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Confirmatory method for macrolide residues in bovine tissues by micro-liquid chromatography–tandem mass spectrometry

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Abstract

A new confirmatory method for three macrolides (tylosin, tilmicosin and erythromycin) in bovine muscle, liver and kidney by micro-LC–MS–MS using an atmospheric pressure ionisation source and an ionspray interface has been developed. Roxithromycin was used as internal standard. The molecular related ions, $[M+2H]^{2+}$, at m/z 435 for tilmicosin, and $[M+H]^{+}$, at m/z 734 and 916 for erythromycin and tylosin, respectively, were the precursor ions for collision-induced-dissociation and two diagnostic product ions for each macrolide were identified for the unambiguous confirmation by selected reaction monitoring LC–MS–MS. Precision values (relative standard deviations) were all below 14.9%, whereas the overall accuracy (relative error) ranged from −17.7 to −9.8% for tylosin, from −17.5 to −10.7% for tilmicosin and from −19.6 to −13.7% for erythromycin, in all the investigated bovine tissues. The limits of quantification were 30 (muscle) or 40 (liver, kidney) μg kg^{-1}, 20 (muscle) or 150 (liver, kidney) μg kg^{-1}, 50 (muscle, liver) or 80 (kidney) μg kg^{-1}, 20 (muscle, liver) or 50 (kidney) μg kg^{-1} for tylosin, tilmicosin, erytromycin and roxithromycin, respectively. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The macrolides are lipophilic molecules having a central lactone ring bearing 12 to 16 atoms to which several amino and/or neutral sugars are bound. They are broad spectrum antibiotics active against gram-positive bacteria and mycoplasmas, as well as some gram-negative organisms and members of the chlamidia group. Macrolides are widely used in veterinary medicine to treat respiratory diseases and enteric infections in cattle, sheep, swine and poultry. However, they are also employed as feed additives to promote animal growth [1,2]. Incorrect use of these antibiotics may leave residues in edible tissues causing toxic effects on consumers, e.g. allergic reactions in hypersensitive individuals, or indirectly, problems through the induction of resistant strains of bacteria [3].

Within the European Union (EU), maximum residue limits (MRLs) in bovines are 50 μg kg^{-1} in muscle and 1000 μg kg^{-1} in liver and kidney for tilmicosin, 100 μg kg^{-1} and 200 μg kg^{-1} in muscle, liver and kidney for tylosin and erythromycin, respectively.

Measures to monitor certain pharmacological substances and residues in live animals and animal products have been established by the EU with Council Directive 96/23/EEC [4]. Routine methods

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to be used for residue control should comply with the requirements of the Commission Decision 93/256/EEC [5], currently under revision.

A number of methods for the detection of macrolides in animal tissues have been proposed. Microbiological assays are used to screen samples for residual amounts of macrolide residues [6–8] although they lack specificity and are unable to identify the exact nature of the antibiotic residues. More specific chemical methods such as liquid chromatography coupled with ultraviolet (LC–UV) [9–11], fluorimetric (LC–FL) [12,13], chemiluminescence (LC–CL) [14] and electrochemical (LC–ED) detection [11,15] have been proposed for determination of macrolides in animal tissues. Gas chromatography coupled to mass spectrometry (GC–MS) [16] has also been reported as confirmatory method for macrolides on account of its high specificity due to the information on the molecular structure of the analyte, obtained by electron impact (EI) ionisation.

Methods based on a combination of liquid chromatography with mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS–MS) using particle beam [17,18], heated nebulizer [19] and electrospray [20–22] interfaces offer a rapid, simplified, specific and sensitive alternative to GC–MS methods for macrolide determination involving simple extraction procedures and avoiding derivatization reactions. Moreover, microbore columns are frequently used in analytical chemistry, as they offer advantages over conventional columns, such as increased mass sensitivity, higher efficiency, reduced chromatographic dilution and lower mobile phase volumetric flow-rates with large reduction in solvent consumption. These factors result in a high demand for microbore techniques although stringent dead volume requirements are needed in the instrumentation.

