Determination of Sulfonamides in Milk by Cloud Point - Salting Out Extraction and Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry

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Abstract

A method by cloud point - salting out extraction (CPSOE) coupled with UHPLC-MS/MS was developed for determination of eleven sulfonamides in milk. In this study, the type and concentration of surfactant, de-emulsification condition, pH value, volume of n-butanol, equilibration temperature and time were optimized. For this developed method, the linear range of SAs was from 0.05 to 50 μg L⁻¹, and the correlation coefficients were higher than 0.997. The average recoveries for SAs were from 61.32 % to 91.67 %, and the LOQs were less than 0.06 μg kg⁻¹.
Introduction

Sulfonamides (SAs) antimicrobials were widely used in stockbreeding due to the low-cost and broad-spectrum antimicrobial activity. The residues of SAs in animal producing foods were a concern due to the side and toxic effects such as allergic reactions and multidrug resistance and so on. Therefore, the maximum residue limits (MRLs) of SAs in related food were stipulated in many countries and regions. In China, the MRLs of sulfamethazine in milk and other animal producing foods were 25 μg kg$^{-1}$ and 100 μg kg$^{-1}$, respectively, while the MRLs of other SAs in sum were 100 μg kg$^{-1}$. In Japan, Milk has the MRLs of sulfapyridine 10 μg kg$^{-1}$, but other foods was 100 μg kg$^{-1}$. Thus, the MRLs of SAs in milk were obviously less than other foods.

Nowadays, liquid chromatography coupled with mass spectrometric detectors (LC-MS) or ultra-high-performance liquid chromatography (UHPLC-MS) is commonly applied to analysis active ingredients or drugs residues including SAs in food due to their high selectivity and sensitivity. Accompanying, kinds of sample preparation methods applied for SAs determination in milk, involving solid phase extraction (SPE), dispersive-solid phase extraction, dispersive liquid–liquid micro extraction and traditional liquid–liquid extraction. However, these approaches suffer from some disadvantage like large amounts usage of organic solvents and time-consuming.

A novel environment friendly technique, cloud-point extraction (CPE), attracts great attention. This method can quickly extract high-concentration analytes into a relatively small amount of coacervate phase without other enrichment procedure such as nitrogen blowing and SPE process. Additionally, CEP was safer than the traditional extraction method because small quantity of surfactant was used instead of a large amount of flammable and highly toxic organic solvent to extract the target substance.
Theoretically, CEP was not suitable to couple with LC–MS because of the signal suppression effect by surfactant, which limited the method sensitivity. Fortunately, a few recently published papers reported the CPE coupled with LC-MS be used to drugs detection in biological matrices. Salting-out was an effective option to precipitate the surfactants and reduce the signal suppression. In this study, a cloud point-salting out extraction (CPSOE) method coupled with UHPLC-MS/MS was developed to bring new hope to SAs analysis in milk.

**Experimental**

**Reagents and chemicals**

Analytical standards of sulfapyridine, sulfadiazine, sulfamethoxazole, sulfisoxazole, sulfamethizol, sulfamethazine, sulfabenzamide, sulfachloropyridazine, sulfadoxine, sulfaphenazole and sulfamonomethoxine were purchased from Sigma Aldrich (St. Louis, MO, USA; purity > 95%). Acetic acid (AC), formic acid, $n$-butyl alcohol, hydrochloric acid, sodium hydroxide and sodium sulfate anhydrous ($\text{Na}_2\text{SO}_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Triton X-114 (TX-114), Triton X-100 (TX-100) and Tween 20 of laboratory grade were obtained from Sigma Chemicals Co., Ltd (Shanghai, China). Methanol and acetonitrile were obtained from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water was yielded by a Milli-Q Gradient system (Millipore, Bedford, MA, USA).

