

Investigation of Antibiotic Residues in Meat, Milk and Egg Samples



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of Philosophy (Ph.D.) in Chemistry**

Submitted by

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CERTIFICATION

We hereby certify that the thesis entitled “**Investigation of Antibiotic Residues in Meat, Milk and Egg Samples**” represents original research carried out by Rafiza Islam (Registration No: 11, Session: 2022-2023) in the Department of Chemistry, University of Dhaka, Bangladesh, under our joint supervision and guidance. It contains no material previously published or written by any other person, except where due references have been acknowledged. Moreover, it has not been submitted previously for any degree. We have reviewed the thesis and found it satisfactory for the award of the degree of Doctor of Philosophy in Organic Chemistry.

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DECLARATION

The experimental work described in this thesis was conducted by the author in the Department of Chemistry, University of Dhaka. In addition, part of the research was carried out at the Institute of National Analytical Research and Service (INARS), Bangladesh Council of Scientific and Industrial Research (BCSIR). This thesis represents an original contribution and has not been submitted for any other academic degree. It has been prepared in fulfillment of the requirements for the degree of Doctor of Philosophy in Organic Chemistry.

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DEDICATION

I dedicate this thesis to the Almighty Allah, who is the Giver of All, the Owner of All, and the Satisfier of All Needs. I also dedicate this thesis to my parents and husband.

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The Author

ABSTRACT

Investigation of Antibiotic Residues in Meat, Milk, and Egg Samples

Broad-spectrum antibiotics are widely administrated in poultry and cattle farms for the prevention and treatment of infectious diseases and in some cases are inappropriately used as feed additives to enhance growth in food-producing animals. The presence of antibiotic residues in animal protein is a public health concern because long-term regular consumption of foods containing antibiotic residues may pose health risks due to continuous exposure to these residual antibiotics. The extensive use of sulfa drugs (SAs), fluoroquinolones (FQs), tetracyclines (TCs), chloramphenicol (CAP), derivatives of nitrofurantoin metabolites (NFs) in cattle and poultry farms production has been recognized as a key contributor of antibiotic resistance. This study aimed to identify and quantify residual amounts of sulfa drugs namely sulfadimethoxine (SMX), sulfadiazine (SDZ), sulfamethazine (SMT), fluoroquinolones like ciprofloxacin (CIP), enrofloxacin (ENR), levofloxacin (LEV), tetracyclines namely tetracycline (TC) oxytetracycline (OTC), chlortetracycline (CTC), chloramphenicol, derivatives of nitrofurantoin metabolites of 3-Amino-2-oxazolidinone (AOZ), 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMMZ), and 1-Aminohydantoin (AHD) in poultry meat, beef, egg, milk, and poultry feed samples using reported methods after modification and validation. Poultry meat, beef, egg, milk, and poultry feed samples were collected from poultry farms, and slaughter houses, different local markets and super shops around Dhaka and Gazipur cities, Bangladesh. Sample preparation was carried out using modified QuEChERS (Quick Easy Cheap Effective Rugged and Safe) method and solid phase extraction using n-hexane, ethyl acetate, methanol, water, acetic acid and other solvents where needed. Clean-up was carried out using primary secondary amine (PSA) and C18 gel (particle form).

Antibiotic residues were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consists of a Triple Quadrupole (QqQ) mass spectrometer equipped with electrospray ionization (ESI) and liquid chromatography (LC) separation system. The mass spectrometer was operated in both positive and negative ion modes with a capillary voltage of 3-4 kV. Method parameters were optimized in different optimized conditions such as desolvation and column oven temperature, pressure, gas flow. Chromatographic separation was performed on C18 column using optimized mobile phase compositions. Identifications were based on LC-

MS/MS analysis, and quantifications were performed by comparing analyte peaks with those corresponding standards. Method linearities were measured within concentration range of 1.25-100 $\mu\text{g L}^{-1}$, 5-200 $\mu\text{g L}^{-1}$, 1.5-100 $\mu\text{g L}^{-1}$, 1.5-100 $\mu\text{g L}^{-1}$, and 1-200 $\mu\text{g L}^{-1}$ for SAs, FQs, TCs, CAP and NFs, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were established as the minimum analyte level that produced a signal to noise ratio (S/N) of 3 and 10, respectively ensuring that the signal could be distinguished from baseline noise with confidence.

Method validations were performed using spiked sample at ppb levels such as 5 (n=3) and 10 (n=3) ppb for SAs and NFs, 20 and 25 ppb for FQs, 10 and 15 ppb for TCs and CAP, respectively. Recoveries were evaluated for poultry meat, beef, egg, milk and poultry feed samples; control samples spiked with two levels of CRM (certified reference materials) for each class of antibiotics, spiking levels 5 and 10 ppb were in the range of 91-101% with RSD% from 6.12-8.22% in poultry meat, 96-103% with RSD% from 3.38-9.85% in beef, 93-98% with RSD% from 4.32-7.55% in egg, 93-97% with RSD% from 4.39-8.73% in milk, and 88-97% with RSD% from 4.98-7.85% in poultry feed for SMX, SDZ, SMT. FQs recoveries were done at two concentration levels of 20 and 25 ppb and found to be in the range of 97-101% with RSD% from 5.40-7.62% in poultry meat, 98-101% with RSD% from 3.82-7.25% in beef, 99-102% with RSD% from 4.31-9.49% in egg, 98-102% with RSD% from 4.24-9.31% in milk, and 93-98% with RSD% from 3.66-5.75% in poultry feed for CIP, ENR, LEV. TCs recoveries were done such as 10 and 15 ppb level with the range 100-103% with RSD% from 4.00-8.79% in poultry meat, 100-108% with RSD% from 4.00-10.90% in beef, 88-102% with RSD% from 6.21-9.70% in egg, 99-102% with RSD% from 5.59-9.01% in milk, and 87-97% with RSD% from 4.58-7.15% in poultry feed for TC, OTC, and CTC. CAP recoveries were done at 10 and 15 ppb level and found in the range 94-100% with RSD% from 8.38-8.52% in poultry meat, 102% with RSD% from 7.47-8.24% in beef, 97-99% RSD% from 7.88-8.17% in egg, 98-99% with RSD% from 8.59-9.97% in milk, and 99-100% with RSD% from 8.03-8.14% in poultry feed. NFs recoveries were done at 5 and 10 ppb level with the range 95-104% with RSD% from 3.26-7.17% in poultry meat, 95-102% with RSD% from 4.63-9.14% in beef, 92-101% RSD% from 5.44-8.02% in egg, 95-103% with RSD% from 4.15-7.86% in milk, and 95-103% with RSD% from 3.97-6.79% in poultry feed for NP-AOZ, NP-AMOX, NP-AHD.

In all of the experiments, recovery values remained within the accepted ranges of 70-120%

specified by CODEX guidelines. World Health Organization (WHO), Food and Agriculture Organization (FAO) and Bangladesh Food Safety Authority (BFSA) have imposed maximum residue limits (MRLs) to protect consumers from harmful exposure to veterinary drug residues. In our present studies, among the targeted 750 analyzed samples, residues were detected in 13 samples collected from different place of Dhaka and Gazipur cities however detected residual concentrations were below MRL values.

CONTENT

Chapter 1: Introduction

No	Title	Page no
1.0	Background	1-4
1.1	Antibiotics and their structural patterns	4-6
1.2	Contamination of antibiotics	7
1.3	Antibiotic resistance	8
1.3.1	Global statistics on antibiotic resistance	8-10
1.4	Routes of antibiotic exposure	11-14
1.5	Drug residues surveillance in livestock	14-16
1.6	Antibiotic residues in food and feed	16-18
1.7	Sulfa drugs (SAs)	18
1.7.1	Sulfa drug (SAs): Literature survey	18-21
1.8	Fluroquinolones (FQs)	21-22
1.8.1	Fluoroquinolones (FQs): Literature survey	22-25
1.9	Tetracyclines (TCs)	25
1.9.1	Tetracyclines (TCs): Literature survey	25-28
1.10	Chloramphenicol (CAP)	28-29
1.10.1	Chloramphenicol (CAP): Literature survey	29-32
1.11	Derivatives of nitrofurans metabolites (NFs)	32-33
1.11.1	Derivatives of nitrofurans metabolites (NFs): Literature survey	33-38
1.12	Maximum Residue Limits (MRL) and Minimum Required Performance Level (MRPL)	38-41
1.13	Research in Bangladesh	41-42
1.14	Different instrumental methods for antibiotic analysis	42-45
1.14.1	Selected instrumental methods for antibiotic analysis	46-47
1.14.2	Ultra-fast high performance liquid chromatography (UFLC-MS/MS): superior to antibiotic analysis	47
1.15	Different extraction methods for antibiotic determination	48-49
1.15.1	Selected sample extraction methods	49-51
1.16	Research gap and novelty	51-52
1.17	Socio-economic importance	52-53
1.18	Importance at national level	53-54
1.19	Aim and objectives of the research	54-55

Chapter 2: Materials and Methods

No	Title	Page no
2.0	Materials and methods	57
2.1	Chemicals, reagents and solvents	57
2.2	Certified standards	57
2.3	Anhydrous sodium sulphate and magnesium sulphate activation	57
2.4	Preparation of 0.2 M HCl, 100 mM 2-NBA, 0.3 M Na ₂ HPO ₄ , 2M NaOH and 0.5 mL CH ₃ COOH solution	58
2.5	Glasses apparatus and laboratory consumables	58
2.6	Minor equipment	58-59
2.7	Major equipment	59
2.7.1	Liquid Chromatography-Tandem Mass Spectrometry (8050)	59-60
2.7.2	Liquid Chromatography-Tandem Mass Spectrometry (6420 LC/TQ)	60
2.8	Principle of ESI-LC-MS/MS	61-62
2.9	Mobile phase filtration and degassing	63
2.10	Optimization and stabilization of MS vacuum conditions	63
2.11	Method optimization and validation parameters	63
2.11.1	Preparation of standard, primary and secondary solutions	63
2.11.2	SCAN mode	64
2.11.3	Multiple reaction monitoring (MRM) Mode	64
2.11.4	Standards and matrix-matched calibration curves	64
2.11.5	Linearity	64-65
2.11.6	Limit of detection (LOD) and limit of quantification (LOQ)	65-66
2.11.7	Standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%)	66
2.11.8	Recovery percentages (%)	66-67
2.11.9	Matrix effects (%)	67-68
2.12	Survey strategy, sampling design and size	68-71
2.13	Sample collection, storage and extraction procedures	71
2.13.1	Collection and storage of control and targeted poultry meat and beef samples	71-76
2.13.1.1	Extraction and clean-up method for SAs, FQs, TCs, and CAP residues in poultry meat and beef samples	77-78
2.13.1.2	Extraction and clean-up method for NFs residues in poultry meat and beef samples	89-79
2.13.2	Collection and storage of control and targeted milk samples	80-82

2.13.2.1	Extraction and clean-up method for residues in milk samples	83-84
2.13.3	Collection and storage of control and targeted egg samples	84-87
2.13.3.1	Extraction and clean-up method for residues in egg samples	87-88
2.13.4	Collection and storage of control and targeted feed Samples	89-90
2.13.4.1	Extraction and clean-up method for residues in feed Samples	90-91
2.14	Preparation of standards, stock and intermediate working solutions	92
2.15	Method stabilization for Sulfa drugs (SAs)	92
2.15.1	Preparation of sulfa drugs (SAs) standards, stock and intermediate Working Solutions	92
2.15.2	LC-MS/MS method optimization for sulfa drugs (SAs) residues Analysis	92-94
2.16	Method stabilization for fluroquinolones (FQs)	94
2.16.1	Preparation of fluroquinolones (FQs) standards, stock and intermediate working solutions	94
2.16.2	LC-MS/MS method optimization for fluroquinolones (FQs) residues analysis	94-96
2.17	Method stabilization for tetracyclines (TCs)	996
2.17.1	Preparation of tetracyclines (TCs) standards, stock and intermediate working solutions	96
2.17.2	LC-MS/MS method optimization for tetracyclines (TCs) residues analysis	97-98
2.18	Method stabilization for chloramphenicol (CAP)	98
2.18.1	Preparation of chloramphenicol (CAP) standards, stock and intermediate working solutions	98
2.18.2	LC-MS/MS method optimization for chloramphenicol (CAP) residues analysis	98-100
2.19	Method stabilization for derivatives of nitrofurans metabolites (NFs)	101
2.19.1	Preparation of derivatives of nitrofurans metabolites (NFs) standards, stock and intermediate working solutions	101
2.19.2	LC-MS/MS method optimization for derivatives of nitrofurans metabolites (NFs) residues analysis	101-103

Chapter 3: Results and Discussion

No	Title	Page no
3.0	Results and discussion	105
3.1	Method optimization and validation of sulfa drugs (SAs)	105
3.1.1	Optimization of sulfa drugs (SAs)	105

3.1.2	Screening of SAs compounds	105
3.1.3	MRM of SAs compounds	105-106
3.1.4	Standard calibration curves of SAs	106
3.1.5	Matrix-matched calibration curves of SAs in poultry meat, beef, egg, milk and poultry feed samples	106-108
3.1.6	Linearity, limit of detection (LOD), limit of quantification (LOQ) & retention time (tR) of SAs	108-109
3.1.7	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), recovery percentage (%) of SAs in samples	110
3.1.7.1	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), recovery percentage (%) of SAs in poultry meat samples	110-112
3.1.7.2	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), recovery percentage (%) of SAs in beef samples	112-114
3.1.7.3	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), recovery percentage (%) of SAs in egg samples	115-116
3.1.7.4	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), recovery percentage (%) of SAs in milk samples	116-118
3.1.7.5	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), recovery percentage (%) of SAs in poultry feed samples	118-120
3.1.8	Matrix effect of SAs	120-121
3.1.9	SAs snalysis in real samples	121-12=2
3.1.10	Chromatograms of SAs	122-124
3.2	Method optimization and validation of fluoroquinolones (FQs)	124
3.2.1	Optimization of fluoroquinolones (FQs)	124
3.2.2	Screening of FQs compounds	124-125
3.2.3	MRM of FQs compounds	125-126
3.2.4	Standard calibration curves of FQs	126
3.2.5	Matrix-matched calibration curves of FQs in poultry meat, beef, egg, milk and poultry feed samples	126-128
3.2.6	Linearity, limit of detection (LOD), limit of quantification (LOQ) & retention time (tR) of FQs	128-130

3.2.7	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of FQs in samples	130-131
3.2.7.1	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of FQs in poultry meat samples	131-133
3.2.7.2	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of FQs in beef samples	133-135
3.2.7.3	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of FQs in egg samples	135-137
3.2.7.4	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of FQs in milk samples	137-139
3.2.7.5	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of FQs in poultry feed samples	139-141
3.2.8	Matrix effect of FQs	141
3.2.9	FQs analysis in real sample	141-142
3.2.10	Chromatograms of FQs	142-146
3.3	Method optimization and validation of tetracyclines (TCs)	147
3.3.1	Optimization of tetracyclines (TCs)	147
3.3.2	Screening of TCs compounds	147
3.3.3	MRM of TCs compounds	147-148
3.3.4	Standard calibration curves of TCs	148-149
3.3.5	Matrix-matched calibration curves of TCs in poultry meat, beef, egg, milk and poultry feed samples	149-150
3.3.6	Linearity, limit of detection (LOD), limit of quantification (LOQ) & retention time (t_R) of TCs	150-152
3.3.7	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of TCs in samples	152-153
3.3.7.1	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of TCs in poultry meat samples	153-155
3.3.7.2	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of TCs in beef samples	155-157

3.3.7.3	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of TCs in egg samples	157-159
3.3.7.4	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of TCs in milk samples	159-160
3.3.7.5	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of TCs in poultry feed samples	161-162
3.3.8	Matrix effect of TCs	163-164
3.3.9	TCs analysis in real samples	164
3.3.10	Chromatograms of TCs	165-170
3.4	Modified method optimization and validation of chloramphenicol (CAP)	170
3.4.1	Optimization of chloramphenicol (CAP)	170
3.4.2	Screening of CAP	170
3.4.3	MRM of CAP	171
3.4.4	Standard calibration curves of CAP	171
3.4.5	Matrix-matched calibration curves of CAP in poultry meat, beef, egg, milk and poultry feed samples	172-173
3.4.6	Linearity, limit of detection (LOD), limit of quantification (LOQ) & retention time (t _R) of CAP	173-174
3.4.7	Average, standard deviation ($\pm$ SD) & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of CAP in samples	174-175
3.4.7.1	Average $\pm$ SD & Intra- and Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of CAP in poultry meat samples	175-176
3.4.7.2	Average $\pm$ SD & Intra- and Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of CAP in beef samples	176-177
3.4.7.3	Average $\pm$ SD & Intra- and Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of CAP in egg samples	178-179
3.4.7.4	Average $\pm$ SD & Intra- and Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of CAP in milk samples	179-180

3.4.7.5	Average $\pm$ SD & Intra- and Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of CAP in poultry feed samples	180-181
3.4.8	Matrix effect of CAP	182
3.4.9	CAP analysis in real samples	182
3.4.10	Chromatograms of CAP	183-184
3.5	Modified method optimization and validation of derivatives of nitrofurans metabolites (NFs)	184
3.5.1	Optimization of derivatives of nitrofurans metabolites (NFs)	184
3.5.2	Screening and MRM of NFs compounds	184-185
3.5.3	Standard calibration curves of NFs	185
3.5.4	Matrix-matched calibration curves of NFs in poultry meat, beef, egg, milk and poultry feed samples	185-186
3.5.5	Linearity, limit of detection (LOD), limit of quantification (LOQ) & retention time (tR) of NFs	187-188
3.5.6	Average, standard deviation ($\pm$ SD), Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of NFs in samples	188
3.5.6.1	Average $\pm$ SD & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of NFs in poultry meat samples	189-190
3.5.6.2	Average $\pm$ SD & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of NFs in beef samples	190-192
3.5.6.3	Average $\pm$ SD & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of NFs in egg samples	192-194
3.5.6.4	Average $\pm$ SD & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of NFs in milk samples	194-196
3.5.6.5	Average $\pm$ SD & Intra- & Inter-day relative standard deviation (RSD%), Intra- & Inter-day recovery percentage (%) of NFs in poultry feed samples	196-198
3.5.7	Matrix effect of NFs	198-199
3.5.8	NFs analysis in real samples	199-200
3.5.9	Chromatograms of NFs	200-202
3.6	Data comparison with international studies	202-204
3.7	Dietary exposure, health hazard quotient and health risk assessment of antibiotics	204-206
3.8	Conclusion	206-210

Chapter 4: References

No	Title	Page no
	References	212-233

List of Figures

No	Title	Page no
1.1	Structures of sulfa drugs, fluroquinolones, tetracyclines, chloramphenicol and derivatives of nitrofurantoin metabolites	6
1.2	Cause of antibiotics contamination	7
1.3	Global deaths count caused by drug resistance	10
1.4	Routes of antibiotic exposure	14
2.1	Solvent filtration system, solvent glass bottles, sample filter (pore size 0.22µm), LC-column and LC-vial	59
2.2	Liquid Chromatography-Tandem Mass Spectrometry (8050)	60
2.3	Liquid Chromatography-Tandem Mass Spectrometry (6420 LC/TQ)	60
2.4	Principle of ESI-LC-MS/MS	62
2.5	Triple quadrupole cell and MRM fragmentation	62
2.6	Targeted matrices (Beef, egg, poultry feed, milk and poultry meat)	72
2.7	Collection sites of Poultry meat sample (Dhaka and Gazipur city)	74
2.8	Collection sites of Beef sample (Dhaka city)	76
2.9	Collection sites of Milk samples (Dhaka city)	81
2.10	Collection sites of Egg samples (Dhaka and Gazipur city)	85
2.11	Collection sites of Feed samples (Dhaka and Gazipur city)	89
3.1	Sulfa drugs screening	105
3.2	Sulfa drugs MRM	106
3.3	Standard calibration curves of SMX, SDZ and SMT	106
3.4	Matrix matched calibration curves of SMX, SDZ and SMT in poultry meat samples	107
3.5	Matrix matched calibration curves of SMX, SDZ and SMT in beef samples	107
3.6	Matrix matched calibration curves of SMX, SDZ and SMT in egg samples	107
3.7	Matrix matched calibration curves of SMX, SDZ and SMT in milk samples	107

3.8	Matrix matched calibration curves of SMX, SDZ and SMT in poultry feed samples	108
3.9	Sulfa drug standards (SMX, SDZ and SMT)	122
3.10	Sulfa drug matrix-matched in poultry meat	122
3.11	Sulfa drug matrix-matched in beef	123
3.12	Sulfa drug matrix-matched in egg	123
3.13	Sulfa drug matrix-matched in milk	123
3.14	Sulfa drug matrix-matched in poultry feed	123
3.15	Targeted SDZ in one beef and in one feed sample	124
3.16	Targeted SMX in one beef sample	124
3.17	FQs screening	125
3.18	FQs (CIP, ENR, LEV) MRM	126
3.19	Standard calibration curves of CIP, ENR and LEV	126
3.20	Matrix matched calibration curves of CIP, ENR and LEV in poultry meat samples	127
3.21	Matrix matched calibration curves of CIP, ENR and LEV in beef samples	127
3.22	Matrix matched calibration curves of CIP, ENR and LEV in egg samples	127
3.23	Matrix matched calibration curves of CIP, ENR and LEV in milk samples	128
3.24	Matrix matched calibration curves of CIP, ENR and LEV in poultry feed samples	128
3.25	Ciprofloxacin (CIP) standard	142
3.26	Enrofloxacin (ENR) standard	142
3.27	Levofloxacin (LEV) standard	142
3.28	Matrix-matched of ciprofloxacin (CIP) in poultry meat	143
3.29	Matrix-matched of enrofloxacin (ENR) in poultry meat	143
3.30	Matrix-matched of levofloxacin (LEV) in poultry meat	143
3.31	Matrix-matched of ciprofloxacin (CIP) in beef	143
3.32	Matrix-matched of enrofloxacin (ENR) in beef	144
3.33	Matrix-matched of levofloxacin (LEV) in beef	144
3.34	Matrix-matched of ciprofloxacin (CIP) in egg	144
3.35	Matrix-matched of enrofloxacin (ENR) in egg	144
3.36	Matrix-matched of levofloxacin (LEV) in egg	145
3.37	Matrix-matched of ciprofloxacin (CIP) in milk	145
3.38	Matrix-matched of enrofloxacin (ENR) in milk	145
3.39	Matrix-matched of levofloxacin (LEV) in milk	145
3.40	Matrix-matched of ciprofloxacin (CIP) in feed	146
3.41	Matrix-matched of enrofloxacin (ENR) in feed	146
3.42	Matrix-matched of levofloxacin (LEV) in feed	146
3.43	Targeted LEV in one milk sample	146

3.44	TCs (TC, OTC CTC) screening	147
3.45	Product ions of TC	148
3.46	Product ions of OTC	148
3.47	Product ions of CTC	148
3.48	Standard calibration curves of TC, OTC and CTC	149
3.49	Matrix matched calibration curves of TC, OTC and CTC in poultry meat samples	149
3.50	Matrix matched calibration curves of TC, OTC and CTC in beef samples	149
3.51	Matrix matched calibration curves of TC, OTC and CTC in egg samples	150
3.52	Matrix matched calibration curves of TC, OTC and CTC in milk samples	150
3.53	Matrix matched calibration curves of TC, OTC and CTC in poultry feed samples	150
3.54	Standard of tetracycline (TC)	165
3.55	Standard of oxytetracycline (OTC)	165
3.56	Standard of chlortetracycline (CTC)	165
3.57	Matrix-matched of tetracycline (TC) in poultry meat	165
3.58	Matrix-matched of oxytetracycline (OTC) in poultry meat	166
3.59	Matrix-matched of chlortetracycline (CTC) in poultry meat	166
3.60	Matrix-matched of tetracycline (TC) in beef	166
3.61	Matrix-matched of oxytetracycline (OTC) in beef	166
3.62	Matrix-matched of chlortetracycline (CTC) in beef	167
3.63	Matrix-matched of tetracycline (TC) in egg	167
3.64	Matrix-matched of oxytetracycline (OTC) in egg	167
3.65	Matrix-matched of chlortetracycline (CTC) in egg	167
3.66	Matrix-matched of tetracycline (TC) in milk	168
3.67	Matrix-matched of oxytetracycline (OTC) in milk	168
3.68	Matrix-matched of chlortetracycline (CTC) in milk	168
3.69	Matrix-matched of tetracycline (TC) in feed	168
3.70	Matrix-matched of oxytetracycline (OTC) in feed	169
3.71	Matrix-matched of chlortetracycline (CTC) in feed	169
3.72	Targeted OTC in two poultry meat sample	169
3.73	Targeted CTC in one beef sample	169
3.74	Targeted OTC in one egg sample	170
3.75	Chloramphenicol screening	170
3.76	Chloramphenicol MRM	171
3.77	Standard calibration curves of CAP	171
3.78	Matrix matched calibration curves of CAP in poultry meat samples	172
3.79	Matrix matched calibration curves of CAP in beef samples	172
3.80	Matrix matched calibration curves of CAP in egg samples	172

3.81	Matrix matched calibration curves of CAP in milk samples	173
3.82	Matrix matched calibration curves of CAP in poultry feed samples	173
3.83	Standard of chloramphenicol (CAP)	183
3.84	Matrix-matched of CAP in poultry meat	183
3.85	Matrix-matched of CAP in beef	183
3.86	Matrix-matched of CAP in egg	183
3.87	Matrix-matched of CAP in milk	184
3.88	Matrix-matched of CAP in poultry feed	184
3.89	Standard calibration curves of NP-AOZ, NP-AMOX and NP-AHD	185
3.90	Matrix matched calibration curves of NP-AOZ, NP-AMOX and NP-AHD in poultry meat samples	185
3.91	Matrix matched calibration curves of NP-AOZ, NP-AMOX and NP-AHD in beef samples	186
3.92	Matrix matched calibration curves of NP-AOZ, NP-AMOX and NP-AHD in egg samples	186
3.93	Matrix matched calibration curves of NP-AOZ, NP-AMOX and NP-AHD in milk samples	186
3.94	Matrix matched calibration curves of NP-AOZ, NP-AMOX and NP-AHD in poultry feed samples	186
3.95	Standards of NFs	200
3.96	NFs Matrix-matched in poultry meat	200
3.97	NFs Matrix-matched in beef	200
3.98	NFs Matrix-matched in egg	201
3.99	NFs Matrix-matched in milk	201
3.100	NFs Matrix-matched in poultry feed	201
3.101	Targeted NP-AOZ in two beef and in one poultry feed sample	201
3.102	Targeted NP-AMOX in one poultry meat and one egg sample	202

List of Tables

No	Title	Page no
1.1	Maximum residue limits (MRLs) and minimum required performance limit (MRPL) of targeted antibiotics	39-41
1.2	Different instrumental methods for residual antibiotic analysis	45-45
1.3	Different extraction methods for residual antibiotic analysis	49
2.1	Poultry meat sample collection areas	73
2.2	Beef sample collection areas	75
2.3	Milk sample collection areas	82
2.4	Egg sample collection areas	86-87

2.5	Poultry feed sample collection areas	90
2.6	Instrumental parameter: Identification of SAs using SCAN mode	93
2.7	Instrumental parameter: SAs method optimization using MRM mode	93
2.8	Instrumental parameter: LC and MS method conditions of SAs	93-94
2.9	Instrumental parameter: Identification of FQs using SCAN mode	95
2.10	Instrumental parameter: FQs method optimization using MRM mode	95
2.11	Instrumental parameter: LC and MS method conditions of FQs	95-96
2.12	Instrumental parameter: Identification of TCs using SCAN mode	97
2.13	Instrumental parameter: TCs method optimization using MRM mode	97
2.14	Instrumental parameter: LC and MS method conditions of TCs	98
2.15	Instrumental parameter: Identification of CAP using SCAN mode	99
2.16	Instrumental parameter: CAP method optimization using MRM mode	99
2.17	Instrumental parameter: LC and MS method conditions of CAP	100
2.18	Instrumental parameter: Identification of NFs using SCAN mode	101
2.19	Instrumental parameter: NFs method optimization using MRM mode	102
2.20	Instrumental parameter: LC and MS method conditions of NFs	102-103
3.1	SMX Linearity, LOD, LOQ and RT	109
3.2	SDZ Linearity, LOD, LOQ and RT	109
3.3	SMT Linearity, LOD, LOQ and RT	109
3.4	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of SAs in poultry meat samples	112
3.5	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of SAs in beef samples	114
3.6	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of SAs in egg samples	116
3.7	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of SAs in milk samples	118
3.8	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of SAs in poultry feed samples	120
3.9	Matrix-induced ionization effects of SAs in different matrices	121
3.10	Amount of targeted SAs compound	122
3.11	CIP Linearity, LOD, LOQ and t_R	129
3.12	ENR Linearity, LOD, LOQ and t_R	130
3.13	LEV Linearity, LOD, LOQ and t_R	130
3.14	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation, (RSD%), Intra- and Inter-day recovery (%) of FQs in poultry meat samples	132-133

3.15	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of FQs in beef samples	134-135
3.16	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of FQs in egg samples	136-137
3.17	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of FQs in milk samples	138-139
3.18	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of FQs in poultry feed samples	140-141
3.19	Matrix-induced ionization effects of FQs in different matrices	141
3.20	Amount of targeted FQs compound	142
3.21	TC Linearity, LOD, LOQ and t_R	152
3.22	OTC Linearity, LOD, LOQ and t_R	152
3.23	CTC Linearity, LOD, LOQ and t_R	152
3.24	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of TCs in poultry meat samples	154-155
3.25	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of TCs in beef samples	156-157
3.26	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of TCs in egg samples	158-158
3.27	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of TCs in milk samples	160
3.28	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter-day recovery (%) of TCs in poultry feed samples	162
3.29	Matrix-induced ionization effects of TCs in different matrices	164
3.30	Amount of targeted TCs compound	164
3.31	CAP Linearity, LOD, LOQ and t_R	174
3.32	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of CAP in poultry meat samples	176
3.33	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation, (RSD%), Intra- and Inter-day recovery (%) of CAP in beef samples	177
3.34	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation, (RSD%) Intra- and Inter- day recovery (%) of CAP in Egg Samples	179
3.35	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation, (RSD%) Intra- and Inter- day recovery (%) of CAP in milk samples	180
3.36	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation, (RSD%), Intra- and Inter-day recovery (%) of CAP in poultry feed samples	181

3.37	Matrix-induced ionization effects of CAP in different matrices	182
3.38	NP-AOZ Linearity, LOD, LOQ and t_R	187
3.39	NP-AMOX Linearity, LOD, LOQ and t_R	188
3.40	NP-AHD Linearity, LOD, LOQ and t_R	188
3.41	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of NFs in poultry meat samples	190
3.42	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of NFs in beef samples	192
3.43	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of NFs in egg samples	194
3.44	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of NFs in milk samples	196
3.45	(Average $\pm$ SD) & Intra- and Inter-day relative standard deviation (RSD%), Intra- and Inter- day recovery (%) of NFs in poultry feed samples	198
3.46	Matrix-induced ionization effects of NFs in different matrices	199
3.47	Amount of targeted NFs compound	200
3.48	Comparison with previous reported methods for analysis of antibiotic residues	202-204

Scheme List

No	Title	Page no
1	Extraction and clean-up for SAs, FQs, TCs, and CAP residues analysis in poultry meat and beef samples	77
2	Extraction and clean-up for NFs residues analysis in poultry meat and beef samples	79
3	Extraction and clean-up for antibiotic residues analysis in milk samples	83
4	Extraction and clean-up for antibiotic residues analysis in egg samples	88
5	Extraction and clean-up for antibiotic residues analysis in feed samples	91

List of Abbreviations

BFSA	Bangladesh Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
WHO	World Health Organization
SPE	Solid Phase Extraction
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry

Chapter 1

1.0 Background

Food safety is a fundamental pillar of public health, helping to prevent infectious diseases and reduce risks of animal and human health across all stages from food production, processing, retails to consumption. According to [Codex Alimentarius Commission 2020](#), food safety consists of a process during food consumption, handling, preparation, and storage (Lee et al., 2022). Modern food safety frameworks address long-term health hazards that arise from chronic effects linked to exposure at low levels of contaminants. It encompasses all activities aimed at maintaining food quality, preventing contamination, and preserving nutritional value, recovery with wide implications for public welfare, economic sustainability ([World Health Organization, 2024](#)), and national food security.

Food contaminants pose a significant threat to food safety by entering the food chain via production, processing, transportation, storage, or as by-products of environmental pollution. According to WHO these contaminants are broadly classified into three categories such as biological, chemical, and physical ([WHO, 2024](#)). Biological contaminants include harmful bacteria (*Salmonella*, *Escherichia coli*), fungi, parasites, and viruses. Chemical contaminants encompass heavy metals (arsenic, lead, mercury etc.), industrial chemicals (dioxins, polychlorinated biphenyls, etc.), mycotoxins, pesticides, and residues of antibiotics such as veterinary drugs. Physical contaminants include foreign materials such as glass, metal shards, plastic, or stones that enter food unintentionally during processing or packaging.

Additionally, emerging contaminants such as antibiotic-resistant bacteria, and their resistance genes, endocrine-disrupting chemicals, microplastics present long-term risks including carcinogenesis and the acceleration of antimicrobial resistance (AMR). Among all these, antibiotic residues are particularly concerning due to their direct contribution to the rise of drug resistance.

In Bangladesh's high populated density intensified pressure on food production, distribution, and retail, making food safety a central public health concern. Although government and non-government organizations regularly run awareness campaign, food related misconceptions remain deeply rooted and persist. Consumers often equate food safety with visual cleanliness such as fresh color, absence of dirt and tidy presentation while ignoring microbial and chemical hazards

that cannot be seen, smelled or tested. Contaminants often require laboratory analysis for accurate detection, yet routine testing is limited and rarely visible to the public. The knowledge gap is worsened by inconsistent and sometimes confusing regulatory communication. When inaccurate or non-scientific evidence-based messages or reports of food adulteration are conveyed from unreliable sources; people turn to informal channels. Social media then becomes a major source of misinformation or risk information where sensational posts and unverified claims spread quickly. As a result, public discussions frequently focus on dramatic narratives of food adulteration such as formalin use in fruits, vegetables and fish, harmful preservatives, excessive pesticides application and antibiotic misuse in animal derived foods without clear distinction between confirmed cases and rumor. Weak law enforcement and lack of transparency across the food supply chain reduce accountability and consumer trust. Inadequate labeling, and clear traceability and unclear guidance on safe handling and storage further contribute to confusion. The gaps ultimately reduce awareness of long-term harms including chronic toxicity and the growing threat of drug resistance related to improper antibiotic use of food animals.

As the demand for animal protein continues to rise in Bangladesh the unregulated use of antibiotics at sub-therapeutic (below therapeutic) doses has increased in livestock and poultry farms to enhance animal's disease resistance and accelerate rapid weight gain. In many cases, antibiotics are used without the supervision of professional veterinarians, resulting in persistent residues in foods of animal origin. Research in the poultry and cattle farms showed that, antibiotics are used at inappropriate dosages and withdrawal periods are frequently ignored, resulting antibiotic residues contamination in meat, milk, eggs, and animal feed ([Izah et al., 2025](#)). Poor feed quality, manufacturing faults, or intentional incorporation of growth-promoting substances into animal feed are the common reasons of food contamination ([Onyeaka et al., 2024](#)). When waste and manure from antibiotic-treated animals are used as fertilizer, antibiotic residues can spread into the soil, water, and surrounding environment, leading to crop contamination. In addition, inadequate food safety practices and a lack of transparency during slaughterhouse operations, milk collection, and packaging allow antibiotic residues to persist throughout the food supply chain.

Meat (poultry and beef), milk, eggs, and poultry feed analysis is crucial for ensuring public health protection considering Bangladeshi's people dietary habits. These foods are major sources of

dietary protein which closely linked to antibiotic misuse in animal production. The parallel and overlapping use of multiple antibiotics such as sulfa drugs (SAs), fluoroquinolones (FQs), tetracyclines (TCs), chloramphenicol (CAP), and derivatives of nitrofurantoin metabolites (NFs) makes health risk assessment more complex. The integrated analysis of meat, milk, eggs, and feed using advanced multi-residue detection LC-MS/MS methods can help determine contamination levels, support health risk assessment and provide guidance for developing public health policies.

This approach aligns with “One Health” framework, which recognizes interconnected nature of human and animal health, environmental health. Significant knowledge gaps still persist regarding the presence of antibiotic residues in Bangladesh’s food supply, despite this being recognized as an urgent public health issues. Regular, systematic, and peer-reviewed surveillance remains limited with existing studies predominantly small-scale or region-specific, narrowly focused on single antibiotic classes, or reliant on traditional analytical methods. In many cases, these studies are not aligned with international standards such as those of Codex Alimentarius or European Union, resulting in weak policy outcomes and a lack of consumer confidence.

Bangladesh Food Safety Authority (BFSA) is mandated to ensure the right to safe food by promoting proper application of scientific approaches. BFSA regulates and monitors activities across food production, importation, processing, storage, distribution, and retail and coordinates the roles and functions of all institutions and agencies involved in national food safety management. Although BFSA is responsible for regulatory oversight, it faces challenges due to limitations in capacity and resources. The determination of antibiotic residues is analytically very complex due to the variability in chemical diversity of antibiotics in different food matrices.

Conventional screening methods such as immunoassays, microbiological inhibition tests done with lack of adequate sensitivity, specificity, and less capability for effective multi-residue detection and surveillance. In contrast, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is internationally recognized for generating reliable and comparable data. LC-MS/MS enables precise and simultaneous identification and quantification of multiple antibiotic residues at very low concentrations, even below internationally established maximum residue limits (MRLs). However, its routine application in Bangladesh remains limited due to financial and

technical constraints. Therefore, the main objective of this study was to determine and quantify the concentration of antibiotic residues in poultry meat, beef, eggs, milk, and poultry feed samples collected from the Dhaka and Gazipur regions of Bangladesh using LC-MS/MS. The findings of this study will provide reliable baseline data, attempt to identify major sources, cause of contaminants, and support the development of evidence-based public health strategies.

In Bangladesh, this study was conducted to strengthen farmers' knowledge and capacity to move away from habit-based antibiotic use and scientific evidence-based practices that they can apply on farms. It aimed to make farmers understand that using antibiotics unnecessarily, incorrectly, or repeatedly without proper diagnosis can create drug-resistant bacteria, reduce animal productivity, and raise disease burden and treatment costs. Such misuse leads to more frequent outbreaks, longer recovery times, greater production losses, and growing dependence on stronger and more expensive antibiotics. Over time, problems reduce farm efficiency, harm profits, and threatens the long-term sustainability of farming systems. In addition, poultry feed analysis showed that poor-quality or contaminated feed, accidental cross-contamination, or illegal antibiotic inclusion can create situations where farmers believe they are not using antibiotics, while residue and resistance risks still persist in reality.

1.1 Antibiotics and their Structural Patterns

Antibiotics are used as prophylaxis to treat and prevent infections caused by bacteria and other infectious microorganisms (Cui et al., 2026). Their primary function is to inhibit bacterial growth (bacteriostatic action) or killing bacteria directly (bactericidal action). Broad-spectrum antibiotics e.g., sulfa drugs (SAs), fluoroquinolones (FQs), tetracyclines (TCs), chloramphenicol (CAP) and nitrofurantoin derivatives (NFs) target a wide range of bacteria, both gram-positive and gram-negative based on bacterial structures or metabolic pathways (Stojković et al., 2023). Antibiotic can be classified based on their origin, chemical structure and molecular formulas (Fig. 1.1), mechanism and spectrum of action, level of efficacy and route of administration, as well as logP and pKa values (Nugrahani et al., 2024).

Minor alterations in chemical structure can be led to significant changes in their properties such as activity, stability, solubility, toxicity and resistance profiles. Side chains determine the

pharmacokinetic behavior of antibiotics such as absorption, distribution, metabolism and elimination, whereas molecular properties such as water and lipids solubility and UV absorbance, ionization and interactions with biological membranes are governed by functional groups present in the compound (Khataei et al., 2022). The functional groups within a compound play a crucial role in the development of analytical methods for examples; in electrospray ionization mass spectrometry, the formation of protonated and deprotonated ions of polar and non-volatile molecules depending on their acidic or basic functional groups, enabling sensitive detection at trace levels (Qin et al., 2023), as well as UV absorption through conjugated double bonds and energy transitions within molecular structures, and partitioning behaviors in extraction and clean-up procedures are all structure-dependent (Khataei et al., 2022).

The extraction procedure selection is based on how the compound dissolves in various standard methodology is influenced by environmental factors such as temperature, humidity, light intensity. Each class of antibiotics has a specific application for example; cyclic antibiotics (Zhang et al., 2025) are effective against gram-negative bacteria. After use these antibiotics and their metabolites can enter different environmental sources leading to environmental persistence and potential human exposure (Khataei et al., 2022). Therefore, understanding antibiotic structural patterns is essential not only for interpreting their antibacterial function, but also designing appropriate extraction strategies, improving analytical detection and support reliable monitoring of residues in food and feed samples.

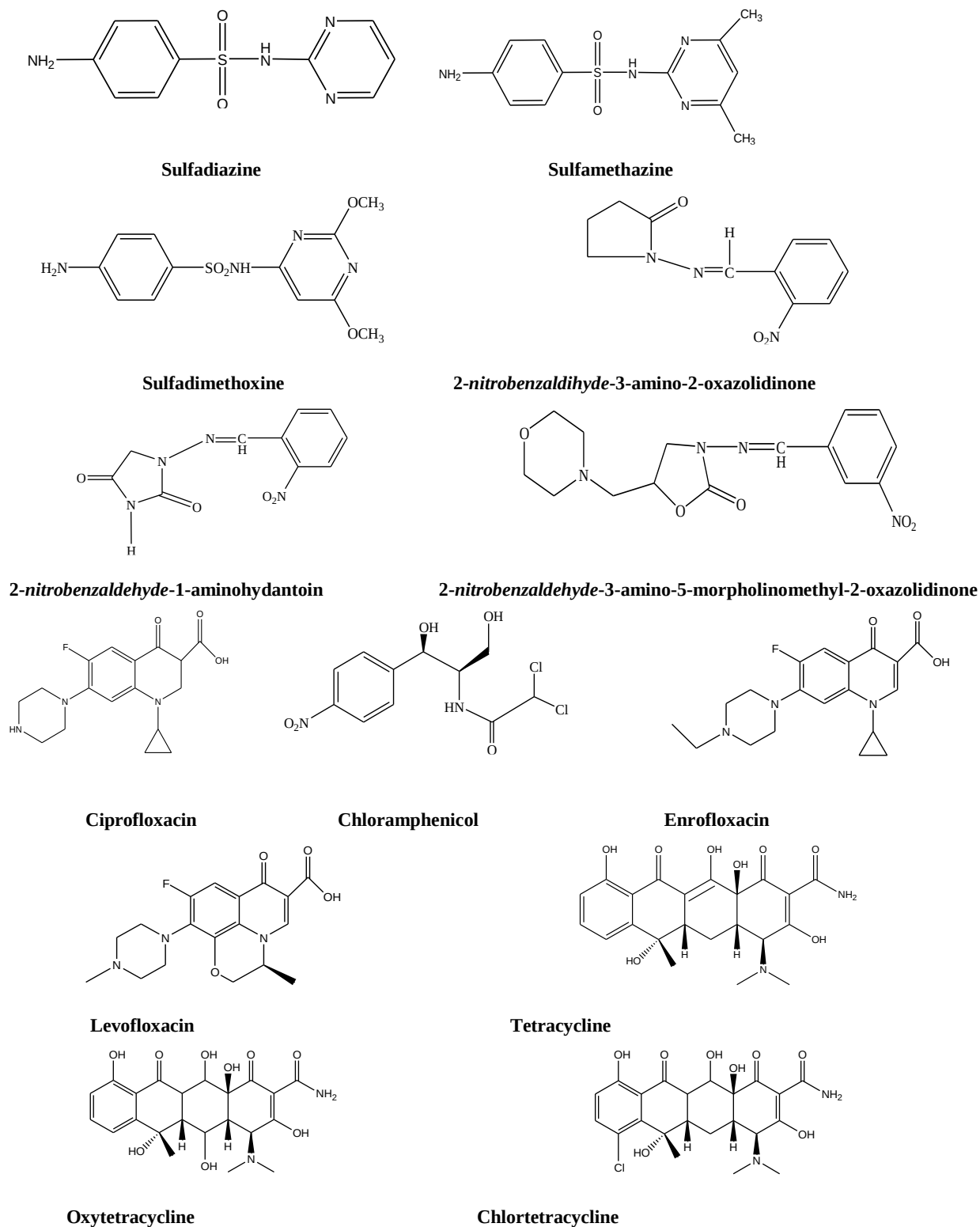


Fig. 1.1: Structures of Sulfa drugs, Fluroquinolones, Tetracyclines, Chloramphenicol and Derivatives of nitrofurans metabolites

1.2 Contamination of Antibiotics

When animals are treated with antibiotics, their metabolites are produced during antibiotic breakdown for detoxification and excretion can accumulate in edible animal tissues either as parent compounds or biotransformation products. These residues may sometimes exceed established maximum residue limits (MRLs), leading to antibiotic residues entering the food chain (Sachi et al., 2019). Incorrect or improper use of antibiotic reduces their effectiveness bacteria can change and survive antibiotic exposure, resulting in antibiotic resistance. Different pathways via unauthorized antibiotic doses, prolong treatment, inadequate collection times, treated sample mixing error, use contaminated feed or water, wrong selling times in farm-to-fork contribute to food contamination and increase antibiotic resistance (Li et al., 2025; Shoeb et al., 2023) (Fig.1.2).

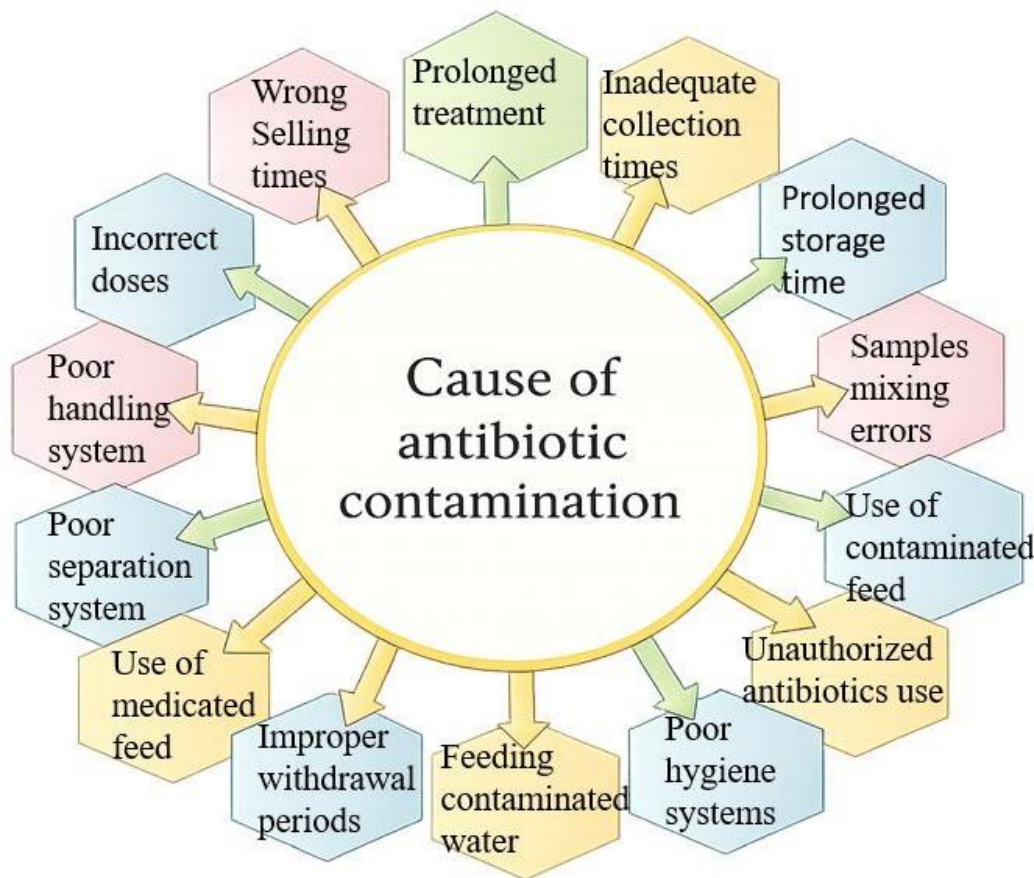


Fig. 1.2: Cause of antibiotics contamination

1.3 Antibiotic Resistance

Antibiotic resistance is a condition in which resistant bacteria can survive and continue to multiply even when exposed to an antibiotic. Antibiotic resistance development or spreading is a growing global health concern resulting from the excessive and improper use of antibiotics or wrong treatment in both humans and animals. It occurs when bacteria adapt to block the effects of antibiotics through multiple mechanisms such as restricting or blocking drug entry into the cell, changing the drug's target site, breaking down or deactivating or neutralizing the drug, and ejecting the drug from the cell (Okaiyeto et al., 2024). In that time, resistant bacteria can multiply through genetic mutations with each other. Resistance plasmids transferred poultry to humans frequently (Wang et al., 2024). These reflections may lead to antibiotic prophylaxis failure, increase clinical death, harder to treat or required for stronger or combined medications, increase treatment costs, prolong hospital time and increased expenses for making more testing cost for antibiotics effectiveness.

1.3.1 Global Statistics on Antibiotic Resistance

Antimicrobial resistance (AMR) may be responsible for 2 million resistance strains related infectious deaths and 5 billion USD losses in health care issues (Stojković et al., 2023), up to 10 million deaths annually by 2050, making it greater threat than cancer (Harun et al., 2024) and for these reason up to 2 trillion USD losses in the world (Li et al., 2025). According to WHO reports in 2025, Antimicrobial resistance (AMR) is the next pandemic in global health. Reports showed that drug-resistant bacterial infections could cause around 5.2 million deaths in 38 countries and areas of the Western Pacific Region between 2020 and 2030. Worldwide, nearly 5 million deaths in 2019 were linked to bacterial AMR. Out of these, about 1.3 million deaths were caused directly by drug-resistant infections (Fig. 1.3). Recent research also indicates that resistant bacteria can be transmitted from mother to newborn, leading to sepsis and significantly contributing to neonatal deaths, especially in low- and middle-income countries. Most critically, they contribute to the transmission of antibiotic-resistant bacteria (Arsène et al., 2022), which are currently responsible for 700,000 deaths worldwide each year (Chandrakar et al., 2023). In 2018, the U.S. Food and Drug Administration (FDA) recorded the sale of about 6,650 tons of key antibiotics like sulfonamides and tetracyclines for use in food animals, while Centers for Disease Control and

Prevention data shows that antimicrobial-resistant infections cause over 2.8 million cases and 35,000 deaths each year in the U.S., leading to greater healthcare expenses, extended hospitalizations, and higher mortality rates (Sarkar et al., 2023). Antibiotic resistant genes were found in poultry and livestock in South Asian countries over the last 10 years (Harun et al., 2024). World Bank modeling suggests that if antimicrobial resistance remains low, global health expenses may reach USD 330 billion, but in the event of high resistance, these costs could soar to USD 1.2 trillion (Muteeb et al., 2023). Research on antibiotic resistance is conducted in developed countries, with China (21.88%), Germany (4.91%), United States (4.46%), Spain (13.39%), and Italy (4.01%) (Sachi et al., 2019). This trend underscores a global research imbalance, where the analysis of antibiotic residues receives far greater attention in developed regions than in developing nations.

Over the last few decades the global major animal protein sources for humans, poultry meat which consumption has undergone tremendous growth. However, many veterinary drugs, particularly antibiotics, are administered to poultry for therapy and prophylaxis, and also used as feed additives to promote growth due to raising poultry meat demand (Fei et al., 2023). In Bangladesh, a study was conducted in Khulna, Satkhira, and Bagerhat districts to assess the presence of antibiotic residues in meat samples. Out of 200 total meat samples, 21% tested positive for antibiotic residues. Among districts, Khulna showed the highest prevalence in both chicken and cattle meat. Satkhira had a lower prevalence in chicken and cattle. In Bagerhat the rates were 28% for chicken and 6.7% for cattle based on 200 samples. Overall, chicken meat showed significantly higher residue levels than cattle meat across all districts, suggesting more frequent antibiotic use in poultry production which raises public health concerns (Matubber et al., 2025).

India's poultry industry, valued at around Rs. 80,000 crores, is rapidly growing, with over 90% of broiler meat sold live and only 5% processed. By 2025, poultry meat and eggs are expected to be the top animal protein sources. Widespread use of antibiotic feed supplements boosts production and disease prevention. Antibiotic use in Indian animal feed is projected to rise by 82%, with usage in chickens expected to triple by 2030 (Chandrakar et al., 2023). Approximately 75% of the 12 million kilograms of antibiotics used globally each year are administered in the animal agriculture sector (Murungi et al., 2024). More critically, misuse contributes to the development

of antibiotic-resistant bacteria, reducing treatment effectiveness and harming animal welfare. Numerous studies have reported antibiotic residues in poultry meat. For example, 33% of broilers in Nigeria out of 70 broiler chicken samples, and 24% of farms in Iran out of 90 broiler farms had detectable residues, with some exceeding maximum residue limits (MRLs). In Turkey out of 80 samples, 45% of chicken samples tested positive for quinolone residues. A broader assessment found that 77% of chicken samples were contaminated with at least one antibiotic, and 53% contained multiple residues. Eggs, which supply 12% of daily recommended protein, are a vital nutritional resource. Between 2000 and 2018, global egg production climbed by 50% to 77 million tons (FAO). India, the world's third-largest eggs producer, generated 138.38 billion eggs in 2022-23, with per capita availability rising to 101 eggs. Haryana, India alone produced 8.18 billion eggs, with per capita availability at 274. Tetracyclines are classified by the WHO as a highly important antimicrobial, faces widespread bacterial resistance, particularly among commensal bacteria. Around 80% of laying flocks are treated with antibiotics during their lives, leading to the presence of antibiotic residues in eggs (Waghamare et al., 2020). These findings highlight the urgent need for stricter regulation and responsible antibiotic use in the poultry sector to ensure food safety and reduce antimicrobial resistance in Bangladesh (Jammoul et al., 2019).

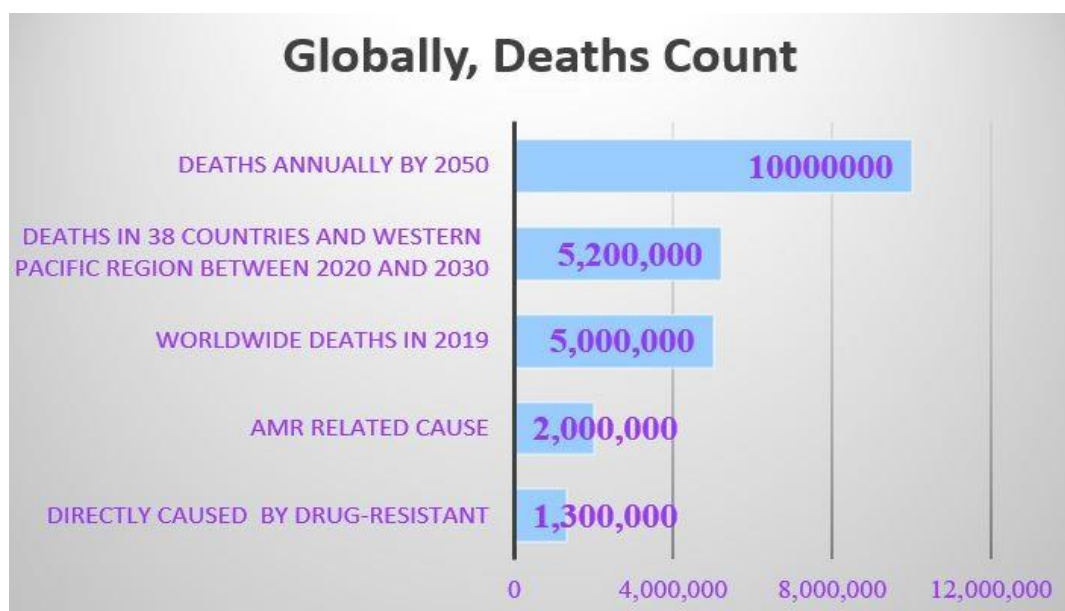


Fig. 1.3: Global deaths count caused by drug resistance

1.4 Routes of Antibiotic Exposure

Humans and animals are exposed to antibiotics through multiple direct and indirect routes. In livestock and poultry farming, antibiotics are frequently administered for various purposes, leading to residues in edible tissues such as meat, milk, eggs, and other animal-derived products (Fig. 1.4). Animals may receive antibiotics directly through feed, water, or injections, and these compounds can accumulate in tissues over time. Consumption of contaminated animal products represents a major route of human exposure, as residues can persist despite cooking or processing. Environmental pathways also contribute to exposure, since manure and waste from treated animals may introduce antibiotics into soil and water systems, which can subsequently contaminate crops and drinking water. Feed treated with antibiotics or containing contaminated additives serves as another source of residues in the food chain. Cross-contamination during processing, storage, or distribution can extend exposure to otherwise unexposed products. Improper use or overuse of antibiotics increases the risk of accumulation in edible tissues, eggs, and milk, creating a potential route for chronic human exposure. Veterinary and farm workers handling treated animals may also experience direct exposure through handling, skin contact, or inhalation. Aquaculture systems using antibiotics in fish farming represent an additional route for environmental and dietary exposure. Antibiotic residues can spread through multiple routes contributing antibiotic-resistant bacteria grow and spread. As a result, drug resistance can also spread through the animal-based food. Understanding, monitoring, and controlling these exposure routes is critical for reducing residue levels, ensuring food safety, protecting public health by minimizing the risks associated with antibiotic resistance, and maintaining the efficacy of clinically important antibiotics.

Antibiotics are used for several reasons, including their primary use as preventive or curative measures against bacterial infection. Others are used as growth promoters for improved animal health, eggs and milk production and feed conversion efficiency, carcass quality, and economic production ([Arsène et al., 2022](#)). Antibiotics are widely preventing infections and various diseases of poultry and cattle. Preventive administration involves giving sub-therapeutic doses to otherwise healthy animals considered at risk of illness. Moreover, relatively low dosages of antibiotics are frequently added to feed to inhibit gut microflora, thereby improving nutrient availability and supporting better growth performance ([Kamouh et al., 2024](#)).

Antibiotic residues in livestock are often associated with gastrointestinal absorption and excretion

pathways. After direct injection or oral administration through medicated feed or water, antibiotics are absorbed in the gut and distributed via systemic circulation due to the antibiotic's hydrophobic or lipophilic properties. However, incomplete absorption or enterohepatic recirculation can result in residues being retained in gut tissues or excreted through feces. These residues may accumulate in the intestinal lining and contribute to antimicrobial resistance within the gut microbiota. Monitoring animal tissues is essential to ensure food safety and to minimize public health risks associated with contaminated poultry meat or beef muscles.

Eggs serve as a vital source of protein and vitamins for all human being; affordable for low-income families and offer a quick source of nutrition and income with minimal expenditure. These systems provide a rapid turnover of animal-source food, such as eggs and meat, which can be consumed or sold to support household needs within a short time. However, poultry in these systems are often raised under inadequate disease control measures and poor management practices, increasing their susceptibility to infections. The lack of biosecurity exposes these chicks to significant risks of infectious diseases, including avian influenza, newcastle disease, salmonellosis, fowl cholera, fowl typhoid, tuberculosis, paratyphoid, and gumboro. Similar biosecurity gaps have been observed in commercial poultry operations as well and may face similar challenges. Additionally, recommended guidelines such as appropriate timing of antibiotic application, adherence to withdrawal periods, and respecting re-entry intervals are frequently overlooked, further compounding food safety and poultry chicken health concerns ([Cornejo et al., 2020](#)).

The lack of biosecurity exposes cattle to significant risks of bacterial diseases, including tuberculosis, paratuberculosis, leptospirosis, mastitis, salmonellosis, respiratory disease and anthrax. So, antibiotic residues in beef and milk represent a pressing public health concern with far-reaching implications. Even low levels of these residues can promote the development of antibiotic-resistant microorganisms. These resistant microbes can spread between individuals either through direct or indirect contact. Although high-quality milk and dairy products are essential for maintaining public health, the presence of antibiotic residues poses significant risks to consumers. Furthermore, antibiotic residues can disrupt the dairy industry by interfering with fermentation processes critical to the production of yogurt and cheese, as they inhibit the activity of beneficial starter cultures, ultimately contributing to the growing problem of antimicrobial

resistance ([Sachi et al., 2019](#)).

Antibiotic residues can enter cow's milk or beef through several connected pathways. For example, when antibiotics are used to treat mastitis, the drug can spread through the bloodstream and residues may pass into beef muscles or milk through the mammary gland, including via intramammary tubes directly. In addition, residues can remain in milk from treated cows if the withdrawal period is kept too short, or if milk is collected or marketed before the withdrawal period ends, or if unapproved antibiotics or incorrect doses are used. Another major cause is treated milk accidentally mixing into the bulk tank (mixing errors) during milk collection, which can contaminate the entire tank. Residues can also appear if medicated feed intended for calves is mistakenly given to lactating cows. Even though contamination of water sources with antibiotics is more indirect, it can increase exposure risks on the farm. Feeding waste milk (milk from treated cows) to calves can also contribute to contamination risk if handling and separation are poor. Finally, if milk storage/transport systems are not kept separate and properly cleaned or flushed, residues can remain in the equipment and carry over into later milk samples. Similar reasons were applicable for eggs contamination via antibiotic residues.

Typically, antibiotic residues found in poultry meat, beef, eggs, milk, and feed is attributed to the application of veterinary medicines, inclusion of antibiotics as feed additives, or the use of unapproved drugs contrary to existing regulations. Antibiotic residues differ by sample types, potentially influenced by factors such as administration methods, metabolic pathways, chemical properties, regulatory enforcement, feeding practices, nutritional inputs, economic significance, and productivity of the livestock. Antibiotics are primarily administered via drinking water and feed in livestock and poultry farming ([Wang et al., 2017](#)). Nonetheless, aquatic products exhibited a higher frequency of antibiotic detection than livestock and poultry meat, with TCs showing an inverse pattern ([Wang et al., 2017](#)).

Metabolic process of antibiotic started after digestion by the liver and kidneys, eventually accumulating and being excreted via feces and urine. In animals, these are directly released into the environment. Notably, certain environmental bacteria, such as *Streptomyces*, naturally produce antimicrobials (e.g., streptomycin) which can accumulate may travel from soil to surface or

groundwater, or be absorbed by plants, eventually circulating through the food chain and water supply system. As antibiotics often occur in mixed forms, they may cause combined effects or negative interactions in the environment. In some cases, their metabolic byproducts can be more toxic than the original compound, with potential carcinogenic and mutagenic effects (Khataei et al., 2022).

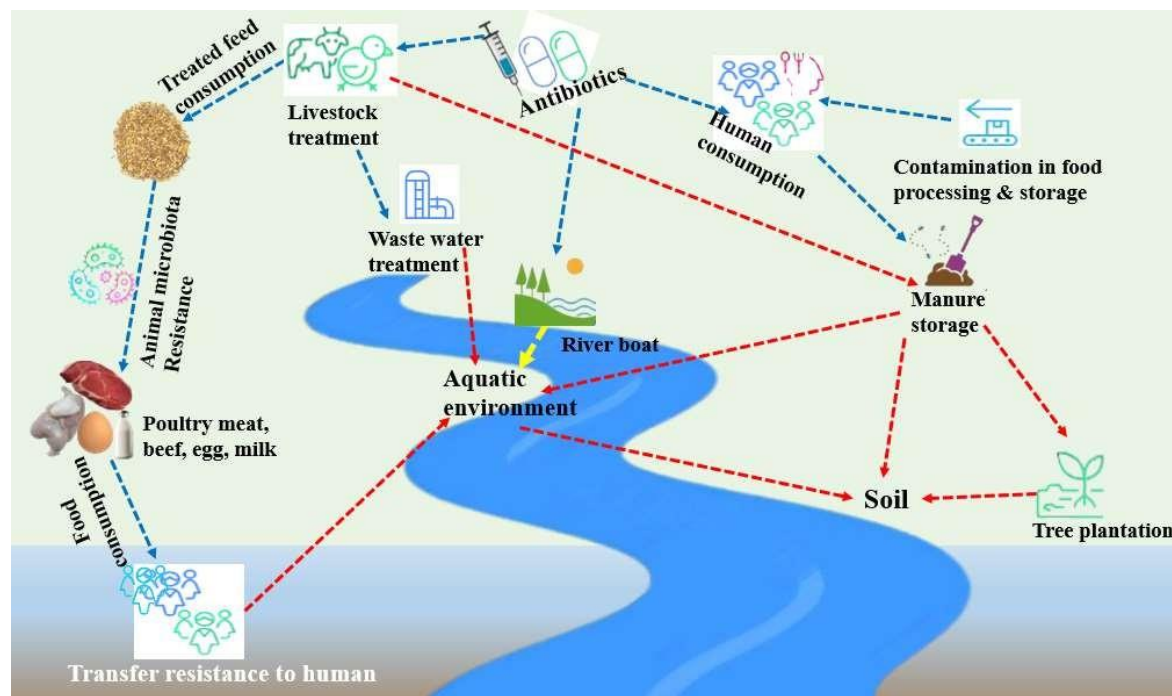


Fig. 1.4: Routes of antibiotic exposure

1.5 Drug Residues Surveillance in Livestock

Globally, broiler poultry is an affordable protein source and broiler feed accounts for 27.6% of global feed tonnage, within a total 2024 feed output of 1.4 billion metric tons; China led production with 315 million metric tons (Alltech feed production survey, 2025). The FAO projects showed global food demand will rise by about 60% by 2050, alongside expansion in animal protein output (~1.7% annually). Within this growth, meat production could increase by nearly 70% and dairy by up to 55%. Supporting this demand, globally feed production already exceeds one billion tons per year, and the commercial feed industry generates an estimated USD 400 billion in annual turnover, expansion is occurring in developing countries as well including Bangladesh (global feed statistics, 2025).

On the other hand, misuse of antibiotics also promotes residue accumulation in edible organs, elevating zoonotic and food-safety risks through food or contact (Harun et al., 2024) and drug resistance has become a major constraint on public health around the world and more concern for the developing densely populated south Asian country, including Bangladesh, India, Nepal, Pakistan, and Sri Lanka, where rural livelihoods are closely tied to poultry and livestock production (Harun et al., 2024). Inadequate farmer knowledge, limited veterinary supervision and inappropriate antibiotic selection and dosing facilitate the emergence and spread of resistant pathogens, with transmission to humans occurring via food contamination and environmental pathways. Poultry disease such as abscesses, yolk sac infection, omphalitis, cellulitis, colibacillosis, coligranuloma, enteric and skin infections, endocarditis, gastroenteritis, swollen head syndrome, septicemia, pneumonia, toxic shock syndrome, and foodborne illness are frequently associated with *Escherichia coli* (*E. coli*), *Salmonella* spp., and *Staphylococcus aureus* increasing production losses, animal and human healthcare costs (Harun et al., 2024). *S. aureus* is increasingly aggressive both animal and human hosts which is the leading cause for human salmonella. *E. coli* transmission and are associated with high resistance to tetracyclines and growing fluoroquinolone resistance, including ciprofloxacin for common poultry farming practices in Bangladesh. Fluroquinolones (FQ) resistance is a major concern, with 11.5% of *E. coli* isolates showing high resistance to ciprofloxacin. High resistance to tetracyclines (TCs) was found in Bangladeshi poultry. Notably, resistance to nitrofurantoin, a broad-spectrum human antibiotic not used in local veterinary practices was also detected. The frequent and unregulated use of antibiotics, including in poultry litter used for crop and fish farming, increases the risk of spreading resistant bacteria to humans and the environment. Around 37% of isolates showed multidrug resistance due to the common use of antibiotic combinations as growth promoters (Hasan et al., 2011). In the study by Manafi et al., 2018, 600 broiler chickens were fed for 42 days and divided into two groups: a control diet and a diet supplemented with tetracyclines (TCs) at 500 mg per kg of feed. Compared with the control group, TCs improved final body weight and feed conversion ratio, but showed mixed effects on carcass quality. The easy availability of chlortetracycline (CTC) in the market of India may be one reason for its widespread use in feed. (Waghamare et al., 2020). According to the European Medicines Agency (2016), tetracyclines comprised 33.4% of all antibiotics used in veterinary practice in 2014. In Bangladesh, 249 registered and 83 unregistered mills in 2020-21, dominated by large firms such as Kazi Farms and Paragon; the top 25 companies

controlled about 81.5% of the market. Rising raw material costs in 2023-2024 reduced demand and pushed many small mills to shut down or operate below half capacity ([poultry tech Bangladesh impact, 2024](#)). Although, regulatory frameworks, such as European rules on medicated feed, treat feed drug mixtures as a controlled delivery route and allow limited variation ($\pm 10\%$) from labeled antimicrobial content but reports of multidrug resistance and potential environmental dissemination via antibiotic-contaminated litter emphasize the need for routine surveillance and stricter control measures in Bangladesh ([Hasan et al., 2011](#)).

1.6 Antibiotic residues in Food and Feed

In antibiotic residues investigation, it is essential to consider the specific concerns for each target matrix such as poultry meat, beef, eggs, milk and feed due to their distinct composition, metabolic pathways, and consumption patterns types. Poultry meat is among the most consumed animal proteins, next to egg and milk, beef and residues in muscle tissues can pose serious antimicrobial resistance, and long-term toxic effects. Milk consumed across child and age groups widely which is particularly sensitive due to its daily intake. Even low levels of antibiotic residues in milk can reduce dairy product quality and fermentation processes, and promote antibiotic-resistant bacteria. Moreover, eggs enriched with essential nutrients which consumed in both rural and urban people. If withdrawal periods are not maintained eggs are vulnerable to contamination during development and feed being the primary route of antibiotic exposure in livestock, plays a critical role in residue transfer. Medicated feed contains subtherapeutic antibiotic doses for growth promotion which is increasing bioaccumulation and environmental risk. Therefore, matrix-specific analysis is needed to determine real scenario of retail and local market also super shops for control strategies of antibiotic residue free food consumption and protecting public health from potential risks associated with residues exceeding international safety limits ([Chowdhury et al., 2015](#)).

The rising demand of poultry and cattle farming, animals are growing in highly dense housing and communal feeding and facilitating rapid diseases spreading, and often necessitate the routine use of antibiotics to prevent productivity losses ([Velazquez-Meza et al., 2022](#)). However, this practice has raised major food safety concerns because indiscriminate antibiotic use may leave in residues in edible poultry meat, beef, eggs, and milk, which are direct pathways of exposure for consumers ([Owusu-Doubreh et al., 2023](#)). Among these foods, eggs are a highly nutritious food, containing a

wide range of bioactive nutrients including essential amino acids, proteins, vitamins, and fats such as cholesterol and monounsaturated fatty acids ([Lima et al., 2024](#)), and egg yolk is rich in fat-soluble vitamins A, D, E, K, and vitamin B-complex (B1, B2, B3, B5, B6, B9, B12), minerals such as calcium, phosphorus, potassium, zinc, magnesium, and selenium ([Sanlier et al., 2021](#)). Despite their nutritional value, eggs containing any level of antibiotic residue are considered risks of antibiotic contamination.

The demand for milk is rising rapidly due to population growth, broader access to education, economic development, and greater nutritional awareness. Antibiotic use in dairy farming especially for mastitis disease control raises concerns about residue contamination. Most antibiotics used in dairy production are administrated through intramammary infusion (92%), followed by injections (6%) and other routes (2%) ([Chowdhury et al., 2015](#)). Residues may enter milk through intentional or unintentional use of antibiotics, often linked to limited knowledge of prudent antibiotic practices and inadequate farm management. Their detection also complex because residues can interfere routine quality assays, including microbial inhibition and fermentation-based tests. In many cases, contamination occurs when farmers fail to follow the recommended withdrawal period before milk is collected. Every trace residue level can facilitate the selection of antibiotic-resistant microorganisms and are associated with adverse health outcomes, including hypersensitivity and allergic reactions (such as serum sickness and anaphylaxis), as well as potential long-term effects like carcinogenicity, chromosomal damage and DNA/RNA interactions, teratogenicity, and infertility. Furthermore, residues suppress beneficial starter cultures, disrupting dairy fermentation processes, reducing yogurt and cheese production, ([Sachi et al., 2019](#)) while also aggravating drug resistance and making infections harder to treat.

Feed may be contaminated with veterinary drug residues through multiple pathways including excessive and inappropriate use of medicines, carry-over from medicated feed to non-medicated feed, and the application of animal manure as fertilizer. According to the 7th ESVAC report, antimicrobials in medicated feed are used in 30 European Union countries to treat and prevent diseases in food-producing animals ([Patyra et al., 2018](#)). Monitoring antibiotics in feed is technically demanding because of the wide range of antibiotics' chemical and physical properties. This diversity creates multiple analytical barriers, such as variation in sample preparation and

extraction methods, matrix interference, and the need to optimize analytical conditions (Kenjerić et al., 2024). In addition, proper management of veterinary drug standards is essential. Animal manure is an important source of agricultural nutrients (especially nitrogen and phosphorus) varies by species, diet, and farm management practices. Manure can therefore indirectly contribute to environmental contamination that may ultimately affect agricultural products. Along with organic and inorganic components, manure may also contain heavy metals, hormones, and antibiotic residues. To improve feed efficiency, antibiotics are often added to animal feed at sub-therapeutic levels can lead to substantial environmental release, with estimated about 75% of these antibiotics are excreted unchanged into the environment, where they can disrupt terrestrial and aquatic ecosystems (Campagnolo et al., 2002). This environmental release further highlights the importance of integrated surveillance and responsible antibiotic use throughout the animal production system. In Bangladesh, different class of antibiotics were used for meat, milk and egg production farms such as sulfa drugs, fluroquinolones, tetracyclines etc to treat bacterial disease control.

1.7 Sulfa Drugs (SAs)

Sulfa drugs (SAs) have a core structure with a sulfur atom linked to two oxygen, a nitrogen and an aniline group with variable side chains. Variations in side chains such as methyl groups (4th and 6th positions) in SMT; 4-amino-N-(4,6-dimethylpyrimidin-2-yl) benzenesulfonamide), methoxy groups (5th and 6th positions) in SMX; 4-amino-N-(5,6-dimethoxypyrimidin-2-yl) benzenesulfonamide, or no substitutions in SDZ; 4-amino-N-(pyrimidin-2-yl) benzenesulfonamide affect their chemical, pharmacokinetics and biological properties (Ovung et al., 2021). SAs work by inhibiting bacterial tetrahydrofolate production, blocking DNA replication, bacterial growth and prevents bacterial multiplication (bacteriostatic). In humans, folic acid (vitamin B9) is crucial for DNA synthesis and repair, promotes cell growth and supports reproductive health like improve sperm count and motility in men, while impaired folate metabolism can increase cancer risk (Ovung et al., 2021).

1.7.1 Sulfa drug (SAs): Literature Survey

Bacteriostatic SAs, a free amino group (-NH₂) is attached to a benzene ring, and a sulfonamide

group ($-\text{SO}_2\text{NH}_2$) is positioned para to the amine group. SAs look similar in structure to *p*-aminobenzoic acid (PABA) which bacteria need to grow. Because of this similarity, SAs compete with PABA and block its conversion into dihydropteroate (folate production key precursor) (Venkatesan et al., 2023). As a result, SAs prevent dihydropteroate, a bacterial survival nutrient for inhibiting bacterial growth, multiplying and reproduction or directly killing bacteria (Ovung et al., 2021; Xu et al., 2016). Most pathogenic bacteria cannot utilize folic acid and thus cannot bypass the inhibition of folic acid synthesis caused by SAs. Modifications to the amino group of SAs alter their antibacterial spectrum. Due to their alkaline nature, most SAs are suitable for oral administration and are typically given by this route (Daeseleire et al., 2017; Tolika et al., 2010).

SAs such as Sulfadimethoxine (SMX), Sulfadiazine (SDZ), and sulfamethazine (SMT) are widely used due to their key advantages, including a broad spectrum of antibacterial activity strong effectiveness, and affordability (Huertas-Pérez et al., 2016). SAs are used for bacterial infections as a first line (Catania et al., 2024) treatment in humans and livestock but frequently misused or overused for animal breeding due to their affordability and antibacterial efficacy, leading to their accumulation in the human body which can result in residues trigger adverse outcomes such as allergic reactions (Velazquez-Meza et al., 2022), antibiotic resistance (Bava et al., 2024; Zhang et al., 2024), toxicity, teratogenic (birth defects) and carcinogenic (cancer) effects (Xu et al., 2016). After being used in hospitals, SAs are excreted by humans into domestic sewage systems. These water-soluble weak acidic drugs (Akili et al., 2021) and their metabolites, reach wastewater treatment plants (WWTPs), which can become the primary entry point for SAs into surface and groundwater once discharged into the environment (Tolika et al., 2010). The presence of SAs in the aquatic environment was linked to their hydrophilic characteristics (Ciglenc̆ki et al., 2021), which facilitates their mobility in water (Suo et al., 2019). Due to their high resistance to degradation and potential human exposure (Izah, S. C. et al., 2025).

