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Residue analysis of macrolides in poultry muscle by liquid chromatography–electrospray mass spectrometry

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Abstract

A liquid chromatography–mass spectrometry method is proposed for the determination of seven macrolides authorised in the EU as veterinary drugs for food-producing animals. Sample treatment involves extraction of the analytes with a water–methanol mixture containing metaphosphoric acid and clean-up by SPE with a cation-exchange cartridge. Separation was carried out in an end-capped silica-based C_{18} column and mobile phases consisting of water/acetonitrile mixtures containing trifluoroacetic acid. A gradient elution, from 28 to 40% acetonitrile was used. Detection was performed by mass spectrometry with electrospray ionisation in the positive mode. Several parameters affecting the mass spectra were studied. The protonated molecular ion was selected for quantitation purposes under selected ion monitoring mode. Detection limits were in the range 1–20 $\mu g\ l^{-1}$. Recoveries ranged between 56 and 93% with RSD lower than 12%. The method has been successfully applied for multiresidue determination of seven macrolides below the MRLs established by the European Union.

Keywords: Food analysis; Multiresidue analysis; Mass spectrometry; Antibiotics; Macrolides

1. Introduction

Macrolide antibiotics are antibacterial agents used as veterinary drugs in food-producing animals with either a curative or prophylactic aim. Residues of these antibiotics in edible animal tissues may therefore occur and this could lead to allergies or bacterial resistance. Consequently, maximum residue limits (MRLs) have been established for these substances in animal products.

Regulatory control programs are often performed in two stages: a simple routine screening that acts as a first sieve, followed by an often more time-consuming and expensive quantitation and confirmation method. Traditionally, screening methods for antibiotics are based on microbiological and immunological assays (ELISA), but they often lack the selectivity and precision required for regulatory purposes. Chromatographic methods, which allows multiresidue analysis, are appropriate alternatives, and several methodologies have been described for

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quantitative analysis of macrolides by liquid chromatography (LC) [1–11]. Although UV absorption is the most common detection system [2–4], some macrolides lack of a suitable chromophore group, and therefore electrochemical detectors [5–8] or fluorescence [9–11], via pre-column derivatization, have also been reported for the determination of some macrolides.

For confirmatory purposes, complementary information allowing analyte identification is essential. Owing to its specificity, liquid chromatography–mass spectrometry (LC–MS) is the preferred technique. Electrospray mass spectrometry (ES-MS) and particle beam mass spectrometry have been coupled to LC for the analysis of some individual macrolides, such as erythromycin [12], roxithromicin [13], spiramycin [14] and tylosin [15] in animal tissues, as well as for the simultaneous analysis of five macrolides [16]. However, this multiresidue method is not directed towards quantitative determination. A method based on LC in combination with tandem MS has recently been reported [17]. It allows quantitation and confirmation of five macrolides in several tissues, but requires expensive, viz. MS–MS instrumentation.

In a previous study [4], we reported a LC method using UV–DAD detection for the determination of seven macrolides: spiramycin (SPI), tilmicosin (TILM), oleandomycin (OLE), erythromycin (ERY), tylosin (TYL), kitasamycin (KIT) and josamycin (JOS). The method proved to be suitable for residue analysis of five of the studied macrolides, whereas for ERY and OLE it lacks the required sensitivity. Moreover, although LC–UV absorption with a diode array detector (DAD) may be used as a simple and economic approach for confirmatory analysis, it is not as powerful as MS to unequivocally assign the identity of the eluting compounds. In the present paper, the LC separation has been coupled to an ES-MS detector to improve sensitivity and to provide specific structural information to be used for confirmatory purposes.

The objectives of this work were to optimise the LC–ES-MS parameters and to assess the capability of the method for the residue analysis of the seven macrolides in animal tissues. The LC separation was adapted to allow macrolides separation while ensuring compatibility with the mass spectrometer and MS detection was optimised. The proposed LC–ES-MS method was successfully applied to the quantitative determination of the seven macrolides, at residue levels, in spiked commercial chicken tissues.

2. Experimental

2.1. Chemicals and solutions

Spiramycin, tylosin tartrate, oleandomycin phosphate, roxithromycin and erythromycin were supplied by Sigma (St. Louis, MO, USA). Tilmicosin, kitasamycin and josamycin were kindly supplied by Elanco Valquímica, (Madrid, Spain), Laboratorios Dr. Esteve, (Barcelona, Spain) and Laboratorios Virbac, (Esplugues de Llobregat, Spain), respectively. The structures of these compounds are shown in Fig. 1.

