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Multiclass analysis of 23 veterinary drugs in milk by ultraperformance liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

An ultraperformance liquid chromatography–electrospray tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous detection and confirmation of 23 veterinary (multiclass) drugs in milk was developed and validated. The analytes were extracted by acetonitrile, evaporated and injected into the UPLC–MS/MS system on a Waters UPLC HSS T3 column in gradient mode. Data acquisition under MS/MS was achieved by applying multiple reaction monitoring (MRM) of two ion transitions per compound to provide a high degree of specificity. Results showed good repeatability, and recoveries for the 12 macrolide, 7 β-lactam and 2 lincosamide antibiotics and 2 other veterinary drugs (morantel, orbifloxacin) used in milk averaged 51.8–139.0%, 51.5–100.6%, 82.4–102.5% and 87.5–99.4%, respectively. The coefficients of variation (C.V.) of the recoveries were less than 15% for intraday and interday precisions. The limits of quantification (LOQs) were all lower than 5 ng/ml. This method was applied to 17 fresh milk samples and only lincomycin was found in milk samples under allowable levels. Overall, this method is a suitable and rapid tool to confirm the presence of 23 veterinary drug residues in milk.

1. Introduction

Veterinary drugs are of great interest because large volumes of these substances are used to treat disease in animals. There is growing concern over the release of these drugs, particularly antibiotics, into the environment because of the potential for the development of antimicrobial resistance among microorganisms. Furthermore, these chemicals can be transformed into different metabolites via microbial action, as well as other physical or chemical processes, resulting in mixtures with higher risks to human health than those of the individual compounds themselves. In particular, the presence of antibiotics in substances such as milk that are consumed daily can create numerous problems for human health [1].

Macrolide antibiotics contain macrocyclic lactones that were first isolated from *Steptomyces* spp. Their chemical structures consist of a 12-, 14-, or 16-membered macrocyclic lactone bound to sugar moieties, including amino and deoxy sugars. Macrolide antibiotics are widely used in veterinary practices to treat respiratory diseases and enteric infections in food-producing animals [2]. According to Taiwan's regulation, the maximum residues limits (MRLs) of erythromycin, spiramycin and tylosin are below 40, 200, and 50 ng/ml in milk, respectively [3]. The MRLs of several macrolide antibiotics in the EU (European Union) are 40, 200, 50, and 50 ng/ml in milk for erythromycin, spiramycin, tilmicosin and tylosin, respectively [4].

β-Lactam antibiotics are the most widely used class in veterinary medicine, especially in lactating cows. There are two types of β-Lactam antibiotics: penicillins and cephalosporins. According to Taiwanese regulations, the MRLs of ampicillin, cefapirin, cloxacillin, dicloxacillin and oxacillin are below 10, 10, 10, 10 and 30 ng/ml in milk, respectively [3]. The respective MRLs for the EU range from 4 to 60 ng/ml [4].

Lincosamides (e.g., lincomycin and clindamycin) are moderate broad-spectrum antibiotics used in veterinary medicines [5]. According to both Taiwanese and EU regulations, the MRL for lincomycin should be below 150 ng/ml in milk [3,4]. Morantel and orbifloxacin belong to the anthelmintic and quinolone classes of veterinary drugs, respectively, and the MRLs in Taiwan and the EU are 100 and 50 ng/ml in milk, respectively [3,4].

Many papers have been published in past years on the different classes of veterinary drugs, such as bioassay techniques [6,7] and liquid chromatography (LC) with UV or photodiode array detection [8–10]. However, these methods are less sensitive for determining drug residues compared to mass spectrometry (MS). MS techniques have advanced considerably, resulting in rugged mass spectrometers as powerful analytical tools for veterinary drug residue determinations. LC coupled with single or triple quadrupole (MS/MS) systems have been well established in recent years, and they have become popular in monitoring single [11–14] or

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multiclass antibiotics in muscle [15–17], honey [18,19] and eggs [20]. Most of these papers have determined single drug classes, and some methods have focused on the determination of multi-residue antibiotic in milk [21–27]. Recently, a method that utilized liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) for the analysis of veterinary drugs in milk matrices was presented [28]. Despite LC/MS/MS having higher sensitivity and repeatability than the LC-TOF/MS method, LC-TOF/MS provided ultimate and unequivocal confirmations of the positive finding and more degradation product identifications because of the TOF's accurate mass measurements. The cost-effectiveness of analytical procedures is becoming an important issue for all experimental designs involved in the residue analysis of food contaminants. The goal is to maximize the number of analytes that can be determined by a single, simple procedure, such as a multi-residue technique [2]. However, it is sometimes difficult to develop a multiclass method due to the presence of a few closely related compounds that typically belong to a single drug class.

This article presents a method for the determination and confirmation of 23 veterinary drugs belonging to multiple drug classes in milk: 12 macrolide, 7 β-lactam and 2 lincosamide antibiotics and 2 other veterinary drugs (morantel, orbifloxacin) compared to several methods for the determination of a single antibiotics groups. The optimized procedure was then used to analyze commercial milk in Taiwan and proved to be a fast and simple method that can be applied in routine laboratory analysis of large numbers of samples containing different families of compounds.

