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Aqueous Two-Phase System–Ion Chromatography for Determination of Thiocyanate in Raw Milk

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Abstract: Thiocyanate could effectively inhibit bacteria in milk and extend the shelf life of milk. However, excessive addition will lead to health risks. Therefore, the determination of thiocyanate in raw milk has received a lot of attention, but the determination could be interfered with by other components in raw milk and the pre-treatment of raw milk is complex. In this study, a new pretreatment method combined with ion chromatography (IC) for rapid and sensitive determination of thiocyanate is proposed. An acetonitrile/(NH₄)₂SO₄ aqueous two-phase system (ATPS) was developed for the separation and enrichment of thiocyanate in raw milk. Response surface methodology was performed to optimize the extraction conditions and an efficient pretreatment were obtained using ATPS composed of 42% acetonitrile (*w/w*) and 16% (NH₄)₂SO₄ (*w/w*), with the pH 4.7, and the recovery of thiocyanate reached 107.24 ± 0.5%, and the enrichment ratio was 10.74 ± 0.03. IC was used to establish a thiocyanate enrichment method. The linear range was from 0.05 to 15 mg/L and R² = 0.998, the limit of detection (LOD) was 0.2 µg/L, the limit of quantification (LQD) was 0.6 µg/L. Hence, it is feasible to combine ATPS with IC for the enrichment and determination of thiocyanate in raw milk.

Keywords: thiocyanate; raw milk; aqueous two-phase system; ion chromatography; extraction

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1. Introduction

Milk has high nutritional value which is easily digested and absorbed, making it one of the important sources of nutrition for human body, but it is highly prone to spoilage. Thiocyanates are known to be effective in prolonging the shelf life of milk through the lactoperoxidase system (LPS), a naturally occurring enzymatic antimicrobial system in raw milk that promotes the oxidation of halides and thiocyanates by hydrogen peroxide, resulting in a series of antimicrobial compounds [1]. Therefore, in countries where cold chain delivery is underdeveloped, trace amounts of thiocyanate ions are allowed to be added in raw milk to artificially activate LPS for the purpose of temporary inhibition of pathogenic microorganisms. However, thiocyanates are toxic substances that produce CN[−] which could bind to Fe³⁺ in the cytochrome oxidase enzyme in the body, thereby inhibiting the enzyme activity. It would lead to hypoxia in human tissues, even significant damage of kidney function. Lower doses of thiocyanate (4.8–6.4 mg/L) can competitively inhibit the transport of sodium iodide in the thyroid gland, thereby impairing iodine uptake. Iodide is a key component in the production of thyroid hormones, which plays a critical role in many physiological functions, especially for brain and nervous system development in fetuses and children. The concentration of thiocyanate in plasma above 120 mg/L usually triggers toxicity, with a lethal concentration of approximately 200 mg/L. In addition, chronic high levels of thiocyanate in physiological fluids could lead to vertigo, nasal bleeding, and

confusion [2–4]. The International Codex Alimentarius Commission (CAC) allows a limit value of 14 mg/kg of sodium thiocyanate (in terms of thiocyanate) to be added to raw milk; however, excessive use of thiocyanate is common in practical applications. It was noted that thiocyanate is also a naturally occurring substance in raw milk, and clinical trials by Reiter et al. [5] demonstrated that risky raw milk was related to excess added thiocyanate and that the content of thiocyanate in raw milk needed to be strictly controlled in industrial production. Therefore, the rapid and accurate determination of thiocyanate content in raw milk is of great importance in food safety and health care.

In order to detect thiocyanate, it is necessary to separate and enrich thiocyanate from raw milk, so that the interference of other components of raw milk in the detection process can be excluded. Among the available methods for thiocyanate enrichment, solid-phase extraction techniques have mostly been studied. Al-Saidi et al. [6] established a headspace sorptive solid-phase microextraction (HS-SPME) technique for the extraction of thiocyanate and cyanide. Under the optimized conditions, the LOD and LOQ were 0.34 and 1.2 mmol/L, respectively. Da Silva et al. [7] separated thiocyanates using capillary electrophoresis. The ranges of LOD and LOQ were 0.03–0.04 and 0.05–0.07 mg/L. Although the electromigration technique is inexpensive and environmentally friendly, it requires a high level of professionalism from the operator and is not easily promoted in factories. Lu et al. [8] extracted thiocyanate ions in the aqueous phase with complexes of Hg^{2+} into methyl isobutyl ketone and the LOD was 1.33 ng/mL. However, the currently reported methods for thiocyanate determination suffer from the disadvantages of cumbersome pre-treatment, low sensitivity, many influencing factors, and high cost. Therefore, a more rapid, sensitive, simple, and economical method for the enrichment and detection of thiocyanate in raw milk is necessary.

