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Rapid and Semiautomated Method for the Analysis of Veterinary Drug Residues in Honey Based on Turbulent-Flow Liquid Chromatography Coupled to Ultrahigh-Performance Liquid Chromatography–Orbitrap Mass Spectrometry (TFC-UHPLC-Orbitrap-MS)

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ABSTRACT: A simple and rapid method is described for the determination of veterinary drug (VD) residues in honey samples using turbulent flow chromatography coupled to ultrahigh-performance liquid chromatography–Orbitrap mass spectrometry (TFC-UHPLC-Orbitrap-MS). Honey samples were diluted with an aqueous solution of Na_2EDTA (0.1 M). Then, they were injected into the chromatographic system including a TFC column. Afterward, the analytes were transferred to an UHPLC analytical column, where they were determined by UHPLC-Orbitrap-MS. Mean recoveries were obtained at three concentration levels (5, 10, and 50 μg/kg), ranging from 68 to 121% for most compounds. Repeatability (intraday precision) and interday precision (expressed as relative standard deviation, RSD) were <25% for most compounds. Limits of quantification (LOQs) ranged from 5 to 50 μg/kg and limits of identification (LOIs) from 0.1 to 50 μg/kg. The developed method was applied in honey samples, and it was fast and nonlaborious.

KEYWORDS: *turbulent flow chromatography (TFC), ultrahigh-performance liquid chromatography (UHPLC), Orbitrap high-resolution mass spectrometry (HRMS), multiclass analysis, veterinary drugs, honey*

INTRODUCTION

Honey is a complex food product rich in sugars, vitamins, and minerals, very popular and highly consumed. It has been generally considered to be a natural and healthy product of animal origin, free of impurities.[1] However, honeybees are also subject to a number of diseases, such as American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*), and veterinary drugs (VDs) are used to protect colonies.[2,3] These drugs can remain in the insects and be transferred to honey, and therefore, VD residue control represents an important issue to ensure consumer protection. Although some organizations, such as the U.S. Food and Drug Administration (FDA)[4] and the Canadian Food Inspection Agency,[5] approve the use of certain VDs, the European Union (EU) does not accept the use of antimicrobial drugs in beekeeping. Thus, no maximum residue limits (MRLs) for these substances are set by European legislation,[6] although some European countries, such as Belgium, France, and Switzerland, have set action limits and tolerance levels for tetracyclines[7,8] and total sulfonamides in honey.[9] In general, there is a zero-tolerance policy to VD residues in honey.

Several analytical methodologies have been previously developed for the determination of single-class VD residues in honey, that is, macrolides,[10] aminoglycosides,[11] tetracyclines,[12,13] and sulfonamides.[14−17] Most of the proposed methods employed liquid chromatography (LC) coupled to fluorescence detection,[9,12,17,18] UV,[19] triple-quadrupole tandem mass spectrometry (QqQ-MS/MS),[11,12,14] or time-of-flight (TOF).[10,18] In relation to the extraction techniques, liquid–liquid extraction (LLE),[12] solid-phase extraction (SPE),[12,14,17,20] and, more recently, molecularly imprinted polymers (MIPs)[7] have been used. Despite their applicability for single-class analysis, these methods have not been capable of determining a high number of compounds belonging to different classes.

To our best knowledge, few methods have been devoted to the multiclass analysis of VDs in honey in the past years,[21−25] and nonautomated procedures have been applied. The use of semiautomated techniques for sample preparation, such as turbulent flow chromatography (TFC), can be considered to increase sample throughput. TFC consists of a sample preparation system based on a column with large and porous stationary particles combined with a high flow rate of mobile phase to exhibit TF properties, which allows a separation of molecules on the basis of their different molecular weights.[25] Therefore, low molecular weight analytes diffuse into the particle pores of the stationary phase, and high molecular weight compounds (e.g., sugars, proteins, ...) are rapidly flushed to the waste. Then, the analytes are eluted toward the analytical

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Table 1. Experimental Conditions Used for the TFC-UHPLC Elution of Veterinary Drugs from Honey Samples

	time (min)			TFC (loading pump)[a]				UHPLC (eluting pump)[b]				
stage	start (min)	step length (min)	flow (mL/min)	A %	B %	C %	D %	flow (mL/min)	gradient mode	E %	F %	comments
1	0.0	1.50	2.00			100		0.30	step	100	0	sample loading onto TFC column
2	1.50	1.50	0.10	100				0.30	step	100	0	sample transfer to AC[c]
3	3.00	1.50	2.00				100	0.30	ramp	75	25	rising TFC column/gradient elution on AC
4	4.50	1.50	2.00		100			0.30	ramp	50	50	rising TFC column/gradient elution on AC
5	6.00	1.00	2.00				100	0.30	ramp	0	100	rising TFC column/gradient elution on AC
6	7.00	1.50	2.00		100			0.30	step	0	100	rising TFC column/gradient elution on AC
7	8.50	1.00	2.00		100			0.30	step	0	100	rising TFC column/gradient elution on AC
8	9.50	1.00	2.00		100			0.30	step	0	100	filling the transfer loop/gradient elution on AC
9	10.50	1.00	2.00	100				0.30	ramp	100	0	equilibrating TFC column/ equilibrating AC
10	11.50	2.00	2.00			100		0.30	step	100	0	equilibrating TFC column/ equilibrating AC

[a]TFC solvents: solvent A, aqueous solution of formic acid (0.05%, v/v); solvent B, methanol/acetonitrile (1:1, v/v) with Na_2EDTA, 0.1%; solvent C, 10 mM ammonium formate in water; solvent D, acetonitrile/acetone:2-propanol (4:3:3, v/v/v). [b]UHPLC solvents: solvent E, 4 mM ammonium formate aqueous solution acidified with 0.1% formic acid; solvent F, 4 mM ammonium formate in methanol acidified with 0.1% formic acid. [c]Analytical column.

column to be chromatographically separated and subsequently analyzed. TFC provides online sample extraction and cleanup, allowing the reduction of the overall analysis time compared to traditional off-line methods.[26−29] However, TFC has not been evaluated for the extraction of a high number of VDs in food samples so far. This methodology has been limited to the extraction of 16 fluoroquinolones[30] and 10 antibiotics belonging to 4 different classes[31] in honey and to the determination of enrofloxacin and ciprofloxacin in edible tissues[32] and 8 VDs belonging to 7 classes in milk.[33] In these studies, TFC was coupled online to LC-QqQ-MS/MS. In the present study, TFC has been coupled to Exactive-Orbitrap mass spectrometry (Orbitrap-MS), which provides higher selectivity than and similar sensitivity to that of QqQ;[34] its mass resolving power (up to 100000 fwhm, m/z 200) and mass accuracy (<5 ppm) provide selective detection of residues at low nanogram per gram level in complex samples, such as honey.[35]

The aim of this study was the development of a multiclass online extraction procedure for the simultaneous determination of several classes of VDs (sulfonamides, quinolones, macrolides, tetracyclines, penicillins, imidazothiazoles, avermectins, and benzimidazoles) in honey employing TFC-UHPLC-Orbitrap-MS. Benzathine was also included in this study as a marker of the presence of penicillin, bearing in mind that it is usually used to stabilize these compounds. The different classes of VDs included in the study were selected on the basis of their worldwide occurrence in honey and their previous study in honey by other authors.[23−25]

The combination of TFC extraction and UHPLC-Orbitrap-MS provides a fast and simple method and increases sample throughput.

