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Determination of sulfonamides in milk by ID-LC-MS/MS

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Abstract: In this study, an ID-LC-MS method was developed and validated for the determination of 14 sulfonamides in milk samples. Recovery results were found to be in the range of 91%-114% for milk samples. The relative measurement uncertainty was between 7.5%- 12.7%. Validated method was performed on milk samples obtained from market and street vendors. The amount of sulfonamides in the analyzed samples was found to be below the legal limits (Sulfamethazine: 6.46±0.76 ng/g and sulfisoxazole: 7.3±0.71 ng/g).

Keywords: Sulfonamides; isotope dilution; LC-MS/MS, method validation; uncertainty estimation.

1. Introduction

Antibacterial sulfonamides (SAs), in general called as ‘‘sulfa drugs’’, are a group of synthetic antibacterial agents that comprise sulfonamide group and widely used in veterinary practice for the treatment of infections and growth of animals that produce food. Due to their low cost, low toxicity, and excellent antibacterial properties against common bacterial diseases make them popular agents [1]. However, uncontrolled use of veterinary drugs and noncompliance with the withdrawal period pave the way for drug residues to remain in animal tissues and transfer into their milk [2–4]. Sulfonamide residues in food is an important issue as they possess a potential risk toward human health by inducing drug-resistant pathogenic strains of bacteria, toxicity and allergic reactions [2,4–6]. Thus, European Union Commission Regulation 37/2010/EEC and Republic of Turkey, Ministry of Food, Agriculture and Livestock announcement No 2011/20 have set the maximum residue limits (MRLs) for total SAs in milk as 100 µg/ kg [7,8].

The methods reported in the literature for the determination of sulfonamide residues comprise enzyme-linked immunosorbent assay (ELISA) [9,10], capillary electrophoresis [11,12], high performance liquid chromatography (HPLC) coupled with diode array detection (DAD)/ florescence detection (FLD) [13–16], gas chromatography-mass spectrometry (GC-MS) [17,18], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [2,19–21] and liquid chromatography-high resolution mass spectrometry (LC-HRMS) [20,22]. In order to fulfill the requirements of food safety regulations, methods developed to determine sulfonamide residues in milk should be sensitive, selective and capable of detecting the residues below the maximum residue limits (MRL). Microbiological assays are quick, inexpensive, selective but

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lacking of structural information that might produce false positive results [2]. LC-MS/MS has become most appropriate method for the residue analysis with its high selectivity, sensitivity, decisiveness and its applicability to determine the polar and/or non-volatile compounds without derivatization, including both electrospray and atmospheric pressure chemical ionization methods [18].

In recent years, although determination of multiclass veterinary drugs in one method has become more popular, they are compromising from the accuracy of the analytes. This study is focused especially on isotope dilution-liquid chromatography mass spectrometry (ID-LCMS) technique because of its high performance on accuracy and repeatability. In IDMS technique, isotopically labelled analogues of all compounds are added to the sample before the extraction processes and allowed to reach equilibrium without any loss or isotopic fractionation. It eliminates all errors at all stages of the method. IDMS technique traceable to International System of Units is a primary method and it is an acceptable alternative to estimate "true value" in the absence of a certified reference material (CRM) [23,24]. IDMS provides high accuracy and precision compared with internal standard addition or external calibration techniques [24].

None of the methods in the literature reported a full IDMS technique for the determination of sulfonamide residues in milk. The available methods provide only 1-3 isotopically labelled sulfonamides to be used as internal standards [25–32]. This study describes the development and validation of a full IDMS technique for all 14 sulfonamides in milk. In order to ensure the reliability of the developed method, recovery, accuracy, precision, LOD and LOQ, intraday and interday repeatability, linearity, robustness were estimated based on the analyses of spiked milk samples. The measurement uncertainty was evaluated by using "top-down approach" as described by EURACHEM CITAC Guide [33] and ISO 21748:2017 [34].

Milk comprises rich proteins and lipids, which make it a complex matrix for extraction and clean up to determine veterinary drugs in it. Different extraction methods have been proposed in the literature for sample preparation and clean-up for the determination of sulfonamides, such as liquid–liquid extraction (LLE) [25], solid-phase extraction (SPE) [5], solid-phase microextraction (SPME) [35], molecularly imprinted polymer extraction [36], pressurized liquid extraction (PLE) [37], QuEChERS [38], cloud point extraction (CPE) [39] and dispersive micro solid-phase extraction [40]. In this study a liquid-liquid extraction was employed, which greatly reduced the operation time and cost. The sulfonamides were extracted from milk samples using acetonitrile:ethyl acetate (6:4) solvent mixture. It was stirred employing a vortex, after which the mixture was centrifuged to separate proteins. The upper layer was separated by pipette and the solvent was evaporated by applying a gentle stream of nitrogen. The residue was vortexed after addition of hexane to remove fat content, after which sulfonamides were transferred into a 10% methanol/water mixture. An aliquot from bottom part (methanol:water) was analyzed by LC-MS/MS.