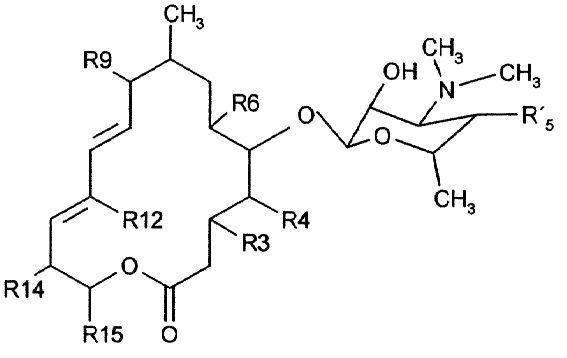
Stock standard solutions (1 g l^{-1}) were prepared by dissolving the compounds in methanol. These solutions were stored in dark glass bottles at 4 °C and were stable for at least 4 months. Aliquots of the standard solutions were diluted with water and final standard solutions were freshly prepared by dilution with a mixture 0.02% trifluoroacetic acid (TFA) (pH 4)/acetonitrile (ACN) (70:30 v/v). Matrix matched standards were prepared similarly, but adding appropriate amounts of an extract from a drug-free chicken tissue.

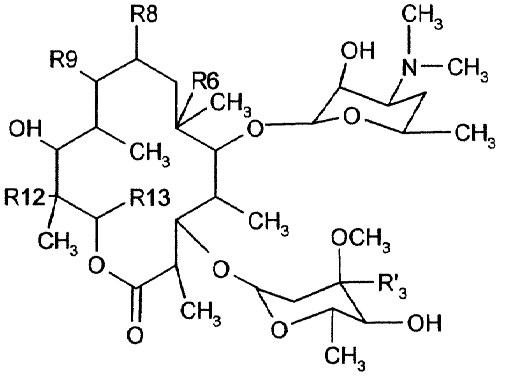
Mobile phase A was a 0.02% TFA aqueous solution and mobile phase B consisted of a mixture of 0.02% TFA and acetonitrile (60:40 v/v). The aqueous solution was filtered through a 0.22-μm Nylon membrane filter (Lida, Kenosha, WI, USA).

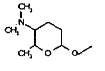
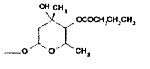
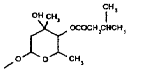
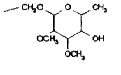
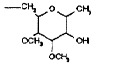
Bond Elut SCX (500 mg) cartridges were purchased from Varian (Harbor City, CA, USA). Hyflo Super-Cel was kindly supplied by World Minerals Division Española (Rubí, Barcelona, Spain). Double-deionized water (Milli-Q, Millipore, Molsheim, France) of 18.2 MΩ cm^{-1} resistivity was used throughout.

2.2. Samples

Chicken samples used for the preparation of spiked muscle were purchased from local groceries. The samples chosen contained no detectable macro-





	R3	R4	R6	R9	R12	R14	R15	R'5
SPI	-OH	-OCH3	-CH2COH		-H	-H	-CH3	
KIT	-OH	-OCH3	-CH2COH	-OH	-H	-H	-CH3	
JOS	-OCOCH3	-OCH3	-CH2COH	-OH	-H	-H	-CH3	
TYL	-OH	-CH3	-CH2COH	=O	-CH3		-CH2CH3	
TILM	-OH	-OCH3		=O	-CH3		-CH2CH3	-OH

	R'3	R6	R8	R9	R12	R13
ROX	-CH3	-OH	-CH3	=NOCH2OCH2CH2OCH3	-OH	-CH2CH3
ERY	-CH3	-OH	-CH3	=O	-OH	-CH2CH3
OLE	-H	-H		=O	-H	-CH3

Fig. 1. Structures of the macrolides studied.

lides. Skin and bones were removed prior grinding the muscle. Minced muscle was kept at −20 °C and thawed before analysis. Spiking was performed by adding a microvolume of an aqueous standard solution containing seven macrolides to each portion of the weighed samples. Spiked samples were left to stand at room temperature for 15 min in the dark before analysis.

2.3. Apparatus

A HP-1100 liquid chromatograph equipped with a HP 1100 diode array detector (Hewlett-Packard, NY, USA) and a 100-μl injection loop was used for the optimisation of the mobile phase. Response was monitored at 204 nm (for OLE, ERY and ROX), 232 nm (for SPI, KIT and JOS) and 287 nm (for TILM and TYL).

LC–MS measurements were carried out with a Waters 2690 separation module, (Waters, Milipore, MA, USA) coupled to a VG Platform II (Fisons Instruments, VG Biotech, Altrincham, UK) quadrupole mass spectrometer equipped with a standard pneumatically assisted electrospray interface (nitrogen flow-rate 20 l h^{-1}).