MATERIALS AND METHODS

Chemical and Reagents. Commercial VD standards (levamisole hydrochloride, marbofloxacin, sulfadimidine, chlorotetracycline hydrochloride, tetracycline hydrochloride, sarafloxacin, doxycycline hyclate, enrofloxacin, danofloxacin, difloxacin, thiabendazole, chlorpyridazine, sulfaquinoxaline, sulfadimethoxine, mebendazole, fenbendazole, emamectin benzoate, and abamectin), with purity >95%, were supplied by Riedel-de Haën (Seelze, Germany). Flumequine, sulfadiazine, and oxolinic acid were from Dr. Ehrenstorfer (Augsburg, Germany) with purity >98%. Oxytetracycline hydrochloride, tylosin phosphate, tilmicosin, erythromycin, josamycin, benzathine, oxfendazole, griseofulvin, ampicillin trihydrate, cloxacillin sodium salt monohydrate, dicloxacillin sodium salt hydrate, oxacillin sodium salt hydrate, penicillin G, and penicillin V potassium salt were purchased from Fluka (Steinheim, Germany) with purity >90%, whereas albendazole, ivermectin, neomycin-trisulfate-x hydrate, and sulfathiazole, with purity >99%, were obtained from Sigma (Madrid, Spain).

Stock standard solutions of individual compounds (with concentrations from 200 to 300 mg/L) were prepared by dissolving the standard material (powder or liquid) in 50 mL of methanol or acetonitrile (LC-MS quality) obtained from Fluka, and they were stored at −20 °C in the dark. A multicompound working standard solution (4 mg/L of each compound) was prepared by appropriate dilutions of the stock solutions with acetonitrile and stored in screw-capped glass bottles at −20 °C in the dark. This solution was stable for 3 weeks.

A 0.1 M disodium ethylenediaminetetraacetic acid (Na_2EDTA) solution was prepared by dissolving 9.3 g of Na_2EDTA (>99%, Merck, Darmstadt, Germany) in 250 mL of water. The pH of this solution was adjusted to 4 with a 0.2 M hydrochloric acid solution (37%, J. T. Baker, Deventer, The Netherlands). Water (LC-MS quality) was obtained from Scharlab (Barcelona, Spain). Ammonium formate (purity > 99%), a solution of ammonium hydroxide (35%), formic acid (purity > 98%), and 2-propanol (purity > 99%) were purchased from Panreac (Barcelona, Spain). Acetone (purity > 99%) was purchased from J. T. Baker.

For accurate mass calibration from m/z 100 to 2000, a mixture of caffeine, Met-Arg-Phe-Ala acetate salt (MRFA), and Ultramark 1600 (ProteoMass LTQ/FT-Hybrid ESI positive mode calibration mix) from Sigma was used in the Orbitrap analyzer.

Instruments. The TFC system consisted of an Aria TLX-1 system (Thermo Fisher Scientific, Franklin, MA, USA) including a CTC HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), which kept the samples at 10 °C, a low-pressure mixing quaternary pump (loading pump), a high-pressure mixing quaternary pump (eluting pump), and a three-valve switching device unit with a six-port valve. The entire system was controlled by software Aria 1.6. The TFC column was a

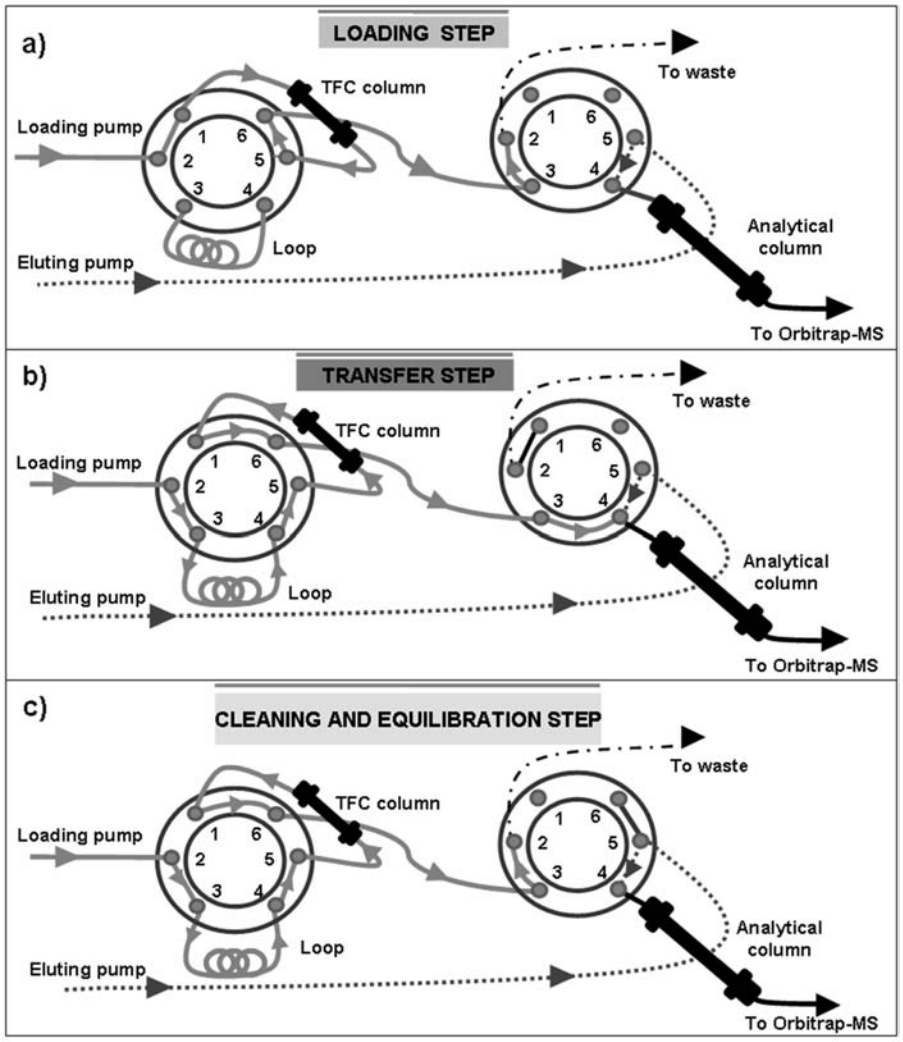

Figure 1. Schematic representation for the different steps of the TFC procedure: (a) loading step; (b) transfer step; (c) cleanup and equilibration steps.

Cyclone P (50 × 0.5 mm, 60 μm particle size, 60 Å pore size) from Thermo Fisher Scientific (Franklin, MA, USA). The chromatographic separation was carried out using an LC system, Transcend 600 (Thermo Fisher Scientific, San Jose, CA, USA), equipped with an analytical column Hypersil GOLD aQ C18 column (100 × 2.1 mm, 1.7 μm particle size) from Thermo Fisher Scientific (San Jose, CA, USA). The LC system was coupled to a benchtop single stage Orbitrap mass spectrometer, Exactive (Thermo Fisher Scientific, Bremen, Germany), operating with a heated electrospray interface (HESI-II; Thermo Fisher Scientific, San Jose, CA, USA).

All data were evaluated and processed by Qual Browser and Quan Browser included in Xcalibur 2.1.1 software (Thermo Fisher Scientific, Les Ulis, France).

