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Simultaneous Determination of Sulfonamides in Porcine and Chicken Muscle Using High Performance Liquid Chromatography with Ultraviolet Detector

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Abstract The present study used the liquid extraction pretreatment method and developed a liquid chromatography-ultraviolet detector (LC-UV) for the simultaneous determination of 14 sulfonamides (SAs) residues in porcine and chicken muscle. Linearity within a range of 50-150 μg/kg was obtained with the correlation coefficient (r^2) of 0.9673-0.9997. The mean recovery of SAs was 55.9-109.7% (relative standard deviations; RSDs 1.7-17.3%) in porcine muscle and 52.8-112.4% (RSDs 2.3-16.9%) in chicken muscle. The limits of detection (LODs) and limits of quantification (LOQs) were 2-32 and 7-96 μg/kg in porcine muscle, and 4-32 and 13-97 μg/kg in chicken muscle, respectively. These values were lower than the maximum residue limits (MRLs) established by the European Union. The sum of all SAs residues present should be less than 100 μg/kg.

Keywords: sulfonamide, porcine muscle, chicken muscle, liquid-liquid extraction (LLE), high performance liquid chromatography-ultraviolet detector (HPLC-UVD)

Introduction

The sulfonamides (SAs) are antimicrobial compounds commonly used as preventive and therapeutic agents for bacterial infective diseases in veterinary medicine (1). They inhibit multiplication of microorganisms by acting as competitive inhibitors of *p*-aminobenzoic acid in the folic acid metabolism cycle. However, the presence of residues of SAs in foodstuffs of animal origin is of wide concern due to their possible harmful effect on human health (2). Therefore, to ensure food safety, many countries within the European Union (EU) has established maximum residue limits (MRLs) for SAs from animal tissue according to which the sum of all SAs residues present should be less than 100 μg/kg (3). Thus, the analytical method for the monitoring of SAs residues in foodstuffs is required to be simple, rapid, sensitive, and capable of detecting residues below the MRLs.

A variety of extraction methods for SAs residues in animal food products, liquid-liquid extraction (LLE) (4), solid-phase extraction (SPE) (5,6), and matrix solid-phase dispersion (MSPD) extraction (7,8) have been reported. The analytical approaches for the multiresidue analysis of SAs were based on different analytical techniques, such as gas chromatography with mass spectrometry (GC-MS) detection (9), and high performance liquid chromatography with ultraviolet (HPLC-UV) detection (10,11), fluorescence (FL) (12,13), and MS detectors (3,14). LC-MS has been proved to be a very sensitive and accurate technique in foodstuffs of animal origin for SAs. However, those methods are not easily available in all analytical laboratories because they are expensive, thus not widely used.

Therefore, the purpose of this work is to develop a novel method for the determination of SAs residues in porcine and chicken muscle by HPLC with UV detection. The developed method was validated according to Food and Drugs Administration (FDA) guideline for bioanalytical assay procedures (15).

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Materials and Methods

Reagent and chemicals Sulfacetamide (SCM), sulfadiazine (SDZ), sulfathiazole (STZ), sulfapyridazine (SPD), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxypyridazine (SMPD), sulfachloropyridazine (SCP), sulfamethoxazole (SMXZ), sulfamonomethoxine (SMONO), sulfisoxazole (SIX), sulfabenzamide (SBZ), sulfadimethoxine (SDM), and sulfaquinoxalin (SQX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and sodium acetate and sodium phosphate monobasic were obtained from Sigma-Aldrich. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). All chemicals and solvents were used in HPLC grade.

Preparation of stock and working standard solutions Individual stock solutions (1 mg/mL) of SAs were prepared in MeOH. Mixed working solutions (10 μg/mL) were prepared by diluting the stock solutions in 200 mM sodium acetate (pH 3.5). The working solutions, used to spike the muscle samples or construct the calibration curves, were prepared by diluting the mixed solutions to a concentration of 50, 80, 100, and 150 ng/mL with 200 mM sodium acetate buffer (pH 3.5)/MeOH (1/1, v/v) on the day of the experiment. These solutions were stored at 4°C in brown volumetric flasks.

