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Multiclass Analysis of Antibiotic Residues in Honey by Ultraperformance Liquid Chromatography−Tandem Mass Spectrometry

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A method has been developed and validated for the simultaneous analysis of different veterinary drug residues (macrolides, tetracyclines, quinolones, and sulfonamides) in honey. Honey samples were dissolved with Na_2EDTA, and veterinary residues were extracted from the supernatant by solid-phase extraction (SPE), using OASIS HLB cartridges. The separation and determination was carried out by ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), using an electrospay ionization source (ESI) in positive mode. Data acquisition under MS/MS was achieved by applying multiple reaction monitoring (MRM) of two ion transitions per compound to provide a high degree of sensitivity and specificity. The method was validated, and mean recoveries were evaluated at three concentration levels (10, 50, and 100 μg/kg), ranging from 70 to 120% except for doxycycline, erythromycin, and tylmicosin with recovery higher than 50% at the three levels assayed. Relative standard deviations (RSDs) of the recoveries were less than 20% within the intraday precision and less than 25% within the interday precision. The limits of quantification (LOQs) were always lower than 4 μg/kg. The developed procedure was applied to 16 honey samples, and erythromycin, sarafloxacin, and tylosin were found in a few samples.

KEYWORDS: Antibiotics; multiclass analysis; honey; UPLC; tandem mass spectrometry

INTRODUCTION

Honey is a complex product that has always been considered as a natural and healthy food, free of contaminants. However, in apiculture, antibiotics are mainly used for the treatment of bacterial brood diseases, such as American foulbrood (*Bacillus larvae*) and European foulbrood (*Streptococcus pluton*) (*1*). Bearing in mind that veterinary residues can be found in honey (*2*, *3*), several surveillance systems have been established to control the presence of antibiotic residues in honey (*4*).

Oxytetracycline was the first antibiotic to be used for the control of American and European foulbrood, but other antibiotics, such as sulfonamides and macrolides such as tylosin, can potentially be used to prevent these diseases (*5*, *6*) because oxytetracycline resistant strains have been found in several countries (*7*). However, no harmonization related to maximum residue limits (MRLs) in honey has been defined worldwide. For instance, the Canadian Food Inspection Agency recommended working residue levels (WRLs) for several antibiotics in honey, setting a WRL of 60 μg/kg for tylosin (*8*), although other antibiotics such as macrolides are not allowed to be present in honey (*2*). However, the US Food and Drug Administration (FDA) only approves the use of tylosin, oxytetracycline, and fumagillin for the treatment of diseases in honey bees (*9*), whereas in Europe, no MRLs have been fixed in honey (*10*), although a MRL of 10 μg/kg for all tetracyclines has been proposed (*11*), and some countries have established action limits or tolerated levels for some antibiotics (*12*).

In general, there is a zero-tolerance policy to drug residues in honey; therefore, it is necessary to develop sensitive analytical methods with low limits of detection (LODs) in order to detect traces of antibiotics in honey. Furthermore, some surveys show that positive samples can be contaminated by more than one class of antibiotics (*13*); therefore, new sensitive multiclass methods are necessary for the simultaneous determination of antibiotics at trace levels in this matrix.

Although immunoassay methods have been proposed for the determination of antibiotics in honey (*14*), liquid chromatography (LC) has been mainly used for the separation of these type of compounds, coupled to ultraviolet (*5*, *15*) and fluorimetric detection (*4*, *12*), although mass spectrometric detection using single (MS) (*2*, *16*), triple quadrupole (MS/MS) (*3*, *7*, *13*, *17*, *18*), or time-of-flight (TOF) (*19*) analysers provides more reliable determination. However, most of the developed analytical methods are multiresidue analyses within a single class of compound (*5*, *18*, *20*), and very few multiclass methods for the determination of antibiotics in honey have been proposed (*13*, *21*).

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Table 1. Retention Time Windows (RTWs) and MS/MS Parameters for the Selected Antibiotics

antibiotic	channel	dwell time (s)	RTW (min)	cone voltage (V)	quantification transition[a]	confirmation transition[a]	ion ratio (%)[b]
marbofloxacin	1	0.025	2.16–2.22	35	363.1 > 320.4 (15)	363.1 > 345.4 (20)	42.8 (12.9)
sulfadimidine	1	0.025	2.37–2.46	35	279.1 > 92.1 (30)	279.1 > 124.2 (20)	65.5 (9.4)
tetracycline	2	0.015	2.55–2.65	28	445.4 > 410.2 (20)	445.4 > 427.3 (13)	54.5 (4.0)
enrofloxacin	2	0.015	2.55–2.62	38	360.3 > 342.3 (20)	360.3 > 316.3 (20)	76.6 (5.2)
danofloxacin	2	0.015	2.57–2.65	38	358.3 > 340.3 (32)	358.3 > 255.2 (38)	28.5 (10.6)
oxytetracycline	2	0.015	2.65–2.73	28	461.4 > 443.3 (13)	461.4 > 426.3 (10)	14.9 (28.4)
difloxacin	2	0.015	2.65–2.79	38	400.3 > 382.3 (23)	400.3 > 356.3 (23)	42.3 (6.8)
sulfachloropyridazine	2	0.015	2.67–2.77	32	285.1 > 156.2 (15)	285.1 > 80.2 (50)	57.0 (9.8)
sarafloxacin	2	0.015	2.78–2.86	45	386.2 > 368.4 (25)	386.2 > 348.4 (30)	12.0 (19.4)
chlorotetracycline	3	0.015	3.24–3.35	35	479.3 > 444.3 (20)	479.3 > 462.3 (18)	73.4 (7.5)
sulfadimethoxine	3	0.015	3.39–3.48	60	311.1 > 156.2 (20)	311.1 > 245.3 (18)	11.8 (10.6)
sulfaquinoxaline	3	0.015	3.55–3.64	32	301.2 > 156.1 (35)	301.2 > 108.1 (30)	2.2 (9.0)
tilmicosin	3	0.015	3.63–3.74	18	870.4 > 174.3 (45)	870.4 > 696.9 (45)	13.0 (28.4)
doxycycline	3	0.015	3.65–3.79	30	445.3 > 428.3 (18)	445.3 > 154.2 (28)	11.3 (5.9)
tylosin	4	0.025	4.06–4.17	35	917.4 > 174.3 (18)	917.4 > 101.1 (45)	7.1 (23.4)
erythromycin	4	0.025	4.23–4.35	55	717.1 > 158.2 (30)	717.1 > 116.2 (45)	19.8 (6.1)
josamycin	4	0.025	4.35–4.46	55	829.3 > 174.2 (32)	829.3 > 109.1 (40)	89.8 (9.5)

