

Separation, Concentration and Determination of Trace Thiamphenicol in Egg, Milk and Honey Using Polyoxyethylene Lauryl Ether-Salt Aqueous Two-Phase System Coupled with High Performance Liquid Chromatography

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As a pretreatment technique to trace analysis, polyoxyethylene lauryl ether (POELE10)-salt aqueous two-phase extraction system (ATPES) is coupled with high performance liquid chromatography to analyse thiamphenicol (TAP) in egg, milk and honey. The analysis of the phase behaviors of the ATPES found that the extraction efficiency and enrichment factor of TAP was influenced by the types of salts, the concentration of salt, the concentration of POELE10, the concentration of 1-propanol and the temperature. The optimized conditions of experiment were determined in the multi-factor experiment by using response surface methodology. The extraction efficiency of TAP is up to 99.59% and the optimal values of enrichment factor of TAP was 27 under the optimized conditions. The limit of detection and the limit of quantification of TAP are 0.5 $\mu\text{g kg}^{-1}$ and 1.5 $\mu\text{g kg}^{-1}$, which meet the needs of determination of trace or ultratrace TAP in food.

Keywords: aqueous two-phase system, polyoxyethylene lauryl ether, trace thiamphenicol, determination

Introduction

Thiamphenicol¹ [D(+)-threo-2-di-chloroacetamido-1-(4-methylsulphonylphenyl) propane-1, 3-diol; (TAP)] is an analogue of chloramphenicol in which the nitro group on the benzene ring is replaced with a methylsulfonyl group (Figure 1).² As a member of chloramphenicol family, TAP is a broad spectrum antibiotics against most of Gram negative and Gram positive pathogens and especially effective against anaerobic organisms. It was reported that TAP has excellent performance in the treatment of infectious diseases, including respiratory infections, bacterial prostatitis and venereal diseases.³ TAP has been widely used in veterinary medicine for the treatments of various infections. TAP has been found in various foods, such as fish, liver, milk, poultry, honey and even egg because of the abuse of TAP in various types of animal farms. In the USA, China and European Union, the clinical applications of TAP is strictly controlled because of the potential for the emergence of drug resistant bacteria and the drug residues in animal tissues.⁴ However, illegal use of TAP in livestock

and aquaculture still exists because of its steady antibiosis effectiveness and low price.

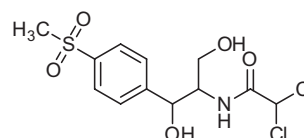


Figure 1. The structure of TAP.

Many different analytical methods have been reported for the determination of TAP in animal tissues, such as liquid chromatography (LC),⁵ gas chromatography (GC),⁶ LC-mass spectrometry (MS),⁷ LC-MS/MS,⁸ and GC-MS.⁹ However, these methods have some defects and insufficiencies such as long sample preparation time, complex analyzing processes and expensive equipment. Therefore, it is necessary to develop a simple, rapid and sensitive method to determine the concentration of TAP in food commodities.

Aqueous two-phase system (ATPS)¹⁰ is a powerful green extraction technique, and it is more and more widely applied in the separation and extraction of the biological materials, including nucleic acids,¹¹ proteins,¹² viruses,¹³

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antibiotics¹⁴ and other biological molecules.¹⁵ The ATPSs mainly divide into four kinds that are the polymer-polymer ATPSs,¹⁶ polymer-salt ATPSs,¹⁷ ion liquid-salt ATPSs¹⁸ and micromolecule alcohol-salt ATPSs.¹⁹ However, there are no public reports on the ATPS separation and enrichment method for TAP. In our previous article,²⁰ it was found that nonionic surfactant polyoxyethylene (10) lauryl ether (POELE10, $C_{32}H_{66}O_{11}$) was an appropriate choice to form polymer-salt ATPS because it consisted of the hydrophobic alkyl domain and hydrophilic polyoxyethylene tail. The phase behaviors of the ATPSs, composed of POELE10 and various salts, were studied and it was found that the critical concentration of POELE10 was quite low for the POELE10-salt ATPS forming. Therefore, the POELE10-salt ATPSs can work well in the isolation and concentration of materials. In this paper, we studied the extraction and determination of TAP by using POELE10-salt ATPSs coupled with high performance liquid chromatography (HPLC).

Experimental

Materials

Salts (K_2HPO_4 , $ZnSO_4$, $(NH_4)_2SO_4$, NaH_2PO_4 , $K_2C_4H_4O_4$, $Na_2C_4H_4O_6$ and $K_2C_2O_4$) and 1-propanol were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), which were analytical grade reagents (GR, min. 99% by mass fraction). Nonionic surfactants POELE10 with a quoted purity of greater than 0.99 mass fractions was obtained from Aladdin reagent company (Shanghai, China). The thiamphenicol standard sample was purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The water used in experiments was double distilled. All reagents were used without further purification.

Apparatus and procedure

An HPLC (Agilent 1200, Agilent, USA) equipped with UV-Vis detector was employed for the qualitative

and quantitative analysis of the TAP. The Agilent ChemStation software was used to control the machine and process data. An analytical balance (BS124S, Beijing Sartorius Instrument Co., China) with an uncertainty of $\pm 1.0 \times 10^{-7}$ kg was used to weigh.

The appropriate amounts of POELE10, salt, 1-propanol and TAP were put into the vessel from stock solutions, and water was added until the total volume is up to 10 mL. The mixed solution was kept for 30 min under mechanical shaking, and then it was placed in the thermostat water bath. The temperature of the thermostat water bath was maintained at the constant temperature ($T = 298.15$ K). The concentration of TAP in the top phase was determined by using HPLC when the system was separated to two phases. An Eclipse XDB-C18 reversed-phase column (serial No. G1314B, 250 mm \times 4.6 mm, 5 μ m) was used for the chromatographic separation, and the column temperature was at 298.15 K. The mobile phase consisted of water and methanol with the ratio of 3:1, and the flow rate was 1.0 mL min^{-1} . The injected volume of sample was 20 μ L, and the column effluent was monitored at the wavelength of 225 nm (Figure 2).

