Deliverable 4.3

Method(s) for polar drug analysis transferred to EU laboratories

Teagasc

Martin Danaher

Draft deliverable

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DELIVERABLE 4.3 – VERSION 1

WORK PACKAGE N° 4

Nature of the deliverable		
ORDP	Open Research Data Pilot	
R	Document, report (excluding the periodic and final reports)	X
DEM	Demonstrator, pilot, prototype, plan designs	
DEC	Websites, patents filing, press & media actions, videos, etc.	
E	Ethics	
OTHER	Software, technical diagram, etc.	

Dissemination Level		
PU	Public, fully open, e.g. web	X
CO	Confidential, restricted under conditions set out in Model Grant Agreement	
CI	Classified, information as referred to in Commission Decision 2001/844/EC	

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EU-China-Safe aims at reducing food fraud and improving food safety through focusing on improving food legislation, food inspection and increasing access to information across Europe and China. State-of–the-art technologies including a virtual laboratory will create a unique space to share and demonstrate best practice. The use of innovative technologies will result in improved detection of adulteration of food products as well as increased traceability and transparency of global supply chains.

The project runs from September 2017 to August 2021. It involves 33 partners and is coordinated by QUB (The Queen’s University of Belfast).

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1. SUMMARY

Deliverable 4.3 addresses a number of objectives of WP4, namely:

- To address current challenges and gaps in the analysis of aminoglycoside residue testing through the implementation of improved analytical methods.
- To transfer analytical methodology for the analysis of aminoglycoside resiues and harmonise testing between China and the EU.
- To improve the safety and quality of food consumed in Chinese and European markets through improved testing for aminoglycoside residues.
- To improve the food safety infrastructure in both China and the EU.

In this task a comprehensive test was developed for the analysis of 14 aminoglycoside residues in pork and the standard operatibg procedure for the method has been transferred to an EU laboratory at Teagasc in Dublin, Ireland.

2. REVISION HISTORY

Version	Date	Revised by	Comment
V0.1	12.10.2020		Draft deliverable

3. INTRODUCTION

This task involves the transfer of methodology for the analysis of aminoglycoside and antiviral drugs from Chinese to EU laboratories.

The aminoglycoside antibiotics are licensed as veterinary drugs to treat infections in various food producing animals. They are notoriously difficult to analyse in food samples because the highly polar nature of these compounds, which pose a number of challenges in both sample preparation and detection methods. Teagasc have found from previous research that these aminoglycosides require a highly acidified extraction solvent to efficiently extract these compounds. Teagasc also previously evaluated a range of different HILIC (hydrophilic interaction liquid chromatography) columns for the analysis of aminoglycosides with detection by tandem mass spectrometry showing that these stationary phases were not sufficiently robust to separate a wide range of these analytes. Thus the most robust approach for the analysis of aminoglycocides is through the use of ion pair LC-MS/MS.

Antviral drugs have potential to be illegally used in poultry to control avian influenza. A number of methods have been developed for the determination of antiviral residues but most are highly specific methods or methods that can analysis six residues in one test. The goal of this work was to develop a method that can analyse >10 influenza drugs using a simple rapid sample preparation method prior to detection by LC-MS/MS.

4. METHOD

Aminoglycosides Method

Researchers at the Beijing-CDC provided a method for the analysis of 15 Aminoglycoside Residues in Porcine Tissues based on LC-MS/MS. In this procedure, aminoglycosides are extracted using 5 % aqueous trichloroacetic acid, followed by ion-paired extraction, defatted *n*-hexane, and purified using two consecutive HLB clean-up SPE steps. Following extraction sample extracts are filtered and separated on a C_{18} column prior to detection by ion pair liquid chromatography coupled to a tandem mass spectrometry detection. The LOQ of the method is typically

less than 20 µg/kg (ppb) for all analytes (Table 1). In routine operation the method measures residues to 0.25 the MRLs.

A standard operation procedure (SOP) for the above method has method has been provided in English along with an open access copy of the paper, where this work was originally published. The SOP is currently being reviewed by Teagasc staff and is being adapted into the ISO17025 format that is being ised in the Teagasc laboratories.

