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The confirmation and quantification of selected aminoglycoside residues in animal tissue and bovine milk by liquid chromatography tandem mass spectrometry

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Abstract: Aminoglycosides are a large class of antibiotics that are characterised by two or more amino sugars linked by glycosidic bonds to an aminocyclitol component. They are water soluble, highly polar thermally labile compounds with no chromophores or fluorophores and so difficult to assay using standard HPLC, GC or GC-MS instrumentation. This paper reports a robust method to confirm and quantify the levels of dihydrostreptomycin, streptomycin, apramycin, neomycin and gentamicin (C_1, C_2 and C_{1a}) present in animal tissue and dihydrostreptomycin, streptomycin, neomycin and gentamicin (C_1, C_2 and C_{1a}) present in bovine milk using liquid chromatography-tandem mass spectrometry. The aminoglycosides were extracted from the samples with perchloric acid, EDTA, phosphate buffer solution, followed by centrifugation and cleanup using weak cation exchange solid phase chromatography. The compounds were separated with a 5 µm C18 HPLC column and a mobile phase consisting of a mixture of acetonitrile, water and 50 mM heptafluorobutyric acid. Matrix matched standards were used to achieve the best accuracy of the method. The Limits of Quantification (LOQ) (0.1-0.5 mg/kg for animal tissue and 0.01-0.1 mg/kg for milk) were based on the requirements of the Australian antibiotic residue monitoring programs. The method was used to measure the levels of aminoglycoside residues in samples submitted by the Australian Regulatory Authorities.

Keywords: Aminoglycoside residues, liquid chromatography-tandem mass spectrometry, quantification, confirmation

Introduction

In Australia, the monitoring of antimicrobial residues in bovine milk and animal tissue is delivered through a number of programs, including the Australian Milk Residue Analysis (AMRA) Survey, the National Residue Survey (NRS), the Targeted Antibacterial Residue Testing program (TART), the Sheep Targeted Antibacterial Residue Testing program (START) and the National Antibacterial Residue Minimisation (NARM) program (DAFF 2009). These programs provide a national, credible, independent monitoring system for agricultural and veterinary chemical residues, with the key aim being to reduce the levels of chemical residues in agricultural produce. Monitoring can also identify potential problems including failure to use chemicals appropriately, and can indicate where follow-up action by regulators is required. The AMRA and NRS Surveys are risk based programs designed to identify and manage potential chemical inputs into Australian Agriculture production that may affect food safety for the domestic market as well as access to international markets. In doing so, the Surveys underpin the export certification requirements of the Australian Quarantine and Inspection Service (AQIS). This includes reference to meeting the requirements of importing countries.

Laboratories contracted to undertake analytical work for the programs must employ fully validated in house methods or verified standard methods for each test. The validation/verification is carried out according to the procedures described in National Association of Testing Authorities (NATA) technical note 17 Guidelines for the validation and verification of Chemical Test Methods (NATA 2006). The analytical methods must also comply with EC Decision 202/657/EC section 2.3. The relative performance and competence to undertake specific chemical analyses are evaluated through numerous proficiency testing programs initiated by the NRS. Trading partners also audit the operation and results of the residue monitoring plans. In the Australian domestic meat market, participation in residue monitoring programs is a general requirement of the Australian Standard for hygienic production of meat for human consumption.

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The aminoglycosides (apramycin, streptomycin, dihydrostreptomycin, neomycin and gentamicin C_1, C_2 and C_{1a}) are one of the many classes of analytes tested for in Australia under the general heading of "Antimicrobials". Other classes include β-lactams (e.g. benzyl G penicillin), cephalosporins (e.g. ceftiofur), tetracyclines (e.g. oxytetracycline), sulphonamides (e.g. sulfadiazine) and macrolides (e.g. erythromycin) as well as dairy/milk specific antimicrobials (e.g. benzimidazoles and chloramphenicol). Monitoring of antibiotic residues is generally a two-stage process. Firstly, a general antibiotic screen is performed on milk or animal tissue to identify the class of compound present (e.g. β-lactam, aminoglycoside, macrolide, tetracycline). When the presence of a class of compounds is identified, confirmation and quantification is carried out by the specific instrumental method appropriate for the class of compound. The LOQ for each residue is based on the requirements of the Australian antibiotic residue monitoring programs. For aminoglycoside residues, the LOQ are 0.1 mg/kg for tissue, except for apramycin (0.5 mg/kg) and 0.1 mg/kg for bovine milk, except for gentamicin (0.01 mg/kg).

