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Development of an Immunoaffinity Chromatography Purification and Ultra Performance Liquid Chromatography Tandem Mass Spectrometry Method for Determination of 12 Sulfonamides in Beef and Milk

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ABSTRACT: A highly selective and sensitive method was developed for the simultaneous determination of 12 sulfonamides in beef and milk by immunoaffinity chromatography purification coupled to ultra performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS). The MS/MS conditions, UPLC mobile phase, injection solution, sample purification process, and matrix effect were studied to optimize the operating conditions. The limits of detection (LODs) of the instrument for the studied sulfonamides ranged from 0.4 to 2.0 μg L^{-1}, being 1.6−8.0 μg kg^{-1} for beef and 1.8−6.4 μg kg^{-1} for milk. The standard solution was diluted with blank beef or milk matrix for the construction of calibration curves, which had a linear range from 10 to 200 μg kg^{-1} and regression coefficients higher than 0.990 (n = 10) for all the studied sulfonamides. Samples spiked at 10, 20, and 100 μg kg^{-1} showed recoveries above 70% and relative standard deviations below 10%.

KEYWORDS: immunoaffinity chromatography (IAC), ultra performance liquid chromatography (UPLC), mass spectrometry (MS), sulfonamide, beef, milk

INTRODUCTION

Sulfonamides (SAs) are among the most widely used veterinary drugs and are used frequently to prevent and treat bacterial infections because of their broad-spectrum antimicrobial activity, effectiveness as growth promoters in livestock, and low cost.[1] Because of excessive and long-term use of antimicrobial substances in animals, food of animal origin can contain residues of these compounds. Human consumption of these contaminated foods can trigger serious health problems, such as allergic or toxic reactions in hypersensitive individuals.[2] Some sulfonamides are also potentially carcinogenic.[3] To protect consumer health, the European Union (EU) has established a maximum residue limit (MRL) of 100 μg kg^{-1} for the total SA concentration in food of animal origin.[4]

Currently, several techniques are used for the analysis of SAs in milk and other matrices, including thin-layer chromatography,[5] immunochemical enzyme-linked immunosorbent assay,[6,7] capilary electrophoresis,[8] gas chromatography,[9] and liquid chromatography coupled with mass spectrometry (MS),[10−12] ultraviolet−visible,[13,14] or fluorescence[15,16] detection. Recently, ultra performance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS)[17−20] has become the preferred method for the detection of veterinary drug residues because of its high sensitivity and specificity. Although these methods are well proven and widely accepted, a quick and widely applicable sample treatment method is not available.

The extraction and cleanup steps are important for the quantitative analysis of SAs. Various sample treatment procedures have been developed for the determination of SAs in foods of animal origin. The main extraction and cleanup techniques currently used for SAs are liquid−liquid extraction,[21,22] solid−liquid extraction,[23] and solid phase extraction.[24−26] Research on the development of immunochemistry for food monitoring was reported.[27] Compared to above sample treatment methods, immunoaffinity chromatography (IAC)[28,29] provides better cleanup of the samples and has higher selectivity. IAC can also simplify the sample treatment process, and a smaller volume of toxic solvent is needed than in the other sample treatment techniques.

The main objective of this work was to develop an IAC and UPLC−MS/MS procedure for the determination of multiple sulfonamides in beef and milk. This method would have a low limit of detection (LOD) and a reduced requirement for toxic solvents compared to the other methods currently available.

MATERIALS AND METHODS

Chemicals and Reagents. Sulfadiazine (SD, 99.5%, CAS: 68-35-9), sulfadimethoxine (SDM, 99.0%, CAS: 122-11-2), sulfamethoxazole (SMZ, 99.0%, CAS: 723-46-6), sulfachloropyridazine (SCP, 99.0%, CAS: 80-32-0), sulfamethazine (SM2, 99.0%, CAS: 57-68-1), sulfaquinoxaline (SQ, 98.0%, CAS: 59-40-5), sulfisoxazole (SIZ, 98.7%, CAS: 127-69-5), and sulfamethoxydiazine (SMD, 99.0%, CAS: 651-06-9) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Sulfapyridine (SPD, no purity information available, CAS: 144-83-2), sulfathiazole (ST, no purity information available, CAS: 72-14-0),

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sulfamerazine (SM1, 99.0%, CAS: 127-79-7), and sulfamonomethoxine (SMM, no purity information available, CAS: 1220-83-3) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (HPLC grade) was purchased from Honeywell Burdick and Jackson (Ulsan, Korea). Ethanol (analytical grade), $Na_2HPO_4 \cdot 12H_2O$ (analytical grade), potassium chloride (analytical grade), KH_2PO_4 (analytical grade), sodium chloride (analytical grade), formic acid (HPLC grade), and Tween-20 (analytical grade) were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Distilled water and ultrapure water were purified using a Milli-Q apparatus (Millipore Direct-Q UV, Bedford, MA).