Table 1 LODs and LOQs of aminoglycoside antibiotics in different matrices

Matrices	muscle		liver		kidney	
Analytes	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Apramycin	4.5	15	3.0	10	3.0	10
Amikacin	4.5	15	6.0	20	4.5	15
Spectinomycin	6.0	20	9.0	30	9.0	30
Neomycin	7.5	25	4.5	15	6.0	20
Tobramycin	3.0	10	1.5	5	1.5	5
Gentamicin C1a	1.5	5	1.5	5	3.0	10
Gentamicin C2	0.9	3	0.9	3	1.5	5
Gentamicin C1	1.5	5	1.5	5	1.5	5
Kanamycin	3.0	10	3.0	10	3.0	10
Hygromycin	7.5	25	9.0	30	9.0	30
Dihydrosteptomycin	3.0	10	3.0	10	3.0	10
Paromomycin	3.0	10	3.0	10	3.0	10
Steptomycin	6.0	20	6.0	20	4.5	15
Netilmicin	1.5	5	1.5	5	3.0	10
Sisomycin	3.0	10	3.0	10	3.0	10

Antiviral drugs Method

Teagasc received a method for the analysis of five antiviral drugs from Beijing CDC. Teagasc have evaluated method and it basically did not include a wide enough range of analytes. Consequently, extensive method development work was carried out to establish a protocol that would analyse a wider range of analytes in one protocol. During method development work several analytical columns were evaluated for the separation of these analytes and best results were obtained on a Waters amide column. In parallel, a simple sample preparation procedure was developed for the isolation of residues from meat samples using a combination of protein precipitation, cold temperature treatment and ultrafiltration. The developed method allows sensitive measurement of these analytes in low to sub-ppb level (refer Table 2). The trueness and accuracy of the method is in the accepatable range of 80-120%, with precision typically less than 10% (RSD values). Prelimknary validation work has been carried out on the method including matrix effects studies and within repeatability validation. Additional isotopically labelled standards have been purchased to improve accuracy and precision where available. Validation work on this method is ongoing.

Table 2 Overview of analytical method for the analysis of antiviral drugs in poultry meat.

Analyte	Calibration Range (µg/kg)
Arbidol	0.1 - 2
Arbidol sulphoxide	0.1 - 2
Arbidol sulphone	0.1 - 2
Oseltamivir	0.1 - 2
Rimantadine	0.1 - 2
Acylclovir	0.5 - 10
Amantadine	0.5 - 10
Ganciclovir	1 - 20
Zanamivir	1 - 20
Viramidine	1 - 20
Oseltamivir acid	1 - 20
Peramivir	1 - 20
Laninamivir	2 - 40
Ribavirin	5 - 100
Favipiravir	5 - 100

5. CONCLUDING REMARKS

A method for the analysis of 15 aminoglycoside residues has been supplied to Teagasc by Beijing CDC. This SOP is being adapted into the ISO17025 format used in the Teagasc laboratories. A draft of the SOP is appended to this report.

A new method was developed for the measurement of 14 antiviral drugs in poultry muscle and method validation is ongoing.

6. ACKNOLWEDGEMENTS

We wish to acknowledge the contribution of all project partners who contributed to the completion of this deliverable.

7. REFERENCES

Zhu, Z., Liu, G., Wang, F., Sasanya, J. J., Cannavan, A. Development of a Liquid Chromatography Tandem Mass Spectrometric Method for Simultaneous Determination of 15 Aminoglycoside Residues in Porcine Tissues. Food Anal. Methods (2016) 9: 2587-2599.

Simultaneous Determination of 15 Aminoglycoside Residues in Porcine Tissues

LC-MS/MS Method

1. Identification

Determination of aminoglycoside residues in porcine tissues (muscle, liver and kidney) using LC-MS/MS method

2. Scope

Method is suitable for the confirmatory analysis of aminoglycoside residues in porcine tissues (muscle, liver and kidney) according to Decision 2002/657/EC.

