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Identification and quantification of five macrolide antibiotics in several tissues, eggs and milk by liquid chromatography–electrospray tandem mass spectrometry

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Abstract

We present an electrospray high-performance liquid chromatographic tandem mass spectrometric (HPLC–MS–MS) method capable of determining in several tissues (muscle, kidney, liver), eggs and milk the following five macrolides: tylosin, tilmicosin, spiramycin, josamycin, erythromycin. Roxithromycin was used as an internal standard. The method uses extraction in a Tris buffer at pH 10.5, followed by protein precipitation with sodium tungstate and clean-up on an Oasis solid-phase extraction column. The HPLC separation was performed on a Purospher C_{18} column (125×3 mm I.D.) protected by a guard column, with a gradient of aqueous 0.1 *M* ammonium acetate–acetonitrile as the mobile phase at a flow-rate of 0.7 ml min^{-1}. Protonated molecules served as precursor ions for electrospray ionisation in the positive ion mode and four product ions were chosen for each analyte for multiple reaction monitoring (MRM). A validation study was conducted to confirm the five macrolides by MRM HPLC–MS–MS analysis of a negative control and fortified samples. All of the samples analysed were confirmed with four ions. The ion ratio reproducibility limit ranged from 2.4 to 15%. All compounds could be detected and quantified at half-maximum residue limits (MRLs). The method is specific, quantitative and reproducible enough to conform to European Union recommendations within the concentration range 0.5 MRL–2 MRL (accuracy: 80 to 110%, relative standard deviation: 2 to 13%). This whole method allows extraction and analysis of up to 50 samples per day. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Tylosin; Tilmicosin; Spiramycin; Josamycin; Erythromycin

1. Introduction

Macrolide antibiotics constitute a very important class of antibacterial compounds widely used in veterinary medicine to treat respiratory diseases, or as feed additives to promote growth. These antibiotics are molecules with a central lactone ring bearing 12 or 16 atoms, to which several amino and/or neutral sugars are bound. They are absorbed well after oral administration and distribute extensively to tissues, especially the lungs, liver and kidneys [1]. Incorrect use of these drugs may leave residues in edible tissues.

Antibiotic residues may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or cause problems indirectly through induction of resistant strains of bacteria [2]. The European Union (EU) has set maximal residue

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limits (MRLs) for some macrolides in tissues, milk and eggs. Therefore, simple and reliable analytical methods are required to monitor these drug residues in edible tissues of livestock animals.

Generally, microbiological assays are used to determine antibiotics [3–5]. These assays excel as qualitative means of screening samples for residual amounts of antibacterial substances, but they tend to lack specificity and it is hard to confirm the exact nature of the drugs remaining in the animal tissues. To overcome these problems, chemical analyses such as high-performance liquid chromatography (HPLC) have been used to determine macrolide antibiotics [6–11]. Yet mass spectrometry (MS) coupled with chromatography is the best technique, combining analyte separation with structural information. Few methods for confirming macrolides involve MS preceded by liquid chromatography (LC) [12–20].

Multiresidue methods [21,22] are preferred for regulatory work because of the simultaneously provided information.

We report here the development and validation of an easy, quick, quantitative method for the simultaneous confirmation of five macrolides (spiramycin, tylosin, tilmicosin, erythromycin and josamycin) at half-MRL concentration in animal tissues (muscle, kidney, liver), bovine milk and hen eggs.

2. Experimental

2.1. Materials

2.1.1. Reference compounds

Reference products were erythromycin (ERY, E6376), tylosin (TYL, T6134), spiramycin (SPI, S9132) from Sigma (St. Louis, MO, USA), josamycin (JOS, RP24693) from Rhône-Poulenc Rorer (Vitry-sur-Seine, France) and tilmicosin (TIL, R50164) from Eli-Lilly (Saint-Cloud, France).

Roxithromycin (ROX, R4393) from Sigma was used as internal standard (I.S.). Roxithromycin is a macrolide antibiotic with an activity spectrum similar to that of erythromycin. It is not used in veterinary medicine.

A standard stock solution (1 g l^{-1}) was prepared by dissolving 10 mg standard in 10 ml ethanol. Secondary standard solutions (10 mg l^{-1}) were obtained by diluting 0.1 ml stock solution in 9.9 ml ethanol. Dilutions were then prepared from this solution to obtain a suitable range of working solutions.

2.1.2. Chemicals

Chemicals and reagents [tris(hydroxymethyl)-aminomethane, ammonium acetate, anhydrous sodium acetate (UCB, Belgium), sodium tungstate dihydrate (Merck, Darmstadt, Germany), and 30% ammonia solution (Carlo Erba, Italy) were of the highest available purity grade.

For extraction, Tris buffer (pH 10.5±0.2; 0.1 *M*) was prepared daily.

For protein precipitation, sodium tungstate dihydrate (0.15 *M*) and sodium acetate buffer (pH 4.6, 3 *M*) were prepared monthly and stored at room temperature.

Solvents such as acetonitrile, methanol, water and hexane used in the various steps of the extraction procedure (see below) were of the highest quality grade (pro-analysis or HPLC grade).

The biological materials were subjected to solid-phase extraction (SPE) on an Oasis HLB column (6 ml/200 mg capacity; Waters, Milford, MA, USA).

2.1.3. Biological specimens

Muscle, liver, kidney, milk and egg samples from untreated swine, cattle and hens were used as blanks and after spiking with the different macrolides as quality control specimens.

Macrolide concentrations in these specimens were equal to half the MRL, the MRL, or twice the MRL for all specimens, in accordance with EU guidelines.

2.1.4. Instrumentation and chromatography conditions

The gradient liquid chromatograph was a Hewlett-Packard (Palo Alto, CA, USA).

Macrolides were separated on a 125×3 mm column (Purospher, C_{18}, 5 μm) protected by a guard column (Purospher, C_{18}, 5 μm) (Merck). A 50×4.6 mm Biomatrix column (Chrompack, The Netherlands) with a Biomatrix guard column was placed before the analytical column to concentrate the

samples at the beginning of the run. The Biomatrix stationary phase consists of a combined phase: a hydrophobic part encapsulated by a hydrophilic outer layer. The latter rejects proteins by exclusion while drugs, owing to their small size, can penetrate this layer and interact with the hydrophobic part. Both columns were thermostated at 30°C.

The HPLC system was operated with a gradient system at a flow-rate of 0.7 ml min^{-1}. The mobile phase consisted of two elements.

Eluent A was filtered HPLC-acetonitrile and eluent B was a 0.1 ml l^{-1} ammonium acetate solution. We used a two-linear-step elution gradient. The initial conditions were A–B (0:100, v/v). These conditions remained for 1 min. During the first gradient step, the percentage of eluent A was increased to 30% over 3 min, while eluent B was decreased to 70% (v/v). During the second gradient step, eluent A was brought to 95% over 3 min, while eluent B gradually reached 5% (v/v). These conditions remained for 4 min. A 3-min post-run was used to return to the initial conditions.

