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ARTICLES

Determination of Five Macrolide Antibiotic Residues in Eggs Using Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry

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A method using liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) for the determination of trace levels of five macrolide antibiotics (spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin) in eggs is presented. Data acquisition under MS/MS was achieved by applying multiple reaction monitoring (MRM) of two or three fragment ion transitions to provide a high degree of sensitivity and specificity for both quantification and confirmation. Matrix-matched standard calibration curves were used to achieve the best accuracy of the method. A fully nested experimental design was used to study the measurement uncertainty arising from intermediate precision and trueness or proportional bias. The overall recoveries, that is, those determined by the nested experiments, of spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin at fortified levels of 60, 100, 200, and 300 μg/kg were 96.8, 98.2, 98.3, 98.8, and 95.4%, respectively. The LC/ESI-MS/MS method detection limits (S/N $\geq$ 3:1) of five macrolides were $<$1.0 μg/kg.

KEYWORDS: Antibiotics; macrolides; spiramycin; tilmicosin; oleandomycin; erythromycin; tylosin; eggs; quantification; confirmation; 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 used in medical and veterinary practices. Incorrect use of these drugs can possibly leave residues in edible tissues or food products, which may have a potential risk to consumers because of allergic reactions of individuals to the antibiotics and/or their metabolites (*1*, *2*). The European Union has set maximum residue limits (MRLs) for macrolides in foods, for example, 200 μg/kg for tylosin A and 150 μg/kg for erythromycin A in eggs. In Canada, macrolide residues in foods have been tested under the Canadian National Chemical Residues Monitoring Program using qualitative screening tests. Therefore, reliable confirmatory methods are required to monitor these drug residues in edible foods such as eggs and to ensure the safety of food supply.

Analytical methods used for the determination of macrolides in animal products and biological samples include 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 analysis (LC/MS/MS) (*2*, *12*, *13*). The method detection limits depended on the techniques that were applied. For example, an LC with UV detection was able to determine as low as 15 μg/kg of tilmicosin and tylosin in porcine, bovine, and poultry muscles (*6*). LC/MS tended to be a sensitive method that was capable of detecting various macrolides in the range from 1 to 10 μg/kg (*11*). LC/MS/MS has become one of the most promising techniques for the analysis of antibiotics in food because it allows the antibiotics to be quantified and their identities to be confirmed at trace levels. For example, one LC/MS/MS method has been reported to determine macrolides in animal tissues, eggs, and milk with detection limits between 0.01 and 37 μg/kg (*13*).

In this paper, we present a validated simple LC/ESI-MS/MS method with liquid-to-liquid partition and solid-phase extraction (SPE) for the quantification and confirmation of five macrolides in eggs. To eliminate the matrix effects on the quantitative results, a comprehensive experiment on the sample cleanup with the SPE was conducted. The extraction procedure presented in this paper provided repeatable LC/ESI-MS/MS quantitative results.

The measurement uncertainty associated with a result is an essential part of quantitative results. Many accreditation bodies

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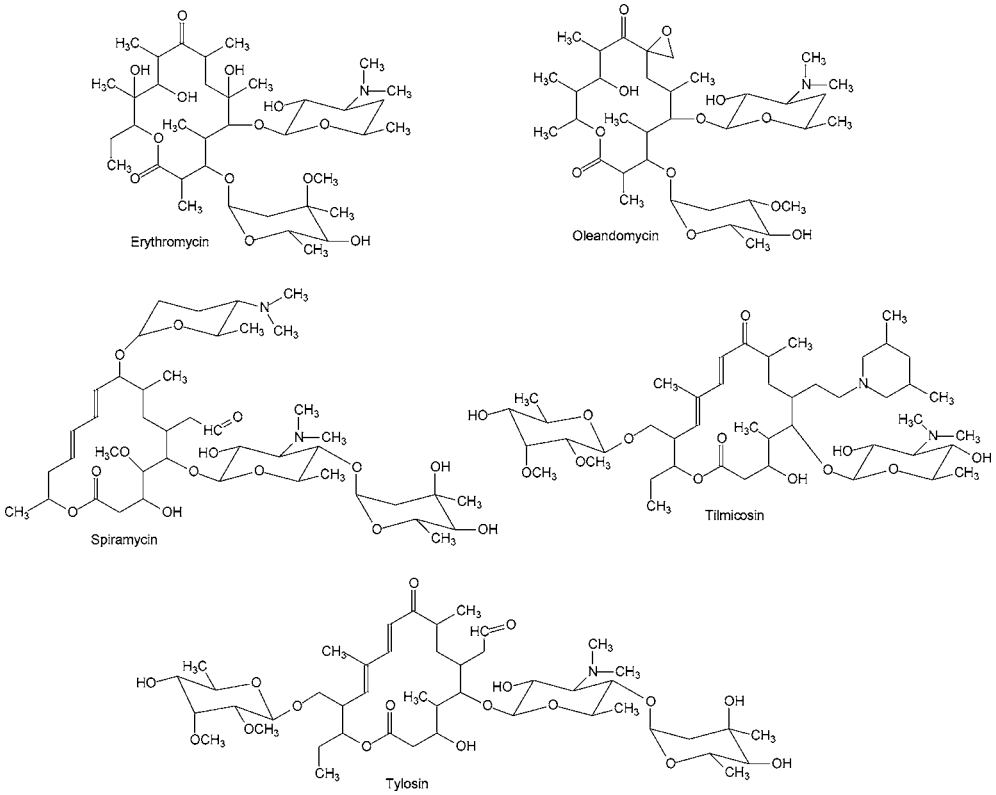

Figure 1. Chemical structures of five macrolides.

are now requiring uncertainty values or estimations when a laboratory implements ISO standard 17025 (*14*). Uncertainty can be obtained by calculating all the sources of uncertainty whenever possible using the "bottom-up" approach proposed by the ISO (*15*). Some other approaches are commonly known as "top-down" methods using information from interlaboratory study (*16*, *17*) and method validation results (*18*−*20*). In the present study, we estimated the uncertainties of the method using the information from intermediate precision and trueness in terms of recovery or proportional bias with a fully nested experimental design. This approach has been used to estimate the expanded uncertainty and different levels of uncertainties of analytical methods elsewhere (*18*, *19*).