[a] Collision energy (eV) is given in parentheses. [b] Relative standard deviation (RSD) is given in parentheses ($n = 10$).

Two main problems can be found when a multiresidue method must be developed. First, chromatographic analysis time can be very long, higher than 20 min (*5, 13*), or when a multiclass determination is developed, a tedious sample preparation treatment is carried out due to the different physicochemical properties of the antibiotics (*21*).

In order to overcome the first problem, the introduction of ultraperformance liquid chromatography (UPLC) has decreased the analysis time, by the reduction of particle size of stationary phase (<2 μm), providing superior chromatographic resolution than conventional LC and higher sensitivity (*8, 22*), decreasing LODs.

It is also difficult to find a single and simultaneous extraction and purification procedure for the determination of several classes of antibiotics with acceptable recoveries. In general, sulfonamides and tetracyclines are extracted using an acid hydrolysis to dissociate sugar-bound antibiotics, followed by a cleanup/enrichment step using solid phase extraction (SPE) (*23*). However, macrolides are usually extracted at basic conditions because they are not stable in acidic media (*2*). Furthermore, the addition of EDTA is necessary to avoid the complexation of macrolides with metal ions that provokes low recoveries of these compounds (*24*).

Among the proposed methods, homogenization of honey with EDTA under mildly acidic conditions and SPE can be a good choice for the simultaneous extraction of several classes of antibiotics in honey, which allows the elimination of interferences as well as the concentration of the compounds, improving LOD of the analytical method.

The aim of the present work was the development of a sensitive method for the simultaneous determination of 17 veterinary drugs belonging to several classes of antibiotics (sulfonamides, quinolones, tetracyclines, and macrolides) in honey, applying SPE and UPLC-MS/MS determination. The proposed method will quantify and confirm veterinary drugs at low concentration levels (<4 μg/kg), indicating that it can be applied in routine analyses to provide a large amount of data related to the presence of antibiotics in honey.

MATERIALS AND METHODS

Chemicals and Reagents. Commercial antibiotic standards (marbofloxacin, sulfadimidine, doxycycline hyclate, enrofloxacin, danofloxacin, difloxacin, tetracycline, chlorotetracycline, sulfaquinoxaline, sulfachloropyridazine, sarafloxacin, and sulfadimethoxine) were supplied by Riedel-de Haën (Seelze, Germany), tylosin phosphate, tilmicosin, erythromycin, and josamycin from Fluka (Steinheim, Germany), and oxytetracycline hydrochloride was supplied by Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions of individual compounds (with concentrations between 200 and 300 mg/L) were prepared by exact weighing of the powder and dissolved in 100 mL of HPLC-grade methanol (Sigma, St. Louis, MO, USA), which were then stored at −20 °C in the dark. A multicompound working standard solution at a concentration of 10 mg/L of each compound was prepared by appropriate dilutions of the stock solutions with methanol and stored in screw-capped glass tubes at −20 °C in the dark. This solution was stable for 3 weeks, after which it was replaced by a new fresh solution.

HPLC-grade acetonitrile (MeCN) was supplied by Sigma. Hexane (purity >99%) was purchased from J. T. Baker (Deventer, Holland), and formic acid (purity >98%) and NH_4OH were obtained from Panreac (Barcelona, Spain). Na_2EDTA (0.1 M) solution was prepared by dissolving Na_2EDTA (Merck, Darmstadt, Germany) in water. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). Oasis HLB SPE (60 and 200 mg) and C_{18} Sep-Pak (200 mg) cartridges were purchased from Waters (Milford, MA, USA).

Apparatus and Software. Chromatographic analyses were performed using an Acquity UPLC system (Waters, Milford, MA, USA), and separations were achieved using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm particle size) from Waters. Mass spectrometry analysis was carried out using a Waters Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using electrospray ionization (ESI). Data acquisition was performed using MassLynx 4.0 software with QuanLynx program (Waters).

Chromatographic separation was carried out with a mobile phase consisting of methanol (eluent A) and 0.05% formic acid in water (eluent B) at a flow rate of 0.3 mL/min.

The elution started at 20% A for 0.5 min and then was linearly increased up to 100% A in 5 min, keeping constant for 1 min before being returned to the initial conditions in 1 min. Finally, the total run time, including the conditioning of the column to the initial conditions was 7.5 min. Injection volume was 5 μL, and the column was maintained at 30 °C.