The stock solution of each SA was prepared by dissolving 10 mg of compound with 10 mL of methanol. The stock solution was stored in frozen. Before use, the stock solutions were mixed with appropriate volume of methanol to prepare standard working solution.
Apparatus

An ultra performance liquid chromatograph coupled with Xevo TQ-S mass spectrometry equipped an ACQUITY UPLC BEH C18 column (100 mm×2.1 mm, 1.7 μm) (Waters, Milford, MA, USA) was applied. The column temperature and sampler temperature set at 40 °C and 10 °C, respectively. The mobile phases consisted of water with 0.1% formic acid (A) and methanol (B) with gradient elution procedure as shown in Table S1. The injection volume was 5 μL. The capillary voltage and cone voltage were set at 3.0 kV and 40 V, respectively. The source temperature and desolvation temperature were 150 °C and 500 °C. The details of acquisition parameter and retention time for SAs were listed in Table 1.

Sample preparation of CPSOE

Ten gram of milk sample and 2.5 mL TX-114 solution (420 g L⁻¹, w/v) were added into a 15 mL centrifuge tube. After mixing for 1 min and ultrasonic extracting for 10 min, 0.15 mL AC and 2 mL saturated Na₂SO₄ (bath at 40 °C for 20 min) was added. After mixing for 1 min, the sample was centrifuged at 10,000 rpm 4 °C for 10 min. Then, 0.5 mL saturated Na₂SO₄ and 0.2 mL n-butyl alcohol was added, after transferring the supernatant into a new centrifuge tube. The pH value was adjusted to 4.0 ± 0.1 by adding appropriate volume of 2 mol L⁻¹ HCl. The mixture was vortexed for 1 min before water bath at 40 °C for 20 min. The mixture was centrifuged for 5 min at 10,000 rpm (10528 × g), and then the 2 mL of micellar coacervated phase (supernatant) was obtained. The 0.5 mL of supernatant was carefully transferred into a 2 mL centrifuge tube, and 1 mL of acetonitrile / water (1:1, v/v) was added. After being vortexed for 30 s and centrifuged at 10,000 rpm (10528 × g), 5 °C for 10 min, the supernatant was ready for UHPLC-MS/MS analysis.
Results and Discussion

Optimization of CPSOE procedure

Firstly, surfactant is the most concerned factor in CPE method. TX-114, TX-100 and Tween 20 were compared in this study because of their high biocompatibility, stability, and low cloud point temperature.\(^{18}\) The recoveries of SAs using TX-114 (40\%, w/v) were the highest in three surfactants at the same concentration (Fig. 1(A)). Further, a perfect CPSOE procedure should get not only high extraction efficiency but also low micellar coacervated phase volume.\(^{18}\) It is required that the minimum amount of surfactant can be used to extract the SAs as much as possible, thereby improving its concentration ability. Therefore, the concentration of TX-114 (30 g L\(^{-1}\) to 80 g L\(^{-1}\) in extracting solution) was studied for the best extraction efficiency. As shown in Fig. 1B, the extraction efficiency of the SAs in milk was significantly regulated by the concentration of TX-114. When the concentration of TX-114 was blow 70 g L\(^{-1}\), the extraction efficiency was significantly increased with increase of concentration of TX-114. While the extraction efficiency was slightly decreased with increase of concentration of TX-114 when the concentration of TX-114 over 70 g L\(^{-1}\). The reason for this phenomenon might be that the low mass transfer rate would decrease the extraction efficiency due to high viscosity of micellar coacervated phase.\(^{19}\) Based on the experimental results, added 2.5 mL 420 g L\(^{-1}\) TX-114, equally 70 g L\(^{-1}\) TX-114 in extracting solution was selected.