2.4. Procedures

2.4.1. LC–MS

Samples and standard solutions were filtered through a 0.22-μm Nylon membrane and injected using a 100-μl injection loop. Separation was performed at room temperature on a Hypurity Elite C_{18} (Hypersyil, Cheshire, UK) analytical column (5 μm particle size, 25 cm×4.6 mm I.D.) equipped with a HyPurity Elite C_{18} (10×4 mm) guard column. Mobile phase flow-rate was set to 1 ml min^{-1}. The gradient elution program used was: from 70 to 91% of mobile phase B in 12 min; then to 100% B at 12.1 min and held until 20 min. The mobile phase returned to the initial conditions in 2 min. It took 10 min to reequilibrate the column.

In LC–ES-MS a split system 1/9 was used to introduce the effluent into the ES. The experimental conditions were the following: drying nitrogen was heated to 120 °C and introduced into the capillary region at a flow rate of 400 l/h. The capillary voltage was held at +4 kV and the ion energy to 4 V. The extraction voltage was set to 55 V.

Full-scan data acquisition was performed from m/z 400 to 1200 in centroide mode and using a cycle time of 1.5 s and an inter scan time of 0.15 s.

For quantitation purposes, the $[M+1]^+$ ion of each compound was monitored in the Selected Ion Monitoring (SIM) mode, with a dwell time of 0.2 s. Calibration was done using ROX as internal standard. The ions monitored for confirmatory analysis are listed in Table 1. Data were processed by means of Mass Lynx software.

2.4.2. Sample treatment

A 2.5-g amount of tissue was manually shaken for 5 min with 17 ml of 0.3% metaphosphoric acid–methanol (7:3, v/v) in a glass tube. The extract was filtered through a 2-mm layer of Hyflo Super-Cel coated on a suction funnel. The filtrate was rotary evaporated to about 12 ml and loaded on a Bond-Elut SCX 500-mg cartridge, previously conditioned with 5 ml of methanol and 10 ml of 0.1 *M* KH_2PO_4 (pH = 4.4). The cartridge was then washed with 10 ml of water and 3 ml of 0.1 *M* K_2HPO_4 (pH 8.8). Elution was carried out with 10 ml of methanol. The extract was rotary evaporated to dryness at 45 °C,

Table 1
Main ions obtained at extraction voltage of 55 V with their tentative assignations

Compound	M_z	m/z	Tentative assignation
SPI	842	843	$[M+H]^+$
		540	$[M+H-C_7H_{13}O_3-C_8H_{16}O_2N]^+$
		422	$[M+2H]^{2+}$
TILM	868	869	$[M+H]^+$
		696	$[M+2H-C_8H_{16}O_3N]^+$
		435	$[M+2H]^{2+}$
OLE	687	688	$[M+H]^+$
		544	$[M+2H-C_7H_{13}O_3]^+$
ERY	733	734	$[M+H]^+$
		576	$[M+2H-C_8H_{15}O_3]^+$
		558	$[M+2H-C_8H_{15}O_3-H_2O]^+$
TYL	915	916	$[M+H]^+$
KIT	771	772	$[M+H]^+$
ROX	836	837	$[M+H]^+$
		679	$[M+2H-C_8H_{15}O_3]^+$
JOS	827	828	$[M+H]^+$

reconstituted with 1 ml of the mixture 0.02% TFA (pH 4)/ACN (70:30, v/v) that contains the internal standard, filtered and injected (100 μl) into the chromatographic system.

3. Results and discussion

3.1. Optimisation of the chromatographic separation

The optimisation of the chromatographic conditions for the separation of the eight macrolides, including ROX, which was used as internal standard, was based on a previous study [4] where separation was achieved with a C_{18} stationary phase and binary gradient elution using water–acetonitrile mixtures acidified to pH about 2 with phosphate buffer. As coupling LC to MS requires volatile mobile phases, TFA and formic acid were used to adjust the pH instead of phosphoric acid buffer. The chromatograms obtained showed that TFA provided more symmetric peaks than formic acid, and the best peak shapes were obtained at TFA concentrations higher than 0.01%. The gradient profile was also adjusted to obtain a good separation within an acceptable analysis time. The best separation was obtained using a binary gradient composed of 0.02% TFA (phase A) and a mixture of TFA 0.02% and ACN (60:40 v/v) (phase B), at a flow rate of 1 ml/min. The selected gradient program is described under the Procedures section.

3.2. Optimisation of the LC–ES-MS method

In order to optimise the ES-MS conditions, different parameters influencing mass spectra were investigated: the drying and auxiliary nitrogen flow-rates, the source temperature, the capillary voltage, the ion energy and cone voltage. These optimum operational parameters were obtained under full-scan conditions in the positive mode from 400 to 1200 Da using a flow injection-ES-MS set up. Individual 5 mg l^{-1} macrolide standard solutions of TYL, OLE and JOS were directly injected (20 μl) into a carrier consisting of 0.02% TFA aqueous solution/ACN (65:35). These analytes were selected as representative compounds since they cover the whole macrolide mass range. Selected conditions for the detection system are summarised in the Procedures section.

