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Article

Quantification of four sulfonamide residues in prawns using ultrasound-assisted matrix solid-phase dispersive extraction coupled with pre-column derivatization and high-performance liquid chromatography with fluorescence detection

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Abstract: Objective: To develop a method for the simultaneous detection of four sulfonamide residues in prawns—sulfadiazine, sulfathiazole, sulfamerazine, and sulfamethazine—using ultrasound-assisted matrix solid-phase dispersion extraction combined with pre-column derivatization and high-performance liquid chromatography (HPLC). **Methods:** By optimizing extraction conditions, ethyl acetate was chosen as the extraction solvent and florisil as the solid dispersion agent. Sulfonamides were extracted from prawns using ultrasound-assisted matrix solid-phase dispersion, then derivatized with fluorescamine and analyzed by HPLC with fluorescence detection. **Results:** The sulfonamides exhibited excellent linearity within the concentration range of 2–100 μg/L, with correlation coefficients greater than 0.999. Detection limits were 0.5 μg/kg and quantification limits were 2 μg/kg. Spike recoveries for blank prawn samples ranged from 84.4% to 93.9% at 2 and 20 μg/kg, with relative standard deviations (n = 3) below 7.7%. **Conclusion:** The method is straightforward, efficient, and highly precise, meeting the standards for residue analysis.

Keywords: ultrasound-assisted matrix solid phase dispersion; pre-column derivation; high performance liquid chromatography; prawn; sulfonamides

Sulfonamides (SAS) are a class of drugs containing p-aminobenzenesulfonamide structure. They are commonly used broad-spectrum antibiotics. Because of its broad antibacterial spectrum. It is widely used in aquaculture because of its strong curative effect [1,2]. People often eat animal derived food with sulfonamides residues, which may cause the gradual accumulation of sulfonamides in the body, and its harm is mainly manifested in bacterial drug resistance. Allergy. Hematopoiesis disorder. Carcinogenesis and hormone like effects [3,4]. Therefore, different countries have set limits on SAS [5,6]. The residual concentration of veterinary drugs in aquatic products is very low, the sample matrix is complex, and there are many interfering substances, so it is difficult to separate from the sample. Purify the residue. Therefore, the separation of samples. Purification is the most time-consuming and labor-intensive step in the analysis of veterinary drug residues. Matrix solid phase dispersion (MSPD)was first proposed by Baker et al. [7] for extraction. This method saves time by purifying drug residues in food samples. Save effort. Rapid and efficient, it is more and more used in the residue analysis of aquatic products [8–13]. The detection methods of SAS residues mainly include enzyme-linked immunosorbent assay [14]. Gas chromatography mass

spectrometry [15]. High performance liquid chromatography [16,17]. High performance liquid chromatography tandem mass spectrometry [13,18]. Capillary electrophoresis [19,20], etc. Enzyme linked immunosorbent assay is mainly used for large-scale screening work, generally not for quantification, which is prone to false positives; although gas chromatography-mass spectrometry has high sensitivity and specificity, it needs to methylate sulfonamides, and the operation is more complex. In recent years, it has been basically no longer used. High performance liquid chromatography Ultraviolet Detection Method and capillary electrophoresis method have low sensitivity and poor specificity, especially in low concentration detection, the sample matrix interference is large, so it is difficult to accurately carry out qualitative confirmation; high performance liquid chromatography tandem mass spectrometry overcomes these problems, but the price of liquid chromatography-mass spectrometry is expensive, which is not conducive to popularization. Method 2 in the agricultural industry standard adopts post column derivatization fluorescence detection[21], which has high sensitivity. The reproducibility is good, but it needs a special post column derivatization system to achieve. Therefore, we established a pre column derivatization fluorescence method to detect sulfa drugs in shrimp. An analytical method for the simultaneous determination of four SAS drug residues in shrimp was established by using Florisil as solid-phase dispersant and ethyl acetate as extraction solvent, extracting by ultrasonic assisted matrix solid-phase dispersion and detecting by fluorescence amine precolumn derivatization fluorescence method. The accuracy of this method. The sensitivity is high and the operation is simple. The quantitative limit of the four SAS is 2 μg/kg, suitable for rapid detection of large quantities of aquatic samples.