A de-emulsification process was usually applied to wipe off protein and fat in order to reduce matrix interference before CPE process.\(^{18}\) In addition, SAs distributed in fat globules could be released by de-emulsification.\(^{19}\) According to previous studies, AC and Na\(_2\)SO\(_4\) were commonly used regent for de-emulsification.\(^{19}\) Na\(_2\)SO\(_4\) could not only assist...
to precipitate milk proteins for promoting the de-emulsification, but also promote the phase separation. Here, the addition amount of AC and Na$_2$SO$_4$ were investigated. Firstly, the mass of Na$_2$SO$_4$ was set as 0.3 g based on the literatures, and the volume of AC ranging from 0.05 to 0.3 mL was investigated. As shown as Fig. 2A, the recoveries of all SAs was remarkably increased when the volume of AC was enhanced from 0.05 to 0.15 mL but marginally decreased with the volume of AC higher than 0.15 mL. Secondly, different volume of saturated Na$_2$SO$_4$ solution (bath at 40°C for 20min) in the ranging from 0.5 to 3 mL was investigated. As shown as Fig. 2B, 2 mL of a saturated Na$_2$SO$_4$ solution is the best adjustment concentration. Above all, 0.15 mL AC and 2 mL saturated Na$_2$SO$_4$ solution (bath at 40°C for 20min) were the optimized de-emulsification condition.

The value of pH is another important influence factor because the value of pH affects the ionisation state of target compound which decide the distribution of analysis between aqueous phase and micellar phase. The results of the effect of pH value were given in Fig. 2C. The optimal pH value is 4.0 regarding the recoveries of SAs.

The cloud point temperature of surfactant would be changed also with addition of organic additive. According to the previous study, the short chain (C1-C3) ethanol increased the cloud point temperature (CPT) while $n$-butanol decreased CPT. However, the recovery of analyte would be reduced if the addition volume of $n$-butanol is too large, although the addition of $n$-butanol promoted the phase separation. In this study, the volume of $n$-butanol was investigated from 0 to 0.25 mL. Good recoveries of target compounds were achieved when the addition amount is 0.2 mL (Fig. 2D), compared that of the other addition volume of $n$-butanol. Finally, the optimized volume of $n$-butanol was 0.2 mL.

In addition, the increase of equilibration temperature can accelerate phase separation and reduce the coacervate-phase volume because the high temperature would
increase the mass transfer rate for analyte and promote the micelles dehydration.\textsuperscript{24,25}

Here, the temperature of equilibration was concerned from 30 to 80ºC. The recoveries for all target compounds (Fig. 2E) at equilibration temperature 40 ºC were optimal, except for sulfaphenazole. When the temperature increased above 40 ºC, the recoveries of SAs gradually decreased. While, there were almost no difference in recoveries of SAs with equilibration time from 10 to 60 min (Fig. 2F). So, the equilibration temperature and time used in the finalized procedure were 40 ºC and 20 min, respectively.

The presence of TX-114 in micellar phase would reduce the ionization of analyte and increase the matrix effect, which greatly narrows the application of CPE in trace analysis by LC-MS method. Here we try to remove the TX-114 from micellar phase using salting-out effect. In this step, 0.5 mL 50 % acetonitrile (ACN), 0.5 mL 80 % ACN and 0.5mL ACN were added into 0.5 mL micellar phase, respectively. While the volume of ACN-water solution was added to 1 mL, the TX-114 was quickly precipitated at the bottom of tube. As the result shown in Fig. 3, the recoveries of SAs with the addition of 1 mL 50 % ACN were significantly higher than that under other conditions, except for sulfapyridine. Finally, 1 mL 50 % ACN was used to precipitate TX-114.

Method validation

Good linearity was obtained for all the SAs from 0.05 μg L\textsuperscript{-1} to 50 μg L\textsuperscript{-1} upon this method, with the coefficient $r^2$ higher than 0.997. In this study, the estimated LOD and LOQ were determined by injecting the lowest concentration (in spiked blank samples) and yielded signal-to-noise (S/N) ratio equal to 3 and 10, respectively. For the developed method, the LOD and LOQ were 0.01 - 0.02 μg kg\textsuperscript{-1} and 0.05 - 0.06 μg kg\textsuperscript{-1}, respectively in Table S2. Average recoveries calculated by matrix-matched calibration curve were from 61.32 % to 91.67 % at three spiked levels. The inter-day and intra-day RSD were no more
than 14.06 % and 6.50 %, respectively. In addition, the matrix effects of SAs in milk were all less than 36 %, as shown in Table S2.