As the most influencing parameter was extraction voltage, this parameter was further studied for each macrolide. To establish the optimum extraction voltage for the analysis of all the compounds, the normalised abundance of the $[M+H]^+$ peak versus extraction voltage, from +30 to +80 V, for each macrolide was studied (Fig. 2). JOS, TYL, KIT, ROX and SPI showed maximum response at 60 V, whereas 70 V was found optimum for TILM and 40 V for OLE and ERY. It was decided to apply the same extraction voltage to all the macrolides, and 55 V was selected as a compromise value that provided appropriate sensitivity for the whole set of analytes. The main ions of each compound obtained at this voltage are listed in Table 1.

The mass spectra of most of the macrolides (TILM, ERY, TYL, KIT, ROX and JOS) (Fig. 3), obtained in the full-scan mode at the selected conditions, showed that the protonated molecular ion $[M+H]^+$ was the predominant ion, except for SPI and OLE. (Table 1). The base peak for SPI was $[M+2H]^{2+}$ ($m/z=422$), whereas the predominant ion for OLE was $[M-C_7H_{13}O_3+2H]$ ($m/z=544$), which corresponds to the loss of sugar moieties from $[M+H]^+$. These fragments were the predominant ions in the mass spectra of SPI and OLE even at 30 V extraction voltage.

The loss of sugar moieties was also observed in the spectra generated at 55 V for TILM, ERY and ROX, but the relative abundance of these ions was lower than the molecular peaks (Fig. 3). TYL, KIT and JOS spectra consisted only of the protonated molecular ion $[M+H]^+$. Increasing the extraction voltage up to 80 V was neither successful to achieve more fragmentation.

The analytical performance characteristics of the LC–ES-MS method were first determined from standard solutions of macrolides in pure solvent. In order to increase sensitivity all measurements were carried out in SIM acquisition mode using the mass corresponding to $[M+H]^+$ ion for each macrolide. ROX, which is a macrolide widely used in human medicine but not as a veterinary drug, was used as internal standard. Data were calculated from both peak height and peak area, and since no significant

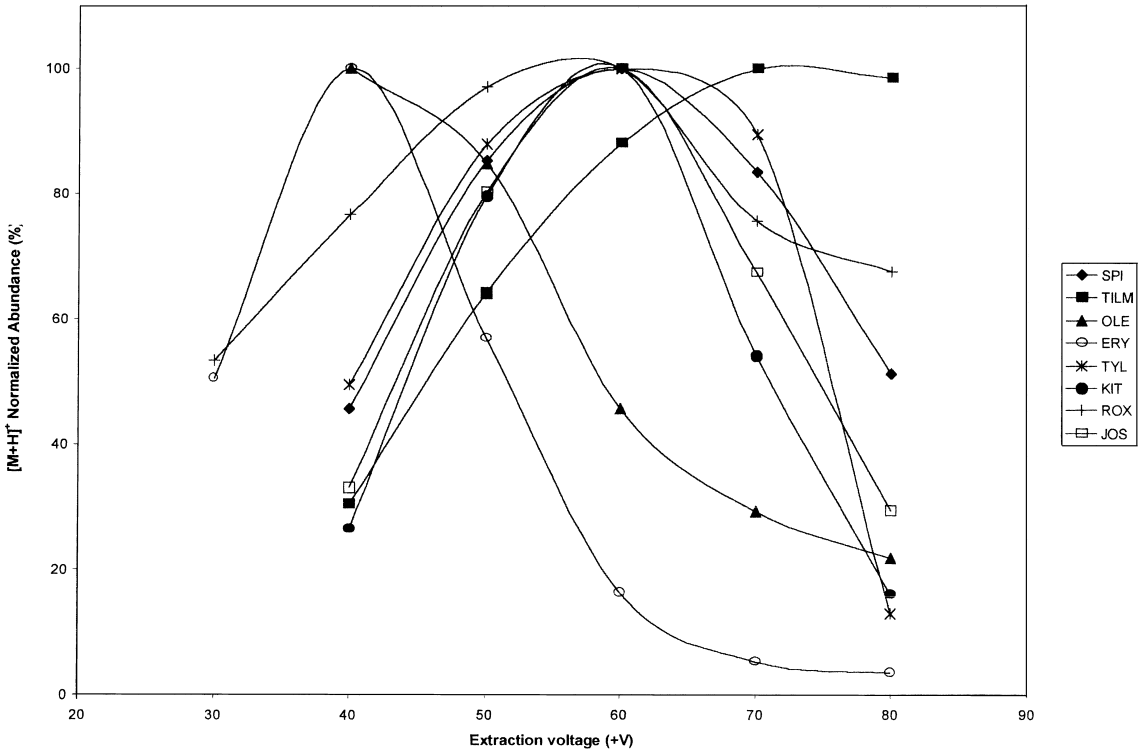


Fig. 2. Variation of the normalised abundance (%) of the $[M+H]^+$ ion of each macrolide versus extraction voltage.

differences were found the former was selected from quantitation.