1. Materials and methods

1.1. Instruments and reagents

Agilent 1200 liquid chromatograph: equipped with quaternary pump. Fluorescent detector. Column temperature box. Autosampler, chromatographic column. Vortex mixer (German IKA company). Ultra pure water machine (American millipore company). Centrifuge (Shanghai Feige company). High speed centrifuge (sigma, Germany). Rotary evaporator (Japan eyela company). Ultrasonic cleaner (Ningbo Xinzhi company), sulfadiazine (SD). Sulfathiazole (st). Sulfamethylpyrimidine (SM_1). Sulfamethazine (SM_2) standard (German Dr. Ehrenstorfer company): purity ≥ 98%, fluoroamine (Shanghai TCI company): purity ≥ 99%, methanol. Acetonitrile. Acetic acid is chromatographically pure (TE dia company of the United States), and n-hexane. Ethyl acetate. Acetone and magnesium sulfate are analytically pure (Guangzhou Reagent Factory). Florisil (60~100 mesh) is burned at 650 ℃ for 5 h. Before use, it is placed overnight in a 130 ℃ oven. After cooling, it is stored in a dryer for standby. The water used is ultra pure water.

1.2. Preparation of standard solution

(1) Preparation of standard stock solution. Accurately weigh the standard and dissolve it in methanol to a volume of 100 mg/L stock solution, and store it under

−20 ℃ for 3 months. (2) Preparation of standard working solution. Dilute the standard stock solution with methanol to the corresponding concentration, and store it below 4 ℃. (3) Preparation of fluoroamine. Weigh 0.04 g fluoroamine standard sample and dissolve it in acetone to form 0.04% derivative reagent. Store it under 4 ℃ for 1 month.

1.3. Method

1.3.1. Chromatographic conditions

Chromatographic column: Xterra C18 (150 mm × 2.1 mm, 3.5 μm. Waters, USA), mobile phase: acetonitrile/2% acetic acid aqueous solution (30/70, v/v), flow rate: 0.6 mL/min; column temperature: 40 ℃, injection volume, 20 μL.

1.3.2. Sample handling

Weigh 5 g (accurate to 0.01 g) of minced meat that has been ground and mixed evenly, add 1 g of Florisil and 15 mL of ethyl acetate into a 50 mL plugged centrifuge tube, vortex mix for 2 min, then add 3 g of anhydrous magnesium sulfate, vortex mix for 0.5 min, ultrasonic dispersion extraction for 10 min, and centrifugation at 4500 rpm/min for 5 min (centrifugation radius = 16.5 cm). Transfer the supernatant to a 100 mL pear shaped bottle. Continue to add 15 mL of ethyl acetate to the remaining samples and repeat the above operations. The combined supernatant is rotated and evaporated to dryness at 40 ℃. Add 2 mL of mobile phase to the pear shaped bottle to dissolve the residue on the inner wall, add 6 mL of n-hexane twice, shake for 1 min, leave it still for layering, discard the n-hexane layer, transfer the lower solution to a 1.5 mL centrifuge tube, and centrifuge for 2 min at 10,000 RPM/min (centrifuge radius = 5 cm). Transfer 0.5 mL of the lower solution to the sample bottle, add 0.2 mL of 0.04% fluoroamine solution, mix for 10 s, and pass 0.45 μM filter membrane, let the filtrate stand for 40 min for HPLC determination.

2. Results and discussion

2.1. Selection and optimization of chromatographic conditions

2.1.1. Optimization of mobile phase

Methanol was selected for this study. Water, acetonitrile and other commonly used solvents were tested in different proportions. When methanol and water are used as the mobile phase, the derivatives of the four SAS cannot be completely separated, and the peak shape is poor. SAS is an amphoteric substance with strong polarity, which is easy to cause peak deformation. Therefore, the acidic mobile phase is used to suppress the ionization of SAS. In this experiment, the effect of different concentrations of acetic acid in the mobile phase on the separation effect was studied, using 1.0% respectively. 2.0% and 3.0% (V/V) acetic acid, the result is that 1.0% acetic acid has tailing phenomenon, while other concentrations are well separated, and the peak shape is symmetrical. Considering that too strong acidity will reduce the life of chromatographic column filler, 2.0% acetic acid is finally selected as the best concentration for ion inhibition.