Aminoglycosides are a large class of antibiotics that are characterised by two or more amino sugars linked by glycosidic bonds to an aminocyclitol component. Many aminoglycosides are composed of a mixture of closely related compounds, e.g. the gentamicin C complex is made up of five compounds with three molecular masses as C_1 (molecular mass 477.59), C_{1a} (molecular mass 449.54) and C_2, C_{2a} and C_{2b} (molecular mass 463.57) (Seidl and Nerad, 1988; Stolker and Brinkman, 2005). Aminoglycosides are water soluble, highly polar compounds and may be efficiently extracted from biological matrices under acidic conditions (McGlinchey *et al*., 2008).

Aminoglycosides contain no chromophores or fluorophores and are thermally labile, making analysis by standard HPLC, GC or GC-MS virtually impossible without derivatisation (Kennedy *et al*., 1998; Preu *et al*., 1998; Isoherranen and Soback 2000). Liquid chromatography tandem mass spectrometry (LC-MS/MS) is currently the instrument of choice to quantify the levels of aminoglycoside residues in biological matrices. 'State of the art' LC-MS/MS instruments are extremely sensitive and robust and the use of LC-MS/MS as the determinative step enables simpler sample clean up procedures to be adopted, reducing not only sample analysis time, but more importantly, method development time. Numerous studies have been reported for the screening and determination of selected aminoglycosides in animal tissue (Babin and Fortier, 2007; Heller *et al*., 2005; Ishii *et al*., 2008) and milk (Bogialli *et al*., 2005; Gaugain-Juhel *et al.*, 2009; Heller *et al.*, 2000; Turnipseed *et al.*, 2009; Van Bruijnsvoort *et al.*, 2004), using a variety of extraction, cleanup and LC-MS conditions. Cherlet reported the determination of streptomycin in both animal tissue and milk using the same extraction and LC-MS/MS conditions (Cherlet *et al*., 2007), and Zhu reported the determination of a number of aminoglycosides in a variety of matrices using a single extraction and LC-MS/MS procedure (Zhu *et al*., 2008).

This paper describes the development of a robust method for confirming and quantifying the levels of the aminoglycosides dihydrostreptomycin, streptomycin, apramycin, neomycin and gentamicin (C_1, C_2 and C_{1a}) in animal tissue and dihydrostreptomycin, streptomycin, neomycin and gentamicin (C_1, C_2 and C_{1a}) in bovine milk using a perchloric acid/ EDTA/ KH_2PO_4 extraction followed by weak cation exchange cleanup and LC-MS/MS quantification. The method complies with NATA technical note No 17 and the requirements of EC Decision 202/657/ EC section 2.3. Data are presented for the analysis of aminoglycoside residues in seventy two samples of animal tissue submitted by the Australian Regulatory Authorities from December 2006 to June 2009.

Materials and Methods

Chemicals and reagents

Dihydrostreptomycin, streptomycin, apramycin, neomycin, gentamicin (mixture of C_1, C_2 and C_{1a}), hygromycin B, tobramycin and heptafluorobutyric acid (HFBA) were supplied by Sigma Chemical Company, Sydney, Australia. HPLC grade acetonitrile and methanol, and AR grade disodium EDTA, potassium dihydrogen orthophosphate, sodium hydroxide, citric acid monohydrate, trisodium citrate dihydrate and hydrochloric acid were supplied by Lomb Scientific, Melbourne, Australia. Oasis WCX (60 mg) cartridges were supplied by Waters, Melbourne, Australia. All water used was deionised water produced from a Milli Q system, (Millipore Australia, Melbourne, Australia).

