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Determination of Five Macrolide Antibiotic Residues in Raw Milk Using Liquid Chromatography−Electrospray Ionization Tandem Mass Spectrometry

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A confirmatory method using liquid chromatography−electrospray ionization tandem mass spectrometry for determination of five macrolide antibiotics including spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin in raw milk is presented. Macrolides were extracted from raw milk by acetonitrile, and sample extracts were further cleaned up using solid-phase extraction cartridges. Data acquisition was achieved using multiple reaction monitoring, that is, two transitions, to provide a high degree of sensitivity and specificity. Matrix-matched standard calibration curves with the use of roxithromycin as an internal standard were utilized to achieve the best accuracy of the method. Both a conventional validation procedure and a designed experiment were applied to study the accuracy and precision of the method. The measurement uncertainty arising from accuracy and precision was estimated. The method accuracy, expressed as a percentage of overall recovery, was ~100%, and its intermediate precision was <10%. LC-ESI/MS/MS method detection limits (S/N ≥ 3:1) of five macrolides were <0.3 μg/kg.

KEYWORDS: Antibiotics; macrolides; raw milk; experimental design; measurement uncertainty; LC-ESI/MS/MS

INTRODUCTION

Macrolides (**Figure 1**) are a group of antibacterial compounds that display antibacterial properties and are active against Gram-positive and some Gram-negative bacteria. They have been widely utilized in medical and veterinary practice. For example, erythromycin is used for the treatment of clinical and subclinical mastitis in lactating cows. Incorrect use of these drugs or insufficient withdrawal time after treatment can possibly lead to the presence of macrolide residues in milk, which increases the potential risk to consumers because of allergic reactions of those sensitive to the antibiotics (*1*, *2*). The European Union (EU) has set maximum residue limits (MRLs) for macrolides, that is, marker residues, in milk, for example, 50 μg/kg for tylosin, 50 μg/kg tilmicosin, and 40 μg/kg for erythromycin. In Canada, macrolide residues in foods have been tested under the Canadian National Chemical Residues Monitoring Program. To ensure the safety of the food supply, sample monitoring programs require improved methods to lower residue detection limits and confirm identities of incurred residues in edible foods such as raw milk.

Analytical methods for the determination of macrolides in animal products, biological samples, and apicultural products include the use of liquid chromatography (LC) with ultraviolet (UV) or fluorometric detection (*3*−*8*), liquid chromatography−mass spectrometry (LC-MS) (*9*−*11*), and liquid chromatography−tandem mass spectrometry (LC-MS/MS) (*2*, *12*−*15*). The method sensitivity depends on the technique that was applied. For example, an LC with UV detection was able to determine tilmicosin and tylosin at levels as low as 15 μg/kg in porcine, bovine, and poultry tissues (*6*). LC-MS, which was capable of detecting various macrolides in the range from 1 to 10 μg/kg (*11*), tended to be more sensitive than LC-UV. LC-MS/MS has become one of the most promising techniques for the analysis of antibiotics in food because it allows quantification of the antibiotics and confirmation of their identities at trace levels. For example, LC-MS/MS methods have been reported to identify macrolides in animal tissues, eggs, honey, and milk with detection limits of <1 μg/kg (*13*−*15*).

In this paper, we present a validated LC-ESI/MS/MS method with liquid-to-liquid partition and solid-phase extraction (SPE) for the quantification and confirmation of five macrolides in raw milk. The extraction procedure and LC profile were modified from our previously published methods (*14*, *15*) to improve the method sensitivity and repeatability for the determination of these compounds in raw milk. The method was validated according to a conventional validation procedure as well as a designed experiment, that is, a nested experimental design, to evaluate its performance criteria. The conventional validation procedure provided data of performance criteria at each level, whereas the designed experiment allowed evaluation of the method as a whole. In addition, the measurement

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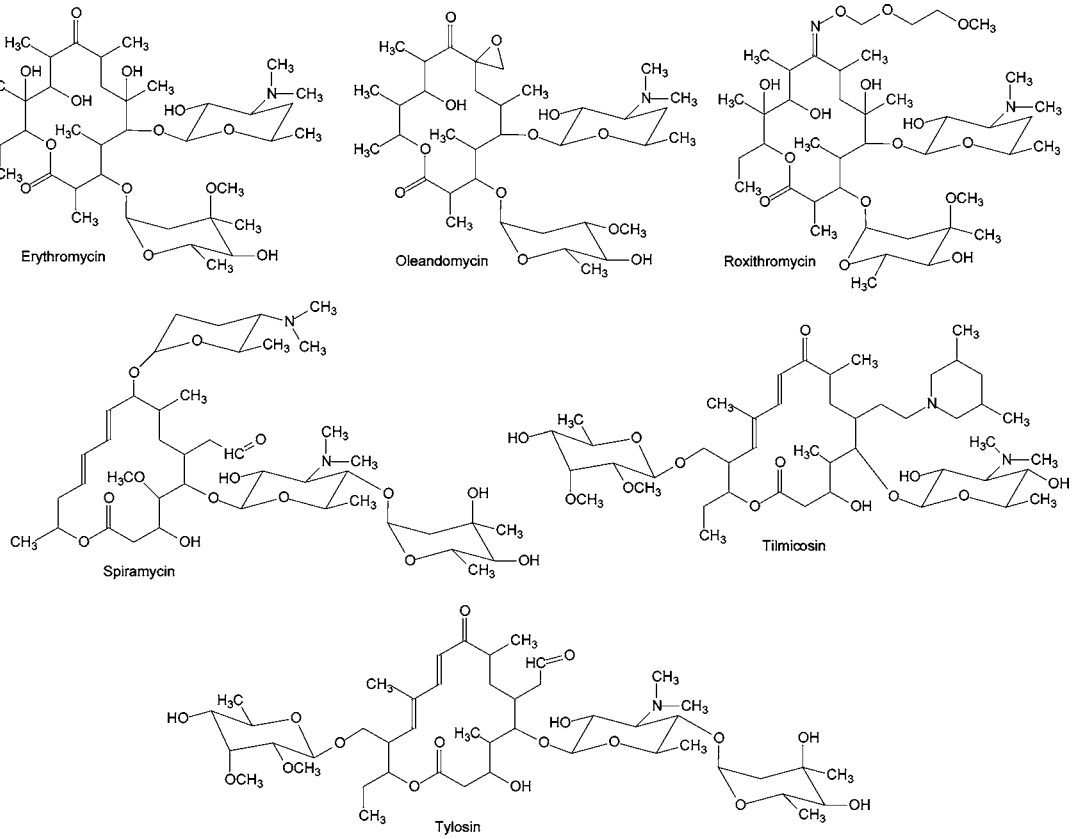