Preparation of real samples

Egg, milk, and honey samples were purchased from the local supermarket. 50 mL of sample and 20 mL of trichloroacetic acid (10%) were placed into a 100 mL tube, and the TAP working solution was added. Then, water was added into the tube until the total volume reached 100 mL. The mixture solution was shaken by using homogenizer-disperser (SJB-S450, Shanghai Siehe Instrument Co., China) until it was thoroughly mixed. The sample was put into centrifugal tube and was centrifuged at 4000 rpm for 30 min (Anke TDL-4, Shanghai Chemical Machinery Plant Co., Ltd., China). The supernatant was taken out and filtered through the microfilter (Fushun filter paper factory, China) with a pore size of 0.45 μ m to remove the proteins. The homogenization and centrifugation was carried out at room temperature. Finally, the filter liquor was stored at 4 $^{\circ}C$.

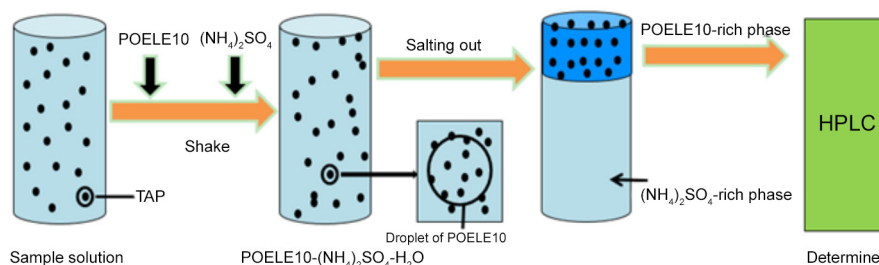


Figure 2. The schematic diagram of separation TAP in POELE10- $(NH_4)_2SO_4$ ATPS.

Determination of the partition parameters of TAP

The enrichment factor and extraction efficiency was used to measure the standard of partition and enrichment efficiency of TAP. The enrichment factor (F) was defined as a ratio of the concentration of TAP in the top phase to that in the initial system.

$$F = \frac{C_t}{C_s} \quad (1)$$

where C_t was the concentration of TAP in the top phase, C_s was the concentration of TAP in the initial system before the two phases were separated. The extraction efficiency ($E\%$) was calculated by the following equation:

$$E\% = \frac{C_t \times V_t}{m_s} \times 100 \quad (2)$$

where C_t was the concentration of TAP in the top phase, V_t was the volume of top phase, m_s was the total mass of TAP added in the initial system. The phase ratio ($R\%$) was defined as a ratio of the volume of top phase to the volume of bottom phase.

$$R\% = \frac{V_t}{V_b} \times 100 \quad (3)$$

where V_t and V_b were the volume of top phase and bottom phase.

Statistical analysis

The range of optimized conditions of the aqueous two phase extraction system for the extraction and determination of trace TAP was obtained by the single factor test. The response surface methodology (RSM) was used to design the multiple-factor and multiple-level experiment to determine the optimized conditions of experiment. RSM includes two steps: the factorial designs and the regression analysis. In this experiment, factorial design of RSM was

performed by a central composite design (CCD) with four factors and five levels. The independent factors were the concentration of $(\text{NH}_4)_2\text{SO}_4$ (X_1), the concentration of POELE10 (X_2), the concentration of 1-propanol (X_3) and the temperature (X_4). The CCD was applied by using Design-Expert 7.0. The four factors at five levels are shown in Table 1.

The regression analysis of experimental data was performed by the response surface regression procedure, and analysis model was conducted according to the following quadratic equation (equation 4).

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j \quad (4)$$

where Y is the response, X_i and X_j represent the factors, β_0 , β_i , β_{ii} and β_{ij} are regression coefficient for the intercept, linear, quadratic and interaction coefficients, respectively. The ranges of i and j is 1 to 4. The evaluation method of statistical significance of model was F-test.

Results and Discussion

The choice of salt for ATPS

In our previous work,²⁰ we have discussed some ATPSs composed of POELE10 and various salts, and found that the salt type is one important factor affecting the phase diagram. This means that salt type will also affect the extraction of TAP. Thus, the study on the extraction efficiency and enrichment factor of TAP using different ATPS that contained POELE10 and various salts (K_2HPO_4 , ZnSO_4 , $(\text{NH}_4)_2\text{SO}_4$, NaH_2PO_4 , $\text{K}_2\text{C}_4\text{H}_4\text{O}_6$, $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$ and $\text{K}_2\text{C}_2\text{O}_4$) was conducted. The extraction efficiency and enrichment factor of the various ATPSs for TAP at three temperatures are shown in Figures 3 and 4. It was found that POELE10- $(\text{NH}_4)_2\text{SO}_4$ ATPS is obviously better than other POELE10-salts ATPSs in terms of the extraction efficiency and enrichment factor (Figures 3 and 4). The main reason for POELE10-salts to present different performance in extraction of TAP is salting-out ability of salt. The salting-out ability of salt is affected by many factors, such as the

Table 1. Factors and their levels of CCD for extraction of TAP using ATPS

Symbol	Factor	Coded level				
		-2	-1	0	1	2
A(X_1)	$(\text{NH}_4)_2\text{SO}_4$ / (g mL ⁻¹)	0.138	0.141	0.144	0.147	0.150
B(X_2)	POELE10 / (g mL ⁻¹)	0.021	0.024	0.027	0.030	0.033
C(X_3)	1-propanol / (g mL ⁻¹)	0	0.05	0.15	0.20	0.25
D(X_4)	Temperature / K	288.15	293.15	298.15	303.15	308.15

valence of ions, the Gibbs free energy of hydration of the ions and the effective excluded volume.²¹ Therefore, the POELE10-(NH₄)₂SO₄ ATPS was used to separate and enrich TAP in this work.