2. Materials and methods

2.1. Chemical, reagents and samples

Antibiotic standards (erythromycin, kitasamycin, leucomycin hydrate, cefoperazone sodium salt, virginiamycin M1, dicloxacillin sodium salt monohydrate, clindamycin hydrochloride, lincomycin hydrochloride, and ampicillin) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Josamycin was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Spiramycin, cefapirin sodium, cloxacillin sodium salt and oxacillin sodium salt monohydrate were from Fluka (Steinheim, Germany), and tylosin, tilmicosin, troleandomycin, clarithromycin, natamycin, morantel tartrate and orbifloxacin were supplied by USP (Rockville, MD, USA). Neospiramycin I, oleandomycin phosphate salt and mecillinum were from Wako Pure Chemical Industries (Osaka, Japan), MP Biomedicals (Ohio, USA), and Riedel-de Haën (Munich, Germany), respectively. Stock standard solutions of individual compounds at 1000 mg/l were prepared by exact weighing of the solid powders. The powders were dissolved in 10 ml of methanol (natamycin), 50% acetonitrile (virginiamycin M1, clindamycin, lincomycin, and orbifloxacin) or acetonitrile (others). These standard solutions were stored at −20 °C and diluted with 50% acetonitrile to prepare working solutions. The working solutions were stable for 3 weeks, after which they were replaced by fresh solutions. Roxithromycin from Sigma–Aldrich was used as an internal standard and spiked at 10 ng/ml per sample to correct for the recovery of each drug. HPLC-grade acetonitrile and formic acid (purity > 99%) were purchased from Merck Ltd. (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

Fresh milk samples (10 for full-cream, 6 for low-fat and 1 for skim milk) were purchased from supermarkets and convenient stores. Samples were stored at 4 °C before analysis (within 10 days). Seventeen samples were analyzed by UPLC–MS/MS.

2.2. Equipment

Chromatographic analyses were performed on an Acquity UPLC system, and separations were achieved on an UPLC HSS T3 column (10 mm × 2.1 mm, 1.8 μm particle size) from Waters (Waters, Milford, MA, USA). The analytes were separated with a mobile phase consisting of 0.05% formic acid in water (eluent A) and acetonitrile (eluent B) at a flow rate of 0.3 ml/min. The gradient profile started at 100% of eluent A until 1.5 min, decreased linearly to 50% at 4 min, then continued to decrease linearly to 20% from 4 min to 7 min. This composition was held for an additional 2 min before decreasing to 5% by 13 min. The composition was then held for an additional 1 min and returned to the initial conditions in 0.1 min.

Table 1
The MS parameters of 23 veterinary drugs.

Compound		Retention time (min)	Parent ion (*m*/*z*)	Transition 1 (CE)	Transition 2 (CE)	Cone voltage	Ion ratio (SD) ** (%)
Clarithromycin	CLA	4.7	748.7	115.9 (44)	**158.0* (32)**	28	26.2 (0.7)
Erythromycin	ERY	4.4	734.6	**158.1* (32)**	576.5 (18)	26	38.4 (1.3)
Josamycin	JOS	4.9	828.7	**109.0* (44)**	174.1(34)	46	84.8 (1.6)
Kitasamycin	KIT	4.6	772.6	**109.0* (44)**	174.1 (32)	46	84.8 (2.5)
Natamycin	NAT	4.4	666.5	463.3 (32)	**503.3* (12)**	54.18	55.9 (4.1)
Neospiramycin	NEO	3.9	699.6	142.1 (22)	**174.1* (30)**	34	29.3 (2.8)
Oleandomycin	OLE	4.3	688.6	**158.0* (28)**	544.5 (16)	24	46.4 (1.8)
Spiramycin	SPI	3.9	843.7	100.9 (40)	**174.0* (38)**	48	27.9 (3.0)
Tilmicosin	TIL	4.1	869.8	132.0 (50)	**174.1* (46)**	70	28.5 (3.2)
Troleandomycin	TRO	5.0	814.7	**200.1* (26)**	158.0 (46)	34	15.3 (0.5)
Tylosin	TYL	4.5	916.8	100.9 (50)	**174.0* (40)**	50	19.5 (1.0)
Virginiamycin M1	VIR	5.4	526.4	337.1 (22)	**355.2* (18)**	24	82.7 (2.0)
Ampicillin	AMP	3.8	350.2	160.0 (12)	**174.0* (16)**	22	96.1 (3.3)
Cefapirin	CEF	3.5	424.2	**292.1* (14)**	152.0 (26)	20	50.0 (1.0)
Cefoperazone	CEO	4.3	646.4	**143.1* (40)**	530.2 (18)	18	63.9 (4.4)
Cloxacillin	CLO	5.5	436.2	160.0 (12)	**277.1* (14)**	16	100.7 (4.7)
Dicloxacillin	DIO	5.9	470.2	**160.0* (12)**	311.1 (14)	16	64.9 (5.8)
Mecillinum	MEC	3.8	326.3	139.1 (30)	**167.1* (22)**	32	13.0 (0.3)
Oxacillin	OXA	5.3	402.3	114.1 (32)	**243.1* (12)**	16	41.9 (4.0)
Clindamycin	CLI	4.1	425.3	377.2 (20)	**389.3* (18)**	30	5.9 (0.3)
Lincomycin	LIN	3.5	407.3	**126.1* (30)**	359.3 (18)	32	7.9 (0.3)
Morantel	MOR	4.0	221.1	**122.9* (34)**	111.0 (26)	42	97.7 (3.0)
Orbifloxacin	ORB	3.9	396.3	226.1 (42)	**295.1* (24)**	32	16.7 (0.4)
Roxithromycin (I.S.)		4.7	837.8	**158.1* (36)**		32	