2.1.2. Selection of detector and detection wavelength

In this study, the detection effects of two detectors, namely fluorescence detector (Flu) and ultraviolet detector (UV), were investigated. Because many compounds in aquatic products are absorbed in the ultraviolet region, they have poor selectivity and are easy to cause interference, which increases the difficulty of purification. The sulfonamides are first derived, and the fluorescence detector is used for detection, which has strong selectivity and high sensitivity, and can simplify the purification process. Therefore, the fluorescence detector is selected for detection in this experiment.

The maximum excitation wavelength of the fluorescence spectra of four sulfonamides is 405 nm, and the maximum emission wavelength is about 495 nm. Therefore, determine λex = 405 nm, λem = 495 nm is the detection wavelength. See **Figure 1**.

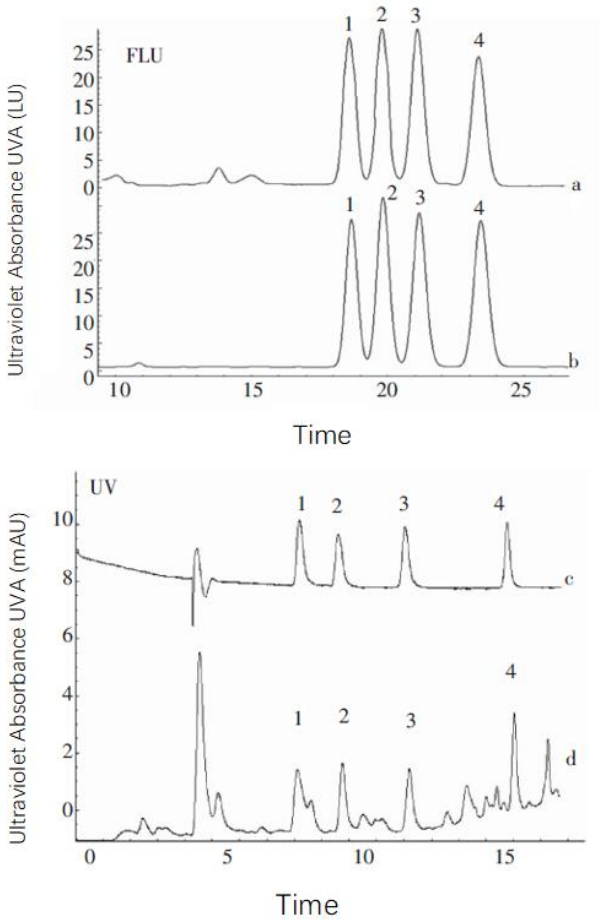

Figure 1. UV detector and fluorescence detector chromatograms of four sulfonamides. Note: flu fluorescent detector; UV: ultraviolet detector; A: standard sample of fluorescence method; B: fluorescence shrimp addition chromatography; C: uv standard sample; D: ultraviolet shrimp addition chromatography; 1-sulfadiazine (SD); 2-sulfathiazole (st); 3-sulfamethylpyrimidine (SM1); 4-sulfamethazine (SM2).

2.2. Optimization of sample extraction conditions

2.2.1. Selection of extraction solvent

SAS belongs to amphoteric compounds with weak polarity, and ethyl acetate is selected. Dichloromethane. Methanol and acetonitrile were used as extraction solvents. Four kinds of SAs with a concentration of 10 μg/kg were added to the minced meat respectively, mixed well, kept in the dark for 2 h, and then 30 mL of extraction solvent was added twice for extraction to compare the extraction effect. Among them, the extraction efficiency of methanol is low, and the average recovery is less than 75%; acetonitrile. The recoveries of ethyl acetate and dichloromethane were more than 80%. However, due to the high boiling point of acetonitrile. The rotary evaporation time is long and explosive, and there is also the problem of dehydration, which increases the steps and difficulty of sample pretreatment, and the reproducibility is poor. When ethyl acetate and dichloromethane are extracted, the extraction efficiency is high and there are few impurities. Due to the toxicity of dichloromethane, ethyl acetate was selected as the extraction solvent in this study.