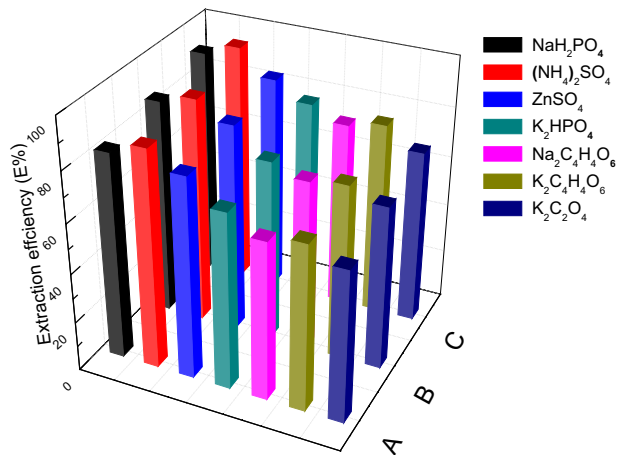


Figure 3. The changes of the extraction efficiency of TAP for the different POELE10-salts ATPSs at three temperatures. A: 15 °C, B: 25 °C and C: 35 °C.

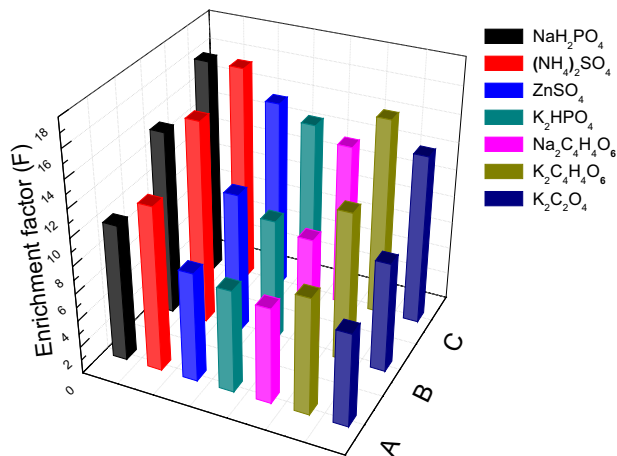


Figure 4. The changes of the enrichment factor of TAP for the different POELE10-salts ATPSs at three temperatures. A: 15 °C, B: 25 °C and C: 35 °C.

TAP distribution in the POELE10-(NH₄)₂SO₄ ATPS

Effect of concentration of (NH₄)₂SO₄ on the distribution

From our previous research,²¹ it was found that the binodal curve of POELE10-salt ATPS moves towards the axis with the increase in the concentration of salt. As an important factor affecting the distribution of TAP in two phases, the concentration of (NH₄)₂SO₄ was discussed. The extraction efficiency and enrichment factor of TAP at three temperatures was studied when the concentration of (NH₄)₂SO₄ ranged from 0.126 g mL⁻¹ to 0.159 g mL⁻¹ and the other parameters were fixed, and the experiment

results are given in Figure 5. It was found that the extraction efficiency and enrichment factor improved with increase of (NH₄)₂SO₄ concentration from Figure 5. That is because the salting-out strength of system enhanced with increase of salt concentration, and therefore, more TAP was excluded to the rich-POELE10 phase from rich-salt phase. Figure 5 showed that the enrichment factor is too low when the concentration of (NH₄)₂SO₄ is below 0.138 g mL⁻¹ and the extraction efficiency and enrichment factor slowly grows when the concentration of (NH₄)₂SO₄ is more than 0.150 g mL⁻¹. Therefore, the ideal range of the concentration of (NH₄)₂SO₄ is from 0.138 g mL⁻¹ to 0.150 g mL⁻¹ for the subsequent multi-factor experiment.

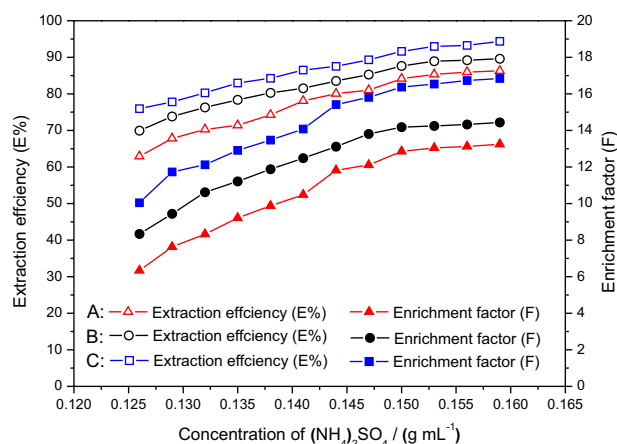


Figure 5. The changes of the extraction efficiency and enrichment factor of TAP with the concentration of (NH₄)₂SO₄. A: 15 °C, B: 25 °C and C: 35 °C.

