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Development of a high performance liquid chromatography method and a liquid chromatography–tandem mass spectrometry method with the pressurized liquid extraction for the quantification and confirmation of sulfonamides in the foods of animal origin

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ABSTRACT

The residues of sulfonamides (SAs) in the foods of animal origin are of the major concern because they are harmful to the consumer's health and could induce pathogens to develop resistance. Rapid and efficient determination methods are urgently in need. A quantitative high performance liquid chromatography method (HPLC) and a confirmative liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the simultaneous determination of 18 sulfonamides such as sulfamidinum, sulfanilamide, sulfisomidine, sulfadiazine, sulfapyridine, sulfathiazole, sulfamerazine, sulfadimidine, sulfamethoxypyridazine, sulfamethoxydiazine, sulfisoxazole, sulfachloropyridazine, sulfamethoxazole, sulfamonomethoxine, sulfadoxine, sulfaclozine, sulfadimethoxine, sulfaquinoxaline in the muscles, livers and kidneys of swine, bovine and chicken were developed and validated. The sample preparation procedures included a pressurized liquid extraction (PLE) with acetonitrile conducted at elevated temperature (70 °C) and pressure (1400 psi). After clean-up with hydrophilic–lipophilic balance cartridge, the extraction solution was concentrated and analyzed by HPLC and LC–MS/MS analysis. 18 SAs were separated by the HPLC with a Zorbax SB-Aq-C18 column and the mobile phase of methanol/acetonitrile/1% acetic acid with a gradient system. The wavelength of UV for the HPLC detection was set at 285 nm. The LC–MS/MS analysis was achieved with a Hypersil Golden column and the mobile phase of acetonitrile and 0.1% formic acid aqueous solution with two gradient systems. The Limits of detection (LOD) and the limits of quantitation (LOQ) were 3 μg/kg and 10 μg/kg, respectively, for both of the HPLC and LC–MS/MS. Linearity was obtained with an average coefficient of determination (R) higher than 0.9980 over a dynamic range from the LOQ value up to 5000 μg/kg. The recoveries of the methods range from 71.1% to 118.3% with the relative standard derivation less than 13%. The peaks of interest with no interferences were observed throughout the chromatographic run. The sample pretreatment provided efficient extraction and cleanup that enables a sensitive and rugged determination of 18 SAs, the obtained results revealed that PLE, in comparison with other sample preparation methods applied, has significantly higher efficacy for SAs isolation from animal tissues.

1. Introduction

The sulfonamides (SAs) are N-substituted derivatives of the sulfanilamide (Fig. 1) that inhibit multiplication of bacteria by acting as competitive inhibitors of p-aminobenzoic acid in the folic acid metabolism cycle. Owing to their wide spectrum against gram-positive and gram-negative organisms as well as their relatively inexpensive in price, SAs are widely used in the food-producing animals. The widespread use of SAs can result in the residues of SAs in the foods of animal origin that are the major concerns of consumers and regulatory bodies due to their adverse reactions such as thyroid follicular tumors [1], allergic reactions [2] and the development of antibiotic resistance [3]. The most countries such as China, USA and the European Union have established a series of maximum residue limits (MRLs) [4–7] for such kind of drugs in foods of animal origin and set up the performance criteria for analytical methods as well as the procedures for method validation [8].

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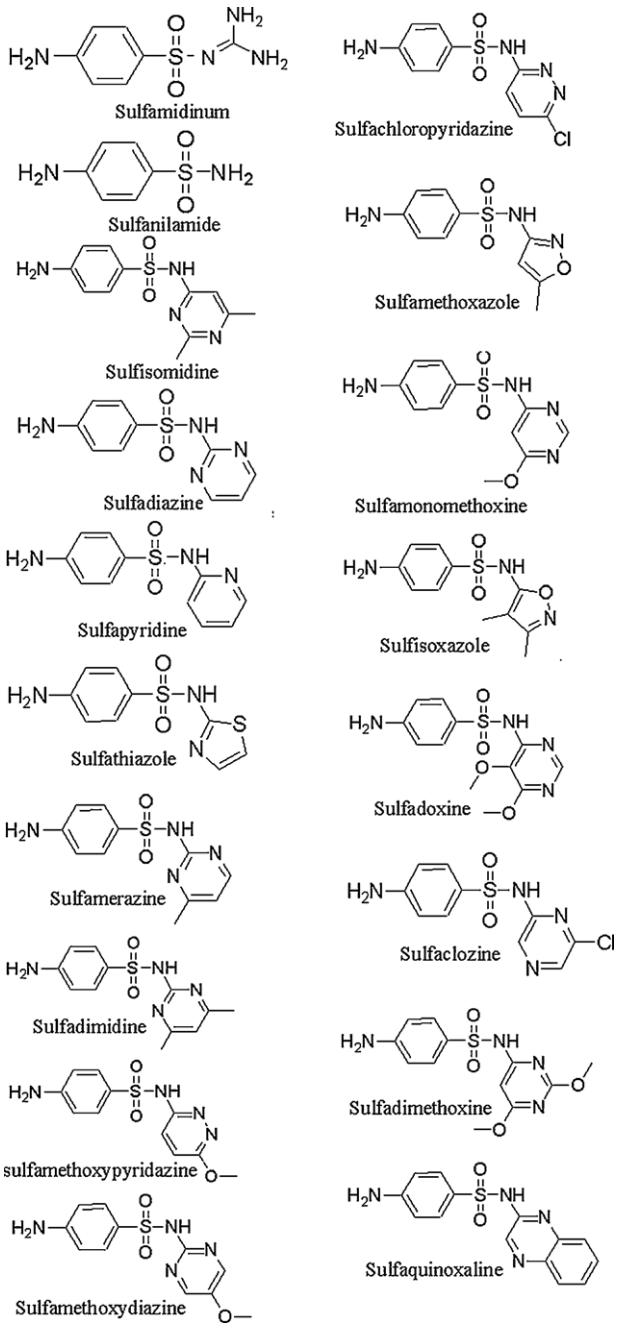

Fig. 1. Structures of 18 sulfonamides.

A lot of analytical methods have been developed for the determination of sulfonamides in animal tissues such as microbiological [9,10], receptor [11,12], immunological techniques [13,14], high-performance liquid chromatography (HPLC) [15–19], gas chromatography (GC) [19,20], capillary electrophoresis (CE) [21,22]. HPLCs with ultraviolet detection are the most widely applied. One of the most common problems faced in the extraction of SAs in biological matrices is natural organic substances in the samples that reduce extraction efficiencies and hinder detection. A broad range of extraction techniques (Soxhlet extraction, percolation, maceration, digestion, extraction under reflux, steam distillation, etc.) are currently used for this purpose, most frequently exhaustive extraction in the Soxhlet apparatus. Although this is a relatively simple method, it suffers from such disadvantages as long extraction time, relatively high solvent consumption and often unsatisfactory reproducibility. Most of the methods published used buffer as a mobile phase for the analysis of SAs, and the matrix used are either feed or other environmental samples such as manure and wastewater [23–25]. An important and interesting employment of pressurized liquid extraction (PLE) is the extraction of chemical constituents from plant and herbal materials where PLE appears to be the most effective sample preparation method.