Figure 1. Chemical structures of five macrolides and the internal standard roxithromycin.

uncertainty associated with a testing result, which is an essential part of quantitative results and is required by ISO standard 17025 (*16*), was also estimated. This approach has been used by others to estimate the method measurement uncertainty and different levels of uncertainties of analytical methods (*15*, *17*, *18*). We continued to demonstrate that the approach is very practical for measurement uncertainty estimation using in-house validation data for newly developed methods. The method we present in this paper can be used for regulatory routine testing, especially for monitoring programs, which have defined MRLs.

MATERIALS AND METHODS

Materials and Reagents. Erythromycin (E6376), oleandomycin (phosphate salt) (O6125), roxithromycin (R4393), spiramycin (S-9132), and tylosin (tartrate) (T6134) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Tilmicosin (90.7%) was a gift from Eli Lilly and Co. (Indianapolis, IN). Formic acid (96%) and sodium dihydrogen orthophosphate or monobasic sodium phosphate (monohydrate) were from BDH Inc. (Ontario, Canada). Acetonitrile, sodium chloride, and ammonium acetate were obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Oasis HLB Plus cartridges (225 mg) were from Waters Corp. (Milford, MA). Raw milk (macrolides free), a total of six batches, was collected from different local farms or at different times in Alberta, Canada, to ensure that testing matrices were representative. Codes such as samples A, B, C, D, E, and F were assigned to six batch raw milk samples and have been used throughout the text. Raw milk samples were kept frozen under −20 °C and were thawed at room temperature prior to weighing. All water used was doubly deionized water (Milli-Q water purification system, Millipore Corp., Bedford, MA). Formic acid (1%) was prepared by adding 10.4 mL of formic acid (96%) into a 1000 mL volumetric flask and making up to volume with water. Sodium chloride solution (2%) was made by dissolving 20 g of NaCl into 900 mL of water and making up to volume in a 1000 mL volumetric flask with water. Phosphate buffer (0.1 M, pH 8.0) was prepared by dissolving 13.8 g of monobasic sodium phosphate (monohydrate) in 900 mL of water in a 1000 mL beaker, adjusting to pH 8.0 with dropwise addition of 10 N NaOH, and finally making up to volume in a 1000 mL volumetric flask with water. Ammonium acetate (0.1 M) was made by dissolving 7.7 g of ammonium acetate into 900 mL of water and making up to volume in a 1000 mL volumetric flask with water. Solvent buffer, which was used to reconstitute the intermediate solution, working solution, and final sample extracts, was prepared by mixing acetonitrile and 0.1 M ammonium acetate in a ratio of 15:85.

Preparation of Standard Solutions. Individual standard stock solutions (1000.0 μg/mL) were prepared by weighing 10 mg of each spiramycin, tilmicosin, oleandomycin, erythromycin, tylosin, and roxithromycin (internal standard, IS) into separate 10 mL volumetric flasks and dissolving in methanol. The standards were corrected for purity to give concentrations as free bases (in case of salts). Stock solutions were refrigerated at 4 °C for not longer than 2 months. An intermediate standard solution (1.0 μg/mL) was prepared by transferring 100 μL of each standard stock solution except roxithromycin to a single 100 mL volumetric flask and diluting to volume with solvent buffer. An intermediate internal standard solution (1.0 μg/mL) was prepared by transferring 100 μL of roxithromycin stock solution to a single 100 mL volumetric flask and diluting to volume with solvent buffer. Working standard solutions, that is, set A, with concentrations of 0.01, 0.04, 0.2, 0.4, 0.6, and 0.8 μg/mL, which were used for the preparation of matrix-matched standard calibration curves, were prepared by transferring 0.1, 0.4, 2, 4, 6, and 8 mL of the intermediate solution into six separate 10 mL volumetric flasks, adding 2 mL of the intermediate internal standard solution to each flask, and diluting to volume. Working standard solutions, that is, set B, with concentrations of 0.05, 0.4, 0.5, and 0.7 μg/mL, which were used for the preparation of spike samples, were prepared by transferring 0.5, 4, 5, and 7 mL of the intermediate solution into four separate 10 mL volumetric flasks, adding 2 mL of the intermediate internal standard solution to each flask, and diluting to volume. All intermediate solutions and working solutions were prepared weekly and stored at 4 °C.

