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Development and Validation of a Primary IDMS Method for the Quantification of 12 Sulfonamides in Meat by Using LC-MS/MS

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Abstract: A new liquid chromatography-tandem mass spectrometry (LC-IDMS) method was developed and validated for simultaneous multi-residue analysis of 12 sulfonamides (SAs) in beef meat. Sulfonamides were isolated from meat with a solvent extraction procedure. Reliable quantitative evaluation was accomplished using each sulfonamide as the internal standard of their isotopes. Matrix-matched calibration curves were used. Studied performance characteristics were linearity, recovery, precision, detection and quantification limits and robustness. Results were compatible with method performance acceptance criteria according to UME. Recovery results were 82-116%. The measurement uncertainty was calculated from the "top-down approach". The relative measurement uncertainty was between 7-14%.

Keywords: Sulfonamides; IDMS; method validation.

1. Introduction

Sulfonamides are N-substituted derivatives of the *p*-amino benzenesulfonic acid. They have been used for the prevention of infectious diseases and growth-promoting purposes in veterinary medicine practice. Extensive usage of these antimicrobials and failure to comply with the waiting period before slaughter can result in residues in edible products. Sulfonamide residues can promote the occurrence of antibiotic-resistant bacteria, allergic reactions and toxic reactions in consumers. Also, some sulfonamides could be carcinogenic [1]. For this reason, sulfonamide residues in food are an important subject as they have significant effects on human health. The European Union has set a maximum residue limit (MRL, 100 µg/kg) for sulfonamides in foods of animal origin [2]. Thus, accurate determination of sulfonamide analytes is necessary for compliance with food safety laws. Many analysis techniques have been investigated in the literature, such as HPLC, LC-MS, enzyme-linked immunosorbent assay (ELISA), gas chromatography (GC), thin-layer chromatography (TLC), high-performance capillary electrophoresis (HPCE), biosensor immunoassay (BIA) and liquid chromatography isotope dilution mass spectrometry (LC-IDMS) method in the determination of sulphonamides [3-9]. However, methods based on

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chromatographic analysis without the use of molecular spectrometric detection aren't suitable for use as confirmatory methods (EU Commission Decision 2002/657/EEC). In this study, we used the LC-IDMS method with isotope conjugates of each sulfonamide. This is a distinctive difference from the literature reviews. IDMS technique provides high precision and accuracy and low uncertainty as it minimizes errors, calibration problems and matrix effects in the sample preparation and analysis process [10]. In addition, it eliminates personal analysis mistakes. In previous years sulfonamide research, which provides only 1-3 isotopically labelled sulfonamides as internal standards, has been frequently mentioned. Many studies focused on determining multiclass veterinary drugs in one method, but they compromise the accuracy of the analytes [11]. This study contributes to the literature with a full IDMS technique for all 12 sulfonamides in meat using an advanced determination method.

In isotope dilution mass spectrometry, a sample with known isotopic composition of the analyte element but unknown element content is mixed with a known amount of spike. This spike contains the analyte element in a different isotopic composition: Ideally enriched in the rarest natural isotope. After complete mixing of sample and spike, the so-called sample-spike blend or isotope diluted sample gained a new isotope ratio being between the isotope ratio of the sample and that of the spike. This blend isotope ratio directly reflects the analyte concentration in the sample [12].

Detection of trace level antibiotic residues in a rich matrix such as meat requires good sample preparation and clean-up procedures. Classical solvent extraction and accelerated solvent extraction (ASE), QuEChERS-based extraction were studied in the past [13]. This study compared ASE and modified solvent extraction using acetonitrile (ACN) solvent for the protein precipitation step, and the best recovery results (75-110%) were obtained by modified solvent extraction.

Method validation is a test and measurement process to determine the performance of a method. In chemical analyses, the application performance of the analysis method depends on various factors. These are laboratory conditions, instruments, used chemicals, standards, operator, and matrix (due to impurity effect). The purpose of validation is; to determine and demonstrate that the method's performance is appropriate for the determined analysis needs. To ensure the quality of the developed method, accuracy, recovery, precision, LOD and LOQ, intra-day and inter-day repeatability were estimated with the analyses of spiked meat samples.