Standard preparation

Stock standard solutions (100 µg/ml): 0.01 g of each aminoglycoside were dissolved in 100 ml of water. The solutions were stored at 4°C and were stable for at least 1 month.

Internal standard mix (10 µg ml): 1 ml of 100 µg/ml tobramycin and hygromycin B stock standard solutions were mixed and diluted to 10 ml with deionised water. The solution was stored at 4°C and was stable for at least 6 months.

Mixed spiking standard solution for tissue (2.5 µg/ml; 12.5 µg/ml for apramycin): 0.25 ml of each stock standard solution (1.25 ml for apramycin) were mixed and diluted to 10 ml with water.

Mixed spiking standard solution for milk (12.5 µg/ml; 1.25 µg/ml for gentamicin): 1.25 ml of each stock standard solution (0.125 ml for gentamicin) were mixed and diluted to 10 ml with water.

Matrix mixed standard solution series were prepared by adding various amounts of mixed spiking standard solutions to a blank tissue or milk extract (analysed previously and shown not to contain aminoglycoside residues above the LOQ). For tissue, the standards ranged from 0.017 µg/ml to 0.33 µg/ml, except for apramycin, which was 5 times greater than these values. For milk, the standards ranged from 0.083 µg/ml to 1.7 µg/ml, except for gentamicin, which was one tenth of these values. Each solution contained the internal standards at concentrations of 0.17 µg/ml.

Sample preparation

Samples of frozen animal (lamb, calf and beef) tissue and frozen bovine milk were received from the regulatory authorities and stored frozen until analysis. For kidney samples, only the outer tissue (cortex) without connective tissue was used for analysis. For small samples of animal tissue, (e.g. calf kidney) the samples were thawed prior to chopping with a knife. For larger samples (e.g. beef kidney), the samples were partially thawed and then sub sampled by randomly cutting thin slices of tissue from each quarter with a knife from the outside. The slices were then finely chopped with a knife.

Sample extraction

Animal tissue

2 g of tissue was spiked with 100 µL of internal standard solution, 14 ml of extracting solution (2.0% perchloric acid, 0.4 mM Na_2EDTA, 10 mM KH_2PO_4) was added and the sample homogenised with an ultra turrax blender. The solution was centrifuged for 10 min at 3500 rpm at 15°C and the supernatant solution decanted into another tube. The tissue pellet was then re-extracted with a further 14 ml of extracting solution, centrifuged, and the supernatants combined.

Bovine milk

10 ml of milk was spiked with 100 µl of internal standard solution, 20 ml of extracting solution (2.9% perchloric acid, 0.6 mM Na_2EDTA and 15 mM KH_2PO_4) was added and the sample shaken for 10 min. The solution was centrifuged for 10 min at 3500 rpm at 15°C and the supernatant solution decanted into another tube.

Extraction and clean-up

The extract was adjusted to pH 6.7 ± 0.1 with 50% NaOH solution. If the solution was cloudy it was centrifuged for 10 min at 3500 rpm at 15°C to obtain a clear solution. A 5 ml aliquot of the solution was loaded onto a WCX SPE cartridge (conditioned with 3 ml of methanol followed by 3 ml of water). The cartridge was attached to a SPE manifold that was equipped with taps to restrict the flow. The flow of solution through the column was maintained at 1 drop per 8 seconds by adjusting the tap beneath the cartridge. When all of the extract had passed through the cartridge, the cartridge was washed with 2 ml of 25 mM sodium citrate buffer followed by 2 ml of methanol. Air was drawn through the cartridge for 5 sec and the analytes eluted slowly with 3 ml of 0.1% HCl in methanol. The solvent was removed under a stream of nitrogen at 45°C and the residue reconstituted in 1 ml of 5 mM HFBA and mixed thoroughly.