*Transitions with bold numbers were used for quantification.
** Relative standard deviation (RSD) is given in parentheses ($n = 18$) and ion ratio is presented by first qualitation/quantification ion ratio.

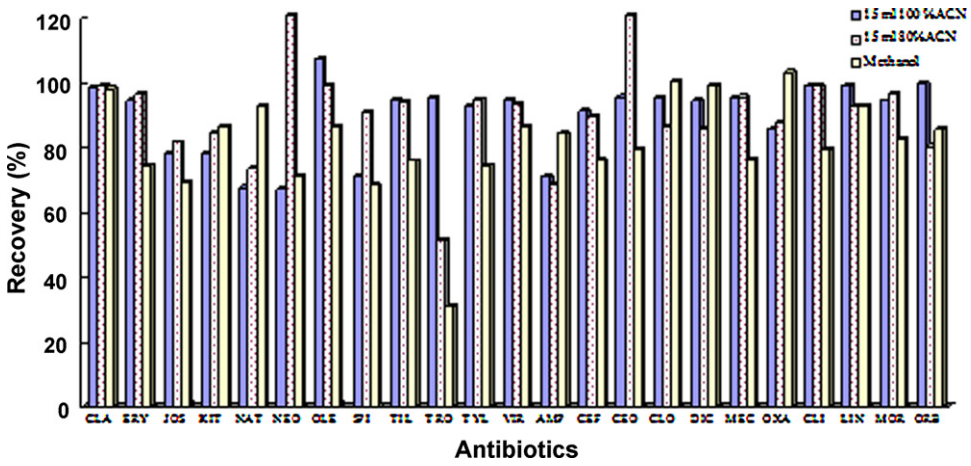

Fig. 1. The different solvents on extraction recoveries of antibiotics from milk.

This was followed by a re-equilibration time at 20 min to give a total run time of 20 min.

Mass spectrometric analysis was conducted on a Waters Zevo quadrupole tandem mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray ionization (ESI) source in the positive ion mode. The mass parameters were as follows: capillary voltage of 2.5 kV, extractor voltage of 3 V, source temperature of 150 °C, desolvation temperature of 600 °C, cone gas (nitrogen) flow of 26 l/h and desolvation gas (also nitrogen) flow of 1200 l/h. The parameters for each antibiotic are shown in Table 1. Data acquisition was performed using the MassLynx 4.1 software with the QuanLynx program (Waters, Manchester, UK).

2.3. Extraction procedure

Two milliliters of blank fresh milk were transferred to polypropylene centrifuge tubes (50 ml) and extracted with 15 ml of acetonitrile. The mixture was then vortexed for 1 min and centrifuged at 4000 rpm for 10 min. The upper layer was removed and evaporated under nitrogen to dryness at 35 °C. The residue was reconstituted in 1 ml of 50% acetonitrile and filtered through a PVDF filter (0.2 μm, Waters, Milford, MA, USA). Finally, 10 μl were injected into the UPLC–MS/MS system under the optimized conditions.

Table 2
Evaluation of matrix effects by comparing the slopes of the calibration curves using matrix-matched calibration and solvent-based standards.

Antibiotic	Solvent	Full-cream	Skim
Clarithromycin	3561	6244	6225
Erythromycin	4535	6153	6524
Josamycin	11,764	8640	9839
Kitasamycin	58,010	4229	4584
Natamycin	463	513	503
Neospiramycin	1447	504	481
Oleandomycin	1734	3860	3909
Spiramycin	262	252	250
Tilmicosin	89	159	141
Troleandomycin	5188	10,854	12,556
Tylosin	11,275	6333	6525
Virginiamycin M1	384	1262	1462
Ampicillin	1258	3032	2872
Cefapirin	240	870	620
Cefoperazone	1336	394	386
Cloxacillin	413	814	1098
Dicloxacillin	104	330	470
Mecillinum	3943	11,640	10,111
Oxacillin	631	776	970
Clindamycin	3471	1955	1996
Lincomycin	7917	19,606	16,899
Morantel	10,229	4044	4359
Orbifloxacin	2190	3503	3426

2.4. Preparation of matrix-matched calibration curves

Blank full-cream milk samples were extracted following the same procedure described above to give matrix-based calibration curves after spiking six concentration levels (12.5, 25, 50, 75, 100 and 150 ng/ml) standard solutions and 10 ng/ml roxithromycin as internal standard (IS). The calibration curves were constructed by calculating the ratio of each peak area relative to an IS.

