JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Multiclass Determination and Confirmation of Antibiotic Residues in Honey Using LC-MS/MS

MAYDA I. LOPEZ,*,† JEFFERY S. PETTIS,‡ I. BARTON SMITH,‡ AND PAK-SIN CHU†

Center for Veterinary Medicine, U.S. Food and Drug Administration, 8401 Muirkirk Road, Laurel, Maryland 20708, and Bee Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

A multiclass method has been developed for the determination and confirmation in honey of tetracyclines (chlortetracycline, doxycycline, oxytetracycline, and tetracycline), fluoroquinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, and sarafloxacin), macrolides (tylosin), lincosamides (lincomycin), aminoglycosides (streptomycin), sulfonamides (sulfathiazole), phenicols (chloramphenicol), and fumagillin residues using liquid chromatography tandem mass spectrometry (LC-MS/MS). Erythromycin (a macrolide) and monensin (an ionophore) can be detected and confirmed but not quantitated. Honey samples (~2 g) are dissolved in 10 mL of water and centrifuged. An aliquot of the supernatant is used to determine streptomycin. The remaining supernatant is filtered through a fine-mesh nylon fabric and cleaned up by solid phase extraction. After solvent evaporation and sample reconstitution, 15 antibiotics are assayed by LC-MS/MS using electrospray ionization (ESI) in positive ion mode. Afterward, chloramphenicol is assayed using ESI in negative ion mode. The method has been validated at the low part per billion levels for most of the drugs with accuracies between 65 and 104% and coefficients of variation less than 17%. The evaluation of matrix effects caused by honey of different floral origin is presented.

KEYWORDS: Antibiotics; honey; multiclass; multiresidue; LC-MS/MS

INTRODUCTION

The U.S. Food and Drug Administration (FDA) and many state laboratories assay honey for the presence of allowed and banned antibiotics as part of their food safety surveillance. The vast majority of the recently developed methods for the analysis of antibiotics in honey are multiresidue analyses within a single class of compounds (*1*–*9*). A few multiclass/multiresidue liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the determination of antibiotics in honey have been presented (*10*–*12*); however, none have yet been published in the literature. A multiclass/multiresidue analytical method for the determination of antimicrobials in honey is needed to speed the drug approval process and for a more cost-effective surveillance. The goal of the research described in this paper was to develop a multiclass/multiresidue LC-MS/MS method for the determination and confirmation of antibiotics in honey at levels that would likely be involved in the regulation and surveillance of these drugs in honey.

MATERIALS AND METHODS

Reagents and Supplies. The following sources of antibiotics were used in this study: chloramphenicol, erythromycin, streptomycin sulfate, and tylosin tartrate (Sigma-Aldrich, St. Louis, MO); danofloxacin, difloxacin hydrochloride, doxycycline hyclate, enrofloxacin, and sarafloxacin hydrochloride (Vetranal, Sigma-Aldrich, Steinheim, Germany); chlortetracycline hydrochloride, ciprofloxacin hydrochloride dihydrate, lincomycin hydrochloride hydrate, monensin sodium salt, oxytetracycline hydrate, sulfathiazole, and tetracycline hydrochloride (USP, Rockville, MD); and fumagillin (WAKO Chemicals, Osaka, Japan). HPLC grade methanol (MeOH), hexane, and acetonitrile (ACN) were purchased from Burdick & Jackson (Muskegon, MI). Formic acid (FA, 95%, reagent grade) was purchased from Sigma-Aldrich and ammonium hydroxide (concentrated, certified) from Fisher Scientific (Pittsburgh, PA). Distilled-deionized water was generated in-house from a Milli-Q-Plus water system.

Strata X (33 μm, 60 mg, 3 mL) solid phase extraction (SPE) columns were obtained from Phenomenex (Torrance, CA). High-speed screw-top polypropylene copolymer centrifuge tubes (Oak Ridge tube Catalog No. 3119-0050, 28.8 × 106.7 mm) were obtained through Fisher Scientific. The fine-mesh nylon fabric (white, 100% nylon, 100 thread count per inch) used to filter the diluted honey samples was purchased at a local retail store.

Preparation of Standard Solutions. All laboratory work, including the preparation of standard solutions, was done under sodium laboratory lights. Many of the antibiotics are light sensitive. Fumagillin readily decomposes upon exposure to sunlight or fluorescent lights (*13*).

Stock Solutions (~1 mg/mL). Between 10 and 12 mg of each antibiotic was weighed into individual scintillation vials. The antibiotics were dissolved in 10.00 mL of MeOH (except where indicated below) using a vortex mixer. A plastic vial and water were used to prepare the streptomycin standard, an amber vial and acetonitrile were used to

* Author to whom correspondence should be addressed. Phone: 301-210-4587. Fax: 301-210-4653. E-mail: mayda.lopez@fda.hhs.gov.
† U.S. Food and Drug Administration.
‡ U.S. Department of Agriculture.

10.1021/jf073236w
Published on Web 02/08/2008

Table 1. Drug and Honey-Equivalent Concentrations in Mixed-Standard Stock Solution

antibiotic	concn. (ng/μL)	honey-equiv[a] concn. (ng/g)
chloramphenicol	0.0200	1.00
chlortetracycline	0.800	40.0
ciprofloxacin	0.800	40.0
danofloxacin	0.400	20.0
difloxacin	0.400	20.0
doxycycline	1.20	60.0
enrofloxacin	0.200	10.0
erythromycin	0.400	20.0
fumagillin	1.20	60.0
lincomycin	0.0800	4.00
monensin	0.800	40.0
oxytetracycline	0.400	20.0
sarafloxacin	0.400	20.0
streptomycin	2.00	100
sulfathiazole	0.0800	4.00
tetracycline	0.200	10.0
tylosin	0.800	40.0

[a] Fortification of 2 g honey sample with100 μL of the standard.

prepare the fumagillin standard, and danofloxacin was diluted in 20.00 mL of MeOH instead of 10 mL. The solutions of chlortetracycline, ciprofloxacin, enrofloxacin, danofloxacin, and fumagillin were sonicated to aid the dissolution. The concentrations were corrected for purity and salt form.

