MULTIRESIDUE DETERMINATION OF QUINOLONES REGULATED BY THE EUROPEAN UNION IN BOVINE AND PORCINE PLASMA. APPLICATION OF CHROMATOGRAPHIC AND CAPILLARY ELECTROPHORETIC METHODOLOGIES.

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ABSTRACT

This paper presents the multiresidue determination of the series of quinolones regulated by the European Union (marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid and flumequine) in bovine and porcine plasma using capillary electrophoresis and liquid chromatography with ultraviolet detection (CE-UV, LC–UV), liquid chromatography–mass spectrometry and –tandem mass spectrometry (LC–MS, LC-MS/MS) methods. These procedures involve a sample preparation by solid-phase extraction (SPE) for clean-up and preconcentration of the analytes before their injection in the separation system. All methods give satisfactory results in terms of linearity, precision, accuracy and limits of quantification. The suitability of the methods to determine quinolones was evaluated by determining the concentration of enrofloxacin and ciprofloxacin in real samples from pig plasma and cow plasma.

Keywords: quinolones; cow and pig plasma; liquid chromatography; capillary electrophoresis; mass spectrometry; tandem mass spectrometry

1. INTRODUCTION

Many classes of antibiotics are widely administered to food-producing animals such as cattle, swine and poultry for the purposes of prevention and treatment of several diseases as well as for promoting growth (Stolker *et al.* 2005). As a result, these substances can be present in food products of animal-origin and may pose a health threat to consumers (Blasco *et al.* 2007; Companyó *et al.* 2009).

There is increasing international concern that the indiscriminate use of quinolones and other antibiotics has led to increased bacterial resistance. Resistant bacteria from animals can infect the human population not only by direct contact but also via food product of animal origin. These resistant bacteria can colonise humans or transfer their resistance genes to other bacteria belonging to the endogenous human flora (Fabrega *et al.* 2008). This is now regarded as a public health hazard, primarily due to the increasing prevalence of treatment failures (Turnidge 2004). The World Health Organisation, has recommended the discontinuing use of antimicrobial growth promoters that belong to an antimicrobial class used in humans. The Health Ministries in the European Union agreed to discontinue the use of all antimicrobial growth promoters by 2002 (Angulo *et al.* 2004). To ensure safety, the European Union (EU) has established safe maximum residue limits (MRLs) for residues of veterinary drugs in those animal tissues that enter the human food chain (Council Regulation (EEC) No. 2377/90) (Comission EU 1990). It is hoped that this will minimize the risks to human health. The development of accurate and sensitive methods in biological fluids as plasma or serum could be of great interest to relate its content with the amount of quinolone in tissue without requiring the immediate slaughter of the animal.

There are numerous studies and reviews in the literature addressing quinolones determination in the veterinary field (Blasco *et al*. 2007; Companyó *et al.* 2009; Moreno-Bondi *et al.* 2009; Boglialli *et al*. 2007; Garcés *et al.* 2006; Van Hoof *et al.* 2005; Pecorelli *et al.* 2005; Hermo *et al.* 2005; Hermo *et al.* 2006; Bailac *et al.* 2004). There are a great number of studies specifically addressing to determination of quinolones in animal tissues. Most papers focus on a specific matrix such as muscle, kidney or liver, but the number of studies specifically addressing quinolones in bovine or pig plasma remains quite low and generally analysing only two or three quinolones (Hernández-Arteseros *et al.* 2002; Idowu *et al.* 2004).

Most of the methodologies currently applied to quinolone sample preparation in either plasma or serum are based on solid-phase extraction (SPE) techniques due to its inherent double function: pre concentration and clean-up of extracts (Samanidou *et al.* 2005; Zoutendam *et al.* 2003).

In the recent decade, separation techniques, primarily liquid chromatography (LC) and capillary electrophoresis (CE) coupled to ultraviolet detection (UV), fluorescence detection (FD), and mass spectrometry (MS) have been widely used for drugs analyses involving complex samples (Hermo *et al.* 2005; Hermo *et al.* 2006; Hernández-Arteseros *et al.* 2002; Jiménez-Lozano *et al.* 2004). The use of electrospray ionization (ESI) sources and tandem MS (MS/MS) instruments has allowed for improvements in sensitivity as well as in reduced analysis time, making it possible to rapidly achieve complete analyte separation (Picó *et al.* 2008; Le Bizec *et al.* 2009; Bogialli *et al.* 2009; Andreu *et al.* 2007; Hermo *et al.* 2008).

The present paper describes the optimization and validation of a new method allowing for the simultaneous identification and quantification of eight quinolones by CE-UV, LC-UV, LC-MS, and LC-MS/MS in plasma samples. Several solid-phase extraction (SPE) cartridges were tested for the clean-up purposes in order to obtain cleaner extracts and better recoveries. Quality control parameters (Commission EU 2002; US Department 2001) were established and compared when the samples of cow and pig plasma were analyzed by LC and CE. The developed method has been applied to determination and quantification of enrofloxacin and its main metabolite ciprofloxacin in pig and cow plasma samples from animals treated with ENR.

EXPERIMENTAL

2.1. Reagents

Quinolones were purchased from various pharmaceutical firms: ciprofloxacin (CIP) (Ipsen Pharma, Barcelona, Spain), sarafloxacin (SAR), difloxacin (DIF) and tosufloxacin (TOS) (Abbot, Madrid, Spain), enrofloxacin (ENR) (Cenavisa, Reus, Spain), danofloxacin (DAN) (Pfizer, Karlsruhe, Germany), marbofloxacin (MAR) (Vetoquinol, Barcelona, Spain), piromidic acid (PIR), oxolinic acid (OXO), flumequine (FLU) and lomefloxacin (LOM) (Sigma, St. Louis, MO, USA), norfloxacin (NOR) (Liade, Boral Quimica, Barcelona, Spain), and pipemidic acid (PIP) (Prodesfarma, Barcelona, Spain). Structures of the studied quinolones are shown in figure 1. TOS, LOM, NOR and PIP were used as an internal standard in liquid chromatography and PIR was used as an internal standard in capillary electrophoresis.

Plasma samples were purchased from *Seguridad y Bienestar Animal* S.L. (Bigues i Riells, Spain). All reagents were of analytical grade. Merck (Darmstadt, Germany) supplied phosphoric, acetic, formic, trifluoroacetic (TFA) and trichloracetic (TCA) acids, ammonium acetate, sodium hydroxide (NaOH), ammonia, hexane, acetonitrile (MeCN) and methanol (MeOH). Citric acid and potassium hydrogenphthalate were supplied by Fluka (Buchs, Switzeland). Ultrapure water generated by the Milli-Q system (Millipore, Billerica, MA, USA) was also used.

