Journal of Chromatography B, xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of 17 macrolide antibiotics and avermectins residues in meat with accelerated solvent extraction by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 24 December 2011
Accepted 7 April 2012
Available online xxx

Keywords:
Macrolide antibiotics
Avermectins
ASE
Animal origin food
LC–MS/MS

ABSTRACT

A method has been developed for simultaneous determination of 17 kinds of macrolide antibiotics and avermectins residues in animal origin foods. Samples were extracted with acetonitrile-methanol using accelerated solvent extraction (ASE) instrument. Parameters such as extraction temperature and pressure were investigated by a fractional factorial design (FFD) and the selected extraction (60 °C, 1500 psi for 10 min in two cycles) was most effective. High correlation coefficients ($r > 0.999$) of 17 macrolide antibiotics and avermectins were obtained within their respective linear ranges (2–400 μg/kg) using roxithromycin as internal standard. The recoveries of them were above 75% at different spiked levels in various samples. Using ASE the method was featured as short extraction times, reduction use of extraction solvent, high extraction yields, with high level of automation.

1. Introduction

Macrolide antibiotics are a group of antibacterial compounds that are active against gram-positive and some gram-negative bacteria, which are widely used to treat respiratory diseases and enteric infections in cattle, sheep, swine and poultry [1]. Avermectins, belonging to macrolide compounds, have also been widespread used as antiparasitic drug for protecting human, animal health and crop [2]. The incorrect use of these drugs can leave residues in food products and this can have undesirable effects on consumer health. To this respect, different actions have been taken by international regulatory bodies, such as the European Union [3], the US Food and Drug Administration (FDA) [4], China and others, setting maximum residue levels (MRLs) for some macrolides in muscle tissue, fat, liver and kidneys. Table 1 listed the values by EU Commission regulation 37/210 [3]. Erythromycin, kitasamycin, spiramycin, tilmicosin, tylosin and avermectin, doramectin are included with MRL (Table 1). Roxithromycin, medecamycin, troleandomycin, josamycin and others have no MRL values.

So far, analytical methods for the determination of macrolides antibiotics in animal products, biological samples, and apicultural products include the use of liquid chromatography (LC) with ultraviolet (UV) or fluorometric detection [5–8], liquid chromatography–mass spectrometry (LC–MS) [9,10], and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [11–16]. For avermectins, only a few works dealing with LC determination have been reported in the literature [17,18]. There were no qualitative and quantitative methods for both macrolides antibiotics and avermectins which belong to different macrolide subgroups residues in edible food.

On the other hand, efforts have been directed to attain high-throughput methods able to extract a large number of samples in a short time. ASE is a technique that uses solvent at a relatively high pressure and temperature without their critical point being reached. This improves efficiency compared to extractions at room temperature and atmospheric pressure. Until now there are only three publications were reported to use accelerating solvent extraction (ASE) to extract macrolide antibiotics from fish [19–21], milk [21] and animal tissues [19,20]. The macrolide antibiotics selected were erythromycin, josamycin, roxithromycin, spiramycin, tilmicosin, troleandomycin and tylosin. All groups remarked on the technology's benefits in providing rapid and reliable analysis. However, to the best of our knowledge, there was no method for 17 macrolides including avermectins residues in meat using ASE by LC–MS/MS.

The present study focuses on developing a robust, simple and practical method capable of simultaneously extracting and

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http://dx.doi.org/10.1016/j.jchromb.2012.04.011

Table 1
The maximum residue limits of macrolides (μg/kg).

Pharmacologically active substance	Marker residue	Animal species	Target tissues	MRL (μg/kg)
Erythromycin	Erythromycin A	All food producing species	Muscle	200
			Fat	200
			Liver	200
			Kidney	200
			Milk	40
			Egg	150
Acetylisovaleryltylosin	Acetylisovaleryltylosin and 3-O-acetylisovaleryltylosin	Porcine	Muscle	50
			Skin + fat	50
			Liver	50
			Kidney	50
Kitasamycin	Kitasamycin	Porcine/poultry	Liver	200
			Kidney	200
			Muscle	200
Tilmicosin	Tilmicosin	Bovine/sheep	Muscle	100
			Fat	100
			Liver	1000
			Kidney	300
		Sheep	Milk	50
		Swine	Muscle	100
			Fat	100
			Liver	1500
			Kidney	1000
		Chicken	Muscle	75
			Skin + fat	75
			Liver	1000
			Kidney	250
Tylosin	Tylosin A Sum of tylosin and 3-O-tylosin	Porcine	Muscle	50
			Skin + fat	50
			Liver	50
			Kidney	50
		Poultry	Skin + fat	50
			Liver	50
Avermectin	Avermectin B1a	Bovine	Fat	100
			Liver	100
			Kidney	50
		Goat	Fat	50
			Liver	25
			Kidney	20
			Muscle	25
Doramectin	Doramectin	All mammalian food producing species	Fat	150
			Liver	100
			Kidney	60
			Muscle	40
Ivermectin	22,23-Dihydroavermectin B1a	Bovine	Muscle	10
			Fat	40
			Liver	100
			Milk	10
		Swine/sheep	Muscle	20
			Fat	20
			Liver	15
Spiramycin	Sum of spiramycin and neospiramycin	Bovine	Muscle	200
			Fat	300
			Liver	300
			Kidney	300
			Milk	200
		Chicken	Muscle	200
			Skin + fat	300
			Liver	400
	Spiramycin 1	Porcine	Muscle	250
			Liver	2000
			Kidney	1000

determining 17 macrolide antibiotics and avermectins in meat. Optimization of ASE (e.g. composition of the extraction solvent, temperature, pressure and number of cycles) was carried out by using an experimental design methodology, consisting of a fractional factorial design (FFD). The present method allows to efficiently extract the target compounds from meat matrices with a significant reduction in the sample treatment time, mainly due to method automation and the elimination of a further SPE clean-up step. The method was successfully applied in the analysis of real tissue sample.

