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Determination of Five Macrolide Antibiotic Residues in Honey by LC-ESI-MS and LC-ESI-MS/MS

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Liquid chromatography−electrospray ionization mass spectrometry methods (LC-ESI-MS and LC-ESI-MS/MS) for the determination of five macrolide antibiotics including spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin in honey are presented. Macrolides were protonated to form singly and/or doubly charged pseudomolecular ions, depending on their chemical structures, in an electrospray positive ionization mode. 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. The recoveries, that is, determined by LC-ESI-MS/MS, of the five macrolides at fortified levels of 6, 16, 40, and 80 μg/kg ranged from 75.5 to 135.7% in light honey and from 42.1 to 111.0% in dark honey. The ion ratios obtained under MS/MS were key criteria to confirm the identity of macrolides in incurred samples. LC-ESI-MS/MS method detection limits of the five macrolides were <0.1 μg/kg.

KEYWORDS: Antibiotics; macrolides; spiramycin; tilmicosin; oleandomycin; erythromycin; tylosin; honey; quantification; confirmation; LC-ESI-MS; LC-ESI-MS/MS

INTRODUCTION

Macrolides (**Figure 1**) are a group of antibacterial compounds that have been widely used in medical and veterinary practices. It is believed that macrolide residues in food may pose a potential risk to consumers because of allergic reactions of individuals to the antibiotics and/or their metabolites (*1*, *2*). American foulbrood (AFB) is a common honeybee brood disease, which can be very destructive to honeybee colonies. Macrolides, for example, tylosin, can potentially be used in AFB disease prevention and treatment (*3*). However, macrolides are not allowed to be present in honey under the Canadian Food and Drugs Act and Regulations (*4*). Honey is currently tested for these drugs under the Canadian National Chemical Residues Monitoring Program.

Liquid chromatography (LC) with ultraviolet (UV) or fluorometric detection (*5*−*10*), liquid chromatography−mass spectrometry (LC-MS) (*11*−*13*), and liquid chromatography−tandem mass spectrometry analysis (LC-MS/MS) (*2*, *14*, *15*) have been used to analyze macrolides in various matrices including animal products and biological samples. One LC-UV method was able to detect as low as 15 μg/kg of tilmicosin and tylosin in porcine, bovine, and poultry muscles (*8*). LC-MS was sensitive enough that it was capable of detecting various macrolides in the range from 0.001 to 0.01 μg/g (*13*). Some LC-MS methods were practically validated and used around maximum residue limits (MRLs) such as 100 μg/kg for tylosin in chicken muscle (*11*). LC-MS/MS is 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 the same time. 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 (*15*).

The Charm II test, microbiological inhibition, and microbial receptor assay (*16*−*19*) were rapid detection techniques that have been developed as screening methods to check residue levels of macrolides in milk and animal tissues. It is possible to use the Charm II test as a screening method to detect tylosin and erythromycin in honey with detection limits of 800 and 200 ppb, respectively (manufacturer's information). Any positive or suspected samples, after screening tests were applied, must have the presence of antibiotics confirmed by a true confirmatory method such as LC-MS/MS.

Currently, there are no analytical or chemical methods reported or available for the determination of macrolides in honey. In this study, LC-ESI-MS and LC-ESI-MS/MS methods are presented for the determination of macrolides in honey with high sensitivity and specificity to meet regulatory requirements. Ionization, calibration considerations, repeatability, accuracy, confirmation, honey matrix effects, and storage treatment are presented and discussed.

MATERIALS AND METHODS

Materials and Reagents. Erythromycin (E6376), oleandomycin (phosphate salt) (O6125), roxithromycin (R4393), and tylosin (tartrate) (T6134) were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

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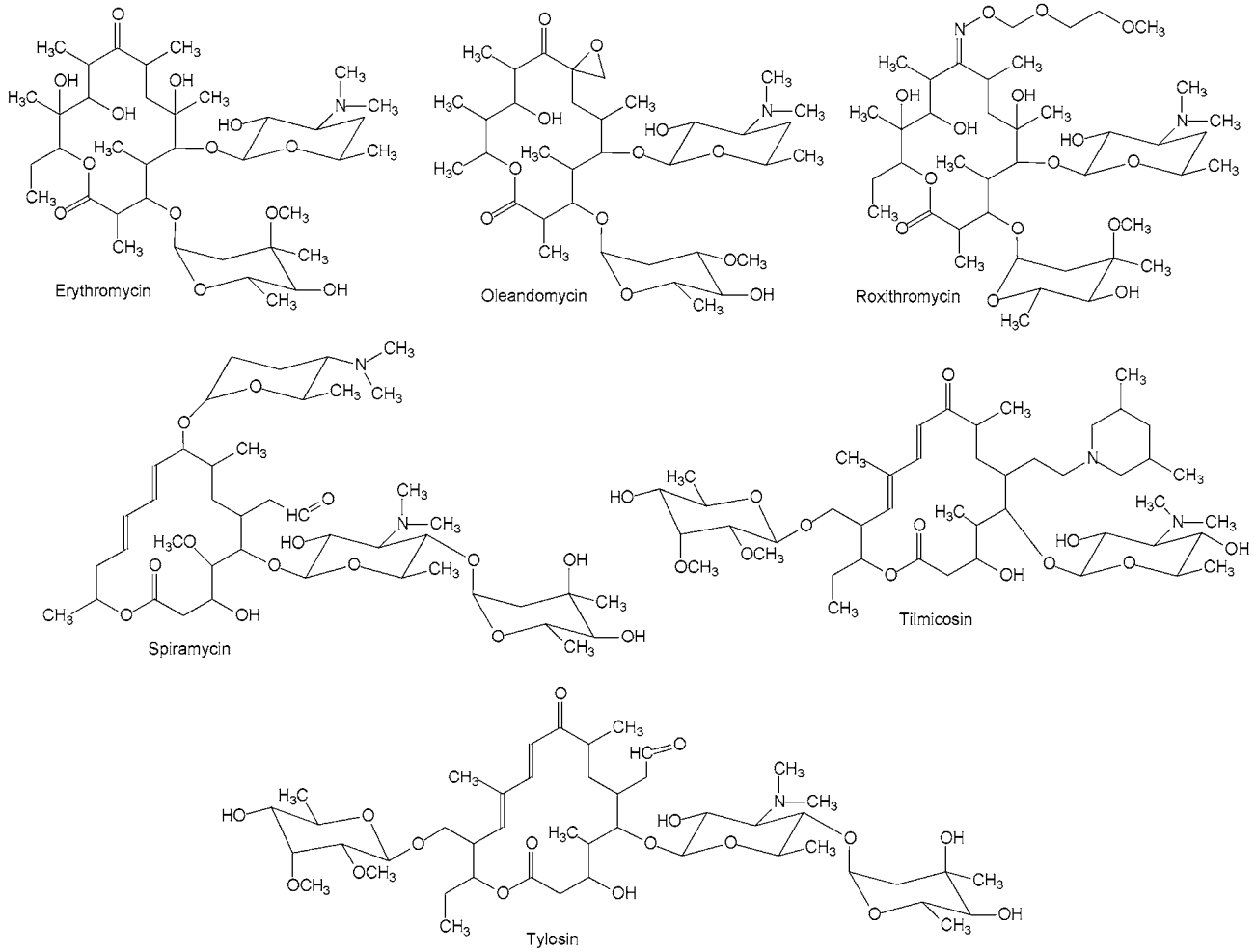

Figure 1. Chemical structures of five macrolides and the internal standard roxithromycin.