3. Description of items to be tested

As outlined in Scope.

4. Apparatus and Equipments

4.1 Foss Tecator 2094 homogeniser (Höganäs, Sweden).

4.2 Vacuum pump (Visiprep™ SPE vacuum manifold DL, 24-port model, Supelco, USA) or equivalent.

4.3 High-speed blender (IKA® T25 digital Ultra-Turrax® with IKA® works S25N-25F dispersing, IKA, Germany) or equivalent.

4.4 Platform shaker (IKA KS 260 control, IKA, Germany) or equivalent.

4.5 Centrifuge (Beckman Coulter Allegra™ X-22R, USA) or equivalent.

4.6 Analysis is carried out on an Agilent 1100 Series HPLC system (Agilent Technologies, USA) coupled to an API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada).

5. Description of Procedure

5.1 Principle

The analytes are extracted by a volume of 10 mL of 5 % TCA (W/V) solution, centrifuged, defatted by a volume of 5 mL of n-hexane, and cleaned up by two consecutive SPE procedure. The extracts are filtered and transferred to an autosampler vial. Aminoglycoside residues are determined on an Atlantis® dC18 column (150 mm × 2.1 mm, particle size 5 μm) using liquid chromatography coupled to a triple quadrupole mass spectrometer.

5.2 Reagents and Materials

Unless otherwise specified, all reagents were of analytical reagent quality or better.

5.2.1 HPLC grade acetonitrile, methanol, glacial acetic acid and N-hexane, Merck (Germany).

5.2.2 Heptafluorobutyric acid (HFBA, >99.5 %), Sigma-Aldrich (USA)

5.2.3 Ultra-pure Water (Millipore 18.2 MΩcm).

5.2.4 Analytical grade trichloroacetic acid (TCA), sodium hydroxide (NaOH) and hydrochloric acid (HCl), Guangzhou Chemical Company (Guangzhou, China).

5.2.5 Oasis HLB cartridges (3 mL/60 mg), Waters (Milford, MA, USA).

5.2.6 Filter membranes (0.45 μm), Jinteng Laboratory Facilities Co. Ltd. (Tianjin, China).

5.2.7 50-mL polypropylene centrifuge tubes.

5.2.8 Apramycin (APRA, ≥98.5%), amikacin (AMIK, ≥99.0%), spectinomycin (SPEC, ≥96.0%), kanamycin A (KANA, ≥94.5%), neomycin (NEO, ≥90.0%), paromomycin (PARO, ≥90.0%), streptomycin (STREP, ≥98.0%), dihydrostreptomycin (DISTREP, ≥99.0%), tobramycin (TOBRA, ≥93.0%) and gentamicin (≥96.5%, consisting of GENT C1 (29.1%), GENT C1a (21.3%) and GENT

C2/2a/2b (49.6%)) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany), while hygromycin B (HYGRO, ≥60.0%) aqueous solution (54 mg/mL), netilmicin (NETIL, ≥93.0%) and sisomicin (SISO, ≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), European Pharmacopoeia (Strasbourg, France) and Toronto Research Chemicals Inc. (Toronto, Canada), respectively.

