Determination of fluoroquinolones in compost by green microwave-assisted extraction followed by ultra performance liquid chromatography tandem mass spectrometry

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Abstract

A novel, simple and straightforward method for determination of fluoroquinolones (FQs) in compost has been developed. The procedure entails a mild microwave-assisted extraction (MAE) carried out by a high performance instrument, in alkaline aqueous solution containing magnesium ions as FQs complexing agent, followed by ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS). Ciprofloxacin (CIP), Enrofloxacin (ENR), Levofloxacin (LEV) and Norfloxacin (NOR), four widely used FQ antibiotics, were simultaneously extracted from compost by a *single* MAE cycle (20 min, 135°C). Due to the absence of certified reference materials, the method was validated using matrix-matched calibration and recovery tests on fortified samples. Quantitative absolute recovery (70-112%, n=3) and suitable precision (RSD<15%, n=3) were observed, at concentration levels ranging from 25 ng g^{-1} to 2,500 ng g^{-1}. Analytes were separated in a 10 min chromatographic run and quantified/confirmed in single reaction monitoring (SRM) mode. UPLC coupled to MS detection allowed to achieve improved sensitivity, and selective detection. Method detection and quantification limits, MDLs and MQLs, were in the range 2.2-3.0 ng g^{-1} and 6.6-9.0 ng g^{-1}, respectively. The procedure proved to be simpler, less expensive, faster, and more green with respect to the few methods currently described in literature, providing at the same time suitable recovery and reproducibility. The analytical method has been applied to the analysis of actual compost samples, wherein FQs have been quantified at concentrations up to 88 ng g^{-1}.

Keywords

Compost; Emerging pollutants; Fluoroquinolones; Green analytical chemistry; Microwave-assisted extraction; UPLC-MS

1. Introduction

In the last two decades the amount of sewage sludge has increased dramatically due to the growth in urban population and thus livestock farming [1]. Besides being regarded as an undesired waste, sewage sludge can be conveniently recycled for agricultural purposes as a low-cost organic biomass. Composting or aerobic biological treatment of organic wastes is a common practice to reduce wastes and to exploit organic matter and inorganic nutrients (e.g. phosphorous, nitrogen); in particular, composting is an economical and sustainable approach for converting sewage sludge into a final product with a high organic content that is largely and conveniently used as soil conditioner and/or fertilizer [1]. On the other side, direct field application of raw sludge is not convenient because of leaching of nutrients and the environmental pollution that would derive from the widespread of untreated sludge [2]. With regard to this, several pharmaceuticals have been determined in sewage sludge at micrograms *per* kilogram levels [3], among which residuals of antibiotics such as fluoroquinolones (FQs), sulfonamides and tetracyclines [4]. It has been demonstrated that, despite biodegradation possibly taking place during the composting process [5], variable amounts of residual drugs can be found also in the final product. In particular, a recent paper [2] reported that Ciprofloxacin (CIP), one of the most widely prescribed FQ in the world [6], shows higher persistence during composting than other pharmaceuticals such as sulfadiazine and chlortetracycline, as a further evidence for FQs resistance to biodegradation [6]. Despite this poses the question of environmental diffusion of pharmaceutically active compounds, the presence of FQs antibiotics in compost has received little attention.

FQs are one of the most commonly employed class of antibacterial agents, adopted both for human and veterinary medicine. The target proteins of FQ drugs are bacterial DNA gyrase and topoisomerase IV enzymes, essential for DNA replication and transcription. In particular, the fluorine atom at C-6 position of the ring provides a more than 10-fold increase in gyrase inhibition and up to 100-fold improvement in minimum inhibitory concentrations, while substituent groups at position C-7 play a key role in determining the antibacterial spectrum and bioavailability; piperazine is frequently used and grants potency against

Gram-negative bacteria. Due to their peculiar pharmacological properties, above all good oral intake and broad activity spectrum, they gained worldwide popularity [7].

At the same time, FQs have been included in the list of the "emerging pollutants", defined as new chemicals that have no regulatory status but may have an adverse impact on the environment and human health [8]. The environmental diffusion of these synthetic drugs has been assessed in the recent years, both in water [9,10] and soil [11] compartments, and essentially is involved by the partial metabolism FQs undergo after ingestion [12], to the partial removal during wastewater treatment [13,14] and land application of livestock/urban compost [15]. As a matter of fact, FQs are one of the most frequently detected pharmaceuticals, together with sulfonamides, tetracycline, and macrolides [12].