Linear calibration graphs ($r^2>0.99$) were obtained up to 1000 μg l^{-1} for all the macrolides studied. Higher concentrations were not tested. The repeatability of the method, was calculated for a standard solution at 75 μg l^{-1} ($n=5$) of each compound, in the same day. The detection limits, calculated by using a signal-to-noise ratio of 3, ranged from 1 to 23 μg l^{-1}, and was about 10–20-fold lower than those obtained from the spectra in full-scan mode. Quality parameters are summarised in Table 2.

3.3. Analysis of chicken tissues

The sample treatment procedure, which was adapted from that proposed by Horie et al. [3], involves lixiviation of macrolides with methanol–water at pH 4–5 (30:70) and clean-up by SPE using a cation-exchange phase (see Section 2.4). To ensure retention of analytes in the SPE cartridge the methanol content of the loading solution must be keep below 5% and the pH about 4–6. Therefore, because of high methanol content of the extracting solution, the extract was partially evaporated. Moreover, to avoid losses due to the washing step, the volume of the washing basic solution had to be limited to 2–3 ml.

Prior to the analysis of chicken tissues, the matrix effect on the chromatographic determination was investigated by comparing LC–MS chromatograms obtained from standard solutions in pure solvent and from matrix matched standards, prepared with extracts from different analyte-free samples. It was observed that the tissue extract matrix led to alterations in the chromatogram, which included additional peaks and variations in some peaks, mainly SPI, TILM and ERY. The additional peaks caused no interference, since they eluted prior to the macrolides and were well resolved. In contrast, the effect on analytes behaviour was significant: slightly longer

