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Manual of Standard Operating Procedures for Veterinary Drug Residue Analysis

Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

VIENNA, 2016

TRAINING COURSE SERIES 63

MANUAL OF STANDARD OPERATING PROCEDURES FOR VETERINARY DRUG RESIDUE ANALYSIS

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INTERNATIONAL ATOMIC ENERGY AGENCY
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FOREWORD

Laboratories are crucial to national veterinary drug residue monitoring programmes. However, one of the main challenges laboratories encounter is obtaining access to relevant methods of analysis. Thus, in addition to training, providing technical advice and transferring technology, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture has resolved to develop clear and practical manuals to support Member State laboratories.

The Coordinated Research Project (CRP) on Development of Radiometric and Allied Analytical Methods to Strengthen Residue Control Programs for Antibiotic and Anthelmintic Veterinary Drug Residues has developed a number of analytical methods as standard operating procedures (SOPs), which are now compiled here. This publication contains SOPs on chromatographic and spectrometric techniques, as well as radioimmunoassay and associated screening techniques, for various anthelmintic and antimicrobial veterinary drug residue analysis. Some analytical method validation protocols are also included.

The publication is primarily aimed at food and environmental safety laboratories involved in testing veterinary drug residues, including under organized national residue monitoring programmes. It is expected to enhance laboratory capacity building and competence through the use of radiometric and complementary tools and techniques. The publication is also relevant for applied research on residues of veterinary drugs in food and environmental samples.

Seventeen collaborating research organizations from fifteen IAEA and FAO Member States that participated in the CRP were involved in drafting the SOPs in this publication. The IAEA officer responsible for this publication was J.J. Sasanya of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

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1. INTRODUCTION

1.1. BACKGROUND

The manual is a product of a Joint FAO/IAEA Coordinated Research Project (CRP) "Development of Radiometric and Allied Analytical Methods and Strategies to Strengthen Residue Control Programs for Antibiotic and Anthelmintic Veterinary Drug Residues" conducted between 2009 and 2014 to support monitoring of selected veterinary antibiotic and anthelmintic drug residues in food and environmental samples in order to safeguard consumer safety.

Mostly multi−residue methods were developed and validated during the research and subsequently applied to national residue programs in some countries. Since only 15 countries participated in the research, it is believed other Member State laboratories can benefit from the project output through dissemination of transferrable techniques in form of Standard Operating Procedures (SOPs) complied as a manual. Though a number of screening and confirmatory techniques are included in the manual, and in view of the extent and diversity of analytical needs around the world, the manual therefore covers a limited scope, informed by the needs identified by participants of the CRP. However, these needs are common among many Member States and it is hoped the manual will be useful to many testing laboratories.

1.2. OBJECTIVE

The purpose of this manual is to aid food and environmental testing laboratories primarily in the routine monitoring and control of residues of some veterinary drugs in animal products but also selected environmental samples. The manual can also support relevant research activities.

1.3. SCOPE

This manual publication consists of analytical methods, in form of SOPs, for testing selected veterinary drug residues in some animal products and environmental samples. It covers a number of chromatographic−spectrometric and antibody based techniques, radio−immunoassay, radioisotopes and liquid scintillation counting technique. These methods were produced based on some needs and research questions raised by 17 institutions that participated in the above CRP and therefore do not cover a very broad range. The information in the manual is also presented as an informative guide in some respect.

1.4. STRUCTURE

The preliminary (and larger) part of the manual consists of chromatographic and spectrometric SOPs for analysis of anthelmintic residues such as benzimidazoles and avermectins, antimicrobial residues such as chloramphenicol and florfenicol, tetracyclines, aminoglycosides, sulfonamides as well as quinolones. An SOP for a radioimmunoassay technique for residues of florfenicol and florfenicol amine is also included in the early part. The manual then concludes with two validation protocols.

2. DETERMINATION OF BENZIMIDAZOLE AND AVERMECTIN RESIDUES IN BOVINE MILK BY LIQUID CHROMATOGRAPHY−TANDEM MASS SPECTROMETRY

2.1. PRINCIPLE

This method is based on the principle of the quick, easy, cheap, effective, rugged and safe (QuEChERS) method [1]. It includes extraction of a representative portion of the sample with acetonitrile (MeCN) followed by salting−out and dispersive solid−phase extraction with a mixture of magnesium sulphate ($MgSO_4$) and C18 material. After clean−up, an aliquot of the supernatant is analyzed by liquid chromatography tandem mass spectrometry (LC−MS/MS).

2.2. SCOPE

This analytical method includes determination of residues of seven benzimidazoles namely, albendazole (ABZ), thiabendazole (TBZ), albendazole−sulphoxide, (ABZ−SO), albendazole−sulphone (ABZ−SO_2), triclabendazole (TCB), triclabendazole−sulphoxide (TCB−SO) and triclabendazole−sulphone (ABZ−SO_2) as well as three avermectins: Abamectin (ABA), emamectin (EMA) and ivermectin (IVE) in bovine milk at concentration levels of 5 ng/g to 500 ng/g.

2.3. MATERIALS

The following reagents and chemical are applicable:

MeCN; High performance liquid chromatography (HPLC) grade Methanol (MeOH); Octadecylsilane sorbent C18; Sodium Chloride (NaCl), analytical grade; Ammonium acetate, analytical grade; Formic acid, analytical grade; Primary secondary amine (PSA) sorbent; Magnesium sulphate anhydrous.

2.3.1. Standards and stock solutions

The analytical standards include: ABZ, 99.6% and IVE, 91.0% from United States Pharmacopoeia (USP); TBZ 98.3%, ABA, 94.4%, EMA, 96.5%; Cyprodinil, 99.5% all from Chem service; ABZ−SO, ABZ−SO_2, TCB−SO and TCB−SO_2 all from Witega; TCB, 99.0% (Dr. Ehrenstorfer); and Triphenyl phosphate 99.9% (Supelco/Sigma Aldrich).

The solutions (and how they are prepared) are as follows:

a) Stock standard solutions (2,000 µg/mL)

Weigh 20 mg of each analytical standard into 10 mL volumetric flask and fill to volume with MeOH (for EMA, IVE and ABA), MeCN (for TCB, TCB−SO and TCB−SO_2) or dimethyl formamide (for ABZ, ABZ−SO and ABZ−SO_2). Store all stock standard solutions at -18°C.

b) Intermediate standard solution (100 µgm/L)

Transfer 500 µL of each stock standard solution (2,000 µgm/L) into 10 mL volumetric flask and fill to volume with MeCN

c) Working Standard solution (0.1 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL and 10 µg/mL)

Transfer 10 µL, 100 µL, 200 µL, 500 µL and 1,000 µL of mixed standard solution (100 µg/mL) into five 10 mL volumetric flasks and fill to volume with MeCN to obtain mixed working standard solutions of 0.1 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL and 10 µg/mL, respectively.

d) Internal Standard (IS): Triphenyl phosphate (TPP) (0.1 µg/mL)

Use TPP as IS for positive ion LC−MS/MS mode. Prepare by weighing 10 mg of TPP into a 10 mL volumetric flask and fill to volume with MeCN (1,000 µg/mL). Transfer 10 µL of this solution into a 10 mL volumetric flask and bring to volume with MeCN.

e) Cyprodinil IS (10 µg/mL)

Use Cyprodinil as a quality control (QC) standard to monitor the performance of the LC−MS/MS system. Prepare by weighing 10 mg of Cypronidil into a 10 mL volumetric flask and fill to volume with MeCN (1,000 µg/mL). Transfer 100 µL of this solution into a 10 mL volumetric flask and bring to volume with MeCN.

2.3.2. Apparatus

The following apparatus are applicable:

Refrigerated centrifuge (Sigma, Model 4K 15C); Vortex mixer, Barnstead Thermolyne. (Model 37600 Mixer); Balance, 0.1 mg sensitivity; Multi Reax shaker (Heidolph); Class A volumetric flasks (10, 25 mL clear glass); Polypropylene centrifuge tubes (50 mL); LC–MS/MS system (Waters Alliance 2695 coupled to Quattro Premier XE, Waters Corporation, USA).

2.4. PROCEDURE

2.4.1. Sample extraction

The following steps are followed during extraction:

(a) Weigh 10 g of homogenized milk samples into 50 mL fluorinated ethylene propylene centrifuge tubes; use 10 mL distilled (H_2O) for a reagent blank
(b) Weigh 20 g of blank milk samples for matrix−matched calibration standards.
(c) Weigh 10 g for QC spikes. Add 100 µL of mixed standard solution (10 µg/mL of the seven benzimidazoles and three avermectins) to obtain 100 ngg^{-1} QC; and let it stand for 15 minutes (min)
(d) Add 100 µL of IS TPP solution (0.1 µg/mL) per 10 g (this results in 1 ng/g equivalent concentration) to all tubes containing sample, QC spike, blank matrix and reagent blank
(e) Add 10 mL of MeCN in each tube
(f) Add 5 g of $MgSO_4$:NaCl [4:1, weight by weight (w/w)] and immediately shake the tubes for 1 min and centrifuge [for 5 min) at 3,500 revolutions per minute (rpm)]
(g) Transfer 2 mL of supernatant into a 5 mL tube containing $MgSO_4$ (300 mg) and 100 mg of C18. Vortex for 1 min and centrifuge at 3,000 rpm for 2 min
(h) Transfer an aliquot of the supernatant (1 mL) into an autosampler vial containing 10 µL of cyprodinil solution
(i) Inject 10 µL of the final solution onto the LC−MS/MS system

2.4.2. Matrix−matched standards calibration

To prepare matrix matched standard curve, transfer 1 mL of the sample extract into each 5 autosampler vial. Add to each 25 µL of 0.1 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL and 10 µg/mL working standard solutions, corresponding to the concentrations of 5 µg/kg, 50 µg/kg, 100 µg/kg, 250 µg/kg and 500 µg/kg, respectively. Add 10 µL of Cypronidil (100 µg/kg) to the 5 vials.

2.4.3. Chromatographic condition

a) System suitability

To evaluate system suitability, inject at least five replicates of an intermediate standard used for the calibration curve. The relative standard deviation (RSD) of the peak response and the retention time should not be greater than 5%.

b) Instrumental condition

As detailed in Table 1, conduct reversed−phase separation of analytes at using an XTerra MS C18 column (150 mm × 2.1 mm I.D., 5 µm particles) maintained at 20°C.

The final mobile phase conditions is (A) 0.1% aqueous formic acid and (B) MeCN as Gradient elution (0.3 mL/min) is used with an initial starting condition of 98:2 [A:B, volume by volume (v/v], and the organic concentration increased to 100% over 5 min; hold for 10.5 min before returning it to the initial starting condition.

Equilibrate the LC system for at least 30 min with mobile phase A:mobile phase (2%:98%, v/v) shown in Table 2 before running samples.

TABLE 1. A SUMMARY OF CHROMATOGRAPHIC CONDITIONS

Chromatographic column	Reversed-phase C18, 150 mm x 2.1 mm, 3.5 µm
Mobile phase	Mobile phase A: Mobile phase B (2:98, v/v)
Flow condition	Gradient
Flow rate	0.2 mL/min
Injection volume	10 µL
Temperature	25°C

TABLE 2. GRADIENT MOBILE PHASE

Time (min)	A	B	Flow (mL/min)	Curve
0	2	98	0.30	1
5	100	0	0.30	6
10.00	0.0	100.0	0.30	1
15.00	98	2	0.30	1

2.4.4. Mass spectrometry (MS)

Here the MS analyses are performed by atmospheric pressure electrospray ionisation in the positive (ESI+) mode at a flow rate of 0.3 mL/min. Use nitrogen gas for desolvation at 350°C and Argon for collision.

Set the source temperature and ion spray voltage at 120°C and 650°C, respectively. Use a tandem MS in multiple reaction monitoring (MRM) with a dwell time of

100−200 milliseconds (msec) to detect the analytes. Set the cone and desolation gases are set at 50 and 800 L/h, respectively.

The MS acquisition is divided into two time periods (0−7.5 min, and 6.5−14 min). The MS conditions are optimized by tuning the analyte−specific parameters, collision energy, and collision cell exit potential for each analyte. Optimize by infusing 1 µg/mL standard solution of each analyte and monitor the two most abundant fragment ions produced from the molecular ion (Table 3).

TABLE 3. MONITORED IONS AND OPTIMIZED MS PARAMETERS FOR EACH ANALYTE

#	Analyte	MS/MS Transition Base peak (1) Second peak (2)		Cone Voltage (V)	Collision Energy (eV)	Dwell Time (sec)	Function
1	TBZ	202.1>175.1 202.1>131.1	(1) (2)	50 50	30 28	0.20	I
2	ABZ	266.2>234.2 266.2>191.1	(1)	40 40	15 30	0.20	I
3	ABZ−SO_2	298.1>265.9 298.1>159.1	(2)	40 40	28 15	0.20	I
4	ABZ−SO	282.1>239.9 282.1>208	(1)	40 40	28 15	0.20	I
5	TCB	359>274.1 359>344.1	(2)	50 50	25 28	0.20	II
6	TCB−SO2	391>242.3	(1)	50	40	0.10	II
7	TCB−SO_2	375>242.2	(2)	30	45	0.10	II
8	EMA	886.5>158.1 886.5>126	(1)	50 50	30 15	0.10 0.10	I
9	ABA	891>305.1 891>567.2	(2)	50 50	30 12	0.10 0.10	II
10	IVE	892.5>569.2 892.5>307.1	(1)	50 50	30 12	0.10 0.10	II
11	Triphenyl phoshate	327>77	(2)	25	20	0.20	II
12	Cyprodinil	226>108	(1)	40	25	0.20	II

3. DETERMINATION OF BENZIMIDAZOLES AND AVERMECTIN RESIDUES IN BOVINE MILK BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTOR

3.1. PRINCIPLE

This method involves sample extraction with organic solvents, clean−up by solid phase extraction (SPE) and derivatization before analysis using HPLC.

3.2. SCOPE

The method includes determination of residues of five avermectins [emamectin (EMA), ivermectin (IVE), eprinomectin (EPRI), doramectin (DORA) and abamectin (ABA) in bovine milk at level of 0.5 ng/g to 100 ng/g.

3.3. MATERIALS

The following reagents and chemicals are required: HPLC grade MeCN, MeOH, and H_2O; C18 cartridges (Sep−Pak®Vac 6 cc, 500 mg); Triethylamine ≥ 99% ; 1−Methylimidazole ≥ 99%; Trifluoroacetic anhydride−Reagent Plus ≥99%. ABA, 95% and EMA, 96% (Chem Service); IVE, 91.0% and EPRI, 91%, USP; DORA, 96% (Dr. Ehrenstorfer).

3.3.1. Standards and solutions

a) Stock standard solutions (2,000 µg/mL)

- Weigh 20 mg of each analytical standard into 10 mL volumetric flask and fill to volume with MeOH (for EMA, IVE, EPRI and DORA) and MeCN (for ABA).
- Store all stock standard solutions at -20°C.

b) Mixed standard solution (50 µg/mL)

Transfer 250 µL of each stock standard solution (2,000 µg/mL) into 10 mL volumetric flask and fill to volume with MeCN.

c) Mixed standard solution (250 ng/mL)

Transfer 50 µL of the mixed standard solution (50 µg/mL) into volumetric flask of 10 mL and fill to volume with MeCN.

d) Working standard solution

- Transfer 5 µL, 10 µL, 50 µL, 100 µL, 300 µL and 600 µL of the mixed standard solution (250 ng/mL) into six 10 mL volumetric flasks and fill to volume with MeCN to obtain the mixed working standard solution of 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 30 ng/mL and 60 ng/mL, respectively.
- Table 4 below summarizes the process of preparing standard solutions.