Intermediate Solutions and Mixed-Standard Stock Solution. Intermediate solutions of the stock standards were prepared in the same solvents used to prepare the stock standards. The concentration of each intermediate standard solution was precalculated to be 50 times its concentration in the final mixed-standard stock solution. The concentrations of each analyte in the mixed-standard stock solution are listed in **Table 1**. To prepare the mixed stock solution, 0.500 mL of each of the intermediate solutions was pipetted into a 25 mL volumetric flask and diluted to the mark with 100% MeOH. After mixing, approximately 1.5 mL aliquots were transferred into amber autosampler vials and stored at −80 °C. The splitting of the mixed-standard stock solution into aliquots was done to avoid the degradation caused by the repeated warming of the solution to room temperature. This mixed-standard stock solution was tested and found stable for 4 months when stored in a freezer at −80 °C. The stability when stored in a –20 °C freezer is 2 days because of the rapid degradation of streptomycin and fumagillin.

Working Standard Solutions. Working standard solutions were prepared on each day of analysis by successive dilutions of the mixed-standard stock solution using 100% MeOH. A vial of the mixed-standard stock solution was brought to room temperature, sonicated 5 min, and vortexed. Used as is, the mixed-standard stock solution is working standard level 5 (L5). The concentration of the antibiotics in levels 1, 2, 3, and 4 (L1−L4) are 1/10, 2/10, 4/10, and 8/10 of that in level 5, respectively. These solutions were kept in the dark at room temperature and were discarded at the end of the day. Samples of honey (2 g) were fortified before extraction with 100 μL of levels 2, 3, and 4 of the working standards to evaluate the method performance. Matrix calibration standards were prepared by adding 100 μL of the working standards to individual extracts of control honey before the last solvent evaporation in the procedure (i.e., post fortification). The honey-equivalent concentration of the antibiotics in level-5 matrix calibration standard is listed in **Table 1**.

LC-MS/MS System. The liquid chromatography system consisted of Agilent 1100 model G1312A binary pumps and a model G1329A autosampler (Agilent Technologies, Inc., Wilmington, DE). The liquid chromatography column was a Phenomenex Polar-RP Synergi, 4 μm, 50 × 2.0 mm (Catalog No. 00B-4336-B0) with a guard column of the same packing, 4 × 2.0 mm (Catalog No. AJ0-6075) and holder (Catalog No. KJ0-4282). A precolumn filter was installed between the autosampler and the guard column (Upchurch, Oak Habour, WA). A Micromass Quattro Micro mass spectrometer equipped with an electrospray ionization (ESI) source was operated in positive and negative ion modes (Waters, Milford, MA).

Other Equipment Used. A TurboVap LV solvent evaporator with a 15 mL test-tube rack (Zymark, Hopkinton, MA), a multitube vortexer (VWR, Bridgeport, NJ), an ultrasonic cleaner (Crest Ultrasonics Corp., Trenton, NJ), and a DR 100 Vitek colorimeter (Hach Co., Loveland, CO) were also used in this study.

Honey Bee Colony Dosing. Work with honey bee colonies (*Apis mellifera*) was carried out at the Bee Research Laboratory in Beltsville, MD. Incursions were conducted in May and June of 2006 and 2007 during the black locust (*Robinia pseudoacacia*) and tulip poplar (*Liriodendron tulipifera*) nectar flows. The colonies were established in two-story nucleus colonies containing 5 frames per box (10 frames total). One day prior to feeding antibiotics to the bees, adult bees in each hive were condensed into one box (5 frames) with the removal of the second-story hive body. Between 2 and 20 mg samples of 3−5 antibiotics were mixed with granulated sugar and honey and fed to individual colonies. The bees consumed the patties within 24 h. Then, a second hive body with 5 new frames was added to each colony above a queen excluder. All upper hive bodies and frames were left on the colonies during the entire incursion study. The colonies were examined periodically, and the frames were marked to identify the locations where honey bees had stored the honey. At the end of the incursion period, the upper hive bodies were removed, and the honey was sampled according to the dated markings on the frames. Each sample of incurred honey received from the Bee Research Laboratory was stored at −80 °C.

Extraction Procedure. All work was conducted under sodium laboratory lights. Control honey received from the Bee Research Laboratory (called USDA honey) and acacia honey purchased at a local grocery store were used as negative control honeys. These negative control honeys were used to prepare fortified samples and matrix standards and to mix with incurred honey to obtain the appropriate antibiotic levels for the method validation.

Homogenized control honey (2.00 ± 0.05 g) was weighed into 50 mL polypropylene copolymer high-speed centrifuge tubes. Fortified samples were prepared by the addition of 100 μL of levels 2–4 of the working standards to the control honey. A negative control, fortified samples, five control honey samples to prepare postfortified matrix standards, and incurred samples (as available) were assayed concurrently. Five standards and 10 samples could be comfortably assayed in an 8 h period.