5.2.9 Mobile Phase A: acetonitrile containing 20 mmol/L HFBA.

5.2.10 Mobile Phase C: acetonitrile/water (5:95, V/V) containing 20 mmol/L HFBA.

5.2.11 Mobile Phase D: acetonitrile/water (50:50, V/V) containing 20 mmol/L HFBA.

5.3 Preparation of Standard Solutions

The analytical standard solutions were prepared in a dilution solution comprising acetonitrile/water/acetic acid (20:78:2, V/V/V). Individual stock standard solutions of all 15 analytes (1000 μg/mL) were prepared by accurately weighing 50 mg of reference substance (calculated as dry free base, except HYGRO, for which 926 μL of the aqueous standard solution was transferred into a 50-mL volumetric flask), dissolved with the dilution solution and made up to volume with the solution. These stock standard solutions were stable for 1 year when stored in plastic tubes at 2–4 °C. Since the solid standard of gentamicin consists of GENT C1, C1a, C2, C2a and C2b, with proportion 29.1 % (C1), 21.3 % (C1a) and 49.6 % (sums of C2, C2a and C2b), so there are three stock standard solutions of gentamicin, corresponding to 1000 $\mu g\ mL^{-1}$ for GENT C1, C1a and C2 (sums of C2, C2a and C2b), respectively. Tuning solutions of each analyte (10 μg/mL) were prepared by dilution of the stock standard solutions. Working mixed standard solutions for fortification/validation experiments were prepared by diluting individual stock standard solutions to the appropriate concentrations. The working mixed standard solutions were stored in plastic tubes at 2–4 °C and remained stable for 1 month.

5.4 Sample

For each sample, around 500 g tissue was first minced using a Foss Tecator 2094 homogeniser and then packaged into a plastic container, capped and stored at −20 °C before analysis. Samples were returned to

cold storage immediately after sub-sampling. Special care was taken while sample handling to prevent accidental contamination or loss of target analytes.

5.5 Procedure

5.5.1 Extraction

Five-gramme aliquots of the homogenised tissue samples were weighed into 50-mL polypropylene centrifuge tubes. For validation purposes, samples were spiked with the working mixed standard solutions at appropriate concentrations and let stand for 1 h. A volume of 10 mL of 5 % TCA (W/V) was added to each centrifuge tube. The mixture was homogenised thoroughly at 10,000 rpm for 1 min using a high-speed blender (IKA® T25 digital Ultra-Turrax® with IKA® works S25N-25F dispersing, IKA, Germany) and then centrifuged at 5 °C, 8000 rpm (6953×G) for 5 min (Beckman Coulter Allegra™ X-22R, USA). The extraction procedure was repeated with 10 mL of 5 % TCA, and the TCA supernatants were combined into another centrifuge tube. A 5 mL volume of 0.2 mol L^{-1} HFBA and 5 mL N-hexane were added to the extracts. After vibration mixing using a platform shaker (IKA KS 260 control, IKA, Germany) at 360 rpm for 30 min and additional centrifuging at 5 °C, 6953×G for 5 min, the upper N-hexane phase was removed and then the residual aqueous extracts were cleaned up as described below.

5.5.2 SPE Clean-up and Concentration

A HLB cartridge was pre-conditioned with 3 mL methanol, 3 mL water and 3 mL of 0.2 mol L^{-1} HFBA by gravity. A 5 mL volume of the extract was transferred onto the cartridge at a flow rate of 1 mL min^{-1}. The total effluent was collected into another tube and adjusted to pH 8.5 ± 0.2 with 100 g/L NaOH (about nine drops) and 0.2 mol/L HCl. Afterwards, the cartridge was dried by a vacuum pump (Visiprep™ SPE vacuum manifold DL, 24-port model, Supelco, USA) for 5 min. Another HLB cartridge was pre-conditioned with 3 mL methanol, 3 mL water, 3 mL of 0.2 mol L^{-1} HFBA and 3 mL of pH 8.5 aqueous NaOH by gravity. Then, the pH 8.5 ± 0.2 effluent was loaded onto the column at 1 mL min^{-1}. After the sample had been passed through, the two HLB cartridges were joined with vacuum joints. The two tandem cartridges were rinsed with 5 mL water and then dried at less than 15 mmHg for 10 min. AG residues were finally eluted with 6 mL acetonitrile/0.15 mol/L HFBA (4:1, V/V), and the eluate was

evaporated to 0.3 mL under a gentle steam of nitrogen at 40 °C. Finally, the residue was reconstituted to 1 mL with 20 mmol L^{-1} HFBA. The resulting solution (filtered through a 0.45-μm membrane first if turbid) was transferred into an LC autosampler vial for LC-ESI-MS/MS analysis.

