Research Article

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Simultaneous Quantification of Multi-Class Antimicrobials in Chicken Kidney and Liver by New Validated UPLC-MS/MS Method

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ABSTRACT

A novel and significant method was developed and validated with a sensitive, rapid, and simultaneous analytical method to determine antimicrobials in chicken tissues such as the kidney and liver. The process involved a unique approach to precipitation extraction. This method has not been widely used in this context, followed by the evaporation of the supernatant and reconstitution with the mobile phase. Antimicrobials, including Azithromycin, Clarithromycin, Erythromycin, Clavulanic acid, Ciprofloxacin, Clofazimine, Fluconazole, Linezolid, and Moxifloxacin were meticulously considered for development and validation in the chicken tissues. These antimicrobials were chosen based on their everyday use in poultry farming and their potential impact on human health. We used Ultra-Performance Liquid Chromatography with triple quad Mass Spectrometry and employed multiple reaction monitoring to detect the analytes of interest. All the compounds were well separated using Atlantis T3, 4.6x50mm, 3 µm. The linear range was set between 25 to 1000 ng/gm. The method was validated following linearity, extraction recovery, matrix effect impact, limit of detection, sensitivity, autosampler and benchtop stability, ensuring the results' reliability and our method's robustness.

Keywords: Antimicrobials, Antimicrobial resistance, Chicken tissues, Method development, Validation.

1. Introduction

Antimicrobials play a pivotal role in modern agriculture, safeguarding the health and well-being of livestock populations while ensuring food safety for consumers. Their judicious use is paramount, as their indiscriminate application can lead to the emergence of antimicrobial-resistant strains, posing a significant threat to both animal and human health [1] . Consequently, precise and consistent analytical methods for quantifying antimicrobial residues in animal tissues are imperative.

The development of antimicrobial resistance via poultry animals is crucial as the consumption of poultry meat has increased predominantly in recent years. One or more antibiotic residues are frequently found in the meat samples. This is an alarming situation to regulate the usage of antibiotics in poultry farms [2,3]. The primary cause of using antibiotics in poultry farms is to save the birds from illness and promote their growth. The most frequently used antibiotics to treat infections that spread across animals are Aminoglycosides, Bambermycin, Beta-lactams, Macrolides, Quinolones, and Sulfonamides. These antibiotics develop resistance among farm animals and humans who consume them [4]. Antimicrobials in poultry must be regulated to fight and eradicate antimicrobial resistance. As an initiative, the Australian government has released a guideline on prescribing antimicrobials for poultry that describes the appropriate usage and best practices in handling antimicrobials [5].

A country-wide residue monitoring program is in place to monitor the residue levels in the meat, which guides the safe usage of antimicrobials, random monitoring of the medicines used in the animals, and appropriate sampling and analysis of the same [6-8]. Post-administration, the drugs are absorbed into the intestine and transported to other tissue parts via blood. This will increase the risk of deposition of residues in the tissues, such as the liver, kidney, spleen, and muscle [9]. To safeguard the consumers, regulatory agencies perform safety evaluations to determine the antibiotic concentrations in the edible tissues. This will ensure the consumers are not exposed to the residual compounds [10].

The analytical methodologies that are being followed currently and in the past decade to determine the antibiotics either qualitatively or quantitatively suggest various technologies that include microbiological assay, immunoassay, physical and chemical assays, and biosensors [11]. Different detection methods, such as UV, PDA, GC, and MS, are used based on sensitivity requirements. The extraction of antibiotics from the meat is widely achieved by the SPE technique, which includes the sample cleanup procedure, and the resultant sample is analysed using LC-MS/MS [12]. pH of the sample plays a vital role in the analysis as the compounds are more stable in acidic conditions. Unlike blood and plasma sample preparation, tissue sample processing requires a buffer medium for homogenising the samples. Extracting the residues from the animal tissues is quite challenging as an appropriate sample pre-treatment procedure must be followed to avoid the loss of recovery. Also, multiple steps of the extraction process are involved, such as homogenising the tissue using the suitable buffer, incubation, processing steps, etc., to keep the sample intact. The sensitivity may be affected by various other parameters like mobile phase composition and pH, column chemistry, flow rate, signal enhancers, matrix interferences, and sample compatibility [13-15].

Many studies have reported the development and validation of antibiotics in chicken muscles according to European Commission Decision 2002/657/EC. European Commission and Codex Alimentarius pointed out the MRLs of frequently used antibiotics in animal tissues [16-17]. Extensive research has been done in various chicken tissues to detect and determine antibiotics. However, there was not much focus on the other two antimicrobials, i.e., antivirals and antifungals, as these classes of compounds also trigger resistance [18-23]. There is a need to determine all three types of antimicrobials in a simultaneous method. The antimicrobial residues reach animal tissues via feed and environmental resources such as surface water, soil, etc., as the environmental resources are contaminated with all these classifications of antimicrobials [24-26]. Hence, developing a separate method to detect the antimicrobials will not solve the purpose.

In this context, we present the development and validation of a cutting-edge UPLC-MS/MS method tailored to simultaneously determine antimicrobial compounds within the complex matrix of chicken kidney and liver tissues. This method is a significant advancement in the field, offering high sensitivity, selectivity, and efficiency in quantifying a diverse range of antimicrobials. In the best of the search, no

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methods are available to determine antibiotics and antifungals in chicken tissues simultaneously using LC-MS/MS. The intended method can be applied to determine various frequently used antimicrobials in the chicken liver and kidneys with a minimal cost compared with other sophisticated processing methodologies.