After the addition of 10 mL of water, all tubes were capped, vortex-mixed to dissolve the honey, and centrifuged for 15 min at 30 °C and 15 000 rpm (RCF ≈ 15500*g*). A 100 μL aliquot of each supernatant was transferred into labeled autosampler vials to assay for streptomycin (continued below under Dilution Procedure for Streptomycin). The remaining supernatant and a 1 mL water rinse of the sample tube were filtered through a fine-mesh nylon fabric placed on top of a 15 mL polypropylene centrifuge tube. A small rubber band was used to hold the fabric in place, and a pocket was made by pushing the fabric into the tube with a blunt object.

Strata X (60 mg, 3 mL) SPE columns were conditioned sequentially with 2 mL of MeOH, 2 mL of ACN, and twice with 2 mL of water. Then, the filtered supernatants were loaded into the SPE columns at a flow no faster than 1 drop/8 s. Each tube was vortexed with 2 mL of water, and the rinse was added to the respective SPE column. A 20 mL reservoir was placed on top of each SPE column, and two 20-mL water washes were done at a flow of ∼1 drop/3 s. After the reservoirs were removed, the columns were vacuum-dried for 5 min at a pressure between −10 and −15 mmHg, washed twice with 3 mL of hexane, and vacuum-dried again for 5 min at a pressure between −10 and −15 mmHg. The analytes were eluted by adding sequentially 3 mL of MeOH, 3 mL of ACN, and 3 mL of freshly prepared 0.04% NH_4OH in MeOH. The eluates from each column were collected into a single 15 mL polypropylene centrifuge tube at a flow of ∼1 drop/4 s.

The evaporation of the organic solvent was carried out in several steps. First, the organic solvent was evaporated to 2 mL using the solvent evaporator at 55 °C. Then, the samples were capped and vortexed for 5 min. The evaporation was continued until the solvent volume in the tubes was ∼0.5 mL. The samples were vortexed again for 15 s, and 50 μL of water was added to all tubes. The matrix standards were prepared by adding 100 μL of the working standards

L1−L5 to the control samples extracted for this purpose. The evaporation of the solvent was continued, and the samples were removed from the evaporator as soon as the solvent level was at ~50 μL.

After evaporation, 50 μL of methanol was added to each sample, and the tubes were vortex-mixed for 10 s and sonicated for 5 min. Then, 500 μL of water was added, and the tubes were vortexed for 5 min. Next, the extracts were poured into microcentrifuge tubes and centrifuged at 13 000 rpm for 15 min. Finally, the extracts were decanted into amber autosampler vials and injected into the LC-MS/MS system.

The assay for all drugs except streptomycin and chloramphenicol was done injecting 10 μL of the extracts by using the positive-mode LC-MS/MS conditions. Two 2 min injections of 50% MeOH (10 μL) were made between samples to reduce carryover and to equilibrate the gradient back to its initial condition. Chloramphenicol was assayed injecting 20 μL of the extracts by using the negative-mode LC-MS/MS conditions. The blank injections between samples are not needed when assaying for chloramphenicol or streptomycin.

Dilution Procedure for Streptomycin. The analysis of streptomycin was continued using the 100 μL aliquots reserved from the above procedure. Exactly 400 μL of water was added to the autosampler vials containing the aliquots of fortified and incurred samples and to one of the negative controls. The vials were capped and vortex-mixed. Diluted working standards of streptomycin in water were prepared by adding 90 μL of L2, 100 μL of L2−L4, and 120 μL of L5 of the methanolic working standards into individual 15 mL polypropylene tubes. Water was then added to the 11.5 mL mark, and the tubes were vortex-mixed. To prepare streptomycin matrix standards ranging from 18 to 120 honey-equivalent ppb, 300 μL of water and 100 μL of the diluted aqueous standards were added to the rest of the autosampler vials containing the 100 μL aliquots of diluted control honey reserved from the extraction procedure above. The autosampler vials were capped and vortex-mixed. The LC-MS/MS assay used the streptomycin gradient, 15 μL injections, and the mass spectrometer was operated using the ESI positive-mode conditions. The streptomycin samples are stable for only 24 h when refrigerated.

Matrix Effects. Honey from various sources was purchased at local grocery stores and assayed using the developed method to evaluate the matrix effects on the response of the 17 antibiotics. These honey samples were from different floral origins and had a wide range of physical characteristics typically found in honey (i.e., crystallized, liquid, clear, containing pollen or comb, and colors from very dark amber to light amber to almost white in crystallized honey). A negative control, a level-3 fortified sample, and a level-3 matrix standard of each honey were assayed concurrently to evaluate matrix effects on the accuracy and precision of the validated method. As described in the method, fortified samples are honey samples spiked before the sample cleanup, and matrix standards are honey samples spiked after the sample cleanup. For each honey, the response of the level-3 fortified sample was quantitated against the level-3 standard prepared in water, against the level-3 matrix standard prepared in the same type of honey, and against the level-3 matrix standard prepared in USDA control honey. A colorimeter was used to rank unfiltered honey solutions (1 g of honey in 10 mL of water) by their percent light transmittance.