Effect of the added volume of POELE10 on the distribution of TAP

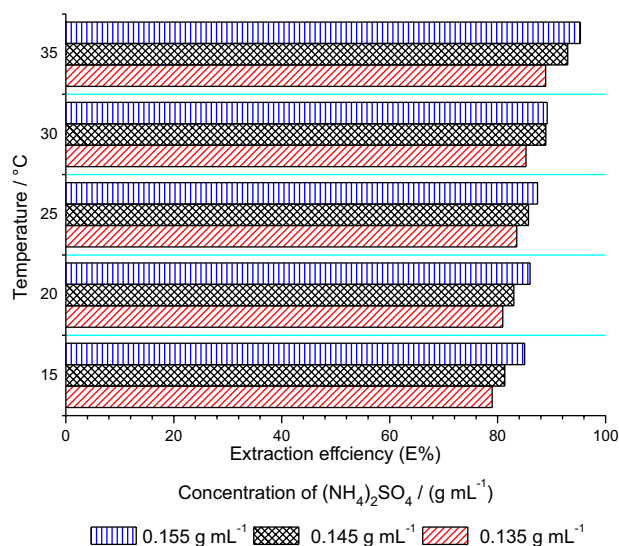
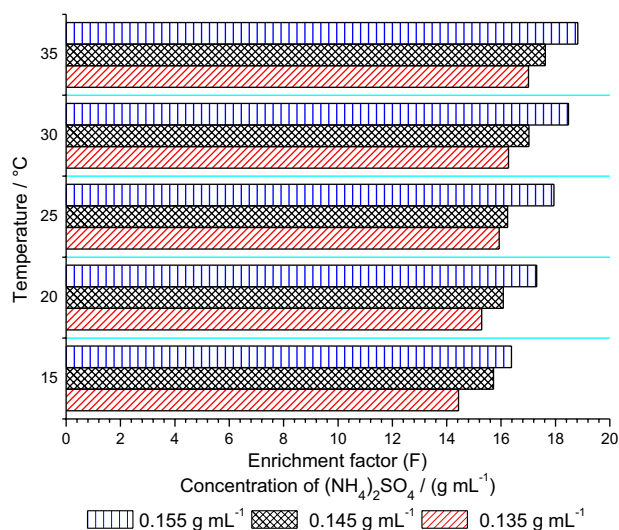
The volume of POELE10 added at the experiment is another important affecting factor for phase diagram, and the two phases area will expand with the added volume of POELE10 rising. We have determined the extraction efficiency (E%), enrichment factor (F) and phase ratio (R%) for the extraction system in which the volume of POELE10 is different, and the result is shown in Table 2. In Table 2, it was found that with the increase in the volume of POELE10, the extraction efficiency of TAP increased, however, the enrichment factor decreased. The reason is that the volume of top phase increased with the increase in the addition content of POELE10, which can be seen from the phase ratio (R%). This will cause the concentration of TAP in top phase to decrease. The concentration of TAP in top phase (C₁) decreased, and the concentration of TAP in total system (C_s) remained the same, and therefore, the enrichment factor (F) would decrease. In the multi-factor experiment, the range of concentration of POELE10 will be set between 0.021 g mL⁻¹ (volume: 1.4 mL) and 0.033 g mL⁻¹ (volume: 2.2 mL) for balancing extraction efficiency and enrichment factor.

Table 2. The effect of the amount of POELE10 on extraction efficiency, enrichment factor and phase ratio of TAP

POELE10 / mL	15 °C			25 °C			35 °C		
	F	E%	R%	F	E%	R%	F	E%	R%
1.0	17.31	74.85	3.08	23.46	81.32	3.13	24.82	85.58	3.20
1.2	16.25	76.69	3.58	22.48	83.12	3.63	23.35	86.36	3.71
1.4	15.03	78.25	4.35	20.25	84.63	4.40	22.14	88.85	4.48
1.6	14.30	79.08	5.45	18.21	85.42	5.49	21.11	89.63	5.57
1.8	13.88	80.83	6.21	16.07	87.12	6.25	18.98	91.31	6.32
2.0	12.29	82.21	6.77	14.47	88.47	6.82	17.40	92.65	6.91
2.2	10.18	82.73	7.26	12.35	88.95	7.30	15.28	93.10	7.38
2.4	8.55	83.08	7.73	10.72	89.26	7.78	13.65	94.40	7.84
2.6	7.81	85.54	8.94	9.46	91.19	8.99	12.41	95.33	9.07
2.8	7.31	86.17	9.31	8.46	91.20	9.34	10.41	96.32	9.41

Effect of temperature on the distribution

Besides the concentration of two forming phase materials, temperature is one important influence on the phase diagram of ATPS.²⁰ It was found in our previous works,²¹ that the phase-forming ability of POELE10-ATPSs enhanced with the increase in temperature. Thus, temperature will influence the extraction efficiency and enrichment factor of TAP. The effect of temperature on the extraction efficiency and enrichment factor was studied and their relationship is shown in Figures 6 and 7. With the rising temperature, the extraction efficiency and enrichment factor increased. The hydrophobicity of POELE10 enhanced with the increase of the system temperature,²¹ which makes TAP more prone to enrich in top phase. Because TAP may be inactivated when the temperature is too high or low and the extraction is easier to implement at near room

**Figure 6.** The changes of the extraction efficiency of TAP with the temperature.**Figure 7.** The changes of the enrichment factor of TAP with the temperature.

temperature, the temperature varies from 15 °C to 35 °C in the multi-factor experiment.

Effect of added amount of ethanol, 1-propanol and 2-propanol on the distribution

The extraction efficiency of POELE10-(NH₄)₂SO₄ ATPS for TAP was up to 95.87%, and in order to increase the extraction efficiency of TAP, ethanol,²² 1-propanol²³ and 2-propanol²⁴ were added in the system to enhance the transferring ability to top phase, which is learned from the successful experience of Pan and Cisneros.²² The effect of added amount of ethanol, 1-propanol and 2-propanol on the extraction efficiency and enrichment factor at 298.15 K was studied, and the results are shown in Figure 8. It was found that the addition of ethanol had no effect on the extraction efficiency of TAP, and the extraction efficiency of TAP slightly increased as the 2-propanol was added, however,

the extraction efficiency of TAP significantly increased as the 1-propanol was added. This may be because the TAP would mix more easily with 1-propanol, and meanwhile, ethanol, 1-propanol and 2-propanol are much likely to come into top phase due to salting-out effect. Therefore, the extraction efficiency of TAP rised with the increase in added amount of 1-propanol.

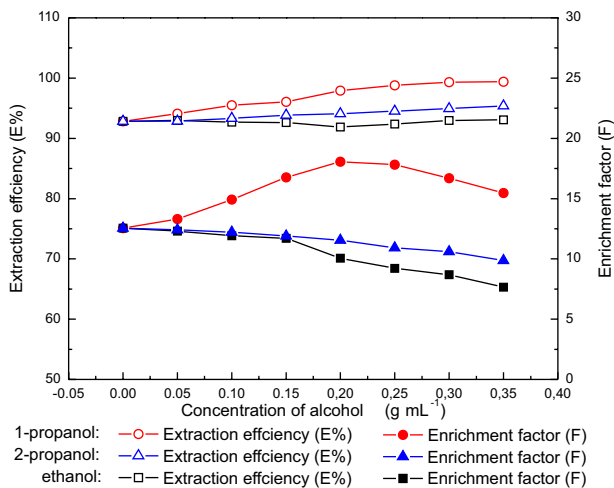


Figure 8. The changes of the extraction efficiency and enrichment factor of TAP with the concentration of ethanol, 1-propanol and 2-propanol.

