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Quantitative High-Throughput Analysis of 16 (Fluoro)quinolones in Honey Using Automated Extraction by Turbulent Flow Chromatography Coupled to Liquid Chromatography−Tandem Mass Spectrometry

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A method making use of turbulent flow chromatography automated online extraction with tandem mass spectrometry (MS/MS) was developed for the analysis of 4 quinolones and 12 fluoroquinolones in honey. The manual sample preparation was limited to a simple dilution of the honey test portion in water followed by a filtration. The extract was online purified on a large particle size extraction column where the sample matrix was washed away while the analytes were retained. Subsequently, the analytes were eluted from the extraction column onto an analytical column by means of an organic solvent prior to chromatographic separation and MS detection. Validation was performed at three fortification levels (i.e., 5, 20, and 50 μg/kg) in three different honeys (acacia, multiflower, and forest) using the single-point calibration procedure by means of either a 10 or 25 μg/kg calibrant. Good recovery (85−127%, median 101%) as well as within-day (2−18%, median 6%) and between-day (2−42%, median 9%) precision values was obtained whatever the level of fortification and the analyte surveyed. Due to the complexity of the honey matrix and the large variation of the MS/MS transition reaction signals, which were honey-dependent, the limit of quantification for all compounds was arbitrarily set at the lowest fortification level considered during the validation, e.g., 5 μg/kg. This method has been successfully applied in a minisurvey of 34 honeys, showing ciprofloxacin and norfloxacin as the main (fluoro)quinolone antibiotics administered to treat bacterial diseases of bees. Turbulent flow chromatography coupled to LC-MS/MS showed a strong potential as an alternative method compared to those making use of offline sample preparation, in terms of both increasing the analysis throughput and obtaining higher reproducibility linked to automation to ensure the absence of contaminants in honey samples.

KEYWORDS: Honey; quinolone; fluoroquinolone; liquid chromatography−tandem mass spectrometry; LC-MS/MS; turbulent flow chromatography

INTRODUCTION

Quinolones belong to a family of synthetic antibiotics structurally related to nalidixic acid (**Figure 1**), itself being the first quinolone used clinically in animals in the early 1960s. Because of their narrow spectrum of activity and bacteria resistance issues, the original class of quinolones was supplanted in the mid 1980s by a new generation of drugs, still structurally related to nalidixic acid, containing a fluorine covalently bound to the carbon in position 6 and a piperazine ring at the number 7 carbon. These 6-fluoroquinolones were shown to have a much broader spectrum of activity, since they were more effective against Gram-negative bacteria and also moderately effective against Gram-positive bacteria. In animal therapy, quinolones belong to the current arsenal of antibiotics developed to treat various infections and are specifically used for respiratory diseases and enteric bacterial infections in cattle, swine, broiler, and turkey as well as for diseases (septicemia, skin/ulcers) in aqua-cultured fish (*1*). In the European Union (EU), several of these drugs have been regulated, and maximum residue limits (MRLs) have been defined for different food matrices of animal origin, i.e., muscle, liver, kidney, fish flesh, egg, and milk (*2*).

In honey, however, such regulations do not exist for quinolone-based antibiotics. Consequently, the presence of such residues must be considered as resulting from wrong beekeeping practices and are illegal. Honey is not only consumed during breakfast but is also largely used in the food industry (bakery and cereal-based goods, baby foods, chocolate, etc). Indeed, on

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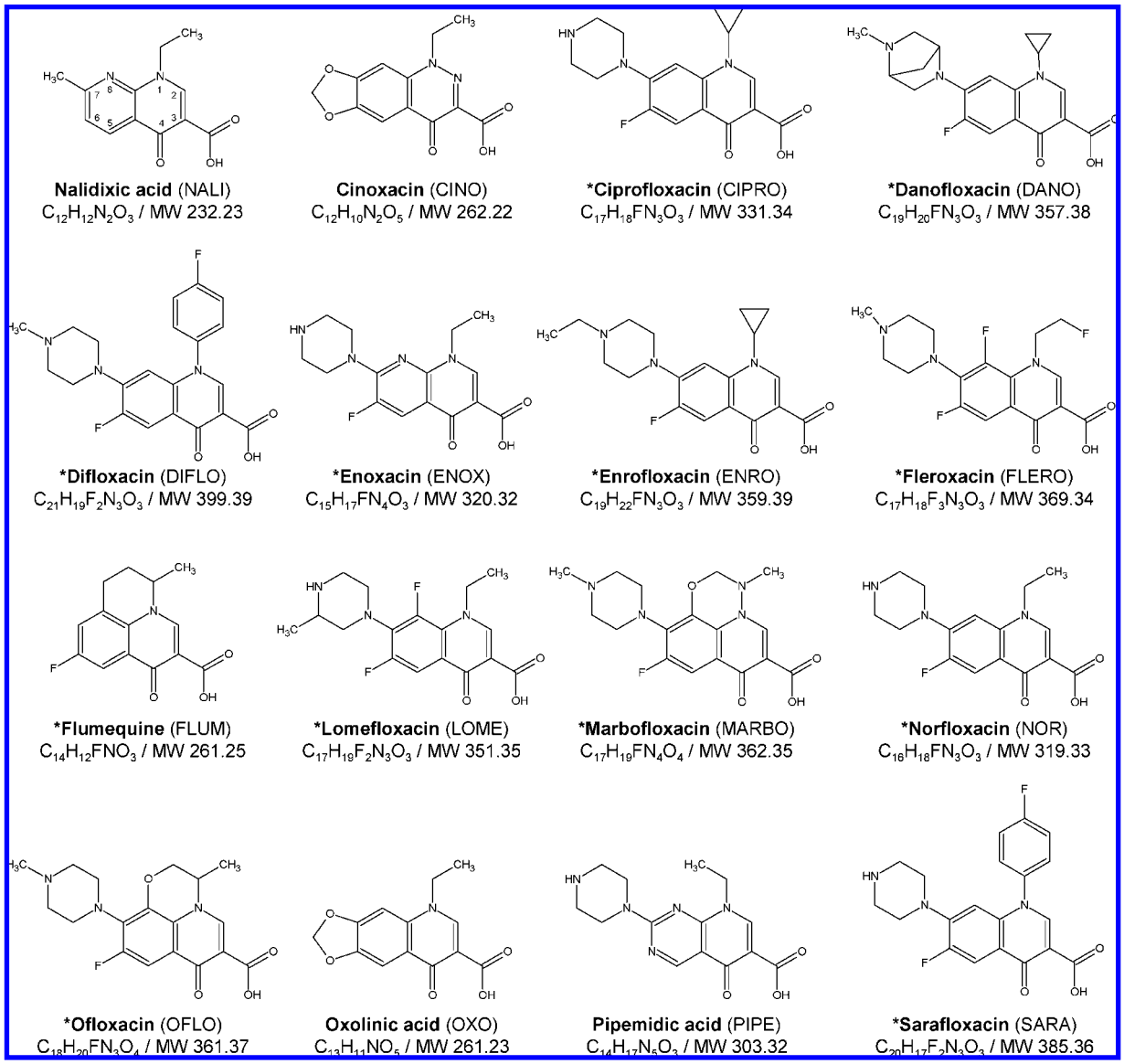


Figure 1. Chemical structures of the 4 quinolones and 12 fluoroquinolones (denoted with asterisks) studied. Nalidixic acid is the progenitor of these compounds.

a yearly basis, about 1.2 million tons of honey is produced worldwide and 400000 tons is traded internationally (*3*). In the last 5 years, the finding of veterinary drug residues (aminoglycosides, tetracyclines, sulfonamides, chloramphenicol, nitrofurans, etc.) in this commodity has had a serious impact on both raw material suppliers and food manufacturers, resulting in rejection and potentially destruction of honey batches. Additionally, this has endangered the image of bee products as natural and clean. Recently, fluoroquinolones (enrofloxacin and its metabolite ciprofloxacin) were found in honey originating from China (*4*), demonstrating that such broad spectrum antibiotics are used by some beekeepers.

