JHWCR JOURNAL OF HEALTH, WELLNESS AND COMMUNITY RESEARCH 3007-0570

Original Article Volume 4, Issue 4

A Rapid and Simple Method for Multi-Residue Sulfonamide Analysis in Poultry Meat

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ABSTRACT

Background: *Sulfonamides are widely used in poultry production for disease prevention and treatment, but their misuse or inadequate withdrawal before slaughter may result in residues in edible tissues. Monitoring these residues requires analytical methods that are sensitive, reliable, and practical for routine use, particularly in laboratories with limited access to advanced mass spectrometry-based instrumentation.* ***Objective:*** *To develop and validate a simple and rapid HPLC-DAD method for the simultaneous determination of five sulfonamide residues in poultry meat, and to assess its applicability to real retail samples.* ***Methods:*** *Five sulfonamides—sulfadiazine, sulfamerazine, sulfadimidine, sulfamethoxazole, and sulfamethizole were extracted from poultry meat using liquid-liquid extraction with ethyl acetate, followed by defatting with n-hexane. Chromatographic separation was performed on a C18 column using isocratic elution with acetonitrile and 0.1% formic acid in water (30:70, v/v), with detection at 270 nm. The method was validated according to ICH Q2(R1) guidelines for specificity, linearity, accuracy, precision, robustness, limit of detection, and limit of quantification. The validated method was then applied to ten poultry meat samples collected from retail markets in Hyderabad, Pakistan.* ***Results:*** *The method showed good linearity over the concentration range of 5-500 ng/mL, with correlation coefficients of 0.99. The limits of detection ranged from 1.2 to 2.8 µg/kg, while the limits of quantification ranged from 3.6 to 8.4 µg/kg, all below the regulatory limit of 100 µg/kg. Mean recoveries ranged from 88.4% to 102.1%, and intra-day and inter-day precision values were below 6% RSD. All five analytes were separated within 8 min, with a total run time of 12 min. Application of the method to retail poultry meat samples showed detectable sulfonamide residues in 38% of samples, with concentrations ranging from 3.4 to 60 µg/kg; none exceeded the regulatory limit.* ***Conclusion:*** *The proposed HPLC-DAD method is simple, rapid, and reliable for the simultaneous determination of five sulfonamide residues in poultry meat. Its satisfactory validation performance, short analysis time, and use of commonly available laboratory equipment make it suitable for routine food safety monitoring, particularly in resource-limited settings.*

Keywords: *Sulfonamides; HPLC-DAD; Poultry meat; Antibiotic residues; Food safety*

INTRODUCTION

The use of sulfonamides as antibacterial agents has become widespread in chicken production since their discovery (1, 2). Farmers use these antibiotics for disease prevention, therapeutic treatment of bacterial infections, and, in some situations, growth promotion (3). Their low cost, broad-spectrum activity, and ease of use make them suitable for intensive poultry production (4). However, the use of these medications close to the time of slaughter can result in residues remaining in edible tissues, thereby contaminating the food chain and posing potential risks to consumer health. Antibiotics administered to poultry may persist in tissues as residues when adequate withdrawal periods are not observed before slaughter (5). Different studies have reported the presence of such residues in meat and liver (6). In response to these concerns, regulatory authorities have established maximum residue limits

Received: 09 January 2026
Revised: 16 January 2026
Accepted: 25 February 2026
Published: 28 February 2026

Citation: Click to Cite

(MRLs), including a limit of 100 µg/kg for sulfonamides in meat, to minimize consumer exposure (7, 8).

The detection of these residues in a complex meat matrix requires a suitable analytical approach. The chemical structures of the most common sulfonamides are shown in Figure 1. Liquid chromatography coupled with mass spectrometry (LC-MS) is the most commonly used technique for sulfonamide analysis because of its high sensitivity and ability to detect multiple compounds in a single run (9-12). However, many laboratories, particularly those operating in resource-limited settings, do not have access to such advanced instrumentation. The high capital cost, continuous maintenance requirements, and need for specialized technical expertise often limit the routine use of LC-MS in quality control and public health laboratories (13). In such circumstances, there is a need for a simple analytical method that relies on more widely available instrumentation while still providing reliable results (14-16).