Spiramycin (spiramycin I, 89.0%) was obtained from the European Directorate for the Quality of Medicines (Council of Europe, Strasbourg Cedex, 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. (ON, 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). Honeys were from a sample analysis program remaining from the year 2001. Three types of honeys (macrolide-free), including light (honeys A and D, wild free range; honeys B and C, clover), extra light (honey E, acacia), and dark (honey F, blueberry), a total of six honey matrices, were used in this study. 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 the pH to 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 μg/mL) were prepared by weighing 10 mg each of spiramycin, tilmicosin, oleandomycin, erythromycin, tylosin, and roxithromycin (internal standard, IS) into separate 10 mL volumetric flasks and dissolving in methanol. Stock solutions were stored at 4 °C for 2 months. A mixture of working standard solution (1 μg/mL) was prepared by transferring 100 μL of each standard (except roxithromycin) stock solution to a single 100 mL volumetric flask and diluting to volume with water. Working standard solution for the internal standard (1 μg/mL) was prepared by transferring 100 μL of roxithromycin stock solution to a single 100 mL volumetric flask and diluting to volume with water. All working solutions were prepared daily.

Extraction of Macrolides from Honey. A honey sample (5.00 ± 0.05 g) was weighed into a 50 mL polypropylene centrifuge tube [polypropylene centrifuge tubes with screw caps (VWR International, Edmonton, AB, Canada)]. The internal standard (200 μL), roxithromycin, working solution (1 μg/mL) was added to the sample, and then 20 mL of 0.1 M phosphate buffer (pH 8.0) was added, followed by shaking for 5 min or until the honey was dissolved. The sample solution was centrifuged at 16400*g* [RC5C Sorvall Instruments (DuPont Canada, Inc. Mississauga, ON, Canada)] for 10 min at room temperature to remove undissolved particles to avoid plugging the Oasis HLB cartridges. 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 supernatant was loaded on the preconditioned Oasis HLB cartridge under vacuum at −2 to −3 inHg 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 with the same flow rate. The cartridge was evacuated continuously to "dryness" for 5 min under vacuum. Finally, macrolides were eluted from the cartridge with 5 mL of methanol at a flow rate of 1−2 mL/min under vacuum into a 15 mL test tube. The eluate was brought to dryness using N-EVAP at 40−50 °C under a stream of nitrogen. Then, 0.5 mL of a mixture of 0.1 M ammonium acetate and acetonitrile (85:15) was added into the dryness residues. 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 and LC-ESI-MS/MS analysis.

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

	MS (SIR)		MS/MS			
compound	*m/z*	cone voltage (V)	MRM transition (*m/z*)	cone voltage (V)	collision energy (eV)	retention time window (min)
spiramycin	843	80	843→174*[a]	80	30	1.0–5.0
			843→318			
			843→540			
tilmicosin	869	80	869→156	80	40	5.0–10.0
			869→174*			
oleandomycin	688	35	688→158*	35	20	5.0–10.0
			688→544			
erythromycin	734	30	734→158*	30	22	5.0–10.0
			734→558			
			734→576			
tylosin	916	30	916→145	30	31	5.0–10.0
			916→174*			
			916→772			
roxithromycin (IS)	837	60	837→158	60	25	5.0–10.0

[a] Predominant ion defined as a base peak. Ion ratios of each individual macrolides are expressed as percentage of the corresponding base peak.

LC-ESI-MS/MS and LC-ESI-MS (SIR). The LC-MS/MS system used was an Alliance 2695 HPLC (Waters) coupled with a Micromass Quattro Ultima tandem mass spectrometer with electrospray interface (LC-ESI-MS/MS) and MassLynx 3.5 software (Micromass, Manchester, U.K.). In this study, the LC-ESI-MS/MS was also set at single-ion recording (SIR) mode in order to develop an LC-ESI-MS or single-quadrupole instrumentation method for the detection of macrolides in honey. Hereafter, the term "LC-ESI-MS (SIR)" represents this specific setting.

LC Profile. Mobile phases 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; 10−12 min, 100% A; and 12−17 min, 20% A and 10% B. Flow rates were 0−10 min, 0.2 mL/min; 10−16 min, 0.3 mL/min; and 16−17 min, 0.2 mL/min. 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).

MS Conditions. MS parameters were set 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 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, MRM, and SIR (*m/z*) are listed in **Table 1**. These settings were able to achieve unit mass resolution. The mass spectrometer was tuned to obtain reasonable responses and ion ratios under MRM for each individual macrolide using flow injection. The flow rate of a syringe pump (Harvard Apparatus, Holliston, MA) was set at 30 μL/min. For the flow injection, macrolides (1 μg/mL) (expect erythromycin) were prepared in a mixture of acetonitrile and water (50:50) containing 0.1% formic acid, and erythromycin (1 μg/mL) was prepared in a mixture of acetonitrile and water (50:50).

Preparation of Calibration Curves and Calculation. Matrix-matched calibration standard curves were utilized in this study for the quantification of macrolides in honey. Honey (5.00 ± 0.05 g) was weighed into six separate 50 mL centrifuge tubes. Then, 200 μL of the internal standard working solution was added, which made 36.5 μg/kg of roxithromycin equivalent in honey. Next, 5, 25, 50, 100, 200, and 500 μL of the working solution of macrolides were transferred into honey sample solutions to provide calibration standards containing about 1, 5, 10, 20, 40, and 100 μg/kg of each macrolide equivalent in honey. Finally, 20 mL of phosphate buffer (0.1 M, pH 8.0) was added, and honey samples were mixed by vortexing until the honey was completely dissolved. These honey samples containing macrolide standards were processed through extraction procedure. The concentration ranges used in this study were 0.9−89.0 μg/kg (ppb) for spiramycin, 0.9−90.7 μg/kg for tilmicosin, 0.8−84.0 μg/kg for oleandomycin, 0.9−94.9 μg/kg for erythromycin, and 0.9−98.2 μg/kg for tylosin.

Concentration [μg/kg (ppb)] versus the ratios of the peak area of each individual macrolide over the unit peak area of the internal standard was plotted to prepare the matrix-matched calibration standard curves for each individual macrolide standard using LC-ESI-MS/MS software (Quanlynx). The equation was $y = ax + b$, where y was the analyte peak area × (IS concentration/IS peak area), x was macrolide concentration in honey [μg/kg (ppb)], a was the slope of the curve, and b was the intercept of the curve. Weighting applied was $1/x$ (*20*). Peak height was used for LC-ESI-MS (SIR) to achieve a good accuracy for the method. Responses for the unknown or fortified samples were compared to the curves to calculate the amount of macrolide residues [μg/kg (ppb)] in honey. 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. Mean comparisons were made with the least significant difference (LSD) test at a 0.05 significance level using SAS software release 8.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISSUSSION

Ionization. Macrolides are a group of nitrogen-containing molecules that were easily protonated to form singly and/or doubly charged pseudomolecular ions in the positive electrospray ionization mode (**Figures 1** and **2**). Spiramycin, tilmicosin, and roxithromycin, containing two nitrogens, formed both singly, $[M + H]^+$, and doubly charged, $[M + 2H]^{2+}$, ions. Oleandomycin, erythromycin, and tylosin, containing one nitrogen, formed only singly charged, $[M + H]^+$, ions. Apparently, the formation of doubly charged molecular ions might be attributed to the presence of two nitrogens in spiramycin, tilmicosin, and roxithromycin. Furthermore, the abundance of doubly charged ions was affected by the cone voltage (V) of the ion source. **Figure 3** indicated that, with the increase of the cone voltage, intensities of doubly charged molecular ions decreased, whereas those of singly charged molecular ions remained constant or increased slightly. Meanwhile, fragment ions, for example, those of spiramycin at *m/z* 174 and 540, were produced when the cone voltage reached certain levels, and afterward their intensities increased as the cone voltage increased. Because the intensities of the singly charged molecular ions remained constant or increased slightly within the cone