Several publications have already reported the analysis of (fluoro)quinolones in foods and have been recently reviewed (*5*). However, none has dealt with the analysis of honey, except that described by Rose et al. (*6*) for the quantification of some (fluoro)quinolones by LC coupled to ultraviolet and fluorescence detection. All reported analytical methods encompass a traditional scheme, i.e., liquid–liquid extraction (LLE) followed by a cleanup/enrichment step using solid-phase extraction (SPE) before final quantification by LC coupled either to ultraviolet, fluorescence, or MS detection. Such methodologies, though efficient at detecting low contamination levels, are time-consuming.

Considering that the global trade of food is continuously expanding with steadily growing numbers of samples to be analyzed for drug residues, the need for analytical procedures allowing high sample throughput has become mandatory. Automation of the sample preparation by means of turbulent flow chromatography (TFC) could be to some extent a way to fulfill this requirement. The concept of TFC was already explored by Pretorius and Smuts (*7*) in the 1960s, but its applicability was only demonstrated in 1997 by Ayrton et al. (*8*), who presented the first TFC tandem mass spectrometry application for the direct analysis of a pharmaceutical compound in plasma, without the requirement of any sample cleanup. The technology is now well established in various clinical and pharmaceutical environments, i.e., for drug discovery, pharmacokinetics, and metabolite profiling (*9–12*), whereas its use in food analysis is scarce and so far has been limited to the analysis of pesticides (*13*) and some antibiotics (*14*) in water. Briefly, TFC makes use of large size particle sorbents which allow high

Table 1. TFC and HPLC Parameters and Plumbing Configuration

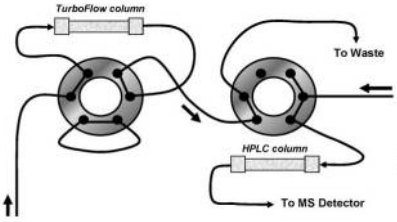
Step No	Time (min)		TFC (loading pumps)						HPLC (eluting pumps)				
	Step Length	Cumulative	Comment	Flow (mL/min)	%A	%B	%C	%D	Comment	Flow (mL/min)	Gradient Mode	%A	%B
1	0.5	0.0	Loading	1.50			100		Conditioning	0.30	Step	100	0
2	4.0	0.5	Transfer	0.05	100				Loading	0.25	Step	100	0
3	3.0	4.5	Cleaning	2.00				100	Elution	0.30	Step	80	20
4	4.0	7.5	Loop filling	1.50		100			Elution	0.30	Ramp	20	80
5	5.0	11.5	Cleaning	1.50		100			Elution	0.30	Ramp	5	95
6	2.0	16.5	Conditioning	1.50	100				Elution	0.30	Ramp	1	99
7	8.0	18.5	Conditioning	1.50			100		Conditioning	0.30	Step	100	0

TFC Conditions (Loading pumps) :

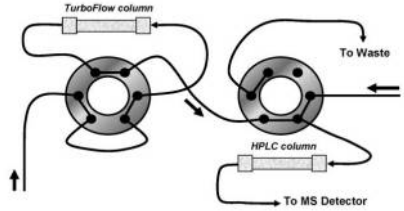
Turbulent flow column : Cyclone HTLC, 50 x 0.5 mm, 60 μm
Injection volume : 4 x 40 μL
Solvent A : 0.1 % formic acid in water
Solvent B : 0.1 % formic acid in acetonitrile
Solvent C : 10 mM ammonium formate in water
Solvent D : acetonitrile/isopropanol/acetone (4:3:3, v/v)

HPLC Conditions (Eluting pumps) :

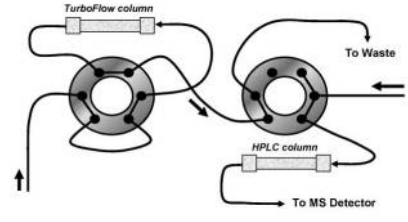
HPLC column : Zorbax SB C_{18}, 50 x 2.1 mm , 1.8 μm
HPLC column temp.: 40 °C
Solvent A : 0.5 % formic acid and 1 mM NFPA in water
Solvent B : 0.5 % formic acid in methanol/acetonitrile (1:1,v/v)



Flow diagram for Steps 1, 5, 6 & 7



Flow diagram for Step 2



Flow diagram for Steps 3 & 4

Table 2. Transition Reactions Monitored by LC-ESI-MS/MS[a] and Their Corresponding Peak Area Ratios

		transition reactions (*m/z*) used for				
		quantification	analyte confirmation			peak area ratios
analyte	RT (min)	SRM1	SRM2	SRM3	DP[b]	rSRM2 and rSRM3[c]
PIPE	9.32	304 → 286 (30)	304 → 217 (32)	304 → 189 (44)	50	0.28 and 0.11
CINO	9.41	263 → 245 (25)	263 → 217 (33)	263 → 189 (39)	40	0.57 and 0.48
MARBO	9.73	363 → 345 (23)	363 → 320 (32)	363 → 277 (31)	58	0.20 and 0.11
ENOX	9.79	321 → 303 (30)	321 → 277 (23)	321 → 257 (28)	50	0.06 and 0.04
FLERO	9.80	370 → 326 (28)	370 → 352 (30)	370 → 269 (38)	58	0.66 and 0.42
OFLO	9.84	362 → 344 (34)	362 → 318 (30)	362 → 261 (47)	56	1.32 and 0.42
NOR	9.85	320 → 302 (32)	320 → 276 (26)	320 → 233 (36)	62	0.13 and 0.04
OXO	9.89	262 → 244 (30)	262 → 216 (42)	262 → 160 (52)	52	0.13 and 0.16
CIPRO	9.94	332 → 314 (31)	332 → 288 (27)	332 → 245 (35)	50	0.15 and 0.06
DANO	9.99	358 → 340 (34)	358 → 314 (26)	358 → 283 (36)	62	0.06 and 0.03
LOME	10.00	352 → 334 (34)	352 → 308 (31)	352 → 251 (34)	76	1.15 and 0.18
ENRO	10.10	360 → 342 (33)	360 → 316 (29)	360 → 245 (41)	64	0.44 and 0.16
DIFLO	10.50	400 → 382 (34)	400 → 356 (28)	400 → 299 (40)	55	0.57 and 0.48
SARA	10.50	386 → 368 (34)	386 → 342 (28)	386 → 299 (38)	45	0.35 and 0.32
NALI	10.60	233 → 215 (24)	233 → 187 (36)	233 → 159 (46)	35	0.93 and 0.40
FLUME	10.70	262 → 244 (31)	262 → 202 (47)	262 → 220 (46)	50	0.44 and 0.03