All antibiotics were detected using ESI in positive mode. The capillary voltage and the extractor voltage were 3.0 kV and 2 V, respectively. The source temperature was 120 °C and desolvation temperature 350 °C. The cone gas (nitrogen) and desolvation gas (also nitrogen) were set at flow rates of 80 L/h and 600 L/h, respectively, and the collision-induced dissociation was performed using argon as the collision gas at the pressure of 4×10^{-3} mbar in the collision cell. The specific MS/MS parameters for each antibiotic are shown in **Table 1**.

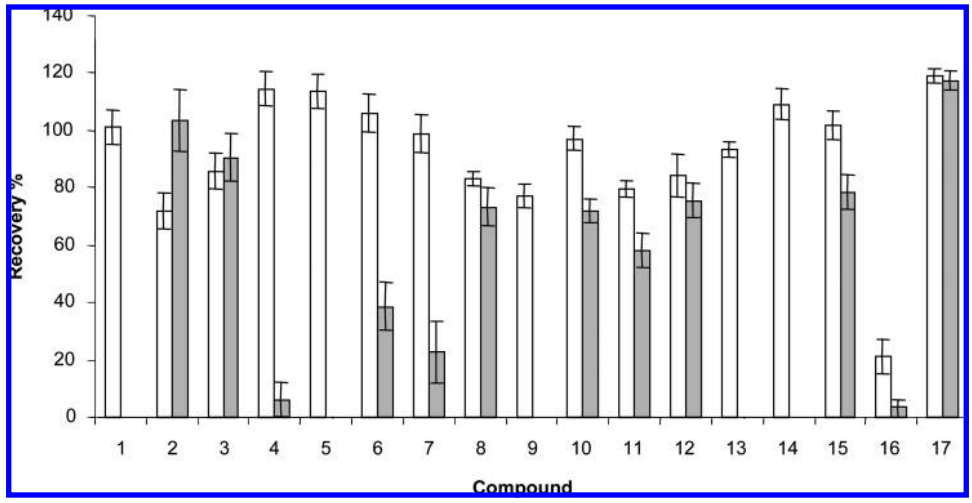

Figure 1. Study of the type of sorbent (□, Oasis HLB and ■, C_{18}) on the recovery of the selected antibiotics spiked at 50 μg/kg in a blank honey sample ($n = 3$). Antibiotic codes are as follows: (1) marbofloxacin, (2) sulfadimidine, (3) tetracycline, (4) enrofloxacin, (5) danofloxacin, (6) oxytetracycline, (7) difloxacin, (8) sulfachloropyridazine (9) sarafloxacin, (10) chlorotetracycline, (11) sulfadimethoxine, (12) sulfaquinoxaline, (13) tilmicosin, (14) doxycycline, (15) tylosin, (16) erythromicin, and (17) josamycin.

Centrifugations were performed in a high-volume centrifuge from Centronic (Barcelona, Spain). A Vortex mixer Heidolph, model Reax 2000 and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. A extraction manifold from Waters connected to a Büchi Vac V-500 (Flawil, Switzerland) vacuum system was used for SPE experiments.

Extraction Procedure. All honey samples were processed according to the following procedure: an aliquot of honey (2 g) was weighed into a 50 mL polypropylene centrifuge tube and was dissolved in 10 mL of Na_2EDTA at pH 4. The mixture was vortexed until the honey was completely diluted. Then, the sample tube was centrifuged at 4500*g* for 5 min. After centrifugation, the remaining supernatant was loaded onto an OASIS HLB (60 mg) cartridge previously conditioned with 2 mL of methanol, 2 mL of MeCN, and twice with 2 mL of water. After the samples were passed through the cartridges, they were washed with 6 mL of water, and they were vacuum-dried for 30 min. Analytes were eluted by sequentially adding 3 mL of MeOH, 3 mL of MeCN, and 3 mL of 0.04% NH_4OH dissolved in MeOH. The extract was evaporated under a stream of nitrogen and redissolved with 1 mL of a mixture of methanol/aqueous solution of formic acid, 0.05% (50:50 v/v). Finally, the extract was then transferred into a vial, and 5 μL was injected into the UPLC system.

Method Validation. Calibration curves, sensitivity, accuracy, intra, and interday precision were performed to validate the whole procedure. Linearity was evaluated using matrix-matched calibration, spiking blank extracts at five concentration levels (from 3 to 150 μg/kg). Precision and accuracy of the method were studied spiking blank samples. Repeatability (intraday precision) was performed spiking blank honey at three concentration levels (10, 50, and 100 μg/kg), using five replicates for each concentration level in one day. To evaluate interday precision (reproducibility), the same concentration levels were studied, spiking blank honey during five consecutive days.

Recovery was studied by analyzing blank samples that were fortified before extraction at three concentration levels (10, 50, and 100 μg/kg). Limits of detection (LODs) and quantification (LOQs) were estimated by fortifying blank honey with antibiotics (0.5, 1, 2, 5, and 10 μg/kg) and applying the extraction procedure prior to chromatographic determination. LODs and LOQs were determined as the amount for which signal-to-noise ratios (*S/N*) were higher than 3 and 10, respectively.

Honey Samples. Multiflower honey samples were obtained from different local markets in Almeria (Spain) and from several beekeepers from different geographical origins (Almeria and Granada, Spain). The samples were stored at room temperature in the dark. One multiflower sample was checked to be free of any of the selected antibiotics and it was used as blank honey for calibration and validation purposes.

RESULTS AND DISCUSSION

Analytes, such as quinolones, sulfonamides, tetracyclines, and macrolides, were selected on the basis of their worldwide occurrence in honey, and the aim of this work was to develop and validate a simple and rapid method for the simultaneous determination of these antibiotics in honey.