Please cite this article in press as: Y. Tao, et al., J. Chromatogr. B (2012), http://dx.doi.org/10.1016/j.jchromb.2012.04.011

2. Experimental

2.1. Reagents and materials

Spiramycin (SPM, 99.0%), troleandomycin (OLE, 99.0%), erythromycin ethylsuccinate (ERE, 96.8%) and doramectin (DOR, 99.0%) were purchased from Labor Dr. Ehrenstorfer GmbH (EQ Laboratories, Atlanta, GA). Ivermectin (IVE, 93.2%), tilmicosin (TIL, 90.0%), erythromycin (ERM, 93.5%), roxithromycin (ROX, 98.2%), clarithromycin (CLA, 98.9%), kitasamycin (KIT, 97.8%), medecamycin (MED, 99.0%), josamycin (JOS, 99.0%), azithromycin (AZI, 98.2%), tylosin (TYL, 91.7%), acetyl spiramycin (ASP, 99.0% including single acetyl spiramycin II, single acetyl spiramycin III, diacetyl spiramycin II) and abamectin (ABA, 98.7%) reference standard were purchased from Sigma (St. Louis, MO, USA).

Methanol and acetonitrile were obtained from Fisher (Bar-Bel, France). Ethylenediaminotetracetic acid sodium salt (Na_2EDTA) and sand (Crystobalite, 40–200 mesh size) were purchased from Beijing Agela Technologies Company (Beijing, China). Before using the sand, metal impurities on the surface sand were blocked by treatment with EDTA. In particular, 120 g of sand was covered with 240 mL of 0.1 mol/L EDTA during 5 min, and sand was completely dried in an oven at 100 °C.

Filter membranes (0.22 μm) provided by Agilent (Palo Alto, USA) were used to filter the extracts before the injection into chromatographic system. De-ionized water (18 MΩ cm) was generated by a Milli-Q water-purification system (Bedford, USA).

2.2. Standard solutions

Stock standard solutions of the macrolide antibiotics and avermectins were prepared by dissolving each of them (1.0 mg/mL) in methanol. Working mixed standard solutions (0.1 mg/mL) were prepared by dilution of the stock standards in methanol. Individual stock solutions of macrolide antibiotics and avermectins (0.1 mg/mL) were prepared in diluted solution. They were stable in plastic tube for 6 months at 2–4 °C. Tuning solutions of each analyte (1.0 mg/L) were prepared by diluting individual stock solutions with methanol. A working solution and a standard mixture used to fortify the samples, were prepared by diluting individual stock solution with diluted solution. When lower fortification mixture was needed, an extra macrolide antibiotics and avermectins dilution was prepared. These solutions were stored in plastic tubes at 2–4 °C and stayed stable for up to 1 month.

2.3. Samples

All samples including swine, bovine tissues (muscle, kidney and liver), were collected from supermarkets and local markets in Wuhan (Hubei, China). Typically, 500 g tissue samples were first minced by a kitchen homogenizer and stored at −18 °C until experiment. For the recovery experiments, homogeneous samples were fortified with right amount of the mixed standard solutions and the internal standard substance with the level of 2 μg/mL solvent containing roxithromycin (ROX) 100 μL, then staying for 1 h before extraction respectively.

2.4. Sample preparation using ASE

Samples were extracted with a Dionex accelerated solvent extractor 200 (Dionex, 99 Sunnyvale, CA, USA). For recovery studies, 2 g meat sample was accurately weighed in a centrifuge tube and spiked with variable volume of stock solutions of macrolide antibiotics and avermectins. The sample was left for 30 min at room temperature to ensure the appropriate distribution of drugs in the matrix. Then, 12 g of EDTA-treated sand was added, and the mixture was blended with the pestle for less than 15 min, until apparently dry material was obtained. This mixture was introduced into a stainless steel extraction cell (22 mL capacity), which was positioned in the system of ASE 200 connected to a four-bottle solvent controller. Nitrogen at pressure of 145 psi was supplied to assist the pneumatic system and to purge the extraction cells. The optimum procedure was to extract the mixtures with acetonitrile/methanol (1/1, v/v) at 60 °C and 1500 psi for 10 min (static time) in two cycles; water volume flushing respect to the cell size in percentage, 60%; preheating for 2 min and purging for 60 s. Finally, each resulting extract was evaporated to dryness under vacuum distillation, and the residue was rinsed with 5 mL of methanol, evaporated to dryness under a nitrogen flow at 45 °C and re-dissolved in 1.0 mL mobile phase, vortexed, and filtered through a 0.22 μm membrane filter and collected into an LC auto sampler vial, and finally injected into LC–MS/MS for analysis.