The SPE cartridges used in this study were as follows: ENV + Isolute (3 cm^3 /200 mg; Isolute Sorbent Technologies, Hengoed, UK), Oasis HLB (3 cm^3 /60 mg; Waters, USA) and Strata X (1 cm^3 /30 mg; Phenomonex, Torrance, USA).

The 0.45 μm pore size nylon filter membranes (Sharlab, Barcelona, Spain) were used to filter the extracts before the injection in the electrophoretic system.

2.2. Instrumentation

The LC-UV equipment by consisted of an HP 1100 series HPLC employing an injection valve with a 20 μL sample loop. Detection was performed using a diode array detector (DAD) at maximum quinolone wavelengths (250 nm for FLU and OXO, 290 nm for LOM and MAR, and

280 nm for the rest of quinolones). A Chemstation for LC 3D Rev. A 08.03(847) software (Agilent Technologies) was used for data analysis.

The LC-MS and LC-MS/MS analysis were performed using an API 3000 triple-quadrupole mass spectrometer (PE Sciex) and data were collected with Analyst 1.4.2 software (Applied Biosystems, Foster City, CA, USA).

Chromatographic separation of the quinolones was performed on a Zorbax Eclipse XDB-C8 (150 mm x 4.6 mm i.d.; Agilent Technologies, Waldbronn, Germany) column protected by a Kromasil C8 column (20 mm x 4.5 mm i.d.; Aplicaciones Analíticas, Barcelona, Spain).

A CE Beckman P/ACE system 5500 (Beckman Instruments, München, Germany) equipped with a photodiode array detector was used. The separation was performed as usual with the cathode at the detector end of the capillary. Detection was performed at the same wavelengths than in LC-UV. P/ACE 5500 software of Beckman was used for data acquisition.

An uncoated fused-silica CE capillary column of 57 cm (50 cm from the inlet to the detector) and 75 μm internal diameter (Polymicro Technologies, Phoenix, USA) was used.

A Crison 2002 potenciometer (±0.1 mV) (Crison, Barcelona, Spain) with an Orion 81025 C Ross combination pH electrode was used to measure the pH of the mobile phase.

A Mikro 20 mini-centrifuge from Hettich Zentrifugen (Hettich Zentrifugen, Germany) was used to centrifuge the final extract. SPE was carried out using a Supelco vacuum 12-cartridge manifold (Bellefonte, PA, USA) connected to a Supelco vacuum tank.

2.3. Standards and stock solutions

Individual quinolone stock solutions of MAR, CIP, DAN, ENR, SAR, DIF and NOR at a concentration of 500 μg mL^{-1} were prepared in 50 mM acetic acid aqueous solution. Individual quinolone stock solutions of OXO and FLU at a concentration of 100 and 500 μg mL^{-1},

161 respectively, were prepared in MeCN. Individual quinolone stock solutions of TOS, LOM and
162 PIP at a concentration of 100 μg mL^{-1} were prepared in 50 mM acetic acid: MeCN (80:20; v/v).
163
164 PIR (internal standard used in CE) was prepared in 50 mM acetic acid-MeCN (20:80; v/v) at a
165 concentration of 100 μg mL^{-1}. Working solutions, containing all quinolones except the internal
166 standard, were prepared using stock solutions diluted with Milli-Q water at a concentration of 40,
167 10, 5, 1, 0.5, 0.05 and 0.01 μg mL^{-1}. These solutions were stored at 4 ºC.
168
169 Standard buffers of potassium hydrogenphtalate (0.05 mol kg^{-1}), prepared in a hydroorganic
170 mixture (9 and 14 % MeCN), with a pH of 4.25 and 4.38, respectively (Barbosa *et al.* 1999),
171 were used to calibrate the pH-meter.
172
173 2.4. Procedures
174
175 2.4.1. Sample treatment
176 0.5 millilitres of cow or pig plasma was placed in a 1.5 mL polypropylene eppendorf tube.
177 Appropriate volumes of working solutions and I.S. were added. When the final extracts were
178 analyzed by LC, the concentration of I.S. was 1000 μg L^{-1}. When the extracts were analyzed by
179 CE, a concentration of 2500 μg L^{-1} of I.S. was used. After gently mixing of samples and in order
180 to improve the retention of quinolones on the SPE cartridge, Milli-Q water was added to obtain a
181 final volume of 1.5 mL.
182
183 2.4.2. Protein precipitation
184 Prior to carrying out SPE extraction, different agents were tested for the protein precipitation. 1
185 mL MeCN or 0.5 mL 10 % TCA was added to the plasma sample and mixed for 1 min using a
186 vortex. The samples were then centrifuged at 12000 rpm for 5 min. Supernatant was transferred
187 to a 15 mL polypropylene tube and 10 mL of Milli-Q water was added to decrease any high
188 concentrations of MeCN or TCA.
189

190 2.4.3. Solid-phase extraction

191 Three different commercial cartridges based on a polymeric sorbent (Strata X, Oasis HLB and
192 ENV+ Isolute) were examined in this study. Strata X are based on a poly(benzylpiperidone)
193 polymer, Oasis HLB on a poly(divinylbenzene-co-N-vinylpyrrolidone) polymer, and ENV+
194 Isolute on a hyper cross-linked polystyrene-divinylbenzene polymer with a hydroxylated surface.
195 In table 1, the optimized SPE conditions (activation, clean-up and elution) are shown. The
196 solution obtained from the SPE was evaporated to dryness at 45 ºC under a stream of air and the
197 residue was dissolved in corresponding mobile phase depending on the separation techniques
198 used.

199

200 2.4.4. Separation

201

202 2.4.4.1 Chromatographic conditions

203 For LC-UV, we used a gradient program featuring a mobile phase that combined solvent A (10
204 mM citric acid: MeCN (91:9, v/v), adjusted with NH_3 and solvent B (MeCN) as follows: from 0
205 to 5 min the mobile phase contained 9% MeCN; from 5.0 to 11.5 min, the percentage of MeCN
206 linearly increased to 12.5%; from 11.5 to 22.0 min, it increased to 27%; from 22.0 to 24.0 min, to
207 39%, maintaining this level until the 26.0 min mark. Finally the MeCN percentage decreased to
208 9% from 26.0 to 29.0 min. The flow rate was 1.5 mL min^{-1}, the injection volume was 20 µL. The
209 separation of the quinolones was obtained in 26 min. The pH of the mobile phase were adjusted
210 at 5 when samples of cow plasma and pH 4.5 when samples of pig plasma were analysed.