TABLE 4. PREPARATION OF THE ANALYTICAL STANDARDS

Level	Vol.(µL) Mix Standard Solution (250 ng/mL)	Final Volume	Concentration of Standard (ng/µL)	Dilution Medium
1	5	2.5	0.5	MeCN
2	10	2.5	1	MeCN
3	50	2.5	5	MeCN
4	100	2.5	10	MeCN
5	300	2.5	30	MeCN
6	600	2.5	60	MeCN

e) Fortified Samples

Add 10 µL, 20 µL and 500 µL of the mixed standard solution to 2.5 g of sample in a test tube, leave to stand as you continue with the sample extraction procedure.

3.3.2. Apparatus

The following apparatus are required for the test: Refrigerated centrifuge (Sigma. Model 4K 15 C); Vortex mixer, Barnstead Thermolyne (Model. 37600 Mixer); Balance, 0.1 mg sensitivity; Multi Reax shaker (Heidolph); Class A volumetric flasks: 10, 25 mL clear glass; Polypropylene centrifuge tubes (50 mL). An LC−FLD system: Waters Alliance 2695 coupled to a Fluorescence detector (FLD, Waters Corporation, USA) is also required.

3.4. PROCEDURE

3.4.1. Sample extraction

(a) Weigh 2.5 g of milk sample into a 50 mL centrifuge tube
(b) Add 10 mL of MeCN for the first extraction, vortex for 30 sec and centrifuge at 2,500 rpm for 3 min. Transfer the supernatant to another 50 mL centrifuge tube
(c) Extract with another 5 mL of MeCN. Combine the extract and mix with 20 mL of H_2O and 40 µL, triethylamine
(d) Clean−up the extract with SPE C18 cartridge, conditioned with 5 mL, MeCN followed by 5 mL MeCN:H_2O (40:60, v/v) containing 0.1% triethylamine; vacuume for 5 min
(e) Wash the cartridge with 3 mL of hexane and vacuum for 5 min. Elute the residue with 10 mL of MeCN into a 12.5 mL amber colored vial. Evaporate the eluate to dryness under nitrogen at 600ºC

3.4.2. Derivatization

(a) Dissolve the residue in 1 mL of MeCN and place in an ultra−sonic bath for 20 min and add 100 µL of N−methylimidazole and 100 µL trifluoroacetic anhydride
(b) Let sample stand and protect from the light for 35 min before transfering an aliquot into an autosampler vial and injecting into the HPLC−FLD

3.4.3. Chromatographic conditions

a) System suitability and instrument conditions

To assess system suitability, inject at least five replicates of an intermediate standard used for the calibration curve. The relative standard deviation (RSD) of the peak response and the

retention time (RT) should not be greater than 5%. Operate the HPLC using the conditions summarized in Table 5 below.

TABLE 5. CHROMATOGRAPHIC CONDITIONS USED

Chromatographic column:	Phase reverse C18, 4.6 x 150 mm, 3.5 µm
Mobile phase:	MeOH:H_2O (97:3, v/v).
Mobile phase flow type:	Isocratic
Flow rate:	1 mL/min
Injection volume	50 µL
Temperature (sample):	25°C
Temperature (column):	30°C
Time of reading:	9 min
Detector:	FLD 2475
Excitation wavelength:	365 nm
Emission wavelength:	465 nm

4. DETERMINATION OF FLORFENICOL IN FISH MUSCLE BY LIQUID CHROMATOGRAPHY

4.1. PRINCIPLE

Florfenicol (FFC) is a broad spectrum antimicrobial agent used to treat bacterial diseases in fish and the corresponding maximum residue limit (MRL) in fish muscle is 1000 µg/kg [2] Samples are extracted in ethyl acetate, cleaned−up by SPE and analysed by HPLC with an ultra−violet detector (UV).

4.2. SCOPE

The method is suitable for analysis of FFC in fish muscle at the limit of quantification (LOQ) of 500 µg/kg.

4.3. MATERIALS

The following consumables are required:

FFC analytical standard; acetonitrile (MeCN) (*), methanol (MeOH) (*), Hexane(*), Ultra−pure water (H_2O); Ethyl acetate, all HPLC grade.

(*) The solvents used during the clean−up process should be analytical or chromatographic (HPLC) grade with a high degree of purity. If a solvent does not meet these specifications, it should be filtered using (at least) 0.45 µm membrane filters. Primary Standard Solution (SNI)−FFC Solution, 100.10^3 µg/L; Secondary Standard Solution (SNII)−FFC solution.

4.3.1. Standards and solutions

a) Standard stock solution of FFC (SNI) 100.10^3 µg/L

- Accurately weigh 10.0 mg (± 0.1 mg) FFC into a 100 mL volumetric flask and dissolve in ~80 mL of HPLC grade MeCN
- Shake and after complete dissolution, fill to the 100 mL mark with MeCN
- The stability of this solution is 3 mn in a freezer

b) Working standard solution 10.10^3µg/L

- Pipette 5 mL of the SNI FFC solution into a 50 mL flask, fill to the mark with MeCN and mix thoroughly
- The stability of this solution is 7 days in a refrigerator
- Keep in the dark

c) MeOH:H_2O (10:90, v/v)

Mix 9 mL of H_2O with a 1 mL of MeOH in a 10 mL tube

4.3.2. Apparatus

The following are necessary:

Vortex mixer; Pasteur pipette; Evaporation system; Conical plastic tubes 15 mL; Flask; Water bath; SPE cartridges (C−18; 500 mg/3 mL clean−up material); Vacuum manifold; HPLC−UV; Micropipette; Dispenser; Disposable 3 mL syringes; Vials with inserts; Analytical balance (0.0001 g); Beakers; 0.45 µm filter unit.

4.4. PROCEDURE

(a) Weigh 5.0 g (± 0.009) of the ground sample into 50 mL plastic conical tubes
(b) Weigh 6 portions of 5.0 g (± 0.009) of blank tissue ground into a 50 mL conical plastic tubes before transfering to a lab for analysis
(c) Prepare the matrix calibration curve:
    (i) Fortify blanks with 10 µgm/L SNII according to the concentrations described in Table 6
    (ii) Use the remaining blank sample as a control. Using a dispensor, add 10 mL of ethyl acetate to weighed samples in test tubes
(d) Vortex for 1 min
(e) Centrifuge at 2,000 rpm for 5 min
(f) Transfer supernatant to another 15 mL plastic tube
(g) Evaporate supernatant using nitrogen, in a water bath at 50ºC−55ºC until an oily residue remains
(h) Add 2 mL of hexane to the tube containing the extract to remove fat
(i) Vortex for 15 sec
(j) Add 5 mL of water to the tube and shake for 30 sec
(k) Centrifuge at 2,000 rpm for 5 min; remove the top layer (hexane) and if necessary repeat this step to defat
(l) Evaporate any remaining hexane under nitrogen in a water bath at 50ºC−55°C for 2 min
(m) Start SPE extraction as follows:
    (i) Prepare the vacuum manifold with the necessary amount of SPE (C18; 500 mg/3 mL) cartridges
    (ii) Condition the cartridges with 5.0 mL of ethyl acetate, 10 mL of MeOH and 10 mL of H_2O
    (iii) Load samples to the extraction cartridge; transfer the samples in the tubes to the cartridge, wash the tubes with 3 mL of H_2O and transfer to the reservoir; Elute the sample and allow the cartridge to dry
    (iv) Sample washing: Add 10 mL of MeOH:H_2O (1:9, v/v), then add 10 mL of H_2O
    (v) Sample elution: Place the cartridge on 15 mL tapered plastic tubes to collect the eluted sample. Elute samples; Add to the cartridge 5.0 mL of ethyl acetate and dry the cartridge
(n) Remove the C−18 cartridges and evaporate the solution in the tube (step m) to drynes under nitrogen in a water bath at 50°C−55°C
(o) Add 1 mL of MeCN:H_2O (40:60, v/v) to the residue and mix well
(p) Press content (step o) through a 0.45 µm filter into an HPLC vial
(q) Inject sample into the HPLC for analyis using the chromatographic conditions described in Table 7

TABLE 6. SAMPLE FORTIFICATION PROCEDURE

Standard concentration µg/kg	Vol (µL) of SNII concentration 1000 µg/kg	Mass (µg) added to 5.0 g sample
250	125	125.10^3
500	250	250.10^3
1,000	500	500.10^3
1,500	750	750.10^3
2,000	1,000	$1{,}000.10^3$

TABLE 7. CHROMATOGRAPHIC CONDITIONS

Chromatographic parameters	
Detector:	UV
Column:	C−18 250 × 4.6 mm, 5 µm
Method set:	FFC-UV
Column Temperature:	50ºC
Wavelength (λ):	230 nm
Mobile phase:	H_2O: MeCN (60:40, v/v)
Run time:	6 min
Injected volume:	20 µL
Flow rate:	1 mL/min
RT:	FFC 4.5 min

4.5. CRITICAL CONTROL

Ensure that:

(a) The analytical column is conditioned for up to 1 h 30 min prior to sample injection and analysis
(b) After each run wash the column with MeOH at a low flow rate (~0.1−0.2. mL/min) for at least 4 h

4.6. ACCEPTABILITY CRITERIA

For method suitability, the ranges in Table 8 are recommended.

TABLE 8. FORTIFICATION LEVELS AND ACCEPTABLE RECOVERY RANGES

Spike concentration (µg/kg)	Recovery interval
Less than 1	50 to 120%
1 to 10	70 to 110%
Over 10	80 to 110%

4.7. MEASUREMENT UNCERTAINTY

The estimated expanded measurement uncertainty should be 5.4%

4.8. LOQ

The method LOQ for FFC in fish is 500 µg/kg

4.9. CC ALPHA AND CC BETA

- The CCα for FFC in fish is 840 µg/kg
- The CCβ is 879 µg/kg

4.10. SUMMARY OF THE SAMPLE PREPARATION PROCEDURE

(a) Weigh 5.0 g of sample into a 50 mL test tube
(b) Prepare matrix−matched curve using standard concentrations 250 µg/kg, 500 µg/kg, 1,000 µg/kg, 1,500 µg/kg, 2,000 µg/kg
(c) Add 10 mL of ethyl acetate and vortex for 1 min
(d) Centrifuge at 2,000 rpm for 5 min
(e) Transfer the supernatant to a clean 15 mL test tube
(f) Evaporate the supernatant under nitrogen in a water bath at 50°C−55°C
(g) Add 2 mL of hexane to the tube containing the extract and shake for 15 sec
(h) Add 5 mL of de−ionized H_2O and shake for 30 sec
(i) Centrifuge at 2,000 rpm for 5 min
(j) Discard the upper layer (hexane)
(k) Evaporate residual hexane using a stream of nitrogen using a water bath set at 50°C−55ºC, for 2 min
(l) Condition SPE column with 5 mL of ethyl acetate, 10 mL of MeOH and 10 mL of de−ionized H_2O
(m) Transfer the content of the 15 mL tube (step e) to a reservoir in the SPE clean−up chamber
(n) Rinse the sample tube with 3 mL of H_2O and transfer content to the reservoir
(o) Elute the sample at a flow rate of 2−3 drops per sec
(p) Add 10 mL of MeOH:H_2O (1:9, v/v) to the reservoir and elute the SPE column
(q) Wash the column with 10 mL of H_2O
(r) Dry the SPE column for 1 min
(s) Use clean 15 mL tubes to collect eluted sample
(t) Elute the sample with 5 mL of ethyl acetate at a flow of 1−2 drops per sec
(u) Remove the SPE column and dry the samples under nitrogen in a water bath at 50°C−55°C
(v) Reconstitute residue with 1.0 mL of MeCN:H_2O (40:60, v/v) and vortex for 1 min
(w) Filter reconstituted residue through a polytetrafluoroethylene (PTFE) membrane, 0.45 µm into a vial before injecting into the HPLC−UV

5. DETERMINATION OF FLORFENICOL AND FLORFENICOL AMINE IN FISH MUSCLE BY RADIOIMUNNOASSAY AND CONFIRMATION BY LC−MS/MS

5.1. PRINCIPLE

Florfenicol (FFC) is commonly used to manage bacterial diseases in fish but its residues must not exceed the MRL of 1,000 µg/kg in fish muscle [2]. Samples are extracted in ethyl acetate, cleaned-up by SPE and analysed by HPLC−UV to establish a procedure for screening FFC residues in fish muscle by radioimmunoassay (RIA) with confirmation of suspect results by LC−MS/MS.

5.2. SCOPE

The method is suitable for detecting FFC in fish to meet the permitted/ acceptable MRL of 1,000 µg/kg.

5.3. MATERIALS

The following consumables are required:

5.3.1. Standards

Primary standard solution (SNI); FFC and Florfenicol Amine (FFA), 100.10^3 μgL^{-1}; Secondary standard solution (SNII)−FFC and FFA, 2.103 μgL^{-1}.

5.3.2. Reagents

KH_2PO_4; Na_2HPO_4PO; NaCl; physiological gelatin; 2,5−Diphenyloxazole (PPO);1,4-Bis(5−phenyl−2−oxazolyl) benzene (POPOP); activated charcoal; dextran T−70; Toluene; triton X−100; Sodium Azide NaN_3; Phosphate buffer saline (PBS). Others include: Acetone(*1); Dichloromethane(*1); Hexane(*1); De−ionized H_2O; MeOH(*1); Toluene; Triton X−100.

- (* [1]) The solvents used during the clean−up process should be analytical or HPLC grade. All solvents used with the equipment (HPLC), must be chromatographic or better grade.

- If a solvent does not meet the specifications, filtration should be done using ~0.45 µm membrane filter.

5.3.3. Standard solutions

a) Standard stock solution of FFC (SNI) 100.10^3 µg/L

- Accurately weigh 10.0 mg (±0.1 mg) of FFC into a 100 mL flask with the actual mass corrected according to the standard's purity
- Dissolve the material in ~80 mL of HPLC grade MeOH, shake and after complete dissolution, bring the solution to the 100 mL mark
- The expiry period is 1 year if stored in a freezer

b) Working standard solution 2.103 µg/L

- Pipette 2 mL of SNI solution into a 100 mL volumetric flask and bring to the mark with MeOH
- Mix thoroughly
- The expiry period is 1 year if stored in freezer

c) PBS 0.1 mol/L; pH=7.4 Buffer

- Weigh 13.609 g of KH_2PO_4, 14.196 g of Na_2HPO_4, 5.844 g of NaCl and 6.501 g of Sodium Azide NaN_3, and dissolve in 800 mL of H_2O before adjusting the pH
- Transfer to a 1,000 mL volumetric flask and fill to the mark with de−ionized H_2O
- This expires in 12 months (mn) if stored in a refrigerator

d) PBS + Gelatin 0.01 mol/L; pH=7.4 buffer

- Weigh 1.0 g of physiological gelatin and dissolve in 800 mL of H_2O
- Transfer into a 1,000 mL volumetric flask and add ~100 mL of (PBS) 0.1 mol/L adjusted to pH = 7.4
- Adjust volume with de−ionized H_2O
- This expires in 12 mn if stored in a refrigerator

e) Scintillation cocktail

- Weight 4.9 g of PPO and 0.1 g of POPOP
- Dissolve with 666 mL of toluene
- After complete dissolution, add 333 mL of triton and mix

f) Charcoal suspension

- Weigh 0.5 g of activated charcoal and 0.05 g of dextran T−70 and dissolve in 100 mL of PBS + Gelatin 0.01 mol/L at pH = 7.4 buffer
- Prepare daily

5.3.4. Apparatus

The following are required: Shaker (vortex); Micropipette; Pasteur pipette; Dispenser; Evaporation system; Disposable 3 mL syringes; 15 mL and 50 mL Conical plastic tubes; Scintillation vials and inserts; Flask; Analytical balance (accuracy of 0.0001 g; Water bath; Beakers; Liquid Scintillation Counter; RIA Tubes; Test tubes.