The aim of this work was to determine the feasibility of micro-high-performance liquid chromatography-tandem mass spectrometry (micro-LC–MS–MS) for the direct detection and quantitation of macrolides in bovine tissues. In this study, a multiresidue confirmatory method for three macrolides (tylosin, tilmicosin and erythromycin) in different bovine matrices (muscle, liver and kidney) by micro-LC–MS–MS using an atmospheric pressure ionisation (API) source and an ionspray (IS) interface was developed.

2. Experimental

2.1. Chemicals and reagents

All the solvents were LC or analytical grade. Erythromycin and roxithromycin were from Sigma (St Louis, MO, USA); tylosin and tilmicosin were from Eli-Lilly (Indianapolis, IN, USA). All the individual and composite standard stock solutions were prepared in methanol. All the solutions were stored at 4°C and were stable for at least 1 month.

2.2. Sample extraction

Extraction of fortified and incurred tissue samples was carried out according to Deléfine et al. [18]. An aliquot of 2 g of homogenised tissues was weighed into a 15 ml glass centrifuge tube and added with 200 ng (muscle) or 400 ng (liver and kidney) of roxithromycin (internal, I.S.) and 800 μl of deionised water. The sample was stirred and mixed with Vortex stirrer for 1 min at maximum speed and allowed to stand for 15 min before extraction. Then, 2 ml of buffer solution (33.46 g $K_2HPO_4 \cdot 3H_2O$, 1.046 g KH_2PO_4 in 1000 ml deionised water, pH≈8) and 10 ml of chloroform were added. Then the sample was mixed with a rotating stirrer for 15 min at 100 rev./min and successively centrifuged at 4000 *g* for 10 min. Phases were completely separated into three layers: aqueous layer at the top, remaining tissue in the middle and chloroform layer at the bottom. The chloroform layer was recovered and filtered through glass wool.

2.3. Solid-phase extraction column cleanup

The sample was purified by a solid-phase extraction (SPE) column using a diol column (Bond Elut diol 100 mg, 1 ml, Varian, Les Ulis, France) previously conditioned with 1 ml chloroform. After sample loading, the column was washed twice with 0.5 ml of chloroform and 0.5 ml of water. Finally, macrolides were eluted with three applications of 200 μl of 0.1 *M* ammonium acetate–methanol

(50:50, v/v). A 1-μl volume of the solution was injected into the LC–MS–MS system.

2.4. Calibration and quantitation

Calibration curves based on blank bovine tissues spiked with mixtures of tylosin, tilmicosin and erythromycin were prepared daily. These curves ranged from 30 (muscle) or 40 (liver, kidney) to 400 μg kg^{-1} for tylosin, from 20 to 200 μg kg^{-1} (muscle) or 150 to 4000 μg kg^{-1} (liver, kidney) for tilmicosin and from 50 (muscle, liver) or 80 (kidney) to 800 μg kg^{-1} for erytromycin. They were constructed by plotting peak area ratios of the analyte to I.S. vs. macrolide concentrations using a least-squares linear regression model. Estimations of the amount of the macrolides in fortified and incurred samples were interpolated from these calibration graphs.

In order to evaluate extraction efficiency, repeatability and accuracy of the analytical method, six replicates of tissue blank control samples, fortified with mixtures of the macrolide antibiotics at four different concentrations [i.e. limit of quantification (LOQ), MRL/2, MRL, 2 MRL] were prepared and analysed on each of 3 days for each concentration. The extraction efficiency of the analytes was determined by comparing the peak areas of the fortified samples with those of the corresponding standards.

2.5. LC–MS and LC–MS–MS

Analyses were performed on a Phoenix 20 CU LC pump (Fisons, Milan, Italy) liquid chromatograph. A Valco (Valco, Houston, TX, USA) injection valve equipped with a 1-μl internal loop was used for the injection by flow injection analysis (FIA)–MS, FIA–MS–MS and LC–MS–MS. Chromatographic separations were obtained under isocratic conditions using a reversed-phase LC-ABZ Supelcosil microbore column (Saint Quentin, Fallavier, France) (300×1 mm I.D., 5 μm) at room temperature, with a mobile phase of acetonitrile–methanol–1% trifluoroacetic acid (60:20:20, v/v/v) and at a flow-rate of 50 μl min^{-1}.