HPLC-MS/MS conditions

The analyses were performed with a Waters Alliance HT 2795 quaternary solvent delivery system with a cooled autosampler (4^0C) (Waters, Milford, MA, USA) interfaced to a QuattroMicro tandem mass spectrometer operating in the positive ion Electrospray Ionization (ESI) mode (Waters Micromass, Manchester, UK). The compounds were separated on a 2.0 x 150 mm 5 µm Luna C18 (2) column (Phenomenex, Sydney, Australia) fitted with a C18 guard column. The column was contained in a thermostated column oven maintained at 30^0C. The mobile phase gradient is displayed in Table 1.

The mass spectrometer capillary voltage was set at 3.2 kV, the source temperature at 110°C, desolvation temperature at 400°C. The nitrogen gas flow rates were 60 L/h for cone gas and 500 L/h for desolvation gas. Argon was used as a collision gas at 3×10^{-3} mbar. Cone voltage, collision energy and Multiple Reaction Monitoring (MRM) transitions for each of the compounds are listed in Table 2. A dwell time of 0.1 sec was used for each MRM transition. Total Ion Chromatograms (sum of all transitions for each compound) were used for the calculation of results. Masslynx V4.1 software was used to process the data.

Method validation

Recovery experiments and proficiency studies were used to validate the method.

Table 1. Mobile phase composition and gradient conditions for the separation of the aminoglycosides by LC-MS/MS

Time (min)	% CH_3CN	% 50 mM HFBA	% H_2O	Flow rate (ml/min)	Waters gradient curve number
0.00	5.0	4.0	91.0	0.2	1
2.00	5.0	4.0	91.0	0.2	6
26.0	41.0	4.0	55.0	0.2	6
26.5	5.0	4.0	91.0	0.4	6
31.5	5.0	4.0	91.0	0.4	6
32.0	5.0	4.0	91.0	0.2	6

Table 2. LC-MS/MS settings

Compound	Retention window (mins)	MRM transitions (m/z)	Cone voltage (Volts)	Collision energy (Volts)
Dihydrostreptomycin	0.0-18.5	548.35→246.2	46	34
		548.35→263.2	46	30
		548.35→176.3	46	36
Streptomycin	14.5-18.5	582.35→246.1	52	36
		582.35→263.2	52	30
		582.35→176.1	52	36
Hygromycin	14.5-18.5	528.25→177.2	38	32
		528.25→352.2	38	22
		528.25→257.1	38	28
Tobramycin	17.5-21.5	468.3→163.2	24	22
		468.3→324.2	24	16
		468.3→145.1	24	22
Apramycin	17.5-21.5	540.3→217.2	30	32
		540.3→378.3	30	16
		540.3→199.2	30	32
Neomycin	17.5-26.5	615.4→161.2	36	30
		615.4→163.2	36	30
		615.4→293.3	36	24
Gentamicin C_1	18.0-24.0	478.4→157.2	24	24
		478.4→322.2	24	14
		478.4→160.1	24	24
Gentamicin C_2	18.0-24.0	464.4→160.2	20	26
		464.4→322.2	20	14
		464.4→163.1	20	24
Gentamicin C_{1a}	18.0-24.0	450.4→160.2	20	26
		450.4→322.2	20	14
		450.4→163.1	20	24

Recovery data

For the recovery experiments, tissue samples were spiked with aminoglycosides at LOQ, 2 × LOQ, 5 × LOQ, 7.5 × LOQ and 10 × LOQ. The analyses were batched (10) and carried out independently by 2 analysts over an 8 week period. Milk samples were spiked with aminoglycosides at LOQ, 2 × LOQ, 5 × LOQ and were assayed independently by 2 analysts in 3 batches over a 32 week period.

Measurement Uncertainty

In house validation data were used to estimate the measurement uncertainty (MU) for each analyte in both matrices. The major sources of uncertainty were homogeneity, method recovery and the LC-MS/MS analytical standard calibration curves. Homogeneity uncertainty was estimated from the relative standard deviation (RSD) of duplicate analyses of incurred tissue samples. Method recovery uncertainty was estimated from the RSD of all spike recoveries from ten batches carried out by 2 different analysts for concentrations from LOQ to 20 × LOQ for tissue and the RSD of all spike recoveries from three batches carried out by 2 different analysts for concentrations from LOQ to 5 × LOQ for milk. The uncertainty of the analytical standard calibration curves were estimated from the RSD of the calibration curve at the concentrations measured.