3. Results and discussion

The development of a simple, sensitive and rapid method to determine multiclass antibiotics in milk is of great interest for residues analysis. Several problems need to be overcome to determine multiclass antibiotics with a single analytical procedure. The chromatographic analysis time will be longer than that for single classes of antibiotics. The introduction of UPLC with MS/MS can decrease this analysis time by using HSS T3 column, which has 1.8 μm small particle sizes that provide good chromatographic resolution and separation simultaneously. The columns are universal silica-based, reversed-phase C18 columns with higher ligand density relevant that not only retain and separate highly polar compounds, but also provide good retention selectivity for other compounds.

This system also allows for high-speed analyses to reduce the overall analysis time. Furthermore, selecting a simple extraction method is crucial to avoid the loss and low recovery of certain compound classes.

3.1. MS/MS conditions

MS optimization was performed by infusing a 1-μg/ml standard solution of each antibiotic in a mixture of 50% acetonitrile in water at a flow rate of 10 μl/min. First, full-scan spectra were acquired to select the most abundant m/z value for each antibiotic. Using the ESI source in positive ionization mode is the common method for analyzing these antibiotics. In all the antibiotics investigated, the $[M+H]^+$ ions were found to be the most abundant, and these ions were selected as the precursor ions. Next, collision energies were evaluated to find the most abundant product ions, and the qualitative and quantitative ions were selected for confirmation and quantification purposes, respectively. Table 1 shows the mass parameters of the 23 veterinary drugs in this study, illustrating the