5.4. PROCEDURE

(a) Weigh 5.0 g (± 0.001 g) of ground sample into 50 mL plastic conical tubes
(b) Weigh 6 portions of 5.0 g (± 0.001 g) blank tissue (samples without analyte in question)into 50 mL conical plastic tubes. Fortify samples according to Table 9 and representing 2 × MRL, 1 × MRL and 0.5 × MRL using SNI solutions
(c) Prepare the calibration curve using the concentration ranges indicated in the Table 10:
(d) Fortify samples with aliquots of working standard at this stage; add 2 mL de−ionized H_2O to tube
(e) Add 8 mL of acetone and homogenize contents

(f) Centrifuge at 1,500 rpm for 10 min. Adjust final volume of combined supernatant, add 20 mL acetone, and decant supernatant into a tube
(g) Mix supernatant well, transfer 8 mL of it to a screw−top tube, and add 6 mL dichloromethane
(h) Cap the tube and mix contents (5 sec) using a vortex mixer
(i) Centrifuge for 5 min at 1,000 rpm to separate the layers
(j) Aspirate and discard upper aqueous layer, and evaporate lower dichloromethane layer to dryness, using nitrogen (maximum temperature, 45ºC). Any fat that is present should be removed with the dichloromethane
(k) Dissolve residue in 1.0 mL of 0.001% acetic acid solution, and extract with 2 mL hexane
(l) Centrifuge at 1,000 rpm for 5 min to separate layers; aspirate and discard upper hexane layer
(m) Measure 250 µL of the solution and bring to 500 µL with physiological buffer
(n) Conduct RIA test
(o) Prepare calibration points at a final concentration range of 250 µg/kg to 2,000 µg/kg using physiological buffer (pH = 7.4)
(p) Add 5,000 to 8,000 counts per minute (cpm) of tritium labelled FFC
(q) Add 100 µL of antisera at a dilution of 1:2,500 to each tube
(r) Incubate for 15 min at 37ºC and then overnight at 4ºC
(s) Add 1 mL of activated charcoal (0.5%) and dextran (0.05%)
(t) Let mixture stand for 10 min at 4ºC
(u) Centrifuge at 10,000 rpm
(v) Remove the aqueous layer
(w) Add 5 mL of scintillation cocktail
(x) Read samples for 4 min using a scintillation counter

TABLE 9. SPIKING CONCENTRATION AND VOLUME

Final Concentration (µg/kg)	Vol (µL) of SNI
500	25
1,000	50
2,000	100

TABLE 10. PREPARATION OF THE CALIBRATION CURVE

Concentration sample (µgkg)	Vol (µL) of SNII	Vol of PBS buffer
250	1,000	1,000
375	1,000	1,000
500	1,000	1,000
750	1,000	1,000
1,000	1,000	1,000
1,500	1,500	500
2,000	2,000	-

5.5. CALCULATIONS

As the final volume of the analytical procedure is 250 µL representing 1 g of tissue, no further calculations are required.

5.6. CRITICAL CONTROL POINTS

It is important to note the following as critical points:

- Evaporation temperature should be 45ºC ± 2ºC
- Pre−incubation of RIA step should be 37 ºC ± 3ºC
- Physiologic buffer pH should be at 7.4 ± 0.1
- Storage temperature for standards as well solutions should be 4ºC−9ºC
- The sample extract will be stable for ~2 days in a freezer

5.7. UNCERTAINTY OF MEASUREMENT

The expanded uncertainty levels for FFC and FFA are 5.9% and 10.2%, respectively.

5.8. LOQ, CC ALPHA AND CC BETA

The limit of quantification for FFC and FFA in fish is 250 µg/kg, while the CCα and CCβ values for both analytes are 100 µg/kg and 250 µg/kg, respectively.

5.9. CONFIRMATORY TEST

Samples with suspect positive results from the RIA assay may be confirmed using LC−MS/MS technique following clean−up.

The following LC−MS/MS conditions are required for the analysis of FFA:

a. LC component

   - Hypersil^{TM} C18−BD, 5 µm, 15 cm × 2.0 mm id, reversed−phase column
   - The mobile phase consisting of de−ionized H_2O + 0.1% acetic acid solution (Solvent A); MeCN + 0.1% acetic acid solution (Solvent B)
   - Equilibrate the LC system for 30 min at column/oven temperature of 40ºC with 100% Solvent A at a flow rate of 0.3 mLmin^{-1}
   - Construct linear gradient as follows: 2% Solvent B for 2 min; to 40% Solvent B in 11 min; hold for 2 min; to 100% Solvent B in 2 min while increasing flow to 0.4 mL/min; to 2% Solvent B in 2 min while decreasing flow to 0.3 mL/min; equilibrate for 11 min

b. The parameters for the MS include:

Ion source: Atmospheric Pressure Chemical Ionization (APCI) in positive mode; Corona = 2 µA; Cone = 25 V; Extractor = 5 V; RF Lens = 0.2V; Source Temperature = 130ºC; APCI probe temperature = 500ºC; Desolvation gas flow 300 L/h; Cone gas flow = 100 L/h. The transitions (mass to charge, m/z) for FFA: m/z 248−230; m/z 248−197; m/z, 248−151; m/z, 248−130.

Interchannel delay, 0.3 sec; span (Dalton, Da), 0.1; FFA ion mode ES+, mass 248.2, dwell time of 0.3; FFC mass ion mode ES- mass 356.1; Source ES+ 0.3 capillary, 3.5 kV; cone, 25.0 V; extractor, 1.0 V; RF lens, 0.0 V; source temperature, 100ºC; Source ES-: capillary,

2.5 kV; cone, 20.0 V; extractor, 5.0 V; RF lens, 0.5 V; source temperature, 100ºC. Other MS conditions are as follows: desolvation temperature, 400^{o}C; cone gas flow, 60 L/h; desolvation gas flow 400 L/h; run time, 30 min.

The LC−MS/MS conditions for FFC below are as reported elsewhere [3] include:

a. HPLC: Zorbax 5 mm, C18 (150 × 2.1 mm); Mobile phase (isocratic mode) consisting of MeCN:H_2O:Ammonium formate at a flow rate of 0.2 mL/min; Oven temperature of 50ºC; Injection volume 10 mL.

b. MS: Ionization − ESI +; Drying gas − Ar (520 L/h, 250ºC); V−cap 3000 V; Collision gas N_2, 25 L/h; Dwell time 0.2 sec; Cone voltage (V) of 45 for m/z 355> 336.0 (qualification ion) and 355.8>185.0 transition ions, whose collision energies are 39 and 50 V, respectively.

6. CONFIRMATORY METHOD FOR THE DETECTION OF AMINOGLYCOSIDES IN MUSCLE, LIVER AND KIDNEY BY LC−ESI−MS/MS

6.1. PRINCIPLE

Residues of aminoglycosides (AGs), commonly broad−spectrum antibiotics, are extracted from samples using an aqueous solution of trichloroacetic acid (TCA). Extracts are passed through Hydrophilic−Lipophilic−Balance (HLB) SPE cartridges and analyzed by LC−ESI−MS/MS.

6.2. SCOPE

This method is suitable for the confirmation and quantification of the following analytes in the Table 11.

TABLE 11. DRUG RESIDUES DETECTABLE USING THIS METHOD

Drug	Marker Residues	Matrix	Species	CCα (μg/kg)	CCβ (μg/kg)	MRL/ Suggesting level for no MRL substances (μg/kg)
Aminoglycosides	Apramycin	muscle	pig	63.2	65.8	60
	Kanamycin			44.7	49.0	40
	Gentamicin C1			54.0	59.2	50
	Gentamicin C2			54.9	59.7	50
	Gentamicin C1a			55.2	59.9	50
	Amikacin			105.1	109.9	/100
	Neomycin			555.0	612.3	500
	Spectinomycin			113.6	125.6	100
	Streptomycin			561.7	626.0	500
	Dihydrostreptomycin			564.7	627.7	500
	Tobramycin			51.9	53.9	/50
	Paromomycin			551.4	599.9	500
	Netilmicin			56.4	62.6	/50
	Sisomicin			54.8	60.0	/50
	Micronomicin			55.2	60.3	/50
	Hygromycin B			549.5	595.8	/500
Aminoglycosides	Apramycin	liver	pig	65.7	72.5	60
	Kanamycin			45.9	50.9	40
	Gentamicin C1			112.0	125	100
	Gentamicin C2			109.0	121.0	100
	Gentamicin C1a			112	125.0	100
	Amikacin			108.0	117	/100
	Neomycin			555	618.7	500
	Spectinomycin			109.3	118	100
	Streptomycin			550	609.0	500
	Dihydrostreptomycin			553	611	500
	Tobramycin			51.6	54.6	/50
	Paromomycin			1631	1759	1,500
	Netilmicin			57.7	66.2	/50
	Sisomicin			54.8	59.6	/50
	Micronomicin			57.8	66.5	/50
	Hygromycin B			542	581.0	/500

Aminoglycosides	Apramycin	kidney	pig	111.0	124	100
	Kanamycin			45.2	50.3	40
	Gentamicin C1			226	253	200
	Gentamicin C2			218	237	200
	Gentamicin C1a			228	257	200
	Amikacin			109	117	/100
	Neomycin			5359	5797	5,000
	Spectinomycin			570	631	500
	Streptomycin			1104	1206	1,000
	Dihydrostreptomycin			1105	1222	1,000
	Tobramycin			56.9	63.3	/50
	Paromomycin			1641	1789	1,500
	Netilmicin			55.5	62.1	/50
	Sisomicin			54.1	59.4	/50
	Micronomicin			57.3	65.1	/50
	Hygromycin B			560	610	/500

6.3. MATERIALS

The following reagents/materials are required: Ultra−pure H_2O (18.2 mega−ohms); HPLC grade Acetic acid, MeOH, MeCN and n−Hexane; Heptafluorobutyric acid (HFBA) >99.5%,; TCA, A.R; NaOH,; HCl, A.R; Disposable filter unit (0.45 μm); Oasis HLB cartridge (3cc/60 mg); pH test kit (pH range 4~10).

6.3.1. Negative control sample

A negative control is considered as a blank sample material ("Blank") previously analyzed with no AGs detected. This should be obtained from animals of known medical/treatment history (if available) or, otherwise pooled from several blank samples of the same matrix and species as the sample to be confirmed.

6.3.2. Pre−extracted spiked matrix standard (PrEMS)

In this procedure, the negative control samples ("Spikes") are spiked with the analytes to be determined at the beginning of the analytical procedure. These are useful for calibration and quantification of target analytes in the samples.

6.3.3. Post−extraction spiked matrix standards (PoEMS)

These are negative control samples taken through the entire extraction procedure and then spiked with the analytes of interest prior to detection (matrix−matched standards). These can be used for determination of analyte recovery.

6.3.4. Reagent blank (or "Procedural blank")

In this case the entire analytical protocol is followed but without the inclusion of any sample material or analytes. The blank is used to check for possible contamination from reagents, apparatus or laboratory environment.

6.3.5. Reference materials

No certified reference materials are necessary for this assay, rather negative controls, PrEMS and PoEMS should be included in this assay to ensure validity.

6.3.6. Solvents

a) 5% (w/v) TCA aqueous solution

- Weigh 50.0 g of TCA into a beaker and dissolve in ~900 mL of ultra−pure H_2O
- Transfer content to a 1 L volumetric flask and fill to the mark with ultra−pure H_2O
- Prepare fresh for each assay

b) 0.2 mol/L HFBA

- Dilute 13 mL HFBA in ultra−pure H_2O to a final volume of 500 mL
- This remains stable up to 6 mn at 4ºC

c) 0.02 mol/L HFBA

- Dilute 1.3 mL HFBA in ultra−pure water to a final volume of 500 mL
- This remains stable up to 6 mn at 4ºC

d) MeCN:0.15M HFBA (4:1, v/v)

- Add 400 mL MeCN and 75 mL 0.2 mol/L HFBA to 25 mL ultra−pure H_2O and mix thoroughly
- Prepare fresh for each assay

e) 100 g/L NaOH aqueous solution

- Dissolve 100 g NaOH in ultra−pure H_2O, and bring to a final volume of 1,000 mL
- Prepare fresh for each assay

f) 0.2 mol/L HCl solution

- Dilute 5 mL HCl in ultra−pure H_2O, and bring to a final volume of 300 mL
- Prepare fresh for each assay

g) NaOH solution (pH 8.5)

- Dilute 100 g/L NaOH to be 10 g/L, and then adjust to pH 8.5 by adding 0.2 mol/L HCl
- Prepare fresh for each assay

h) Mobile phases

- MeCN containing 20mM HFBA; prepare by adding 1.3 mL of HFBA to 500 mL of MeCN and mixing thoroughly
- MeCN:H_2O (5:95, v/v) containing 20mM HFBA prepare by adding 2.6 mL of HFBA to 50 mL of MeCN and 950 mL H_2O and mixing thoroughly
- MeCN:H_2O (50:50, v/v) containing 20mM HFBA; prepare by adding 2.6 mL of HFBA to 500 mL MeCN and 500 mL water and mixing thoroughly
- Prepare all the above fresh for each assay
-

6.3.7. Standard and standards solutions

Analytical standards of high purity are required (Table 12).

TABLE 12. LIST OF DRUGS UNDER STUDY, THEIR PURITY AND POSSIBLE SOURCE

Compound	Purity	Producer/Source
Apramycin sulphate	98.5%	Dr.Ehrenstorfer GmbH, Germany
Kanamycin sulphate	94.5%	Dr.Ehrenstorfer GmbH, Germany
Gentamicin-2,5-sulfate hydrate (mixture of Gentamicin C1, C2 and C1a, with proportion 29.1%, 21.3% and 49.6%)	96.5%	Dr.Ehrenstorfer GmbH, Germany
Amikacin hydrate	99.0%	Dr.Ehrenstorfer GmbH, Germany
Neomycin sulphate	90.0%	Dr.Ehrenstorfer GmbH, Germany
Spectinomycin sulphate hydrate	96.0%	Dr.Ehrenstorfer GmbH, Germany
Streptomycin sulphate	98.0%	Dr.Ehrenstorfer GmbH, Germany
Dihydrostreptomycin sesquisulfate hydrate	99.0%	Dr.Ehrenstorfer GmbH, Germany
Tobramycin	93.0%	Dr.Ehrenstorfer GmbH, Germany
Paromomycin sulphate	90.0%	Dr.Ehrenstorfer GmbH, Germany
Netilmicin sulphate	93.0%	European pharmacopoeia, France
Sisomicin	98.0%	Toronto Research Chemicals Inc., Canada
Micronomicin sulphate	60.7%	International laboratory, USA
Hygromycin B	60.0%	Sigma-Aldrich, USA

a) Stock solution

The following should be noted:

- For standards supplied as salts and hydrates, adjust the mass weighed so that only the mass of the free base is considered
- Prepare dilution solution by mixing MeCN:H_2O:acetic acid (20:78:2, v/v/v). Prepare individual stock solutions of all 16 AGs (100 µg/mL except gentamicin 80 µg/mL) in dilution solution of MeCN:H_2O:acetic acid (20:78:2, v/v/v)
- Transfer the standard stock solutions to plastic tubes and store at 2°C~4°C for up to 6 mn
- Prepare tuning solution of each analyte (10 µgm/L) by diluting individual stock solutions with a mixture of MeCN:H_2O:acetic acid (20:78:2, v/v/v)

b) Spiking standard solution

- Prepare spiking standard mixture (to fortify three matrices/samples) by diluting individual stock solution of the 16 AGs with MeCN:H_2O:acetic acid (20:78:2, v/v/v) in appropriate concentrations to a final volume of 50 mL
- When a lower fortification mixture is needed, prepare an extra dilution of the AGs
- Store solutions in plastic tubes at 2~4°C; these are stable for 1 mn.

c) Spiking standard mixture of target AGs for porcine muscle

The standard solution for spiking porcine muscle is indicated in Table 13.