MATERIALS AND METHODS

Materials and Reagents. Erythromycin (E6376), oleandomycin (phosphate salt) (O6125), and tylosin (tartrate) (T6134) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Spiramycin (spiramycin I, 89.0%) was obtained from the European Directorate for the Quality of Medicines (Council of Europe, Strasbourg, France). 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 (225 mg) was from Waters Corp. (Milford, MA). Shell eggs (macrolides free) were purchased from a local market. Egg samples were kept under −20 °C after they were homogenized from at least a dozen eggs in the laboratory. Egg samples were thawed at room temperature prior to weighing. A total of four different brands of eggs were used in this study. Samples were assigned codes as samples A, B, C, and D, which are used throughout the text. 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.

Preparation of Standard Solutions. Individual standard stock solutions (1000.0 μg/mL) were prepared by weighing 10 mg each of spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin into separate 10 mL volumetric flasks and dissolving in methanol. The standards were corrected for purity to give concentrations as free bases (in the case of salts). Stock solutions were stored at 4 °C for 2 months. A mixture of working standard solution (1) (1.0 μg/mL) was prepared by transferring 100 μL of each standard stock solution to a single 100 mL volumetric flask and diluting to volume with water for analytical ranges from 1.0 to 50.0 μg/kg. A mixture of working standard solution (2) (2.0 μg/mL) was prepared by transferring 100 μL of each standard stock solution to a single 50 mL volumetric flask and diluting to volume with water for analytical ranges from 50.0 to 350.0 μg/kg. All working solutions were prepared daily.

Extraction of Macrolides from Egg Samples. An egg 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)]. Acetonitrile (3 mL) was added and vortexed for 15 s, followed by the addition of 9 mL of acetonitrile. 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 containing ∼2 g of NaCl, followed by the addition of 10 mL of hexane. The centrifuge tube was capped and shaken again for 15 min on the shaker. The above sample mixture was then centrifuged at 3210*g* for 15 min at room temperature. The top hexane layer was removed, and the acetonitrile layer was transferred into a 16 × 125 mm culture tube. Acetonitrile was removed using a stream of

Table 1. LC/ESI-MS/MS Parameters for Five Macrolides

compound	MRM transition (*m*/*z*)	cone voltage (V)	collision energy (eV)	retention window (min)
spiramycin	843 → 174*[a]	80	30	1.0–5.0
	843 → 540			
	843 → 318			
tilmicosin	869 → 156	80	40	5.0–10.0
	869 → 174*			
oleandomycin	688 → 158*	35	20	5.0–10.0
	688 → 544			
erythromycin	734 → 158*	30	22	5.0–10.0
	734 → 558			
	734 → 576			
tylosin	916 → 145	30	31	5.0–10.0
	916 → 174*			
	916 → 772			

[a] Predominant ion defined as a base peak.

nitrogen at 50 °C on an N-EVAP nitrogen evaporator (Organomation Associates Inc., Berlin, MA). The dry residues were redissolved in 7 mL of 0.1 M phosphate buffer (pH 8.0). 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, the 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 to dryness again using an N-EVAP nitrogen evaporator at 50 °C. Then, 0.5 (for analytical ranges of 1.0−50.0 μg/kg) or 2 mL (for analytical ranges of 5.0−350 μg/kg) of a mixture of 0.1 M ammonium acetate and acetonitrile (85:15) was added to the dry residue. The extract was vortexed for 30 s to dissolve the residues and 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 (Waters, Milford, MA) coupled with a Micromass Quattro Ultima tandem mass spectrometer with electrospray interface (LC/ESI-MS/MS) and MassLynx 4.0 software (Waters).

(a) LC Profile. Mobile phase components were acetonitrile (solvent A), 1% formic acid (solvent B), and water (solvent C). The linear gradient profile was as follows: 0−8 min, 20−80% A and 10% B; 8−10 min, 80% A and 10% B; 10−12 min, 100% A; 12−17 min, 20% A and 10% B. Flow rates were 0.2 mL/min at 0−10 min, 0.3 mL/min at 10−16 min, and 0.2 mL/min at 16−17 min. The injection volume was 20 μL. Retention time windows for data acquisition are listed in **Table 1**. The LC column was a YMC ODS-AQ S-3 120 Å 50 × 2 mm cartridge with 2.0 mm YMC Endfittings and YMC Direct Connect Endfitting (Waters). The guard column was a YMC ODS-AQ S-3 120 Å 20 × 2 mm guard cartridge (Waters).

(b) MS conditions were as follows: ionization mode, electrospray positive ion mode; capillary voltage, 3.25 kV; source temperature, 130 °C; desolvation temperature, 280 °C; nebulizer nitrogen flow rate, 95 L/h; desolvation nitrogen gas flow rate, 610 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, 0.15 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 30 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.

Preparation of Calibration Curves and Calculation. Matrix-matched calibration standard curves were utilized in this study for the quantification of macrolides in eggs. A blank egg sample (5.00 ± 0.05 g) was weighed into each of six separate 50 mL centrifuge tubes. For analytical ranges from 1.0 to 50.0 μg/kg, 5, 50, 100, 150, 200, and 250 μL of the working solution (1) of macrolides were transferred into egg samples to provide calibration standards containing about 1, 10, 20, 30, 40, and 50 μg/kg of each of macrolide equivalent in eggs. For analytical ranges from 5.0 to 350.0 μg/kg, 125, 275, 425, 575, 725, and 875 μL of the working solution (2) of macrolides were transferred into egg samples to provide calibration standards containing about 50, 110, 170, 230, 290, and 350 μg/kg of each of macrolide equivalent in eggs. These egg samples containing macrolide standards were processed through the complete extraction procedure.