5.6 Determination

5.6.1 LC conditions

Liquid chromatography was performed using an Agilent 1100 Series HPLC system equipped with an automatic degasser, a quaternary pump and an autosampler. Chromatographic separation was carried out using an Atlantis® dC18 column (150 mm × 2.1 mm, particle size 5 μm) at 30 °C. The flow rate of mobile phase was maintained at 0.4 mL min^{-1}, and the injection volume was 30 μL. Mobile phase A was acetonitrile containing 20 mmol L^{-1} HFBA, mobile phase C was acetonitrile/water (5:95, V/V) containing 20 mmol L^{-1} HFBA and mobile phase D was acetonitrile/water (50:50, V/V) containing 20 mmol L^{-1} HFBA. The gradient elution program is summarised in Table 1.

Table 1 HPLC gradient elution program for the separation of monitored aminoglycosides

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

5.6.2 MS Conditions

The HPLC system was connected to an API 3000 triple quadrupole tandem mass spectrometer, equipped with a turbo ion spray source and a syringe pump. The electrospray ionisation mode (positive) was adopted. Optimisation of the ionisation parameters for each analyte was achieved by infusing each compound separately at a flow rate of 10 μL min^{-1}. For sample analysis, the instrument was operated in

multiple reaction monitoring (MRM) mode and two transitions were monitored for each compound. The higher intensity transition was selected for quantitation, and the resolution was set at 0.7 U. The analyst 1.4.1 software was used for instrument control and data acquisition. Mass parameters for each analyte including precursor ion (Q1), product ion (Q3), declustering potential (DP), entrance potential (EP), cell exit potential (CXP) and collision energy (CE) are summarised in Table 2. The focusing potential (FP) and dwell time (DT) for all analytes were 350 V and 40 ms, respectively. The MS ion source parameters including nebuliser gas (NEB), curtain gas (CUR), collision gas (collision-activated dissociation (CAD)), ion spray voltage (IS) and ion source temperature (TEM) were 12 psi, 8 psi, 6 L min^{-1}, 3500 V and 500 °C, respectively.

Table 2 Optimised MRM parameters for analytes using LC-MS/MS in ESI^+ mode

Analytes	MW	Q1(*m/z*)	Q3(*m/z*)	DP(V)	EP(V)	CXP(V)	CE(V)
APRA	539.6	540.4	*378.3	105	4.2	23	25
			217.2			13	40
AMIK	585.6	586.3	*425.2	90	4.2	27	29
			264.1			17	38
SPEC	332.3	351.3	*333.2	60	10.0	23	26
			98.2			6	44
NEO	614.6	615.4	*161.2	155	4.3	10	44
			293.0			17	36
TOBRA	467.5	468.3	*163.2	65	10.0	10	36
			324.1			19	23
GENT C1a	449.5	450.3	*160.1	85	5.0	9	34
			322.1			20	20
GENT C2	463.6	464.3	*322.1	85	5.0	20	20
			160.1			9	34
GENT C1	477.6	478.3	*157.2	100	4.5	10	30
			322.2			20	21
KANA	484.5	485.3	*163.2	80	4.3	9	39
			324.1			19	25
HYGRO	527.5	528.2	*177.2	95	10.0	10	44
			352.2			20	35
DISTREP	583.6	584.2	*263.1	145	9.5	14	46
			246.2			14	56
PARO	615.6	616.3	*163.2	135	9.0	11	52
			293.0			17	35

STREP	581.6	600.3	*582.2	125	4.5	34	26
			263.1			16	52
NETIL	475.6	476.4	*299.5	65	7.2	21	31
			191.4			11	36
SISO	447.5	448.5	*322.4	50	7.0	20	20
			271.5			19	27

Note: * Selected as quantitative ion.

5.7 Confirmation of analytes

5.7.1 An analyte peak must have a S:N of ≥3 before it is deemed a valid peak.

5.7.2 Compared with the retention time of the corresponding standard chromatographic peak, the retention time of the target compound in the sample should be within ± 2.5%.

5.7.3 The mass spectrum qualitative ion of each compound shall appear, including at least one parent ion and two fragment ions. For the same compound, the relative abundance ratio of two fragment ions of the target compound in the sample shall not exceed the range specified in Table 3 when compared with the standard solution with the same concentration.