An aqueous two-phase system (ATPS) is formed mainly by the partitioning of two immiscible solutions. In some fields, ATPS is a novel alternative technique to conventional solvent extraction. ATPS has successfully been used for the extraction and purification of peptides [9], polysaccharides [10], enzymes [11], heavy metals [12], proteins [13], amino acids [14], cells [15], and cytochromes [16], and has also been used for the separation and enrichment of neutral, anionic, and cationic ions [17]. In recent years, some novel, low-cost, and efficient ATPSs have appeared that are different from typical polymer/salt and ionic liquid/salt systems. These ATPSs have received increasing attention due to high extraction efficiency, fast phase separation, low viscosity, mild environmental effects, and recyclability, such as alcohol/inorganic salt systems [18], propanol/inorganic salt systems [19], acetonitrile/inorganic salt systems [20], etc. These ATPSs allow the recovery of small organic molecules from the ATPSs by evaporation and crystallization, making it easy to separate the extract from the rich organic phase, thus reducing the cost of extraction and enrichment, and simplifying the subsequent production process for easy application in downstream production [11]. In analytical applications, such systems easily exclude the possible interference of background in the determination of target substances. Since the introduction of ion chromatography (IC) by Shapiro et al. [21] in 1975, the method has become the preferred method for the determination of small inorganic and organic ions. Thienpont et al. [22] reported that IC could determine the total sodium and potassium concentrations in human serum. Charles et al. [23] proposed a method of ion chromatography-mass spectrometry (IC-MS) to determine bromate ions in water. Fernandes et al. [24] used IC combined with UV detection to detect four bisphosphonates in pharmaceuticals or bulk materials. Now, the determination of thiocyanate in the industry is mostly based on the spectrophotometric method, which is based on the principle that SCN^- can generate blood-red iron thiocyanate complex ion ($[\text{Fe}(\text{SCN})_n]^{m\pm}$) with Fe^{3+} and $[\text{Fe}(\text{SCN})_n]^{m\pm}$ has the maximum absorption peak at 450 nm. The limit of detection mostly approaches 0.05 mg/L. Because of the disadvantages of the spectrophotometric methods such as large interference factors, low sensitivity, and low efficiency, this method is receiving less and less attention.

The combination of ATPS with ion chromatography (IC) using an anion-exchange column for separation and an amperometric detector or ultraviolet (UV) detector for determination offers the advantages of rapidity and simplicity, and high selectivity and sensitivity. ATPS was applied to the separation and enrichment of thiocyanate in raw milk for the first time in this study. Enrichment of thiocyanate from raw milk using ATPS has the advantages of being rapid, simple, inexpensive, low interference, and environmentally friendly, providing a new pre-treatment method for the determination of thiocyanate. In this study, acetonitrile/ $(\text{NH}_4)_2\text{SO}_4$ ATPS was compared with acetone/ammonium ATPS for the extraction of thiocyanates from raw milk, and the best extraction system was selected. The extraction conditions were optimized by response surface methodology (RSM) and the extraction mechanism was initially investigated. IC was combined with ATPS for the determination of thiocyanate in raw milk and the feasibility of the method was evaluated using the limit of detection (LOD) and quantification (LOQ), precision, spike recovery, and interference analysis.

2. Materials and Methods

2.1. Materials

Acetonitrile (ACN) was HPLC grade, KBr was SP grade, and all other reagents were AR grade. Sodium thiocyanate was obtained from Aladdin Industrial Corporation (Shanghai, China). ACN was provided by Dikma Technology Co. Ltd. (Beijing, China). KBr was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Other reagents were from Fuchen Chemical Reagents Factory (Tianjin, China).

2.2. Equipment

IC analysis was performed on a 930 (Metrohm AG, Herisau, Switzerland) ion chromatography system. Chromatographic separation of sodium thiocyanate was carried out using a Metrosep A Supp5 (250 mm $\times$ 4.0 mm, 5 μm) anion chromatographic column, which utilized 5 mM Na_2CO_3 containing 5% (v/v) acetone as eluent under isocratic conditions at a flowrate of 1.0 mL/min. The temperature of column and detector was maintained at 35 $^\circ\text{C}$ using a chromatography oven. The suppressor regeneration solution was H_2SO_4 solution (10 M). A SC-3610 low speed centrifuge (Zhongke Zhongjia Scientific Instrument Co., Ltd., Hefei, China) and 0.22 μm water membrane filters (Jinteng Laboratory Equipment Co., Ltd., Tianjin, China) were used for sample treatment. IR spectra were examined with an ALPHA-T Fourier transform infrared spectrometer (Bruker Daltonics, Karlsruhe, Germany).