[a] Collision energies in eV are reported within parentheses. [b] Declustering potential (V). [c] Area ratio of SRM2 and SRM1 and area ratio of SRM3 and SRM1.

solvent flow rates to be considered with moderate back-pressure in the system. The solvent is no longer exhibiting laminar flow, as in conventional HPLC, but behaves in a turbulent manner, leading to the formation of eddies which promote cross-channel mass transfer and control effective diffusion of the solutes. As small molecular weight molecules diffuse more extensively than macromolecules (i.e., proteins, sugars), they are driven into the pores of the packing material while the large compounds, due to the high flow rate, are flushed to waste before they have an opportunity to diffuse into the particle pores. Roughly, TFC can be considered as the simultaneous contribution of size exclusion and chromatographic adsorption. Once trapped onto the TFC column, a back-flush operation using a polar organic solvent allows the analytes to be desorbed and driven to the normal analytical HPLC column for further chromatographic separation and subsequent MS/MS detection.

The present paper describes an online extraction procedure for the determination of 16 (fluoro)quinolones including 4 4-quinolones and 12 6-fluoroquinolones in honey, employing high turbulent flow liquid chromatography−tandem mass spectrometry (HTLC-MS/MS).

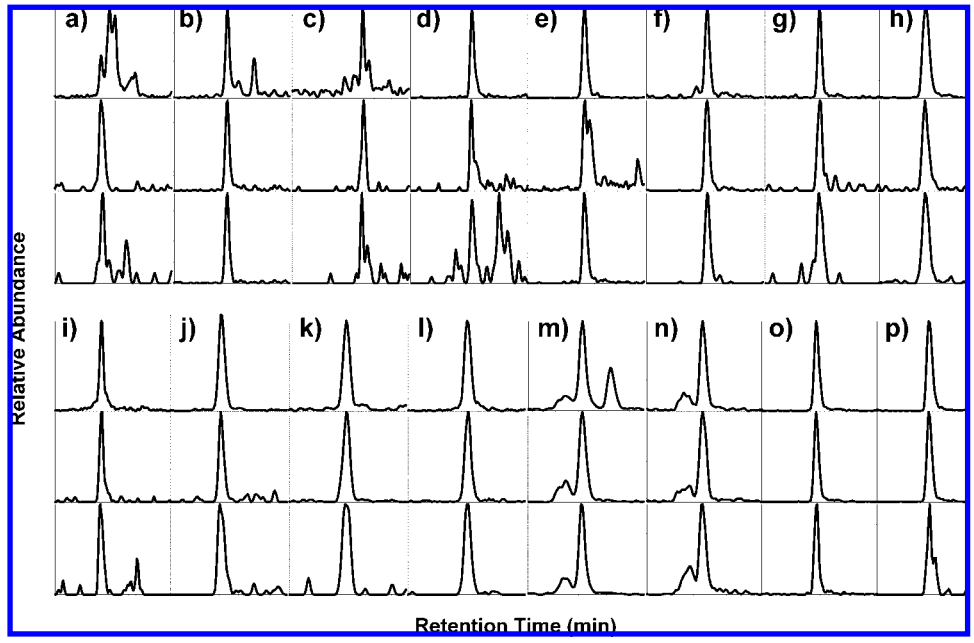


Figure 2. SRM chromatograms of an acacia honey fortified with 16 (fluoro)quinolones at the 5 μg/kg level: (**a**) PIPE (RT 9.32 min), (**b**) CINO (9.41), (**c**) MARBO (9.73), (**d**) ENOX (9.79), (**e**) FLERO (9.80), (**f**) OFLO (9.84), (**g**) NOR (9.85), (**h**) OXO (9.89), (**i**) CIPRO (9.94), (**j**) DANO (9.99), (**k**) LOME (10.00), (**l**) ENRO (10.10), (**m**) DIFLO (10.50), (**n**) SARA (10.50), (**o**) NALI (10.60), and (**p**) FLUME (10.70). For each analyte, three transition reactions were considered: (from top to bottom) one for quantification (SRM1) and two for peak confirmation (SRM2 and SRM3). The time window is 2 min.

MATERIALS AND METHODS

Chemicals and Reagents. Cinoxacin (CINO), enoxacin (ENOX), lomefloxacin (LOME) hydrochloride, and pipemidic acid (PIPE) were obtained from Sigma (Buchs, Switzerland). Ciprofloxacin (CIPRO), danofloxacin (DANO), difloxacin (DIFLO) hydrochloride, enrofloxacin (ENRO), fleroxacin (FLERO), flumequine (FLUM), marbofloxacin (MARBO), norfloxacin (NOR), ofloxacin (OFLO), and sarafloxacin (SARA) hydrochloride were supplied by Riedel-de-Haën (Fluka, Buchs, Switzerland) whereas nalidixic acid (NALI) and oxolinic acid (OXO) were from Fluka (Buchs, Switzerland). All compounds were above 98% purity. Methanol, acetonitrile, acetone, 2-propanol (HPLC grade), ammonium formate, and concentrated formic acid (98%) were obtained from Merck (Darmstadt, Germany). Nonafluoropentanoic acid (NFPA) (97%) was from Acros Organics (Geel, Belgium). Deionized and distilled water was obtained from a Milli-Q water purification apparatus (Millipore, Bedford, MA).

Standard Solutions. Individual stock standard solutions at the 100 μg/mL concentration level were prepared by dissolving the appropriate amount of each drug in methanol. A composite standard solution of the 16 compounds at the 1 μg/mL concentration level was then obtained by dilution with water−methanol (84:16 v/v). All solutions were stored at −20 °C and allowed warming at room temperature before use.

Honey Samples. Honeys of different flowers and of different geographical origins were collected from various suppliers or purchased from retail outlets in Switzerland. A particular set of 34 honeys of Chinese origin, collected during the Sept 2006−Feb 2007 period was also included. Samples were stored under dry conditions in the dark at 4 °C until analysis.

Sample Preparation. Honey samples (at least 50 g) were first heated in a water bath at 40 °C and mixed to a homogeneous mixture before considering a test portion. A 1.0 g portion of the homogenized honey was then weighed into a 2 mL Eppendorf tube (Hamburg, Germany) to which 1.0 mL of water was added. The resulting solution was thoroughly vortexed for 1 min and, when needed, gently heated (microwave oven; Koenig, Zurich, Switzerland; 800 W, 5–10 s) until the achievement of a homogeneous slurry and then filtered through a 0.22 μm polyether sulfone membrane syringe filter (diameter 33 mm; Millipore, Cork, Ireland) directly into a HPLC vial.