Given their physiological significance in drug metabolism and excretion, including kidney and liver tissues in our analytical approach is essential. Ensuring accurate quantification in these matrices is critical for comprehensive residue monitoring and regulatory compliance. Moreover, this study addresses a crucial gap in the analytical methodologies, particularly in simultaneous determination. The ability to assess multiple antimicrobials concurrently provides a comprehensive snapshot of residue levels, reflecting real-world scenarios of complex drug regimens and potential interactions. This research contributes to the analytical toolkit for veterinary drug residue analysis by establishing a robust and validated UP-LC-MS/MS method. It holds implications for food safety, public health, and regulatory enforcement. The potential applications of this method are far-reaching, encompassing routine surveillance of antimicrobial residues and investigative studies on drug pharmacokinetics and pharmacodynamics in avian species.

As per the Codex Alimentarius and the National Pharmaceutical Regulatory Agency, the maximum residue levels of some key chemical classifications in poultry products are macrolides with 600-800 µg/kg, quinolones with 30-80 µg/kg, azole derivatives with 50 µg/kg, antibacterials with 100 µg/kg, etc.,

In the subsequent sections, we elaborate on the methodology employed in developing and validating this analytical approach, present our study's key findings, and discuss their broader implications for animal husbandry and food safety.

2. Material and Methods

2.1. Chemicals and reagents:

Methanol and acetonitrile were procured from Merck (Germany), and water, an LC-MS grade, was purchased from the RCI labscan limited. Formic acid, an analytical grade, was procured from Sigma Aldrich. Ammonium acetate was procured from Merck. Centrifuge tubes for processing were from Abdos (Eppendorf, Germany) and the weighing balances were purchased from Mettler Toledo (Ohio, USA). The tube vortexer was purchased from Remi (Maharashtra, India). Atlantis T3, 4.6x50mm, 3 µm was procured from Waters Inc. Other solutions and reagents were equivalent to analytical grade or higher than that and were purchased from renowned suppliers. UPLC and Xevo TQD were from Waters Corporation, USA. Naïve chicken tissues were procured from a farm grown in an antibiotic-free environment. Phosphate-buffered saline tablets were purchased from Sigma Aldrich, USA. The Incubator was procured from Thermo. The homogeniser/blender was procured from Pro Scientific Inc., USA. Reference standards were procured from Clearsynth, Sigma Aldrich, TCI chemicals, and BLD pharm, India. The reference standards are Azithromycin (AZI), Clarithromycin (CLAR), Clavulanic acid (CLAV), Ciprofloxacin (CIPRO), Clofazimine (CLOF), Erythromycin (ETH), Fluconazole (FLU), Linezolid (LIN), Moxifloxacin (MOXI), and Sulphaphenazole (Internal Standard).

2.2. Preparation of stock and working solutions of standards and quality control samples

Two individual reference standards were weighed for standards and quality control samples and dissolved using dimethyl sulfoxide to achieve a stock concentration of 1 mg/mL. The intermediate stock solutions for standards and quality controls were made using acetonitrile/water (80: 20 ratio) as a diluent. The final spiked concentration of standards ranged from 25, 50, 100, 200, 250, 500, 750, and 1000 ng/gm and three levels of QCs with working concentrations of 45, 475, and 775 ng/gm, respectively, in the chicken tissues.

Sulphaphenazole was used as an internal standard in the assay. The compound was weighed and dissolved using dimethyl sulfoxide for a final 1mg/mL concentration. The final working solution concentration for ISTD was 500 ng/mL, prepared by diluting 100µL of the stock into a 200 mL container containing acetonitrile with 0.1% formic acid. The prepared stock and working solutions were kept in a refrigerator at 2-8°C until the subsequent use.

2.3. LC-MS/MS conditions

The Waters UPLC system was equipped with a pump, column oven, degasser, and autosampler. It is connect-

ed with a Xevo triple quad mass spectrometer. Positive electrospray ionization was used as a primary ionization technique to detect all the compounds. Multiple reaction monitoring mode was used to detect the ions at a unit resolution. The chromatographic separations were achieved using a C18 column, i.e., Atlantis T3, 4.6x50mm, 3 µm. Mobile phase A consists of ammonium acetate buffer with 0.1% formic acid, whereas mobile phase B consists of acetonitrile with 0.1% formic acid with a flow rate of 0.250 mL/min. A gradient flow was followed to achieve the desired compound separation and elution. Setting the column oven temperature to 40°C and the autosampler temperature to 15°C leads to an appropriate peak shape. The column was loaded with a 5 µL injection volume, ensuring the volume was sufficient to achieve the desired peak response. All the analytes were eluted within a 4-minute run time. Mass parameters such as gas, temperature, collision energy, cone voltage, etc., were determined and optimised using an auto-tune mode. However, the tune parameters were set manually. Mass Lynx software was used to perform the data acquisition and regression. The mass and mass spectrometer parameter details are given in Table 1.

2.4. Sample preparation and extraction procedure

One phosphate buffer tablet was dissolved in a specified quantity of deionised water. The saline solution's pH needed to be maintained between 7.2 and 7.4 to prevent any possible interactions with tissues. A 1 gm sample of chicken tissues was weighed and placed in an individual tube. Subsequently, 5 mL of saline solution was added, and the entire content was homogenised using a hand blender. The homogenised tissues were spiked with intermediate standards and quality controls to achieve the desired concentration. The spiked samples were vortexed to ensure thorough mixing. They were incubated in tubes in a controlled environment at 37 ºC for 30 minutes, allowing the analytes to bind effectively with the chicken tissues.

Following incubation, 500 µL of the spiked samples were aliquoted into another tube. 2.5 mL of the internal standard working solution was transferred to precipitate the sample. The mixture was vortexed for 5 minutes. Subsequently, the samples were centrifuged at 13000 rpm for 10 minutes at 4 ºC. The supernatant was aliquoted into another tube and dried by evaporation under nitrogen pressure at 40 ºC. The dried samples were resuspended using a reconstitution solution containing acetonitrile: water mixture with 0.1% formic acid and loaded into autosampler vials for analysis. A zero-concentration sample was also processed to identify and assess potential analyte contamination. Figure 1 shows a schematic extraction technique.