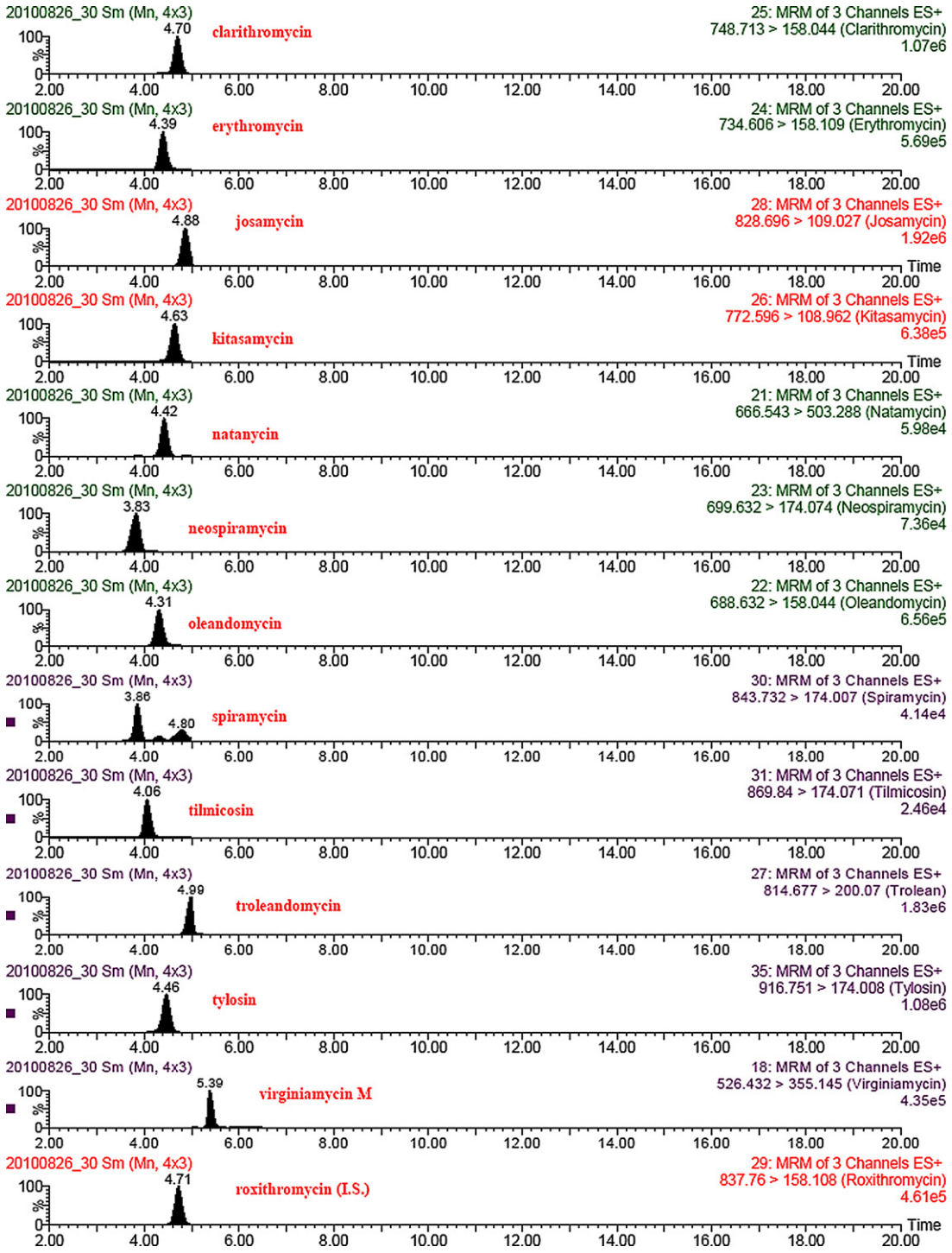

Fig. 2. Chromatograms of the 12 macrolide antibiotics spiked at 25 ng/ml and the I.S. in milk.

typical fragmentations for macrolides (m/z 109, 158, 174) and β-lactams (m/z 160). These transitions were used for confirmation and quantification. Finally, parent ions and fragmentations from electrospray ionization in positive mode were chosen for erythromycin, josamycin, tilmicosin and tylosin similar to a previous report [22].

3.2. Chromatographic separation

Chromatographic conditions were optimized for the best separation of each antibiotic. UPLC with MS/MS can increase column efficiency, yielding good separation and narrow peak widths. Several separation programs were tested initially by selecting different organic solvents and water. Better separation was achieved with water and acetonitrile in a gradient program compared to water and methanol. Finally, optimal results were obtained when acetonitrile was used as organic modifier in an aqueous solution of 0.005% formic acid in water.

3.3. Optimization of the extraction procedure

Several single class and multi-class LC/MS/MS methods based on SPE [13,23], QuEChERS [22] and matrix solid-phase