The individual uncertainties were combined using the following equation:

$$u_c = 100 \times \sqrt{\left(\frac{u_{hom}}{1}\right)^2 + \left(\frac{u_{rec}}{rec}\right)^2 + \left(\frac{u_{cal}}{cal}\right)^2}$$

Where: u_c = combined uncertainty (%)

u_{hom} = homogeneity uncertainty (normalised)

u_{rec} = method recovery uncertainty

u_{cal} = calibration curve uncertainty at given sample extract concentration

rec = mean recovery (%)

cal = concentration of sample extract (μg ml^{-1})

Results and Discussion

Sample extraction and HPLC-MS/MS optimisation

This method was initially developed for the analysis of tissue samples and was based on aspects of previously published procedures (Cherlet *et al*., 2000; Heller *et al*., 2000; Hornish and Wiest, 1998; USDA 2005). A combination of 2% perchloric acid, 10 mM KH_2PO_4 and 0.4 mM EDTA gave the best overall recoveries of the aminoglycosides from spiked tissue. For milk, the concentrations of perchloric acid, KH_2PO_4 and EDTA were increased to compensate for the amount of water in the sample. Optimal recoveries from SPE were achieved when the pH of the extract was adjusted to 6.7 and 0.1% HCl in methanol was used as the SPE eluant. Significantly higher recoveries of neomycin and gentamicin were observed with the polymeric Oasis WCX SPE cartridges compared to silica-based WCX SPE cartridges.

For LC-MS/MS confirmation and quantification, HILIC and reversed phase (C18) HPLC columns were trialled, along with a number of mobile phase organic modifiers. HILIC columns were promising as the high organic content of the mobile phase gave large peak areas when used without modifiers, but they required high concentrations of ammonium acetate to achieve reasonable peak shapes, and this greatly suppressed the response from the mass spectrometer. A suitable reversed phase separation of a standard mixture (Figure 1) was achieved with a 2.0 × 150 mm 5 μm C18 HPLC column using a mobile phase gradient detailed in Table 1 and a flow rate of 0.2 ml/min. This combination gave the best compromise of peak shape, resolution and detector response. The flow rate was increased to 0.4 ml/min after all analytes had eluted from the column to facilitate column equilibration and reduce the overall run time.

Signal enhancement/suppression, commonly called the "matrix effect", can dramatically affect the quality of the results obtained for LC-MS assays. The "matrix effect" can be overcome by using isotopically labelled internal standards, or by preparing standard solutions in the sample matrix that has previously been shown not to contain the analytes of interest (matrix matched standards). Matrix matched

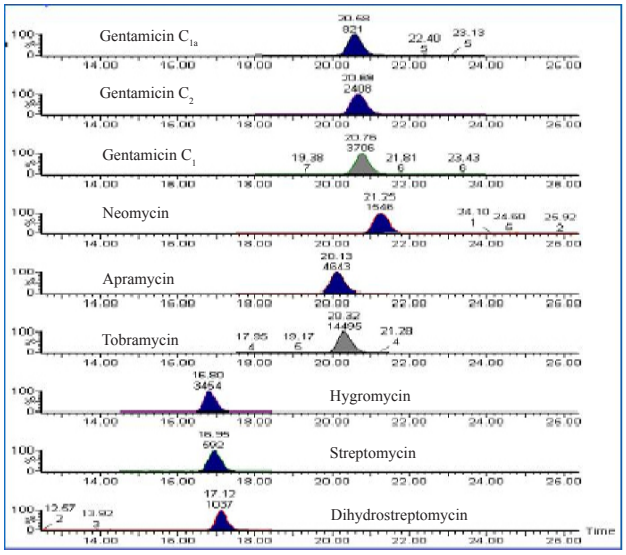

Figure 1. LC-MS/MS chromatogram showing the separation of gentamicin C_{1a}, gentamicin C_2, gentamicin C_1, neomycin, apramycin, tobramycin (IS), hygromycin (IS) streptomycin and dihydrostreptomycin using the conditions described in the Experimental section.