Mass spectral analyses were performed on a PE-Sciex API III plus triple-quadrupole (PE-Sciex, Thornhill, Canada) equipped with an API source and an I.S. interface set at a voltage of 5500 V. Ultra-high-purity nitrogen was used as curtain gas (0.6 l min^{-1}) and nebulizer gas (400 kPa) in the I.S. interface. The orifice potential voltage (OR) was set at 80 V for erythromycin and tilmicosin, at 50 V for tylosin and at 60 V for roxithromycin. The standard software packages (PE-Sciex) were used for instrument control data acquisition and data elaboration.

Full-scan mass spectra were acquired in single MS positive-ion mode over the mass range m/z 300–1100. In the MS–MS experiments, product ion mass spectra were acquired in positive ion mode by colliding the quadrupole 1 (Q1) selected precursor ion, with argon (gas thickness $300 \cdot 10^{13}$ molecules cm^{-2}) in quadrupole 2 (Q2) operated in radio frequency (RF)-only mode and scanning the third quadrupole mass spectrometer, Q3, from m/z 50–1000. Collision energies of 25 eV for erythromycin, tilmicosin, and roxithromycin and 40 eV for tylosin were used to carry out the collision-induced dissociation (CID) experiments. The molecular related ions, $[M+2H]^{2+}$, at m/z 435 for tilmicosin, and $[M+H]^{+}$, at m/z 734, 916 and 837 for erythromycin, tylosin and roxithromycin, respectively, were the precursor ions for CID and two diagnostic product ions for each analyte were identified to carry out selected reaction monitoring (SRM) LC–MS–MS analyses. The analyses were carried out using the double precursor-product ion combinations of m/z 435→99 and m/z 435→174 for tilmicosin, m/z 734→158 and m/z 734→576 for erythromycin, m/z 916→174 and m/z 916→772 for tylosin, m/z 837→158 and m/z 837→679 for roxythromycin (I.S.).

Peak areas of the analytes were computed using MacQuan version 1.3 software from PE-Sciex.

3. Results and discussion

The aim of this research was to develop a specific, sensitive and reliable LC–API-MS–MS method using an ionspray interface for the measurement of antibiotic macrolide residues (tylosin, tilmicosin and erythromycin) in bovine tissues. Due to the unavailability of an isotope-labelled form of one macrolide, roxithromycin, a macrolide structurally

related to the analyte under investigation and not used in veterinary medicine, was used as internal standard [21].

Preliminary experiments were carried out by FIA on individual macrolide standard solutions in order to screen the solvent system and ionisation parameters to obtain suitable ionisation of the compounds. Ionspray full-scan mass spectra (mass range m/z 300–1100) were obtained by FIA in the MS positive ion mode for the analytes under investigation, by adopting a mobile phase of acetonitrile–methanol–1% trifluoroacetic acid (60:20:20, v/v/v) and at a flow-rate of 50 μl min^{-1}. The selective formation of the molecular related ions, $[M+2H]^{2+}$, at m/z 435 for tilmicosin, and $[M+H]^{+}$, at m/z 734, 916 and 837 for erythromycin, tylosin and roxithromycin, respectively, was observed in these spectra with negligible fragmentation (data not shown). Individual optimisation of the MS parameters for each macrolide was then performed by selected ion monitoring (SIM) FIA–MS analyses at m/z 435, 734, 837 and 916 in order to obtain spectra with maximum intensities of the molecular related ions. The orifice OR was also optimised and OR of 80 V for erythromycin and tilmicosin, 50 V for tylosin and 60 V for roxithromycin were chosen as the best compromise in terms of S/N.