Then the enrichment factor of TAP decreased as ethanol or 2-propanol was added, because the addition of ethanol and 2-propanol has nothing to promote TAP into top phase. However, the volume of top phase increases, and the enrichment factor of TAP decreases. The enrichment factor of TAP first increased and then decreased along with the increasing amount of added 1-propanol. The reason may be that the concentration of TAP in top phase rised with the increase in the added amount of 1-propanol at first, and when most of TAP have been in top phase and the concentration of TAP in top phase decreased with the increase in the added amount of 1-propanol. Therefore, it was concluded that adding suitable amount of 1-propanol can improve the extraction efficiency and enrichment factor of TAP.

The multi-factor experiment by using RSM

Factors affecting enrichment of TAP

The specific design for the multi-factor experiment by using CCD with four factors at five levels and the experimental results are given in Table 3. In order to test the statistical analysis of the extraction efficiency and enrichment factor by using RSM, the Fisher's F-test was used for the analysis of variance and the results are given in Tables 4 and 5. The probability equals to the proportion

of the area under the curve of the F-distribution that lies beyond the observed F-value was expressed as the "Prob > F" value. The small probability values called for the rejection of the null hypothesis, in other words, the particular term was statistically significant.²⁵ The value of "Prob > F" is less than 0.05 and it indicates that the model terms are significant, and the value is greater than 0.1, that indicates the model terms are not significant. In this work, the factors B, C, D have an important effect on extraction efficiency of TAP as shown in Table 3. On the other hand, the factors B, C, D as well as the interaction of factors CD, and C² were significant in affecting enrichment factor of TAP according to the data in Table 4.

Regression analysis

The response function (equation 4) was fit by the equation regression analysis to predict the outcome of enrichment of TAP. The multiple regression analysis was performed according to the experimental data of extraction efficiency of TAP (Table 3), and the coefficients of the model were evaluated for the significance. The equation for predicting the extraction efficiency of TAP was obtained, and it is given as follows:

$$Y_{E\%} = 89.42 + 0.77X_A + 2.50X_B + 8.08X_C + 1.06X_D \quad (5)$$

For the enrichment factor of TAP, the final predictive equation was also obtained according to the experimental data of enrichment factor of TAP in Table 3.

$$Y_F = 21.45 + 0.39X_A - 1.09X_B - 2.19X_C + 1.29X_D - 0.23X_AX_B - 0.47X_AX_C + 0.16X_AX_D + 0.57X_BX_C + 0.63X_BX_D - 0.88X_CX_D - 0.037X_A^2 + 0.15X_B^2 - 1.23X_C^2 + 0.68X_D^2 \quad (6)$$

In the above mentioned two equations, $Y_{E\%}$ represents the extraction efficiency of TAP, Y_F represents the enrichment factor of TAP, and X_A , X_B , X_C , X_D are four factors. The proportion of total variation attributed to each fit can be evaluated by the value of R-square. The R-square value was 0.9869 for the extraction efficiency of TAP, which indicated that the fitting between the model and the experimental results were good. However, for the enrichment factor of TAP, lower R-square value of 0.9345 was obtained.

Response surface plot

The factors influencing the extraction efficiency of TAP and enrichment factor of TAP were shown as the response surface plots in Figures 9 and 10. The relationship

Table 3. The extraction efficiency and enrichment factor in the upper phase for each experiment of the CCD

Run	A	B	C	D	Y ₁	Y ₂
	(NH ₄) ₂ SO ₄ / (g mL ⁻¹)	POELE10 / (g mL ⁻¹)	1-propanol / (g mL ⁻¹)	Temperature / K	Extraction efficiency (E%)	Enrichment factor (F)
1	0.147	0.03	0.15	303.15	99.50	17.82
2	0.144	0.027	0.1	298.15	89.66	21.87
3	0.144	0.027	0.1	308.15	93.41	26.70
4	0.147	0.03	0.05	303.15	85.59	23.13
5	0.141	0.03	0.15	303.15	99.59	18.20
6	0.147	0.03	0.05	293.15	82.45	17.58
7	0.141	0.024	0.05	293.15	75.50	21.60
8	0.141	0.024	0.15	293.15	97.56	18.20
9	0.144	0.033	0.1	298.15	98.19	20.22
10	0.141	0.03	0.15	293.15	99.26	16.51
11	0.141	0.024	0.05	303.15	75.40	21.55
12	0.144	0.027	0.1	288.15	85.02	20.01
13	0.147	0.024	0.05	303.15	76.72	26.70
14	0.141	0.03	0.05	293.15	78.24	17.30
15	0.147	0.024	0.05	293.15	76.62	22.20
16	0.147	0.024	0.15	303.15	96.21	17.05
17	0.147	0.024	0.15	293.15	98.84	17.80
18	0.144	0.027	0.2	298.15	99.46	13.03
19	0.141	0.03	0.05	303.15	85.59	23.13
20	0.144	0.027	0.1	298.15	85.86	19.37
21	0.144	0.027	0	298.15	78.39	23.14
22	0.15	0.027	0.1	298.15	95.23	24.09
23	0.141	0.024	0.15	303.15	97.78	18.26
24	0.138	0.027	0.1	298.15	89.13	21.65
25	0.147	0.03	0.15	293.15	99.16	17.02
26	0.144	0.021	0.1	298.15	85.61	26.99

Table 4. Results of the analysis of variance (ANOVA) performed to the extraction efficiency models

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	1757.534	4	439.3836	48.99841	< 0.0001	significant
A	14.08917	1	14.08917	1.571171	0.2216	–
B	149.5228	1	149.5228	16.67422	0.0004	–
C	1566.753	1	1566.753	174.7184	< 0.0001	–
D	27.16971	1	27.16971	3.029864	0.0940	–
Residual	224.1826	25	8.967302	–	–	–
Lack of fit	212.1494	20	10.60747	4.407603	0.0536	not significant
Pure error	12.03315	5	2.40663	–	–	–
Cor total	1981.717	29	–	–	–	–

Table 5. Results of the analysis of variance (ANOVA) performed to the enrichment factor models

