

## Improving the efficiency of a pre-treatment method for sulphonamide detection in raw chicken meat by high-performance liquid chromatography-tandem mass spectrometry

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### Abstract

The increasing use of sulphonamides in poultry production raises food safety concerns due to potential health risks and the emergence of antimicrobial resistance. This study aimed to validate an efficient sample pre-treatment method for the determination of eight sulphonamide residues in raw chicken meat using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS), focusing on simplifying the extraction process. The improved method was applied to simplify the extraction process by eliminating the need for solid-phase extraction, thereby reducing cost and analysis time. Method validation in accordance with European Commission Decision 2002/657/EC to evaluate parameters such as specificity, linearity, matrix effect, precision, trueness, limit of detection (LOD), and limit of quantification (LOQ). The results showed good specificity and linearity ( $R^2 > 0.99$ ), acceptable trueness (80.21–103.39%), and precision with HorRat values ranging from 0.301 to 0.687. All analytes exhibited higher sensitivity LODs below 0.6  $\mu\text{g}/\text{kg}$  and LOQs below 5  $\mu\text{g}/\text{kg}$  as compared to the current method. Significant matrix effects were observed, justifying the use of matrix-matched calibration for accurate quantification. The validated method offers a rapid, cost-effective, and sensitive approach suitable for routine screening of sulphonamide residues in poultry meat, contributing to improved food safety monitoring and regulatory compliance.

## 1. Introduction

The increasing global usage of antimicrobial agents in animal production is a growing concern for public health. In poultry farming, the average annual usage of antimicrobial agents globally had already reached approximately 148 mg/kg (Van Boeckel *et al.*, 2015; Ma, Xu, Tang *et al.*, 2020), which is equivalent to 28.40 mg/day, and approximately 10.36 g per year if scaled to a 70 kg human.

The administration of antimicrobial agents is expected to increase as the global consumption of chicken meat has been steadily increasing over the years. In 2021, the total production reached 25.8 billion chickens, which is up from 14.38 billion chickens in

2000 (Shahbandeh, 2023). One of the main concerns of antibiotic usage in livestock animals is the regulatory and control inconsistency by responsible agencies across different countries. This inconsistency has led to the accumulation of drug residue concentration in animal products, which poses significant health risks (Okocha *et al.*, 2018).

Drug residues (including the compound itself or its metabolites) could pose potentially adverse health effects towards humans and animals, such as cancers, reproductive disorders, mutagenicity, severe allergy, and disruption of microbiota in the digestive tract (Mund *et al.*, 2016; Canton *et al.*, 2021). In addition, these drug residues, even at low concentrations, can contribute to

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the development of antimicrobial resistance (AMR) that presents a significant prolonged health threat (Andersson and Hughes, 2012; Chow *et al.*, 2015). Therefore, effective and reliable detection methods are crucial for monitoring antimicrobial residues, which would help determine the safe legal limit of their concentration to ensure food safety (Wang *et al.*, 2021).

Current analytical methods for detecting sulphonamide residues in food matrices are primarily based on advanced chromatographic techniques coupled with various detectors. These include instrument analysis (liquid chromatography mass spectrometry (LC-MS), liquid chromatography time-of-flight (LC-TOF) mass spectrometry (MS) and liquid chromatography-ultraviolet (LC-UV) detection), immunoassay (immunosorbent technology (ELISA) and fluorescence immunoassay technology), molecular biological technique (biochip and molecular imprinting technology), as well as biosensor, which have shown good performance in detection of residues (Chen, 2021). However, these techniques often require a pre-treatment method to reduce noise and matrix interferences. These pre-treatment steps include liquid-liquid extraction, solid-phase extraction (SPE) and Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS), but they are associated with high costs, long processing times and extensive manpower, which makes them inaccessible for routine screening. This is because liquid-liquid extraction involves a large workload, while solid-phase extraction is accurate but costly, and QuEChERS often provides insufficient purification and is prone to matrix interferences (Wu *et al.*, 2023).

Therefore, in response to the limitation of current pre-treatment, particularly the time-consuming and costly nature, there is a need for innovative approaches that could be faster, cheaper, and more sensitive to improve throughput. The objective of the present study was to improve the sulphonamide detection method to achieve shorter analysis time, reduce solvent consumption (cost-saving), increase simplicity by eliminating the need for SPE and other clean-up treatment, and enhance sensitivity to detect drug residues of sulphonamides. The impact of the present study would be able to aid in detecting drug residues rapidly, and that would help in monitoring, enhancing food safety, and supporting regulatory compliance.

## 2. Materials and methods

### 2.1 Standard solution preparation

The internal standard (ISTD) stock solution (1,000  $\mu\text{g/mL}$ ) was prepared by weighing 10 mg of SDM-d6 and dissolving it in 10 mL of a 10 mL volumetric flask

with acetonitrile, and stored at 4°C. Then, the ISTD working standard solution (2  $\mu\text{g/mL}$ ) was prepared by diluting 20  $\mu\text{L}$  from stock solution 1,000  $\mu\text{g/mL}$  in a 20 mL volumetric flask with acetonitrile, and stored at 4°C.