The simplicity of the FIA–MS spectra is useful for the identification of the analytes based on their molecular-related ions, although they do not provide further structural information. Tandem mass spectrometry was therefore used in order to obtain additional structural information by detecting diagnostic product ions obtained by CID of the precursor ion. The molecular related ions, $[M+2H]^{2+}$, at m/z 435 for tilmicosin, and $[M+H]^{+}$, at m/z 734, 916 and 837 for erythromycin, tylosin and roxithromycin, respectively, served as precursor ions for CID in the MS–MS experiments, carried out by FIA–MS–MS on the individual macrolide standard solutions. Fig. 1 shows the positive ionspray product ion mass spectra (mass range m/z 50–1000) of the molecular related ion of each macrolide. The most abundant product ions were observed at m/z 174 and 99 for tilmicosin, at m/z 576 and 158 for erythromycin, at m/z 772 and 174 for tylosin, at m/z 679 and 158 for roxithromycin. Transitions of the respective molecu-

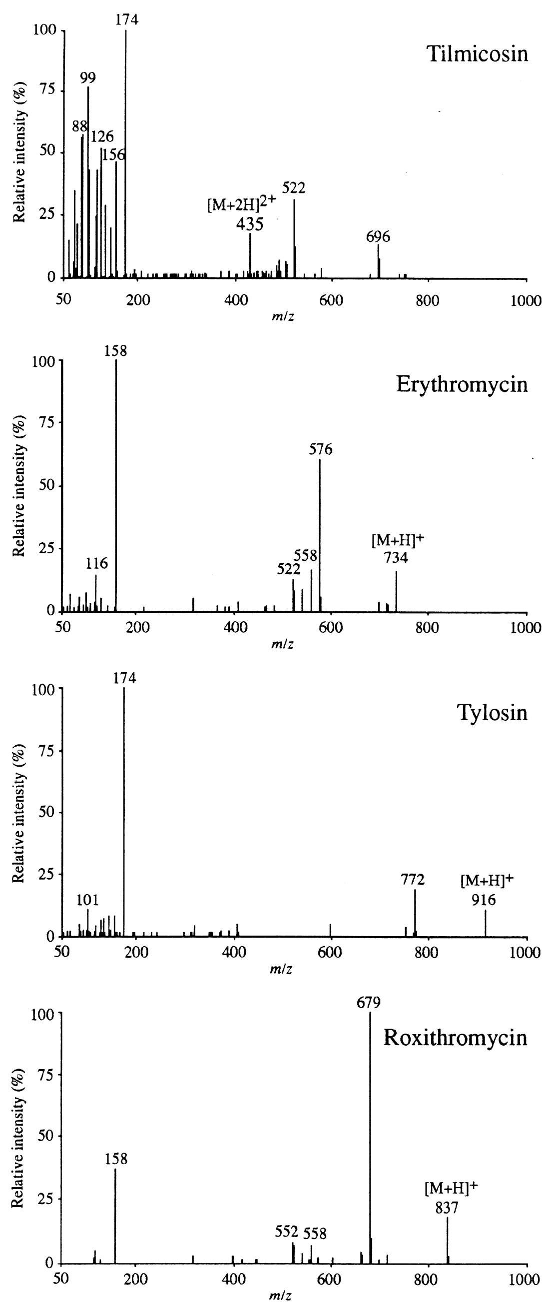

Fig. 1. Positive product ion mass spectra of tilmicosin, erythromycin, tylosin and roxithromycin (I.S.). Conditions: FIA; mobile phase: acetonitrile–methanol–1% trifluoroacetic acid (60:20:20, v/v/v); flow-rate 50 μl min^{-1}; OR was set at 80 V for tilmicosin and erythromycin, at 50 V for tylosin and at 60 V for roxithromycin. Argon was used as the collision gas. CID was carried out with collision energies of 25 eV for tilmicosin, erythromycin and roxithromycin and at 40 eV for tylosin.

lar related ions to these product ions were therefore selected according to the SRM technique.

In order to achieve targeted analyses and maximum sensitivity as well as for quantitative purposes, SRM LC–MS–MS analyses were finally performed under isocratic conditions using a Supelco LC-ABZ column at room temperature, with a mobile phase of acetonitrile–methanol–1% trifluoroacetic acid (60:20:20, v/v/v) and at a flow-rate of 50 μl min^{-1}.

Although the separation of tilmicosin (3.0 min), erythromycin (3.7 min), tylosin (3.5 min) and roxithromycin (4.0 min) was not excellent under the adopted conditions, the specificity of MS–MS reduces the need for complete chromatographic resolution of individual compounds. The focus in developing the confirmatory LC–MS–MS method was rather on providing simple and fast treatment of samples and reducing the analytical run time by API-compatible mobile phases.