A vortex mixer (model WX, Velp Scientifica, Usmate, Italy), a pH-meter equipped with a combined AgCl–glass electrode assembly (Crison, Barcelona, Spain), and an analytical balance (AB204-S, Mettler Toledo, Greifensee, Switzerland) were used.

Honey Samples. Different types of honey samples were purchased from several local markets in Almeria (Spain), and one was obtained from a local beekeeper. All samples were stored at room temperature in the dark. A multiflower honey sample was used for all optimization procedures and validation purposes after it was verified to be free of the selected VDs.

Sample Preparation. Honey sample (1.0 g) was weighed in a 15 mL polypropylene tube and introduced into a heated water bath (~50 °C) to decrease its viscosity and facilitate its dilution with 1 mL of 0.1 M Na_2EDTA (pH 4). The mixture was shaken in a vortex to homogenize it, and then it was filtered through a Millex-GN nylon filter (0.20 μm, Millipore, Carrightwohill, Ireland) directly into a vial prior to online extraction procedure.

Online Sample Extraction by TFC. The TFC procedure can be divided into four general steps: (1) loading, (2) transfer, (3) cleaning, and (4) equilibration. The solvents employed in the TFC procedure were an aqueous solution of formic acid (0.05%, v/v, solvent A); methanol/acetonitrile (1:1, v/v) with Na_2EDTA, 0.1% (solvent B); ammonium formate 10 mM (solvent C); and acetonitrile/acetone/2-propanol (4:3:3, v/v/v, solvent D). A summary of all parameters of each step is listed in Table 1. For the loading step (1) (stage 1 in Table 1), 10 μL of the homogenized sample was injected into the TFC column under TF conditions. The matrix components were washed out using solvent C (2 mL/min), whereas the analytes were retained on the TFC column. During this step (1.5 min), the TFC column and the analytical column were not connected, and the matrix components were flushed to the waste (Figure 1a). Then, during the transfer step (2) (stage 2 in Table 1), the six-port valve was switched and the TFC column was placed online with the UHPLC-Orbitrap-MS system, and the analytes were transferred to the analytical column (Figure 1b). In this transfer step, the analytes were eluted from the TFC column to the analytical column using the solvent contained in the elution loop (100 μL of solvent B), which was pushed by an aqueous solution of formic acid, 0.05% v/v (solvent A, 0.1 mL/min for 1.5 min). Once the analytes were transferred to the head of the analytical column, they

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were eluted by applying the gradient shown in Table 1 (stages 3−8). During the analytical separation, the TFC column was washed sequentially with solvent B and solvent D, at 2 mL/min (stages 3−7 in Table 1). Concurrently, the elution loop (100 μL) was filled again with solvent B for the next injection (stage 8 in Table 1). Finally, the TFC-UHPLC-Orbitrap-MS system was equilibrated for the next run during 3 min (stages 9 and 10 in Table 1). During the cleaning and equilibration steps, the TFC column and the analytical column were not connected (Figure 1c).

UHPLC-Orbitrap-MS Analysis. The chromatographic separation was carried out employing 4 mM ammonium formate aqueous solution acidified with 0.1% formic acid (eluent E) and 4 mM ammonium formate in methanol acidified with 0.1% formic acid (eluent F). The elution was carried out as described in Table 1 (stages 3−11), including cleaning and equilibration steps. The total run time of the chromatographic separation was 10.5 min (13.5 min, including TFC extraction). The temperature of the analytical column was set at 30 °C.

The detection was carried out in positive ionization mode (ESI+) using the following operational parameters: spray voltage, 4 kV; sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); skimmer voltage, 18 V; tube lens voltage, 95 V; heater temperature, 305 °C; and capillary temperature, 300 °C. The full scan spectra were acquired using an acquisition function as follows: resolution, high (equivalent to a mass resolving power of 50000 fwhm at m/z 200); automatic gain control (AGC), balance target value of 1×10^6; and scan speed, 2 Hz. The mass range of the full mass scan positive ion mode was m/z 150−2000 for detection acquisition. For identification purposes, ion fragments were produced in the higher energy collision-induced dissociation cell (HCD), operating with N_2 (>95%) and employing a collision energy of 30 eV (scans from m/z 90 to 1000). The HCD acquisition event had the same values of resolution, AGC, balance target value, and scan speed that were employed for full scan MS spectra. Full scan MS spectra and fragments obtained by HCD spectra were therefore acquired in the same injection. All of the analyses were performed without lock mass. The Orbitrap was calibrated once a week by using a mixture of caffeine, MRFA peptide, and Ultramark 1600. Data were acquired using external calibration mode. Data acquisition and processing were carried out using Xcalibur version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Qual and Quan Browser. Genesis peak detection was applied.

Method Validation. The method was validated in terms of linearity, trueness, repeatability, interday precision, limits of quantification (LOQs), and limits of identification (LOIs). Herein, we observed the need for defining LOQs and LOIs considering that the detection/identification process in Exactive-Orbitrap is different from that in typical QqQ instruments. LOQs were established considering the minimum concentration to determine adequately the base peak (normally $[M + H]^+$). LOIs were established as the minimum concentration in which identification using fragments or isotopic patterns was possible regarding the established criteria in the Commission Decision 2002/657/EC.[36]

Linearity was assayed by spiking honey samples with the target compounds at six concentrations, ranging from 5 to 250 μg/kg. Calibration curves were obtained by least-squares linear regression analysis of the peak area versus concentration.

Trueness was evaluated through recovery studies at three concentration levels (5, 10, and 50 μg/kg) using spiked blank honey samples (n = 7). Repeatability (intraday precision) and interday precision (evaluated in consecutive days) were evaluated at the same concentration levels (n = 5).

LOQs were estimated by analyzing matrix-matched standards at the same levels of concentration used in the linearity study, and they were defined as the lowest concentration to which the method was linear. LOIs were estimated by analyzing spiked honey samples at a wide range of concentration levels (from 0.1 to 50 μg/kg). They were calculated as the minimum concentration for which the accurate mass error was <5 ppm for the characteristic ion of the selected compounds, $[M + H]^+$ or $[M + Na]^+$, and one fragment ion, and the experimental isotopic pattern matched with the theoretical isotopic pattern for these ions.

Taking into account that erythromycin suffers a rapid degradation in acidic aqueous solutions such as honey at high temperatures,[37] we decided to monitor both erythromycin and anhydroerythromycin (main transformation product of erythromycin in honey) in each sample employed for determination of validation parameters.

RESULTS AND DISCUSSION

Method Development. Before the application of the TFC procedure, a manual sample preparation involving dilution and filtration of the sample was performed. Bearing in mind the different properties of the target VDs, such as the bond of sulfonamides with sugars,[15] the instability of macrolides at acidic conditions,[38] or the high affinity for the cations showed by macrolides and tetracyclines,[39] we used a Na_2EDTA solution (0.1 M) at pH 4 to dissolve honey samples.[21] Although acid hydrolysis can improve liberation of sulfonamides bound to sugars, macrolides are unstable in acidic solutions (pH >4).[10] Different Na_2EDTA volumes were investigated, and 1 mL of Na_2EDTA solution was the best volume to dissolve 1 g of honey. To facilitate the dissolution of the samples, it was necessary to introduce them in a heated water bath at 50 °C. Finally, the diluted samples were filtered to be collected in a vial and injected into the TFC system.