The external standard (ESTD) stock solutions (100  $\mu\text{g/mL}$ ) were prepared individually by weighing 10 mg of each antibiotic standard (sulfamerazine (SMR), sulfamonomethoxine (SMM), sulfadimethoxine (SDM), sulfaquinoxaline (SQX), sulfadiazine (SDZ), sulfapyridine (SPD), sulfadimidine (SDD) and sulfathiazole (STZ)), and diluting to 100 mL in a 100 mL volumetric flask with acetonitrile, and stored at 4°C. The mix ESTD standard solution (2  $\mu\text{g/mL}$ ) was prepared by pipetting 200  $\mu\text{L}$  of each 100  $\mu\text{g/mL}$  standard solution into 10 mL volumetric flask, and making up to volume with acetonitrile. Meanwhile, the mix ESTD standard solution (0.1  $\mu\text{g/mL}$ ) was prepared by pipetting 10  $\mu\text{L}$  of each 100  $\mu\text{g/mL}$  standard solution into 10 mL volumetric flask, and making up to volume with acetonitrile.

Each standard solution was spiked into the chicken meat sample to construct the calibration curve (Table 1).

Table 1. Preparation of matrix-matched standard calibration.

Matrix-matched standard level	ESTD concentration taken ( $\mu\text{g/mL}$ )	Volume taken ( $\mu\text{L}$ )	Expected concentration ( $\mu\text{g/kg}$ )
Blank	0	0	0
1	0.1	20	1
2	0.1	100	5
3	0.1	200	10
4	2.0	20	20
5	2.0	100	100
6	2.0	200	200

### 2.2 Sample pre-treatment (sulphonamide extraction)

The method used in this study was adapted with modifications from the protocol described by Marni *et al.* (2017). Finely ground samples of chicken meat purchased from supermarkets in Klang Valley, Malaysia (2.00 $\pm$ 0.02 g) were weighed in 50 mL polypropylene centrifuge tubes. All the tubes were spiked with 20  $\mu\text{L}$  of 2  $\mu\text{g/mL}$  SDM-d6 as ISTD, while the matrix-matched samples were spiked with a proper amount of sulphonamides to achieve the concentration as listed in Table 1. All of the tubes were shaken vigorously for 5 min at room temperature by a Vortex laboratory mixer (BenchMixer™, Germany), and were placed on a bench for 15 min to allow the incorporation of the respective drug into the sample. Then, 5 mL of acetonitrile was added to the polypropylene tube, and the tubes were rotated for 10 min by an overhead shaker (Heidolph, Germany). The polypropylene tubes were then centrifuged (Hettich Zentrifugen Universal 320 R centrifuge, Germany) at 4°C at 4,000 rpm for 10 min.

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The supernatants were transferred to a glass test tube and dried at 40°C using nitrogen gas. The glass test tube was then reconstituted with 500 µL of 0.1% formic acid in deionised water (dH<sub>2</sub>O). The solution was transferred into a 1.5 mL microcentrifuge tube, then 500 µL of hexane was added to remove the fat. The solution was then centrifuged at 13,000 rpm for 5 min (IKA G-L centrifuge, Germany). The lower layer was collected, filtered through a 0.2-µm polyvinylidene fluoride (PVDF) filter, and transferred into an injection vial (10 µL). The internal quality control also included a negative (blank with only reagent) and a positive sample (spiked at a known concentration, 50 µg/kg).

## 2.2 High-performance liquid chromatography coupled with tandem mass spectrometry determination

### 2.2.1 Chromatographic conditions

The HPLC-MS/MS system consisted of a binary liquid chromatography (LC) pump linked to an autosampler (Agilent 1200 Model, Agilent Technologies, USA) and coupled to a Triple Quadrupole LC-MS (Agilent 6410, Agilent Technologies, USA). The separation was achieved using a reversed-phase HPLC column, Zorbax Eclipse Plus C18 (3.5 µm; 2.1x150mm; Agilent Technologies, USA), and maintained at 30°C using an oven. The flow rate and injection volumes were 0.2 mL/min and 10 µL, respectively. The mobile phases used were (A) 0.1% formic acid in dH<sub>2</sub>O and (B) acetonitrile. The isocratic eluent program was applied throughout 20 min with maintaining 90% mobile phase A (0.1% formic acid in dH<sub>2</sub>O) and 10% mobile phase B (acetonitrile).

### 2.2.2 Mass spectrometry condition

Electrospray Ionisation (ESI) was used in MS positive mode for all analytes. The source parameters were gas temperature at 350°C, gas flow at 10 L/min, capillary voltage of 4,000 V, and nebuliser pressure of 45 psi. The analysis was carried out in the Multiple Reaction Monitoring (MRM) condition as listed in Table 2.