TABLE 13. PREPARATION OF FORTIFICATION SOLUTION FOR PORCINE MUSCLE

Compound	MRL/Suggesting level − no MRL substances (μg/kg)	Concentration of individual solution (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)
Streptomycin	500	100	6.25	12.5
Dihydrostreptomycin	500	100	6.25	12.5
Neomycin	500	100	6.25	12.5
Paromomycin	500	100	6.25	12.5
Kanamycin	40	100	0.50	1.0
Amikacin	/ (100)	100	1.25	2.5
Tobramycin	/ (50)	100	0.625	1.25
Spectinomycin	100	100	1.25	2.5
Apramycin	60	100	0.75	1.5
Gentamicin C1	50	80（total GENT）	2.50	1.16
Gentamicin C2	50			1.98
Gentamicin C1a	50			0.85
Hygromycin B	/ (500)	100	6.25	12.5
Netilmicin	/ (50)	100	0.625	1.25
Sisomicin	/ (50)	100	0.625	1.25
Micronomicin	/ (50)	100	0.625	1.25

d) Spiking standard mixture of target AGs for porcine liver

Prepare the standard solution for spiking porcine liver as indicated in Table 14.

TABLE 14. PREPARATION OF FORTIFICATION SOLUTION FOR PORCINE LIVER

Compound	MRL/ Suggesting level−no MRL (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)	
Streptomycin	500	100	5	10
Dihydrostreptomycin	500	100	5	10
Neomycin	500	100	5	10
Paromomycin	1500	100	15	30
Kanamycin	40	100	0.4	0.8
Amikacin	/ (100)	100	1	2
Tobramycin	/ (50)	100	0.5	1
Spectinomycin	100	100	1	2
Apramycin	100	100	0.6	1.2
Gentamicin C1	100	80 (total GENT)	4	1.86
Gentamicin C2	100			3.17
Gentamicin C1a	100			1.36
Hygromycin B	/ (500)	100	5	10
Netilmicin	/ (50)	100	0.5	1
Sisomicin	/ (50)	100	0.5	1
Micronomicin	/ (50)	100	0.5	1

e) Spiking standard mixture of target AGs for porcine kidney

Prepare the standard solution for spiking porcine kidney as detailed in Table 15.

TABLE 15. PREPARATION OF FORTIFICATION SOLUTION FOR PORCINE KIDNEY

Compound	MRL/Suggesting level−no MRL substances (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)	
Streptomycin	1,000	100	2.5	5
Dihydrostreptomycin	1,000	100	2.5	5
Neomycin	5,000	100	12.5	25
Paromomycin	1,500	100	3.75	7.5
Kanamycin	40	100	0.1	0.2
Amikacin	/ (100)	100	0.25	0.5
Tobramycin	/ (50)	100	0.125	0.25
Spectinomycin	500	100	1.25	2.5
Apramycin	100	100	0.25	0.5
Gentamicin C1	200	80 (total GENT)	2	0.93
Gentamicin C2	200			1.59
Gentamicin C1a	200			0.68
Hygromycin B	/ (500)	100	1.25	2.5
Netilmicin	/ (50)	100	0.125	0.25
Sisomicin	/ (50)	100	0.125	0.25
Micronomicin	/ (50)	100	0.125	0.25

6.3.8. Apparatus

Polypropylene screwed cap tubes (15 mL and 50 mL); Analytical balance (0.1 mg and 0.01g); High speed blender (IKA® T25 digital ultra−turrax®); Dispersing (IKA® works S25N−25F); Calibrated microliter pipettes (10 µL−1,000 µL); Vortex mixer; Flatbed shaker (IKA−KS260; Ultra−sonicator; pH meter (sensION+DO6); High speed refrigerated centrifuge(Beckman Coulter AllegraTM X−22R); Nitrogen Evaporator(N−EVAPTM 112); SPE vacuum manifold with PTFE flow control; Vacuum pump (GM−0.33A); Ultra−pure H_2O production system (Milli−Q Plus pure system); Volumetric flasks (5 mL, 10 mL, 25 mL, 50 mL and 1,000 mL); Aluminium foil; Amber colored autosampler vials and PTFE lined caps; LC−ESI−MS/MS system.

The method optimum LC and MS conditions as well as physicochemical characteristics for the analytes are summarised in Tables 16 and 17.

TABLE 16. OPTIMUM LC AND MS CONDITIONS USED

System	Agilent 1100 series system equipped with an automatic degasser, a quaternary pump and an autosampler; API 3000 LC−MS/MS		
Column	Waters Atlantis® dC18 (2.1×150 mm, 5 µm)		
Injection volume	30 µL		
Column temperature	30ºC		
Flow rate	0.4 mL/min		
MS mode	ESI^+		
Mobile phase	A: MeCN containing 20mM HFBA C: MeCN:H_2O (5:95, v/v) containing 20mM HFBA D: MeCN:H_2O (50:50, v/v) containing 20mM HFBA		
Time (min)	C (%)	D (%)	A (%)
0.00	90	10	0
1.00	90	10	0
5.00	50	50	0
8.00	50	50	0
11.00	35	65	0
11.10	0	5	95
13.90	0	5	95
14.00	90	10	0
18.00	90	10	0

TABLE 17. MOLECULAR WEIGHTS (MW), ION TRANSITIONS AND ASSOCIATED MS VOLTAGES OF THE AGS

Compound	MW	Precursor ion *(m/z)*	Product ion *(m/z)*	DP (V)	EP (V)	CXP (V)	CE (V)
Apramycin	539.6	540.4	*378.3	105	4.2	23	25
			217.2			13	40
Amikacin	585.6	586.3	*425.2	90	4.2	27	29
			264.1			17	38
Spectinomycin	332.3	351.3	*333.2	60	10.0	23	26
			98.2			6	44
Neomycin	614.6	615.4	*161.2	155	4.3	10	44
			293.0			17	36
Tobramycin	467.5	468.3	*163.2	65	10.0	10	36
			324.1			19	23
Gentamicin C1a	449.5	450.3	*160.1	85	5.0	9	34
			322.1			20	20
Gentamicin C2	463.6	464.3	*322.1	85	5.0	20	20
			160.1			9	34
Gentamicin C1	477.6	478.3	*157.2	100	4.5	10	30
			322.2			20	21
Kanamycin	484.5	485.3	*163.2	80	4.3	9	39
			324.1			19	25
Hygromycin B	527.5	528.2	*177.2	95	10.0	10	44
			352.2			20	35
Dihydrostreptomycin	583.6	584.2	*263.1	145	9.5	14	46
			246.2			14	56
Paromomycin	615.6	616.3	*163.2	135	9.0	11	52
			293.0			17	35
Streptomycin	581.6	600.3	*582.2	125	4.5	34	26
			263.1			16	52
Netilmicin	475.6	476.4	*299.5	65	7.2	21	31
			191.4			11	36
Sisomicin	447.5	448.5	*322.4	50	7.0	20	20
			271.5			19	27
Micronomicin	463.6	464.6	*322.4	90	7.6	22	21
			160.3			15	33
*Selected as quantitative ion							
Data handling	Analyst 1.4.1 or higher						

Additional conditions include: Dwell time of 40 msec; Focusing potential of 350 V; Nebulizer gas 12 psi; Curtain gas = 8 psi; Collision gas = 6 L/min; Ion spray voltage = 3,500 V; Ion source temperature = 500ºC.

6.4. ENVIRONMENTAL CONTROL

- Extraction and clean−up should be carried out under non−UV lighting (yellow light)
- When taken out of the yellow light area, extracts should be protected from UV light (for instance by using amber coloured glass or foil wrapping)

6.5. SAMPLE PREPARATION AND ANALYSIS

Adopt the sample preparation and analysis procedure outlined below including use of concentrations in Table 18.

(a) Homogenize porcine tissue (muscle, liver, and kidney) samples to paste using a high speed blender. Store samples at -20ºC ± 5ºC in plastic containers and analyze within 3 mn. The sample should be returned to cold storage immediately after

sub−sampling. Analysis should be performed as soon as possible after sub−sampling or else samples should be re−stored at -20ºC ± 5 ºC

(b) Weigh 5 g ± 0.1 g of prepared sample in duplicate and transfer into a 50 mL polypropylene tube

(c) For negative controls and PrEMS, weigh as in *step b* and spike with appropriate volumes of fortification standard. A minimum of four calibration levels (plus blank) should be used, with a duplicate PrEMS at each level. The concentration levels should be spaced equidistant, and be chosen such that the expected sample concentration falls in the middle of the calibration curve

(d) For each PrEMS extract, an additional negative control taken through the extraction procedure (PoEMS), should be prepared and labelled as PoEMS

(e) Add 10 mL of 5% TCA (w/v) to the sample and homogenize thoroughly at 10, 000 rpm for 1 min and then centrifuge at 8,000 rpm for 5 min. Transfer extract into another 50 mL polypropylene tube

(f) Repeat the extraction procedure using 10 mL of 5% TCA, and combine the TCA supernatants in the 50 mL polypropylene tube

(g) Add 5 mL each of 0.2M HFBA and n−hexane to the TCA supernatants; Cover the tubes with aluminium foil, place on a flatbed shaker and mix for 30 min at ≥ 360 rpm

(h) Centrifuge tubes at 8,000 rpm for 5 mins at room temperature, remove the upper n−hexane phase and use it for clean−up

(i) Place a HLB SPE cartridge on the vacuum manifold, condition it with 3 mL MeOH, 3 mL H_2O, and 3 mL 0.2 M HFBA and let the solvents flow by gravity. Discard the eluate

(j) Load 5 mL of the upper layer extract from (step h) onto the conditioned cartridge, and allow to run through at 1 mL/min. Collect the extract into a 15 mL polypropylene tube, and adjust pH to 8.5±0.2 with 100 g/L NaOH (about 9 drops) and 0.2 mol/L HCl

(k) Place another HLB SPE cartridge on the vacuum manifold, condition with 3 mL MeOH, 3 mL H_2O, 3 mL 0.2M HFBA and 3 mL pH 8.5 NaOH solution and let elution flow by gravity. Discard the eluate

(l) Load the pH 8.5±0.2−extract from (step k) onto the conditioned cartridge, and allow to run at 1 mL/min. Discard the eluate

(m) Hyphenate the two HLB cartridges with vacuum joints and rinse with 5 mL ultra−pure H_2O. Dry these two cartridges at less than 15 mmHg for 10 min

(n) Elute AG residues from the two cartridges with 6 mL MeCN:0.15M HFBA (8:2, v/v) at 1 mL/min, and collect the eluate into a new 15 mL polypropylene tube

(o) Reduce the collected eluate to approximately 0.3 mL using nitrogen evaporator at a temperature setting of 40°C

(p) To prepare PoEMS, take the residual 0.3 mL samples from (step d) labelled PoEMS and add spiking standard to match the PrEMS prepared in (step c) as detailed in Table 18

(q) Reconstitute residue (in step o) to 1 mL by adding 20mM HFBA and vortex for 30 sec. Transfer sample to a 2 mL screw caped auto−sampler vial

(r) Run the samples and controls on the LC−ESI−MS/MS in the following sequence:
  (i) Inject PoEMS in increasing order of concentration
  (ii) Inject PrEMS also in increasing order of concentration
  (iii) Flush column by injecting a blank
  (iv) Inject samples
  (v) After the 10^{th} sample, inject a PrEMS followed by another flush (blank injection)

(vi) Inject another batch of PoEMS (in increasing order of concentration)
(vii) Inject another batch of PrEMS also in increasing order of concentration
(viii) Inject a final flush

TABLE 18. PREPARATION OF CONTROLS STANDARDS AND REFERENCE LEVELS

Control Identification	PrEMS Spiking Level (μg/kg)
Reagent Blank	0
PrEMS 0	0
PrEMS 1	0.5 MRL/Suggesting level
PrEMS 2	1.0 MRL/Suggesting level
PrEMS 3	1.5 MRL/Suggesting level
PrEMS 4	2.0 MRL/Suggesting level

6.6. PERFORMANCE QUALIFICATION

Before starting the LC−ESI−MS/MS sequence, check that the following are met:

a) Approximate baseline width of PrEMS 1 for each analyte: < 1
b) Approximate signal−to−noise ratio of PrEMS 1 for each analyte > 5:1
c) Resolution of gentamicin C2/micronomicin: > 50%
d) RT drift (PrEMS 1 from start to end of run) < 5%
e) Peak area drift (PrEMS 1 from start to end of run): < 30%
f) Regression coefficient of PrEMS (r^2) for each analyse > 0.97

6.7. CALCULATION OF RESULTS

Note the following:

(a) Prepare PrEMS calibration curves from the peak area versus concentration for each analyte/transition and calculate the r^2 value
(b) Prepare PoEMS response curves from the peak area versus concentration for each analyte/transition
(c) Calculate recoveries by comparing the slopes of the PrEMS and PoEMS curves.
(d) Perform system suitability checks using streptomycin as a marker
(e) If the sample contains a peak at the retention time (RT) typical for any of the analytes, calculate the concentration of the analytes from the PrEMS calibration curves by using the formula:

$$Y = mx + c \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where

Y is the peak area; m the slope and x the concentration; c the intercept

Use the calculation: $Concentration\ (\mu g kg - 1) = \frac{peak\ area\ of\ sample - c}{m} \ldots\ldots (2)$

(f) Compare the difference between the result from duplicate samples with the repeatability stated in the validation document

(g) Compare the ratio of the concentrations calculated from each plot with the tolerances listed in Commission Decision 2002/657/EC [4]. If the ratios are within the specified tolerances for the required number of identification points, then the identity of the analyte is confirmed.

Detailed method validation information is presented in Table 19.