Accredited laboratories estimate and report uncertainty according to the requirements of ISO/IEC 17025. In this top-down study, an approach was applied to obtain the measurement uncertainty [14]. The top-down approach has all effects within calculation; realistic uncertainty values are also calculated. Previous studies in sulfonamides uncertainty calculations were based on a bottom-up approach, and our study filled the absence of review in this respect.

In this study, metrological traceability ensured that the primary method to determine 12 sulfonamides in beef meat samples was developed using LC-IDMS. In addition, the extraction method of the developed method was simpler than the QuEChERS, ASE and SPE extraction methods. For these reasons, this method can be used in field studies.

2. Materials and Methods

2.1. Chemicals and Reagents

All the reagents used were of analytical grade. LC-grade ACN, methanol (MeOH) and, hexane, ethyl-acetate were supplied by Merck. Formic acid was purchased from Fluka. The TÜBİTAK UME laboratory generated ultrapure water.

Sulfathiazole (STZ), sulfapyridine (SPY), sulfamethazine (SMZ), sulfamerazine (SMR), sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfadoxine (SDX), sulfisoxazole (SSX), sulfaquinoxaline (SQX), sulfachloropyridazine (SCP), sulfamethizole (SME), sulfamethoxypyridazine (SMP) and conjugate of these materials sulfamethoxypyridazine-d_3, sulfamethizole-(fenil $^{13}C_6$), sulfadoxine-d_3, sulfaquinoxaline-(fenil $^{13}C_6$), sulfapyridine-(fenil $^{13}C_6$), were purchased from Sigma Aldrich. From conjugates, sulfathiazole-d_4, sulfamethazine-d_4, sulfamerazine-d_4, sulfadiazine-d_4, sulfamethoxazole-d_4, sulfisoxazole-d_4, sulfachloropyridazine-d_4, were synthesized in TÜBİTAK organic chemistry laboratory and characterized by NMR and HPLC/DAD.

2.2. Standard Preparation

Stock solutions of single analytes were prepared at a concentration of 10-1000 µg/g in MeOH gravimetrically at Mettler Toledo XP205 balance (d:0.01 mg).

Stock Solution 1 (1000 µg/g): A 30 mg amount of each sulfonamide standard was accurately weighed. 30 mg MeOH (23,73 g) was added. Only sulfaquinoxaline was prepared in acetone because of its solubility. Stock solution 1 was stored at 4°C.

Stock Solution 2 (10 µg/g): 150 µl of stock solution 1 was weighed, and 14,85 ml MeOH was added. Stock solution 2 was stored at 4°C.

Working solutions were prepared freshly by dilution with MeOH: Water (1:9) mixture.

2.3. Instrumentation

Analyses were performed by a Tandem Gold LC-MS-MS triple quadrupole analyser and heated electrospray ionisation source. The detector was 1600 V, needle voltage 5500 V, spray shield voltage was 600 V, spray chamber temperature was 55ºC, drying gas temperature was 350 ºC, vortex gas temperature was 120 ºC, and nebulizing gas pressure was 50 psi drying gas temperature was 30 psi and vortex gas pressure 25 psi. The analytical column was Phenomenex Synergi 4u Max RP 80A 250mm x 3mm x 4 µm). The column oven temperature was kept at 25 ºC. The mobile phases were 0.1 % (v/v) formic acid in water-ACN (95:5 v/v) (A) and 0.1 % (v/v) formic acid in water-ACN (5:95 v/v) (B). The gradient of the pump program is presented in Table 1.

Table 1. The gradient of the pump program

Time	A%	B%	Flow (µL/min)
0:0	95	5	400
0:30	95	5	400
6:00	85	15	400
12:00	65	35	400
16:00	65	35	400
18:00	45	55	400
21:00	45	55	400
22:00	95	5	400
30:00	95	5	400