## 2.4 Method validation

This step was to determine the method performance parameters and the minimum requirements of acceptance that must be fulfilled to be considered as valid and accurate. The protocol of the European Community (EU, 2002) was used as a guideline to perform validation of the methods. The HPLC-MS/MS method was validated for the determination of the selected analytes (SQX, SDM, SMR, SDD, SMM, SDZ, SPD, and STZ) by assessing its specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), trueness, and matrix effect. Sulfadimethoxine-d6 (SDM-d6) was used

Table 2. MS/MS conditions for each sulphonamide analyte.

Analyte	Precursor ion	Product ion	Fragmenter (V)	Collision energy (CE) (V)
SQX	301	208	110	20
	301	156	110	10
SDM	311	245	110	20
	311	156	110	20
SMR	265	172	110	10
	265	156	110	20
SDD	279	186	110	20
	279	156	110	20
SMM	281	188	110	20
	281	156	110	20
SDZ	251	156	110	20
	251	108	110	20
SPD	250	184	100	20
	250	156	100	10
STZ	256	156	110	10
	256	108	110	25

as an ISTD for rescaling the analyte's signal to the standard's signal, in order to compensate for the fluctuations and changes occurring during sample extraction (Joseph *et al.*, 2022; Cheng *et al.*, 2023).

### 2.4.1 Specificity

The purpose of specificity is to ensure the absence of any interference within the retention time segment of each product ion (Guillén *et al.*, 2016). Specificity of the method was performed by injecting ten blank samples (matrix without analyte), and ten samples spiked at the limit of quantification (LOQ) level (matrix with analyte). The method, showing good specificity, can accurately distinguish the desired analytes even when other substances are present that might cause interference.

### 2.4.2 Linearity

The purpose of linearity is to examine the correlation between the calibration plot of response with concentration to form an approximately straight line (Ravisankar *et al.*, 2015). Linearity was performed by analysis of three batches of samples spiked at six concentration levels (5, 10, 20, 50, 100, 150 µg/kg). The standard calibration curves were constructed by plotting concentrations (µg/kg) versus response (peak area ratio of analyte to SDM-d6). The analysis of linearity was carried out by the method of least squares and expressed in  $R^2$ , the coefficient of determination. A high  $R^2$  of 0.99 is often used as a criterion of linearity (UNODC, 2009).

### 2.4.3 Matrix effect

A matrix effect is defined as a change in the analyte's response caused by interfering components present in the sample matrix (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), 2022). This can lead to inaccurate results during quantitative analysis. Matrix effects were carried out due to the complexity of the samples used (chicken meat) by comparing the matrix-matched calibration to the standard solution calibration. For the matrix-matched curve, chicken meat was spiked with a selected concentration of the sulphonamides solution (Table 1), and SDM-d6 was used as an ISTD at 20 µg/kg (20 µL taken from 2 µg/mL). Matrix-matched calibration was built at six concentration levels (5, 10, 20, 50, 100, and 150 µg/kg), and the responses (sulphonamide/SDM-d6 peak ratio) versus concentration were plotted. For slope difference, an F-test was applied; if  $F_{critical} < F_{calculated}$ , the two curves are considered having similar variances. Besides, matrix effects were also expressed as the ratio between the calibration curve slopes of matrix-matched standards and solvent-based standards (Matrix effect =  $\frac{\text{slope}_{\text{spiked sample}}}{\text{slope}_{\text{standard solution}}}$ ) (Romero-González *et al.*, 2011). The slope ratio should fall between 0.9 – 1.1 to prove the insignificant difference of analyte recoveries between spiked samples and standard solution (Hoff *et al.*, 2015). If matrix effects were observed, ISTD should be added in the very beginning of the sample pre-treatment (sample extraction) procedures to compensate for the loss/gain during the process (Zhou *et al.*, 2017). Besides, calibration standards can be prepared in the same matrix as the test samples to account for interference effects.

#### 2.4.4 Precision (repeatability)

The precision of a method refers to the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under similar analytical conditions (Ravisankar *et al.*, 2015). The purpose of determining precision is to ensure that repeated measurements yield similar results, confirming the method's consistency. Precision (repeatability) was performed by spiking at three concentration levels (10, 50, and 150 µg/kg), and following the standard protocol method; each sample was run in duplicates. A total of ten repetitions (20 measurements) were prepared for each concentration level. Repeatability ( $r$ ) is the value below which the absolute difference between duplicate samples acquired might be expected to achieve 95% probability. Repeatability limit ( $r$ ), repeatability standard deviation (SD) ( $s_r$ ), relative SD of repeatability ( $RSD_r$ ), calculated relative SD of repeatability ( $RSD$  calculated), and Horwitz ratio of repeatability ( $HorRat_r$ ) were calculated using Equations 1-5. The precision was also evaluated by

$HorRat_r$ , at which the acceptable values for  $HorRat_r$  should be between 0.3 to 1.3 (AOAC, 2002).

$$r = 2.8 S_r \quad (1)$$

(ISO 5725, 1994)

$$S_r = \sqrt{\frac{\sum W^2}{2n}} \quad (2)$$

Where,  $S_r$  = repeatability SD,  $W$  = absolute differences between duplicates, and  $n$  = total number of repetitions analysed in duplicate (in this study,  $n = 10$ ).