UPLC-MS/MS Determination. The chromatographic procedure was adopted from Aguilera-Luiz et al. (*25*), and the main goal was to achieve a short overall run time for the simultaneous determination of the selected classes of antibiotics. Several gradient profiles were studied in order to elute the analytes within a short run time and provide good peak shape. It was observed that starting with 20% of organic solvent (methanol) and increasing this percentage up to 100%, good separation can be obtained in less than 5 min. Retention times of the analytes were reproducible and ranged from 2.19 (marbofloxacin) to 4.40 (josamycin). Although honey contains low polar substances such as lipids and waxes at low concentrations, it is necessary to raise the content of organic solvent during the gradient profile to avoid an increase of the contamination of the column due to these poorly eluting matrix constituents at low concentrations of organic solvent. Total gradient run time was 7.5 min, including the cleaning and preconditioning step.

For MS/MS determination, ESI in positive mode was used, and two transitions per antibiotic were monitored. **Table 1** indicates the two transitions used for the identification and quantitation for each compound. For instance, it can be observed that tetracyclines show a common fragmentation pattern with characteristic losses of water, $[M + H\text{-}H_20]^+$ (oxytetracycline), ammonia, $[M + H\text{-}NH_3]^+$ (doxycyclin), and the combination of both losses $[M + H\text{-}H_2O\text{-}NH_3]^+$ (tetracycline and chlorotetracycline).

Using these conditions, the analytes were distributed in four overlapping functions, and good peak shape was obtained when a dwell time of 0.015 s was used, except for marbofloxacin, sulfadimidine, josamycin, erythromicyn, and tylosin, which were monitored using a dwell time of 0.025 s.

Optimization of the Extraction Procedure. Because honey is a matrix that contains sugars and other substances such as pigments and phenolic compounds that have to be removed before chromatographic analysis, a preconcentration and purification step is necessary. Furthermore, the development of a

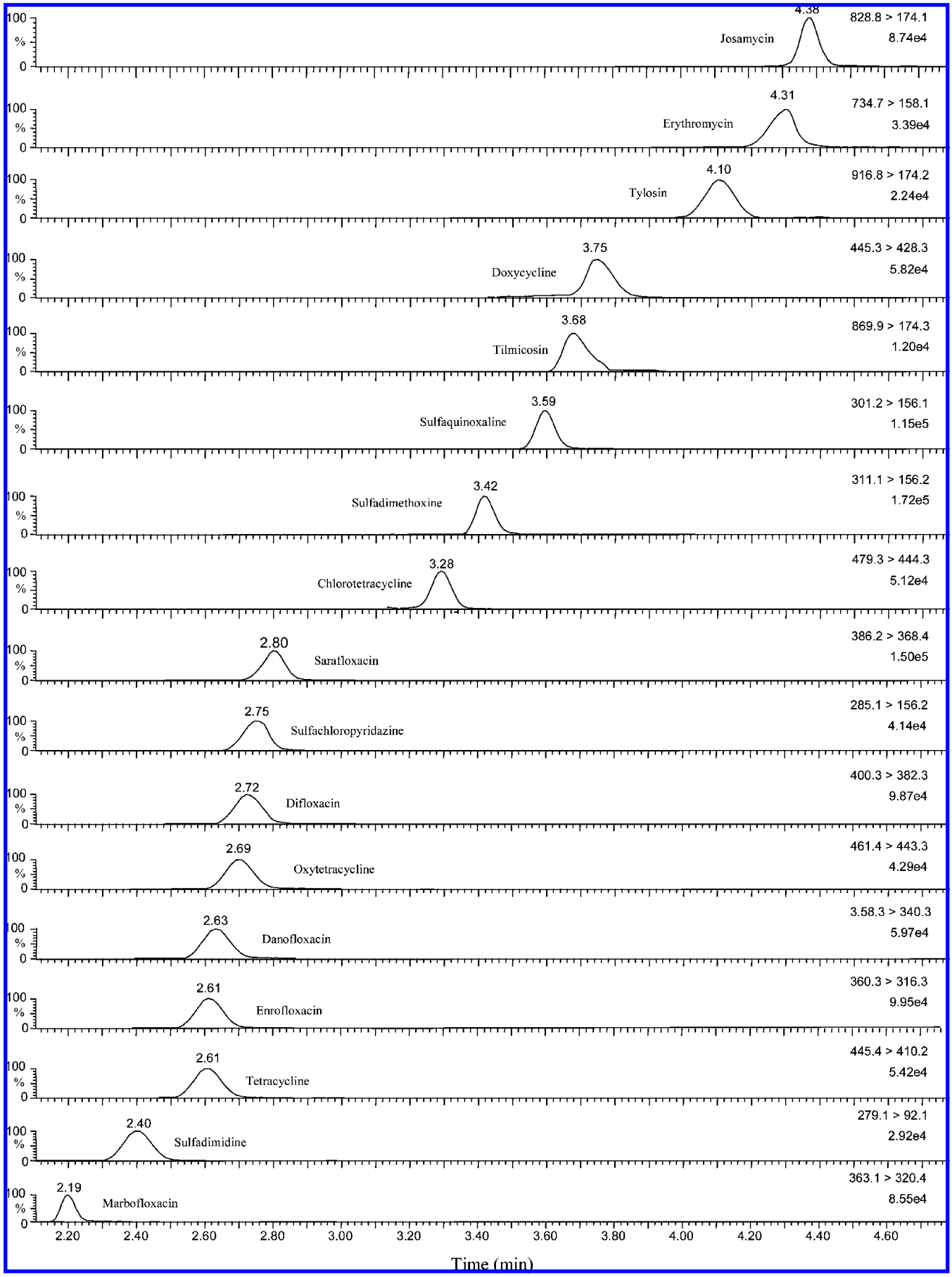

Figure 2. UPLC-MS/MS chromatograms obtained from a blank honey sample spiked at 25 μg/kg.