Turbulent Flow and Liquid Chromatography Conditions. A high turbulent liquid chromatograph (HTLC) Aria TLX1 system (Thermo Fisher Scientific, Franklin, MA) was used for these experiments and

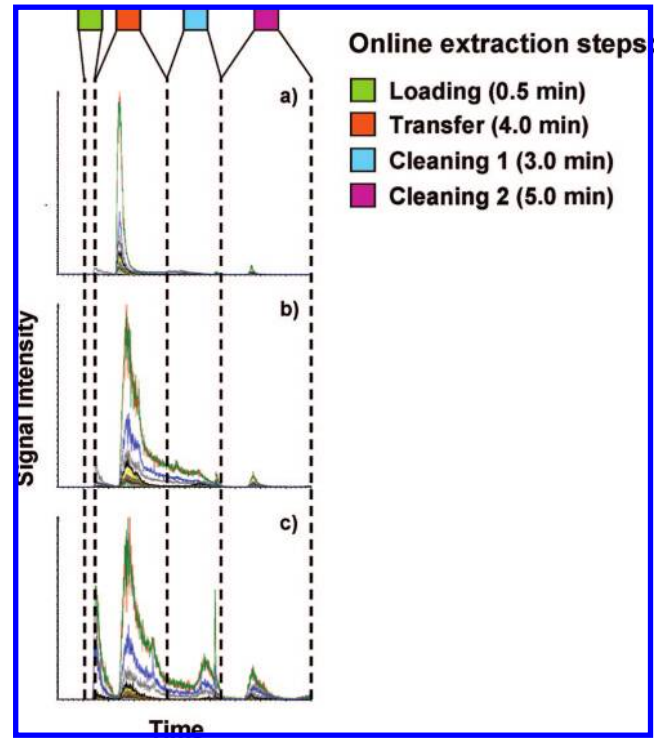


Figure 3. Typical MS chromatograms obtained at different steps during the optimization of the online extraction parameters for three TFC columns: (**a**) Cyclone (polymer based); (**b**) C_{18} (silica based), and (**c**) PolarPlus (silica based).

was comprised of a PAL thermostated autosampler (CTC Analytics, Zwingen, Switzerland), which was set up at a temperature of 8 °C, a low-pressure mixing quaternary pump (loading pump), a high-pressure mixing binary pump (eluting pump), a multiple column module (MCM), and a three-valve switching device unit with six-port Valco. The entire system was controlled via Aria software. The TurboFlow HTLC column was made of a styrene−divinylbenzene copolymer (Cyclone, 50 × 0.5

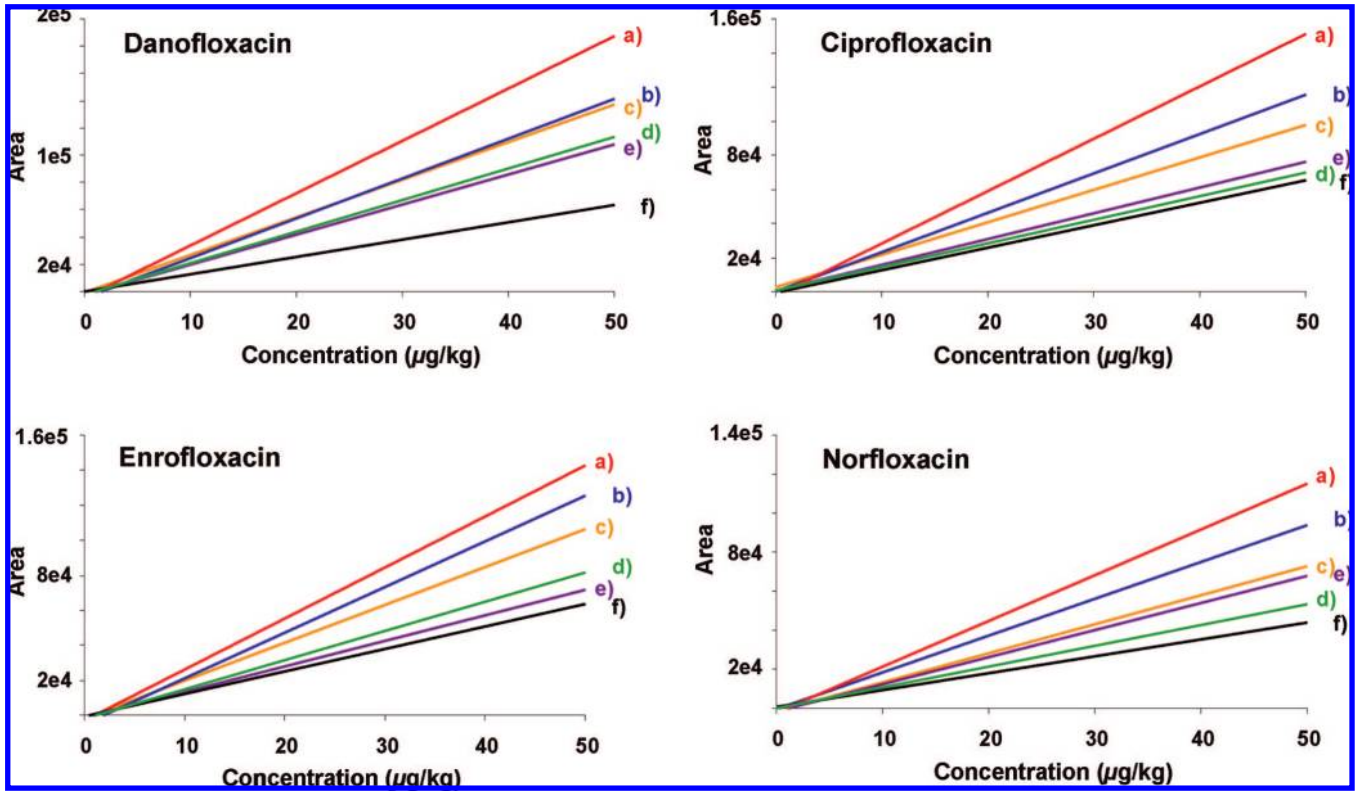


Figure 4. Matrix-matched calibration curves for four fluoroquinolones in different honey samples: (**a**) lime, (**b**) clover, (**c**) acacia, (**d**) forest, (**e**) sunflower (Swiss origin), and (**f**) sunflower (French origin). For clarity reasons, individual data points are not shown.

mm, 60 μm particle size, 60 Å pore size; Thermo Fisher Scientific) whereas the analytical HPLC column was a Zorbax SB C_{18} (50 × 2.1 mm, 1.8 μm; Agilent, Geneva, Switzerland). The temperature of the analytical column was maintained at 40 °C using a TC-50 controller (FIAtron Systems Inc., Oconomowoc, WI). The injection volume was 160 μL, delivered by four portions of 40 μL using an enrichment procedure available from the Aria software. **Table 1** lists the parameters used for both online TFC cleanup and HPLC elution steps.