2.2.2. Selection of extraction method and dispersed phase

Generally, the extraction method is homogenization. Vortex oscillation. Ultrasonic vibration. Matrix solid-phase dispersion, etc. The homogenized sample can completely extract the drugs from the tissue by ultrasonic method, but it is easy to produce cross contamination. If the vortex oscillation combined with ultrasonic extraction method is used, it is found that the sample minced meat will agglomerate, and the SAS extraction is insufficient. This is because the protein content in the muscle tissue of aquatic products is as high as 10% to 30%. The added organic solvent will denature the protein, thus wrapping the sample tissue and poor dispersion, preventing further extraction, resulting in low recovery. Adding dispersed phase into minced meat can increase the specific surface area of the sample and improve the extraction efficiency. Ultrasonic extraction (UAE) uses the energy generated by ultrasound to transfer from the outside to the inside, so that the solution forms bubbles to enhance the chemical reaction. At the same time, the high-frequency oscillation of ultrasound can disperse the solid sample, increase the contact area between the sample and the solvent, improve the mass transfer rate, and improve the extraction efficiency. In this study, matrix dispersion extraction and ultrasonic assisted extraction are combined. The specific steps are to add dispersion phase (agent) and ethyl acetate vortex vibration to the minced meat until the minced meat sample becomes loose, then add anhydrous magnesium sulfate ($mgso_4$) to remove water and some water-soluble impurities, and finally ultrasonic extraction.

Using ethyl acetate as the extraction solvent, four dispersed phases (agents) (C18) were investigated. Floric silica. Neutral alumina and nacl solution) on the recovery of SAS spiked. Add a certain amount of dispersed phase (agent) to the spiked sample, and then add ethyl acetate for ultrasonic extraction. It was found that when sodium chloride (nacl) solution was used as dispersant, the sticky minced meat was easy to gel and become elastic gelatinous. This is because the salt soluble protein in fish meat will dissolve after adding salt, and the fish meat will become a very viscous sol [22]. When the other three solid dispersants are selected, the dispersibility is better. Compared with the spiked recovery rate, Florisil has the

highest recovery rate. See **Figure 2**.

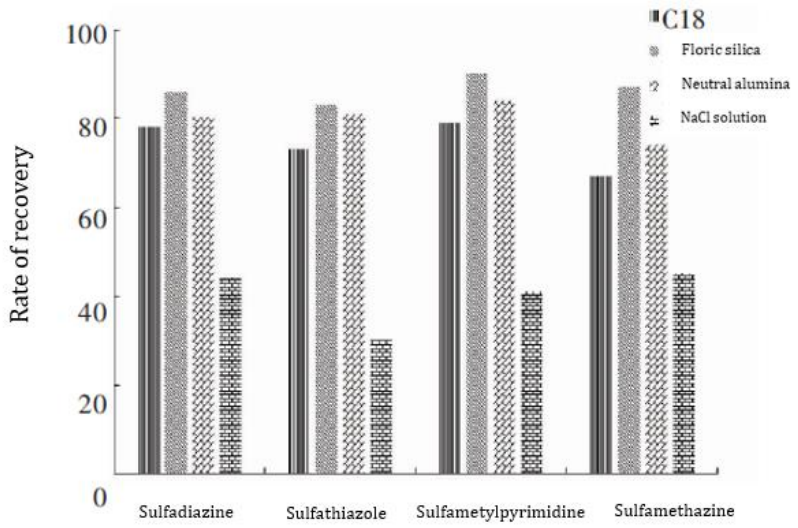

Figure 2. Effect of different dispersants on the recoveries of four sulfonamides.