2.5. LC–MS/MS analysis

Analysis was carried out by using a thermoelectron TSQ quantum access triple quadrupole mass spectrometer coupled to a surveyor LC pump and surveyor LC autosampler. The separation was achieved using a Thermo Hypersil Gold C_{18}, 100 mm × 2.1 mm, 5 μm column at 35 °C. The mobile phase A was methanol, while the mobile phase B was formate buffer (containing 5 mM formate ammonium and 0.1% formic acid). The gradient is, 0.0 min: B/A (70/30, flow rate: 0.25 mL/min); 5.0 min: B/A (5/95, flow rate: 0.25 mL/min); 15.1 min: B/A (70/30, flow rate: 0.4 mL/min); 20 min: B/A (70/30, flow rate: 0.4 mL/min). The divert valve was switched to the MS at 5.0 min and to waste at 15.0 min. Each elution was followed by a 5 min column re-equilibration period with flow rate at 0.4 mL/min. This was done to minimize changes in retention times caused by matrix loading on the column. The mass spectrometer was tuned using a syringe pump to introduce tylosin standard solution (10 mg/L in methanol) at a rate of 10 μL/min into a stream of mobile phase (200 μL/min) via a T-union. The source parameters were optimized by monitoring the MS and MS/MS spectra of the residues. Multiple reaction monitoring (MRM) was performed on each of the analyte protonated molecular ions using the parameters: source voltage was 4.5 kV, capillary temperature 350 °C, sheath gas (nitrogen) 35, auxiliary gas 5, Q1 peak width 0.70 amu, Q3 peak width 0.50 amu, collision gas (argon) 1.50 mTorr, scan width 1–2 amu, and scan time 0.3–0.5 s. Collision energies were set at the maximum for each transition, ranging from 18 to 30 eV. These parameters (see Table 2) were optimized for matrix extracts to confirm macrolide residues.

3. Results and discussion

3.1. Optimization of LC–MS/MS conditions

The chromatography conditions were adjusted in order to develop a quick method and to improve the analytes separation. A gradient proved quite efficient, as only 15 min were required to elute the analytes. Taking into account the 5 min post-run, the total time of the analysis was 20 min. Yet a switch valve system was proved to be very useful. The 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. Good reproducibility was achieved using a mobile phase consisting primarily of 0.1% formic acid and 0.005 M ammonium acetate.

The macrolide antibiotics are characterized by a common chemical structure including a macrocyclic lactone ring with aminosugars, positive ESI mode was suitable for analysis of macrolide antibiotics. Monitoring of the $[M+Na]^+$ ion tended to

Table 2
The optimized parameters of selected reaction monitoring.

Compound	M_W	Retention time (min)	Precursor ion (m/z)	Qualitative ions (m/z)	Quantitative ions (m/z)	Collision energy
Tilmicosin	868.0	8.06	435.5	125.8; 339.9	339.9	33; 18
Spiramycin	847.6	7.13	425.5	173.9; 335.8; 366.1	173.9	21; 13; 14
Erythromycin A	733.0	8.90	734.0	157.9; 558.1; 679.0	679.0	23; 17; 21
Ethylsuccinate	733.7	8.86	734.7	157.8; 558.2; 576.1	576.1	29; 20; 20
Azithromycin	748.5	7.53	749.5	82.8; 157.8; 590.9	590.9	35; 33; 25
Kitasamycin	771.0	8.77	772.0	109.4; 174.1; 558.5	174.1	37; 26; 28
Clarithromycin	748	9.46	748.5	157.8, 589.9	589.9	32, 17
Troleandomycin	771.5	9.05	772.5	116.0; 158.0; 585.9	585.9	35; 24; 21
Medecamycin	813.5	9.17	814.5	108.7; 173.8	173.8	27; 30
Josamycin	827.5	9.28	828.5	108.6; 174.0; 185.2	174.0	13; 31; 40
Roxithromycin	836.5	9.60	837.5	157.7; 557.9; 679.0	679.0	32; 22; 22
Tylosin	915.6	8.79	916.6	100.9; 155.9; 173.8	173.8	33; 43; 37
Single acetyl spiramycin II	926.6	7.56	927.6	108.9; 173.7	173.7	37; 37
Single acetyl spiramycin III	940.0	8.01	941.6	109.2; 173.7	173.7	46; 33
Diacetyl spiramycinII	968.6	7.99	969.6	108.9; 173.8; 581.4	173.8	31; 37
Abamectin	872.5	12.55	890.6	305.1; 567.2; 642.9	567.2	26; 19; 17
Ivermectin	875.4	14.01	892.6	307.0; 551.0; 569.2	569.2	25; 24; 14
Doramectin	898.8	13.18	916.6	330.8; 593.3; 736.2	679.0	27; 16; 41

produce a very non linear response, which was attributed to traces of sodium present in the extracts from the matrix. To overcome the sodium adduct and linearity problems, several methods have been reported using LC–MS with atmospheric pressure chemical ionization (APCI) with positive ion detection in which either the $[M+H]^+$ [17], the $[M+NH_4]^+$ [18] were monitored. Second order polynomial calibration curves were found to be more appropriate for quantification using the $[M-H]^-$ ions formed by negative ion APCI with a wide range of analyte concentrations.