ESI-MS/MS Conditions. MS analyses were performed on a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray source. MS tuning was performed in positive electrospray ionization mode (ESI) by infusing separately a solution of each analyte (5 μg/mL in methanol) at a flow rate of 10 μL/min mixed with a HPLC flow made of solvents A and B (50/50 v/v; 0.3 mL/min) using a T-connector. The source block temperature was maintained at 600 °C, and the electrospray capillary voltage was set at 5.0 kV. Other set values were as follows: curtain gas, 25 psi; nebulizer gas, 30 psi; turbo gas, 50 psi; collision gas, 0.3 mTorr. Quantitative analysis was performed using tandem MS/MS in selective reaction monitoring (SRM) mode alternating three transition reactions for each compound with a constant dwell time of 25 ms (**Table 2**). The most intense transition reaction (SRM1) was used for quantification purpose while the second and third ones (SRM2 and SRM3) were employed for analyte confirmation. Exceptions were lomefloxacin (LOME) and ofloxacin (OFLO) for which the second transition reaction was used for quantification since it showed a better signal-to-noise ratio than the first. Peak area integrations were done using Analyst software (version 1.4.2), and the raw data were then exported and processed through a homemade visual basic Microsoft Excel Macro, allowing automatic calculations of final concentrations as well as confirmation of the analytes based on peak area ratios and retention time.

Method Validation. Incurred-like samples were obtained by fortifying three honeys (acacia, multiflower, and forest), each at three different concentration levels, i.e., 5, 20, and 50 μg/kg, and each level 9-fold. Samples were then gently heated and thoroughly mixed to allow an optimal integration of the analytes into the matrix and eventually left standing overnight at room temperature.

Quantification was performed by the single-point matrix matched calibration procedure (*15*), which usually requires each sample to be extracted in duplicate: one as such and a second containing an amount of analyte standard added, named as the calibrant. It is thus a simplified approach of the standard addition quantification method. In our validation, both 10 and 25 μg/kg additional calibrant spikes were considered for comparative purposes, and triplicate extractions were performed at each unspiked and spiked level. Therefore, the equation used for quantification was

$$\text{(fluoro)quinolone concn } (\mu\text{g/kg}) = \frac{\text{CAL} \times A}{A_{\text{spike}} - A} \quad (1)$$

where CAL is the concentration of the single-point matrix matched calibrant (either 10 or 25 μg/kg), A is the peak area in the original sample, and A_{spike} is the peak area in the spiked sample (either with a 10 or 25 μg/kg added amount).

Thus, for an incurred-like honey at the 5 μg/kg level, the following samples were analyzed: three samples without any additional calibrant spike, three samples with a 10 μg/kg additional calibrant spike, and three samples with a 25 μg/kg additional calibrant spike. The two other fortification levels (i.e., 20 and 50 μg/kg) were treated similarly. This sequence was repeated 3-fold for each honey considered, representing thus a total of 243 honey extracts, analyzed over a 1 month period.

Precisions (within- and between-day) based on peak area values were calculated following ISO recommendations (*16*) at each fortification level (i.e., 5, 20, and 50 μg/kg) from three sets of data obtained at different weeks, each set containing peak areas from triplicate independent extractions. The following equations were used:

$$\text{within-day precision (WD, \%)} = \frac{100}{\text{mean}} \sqrt{\frac{\text{Var}_1 + \text{Var}_2 + \text{Var}_3}{3}} \quad (2)$$

where Var_i is the variance of results ($n = 3$) obtained at day i and mean is the average value of all measurements ($n = 9$).

between-day precision (BD, %) =

$$\frac{100}{\text{mean}}\sqrt{\text{SD}^2(u)+\left(\frac{n-1}{n}\right)\times \text{SD}^2{}_{\text{within-day}}} \quad (3)$$

where $SD^2(u)$ is the variance of the every-day mean, n is the number of replicates per day ($n = 3$), and mean is the average value of all measurements ($n = 9$).

Recovery values at the fortified concentrations (i.e., 5, 20, and 50 μg/kg) were calculated from the average and standard deviation (SD) of three data sets performed over three distinct weeks, each set resulting from triplicate independent extractions at each fortification level, using either the 10 or 25 μg/kg calibrant.

Confirmation criteria as defined in EU Commission Decision 2002/657/EC (*17*) (at least four identification points, peak area ratios for the different transition reactions recorded within the required limits of acceptance, signal-to-noise ratio of these ions above 3, retention time within 2.5% of the retention time of a standard injected on that day) were followed to unambiguously confirm the presence of an analyte within the matrix.

RESULTS AND DISCUSSION

LC-ESI-MS/MS. In the positive electrospray ionization mode, protonated molecules $(M + H)^+$ were obtained for all (fluoro)quinolones surveyed. Collision-induced dissociation (CID) mass spectra were then recorded for each analyte at various collision energies before selecting the optimal MS/MS transition reactions and electronic parameters. The CID fragmentation pathways of the analytes surveyed have been described previously (*18, 19*) and will not be elaborated further. Three SRM transition reactions were chosen for each analyte (giving a total of 5.5 identification points), though two are said to be sufficient to fully confirm the presence of a contaminant (*17*). However, the choice of considering several transition reactions was proven adequate when quantifying/identifying some analytes, i.e., pipemidic acid, marbofloxacin, enoxacin, and fleroxacin (**Figure 2a**,**c**,**d**,**e**), when one of the analyte-related signals was not well resolved from that of a near-eluting peak. In a decreasing order, the best MS/MS response (peak area comparison) for the transition reaction giving the highest response was observed for FLUM > OXO > NALI > DANO > ENRO > SARA > DIFLO > CINO > ENOX > FLERO > CIPRO > NOR > OFLO > LOME > PIPE > MARBO, showing a ratio of 17 between the highest and weakest response. Concentration−MS response relationships of six-point matrix-matched calibration curves were shown to be linear up to 100 μg/kg (equivalent in sample) for all compounds, and their linearity was checked by calculating the relative standard deviation (RSD) of the average of response factors (RFs; RF = peak area/concentration), which should be below 15% (*20*). Since several analytes have similar or close SRM1 transition reactions (Δ amu = 1), i.e., flumequine and oxolinic acid (m/z 262 → 244) and cinoxacin (m/z 263 → 245) or ofloxacin (m/z 362 → 344) and marbofloxacin (m/z 363 → 345), a good chromatographic separation was mandatory to avoid misquantifications. Through chromatographic separation as well as MS detection, the signals of these analytes were well separated in 2 min. The total run time was 26.5 min, including both TFC loading and transfer steps as well as the reequilibration of the HPLC analytical column.

Nonafluoropentanoic acid (NFPA) was used as an ion pairing agent in solvent A only and was already reported to be effective for the retention and separation of aminoglycosides onto C_{18}-based HPLC packing materials (*21*). Though not needed for the analysis of the 16 (fluoro)quinolones, this compound was still included within the HPLC mobile phase A, as the final goal of our methodology is the simultaneous survey of >50 veterinary drugs in honey (*22*).