In humans, exposure can result in adverse effects like acute hemolytic anemia, nausea, vomiting and anorexia occur in 1-2% of patients (Zhang et al., 2012), hypersensitivity reactions, Stevens-Johnson syndrome, chronic bacterial infections (Zhang et al., 2012), urinary tract infections, fever, serum sickness, enzyme activity suppression, hepatocellular dysfunction and necrosis, sometimes carcinogenic. Clinically, SAs are utilized to treat conditions such as bronchitis, bacterial

meningitis, ocular infections, pneumocystis carinii pneumonia, ulcerative colitis, rheumatoid arthritis (Tolika et al., 2010), central nervous system disorder, and extreme touchiness responses (Ovung et al., 2022). SAs were the first therapeutic agents used for a range of conditions, including infections, inflammation, but they can also cause strong allergic reactions such as Stevens-Johnson syndrome and toxic epidermal necrolysis in about 3-8% of people. SDZ is especially important for treating toxoplasmosis in humans and burn wound infections. These drugs are known for their resistance to biodegradation, and multiple studies have examined their environmental impact, antibacterial effects, and how they interact with biomacromolecules (Ovung et al., 2021). Sulfadiazine (SDZ), an intermediate-acting sulfa drugs with a 12-hour half-life, is commonly employed in the treatment of toxoplasmosis, characterized by rapid absorption, hepatic metabolism, renal excretion, notable distribution in cerebrospinal fluid, and 45-55% protein binding. Sulfamerazine (SMZ) can trigger allergic reactions and may lead to hypothyroidism or thyroid tumors in rodents, raising concerns that it might be carcinogenic (Zhang et al., 2012).

According to the review by Malintan et al., 2006, multiple studies have shown that environmental antibiotic contamination often originates from wastewater treatment plants surrounded by animal farming activities, as well as wastewater discharge from animal feed production and livestock farming operations. The study also reports that elevated levels of SMT have been detected in intensive farming areas, exceeding the concentrations found in nearby wastewater treatment facilities. Researchers detected SMT and SMX in groundwater close to confined animal feeding operations (CAFOs), another research further confirmed the traces in groundwater and water sources near poultry farms. These findings collectively highlight that animal agriculture, alongside those from human medicine, significantly contribute to environmental contamination (Malintan et al., 2006).

SAs are used in poultry frequently to halt bacterial multiplication by acting as competitive inhibitors of p-aminobenzoic acid within the folic acid metabolic synthesis pathway (Cheong et al., 2010) for therapeutic, prophylactic, therapeutics or growth-promoting purposes (Cheong et al., 2010). However, SAs resistant strains of bacteria has increased and are present in the commercially available meat for improper use such as failure to maintain adequate withdrawal periods. The widespread application of SAs has also raised serious concerns regarding their carcinogenic

potential (Errayess et al., 2017) and contribution to SAs resistance, posing notable risks to public health like carcinogen (Cheong et al., 2010). In poultry, SAs exert their antimicrobial effect by competitively inhibiting dihydropteroate synthase, an enzyme critical for the biosynthesis of folic acid in microbes. SAs are categorized as short and intermediate acting agents are employed to treat protozoal and helminthic diseases in poultry including coccidiosis, histomoniasis, ascariasis, and capillariasis, which manifest as diarrhea, impaired growth, increased mortality, blackhead, intestinal blockages, and defective feathering. SAs have a slow metabolism and tend to remain in body for a long time, leading to excess residues in livestock and poultry (Zuo et al., 2021); although they undergo hepatic metabolism, a significant portion is excreted unchanged in urine (Tolika et al., 2010). Antibacterial SAs like SMT and SDZ, which contain an aromatic amine group, are used to treat livestock gastrointestinal and respiratory infections, while non-antibacterial SAs lacking this group generally have a lower risk of allergic reactions and are considered safer. SMZ is also unauthorizedly used as a growth promoter in animal feed. In livestock production, approximately 50-80% of antibiotics in the European Union and United States are administered to animals, with the primary purpose being to enhance growth and guard against bacterial infections (Xu et al., 2022). The administration of SAs to poultry is prohibited during the laying period, whether as veterinary medicine or feed supplements (Suo et al., 2022).

Failure to observe proper withdrawal periods contributes not only to food contamination but also to the development of SAs resistance (Odundo et al., 2023; Suo et al., 2022; Sumaiya et al., 2024; Sani et al., 2023). SAs' resistance to degradation and their hydrophilic nature enables their persistence in animal products and environmental systems, increasing the risk of indirect human exposure. The misuse of sulfonamides in livestock for both therapeutic and prophylactic purposes, without proper regulation, further exacerbates this public health concern.

1.8 Fluoroquinolones (FQs)

Synthetic and broad-spectrum fluoroquinolones (FQs) e.g., ciprofloxacin (CIP), enrofloxacin (ENR) and levofloxacin (LEV) are widely employed in humans' treatment and animal husbandry practices to treat bacterial infections in livestock, poultry, and aquaculture. However, their extensive and indiscriminate use has contributed to concerns regarding antibiotic residues in

animal-derived food products and the global threat of FQs resistance. Understanding the mechanism and mode of action of CIP, ENR and LEV are fundamental to evaluating their efficacy, safety, and impact on public health. Fluroquinolones (FQs) are derivatives of nalidixic acid, structurally characterized by a quinolone core with a fluorine atom at the 6th position. The fluorine atom significantly enhances their antibacterial activity, spectrum, and pharmacokinetic properties, making them effective against a wide range of gram-negative and gram-positive bacteria. CIP has linked 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. The structure includes a quinolone core (bicyclic ring system), a fluorine atom at 6th position, a cyclopropyl group at 1st position (enhances gram-negative activity), and a piperazine ring at 7th position (broadens spectrum). CIP has good oral bioavailability, high tissue penetration activity and broad-spectrum character which make it popular for treating urinary tract, respiratory, and gastrointestinal infections.

ENR is chemically linked with 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3 quinoline carboxylic acid. The quinolone core and fluorine at 6th position are retained. The cyclopropyl group at 1st position is similar to CIP, but the piperazine ring at 7th position carries an ethyl group at the 4th position. ENR is metabolized and contributes to its antimicrobial effects in humans and animals. It is used extensively in food-producing animals (poultry and cattle) for treating respiratory, urinary, and systemic infections. LEV is belonging to the 3rd generation of

FQs which linked to (S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid. It features a fluorine atom at 6th position, a methyl group at 3rd position, and a piperazinyl group at 7th position. The presence of the L-isomer configuration enhances binding to bacterial enzymes.

1.8.1 Fluoroquinolones (FQs): Literature Survey

Fluroquinolones (FQs) primary target is bacterial DNA replication, specifically the enzymes DNA gyrase (topoisomerase II and IV) introduces negative supercoils into structure of bacterial DNA, a prerequisite for DNA helix replication, transcription, and repair. FQs bind to the gyrase-DNA complex, stabilizing the DNA breakage-reunion intermediate. This prevents the resealing of DNA breaks, ultimately blocking DNA replication and leading to cell death. DNA gyrase is the main

target in gram-negative bacteria. Topoisomerase IV is separating replicated chromosomal DNA into daughter cells during cell division. In gram-positive bacteria, FQs inhibit topoisomerase IV more efficiently than DNA gyrase, thereby impeding chromosome segregation and bacterial proliferation. This broad and potent activity such as rapid cell death by causing irreversible damage to bacterial DNA underpins FQs popularity for treating bacterial infections.

The widespread use of FQs raises significant concerns regarding food safety and public health. FQs residues can persist in animal-derived foods, potentially causing adverse effects in consumers, such as hypersensitivity reactions, genotoxicity, carcinogenicity, neurotoxic effects, phototoxic skin reactions, immunotoxicity, and endocrine disruption. Furthermore, the ingestion of FQs residues through the food matrices may contribute to the development of FQs resistance in human pathogens, thereby reducing the effectiveness of clinical antibiotics and altering the ecology of the consumer's intestinal flora. Fluoroquinolones (FQs) show damage to the juvenile joint, the kidney, the eye, and the central nervous system (Er et al., 2013). LEV has a broader spectrum of activity on gram-positive bacteria, especially *Streptococcus pneumoniae*. It is widely used in human respiratory, urinary and skin infections diseases and also improved pharmacokinetic properties and safety profiles.

FQs have been reported to introduce chondrotoxic effects in young animals and are associated with an increased risk of tendon rupture. ENR is commonly administered orally to chickens and cattle or via subcutaneous injection. Due to its low ionization and high lipophilicity, ENR is rapidly absorbed and widely distributed throughout animal tissues. Consequently, ENR is used in poultry, cattle, and fish for the treatment of infectious diseases and in some settings for disease prevention and growth promotion. In poultry production, ENR is considered effective against bacterial infections such as mycoplasmas, colibacillosis, and pasteurellosis (Moghadam et al., 2018). Unauthorized FQs doses in both clinical and animal husbandry practices have accelerated the development of resistant bacterial strains, decreased drug uptake due to changes in bacterial outer membrane permeability, and the active efflux of the drug from bacterial cells. These resistances reduce the efficacy of FQs and pose a significant challenge to public health. Residues of FQs in poultry meat, milk, eggs, and animal feed can expose consumers to subtherapeutic antibiotic levels, increasing the development of resistant pathogens in the human gut microbiota.

Therefore, monitoring and controlling veterinary drug residues in food-producing animals is the safeguard for protecting public health.

CIP and ENR are used in animal farming for disease prevention and growth promotion, sometimes even added to animal feed. They are usually prescribed for the treatment and prevention of infectious diseases in farm animals. CIP is effective against microorganisms which are resistant to other antimicrobial agents, such as tetracyclines, macrolides and β -lactams and ENR is partly metabolized into CIP in cattle, making both detectable in meat products. These antibiotics are effective against bacteria resistant to other drugs, but their misuse can lead to residue accumulation in animal tissues, particularly the liver. Long-term exposure to doses of antibiotic residue in the body could lead to acute or chronic toxicity to the organs and the entire body. This poses serious health risks, including antibiotic resistance, allergic reactions, clinical mastitis, cancer and hypersensitivity reaction (Sachi et al., 2019), neonatal diarrhea (Chiesa et al., 2021), urinary tract and enteric infections (Sultan, 2014), and organ toxicity (Ramatla et al., 2017). CIP, a light sensitive, synthetic antibiotic from the fluoroquinolone class, demonstrates potent antibacterial effects against gram-negative bacteria by inhibiting their DNA and protein synthesis. It is used to treat for anthrax, urinary tract, gastrointestinal tract, respiratory tract and skin infection (Demeiry et al., 2020). Despite the detection of antibiotic residues from all the organs, the liver stood out as the organ with the highest CIP detection level (30%) (Ramatla et al., 2017) and this could be as a result of the roles the liver plays in animals and humans. The liver helps in protein synthesis and also in metabolism and detoxification processes. Most of the toxic substances and residues are metabolized in the liver so acute liver injury has been described as the leading cause of drug withdrawal based on safety grounds. The presence of FQs residues coupled with multidrug residues in some of the meat samples calls for concern, as this could pose serious public health risks to humans and animals, such as toxicity and FQs resistance development (Ramatla et al., 2017). Key contributors to the presence of FQs residues in milk include dry cow therapy, treatment of mastitis, and post-surgical infection management in cattle. Fluroquinolones (FQs) in animal feed at levels exceeding the established MRLs create a secondary source of antibiotic contamination in milk and dairy products, posing serious health risks to consumers, including allergic reactions, toxicity, and drug resistance (Sachi et al., 2019). ENRs are administered at sub-therapeutic levels to promote livestock growth and at therapeutic levels to treat infections in

humans, with a significant proportion excreted unmetabolized or as conjugates that can revert to parent compounds, while environmental degradation of FQs through photolysis and chemical oxidation plays a key role in their fate (Renew et al., 2004). The FQs exhibit increased antibacterial activity against the Enterobacteriaceae and other gram-negative bacteria such as *Pseudomonas aeruginosa*, and exert some activity against certain gram-positive cocci (Hubicka et al., 2013).

1.9 Tetracyclines (TCs)

The core structure of broad-spectrum tetracyclines comprising tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) features a robust, fused tetracyclic nucleus of four condensed rings, resulting in an octahydrotetracene-2-carboxamide skeleton typical of polyketide antibiotics. This nucleus is characterized by functional groups at C10 (phenolic), C11-C12 (keto-enol conjugation), C12a (hydroxyl), and C1-C3 (diketo) (Borghi et al., 2014), with the C4 dimethylamino substituent being crucial for antibacterial activity. Tetracycline (TC) contains a C6 methyl group along with multiple hydroxyl and keto groups. Oxytetracycline (OTC) differs from TC by an additional hydroxyl group at C5, making it a hexahydroxy derivative that influences solubility and pharmacokinetics. CTC, on the other hand, is distinguished by a chlorine atom at the C7 position, which affects its binding properties and serum protein interactions. Recognizing these variations in side chain chemistry is essential for assessing how residues behave in poultry meat, beef, eggs milk, and feed, as such differences affect their stability, binding properties, and biological effects.

1.9.1 Tetracyclines (TCs): Literature Survey

Tetracyclines (TCs) are widely used in farm animals for the prevention and treatment of various infectious diseases due to their effectiveness against both gram-positive and gram-negative bacteria (Venkatesh et al., 2013; Bahmani et al., 2020), mycoplasma and several protozoans (Koutsoumanis et al., 2021) and their affordability. TCs at therapeutic doses rapidly induce both transmissible and adaptive resistance by targeting the ribosomal complexes of various pathogenic and commensal bacteria (Koutsoumanis et al., 2021). Once inside the bacterial cell, TCs bind reversibly to the 16S rRNA of the 30S ribosomal subunit (Patyra et al., 2014), blocking the entry of aminoacyl-tRNA (Borghi et al., 2014) at the A-site. This action inhibits protein synthesis and

bacterial growth, demonstrating a primarily bacteriostatic mechanism (Bahmani et al., 2020). However, the most lipophilic TCs can also disrupt bacterial membranes, leading to a bactericidal effect. Tetracyclines resistance mechanism in bacteria (Koutsoumanis et al., 2021) occurs through several strategies including pumping the drug out (efflux) (Arsène et al., 2022), decreasing drug uptake, protecting the ribosome, and enzymatic inactivation. Both gram-positive and gram-negative bacteria exhibit these mechanisms, often by acquiring resistance genes via plasmids, transposons, or mutations of native genes. New resistance genes including those that inactivate even advanced TCs are still being identified, highlighting antimicrobial resistance (Borghi et al., 2014; Patyra et al., 2014; Bahmani et al., 2020). The unique chemical characteristics of tetracyclines affect their capacity to penetrate biological membranes and gain entry into bacterial cells. Their 11,12-beta-diketone structure allows TCs to chelate with divalent metal ions, functioning as potent chelating agents that are vital for antimicrobial efficacy (Venkatesh et al., 2013). Tetracyclines represent one of the most extensively utilized groups in veterinary practice. In 2010, livestock received around 63,151 tons of these drugs, and usage is expected to rise by 67% by 2030, reaching 105,596 tons. This makes TC, OTC, and CTC the 2nd most widely used antibiotics worldwide a status attributed to their low cost, broad-spectrum efficacy (Borghi et al., 2014) and excellent water solubility (Santás-Miguel et al., 2020).

Tetracyclines (TCs) are sometimes illegally administered to poultry or livestock before sale, either through drinking water or by injection, and they can persist as residues in the animal's body for a prolonged time due to enterohepatic circulation. The metabolism rate of TCs in dairy cattle ranges from 25% to 75% (Abbasi et al., 2011). Improper administration (Borghi et al., 2014) or failure to observe proper withdrawal periods (Waghamare et al., 2020) can result in TCs residues entering meat, milk, or eggs posing significant health risks such as disruption of gut microflora, metabolic disturbances, and tooth discoloration in children (Tian et al., 2016).

TCs are illegally added to animal feed to reduce pathogens and subclinical infections, improve feed efficacy, promote weight gain in livestock and poultry, enhance feed conversion ratio, support disease prevention, and increase overall productivity; however, careful management is essential to balance these benefits against public health risk, as TCs residues in feed can accelerate antimicrobial resistance and create serious health threats (Ibrahim et al., 2018). To reduce these

risks and prevent the spread of resistant microbes, it is essential to regulate the unnecessary application of TCs (Venkatesh et al., 2013).

Cattle may ingest antibiotics through contaminated water or feed and backyard chickens can be indirectly exposed by consuming antibiotic-containing cattle manure which may result in residue in their eggs. The method of administration can lead to drug accumulation in adipose tissue, slowing metabolism and elimination, and causing residues to persist in animal tissues even after slaughter (Arsène et al., 2022). Extensive research has documented the presence of TC residues in beef and eggs from conventional markets (Sarkar et al., 2023). Consumption of contaminated eggs can cause allergic reactions, promote antibiotic-resistant bacteria, and trigger phototoxic dermatitis. The widespread use of TCs in poultry farming (Waghamare et al., 2020) often leads to residue accumulation in eggs, raising significant health concerns and accelerating the problem of antibiotic resistance. However, the detection of CTC and TC residues above this limit, especially from therapeutic use (Borghi et al., 2014) in layer birds, is a significant concern at layer farms in Haryana, India. This situation emphasizes the pressing need for enhanced farmer awareness and tighter regulatory measures, as ensuring food safety has become a major public health challenge worldwide. (Bishnoi et al., 2025). Tetracyclines are primarily used in food-producing animals to treat respiratory infections in cattle caused by *Pasteurella multocida*, *Mannheimia haemolytica*, *Mycoplasma spp.*, and *Pasteurella multocida*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae* in poultry as well as indicated for managing secondary bacterial infections such as chlamydiosis, Lawsonia proliferative enteropathy, rickettsial diseases, and salmonellosis (Koutsoumanis et al., 2021).

Exposure to antibiotic residues in food can cause a range of toxic effects in humans, such as allergies, immune disorders, reproductive issues, kidney and liver toxicity, carcinogenic and mutagenic risks, anaphylactic shock and mastitis, arthritis (Arsène et al., 2022), respiratory diseases, and gastrointestinal infections, adversely affect human fertility, produce reproductive disorders, and teratogenic effects (Arsène et al., 2022). Common side effects of tetracyclines include photosensitivity, urticaria, headache, abdominal pain, fever, nausea, vomiting, diarrhea, hypertension, mild leukopenia, anemia, and thrombocytopenia (Borghi et al., 2014). OTC is commonly administered in cattle to treat conditions such as actinobacillosis, bacterial enteritis,

pneumonia, respiratory disease, diphtheria, keratoconjunctivitis (pink eye), leptospirosis (urin infection), metritis (inflammation), anaplasmosis (flu or fever), foot-rot, black leg, navel and joint ill, heartwater (fluid accumulation), dermatophilosis, wound infections (tissue injury), mycoplasmas (ear infection), shipping fever, and infections following surgery or parturition, while in poultry it is used to manage diseases including fowl cholera, fowl typhoid, and complicated chronic respiratory disease (CRD). The use of tetracyclines (TCs) in poultry is higher during winter than summer in Iran, likely because respiratory problems are more prevalent in colder seasons, leading to increased reliance on TCs (Shahbazi et al., 2015).

Tetracyclines can be hazardous to non-target environmental organisms and may disrupt soil bacterial communities with OTC and CTC toxicity varying across soils with different characteristics, raising environmental and public health (Santás-Miguel et al., 2020). TCs discharge can pollute and degrade soil and water resources and harm aquatic ecosystems (Borghi et al., 2014; Conde-Cid et al., 2019). The rising food demand in developing countries is driving growth in livestock production, alongside increased use of organic and inorganic fertilizers, agricultural wastes and routine veterinary antibiotics for treatment, prevention, and growth promotion. Among these, TCs are most frequently used antibiotics, and about 90% of the administered dose is excreted unchanged (Borghi et al., 2014) in animal urine, gastrointestinal tract (Borghi et al., 2014) as well as feces and bile (Koutsoumanis et al., 2021; Arsène et al., 2022) due to their low absorption rates. But the biliary excretion has little effect on intestinal concentrations (Koutsoumanis et al., 2021). Antibiotics have emerged as a significant source of soil contamination. The persistence of antibiotics in the environment is influenced by their chemical structure, molecular size, solubility, and hydrophobicity, as well as soil properties such as pH, organic matter, clay, and amorphous mineral content (Conde-Cid et al., 2019).

1.10 Chloramphenicol (CAP)

Chloramphenicol (CAP) is a broad-spectrum antibiotic structurally distinct from most other classes, characterized by a nitrobenzene core (*p*-nitrophenyl) linked to a dichloroacetamide group and a propanediol moiety. Its unique structure consists of 2, 2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl] acetamide.

CAP is a broad-spectrum antimicrobial agent, synthetic (Sun et al., 2023; El-Alfy et al., 2024) antibiotic belonging to the amphenicol class. Structurally, CAP is characterized by a para-nitrobenzene ring that is attached to a chiral propanediol (glycerol) backbone and a dichloroacetamide side chain. The molecule's core features include a nitro-substituted aromatic ring, two vicinal hydroxyl groups on the propanediol moiety, and an amide linkage with two chlorine atoms at the α -carbon of the acetamide group. This specific arrangement of functional groups is crucial for both the drug's pharmacological activity and its physical-chemical properties. The dichloroacetyl group and the para-nitro group are especially critical to chloramphenicol's antibacterial function. These groups facilitate the molecule's ability to bind with high affinity to the peptidyl transferase center of the bacterial 50S ribosomal subunit (Jung et al., 2022) inhibits protein synthesis in bacteria by binding to the bacterial ribosomal subunit, specifically blocking peptide bond formation. This interference with protein production halts bacterial growth and ultimately leads to bacterial death (El-Alfy et al., 2024). Recent structural studies using cryo-electron microscopy have further clarified the molecular interactions between CAP and the ribosomal target, confirming the essential role of these substituents in mediating antibacterial activity.

The synthetic origin and unique chemical architecture of CAP also contribute to its stability and widespread detection as a residue in animal-derived food products. Research highlights the importance of understanding CAP's molecular structure not only to appreciate its mechanism of action but also to assess its persistence and potential risks when present as a contaminant in meat, milk, eggs, and poultry feed.

1.10.1 Chloramphenicol (CAP): Literature Survey

Chloramphenicol (CAP) depending on physicochemical properties and patterns of therapeutic and prophylaxis use, is applied in both human and veterinary sectors (Karaseva et al., 2021); however, CAP residues can be widely transmitted through aquatic environments like natural water system or in the water treatment facilities and may indirectly exposed humans via contaminated food and drinking water supplies (Malintan et al., 2006).

In response of drug-resistance, the European Union (EU) banned (Khataei et al., 2022)

nontherapeutic, growth-promoting uses of broad-spectrum antimicrobials from 2006 (European Commission, 2003), while regulatory agencies and Codex Alimentarius have defined maximum residue limits (MRLs) for veterinary drugs in animal-derived products (CODEX, 2016) (Ngoc Do et al., 2016). Developing nations are witnessing a significant increase in veterinary drug violations because of weak regulation and limitations in analytical capabilities, further aggravated by increasing food demand. Nevertheless, this scenario contributes to environmental contamination, accelerates disease transmission among animals, and makes disease management increasingly difficult. As a result, farmers heavily depend on antibiotics, often acquiring them without prescriptions from veterinary drug stores for therapeutic treatment, prophylactic, and growth enhancement purposes (Ngoc Do et al., 2016). Several studies have focused on the persistence of CAP residues in animal-derived foods which reveal that slight modifications in its structural groups, such as halogen substitutions or nitro reduction, can significantly affect its environmental stability, residue behavior, and detection sensitivity in meat, milk, eggs, and poultry feed (Rejtharova et al., 2009) samples.

CAP exhibits high lipid solubility, which enables rapid absorption and extensive distribution throughout the body. This property allows the drug to readily penetrate nearly all tissues, where it exerts its antibacterial effect by inhibiting protein synthesis (Sanga et al., 2024). However, this broad tissue penetration is also associated with severe adverse effects on human body such as aplastic anemia, urinary infections (Adane et al., 2023), stem cells damage (Samouris et al. 1998), bone marrow suppression (El-Alfy et al., 2024), growth retardation (Wu et al., 2025), leukemia, genital infections (Adane et al., 2023), pediatric meningitis (El-Alfy et al., 2024), agranulocytosis, allergic reactions (Karaseva et al., 2021), decreased cell membrane permeability (Ayed et al., 2024), muscle pain, abdominal infections (Adane et al., 2023), tendon rupture, gastrointestinal infections (Adane et al., 2023), hyperactivity, cancer (Ayed et al., 2024), cardiovascular failure, as well as digestive and heart diseases, liver and kidney toxicity (Ayed et al., 2024). In neonates, elevated levels of CAP can disrupt the circulatory system, resulting in the potentially fatal “gray baby syndrome” (Sanga et al., 2024). Additional side effects may include optic neuritis, visual disturbances such as scotoma, impaired vision, and an increased risk of cleft lip formation. The FDA classifies CAP as a category C (Sanga et al., 2024) drug for use during pregnancy, and advises heightened caution during pregnancy and breastfeeding due to potential risks to the fetus

or infant. Chloramphenicol (CAP) demonstrates efficacy against also rickettsia (Vuran et al., 2021), and mycoplasma (Vuran et al., 2021) by interfering with microbial protein synthesis (Vuran et al., 2021). However, because of its numerous adverse effects, its use is now largely restricted to treating severe and life-threatening infections such as cholera (Vuran et al., 2021), typhoid (Tao et al., 2025), paratyphoid (Tao et al., 2025) and abortion in pregnant women (Bişgin et al., 2025), low blood pressure, nausea and vomiting, diarrhea (Bişgin et al., 2025).

Several studies reported that, CAP is frequently overused and misused in poultry farm and livestock sector to treat bacterial infections, including superficial eye infections (Ayed et al., 2024) in animals. CAP is widely used in animals and poultry to treat infections caused by fungi, protozoa, microalgae, viruses, and bacteria (Adane et al., 2023). In addition to its therapeutic use, CAP is also administered at sub-therapeutic doses in livestock and poultry farming to promote growth by altering the gut microbiota. The antibiotic acts by inhibiting DNA synthesis, blocking the elongation of nascent RNA (Adane et al., 2023), restricting the transcription cycle, and eliminating microbial communities. However, the widespread and sometimes improper use of CAP can lead an adaptive genetic mechanism in bacteria to the development of antibiotic resistance. Moreover, when chloramphenicol (CAP) is used as a preservative (Adane et al., 2023) in animal-derived food products and dairy, both the chemical and its residues can enter the food chain and accumulate in food, further increasing health risks to consumers.

In livestock, CAP is used for medicinal treatment purposes when severe bacterial infections occur, which are characterized by submandibular swelling, fever, loss of appetite, nasal discharge, excessive salivation, and difficulty breathing. CAP has been used in Asia and Africa to control respiratory tract infections, hemorrhagic septicemia (Lestari et al., 2025), mastitis (Wu et al., 2025), enteritis, and other serious bacterial infections. This leads to monitoring and measuring CAP levels in animal-derived food products and tissue samples (Mottier et al., 2003) is extremely important for ensuring food safety and protecting public health (Wu et al., 2025). Milk contains proteins, lipids, carbohydrates, vitamins, and minerals (Ayed et al., 2024), all of which play important roles in growth, immunity, and development. As a result, there is a growing demand for safe and healthy foods, making milk especially important. However, the presence of antibiotic residues, veterinary drugs, cleaning agents, or chemicals used in farm management (Tao et al.,

2025) can negatively affect both the nutritional value and quality of milk (Ronning et al., 2006).

Bacteriostatic CAP (Li et al., 2021) remains highly effective against a variety of diseases caused by both gram positive (Rønning et al., 2006; Li et al., 2021) and gram-negative bacteria (Samouris et al., 1998). Due to its low cost (Ayed et al., 2024) and strong antibacterial activity (Wu et al., 2025), CAP has been extensively used for the treatment of animal diseases as well as a growth promoter in agriculture—a widespread global practice. While this approach enhances productivity and animal health, it also raises concerns regarding the accumulation of CAP residues in animal-derived food such as poultry meat, beef, eggs, milk (Samouris et al. 1998) reported that, following CAP administration in the daily feed of experimental hens, the antibiotic was detected in the egg albumen from the first day and in the yolk from the second day (Ronning et al., 2006; Xie et al., 2018), with some variation in timing among dosing groups. The presence of such residues poses potential health risks to consumers, highlighting the critical need for strict regulation and routine monitoring (Viñas et al., 2006) to ensure food safety.

Recent findings by El-Alfy et al. 2024 indicate that exposure to chloramphenicol (CAP) poses significant risks to both the environment and human health. CAP is generally reserved for life-threatening infections where safer antibiotic alternatives are unavailable. It inhibits mRNA translation on prokaryotic 70S ribosomes but does not affect eukaryotic 80S ribosomes. However, mitochondrial ribosomes, which resemble bacterial ribosomes, can be impacted, leading to side effects. In Asia, CAP eye drops are still used for treating ophthalmic infections, but its clinical application is limited due to severe adverse effects such as neurotoxicity and hematological complications. Hosain et al. 2022 showed that, due to its pharmacokinetic properties, CAP is effective in improving feed efficiency in food-producing animals. Poultry meal is produced from the clean, rendered parts of slaughtered poultry such as heads, necks, undeveloped eggs, gizzards, intestines, and feet and serves as an important protein source for animals and birds.

1.11 Derivatives of Nitrofuran Metabolites (NFs)

Nitrofurans (NFs), including furazolidone (FZD), furaltadone (FTD), nitrofurazone (NFZ), and nitrofurantoin (NFT), are synthetic antibacterial agents formerly used in veterinary medicine, particularly for treating microbial infections in food-producing animals. However, due to their

carcinogenic and teratogenic risks, these compounds have been banned. After administration, NFs share furan ring bearing a nitro group, but they rapidly degrade and bind to tissue macromolecules, forming stable marker residues that are difficult to detect directly. Their metabolites-3-Amino-2-oxazolidinone (AOZ, from FZD), 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ, from FTD), 1-Aminohydantoin (AHD, from NFT), and semicarbazide (SEM, from NFZ)-persist in animal tissues for extended periods and serve as marker residues. Structurally, AOZ and AMOZ contain an oxazolidinone ring with an amino group at the 3rd position, while AMOZ includes a morpholinomethyl side chain in 5th position that enhances water solubility. AHD, derived from nitrofurantoin, features a hydantoin core with distinct hydrogen bonding properties (Wang et al., 2022). These structural features influence their polarity, stability, extraction efficiency, and detectability in food matrices. Traditional analysis requires derivatization with 2-nitrobenzaldehyde (2-NBA) to overcome low retention in reversed-phase HPLC and MS ion suppression, though this process is time-consuming. Recently, 5-nitro-2-furaldehyde (5-NFA) has been proposed as a more efficient and cost-effective derivatizing agent due to their antimicrobial activity (Draiscim et al., 1997), allowing for faster sample preparation using readily available NF standards (Melekhin et al., 2021). Given the genotoxic and carcinogenic potential of these residues, detailed knowledge of their chemical structures is essential for developing sensitive analytical techniques (Wang et al., 2022) and ensuring effective regulatory monitoring of animal-derived food products.

1.11.1 Derivatives of Nitrofuran Metabolites (NFs): Literature Survey

In modern farming, poultry producers in intensive farming systems commonly use antibiotics in feed to prevent infections, enhance growth production, and for prophylactic treatment in order to reduce disease outbreaks. Antibiotics enhance weight and nutrient absorption by reducing bacterial populations, improving animal growth, and lowering disease mortality. While once used against diseases such as fowl cholera and coccidiosis, polar organic NFs are now banned as feed additives because their metabolites pose (Shoeb et al., 2009) serious health hazards. Regular exposure to low doses of these drugs creates selective pressure for resistant microorganisms and allows resistant bacteria to survive and proliferate, leading to an increased prevalence of antimicrobial resistance (AMR) in bacterial populations. When antibiotics are used inappropriately

for treating infections fail to completely eliminate them, the most resistant bacteria can survive and replicate, further complicating future treatment strategies.

The parent NFs drugs; furazolidone, nitrofurazone, and nitrofurantoin are rapidly metabolized after administration, converting into their respective tissue-bound metabolites. Due to this instability, effective monitoring of the illegal use of these drugs is challenging. The parent compounds have a very short half-life in the body, leading to rapid depletion of NFs from blood and tissues (Vass et al., 2008). However, through cleavage of the nitrofur ring, a specific tail group forms a covalent bond with tissue proteins, and the resulting metabolites (AOZ, AMOZ, AHD, and SEM) remain bound to tissue proteins for several weeks after treatment (Vass et al., 2008). Studies on the bioavailability of NFs metabolites have shown that these residues can be transferred to secondary species through the consumption of contaminated meat, animal products, and eggs, as well as through transfer to poultry (Vass et al., 2008). Even after cooking, frying, grilling, roasting, or microwaving, 67% to 100% of the residues remain in the tissue. Another study found that AOZ metabolites in eggs remain stable for at least 12 months when stored at 4 °C, with 78% of AOZ present in the yolk, which is much higher than in the albumin (Vass et al., 2008). Additionally, endogenous nitro reductase in *E. coli* is responsible for the *in vitro* reduction of NFs, leading to the formation of DNA adducts after bacterial replication and the induction of faulty DNA repair processes (Vass et al., 2008). Exposure to furazolidone has also been shown to cause irreversible DNA damage and hormonal imbalance (endocrine dysfunction) in human epithelial cells (HEp-2). In mice, benign tumors, granulosa cell tumors, and osteoporosis have also been observed (Vass et al., 2008).

NFs and their stable metabolites can persist in the environment for extended periods and pose multiple health risks to humans, including dermatological reactions, itching, nausea, gastrointestinal issues (Barbosa et al., 2007), diarrhea, dizziness, urinary tract infections, colitis and infectious diarrhea, bladder infections, and fungal infections such as candidiasis (Khataei et al., 2022). They can also increase the risk of diseases caused by *Salmonella spp.*, coliforms, *Mycoplasma spp.*, and protozoa such as *Coccidia spp.* (Barbosa et al., 2007; Fernando et al., 2015; Jarosł et al., 2019). Additionally, pulmonary fibrosis may develop as a result of respiratory toxicity (Jarosł et al., 2019). Nitrofurazone is used to treat burns and skin infections, furazolidone is used

for cholera, bacterial diarrhea, and giardiasis, and nitrofurantoin is prescribed for urinary tract infections (Vass et al., 2008). Before the ban on NFs, the concentration of NFs in blood and tissues was measured for residue monitoring. To control illegal NFs use, specific metabolic structures of the drugs have been established as marker residues for determining residue levels in tissues. Despite legal restrictions, NFs are still used in both veterinary and human medicine due to their highly effective antibacterial activity (Vass et al., 2008), low cost, and proven efficacy in promoting animal growth and preventing infectious diseases (Vass et al., 2008; Melekhin et al., 2021; Kim et al., 2015).

In the past, NFs were used as feed additives in animal husbandry, particularly in poultry and cattle, to treat and prevent bacterial and protozoan infections caused by *Escherichia coli* and *Salmonella* spp., as well as gastrointestinal enteritis, fowl cholera, coccidiosis, and blackhead disease (Vass et al., 2008). They were also used for the prevention and control of mastitis in dairy cattle (Raza et al., 2017). Administration of NFs at sub-therapeutic levels as prophylactic agents in feed has been shown to promote rapid growth in animals (Kim et al., 2015; Vass et al., 2008), resulting in a positive effect on weight gain through preventive use (John Points et al., 2015). Moreover, mutagenic activity has been observed in yeast, fungi, bacteria, and sub-mammalian systems (Cooper et al., 2007), and NFs have also been used for the management of gastrointestinal infections and to enhance animal growth (Cooper et al., 2007).

Artificially synthesized broad-spectrum NFs compounds (Fernando et al., 2015; Yuana et al., 2020) have been widely used to treat infections caused by gram-positive and gram-negative bacteria (John Points et al., 2015), as well as fungi and protozoa (Park et al., 2017; Yuana et al., 2020). In addition, NFs have been used as feed additives in animal husbandry, particularly in poultry and livestock, to promote rapid growth and control diseases especially those caused by *Escherichia coli* and *Salmonella* (Finzi et al., 2005; Park et al., 2017; Melekhin et al., 2021) often at sub-therapeutic levels (Zhang et al., 2016). NFs are also used in human medicine to treat infectious diseases such as bacterial diarrhea and skin infections (Yuana et al., 2020). However, NFs and their tissue-bound metabolites have been suggested to possess carcinogenic and mutagenic properties (Kaufmann et al., 2014; Park et al., 2017).

The global issue of NFs contamination in food production was first recognized in 2001 (Cooper et al., 2007). By 2007, inspections carried out by the European Union revealed significant NFs residue contamination in food products, particularly in shrimp and canned meats sourced from countries such as India (37%), China (37%), Bangladesh (10%), and Thailand (5%) (European Commission, 2008). To manage these risks in Bangladesh, residue monitoring laboratories process large numbers of shrimp samples within short periods, utilizing rapid screening techniques in both governmental and industrial sectors. In response to the health and safety risks including toxicity, metabolic concerns, regulatory restrictions, and the carcinogenic potential of NFs the European Union prohibited their use in food animals in 1995. EU regulations mandate that all food products destined for the European market, regardless of origin, must meet strict residue standards (Commission Decision, 2003). Similar NFs free requirements are enforced in countries like Australia, the United States, the Philippines, and Thailand (Vass et al., 2008). Between 2005 and 2009, Bangladesh experienced the rejection of almost 100 shrimp consignments by the EU due to NFs residues, resulting in export losses exceeding \$500 million (Shoeb et al., 2009). Investigations attributed this contamination primarily to the use of poultry feed in shrimp ponds and hatcheries, as well as in shrimp feed formulations (Shoeb et al., 2009).

Kennedy et al. (2007) reported that, in order to identify optimal markers and confirmatory methods for NFs detection, residues of the four main NFs in chicken eggs were evaluated. Their findings revealed that furazolidone (FZD) can accumulate as the intact parent compound in eggs, reaching stable levels of approximately 300 µg/kg after four days of treatment and remaining detectable up to nine days after withdrawal. According to Commission Decision 2002/657/EC, monitoring both parent and side-chain metabolites in yolk, albumen, and shell demonstrates that direct analysis of the parent compound in poultry eggs is feasible (Kennedy et al., 2007). Eggshell, widely used as a calcium source in food and industry, can also accumulate NFs. Thus, the utilization of eggshell waste may pose an additional route for consumer exposure to NFs residues.

After entering the human body, NFs are rapidly metabolized within a few hours, and their protein-bound metabolites acquire greater physicochemical stability. As a result, the concentration of the parent drug in the blood decreases significantly often dropping below the detection limit of modern analytical methods. Therefore, detecting and accurately quantifying the original drug in most

animal tissues is quite challenging. For this reason, most analytical approaches for NFs detection are based on identifying their metabolites or protein-bound residues (Park et al., 2017). Studies have shown that when [^{14}C]-labeled furazolidone is used, NFs form covalent bonds with tissue proteins. Through acid hydrolysis, these covalently bound drugs can be partially released, after which they are derivatized with 2-nitro-benzaldehyde (NBA). Acid hydrolysis does not liberate the entire parent drug; instead, only a structural fragment of the original molecule is released. The application of this hydrolysis and derivatization-based method has enabled the identification of numerous non-compliant samples containing furazolidone metabolites (Kaufmann et al., 2014).

To meet the growing demand for milk and dairy products and to maintain milk quality, the use of various chemicals such as antibiotics, pesticides, polycyclic aromatic hydrocarbons, urea, and hormones has become increasingly prevalent, although these substances can pose serious health risks to both animals and consumers (Raza et al., 2017). Animal milk is an excellent source of nutrition for children, adolescents, and adults, as it contains high levels of micro- and macronutrients, calcium, magnesium, selenium, riboflavin, zinc, vitamin B12, and pantothenic acid. With around 400 types of fatty acids present in milk fat, it is considered one of the most complex natural fats. However, animal feed can be contaminated by various sources such as farm-used sprays, mastitis, systemic diseases (e.g., bovine tuberculosis), carriers (such as flies), human handlers, feces, regular injections of stimulants to boost milk production, contaminated grass (or hay), or polluted water. These contaminants can transfer to the milk (Raza et al., 2017) and exert biological effects on milk consumers.

In some cases, unscrupulous farmers intentionally adulterate milk with substances like unclean water, salt, blotting paper, baking soda, washing soda, melamine, caustic soda, urea, hydrogenperoxide, formalin, and more. Additionally, harmful substances can enter milk and dairy products through industrial processes, animal feed, livestock management, and the environment. Various chemical additives such as stabilizers, emulsifiers, colorants, thickeners, preservatives, and flavoring agents are used to enhance the taste, texture, and shelf life of animal feed and products, and both short-term and long-term effects may result from processing ingredients. Sometimes, due to lack of refrigeration, antimicrobials are added as preservatives to dairy products. Similarly, growth promoters such as rBST (recombinant bovine somatotropin) are used

to increase milk production in dairy cows (Raza et al., 2017). NFs antibiotics are prohibited for use in food production animals (Cooper et al., 2007) because their drug residues persist in muscle and liver tissues.

NFs are typically administered to animals via enteral or parenteral routes, resulting in the inevitable accumulation of their residues in various tissues and milk (Zhang et al., 2016). In dairy cows, NFs can inhibit the normal growth of lactic acid-producing bacteria, which are essential for milk production (Zhang et al., 2016). These toxic metabolites can remain in animal tissues for weeks or even months. According to data from the European Rapid Alert System for Food and Feed (RASFF), the presence of NFs residues in meat, aquatic animals, and other food products especially those originating from non-European countries has been well documented in recent years (Vass et al., 2008).

1.12 Maximum Residue Limits (MRL) and Minimum Required Performance Level (MRPL)

Maximum Residue Limit (MRL)

Maximum Residue Limit (MRL) is the maximum allowable concentration of a residue legally tolerated in or on food or feed obtained from an animal that has received a veterinary medicine correctly according to Bangladesh Food Safety Authority (BFSA, 2017), CODEX ALIMENTARIUS (2-2018) and Commission Regulation (European Union, No 37/2010, Updated 2018). To ensure that antibiotic residues in poultry meat, beef, milk, and eggs remain within safe limits for human consumption, many countries have established legally binding MRLs for strengthened monitoring and regulatory control measures (Zhao et al., 2014) (Table 1.1) (Fernandes et al., 2015).

Both the European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have defined MRLs for antibiotic residues. European Union initially introduced MRLs through Council Regulation 2377/90/EC and later updated and standardized under Regulation 37/2010/EC, which sets standardized tolerance levels (Zhang et al., 2016). Food Safety and Standards Authority of India (FSSAI) (Waghmare et al., 2020; Bishnoi et al., 2025) in coordination with Export Inspection Council of India enforce monitoring programs and align unified MRLs with European Union (EU) standards (Govind et al., 2018). Similarly, Turkish Food Codex (TFC) specifies limits for antibiotic residues (Er et al., 2013; Sultan, 2014). In addition,

Codex Alimentarius Commission ([Bahmani et al., 2020](#); [Chandrakar et al., 2023](#)) and United States Food and Drug Administration (FDA) have established MRLs values for antibiotic residues across diverse matrices ([Abbasi et al., 2011](#); [Uekane et al., 2011](#)).

Minimum Required Performance Limit (MRPL)

Minimum Required Performance Limit (MRPL) means the minimum concentration of an analyte in a sample that an analytical method must, at a minimum, be able to detect and confirm. It is intended to harmonize analytical method performance for substances for which no legal permitted limit has been established often for unauthorized or prohibited antibiotics. According to European Union Commission (EU 37/2010) and European Union Commission (SANCO/2004/2726-rev 2008), MRPL has been established for multiple prohibited antibiotics (Table 1.1).

Table 1.1: Maximum Residue Limits (MRLs) and Minimum Required Performance Limit (MRPL) of Targeted Antibiotics

Authority	Antibiotics	MRL (μgkg^{-1})	Target matrices	References
Codex Alimentarius Commission	Sulfa drugs	100	muscle	CXM 2-2018, Regulation
	Fluoroquinolones	100	muscle	CXM 2-2018, Regulation
	Tetracyclines	200	muscle	CXM 2-2018, Regulation
		100	milk	Doyuk et al., 2023
		400	eggs	CXM 2-2024, Regulation
	Tetracyclines	100	cow milk edible meat	Doyuk et al., 2023
Canadian Food Inspection Agency (CFIA)	Tetracyclines	200	muscle	Bahmani et al., 2020 Waghamare et al., 2020 Chandrakar et al., 2023 Health Canada, Government of Canada, Updated 2025
		400	eggs	
		100	milk of cattle	
	Ciprofloxacin	200	muscle of cattle	
	Oxytetracycline	200	muscle	
	Sulfa drugs	100	muscle	
European Union	Sulfa drugs	100	muscle tissue milk	Malintan et al., 2006 Zhao et al., 2014
	Fluoroquinolones	100	muscle tissue	Zhang et al., 2016 Ramatla et al., 2017 Moghadam et al., 2018

				Doyuk et al., 2023
	Tetracyclines	100	food products	Abbasi et al., 2011 Uekane et al., 2011 Gajda et al., 2015 Tavakoli et al., 2015 Bahmani et al., 2020
Bangladesh Food Safety Regulations, 2017	Sulfa drugs	100	muscle	BFSA, 2017 Regulation
	Tetracyclines	200	muscle	BFSA, 2017 Regulation
		400	eggs	BFSA, 2017 Regulation
	Fluoroquinolones	100	muscle	BFSA, 2017 Regulation
		-	milk	BFSA, 2017 Regulation
Food Safety and Standards Authority of India (FSSAI)	Sulfa drugs	100	food products	Govind et al., 2018
	Tetracyclines	400	egg	Bishnoi et al., 2025
Turkish Food Codex Regulation (TFCR), 2017	Fluoroquinolones	100	muscle milk	Er et al., 2013 Sultan, 2014 Doyuk et al., 2023
		100	muscle tissue	Doyuk et al., 2023
		200	eggs	Doyuk et al., 2023
United States Food and Drug Administration (FDA)	Tetracyclines	300	muscle tissue	Abbasi et al., 2011 Uekane et al., 2011
Commission regulation (EU) 2019/1871	Chloramphenicol	Banned	food producing animals edible tissues animal feed	Rizzo et al., 2020 Salma Akter Mou et al., 2021 Jung et al., 2022 Khataei et al., 2022 Li et al., 2024 Bişgin et al., 2025 Li et al., 2025 Semeraro et al., 2024 Wu et al., 2025
USA Food and drug administration (FDA)	Nitrofurantoin metabolites derivative	Banned	food producing animals	Cooper et al., 2007 Bock et al., 2007 Vass et al., 2008 John Points et al., 2015 Yang et al., 2024

Authority	Antibiotics	MRPL (μgkg^{-1})	Target matrices	References
European Union	Chloramphenicol	0.3	meat eggs milk urine fish animal origin	Viñas et al., 2006 Wang et al., 2008 Rejtharová et al., 2009 Karaseva et al., 2021 Li et al., 2025 Rønning et al., 2006 Scortichini et al., 2005
Russian Food Regulations	Chloramphenicol	10	meat beef milk eggs	Rejtharová et al., 2009 Zhaoa et al, 2021
European Union	Nitrofurans and their metabolites	1	animal origin	Vass et al., 2008 Shoeb et al., 2009 Kim et al., 2015 Alkan et al., 2016 Kwon et al., 2017 Melekhin et al., 2021

1.13 Research in Bangladesh

Antibiotic resistance in Avian colibacillosis is a common problem around the year in Bangladesh poultry industry caused by antibiotic-resistant *E. coli*, leading to significant losses in poultry industry. Antibiotics are the major weapon to reduce the incidence. Although their extensive use as feed additives for growth promotion and disease prevention agents has triggered alarming levels of resistance. This poses negative implications for animal and human health, as resistant bacteria can transfer from animals to humans. 89% of rural households raise poultry as a primary protein source in developing nations according to national statistics from Bangladesh ([Hasan et al., 2011](#)). Farmers want to prevent unexpected death using unauthorized prescription combined with SAs, FQs, TCs, CAP and NFs in Bangladesh ([Hasan et al., 2011](#)). According to our questionnaire, the vast majority of Bangladeshi farmers habitually administer broad-spectrum tetracycline and ciprofloxacin to enhance growth and prevent diseases in poultry yet there are no government records that detail how much antibiotics are used each year. In Bangladesh there are over 95 authorized companies overseeing animal feed production however, the presence of

unauthorized feed manufacturers remains a growing concern across the country (Hasan et al., 2011). In order to assess antibiotic residue levels in milk and eggs from local farms in Raozan Upazila and the Chittagong Metropolitan area, approximately 200 egg samples underwent microbial inhibition (MIT) and TLC testing. The results revealed tetracycline residues in 7% and ciprofloxacin residues in 9% of the eggs (Chowdhury et al., 2015). Rahman et al. 2021 analyzed 120 poultry meat and organ samples from different farms of Dhaka and found that 55% of muscle samples exceeded BFSA MRL limit, with the highest residues at Palashi and Karwan Bazar and the lowest at Hatirpool, indicating frequent withdrawal-period noncompliance. Other evidence shows continued unregulated antibiotic use in Bangladesh, including oxytetracyclines (OTC) residues in 23% of poultry breast muscle samples (Sarker et al., 2018), raising public health concerns and limiting export potential. In Bangladesh, although CAP has been illegally used to treat intestinal infections such as *Salmonella* and *E. coli* in poultry and livestock, its use is now banned to protect public health. However, due to the illegal use of CAP and its presence in the environment, poultry meal may still contain CAP residues.

1.14. Different Instrumental Methods for Antibiotic Analysis

Several instrumental methods (Table 1.2) are employed successfully to detect antibiotic residues including sulfa drugs (SAs), fluoroquinolones (FQs), tetracyclines (TCs), chloramphenicol (CAP) and derivatives of nitrofurantoin metabolites (NFs) in animal-derived foods and feeds (Malintan et al., 2006). For instance, Malintan et al. 2006 demonstrated the detection of different antibiotic groups in hospital sewage and domestic wastewater, while Zhao et al. (2014) determined 18 SAs in poultry meat and eggs using the more advanced LC-MS/MS technique. However, SAs are not well-suited to GC (Guo et al., 2010) and GC-MS analysis (Guo et al., 2010) while derivatization can address this limitation like SAs low volatility, it significantly increases sample preparation time, particularly when processing large batches. In contrast, UV/Visible spectrophotometry (Errayess et al., 2017), biosensor immunoassay (BIA) (Guo et al., 2010), HPLC methods (Zhang et al., 2012) has been effectively used to detect SAs in animal products and feeds for both research or commercial purposes effectively while UFLC-MS/MS is preferred for its superior sensitivity and capability to confirm compound identities. Due to the complexity and cost of HPLC and the operational difficulties of ELISA, immunochromatographic assays are preferred for detecting SMT and SDZ residues in eggs and chicken muscle because they are simpler and more accessible

(Guo et al., 2010). An immunochromatographic assay (ICA) was developed and validated for detecting SMZ and SDZ in chicken breast muscle and egg samples (Guo et al., 2010). Different types of veterinary drugs have been administered to support increased livestock production and consumption, to determine the drug residues, various analytical methods were developed using advanced equipment. These instruments have improved in functionality, enabling the detection of a greater number of targeted compounds with higher accuracy. However, these methods are limited in their ability to simultaneously analyze different food matrices, as they are often developed for specific food products. FQs, being highly fluorescent, are commonly analyzed using reversed-phase liquid chromatography with a fluorometric detector (Esponda et al., 2009) but LC-MS/MS is more sensible for it. Other widely employed analytical techniques for the detection of tetracyclines, chloramphenicol, and derivatives of nitrofurantol metabolites in food-producing animals include HPLC, HPLC with spectroscopic fluorometric detection (HPLC-RF) (Ramatla et al., 2017), thermal analysis (TA) (El-Gamel et al., 2011), electrochemistry (Demeiry et al., 2020), TLC (Sultan, 2014), LC-MS, and LC-MS/MS (Esponda et al., 2009). However, Microbiological assays are limited for TCs determination by lower specificity and lack structural information, resulting in only semi-quantitative data and a higher risk of false positives (Abbasi et al., 2011). A nanoflow liquid chromatography high resolution mass spectrometry (LC-HRMS) method has been used for drug residue determination, but this approach often results in long retention times, averaging around 35 minutes. As a result, the development of reliable and accurate multi-residue analysis methods for food matrices remains a significant challenge (Park et al., 2022).

Advancements in analytical instrumentation have significantly improved the accuracy, sensitivity, and speed of residue detection. Compared to traditional HPLC, ultrahigh performance liquid chromatography (UHPLC) coupled with a triple quadrupole (QqQ) mass analyzer (MS/MS) offers significantly faster analysis, enhanced sensitivity, greater sample throughput, improved separation efficiency, and making it popular in food safety testing (Xu et al., 2016). Although conventional microbiological, enzymatic, and immunological methods are still used for preliminary screening, microbiological assays have largely been replaced due to their lack of specificity, semi-quantitative results, and potential for false positives (Chowdhury et al., 2015). Currently, ultra-fast liquid chromatography (UFLC) coupled with tandem mass spectrometric detectors is favored for its high sensitivity and ability to provide both identification and confirmation of antibiotic

residues. LC-MS/MS is a preferred technique because it provides good structural information from minimal sample quantities, utilizing a combination of competitive and consecutive fragmentation reactions. When molecules are ionized at 70 eV, the fragmentation spectra become highly intricate, complicating the interpretation of all the possible and overlapping breakdown pathways (El-Gamel et al., 2011).

Table 1.2: Different instrumental methods for residual antibiotic analysis

Different Instrumental Methods	References
Capillary electrophoresis (CE)	Demeiry et al., 2020 Adane et al., 2023 Ayed et al., 2024
Enzyme-linked immunosorbent assay (ELISA)	Arslanbaş et al., 2018 Kulikovskii et al., 2019 Bahmani et al., 2020 Sarkar et al., 2023 Li et al., 2024 Wu et al., 2025
Microbiological assays or microbiological inhibitory screening test	Murielle et al., 2009 Shahbazi et al., 2015 Bahmani et al., 2020
Thin layer chromatography (TLC)	Draiscim et al., 1997 Tong et al., 2009 Chowdhury et al., 2015 Raza et al., 2017 Ibrahim et al., 2018
Ultraviolet (UV)	Uekane et al., 2011 Patyra et al., 2014 Huang et al., 2019 Arsène et al., 2022
Gas chromatography (GC)	Xie et al., 2018

	Vuran et al., 2021 Khataei et al., 2022
Gas chromatography mass spectrometry (GC-MS)	Zhaoa et al., 2021 Wu et al., 2025
High-performance liquid chromatography (HPLC)	Gantverg et al., 2003 Abbasi et al., 2011; Tavakoli et al., 2015 Errayess et al., 2017 Huang et al., 2019 Bahmani et al., 2020 Li et al., 2021 Arsène et al., 2022 Chandrakar et al., 2023 Ayed et al., 2024 Wu et al., 2025
Liquid chromatography-mass spectrometry (LC-MS)	Xie et al., 2018 Li et al., 2021 Jung et al., 2022 Wu et al., 2025
Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS)	Leitner, A. et al., 2001 Venkatesh et al., 2013 Kim et al., 2015 Fernando et al., 2015 Kim et al., 2015 Errayess et al., 2017 Arslanbas et al., 2018 Wang et al., 2022 El-Demerdash et al., 2019

1.14.1 Selected Instrumental Methods for Antibiotic Analysis

The detection of antibiotic residues in animal-derived products typically employs two analytical approaches: screening tests and confirmatory or quantitative methods. Early antibiotic residue detection methods, notably cost-effective simple microbial inhibition tests such as multi-plate and tube assays, remain popular for the simplicity. These assays detect antimicrobial residues by observing the inhibition of bacterial growth, for example, a clear inhibition zone on nutrient agar plates or in tubes indicates the presence of residues exceeding permissible concentrations. While these methods can identify a range of antimicrobial groups; their lengthy incubation times and labor-intensive culture preparation limit their utility for large-scale screening. Conversely, commercial tube tests offer rapid, on-site screening options that are suitable for locations like slaughterhouses (Ngoc Do et al., 2016).

Compared to traditional methods, continuous flow analytical techniques offer several advantages, including quicker and more precise results, more affordable instruments, reduced contamination risk, and decrease use of smaller amounts of reagents and samples thus making more environmentally sustainable analysis. A large number of studies on antibiotics detection in food are limited to screening applications, owing to limitations in sensitivity and selectivity. Additionally, Commission Decision 2002/657/EC specifies the use of MS paired with proper LC separation. Within this context, LC-MS is fit for confirmatory analysis of polar and non-volatile veterinary drugs. Unlike GC-MS, LC-MS does not require samples to undergo preliminary derivatization, making it particularly effective for analyzing such compounds and for regulatory validation according to the requirements of Commission Decision 2002/657/EC (Forti et al., 2009).

A reliable, accurate, sensitive flow injection LC-MS/MS (Zhao et al., 2014; Fernandes et al., 2015; Huertas-Pérez et al., 2016 and Errayess et al., 2017) method is a simultaneous determination of polar and/or non-volatile antibiotics that are used as therapeutic and prophylaxis (Malintan et al., 2006), to increase the frequent detection of antibiotic residues in poultry meat, beef, eggs, milk (Fernandes et al., 2015) and poultry feed samples. The growing demand for domestic livestock products has driven the use of various veterinary drugs and prompting the development of advanced analytical methods and equipment. These equipment's functions have improved the detection of a larger number of target compounds with more accurate results (Park et al., 2022).

The persistence identification of antibiotic residues in diverse food matrices and poultry feed is a global concern, particularly due to the associated risk of antimicrobial resistance arising from veterinary use.

1.14.2 Ultra-fast High Performance Liquid Chromatography (UFLC-MS/MS): Superior to Antibiotic Analysis

A high-performance triple quadrupole liquid chromatography-tandem mass spectrometer (LC-MS/MS) is manufactured for highly sensitive and reproducible quantitative analysis. It integrates ultrafast scanning, optimized ion optics, and collision-induced dissociation (CID), making it highly effective for detecting for trace level antibiotic residues in complex matrices. Its multi-analyte detection capability with high precision is crucial for food safety monitoring and compliance with maximum residue limits (MRLs). The LC-MS/MS employs a electrospray ionization (ESI) probe, combining high-temperature drying gas with a fine nebulizer spray to enhance desolvation and ionization. This configuration supports both polar and non-polar compounds, producing $[M+H]^+$ and $[M-H]^-$ ions in positive and negative modes (Kim et al., 2024), respectively. Ionized analytes are directed into the triple quadrupole system for MS/MS analysis (Li et al., 2003). These ions enter the high-efficiency collision cell (Q2), where they undergo collision-induced dissociation (CID) with argon gas under controlled collision energies (Chang et al., 2019) for fragmented ions are selected for quantification. The third quadrupole (Q3) isolates these product ions for multiple reaction monitoring (MRM) (Kenjeric et al., 2024). Ion source parameters, including desolvation temperature and gas flow rates, are tuned (Wang et al., 2023) in real time to maximize signal intensity. In LC-MS/MS, MRM mode is combined with full scan and neutral loss scanning to achieve high specificity quantitative analysis alongside broad-spectrum detection. MS features such as hyperbolic quadrupoles ensure stable ion trajectories while ultrafast detectors enable reliable, high-resolution data acquisition for high-performance UFLC peaks. Ion focusing electrodes reduce contamination and signal drift, and the mass spectrometry system enables simultaneous rapid data acquisition, polarity switching, and real-time processing, thereby delivering consistent and high-quality results. LC-MS/MS routinely achieves limits of detection (LOD) and limits of quantification (LOQ) in the low ppb to ppt range, well below Codex Alimentarius MRLs for veterinary drug residues.

1.15 Different Extraction Methods for Antibiotic Determination

Most antibiotics exhibit environmental persistence and retain biological activity, allowing them to remain in environmental compartments and enter the food chain. Accordingly, reliable surveillance of antibiotic residues in animal-derived matrices required effective sample pretreatment to overcome matrix complexity and achieve selective enrichment of trace analytes. A wide range of preparation strategies including extraction, clean-up, and concentration has therefore been extensively applied for routine screening (Table 1.3). In recent times, emphasis has shifted towards greener sample preparation approaches that reduce solvent consumption and hazardous waste while maintaining high analytical performance (Moghadam et al., 2018). Methods are used for SAs analysis for biological samples including muscle, fat, liver (Tolika et al., 2010), kidney, hair, milk, honey, eggs, blood, urine, bile, cerebrospinal and prostatic fluid (Tolika et al., 2010), sputum. The determination of SAs and FQs residues is challenging due to their low concentrations in poultry meat, beef, eggs, and milk further complicated by the complex composition of these matrices, which are rich in proteins and fats (Chen et al., 2020). Tetracyclines present additional difficulties, as they tend to chelate with divalent cations (Ca^{2+} , Mg^{2+}) abundant in milk and animal tissues, forming insoluble complexes. Their high-water solubility, sensitivity to light, and strong binding to proteins make their complete extraction particularly problematic. CAP being highly polar, is also difficult to extract and detect; moreover, it is susceptible to degradation by endogenous enzymes, posing further analytical challenges. For NFs residues are often found at extremely low levels and are typically protein-bound, requiring chemical derivatization with 2-nitrobenzaldehyde to render the metabolites detectable by LC-MS/MS. Accurate determination of contaminants in animal products requires sample preparation techniques capable of efficiently separating analytes from interfering lipids and proteins.

Typically, a combination of liquid and liquid-solid extraction methods is used. After extraction, analytes are enriched and cleaned up through solid-phase or dispersive solid-phase extraction to remove matrix interferences that may impair analytical performance. Therefore, developing simplified sample preparation methods that eliminate complex purification steps is highly desirable (Zhang et al., 2016). Due to the presence of CAP in trace amounts within complex food matrices and the interfering effects of matrix components, various analytical procedures are now necessary. For the determination of NFs, several extraction methods were applied and separation

were done by using reversed-phase chromatographic columns. NFs are associated with food proteins, so their isolation from food is possible only after acid hydrolysis. Due to low molecular weight and the absence of chromophores in composition of these substances and sensitivity of their determination is low using both UV and mass spectrometric detectors; therefore, their preliminary derivatization is necessary. Due to short half-lives, they fall into protein-bound metabolites and showing undetectable, therefore different extractions were suitable to determine NFs in food and feed samples. And finally, one more problem is associated with the high cost of the analytical standards of the metabolites and products of their derivatization (Melekhin et al., 2021).

Table 1.3: Different extraction methods for residual antibiotic analysis

Different extraction Methods	References
Accelerated solvent extraction (ASE)	Zhao et al., 2014
Dispersion solid-phase matrix (DSPM)	Xu et al., 2016
Matrix solid-phase dispersion (MSPD)	Wang et al., 2008 Zhang et al., 2012
Liquid membrane (hollow fiber supported liquid membrane)	Moghadam et al., 2018
Liquid-liquid extraction (LLE)	Leitner, A. et al., 2001 Zhao et al., 2014 Ayed et al., 2024 Govind et al., 2018
Magnetic solid-phase extraction (MSPE)	Melekhin et al., 2021 Ayed et al., 2024
Quick, easy, cheap, effective, rugged, and safe (QuEChERS)	Zhang et al., 2016
Solid-phase extraction (SPE)	Vass et al., 2008 Zhao et al., 2014 Alkan et al., 2016 Ayed et al., 2024

1.15.1 Selected Sample Extraction Methods

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) is a method that combines liquid-liquid extraction with dispersive solid-phase extraction (d-SPE) for sample clean-up. It is

particularly effective for extracting both polar and non-polar compounds from a wide range of matrices in food safety testing, thereby enhancing the accuracy of detection (Casado et al., 2022). The method was first introduced in 2003 and later validated in 2005 (Veiga-del-Bano et al., 2024). Due to its high extraction efficiency and alignment with green analytical chemistry principles such as avoiding chlorinated solvents, using minimal solvent, and generating very little waste (Veiga-del-Bano et al., 2024) this method has seen widespread application in the detection of residues in various food matrices (Perumal et al., 2024; Wang et al., 2025). QuEChERS method including the original QuEChERS, AOAC, and EN methods (Musarurwa et al., 2019) but recently also modified QuEChERS approaches have proven to be effective such as facilitating alkaline hydrolysis with salt mixtures for acidic compounds and simplifying phase separation (Tongchai et al., 2025), or including a freeze-out step for high-fat samples. In the clean-up stage, dispersive solid-phase extraction (dSPE) is applied using sorbents like primary secondary amine (PSA) and anhydrous magnesium sulfate (MgSO_4). For more complex matrices, additional sorbents such as C18 are used. As a multiresidue method, QuEChERS is particularly suitable for the qualitative analysis of dairy products when properly applied and optimized for sulfa drug (SAs), fluoroquinolones (FQs), and tetracyclines (TCs). To achieve more accurate, rapid, and reliable safety assessments of dairy products, the QuEChERS technique combined with chromatography-mass spectrometry enables the simultaneous analysis of hundreds of target analytes, making it highly effective for comprehensive quality and safety testing in dairy products. (Wang et al., 2025). According to data from the Science Citation Index Expanded (SCIE), approximately 4,789 research articles on the QuEChERS method have been published since 2003 in journals including the Journal of AOAC International. This indicates a linear trend in the growth of scientific literature on the QuEChERS method. Based on the prediction by Veiga-del-Bano et al. 2024, the number of publications is expected to increase to 1.5 times in 2022 count by 2025, and to double by 2028 (Veiga-del-Bano et al., 2024). Hence, effective application and optimization of the QuEChERS method plays a significant role in CAP analytical research involving food matrices due to its versatility, ability to minimize matrix interference, high extraction efficiency for both polar and nonpolar compounds, and low solvent usage. Liquid-liquid extraction (LLE) is more suitable for egg samples, whereas both LLE and solid-phase extraction (SPE) are preferred for feed samples. The aim of this study was to determine SAs residues in poultry meat, beef, eggs, milk and feed samples to provide a scenario of the latest status of getting the type of SAs variation

in Dhaka and Gazipur area.

1.16 Research Gap and Novelty

Bangladeshi poultry and dairy sectors are characterized by highly decentralized. Despite their growing demand in ensuring national food security, significant challenges persist in establishing antibiotic residue surveillance systems effectively. Many local laboratories lack advanced infrastructure and technical capacity required for the identification and quantification of antibiotic residues in complex food and feed matrices.

In Bangladesh, a critical research gap exists in the limited availability of robust, peer-reviewed data on antibiotic residues in animal-derived foods and animal feed due to limited funding, short project durations, insufficient budgets for analytical instruments and highly pure solvents, lower capability to conduct wide range study across the entire poultry and dairy farming chain. As a result, previous studies do not represent longitudinal surveillance and fails to provide the long-term variation of antibiotic usages over years which may fluctuate due to seasonal disease outbreaks in poultry sector. Furthermore, existing research outputs are fragmented and lack harmonized methodological standards which make difficult to compare findings at a national level study or to reliable assess trends. In addition, conventional screening methods may suffer by low sensitivity and quantitative accuracy, false positive results and less precise data, and limited trace-level detection capacity for antibiotic residues monitoring while LC-MS/MS represents more sensitive and selective data for residual antibiotic analysis. But the antibiotic residue investigation in food and feed matrices by LC-MS/MS remains limited.

Another significant gap exists in lack of multi-matrix analytical methods application across poultry meat, beef, egg, milk and poultry feed. These matrices present more analytical challenges due to physicochemical properties and matrix interferences including co-extracted lipids, proteins, and carbohydrates may trigger ion suppression or enhancement during LC-MS/MS analysis. In previous studies, LC-MS/MS protocols have generally been optimized for a single matrix or a limited number of matrices, their integrated application across diverse food and feed matrices has not yet been adequately established ([Steiner et al., 2020](#); [Moretti et al., 2016](#)). To address these gaps, this study introduces a novel and integrated analytical approach utilizing cost effective

sample preparation strategies and environmentally sustainable LC-MS/MS methods for simultaneous detection of antibiotic residues across multiple food and feed matrices adhering green chemistry principles for a large-scale monitoring. Analytical performance was ensured using validated criteria including limits of detection, limit of quantification, recovery, and precision to ensure results in complex matrices.

The novelty of this research exits in the application of a unified multi-matrix analytical workflow, incorporation of cost-effective modified sample preparation and analytical methods within short time, minimize matrix effects, enhancement of analytical sensitivity and specificity while reducing solvent consumption, minimize environmental hazards. The analytical methods are also highly sensitive allowing for reliable identification and quantification at or below established MRLs, and are readily adaptable to diverse matrices. Moreover, these standardized workflows and robust validation make them easy to transfer and implement in other laboratories. This study provides a practical framework for strengthening harmonized antibiotic residue surveillance in Bangladesh. The findings are expected to support evidence-based policymaking, promote responsible antibiotic use, and enhance the safety of animal-derived food and feed.

1.17 Socio-economic Importance

Bangladesh's livestock and poultry sectors are vital to gross domestic product (GDP), national food security, and rural livelihoods. However, meeting the growing demand for animal protein has, in many cases, led to widespread and weakly regulated use of antibiotics, rising major concerns for food safety, long-term economic sustainability, and international trade. Poultry meat, beef, eggs, and milk are the main animal-source foods across all socioeconomic groups; however, when these products contain antibiotic residues, consumer confidence declines, and domestic demand may fall due to non-compliance with international food safety standards. In addition, antibiotic residues in food accelerates drug resistance, which is linked to a greater persistence of chronic illness, higher treatment costs, longer hospital stays-burdens that fall especially on low- and middle-income communities where access to quality healthcare is limited.

On the other hand, feed safety is equally essential for maintaining animal health and productivity because antibiotic residues in feed can bioaccumulate in animal tissues and indirectly enter the

human food chain. This can weaken existing regulatory efforts to control veterinary drug use at the farm level and create a recurring cycle of contamination that is costly to monitor and manage. The resulting economic exposure extends beyond farmers to processors, the broader agro-based industry value chain, public health authorities, and private producers. In this context, evidence-based research on the prevalence and distribution of antibiotic residues across different matrices along with strong surveillance and enforcement of Maximum Residue Limits (MRLs) would increase awareness among farmers, veterinarians, and consumers about responsible antibiotic practices. Ultimately, reliable residue detection using validated LC-MS/MS methods can help address key knowledge gaps in national surveillance, expand market access for animal-source foods, increase economic opportunities and foreign exchange earnings, improve public health, and build a safe food system that supports the sustainable development of the livestock and poultry sectors.

1.18 Importance at National Level

At the national level, this research is highly significant for protecting public health and supporting Bangladesh's commitment to the Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger), SDG 3 (Good Health and Well-being), and SDG 12 (Responsible Consumption and Production). Generating reliable antibiotic-residue data is essential for enforcing food safety standards and reducing population exposure to harmful contaminants. Achieving SDG 2 is not limited to ensuring access to sufficient food; it emphasizes establishing safe, nutritious, and sustainable food systems that provide affordable, safe foods consistently. In Bangladesh, increasing the production of poultry meat, beef, milk, eggs, and poultry feed is crucial for meeting dietary protein needs; however, this growth must be accompanied by safeguards that ensure product safety and preserve consumer confidence. Strengthening residue surveillance and promoting responsible antibiotic stewardship can protect food quality while supporting sustainable livestock and poultry production. In addition, sensitive LC-MS/MS-based multi-residue methods enable accurate detection of antibiotics in complex food and feed matrices, helping to identify non-compliance early and reducing the risk of production losses, product rejection, and market disruption associated with food safety failures.

Uncontrolled antibiotic use for therapeutic, prophylactic, and growth-promoting purposes may increase the risk of antimicrobial resistance (AMR), posing a serious threat to public health.

Antibiotic-resistant bacteria can spread through contact with infected humans or animals, as well as via the food chain and the environment, increasing community exposure and complicating treatment outcomes. Consequently, national-level residue surveillance, risk assessment, and effective regulatory control measures are vital. In line with SDG 3, controlling antibiotic residues supports preventive public health action by reducing avoidable exposure, strengthening consumer protection, and promoting healthier living.

By determining antibiotic residues and generating baseline data across multiple matrices such as poultry meat, beef, eggs, milk, and poultry feed, this study is also consistent with the objectives of SDG 12. SDG 12 emphasizes strengthening scientific capacity and advancing responsible consumption and production in developing countries. The evidence generated can inform national residue monitoring programs, guide the formulation of standard operating procedures (SOPs) for veterinary drug use, and support targeted awareness and training for farmers, veterinarians, and feed manufacturers, thereby reinforcing the foundations of responsible production and safe consumption. Overall, this study will help fill critical evidence gaps in Bangladesh's food chain, strengthen policy-making and regulatory frameworks, and make a meaningful contribution to enhancing food safety and protecting public health.

1.19 Aim and Objectives of the Research

Investigating antibiotic residues in poultry meat, beef, eggs, milk, and feed is essential for maintaining food safety standards. It not only helps determine whether residue levels remain within the permissible limits setting but also promotes good veterinary practices, and sustainable farming. This research aims to bridge the gap between food producers and consumers by ensuring a responsible food chain that prevents the harmful effects on both humans and animals while supporting healthy farming systems.

The aim of this study was to optimize and validate a highly sensitive modified LC-MS/MS analytical method for the detection of antibiotic residues in 750 food and feed samples (150 each of poultry meat, beef, eggs, milk, and poultry feed samples) collected from various retail outlets, local markets, and super shops in Dhaka and Gazipur cities, Bangladesh. The study also evaluated method accuracy and compliance with standards through rigorous assessment of limits of detection (LOD) and limit of quantification (LOQ), determined intra- and inter-day recoveries to ensure

repeatability and reproducibility, achieved accurate and reliable quantification of targeted antibiotic residues, and assessed matrix effects to account for potential interferences across different sample types. The objectives of this research work are given below:

- i. Five modified LC-MS/MS methods optimization and validation for the determination of five different classes of antibiotic residues such as sulfa drugs: sulfadimethoxine (SMX), sulfadiazine (SDZ), and sulfamethazine (SMT), fluoroquinolones: ciprofloxacin (CIP), enrofloxacin (ENR) and levofloxacin (LEV), tetracyclines: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), chloramphenicol (CAP), and nitrofurantoin metabolites: 2-NP-AOZ, 2-NP-AMOZ and 2-NP-AHD across targeted food and feed samples
- ii. To evaluate accuracy, precision, and reliability of the modified LC-MS/MS method by performing validation for antibiotic residues in poultry meat, beef, eggs, milk and poultry feed samples
- iii. Identification and quantification of these antibiotic residues in poultry meat, beef, eggs, milk and poultry feed samples

Chapter 2

2.0 Materials and Methods

2.1 Chemicals, Reagents and Solvents

Analytical grade magnesium sulphate (anhydrous MgSO_4) and sodium sulphate (anhydrous Na_2SO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide (NaOH), sodium chloride (NaCl), 2-nitrobenzaldehyde ($\text{C}_7\text{H}_5\text{NO}_3$, 2-NBA), hydrochloric acid (HCl), ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$), ethylenediaminetetraacetic acid (EDTA; $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$), n-hexane (C_6H_{14}) were purchased from Scharlau (Spain), Sigma-Aldrich (Germany), and Merck (Germany), respectively. Formic acid (HCOOH), LC-MS/MS grade Methanol (CH_3OH , $\geq 99.99\%$) & acetonitrile (CH_3CN ; $\geq 99.99\%$) and Glacial acetic acid (CH_3COOH) were purchased from Shantou (China), Honeywell (Germany) and Sigma-Aldrich (USA), and India, respectively.

2.2 Certified Standards

Certified analytical grade standards of all antibiotics (99% purity) were purchased from Sigma-Aldrich (USA) including-

- Sulfa drugs (**SAs**): Sulfadimethoxine (SMX, $\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_4\text{S}$), Sulfadiazine (SDZ, $\text{C}_{10}\text{H}_{10}\text{N}_4\text{O}_2\text{S}$), Sulfamethazine (SMT, $\text{C}_{12}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$)
- Fluroquinolones (**FQs**): Ciprofloxacin (CIP), Enrofloxacin (ENR), Levofloxacin (LEV)
- Tetracyclines (**TCs**): Tetracycline (TC), Oxytetracycline (OTC), Chlortetracycline (CTC)
- Chloramphenicol (**CAP**)
- Derivatives of nitrofurantoin metabolites (**NFs**): (3-amino-2-oxazolidinone (2-NP-AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (2-NP-AMTZ) and 1-aminohydantoin (2-NP-AHD)

2.3 Anhydrous Sodium Sulphate and Magnesium Sulphate Activation

Analytical grade anhydrous magnesium sulfate (MgSO_4) and sodium sulfate (Na_2SO_4) were activated by heating in a furnace for 8 hours at 300°C and kept in a desiccator until use.