2. Experimental

2.1. *Chemicals and Reagents*

Acetonitrile, ethyl acetate, methanol and n-hexane were LC or GC grade and supplied from Merck (USA). Formic acid was purchased from Fluka (USA). Ultrapure water was generated by ELGA water purification system. Sulfathiazole (STZ), sulfapyridine (SPD), sulfamethazine (SMZ), sulfamerazin (SMR), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfamethoxazole (SMX), sulfadoxine (SDX), sulfisoxazole (SSX), sulfaphenazole (SPA), sulfaquinoxaline (SQX), sulfachloropyridazine (SCP), sulfamethizole (SME), sulfamethoxypyridazine (SMP), sulfamethoxypyridazine-d_3, sulfamethizole-(phenyl-$^{13}C_6$), sulfadoxine-d_3, sulfaquinoxaline-(phenyl-$^{13}C_6$), sulfaphenazole-(phenyl-$^{13}C_6$), sulfapyridine-(phenyl-$^{13}C_6$) and sulfadimethoxine-(phenyl-$^{13}C_6$) were purchased from Sigma Aldrich. Sulfathiazole-d_4, sulfamethazine-d_4, sulfamerazine-d_4, sulfadiazine-d_4, sulfamethoxazole-d_4, sulfisoxazole-d_4 and sulfachloropyridazine-d_4 were synthesized in TUBITAK UME Organic Chemistry Laboratory and characterized by NMR and HPLC/DAD.

Stock solutions of single analytes were prepared gravimetrically at a concentration of 1000 ng/g in methanol at Mettler Toledo XP205 balance (d:0.01 mg) and stored at 4 ºC. Mixed standard solutions were prepared by dilution of stock solutions with methanol gravimetrically and stored at 4 ºC. Working solutions were prepared freshly by methanol:water (1:9) mixture.

Table 1. MS parameters of sulfonamide compounds

Sulfonamides	Parent Ion	Quantitative Ion	Capillary Energy	Collision Energy	Retention Time (min)
Sulfamethizole	271	156	100	12	14.401
Sulfamethazine	279	186	110	20	14.014
Sulfachloropyridazine	284.9	156	100	14	15.909
Sulfaquinoxaline	301	156	100	15	19.246
Sulfadoxine	311	156	100	18	16.305
Sulfadimethoxine	311	156	100	20	19.048
Sulfapyridine	250	156	100	20	12.895
Sulfadiazin	251	156	80	15	11.349
Sulfamethoxazole	254	156	80	18	16.681
Sulfathiazole	256	155.9	80	14	12.127
Sulfamerazine	265	156	80	20	13.172
Sulfisoxazole	268	156	80	10	17.335
Sulfaphenazole	315	160	60	20	19.745
Sulfamethoxypyridazine	281	155.9	100	17	14.434
Sulfamethizole-$^{13}C_6$	276.9	161.9	100	12	14.398
Sulfamethazine-d_4	283	186	110	18	13.953
Sulfachloropyridazine- d_4	289	160	100	14	15.863
Sulfaquinoxaline-$^{13}C_6$	307	161.9	100	15	19.243
Sulfadoxine- d_3	314	155.9	100	16	16.241
Sulfadimethoxine-$^{13}C_6$	317	155.9	100	20	19.055
Sulfapyridine-$^{13}C_6$	256	113.9	100	16	12.135
Sulfadiazin- d_4	255	160	80	15	11.262
Sulfamethoxazole- d_4	258	160	80	16	16.630
Sulfathiazole- d_4	260	160	80	14	12.039
Sulfamerazine- d_4	269	160	80	18	13.091
Sulfisoxazole- d_4	272	160	80	13	17.279
Sulfaphenazole-$^{13}C_6$	321	158	60	22	19.746
Sulfamethoxypyridazine- d_3	284	128.9	100	16	14.391