Method Development. Online extraction includes loading, transferring, washing, and equilibration steps. The loading step involves sufficient washing of the matrix from the extraction column to maintain its efficiency while not affecting the recovery of analytes. Consequently, the mobile phase used during this step should be suitable to make the matrix bulk components soluble to facilitate their transport out of the TFC column. An aqueous 10 mM ammonium formate was proven adequate to fulfill this task. The analyte transfer is performed by allowing a polar organic mixture (previously contained in a closed 100 μL loop) to desorb the analytes from the TFC column onto the HPLC column in a back-flush operation. At this stage, the TFC flow rate (containing the polar mixture) must be dramatically decreased before its in-line mixing with the low organic content HPLC mobile phase to avoid diffusion of the analytes onto the HPLC column leading to peak broadening. Various loop compositions containing water and acetonitrile/formic acid, 0.1%, in different ratios (from 50% to 100% of the acidified organic solvent) were tested, and 100% of the acidified organic solvent was needed to completely desorb the analytes from the selected TFC column. Finally, the composition of the washing solvents as well as their time of action was carefully set up to avoid carry-over effects. The optimization of these online extraction parameters (choice of the TFC sorbent, composition of the mobile phases, step time, etc) was conducted by appropriate column switching (set in Aria software) in an automatic optimization procedure using the quick elute mode (i.e., without any analytical column) connected to the MS detector. MS signals of the 16 analytes resulting from the injection of a spiked honey were thus constantly recorded during the different extraction steps. **Figure 3** presents an example of this optimization procedure during the selection of the most adapted TFC column, showing the Cyclone column stationary phase as the most appropriate to ensure minimal (fluoro)quinolone losses during loading and cleaning steps.

The manual sample preparation was reduced to a simple dilution and filtration of the honey sample, allowing 12 samples to be prepared within 30 min. However, some key points of this preparative step were highlighted, and the first one concerned the homogenization of the honey slurry. Indeed, as some types of honey (forest, multiflower, etc.) are highly viscous or even solid at room temperature, heating them to help for dilution in water could have led to the decomposition of one or several of the 16 analytes under survey. To assess this potential issue, samples previously spiked at the 10 μg/kg level were first heated at 85 °C in an oven for 0, 15, 30, and 60 min, respectively, and then analyzed as described previously. No significant decrease of the corresponding peak areas was noticed whatever the heating time, demonstrating thus the heat stability of the (fluoro)quinolones considered in the honey matrix. The second point concerned the filtration of the diluted extracts, which was shown to be mandatory to avoid rapid TFC column clogging and, thus, to extend column lifetime. Initially, this filtration was first conducted by means of 0.22 μm nylon filters. When using this material, some compounds (flumequine, nalidixic acid, and oxolinic acid) were trapped by the nylon filter, leading to up a 50% loss of signal. On the contrary, this effect was not observed when using a polyether sulfone-based filter. The reason for these selective adsorptions onto the nylon-based material could not be explained so far.

The proposed methodology was shown to be satisfactory in terms of solvent consumption as only 44 mL per sample was

Table 3. Performance Data of the HTLC-MS/MS Method for Analysis of 16 (Fluoro)Quinolones in Three Honeys: (a) Acacia, (b) Forest, and (c) Multiflower