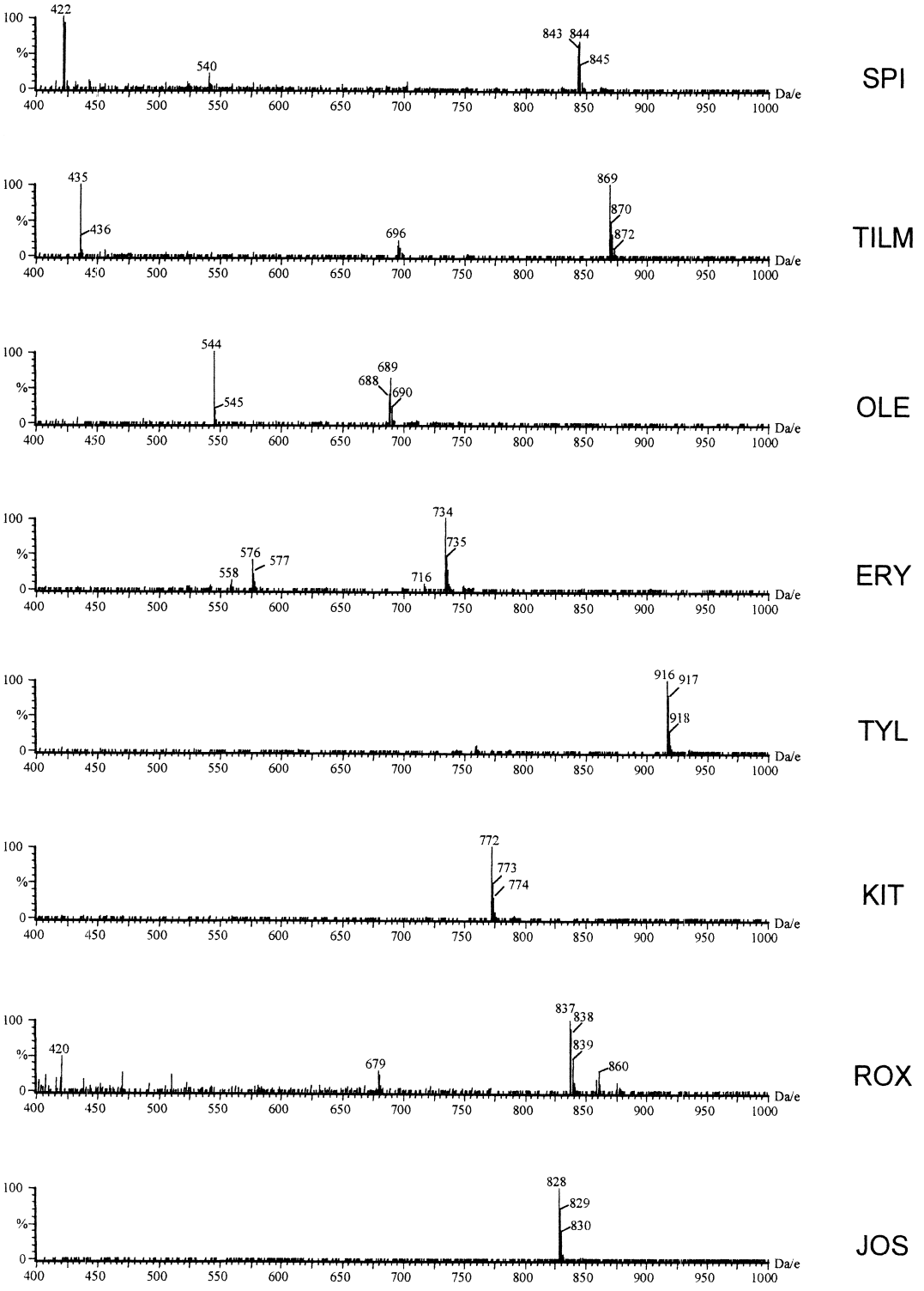


Fig. 3. ES mass spectra obtained at +55 V.

Table 2
Quality parameters of the LC–ES-MS method

	Repeatability[a]		LOD[b] ($\mu g\ l^{-1}$)
	tr (min)	Area	
SPI	0.46	9	23
TILM	0.26	6	8
OLE	0.15	4	6
ERY	0.28	8	3
TYL	0.36	2	1
KIT	0.21	3	4
JOS	0.06	1	1

[b] Calculated using a signal-to-noise of 3.
[a] $n=5$, at 75 $\mu g\ l^{-1}$ for each macrolide. Expressed as %RSD.

retention times, and higher peaks. Changes on retention time were about 10, 4 and 3% for SPI, TILM and OLE, respectively. The observed increase in the peak intensities, which obviously led to an increase in the slope of the calibration curves, was significant for SPI and TILM (about 25%), and dramatic (about 100%) for ERY (Table 3). Variation in peak intensities are presumably due to some matrix components that coelute with analytes and cause changes in the electrospray ionisation in comparison with standard solutions in pure solvent. To minimise variability due to this effect, calibration should be carried out by using matrix matched standards or the standard addition method, and the former was chosen since standard addition is more tedious for a large number of samples. Matrix extracts from three samples from different origins led to reproducible retention times and calibration curves. The matrix effect did not result in significant changes in the detection limits, and the LOD values obtained from matrix matched standards were lower than 4 $\mu g\ l^{-1}$ for all macrolides, except for TILM (8 $\mu g\ l^{-1}$) and SPI (35 $\mu g\ l^{-1}$).

Table 3
Calibration equations from aqueous standards and matrix matched standards

	Water	Chicken tissue extract
SPI	$y=0.106x+0.001$ $r^2=0.9986$	$y=0.129x-5e-5$ $r^2=0.9965$
TILM	$y=0.272x-0.004$ $r^2=0.9967$	$y=0.342x+0.027$ $r^2=0.9939$
OLE	$y=0.483x+0.019$ $r^2=0.9995$	$y=0.476+0.024$ $r^2=0.9988$
ERY	$y=0.312x-0.01$ $r^2=0.9973$	$y=0.731x+0.044$ $r^2=0.9970$
TYL	$y=0.622x+0.015$ $r^2=0.9985$	$y=0.652x+0.004$ $r^2=0.9996$
KIT	$y=0.409x+0.006$ $r^2=0.9993$	$y=0.426x+0.159$ $r^2=0.9977$
JOS	$y=1.770x-0.041$ $r^2=0.9988$	$y=1.689x+0.012$ $r^2=0.9991$

To evaluate recoveries, the proposed method (see Procedures section) was applied to the analysis of spiked macrolide-free samples of chicken tissue. Three samples of different animals were each spiked with the seven macrolides, and each sample was spiked at three levels. The spiking levels, which ranged from 40 to 800 $\mu g\ Kg^{-1}$, were chosen depending on the MRL of the analyte, (0.5, 1 and 2 times the MRLs established for each macrolide). Typical chromatograms from extracts of a blank chicken muscle and the same sample spiked with the seven macrolides, at the MRL level, are shown in Fig. 4. Data from the nine analysed samples led to recovery rates ranging from 56 to 93% (Table 4). Although the method has low recoveries for OLE and TYL (57 and 56%), they can be considered acceptable since data are reproducible, and thus a correction for recovery can be applied. The results indicate that this LC–ES-MS method is suitable for the analysis of residues of macrolides in edible animal tissues below their MRLs.