Table 1. Summary of the compound parameters

S. no.	Antimicrobials	MW	Chemical formula	Parent ion/daughter ion (m/z)	MS Collision Energy (eV)	MS Cone Voltage (V)
1	AZI	749.00	$C_{38}H_{72}N_2O_{12}$	749.7/158.1	30	44
2	CLAR	747.95	$C_{38}H_{69}NO_{13}$	748.7/83.0	45	32
3	CLAV	199.16	$C_8H_9NO_5$	200.0/76.9	32	86
4	CIPRO	331.34	$C_{17}H_{18}FN_3O_3$	332.1/288.2	18	38
5	CLOF	473.40	$C_{27}H_{22}C_{l2}N_4$	473.2/283.4	72	64
6	ETH	733.93	$C_{37}H_{67}NO_{13}$	734.7/158.1	22	32
7	FLU	306.27	$C_{13}H_{12}F_2N_{6O}$	307./238.1	16	28
8	LIN	337.35	$C_{16}H_{20}FN_3O_4$	338.2/296.2	18	36
9	MOXI	401.43	$C_{21}H_{24}FN_3O_4$	402.3/110.0	22	44
10	Sulphaphenazole	314.36	$C_{15}H_{14}N_4O_2S$	315.0/91.9	38	32

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3. Results and Discussion

3.1. UPLC-MS/MS method optimization

3.1.1. Optimization of mass spectrometry

A 20 µg/mL tuning solution containing all the analytes, including ISTD, was prepared using a mixture of 80% acetonitrile in water with 0.1% formic acid as a diluent. Formic acid was added to increase the mass signal during the tuning process. The parent and the daughter ions were determined through the infusion process for ESI+ mode. The positive mode was selected as all the compounds exhibit greater signal strength in this mode of ionization to determine the analytes in the chicken tissues quantitatively. The predominant daughter ions were selected to quantitate the analytes. The parent and daughter ions were given in Table 1. All the compounds exhibit various collision energy and cone voltage based on the individual compounds' response. However, the gas parameters, such as cone and desolvation gas, and temperature parameters, such as source and desolvation temperature, remain the same. The various optimized instrument parameters were selected based on the molecules' intensity. The optimum parameters show no ion enhancement and or suppression. The representative chromatograms are given in Figure 2.

3.1.2. Chromatography

A mobile phase with a gradient flow was selected for the elution of the compounds. The gradient is as follows- mobile phase A: 90% till 0.8 minutes and switch to 10% from 0.8 to 3 minutes and back to 90% from 3 to 4 minutes. More aqueous in the initial run time elutes all matrix impurities, and switching the run to more organic separates the non-polar compounds. Individual samples were initially eluted to ensure the respective retention time. After that, a mix of standard solutions was prepared for simultaneous elution. Since pH plays a significant role, the intermediate stock dilutions, mobile phase, and the