The first step in developing the detection method was to select the precursor ion to be fragmented in the collision. The full scan mass spectrum for macrolide antibiotics was dominated by ions with the following characteristics: $[M+H]^+$, for clarithromycin and erythromycin, $[M+2H]^{2+}$ and $[M+H]^+$, for tilmicosin, spiramycin, roxithromycin and azithromycin, $[M+H]^+$ and $[M+CH_3OH]^+$ for tylosin, kitasamycin, medecamycin, and josamycin, and $[M+H]^+$ and $[M+2H-COCH_3]^+$ for troleandomycin. The molecular ions for each macrolide antibiotics served as precursor ion for CID in the MS–MS experiments on the individual macrolide antibiotics standard solution. Different transitions of the molecular related ions to respective product ions for each macrolide antibiotics were therefore selected according to the MRM technique.

Ammonium adducts were obtained by the addition of 5 mM of ammonium acetate into the infusion solution, a peak at m/z 890.5 corresponding to $[M+NH_4]^+$ for abamectin, m/z 892.5 for ivermectin and m/z 916.5 for doramectin. The higher affinity of avermectins for Na^+ than NH_4^+, showing an abundant $[M+Na]^+$ signal at m/z 895.5, 897.5 and 921.5, which was even present more stronger signal than $[M+NH_4]^+$ after addition of ammonium acetate. However, the MS/MS spectra of $[M+NH_4]^+$ presented two satisfied abundant fragments with higher relative intensities than those obtained from the sodium adduct for each avermectin. In this case, the lower affinity of the analyte for NH_4^+ may facilitate the fragmentation increasing the sensitivity of the product ions. Thus, the best approach for analysis of avermectins was using $[M+NH_4]^+$ as a precursor ion by ESI+. The results of the optimized parameters of selected reaction monitoring and the retention times were collected in Table 2.

3.2. Optimization of ASE procedure

Sample extraction and preparation are important parts of this method. With the aim to find the most effective conditions, the influence of solvent proportion, temperature, pressure and extraction cycles were investigated. Referring the document of Juan et al. [21] different amount of sea sand, which facilitate the dispersion of the sample and permit the contact between the extract solvent and the sample, and several quantities of EDTA were studied. In this study, 120 g of sand was covered with 240 mL of 0.1 mol/L EDTA during 5 min, and sand was completely dried in an oven at 100 °C. For 2 g meat, 12 g of sand mixed with EDTA were used.

The other challenge for developing the ASE method is to choose an appropriate extraction solvent. According to Berrada et al. [20] and Juan et al. [21], acidic water at pH 3.6, methanol, acetonitrile and mixtures of methanol and water were tested to choose the best extracting solvent. Pure organic solvents, such as acetonitrile or methanol could obtain good recoveries, so the experiments using acetonitrile, methanol, acetonitrile/methanol (v/v, 1/2), acetonitrile/methanol (v/v, 1/1) and acetonitrile/methanol (v/v, 2/1) were tested. Using acetonitrile/methanol (v/v, 1/1) to extract macrolides and avermectins from swine liver since it recovered over 72% of them, except for ivermectin and abamectin (see Table 3).

Extractions of macrolides and avermectins at various temperatures (40–90 °C) were examined in some detail with swine muscle. The influence of temperature on the recoveries of the antibiotics is shown in Fig. 1(a). The temperature effect on antibiotic recoveries was evaluated by performing extractions at various temperatures. Raising the temperature from 40 to 90 °C improved the extraction yield of all the studied compounds. Extraction efficiency showed an optimum at 60 °C, with recoveries ranging from 72% to 91% and RSDs below 8% ($n = 5$). The variable pressures of the extracting system have also been investigated from 500 to 2500 psi (see Fig. 1(b)). Best recoveries (between 75% and 94% ($n = 5$)) were obtained at a pressure of 1500 psi which was in accordance with Berrada et al. [20] and Juan et al. [21]. The lengthy of exposure to solvents allows the matrix to swell and improve the penetration of the solvent into the sample interstices and the contact of the solvent with the analytes. The extraction time was set at 10 min to assure a rapid extraction. The number of extraction cycles (from 1 to 3) was checked. The highest recoveries reached at 80% flush, and two extraction cycles (see Fig. 1(c)).

In view of the different parameters (e.g. solvent volume, temperature, pressure and number of extraction cycles) affecting the ASE extraction process together with a desirability function, a fractional factorial design (FFD) has been used to optimize the values of the significant parameters to achieve the highest global recovery for the representational drugs (erythromycin, kitasamycin, tilmicosin, tylosin, medecamycin, doramectin, ivermectin). The optimized ASE conditions were further applied for method development and validation. Fractional factorial (FFD) designs were applied: Flush volume (%), temperature (T), pressure (P) and number of

Table 3
Solvent influence on the extraction recovery of studied macrolides from swine liver ($n = 5$).