2.4 Preparation of 0.2 M HCl, 100 mM 2-NBA, 0.3 M Na₂HPO₄, 2M NaOH and 0.5 mL CH₃COOH Solution

0.2 M hydrochloric acid (HCl) solution was prepared by diluting 8.33 mL of concentrated 12 M HCl with 491.67 mL of deionized water (H₂O). The solution of 100 mM 2-nitrobenzaldehyde (2-NBA) was prepared by dissolving 1.512 g of 2-nitrobenzaldehyde (C₇H₅NO₃, molecular weight (M) = 151.12 g mol⁻¹) in 100 mL of LC-MS/MS grade methanol (CH₃OH).

For the preparation of a 0.3 M disodium hydrogen phosphate (Na₂HPO₄) solution, 21.294 g of Na₂HPO₄ was dissolved in 500 mL of deionized H₂O where Na₂HPO₄ molecular weight 141.96. And the solution of 2 M sodium hydroxide (NaOH) was prepared by dissolving 8 g of NaOH (M = 40 g mol⁻¹) in 100 mL of deionized H₂O. Finally, a 0.5 mL acetic acid (CH₃COOH) solution was prepared by measuring 500 µL of acetic acid (M = 60.05 g mol⁻¹).

2.5 Glass Apparatus and Laboratory Consumables

All glass apparatus; conical flasks (50, 100, 250 mL), funnels, graduated test tubes (10 mL), micropipettes (200, 1000 mL), plastic syringes (5mL), pipettes (10 and 20 mL), round-bottom flasks (50, 100 mL), teflon tubes (50 mL) sample vials (5, 20 mL), and volumetric flasks (5, 10, 20, 25, 50, 100 and 250 mL) were purchased from Borosil, (India), Quickfit (England) and Sigma-Aldrich (USA), respectively.

One pipettes (10 mL) and one volumetric flask (25 mL) calibrated internally related with respect to the calibrated by Bangladesh Standards and Testing Institute (BSTI). All required glass apparatus were thoroughly cleaned with detergent and water, followed by rinsing with distilled water and finally rinsed with acetone. All glassware was heated in an oven at 60 °C overnight, allowed to cool and covering with aluminum foil. Prior to use, all glass apparatus was rinsed again with the appropriate solvent relevant to the extractions.

2.6 Minor Equipment

Furnace (GSM 11/8, Hope Valley, England), oven (JSOF-250, Korea), rotary vacuum evaporators (Heidolph, Germany), vortex mixer (3000 rpm, Taiwan and Vortex machine (2-16P), Sigma Aldrich, USA), kitchen blender (Miyako Chopper, Japan) and ultrasonic bath (Power Sonic,

China) were used as a part of the experimental work. Two centrifuge machines were utilized for centrifugation (SIGMA 2-16P, Benchtop, 10000 rpm and Cowbell, 2000 rpm), LC vials (1.5 and 2 mL) from Sigma Aldrich (USA). Vacuum filtration system set was brought from Sartorius (France) (Fig. 2.1). HPLC grade ultra-pure water purification system from Millipore (Automatic Plus 1+2 GRUF, Wasserlab, Spain) was purchased to remove cations, anions, and hydrocarbons. Nitrogen gas generator (Peak Scientific, India), argon gas cylinder (Linde, India), and UPS (Win power, India) were bought with LC-MS/MS supplier Shimadzu. A four-digit analytical balance (ATY124; Shimadzu, Japan) was calibrated by Bangladesh Standards and Testing Institution (BSTI). O-ring filter paper and nylon membrane filter (0.22 μm) were purchased from Pall Life sciences (India) and Restek (Germany), respectively.



Fig. 2.1. Solvent filtration system, solvent glass bottles, sample filter (pore size 0.22 μm), LC-column and LC-vial

2.7 Major Equipment

2.7.1 Liquid Chromatography-Tandem Mass Spectrometry (8050)

A triple quadrupole Ultra-Fast Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS, 8050) coupled with electrospray ionization (ESI) integrates with LC part and triple quadrupole (QqQ) mass analyzer which consists of a degassing Unit (DGU-20A3R), Solvent Delivery Unit (Pump-A and Pump-B)-Prominence liquid chromatograph (LC-20AD), a Prominence Auto Sampler (SIL-20AHT), Column Oven (CTO-20AC), and Communication Bus

Module (CBM-20A) (Fig. 2.2). In this system, Nebulizing and drying gases were N₂. Collision and heating gas argon (Ar) and air, respectively. A Shim-pack GISS C18 column (particle size 5 μ m) was used to achieve analytical separations. MS consists of ion source, two mass analyzer (MS1 and MS2) separated by a collision cell (Q2), and detector.



Fig. 2.2 Liquid Chromatography-Tandem Mass Spectrometry (8050)

2.7.2 Liquid Chromatography-Tandem Mass Spectrometry (6420 LC/TQ)

Triple quadrupole LC/TQ system integrates UPLC with MS/MS and coupled with ESI ionization system. This analyzer consists of degasser, binary pump, multi sampler, column oven, Triple Quadrupole Mass Spectrometer, nebulizer chamber (maximum pressure 1300 bar), C18 reverse-phase column (Zorbax SB-C18; 2.1X 50 mm, 1.8 μ m, 80A), MS heater, detector and pre-loaded Mass Hunter software (Fig. 2.3).



Fig. 2.3 Liquid Chromatography-Tandem Mass Spectrometry (6420 LC/TQ)

2.8 Principle of ESI-LC-MS/MS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with electrospray (ESI) soft ionization technique involved with a multi-stage ion separation and detection process (Fig. 2.4). ESI involves generating charged droplets from a liquid analyte as it exits a capillary tip under a high applied voltage (typically $\sim 2.5\text{--}6.0$ kV) between the LC effluent and the mass spectrometer inlet. The resulting fine aerosol undergoes desolvation of the droplets (evaporation of neutral solvent) and an associated droplet size reduction. A nebulizing gas (e.g., nitrogen) aids droplet formation and allows higher liquid flow rates, while source heating and/or drying gas continuously removes solvent and reduces droplet size and rises surface charge density. These droplets increased charge repulsion leads to Coulombic fission between like charges and gas phase ion formation from small or highly charged droplets. These ions extracted through a skimmer cone and directed into the mass analyzer.

In MS/MS (triple quadrupole), the ion molecules were initially scanned and recorded by the first quadrupole (Q1, mass analyzer) ion separator across a specific precursor ion from the sample based on its mass-to-charge ratio (m/z). Then the selected ions were focused and transmitted into the collision cell (q2) where inert gas argon into of the selected ion and fragmented by collision-induced dissociation (CID) to generated specific fragmented product ions. Subsequently specific product ions were separated by the third quadrupole (Q3, mass analyzer). Finally, the selected product ions reach the detector where the signal is recorded and quantitative data were acquired using multiple reaction monitoring (MRM) mode.

Identification of targeted analytes was done by Q1 scanning of recorded precursor ions $[M+H]^+$ and or $[M-H]^-$ and Q3 scanning of three product-ions (m_1 , m_2 & m_3) of standards by multi-reaction monitoring (MRM) method and quantification was done by making a calibration curve of the target product ion peak (m_1). LabSolutions software was employed for instrument control, data acquisition, and data analysis and processing.

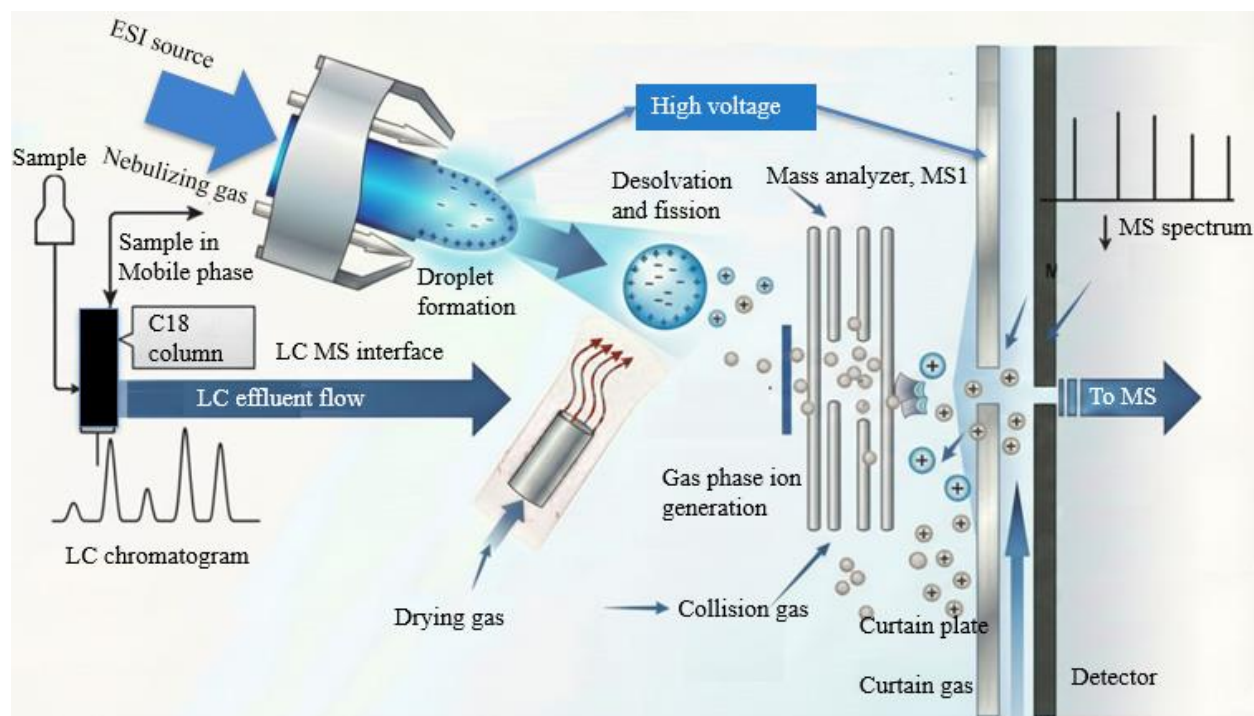


Fig. 2.4: Principle of ESI-LC-MS/MS

Targeted ions m_1 , m_2 , and m_3 were used for identification, while quantification was carried out by plotting calibration curves for m_1 at different analyte concentrations. For a sensitive compound, especially in forensic analysis, the ratio of m_1 to m_3 should remain constant (Fig. 2.5).

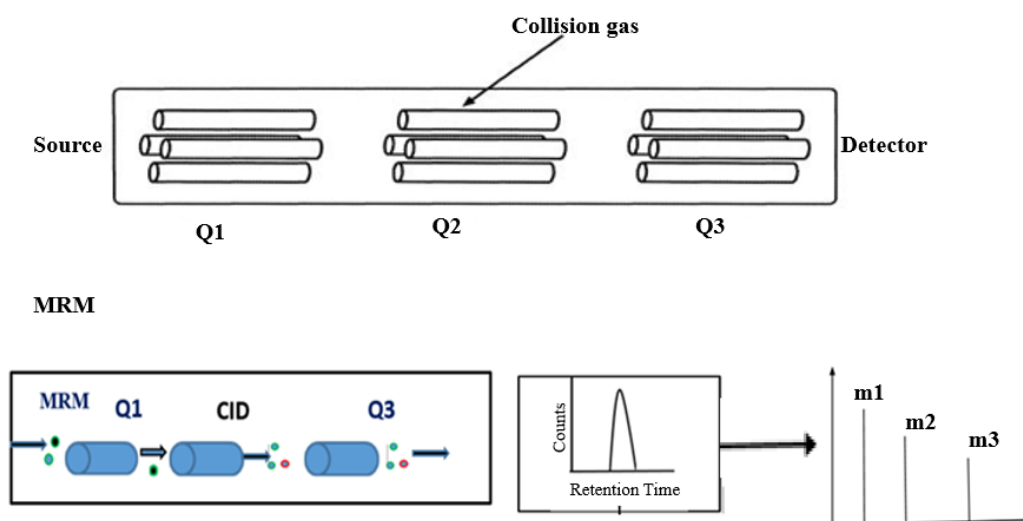


Fig. 2.5 Triple quadrupole cell and MRM fragmentation

2.9 Mobile Phase Filtration and Degassing

LC-MS/MS grade acetonitrile (CH_3CN) and HPLC grade methanol (CH_3OH), H_2O (Milli-Q water) were passed through and filtered using a Sartorius vacuum pump filtration system (mobile phase vacuum filtration apparatus set; 1000 mL capacity) and subsequently degassed for approximately 30 minutes in an ultrasonic sonicator prior to LC-MS/MS analysis.

2.10 Optimization and Stabilization of MS Vacuum Conditions

LC-MS/MS analysis was performed only after the MS system reached a stable high-vacuum condition to ensure efficient ion transmission from the source to the detector. High vacuum reduces ion scattering and ion-molecule interactions, thereby improving sensitivity and resolution. Once the vacuum was stable, the mobile phases (CH_3OH , CH_3CN , and H_2O) were passed through the system with and without the C18 column until a smooth baseline was achieved. Column oven temperature was stable at 40 °C.

2.11 Method Optimization and Validation Parameters

2.11.1 Preparation of Standard, Primary and Secondary Solutions

All type of standard stock solutions containing target analytes at a concentration of $1000\ \mu\text{g mL}^{-1}$ were prepared in LC-MS/MS grade CH_3OH or CH_3CN . Each standard (0.001 gm) was individually dissolved in 1.5 mL vial. The stock standards were tightly sealed with foil paper, kept individually in zip-locked bags, and stored in a freezer at -20 °C. Each intermediate standard solution was diluted with the required mobile phase to achieve a final concentration of $100\ \mu\text{g mL}^{-1}$ in 10 mL volumetric flasks, then placed at -20 °C. Each working standard solution was a mixture of antibiotics within their respective class, prepared at $250\ \mu\text{g mL}^{-1}$ or $200\ \mu\text{g mL}^{-1}$ following the specific method required mobile phase and were further diluted to concentration levels ranging from $250\ \mu\text{g mL}^{-1}$ to $0.13\ \mu\text{g mL}^{-1}$ and stored until residues analysis was performed. Metabolites of nitrofurantoin derivatives were prepared using amber vials and volumetric flasks to avoid photo degradation.

2.11.2 SCAN Mode

In LC-MS/MS triple quadrupole (QqQ) systems operated in full-scan mode, the combination of rapid acquisition rates and high-resolution methodologies enables the generation of high-quality MS1 spectra which helps reliably identify all targeted and non-targeted analytes using LC-MS system that are strongly co-eluted or heavily overlapped with unknown interferences in a sample.

2.11.3 Multiple Reaction Monitoring (MRM) Mode

The multiple reaction monitoring (MRM) technique using triple quadrupole mass spectrometry has become the predominant approach in LC-MS/MS for quantifying multiple target analytes, due to its exceptional sensitivity, wide dynamic range, robust quantitative precision, and stability. This targeted method enables reliable quantification of pre-selected analytes by fragmenting and detecting all ion products of the analytes eluting at a separation run time. Furthermore, the accurate quantitative outcomes can be achieved by integrating chemometric algorithms with single-quadrupole mass spectrometers. During the LC separation, analytes are ionized under optimized ionization modes, while flexible and variable quadrupole voltages (CVs) are applied to maximize ionization efficiency and enhance analytical performance.

2.11.4 Standards and Matrix-matched Calibration Curves

Standards were prepared from certified reference materials and used to generate LC-MS/MS calibration curves across the targeted concentration range for each analyte using serial dilution. To account for matrix effects (e.g., ion suppression or enhancement), matrix-matched calibration curves were generated by spiking known quantities of the analytes into blank matrix extracts. The diluted standards from low to higher concentrations were injected into the LC-MS/MS and the peak areas were recorded. Peak areas were plotted against the corresponding standards or analytes concentrations to generate the calibration curves.

2.11.5 Linearity

The linearity of the calibration standards was rigorously evaluated to ensure the reliability and quantitative accuracy of the analytical method. A series of standard solutions was prepared to comprehensively cover the concentration range of the target analytes (1-250 $\mu\text{g kg}^{-1}$), reflecting

residues level expected in real sample matrices. Each calibration point was analyzed under identical instrumental conditions, and peak areas were plotted against their corresponding concentrations to making individual calibration curves for each analyte. The resulting curves exhibited excellent linear behavior across the entire tested range, demonstrating that the instrument response increased proportionally with the analyte concentration. This proportionality is essential in quantitative residue analysis, as it ensures that variations in instrumental signal accurately represent differences in actual analyte levels. The correlation coefficients (R^2 values approaching up to 0.9999. Such strong linearity provides confidence that the analytical method is capable of producing precise and reliable quantification over a wide concentration range, making it suitable for routine monitoring, regulatory screening, and confirmatory analysis of residues in complex food matrices. Linear regression (R^2) formula of calibration curves:

$$y = mx + C$$

Where, y = peak area, x = concentration, m = slope of the calibration curve, C = intercept

2.11.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

In analytical method validation, determining the limit of detection (LOD) and limit of quantification (LOQ) involved a critical role in assessing the capability and sensitivity of an instrumental method to identify and measure target analytes at very low concentrations. In this study, the LOD was established as the minimum analyte level that produced a signal-to-noise ratio (S/N) of 3, ensuring that the signal could be distinguished from baseline noise with confidence. The LOQ was defined as the lowest concentration achieving an S/N ratio of 10, representing the threshold at which quantitative measurements could be performed with acceptable precision and accuracy (Mou et al., 2021). LC-MS/MS method developed in this study exhibited remarkably low LOD and LOQ values for all analyzed antibiotics. These findings demonstrate the high analytical sensitivity of the method, enabling reliable detection of residues even at trace levels. Such sensitivity is particularly important for complex food matrices, including poultry meat, beef, milk, eggs, and poultry feed, where matrix interferences may affect detection performance. The capability to identify extremely low concentrations underscores the suitability of the method for rigorous surveillance of antibiotic residues. Moreover, it supports its application in regulatory

monitoring programs aimed at ensuring food quality, consumer safety, regulatory compliance, and conducting risk assessments related to antibiotic residues in food products, thereby contributing to public health protection. The formula which used for LOD and LOQ analysis below:

$$\text{LOD} = \frac{3.3 \times \text{SD of intercept}}{\text{Slope}}$$

$$\text{LOQ} = \frac{10 \times \text{SD of intercept}}{\text{Slope}}$$

Where, SD of intercept = Standard error of intercept $\times \sqrt{n}$;[n=number of tests]

2.11.7 Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%)

The mean concentration (Mean $\pm$ SD) of antibiotic residues provides a quantitative characterization of analyte occurrence across distinct sample matrices, facilitating comparative evaluation of contamination levels. These mean values help illustrate the distribution pattern of antibiotics in each matrix and allow comparison of contamination levels between sample types. The standard deviation (SD) reflects the extent of variability among replicate measurements, reflecting analytical precision and potential matrix-dependent effects. Method performance was further assessed through intra-day RSD% to determine repeatability within a single analytical run, and inter-day RSD% to evaluate reproducibility across multiple days. Maintaining these metrics within acceptable limits confirms the method's robustness, reliability, and suitability for multi-matrix residue analysis, while minimizing analytical uncertainty associated with complex food matrices.

2.11.8 Recovery Percentages (%)

Recovery percentage is an essential parameter in analytical method validation, providing a quantitative measure of the method's accuracy and efficiency in extracting analytes, particularly when analyzing complex matrices. Recovery assesses the method's ability to extract and quantify the analyte of interest from a given sample without significant loss or interference. It means the reflects how much of the known added analyte can be successfully detected after sample preparation and analysis, thereby evaluating potential losses or matrix-induced interferences. A high recovery percentage, typically within 70-120% ([SANTE/11312/2021](#); [CODEX guideline](#),

2017) for most food matrices, indicates that the method can efficiently recover the analyte with minimal matrix interference or analyte loss during sample preparation and analysis. So, achieving consistent recovery within an acceptable range indicates that the method can reliably quantify the target analytes without significant loss or bias. In this study, recovery experiments were conducted for all targeted antibiotics in poultry meat, beef, milk, eggs, and poultry feed to validate the LC-MS/MS method. The consistently high recovery percentages demonstrate the method's robustness, accuracy, and suitability for quantitative analysis of antibiotic residues in complex food matrices, supporting its application in regulatory and research settings. But the recovery values were within the acceptable range, confirming the method's accuracy. It is typically calculated using the formula:

$$\text{Recovery (\%)} = \frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \times 100$$

Where, C_{spiked} is the measured concentration in the spiked sample and C_{unspiked} is the measured concentration in the unspiked sample, and C_{added} represents the known amount of analytes added.

2.11.9 Matrix Effects (%)

In LC-MS/MS-based quantitative analysis, the matrix effect (ME) is a critical phenomenon that can significantly influence the accuracy, precision, and reliability of analytical measurements, particularly in complex biological and food matrices. ME occur when co-extracted substances from complex sample matrices or other endogenous compounds interfere with the ionization efficiency of target analytes during mass spectrometric detection, leading to either ion enhancement or ion suppression. These effects can result in overestimation or underestimation of the actual analyte concentration, potentially causing inaccurate quantification. A matrix-matched calibration curve is constructed by spiking known concentrations of analytes into blank sample extracts, whereas a solvent-matched calibration curve is prepared in pure solvent. Comparison of the slopes of these curves allows the quantification of signal enhancement or suppression caused by the matrix, which is essential for ensuring method accuracy and reproducibility. A positive matrix effect value indicates ion enhancement, where the presence of matrix components increases the analyte signal relative to the solvent. Conversely, a negative value indicates ion suppression, where co-eluting matrix constituents reduce the analyte response. Ion

suppression is particularly common in complex food matrices such as poultry meat, beef, milk, eggs, and poultry feed, where endogenous compounds compete for charge during electrospray ionization, resulting in lower observed signals. Ion enhancement, although less frequent, can occur when matrix components improve analyte ionization efficiency. Evaluating and correcting matrix effects is therefore essential for robust method validation. The use of matrix-matched calibration curves effectively compensates for these effects, ensuring accurate, precise, and reliable quantification of trace-level residues in complex food matrices. Monitoring and correcting for matrix effects is a crucial step for the credibility of LC-MS/MS methods applied in food safety and residue analysis studies. To evaluate and quantify the extent of matrix effects in this study, the following formula was applied:

$$\text{Matrix effect, ME (\%)} = \frac{\text{Slope of MM} - \text{slope of SM}}{\text{Slope of SM}} \times 100$$

Where, MM= Slope of matrix matched calibration curve and SM= Slope of solvent matched calibration curve

2.12 Survey Strategy, Sampling design and Size

Survey strategy and sampling design and prioritized contamination sources traceable and comparable sampling across matrices, and a sample size sufficient to support reliable residue detection and inter-matrix comparisons. Initially, a comprehensive list of potential poultry meat, beef, egg, milk and feed sample sources was made based on literature reviews and field investigations. The preparatory phase was essential for identifying intensive poultry production zones, dairy collection routes, wholesale distribution hubs and retailers where veterinary drug use and product mixing can occur. Based on this mapping, super shops, wholesale markets, local dairy and poultry farm and local markets in Dhaka and Gazipur cities were visited. Because residue occurrence is strongly influenced by on-farm dosing practices and withdrawal-period compliance. Day-long discussions were conducted with wholesalers, poultry farmers, and dairy farmers regarding antibiotic application over multiple days, to understand the frequency of treatment, common drug classes used, and disease-driven peak usage periods. Sampling points were finalized through the combination of insights from these discussions on real field sincere where

contamination risk was expected. Information was collected to evaluate patterns of antibiotic use in small-scale farms. The key areas of data collection included: (i) farmers' knowledge about antibiotic residues, (ii) duration of antibiotic use and the withdrawal period, (iii) purpose of antibiotic use (therapeutic vs. prophylactic), (iv) types of antibiotics used, and (v) routes of administration.

The survey indicated that poultry and cattle farms commonly experience diseases such as gumboro, avian influenza (bird flu), fowl cholera, fowl typhoid, diarrhea, cholera-like enteric illness, respiratory diseases, and mycoplasmosis. In contrast, cattle farms may face a wide range of conditions, including anthrax, brucellosis, salmonellosis, tuberculosis, mastitis, pneumonia, parainfluenza, *Histophilus somni* infections, cholera-like illness, diarrhea, foot and-mouth disease, hemorrhagic septicemia, black quarter, lumpy skin disease (LSD), and edema/swelling-related conditions. These diseases can result in reproductive problems such as abortion and infertility, reduced milk production, sudden death, and bleeding from natural body openings. As a result, antibiotics are often used for treatment. However, the risk of antibiotic residues in meat and milk increases when antibiotics are administered at unauthorized or incorrect doses. In particular, failure to follow the required withdrawal period after treatment can lead to drug residues remaining in milk or meat, egg sold in the market or super shops, posing potential risks to consumers.

The survey further showed that although many farmers have a general understanding of antibiotic residues but awareness of withdrawal periods is very low. Most farms use multiple antibiotics (multidrug use). Commonly used drugs include ciprofloxacin, enrofloxacin, levofloxacin, tetracycline, oxytetracycline, chlortetracycline, and sulfa drugs. By class, the most frequently used antibiotics were fluoroquinolones, followed by tetracyclines and sulfa drugs, and sometimes chloramphenicol for the treatment purpose intentionally. Farmers reported using antibiotics for both therapeutic and prophylactic purposes, most commonly administering them through drinking water. Some farmers also self-administer antibiotics based on social media, personal experience, or old prescriptions because antibiotics can be easily obtained from shops or dealers with weak regulatory control. The discussion also revealed that many farmers do not properly follow withdrawal periods. Farmers reported that veterinarians often do not provide adequate guidance on withdrawal times, and government veterinary services may be difficult to access. Additionally,

due to lack of insurance or government support, many farmers are unwilling to stop selling products during the withdrawal period, which increases the risk of residues.

Moreover, weak biosecurity and inadequate antibiotic management increase the likelihood of repeated infections, which further drives antibiotic use. Antibiotic use often increases during outbreaks, and some farmers use antibiotics routinely without any specific disease indication. So, several research showed that derivatives of nitrofurans metabolites were determined in different matrices. Together, indiscriminate use, possible low-quality drugs, weak regulation of authorized sales, and unnecessary use of broad-spectrum antibiotics can accelerate the development of antimicrobial resistance (AMR). In summary, although the survey does not represent the entire poultry industry of Bangladesh, it clearly highlights real-world antibiotic use practices in small-scale farms and identifies key reasons for poor awareness and management of antibiotic residues. Initially, a comprehensive list of potential poultry meat, beef, egg, milk and feed sample sources was made in different super shops, wholesale markets, local dairy and poultry farms and local markets in Barisal, Dhaka and Gazipur cities were visited.

Kaptan bazar and Kawran bazar were selected for poultry meat sample collection as they serve as major retail and distribution source supplying poultry meat and eggs across Dhaka city. Kapasia and Gazipur chowrasta bazar were included because they function as main distribution hubs for both poultry meat and poultry feed samples in Gazipur region supplying surrounding local markets. In Dhaka city, most of the people collected poultry meat, beef, egg, milk samples from nearby super shops and slaughterhouses. Therefore, the selection of these super shops and markets ensures that the collected samples are representative of the supply chains in both cities.

Sampling points were identified through the combination of insights from these discussions on real field sincere. Ultimately, thirty-one (31) places: Aftab Nagar, Adabar, Ananda Bazar, Badda, Barisal, BUET Campus-Polashi, Banasree, Chankharpul, Dhanmondi, Dholaipar, Chowrasta-Gazipur, Kapasia-Gazipur, Nimtoli-Gulistan, Fulbaria-Gulistan, Hazaribag, Harinal-Gazipur, Jatrabari, Jigatola, Joydebpur-Gazipur, Kakrail, Kaptan Bazar, Kawran Bazar, Kazla, Moghbazar, Mohammadpur, Rayerbag, Shantinagar, Segunbagicha, Sonir Akhra, Thatari Bazar, Tikatuli, and Wari were randomly selected for the sample collection. The determined sample size for this research comprised after survey. A total of 5 control samples (n=5) were collected, representing

different matrices, including poultry meat and beef from Mahiskata, Barisal, milk from Aftab Nagar, egg from Kapasia (Gazipur) and feed from Gazipur. In this study, five classes of antibiotic including sulfa drugs (SAs), fluoroquinolones (FQs), tetracyclines (TCs), chloramphenicol (CAP), and derivatives of nitrofurantoin metabolite (NFs) were investigated across five matrices (poultry meat, beef, egg, milk and feed) (Fig.2.6). For each antibiotic class, 30 samples were systematically collected and analyzed from each matrix, resulting 150 samples per antibiotic group. The design led to an overall 750 samples ensuring comprehensive representation across all matrices under a balanced approach to the investigation and allowing for a thorough evaluation of antibiotic contamination across diverse food sources.

2.13 Sample Collection, Storage and Extraction Procedures

2.13.1 Collection and Storage of Control and Targeted Poultry Meat and Beef Samples

Poultry meat and beef samples were systematically collected from sixteen different sources located in different areas of Dhaka and Gazipur cities to investigate the presence of antibiotic residues levels. Control sample of one poultry meat and single beef sample were collected as control sample from Mahiskata, Barisal. All of control and collected sample were transported from the collection sites to the laboratory using shopping bags, with temperature optimization ensured during transit. Upon arrival, the whole muscle portion of the sample was processed by chopping and homogenizing them using a kitchen blender, excluding skin and bones. Each prepared sample was sealed individually in a zip-lock bag with proper labeling and stored at -20°C until further analysis.



Fig. 2.6: Targeted matrices (Beef, egg, poultry feed, milk and poultry meat)

A total of thirty poultry meat samples (n=30; PM1-PM30) were sourced from super shops (Banasree, Chankharpul, Dhanmondi, Jigatola, Kakrail, Kazla, Moghbazar, Mohammadpur, Segunbagicha, Shantinagar, Tikatuli and Wari), wholesale markets (Kaptan Bazar, Kawran Bazar, Thatari Bazar) and different local markets in Dhaka city (Jatrabari, Mohammadpur) and Gazipur city (Harinal Bazar) to clarify the presence of antibiotic residues in poultry meat in (Fig. 2.7; Table 2.1).

A total of thirty beef samples (BM1-BM30) were collected from super shops, slaughter houses, wholesale markets, and local markets across Dhaka city. The super shop locations included Banasree, Dhanmondi, Kakrail, Malibag, Moghbazar, Mohammadpur, Rampura, Shantinagar, Segunbagicha, Tikatuli, Wari and Gazipur. The slaughter houses and wholesale markets were Kaptan, Kawran, and Thatari Bazar, while the local markets included Adabor, Mohammadpur and Gazipur Chowrasta in (Fig. 2.8; Table. 2.2).

Table 2.1: Poultry meat sample collection areas

Poultry Sample ID	Collection Source	Collection point	Places
Control	Poultry meat Control sample, Mahiskata, Barisal		
PM1	Super shops Dhaka	Agora	Dhanmondi
PM2			Kakrail
PM3			Moghbazar
PM4			Mohammadpur
PM5			Segunbagicha
PM6		Meena Bazar	Dhanmondi
PM7			Dhanmondi
PM8			Mohammadpur
PM9			Shantinagar
PM10			Wari
PM11		Shwapno	Banasree
PM12			Chankharpul
PM13			Jigatola
PM14			Kazla
PM15			Tikatuli
PM16-PM18	Wholesale markets Dhaka	Murgir Aarot/Chicken market	Kaptan Bazar
PM19-PM21		City Corporation Market	Kawran Bazar
PM22-PM23		Thatari Bazar chicken market	Thatari Bazar
PM24-PM25	Local markets Dhaka	Krishi Market	Mohammadpur
PM26		Geneva Camp Kacha Bazar	
PM27		Adabor Kacha Bazar	
PM28		Kacha Bazar	Jatrabari
PM29	Local markets Gazipur	Harinal Bazar	Gazipur
PM30		Harinal Bazar	

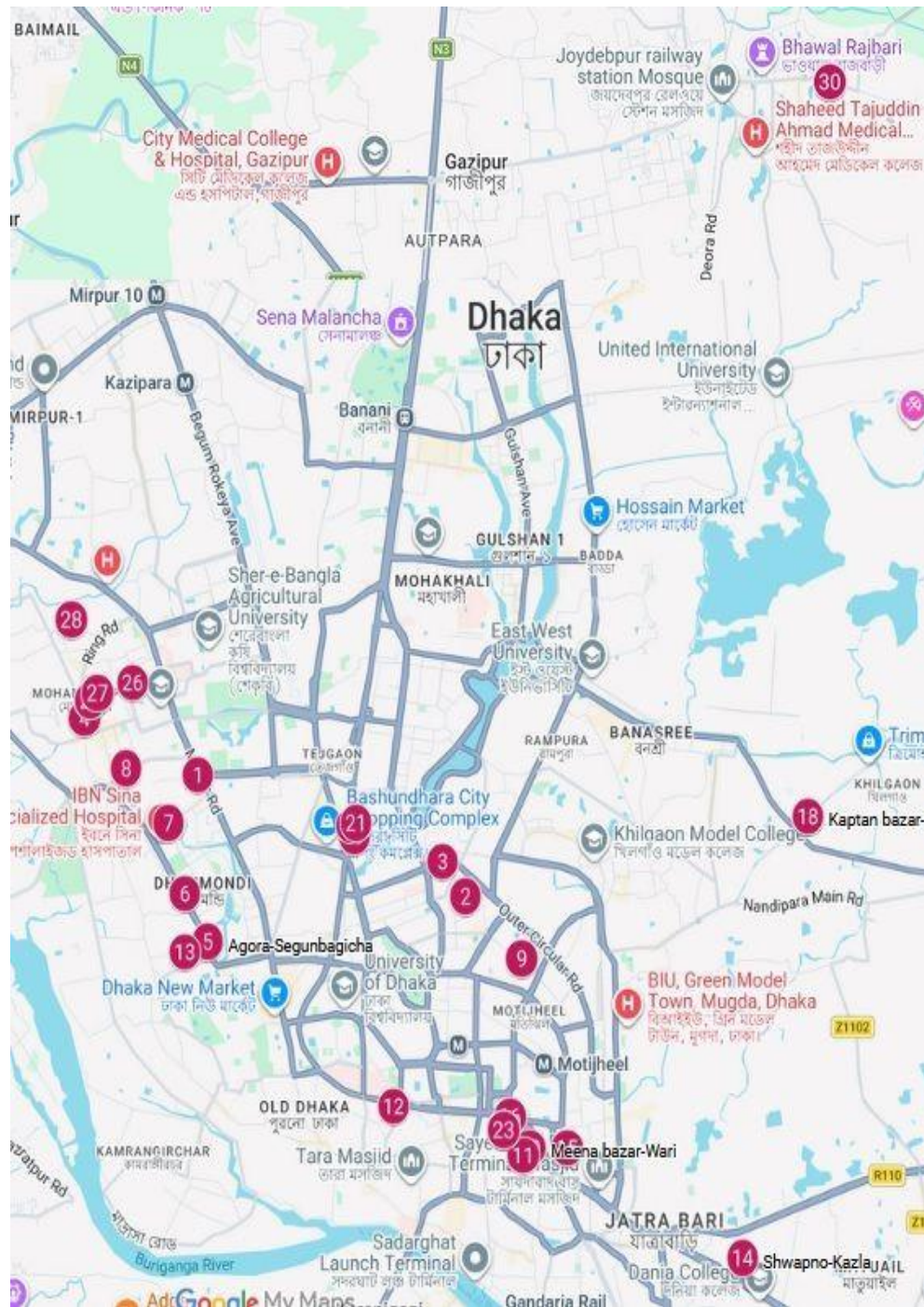


Fig. 2.7: Collection sites of poultry meat sample (Dhaka and Gazipur city)

Table 2.2: Beef sample collection areas

Beef Sample ID	Collection Source	Collection point	Places
Control	Control sample, Mahiskata, Barisal		
BM1	Super shops Dhaka	Agora	Dhanmondi
BM2			Kakrail
BM3			Moghbazar
BM4			Mohammadpur
BM5			Segunbagicha
BM6		Meena Bazar	Dhanmondi
BM7			Dhanmondi
BM8			Mohammadpur
BM9			Shantinagar
BM10			Wari
BM11		Shwapno	Banasree
BM12			Rampura
BM13			Malibag
BM14			Tikatuli
BM15			Wari
BM16-BM18	Wholesale markets Dhaka	Kaptan Bazar beef market	Kaptan Bazar
BM19		City Corporation Market	Kawran Bazar
BM20-BM22		Whole Sale Meat Market	
BM23		Kawran Bazar Road	
BM24		Thatari Bazar market	Thatari Bazar
BM25		Thatari Bazar market	
BM26	Local markets Dhaka	Krishi Market	Mohammadpur
BM27		Geneva Camp Kacha Bazar	
BM28		Notun Kacha Bazar	
BM29		Adabor Kacha Bazar	Adabor
BM30	Gazipur	Gazipur chowrasta	Gazipur

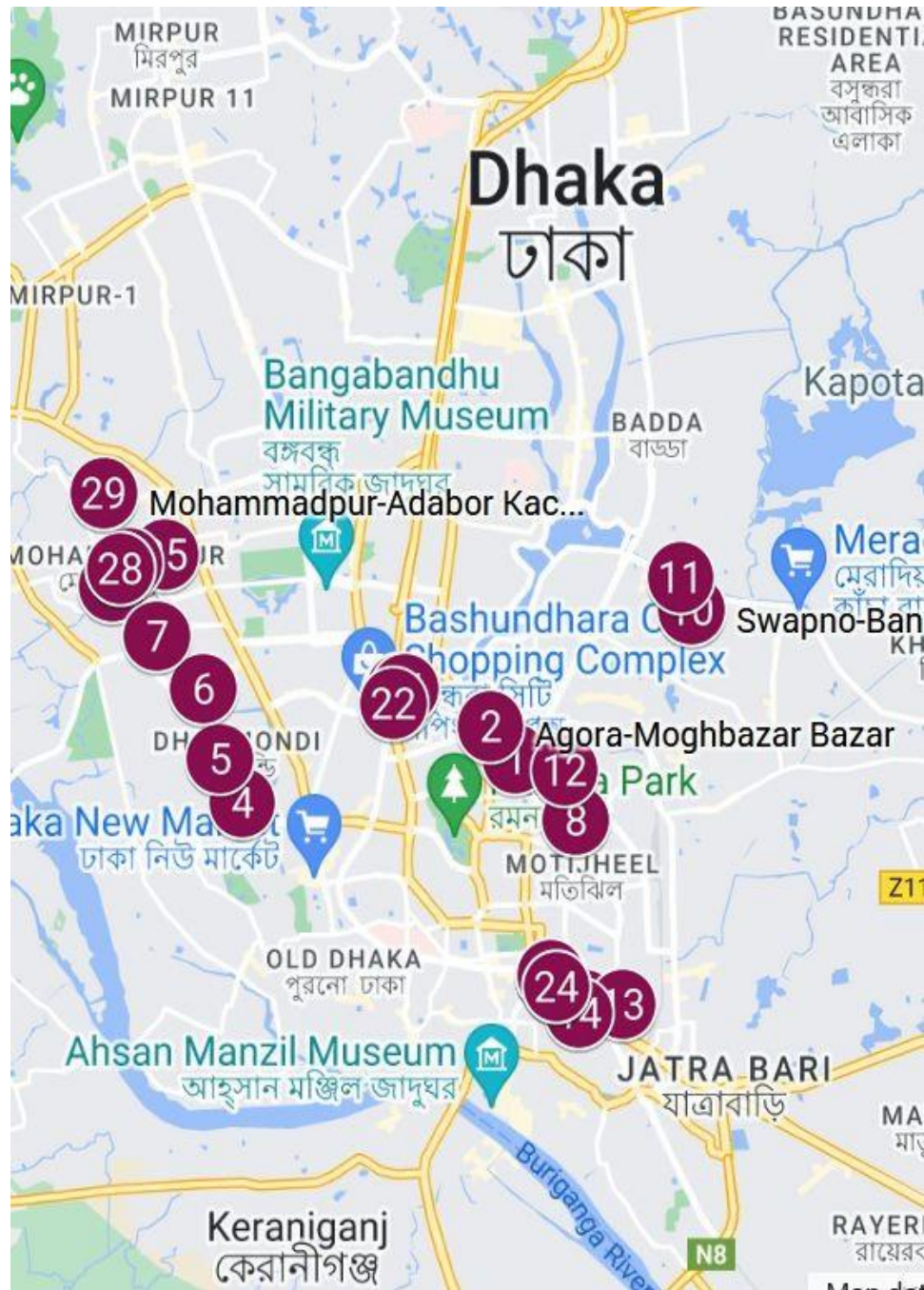
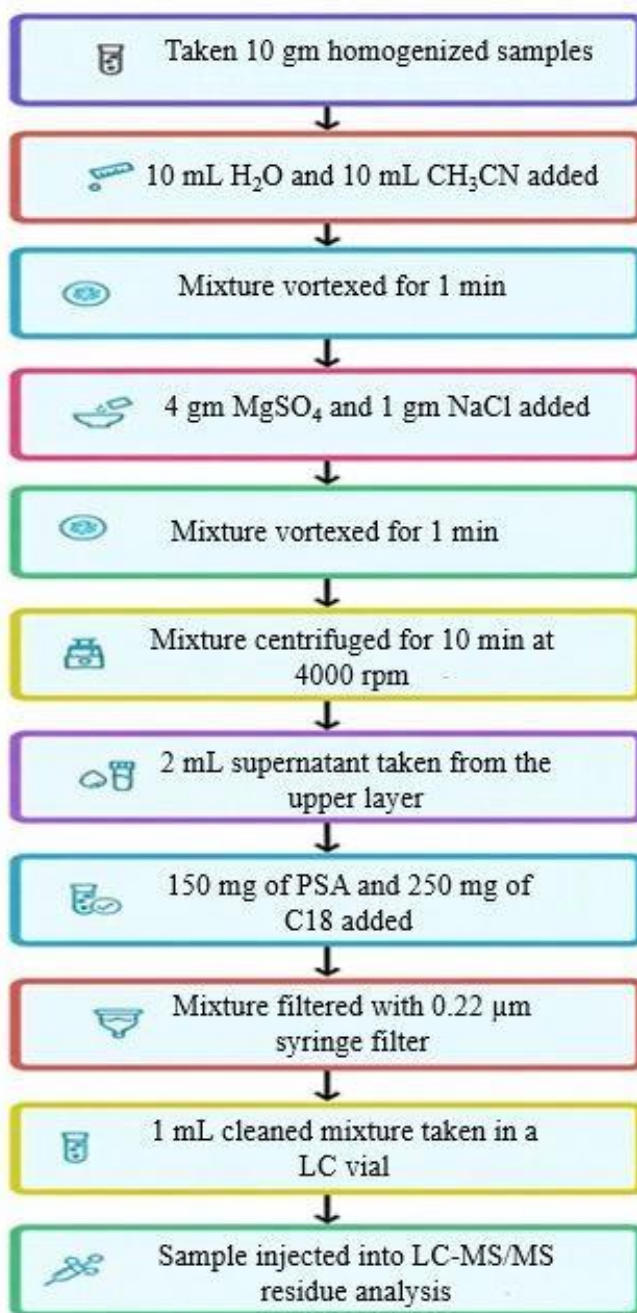


Fig. 2.8: Collection sites of beef sample (Dhaka city)

2.13.1.1 Extraction and Clean-up Method for SAs, FQs, TCs, and CAP Residues in Poultry Meat and Beef Samples

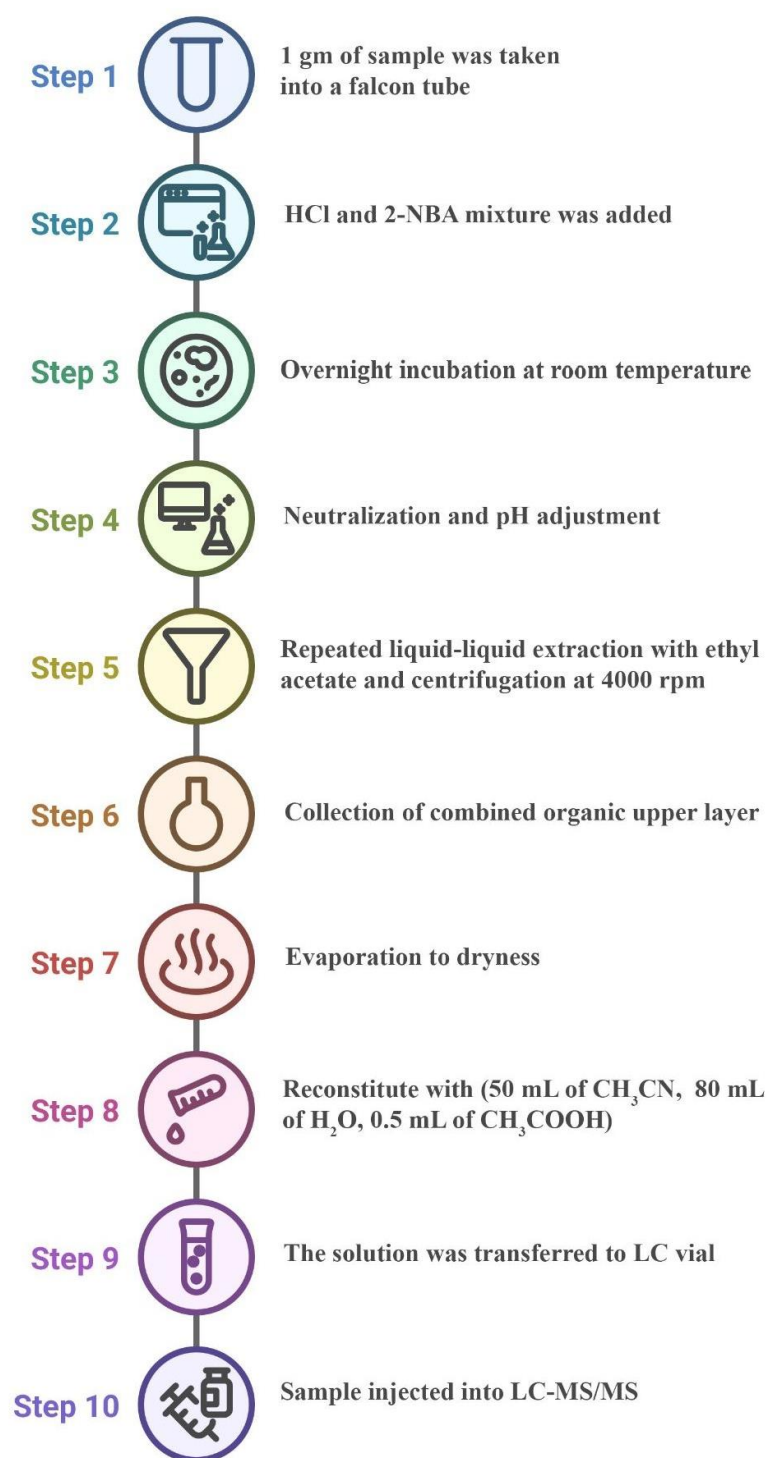


Scheme 1: Extraction and clean-up for SAs, FQs, TCs, and CAP residues analysis in poultry meat and beef samples

Following the modified QuEChERS method ([Anastassiades et al., 2003](#)) (Scheme 1), poultry meat or beef sample (10 gm) was taken into a Teflon tube of 50 mL by weighing analytical balance. Then LC-MS/MS grade 10 mL of acetonitrile (CH_3CN) and 10 mL of H_2O (20 mL; 1:1 ratio) were added in same tube. The Teflon tube was vortexed for 5 minutes to enhance better interaction between sample and solvents. Extraction for better separation, 4 gm of MgSO_4 and 1 gm of NaCl were added again with sample tube and shaken continuously by hand to ensure proper mixing. The solution was centrifuged for 10 mins at 4000 rpm. After that the upper acetonitrile layer (CH_3CN layer) called supernatant was collected from this solution transferred into a new screw cap test tube. Then, 150 mg of PSA and 250 mg of C18 were added in this CH_3CN layer and centrifuged at 2000 rpm for 1 minutes. The concentrated supernatant was filtered into the LC vial using nylon syringe filter (0.22 μm).

2.13.1.2 Extraction and Clean-up Method for NFs Residues in Poultry Meat and Beef Samples

According to modified method ([Violante et al., 2018](#)) (Scheme 2), Homogenized poultry meat or beef (1 gm) sample was transferred into a 50 mL falcon tube. Subsequently, 5 mL of 0.2 M HCl and 50 μL of o-nitrobenzaldehyde mixture (2-NBA, 100 mM in methanol; CH_3OH) were added. The sample was incubated at room temperature overnight. The solution was then neutralized using 500 μL of 0.3 M Na_3PO_4 in water, and the pH was adjusted to 7 with 2M NaOH . Liquid-liquid extraction was performed by adding 4 mL of ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$), followed by centrifugation at 4000 rpm for 10 min. The organic upper layer was collected and transferred to a round-bottom flask then repeated extraction was carried out using another 4 mL aliquot of $\text{C}_4\text{H}_8\text{O}_2$, followed by centrifugation under identical conditions. The resulting organic layer was combined with the previous collected fraction. The combined organic extract was evaporated to dryness. The residue was reconstituted in 1500 μL solvent (composed of 50 mL acetonitrile CH_3CN ; 80 mL H_2O and 0.5 mL acetic acid, CH_3COOH solution). A 1.5 mL aliquot of the reconstituted sample was transferred into an LC vial. Finally, the solution was injected into an LC-MS/MS system for residue analysis.



Scheme 2: Extraction and clean-up for NFs residues analysis in poultry meat and beef samples

2.13.2 Collection and Storage of Control and Targeted Milk Samples

Collected milk control samples and targeted samples were transported to the laboratory under temperature-controlled conditions to minimize any degradation or compositional changes prior to analysis. Immediately after collection, each sample was transferred into clean, sealed PET bottles to prevent leakage, or exposure to external contaminants during handling and transit. Throughout transportation, the samples were kept consistently cool using an insulated carrier with ice packs to slow down microbial growth that could alter milk quality and potentially interfere with residue detection. Upon arrival at the laboratory, all bottles were inspected for clearly labeled with relevant identifiers and stored in a freezer at -20 °C until further processing. Freezing is a widely used preservation step for dairy matrices because it helps reduce bacterial proliferation and slows down chemical reactions that may affect sensitive analytes. For extended storage and to strengthen sample preservation prior to analysis, the collected samples were maintained at -20 °C until further testing, which further minimized the risk of analyte loss or degradation.

To evaluate potential antibiotic contamination, milk samples were obtained from ten distinct locations across Dhaka and Gazipur including local milk markets and dairy farms, where production, handling, and distribution practices may differ (Fig. 2.9). In total, 31 milk samples were collected for this research. One sample served as the control was obtained from Aftab Nagar, Dhaka. The remaining 30 samples were assigned unique identifiers (MM1–MM30) and collected from multiple local markets and dairy farms across different areas of Dhaka city. Specifically, most of samples were gathered from Aftab Nagar, Chankharpul, Dolaipar, Hazaribag, Mohammadpur, Polashi Bazar, and the Rayerbag, Sonir Akhra in Dhaka city and others from Harinal, Gazipur city.

All collected milk samples were treated as analytical specimens intended for residue evaluation. After collection, each sample was stored under frozen conditions to preserve its matrix characteristics and to maintain the integrity of target analytes prior to laboratory testing following appropriate laboratory protocols (Table 2.3).

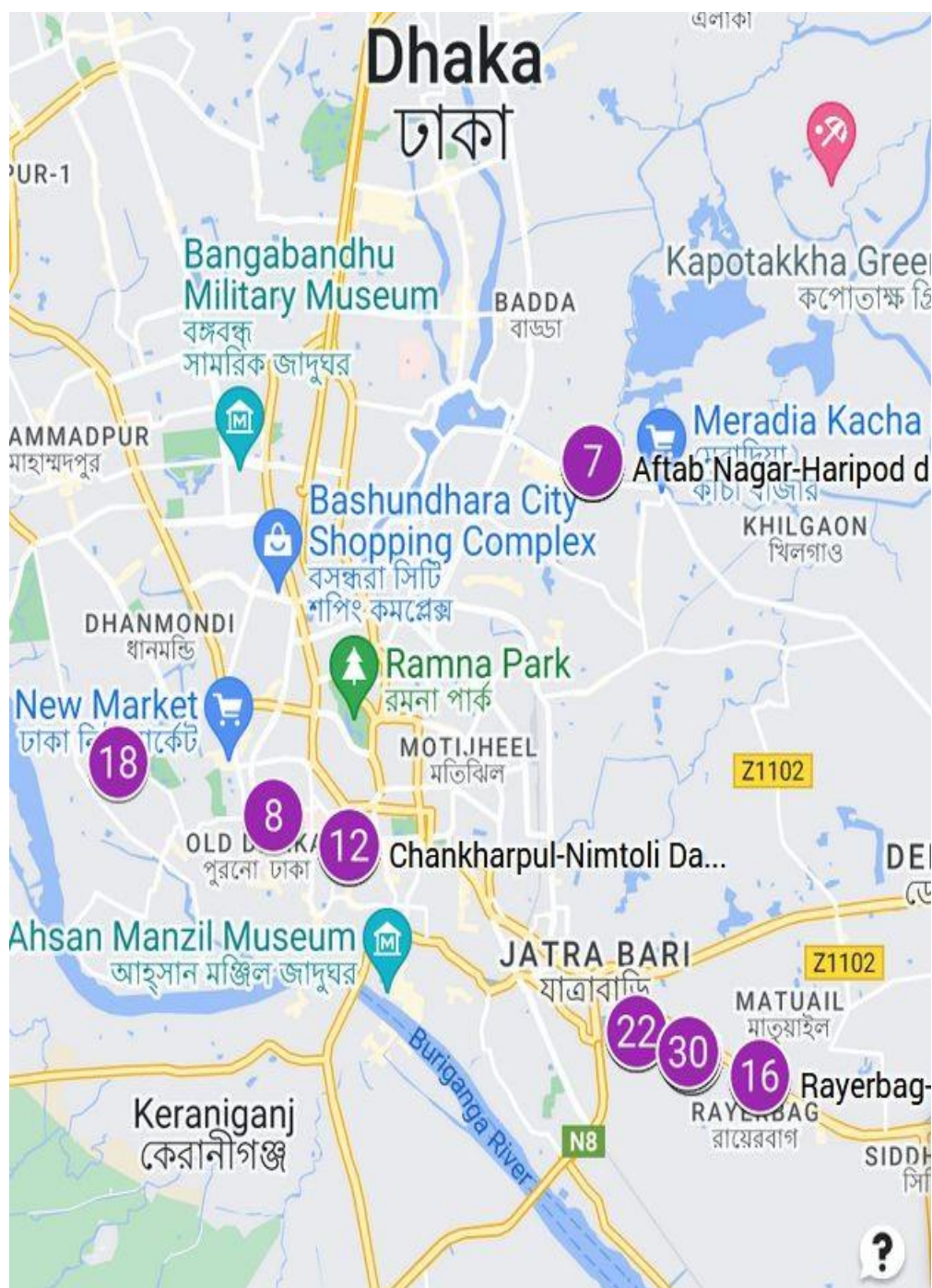
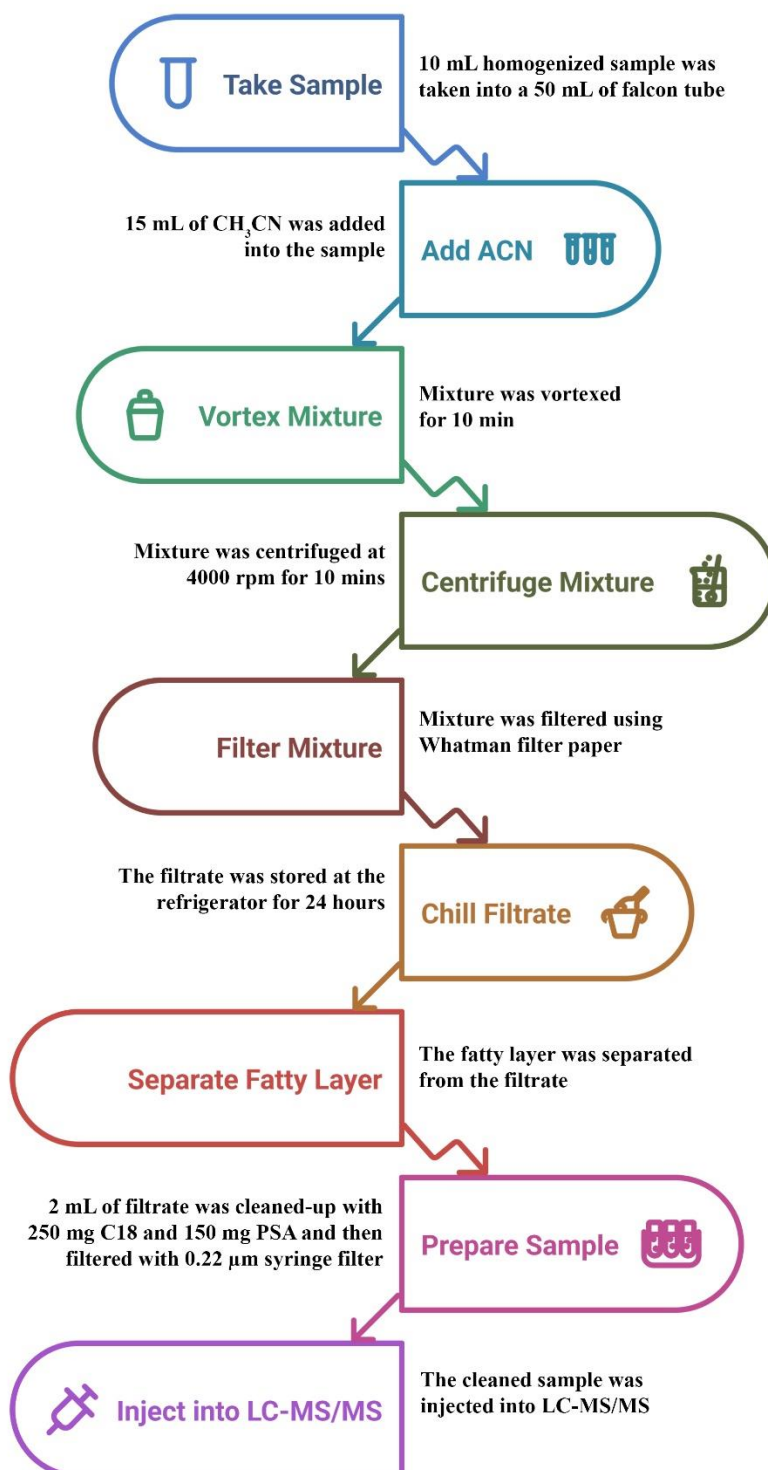


Fig 2.9: Collection sites of milk samples (Dhaka city)

Table 2.3: Milk sample collection areas

Milk Sample ID	Collection Source	Collection point	Places
Control	Control sample, Aftab Nagar, Dhaka		
MM1	Milk market	Haripod dairy farm	Aftab Nagar
MM2		Joya dairy farm	
MM3		MS/ Rozina dairy farm	
MM4		Onik dairy farm	
MM5		Badol dairy farm	
MM6		Alo dairy farm	Mohammadpur
MM7			
MM8	Milk shop	Baghabari Sweets	Polashi market, BUET
MM9	Dairy farm	Nimtoli dairy farm	Chankharpul
MM10			
MM11			
MM12	Dairy farm	Kalam dairy farm, Rayerbag kacha Bazar	Rayerbag
MM13			
MM14			
MM15	Milk market	Nilambar Saha dairy, Hazaribag Bazar	Hazaribag
MM16			
MM17	Milk market	Dholaipar dairy, Dholaipar Bazar	Dholaipar
MM18			
MM19			
MM20			
MM21	Milk market	Dania dairy, Dania Bazar	Dania
MM22			
MM23			
MM24	Milk market	Goyalbari dairy, Goyalbari mor	Sonir Akhra
MM25			
MM26	Milk market	Harinal dairy	Gazipur
MM27			
MM28			
MM29			
MM30			

2.13.2.1 Extraction and Clean-up Method for Residues in Milk Samples



Scheme 3: Extraction and clean-up for antibiotic residues analysis in milk samples

Following the modified method (Jank et al., 2012), milk sample (10 mL) was taken into a 50 mL falcon tube. Acetonitrile (CH_3CN) of 15 mL was added into the sample. The mixture was vortexed for 10 minutes then centrifuged at 4000 rpm for 10 minutes. After centrifugation, the sample was filtered using Whatman filter paper. The filtrate was then kept in the refrigerator for 24 hours to chill and allowing the fatty layer to separate from the filtrate (Scheme 3).

Aliquot of the filtrate (2 mL) was transferred into a screw-capped test tube, followed by the addition of 250 mg of C18 and 150 mg of PSA. The mixture was then filtered using 0.22 μm syringe filter. The cleaned sample was transferred into an LC vial and injected into the LC-MS/MS for antibiotic residue analysis.

2.13.3 Collection and Storage of Control and Targeted Egg Samples

Egg samples were collected from five selected sites located within wholesale markets in Dhaka and Gazipur cities. The control egg sample was obtained from Kapasia, Gazipur city while the tested egg samples were securely purchased in a paper box and bought into the laboratory. A total of 31 egg samples were collected for this study. The control sample was sourced from Kapasia, Gazipur city, while the remaining 30 egg samples (EM1-EM30) were obtained from wholesale such as Charulata market, Merul badda Bazar, Jatrabari and Kawran Bazar markets in Dhaka and Gazipur cities (Fig. 2.10). To analyze CAP residues, all collected samples were frozen at $-20\text{ }^{\circ}\text{C}$ until analysis (Table 2.4).



Fig. 2.10: Collection sites of egg samples (Dhaka and Gazipur city)

Table 2.4: Egg sample collection areas

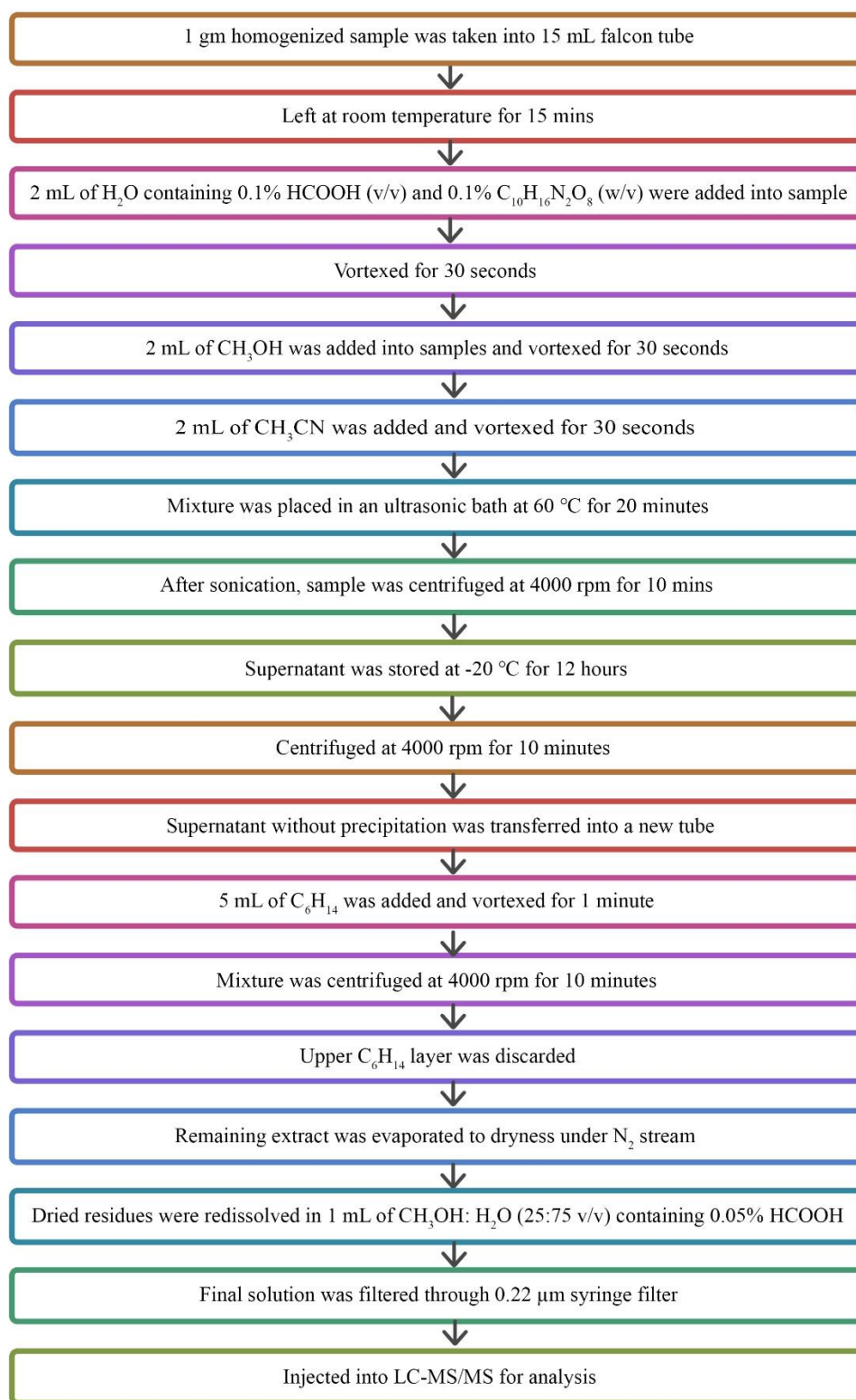
Egg Sample ID	Collection Source	Collection point	Places
Control	Control sample, Aftab Nagar, Dhaka		
EM1	Wholesale egg market	Kapasias Bazar	Gazipur
EM2			
EM3			
EM4			
EM5			
EM6			
EM7			
EM8			
EM9	Dimer aarot	Charulata market	Moghbar
EM10			
EM11			
EM12			
EM13			
EM14	Dimer aarot	Merul badda	Badda
EM15			
EM16			
EM17			
EM18			
EM19	Boro Bazar aarot	Jatrabari market	Jatrabari
EM20			
EM21			
EM22			
EM23			
EM24	Wholesale egg market	Kawran Bazar market	Kawran Bazar
EM25			

EM26			
EM27			
EM28			
EM29			
EM30			

2.13.3.1 Extraction and Clean-up Method for Residues Analysis in Egg Samples

Following modified method ([Dasenaki et al., 2015](#)), homogenized egg (1 g) sample was placed into a 15 mL falcon tube and left at room temperature for 15 minutes. 2 mL of H₂O containing 0.1% formic acid (HCOOH) (v/v) and 0.1% EDTA (C₁₀H₁₆N₂O₈) (w/v) were added into the sample, followed by vertexing for 30 seconds. Subsequently, 2 mL of methanol (CH₃OH) was added and vortexed for another 30 seconds. This was followed by the addition of 2 mL acetonitrile (CH₃CN) which was also vortexed for 30 seconds. The prepared mixture was then placed in an ultrasonic bath at 60 °C for 20 minutes, ensuring thorough extraction. After sonication, the sample was centrifuged at 4000 rpm for 10 minutes, then resulting supernatant was stored at -20 °C for 12 hours to facilitate lipid and protein precipitation.

Then it was centrifuged again at 4000 rpm for 10 minutes. Supernatant (without precipitation) was carefully transferred into a new tube (Scheme 4). 5 mL of hexane (C₆H₁₄) was added and vortexed for 1 minute for defatting. The mixture was then centrifuged at 4000 rpm for 10 minutes, after which the upper C₆H₁₄ layer was discarded. The remaining extract was evaporated to dryness under a gentle stream of N₂, with the temperature not exceeding 45 °C. The dried residues were redissolved in 1 mL of CH₃OH: H₂O (25:75 v/v) containing 0.05% HCOOH. The final solution was filtered through 0.22 µm syringe filter to remove any remaining particles before being injected into LC-MS/MS for analysis.



Scheme 4: Extraction and clean-up for antibiotic residues analysis in egg samples

2.13.4 Collection and Storage of Control and Targeted Feed Samples

In order to assess the extent of antibiotic contamination, feed samples were collected from four representative locations throughout Dhaka and Gazipur city (Fig. 2.11). The control feed sample was collected from Joydebpur Bazar, Gazipur city while the test feed samples were purchased and stored in an airtight zip-lock bag. Before storage, the feed samples were finely ground until fully homogenized to ensure uniformity. The processed sample were then stored at room temperature under controlled conditions for further analysis. In this study, 31 feed samples were collected for analysis. One control sample (FC) was obtained from Joydebpur Bazar, Gazipur, while the other 30 feed samples (FM1-FM30) were sourced from wholesale and local markets in Dhaka and Gazipur cities. All samples were stored at room temperature to preserve them until residues analysis (Table 2.5).



Fig 2.11: Collection sites of feed samples (Dhaka and Gazipur city)

Table 2.5: Poultry Feed sample collection areas

Feed Sample ID	Collection Source	Collection point	Places
Feed Control	Control sample, Joydebpur Bazar, Gazipur		
FM1-FM4	Broiler	Baby starter, baby chick Feed	Joydebpur Bazar, Gazipur
FM5-FM8	Broiler	Grower, after 20 Days	Gazipur Chowrasta
FM9-FM12	Broiler	Finisher, after 35 Days	Joydebpur Bazar, Gazipur
FM13-FM16	Layer	Layer chick feed, 0 to 4 Weeks	Gazipur Chowrasta
FM17-FM20	Sonali	Sonali chick feed, after 30 Days	Joydebpur Bazar, Gazipur
FM21-FM22	Broiler	Broiler house feed	Nimtoli municipal market, Gulistan, Dhaka
FM23-FM24	Broiler	Broiler house feed	Ananda Bazar, Dhaka
FM25	Layer	Layer Baby starter feed	Nimtoli municipal market, Gulistan, Dhaka
FM26	Layer	Layer mash feed	Nimtoli municipal market, Fulbaria, Gulistan, Dhaka
FM27	Broiler	Baby starter feed	
FM28		Broiler house feed	
FM29	Sonali	Sonali baby starter feed	
FM30		Sonali grower feed	

2.13.4.1 Extraction and Clean-up Method for Residues in Feed Samples

Following the modified method of [Leitner et. Al., 2001](#), homogenized sample (5 gm) was placed in a stoppered conical flask containing 30 mL hexane (C_6H_{14}), ultra-sonication for 1 minute and then left to stand overnight at room temperature. Hexane (C_6H_{14}) soluble portion was discarded. Next, 20 mL of ethyl acetate ($C_4H_8O_2$) was added to the sample residues and manually shaken for 1 hour before being filtered. The resulting filtrate was collected in a round-bottomed flask and evaporate to dryness. A mixture of $C_6H_{14}:H_2O$ (1:1; 2 mL) was added to the dried sample, ultrasonicated, transferred to a test tube, and vortexed for 1 minute then C_6H_{14} layer was discarded. The aqueous portion was then partitioned with 2 mL of $C_4H_8O_2$ and the organic layer was collected

and dehydrated by passing through a cotton filter over anhydrous sodium sulphate (Na_2SO_4) to remove any remaining H_2O . After evaporation, the sample was re-dissolved in 2 mL of $\text{C}_4\text{H}_8\text{O}_2$ in a pear-shaped flask (Scheme 5). For cleanup, ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$) fraction was applied to a preconditioned C18 reversed-phase silica gel column and eluted with 3 mL of acetonitrile (CH_3CN). The eluate was then evaporated to a final volume of 1.5 mL under a stream of N_2 . Then the resulting solution was ready to inject into LC-MS/MS.



Scheme 5: Extraction and clean-up for antibiotic residues analysis in poultry feed samples

2.14 Preparation of Standards, Stock and Intermediate Working Solutions

All types of standard stock solutions containing target analytes at a concentration of $1000\text{ }\mu\text{g mL}^{-1}$ were prepared in LC-MS/MS grade MeOH or ACN. A 0.001 gm of each standard was individually dissolved in 1.5 mL vial. The stock standards were tightly sealed with foil paper, kept individually in zip-locked bags, and stored in a freezer at $-20\text{ }^{\circ}\text{C}$. Each intermediate standard solution was diluted with the required mobile phase to achieve a final concentration of $100\text{ }\mu\text{g mL}^{-1}$ in 10 mL volumetric flasks, then placed at $-20\text{ }^{\circ}\text{C}$. Each working standard solution was a mixture of antibiotics within their respective class, prepared at $250\text{ }\mu\text{g mL}^{-1}$ or $200\text{ }\mu\text{g mL}^{-1}$ following the specific method required mobile phase and were further diluted to concentration levels ranging from $250\text{ }\mu\text{g mL}^{-1}$ to $0.13\text{ }\mu\text{g mL}^{-1}$ and stored until residues analysis was performed. Derivatives of nitrofurans metabolites were prepared using amber vials and volumetric flasks to avoid photo degradation.

2.15 Method Stabilization for Sulfa Drugs (SAs)

2.15.1 Preparation of Sulfa Drugs (SAs) Standards, Stock and Intermediate Working Solutions

A sulfa drug (SAs) mixture including sulfadimethoxine (SMX), sulfadiazine (SDZ), and sulfamethazine (SMT), with concentration ranging from $250\text{ }\mu\text{g mL}^{-1}$ to $1.25\text{ }\mu\text{g mL}^{-1}$ was prepared using CH_3OH for SAs residues analysis in poultry meat, beef, egg, milk and feed samples by LC-MS/MS

2.15.2 LC-MS/MS Method Optimization for Sulfa Drugs (SAs) Residues Analysis

SAs including sulfadimethoxine (SMX), sulfadiazine (SDZ), and sulfamethazine (SMT) were identified in positive scan mode (Table 2.6). SAs residues analysis modified method was optimized by LC-MS/MS for product ions using multi reaction monitoring (MRM) mode (Table 2.7). 0.1% HCOOH containing H_2O and CH_3OH (60:40) were used as a mobile phase using LC-MS/MS 8050 separation (Table 2.8).

Table 2.6: Instrumental parameter: Identification of SAs using SCAN mode

Compound Name	Molar mass, [M]	Ionization mode	Precursor ion [M+H] ⁺
Sulfadimethoxine (SMX)	278	+	279
Sulfadiazine (SDZ)	250	+	251
Sulfamethazine (SMT)	310	+	311

Table 2.7: Instrumental parameter: SAs method optimization using MRM mode

Event	Ch	Precursor ions	Product ions	Dwell Time	Voltage		
				(msec)	Q1 Pre Bias (V)	Collision energy (CE)	Q3 Pre Bias (V)
1	Ch1	311	156	100	-18	-18	-30
	Ch2	311	92	100	-10	-30	-18
	Ch3	311	108	100	-18	-29	-23
2	Ch1	251	92	100	-15	-26	-20
	Ch2	251	156	100	-14	-14	-17
	Ch3	251	108	100	-15	-44	-27
3	Ch1	279	92	100	-16	-16	-17
	Ch2	279	108	100	-17	-51	-27
	Ch3	279	156	100	-16	-16	-20

Table 2.8: Instrumental parameter: LC and MS method conditions of SAs

Parameter	Values
MS	Triple Quadrupole LC-MS/MS
SCAN	Positive
MRM	Positive
Ionization source	ESI
Nebulizing gas flow	3 L min ⁻¹
Heating gas flow	10 L min ⁻¹

Drying gas flow	10 L min ⁻¹
Collision gas	17 kPa
Interface temperature	300 °C
DL temperature	250 °C
Heat block temperature	400 °C
Column oven temperature	40 °C
Mobile phase A	H ₂ O with 0.1% HCOOH; (60%)
Mobile phase B	CH ₃ OH; (40%)
Column	Shim- Pack GIST C18 column, particle size 5 µm
Column dimension	4.6 × 150 mm
Injection volume	20 µL
Flow rate	1 mL min ⁻¹
Acquisition time	0-8 min
LC stop time	8 min

2.16 Method Stabilization for Fluoroquinolones (FQs)

2.16.1 Preparation of Fluoroquinolones (FQs) Standards, Stock and Intermediate Working Solutions

Fluoroquinolones (FQs) mixtures carrying ciprofloxacin (CIP), enrofloxacin (ENR), and levofloxacin (LEV) were prepared in ACN at concentrations ranging from 200 µg mL⁻¹ to 5 µg mL⁻¹ for the analysis of FQs residues in poultry meat, beef, egg, milk and feed samples by LC-MS/MS.

2.16.2 LC-MS/MS Method Optimization for Fluoroquinolones (FQs) Residues Analysis

Fluoroquinolones (FQs) combining ciprofloxacin (CIP), enrofloxacin (ENR), and levofloxacin (LEV) were detected in positive scan mode (Table 2.9) and optimized MRM mode (Table 2.10) for product ions were used with 0.1% HCOOH containing H₂O and CH₃CN (80:20) as a mobile phase on LC-MS/MS (Table 2.11).