Table 2. Evaluation of Matrix Effects by Comparing the Slopes of the Calibration Curves Using Solvent-Based Standards and Honey Matrix-Matched Calibration

antibiotic	slope (au · kg/μg)[a] solvent	honey	P (%)[b]
marbofloxacin	8.6	18.0	0.5
sulfadimidine	4.9	22.8	0.2
tetracycline	20.9	37.0	0.8
enrofloxacin	37.0	59.5	0.9
danofloxacin	18.9	34.5	0.5
oxytetracycline	3.9	9.3	0.5
difloxacin	29.3	59.1	0.6
sulfachloropyridazine	27.6	45.0	0.7
sarafloxacin	44.9	68.2	1.1
chlorotetracycline	6.4	24.0	0.4
sulfadimethoxine	34.5	72.9	0.2
sulfaquinoxaline	18.2	32.4	0.5
tilmicosin	8.5	8.3	82.5
doxycycline	19.4	25.3	4.9
tylosin	14.7	25.9	1.0
erythromycin	30.9	77.7	0.3
josamycin	28.6	31.0	9.0

[a] au: arbitrary units for peak area. [b] The p-value was obtained (in %) using the procedure indicated in ref *31*, which was based on a t-test and permits the detection of the presence of any matrix effect during the quantification of the analytes.

common extraction method for the simultaneous extraction of several classes of antibiotics can be a difficult task, taking into account the different properties of the antibiotics included in the survey. For instance, an acid hydrolysis step is necessary to disassociate sugar-bound sulfonamides (*26*), whereas macrolides are not stable at acidic conditions (*2*).

First, honey was dissolved in Na_2EDTA solution at pH 4 because EDTA has a greater affinity for the cations than macrolides and tetracyclines, which can form complexes with transition metal ions (*27*), and the addition of EDTA can increase recoveries of both classes of veterinary drugs.

Although liquid−liquid extraction can be applied (*21*) during the purification step, SPE has been mainly used. Typically, C_{18} bonded silica or Waters Oasis HLB are used as stationary phase for this purpose (*28*). Both cartridges were preconditioned with methanol, MeCN, and water, and for the C_{18} cartridge, 10 mL of Na_2EDTA (0.1 M) was necessary in order to avoid the interactions of tetracyclines with silanol groups (*29*). Both cartridges were washed with water in order to remove sugars, and then the compounds were eluted as was indicated in the Materials and Methods section. In this sense, it must be indicated that the combination of methanol and acetonitrile as well as the addition of NH_4OH allow the quantitative extraction of macrolides (*18*) and sulfonamides (*12*), respectively.

The obtained results are shown in **Figure 1**, and it can be observed that better recoveries were obtained when Oasis HLB was used. Moreover, some antibiotics such as marbofloxacin, danofloxacin sarafloxacin, tilmicosin, and doxycycline were not extracted when the C_{18} cartridge was used, indicating that Oasis HLB is more suitable for the extraction of a wide range of compounds than C_{18} due to its hydropholic and hydrophobic retention mechanisms.

Sixty and 200 mg cartridges of equal particle size HLB were assayed in order to determine optimum sorbent amount. Slightly better recoveries were obtained with the 60 mg cartridge over the 200 mg cartridge, and the 60 mg size was chosen for further experiments.

Finally, **Figure 2** shows a representative chromatogram obtained from a blank sample fortified at 25 μg/kg, and clean extracts can be observed without interferences. Furthermore, it