2.1.5. MS–MS parameters

The system used was a Micromass Quattro II mass spectrometer (Micromass UK, Altrincham, UK) with electrospray ionisation (ESI). The analysis was performed in the positive ion mode.

Nitrogen was used as a drying gas with a flow-rate of 300 l h^{-1} and also as a nebulising gas with a flow-rate of 15 l h^{-1}.

The source temperature was 150°C. The HPLC flux was split 4.5:1, resulting in an approximate spray flux of 150 μl min^{-1}.

The cone voltage generally varied from 30 to 50 V, depending on the best signal of the ionisation products.

MS–MS parameters were optimised in the continuous flow mode as follows: after determination of the best conditions for isolating the precursor ion (usually a pseudo-molecular ion), the ion spray voltage, quadrupole, and lens conditions for argon-collision-induced dissociation were optimised.

Product ion scans were studied so as to choose the best ions for monitoring each molecule in multiple reaction monitoring (MRM). The dwell times were 0.08 ms.

2.2. Sample preparation

2.2.1. Primary extraction

A 5-g aliquot of a blank or spiked minced tissue sample (muscle, kidney, liver tissue) was mixed with a small volume (100 μl) of internal standard (roxithromycin, 1 μg).

After a 15-min equilibration period, the tissues were mixed vigorously for 15 min with 25 ml Tris buffer.

After a 10-min centrifugation at 3000 *g* and 4°C, the supernatant was transferred to a polypropylene tube and the solid residue extracted a second time with 25 ml Tris buffer (for muscle) or 20 ml Tris buffer (for liver and kidney).

Acetic acid (600 μl) and 5 ml sodium tungstate buffer were added to precipitate the proteins. After equilibration for 1 h at 4°C, the samples were centrifuged at 3000 *g* for 10 min. The supernatants were further filtered through a plug of glass wool.

The same procedure was used for egg except that the whole egg was extracted only once with 35 ml Tris buffer and that 1 ml acetic acid was added before protein precipitation step.

For milk, aliquots (10 ml) of pooled samples (biological blanks or spiked specimens) were added to a small volume of roxithromycin (I.S.).

After equilibration at room temperature for 15 min, the samples were centrifuged for 10 min at 3000 *g* and 4°C. The floating fat layer was eliminated.

A 5-ml volume of residual skimmed milk phase was diluted with 20 ml Tris buffer. The homogenised milk was then shaken gently and horizontally for 10 min.

The subsequent steps were identical to those described for tissues except that 15 ml 3 *M* sodium acetate buffer replaced the acetic acid in the protein precipitation step.

2.2.2. Solid-phase extraction: tissues, milk, and egg

The 6-cm^3 HLB Oasis extraction cartridges (200 mg) were prepared and conditioned with 10 ml methanol and 10 ml water. The biological samples were placed at the top of the column. Two wash solutions were applied before macrolide elution: 20 ml methanol–water (5:95, v/v) and 5 ml hexane.

After the last washing step, the Oasis columns were vacuum-dried for 10 min. The macrolides were finally eluted with 5 ml methanol–30% ammonia (95:5, v/v) and evaporated dry under a nitrogen flow. The extracts were dissolved in 500 μl NH_4AC–ACN (80:20, v/v), transferred to Eppendorf tubes, and centrifuged at 3000 *g* for 10 min. Aliquots of the supernatant (450 μl) were transferred to conical autosampler vials and a 100-μl loop was used to inject the extract into the LC–MS–MS system.

2.3. Validation tests

Over 3 consecutive days, 14 blank samples and 18 spiked samples were extracted for each matrix.

Blanks consisted of extracts of muscle, kidney, liver, milk, eggs from drug-free animals. The blanks were assayed. The chromatograms were routinely inspected for peaks that might correspond to one or more of the five macrolides or to the internal standard.

Six samples were spiked with an amount corresponding to half the MRL, six others with an amount corresponding to the MRL, and six with an amount corresponding to twice the MRL. A 1-μg amount of roxithromycin was added to each spiked sample.

The following parameters were studied on the basis of the results obtained: the limit of detection (LOD), the (intra- and inter-day) precision, and the accuracy. Post-extraction yields of the five macrolides were determined for all three initial concentrations (0.5 MRL, MRL, 2 MRL). In each case, the response observed when a macrolide was added to the blank prior to extraction, was compared with the response of the standard. Recovery of the I.S. was determined similarly.

3. Results and discussion

The aim of this work was to develop a multiresidue method for quantifying and confirming five macrolides in different matrices. For regulatory needs, the method must give good results for concentrations near the MRL. The objective was thus to validate the method for confirmation and quantification at concentrations near this limit (MRL/2, MRL, 2 MRL) in spiked blanks obtained from muscle, kidney, liver, milk and eggs. This requires simple and efficient extraction, chromatographic separation, and a suitable internal standard for quantification and for optimising the MS–MS conditions to be used to confirm each macrolide.

In the absence of an isotope-labelled form of one macrolide, particularly suited for detection by MS, we used roxithromycin as an internal standard. Roxithromycin is a macrolide structurally related to those we sought to detect and not used in veterinary medicine.

3.1. Extraction

Instead of organic solvents (acetonitrile, chloroform, dichloromethane, etc.) we used a Tris buffer at pH 10.5 to extract the macrolides. This extraction followed by protein precipitation with sodium tungstate and a clean-up on an Oasis SPE column proved more satisfactory than other methods, i.e., the samples were cleaner than with acetonitrile extraction or with clean-up by liquid–liquid partitioning using dichloromethane. This latter method proved applicable to clean-up of muscle samples, but not suitable for liver and kidney samples owing to extensive emulsion formation. In addition, chlorinated solvents may raise safety concerns and create expensive waste-disposal problems. The extraction yield obtained with Tris buffer was acceptable for all five macrolides and the internal standard except for erythromycin in milk and egg. Results are however good as stated later at the time of the reproducibility and exactitude study. The described method proved suitable for liver, muscle, kidney and egg samples. In addition, it requires less time and effort than other methods, an advantage when many samples have to be analysed. The mean recovery values calculated for muscle, liver, kidney, milk and egg samples for each macrolide are reported in Table 1.