Extraction solvent	% Recovery (% RSD)														
	ERM	TYL	TIL	KIT	ABA	IVE	DOR	CLA	AZI	SPM	OLE	MED	JOS	ROX	ERE	ASP
Acetonitrile	40(12)	81(9)	51(13)	52(14)	40(15)	31(11)	64(8)	56(10)	52(12)	43(9)	53(10)	60(11)	61(13)	70(11)	47(6)	41(10)
Methanol	70(13)	65(12)	81(15)	67(15)	43(14)	39(9)	72(6)	63(7)	68(7)	51(6)	69(9)	58(8)	57(10)	62(9)	67(9)	62(5)
Acetonitrile/methanol (v/v, 1/2)	75(10)	70(11)	83(9)	70(13)	50(11)	49(10)	78(9)	75(9)	77(8)	66(12)	70(8)	72(9)	73(7)	80(12)	81(10)	66(8)
Acetonitrile/methanol (v/v, 1/1)	80(9)	85(7)	91(10)	77(12)	53(8)	59(8)	84(6)	96(8)	92(12)	73(9)	93(5)	87(10)	97(11)	91(10)	87(8)	72(9)
Acetonitrile/methanol (v/v, 2/1)	76(14)	77(10)	85(8)	65(14)	65(9)	44(6)	77(8)	71(5)	81(10)	69(6)	82(11)	74(8)	82(9)	81(6)	72(7)	80(12)

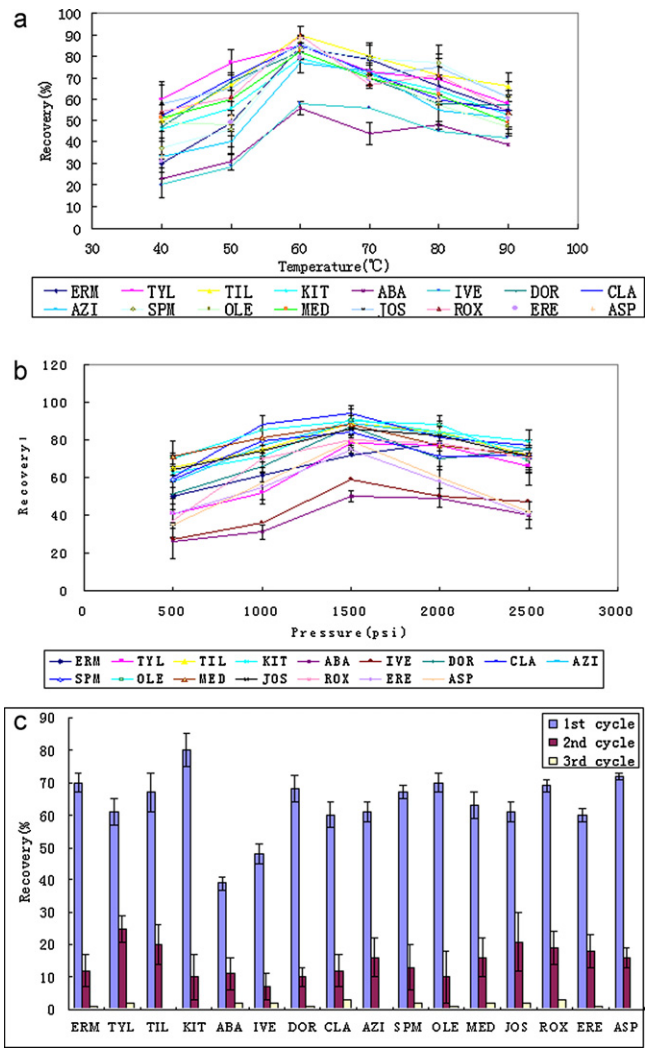

Fig. 1. Effects of temperature (a), of pressure (b) and cycles (c) on the extraction efficiency.

extraction cycles (C). A fractional factorial design was defined by an experimental domain constituted by a central point and two levels corresponding to the maximum and the minimum values for each extraction parameter. The experimental domain and the resulting FFD design matrix are shown in Table 4. The design consisted of 8 experiments performed by duplicate and 5 replicates for the central point. The results, in terms of average recoveries, are collected in Table 4. The average recoveries collected in Table 4 show that, the best extraction yields were obtained using solvent (acetonitrile/methanol) (v/v, 1/1) at 60 °C and 1500 psi for 10 min (static time) in two cycles. The flush volume was kept constant at 80% of the cell volume; preheating for 2 min and purging for 60 s. Under the optimized conditions, the method reduces the overall analytical time and offers good accuracy and precision.

3.3. Method validation

The specificity of the method was checked by analyzing different types of blank edible tissue samples. Typical chromatograms of tissue samples spiked ERM, TYL, TIL, KIT, ABA, IVE, DOR, CLA, AZI, SPM, OLE, MED, JOS, ERE, ASP (three kinds) at the level of 5 μg/kg are shown in Fig. 2. The obtained chromatograms did not show any interference, as no detectable matrix peaks were eluted in the retention time of the target compounds.

Table 4
Fractional factorial designs design matrix for the ASE parameters optimized for macrolide antibiotics and avermectins residues in meat.