Table 2.9: Instrumental parameter: Identification of FQs using SCAN mode

Compound Name	Molar mass, [M]	Ionization mode	Precursor ions [M+H] ⁺
Ciprofloxacin (CIP)	331	+	332
Enrofloxacin (ENR)	359	+	360
Levofloxacin (LEV)	361	+	362

Table 2.10: Instrumental parameter: FQs Method optimization using MRM mode

Event	Ch	Precursor ions	Product ions	Dwell Time	Voltage		
				(msec)	Q1 Pre Bias (V)	Collision energy (CE)	Q3 Pre Bias (V)
1	Ch1	332	231	100	-18	-40	-17
	Ch2	332	288	100	-19	-17	-15
	Ch3	332	314	100	-10	-21	-23
2	Ch1	360	245	100	-11	-29	-13
	Ch2	360	316	100	-10	-20	-23
	Ch3	360	342	100	-12	-20	-26
3	Ch1	362	221	100	-12	-37	-14
	Ch2	362	261	100	-11	-27	-11
	Ch3	362	318	100	-12	-17	-17

Table 2.11: Instrumental parameter: LC and MS method conditions of FQs

Parameter	Values
MS	Triple Quadrupole LC-MS/MS
SCAN	Positive
MRM	Positive
Ionization source	ESI
Nebulizing gas flow	3 L min ⁻¹
Heating gas flow	10 L min ⁻¹
Drying gas flow	10 L min ⁻¹

Collision gas	17 kPa	
Interface temperature	300 °C	
DL temperature	250 °C	
Heat Block temperature	400 °C	
Column oven temperature	40 °C	
Mobile phase A	H ₂ O with 0.1% HCOOH	
Mobile phase B	CH ₃ CN	
LC time program	Time (min)	Pump B concentration values
	1	20
	6	40
	8	100
	10	100
	12	1
	15	1
Column	Shim- Pack GIST C18 column, particle size 5 µm	
Column dimension	4.6 × 150 mm	
Injection volume	10 µL	
Flow rate	0.20 mL min ⁻¹	
Acquisition time	0-15 min	
LC stop time	15 min	

2.17 Method Stabilization for Tetracyclines (TCs)

2.17.1 Preparation of Tetracyclines (TCs) Standards, Stock and Intermediate Working Solutions

Tetracyclines working standards (TCs) containing tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) were utilized at concentrations ranging from 100 µg mL⁻¹ to 1.5 µg mL⁻¹ using MeOH for residues analysis of TCs in poultry meat, beef, egg, milk and feed samples by LC-MS/MS.

2.17.2 LC-MS/MS Method Optimization for Tetracyclines (TCs) Residues Analysis

Tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) were detected in positive ESI mode (Table 2.12), and optimized MRM transitions (Table 2.13) for their corresponding product ions were employed. The analytes were chromatographically separated using a mobile phase composed of 0.1% HCOOH in H₂O and MeOH (60:40, v/v), providing enhanced ionization efficiency and stable mass-spectrometric response (Table 2.14).

Table 2.12: Instrumental parameter: Identification of TCs using SCAN mode

Compound Name	Molar mass, [M]	Ionization mode	Precursor ion, [M+H] ⁺
Tetracycline (TC)	444	+	445
Oxytetracycline (OTC)	460	+	461
Chlortetracycline (CTC)	478	+	479

Table 2.13: Instrumental parameter: TCs method optimization using MRM mode

Event	Ch	Precursor ions	Product ions	Dwell Time	Voltage		
				(msec)	Q1 Pre Bias (V)	Collision energy (CE)	Q3 Pre Bias (V)
1	Ch1	445	427	100	-15	-13	-30
	Ch2	445	410	100	-15	-19	-30
	Ch3	445	154	100	-16	-12	-24
2	Ch1	461	426	100	-10	-19	-22
	Ch2	461	443	100	-12	-13	-23
	Ch3	461	337	100	-12	-37	-23
3	Ch1	479	444	100	-16	-20	-23
	Ch2	479	154	100	-10	-27	-17
	Ch3	479	461	100	-17	-41	-19

Table 2.14: Instrumental parameter: LC and MS method conditions of TCs

Parameter	Values	
MS	Triple Quadrupole LC-MS/MS	
SCAN	Positive	
MRM	Positive	
Ionization source	ESI	
Nebulizing gas flow (N ₂)	3 L min ⁻¹	
Heating gas flow (N ₂)	10 L min ⁻¹	
Drying gas flow (Air)	10 L min ⁻¹	
Collision gas (Ar)	17 kPa	
Interface temperature	300 °C	
DL temperature	250 °C	
Heat block temperature	400 °C	
Column oven temperature	40 °C	
Applied voltage	4 kV	
Mobile phase A	H ₂ O with 0.1% HCOOH	
Mobile phase B	CH ₃ OH	
LC time program	Time (min)	Pump B concentration values
	5	40
	7	100
	8	100
	9	0
	12	0
	15	Stop
Column	Shim- Pack GIST C18 column, particle size 5 µm	
Column dimension	250 × 4.6 mm	
Injection volume	20 µL	
Flow rate	1 mL min ⁻¹	
Acquisition time	0-15 min	
LC stop time	15 min	

2.18 Method Stabilization for Chloramphenicol (CAP)

2.18.1 Preparation of Chloramphenicol (CAP) Standards, Stock and Intermediate Working Solutions

Chloramphenicol (CAP) standard was prepared at concentrations ranging from 250 $\mu\text{g mL}^{-1}$ to 1.5 $\mu\text{g mL}^{-1}$ with using MeOH used as the solvent for CAP residues analysis in poultry meat, beef, egg, milk and feed samples by LC-MS/MS.

2.18.2 LC-MS/MS Method Optimization for Chloramphenicol (CAP) Residues Analysis

Chloramphenicol (CAP) was detected in negative scan mode (Table 2.15) and optimized MRM mode (Table 2.16) for product ions were used with 0.1% HCOOH containing H₂O and MeOH (70:30) as a mobile phase on LC-MS/MS (Table 2.17).

Table 2.15: Instrumental parameter: Identification of CAP using SCAN mode

Compound Name	Molar mass, [M]	Ionization mode	Precursor ion, [M-H] ⁻
Chloramphenicol (CAP)	322	-	321

Table 2.16: Instrumental parameter: CAP Method optimization using MRM mode

Event	Ch	Precursor ions	Product ions	Dwell Time	Voltage		
		(m/z)	(m/z)	(msec)	Q1 Pre Bias (V)	Collision energy (CE)	Q3 Pre Bias (V)
1	Ch1	321	152	100	11	18	25
	Ch2	321	257	100	23	12	10
	Ch3	321	194	100	21	14	30

Table 2.17: Instrumental parameter: LC and MS method conditions of CAP

Parameter	Values	
MS	Triple Quadruple LC-MS/MS	
SCAN	Negative	
MRM	Negative	
Ionization source	ESI	
Nebulizing gas flow	3 L min ⁻¹	
Heating gas flow	10 L min ⁻¹	
Drying gas flow	10 L min ⁻¹	
Collision gas	17 kPa	
Interface temperature	300 °C	
DL temperature	250 °C	
Heat block temperature	400 °C	
Column oven temperature	40 °C	
Interface voltage	3 kV	
Mobile phase A	H ₂ O with 0.1% HCOOH; (70%)	
Mobile phase B	CH ₃ OH; (30%)	
Column	Shim- Pack GIST C18 column, particle size 5 µm	
LC time program	Time (min)	Pump B concentration values
	5	30
	11	95
	13	30
	15	0
Column dimension	4.6 × 150 mm	
Injection volume	20 µL	
Flow rate	1 mL min ⁻¹	
Acquisition time	0-15 min	
LC stop time	15 min	

2.19 Method Stabilization for Derivatives of Nitrofuran Metabolites (NFs)

2.19.1 Preparation of Derivatives of Nitrofuran Metabolites (NFs) Standards, Stock and Intermediate Working Solutions

Metabolites of nitrofuran derivatives (NFs) mixtures containing 3-amino-2-oxazolidinone (2-NP-AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (2-NP-AMTZ), and 1-aminohydantoin (2-NP-AHD) with concentration ranging $1 \mu\text{g mL}^{-1}$ to $200 \mu\text{g mL}^{-1}$ were made with ACN which were utilized for the quantitative analysis of NFs residues in poultry meat, beef, egg, milk and feed samples using LC-MS/MS.

2.19.2 LC-MS/MS Method Optimization for Derivatives of Nitrofuran Metabolites (NFs) Residues Analysis

NFs containing 3-Amino-2-oxazolidinone (2-NP-AOZ), 3-Amino-5-morpholinomethyl-2-oxazolidinone (2-NP-AMTZ), and 1-Aminohydantoin (2-NP-AHD) were found in positive scan mode (Table 2.18). NFs residues analysis method was optimized and developed by LC-MS/MS for product ions using multi reaction monitoring (MRM) mode (Table 2.19). 0.1% HCOOH containing H₂O and 0.1% HCOOH containing ACN (50:50) were used for LC separation using Agilent 6420 LC/TQ (Table 2.20).

Table 2.18: Instrumental parameter: Identification of NFs using SCAN mode

Compound Name	Molar mass, [M]	Ionization mode	Precursor ion [M+H] ⁺
3-Amino-2-oxazolidinone (2-NP-AOZ)	235	+	236
3-Amino-5-morpholinomethyl-2-oxazolidinone (2-NP-AMTZ)	334	+	335
1-Aminohydantoin (2-NP-AHD)	248	+	249

Table 2.19: Instrumental parameter: NFs method optimization using MRM mode

Event	Precursor ions	Product ions	Dwell Time	Fragmentor	Collision energy (CE)	Cell accelerator voltage
			(msec)			
1	236	134	200	110	5	4
	236	104	200	110	5	4
2	335	291	200	110	5	4
	335	262	200	110	5	4
3	249	134	200	110	5	4
	249	104	200	110	5	4

Table 2.20: Instrumental parameter: LC and MS method conditions of NFs

Parameter	Values
LC	LC system (1290 Infinity II)
MS	Agilent 6420 LC/TQ
MRM mode	(+) ESI
Gas flow	10 L min ⁻¹
Gas temperature	350 °C
MS 1 Heater	100 °C
MS 2 Heater	100 °C
Draw speed	100 µL/min
Wait Time After Draw	1.2 s
Flow rate	0.5 mL/min
Sheath gas flow	0 L/min
Eject speed	400 µL/min
Injection volume	5 µL
High Vac	3.73E-5 Torr
Rough Vac	NP-AOZ: 1.17E+0 Torr NP-AMAZ: 1.15E+0 Torr NP-AHD: 1.16E+0 Torr
Cell Accelerator Voltage	4
Collision Energy	5

Dwell time for all product ions	200
Spectrum pane	QqQ
Sample flash-Out Factor	5 times Injection Volume
Fragmentor	110
Polarity	Positive
Turbo 1 Speed	100%
Maximum pressure limit	1300 bar
Mobile phase A	H ₂ O with 0.1% HCOOH: 50%
Mobile phase B	CH ₃ CN with 0.1% HCOOH: 50%
Column	1µL (50pg/µL) on column injection, LC gradient, Zorbax SB-C18
Column dimension	(2.1x 50 mm, 1.8 µm)

Chapter 3

3.0 Results and Discussion

3.1 Method Optimization and Validation of Sulfa Drugs (SAs)

3.1.1 Optimization of Sulfa Drugs (SAs)

The method optimization for sulfa drugs (SAs) on the MS system was performed for sulfadimethoxine (SMX), sulfadiazine (SDZ) and sulfamethazine (SMT) using ESI (+) mode at low concentrations in complex food and feed matrices. C18 reverse-phase column (Shim-Pack GIST C18, particle size 5 μm , 4.6 $\times$ 150 mm) was employed for SAs separation using LC-MS/MS. Mobile phase was prepared by H₂O with 0.1% formic acid (HCOOH) and methanol (CH₃OH) in 60:40 ratio. Mass spectrometric transitions were optimized through a combination of preliminary full scan (SCAN) and Multiple Reaction Monitoring (MRM) methods.

3.1.2 Screening of SAs Compounds

The optimized molecular ions at m/z 310, 250, and 278 for SMX, SDZ and SMT were scanned to identify their corresponding precursor ions at m/z 311, 251, and 279 (Fig. 3.1). For this purpose, the analytes were introduced into the LC system using SA standards, passed through a C18 column. A 250 $\mu\text{g L}^{-1}$ concentrated standard was used for screening using the SCAN method.

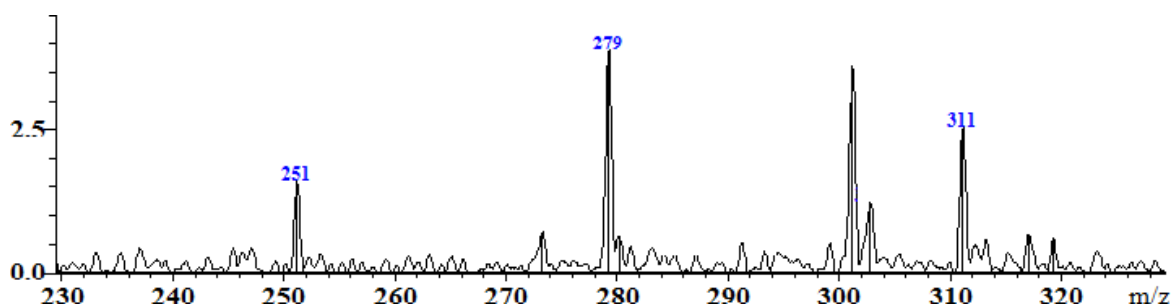


Fig. 3.1: Sulfa drugs screening

3.1.3 MRM of SAs Compounds

For MRM purpose, the mixture of SAs standards (250 $\mu\text{g L}^{-1}$) were injected into the LC system, passed through a C18 column, and then transferred via the MS interface to the MS/MS fragmentor. In MRM mode, these precursor ions of SAs were fragmented to their desire product ions m/z 92, 108 and 156 for each SMX, SDZ and SM, which were used for quantification (Fig. 3.2).

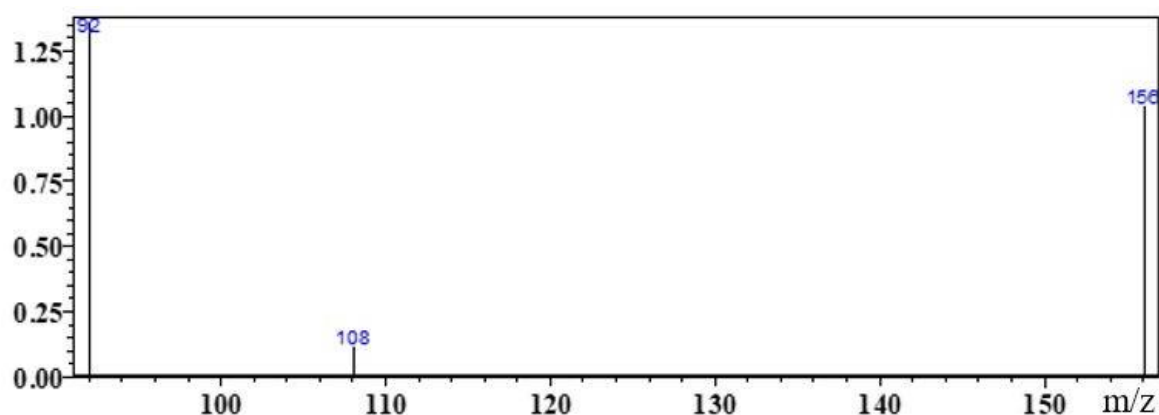


Fig. 3.2: Sulfa drugs MRM

3.1.4 Standard Calibration Curves of SAs

Standards of sulfa drugs e.g., SMX, SDZ and SMT were initially prepared at a concentration of $1000 \mu\text{g L}^{-1}$. From these stock solutions, $250 \mu\text{g L}^{-1}$ intermediate standard solution was prepared subsequently for a series of working standards were prepared for calibration curves over a concentration range of 1.25 - $100 \mu\text{g L}^{-1}$, ensuring accurate quantification of analytes in subsequent analyses (Fig. 3.3).

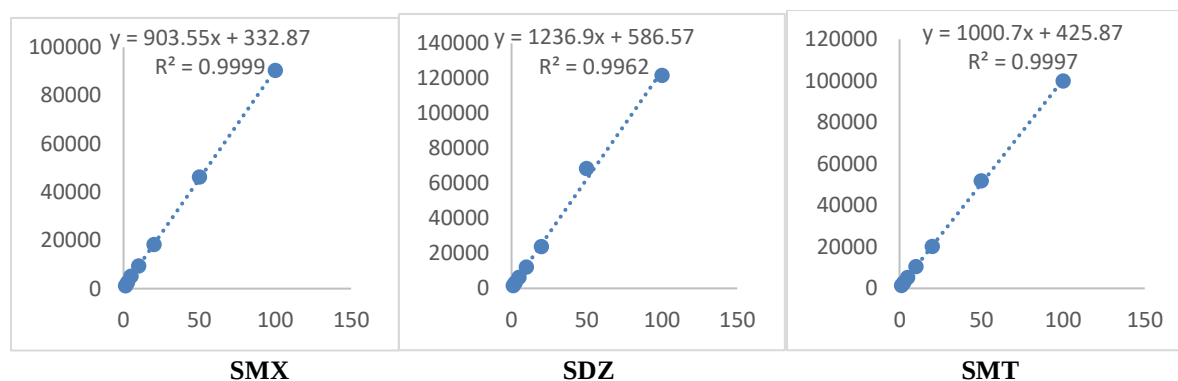


Fig. 3.3: Standard calibration curves of SMX, SDZ and SMT

3.1.5 Matrix-matched Calibration Curves of SAs in Poultry meat, Beef, Egg, Milk and Poultry Feed samples

A mixture of SA standards (SMX, SDZ and SMT) was spiked into poultry meat, beef, egg, milk, and poultry feed samples. Following extraction, the resulting matrix-matched samples were analyzed over a concentration range of 1.25 - $100 \mu\text{g kg}^{-1}$ to generate matrix-matched calibration curves (Fig. 3.4-3.8).

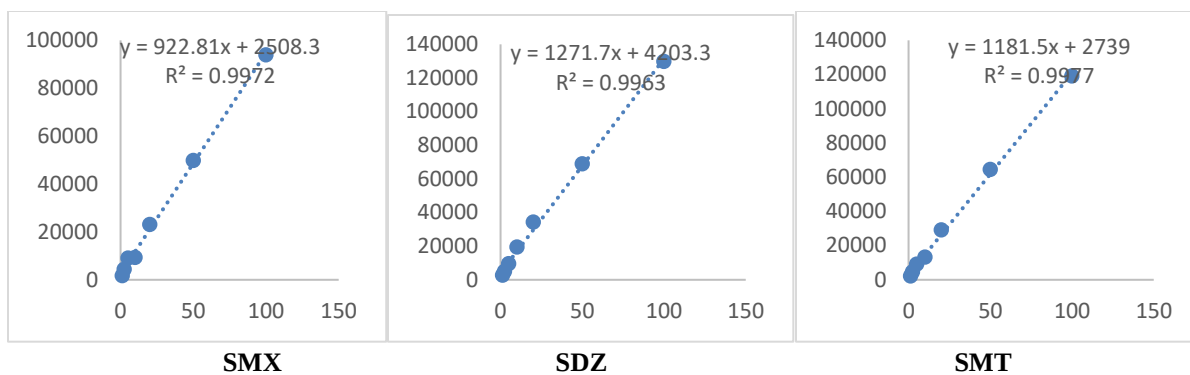


Fig. 3.4: Matrix matched calibration curves of SMX, SDZ and SMT in poultry meat samples

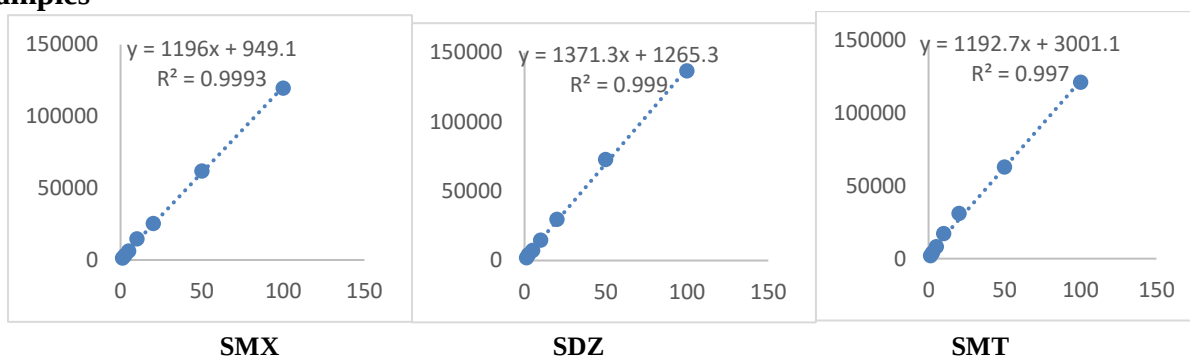


Fig. 3.5: Matrix matched calibration curves of SMX, SDZ and SMT in beef samples

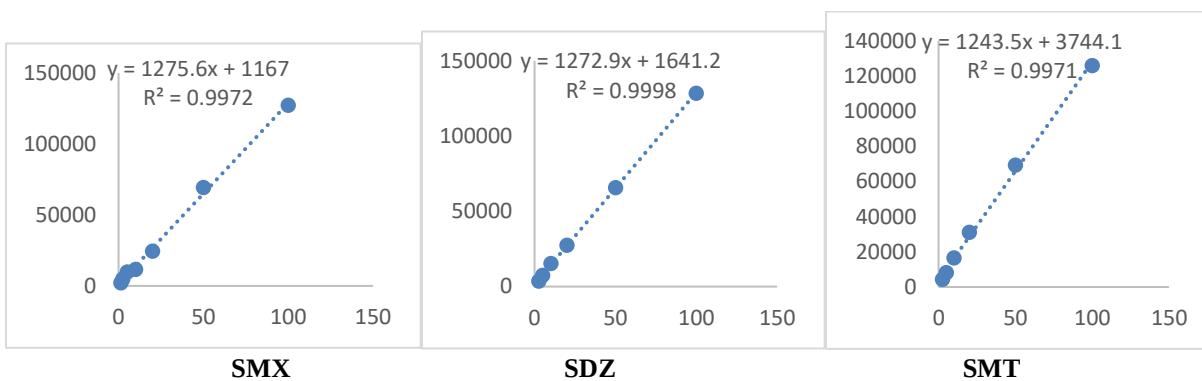


Fig. 3.6: Matrix matched calibration curves of SMX, SDZ and SMT in egg samples

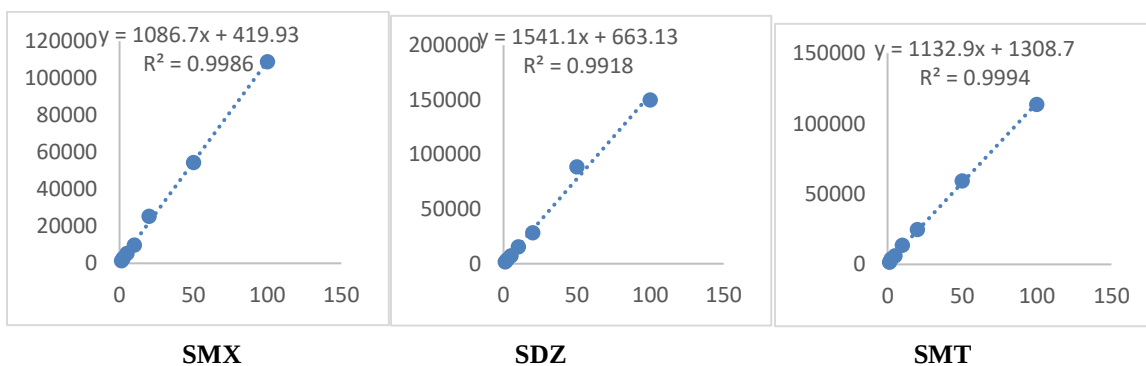


Fig. 3.7: Matrix matched calibration curves of SMX, SDZ and SMT in milk samples

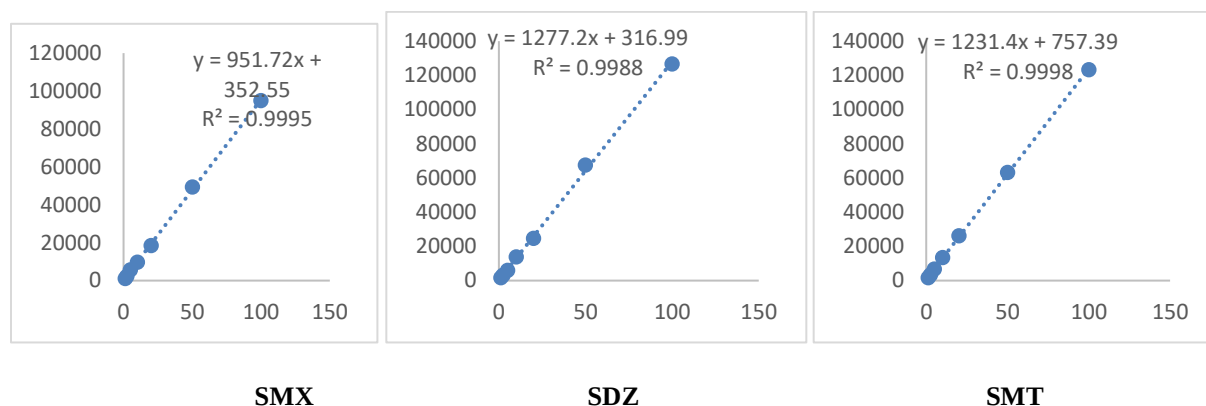


Fig. 3.8: Matrix matched calibration curves of SMX, SDZ and SMT in poultry feed samples

3.1.6 Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ) & Retention time (t_R) of SAs

The linearity of SMX, SDZ and SMT was determined using matrix-matched calibration curves prepared over the concentration range 1.25-100 $\mu\text{g kg}^{-1}$ in poultry meat, beef, egg, milk and poultry feed. All three analytes exhibited excellent linear responses, with correlation coefficients (R^2) spanning 0.9918-0.9999, demonstrating a strong proportional relationship between detector response (peak area) and analyte concentration in every matrix and confirming the suitability of the curves for quantitative purposes. The method also showed high sensitivity with LOD values ranging from 0.1-0.6 $\mu\text{g kg}^{-1}$ for SMX, 0.1-0.9 $\mu\text{g kg}^{-1}$ for SDZ and 0.1-0.7 $\mu\text{g kg}^{-1}$ for SMT, and corresponding LOQs of 0.3-1.8 $\mu\text{g kg}^{-1}$ for SMX, 0.3-2.7 $\mu\text{g kg}^{-1}$ for SDZ and 0.3-2.1 $\mu\text{g kg}^{-1}$ for SMT. These low values indicate that the LC-MS/MS procedure is capable of detecting and quantifying trace levels of SAs in complex food and feed samples (Table 3.1-3.3).

Under the optimized conditions, SMX, SDZ and SMT eluted at well-resolved and highly reproducible retention times (t_R), ensuring clear peak separation and minimizing the risk of co-elution or ion interference. SMX eluted between 5.7-5.9 min in the matrices compared with 5.9 min for the standard; SDZ showed a standard t_R of 2.3 min, with matrix t_R slightly shifted to 2.8-2.9 min due to matrix interferences and SMT was consistently retained at 3.3 min in all matrices except milk, where it appeared at 3.2 min. The distinct t_R , together with the excellent linearity and low LOD/LOQ values, demonstrate that the developed LC-MS/MS method is accurate, sensitive and reliable for quantifying SMX, SDZ and SMT residues in diverse food and feed matrices.

Table 3.1: SMX Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std SMX	5.9	1.25-100	-	-	$y = 903.55x + 332.87$	0.9999
Poultry meat	5.7		0.1	0.3	$y = 922.81x + 2508.3$	0.9972
Beef	5.9		0.1	0.3	$y = 1196x + 949.1$	0.9993
Egg	5.8		0.6	1.8	$y = 1275.6x + 1167$	0.9972
Milk	5.7		0.2	0.6	$y = 1086.7x + 419.93$	0.9986
Poultry feed	5.7		0.1	0.3	$y = 951.72x + 352.55$	0.9995

Table 3.2: SDZ Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std SDZ	2.3	1.25-100	-	-	$y = 1236.9x + 586.57$	0.9962
Poultry meat	2.8		0.2	0.6	$y = 1271.7x + 4203.3$	0.9963
Beef	2.3		0.1	0.3	$y = 1371.3x + 1265.3$	0.9990
Egg	2.9		0.9	2.7	$y = 1272.9x + 1641.2$	0.9998
Milk	2.8		0.3	0.9	$y = 1541.1x + 663.13$	0.9918
Poultry feed	2.3		0.5	1.5	$y = 1277.2x + 316.99$	0.9988

Table 3.3: SMT Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std SMT	3.3	1.25-100	-	-	$y = 1000.7x + 425.87$	0.9997
Poultry meat	3.3		0.6	1.8	$y = 1181.5x + 2739$	0.9977
Beef	3.3		0.1	0.3	$y = 1192.7x + 3001.1$	0.9970
Egg	3.3		0.7	2.1	$y = 1243.5x + 3744.1$	0.9971
Milk	3.2		0.3	0.9	$y = 1132.9x + 1308.7$	0.9994
Poultry feed	3.3		0.2	0.6	$y = 1231.4x + 757.39$	0.9998

3.1.7 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of SAs in Samples

The mean concentration (average $\pm$ SD) of SAs residues provides a quantitative characterization of analyte occurrence across distinct sample matrices, facilitating comparative evaluation of contamination levels. These mean values help illustrate the distribution pattern of SAs in each matrix and allow comparison of contamination levels between sample types. Standard deviation (SD) reflects the extent of variability among replicate measurements, reflecting analytical precision and potential matrix-dependent effects. Method performance was further assessed through intra-day RSD% to determine repeatability within a single analytical run, and inter-day RSD% to evaluate reproducibility across multiple days. Maintaining these metrics within acceptable limits confirms the method's robustness, reliability, and suitability for multi-matrix residue analysis, while minimizing analytical uncertainty associated with complex food matrices. The recovery percentage of sulfa drugs such as SMX, SDZ, and SMT demonstrated acceptable accuracy, indicating that the analytical method reliably quantified these compounds in the tested matrices.

3.1.7.1 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of SAs in Poultry Meat Samples

The quantitative evaluation of SMX, SDZ, and SMT in spiked poultry meat samples at 5 and 10 $\mu\text{g L}^{-1}$ demonstrates strong precision and reliability of the LC-MS/MS method (Table 3.4). At 5 $\mu\text{g kg}^{-1}$ level, SMX showed mean concentrations of 4.98-5.15 $\mu\text{g kg}^{-1}$ with intra-day RSD% values of 5.25-7.66% and an inter-day RSD% of 6.46%, indicating accurate recovery and acceptable variability for low-level determination in a complex matrix. SDZ exhibited mean concentrations of 4.28-5.07 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 5.76-6.78% and an inter-day RSD% of 6.14%, reflecting stable and reproducible performance. SMT showed mean concentrations of 4.75-5.23 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 6.54-8.84% and an inter-day RSD% of 7.91%, also indicating good precision at this spiking level. At 10 $\mu\text{g kg}^{-1}$ spiking level, SMX maintained mean concentrations values 9.41-10.31 $\mu\text{g kg}^{-1}$ with intra-day RSD% of 5.10-8.15% and an inter-day

RSD% of 6.12%, confirming consistent repeatability at the higher concentration. SDZ yielded mean concentrations of around 8.59-10.24 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 9.23-9.33% and an inter-day RSD% of 9.27%, suggesting slightly higher but still acceptable variability. SMT showed mean recoveries of 9.45-10.43 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 5.58-9.80% and an inter-day RSD% of 8.22%, again indicating reliable quantification despite matrix complexity. The consistency between measured concentrations and target spiking levels, together with intra- and inter-day RSD% values generally below 10% confirms that the method provides robust precision and is suitable for routine multi residue monitoring of sulfa drugs in poultry meat.

At 5 $\mu\text{g kg}^{-1}$ spiking level in poultry meat, SMX showed intra-day recoveries 99-103%, with an inter-day recovery of 101%, indicating efficient extraction and excellent day-to-day reproducibility. SDZ exhibited intra-day recoveries of 85-101%, and an inter-day recovery of 91%, reflecting slightly greater variability but still acceptable accuracy for trace-level analysis (Table 3.4). SMT showed intra-day recoveries of 94-104%, with an inter-day recovery of 101%, demonstrating stable performance with minimal matrix interference.

At 10 $\mu\text{g kg}^{-1}$ level, SMX intra-day recoveries of 94-103% and an inter-day recovery of 98% confirm that accuracy and precision are maintained at the higher concentration (Table 3.4). SDZ showed intra-day recoveries 85-102%, with an inter-day recovery of 91%, again indicating acceptable but slightly lower than those of the other analytes. SMT displayed intra-day recoveries of 97-104%, and an inter-day recovery of 98%, further supporting good reproducibility across multiple days.

Overall, recoveries for SMX, SDZ and SMT at both spiking levels cluster around 100% and fell within accepted typical validation limits 70-120% ([SANTE/11312/2021](#); [CODEX guideline, 2017](#)), confirming that LC-MS/MS method provides accurate and reproducible quantification of sulfa drugs residues in poultry meat.

Table 3.4: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter- day Recovery (%) of SAs in Poultry meat Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
SMX	5	5.1423 ± 0.3941	7.66	6.46	102	101
		4.9891 ± 0.2619	5.25		99	
		5.1518 ± 0.3332	6.46		103	
SDZ		4.2825 ± 0.2516	5.87	6.14	85	91
		5.0770 ± 0.3446	6.78		101	
		4.3278 ± 0.2494	5.76		86	
SMT		4.7487 ± 0.4027	8.84	7.91	94	101
		5.2331 ± 0.4565	8.72		104	
		5.2152 ± 0.3412	6.54		104	
SMX	10	9.9380 ± 0.8099	8.15	6.12	99	98
		10.3084 ± 0.5285	5.12		103	
		9.4187 ± 0.4812	5.10		94	
SDZ		8.5961 ± 0.7962	9.26	9.27	85	91
		10.2476 ± 0.9459	9.23		102	
		8.6538 ± 0.8075	9.33		86	
SMT		10.4323 ± 0.9702	9.30	8.22	104	98
		9.4513 ± 0.5274	5.58		94	
		9.7824 ± 0.9594	9.80		97	

3.1.7.2 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of SAs in Beef Samples

The quantitative evaluation of SMX, SDZ and SMT in beef showed good accuracy and precision at both spiking levels (Table 3.5). At 5 $\mu\text{g L}^{-1}$ spiking level, SMX yielded mean concentrations of 5.00-5.63 $\mu\text{g kg}^{-1}$ with intra-day RSD% of 2.32-5.00% and an inter-day RSD% of 3.38%, indicating accurate recovery and low variability at the lower concentration. SDZ showed mean concentrations of 4.66-5.40 $\mu\text{g kg}^{-1}$, with intra-day RSDs% ranging from 1.33-7.26% and an inter-day RSD% of 3.93%, reflecting stable and reproducible measurements. SMT showed mean values of 4.63-5.03 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 2.23-7.54% and an inter-day RSD% of 5.30%, confirming acceptable precision at 5 $\mu\text{g kg}^{-1}$.

At 10 $\mu\text{g kg}^{-1}$ spiking level, SMX showed mean recoveries of 10.00-11.18 $\mu\text{g kg}^{-1}$ with intra-day RSD% of 4.19-15.15% and an inter-day RSD% of 9.84%; although one replicate showed higher variability, overall precision remained within validation limits. SDZ showed mean concentrations of 9.33-10.81 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 3.55-9.51% and an inter-day RSD% of 5.87%, indicating good repeatability and reproducibility. SMT showed mean recoveries of 9.28-10.07 $\mu\text{g kg}^{-1}$, with intra-day RSDs% of 8.80-11.79% and an inter-day RSD% of 9.85%, again reflecting acceptable precision at the higher concentration.

The precision indicators for beef samples characterized by low to moderate intra- and inter-day RSD% values together with mean concentrations close to the nominal spiking levels demonstrate strong analytical reliability of the LC-MS/MS method for SMX, SDZ and SMT. SMX shows generally good precision, with slightly higher variability at 10 $\mu\text{g L}^{-1}$ level, whereas SDZ and SMT maintain consistent recoveries and reproducibility across both fortification levels, indicating stable method performance in this matrix. All three analytes remain well within commonly accepted validation limits, confirming the suitability of the method for routine SAs residue monitoring in beef.

At 5 $\mu\text{g kg}^{-1}$ spiking level in beef, SMX showed intra-day recoveries of 99-111%, indicating very good accuracy with only slight variability, which confirms that the extraction and quantification procedures perform reliably at low concentrations in this matrix (Table 3.5). The inter-day recovery of 103% supports this observation, demonstrating stable and reproducible results across different analytical days. For SDZ, intra-day recoveries of 93-108% reflect consistent precision and satisfactory accuracy close to the ideal range, while an inter-day recovery of 98% further confirms dependable performance with limited day-to-day fluctuation. SMT presented intra-day recoveries of 92-108, and 96%, suggesting generally accurate quantification with a combination of slight over- and under-recovery that may be related to normal matrix effects; its inter-day recovery of 96% nonetheless remains well within acceptable validation criteria, indicating reliable long-term method performance.

At 10 $\mu\text{g kg}^{-1}$ spiking level, SMX exhibited intra-day recoveries of 100-105%, showing good accuracy with mildly increased variability compared with the lower level, while an inter-day recovery of 105% confirms that reproducibility remains strong and is not adversely affected by the

higher concentration (Table 3.5). SDZ gave intra-day recoveries of 93-108%, which indicate accurate but slightly more variable behavior, possibly due to subtle concentration-dependent matrix interactions; the inter-day recovery of 98% demonstrates that this variability is still controlled across runs. SMT produced intra-day recoveries of 92-100%, reflecting acceptable accuracy and precision for a complex beef matrix, and its inter-day recovery of 98% confirms robust reproducibility.

Overall, SMX, SDZ and SMT all display strong accuracy and consistent reproducibility at both spiking levels in beef, confirming that the developed LC-MS/MS method is robust and suitable for multi-residue sulfa drug analysis in this matrix. All recoveries remain within commonly accepted validation limits, supporting the reliability of the method for routine quantitative applications.

Table 3.5: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of SAs in Beef Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
SMX	5	5.0268 $\pm$ 0.1415	2.81	3.38	99	103
		5.6300 $\pm$ 0.1311	2.32		111	
		5.0069 $\pm$ 0.2506	5.00		100	
SDZ		4.6555 $\pm$ 0.1487	3.19	3.93	93	98
		4.7797 $\pm$ 0.3473	7.26		95	
		5.4045 $\pm$ 0.0723	1.33		108	
SMT		4.6319 $\pm$ 0.2461	5.31	5.30	92	96
		5.0380 $\pm$ 0.1128	2.23		100	
		4.8291 $\pm$ 0.3642	7.54		96	
SMX	10	10.0019 $\pm$ 1.5551	15.15	9.84	100	105
		11.1786 $\pm$ 0.4692	4.19		111	
		10.5280 $\pm$ 1.0298	9.78		105	
SDZ		9.3354 $\pm$ 0.4239	4.54	5.87	93	98
		10.8188 $\pm$ 0.3847	3.55		108	
		9.3364 $\pm$ 0.8883	9.51		93	
SMT		9.2761 $\pm$ 0.8164	8.80	9.85	92	98
		10.0664 $\pm$ 0.9034	8.97		100	
		10.0577 $\pm$ 1.1859	11.79		100	

3.1.7.3 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of SAs in Egg Samples

The quantitative assessment of SMX, SDZ and SMT in egg at spiking levels at 5 $\mu\text{g kg}^{-1}$ level, SMX produced mean concentrations of about 4.66-5.09 $\mu\text{g kg}^{-1}$ with intra-day RSD% values between 5.68 and 10.36% and an inter-day RSD% of 7.36%, reflecting acceptable repeatability for low-level analysis in a complex matrix such as egg (Table 3.6). SDZ showed mean values of approximately 3.89-5.46 $\mu\text{g kg}^{-1}$, with intra-day RSD% ranging from 2.25-8.39% and an inter-day RSD% of 4.32%, indicating comparatively stable and precise measurements. SMT gave mean concentrations of 4.71-5.11 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 4.38-7.23% and an inter-day RSD% of 5.73%, again demonstrating good precision at the lower spiking level.

At 10 $\mu\text{g kg}^{-1}$ level, SMX recoveries ranged from 9.31-10.19 $\mu\text{g kg}^{-1}$, with intra-day RSD% between 3.48 and 13.64% and an inter-day RSD% of 7.55%; although one replicate displayed higher variability 13.64%, overall precision remained within acceptable validation limits (Table 3.6). SDZ exhibited mean concentrations of 7.77-10.83 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 4.12-8.89% and an inter-day RSD% of 6.66%, confirming reliable repeatability and reproducibility at the higher spiking level. SMT showed mean values of 9.47-10.26 $\mu\text{g kg}^{-1}$, with intra-day RSD% in the range 5.99-8.73% and an inter-day RSD% of 7.13%, indicating consistent performance and good analytical precision.

The mean concentrations close to the nominal spiking levels and intra- and inter-day RSD% values predominantly below 10% confirm that the method affords robust precision and reliable quantification of SMX, SDZ and SMT residues in egg samples, supporting its suitability for routine SAs residue monitoring in eggs.

At 5 $\mu\text{g kg}^{-1}$ spiking level in egg samples, SMX showed intra-day recoveries of approximately 93-101%, with an inter-day recovery of 98%, indicating accurate extraction with only slight variability and good day-to-day reproducibility (Table 3.6). SDZ exhibited intra-day recoveries of 93-109% and an inter-day recovery of 93%, reflecting generally acceptable accuracy with some matrix-related fluctuation. SMT showed intra-day recoveries of 94-102% and an inter-day recovery of 98%, demonstrating accurate quantification and consistent behavior in the egg matrix.

At 10 $\mu\text{g kg}^{-1}$ level, SMX again provided recoveries near the targeted intra-day 93, 101 and 94%,

inter-day 96%), confirming that precision and accuracy are maintained at the higher concentration (Table 3.6). SDZ showed intra-day recoveries of 108, 77 and 94% with an inter-day value of 93%, while SMT showed intra-day recoveries of 94, 102 and 99% and an inter-day recovery of 98%. Overall, intra- and inter-day recoveries for all three sulfa drugs were confirming that the LC-MS/MS method affords accurate and reproducible quantification of SMX, SDZ and SMT in egg samples.

Table 3.6: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of SAs in Egg Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
SMX	5	5.0923 $\pm$ 0.2894	5.68	7.36	101	98
		5.0569 $\pm$ 0.5240	10.36		101	
		4.6594 $\pm$ 0.2814	6.04		93	
SDZ		5.4558 $\pm$ 0.1230	2.25	4.32	109	93
		3.8895 $\pm$ 0.3263	8.39		77	
		4.6980 $\pm$ 0.1096	2.33		93	
SMT		4.7085 $\pm$ 0.2063	4.38	5.73	94	98
		5.1056 $\pm$ 0.2855	5.59		102	
		4.9401 $\pm$ 0.3572	7.23		98	
SMX	10	9.3145 $\pm$ 0.5170	5.55	7.55	93	96
		10.1883 $\pm$ 0.3549	3.48		101	
		9.4841 $\pm$ 1.2938	13.64		94	
SDZ		10.8327 $\pm$ 0.4467	4.12	6.66	108	93
		7.7748 $\pm$ 0.6913	8.89		77	
		9.4008 $\pm$ 0.6547	6.96		94	
SMT		9.4691 $\pm$ 0.5677	5.99	7.13	94	98
		10.2551 $\pm$ 0.6832	6.66		102	
		9.9082 $\pm$ 0.8651	8.73		99	

3.1.7.4 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of SAs in Milk Samples

The quantitative assessment of SMX, SDZ and SMT in milk at 5 $\mu\text{g L}^{-1}$ spiking levels, SMX gave mean concentrations of approximately 4.37-5.17 $\mu\text{g L}^{-1}$, with intra-day RSD% values ranging from 4.93-12.94% and an inter-day RSD% of 8.65%; the slightly higher RSD% at this low level reflects typical variability associated with trace-level quantification in a complex matrix, but remains

within acceptable limits (Table 3.7). SDZ showed mean values of 4.38-5.25 $\mu\text{g L}^{-1}$, accompanied by lower intra-day RSD% of 2.88-6.11% and an inter-day RSD% of 4.39%, indicating comparatively stable values. SMT yielded mean concentrations of 4.42-5.02 $\mu\text{g L}^{-1}$, with intra-day RSD% between 6.28 and 8.88% and an inter-day RSD% of 7.23%, also confirming good repeatability at the lower spiking level. At 10 $\mu\text{g L}^{-1}$ level, SMX recoveries ranged from 8.77-10.36 $\mu\text{g L}^{-1}$, with intra-day RSD% of 2.99-9.62% and an inter-day RSD% of 7.01%, demonstrating improved or comparable precision at the higher concentration. SDZ displayed mean concentrations of 8.74-10.44 $\mu\text{g L}^{-1}$, with intra-day RSD% of 3.11-7.83% and an inter-day RSD% of 6.08%, reflecting robust repeatability and reproducibility. SMT showed mean values of 9.43-9.71 $\mu\text{g L}^{-1}$, with intra-day RSD% of 7.04-10.93% and an inter-day RSD% of 8.73%, indicating acceptable precision and consistent performance across days.

Overall, in the mean concentrations and the nominal spiking levels, together with intra- and inter-day RSD% values mostly below 10%, demonstrates that the method provides precise and reliable quantification of SMX, SDZ and SMT residues in milk, confirming its suitability for routine SAs residue monitoring in milk.

At 5 $\mu\text{g L}^{-1}$ fortification level in milk, SMX showed intra-day recoveries of 87, 100 and 103%, indicating good accuracy with slightly broader variability at low concentration but still well within acceptable validation limits (Table 3.7). The inter-day recovery of 97% confirms that stable and reproducible SMX values across different days. SDZ exhibited intra-day recoveries of 104, 87 and 88%, reflecting a mix of slight over- and under-recovery that may be related to matrix effects, yet the inter-day recovery of 93% demonstrates that overall performance remains close to the nominal value with limited day-to-day fluctuation. SMT showed intra-day recoveries of 100, 88 and 94%, indicating generally accurate extraction with minor variability, while an inter-day recovery of 94% supports reliable long-term performance at this lower level.

At 10 $\mu\text{g L}^{-1}$ spiking level, SMX displayed intra-day recoveries of 87, 103 and 90%, showing acceptable accuracy with moderate variability typical of higher fortification in complex matrices; the inter-day recovery of 93% confirms that reproducibility is maintained at the elevated concentration (Table 3.7). SDZ presented intra-day recoveries of 87, 104 and 95%, again suggesting a balance of slight over- and under-recovery, and an inter-day recovery of 95%, which

indicates stable day-to-day behavior. SMT produced intra-day recoveries of 97, 94 and 94%, demonstrating strong accuracy with small fluctuations, while its inter-day recovery of 95% further confirms robust reproducibility.

Table 3.7: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of SAs in Milk Samples

Compound Name	Spiking Level ($\mu\text{g L}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
SMX	5	4.3727 $\pm$ 0.5661	12.94	8.65	87	97
		5.0242 $\pm$ 0.2480	4.93		100	
		5.1692 $\pm$ 0.4176	8.07		103	
SDZ		5.2470 $\pm$ 0.3209	6.11	4.39	104	93
		4.3843 $\pm$ 0.1265	2.88		87	
		4.4149 $\pm$ 0.1843	4.17		88	
SMT		5.0229 $\pm$ 0.4461	8.88	7.23	100	94
		4.4153 $\pm$ 0.2881	6.52		88	
		4.7295 $\pm$ 0.2973	6.28		94	
SMX	10	8.7651 $\pm$ 0.2624	2.99	7.01	87	93
		10.3595 $\pm$ 0.8713	8.41		103	
		9.0165 $\pm$ 0.8680	9.62		90	
SDZ		8.7352 $\pm$ 0.6381	7.30	6.08	87	95
		10.4397 $\pm$ 0.3250	3.11		104	
		9.5246 $\pm$ 0.7458	7.83		95	
SMT		9.7123 $\pm$ 0.6846	7.04	8.73	97	95
		9.4347 $\pm$ 1.0313	10.93		94	
		9.4551 $\pm$ 0.7776	8.22		94	

3.1.7.5 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of SAs in Poultry Feed Samples

In poultry feed, the precision study for SMX, SDZ and SMT at 5 $\mu\text{g kg}^{-1}$ spiking level, SMX recoveries ranged from about 4.65 to 5.08 $\mu\text{g kg}^{-1}$, with intra-day RSD% values of 6.03-9.15% and an inter-day RSD% of 7.85%, indicating good repeatability with moderate variability typical of low spiking level (Table 3.8). SDZ showed mean concentrations of 3.98-5.41 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 4.72-9.30% and an inter-day RSD% of 6.33%, reflecting

slightly higher matrix-dependent variability but still within acceptable limits. SMT showed mean values of 4.68-5.06 $\mu\text{g kg}^{-1}$ with lower intra-day RSD% 3.78-6.20% and an inter-day RSD% of 4.98%, suggesting comparatively stable performance. At 10 $\mu\text{g kg}^{-1}$ spiking level, SMX concentrations remained close to the target 9.35-10.16 $\mu\text{g kg}^{-1}$ with intra-day RSD% of 4.87-8.13% and an inter-day RSD% of 6.08%, confirming consistent repeatability at higher concentration. SDZ showed mean recoveries between 7.90 and 10.88 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 4.89-7.94% and an inter-day RSD% of 6.71%, again indicating acceptable precision in poultry feed. SMT produced mean values of 9.39-10.12 $\mu\text{g kg}^{-1}$, with intra-day RSD% ranging from 3.26 to 12.51% and an inter-day RSD% of 7.14%; despite one higher intra-day RSD%, overall precision remained within typical validation criteria.

Taken together, recoveries close to the nominal levels and intra- or inter-day RSD% below 10% demonstrate that the method provides robust precision and reliable quantification of SMX, SDZ and SMT in poultry feed, supporting its use for routine SAs residue surveillance in this matrix.

At 5 $\mu\text{g kg}^{-1}$ fortification level in poultry feed, SMX showed intra-day recoveries of 93, 96 and 101%, reflecting good accuracy and low variability, indicating that the extraction and quantification procedures perform reliably at low analyte concentrations in this matrix (Table 3.8). The inter-day recovery of 97% reinforces this observation, demonstrating stable and reproducible performance across different analytical days. For SDZ, intra-day recoveries of 79, 108 and 100% reveal acceptable precision, with values slightly below and above 100% but still within typical recovery limits; its inter-day recovery of 96% confirms dependable behavior with limited day-to-day fluctuation. SMT exhibited intra-day recoveries of 93, 101 and 101%, indicating good accuracy and consistent response, while the inter-day recovery of 98% remains well within validation criteria, supporting reliable long-term performance.

At 10 $\mu\text{g kg}^{-1}$ spiking level, SMX displayed intra-day recoveries of 93, 101 and 98%, indicating strong accuracy with minimal variability and no evidence of concentration-dependent deterioration in method performance; the inter-day recovery of 97% further confirms robust reproducibility at the higher concentration (Table 3.8). SDZ showed intra-day recoveries of 79, 108 and 77%, suggesting somewhat greater matrix-related variability and reduced extraction efficiency in some replicates, with an inter-day recovery of 88% indicating that this behavior persists across days but

still falls within acceptable analytical limits. SMT produced intra-day recoveries of 93, 101 and 98%, demonstrating excellent accuracy and stability, and an inter-day recovery of 97%, confirming very good reproducibility and overall method robustness. All three analytes SMX, SDZ and SMT exhibited acceptable accuracy for multi-residue determination of SAs in poultry feed. Although SDZ showed somewhat stronger matrix-dependent effects at the higher fortification level due to ion suppression, all recoveries remained within commonly accepted validation criteria, supporting the method's reliability for routine quantitative applications.

Table 3.8: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of SAs in Poultry Feed Samples

Compound Name	Spiking Level (μg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
SMX	5	4.6549 ± 0.2807	6.03	7.85	93	97
		4.8176 ± 0.4412	9.15		96	
		5.0834 ± 0.4251	8.36		101	
SDZ		3.9845 ± 0.1989	4.99	6.33	79	96
		5.4143 ± 0.2558	4.72		108	
		5.0144 ± 0.4663	9.30		100	
SMT		4.6790 ± 0.2903	6.20	4.98	93	98
		5.0540 ± 0.2506	4.95		101	
		5.0591 ± 0.1913	3.78		101	
SMX	10	9.3473 ± 0.7600	8.13	6.08	93	97
		10.1612 ± 0.4952	4.87		101	
		9.8264 ± 0.5167	5.25		98	
SDZ		7.9026 ± 0.3871	4.89	6.71	79	88
		10.8838 ± 0.8647	7.94		108	
		7.7752 ± 0.5674	7.29		77	
SMT		9.3938 ± 0.5316	5.65	7.14	93	97
		10.1177 ± 0.3300	3.26		101	
		9.8518 ± 1.2324	12.51		98	

3.1.8 Matrix effect of SAs

The matrix-induced ionization effects of SAs varied notably among the different food and feed matrices, indicating matrix- and analyte-dependent behavior (Table 3.9). For SMX, ion

enhancement was very low in poultry meat (2.13%) and poultry feed (5.33%), but much higher in beef (32.36%), egg (41.17%) and milk (20.27%), suggesting that these latter matrices contain more co-eluting components that promote ionization and increase signal intensity. SDZ was less affected overall, showing relatively small matrix effects in poultry meat (2.81%), egg (2.91%) and poultry feed (3.25%), but moderate enhancement in beef (10.86%) and particularly in milk (24.59%), where matrix constituents appear to exert a stronger influence on its ionization efficiency. SMT experienced consistently higher matrix effects across all matrices, with enhancement ranging from 13.21% in milk, 18.06 in poultry meat, 19.18 in beef, 24.26% in egg and 23.05% in poultry feed, indicating that this analyte is more sensitive to co-extracted matrix components.

Overall, these results show that matrix effects are both compound-specific and matrix-specific, with SMX and SMT exhibiting pronounced enhancement in several matrices and SDZ being comparatively less affected. These findings underline the necessity of matrix-matched calibration to correct for ionization differences and ensure accurate quantification of SAs in complex food and feed samples.

Table 3.9: Matrix-Induced Ionization Effects of SAs in Different Matrices

Matrix	Matrix effects (%)		
	SMX	SDZ	SMT
Poultry meat	2.13	2.81	18.06
Beef	32.36	10.86	19.18
Egg	41.17	2.91	24.26
Milk	20.27	24.59	13.21
Poultry feed	5.33	3.25	23.05

3.1.9 SAs Analysis in Real Samples

A total of 150 samples were analyzed for the presence of SAs (Fig. 3.9-3.14). Each category of samples (n=30) was extracted using method-specific extraction procedures to ensure recovery and accurate quantification. The results showed that all detected SAs residues were below their respective MRL values (Table 3.10). Among the analytes, SDZ was detected in one beef sample at a concentration of 61 $\mu\text{g kg}^{-1}$ and in one feed sample at the same concentration 61 $\mu\text{g kg}^{-1}$ (Fig. 3.15). Additionally, SMX was identified in one beef sample, with a measured level of 43 $\mu\text{g kg}^{-1}$

(Fig. 3.16). No other SAs residues were detected in the remaining samples, indicating a generally low occurrence of SAs contamination across the tested materials.

Table 3.10: Amount of Targeted SAs Compound

Antibiotic name	Matrix	No of positive samples	Amount (μgkg^{-1})	Sample detection frequency (%)
SDZ	Beef	1	61	3
SDZ	Feed	1	61	3
SMX	Beef	1	43	3

3.1.10 Chromatograms of SAs

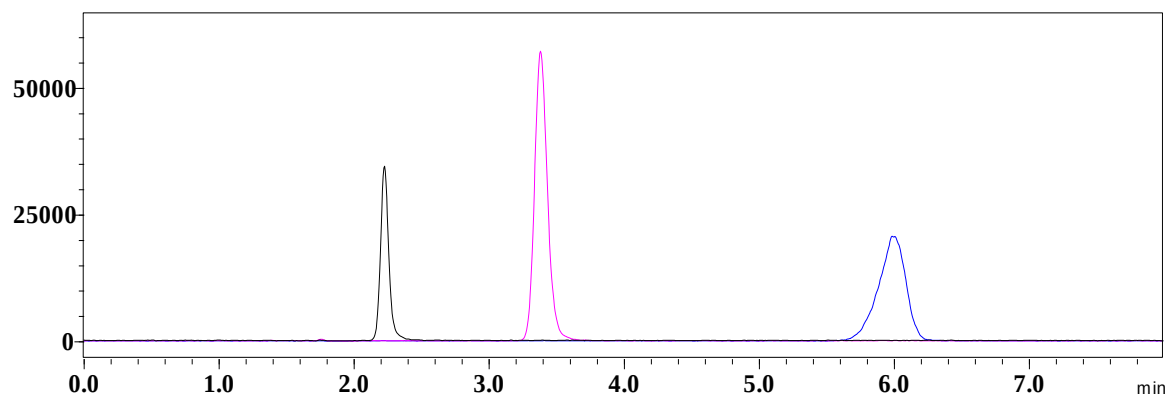


Fig. 3.9: Sulfa drug standards (SDZ, SMT and SMX)

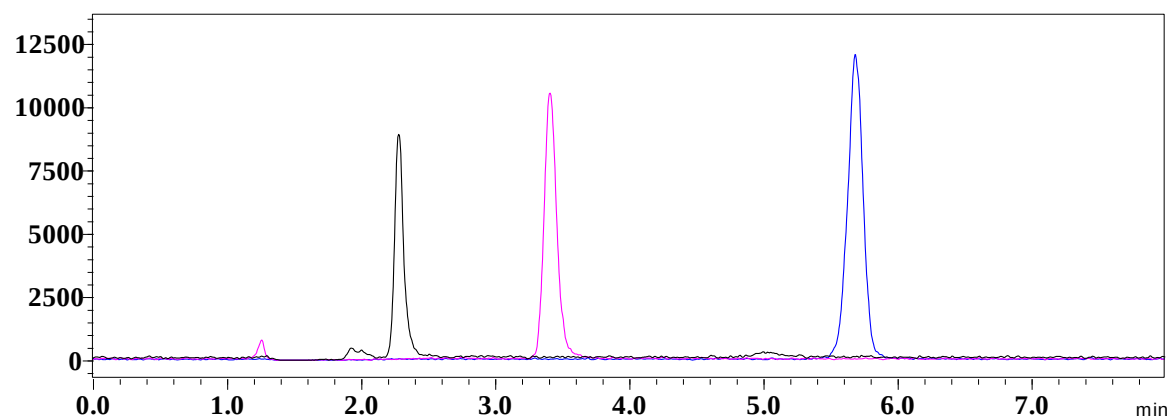


Fig. 3.10: Sulfa drug matrix-matched in poultry meat

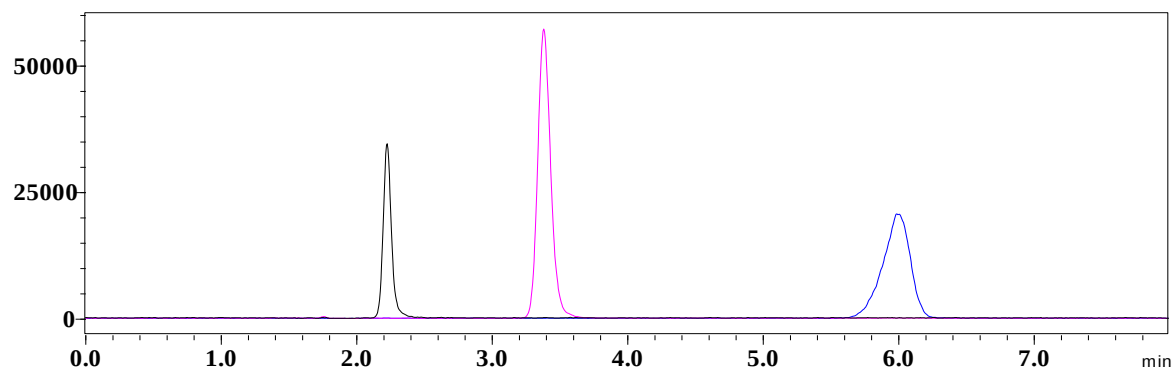


Fig. 3.11: Sulfa drug matrix-matched in beef

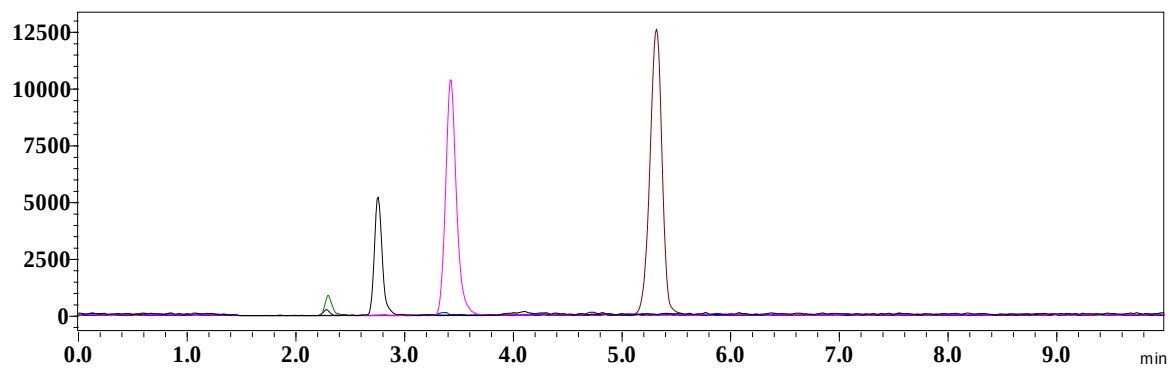


Fig. 3.12: Sulfa drug matrix-matched in egg

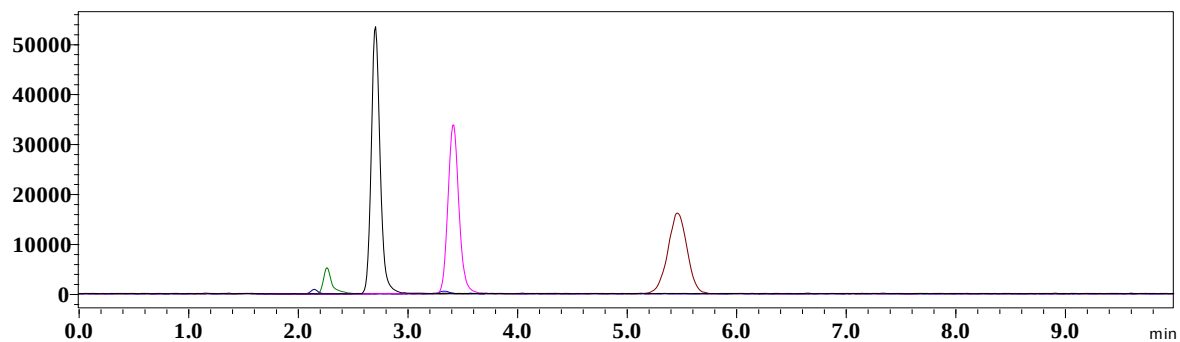


Fig. 3.13: Sulfa drug matrix-matched in milk

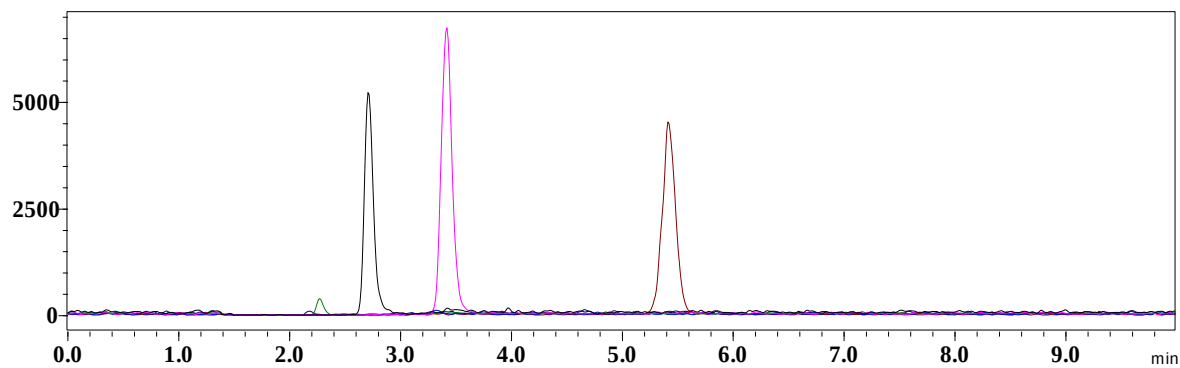


Fig. 3.14: Sulfa drug matrix-matched in poultry feed

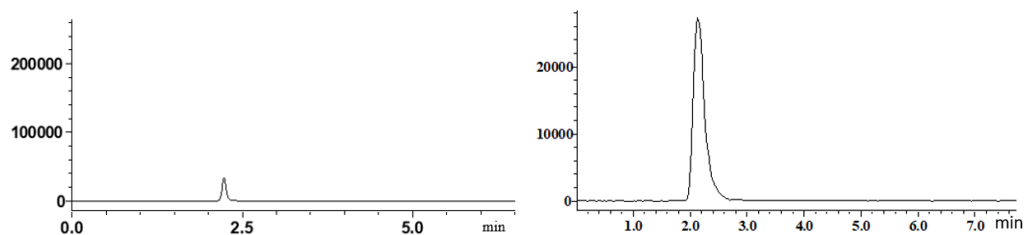


Fig. 3.15: Targeted SDZ in one beef and in one feed sample

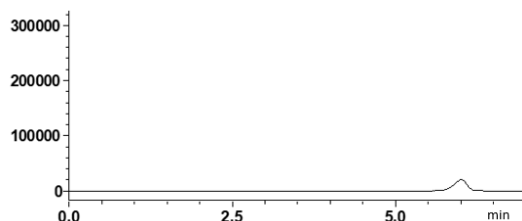


Fig. 3.16: Targeted SMX in one beef sample

3.2 Method Optimization and Validation of Fluoroquinolones (FQs)

3.2.1 Optimization of Fluoroquinolones (FQs)

The method optimization for fluoroquinolones (FQs) on the MS system was conducted for ciprofloxacin (CIP), enrofloxacin (ENR) and levofloxacin (LEV) in ESI (+) mode at low concentrations within complex food and feed matrices. Separation of the FQs was achieved on a C18 reverse-phase column (Shim-Pack GIST C18, particle size 5 μm , 4.6 $\times$ 150 mm) using LC-MS/MS. The mobile phase consisted of H₂O with 0.1% HCOOH and CH₃CN in LC-gradient mode. Mass spectrometric transitions were optimized using a combination of SCAN and MRM approaches.

3.2.2 Screening of FQs Compounds

The optimized molecular ions at m/z 331, 359, and 361 for CIP, ENR and LEV were scanned to determine their corresponding precursor ions at m/z 332, 360, and 362. To achieve this, the analytes introduced into the LC system using FQ standards were passed through a C18 column (Fig. 3.17). 250 $\mu\text{g L}^{-1}$ standard solution was employed for screening using the SCAN acquisition method.

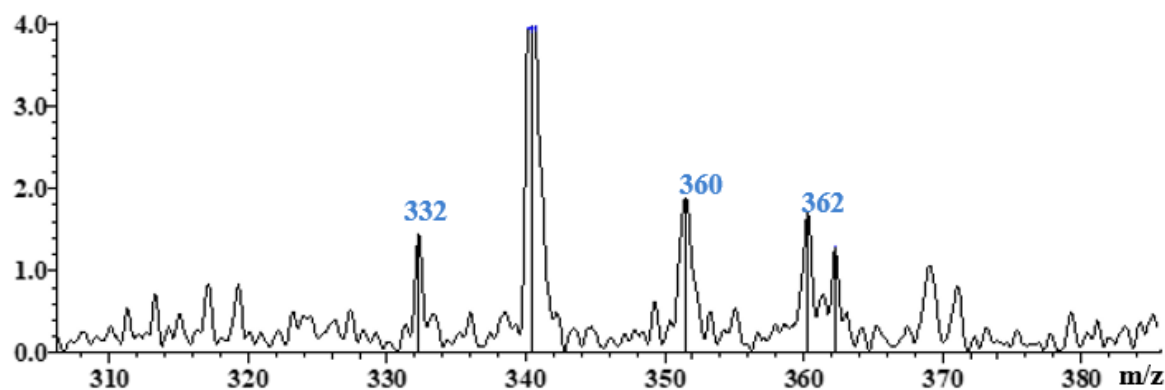
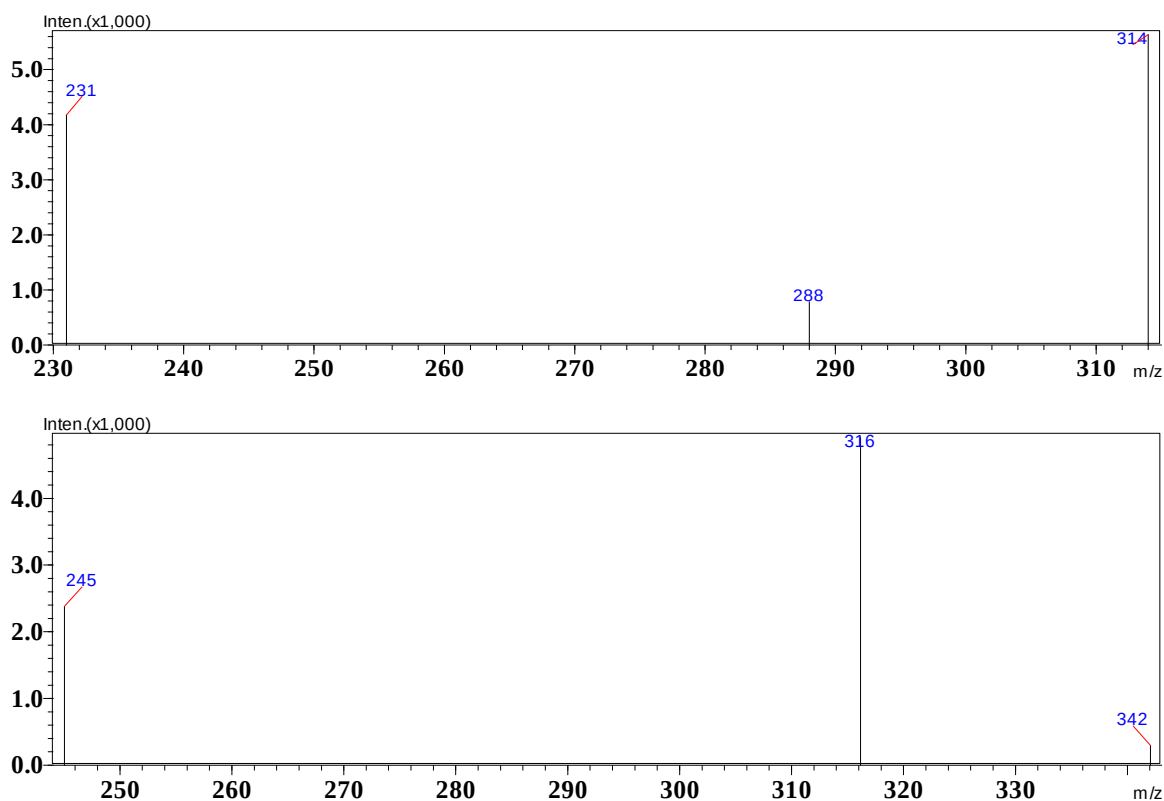


Fig.

3.17: FQs screening

3.2.3 MRM of FQs Compound

For MRM analysis, the analytes were introduced into the LC system using FQ standards, separated on a C18 column, and subsequently directed through the MS interface to the MS/MS fragmentor. In MRM mode, the selected precursor ions were fragmented to yield product ions for each CIP (231, 288, 314), ENR (245, 316, 342) and LEV (221, 261, 318), respectively, which served as the basis for quantification (Fig. 3.18).



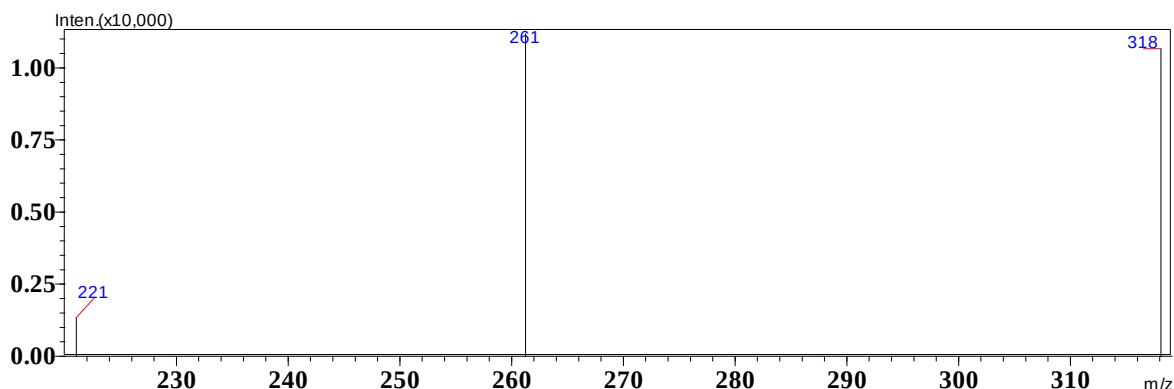


Fig. 3.18: FQs (CIP, ENR, LEV) MRM

3.2.4 Standard Calibration Curves of FQs

FQ standards were prepared as a stock solution at a concentration of $1000 \mu\text{g L}^{-1}$. From these, an intermediate standard of $250 \mu\text{g L}^{-1}$ was prepared, and subsequently used to prepare by serial dilution a series of working standard standards. These working standards, ranging concentrations from $5\text{--}200 \mu\text{g L}^{-1}$ were used to construct calibration curves for accurate quantification of the analytes in subsequent analyses (Fig. 3.19).

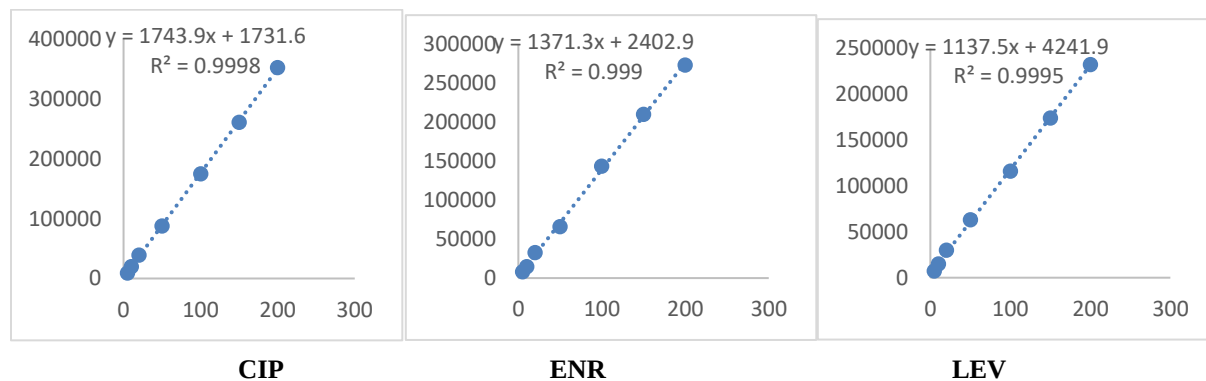


Fig. 3.19: Standard calibration curves of CIP, ENR and LEV

3.2.5 Matrix-matched Calibration Curves of FQs in Poultry meat, Beef, Egg, Milk and Poultry Feed samples

A combined solution of FQ standards was spiked to samples of poultry meat, beef, egg, milk, and poultry feed samples. After extraction, the matrix-matched samples were analyzed across a concentration range of $5\text{--}200 \mu\text{g kg}^{-1}$ (Fig. 3.20-3.24) to establish the corresponding matrix-matched calibration curves.