(OIV, 2005)

$$RSD_r = \frac{s_r}{\bar{x}} \times 100 \quad (3)$$

where,  $RSD_r$  = relative SD of repeatability,  $S_r$  = repeatability standard deviation, and  $\bar{x}$  = mean of all of the measurements.

(ISO 5725, 1994)

$$RSD(\text{calculated}) = C^{-0.15} \quad (4)$$

Where  $RSD(\text{calculated})$  = predicted relative SD of repeatability, which varies with the concentration, and  $C$  = concentration of analyte expressed as MS fraction.

(AOAC, 2002)

$$HorRat_r = RSD_r / RSD(\text{calculated}) \quad (5)$$

Where,  $HorRat_r$  value = ratio of  $RSD_r$  (obtained from laboratory data) to the  $RSD(\text{calculated})$  from the Horwitz equation, and  $C$  = concentration of analyte expressed as mass fraction.

(AOAC, 2016)

#### 2.4.5 Trueness (recovery)

The trueness of a method refers to the closeness of agreement between the average of replicate measured values and a reference quantity value (Prenezi and Gosmaro, 2014). The purpose of determining trueness is to ensure that the method provides accurate measurements, thereby guaranteeing the reliability of the results. Trueness was performed by injecting three different concentration levels (10, 50, and 150 µg/kg) on three different days to spike chicken samples. The replicate analysis (four for each level) was repeated on separate days for a total of  $n = 60$  experiments. In order to compare the difference between the mean of spiked ( $\bar{x}_{\text{spike}}$ ) and blank ( $\bar{x}_{\text{blank}}$ ) value (non-spiked) with the added concentration, Equation 6 was used for the determination of recovery:

$$\text{Trueness (\%)} = \frac{\bar{x}(\text{spike}) - \bar{x}(\text{blank})}{\text{Spiking Concentration}} \times 100\% \quad (6)$$

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(Magnusson and Örnemark, 2014)

The European Commission (2002) suggested the following limits for residues of veterinary drugs in foods (Table 3).

Table 3. Accepted value of trueness value (%) for different concentration ranges according to the European Commission (2002).

Concentration ( $\mu\text{g}/\text{kg}$ )	Acceptable range (%)
$\leq 1$	50-120
1-10	70-110
$\geq 10$	80-110

#### 2.4.6 Limit of detection and limit of quantification

The LOD is a concentration where exceeding by a measured value indicates that the sample contains the desired analytes (Huber, 2003). LOD was performed by analysis of three batches of chicken samples spiked at six different concentration levels (0.25, 0.5, 1, 2, 4, 8  $\mu\text{g}/\text{kg}$ ). LOD was calculated based on the SD of the intercepts ( $S_{\text{intercept}}$ ) of the calibration curve, and the slope of the calibration curve (S) using Equation 7:

$$\text{LOD} = 3.3 S_{\text{intercept}}/S \quad (7)$$

Where  $S_{\text{intercept}}$  = SD of intercepts of calibration curves, and S = the slope of the calibration curve.

(ICH, 2005)

The LOQ is the minimum concentration at which the analytes can be quantified with acceptable accuracy and precision (Epshtein, 2021). LOQ was calculated based on the SD of the response ( $S_{\text{response}}$ ) curve and the slope of the calibration curve (S) using Equation 8. The values obtained for  $S_{\text{intercept}}$ ,  $S_{\text{response}}$ , and the slope (S) were generated by the LINEST function in MSExcel:

$$\text{LOQ} = 10 S_{\text{response}}/S \quad (8)$$

Where,  $S_{\text{response}}$  = SD of response, and S = Mean of slopes of the calibration curves.