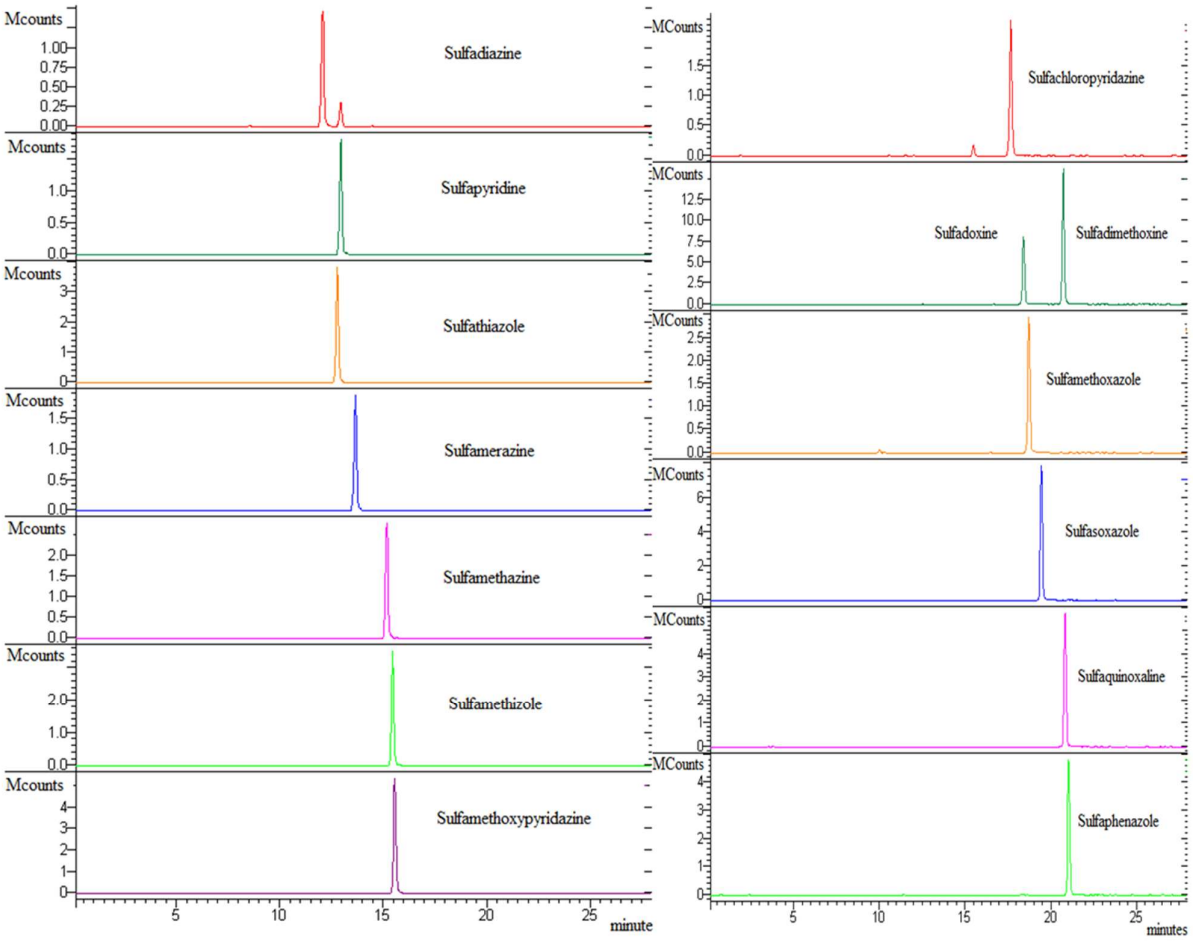

Figure 1. LC-MS/MS chromatogram of sulfonamides at 20 ng/g level.

2.2. Instrumentation

Analyzes were performed by ZIVAK Tandem Gold LC-MS-MS equipped with triple quadrupole analyzer and heated electro spray ionization source. Detector was 1600 V, needle voltage was 5500 V, spray shield voltage was 600 V, spray chamber temperature was 55 ºC, drying gas temperature was 350 ºC, vortex gas temperature was 120 ºC, nebulizing gas pressure was 50 psi, drying gas pressure was 30 psi and vortex gas pressure was 25 psi. The analytical column was Phenomenex Synergi 4u Max RP 80A (250 mm X 3 mm, 4 µm). Column oven temperature was kept at 25 ºC. The mobile phases were 0.1% (v/v) formic acid in water-acetonitrile (95:5 v/v) (A) and 0.1% (v/v) formic acid in water-acetonitrile (5:95 v/v) (B). A 30 min gradient of the LC method was set as follows (0–0.5 min) 95% A and 5% B, (0.5–6 min) 85% A and 15% B, (6–12 min, hold 4 min) 65% A and 35% B, (16–18 min, hold 3 min) 45% A and 55% B, (21–22 min, hold 8 min) 95% A and 5% B. MRM method details of the analytes are given in Table 1, and a representative chromatogram at 20 ng/g level is given in Figure 1.