Source	Sum of squares	df	Mean square	F value	<i>p</i> -value Prob > F	
Model	262.3053	14	18.73609	5.401335	0.0012	significant
A	3.716025	1	3.716025	1.071275	0.3171	–
B	28.64458	1	28.64458	8.257805	0.0116	–
C	115.0153	1	115.0153	33.1572	< 0.0001	–
D	40.09161	1	40.09161	11.55781	0.0040	–
AB	0.866028	1	0.866028	0.249663	0.6246	–
AC	3.534845	1	3.534845	1.019043	0.3288	–
AD	0.411509	1	0.411509	0.118632	0.7353	–
BC	5.227116	1	5.227116	1.5069	0.2385	–
BD	6.362108	1	6.362108	1.834101	0.1957	–
CD	12.30139	1	12.30139	3.546309	0.0792	–
A ²	0.036572	1	0.036572	0.010543	0.9196	–
B ²	0.593457	1	0.593457	0.171085	0.6850	–
C ²	41.67487	1	41.67487	12.01424	0.0035	–
D ²	0.194092	1	0.194092	0.055954	0.8162	–
Residual	52.03183	15	3.468789	–	–	–
Lack of fit	46.82346	10	4.682346	4.495022	0.0555	not significant
Pure error	5.208369	5	1.041674	–	–	–
Cor total	314.3371	29	–	–	–	–

between response (E% and F) and experimental levels of each variables was visualized in the 3D response surface plot, which, meanwhile, provides a method to directly observe the interactions between two test variables.²⁶ The interactions between variables and their optimum ranges can be well observed by studying the response surface plots. The analysis of Figures 9 and 10 and Table 3 shows that the optimal value of extraction efficiency of TAP (E%) was 99.59%, and it occurred when the concentration of (NH₄)₂SO₄, the concentration of POELE10, the concentration of 1-propanol and temperature were 0.141 g mL⁻¹, 0.030 g mL⁻¹, 0.15 g mL⁻¹ and 303.15 K, respectively. The optimized condition for the enrichment factor of TAP (F) was 26.99 under the following conditions: the concentration of (NH₄)₂SO₄ was 0.144 g mL⁻¹; the concentration of POELE10 was 0.021 g mL⁻¹; the concentration of 1-propano was 0.10 g mL⁻¹ and temperature was 298.15 K.

Samples analysis

This POELE10-(NH₄)₂SO₄ aqueous two-phase extraction technique was applied to separate and enrich TAP in egg, milk and honey under the optimal conditions as previously discussed in this paper. After POELE10-(NH₄)₂SO₄ ATPS separation procedures, TAP in real samples was separated to top phase, and then it

was determined by HPLC-UV method mentioned above. In the linear range of 0.5-3 µg mL⁻¹, TAP in real samples was analyzed by using this extraction method. The limit of detection (LOD) and the limit of quantification (LOQ) are 0.5 µg mL⁻¹ and 1.5 µg mL⁻¹ with the standard deviation of 0.9984 and 0.9991. The extraction efficiency of TAP and HPLC spectrogram is shown in Table 6 and Figure 11. It was found that the recovery of TAP was 96.80-99.89% with a RSD of 0.46-2.47% when the concentration of the added TAP was 10-100 ng mL⁻¹ as shown in Table 6. All this illustrated that this extraction technique has a satisfactory recovery and reproducibility for the determination of the TAP in a wide range of concentrations. Thus, this method can be applied to the quantitative analysis of residual trace TAP in the food and environment.

Conclusions

In this paper, the POELE10-(NH₄)₂SO₄ ATPS was applied to separate and purify trace of TAP in food. The RSM was used to design multi-factor experiment in order to determine the optimized conditions for the extraction efficiency of TAP (E%) and enrichment factor of TAP (F). Under the optimized conditions, the trace TAP in egg, milk and honey was successfully quantitatively determined by this preprocessing method with HPLC. The LOD and LOQ