2.3. Pretreatment of Raw Milk

Raw milk (40 g) was weighed into a colorimetric tube, where 9 g of trichloroacetic acid aqueous solution (50%, w/w) and 1 g of hydrogen peroxide aqueous solution (1%, w/w) were added and mixed. Then, the sample was centrifuged (5000 rpm, 10 min), the supernatant was filtered by a 0.22 μm water membrane filter [25], and the filtrate was reserved.

2.4. Preparation of Solution

The sodium thiocyanate standard was dried in an oven at 80 $^\circ\text{C}$ for 3 h. The dried sodium thiocyanate was accurately weighed to 1.397 g in a 1000 mL volumetric flask, fixed to the mark with ultrapure water. Then, the solution was mixed and stored at 4 $^\circ\text{C}$. It was valid for 3 months.

2.5. Preparation of ATPSs

Phase systems were prepared by adding predetermined quantities of organic solvent (acetonitrile and acetone), $(\text{NH}_4)_2\text{SO}_4$, and the filtrate. The pH of the systems was adjusted with hydrochloric acid or sodium hydroxide. The compositions of ATPS are shown in Table 1. The phase-forming components were mixed using a vortex mixer for 2 min, then

performed centrifugal separation at 2000 rpm for 3 min. The volume of the top phase of ATPS was recorded. The top phase was collected and concentrated by nitrogen blowing, then the volume of the concentrated solution was recorded and the concentrated solution filtered with the organic filtration (0.22 µm). Finally, IC was used to detect the SCN[−] content in the concentrated top phase solution. The average of three replicates is reported.

Table 1. The compositions of ATPSs.

Organic/(NH ₄) ₂ SO ₄ ATPS	Organic Solvent (%)	(NH ₄) ₂ SO ₄ (%)	pH	Temperature (°C)
acetonitrile/(NH ₄) ₂ SO ₄	30			
	32			
	34	10	2.5	25
	36	12	3.5	40
	38	14	4.5	55
	40	16	5.5	70
	42	18	7.0	80
	44	20		
	46			
acetone/(NH ₄) ₂ SO ₄	30	10	2	
	32	12	3	25
	34	14	4	32
	36	16	5	40
	38	18	6	50
	40	20	7	55
	42		8	

2.6. Evaluation Index of ATPS

The separation and enrichment efficiency of SCN[−] by ATPS were investigated by the recovery rate (*Y*) and enrichment factor (*CF*) of SCN[−], respectively. The calculation formula was as follows:

$$Y = \frac{c_0 \times V \times 1000}{m \times 1000} \times 100\% \quad (1)$$

$$CF = \frac{c_0}{c_i} \quad (2)$$

where *C*₀ is the SCN[−] concentration of the top phase after nitrogen blowing (mg/L); *V* is the top phase volume of ATPS after nitrogen blowing (mL); *m* is the added mass of SCN[−] in the system (mg); 1000 is the unit conversion factor; and *C*_{*i*} is the concentration of SCN[−] before enrichment (mg/L).

2.7. RSM Optimization

Response surface methodology (RSM) was used to analyze the interaction between parallel factors (acetonitrile, ammonium sulfate, pH, and temperature), and Box–Behnken experimental design (BBD) was used to design the experiment. The results are shown in Table 2. Statistical analysis was performed by analysis of variance (ANOVA), the formula listed below was applied to estimate the optimal parameters.

$$Y = A_0 + A_i \sum x_i + A_{ij} \sum x_i x_j + A_j \sum x_j^2 (i \neq j) \quad (3)$$

where *Y* is the response; *X_i* and *X_j* are the arguments studied; and *A*₀, *A_i*, *A_{ii}*, and *A_{ij}* are the constants of nodal increment, linearizing, quadratic, and cross-product terms, respectively. The range of *i* and *j* is 1 to 3. *F* test was used to evaluate the statistical significance of the model.

Table 2. Factors and levels of code values in the response surface design.