Although photochemical degradation can alleviate their accumulation in natural ecosystems [16-19], resistance to biodegradation and strong adsorption on solid matrices are responsible for their enhanced persistence [20]. From the environmental viewpoint, FQs diffusion is reason of great concern due to their capability to induce bacterial resistance [21-23], genotoxicity [24] and ecotoxicity [25-27]; moreover, their overall environmental impact is also due to the formation of various photoproducts able to exert themselves antibiotic activity [12,28]. For these reasons the development of novel treatments for environmental remediation is of overwhelming importance [29-31].

Presently, no trigger values exist for FQs in environmental matrices, although the general concentration of drugs should not exceed 0.1 $\mu g\ L^{-1}$ in groundwater, 100 $\mu g\ kg^{-1}$ in manure and 10 $\mu g\ kg^{-1}$ in soil [32]. Notice that CIP has been determined at far higher concentrations in manure, up to ca. 30 $mg\ kg^{-1}$ [2], and it has been demonstrated that up to 30% of the CIP initial amount remains in the composting mass after manure storage [2].

Although only very few data are available in the literature, FQs have been detected in compost, namely CIP and Norfloxacin (NOR) [1]. It seems evident that using compost in agriculture represents an important route for FQs environmental pollution. This highlights the need for analytical tools suitable for monitoring the levels of such pharmaceuticals in this very complex, from the analytical point of view, humus-like matrix, and more generally to deeper assess the fate of these pharmaceuticals alongside sludge treatment [3].

The main challenge in trace-level determination in compost is to find out working strategies to minimize matrix interferences. This means improving selectivity, both in sample preparation and in detection, and sensitivity towards the target compounds.

For FQs determination in compost samples, Lillenberg et al. [4] applied the analytical method initially designed and validated on sewage sludge. The procedure entailed 5 consecutive pressurized liquid extraction (PLE) cycles followed by solid-phase extraction (SPE) cleanup prior liquid-chromatography tandem mass spectrometry (LC-MS). A modification of the USEPA method 1694 was applied by Selvam et al. [2] to extract CIP from compost, performing a 3-cycles ultrasonic-assisted extraction (UAE) followed by SPE and evaporation of the SPE extract prior LC-MS. To the authors' best knowledge, no other analytical method is currently available in the literature for the determination of FQs in compost.

On the basis of this background, we developed a straightforward analytical method for determination of four widely used FQs – CIP, Enrofloxacin (ENR), Levofloxacin (LEV) and NOR – in compost, at the nano/micro-grams *per* gram concentration levels, based on a mild and highly efficient microwave-assisted extraction (MAE) followed by SPE, and ultra performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS) determination. The analytical figures of merit of the method (selectivity, linearity, sensitivity, recovery, intra/inter-day precision) have been explicated and, after proper validation, the final procedure has been applied to the analysis of commercial compost samples.

2. Experimental

2.1. Chemicals and materials

All the chemicals employed were reagent grade or higher in quality and used with no further purification. All FQs (CIP, ENR, LEV, NOR), HCOOH (≥96%), methanol (≥99.9%), UPLC-MS grade methanol, UPLC-MS grade HCOOH and hexahydrate $Mg(NO_3)_2$ (97%) were supplied by Sigma–Aldrich (Milan, Italy). HPLC gradient grade acetonitrile (ACN) was purchased by VWR (Milan, Italy). Ultra-pure water (resistivity 18.2 MΩ cm^{-1} at 25°C) was produced in laboratory by a Millipore Milli-Q system. Anhydrous NaOH pellets (97%), H_3PO_4 (85%, w/w) and NH_3 (30% v/v) were obtained from Carlo Erba Reagents (Milan, Italy). Oasis® HLB (60 mg) cartridges were purchased from Waters (Milan, Italy). FQs stock solutions of 300 μg mL^{-1} were prepared in methanol containing 0.1% (v/v) 1 M NaOH, and stored in the dark at 4°C for a

maximum of three months. FQs working solutions of 0.04-4 μg mL^{-1} in methanol were renewed daily. All the laboratory operations involving use of standard solutions were conducted under red light.