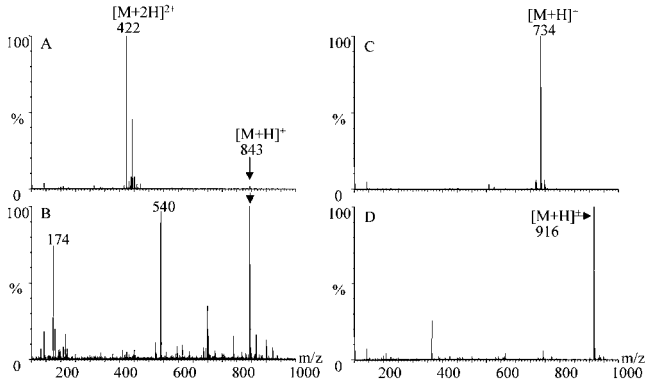

Figure 2. ESI-MS spectra of spiramycin, erythromycin, and tylosin: (**A**) spiramycin, 1.0 μg/mL in acetonitrile/water (50:50) with 0.1% formic acid, cone voltage = 30 V, $[M + 2H]^{2+}$, *m/z* 422, doubly charged molecular ion; $[M + H]^+$, *m/z* 843, singly charged molecular ion; (**B**) spiramycin, 1.0 μg/mL in acetonitrile/water (50:50) with 0.1% formic acid, cone voltage = 80 V, fragment ions, *m/z* 174 and 540; (**C**) erythromycin, 1.3 μg/mL in acetonitrile/water (50:50), $[M + H]^+$, *m/z* 734, singly charged molecular ion; (**D**) tylosin, 1.0 μg/mL in acetonitrile/water (50:50) with 0.1% formic acid, $[M + H]^+$, *m/z* 916, singly charged molecular ion. Each standard solution was infused into the mass spectrometer by flow injection. Spectra were recorded in ESI positive ion mode by scanning a mass range from *m/z* 100 to 1000 with a scan time of 1 s, an inter scan time of 0.02 s, and a run duration of 0.5 min. Multiplier voltage was set at 500 V.

voltage ranges as shown in **Figure 3**, fragments, presumably, resulted from the cleavage of the doubly charged molecules of spiramycin, tilmicosin, and roxithromycin caused by the in-source collision-induced dissociation (CID). Therefore, the doubly charged molecules might be more easily dissociated than their corresponding singly charged ions. In theory, both singly and doubly charged molecular ions could be used for data acquisition. However, as the molecular mass (*m*/*z*) of the analyte increases, the probability of interfering compounds of similar mass becomes less and interference may be rare for analytes with masses (*m*/*z*) greater than, for example, 500. Therefore, the specificity of the method with a single-quadrupole mass spectrometer was increased if only singly charged molecular ions were monitored for data acquisition. For the simplicity and the increased specificity, the LC-ESI-MS and LC ESI-MS/MS methods were validated using singly charged monitoring in this study.

Extraction. Honey contains sugars and other substances such as pigments and phenolics that have to be removed before the LC-ESI-MS (SIR) or LC-ESI-MS/MS analysis of macrolides. Honey samples were first dissolved in 0.1 M phosphate buffer (pH 8.0) and then cleaned up on Oasis HLB solid-phase extraction (SPE) cartridges. Because the macrolides were unstable in acidic solutions (*1*), that is, pH <4.0, the extraction was done under basic condition (pH 8.0). Sugar was removed in the first step by washing with water. However, there was still a substantial amount of pigments or color compounds retained on the cartridges, which might interfere with the detection of the macrolides. To remove the pigments, a further experiment was conducted to obtain optimum cleanup conditions by using different portions of methanol. Cartridges, after being loaded with samples, were sequentially washed with 5 mL of 0, 5, 15, 25, 40, 60, 80, and 100% of methanol in water. All macrolides were washed from the cartridges with ≥60% of methanol. Further experiments, using 40, 45, 50, 55, 60, and 100% of methanol in water, confirmed that macrolides were eluted from the cartridges only when the concentration of methanol reached ≥55%. Therefore, to ensure the maximum

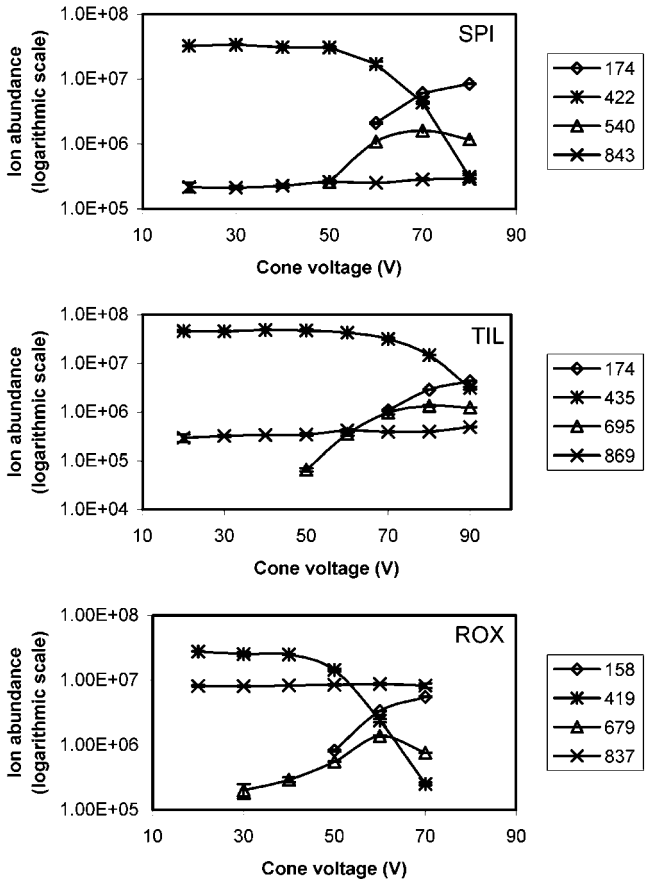

Figure 3. Effect of cone voltage on the formation of singly, $[M + H]^+$, and doubly, $[M + 2H]^{2+}$, charged molecular ions of spiramycin (1.0 μg/mL), tilmicosin (0.91 μg/mL), and roxithromycin (1.0 μg/mL). Standards were prepared in acetonitrile/water (50:50) with 0.1% formic acid. Each standard solution was infused into a mass spectrometer (Quattro Ultima) by flow injection. Spectra were recorded in ESI positive ion mode by scanning a mass range from *m/z* 100 to 1000 with a scan time of 1 s, an inter scan time of 0.02 s, and a run duration of 0.5 min. Multiplier voltage was set at 500 V. SPI: spiramycin, singly charged molecular ion, *m/z* 843; doubly charged molecular ions, *m/z* 422; and fragments, *m/z* 174 and 540. TIL: tilmicosin, singly charged molecular ion, *m/z* 869; doubly charged molecular ions, *m/z* 435; and fragments, *m/z* 174 and 695. ROX: roxithromycin, singly charged molecular ion, *m/z* 837; doubly charged molecular ions, *m/z* 419; and fragments, *m/z* 158 and 679.

recovery of macrolides from the SPE, 40% of methanol was utilized to remove some of the pigments, and, thereafter, the macrolides were eluted with 100% methanol. The specified SPE procedures generated interference-free chromatograms at retention times of five macrolides and the internal standard roxithromycin. **Figures 4** and **5** show typical LC-ESI-MS and LC-ESI-MS/MS chromatograms of a blank honey extract (**Figures 4A** and **5A**) and a honey extract fortified with the five macrolides and the internal standard (**Figures 4B** and **5B**) as a result of Oasis HLB cleanup and extraction. No interference for any of the macrolides was observed in honey extracts after the extraction.