Variables	Coded Variable Levels		
	−1 ^a	0 ^b	+1 ^c
x_1 acetonitrile (w/w)%	41	42	43
x_2 (NH ₄) ₂ SO ₄ (w/w)%	15	16	17
x_3 pH	3.5	4.5	5.5

^a high level; ^b middle level; ^c low level.

2.8. Sample and Result Analysis

The content of SCN[−] in the sample (C) was calculated according to the following formula:

$$C = \frac{\rho V f \times 1000}{m \times 1000} \quad (4)$$

where C is the concentration of SCN[−] in the raw milk (mg/kg); ρ is the concentration of SCN[−] in the top phase of ATPS (mg/L) measured from the standard curve; V is the volume of the top phase of ATPS (mL); f is the dilution factor of the sample solution; m is the sampling mass of the filtrate (g); and 1000 is the unit conversion factor.

The recovery rate of standard addition in the spiked sample (P) is calculated according to the following formula:

$$P = \frac{c_2 - c_1}{c_3} \times 100\% \quad (5)$$

where P is the recovery rate in the spiked sample (%); C₁ is the concentration of SCN[−] in the sample to be tested (mg/kg); C₂ is the concentration of the spiked sample to be measured (mg/kg); and C₃ is the spiked amount (mg/kg).

2.9. Analysis of FTIR

A measure of 20 mg of powdered potassium bromide (KBr) was ground using a mortar and pestle. The background spectra were recorded using 20 mg of powdered KBr. A suitable amount of the top phase sample was taken and dropped onto a KBr wafer and the sample was allowed to evaporate before being scanned by FTIR at 4000–400 cm^{−1}.

2.10. Statistical Analysis

All experiments were carried out 3 times, and the data are expressed as the mean ± standard deviation. All data were analyzed by the analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means were identified by the least significant difference calculations.

3. Results

3.1. Influencing Factors of SCN[−] Isolation

3.1.1. ATPS of Acetonitrile/(NH₄)₂SO₄

The effects of acetonitrile, ammonium sulfate, pH, and temperature on the separation and enrichment of SCN[−] were investigated using the recovery (Y) and enrichment factor (CF) as evaluation indicators. As shown in Figure 1A, with increasing mass fraction of acetonitrile, the Y value gradually increased, then stabilized and reached the maximum value at 42% (w/w), while the CF value continued to decrease. The reason for the increase of Y value was the increase in the mass fraction of acetonitrile, which reduced the water content of the system, increased the electrostatic repulsion of SO₄^{2−} and SCN[−], and promoted the retention of SCN[−] in the top phase. When acetonitrile reached 42% (w/w), SCN[−] had been largely enriched in the top phase.

Figure 1. Effect of system composition on SCN^- extraction efficiency. (A) acetonitrile (30%, 32%, 34%, 36%, 38%, 40%, 42%, 44%, and 46%), (B) ammonium sulfate (10%, 12%, 14%, 16%, 18%, and 20%), (C) pH (2.5, 3.5, 4.5, 5.5, and 7.0), and (D) temperature (25 °C, 40 °C, 55 °C, 70 °C, and 80 °C).

The effects of inorganic salts on ATPS were as shown in Figure 1B. As the mass fraction of ammonium sulfate increased, the Y value gradually increased and then plateaued and the CF was generally stabilized. The reason for this was that ammonium sulfate was a salt of strong acid and weak base, which could ionize SO_4^{2-} [26]. When the mass fraction of ammonium sulfate reached 16%, the charge repulsion between SO_4^{2-} and SCN^- was maximum. However, flocculation was observed in the system when the mass fraction exceeded 20% and the phase separation interface was not significant. In summary, when the ammonium sulfate mass fraction was 16%, the maximum Y and CF of SCN^- in the bottom phase were obtained.

Figure 1C shows that, as the pH increased, both Y and CF increased and then decreased, reaching a maximum at pH 4.5. This is because as the pH increased, SO_4^{2-} was more favorably assigned to the phase with higher hydrophobicity [27]. When the pH in the system exceeded 4.5, the concentration of H^+ decreased, which resulted in a decrease in the ability of the organic solvent to bind to water [28], and the SCN^- reverted to the bottom phase, resulting in a decrease in both Y and CF .

With the change of temperature, Y and CF changed less. It indicated that the temperature of the system had no significant effect on the separation and enrichment of SCN^- , so the extraction temperature was not further investigated in the subsequent experiments.

In summary, the optimal extraction conditions for the ATPS of acetonitrile and ammonium sulfate were 42% (w/w) acetonitrile, 16% (w/w) ammonium sulfate, pH 4.5, and room temperature.