Specificity of the SRM LC–MS–MS method was proved by processing and analysing muscle (Fig. 2A), kidney and liver blank samples. No interference was noticed around the macrolides retention times in the matrices under investigation (data not shown).

Calibration curves were prepared daily by spiking blank control samples of bovine tissues with mixtures of macrolides. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) greater than 0.995 for all curves.

Fortified bovine tissue samples at four different concentrations for each analyte/matrix combination were prepared and analysed to determine the extraction efficiency of the macrolides and to evaluate the precision and the accuracy of the analytical method. Representative chromatograms of fortified bovine muscle are reported in Fig. 2B. The relative retention time for each analyte/matrix combination corresponded to that of the standard within a tolerance of ±0.6%.

The extraction efficiency of the analytes was determined by comparing the peak areas of fortified samples with those of the corresponding standards. Six replicates at each concentration were analysed on each day for 3 days ($n=18$) and mean percentages of extraction efficiency were estimated for bovine muscle, liver and kidney. Extraction efficiency values greater than 72.8±15.6, 73.5±16.3 and 70.2±15.2%, for tylosin, tilmicosin and erythromycin, respectively, were found for all bovine tissues.

The precision (repeatability) and accuracy of the method were obtained by analysing six replicates for each of the four tested fortification levels on each of 3 days for the bovine tissues under investigation. Precision was determined by calculating the relative standard deviation (RSD, %) for the replicated measurements and the accuracy (relative error, RE, %) was calculated by the agreement between the measured and the nominal concentrations for the fortified samples. Precision values were all below 14.9% (RSD), while the overall accuracy ranged from −17.7 to −9.8% for tylosin, from −17.5 to −10.7% for tilmicosin and from −19.6 to −13.7% for erythromycin, for all the investigated bovine tissues (Table 1). These values could be considered satisfactory, on account of the complexity of the biological matrices. The LOQs were 30 (muscle) or 40 (liver, kidney) μg kg^{-1}, 20 (muscle) or 150 (liver, kidney) μg kg^{-1}, 50 (muscle, liver) or 80 (kidney) μg kg^{-1}, for tylosin, tilmicosin and erytromycin, respectively. These were at least 2.5 times below the MRLs set for the investigated macrolide residues in the bovine tissues, thus complying the requirements of methods to be used for confirmatory purposes. Representative chromatograms of a muscle sample spiked with macrolides at concentrations equivalent to the LOQs (20 μg kg^{-1} for tilmicosin, 50 μg kg^{-1} for erythromycin and 30 μg kg^{-1} for tylosin) and to 100 μg kg^{-1} for roxithromycin are shown in Fig. 2B.

The SRM LC–MS–MS method was finally used to analyse real samples collected as part of the national program for veterinary drug residue control in Italy. Representative SRM LC–MS–MS chromatographic profiles of a violative bovine muscle sample for which tilmicosin level (82 μg kg^{-1}) resulted above the MRL is shown in Fig. 2C. In the violative bovine muscle sample, the relative intensities of the detected ions for tilmicosin, expressed as a percentage of the intensity of the most intense transition, correspond to those of the standard analyte from spiked samples, at comparable concentrations, within the maximum permitted tolerances