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 given in parentheses: spiramycin (3.4 min), tilmicosin (6.5 min), oleandomycin (7.0 min), erythromycin (7.3 min), tylosin (7.7 min), and internal standard roxithromycin (8.3 min) (**Figures 4B** and **5B**). The tolerance of retention time matching did not exceed 5% relative to the retention time of standards. Note that

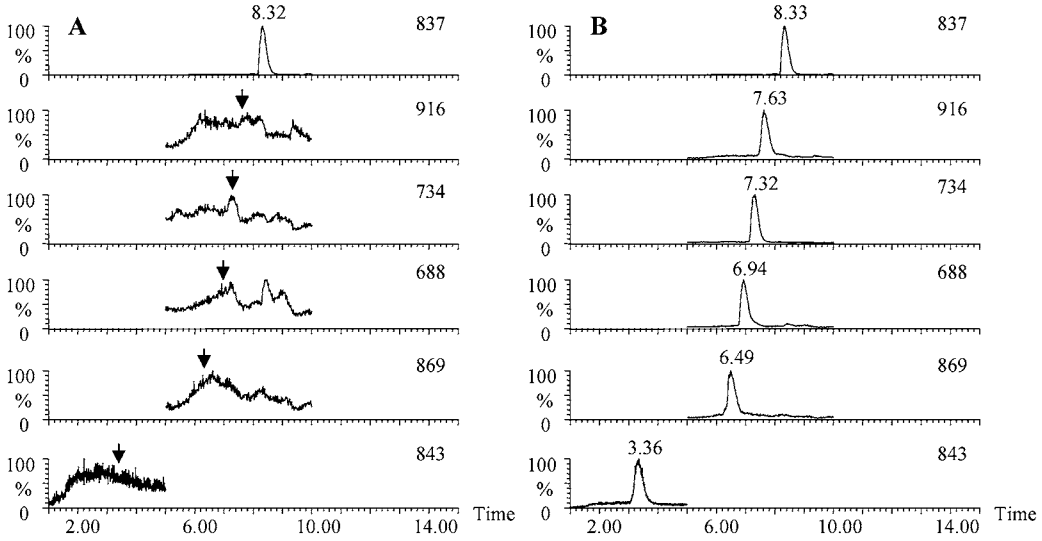

Figure 4. LC ESI-MS (SIR) chromatograms of a blank honey extract (**A**) and a honey extract fortified with five macrolides and the internal standard roxithromycin (**B**): from bottom to top, spiramycin (8.2 μg/kg), tilmicosin (7.4 μg/kg), oleandomycin (7.8 μg/kg), erythromycin (9.1 μg/kg), tylosin (5.1 μg/kg), and roxithromycin (IS) (36.5 μg/kg) in honey.

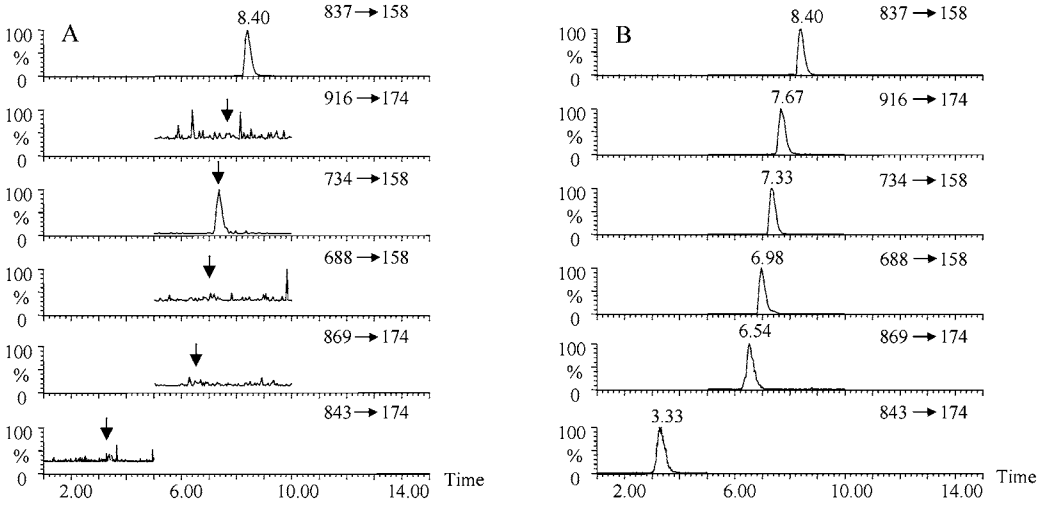

Figure 5. LC ESI-MS/MS chromatograms of a blank honey extract with the internal standard (**A**) and a honey extract fortified with macrolides and the internal standard roxithromycin (**B**): from bottom to top, spiramycin (8.2 μg/kg), tilmicosin (7.4 μg/kg), oleandomycin (7.8 μg/kg), erythromycin (9.1 μg/kg), tylosin (5.1 μg/kg), and roxithromycin (IS) (36.5 μg/kg) in honey.

roxithromycin is a semisynthetic erythromycin derivative; therefore, it may contain a very small amount of erythromycin. For example, roxithromycin used in our experiment (Sigma, R4393, lot 84H0666) contained 0.61% (RSD = 4.3%, $n = 3$) of erythromycin. At a spike level of 36.5 μg/kg of roxithromycin equivalent in honey, that is, 200 μL of roxithromycin working solution (1 μg/mL) in 5 g of honey, a sample might contain 0.19 μg/kg of erythromycin (RSD = 3.1%, $n = 3$, determined by LC-ESI-MS/MS) (**Figures 4A** and **5A**).

LC-ESI-MS (SIR). *Recovery and Calibration.* The accuracy of quantitative results of a method depends on the calibration. The recoveries of macrolides, spiked around 20 and 40 μg/kg levels, were studied, and the results are shown in **Table 2**. Recoveries (percent) were determined by comparing the peak heights of macrolides extracted from spiked samples to those of standards injected directly under LC-ESI-MS (SIR). The apparent recoveries of oleandomycin, erythromycin, and tylosin were ~60% and were close to that of the internal standard, roxithromycin (**Table 2**). Tilmicosin had a recovery of 30 or 39% from either singly or doubly charged ion monitoring. Spiramycin showed average recoveries of 141.8 and 29.8% from either singly or doubly charged ion monitoring. Apparently, the recoveries of macrolides were affected by the extraction efficiency of individual macrolides from honey and ion enhancement and/or suppression by endogenous impurities in honey extracts during ionization process. A good example of possible ion enhancement and suppression was seen from spiramycin, where the ionization of singly charged molecular ions was enhanced and that of doubly charged molecular ions might be suppressed as denoted by the apparent recoveries (**Table 2**). The difference in apparent recoveries indicated the different performance of macrolides in the extraction and ionization process. The same phenomena were observed when data were acquired under the LC-ESI-MS/MS (data not shown). Therefore, to compensate for the effects of extraction and ionization including ion suppression and/or enhancement, matrix-matched calibration standard curves, as described under Materials and Methods, were used to quantify macrolides in honey and to achieve the best accuracy of the method. All matrix-matched calibration standard curves were prepared with honey A, and the correlation of coefficient values (R^2) were consistently >0.99.

Table 2. Apparent Recovery of Macrolides from Honey[a]

	spike level (μg/kg)	apparent recovery[b] (%)	RSD (%)
spiramycin	27.4	154.2	4.4
	54.8	129.4	4.7
mean[c]		141.8	
spiramycin^{2+} [d]	27.4	28.0	1.5
	54.8	31.5	4.4
mean[c]		29.8	
tilmicosin	24.7	37.6	14.9
	49.3	38.4	10.7
mean[c]		38.0	
tilmicosin^{2+} [d]	24.7	30.9	13.8
	49.3	30.7	9.1
mean[c]		30.8	
oleandomycin	29.3	54.6	5.9
	58.5	63.1	4.1
mean[c]		58.8	
erythromycin	30.4	51.7	6.2
	60.7	61.4	2.7
mean[c]		56.5	
tylosin	17.6	50.1	4.1
	35.3	59.1	2.3
mean[c]		54.6	
roxithromycin	18.3	57.6	3.7
	36.5	64.7	7.5
mean[c]		61.1	

[a] Honey A was used for extraction efficiency study, and data were acquired using LC-ESI-MS (SIR). An internal standard was 36.5 μg/kg of roxithromycin equivalent in honey. [b] Means of triplicates (n = 3). [c] Means of the average recoveries from two spike levles (n = 2). [d] Doubly charged ions, i.e., spiramycin^{2+} at m/z 422 and tilmicosin^{2+} at m/z 435, were monitored for data acquisition. Cone volatge was set at 30 V.