Table 3 Maximum allowable deviation of relative ion abundance in qualitative analysis

Relative ion abundance	>50%	20%~50%	10%~20%	≤10%
Allowable relative deviation	±20%	±25%	±30%	±50%

5.8 Calibration Curves

Calibration was performed using matrix-matched standards prepared by adding the appropriate amounts of the aminoglycoside-mixed standard solutions to each blank matrix at six concentration levels, corresponding to 0, 0.25, 0.5, 1.0, 2.5 and 5.0 times MRL or suggested level (for compounds HYGRO, AMIK, TOBRA, NETIL and SISO, of which there was no MRL in porcine tissue matrices) for muscle; 0, 0.2, 0.4, 0.8, 2.0 and 4.0 times MRL or suggested level for liver; and 0, 0.2, 0.5, 1.0, 2.5 and 5.0 times MRL or suggested level for kidney (Tables 4-6). Those samples fortified with AGs were operated with entire extraction and purification procedure and finally injected to the LC-ESI-MS/MS system. The calibration curves were constructed using linear regression of the peak areas from the six concentration

levels versus the concentration of analytes. Thus, there were always 15 different matrix-matched calibration curves for each test sample (one calibration curve per analyte).

Table 4 Preparation of standard stock solution for muscle

Analytes	MRL/suggested level (μg/kg)	Concentration（mg/L）	Volume added (mL)	Concentration in mixed solution for each analyte (mg/L)
STREP	500	100	6.25	12.5
DISTREP	500	100	6.25	12.5
NEO	500	100	6.25	12.5
PARO	500	100	6.25	12.5
KANA	40	100	0.50	1
AMIK	/ (100)	100	1.25	2.5
TOBRA	/ (50)	100	0.625	1.25
SPEC	100	100	1.25	2.5
APRA	60	100	0.75	1.5
GENT C1	50			1.16
GENT C2	50	80 (total GENT)	2.50	1.98
GENT C1a	50			0.86
HYGRO	/ (500)	100	6.25	12.5
NETIL	/ (50)	100	0.625	1.25
SISO	/ (50)	100	0.625	1.25
Diluting to 50 ml with acetonitrile / water / acetic acid (20:78:2, V/V/V) solution				

Table 5 Preparation of standard stock solution for liver

Analytes	MRL/ suggested level (μg/kg)	Concentration（mg/L）	Volume added (mL)	Concentration in mixed solution for each analyte (mg/L)
STREP	500	100	5	10
DISTREP	500	100	5	10
NEO	500	100	5	10
PARO	1500	100	15	30
KANA	40	100	0.4	0.8
AMIK	/ (100)	100	1	2
TOBRA	/ (50)	100	0.5	1
SPEC	100	100	1	2
APRA	100	100	0.6	1.2
GENT C1	100	80 (total GENT)	4	1.86

GENT C2	100			3.17
GENT C1a	100			1.37
HYGRO	/ (500)	100	5	10
NETIL	/ (50)	100	0.5	1
SISO	/ (50)	100	0.5	1
Diluting to 50 ml with acetonitrile / water / acetic acid (20:78:2, V/V/V) solution				

Table 6 Preparation of standard stock solution for kidney

Analytes	MRL/ suggested level (μg/kg)	Concentration（mg/L）	Volume added (mL)	Concentration in mixed solution for each analyte (mg/L)
STREP	1000	100	2.5	5
DISTREP	1000	100	2.5	5
NEO	5000	100	12.5	25
PARO	1500	100	3.75	7.5
KANA	40	100	0.1	0.2
AMIK	/ (100)	100	0.25	0.5
TOBRA	/ (50)	100	0.125	0.25
SPEC	500	100	1.25	2.5
APRA	100	100	0.25	0.5
GENT C1	200			0.93
GENT C2	200	80 (total GENT)	2	1.59
GENT C1a	200			0.68
HYGRO	/ (500)	100	1.25	2.5
NETIL	/ (50)	100	0.125	0.25
SISO	/ (50)	100	0.125	0.25
Diluting to 50 ml with acetonitrile / water / acetic acid (20:78:2, V/V/V) solution				

5.9 Determination of sample solution

Inject 30 μL of sample solution into LC-MS/MS to determine aminoglycoside residues. Calculate the concentration of target compound in the solution according to the corresponding matrix matching standard curve.