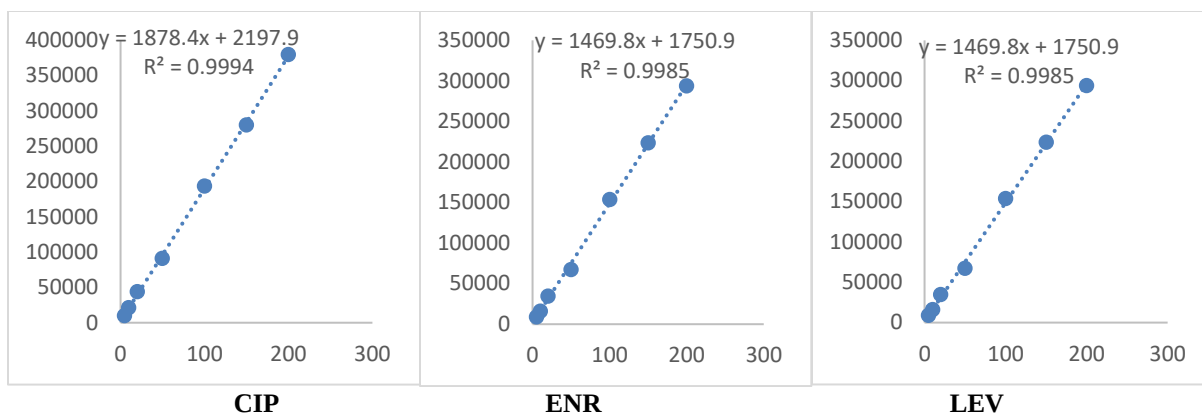


Fig. 3.20: Matrix matched calibration curves of CIP, ENR and LEV in poultry meat samples

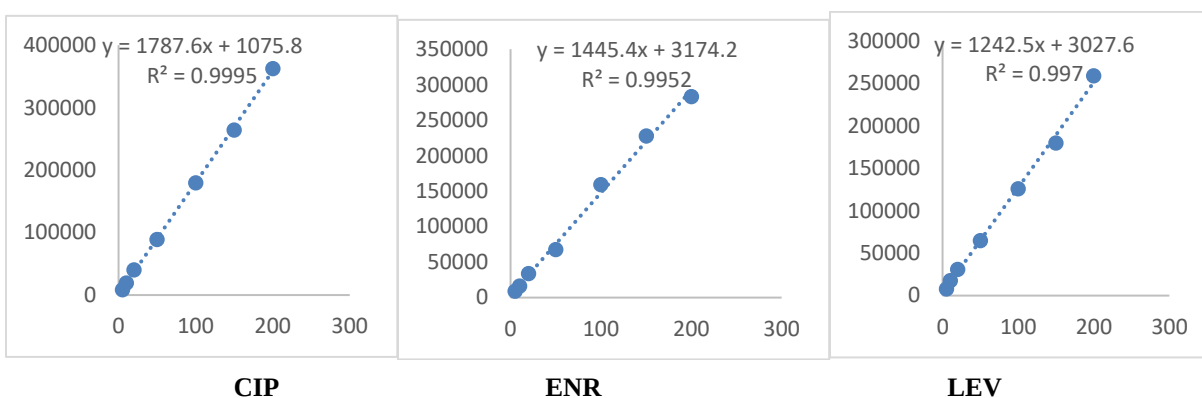


Fig. 3.21: Matrix matched calibration curves of CIP, ENR and LEV in beef samples

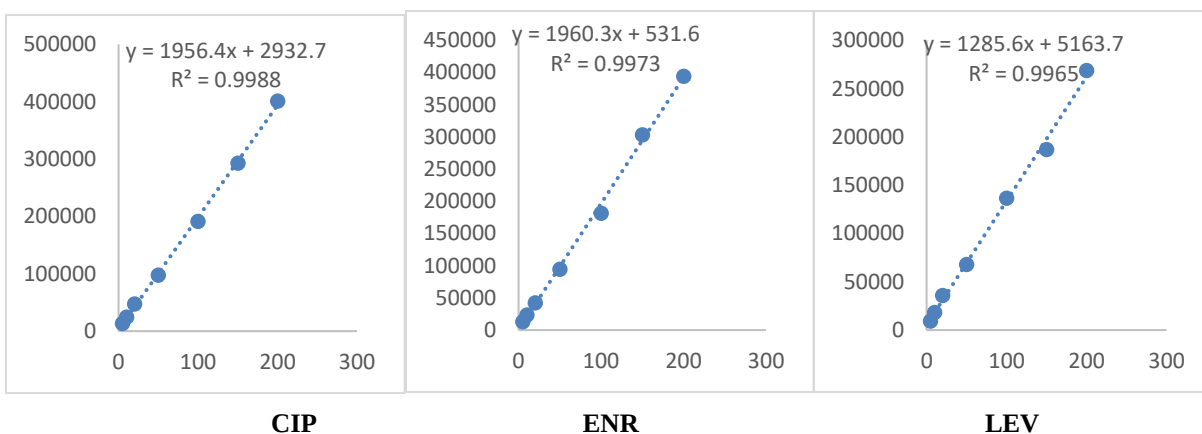


Fig. 3.22: Matrix matched calibration curves of CIP, ENR and LEV in egg samples

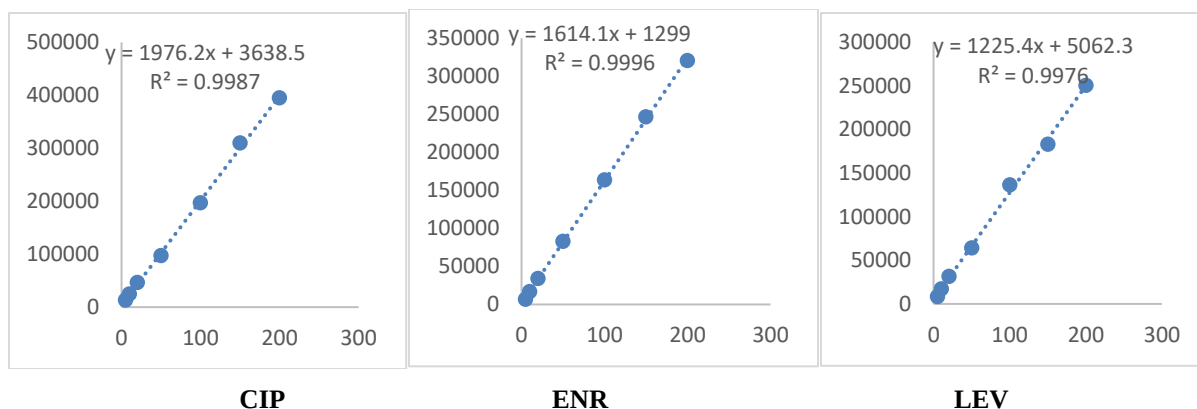


Fig. 3.23: Matrix matched calibration curves of CIP, ENR and LEV in milk samples

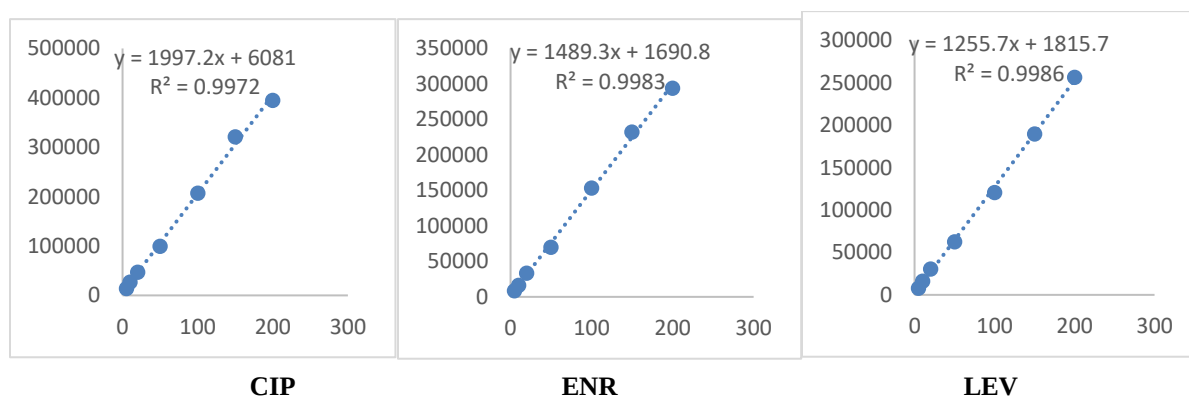


Fig. 3.24: Matrix matched calibration curves of CIP, ENR and LEV in poultry feed samples

3.2.6 Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ) & Retention time (t_R) of FQs

The linearity, LOD, LOQ and retention time data for CIP, ENR and LEV demonstrate that the developed LC-MS/MS method is highly suitable for multiresidue analysis in all tested matrices (Table 3.11-3.13). Matrix-matched calibration curves for each fluoroquinolone were constructed over the working range of 5-200 $\mu\text{g kg}^{-1}$, and all three analytes showed excellent linearity between concentration and signal was obtained for each analyte in all matrices, with R² values between about 0.994 and 0.9999 in poultry meat, beef, egg, milk and poultry feed. The similarity of the slopes for the matrix-matched curves to those of the corresponding standards indicates that matrix effects were effectively compensated. The method also exhibited high sensitivity. For CIP, LOD values ranged from 0.2-0.8 $\mu\text{g kg}^{-1}$ and LOQs from 0.6-2.6 $\mu\text{g kg}^{-1}$ across the different matrices.

ENR showed even lower limits, with LODs of 0.1-0.5 $\mu\text{g kg}^{-1}$ and LOQs of 0.3-1.6 $\mu\text{g kg}^{-1}$, while LEV presented LODs between 0.1 and 0.8 $\mu\text{g kg}^{-1}$ and LOQs of 0.3-2.6 $\mu\text{g kg}^{-1}$. These low LOD/LOQ values confirm that trace levels of FQ residues can be reliably quantified in complex samples. Although slightly higher LOQ values were observed in some of the more complex matrices, e.g. beef and poultry feed, all three compounds could still be quantified at low $\mu\text{g kg}^{-1}$ levels.

Retention time (t_R) was used as a confirmatory parameter to ensure accurate peak identification in complex matrices such as poultry meat, beef, eggs, milk, and poultry feed. Under the optimized chromatographic conditions, CIP, ENR, and LEV eluted at distinct and reproducible t_R across the tested matrices, which were consistent across multiple injections, demonstrating the method's precision and stability. The clear separation of t_R prevented co-elution and minimized potential ion suppression or interference from other matrix components. CIP, ENR and LEV eluted within approximately 3.7-3.9 min, with only minor shifts between poultry meat, beef, egg, milk and poultry feed, indicating robust chromatographic performance and the absence of major co-eluting interferences.

Overall, the combination of strong linearity, low LOD/LOQ, and consistent t_R values validates the developed method's suitability for quantitative analysis in complex food and feed matrices.

Table 3.11: CIP Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std CIP	3.9	5-200	-	-	$y = 1743.9x + 1731.6$	0.9998
Poultry meat	3.7		0.2	0.6	$y = 1878.4x + 2197.9$	0.9994
Beef	3.9		0.8	2.6	$y = 1787.6x + 1075.8$	0.9995
Egg	3.8		0.5	1.6	$y = 1956.4x + 2932.7$	0.9988
Milk	3.8		0.3	0.9	$y = 1976.2x + 3638.5$	0.9987
Poultry feed	3.9		0.7	2.3	$y = 1997.2x + 6081$	0.9972

Table 3.12: ENR Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std ENR	3.9	5-200	-	-	$y = 1371.3x + 2402.9$	0.9990
Poultry meat	3.9		0.3	0.9	$y = 1469.8x + 1750.9$	0.9985
Beef	3.7		0.1	0.3	$y = 1445.4x + 3174.2$	0.9952
Egg	3.8		0.4	1.3	$y = 1960.3x + 531.6$	0.9973
Milk	3.7		0.3	0.9	$y = 1614.1x + 1299$	0.9996
Poultry feed	3.9		0.5	1.6	$y = 1489.3x + 1690.8$	0.9983

Table 3.13: LEV Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std LEV	3.8	5-200	-	-	$y = 1137.5x + 4241.9$	0.9995
Poultry meat	3.9		0.2	0.6	$y = 1252.3x + 1094.9$	0.9944
Beef	3.8		0.8	2.6	$y = 1242.5x + 3027.6$	0.9970
Egg	3.9		0.6	1.9	$y = 1285.6x + 5163.7$	0.9965
Milk	3.7		0.1	0.3	$y = 1225.4x + 5062.3$	0.9976
Poultry feed	3.7		0.6	1.9	$y = 1255.7x + 1815.7$	0.9986

3.2.7 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of FQs in Samples

The mean concentration (average $\pm$ SD) of FQs residues quantitatively describes analyte levels in each matrix and allows direct comparison of contamination between matrices. These values summarize the central tendency of FQs residues within each sample type and support evaluation of differences in residue occurrence among matrices. Standard deviation (SD) quantifies variability among replicate measurements, reflecting analytical precision and potential matrix effects. Method precision was further characterized by intra-day RSD% for repeatability within a single run and inter-day RSD% for reproducibility across different days. Keeping SD and RSD% values within accepted limits confirms the method's robustness and suitability for multi-matrix residue analysis, while limiting uncertainty associated with complex food matrices. Recovery percentages of the fluoroquinolone drugs CIP, ENR and LEV were within acceptable ranges,

confirming that the analytical method accurately quantified these compounds in the examined matrices.

3.2.7.1 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of FQs in Poultry Meat Samples

The quantitative assessment of CIP, ENR and LEV in poultry meat at spiking levels of 20 and 25 $\mu\text{g kg}^{-1}$ demonstrates good analytical precision and reliability of the LC-MS/MS method (Table 3.14). At 20 $\mu\text{g kg}^{-1}$ level, CIP showed mean concentrations of approximately 18.10-21.03 $\mu\text{g kg}^{-1}$ with intra-day RSD% values between 3.70 and 9.03% and an inter-day RSD% of 6.12%, indicating satisfactory repeatability at this concentration. ENR exhibited mean values of about 18.62-21.13 $\mu\text{g kg}^{-1}$, with intra-day RSD% ranging from 5.93 to 8.23% and an inter-day RSD% of 6.86%, reflecting stable measurement performance. LEV gave mean concentrations of roughly 17.51-21.31 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 5.03-7.55% and an inter-day RSD% of 6.58%, which corresponds to good precision in this matrix.

At 25 $\mu\text{g kg}^{-1}$ spiking level, CIP yielded mean recoveries of about 22.54-26.43 $\mu\text{g kg}^{-1}$, with intra-day RSD% between 3.39 and 9.00% and an inter-day RSD% of 5.40%, confirming improved repeatability at the higher concentration. ENR displayed mean concentrations of 23.44-26.48 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% values of 7.20-9.97% and an inter-day RSD% of 8.63%, indicating acceptable precision with some matrix-related variability. LEV showed mean recoveries in the range of 21.86-26.55 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 5.26-9.30% and an inter-day RSD% of 7.62%, again demonstrating reliable quantification despite the complexity of the poultry meat. The combination of mean concentrations closes to the nominal spiking levels and intra- and inter-day RSD% values generally below 10% confirms that the method provides robust precision and acceptable variability for all three FQs at both evaluated fortification levels. These findings indicate that the LC-MS/MS procedure is suitable for routine monitoring of fluoroquinolone residues in poultry meat under realistic matrix conditions.

At 20 $\mu\text{g kg}^{-1}$ level, the intra-day recoveries for CIP in poultry meat 90, 105 and 100% indicate good accuracy with only slight variation around the nominal value, suggesting efficient extraction

and reliable quantification at this concentration (Table 3.14). The inter-day recovery of 98% confirms strong reproducibility, showing that CIP measurements remain stable across different analytical days. ENR intra-day recoveries 93, 105 and 104% are consistently close to 100% that may reflect mild matrix-induced signal enhancement; the inter-day recovery of 101% supports good day-to-day stability. LEV shows intra-day recoveries of 87, 106 and 104%, indicating overall acceptable accuracy with one slightly low value that could be related to minor ion suppression or extraction variability, while the inter-day recovery of 99% demonstrates that long-term performance remains robust. At 25 $\mu\text{g kg}^{-1}$, CIP exhibits intra-day recoveries of 90, 105 and 105%, again showing accurate quantification with a small proportion of values above 100%, and an inter-day recovery of 100%, which indicates excellent reproducibility and no concentration-dependent bias (Table 3.14). ENR recoveries at this level 99, 93 and 105% are well balanced around 100%, suggesting reliable extraction and minimal matrix interference; the inter-day recovery of 99% further confirms stable performance over multiple days. LEV intra-day recoveries 98, 87 and 106% remain within typical validation limits, with both slight under- and over-recoveries pointing to minor, but acceptable, matrix effects; the inter-day recovery of 97% indicates that these variations do not significantly impact overall method reproducibility. The intra- and inter-day recovery percentages for CIP, ENR and LEV at both 20 and 25 $\mu\text{g kg}^{-1}$ fall within commonly accepted validation criteria 70-120% (SANTE/11312/2021; CODEX guideline, 2017), demonstrating that the LC-MS/MS method provides accurate, precise and reproducible quantification of FQs residues in poultry meat with limited susceptibility to matrix-induced bias.

Table 3.14: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation, (RSD%), Intra- and Inter-day Recovery (%) of FQs in Poultry Meat Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CIP	20	18.0988 ± 1.6358	9.03	6.12	90	98
		21.0302 ± 0.7782	3.70		105	
		20.0592 ± 1.1275	5.62		100	
ENR		18.6171 ± 1.5326	8.23	6.86	93	101
		21.1289 ± 1.2538	5.93		105	
		20.9599 ± 1.3447	6.41		104	
LEV		17.5115 ± 0.8813	5.03	6.58	87	99
		21.3131 ± 1.6091	7.55		106	
		20.8366 ± 1.4959	7.17		104	

CIP	25	22.5350 ± 2.0291	9.00	5.40	90	100
		26.2461 ± 0.8911	3.39		105	
		26.4276 ± 1.0051	3.80		105	
ENR		24.9811 ± 1.7989	7.20	8.63	99	99
		23.4420 ± 2.0442	8.72		93	
		26.4813 ± 2.6412	9.97		105	
LEV		24.5298 ± 2.2833	9.30	7.62	98	97
		21.8646 ± 1.1505	5.26		87	
		26.5501 ± 2.2049	8.30		106	

3.2.7.2 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of FQs in Beef Samples

The quantitative evaluation of CIP, ENR and LEV in beef at spiking levels of 20 and 25 $\mu\text{g kg}^{-1}$ indicates good analytical precision and reliability of the LC-MS/MS method in this matrix (Table 3.15). At 20 $\mu\text{g kg}^{-1}$ level, CIP showed mean concentrations of approximately 17.71-21.02 $\mu\text{g L}^{-1}$ with intra-day RSD% values between about 2.76 and 4.50% and an inter-day RSD% of 3.82%, demonstrating satisfactory repeatability and accuracy close to the nominal level. ENR exhibited mean values of roughly 19.28-21.09 $\mu\text{g kg}^{-1}$, with intra-day RSD% ranging from 2.59 to 5.64% and an inter-day RSD% of 3.98%, reflecting stable and precise measurements. LEV gave mean concentrations of about 19.25-20.52 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSDs% of 4.45-10.74% and an inter-day RSD% of 6.70%, which still represents acceptable precision, although with slightly higher variability for some replicates. At 25 $\mu\text{g kg}^{-1}$ spiking level, CIP yielded mean recoveries of approximately 22.01-26.36 $\mu\text{g kg}^{-1}$ with intra-day RSD% values between 1.98 and 6.87% and an inter-day RSD% of 4.03%, confirming good repeatability at the higher concentration. ENR showed mean concentrations in the range of 24.14-26.36 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 7.68-10.45% and an inter-day RSD% of 8.84%, indicating acceptable precision with some matrix-related variability. LEV displayed mean recoveries of about 24.11-25.40 $\mu\text{g kg}^{-1}$, with intra-day RSD% between 5.13 and 10.13% and an inter-day RSD% of 7.25%, again supporting reliable quantification in beef.

Overall, the combination of mean concentrations closes to the fortification levels and intra- and inter-day RSD% values generally below 10% confirms that the method offers robust precision and acceptable variability for all three fluoroquinolones in beef, making it suitable for routine residue monitoring in beef.

At 20 $\mu\text{g kg}^{-1}$ spiking level in beef, the intra-day recoveries for CIP 88, 105 and 103% show good overall accuracy with one slightly low value, suggesting minor variability in extraction or matrix effects but still within acceptable validation limits (Table 3.15). The inter-day recovery of 98% confirms that CIP can be quantified reproducibly over multiple days without significant loss of accuracy. ENR exhibits intra-day recoveries of 105, 96 and 102%, tightly clustering around 100%, which indicates efficient extraction and minimal ion suppression; the inter-day recovery of 101% further supports excellent day-to-day stability. LEV shows intra-day values of 102, 96 and 101%, also very close to the nominal value, and an inter-day recovery of 99%, demonstrating accurate and consistent performance in the beef matrix.

At 25 $\mu\text{g kg}^{-1}$ spiking level, CIP yields intra-day recoveries of 105, 103 and 88%, reflecting predominantly accurate quantification with one slightly lower recovery that may reflect normal matrix-related variability; the inter-day recovery of 98% indicates that these fluctuations do not compromise longer-term reproducibility (Table 3.15). ENR intra-day recoveries 105, 96 and 102% again cluster near 100%, and the inter-day recovery of 101% confirms robust reproducibility at the higher concentration. LEV shows intra-day recoveries of 101, 101 and 96%, with an inter-day recovery of 99%, indicating stable and accurate measurements across different runs.

Overall, the intra- and inter-day recoveries for CIP, ENR and LEV at both 20 and 25 $\mu\text{g kg}^{-1}$ remain close to the nominal spiking levels and fall well within commonly accepted validation criteria. These results demonstrate that the LC-MS/MS method provides accurate, precise and reproducible quantification of fluoroquinolone residues in beef, with limited susceptibility to matrix-induced bias or day-to-day variability.

Table 3.15: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of FQs in Beef Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CIP	20	17.7053 ± 0.7978	4.50	3.82	88	98
		21.0209 ± 0.8811	4.19		105	
		20.6358 ± 0.5711	2.76		103	
ENR		21.0893 ± 1.1901	5.64	3.98	105	101
		19.2846 ± 0.5010	2.59		96	
		20.5173 ± 0.7631	3.71		102	

LEV		20.4637 ± 0.9109	4.45	6.70	102	99
		19.2487 ± 0.9444	4.90		96	
		20.2322 ± 2.1742	10.74		101	
CIP	25	26.360 ± 0.5245	1.98	4.03	105	98
		25.8410 ± 0.8383	3.24		103	
		22.0089 ± 1.5130	6.87		88	
ENR		26.3605 ± 2.0256	7.68	8.84	105	101
		24.1443 ± 2.0298	8.40		96	
		25.5206 ± 2.6687	10.45		102	
LEV		25.3994 ± 1.6507	6.49	7.25	101	99
		25.3472 ± 1.3027	5.13		101	
		24.1070 ± 2.4425	10.13		96	

3.2.7.3 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of FQs in Egg Samples

The quantitative assessment of CIP, ENR and LEV in egg samples at spiking levels of 20 and 25 $\mu\text{g kg}^{-1}$ demonstrates that the LC-MS/MS method provides generally good precision in this matrix (Table 3.16). At 20 $\mu\text{g L}^{-1}$ level, CIP gave mean concentrations of about 18.61-21.09 $\mu\text{g kg}^{-1}$, with intra-day RSD% values between 2.74 and 6.82% and an inter-day RSD% of 5.14%, indicating repeatable and accurate measurements close to the nominal level. ENR showed mean values of roughly 17.90-22.08 $\mu\text{g kg}^{-1}$, with intra-day RSD% ranging from 2.75 to 6.23% and an inter-day RSD% of 4.31%, reflecting stable performance with low day-to-day variation. LEV yielded mean concentrations of around 19.68-21.27 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 6.59-12.54% and an inter-day RSD% of 9.31%; although variability was somewhat higher for LEV, the values remain acceptable for residue analysis in a complex matrix such as egg.

At 25 $\mu\text{g kg}^{-1}$ spiking level, CIP produced mean concentrations of approximately 23.37-26.36 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 7.89-11.13% and an inter-day RSD% of 9.49%, confirming reasonable precision at the higher fortification level. ENR showed mean recoveries from 22.33 to 27.60 $\mu\text{g kg}^{-1}$, with intra-day RSD% values between 3.24 and 7.58% and an inter-day RSD% of 5.49%, indicating good repeatability and reproducibility. LEV exhibited mean concentrations of about 24.59-26.62 $\mu\text{g kg}^{-1}$, with notably low intra-day RSD% of 2.53-3.07% and an inter-day RSD% of 7.94%, demonstrating particularly strong precision for this analyte at 25 $\mu\text{g kg}^{-1}$.

The consistency of the measured values means to the nominal spiking levels, together with intra-

and inter-day RSD% values generally below 10%, confirms that the method offers robust and acceptable precision for the determination of FQs residues in egg samples and is suitable for routine monitoring in eggs.

At 20 $\mu\text{g kg}^{-1}$ spiking level in egg samples, intra-day recoveries for CIP 105, 93 and 101% indicate good accuracy with values fluctuating slightly around the nominal level, suggesting efficient extraction and only minor matrix-related variability (Table 3.16). The inter-day recovery of 100% confirms excellent reproducibility, showing that CIP can be measured consistently across different analytical days. ENR exhibits intra-day recoveries of 89, 110 and 97%, reflecting a balance of slight under and over-recoveries that may be attributed to moderate ion suppression or enhancement in the egg matrix; inter-day recovery of 99% demonstrates that these effects remain stable over time and do not compromise overall method performance. LEV shows intra-day recoveries of 106, 98 and 102%, indicating slightly positive bias but still close to the nominal concentration, while an inter-day recovery of 102% supports strong day-to-day stability and reliable quantification.

At 25 $\mu\text{g kg}^{-1}$ level, CIP yields intra-day recoveries of 105, 103 and 93%, again centered near 100% with one lower value, suggesting generally accurate extraction with limited variability; the inter-day recovery of 100% confirms that method reproducibility remains high at the higher spiking level (Table 3.16). ENR intra-day recoveries 89, 97 and 110% show a similar pattern to that observed at 20 $\mu\text{g kg}^{-1}$, with both slight under- and over-recovery consistent with typical matrix effects in complex egg samples, while the inter-day recovery of 99% indicates robust long-term stability. LEV presents intra-day recoveries of 106, 98 and 101% and an inter-day recovery of 102%, demonstrating accurate measurement with a small but acceptable positive bias.

Table 3.16: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter- day Recovery (%) of FQs in Egg Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CIP	20	21.0928 $\pm$ 1.2362	5.86	5.14	105	100
		18.6089 $\pm$ 0.5111	2.74		93	
		20.3134 $\pm$ 1.3874	6.82		101	

ENR	25	17.8966 ± 1.1161	6.23	4.31	89	99
		22.0797 ± 0.6082	2.75		110	
		19.5138 ± 0.7702	3.94		97	
LEV		21.2690 ± 1.4026	6.59	9.31	106	102
		19.6775 ± 2.4683	12.54		98	
		20.4597 ± 1.7994	8.79		102	
CIP		26.3558 ± 2.4879	9.43	9.49	105	100
		25.9713 ± 2.0504	7.89		103	
		23.3720 ± 2.6025	11.13		93	
ENR		22.3253 ± 1.6927	7.58	5.49	89	99
		24.4201 ± 0.7918	3.24		97	
		27.5950 ± 1.5609	5.65		110	
LEV		26.6170 ± 2.5336	2.53	7.94	106	102
		24.5881 ±2.7659	2.76		98	
		25.4392 ± 0.7833	3.07		101	

3.2.7.4 Average, Standard Deviation (±SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of FQs in Milk Samples

The quantitative assessment at 20 µg L⁻¹ level, CIP gave mean concentrations of approximately 19.25-20.87 µg L⁻¹, with intra-day RSD% between 1.31 and 7.04% and an inter-day RSD% of 3.68%, indicating repeatable measurements close to the nominal value (Table 3.17). ENR showed mean recoveries of about 13.90-17.08 µg L⁻¹, with intra-day RSD% of 3.58-8.21% and an inter-day RSD% of 5.70%, reflecting acceptable precision but with some under-recovery in milk. LEV showed mean values of roughly 19.68-21.27 µg L⁻¹, accompanied by intra-day RSD% of 6.59-12.54% and an inter-day RSD% of 9.31%, suggesting somewhat higher variability, yet still within a range generally considered acceptable for residue analysis in complex matrices. At 25 µg L⁻¹ spiking level, CIP yielded mean concentrations of around 23.94-25.97 µg L⁻¹, with intra-day RSD% of 5.16-11.36% and an inter-day RSD% of 8.11%, confirming reasonable repeatability at the higher spiking level. ENR produced mean recoveries of 17.35-21.23 µg L⁻¹, with intra-day RSD% between 2.21 and 7.72% and an inter-day RSD% of 4.24%, indicating comparatively stable performance and good reproducibility. LEV showed mean concentrations of about 22.91-25.25 µg L⁻¹, with intra-day RSD% of 6.60-8.70% and an inter-day RSD% of 7.34%, again demonstrating acceptable precision in milk. The intra- and inter-day RSD% values largely below 10%, confirms that the method acceptable precision for FQs determination in milk and is suitable for routine residue monitoring in all matrix.

At 20 µg L⁻¹ spiking level in milk, intra-day recoveries for CIP 101, 93 and 101% show good accuracy with only moderate variation around the nominal value, and an inter-day recovery of 98% confirms stable quantification over multiple days (Table 3.17). ENR exhibits intra-day recoveries of 101, 100 and 82%, indicating generally satisfactory accuracy with one lower value that suggests some matrix-related under-recovery; the inter-day recovery of 94% shows that this effect is consistent but remains within acceptable validation limits. LEV shows higher intra-day recoveries 111, 103 and 107%, pointing to a slight positive bias that may be attributed to mild matrix-induced signal enhancement, while the inter-day recovery of 107% still lies within typical recovery criteria for residue methods.

At 25 µg L⁻¹ level, CIP yields intra-day recoveries of 93, 101 and 101%, with an inter-day recovery of 98%, indicating accurate extraction and excellent reproducibility at the higher concentration (Table 3.17). ENR presents intra-day values of 82, 101 and 99%, again showing mostly accurate performance with some under-recovery, while the inter-day recovery of 94% confirms acceptable day-to-day stability. LEV shows intra-day recoveries of 106, 96 and 104%, and an inter-day recovery of 102%, reflecting slightly elevated but consistent recoveries that suggest limited, manageable matrix effects.

Table 3.17: (Average± SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter- day Recovery (%) of FQs in Milk Samples

Compound Name	Spiking Level (µg L ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CIP	20	20.8654 ± 0.4872	2.33	3.68	101	98
		19.2498 ± 0.2522	1.31		93	
		20.8606 ± 1.5438	7.04		101	
ENR		17.0804 ± 1.4037	8.21	5.70	101	94
		16.9785 ± 0.6082	3.58		100	
		13.9024 ± 0.7393	5.31		82	
LEV		21.2690 ± 1.4026	6.59	9.31	111	107
		19.6775 ± 2.4683	12.54		103	
		20.4597 ± 1.7994	8.79		107	
CIP	25	23.9439 ± 1.8702	7.81	8.11	93	98
		25.9684 ± 2.9519	11.36		101	
		25.9494 ± 1.3395	5.16		101	
ENR		17.3455 ± 1.3404	7.72	4.24	82	94

LEV		21.2271 ± 0.4693	2.21	7.34	101	102
		20.9559 ± 0.5862	2.79		99	
		25.2521 ± 1.6971	6.72		106	
		22.9068 ± 1.9945	8.70		96	
		24.9300 ± 1.6460	6.60		104	

3.2.7.5 Average, Standard Deviation (±SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of FQs in Poultry Feed Samples

The evaluation of CIP, ENR and LEV in poultry feed at spiking levels of 20 µg kg⁻¹ level, CIP gave mean concentrations of 15.89-21.05 µg kg⁻¹, with intra-day RSD% values between 1.91 and 6.52% and an inter-day RSD% of 3.66%, showing repeatable measurements reasonably close to the target level (Table 3.18). ENR showed mean recoveries of about 15.28-22.29 µg kg⁻¹, with intra-day RSD% of 2.06-5.06% and an inter-day RSD% of 4.06%, reflecting stable performance and low day-to-day variability. LEV at the same level produced mean concentrations of 17.49-21.21 µg kg⁻¹, with intra-day RSD% ranging from 4.57 to 11.02% and an inter-day RSD% of 6.87%, which is still acceptable for residue analysis in feed despite slightly higher variability in some runs.

At 25 µg kg⁻¹ spiking level, CIP yielded mean concentrations of around 19.84-26.28 µg kg⁻¹, with intra-day RSD% between 2.32 and 9.73% and an inter-day RSD% of 5.75%, confirming satisfactory repeatability at the higher spiking level. ENR displayed mean values of 19.05-27.77 µg kg⁻¹, accompanied by intra-day RSD% of 2.63-5.10% and an inter-day RSD% of 3.59%, indicating particularly good precision and reproducibility. LEV showed mean recoveries of about 21.85-26.51 µg kg⁻¹, with intra-day RSD% in the range of 3.62-5.62% and an inter-day RSD% of 4.70%, again demonstrating reliable quantification in poultry feed. The acceptable intra- and inter-day RSD% values largely below 10% confirm that the method affords robust precision and acceptable variability for FQs determination in poultry feed, supporting its suitability for routine residue monitoring in complex matrix.

At 20 µg kg⁻¹ spiking level in poultry feed, the intra-day recoveries for CIP 100, 79 and 105% demonstrate acceptable accuracy, with one lower recovery likely caused by minor matrix suppression (Table 3.18). The inter-day recovery of 95% confirms stable reproducibility across

multiple analytical runs. ENR exhibited intra-day recoveries of 111, 76 and 97%, showing slight over- and under-recoveries, possibly due to heterogeneous feed composition, while the inter-day recovery of 95% indicates consistent method performance over time. LEV displayed intra-day recoveries of 106, 87 and 99%, showing good precision within acceptable analytical limits, with an inter-day recovery of 99% that supports robust day-to-day repeatability.

At 25 $\mu\text{g kg}^{-1}$ spiking level, CIP recoveries 105, 105 and 79% remained close to the target level overall, with an inter-day recovery of 93%, suggesting slightly higher variability at the upper concentration but still within acceptable recovery limits (Table 3.18). ENR yielded intra-day recoveries of 96, 76 and 102%, with an inter-day recovery of 96%, reflecting stable and reliable quantification with moderate matrix influence. LEV intra-day recoveries 111, 106, 87 and 102% indicate high analytical accuracy with slight signal enhancement, while the inter-day recovery of 98% demonstrates consistent performance across days.

Both intra- and inter-day recoveries for CIP, ENR and LEV fall within the acceptable ranges, confirming that the LC-MS/MS method provides accurate, reproducible, and reliable quantification of fluoroquinolone residues in poultry feed matrices, even under complex matrix conditions.

Table 3.18: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of FQs in Poultry Feed Samples

Compound Name	Spiking Level (μg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CIP	20	20.1908 ± 0.3864	1.91	3.66	100	95
		15.8858 ± 1.0362	6.52		79	
		21.0515 ± 0.5411	2.57		105	
ENR		22.2903 ± 1.1276	5.05	4.06	111	95
		15.2849 ± 0.7742	5.06		76	
		19.5458 ± 0.4031	2.06		97	
LEV		21.2062 ± 0.9706	4.57	6.87	106	99
		17.4921 ± 1.9292	11.02		87	
		21.1852 ± 1.0638	5.02		105	
CIP	25	26.2791 ± 0.6098	2.32	5.75	105	93
		19.8412 ± 1.9313	9.73		79	
		24.0680 ± 1.2540	5.21		96	

ENR		19.0466 ± 0.5023	2.63	3.59	76	96
		25.5593 ± 1.3055	5.10		102	
		27.7737 ± 0.8455	3.04		111	
LEV		26.5064 ± 1.4899	5.62	4.70	106	98
		21.8465 ± 1.0666	4.88		87	
		25.5662 ± 0.9265	3.62		102	

3.2.8 Matrix effect of FQs

The matrix-induced ionization effects of FQs varied clearly among the different food and feed matrices, reflecting matrix-dependent interactions with the analytes (Table 3.19). CIP showed relatively mild ion enhancement in poultry meat (7.71%) and beef (2.50%), but stronger effects in egg (12.18%), milk (13.32%) and poultry feed (14.52%), indicating that these matrices contain co-extractives that increase its signal intensity. ENR was more influenced by matrix composition, with enhancement in poultry meat (7.18%), beef (5.40%), poultry feed (8.60%) and milk (17.70%), and a particularly high effect in egg (42.95%), suggesting substantial co-eluting components that strongly promote its ionization. LEV exhibited fairly consistent enhancement across all matrices, ranging from 7.72% in milk to 13.01% in egg, implying relatively stable ionization behavior with limited but noticeable matrix influence. Overall, these results demonstrate that matrix effects for FQs are both analyte- and matrix-specific, supporting the need for matrix-matched calibration to achieve accurate quantification in complex samples.

Table 3.19: Matrix-Induced Ionization Effects of FQs in Different Matrices

Matrix	Matrix effects (%)		
	CIP	ENR	LEV
Poultry meat	7.71	7.18	10.09
Beef	2.50	5.40	9.23
Egg	12.18	42.95	13.01
Milk	13.32	17.70	7.72
Poultry feed	14.52	8.60	10.39

3.2.9 FQs Analysis in Real Sample

All collected samples (150 in total; 30 from each matrix) were analyzed for the presence of FQs residues (Fig. 3.25-3.42). Sample extraction and clean-up were carried out using the validated LC-MS/MS method to ensure reliable recovery and quantification in each matrix. The results revealed (Table 3.20) that only one sample was tested positive for the targeted compound at below MRL

value, LEV was detected in a single milk sample at a concentration of $12 \mu\text{g kg}^{-1}$ (Fig. 3.43). No LEV residues were found in any of the remaining milk, meat, egg or feed samples, and no other FQs were detected. Overall, these findings indicate a very low occurrence of LEV contamination in the tested materials.

Table 3.20: Amount of Targeted FQs Compound

Antibiotic name	Matrix	No of positive samples	Amount ($\mu\text{g kg}^{-1}$)	Sample detection frequency (%)
LEV	Milk	1	12	3

3.2.10 Chromatograms of FQs

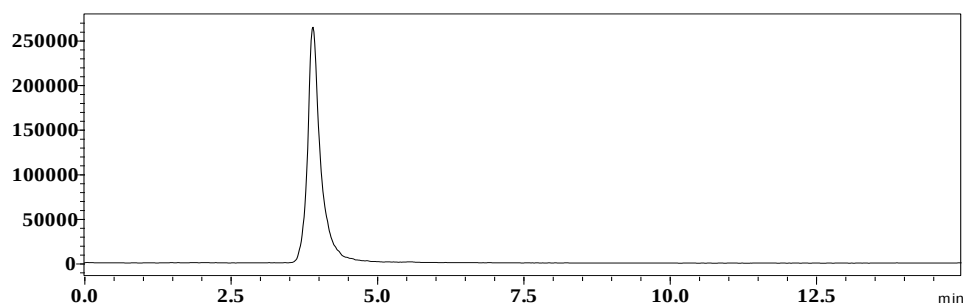


Fig. 3.25: Ciprofloxacin (CIP) standard

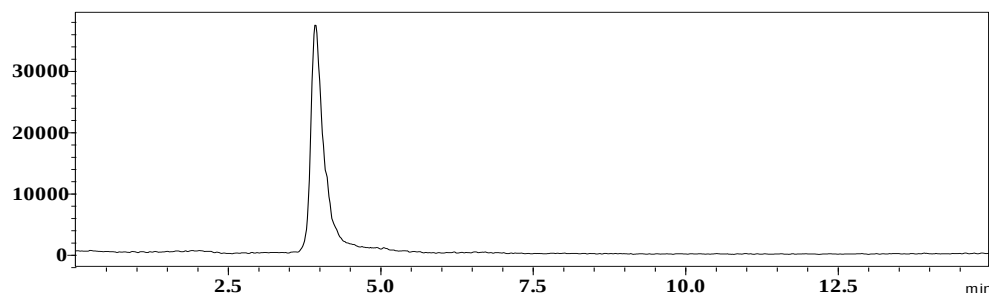


Fig. 3.26: Enrofloxacin (ENR) standard

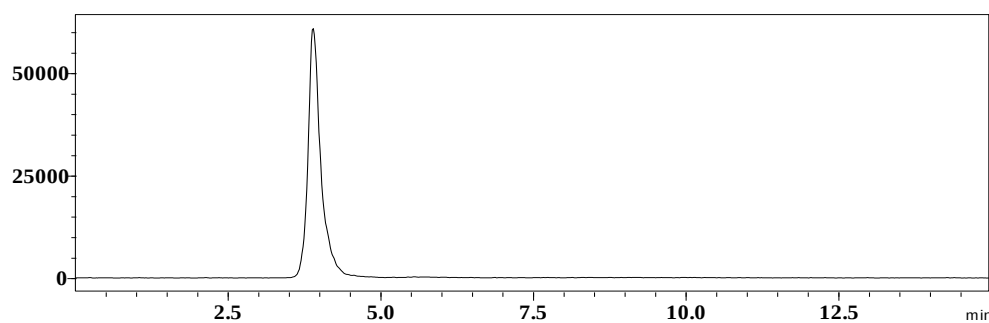


Fig. 3.27: Levofloxacin (LEV) standard

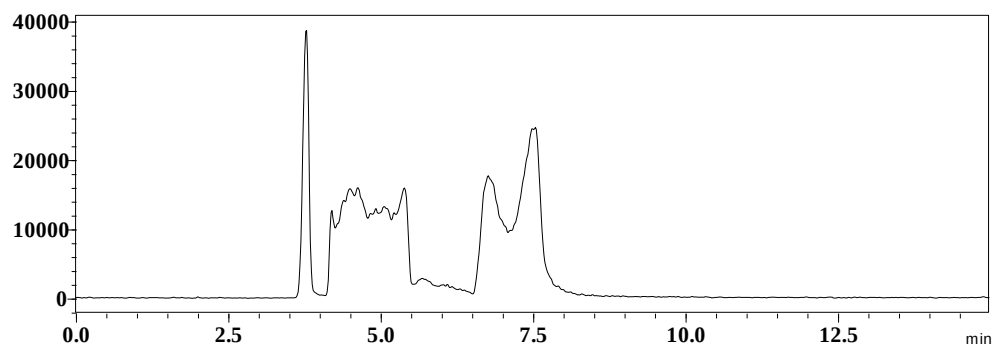


Fig. 3.28: Matrix-matched of Ciprofloxacin (CIP) in poultry meat

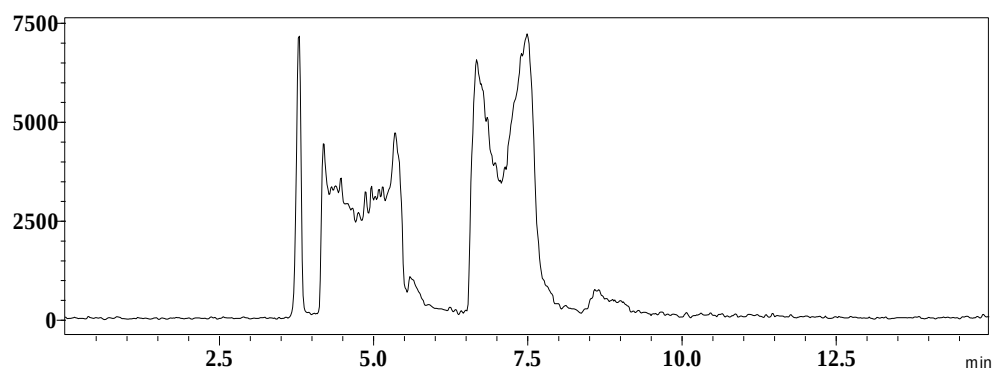


Fig. 3.29: Matrix-matched of Enrofloxacin (ENR) in poultry meat

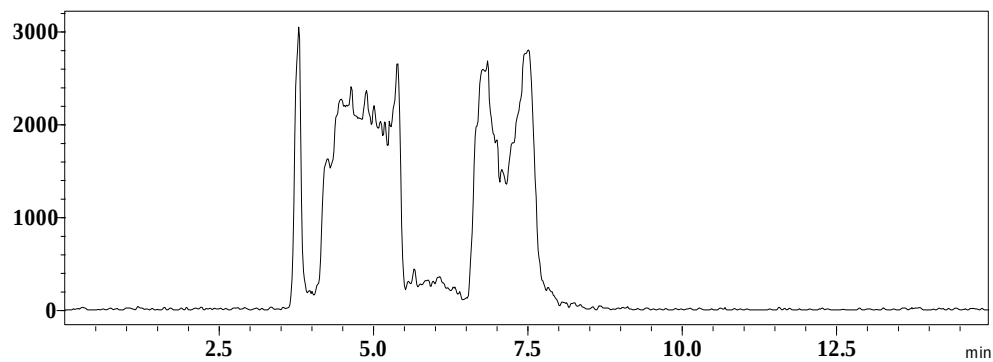


Fig. 3.30: Matrix-matched of Levofloxacin (LEV) in poultry meat

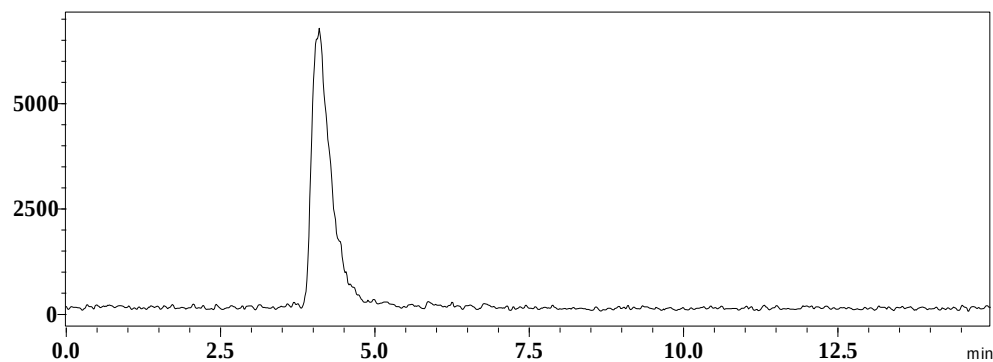


Fig. 3.31: Matrix-matched of Ciprofloxacin (CIP) in beef

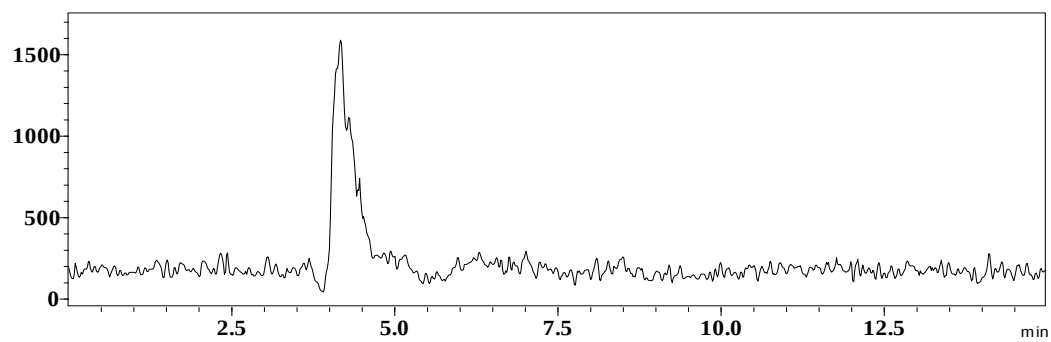


Fig. 3.32: Matrix-matched of Enrofloxacin (ENR) in beef

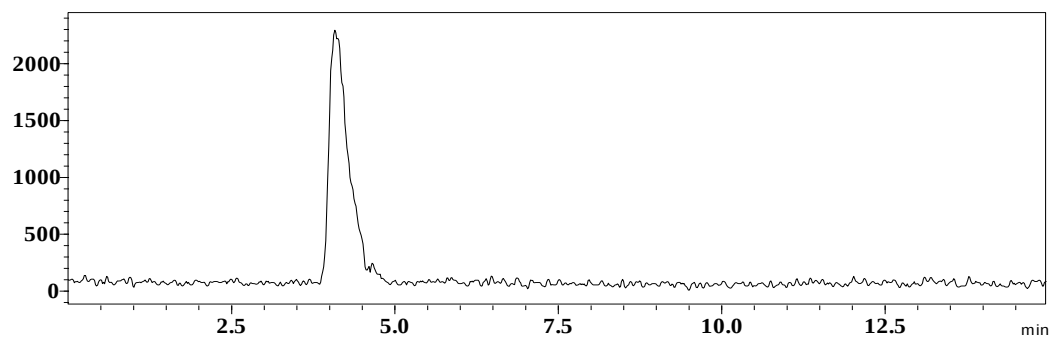


Fig. 3.33: Matrix-matched of Levofloxacin (LEV) in beef

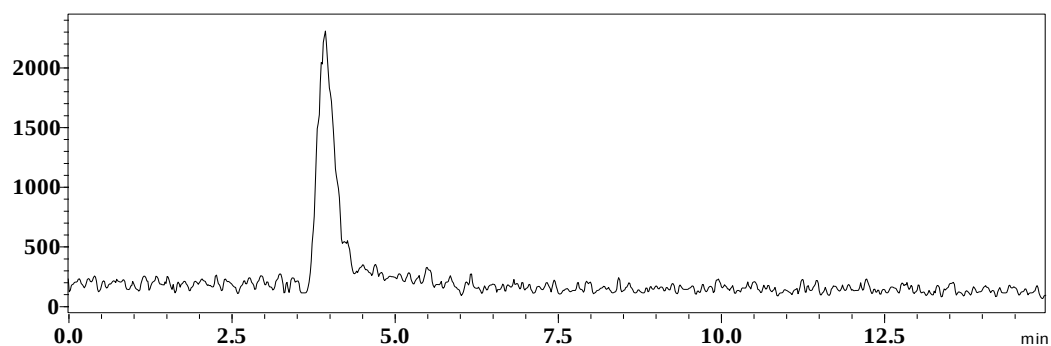


Fig. 3.34: Matrix-matched of Ciprofloxacin (CIP) in egg

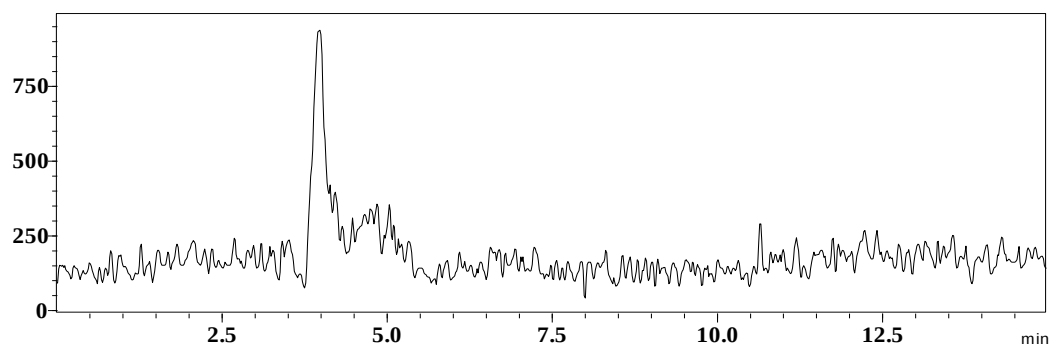


Fig. 3.35: Matrix-matched of Enrofloxacin (ENR) in egg

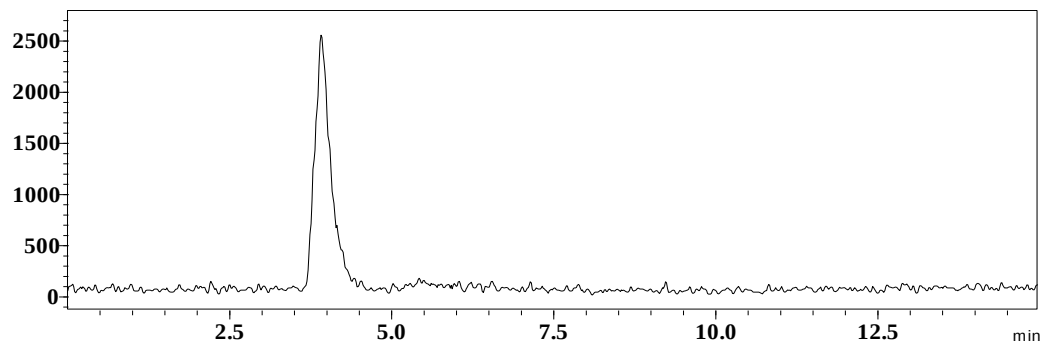


Fig. 3.36: Matrix-matched of Levofloxacin (LEV) in egg

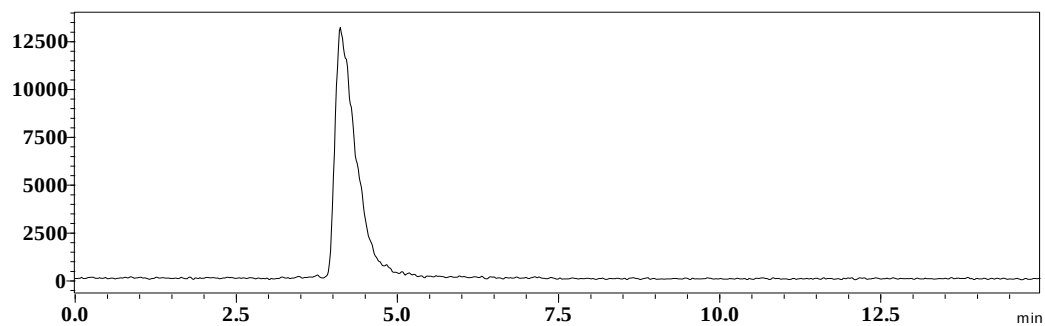


Fig. 3.37: Matrix-matched of Ciprofloxacin (CIP) in milk

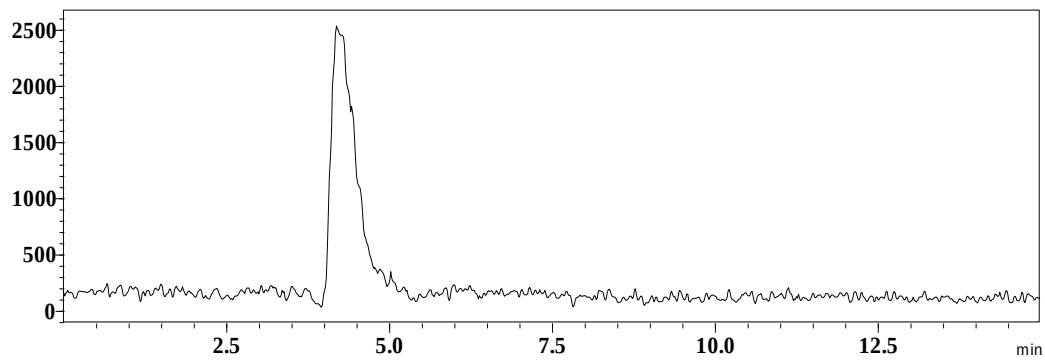


Fig. 3.38: Matrix-matched of Enrofloxacin (ENR) in milk

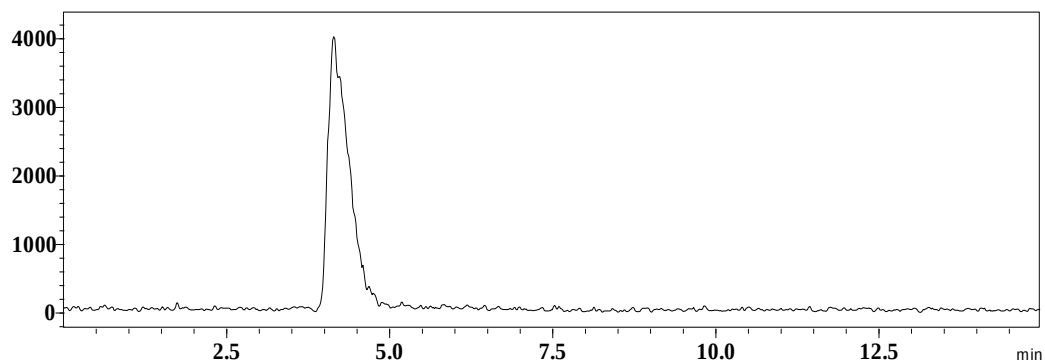


Fig. 3.39: Matrix-matched of Levofloxacin (LEV) in milk

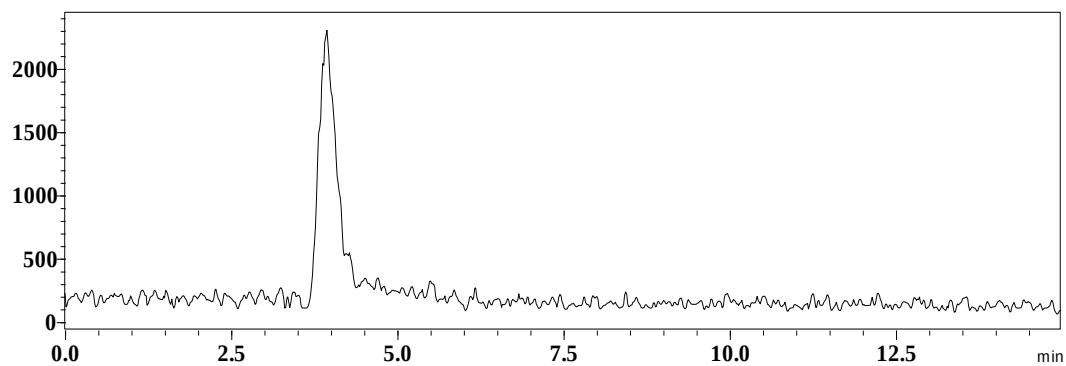


Fig. 3.40: Matrix-matched of Ciprofloxacin (CIP) in feed

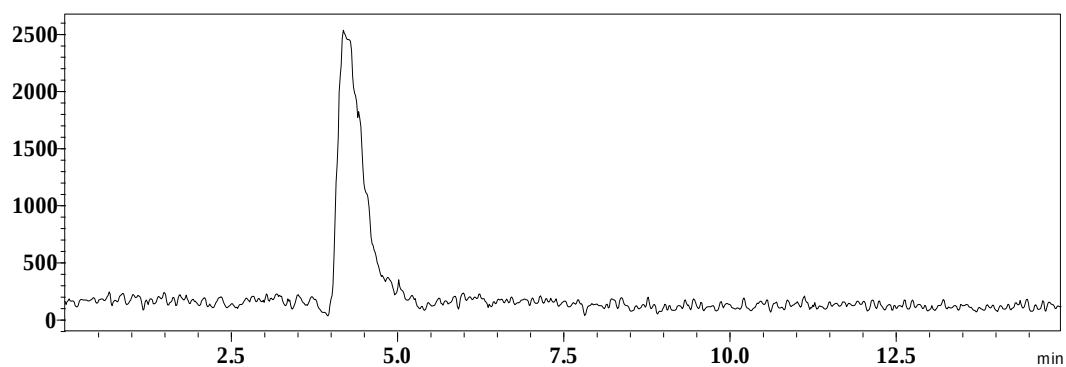


Fig. 3.41: Matrix-matched of Enrofloxacin (ENR) in feed

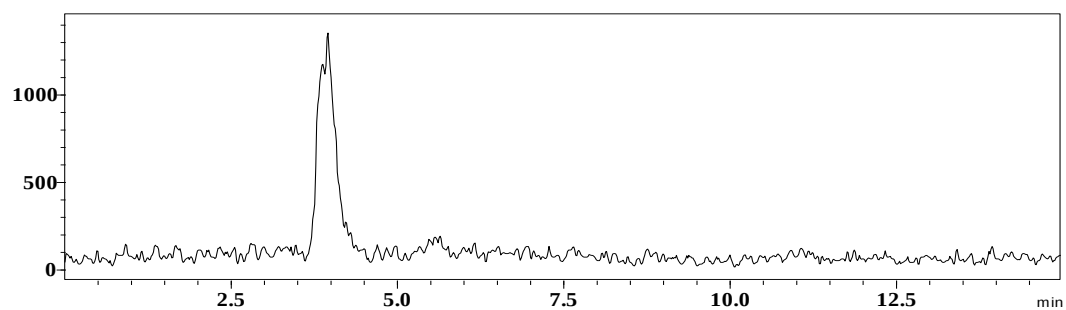


Fig. 3.42: Matrix-matched of Levofloxacin (LEV) in feed

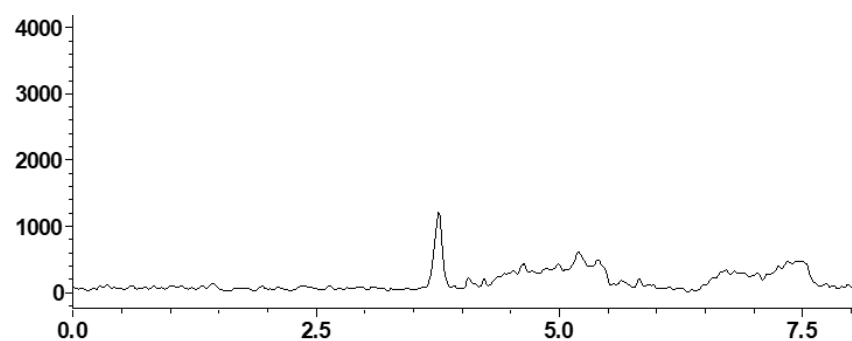


Fig. 3.43: Targeted LEV in one milk sample

3.3 Method Optimization and Validation of Tetracyclines (TCs)

3.3.1 Optimization of Tetracyclines (TCs)

The method optimization for tetracyclines (TCs) on the MS system was performed for tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) using ESI (+) mode at low concentrations in complex food and feed matrices. C18 reverse-phase column (Shim-Pack GIST C18, particle size 5 μm , 250 $\times$ 4.6 mm) was employed for TCs separation using a LC-MS/MS. The mobile phase consisted of water containing 0.1% HCOOH and CH₃OH in a different (v/v) ratio, and separation was achieved using a gradient LC program. Mass spectrometric transitions were optimized using a combination of preliminary SCAN experiments and Multiple Reaction Monitoring (MRM) analysis.

3.3.2 Screening of TCs Compounds

The optimized molecular ions at m/z 444, 460, and 478 for TC, OTC and CTC were scanned to identify their corresponding precursor ions at m/z 445, 461, and 479 (Fig. 3.44). For this purpose, the analytes were introduced into the LC system using TC standards, passed through a C18 column. A 250 $\mu\text{g L}^{-1}$ concentrated standard was used for screening using the SCAN method.

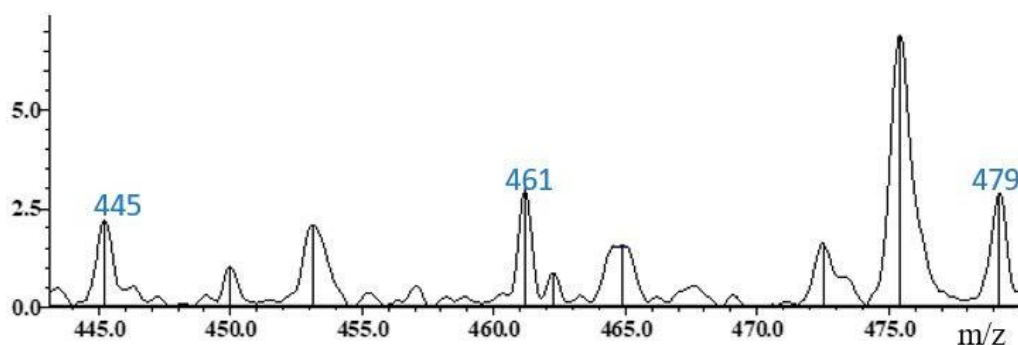


Fig. 3.44: TCs (TC, OTC CTC) screening

3.3.3 MRM of TCs Compounds

For MRM purpose, the analytes were introduced into the LC system using TC standards, passed through a C18 column, and then transferred via the MS interface to the MS/MS fragmentor. In MRM mode, these precursor ions were further fragmented to the product ions m/z 410, 426 and 444 for each TC, OTC and CTC, which were used for quantification (Fig. 3.45-3.47).

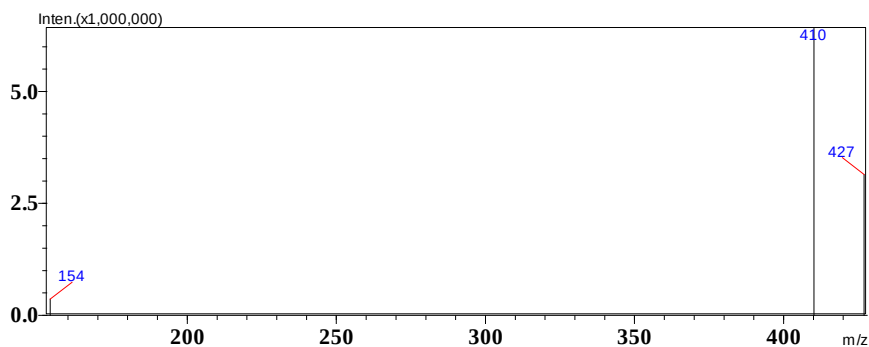


Fig. 3.45: Product ions of TC

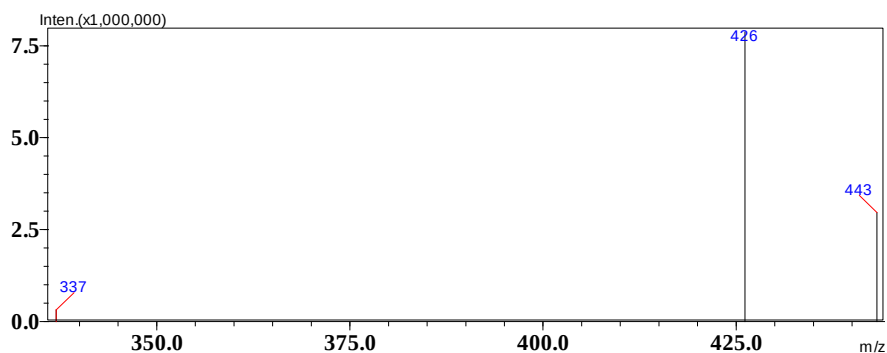


Fig. 3.46: Product ions of OTC

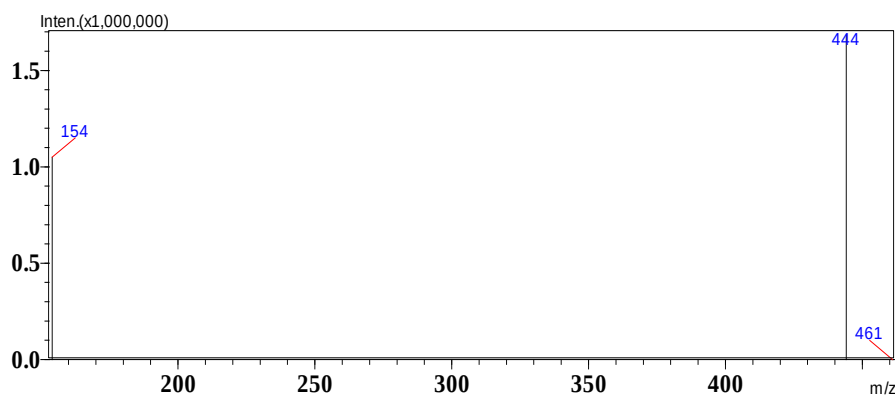


Fig. 3.47: Product ions of CTC

3.3.4 Standard Calibration Curves of TCs

All of TC standards were initially prepared at a concentration of $1000 \mu\text{g L}^{-1}$. An intermediate standard of $250 \mu\text{g L}^{-1}$ was then prepared from the stock solution, which was subsequently used to prepare a series of working standards. These working standards, covering a concentration range of $1.5\text{--}100 \mu\text{g L}^{-1}$, were employed to construct calibration curves, ensuring accurate quantification of the analytes in the subsequent analyses (Fig. 3.48).

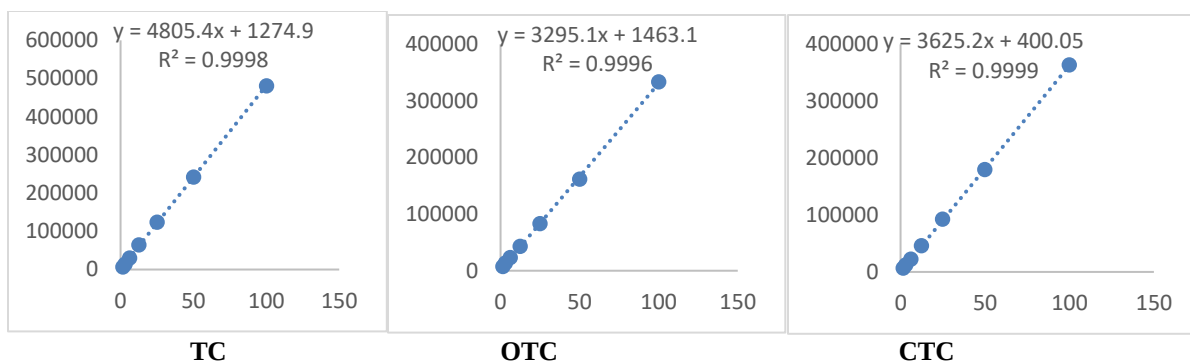


Fig. 3.48: Standard calibration curves of TC, OTC and CTC

3.3.5 Matrix-matched Calibration Curves of TCs in Poultry meat, Beef, Egg, Milk and Poultry feed Samples

Poultry meat, beef, egg, milk, and poultry feed samples were spiked with a mixture of TC standards. After extraction, the matrix-matched samples were analyzed across a concentration range of $1.5\text{--}100\ \mu\text{g L}^{-1}$ (Fig. 3.49-3.53) to construct matrix-matched calibration curves.

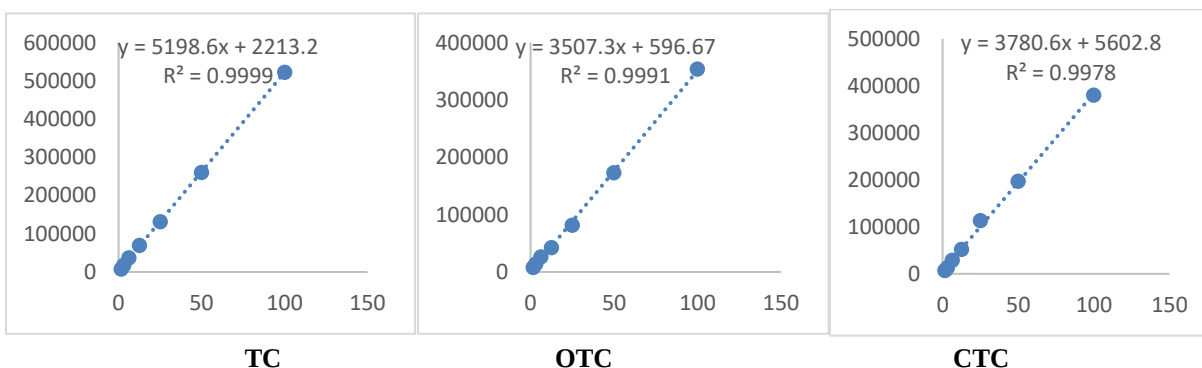


Fig. 3.49: Matrix matched calibration curves of TC, OTC and CTC in poultry meat samples

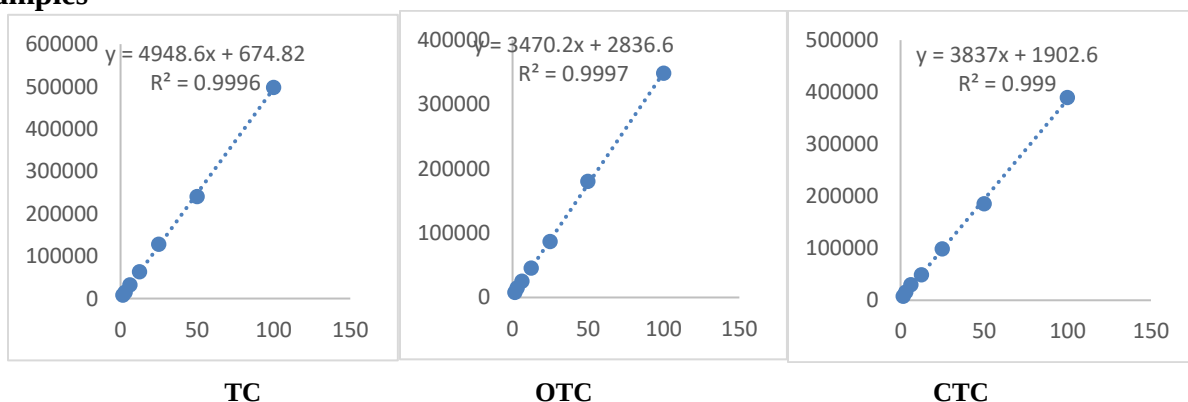


Fig. 3.50: Matrix matched calibration curves of TC, OTC and CTC in beef samples

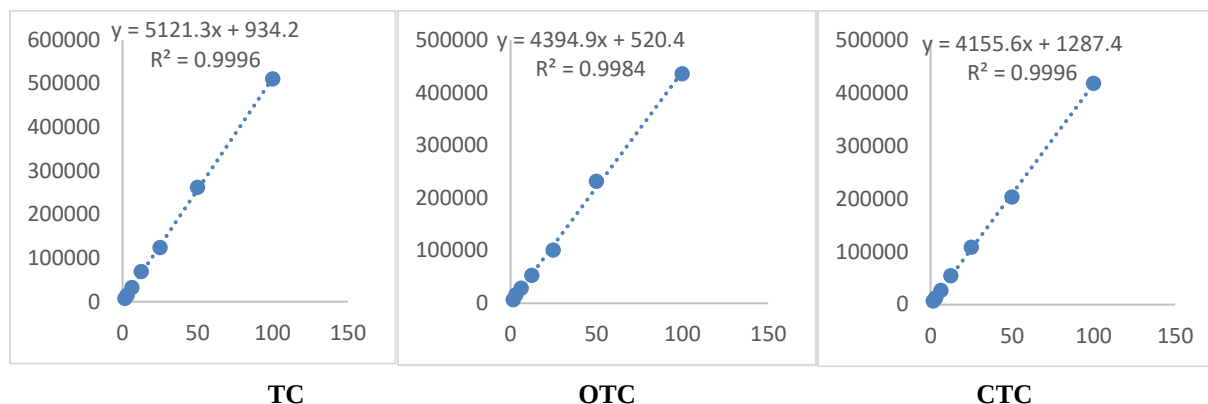


Fig. 3.51: Matrix matched calibration curves of TC, OTC and CTC in egg samples

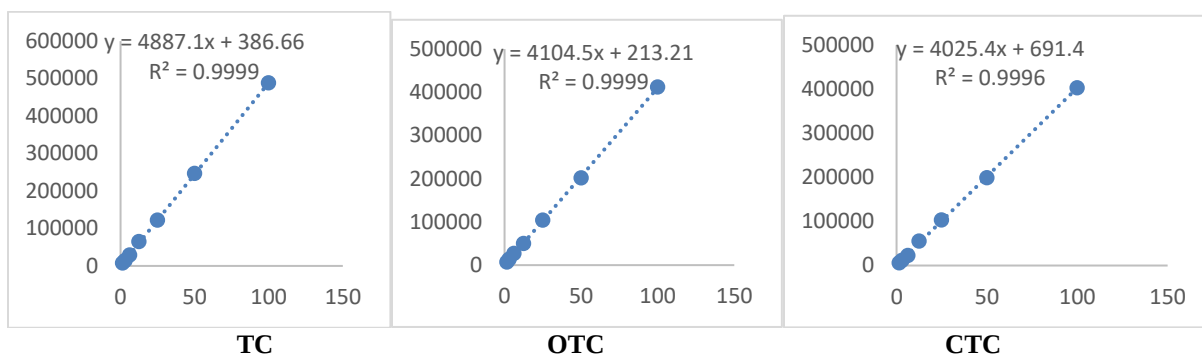


Fig. 3.52: Matrix matched calibration curves of TC, OTC and CTC in milk samples

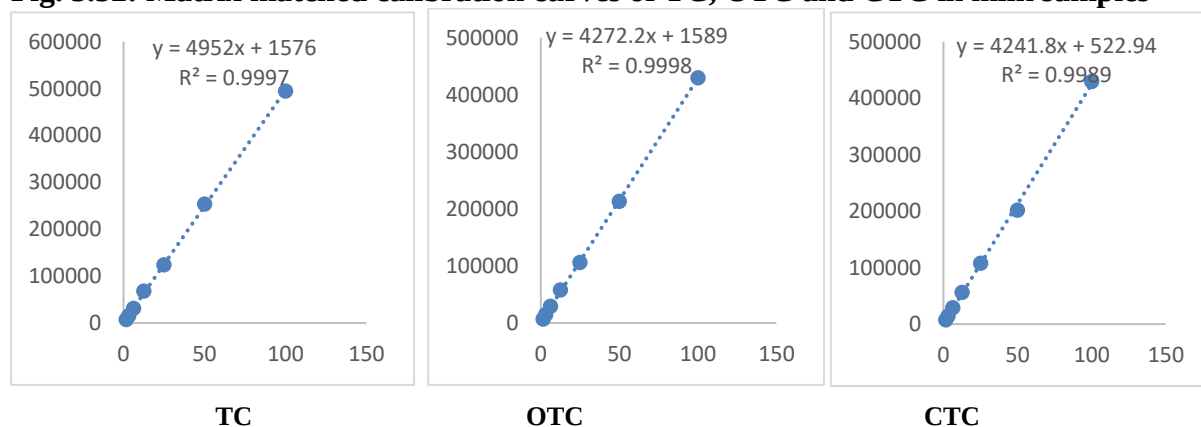


Fig. 3.53: Matrix matched calibration curves of TC, OTC and CTC in poultry feed samples

3.3.6 Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ) & Retention time (t_R) of TCs

The analytical validation performance of TCs across various food and feed matrices demonstrated excellent linearity, high sensitivity, and stable separation performance (Table 3.21-3.23). Matrix-matched calibration for TC yielded excellent linearity, with correlation coefficients (R^2) between

0.9998 and 0.9999, demonstrating a very strong linear relationship between peak area and TC concentration and confirming the robustness of the quantitative model. In beef, a validated linear working range of 1.5-100 $\mu\text{g kg}^{-1}$ was established, and the remaining matrices poultry meat, egg, milk and poultry feed followed the same calibration trend with similarly strong regression fits. The LC-MS/MS method also showed high sensitivity, with LOD values from 0.03-0.27 $\mu\text{g kg}^{-1}$ and LOQ values between 0.17 and 0.99 $\mu\text{g kg}^{-1}$, enabling reliable detection of low-level TC residues in all tested matrices. Retention times were highly reproducible, ranging from 4.6-4.8 min and closely matching the standard TC t_R of 4.7 min, which indicates stable chromatographic behavior and negligible matrix-induced shifts. Matrix-matched calibration curves for OTC exhibited excellent linearity in all matrices, with R^2 ranging from 0.9984 to 0.9999, confirming a highly reliable proportional relationship between OTC concentration and detector response. Beef showed a validated linear range of 1.25-100 $\mu\text{g kg}^{-1}$, and the remaining matrices followed the same linear behavior, indicating consistent quantitative performance across poultry meat, egg, milk and poultry feed. The method was highly sensitive, with LOD values between 0.1 and 0.6 $\mu\text{g kg}^{-1}$ and LOQ values between 0.3 and 1.8 $\mu\text{g kg}^{-1}$, allowing detection of trace OTC residues in all tested matrices. Retention times were very consistent, spanning 4.7-4.9 min and closely matching the standard OTC t_R of 4.8 min, demonstrating robust separation stability with minimal matrix-induced shifts. The comparable slopes of the matrix-matched calibration equations further indicate uniform instrument response among matrices, supporting the accuracy and reliability of OTC quantification by the LC-MS/MS method. Matrix-matched calibration curves for CTC showed excellent linearity in all matrices, with R^2 ranging from 0.9978 to 0.9999, confirming a precise and stable proportional relationship between CTC concentration and detector response. Beef displayed a validated linear range of 1.25-100 $\mu\text{g kg}^{-1}$, and the calibration equations, such as $y = 3780.6x + 5602.8$ for poultry meat and $y = 3837x + 1902.6$ for beef, indicate consistent instrument sensitivity and dependable quantitative performance across matrices. The method also exhibited high analytical sensitivity, with LOD values between 0.1 and 0.6 $\mu\text{g kg}^{-1}$ and LOQ values from 0.3-1.8 $\mu\text{g kg}^{-1}$, enabling accurate detection of trace CTC residues in complex samples including egg, milk and poultry feed. Retention times were highly reproducible, spanning 6.2-6.3 min and closely matching the standard CTC t_R of 6.3 min, which demonstrates stable separation performance with minimal matrix-induced variation. Collectively, the outstanding linearity, low detection/quantification limits, and consistent t_R profiles confirm that the developed LC-MS/MS

method is accurate, sensitive, and reliable for TCs determination in a variety of food and feed samples.