TABLE 19. METHOD VALIDATION EXPERIMENT AND DATA

	SPECTINOMYCIN	**HYGROMYCIN**	**STREPTOMYCINE**	**DIHYDROSTREPTOMYCIN**	**AMIKACIN**	**KANAMYCIN**	**APRAMYCIN**	**PAROMOMYCIN**	**TOBRAMYCIN**	**SISOMICIN**	**GENTAMYCIN C1a**	**GENTAMYCINE C2**	**MICRONOMYCIN**	**NETILMICIN**	**GENTAMYCIN C1**	**NEOMYCIN**
Muscle																
Intra−day RSD (%) n=21	3.9-9.5	4.1-8.7	4.8-10.6	5.2-8.0	3.5-9.6	4.1-9.4	4.3-12.2	3.0-11.2	3.5-8.6	4.1-11.1	3.7-10.1	3.3-9.7	4.4-12.1	4.7-12.6	4.2-11.7	5.2-10.3
Inter−day RSD (%) n=63	5.0-10.4	7.4-10.1	8.2-11.1	5.3-8.2	5.5-8.4	6.4-7.8	5.7-10.5	5.6-8.9	6.4-8.2	5.9-9.5	5.4-8.8	7.0-10.3	4.8-9.3	9.1-12.2	5.6-9.6	5.5-9.2
Recovery (%)	60-85	51-78	64-98	74-107	60-90	66-88	66-101	78-107	76-108	76-107	76-106	76-111	76-110	74-112	76-111	75-102
MRL/suggested level (μg/kg)	100	/500	500	500	/100	40	60	500	/50	/50	50	50	/50	/50	50	500
CCα (μg/kg)	113.6	549.5	561.7	564.7	105.1	44.7	63.2	551.4	51.9	54.8	55.2	54.9	55.2	56.4	54.0	555.0
CCβ (μg/kg)	125.6	595.8	626.0	627.7	109.9	49.0	65.8	599.9	53.9	60.0	59.9	59.7	60.3	62.6	59.2	612.3
LOD (μg/kg)	6.0	15	9.0	8.0	6.0	3.0	3.0	9.0	4.0	4.5	3.0	3.0	4.5	3.0	3.0	9.0
LOQ (μg/kg)	20	50	30	25	20	10	10	30	13	15	10	10	15	10	10	30
Liver																
Intra−day RSD (%) n=21	3.3-9.1	4.0-7.2	5.4-9.8	4.6-8.5	4.1-7.8	3.2-10.0	4.2-9.8	4.5-9.5	3.4-7.1	3.4-13.3	3.4-10.2	4.6-10.7	3.6-11.3	2.8-11.0	4.2-9.5	4.9-10.3
Inter−day RSD (%) n=63	5.4-9.4	6.2-6.8	8.4-9.7	8.8-10.6	8.8-10.0	7.1-8.6	4.9-10.4	5.5-8.6	5.6-10.7	6.6-10.6	5.5-8.8	7.2-10.4	7.5-11.5	6.7-10.5	7.4-8.9	7.1-10.7
Recovery (%)	59-85	56-72	61-92	69-102	60-87	59-84	66-95	75-100	63-101	69-100	73-110	69-106	70-105	73-109	77-109	71-100
MRL/suggested level(μg/kg)	100	/500	500	500	/100	40	60	1500	/50	/50	100	100	/50	/50	100	500
CCα (μg/kg)	109.3	541.5	549.8	552.5	108.0	45.9	65.7	1630.8	51.6	54.8	111.6	109.4	57.8	57.7	112.0	555.2
CCβ (μg/kg)	117.6	581.0	609.0	610.9	116.6	50.9	72.5	1758.9	54.6	59.6	125.4	121.3	66.5	66.2	124.9	618.7
Limit of detection (LOD, μg/kg)	8.0	18	9.0	9.0	6.0	3.0	3.0	12	3.0	4.5	2.5	4.0	5.5	6.0	3.0	6.0
Limit of quantification (LOQ, μg/kg)	25	60	30	30	20	10	10	40	10	15	8.0	12	18	20	10	20
Kidney																

	SPECTINOMYCIN	**HYGROMYCIN**	**STREPTOMYCINE**	**DIHYDROSTREPTOMYCIN**	**AMIKACIN**	**KANAMYCIN**	**APRAMYCIN**	**PAROMOMYCIN**	**TOBRAMYCIN**	**SISOMICIN**	**GENTAMYCIN C1a**	**GENTAMYCINE C2**	**MICRONOMYCIN**	**NETILMICIN**	**GENTAMYCIN C1**	**NEOMYCIN**
Intra−day RSD (%) n=21	4.7-8.4	4.2-6.9	3.3-9.6	3.5-8.0	2.9-8.3	3.8-8.8	4.4-10.1	3.8-8.9	5.0-9.5	3.5-9.5	4.2-10.2	3.5-8.1	3.8-13.8	2.8-9.3	4.2-8.2	4.7-10.2
Inter−day RSD (%) n=63	5.6-10.4	7.0-7.9	8.3-8.6	8.3-10.8	6.1-7.6	9.0-9.7	5.5-8.1	5.6-8.0	6.0-8.7	9.6-10.1	6.6-10.3	5.8-7.3	5.0-9.8	6.6-9.6	7.0-8.6	10.1-11.4
Recovery (%)	60-91	54-77	68-100	70-106	74-100	61-88	76-101	76-103	74-103	68-101	68-103	76-102	69-105	69-98	75-107	67-99
MRL/suggested level (μg/kg)	500	/500	1000	1000	/100	40	100	1500	/50	/50	200	200	/50	/50	200	5000
CCα (μg/kg)	570.3	559.9	1104.2	1104.9	108.9	45.2	111.0	1640.5	56.9	54.1	227.8	217.5	57.3	55.5	226.3	5357.5
CCβ (μg/kg)	630.7	609.6	1206.2	1221.9	117.3	50.3	123.7	1788.8	63.3	59.4	257.3	237.3	65.1	62.1	252.7	5795.3
LOD (μg/kg)	6.0	15	7.5	6.0	8.0	2.5	3.0	9.0	4.5	3.0	3.0	4.0	4.5	4.5	3.6	8.0
LOQ (μg/kg)	20	50	25	20	25	8.0	10	30	15	10	10	12	15	15	12	25

Note: Spiked levels are 0.5, 1.0 and 1.5 × MRL/suggested levels

7. LC−MS/MS METHOD FOR DETERMINATION OF BENZIMIDAZOLE RESIDUES IN ANIMAL PRODUCTS

7.1. PRINCIPLE

Samples are extracted with potassium carbonate and ethyl acetate and defatted using hexane. Qualitative and quantitative measurement of the residues is done by LC−ESI−MS/MS with or without IS ($^{13}C_6$−thiabendazole).

7.2. SCOPE

This LC−ESI−MS/MS method is suitable for determination of benzimidazole (BZs), pro−benzimidazoles and their metabolites in animal products including pork, mutton, liver, milk and fish. The target BZs, pro−benzimidazole and their metabolites include 5−hydroxy−thiabendazole (TBZ−5−OH), thiabendazole (TBZ), albendazole−2−aminosulfone (ABZ−NH_2−SO_2), albendazole sulfoxide (ABZ−SO), oxibendazole (OXI), oxfendazole (OXF), albendazole sulfone (ABZ−SO_2), albendazole (ABZ), febantel, thiophenate−ethyl, fenbendazole sulfone (FBZ−SO_2) and fenbendazole (FBZ). The LOD and LOQ are 0.75 µg/kg and 2.5 µg/kg, respectively.

7.3. MATERIALS

The following are required: Analytical grade H_2O; HPLC grade MeCN, n−hexane, ethyl acetate, MeOH, and acetone; formic acid, acetic acid, GR; anhydrous $MgSO_4$, anhydrous sodium acetate, AR; SPE adsorbent (PSA); Analytical standards including ABZ, 99.0%, ABZ−SO, 98.5%, ABZ−SO_2, 99.0%, ABZ−NH_2−SO_2, >99%, TBZ, 98.5%, TBZ−5−OH, 99.5%, OXI, 98.5%, OXF, 99.0%, FBZ, 99.0%, FBZ−SO_2, >99%, febantel, 99.0% and thiophenate−ethyl, 99.0%. Others are: filter membrane, 0.22 µm; HPLC−ESI−MS/MS; homogenizer; vortex mixer; super−centrifuge; ultra−sonicator; rotary/nitrogen evaporator; and analytical balance.

7.3.1. Standard solutions

- Prepare 100 mg/L mixed stock solution in MeOH and dilute to working standard solutions (~ 10 mg/L) immediately before use
- Store stock standard solutions in a refrigerator at 4°C
- Also prepare matrix−matched working standards using blank liver sample

7.4. PROCEDURE

7.4.1. Extraction and clean−up of muscle and liver tissues

(a) Add 50 µg/L IS, 3 g of sodium sulphate, 3 mL 2M potassium carbonate and 15 mL of ethyl acetate to 5 g of homogenized muscle or liver tissue, in a 50 mL centrifuge tube
(b) Shake on a vortex mixer for 2 min and centrifuge for 5 min at 5,000 rpm
(c) Decant the supernatant into 100 mL a distillation flask
(d) Repeat the extraction procedure once using 15 mL of ethyl acetate
(e) Evaporate the collected organic phases to dryness under nitrogen (at 45°C)
(f) Add 3 mL MeCN and 5 mL n−hexane to the dried residue and shake for 2 min with an ultra−sonicator before transferring content into a 10 mL centrifuge tube
(g) Centrifuge for 5 min at 5,000 rpm and, discard the upper layer

(h) Add 5 mL of n−hexane to the remaining layer to defat the extract
(i) Evaporate the MeCN layer to dryness under nitrogen (at 45°C) and redissolve the residue in 1 mL of MeCN
(j) Pipet 100 µL (step i) solution into 900 µL MeCN:H_2O (30:70, v/v) and press the material through a 0.22 µm filter material
(k) Inject into an LC−MS/MS for analysis.

7.4.2. Extraction and clean−up of milk samples

(a) Weigh 5 mL of milk sample into 50 mL polypropylene centrifuge tubes
(b) Add 50 µg/L IS, 20 mL of MeCN containing 0.5% acetic−acid
(c) Shake on a vortex mixer for 2 min
(d) Add anhydrous sodium acetate (1.5 g) and anhydrous sodium sulfate (6.0 g) to each tube and shake vigorously for 2 min
(e) Centrifuge for 5 min at 5,000 rpm
(f) Aspirate 4 mL of the supernatant and evaporate to dryness under nitrogen (at 45°C)
(g) Dissolve residue in 2 mL of MeOH and add 50 mg PSA
(h) Vortex for 2 min
(i) Centrifuge again for 5 min at 5,000 rpm.
(j) Evaporate an aliquot of 1 mL supernatant to dryness under nitrogen (at 45°C) and reconstitute residues in 1 mL MeCN:H_2O (30:70, v/v)
(k) Press the material through a 0.22 µm filter
(l) Inject content into an LC−MS/MS for analysis.

7.4.3. HPLC conditions

These include: Chromatographic column−Eclipse x DB−C18 analytical column (50 mm × 4.6 mm 1.8 µm); flow rate of 0.3 mL/min; Injection volume of 10 µL; Column temperature: 26°C; Mobile phase: MeCN and 0.005 M formic acid solution in gradient mode (Table 20).

TABLE 20. HPLC MOBILE PHASE GRADIENT FLOW

Time (min)	A: MeCN (%)	B：0.005 M formic acid (%)
0	85	15
5	20	80
8.5	20	80
8.6	85	15
14	85	15

7.4.4. MS parameters

- Use the ESI ionization mode with MRM scan with nitrogen as nebulizer, collision, curtain, and heater gas.

- The optimized ESI (+)−MS/MS operating conditions include: focusing potential: 400 V; entrance potential: 10 V; collision cell exit potential: 4 V; temperature: 450°C; ionspray voltage: 4500 V; gas 1: 50 psi; gas 2: 60 psi; curtain gas: 30 psi; collision gas: 10 psi; dwell time: 50 ms.

- The tolerance and MS/MS parameters for the benzimidazoles are shown in Table 21 and Table 22, respectively.

7.4.5. Evaluation of results

a) Qualitative determination

- For each compound select one parent ion and two sub-ions.
- Maintain the retention time (RT) at ±2.5% of the respective standard ions, and the maximum permissible errors of the relative ion abundances as shown in Table 21

TABLE 21. RELATIVE ION ABUNDANCIES (%) AND MAXIMUM PERMISSIBLE ERRORS

The relative ion abundances %	>50	20−50	10−20	≤10
The maximum permissible errors %	±20	± 25	±30	±50

b) Quantitative determination

- Prepare standard curves for each analyte by analysing negative samples spiked in duplicate with standard solutions of each of the 12 analytes at 7 different concentration levels.
- Quantify each compound by making reference to the peak area response of the IS.

c) Calculation

Express the analyte concentrations as *X* (mg/kg) calculated inform the formula:

$$X = \left[\frac{ml \times 1000}{m \times 1000}\right] \times n \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

Where

ml is the analyte weight in calibration curve (µg); m the sample weight (g) and; *n* the dilution factor.

7.4.6. Acceptability criteria

- Blank and reference samples are recommended for use in routine analysis.
- Only results with recoveries between 70%−110% are accepted, otherwise, analysis should be stopped and source of problems identified.

TABLE 22. QUALITATIVE AND QUANTITATIVE IONS* AND RELEVANT ANALYTICAL PARAMETERS

Compound	Transition (m/z)	Desolvation Potential /v	Collision Energy /v	RT /min
ABZ	226/234.1*	45	31	8.24
	222/191.1		48	
ABZ−SO	282.3/208.1*	33	37	6.26
	282.3/191.1		45	
ABZ−SO_2	298.3/159.1*	44	53	7.19
	298.3/224.1		40	
TBZ	202.2/175.1*	50	37	3.61
	202.2/131.2		48	
TBZ−5−OH	218.1/191.1*	48	37	1.93
	218.1/147.2		50	
OXI	250.2/218.0*	41	30	7.07
	250.2/176.2		43	
OXF	316.3/159.1*	48	51	7.07
	316.3/191.2		32	
FBZ	300.3/268.1*	44	35	8.89
	300.3/159.1		52	
FBZ−SO_2	332.2/159.1*	50	58	7.82
	332.2/300.2		36	
ABZ−NH_2−SO_2	240.1/133.2*	45	44	2.72
	240.1/198.2		30	
Febantel	447.2/280.1*	37	48	10.2
	447.2/227.1		48	
Thiophenate−ethyl	371.3/151.0*	34	34	9.11
	371.3/104.0		48	

8. SCREENING OF ALBENDAZOLE RESIDUES IN MEAT

8.1. PRINCIPLE

Albendazole (ABZ) extracted and purified from meat samples competes with ABZ conjugated with horseradish (HRP) on an Enzyme Immunosorbent assay (ELISA) plate. After washing, residues of HRP catalyse the substrate, resulting in a colour change. Colour intensity is inversely proportional to the concentration of ABZ in samples.

8.2. SCOPE

This method is suitable for screening ABZ and its metabolites in animal foodstuff including pork, mutton, liver, milk and fish. The target residues are albendazole sulfoxide (ABZ−SO), albendazole sulfone ($ABZ-SO_2$) and ABZ.

8.3. MATERIALS

Components of the analytical kit include:

A 96 well microtitre plate coated with rabbit immunoglobulin g (IgG) against ABZ; ABZ standard solutions; Conjugated peroxidase; Wash buffer 10 × concentrate; Citrate buffer; Substrate; Diluent solution 20 × concentrate; Stop solution; Plastic envelope; Box insert. Other reagents required for the immunoassay include: HCl 0.01 M; NaOH 5 M; HCl 5 M; Distilled H_2O; dimethyl sulfoxide (DMSO); Phosphate Buffered Saline (PBS) solution.

8.3.1. Apparatus

The following are required: Homogenizer; Super−centrifuge; 50 µL, 100 µL and 200 µL precision micropipettes; 50 µL−200 µL multichannel micropipette; Microtitre plate reader with 450 nm filter.