Concentration, micrograms per kilogram (parts per billion), versus the peak area of each individual macrolide was plotted to prepare the matrix-matched calibration standard curves for each individual set of macrolide standards using LC/ESI-MS/MS software (QuanLynx of MassLynx 4.0). The equation was $y = ax + b$, where y was the analyte peak area, x was the macrolide concentration in eggs, μg/kg (ppb), a was the slope of the curve, and b was the intercept of the curve. Weighting applied was $1/x$ (*21*). Matrix-matched calibration standard curves were prepared fresh for each day's samples.

Statistics. Means and standard deviations were calculated using Microsoft Excel 97 (Microsoft Office 97). Linear regression and coefficients of correlation (R^2) were generated using Quanlynx of MassLynx 4.0. Mean recoveries and variances of the nested experimental design were calculated using SAS software release 8.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

MS/MS Data Acquisition. The ionization of macrolides in the positive electrospray was studied and discussed elsewhere (*22*). Macrolides were protonated in the positive electrospray mode to form singly and/or doubly charged pseudomolecular ions based on their chemical structures (*22*). For example, spiramycin and tilmicosin, containing two nitrogens, formed both singly, $[M + H]^+$, and doubly charged, $[M + 2H]^{2+}$, ions, whereas oleandomycin, erythromycin, and tylosin, containing one nitrogen, formed only singly charged, $[M + H]^+$, ions. Compared to the doubly charged ones, the singly charged molecular ions showed constant responses and were not easily affected by the cone voltages (*22*). The singly charged molecular ions, therefore, were monitored for data acquisition in this study.

Extraction. Eggs contain proteins, lipids, and other substances that have to be removed to eliminate the matrix effects before the LC/ESI-MS/MS analysis of macrolides. Acetonitrile and hexane were used to precipitate proteins and to remove lipids in samples, respectively. Samples were further cleaned up on Oasis HLB solid-phase extraction cartridges. Because the macrolides were unstable in acidic solutions (*1*), that is, pH <4.0, the extraction, therefore, was conducted under basic conditions (pH 8.0). Initially, after a sample solution was loaded on a cartridge, two wash solutions, that is, 5 mL of water and 5 mL of 5% methanol in water, were applied before macrolide elution. Satisfactory LC chromatograms were obtained for tilmicosin, oleandomycin, erythromycin, and tylosin and less than satisfactory chromatograms for spiramycin. As shown in **Figure 2A**, the spiramycin peak was split into two peaks, which were further evidenced from its second (843 → 540) and third (843 → 318) transitions and doubly charged ion transitions. Ion

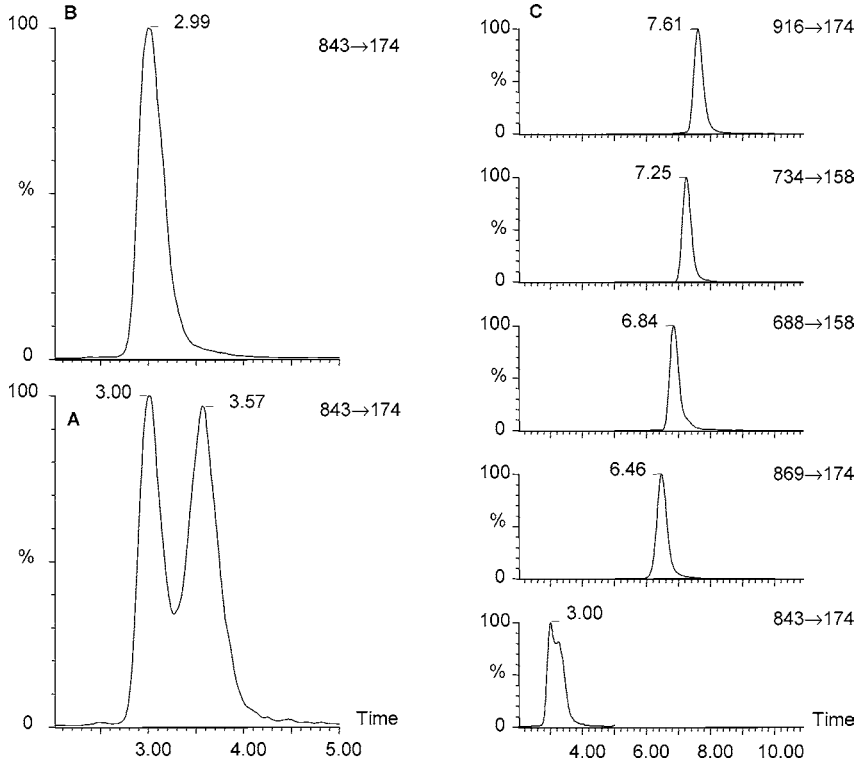

Figure 2. LC/ESI-MS/MS chromatograms of an egg sample fortified with macrolides: (**A**) sample fortified with spiramycin (36.7 μg/kg) treated with 5 mL of water and 5 mL of 5% methanol in water wash; (**B**) sample fortified with spiramycin (36.7 μg/kg) treated with 5 mL of water and 5 mL of 60% methanol in water wash; (**C**) sample fortified with macrolides treated with 5 mL of water and 5 mL of 40% methanol in water wash [from bottom to top, spiramycin (36.7 μg/kg), tilmicosin (37.4 μg/kg), oleandomycin (31.6 μg/kg), erythromycin (41.0 μg/kg), and tylosin (33.7 μg/kg) in eggs].

ratios of transitions from the split peaks matched those of a single peak of spiramycin prepared in a solvent. Apparently, the second peak (retention time at 3.57 min, **Figure 2A**) was not an interference peak. The split peak might be attributed to the interaction between egg matrices and spiramycin, which changed the characteristics of spiramycin and, consequently, affected the LC separation. The procedure according to Dubois et al. (*13*) that used Tris buffer, acetic acid, and sodium tungstate solution was unable to solve the problem either. Therefore, further experiments were conducted to explore the cause of the observed phenomenon. Samples (spiked at ~40 μg/kg equivalent of macrolides), before the SPE step, were first prepared as described under Materials and Methods, and then 10 individual cartridges were loaded with sample solutions separately. Next, each cartridge was washed with 5 mL of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 95% methanol in water, respectively. The wash profile is shown in **Figure 3A**. Finally, after the wash step, macrolides were eluted from the cartridges using 95% methanol in water. The elution profile is shown in **Figure 3B**. After the 60% methanol wash, spiramycin showed one peak (**Figure 2B**), and the loss of spiramycin was <0.5%. Meanwhile, 4% tylosin loss was noted with the 60% methanol wash, resulting in the poor repeatability of the method for tylosin. Therefore, to ensure the maximum recovery of macrolides from the SPE cleanup and extraction and the minimum effects from the egg matrices on the LC peak of spiramycin, 40% methanol in water was utilized for cleanup, and, thereafter, the macrolides were eluted with 95% methanol. The specified SPE extraction procedures generated interference-free chromatograms at retention times of five macrolides. **Figure 2C** shows typical LC/ESI-MS/MS chromatograms of an egg sample fortified with the five macrolides as a result of Oasis HLB cleanup and extraction. Macrolides were separated on a reverse phase LC column under