PLE is a recent advance in sample preparation for trace analytes and this technique uses conventional solvents at elevated pressures and temperatures to extract solid samples quickly. The process takes advantage of the increasing solubility of analyte at temperatures and pressures well above the common, raising the diffusion rate and decreasing the viscosity and surface tension, so the kinetic processes for analytes desorbing from the matrix are accelerated. The number of methods described in the literature using the more advanced PLE [26] has grown considerably in recent years. Because of its better repeatability, lower solvent consumption and reduced time for sample pre-treatment. Jacobsen et al. [27] have recently examined the potential of PLE for extracting selected antimicrobials from environmental solid samples. It also has been used to extract other antibiotics in foodstuffs [28–31]. Thus, the success of a quantitative determination of trace-level SAs in complicated matrices is largely determined by the success in the extraction step. However, in the previous work, only two papers described the applicability of PLE for the extraction of SAs in animal tissues [32,33].

In the present study, a rapid, sensitive, and reliable method for simultaneous determination of 18 SAs in chicken, swine and bovine muscle and liver by PLE has been developed. The sample preparation procedure is consisted of a PLE with acetonitrile conducted at elevated temperature (70 °C) and pressure (1400 psi). After clean-up with hydrophilic–lipophilic balance cartridge, the extraction solution was concentrated and analyzed by HPLC and LC–MS/MS analysis. The ASE extraction conditions, including extraction solvents, temperature, pressure and extraction cycles, high extraction efficiency, have been optimized. The extracted samples can be analyzed easily using HPLC, and the robustness of the method has been tested according to real samples analysis.

2. Experimental

2.1. Chemicals and reagents

Sulfamethoxydiazine, sulfisomidine, sulfamethoxazole, sulfamonomethoxine, sulfisoxazole, sulfaclozine, sulfadimethoxine, sulfaquinoxaline, sulfaguanidine, sulfanilamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine and sulfadoxine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were obtained from Fisher (Bar-Bel, France). Citric acid, ammonium acetate, acetic acid and ammonia solution (25%) were supplied by Beijing Chemical Company (Beijing, China). The cartridges used for solid-phase extraction were Oasis hydrophilic–lipophilic balance (HLB) cartridges (3 cm^3/60 mg) from Waters (Milford, MA, USA). Filter membranes (0.22 μm) used to filter the extracts before injection into the chromatographic system was provided by Agilent (Palo Alto, CA, USA). Separation columns for HPLC and LC–MS/MS were ZORBAX SB-aq-C18 (150 mm × 4.6 mm I.D., 5 μm) (Agilent Technology, USA) and Hypersil Golden (100 mm × 2.1 mm, 3.5 μm) (Thermo scientific, Germany), respectively. Unless indicated otherwise, all reagents used in this study were analytically pure substances and HPLC-grade. Deionized water (18 MΩ m) was generated by a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

2.2. Blank sample

The muscle, liver and kidney samples of swine, bovine, muscle and liver of chicken were purchased from local market, homogenized in a high-speed food blender, and stored at below −20 °C until the time of analysis.

2.3. Sample preparation

2.3.1. Pressurized liquid extraction

ASE was carried out using a Dionex accelerated solvent extractor 200 (Dionex, Sunnyvale, CA, USA) equipped with 11 ml stainless-steel extraction cells. For each cell, 5 g tissue sample was placed in cellulose filters (Dionex) which were in turn placed in the stainless steel extraction cells. All the cells were heated in a water bath at 40 °C to improve and facilitate the handling of the mixture, using acetonitrile as solvents. Optimized extraction conditions were obtained by sequentially varying one experimental parameter while all other parameters remained fixed. Final conditions used in the extraction for SAs were as follows: time heating cell 2 min, time of solvent in contact with the sample 2 min (static time), pressure at 65 bar, temperature at 60 °C, time purging with nitrogen to expulse rest of solvent in the cell 60 s, water volume flushing in respect to the cell size in percentage 50%, and 1 cycle. The extraction solution was evaporated to dryness (under nitrogen flow) at 40 °C and reconstituted in 10 ml of 2% acetic acid solution.

2.3.2. Clean-up by solid-phase extraction

HLB SPE cartridge was activated with 3 ml of methanol and 3 ml of water. Then, the solution obtained in the sample extraction section was passed through the column. The cartridge was washed twice with 5 ml of methanol:water (10:90, v/v) and then dried by applying vacuum for 1 min. The compounds of interest were eluted with 4 ml of methanol and evaporated to dryness (under nitrogen flow) at 40 °C. The residue was dissolved in 1 ml of HPLC initial mobile phase. After filtration, this solution was injected into HPLC or LC–MS/MS for analysis.

2.3.3. HPLC conditions

The HPLC system consists of a Waters 2695 separations module and 2487 dual wavelength absorbance detector (Waters, USA). A Zorbax SB-Aq-C18 (250 mm × 4.6 mm I.D., 5 μm) HPLC column was used for separating SAs. The operating temperature of the column was set at 35 °C. The injection volume was 50 μl. The mobile phase used was a three gradient system with methanol/acetonitrile/1% acetic acid. The starting mobile phase composition at 0 min was 5/8/87 and maintained for 15 min, it was switched to 15/10/75 at 45 min, and then it was switched to 25/20/55 at 60 min. The flow rate was 0.8 ml/min. The wavelength of UV detector was set at 285 nm.

2.4. LC–MS/MS analysis

Analysis was carried out using a Thermo-electron TSQ Quantum Access triple quadrupole mass spectrometer coupled with a Surveyor LC pump and an autosampler. The separation was achieved by a Hypersil Golden (150 mm × 2.1 mm, 3.5 μm). Mobile phase A was acetonitrile and B was 0.1% formic acid aqueous solution. The gradient composition of mobile phase was initially 0.5/99.5, and then programmed to 10/90 at 5 min, finally switched to 50/50 at 30 min. The flow rate was 0.2 ml/min and injection volume was 10 μl. The source parameters were optimized by monitoring the MS/MS spectra of the analytes. The ion mode was positive. Selected reaction monitoring (SRM) was performed on each of the analyte protonated molecular ions using the parameters: source voltage was 5 kV, capillary temperature 350 °C, sheath gas (nitrogen) 35 psi, auxiliary gas 5 arb, Q1 peak width 0.70 amu, Q3 peak width 0.50 amu, collision gas (argon) 1.50 mTorr, scan width 1–2 amu, and scan time 0.3–0.5 s. Collision energies were set at the maximum for each transition, and ranged from 18 to 30 eV. These parameters were optimized for matrix extracts to confirm analyte residues.