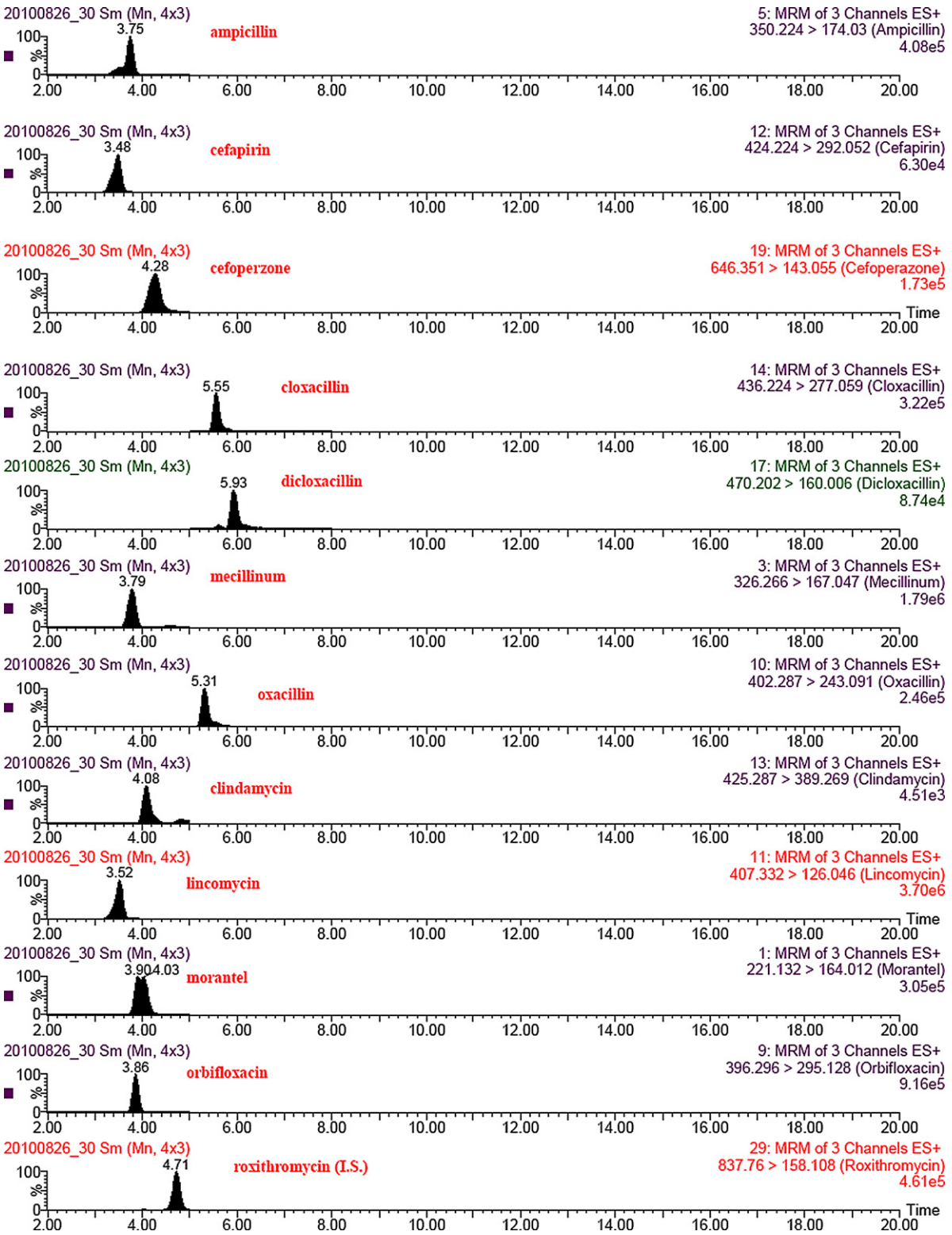

Fig. 3. Chromatograms of the 7 β-lactam and 2 lincosamide antibiotics and 2 other classes of veterinary drugs (morantel, orbifloxacin) spiked at 25 ng/ml and the I.S. in milk.

dispersion (MSPD) [21] have been developed to analyze milk extracts. The main problem with these methods lies in removing and avoiding protein and lipid matrix, which can interfere with the analytical process. Precipitating the milk protein and extracting the drug residues with acetonitrile was found to be efficient for milk samples. The recoveries of each compounds using 3 different extractions were showed in Fig. 1. Higher recoveries were observed with acetonitrile relevant to methanol. Among different percentage of acetonitrile, 100% acetonitrile was chosen as the extraction solvent as the evaporation time is less than 80% acetonitrile. N-hexane had no effect on extraction recoveries (data not shown), and thus, the use of n-hexane to remove the fat was unnecessary. Certain antibiotics (i.e., tilmicosin, cloxacillin and dicloxacillin) had recoveries that were too high, while others (i.e., josamycin, kitasamycin

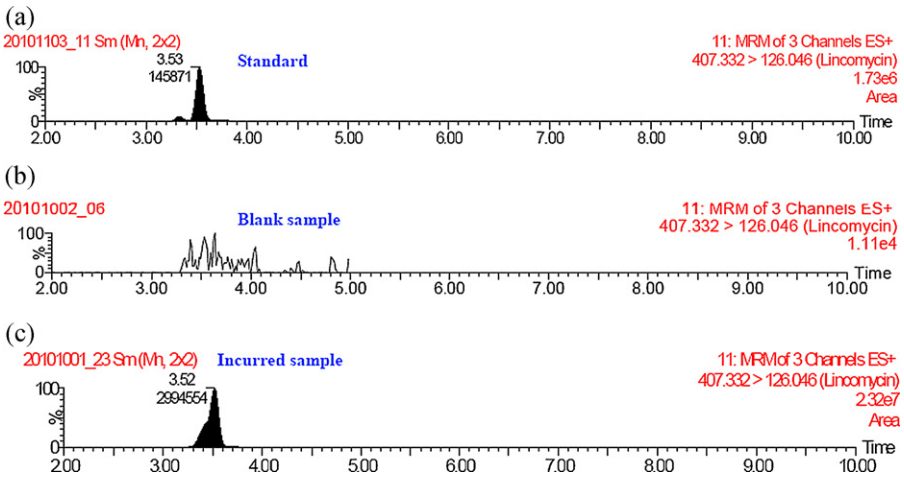

Fig. 4. Chromatograms of lincomycin in (a) standard solutions (25 ng/ml), (b) a blank and (c) a purchased milk sample (86.9 ng/ml of lincomycin were detected on *m*/*z* 126.1).

and cefoperazone) had lower recoveries when n-hexane was used. The highest recoveries were obtained with acetonitrile alone compared to the addition of n-hexane to the extraction. The total extraction procedure for one sample was less than 12 min. Therefore, this approach could be used to detect the target antibiotics in milk quickly and reliably.

3.4. Evaluation of matrix effects

Matrix effects will cause enhancement or inhibition of target analytes by enhancing or suppressing their signal in UPLC–MS/MS using ESI as the ionization technique. The influence of matrix effects on response must be evaluated and is compound-dependent because of interactions between the co-eluting matrix components and target compounds in the ionization step. Two different types of milk (full-cream and skim) were tested and compared to a pure solvent solution at seven concentrations (5–300 ng/ml). The slopes are shown in Table 2. The matrix enhanced the signal for clarithromycin, erythromycin, oleandomycin, tilmicosin, troleandomycin, virginiamycin M1, ampicillin, cefapirin, cloxacillin, dicloxacillin, oxacillin, clindamycin, lincomycin and orbifloxacin, while it suppressed the signal for josamycin, kitasamycin, neospiramycin, tylosin, cefoperazone, mecillinum and morantel. For those compounds with which a matrix effect was detected, matrix-matched calibrations were used to avoid this effect.