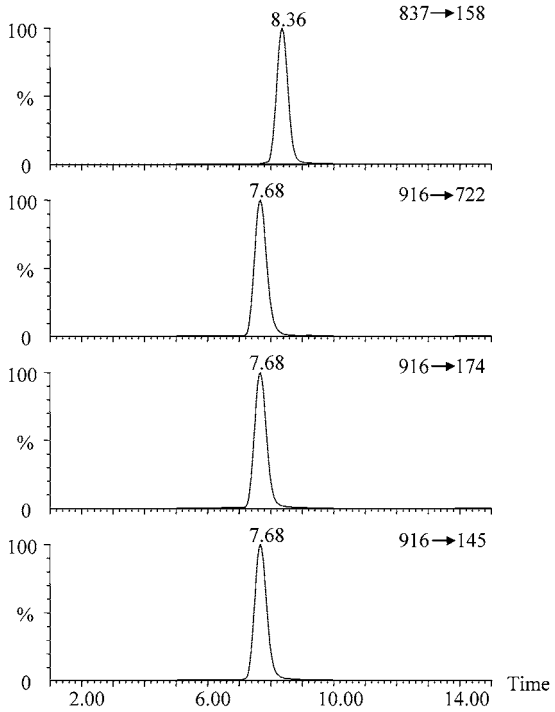

Figure 6. LC ESI-MS/MS chromatograms of an incurred sample containing 22.1 μg/kg (RSD = 5.2%, n = 3) of tylosin (transitions: 916→145, 916→174, and 916→722). The ion ratios 145/174 and 772/174 of 13.2 and 11.4 corresponded to those from the calibration standard (tylosin), 14.1 (RSD = 6.7%, n = 6) and 11.4 (RSD = 4.7%, n = 6), respectively, measured under the same conditions. Roxithromycin (transition: 837→158) (IS): 36.5 μg/kg in honey.

Repeatability. The LC-ESI-MS (SIR) method was tested for its inter- and intra-assay repeatability to determine its accuracy and precision, and results are shown in **Table 3**. Macrolides were fortified in honey A at levels of 6, 16, 40, and 80 μg/kg on four different days and within the same day. Macrolides were then extracted and analyzed using LC-ESI-MS (SIR). The recoveries of macrolides of the inter-assay, that is, fortified on four different days, ranged from 97.8 to 109.3% with relative standard deviations (RSD) of typically <12%. The recoveries of macrolides of the intra-assay, that is, fortified within the same

Table 3. LC-ESI-MS (SIR) Repeatability of the Method for the Determination of Macrolides Spiked in Honey[a]

	spike level	inter-assay (honey A)			intra-assay (honey A)		
compound	(μg/kg)	assay[b] (μg/kg)	RSD (%)	recovery (%)	assay[c] (μg/kg)	RSD (%)	recovery (%)
spiramycin	8.2	8.5	7.0	102.8	8.3	2.3	100.6
	21.9	23.2	5.5	105.7	23.8	1.6	108.6
	54.8	55.9	6.5	101.9	57.8	2.5	105.4
	109.6	109.6	4.9	100.0	118.5	4.9	108.1
tilmicosin	7.4	7.6	6.7	102.7	7.4	1.5	100.6
	19.7	21.6	10.3	109.2	22.1	9.0	112.1
	49.3	55.3	11.5	112.2	63.0	4.3	127.7
	98.7	110.7	7.6	112.1	125.9	7.5	127.6
oleandomycin	7.8	8.0	4.6	103.6	8.3	6.9	106.4
	20.7	21.7	4.9	104.6	21.3	2.7	102.8
	51.7	51.7	5.0	99.9	50.3	1.8	97.2
	103.5	102.3	9.7	98.9	97.4	2.4	94.2
erythromycin	9.1	9.8	5.4	107.4	9.9	6.7	109.2
	24.3	26.6	4.7	109.3	25.9	3.2	106.7
	60.7	63.4	2.3	104.4	62.9	1.5	103.5
	121.5	122.2	10.7	100.6	118.8	2.6	97.8
tylosin	5.1	5.3	3.0	102.8	5.5	3.8	106.4
	13.7	14.0	3.0	102.7	14.4	1.3	105.0
	34.2	33.5	3.7	98.1	34.4	1.1	100.7
	68.4	66.9	7.2	97.8	65.4	3.2	95.7

[a] Data were acquired using LC-ESI-MS (SIR). An internal standard was 36.5 μg/kg of roxithromycin equivalent in honey. [b] Means of four replicates (n = 4). [c] Means of triplicates (n = 3).

Table 4. LC-ESI-MS/MS Repeatability of the Method for the Determination of Macrolides Spiked in Honey Samples

		inter-assay[a] (honey A)							intra-assay[b] (honey A)						
					ion ratio (%)							ion ratio (%)			
compound	spike level (μg/kg)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)
spiramycin					(843→318/ 843→174)		(843→540/ 843→174)					(843→318/ 843→174)		(843→540/ 843→174)	
	8.2	8.6	10.8	104.0	6.4	17.4	7.9	17.3	8.7	4.9	105.4	5.5	3.8	7.4	2.3
	21.9	22.1	3.1	100.8	6.3	17.0	8.1	11.3	22.8	2.4	104.1	5.2	3.3	7.9	7.0
	54.8	54.5	3.5	99.4	6.2	10.0	8.1	10.0	57.6	3.4	105.0	5.7	1.0	7.9	1.5
	109.6	107.8	3.9	98.3	6.5	10.3	8.3	12.4	117.2	6.9	106.9	5.9	1.0	7.9	1.9
tilmicosin					(869→156/ 869→174)							(869→156/ 869→174)			
	7.4	7.7	4.5	103.6	19.9	13.7			7.8	5.8	104.9	20.6	15.0		
	19.7	20.9	9.9	105.9	17.8	7.1			22.7	8.9	114.8	18.2	6.9		
	49.3	56.6	12.4	114.6	17.5	6.9			64.7	7.3	131.2	19.1	3.1		
	98.7	108.5	12.3	110.0	17.5	8.5			132.4	12.4	134.2	19.0	4.0		
oleandomycin					(688→544/ 688→158)							(688→544/ 688→158)			
	7.8	8.3	7.0	106.5	38.8	23.9			8.5	4.4	109.2	39.5			
	20.7	21.9	3.7	105.6	39.1	30.0			21.6	2.0	104.3	39.1			
	51.7	50.8	0.4	98.3	37.9	30.1			50.7	1.4	97.9	38.5			
	103.5	101.7	9.2	98.2	37.8	28.0			96.4	1.8	93.1	37.8			
erythromycin					(734→558/ 734→158)		(734→576/ 734→158)					(734→558/ 734→158)		(734→576/ 734→158)	
	9.1	9.4	6.2	103.0	9.6	11.0	39.6	9.5	9.7	8.0	107.0	8.7	4.0	37.9	3.1
	24.3	25.5	2.6	105.0	9.2	11.2	39.0	9.4	25.2	2.1	103.7	8.9	1.1	37.9	1.4
	60.7	61.2	1.0	100.8	9.3	12.9	38.2	9.2	60.5	0.6	99.6	9.0	1.7	37.3	1.0
	121.5	119.3	8.1	98.2	9.2	10.6	37.9	7.5	113.3	3.2	93.3	8.9	2.3	37.6	0.7
tylosin					(916→145/ 916→174)		(916→772/ 916→174)					(916→145/ 916→174)		(916→772/ 916→174)	
	5.1	5.3	4.2	102.5	13.9	7.6	10.0	7.0	5.3	3.5	102.8	14.7	2.2	9.9	6.3
	13.7	14.2	6.5	104.1	13.7	8.9	10.0	7.6	14.5	0.8	105.8	14.4	1.4	10.2	2.0
	34.2	34.3	3.9	100.2	13.9	2.2	9.7	10.5	35.5	2.1	103.8	13.9	2.9	9.9	1.7
	68.4	67.4	3.9	98.6	13.7	3.1	9.7	8.7	68.2	1.2	99.8	13.7	3.7	9.8	1.6

[a] Means of four replicates ($n = 4$). [b] Means of triplicates ($n = 3$).

day, ranged from 94.2 to 127.7% with RSDs of <9%. The recoveries and RSDs indicated good accuracy and precision of the method and the possibility of it being used to screen and quantify macrolides in honey. The LC-ESI-MS (SIR) method was tested for its feasibility for different honey matrixes, and reasonable recoveries reflected the accuracy of the method (data not shown).