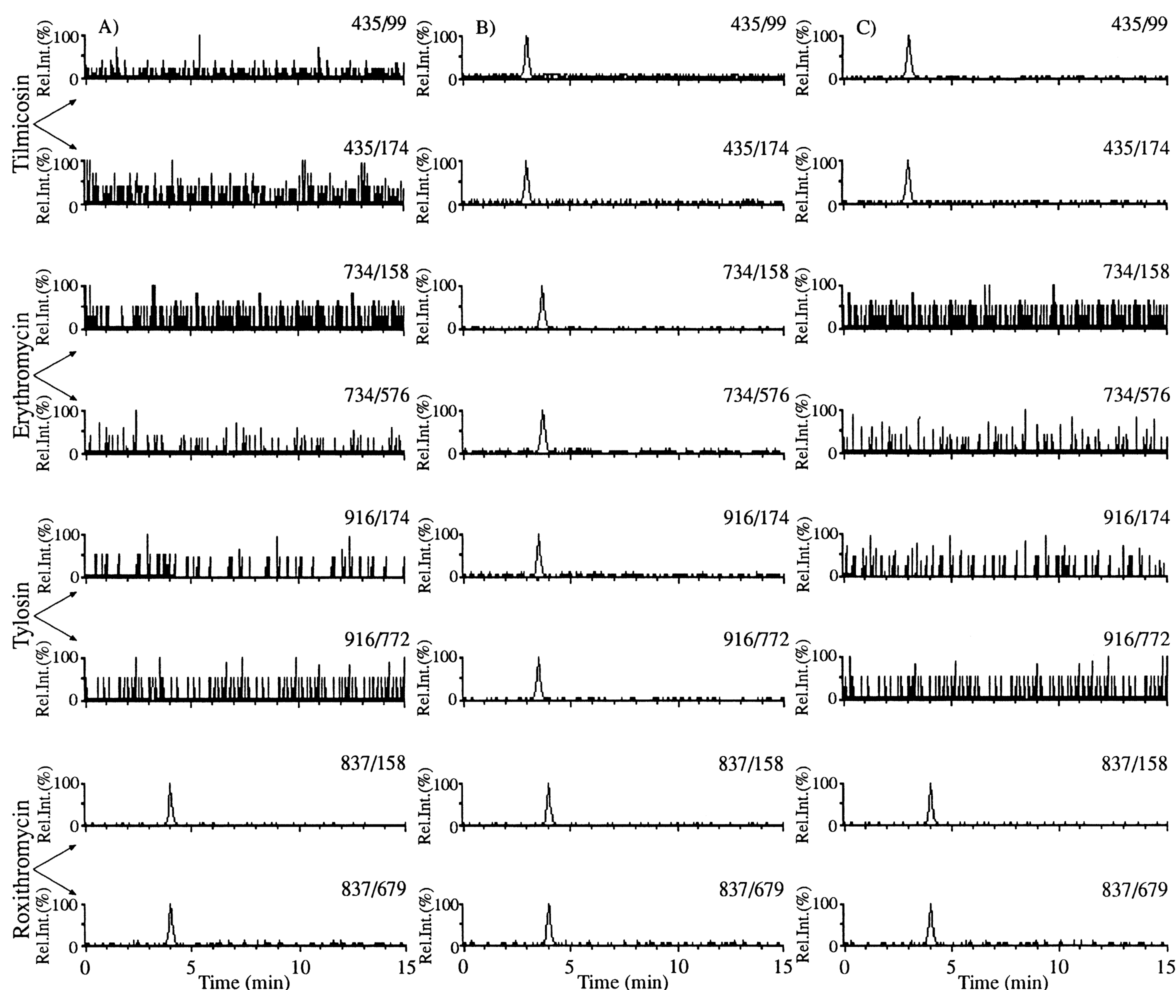

Fig. 2. SRM LC–MS–MS chromatograms of: (A) extract of blank bovine muscle spiked with roxithromycin (100 μg Kg^{-1}) as I.S.; (B) extract of bovine muscle spiked with macrolides at concentrations equivalent to the LOQ (i.e. 20 μg kg^{-1} for tilmicosin, 50 μg kg^{-1} for erythromycin and 30 μg kg^{-1} for tylosin) and with roxithromycin (100 μg kg^{-1}) as I.S.; (C) extract of a violative bovine muscle sample containing tilmicosin (82 μg kg^{-1}) and roxithromycin (100 μg kg^{-1}) as I.S. Precursor–product ion combinations used in SRM detection are shown. Conditions: isocratic HPLC analysis; RP LC-ABZ Supelcosil microbore column (300×1 mm, 5 μm); mobile phase: acetonitrile–methanol–1% trifluoroacetic acid (60/20/20, v/v/v); flow-rate 50 μl min^{-1}; 1 μl injection; OR was set at 80 V for tilmicosin and erythromycin, at 50 V for tylosin and at 60 V for roxithromycin. Argon was used as the collision gas. CID was carried out with collision energies of 25 eV for tilmicosin, erythromycin and roxithromycin and at 40 eV for tylosin.