Table 3.21: TC Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std TC	4.7	1.5-100	-	-	$y = 4805.4x + 1274.9$	0.9998
Poultry meat	4.7		0.27	0.83	$y = 5198.6x + 2213.2$	0.9999
Beef	4.7		0.06	0.17	$y = 4948.6x + 674.82$	0.9996
Egg	4.8		0.03	0.99	$y = 5121.3x + 934.2$	0.9996
Milk	4.7		0.15	0.48	$y = 4887.1x + 386.66$	0.9999
Poultry feed	4.6		0.2	0.66	$y = 4952x + 1576$	0.9997

Table3.22: OTC Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std OTC	4.8	1.25-100	-	-	$y = 3295.1x + 1463.1$	0.9996
Poultry meat	4.8		0.1	0.3	$y = 3507.3x + 596.67$	0.9991
Beef	4.8		0.1	0.3	$y = 3470.2x + 2836.6$	0.9997
Egg	4.9		0.6	1.8	$y = 4394.9x + 520.4$	0.9984
Milk	4.8		0.2	0.6	$y = 4104.5x + 213.21$	0.9999
Poultry feed	4.7		0.1	0.3	$y = 4272.2x + 1589$	0.9998

Table3.23: CTC Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std CTC	6.3	1.25-100	-	-	$y = 3625.2x + 400.05$	0.9999
Poultry meat	6.3		0.1	0.3	$y = 3780.6x + 5602.8$	0.9978
Beef	6.3		0.1	0.3	$y = 3837x + 1902.6$	0.9990
Egg	6.3		0.6	1.8	$y = 4155.6x + 1287.4$	0.9996
Milk	6.2		0.2	0.6	$y = 4025.4x + 691.4$	0.9996
Poultry feed	6.2		0.1	0.3	$y = 4241.8x + 522.94$	0.9989

3.3.7 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of TCs in Samples

The mean concentrations (average $\pm$ SD) of TC, OTC, and CTC residues provide a clear

quantitative measure of analyte levels across matrices, enabling direct comparison of contamination patterns. The associated standard deviations indicate the variability of replicate measurements and reflect both analytical precision and matrix influence. Intra-day and inter-day RSD% values further assess method repeatability and reproducibility, ensuring consistent performance across runs and days. When these parameters remain within accepted limits, they confirm the robustness and reliability of the method for multi-matrix residue analysis, while minimizing analytical uncertainty in complex food systems. The recovery results for the tetracyclines TC, OTC, and CTC showed satisfactory accuracy, confirming that the analytical procedure consistently and reliably quantified these antibiotics across all evaluated matrices.

3.3.7.1 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of TCs in Poultry Meat Samples

At 10 $\mu\text{g kg}^{-1}$ spiking level in poultry meat, tetracycline (TC) gave mean concentrations of 9.99-10.68 $\mu\text{g kg}^{-1}$ with intra-day RSD% values of 3.33-5.31% and an inter-day RSD% of 4.00%, demonstrating good precision and stable day-to-day reproducibility (Table 3.24). Oxytetracyclines (OTC) showed slightly higher dispersion, with mean concentrations of 9.68-10.47 $\mu\text{g kg}^{-1}$, intra-day RSD% of 3.33-5.59%, and an inter-day RSD% of 4.63%, indicating generally acceptable precision with minor matrix-related variation. Chlortetracycline (CTC) exhibited higher recoveries, with mean values of 11.31-12.47 $\mu\text{g kg}^{-1}$, intra-day RSD% of 3.09-6.83%, and an inter-day RSD% of 5.18%, reflecting moderate variability but still within acceptable analytical limits. At 15 $\mu\text{g kg}^{-1}$ level, TC produced mean concentrations of 14.74-15.94 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 3.64-6.73% and an inter-day RSD% of 5.04%, confirming good repeatability and reproducibility at the higher fortification level (Table 3.24). OTC showed mean values of 14.48-15.97 $\mu\text{g kg}^{-1}$, intra-day RSD% of 5.72-11.15%, and an inter-day RSD% of 7.87%, indicating acceptable precision but with somewhat greater variability in this matrix. CTC yielded mean concentrations of 14.38-15.92 $\mu\text{g kg}^{-1}$, intra-day RSD% of 7.00-11.82%, and an inter-day RSD% of 8.79%, demonstrating reliable quantification despite slightly higher dispersion.

Overall, mean concentrations for TC, OTC and CTC closely matched the fortified levels at both 10 and 15 $\mu\text{g L}^{-1}$, and intra- and inter-day RSD% values were generally below or around 10%,

indicating satisfactory repeatability and reproducibility and these results confirm that the method provides reliable quantification of TCs residues in poultry meat monitoring.

The recovery results for TCs in poultry feed indicate high analytical accuracy and precision at both 10 and 15 $\mu\text{g kg}^{-1}$ spiking levels. At 10 $\mu\text{g kg}^{-1}$, TC showed intra-day recoveries of 99-106% with an inter-day recovery of 103%, demonstrating excellent repeatability and reproducibility (Table 3.24). OTC produced intra-day recoveries of 96-106% and an inter-day value of 100%, indicating stable performance with only slight matrix-related variation. CTC gave intra-day recoveries of 95-106% and an inter-day recovery of 100%, suggesting minor signal enhancement but still well within accepted validation criteria. At 15 $\mu\text{g kg}^{-1}$ level, TC again exhibited robust behavior, with intra-day recoveries of 98-106% and an inter-day recovery of 101%, confirming consistent accuracy at the higher concentration (Table 3.24). OTC displayed intra-day recoveries of 96-106% and an inter-day value of 101%, while CTC showed intra-day recoveries of 95-106% and an inter-day recovery of 100%, all indicating reliable quantification across different days.

Overall, the intra- and inter-day recovery data for TCs remain tightly clustered around 100%, confirming that the LC-MS/MS method provides accurate and precise determination of tetracyclines in poultry feed, with minimal concentration-dependent or matrix-induced bias.

Table 3.24: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter- day Recovery (%) of TCs in Poultry meat Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
TC	10	10.6778 ± 0.3563	3.33	4.00	106	103
		9.9863 ± 0.3374	3.37		99	
		10.3912 ± 0.5521	5.31		103	
OTC		10.4663 ± 0.5192	4.96	4.63	106	100
		9.6803 ± 0.3224	3.33		96	
		9.7330 ± 0.5449	5.59		97	
CTC		11.3110 ± 0.3497	3.09	5.18	95	100
		12.4729 ± 0.7012	5.62		106	
		11.7950 ± 0.8064	6.83		99	
TC		14.7381 ± 0.9924	6.73	5.04	98	101

	15	15.9366 ± 0.5810	3.64		106	
		14.9189 ± 0.7089	4.75		99	
OTC		14.4788 ± 1.6154	11.15	7.87	96	101
		15.3939 ± 0.8814	5.72		102	
		15.9738 ± 1.0748	6.72		106	
CTC	15	14.3788 ± 1.6997	11.82	8.79	95	100
		15.9239 ± 1.2053	7.56		106	
		14.7464 ± 1.0334	7.00		98	

3.3.7.2 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of TCs in Beef Samples

At 10 $\mu\text{g kg}^{-1}$ spiking level in beef, TC gave mean concentrations of 9.53-11.30 $\mu\text{g kg}^{-1}$, with intra-day RSD% values ranging from 1.65 to 5.30% and an inter-day RSD% of 4.00%, indicating good repeatability performance (Table 3.25). OTC showed mean recoveries of 9.96-10.83 $\mu\text{g kg}^{-1}$, with intra-day RSD% between 3.42 and 13.98% and an inter-day RSD% of 6.29%, reflecting generally acceptable precision with some increased variability in one replicate. CTC produced mean concentrations of 9.83-10.59 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 3.59-8.82% and an inter-day RSD% of 6.94%, demonstrating moderate but acceptable variability across analytical days.

At 15 $\mu\text{g kg}^{-1}$ level, TC exhibited mean concentrations of 13.86-16.35 $\mu\text{g kg}^{-1}$, with intra-day RSD% values of 4.25-5.13% and an inter-day RSD% of 4.71%, confirming good precision and reproducibility at the higher spiking level (Table 3.25). OTC showed mean values of 14.88-16.22 $\mu\text{g kg}^{-1}$, with higher intra-day RSD% of 9.72-12.43% and an inter-day RSD% of 10.90%, indicating greater matrix-related variability but still within typical validation limits. CTC yielded mean concentrations of 14.82-15.76 $\mu\text{g kg}^{-1}$, with intra-day RSD% between 3.28 and 5.76% and an inter-day RSD% of 4.35%, reflecting strong repeatability and reproducibility. TCs all showed mean recoveries close to their respective spiking levels at both 10 and 15 $\mu\text{g kg}^{-1}$, confirming good analytical accuracy. Intra-day RSD% values were mostly below 10%, and inter-day RSD% were generally below or around this threshold, indicating acceptable repeatability and reproducibility. These findings demonstrate that the LC-MS/MS method provides reliable quantification of TCs residues in beef.

At 10 $\mu\text{g kg}^{-1}$ spiking level in beef, TC showed intra-day recoveries ranging from 92-109%, with an inter-day recovery of 100%, indicating accurate quantification with slight positive bias and good day-to-day reproducibility (Table 3.25). OTC exhibited intra-day recoveries of 99, 99 and 108% and an inter-day value of 108%, suggesting minor signal enhancement but still well within acceptable validation limits. CTC gave intra-day recoveries of 98-105% with an inter-day recovery of 100%, reflecting stable performance across replicate runs.

At 15 $\mu\text{g kg}^{-1}$ level, TC again demonstrated robust behavior, with intra-day recoveries of 92-109% and an inter-day recovery of 100%, confirming consistent accuracy at the higher concentration (Table 3.25). OTC produced intra-day recoveries of 99, 100 and 108% and an inter-day recovery of 102%, indicating reliable precision and only modest matrix effects. CTC showed intra-day recoveries between 98 and 105% with an inter-day recovery of 100%, further supporting strong reproducibility. Collectively, these recoveries highlight for TCs at both spiking levels clustered closely around 100%, confirming high analytical accuracy and precision of the LC-MS/MS method for TCs residue determination in beef samples across both concentration levels, with no significant concentration-dependent or matrix-induced performance issues observed and supporting its suitability for routine monitoring.

Table 3.25: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of TCs in Beef Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
TC	10	11.3016 ± 0.5997	5.30	4.00	109	100
		9.5312 ± 0.4823	5.06		92	
		10.5093 ± 0.1734	1.65		101	
OTC		9.9980 ± 0.3421	3.42	6.29	99	108
		9.9553 ± 1.3919	13.98		99	
		10.8281 ± 0.3664	3.38		108	
CTC		10.5879 ± 0.8967	8.46	6.94	105	100
		9.8345 ± 0.3536	3.59		98	
		9.8682 ± 0.8711	8.82		98	
TC	15	15.1582 ± 0.6453	4.25	4.71	101	100
		16.3517 ± 0.8401	5.13		109	
		13.8591 ± 0.6598	4.76		92	
OTC		14.8828 ± 1.4476	9.72	10.90	99	102

CTC		15.0563 ± 1.8718	12.43	4.35	100	100
		16.2213 ± 1.7136	10.56		108	
		14.8171 ± 0.8543	5.76		98	
		15.7592 ± 0.6314	4.00		105	
		14.8187 ± 0.4861	3.28		98	

3.3.7.3 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of TCs in Egg Samples

At 5 $\mu\text{g kg}^{-1}$ spiking level in egg samples, TC gave mean concentrations of approximately 4.01-5.08 $\mu\text{g kg}^{-1}$ with intra-day RSD% values of 4.48-14.77% and an inter-day RSD% of 9.46%, indicating acceptable precision, although one replicate showed higher variability typical of low-level analysis in a complex matrix (Table 3.26). OTC showed mean recoveries of 4.90-5.21 $\mu\text{g kg}^{-1}$, with tighter intra-day RSD% of 4.50-9.81% and an inter-day RSD% of 7.22%, reflecting generally stable analytical performance. CTC produced mean concentrations of 4.69-5.40 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 8.27-10.94% and an inter-day RSD% of 9.70%, demonstrating moderate but still acceptable precision at the lower spiking level.

At 10 $\mu\text{g L}^{-1}$ spiking level, TC yielded mean concentrations ranging from 7.83-10.13 $\mu\text{g kg}^{-1}$, with intra-day RSD% values of 6.32-12.03% and an inter-day RSD% of 9.26%, suggesting somewhat higher variability but remaining within common validation limits (Table 3.26). OTC showed mean values of 9.44-10.41 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 4.57-6.20% and an inter-day RSD% of 5.48%, indicating good repeatability and reproducibility. CTC gave mean concentrations of 9.75-10.74 $\mu\text{g kg}^{-1}$, with intra-day RSD% values between 4.44 and 8.98% and an inter-day RSD of 6.21%, supporting consistent quantification at the higher level.

Across both spiking levels, TC, OTC and CTC provided mean recoveries close to the nominal concentrations, confirming satisfactory accuracy in the egg matrix. Intra-day RSD% values were mostly at or below 10% which indicating acceptable repeatability and reproducibility. These results demonstrate that the LC-MS/MS method affords reliable quantification of TCs residues in egg samples monitoring.

At 5 $\mu\text{g kg}^{-1}$ spiking level in egg samples (Table 3.26), TC showed intra-day recoveries of 80, 101 and 83%, indicating moderate variability and slight under-recovery that can be attributed to matrix-

induced ion suppression in the complex egg matrix. The inter-day recovery of 88% confirms acceptable reproducibility, though with reduced accuracy compared with other matrices. OTC performed better at this level, with intra-day recoveries of 94, 97 and 104% and an inter-day value of 97%, suggesting minimal suppression. CTC also showed reliable performance, with intra-day recoveries of 97, 107 and 93% and an inter-day recovery of 99%, reflecting slight ion enhancement but still remaining within standard validation limits. At 10 $\mu\text{g kg}^{-1}$ level, TC produced intra-day recoveries of 80, 101 and 78% and an inter-day recovery of 86%, again indicating acceptable accuracy but suggesting persistent ion suppression or incomplete extraction at the higher concentration (Table 3.26).

In contrast, OTC exhibited intra-day recoveries of 98, 104 and 94% with an inter-day recovery of 98%, implying negligible suppression and excellent reproducibility. CTC maintained high accuracy, with intra-day recoveries of 97, 107 and 102% and an inter-day value of 102%, indicating that suppression effects are minimal for this compound.

Overall, the data showed that the LC-MS/MS method was reliable for quantification of TCs in egg, with OTC and CTC largely unaffected by ion suppression, while TC experiences moderate suppression yet still remains within acceptable recovery limits.

Table 3.26: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of TCs in Egg Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
TC	5	4.0135 ± 0.3663	9.12	9.46	80	88
		5.0773 ± 0.2275	4.48		101	
		4.1822 ± 0.6179	14.77		83	
OTC		4.8992 ± 0.4808	9.81	7.22	94	97
		5.2068 ± 0.2344	4.50		97	
		4.8993 ± 0.3609	7.36		104	
CTC		4.8708 ± 0.5333	10.94	9.70	97	99
		5.3976 ± 0.4465	8.27		107	
		4.6876 ± 0.4635	9.88		93	
TC	10	8.0502 ± 0.9691	12.03	9.26	80	86
		10.1278 ± 0.6409	6.32		101	
		7.8328 ± 0.7383	9.42		78	

OTC		9.7041 ± 0.5500	5.66	5.48	98	98
		10.4131 ± 0.4760	4.57		104	
		9.4353 ± 0.5851	6.20		94	
CTC		9.7478 ± 0.5074	5.20	6.21	97	102
		10.7430 ± 0.4775	4.44		107	
		10.2210 ± 0.9186	8.98		102	

3.3.7.4 Average, Standard Deviation (±SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Recovery Percentage (%) of TCs in Milk Samples

At 5 µg L⁻¹ spiking level in milk, TC gave mean concentrations of 4.80-5.46 µg L⁻¹, with intra-day RSD% values of 2.95-8.33% and an inter-day RSD% of 6.04%, indicating good precision and acceptable reproducibility in this complex matrix (Table 3.27). OTC showed mean recoveries of 4.85-5.16 µg L⁻¹, accompanied by intra-day RSD% of 7.90-10.05% and an inter-day RSD% of 9.01%, reflecting slightly higher variability but still within typical validation limits. CTC produced mean concentrations of 4.98-5.29 µg L⁻¹, with intra-day RSD% between 7.17 and 10.03% and an inter-day RSD% of 8.48%, indicating moderate yet acceptable precision at the lower fortification level.

At 10 µg L⁻¹ level, TC yielded mean concentrations of 9.66-10.83 µg L⁻¹, with intra-day RSD% of 2.41-8.13% and an inter-day RSD% of 5.59%, demonstrating improved repeatability and reproducibility at the higher concentration (Table 3.27). OTC showed mean values of 9.73-10.20 µg L⁻¹, with intra-day RSD% of 5.91-9.44% and an inter-day RSD% of 7.64%, indicating consistent quantification with manageable matrix influence. CTC gave mean concentrations of 9.06-10.60 µg L⁻¹, accompanied by intra-day RSD% of 4.27-9.71% and an inter-day RSD of 6.13%, confirming stable performance across analytical days. Overall, TCs showed recoveries close to their nominal spiking levels at both 5 and 10 µg L⁻¹, with intra- and inter-day RSD% values below 10%, confirming reproducible and reliable quantification of TCs residues in milk and supporting their suitability for routine residue monitoring in dairy matrices.

At 5 µg L⁻¹ spiking level in milk, TC showed intra-day recoveries of 96-108% with an inter-day recovery of 100%, indicating accurate quantification with only slight deviations around 100% (Table 3.27). Recoveries below 100% suggest minor ion suppression, whereas those slightly above

100% indicate mild ion enhancement, but overall matrix effects remain well controlled. OTC displayed intra-day recoveries of 97-103% and an inter-day recovery of 100%, and CTC gave 99-105% intra-day with 101% inter-day recovery, both pointing to minimal suppression/enhancement and very stable performance.

At 10 $\mu\text{g L}^{-1}$ level, TC produced intra-day recoveries of 96-108% with an inter-day value of 102%, again reflecting only small enhancement effects and negligible suppression (Table 3.27). OTC showed intra-day recoveries of 97-101% and an inter-day recovery of 99%, while CTC ranged from 90-105% intra-day with an inter-day recovery of 99%; the single lower value (90%) indicates localized ion suppression, but the overall pattern still lies within accepted validation limits. Overall, these recovery patterns for TC, OTC and CTC in milk demonstrate that matrix-induced ion suppression or enhancement is minimal, despite the complexity and high-protein content of the matrix, and that the LC-MS/MS method provides reliable, unbiased quantification of TC, OTC and CTC across both spiking levels.

Table 3.27: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of TCs in Milk Samples

Compound Name	Spiking Level ($\mu\text{g L}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
TC	5	4.8048 $\pm$ 0.4006	8.33	6.04	96	100
		4.8501 $\pm$ 0.3320	6.84		97	
		5.4597 $\pm$ 0.1612	2.95		108	
OTC		4.8529 $\pm$ 0.4412	9.09	9.01	97	100
		5.0750 $\pm$ 0.4011	7.90		101	
		5.1551 $\pm$ 0.5181	10.05		103	
CTC		4.9776 $\pm$ 0.4995	10.03	8.48	99	101
		4.9799 $\pm$ 0.4109	8.25		99	
		5.2937 $\pm$ 0.3800	7.17		105	
TC	10	9.6637 $\pm$ 0.6037	6.24	5.59	96	102
		10.1831 $\pm$ 0.8286	8.13		101	
		10.8270 $\pm$ 0.2613	2.41		108	
OTC		9.8111 $\pm$ 0.5804	5.91	7.64	98	99
		9.7288 $\pm$ 0.7383	7.58		97	
		10.1972 $\pm$ 0.9626	9.44		101	
CTC		10.5986 $\pm$ 0.4678	4.41	6.13	105	99
		10.1305 $\pm$ 0.9843	9.71		101	
		9.0601 $\pm$ 0.3875	4.27		90	

3.3.7.5 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Recovery Percentage (%) of TCs in Poultry Feed Samples

At 12.5 $\mu\text{g kg}^{-1}$ spiking level in poultry feed, TC gave mean concentrations between 11.84 and 12.75 $\mu\text{g kg}^{-1}$, with intra-day RSD% values of 5.27-9.12% and an inter-day RSD% of 7.15%, indicating acceptable precision and reproducibility in this matrix (Table 3.28). OTC showed mean recoveries of 10.77-13.05 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 3.28-5.93% and an inter-day RSD% of 4.88%, reflecting stable quantification with limited matrix-related variability. CTC at the same level yielded mean values of 10.11-11.75 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 4.44-4.77% and an inter-day RSD% of 4.58%, confirming good precision and day-to-day stability.

At 25 $\mu\text{g kg}^{-1}$ spiking level, TC produced mean concentrations from 23.61 to 25.52 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 2.64-6.71% and an inter-day RSD% of 4.31%, demonstrating consistent performance at the higher spiking level (Table 3.28). OTC gave mean recoveries of 21.74-26.01 $\mu\text{g kg}^{-1}$, with intra-day RSD% values of 2.97-6.91% and an inter-day RSD of 5.37%, indicating robust quantification across analytical days. CTC showed mean concentrations of 20.14-23.68 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 2.97-10.35% and an inter-day RSD% of 6.51%; the slightly higher RSD% values for CTC suggest stronger matrix effects at this level, but the results remain within commonly accepted validation limits.

Overall, across both fortification levels, TCs exhibited recoveries that closely matched the nominal spiking concentrations, confirming good analytical accuracy. Intra-day precision was generally within 3-10%, and inter-day RSD% were mostly <8%, indicating stable method performance and supporting the suitability of the LC-MS/MS method for routine TCs residue monitoring in poultry feed.

At 12.5 $\mu\text{g kg}^{-1}$ spiking level in poultry feed, TC showed intra-day recoveries of 94, 102 and 96% with an inter-day recovery of 97%, indicating only minor matrix-induced ion suppression/enhancement and overall accurate quantification (Table 3.28). OTC exhibited a slightly wider range of intra-day recoveries 104, 86 and 101%, where the value of 86% suggests some ion suppression in certain injections, but the inter-day recovery of 97% shows that these effects average out and remain within acceptable limits. CTC displayed more pronounced

suppression, with intra-day recoveries of 80, 94 and 88% and an inter-day value of 87%, pointing to stronger matrix-related signal loss, though still inside typical validation criteria 70-120% (SANTE/11312/2021; CODEX guideline, 2017).

At 25 $\mu\text{g kg}^{-1}$ level, TC again performed robustly, with intra-day recoveries of 94, 102 and 97% and an inter-day recovery of 98%, indicating that ion suppression or enhancement is minimal at the higher concentration (Table 3.28). OTC yielded intra-day values of 86, 104 and 98% and an inter-day recovery of 96%; the single low value reflects localized suppression, but overall accuracy remains high. CTC showed intra-day recoveries of 94, 91 and 80% with an inter-day recovery of 88%, confirming persistent but manageable ion suppression in this matrix.

These data show that although CTC is more susceptible to matrix-induced ion suppression than TC and OTC in poultry feed, all three TCs still exhibit recoveries within acceptable validation limits, and the LC-MS/MS method delivers reliable quantification across both spiking levels in this challenging multi-component feed matrix.

Table 3.28: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of TCs in Poultry Feed Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
TC	12.5	11.8367 $\pm$ 0.6239	5.27	7.15	94	97
		12.7523 $\pm$ 1.1633	9.12		102	
		12.1599 $\pm$ 0.8585	7.06		96	
OTC		13.0512 $\pm$ 0.4285	3.28	4.88	104	97
		10.7660 $\pm$ 0.6386	5.93		86	
		12.6827 $\pm$ 0.6915	5.45		101	
CTC		10.1059 $\pm$ 0.4493	4.44	4.58	80	87
		11.7546 $\pm$ 0.5612	4.77		94	
		11.0098 $\pm$ 0.4981	4.52		88	
TC	25	23.6119 $\pm$ 1.5861	6.71	4.31	94	98
		25.5193 $\pm$ 0.6753	2.64		102	
		24.4811 $\pm$ 0.8769	3.58		97	
OTC		21.7430 $\pm$ 1.5029	6.91	5.37	86	96
		26.0138 $\pm$ 1.6215	6.23		104	
		24.6580 $\pm$ 0.7345	2.97		98	
CTC		23.6750 $\pm$ 1.4699	6.20	6.51	94	88
		22.8554 $\pm$ 2.3657	10.35		91	
		20.1421 $\pm$ 0.5988	2.97		80	

3.3.8 Matrix effect of TCs

The matrix-induced ionization effects for TCs vary notably across the evaluated food and feed matrices, confirming that matrix composition has a clear impact on ESI response in LC-MS/MS (Table 3.29). For TC, matrix effects are relatively low and consistent, ranging from 1.70-8.18%, corresponding to slight ion enhancement. Poultry meat shows the highest enhancement 8.18%, whereas milk exhibits the lowest 1.70%, suggesting that protein-rich animal matrices exert only a modest influence on TC ionization and that TC is comparatively robust to matrix effects. In egg, the TC matrix effect of about 6.56% indicates mild enhancement: egg components slightly increase the TC signal in the ESI source, but TC remains relatively stable in this matrix. In milk, matrix effects for the three TCs are generally low to moderate.

TC shows only 1.70%, while OTC and CTC exhibit 24.56% and 11.03%, respectively. All values are positive, meaning ion enhancement rather than suppression. This suggests that some co-extracted milk components slightly boost the ESI signal, particularly for OTC. However, these levels remain within an acceptable range, indicating that TC and other TCs can be quantified reliably in milk, especially when modest matrix effects are controlled by matrix-matched calibration. In poultry feed, matrix effects are more pronounced, reflecting the high complexity of this multi-component matrix. TC showed only modest enhancement 3.05%, but OTC and CTC exhibit much larger effects 33.37% and 17.00%, indicating substantial ion enhancement likely caused by a mixture of co-eluting substances that alter droplet formation and charge distribution in the ESI source. OTC is generally more affected by co-extracted components, with enhancement values spanning 5.31-33.37%. CTC behaves intermediately between TC and OTC, with enhancement from 4.27-17.00%.

Overall, poultry and beef matrices cause only minor ion enhancement for all TCs, whereas egg, milk and especially poultry feed generate stronger effects, most prominently for OTC and CTC. These observations highlight the need for matrix-matched calibration and/or suitable internal standards to correct for matrix effects and ensure accurate, unbiased TC residue determination across diverse sample types.

Table 3.29: Matrix-Induced Ionization Effects of TCs in Different Matrices

Matrix	Matrix effects (%)		
	TC	OTC	CTC
Poultry meat	8.18	6.43	4.27
Beef	2.97	5.31	5.84
Egg	6.56	33.37	14.63
Milk	1.70	24.56	11.03
Poultry feed	3.05	29.65	17.00

3.3.9 TCs Analysis in Real Samples

A total of 150 samples were analyzed for the presence of TCs (Fig. 3.54-3.71). Each category of samples (n=30) was extracted using method-specific extraction procedures to ensure optimal recovery and accurate quantification. The results showed that all TC was not found in 150 samples but OTC and CTC were found in below their respective MRL values including detected samples (Table 3.30). Among the analytes,

OTC was found in two poultry meat samples at a concentration of 46 and 32 μgkg^{-1} and in one egg sample at a concentration of 37 μgkg^{-1} (Fig. 3.72 and 3.74). Additionally, CTC was identified in one beef sample, with a measured level of 48 μgkg^{-1} (Fig.3.73). No other TCs residues were detected in the remaining samples, indicating a generally low occurrence of TCs contamination across the tested materials.

Table 3.30: Amount of Targeted TCs Compound

Antibiotic name	Matrix	No of positive samples	Amount (μgkg^{-1})	Sample detection frequency (%)
OTC	Poultry meat	1	46	3
	Poultry meat	1	32	3
	Egg	1	37	3
CTC	Beef	1	48	3

3.3.10 Chromatograms of TCs

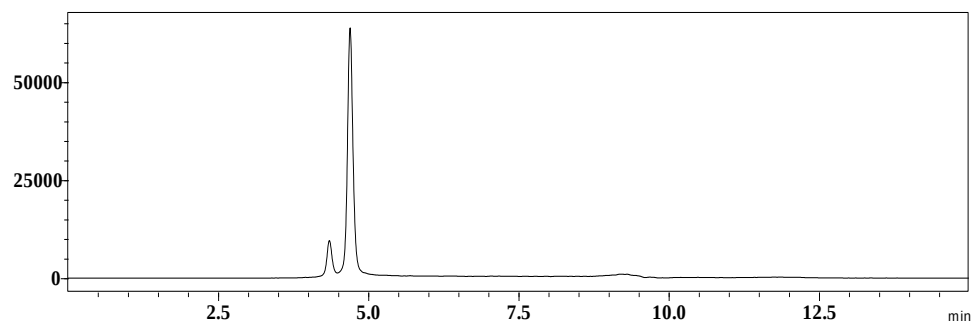


Fig. 3.54: Standard of Tetracycline (TC)

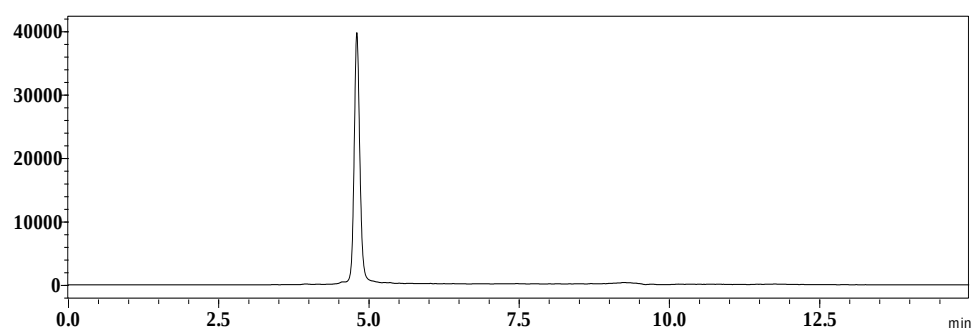


Fig. 3.55: Standard of Oxytetracycline (OTC)

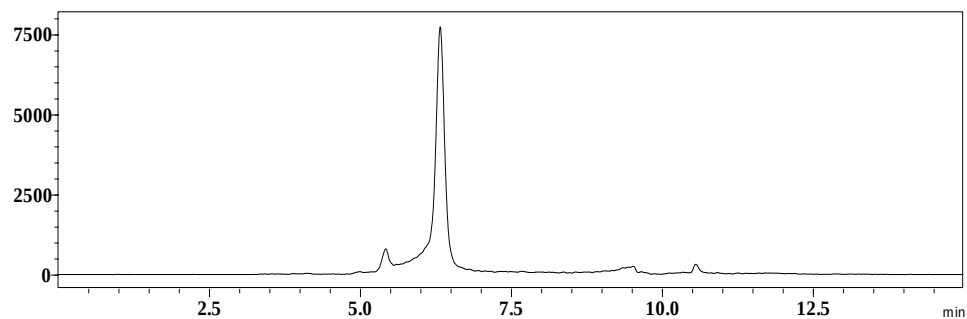


Fig. 3.56: Standard of Chlortetracycline (CTC)

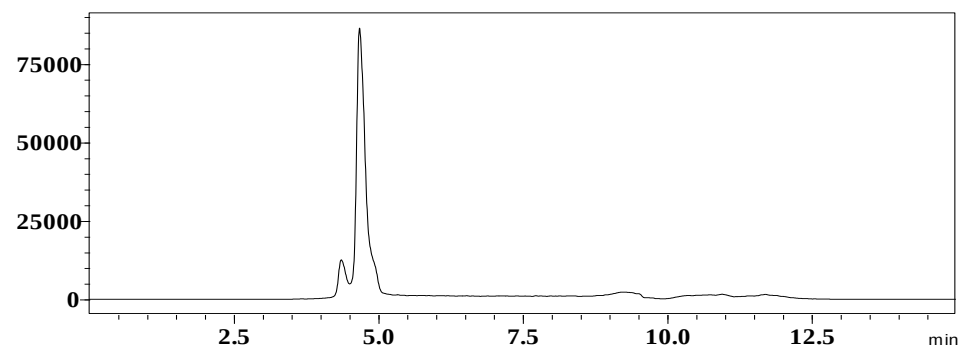


Fig. 3.57: Matrix-matched of Tetracycline (TC) in poultry meat

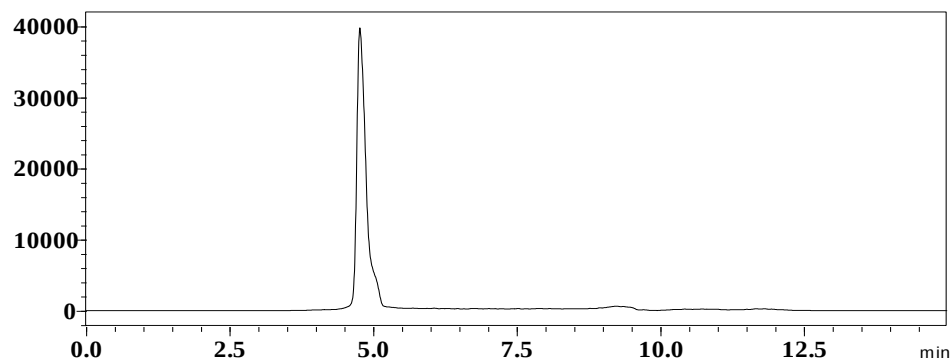


Fig. 3.58: Matrix-matched of Oxytetracycline (OTC) in poultry meat

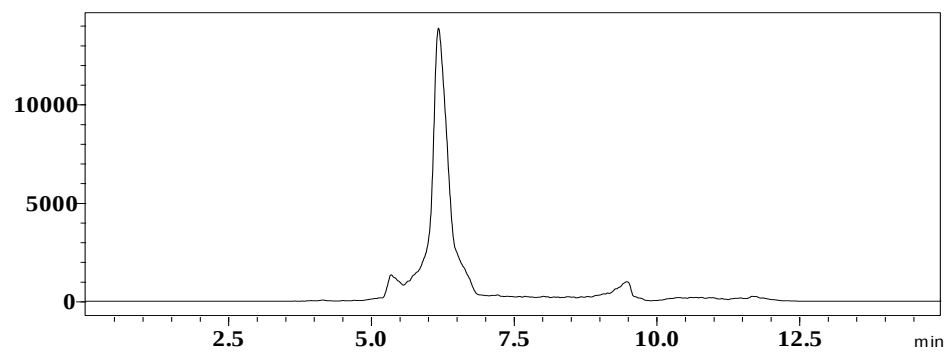


Fig. 3.59: Matrix-matched of Chlortetracycline (CTC) in poultry meat

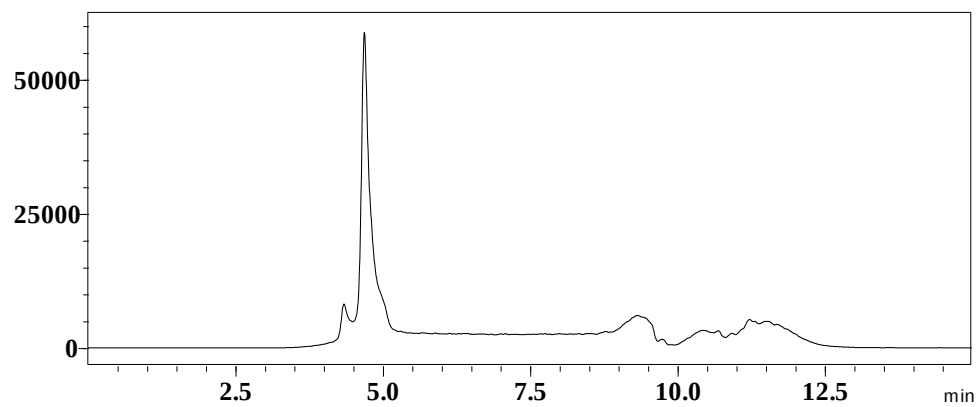


Fig. 3.60: Matrix-matched of Tetracycline (TC) in beef

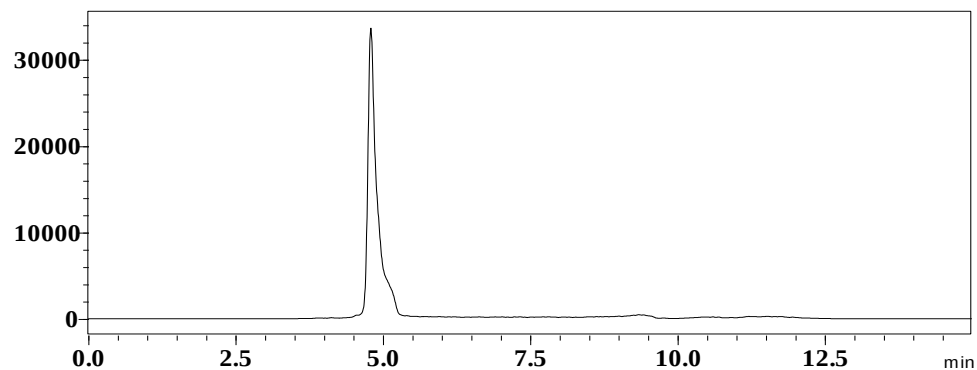


Fig. 3.61: Matrix-matched of Oxytetracycline (OTC) in beef

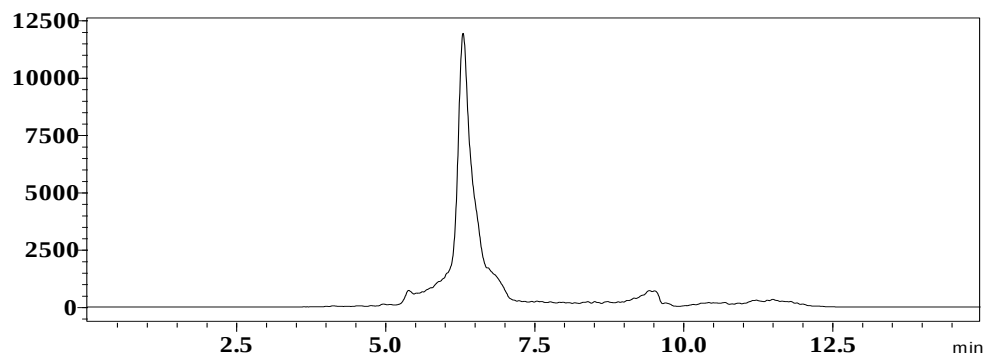


Fig. 3.62: Matrix-matched of Chlortetracycline (CTC) in beef

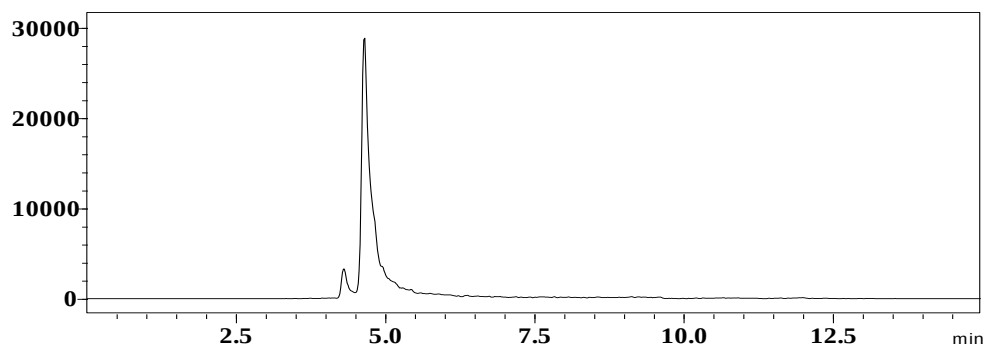


Fig. 3.63: Matrix-matched of Tetracycline (TC) in egg

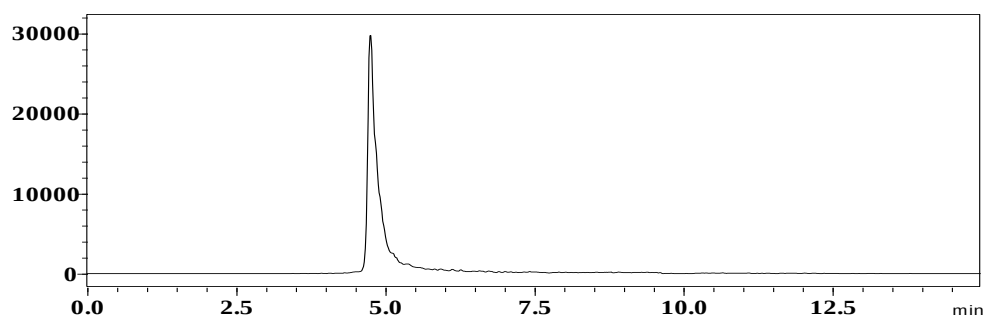


Fig. 3.64: Matrix-matched of Oxytetracycline (OTC) in egg

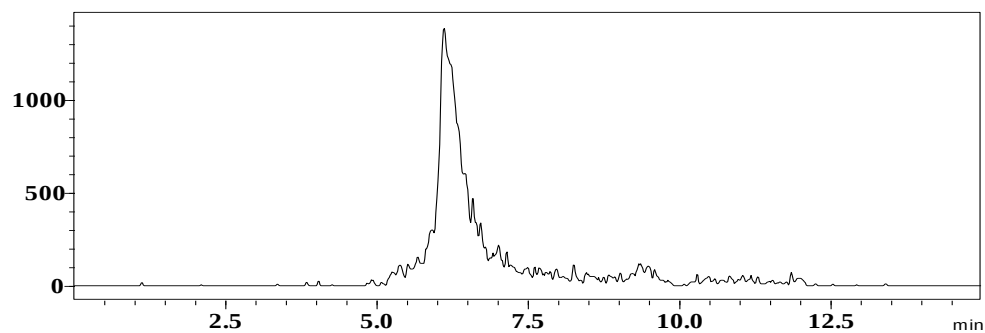


Fig. 3.65: Matrix-matched of Chlortetracycline (CTC) in egg

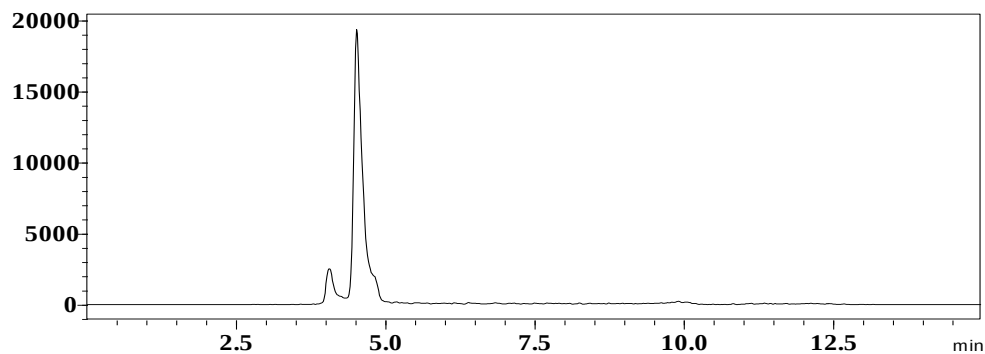


Fig. 3.66: Matrix-matched of Tetracycline (TC) in milk

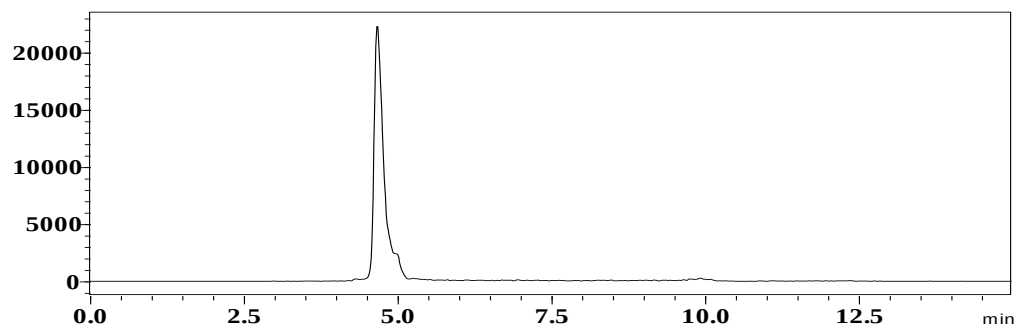


Fig. 3.67: Matrix-matched of Oxytetracycline (OTC) in milk

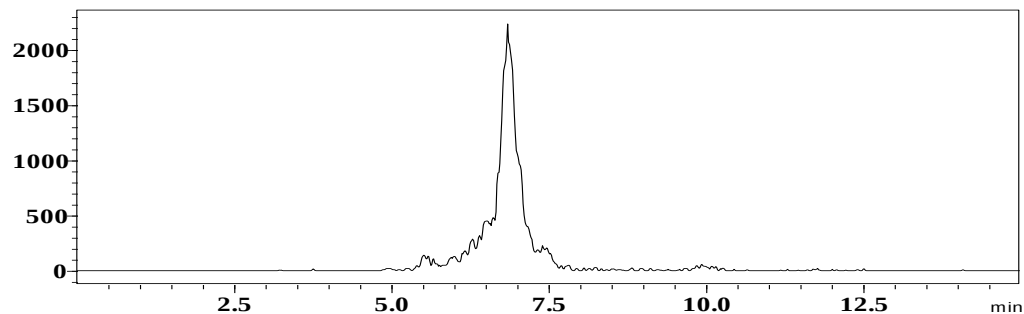


Fig. 3.68: Matrix-matched of Chlortetracycline (CTC) in milk

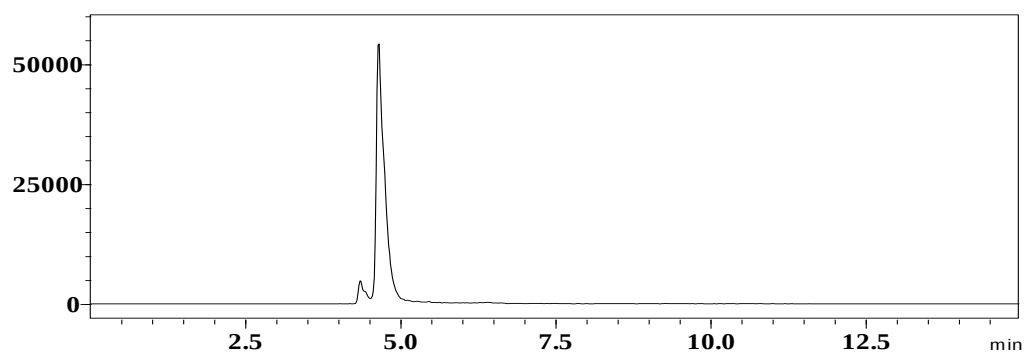


Fig.3.69: Matrix-matched of Tetracycline (TC) in feed

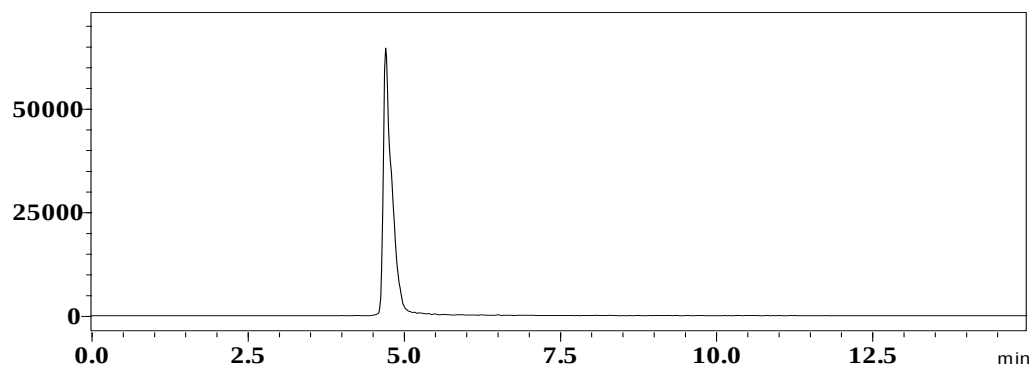


Fig. 3.70: Matrix-matched of Oxytetracycline (OTC) in feed

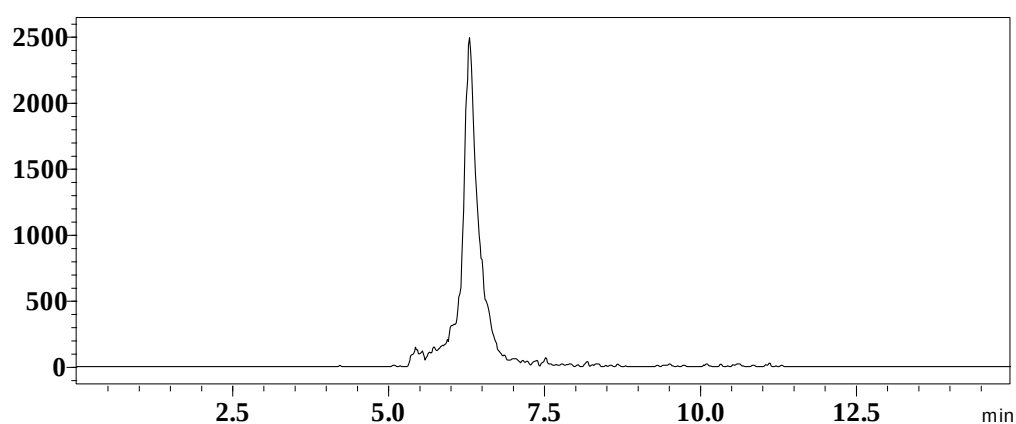


Fig. 3.71: Matrix-matched of Chlortetracycline (CTC) in feed

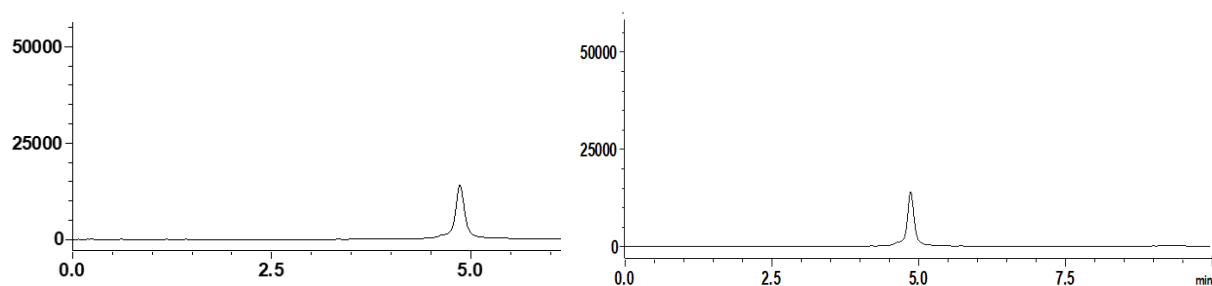


Fig. 3.72: Targeted OTC in two poultry meat sample

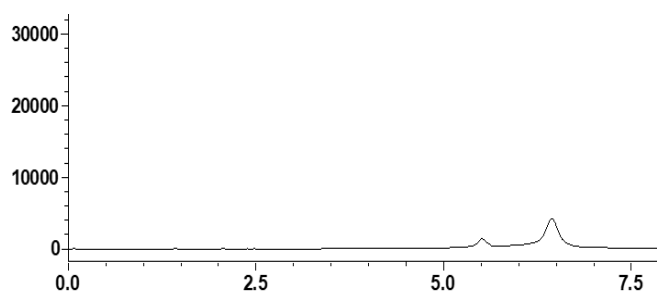


Fig. 3.73: Targeted CTC in one beef sample

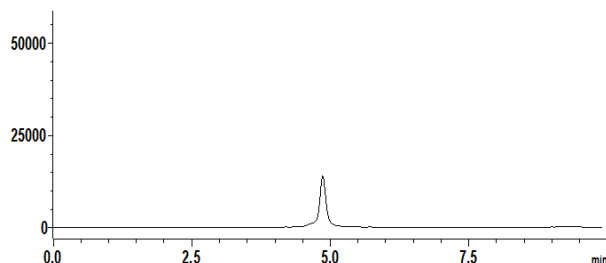


Fig. 3.74: Targeted OTC in one egg sample

3.4 Method Optimization and Validation of Chloramphenicol (CAP)

3.4.1 Optimization of Chloramphenicol (CAP)

Method optimization for chloramphenicol (CAP) in the mass spectrometry system was carried out using the ESI (-) ionization mode at low analyte concentrations within complex food and feed matrices. A C18 reverse-phase column (Shim-Pack GIST C18; 5 μm , 4.6 $\times$ 250 mm) was utilized for chromatographic separation in the LC-MS/MS system. The mobile phase consisted of water with 0.1% HCOOH and methanol, and separation was performed under a gradient elution program. Optimization of mass spectrometric parameters was conducted through preliminary SCAN experiments followed by refinement using Multiple Reaction Monitoring (MRM) transitions.

3.4.2 Screening of CAP

The optimized molecular ion of CAP at m/z 323 was scanned to determine its corresponding precursor ion at m/z 321 (Fig. 3.75). For this analysis, the CAP standard solution was introduced into the LC system and chromatographically separated using a C18 column. A standard solution with a concentration of 250 $\mu\text{g L}^{-1}$ was employed for screening under the SCAN acquisition mode.

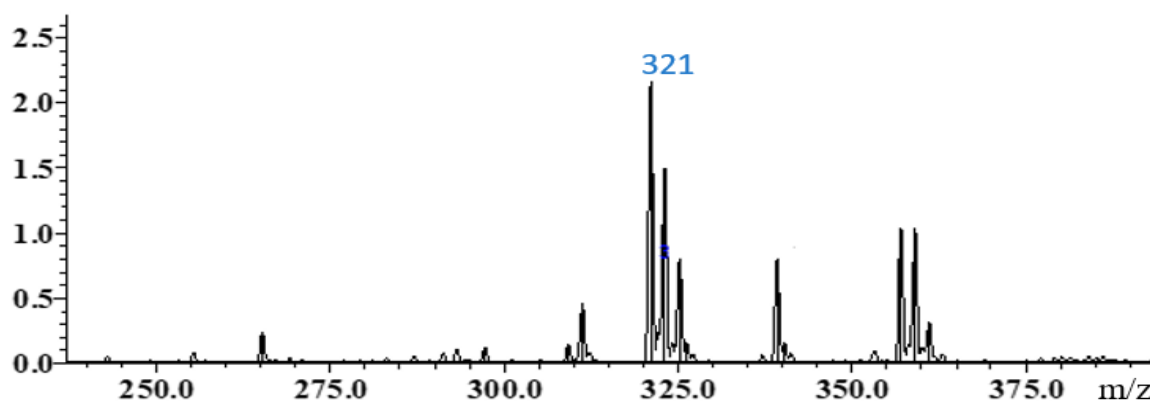


Fig. 3.75: Chloramphenicol screening

3.4.3 MRM of CAP

For MRM analysis, the analyte was introduced into the LC system using the CAP standard, separated on a C18 column, and subsequently directed through the MS interface into the MS/MS detector. In MRM mode, the selected precursor ion of CAP was subjected to collision-induced fragmentation, generating the product ions at m/z 152, 257, and 194, which were used for quantitative determination (Fig. 3.76).

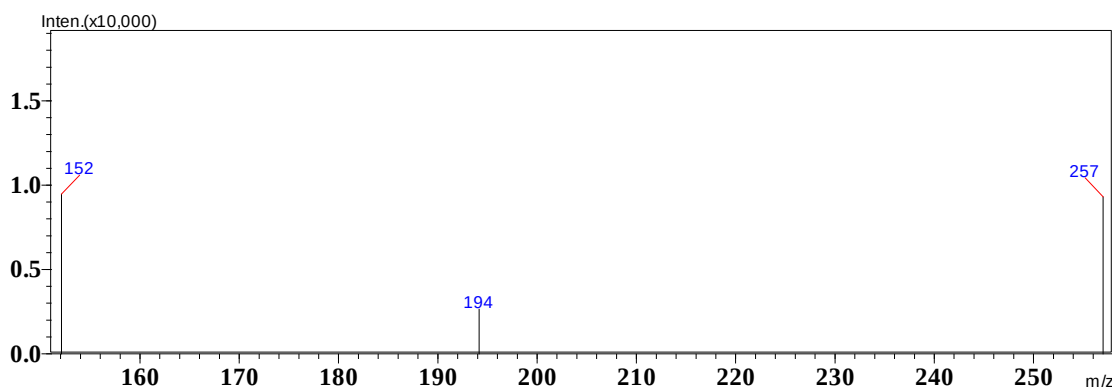


Fig. 3.76: Chloramphenicol MRM

3.4.4 Standard Calibration Curves of CAP

Primary stock solution of chloramphenicol (CAP) was prepared at a concentration of $1000 \mu\text{g L}^{-1}$. From this stock, an intermediate standard solution of $250 \mu\text{g L}^{-1}$ was prepared and subsequently used to generate a series of working standards. These working standards, spanning concentrations from 1.5 to $100 \mu\text{g L}^{-1}$ were utilized to construct calibration curves for reliable and accurate quantification of the analyte in the analytical measurements (Fig. 3.77).

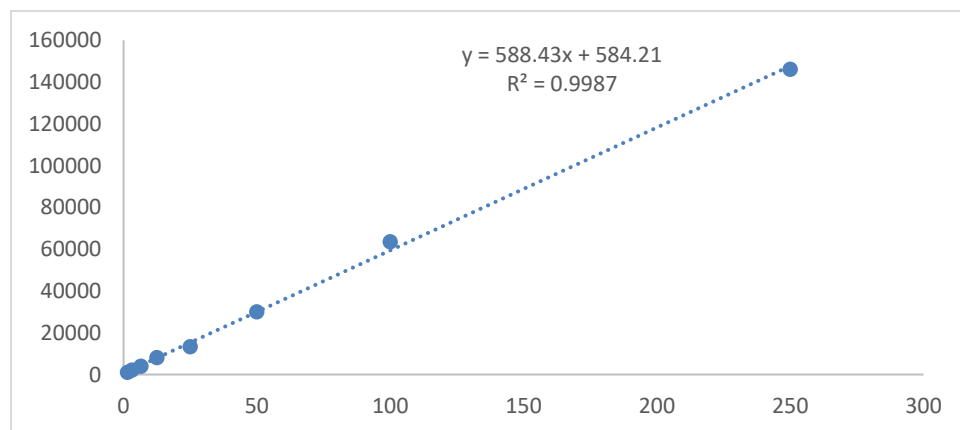


Fig. 3.77: Standard calibration curves of CAP

3.4.5 Matrix-matched Calibration Curves of CAP in Poultry meat, Beef, Egg, Milk and Poultry Feed samples

CAP standard was spiked into poultry meat, beef, egg, milk, and poultry feed samples. After extraction, the fortified matrix extracts were analyzed across a concentration range of 1.5-100 $\mu\text{g kg}^{-1}$ (Fig. 3.78-3.82) to construct matrix-matched calibration curves.

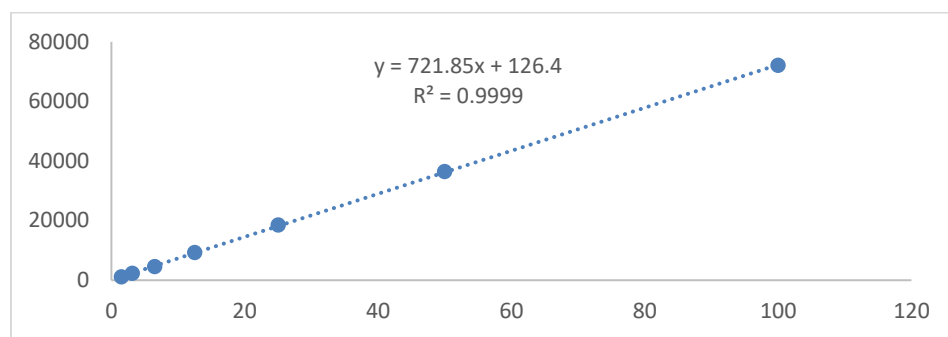


Fig. 3.78: Matrix matched calibration curves of CAP in poultry meat samples

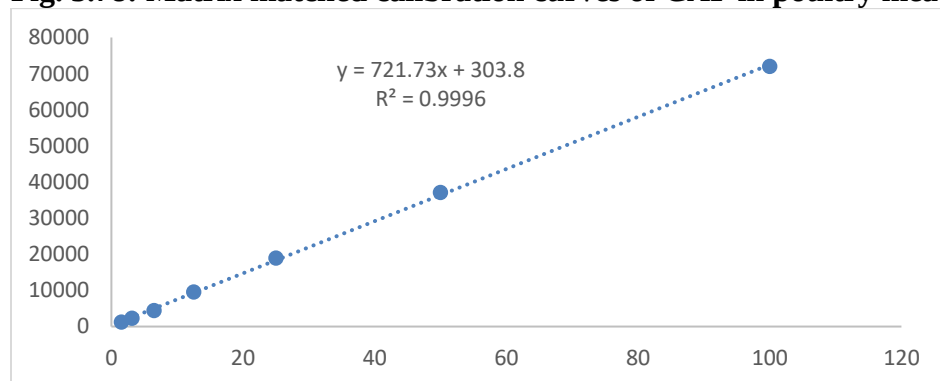


Fig. 3.79: Matrix matched calibration curves of CAP in beef samples

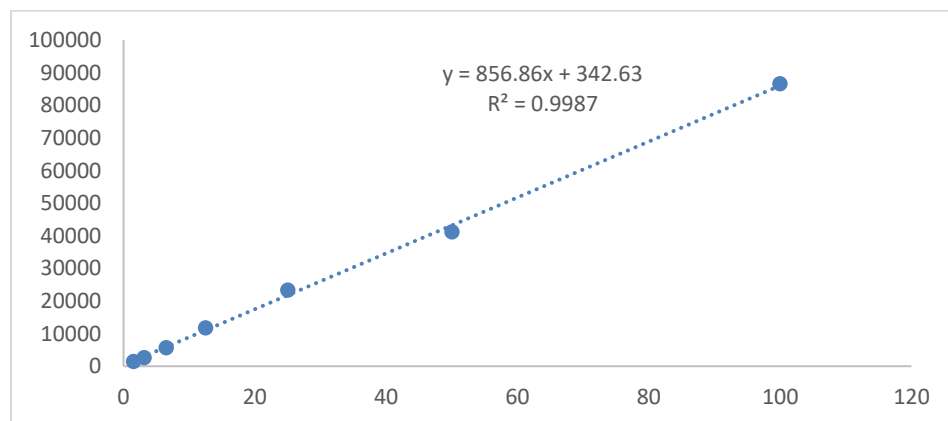


Fig. 3.80: Matrix matched calibration curves of CAP in egg samples

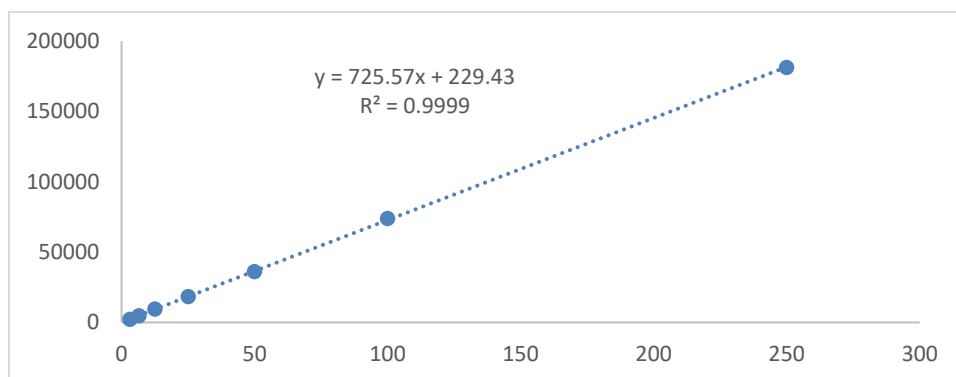


Fig. 3.81: Matrix matched calibration curves of CAP in milk samples

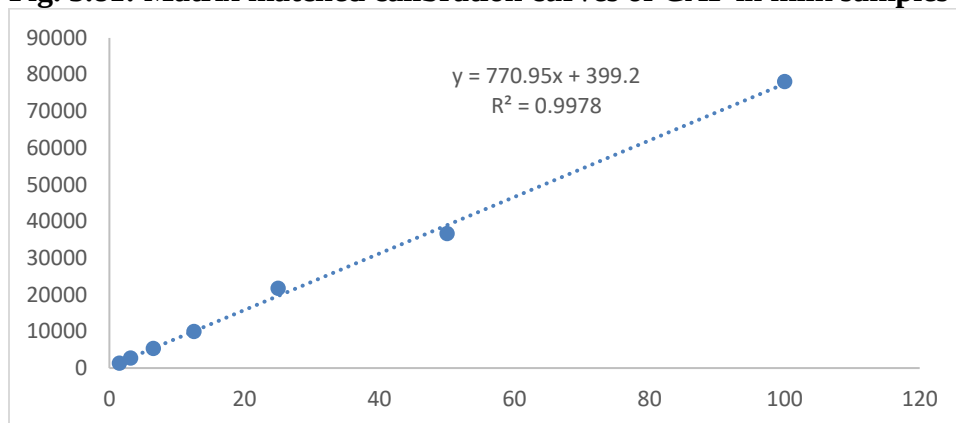


Fig. 3.82: Matrix matched calibration curves of CAP in poultry feed samples

3.4.6 Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ) & Retention time (t_R) of CAP

The linearity, sensitivity, and chromatographic pattern of CAP in different matrices demonstrate that the LC-MS/MS method is highly fit for quantitative residue analysis (Table 3.31). Matrix-matched calibration curves for CAP showed excellent linearity, with correlation coefficients (R^2) ranging from 0.9978 to 0.9999 for the standard solution and all food or feed matrices. These values confirm a very strong linear relationship between concentration and peak response and indicate that the calibration model is robust across poultry meat, beef, egg, milk, and poultry feed. Beef was validated over a 1.5-100 $\mu\text{g L}^{-1}$ range, and the similar slopes of the calibration equations (e.g., $y = 721.85x + 126.4$ for poultry meat, $y = 721.73x + 303.8$ for beef, $y = 725.57x + 229.43$ for milk) indicate uniform detector response and consistent ionization efficiency among matrices.

The method also exhibits high sensitivity, as reflected by low LOD values of 0.06-0.7 $\mu\text{g kg}^{-1}$ and LOQ values of 0.1-0.6 $\mu\text{g kg}^{-1}$. Poultry meat and beef show particularly low LOD 0.06-0.1 $\mu\text{g kg}^{-1}$.

kg⁻¹, while egg, milk, and poultry feed maintain suitably low LOD or LOQ values, allowing reliable detection of CAP at trace levels. Chromatographic performance is consistent, with the CAP standard eluting at 3.9 min. Beef, milk, and poultry feed share the same t_R 3.9 min, egg elutes slightly earlier at 3.8 min, and poultry meat at 3.5 min, suggesting minor matrix-dependent interactions with the stationary phase. Nonetheless, all retention times fall within a narrow window, ensuring good peak separation and minimizing the risk of co-elution and matrix-induced ion suppression.

Taken together, the strong linearity, low LOD or LOQ values, and stable t_R demonstrate that the developed LC-MS/MS method provides accurate, precise, and robust quantification of CAP across a wide range of food and feed matrices, making it well suited for routine surveillance and regulatory monitoring of CAP residues.

Table 3.31: CAP Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	Correlation coefficient, R^2
Std CAP	3.9	1.5-100	-	-	$y = 588.43x + 584.21$	0.9987
Poultry meat	3.5		0.06	0.1	$y = 721.85x + 126.4$	0.9999
Beef	3.9		0.1	0.3	$y = 721.73x + 303.8$	0.9996
Egg	3.8		0.13	0.3	$y = 856.86x + 342.63$	0.9987
Milk	3.9		0.2	0.6	$y = 725.57x + 229.43$	0.9999
Poultry feed	3.9		0.7	0.2	$y = 770.95x + 399.2$	0.9978

3.4.7 Average, Standard Deviation ($\pm$ SD) & Intra- & Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of CAP in Samples

The mean concentrations (Mean $\pm$ SD) of CAP residues provide a clear quantitative measure of analyte levels across different matrices, allowing direct comparison of contamination patterns. The associated standard deviations indicate the variability of replicate measurements and reflect both analytical precision and matrix influence. Method precision was further verified through intra-day RSD% for evaluating repeatability and inter-day RSD% for assessing reproducibility across multiple runs. The acceptable ranges of SD and RSD% values confirm the robustness and reliability of the analytical method, supporting its suitability for accurate multi-matrix residue

determination with minimal analytical uncertainty. CAP recoveries were within acceptable limits, verifying that the method consistently delivers accurate quantification across diverse matrices.

3.4.7.1 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- & Inter-day Recovery Percentage (%) of CAP in Poultry Meat Samples

The precision and accuracy of CAP quantification in poultry meat samples were evaluated through average concentrations (Mean $\pm$ SD) and intra- and inter-day RSD% values at two spiking levels 10 and 15 $\mu\text{g kg}^{-1}$ (Table 3.32). At 10 $\mu\text{g kg}^{-1}$ level, the measured averages 9.9538 ± 0.9352 , 9.9319 ± 0.8680 , and 10.1209 ± 0.7094 closely aligned with the nominal concentration, indicating good analytical accuracy. The corresponding intra-day RSD% values 9.39, 8.74, and 7.00% demonstrate acceptable repeatability, while the inter-day RSD% of 8.38% confirms consistent method performance across different analytical sessions.

At 15 $\mu\text{g kg}^{-1}$ spiking level, the average values 14.4157 ± 1.0712 , 14.3453 ± 1.1197 , and 13.8017 ± 1.4256 again showed close agreement with the target concentration, supporting reliable quantification. Intra-day RSD% values ranged from 7.43-10.33%, indicating moderate but acceptable variability, whereas the inter-day RSD% of 8.52% reflects stable reproducibility over time. Overall, the mean concentration values, together with controlled intra- and inter-day variability, confirm that the analytical method provides robust precision and accuracy for CAP detection in poultry meat samples.

At 10 $\mu\text{g kg}^{-1}$ spiking level, CAP demonstrated highly consistent intra-day recoveries of 99, 99, and 101%, indicating excellent analytical accuracy and very low variability (Table 3.32). These closely aligned values reflect efficient extraction and stable instrument response at this concentration. The inter-day recovery of 100% further supports the strong reproducibility of the method, confirming that day-to-day performance remains consistent without significant drift or matrix-related interference. At 15 $\mu\text{g kg}^{-1}$ level, CAP produced intra-day recoveries of 96, 95, and 92%, showing slightly greater variability than the lower level but still maintaining acceptable accuracy (Table 3.32). These values fall well within typical validation limits, demonstrating that the method performs reliably even at elevated concentrations. The corresponding inter-day

recovery of 94% indicates robust intermediate precision, confirming that reproducibility across analytical days remains strong.

CAP recovery pattern in poultry meat across both concentration levels demonstrates that the LC-MS/MS method provides accurate, precise, and reproducible quantification. Minor fluctuations at the higher spiking level do not impact method reliability, and all recoveries comfortably meet standard validation criteria for residue analysis.

Table 3.32: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter- day Recovery (%) of CAP in Poultry meat Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CAP	10	9.9538 $\pm$ 0.9352	9.39	8.38	99	100
		9.9319 $\pm$ 0.8680	8.74		99	
		10.1209 $\pm$ 0.7094	7.00		101	
CAP	15	14.4157 $\pm$ 1.0712	7.43	8.52	96	94
		14.3453 $\pm$ 1.1197	7.81		95	
		13.8017 $\pm$ 1.4256	10.33		92	

3.4.7.2 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of CAP in Beef Samples

At 10 $\mu\text{g kg}^{-1}$ spiking level in beef, CAP gave mean concentrations of 9.08-11.24 $\mu\text{g kg}^{-1}$, indicating recoveries reasonably close to the nominal level, with a slight under- and overestimation in individual replicates (Table 3.33). The intra-day RSD% 4.37-10.99% showed acceptable repeatability, although one run exhibits somewhat higher variability, which is still within typical validation limits for residue analysis. The inter-day RSD% of 8.24% confirms that day-to-day performance of the method in the beef matrix remains stable.

At 15 $\mu\text{g kg}^{-1}$ spiking level, CAP produced mean concentrations ranging from 13.62 to 16.85 $\mu\text{g kg}^{-1}$, again in good agreement with the spiking level (Table 3.33). The intra-day RSD% values 5.65-10.77% indicate generally good repeatability, with slightly increased dispersion in one replicate, likely reflecting matrix effects at the higher concentration. The inter-day RSD% of 7.47% demonstrates consistent quantification over multiple days and supports robust intermediate precision.

Taken together, the results at both 10 and 15 $\mu\text{g kg}^{-1}$ show that CAP achieves accurate recoveries, acceptable intra-day precision, and strong inter-day reproducibility ($\text{RSD\%} < 10\%$) in beef samples, confirming the reliability of the LC-MS/MS method for routine CAP residue monitoring in this matrix.

At 10 $\mu\text{g kg}^{-1}$ spiking level, CAP yielded intra-day recoveries of 111, 112, and 102%, indicating reliable quantification with minor enhancement effects that may arise from matrix-induced ionization (Table 3.33). These values reflect strong method accuracy and stable performance under single-day analytical conditions. The inter-day recovery of 102% further demonstrates that the method provides consistent results across multiple days, confirming robust reproducibility and minimal day-to-day variation. At 15 $\mu\text{g kg}^{-1}$ level, the intra-day recoveries of 90, 112, and 104% show slightly greater variability, suggesting modest concentration-dependent influences or matrix effects (Table 3.33). Nevertheless, all values remain within the acceptable recovery range, indicating that the method maintains suitable accuracy even at increased analyte levels. The inter-day recovery of 102% supports dependable intermediate precision, showing that the method continues to perform consistently across different analytical sessions.

Collectively, the recovery profiles at both concentrations affirm that the method is accurate, reproducible, and fit for routine CAP residue determination, with all results complying with established validation criteria.