The optimization of the TFC procedure was developed by employing spiked blank honey samples at 500 μg/kg. For the optimization of the TFC procedure, it was necessary to elute the analytes directly from the TFC column to the detector and, consequently, the UHPLC system was bypassed. As aforementioned, the TFC extraction consists of four steps (loading, transfer, cleaning, and equilibration), and several parameters need to be optimized. The number of stages, mobile phase composition, flow rate, and time of each step were evaluated to allow the retention of the compounds during the loading step, their transfer from the TFC column to the analytical column, and the cleanup and equilibration of the TFC system.

Optimization of Loading and Transfer Steps. For the optimization of this procedure the analytes should elute directly from the TFC column to the analyzer to check that all of the compounds were retained in the TFC column during the loading step and that they were eluted during the transfer step. In addition, no peaks should be observed in the cleaning step, indicating that all of the compounds were totally eluted during the transfer step. To carry this out, loading and transfer time were optimized using an aqueous solution of ammonium formate 10 mM in the loading step[30] and a mixture of methanol/acetonitrile (1:1, v/v) in the transfer step.[21] The tested times for each step were 1.0 and 1.5 min, observing that 1.5 min was necessary to totally load the sample onto the TFC column (data not shown). When 1 min was used as loading time, the analytes were not completely loaded into the column (intense peak at 0.20 min), and as a consequence, 1.5 min was required. It can be noted that when low loading and/or transfer times were used, the analytes were not properly retained in the TFC column or the analytes did not easily elute from the TFC column to the analytical column.

After that, the mobile phase composition of the loading and transfer steps was optimized. During the loading step, an aqueous solution of ammonium formate 10 mM (solvent C) was initially employed because it was reported as adequate solvent to load the analytes and remove honey matrix.[30] A mixture of 2-propanol/acetone (1:1, v/v)[33] was also tested as

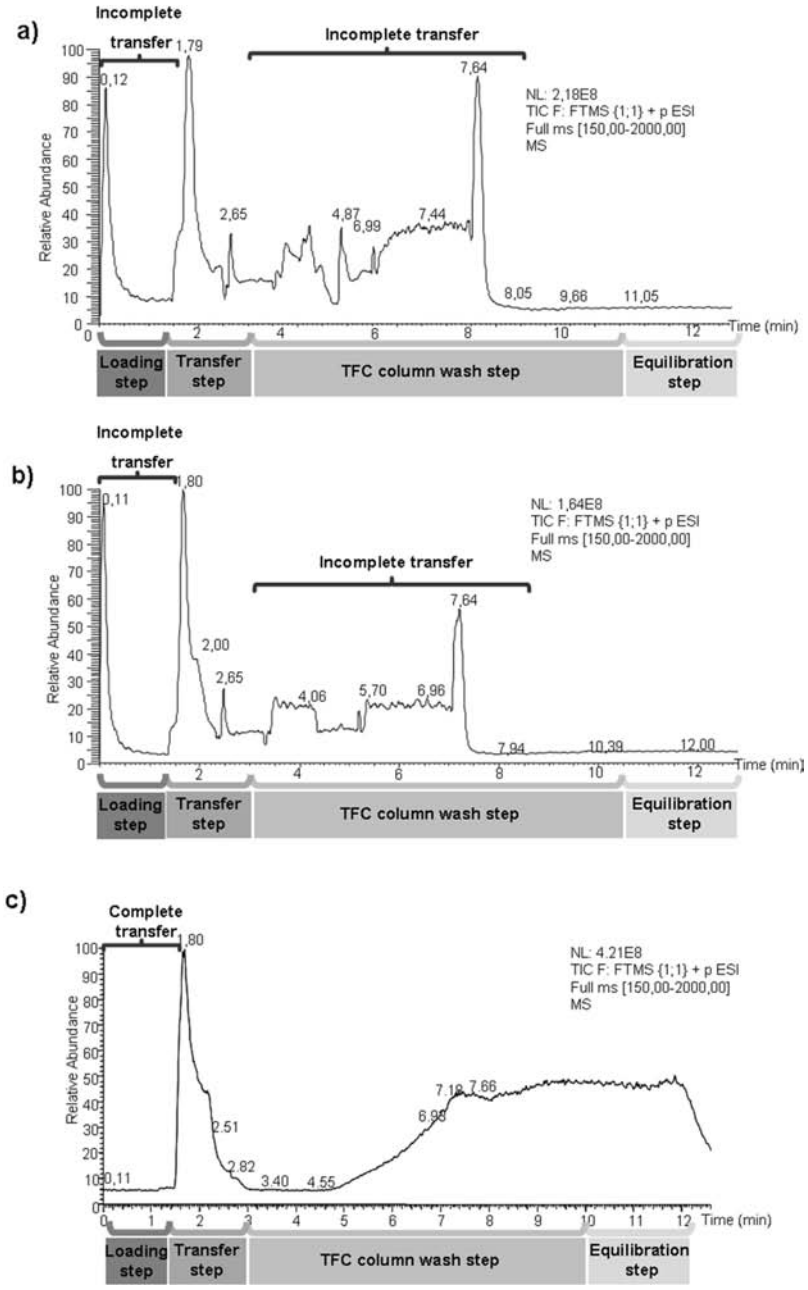

Figure 2. MS chromatograms obtained from a blank honey sample spiked at 500 μg/kg during the optimization of the solvent employed in the transfer step setting 1.5 min in the loading step and 1.5 min in the transfer step: (A) methanol at 0.1% of Na_2EDTA solution (pH 4, 0.1 M); (B) a mixture of methanol/acetonitrile (1:1, v/v); (C) a mixture of methanol/acetonitrile (1:1, v/v) with 0.1% of Na_2EDTA solution (pH 4, 0.1 M).

solvent C, but ammonium formate 10 mM was indeed more efficient in carrying out the loading step. For the analyte transfer, different organic compositions to fill in the 100 μL loop were tested: methanol/acetonitrile (1:1, v/v), methanol with 0.1% of Na_2EDTA solution (pH 4, 0.1 M), and methanol/acetonitrile (1:1, v/v) with 0.1% of Na_2EDTA solution (pH 4, 0.1 M) (Figure 2), observing that the latter mixture was the most suitable, as solvent B, to perform the transfer stage (Figure 2c). The other evaluated solvents generated an incomplete transfer of the target compounds (Figure 2a,b). Because it is possible to add pushing solvent (solvent A) in the loop, this option was also studied, and different percentages of solvents A and B were tested to fill in the loop (from 50 to 100% of solvent B); the best results were obtained when 100% of solvent B was used. This can be explained considering that this percentage of solvent B allowed the elution of all analytes during the transfer step and no residual signal of the analytes was observed in the following step. Finally, the flow rate during the transfer step was also evaluated, testing flow rates from 0.1 to 0.5 mL/min and obtaining the best results at 0.1 mL/min (data not shown).