2.5. Validation procedure

The method was validated with reference to the validation procedure for residues in food animal products as described in the EU Commission Decision 2002/657/EC under Council Directive 96/23/EC [8]. The validation of specificity, linearity, decision limits (CCα) and the detection capability (CCβ), recovery and precision for the method were determined. The blank tissue samples were spiked with SAs at each of six concentrations (5, 10, 50, 100, 200 and 500 μg/kg), and the linearity of these matrices spiked curves was established.

2.5.1. Specificity

Specificity was performed by analyzing each type of 10 blank tissue samples from different sources to evaluate possible endogenous interferences in samples. The sample preparation and chromatographic condition were optimized to guarantee that no interferences incurred at the retention time of the tested compounds.

2.5.2. Decision limits (CCα) and the detection capability (CCβ)

CCα was calculated by analyzing 20 blank samples fortified with SAs at their MRLs (100 μg/kg). CCβ was calculated as the decision limit plus 1.64 times the corresponding standard deviation ($\beta = 5\%$), supposing that standard deviation at CCα level equals standard deviation at the MRL level.

2.5.3. Recovery

Sample recovery was determined with blank muscle and liver samples that were spiked at the levels of 0.5, 1 and 1.5 times of MRL. The spiked samples were analyzed and the recoveries were calculated by comparing the peak area of measured concentration to the peak area of the spiked concentration.

2.5.4. Repeatability and reproducibility

Six sets of samples that have been spiked with SAs at three different concentrations as described above were analyzed on the same day with the same instrument and operator. The mean concentration and the relative standard deviation (RSD) were calculated as repeatability. Separately, samples that have been spiked with SAs at three different concentrations were analyzed for 3 days with the same instrument and operator. The overall mean concentration and RSD were calculated as reproducibility.

3. Results and discussion

3.1. HPLC separation

The zwitterionic nature of sulfonamides makes their separation difficult, the weak basic characteristic is capable to take a proton from the medium, which implies the use of acidic eluent. According to our research, when using an isocratic elution, sulfanilamide and sulfadiazine were eluted almost at the same time, and the peak of sulfamethazine showed as a "broad peak", which indicated that there existed serious interaction between sulfamethazine and silanol groups on the separation. Our experiment revealed that acidic water and acetonitrile worked as effective as the buffer and other solvents combination. Furthermore, it avoided the use of buffer, from which, salt traces tend to crystallise in the column and reduced its separation capacity. 1% acetic acid and acetonitrile as

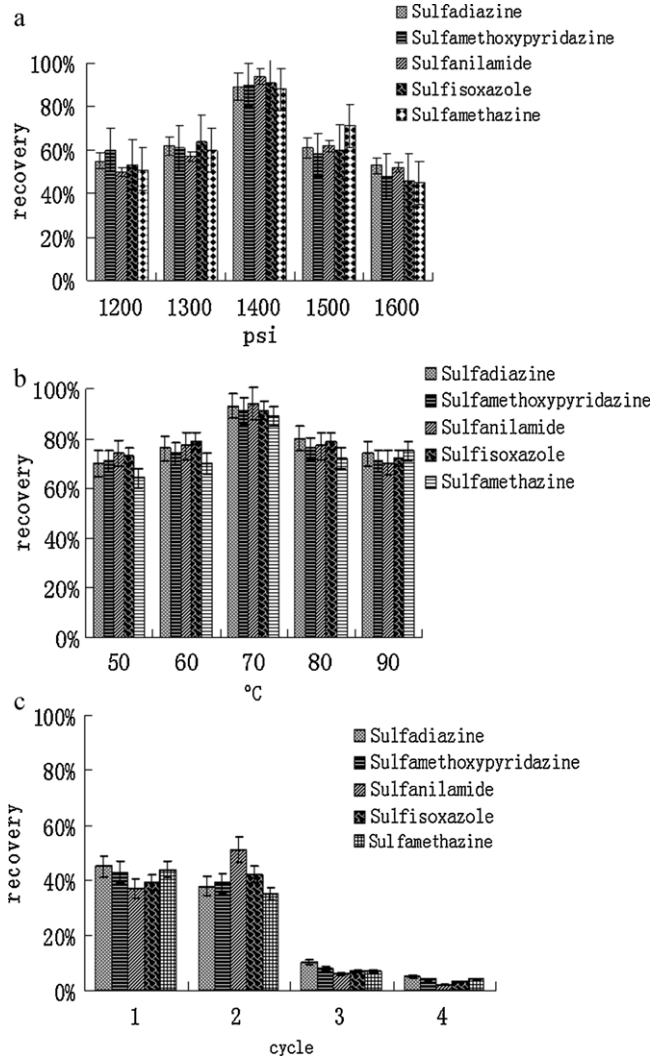

Fig. 2. Effect of temperatures, pressures and extraction cycle on the recovery.

mobile phases was used to separate 18 SAs and gradient elution was involved.

3.2. Optimization of PLE conditions

To explore the most effective PLE conditions, three independent series of multiple-PLE of tissue differing in extraction temperature, pressure and cycle were performed. After the matrix investigation, swine was selected as the typical sample since it is more complicated. Their results are presented in Fig. 2, which contains also the total amounts of SAs obtained at all extraction steps carried out within each series.

Pure organic solvents such as methanol and acetonitrile can be used to extract antibiotics, however, the drawback of using these solvent systems is that many other organic impurities are also extracted, causing problems for later HPLC analysis, and this may influence the separation of drugs on the column and further affect the recovery. After the optimization of aqueous phase, when this biological matrix was treated with acetonitrile, higher extraction efficiencies for all the 18 analytes were obtained in tissues, followed by SPE clean up, the interference was eliminated and the recoveries were stable.

Extractions of SAs at various temperatures (50–90 °C) were examined in some detail with muscle and liver. Recovery rates arranged from 67% to 95% (n = 3; RSD = 4–9%). Extraction efficiency showed an optimum at 70 °C. It appears from Fig. 2a that, above it recoveries decreased, probably due to that in high temperature, the extractant was not clear which may be due to the matrix dispersion of the tissues. Recoveries were also low at below 70 °C, most likely due to the inefficient desorption and dissolution of the SAs (see Fig. 2a).

The variable pressures of the extracting system have also been investigated from 1200 to 1600 psi. The recovery was obtained between 45% and 94% with RSD of 4–7%. It was found that the best recovery could be obtained at the pressure of 1400 psi (see Fig. 2b) and this pressure is considerably above the minimum pressure to keep the solvent liquid. Probably the elevated pressure used in PLE squeezes the tissue matrix making the diffusion of SAs from the inside to the outside of the matrix difficult or hindering the penetration of the inner matrix by the extracting solvent.