211

212 For LC-MS and LC-MS/MS, the citric acid from the mobile phase was replaced by ammonium
213 acetate to obtain a more volatile mixture. The initial mobile phase used in LC–MS and LC–
214 MS/MS was MeCN:water (14:86,v/v) with 5 mM ammonium acetate and adjusted to a pH 2.5
215 with formic acid. In this case, was not necessary to achieve complete separation if the ions
216 monitorized are different. Because of this reason, the chromatographic gradient used was as
217 follows: from 0 to 5.0 min, the MeCN percentage linearly increased to 21%; from 5.0 to 6.0 min,
218 it increased to 24%; from 6.0 to 7.5 min, to 25%; and from 7.5 to 9.0 min, to 54% where it
219 remained until 11.0 min. Finally the MeCN percentage decreased to 14% from 11.0 to 14.0 min.

The flow rate was 1.0 mL min^{-1}. Under these conditions, the chromatographic separation of the 10 quinolones was achieved in approximately 12 min.

2.4.4.2. Electrophoretic conditions

50 mM of H_3PO_4 adjusted at pH 8.4 with NaOH was used to prepare the electrophoretic solutions. All running buffer solutions, were prepared weekly and stored in a refrigerator before use and were filtered through a membrane filter (0.45 μm).

When a new capillary was used, the capillary was activated for 30 min with 1 M NaOH solution, followed by 30 min with Milli-Q water. For preconditioning, the capillary was rinsed with 1 M NaOH and Milli-Q water for 15 min each, and then equilibrated with running buffer for 15 min applying a voltage of 15 kV. After each injection, the capillary was washed for 1 min with 1 M NaOH, 1 min with Milli-Q and 1 min running buffer to maintain proper reproducibility of run-to-run injections. Sample injections were done in a hydrodynamic mode over 2 s under a pressure of 0.5 psi at 25 ºC and separation voltage was 15 kV, in order to avoid high intensities.

2.4.5. ESI–MS and ESI–MS/MS parameters

The MS conditions were optimized for the quinolones studied, directly injecting each compound individually at a flow-rate of 0.05 mL min^{-1} (Hermo *et al.* 2006).

When samples were analyzed, a post-column LC split (3:1) was used to reduce the flow-rate entering into the electrospray ionization source. A turbo ion-spray source in positive mode was used, since the amino group present in most quinolones is easily protonated in acidic media. Thus, we used the following settings: capillary voltage 4500 V; temperature 400 °C; nebulizing gas (NEB) (N_2) 10 (arbitrary units); curtain gas (CUR) (N_2) 12 (arbitrary units); collision gas (N_2) 15 (arbitrary units); focusing potential (FP) 200 V; and entrance potential (EP) 10 V. In MS, Single-ion monitoring mode (SIM) experiments were performed using a dwell time of 200 ms to detect ions. Ions monitored by SIM mode and cone voltage for all compounds are displayed in table 2.

MS–MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions, in the collision cell of the triple quadrupole mass spectrometer. They were then

mass-analyzed using the second analyzer of the instrument. In all experiments, CAD gas (nitrogen) of 4 (arbitrary units) was used. Multiple reaction monitoring (MRM) mode was chosen for the experiments in MS-MS. The specific transition and collision energies used to quantify and confirm the quinolones in plasma samples are also shown in table 2.

2.5. Quality parameters

Analyte stability for a given matrix is relevant only to that matrix and should not be extrapolated to other matrices. For this reason, it is regarded as a fundamental parameter for the validation of bioanalytical methods (Commission EU 2002; US Department 2001). Since the analysis is not performed immediately after sample collection, it is very important to know if drugs are stable at least during the storage time before the analysis.

Quinolone stability in plasma was evaluated based on the freeze-thaw (F/T) cycles, storage stability in the refrigerator (holding time), and autosampler stability. F/T cycle consisted of the following: plasma samples containing each quinolone were stored at -20 ºC. Samples were removed from deepfreeze, equilibrated to room temperature and analyzed in duplicate. This process was repeated three times and the results were compared with control samples that had not undergone freezing. Storage stability in the refrigerator (holding time) of quinolones in plasma at 4 ºC was evaluated in spiked samples. Samples were periodically removed from the refrigerator (at 0, 6, 12, 24, 48, 72, and 96 h), equilibrated to room temperature, and analyzed in duplicate. The results were then compared to determine refrigerator stability. Auto sampler stability of quinolones in the reconstituted plasma extracts was evaluated for 36 h in cow plasma and for 50 h in pig plasma.

Quality parameters were established for the LC-UV, LC-MS and LC-MS/MS techniques for the developed method in cow and pig plasma, while CE-UV techniques were used for the analysis in pig plasma (Commission EU 2002; US Department 2001). To determine LOD and LOQ values, the plasma were spiked with working solutions at different concentration levels (between 5 and 50 $\mu g\ L^{-1}$ for LC-UV, between 100 and 500 $\mu g\ L^{-1}$ for CE-UV, and between 0.1 and 25 $\mu g\ L^{-1}$ for LC-MS and LC-MS/MS) in duplicate and subsequently injected in the separation system. The LOD and LOQ values were calculated by using a signal-to-noise ratio of 3 and 10, respectively. The LODs in LC-MS were obtained in single ion monitoring (SIM) mode using the most

282 abundant ion available. The LODs in LC-MS/MS were obtained with the transition of higher S/N
283 reaction monitoring (MRM) mode.
284
285 In order to establish calibration curves by LC-UV, LC-MS and LC-MS/MS, between seven and
286 twelve concentration levels of spiked cow and pig plasma, samples (LOQ-2500 μg L^{-1}) were
287 prepared in duplicate and injected in the separation system. By CE-UV, eight concentration levels
288 of spiked pig plasma samples were prepared in triplicate (LOQ-10000 μg L^{-1}) due to dispersion
289 dates. The results were presented with response of quinolone/ internal standard ratio vs. the
290 quinolone/internal standard concentration ratio. Linear calibration curves were established using
291 TOS as internal standards in LC and using PIR by CE.
292
293 In addition, the composition of these samples is rather complex with high protein and lipid
294 contents, as well as other nutrients such as vitamins or mineral elements. Such a complex matrix
295 requires extensive sample preparation procedures in order to quantitatively extract the antibiotics,
296 avoiding matrix interferences. The observation of signal suppression (matrix effect) for some
297 fluoroquinolones in ESI LC-MS and LC-MS/MS is a notable problem to discuss, due to the co-
298 eluting interferences. For evaluate the presence or absence of matrix effect, calibrations curves
299 prepared in matrix and subjected to the sample treatment were compared with calibrations curves
300 obtained when the plasma was substituted by water.
301
302 Recovery experiments were performed by comparing the analytical results of extracted standard
303 samples (internal standards added prior to extraction) with those with plasma samples spiked
304 after the extraction procedure, which exhibited 100% recovery in the concentration range where
305 calibration curves were established.
306
307 To evaluate the intra-day-precision, five spiked plasma samples at three concentration levels each
308 (50, 1000 and 2500 μg L^{-1}) were prepared and analysed by LC-UV. When LC-MS and LC-
309 MS/MS are the techniques used the high level were decreased until 2000 μg L^{-1}. When CE-UV is
310 the technique used the levels of concentrations are 1000, 4000 and 8000 μg L^{-1}. The procedure
311 was repeated on three different days to determine the inter-day-precision.
312

313 2.6. Positive pig and cow plasma samples.