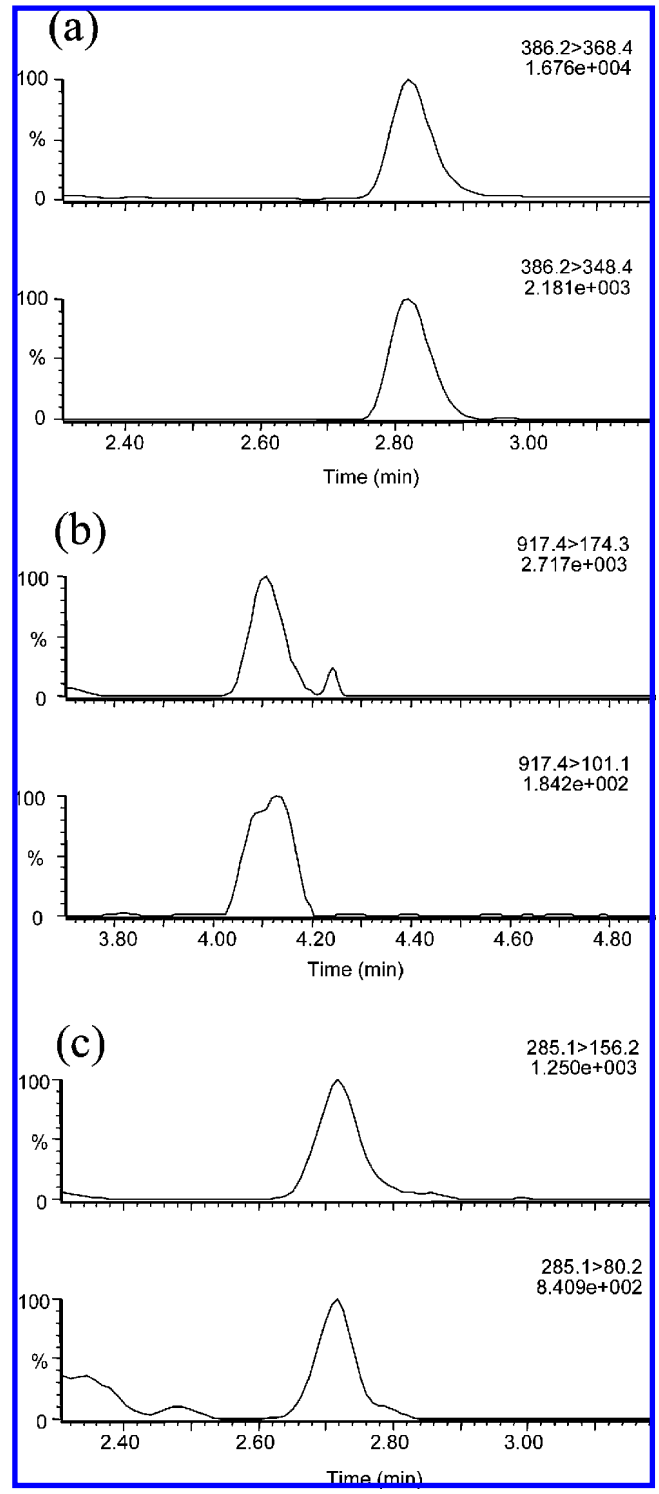

Figure 3. UPLC-MS/MS MRM chromatogram for a honey sample containing (**a**) sarafloxacin at 14.6 μg/kg, (**b**) tylosin at 3.2 μg/kg, and (**c**) sulfachloropyridazine at trace levels.

can be noted that although complete resolution is not obtained for some of the antibiotics, MS/MS allows the selective determination of the compounds since unique transitions are monitored.

Validation of the Proposed Method. Method validation was performed in terms of selectivity, linearity, accuracy, repeatability (intraday precision), interday precision, LODs, and LOQs.

Identification of the antibiotics was carried out by searching in the appropriate retention time windows (RTWs), defined as the retention time ± three standard deviations of the retention

Table 3. Validation Parameters of the Developed Method

	recovery[a]			interday precision			
antibiotic	10 (μg/kg)	50 (μg/kg)	100 (μg/kg)	10 (μg/kg)	50 (μg/kg)	100 (μg/kg)	LOQ (μg/kg)
marbofloxacin	81 (13)	82 (7)	88 (6)	15	12	6	0.3
sulfadimidine	71 (14)	92 (11)	72 (5)	23	15	9	1.0
tetracycline	75 (10)	90 (10)	91 (11)	23	10	5	0.3
enrofloxacin	110 (17)	96 (8)	93 (6)	17	19	12	2.0
danofloxacin	71 (15)	84 (8)	76 (12)	16	7	10	0.3
oxytetracycline	108 (10)	92 (12)	73 (7)	25	21	5	3.3
difloxacin	94 (14)	83 (12)	88 (3)	14	12	11	2.0
sulfachloropyridozine	101 (9)	118 (2)	86 (2)	13	14	10	3.3
sarafloxacin	82 (19)	81 (11)	83 (4)	20	5	4	0.7
chlorotetracycline	73 (6)	80 (4)	82 (6)	5	4	3	0.3
sulfadimethoxine	92 (8)	70 (6)	74 (3)	9	6	3	0.7
sulfaquinoxaline	77 (18)	108 (13)	109 (13)	20	16	5	0.3
tilmicosin	72 (17)	63 (3)	55 (3)	19	8	3	0.7
doxycycline	62 (18)	78 (8)	77 (1)	19	8	4	0.3
tylosin	97 (6)	94 (6)	115 (2)	20	14	8	1.0
erythromycin	56 (11)	78 (11)	53 (5)	20	16	9	0.3
josamycin	84 (5)	95 (2)	87 (1)	14	8	7	0.7

[a] Repeatability values, expressed as RSD, are given in parentheses ($n = 5$).

time of 10 blank honey samples spiked at 25 μg/kg for each antibiotic (**Table 1**), and confirmation was carried out by comparison of the signal intensity ratios of the two transitions (quantification and confirmation) with those obtained using fortified blank honey samples. Confirmation was considered reliable if the ratio was within the criteria laid down in the European Commission Decision (*30*), which were based on relative abundance criteria that are dependent on the relative intensities of the two transitions; **Table 1** shows the obtained ion ratios.

Furthermore, the selectivity of the proposed method was evaluated by the analysis of blank samples. The absence of any chromatographic signal at the same retention time as the target antibiotics indicated that no matrix or chemical compounds are extracted and give a false positive signal, indicating the good performance of the proposed analytical method.

When ESI is used, it is well-known that the presence of matrix components such as sugars and carbohydrates can affect the ionization of the target compounds, reducing or enhancing the response compared with standards in solvents, and the influence of the matrix effect on the response must be studied. The best way to compensate the matrix effect is the use of isotope internal standards. However, these compounds are not available for most antibiotics, and other approaches such as matrix-matched calibration can be used. To evaluate the presence and extension of the matrix effect, several concentrations (from 5 to 100 μg/kg) were analyzed in pure solvent and in blank honey samples, and the slopes of the calibration curve were compared using the procedure indicated by Cuadros-Rodríguez et al. (*31*). **Table 2** shows the obtained results, and it can be observed that slopes are statistically different (*p*-value lower than 5%) for most of the antibiotics assayed, except for tilmicosin and josamycin, indicating that a matrix effect was detected, and matrix-matched calibration should be used to compensate for these matrix effects.