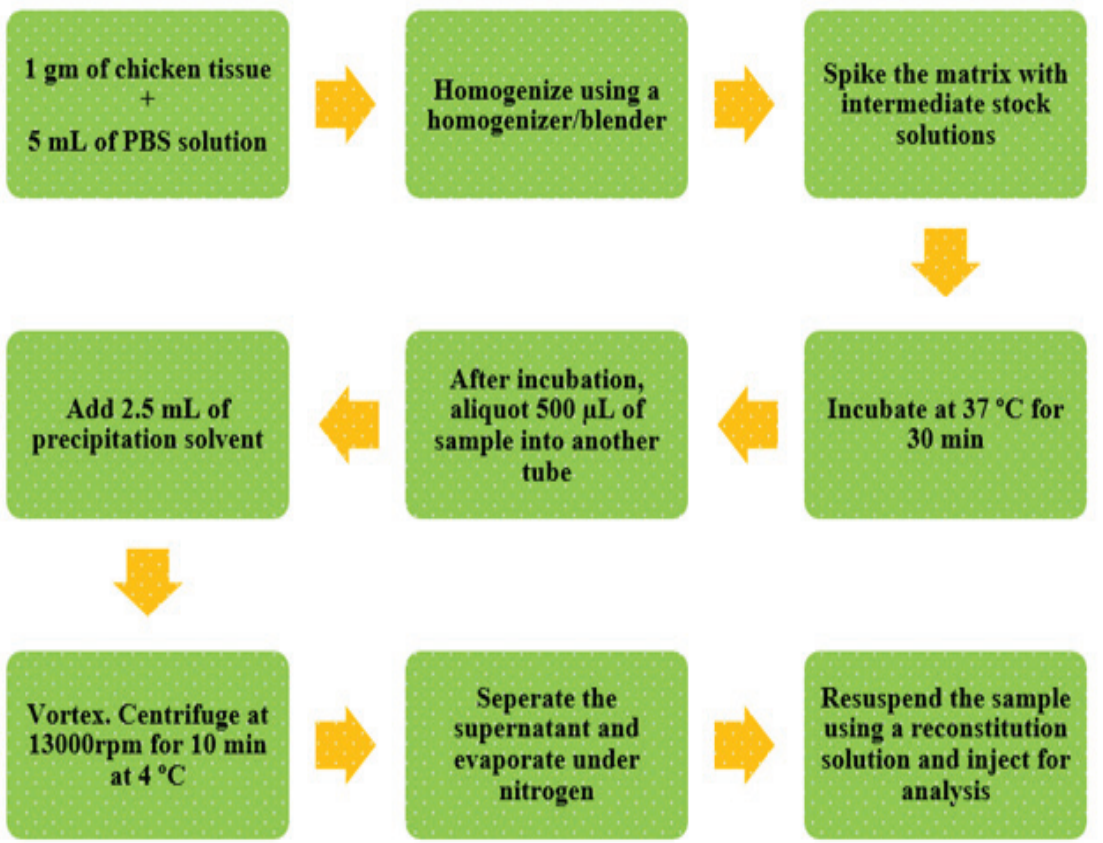

Figure 1. Schematic diagram of the sample preparation and extraction procedure

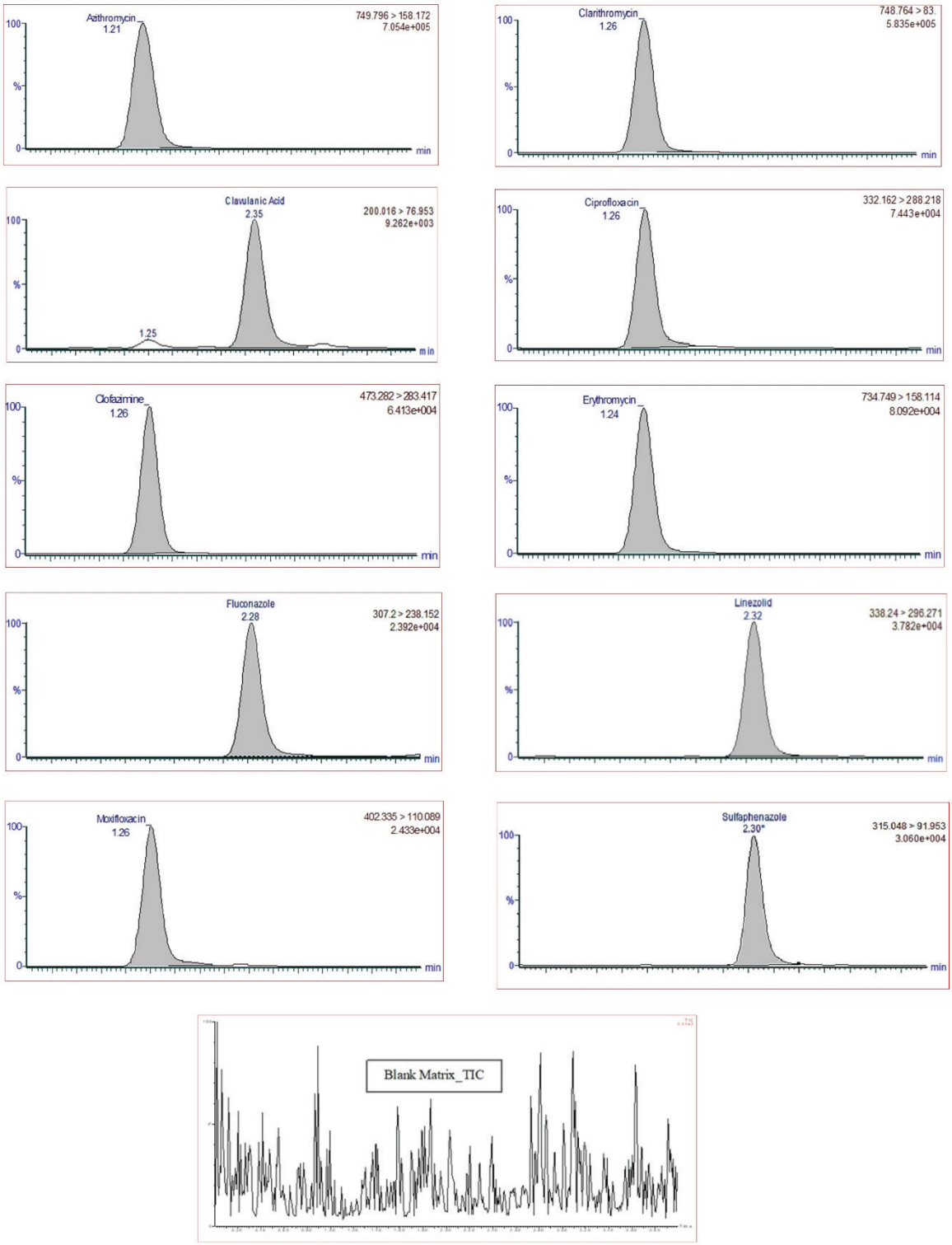

Figure 2. Representative chromatograms for antimicrobials

internal standard working solution were acidified with 0.1% formic acid to make the sample compatible for an appropriate ionization. The proposed formic acid strength gives an enhanced signal intensity compared with 0.05% and 0.2% formic acid, as these two formic acid strengths suppressed the ionization.

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A non-polar column was selected to ease the compounds' elution as the C18 column exhibits an appropriate separation. The 0.250 mL/min flow rate enhances the sensitivity of compounds compared with 0.3 and 0.4 mL/min. A solid organic wash containing 10% water in acetonitrile with 0.1% formic acid and a weak organic wash containing 90% water in acetonitrile with 0.1% formic acid ensures no carryover. Each compound was well separated when the column temperature was set at 40 ºC compared with various other temperatures at 25 ºC and 30 ºC, as peak tailing was observed. Using deuterated internal standards will be expensive for individual compounds. Hence, Sulphaphenazole was used, which was compatible with the analytical method and had little to no variation.

3.2. Method validation

With the proposed sample processing method and instrument conditions, method validation was performed with parameters such as linearity, matrix factor, recovery, sensitivity, limit of detection, autosampler stability, and bench-top stability.

3.2.1. Calibration curve

Three precision and accuracy batches were performed individually for the chicken tissues on two consecutive days. The r^2 is the square of the correlation coefficient and provides information about how close the calculated points are to the line. The R-value, which is near to 1, is desirable. All the antimicrobials exhibit r^2 values between 0.9900 and 0.9925 in chicken kidney and liver tissues. The results are provided in Table 2. The calibration curve for both tissues is given in Figure 3.