2.2. Instruments and apparatus

A sequential microwave solvent extraction system, equipped with a volume independent IR temperature sensor, electromagnetic stirring and cooling device (Discover SP, CEM S.r.l., Cologno al Serio, Italy) was employed. A Sigma 2-16P centrifuge (Celbio S.p.a., Pero, Italy) was used after sample extraction.

The chromatographic analysis was performed with a JASCO (Lecco, Italy) X-LC system interfaced with a Thermo Scientific (Milan, Italy) LTQ XL HESI-MS/MS system. An Agilent EC-C18 Poroshell column (2.1 mm × 50 mm, 2.7 μm) equipped with a similar pre-column was used.

2.3 Sample collection and storage

Compost samples were purchased from a composting plant located in northern Italy, and their physical-chemical parameters can be found in Table S1 (Supplementary Data). Samples were left to dry at room temperature, homogenized, sieved (2 mm) and stored in the dark at 4°C until analysis. For method validation (see Section 2.5), a blank compost sample was used; the native FQs content, determined according to literature [4], was lower than method detection limits (MDLs). Sample aliquots (0.3 g) were fortified at different concentration levels into 5 mL weight-boats and stored in the dark overnight before analysis, to allow solvent evaporation and FQs adsorption to the matrix sites.

2.4 Procedures

2.4.1. Microwave-assisted extraction

10 mL of an aqueous solution 40% (w/v) $Mg(NO_3)_2 \cdot 6\ H_2O$ and 4% (v/v) NH_3 were added to 0.3 g of compost in a 35 mL Pyrex tube and introduced into the microwave cavity. After 15 min pre-stirring, microwave irradiation was provided (200 W, PowerMAX™ Simultaneous Cooling, 135°C, 15 min, stirring). The cooled suspension was centrifuged for 10 min at 4500 rpm, and the supernatant was filtered (0.22 μm) and acidified with HCOOH (1:5) before UPLC-MS analysis. In the case of the lowest spikes (25-50 ng g^{-1}),

the MAE extract was diluted to 25 mL with water (pH 3) and pre-concentrated on HLB, according to a previous work [33]. The eluate, 2 mL 0.1% HCOOH-ACN (80:20), was submitted to UPLC-MS.

2.4.2. Chromatographic determination

After an equilibration period of 3 min, 20 μL of each sample were injected in the UPLC system. Isocratic elution was performed with methanol/water–0.2% HCOOH (18:82) as the mobile phase, at a flow rate of 1 mL min^{-1}. The column temperature was maintained at 40°C, and the total run time was 16 min (including a short column washing). Mass spectrometer used an HESI (Heated Electro Spray Ionization) Probe (5.0kV) and the spray gas was N_2 with a desolvation temperature of 150°C. Gas flows were adjusted to enhance signals. Internal potentials are shown in Table 1 footnotes.

2.5. MAE followed by UPLC-MS: method validation

Due to the absence of certified reference materials, the method was validated by matrix-matched calibration and recovery tests on fortified samples [34].

Selectivity was evaluated on the basis of the UPLC-MS chromatograms obtained by MAE of blank compost samples [35].

Linearity was determined in SRM mode by three independent six-point calibration curves generated for each analyte in the range 10-100 μg L^{-1} using matrix-matched standards [34], prepared in the MAE extract of blank compost samples.

MDLs and MQLs, were calculated on the basis of the instrumental detection and quantification limits (IDLs and IQLs, respectively) evaluated from linear regression parameters [36].

Recovery was evaluated on blank compost samples independently fortified with different amounts of FQs, at concentrations ranging from values (25 ng g^{-1}) near the calculated MQLs, to 2,500 ng g^{-1} (n=3).

The intraday precision (repeatability) was evaluated on blank compost samples spiked with 500 ng g^{-1} (n=3), while the inter-day precision (within-laboratory reproducibility) was assessed on compost samples independently fortified at concentration levels ranging from 25 to 2,500 ng g^{-1} of each FQ (n=3). Precision was calculated as RSD%.