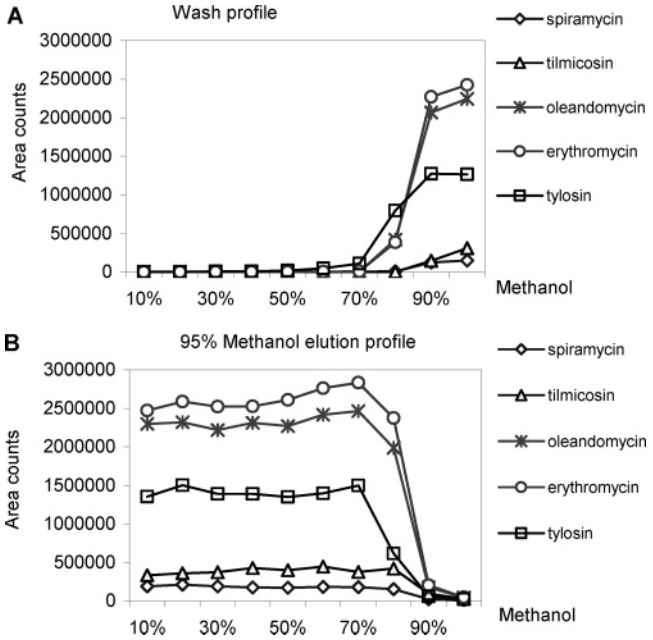

Figure 3. Wash and elution profiles of macrolides from Oasis HLB cartridges: (**A**) wash profile; (**B**) 95% methanol elution profile after the wash step. The *x*-axis is the percentage of methanol in water used to wash the cartridges before the 95% methanol elution.

the given gradient conditions within 10 min. The elution profile was in the following order with typical retention times given in parentheses: spiramycin (3.99 min), tilmicosin (6.46 min), oleandomycin (6.84 min), erythromycin (7.25 min), and tylosin (7.61 min) (**Figure 2C**). The tolerance of retention time matching did not exceed 5% relative to the retention time of

Table 2. LC/ESI-MS/MS Repeatability of the Method for Determination of Macrolides Spiked in Eggs[a]

	intra-assay[b]							intra-assay[c]						
				ion ratio[d] (%)							ion ratio[d] (%)			
compound	spike level (μg/kg)	recovery (%)	RSD (%)		RSD (%)		RSD (%)	spike level (μg/kg)	recovery (%)	RSD (%)		RSD (%)		RSD (%)
				(843 → 318)/ (843 → 174)		(843 → 540)/ (843 → 174)					(843 → 318)/ (843 → 174)		(843 → 540)/ (843 → 174)	
spiramycin	4.5	105.9	6.1	2.7	12.8	4.8	7.9	57.3	105.7	7.7	3.1	8.5	5.7	16.9
	13.5	110.4	4.7	2.9	5.2	4.6	14.6	95.5	106.5	7.5	3.3	8.0	4.9	5.4
	22.6	111.9	2.6	3.3	7.7	5.0	2.0	191.0	93.5	3.2	3.4	4.5	5.2	3.0
	40.6	117.3	9.6	3.2	11.1	4.8	5.2	286.5	99.0	6.8	3.4	4.5	5.1	5.2
				(869 → 156)/ (869 → 174)							(869 → 156)/ (869 → 174)			
tilmicosin	4.7	105.0	13.2	16.1	14.8			57.1	107.2	7.6	15.6	13.0		
	14.0	102.6	4.0	15.8	9.9			95.2	102.4	6.8	16.1	8.1		
	23.4	106.0	2.1	17.5	5.6			190.5	97.8	2.6	15.9	5.0		
	42.0	104.7	2.6	16.6	8.0			285.7	96.7	1.7	15.8	2.4		
				(688 → 544)/ (688 → 158)							(688 → 544)/ (688 → 158)			
oleandomycin	3.9	109.4	9.5	58.2	3.4			53.4	107.4	7.3	60.5	3.3		
	11.8	113.0	3.0	55.6	3.8			89.0	103.4	6.5	61.4	5.6		
	19.7	108.3	2.8	56.8	1.5			178.1	104.0	4.0	58.4	3.0		
	35.5	103.2	2.9	57.6	1.2			267.1	98.6	7.0	60.8	7.0		
				(734 → 558)/ (734 → 158)		(734 → 576)/ (734 → 158)					(734 → 558)/ (734 → 158)		(734 → 576)/ (734 → 158)	
erythromycin	5.1	111.8	3.5	12.7	8.5	50.3	3.4	67.8	107.3	2.2	12.4	4.1	50.6	1.2
	15.4	112.6	5.8	12.9	0.9	50.9	1.2	113.1	107.0	5.3	12.8	2.8	50.1	2.5
	25.7	109.9	1.2	13.3	2.7	50.6	1.8	226.1	100.3	5.3	13.0	2.4	50.9	4.0
	46.2	104.4	3.3	12.8	3.6	51.2	4.3	339.2	99.9	1.5	12.6	1.4	49.6	1.0
				(916 → 145)/ (916 → 174)		(916 → 772)/ (916 → 174)					(916 → 145)/ (916 → 174)		(916 → 772)/ (916 → 174)	
tylosin	4.2	113.5	8.5	12.4	7.0	15.2	5.4	56.2	99.1	6.2	11.9	3.4	15.5	3.6
	12.6	104.0	4.3	13.3	4.4	15.8	1.9	93.7	103.4	6.1	12.2	4.3	15.1	1.0
	21.1	109.8	1.7	13.1	3.8	14.5	1.6	187.5	92.7	3.0	12.2	4.1	14.4	2.4
	37.9	103.2	1.3	13.3	2.0	15.3	4.9	281.2	95.4	3.4	12.5	6.0	14.9	2.1