Linearity was then evaluated by spiking blank honey samples with different concentrations of antibiotics from 3 to 150 μg/kg. Good linearity was obtained when peak area was used as the analytical response, and determination coefficients were higher than 0.990.

LODs ranged from 0.1 μg/kg (marbofloxacin, danofloxacin, tetracycline, chlorotetracycline, sulfaquinoxaline, doxyclyne, and erythromycin) to 1.0 μg/kg (oxytetracyline and sulfachloropyridozine), whereas LOQs ranged from 0.3 μg/kg to 3.3 μg/kg as can be observed in **Table 3**.

Accuracy was estimated through recovery studies. Satisfactory results were found, with recoveries higher than 70% for all the assayed compounds at the three concentration levels, except for doxycycline at 10 μg/kg, tilmicosin at 50 μg/kg, and erythromycin at 10 and 100 μg/kg, with recoveries always higher than 50% (**Table 3**). Although these recoveries are not close to 100%, they can be considered acceptable since they were reproducible (see **Table 3**).

Precision of the overall method was studied by performing repeatability (intraday precision) and interday precision experiments; the results obtained are shown in **Table 3**. It can be observed that relative standard deviations (RSDs) were always lower than 20% for all the antibiotics and concentration levels assayed, except for sulfadimidine, tetracycline, and oxytetracycline, which presented values slightly higher than 20%, (<25%) in reproducibility studies, indicating the stability of the developed method.

Application to Real Samples. To evaluate the applicability of the proposed method in real samples, 16 samples were analyzed. Eleven samples were purchased from local supermarkets in Almeria (Spain), and 5 samples were obtained from different beekeepers from the southeast of Spain (Almeria and Granada). In order to ensure the quality of the results when the proposed method was applied, an internal quality control was carried out in every batch of samples. This quality control implies a matrix-matched calibration, a reagent blank and a spiked blank sample at 10 μg/kg in order to evaluate the stability of the proposed method. Furthermore, the relative ion intensities of the detected ions in real samples were compared to those of corresponding calibration standards in the same batch to confirm the identity of the detected antibiotics using the criteria established by EU (*30*) and indicated in **Table 1**.

Among the samples analyzed, only three of them contained traces of antibiotics. First, one commercial sample contained erythromicyn (8.6 μg/kg), whereas traces of sarafloxacin were detected in another commercial sample. In relation to the samples obtained from beekeepers, several antibiotics were detected in only one sample. Sarafloxacin and tylosin were quantified at 14.6 and 3.2 μg/kg, respectively, and traces of sulfadimidine and sulfachloropyridozine (concentrations lower than LOQs) were detected. The detection of several classes of antibiotics in

the same sample indicates the usefulness of the developed multiclass method for the simultaneous analysis of several classes of veterinary drugs in honey. Bearing in mind the zero-tolerance policy to drug residues in honey, these three samples come from wrong beekeeping practices, indicating that more work should be carried out in order to achieve samples without antibiotic residues or, at least, with lower concentrations. Furthermore, the continuous reduction of the LODs allows the detection of positive samples that were not detected with other techniques that provide higher LODs.

Figure 3 shows the positive sample of honey, which contains several traces of antibiotics, observing that no interfering peaks appear on the chromatograms. It can be noted that a reliable detection and quantification can be carried out even at these trace levels.

In conclusion, this work presents a suitable method for the extraction, detection, and quantification of tetracyclines, macrolides, sulfonamides, and quinolones by UPLC-MS/MS. Bearing in mind the difficulties in developing a generic SPE clean up procedure and MS-MS determination for multiclass determination, good validation results were obtained. The method can be applied in routine analysis due to sample throughput as well as in surveillance programs to control the presence of antibiotic residues in honey samples.

LITERATURE CITED

(1) Martel, A. C.; Zeggane, S.; Drajnudel, P.; Faucon, J. P.; Aubert, M. Tetracycline residues in honey after hive treatment. *Food Addit. Contam.* **2006**, *23*, 265–273.

(2) 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.

(3) Verzegnassi, L.; Savoy-Perroud, M.-C.; Stadler, R. H. Application of liquid chromatography-electrospray ionization tandem mass spectrometry to the detection of 10 sulfonamides in honey. *J. Chromatogr., A* **2002**, *977*, 77–87.

(4) Pena, A.; Pelantova, N.; Lino, C. M.; Silveira, M. I. N.; Solich, P. Validation of an analytical methodology for determination of oxytetracycline and tetracycline residues in honey by HPLC with fluorescence detection. *J. Agric. Food Chem.* **2005**, *53*, 3784–3788.

(5) Zotou, A.; Vasiliadou, C. Selective determination of sulphonamide residues in honey by SPE-RP-LC with UV detection. *Chromatographia* **2006**, *64*, 307–311.

(6) Benetti, C.; Dainese, N.; Biancotto, G.; Piro, R.; Mutinelli, F. Unauthorised antibiotic treatments in beekeeping. Development and validation of a method to quantify and confirm tylosin residues in honey using liquid chromatography-tandem mass spectrometric detection. *Anal. Chim. Acta* **2004**, *520*, 8–92.

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

(8) Wang, J.; Leung, D. Analyses of macrolide antibiotic residues in eggs, raw milk, and honey using both ultra-performance liquid chromatography/quadruple time-of-flight mass spectrometry and high performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3213–3222.

(9) 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: Washington, DC, April 1, 2007.

(10) Council Regulation (EEC) No. 2377/90 of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Off. J. Eur. Commun.* L224/1, 1990: consolidated version of the Annexes I to IV up to July, 3, 2008, obtained from http://www.emea.europa.eu/htms/vet/mrls/a.htm.