The method was also tested for compatibility with a true single-quadrupole mass spectrometer, that is, an LC-ESI-MS (ZQ) system (Waters). The LC-ESI-MS (ZQ) coupled with Oasis HLB extraction was able to accurately quantify the five macrolides in honey in a range from 2 to 100 μg/kg with method limits of detection (LOD) (signal-to-noise, S/N ≥ 3) of <1 μg/kg (data not shown). All of this demonstrated that LC-ESI-MS or a single-quadrupole mass spectrometry system is applicable for the determination of macrolides in honey.

LC-ESI-MS/MS. *MS/MS Data Acquisition.* The pseudomolecular ions, $[M + H]^+$, were selected and fragmented by collision-induced fragmentation to generate compound-specific daughter ions under MS/MS. The formation of at least two fragment ions under MS/MS conditions enabled the acquisition of two or more MRM transitions for each of macrolides. Usually, two or three fragment ion transitions per macrolide were used for data acquisition (**Table 1**). The predominant ion provided the method with high sensitivity for quantification and diminished false-negative results because of the high specificity of the transitions. The ratios of daughter ions allowed the identity of macrolides to be confirmed (**Tables 1**, **4**, and **5**). For example, the pseudomolecular ion of tylosin, $[M + H]^+$ at *m*/*z* 916, was selected and fragmented in the collision cell with argon gas. Fragmentation of tylosin led to the cleavage of the molecule and gave rise to the specific fragment ions at *m*/*z* 145, 174, and 722. The ion *m*/*z* 175 was the predominant ion and was defined as the base peak that was used for quantification. The ion ratios, expressed as percentage of the base peak, of 145/174 and 722/174, were around 14 and 10, respectively, which provided confidence to the identity of tylosin (**Tables 4** and **5**) and reduced the risk of false-positive results. **Figure 6** shows the chromatograms acquired under MRM with three transitions from an incurred honey sample containing 22.1 μg/kg (RSD = 5.2%, $n = 3$) of tylosin. The ion ratios of 145/174 and 772/174 were 13.7 (RSD = 5.3%, $n = 3$) and 11.1 (RSD = 2.3%, $n = 3$), which corresponded to those from the calibration standard (tylosin), 14.1 (RSD = 6.7%, $n = 6$) and 11.4 (RSD = 4.7%, $n = 6$), respectively, measured under the same conditions. In practice, positive quantification and confirmation of macrolides in any incurred samples were reported only if all chosen MRM transitions were detected with correct daughter ion ratios and retention times from the LC column compared to the standards.

Repeatability. As discussed above, endogenous impurities in honey extracts affected quantitative results of macrolides. Matrix-matched calibration standard curves were used for LC-ESI-MS/MS to quantify macrolides in honey and to achieve the best accuracy of the method. All matrix-matched calibration standard curves were prepared with honey A, and the correlation of coefficient values (R^2) were consistently >0.99.

Table 5. Validation Results for LC-ESI-MS/MS Determination of Macrolides Spiked in Different Honey Matrices[a]

		honey sample B							honey sample C						
					ion ratio (%)							ion ratio (%)			
compound	spike level (μg/kg)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)
spiramycin					(843→318/ 843→174)		(843→540/ 843→174)					(843→318/ 843→174)		(843→540/ 843→174)	
	8.2	7.6	4.3	92.6	6.8	0.8	7.3	9.0	9.2	9.4	111.5	5.5	3.1	9.2	2.9
	21.9	22.2	3.4	101.2	7.0	2.2	7.4	3.4	25.5	10.3	116.4	5.8	5.2	9.6	4.5
	54.8	53.0	2.0	96.7	7.2	2.1	7.7	1.3	63.5	5.5	115.8	5.8	4.0	9.6	1.6
	109.6	103.4	1.2	94.3	7.1	2.8	8.0	3.2	121.6	3.5	110.9	6.0	1.0	9.6	3.3
	calibration standard[b]				6.4	4.0	7.7	6.5				6.0	3.9	9.4	2.6
tilmicosin					(869→156/ 869→174)							(869→156/ 869→174)			
	7.4	9.5	3.8	128.2	18.6	5.9			8.0	5.3	107.9	17.4	3.7		
	19.7	23.5	15.4	119.1	17.2	1.2			26.3	12.8	133.3	16.7	2.6		
	49.3	62.4	11.7	126.5	17.4	4.1			63.9	6.4	129.6	17.2	3.0		
	98.7	136.3	2.9	138.1	17.1	2.7			115.2	4.5	116.7	17.0	1.8		
	calibration standard[b]				18.0	3.7						18.0	4.2		
oleandomycin					(688→544/ 688→158)							(688→544/ 688→158)			
	7.8	8.2	4.6	105.8	31.6	3.6			8.2	3.3	106.3	55.0	2.3		
	20.7	21.7	1.0	104.9	31.1	3.2			20.6	4.6	99.4	54.9	3.0		
	51.7	51.5	2.8	99.5	30.9	3.3			48.1	1.4	93.0	54.4	1.7		
	103.5	101.9	2.3	98.5	30.6	1.1			93.4	3.8	90.2	54.1	2.7		
	calibration standard[b]				33.0	2.4						55.0	2.7		
erythromycin					(734→558/ 734→158)		(734→576/ 734→158)					(734→558/ 734→158)		(734→576/ 734→158)	
	9.1	9.7	3.9	106.4	9.0	1.7	37.4	2.1	9.9	3.8	108.7	10.8	2.4	42.5	1.8
	24.3	26.3	2.0	108.1	9.2	3.8	37.1	2.7	24.9	2.1	102.5	10.6	2.9	42.5	2.4
	60.7	60.8	1.8	100.1	9.1	3.8	38.5	1.8	57.9	1.4	95.3	11.0	0.9	42.9	1.6
	121.5	122.5	1.4	100.9	9.0	1.1	38.3	1.5	109.2	3.1	89.9	10.8	0.9	42.6	1.3
	calibration standard[b]				9.1	4.1	37.0	2.8				11.0	1.7	44.0	4.2
tylosin					(916→145/ 916→174)		(916→772/ 916→174)					(916→145/ 916→174)		(916→772/ 916→174)	
	5.1	5.9	1.4	114.4	13.2	3.9	9.2	3.5	5.6	1.0	109.1	13.9	5.9	10.7	4.8
	13.7	15.5	1.0	113.5	13.0	2.8	9.3	2.7	14.8	6.1	108.3	13.9	3.3	10.7	1.9
	34.2	38.1	1.1	111.3	13.1	0.8	9.8	0.6	37.2	4.1	108.9	13.2	1.8	10.6	0.9
	68.4	75.9	1.0	111.0	13.3	3.8	9.5	2.6	71.2	2.6	104.2	13.5	0.7	10.9	2.8
	calibration standard[b]				14.0	9.9	9.6	5.7				13.0	10.4	12.0	7.7