LC–ES-MS also provides structural data about the compounds, which is a very useful tool for confirmatory purposes. However, since fragmentation achieved with ES interface is rather limited, it is often difficult to meet the criteria accepted in the frame of the EU for residue analysis in order to

Table 4
Recovery data for chicken muscle ($n=9$)

	MRL (μg/Kg)	Spiking level (μg/Kg)	Recovery ($\pm$RSD (%))
SPI	200	100–400	83±4
TILM	75	40–150	93±9
OLE	–	100–400	57±12
ERY	400	200–800	65±11
TYL	100	50–200	56±9
KIT	–	100–400	70±2
JOS	200	100–400	67±4

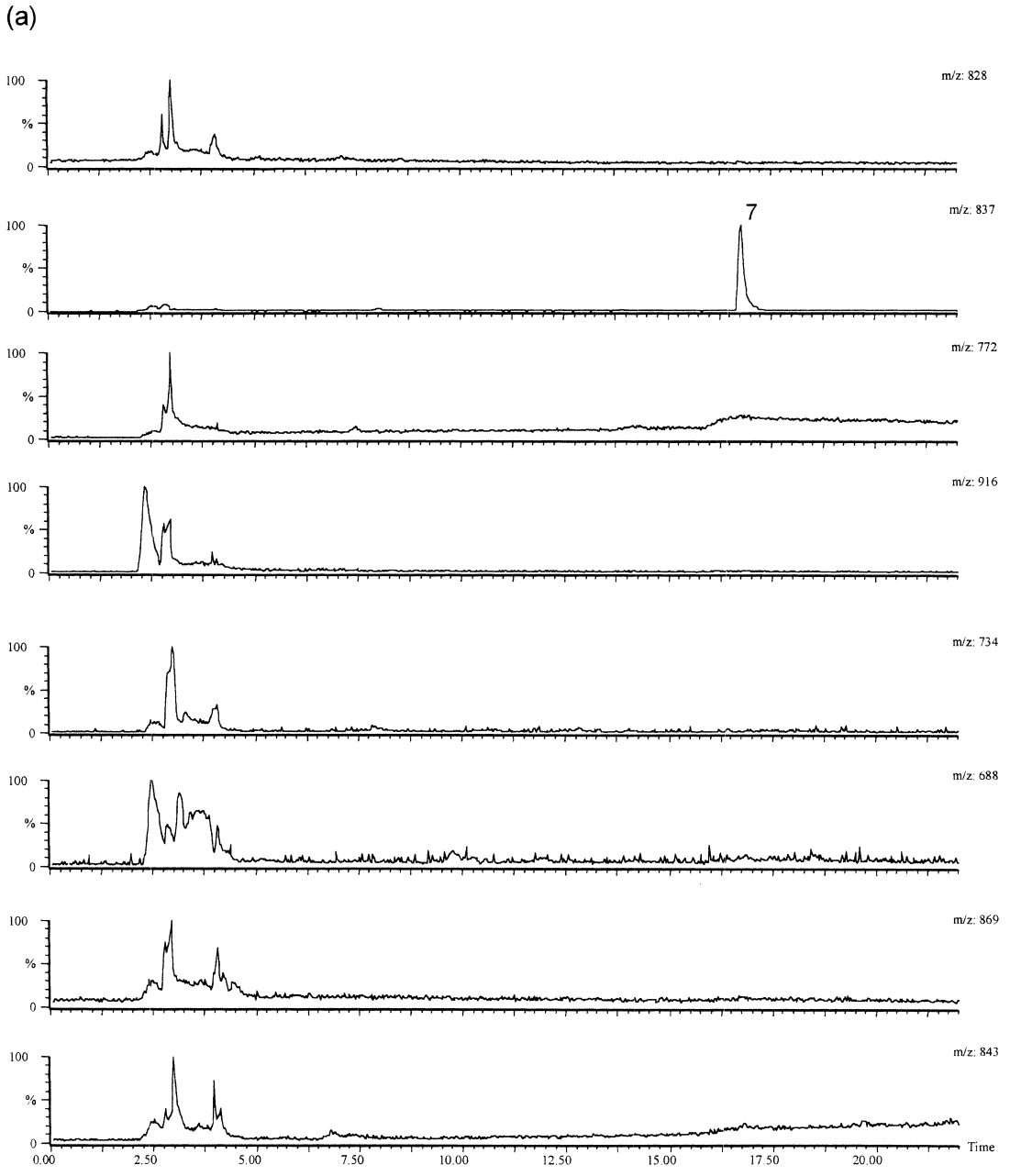


Fig. 4. LC–ES-MS chromatograms of a blank tissue (a) and a spiked tissue chicken muscle at 1 MRL level for each macrolide (b). Peaks: 1: SPI, 2: TILM, 3: OLE, 4: ERY, 5: TYL, 6: KIT, 7: ROX (Internal standard), 8: JOS.

satisfactorily confirm the identity of the analytes [18].

