

Analytical Performance of the Method

To determine the extraction efficiency, repeatability, and reproducibility, SPAs were spiked into cake at 3 different concentrations (20, 50 and 80 mg kg^{-1}), the measurements were made under the optimum conditions described above, and six replicates were analyzed per concentration level in two independent analytical runs under the established chromatographic conditions.

Analytical Characteristics

Table-1 summarizes the analytical characteristic data of the present system for the four SPAs. Linear calibration curves obtained by plotting the peak area against the concentration of the respective compounds were found to be linear over the range of 1 to 600 μg mL^{-1} for all the SPAs. The correlation coefficients for SPAs were all more than 0.99. The sensitivity was also evaluated in terms of LOD as concentration giving the signal-to-noise ratio of 3 (S/N=3). The LOD for PG was 1.5ng mL^{-1}, for TBHQ was 3.6 ng mL^{-1}, for BHA 2.9 ng mL^{-1}, and for OG 0.8 ng mL^{-1}, respectively. The analytical characteristics such as the recovery, repeatability, and reproducibility varied from 92% to 99%, from 1.1% to 2.2%, and from 1.2% to 2.0% (CV_R), respectively. Data are listed in Table-2. All the data were obtained under the optimized conditions.

Table-1: Analytical curves of the four SPA solutions.

SPA	Linear equation	R^2	Linear range (mg mL^{-1})	LOD (ng mL^{-1})
PG	y=61.6362x+59.2551	0.9999	1.0—600.0	1.5
TBHQ	y=25.6631x+49.8238	0.9998	1.0—600.0	3.6
BHA	y=22.4670x+54.0024	0.9999	1.0—600.0	2.9
OG	y=66.1653x+151.1662	0.9999	1.0—600.0	0.8

y is the peak area, and x is the concentration (µg mL^{-1})

Table-2: Recovery, reproducibility, and R.S.D. of cake spiked with SPAs

	Found (mg kg^{-1}), R.S.D. (%)	Found (mg kg^{-1}), R.S.D. (%)			
	Added (mg kg^{-1})	Day1 (n=6)	Day2 (n=6)	CV_R (%)	Recovery (%)
PG	20	19(1.9)	18(1.7)	1.8	93
	50	45(2.1)	48(1.7)	1.9	93
	80	75(1.5)	72(1.9)	1.7	92
TBHQ	20	19(1.1)	19(1.3)	1.2	95
	50	46(1.7)	47(1.7)	1.7	93
	80	73(1.9)	78(2.1)	2.0	94
BHA	20	20(2.0)	19(1.8)	1.9	98
	50	48(2.1)	47(1.7)	1.9	95
	80	79(1.5)	76(1.7)	1.6	97
OG	20	19(1.2)	20(1.4)	1.3	98
	50	49(1.8)	48(1.8)	1.8	97
	80	79(1.8)	79(2.2)	2.0	99

R.S.D. represents means of per concentration level in each day; CV_R represents means of per concentration level in 12 times

Application of this Method in Commercial Matrices of Foods

To evaluate the effectiveness of the established method in larger range of cakes, it was applied to the analysis of a total of 8 samples of different kinds of cakes (Table-3). SPAs were found at concentration levels near the limit of detection in 7 samples. Furthermore, the total content of SPAs in all of the samples was below the critical value defined in Chinese National Standard for all of the SPA-positive samples.

Table-3: Levels of SPAs found in food samples.

Sample type	Manufacturing country	SPAS (mg kg^{-1})				
		PG	TBHQ	BHA	OG	Total
Creame cake	China	—	—	—	10	10
Creame cake	China	—	15	—	8	23
Meat floss cake	China	—	—	—	—	—
Meat floss cake	China	12	—	—	11	23
Chocolate cake	China	8	10	—	—	18
Chocolate cake	China	—	—	9	5	14
Jam cake	China	—	11	—	—	11
Jam cake	China	—	—	6	7	13

"—" represents no SPAs were found

Conclusion

Mixed micelle-mediated cloud point extraction (MMCPE) using two kinds of nonionic surfactants NP-7 and NP-9 coupled with HPLC has been proven to be an effective preconcentration method for the analysis of SPAs in food samples. The technique is higher in extraction efficiency and provides efficient phase separation without using salt and centrifugation and proven to be more simple, rapid and reliable compared with traditional CPE. In this method, only a small amount of surfactant is used, which is safer and more environmental-friendly. In a wider perspective, handling food samples with the aid of MMCPE is a potential field of investigation, which could be applied to monitoring of other chemical compositions.

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