314 The method was applied to analyze treated pig plasma samples obtained from four pig specimens
315 (A-D) orally medicated with 200 mg of ENR by kg of cattle feed, during five consecutive days.
316 Plasma was stored at -20ºC until its analysis. The positive samples were analysed by LC-UV and
317 CE-UV. ENR and CIP concentrations were quantified by a daily prepared calibration curve.
318 Samples from pig intramuscular medicated with 2.5 mg kg^{-1} ENR were analysed after plasma
319 samples were collected on 0h, 2h, 6h, 12h and 24h after treatment. Positive cow plasma samples
320 were obtained from 3 cow specimens (A-C) orally medicated during 5 days with 1.5 g of ENR
321 dissolving in drink water. The samples were collected while the animal was undergoing medical
322 treatment.
323 Plasma was obtained by centrifugation and stores at -20ºC until its analysis. The positive samples
324 were analysed with the techniques more sensitive and selective, LC-MS and LC-MS/MS, in order
325 to identify the antibiotics ENR and its main metabolite CIP.
326

RESULTS AND DISCUSSION

3.1. Optimization of LC conditions

In previous studies, analyses of quinolones from food samples by LC-UV were performed with 10 mM citric acid with different percentages of MeCN at pH 4.5 by using a gradient elution timetable (Garcés *et al.* 2006; Hermo *et al.* 2006; Bailac *et al.* 2004). When the biological sample is cow or pig plasma, the pH of the mobile phase should to be changed to pH 5.0 in order to obtain better resolution between peaks of SAR and DIF. The maximum MeCN percentage value was set at 39%. Any increase above 40% resulted in a corresponding baseline increase (occurring between 25 and 27 min), which caused problems with FLU detection. Figure 2 shows the separation of quinolones in spiked cow plasma at a concentration of 1000 μg L^{-1} (for each quinolone), with the optimized analysis timetable. In blank samples, both LOM and TOS I.S. used are observed. Optimal separation of the eight quinolones and the two I.S. was achieved in less than 30 min.

As a more volatile mobile phase is needed for LC-MS and LC-MS/MS methods, citric acid from the mobile phase was replaced by ammonium acetate. In this case, as full separation of the quinolones was unnecessary, higher MeCN concentrations were used in order to reduce the analysis time. Under these new conditions, separation of eight quinolones from plasma as well as of the two internal standards used was achieved in 12 min, as can be observed in figure 3. Figure 4 shows the resulting ion-reconstituted chromatogram when bovine spiked plasma was analyzed using the MRM mode. Table 2 depicts the most abundant product ions that corresponded to $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-CO_2]^+$. In this table, we also show the quantification transition of the studied quinolones, including the I.S. The transition $[M+H]^+ \rightarrow [M+H-H_2O]^+$ was used for CIP, DAN, SAR, DIF, OXO, FLU, and both I.S., TOS and LOM, while $[M+H]^+ \rightarrow [M+H-CO_2]^+$ was used for MAR, and ENR. The identification transitions are also shown in table 2. As an illustrative example, the product ions from TOS and MAR, as well as our spectra interpretations, are shown in figure 5.

357 3.2. Optimization of CE separation

358 Using CE methodologies Jiménez-Lozano *et al.* 2004), a simple and rapid method of separating
359 and identifying the series of quinolones was developed. The optimization of the separation was
360 carried out using pig plasma samples fortified at 10 mg L^{-1} of each quinolone. For this purpose,
361 two different length of the capillary (Lt 47 and 57 cm) were tried, with different times of
362 hydrodynamic injection (2, 4 and 6s). Best results were obtained with a capillary length of 57 cm
363 and 2 s hydrodynamical injection at 50 psi. A 15 kV voltage with a normal polarity was applied.
364 Under these conditions, the time need for the total separation of the compounds was 14 min.
365
366 3.3. Selection of the SPE cartridge
367 Various hydrocarbon chain-, polymeric-, and mixed sorbent-based cartridges have been
368 previously evaluated for quinolones determination in different matrices (Hermo *et al.* 2005;
369 Jiménez-Lozano *et al.* 2004). In plasma, different types of sorbents were tested in this work
370 (Strata X, Oasis HLB and ENV+ Isolute). To detect interfering substances from the matrix, blank
371 bovine plasma was analyzed using these cartridges. Similar chromatograms were obtained with
372 the three sorbents, although interfering substances were present to a lesser extent when Strata X
373 were used. Good recoveries (higher than 90%) were obtained with the three sorbents. In table 1
374 the optimized experimental conditions for each sorbent are shown. To obtain good recoveries
375 with ENV+ Isolute and Oasis HLB, a greater elution solution volume than that provided by Strata
376 X was needed. As a consequence, evaporation time and sample preparation time was higher with
377 ENV+ Isolute and Oasis HLB. When the elution solution volume added to ENV+ Isolute and
378 Oasis HLB was the solution used for Strata X (2 mL of 1% TFA:MeCN (25:75, v/v)), decrease
379 evaporation times, but quinolone recoveries also decreased (≈ 70% and 80%, respectively). To
380 decrease sample preparation time and organic solvent consumption, thereby ensuring high
381 recoveries, Strata X was selected as the best cartridge for the analysis of plasma samples.
382
383 Although endogenous plasma interference was minimized with Strata X, the presence of
384 interfering substances was not eliminated. To obtain cleaner baselines, we tried different washing
385 steps in SPE and also introduced a protein precipitation step before SPE application. However,
386 regardless of the additional washing solution used [1 mL 0.5 % TFA:MeCN (90:10), 2 mL 0.5 %
387 TFA:MeOH (90:10), 2 mL 0.5 % TFA:MeOH (80:20) and 3 mL hexane], there were no