standards were used in this study as isotopically labelled aminoglycosides were not readily available. Two other aminoglycosides not registered for use in Australia were also used as internal standards to correct for variations in the detector response over time (as the ion source becomes dirty), and also for correcting for incomplete recovery of the analytes during the extraction procedure. Hygromycin B was used as the internal standard for the early eluting aminoglycosides (streptomycin and dihydrostreptomycin), and tobramycin was used as the internal standard for the late eluting aminoglycosides (apramycin, neomycin and gentamicin).

The mass spectrometer was optimised by infusing 5 µg/ml solutions of each standard in the HPLC mobile phase and altering conditions to achieve the maximum response. The mass spectrometer response was linear in the concentration range of the matrix matched standards used, which was equivalent to residue levels within the range 0.5 × LOQ - 10 × LOQ for each analyte. This range covered the expected levels of residues in the samples submitted by the regulatory authorities.

The LC-MS/MS chromatogram of a tissue sample containing incurred neomycin is shown in figure 2. The figure also shows the MS/MS response to the three MRM transitions for neomycin (m/z 615.4→293.3, m/z 615.4→163.2, m/z 615.4→161.2), which were used to confirm the identity of the compound.

Method validation

Recovery experiments and proficiency studies were used to validate the method.

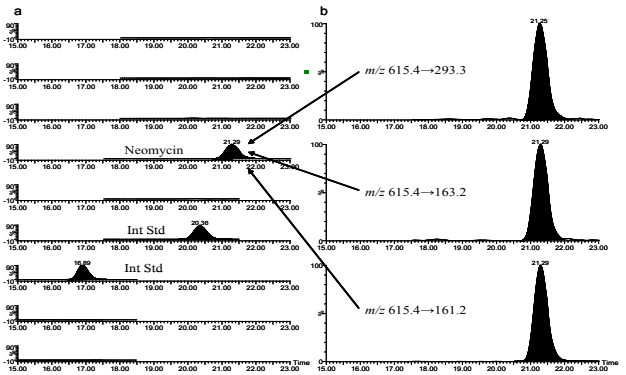

Figure 2. LC-MS/MS chromatogram of a tissue sample found to contain 0.27 mg/kg of neomycin, showing (a) the total ion chromatograms for the sample and (b) the three MRM transitions which make up the total ion chromatogram for neomycin

Recovery data

The spiked recovery data for 51 assays of animal tissue are summarised in Table 3. The mean recoveries of added analytes were respectable (70 - 107%). The standard deviation (SD) for apramycin recoveries was found to be significantly lower than those of the other aminoglycosides, presumably due to the negation of the matrix effects due to the similarities of the retention times between apramycin and the internal standard. The acceptable range for recoveries during routine analysis was defined as the mean ±2 SD.

Table 3. Summary of analyte recoveries at all levels for animal tissue

Analyte	N*	Concentration range (mg/kg)	Mean % Recovery	SD	Acceptable range
Dihydrostreptomycin	51	0.1-2	107	8.9	86-122
Streptomycin	51	0.1-2	70	10.1	51-91
Apramycin	51	0.5-10	96	3.1	90-103
Neomycin	51	0.1-2	86	6.4	73-99
Gentamicin C_1	51		102	8.1	85-118
Gentamicin C_2	51	0.1-2#	102	10.7	81-123
Gentamicin C_{1a}	51		100	11.8	76-123

N* number of samples
gentamicin standard consists of three isomers (C_1, C_2 and C_{1a}). % recoveries were determined with each isomer.

The spiked recovery data for 49 assays of milk are summarised in table 4. The mean recoveries of added analytes were good (91-139%); the mean recoveries of dihydrostreptomycin and gentamicin were quite high (>120%) and reflect the fact that these compounds were more efficiently extracted from the milk matrix than the internal standards. The acceptable range for recoveries during routine analysis was defined as the mean ±2 SD.