Parameter	Code	Level		
		Minimum	Central	Maximum
Flush volume (%)	*V*	70	80	90
Temperature (°C)	*T*	50	60	70
Pressure (psi)	*P*	1000	1500	2000
Number of cycles	*C*	1	2	3

Recovery (%) ± RSD (%)

Experiment	*V*	*T*	*P*	*C*	Erythromycin	Kitasamycin	Tilmicosin	Tylosin		Medecamycin	Ivermectin
1	70	50	1000	1	42.3 ± 17.2	50.8 ± 14.9	39.2 ± 18.1	48.7 ± 10.9	52.3 ± 11.8	40.7 ± 9.9	30.1 ± 16.8
2	70	60	1500	2	82.3 ± 10.4	79.6 ± 8.9	80.2 ± 7.6	77.1 ± 14.8	73.7 ± 10.5	76.8 ± 14.7	42.2 ± 17.9
3	70	70	2000	3	60.1 ± 13.7	51.6 ± 11.8	62.8 ± 12.7	58.2 ± 9.5	57.4 ± 13.8	61.3 ± 17.3	32.9 ± 10.1
4	80	70	1000	2	69.6 ± 11.6	70.8 ± 8.4	80.0 ± 13.3	70.5 ± 7.2	76.2 ± 7.8	71.5 ± 10.3	43.9 ± 7.9
5	80	60	1500	2	90.5 ± 5.8	85.1 ± 2.9	89.8 ± 7.4	86.9 ± 6.5	94.2 ± 3.8	91.8 ± 2.7	57.1 ± 8.3
6	80	60	1000	3	70.5 ± 6.7	72.9 ± 7.1	68.3 ± 11.8	72.9 ± 3.6	75.7 ± 6.3	66.2 ± 7.8	40.6 ± 12.5
7	90	50	2000	2	50.6 ± 14.8	42.6 ± 8.2	57.5 ± 14.3	62.0 ± 13.4	53.8 ± 11.2	57.1 ± 16.8	32.7 ± 15.1
8	90	70	1500	2	65.8 ± 8.3	63.9 ± 6.1	70.7 ± 8.4	71.5 ± 9.9	69.2 ± 7.5	60.3 ± 5.9	36.3 ± 7.8
9	90	60	2000	1	56.9 ± 10.9	51.4 ± 10.7	45.1 ± 12.6	48.2 ± 11.6	51.2 ± 12.9	52.1 ± 14.7	27.0 ± 11.7

Since some internal standards, such as deuterium internal standard, could not be obtained commercially, we have used ROX as internal standard for quantification. The matrix-match calibration curves were made by fortified tissues with the seventeen compounds at each of six concentrations (2, 5, 10, 50, 100, 200 and 400 μg/kg) of macrolide antibiotics and avermectins and ROX at the level of 100 μg/kg before the extraction procedure. The matrix-match calibration curves were constructed using the peak area ratio of ERM, TYL, TIL, KIT, ABA, IVE, DOR, CLA, AZI, SPM, OLE, MED, JOS, ERE, ASP (three kinds) versus that of ROX versus the concentration ratio of the seventeen compounds versus that of ROX. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients greater than 0.999 for all curves.

Sensitivity was evaluated by limit of detection (LOD) and limit of quantification (LOQ) values. The LOD was estimated from blank extract, spiked with decreasing concentrations of the analytes, where the response of the qualitative ion was equal to 3 times the response of the blank extract. Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LOQ was estimated in the same way as the LOD, but using the criterion of S/N ≥ 10 for the qualitative ion. Also the LOQ (coefficient of variation less than 19% and an accuracy more than 70 ± 19%) was later estimated. LODs and LOQs were calculated analyzing fortified meat sample and the results obtained are shown in Table 5. The LOD of ERM, TYL, TIL, KIT, ABA, IVE, DOR, CLA, AZI, SPM, OLE, MED, JOS, ERE, ASP (three kinds) were lower than 0.55 μg/kg. The LOQ was established by analyzing samples of muscle, liver and kidney were spiked with serial different concentrations (1, 2, 5, 10 and 20 μg/kg, $n = 5$). After the sample preparation using ASE, the data of accuracy and precision was analyzed. The results showed that the LOQ of selected analytes were less than 5 μg/kg for all tissues except ASP (three kinds) less than 10 μg/kg. In the same table are indicated the maximum levels recommended by EU [3] to compare the results obtained. In all these cases, LOQs were always lower than the MRLs established by EU.

Trueness and precisions (intra-day, inter-day, and within-laboratory) were calculated from the determination of five aliquots each tissue fortified at four levels (5, 20, 100 and 200 μg/kg). The analyses were finished by the same operator in triplicate in a 2-week period. Within-laboratory was carried out in the same laboratory, but performed by two different operators. The recovery was calculated by the following formula: (the measured level/the fortified level) × 100%. The precision was expressed as the RSD. Table 5