2.2.3. Quality of dispersed phase

The amount of dispersed phase depends on the amount of sample. The content of oil and protein in the sample and the properties of the target substance. If the dosage is too small, the dispersity is not enough, and if the dosage is too large, the target will be adsorbed and lost. The effect of using ethyl acetate as the extraction solvent on the sensitivity when using different masses of Florisil (0.5, 1, 2, 5 and 10 g) as the solid phase dispersant was compared. It is found that the sensitivity will be lost after the addition of Florisil exceeds 1 g, which may be due to the adsorption of SAS. Therefore, its dosage is determined to be 1 g.

2.2.4. Ultrasonic extraction time

Sufficient ultrasonic time can improve the extraction and enrichment effect, but because ultrasonic will generate heat, SAS is thermally unstable, and too long ultrasonic time may cause SAS decomposition and reduce sensitivity. After adding the extraction solvent and dispersed phase to the spiked sample, the vortex was mixed evenly, and different ultrasonic times (2, 5, 10, 15 and 20 min respectively) were investigated, other conditions remain unchanged. In the first 10 min, the response of the four SAS increased gradually with the extension of ultrasonic time, and decreased after 15 min. Therefore, the ultrasonic time in this experiment is 10 min. See **Figure 3**.

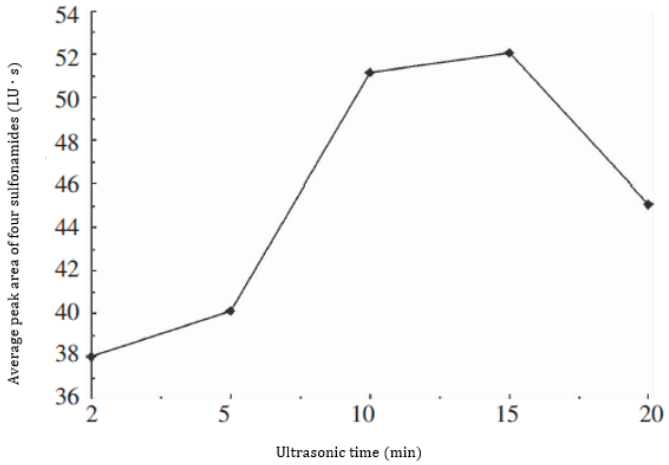

Figure 3. Effect of ultrasonic time on sulfonamide peak area.

2.3. Determination of derivatization conditions

Fluoroamine is a fluorescent reagent, which has specificity for primary aliphatic amines and aromatic amines. It combines with sulfonamides to produce high fluorescence effect and selective phosphor, which has excitation emission spectrum characteristics, while fluoroamine and its hydrolysates have no fluorescence. Taking advantage of this characteristic, SAS was used in this study for fluorescence detection after fluorescence amine pre column derivatization, which avoided excessive purification steps in sample processing and eliminated matrix interference.

The optimization of derivation conditions is mainly to optimize the derivation time. If the time is too short, the reaction is incomplete, and the time is too long, the unstable SAS derivatives will decompose. By comparing the derivatization times of 5, 10, 20, 30, 40, 50, 60 and 70 min, it was found that the reaction was not complete before 40 min, and the peak areas of the four SAs remained basically unchanged between 40 and 50 min, reaching equilibrium, and the area of sulfanilamide tends to decrease after 50 min. See **Figure 4**.