Table 4. Summary of analyte recoveries at all levels for bovine milk

Analyte	N*	Concentration range (mg/kg)	Mean % Recovery	SD	Acceptable range
Dihydrostreptomycin	49	0.1-.5	139	17.2	105-173
Streptomycin	49	0.1-.5	113	14.7	83-142
Neomycin	49	0.1-.5	91	10.9	70-113
Gentamicin C_1	49		133	12.6	107-158
Gentamicin C_2	49	0.01-0.05#	130	14.2	101-158
Gentamicin C_{1a}	49		118	15.3	87-148

N* number of samples
gentamicin standard consists of three isomers (C_1, C_2 and C_{1a}). % recoveries were determined with each isomer.

Proficiency study data

The method was further validated using proficiency study data and these data are displayed in table 5. Due to the low number of participants, Z-scores were not calculated however the results were deemed 'satisfactory' by the proficiency study provider. Excellent correlations between spiked and expected levels over a three year period demonstrate the robustness of the procedure. All results are recovery corrected, as required by the residue testing programs.

Table 5. Proficiency study data for selected aminoglycosides added to samples of animal tissue and bovine milk

Proficiency study[#]	Sample[#]	Analyte	Expected result (mg/kg)	Reported result (mg/kg)	Reported/ Expected %
Animal tissue					
A	1	Dihydrostreptomycin	0.16	0.17	106
	2	Apramycin	0.55	0.48	87
		Neomycin	0.14	0.15	107
	3	Gentamicin C_1	0.19	0.11*	58*
		Gentamicin C_2	0.19	0.11*	58*
		Gentamicin C_{1a}	0.19	0.20*	105*
B	1	Streptomycin	0.17	0.18	106
	2	Neomycin	0.14	0.16	114
	3	Apramycin	0.76	0.66	87
C	1	Neomycin	0.14	0.11	79
	2	Streptomycin	0.22	0.21	95
D	1	Dihydrostreptomycin	0.17	0.15	88
	2	Apramycin	0.45	0.37	82
E	1	Streptomycin	0.14	0.11	79
	2	Apramycin	0.35	0.31	89
F	1	Streptomycin	0.17	0.15	88
G	1	Streptomycin	0.18	0.26	144
	2	Neomycin	0.13	0.14	108
H	1	Dihydrostreptomycin	0.22	0.21	95
	2	Apramycin	0.25	0.28	112
I	1	Gentamicin	0.19	0.17	89
Bovine milk					
J	1	Streptomycin	0.18	0.16	89
	2	Gentamicin	0.082	0.089	109
	3	Streptomycin	0.13	0.11	85
K	1	Streptomycin	0.19	0.21	111
	2	Neomycin	0.56	0.46	82

[#] Proficiency studies were conducted by the NRS over a period of time. The proficiency studies are designated A-I for animal tissue and J-K for bovine milk. Each proficiency study had a number of different samples, designated numerically, e.g. proficiency study A comprised 3 different samples.
* Unable to accurately quantify due to differing isomer ratios between the participating laboratory standard mixture and standard mixture used by the proficiency study originator.

Measurement Uncertainty

In house validation data were used to estimate the measurement uncertainty (MU) for each analyte in both matrices. The major sources of uncertainty were homogeneity, method recovery and the LC-MS/MS analytical standard calibration curves. The purity of the analytical standards was shown to be insignificant for most analytes and so was not included. The purity of the gentamicin standard would most likely have some contribution to the uncertainty due to the multiple isomers, however, no certified standard was available at the time the method was being developed.

Homogeneity uncertainty was estimated from the relative standard deviation (RSD) of duplicate analyses of incurred tissue samples. Limited data were available for dihydrostreptomycin (8 duplicates), streptomycin (12 duplicates) and neomycin (24 duplicates) and no data were available for apramycin or gentamicin. A t-test showed there was no significant difference between data sets, so the data were pooled (44 duplicates), normalised and used for all compounds resulting in a RSD of 0.0869. The data were also used to calculate the milk uncertainty as it was the best estimation available.