Optimization of the Cleaning Step. The number of stages to carry out the cleaning of the system was carefully optimized. For this aim, two different solutions were tested as solvent D: (i) an aqueous solution containing 2% of acetonitrile and 0.1% of ammonium hydroxide and (ii) an organic solution of acetonitrile/2-propanol/acetone (4:3:3, v/v/v). Finally, this strong mixture was employed to wash the TFC column and avoid carry-over (solvent D). The optimized number of stages to wash the TFC system is shown in Table 1, indicating that

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Table 2. Retention Time Windows (RTWs) and *m/z* Ions Selected for the Identification and Confirmation of the Target Compounds in the Orbitrap System[a]

compound	family[b]	RTW (min)	elemental compos	theor mass (*m/z*)	mass error (ppm)	elemental compos (fragment 1)	theor mass (*m/z*)	elemental compos (fragment 2)	theor mass (*m/z*)
sulfadiazine	SF	3.90−4.00	$C_{10}H_{11}O_2N_4S$[c]	251.05972	0.3	$C_6H_6O_2NS$	156.01138	NA[d]	NA
sulfathiazole	SF	6.38−6.64	$C_9H_{10}N_3O_2S_2$	256.02089	−0.1	$C_6H_6O_2NS$	156.01138	NA	NA
levamisole	IMT	6.75−7.15	$C_{11}H_{13}N_2S$	205.07940	0.7	$C_{10}H_{12}NS$	178.06850	C_7H_7S	123.02630
benzathine	P	6.89−7.03	$C_{16}H_{21}N_2$	241.16992	0.3	$C_9H_{11}N$	133.08860	NA	NA
marbofloxacin	Q	7.16−7.46	$C_{17}H_{20}FN_4O_4$	363.14631	−0.4	$C_{17}H_{18}O_3N_4F$	345.13574	$C_{16}H_{21}O_2N_4F$	320.16431
sulfadimidine	SF	7.28−7.41	$C_{12}H_{15}O_2N_4S$	279.09102	0.2	$C_6H_6O_2NS$	156.01138	NA	NA
ampicillin	P	7.34−7.68	$C_{16}H_{20}N_3O_4S$	350.11690	0.6	$C_6H_{10}O_2NS$	160.04267	$C_{10}H_{11}O_2N_2$	191.08150
thiabendazole	BNZ	7.40−7.66	$C_{10}H_8N_3S$	202.04334	0.4	$C_9H_7N_2S$	175.03245	$C_8H_7N_2$	131.06037
tetracycline	TC	7.56−7.74	$C_{22}H_{25}N_2O_8$	445.16054	−0.1	$C_{22}H_{20}O_7N$	410.12343	$C_7H_8O_3N$	154.04987
oxytetracycline	TC	7.56−7.74	$C_{22}H_{25}N_2O_9$	461.15546	−0.5	$C_{22}H_{20}O_8N$	426.11834	$C_7H_8O_3N$	154.04987
sulfachlorpyridazine	SF	7.59−7.74	$C_{10}H_{10}ClN_4O_2S$	285.02075	−0.2	$C_6H_6O_2NS$	156.01138	NA	NA
enrofloxacin	Q	7.67−7.76	$C_{19}H_{23}FN_3O_3$	360.17180	−0.9	$C_{18}H_{23}ON_3F$	316.18197	$C_{19}H_{21}O_2N_3F$	342.16123
danofloxacin	Q	7.69−7.77	$C_{19}H_{21}FN_3O_3$	358.15615	0.3	$C_{19}H_{19}O_2N_3F$	340.14558	$C_{18}H_{21}ON_3F$	314.16632
difloxacin	Q	7.88−7.91	$C_{21}H_{20}F_2N_3O_3$	400.14672	−0.3	$C_{20}H_{20}ON_3F_2$	356.15690	$C_{21}H_{18}O_2N_3F_2$	382.13616
sarafloxacin	Q	7.98−7.98	$C_{20}H_{18}F_2N_3O_3$	386.13107	−0.3	$C_{20}H_{16}O_2F_2N_3$	368.12051	$C_{19}H_{18}ON_3F_2$	342.14125
neomycin	AGL	8.19−8.21	$C_{23}H_{47}O_{13}N_6$	615.31956	−4.5	$C_{17}H_{35}O_{10}N_4$	455.23477	$C_6H_{13}O_3N_2$	161.09207
chlorotetracycline	TC	8.33−8.40	$C_{22}H_{24}ClN_2O_8$	479.12157	−0.2	$C_{22}H_{19}O_7NCl$	444.08446	$C_7H_8O_3N$	154.04987
sulfadimethoxine	SF	8.37−8.37	$C_{12}H_{15}N_4O_4S$	311.08085	−0.1	$C_6H_{10}O_2N_3$	156.07675	C_6H_6ON	108.04439
oxalinic acid	Q	8.40−8.48	$C_{13}H_{12}NO_5$	262.07100	−0.2	$C_{13}H_{10}O_4N$	244.06043	$C_{10}H_6O_3N$	188.03422
sulfaquinoxaline	SF	8.41−8.48	$C_{14}H_{13}N_4O_2S$	301.07537	−0.4	$C_6H_6O_2NS$	156.01138	C_6H_6ON	108.044339
doxycycline	TC	8.59−8.62	$C_{22}H_{25}O_8N_2$	445.16054	0.1	$C_{22}H_{22}O_8N$	428.13399	$C_7H_8O_3N$	154.04987
oxfendazole	BNZ	8.63−8.66	$C_{15}H_{14}N_3O_3S$	316.07504	−0.1	$C_8H_5O_3N_3$	191.03254	$C_{14}H_{10}O_2N_3S$	284.04882
tilmicosin	M	8.63−8.66	$C_{46}H_{81}N_2O_{13}$	869.57332	−1.0	$C_{38}H_{66}O_{10}N$	696.46812	$C_{30}H_{52}O_6N$	522.37891
penicillin G	P	8.70−8.73	$C_{16}H_{19}N_2O_4S$	335.10600	−0.1	$C_{10}H_{10}O_2N$	176.07060	$C_6H_{10}NO_2S$	160.04267
oxacillin	P	8.71−9.06	$C_{19}H_{20}N_3O_5S$	402.11182	0.1	C_9H_6NO	144.04439	NA	NA
abamectin	AV	8.79−8.84	$C_{48}H_{72}O_{14}Na$[e]	895.48143	−1.2	$C_{13}H_{21}O$	193.15963	NA	NA
tylosin	M	8.81−8.84	$C_{46}H_{78}NO_{17}$	916.52643	−0.2	$C_{18}H_{28}O_6N$	318.19110	$C_{39}H_{66}O_{14}N$	772.44778
flumequine	Q	8.83−8.86	$C_{14}H_{13}FNO_3$	262.08740	−0.1	$C_{11}H_7O_3NF$	220.04045	$C_{14}H_{11}O_2NF$	244.07683
penicillin V	P	8.85−8.88	$C_{16}H_{19}N_2O_5S$	351.10092	0.1	$C_{10}H_8NO_3$	190.04987	$C_6H_{10}NO_2S$	160.042675
cloxacillin	P	8.89−8.92	$C_{19}H_{19}ClN_3O_5S$	436.07285	−0.1	$C_{14}H_{12}ClN_2O_2$	277.03745	C_9H_5ClNO	178.00542
erythromycin	M	8.91−8.94	$C_{37}H_{68}NO_{13}$	734.46852	−0.1	$C_8H_{16}O_2N$	158.11756	$C_{29}H_{54}O_{10}N$	576.37422
anhydroerythromycin A	M	8.98−9.01[f]	$C_{37}H_{66}NO_{12}$	716.45795	−0.9[f]	$C_8H_{16}O_2N$	158.11756	NA	NA
mebendazole	BNZ	8.93−8.96	$C_{16}H_{14}N_3O_3$	296.10297	0.2	$C_{15}H_{10}O_2N_3$	264.07675	C_7H_5O	105.03349
griseofulvin	P	8.95−8.98	$C_{17}H_{18}ClO_6$	353.07864	0.4	NA	NA	NA	NA
emamectin	AV	8.96−9.01	$C_{49}H_{76}NO_{13}$	886.53894	−0.1	$C_8H_{16}O_2N$	158.11756	$C_7H_{12}ON$	126.09134
dicloxacillin	P	8.96−9.02	$C_{19}H_{18}Cl_2N_3O_5S$	470.03387	0.4	$C_{13}H_9N_2Cl_2O_3$	310.99847	$C_6H_{10}NO_2S$	160.04267
josamycin	M	9.02−9.05	$C_{42}H_{70}NO_{15}$	828.47400	−0.1	$C_8H_{16}O_3N$	174.11247	$C_{12}H_{21}O_4$	229.14344
albendazole	BNZ	9.14−9.16	$C_{12}H_{16}N_3O_2S$	266.09577	0.4	$C_{11}H_{12}ON_3S$	234.06956	$C_8H_5ON_3S$	191.01478
fenbendazole	BNZ	9.34−9.36	$C_{15}H_{14}N_3O_2S$	300.08012	0.4	$C_{14}H_{10}ON_3S$	268.05391	$C_8H_5ON_3$	159.04271
ivermectin	AV	9.89−9.94	$C_{48}H_{78}O_{14}N$[g]	892.54168	−1.9	NA	NA	NA	NA