Moreover, in the case of animal tissue, most of SAs (more than 59.9%) is extracted in the first cycle of the PLE process performed in conditions. Hence, it can be concluded that different kinetics of SAs isolation in the PLE process of animal tissue is connected with the properties (rigidity) of matrices. According to our study, the number of extracting cycle was not the most important factor. After 2 cycles of extraction with 4 min of each one, the recovery could not increase any more (see Fig. 2c), on the contrary, the impurity raised and this result was consisted to a prior study [34].

3.3. Clean-up

The difference in the p*K*a of the sulfonamides makes sample clean-up difficult. The published methods indicated SAs were best clean-up in acidic condition for further clean-up [35–37]. This could be due to the fact that some SAs had a dissociation constant (p*K*a) value of 10.43 which is favoured in alkaline condition. However, if the extraction were carried out in an alkaline condition, the recoveries for the rest of the compounds will be reduced. In this study, the final extraction solution was dried and reconstituted in 10 ml of 2% acetic acid solution. The recoveries were found to be very good. The recoveries were much improved with Oasis HLB cartridges.

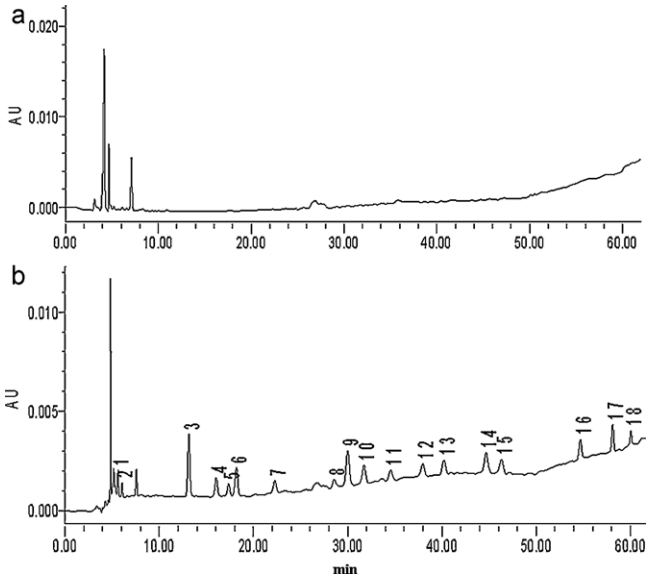

Fig. 3. HPLC chromatograms of 18 SAs of blank (a) and spiked at a concentration of each drug 10 μg/kg in the liver of swine (b).

Table 1
The limits of detection (LODs), limits of quantitation (LOQs), maximum residue limits (MRLs), decision limits (CCα) and detection capability (CCβ) of the 18 sulfonamides in the liver of swine in HPLC.

Analyte	LODs (μg/kg)	LOQs (μg/kg)	MRLs (μg/kg)	CCα (μg/kg)	CCβ (μg/kg)
Sulfamidinum	3	10	100	103	109
Sulfanilamide	3	10	100	106	110
Sulfisomidine	3	10	100	105	109
Sulfadiazine	3	10	100	105	108
Sulfapyridine	3	10	100	104	109
Sulfathiazole	3	10	100	103	107
Sulfamerazine	3	10	100	103	109
Sulfadimidine	3	10	100	103	109
Sulfamethoxypyridazine	3	10	100	103	109
Sulfamethoxydiazine	3	10	100	107	113
Sulfachloropyridazine	3	10	100	103	108
Sulfamethoxazole	3	10	100	103	108
Sulfamonomethoxine	3	10	100	104	109
Sulfisoxazole	3	10	100	103	107
Sulfadoxine	3	10	100	106	107
Sulfaclozine	3	10	100	105	108
Sulfadimethoxine	3	10	100	102	109
Sulfaquinoxaline	3	10	100	103	108

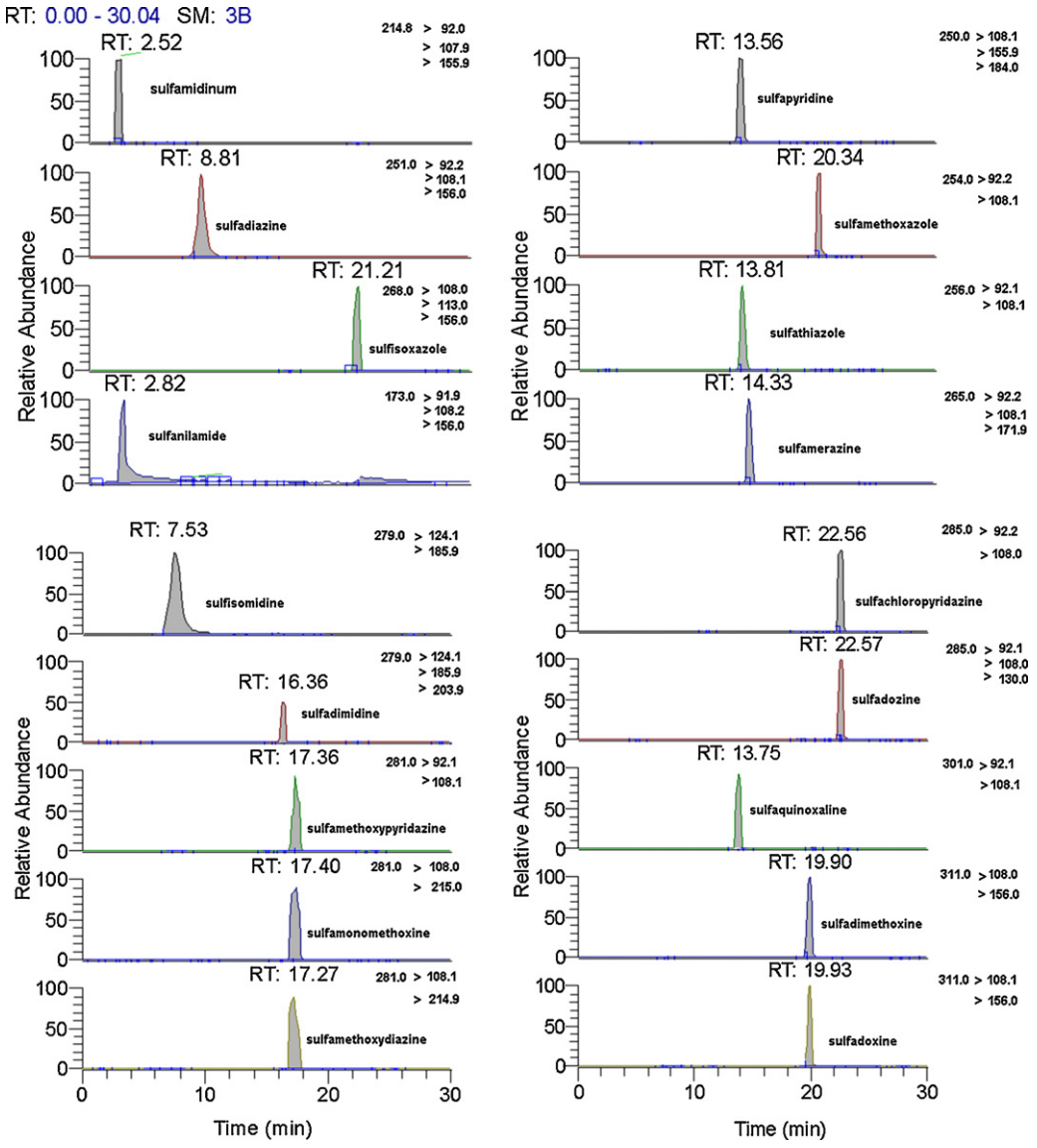

Fig. 4. TIC LC–MS/MS chromatograms of 18 sulfonamides in standard solution at 5 μg/l.