Method recovery uncertainty was estimated from the RSD of all spike recoveries from ten batches carried out by 2 different analysts for concentrations from LOQ to 20 × LOQ for tissue and the RSD of all spike recoveries from three batches carried out by 2 different analysts for concentrations from LOQ to 5 × LOQ for milk. The uncertainty of the analytical standard calibration curves were estimated from the RSD of the calibration curve at the concentrations measured.

An example of the MU calculation for streptomycin in animal tissue is shown in table 6. Using a coverage factor of 2 for a confidence level of 95%, the expanded measurement uncertainties for each analyte at the LOQ are: dihydrostreptomycin, tissue ± 25%, milk ± 20%; streptomycin, tissue ± 35%, milk ± 20%; apramycin tissue ± 20%; neomycin, tissue ± 20%, milk ± 20%; gentamicin C_1, tissue ± 25%, milk ± 20%, gentamicin C_2, tissue ± 30%, milk ± 20% and gentamicin C_{1a}, tissue ± 30%, milk ± 30%. For concentrations of 2 x LOQ and above, all uncertainties are ± 20%.

Measurement uncertainties were also calculated taking into account the bias due to analyte recoveries, which would apply where recovery correction is not used. For tissue, this increased the expanded measurement uncertainties for streptomycin to 70% (LOQ) and 60% (2 x LOQ and above) and neomycin to 35%. For milk, the expanded measurement uncertainties became: dihydrostreptomycin, ± 80%; streptomycin, ± 30%; neomycin, ± 25%; gentamicin C_1, ± 70%, gentamicin C_2, ± 60% and gentamicin C_{1a}, ± 50% (LOQ) and 40% (2 x LOQ and above).

Table 6. Measurement uncertainty calculation for streptomycin in animal tissue

Component	Value, x	$\mu(x)$	$\mu(x)/x$	$[\mu(x)/x]^2$	
Homogeneity (u_{hom})	1.000	0.087	0.087	0.0076	
Recovery (u_{rec})	71	1.4	0.020	0.0004	
Calibration (u_{cal})	0.0107	0.0017	0.155	0.0239	
			Sum$[\mu(x)/x]^2$ =	0.0319	
			$\sqrt{\text{Sum}[\mu(x)/x]^2}$ =	0.1785	$\mu_{combined}$
			2 x $\sqrt{\text{Sum}[\mu(x)/x]^2}$ =	0.3569	$\mu_{expanded}$

Analysis of samples

Seventy two samples of animal tissue suspected to contain aminoglycoside residues were assayed by LC-MS/MS between 2006-2009. These consisted of bovine kidney (50), ovine kidney (18) and poultry liver (4). Three aminoglycosides were detected in these samples; neomycin, streptomycin and dihydrostreptomycin. Of these samples, 41 had incurred residues above the LOD, and 33 samples had levels above the LOR. However, only one sample

had a level of neomycin > Maximum Residue Limit (MRL: 10 mg/kg), with five samples having levels of streptomycin > MRL: 0.3 mg/kg and six samples having levels of dihydrostreptomycin > MRL: 0.3 mg/kg.

Conclusion

A robust method had been developed to confirm and quantify the levels of dihydrostreptomycin, streptomycin, apramycin, neomycin and gentamicin (C_1, C_2 and C_{1a}) in animal tissue and dihydrostreptomycin, streptomycin, neomycin and gentamicin (C_1, C_2 and C_{1a}) in bovine milk. The extraction, SPE and LC-MS/MS conditions were selected to achieve the desired Limit of Quantification (0.1-0.5 mg/kg for animal tissue and 0.01-0.1 mg/kg for milk) specified by the Australian antibiotic residue monitoring programs. Measurement uncertainties for each compound in milk and tissue samples determined at the LOQ varied between ± 20% and ± 35% respectively. The method was used to measure the levels of aminoglycoside residues in samples submitted by Australian Regulatory Authorities.

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