Table 3.33: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of CAP in Beef Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CAP	10	9.0828 $\pm$ 0.9986	10.99	8.24	111	102
		11.2402 $\pm$ 0.4912	4.37		112	
		10.2919 $\pm$ 0.9644	9.37		102	
CAP	15	13.6181 $\pm$ 0.8177	6.00	7.47	90	102
		16.8453 $\pm$ 0.9532	5.65		112	
		15.7197 $\pm$ 1.6934	10.77		104	

3.4.7.3 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of CAP in Egg Samples

At 15 $\mu\text{g kg}^{-1}$ spiking level, CAP exhibited mean concentrations ranging from 13.74 to 14.12 $\mu\text{g kg}^{-1}$, showing very good agreement with spiking level (Table 3.34). The intra-day RSD% values 2.98-6.41% indicate acceptable repeatability within the same analytical run. The inter-day RSD% of 5.15% further confirms stable reproducibility across different days, demonstrating reliable analytical performance in the egg matrix. At 25 $\mu\text{g kg}^{-1}$ spiking level, CAP produced mean concentrations between 21.94 and 23.88 $\mu\text{g kg}^{-1}$, closely corresponding to the expected spiking concentration (Table 3.34). Intra-day precision showed RSD% values of 3.65-8.62%, with the slightly higher RSD% likely attributed to matrix complexity at this elevated concentration. The inter-day RSD% of 6.74% demonstrates reliable day-to-day precision, supporting method stability for CAP quantification in eggs. Across both spiking levels, CAP showed consistently accurate recoveries, with mean concentrations closely matching the intended spiking levels. The intra-day RSD% values remained below 9%, demonstrating reliable repeatability within each analytical run, while the inter-day RSD% values stayed under 7%, confirming strong day-to-day reproducibility. Together, these performance indicators verify that the LC-MS/MS method is robust, precise, and fully suitable for routine determination of CAP residues in egg samples, even when dealing with the inherent complexity of this biological matrix.

At 15 $\mu\text{g kg}^{-1}$ spiking level, CAP showed intra-day recoveries of 93, 91, and 94%, demonstrating strong accuracy with minimal variability (Table 3.34). These closely clustered values indicate effective extraction and stable instrument response at this concentration. The inter-day recovery of 93% further confirms excellent reproducibility, reflecting consistent analytical performance across multiple days with negligible day-to-day variation.

At 25 $\mu\text{g kg}^{-1}$ level, the intra-day recoveries of 95, 90, and 87% highlight similarly high accuracy and reliable quantification, with recoveries remaining well within accepted analytical limits (Table 3.34). The inter-day recovery of 91% supports strong intermediate precision, demonstrating that the method maintains robust reproducibility over extended analytical periods. Taken together, the recovery data at both spiking levels confirm that the method is accurate, precise, and well-suited for routine CAP residue analysis. All results fall comfortably within established validation criteria, affirming the reliability of the method for quantitative applications.

Table 3.34: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of CAP in Egg Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CAP	15	14.0655 $\pm$ 0.8523	6.06	5.15	93	93
		13.7429 $\pm$ 0.8813	6.41		91	
		14.1252 $\pm$ 0.4212	2.98		94	
CAP	25	23.8848 $\pm$ 0.8734	3.65	6.74	95	91
		22.6988 $\pm$ 1.9579	8.62		90	
		21.9475 $\pm$ 1.7473	7.96		87	

3.4.7.4 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of CAP in Milk Samples

At 15 $\mu\text{g L}^{-1}$ spiking level, CAP produced mean concentrations between 14.03 and 16.67 $\mu\text{g L}^{-1}$, showing good alignment with the fortified amount and confirming accurate recovery (Table 3.35). The intra-day RSD% values ranged from 6.58-11.46 %, indicating acceptable repeatability despite moderate variability in one replicate. The inter-day RSD% of 8.59% demonstrates reliable reproducibility across different analytical days, suggesting stable instrument performance within the milk matrix. At 25 $\mu\text{g L}^{-1}$ level, CAP showed mean concentrations of 20.38-22.03 $\mu\text{g L}^{-1}$, again confirming accurate quantification relative to the spiking concentration (Table 3.35). Intra-day RSD% values varied from 6.29 to 14.42%, with the slightly higher RSD% likely attributable to stronger matrix effects or increased sample complexity at higher concentrations. The inter-day RSD% of 9.97% indicates consistent day-to-day precision within acceptable analytical limits for complex dairy matrices.

Across both fortification levels, CAP exhibited accurate recoveries, with mean concentrations closely matching their spiking values. Acceptable intra-day precision, generally remaining below 12%. Strong inter-day reproducibility (<10%), confirming stable method performance of challenging nature of the dairy matrix.

At 15 $\mu\text{g L}^{-1}$ spiking level, CAP exhibited intra-day recoveries of 111, 94, and 94%, indicating moderate variability with two values slightly above 100%, suggesting possible mild enhancement effects (Table 3.35). Despite this variation, the overall recovery pattern reflects acceptable analytical accuracy at this concentration. The inter-day recovery of 99% confirms strong

reproducibility across multiple analytical sessions, demonstrating stable day-to-day performance. At 25 $\mu\text{g L}^{-1}$ level, the intra-day recoveries of 101, 94, and 101% indicate consistent and reliable quantification, with values largely clustered near 100% (Table 3.35). The two elevated values 101% may reflect slight ionization enhancement, yet the recoveries remain within acceptable method validation limits. The inter-day recovery of 98% further supports robust intermediate precision, confirming excellent reproducibility across days.

Taken together, the recovery results across both spiking levels demonstrate that the method is accurate, precise, and well-suited for routine CAP residue determination. All values fall within standard validation criteria, reinforcing the method's reliability for quantitative analysis.

Table 3.35: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of CAP in Milk Samples

Compound Name	Spiking Level ($\mu\text{g L}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CAP	15	16.6723 $\pm$ 1.0973	6.58	8.59	111	99
		14.0702 $\pm$ 1.0869	7.72		94	
		14.0278 $\pm$ 1.6086	11.46		94	
CAP	25	22.0336 $\pm$ 2.0267	9.19	9.97	101	98
		20.3840 $\pm$ 2.9405	14.42		94	
		22.0336 $\pm$ 1.3865	6.29		101	

3.4.7.5 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of CAP in Poultry Feed Samples

At 15 $\mu\text{g kg}^{-1}$ spiking level, CAP produced mean concentrations ranging from 13.75-14.82 $\mu\text{g kg}^{-1}$, showing good agreement with the fortified values and confirming accurate analyte recovery in the feed matrix (Table 3.36). The intra-day RSD% values 4.24-9.86% indicate acceptable repeatability within daily analytical runs. The inter-day RSD% of 8.20% further demonstrates consistent day-to-day performance, suggesting that the method maintains stable precision despite the complex nature of poultry feed samples. At 25 $\mu\text{g kg}^{-1}$ spiking level, CAP yielded mean concentrations between 22.56 and 23.28 $\mu\text{g kg}^{-1}$, again confirming accurate recovery at the higher spiking level (Table 3.36). Intra-day RSD% values ranged from 2.48-4.64%, with slight variability likely due to matrix interference typical of heterogeneous feed samples. The inter-day RSD% of

3.41% indicates robust reproducibility over multiple analytical sessions, remaining well within acceptable validation limits. These findings confirm that acceptable intra-day precision <10%, confirming reliable repeatability and inter-day reproducibility <9%, indicating stable analytical performance of highly variable and challenging matrix. At 15 $\mu\text{g kg}^{-1}$ spiking level, CAP exhibited intra-day recoveries of 96, 89, and 93%, indicating moderate variability across replicates (Table 3.36). The value of 99% suggests slight signal enhancement, while the 89% result reflects mild under-recovery, likely due to normal matrix-related fluctuations. Nonetheless, these values remain within acceptable analytical limits 70-120% (SANTE/11312/2021; CODEX guideline, 2017) confirming that the method maintains satisfactory accuracy at this concentration. The inter-day recovery of 92% demonstrates good reproducibility, indicating consistent performance across different analytical days.

At 25 $\mu\text{g kg}^{-1}$ level (Table 3.36), CAP showed intra-day recoveries of 92, 90, and 93%, reflecting strong accuracy and tight clustering of values around 93%. The minor elevation to 90% suggests slight positive bias, but overall, the recoveries indicate efficient extraction and stable ionization at higher analyte concentrations. The inter-day recovery of 91% confirms robust intermediate precision, demonstrating that the method provides consistent results over extended analytical sessions. Overall, the recovery data across both spiking levels illustrate that the method is accurate, precise, and reliable for CAP quantification. All intra-day and inter-day recoveries fall within standard validation criteria, supporting the suitability of the method for routine analysis of CAP residues in feed matrices.

Table 3.36: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of CAP in Poultry Feed Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
CAP	15	14.8240 $\pm$ 1.4624	9.86	8.20	96	92
		13.7599 $\pm$ 0.5987	4.35		89	
		13.7599 $\pm$ 0.5837	4.24		93	
CAP	25	23.0227 $\pm$ 0.7188	3.12	3.41	92	91
		22.5607 $\pm$ 0.5605	2.48		90	
		23.2821 $\pm$ 1.0823	4.64		93	

3.4.8 Matrix effect of CAP

CAP experiences little matrix influence in poultry meat, beef, and milk, as all three matrices gave the same low matrix-effect value 23 (Table 3.37). This suggests that co-extracted compounds from these samples have minimal impact on CAP ionization under the optimized LC–MS/MS conditions. In contrast, egg and feed showed a much higher matrix effect 46 and 31, indicating a stronger influence from the egg and feed matrix. Overall, CAP can be measured reliably in meat and milk with low matrix-related bias, while egg and feed measure higher matrix-related bias.

Table 3.37: Matrix-Induced Ionization Effects of CAP in Different Matrices

Matrix	Matrix effects
Poultry meat	23
Beef	23
Egg	46
Milk	23
Poultry feed	31

3.4.9 CAP Analysis in Real Samples

A total of 150 samples were subjected to CAP residue analysis to assess the occurrence of this prohibited antimicrobial agent in various food and feed matrices. Each sample category (n=30 per matrix) including poultry meat, beef, eggs, milk, and poultry feed was processed using matrix-optimized extraction procedures designed to maximize analyte recovery and ensure reliable quantification. The analytical method demonstrated high sensitivity and selectivity, enabling the detection of CAP at trace levels well below internationally established MRLs.

Importantly, no detectable levels of CAP were found in any of the analyzed samples, indicating full compliance with regulatory food safety standards. These findings suggest that CAP contamination was absent across all tested matrices, further confirming the effectiveness of current regulatory controls, good agricultural practices, and proper feed management strategies in preventing the use or accidental introduction of this banned antibiotic.

3.4.10 Chromatograms of CAP

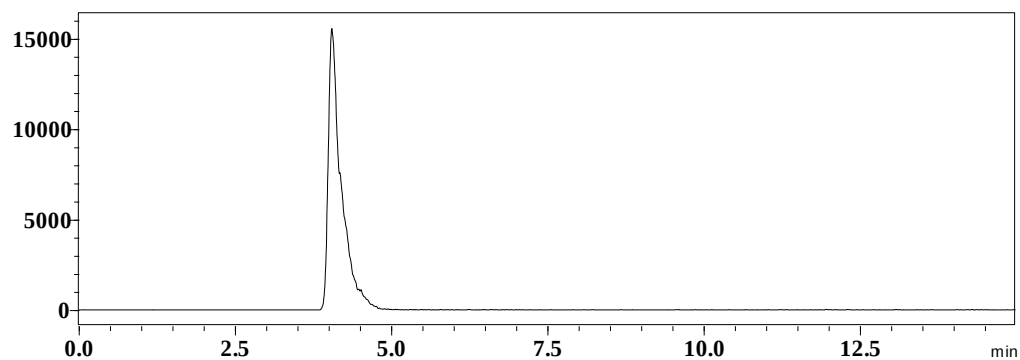


Fig. 3.83: Standard of Chloramphenicol (CAP)

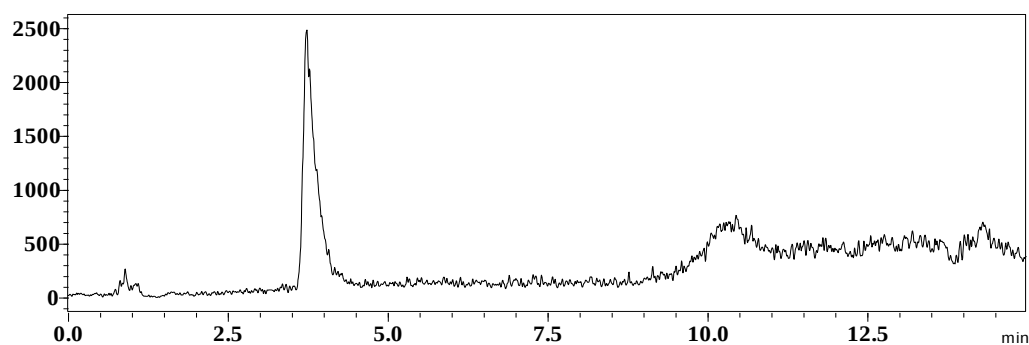


Fig. 3.84: Matrix-matched of CAP in poultry meat

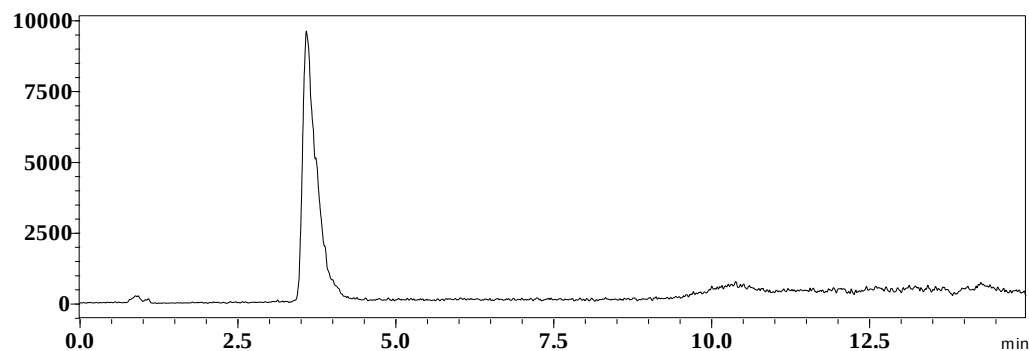


Fig. 3.85: Matrix-matched of CAP in beef

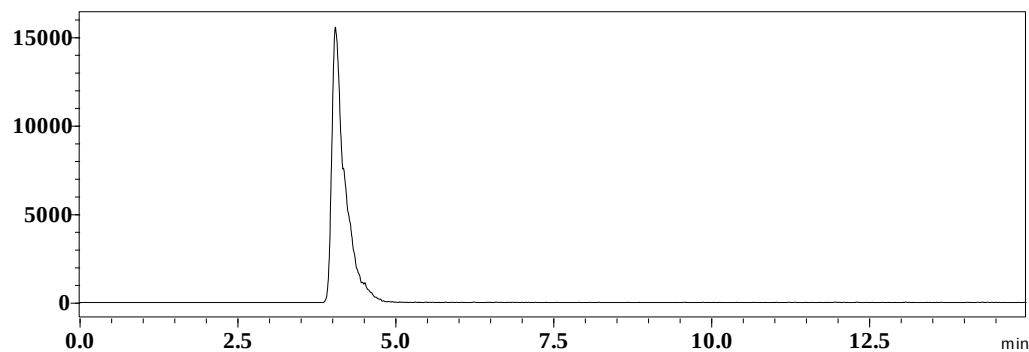


Fig. 3.86: Matrix-matched of CAP in egg

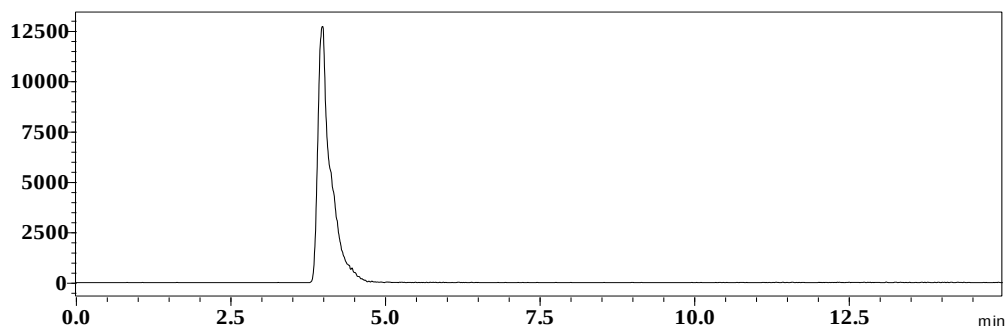


Fig. 3.87: Matrix-matched of CAP in milk

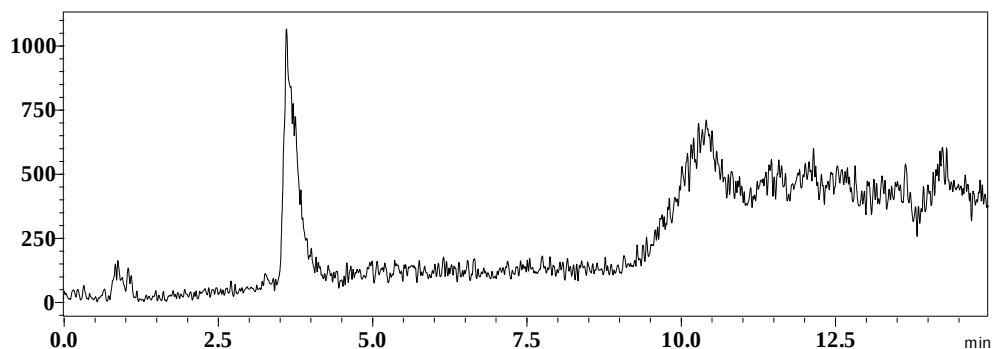


Fig. 3.88: Matrix-matched of CAP in poultry feed

3.5 Method Optimization and Validation of Derivatives of Nitrofurantol Metabolites (NFs)

3.5.1 Optimization of Derivatives of Nitrofurantol Metabolites (NFs)

The method optimization for derivative of nitrofurantol metabolites (NFs) on the Agilent 6420 LC/TQ MS system was performed for NP-AMOZ, NP-AHD, and NP-AOZ using ESI (+) mode at low concentrations in complex food and feed matrices. C18 reverse-phase column (Zorbax SB-C18) was employed for NFs separation using an Agilent 1290 LC system (1290 Infinity II). A mobile phase consisted of H₂O and ACN in 50:50 ratio, with 0.1% HCOOH added to both phases. Mass spectrometric transitions were optimized through a combination of preliminary SCAN and Multiple Reaction Monitoring (MRM) methods.

3.5.2 Screening and MRM of NFs Compounds

The optimized molecular ions at m/z 235, 334, and 248 for NP-AOZ, NP-AMOZ, NP-AHD, and were scanned to identify their corresponding precursor ions at m/z 335, 249, and 236. For this purpose, the analytes were introduced into the LC system using NF standards, passed through a C18 column. A 200 $\mu\text{g L}^{-1}$ concentrated standard was used for screening using the SCAN method.

For MRM purpose, the analytes were introduced into the LC system using NF standards, passed through a C18 column, and then transferred via the MS interface to the MS/MS fragmentor. In MRM mode, these precursor ions were further fragmented to the product ions m/z 134 and 104 for NP-AOZ, m/z 291 and 262 for NP-AMTZ, and m/z 134 and 104 for NP-AHD, which were used for quantification.

3.5.3 Standard Calibration Curves of NFs

Derivative standards of all NF metabolites were initially prepared at a concentration of 1 mg L^{-1} . From these stock solutions, a series of working standards was subsequently prepared to construct calibration curves over a concentration range of $1\text{--}200 \mu\text{g L}^{-1}$ (Fig. 3.89), ensuring accurate quantification of analytes in subsequent analyses.

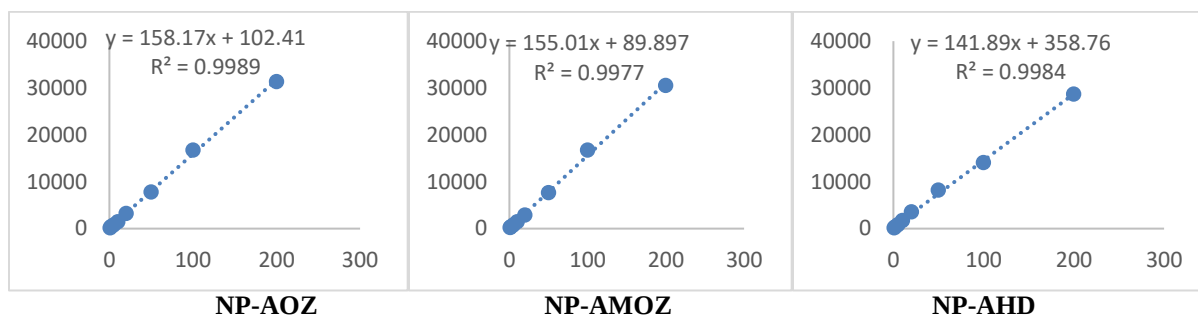


Fig. 3.89: Standard calibration curves of NP-AOZ, NP-AMTZ and NP-AHD

3.5.4 Matrix-matched Calibration Curves of NFs in Poultry Meat, Beef, Egg, Milk and Poultry Feed Samples

A mixture of NF standards was spiked into poultry meat, beef, egg, milk, and poultry feed samples. Following extraction, the resulting matrix-matched samples were analyzed over a concentration range of $1\text{--}200 \mu\text{g kg}^{-1}$ (Fig. 3.90-3.94) to generate matrix-matched calibration curves.

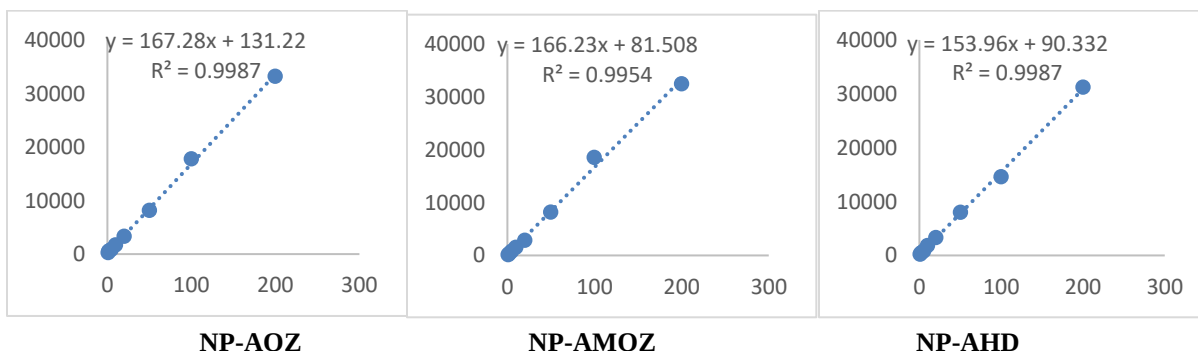


Fig. 3.90: Matrix matched calibration curves of NP-AOZ, NP-AMTZ and NP-AHD in poultry meat samples

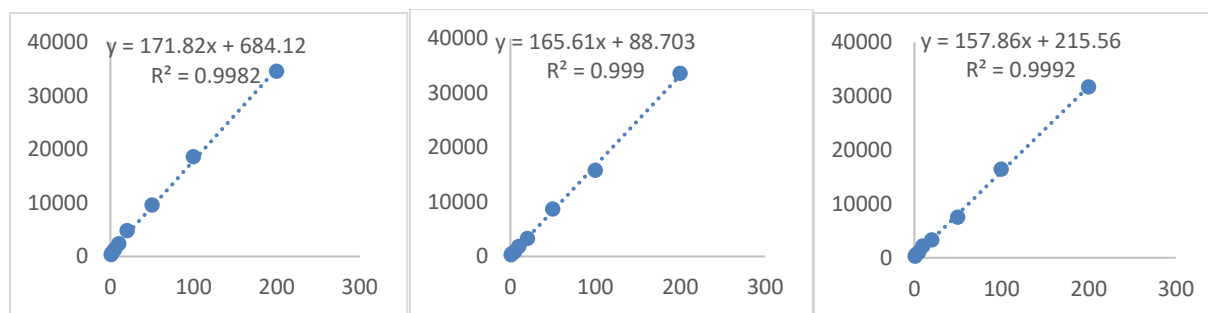


Fig. 3.91: Matrix matched calibration curves of NP-AOZ, NP-AMAZ and NP-AHD in beef samples

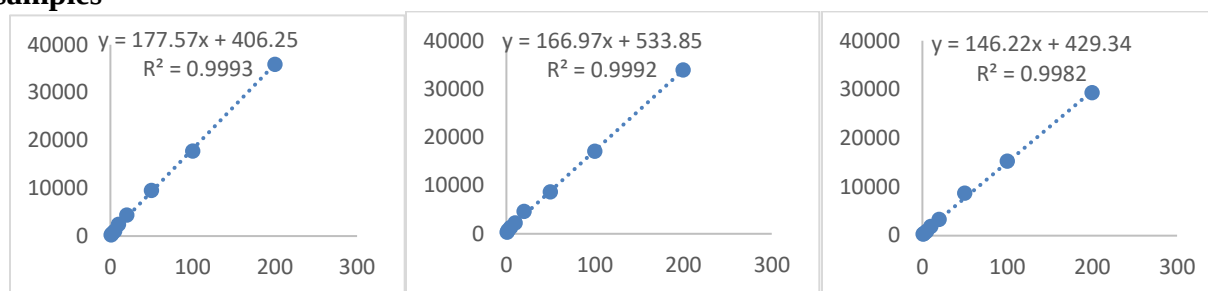


Fig. 3.92: Matrix matched calibration curves of NP-AOZ, NP-AMAZ and NP-AHD in egg samples

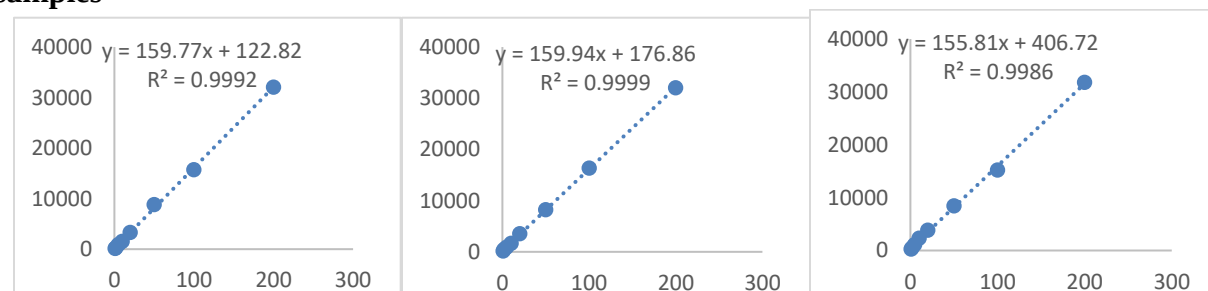


Fig. 3.93: Matrix matched calibration curves of NP-AOZ, NP-AMAZ and NP-AHD in milk samples

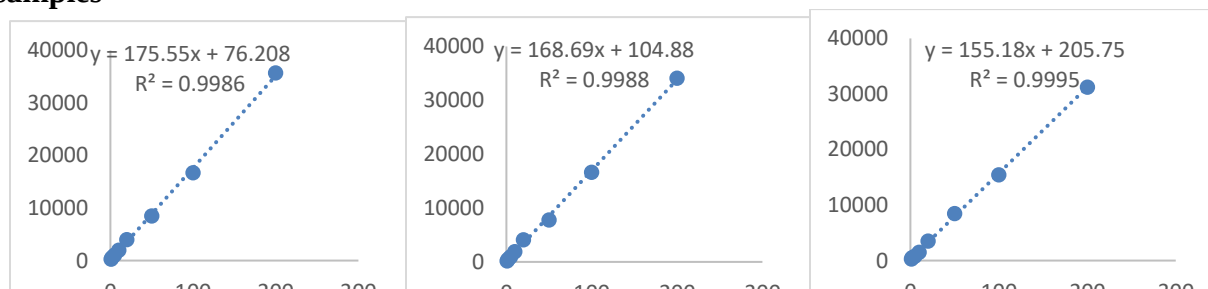


Fig. 3.94: Matrix matched calibration curves of NP-AOZ, NP-AMAZ and NP-AHD in poultry feed samples

3.5.5 Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ) & Retention time (t_R) of NFs

The linearity, sensitivity, and chromatographic behavior of NP-AOZ, NP-AMTZ and NP-AHD in the different matrices indicate that the developed LC-MS/MS method is highly suitable for quantitative residue analysis (Table 3.38-3.40). Matrix-matched calibration curves for NP-AOZ, NP-AMTZ and NP-AHD showed excellent linearity, with correlation coefficients (R^2) ranging from 0.9987-0.9993, 0.9954-0.9999 and 0.9982-0.9994 respectively, for the standard solution and all food or feed matrices. These values confirm a very strong linear relationship between analyte concentration and peak response and demonstrate that the calibration model is robust across poultry meat, beef, egg, milk and poultry feed. The linear range of the method was established between 1-200 $\mu\text{g kg}^{-1}$, ensuring reliable quantification across the expected sample concentrations.

All analytes exhibited low LOD ranging from 0.09-0.7, 0.1-0.7 and 0.5-1.1 respectively, and LOQ 0.2-2.3, 0.3-2.3 and 1.3-3.6 respectively, highlighting the high sensitivity of the LC-MS/MS method for detecting at trace levels of nitrofurans metabolites in complex, protein and fat rich matrices. Retention time (t_R) were recorded for each compound under optimized chromatographic conditions.

All NFs eluted at distinct and reproducible t_R , ensuring clear separation. Retention time across matrices suggests minimal matrix-dependent interactions with the stationary phase, ensuring stable peak shapes, good separation from potential interferences and a low risk of co-elution or matrix-induced ion suppression.

Table 3.38: NP-AOZ Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	R^2
Std NP-AOZ	1.9	1-200	-	-	$y = 158.17x + 102.41$	0.9989
Poultry meat	1.9		0.7	2.3	$y = 167.28x + 131.22$	0.9987
Beef	1.9		0.6	1.9	$y = 171.82x + 684.12$	0.9982
Egg	1.9		0.4	1.3	$y = 177.57x + 406.25$	0.9993
Milk	1.9		0.09	0.2	$y = 159.77x + 122.82$	0.9992
Poultry feed	1.8		0.7	2.3	$y = 175.55x + 76.208$	0.9986

Table 3.39: NP-AMTZ Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	R^2
Std NP-AMTZ	1.3	1-200	-	-	$y = 155.01x + 89.897$	0.9977
Poultry meat	1.6		0.1	0.3	$y = 166.23x + 81.508$	0.9954
Beef	1.3		0.7	2.3	$y = 165.61x + 88.703$	0.9990
Egg	1.3		0.6	1.9	$y = 166.97x + 533.85$	0.9992
Milk	1.3		0.14	0.4	$y = 159.94x + 176.86$	0.9999
Poultry feed	1.6		0.22	0.6	$y = 168.69x + 104.88$	0.9998

Table 3.40: NP-AHD Linearity, LOD, LOQ and t_R

Matrix	Retention time (t_R) min	Linearity ($\mu\text{g kg}^{-1}$)	LOD	LOQ	Matrix-matched calibration curves ($y=mx+C$)	R^2
Std NP-AHD	1.6	1-200	-	-	$y = 141.89x + 358.76$	0.9984
Poultry meat	1.6		0.5	1.6	$y = 153.96x + 90.332$	0.9987
Beef	1.9		0.8	2.6	$y = 157.86x + 215.56$	0.9992
Egg	1.5		0.7	2.3	$y = 146.22x + 429.34$	0.9982
Milk	1.5		1.1	3.6	$y = 155.81x + 406.72$	0.9986
Poultry feed	1.5		0.4	1.3	$y = 155.18x + 205.75$	0.9995

3.5.6 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of NFs in Samples

The average concentrations (mean $\pm$ SD) of nitrofurantoin derivative metabolites in the analyzed samples provide a quantitative estimate of residue levels and allow comparison of contamination between different matrices. The associated standard deviation ($\pm$ SD) values describe the spread of replicate measurements and therefore reflect the precision of the method and any influence of matrix complexity. Evaluation of intra-day and inter-day RSD% further confirms the repeatability and reproducibility of the procedure, demonstrating that the LC-MS/MS system produces stable responses over time. In parallel, the obtained recovery (%) values indicate the efficiency of the extraction and clean-up steps and assess the trueness of the method by comparing measured and spiking concentrations. When mean values, SD, RSD%, and recovery all fall within commonly accepted validation criteria, they collectively verify that the method is robust, accurate, and precise for routine determination of nitrofurantoin metabolite residues in diverse food and feed samples.

3.5.6.1 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of NFs in Poultry Meat Samples

At 5 $\mu\text{g L}^{-1}$ spiking level in poultry meat, NP-AOZ yielded mean concentrations of 4.40-5.37 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 5.02-9.14% and an inter-day RSD of 7.17%, indicating good precision and stable day-to-day reproducibility (Table 3.41). NP-AMTZ showed mean concentrations of 4.31-5.56 $\mu\text{g kg}^{-1}$, intra-day RSD% of 4.49-6.05%, and an inter-day RSD% of 5.25%, reflecting generally acceptable precision with slight matrix-related variability. NP-AHD presented higher recoveries, with mean values of 4.44-5.61 $\mu\text{g kg}^{-1}$, intra-day RSDs% of 4.65-6.91%, and an inter-day RSD% of 6.75%, indicating moderate but still acceptable variability.

At 10 $\mu\text{g kg}^{-1}$ level, NP-AOZ produced mean concentrations of 8.83-10.73 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 4.62-7.95% and an inter-day RSD% of 6.23%, confirming good repeatability and reproducibility at the higher spiking level (Table 3.41). NP-AMTZ showed mean values of 8.68-11.40 $\mu\text{g kg}^{-1}$, intra-day RSD% of 1.91-4.94%, and an inter-day RSD% of 3.53%, indicating high precision with slightly increased variability in this matrix. NP-AHD yielded mean concentrations of 8.89-11.54 $\mu\text{g kg}^{-1}$, intra-day RSD% of 2.38-5.25%, and an inter-day RSD% of 3.26%, demonstrating reliable quantification with only minor dispersion.

The mean concentrations for NP-AOZ, NP-AMTZ, and NP-AHD were in close agreement with the fortified levels at both 5 and 10 $\mu\text{g kg}^{-1}$, and intra- and inter-day RSD% values were generally below or around 10%. These findings confirm that the method affords satisfactory repeatability and reproducibility, providing reliable quantification of NFs residues in poultry meat.

The recovery data for the nitrofurans metabolites (NP-AOZ, NP-AMTZ, and NP-AHD) demonstrated acceptable accuracy, indicating that the analytical method quantified these compounds consistently and reliably in all tested matrices.

The intra- and inter-day recovery (%) of NP-AOZ, NP-AMTZ, and NP-AHD in poultry meat at spiking levels of 5 and 10 $\mu\text{g kg}^{-1}$, illustrating the accuracy of the method (Table 3.41). At 5 $\mu\text{g kg}^{-1}$, intra-day recoveries for NP-AOZ of 88, 92 and 107%, NP-AMTZ of 86, 97 and 111% and NP-AHD of 98, 88 and 112 % all fall within the commonly accepted 80-120% range, while the corresponding inter-day recoveries are 95, 98 and 99%, respectively, indicating consistent accuracy across different days. At the higher 10 $\mu\text{g kg}^{-1}$ level, intra-day recoveries remain

satisfactory, ranging from 88-107% for NP-AOZ, 86-114% for NP-AMOZ, and 88-115% for NP-AHD, with inter-day recoveries of 99, 100 and 104%, respectively.

Collectively, both intra-day and inter-day recovery values are clustered close to 100%, confirming that the analytical procedure provides accurate and reliable quantification of nitrofurán metabolites in poultry meat samples, with minimal concentration-dependent or matrix-induced bias.

Table 3.41: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of NFs in Poultry Meat Samples

Compound Name	Spiking Level ($\mu\text{g kg}^{-1}$)	Average $\pm$ SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
NP-AOZ	5	4.4008 $\pm$ 0.3240	7.36	7.17	88	95
		4.6256 $\pm$ 0.4228	9.14		92	
		5.3717 $\pm$ 0.2701	5.02		107	
NP-AMOZ		4.3138 $\pm$ 0.2613	6.05	5.25	86	98
		4.8673 $\pm$ 0.2186	4.49		97	
		5.5699 $\pm$ 0.2911	5.22		111	
NP-AHD		4.9199 $\pm$ 0.3400	6.91	6.75	98	99
		4.4405 $\pm$ 0.3858	8.68		88	
		5.6187 $\pm$ 0.2614	4.65		112	
NP-AOZ	10	8.8377 $\pm$ 0.4089	4.62	6.23	88	99
		10.7327 $\pm$ 0.6561	6.11		107	
		10.2736 $\pm$ 0.8175	7.95		102	
NP-AMOZ		8.6825 $\pm$ 0.1664	1.91	3.53	86	100
		10.0889 $\pm$ 0.4993	4.94		100	
		11.4004 $\pm$ 0.4278	3.75		114	
NP-AHD		10.8695 $\pm$ 0.3521	3.23	3.26	108	104
		11.5463 $\pm$ 0.2750	2.38		115	
		8.8923 $\pm$ 0.3706	4.16		88	

3.5.6.2 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of NFs in Beef Samples

At 5 $\mu\text{g kg}^{-1}$ spiking level in beef, NP-AOZ yielded mean concentrations of 4.42-5.31 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 5.59-8.74% and an inter-day RSD% of 7.76%, indicating good precision and consistent performance between days (Table 3.42). NP-AMOZ gave mean concentrations of

4.30-5.71 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 3.25-8.79% and an inter-day RSD% of 6.32%, suggesting acceptable precision with only minor matrix effects. For NP-AHD, mean values of 4.43-5.75 $\mu\text{g kg}^{-1}$, intra-day RSD% of 3.32-8.64%, and an inter-day RSD% of 5.84% indicated moderate, yet analytically acceptable, variability.

At 10 $\mu\text{g kg}^{-1}$ level, NP-AOZ showed mean concentrations of 8.81-10.40 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 8.79-9.36% and an inter-day RSD% of 9.14%, supporting good repeatability and day-to-day reproducibility at the higher spiking level (Table 3.42). NP-AMTZ presented mean concentrations of 8.60-11.40 $\mu\text{g kg}^{-1}$, intra-day RSD% of 4.09-8.59%, and an inter-day RSD% of 6.35%, reflecting generally sound precision despite somewhat greater variability in beef. NP-AHD produced mean concentrations of 8.89-11.56 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 4.99-6.29% and inter-day RSD% of 4.63%, confirming robust quantification with limited dispersion.

Taken together, the mean concentrations of NP-AOZ, NP-AMTZ, and NP-AHD were in close agreement with the nominal 5 and 10 $\mu\text{g kg}^{-1}$ levels, and both intra- and inter-day RSD% generally remained at or below 10%. These findings demonstrate satisfactory repeatability and reproducibility, confirming the method's reliability for monitoring NF residues in beef.

The intra- and inter-day recovery (%) of NP-AOZ, NP-AMTZ and NP-AHD in beef samples at 5 and 10 $\mu\text{g kg}^{-1}$ spiking levels, illustrating the accuracy and robustness of the method (Table 3.42). At 5 $\mu\text{g kg}^{-1}$, intra-day recoveries for NP-AOZ of 88, 90 and 106 %, NP-AMTZ of 86, 101 and 114% and NP-AHD of 88, 115 and 93% are generally within the acceptable 70-120% range ([SANTE/11312/2021; CODEX guideline, 2017](#)), while the corresponding inter-day recoveries of 95, 100 and 99% demonstrate good accuracy across different days. At the higher 10 $\mu\text{g kg}^{-1}$ level, intra-day recoveries remain satisfactory for all analytes, ranging from 88-104% for NP-AOZ, 86-114% for NP-AMTZ and 88-115% for NP-AHD, with inter-day recoveries of 93, 100 and 102%, respectively. Overall, these recoveries at both spiking levels are clustered close to 100%, confirming that the analytical method provides accurate and reproducible quantification of nitrofurans metabolites in beef matrices across both concentration levels, with no significant concentration-dependent or matrix-induced performance issues observed and supporting its suitability for routine monitoring.

Table 3.42: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of NFs in Beef Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
NP-AOZ	5	4.4271 ± 0.3873	8.74	7.76	88	95
		4.5214 ± 0.4050	8.95		90	
		5.3176 ± 0.2977	5.59		106	
NP-AMTZ		4.3034 ± 0.3782	8.79	6.32	86	100
		5.0956 ± 0.3532	6.93		101	
		5.7103 ± 0.1860	3.25		114	
NP-AHD		4.4358 ± 0.3834	8.64	5.84	88	99
		5.7597 ± 0.1914	3.32		115	
		4.6904 ± 0.2613	5.57		93	
NP-AOZ	10	8.8911 ± 0.8244	9.27	9.14	88	93
		8.8166 ± 0.7757	8.79		88	
		10.4043 ± 0.9743	9.36		104	
NP-AMTZ		8.6027 ± 0.7391	8.59	6.35	86	100
		10.0350 ± 0.6413	6.39		100	
		11.4093 ± 0.4667	4.09		114	
NP-AHD		8.8954 ± 0.5600	6.29	4.63	88	102
		10.2523 ± 0.5118	4.99		102	
		11.5662 ± 0.3017	2.60		115	

3.5.6.3 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of NFs in Egg Samples

At 5 $\mu\text{g kg}^{-1}$ spiking level in egg samples, NP-AOZ yielded mean concentrations of 4.31-5.06 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 7.15-8.40% and an inter-day RSD% of 7.82%, indicating good precision and consistent performance across days (Table 3.43). NP-AMTZ presented mean concentrations of 4.58-5.67 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSDs% of 3.50-8.74% and an inter-day RSD% of 6.60%, suggesting generally acceptable precision with only slight matrix effects. For NP-AHD, mean values ranged from 4.23-5.79 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 4.24-8.80% and an inter-day RSD% of 6.13%, reflecting moderate but still acceptable variability.

At the higher 10 $\mu\text{g kg}^{-1}$ spiking level, NP-AOZ gave mean concentrations of 8.69-10.15 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 6.03-9.95% and an inter-day RSD% of 8.02%, confirming satisfactory repeatability and day-to-day reproducibility (Table 3.43). NP-AMTZ showed mean concentrations

of 9.13-11.30 $\mu\text{g kg}^{-1}$, intra-day RSD% of 2.87-8.23%, and an inter-day RSD% of 5.44%, indicating good precision despite slightly increased variability. NP-AHD produced mean concentrations of 8.46-11.52 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 3.36-8.17% and an inter-day RSD% of 5.85%, demonstrating reliable quantification with only slight dispersion.

Overall, the mean concentrations for NP-AOZ, NP-AMTZ, and NP-AHD were in close agreement with the nominal 5 and 10 $\mu\text{g kg}^{-1}$ levels, and intra- and inter-day RSD% values were generally at or below 10%. These results indicate satisfactory repeatability and reproducibility and confirm that the method is suitable for reliable quantification of NF residues in egg monitoring.

At 5 $\mu\text{g kg}^{-1}$ spiking level in egg samples, NP-AOZ showed intra-day recoveries of 86, 101 and 89%, with an inter-day recovery of 92%, indicating generally good accuracy with only slight under recovery that can be ascribed to mild matrix effects in the egg extract (Table 3.43). NP-AMTZ performed comparably well, giving intra-day recoveries of 113, 94 and 91% and an inter-day recovery of 99%, suggesting a balance between minor ion enhancement and suppression while remaining comfortably within typical validation limits. NP-AHD displayed intra-day recoveries of 84, 91 and 115% and an inter-day value of 97%, reflecting somewhat broader variability but still acceptable accuracy for residues in this matrix.

At 10 $\mu\text{g kg}^{-1}$ level, NP-AOZ yielded intra-day recoveries of 101, 100 and 86% with an inter-day recovery of 96%, again indicating slight suppression at higher concentration but overall satisfactory trueness and reproducibility (Table 3.43). NP-AMTZ showed intra-day recoveries of 113, 91 and 100% and an inter-day recovery of 101%, pointing to negligible net suppression and stable chromatographic response over time. NP-AHD gave intra-day recoveries of 115, 91 and 84% with an inter-day recovery of 97%, consistent with modest matrix influence but adequate method robustness.

Overall, the recovery data demonstrate that the chromatographic method provides reliable quantification of NP-AOZ, NP-AMTZ and NP-AHD in egg samples, with all recoveries falling within accepted ranges and only minor matrix-induced suppression or enhancement.

Table 3.43: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of NFs in Egg Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
NP-AOZ	5	4.3168 ± 0.3089	7.15	7.82	86	92
		5.0613 ± 0.4253	8.40		101	
		4.4802 ± 0.3550	7.92		89	
NP-AMTZ		5.6701 ± 0.1985	3.50	6.60	113	99
		4.7191 ± 0.4124	8.74		94	
		4.5885 ± 0.3472	7.56		91	
NP-AHD		4.2337 ± 0.3729	8.80	6.13	84	97
		4.5620 ± 0.2443	5.35		91	
		5.7957 ± 0.2457	4.24		115	
NP-AOZ	10	10.1557 ± 0.8215	8.08	8.02	101	96
		10.0960 ± 0.6095	6.03		100	
		8.6926 ± 0.8651	9.95		86	
NP-AMTZ		11.3071 ± 0.3255	2.87	5.44	113	101
		9.1318 ± 0.4772	5.22		91	
		10.0398 ± 0.8270	8.23		100	
NP-AHD		11.5200 ± 0.3880	3.36	5.85	115	97
		9.1961 ± 0.5546	6.03		91	
		8.4671 ± 0.6920	8.17		84	

3.5.6.4 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of NFs in Milk Samples

At 5 $\mu\text{g L}^{-1}$ spiking level in milk samples, NP-AOZ yielded mean concentrations of 4.11-5.51 $\mu\text{g L}^{-1}$, with intra-day RSD% of 5.26-7.37% and an inter-day RSD% of 6.33%, indicating good precision and consistent day-to-day performance (Table 3.44). NP-AMTZ showed slightly greater variability, giving mean concentrations of 4.85-5.33 $\mu\text{g L}^{-1}$, intra-day RSDs% of 6.52-7.38%, and an inter-day RSD% of 7.86%, which still reflects acceptable precision with minor matrix-related effects. NP-AHD displayed comparatively higher recoveries, with mean values of 4.91-5.53 $\mu\text{g L}^{-1}$, intra-day RSD% of 2.37-6.62%, and an inter-day RSD% of 5.18%, indicating moderate but analytically acceptable variability.

At 10 $\mu\text{g L}^{-1}$ level, NP-AOZ provided mean concentrations of 9.45-11.01 $\mu\text{g L}^{-1}$, along with intra-day RSD% of 2.90-4.94% and an inter-day RSD% of 4.15%, confirming good repeatability and

reproducibility at the higher spiking level (Table 3.44). NP-AMTZ produced mean concentrations of 9.76-10.62 $\mu\text{g L}^{-1}$, with intra-day RSD% of 2.95-9.79% and an inter-day RSD% of 5.52%, demonstrating acceptable precision despite somewhat increased variability in this matrix. NP-AHD gave mean concentrations of 9.67-11.02 $\mu\text{g L}^{-1}$, with intra-day RSD% of 6.59-8.93% and an inter-day RSD% of 7.57%, showing reliable quantification even with slightly higher dispersion. Overall, the mean concentrations for NP-AOT, NP-AMTZ, and NP-AHD were in close agreement with the nominal 5 and 10 $\mu\text{g L}^{-1}$ fortification levels, and intra- and inter-day RSD% values generally remained at or below 10%. These findings indicate satisfactory repeatability and reproducibility and confirm that the method enables reliable quantification of NFs residues in milk monitoring.

At 5 $\mu\text{g L}^{-1}$ spiking level in milk samples, NP-AOT gave intra-day recoveries of 82, 99 and 110%, with an inter-day recovery of 97%, indicating generally good accuracy with a slight tendency to ion suppression at the lower value and mild enhancement at the upper value (Table 3.44). NP-AMTZ showed intra-day recoveries of 97, 97 and 106% and an inter-day recovery of 103%, suggesting near-quantitative extraction and only minor ion enhancement in the LC-MS/MS system. NP-AHD exhibited intra-day recoveries of 98, 102 and 110% and an inter-day value of 103%, reflecting consistently high accuracy with a small positive bias that remains within standard validation limits.

At 10 $\mu\text{g L}^{-1}$ level, NP-AOT produced intra-day recoveries of 82, 110 and 94%, with an inter-day recovery of 95%, again showing acceptable accuracy with slight matrix-related suppression/enhancement but stable day-to-day performance (Table 3.44). NP-AMTZ yielded intra-day recoveries of 97, 102 and 106% and an inter-day recovery of 102%, indicating excellent trueness and reproducibility with negligible suppression. NP-AHD showed intra-day recoveries of 110, 96 and 104% and an inter-day value of 103%, confirming robust quantification with only modest enhancement.

Recoveries for all three nitrofurans metabolites fall within the typical 70-120% acceptance window ([SANTE/11312/2021](#); [CODEX guideline, 2017](#)) and inter-day values remain close to 100%, demonstrating that the chromatographic method provides reliable, accurate and reproducible determination of NFs in milk.

Table 3.44: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of NFs in Milk Samples

Compound Name	Spiking Level (µg L ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
NP-AOZ	5	4.1157 ± 0.3035	7.37	6.33	82	97
NP-AMOZ		4.9595 ± 0.3163	6.37		99	
		5.5128 ± 0.2901	5.26		110	
		4.8539 ± 0.3166	6.52	97	103	
5.3328 ± 0.5158		9.67	7.86	97		
5.3341 ± 0.3939		7.38		106		
NP-AHD		4.9116 ± 0.3252	6.62	5.18	98	103
		5.1221 ± 0.3358	6.55		102	
		5.5303 ± 0.1313	2.37		110	
NP-AOZ	10	8.2642 ± 0.4089	4.94	4.15	82	95
NP-AMOZ		11.0182 ± 0.3205	2.90		110	
		9.4522 ± 0.4361	4.61		94	
		9.7657 ± 0.2890	2.95	5.52	97	102
10.2272 ± 1.0019		9.79	102			
10.6211 ± 0.4067		3.82	106			
NP-AHD		11.0216 ± 0.7272	6.59	7.57	110	103
		9.6738 ± 0.8641	8.93		96	
		10.4529 ± 0.7523	7.19		104	

3.5.6.5 Average $\pm$ SD & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery Percentage (%) of NFs in Poultry Feed Samples

At 5 $\mu\text{g kg}^{-1}$ spiking level in beef samples, NP-AOZ showed mean concentrations of 4.41-5.36 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 2.75-7.72% and an inter-day RSD% of 3.97%, indicating good precision and consistent performance between days (Table 3.45). NP-AMOZ displayed somewhat greater variability, with mean concentrations of 4.34-5.70 $\mu\text{g kg}^{-1}$, intra-day RSD% of 3.14-9.94%, and an inter-day RSD% of 6.79%, which still reflects generally acceptable precision with minor matrix effects. NP-AHD gave higher recoveries, with mean values of 4.14-5.51 $\mu\text{g kg}^{-1}$, intra-day RSD% of 3.90-7.79%, and an inter-day RSD% of 5.71%, indicating moderate but analytically acceptable variability.

At 10 $\mu\text{g kg}^{-1}$ level, NP-AOZ yielded mean concentrations of 8.06-9.79 $\mu\text{g kg}^{-1}$, accompanied by intra-day RSD% of 1.99-6.16% and an inter-day RSD% of 4.21%, confirming good repeatability

and reproducibility at the higher spiking level (Table 3.45). NP-AMTZ produced mean values of 8.69-11.36 $\mu\text{g kg}^{-1}$, with intra-day RSD% of 3.15-8.46% and an inter-day RSD% of 5.09%, demonstrating acceptable precision despite somewhat increased variability in this matrix. NP-AHD showed mean concentrations of 8.99-11.65 $\mu\text{g kg}^{-1}$, intra-day RSD% of 2.02-8.65%, and an inter-day RSD% of 5.05%, indicating reliable quantification even with slightly higher dispersion. Overall, the mean concentrations for NP-AOT, NP-AMTZ, and NP-AHD were close to the nominal 5 and 10 $\mu\text{g kg}^{-1}$ levels, and intra- and inter-day RSD% values were generally at or below 10%, confirming satisfactory repeatability, reproducibility, and reliable quantification of NF residues in this matrix.

At 5 $\mu\text{g kg}^{-1}$ spiking level in poultry feed, NP-AOT showed intra-day recoveries of 107, 92 and 88% with an inter-day recovery of 95%, indicating only mild matrix-induced ion suppression/enhancement and overall accurate quantification (Table 3.45). NP-AMTZ exhibited intra-day recoveries of 86, 107 and 114%; the low value of 86% points to some suppression in individual injections, whereas the higher values suggest slight ion enhancement, but the inter-day recovery of 102% confirms that these effects average out and remain well within accepted validation limits. NP-AHD gave intra-day recoveries of 88, 115 and 103% and an inter-day value of 102%, reflecting a tendency toward slightly enhancement yet still providing reliable accuracy at this lower spike level. At 10 $\mu\text{g kg}^{-1}$ level, NP-AOT again performed robustly, with intra-day recoveries of 88, 107 and 97% and an inter-day recovery of 97%, indicating that matrix effects are limited and the response of the separation system remains stable at the higher concentration (Table 3.45). NP-AMTZ produced intra-day recoveries of 86, 113 and 104% and an inter-day recovery of 101%; although the 86% recovery suggests localized suppression, overall trueness is high. NP-AHD showed intra-day recoveries of 88, 106 and 115% with an inter-day value of 103%, confirming consistent, slightly enhanced signal but still comfortably within typical 80-120% validation criteria.

These results indicate that while minor matrix-related suppression or enhancement can occur particularly for NP-AMTZ and NP-AHD the recoveries for NP-AOT, NP-AMTZ and NP-AHD all fall within acceptable validation limits at both 5 and 10 $\mu\text{g L}^{-1}$, demonstrating that the LC-MS/MS method affords reliable and accurate quantification of nitrofurans residues in poultry feed.

Table 3.45: (Average $\pm$ SD) & Intra- and Inter-day Relative Standard Deviation (RSD%), Intra- and Inter-day Recovery (%) of NFs in Poultry Feed Samples

Compound Name	Spiking Level (µg kg ⁻¹)	Average ± SD	Intra-day RSD%	Inter-day RSD%	Intra-day Recovery (%)	Inter-day Recovery (%)
NP-AOZ	5	5.3670 ± 0.0768	1.43	3.97	107	95
		4.6026 ± 0.1266	2.75		92	
		4.4169 ± 0.3412	7.72		88	
NP-AMOZ		4.3471 ± 0.4323	9.94	6.79	86	102
		5.3643 ± 0.3908	7.28		107	
		5.7094 ± 0.1794	3.14		114	
NP-AHD		4.1423 ± 0.2250	5.43	5.71	88	102
		5.5120 ± 0.2151	3.90		115	
		4.9044 ± 0.3823	7.79		103	
NP-AOZ	10	8.0683 ± 0.4973	6.16	4.21	88	97
		9.7920 ± 0.1954	1.99		107	
		8.8680 ± 0.3969	4.47		97	
NP-AMOZ		8.6959 ± 0.7362	8.46	5.09	86	101
		11.3683 ± 0.3587	3.15		113	
		10.4968 ± 0.3856	3.67		104	
NP-AHD		8.9953 ± 0.3925	4.36	5.05	88	103
		10.7330 ± 0.9288	8.65		106	
		11.6560 ± 0.2363	2.02		115	

3.5.7 Matrix effect of NFs

The matrix-induced ionization effects for NP-AOZ, NP-AMOZ and NP-AHD (Table 3.46) show clear variation among the different food and feed matrices, confirming that matrix composition noticeably influences the ESI response in LC-MS/MS. Overall, the effects are moderate and positive 1.01-12.26%, indicating ion enhancement rather than suppression. For NP-AOZ, matrix effects range from 1.01% in milk to 12.26% in egg. The very low value in milk suggests that this matrix has minimal impact on NP-AOZ ionization, whereas the higher enhancement in egg and poultry feed (10.98%) reflects the presence of co-extracted components that slightly boost signal intensity. NP-AMOZ shows a similar pattern, with matrix effects between 3.18% (milk) and 8.72% (poultry feed), indicating mild to moderate enhancement across all matrices but no severe interference. NP-AHD tends to be somewhat more affected in certain matrices, with values of 8.50% in poultry meat, 11.17% in beef and 9.81% in milk, while egg shows the lowest effect 3.05%.

Taken together, poultry meat, beef and milk cause low to moderate ion enhancement for all three nitrofurans, whereas egg and poultry feed generally produce stronger matrix effects, particularly for NP-AOZ and NP-AHD. Although these percentages remain within typical acceptable limits, they underline the importance of using matrix-matched calibration and/or isotopically labeled internal standards to compensate for matrix-induced ionization changes and to ensure accurate, unbiased quantification of nitrofuran residues in these diverse sample types.

Table 3.46: Matrix-Induced Ionization Effects of NFs in Different Matrices

Matrix	Matrix effects (%)		
	NP-AOZ	NP-AMTZ	NP-AHD
Poultry meat	5.75	7.24	8.50
Beef	8.63	6.83	11.17
Egg	12.26	7.67	3.05
Milk	1.01	3.18	9.81
Poultry feed	10.98	8.72	9.50

3.5.8 NFs Analysis in Real Samples

Out of 150 samples of poultry meat, beef, egg and milk collected from super shops and local markets in Dhaka and Gazipur, only a small number were found positive for the targeted nitrofuran metabolites, although measurable residues were present in several matrices (Table 3.47). All the samples were matched with standards properly in this method (Fig. 3.95-3.100). NP-AOZ was detected in a single poultry meat sample at $18 \mu\text{gkg}^{-1}$ and in two beef samples at 15 and $17 \mu\text{gkg}^{-1}$, indicating low to moderate contamination levels in these animal tissues (Fig. 3.101). NP-AMTZ was found at slightly higher concentrations, with $22 \mu\text{gkg}^{-1}$ in poultry meat and a maximum amount of $37 \mu\text{gkg}^{-1}$ in egg, suggesting occasional exposure to this metabolite through these products (Fig. 3.102). In contrast, NP-AHD was not detected in any sample, and all analyzed milk samples were free of detectable nitrofuran residues, highlighting that contamination with these compounds was limited and matrix-specific within the surveyed area.

Overall, the results demonstrate that, although the number of contaminated samples was small, residues of NP-AOZ and NP-AMTZ can occur in edible animal products, underlining the need for continued monitoring of nitrofuran metabolites in poultry meat, beef and eggs.

Table 3.47: Amount of targeted NFs compound

Compound name	Matrix	No of Samples	Amount (μgkg^{-1})	Sample detection frequency (%)
NP-AOZ	Poultry meat	1	18	3
NP-AOZ	Beef	1	15	3
NP-AOZ	Beef	1	17	3
NP-AMOX	Poultry meat	1	22	3
NP-AMOX	Egg	1	37	3

3.5.9 Chromatograms of NFs

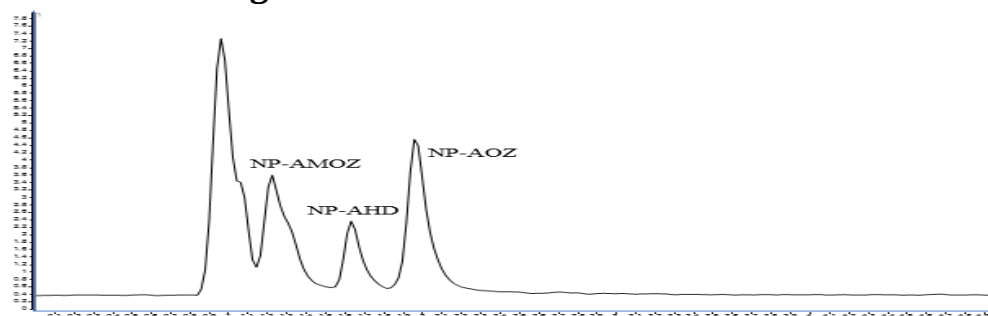


Fig. 3.95: Standards of NFs

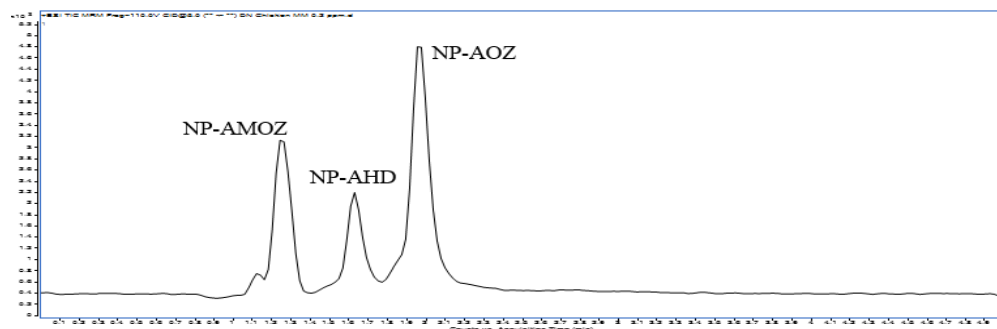


Fig. 3.96: NFs Matrix-matched in poultry meat

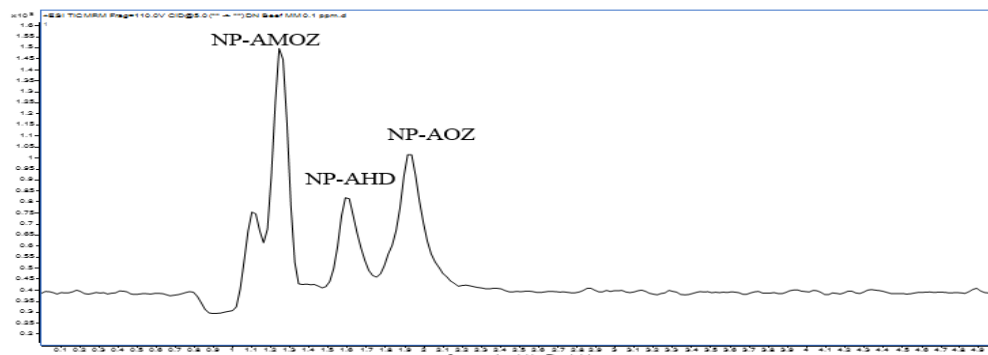


Fig. 3.97: NFs Matrix-matched in beef

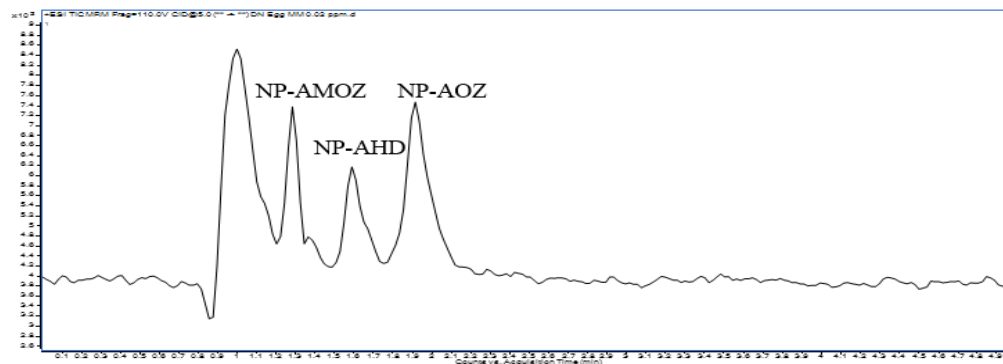


Fig. 3.98: NFs Matrix-matched in egg

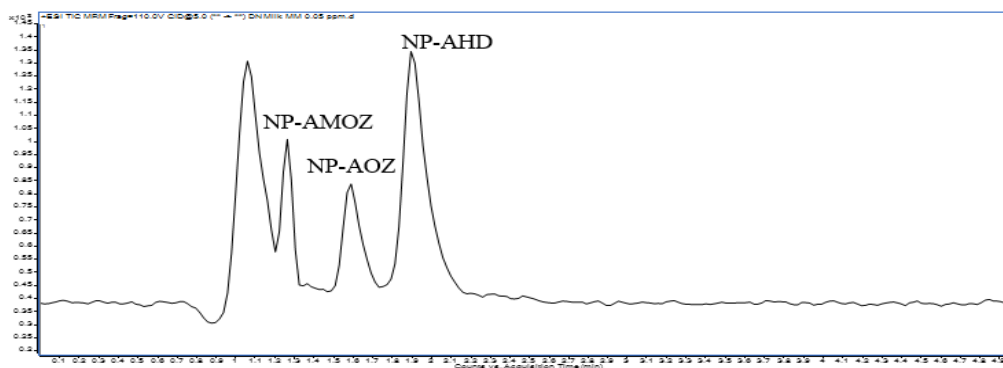


Fig.3.99: NFs Matrix-matched in milk

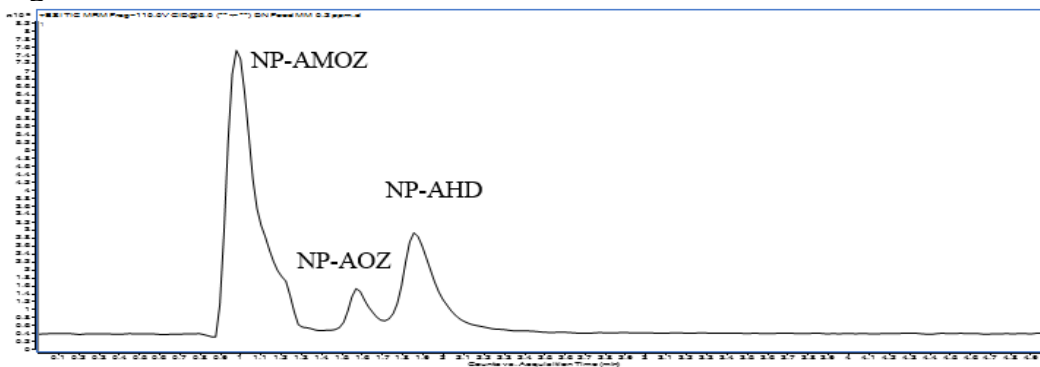


Fig.3.100: NFs Matrix-matched in poultry feed

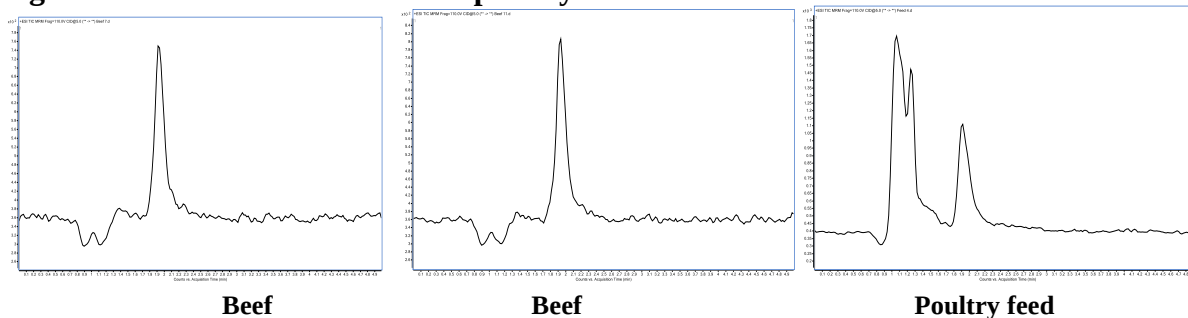


Fig.3.101: Targeted NP-AOZ in two beef, and in one poultry feed

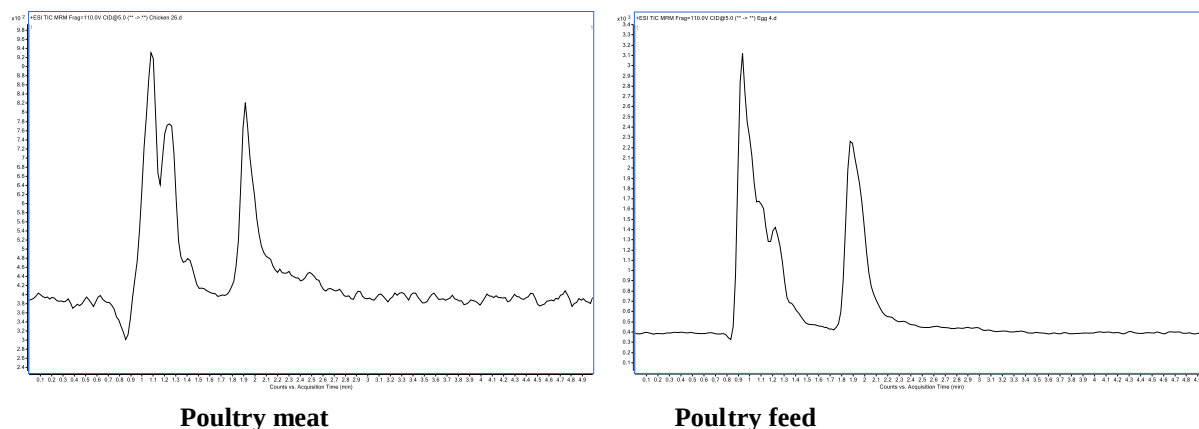


Fig. 3.102: Targeted NP-AMAZ in one poultry meat and one egg

3.6 Data Comparison with International Studies

The validated methods were systematically compared with previously reported approaches for all targeted class of antibiotic residues determination in poultry meat, beef, milk, egg and poultry feed samples to consider method variation, matrix-specific comparisons.

Table 3.48: Comparison with Previous reported methods for analysis of Antibiotic Residues

Sample	Instrument	Targeted Analytes	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	Range (µg/kg)	Ref.
Poultry Meat	LC-MS/MS	SMX	0.1	0.3	99-103	1.25-100	This study
		SDZ	0.2	0.6	85-102	5-200	
		SMT	0.6	1.8	94-104		
		CIP	0.2	0.6	90-105		
		ENR	0.1	0.3	93-105		
		LEV	0.8	2.6	87-106		
		TC	0.27	0.83	98-106		
		OTC	0.1	0.3	96-106	1.5-100	
		CTC	0.1	0.3	95-106		
		CAP	0.06	0.1	92-101		
		NP-AOZ	0.7	2.3	88-107		
		NP-AMOZ	0.1	0.3	86-114		
		NP-AHD	0.5	1.6	98-115		
	LC-MS/MS	CTC	0.32-			0.003-	(Lee et al., 2018)
		ENR	0.40			0.02	
		TC					
	LC-MS/MS	CIP	0.28–0.61				

Beef	LC-MS/MS	SMX	0.1	0.3	99-111	1.25-100	This study
		SDZ	0.1	0.3	93-108		
		SMT	0.1	0.3	92-100		
		CIP	0.8	2.6	88-105	5-200	
		ENR	0.1	0.3	96-102		
		LEV	0.8	2.6	96-102		
		TC	0.06	0.17	92-109	1.5-100	
		OTC	0.1	0.3	99-108		
		CTC	0.1	0.3	98-105		
		CAP	0.1	0.3	90-112	1.5-100	
Egg	LC-MS/MS	NP-AOZ	0.6	1.9	88-106	1-200	This study
		NP-AMOX	0.7	2.3	86-114		
		NP-AHD	0.8	2.6	88-115		
	HPLC	SDM	-	-	-	0.003–0.146	
	HPLC	SMZ					
		ENR	-	-	-	52.0-91.5	
		CIP					
		OTC					
	LC-MS/MS	SMX	0.6	1.8	93-101	1.25-100	This study
		SDZ	0.9	2.7	77-109		
		SMT	0.7	2.1	94-102		
		CIP	0.5	1.6	93-105	5-200	
		ENR	0.4	1.3	97-110		
		LEV	0.6	1.9	98-106		
		TC	0.03	0.99	78-101	1.5-100	
		OTC	0.6	1.8	94-104		
		CTC	0.6	1.8	93-107		
		CAP	0.13	0.3	87-95	1.5-100	
Egg	LC-MS/MS	NP-AOZ	0.4	1.3	86-101	1-200	This study
		NP-AMOX	0.6	1.9	91-113		
		NP-AHD	0.7	2.3	84-115		
	UHPLC-MS/MS	SMX	0.002	0.008	93.02-98.55	0.01-1.0	
	LC-MS/MS	TC	1.5-3.0	5.0-10.0	80.1-96.5	6.44-347.50	
		OTC					
		CTC					
	UPLC-FLD	OTC	5.5-10.5	19.2-35.3	Above 83.5	50.1-54.0	
		CTC					

Milk	LC-MS/MS	SMX	0.2	0.6	87-103	1.25-100	This study
		SDZ	0.3	0.9	87-104		
		SMT	0.3	0.9	88-100		
		CIP	0.3	0.9	93-101	5-200	
		ENR	0.3	0.9	82-101		
		LEV	0.6	1.9	103-111		
		TC	0.15	0.48	96-108	1.5-100	
		OTC	0.2	0.6	97-103		
		CTC	0.2	0.6	90-105		
		CAP	0.2	0.6	94-111	1.5-100	
		NP-AOZ	0.09	0.2	82-110	1-200	
		NP-AMOX	0.14	0.4	97-106		
		NP-AHD	1.1	3.6	96-110		
	HPLC	TC	0.027-0.060	0.089-0.110	99.25-100.75	-	(Rinky et al., 2025)
		OTC					
		CTC					
		ENR					
Poultry feed	LC-MS/MS	SMX	0.1	0.3	93-101	1.25-100	This study
		SDZ	0.5	1.5	79-108		
		SMT	0.2	0.6	93-101		
		CIP	0.7	2.3	79-105	5-200	
		ENR	0.5	1.6	76-111		
		LEV	0.6	1.9	87-106		
		TC	0.2	0.66	94-102	1.5-100	
		OTC	0.1	0.3	86-104		
		CTC	0.1	0.3	80-94		
		CAP	0.7	2.3	89-96	1.5-100	
		NP-AOZ	0.7	2.3	88-107	1-200	
		NP-AMOX	0.22	0.6	86-114		
		NP-AHD	0.4	1.3	88-115		
	QTrap 5500 MS/MS	CAP	11.0	39.0	50-60		(Kenjeri et al., 2024)
		ENR	14.4	43.1			
		TC	11.8	49.4			
		OTC	12.3	41.1			
		CIP	14.3	38.9			
		ENR	13.4	36.3			
		SDZ	2.1	6.3			
		SMX	6.9	20.8			
		SMT	10.3	30.9			

3.7 Dietary Exposure, Health Hazard Quotient and Health Risk Assessments of Antibiotics

Dietary exposure to antibiotic residues was assessed through the estimated daily intake (EDI) of commonly identified antibiotics namely sulfadimethoxine (SMX), sulfadiazine (SDZ), sulfamethazine (SMT), ciprofloxacin (CIP), enrofloxacin (ENR), levofloxacin (LEV), tetracycline (TC), oxytetracycline (OTC),

chlortetracycline (CTC), chloramphenicol (CAP) and metabolites of nitrofurantoin derivatives (NP-AOZ, NP-AMOZ and NP-AHD) through the consumption of edible animal-derived foods including poultry meat, beef egg and milk to evaluate human exposure levels (Moudgil et al. 2019).

$$EDI = \frac{C \times FCR}{BW}$$

Where C is the mean residue level of antibiotics in poultry meat, beef, egg and milk (in micrograms per gram), FCR is the food consumption rate (in grams per person per day), and BW is the average body weight 60 kg (adult) for Bangladeshi peoples.

The acceptable daily intake (ADI) values applied in this study were as follows: 50 µg/kg bw for SMX, SDZ, SMT; 20 µg/kg b w for CIP, ENR, LEV; 30 µg/kg bw for TC, OTC, CTC, respectively. CAP and metabolites of nitrofurantoin derivatives (NP-AOZ, NP-AMOZ and NP-AHD) have no established ADI values according to Jahan et al., 2025 and FAO/WHO 2025 guidelines.

The health hazard quotient (HQ) presents the long-term health hazard risk assessment to characterize the potential for non-carcinogenic health effects resulting from exposure with the consumption of residual antibiotics contaminated food. It is determined by comparing the estimated daily intake (EDI) with its corresponding toxicological reference values of acceptable daily intake (ADI) (Jahan et al., 2025).

$$HQ = \frac{EDI}{ADI} \times 100\%$$

where ADI values are obtained from the committee for veterinary medicinal products for a standard body weight (60 kg per person).

Dietary intake data for the Bangladeshi adult population were considered based on Household Income and Expenditure Survey (HIES) Report, 2022. According to HIES-2022 report, an average Bangladeshi adult with a body weight of 60 kg consumes approximately 26.17 g, 11.66 g, and 12.73 g of poultry meat, beef and eggs, respectively per day along with 34.10 g of milk.

In addition, antibiotic residues related health risks were assessed based on the maximum residue limits (MRLs) and guideline provided by the Codex Alimentarius, from which ADI values were also obtained. Sample concentrations were compared against MRLs to evaluate compliance, while detection and exceedance rates were quantified (Zhung et al., 2021).

$$\text{Detection frequency (\%)} = \frac{\text{Number of positive samples}}{\text{Total samples}} \times 100$$

$$\text{Exceeding rate (\%)} = \frac{\text{Samples exceeding MRLs}}{\text{Total samples}} \times 100$$

Overall dietary risk was interpreted using hazard index (HI). If $HI < 100\%$, no significant health risk is indicated whereas $HI \geq 100\%$ found in tested samples suggest a potential health risk. In this study, all calculated HI values were below 100%, indicating a negligible dietary risk in these analyzed food and poultry feed samples.