Chromatographic Conditions. The LC mobile phase composition and the gradients used are presented in **Table 2**. The autosampler temperature was set at 4 °C, and the column compartment temperature was set at 25 °C. The LC column was equilibrated at the first step of the gradient for at least 1 h prior to injecting samples. During this time, the collision gas and the instrument electronics were also equilibrated. Five injections of a solution standard were made to equilibrate the gradient and to check the instrument response. A typical injection sequence was as follows: a solvent blank, duplicate injections of level-1 matrix standard, the level-5 matrix standard (as quality control (QC) standard), a solvent blank (for carryover check), and the negative control. Next, the fortified, incurred, or unknown samples were injected between the matrix standards arranged from low to high concentration (i.e., blank, L1 std., L1 std., L5 std., blank, negative control, positive control, L2 std., test samples, L3 std., test samples, L4 std., test samples, and L5 std.). The assay using the positive-mode gradient required two 2-min wash injections between samples to reduce carryover and to re-equilibrate the gradient to its initial condition. Two 10 μL injections of 50% aqueous MeOH were made using the initial conditions of the positive-mode gradient. Monensin and fumagillin still had less than 1% carryover. After each batch, the column was flushed for 1 h with 50% ACN/50% H_2O at a flow of 0.200 mL/min to remove the formic acid and other retained materials from the column.

Table 2. Mobile Phase Gradients

time (min)	% A	% B	flow (mL/min)
	Streptomycin Gradient[a]		
0.00	0	100	0.200
0.10	20	80	0.200
0.20	60	40	0.200
3.00	60	40	0.200
3.10	50	50	0.200
10.0	50	50	0.200
10.5	0	100	0.300
14.9	0	100	0.300
15.0	0	100	0.200
	Positive Mode Gradient[b]		
0.0	90	10	0.300
4.0	85	15	0.300
10.0	70	30	0.300
13.0	50	50	0.300
14.5	20	80	0.300
18.5	10	90	0.300
18.6	90	10	0.300
20.0	90	10	0.300
	Negative Mode Gradient[c]		
0.0	80	20	0.200
2.0	65	35	0.200
3.0	30	70	0.200
3.1	30	70	0.300
6.0	30	70	0.300
6.5	80	20	0.300
10.0	80	20	0.300

[a] A = 0.1% FA in water, B = 0.1% FA in ACN. [b] A = 0.1% FA in water, B = 0.1% FA in ACN. [c] A = 100% water, B = 100% ACN.

Table 3. Quattro Micro Operating Parameters

parameters	positive mode	negative mode
Tune Parameters		
ESI source	positive	negative
capillary (kV)	0.83	0.83
cone (V)	**Table** 4	**Table** 4
extractor (V)	3.00	4.00
RF lens (V)	0.3	0.1
source temp. (°C)	125	125
desolvation temp. (°C)	400	400
cone gas flow (L/h)	10	10
desolvation gas (L/h)	600	600
Analyzer Parameters		
LM 1 resolution	13.0	13.0
HM 1 resolution	13.0	13.0
ion energy 1	0.4	0.7
entrance	−5	1
collision energy	**Table** 4	**Table** 4
exit 1	1	1
LM 2 resolution	14.0	15.0
HM 2 resolution	14.0	15.0
ion energy 2	2.3	2.3
multiplier (V)	650	650
gas cell Pirani (mbar)	3×10^{-3}	3×10^{-3}
interchannel delay (s)	0.020	0.020
interscan delay (s)	0.100	0.100

MS/MS Analysis. The operating parameters for the mass spectrometer are listed in **Table 3**. The precursor ions used for collision-induced dissociation and the selected reaction monitored transitions (SRM) for the LC-MS/MS analysis are listed in **Table 4**. There are eight acquisition segments in the positive-mode LC-MS/MS assay (0–2.5,

Table 4. SRM Transitions and Abundance Ion Ratios

antibiotic	retention time (min)	precursor ion (Da)	product ion (Da)	cone (V)	collision energy (eV)	ion ratio
lincomycin	1.8	407.20	126.20	32	26	0.06
			359.20	32	19	
sulfathiazole	4.6	256.00	156.0	25	16	0.54
			92.00	25	24	
oxytetracycline	4.9	461.15	426.15	22	19	0.18
			444.20	22	17	
tetracycline	7.1	445.15	410.15	18	18	0.83
			154.15	18	26	
ciprofloxacin	8.8	332.05	288.15	31	18	0.28
			254.15	31	19	
danofloxacin	9.0	358.15	314.15	33	18	0.43
			283.15	33	23	
enrofloxacin	10.3	360.10	316.15	31	19	0.22
			245.15	31	24	
sarafloxacin	11.5	386.10	342.15	31	19	0.77
			299.15	31	26	
doxycycline	11.6	445.20	321.10	27	30	0.65
			410.20	27	25	
difloxacin	11.8	400.15	356.15	32	19	0.56
			299.10	32	24	
chlortetracycline	10.8	479.15	444.20	22	22	0.54
			462.20	22	20	
erythromycin	13.4	734.70	576.55	27	18	0.18
			522.50	27	21	
tylosin	14.6	916.55	772.55	55	29	0.47
			174.20	55	23	
fumagillin	17.3	459.10	233.30	18	13	0.59
			215.30	18	13	
monensin	18.3	693.50	461.40	53	51	0.86
			479.40	53	51	
streptomycin	5.6	582.20	263.20	52	30	0.52
			246.20	52	35	
chloramphenicol	5.0	321.00	152.10	20	17	0.15
			194.20	20	14	

2.5–6.0, 6.0–7.75, 7.75–9.8, 9.8–12.75, 12.75–13.8, 13.8–16.0, and 16.0–20.0 min). The analytes included in each of the acquisition segments can be identified from their retention times in **Table 4**.