Prior to extraction, milk samples were decreamed. Because of the different physico-chemical phases present in milk, drugs will sometimes be distributed unevenly and may remain predominantly in one phase after acidification or decreaming, for instance. Ziv and Rasmussen [23] have reported data on the distribution of a number of antimicrobials over cream, casein and whole milk, as established in

Table 1
Macrolide recoveries observed for the five studied matrices[a]

Matrix	Recovery (%)					
	Tylosin	Tilmicosin	Spiramycin	Josamycin	Erythromycin	Roxithromycin
Muscle	93	115	85	99	68	74
Kidney	64	56	57	61	57	58
Liver	78	47	58	79	78	74
Egg	66	92	53	55	44	76
Milk	115	53	103	101	30	70

[a] Negative samples were spiked with three concentrations (0.5 MRL, MRL, 2 MRL) of each of the macrolides. The main product ions were monitored for each. The response observed when a macrolide was added to the blank prior to extraction was compared with the response of the standard.

radiolabelling studies with goats. The distribution depends both on the residue concentration range and the route of administration. For spiramycin, the concentration ratio for skim versus whole milk was 0.9 after intramammary infusion and 0.8 after intramuscular injection. This phenomenon can be explained by trapping of the more lipophilic compounds during formation of fat globules in the milk-secreting cells of the udder alveoli. When the drug is added after fat globules are formed, as in the case of intramammary injection and also when a sample is spiked, enrichment in the fat globules apprecursorly does not take place to the same extent. The distribution of a drug residue over the various phases should be established for each individual drug. To determine whether the milk is contaminated, it would thus be best to determine the total drug content, but this means developing, in the future, a clean-up procedure for whole milk.

3.2. Liquid chromatography

The chromatography conditions were adjusted with two aims in mind: to develop a quick method and to improve sample purification in order to minimise the frequency at which the mass spectrometer must undergo cleaning operations. These two aims were reached by using a gradient, a Biomatrix column and a switch column.

Gradient elution gave rather poor separation: several analytes were found to co-elute. This is not a problem, thanks to the mass selectivity afforded by MS detection.

A gradient proved quite efficient, as only 11 min were required to elute the analytes. Taking into account the 3-min post-run, the total analysis was 14 min. Yet an additional purification system proved indispensable: when the solvent flowed through the mass spectrometer throughout the run, the instrument displayed decreased sensitivity after only 20 samples. To improve the situation, two elements were added to the LC system.

First, upstream from the analytical column (C_{18}), we placed a Biomatrix column in order to eliminate most of the macromolecules remaining in the sample. A ''switch column'' allowed the flow to pass solely through the Biomatrix column for 1 min, then through the analytical column and mass spectrometer. The sample was thus purified before reaching the analytical column. This additional purification made it possible to inject about 50 samples before having to clean the source of the mass spectrometer. It also extended the lifetime of the analytical column. Lastly, a switching valve was placed between the analytical column and the mass spectrometer, allowing the flow to pass through the mass spectrometer only during analyte elution. The combined measures made it possible to analyse about a hundred samples before having to clean the instrument.

Variation of the relative retention times of the five macrolides with respect to the retention time of the internal standard did not exceed 1%.

3.3. Mass spectrometry

The electrospray interface was used between the analytical column and the triple quadrupole mass spectrometer. As electrospray gave good sensitivity, fragmentation, and linearity, we have not yet tested atmospheric pressure ionisation (APCI).

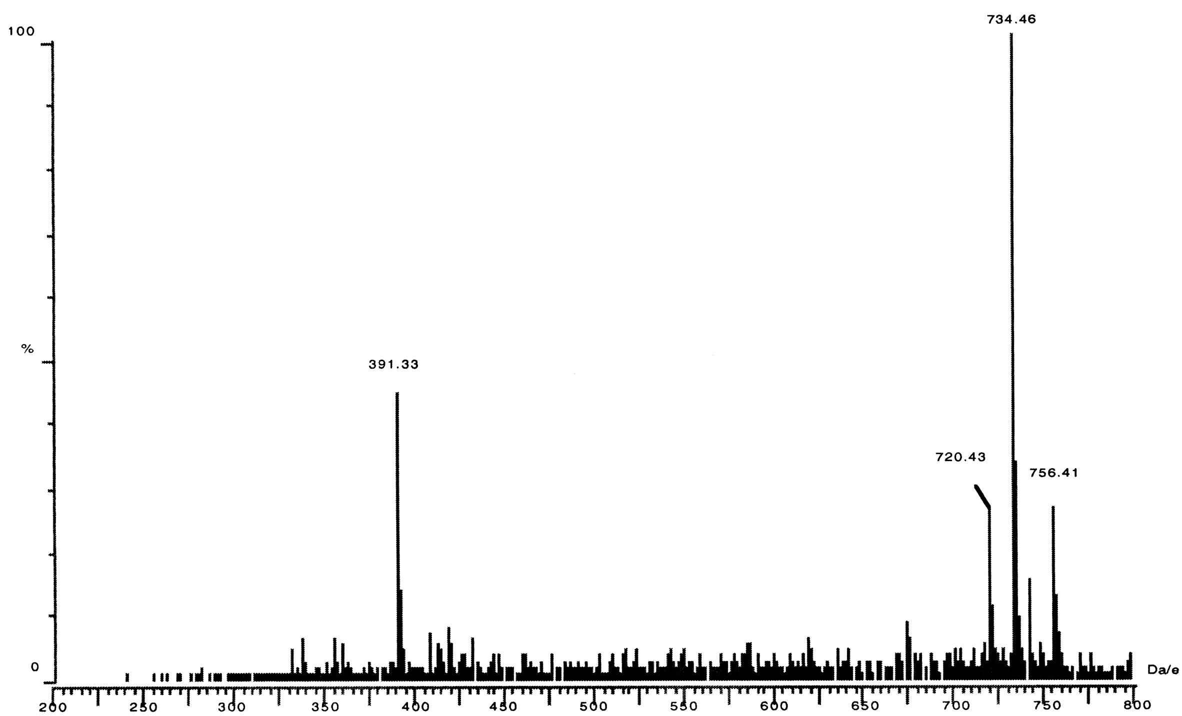

Fig. 1. Full scan spectra of erythromycin. Erythromycin at 100 ng ml^{-1} concentration in acetonitrile–water (50:50, v/v) was infused at a flow-rate of 2 ml.h^{-1}.