Extraction of Macrolides from Raw Milk Samples. For routine testing samples, a raw milk sample (5.00 ± 0.05 g) was weighed into a 50 mL centrifuge tube [polypropylene centrifuge tubes with screw

caps (VWR International, Edmonton, AB, Canada)], to which 100 μL of the intermediate internal standard solution was added. For spike samples, 500 μL of working solutions in set B at each concentration was added to blank raw milk samples to produce spike levels at 5, 40, 50, and 70 μg/kg of macrolides equivalent in samples. Acetonitrile (15 mL) was added, and the centrifuge tube was capped and shaken for 15 min on an Eberbach shaker (Eberbach Corp., Ann Arbor, MI). Then the sample was centrifuged [Allegra 6 centrifuge (Beckman Coulter, Inc. Fullerton, CA)] at 3210*g* for 15 min at room temperature. The supernatant was transferred into another 50 mL centrifuge tube, and 20 mL of hexane was added. The centrifuge tube was capped and shaken again for 15 min. The above sample mixture was centrifuged again at 3210*g* for 15 min at room temperature. The top hexane layer was removed, and the middle layer (~18 mL), that is, a mixture of acetonitrile and water containing macrolide residues, was transferred into a 16 × 125 mm test tube. Acetonitrile was removed using a stream of nitrogen at 50 °C on an N-EVAP nitrogen evaporator (Organomation Associates Inc., Berlin, MA). After ~90 min, ~3 mL aqueous remained in the test tube, to which 15 mL of 0.1 M phosphate buffer (pH 8.0) was added to reconstitute sample extracts. Oasis HLB cartridges were preconditioned sequentially with 10 mL of methanol, 10 mL of water, 10 mL of 2% NaCl, and 2 mL of 0.1 M phosphate buffer (pH 8.0). The reconstituted solution was loaded on the preconditioned Oasis HLB cartridge under vacuum at −6 to −10 kPa with a flow rate of ~1 mL/min. The cartridge was then rinsed with 5 mL of water at a flow rate of ~2 mL/min, followed by 5 mL of 40% methanol in water at the same flow rate. The cartridge was dried under vacuum for 5 min. Finally, macrolides were eluted from the cartridge under vacuum with 5 mL of 95% methanol at a flow rate of 1−2 mL/min into a 15 mL test tube. The eluate was brought close to dryness using an N-EVAP nitrogen evaporator at 50 °C with attention that the bottom of a test tube appeared to be just dry (~45 min). Any condensation around the mouth of a test tube and/or on the stainless steel luer needle of the evaporator was ignored. Excess evaporation or heating resulted in poor quantitative results for tilmicosin. The sample was reconstituted by the addition of 1 mL of solvent buffer to extracted residues in the test tube. The test tube was vortexed for 30 s to dissolve the residues, and extracts were then filtered through Mini-UniPrep syringeless filter vials (PVDF 0.45 μm) (Whatman Inc., Clifton, NJ) for LC-ESI/MS/MS analysis.

LC-ESI/MS/MS. The LC-ESI/MS/MS system used was an Alliance 2695 HPLC coupled with a Micromass Quattro Ultima Pt tandem mass spectrometer utilizing electrospray interface (LC-ESI/MS/MS) and MassLynx 4.0 software (Waters, Milford, MA).

(a) LC Profile. Mobile phase components were acetonitrile (solvent A), 1% formic acid (solvent B), and water (solvent C). The linear gradient profile consisted of 0−8 min, 20−80% A and 10% B; 8−10 min, 80% A and 10% B; 10−18 min, 100% A; and 18−25 min, 20% A and 10% B. Flow rates were, at 0−10 min, 0.2 mL/min; 10−18 min, 0.35 mL/min; 18−22 min, 0.3 mL/min; and 22−25 min, 0.2 mL/min. The injection volume was 20 μL. Retention time windows for data acquisition are listed in **Table 1**. The LC analytical column utilized was a YMC ODS-AQ S-3 120 Å 50 × 2 mm cartridge coupled with a YMC ODS-AQ S-3 120 Å 20 × 2 mm guard cartridge with 2.0 mm YMC Endfittings and YMC Direct Connect Endfitting (Waters).

(b) MS conditions: ionization mode, electrospray positive ion mode; capillary voltage, 3.20 kV; source temperature, 120 °C; desolvation temperature, 300 °C; nebulizer nitrogen flow rate, 150 L/h; desolvation nitrogen gas flow rate, 650 L/h; collision gas argon pressure, 2.5 × 10^{-3} mbar; LM 1 resolution, 14.0; HM 1 resolution, 14.0; ion energy 1, 0.8 V; entrance voltage, −2 V; exit voltage, 1 V; LM 2 resolution, 14.0; HM 2 resolution, 14.0; ion energy 2, 1.0 V; multiplier voltage, 650 V; dwell time, 0.08 s. Cone voltage, collision energy, and multireaction monitoring (MRM) are listed in **Table 1**. These settings were able to achieve unit mass resolution. For each individual macrolide, the mass spectrometer was optimized using flow injection to provide the best responses for quantification and reasonable ion ratios for confirmation under MRM. The flow rate of a syringe pump (Harvard Apparatus, Holliston, MA) was set at 10 μL/min. For the flow injection, macrolides (1.0 μg/mL) (except erythromycin) were prepared in a mixture of acetonitrile and water (50:50) containing 0.1% formic acid, and erythromycin (1.0 μg/mL) was prepared in a mixture of acetonitrile and water (50:50) without the presence of 0.1% formic acid because it degraded significantly under the acidic condition in a few hours.

Table 1. ESI-MS/MS Parameters for the Five Macrolides and the Internal Standard

analyte	MRM transition (*m*/*z*)	cone voltage (V)	collision energy (eV)	retention time window (min)
spiramycin	843 → 174*[a]	35	35	1.6–4.0
	843 → 142			
spiramycin^{2+}[b]	422 → 101*	35	18	1.6–4.0
	422 → 142			
tilmicosin	869 → 174*	35	40	4.0–9.0
	869 → 132			
oleandomycin	688 → 158*	35	20	4.0–9.0
	688 → 544		14	
erythromycin	734 → 158*	35	30	4.0–9.0
	734 → 576		20	
tylosin	916 → 174*	35	35	4.0–9.0
	916 → 145			
roxithromycin (IS)	837 → 158	35	30	4.0–9.0

[a] The predominant ion defined as a base peak for quantification is marked by an asterisk. [b] Doubly charged ion was used for data acquisition.