Table 1. Retention Times (n = 10) and Acquisition MRM Parameters of the Sulfonamides

compound	retention time ± SD[a] (min)	precursor/ production (m/z)	cone voltage (V)	collision energy (eV)
SD	4.04 ± 0.02	251.0/92.0[b]	34	30
		251.0/107.8	34	25
SPD	5.34 ± 0.03	250.2/92.1[b]	30	30
		250.2/108.0	30	28
SMZ	9.26 ± 0.03	254.0/92.0[b]	35	25
		254.0/108.0	35	35
ST	4.89 ± 0.03	256.1/156.0[b]	35	15
		256.1/92.0	35	35
SM1	5.83 ± 0.04	265.0/108.0[b]	32	32
		265.0/91.7	32	30
SIZ	10.43 ± 0.02	268.3/156.0[b]	24	14
		268.3/92.0	24	34
SM2	7.66 ± 0.04	279.0/92.0[b]	40	45
		279.0/65.0	40	52
SMM	9.42 ± 0.03	281.0/91.9[b]	40	35
		281.0/156.0	40	20
SMD	7.13 ± 0.03	281.2/91.9[b]	40	30
		281.2/107.9	40	30
SCP	8.75 ± 0.04	285.0/156.0[b]	32	18
		285.0/91.7	32	32
SQ	14.76 ± 0.02	301.0/156.0[b]	36	20
		301.0/108.1	36	26
SDM	13.88 ± 0.01	311.2/155.9[b]	36	26
		311.2/107.8	36	40

[a] Standard deviation, not to be confused with compound SD. [b] Quantitative ion.

Individual stock standard solutions for each sulfonamide (1000 mg L^{-1}) were prepared in methanol. A multicomponent solution containing all the compounds was prepared using the individual standard solutions, diluted with methanol to a final concentration of 10 mg L^{-1}. These solutions were stored at 4 °C. Phosphate buffer solution (pH = 7.4) was prepared using 0.20 g of potassium chloride, 0.24 g of KH_2PO_4, 2.90 g of $Na_2HPO_4 \cdot 12H_2O$, and 8.00 g of sodium chloride in 1 L of ultrapure water.

Apparatus. Immunoaffinity columns for sulfonamides (IAC-SULF, Clover Technology Group Inc., Beijing, China) were used for sample purification and stored at 4 °C; the type of columns was IAC305, the volume was 1 mL, and the immunoaffinity columns were made of the simultaneously covalent connection of 12 monoclonal antibodies (all of the studied sulfonamides) and activated Sepharose 4B. Glass microfiber filter paper was obtained form Clover Technology Group Inc. (Beijing, China). A Waters ACQUITY UPLC coupled with a TQ Detector MS was used for the analysis. A S 900H Elmasonic (Elma, Germany) was used for ultrasonic extraction. An Eppendorf centrifuge 5801R (Hamburg, Germany), an 18780 Reacti-Vap nitrogen evaporator (Thermo Scientific, Rockford, IL), and a Barnstead Vortex Maxi Mix II (Dubuque, IA) were used.

Samples. Twenty raw beef loin samples and 20 full milk samples used for method development and validation were collected from local markets, and they were homogenized and stored in a refrigerator at 4 °C.

Immunoaffinity Chromatography (IAC) Method. A portion of the homogenized sample (5.000 g) was accurately weighed and placed in a 50 mL centrifuge tube. For recovery tests, the blank samples (5.000 g) were spiked with standard analytes at three concentration levels (10, 20, and 100 μg kg^{-1}) and stabilized for 30 min. Twenty milliliters of the extraction solvent (ultrapure water/ethanol, 20:80 v/v) was added, and then the homogenate was vortexed for 3 min and ultrasonicated for 30 min. After centrifugation at 3000 rpm for 5 min, the clear supernatant (5 mL) was diluted to 50 mL in a volumetric flask with