3. Results and Discussion

3.1. Development of the MAE procedure

In the recent years, use of microwaves for extraction of pollutants from environmental matrices has gained great interest. MAE has successfully been adopted to various classes of micro-pollutants (e.g. flame retardants, surfactants, pharmaceutical and personal care products) due to the small number of conditions affecting extraction, speed, reduction of organic solvent consumption, relatively low cost and increased sample throughput [37]. Various methods have been proposed also for determination of organic pollutants, including FQs, in solid environmental matrices [11,38], but presently no report is available in the literature about microwave-mediated extraction of FQs from compost.

In the present work, a focused single mode microwave extraction system was used. The pyrex pressure vessel, equipped with silicone/PTFE patented ActiVent cap, was inserted in the circular cavity. This was specially designed to maximize the microwave energy input to the sample in a high-density field allowing the extraction to proceed faster than other techniques and reducing solvent usage. A pre-stirring step was mandatory to homogenize the suspension and to favour the diffusivity of the aqueous solution into the matrix considered; the stirring was maintained during all the irradiation time.

With the aim of developing a green procedure for determination of such drugs in compost, avoiding use of organic solvents and exploiting the high stability of the FQ-Mg^{2+} complex [39], a first series of experiments was carried out on 0.3 g compost samples spiked with 2,500 ng g^{-1} FQs. The extraction was performed by 10 mL hexahydrate $Mg(NO_3)_2$ and NH_3 aqueous solution. This kind of mixture was selected on the basis of the results previously obtained by conventional MAE of FQs from soil samples [36].

For microwave irradiation, a dynamic method was selected. This allows to apply the microwave power until the actual temperature reaches the temperature control point, then the power is automatically adjusted to maintain this set point for the programmed time period. The best yield was reached when the mixture was in situ cooled during microwave irradiation under the same extraction conditions. The simultaneous cooling (PowerMAX™) prevents microwave overheating by continuously removing latent heat of the reaction (or extraction), allowing a higher level of microwave output power to be directly transferred to the reaction (or extraction) mixture.

193 The effect of temperature was investigated in the range 80-135°C. Results showed an improvement of the
194 recovery rates by increasing the extraction temperature; specifically, working at 80°C extraction yields in the
195 range 30-49% (RSD<9%, *n*=3) were obtained, while the highest recovery (up to 91%, see Table 2) was
196 attained at 135°C. Higher temperatures did not provide statistically different results (p = 0.05), as well as
197 longer extraction times (>15 min). The improved extraction yield is reasonably due to the increase of
198 pressure that is strictly dependent on temperature [40]. A temperature of 135°C (ramp time 5 min,
199 temperature holding time 15 min), leading to a pressure of 60 psi, was therefore selected. The profiles of
200 temperature, pressure and power *vs* time are reported in Fig. S1 (Supplementary data).
201 After extraction, the sample was rapidly cooled by means of a nitrogen flow directed onto the vessel in the
202 system cavity, drastically reducing analysis time.
203 On the basis of the quantitative extraction yields obtained for high FQs concentration, further experiments
204 were carried out on compost samples spiked with 500 ng g^{-1} and 100 ng g^{-1} FQs. Results reported in Table 2
205 showed quantitative recoveries also at these concentration levels.
206 To achieve accurate quantification of the lowest concentrations (25, 50 ng g^{-1}), a pre-concentration step after
207 MAE was performed, as described in Section 2.4.1. The adsorption of FQs on the HLB cartridge from the
208 MAE solution was first verified by SPE of blank compost MAE samples spiked with 0.5 μg L^{-1} FQs. The
209 recovery from the cartridge was quantitative, with mean values in the range 83-116% (*n*=3, RSDs<6%).
210 The overall MAE-SPE recoveries obtained by these experiments are reported in Table 2. As it can be seen,
211 the procedure here developed provided satisfactory absolute recovery of the four drugs, in the concentration
212 range 25-2,500 ng g^{-1}.
213 Under the selected experimental conditions, a *single* MAE cycle (20 min, 135°C) has proved useful to attain
214 extraction efficiency as high as 112%, differently from other techniques recently adopted for FQs
215 determination in compost, such as PLE [1] and UAE [2]. Indeed, the procedure selected by Lillenberg et al.
216 [1] was quite laborious, involving 5 consecutive PLE cycles, consumption of large volumes of organic
217 solvent, dilution of the PLE extract before cleanup, and finally evaporation of the SPE extract prior LC-MS
218 analysis, with recoveries in the range 58-84% (spike 12-50 ng g^{-1}). Recovery around 70% (spike 1,000-
219 10,000 ng g^{-1}) was attained by the UAE method applied by Selvam et al. [2], requiring 3 extraction cycles in
220 phosphate buffer-acetonitrile mixture, followed by SPE and evaporation of the SPE extract prior LC-MS.