3.1.2. ATPS of Acetone/ $(\text{NH}_4)_2\text{SO}_4$

As shown in Figure 2A, with the increase of acetone mass fraction, Y increased and then decreased, and reached the maximum value when the mass fraction of acetone was about 34%, while CF firstly tended to stabilize and then decreased sharply. Because of the hydrophilicity of the organic solvent, some of the water molecules in the bottom phase were competitively adsorbed to the top phase, which made the volume of the bottom phase gradually decrease and the volume of the top phase gradually increase. Moreover, the concentration difference between the top and bottom phase prompted SCN^- to enter the top phase with water molecules, so the Y value increased and the CF value stabilized. In addition, the decrease of the volume of the bottom phase led to the increase of the salt concentration in the bottom phase and the salting effect, which prompted the increase of the Y value. Therefore, when the mass fraction of acetone was 34%, Y and CF values reached maximum.

Figure 2. Effect of system composition on SCN^- extraction efficiency. (A) acetone (30%, 32%, 34%, 36%, 38%, and 40%), (B) ammonium sulfate (10%, 12%, 14%, 16%, 18%, and 20%), (C) pH (2, 3, 4, 5, 6, 7, and 8.0), and (D) temperature (25 °C, 32 °C, 40 °C, 50 °C, and 55 °C).

Figure 2B showed the changes of CF and Y in the mass fraction of ammonium sulfate range of 8–22%. With the increase of the mass fraction of ammonium sulfate, the Y value at first increased and then decreased, reaching the maximum at about 14%, whereas CF continued to increase and stabilized after the mass fraction of ammonium sulfate was 16%. The recoveries of SCN^- were $99.77 \pm 0.17\%$ and $98.96 \pm 0.06\%$ at 14% and 16% (w/w) ammonium sulfate, respectively, with no significant difference, while the enrichment multiple was relatively larger at 16%. Thus, the 16% mass fraction of ammonium sulfate was chosen as the best condition for the next experiment.

In Figure 2C, Y and CF both increased and later decreased with increasing pH, which was probably due to charge and hydrophobic interactions [29]. While the maximum Y was obtained at around pH 4.0, CF was maximum at around pH 6.0, but CF was less variable between pH 4.0 and 6.0. As shown in Figure 2D, the Y value and CF gradually increased with increasing temperature. Since the boiling point of acetone was 56.53 °C, 55 °C was chosen as the best extraction condition. In summary, the optimal extraction conditions for the ATPS of acetone and ammonium sulfate were 34% (w/w) acetone, 16% (w/w) ammonium sulfate, pH 4.0, and 55 °C.

3.2. Comparison of the ATPSs

Under optimal conditions, the mass fraction of ammonium sulfate was 16% for both systems and the pH difference was not significant, while the mass fraction of the organic phase was significantly different. The phase separation ability in the ATPS of organic is related to the polarity of the organic compound, where the greater the polarity, the worse the phase separation ability. Acetonitrile polarity (5.8) is higher than acetone polarity (5.4), which requires more acetonitrile up to the component phase. The results of the single-factor test showed that the Y of the acetonitrile/ammonium sulfate system was $104.23 \pm 0.16\%$ and the CF was 11.22 ± 0.02 under the optimal conditions, and the Y of the acetone/ammonium sulfate system was $103.13 \pm 0.25\%$ and the CF was 10.38 ± 0.02 . For both systems, the Y of SCN^- were not significantly different, while the CF of the acetonitrile/ammonium sulfate system was larger than that of the acetone/ammonium sulfate system, and the experimental steps were relatively cumbersome because the ATPS of acetone and ammonium needed to be treated with heating. Therefore, RSM was conducted for the ATPS of acetonitrile and ammonium sulfate on the basis of single-factor tests.

3.3. RSM Optimization of ATPS Conditions

3.3.1. Model Fitting and Statistical Analysis

BBD and RSM were performed to optimize the process parameters for the extraction of SCN^- from the ATPS of acetonitrile and ammonium sulfate. The effects of acetonitrile

mass fraction (15–17%), ammonium sulfate mass fraction (41–43%) and system pH (3.5–5.5) on the Y and CF values of SCN^- in the top phase of ATPS were investigated.