8.4. PROCEDURE

8.4.1. Sample preparation/extraction and analysis

8.4.1.1.Liver

(a) Weigh 1 g of liver in a 50 mL centrifugation tube
(b) Add 10 mL of PBS containing 10% DMSO
(c) Homogenize for 30 min with an ultra−turrax or similar equipment
(d) Centrifuge homogenate at 4,500 rpm for 5 min
(e) Transfer supernatant to a new test tube
(f) Repeat steps b−e
(g) Combine supernatants and mix well
(h) Centrifuge 1 mL of the supernatant at 10,000 rpm for 5 min
(i) The clean supernatant is ready for detection

8.4.1.2.Meat including fish

(a) Weigh 2 g of meat sample into a 50 mL centrifugation tube
(b) Add 10 mL of PBS containing 10% DMSO

(c) Homogenize for 30 min with an ultra−turrax or similar equipment
(d) Centrifuge homogenate at 4,500 rpm for 5 min
(e) Transfer supernatant to another test tube
(f) Repeat the steps a−e
(g) Combine supernatants and centrifuge (1 mL) at 10,000 rpm for 5 min
(h) The clean supernatant is ready for detection

8.4.1.3.Milk

(a) Measure 1 mL of milk sample into a 50 mL tube
(b) Add 10 mL of PBS containing 10% DMSO
(c) Homogenize for 30 min with ultra−turrax or similar equipment
(d) Centrifuge homogenate at 4,500 rpm for 5 min
(e) Transfer supernatant into another test tube
(f) Centrifuge 1 mL of the supernatants at 10,000 rpm for 5 min
(g) The clean supernatant is ready for analysis/detection

For analysis

(h) Allow all reagents to reach room temperature. Once started, complete all steps without interruption
(i) Add 200 µL of distilled H_2O into the blank wells
(j) Add 50 µL of distilled H_2O into the Maximum Binding wells
(k) Add 50 µL of each standard into the standard wells
(l) Add 50 µL of each sample into the sample wells
(m) Add 50 µL of enzyme conjugate to each well except the blank wells
(n) Shake the plate gently
(o) Incubate plate for 30 min at room temperature (20°C−25°C)
(p) Empty all wells after incubation

8.4.1.4.Washing sequence

(a) Fill all wells completely with wash solution; empty wells by inverting the plate, squeezing the plastic frame at the centre to prevent the strips from falling out of the frame
(b) Repeat the washing procedure 4 times
(c) Remove residual droplets by vigorous knocking on a paper towel

8.4.1.5.Development

(a) Prepare the developing solution by diluting 1 part of chromogen with 9 parts of citrate buffer
(b) Using a multichannel micropipette, add 200 µL of developing solution to each well, including the blank wells
(c) Incubate for 30 min at room temperature in the dark
(d) Add 50 µL of stop solution into each well. Colour should turn from blue to yellow. Mix thoroughly

8.4.1.6.Reading

- After thorough mixing, measure the absorbance using a microplate reader at 450 nm
- Take the measurement immediately after stopping the development process

8.4.2. Evaluation of results

Use a calibration curve to determine the unknown benzimidazole concentrations.

(a) Calculate the mean absorbance value for the blank and subtract it from the absorbance value of all the wells
(b) Calculate the mean absorbance value for the Maximum Binding (B_o), the standards and the samples
(c) Divide the mean absorbance value of standards and samples (B) by the mean absorbance value of the Maximum Binding (B_o) and multiply by 100.
(d) Maximum binding thus equals 100% and quote the absorbance values in percentages
(e) Enter the B/Bo (%) values calculated for each standard in a semi−logarithmic system of coordinates against the standard benzimidazole concentration; draw the standard curve
(f) Take the B/Bo (%) value for each sample and interpolate the corresponding concentration from the calibration curve

9. ANALYTICAL METHOD FOR SULFONAMIDES AND TETRACYCLINES IN SOLID/SEMI−SOLID AND AQUEOUS ENVIRONMENTAL SAMPLES FROM BY LC−MS/MS

9.1. PRINCIPLE

Samples are prepared by solid−liquid extraction including SPE clean−up using HLB cartridge and concentrated under a stream of nitrogen before LC−MS/MS analysis involving MRM or selected−reaction monitoring (SRM), product ion scan, precursor ion scan, and constant neutral loss scans.

9.2. SCOPE

This SOP is for determination of sulphonamide and tetracycline drug residues in multiple environmental samples by LC−MS/MS.

The test matrices are divided into solid/semi−solid and aqueous. Solid/semi−solid matrices are mainly soil, animal manure and manure compost, and sediment while aqueous matrices are fresh ground and stream H_2O as well as marine saline H_2O.

The target analytes are antimicrobials (such as tetracyclines, and sulfonamides) used in animal farming and aquaculture practices. The target concentration is as low as reasonably achievable.

9.3. MATERIALS

The following chemicals, reagents and supplies are required:

Aqueous/(semi−) solid samples – Sample bottle, PTFE or equivalent plastic ware resistant to organic solvent with screw cap; Bottle caps (lined with fluoropolymer); Analytical grade reagents

9.3.1. Standards/standard solutions

(a) Standard solutions
- Use materials of known purity and composition or purchase as solutions/mixtures with certification of their purity, concentration, and authenticity
- For standards of ≥98% purity, weight correction when calculating concentration is not necessary

(b) Stock solution
- Dissolve an appropriate amount of assayed reference material/pure standard in suitable solvent (10 mg in a 10 mL ground−glass−stoppered volumetric flask and fill to the mark with MeOH)
- Store standard solutions in the dark below -10ºC in screw-capped vials with fluoropolymer−lined caps or under a non−reactive gas (esuch as nitrogen)
- Place a mark on the flask at the level of the solution so that solvent loss by evaporation can be detected
- Replace the solution if some solvent has evaporated.

(c) Spiking solution
- Prepare at a practical quantification limit or reporting limit.

- In this protocol, this is determined as

$$amount\ (ng) \times\ dilution\ vol\ (mL)\ \div \\ Injection\ vol\ (\mu l) x \frac{1}{sample} size\ (g) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

(d) Isotope labeled standard solutions
- Prepare corresponding internal standards in the same way as the analytical standard under study.

9.3.2. Apparatus

The following are required: Tissue homogenizer, Vortex mixer; Ultra−sonic mixer; Oven; Desiccator; Analytical balance (Capable of weighing 0.1 mg−10 mg); pH meter; pH paper (wide range); Ultra−sonicator; SPE cartridge HLB/Mixed mode; Cation Exchange (MCX) 60 mg, Waters Oasis, 20 cc/1 g LP, 60 µm, or equivalent; Solvent filtration apparatus; Vacuum filtration apparatus including vacuum manifold; Glass−fibre filter–Whatman glass microfiber filter grade C (GF/C) 0.45 µm pore size; Centrifuge; Pipets/micro−pipets; Rotary evaporator; Nitrogen evaporation apparatus.

9.4. PROCEDURE

9.4.1. Sample preparation

(a) Samples, especially soil with particle sizes less than 2 mm, are used for analysis (otherwise homogenize/blend samples to reduce size)
(b) For solid manure/manure compost, obtain antimicrobial−free solid manure and manure compost from organic animal farms
(c) Liquid manure may be prepared from solid manure using distilled H_2O. Mix solid manure and distilled H_2O at 1:1 (w/w) and shake for 5 h; collect filtrate
(d) Soil should be antimicrobial−free (where veterinary drugs and manure have not been applied)
(e) Soil, sediment and manure/manure compost for analysis should not contain debris. As much H_2O as possible should be removed by centrifugation or vacuum filtration. Sample should be dried for ≥ 12 h at 110ºC
(f) Filter aqueous sample for analysis through 0.45 µm pore size GF/C under vacuum

9.4.2. Extraction and concentration

(a) Use SPE to extract the target analytes from aqueous samples
(b) Use ultra−sonic extraction with MeOH:EDTA−Mcilvaine Buffer pH = 6 (90:10, v/v) to extract solid samples
(c) To remove co−extracts/interferences in solid extracts, use the same SPE procedure employed for the aqueous samples
(d) Use EDTA−Mcilvaine buffer (pH 6) as an extraction solvent to avoid chelation especially of tetracyclines
(e) Filter soil samples using celite 545 material (less than 1 g)

(f) Use plastic ware during sample preparation and handling to avoid adsorption that could contribute to poor recoveries
(g) Use C18 material for sample clean−up

9.4.3. Summary of sample preparation

a) Measure 3 mg−5 mg solid sample (solid manure)

b) Add MeOH:EDTA−Mcilvaine buffer pH= 6 (90:10, v/v); 3×30 mL, and ultra−sonicate for 20 min

c) Perform vacuum filtration with GF/C and Celite 545 material

d) Load 1 g SPE C18 on a manifold (activate and pre−treat with 5 mL MeOH, 5 mL EDTA−Mcilvaine buffer pH = 4)

e) Filter sample

f) Elute sample using 10 mL H_2O; discard and add 20 mL of 0.01 M oxalic acid in MeOH

g) Dry sample under N_2 and add MeCN:H_2O (10:90, v/v)

h) Inject solid sample into LC−MS/MS

i) Measure ≤ 100 mL aqueous sample (H_2O/liquid manure)

j) Perform vacuum filtration with GF/C and Celite 545 material

k) Load 1 g SPE C18 on a manifold (activate and pre−treat with 5 mL MeOH, 5 mL EDTA−Mcilvaine buffer pH = 4)

l) Filter sample

m) Elute sample with 10 mL H_2O; discard, 20 mL 0.01 M Oxalic acid in MeOH

n) Dry sample under N_2 and add MeCN:H_2O (10:90, v/v)

o) Inject aqueous sample into LC−MS/MS

9.4.4. Measurement

Instrument conditions include:

- A C18 Acquity ultra−performance liquid chromatography (UPLC), ethylene bridged hybrid column (100 mm x 2.1 mm; 1.7 μm particle diameter); programmable 20mM NH_4HCO_2/20% MeOH with 20mM NH_4HCO_2/95% MeOH.
- MRM transitions (m/z): Chlortetracycline (CTC) 479>444, 479>462, Oxytetracycline (OTC) 461>426, 461>443, Sulfamethazine (SMT) 279>124, 279>186, Sulfamethoxazole (SMTZ) 254>108, 254>156 (99), Sulfathiazole (STZ) 256>108, 255>156 (92) and as ISs SMTZ−$^{13}C_6$ 260>162, and SMT−$^{13}C_6$ 285>124 (Table 23).

- Prepare calibration curves using standards in solvent/buffer, in control matrix extract and in matrix processed through the extraction procedure.

TABLE 23. MS/MS PARAMETERS FOR ANALYTES AND INTERNAL STANDARDS

Generic Name	Class	MRM transition (m/z) Precursor ion → Product ion	Cone Voltage (V)	CE (eV)
CTC	Tetracyclines	479>444, 479>462	36	18, 18
4-epichlortetracyclline (ECTC)	Tetracyclines	479>444, 479>462	36	18, 18
4-epi-anydrochlotetracycline (EACTC)	Tetracyclines	461>154, 461>444	36	18, 18
OTC	Tetracyclines	461>426, 461>443	34	18, 14
4-Epioxytetracycline (EOTC)	Tetracyclines	461>426, 461>444	34	18, 14
SMT	Sulfa drugs	279>124, 279>186	40	26, 16
SMT–$^{13}C_6$	Sulfa drugs	285>124	36	26
SMTZ	Sulfa drugs	254>108, 254>156 (99)	36	24, 16
SMTZ–$^{13}C_6$	Sulfa drugs	260>162	30	16
STZ	Sulfa drugs	256>108, 255>156 (92)	34	20, 14

9.4.5. Evaluation of results

- Recovery ratio (%) of the tetracyclines and sulfonamides in the aqueous samples (water and liquid manure) should be higher 70% (79.4−116 and 76.46−110 for solid/semi-solid samples) as detailed in Table 24.

- Reporting limits of the tetracyclines and sulfonamides in soil should be <0.05 ngg^{-1}, solid compost <0.1 ngg^{-1} (except SMTZ) and liquid manure/water 0.0025 ngg^{-1} (Table 25).

TABLE 24. MEAN RECOVERY OF VETERINARY DRUG RESIDUES IN SOLID/SEMI−SOLID AND AQUEOUS ENVIRONMENTAL SAMPLES

Generic Name	Soil/compost		Water/liquid samples	
	Mean (%)	Coefficient of variation (cv %)	Mean (%)	CV (%)
CTC	110.0	7.5	108.0	4.3
ECTC	71.3	8.2	78.2	5.4
EACTC	72.3	9.3	76.6	5.7
OTC	92.5	7.3	79.4	7.2
EOTC	80.3	6.4	82.4	6.7
SMT	101.0	8.4	101.0	7.3
SMTZ	88.7	8.2	89.5	6.3
STZ	77.5	9.4	81.2	7.8

TABLE 25. CC ALPHA, CC BETA, AND REPORTING LIMIT FOR TETRACYCLINES AND SULFONAMIDES RESIDUES

Generic Name	CC_α (µg/kg)	CC_β (µg/kg)	Reporting Limit (µg/kg)	Remark (m/z)
CTC	14.9	25.3	<0.05 (soil) <0.1 (Solid Compost)	479>444
OTC	19.2	32.7	<0.05 (soil) <0.1 (Solid Compost)	461>426
SMT	0.4	0.7	<0.05 (soil) <0.1 (Solid Compost)	279>124
SMTC	0.5	0.9	<0.05 (soil) N/A (Solid compost)	254>108
STZ	0.5	0.9	<0.05 (soil) <0.1 (Solid Compost)	256>108

- Report results of aqueous samples in ng/L and the solids (aqueous samples containing visible particles, solids, soils, sediments, filter cake, compost) in µg/kg based on the dry weight basis of the sample.

9.4.6. Acceptability criteria

Acceptability criteria should base on a European Commission Decision [4] concerning the performance of analytical methods and the interpretation of results.

10. DETECTION OF CHLORAMPHENICOL RESIDUES IN MEAT, CASINGS AND HERB BY ELISA

10.1. PRINCIPLE

This is a modification of a standard method [5] where meat, casings and plants are extracted with H_2O. After centrifugation an aliquot of the supernatant is cleaned−up by Extrelut® NT3 columns. After incubation, chloramphenicol (CAP) is then extracted with dichloromethane and the final eluate diluted in PBST.

The detection principle is Enzyme Immuno Assay based on antigen−antibody reaction. Rabbit Polyclonal Antibodies (PCA) are prepared against Bovine Serum Albumin (BSA) coupled CAP. The wells of the microtiter plate are coated with anti−rabbit antibodies against goat.

10.2. SCOPE

This screening method can detect CAP in meat and casings (at 0.3 µg/kg) and plants at 0.5 µg/kg. The method is also suitable for detection of CAP glucuronide.

10.3. MATERIALS

10.3.1. Chemicals/reagents

Chemicals required include: Ethyl acetate; Iso−octane; Trichloromethane; n−hexane; De−ionized H_2O.

10.3.2. Apparatus

The following are also required: Universal plastic bottles and lids (25 mL and 50 mL); Reagent sealer (50 mL); Glass tubes (12 mL); Glass beaker; Pipette tips (10 µL−200 µL; 100 µL−1,000 µL and 500 µL−5,000 µL); Analytical balance; Multi speed vortex; Refrigerated centrifuge; Homogenizer; Sample concentrator; Water bath; ELISA reader; Microplate shaker; Single channel pipette (10 µL−100 µL; 100 µL−1000 µL and 500 µL−5000 µL); Multi−channel pipette (25 µL−300 µL).

10.4. PROCEDURE

10.4.1. Specificity and sensitivity

- This ELISA technique utilizes a specific antibody raised in rabbits against protein conjugated CAP and it is important to note cross reactivity.
- Prepare a linear calibration curve in the range 0.025 ng/mL−2 ng/mL.
- No sample hydrolysis is required because of the cross−reactivity between CAP and CAP−glucuronide.

10.4.2. Sample treatment

a) Tissue samples (meat/casings)

- Homogenize ~10 g of tissue
- Weigh 3 g of the homogenized sample and transfer into a glass tube
- Add 6 mL of ethyl acetate and mix for 10 min
- After centrifugation (10 min, 2,000g) pipette 4 mL of the ethyl acetate into a glass tube and evaporate the ethyl acetate at 50ºC under a mild stream of nitrogen.
- Disolve the fatty residue in 1 mL of iso−octane/trichloromethane (2:3, v/v) and add 1.0 mL of sample dilution buffer. Shake the mixture for 1 min and centrifuge (10 min, 2,000g)
- In case of an emulsion in the upper layer, place the test tube in a water bath (80ºC) for 5 min and centrifuge again. Pipette 50 µL portion of the upper layer into a test tube
- Iso−octane/trichloromethane or n−hexane may be used in the sample preparation. When iso−octane/trichloromethane is used, pipette 50 µL of the upper layer; take 50 µL of the layer underneath when n−hexane is used. A better recovery may be obtained when iso−octane/trichloromethane is used rather than n−hexane.

b) Herb sample

- Grind 10 g to 100 g of herb
- Homogenize 5 g of the ground herb in 20 mL of distilled H_2O
- Pipette 5 mL of this mixture into a glass tube
- Add 10 mL of ethyl acetate and mix for 30 min
- Centrifuge for 10 min at 2,000g
- Pipette 5 mL of ethyl acetate (upper layer) into a glass tube and evaporate at 50ºC under a mild stream of nitrogen
- Dissolve the fatty residue in 0.5 mL of iso−octane/trichloromethane (2:3, v/v) and add 0.5 mL of sample dilution buffer
- Vortex the mixer for 1 min and centrifuge (10 min, 2,000g)
- Take 50 µL aliquot of the upper layer for the ELISA test.