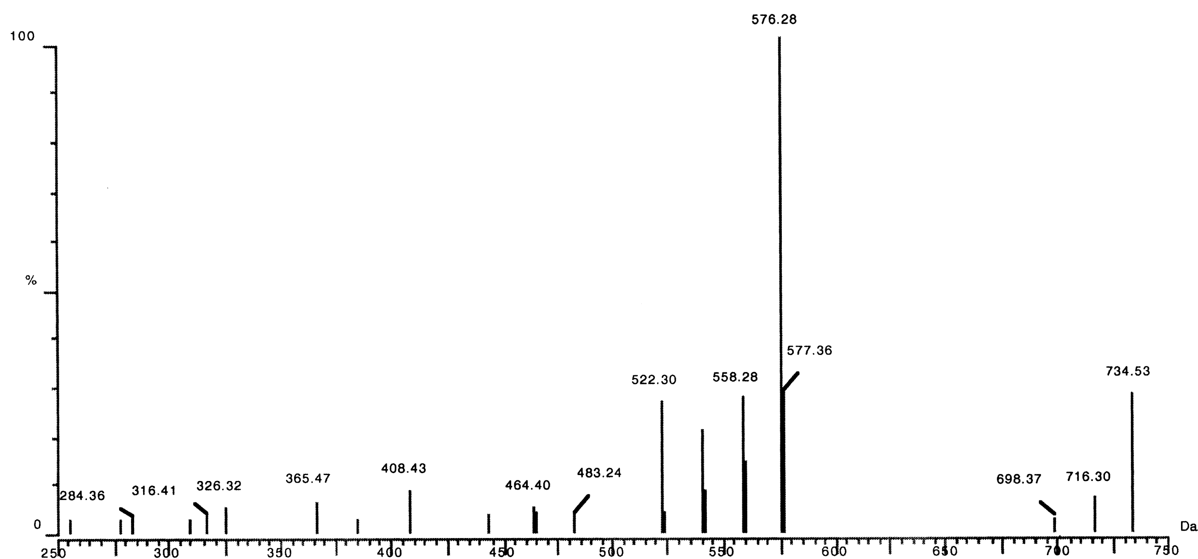

Fig. 2. Product ion LC–ESI-MS–MS spectra of erythromycin. The pseudo-molecular ion (da/e 734) is the precursor. Erythromycin at 100 ng ml^{-1} concentration in acetonitrile–water (50:50, v/v) was infused in the electrospray interface at a flow-rate of 2 ml.h^{-1}.

Table 2
Precursor ions, product ions and additional ions used for detection, quantification and confirmation of the macrolides in MS–MS with the electrospray source in the positive ion mode

Molecule	Precursor ion (Da)	Product ion for detection and quantification	Additional ions for confirmation (da/e)	Cone voltage (V)	Collision energy (eV)
Tylosin	916.3	772.2	318.4; 407.4; 598.4	40	33
Tilmicosin	869.6	696.6	522.2; 678.9; 505.7	50	40
Spiramycin	843.4	540.2	318.2; 700.2; 522.5	40	32
Josamycin	828.7	174	109; 600.4; 228.8	40	30
Erythromycin	734.3	576.3	558.3; 540.3; 522.3	30	25
Roxithromycin (internal standard)	837.4	158.1	None	45	45

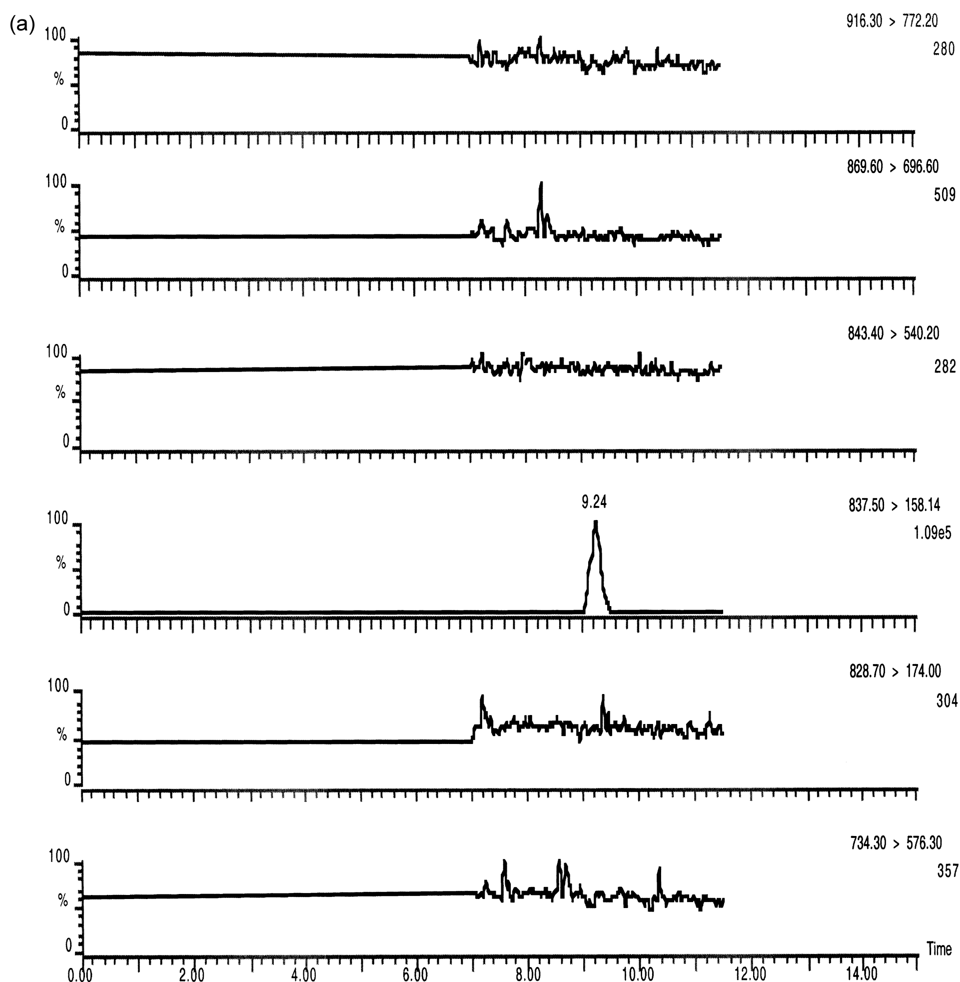

Fig. 3. (a) Chromatogram (MRM) of an extracted blank muscle sample. (b) Multiple reaction monitoring chromatogram of a standard (5 ng on the column). (c) Chromatogram (MRM) of a spiked muscle sample. The concentration of each macrolide was 0.5 MRL: tylosin: 50 μg kg^{-1}, tilmicosin: 25 μg kg^{-1}, spiramycin: 150 μg kg^{-1}, josamycin: 100 μg kg^{-1}, erythromycin: 200 μg kg^{-1}. (The internal standard concentration was 200 μg kg^{-1}).

The first step in developing the detection method was to select the precursor ion to be fragmented in the collision cell. The MS1 spectrum for erythromycin was dominated by ions with the following characteristics: $(M+H^+)$ 734.5, $(M+Na^+)$ da/e 756.5 and two other ions (720.4; 391.3) (Fig. 1) that remain unexplained. The pseudo-molecular ion (da/e 734.5) was chosen as the precursor ion for erythromycin. The pseudo-molecular ion was also selected as the precursor ion for all the other macrolides.