The experimental design and results of BBD were shown in Table 3. The regression equation was obtained using Design Expert (Version 8.0.6) software (Statease, Minneapolis, MN, USA), and the fitted equation was as follows.

$$CF = 10.86 + 0.060A + 0.14B + 0.32C + 0.045AB + 0.47AC + 0.32BC - 0.34A^2 - 0.79B^2 - 1.15C^2 \quad (6)$$

$$Y = 106.62 + 0.90A + 1.09B + 1.94C - 0.11AB + 3.06AC + 2.77BC - 1.82A^2 - 5.13B^2 - 9.02C^2 \quad (7)$$

where A , B , and C are the acetonitrile concentration, $(NH_4)_2SO_4$ concentration, and pH, respectively.

Table 3. Experimental design and results for BBD.

Number	<i>A</i>	<i>B</i>	<i>C</i>	<i>CF</i>	<i>Y (%)</i>
	x_1 Acetonitrile (<i>w/w</i>)%	x_2 $(NH_4)_2SO_4$ (<i>w/w</i>)%	x_3 pH		
1	0	0	0	10.98	107.13
2	−1	−1	0	9.30	95.20
3	1	0	−1	8.46	90.22
4	0	0	0	10.74	106.42
5	−1	0	−1	9.54	96.70
6	1	0	1	10.14	100.99
7	0	0	0	10.56	105.64
8	0	1	1	9.48	96.59
9	0	1	−1	8.34	87.93
10	0	0	0	10.56	105.07
11	0	−1	1	8.88	91.47
12	1	1	0	10.26	103.92
13	0	−1	−1	9.00	93.91
14	−1	0	1	9.36	95.22
15	0	0	0	11.46	108.83
16	−1	1	0	9.78	100.20
17	1	−1	0	9.60	99.37

3.3.2. Variance Analysis

The regression model was significant ($p < 0.05$) as seen in Tables 4 and 5, which indicates that the regression equation was ideal. None of the misfit term tests proved to be significant ($p_1 = 0.5422 > 0.05$ and $p_2 = 0.1176 > 0.05$), suggesting that the model could make good numerical predictions. Combined with Figure 3, the correlation between the predicted and true values of the CF and Y prediction models was relatively good, and coefficients of variation (CV) in this test were 3.71% and 2.17%, respectively. This demonstrated a high correlation between the predicted and actual values, as well as a high-quality fit.

Table 4. The analysis of variance of the fitting quadratic polynomial prediction model of CF .

Source	Sum of Squares	df	Mean Square	f_1 -Value	p_1 -Value
Model	11.66	9.00	0.036	9.82	0.0033
A-acetonitrile	0.029	1.00	0.0008	0.218	0.6545
B- $(NH_4)_2SO_4$	0.15	1.00	0.0041	1.105	0.3280
C-pH	0.79	1.00	0.0221	6.018	0.0439
Residual	0.92	7.00	0.004	—	—
Lack of fit	0.35	3.00	0.003	0.83	0.5422
Pure error	0.57	4.00	0.004	—	—
Cor total	12.58	16.00	—	—	—
$CV_1\%$	—	—	3.71	—	—
R_1^2	—	—	0.93	—	—

Table 5. The analysis of variance of the fitting quadratic polynomial prediction model of Y .

Source	Sum of Squares	df	Mean Square	f_2 -Value	p_2 -Value
Model	617.82	9.00	68.65	14.78	0.0009
A-acetonitrile	6.4441	1.00	6.4441	1.3873	0.2774
B-(NH ₄) ₂ SO ₄	9.4395	1.00	9.4395	2.0321	0.1970
C-pH	30.0700	1.00	30.0700	6.4734	0.0384
Residual	32.52	7.00	4.65	—	—
Lack of fit	23.97	3.00	7.99	3.74	0.1176
Pure error	8.55	4.00	2.14	—	—
Cor total	650.34	16.00	—	—	—
CV ₂ %	—	—	2.17	—	—
R_2^2	—	—	0.95	—	—

Figure 3. Correlation between predicted value and true value of model CF and Y .

3.3.3. Interactive Analysis

The response surfaces of the model are shown in Figure 4. The interaction of (NH₄)₂SO₄ mass fraction and pH had the most significant effect on recovery and enrichment multiplicity of SCN[−], which is shown in Figure 4e,f. Both a lower mass fraction of (NH₄)₂SO₄ and pH were not favorable for SCN[−] extraction, however, with increasing mass fraction of (NH₄)₂SO₄ and pH, the repulsive force between SO₄^{2−} and SCN[−] increased, which resulted in SCN[−] remaining more easily in the top phase. However, as the mass fraction of (NH₄)₂SO₄ continued to increase, the system water molecules were reduced and the pH was increased, which caused the SCN[−] to retransfer to the bottom phase with the water molecules, resulting in the decrease of the CF and Y of SCN[−].