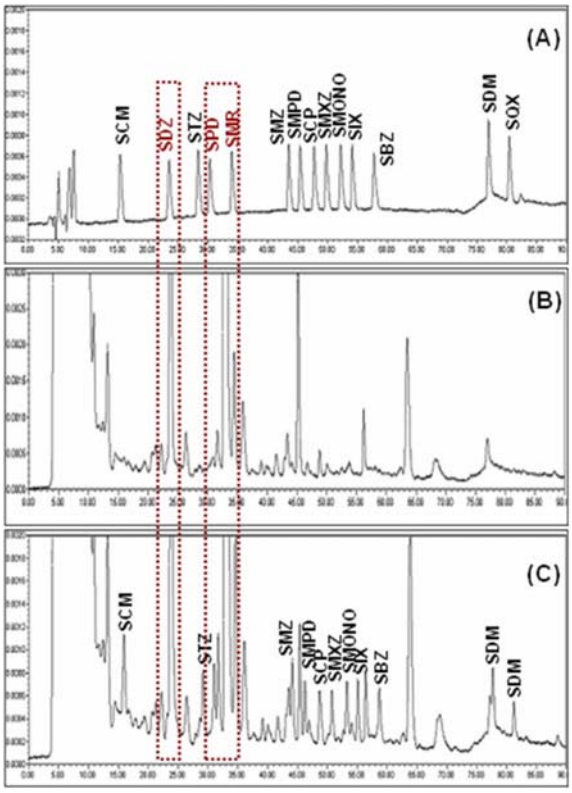

Fig. 1. HPLC chromatograms of (A) a standard mixture of the selected sulfoamides (SAs), (B) an unspiked porcine muscle, and (C) a spiked porcine muscle at 100 μg/kg of SAs.

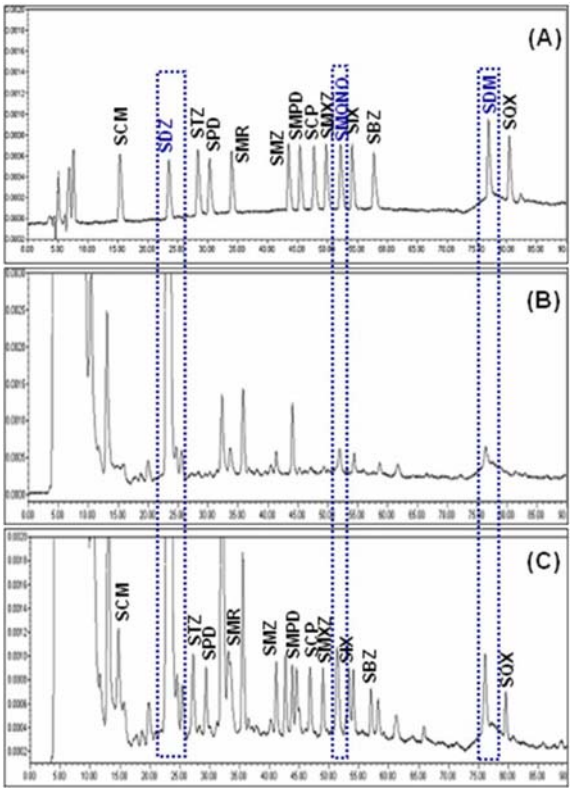

Fig. 2. HPLC chromatograms of (A) a standard mixture of the selected sulfoamides (SAs), (B) an unspiked chicken muscle, and (C) a spiked chicken muscle at 100 μg/kg of SAs.

Chromatographic conditions The HPLC system was consisted of a Waters 2690 (Waters Co., Miliford, MA, USA) separations module coupled with a Waters 486 UV detector. Chromatographic separation of the SAs was performed on a Union C18 column (250×3.0 mm, 5 μm) from Imatakt (Tokyo, Japan). The mobile phase consisted of 200 mM sodium acetate buffer (pH 3.5)/MeOH (9/1, v/v) (mobile phase A) and MeOH (mobile phase B) and was run at a flow rate of 0.3 mL/min with gradient program as follows; 10% B (0-10 min), 10-30% B (10-50 min), 30% B (15 min), 30-40% B (65-70 min), 40% B (15 min), 40-100% B (85-90 min), 100% B (10 min). After completing the chromatographic elution, the mobile phase was programmed to its initial condition within 5 min, and the 25 min reconditioning time was set before next injection. The injection volume was 10 μL, and the column temperature was maintained at 40°C, and the SAs were detected at 270 nm.