388 significant differences between chromatograms involving washing solely with water and those
389 including additional steps. When we introduced protein precipitation steps involving MeCN or 10
390 % TCA, cleaner baselines were obtained, although quinolones recoveries were 70% for MAR and
391 CIP and 50% for the others. In both cases, a part of quinolones elute in the organic solvent and
392 the acid fraction, respectively and thus, to obtain high recoveries with maximum simplicity, the
393 protein precipitation step was not considered, and 2 x 1 mL Milli-Q water was selected as the
394 ideal wash solvent. Indeed, it removed the majority of impurities without affecting quinolone
395 retention and recovery.
396
397 Eluates were evaporated to dryness at 45 ºC under a stream of air and residue was redissolved in
398 200 µL of mobile phase when the LC was used and 100 µL of MeCN:Milli-Q water (50:50;v/v)
399 was added for analysis by CE.
400
401 3.4. Selection of the internal standard
402 For the selection of the I.S. in this work, two requirements were considered: firstly, the time
403 retention of the I.S. was proper to every quinolones studied and, the second requirements consist
404 to minimize the interfering substances from the plasma matrix with the standard internal. Under
405 these premise, four different quinolones were tested as potential I.S. for LC-UV quantification:
406 NOR, PIP, LOM and TOS. NOR has not been used as an I.S. in cow plasma due to an interfering
407 substance that appears at the same retention time than NOR. PIP was the first substance to be
408 separated from the mixture at the 5.7 min mark, far below that of the next substance, whose
409 retention time was 10 min. On the other hand, LOM and TOS present adequate retention times
410 and minimum interferences. At 13.4 min, LOM fell between CIP and DAN, while at 20 min,
411 TOS fell between DIF and OXO. Therefore, we used both substances LOM and TOS as I.S.
412
413 Table 3 shows as an example, the slope, intercept and recovery of quinolones for both I.S. in cow
414 plasma, when LC-UV is used. A two-factor ANOVA test was applied to evaluate the recoveries
415 values, obtained from the two I.S. selected (LOM and TOS) for all quinolones. The ANOVA test
416 revealed differences in recoveries when both internal standards were used. As can be seen in
417 table 3, the calibration curves are also different using both I.S. Ratio of 1,8 between slopes are
418 obtained. As a similar working concentration of I.S. had been used, we attributed this variance to

419 the different absorbance levels of LOM and TOS in UV. These differences between I.S. reflect
420 the high response of LOM, which decreased the area of quinolone/area I.S. ratio. Consequently,
421 there was a decrease in the slope value of the calibration curve. When LOM and TOS were
422 analyzed by LC-MS also a different signal is obtained as can be seen in figure 6A, where ions
423 monitored by LC-MS for LOM and TOS are shown at the same concentration of 1000 μg L^{-1}.
424 Figure 6B shows the different intensity of the quantification transitions for LOM and TOS when
425 quinolones were analyzed by LC-MS/MS. The slope values revealed that significantly greater
426 sensitivity was obtained in these techniques when TOS served as the I.S. Thus, we recommend
427 TOS as the I.S. when cow or pig plasma were analyzed by LC.
428
429 Using CE-UV, different quinolones were tested as potential internal standards for quantification.
430 TOS and PIR present the best characteristics, but TOS migrates between CIP and MAR, making
431 difficult the quantification of CIP and MAR, so PIR was selected as I.S. in the CE analysis.
432
433 3.5. Quality parameters
434 The quinolones assay was validated with respect to stability, LOD and LOQ, linearity, precision,
435 accuracy and selectivity (Commission EU 2002; US Department 2001).
436
437 Antibiotic stability was evaluated in cow and pig plasma and analyzing by LC-UV. Quinolone
438 stability (at 1000 μg L^{-1} per compound) was evaluated based on the F/T cycles, storage stability
439 in refrigerator (holding time) and autosampler stability. These experiments were performed as
440 described in Section 2.5. Similar results were obtained for F/T cycles. Recoveries obtained in this
441 study were similar to those obtained from control samples without freezing. In relation to the
442 storage stability in the refrigerator, only some differences are obtained for CIP and SAR
443 depending on the kind of plasma (pig or cow). Figure 7, shows the recovery vs. time that plasma
444 samples stayed in the refrigerator at 4ºC. Both CIP and SAR in cow plasma present similar
445 recoveries over the period of time studied. When pig plasma is considered CIP and SAR are
446 stable at least for 24-48 h, and after this time recovery clearly decrease. The autosampler stability
447 of the extracts was also studied for 36 h with extracts from cow plasma and for a long period of
448 time with extracts from pig plasma. Similar results were obtained to those obtained from control
449 samples. A one-factor ANOVA test was applied to evaluate the concentration of each quinolone

450 in the three stability studies. The results obtained showed no statistically significant differences
451 between measurements ($p_{calculated} > 0.05$) and because of this, we concluded that the quinolones
452 were stable throughout 3 F/T cycles and in mobile phase solution for 36 h stayed in the
453 autosampler. All quinolones were also stable at least for 96h in the refrigerator except for CIP
454 and SAR in pig plasma, whose concentration decreases from 24 to 48 h.
455
456 The LOD and LOQ of quinolones in cow and pig plasma by LC and CE were estimated based on
457 the results of two plasma replicates spiked at different levels of concentration. LOD and LOQ
458 values are shown in table 4. Lowest LOD and LOQ values were obtained when MS-MS was used
459 as the detection technique. The LOD and LOQ values obtained from LC-MS were from 5 to 20
460 times below those determined by LC–UV, while for LC-MS/MS, the LOD were at least 50 times
461 less than those of LC–UV. CE-UV had offer lower sensitivity in terms of LOD and LOQ that are
462 between 7 and 15 times higher than the obtained values using LC-UV.
463
464 The calibration curves established for all compounds yielded high correlation coefficients, all of
465 them higher 0.91, as can be seen in table 4. The calibration curve generated for the some of the
466 quinolones by LC-MS and LC-MS/MS revealed a loss of linearity at high levels of concentration
467 (above 2000 $\mu g\ L^{-1}$). In evaluating the slope of calibration curves, similar values in the slope of
468 the curves from pig and cow plasma in LC-UV (except for OXO) were obtained and in
469 conclusion a similar behavior of quinolones in the two matrixes is observed.
470
471 One significant drawback of electrospray mass spectrometry is that the ionization source is highly
472 susceptible to co-extracted matrix component. It may also produce erratic quantitative results in
473 LC-ESI-MS analysis due to the “matrix effect”. Because of this, the variation instrument
474 response (matrix effects) on the signal intensities of target antibiotics in LC-ESI-MS was
475 investigated. For evaluate this effect, it was prepared calibration curves where the plasma was
476 substituted by water. The slope of this calibration curves where compared with the slope of the
477 calibration curves prepared in plasma. Table 5 shows the results of the curves prepared in water
478 and registered by ESI-MS and ESI-MS/MS. Comparing these data with those obtained in plasma
479 (table 4) and applying a two vies ANOVA test, we concluded that the calibration curves (in
480 matrix) and standard curve (in water) registered in MS and MS/MS are different and a matrix