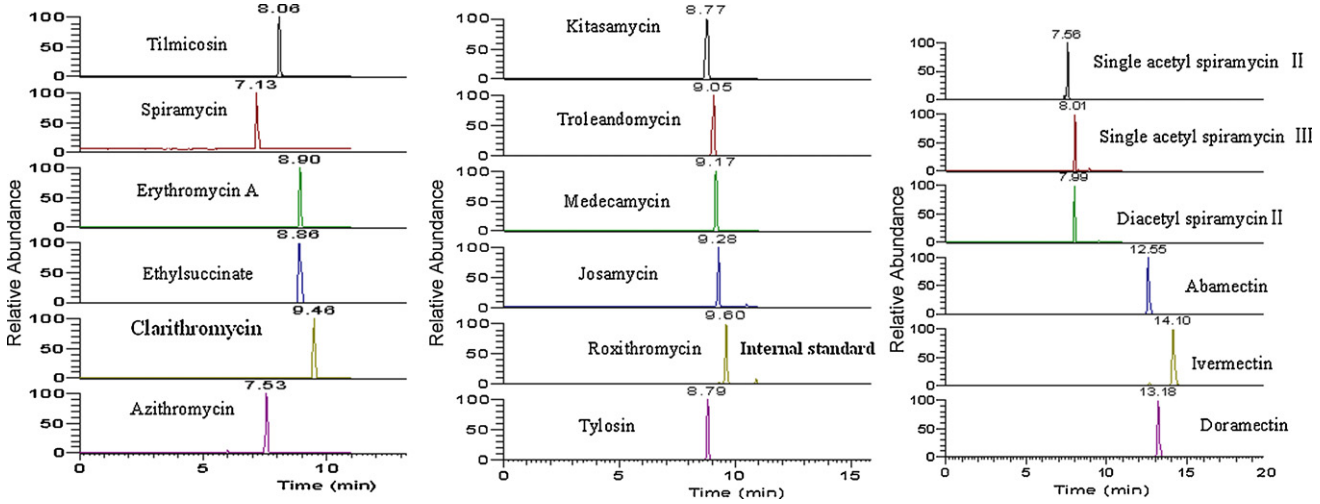

Fig. 2. LC–MS/MS chromatograms of: extract of blank bovine liver spiked with macrolides equivalent to the LOQ (5 μg/kg for erythromycin, tylosin, tilmicosin, clarithromycin, azithromycin, spiramycin, josamycin, ethylsuccinate, single acetyl spiramycin II, single acetyl spiramycin III, diacetyl spiramycin II, erythromycin ethylsuccinate, kitasamycin, abamectin, ivermectin, doramectin, medecamycin).

Table 5
The validation results and MRLs levels of 17 macrolide antibiotics and avermectins in different animal-origin foods.

	ERM	TYL	TIL	KIT	ABA	IVE	DOR	CLA	AZI	SPM	OLE	MED	JOS	ERE	ASP (3 kinds)
M*															
Intra-day RSD (%)	8–10	7–11	6–7	7–10	9–12	8–11	8–10	6–9	8–12	6–10	5–11	7–11	5–8	8–10	8–11
Inter-day RSD (%)	9–12	9–14	7–10	8–13	10–13	9–13	8–12	9–11	10–14	8–14	7–12	8–13	10–12	9–12	10–13
Accuracy (%)	85–94	86–93	85–95	90–96	76–82	80–85	88–96	89–97	88–90	87–98	87–96	89–93	87–91	87–89	90–95
MRL	200	50	50	200	25.0	20	10	–	–	–	–	–	–	–	–
LOD (μg/kg)	<0.21	<0.32	<0.36	<0.20	<0.31	<0.21	<0.33	<0.28	<0.27	<0.41	<0.5	<0.29	<0.33	<0.29	<0.35
LOQ (μg/kg)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	10
L*															
Intra-day RSD (%)	3–5	4–7	5–8	8–12	7–10	2–6	7–10	5–9	6–11	6–8	4–8	5–9	4–7	7–10	2–7
Inter-day RSD (%)	8–9	6–10	6–11	9–13	9–12	8–11	8–12	6–10	10–13	10–12	6–9	6–10	5–8	9–12	5–10
Accuracy (%)	86–100	85–96	87–103	85–90	77–84	76–88	89–101	87–98	86–101	87–91	89–105	87–98	85–101	86–99	88–92
MRL	200	50	1000	1000	25.0	100	100	–	–	–	–	–	–	–	–
LOD (μg/kg)	<0.22	<0.23	<0.35	<0.31	<0.20	<0.24	<0.29	<0.28	<0.31	<0.41	<0.31	<0.27	<0.31	<0.31	<0.45
LOQ (μg/kg)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	10
K*															
Intra-day RSD (%)	4–6	7–9	6–9	6–12	5–8	5–9	4–11	8–10	7–8	6–8	3–6	7–8	8–10	5–8	4–9
Inter-day RSD (%)	5–9	8–11	7–14	9–13	6–10	6–11	7–13	7–11	9–12	8–12	7–11	9–13	8–13	7–12	9–14
Accuracy (%)	90–94	86–101	90–102	87–94	76–87	78–85	87–96	86–98	89–100	86–106	90–102	90–103	90–97	87–99	89–102
MRL	200	50	250	200	20	20	60	–	–	–	–	–	–	–	–
LOD (μg/kg)	<0.33	<0.25	<0.28	<0.31	<0.25	<0.33	<0.25	<0.27	<0.30	<0.31	<0.25	<0.22	<0.56	<0.61	<0.31
LOQ (μg/kg)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	10

M*: muscle of swine, bovine and sheep; L*: liver of swine, bovine and sheep; K*: kidney of swine, bovine and sheep.

Table 6
Confirmatory and quantitative LC–MS/MS analysis of incurred samples.