221 The good extraction yield here achieved by a single MAE cycle is attributable to the high performance and
222 energy-efficient microwave instrument, and reasonably due to the strong complexes deprotonated FQs form
223 with the Mg^{2+} ions. This enables a consistent extraction of the target compounds from the matrix and, at the
224 same time, highly improves the selectivity of the aqueous extracting solution with respect to organic solvents
225 [11].
226 Compared with the two above described procedures [1,2], shorter analysis time and no use of organic solvent
227 make the present procedure simpler, less expensive and more environmentally friendly.
228
229 *3.2. Analytical figures of merit*
230 *3.2.1. Selectivity*
231 The selectivity of the proposed method was assessed by analysis of blank samples. The absence of
232 chromatographic peaks in the blank compost extracts at the retention times of the analytes excluded the
233 interference of matrix compounds accountable for false positive signals. Firm identification of the analytes in
234 the actual samples was assured by performing the analysis in SRM mode.
235
236 *3.2.2. Linearity and sensitivity*
237 As reported in Table 3, the linear regression equations, mean of three independent calibration lines, showed
238 good linearity in the studied concentration range for all FQs (r^2> 0.9995). IDLs and IQLs were between 2.3-
239 3.1 μg L^{-1} and 6.9-9.4 μg L^{-1}, respectively. The method sensitivity resulted suitable for the determination of
240 FQs in compost, being the MQLs around 10 ng g^{-1} (see Table 3).
241
242 *3.2.3. Recovery and precision*
243 All the mean absolute recoveries (%) obtained in blank compost samples spiked at different concentrations
244 (25-2,500 ng g^{-1}) are gathered in Table 2. Recovery has been calculated as the ratio between the
245 concentration determined in the MAE extract and that expected after extraction, calculated considering the
246 initial amount of analyte; in the case of the lowest spikes also the enrichment factor due to pre-concentration
247 was considered. MAE recoveries, evaluated at three concentration levels (100, 500 and 2,500 ng g^{-1}), were in

248 the range 70-112%, while the overall MAE-SPE recoveries, studied at 25 and 50 ng g^{-1}, ranged from 71% to
249 104%.
250 Precision was evaluated by calculating the RSD associated to the mean recovery obtained for each
251 concentration level (see Table 2). The intra-day precision (repeatability), calculated for 500 ng g^{-1} spike,
252 showed RSDs<6% (*n*=3); as reported in Table 2, for spike levels in the range 25-2,500 ng g^{-1} the inter-day
253 precision (within-laboratory reproducibility) showed RSDs<15% (*n*=3).
254
255 *3.3. Analysis of actual samples*
256 The analytical procedure was finally applied for the determination of FQs in real compost samples; after
257 MAE of three independent sub-samples, the extracts were pre-concentrated on HLB obtaining RSDs<11%.
258 Two different types of compost were analyzed; the CV sample mainly derived from plant debris, while the
259 CM sample was obtained by composting of vegetal wastes, urban organic wastes and sludge from
260 wastewater treatment plants. Generally, the antibiotics were quantified at concentration levels of some tens
261 ng g^{-1}. As it was reasonable to expect, the three human use FQs were not detected in the CV sample, while
262 ENR was determined at a mean concentration level of 85 ng g^{-1}. Differently, in the CM sample, 88 ng g^{-1}
263 LEV and 51 ng g^{-1} CIP were found. Typical chromatograms are shown in Figs. 1a and 1b.
264 These results are consistent with those found by Lillenberg et al. [1], reporting concentrations of CIP and
265 NOR around 20 ng g^{-1}, and further confirm that such compounds are not completely eliminated during
266 composting. This implies that land application of compost as fertilizer could involve an environmental
267 impact on the soil ecosystem.
268
269 **4. Conclusions**
270 A novel analytical method for FQs determination in compost has been developed. The proposed procedure
271 entails an efficient microwave-assisted extraction carried out by a highly performant microwave system, not
272 yet used for FQs extraction from compost. Quantitative recovery was achieved by a single extraction cycle
273 (20 min, 135°C) using aqueous Mg^{2+} as the extracting agent, thus avoiding use of any organic solvent.
274 Analytes have been quantified and confirmed by UPLC-MS. The method showed good performance in terms
275 of linearity, recovery and intra/inter-day precision. Sensitivity was suitable for determination of FQs in