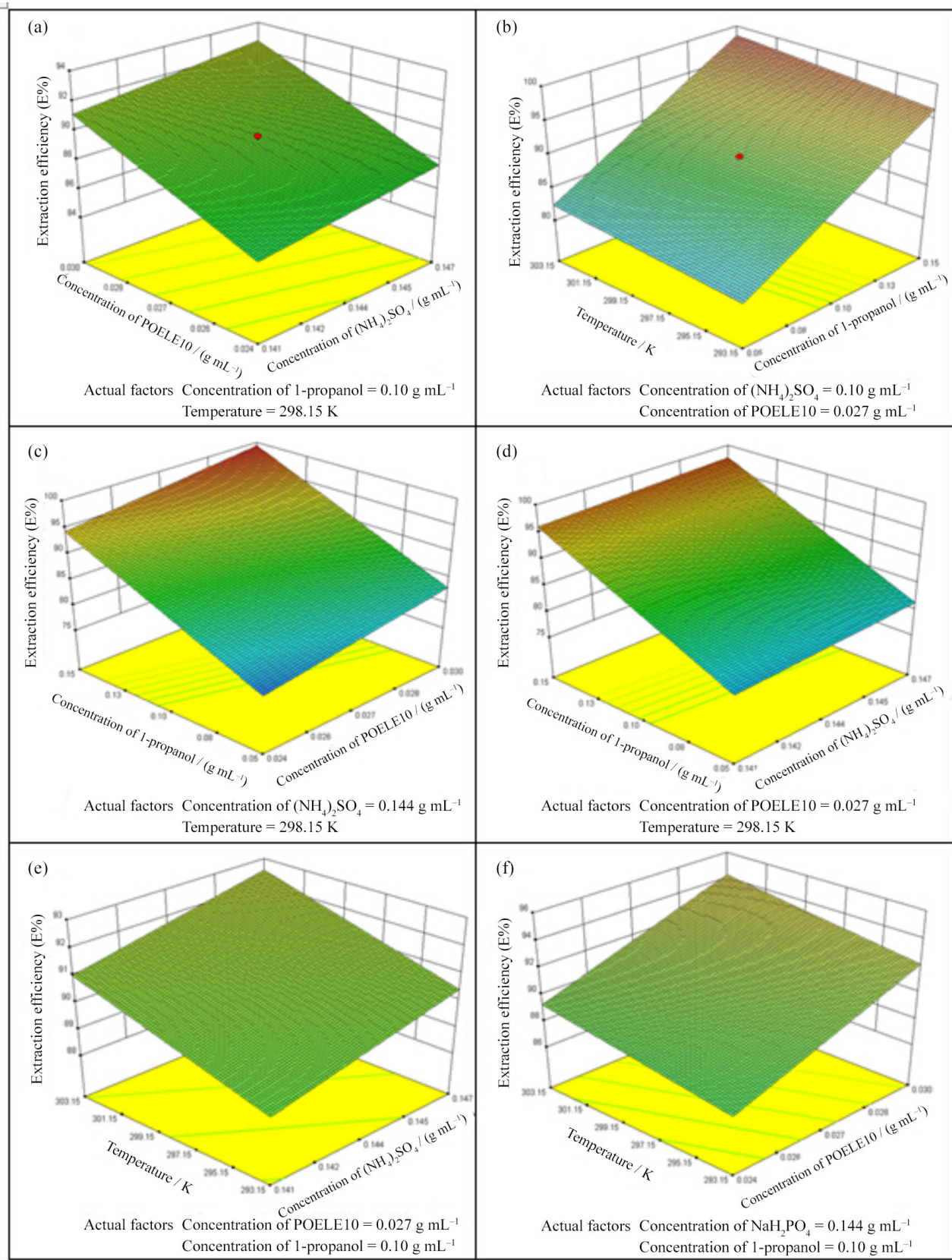


Figure 9. The 3D response surface plot for the interactive effect of (a) concentration of (NH₄)₂SO₄ and concentration of POELE10; (b) temperature and concentration of 1-propanol; (c) concentration of 1-propanol and concentration of POELE10; (d) concentration of 1-propanol and concentration of (NH₄)₂SO₄; (e) temperature and concentration of (NH₄)₂SO₄; and (f) temperature and concentration of POELE10 on the extraction efficiency of TAP, while other variables are set at the fixed value.

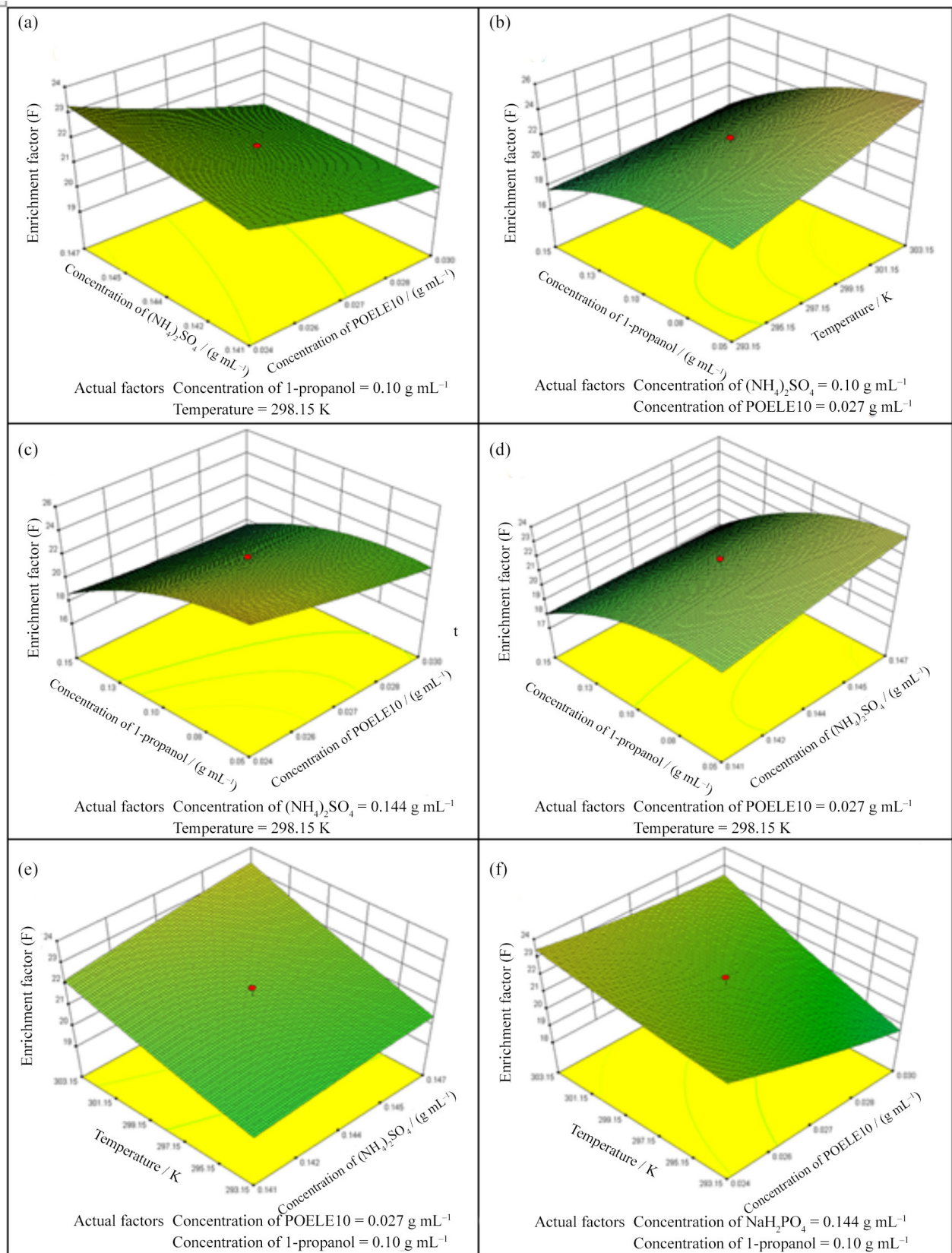
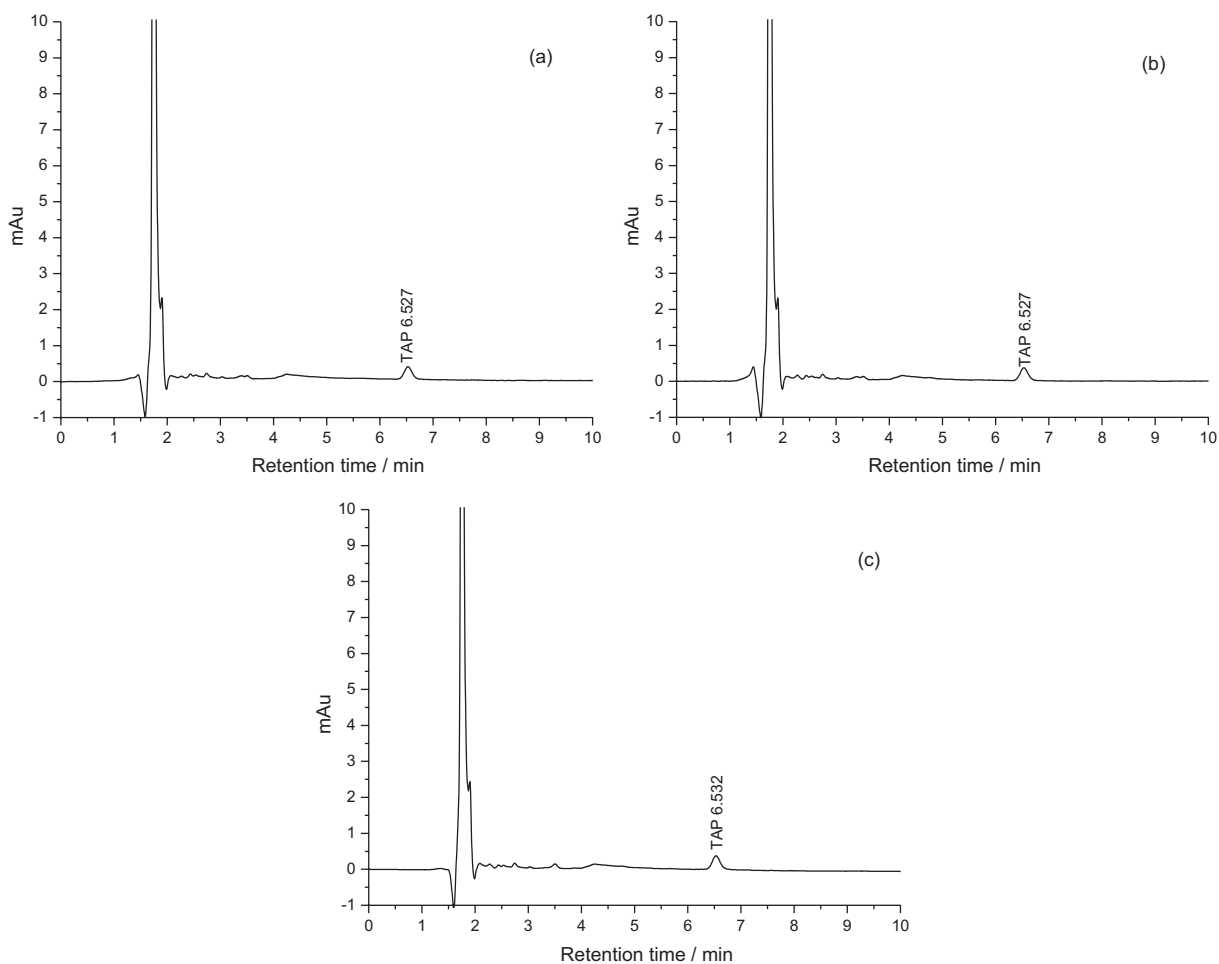