Conclusions

In this study, CPSOE coupled with UHPLC-MS/MS method was a fast, simple and efficient method for the determination of SAs in milk. Comparing to the SPE or QuEChERS procedures, this method presents comparable or higher sensitivity and significantly less usage of organic solvent. Especially, CPSOE was a simple and fast method compared to SPE technology. Further, the application of salting-out effectively decreased the matrix effect and widen the application of CPE technology coupled with LC-MS/MS. CPSOE is a cost-effective, environmentally friendly, simple method which enables to selective simultaneous extraction of SAs. It might hopefully be a routine method for determination SAs in milk.

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| Compound               | Retention Time/ min | Precursor Ion, m/z | Quantitation Product Ion, m/z | Confirmation Product Ion, m/z | CE a / ev |
|------------------------|---------------------|--------------------|--------------------------------|--------------------------------|-----------|
| Sulfapyridine          | 3.70                | 250.0              | 156.0 28                       | 91.9 20                        |
| Sulfadiazine           | 3.05                | 251.0              | 156.0 16                       | 91.9 32                        |
| Sulfamethoxazole       | 5.97                | 254.0              | 156.0 14                       | 108.0 24                       |
| Sulfisoxazole          | 6.68                | 268.1              | 156.0 12                       | 112.9 14                       |
| Sulfamethizol          | 4.83                | 271.1              | 156.0 14                       | 91.9 26                        |
| **Sulfamethazine**     | 4.91                | 279.1              | 185.8 20                       | 91.9 32                        |
| Sulfabenzamide         | 7.29                | 276.9              | 156.0 15                       | 108.0 20                       |
| Sulfachloropyridazine  | 5.66                | 285.2              | 156.0 19                       | 91.9 12                        |
| Sulfadoxine            | 6.56                | 311.6              | 156.0 15                       | 108.0 15                       |
| Sulfaphenazole         | 8.22                | 315.2              | 156.0 25                       | 108.0 24                       |
| Sulfamonomethoxine     | 5.91                | 281.0              | 156.0 16                       | 126.0 16                       |

a. CE, collision energy
**Figure Captions**

Fig. 1 Effects of (A) type of non-ionic surfactants and (B) concentration of TX-114 on the recoveries of SAs.

Fig. 2 Effects of (A) volume acetic acid, (B) volume of saturated sodium sulfate solution, (C) pH, (D) volume of n-butyl alcohol, (E) equilibration temperature, (F) equilibration time on the recoveries of SAs. (No. designation: 1. sulfapyridine; 2. sulfadiazine; 3. sulfamethoxazole; 4. sulfisoxazole; 5. sulfamethizol; 6. sulfamethazine; 7. sulfabenzamide; 8. sulfachloropyridazine; 9. sulfadoxine; 10. sulfaphenazole; 11. sulfamonomethoxine.)

Fig. 3 Effects of salting-out procedure on the recoveries of SAs.
Fig. 1 Effects of (A) type of non-ionic surfactants and (B) concentration of TX-114 on the recoveries of SAs (n=3)
Fig. 2 Effects of (A) volume acetic acid, (B) volume of saturated sodium sulfate solution, (C) pH, (D) volume of n-butyl alcohol (E) equilibration temperature (F) equilibration time on the recoveries of target compounds (n=3); No. designation: 1. sulfapyridine; 2. sulfadiazine; 3. sulfamethoxazole; 4. sulfisoxazole; 5. sulfamethizol; 6. sulfamethazine; 7. sulfabenzamide; 8. sulfachloropyridazine; 9. sulfadoxine; 10. sulfaphenazole; 11. sulfamonomethoxine.)
Fig. 3 Effects of salting-out solutions on the recoveries of SAs ($n=3$).
Graphical Abstract