Sample	Analyte detected	Incurred samples			Matrix-matched standard		Level (μg/kg)
		Product ions	Ion ratio (%)	Retention time (min)	Ion ratio (%)	Retention time (min)	
Swine liver 5	Tylosin	916.6 > 173.8	70 ± 5.7	8.77	71 ± 6.3	8.79	38.8 ± 0.5
Swine liver 28	Tylosin	916.6 > 173.8	70 ± 5.7	8.77	46 ± 6.3	8.79	19.2 ± 0.7
Swine liver 51	Tilmicosin	435.5 > 339.9	81 ± 6.8	7.99	86 ± 5.7	8.06	54.1 ± 0.3
Bovine muscle 23	Erythromycin A	733.0 > 679.0	73 ± 4.8	8.92	76 ± 3.9	8.90	63.2 ± 0.8
Bovine muscle 66	Erythromycin A	733.0 > 679.0	73 ± 4.8	8.94	70 ± 6.5	8.90	22.3 ± 0.9

Please cite this article in press as: Y. Tao, et al., J. Chromatogr. B (2012), http://dx.doi.org/10.1016/j.jchromb.2012.04.011

gives the results of the recovery and repeatability of the method. It was reflected that the method provided a wide concentration range over which to assess the performance of the developed method. The recoveries were higher than 85%, except ivermectin and abamectin (above 76%), and the overall relative standard deviation (RSD) were lower than 14%.

3.4. Method application

Around 100 swine liver and 100 bovine meat samples were bought from different supermarkets in the city of Wuhan (China). All the samples were transferred to our laboratory and pretreated. Table 6 summarizes the confirmatory and the quantitative analysis of the samples and the results were: two swine livers contained tylosin; one swine livers contained tilmicosin and two bovine meat contained erythromycin A. The average amount of tylosin was lower than 38.8 μg/kg and tilmicosin 54.1 μg/kg and erythromycin A lower than 63.2 μg/kg respectively. The total level of residues found did not exceed 100 μg/kg, the set MRL. It indicated that tylosin, tilmicosin and erythromycin A have been widely used in animal husbandry.

4. Conclusions

A convenient analytical method developed for seventeen macrolide antibiotics and avermectins in meat was based on ASE followed by an LC–MS/MS analysis has been successfully optimized. ASE permits an automatic extraction and successful purification of analytes in short time, minimizing the risk of contamination by reducing the manipulation of the sample. Also it generates less hazardous waste and was more benign to the environment. The method validation in meat showed that it is rapid, simple and rugged and permits good linearity, accuracy, repeatability, selectivity, with recoveries higher than 75%, and offers quantization and confirmation at concentration between 4 and 40 times lower than the MRL. Therefore, the method was useful for identification and quantification of macrolide antibiotics and avermectins residues in foods of animal origin.

Acknowledgements

The authors are grateful to the Ministry of Agriculture the People's Republic of China for the financial support, which enabled this work to be carried out.

References

[1] S. Omura, K. Shiomi, Pure Appl. Chem. 79 (2007) 581.
[2] T. Pitterna, J. Cassayre, O. Huter, Bioorg. Med. Chem. 17 (2009) 4085.
[3] Official Journal of the European Union, (EU) No 37/2010, December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.
[4] USDA Foreign Agricultural Service. Veterinary drug MRL database. http://www.mrldatabase.com/default.cfm.
[5] M. Horie, K. Saito, R. Ishii, T. Yoshida, Y. Haramaki, H. Nakazawa, J. Chromatogr. A 812 (1998) 295.
[6] P. Edder, L. Coppex, A. Cominoli, C. Corvi, Food Addit. Contam. 19 (2002) 232.
[7] C. Prats, R. Francesch, M. Arboix, B. Perez, J. Chromatogr. B 766 (2002) 57.
[8] F. Kees, S. Spangler, M. Wellenhofer, J. Chromatogr. A 812 (1998) 287.
[9] M. Juhel, B. Anger, M. Laurentie, J. AOAC Int. 82 (1999) 1046.
[10] J.W. Moran, J.M. Turner, M.R. Coleman, J. AOAC Int. 80 (1997) 1183.
[11] C. Stobba, J. Chang, D. Elsbury, J. Moran, J. Turner, R. Readnour, J. AOAC Int. 83 (2000) 837.
[12] R. Codony, R. Compano, M. Granados, J. Garcia-Regueiro, M. Prat, J. Chromatogr. A 959 (2002) 131.
[13] B. Delepine, D. Hurtaud-Pessel, P. Sanders, J. AOAC Int. 79 (1996) 397.
[14] Y. Hwang, J. Lim, B. Park, H. Yun, J. Vet. Sci. 3 (2002) 103.
[15] M. Dubois, D. Fluchard, E. Sior, P. Delahaut, J. Chromatogr. B: Biomed. Sci. Appl. 753 (2001) 189.
[16] B. Sara, C. Cecilia, C. Roberta, D. Antonio, A. Laganà, J. Chromatogr. A 1216 (2009) 6810.
[17] M. Danaher, M. O'keeffe, J. Glennon, L. Howells, Analyst 126 (2001) 576.
[18] M. Pollmeier, S. Maier, K. Moriarty, P. Demontigny, J. Chromatogr. B 772 (2002) 99.
[19] V. Carretero, C. Blasco, Y. Picó, J. Chromatogr. A 1209 (2008) 162.
[20] H. Berrada, F. Borrull, G. Font, R. Marcé, J. Chromatogr. A 1208 (2008) 83.
[21] C. Juan, J. Carlos Moltó, J. Mañes, G. Font, Food Control 21 (2010) 1703.