481 effect was found for most antibiotics in cow and pig plasma. An ANOVA test made to compare
482 cow and pig plasmas show no difference between these plasmas, and in conclusion these samples
483 present similar matrix effect.
484
485 Recoveries of quinolones from plasma samples are shown in table 6. In all analysis techniques,
486 recovery values were higher than 80 %. Moreover, in all cases, comparable results were obtained
487 regardless of the method employed, as has been discussed above.
488
489 The intra- and inter-day precision was evaluated at three concentration levels as has been
490 explained in section 2.5. Based on FDA for bioanalytical validation (US Department 2001), the
491 %RSD values from repeated analyses of spiked samples should not exceed 15%. The RSD%
492 values of intra- and inter-day are also shown in table 6. The RSD percentage values of the
493 methods are below 15% excepted for FLU by CE with a value of 16%; which indicates that, in
494 general, they boast suitable intra- and inter-day precision.
495
496 3.6. Application of the developed methods to positive plasma samples obtained from medicated
497 animals
498 In order to test the efficiency of the proposed methods and compare CE-UV and LC-UV, we have
499 applied the developed methods to the determination of ENR and its main metabolite CIP in pig
500 plasma samples from four animals orally medicated with ENR during five consecutive days.
501
502 Figure 8 shows the differences between plasma concentration-time profiles obtained by LC-UV
503 versus CE-UV in the analysis of ENR in four pig plasma of treated animals (A-D). The
504 concentration obtained of ENR in pig plasma is around 1000 $\mu g\ L^{-1}$ using both techniques.
505
506 Figure 9A shows: (a) a blank pig plasma sample and (b) an electropherogram of a pig plasma
507 sample spiked at a concentration of 1000 $\mu g\ L^{-1}$ of each quinolone. Figures 9B and 9C show the
508 UV peaks corresponding to the analysis of ENR and I.S. in a positive plasma sample obtained
509 from a treated animal with ENR. In figure 9B, obtained by CE-UV, the peak corresponding to
510 CIP doesn't appear because its concentration, in the real sample, is lower than the LOD obtained
511 with this technique. However, using LC-UV (figure 9C), the obtained chromatogram permits to

512 know the presence of ENR but also of its metabolite CIP, whereas at a concentration lower than
513 the LOQ.
514
515 In order to test the efficiency of the proposed methods and also quantify and identify the positive
516 quinolones samples, LC-MS and LC-MS/MS has been used for the determination of ENR and its
517 main metabolite CIP in pig and cow plasma samples obtained from animals medicated with ENR.
518
519 Figure 10 shows the pharmacokinetic profile for the ENR and CIP contents, in the analysis of
520 plasma samples at five different times (0 h to 24 h), from pig plasma of treated animals using LC-
521 MS and LC-MS/MS. Between 2 and 6 h, the highest value of ENR in plasma samples is
522 observed. After 6 h the concentration of ENR decrease and a part of ENR is metabolized to CIP,
523 as can be observed in figure 10. Similar concentration values are obtained by LC-MS and LC-
524 MS/MS.
525
526 Figure 11 shows, as an example, the results obtained in the analysis by LC-MS/MS of a positive
527 pig plasma sample from an animal treated with ENR. This figure shows: (11a) a chromatogram
528 obtained before the treatment (0 h) and (11b) after 6 h. The use of LC-MS/MS allows the
529 quantification of ENR and CIP using the corresponding transition in MRM mode and also the
530 univocal confirmation of the presence of ENR and CIP by the transition of identification, as can
531 be shown in figure 11c.
532
533 In this work we have also studied the application of MS methodologies to the analysis of
534 quinolones in positive cow plasma samples, from three cow specimens (A, B and C) medicated
535 with ENR. Comparable results are obtained by LC-MS and LC-MS/MS for ENR and CIP. The
536 values in parenthesis are the standard deviation obtained. The different results obtained for A, B
537 and C specimens can be explained taking into account that the concentration found depends on
538 the volume of water that each animal has drunk.
539
540 The results show that CE-UV is satisfactorily applied when relatively high values of
541 concentration of quinolones are present, but techniques as LC-MS or LC-MS/MS are needed in

542 order to quantify low levels of analytes and also to quantify the metabolite CIP in plasma samples
543 after the treatment with ENR.
544

4. CONCLUSIONS

LC-UV, LC-MS, LC–MS/MS and CE-UV methods were developed and applied in order to identify and quantify simultaneously a series of quinolones in cow and pig plasma samples. A rapid and efficient SPE method was optimized for extraction and clean-up of quinolones from plasma. CE-UV, LC-MS and LC-MS/MS allowed us to greatly reduce the separation run-time, compared with LC-UV.

Detailed validation studies were performed to evaluate the effectiveness of these methods based on FDA regulations, obtaining adequate sensitivity, linearity and precision results. The precision of each method was below the 15% required by EU regulation excepted for DIF and FLU in CE with a value of 16 %. The recoveries of the quinolones were higher than 80% in all methods. The lowest LOQ were obtained with LC-MS and LC-MS/MS. Similar parameters are obtained with both pig and cow plasma in LC-UV.

The studies made show a matrix effect cow and pig in plasma, showing different slopes between calibration curves prepared in plasma and prepared in water. In conclusion to analyze positive samples the calibration curves showed to be prepared in the corresponding plasma.

Despite the low sensitivity of the CE-UV, this technique has been satisfactorily applied to identify and quantify ENR in plasma samples from animals orally medicated, obtaining similar results than using LC-UV. However, techniques as the LC-MS or LC-MS/MS are needed when low concentration of ENR and CIP are present in the samples.