3.8 Conclusion

This research work was focused on modifying and optimizing an LC-MS/MS methods, validating its performance, and applying it to multi-residue detection of five major veterinary drugs classes namely sulfa drugs (SAs), fluoroquinolones (FQs), chloramphenicol (CAP), and derivatives of nitrofurantoin metabolites (NFs) in poultry meat, beef, egg, milk and poultry feed samples. Each method was optimized and subsequently applied to 150 field samples (30 per matrix) collected from super shops and local markets in Dhaka and Gazipur cities. The results demonstrated that well designed LC-MS/MS approaches can deliver sensitive and selective quantification of antibiotics in highly complex food and feed matrices. For all drug classes except CAP, electrospray ionization in positive mode ESI (+) combined with C18 reversed-phase chromatography, enabled efficient separation and ionization; CAP was quantified using ESI (-) mode because of its acidic character. Gradient or isocratic mobile phases composed of H_2O (0.1% $HCOOH$) and CH_3OH or CH_3CN were selected to each analyte group to ensure optimal retention, peak shape and run time. In every case, multiple-reaction monitoring (MRM) transitions were optimized from precursor ions obtained in SCAN mode, with product ions per analyte used for confirmation, ensuring high selectivity and accuracy.

Chromatographic retention times were provided highly reproducible across matrices: for example, SAs eluted at 2.3-6.3 min, FQs at 3.7-3.9 min, TCs at 4.6-6.3 min, CAP at 3.5-3.9 min and NFs at 1.3-1.9, with non-overlapping retention times. Clean chromatograms with fully resolved peaks demonstrated excellent chromatographic selectivity and minimal the risk of co-elution and qualitative misidentification. To compensation for matrix-induced ionization effects, matrix-matched calibration was employed across all analyte groups. The validated methods showed excellent linearity for all analyte classes, with sulfa drugs (SMX, SDZ, SMT) linear over $1.25-100 \mu g kg^{-1}$ with $R^2 = 0.9918-0.9999$, fluoroquinolones (CIP, ENR, LEV) over $5-200 \mu g kg^{-1}$ with R^2 typically $0.944-0.9999$, tetracyclines (TC, OTC, CTC) over $1.5-100 \mu g kg^{-1}$ and $1.25-100 \mu g kg^{-1}$ for OTC, CTC in beef with $R^2 = 0.9978-0.9999$, chloramphenicol (CAP) over

1.5-100 $\mu\text{g kg}^{-1}$ with $R^2 = 0.9978-0.9999$, and derivatives of nitrofurans metabolites (NP-AOZ, NP-AMOZ, NP-AHD) over 1-200 $\mu\text{g kg}^{-1}$ with R^2 values mostly above 0.9954. The excellent correlation coefficients across all matrices confirm that signal response is proportional to concentration and that the calibration curves are robust for quantitative work. The analytical methods exhibited high sensitivity, enabling trace-level monitoring well below established MRLs. SAs exhibiting LODs of 0.1-0.9 $\mu\text{g kg}^{-1}$ and LOQs of 0.3-2.7 $\mu\text{g kg}^{-1}$ (depending on analyte and matrix), FQs showing LODs of 0.1-0.8 $\mu\text{g kg}^{-1}$ and LOQs of 0.3-2.6 $\mu\text{g kg}^{-1}$, TCs achieving LODs of 0.03-0.6 $\mu\text{g kg}^{-1}$ and LOQs of 0.17-1.8 $\mu\text{g kg}^{-1}$, CAP displaying LODs of 0.06-0.7 $\mu\text{g kg}^{-1}$ and LOQs of 0.1-0.6 $\mu\text{g kg}^{-1}$, and NFs presenting LODs of 0.09-1.1 $\mu\text{g kg}^{-1}$ and LOQs of 0.2-3.6 $\mu\text{g kg}^{-1}$. These figures demonstrate that the LC-MS/MS procedures are capable of detecting and quantifying residues at levels significantly below regulatory threshold or, in case of CAP and NFs, at very low level of Minimum Required Performance Limits (MRPL) appropriate for banned substances.

Across all matrices, intra-day precision (repeatability) was evaluated via replicate analyses at two spiking levels, while inter-day precision (reproducibility) was assessed over multiple days. For the great majority of analyte-matrix combinations, intra-day RSD% were typically <10%, with occasional values up to ~14% at the lowest spiking levels or in particularly challenging matrices such as egg and poultry feed, whereas inter-day RSD% generally remained below 10%, confirming stable day-to-day method performance. Where slightly higher RSD% occurred for certain analytes in egg, milk or poultry feed, the values still remained within acceptable residue analysis criteria and reflected realistic variability associated with highly complex matrices rather than fundamental method instability. The comprehensive recovery studies confirmed that the optimized extraction and clean-up procedures tailored to each matrix were efficient and analytically reliable for all target compounds.

Mean measured concentrations were consistently close to spiking levels for SAs, FQs, TCs, CAP and NFs in all five matrices, demonstrating good trueness of the methods. This was confirmed by recovery experiments, expressed as intra- and inter-day recovery percentages: for sulfa drugs (SMX, SDZ, SMT), recoveries were mostly within 79 -111% in every matrix, with inter-day values clustering around 87-109%; for fluoroquinolones (CIP, ENR, LEV), intra-day recoveries at 20 and 25 $\mu\text{g kg}^{-1}$ were usually 79-111%, and inter-day recoveries ranged from 93-107% across poultry meat, beef, egg, milk and poultry feed. Tetracyclines (TC, OTC, CTC) showed typical recoveries of 80-109%; TC occasionally exhibited modest under-recovery in egg, whereas OTC and CTC performed very well in all matrices. Chloramphenicol (CAP) displayed intra-day recoveries of 90-112% and inter-day values of 94-102% for all matrices and spiking levels. For derivatives of nitrofurans metabolites (NP-AOZ, NP-AMOZ, NP-AHD), recoveries were generally 82-115%, with inter-day recoveries between 92-104% in all matrices.

Collectively, these precision and recovery data fall within commonly accepted validation criteria (typically 70-120% recovery ([SANTE/11312/2021](#); [CODEX guideline, 2017](#)) and $\leq 20\%$ RSD% for residue analysis, even at the lower spiking levels, demonstrating good repeatability and reproducibility across runs, days and matrices), confirming that the methods are both accurate and precise for multi-residue determination in complex animal-derived matrices.

A detailed evaluation of matrix-induced ionization effects revealed that electrospray responses are both compound and matrix-dependent, but remain analytically manageable when matrix-matched calibration is applied. Systematic evaluation of matrix-induced ionization effects highlighted that ESI responses were both analyte and matrix-dependent, but generally moderate and positive (ion enhancement) rather than suppressive. Among the sulfa drugs, SMX exhibited comparatively stronger positive matrix effects, with ion enhancement up to $\sim 40\%$ in egg, whereas SDZ, SMT were less influenced. For the FQs, matrix effects ranged from modest ($\sim 2\text{-}14\%$) enhancement for CIP, ($\sim 7\text{-}10\%$) for LEV to substantial for ENR in egg ($\sim 43\%$), demonstrating that certain FQs are more susceptible to co-eluting components. Within the TCs, TC showed only modest enhancement ($\sim 1\text{-}8\%$), while OTC and CTC were more affected, particularly in egg and poultry feed where enhancement reached ($\sim 33\%$) for OTC. In contrast, CAP displayed slightly higher matrix effects ($\sim 22\text{-}45\%$), indicating that it is largely insensitive to co-extractives under the conditions used. For the derivatives of nitrofurans metabolites, matrix effects were moderate and positive ($\sim 1\text{-}12\%$), with slightly higher enhancement for NP-AOZ and NP-AHD in egg, beef and poultry feed. All observed matrix effects were enhancing rather than suppressive and remained within ranges that can be effectively controlled by matrix-matched calibration and they did not compromise the linearity, precision or accuracy of the validated methods. Collectively, SAs, FQs and CAP showed more pronounced enhancement in some matrices such as egg, milk, poultry feed, whereas TCs and NFs typically experienced low to moderate enhancement matrix effects.

The validated methods were applied to 750 samples across all five classes where a total of 13 positive findings were obtained when individual residues in specific samples are counted; however, at the level of sample numbers, contamination frequencies remained low and concentrations were generally below the relevant MRLs (or low but quantifiable of banned compounds). Residue monitoring across the five antibiotic classes showed a low level of contamination in the analyzed samples. For sulfa drugs, SDZ was detected in one beef sample (BM18, Kaptan bazar, Dhaka) and one poultry feed sample (FM22, broiler house feed, Nimtoli municipal market, Gulistan, Dhaka) where detected amount of both $61\text{ }\mu\text{g kg}^{-1}$, while SMX was found in a single beef sample (BM29, Adabor kancha bazar, Adabor, Dhaka) at $43\text{ }\mu\text{g kg}^{-1}$; all

concentrations were below their respective MRLs, and SMT was not detected in any sample. Among the fluoroquinolones, only one milk sample (MM10, Nimtoli dairy farm, Chankharpul, Dhaka) contained LEV at $12 \mu\text{g kg}^{-1}$, and neither CIP nor ENR was detected in any matrix. For tetracyclines, TC itself was absent from all 150 samples, but OTC was found in two poultry meat samples (PM17, Chicken market Kaptan bazar, Dhaka and PM24, krishi market, Mohammadpur, Dhaka) at 46 and $32 \mu\text{g kg}^{-1}$ and one egg sample (EM11, Charulata market, Maghbazar, Dhaka) at $37 \mu\text{g kg}^{-1}$, while CTC was detected in one beef sample (BM26, krishi market, Mohammadpur, Dhaka) at $48 \mu\text{g kg}^{-1}$; all TC residues remained below established MRLs. Chloramphenicol was not detected in any poultry meat, beef, egg, milk or poultry feed sample, indicating full compliance with the ban on this drug. Regarding derivatives of nitrofurantoin metabolites, NP-AOZ was detected in one poultry meat sample (PM19, City corporation market, Kawran bazar, Dhaka) at $18 \mu\text{g kg}^{-1}$ and two beef samples (BM16 and BM17, Kaptan bazar, Dhaka) at 15 and $17 \mu\text{g kg}^{-1}$, and NP-AMOZ was found in one poultry meat sample (PM18, Chicken market Kaptan bazar, Dhaka) at $22 \mu\text{g kg}^{-1}$ and one egg sample (EM13, Charulata market, Maghbazar, Dhaka) at $37 \mu\text{g kg}^{-1}$, whereas NP-AHD and all NFs in milk were below limit.

All these findings indicate that, Antibiotic residues were not detected in any of the samples collected from super shops, which may be attributed to the adoption of good veterinary practices and compliance with recommended withdrawal periods. Although residues of several antibiotic classes do occur occasionally in edible animal products and poultry feed, the overall contamination burden is low and generally within regulatory limits, while still underscoring the need for continued surveillance and adherence to good veterinary and agricultural practices. These occurrence data demonstrate that, within the surveyed area and sampling frame, residue levels are generally low, yet the occasional presence of NFs banned compounds. Residues may also be influenced by matrix-dependent residue distribution such as lower residues in muscle compared with liver or kidney.

These analytical establishes a comprehensive multi-class, multi-matrix capability, demonstrating that LC-MS/MS can be systematically optimized to quantify five structurally diverse drug classes across five matrices, including poultry meat, beef, egg, milk and poultry feed. It presents full method validation according to analyte-matrix combinations evaluated for linearity, working range, LOD, LOQ, selectivity via retention time and MRM transitions, accuracy (recovery), precision (intra- and inter-day RSD%), and matrix effects, consistently meeting widely accepted validation criteria and confirming the methods fitness purpose for residue monitoring.

A quantitative assessment of matrix effects was obtained by determining ion suppression and enhancement for each analyte in each matrix, providing a sound basis for selecting an appropriate matrix-matched calibration strategy and for reliably interpreting LC–MS/MS residue data in complex matrices. The use of a C18 stationary phase, an acidified aqueous–organic mobile phase, and carefully optimized MRM transitions ensured clear chromatographic separation and selective detection for all target compounds, while matrix-matched calibration minimized matrix-induced bias. Although the overall occurrence of residues in field samples from Dhaka and Gazipur was low, it was not zero, underscoring the continued need for routine monitoring within existing control frameworks. Such surveillance will support effective management of veterinary drug residues and contribute to improved food safety and public health protection.

This analysis aimed to identify and quantify antibiotic residues. Residues can persist in meat, milk, eggs, and feed due to ignoring withdrawal periods which raising consumer health risks, undermining consumer confidence, and reducing market acceptance. This study emphasized the long-term consequences of incorrect antibiotic selection, improper dosing, repeated use without proper diagnosis, and failure to comply with withdrawal periods. Overall, this study encourages farmers to keep animal-derived foods and poultry feed safe and residue-free, protect consumer trust, strengthen a sustainable livestock and poultry sector, and reduce long-term drug resistance risks in Bangladesh.

Chapter 4

References

- Abbasi, M. M.; Babaei, H.; Ansarin, M.; Nourdadgar, A. O. S.; Nemati, M. (2011). Simultaneous Determination of Tetracyclines Residues in Bovine Milk Samples by Solid Phase Extraction and HPLC-FL Method. *Advance Pharmaceutical Bulletin*. 1(1), 34-39. doi: 10.5681/apb.2011.005
- Adane, W. D.; Chandravanshi, B. S.; Tessema, M. (2023). Highly sensitive and selective electrochemical sensor for the simultaneous determination of tinidazole and chloramphenicol in food samples (egg, honey and milk). *Sensors and Actuators B: Chemical*, 390, 134023. <https://doi.org/10.1016/j.snb.2023.134023>
- Alkan, F.; Kotan, A.; Ozdemir, N. (2016). Development and validation of confirmatory method for analysis of nitrofurantoin metabolites in milk, honey, poultry meat and fish by Liquid Chromatography-Mass Spectrometry. *Macedonian veterinary review*, 39 (1): 15-22. <http://dx.doi.org/10.1515/macvetrev-2015-0060>
- Akili, S.; Hadda, D. B.; Bitar, Y.; Balash, A.; Chehna, M. F. (2021). Design, Synthesis and Characterization of Novel Sulfonamides Derivatives as Anticancer Agent Targeting EGFR TK, and Development of New Methods of Synthesis by Microwave Irradiation. *International Journal of Organic Chemistry*, 11(4), doi: 10.4236/ijoc.2021.114014
- Anastassiades M.; Lehotay, S. J.; Stajnbaher, D.; and Schenck, F. j. (2003). Fast and easy multiresidue method employing acetonitrile extraction/ portioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. *J. AOAC Int*, 86: 412-431
- Arslanbas, E.; Şahin, S.; Kalin, R.; Mogulkoc, M. N.; Gungor, H. (2018). Determination of Some Antibiotic Residues by HPLC Method in Chicken Meats Prepared for Consumption. *Araştırma Makalesi*, 15(3), 247-252. DOI: 10.32707/ercivet.477315
- Arsène, M.M. J.; Davares, A. K. L.; Viktorovna, P. I.; Andreevna, S. L.; Sarra, S.; Khelifi, I.; Sergueïevna, D. M. (2022). The public health issue of antibiotic residues in food and feed: Causes, consequences, and potential solutions. *Vet World*, 15(3):662-671. doi: 10.14202/vetworld.2022.662-671
- Ayed, A. B.; Ulusoy, H. I.; Polat, U.; Ulusoy, S.; Locatelli, M.; Kabir, K.; Khemakhem, H. (2024). A facile fabric phase sorptive extraction method for monitoring chloramphenicol residues in milk samples. *Journal of Pharmaceutical and Biomedical Analysis*, 239, 115913. <https://doi.org/10.1016/j.jpba.2023.115913>
- Bahmani, K.; Shahbazi, Y.; Nikousefat, Z. (2020). Monitoring and risk assessment of tetracycline

residues in foods of animal origin. *Food Science Biotechnology*, 29(3):441-448.
<https://doi.org/10.1007/s10068-019-00665-x>

Barbosa, J.; Moura, S.; Barbosa, R.; Ramos, F.; Silveira, M. I. N. D. (2007). Determination of nitrofurans in animal feeds by liquid chromatography-UV photodiode array detection and liquid chromatography-ionspray tandem mass spectrometry. *Analytica Chimica Acta* 586, 359-365.
[doi:10.1016/j.aca.2006.11.053](https://doi.org/10.1016/j.aca.2006.11.053)

Bava, R.; Castagna, F.; Lupia, C.; Poerio, G.; Liguori, G.; Lombardi, R.; Naturale, M. D.; Mercuri, C.; Bulotta, R. M.; Britti, D.; Palma, E. (2024). Antimicrobial Resistance in Livestock: A Serious Threat to Public Health. *Antibiotics*, 13(6), 551.
<https://doi.org/10.3390/antibiotics13060551>

Bişgin, A. T.; Elik, A.; Altunay, N. (2025). Investigation of magnetic fluids in a chemometrically designed green microextraction method for determining chloramphenicol in fortified honey, milk and chicken meat. *Food Chemistry*, 492(2), 145465.
<https://doi.org/10.1016/j.foodchem.2025.145465>

Bishnoi, K.; Moudgil, P.; Soni, D.; Jadhav, V.J. (2025). Occurrence and Risk Assessment of Tetracycline Residues in Layer Eggs in Haryana, India. *Journal of Food Protection*, 88 (2), 100449.
<https://doi.org/10.1016/j.jfp.2025.100449>

Bock, C.; Gowik, P.; Stachel, C. (2007). Matrix-comprehensive in-house validation and robustness check of a confirmatory method for the determination of four nitrofurans metabolites in poultry muscle and shrimp by LC-MS/MS. *Journal of Chromatography B*, 856, 178-189.
[doi:10.1016/j.jchromb.2007.05.044](https://doi.org/10.1016/j.jchromb.2007.05.044)

Borghi, A. A.; and Palma, M. S. A. (2014). Tetracycline: production, waste treatment and environmental impact assessment. *Brazilian Journal of Pharmaceutical Sciences*, 50(1).
<http://dx.doi.org/10.1590/S1984-82502011000100003>

Bushra, R., Roy, H., Maysha, T. I., Rahman, S., Saha, W., Ferdous, F. B., Rahman, S., Alam, M. M. (2026). Antibiotic Residues in Animal-Derived Foods: A Growing Food Safety Crisis and Regulatory Dilemma. *Journal of Food Protection*. 89(6), 100783.
<https://doi.org/10.1016/j.jfp.2026.100783>

Campagnolo, E. R.; Johnson, K. R.; Karpati, A.; Rubin, C. S.; Kolpin, D. W.; Meyer, M. T.; Esteban, J. E.; Currier, R. W.; Smith, K.; Thu, K. M.; McGeehin, M. (2002). Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. *The Science of the Total Environment*, 299, 89-95

- Catania, A. M.; Stella, M. C.; Cimino, F.; Zoppi, S.; Grego, E. (2024). Sulfonamide resistance evaluation in five animal species and first report of *sul4* in companion animals. *Veterinary Microbiology*, 296, 110170. <https://doi.org/10.1016/j.vetmic.2024.110170>
- Casado, N.; Morante-Zarcelo, S.; Sierra, I. (2022). Application of the QuEChERS Strategy as a Useful Sample Preparation Tool for the Multiresidue Determination of Pyrrolizidine Alkaloids in Food and Feed Samples: A Critical Overview. *Applied Sciences*, 12(9), 4325, doi: <https://doi.org/10.3390/app12094325>
- Chandrakar, C.; Shakya, S.; Patyal, A.; Bhonsle, D.; Pandey, A. K. (2023). Detection of antibiotic residues in chicken meat from different agro-climatic zones of Chhattisgarh, India by HPLC-PDA and human exposure assessment and risk characterization. *Food Control*, 148, 109667. <https://doi.org/10.1016/j.foodcont.2023.109667>
- Chang, S. H.; Lai, Y. H.; Huang, C. N.; Peng, G. J.; Liao, C. D.; Kao, Y. M.; Tseng, S. H.; Wang, D. Y. (2019). Multi-residue analysis using liquid chromatography tandem mass spectrometry for detection of 20 coccidiostats in poultry, livestock, and aquatic tissues. *Journal of Food and Drug Analysis*. 27(3), 703-716. <https://doi.org/10.1016/j.jfda.2019.02.004>
- Cheong, C.K.; Hajeb, Parvaneh.; Jinap, S.; and Ismail-Fitry, M.R. (2010). Sulfonamides determination in chicken meat products from Malaysia. *International Food Research Journal* 17: 885-892
- Chen, Y.; Xia, S.; Han, X.; Fu, Z. (2020). Simultaneous Determination of Malachite Green, Chloramphenicols, Sulfonamides, and Fluoroquinolones Residues in Fish by Liquid Chromatography-Mass Spectrometry. *Journal of Analytical Methods in Chemistry*. 20(12), 3725618. <https://doi.org/10.1155/2020/3725618>
- Chiesa, L. M.; Cesare, F. D.; Nobile, M.; Villa, R.; Decastelli, L.; Martucci, F.; Fontana, M.; Pavlovic, R.; Arioli, F.; Panseri, S. (2021). Antibiotics and Non-Targeted Metabolite Residues Detection as a Comprehensive Approach toward Food Safety in Raw Milk. *Foods* 2021, 10(3), 544; <https://doi.org/10.3390/foods10030544>
- Chowdhury, S.; Hassan, M. M.; Alam, M.; Sattar, S.; Bari, M. S.; Saifuddin, A. K. M.; Hoque, M. A. (2015). Antibiotic residues in milk and eggs of commercial and local farms at Chittagong, Bangladesh. *Veterinary World*, EISSN: 2231-0916. Vol 8, DOI: 10.14202/vetworld.2015.467-471
- Ciglencečki, I.; Orlović-Leko, P.; Vidović, K.; Tasić, V. (2021). The possible role of the surface-active substances (SAS) in the airborne transmission of SARS-CoV-2. *Environmental research*, 198:111215. doi: 10.1016/j.envres.2021.111215

Commission Regulation (European Union, No 37/2010, Updated 2018

CODEX ALIMENTARIUS, Maximum Residue Limits (MRLs) and Risk Management Recommendations (RMRs) for Residues of Veterinary Drugs in Foods. CX/MRL 2-2018

CODEX ALIMENTARIUS, Maximum Residue Limits (MRLs) and Risk Management Recommendations (RMRs) for Residues of Veterinary Drugs in Foods. CX/MRL 2-2024

CODEX ALIMENTARIUS, Guidelines on Performance Criteria for Methods of Analysis for the Determination of Pesticide Residues in Food and Feed, CXG 90-2017, Adopted in 2017

Commission Decision 181/2003/EC, March 13, 2003, amending decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. Official Journal European Communities 2003, L71,17-18

Codex Alimentarius Commission. General Principles of Food Hygiene; FAO: Rome, Italy; WHO: Geneva, Switzerland, 2020

Cooper, K. M.; Kennedy. D. G. (2007). Stability studies of the metabolites of nitrofurantoin antibiotics during storage and cooking. Food Additives and Contaminants, 24 (09), 935-942. Doi: 10.1080/02652030701317301

Conde-Cid, M.; Ferreira-Coelho, G.; Núñez-Delgado, A.; Fernández-Calviño, D.; Arias-Estévez, M.; Álvarez-Rodríguez, E.; Fernández-Sanjurjo, M. J. (2019). Competitive adsorption of tetracycline, oxytetracycline and chlortetracycline on soils with different pH value and organic matter content. Environmental Research, 178, 108669. <https://doi.org/10.1016/j.envres.2019.108669>

Cornejo, J.; Pokrant, E.; Figueroa, F.; Riquelme, R.; Galdames, P.; Pillo, F. D.; Jimenez-Bluhm, P.; Hamilton-West, C. (2020). Assessing Antibiotic Residues in Poultry Eggs from Backyard Production Systems in Chile, First Approach to a Non-Addressed Issue in Farm Animals. Animals (Basel), 10(6):1056. doi: 10.3390/ani10061056

Cui, W.; Niu, M.; Li, M.; Zhang, N.; Lv, E.; Wang, R.; Li, C.; Li, Q.; Qiao, M.; Li, W.; Xu, S. (2026). A novel fluorescence-colorimetric dual-mode aptasensor for sensitive antibiotic detection in food samples. Microchemical Journal, 221, 117021. <https://doi.org/10.1016/j.microc.2026.117021>

Daeseleire, E.; Pamel, E. V.; Poucke, C. V.; Croubels, S. (2017). Veterinary Drug Residues in Foods (Chapter 6), Chemical contaminations and residues in food (2nd edition). Woodhead Publishing Series in Food Science, Technology and Nutrition, 117-153.

<https://doi.org/10.1016/B978-0-08-100674-0.00006-0>

Dasenaki, M. E.; and Thomaidis, N. S. (2015). Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography–tandem mass spectrometry. *Analytica Chimica Acta* 880: 103–121. <http://dx.doi.org/10.1016/j.aca.2015.04.013>

Demeiry, M. E.; Alib, A.; Abouleila, Y.; Shimizud, Y.; Masujimab, T.; Salam, R. A.; Hadade, G.; Emara, S. (2020). Quantification and targeted detection of ciprofloxacin in dosage form and human urine by direct injection nano-electrospray ionization multi-stage mass spectrometry. *Microchemical Journal*, 153. <https://doi.org/10.1016/j.microc.2019.104534>

Doyuk, F.; and Dost, K. (2023). Simultaneous determination of six antibiotics belonging to four different classes in chicken meat BY HPLC/DAD and verification BY LC-MS/MS. *Food Chemistry*, 426, 136549. <https://doi.org/10.1016/j.foodchem.2023.136549>

Draiscim, R.; Giannetti, L.; Lucentini, L.; Palleschi, L.; Brambilla, G.; Serpe, L.; Gallo, P. (1997). Determination of nitrofurans residues in avian eggs by liquid chromatography-UV photodiode array detection and confirmation by liquid chromatography-ionspray mass spectrometry. *Journal of Chromatography A*, 777(1), 201-211

El-Demerdash, A.; Song, F.; Reel, R. K.; Hillegas, J.; Smith, R. E. (2019). Simultaneous Determination of Nitrofurans Metabolites and Chloramphenicol in Shrimp with a Single Extraction and LC-MS/MS Analysis. *Journal of AOAC INTERNATIONAL*, 98(3), 595-601. <https://doi.org/10.5740/jaoacint.14-261>

El-Gamel, N. E. A.; Hawash, M.; Fahmey, M. A. (2011). Structure characterization and spectroscopic investigation of ciprofloxacin drug. *Journal of Thermal Analysis and Calorimetry*, 108(1). DOI: 10.1007/s10973-011-1584-8

El-Alfy, N. J. I.; Emam, A. A. K.; Mahmoud, M. F.; Morgan, O. N. M.; El-Ashry, S. R. G. E. (2024). Potential protection by vitamin D against DNA fragmentation and bone marrow cytotoxicity induced by chloramphenicol. *Toxicology Reports*, 13, 101828. <https://doi.org/10.1016/j.toxrep.2024.101828>

Er, B.; Onurdağ, F. K.; Demirhan, B.; Özgacar, S. Ö.; Öktem, A. B.; Abbasoğlu, U. (2013). Screening of quinolone antibiotic residues in chicken meat and beef sold in the markets of Ankara, Turkey. *Poultry Science* 92 :2212-2215. <http://dx.doi.org/10.3382/ps.2013-03072>

Esponda, S. M.; Padrón, M. E. T.; Ferrera, Z. S., Rodríguez, J. J. S. (2009). Solid-phase

microextraction with micellar desorption and HPLC-fluorescence detection for the analysis of fluoroquinolones residues in water samples. *Analytical Bioanalytical Chemistry*, 394:927-935. DOI 10.1007/s00216-009-2629-8

Errayess, S. A.; Lahcen, A. A.; Idrissi, L.; Marcoaldi, C.; Chiavarini, S.; Amine (2017). A sensitive method for the determination of Sulfonamides in seawater samples by Solid Phase Extraction and UV-Visible spectrophotometry. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. 181, 276-285. <http://dx.doi.org/10.1016/j.saa.2017.03.061>

European Commission, Health & Consumer Protection Directorate-General, Guidelines for the Implementation of Decision 2002/657/EC (SANCO/2004/2726-rev 2008)

Fei, Z.; Song, S.; Gao, J.; Song, Y.; Xiao, X.; Yang, X.; Jiang, D.; Yang, D. (2023). Antibiotic residues in chicken meat in China: Occurrence and cumulative health risk assessment. *Journal of Food Composition and Analysis*, 116, 105082. <https://doi.org/10.1016/j.jfca.2022.105082>

Fernandes, F. C. B.; Silva, A. S.; Rufino, J. L.; Pezza, H. R.; Pezza, L (2015). Screening and determination of sulphonamide residues in bovine milk samples using a flow injection system. *Food Chemistry* 166, 309-315. <http://dx.doi.org/10.1016/j.foodchem.2014.06.034>

Fernando, R.; Munasinghe, D.M.S.; Gunasena, A.R.C.; Abeynayake, P. (2015). Determination of nitrofurantoin metabolites in shrimp muscle tissue by liquid chromatography-photo diode array detection. *Food Control*, 72 Part B, 300-305. <https://doi.org/10.1016/j.foodcont.2015.08.044>

Finzi, J. K.; Donato, J. L.; Sucupira, M.; Nucci, G. D. (2005). Determination of nitrofurantoin metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, 824 (2005) 30-35. doi:10.1016/j.jchromb.2005.05.012

Forti, A.F.; and Scortichini, G. (2009). Determination of ten sulphonamides in egg by liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta* 637, 214-219. doi:10.1016/j.aca.2008.11.007

Food Safety Regulation, Bangladesh Food Safety Authority, 2017

Gantverg, A.; Shishani, I.; Hoffman, M. (2003). Determination of chloramphenicol in animal tissues and urine: Liquid chromatography-tandem mass spectrometry versus gas chromatography-mass spectrometry. *Analytica Chimica Acta*, 438(1-2), 125-135. [https://doi.org/10.1016/S0003-2670\(02\)01566-0](https://doi.org/10.1016/S0003-2670(02)01566-0)

Gajda, A.; and Posyniak, A. (2015). Liquid chromatography-tandem mass spectrometry method for the determination of ten tetracycline residues in muscle samples. *Bulletin Vet Inst Pulawy*

59, 345-352. DOI:10.1515/bvip-2015-0051

Govind, V. R.; Babu, N.; Rao, V. A.; Sriram, P.; Kumar, T. M. A. S. (2018). Determination of sulfadoxine residues in poultry meat by liquid chromatography and tandem mass spectrometry. *Journal of Entomology and Zoology Studies*, 6(2), 2580-2584

Guo, Y.; Ngom, B.; Le, T.; Jin, X.; Wang, L.; Shi, D.; Wang, X.; Bi, D. (2010). Utilizing Three Monoclonal Antibodies in the Development of an Immunochromatographic Assay for Simultaneous Detection of Sulfamethazine, Sulfadiazine, and Sulfaquinoxaline Residues in Egg and Chicken Muscle. *Analytical Chem.* 82 (18), 7550-7555. doi: 10.1021/ac101020y

Guo, Y., He, z., Chen, J., Chen, L., Xie, K., Zhang, T., Zhang, G., Dai, G. (2021). Simultaneous determination of tetracyclines and fluoroquinolones in poultry eggs by UPLC integrated with dual-channel-fluorescence detection method. *Molecules* 26 (18). <https://doi.org/10.3390/molecules26185684>

Hasan, B.; Faruque, R.; Drobni, M.; Waldenstroöm, J.; Sadique, A.; Ahmed, K. U.; Islam, Z.; Parvez, M. B. H.; Olsen, B.; Alam, M. (2011). High Prevalence of Antibiotic Resistance in Pathogenic *Escherichia coli* from Large- and Small-Scale Poultry Farms in Bangladesh, *AVIAN DISEASES* 55:689-692. DOI: 10.1637/9892-968611-DIGEST.1

Harun, A. B.; Khatri, B.; Karim, M. R. (2024). Phenotypic and genotypic patterns of antimicrobial resistance in livestock and poultry in South Asia: A systematic review and meta-analysis. *Food Control*, 164, 110575. <https://doi.org/10.1016/j.foodcont.2024.110575>

Hosain, M. Z.; Islam, S. M. S.; Kamal, M. M. (2022). Analytical Method Validation for Quantification of Chloramphenicol Residues in Poultry Meal Using a Liquid Chromatography-Tandem Mass Spectrometry. *American Journal of Chemical and Biochemical Engineering*, 6(2): 44-50. doi: 10.11648/j.ajcbe.20220602.11

Household income and expenditure survey HIES 2022.
<https://knowledgehub.pksf.org.bd/collections/file/c2tnMHLzMGxsNERHTjErb1puR3hGQT09>

Huang, W.; Qiu, Q.; Chen, M.; Shi, J.; Huang, X.; Q.; Kong, Long, D.; Chen, Z.; Yan, S. (2019). Determination of 18 antibiotics in urine using LC QqQ-MS/MS. *Journal of Chromatography B*, 1105(15), 176-183. doi:10.1016/j.jchromb.2018.12.019

Hubicka, U.; Zmudzki, P.; Zuromska-Witek, B.; Zajdel, P.; Pawłowski, M.; Krzek, J. (2013). Separation and characterization of ciprofloxacin, difloxacin, lomefloxacin, norfloxacin, and ofloxacin oxidation products under potassium permanganate treatment in acidic medium by

UPLC-MS/MS. *Talanta*, 109, 91-100. doi: 10.1016/j.talanta.2013.01.055

Huertas-Pérez, J., F.; Arroyo-Manzanares, N.; Havlíková, L.; Gámiz-Gracia, L.; Solich, P.; García-Campaña, A. M. (2016). Method optimization and validation for the determination of eight sulfonamides in chicken muscle and eggs by modified QuEChERS and liquid chromatography with fluorescence detection. *Journal of Pharmaceutical and Biomedical Analysis* 124, 261-266. <http://dx.doi.org/10.1016/j.jpba.2016.02.040>

Ibrahim, I. G.; Sabiel Y. A.; Thoria O. O.; Khalafalla A. E.; Safa O. S.; Salwa, Khogali, M. E.; Elrayah, H. A.; Salih, M. H.; Muna E. A. (2018). Microbiological and hplc assays for detection of tetracycline residues in chicken meat. *Asian Journal of Science and Technology*, 6(2) 1020-1022, ISSN: 0976-3376

Izah, S. C.; Nurmahanova, A.; Ogwu, M. C.; Toktarbay, Z.; Umirbayeva, Z.; Ussen, K.; Koibasova, L.; Nazarbekova, S.; Tynybekov, B.; Guo, Z. (2025). Public health risks associated with antibiotic residues in poultry food products. *Journal of Agriculture and Food Research* 21, 101815. <https://doi.org/10.1016/j.jafr.2025.101815>

Jahan, T.; Yasmin, S.; Rahman, M. S.; Tabassum, B. B.; Lisa, S. A.; Kabir, M. A.; Kabir, M. H. (2025). Occurrence and risk assessment of tetracycline residues in poultry meat of Dhaka, Bangladesh: A sensitive and reliable analytical method development approach. *Food Chemistry Advances* 9, 101120 <https://doi.org/10.1016/j.focha.2025.101120>

Jahan, T., Yasmin, S., Pathan, M. H., Kabir, M. A., Moniruzzaman, M., Kabir, M. H. (2026). Prevalence of tetracyclines residues in poultry eggs using LC-MS/MS with methanol extraction: Implications for consumer health. *Journal of Food Composition and Analysis*. 153, 109150. <https://doi.org/10.1016/j.jfca.2026.109150>

Jammoul, A.; and Darra, N. E. (2019). Evaluation of Antibiotics Residues in Chicken Meat Samples in Lebanon. *Antibiotics (Basel)*, 8(2):69. doi: 10.3390/antibiotics8020069

Jank, L.; Hoff, R. B.; Tarouco, P. C.; Barreto, F.; Pizzolato, T. M. (2012). β -lactam antibiotics residues analysis in bovine milk by LC-ESI-MS/MS: a simple and fast liquid-liquid extraction method. *Food Additives & Contaminants: Part A*, 29(4), 497–507. <https://doi.org/10.1080/19440049.2011.604044>

Jarosz, L.; Rogacz D.; Rychter P. (2019). Hazardous ecotoxicological impact of two commonly used nitrofurantoin-derived antibacterial drugs: Furazolidone and nitrofurantoin, *Chemosphere*. *Chemosphere*, 222, 381-390. doi: <https://doi.org/10.1016/j.chemosphere.2019.01.144>

References

- John Points, D.; Burns, T.; Walker, M. J. (2015). Forensic issues in the analysis of trace nitrofuran veterinary residues in food of animal origin Food Control, 92-103. <https://doi.org/10.1016/j.foodcont.2014.08.037>
- Jung, H. N.; Park, D. H.; Choi, Y. J.; Kang, S. H.; Cho, H. J.; Choi, J. M.; Shim, J. H.; Zaky, A. A.; El-Aty, A. M. A.; Chul-Shin, H. (2022). Simultaneous Quantification of Chloramphenicol, Thiamphenicol, Florfenicol, and Florfenicol Amine in Animal and Aquaculture Products Using Liquid Chromatography-Tandem Mass Spectrometry. Frontiers in Nutrition, Food Chemistry, 8(2021), <https://doi.org/10.3389/fnut.2021.812803>
- Kaufmann, A.; Butcher, P.; Maden, K.; Walker, S.; Widmer, M. (2014). Determination of Nitrofuran and Chloramphenicol Residues by High Resolution Mass Spectrometry versus Tandem Quadrupole Mass Spectrometry, Analytica Chimica Acta. 862, 41-52. <http://dx.doi.org/10.1016/j.aca.2014.12.036>
- Kamouh, H. M.; Abdallah, R.; Kirrella, G. A.; Mostafa, N. Y.; Shafik, S. (2024). Assessment of antibiotic residues in chicken meat. Open Veterinary Journal, 14(1): 438-448. Doi: 10.5455/OVJ.2024.v14.i1.40
- Karaseva, N.A.; and Ermolaeva, T.N. (2012). A piezoelectric immunosensor for chloramphenicol detection in food. Talanta, 93, 44-48. <https://doi.org/10.1016/j.talanta.2011.12.047>
- Kenjeric, L.; Sulyok, M.; Malachova, A.; Greer, B.; Kolawole, O.; Quinn, B.; Elliott, C. T.; Krska, R. (2024). Extension and interlaboratory comparison of an LC-MS/MS multi-class method for the determination of 15 different classes of veterinary drug residues in milk and poultry feed. Food Chemistry, 449, 138834. <https://doi.org/10.1016/j.foodchem.2024.138834>
- Kennedy, D. G.; and Mccracken, R.J. (2007). The detection, accumulation and distribution of nitrofuran residues in egg yolk, albumen and shell. Food Additives and Contaminants, 24 (01), 26-33. DOI: 10.1080/02652030600967214
- Khataei, M. M.; Shazalatun, B. H. E.; Lood, R.; Spégel, P.; Yamini, Y.; Turner, C. (2022). A review of green solvent extraction techniques and their use in antibiotic residue analysis. Journal of Pharmaceutical and Biomedical Analysis. 209, 114487. <https://doi.org/10.1016/j.jpba.2021.114487>
- Kim, D.; Kim, B.; Hyung, S.W.; Lee, C. H.; Kim, J. (2015). An optimized method for the accurate determination of nitrofurans in chicken meat using isotope dilution-liquid chromatography/mass spectrometry, Journal of Food Composition and Analysis. Journal of Food Composition and

Analysis, 40, 24-31. <http://dx.doi.org/10.1016/j.jfca.2014.12.005>

Kim, Y. R.; Park, S. Y.; Kim, J. Y.; Choi, J. D.; Moon, G. I. (2024). Simultaneous determination of 31 Sulfonamide residues in various livestock matrices using liquid chromatography-tandem mass spectrometry. *Applied Biological Chemistry* 67, 13. <https://doi.org/10.1186/s13765-024-00864-z>

Koutsoumanis, K.; Allende, A.; Alvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; Cesare, A. D.; Herman, L.; Hilbert, F.; Lindqvist, R.; Nauta, M.; Ru, G.; Simmons, M.; Skandamis, P.; Suffredini, E.; Andersson, D. I.; Bampidis, V.; Bengtsson-Palme, J.; Bouchard, D.; Ferran, A.; Kouba, M.; Puente, S. L.; López-Alonso, M.; Nielsen, S. S.; Pechová, A.; Petkova, M.; Girault, S.; Broglia, A.; Guerra, B.; Innocenti, M. L.; Liébana, E.; López-Gálvez, G.; Manini, P.; Stella, P.; Peixe, L. (2021). Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 12: Tetracyclines: tetracycline, chlortetracycline, oxytetracycline, and doxycycline. *European food safety journal*, <https://doi.org/10.2903/j.efsa.2021.6864>

Kulikovskii, A.; Gorlova, I. F.; Slozhenkina, M. I.; Vostrikova, N.; Ivankin, A. N.; Kuznetsova, O. (2019). Determination of Nitrofurantoin Metabolites in Muscular Tissue by High-Performance Liquid Chromatography with Mass Spectrometric Detection. *Journal of Analytical Chemistry*, 74(9), 906-912, ISSN 1061-9348, DOI:10.1134/S106193481907013X

Kwon, J. W. (2017). Semicarbazide: Natural occurrence and uncertain evidence of its formation from food processing. *Food Control*, (72, part B), 268-275. <https://doi.org/10.1016/j.foodcont.2016.02.022>

Lee, J.; Onuigbo-Chatta, N.; Okoruwa, A.; Monterrosa, E.; Lambertini E.; and Pelto, G. H. (2022). What Is Safe and How Much Does It Matter? Food Vendors' and Consumers' Views on Food Safety in Urban Nigeria, *Foods*, 11(2), 225; <https://doi.org/10.3390/foods11020225>

Lee, H. J., Cho, S. H., Shin, D., Kang, H. S. (2018). Prevalence of Antibiotic Residues and Antibiotic Resistance in Isolates of Chicken Meat in Korea. *Korean Journal for Food Science of Animal Resource*. 38(5):1055–1063. doi: 10.5851/kosfa.2018.e39

Leitner, A.; Zöllner, P.; Lindner, W. (2001). Determination of the metabolites of nitrofurantoin antibiotics in animal tissue by high-performance liquid chromatography-tandem mass spectrometry. *Journal of chromatography. A*, 939(1-2), 49-58. [https://doi.org/10.1016/s0021-9673\(01\)01331-0](https://doi.org/10.1016/s0021-9673(01)01331-0)

Lestari, T. D.; Khairullah, A. R.; Damayanti, R.; Mulyati, S.; Rimayanti, R.; Hernawati, T.; Utama,

- S.; Wardhani, B. W. K.; Wibowo, S.; Kurniasih, D. A. A.; Ma'ruf, I.F.; Moses, I. B.; Lisnanti, E. F.; Ahmad, R. Z.; I.; Fauziah, Amalia, N.; Fauzia, K. A.; Kusala, M. K. J. (2025). Hemorrhagic septicemia: A major threat to livestock health. *Open Veterinary Journal*, 15(2), 519-532. DOI: 10.5455/OVJ.2025.v15.i2.3
- Lima, É.; Leite, M.; Oliveira, B.; Freitas, A. (2024). Antibiotics in eggs: An analytical approach based on low- and high-resolution mass spectrometry techniques. *Journal of Food Composition and Analysis*, 133- 106429. <https://doi.org/10.1016/j.jfca.2024.106429>
- Li, F.; Xiong, S.; Wu, Z.; Dong, P.; Li, H.; Bao, H.; Yang, X. (2024). A simple lable-free aptasensor based on liquid exfoliated graphene modified electrode for chloramphenicol detection in milk. *Microchemical Journal*, 207, 112175. <https://doi.org/10.1016/j.microc.2024.112175>
- Li, Y.; Wang, L.; Zhao, L.; Li, M.; Wen, Y. (2021). An fluorecence resonance energy transfer sensing platform based on signal amplification strategy of hybridization chain reaction and triplex DNA for the detection of Chloramphenicol in milk. *Food Chemistry*, 357, 129769. <https://doi.org/10.1016/j.foodchem.2021.129769>
- Li, Y.; Wu, Y.; Shao, J.; Shi, J.; Sun, L.; Hong, Y.; Wang, X. (2025). Stresses in the food chain and their impact on antibiotic resistance of foodborne pathogens: A review. *Food Microbiology*, 128, 104741. <https://doi.org/10.1016/j.fm.2025.104741>
- Li, X.; He, M.; W.; Sun, J. J.; Zhang, Y.; Tan, B.; Wang, G.; Zhu, G. (2025). Detection of chloramphenicol by nucleic acid cyclic amplification fluorecence analysis and lateral flow assay assisted with CRISPR/Cas12a and exonuclease III. *Food Bioscience*, 68, 106477. <https://doi.org/10.1016/j.fbio.2025.106477>
- Li, W.; Sun, Y.; Liang, W.; Fitzloff J. F.; Breemen, R. B. V. (2003). Identification of caffeic acid derivatives in *Actea racemosa* (*Cimicifuga racemosa*, black cohosh) by liquid chromatography/tandem mass spectrometry. *Rapid communications in mass spectrometry*. 17: 978-982, DOI: 10.1002/rcm.1008
- Malintan, N. T.; and Mohd, M. A. (2006). Determination of sulfonamides in selected Malaysian swine wastewater by high-performance liquid chromatography. *Journal of Chromatography A*, 1127, 154-160. doi: 10.1016/j.chroma.2006.06.005
- Melekhin, A. O.; Tolmacheva, V. V.; Shubina, E. G.; Dmitrienko, S. G.; Apyari, V. V.; Grudev, A. I. (2021). Determination of nitrofurantol metabolites in honey using a new derivatization reagent, magnetic solid-phase extraction and LC-MS/MS. *Talanta*, 230, 122310. <https://doi.org/10.1016/j.talanta.2021.122310>

- Matubber, B.; Rahman, S. M. E.; Akter, S.; Farzana, F.; Rahman, M. A.; Wang, J.; Oh, D. (2025). Prevalence of antibiotic resistance and residue in meats in southwest region of Bangladesh. *Food and Humanity*, 4, 100636
- Moghadam, N. R.; Arefhosseini, S. R.; Javadi, A.; Lotfipour, F.; Ansarin, M.; Tamizi, E.; Nemati, M. (2018). Determination of Enrofloxacin and Ciprofloxacin Residues in Five Different Kinds of Chicken Tissues by Dispersive Liquid-Liquid Microextraction Coupled with HPLC. *Iran Journal of Pharmaceutical Research*. 17(4):1182-1190
- Moretti, S.; Cruciani, G.; Romanelli, S.; Rossi, R.; Salutia G.; Galarini, R. (2016). Multiclass method for the determination of 62 antibiotics in milk. *Journal of Mass Spectrometry*, Wiley. DOI 10.1002/jms.3834
- Mottier, P.; Parisod, V.; Gremaud, E.; Guy, P. A.; Stadler, R. H. (2003). Determination of the antibiotic chloramphenicol in meat and seafood products by liquid chromatography-electrospray ionization tandem mass spectrometry, *Journal of Chromatography A*, 994(1-2), 75-84. [https://doi.org/10.1016/S0021-9673\(03\)00484-9](https://doi.org/10.1016/S0021-9673(03)00484-9)
- Mou, S. A.; Islam, R.; Shoeb, M.; Nahar, N. (2021). Determination of chloramphenicol in meat samples using liquid chromatography-tandem mass spectrometry. *Food Science & Nutrition*, 9(10), 5670-5675. doi: 10.1002/fsn3.2530
- Moudgil, P.; Bedi, J. S.; Aulakh, R. S.; Gill, J. P. S. Analysis of antibiotic residues in raw and commercial milk in Punjab, India vis-à-vis human health risk assessment. *Journal of Food Safety* 2019, 39 (4), e12643. <https://doi.org/10.1111/jfs.12643>
- Murungi, M.; Vudriko, P.; Ndagije, H. B.; Kesi, D. N.; Serwanga, A.; Rajab, K.; Manirakiza, L.; Waswa, J. P.; Kasujja, H.; Barigye, M.; Adegbeye, D. M. J.; Adetuyi, B. O.; Igirigi, A. I.; Adisa, A.; Palangi, V.; Aiyedun, S.; Alvarado-Ramírez, E. R.; Elghandour, M. M.M.Y.; Molina, O. M.; Oladipo, A. A.; Salem, A. Z. M. (2024). Comprehensive insights into antibiotic residues in livestock products: Distribution, factors, challenges, opportunities, and implications for food safety and public health. *Food control*, 163, 110545
- Musarurwa, H.; Chimuka, L.; Pakade, V. E.; Tavengwa, N. T. (2019). Recent developments and applications of QuEChERS based techniques on food samples during pesticide analysis. *Journal of Food Composition and Analysis*. 84, 103314. <https://doi.org/10.1016/j.jfca.2019.103314>
- Murielle, G. J.; Bernard, D.; Sophie, G.; Marie-Pierre, F.; Valerie, G.; Dominique, H. P.; Eric, V.; Pascal, S. (2009). Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: qualitative approach. *Food Additives and Contaminants*,

6(11), 1459-1471. Doi: 0.1080/02652030903150575

Muteeb, G.; Rehman, M. T.; Shahwan, M.; Aatif, M. (2023). Origin of Antibiotics and Antibiotic Resistance, and Their Impacts on Drug Development: A Narrative Review. *Pharmaceuticals* (Basel). 16(11):1615. doi: 10.3390/ph16111615

Ngoc Do M.H.; Yamaguchi T.; Okihashi M.; Harada K.; Konishi Y.; Uchida K.; Bui L.T.; Nguyen T.D.; Phan H.B.; Bui H.D.T.; Nguyen P.D.; Kajimura K.; Kumeda Y.; Van Dang C.; Hirata K.; Yamamoto Y. (2016). Screening of antibiotic residues in pork meat in Ho Chi Minh City, Vietnam, using a microbiological test kit and liquid chromatography/tandem mass spectrometry, *Journal of Food Control*. doi: 10.1016/j.foodcont.2016.05.004

Nugrahani, I.; Aini, A. F. N.; Wibowo, M. S. (2024). Solid-state properties and antibiotic potency of levofloxacin dicarboxylate salts prepared by the green method. *Heliyon*, 10(22), e40373. <https://doi.org/10.1016/j.heliyon.2024.e40373>

Odundo, F.; Ngigi, A.; Magu, M. (2023). Sulfonamides and β -lactam antibiotic residues and human health risk assessment in commercial chicken meat sold in Nairobi City, Kenya. *Heliyon*, 9(8):e18810. doi: 10.1016/j.heliyon.2023.e18810

Okaiyeto, S.A.; Sutar, P. P.; Chen, C.; Ni, J.; Wang, J.; Mujumdar, A. S.; Zhang, J.; Xu, M.; Fang, X.; Zhang, C.; Xiao, H. (2024). Antibiotic resistant bacteria in food systems: Current status, resistance mechanisms, and mitigation strategies. *Agriculture Communications* 2(1), 100027. <https://doi.org/10.1016/j.agrcom.2024.100027>

Onyeaka, H.; Ghosh, S.; Obileke, K.; Miri, T.; Odeyemi, O. A.; Nwaiwu, O.; Tamasiga, P. (2024). Preventing chemical contaminants in food: Challenges and prospects for safe and sustainable food production. *Food Control*. 155, 110040. <https://doi.org/10.1016/j.foodcont.2023.110040>

Ovung, A.; Rupreo, V.; Bhattacharyya, J. (2022). Molecular insight into sulfa antibiotic drug binding with human proteins. *Materials Today: Proceedings*, 68, 171-176. <https://doi.org/10.1016/j.matpr.2022.07.203>

Ovung, A.; and Bhattacharyya, J. (2021). Sulfonamide drugs: structure, antibacterial property, toxicity, and biophysical interactions. *Biophysical Reviews*, 13, 259-272. <https://doi.org/10.1007/s12551-021-00795-9>

Owusu-Doubreh, B.; Appaw, W. O.; Abe-Inge, V. (2023). Antibiotic residues in poultry eggs and its implications on public health: A review. *Scientific African*. 19-e01456. <https://doi.org/10.1016/j.sciaf.2022.e01456>

Park, H.; Choi, S. Y.; Kang, H. S.; Kwon, N. J. (2022). Multi residue determination of 96 veterinary drug residues in domestic livestock and fishery products in South Korea. *Aquaculture*, 553, 738064. doi: 10.1016/j.aquaculture.2022.738064

Park, M. S.; Kim, K. T.; Kang, J. S. (2017). Development of an analytical method for detecting nitrofurans in bee pollen by liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Chromatography B*, 1046:172-176. DOI: 10.1016/j.jchromb.2016.11.042

Park, H.; Choi, S. Y.; Kang, H. S.; Kwon, N. J. (2022). Multi residue determination of 96 veterinary drug residues in domestic livestock and fishery products in South Korea. *Aquaculture* 553, 738064

Patyra, E.; Kowalczyk, E.; Kwiatek, K. (2014). Screening method for the determination of selected tetracyclines in water by liquid chromatography with diode array detector. *Bulletin of the Veterinary Institution in Pulawy*, 58, 65-70, DOI: 10.2478/bvip-2014-0010

Patyra, E.; Nebot, C.; Gavilán, R. E.; Cepeda, A.; Kwiatek, K. (2018). Development and validation of an LC-MS/MS method for the quantification of tiamulin, trimethoprim, tylosin, sulfadiazine and sulfamethazine in medicated feed. *Food Additives & Contaminants: Part A*, Taylor & Francis Informa & UK Limited. ISSN: 1944-0049 (Print) 1944-0057 (Online). <https://doi.org/10.1080/19440049.2018.1426887>

Perumal, S.; Kottadiyil, D.; Thasale, R.; Mehta, T. (2024). Optimization of QuEChERS method for determination of pesticide residues in vegetables and health risk assessment. *Environmental science and pollution research international*, 31(23), 34355-34367. doi: 10.1007/s11356-024-33345-3

Qin, M.; Qian, Y.; Huang, L.; Zhong, C.; Li, M.; Yu, J.; Chen, H. (2023). Extractive electrospray ionization mass spectrometry for analytical evaluation and synthetic preparation of pharmaceutical chemicals *Frontiers Pharmacology*, 14. <https://doi.org/10.3389/fphar.2023.1110900>

Rahman, A.; Naznin, M.; Bari, L. (2021). Simultaneous estimation of multiple antibiotic residues from chicken samples using High Performance Liquid Chromatography (HPLC), *Journal of Agriculture, Food and Environment (JAFE)*, 2(1), 20-26. <http://doi.org/10.47440/JAFE.2021.2104>

Ramatla, T.; Ngoma, L.; Adetunji, M.; Mwanza, M. (2017). Evaluation of Antibiotic Residues

in Raw Meat Using Different Analytical Methods. *Antibiotics*, 6, 34; doi:10.3390/antibiotics6040034

Raza, N.; and Kim, K. H. (2017). Quantification techniques for important environmental contaminants in milk and dairy products. *Trends in Analytical Chemistry*, 98, 79-94. <https://doi.org/10.1016/j.trac.2017.11.002>

Rejtharová, M.; and Rejthar, L. (2009). Determination of chloramphenicol in urine, feed water, milk and honey samples using molecular imprinted polymer clean-up. *Journal of Chromatography A*, 1216, 8246-8253. <https://doi.org/10.1016/j.chroma.2009.07.037>

Renew, J. E.; and Huang, C. H. (2004). Simultaneous determination of fluroquinolone, sulfonamide and trimethoprim antibiotics in wastewater using tandem solid phase extraction and liquid chromatography-electrospray mass spectrometry. *Journal of chromatography A*, 1042, 113-121. doi: 10.1016/j.chroma.2004.05.056

Rizzo, S.; Russo, M.; Labra, M.; Campone, L.; Rastrelli, L. (2020). Determination of Chloramphenicol in Honey Using Salting-Out Assisted Liquid-Liquid Extraction Coupled with Liquid Chromatography-Tandem Mass Spectrometry and Validation According to 2002/657 European Commission Decision. *Molecules*, 25(3481), doi:10.3390/molecules25153481

Rinky, F., Rahman, A., Reza, S., Nowar, A., Alim, S. R. (2025). Residual antibiotics in milk samples: Assessing the risk and prevalence in Bangladesh. *Heliyon*. 11, e41422. <https://doi.org/10.1016/j.heliyon.2024.e41422>

Ronning, H. T.; Einarsen, K.; Asp, T. N. (2006). Determination of chloramphenicol residues in meat, seafood, egg, honey, milk, plasma and urine with liquid chromatography-tandem mass spectrometry, and the validation of the method based on 2002/657/EC. *Journal of Chromatography A*, 1118, 226-233. <https://doi.org/10.1016/j.chroma.2006.03.099>

Sachi, S.; Ferdous, J.; Sikder, M. H.; Hussani, S. M. A. K. (2019). Antibiotic residues in milk: Past, present, and future. *Journal of advanced veterinary and animal research*, ISSN 2311-7710, <http://doi.org/10.5455/javar.2019.f350>

SANTE/11312/2021, Main changes introduced in Document N° SANTE/11312/2021v2026 with respect to the previous version (Document N° SANTE/11312/2021-v2)

References

- Santás-Miguel, V.; Arias-Estévez, M.; Díaz-Raviña, M.; Fernández-Sanjurjo, M. J.; Álvarez-Rodríguez, E.; Núñez-Delgado, A.; Fernández-Calviño, D. (2020). Effect of Oxytetracycline and Chlortetracycline on Bacterial Community Growth in Agricultural Soils. *Agronomy*, 10(7), 1011, <https://doi.org/10.3390/agronomy10071011>
- Samouris, G.; Tsoukali-Papadopoulou, H.; Nathanael, B.; Mirtsou-Fidani, V. (1998). Chloramphenicol residues in albumen and yolk of hen's eggs after experimental administration Rückstände von Chloramphenicol in Eiklar und Eigelb von Hühnereiern nach experimenteller Verabreichung. *European Poultry Science*, 62(2), 83-85. [https://doi.org/10.1016/S0003-9098\(25\)00665-4](https://doi.org/10.1016/S0003-9098(25)00665-4)
- Sanlier, N.; and Üstün, D. (2021). Egg consumption and health effects: A narrative review. *Journal of Food Science*, 86(10):4250-4261. doi: 10.1111/1750-3841.15892
- Sani, A. A.; Rafiq, K.; Hossain, M. T.; Akter, F.; Haque, A.; Hasan, M. I.; Sachi, S.; Mustari, A.; Islam, M. Z.; Alam, M. M. (2023). Screening and quantification of antibiotic residues in poultry products and feed in selected areas of Bangladesh. *Veterinary World*, 16(8): 1747-1754. <https://doi.org/10.14202/vetworld.2023.1747-1754>
- Sanga, B.; Kharel, M. K. (2024). Chloramphenicol, *Encyclopedia of Toxicology (Fourth Edition)*, 2, 825-830. <https://doi.org/10.1016/B978-0-12-824315-2.00349-3>
- Sarkar, S.; Souza, M. J.; Martin-Jimenez, T.; Abouelkhair, M. A.; Kania, S. A.; Okafor, C. C. (2023). Tetracycline, Sulfonamide, and Erythromycin Residues in Beef, Eggs, and Honey Sold as “Antibiotic-Free” Products in East Tennessee (USA) Farmers’ Markets. *Vet. Science*, 10(4), 243; <https://doi.org/10.3390/vetsci10040243>
- Sarker, Y. A.; Hasan, M. M.; Paul, T. K.; Rashid, S. Z.; Alam, M. N.; Sikder, M. H. (2018). Screening of antibiotic residues in chicken meat in Bangladesh by thin layer Chromatography. *Journal of Advanced Veterinary and Animal Research*, 5(2), 140-145, ISSN 2311-7710. <http://doi.org/10.5455/javar.2018.e257>
- Scortichini, G.; Annunziata, L.; Haouet, M.N.; Benedetti, F.; Krusteva, I.; Galarini, R. (2005). ELISA qualitative screening of chloramphenicol in muscle, eggs, honey and milk: method validation according to the Commission Decision 2002/657/EC criteria. *Analytica Chimica Acta*, 535(1-2), 43-48. <https://doi.org/10.1016/j.aca.2004.12.004>
- Shahbazi, Y.; Ahmadi, F.; Karami, N. (2015). Screening determination and confirmation of tetracycline residues in chicken tissues using four-plate test, ELISA and HPLC-UV methods:

comparison between correlation results, *Food and Agricultural Immunology*, 26:6, 821-834, DOI: 10.1080/09540105.2015.1036357

Semeraro, A.; Morelli, S.; Pasquale, M. D.; Turco, A. C.; D'Amato, M.; Giacomozzi, C.; Stacchini, P.; Sorbo, A. (2024). Control of residues of non-authorized pharmacologically active substance in food of animal origin. Results of a proficiency test for determination of chloramphenicol in honey organized by the Italian National Reference Laboratory for Residues. *Journal of Food Composition and Analysis*, 135, 106656. <https://doi.org/10.1016/j.jfca.2024.106656>

Shoeb, M.; Nahar, N.; Mandal, S.; Sarma, H. M.; Seraji, M. S. I. (2009). Nitrofurantoin antibiotics and organochlorine pesticides in fish and poultry feed, feed ingredients and water samples collected from shrimp cultivation areas. *Journal of Bangladesh Chemical Society*, 22 (1): 1-8

Shoeb, M., Islam, R.; Parvin, N. (2023). Antibiotic Resistance: A Global Threat to Humanity, Chapter 5, page: 24, *Transcending Humanitarian Engineering Strategies for Sustainable Futures*, A volume in the *Advances in Environmental Engineering and Green Technologies (AEEGT) Book Series*. IGI Global, DOI: 10.4018/978-1-6684-5619-4.ch005

Steiner, D.; Malachová, A.; Sulyok, M.; Krska, R. (2020). Challenges and future directions in LC-MS-based multiclass method development for the quantification of food contaminants. *Analytical and Bioanalytical Chemistry*, 413(1):25-34. doi: 10.1007/s00216-020-03015-7

Stojković, D.; Petrović, J.; Carević, T.; Soković, M.; Liaras, K. (2023). Synthetic and Semisynthetic Compounds as Antibacterials Targeting Virulence Traits in Resistant Strains: A Narrative Updated Review. *Antibiotics (Basel)* 12(6):963. DOI: 10.3390/antibiotics12060963

Sultan, I. A. (2014). Detection of Enrofloxacin Residue in Livers of Livestock Animals Obtained from a Slaughterhouse in Mosul City. *J Veterinar Sci Technol* 2014, 5:2. DOI: 10.4172/2157-7579.1000168

Suo, D.; Wang, P.; Xiao, Z.; Zhang, S.; Zhuang, H.; Li, Y.; Su, X. (2019). Multiresidue Determination of 27 Sulfonamides in Poultry Feathers and Its Application to a Sulfamethazine Pharmacokinetics Study on Laying Hen Feathers and Sulfonamide Residue Monitoring on Poultry Feathers. *Journal of Agricultural and Food Chemistry*, DOI: 10.1021/acs.jafc.9b02782

Suo, D.; Zhang, S.; Song, Z.; Wang, S.; Li, Y.; Fan, X. (2022). Simultaneous Determination of 21 Sulfonamides in Poultry Eggs Using Ionic Liquid-Modified Molecularly Imprinted Polymer SPE and UPLC-MS/MS. *Molecules*, 27(15), 4953; <https://doi.org/10.3390/molecules27154953>

References

- Sumaiya, K. A.; Alka, A.; Mariya, A. H.; Razna, A. M. (2024). Screening of veterinary drug residues in marketed milk and eggs in Oman. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, 14(3),373-378, doi: 10.4103/ijnpnd.ijnpnd_79_24
- Sun, Y.; Waterhouse, G. I. N.; Qiao, X.; Xiao, J.; Xu, Z. (2023). Determination of chloramphenicol in food using nanomaterial-based electrochemical and optical sensors-A review. *Food Chemistry*.410, 135434. <https://doi.org/10.1016/j.foodchem.2023.135434>
- Tao, J.; Feng, N.; Zhang, Y.; Chen, Y.; Zeng, Z.; Jiang, H.; Ma, J. (2025). Ultrabright fluorescence probe mediated biosensor for rapid and ultrasensitive quantification of chloramphenicol in milk. *Microchemical Journal*, 209, 112883. <https://doi.org/10.1016/j.microc.2025.112883>
- Tavakoli, H. R.; Firouzabadi, M. S. S.; Afsharfarnia, S.; Jafari, N. J.; Saadat, S. (2015). Detecting antibiotic residues by HPLC method in chicken and calves meat in diet of a military center in Tehran. *Acta Medica Mediterranea*, 31: 1427-1433
- Tian, H.; Wang, J.; Zhang, Y.; Li, S.; Jiang, J.; Tao, D.; Zheng, N. (2016). Quantitative multiresidue analysis of antibiotics in milk and milk powder by ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometry. *Journal of Chromatography B*, 15:1033-1034:172-179. doi: 10.1016/j.jchromb.2016.08.023
- Tolika, E. P.; Samanidou, V. F.; Papadoyannis, I. N. (2010). An Overview of Chromatographic Analysis of Sulfonamides in Pharmaceutical Preparations and Biological Fluids. *Current Pharmaceutical Analysis*, 6(3), 198-212
- Tongchai, P.; Sawarng, N.; Wongta, A.; Jaitham, U.; Sutan, K.; Kawichai, S.; Jung, C.; Chuttong, B.; Hongsibsong, S. (2025). Optimization and Validation of a QuEChERS-Based Method Combined with Gas Chromatography-Tandem Mass Spectrometry for Analyzing Pesticide Residues in Edible Insect Samples. *Molecule*, 30(11), 2293, doi: <https://doi.org/10.3390/molecules30112293>
- Tong, J.; Rao, O.; Zhu, K.; Jiang, Z.; Ding, S. (2009). Simultaneous determination of five tetracycline and macrolide antibiotics in feeds using HPCE. *Journal of separation science*, 32(23-24), 4254-4260. <https://doi.org/10.1002/jssc.200900415>
- Uekane, T. M.; Neto, F. R. A.; Gomes, L.N. F. (2011). 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*, 34(1), 43-48. <https://doi.org/10.1590/S0100-40422011000100009>

Vass, M.; Hruska, K.; Franek, M. (2008). Nitrofurantoin antibiotics: a review on the application, prohibition and residual analysis. *Vet Med - Czech*, 53(9), 469-500. DOI: 10.17221/1979-VETMED

Venkatesh, P.; Kumar, N. A.; Prasad, H.; Krishnamoorthy, K.B.; Prasath, K. H.; Soumya, V. (2013). LC-MS/MS analysis of tetracycline antibiotics in prawns (*Penaeus monodon*) from south India coastal region. *Journal of Pharmacy Research* 6(1):48-52. DOI:10.1016/j.jopr.2012.11.012

Venkatesan, M.; Fruci, M.; Verellen, L. A.; Skarina, T.; Mesa, N.; Flick, R.; Pham, C.; Mahadevan, R.; Stogios, P. J.; Savchenko, A. (2023). Molecular mechanism of plasmid-borne resistance to sulfonamide antibiotics. *Journal of Natural Communications*. 14:4031. doi: 10.1038/s41467-023-39778-7

Veiga-del-Baño, J. M.; Andreo-Martínez, P.; Pérez-Lucas, G.; Navarro, S. (2024). Overview of the Evolution and Trends of the QuEChERS Sample Preparation Procedure. *Reviews of Environmental Contamination and Toxicology*, 262(22). <https://doi.org/10.1007/s44169-024-00073-1>

Velazquez-Meza, M. E.; Galarde-López, M.; Carrillo-Quiróz, B.; Alpuche-Aranda, C. M. (2022). Antimicrobial resistance: One Health approach, *Vet World*. 15(3):743-749. doi: 10.14202/vetworld.2022.743-749

Viñas, P.; Balsalobre, N.; Hernández-Córdoba, M. (2006). Determination of chloramphenicol residues in animal feeds by liquid chromatography with photo-diode array detection. *Analytica Chimica Acta*, 558(1-2), 11-15. <https://doi.org/10.1016/j.aca.2005.10.059>

Violante, F. G. M.; Rosas, C. D. O.; Guimarães, E. D. F.; Vital, H. D. C.; Zúñiga, N. D. C.; Neto, F. R. D. A. (2018). Feasibility study for the development of a certified reference material of nitrofurantoin metabolites in chicken breast muscle from incurred samples. *Measurement*, 129:368-374. <https://doi.org/10.1016/j.measurement.2018.07.027>

Vuran, B.; Ulusoy, H. I.; Sarp, G.; Yilmaz, F.; Morgül, U.; Kabir, A.; Tartaglia, A.; Locatelli, M.; Soylak, M. (2021). Determination of chloramphenicol and tetracycline residues in milk samples by means of nanofiber coated magnetic particles prior to high-performance liquid chromatography-diode array detection. *Talanta*, 230, 122307. <https://doi.org/10.1016/j.talanta.2021.122307>

Wang, C.; Song, Y.; Liang, J.; Wang, Y.; Zhang, D.; Zhao, Z. (2024). Antibiotic resistance genes are transferred from manure-contaminated water bodies to the gut microbiota of animals through the food chain. *Environmental Pollution*, 363 (1), 125087.

<https://doi.org/10.1016/j.envpol.2024.125087>

Wang, H.; Wang, N.; Qian, J.; Hu, L.; Huang, P.; Su, M.; Yu, X.; Fu, C.; Jiang, F.; Zhao, Q.; Zhou, Y.; Lin, H.; He, G.; Chen, Y.; Jiang, Q. (2017). Urinary antibiotics of pregnant women in Eastern China and cumulative health risk assessment. *Environmental Science & Technology*, doi: 10.1021/acs.est.6b06474

Wang, J.; Yang, H. Y.; Wang, X. D.; Lv, Y. F.; Wei, N. (2025). Application of QuEChERS for Analysis of Contaminants in Dairy Products: A Review. *Journal of Food Protection*, 88 (3),100453. <https://doi.org/10.1016/j.jfp.2025.100453>

Wang, K.; Wang, M.; Kou, Y.; Guo, C.; Ma, X.; Tian, B.; Qi, Y.; Wang, W.; Wang, J. (2022). Determination of nitrofurans metabolites in meat products by UHPLC-fluorescence with ultrasonic-assisted derivatization. *Journal of Food Composition and Analysis*, 107, 104375. <https://doi.org/10.1016/j.jfca.2021.104375>

Wang, H.; Ren, L.; Yu, X.; Hu, J.; Chen, Y.; He, G.; Jiang, Q. (2017). Antibiotic residues in meat, milk and aquatic products in Shanghai and human exposure assessment. *Food Control*. DOI: 10.1016/j.foodcont.2017.04.034

Wang, L.; Yang, H.; Zhang, C.; Mo, Y.; Lu, X. (2008). Determination of oxytetracycline, tetracycline and chloramphenicol antibiotics in animal feeds using subcritical water extraction and high-performance liquid chromatography. *Analytica Chimica Acta*, 619(1), 54-58. <https://doi.org/10.1016/j.aca.2008.01.026>

Wang, R., Zhang, C. X., Li, Z. Y., Zheng, Z. Y., Xiang, Y., Liu, Y., Zhao, R. F., Fang, J. (2022). Detection of fluoroquinolone and sulfonamide residues in poultry eggs in Kunming city, southwest China. *Poultry Science*. 101(6), 101892. <https://doi.org/10.1016/j.psj.2022.101892>

Waghmare, R.; Paturkar, A. M.; Vaidya, V.; Zende, R. (2020). Screening of Antimicrobial Residues and Confirmation of Doxycycline in Samples Collected from Chicken Farms and Processing Units Located Around Mumbai, India. *Indian Journal of Animal Research*, DOI:10.18805/ijar.B-3899

World Health Organization (WHO) 2024, <https://www.who.int/news-room/fact-sheets/detail/food-safety>

Wu, S.; Zhang, Y.; Jia, X.; Mao, J.; Wu, H.; Wang, S.; Guo, H. (2025). Molecularly imprinted electrochemical sensor for ultrasensitive determination of chloramphenicol in milk and egg samples. *Food Chemistry*, 489, 145024. <https://doi.org/10.1016/j.foodchem.2025.145024>

References

- Xie, X.; Wang, B.; Pang, M.; Zhao, X.; Xie, X.; Zhang, Y.; Wang, Y.; Guo, Y.; Liu, C.; Bu, X.; Wang, R.; Shib, H.; Zhang, G.; Zhang, T.; Dai, G.; Wang, J. (2018). Quantitative analysis of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in eggs via liquid chromatography-electrospray ionization tandem mass spectrometry. *Food Chemistry*, 269, 542-548. <https://doi.org/10.1016/j.foodchem.2018.07.045>
- Xu, C.; Kong, L.; Gao, H.; Cheng, X.; Wang, X. (2022). A Review of Current Bacterial Resistance to Antibiotics in Food Animals. *Frontiers in Microbiology*, 13:822689. doi: 10.3389/fmicb.2022.822689
- Xu, X.; Xiao, Y.; Hu, F.; Geng, D. (2016). Rapid Determination of Trace Multiresidues of 18 Sulfonamides in Chicken Eggs Using a Modified QuEChERS Method Coupled with Ultrahigh Performance Liquid Chromatography. *Journal of Food Protection*, 79(9), 1549-1555. doi:10.4315/0362-028X.JFP-15-509
- Yang, G.; Ding, S.; Zhang, J.; Gu, L.; Zhai, W.; Kong, C. (2024). Research progress on metabolites of nitrofurazone in aquatic products. *Heliyon*, 10 (8), e29735. <https://doi.org/10.1016/j.heliyon.2024.e29735>
- Yuana, G.; Zhua, Z.; Yang, P.; Lu, S.; Liu, H.; Liu, W.; Guihua Liu (2020). Simultaneous determination of eight nitrofurans residues in shellfish and fish using ultra-high performance liquid chromatography-tandem mass spectrometry. *Journal of Food Composition and Analysis*, 92, 103540. <https://doi.org/10.1016/j.jfca.2020.103540>
- Zhang, Y.; Xu, X.; Liu, H.; Zhai, Y.; Sun, Y.; Sun, S.; Zhang, H.; Yu, A.; Wang, Y. (2012). Matrix Solid-Phase Dispersion Extraction of Sulfonamides from Blood. *Journal of Chromatographic Science*, 50, 131-136. doi:10.1093/chromsci/bmr040
- Zhang, T.; Nickerson, R.; Zhang, W.; Peng, X.; Shang, Y.; Zhou, Y.; Luo, Q.; Wen, G.; Zhenyu, C. (2024). The impacts of animal agriculture on One Health-Bacterial zoonosis, antimicrobial resistance, and beyond. *One Health*, 18, 100748. <https://doi.org/10.1016/j.onehlt.2024.100748>
- Zhang, D.; Park, Z. Y.; Park, J. A.; Kim, S. K.; Jeong, D.; Cho, S. H.; Shim, J. H.; Kim, J. S.; El-Aty, A. M. A.; Shin, H. C. (2016). A combined liquid chromatography-triple-quadrupole mass spectrometry method for the residual detection of veterinary drugs in porcine muscle, milk, and eggs. *Environ Monitoring and Assessment*, 188: 348. doi.org/10.1007/s10661-016-5344-x
- Zhang, F.; Zhang, F.; Yu, X.; Sun, R.; Wu, Y.; Zhou, Y.; Li, L. (2025). Discovery of a Novel Family of Gram-Negative-Active Cationic Cyclolipopeptides by Motif Search-Guided Chemical Synthesis. *Chinese chemical Society*, <https://doi.org/10.31635/ccschem.024.202404925>

References

Zhang, Y.; Lu, J.; Yan, Y.; Liu, J.; Wang, M. (2021). Antibiotic residues in cattle and sheep meat and human exposure assessment in southern Xinjiang, China. *Food Science & Nutrition*. 9 (11), 6152–6161. <https://doi.org/10.1002/fsn3.2568>

Zhao, H.; Ding, M.; Gao, Y.; Deng, W. (2014). Determination of Sulfonamides in Pork, Egg, and Chicken Using Multiwalled Carbon Nanotubes as a Solid-Phase Extraction Sorbent Followed by Ultra-Performance Liquid Chromatography/Tandem Mass Spectrometry. *Veterinary drug residues, Journal of AOAC International*, 97(5), 1481-1488. DOI: 10.5740/jaoacint.13-133

Zhao, M.; Li, X.; Zhang, Y.; Wang, Y.; Wang, B.; Zheng, L.; Zhang, D.; Zhuang, S. (2021). Rapid quantitative detection of chloramphenicol in milk by microfluidic immunoassay. *Food Chemistry*, 339, 127857. <https://doi.org/10.1016/j.foodchem.2020.127857>

Zuo, X.; and Ai-yun, H. (2021). The Residues and Risk Assessment of Sulfonamides in Animal Products. *Journal of food Quality. Applications of Mass Spectrometry in the Analysis of Food Composition and Contaminants*. <https://doi.org/10.1155/2021/5597755>

<https://www.canada.ca/en/health-canada/services/drugs-health-products/veterinary-drugs/maximum-residue-limits-mrls/list-maximum-residue-limits-mrls-veterinary-drugs-foods.html>

<https://www.alltech.com/press-release/2025-alltech-agri-food-outlook-shares-global-feed-production-survey-data>
(www.alltech.com) <https://ifif.org/global-feed/statistics/>

<https://lightcastlepartners.com/news/poultrytechbangladesh-impact-tour-2024/>

<https://www.who.int/westernpacific/newsroom/commentaries/detail/the-next-pandemic-is-already-here--antimicrobial-resistance-is-upending-a-century-of-achievements-in-global-health>

<https://apps.who.int/food-additives-contaminants-jecfa-database/>