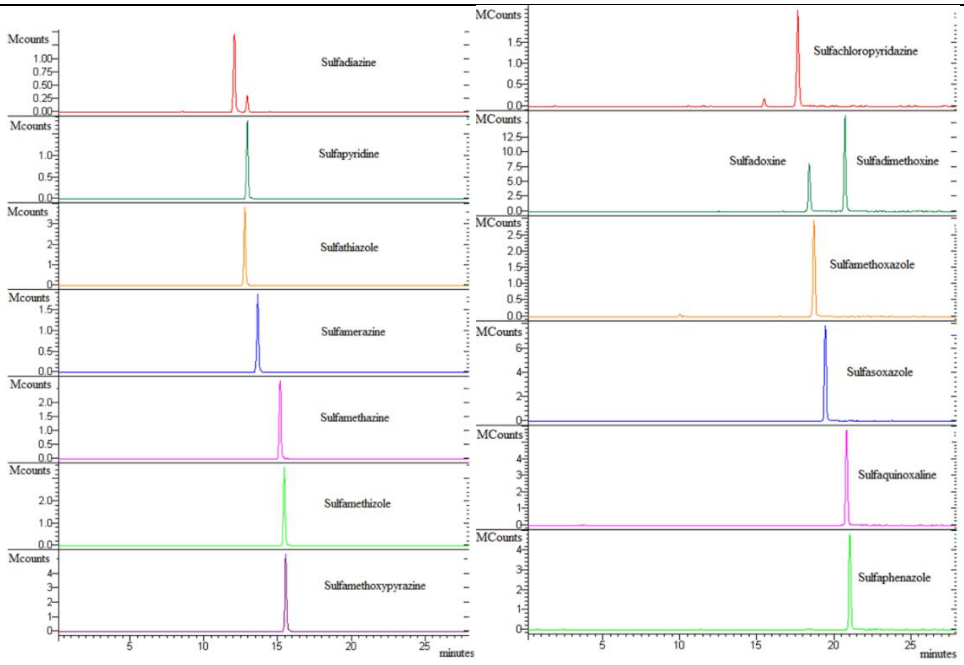

Figure 1. The chromatogram images of the singular sulfonamides

The compounds were identified by their precursor ion and fragments through tandem mass spectrometry analysis. Details of retention time, precursor ion, quantifier ions, fragmentor voltages, and collision energies used for the sulfonamides are listed in Table 2.

Table 2. MS-MS parameters of sulfonamide ions

Sulfonamides [a]	Parent Ion	Quantitative Ion	Capillary Energy	Collision Energy	Retention Time (min)
SMR	265.0	156.0	80	20	13.172
SDZ	251.0	156.0	80	15	11.349
SSX	268.0	156.0	80	10	17.335
SMX	254.0	156.0	80	18	16.681
STZ	256.0	155.9	80	14	12.127
SPY	250.0	156.0	100	20	12.895
SME	271.0	156.0	100	12	14.401
SDX	311.0	156.0	100	18	16.305
SCP	284.9	156.0	100	14	15.909
SMZ	279.0	186.0	110	20	14.014
SQX	301.0	156.0	100	15	19.246
SMP	281.0	155.9	100	17	14.434
SME-$^{13}C_6$	276.9	161.9	100	12	14.398
SMZ-d_4	283.0	186.0	110	18	13.953
SCP-d_4	289.0	160.0	100	14	15.863
SQX-$^{13}C_6$	307.0	161.9	100	15	19.243
SDX-d_3	314.0	155.9	100	16	16.241
SPY-$^{13}C_6$	256.0	113.9	100	16	12.135
SDZ-d_4	255.0	160.0	80	15	11.262
SMX-d_4	258.0	160.0	80	16	16.630
STZ-d_4	260.0	160.0	80	14	12.039
SMR-d_4	269.0	160.0	80	18	13.091
SSX-d_4	272.0	160.0	80	13	17.279
SMP-d_3	284.0	128.9	100	16	14.391

[a] **STZ**: Sulfathiazole, **SPY**: Sulfapyridine, **SMZ**: Sulfamethazine, **SMR**: Sulfamerazine, **SDZ**: Sulfadiazine, **SMX**: sulfamethoxazole, **SDX**: Sulfadoxine, **SSX**: Sulfisoxazole, **SQX**: Sulfaquinoxaline, **SCP**: Sulfachloropyridazine, **SME**: Sulfamethizole, **SMP**: Sulfamethoxypyridazine

Complete separations of the peaks of the sulfonamides were achieved by this method specified in meat (Figure 1).

2.4. Extraction Procedure

Meat is a complex matrix. Many methods have been investigated to separate sulfonamides from meat samples. Because of the presence of structural substances such as proteins, carbohydrates, lipids, and minerals in the meat matrix. In today's technology, it is not possible to analyse sulfonamide compounds directly from meat. Therefore, classic solvent extraction and accelerated solvent extraction were applied. The method was developed with the extraction method, which gave the best recovery results. ACN was found to be the best extraction solvent. In sample preparation, ultra-turrax and vortex fragmentation effectiveness was compared, and no significant difference was observed. ASE extraction was also done, but better recovery results were achieved using the following modified solid-liquid extraction.