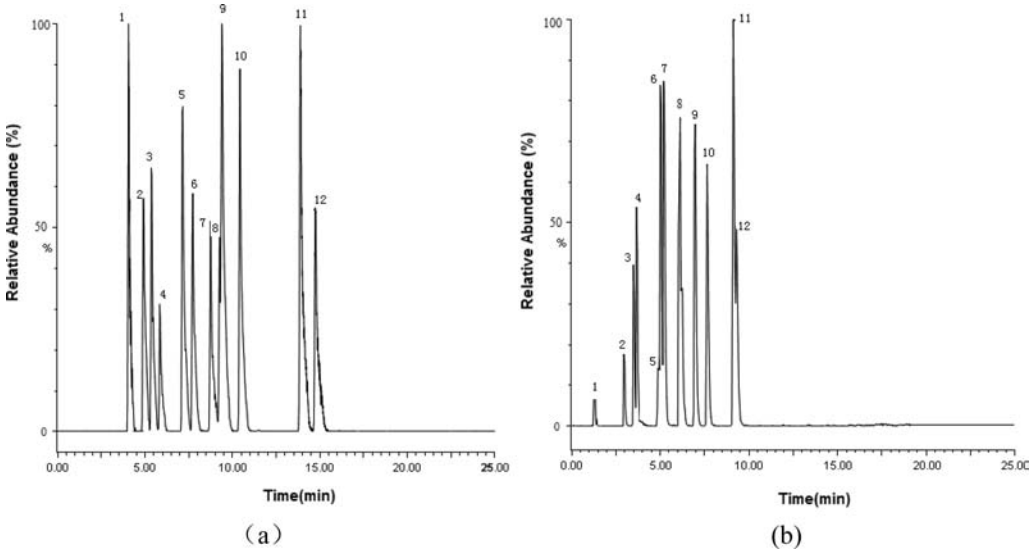

Figure 1. UPLC–MS total-ion chromatogram of the standard mixture of sulfonamides at 100 μg L^{-1} with different mobile phases: (a) methanol/ultrapure water containing 0.1% formic acid and (b) acetonitrile/ultrapure water containing 0.1% formic acid (flow rate: 0.25 mL min^{-1}; analytes: 1 SD, 2 ST, 3 SPD, 4 SM1, 5 SMD, 6 SM2, 7 SCP, 8 SMZ, 9 SMM, 10 SIZ, 11 SDM, 12 SQ).

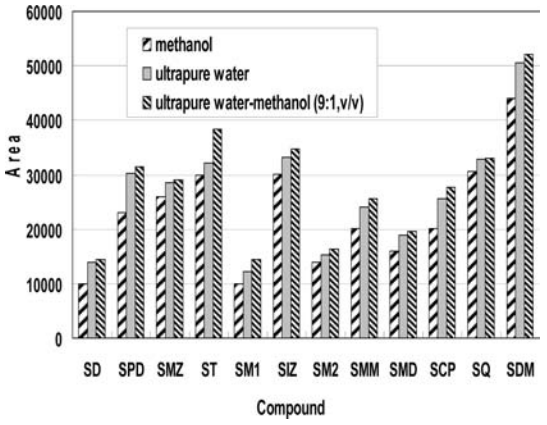

Figure 2. UPLC−MS/MS quantitative ion peak areas with different injection solutions (mobile phases: ultrapure water containing 0.1% formic acid (v/v) and methanol; flow rate: 0.25 mL min^{-1}).

ultrapure water. The mixture (50 mL) was filtered through a glass microfiber filter paper and collected in a clean beaker. Twenty milliliters of the filtrate was passed through the IAC-SULF at a rate of about 1 drop/s until air came through the column. Ten milliliters of phosphate buffer solution containing 0.1% (v/v) Tween-20 were passed through the column, and it was then washed with 10 mL of ultrapure water at a rate of about 1−2 drops/s until air came through the column. This column effluent was discarded. The sample was eluted from the column at a rate of 1 drop/s by passing 2 mL of methanol through the column and collecting all of the sample eluate in a cuvette. The sample eluate was then evaporated to dryness in a 50 °C water bath under a gentle flow of nitrogen, and the residue was reconstituted with 500 μL of injection solution (ultrapure water/methanol (9:1, v/v)). After mixing thoroughly, the reconstituted residue was filtered through a 0.22 μm nylon filter membrane for analysis by UPLC−MS/MS.

UPLC−MS/MS Conditions. The UPLC separation was performed on an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm I.D., 1.7 μm particle size) at 40 °C. The injection volume was 10 μL. Ultrapure water containing 0.1% formic acid (v/v) and methanol were used as the mobile phases, and the flow rate was held at 0.25 mL min^{-1} throughout the analysis. The solvent gradient was increased linearly from 5 to 50% methanol in 22 min, returned to 5% methanol in 1 min, and held at this level for 2 min so that the system could re-equilibrate before the next injection.