3.5. Validation

The developed method was validated in terms of accuracy, intra-day and interday precision and linearity. The retention times of all the antibiotic standards are presented in Table 1. In addition, five standards for each antibiotic at concentrations ranging between 25 and 100 ng/ml and were spiked to analyte-free milk blank extracts and analyzed with UPLC/MS/MS to make a matrix-matched

Table 3
Recoveries of the developed UPLC–MS/MS method for determination of 23 drugs in milk.

Drug/spiked standard (ng/ml)	Recovery (%), $n = 5$				
	25	50	75	100	Average
Macrolides					
Clarithromycin	119.5	110.8	105.4	104.0	109.9
Erythromycin	87.2	77.6	75.7	77.4	79.5
Josamycin	96.8	93.5	88.0	86.6	91.2
Kitasamycin	90.8	80.8	71.9	67.0	77.6
Natamycin	92.4	77.0	83.3	78.9	82.9
Neospiramycin	65.3	58.5	55.9	50.5	57.5
Oleandomycin	133.3	122.3	117.8	117.7	122.8
Spiramycin	79.3	70.1	67.3	64.9	70.4
Tilmicosin	118.2	120.7	119.3	126.2	121.1
Troleandomycin	140.3	135.6	117.4	117.1	127.6
Tylosin	90.0	96.5	92.9	89.9	92.3
Virginiamycin M1	94.5	90.9	89.6	84.9	90.0
β-Lactam					
Ampicillin	75.9	71.8	70.2	64.4	70.6
Cefapirin	67.5	55.2	53.0	52.0	56.9
Cefoperazone	70.8	71.8	66.6	75.7	71.2
Cloxacillin	94.7	82.2	79.5	74.9	82.8
Dicloxacillin	103.9	80.9	74.6	72.7	83.0
Mecillinum	85.7	72.1	69.8	70.4	74.5
Oxacillin	108.7	83.6	80.9	76.5	87.4
Licosamides					
Clindamycin	94.8	98.4	98.3	97.0	97.1
Lincomycin	103.4	87.8	84.1	83.9	89.8
Miscellaneous					
Morantel	95.7	96.0	94.8	93.3	94.9
Orbifloxacin	97.3	97.7	97.4	95.2	96.9

Table 4
C.V. of the developed UPLC–MS/MS method for determination of 23 drugs in milk.

Drug/Spiked standard (ng/ml)	C.V. (%) of intra-day, $n = 3$/inter-day, $n = 9$				
	25	50	75	100	Average
Macrolides					
Clarithromycin	12.3/1.3	8.5/2.7	13.5/2.5	13.1/1.2	11.9/1.9
Erythromycin	10.0/1.4	8.2/1.1	8.1/2.8	6.7/1.8	8.3/1.8
Josamycin	2.4/1.5	5.4/3.6	4.9/2.7	6.1/5.3	4.7/3.3
Kitasamycin	6.8/2.8	4.4/3.8	6.2/2.9	8.7/5.6	6.6/3.8
Natamycin	9.4/8.3	11.7/9.8	12.4/7.5	13.0/1.7	11.6/6.8
Neospiramycin	4.9/5.5	3.4/4.1	11.3/10.6	16.7/2.8	9.1/5.8
Oleandomycin	11.0/0.4	10.7/3.8	9.5/4.6	11.1/0.4	10.6/2.3
Spiramycin	6.6/9.7	4.1/11.4	2.0/4.3	5.0/7.9	4.4/8.3
Tilmicosin	6.3/7.4	6.1/9.7	10.4/8.8	8.0/5.2	7.7/7.8
Troleandomycin	6.4/1.2	10.5/4.2	7.9/3.6	7.3/6.0	8.0/3.7
Tylosin	8.5/2.4	3.0/3.7	2.0/4.3	7.8/1.5	5.3/3.0
Virginiamycin M1	7.8/3.7	1.7/3.0	6.2/2.0	4.7/3.2	5.1/3.0
β-Lactam					
Ampicillin	6.7/3.1	3.2/5.4	8.6/5.5	15.9/0.8	8.6/3.7
Cefapirin	10.7/4.5	11.8/16.8	8.2/16.0	9.5/8.1	10.0/11.4
Cefoperazone	9.1/3.3	2.1/5.8	1.9/8.7	8.5/1.6	5.4/4.9
Cloxacillin	6.8/8.7	2.3/9.1	5.9/3.6	4.6/7.6	4.9/7.2
Dicloxacillin	12.5/4.2	10.7/1.9	13.0/5.2	10.1/2.3	11.6/3.4
Mecillinum	7.7/4.1	10.9/6.8	6.2/7.7	8.5/1.0	8.3/4.9
Oxacillin	8.4/3.7	3.4/2.6	6.7/10.1	8.9/1.0	6.9/4.3
Licosamides					
Clindamycin	7.6/1.8	2.8/3.5	5.5/2.9	4.9/1.6	5.2/2.4
Lincomycin	10.7/3.8	5.9/12.9	5.4/12.8	5.6/4.6	6.9/8.5
Miscellaneous					
Morantel	7.5/3.1	4.6/1.7	7.8/2.8	10.1/3.5	7.5/2.8
Orbifloxacin	6.6/2.4	4.2/1.3	8.3/1.7	7.8/1.5	6.7/1.7