To ensure proper identification of the detected molecules, a highly selective fragmentation technique is recommendable. MS–MS was the chosen technique. We introduced argon gas into the collision cell (Q2) to collide with and fragment the incoming ions from Q1. We adjusted the cone voltage and collision energy to achieve good fragmentation. Good results were obtained with a cone voltage of 30–50 V and a collision energy of 25–50 eV.

For erythromycin, the product-ion spectrum of da/e 734.5 was then further collected in the product scan. A significant product ion was observed at da/e

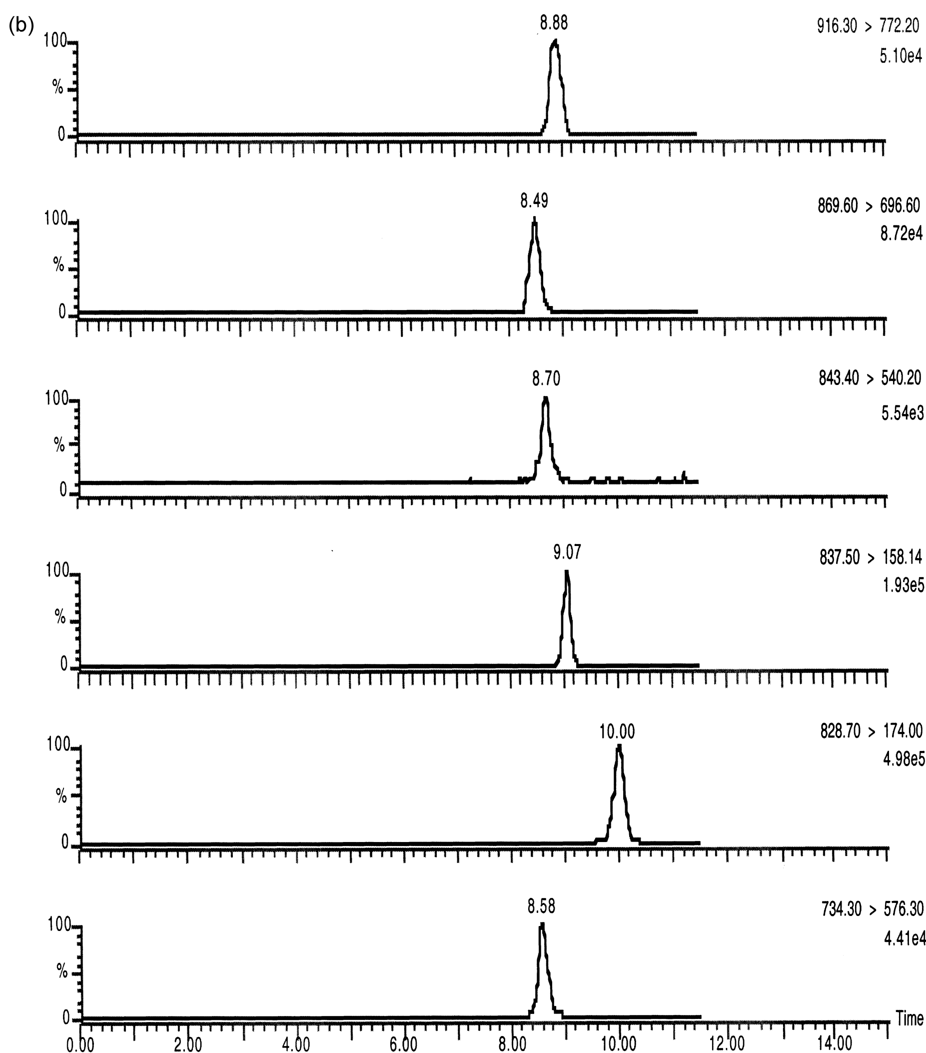

Fig. 3. (*continued*)

576.3 (Fig. 2); it was a fragmentation ion having lost a single sugar moiety as compared to the precursor ion da/e 734.5.

For erythromycin, the precursor ion da/e 734.5 (as the precursor ion) and a characteristic product ion da/e 576.3 (as the product ion) were chosen as the transition pair for MRM detection to measure the erythromycin concentration. The transition pairs of the other macrolides and the internal standard obtained in the positive ion mode were chosen in the same manner and are listed in Table 2.

Fig. 3 shows the MRM chromatograms obtained after injection of 10 μl mixed standard solution (concentration of each test macrolide and of the internal standard: 500 ng ml^{-1}), blank muscle extract, or muscle extract spiked with the various macrolides. The chromatograms are relatively free of interference from other compounds. Liver, kidney, milk and egg from 20 different sources were also found to be free of any interference at the retention time of each macrolide and roxithromycin.