ACKNOWLEDGEMENTS

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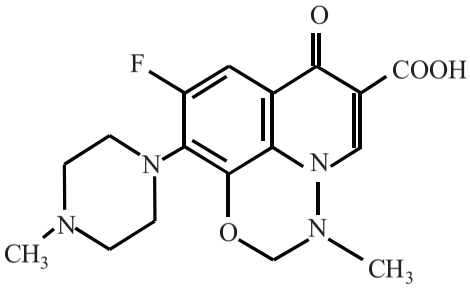
653

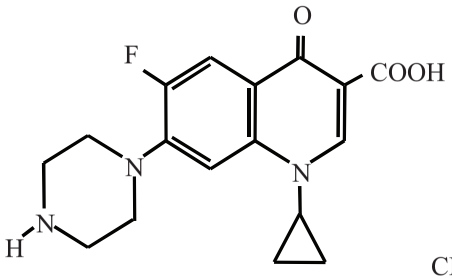
654 **FIGURE CAPTIONS**

655 Figure 1. Chemical structure of the studied quinolones and the I.S. used.
656
657 Figure 2. LC-UV chromatograms of spiked cow plasma samples at 1000 μg L^{-1} of quinolone (a,
658 b, and c) and blank plasma (d, e, and f). IS (LOM and TOS) at 1000 μg L^{-1}. Extracts were
659 analyzed at 280 nm (a and d), 250 nm (b and e) and 300 nm (c and f).
660 Peaks: (1) MAR; (2) CIP; (3) LOM (IS); (4) DAN; (5) ENR; (6) SAR; (7) DIF; (8) TOS (IS); (9)
661 OXO; and (10) FLU.
662
663 Figure 3. LC-MS chromatogram obtained from the analysis of spiked cow plasma samples at 100
664 μg L^{-1} per quinolone in SIM mode. Peaks as in figure 2.
665
666 Figure 4. Ion reconstituted chromatogram obtained for the analysis of spiked cow plasma samples
667 at 50 μg L^{-1} per quinolone by LC–MS/MS in MRM mode.
668
669 Figure 5. Mass spectrum in product-ion scan mode for A) Tosufloxacin: m/z 405 at collision-
670 induced dissociation in q2 at 32V and the proposed fragmentation pathways, B) Marbofloxacin:
671 m/z 363 at collision-induced dissociation in q2 at 30V and the proposed fragmentation pathways.
672
673 Figure 6. A) Comparison between the responses of LOM and TOS by LC-MS at 1000 μg L^{-1}. B)
674 Comparison between the responses of LOM and TOS by LC-MS/MS at 1000 μg L^{-1}.
675
676 Figure 7. Comparison between storage stability at 4ºC in the refrigerator for CIP and SAR in cow
677 and pig plasma.
678
679 Figure 8. Concentration (μg L^{-1}) of ENR in pig plasma from four pigs medicated with ENR (time
680 of extraction of 24 h), obtained by LC-UV and CE-UV.
681
682 Figure 9. A) Electropherograms (260 nm) of blank pig plasma samples (a), pig plasma samples
683 spiked at a concentration of 1000 μg $L^{-1}$of each quinolone and I.S. (PIR and TOS) at 2500 μg L^{-1}

684 (b). B) Electropherogram (260 nm) of a pig plasma sample from medicated animal with ENR by
685 CE-UV. PIR at 2500 μg L^{-1}. C) Chromatogram (280 nm) of pig plasma sample, from medicated
686 animal with ENR obtained by LC-UV. TOS at 1000 μg L^{-1}. Peaks: (1) DAN; (2) CIP: (3) MAR;
687 (4) ENR; (5) DIF; (6) PIR (IS); (7) OXO; (8) FLU; (9) TOS (IS).
688
689 Figure 10. Concentration-time profile of (a) ENR and (b) CIP in plasma samples obtained from
690 pig animals after medication with ENR and analyzing by LC-MS (SIM mode) and LC-MS/MS
691 (MRM mode).
692
693 Figure 11. Ion reconstituted chromatogram obtained for the analysis of pig plasma sample
694 corresponding to an animal treated with ENR (a) before the medical treatment, (b) 6 h after the
695 intramuscular treatment and (c) the confirmatory chromatogram of the positive sample by LC-
696 MS/MS.
697



MARBOFLOXACIN



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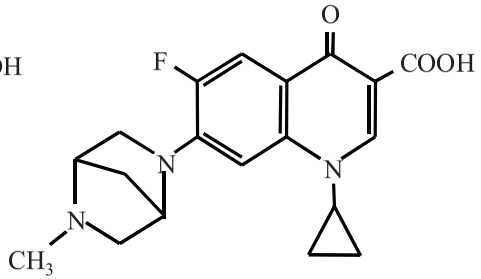

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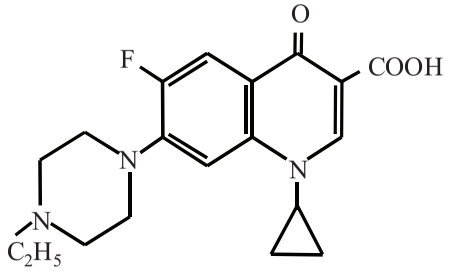

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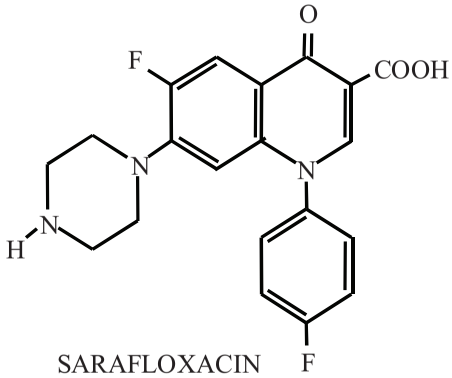

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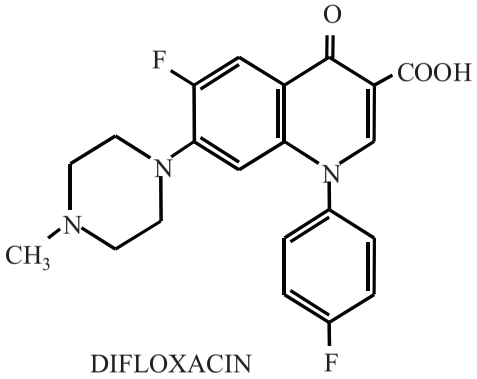

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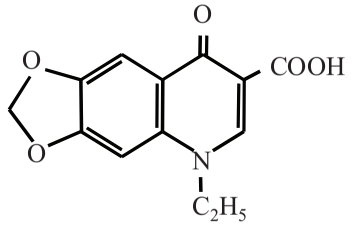
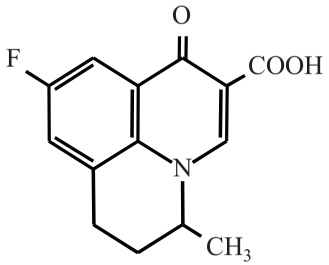
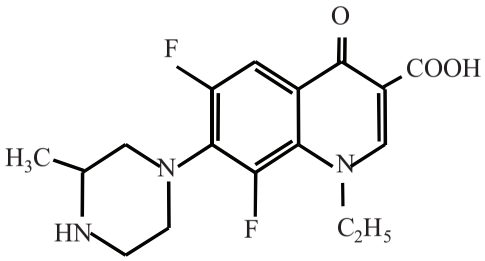