2.3. Sample Preparation Procedure

Five mL milk sample was transferred into a 50 mL polypropylene centrifuge tube and spiked with internal standard solution. After the addition of 10 mL ACN:EA (6:4) (v/v), the mixture was well mixed using a vortex and then centrifuged at 10000 rpm for 10 min. After centrifugation, 6 mL of the supernatant from the top was transferred to a clean 15 mL centrifuge tube and dried thoroughly under a gentle nitrogen stream. 1.5 mL of n-hexane was added into the centrifuge tube and vortexed for 1 minute, then 1.5 mL, 10% (v/v) aqueous MeOH was added and vortexed for 1 minute. 1 mL from the bottom part of the biphasic solution was centrifuged at 14000 rpm for 5 minutes, after which 500 µL from the top layer was transferred to a LC vial for analysis.

2.4. Method Validation

The method validation was performed by spiking blank milk with a working solution. Matrix-matched calibration standards were prepared by spiking on blank extracts (10, 15, 20, 30, 40, 50 ng/g, each contained 20 ng/g of ISTD) to generate six-point calibration curve, which was plotted by peak area ratio (analyte/ISTD) versus concentration ratio (analyte/ISTD). The LOD and LOQ values of the individual analytes were estimated from the standard deviation of 10 samples prepared by spiking blank samples with sulfonamides (10 ng/g of analyte and 20 ng/g of ISTD). LOD was estimated as 3 times of standard deviation and LOQ as 10 times of standard deviation. The precision and accuracy were expressed as RSD and recoveries. The recoveries of all analytes were calculated by the measured content/the fortified level times 100. The linearity of the measurements in the range of 10-50 ng/g was evaluated by applying a linear regression analysis. The robustness of the method was investigated with the amount of acetonitrile, milk and hexane. Measurement uncertainties of the analytes were estimated according to EUROCHEM/CITAC and ISO measurement uncertainty description guideline (GUM).

3. Results and discussion

3.1. Optimization of Spectrometry and LC Conditions

Since MS/MS fragmentation of sulfonamides generally results in m/z 156, and LC/MS/MS used in this study is not of high resolution, we had the issue of separation for closer MW analytes and their ISTDs such as sulfapyridine/sulfadiazine and sulfadoxine-d_3/sulfachloropyridazine. To resolve the problem, as mentioned above (section 2.2), a 250 mm HPLC column was used in place of 150 mm column. Regarding sulfathiazole/sulfapyridine-C_6 and sulfamethoxypyridazine-d_3/sulfachloropyridazine, different quantification ions were identified as listed in Table 1.

For chromatographic separation of analytes, methanol-water and acetonitrile-water gradient elution programs were applied, and acetonitrile-water mixture was found to be the best separation solvent system. Three different concentrations of formic acid (0.1, 0.5 and 1%, v/v) to improve ionization and peak shape were also investigated and 0.1% v/v was found to be the best formic acid content.

3.2. Optimization of Sample Preparation

Different extraction methods to extract sulfonamides from the challenging milk matrix, containing protein and fat, were investigated. Initially a liquid-liquid extraction method, developed by Sun *et. al.*, [41] as an Agilent application note, where water, acetonitrile, sodium sulfate and sodium chloride were used. As it resulted in around 40-45% recoveries, this method was abandoned. Second method involved an Agilent application note, developed by Gonzalez *et. al.* [42], where acetonitrile extraction and SPE clean up (Agilent SampliQ SCX) were employed. It resulted in around 50-60% recoveries. Third one was a modified QuEChERS method, developed by Parab and Amritkar [3], which gave around 40% recoveries. Finally, the method developed by our group, which was modified from the method-used by Cai *et. al.* for the extraction of sulfonamides in meat samples, was applied. Its details are given in section 2.3., recoveries were in the range of 91-114%.

Regarding the extraction solvent, ethyl acetate, acetonitrile and different proportions of acetonitrile and ethyl acetate mixtures were investigated. Acetonitrile:ethyl acetate (6:4, v/v) mixture was found to be the best extraction solvent combination.

3.3. Matrix Effects

The matrix effect of each compound was determined by comparing the peak area of standard solution and blank milk sample extract spiked at 100 ng/g concentration before submitting to LC-MS/MS. The matrix effect observed was ranging from 12% signal suppression for sulfapyridine and 11% signal enhancement for sulfadiazine. Although IDMS technique was used, in order to be more confident about the results, matrix matched calibration solutions, prepared by adding standard solutions to negative milk extracts to compensate signal enhancement and suppression were applied.