The MS was operated in positive electrospray ionization (ESI) mode $[M + H]^+$, and the data were collected in multiple reaction monitoring (MRM) mode. The optimized ESI parameters were as follows: capillary voltage 3.5 kV, source temperature 125 °C, and a desolvation gas (N_2) flow rate of 800 L h^{-1} at 400 °C. Nitrogen (99.99% purity) was supplied by an N_2 LC−MS nitrogen generator (Taylor-Wharton Gas Equipment Ltd., Malaysia) and was used as the cone gas at a flow rate of 50 L h^{-1}. Argon (≥99.999% purity; Beijing Yanan Gas Co., Ltd., Beijing, China) was used as the collision gas.

RESULTS AND DISCUSSION

Selection of Detected Ions for MS/MS. The optimum instrument conditions for the MRM transitions, individual cone voltage, and collision energy for each sulfonamide were established using a 100 μg L^{-1} standard solution and the combined injection mode, with a mobile phase of methanol/ultrapure water containing 0.1% formic acid (5:95, v/v). The optimized parameters are shown in Table 1. Two MRM transitions were used to determine the most abundant product ions. The most sensitive transition was selected for quantification, and the other transition was used for confirmation.

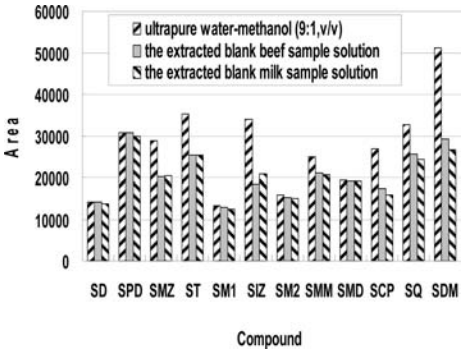

Figure 3. Matrix effect with different injection solutions (mobile phases: ultrapure water containing 0.1% formic acid (v/v) and methanol; flow rate: 0.25 mL min^{-1}).

Table 2. Recoveries for Repeatedly Utilizing (5 Times) the IAC-SULF

	recovery (%) (spiked at 100 μg kg^{-1})				
compounds	first time	second time	third time	fourth time	fifth time
SD	103	101	97	63	27
SPD	99	97	90	53	30
SMZ	98	96	94	51	32
ST	107	105	102	40	18
SM1	104	104	98	60	29
SIZ	92	91	88	43	21
SM2	104	107	98	48	32
SMM	96	92	93	38	10
SMD	85	87	81	20	7
SCP	103	101	98	42	13
SQ	103	99	98	32	10
SDM	91	90	82	43	10

Mobile Phase Selection for UPLC−MS/MS. Methanol/ultrapure water containing 0.1% formic acid and acetonitrile/ultrapure water containing 0.1% formic acid were compared as the mobile phase. When methanol/ultrapure water containing 0.1% formic acid was used as the mobile phase, the quantitative and qualitative ion peak responses were higher and the sulfonamides were separated better than when using the acetonitrile/ultrapure water containing 0.1% formic acid as the mobile phase. Therefore, methanol/ultrapure water containing 0.1% formic acid was selected as the mobile phase. The total-ion chromatogram of the standard mixture of sulfonamides at 100 μg L^{-1} is shown in Figure 1.

Selection of Injection Solution. Methanol, ultrapure water, and ultrapure water/methanol (9:1, v/v) were compared as injection solutions for diluting the working standard mixture of sulfonamides (100 μg L^{-1}) for detection by UPLC−MS/MS. As shown in Figure 2, when ultrapure water/methanol (9:1, v/v) was used as the injection solution, the peak areas for the quantitative ion were larger than with the other solvents. Therefore, a mixture of ultrapure water/methanol (9:1, v/v) was selected as the injection solution.

Table 3. Limits of Detection (LOD) and Limits of Quantification (LOQ) for the 12 Sulfonamides Studied[a]

	instrument		matrix (beef)		matrix (milk)	
compounds	LOD (μg L^{-1})	LOQ (μg L^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
SD	0.5	1.2	5.8	10.0	5.0	9.5
SPD	0.5	1.0	2.0	5.8	4.7	9.0
SMZ	0.4	1.0	2.2	5.8	5.2	10.0
ST	0.5	1.0	5.0	9.5	2.0	5.0
SM1	0.5	1.0	3.0	9.0	3.0	7.0
SIZ	0.4	1.0	1.6	4.5	1.8	4.0
SM2	2.0	5.0	4.0	9.8	6.0	10.0
SMM	1.0	3.0	5.0	9.8	3.2	8.6
SMD	2.0	3.0	7.5	10.0	6.4	10.0
SCP	1.0	2.8	5.2	9.8	4.7	9.0
SQ	0.5	1.0	2.8	8.0	3.0	8.0
SDM	0.5	0.8	8.0	10.0	6.4	10.0

[a] The determinations are based on 20 samples.