10.4.3. Preparation of reagents in ELISA kit

The following are important to note:

(a) The reagents included in the test−kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample should be analysed in duplicate
(b) Ready−to−use standards are prepared in dilution buffer. When an alternative sample matrix is used, standards or spikes should be prepared in the sample matrix using the enclosed 100 ng/mL standard solution
(c) Before starting the test, bring up all reagents to ambient temperature
(d) Any reagents not used should be stored immediately at +2ºC to +8ºC. Keep standard solutions in the dark and store at +2ºC to +8ºC
(e) Rinsing buffer: The rinsing buffer is delivered 20 times concentrated. Therefore prepare dilutions freshly before use. Prepare 40 mL of diluted rinsing buffer (2 mL concentrated rinsing buffer + 38 mL distilled H_2O)

(f) Substrate solution: The redy−to−use substrate solution precipitates at 40 ºC. Ensure that the vial containing this solution is at room temperature (in dark); mix the content before pipetting into the wells
(g) Conjugate solution: Reconstitute the lyophilized CAP conjugate with 4 mL of reconstitution/zero standard buffer; mix thoroughly and keep in the dark until use
(h) Antibody solution: Reconstitute the lyophilised antibodies supplied in a vial with 4 mL of reconstitution/zero standard buffer; mix thoroughly and keep in dark until use
(i) Sample dilution buffer (4 x concentrated): Before dilution (20 mL buffer + 60 mL distilled H_2O) bring the concentrated buffer to room temperature and mix thoroughly. Mix well before dilution with distilled H_2O. The 4 x diluted buffer may be stored in a refrigerator (+2ºC to +8ºC) until the expiry
(j) Standard solution (100 $ngmL^{-1}$): To prepare standards in the appropriate matrix or to prepare spikes, use the standard solution containing 100 ng CAP per mL. Dilute the standard solution in the appropriate matrix to make a dilution range of 2 ng/mL, 0.5 ng/mL, 0.2 ng/mL, 0.1 ng/mL, 0.05 ng/mL, 0.025 ng/mL. The concentration of 0.0125 ng/mL may be included as an option. The zero standard should be prepared from the same matrix under study.

10.4.4. Assay protocol

(a) Prepare sample and ensure microtiter plate (Table 26) is ready to use
(b) Pipette 100 µL of the reconstitution/zero standard buffer in duplicate (well A1, A2). Pipette 50 µL of the reconstitution/zero standard buffer in duplicate (well B1, B2)
(c) Pipette 50 µL of each standard dilution in duplicate (well C1, 2 to H1, 2 that is 0.025 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL and 2 ng/mL)
(d) Pipette 50 µL of each sample solution in duplicate into the remaining wells of the microtiter plate
(e) Add 25 µL of CAP conjugate into all wells, except wells A1 and A2
(f) Add 25 µL of antibody solution into all wells, except wells A1 and A2
(g) Seal the microtiter plate and shake the plate for 1 min
(h) Incubate plate for 1 h in the dark at 40ºC (acceptable range of 20ºC to 80ºC)
(i) Discard the solution from the microtiter plate and wash 3 times with rinsing buffer
(j) Empty the contents of each well by turning plate upside down followed by a firm short vertical movement
(k) Fill all the wells to the rims with rinsing solution (300 µL)
(l) Repeat the rinsing 3 times
(m) Turn the plate upside down and empty the wells by a firm short vertical movement
(n) Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells
(o) Ensure none of the wells dries before the next reagent is dispensed
(p) Discard the solution from the microplate and wash 3 times with rinsing buffer
(q) When using an automatic plate washer, check that all wells can be aspirated completely and that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to perform 3 rinsing cycles
(r) Pipette 100 µL of substrate solution into each well. Incubate plate for 30 min at room temperature (+20ºC to +25ºC)
(s) Add 100 µL of stop solution into each well

(t) Immediately read the absorbance values at 450 nm.

While interpreting results, note:

(a) Test all the control samples, blank samples and spiked samples together
(b) A blank should have no response for CAP
(c) For a suspected sample (sample containing CAP) confirmation by LC−MS/MS is necessary.
(d) For meat and plant samples: Using the extraction in ethyl acetate followed by the clean−up, the CAP equivalents calculated from the calibration curve have to be divided by 2 to express the concentration (ng/g) in tissue.
(e) For herb samples: The CAP equivalents should be read directly from the calibration curve. Spot samples on the ELISA well as demonstrated in Table 26.

TABLE 26. A SKETCH OF THE 96 WELL MICROTITRE ELISA PLATE AND SPOTTING SCHEME

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Sample 1	Sample 1								
B	Standard 1	Standards 1	Samples 2	Samples 2								
C	Standard 2	Standard 2	Sample 3	Sample 3								
D	Standard 3	Standard 3	Sample 4	Sample 4								
E	Standard 4	Standard 4	Sample 5	Sample 5								
F	Standard 5	Standard 5	Sample 6	Sample 6								
G	Standard 6	Standard 6	Samples 7	Sample 7								
H	Standard 7	Standard 7	Sample 8	Sample 8								

11. DETECTION OF SULFONAMIDES IN CHICKEN MUSCLE BY THIN LAYER CHROMATOGRAPY

11.1. PRINCIPLE

Homogenized poultry muscle is extracted with ethyl acetate. The extract is evaporated to dryness, and the residue dissolved in MeOH:H_2O. Samples are defatted using petroleum sprit and 25 µL of extract of each sample spotted on a Whatman AL SIL G/UV thin layer chromatography (TLC) plate and developed using chloroform: n−butanol (9:1, v/v). The plate is dried and observed at 366 nm after treating with fluorescamine using CAMAG viewer followed by scanning the analyte spots using a CMAC scanner (where necessary).

11.2. SCOPE

This method is suitable for analysis of sulfonamides in chicken muscle at the MRL of 100 µg/kg for the combined residues of all substances in the group. The SOP can be used to screen residues of sulfadiazine (SDZ), sulfathiazole (STZ), sulfadoxine (SD), sulfamethazine (SMZ) and sulfaquinoxaline (SQ) in poultry meat at their MRL.

11.3. MATERIALS

11.3.1. Chemicals/Reagents

Acetic acid, glacial, ethyl acetate, petroleum spirit, HCl, 0.1 M, HPLC grade MeOH (Sigma, HPLC grade), chloroform and n-butanol, acetone, fluorescamine, NaOH.

Other materials include: Nitrogen gas, Milli Q H_2O; Thin Layer Chromatography (TLC) plates (Whatman AL SIL G/UV); sulfonamide standards: SDZ (Sigma), STZ (Sigma), SMZ (Sigma), SD (Sigma), SQ (Sigma).

11.3.2. Equipment/glassware

The following are required: Ultra−turrax homogenizer; Centrifuge; Analytical balance (0.01 g); CAMAG TLC scanning densitometer with data analysing system and printer; TLC viewing chamber; Standard laboratory glassware; Centrifuge tubes (15 mL); Amber colour glass bottles (100 mL); Pasteur pipettes; TLC developing tank; capillary tubes (25 µL).

11.3.3. Solutions

(a) Fluorescent solution (0.1M). Dissolve 10 mg in 100 mL acetone. Prepare solutions freshly

(b) TLC solvent system: chloroform/n−butanol (90:10, v/v). Add 9 mL chloroform, 1 mL n−butanol and 10 mL de−ionized H_2O to a separatory funnel. Equilibrate by shaking for 30 sec and allow phases to separate. Collect the bottom solvent mixture. Prepare solvents freshly

(c) Stock standards (1 mg/mL). Dissolve 10 ± 0.1 mg of sulfonamide standard except SDZ and SQ in MeOH and make up to the 10 mL mark in a 10 mL volumetric flask. Dissolve SDZ and SQ in 0.1M NaOH. Store at -20°C in amber coloured vials. Prepare every 12 mn

(d) Mixed intermediate standard (100 µg/mL): Pipette aliquots (1 mL) of stock sulfonamide solutions into a 10 mL volumetric flask and fill to the mark with MeOH. Store at 4°C in an amber coloured vial. Prepare every 3 mn.
(e) Working standards (500 ng/mL, 1,000 ng/mL, 2,000 ng/mL, 3,000 ng/mL). Measure 100 µg/mL of mixed standard (Table 27) into a 5 mL volumetric flask and fill to the mark with MeOH. Prepare weekly. Store in amber coloured vials at 4°C in a refrigerator.

TABLE 27. PREPARATION OF SOLUTIONS FOR THE STANDARD CURVE

Standard concentration (ng/mL)	Volume of 100 µg/mL Standard (to 5 mL final volume)	Standard Concentration (ng/spot)	Standard Equivalent (µg/kg)
0	0	0	0
500	25 µL	12.5	25
1,000	50 µL	25	50
2,000	100 µL	50	100
3,000	150 µL	75	150

11.4. PROCEDURE

Store study samples at -20°C until testing. Samples should be properly thawed at room temperature and weighed (~ 5 g tissue) into a homogenization vials.

11.4.1. Preparation of control sample

(a) Weigh two portions (3.00 ± 0.01 g) of minced blank tissue (without sulfonamides) into 15 mL centrifuge tubes
(b) Add 30 µL of the mixed intermediate standard (10 µg/mL) to one sample. This is equivalent to 100 µg/kg of tissue
(c) Take the unspiked sample through the preparation procedure as a blank
(d) Let spiked tissue samples stand for 15 min before extraction

11.4.2. Extraction

(a) Add 0.5 mL HCl (0.1M) to sample and vortex for about 20 sec
(b) Add 3 mL Milli−Q H_2O and vortex at a low speed for 10 sec
(c) Homogenize samples using ultra−turrax for 2 min
(d) Add 4.5 mL of ethyl acetate and mix the samples for 10 min using the sample rotator
(e) Centrifuge for 10 min at 3,000 rpm
(f) Transfer the supernatant using a pasture pipette into a test tube
(g) Repeat steps d, e and f
(h) Evaporate the supernatant to dryness under a stream of nitrogen at 55°C
(i) Dissolve the dry residue in 1 mL of MeOH:H_2O (72:25, v/v) and vortex vigorously
(j) Add 1 mL of petroleum spirit and mix gently
(k) Discard the petroleum layer
(l) Repeat steps j−k
(m) Transfer the remaining aqueous layer into microvials

11.5. MEASUREMENT

11.5.1. Application of samples

(a) Cut the TLC plate according to the requirement (10 cm × 10 cm)
(b) Apply 25 µL of standards and samples or control samples onto the TLC plate under N_2 flow
(c) Dry the plate using a dryer

11.5.2. Development

(a) Add the TLC solvent system into the TLC chamber
(b) Keep the TLC chamber at room temperature for 30 min
(c) Develop the TLC plate under one−dimensional linear ascending mode
(d) Keep the TLC plate until the solvent front reaches the 9 cm margin of the plate
(e) Withdraw plate and dry it using a dryer

11.5.3. Derivatization

(a) Spray the TLC plate with 0.1 mg/mL fluorescamine solution
(b) Dry the TLC plate using a dryer

11.5.4. Detection

(a) Observe the developed TLC plates under the viewing chamber
(b) Scan the TLC plate using a TLC scanner
(c) View/scan plates at 366 nm

11.5.5. Calculations/evaluation

(a) Identify each sulfonamide using the retention factor (Rf) value by comparing with the position of the respective sulfonamide standards on the plate
(b) Scan spots and compare peak area of the relevant sulfonamide against the peak area of the standard control set at maximum residue limit (MRL).

11.5.6. Acceptance criteria

The results must fulfil the following conditions:

(a) The spots of the standards (equivalent to MRL level) must be clearly visible. The scan value must be between the average and ± 2 × standard deviation (SD) of reference values
(b) There must be no responses in the negative control samples exceeding 10% of the MRL equivalent response
(c) The response in the positive control sample (MRL−equivalent) should exceed 50% of the response of the corresponding standard (MRL-equivalent)
(d) Values for the positive control must be recorded on the quality chart.

Samples with a response greater than MRL plus 2 × relative standard deviation (RSD, the repeatability validation experiment, are considered positive.

12. VALIDATION OF IMMUNOASSAYS

Abstract

Practical guidance is provided for estimation of method performance characteristics, including specificity, cross-reactivity in matrix, limit of detection (LOD), detection capability (CCβ), repeatability, trueness and recovery, stability of the analyte, robustness, and acceptability criteria of immunoassay techniques for veterinary drug residues. The protocol can be adapted to individual laboratory conditions and used as standard deviation (SOP).

12.1. INTRODUCTION

Validation is the means by which proof is obtained that an analytical method is fit for the purpose for which it is to be applied. The validation procedure used for an immunoassay will reflect that immunoassay is a screening test and whether or not the test is used to generate quantitative results. Screening tests are designed to give a low incidence of false compliant (false negative) results at the concentration of interest. In contrast, a proportion of false non−compliant (false positive) findings can be tolerated. The validation procedure adopted for an immunoassay should reflect this important distinction in performance criteria. A number of different authorities have prepared guidelines for validating analytical tests used in residue analysis such as the EU Commission Decision 2002/657/EC [4].

12.1.1. Method Specificity

Specificity here refers to the ability to distinguish between the analyte being measured and other potential interferents. In immunoassay, the specificity of the procedure is a characteristic of the antibody used in the test. Compounds with similar chemical structures to the analyte (s) of interest must be tested for their cross−reactivity (CR) against the antibody.

12.1.1.1. Cross−reactivity evaluation in matrix

The presence of matrix in an assay greatly affects the interaction that occurs between an antibody and an analyte and can have pronounced effects on antibody CR.

Evaluate individual CR for each matrix to which the assay will be applied.

12.1.2. Assay LODs

- The LOD in this method is a value at or above which it can be concluded that the analyte is present in a sample. It can be determined by the analysis of at least 20 blank samples by the procedure being validated. The apparent mean concentration in the blank population plus 3 times the SD of the mean is taken as the LOD. This can be used for quantitative or qualitative tests.

- To illustrate, 20 poultry muscle samples are assayed for the presence of CAP by an ELISA method and the mean concentration detected is 0.05 with a SD of 0.04 μg/kg. The LOD would then be 0.17 μg/kg.

12.1.2.1. The CCβ

- The CCβ is the smallest content of a substance that may be detected in a sample with an error probability of β. A false compliant rate (error probability) of 5% or less is permitted in screening tests according to the EU guidelines [4]

- To illustrate, spike 20 blank samples at different CAP concentrations (0.15 µg/kg, 0.20 µg/kg and 0.25 µg/kg). If the LOD was 0.17 µg/kg and 19 out of the 20 samples (95%) analysed are non−compliant, the CCβ can be assigned the approximate value of 0.20 µg/kg

- The CCβ value must be determined for each matrix individually and wide differences.