Table 2. Validation data summarized.

Compound	Correlation Coefficient	LOD (ng/g)	LOQ (ng/g)	Recovery			RSD (intra-day)	RSD (inter-day)
				15 (ng/g)	25 (ng/g)	40 (ng/g)		
SMR	0.996	2.45	8.16	104.15	101.77	109.75	7.72	4.20
SDZ	0.997	2.38	7.93	108.66	104.72	106.21	5.72	2.88
SSX	0.999	2.19	7.30	105.02	97.10	94.61	6.41	7.33
SMX	0.996	2.56	8.53	100.57	99.07	102.03	7.50	8.65
STZ	0.996	1.82	6.07	107.73	98.95	96.37	5.02	5.13
SDM	0.996	2.74	9.13	110.33	101.30	102.00	3.73	14.25
SPD	0.996	2.28	7.61	109.94	103.03	101.58	6.70	3.40
SME	0.999	2.66	8.87	99.28	95.53	94.13	7.08	2.85
SDX	0.997	1.99	6.66	113.86	108.00	112.78	8.32	7.23
SCP	0.998	2.41	8.05	102.22	96.24	98.13	8.31	11.68
SPA	0.998	2.41	8.04	108.38	96.02	93.07	5.77	8.77
SMZ	0.996	2.45	8.16	99.99	98.58	90.85	8.05	7.87
SQX	0.996	2.63	8.75	103.04	98.85	96.75	4.26	1.82
SMP	0.996	2.92	9.73	102.36	98.31	93.94	5.55	3.00

3.4. Method Validation

The full IDMS method proposed herein was validated in terms of its performance through limit of detection (LOD), limit of quantification (LOQ), working range, precision, trueness, measurement uncertainty and ruggedness. Results are summarized in Table 2. LODs were estimated at 1.82-2.92 ng/g. A good linearity was obtained (regression coefficient > 0.996) in the range of 10-50 ng/g. Intra-day precision was determined at three different spiked concentrations (15, 25 and 40 ng/g each, n=3), and inter-day precision was obtained at a spiked concentration of 25 ng/g on each day (n=3) in 5 different days. Precision values were calculated by one-way analysis of variance (ANOVA) and are summarized in Table 2. The accuracy expressed as recovery determined at three different spiked concentrations (15, 25 and 40 ng/g each, n=3) ranging from 90.85% to 113.86%. The robustness of the method was tested applying three important parameters, i. e. amount of extraction solvent, amount of milk sample and amount of n-hexane used to remove fat content. Evaluation of robustness was performed by F-test and sulfachloropyridazine and sulfaphenazole, which

showed significant difference for the amount of milk. Measurement uncertainty of the method was estimated according to EURACHEM/CITAC Guide (Third Edition) and Guide to the Expression of Uncertainty in Measurement (ISO/IEC Guide 98-3:2008), at 95% confidence level expanded uncertainties ranging from 7.46% to12.71%.

3.5. Analysis of Real Samples

To test the capability of the developed method, occurrence of sulfonamides on different types of milk in local Turkish markets and also 6 raw milk samples from street vendors were examined. The method was applied to six UHT milk samples and six pasteurized milk samples. Although no sulfonamides were detected in preserved milks, one sample from street vendor contains 7.3 ng/g sulfisoxazole and another one contains 6.46 ng/g sulfamethazine.

4. Conclusion

In this study, an IDMS method with high accuracy and good precision was developed for sulfonamides in milk, which is a complex matrix of protein, fat and carbohydrate. Sulfonamides are ampholytes having weak basic and acidic characters (pKa 5-7.6). While their acidic character arises from the N-H linkage of the sulfonamide group, the basic character is due to presence of the nitrogen in aniline group. As the acidic character is more dominant, formic acid is added (0.1%) to the mobile phase to assist the ionization of the molecules with heated ESI source. This study presents, for the first time, a fully isotope dilution mass spectrometry method, applied for sulfonamides in milk and evaluated by linearity, recovery, day-to-day variation, robustness, limits of detection and quantification, and measurement uncertainty. Recoveries range from 96% to 108% at the spiked level of 25 ng/g. This method offers high accuracy, easy operation and low cost, without complex clean up procedures. It was tested on local market milk samples, except two samples from street vendors, which were free of sulfonamides. This method can serve as an effective and easy approach for the determination of sulfonamide residues in milk.

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