[a]Experimental data obtained when a solvent-based standard mixture of studied VDs at 250 μg/kg was monitored (n = 10). [b]Family code: aminoglycosides (AGL), avermectins (AV), benzimidazoles (BNZ), imidazothiazoles (IMT), macrolides (M), penicillins (P), quinolones (Q), sulfonamides (SF), and tetracyclines (TC). [c]Elemental composition corresponding to $[M + H]^+$. [d]NA, not applicable. [e]Elemental composition corresponding to $[M + Na]^+$. [f]Experimental data obtained when a spiked honey sample at 250 μg/kg was monitored (n = 10). [g]Elemental composition corresponding to $[M + NH_4]^+$.

while the LC gradient is running, the TFC column is being cleaned.

Finally, it is important to highlight that TFC was needed to inject the samples into the UHPLC-Orbitrap without any sample pretreatment due to the fact that the direct injection of the samples into the LC-MS system (without coupling to the TFC system) provoked several problems with the analyzer, such as the capillary was blocked and it was not possible to operate with the LC-MS normally. Moreover, samples could not be injected into the UHPLC-Orbitrap system as long as they were not subjected to a cleanup step to remove sugars and other matrix components, in this case, in the TFC stage. As a consequence, this online cleanup system is an easy and straightforward way to analyze these types of samples without previous offline sample handling.

UHPLC-Orbitrap-MS. The general conditions for the ion source and ion transmission employed in our previous method[40] were evaluated for the new compounds included in this study. A solvent standard solution of VDs at 100 μg/L was infused to optimize the source conditions (see UHPLC-Orbitrap-MS Analysis).

In relation to the chromatographic separation, generic eluents were used,[41] considering that the selected compounds belong to several families with different properties. Bearing in mind that the chromatographic gradient has to be synchronized with the cleaning, loop filling, and re-equilibration steps of the TFC system, different gradient profiles were assayed to provide

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good peak shape. All of the compounds were properly eluted using the gradient profile indicated in Table 1. The optimized conditions (see UHPLC-Orbitrap-MS Analysis) provided reproducible retention times (RTs), which ranged from 3.95 min (sulfadiazine) to 9.89 min (ivermectin) (Table 2). The total running time, including online TFC extraction (3.0 min) and chromatographic separation of VDs (10.5 min), was 13.5 min.

The detection of the target compounds was based on the retention time window (RTW) and the measurement of the accurate mass (mass error < 5 ppm) for each compound as well as the isotopic pattern of each ion. Most of the VDs included in this study do not have chlorine atoms or other characteristic atoms useful for the evaluation of the isotopic pattern. Therefore, the relative intensities of the A + 1 isotope peak (A being the corresponding $[M + H]^+$ or $[M + Na]^+$ for abamectin and $[M + NH_4]^+$ for ivermectin), which is mainly due to the presence of ^{13}C, and A + 2 obtained for ^{34}S were considered. However, for sulfachlorpyridazine (1 chlorine and 1 sulfur atom), chlorotetracycline, griseofulvin, and cloxacillin (1 chlorine atom), the relative intensity of the A + 2 isotope peak was used for their detection due to ^{37}Cl.

The identification of the VDs was carried out by the HCD acquisition event of the MS method using the generated fragments and calculating their corresponding mass error. Table 2 shows the elemental composition and theoretical and experimental masses of the monitored ion fragment (except for ivermectin, for which fragments were not found).

Finally, Figure 3 shows some compounds extracted from a honey sample spiked at 25 μg/kg with the optimized TFC method.

Validation of the Optimized Methodology. Two different types of honey (multifloral and forest) were selected for the evaluation of matrix effect. Several concentrations (from 5 to 250 μg/kg) were analyzed in solvent and in each type of honey, and the slopes of the calibration curves were compared. A strong matrix effect was observed for the majority of compounds (mainly ion suppression). However, no significant differences were found between the slopes of multifloral and forest honey curves. Therefore, multifloral honey was selected as representative matrix to carry out the validation procedure to multifloral and forest honey (data not shown).

As a consequence, calibration curves were performed using matrix-matched standards. Good linearity was obtained when peak area was used as the analytical response, and determination coefficients (R^2) were >0.9800 (except for cloxacillin and ivermectin). Table 3 shows the R^2 value and linear range for each compound.

Apart from matrix effect, memory effect in the TFC system was also evaluated considering the analyte area at the lowest calibration level and the analyte area, if present, in a methanol injection performed after a standard injection. Memory effect is considered if analyte area in the methanol injection is >10% of the analyte area in the standard. Using the conditions optimized previously, no memory effect was observed.

The recovery values were obtained by comparison of the areas of the spiked samples with the area of the single-point matrix-matched calibration levels.[30] Taking into account the absence of MRLs established by the EU and the zero-tolerance policy of VD residues in honey, recovery was evaluated at the lowest concentration,[25] being in this case 5 μg/kg, which is smaller than the values used for VDs in other multiclass methods developed for honey samples.[21−24] In Table 3, it can