compost at the actual concentration levels. Analysis of real compost samples showed FQs concentrations up to ca. 90 ng g^{-1}, evidencing that such antibiotics are not completely removed during composting. Compared with the procedures reported in the literature (PLE, UAE), the present method proved to be simpler, faster, costless and more environmentally friendly.

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Figure captions

Figure 1 UPLC-MS SRM chromatograms obtained for CV (a) and CM (b) actual compost samples.

Table captions

Table 1 Optimized SRM conditions for the target FQs.

Table 2 Mean absolute recoveries (%) and RSD (%) values for the inter-day precision (within-laboratory reproducibility) obtained on compost samples spiked with FQs (n=3).

Table 3 Calibration curves and correlation coefficients obtained in SRM mode for each FQ in matrix-matched solution.

Supplementary Data.

Table S1. Physical-chemical parameters of the compost samples.

Figure S1. Profiles of temperature, pressure and power *vs* time.

Figure 1

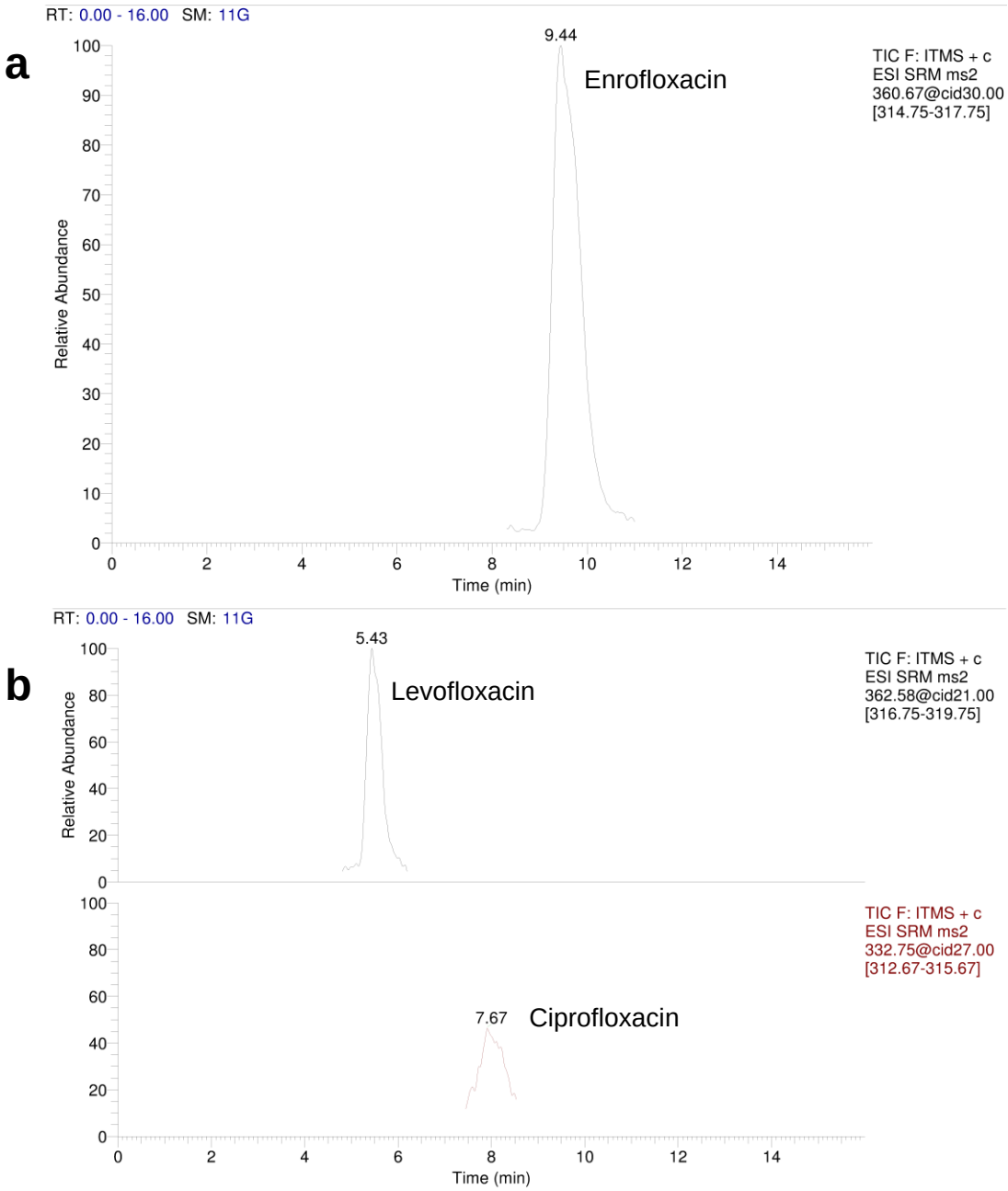
a
RT: 0.00 - 16.00 SM: 11G
9.44
Enrofloxacin
TIC F: ITMS + c
ESI SRM ms2
360.67@cid30.00
[314.75-317.75]
Relative Abundance
Time (min)
b
RT: 0.00 - 16.00 SM: 11G
5.43
Levofloxacin
TIC F: ITMS + c
ESI SRM ms2
362.58@cid21.00
[316.75-319.75]
TIC F: ITMS + c
ESI SRM ms2
332.75@cid27.00
[312.67-315.67]
7.67
Ciprofloxacin
Relative Abundance
Time (min)