(11) Xu, J. Z.; Ding, T.; Wu, B.; Yang, W. Q.; Zhang, X. Y.; Liu, Y.; Shen, C. Y.; Jiang, Y. Analysis of tetracycline residues in royal jelly by liquid chromatography-tandem mass spectrometry. *J. Chromatogr., B* **2008**, *868*, 42–48.

(12) Maudens, K. E.; Zhang, G. F.; Lamber, W. E. Quantitative analysis of twelve sulfonamides in honey after acidic hydrolysis by high-performance liquid chromatography with post-column derivatization and fluorescence detection. *J. Chromatogr., A* **2004**, *1047*, 85–92.

(13) Lopez, M. I.; Pettis, J. S.; Smith, B.; Chu, P. S. Multiclass determination and confirmation of antibiotic residues in honey using LC-MS/MS. *J. Agric. Food Chem.* **2008**, *56*, 1553–1559.

(14) Jeon, M.; Paeng, I. R. Quantitative detection of tetracycline residues in honey by a simple sensitive immunoassay. *Anal. Chim. Acta* **2008**, *626*, 180–185.

(15) Viñas, P.; Balsalobre, N.; López-Erroz, C.; Hernández-Córdoba, M. Liquid chromatography with ultraviolet absorbance detection for the analysis of tetracycline residues in honey. *J. Chromatogr., A* **2004**, *1022*, 125–129.

(16) Thompson, T. S.; Noot, D. K.; Calvert, J.; Pernal, S. F. Determination of lincomycin and tylosin residues in honey using solid-phase extraction and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr., A* **2003**, *1020*, 241–250.

(17) Alfredsson, G.; Branzell, C.; Granelli, K.; Lundstr̈; om, A. Simple and rapid screening and confirmation of tetracyclines in honey and egg by a dipstick test and LC-MS/MS. *Anal. Chim. Acta* **2005**, *529*, 47–51.

(18) Nozal-Nalda, M. J.; Bernal-Yagüe, J. L.; Martín-Gómez, M. T.; Jiménez-Sevilla, J. J.; Bernal del Nozal, J.; Higes Pascual, M. Trace analysis of antibacterial tylosin A, B, C and D in honey by liquid chromatography-electrospray ionization-mass spectrometry. *J. Sep. Sci* **2006**, *29*, 405–413.

(19) Carrasco-Pancorbo, A.; Casado-Terrones, S.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Reversed-phase high-performance liquid chromatography coupled to ultraviolet and electrospray time-of-flight mass spectrometry on-line detection for the separation of eight tetracyclines in honey simples. *J. Chromatogr., A* **2008**, *1195*, 107–116.

(20) Mottier, P.; Hammel, Y. A.; Gremaud, E.; Guy, P. A. Quantitative high-throughput analysis of 16 (fluoro)quinolones in honey using automated extraction by turbulent flow chromatography coupled to liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* **2008**, *56*, 35–43.

(21) Hammel, Y. A.; Mohamed, R.; Gremaud, E.; LeBreton, M. H.; Guy, P. A. Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2008**, *1177*, 58–76.

(22) Pastor-Montoro, E.; Romero-González, R.; Garrido-Frenich, A.; Hernández-Torres, M. E.; Martínez-Vidal, J. L. Fast determination of herbicides in waters by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3585–3592.

(23) Koesukwiwat, U.; Jayanta, S.; Leepipatpiboon, N. Solid-phase extraction for multiresidue determination of sulfonamides, tetracyclines, and pyrimethamine in Bovine's milk. *J. Chromatogr., A* **2007**, *1149*, 102–111.

(24) Yang, S.; Cha, J.; Carlson, K. Trace analysis and occurrence of anhydroerythromycin and tylosin in influent and effluent wastewater by liquid chromatography combined with electrospray tandem mass spectrometry. *Anal. Bioanal. Chem.* **2006**, *385*, 623–636.

(25) Aguilera-Luiz, M. M.; Martínez Vidal, J. L.; Romero- González, R.; Garrido Frenich, A. Multi-residue determination of veterinary drugs in milk by ultra- high pressure liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2008**, *1205*, 10–16.

(26) Sheridan, R.; Policastro, Thomas, S.; Rice, D. Analysis of occurrence of 14 sulfonamide antibacterials and chloramphenicol in honey by solid-phase extraction followed by LC/MS/MS anaysis. *J. Agric. Food Chem.* **2008**, *56*, 3509–3516.

(27) Yang, S.; Cha, J.; Carlson, K. H. Trace analysis and occurrence of anhydroerythromycin and tylosin in influent and effluent wastewater by liquid chromatography combined with electrospray tandem mass spectrometry. *Anal. Bioanal. Chem.* **2006**, *385*, 623–636.

(28) Debayle, D.; Dessalces, G.; Grenier-Loustalot, M. F. Multi-residue analysis of traces of pesticides and antibiotics in honey by HPLC-MS-MS. *Anal. Bioanal. Chem* **2008**, *391*, 1011–1020.

(29) Casado-Terrones, S.; Segura-Carretero, A.; Busi, S.; Dinelly, G.; Fernández-Gutiérrez, A. Determination of tetracycline residues in honey by CZE with ultraviolet absorbance detection. *Electrophoresis* **2007**, *28*, 2882–2887.

(30) Commission Decision 2002/657/EEC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.*, L221, 8−36.

(31) Cuadros-Rodríguez, L.; García-Campaña, A.; Jiménez-Linares, C.; Alés-Barrero, F.; Román-Ceba, M. Validation of an analytical instrumental method by standard addition methodology. *J. Assoc. Off. Anal. Chem.* **1995**, *78*, 471–476.

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