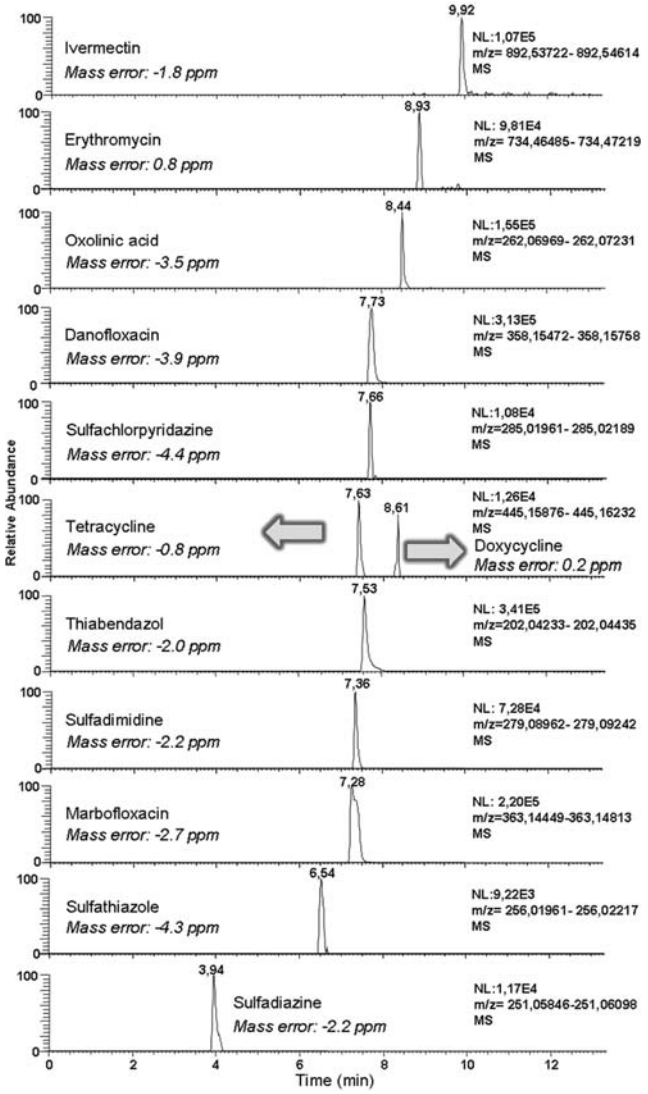

Figure 3. Chromatograms of some veterinary drugs detected in a honey sample spiked at 25 μg/kg analyzed by the optimized TFC-UHPLC-Orbitrap-MS method.

be observed that 15 compounds showed recoveries from 87% (tetracycline) to 121% (flumequine) at 5 μg/kg, except benzathine (68%). At 10 μg/kg, 20 compounds showed recoveries from 75% (doxycycline) to 116% (benzathine, oxytetracycline, and josamycin), and at 50 μg/kg, 34 compounds showed recoveries from 72% (sulfachlorpyridazine) to 120% (sulfadiazine), except sulfaquinoxalin (68%). Repeatability and interday precision values (expressed as relative standard derivation, RSD, %) obtained for each concentration level are shown in Table 3. It can be observed that repeatability and interday precision values were always ≤25% for the majority of compounds.

The LOIs and LOQs obtained for the studied VDs (Table 3) were estimated according to the criteria indicated previously (see Method Validation). In this case, when the identification by fragments and isotopic pattern was evaluated, sensitivity problems were observed at low concentrations. Ion fragments and other ions in isotopic clusters showed lower intensities than the $[M + H]^+$ used in the establishment of the LOQ. For instance, sensitivity problems were detected at the lowest assayed concentration (e.g., 5 μg/kg) for some compounds,

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Table 3. Validation Parameters of the Target Compounds Using the Optimized TFC-UHPLC-Orbitrap-MS Method

	calibration curve		recovery[a]			interday precision[b]				
compound	linear range (μg/kg)	R^2	5 μg/kg	10 μg/kg	50 μg/kg	5 μg/kg	10 μg/kg	50 μg/kg	LOQ (μg/kg)	LOI (μg/kg)
sulfadiazine	25−250	0.9995	NA[c]	NA	120 (2)	NA	NA	13	25	25
sulfathiazole	25−250	0.9929	NA	NA	97 (5)	NA	NA	20	25	25
levamisole	5−250	0.9989	95 (2)	103 (4)	98 (2)	10	6	5	5	0.5
benzathine	5−250	0.9997	68 (17)	116 (15)	98 (16)	25	20	13	5	5
marbofloxacin	5−250	0.9994	95 (9)	100 (2)	107 (1)	11	9	10	5	0.5
sulfadimidine	10−250	0.9976	NA	84 (5)	92 (2)	NA	18	2	10	10
ampicillin	25−250	0.9994	NA	NA	108 (17)	NA	NA	17	25	25
thiabendazole	5−250	0.9967	118 (16)	104 (3)	98 (2)	20	12	10	5	0.1
tetracycline	5−250	0.9997	87 (25)	104 (9)	97 (11)	25	14	12	5	5
oxytetracycline	5−250	0.9990	114 (25)	116 (11)	115 (7)	NA	15	14	5	5
sulfachlorpyridazine	25−250	0.9932	NA	NA	72 (20)	NA	NA	18	25	25
enrofloxacin	5−250	0.9988	94 (5)	97 (4)	98 (2)	7	6	5	5	0.1
danofloxacin	5−250	0.9985	95 (7)	102 (2)	99 (2)	8	8	6	5	0.1
difloxacin	5−250	0.9990	96 (2)	100 (2)	98 (1)	9	6	6	5	0.1
sarafloxacin	5−250	0.9994	99 (10)	107 (5)	96 (4)	8	11	9	5	0.1
chlorotetracycline	25−250	0.9999	NA	NA	82 (18)	NA	NA	17	25	50
sulfadimethoxine	50−250	1.0000	NA	NA	91 (8)	NA	NA	12	50	50
oxolinic acid	5−250	0.9982	117 (20)	102 (2)	104 (16)	23	19	15	5	5
sulfaquinoxaline	25−250	0.9975	NA	NA	68 (1)	NA	NA	16	25	25
doxycycline	10−250	0.9970	NA	75 (25)	93 (10)	NA	3	11	10	10
oxfendazole	25−250	0.9931	NA	NA	103 (6)	NA	NA	21	25	50
tilmicosin	10−250	0.9981	NA	101 (7)	97 (2)	NA	4	9	10	10
penicillin G	25−250	0.9947	NA	NA	98 (9)	NA	42	9	25	25
oxacillin	50−250	0.9860	NA	NA	100 (4)	NA	NA	5	50	50
tylosin	5−250	0.9999	102 (5)	110 (4)	98 (2)	13	16	9	5	10
flumequine	5−250	0.9992	121 (17)	111 (9)	108 (13)	29	19	19	5	0.5
penicillin V	10−250	0.9991	NA	<60 (34)	73 (8)	NA	24	21	10	10
cloxacillin	50−250	0.8637	NA	NA	104 (6)	NA	NA	6	50	50
erythromycin	10−250	0.9990	NA	102 (23)	89 (6)	NA	28	13	10	10
mebendazole	25−250	0.9990	NA	NA	117 (8)	NA	NA	21	25	25
griseofulvin	10−250	0.9988	NA	107 (25)	95 (21)	NA	23	21	10	10
josamycin	5−250	0.9998	102 (13)	116 (4)	105 (6)	15	16	6	5	5
albendazole	50−250	0.9646	NA	NA	106 (23)	NA	NA	29	50	50
ivermectin	5−250	0.8068	98 (2)	102 (1)	95 (2)	7	9	5	5	0.1

[a]Repeatability values, expressed as RSD, are shown in parentheses (n = 7). [b]Reproducibility values expressed as RSD. Samples were analyzed on 5 consecutive days. [c]NA, not applicable.

which made difficult adequate identification using the isotopic pattern. Although the procedures to evaluate both lower limits were based on different approaches, it can be noted that they were equal for some compounds. However, other VDs showed different values of these parameters. This can be explained bearing in mind that LOI (as defined here) is mainly related to the capabilities of the method for the reliable identification of the compound, whereas LOQ indicates the minimum concentration of compound that can be quantified using the protonated molecule within a linear range. As it can be seen in Figure 4, following the criteria established to define the LOI, enrofloxacin can be identified at 0.1 μg/kg, because both the $[M + H]^+$ and one fragment (F1) can be monitored at this level. However, the LOQ for this compound is 5 μg/kg, corresponding to the lowest point of the calibrated linear range. On the other hand, some compounds (chlorotetracycline, oxfendazol, and tylosin) have LOIs higher than LOQs. This fact could be explained due to these compounds requiring a higher concentration to be unambiguously identified with at least one fragment apart from the characteristic ion $[M + H]^+$ or $[M + Na]^+$, which was employed to quantify the compounds. As a consequence, although these compounds can be quantified from their LOQ, they cannot be identified at this level. Therefore, these compounds should be quantified from their LOI level. In the case of erythromycin, it was not detected at low concentration levels (<10 μg/kg) because it was degraded as indicated previously (see Method Validation). Therefore, anhydroerythromycin ($C_{37}H_{66}NO_{12}$ m/z 716.4579) and its fragment ($C_8H_{16}O_2N$ m/z 158.1176) were monitored and showed a LOC of 10 μg/kg.