		honey sample D							honey sample E						
					ion ratio (%)							ion ratio (%)			
compound	spike level (μg/kg)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)
spiramycin					(843→318/ 843→174)		(843→540/ 843→174)					(843→318/ 843→174)		(843→540/ 843→174)	
	8.2	6.4	3.8	77.6	6.8	10.6	7.9	4.6	10.9	3.0	132.5	6.9	3.3	7.9	3.2
	21.9	18.1	4.9	82.6	6.8	4.4	8.1	3.1	29.8	2.2	135.7	6.6	0.0	7.9	2.5
	54.8	46.8	5.4	85.4	6.5	7.1	7.7	0.7	65.8	2.2	120.1	7.2	4.0	8.0	1.9
	109.6	91.5	1.2	83.5	6.7	3.9	8.1	1.9	128.8	1.7	117.4	7.1	1.6	7.7	0.8
	calibration standard[b]				7.0	1.8	7.5	4.8				7.3	9.2	6.9	12.9
tilmicosin					(869→156/ 869→174)							(869→156/ 869→174)			
	7.4	5.6	9.8	75.5	17.0	8.5			9.4	3.3	127.5	18.2	2.5		
	19.7	15.2	7.4	77.1	17.0	5.6			26.4	6.5	133.8	17.8	7.3		
	49.3	39.2	10.1	79.4	17.0	3.2			61.4	2.1	124.4	17.1	2.7		
	98.7	80.7	10.7	81.8	17.7	4.3			123.3	1.8	125.0	17.1	1.2		
	calibration standard[b]				17.0	9.1						16.0	8.7		
oleandomycin					(688→544/ 688→158)							(688→544/ 688→158)			
	7.8	9.6	6.0	123.5	33.4	1.1			8.1	1.8	104.9	33.7	2.5		
	20.7	25.4	3.5	122.5	34.5	2.9			21.8	2.3	105.2	30.9	4.3		
	51.7	59.8	9.8	115.5	34.2	2.0			48.4	1.5	93.6	30.7	2.8		
	103.5	111.0	5.5	107.3	34.8	3.6			93.5	2.7	90.3	30.0	1.9		
	calibration standard[b]				31.0	6.6						27.0	4.6		
erythromycin					(734→558/ 734→158)		(734→576/ 734→158)					(734→558/ 734→158)		(734→576/ 734→158)	
	9.1	11.2	5.1	122.7	9.2	1.1	37.4	1.8	10.5	1.7	115.3	8.7	3.0	35.8	1.9
	24.3	30.4	1.6	125.0	9.1	1.9	37.3	0.9	27.0	2.1	111.0	9.1	3.4	36.4	1.2
	60.7	70.6	7.1	116.2	9.1	2.3	36.4	3.7	61.5	2.0	101.2	8.5	1.2	37.0	0.4
	121.5	131.9	4.7	108.5	8.9	1.7	36.6	2.6	115.2	3.3	94.8	9.0	1.9	37.7	2.7
	calibration standard[b]				9.2	2.5	38.0	2.4				8.7	3.5	39.0	4.1
tylosin					(916→145/ 916→174)		(916→772/ 916→174)					(916→145/ 916→174)		(916→772/ 916→174)	
	5.1	6.2	2.1	121.3	14.0	3.6	9.9	3.6	6.9	3.0	134.3	14.0	2.6	9.5	3.2
	13.7	16.7	0.5	122.0	13.8	6.6	9.7	4.9	17.8	1.8	130.2	13.6	0.4	9.2	1.9
	34.2	41.0	8.0	120.0	13.6	2.2	9.5	3.0	40.6	0.1	118.7	13.6	2.7	9.6	4.7
	68.4	80.7	2.0	118.1	13.8	1.9	9.6	3.4	77.9	0.8	113.9	13.4	1.3	9.4	1.6
	calibration standard[b]				14.0	4.2	9.4	2.5				14.0	5.2	8.9	4.0

Table 5 (Continued)

		honey sample F						
					ion ratio (%)			
compound	spike level (μg/kg)	assay (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)
spiramycin					(843→318/ 843→174)		(843→540/ 843→174)	
	8.2	3.9	3.8	47.7	6.3	16.3	7.3	4.8
	21.9	9.7	5.6	44.2	7.4	3.1	7.9	4.6
	54.8	23.1	6.9	42.1	7.4	2.1	7.5	6.9
	109.6	47.7	3.6	43.5	6.6	6.2	7.3	3.6
		calibration standard[b]			7.0	5.3	7.5	5.4
tilmicosin					(869→156/ 869→174)			
	7.4	6.7	3.0	91.0	17.9	16.5		
	19.7	17.4	3.8	88.0	17.7	4.6		
	49.3	38.8	2.7	78.7	18.1	5.8		
	98.7	82.7	5.4	83.8	17.6	1.3		
		calibration standard[b]			19.0	12.7		
oleandomycin					(688→544/ 688→158)			
	7.8	7.2	2.8	92.6	28.7	0.7		
	20.7	19.2	2.5	92.7	28.6	3.4		
	51.7	47.9	4.0	92.6	28.2	1.6		
	103.5	90.7	2.5	87.6	30.4	4.1		
		calibration standard[b]			30.0	7.2		
erythromycin					(734→558/ 734→158)		(734→576/ 734→158)	
	9.1	9.3	2.4	101.7	9.0	6.3	37.5	2.5
	24.3	24.9	0.6	102.7	8.6	2.3	37.5	1.2
	60.7	61.6	5.2	101.4	8.9	1.7	37.5	3.4
	121.5	113.1	0.8	93.1	8.7	0.7	36.8	1.9
		calibration standard[b]			8.5	5.9	37.0	3.2
tylosin					(916→145/ 916→174)		(916→772/ 916→174)	
	5.1	5.4	1.4	105.0	13.6	2.4	9.4	6.9
	13.7	14.7	1.5	107.9	13.8	1.9	9.4	6.2
	34.2	38.0	3.6	111.0	13.1	0.4	9.2	3.8
	68.4	69.2	1.4	101.2	13.4	1.1	8.8	2.4
		calibration standard[b]			14.0	11.3	9.4	3.0

[a] Data are means of triplicates ($n = 3$). [b] Ion ratios of macrolides from six-point matrix-matched calibration curves. Data are means of replicates at six calibration levels ($n = 6$).

The LC-ESI-MS/MS method was tested for its inter- and intra-assay repeatabilities to determine its accuracy and precision, and results are shown in **Table 4**. Macrolides were fortified in honey A at levels of 6, 16, 40, and 80 μg/kg on four different days and within the same day. Macrolides were then extracted and analyzed using LC-ESI-MS/MS. The recoveries of macrolides of the inter-assay, that is, fortified on four different days, ranged from 98.3 to 114.6% with RSDs of <13%. The recoveries of macrolides of the intra-assay, that is, fortified within the same day, ranged from 93.1 to 134.2% with RSDs of <13%. The method demonstrated good accuracy and precision for quantifying macrolides in honey samples.

Ion ratios of spiramycin and oleandomycin varied significantly from day to day as indicated by large RSDs (**Table 4**, under "inter-assay"). Ion ratios of macrolides obtained within the same day, however, remained very consistent, and RSDs were usually ≤10% (**Table 4**, under "intra-assay"). Therefore, 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 the macrolides. **Table 5** and **Figure 6** show the comparison of ion ratios between the detected ions from spike samples or an incurred sample and those of corresponding calibration standards. Ion ratios acquired under the same conditions lay within tolerances as recommended by European Commission Decision 2002/657/EC (*21*).

Matrix Effects. Honey composition changes with its origin, and these differences may have significant effects on LC-ESI-MS/MS quantitative results. Therefore, the method was tested for its feasibility in quantitative analysis of macrolides in various honey matrices. In this study, six honeys (honeys A, B, C, D, E, and F) were fortified with macrolides at levels of 6, 16, 40, and 80 μg/kg in triplicate. Quantitative results of macrolides from the fortified samples are summarized in **Table 4** (under "intra-assay") and **Table 5**. In light honey (honeys A−E), recoveries ranged from 77.6 to 135.7% for spiramycin, from 75.5 to 138.1% for tilmicosin, from 90.2 to 123.5% for oleandomycin, from 89.9 to 125.0% for erythromycin, and from 99.8 to 134.3% for tylosin. This would indicate that the method was able to accurately quantify the five macrolides in light honey. In a dark honey (honey F), recoveries ranged from 42.1 to 47.7% for spiramycin, from 78.7 to 91.0% for tilmicosin, from 87.6 to 92.7% for oleandomycin, from 93.1 to 102.7% for erythromycin, and from 101.2 to 111.0% for tylosin. The recoveries of spiramycin were relatively low, but those of tilmicosin, oleandomycin, erythromycin, and tylosin reasonably reflected the accuracy of the method. The low recoveries of spiramycin possibly resulted from their losses during the extraction procedure or were due to matrix effects in the ionization process. To achieve accurate results for spiramycin in dark honeys, matrix-matched calibration standard curves can be prepared using a dark blank honey to compensate for the matrix effects.