calibration curve. The quantitative ions used to calculate accuracy and recoveries are shown in Figs. 2 and 3. 17 market milk samples with different brands include skim, low-fat, and full-cream had been analyzed using the multiclass analysis of 23 veterinary drugs. None of these veterinary drugs had been detected in these samples except lincomycin. The chromatograms of lincomycin in blank milk, milk spiked at 100 ng/ml, and purchased milk samples had shown in Fig. 4. Quantification was performed using blank samples spiked at different concentrations: 25, 50, 75 and 100 ng/ml. The values obtained from a matrix-matched curve (6 data points) were used to evaluate the quantification results. The recovery data for all experiments are shown in Table 3. The linearity of the LC/MS–MS response was evaluated by constructing calibration curves in the concentration range of 0–100 ng/ml. The R^2 values of the matrix-matched calibration curves were >0.99 for all of the antibiotics tested (data not shown). The curves were considered to be linear in the range tested, and they were used in this study to determine concentrations of these drugs. Mostly the average recoveries from the milk samples were greater than 70% besides neospiramycin and cefapirin.

In terms of repeatability, Table 4 shows the coefficients of variation (C.V.) for the developed UPLC–MS/MS method for the determination of the 23 drugs in milk. Most of the intra-day and inter-day C.V. values were lower than 15%, which indicates good repeatability for both intra-day and inter-day analyses.

The accuracy of the method was assessed by selecting full-cream as the sample matrix and 3 different concentrations of 25, 50, and 75 ng/ml which cover most range of MRLs. Mean corrected recovery ($n = 5$) of the analytes, determined in three separated assays which is shown in Table 5.

3.6. Method detection limit studies

Limits of detection (LODs) and quantification (LOQs) were calculated as the lowest concentrations of the analyte for which the signal-to-noise (S/N) ratios were over 3 and 10, respectively, from the analysis of blank samples spiked at 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 20 ng/ml. The S/N ratios were calculated using the MassLynx software version 4.1. LODs and LOQs ranged from 0.1 to 2.5 ng/ml and 0.1 to 5 ng/ml, respectively (Table 5).

3.7. Application to real samples

An analyte was considered to be positively identified based on the following criteria: (a) the ratio of the chromatographic

Table 5
MRL, LOD, LOQ and accuracy values for milk samples.

Drug	MRL (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)	Accuracy (%)
Macrolides				
Clarithromycin	–	0.25	0.5	101.7
Erythromycin	40	0.1	0.1	99.5
Josamycin	–	0.1	0.25	100.4
Kitasamycin	–	0.25	0.5	110.4
Natamycin	–	2.5	5	94.3
Neospiramycin	–	1	2.5	103.6
Oleandomycin	–	0.25	0.5	95.6
Spiramycin	200	1	2.5	95.2
Tilmicosin	–	2.5	5	107.2
Troleandomycin	–	0.1	0.25	102.9
Tylosin	50	2.5	5	100.6
Virginiamycin M1	*	0.5	1	102.4
β-Lactam				
Ampicillin	10	1	2.5	103.2
Cefapirin	10	2.5	5	102.3
Cefoperazone	–	2.5	5	101.1
Cloxacillin	10	2.5	5	102.1
Dicloxacillin	–	2.5	5	100.2
Mecillinum	–	0.5	1	102.5
Oxacillin	30	1	2.5	99.3
Licosamides				
Clindamycin	–	1	2.5	105.2
Lincomycin	150	0.1	0.25	105.3
Miscellaneous				
Morantel	100	1	2.5	104.4
Orbifloxacin	–	0.25	0.5	100.4

MRL according to Taiwan regulation DOH Food No. 0991300382 Amended, 3/10/2010.
(–) Means shall not be detected in milk.
* Means not required.

retention time of the analyte to that of the same analyte in the standard solution was within ±2.5% tolerance, (b) the presence of a signal at each of the three transition ions for the analyte, and (c) the peak ion ratio of the quantitative ion against other transition ions was within the tolerance cited by the EU criteria [28]. Seventeen fresh milk samples were tested, and no residues were detected aside from lincomycin. The matrix-matched calibration curve was used to calculate the concentration of these samples. 16 out of the 17 milk samples contained residues of lincomycin, with concentrations ranging from 6.9 to 92.3 ng/ml. According to Taiwanese regulation, lincomycin is not permitted to exceed to 150 ng/ml. Based on these data, the fresh milk in Taiwan's markets remains within safe limits.

4. Conclusions

This paper describes a multi-class residue analysis in milk with a rapid extraction procedure and UPLC/MS/MS analysis that exhibited good precision, LODs, LOQs, linearity and recovery. For example, the LOQs were less than 5 ng/ml for each drug. The proposed method is able to extract more than 30 samples in less than 1 h. The method is able to quickly confirm the presence of 23 veterinary drug residues in milk in a single run and also provides quantitative data on these drug classes.

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