If the proposed method is compared with other published multiclass methods for the determination of VDs in honey,[21−25] it can be seen that the developed method is simpler and faster than previous methods. Moreover, the presented methodology increased sample throughput, because it requires only a manual dilution of the honey sample and the other steps are automated. In relation to the validation results, the developed method allowed the extraction and quantification of some VDs (quinolones, macrolides, benzimidazoles, and some tetracyclines) at low levels (5 μg/kg), improving the results obtained in previous works.[21,24] However, it showed worse results for other families of compounds such as sulfonamides or penicillins, which showed adequate recoveries at 25 μg/kg, which is slightly higher than in other methods.[21,24]

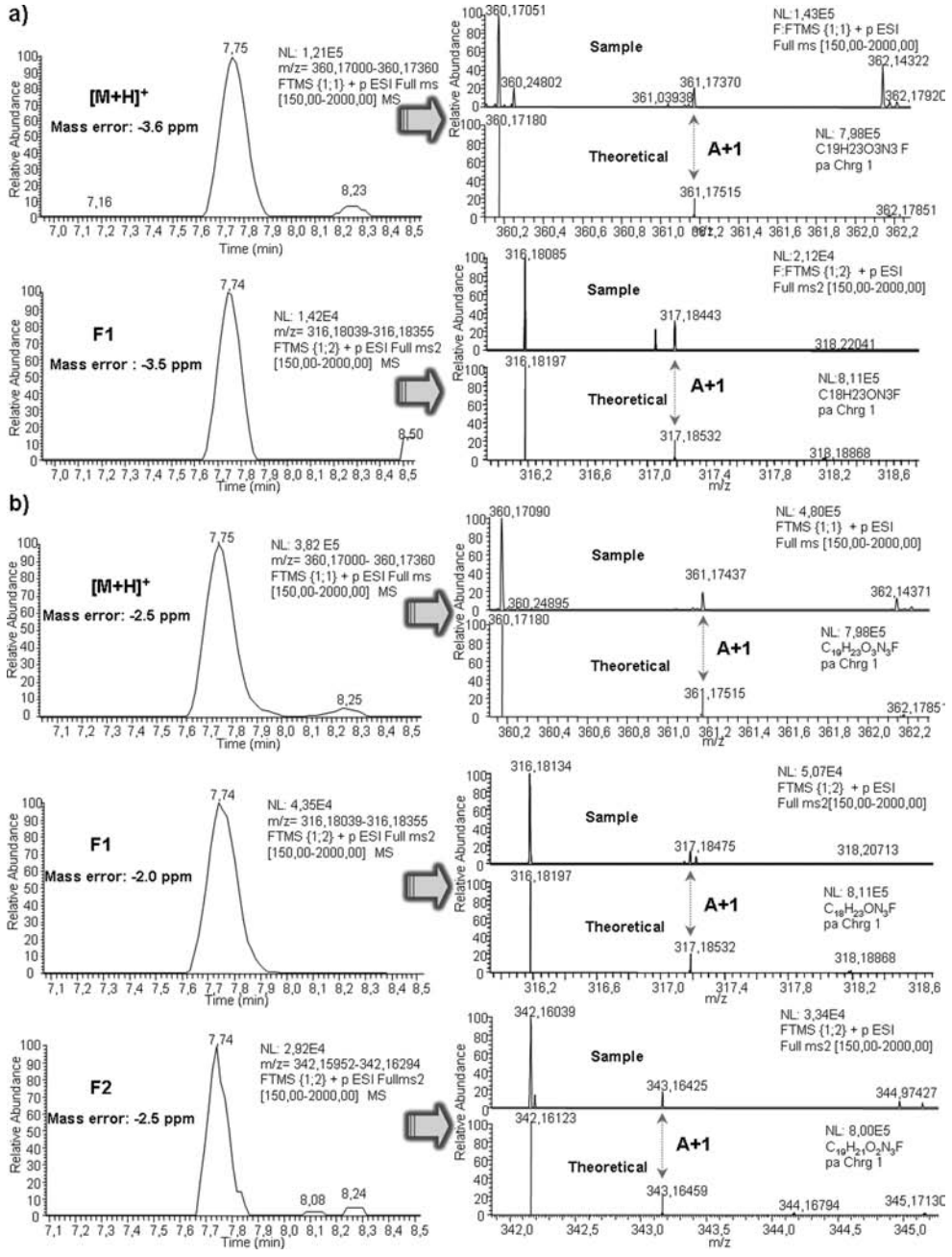

Figure 4. Chromatograms and spectra of enrofloxacin showing the $[M + H]^+$ (m/z 360.17180) and its fragments at (A) the LOI (0.1 μg/kg) and (B) the LOQ (5 μg/kg). F1, fragment 1 (m/z 316.18197).

Analysis of Samples. The optimized method was applied to 15 samples of honey, including 10 floral and multifloral honeys, 2 forest honeys, 2 organic honey samples, and 1 honey sample obtained from a local beekeeper to evaluate the applicability of the method. An internal quality control was carried out for every batch of samples to check if the system is under control, and it employs a matrix-matched calibration, a reagent blank, a matrix blank, and a spiked blank sample at 50 μg/kg.

Five samples contained traces of VDs. Danofloxacin was detected at trace levels (<LOQ) in three samples, and tetracycline was detected in two samples. It was detected at trace levels (<LOQ) in one sample, whereas it was detected at a concentration slightly higher than the LOQ (7 μg/kg) in the other sample.

The results showed the suitability of the proposed method for the detection and quantification of VDs in honey samples at low concentration levels with minimum sample preparation.

In conclusion, a rapid, semiautomated, and quantitative method has been developed and validated for the simultaneous determination of several classes of VDs in honey. The utilization of the TFC system during sample preparation and the use of UHPLC-Orbitrap-MS to carry out the final determination allowed a total analysis time of <14 min (including sample preparation and analytical determination), reducing sample handling. In general, adequate performance characteristics for linearity, trueness, repeatability, interday precision, and lower limits were obtained. Certain sensitivity problems were detected at the lowest assayed concentration (e.g., 5 μg/kg) for some compounds, which made difficult the

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adequate confirmation using the isotopic pattern or fragments. Because of the difficulties in developing a multiclass, generic, rapid, and selective methods for the detection of VDs at low concentrations, this study showed a new approach in semiautomated methodologies for that purpose. Therefore, the proposed method could be applied to the determination and quantification of multicomponent VDs in routine analysis, in which a large number of samples must be analyzed. Despite the variety of compounds that could be determined by the current method, further experiments are needed to include the analysis of aminoglycosides, which could be frequently found in honey samples.

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Notes

The authors declare no competing financial interest.

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