Preparation of Standard Calibration Curves and Calculation. Matrix-matched calibration standard curves were utilized in this study for the quantification of macrolides in raw milk. A raw milk sample (5.00 ± 0.05 g) was weighed into each of six separate 50 mL centrifuge tubes. Five hundred microliters of working solutions in set A at each concentration was added to blank raw milk samples to provide 1, 4, 20, 40, 60, and 80 μg/kg of macrolides equivalent in samples to cover an analytical range from 1.0 to 80 μg/kg. Raw milk samples containing macrolide standards were processed through the complete extraction procedure. Sample A was used to prepare matrix-matched standard calibration curves, which were prepared fresh for each day's samples, through the study.

Quantitative results including ion ratios were calculated using QuanLynx software bundled with MassLynx 4.0. Linear function was used for the quantification of spiramycin and tilmicosin. Quadratic function was used for the quantification of oleandomycin, erythromycin, and tylosin. The fit weighting $^1/_x$ was applied (*19*). Recovery in this study means apparent recovery due to the use of matrix-matched calibration standard curves.

Statistics. Means and standard deviations were calculated using Microsoft Excel 2002 (Microsoft Office 2002). Linear regression or quadratic function and coefficients of correlation (R^2) were generated using QuanLynx MassLynx 4.0. Mean recoveries and variances of the nested experimental design were calculated using the SAS Software release 9.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Extraction and Data Acquisition. The method, which used liquid-to-liquid partition and solid-phase extraction to extract macrolides in eggs, was previously developed and reported by Wang et al. (*15*). The same extraction procedure when utilized to extract macrolides from raw milk, however, ended in poor recovery for spiramycin and tilmicosin because of their high solubility in aqueous solution or relatively high polarity. Therefore, the method was modified accordingly by omitting sodium chloride, which was previously used to salt-out macrolides into the acetonitrile layer, and carrying the whole mixture, that is, acetonitrile and water, through the extraction steps. Second, to avoid generating an emulsion in the hexane extraction step, a gel-like solution found at the bottom of the centrifuge tubes was not transferred with the extracts. Finally, the residues were not permitted to run dry completely on the

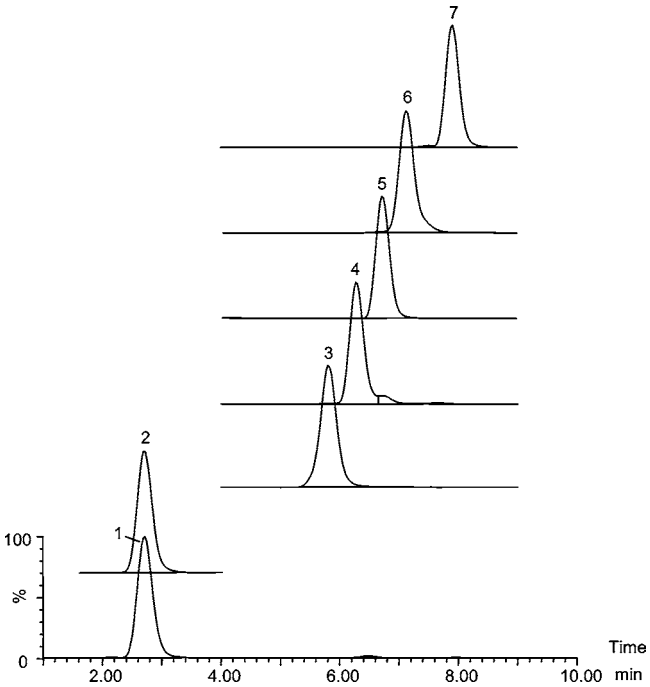

Figure 2. LC-ESI/MS/MS chromatograms of a blank raw milk fortified with five macrolides (5 μg/kg per analyte) and the internal standard roxithromycin (20 μg/kg): 1, spiramycin (843 → 174); 2, spiramycin [doubly charged ion; (422 → 101)]; 3, tilmicosin (869 → 174); 4, oleandomycin (688 → 158); 5, erythromycin (734 → 158); 6, tylosin (916 → 174); 7, roxithromycin (IS; 837 → 158).

N-EVAP nitrogen evaporator to remove methanol, as excessive evaporation was found to result in poor repeatability for tilmicosin.

Macrolides were ionized in protonated form in electrospray positive ion mode to form singly and/or doubly charged pseudomolecular ions as shown by their chemical structures and proton affinity (*14*). Oleandomycin, erythromycin, and tylosin have one nitrogen (**Figure 1**), which formed singly charged, $[M + H]^+$, ions. Spiramycin, tilmicosin, and roxithromycin (IS) contain two nitrogens **(Figure 1)**, which formed both singly, $[M + H]^+$, and doubly charged, $[M + 2H]^{2+}$, ions. In this study, singly and/or doubly charged molecular ions were monitored for data acquisition except for tilmicosin because an interference was observed at its doubly charged ion transition and retention time.

Typical LC-ESI/MS/MS chromatograms are shown in **Figure 2**. Macrolides were separated on a reverse phase LC column under the given gradient conditions within 10 min. The elution profile was in the following order with typical retention times: spiramycin (2.7 min), tilmicosin (5.8 min), oleandomycin (6.3 min), erythromycin (6.7 min), tylosin (7.1 min), and internal standard roxithromycin (7.9 min) (**Figure 2**). The tolerance of retention time matching did not exceed ±2.5% relative to the retention time of matrix-matched standards. To avoid spiramycin and tilmicosin's retention time drift, the column must be regenerated according to the procedure described under Materials and Methods. Although a total of 25 min of chromatography cycle time per injection was a little longer than expected, this provided consistent results to meet the retention time tolerance criterion. Shorter run times may be achieved using different brands of LC columns.