Table 4. Linear Regression Parameters (Linear Range 10−200 μg kg^{-1}, n = 10) of All the Studied Sulfonamides

	beef			milk		
	linear parameters			linear parameters		
compounds	slope (RSD/%)	y-intercept (RSD/%)	regression coefficient / R^2	slope (RSD/%)	y-intercept (RSD/%)	regression coefficient / R^2
SD	106.36(8.2)	8.96(3.4)	0.993	109.70(4.4)	9.77(6.1)	0.995
SPD	415.27(7.9)	284.09(6.1)	0.994	201.67(7.8)	90.87(8.4)	0.992
SMZ	245.33(5.5)	20.96(5.0)	0.997	139.69(3.5)	309.77(9.2)	0.991
ST	329.12(7.2)	63.40(6.5)	0.994	177.50(6.3)	175.01(4.7)	0.997
SM1	128.67(5.3)	95.10(9.1)	0.993	113.51(8.8)	27.86(7.0)	0.993
SIZ	467.79(6.4)	55.73(7.0)	0.996	236.29(2.9)	34.19(6.0)	0.996
SM2	204.58(3.7)	81.68(8.1)	0.997	248.98(5.1)	39.25(1.7)	0.995
SMM	305.29(7.2)	72.86(5.8)	0.998	257.15(3.3)	19.02(6.9)	0.996
SMD	205.70(6.9)	91.58(6.3)	0.991	112.16(7.9)	194.49(8.0)	0.993
SCP	187.80(2.8)	100.74(5.9)	0.995	102.72(4.8)	179.67(9.2)	0.990
SQ	332.79(7.5)	46.53(5.8)	0.995	175.55(5.0)	107.16(1.4)	0.990
SDM	420.50(9.0)	18.14(2.8)	0.992	153.54(8.5)	373.11(2.7)	0.993

Optimization of the Purification Process. The sample purification process was initially performed as described (Immunoaffinity Chromatography (IAC) Method), but without the 10 mL of phosphate buffer solution containing 0.1% (v/v) Tween-20. Using this method, SIZ, SCP, and SQ were not detected. When 10 mL of phosphate buffer solution containing 0.1% (v/v) Tween-20 was used to flush the column, peaks for SIZ, SCP, and SQ appeared. This indicated that the addition of 10 mL of phosphate buffer solution containing 0.1% (v/v) Tween-20 was required for optimal sample purification.

Effect of the Matrix. One significant drawback of electrospray mass spectrometry is that the ionization source is highly susceptible to the matrix effect, which can cause a signal decrease or an enhancement of the analyte. The effect of the matrix was investigated by comparing the results for the standard mixture of sulfonamides (100 μg L^{-1}) dissolved in ultrapure water/methanol (9:1, v/v) and for the extracted blank sample solution. The peak areas obtained are shown in Figure 3. The matrix decreased the signals of SMZ, ST, SIZ, SMM, SCP, SQ, and SDM, but it did not decrease the signals of the other sulfonamides. In addition, beef and milk had different matrix effects. Therefore, the standard curve solutions were prepared in the extracted blank solution of beef sample or milk sample to compensate for interference of the matrix and to ensure the accuracy of qualitative and quantitative analysis.

Stability of the Studied Chemicals. Insufficient stability of the calibration standard in solution may produce significant deviations in the results. Standard solution (100 μg L^{-1}) was prepared from stock standard solution (10 mg L^{-1}) by dilution with ultrapure water/methanol (9:1, v/v) and analyzed by UPLC−MS/MS over 15 days. All the sulfonamides studied in this work were stable.

Study on IAC-SULF Regeneration. To reduce the cost of this protocol, the regeneration of the IAC-SULF was investigated. IAC-SULF was used five times in repeat experiments by spiking the blank sample with a standard mixture of sulfonamides at 100 μg kg^{-1}. In each experiment, the column was eluted with 2 mL of methanol, and then 10 mL of ultrapure water was passed through the column at a rate of 3−4 drops/s, which was followed by 10 mL of phosphate buffer solution at a rate of about 1 drop/s.