Quantitation. The criteria to accept a set of samples for quantitation were as follows: any coeluting interference peaks in the negative control had a response (i.e., peak area) less than 10% of the response of the analyte in the level-1 matrix standard, carryover from the level-5 standard to the following blank injection was less than 1.5%, and the response difference between the level-5 matrix standard (QC standard) injected at the beginning of the sequence and the level-5 matrix standard injected at the end of the sequence was less than 20%. For quantitation, the most intense product ion of an analyte had to be present with a signal-to-noise ratio equal to or greater than 10 and a retention time within ±5% (relative) of its mean retention time in the standards if the retention time was 5 min or less, ±3% if the retention time was between 5 and 10 min, and ±2% if the retention time was 10 min or longer.

Calibration curves (no weighting or forced zero) were prepared by plotting the peak area of the most intense product ion of each analyte in the standards versus its nominal concentration (ng/g). The correlation coefficients (r^2) for each of the calibration curves were larger than 0.99. The concentration of the analytes in the fortified and incurred samples was interpolated from the equations of the linear regression. The lower limit of quantitation for each analyte was based on the concentration of the lowest calibration standard used (i.e., level 1). The measured analyte concentrations (except for the controls and the fortified samples) were corrected for the mass difference of the individual samples to the nominal 2 g sample used to calculate the concentration of the analytes in the calibration standards.

Confirmation. The following criteria were used to confirm the presence of an analyte in a sample: the two product ions associated with the analyte were present and exceeded a signal-to-noise ratio of 3, the retention time of the analyte in the sample matched the mean retention time of the analyte in the standards using the same criteria given for quantitation, carryover from the level-5 standard into the following blank injection was less than 1.5%, and the abundance ion ratio of the analyte in the samples was within 10% (absolute) of the average abundance ion ratio in the standards. For example, if the average abundance ion ratio of an analyte in the standards was 0.77, the acceptance range for the abundance ion ratio in the samples was 0.67–0.87. The calculation of the abundance ion ratio was done using the most intense ion as the denominator. The lowest limit of confirmation for the analytes was set at the area of the lowest calibration standard used (i.e., the area of each of the analyte ions in the sample must be equal to or greater than that in level-1 standard). For monensin and erythromycin, the limit of confirmation is 20% of the response of the lowest standard (approximately equal to the concentration of the level-2 fortified sample) because the recovery for these drugs is very low.

RESULTS

Fortified Honey Samples. Figure 1 shows the SRM chromatograms of the 17 antibiotics fortified at level 2 (i.e., 2/10 the concentration listed in **Table 1**). Very little or no interference was observed in the negative controls at the retention times of the analytes. **Table 5** presents the quantitative validation results collected by one analyst using the same equipment and reagents on three or more separate assay sets. The range of average accuracies ($6 < n < 12$) is based on three fortified levels at 2/10, 4/10, and 8/10 the concentration of the level-5 matrix standard. The day-to-day repeatability error, or coefficient of variation (CV), reported is the highest obtained for the three fortified levels. For most of the analytes, the repeatability errors were less than 17%, and the accuracies were between 65 and 104%, which met FDA performance requirements (*14*) for analytical methods at concentration levels under 100 ng/g. The exceptions were erythromycin and monensin. Because of low recoveries, these compounds can only be detected and confirmed according to FDA guidelines (*14*).

Incurred Honey Samples. The performance of the method was evaluated with incurred honey obtained from treated beehives. For most of the analytes, two incurred levels ($n = 5$) were assayed. Fumagillin and oxytetracycline residues were detected in the incurred honey, but their levels were below the validated range of the method. The highest CVs of the incurred levels are reported in **Table 5**. The repeatability errors for the tested analytes in the incurred honey were less than 14%.

Confirmation. When the confirmation criteria described earlier were applied, all the negative controls failed confirmation. Either no analyte peaks or interference peaks were present in the negative controls, or if peaks were present, they failed the signal-to-noise criterion or the ion-ratio criterion. All the fortified and incurred samples were confirmed positive for all analytes except for lincomycin. One level-2 fortified sample and one incurred sample of lincomycin failed the retention time confirmation criterion of ±5% of the average retention time of the standards. The retention time of lincomycin (~1.8 min) is the shortest of all the analytes, with the ±5% range allowing only a ±0.09 min variation in the retention time. A total of 33 fortified and incurred samples were assayed for lincomycin. The number of false negative results for lincomycin is below the 10% failure rate allowed during a method validation (*15*).

Matrix Effects. Honey from 15 different sources was assayed to evaluate matrix effects on the accuracy and precision of the validated method. For each type of honey, a negative control, a level-3 matrix standard, and a level-3 fortified sample were assayed concurrently. If the amount of an antibiotic present in the negative control of a test honey was equal to or greater than 5% of the level-3 fortified sample, that honey was not used to evaluate the matrix effects on the particular analyte. Only three of the 15 retail honeys tested were totally free from all 17