3.2.2. Precision and accuracy, carry-over impact

The carry-over impact was determined by injecting a blank extracted sample before and after the lower and higher concentrations. No significant peak response was detected in the blank extracted samples, which confirms no potential carry-over for the proposed method. To establish the precision and accuracy of the extraction method in both tissues, five replicates of three concentrations, i.e., LQC, MQC, and HQC, were injected, followed by a standard curve. The precision and accuracy of the standard curve were observed at 0.7 to 12.2% and 87.2 to 108.6%, respectively, for chicken kidney tissue. The same for chicken liver tissue was observed at 0.3 to 9.5% and 87.7 to 108.7%, respectively. The precision and accuracy of the three QCs for chicken kidneys were observed at 2.7 to 9.9% and 90.7 to 108.6%. The same for chicken liver was observed at 3.9 to 10.7% and 91.1 to 108.7%, respectively. The results met the

Table 2. Summarised standard curve correlation coefficient (r^2)

S. no.	Drug	CC range (ng/gm)	Average r2	
			Kidney	Liver
1	AZI	25 - 1000	**0.9910**	**0.9925**
2	CLAR	25 - 1000	**0.9920**	0.9913
3	CLAV	25 - 1000	**0.9930**	**0.9913**
4	CIPRO	25 - 1000	**0.9900**	**0.9913**
5	CLOF	25 - 1000	**0.9900**	**0.9907**
6	ETH	25 - 1000	**0.9900**	0.9907
7	FLU	25 - 1000	**0.9910**	**0.9907**
8	LIN	25 - 1000	**0.9923**	**0.9923**
9	MOXI	25 - 1000	**0.9917**	**0.9910**

[a]Average r^2 was derived from at least three precision and accuracy batches.

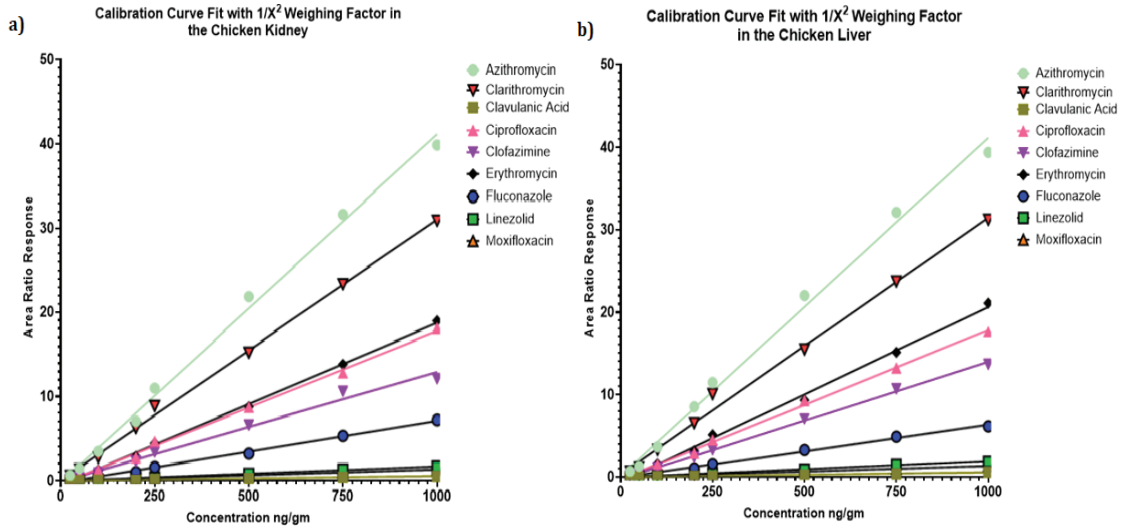

Figure 3. Calibration curve of antimicrobials in a) chicken kidney tissue, b) chicken liver tissue

acceptance criteria with ±15% accuracy and ≤15% precision. Summarised results are shown in Table 3.

3.2.3. Recovery from matrix, limit of detection, and matrix effect

Recovery, i.e., extraction efficiency and matrix effect, was determined at LQC and HQC levels in the respective chicken tissues. While the recovery experiment was performed to confirm the extraction efficiency, the impact of the matrix effect was established to verify that no suppression and enhancement of the ions were detected in the intended method. In chicken kidney tissues, the recovery ranged between 85.16 and 102.14 %, and the matrix effect was observed from 84.15 to 119.64 %, respectively. Meanwhile, in chicken liver, the recovery was between 59.18 and 103.17 %, and the matrix effect was between 76.53 and 109.50 %, respectively. The recovery of Ciprofloxacin, fluconazole, and moxifloxacin in chicken liver tissues was reduced by less than 70%. This may be due to the irreversible binding nature of compounds in particular tissues.

The signal-to-ratio produced by the mass spectrometer was used to determine the limit of detection. The limit of detection is the lower limit of concentration, making the S/N ratio at three values. The results of the matrix effect, recovery, and limit of detection are given in Table 4 and Table 5. The average recovery from matrix and matrix effect of antimicrobials in various chicken tissues are presented in Figure 4 a, b, c, and d.

3.2.4. Sensitivity

Samples with a concentration of lower limit quantitation were determined under the calibration standards. The average accuracy for the lower limit of quantitation for all the antimicrobials was observed between 80 and 120% of the actual concentration, as shown in Table *6*.

3.2.5. Autosampler and benchtop stability

The autosampler and benchtop stability of the antimicrobials extracted from the respective chicken tissues were established for at least 23 hrs 14 min and 7 hrs 18 min, respectively. For the autosampler stability, the autosampler temperature was maintained at 15°C. The spiked samples were stored on a benchtop at a controlled room temperature for benchtop stability. Both stability experiments were established at low and high QC samples against freshly prepared calibration curve standards and quality control samples. The resulting bias is given in Table 7 and Table 8.