5.10 Expression of Results

Calculate the amount of analyte present as outlined in Equation I.

$$X = \frac{c \times V}{m} \times f$$ Equation I

Note : b = intercept and a = slope of the calibration curve.

X = Amount of aminoglycoside antibiotics to be tested in the sample, expressed as μg/kg;

c= Concentration of aminoglycoside antibiotics in the sample solution to be tested obtained from the standard curve, expressed as μg/kg;

V - The final volume of the sample solution to be determined, expressed in ml;

m - The mass of the sample, expressed as g;

f- Dilution factors, the value of 5 was obtained in this procedure.

Where two results are obtained by replicated measurements, the mean results should be reported. The data is expressed by the arithmetic mean of two independent determination results obtained under repeated conditions.

5.11 Method recovery and precision

Porcine muscle, liver and kidney known to be compliant served as blank matrices. Recoveries and precision (intra-day, inter-day) were calculated from the determination of seven aliquots of each sample fortified at three levels (0.5, 1.0 and 1.5 times MRL or 0.5, 1.0 and 1.5 times suggested level for no MRL substances). Seven blank samples for each matrix were included in each series, taken through the entire extraction and purification procedure. After being processed, five of these blank samples were used to prepare matrix-matched recovery standards for recovery calculation. Aminoglycoside-mixed standard solutions were added to the dried extracts to obtain concentrations corresponding to 0.25, 0.5, 1.0, 1.5 and 2.0 times MRL/suggested level for muscle, liver and kidney. The analyses were performed by the same operator in triplicate within a 1-month period. The recoveries were calculated by the measured content/the fortified level×100, and the precision was expressed as the relative standard deviation (RSD). The results shows that the recoveries ranged from 47% to 93 % with overall precisions of 2.9–15.4 %.

5.12 LOD and LOQ

The LODs and LOQs of aminoglycoside antibiotics in this method are shown in Table 7.

Table 7 LODs and LOQs of aminoglycoside antibiotics in different matrices

Matrices	muscle		liver		kidney	
Analytes	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
APRA	4.5	15	3.0	10	3.0	10
AMIK	4.5	15	6.0	20	4.5	15
SPEC	6.0	20	9.0	30	9.0	30
NEO	7.5	25	4.5	15	6.0	20
TOBRA	3.0	10	1.5	5	1.5	5
GENT C1a	1.5	5	1.5	5	3.0	10
GENT C2	0.9	3	0.9	3	1.5	5
GENT C1	1.5	5	1.5	5	1.5	5
KANA	3.0	10	3.0	10	3.0	10
HYGRO	7.5	25	9.0	30	9.0	30
DISTREP	3.0	10	3.0	10	3.0	10
PARO	3.0	10	3.0	10	3.0	10
STREP	6.0	20	6.0	20	4.5	15
NETIL	1.5	5	1.5	5	3.0	10
SISO	3.0	10	3.0	10	3.0	10

6. Notes on Procedure

Storage of all aminoglycoside standards:

The optimum conditions for storage of all aminoglycoside standards were as follows: the compounds (1000 μg mL^{-1}) should be dissolved with acetonitrile/water/acetic acid (20:78:2, V/V/V) and stored in plastic tubes at 4 °C. The stock solutions and lower concentration solutions (100 ng mL^{-1}) are stable in plastic tubes at 4 °C for at least 1 year and for 1 week, respectively.

7. References

Zhu, Z., Liu, G., Wang, F., Sasanya, J. J., Cannavan, A. Development of a Liquid Chromatography Tandem Mass Spectrometric Method for Simultaneous Determination of 15 Aminoglycoside Residues in Porcine Tissues. Food Anal. Methods (2016) 9: 2587-2599.