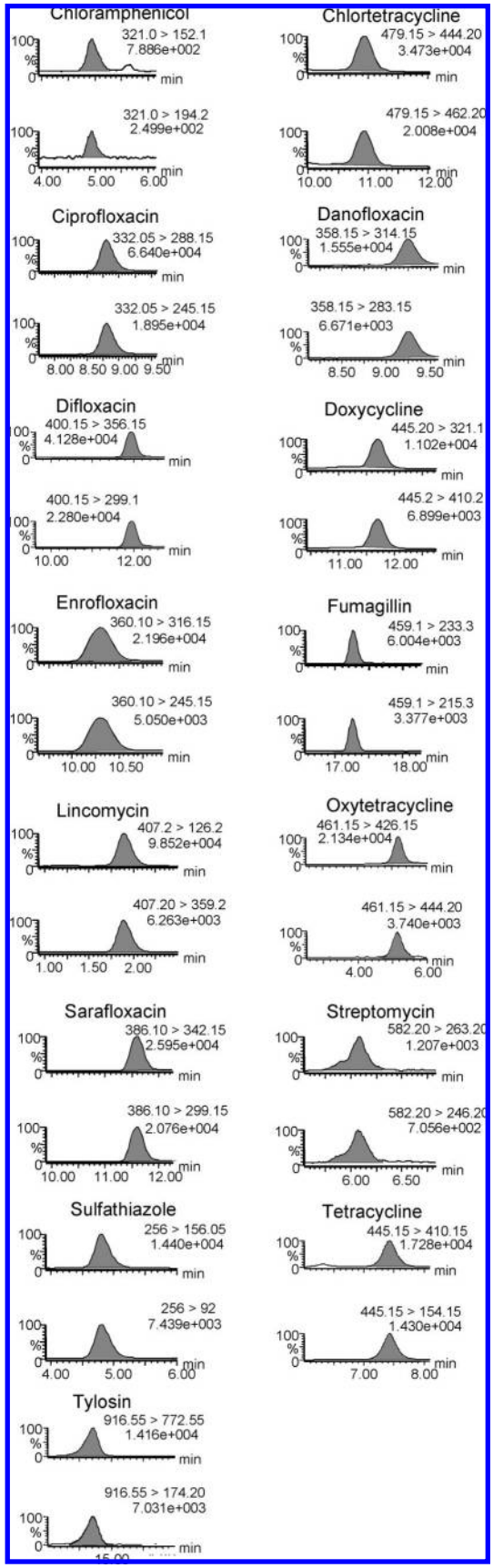

Figure 1. Chromatograms of the 17 antibiotics fortified at level 2 using USDA honey. The quantitation peak for each analyte is the top trace.

antibiotics. The majority of the honeys contained either one or two of the antibiotics, and one sample had as many as four different antibiotics. The positive findings are as follows: tylosin (*8*), lincomycin (*3*), oxytetracycline (*2*), chloramphenicol (*2*), streptomycin (*2*), sulfathiazole (*1*), tetracycline (*1*), and danofloxacin (*1*). **Table 6** lists the absolute matrix effects (suppression or enhancement) observed when the response of the standards prepared in honey extracts is compared to the response of a standard prepared in water at the same concentration. The table shows the accuracy and precision of the method using true-match matrix standards (prepared in the same honey as the fortified samples) and the accuracy and precision of the method using a surrogated-match matrix standard (prepared in the USDA control honey). When a true-match matrix standard is used for quantitation, the variability is reduced (i.e., the precision improves). The average accuracies determined using true-match matrix standards are somewhat higher than those determined using the surrogated honey.

Table 5. Validation Results

antibiotic	average accuracy (%)	fortified honey CV (%)	incurred honey CV (%)
chloramphenicol	93–97	9	10
chlortetracycline	86–89	9	6
ciprofloxacin	69–83	10	8
danofloxacin	94–95	7	5
difloxacin	90–93	9	10
doxycycline	92–93	10	5
enrofloxacin	91–93	8	5
erythromycin	24–29		confirmatory
fumagillin	68–70	16	
lincomycin	69–75	15	4
monensin	31–33		confirmatory
oxytetracycline	95–97	8	
sarafloxacin	65–75	10	7
streptomycin	101–104	10	4
sulfathiazole	81–84	13	13
tetracycline	91–93	12	7
tylosin	91–92	12	9

The matrix effect on the accuracy of the method is shown in **Figure 2**. The figure compares the average accuracy for all 17 antibiotics in honey of different floral origins and/or different physical states to standards prepared in water (top curve), to true-match matrix standards (middle curve), and to a surrogated-match matrix standard prepared in the USDA control honey (bottom curve) plotted versus the percent light transmittance of the unfiltered honey. The light transmitted varied inversely with the intensity of the amber color and the amount of suspended matter in the unfiltered honey. Dark honeys with a lot of pollen had the lowest percent transmittance. The honeys listed from low to high percent light transmittance are buckwheat, chestnut, summer flowers, tupelo, wildflowers, blueberry, desert mesquite, crystallized raw honey, liquid clover, crystallized clover, orange blossom, crystallized alfalfa, sage, liquid alfalfa, acacia, and USDA control. Matrix effects are observed the most when solution standards are used for quantitation. The antibiotics had average accuracies above 100%, and tetracyclines, fluoroquinolones, and tylosin were the most affected by matrix effects. Matrix effects on the accuracy of the method are best compensated using true-match matrix standards (middle curve). The average accuracy of the 17 analytes does not vary significantly among the different honey types. This result agrees with the low CVs reported in **Table 6** for the quantitation of the individual antibiotic in different honey types using true-match matrix standards. When a light amber honey (USDA honey) was used to prepare the surrogated-match matrix standard (bottom curve), the darker honeys had much lower accuracies than the light amber ones. The actual recovery in both light and dark honeys is similar on the basis of the true-match results

Table 6. Matrix Effects on Accuracy and Precision

antibiotic	absolute matrix effects[a]	total honey samples	true match standard			surrogated standard		
			average accuracy	standard deviation	CV (%)	average accuracy	standard deviation	CV (%)
chloramphenicol	S	13	101	11	11	83	24	29
chlortetracycline	E	15	70	7	9	65	14	22
ciprofloxacin	E	15	76	10	13	68	17	24
danofloxacin	E	14	88	9	10	81	18	22
difloxacin	E	15	87	8	10	75	18	24
doxycycline	E	15	75	9	12	76	18	24
enrofloxacin	E	15	86	9	11	78	15	20
erythromycin	N	15	34	13	38	36	13	37
fumagillin	S	15	67	7	11	64	14	22
lincomycin	S	12	87	5	6	69	18	26
monensin	E	15	27	7	26	26	7	26
oxytetracycline	E	13	83	8	10	75	18	23
sarafloxacin	E	15	76	9	12	66	18	27
streptomycin	E	13	116	15	13	113	21	18
sulfathiazole	S	14	78	8	11	61	18	30
tetracycline	E	14	76	6	8	67	15	22
tylosin	E	7	82	11	13	89	8	9

[a] S, suppression; E, enhancement; and N, no effect.