[a] Sample A was used in the intra-assay study. [b] Means of triplicates ($n = 3$). Analytical ranges were 1–50 μg/kg of macrolides equivalent in eggs. [c] Means of triplicates ($n = 3$). Analytical ranges were from 50–350 mg/kg of macrolides equivalent in eggs. [d] Ion ratios of each individual macrolide are expressed as percentage of the corresponding base peak.

standards. Although there was still a LC peak shoulder of spiramycin, the quantitative results and ion ratios for confirmation were not affected by the peak shoulder.

Intra-assay Precision and Trueness. As discussed elsewhere, endogenous impurities in sample extracts affected quantitative results of macrolides (*22*). Matrix-matched calibration standard curves were used for LC/ESI-MS/MS to quantify macrolides in eggs and to achieve the best accuracy of the method. Hereafter, recovery in this paper means apparent recovery. All matrix-matched calibration standard curves were prepared with sample A, and the correlation of coefficient values (R^2) were consistently above 0.99.

The LC/ESI-MS/MS method was first tested for its intra-assay repeatability to determine its trueness expressed as recovery and precision within a day, and results are shown in **Table 2**. Macrolides were fortified in sample A in two analytical ranges at levels of 5, 15, 25, and 45 μg/kg and 60, 100, 200, and 300 μg/kg. Macrolides were then extracted and analyzed using LC/ESI-MS/MS. The recoveries of macrolides of the intra-assay for the two analytical ranges ranged from 92.7 to 117.3% with RSDs of <15%.

Ion ratios of spiramycin and oleandomycin varied significantly from day to day, whereas those obtained within the same day, remained very consistent (*22*). Therefore, ion ratios from the intra-assay are presented in **Table 2**, and their RSDs were usually <17%. In general, the relative ion intensities of the detected ions from incurred samples are 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 the macrolides. Ion ratios acquired under the same conditions shall fall within tolerances as recommended by the European Commission Decision 2002/657/EC (*23*).

Nested Experiments. The main factors of variances associated with uncertainties of an in-house validated method using the spiked samples should include concentrations or spike levels of analytes, matrix effects, day-to-day variation, and within-day variation of the method. The last two factors are designated the intermediate precision. The fully nested experiment of four factors, that is, concentrations, matrices, days, and replicates, was conducted, and the variances of various factors were calculated according to previous publications (*18, 19, 24*). **Table 3** shows the ANOVA table and the equations for expression of uncertainties. Analytical ranges were focused on from 50 to 350 μg/kg because the MRLs of macrolides in foods were likely set within this range. Concentrations ($l = 4$) included four fortified levels, that is, 60, 100, 200, and 300 μg/kg. For each concentration, the recovery was estimated in four different matrices or brand eggs ($p = 4$). For each matrix, the analysis was carried out on two different days ($n = 2$), and samples in triplicates ($r = 3$), that is, three separate extractions, were

Table 3. ANOVA Table for a Nested Experimental Design and Expression of Uncertainty

source	levels	mean squares	uncertainty
concentrations	$l = 4$	MS_{conc}	$u(C)^2 = \frac{MS_{conc} - MS_m}{pnr}$
matrices (concentrations)	$p = 4$	MS_m	$u(M)^2 = \frac{MS_m - MS_d}{nr}$
days (concentrations × matrices)	$n = 2$	MS_d	$u(D)^2 = \frac{MS_d - MS_i}{r}$
replicates	$r = 3$	MS_i	$u(i)^2 = MS_i$

analyzed on each day. All equations on uncertainty are referred to references *18* and *19*.

Recovery (*R*) and Its Uncertainty [*u*(*R*)]. Recovery, *R*, of a spiked sample consisted of the sum of three components as expressed in eq 1.

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

The first component ($\bar{\bar{R}}_m$) is the overall recovery; the second one (ΔR_M) considers the variation of recovery caused by the different matrices, that is, matrix effects, which is usually the major source of uncertainty for the LC/ESI-MS/MS technique; and the last item (ΔR_C) is the variation of recovery due to the amount of an analyte spiked in samples. The uncertainty of the recovery can be calculated from eq 2

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

where $u(\bar{\bar{R}}_m)$ is the uncertainty of the overall recovery and proportional bias is estimated in terms of the overall recovery. $u(M)$ and $u(C)$ are uncertainties associated with matrix effects (ΔR_M) and concentration variability (ΔR_C). The calculations of $u(M)$ and $u(C)$ are expressed in **Table 3**.

Table 4 shows the variances, that is, mean squares of factors, and uncertainties of the LC/ESI-MS/MS quantitative results of spiramycin in spiked egg samples. The uncertainties for other four macrolides were calculated in the same way, and the data are summarized in **Table 5**. The overall recovery, $\bar{\bar{R}}_m$, is an estimation of the "method recovery" and was calculated using eq 3 from macrolide recoveries in **Table 6**.

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

In eq 3 $\bar{\bar{R}}_i$ is the mean recovery for a given amount $X_{a,i}$ of an analyte added to each of four egg samples and l is the number of concentration levels. The overall recoveries of five macrolides range from 0.954 to 0.988 (**Table 5**), and they were also tested if they were statistically significant different from one using eq 4

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

where $t_{\alpha/2,\mathrm{eff}}$ is the two-sided tabulated t value for the effective degrees of freedom associated with $u(\bar{\bar{R}}_m)$ and $u(\bar{\bar{R}}_m)$ is the uncertainty of the overall recovery expressed in eq 5.

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

The uncertainty of mean recoveries, $u(\bar{\bar{R}}_i)$, can be calculated from either relative intermediate precision (eq 6) or the standard deviation of mean recoveries (eq 7).