Conventional Validation Procedure. The method was validated and data were organized according to a conventional validation procedure to study the repeatability, accuracy, and matrix effects as described below. This approach provides data on these performance criteria at each level and allows evaluation of a method through the observation of individual factors. Matrix-matched standard calibration curves with the use of roxithromycin as an internal standard were utilized to achieve the best accuracy of the method. All matrix-matched calibration standard curves with either linear or quadratic function resulted in the correlation of coefficient values (R^2) consistently above 0.99.

Accuracy and Precision. The LC-ESI/MS/MS method was tested for its accuracy, expressed as recovery, and precision, expressed as inter- and intra-assay repeatability, and results are shown in **Table 2**. Macrolides were fortified in sample A at levels of 5, 40, 50, and 70 μg/kg in triplicate, considering tilmicosin and tylosin's EU MRL at 50 μg/kg and erythromycin's MRL at 40 μg/kg, on four different days for interassay repeatability study, in addition to the same day for intra-assay repeatability study. Macrolides were then extracted and analyzed using LC-ESI/MS/MS. The recoveries of macrolides of the interassay, that is, fortified on four different days, ranged from 92.0 to 107.6% with relative standard deviations (RSDs) of <10%. The recoveries of macrolides of the intra-assay, that is, fortified within the same day, ranged from 95.9 to 114.1% with RSDs of <10%. The method demonstrated good accuracy and precision for quantifying macrolides in raw milk samples.

Matrix Effects. Quantitative results can be significantly different from sample to sample due to variable matrix effects. Therefore, the method was tested for its feasibility in quantifying macrolides in various raw milk matrices. In this study, six batches of raw milk from different farms (samples A, B, C, D, E, and F) were fortified with macrolides at levels of 5.0, 40.0, 50.0, and 70.0 μg/kg in triplicate. Quantitative results of macrolides from the fortified samples are summarized in **Table 3**. Mean recoveries, which were calculated from spiked samples that were prepared in triplicate by two analysts, ranged from 0.889 (88.9%) to 1.168 (116.8%) with the RSDs of not greater than 19% and, most of the time, less than 10%. Because experiments were conducted by two analysts for each of matrices, small RSDs (**Table 3**) also demonstrated exceptional robustness of the method. In general, the developed method was able to accurately and precisely quantify the five macrolides in different raw milk matrices.

Experimental Design. As an alternative validation model, a designed experiment, that is, a nested experimental design (*15*, *17*, *18*, *20*), was used to study and evaluate performance criteria including accuracy expressed as overall recovery, intermediate precision, and measurement uncertainty of the method as a whole. Performance criteria of a method were evaluated on the basis of concentrations or spike levels of analytes, matrix effects, day-to-day variation due to analysts, reagents, analytical columns, and instrument and within-day variation. These factors were the same as those studied in the conventional validation procedure above. However, with a nested experiment, the data can be manipulated using statistics software to calculate variances of individual factors. Such outcomes can then be used for further calculation including estimation of the intermediate precision and measurement uncertainty.

With the designed experiment, four concentration levels ($l = 4$) were included, that is, four fortified levels at 5, 40, 50, and 70 μg/kg. For each concentration, the recovery was estimated with six different raw milk matrices. For each matrix, the analysis was carried out on two different days by two analysts using different reagents and analytical columns, and each sample was prepared in triplicate ($r = 3$), that is, three

Table 2. LC-ESI/MS/MS Accuracy and Repeatability of the Method for Determination of Macrolides Spiked in Raw Milk Samples

	spike level	intra-assay[a]				inter-assay[b]			
analyte	(μg/kg)	recovery (%)	RSD (%)	ion ratio[c] (%)	RSD (%)	recovery (%)	RSD (%)	ion ratio[c] (%)	RSD (%)
				(843 → 142)/ (843 → 174)				(843 → 142)/ (843 → 174)	
spiramycin	5.0	96.9	1.5	31.7	6.0	92.0	7.0	32.4	3.2
	40.0	102.7	2.6	32.4	1.9	100.0	4.3	33.1	2.9
	50.0	103.3	1.3	31.9	0.5	98.8	3.6	32.2	1.7
	70.0	106.6	1.8	32.3	1.4	101.3	6.1	32.3	1.2
				(422 → 142)/ (422 → 101)				(422 → 142)/ (422 → 101)	
spiramycin^{2+}	5.0	100.1	2.7	33.3	1.4	98.8	2.2	32.1	3.7
	40.0	100.4	1.3	30.9	0.2	99.1	1.9	30.4	2.0
	50.0	99.1	3.4	30.8	0.9	97.0	1.0	30.1	1.2
	70.0	95.9	2.3	30.6	0.7	94.3	1.7	29.7	1.8
				(869 → 132)/ (869 → 174)				(869 → 132)/ (869 → 174)	
tilmicosin	5.0	114.1	3.5	37.5	2.0	101.8	7.8	36.7	3.1
	40.0	103.5	8.8	36.9	2.1	98.4	7.6	37.1	3.2
	50.0	98.3	6.1	37.0	1.5	96.8	3.5	36.0	2.0
	70.0	97.2	8.4	37.0	2.4	92.7	3.3	36.4	2.9
				(688 → 544)/ (688 → 158)				(688 → 544)/ (688 → 158)	
oleandomycin	5.0	104.0	1.0	76.9	1.6	102.1	3.2	77.2	3.2
	40.0	102.2	1.9	76.9	1.3	100.2	1.5	77.6	2.2
	50.0	102.7	1.6	77.5	0.3	100.0	2.3	77.2	2.5
	70.0	102.1	0.9	77.8	0.3	99.8	1.2	77.1	1.1
				(734 → 576)/ (734 → 158)				(734 → 576)/ (734 → 158)	
erythromycin	5.0	106.6	1.2	34.2	1.2	107.6	2.6	33.7	3.5
	40.0	103.0	2.4	33.8	2.0	103.6	1.1	33.3	3.5
	50.0	105.7	1.9	33.9	0.3	103.1	2.0	33.4	2.7
	70.0	101.7	1.6	34.1	1.2	100.1	2.2	33.5	1.5
				(916 → 145)/ (916 → 174)				(916 → 145)/ (916 → 174)	
tylosin	5.0	105.2	0.3	10.4	2.4	105.5	1.7	10.9	1.6
	40.0	102.4	1.7	10.5	0.5	103.0	2.0	10.9	2.9
	50.0	105.3	2.3	10.3	1.9	105.1	2.0	10.7	2.5
	70.0	100.8	0.3	10.4	2.0	99.4	2.8	10.7	1.6