To further increase sensitivity and to avoid false

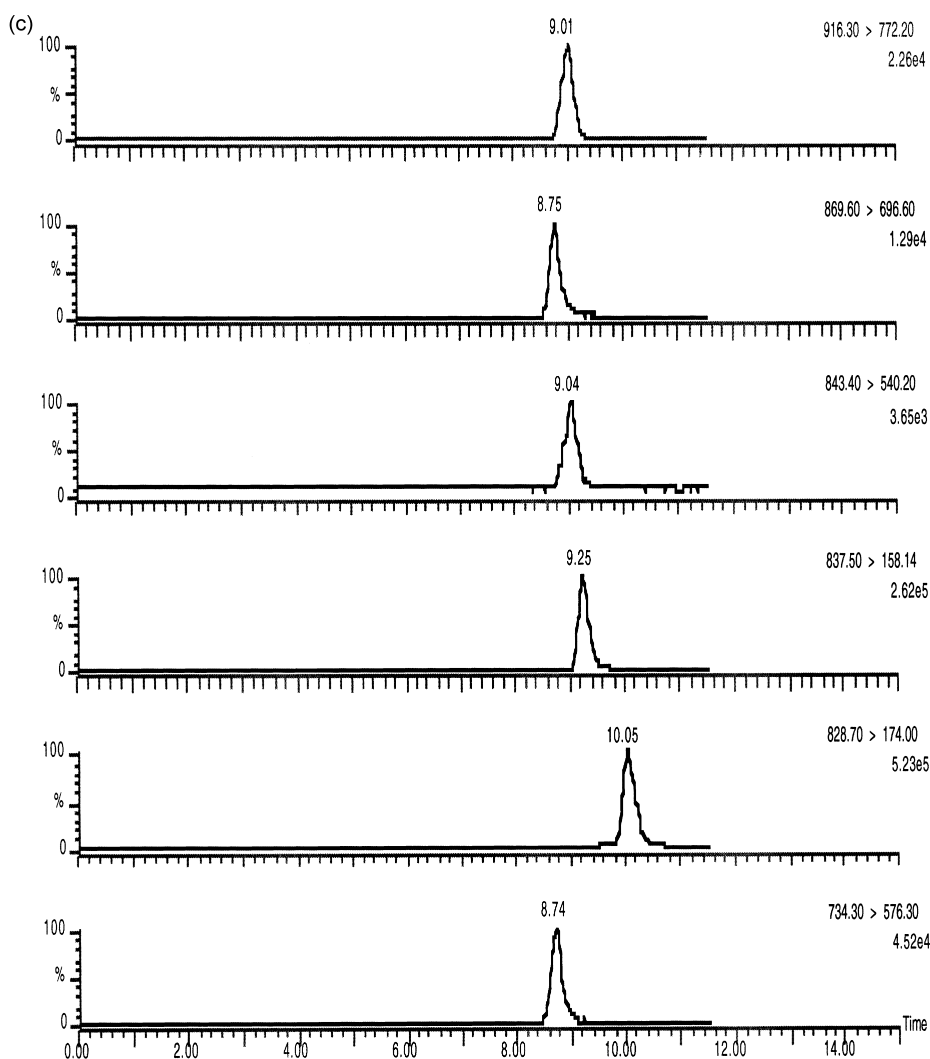

Fig. 3. (*continued*)

positives, we developed an MS–MS program for each macrolide, based on detection of four product ions issued from the same precursor ion (Table 2).

In practice, our approach to analysing unknown samples was first to monitor one characteristic transition for each macrolide so as to quantify the substance. If a peak was found for any macrolide, a repeat injection was done and four ions were monitored for that particular macrolide. This enabled us to measure ion ratios. This confirmatory assay required no special manipulations or extra sample preparation steps.

3.4. Validation

3.4.1. Quantification step

The aim of this study was to develop a method for simultaneous detection and quantification of tylosin, tilmicosin, spiramycin, josamycin and erythromycin. The chosen detection mode was MRM. Molecule detection programs based on one product ion were used to quantify substances in a sample.

The most important parameters for assessing a quantitative method are its accuracy and precision, limit of detection and limit of quantification.

The method was validated at MRL/2, MRL and 2 MRL, according to EU guidelines, for muscle, kidney, liver, eggs and milk.

Assay accuracy and repeatability were tested over 3 days by analysing each day 18 different samples (six quality controls, QCs/concentration) of a same spiked pooled biological specimen.

The experimental data are reported in Tables 3 and 4, where all data derive directly from calibration curves. Tylosin, tilmicosin, spiramycin, josamycin and erythromycin were quantified by means of external standard curves constructed from eight points spanning the concentration range from 0 ppb

Table 3
Inter- and intra-assay validation for tylosin, tilmicosin and spiramycin for the five studied matrices using the proposed method[a]

Matrix	MRL	Mean value (ppb)								
		Tylosin			Tilmicosin			Spiramycin		
		Concentration added ($\mu g\ kg^{-1}$)	Concentration found ($\mu g\ kg^{-1}$)	Accuracy (RSD) (%)	Concentration added ($\mu g\ kg^{-1}$)	Concentration found ($\mu g\ kg^{-1}$)	Accuracy (RSD) (%)	Concentration added ($\mu g\ kg^{-1}$)	Concentration found ($\mu g\ kg^{-1}$)	Accuracy (RSD) (%)
Muscle	MRL 1/2	50	53	+6 (3.6)	25	25	0 (4.8)	150	156	+4 (3.4)
	MRL	100	99	−1 (4.6)	50	49	−2 (5.3)	300	297	−1 (5.5)
	MRL 2	200	190	−5 (3.5)	100	104	+4 (7.0)	600	590	−1.7 (7.8)
Kidney	MRL 1/2	50	49	−2 (5.7)	500	505	+1 (6.0)	150	151	+0.7 (5.5)
	MRL	100	101	+1 (2.9)	1000	989	−1.1 (6.5)	300	288	−0.7 (4.4)
	MRL 2	200	201	+0.5 (3.4)	2000	1969	−1.6 (5.9)	600	569	−5.2 (4.7)
Liver	MRL 1/2	50	45	−10 (4.7)	500	428	−14.4 (5.2)	300	269	−10.3 (5.1)
	MRL	100	102	+2 (4.4)	1000	1000	0 (8.7)	600	616	+2.7 (6.2)
	MRL 2	200	202	+1 (3.3)	2000	2141	+7.1 (2.8)	1200	1278	+6.5 (3.1)
Egg	MRL 1/2	50	47	−6 (4.7)	25	23	−8 (8.2)	100	98	−2 (9.4)
	MRL	100	98	−2 (6.1)	50	47	−6 (6.6)	200	195	−2.5 (6.6)
	MRL 2	200	204	+2 (3.5)	100	103	+3 (4.5)	400	409	+2.3 (4.6)
Milk	MRL 1/2	25	25	0 (5.5)	25	25	0 (6.1)	100	106	+6 (5.7)
	MRL	50	49	−2 (6.8)	50	45	−10 (3.1)	200	189	−5.5 (2.6)
	MRL 2	100	97	−3 (7.5)	100	93	−7 (6.6)	400	340	−15 (5.2)

[a] Negative samples were spiked with each macrolide at MRL/2, MRL and 2 MRL. Six replicates were measured at each level on 3 different days. The accuracy is defined as the percentage deviation from the added concentration.

Table 4
Inter- and intra-assay validation for josamycin and erythromycin for the five studied matrices using the proposed method[a]

Matrix	MRL	Mean value (ppb)					
		Josamycin			Erythromycin		
		Concentration added (μg kg^{-1})	Concentration found (μg kg^{-1})	Accuracy (RSD) (%)	Concentration added (μg kg^{-1})	Concentration found (μg kg^{-1})	Accuracy (RSD) (%)
Muscle	MRL 1/2	100	104	+4 (5.3)	200	190	−5 (6.5)
	MRL	200	188	−6 (3.8)	400	395	−1.3 (3.6)
	MRL 2	400	358	−10.5 (3.8)	800	807	+0.9 (5.5)
Kidney	MRL 1/2	200	197	−1.5 (9.2)	200	196	−2 (5.2)
	MRL	400	423	+5.8 (3.2)	400	411	+3 (4.4)
	MRL 2	800	760	−5 (3.2)	800	800	0 (2.4)
Liver	MRL 1/2	100	88	−12 (4.3)	200	177	−12 (6.1)
	MRL	200	206	+3 (4.6)	400	426	+7 (2.9)
	MRL 2	400	404	+1 (5.8)	800	833	+4 (2.4)
Egg	MRL 1/2	100	84	−16 (4.7)	100	83	−17 (4.4)
	MRL	200	206	+3 (7.6)	200	192	−4 (5.6)
	MRL 2	400	402	+0.5 (13.0)	400	435	+9 (2.0)
Milk	MRL 1/2	100	99	−1 (7.3)	20	20	0 (8.0)
	MRL	200	202	+1 (5.3)	40	39	−2.5 (8.1)
	MRL 2	400	363	−9.3 (5.5)	80	75	−6.3 (7.5)

[a] Negative samples were spiked with each macrolide at MRL/2, MRL and 2 MRL. Six replicates were measured at each level on 3 different days. The accuracy is defined as the percentage deviation from the added concentration.

to twice the MRL (0 MRL; 0.25 MRL; 0.5 MRL; MRL; MRL; 1.5 MRL and 2 MRL). The macrolide-to-roxithromycin peak area ratios and concentrations were fitted to a linear equation over the concentration range 0.25 MRL–2 MRL. The correlation coefficients (r) obtained for all separate calibration curves were above 0.99 for each macrolide. The method proved very reproducible in terms of peak shape, peak area and retention time.