The modified solid-liquid sample preparation procedure was as follows: 5 g of meat samples was transferred to a polypropylene centrifuge tube (50 mL) and spiked with 200 µL of the analyte and internal standard mix solution. 10 mL ACN was added and vortexed, then centrifuged at 11400 rpm for 10 min at 4 °C. 6 mL of upper phase was taken and fully dried under gentle nitrogen flow. 1,5 mL n-hexane was added and vortexed. Then 1,5 mL 20 (v/v) % MeOH aqueous solution was added and vortexed. The solution was centrifuged again. Received bottom-phase carefully transferred to LC vial and analysed by LC-MS-MS.

2.5. Quantitative Analyses

Every analyte had its isotope-labelled analogue. Matrix-matched calibration was done. Calibration solutions were prepared gravimetrically. Calibration curves were constructed as the native/labelled standard chromatographic peak area ratio versus native/labelled concentration. Firstly, calibration levels were determined as 10, 15, 20, 30, 40, and 50 ppb and then the calibration graph was plotted. Linear regression analysis was applied to get the regression coefficients and to obtain the linearity of the method.

Linear relations were good. Linearity was assessed on the basis of the linear regression and squared correlation coefficient, R^2, which is required to be $\geqslant$0.9950. The response factor was calculated by Eq. (1). Coefficients of determination (r^2= 0,995-0,999) were reached over the concentration range of 10-50 ng/g (Table 3).

$$RF = \frac{A_{ABX} \times C_{ISX}}{A_{ISX} \times C_{ABX}} \quad \text{Eq.1.}$$

RF: Response factor
C_{ABX}: Concentration of native compound (ng/g)
$A_{ABX:}$ Peak area of native compound
A_{ISX}: Peak area of the labelled compound
C_{ISX}: Concentration of labelled compound (ng/g)

Table 3. Validation data summarized.

Sulfonamides	**Regression coefficients**	**LOD**	**LOQ**	**RSD** $_{repeatability}$	**RSD$_{intermediate}$ precision**	**Average Recovery (%)**		
Analytes	**(r^2)**	**(ng/g)**	**(ng/g)**			**15 ng/g**	**25 ng/g**	**40 ng/g**
SMR	0.998	2.84	9.46	8.19	5.05	82.98	82.93	82.30
SDZ	0.995	2.87	9.55	2.87	9.55	88.01	115.79	90.60
SSX	0.996	2.09	6.95	2.09	6.95	90.49	93.22	97.30
SMX	0.996	2.68	8.92	2.68	8.92	86.38	93.87	94.66
STZ	0.997	2.98	9.96	4.51	1.44	89.20	94.46	99.52
SP	0.998	2.66	8.85	5.99	3.53	108.92	104.57	104.47
SME	0.999	2.66	8.85	6.11	2.39	87.88	95.88	94.45
SDX	0.996	2.53	8.42	7.83	2.10	96.89	100.94	113.7
SCP	0.996	2.05	6.82	7.54	4.38	94.37	97.00	104.7
SMZ	0.997	2.37	7.89	8.64	5.11	112.79	105.88	101.15
SQX	0.997	2.42	8.06	9.88	1.00	96.52	96.19	96.35
SMP	0.995	2.99	9.96	7.81	4.31	86.45	93.77	92.65

3. Results and Discussion

3.1. Validation

The method was validated following the accepted criteria for analytical method validation, as indicated in the decision 2002/657/EC for quantitative confirmatory methods [2]. Linearity, accuracy,

limit of detection (LOD), the limit of quantification (LOQ), inter-day and intra-day precision and robustness were performed to validate the developed method. In this study, we validated the LC-MS/MS method according to the requirements of the European Union.

3.2. Method Precision

The precision of the developed method was tested by repeatedly analysing the spiked meat samples. Intra assay and intermediate precision were evaluated by analysing spiked meat samples at 15 ng/g , 25 ng/g, and 40 ng/g.