(ICH, 2005)

### 3. Results and discussion

The method was validated following the criteria specified in European Commission Decision 2002/657/EC for a quantitative method. The validation criteria included specificity, linearity, matrix effect, precision, trueness, LOD, and LOQ. The performance of the improved analytical method was assessed by verifying the identification criteria for the presence of two transitions at the same retention time, the signal-to-noise ratio of  $\geq 10$ , the relative retention time of the analyte

falling within a tolerance of 2.5%, and the relative ion intensity ratio within a tolerance defined by the EU Commission Decision 2002/657/EC. The ion ratios for the matrix are shown in Table 2. For specificity, each of the analytes, two ion transitions were monitored (Table 1), for example, SQX was monitored for two pairs of precursor ion-to-product ion, which were 301>156 and 301>208, in order to ensure the accurate and specific analyte (De la Cruz *et al.*, 2011). The chromatograms of the reference standards of all the analytes are shown in Figure 1. All of the peaks were clearly plotted at their respective retention time, showing that the method could accurately detect the target analyte. There were no interfering peaks at the same retention time, confirming that the signal observed was solely attributed to the intended analytes, without being affected by other substances in the sample. Additionally, the clear separation of peaks further emphasised the method's ability to detect the individual analyte.

For linearity, matrix-matched calibration curves were used in this study due to the complexity of the sample matrix. Linearity was evaluated in triplicate in matrix-matched calibration curves. The least-squares linear regression was carried out by plotting the concentrations versus response (peak area ratio of sulphonamide analytes to SDM-D6). Table 4 depicts the linear regression data for all analytes, and the data showed that all  $R^2$  values were above 0.99, indicating a strong and reliable linear relationship between the responses and the analytes' concentration within the tested range (Lopes *et al.*, 2012). Therefore, all regressions were displaying good linearity within the tested range, making the method suitable for accurate quantification.

Table 4. Regression equation and  $R^2$  for sulphonamide analytes.

Analyte	Regression equation	$R^2$
SDZ	$y = 1.0447 x - 1.01$	0.9951
SMM	$y = 0.9663 x + 1.28$	0.9954
SDD	$y = 0.9677 x + 0.77$	0.9954
STZ	$y = 0.9794 x + 0.52$	0.9959
SMR	$y = 1.0592 x - 1.27$	0.9916
SQX	$y = 1.0406 x - 0.04$	0.9924
SPD	$y = 0.9786 x + 0.95$	0.9954
SDM	$y = 1.0293 x - 0.42$	0.9951

SQX: sulfaquinoxaline, SDM: sulfadimethoxine, SMR: sulfamerazine, SDD: sulfadimidine, SMM: sulfamonomethoxine, SDZ: sulfadiazine, SPD: sulfapyridine, STZ: sulfathiazole

According to the International Union of Pure and Applied Chemistry (IUPAC), matrix effect is defined as the combined effect of all components of the sample