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

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

Table 4. Variances and Uncertainities from the Nested Experiment of Spiramycin-Spiked Egg Samples

source	levels	df	MS[a]	source	df	57.3 μg/kg MS[a]	95.5 μg/kg MS[a]	191.0 μg/kg MS[a]	286.5 μg/kg MS[a]
concentrations	$l = 4$	3	0.11441806						
matrices (concentrations)	$p = 4$	12	0.02215903	matrices	3	0.02394861	0.01237778	0.02297083	0.02813889
days (concentrations × matrices)	$n = 2$	16	0.02521458	days (concentrations × matrices)	4	0.05021250	0.03816667	0.00477083	0.00770833
replicates	$r = 3$	64	0.00577500	replicates	16	0.00448750	0.01007500	0.00192917	0.00660833
uncertainty									
$u(C)^2$			0.00384413						
$u(M)^2$			–0.00050926			–0.00437732	–0.00429815	0.00303333	0.00340509
$u(D)^2$			0.00647986			0.01524167	0.00936389	0.00094722	0.00036667
$u(i)^2$			0.00577500			0.00448750	0.01007500	0.00192917	0.00660833
$u(R_I)^2$			0.01225486			0.01972917	0.01943889	0.00287639	0.00697500
$u(R_I)^{2a}$						0.00082205	0.00080995	0.00011985	0.00029062
$u(R_I)^{2b}$						0.00099786	0.00056574	0.00095712	0.00117245
$u(\bar{\bar{R}}_m)^{2b}$			0.00012765						
$u(\bar{\bar{R}}_m)^{2c}$			0.00023082						

[a] Mean square. [b] Using information from the intermediate precision according to the method of Dehouck et al. (*18*). [c] Using the standard deviation of mean recoveries of four spike levels according to the method of Maroto et al. (*19*).

Table 5. Overall Recovery and Uncertainty Arising from the Precision and Trueness of Five Macrolides Spiked in Egg Samples

compound	$\bar{\bar{R}}_m$	t	$u(P)$	$u(R)^a$	$u(R)^b$	spike level (μg/kg)	$\frac{x_{a,i}^2 \times u(R_I)^2}{\bar{\bar{R}}_m^4}$	$\frac{x_{a,i}^2 \times u(R)^2}{\bar{\bar{R}}_m^2}$	U^b ($k = 2$)	U/X (%)	RSDR^c(%)
spiramycin	0.968	2.07	1.1×10^{-1}	6.3×10^{-2}	6.4×10^{-2}	57.3	50.1	15.6	16.2	27.0	24.6
						95.5	139.3	43.4	27.0	27.0	22.8
						191.0	557.1	173.8	54.1	27.0	20.5
						286.5	1253.4	391.0	81.1	27.0	19.3
tilmicosin	0.982	1.64	8.9×10^{-2}	4.6×10^{-2}	4.7×10^{-2}	57.1	29.9	8.1	12.3	20.6	24.6
						95.2	83.2	22.5	20.6	20.6	22.8
						190.5	332.7	90.0	41.1	20.6	20.5
						285.7	748.5	202.4	61.7	20.6	19.3
oleandomycin	0.983	2.81	4.6×10^{-2}	3.1×10^{-2}	3.1×10^{-2}	53.4	7.9	3.7	6.8	11.4	24.9
						89.0	22.0	10.2	11.4	11.4	23.0
						178.1	88.2	40.7	22.7	11.4	20.7
						267.1	198.4	91.6	34.1	11.4	19.5
erythromycin	0.988	1.75	5.2×10^{-2}	3.4×10^{-2}	3.5×10^{-2}	67.8	9.9	4.4	7.6	12.6	24.0
						113.1	27.5	12.3	12.6	12.6	22.2
						226.1	109.8	49.2	25.2	12.6	20.0
						339.1	247.1	110.7	37.8	12.6	18.8
tylosin	0.954	3.33	6.3×10^{-2}	6.2×10^{-2}	6.2×10^{-2}	56.2	15.8	15.5	11.2	18.7	24.7
						93.7	44.0	43.1	18.7	18.7	22.9
						187.5	176.1	172.4	37.3	18.7	20.6
						281.2	396.2	388.0	56.0	18.7	19.4

[a] Using information from the intermediate precision according to the method of Dehouck et al. (*18*). [b] Using the standard deviation of mean recoveries for four spike levels according to the method of Maroto et al. (*19*). [c] RSDR was calculated using the Horwitz equation (*26*). RSDR $= 2^{(1-0.5\log c)}$. C is the concentration expressed as fractions.

Table 6. Macrolide Recoveries Determined by LC/ESI-MS/MS from Spiked Egg Samples under Intermediate Precision Conditions[a]

compound	spike level (μg/kg)	sample A		sample B		sample C		sample D	
		recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)	recovery	RSD (%)
spiramycin	57.3	1.018	7.1	1.138	11.0	1.037	13.9	0.995	11.4
	95.5	1.007	8.2	1.053	8.3	0.940	13.5	1.020	17.6
	191.0	0.927	3.8	0.977	5.1	0.845	7.5	0.857	5.6
	286.5	0.970	6.3	0.982	7.5	0.835	7.7	0.897	13.2
tilmicosin	57.1	1.052	5.5	1.050	8.5	1.060	9.3	1.023	11.4
	95.2	0.978	7.0	0.978	8.1	0.993	5.5	1.040	9.5
	190.5	0.945	5.2	0.950	6.5	0.857	9.6	0.990	8.1
	285.7	0.935	4.1	1.017	11.0	0.882	9.6	0.967	9.8
oleandomycin	53.4	1.007	9.0	0.973	4.4	1.028	4.0	0.992	3.0
	89.0	1.015	5.4	0.992	3.5	1.030	4.2	1.032	2.1
	178.1	1.012	4.5	0.953	2.5	0.937	4.5	0.967	1.6
	267.1	0.978	4.4	0.947	2.8	0.930	3.6	0.932	5.0
erythromycin	67.8	0.993	9.2	0.958	2.9	1.023	5.6	0.997	5.4
	113.1	1.028	5.7	1.015	2.6	1.037	5.7	1.067	3.7
	226.1	1.000	3.6	0.980	2.8	0.928	5.8	0.977	1.1
	339.2	0.992	1.7	0.953	4.0	0.922	5.9	0.938	1.8
tylosin	56.2	0.963	6.3	1.027	6.7	0.945	8.4	0.983	8.0
	93.7	1.025	3.8	1.050	2.8	0.968	5.6	1.025	8.7
	187.5	0.943	4.8	1.002	4.9	0.860	5.4	0.885	5.5
	281.2	0.965	4.8	0.955	6.4	0.812	8.2	0.915	5.4

[a] Recoveries were calculated from data obtained on two different days and prepared in triplicate on each day ($n = 6$).