12.1.2.2. CCβ, MRL and Minimum Required Performance Limits (MRPL)

- Before method validation is attempted, determine what the CCβ should be for MRL and MRPL substances

- For MRL compounds, CCβ should be approximately 50% of the MRL

- For MRPL substances, the CCβ should be as low as possible and certainly less than the MRPL.

12.1.3. Repeatability

Repeatability data are only required for quantitative and semi−quantitative tests.

- Typically, measure by spiking a number (≥ 10) of samples with the same concentration of analyte
- Extract the samples and measure the concentration of drug detected in a single immunoassay procedure (determination of within−assay variation)
- Repeat this over at least two more days to calculate the between−assay variation
- Express results as % coefficients of variations (CVs), the percent ratio of the mean to SD
- For MRL substances, spike each species/matrix combination, at 0.5, 1.0 and 1.5 times the MRL (n > 5)
- Record results following analysis as mean and CV for each species/matrix combination
- For MRPL substances, spike each species/matrix combination at 1.0, 1.5 and 2 times CCβ (n> 5)
- Record results following analysis as mean and CV for each species/matrix combination.

12.1.4. Trueness and recovery

A calibration curve may be prepared by spiking blank samples with the calibrants and extracting these samples by the same procedure used for unknown samples.

- For true recovery, prepare two calibration curves. In one set, add the calibrants prior to sample extraction, and in the second add the calibrants after sample extraction (that is, 100% value).

- Recoveries can be calculated for the first set of calibrants against a calibration curve constructed from the second set.

12.1.5. Stability of the analyte

Devise a protocol to determine the stability of the standards prepared in both light and dark conditions and at a range of temperatures as wide as practicable (such as -17ºC to 23ºC and +4 to + 8ºC).

During the experiments:

- Where possible use an LC−MS/MS as a reference method
- When attempting to use an immunoassay to determine stability of an analyte, a series of calibration curves may be used to compare results e.g. using the same set of standards and with reagents stored in the dark for 1, 2 and 4 weeks at +4°C
- Perform stability test of the analyte in matrix preferably using incurred material
- In the absence of incurred material, measurements may be performed using spiked samples
- Spike samples at a "meaningful" concentration (for example at MRL or MRPL)
- Store spiked samples in a freezer and assay at regular intervals over a period of time
- In case of delays between preparation of sample extracts and their subsequent analysis, data should be generated to prove that significant analyte loss does not occur during this lag period
- Spike and extract a range of samples with analyte (to determine loss). Half of the extracts should be assayed immediately and the other half stored at +4°C and assayed after 24 h.

12.1.6. Robustness

- Measure the sensitivity of the assay to other variables such as operator, equipment, laboratory environment, and reagents
- Repeat assays at least three times using, wherever possible, different operators, different equipment and different batches of reagents.

12.1.7. Acceptability criteria

Include acceptability criteria in the SOP for the ELISA test (once assay has been fully validated and deemed sufficiently sensitive and specific for routine use).

Possible criteria that could be incorporated include:

- Absorbance of the zero standard greater than 0.5 and less than 1.6 optical density units.
- Target optical density of 1.0 with a development time ranging from 8 to 16 min (target: 12 min).
- Absorbance of the mid−point calibrant relative to the zero standard calculated as follows: Mid−point percentage=100 times absorbance of mid−point calibrant, divided by the absorbance of the zero standard.

- Reduction in optical density greater than 60%. (a strong indication that the method sensitivity is sufficient for the analysis)
- Examine the replication of each sample using the % CV. The % CV should be less than or equal to 20. When the % CV exceeds 20 and all the replicates are either above or below the action level, then the mean should be accepted. When the % CV is greater than 20 and the concentrations straddle the action level, the analysis should be repeated.
- Control samples should give the correct interpretation (a negative control gives a negative result).

13. VALIDATION OF SCREENING METHODS FOR THE DETECTION OF VETERINARY DRUG RESIDUES

13.1. SCOPE

The range of analytical screening methods used for veterinary drug residues currently is varied and includes among others, techniques such as immunoassay, microbiological inhibition, bioassays, high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Screening methods may be qualitative, semi−quantitative or quantitative procedures. The following information is a guide to the minimum level of validation required to demonstrate fitness−for−purpose for all screening methods. The elements chosen for the validation of qualitative and semi−quantitative methods will vary depending on each individual method. The EU Commission has laid down performance criteria for analytical methods for the detection of residues of certain substances [4].

13.2. THE VALIDATION PLAN

- The validation plan is applicable to all methods whether existing or in a process of development.
- The method should be in an advanced stage of development and the procedure should be documented in a standard operating procedure (SOP)
- Identify method scope and specify validation in the validation plan. The scope will include the concentration range over which the method is to be used and the tissue type and species to be tested
- Additional parameters may be added with time and validation plan amended as necessary
- For methods in use existing data can support the validation (provided the conditions specified in the SOP and the validation plan have been fulfilled)
- As applicable, once validation is complete, the validation plan and the data will be submitted to a nominated person for audit
- Revise SOP (and associated quality parameters accordingly) following the audit
- If changes are made, their influence must be documented and if necessary a new validation carried out.

13.3. VALIDATION BY MATRIX AND SPECIES

- The method should be validated in the matrix or matrices for which the assay is intended.
- If used for analysis of one or more species, and multiple matrices, key elements of the validation such as standard curve, recovery and inter−assay variation must be compared for each species
- If there are no significant differences apply method for these species and conduct validation for the principal species/matrix
- Conduct full validation for each species/matrix in case of significant differences.

13.3.1. Specificity/selectivity

13.3.1.1. Immunoassays

For immunoassays:

- Validation helps assess antiserum's ability to cross−react with any interfering compound in the assay. Conduct antiserum cross−reactivities in the sample matrix or matrices for which the method is intended
- Validation will also assess the method's ability to detect a number of drugs within a family
- Select drugs to be investigated noting relevant factors (for example likely prevalence of the compound in the matrix, their availability for use in animal production and possible analogues).

13.3.1.2. TLC and HPLC

- Assess the specificity of chemical methods such as HPLC and TLC by determining factors such as retention times (RTs), retention factor (rf) values and spectra
- Initial determinations can be carried out in buffer, solvent or mobile phase as appropriate
- Then carry out further determinations in the matrix for which the method is to be applied (compare results with the standard profile for the assay)
- Spike a range of drugs into the appropriate tissue/matrix and analyse as normal
- Select drugs taking into account the likely prevalence of the compound in the matrix, availability for use in animal production and possible analogues.

13.3.1.3. Microbial Inhibition Assays and Bioassays

- Determine specificity/selectivity with regard to factors such as environmental, disinfectants and natural enzymes
- Assess effect by testing a range of known drug−free samples for each of the matrices to be tested
- For the determination of specificity for quantitative inhibition methods (for example antimicrobials in animal feeding stuffs), preferably calculate the parallelism of the standard range and standard ranges containing other drugs likely to occur as interfering substances.

13.3.2. CC α for banned substances

- For banned substances consider the CCα as the lowest concentration level at which a method can discriminate with a statistical certainty of 1-α that the identified analyte is present
- Analyze 20 negative samples for each matrix and calculate the LOD using the mean + three standard deviations (SDs)
- Ensure the negative samples are from different animals and where possible confirmed negative by an alternative method.

13.3.3. CC α for substances with MRL

- For substances with an established MRL, analyze at least 20 negative samples for each matrix fortified with the analyte at the MRL

- The mean concentration at the MRL plus 1.64 times the corresponding SD equals the CCα (α =5%).

13.3.4. CC βeta for banned substances

- Test 20 representative blank samples fortified at the level of interest based on the CCα for the method
- If all fortified samples are declared positive, that is, greater than the CCα, then CCβ is less than the level of fortification
- If 19 of the fortified samples are declared positive, then CCβ equals the level of fortification
- If 18 or less of the fortified samples are declared positive, then CCβ is greater than the level of fortification.

13.3.5. CC βeta for substances withe MRLs

- Test 20 representative blank samples fortified at the MRL or below (e.g. half MRL where possible).
- If all fortified samples are declared positive (greater than the CCα) then CCβ is less than the level of fortification.
- If 19 of the samples are declared positive, then CCβ equals the level of fortification.
- If 18 or less of the fortified samples are declared positive, then CCβ is greater than the level of fortification.

13.3.6. Stability of the analyte in solution

- Prepare fresh stock solutions of the analyte(s) and dilute to give the standard and control concentrations as specified in the method SOP
- Analyze the standards after preparation and then add appropriate volumes into suitable containers, label and store as follows: Ten aliquots in the dark and light at -20^{o}C, 4^{o}C and 20^{o}C
- Test the aliquots by comparing against a fresh standard and control series one, two, three and four weeks after preparation
- Ensure that the time scale for the study reflects the normal storage conditions of the standards and length of storage.

13.3.7. Stability of the analyte in matrix

- Preferably use incurred material but if not available then spiked material can be used.
- Select a suitable concentration or range e.g. CCβ or MRL
- Dispense samples into 5 aliquots for each concentration and spike at the selected level
- Analyse one set immediately and then after 1, 2, 4 and 20 weeks of storage at -20 ^{o}C.

13.3.8. Stability of the analyte in extract

- Assess the stability in methods where the analyte is extracted into a final solution
- Determine stability in the analytical solution and any other stage the sample is left at
- Extract three sets of controls (6 replicates of each) per assay. A set should be finished within a day
- Store the other sets and complete on other dates reflecting likely use of the method in normal testing conditions
- Compare the results between the different time periods and determine any significant difference.

13.4. CALIBRATION CURVE

- Use between−assay and within−assay variation of the standard curve (to establish acceptability values for each standard point)
- Assay three curves in the same analysis to determine within−assay variation
- Establish between−assay variation as the validation progresses
- Describe the mathematical formula of the curve and describe the degree of fit in the validation data.

13.5. WITHIN LABORATORY REPRODUCIBILITY

- Prepare a set of samples and spike with the analyte(s) to give a suitable range of concentrations [that is, 1, 1.5 and 2 times the CCβ or 0.5, 1 and 1.5 times MRL]
- Analyze six replicates at each level
- Repeat these steps at least twice using different operators and different environmental conditions (for example batches of reagents, standards, instruments etc.)
- Calculate the mean and % CVs for the spiked samples.

13.6. INTER−ASSAY AND INTRA−ASSAY VARIATION

- Prepare a set of samples and spike with the analyte (s) for concentrations equivalent to 1, 1.5 and 2 times the CCβ or 0.5, 1 and 1.5 times the MRL
- Analyze at least six replicates at each level
- Repeat these steps again on at least two other occasions
- Calculate the mean and % CVs for the spiked samples
- Concentration of the analyte may be estimated from other available parameters such as zone size, optical density or % absorbance
- Specify analytes and their concentration for study and include in the validation plan.

13.7. RECOVERY

For the recoveries:

- Use certified reference material (if available) or spike blank samples

- Select 18 aliquots of a blank matrix and spike six aliquots at 1, 1.5 and 2 times the CCβ or 0.5, 1 and 1.5 times the MRL
- Add analyte to the negative matrix before any solvent and allow interaction with the sample for a period specified in the SOP prior to assay
- Analyse the samples and calculate the concentration present in each sample
- Calculate the mean recovery (% ratio of measured concentration to the spike concentration) and CV from the six results at each level.

13.8. ROBUSTNESS

- Estimate the effect of minor reasonable variations on the method
- Select factors such as changes in the analyst, the source and age of reagents, solvent, standards and sample extracts, the rate of heating, the temperature, the pH value, equipment used or any other factor which may affect the method in any way
- Identify possible factors that could influence the results
- Vary each factor slightly
- You can refer to validation data
- Conduct further experiments in case a factor influences the measurement result significantly.

REFERENCES

[1] ANASTASSIADES, M., LEHOTAY, S.J., STAJNBAHER, D., SCHENCK, F.J., Fast and Easy Multi−residue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid−Phase Extraction" for the Determination of Pesticide Residues in Produce. J AOAC Int., **86** 2 (2003) 412−431.

[2] COMMITTEE FOR VETERINARY MEDICINAL PRODUCTS. A summary report on Florfenicol in Fish. The European Agency for The Evaluation of Medicinal Products, Veterinary Medicine Evaluation Unit. EMEA/MRL/251/97−Final, July 2997

[3] GRANJA, R.H., de LIMA, A.C., PATEL, R.K., SALERNOA, A.G., WANSCHEL, A.C., Monitoring of Florfenicol residue in fish muscle by HPL-UV with confirmation of suspect results by LC−MS/MS. Drug Test Anal., **4** Suppl 1 (2012) 125−129.

[4] COMMISSION DECISION. 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJEU, L 221 **45** (2002) 8.

[5] EURO-PROXIMA B.V., A microtiter plate based competitive enzyme immuno assay for screening and quantitative analysis of Chloramphenicol in various matrices Chloramphenicol Fast Elisa 5091 CAPF. Arnhem, The Netherlands.

ABBREVIATIONS AND ACRONYMS

%	Percent
~	Approximate
°C	Degrees Celsius
µA	Microamp
µg/kg	Microgram per kilogram
µm	Micrometre (microns)
AG	Aminoglycoside
APCI	Atmospheric pressure chemical ionization
Ar	Argon
B_o	Maximum binding
C18	Carbon 18
CCα	Decision limit
CCβ	Detection capability
CPM	Counts per minute
CRP	Coordinated Research Project.
CV	Coefficient of variation
Da	Dalton
DMSO	Dimethyl sulfoxide
e.g.	For example
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immune sorbent assay
ESI	Electro Spray Ionization
eV	Electron volt
FAO	Food and Agricultural Organization of the United Nation.
FFA	Florfenicol amine
FFC	Florfenicol
FLD	Fluorescent detector
g	Gram
g/L	Grams per litre
h	Hour
H_2O	Water
HCl	Hydrochloric acid
HFBA	Hepta fluoro butyric acid
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
HRP	Horse radish
i.e	That is
IAEA	International Atomic Energy Agency
MeCN	Acetonitrile
IgG	Immunoglobulin G
IS	Internal Standard
kg	Kilogram
kV	Kilovolts
L/h	Litres per hour
LC−MS/MS	Liquid Chromatography Tandem Mass Spectrometry
m/z	Mass to charge ratio
MeOH	Methanol
mg	Milligram
$MgSO_4$	Magnesium sulphate

min	Minutes
mL	Millilitre
mL/min	Millilitres per minute
mm	Millimetres
mM	Millimolar
mmHg	Millimetres of Mercury
mn	Months
mol/L	Mole per litre
mol/L	Mole per litre
MRL	Maximum recommended residue limits
MRM	Multiple Reaction Monitoring
msec	Milliseconds
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng/µL	Nanogram per microliter
ng/mL	Nanogram per milliliter
ng/g	Nanogram per gram
nm	Nanometre
PBS	Phosphate buffered saline
PoEMS	Post−extraction spiked matrix standards
POPOP	1, 4 – Bis (5 phenyl−2−oxazolyl benzene)
PPO	2, 5, Diphenyl oxazole
PREMS	Pre−extracted spiked matrix standarads
PSA	Primary secondary amine
psi	Pounds per square inch
PTFE	Poly tetra fluoro ethylene
QC	Quality Control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe.
r^2	Coefficient of regression
RIA	Radio immune assay
rpm	Revolutions per minute
RSD	Relative Standard Deviation
tR	Retention time
Sec	Second
SOP	Standard Operating Procedure
SPE	Solid phase extraction
SSNI	Secondary standard solution one
SSNII	Secondary standard solution two
TCA	Trichloro acetic acid
TPP	Triphenyl phosphate
USA	United States of America
USP	United States Pharmacopoeia
UV	Ultra−violet
V	Voltage
v/v	Volume by volume
v/v/v	Volume by volume by volume
Vol	Volume
w/w	Weight by weight
µg/mL	Microgram per millilitre
µL	Microlitre

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