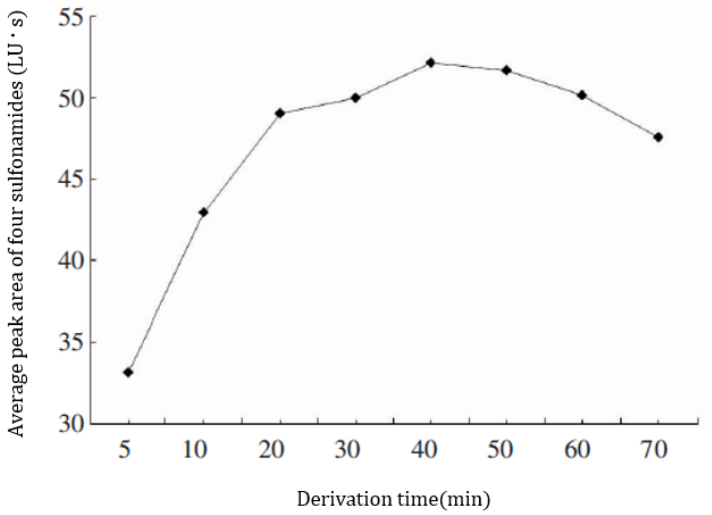

Figure 4. Effect of derivation time on peak area of sulfonamide.

2.4. Linear range of the method, detection limit and quantitation limit

Take 0.5 mL of SAs mixed standard working solution with concentrations of 2.5, 20, 50 and 100 μg/L, respectively, add 0.2 mL of 0.04% fluorescamine solution and mix for 10 s, and let stand for 40 min for HPLC analysis. Take the peak area as the ordinate and the corresponding concentration as the abscissa to perform linear regression analysis and draw a calibration curve. When the signal-to-noise ratio (S/N) is 3, calculate the lowest limit of detection (LOD), signal-to-noise ratio (S/N) The lower limit of quantitation (LOD) was calculated at 10. The four SAs had a good linear relationship between 2 and 100 μg/L, the LOD was 0.5 μg/kg, and the LOQ was 2 μg/kg. See **Table 1**.

Table 1. Linear regression equation and correlation coefficient of SAS working curve.

Chemical compound	Linear equation	Correlation coefficient *r*	Detection limit (μg/kg)	Limit of quantitation (μg/kg)
SD	Y = 5.902*X* + 17.554	0.9992	0.5	2
ST	Y = 6.241*X* + 11.184	0.9998	0.5	2
SM_1	Y = 6.541*X* + 7.720	0.9993	0.5	2
SM_2	Y = 6.220*X* + 8.062	0.9997	0.5	2

Note: SAS: sulfonamides; SD: sulfadiazine; st: sulfathiazole; SM_1: Sulfamethylpyrimidine; SM_2: sulfamethazine.

2.5. Recovery and precision

The recovery and precision of spiked prawn samples without four SAS drugs were tested. The sample pretreatment is the same as that in section 1.3.2. The addition amount of four SAS is 2 and 20 μg/kg, repeat the experiment for 3 times at each addition level, and calculate the recovery and relative standard deviation (RSD). The spiked recoveries of the four SAS in the sample are between 84.4% and 93.9%, and the RSD (n = 3) is between 3.7% and 7.7%, indicating that the accuracy and precision of the method are good and meet the requirements of trace analysis. See T**able 2**.

Table 2. Standard addition recovery and relative standard deviation of SAS (n = 3).

Chemical compound	Addition amount (μg/kg)	Recovery rate (%)	Precision RSD (%)	Addition amount (μg/kg)	Recovery rate (%)	Precision RSD (%)
SD	2	93.9	5.5	20	92.1	3.7
ST	2	84.4	7.7	20	86.2	5.8
SM_1	2	90.9	5.7	20	91.5	4.9
SM_2	2	87.1	6.9	20	86.8	4.6

Note: SAS: sulfonamides; SD: sulfadiazine; st: sulfathiazole; SM_1: sulfamethylpyrimidine; SM_2: sulfamethazine; RSD: relative standard deviation.

3. Conclusion

An ultrasonic assisted solid phase dispersion extraction precolumn derivatization high performance liquid chromatography method was established for the determination of four SAS in shrimp. Through the screening and optimization of experimental conditions, the extraction solvent was determined. Dispersed phase. Derivatization conditions and chromatographic conditions. Compared with the

traditional method, this method has the advantages of simple sample processing method. It has the advantages of fast, high extraction efficiency, less solvent consumption, no special equipment, low cost of adsorbent and easy popularization and application. It is suitable for the determination of SAS residue in shrimp, and also suitable for the rapid detection of large quantities of aquatic samples.

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