In this case, LC–ES-MS supplies information about the molecular ion, which is very valuable to check the identity of the compound, especially when its molecular mass is relatively high, as for macrolides. However, only SPI, TILM and ERY spectra provides the three diagnostic ions (or identification

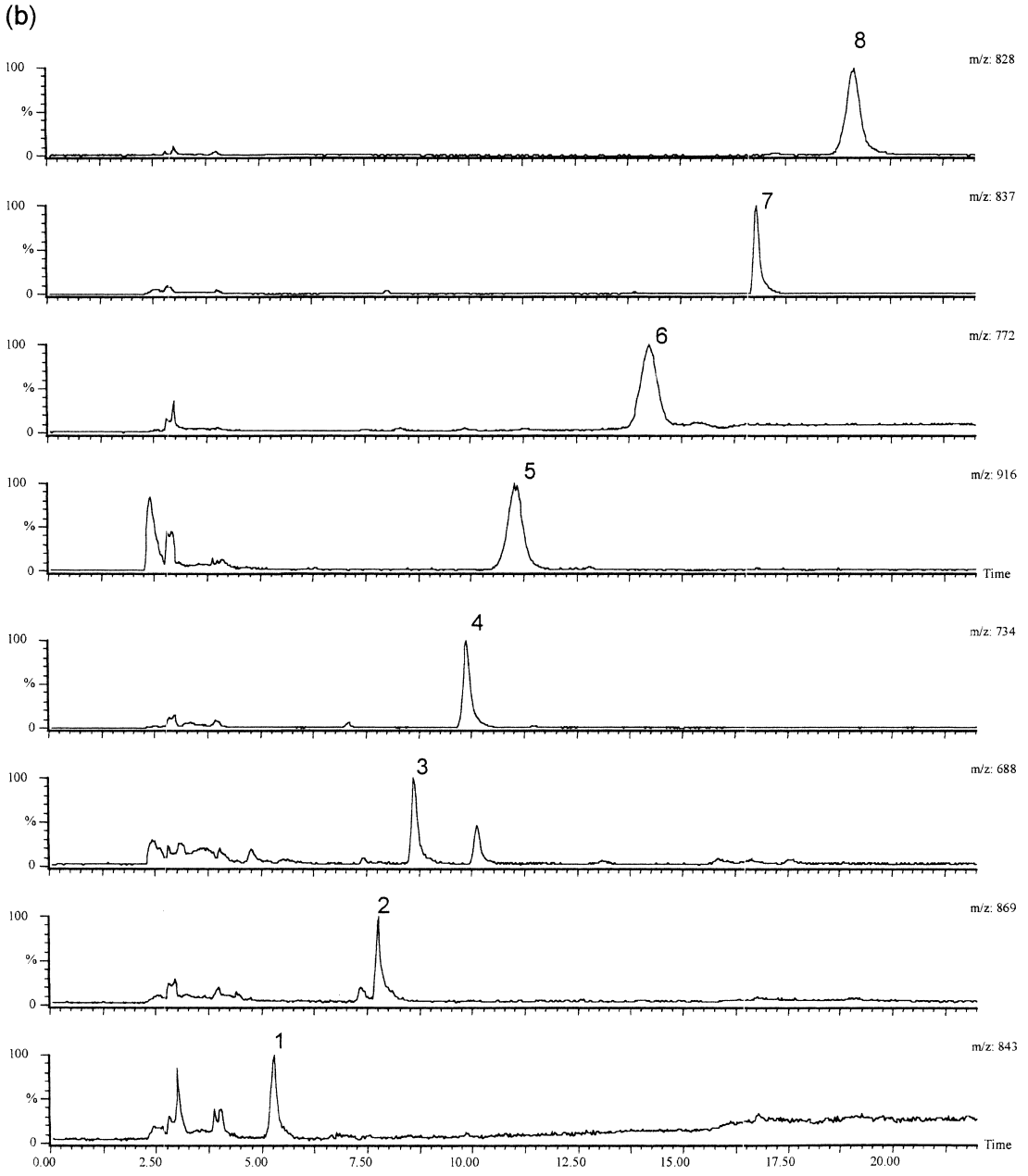


Fig. 4. (*continued*)

points) required for confirmation of the identity of compounds with an established MRL. The ions selected for confirmatory purposes for each analyte are listed in Table 1. Repeated assays were carried out with spiked muscles at several macrolide concentrations, and differences between expected and experimental relative abundances ratios were below 15%.

As pointed out in the previous section, no fragmentation was obtained for TYL, KIT, JOS, and only two diagnostic ions could be used for OLE and ROX. Therefore, a complementary technique should

be used for the confirmation of these macrolides according to the EU guidelines [18].

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