[5]. The suitability of the SRM LC–MS–MS to identify violative samples of macrolides in bovine tissues was therefore shown.

4. Conclusion

A specific, sensitive and reliable LC–API-MS–MS method using an ionspray interface for the measurement of antibiotic macrolide residues (tylosin, tilmicosin and erythromycin) in bovine tissues was developed.

The unambiguous confirmation of the presence of the macrolides in bovine muscle, liver and kidney in the proposed method results from the retention time information, the presence of the molecular related

Table 1
Precision (RSD, %) and accuracy (RE, %) for macrolides in bovine tissue samples

Analyte	Tissue matrix	MRL (μg^{-1}/kg)	LOQ (μg^{-1}/kg)	Spike level (μg^{-1}/kg)	Measured concentration (mean±SD) (μg^{-1}/kg)	RSD (%)	RE (%)
Tylosin	Muscle	100	30	30	26.2±3.7	14.2	−12.7
				50	44.3±6.2	14.0	−11.4
				100	89.7±11.5	12.8	−10.3
				200	180.5±20.8	11.5	−9.8
	Liver	100	40	40	33.2±4.8	14.4	−17.0
				50	42.8±6.2	14.5	−14.4
				100	86.1±11.6	13.5	−13.9
				200	170.5±19.8	11.6	−14.8
	Kidney	100	40	40	32.9±3.6	14.7	−17.7
				50	41.5±6.2	14.9	−16.9
				100	85.2±11.1	13.0	−14.8
				200	171.4±20.2	11.6	−14.3
Tilmicosin	Muscle	50	20	20	16.5±2.4	14.5	−17.5
				25	21.2±3.1	14.7	−15.2
				50	42.4±6.1	14.4	−15.1
				100	85.4±10.8	12.6	−14.6
	Liver	1000	150	150	129.5±19.2	14.8	−13.7
				500	437.0±49.8	11.4	−12.6
				1000	893.3±91.1	10.2	−10.7
				2000	1724.3±168.9	9.8	−13.8
	Kidney	1000	150	150	125.6±18.5	14.7	−16.3
				500	423.4±47.5	11.2	−15.3
				1000	858.9±89.4	10.4	−14.1
				2000	1702.4±171.8	10.1	−14.9
Erythromycin	Muscle	200	50	50	40.5±4.8	11.9	−19.0
				100	84.6±11.5	13.6	−15.4
				200	172.6±19.9	11.5	−13.7
				400	335.1±42.9	12.8	−16.2
	Liver	200	50	50	40.1±5.2	13.0	−19.8
				100	83.9±11.7	13.9	−16.1
				200	169.3±19.6	11.6	−15.4
				400	334.5±41.6	12.4	−16.4
	Kidney	200	80	80	64.3±9.0	14.0	−19.6
				100	81.7±11.9	14.6	−18.3
				200	166.5±19.8	11.9	−16.8
				400	334.9±42.8	12.8	−16.3
Roxithromycin	Muscle	n.a.	20	50	40.1±5.4	13.5	−19.8
				100	83.6±10.8	12.9	−16.4
	Liver	n.a.	20	100	82.9±11.2	13.5	−17.1
				200	167.5±18.5	11.0	−16.2
	Kidney	n.a.	50	100	80.8±11.4	14.1	−19.2
				200	165.0±18.7	11.3	−17.5

Each value is the mean of 18 samples (six per day for 3 days). n.a., not available.

ions of the analyte, and the employment of a double precursor-product ion reaction. The developed method complies with the criteria proposed by the Commission Decision 93/256/EEC, currently under revision [5], for confirmatory methods of substances listed in Group B of Annex I of Council Directive 96/23/EC [4], such as macrolides, requiring the identification of the analyte based on the presence of at least one precursor and two transition product ions for the LC–MS–MS technique.

The ability to perform multi-residue analyses involving simple extraction procedures and highly selective and sensitive determination by SRM LC–MS–MS, along with the wide-spread presence of benchtop LC–multiple MS apparatus in laboratories, makes this analytical method particularly valuable for routine control of the extralabel use of antibiotics.

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