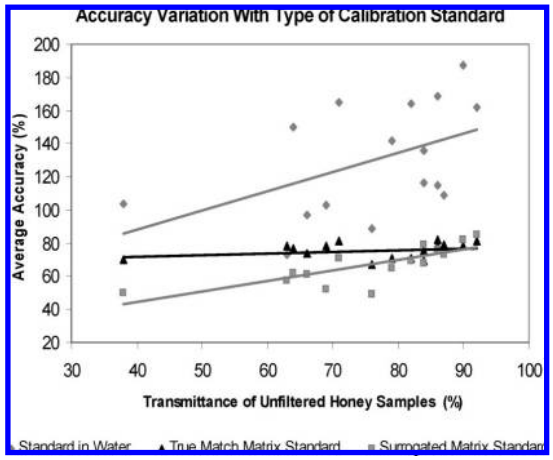

Figure 2. Average accuracy of the 17 antibiotics in different types of honey quantitated against the response of standards in water (top curve), true-match matrix standards (middle curve), and surrogated-match matrix standards (bottom curve) plotted versus the percent light transmittance of the unfiltered honeys.

(**Table 6**). Because of matrix effects on the instrument response, the recovery of the method in dark honeys is underestimated when a light amber honey is used to prepare the matrix standards. Because honey samples have different floral and geographical origins, it is not feasible to have a universal surrogated honey to prepare matrix standards. By observing the general trend of the bottom curve in **Figure 2**, it seems that a reasonable alternative is to prepare matrix standards using a control honey that is not too different in color from the honey in the samples. The control to prepare the matrix standards for honey samples lighter in color than clover honey most likely should be a light-amber honey such as acacia or alfalfa. The use of blueberry honey or crystallized clover honey as surrogates to prepare matrix standards for the assay of clover honey and darker honeys looks promising but needs further investigation.

DISCUSSION

Metabolites, degradants, bound residues, or epimers of the antibiotics included in this study were not considered during the development of the method. Tylosin, oxytetracycline, and fumagillin are the only antibiotics approved for use in the United States for the treatment of diseases in honey bees (*16*). Tolerances have not been established for these three antibiotics in honey. In honey, tylosin degrades to desmycosin with a half-life of 4 months (*17*), and fumagillin readily degrades to neofumagillin upon exposure to light (*13, 18*). However, analytical standards of desmycosin and neofumagillin are not available commercially. In a study conducted by Khong et al. (*4*), oxytetracycline only showed a 3% epimerization during sample preparation. Such a finding correlates well with the 96% recovery observed for fortified samples in our study. The presence of any amounts of banned antibiotics in honey is violative. The method presented in this paper is capable of detecting and confirming residues of banned antibiotics in honey at levels much lower than their listed tolerances in food originating from other sources (*16*).

During the development of the method, several critical steps were identified. Some of the antibiotics are light sensitive, and fumagillin readily decomposes upon exposure to sunlight and fluorescent light. Therefore, all work must be carried out using sodium laboratory lights. To reduce the time it takes to extract a set of samples, the honey may be weighed in advance, and the analysis of streptomycin may be carried out on a separate set of samples another day. When the streptomycin assay is done separately, more unknown samples can be assayed, and a single control sample may be used to prepare all five matrix standards and the negative control.

To avoid clogging the SPE columns, all samples should be filtered using a fine-mesh nylon fabric, even if they appear not to contain wax, pollen, or comb residues. A critical step in the procedure is the loading of the sample onto the SPE column. A faster flow than that recommended in the procedure will result in low recoveries for fumagillin. During the hexane washes of the SPE column, the flow may be started by applying positive pressure using a 3 mL luer-lock plastic syringe. Samples should never be evaporated to dryness or heated longer than necessary because this will result in low recoveries for most of the antibiotics. Many of the drugs have low solubility in 100% water, but increasing the MeOH concentration in the reconstituted extract results in a poor peak shape for several of the analytes. As the amount of water used to wash the SPE column is increased, a significant decrease in matrix effects is seen, but the amount of erythromycin and monensin recovered from the sample decreases. The amount of water used to wash the SPE column in the method is a compromise driven by the need to

have a method that can give reliable data when applied to honey of different sources.

During the study, the stability of the compounds in the sample extract was investigated. Once the streptomycin samples are prepared, they must be assayed immediately as the extracts are not stable. The extracts of the other antibiotics can be stored in a freezer for 1 day with no decrease in recovery or degradation for most of the analytes. The extracts can be stored in a freezer for 1 week pending the assay for chloramphenicol.

The recommendations that follow help improve the sensitivity and repeatability of the method. The same HPLC column is used for all three assays (positive mode, negative mode, and streptomycin assay). The positive-mode gradient is primarily aqueous and acidic, the negative-mode gradient is aqueous but neutral, and the streptomycin gradient is mostly acidic acetonitrile. Therefore, it is imperative to equilibrate the column with the first step of the gradient for at least 1 h before stabilizing the gradient. After assaying a set of samples, the HPLC column must be flushed with 50% ACN/50% water at a flow of 0.200 mL/min for 1 h to remove the formic acid and other retained materials.