4. Conclusion

Antibiotics in chicken tissues significantly influence the development of antimicrobial resistance, underscoring the need for a sensitive and comprehensive analytical method to detect multiple antibiotics. This study introduces a simple, accurate, sensitive, precise, and reproducible LC-MS/MS method developed and validated to detect nine different antibiotics

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Table 3. Summarised precision and accuracy

S. No.	Compounds	Chicken Kidney						Chicken Liver					
		Precision (%RSD)[a]			% Accuracy[b]			Precision (%RSD)[a]			% Accuracy[b]		
		LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
1	AZI	5.6	2.7	3.8	103.0	108.5	92.9	9.2	5.1	6.4	99.6	108.0	93.6
2	CLAR	8.5	4.6	4.7	100.9	102.7	90.7	8.6	6.0	4.4	101.2	105.2	91.1
3	CLAV	5.9	5.4	6.0	107.5	100.0	103.4	8.4	3.9	10.1	102.2	102.7	98.0
4	CIPRO	9.3	5.0	6.3	94.4	105.5	103.9	5.0	4.4	8.5	92.9	108.8	96.3
5	CLO	9.1	3.9	4.9	96.3	108.6	106.2	10.7	4.1	4.7	97.6	108.2	97.7
6	ETH	9.9	4.5	4.0	98.2	107.9	107.6	10.0	5.2	8.3	95.4	104.0	100.9
7	FLU	6.8	5.1	5.3	101.7	105.9	106.5	8.2	5.0	6.3	96.2	104.2	104.3
8	LIN	8.8	8.1	6.6	103.3	103.5	104.5	9.9	6.4	5.8	100.2	103.3	102.0
9	MOXI	9.6	6.4	7.8	97.6	99.0	100.8	10.1	4.9	8.7	95.6	100.4	98.4

[a] %RSD was calculated from precision and accuracy batches. [b]Accuracy was determined from the nominal concentration of the QC concentrations, respectively.

Table 4. Summarised extraction recovery and matrix effect

S. No.	Antimicrobials	% Recovery in Chicken Kidney[a]		% Recovery in Chicken Liver[b]		% Matrix Effect in Chicken Kidney[a]		% Matrix Effect in Chicken Liver[b]	
		LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
1	AZI	91.04	102.14	74.90	100.25	90.62	98.96	98.16	96.84
2	CLAR	92.09	104.98	83.34	98.75	96.87	101.37	98.91	98.36
3	CLAV	90.51	97.11	71.46	94.26	108.91	119.64	76.53	98.68
4	CIPRO	87.83	98.54	69.37	96.56	97.53	102.62	90.79	100.52
5	CLO	90.85	98.17	79.93	100.01	90.40	101.86	99.46	102.50
6	ETH	85.16	100.25	88.75	98.68	94.72	100.91	99.18	102.29
7	FLU	96.83	99.16	61.29	103.17	99.56	104.99	95.28	99.84
8	LIN	92.84.	101.72	94.50	99.42	84.15	99.60	100.28	101.24
9	MOXI	85.76	99.1	59.18	95.14	90.20	102.68	109.50	103.04

[a,b] Average of five replicates.

from various classes in chicken tissues, specifically the kidney and liver. The technique demonstrates precision with ≤15% variability and accuracy within ±15%. The protein precipitation technique, followed by evaporation and reconstitution using the mobile phase, effectively showcased the extraction efficien-

Table 5. Summary of the limit of detection in chicken tissues

S.no.	Antimicrobials	LOD (ng/mL)	
		Kidney	Liver
1	AZI	10.80	9.74
2	CLAR	6.60	5.49
3	CLAV	24.19	18.60
4	CIPRO	14.87	14.86
5	CLO	12.89	15.66
6	ETH	9.80	8.51
7	FLU	12.63	6.09
8	LIN	8.05	12.84
9	MOXI	8.35	10.58