Therefore, the aim of this study was to develop a simple and rapid HPLC-DAD method for the simultaneous determination of five sulfonamides—sulfadiazine, sulfamerazine, sulfadimidine, sulfamethoxazole, and sulfamethazine—commonly used in poultry production in Pakistan. In addition, the performance characteristics of the method were evaluated according to ICH Q2(R1) guidelines, and its practical applicability was assessed by analyzing real poultry meat samples.

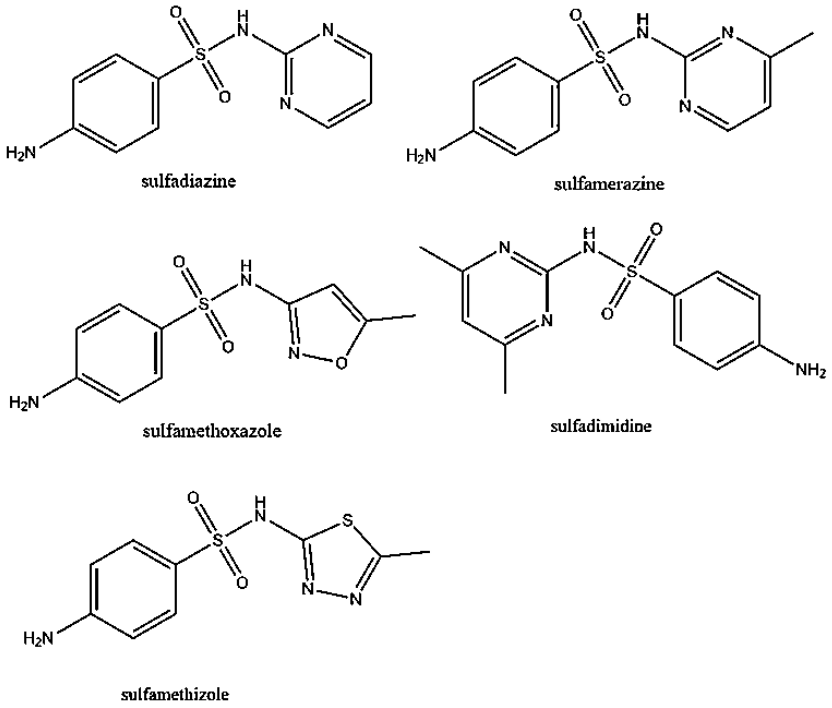

Figure 1 Chemical Structure of five sulfonamides

MATERIALS AND METHODS

Chemicals and instrumentation

Pure standards of sulfadiazine, sulfamerazine, sulfadimidine, sulfamethoxazole, and sulfamethizole were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade n-hexane, ethyl acetate, methanol, acetonitrile, and formic acid were purchased from Fisher Scientific (Loughborough, UK). Analytical-grade potassium chloride, sodium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Ultra-pure water was prepared in-house using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic analysis was

performed using a Shimadzu HPLC system equipped with an LC-20AT solvent delivery pump, SIL-20AC autosampler, CTO-20AC column oven, and SPD-M20A photodiode array detector. Separation was achieved using a Hypersil Gold C18 column (250 mm × 4.6 mm internal diameter, 5 µm particle size) purchased from Thermo Scientific. Equipment used for sample preparation included a Hermle Z200A centrifuge with a maximum capacity of 5000 rpm, a Vortex-Genie 2 mixer (Scientific Industries), and a Remi R-300 rotary evaporator (Thermo Scientific). Samples were weighed using an analytical balance (Sartorius TE214S).

Sample preparation and extraction

Calibration standards of 5, 10, 25, 50, 100, 250, and 500 ng/mL were prepared by serial dilution from a 100 µg/mL mixed sulfonamide stock solution prepared in methanol and stored at −20°C until analysis. Sulfonamide extraction was performed using a liquid-liquid extraction procedure adapted from Premarathne et al. (2017) (17). Briefly, approximately 5.0 g of homogenized meat tissue was weighed into a 50 mL polypropylene centrifuge tube, and 5 mL of phosphate-buffered saline (pH 7.4) was added. A total of 20 mL of ethyl acetate was then added, and the mixture was vortexed for 5 min, followed by centrifugation at 4,000 × g for 10 min. The upper organic layer was carefully collected, and the extraction was repeated twice using fresh 10 mL aliquots of ethyl acetate. The combined extracts were evaporated to dryness at 40°C using a rotary evaporator. The dried residue was reconstituted in 2.0 mL of mobile phase, and fat removal was performed by adding 1.0 mL of n-hexane, after which the lower aqueous-organic layer was retained. The final extract was filtered through a 0.45 µm PTFE membrane syringe filter.