The intermediate precision, $u(R_I)$, is calculated from eq 8, where $u(D)$ represents the variance between days and $u(r)$ is the variance of triplicates.

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

In the significance test of the overall recovery, the two-sided z value ($\alpha = 0.05$), that is, 1.96, was used instead of the $t_{critical}$ value ($\alpha = 0.05$) because of the considerable number of degrees of freedom associated with the uncertainty of the overall recovery (*19*). The t values of five macrolides are listed in **Table 5**. The t values of tilmicosin and erythromycin were <1.96, which means that their recoveries were not significantly different from 1. For the determination of spiramycin, oleandomycin, and tylosin, the t values 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 (*19*). Nevertheless, to avoid the underestimation of the uncertainty associated with the nonsignificant proportional bias, $u(\bar{\bar{R}}_m)$ should be included in the uncertainty budget of an in-house validated method (*25*).

The uncertainties of recoveries [$u(R)$] of five macrolides are listed in **Table 5**. Because the uncertainty of the overall recovery [$u(\bar{\bar{R}}_m)$] was calculated on the basis of information from either the relative intermediate precision (*18*) or the standard deviation of mean recoveries at four spiked levels (*19*), two sets of $u(R)$

are presented, and their values are very close. Under all circumstances of the nested experiments, the matrix and concentration effects were statistically significant ($p < 0.05$), which indicated that uncertainties, that is, $u(M)$ and $u(C)$, associated with matrix and concentration variability should be also included in the uncertainty budget.

Precision and Its Uncertainty [$u(P)$]. 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 is divided by the overall recovery.

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

The results are listed in **Table 5**. The method showed better intermediate precision for oleandomycin, erythromycin, and tylosin [$u(P) < 7\%$] than for spiramycin and tilmicosin [$u(P) > 8\%$]. The doubly charged ions of spiramycin or tilmicosin may be attributed to the poor intermediate precision of the method.

Combined Standard Uncertainty and Expanded Uncertainty. The combined standard uncertainty of the quantitative result, $u(X_{a,i})$, of a sample spiked with an amount $X_{a,i}$ was calculated using eq 10 modified from the equation of Maroto et al. (*19*, *25*), and this uncertainty from in-house validation data can be applied to future sample testing results.

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

The first term of the eq 10 considers the uncertainty arising from the experimental variability of the method, that is, intermediate precision, at fortified levels, and the second one takes into account the uncertainty associated with the estimation of recovery including matrix effects and concentration variability as well. The uncertainties due to the pretreatments (lack of homogeneity) of samples and the constant bias were not studied in this paper. Especially, due to the high specificity of an LC/ESI-MS/MS technique and the relatively high concentrations of macrolides in spiked samples, the uncertainty of constant bias is expected to be low. The expanded uncertainty, U, was then calculated using the coverage factor $k = 2$, and results are listed in **Table 5**. Apparently, uncertainty due to the precision of the method (first term of eq 10) is the major source of the uncertainty in the budget compared to that of the recovery (second term of eq 10) (**Table 5**). The low uncertainty of the recovery also indicated the 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 to the method. The relative uncertainties, U/X (%), obtained at the four fortified levels of individual macrolides were apparently the same because the uncertainty of constant bias was not included in the budget. For comparison, the between-laboratory relative standard deviations (RSDR, %) according to the Horwitz equation were also calculated, the within-laboratory relative standard deviations (RSDr, %) should be half to two-thirds RSDR (%) (*26*). The relative uncertainties, U/X (%), in **Table 5**, of macrolides fortified at four different levels, were close to the predicated values of half to two-thirds RSDR (%).

Method Limits of Detection (LOD). The method LOD (signal-to-noise, S/N $\geq$ 3) were determined by evaluating the MRM transition that provided the most intense analyte signal for the detection of macrolides. Under the conditions specified in the method, the method LODs (micrograms per kilogram) of spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin were 0.9, 0.3, 0.1, 0.2, and 0.1, respectively.

In conclusion, LC/ESI-MS/MS was found to be a sensitive technique for the determination of macrolides in eggs 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 eggs so as to extract and concentrate the 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 eggs in ranges from 1 to 50 μg/kg and from 50 to 350 μg/kg. The LC/ESI-MS/MS method LOD for five macrolides were <1.0 μg/kg (ppb). The overall recoveries of 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 proportional bias including matrix effects and concentration variability were comparable to the predicted values of RSDr (%) from the Horwitz equation. The validated LC/ESI-MS/MS method can thus be employed to determine macrolides in eggs for regulatory purposes, especially when it is important to confirm the identities of macrolides in incurred samples and to report the measurement uncertainty.

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LITERATURE CITED

(1) Horie, M. Chapter 6. Chemical Analysis of Macrolides. In *Chemical Analysis for Antibiotics Used in Agriculture*; Oka, N., Nakazawa, H., Harada, K., MacNeil, J. D., Eds.; AOAC International: Arlington, VA, 1995; pp 165−205.

(2) Draisci, R.; Palleschi, L.; Ferretti, E.; Achene, L.; Cecilia, A. Confirmatory method for macrolide residues in bovine tissues by micro-liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2001**, *926*, 97−104.