Table 6. Effect of Storage Treatment on the Method[a]

		honey A (extracts at −20 °C, 5 days)							honey B (extracts at 5 °C, 4 days)						
					ion ratio (%)							ion ratio (%)			
compound	spike level (μg/kg)	assay[c] (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)	assay[d] (μg/kg)	RSD (%)	recovery (%)		RSD (%)		RSD (%)
spiramycin					(843→318/ 843→174)		(843→540/ 843→174)					(843→318/ 843→174)		(843→540/ 843→174)	
	8.2	8.0	3.3	96.8	6.6	12.4	7.8	6.1	8.0	3.2	96.8	6.1	2.5	7.3	5.2
	21.9	22.6	2.0	102.9	6.5	3.2	7.4	4.1	22.1	4.0	100.8	5.9	4.5	7.6	2.0
	54.8	59.3	4.2	108.2	6.9	2.2	7.3	4.2	51.5	2.1	94.0	6.2	3.3	7.6	2.0
	109.6	111.5	6.2	101.7	6.7	3.9	7.5	3.5	101.2	0.7	92.3	6.4	0.0	7.8	2.0
	calibration standard[b]				6.3	6.9	7.5	11.4				5.6	9.8	7.1	7.5
tilmicosin					(869→156/ 869→174)							(869→156/ 869→174)			
	7.4	7.2	3.5	96.7	16.3	4.4			9.1	4.2	122.4	17.5	3.6		
	19.7	21.0	1.3	106.3	18.4	4.4			23.1	13.7	117.0	18.4	2.8		
	49.3	58.7	2.2	118.9	18.6	2.5			59.1	14.1	119.7	17.2	3.5		
	98.7	113.9	5.4	115.5	17.6	2.0			125.6*	2.6	127.3	17.3	0.3		
	calibration standard[b]				18.0	5.2						18.0	4.7		
oleandomycin					(688→544/ 688→158)							(688→544/ 688→158)			
	7.8	8.1	3.7	104.5	30.0	4.1			7.9	4.1	102.0	33.8	1.6		
	20.7	21.3	4.9	103.0	29.5	1.7			21.7	1.7	105.0	33.0	4.8		
	51.7	51.2	2.5	99.0	29.3	1.9			50.4	5.5	97.3	32.9	3.7		
	103.5	95.4	5.6	92.2	29.2	3.5			98.2	2.2	94.9	31.9	0.6		
	calibration standard[b]				29.9	4.4						34.5	3.1		
erythromycin					(734→558/ 734→158)	(734→576/ 734→158)					(734→558/ 734→158)		(734→576/ 734→158)		
	9.1	9.4	1.9	103.1	8.6	0.7	35.8	2.0	9.4	4.2	103.7	8.6	2.7	34.7	2.2
	24.3	24.8	3.8	102.1	8.6	2.0	36.5	2.0	26.0	2.1	107.0	8.8	2.9	35.9	1.8
	60.7	61.5	1.2	101.3	8.4	2.7	36.6	0.9	60.4	2.1	99.4	8.7	2.4	35.8	2.6
	121.5	110.9	4.0	91.3	8.5	5.1	36.5	2.3	117.8	2.7	97.0	8.8	0.7	35.2	1.6
	calibration standard[b]				8.5	2.4	36.2	2.4				8.7	4.4	35.3	3.1
tylosin					(916→145/ 916→174)		(916→772/ 916→174)					(916→145/ 916→174)		(916→772/ 916→174)	
	5.1	5.3	1.7	104.3	14.2	5.3	9.0	2.2	5.6	4.7	109.3	14.6	6.2	9.6	5.3
	13.7	14.5	4.4	105.8	13.7	3.3	8.9	4.9	15.6	0.9	113.9	13.6	1.1	9.4	3.2
	34.2	34.9	1.6	102.2	13.5	2.7	9.1	1.7	37.6	2.4	110.1	13.7	6.1	9.4	3.2
	68.4	65.3	4.0	95.6	13.6	4.7	9.1	5.0	74.2	3.2	108.6	13.3	0.9	9.3	0.6
	calibration standard[b]				14.2	7.8	9.2	3.4				14.5	5.5	9.3	1.7

[a] Data are means of triplicates ($n = 3$). [b] Ion ratios of macrolides from six points of each matrix-matched standard calibration curve. Data are means of replicates at six concentration levels ($n = 6$). [c] Data in "assay" column show no significant difference from those in **Table 4** honey A (under intra-assay) obtained as such after sample preparation ($p > 0.05$). [d] Data in "assay" column with "*" denote significant difference from those in **Table 5** (under honey B) obtained as such after sample preparation ($p < 0.05$). Otherwise, there is no significant difference in storage treatment ($p > 0.05$).

Honey matrices did not affect the ion ratios and the fragmentation of the macrolides. The ion ratios of the macrolides from the fortified honey corresponded to those of the calibration standards (**Table 5**). The RSDs of the ion ratios were typically <10%. The consistency of ion ratios plus LC retention time is thus essential in the determination of macrolides in various honey samples.

Storage Treatment. The effect of storage treatment on analytical results of the method, which was related to the stability of macrolides in honey extracts, was studied. In experiment I, the extracts were first analyzed as such after sample preparation, and then the extracts were kept at −20 °C for 5 days. The extracts were thawed at room temperature and analyzed again. No significant difference ($p > 0.05$) was observed in the quantification of macrolides under the storage treatment at −20 °C for 5 days (**Tables 4** and **6**). In experiment II, the extracts were first analyzed as such after sample preparation, and then the extracts were kept at 5 °C for 4 days. The extracts were then analyzed again. There was no significant difference ($p > 0.05$) observed under the storage treatment at 5 °C for 4 days (**Tables 5** and **6**) except for tilmicosin fortified at the 98.7 μg/kg level. Therefore, honey extracts containing macrolides can be stored at −20 or 5 °C for further analysis if they cannot be analyzed after sample preparation and there is, in general, no significant effect of the storage on the quantitative results of the method.

Method Limits of Detection and Confirmation. The method limit of detection (LOD) (signal-to-noise, S/N ≥ 3) was determined by evaluating the MRM transition that provided the most intense analyte signal for the detection of macrolides. The limit of confirmation (LOC) (S/N ≥ 3) was determined through the MRM transition with the lowest analyte response so as to achieve the least reasonable ion ratios for confirmation of macrolides. Seven fortified samples (honey A) at the LOD and LOC were used to determine the LOD and LOC. Under the conditions specified in the method, the method LODs of spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin were 0.02, 0.07, 0.02, 0.02, and 0.01 μg/kg, respectively. The method LOCs of spiramycin, tilmicosin, oleandomycin, erythromycin, and tylosin were 0.8, 0.9, 0.7, 0.5, and 0.4 μg/kg, respectively. The ion ratios of macrolides fortified at LOC levels in honey corresponded to those of the calibration standards.

In conclusion, both LC-ESI-MS and LC ESI-MS/MS were sensitive methods for the determination of macrolides in honey at trace levels. Solid-phase extraction with Oasis HLB cartridges served as a simple and rapid method to remove sugar and other substances in honey 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 honey in a range from 1 to 100 μg/kg. The LC-ESI-MS/MS method limits of detection for five macrolides were all below 0.1 μg/kg (ppb). Both LC-ESI-MS and LC-ESI-MS/MS can thus be employed to determine macrolides in honey for regulatory purposes, especially when it is important to confirm the identity of macrolides in incurred honey samples.

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