Table 2
Recovery and reproducibility of the 18 SAs in the muscle, liver and kidney of swine in HPLC ($n = 18$).

Number	Drug	Spiked level (μg/kg)	Muscle		Liver		Kidney	
			Overall recovery (%)	RSD (%)	Overall recovery (%)	RSD (%)	Overall recovery (%)	RSD (%)
1	Sulfamidinum	50	74.3 ± 2.0	2.2	81.2 ± 2.8	3.5	110.0 ± 1.8	1.6
		100	87.1 ± 1.5	1.8	86.0 ± 1.5	1.8	100.4 ± 3.1	2.9
		150	90.2 ± 4.2	4.7	82.1 ± 13.7	16.6	102.0 ± 1.6	1.6
2	Sulfanilamide	50	96.4 ± 4.9	5.1	80.3 ± 4.7	5.9	85.0 ± 3.6	4.2
		100	93.7 ± 3.1	3.3	97.6 ± 3.4	3.5	107.0 ± 4.6	4.3
		150	93.2 ± 7.2	7.7	80.1 ± 8.2	10.2	101.7 ± 3.4	3.3
3	Sulfisomidine	50	98.7 ± 4.3	4.4	73.1 ± 4.0	5.5	92.5 ± 8.1	8.7
		100	109.3 ± 3.0	2.7	105.6 ± 3.5	3.3	100.4 ± 4.0	4.0
		150	96.8 ± 12.3	12.7	96.6 ± 10.0	10.4	87.5 ± 3.3	3.7
4	Sulfadiazine	50	88.5 ± 6.7	7.6	89.3 ± 6.2	6.9	103.3 ± 11.7	11.4
		100	91.5 ± 4.2	4.6	80.8 ± 4.1	5.1	89.9 ± 6.1	6.8
		150	93.3 ± 6.1	8.2	89.3 ± 4.4	4.9	83.1 ± 3.8	4.6
5	Sulfapyridine	50	107.5 ± 5.0	4.7	72.2 ± 7.4	10.2	105.7 ± 4.2	4.0
		100	88.2 ± 8.6	9.8	103.3 ± 5.5	5.4	85.3 ± 5.4	6.3
		150	96.0 ± 5.3	5.5	84.5 ± 5.7	6.7	102.6 ± 7.1	6.9
6	Sulfathiazole	50	102.5 ± 4.7	4.6	71.7 ± 5.1	7.1	96.0 ± 5.5	5.8
		100	103.8 ± 4.4	4.2	93.8 ± 5.4	5.8	105.1 ± 5.0	4.8
		150	81.4 ± 2.3	2.8	95.6 ± 2.4	2.5	100.2 ± 5.4	5.4
7	Sulfamerazine	50	81.8 ± 2.8	3.4	104.6 ± 2.2	2.1	110.5 ± 2.7	2.5
		100	84.2 ± 1.7	2.0	91.0 ± 1.6	1.8	99.1 ± 1.6	1.6
		150	82.7 ± 3.9	4.7	84.6 ± 4.3	5.1	95.0 ± 3.8	4.0
8	Sulfadimidine	50	72.6 ± 4.7	6.5	74.1 ± 3.1	4.2	79.7 ± 4.7	5.8
		100	92.6 ± 3.5	3.8	96.5 ± 3.5	3.7	85.3 ± 3.4	4.0
		150	86.9 ± 8.3	9.5	96.9 ± 7.6	7.8	102.6 ± 8.2	8.0
9	Sulfamethoxypyridazine	50	82.3 ± 4.3	5.2	75.6 ± 4.4	5.8	87.9 ± 4.1	4.6
		100	86.3 ± 3.3	3.8	107.5 ± 4.2	3.9	109.8 ± 3.4	3.1
		150	90.3 ± 2.0	2.2	102.5 ± 10.5	10.3	83.7 ± 2.0	2.4
10	Sulfamethoxydiazine	50	84.0 ± 6.4	7.6	75.1 ± 4.6	6.2	73.6 ± 6.2	8.4
		100	90.8 ± 3.8	4.2	94.3 ± 6.9	7.3	97.6 ± 3.8	3.9
		150	97.1 ± 4.3	4.4	89.1 ± 5.0	5.6	85.7 ± 4.3	5.0
11	Sulfachloropyridazine	50	74.3 ± 5.3	7.2	83.8 ± 4.5	5.4	101.2 ± 5.6	5.6
		100	75.1 ± 7.2	9.6	99.9 ± 7.9	7.9	87.6 ± 7.3	8.3
		150	81.1 ± 5.5	6.8	94.1 ± 5.2	5.6	101.2 ± 5.5	5.5
12	Sulfamethoxazole	50	89.3 ± 5.2	5.8	80.9 ± 5.1	6.3	64.3 ± 5.1	7.9
		100	88.9 ± 5.5	6.2	102.3 ± 7.4	7.3	107.0 ± 5.4	4.9
		150	70.8 ± 2.5	3.5	86.5 ± 2.0	2.3	101.9 ± 2.4	2.4
13	Sulfamonomethoxine	50	91.3 ± 2.7	2.9	79.1 ± 2.3	2.9	76.8 ± 3.4	4.5
		100	82.8 ± 1.5	1.8	91.8 ± 1.7	1.8	88.4 ± 0.2	0.2
		150	81.8 ± 3.7	4.5	82.2 ± 4.8	5.8	81.9 ± 7.3	8.9
14	Sulfisoxazole	50	78.2 ± 4.6	5.9	71.3 ± 3.9	5.5	81.2 ± 4.6	5.7
		100	79.0 ± 3.4	4.4	84.7 ± 3.8	4.5	104.5 ± 3.1	3.0
		150	85.2 ± 8.2	9.6	84.8 ± 7.3	8.7	80.7 ± 8.2	10.1
15	Sulfadoxine	50	85.2 ± 4.1	4.8	84.2 ± 4.5	5.3	71.6 ± 4.2	5.8
		100	106.3 ± 3.4	3.2	102.8 ± 4.0	3.9	83.5 ± 3.4	4.1
		150	97.2 ± 6.1	6.3	97.4 ± 9.5	9.8	84.9 ± 8.1	9.5
16	Sulfaclozine	50	81.9 ± 6.2	7.6	77.0 ± 5.9	7.7	75.1 ± 6.2	8.3
		100	87.4 ± 3.9	4.5	85.4 ± 3.9	4.6	81.5 ± 3.9	4.8
		150	85.2 ± 4.5	5.3	95.0 ± 5.4	5.7	85.1 ± 4.4	5.2
17	Sulfadimethoxine	50	97.0 ± 5.6	5.8	90.7 ± 6.6	7.3	99.5 ± 5.5	5.5
		100	89.7 ± 7.4	10.6	83.4 ± 3.7	4.4	80.3 ± 6.3	7.9
		150	88.8 ± 5.8	6.5	87.5 ± 5.7	6.5	87.6 ± 5.7	6.5
18	Sulfaquinoxaline	50	80.9 ± 5.1	6.3	71.8 ± 7.9	11.1	78.8 ± 5.1	6.4
		100	95.4 ± 5.5	5.7	92.0 ± 5.3	5.7	97.1 ± 5.4	5.6
		150	109.3 ± 2.4	2.2	80.8 ± 2.8	3.5	82.8 ± 2.3	2.8