As the calibration curves were prepared with pure standard solutions and not with extracted samples, a correction factor had to be introduced into the chromatogram integration method. Thus, three blank samples spiked with known amounts (0.5 MRL, MRL, 2 MRL) of each substance and 1 μg roxithormycin were extracted, analysed by LC–MS–MS, and compared with the expected responses on the calibration curves.

The accuracy expressed as the percentage of deviation from the added concentration was in the range −20 to +10%.

The overall relative standard deviation (RSD) ranged from 2 to 13%.

We conclude from these data that the within-laboratory reproducibility and accuracy are excellent in the tested concentration range for the tested biological specimens and meet the EU accuracy and precision standards (Regulation 93/256/EC) [24].

After analysing the background noise by integrating the ion chromatogram of the control sample into the time window of each macrolide peak, we calculated the detection limit for each macrolide as the mean baseline value of 42 blanks plus three times the standard deviation. Detection limits are influenced by a number of factors (cleanliness of the source, tuning state of the LC–MS system, presence of interfering ions in the mobile phase or sample, etc.), but in our experiments were generally between

0.01 and 37 μg kg^{-1}. All limits were well below the MRL. The highest LOD found was that for spiramycin in milk (37 μg kg^{-1}), but for this compound the MRL is 200 μg kg^{-1}. The LOD of spiramycin in muscle was 26 μg kg^{-1} but the MRL is 300 μg kg^{-1}.

The limit of quantification, defined as the lowest level at which the assay was validated, was equiva-

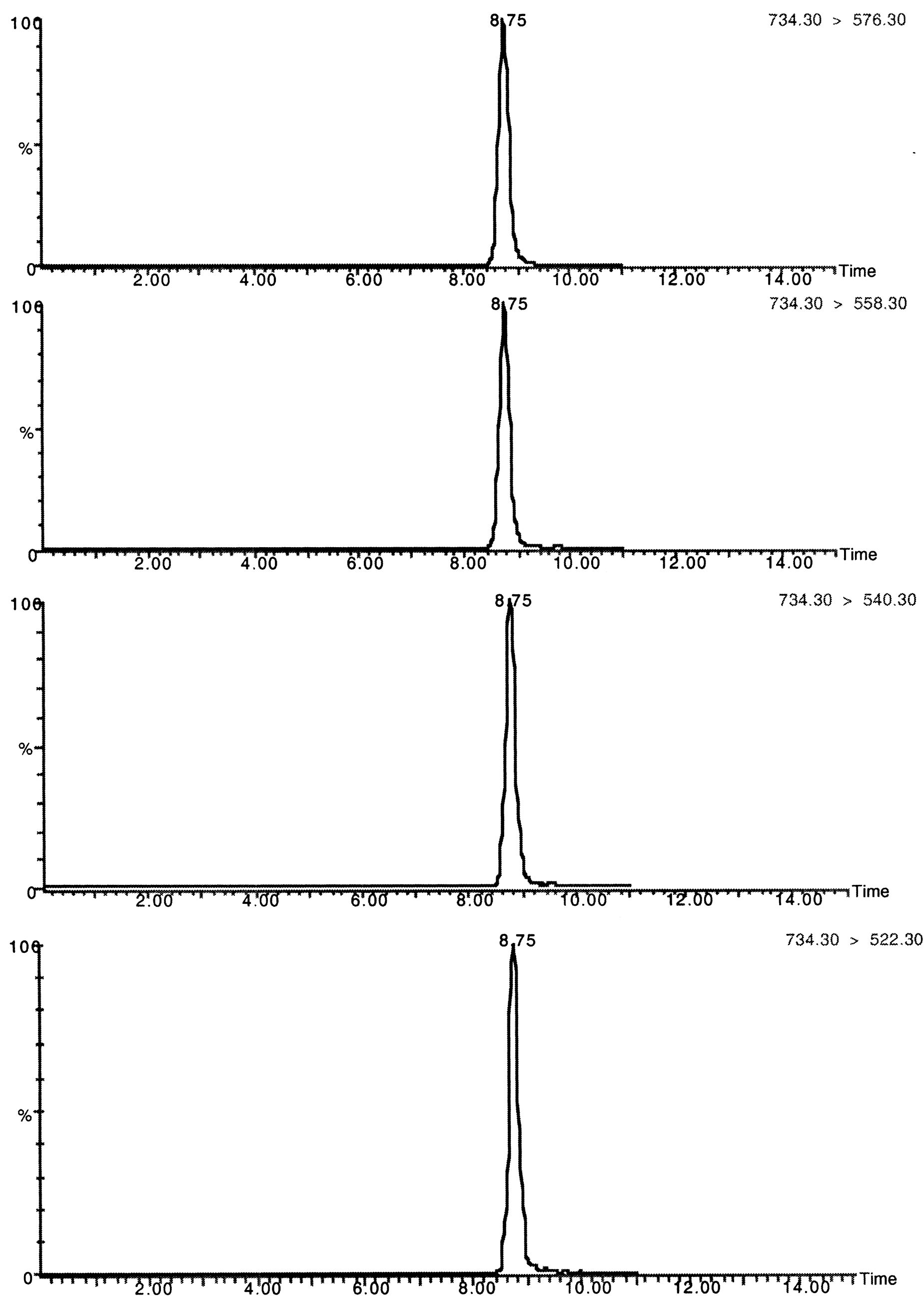

Fig. 4. Confirmation over four ions for a negative muscle sample spiked with erythromycin (200 μg kg^{-1}). The pseudo-molecular ion is the precursor for each MRM trace.