The 3D response surface plots of the interaction between acetonitrile and pH on CF and Y values were shown in Figure 4c,d. The lower mass fraction of acetonitrile and pH were not conducive to the transfer of SCN[−] to the top phase. With the increase of acetonitrile mass fraction, the concentration difference of SCN[−] in the top and bottom phases was increased, which led to the transfer of SCN[−] to the top phase. Moreover, with the increase of pH, the protonation of SO₄^{2−} was decreased and the charge repulsion between SO₄^{2−} and SCN[−] was increased, so SCN[−] tended to move to the top phase. However, when the acetonitrile content in the system was too high or the pH was too large, SCN[−] would re-enter the lower phase, which led to a decrease in both the recovery and the enrichment multiplicity of SCN[−] [30].

As shown in Figure 4a,b, the interaction between the mass fraction of (NH₄)₂SO₄ and the mass fraction of acetonitrile was not significant on the recovery and enrichment multiplicity of SCN[−].

Figure 4. The plots of response surface for CF (a,c,e) and Y (b,d,f) of SCN^- .

3.3.4. Optimal Conditions and Verification

After optimization by RSM, its predicted optimal extraction process parameters were composed of room temperature, 42.31% acetonitrile (w/w), 16.14% $(\text{NH}_4)_2\text{SO}_4$ (w/w), and pH 4.7. Under these conditions, the predicted values of enrichment multiplicity and recovery of SCN^- were 10.92 and 107.06%, respectively. To facilitate the experimental operation, the predicted conditions were rationalized: at room temperature, 42% acetonitrile (w/w), 16% $(\text{NH}_4)_2\text{SO}_4$ (w/w), and pH 4.7. After experimental verification, the actual enrichment and recovery of the top-phase SCN^- were 10.74 ± 0.03 and $107.24 \pm 0.5\%$, respectively, indicating that the optimization results of the response surface experiment were good.

3.4. Mechanism Analysis

In this paper, the mechanism of SCN^- extraction by ATPS of acetonitrile and $(\text{NH}_4)_2\text{SO}_4$ was initially explored using FTIR. The comparative IR spectra of the blank ATPS top phase and the ATPS top phase after SCN^- enrichment were analyzed separately, and the results are shown in Figure 5. Both top phase solutions showed a characteristic absorption peak of acetonitrile at 2292.45 cm^{-1} and 2253.89 cm^{-1} , which was produced by the stretching vibration of the $-\text{C}\equiv\text{N}$ group in acetonitrile [31]. The non-occurrence of other new absorption peaks within $4000\text{--}500 \text{ cm}^{-1}$ indicated that no new chemical bonds were produced and no weaker interactions between acetonitrile and SCN^- were present. The resulting transfer of the SCN^- from the bottom to the top phase was caused by the electrostatic repulsion between SO_4^{2-} and SCN^- , as well as the effect of the concentration difference, rather than the formation of a new compound between acetonitrile and SCN^- .

Figure 5. Infrared spectra of acetoneitrile/ammonium sulfate ATPS for separation and enrichment of SCN^- .

3.5. Interference Analysis

The raw milk samples contained many coexisting ions, such as F^- , Cl^- , NO_2^- , NO_3^- , SO_4^{2-} , PO_4^{3-} , and other anions. Since the content of SCN^- was very low and easily interfered by the coexisting ions, it was necessary to do ion interference experiments. The results in Figure 6 show that the coexisting anions in the sample would not interfere with the determination of SCN^- , because the retention capacity of SCN^- on the column was much higher than that of F^- , Cl^- , NO_2^- , NO_3^- , SO_4^{2-} , and PO_4^{3-} , and the peak time was much later than them.

Figure 6. Interference analysis of coexisting ions.

3.6. Method Validation

The linearity, precision, and sensitivity of the coupled ATPS-IC technique were validated. All sample determinations were corrected using a blank sample. A representative standard chromatogram is shown in Figure 7. In the range of 0.05–15 mg/L, the concentration of SCN^- and the peak area exhibited satisfactory linearity with correlation coefficients ($y = 223.42 \times -1.39$, $R^2 = 0.998$). The limit of detection (LOD) and quantification (LOQ) were measured using a series of blank-spiked sample solution. When the peak height of the analytes could be detected to produce a significant response at three-fold and tenfold of the peak height of baseline noise, the concentrations of the analytes were their LODs and LOQs, respectively. LODs and LOQs for SCN^- were 0.2 $\mu\text{g/L}$ and 0.6 $\mu\text{g/L}$, respectively, and RSDs of intraday and interday were 1.6% and 4.3%, respectively.