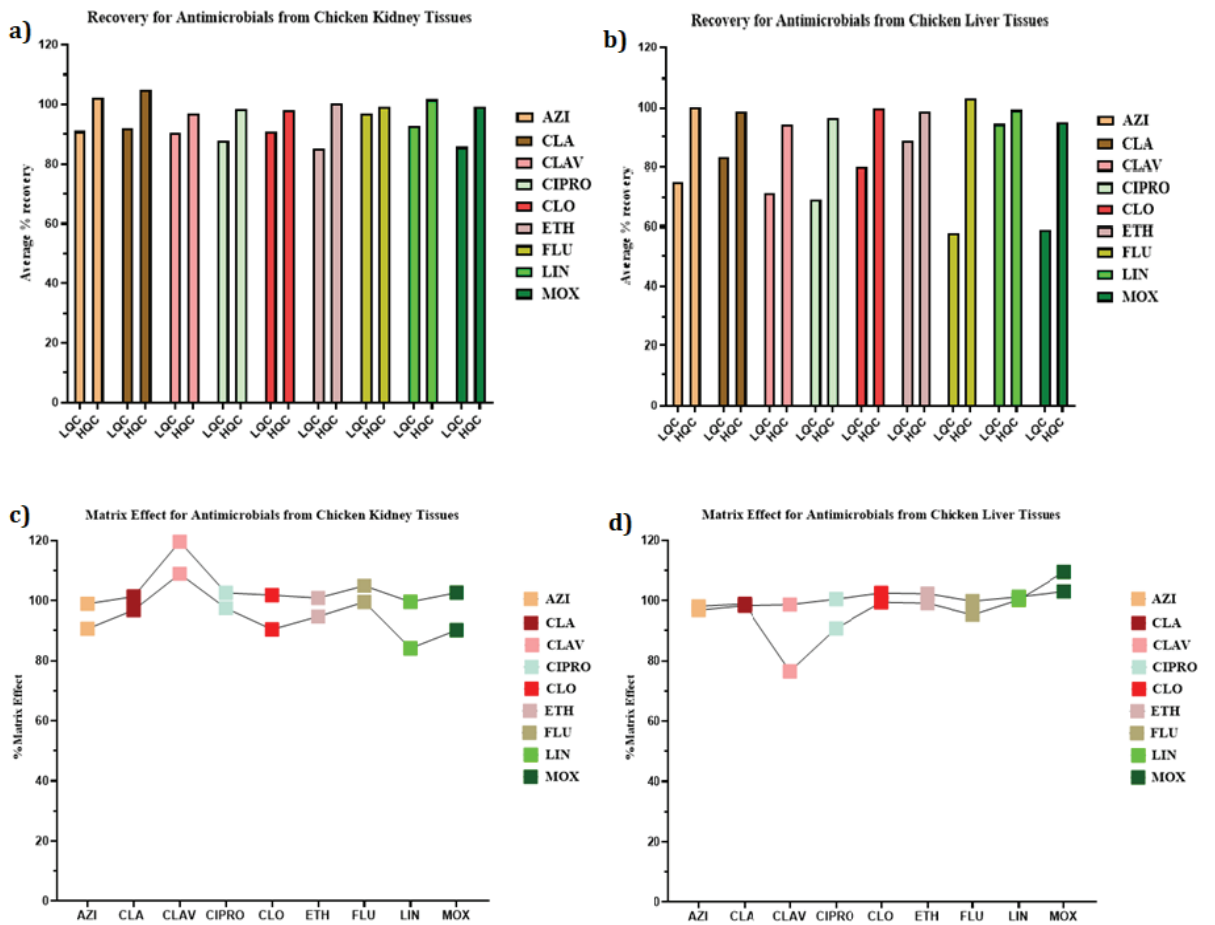

Figure 4. The average recovery of antimicrobials in a) chicken kidney b) chicken liver, Matrix effect of antimicrobials in c) chicken kidney, d) chicken liver.

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Table 6. Summarised sensitivity data in chicken tissues

S. no.	Antimicrobials	The average % accuracy	
		Chicken Kidney	Chicken Liver
1	AZI	97.18	108.70
2	CLAR	104.16	96.78
3	CLAV	111.36	102.14
4	CIPRO	112.24	103.48
5	CLO	108.55	104.68
6	ETH	102.03	102.04
7	FLU	108.30	107.20
8	LIN	102.38	106.96
9	MOXI	111.36	104.94

Table 7. Summary of autosampler stability

Antimicrobials	Kidney		Liver	
	[a]LQC	[b]HQC	[a]LQC	[b]HQC
AZI	4.52	-1.56	-10.85	7.64
CLAR	0.67	0.31	-10.32	-7.41
CLAV	-10.43	-10.16	10.78	5.79
CIPRO	-5.65	-2.33	-5.09	5.28
CLO	-3.59	-2.9	-1.09	-9.85
ETH	5.02	-11.31	-2.54	-3.53
FLU	-2.19	-9.24	-12.4	-8.08
LIN	-6.28	-7.44	5.00	-1.24
MOXI	-2.60	-9.16	-3.16	-5.87

[a,b] Average of five QCs.

cy. Both autosampler and benchtop stability tests confirmed the compounds' stability before and after processing. All compounds were eluted within the specified run time, exhibiting minimal matrix effect. The validated parameters confirm that this method is reliable for detecting antimicrobials in chicken tissues such as kidneys and liver.

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Table 8. Summary of benchtop stability

Antimicrobials	Kidney		Liver	
	[a]LQC	[b]HQC	[a]LQC	[b]HQC
AZI	-5.57	1.28	-8.99	-7.59
CLAR	-5.29	-5.20	-1.4	-8.35
CLAV	0.68	-3.84	-8.88	-2.02
CIPRO	-10.92	-4.61	-2.64	0.45
CLO	-3.79	-7.19	-10.28	-4.12
ETH	-3.42	-7.37	1.86	-4.84
FLU	-12.19	-11.29	-0.26	-8.16
LIN	-5.47	-3.42	-3.60	0.66
MOXI	-9.27	0.20	-2.75	-1.8

[a,b] Average of five QCs.

Conflicts of Interest

The authors declare no conflicts of interest, financial or otherwise, to declare.

Statament of Contribution of Researchers

Data Collection, Analysis, Interpretation, Literature Search, Draft preparation: S.S.S. Concept, Supervision, Review and Revision: S.K.K., S.N., R.K.S.

References

1. Agyare C, Boamah VE, Zumbi CN and Osei FB. Antibiotic use in poultry production and its effects on bacterial resistance. In: Yashwant K, editor. Antimicrobial resistance—A global threat, IntechOpen; 2019. pp.33-51. http://dx.doi.org/10.5772/intechopen.79371
2. Amit Khurana, Antibiotics in Chicken. [cited November 2024]. Available from: https://www.cseindia.org/latest-study-by-cses-pollution-monitoring-lab-finds-antibiotic-residues-in-chicken-8498.
3. Abdelshakour MA, Mostafa AE, Hadad GM, Hamed DM, El-Darder OM, El-Gindy A, et al. Experimental design assisted HPLC/UV and LC-MS/MS for simultaneous determination of selected veterinary antibiotics in broiler chicken. Separations. 2022;9(12):427. https://doi.org/10.3390/separations9120427
4. Chicken antibiotics: why are antibiotics given to chickens? The Human League. [cited November 2024]. Available from: https://thehumaneleague.org/article/chicken-antibiotics.
5. Gray P, Jenner R, Norris J, Page S, Browning G. Antimicrobial prescribing guidelines for poultry. Aust Vet J. 2021;99(6):181. https://doi.org/10.1111/avj.13034.
6. Susan K. Urahn, Allan Coukell, Sandra Eskin, and Karin Hoelzer, The National Residue Program for Meat, Poultry, and Egg Products, A report from the Pew Charitable Trust. [cited November 2024] Available from: https://www.pewtrusts.org/-/media/assets/2016/03/the_national_residue_program_for_meat_poultry_and_egg_products.pdf.
7. Directorate-General for Health and Food Safety, Control plans for residues of veterinary medicines, pesticides and contaminants, Guidelines on EU requirements for entry of animals and products of animal origin, 2024. [cited November 2024] Available from: https://food.ec.europa.eu/document/download/a2661e60-c1cc-4b0f-98bc-ee5edcf17c9c_en.
8. Animal product residue monitoring. [cited November 2024] Available form: https://www.agriculture.gov.au/agriculture-land/farm-food-drought/food/nrs/animal-residue-monitoring.
9. Mund MD, Khan UH, Tahir U, Mustafa BE, Fayyaz A. Antimicrobial drug residues in poultry products and implications on public health: A review. Int. J. Food Prop. 2017;20(7):1433-46. https://doi.org/10.1080/10942912.2016.1212874
10. Reyes-Herrera I, Donoghue DJ. Antibiotic residues distribute uniformly in broiler chicken breast muscle tissue. J.