	fortification levels											
	5 μg/kg				20 μg/kg				50 μg/kg			
			recovery[c]				recovery[c]				recovery[c]	
analyte	WD[a]	BD[b]	CAL 10	CAL 25	WD[a]	BD[b]	CAL 10	CAL 25	WD[a]	BD[b]	CAL 10	CAL 25
						(a) Acacia						
CINO	7.0	7.9	110 ± 3	112 ± 5	10.5	13.5	106 ± 21	99 ± 2	2.8	7.7	101 ± 21	112 ± 2
CIPRO	4.4	6.5	117 ± 13	120 ± 5	7.2	7.0	102 ± 31	107 ± 16	6.1	6.5	169 ± 53	127 ± 12
DANO	5.0	8.0	95 ± 13	98 ± 11	6.6	10.9	96 ± 16	97 ± 1	3.0	7.4	83 ± 5	96 ± 7
DIFLO	5.1	6.1	103 ± 1	112 ± 6	5.4	10.6	91 ± 3	94 ± 3	2.7	8.5	79 ± 7	88 ± 7
ENOX	8.7	8.2	106 ± 9	103 ± 11	7.2	7.7	105 ± 9	107 ± 5	5.2	5.9	120 ± 10	115 ± 14
ENRO	5.6	8.5	92 ± 9	95 ± 14	3.7	4.5	98 ± 23	96 ± 13	2.1	6.9	93 ± 17	93 ± 12
FLERO	4.4	6.1	103 ± 10	100 ± 8	9.6	10.7	94 ± 14	95 ± 8	2.5	9.0	92 ± 16	104 ± 7
FLUM	3.7	4.0	102 ± 6	105 ± 9	6.6	8.9	96 ± 9	96 ± 5	3.7	7.6	86 ± 15	100 ± 4
LOME	10.9	13.0	108 ± 11	107 ± 18	5.0	7.7	102 ± 20	97 ± 11	4.8	10.7	108 ± 53	108 ± 10
MARBO	10.0	10.7	87 ± 4	97 ± 8	4.4	6.2	97 ± 12	96 ± 14	5.8	13.2	108 ± 28	97 ± 8
NALI	4.0	8.1	97 ± 11	100 ± 13	6.4	11.0	97 ± 17	102 ± 5	3.6	6.8	91 ± 13	101 ± 7
NOR	6.6	7.0	105 ± 4	101 ± 7	7.0	6.3	110 ± 28	106 ± 12	5.0	6.0	133 ± 31	117 ± 5
OFLO	7.5	7.6	98 ± 9	98 ± 4	6.6	7.5	107 ± 39	101 ± 13	3.0	10.9	90 ± 4	91 ± 5
OXO	8.4	29.6	97 ± 8	97 ± 8	6.6	8.5	97 ± 10	95 ± 6	4.4	5.4	93 ± 25	103 ± 14
PIPE	12.8	17.4	90 ± 11	93 ± 12	7.1	7.8	107 ± 12	106 ± 11	3.8	7.9	105 ± 16	115 ± 4
SARA	11.1	11.0	113 ± 10	117 ± 13	4.4	6.5	94 ± 20	95 ± 10	2.7	10.4	88 ± 18	97 ± 2
						(b) Forest						
CINO	5.6	6.5	125 ± 9	127 ± 3	6.3	7.8	95 ± 6	99 ± 3	4.5	3.8	95 ± 22	115 ± 22
CIPRO	6.5	18.6	108 ± 25	108 ± 22	6.4	7.4	102 ± 14	104 ± 4	4.3	9.0	114 ± 42	102 ± 23
DANO	8.1	7.3	110 ± 5	113 ± 7	5.5	8.4	90 ± 8	95 ± 9	3.3	4.0	81 ± 18	90 ± 7
DIFLO	6.2	23.4	107 ± 10	109 ± 11	5.7	20.5	109 ± 11	107 ± 9	6.4	24.6	96 ± 16	114 ± 11
ENOX	9.8	11.5	109 ± 16	106 ± 6	3.2	10.8	98 ± 5	104 ± 10	3.3	8.2	98 ± 16	115 ± 9
ENRO	9.5	10.6	105 ± 8	100 ± 4	2.6	6.3	95 ± 7	100 ± 8	4.5	5.1	82 ± 8	101 ± 15
FLERO	10.5	15.7	99 ± 14	93 ± 11	5.3	7.0	80 ± 2	92 ± 2	5.3	4.7	98 ± 30	98 ± 12
FLUM	5.2	5.6	104 ± 7	99 ± 1	3.1	5.9	99 ± 6	103 ± 3	1.7	3.8	97 ± 11	106 ± 6
LOME	13.5	14.4	103 ± 8	102 ± 9	7.5	12.8	89 ± 16	96 ± 13	5.4	4.6	85 ± 22	85 ± 13
MARBO	18.4	15.4	108 ± 14	96 ± 9	8.0	10.1	96 ± 18	103 ± 21	9.8	9.3	73 ± 6	110 ± 18
NALI	4.4	5.9	100 ± 7	95 ± 2	4.8	10.3	92 ± 6	101 ± 12	3.5	5.4	92 ± 5	103 ± 5
NOR	16.0	22.4	114 ± 29	107 ± 22	7.9	10.2	107 ± 20	119 ± 15	5.4	7.7	105 ± 25	104 ± 8
OFLO	11.7	15.5	114 ± 17	112 ± 10	3.3	9.5	93 ± 18	97 ± 11	4.3	5.0	87 ± 13	91 ± 1
OXO	5.7	11.0	103 ± 15	103 ± 13	2.9	5.8	98 ± 4	104 ± 5	2.4	2.0	101 ± 15	106 ± 6
PIPE	8.2	24.4	105 ± 22	106 ± 23	7.2	9.8	113 ± 16	106 ± 11	3.9	9.6	95 ± 20	101 ± 9
SARA	8.9	23.9	106 ± 7	104 ± 10	5.4	21.5	98 ± 12	105 ± 18	3.2	23.0	99 ± 16	105 ± 6
						(c) Multiflower						
CINO	9.5	8.1	98 ± 9	100 ± 8	5.8	5.7	94 ± 17	95 ± 6	4.7	4.5	86 ± 8	106 ± 11
CIPRO	7.0	19.5	102 ± 31	91 ± 18	7.6	11.4	102 ± 14	113 ± 8	4.9	11.0	105 ± 16	100 ± 17
DANO	4.3	24.1	120 ± 49	105 ± 30	5.8	10.8	85 ± 5	87 ± 6	3.5	6.6	92 ± 16	94 ± 6
DIFLO	7.0	15.4	110 ± 22	104 ± 20	5.1	6.3	89 ± 9	90 ± 2	4.5	8.9	96 ± 7	105 ± 16
ENOX	5.0	17.8	122 ± 42	104 ± 28	7.3	11.7	96 ± 14	104 ± 12	9.1	7.8	116 ± 5	109 ± 23
ENRO	9.1	15.4	97 ± 23	91 ± 16	5.4	7.9	92 ± 12	95 ± 5	3.2	7.9	90 ± 7	99 ± 17
FLERO	6.5	19.7	97 ± 26	89 ± 20	6.7	10.9	86 ± 14	88 ± 9	2.3	8.5	88 ± 4	97 ± 10
FLUM	6.0	10.2	98 ± 11	99 ± 13	5.4	6.1	89 ± 10	94 ± 4	2.5	5.2	94 ± 12	107 ± 16
LOME	12.7	19.9	97 ± 18	96 ± 16	8.0	9.1	83 ± 7	88 ± 6	5.3	8.0	74 ± 3	90 ± 10
MARBO	9.8	41.6	105 ± 21	99 ± 24	11.8	17.2	93 ± 9	91 ± 8	3.3	19.8	88 ± 26	93 ± 10
NALI	5.1	7.9	103 ± 1	99 ± 5	7.0	6.2	90 ± 13	96 ± 3	3.6	6.7	99 ± 9	109 ± 16
NOR	15.0	24.4	129 ± 44	105 ± 23	8.2	13.7	98 ± 17	99 ± 13	3.8	9.5	108 ± 8	102 ± 9
OFLO	14.0	22.9	102 ± 23	89 ± 18	7.2	9.3	102 ± 12	92 ± 6	3.0	5.8	91 ± 18	101 ± 16
OXO	7.1	14.5	116 ± 14	107 ± 11	7.9	7.9	94 ± 11	95 ± 5	5.1	7.8	99 ± 7	104 ± 20
PIPE	14.4	19.3	98 ± 30	90 ± 27	8.7	8.9	102 ± 3	98 ± 7	3.2	6.7	122 ± 27	106 ± 12
SARA	7.7	18.5	104 ± 20	101 ± 18	7.1	7.3	98 ± 13	94 ± 6	3.5	8.8	95 ± 6	101 ± 15

[a] WD, within-day precision (%), $n = 9$. [b] BD, between-day precision (%), $n = 9$. [c] Recovery and standard deviation (%) obtained by the single-point matrix matched calibration procedure using either a 10 or a 25 μg/kg calibrant.

needed for both online sample cleanup and chromatographic separation, including 23 and 21 mL of organic-based and water-based solvents, respectively. Additionally, turbulent flow chromatography proved to be a robust technique with over 400 injections of honey extracts without any TFC column deterioration and, consequently, a very good stability of analyte retention times (RT) on the HPLC column (RSD of the mean RT < 0.5%), no soiling of the mass spectrometer interface, and no significant reduction in the detector response.

Method Performance Characteristics. The accuracy of quantitative results provided by an analytical method depends on the calibration. When no isotopically labeled internal standard is available (or too expensive to be considered for routine analysis) for each analyte under survey, quantification by means of matrix-matched calibration curves is usually considered as the best option to compensate for both losses during extraction and matrix effects generated during the ionization of the analyte. However, this procedure shows its limits when a blank matrix similar to the one to be analyzed is not available. Wang et al. (*23*) have demonstrated this problematic when analyzing pesticides during a survey of apple-based infant foods. Organic apples, free of any contaminants, were homogenized, and this resulting blank matrix was subsequently employed to construct "pseudo" matrix-matched standard calibration curves. The low