3.4. Method validation

3.4.1. Specificity and linearity

The specificity of the method was checked by analyzing different types of blank edible tissue samples. As shown in the chromatograms of blank (Fig. 3a) and spiked sample (Fig. 3b), none interfering peaks could be detected at the retention time of the 18 analytes. As some internal standards such as substituted could not be obtained commercially, we used external reference method for quantification. The linearity and regression study were performed according to Section 2.5. The linear range was calculated by matrix-matched calibration standard curves when concentrations were between 10 and 2000 μg/kg of the 18 compounds. The high correlation coefficients ($r = 0.9981–0.9990$) indicated good correlations and it also indicated that this method can be used to determine 18 compounds at a wide contamination level range.

3.4.2. Decision limits (CCα) and the detection capability (CCβ)

The decision limit (CCα) and the detection capability (CCβ) of the method were determined on different animal muscle and liver types according to Section 2.5.2. Part of the representative results (from swine liver) was summarized in Table 1. The limits of detection (LOD) were experimentally calculated from the analysis of samples spiked with a standard mixture of the analytes at serially diluted concentrations, and the minimum concentration which gave a signal to noise ratio higher than 3 is LOD (the signal-to-noise ratio is a measure of how the signal from the analyte

Table 3
Recovery and reproducibility of the 18 SAs in the muscle, liver and kidney of bovine in HPLC ($n = 18$).

Number	Drug	Spiked level (μg/kg)	Muscle		Liver		Kidney	
			Overall recovery (%)	RSD (%)	Overall recovery (%)	RSD (%)	Overall recovery (%)	RSD (%)
1	Sulfamidinum	50	94.1 ± 5.2	5.5	78.7 ± 2.3	2.9	93.6 ± 2.5	2.7
		100	94.1 ± 5.6	6.0	82.8 ± 1.5	1.8	93.8 ± 1.6	1.7
		150	96.9 ± 2.5	2.6	87.6 ± 4.1	4.7	97.1 ± 13.9	14.3
2	Sulfanilamide	50	97.4 ± 2.6	2.7	86.9 ± 4.7	5.4	97.3 ± 4.7	4.8
		100	83.8 ± 1.6	1.9	84.4 ± 2.8	3.3	79.5 ± 2.8	3.6
		150	88.8 ± 4.1	4.6	87.6 ± 8.4	9.6	97.4 ± 8.3	8.6
3	Sulfisomidine	50	95.5 ± 4.8	5.0	75.8 ± 4.2	5.5	77.5 ± 5.0	7.5
		100	99.6 ± 2.3	2.3	83.5 ± 3.8	4.6	103.0 ± 3.7	3.5
		150	97.4 ± 7.7	7.9	86.5 ± 8.1	9.4	96.4 ± 12.1	12.5
4	Sulfadiazine	50	96.2 ± 4.2	4.4	86.0 ± 6.2	7.2	77.9 ± 2.3	3.4
		100	97.5 ± 3.6	3.7	85.0 ± 3.8	4.5	97.4 ± 3.9	4.0
		150	96.5 ± 2.4	2.5	93.3 ± 4.2	4.5	97.1 ± 4.4	4.5
5	Sulfapyridine	50	97.4 ± 6.3	6.5	85.7 ± 5.5	6.4	72.7 ± 8.5	11.6
		100	97.3 ± 4.0	4.1	81.3 ± 7.8	9.6	98.2 ± 7.4	7.5
		150	97.5 ± 4.4	4.5	94.7 ± 5.4	5.7	97.4 ± 5.6	5.8
6	Sulfathiazole	50	97.8 ± 5.6	5.8	76.5 ± 5.1	6.7	97.2 ± 5.1	5.2
		100	98.3 ± 7.4	7.5	88.2 ± 5.6	6.3	98.0 ± 5.5	5.6
		150	97.4 ± 5.7	5.9	88.8 ± 2.5	2.8	98.0 ± 2.5	2.5
7	Sulfamerazine	50	93.4 ± 2.5	2.7	86.7 ± 1.8	2.1	77.6 ± 7.3	12.7
		100	96.0 ± 1.6	1.6	89.1 ± 2.6	2.9	81.7 ± 5.4	8.8
		150	98.5 ± 11.9	12.1	81.4 ± 1.6	2.0	95.2 ± 4.1	4.3
8	Sulfadimidine	50	99.1 ± 4.7	4.8	75.8 ± 7.2	9.5	76.2 ± 5.6	8.5
		100	98.0 ± 3.4	3.5	85.1 ± 4.8	5.6	85.7 ± 2.6	3.9
		150	98.5 ± 8.3	8.4	82.6 ± 2.8	3.4	97.7 ± 6.3	6.5
9	Sulfamethoxypyridazine	50	98.3 ± 4.2	4.3	77.4 ± 8.5	11.0	73.0 ± 1.5	2.4
		100	99.3 ± 3.5	3.5	89.5 ± 4.2	4.7	80.3 ± 14.1	10.7
		150	99.0 ± 12.2	12.3	99.3 ± 12.4	12.5	93.7 ± 6.3	6.8
10	Sulfamethoxydiazine	50	98.2 ± 6.2	6.3	79.7 ± 6.4	8.0	86.1 ± 3.4	5.2
		100	98.5 ± 3.9	3.9	89.2 ± 3.9	4.4	97.2 ± 5.6	5.7
		150	98.0 ± 4.4	4.4	89.5 ± 4.5	5.0	87.6 ± 4.0	5.9
11	Sulfachloropyridazine	50	98.2 ± 5.5	5.6	72.5 ± 5.7	7.9	75.6 ± 3.5	5.4
		100	98.0 ± 7.2	7.4	80.5 ± 7.5	9.3	86.6 ± 9.2	10.2
		150	97.5 ± 5.5	5.7	99.9 ± 5.7	5.7	84.5 ± 6.2	9.7
12	Sulfamethoxazole	50	98.9 ± 5.1	5.1	100.4 ± 5.2	5.2	81.1 ± 3.7	5.5
		100	99.6 ± 5.5	5.5	101.4 ± 5.7	5.6	88.2 ± 4.5	6.5
		150	98.9 ± 2.5	2.5	89.2 ± 2.5	2.8	89.9 ± 5.5	7.8
13	Sulfamonomethoxine	50	112.5 ± 5.1	4.5	83.2 ± 3.9	4.7	104.7 ± 4.2	4.0
		100	97.8 ± 5.4	5.6	94.0 ± 4.5	4.8	95.6 ± 3.4	3.6
		150	97.9 ± 2.4	2.5	94.3 ± 5.6	5.9	94.4 ± 12.1	12.8
14	Sulfisoxazole	50	98.1 ± 2.8	2.9	73.5 ± 7.4	10.1	76.3 ± 4.7	7.1
		100	97.7 ± 1.5	1.6	94.0 ± 5.8	6.2	94.0 ± 3.9	4.1
		150	97.9 ± 3.7	3.8	93.9 ± 5.1	5.4	94.7 ± 2.7	2.9
15	Sulfadoxine	50	95.7 ± 4	4.9	82.3 ± 5.5	6.7	94.0 ± 1.5	1.6
		100	97.1 ± 3.4	3.5	92.5 ± 2.4	2.6	93.7 ± 3.6	3.8
		150	98.0 ± 8.2	8.4	94.4 ± 2.7	2.9	95.1 ± 4.6	4.9
16	Sulfaclozine	50	97.8 ± 4.1	4.2	74.1 ± 1.5	2.0	96.0 ± 3.4	3.6
		100	99.2 ± 3.5	3.5	93.2 ± 10.7	11.5	93.5 ± 8.2	8.8
		150	98.0 ± 10.0	10.2	94.9 ± 4.6	4.8	94.9 ± 4.4	4.7
17	Sulfadimethoxine	50	97.0 ± 6.2	6.4	74.3 ± 3.4	4.6	93.2 ± 5.5	5.9
		100	98.3 ± 4.0	4.1	94.9 ± 8.2	8.6	93.4 ± 7.3	7.8
		150	98.3 ± 4.4	4.5	96.5 ± 4.1	4.2	94.9 ± 5.7	6.0
18	Sulfaquinoxaline	50	97.7 ± 5.5	5.7	94.7 ± 3.4	3.6	94.5 ± 5.1	5.4
		100	97.8 ± 7.4	7.5	84.3 ± 9.1	10.8	94.0 ± 5.4	5.8
		150	97.5 ± 5.7	5.8	94.1 ± 6.2	6.6	90.2 ± 2.3	2.6