The relative standard deviation values were calculated using the following equations:

$$\mathrm{RSD}_{\mathrm{repeatability}} = \frac{\sqrt{\mathrm{MS}_{intra-day}}}{\mathrm{W}_{\mathrm{average}}} \text{ x } 100 \qquad \text{Eq2.}$$

$$\mathrm{RSD}_{intermediate\ precision} = \frac{\sqrt{\mathrm{MS}_{\mathrm{inter-day}} - \mathrm{MS}_{\mathrm{intra-day}}}}{\mathrm{W}_{\mathrm{average}}} \text{ x } 100 \qquad \text{Eq3.}$$

In the equations,
$MS_{intra\text{-}day}$: the average of the intra-day results square
$MS_{inter\text{-}day}$: the average of the inter-day results square
W: mass fraction of the analyte.
Obtained results are given in Table 3.

Our results were less than the defined method acceptance criteria <15 %, so the criteria provided by this way. In general, the precisions of assays are in the range of RSD 10 %. Even the results obtained by isotope dilution mass spectrometry (IDMS) showed similar precision.

3.3. Method Accuracy

The accuracy of the method was accepted as the mean recoveries of spiked analytes in the meat matrix at three concentration levels. Reproducibility of recovery was studied by repeating the spiking experiment on the second, third and fourth day and comparing results with the recovery results obtained on the first day. Recovery calculations were calculated using the following Eq. 4:

$$\text{Recovery } \% = \frac{W_{measured}}{W_{added}} \times 100 \qquad \text{Eq.4.}$$

In this equation;
$W_{measured}$: The difference between measurement results of spiked sample and blank sample (ng/g)
W_{added}: Amount of added analyte (ng/g)
The average recovery values obtained are shown in Table 3.

All recovery results obtained by medium level spike samples are presented in Table 4.

In a study which analysed 62 antibiotics, including sulfonamides, with LC-HRMS/MS in meat, all antibiotics (3.3 µg/kg-150 µg/kg) were validated. Recovery results were 71-95% [15]. Abdallah Hiba et al. analysed 22 sulfonamide compounds and their metabolites using the QuEChERS extraction method in meat (beef, sheep, chicken, pig) in a high-resolution mass spectrometer. Linearity was stated as R^2<99, LOD, 3-26 µg/kg, LOQ; 11-88 µg/kg. Recovery results were 88%-112%, intra-day and inter-day accuracy were found 1%-4% and 1%-17% [16]. Cai et al. investigated 24 sulfonamide compounds in meat by UPLC tandem mass spectrometry. The lipid extraction method was used as a pre-treatment. Calibrations were performed with the sample matrix, and the isotope dilution method and the effect of the sample matrix on ionization were investigated. In the isotope dilution method, three sulfonamide isotope compounds were used as internal standards. These isotopes were sulfadiazine-d_4 (SD-d_4), sulfamethazine-d_4 (SMZ-d_4) and sulfadimethoxine-d_4. R^2 was found to be 0.991-0.999 in the 0.2-50 µg/kg. Recovery

results were found 67.8-113.9 % [17]. Gentili et al. extracted sulfonamides with accelerated solvent extraction and analyzed them in LC-MS-MS. Recovery results were found 70-101 %. Intra-day precision of <8.5% and LOD values were found at <2.6 ppb [18]. Our recovery results complied with the EU guidelines, establishing a range of 80–120% for this concentration level [2].

Table 4. Recovery results for the intermediate level spike samples

	SMR	**SDZ**	**SSX**	**SMX**	**STZ**	**SP**	**SME**	**SDX**	**SCP**	**SMZ**	**SQX**	**SMP**
Day 1	79.11	84.73	91.24	93.30	90.35	100.62	91.13	110.44	100.07	100.22	93.13	88.25
Day 2	81.75	90.60	93.13	91.79	93.14	94.68	96.49	96.45	93.48	113.67	90.60	105.29
Day 3	88.07	88.57	97.64	105.59	98.91	99.01	104.30	99.12	98.97	112.71	101.72	94.42
Day 4	81.93	87.61	93.76	90.05	97.11	108.48	95.55	98.68	103.06	108.48	99.35	100.71
Day 5	83.82	87.46	90.36	90.42	92.78	103.85	91.93	99.99	89.43	94.33	96.15	94.58
Average	82.93	87.80	93.22	94.23	94.46	101.33	95.88	100.94	97.00	105.88	96.19	96.65
SD	3.32	2.12	2.83	6.48	3.47	5.19	5.23	5.47	5.47	8.36	4.51	6.54

3.4. Limits of Detection and Limits of Quantification

The limit of detection (LOD) is the smallest value of the concentration of an analyte which can be detected. The limit of quantification (LOQ) is the smallest value of the concentration of an analyte which can be quantified. LOD and LOQ calculations were done by standard deviation determinations. First, 10 ppb native and 20 ppb isotope was added to the blank sample. LOD value is three times the standard deviation, while LOQ is 10 times the standard deviation of the response obtained for ten samples. Next, LODs were done by spiking the starting material at a 10 ppb concentration level for all analytes. LOD and LOQ values are presented in Table 3.