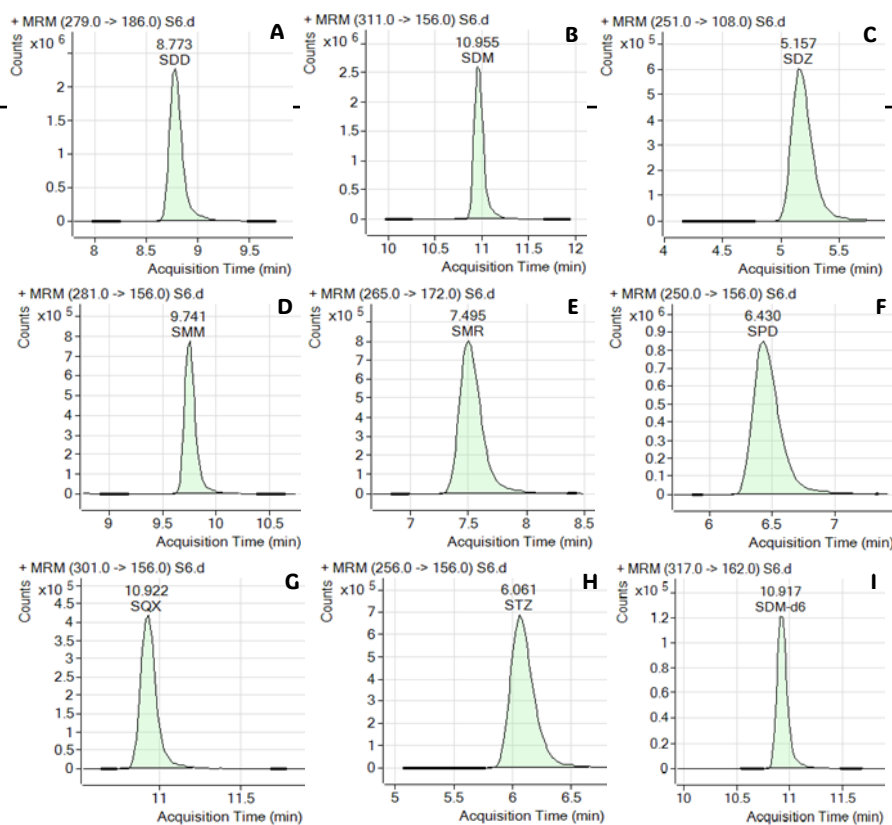


Figure 1. Chromatograms of sulphonamide analyte. A - I show respective peaks that represent each of the sulphonamide analytes at their respective retention time. A is SDD peak ( $m/z$ : 279>186) at retention time of 8.733 min; B is SDM peak ( $m/z$ : 311>156) at retention time of 10.955 min; C is SDZ peak ( $m/z$ : 251>108) at retention time of 5.157 min; D is SMM peak ( $m/z$ : 281>156) at retention time of 9.741 min; E is SMR peak ( $m/z$ : 265>172) at retention time of 7.495 min; F is SPD peak ( $m/z$ : 250>156) at retention time of 6.430 min; G is SQX peak ( $m/z$ : 301>156) at retention time of 10.922 min; H is STZ peak ( $m/z$ : 256>156) at retention time of 6.061 min; I is SDM-D6 peak ( $m/z$ : 317>162) at retention time of 10.917 min. SQX: sulfaquinoxaline, SDM: sulfadimethoxine, SMR: sulfamerazine, SDD: sulfadimidine, SMM: sulfamonomethoxine, SDZ: sulfadiazine, SPD: sulfapyridine, STZ: sulfathiazole.

other than the analyte on the measurement of the quantity. If a specific component can be identified that causes an unwanted effect, then this is referred to as an interference (Zhu *et al.*, 2018). This definition represents the complex phenomenon that exists in reality within samples. It can be observed in ion suppression or enhancement caused by the alteration of ionisation efficiency in the ionisation source. The main reason for this effect was the presence of endogenous substances that were present natively in the sample, which were not removed by the extraction process, and remained prior to analysis (Hoff *et al.*, 2015). Using the ratio equation (Matrix effect = slope<sub>spiked sample</sub> / slope<sub>standard solution</sub>), the differences between the two slopes (spiked sample and standard solution) were determined and recorded. Table 5 depicts the slope ratio and F-test result for the sulphonamide analyte. All of the analytes demonstrated slope ratios out of the range of 0.9 – 1.1, indicating there was a significant difference between slope<sub>spiked sample</sub> and slope<sub>standard solution</sub>, and the analytes analysis was interfered with by the effects of the matrix. Additionally, the F-test result for all the analytes showed  $F_{critical} > F_{calculated}$ , indicating the variation between slope<sub>spiked sample</sub> and slope<sub>standard solution</sub>. These results proved that there was a presence of matrix effect in this experiment, and the

calibration curves must be determined in spiked samples for accurate quantification of sulphonamide in chicken meat samples.

Table 5. Slope ratio and F-test results for sulphonamide analytes.

Analyte	Slope ratio	$F_{calculated}$	$F_{critical}$	Variation significant
SDZ	8.8	0.007	5.05	True
SMM	7.6	0.002	5.05	True
SDD	8.4	0.002	5.05	True
STZ	7.0	0.024	5.05	True
SMR	10.5	0.004	5.05	True
SQX	0.6	0.138	5.05	True
SPD	9.0	0.005	5.05	True
SDM	2.8	0.030	5.05	True

\*none of the analyte gives a slope ratio between 0.9-1.1. SQX: sulfaquinoxaline, SDM: sulfadimethoxine, SMR: sulfamerazine, SDD: sulfadimidine, SMM: sulfamonomethoxine, SDZ: sulfadiazine, SPD: sulfapyridine, STZ: sulfathiazole.

Table 6 presents the repeatability ( $r$ ) and HorRat<sub>r</sub> values for all sulphonamide analytes. Repeatability limit at 10  $\mu\text{g}/\text{kg}$  ranged from 1.522 – 2.522, at 50  $\mu\text{g}/\text{kg}$  ranged from 8.112 – 10.181, and at 150  $\mu\text{g}/\text{kg}$  ranged from 16.651 – 27.425. As the concentration increased, the repeatability limit also increased, demonstrating the concentration-dependent nature of the repeatability. The HorRat<sub>r</sub> value obtained was in the range from 0.301 to 0.687, which fell within the acceptable Horwitz range, indicating a successful collaborative trial and good method performance. Precision (repeatability) is referred to as the intra-laboratory coefficient of variation for the repetitive analysis of a fortified material or a standard. Repeatability limit ( $r$ ) is the value below which the absolute difference between two single test results, obtained under repeatability conditions, is expected to lie with a probability of 95% (confidence level of 0.95) (Massart et al, 1998). If the absolute difference between two results exceeds the repeatability limit ( $r$ ), the test results are considered unacceptable, and the tests should be repeated (Pang, 2018). The repeatability limit is calculated based on concentration and will be applied in

Table 6. Repeatability limit and HorRat<sub>r</sub> of sulphonamide analytes.