[a] Means of triplicates ($n = 3$). [b] Means of four replicates ($n = 4$). [c] Ion ratios of individual macrolides were expressed as percentage of the corresponding base peak.

separate extractions, which were analyzed each day. Detailed calculations on overall recoveries, variances, intermediate precision, and measurement uncertainty were described elsewhere (*15*, *17*, *18*), and Appendix A also lists equations that were presented in the paper by Wang et al. (*15*) for reference.

Recovery (R) (Equation 1) and Its Uncertainty [u(R)] (Equation 2). The overall recovery ($\bar{\bar{R}}_m$) (eq 3), that is, an estimation of the accuracy of the method for each individual macrolide, was calculated from mean recoveries listed in **Table 3** and is shown in **Table 4**. The overall recoveries of five macrolides range from 0.993 (99.3%) to 1.092 (109.2%) (**Table 4**). A test was conducted to determine if the recovery was significantly different from 1. The *t* values (eq 4) of five macrolides are listed in **Table 4.** The *t* values of spiramycin^{2+} (doubly charged ion), tilmicosin, erythromycin, and tylosin were <1.96 (two-sided *z* value, $\alpha = 0.05$), which means that their recoveries were not significantly different from 1. The *t* values of spiramycin and oleandomycin were >1.96; therefore, their recoveries were found to be statistically different from 1, the method has a significant bias, and, as a result by theory, a correction factor expressed as recovery can be applied to correct the analytical results (*18*). Normally, analytical results were not corrected by a correction factor in a regulatory environment, and in this study, because the overall recoveries were very close to 1, the correction was not applied, but the uncertainty associated with the overall recovery was included in the uncertainty budget of the in-house validated method to avoid underestimation of the uncertainty due to the nonsignificant proportional bias, $u(\bar{\bar{R}}_m)$ (eq 5) (*21*).

In general, three components contributed to the uncertainty [$u(R)$] (eq 2) due to the recovery (R) (eq 1) (*15*). The three components were overall recoveries, the variation of recovery caused by the different matrices, and the variation of recovery due to the amount of an analyte spiked in samples (eq 1). The uncertainties of recoveries [$u(R)$] (eq 2) of five macrolides, which were <4%, are listed in **Table 4**. Because the uncertainty of overall recoveries were calculated on the basis of information from either the relative intermediate precision (eq 6) (*17*) or the standard deviation of mean recoveries at four spiked levels (eq 7) (*18*), two sets of $u(R)$ are presented, and their values were very similar (**Table 4**). Furthermore, the matrix and concentration effects were statistically significant ($p < 0.05$), which indicated that uncertainties associated with matrix [$u(M)$] and concentration [$u(C)$] were the main sources of uncertainty in the recovery uncertainty budget [$u(R)$] (eq 2).

Precision and Its Uncertainty [u(P)] (eq 9). The uncertainty arising from the precision of the method is expressed as a relative intermediate standard deviation and calculated using eq 9; that is, the intermediate precision $u(R_I)$ (eq 8) is divided by the overall recovery $\bar{\bar{R}}_m$ (eq 3). The results are listed in

Table 3. Macrolide Mean Recoveries Determined by LC-ESI/MS/MS from Spiked Raw Milk Samples under Intermediate Precision Condition