To evaluate the accuracy and recovery of the extraction method, blank homogenized chicken muscle samples (5.0 g ± 0.05) were fortified with a mixed sulfonamide working standard solution at three concentration levels: 10, 100, and 400 ng/g. Fortified samples were allowed to equilibrate at room temperature for 30 min prior to extraction to allow adequate binding of the analytes to the matrix, after which they were subjected to the full extraction procedure described above.

Optimization and validation

To optimize the chromatographic separation of the five sulfonamides, different mobile-phase compositions, column temperatures, and detection wavelengths were evaluated. The best separation was achieved using a mobile phase consisting of acetonitrile and 0.1% (v/v) formic acid in water (30:70, v/v), delivered at a constant flow rate of 1.0 mL/min, with the column temperature maintained at 30°C and an injection volume of 20 µL. After evaluating the absorption maxima of each drug, 270 nm was selected as the optimal detection wavelength. All five compounds eluted between 4 and 8 min, with sufficient resolution between all peak pairs during the 12-min run time.

Specificity of the method was assessed by analyzing blank meat samples spiked at the MRL level. Linearity over the range of 5–500 ng/mL was evaluated, and the correlation coefficient, slope, y-intercept, and residual plots were calculated according to ICH guidelines (18, 19). Accuracy was evaluated by recovery studies at three spike levels (10, 100, and 400 ng/g), using six replicates at each level (20). Precision was assessed in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). The limit of detection (LOD) and limit of quantification (LOQ) were determined using the signal-to-noise ratio approach.

Robustness was evaluated by deliberately varying method parameters within reasonable limits, including flow rate (±0.1 mL/min), column temperature (±2°C), and mobile-phase organic content (±2%), as described previously (21). The applicability of the developed

method was assessed by determining sulfonamide residues in ten poultry meat samples obtained from retail markets in Hyderabad.

Statistical analysis

Calibration curves were generated by least-squares regression, and significance was tested by ANOVA ($p < 0.05$). Validation was performed according to ICH guidelines, with acceptance criteria of 80–120% recovery, $R^2 > 0.99$, and precision expressed as RSD <15% at the LOQ and <10% at higher concentration levels. Samples were considered positive when concentrations exceeded the LOQ. Data analysis was carried out using OriginPro 2021 and Excel 2016 (20, 22).

RESULTS AND DISCUSSION

Method Development and Optimization

To develop a chromatographic separation that balanced resolution, analysis time, and sensitivity, several parameters were systematically evaluated, starting with different mobile phases. Initially, methanol–water combinations were tested, but these produced asymmetric peaks. Peak shape improved after switching to acetonitrile as the organic modifier, which reduced tailing for all five sulfonamides, and was further improved by adding 0.1% formic acid to the aqueous component. This may have reduced silanol interactions with the basic sulfonamide functional groups (14). Both isocratic and gradient elution approaches were investigated. Although gradient elution theoretically allows compounds to be separated over a wider polarity range, the practical benefit did not justify the additional complexity for the five target sulfonamides (23). Baseline separation of all analytes was achieved within 8 min using isocratic elution with 30% acetonitrile (12). Under the optimized conditions, the retention times of sulfadiazine, sulfamerazine, sulfadimidine, sulfamethoxazole, and sulfamethizole were 4.12, 4.89, 5.67, 6.34, and 7.21 min, respectively. This compares favorably with the 15–20 min run times reported in similar studies (24).

Specificity was evaluated by analyzing blank meat samples, and no interfering peaks were observed at the retention times of the target sulfonamides in the chromatograms (12, 18). Linearity was assessed over the concentration range of 5–500 ng/mL, spanning two orders of magnitude (25). Samples containing residues at the MRL of 100 µg/kg would be expected to produce responses near the midpoint of this calibration range, supporting accurate quantification, as shown in Table 1. According to ICH criteria, all five compounds showed good linearity, with correlation coefficients of 0.99.

Table 1. Calibration curve parameters for five sulfonamides

Compound	R2	Range (ng/mL)	LOD (µg/kg)	LOQ (µg/kg)
Sulfadiazine	0.99	5–500	1.2	3.6
Sulfamerazine	0.99	5–500	1.5	4.5
Sulfadimidine	0.99	5–500	1.8	5.4
Sulfamethoxazole	0.99	5–500	2.8	8.4
Sulfamethizole	0.99	5–500	1.4	4.2

R2 = Correlation coefficient; LOD = Limit of detection; LOQ = Limit of quantification.