Figure 7. Standard chromatogram of SCN^- (0.45 mg/L).

3.7. Application

The method was applied to determine SCN^- contents in raw milk. Analysis was conducted in triplicate. Samples that were spiked with three different concentrations of analytes (1, 5, 10 mg/L) were adopted to examine the recovery of the method. Figure 8 shows the representative chromatograms of SCN^- . The acetonitrile and $(\text{NH}_4)_2\text{SO}_4$ extraction system did not interfere with the determination of inorganic anions. The recoveries of the method were in the range of 81–119% with the relative standard deviations (RSDs) less than 3.7%, indicating that the method was reliable for the determination of SCN^- content in raw milk. The recovery results were all presented in Table 6.

Figure 8. IC chromatograms of SCN^- standard (0.45 mg/L) and the top phase of ATPS.

Table 6. Result of spike recovery experiment.

Addition of Thiocyanate (mg/kg)	Found ₁ ^b (mg/kg)	Found ₂ ^c (mg/kg)	Found ₃ ^d (mg/kg)	Recovery (%)	Average (%)	RSD (% , n = 3)
1.00	1.17	2.13	0.96	96	90 ± 8	8.8
		1.98	0.81	81		
		2.10	0.93	93		
5.00		6.84	5.67	113	114 ± 5	4.5
		7.14	5.97	119		
		6.63	5.46	109		
10.00		11.65	10.48	104.8	108.5 ± 4.0	3.7
		11.98	10.81	108.1		
		12.44	11.27	112.7		

^b Corrected by blank sample; ^c Corrected by actual sample; ^d The value of Found₃ was Found₂ minus Found₁.

3.8. Comparison

The new method developed in this study was compared with the reported methods for the determination of thiocyanate in raw milk in terms of LOD and RSD, and the results are shown in Table 7. The LOD of acetonitrile/ $(\text{NH}_4)_2\text{SO}_4$ ATPS-IC was significantly lower

compared with the results of IC and HPLC without the extraction pretreatment of ATPS, demonstrating that the pretreatment of the ATPS technique could effectively improve the sensitivity of the thiocyanate detection. In addition, the method developed in this study is simpler, faster, and less costly than other detection methods, enabling highly sensitive and rapid thiocyanate enrichment and detection.

Table 7. Comparison of the present method with other methods for the analysis of thiocyanate.

Detection Method	Sample	LOD	RSD	Literature
acetonitrile/(NH ₄) ₂ SO ₄ ATPS-IC	Raw milk	0.20 µg/L	1.6%	This study
IC (UV)	Emulsion	0.08 mg/L	0.40%	[32]
Ion Pair Chromatography	Ionic liquid	0.96 mg/L	1.40%	[33]
High Performance Liquid Chromatography (UV)	Emulsion	0.06 mg/L	1.20%	[34]
Colorimetric sensor	Fossil and drill waters	0.60 mg/L	4.50%	[34]
Gold nanoparticles colorimetry	Saliva/environmental water	11.60 µg/L	3.2%	[35]
Surface Enhanced Raman Spectroscopy	Emulsion	0.04 mg/L	<10%	[36]
Methyl				
Isobutyl Ketone Extraction-Atomic Fluorescence Spectrometry	Seawater	1.33 µg/L	2.10%	[8]
Spectrophotometry	Blood	1.80 mg/L	<7%	[37]
Electrode electrochemical method	Saliva	0.58 µg/L	2.20%	[38]

4. Conclusions

In this study, a highly sensitive and rapid determination of thiocyanate was achieved by using ATPE of acetonitrile/(NH₄)₂SO₄ for the separation and enrichment of thiocyanate, combined with ion chromatography. Optimization of the extraction conditions of the acetonitrile/(NH₄)₂SO₄ system by RSM resulted in a recovery of $107.24 \pm 0.50\%$ and an enrichment multiple of 10.74 ± 0.03 for thiocyanate. The extraction mechanism was initially explored by FTIR. The constructed acetonitrile/(NH₄)₂SO₄ ATPS-IC method showed the linear range of the method was from 0.05 mg/L to 15 mg/L, and $R^2 = 0.998$, the LOD was 0.2 µg/L, the LQD was 0.6 µg/L, the intraday precision was 1.6%, the interday precision was 4.3%, and the recovery rates of standard addition were between 81% and 119%.

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