compound	spike level (μg/kg)	sample A[a]		sample B[a]		sample C[a]		sample D[a]		sample E[a]		sample F[a]	
		recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)
spiramycin	5.0	1.122	6.3	1.110	9.7	1.132	11.5	1.131	9.9	1.104	8.7	0.932	5.3
	40.0	1.072	1.8	1.168	10.6	1.166	7.9	1.117	6.0	1.097	3.3	0.993	4.2
	50.0	1.060	3.4	1.144	10.2	1.151	6.2	1.116	4.4	1.083	4.5	1.012	2.5
	70.0	1.054	3.6	1.112	4.2	1.099	2.8	1.065	3.6	1.120	4.2	1.037	4.0
spiramycin^{2+b}	5.0	0.983	4.2	1.074	9.5	1.056	8.7	0.971	3.1	1.042	2.0	0.989	2.5
	40.0	0.961	3.8	1.051	5.9	1.071	6.2	0.960	1.2	1.024	4.5	0.994	1.9
	50.0	0.919	5.8	1.026	4.2	1.037	9.7	0.986	6.3	0.988	2.9	0.979	2.7
	70.0	0.905	3.3	1.012	0.9	0.992	8.9	0.948	3.2	0.974	3.1	0.947	2.5
tilmicosin	5.0	1.141	8.0	0.989	8.9	1.063	11.8	0.983	9.0	0.932	7.8	1.048	10.0
	40.0	1.036	12.5	1.024	5.2	1.065	6.8	0.989	8.0	1.032	4.2	0.988	9.1
	50.0	1.020	5.9	1.011	8.0	1.107	3.1	0.979	7.5	0.986	10.7	0.986	4.1
	70.0	1.015	4.2	0.938	4.6	1.026	4.7	0.939	12.8	0.889	19.0	0.971	5.8
oleandomycin	5.0	1.102	6.6	0.996	2.0	0.999	2.2	1.005	4.0	1.053	3.8	1.032	1.9
	40.0	1.043	4.1	1.002	2.4	1.015	1.3	0.965	1.8	1.005	2.6	1.008	2.2
	50.0	1.022	1.9	0.999	2.4	1.009	1.8	0.981	6.6	1.019	3.9	1.014	2.1
	70.0	1.043	2.6	1.014	2.5	1.010	2.7	0.951	1.3	1.003	2.9	1.013	1.9
erythromycin	5.0	1.015	4.8	1.036	1.4	0.975	3.4	1.000	2.4	1.059	3.5	1.062	1.4
	40.0	0.987	3.0	1.038	3.4	0.989	4.9	0.958	2.6	0.998	2.1	1.029	1.9
	50.0	0.970	1.8	1.010	2.2	0.977	2.9	0.978	4.5	0.987	2.2	1.040	2.5
	70.0	0.958	2.2	1.013	3.9	0.963	5.0	0.984	2.6	0.991	2.6	1.006	1.6
tylosin	5.0	0.955	4.8	1.065	2.8	0.963	2.3	0.980	2.8	1.002	3.1	1.067	1.6
	40.0	0.946	4.4	1.040	3.1	0.978	4.5	0.953	1.7	0.988	4.5	1.038	2.3
	50.0	0.929	1.9	1.038	4.7	0.960	2.5	0.945	1.5	0.991	5.5	1.047	2.0
	70.0	0.918	2.7	1.047	2.8	0.972	3.8	0.995	3.3	1.011	9.3	1.006	1.1

[a] Samples were prepared by two analysts separately on two different days in triplicate ($n = 6$). [b] Doubly charged ion was used for data acquisition.

Table 4. Overall Recoveries and Measurement Uncertainty Arising from the Accuracy and Precision of Five Macrolides Spiked in Raw Milk Samples

compound	$\bar{\bar{R}}_m$	t	$u(P)$	$u(R)^a$	$u(R)^b$	spike level (μg/kg)	$x_{a,i}^2 \times u(R_i)^2/\bar{R}_m^4$	$x_{a,i}^2 \times u(R)^2/\bar{R}_m^2$	U^a ($k = 2$)	U/X (%)	$^1/_2$ RSDR[c] (%)	$^2/_3$ RSDR[c] (%)
spiramycin	1.092	12.92	7.8×10^{-2}	2.7×10^{-2}	2.9×10^{-2}	5.0	0.1	0.0	0.8	15.1	17.8	23.7
						40.0	8.1	1.0	6.0	15.1	13.0	17.3
						50.0	12.7	1.6	7.6	15.1	12.6	16.7
						70.0	24.9	3.1	10.6	15.1	11.9	15.9
spiramycin^{2+}	0.995	0.89	6.3×10^{-2}	2.1×10^{-2}	2.2×10^{-2}	5.0	0.1	0.0	0.7	13.4	17.8	23.7
						40.0	6.5	0.7	5.4	13.4	13.0	17.3
						50.0	10.2	1.1	6.7	13.4	12.6	16.7
						70.0	19.9	2.2	9.4	13.4	11.9	15.9
tilmicosin	1.007	0.85	8.9×10^{-2}	3.8×10^{-2}	3.9×10^{-2}	5.0	0.2	0.0	1.0	19.2	17.8	23.7
						40.0	12.4	2.4	7.7	19.2	13.0	17.3
						50.0	19.4	3.7	9.6	19.2	12.6	16.7
						70.0	38.1	7.2	13.5	19.2	11.9	15.9
oleandomycin	1.013	4.29	3.4×10^{-2}	2.2×10^{-2}	2.2×10^{-2}	5.0	0.0	0.0	0.4	8.1	17.8	23.7
						40.0	1.9	0.8	3.3	8.1	13.0	17.3
						50.0	3.0	1.2	4.1	8.1	12.6	16.7
						70.0	5.8	2.3	5.7	8.1	11.9	15.9
erythromycin	1.001	0.31	3.4×10^{-2}	2.4×10^{-2}	2.4×10^{-2}	5.0	0.0	0.0	0.4	8.3	17.8	23.7
						40.0	1.8	0.9	3.3	8.3	13.0	17.3
						50.0	2.9	1.5	4.2	8.3	12.6	16.7
						70.0	5.6	2.9	5.8	8.3	11.9	15.9
tylosin	0.993	1.94	4.3×10^{-2}	3.8×10^{-2}	3.9×10^{-2}	5.0	0.0	0.0	0.6	11.5	17.8	23.7
						40.0	3.0	2.3	4.6	11.5	13.0	17.3
						50.0	4.7	3.6	5.8	11.5	12.6	16.7
						70.0	9.3	7.1	8.1	11.5	11.9	15.9

[a] Using information from the intermediate precision according to the method of Dehouck et al. (*17*). [b] Using the standard deviation of mean recoveries of four spike levels according to the method of Maroto et al. (*18*). [c] RSDR was calculated using the Horwitz equation (*22*). RSDR $= 2^{(1-0.5\log C)}$. C is the concentration expressed as fractions.

Table 4. The method showed better intermediate precision for oleandomycin, erythromycin, and tylosin [$u(P) < 5\%$] than for spiramycin and tilmicosin [$u(P) > 7\%$]. Overall, the method demonstrated very good intermediate precision.