The high-speed centrifuge tubes are expensive but may be re-used. The tubes were brushed using hot water, vortexed with fresh hot water, vortexed twice with ~5 mL of MeOH, rinsed several times with deionized water to remove all the MeOH, and hung upside down to dry overnight. If the tubes are not washed and dried properly, cross contamination may occur, or alcohol residues in the tube will cause loss of analytes during the SPE column cleanup. The sorbent bed of the SPE columns is very small. Excess alcohol in the sample or residues of organic solvents from the column-conditioning step will cause loss of analytes, particularly of lincomycin and ciprofloxacin.

ACKNOWLEDGMENT

We gratefully acknowledge the contribution of Ms. Cristina Nochetto to this study. She provided technical advice during method development and conducted an audit of the study data.

LITERATURE CITED

(1) Bogdanov, S. Current state of analytical methods for the detection of residues in bee products. *Trakia J. Sci.* **2003**, *1* (3), 19–22.

(2) Thompson, T. S.; Noot, D. K. Determination of sulfonamides in honey by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **2005**, *551*, 168–176.

(3) Kaufman, A.; Roth, S.; Ryser, B.; Widmer, M.; Guggisberg, D. Quantitative LC/MS-MS determination of sulfonamides and some other antibiotics in honey. *J. AOAC Int.* **2002**, *85*, 853–860.

(4) Khong, S.-P.; Hammel, Y.-A.; Guy, P. A. Analysis of tetracyclines in honey by high-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 493–502.

(5) Quon, D.; Carson, M.; Nochetto, C.; Heller, D. N.; Butterworth, F. Peer validation of a method to confirm chloramphenicol in honey by liquid chromatography-tandem mass spectrometry. *J. Assoc. Off. Anal. Chem.* **2006**, *89* (2), 586–593.

(6) 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.

(7) Bruijnswoort, M.; Ottink, S. J. M.; Jonker, K. M.; Boer, E. Determination of streptomycin and dihydrostreptomycin in milk and honey by liquid chromatography with tandem mass spectrometry. *J. Chromatogr., A* **2004**, *1058*, 137–142.

(8) Lopez, M. I.; Feldlaufer, M. F.; Williams, A. D.; Chu, P.-S. Determination and confirmation of nitrofuran residues in honey using LC-MS/MS. *J. Agric. Food Chem.* **2007**, *55*, 1103–1108.

(9) Heering, W.; Usleber, E.; Dietrich, R.; Martlbauer, E. Immunochemical screening for antimicrobial drug residues in commercial honey. *Analyst* **1998**, *123*, 2759–2762.

(10) Xu, J.; Ding, T.; Wu, Z.; Chen, C.; Wu, B.; Jiang, Y.; Liu, F.; Wang, K. Simultaneous detection of forty five drug residues in honey using solid phase extraction and liquid chromatography-tandem mass spectrometry. Poster B 105. Presented at the 5th International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerp, Belgium, May 2006.

(11) Hammel, Y.-A.; Gremaud, E.; Kieser, B.; Impey, G.; Lisyansky, A.; Guy, A. G. Multi-screening method to monitor the presence of antibiotic residues in honey by LC-ESIMS/MS. Poster 183. *Proceedings of the 53th ASMS Conference on Mass Spectrometry and Allied Topics, San Antonio, TX, June 2005*; American Society for Mass Spectrometry: Santa Fe, NM, 2005.

(12) Hammel, Y.-A.; Davies, A.; Kieser, B.; Impey, G.; Guy, A. G. A shake and shoot approach for screening antibiotic residues in honey using turbulent flow liquid chromatography with tandem mass spectrometry. Poster 03:10. *Proceedings of the 54th ASMS Conference on Mass Spectrometry and Allied Topics, Seattle, WA, May 2006*; American Society for Mass Spectrometry: Santa Fe, NM, 2006.

(13) Kochansky, J.; Nasr, M. Laboratory studies on the photostability of fumagillin, the active ingredient of Fumidil B. *Apidologie* **2004**, *35*, 301–310.

(14) Guidance for Industry 3: General Principles for Evaluating the Safety of Compounds used in Food Producing Animals−Section IV: Guidance for Approval of a Method of Analysis for Residues; Center for Veterinary Medicine, U.S. Food and Drug Administration: Rockville, MD, 2006. Available at www.fda.gov/cvm/Guidance/GFI003.htm (Last Accessed August 2007).

(15) Guidance for Industry 118: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues-Final Guidance; Center for Veterinary Medicine, U.S. Food and Drug Administration: Rockville, MD, 2003. Available at www.fda.gov/cvm/Guidance/guide118.htm (Last Accessed August 2007).

(16) Tolerances for residues on new animal drugs in food. *Code of Federal Regulations, Food and Drugs*, Part 556, Title 21; Office of the Federal Register, National Archives and Records Administration, U.S. Government Printing Office: Washington, DC, April 1, 2007.

(17) Kochansky, J. Degradation of tylosin residues in honey. *J. Apic. Res.* **2004**, *43*, 65–68.

(18) Assil, H. I.; Sporns, P. ELISA and HPLC methods for analysis of fumagillin and its decomposition products in honey. *J. Agric. Food Chem.* **1991**, *39*, 2206–2213.

Received for review November 5, 2007. Revised manuscript received December 21, 2007. Accepted December 28, 2007.

JF073236W