ISSN: 2458 - 8806

Food Prot. 2008;71(1):223-5. https://doi.org/10.4315/0362-028X-71.1.223

11. Parthasarathy R, Monette CE, Bracero S, S. Saha M. Methods for field measurement of antibiotic concentrations: Limitations and outlook. FEMS Microbiol. Ecol. 2018;94(8):fiy105. https://doi.org/10.1093/femsec/fiy105
12. Moga A, Vergara-Barberán M, Lerma-García MJ, Carrasco-Correa EJ, Herrero-Martínez JM, Simó-Alfonso EF. Determination of antibiotics in meat samples using analytical methodologies: A review. CRFSFS. 2021;20(2):1681-716. https://doi.org/10.1111/1541-4337.12702
13. Lakew A, Assefa T, Woldeyohannes M, Megersa N, Chandravanshi BS. Development and validation of liquid chromatography method for simultaneous determination of multiclass seven antibiotic residues in chicken tissues. BMC Chem. 2022;16(1):5. https://doi.org/10.1186/s13065-022-00797-y
14. Uekane TM, Aquino Neto FR, Gomes LN. Development and validation of a method for the analysis of tetracyclines in chicken-muscle by liquid chromatography-electrospray-mass spectrometry in tandem (LC-ESI-MS/MS). Quim. Nova. 2011;34:43-8. https://doi.org/10.1590/S0100-40422011000100009.
15. Chen L, Wang B, Diao Z, Zhao M, Xie K, Zhang P, Wang X, Zhang T, Wang J. Development and validation of an hplc-esi/ms/ms method for the determination of amoxicillin, its major metabolites, and ampicillin residues in chicken tissues. Molecules. 2019;24(14):2652. https://doi.org/10.3390/molecules24142652
16. Codex Alimentarius, International food standards, Food and Agricultural Organization of the UN and WHO, maximum residue limits (MRLs) and risk management recommendations (RMRs) for residues of veterinary drugs in foods. [cited November 2024] Available form: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/it/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXM%2B2%252FMRL2e.pdf.
17. European Commission Regulation Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin; European Union: Brussels, Belgium, 2010. [cited November 2024] Available form: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010R0037.
18. Jammoul A, El Darra N. Evaluation of antibiotics residues in chicken meat samples in Lebanon. Antibiotics. 2019;8(2):69. https://doi.org/10.3390/antibiotics8020069
19. Moghaddam NR, Nemati M, Lotfipur F. Determination of antibiotic residues in chicken tissues using four plate test method. IJABBR. 2022;10(3):194-201. https://doi.org/10.22034/ijabbr.2022.549179.1384
20. Nikolaidou KI, Samanidou VF, Papadoyannis IN. Development and validation of an HPLC method for the determination of seven tetracycline antibiotics residues in chicken muscle and egg yolk according to 2002/657/EC. J Liq Chromatogr R T. 2008;31(14):2141-58. https://doi.org/10.1080/10826070802225445
21. Maddaleno A, Pokrant E, Yanten F, San Martin B, Cornejo J. Implementation and validation of an analytical method for lincomycin determination in feathers and edible tissues of broiler chickens by liquid chromatography tandem mass spectrometry. J Anal Methods Chem. 2019;2019(1):4569707. https://doi.org/10.1155/2019/4569707
22. Samanidou VF, Christodoulou EA, Papadoyannis IN. Determination of fluoroquinolones in edible animal tissue samples by high performance liquid chromatography after solid phase extraction. J Sep Sci. 2005;28(6):555-65. https://doi.org/10.1002/jssc.200401910
23. Oyedeji AO, Msagati TA, Williams AB, Benson NU. Detection and quantification of multiclass antibiotic residues in poultry products using solid-phase extraction and high-performance liquid chromatography with diode array detection. Heliyon. 2021;7(12). https://doi.org/10.1016/j.heliyon.2021.e08469
24. Kolaczkowska A, Kolaczkowski M, Sokolowska A, Mieczkowska H, Kubiak A, Rolka K, Polanowski A. The antifungal properties of chicken egg cystatin against Candida yeast isolates showing different levels of azole resistance. Mycoses. 2010;53(4):314-20. https://doi.org/10.1111/j.1439-0507.2009.01722.x
25. Chan D, Tarbin J, Sharman M, Carson M, Smith M, Smith S. Screening method for the analysis of antiviral drugs in poultry tissues using zwitterionic hydrophilic interaction liquid chromatography/tandem mass spectrometry. Anal. Chim. Acta. 2011;700(1-2):194-200. https://doi.org/10.1016/j.aca.2010.11.015
26. Abdelwhab ES, Hafez HM. Control of Avian Influenza in Poultry with Antivirals and Molecular Manipulation. Epidemiology II: Theory, Research and Practice; iConcept Press: Hong Kong, China. 2015.
27. Meesters R, Voswinkel S. Bioanalytical method development and validation: from the USFDA 2001 to the USFDA 2018 guidance for industry. J Appl Bioanal. 2018;4(3):67-73. https://doi.org/10.17145/jab.18.010