(3) Horie, M.; Saito, K.; Ishii, R.; Yoshida, T.; Haramaki, Y.; Nakazawa, H. Simultaneous determination of five macrolide antibiotics in meat by high-performance liquid chromatography. *J. Chromatogr. A* **1998**, *812*, 295−302.

(4) Edder, P.; Coppex, L.; Cominoli, A.; Corvi, C. Analysis of erythromycin and oleandomycin residues in food by high-performance liquid chromatography with fluorometric detection. *Food. Addit. Contam.* **2002**, *19*, 232−240.

(5) Prats, C.; Francesch, R.; Arboix, M.; Perez, B. Determination of tylosin residues in different animal tissues by high performance liquid chromatography. *J. Chromatogr. B* **2001**, *766*, 57−65.

(6) Juhel-Gaugain, M.; Anger, B.; Laurentie, M. Multiresidue chromatographic method for the determination of macrolide residues in muscle by high-performance liquid chromatography with UV detection. *J. AOAC Int.* **1999**, *82*, 1046−1053.

(7) Moran, J. W.; Turner, J. M.; Coleman, M. R. Determination of tilmicosin in bovine and porcine sera by liquid chromatography. *J. AOAC Int.* **1997**, *80*, 1183−1189.

(8) Stobba-Wiley: C. M.; Chang, J. P.; Elsbury, D. T.; Moran, J. W.; Turner, J. M.; Readnour, R. S. Determination of tilmicosin residues in chicken, cattle, swine, and sheep tissues by liquid chromatography. *J. AOAC Int.* **2000**, *83*, 837−846.

(9) Codony, R.; Compano, R.; Granados, M.; Garcia-Regueiro, J. A.; Prat, M. D. Residue analysis of macrolides in poultry muscle by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **2002**, *959*, 131−141.
(10) Delepine, B.; Hurtaud-Pessel, D.; Sanders, P. Multiresidue method for confirmation of macrolide antibiotics in bovine muscle by liquid chromatography/mass spectrometry. *J. AOAC Int.* **1996**, *79*, 397−404.
(11) Hwang, Y. H.; Lim, J. H.; Park, B. K.; Yun, H. I. Simultaneous determination of various macrolides by liquid chromatography/mass spectrometry. *J. Vet. Sci.* **2002**, *3*, 103−108.
(12) Li, Y. X.; Neufeld, K.; Chastain, J.; Curtis, A.; Velagaleti, P. Sensitive determination of erythromycin in human plasma by LC-MS/MS. *J. Pharm. Biomed. Anal.* **1998**, *16*, 961−970.
(13) Dubois, M.; Fluchard, D.; Sior, E.; Delahaut, P. Identification and quantification of five macrolide antibiotics in several tissues, eggs and milk by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. B: Biomed. Sci. Appl.* **2001**, *753*, 189−202.
(14) *General Requirements for the Competence of Testing and Calibration Laboratories*; ISO 17025; International Standards Organization: Geneva, Switzerland, 2000.
(15) *Guide to the Expression of Uncertainty in Measurement*; International Standards Organization: Geneva, Switzerland, 1993.
(16) Dehouck, P.; Vander Heyden, Y.; Smeyers-Verbeke, J.; Massart, D. L.; Marini, R. D.; Chiap, P.; Hubert, Ph.; Crommen, J.; Van de Wauw, W.; De Beer, J.; Cox, R.; Mathieu, G.; Reepmeyer, J. C.; Voigt, B.; Estevenon, O.; Nicolas, A.; Van Schepdael, A.; Adams, E.; Hoogmartens, J. Interlaboratory study of a liquid chromatography method for erythromycin: determination of uncertainty. *J. Chromatogr. A* **2003**, *1010*, 63−74.
(17) Dehouck, P.; Vander Heyden, Y.; Smeyers-Verbeke, J.; Massart, D. L.; Crommen, J.; Hubert, Ph.; Marini, R. D.; Smeets, O. S. N. M.; Decristoforo, G.; Van de Wauw, W.; De Beer, J.; Quaglia, M. G.; Stella, C.; Veuthey, J. L.; Estevenon, O.; Van Schepdael, A.; Rotes, E.; Hoogmartens, J. Determination of uncertainty in analytical measurements from collaborative study results on the analysis of a phenoxymethylpenicillin sample. *Anal. Chim. Acta* **2003**, *481*, 261−272.
(18) Dehouck, P.; Van Looy, E.; Haghedooren, E.; Deckers, K.; Vander Heyden, Y.; Adams, E.; Roets, E.; Hoogmartens, J. Analysis of erythromycin and benzoylperoxide in topical gels by liquid chromatography. *J. Chromatogr. B.* **2003**, *794*, 293−302.
(19) Maroto, A.; Boque, R.; Riu, J.; Rius, F. X. Measurement uncertainty in analytical methods in which trueness is assessed from recovery assays. *Anal. Chim. Acta* **2001**, *440*, 171−184.
(20) Barwick, V. J.; Ellison, L. R. Measurement uncertainty: Approaches to the evaluation of uncertainties associated with recovery. *Analyst* **1999**, *124*, 981−990.
(21) Almeida, A. M.; Castel-Branco, M. M.; Falcao, A. C. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J. Chromatogr. B* **2002**, *774*, 215−222.
(22) Wang, J. Determination of five macrolide antibiotic residues in honey by LC-ESI-MS and LC-ESI-MS/MS. *J. Agric. Food Chem.* **2004**, *52*, 171−181.
(23) Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. 2002/657/EC. *Off. J. Eur. Communities* **2002**, *L221*, 8−36.
(24) Vander Heyden, Y.; De Braekeleer, K.; Zhu, Y.; Roets, E.; Hoogmartens, J.; De Beer, J.; Massart, D. L. Nested designs in ruggedness testing. *J. Pharm. Biomed. Anal.* **1999**, *20*, 875−887.
(25) Maroto, A.; Boque, R.; Riu, J.; Rius, F. X. Effect of non-significant proportional bias in the final measurement uncertainty. *Analyst* **2003**, *128*, 373−378.
(26) Boyer, K. W.; Horwitz, W.; Albert. R. Interlaboratory variability in trace element analysis. *Anal. Chem.* **1985**, *57*, 454−459.

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