Table 5
Reproducibility of ion ratio measurements for tylosin, tilmicosin and spiramycin[a]

	Tylosin			Tilmicosin			Spiramycin		
	598/772	407/772	318/772	618.9/696.6	522.2/696.6	505.7/696.6	318.2/540.2	522.5/540.2	700.2/540.2
Mean	10.2	9.4	9.4	5.6	4.7	1.2	42.1	13.3	11.9
SD	1.14	0.48	0.49	0.51	0.70	0.17	1.92	0.55	0.95
RSD (%)	11.2	5.1	5.2	9.2	15.0	13.4	4.6	4.1	8.0
n	9	9	9	9	9	9	9	9	9

[a] Each day, three replicates of negative muscle were spiked at MRL/2 concentration and taken through the assay. Muscles extracts were run on 3 different days. Four ions were monitored for each macrolide in the MRM mode. The results show the reproducibility of ion ratios.

lent to half the MRL for each macrolide in the different matrices.

3.4.2. Final confirmation step

During the quantification step, if a peak was found for any of the macrolides, then a repeat injection was done, and four ions were monitored for that particular macrolide. This enabled us to carry out ion ratio measurements.

An example of this, pertaining to a muscle extract spiked with 200 μg kg^{-1} erythromycin, is shown in Fig. 4.

Over 3 consecutive days, three negative samples of muscle, liver, kidney, milk, and egg extract spiked at the MRL/2 were injected into the LC–MS–MS system. Four ions were monitored for each macrolide in the MRM mode. Tables 5 and 6 show the reproducibility of the ion ratio measurements for spiked muscle extract.

The RSDs of the ion ratios ranged from 2.4 to 15%. This shows that the technique is suitable for confirming the presence of macrolides by ion ratio measurements.

For the other tissues, egg and milk, the reproducibility was also good, with RSD values below 20%.

4. Conclusions

We have developed a highly quantitative, linear and specific method for determining five macrolides in tissue, milk and egg samples by LC–MS–MS. We show that the precision and accuracy of the assay are good. We observed no endogenous substances liable to interfere with the assay. The method is specific, quantitative, and reproducible enough to conform to EU recommendations.

Furthermore, since the method requires only a simple extraction without organic solvents and with a short run time, large sample batches (more than 50 samples) can be processed daily.

LC–MS–MS appears as a solution to previous problems, i.e., the low specificity and inaccuracy encountered with both microbiological and HPLC-based assays. Confirmation can be achieved easily by monitoring four ions. The method is quantitative and reproducible within the concentration range 0.5 MRL–2 MRL in muscle, liver, kidney, milk and egg samples.

We thus recommend the present method for routine analysis of residues of the five macrolides erythromycin, spiramycin, tylosin, tilmicosin and josamycin in livestock products.

Table 6
Reproducibility of ion ratio measurements for josamycin and erythromycin[a]

	Josamycin			Erythromycin		
	109/174	228.8/174	600.4/174	558.3/576.3	522.3/576.3	540.3/576.3
Mean	80.1	20.9	14.4	23.1	19.3	16.1
SD	1.89	1.27	0.88	0.95	1.36	1.03
RSD (%)	2.4	6.1	6.09	4.1	7.1	6.4
n	9	9	9	9	9	9

[a] Each day, three replicates of negative muscle were spiked at MRL/2 concentration and taken through the assay. Muscles extracts were run on 3 different days. Four ions were monitored for each macrolide in the MRM mode. The results show the reproducibility of ion ratios.

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References

[1] K. Ninomiya, in: Antibiotics for Animals, Youkendo Press, Tokyo, 1987, p. 307.
[2] W.A. Moats, M.B. Medina (Eds.), Veterinary Drug Residues, ACS Symposium Series 636, American Chemical Society, Washington, DC, 1996, p. 5.
[3] 43rd of the Joint FAO/WHO Expert Committee on Food Additives, in: Evaluation of Certain Veterinary Drug Residues in Food, WHO Tech. Rep. Ser., No. 855, 1995, p. 38.
[4] Official Methods of Analysis of AOAC International, 16th ed., AOAC International, Arlington, VA, 1995.
[5] Official Methods for Residual Substances in Livestock Products, Ministry of Health and Welfare, Japan, 1994.
[6] L.J. Keng, J.O. Boison, J. Liq. Chromatogr. 15 (1992) 2025.
[7] M. Janecek, M.A. Quilliam, M.R. Bailey, D.H. North, J. Chromatogr. 619 (1993) 63.
[8] W. Chan, G.C. Gerhardt, C.D.C. Salisbury, J. AOAC Int. 77 (1994) 331.
[9] L. Renard, P. Henry, P. Sanders, M. Laurentie, J.M. Delmas, J. Chromatogr. 657 (1994) 219.
[10] M. Horie, K. Saito, N. Nose, H. Oka, H. Nakazawa, J. Chromatogr. 655 (1994) 47.
[11] M. Horie, K. Saito, R. Ishii, T. Yosida, Y. Haramaki, H. Nakazawa, J. Chromatogr. A 812 (1998) 295.
[12] S. Pleasance, J. Kelly, M.D. Leblanc, M.A. Quilliam, R.K. Boyd, D.D. Kitts, K. McErlane, R.M. Bailey, D.H. North, Biol. Mass Spectrom. 21 (1992) 675.
[13] C.E. Parker, J.R. Perkins, K.B. Tomer, Y. Shida, K. O'Hara, M. Kono, Am. Soc. Mass Spectrom. 3 (1992) 563.
[14] D.N. Heller, F.J. Schenck, Biol. Mass Spectrom. 22 (1993) 184.
[15] P. Sanders, B. Delepine, Biol. Mass Spectrom. 23 (1994) 369.
[16] B. Delépine, D. Hurtaud, P. Sanders, Analyst 119 (1994) 2717.
[17] B. Delépine, D. Hurtaud-Pessel, P. Sanders, J. AOAC Int. 79 (1996) 397.
[18] D.E. Kielhl, A. Kennington, Mass Spectrom. 9 (1995) 1297.
[19] Y. Kikuchi, T. Teramura, S. Higuchi, J. Chromatogr. B 720 (1998) 81.
[20] Y. Yong-Xili, K. Neufeld, P. Velagaleti, J. Pharm. Biomed. Anal. 16 (1998) 961.
[21] M.E.P. Hows, N.G. Coldham, M.J. Sauer, in: L.A. Van Ginkel, A. Ruiter (Eds.), Proceedings of the Euroresidue IV Conference, Veldhoven, University of Utrecht, Utrecht, 8–10 May 2000, p. 581.
[22] C. Leal, R. Codony, M. Granados, M. Dolors Prat, R. Compano, in: L.A. Van Ginkel, A. Ruiter (Eds.), Proceedings of the Euroresidue IV Conference, Veldhoven, University of Utrecht, Utrecht, 8–10 May 2000, p. 723.
[23] G. Ziv, F. Rasmussen, J. Dairy Sci. 58 (1975) 938.
[24] Commission Decision 93/256/EEC of 14 April 1993, Off. J. Eur. Commun. 1993, No. L 118, 64 and Revision of Commission Decision 93/256/EC. (Recently a draft criteria document was prepared that is intended to replace this reference).