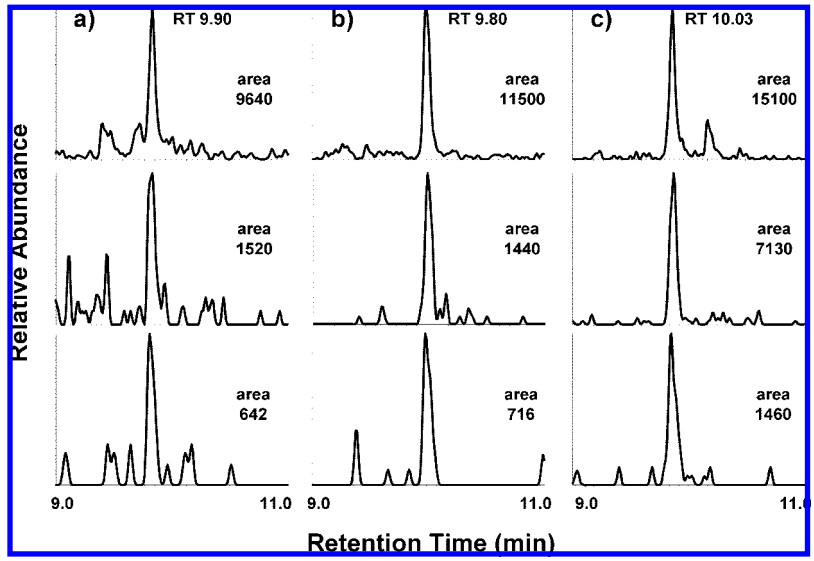


Figure 5. SRM chromatograms of three incurred honeys containing (**a**) ciprofloxacin (1.5 μg/kg), (**b**) norfloxacin (2.3 μg/kg), and (**c**) enrofloxacin (1.4 μg/kg). For each analyte, three transition reactions were considered: (from top to bottom) one for quantification (SRM1) and two for peak confirmation (SRM2 and SRM3).

and variable recoveries obtained afterward indicated that the matrix blank was not able to match the real samples in character. When analyzing sulfonamide-related antimicrobial agents in honey by LC-ESIMS/MS, Verzegnassi et al. (*24*) highlighted a strong matrix effect with slopes of matrix-matched calibration curves differing not only from a solvent-based curves but also among honey extracts of different origin, despite an extensive cleanup (including an acidic hydrolysis followed by several LLE steps) was performed. The same effects were also observed by Khong et al. (*25*) during the analysis of tetracyclines. In this context, the cleanup efficiency of the turbulent flow chromatography was evaluated to check whether matrix-matched calibration curves obtained from one single honey could be used for any other honeys. Thus, samples of different nature, i.e., lime, clover, acacia, forest, and two sunflower honeys of different origins, were spiked over the 0−50 μg/kg concentration range with four representative fluoroquinolones, namely, danofloxacin, ciprofloxacin, enrofloxacin, and norfloxacin, and matrix-matched calibration curves were constructed afterward. As shown in **Figure 4**, matrix effects were still honey-dependent as demonstrated by the large slope variations (statistically different) of the different calibration curves, demonstrating that both extraction and cleanup by turbulent flow chromatography were still insufficient to "normalize" the matrix effects observed between the different honeys analyzed. Therefore, calibration by the standard addition procedure was the unique remaining option. Ideally, this procedure requires that three additional extracts of the matrix under survey are spiked at levels corresponding to 50%, 100%, and 150% of the estimated content of the analyte. A plot of concentrations vs their responses is then constructed, and the concentration in the original extract corresponds to the absolute value at the *x*-intercept. Drawbacks of this quantification route are as follows: (a) the knowledge of the extent of the contamination is rarely known in advance, and deviations from the "50%, 100%, and 150% additional spike" rule may produce incorrect results; (b) the range of concentrations covered must be linear; and (c) several extractions are needed to get one single result. To shorten this lengthy procedure, the single-point matrix matched calibration was considered in our study, using either a 10 or 25 μg/kg calibrant, which was spiked at the beginning of the sample workup. This simplified procedure is currently used in the field of pesticide residue analysis and said to be more accurate than the multilevel approach when the detector response is variable with time (*26*). **Table 3** summarizes the overall performance data of the method for the analysis of 16 (fluoro)quinolones in the three honeys considered for validation using the single-point matrix matched calibration. Within-day precisions were <20% (minimum 2%, maximum 18%, median 6%) whatever the analyte or the honey or the fortification level considered whereas between-day precision data were minimum 2%, maximum 42%, and median 9%, the highest value being related to marbofloxacin, which showed the weakest MS/MS response. The mean recovery values were scored as satisfactory considering the basic quantification procedure used. Better results were obtained when the 25 μg/kg calibrant was used (101 ± 8%, minimum 85%, maximum 127%) compared to the 10 μg/kg one (98 ± 12%, minimum 73%, maximum 169%), whatever the fortification level. As said previously, honey is a particular matrix considering its large variety of composition and origin, which lead to different degrees of matrix effects for the same analyte (**Figure 4**). This fact was corroborated when tentatively estimating the limits of quantification (LOQ) by extrapolation of the signal-to-noise ratios obtained at each analyte transition reaction using an extract spiked at the 5 μg/kg level in the three honeys considered for validation. Enoxacin, used as example, was shown to have a LOQ ranging from 1 μg/kg (acacia) to 3 μg/kg (forest honey). Therefore, a common LOQ per analyte for all honeys could not be set up. However, as all analytes were detected at the lowest level of fortification, i.e., 5 μg/kg, this value was arbitrarily considered as the "method LOQ", with the knowledge that lower amounts could be quantified, when all confirmation criteria were fulfilled.

The stability of the 16 (fluoro)quinolones in the final honey extract was assessed by injecting once a day for 10 consecutive days the same sample (spiked at the 10 μg/kg level) left onto the autosampler at 8 °C. In parallel, a second extract was analyzed after 10 days of storage at −20 °C. No significant decrease (peak area comparison) was observed for any of the compounds considered. However, it was noticed that standard solutions of (fluoro)quinolone made in pure water were not stable, even when stored at −20 °C for less than 2 days.

Minisurvey of Honeys. The applicability of the method in routine quality controls was further demonstrated by the analysis

of a set of Chinese honeys, collected after an U.S. Food and Drug Administration (FDA) import alert issued in February 2006 and related to the adulteration of Chinese honeys by ciprofloxacin and enrofloxacin (*4*). Out of the 34 samples analyzed and for the 16 (fluoro)quinolones surveyed, only ciprofloxacin, norfloxacin, and enrofloxacin were detected. Ciprofloxacin was found in 23 honeys (maximum content: 225 μg/kg) whereas norfloxacin was detected in 17 honeys (maximum content: 31 μg/kg) and enrofloxacin only in 1 honey (1.4 μg/kg). Both ciprofloxacin and norfloxacin were found simultaneously in 12 samples. Only 5 honeys (14.7%) were free of any of the 16 analytes surveyed. **Figure 5** shows SRM chromatograms of three Chinese honey extracts in which the lowest levels of either ciprofloxacin (1.5 μg/kg), norfloxacin (2.3 μg/kg), or enrofloxacin (1.4 μg/kg) had been detected. Even at these trace levels, unambiguous peaks were observed for each transition monitored with adequate S/N ratio to quantify and confirm these compounds.

This first application in food analysis opens a wide new interesting field in residue analysis to ensure food safety and product compliance. Indeed, our preliminary trials have already shown that this online extraction method coupled to a sensitive MS/MS detector is adequate for the screening of a larger number of veterinary drugs (ca. 50) in honey but also in milk-based matrices, including raw milk and milk powders.

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