compares to other background reflections (categorized as "noise"). In the HPLC instrument, the signal-noise ratio (S/N) is calculated at the time window in which the analyte is expected). The LOD were between 5 and 10 μg/kg for different compounds and LOQ were no more than 15 μg/kg. It showed that the method could be useful to determine SAs residues in contaminated animal muscle and liver.

3.4.3. Recovery, repeatability and reproducibility

Tables 2–4 gave the results of the recovery and reproducibility of the method over the concentration range (from 50 to 150 μg/kg) on three separate days. It was also reflected that the method provided a wide concentration range over which to assess the performance of the developed method. Recoveries from edible tissue samples were more than 80%, and between-day RSD were lower than 10%.

3.4.4. Confirmation of LC–MS/MS

For a compound to be positively confirmed, its retention time has to match to that of a standard within 5%. In addition, the ion transitions monitored have to be present at a signal-to-noise of greater than 10, and the relative abundances of the integrated peaks for each transition have to match that observed in a matrix standard by ±10% (i.e. if the relative abundance of an ion transition is 40% in the standard, the relative abundance has to be between 30 and 50% in the sample) [31]. Using positive ion electrospray ionization, triple quadrupole mass spectrometer parameters were

Table 4
Recovery and reproducibility of the 18 SAs in the muscle and liver of chicken in HPLC ($n = 18$).

Number	Drug	Spiked level (μg/kg)	Chicken muscle		Chicken liver	
			Overall recovery (%)	RSD (%)	Overall recovery (%)	RSD (%)
1	Sulfamidinum	50	104.7 ± 1.7	1.6	76.0 ± 2.7	4.0
		100	104.6 ± 12.9	12.2	74.6 ± 1.6	2.5
		150	98.8 ± 4.7	4.7	87.6 ± 4.4	5.0
2	Sulfanilamide	50	98.5 ± 3.5	3.5	75.1 ± 4.7	7.2
		100	99.5 ± 8.3	8.3	83.6 ± 3.4	5.3
		150	98.5 ± 4.3	4.3	87.6 ± 8.6	12.7
3	Sulfisomidine	50	100.4 ± 3.3	3.2	82.9 ± 4.0	6.4
		100	94.0 ± 12.0	12.8	84.1 ± 3.4	4.0
		150	97.8 ± 6.4	6.5	85.7 ± 10.2	11.6
4	Sulfadiazine	50	100.6 ± 3.8	3.8	75.5 ± 6.3	9.6
		100	97.7 ± 4.3	4.4	71.1 ± 4.2	5.9
		150	96.5 ± 5.3	5.5	89.9 ± 4.0	4.7
5	Sulfapyridine	50	95.4 ± 7.2	7.6	74.5 ± 5.5	8.5
		100	98.6 ± 5.5	5.6	101.1 ± 5.5	5.4
		150	98.3 ± 5.2	5.3	98.5 ± 5.0	5.1
6	Sulfathiazole	50	76.2 ± 7.4	11.1	72.5 ± 4.8	7.7
		100	99.5 ± 2.5	2.5	95.9 ± 3.3	3.4
		150	100.5 ± 2.7	2.7	98.9 ± 11.5	11.6
7	Sulfamerazine	50	77.4 ± 1.8	2.3	73.5 ± 2.4	3.8
		100	86.2 ± 3.1	3.6	99.5 ± 1.6	1.6
		150	111.3 ± 1.6	1.4	108.3 ± 3.1	2.9
8	Sulfadimidine	50	83.8 ± 3.6	4.3	76.5 ± 5.6	8.3
		100	100.5 ± 4.6	4.6	97.6 ± 4.5	4.6
		150	80.0 ± 3.4	4.4	97.8 ± 8.0	8.2
9	sulfamethoxypyridazine	50	89.2 ± 8.1	9.1	73.5 ± 5.4	7.4
		100	115.6 ± 4.0	3.5	97.5 ± 3.3	3.4
		150	86.3 ± 3.3	3.8	96.7 ± 11.6	11.9
10	Sulfamethoxydiazine	50	84.5 ± 1.7	2.0	99.7 ± 6.1	6.1
		100	104.3 ± 6.1	5.8	93.9 ± 3.6	3.8
		150	84.2 ± 3.8	4.5	97.6 ± 4.2	4.3
11	Sulfachloropyridazine	50	74.7 ± 4.2	5.6	93.4 ± 5.1	5.4
		100	98.6 ± 4.1	4.1	101.4 ± 7.2	7.1
		150	104.6 ± 7.1	6.8	101.8 ± 5.6	5.5
12	Sulfamethoxazole	50	107.7 ± 5.5	5.1	101.0 ± 5.0	5.0
		100	89.7 ± 5.0	5.6	101.3 ± 5.4	5.4
		150	86.0 ± 5.4	6.3	98.3 ± 2.4	2.4
13	Sulfamonomethoxine	50	100.9 ± 2.7	2.7	107.8 ± 2.9	2.7
		100	95.6 ± 1.6	1.7	101.7 ± 1.7	1.7
		150	96.1 ± 3.8	4.0	99.4 ± 4.1	4.1
14	Sulfisoxazole	50	85.9 ± 4.7	5.5	73.3 ± 2.7	3.7
		100	95.8 ± 3.4	3.6	98.3 ± 3.5	3.6
		150	96.1 ± 8.2	8.6	71.1 ± 6.1	8.5
15	Sulfadoxine	50	93.6 ± 4.1	4.3	98.7 ± 4.3	4.3
		100	94.4 ± 3.4	3.5	94.4 ± 3.3	3.5
		150	95.6 ± 12.0	12.6	98.8 ± 12.3	12.4
16	Sulfaclozine	50	85.8 ± 6.2	7.2	101.0 ± 6.5	6.4
		100	93.9 ± 3.8	4.0	97.6 ± 3.9	4.0
		150	94.3 ± 4.3	4.5	98.6 ± 4.4	4.5
17	Sulfadimethoxine	50	88.5 ± 5.6	6.3	100.1 ± 5.7	5.7
		100	97.8 ± 7.3	7.5	100.8 ± 7.5	7.5
		150	95.8 ± 5.5	5.8	98.5 ± 5.6	5.7
18	Sulfaquinoxaline	50	87.6 ± 5.1	5.8	101.5 ± 5.3	5.2
		100	96.4 ± 5.4	5.6	102.4 ± 5.7	5.6
		150	95.1 ± 2.4	2.5	99.8 ± 2.5	2.5