Table 1

	Parent Peak[a,b] (m/z)	MS2[c] (indentity)	Normalized Collision Energy	RT (min)
CIP	332.75 $[M+H]^+$	314.17 (MH^+ - H_2O)	21	7.20-8.00
ENR	360.67 $[M+H]^+$	316.25 (MH^+ - CO_2)	30	8.80-10.90
LEV	362.58 $[M+H]^+$	318.25 (MH^+ - CO_2)	28	5.00-6.00
NOR	320.58 $[M+H]^+$	302.08 (MH^+ - H_2O)	27	6.20-7.20

[a] HESI Probe: Gas=N_2, T=150°C, Voltage=5.0kV; Capillary T=275°C, Voltage=48V, Tube Lens=70V
[b] Tune Settings: Multipole 00 Offset = -2.5V, Lens 0 = -4.5V, Multipole 0 Offset = -5.25V, Lens 1 = -9.00, Gate Lens = -66.0V, Multipole 1 Offset = -6.5V, Multipole RF Amplitude (p-p)= 400V, Front Lens = -6.0V.
[c] Settings for MS2: SRM detection by CID (Collision Induced Dissociation); Isolation Width: ±2.5d; Activation Q: 0.250; Activation Time 30.0 msec. Isolation width for quantitation ±3.0d.

Table 2

	Mean absolute recovery (%) and inter-day precision [a]				
Spike (ng g^{-1})	2,500	500	100	50[b]	25[b]
CIP	81(11)	80(10)	70(10)	73(13)	72(10)
ENR	85(10)	90(8)	104(9)	84(14)	71(12)
LEV	91(9)	99(11)	94(10)	74(12)	103(13)
NOR	80(8)	88(12)	112(11)	84(13)	104(11)

[a] Reported in parentheses as RSD%.
[b] MAE-SPE recovery.

Table 3

	Equation[a]	Linearity (r^2)	IDL ($\mu g\ L^{-1}$)	IQL ($\mu g\ L^{-1}$)	MDL[b] ($ng\ g^{-1}$)	MQL[b] ($ng\ g^{-1}$)
CIP	y=142(2) x + 365(128)	0.9995	3.1	9.4	3.0	9.0
ENR	y=238(3) x - 214(155)	0.9997	2.6	8.0	2.5	7.6
LEV	y=282(4) x - 703(204)	0.9997	3.0	9.1	2.9	8.6
NOR	y=150(2) x - 323(84)	0.9998	2.3	6.9	2.2	6.6

[a] Calculated as peak area (y) vs. FQs concentration (x); in parentheses slope and intercept uncertainties obtained by OLLSR (Ordinary Linear Lowest Squares Regression).
[b] Calculated from OLLSR parameters.

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