Sample extraction Minced by BÜCHI Mixer B-400 (BÜCHI, Flawil, Switzerland) muscle tissue (2 g) (boned rib of the pork and drumstick of the chicken) was placed in a 50-mL polypropylene centrifuge tube containing 2 g sodium phosphate monobasic and spiked with 2 mL of the working standard solutions. To perform the extraction process, 10 mL of ACN saturated with hexane was added and the tube was mixed for 10 sec, and then allowed to stand for 10 min before centrifugation at 3,000×g for 10 min. The supernatant was transferred into a 25-mL glass tube, and then 10 mL of MeOH was added to residue. The above extraction process was then repeated and supernatant was merged together. Then, the supernatant was defatted with 20 mL of *n*-hexane. The upper layers were removed, and 10 mL of lower layers were transferred into 25-mL glass tube and evaporated to dryness at 35°C under a stream of nitrogen purge. The residue was reconstituted in 1 mL of 200 mM sodium acetate buffer (pH 3.5)/MeOH (1/1, v/v). Finally, the sample was passed through a 0.45-μm polyvinylidene fluoride (PVDF) acrodisc filter (13-mm, Whatman, Maidstone, UK) before injection to the HPLC system.

Validation procedure After selection of the optimum conditions for the sample preparation step and the LC-UV detector (UVD) measurements, validation of method was performed according to FDA guideline for bioanalytical assay procedures (15).

The selectivity of the method was assessed by investigating the presence or absence of any interference during the same chromatographic run as the examined SAs, with the established method for porcine and chicken muscle.

Linearity was examined using a calibration curve, obtained by analyzing blank edible muscle spiked with SAs at 4 different concentrations (50, 80, 100, and 150 μg/kg), with 3 replicates/concentration. Linear regression analysis was performed using analyte peak area vs. analyte concentration.

The limit of detection (LOD) and the limit of quantification (LOQ) of the method as 3.3σ/S and as 10σ/S, respectively were based on the standard deviation (SD), which was y-

intercepts of regression analysis (σ) and the slope (S) of the calibration curve (16).

Precision and accuracy were assessed by analyzing spiked porcine and chicken muscle at concentration levels of 50, 80, and 100 μg/kg. The 3 independent analyses were conducted each day for repeatability tests (n=3, intra-day precision) at 3 concentration levels (50, 80, and 100 μg/kg). This was repeated for 3 consecutive days for the purposes of the reproducibility test (n=9, inter-day precision) at same concentration levels.

Results and Discussion

Optimization of the chromatographic procedure In several HPLC methods which were used before, potassium- or sodium phosphate as mobile phase buffer were used to separate the SAs from foodstuffs (17-19). But potassium- or sodium-phosphate buffer were caused to drift baseline followed by increase organic solvent at gradient elution. Moreover, these buffers were caused to block the line filter and cell of UVD in HPLC. Therefore potassium- and sodium-phosphate was substituted with sodium acetate to provide volatility of buffer. To get better separation of SAs, Union C18 column (250×3.0 mm i.d., 5 μm) was chosen, which has longer length and narrower inner diameter than that ordinary used. Also, the pH of the mobile phase was a critical factor in achieving the chromatographic separation of the SAs. From 10 to 100 mM sodium acetate aqueous solution-MeOH used as the mobile phase could lead to tailing peaks. For the best resolution of analytes, as in this study, 200 mM sodium acetate aqueous solution-MeOH was chosen as the mobile phase. At pH 3.5 baseline separations was achieved for 14 analytes. Typical HPLC chromatograms of a mixed standard of SAs, unspiked (blank) edible muscle, and spiked edible muscle are shown in Fig. 1 and 2, respectively. However SDZ, SPD, and SMR in porcine muscle and then the SDZ, SMONO, and SDM in chicken muscle were not found, respectively because interference overlaid retention time of the standard peak by LC-UVD.

Optimization of the sample preparation procedure Traditionally, SAs are extracted by treating the sample with an organic solvent, and clean-up in solid-phase by passage through disposable cartridges (6). But, this extraction procedure was produced low recoveries less than 57.1% and required expert skills. Therefore, in order to improve low recovery, we tried to find out an optimal LLE process. As the recommended method by other researcher, ACN combined with the defatting process with n-hexane was a more efficient solvent for extraction of SAs residue from animal muscle (20). Because it can give high recoveries of relative volatile SAs, such as SIX and SBZ, and the lipid interference could readily be removed from the extraction solution by introducing n-hexane. Also, the reconstituted solvent was chosen to 200 mM sodium acetate buffer/MeOH (1/1, v/v), because of increasing recoveries more than 8.7% of SIX and SBZ comparison with MeOH.