optimized with 18 standards. Selected reaction monitoring (SRM) was used to measure the transitions from the protonated molecular ions to product ions that have been described in previous electrospray LC–MS/MS methods for these compounds [32,33]. Typically, a sulfonamide fragment loss, in positive-ion mode, is detected at m/z 156, which results from the cleavage of the S–N bond yielding the stable sulfanilamide moiety. This common fragment ion, arising from the biologically active part of the molecule, provides the best basis for the MS^2 analysis of the whole class of sulfonamides, although the optimal collision conditions for forming the m/z 156 fragment vary with the sulfonamide. Fig. 4 shows typical SRM chromatograms for confirmation of 18 SAs. Using these criteria, analytes were confirmed in animal tissues at spiked level 10 μg/kg. Since there are numerous matrixes, swine kidney was chosen as a typical one because it is the most complex matrix according to our previous findings. All of the SRM transitions met relative abundance criteria for these samples as shown in Table 5.

3.4.5. Stability

Stabilities of 18 analytes in different solutions as stock solutions, standard working solution (100 mg/l) and resulting extracts (blank sample was spiked at levels of 50 μg/kg and then extracted) were studied. Using the same calibration set, after comparing with the background noise in various matrices, the results demonstrated that, there were no interfering peaks that could be detected on the expected retention time for these target analytes (within 2.5%). Consequently, stock solutions were found to be stable for at least

Table 5
MS/MS parameters on the parent and quantitative daughter ion (m/z) and collision energy of 18 Sulfonamides.

Number	Drugs	Parent ion, m/z	Quantitative daughter ion, m/z (relative ratio)	Collision energy (eV)	Qualitative daughter ion, m/z (relative ratio)	Collision energy (eV)	RT (min)
1	Sulfamidinum	214.8	92.0 (100)	10	107.9 (61) 155.9 (56)	21	2.5
2	Sulfanilamide	173.0	91.9 (100)	13	108.1 (58) 156.0 (68)	26	2.8
3	Sulfisomidine	279.0	124.1 (100)	21 16	92.1 (57) 185.9 (40)	30	7.5
4	Sulfadiazine	251.0	92.2 (100)	14	108.1 (58) 155.9 (52)	26	8.8
5	Sulfapyridine	250.0	155.9 (100)	15	108.1 (63) 184.0 (59)	25	13.6
6	Sulfamethizol	270.9	156.0 (100)	24	92.1 (57) 108.1(48)	30	13.8
7	Sulfamerazine	265.0	156.1 (100)	17	109.9(69) 171.9(80)	29	14.3
8	Sulfadimidine	279.0	124.1 (100)	16	185.9 (61) 203.9 (55)	27	7.5
9	sulfamethoxypyridazine	281.0	156.1 (100)	17	92.0 (72) 108.1(60)	25	17.3
10	Sulfamethoxydiazine	281.0	156.0 (100)	17	108.0 (48) 215.0 (36)	27	17.3
11	Sulfachloropyridazine	285.0	156.0 (100)	16	92.2 (68) 108.1(58)	30	22.6
12	Sulfamethoxazole	254.0	156.0 (100)	16	92.1 (88) 108.1(79)	25	20.3
13	Sulfamonomethoxine	281.0	156.0 (100)	15	108.1 (49) 214.9 (31)	26	17.4
14	Sulfisoxazole	256.0	158.3 (100)	10	132.2 (71) 212.5 (63)	27	21.2
15	Sulfadoxine	311.0	156.0 (100)	17	92.1 (58) 108.1(49)	25	19.9
16	Sulfathiazole	256.0	92.0 (100)	13	108.0 (66) 156.1 (75)	25	13.8
17	Sulfadimethoxine	311.0	156.0 (100)	20	92.2 (75) 108.1(64)	30	19.9
18	Sulfaquinoxaline	301.0	156.0 (100)	16	92.1 (78) 108.1(64)	29	13.7

3 months in plastic tube at −20 °C, standard working solution for 2 week and resulting extracts for 1 week at 4 °C.

4. Conclusion

The described procedure provided rapid, accurate and sensitive methods for the determination of 18 SAs in animal edible tissues. For the extraction step, various solvents were investigated. The simple method reduced the time for sample pretreatment, improved the separation conditions, ensured higher throughput and met the requirement for SAs residue analysis. It also generates less hazardous waste and is friendly to the environment. There real sample analysis results demonstrated that the method was a reliable tool and could be applied to the testing of SAs in porcine liver for surveillance programs.

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