Combined Standard Uncertainty and Expanded Uncertainty. The combined standard uncertainty of the quantitative result, $u(X_{a,i})$ (eq 10), of a sample spiked with an amount $X_{a,i}$ was calculated using eq 10, and this uncertainty from in-house validation data can be applied to future sample testing results. The first term of eq 10 is associated with the uncertainty arising from the experimental variability of the method, that is, intermediate precision, and the second term takes into account the uncertainty associated with uncertainties due to overall recoveries, matrix effects, and concentration variability. There-

fore, the combined standard uncertainty basically covered uncertainties arising from intermediate precision and recovery or accuracy of the method including matrix effects. The expanded uncertainty, *U*, was then calculated using the coverage factor $k = 2$, and results are listed in **Table 4**. Apparently, uncertainty due to the precision of the method (the first term of eq 10) is the major source of the uncertainty in the budget compared to that of the recovery (the second term of eq 10) (**Table 4**). The relatively low uncertainty of the recovery (<4%) also indicated that matrix effects, a key factor that usually causes large variation of the LC-ESI/MS/MS quantitative results due to ion suppression or enhancement, were not a major source of uncertainty in the method. This could simply result from the use of matrix-matched standard calibration curves with roxithromycin as an internal standard for quantification. The between-laboratory relative standard deviations (RSDR, %) according to the Horwitz formula were also calculated for comparison; the within-laboratory relative standard deviations (RSDr, %) should be half to two-thirds RSDR (%) (*22*). The relative uncertainties, *U*/*X* (%), in **Table 4**, of macrolides fortified at four different levels were close to the predicted values of half to two-thirds RSDR (%) in certain cases.

Confirmation. Ion ratios and retention times are two criteria used for confirmation. Ion ratios of macrolides obtained within the same day remained very consistent, and RSDs were usually ≤10% (**Table 2**, under intra-assay). Although ion ratios between days tended to be consistent, it was preferable that the relative ion intensities of the detected ions from incurred samples were essentially compared to those of corresponding calibration standards measured under the same conditions, that is, in the same batch of runs, so as to confirm the identity of macrolides. Ion ratios acquired under the same conditions lay within tolerances as recommended by European Commission Decision 2002/657/EC (*23*). The consistency of ion ratio plus LC retention time is thus essential in the confirmation of macrolides in various raw milks.

Method Limits of Detection (LOD). The method LOD (signal-to-noise, S/N ≥ 3) was determined using the MRM transition that provided the most intense analyte signal in macrolide-spiked samples. Under the conditions specified in the method, the method LODs of spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin were 0.08, 0.3, 0.09, 0.07, and 0.06 μg/kg, respectively.

In conclusion, LC-ESI/MS/MS proved to be a sensitive technique for the quantification and confirmation of five macrolides in raw milk at trace levels. Liquid-to-liquid partition and solid-phase extraction with Oasis HLB cartridges served as a simple and rapid method to remove proteins, lipids, and other substances in raw milk so as to extract and concentrate macrolides from the matrix for further analysis. The LC-ESI/MS/MS method reported in this paper was able to quantify and confirm five macrolides in raw milk in a range from 1 to 80 μg/kg with method LODs for the five macrolides of <0.3 μg/kg. The conventional validation procedure and the designed experiment described in this paper are both practical in method validation for evaluating method performance criteria such as accuracy, precision, and matrix effects. The conventional validation procedure provides data of performance criteria at each level or component as required, whereas the designed experiment allows evaluation of the method as a whole. The designed experimental data seemed to be practical for estimating the measurement uncertainty of the method. Overall recoveries of the five macrolides are all >90%. The major source of uncertainty of the method is from the intermediate precision of the method. The expanded uncertainty due to the intermediate precision and recovery including matrix effects and concentration variability were comparable to the predicated values of RSDr from the Horwitz equation. The validated LC-ESI/MS/MS method can thus be employed to determine five macrolides in raw milk for regulatory purposes, especially when it is important to confirm the identity of macrolides in incurred samples and to report the measurement uncertainty.

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APPENDIX A

$$R = \bar{\bar{R}}_m + \Delta R_M + \Delta R_C \quad (1)$$

$$u(R) = \sqrt{u(\bar{\bar{R}}_m)^2 + u(M)^2 + u(C)^2} \quad (2)$$

$$\bar{\bar{R}}_m = \frac{\sum_{i=1}^{l} \bar{R}_i}{l} \quad (3)$$

$$|\bar{\bar{R}}_m - 1| \le t_{\alpha/2,\mathrm{eff}} u(\bar{\bar{R}}_m) \quad (4)$$

$$u(\bar{\bar{R}}_m) = \sqrt{\frac{\sum_{i=1}^{l} u(\bar{\bar{R}}_i)^2}{l^2}} \quad (5)$$

$$u(\bar{\bar{R}}_i)^2 = \frac{u(R_I)^2}{npr} \quad (6)$$

$$u(\bar{\bar{R}}_i)^2 = \frac{\sum_{j=1}^{p} (\bar{R}_{ij} - \bar{\bar{R}}_i)^2}{p(p-1)} \quad (7)$$

$$u(R_I)^2 = u(r)^2 + u(D)^2 \quad (8)$$

$$u(P) = \frac{u(R_I)}{\bar{\bar{R}}_m} \quad (9)$$

$$u(X_{a,i}) = \frac{1}{\bar{\bar{R}}_m} \sqrt{\frac{x_{a,i}{}^2 \times u(R_I)^2}{\bar{\bar{R}}_m{}^2} + x_{a,i}{}^2 \times u(R)^2} \quad (10)$$

Symbols are explained in the text. Otherwise, refer to the publication by Wang et al. (*15*).

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