Table 1. Linearity and sensitivity data of the developed method for the determination of sulfonamides in spiked animal muscle

Analytes	Concentration range (μg/kg)	Slope	Intercept	r^2	Limit of detection (μg/kg)	Limit of quantification (μg/kg)
Porcine muscle						
Sulfacetamide	50-150	123.87±5.00	-698.69±527.63	0.9750	14	43
Sulfathiazole	50-150	114.51±11.60	1,011.32±409.16	0.9869	12	36
Sulfamethazine	50-150	189.71±22.10	-7,703.14±1814.85	0.9673	32	96
Sulfamethoxypyridazine	50-150	71.84±21.75	980.92±88.83	0.9832	4	12
Sulfachloropyridazine	50-150	106.46±5.83	-584.24±118.70	0.9717	4	11
Sulfamethoxazole	50-150	65.33±3.71	2,406.68±330.84	0.9941	17	51
Sulfamonomethoxine	50-150	80.52±8.76	1,452.87±57.61	0.9754	2	7
Sulfisoxazole	50-150	75.82±15.15	560.16±79.97	0.9791	4	11
Sulfabenzamide	50-150	104.85±16.63	-907.53±172.01	0.9815	5	16
Sulfadimethoxine	50-150	88.43±1.25	2,523.50±392.63	0.9897	15	44
Sulfaquinoxalin	50-150	47.04±6.60	1,132.51±59.33	0.9939	4	13
Chicken muscle						
Sulfacetamide	50-150	127.93±11.22	-364.23±262.76	0.9979	7	21
Sulfathiazole	50-150	131.17±9.69	-251.83±505.00	0.9997	13	39
Sulfapyridazine	50-150	94.10±12.71	950.64±396.04	0.9963	14	42
Sulfamerazine	50-150	120.67±11.52	-594.36±766.79	0.9953	21	64
Sulfamethazine	50-150	98.05±9.20	355.24±755.02	0.9982	25	77
Sulfamethoxypyridazine	50-150	84.71±4.26	58.93±337.45	0.9975	13	40
Sulfachloropyridazine	50-150	88.50±5.23	1,500.79±114.83	0.9928	4	13
Sulfamethoxazole	50-150	82.63±14.36	1,157.73±801.33	0.9853	32	97
Sulfisoxazole	50-150	90.62±8.95	257.25±323.56	0.9985	12	36
Sulfabenzamide	50-150	66.35±8.53	1,210.29±564.88	0.9961	28	85
Sulfaquinoxalin	50-150	47.88±1.90	826.89±260.82	0.9934	18	55

Consequently, compared to the SPE method (6), this LLE method not only greatly reduced the operation time and cost of pretreatment but increased the efficiency and recovery.

Method validation selectivity As is shown in Fig. 1 and 2, interferences made impossible to match SDZ, SPD, and SMR from porcine muscle and then the SDZ, SMONO, and SDM from chicken muscle all analytes of 14 SAs with only retention time by HPLC-UVD.

Linearity, LOD, and LOQ Calibration curves were obtained by least-squares linear regression analysis using the analyte peak area against analyte concentration.

All calibration data, as well as LOD and LOQ are presented in Table 1. Linearity is evaluated using concentration levels of 50, 80, 100, and 150 μg/kg. Regression analysis revealed a correlation coefficient (r^2) of between 0.9673 and 0.9941 for porcine muscle, and 0.9853-0.9997 for chicken muscle. The LODs of the various SAs extracted from porcine muscle ranged from 2 to 32 μg/kg, whereas in chicken muscle these values ranged from 4 to 32 μg/kg. The LOQs obtained from porcine and chicken muscles are below the MRLs established in the Council Regulation 2377/90 of the EU (21).

Precision and accuracy The precision based on intra-day repeatability was assessed by replicate (n=3) measurements from 3 spiked muscle at 3 different concentration levels of 50, 80, and 100 μg/kg. Statistical evaluation revealed relative standard deviations (RSDs %) at different values in the range of 1.7-15.2% for porcine muscle, and 2.3-12.4% for chicken muscle (Table 2). The inter-day precision was established using edible muscle samples at the same concentration range as above. A triplicate determination of each concentration over a period of 3 consecutive days was

Table 2. Intra-day (n=3) and inter-day (over a period of 3 consecutive days) precision and accuracy data for the determination of sulfonamides in spiked animal muscle