Method performance characteristics, including limits of detection and quantification, are presented in Table 1. Sulfadiazine showed the lowest detection limit at 1.2 µg/kg. These values place the sensitivity of the method well below the MRL of 100 µg/kg, providing a 12- to 28-

fold margin relative to the regulatory limit (26). Accuracy was assessed by spiking blank samples at three concentration levels, and the results are presented in Table 2. Mean recoveries ranged from 88.4% to 102.1%, which falls within the 80–120% acceptability range recommended in international residue analysis guidelines (22). Among the five analytes, sulfamethoxazole consistently showed the lowest recoveries. Its relatively higher lipophilicity may have contributed to greater partitioning into the n-hexane phase during defatting (27). However, the consistency of recovery across all spike levels indicates that these losses were still acceptable and reproducible (5).

Table 2. Recovery and precision data for sulfonamides in poultry matrix

Compound	Spiking at 10 ng/g Recovery %	Spiking at 100 ng/g Recovery %	Spiking at 400 ng/g Recovery %	Mean (%)
Sulfadiazine	94.2 ± 4.8	96.5 ± 3.2	97.8 ± 2.8	96.2 ± 3.6
Sulfamerazine	92.8 ± 5.2	95.4 ± 3.5	96.2 ± 3.0	94.8 ± 3.9
Sulfadimidine	97.5 ± 3.8	98.2 ± 3.0	99.6 ± 2.5	98.4 ± 3.1
Sulfamethoxazole	86.4 ± 6.1	89.2 ± 4.8	89.7 ± 4.5	88.4 ± 5.1
Sulfamethizole	100.4 ± 3.9	102.8 ± 3.1	103.2 ± 2.6	102.1 ± 3.2

Values are expressed as mean ± SD. The intra-day precision (repeatability) of the five compounds showed RSD values ranging from 2.9% to 4.1%, as presented in Table 3. This level of precision indicates that the method produces reliable results when applied under the same conditions on the same day. Inter-day precision values ranged from 4.6% to 5.8%, reflecting day-to-day variation over three different days. The slight increase compared with intra-day precision is consistent with normal laboratory variation but remains well within the acceptable limits outlined in European Commission Decision 2002/657/EC (28). These results confirm that the method performs reliably with respect to intermediate precision, as required by ICH Q2(R1).

Table 3. Intra-day and inter-day precision data

Compound	Intra-day RSD (%)	Inter-day RSD (%)
Sulfadiazine	3.2	5.1
Sulfamerazine	3.5	4.8
Sulfadimidine	3.8	5.2
Sulfamethoxazole	4.1	5.8
Sulfamethizole	2.9	4.6

RSD = Relative standard deviation; concentration = 100 ng/mL. Robustness was tested by deliberately varying the flow rate (±0.1 mL/min), column temperature (±2°C), and mobile-phase composition. Changes in flow rate shifted retention times by approximately 0.08 min with negligible effect on peak area. Variations in column temperature caused retention shifts of about 0.05 min with no effect on quantification. Changes in mobile-phase composition produced the greatest variation; increasing acetonitrile by 2% reduced all retention times by

0.10–0.15 min, although resolution remained >1.4 and recovery remained within acceptable limits, consistent with previously reported findings (29). Overall, these results indicate that typical operational variations are unlikely to adversely affect method performance during routine analysis.