Analyte	Concentration	Repeatability limit ( $r$ )	HorRat <sub>r</sub>
SDZ	10 $\mu\text{g}/\text{kg}$	1.616	0.376
	50 $\mu\text{g}/\text{kg}$	10.053	0.687
	150 $\mu\text{g}/\text{kg}$	16.651	0.445
SMM	10 $\mu\text{g}/\text{kg}$	2.481	0.630
	50 $\mu\text{g}/\text{kg}$	9.850	0.627
	150 $\mu\text{g}/\text{kg}$	25.161	0.576
SDD	10 $\mu\text{g}/\text{kg}$	2.522	0.629
	50 $\mu\text{g}/\text{kg}$	8.238	0.516
	150 $\mu\text{g}/\text{kg}$	20.434	0.518
STZ	10 $\mu\text{g}/\text{kg}$	2.034	0.520
	50 $\mu\text{g}/\text{kg}$	8.112	0.535
	150 $\mu\text{g}/\text{kg}$	21.049	0.537
SMR	10 $\mu\text{g}/\text{kg}$	1.829	0.514
	50 $\mu\text{g}/\text{kg}$	9.815	0.634
	150 $\mu\text{g}/\text{kg}$	27.425	0.676
SQX	10 $\mu\text{g}/\text{kg}$	2.522	0.550
	50 $\mu\text{g}/\text{kg}$	10.181	0.661
	150 $\mu\text{g}/\text{kg}$	21.551	0.503
SPD	10 $\mu\text{g}/\text{kg}$	2.105	0.561
	50 $\mu\text{g}/\text{kg}$	8.262	0.567
	150 $\mu\text{g}/\text{kg}$	23.911	0.643
SDM	10 $\mu\text{g}/\text{kg}$	1.535	0.301
	50 $\mu\text{g}/\text{kg}$	8.386	0.508
	150 $\mu\text{g}/\text{kg}$	21.420	0.497

SQX: sulfaquinolaxaline, SDM: sulfadimethoxine, SMR: sulfamerazine, SDD: sulfadimidine, SMM: sulfamonomethoxine, SDZ: sulfadiazine, SPD: sulfapyridine, STZ: sulfathiazole.

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the routine analysis as one of the criteria to judge the precision of the particular test results.

The precision is also evaluated by HorRat<sub>r</sub>, at which the acceptable values for HorRat<sub>r</sub> range from 0.3 to 1.3 (AOAC, 2016). Values at the extremes must be interpreted with caution. The Horwitz equation was used to calculate the exponential relationship between RSD and the analyte concentration, providing a robust framework for evaluating method performance. The precision is better than expected if the HorRat<sub>r</sub> value is less than 1, and poorer if greater than 1 (Horwitz *et al.*, 2006). On the other hand, the HorRat<sub>r</sub> value obtained in this study indicated that the method used was under statistical control and acceptable with respect to precision.

Table 7 shows the mean of trueness and its SD. The acceptable range determined by the European Commission (2002) is between 80-110% and SD ( $\pm$ ) below 10. All the sulphonamide analytes showed an acceptable range and consistent trueness data, which were between 80.21% and 103.39% with SD ( $\pm$ ) of 2.27 to 9.69. Trueness is usually expressed as bias and

Table 7. Trueness and its deviation for sulphonamide analytes.

Analyte	Concentration	Trueness	SD
SDZ	10 $\mu\text{g}/\text{kg}$	96.74	9.69
	50 $\mu\text{g}/\text{kg}$	83.99	5.31
	150 $\mu\text{g}/\text{kg}$	84.37	4.80
SMM	10 $\mu\text{g}/\text{kg}$	88.78	9.59
	50 $\mu\text{g}/\text{kg}$	90.18	6.99
	150 $\mu\text{g}/\text{kg}$	98.50	8.04
SDD	10 $\mu\text{g}/\text{kg}$	90.41	9.05
	50 $\mu\text{g}/\text{kg}$	91.53	6.98
	150 $\mu\text{g}/\text{kg}$	88.96	8.57
STZ	10 $\mu\text{g}/\text{kg}$	88.06	9.63
	50 $\mu\text{g}/\text{kg}$	86.99	5.29
	150 $\mu\text{g}/\text{kg}$	88.38	5.87
SMR	10 $\mu\text{g}/\text{kg}$	80.21	6.19
	50 $\mu\text{g}/\text{kg}$	88.80	8.34
	150 $\mu\text{g}/\text{kg}$	91.48	8.41
SQX	10 $\mu\text{g}/\text{kg}$	103.39	7.94
	50 $\mu\text{g}/\text{kg}$	88.37	7.03
	150 $\mu\text{g}/\text{kg}$	96.68	7.10
SPD	10 $\mu\text{g}/\text{kg}$	84.61	8.99
	50 $\mu\text{g}/\text{kg}$	83.64	5.75
	150 $\mu\text{g}/\text{kg}$	83.89	7.12
SDM	10 $\mu\text{g}/\text{kg}$	98.58	3.18
	50 $\mu\text{g}/\text{kg}$	94.75	2.27
	150 $\mu\text{g}/\text{kg}$	97.24	4.45

SQX: sulfaquinolaxaline, SDM: sulfadimethoxine, SMR: sulfamerazine, SDD: sulfadimidine, SMM: sulfamonomethoxine, SDZ: sulfadiazine, SPD: sulfapyridine, STZ: sulfathiazole.