Analytes	Added (μg/kg)	Recovery (%)	Intra-day (RSD%)	Inter-day (RSD%)	Analytes	Added (μg/kg)	Recovery (%)	Intra-day (RSD%)	Inter-day (RSD%)
Porcine muscle					Chicken muscle				
Sulfacetamide	50	86.4	6.4	6.3	Sulfaetamide	50	89.9	9.7	11.7
	80	88.7	11.2	11.1		80	95.0	4.8	6.2
	100	109.7	6.4	7.4		100	92.0	2.8	9.5
Sulfathiazole	50	98.9	10.9	15.9	Sulfathiazole	50	100.1	4.4	6.6
	80	108.8	11.4	15.1		80	101.2	4.1	5.6
	100	96.3	12.7	13.1		100	101.7	3.5	5.7
Sulfamethazine	50	105.8	10.3	13.7	Sulfapyridazine	50	112.4	2.9	6.4
	80	93.8	15.2	16.1		80	100.2	2.8	5.5
	100	93.6	13.2	16.7		100	102.4	5.2	12.4
Sulfamethoxypyridazine	50	78.9	5.5	16.7	Sulfamerazine	50	98.3	6.3	6.2
	80	65.3	4.3	12.9		80	107.6	5.1	8.0
	100	71.3	6.8	7.2		100	102.2	4.6	5.7
Sulfachloropyridazine	50	66.2	10.6	13.3	Sulfamethazine	50	89.3	6.2	12.5
	80	102.3	10.7	14.4		80	85.1	2.7	7.8
	100	98.7	11.6	14.7		100	87.8	4.6	9.3
Sulfamethoxazole	50	83.2	9.7	17.2	Sulfamethoxypyridazine	50	82.6	12.4	16.9
	80	72.7	6.0	12.4		80	75.1	2.9	9.4
	100	74.1	9.5	15.8		100	80.6	2.8	6.6
Sulfamonomethoxine	50	88.3	3.5	13.8	Sulfachloropyridazine	50	111.2	4.1	15.4
	80	79.7	7.9	9.7		80	87.9	4.0	5.5
	100	75.1	4.0	12.7		100	90.7	5.1	9.8
Sulfisoxazole	50	95.0	6.6	17.3	Sulfamethoxazole	50	108.8	4.9	14.5
	80	71.1	8.1	14.5		80	79.6	4.8	6.0
	100	70.7	1.7	4.1		100	85.8	4.9	5.4
Sulfabenzamide	50	76.7	4.1	14.3	Sulfisoxazole	50	84.9	4.0	6.1
	80	83.7	7.0	15.7		80	81.5	3.5	9.4
	100	92.1	9.6	14.1		100	84.3	4.5	9.2
Sulfadimethoxine	50	109.7	4.1	4.4	Sulfabenzamide	50	80.0	9.2	9.8
	80	108.1	6.4	10.3		80	72.6	2.3	4.1
	100	107.4	2.7	12.1		100	73.8	4.1	7.3
Sulfaquinoxalin	50	62.4	6.0	12.4	Sulfaquinoxalin	50	60.6	7.7	9.9
	80	63.6	4.4	7.2		80	52.8	8.1	8.4
	100	55.9	7.6	9.8		100	54.3	5.7	9.4

followed by same experimental procedures. RSD values for all the examined SAs were reported between 4.1 and 17.3% for porcine muscle, and 4.1 and 16.9% for chicken muscle.

The measurements for the recovery were performed on spiked samples of porcine and chicken muscle tissues at the concentrations of 50, 80, and 100 μg/kg. The recoveries for porcine muscle varied between 65.3 and 109.7%, and for chicken muscle between 72.6 and 112.4% except SQX (Table 2). The mean precision and accuracy for the limits value was found to be less than maximum tolerable RSDs of 20% under 100 μg/kg concentration and recovery was within 60-110% at the same concentration in accordance with FDA guidance (22) except SQX.

There is a variety of methods using HPLC-UVD to analyze SAs. These methods using HPLC-UVD had high sensitivity and good repeatability. But HPLC-UV methods had disadvantage of poor recoveries and poor selectivity. Some HPLC-UV method used SPE for clean-up step and used 10g animal tissue for test sample to analyze SAs (6). That method had high sensitivity and good purity. But it was reason of poor recoveries to use SPE for clean-up too many interference compounds in animal tissue. In this work, we used 2 g sufficiently homogenized animal muscle tissue and then used LLE for clean-up interferences. That makes possible to develop an analytical HPLC-UV methodology with a better recoveries, high sensitivity, and sufficient repeatability. However, at a point of view for purity, this method needs improvements to separate and quantitate 14 SAs in animal tissues.

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