Table 5. Mean Recovery and Precision (RSD) in the Three Fortified Levels (n = 6) for Beef and Milk

	mean recovery/% (RSD/%)					
	fortified concentration 10 μg kg^{-1}		fortified concentration 20 μg kg^{-1}		fortified concentration 100 μg kg^{-1}	
compounds	beef	milk	beef	milk	beef	milk
SD	87(8.7)	82(5.0)	101(2.8)	93(2.3)	104(2.8)	95(5.4)
SPD	80(6.3)	72(2.0)	87(9.3)	73(2.0)	100(4.6)	85(4.5)
SMZ	82(8.7)	72(3.5)	86(9.9)	71(4.2)	91(6.5)	87(4.9)
ST	98(8.9)	82(3.0)	98(3.6)	100(4.3)	105(3.6)	106(1.4)
SM1	99(7.8)	71(6.5)	104(5.2)	80(7.6)	103(5.5)	88(2.2)
SIZ	71(3.6)	75(6.5)	73(5.0)	82(9.7)	87(2.6)	90(2.3)
SM2	101(7.9)	75(6.7)	107(1.5)	88(3.9)	104(3.2)	92(4.7)
SMM	70(3.8)	74(6.1)	72(2.5)	88(2.8)	82(1.2)	92(6.2)
SMD	71(5.7)	75(6.6)	76(5.4)	80(6.4)	84(2.4)	86(2.9)
SCP	93(9.1)	71(5.8)	106(3.4)	72(2.8)	101(5.3)	86(3.8)
SQ	103(4.6)	91(2.0)	99(5.3)	92(6.7)	102(1.6)	101(7.0)
SDM	73(4.7)	73(6.7)	76(3.7)	83(5.5)	85(4.5)	91(5.7)

After the last 1 mL of phosphate buffer solution had permeated through the column, the ends of the IAC-SULF were sealed. It was then used again after storage at 4 °C in a refrigerator for 2 h. The recoveries obtained are shown in Table 2. These results show that IAC-SULF can be used repeatedly up to three times. Further detailed studies are required on IAC-SULF regeneration.

Method Validation. *Limit of Detection (LOD) and Limit of Quantitation (LOQ).* The LOD and LOQ of the instrument were determined as the concentrations giving responses 3 (LOD) and 10 (LOQ) times the average baseline noise with 20 duplicate injections of the stock standard solution. The LODs of the instrument for the studied sulfonamides ranged from 0.4 to 2.0 μg L^{-1}, and the LOQs of the instrument ranged from 0.8 to 5.0 μg L^{-1}. The LOD and LOQ of the method were estimated based on the concentrations giving responses 3 (LOD) and 10 (LOQ) times the average baseline noise with injections of the spiked samples. The LODs of the method for the studied sulfonamides ranged from 1.6 to 8.0 μg kg^{-1} for beef and 1.8 to 6.4 μg kg^{-1} for milk, and the LOQs of the method ranged from 4.5 to 10.0 μg kg^{-1} for beef and 4.0 to 10.0 μg kg^{-1} for milk. These data are summarized in Table 3. The values obtained in this study are much lower than in other reports.[2,30]

Linearity. The calibration curves for the quantification were obtained by spiking the extracted solution of blank beef or milk sample with five different concentrations (10, 20, 50, 100, and 200 μg kg^{-1}) of analytes. Low RSDs (<10%) of y-intercept and slope of calibration curves indicated good stability of the calibration standards in the matrix. All analyte responses were linear over the concentration range investigated, and their regression coefficients were $R^2 > 0.99$ in all cases (Table 4).

Repeatability of the Method. The validation of the method was performed after optimizing the factors affecting the analysis procedure. The repeatability of the method was investigated by spiking the blank beef sample (5.000 g) or the blank milk sample (5.000 g) with standard analytes at three concentration levels (10, 20, and 100 μg kg^{-1}) in six replicates. The recoveries for all the studied sulfonamides were between 70 and 107%, with relative standard deviations between 1.2 and 9.9% (Table 5). All of these results indicate that the developed method performed well.

Application to Real Samples. The developed method was applied to analyze 20 beef samples (6 loins, 6 ribs, and 8 tendons) and 30 milk samples (18 full milk samples and 12 skim milk samples) bought in local supermarkets in Taiyuan (Shanxi, China) for the sulfonamides. No sulfonamides were detected in these samples.

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