Figure 10. The 3D response surface plot for the interactive effect of (a) concentration of $(\text{NH}_4)_2\text{SO}_4$ and concentration of POELE10; (b) temperature and concentration of 1-propanol; (c) concentration of 1-propanol and concentration of POELE10; (d) concentration of 1-propanol and concentration of $(\text{NH}_4)_2\text{SO}_4$; (e) temperature and concentration of $(\text{NH}_4)_2\text{SO}_4$; and (f) temperature and concentration of POELE10 on the enrichment factor of TAP, while other variables are set at the fixed value.

Table 6. Analysis results (mean \pm SD, n = 5) for TAP in real samples

Sample	Added TAP / (ng mL ⁻¹)	Determined TAP / (ng mL ⁻¹)	Extraction efficiency (E%)	RSD
Egg	0	ND	–	–
	10	9.94 \pm 0.21	99.40 \pm 2.07	2.09
	25	24.86 \pm 0.11	99.44 \pm 0.45	0.46
	50	49.94 \pm 0.42	99.89 \pm 0.85	0.85
	100	99.78 \pm 1.16	99.79 \pm 1.16	1.16
Milk	0	ND	–	–
	10	9.68 \pm 0.24	96.80 \pm 2.39	2.47
	25	24.64 \pm 0.42	98.56 \pm 1.66	1.69
	50	49.80 \pm 0.66	99.60 \pm 1.31	1.32
	100	99.14 \pm 1.16	99.14 \pm 1.16	1.17
Honey	0	ND	–	–
	10	9.82 \pm 0.15	98.20 \pm 1.48	1.51
	25	24.84 \pm 0.11	99.36 \pm 0.46	0.46
	50	49.76 \pm 0.74	99.52 \pm 1.49	1.49
	100	99.64 \pm 0.87	99.64 \pm 0.87	0.87

ND: not found.

**Figure 11.** HPLC chromatograms with UV detection of real sample added with 10 ng mL⁻¹ TAP after ATPS extraction. Real samples: (a) egg; (b) milk and (c) honey.

of TAP are $0.5 \mu\text{g mL}^{-1}$ and $1.5 \mu\text{g mL}^{-1}$, and it coincides with the requirement of detecting the trace TAP in food.²⁷ It also gives us an enlightenment for the future research on whether the POELE10-(NH₄)₂SO₄ ATPE is more applicable to the extraction of trace TAP in environment samples.

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