The concentration of the lowest level calibration solution ($\leqslant$10 ppb). In this context, the highest determined LOD and LOQ values were 2.99 ppb and 9.96 ppb, respectively. LOD values were in the range of 2.05-2.99 ppb, and LOQ values were in the range of 6,82-9,96 ppb. It is seen that LOD and LOQ values targeted by the developed method are provided.

3.5. Method Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small changes in method parameters, ensuring that the method is reliable during use [19]. In our study, extraction was the critical stage. In order to test the method's robustness, one parameter was changed while the others were constant. Extraction solution amount, meat amount and hexane amount were changed, and the method was applied. Changing and constant parameters are given in Table 5.

Table. 5 Changing and constant parameters in method stages

	ACN	**Meat**	**Hexane**
Solvent and matrix amount	10.2 mL ACN	5.5 g	1.7 mL
	9.8 mL ACN	4.5 g	1.3 mL

Every parameter and sample solution prepared for parameter change values were obtained after three free samples. *t*-test was done to question whether a significant difference occurred between the measurements. Results were evaluated statistically using the F test to question the accuracy of the results. When we evaluate results statistically, generally $t_{experimental} \leqslant t_{critical}$; therefore, small changes made to the critical stages of the method didn't create a significant result. From the point of results, precision

$F_{experimental} \leqslant F_{critical}$ except for two compounds. Sulfathiazole and sulfamethizole were the compounds with significant differences seen. ACN amount was the parameter change seen for sulfathiazole. Hexane amount was the parameter change seen for sulfamethizole. Except for these compounds, method performance criteria and acceptance were obtained.

3.6. Measurement Uncertainty Calculations

The measurement uncertainty was calculated according to EUROCHEM/CITAC and ISO measurement uncertainty description guideline (GUM). The approach of validating the method was used to calculate the measurement uncertainty. Results of uncertainty calculations of 12 sulfonamide compounds are presented in Table 6.

Table 6. Uncertainty calculations of 12 sulfonamide compounds in meat

	SMR	SDZ	SSX	SMX	STZ	SP	SME	SDX	SCP	SMZ	SQX	SMP
$\mathbf{U_{cal}}$	0.45	0.45	0.39	0.65	0.43	0.41	0.20	0.45	0.49	0.61	0.43	0.68
$\mathbf{U_{repeatability}}$	1.54	1.71	1.57	1.54	0.96	1.31	1.30	1.79	1.62	1.93	2.06	1.50
$\mathbf{U_{intermediate\ precision}}$	0.32	0.39	0.20	0.08	0.03	0.09	0.17	0.16	0.31	0.13	0.07	0.28
$\mathbf{U_{trueness}}$	0.87	0.96	0.90	0.89	0.55	0.73	0.74	1.03	0.91	1.08	1.19	0.85
u(x), %(1)	1.85	2.05	1.86	1.90	1.19	1.56	1.52	2.12	1.95	2.30	2.42	1.87
U(x), %(2)	3.71	4.10	3.72	3.79	2.37	3.11	3.04	4.24	3.89	4.60	4.84	3.75

(1) Relative standard measurement uncertainty
(2) Relative combined measurement uncertainty

As a result, the target measurement uncertainty value for all analytes K=2 (95% confidence level) was less than 14%. Relative combined measurement uncertainty values were suitable for identified UME acceptance criteria.

In this study, a primary method was developed using conjugated isotopes of 12 sulfonamide compounds in meat samples. Extraction was carried out by the solid-liquid extraction method, which was simpler than QuEChERS, ASE, and SPE extraction methods. Errors and deviations in all analysis steps were minimized by the IDMS technique. The top-down validation calculations and the uncertainty budget were calculated differently from many studies in the literature. Eventually, this method can be used in laboratories making routine analyses. Its superiority over other routine analysis methods is fast, reliable and especially economical in terms of analysis costs.

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