The key performance characteristics of the present method were compared with selected previously reported methods for sulfonamide determination, as shown in Table 4. Premarathne et al. (2017) reported mean recoveries of 86–108%, with repeatability and within-laboratory reproducibility expressed as RSD below 15%, using an isocratic mobile phase (17). The recovery and precision values obtained in the present study are comparable to those results, while separation was achieved in a shorter total run time of 12 min. Deng et al. employed a DSPE-DLLME coupled HPLC method for four sulfonamides in chicken liver, achieving LODs in the range of 0.0004–0.0084 mg/kg and linearity over 0.006–4.00 mg/kg (30). Although that method achieved lower detection limits, it involved a more elaborate multi-step extraction procedure and was more complex than the simple liquid-liquid extraction used in the present study. Tolika et al. developed an HPLC-DAD method for veterinary antibiotic residues in food and environmental matrices, reporting LODs of 0.05–0.17 µg/kg and precision values (%RSD) of 1.02–3.40 for intra-day and 2.14–8.23 for inter-day measurements, achieved through advanced microextraction pre-concentration techniques (31). Although those figures indicate superior sensitivity, they were obtained using considerably more complex and resource-intensive sample preparation procedures. The detection limits achieved in the present study, while less sensitive than those of methods employing solid-phase extraction or multi-step microextraction, remain within the range reported for comparable HPLC-DAD methods and are adequate for regulatory monitoring at and below the EU MRL of 100 µg/kg for sulfonamides in meat. The practical advantages of the present method are particularly relevant for routine food safety monitoring in resource-limited laboratories. The isocratic elution strategy avoids the need for gradient pumping systems and associated maintenance requirements, while the liquid-liquid extraction procedure relies on common laboratory glassware and widely available solvents—ethyl acetate, n-hexane, and methanol—without the need for expensive solid-phase extraction cartridges or sorbents. With a total analysis time of 12 min per sample, the method provides adequate throughput for routine monitoring programs.

Table 4. Comparison of the present method with selected reported methods

Method	Matrix	Detection	LOD (µg/kg)	Recovery (%)	RSD (%)	Run Time (min)
Present method	Meat	HPLC-DAD	1.2–2.8	88–102	2.9–5.8	12
Premarathne et al. 2017 (17)	Meat	HPLC-DAD	129–140 (CCβ)	86–108	<15	~30
Deng et al. 2016 (30)	Liver	HPLC-DAD (DSPE-DLLME)	0.4–8.4	70–91	5.3	30
Tolika et al. 2012 (31)	Meat	HPLC-DAD	30 (CCβ)	90–115	<10.0	36

The developed method was applied to determine sulfonamide residues in ten poultry meat samples collected from Hyderabad. Sulfonamide residues were detected in 38% of the samples, with concentrations ranging from 3.4 to 60 µg/kg and a mean concentration of 12 µg/kg. None of the analyzed samples exceeded the EU MRL of 100 µg/kg. These findings indicate the presence of sulfonamide residues in locally available poultry meat and support the need for continued monitoring. The results are consistent with the pattern of

sulfonamide occurrence reported previously in Pakistan. A surveillance study on chicken meat and eggs collected from sale points in Rawalpindi, Pakistan, reported that 43% of meat samples contained sulfonamide residues, with concentrations ranging from 0.02 to 0.8 µg/g (12). The positivity rate and mean concentration observed in the present study are broadly comparable with those findings, suggesting that sulfonamide contamination remains a recurring issue in the Pakistani poultry supply chain. A study in Lahore reported that 73.3% of commercially available broiler chicken samples were positive for antimicrobial activity, with sulfonamides accounting for 69.6% of detected residues in liver and muscle tissues, further indicating the widespread use of this drug class in commercial poultry farming in Pakistan (32).

CONCLUSION

This study presents a simple and reliable HPLC-DAD method for the simultaneous determination of five sulfonamide antibiotics in poultry breast muscle. Built around liquid-liquid extraction and isocratic elution, the method is compatible with standard laboratory equipment and does not require specialized instrumentation. Validation in accordance with ICH Q2(R1) guidelines confirmed good linearity, mean recoveries of 88–102%, precision below 6% RSD, and detection limits of 1.2–2.8 µg/kg, all within accepted performance criteria and well below the regulatory threshold of 100 µg/kg. Application of the method to field samples from Hyderabad, Pakistan, revealed detectable sulfonamide residues in a proportion of the samples tested, although none exceeded the permitted limit. These findings highlight the continued relevance of antibiotic residue surveillance in commercial poultry. The method offers a practical and cost-effective tool for routine food safety monitoring in laboratories where access to advanced mass spectrometry infrastructure remains limited.

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DECLARATIONS

Ethical Approval: This study involved the analysis of commercially available food products and did not require human or animal ethics committee approval.

Informed Consent: Informed Consent was taken from participants.

Authors' Contributions:

Concept: ZHS; Design: MKC; Data Collection: MAB; Analysis: HB; Drafting: SN

Conflict of Interest: The authors declare no conflict of interest.

Funding: This research received no external funding.

Data Availability: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors thank the staff of the Dr. M.A. Kazi Institute of Chemistry for technical assistance and access to HPLC facilities. The authors also acknowledge the National Centre of Excellence in Analytical Chemistry for providing reference standards and laboratory space for validation studies.

Study Registration: Not applicable.