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assessed by assuming that the bias is the same in the whole concentration range. Spiked samples were compared with the non-spiked same sample (blank sample) to assess the net recovery of the added sulphonamides.

The trueness data within 10% SD indicates that the analytes were successfully extracted, demonstrating good accuracy for the pre-treatment method. At low concentration (10 µg/kg), trueness exhibits greater variability in SD; however, most of the Maximum Residue Levels (MRL) are set at 100 µg/kg, where the method demonstrates good precision, ensuring that the safety threshold is well addressed. The current extraction method demonstrated relatively higher recoveries of sulphonamides compared to other extraction protocols. The recovery of sulphonamides ranged from 38.4% to 103.6% using QuEChERS (Nunes *et al.*, 2018); 67.8% to 113.9% with liquid-liquid extraction (Cai *et al.*, 2008); and 60.0% to 92.0% when combining liquid-liquid extraction with SPE (Mehtabuddin *et al.*, 2012).

Table 8 shows the LOD and LOQ of the sulphonamide analytes. Collectively, the LOD obtained was below 0.6 µg/kg, while the LOQ was below 5 µg/kg, demonstrating the high sensitivity of the method used. These threshold concentrations are important for ensuring compliance with regulatory standards as outlined in the Food Regulations 1985. The determination of LOD and LOQ follows the recommendations provided in ICH Q2 (R1) guidelines (ICH, 2005), which list a few approaches suitable for different analytical methods. These approaches include visual evaluation, signal-to-noise ratio, the SD of the response, and the slope's SD of the calibration curve. In this analysis, the SD of the intercept and the SD slope were used for the determination of LOD and LOQ, respectively. The choice of method depends on the instrument, such as the analyte's nature, the instrument's sensitivity, and the suitability of the method. The LOD

Table 8. LOD and LOQ of sulphonamide analytes.

Analyte	LOD	LOQ
SDZ	0.285	2.631
SMM	0.375	3.457
SDD	0.258	2.381
STZ	0.187	1.727
SMR	0.307	2.832
SQX	0.352	3.247
SPD	0.335	3.095
SDM	0.522	4.815

SQX: sulfaquinolaxaline, SDM: sulfadimethoxine, SMR: sulfamerazine, SDD: sulfadimidine, SMM: sulfamonomethoxine, SDZ: sulfadiazine, SPD: sulfapyridine, STZ: sulfathiazole

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and LOQ values obtained in this study were consistent with those reported in several other studies. For instance, Ma, Fan, Sun *et al.* (2020) achieved similar results using online SPE, with LODs ranging from 0.125 to 2.00 µg/kg, and LOQs from 0.25 to 5 µg/kg. Similarly, Yu and Hu (2012) reported comparable LODs (0.9–10.5 µg/L) using stir bar absorptive extraction.

A comparison between the present study and existing literature methods in terms of sample preparation, solvent usage, preparation time, cost, and analytical sensitivity is summarised in Table 9. Sample preparation methods for sulphonamide residue analysis commonly involve Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE) (Amvrazi and Albanis, 2006; Chitescu *et al.*, 2011; Shi *et al.*, 2011; Patyra *et al.*, 2019; Chandrakar *et al.*, 2023), which are known for moderate to high solvent consumption and longer preparation times due to multiple procedural steps. These methods also incurred higher costs due to the use of SPE cartridges and multiple solvents. In contrast, the present study adopted a simple technique, which required minimal solvent usage, significantly reduced preparation time, and lowered overall analytical costs. Moreover, the present method demonstrated good sensitivity with LOD ranging from 0.187 to 0.375 µg/kg and LOQ between 1.727 and 4.815 µg/kg. These values are markedly lower than those reported in previous literature, indicating enhanced analytical performance of the current method. The comparable performance of the current method to these well-established techniques underscores its suitability for achieving precise and reliable detection and quantification of analytes.

#### 4. Conclusion

The improved sulphonamide residue analysis method in the present study demonstrated an efficient and rapid approach due to its simple extraction procedure, which requires only basic laboratory equipment, a small sample size, and a simple mobile phase for quick separation. Validation result demonstrated good performance characteristics, including strong linearity (all  $R^2 > 0.99$ ), good repeatability (HorRat<sub>x</sub> value between 0.301 and 0.687), acceptable trueness (recoveries between 80.21% and 103.39%) and high sensitivity (LOD < 0.6 µg/kg; LOQ < 5 µg/kg). Overall, these findings confirm that the method is a robust, time-efficient, and cost-effective approach for the determination of sulphonamide residues in raw chicken meat.

#### Conflict of interest

The authors declare no conflict of interest.

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