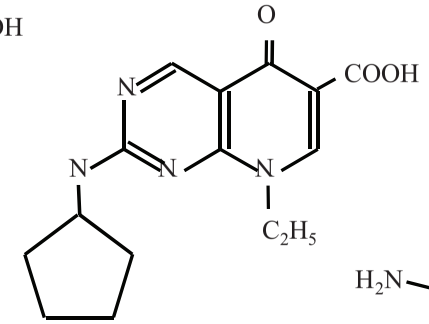
OXOLINIC ACID



FLUMEQUINE



LOMEFLOXACIN (I.S.)



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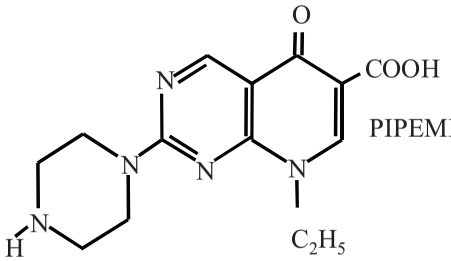

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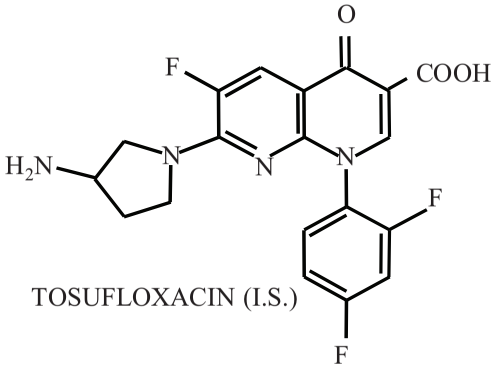

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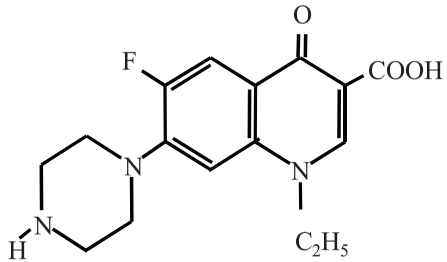


NORFLOXACIN (I.S.)

Figure 1

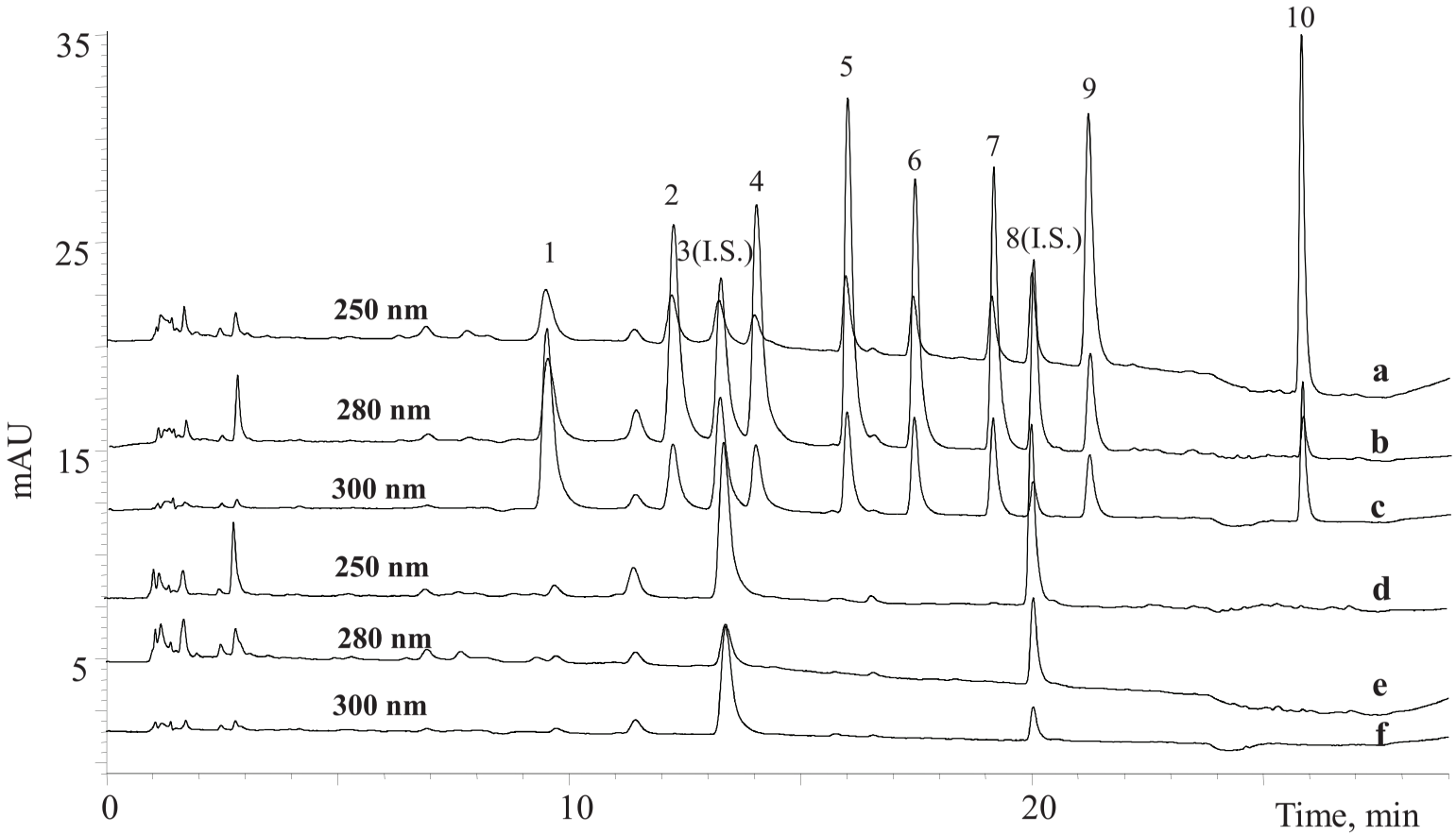

35
25
15
5
mAU
0
10
20
Time, min
1
2
3(I.S.)
4
5
6
7
8(I.S.)
9
10
250 nm
280 nm
300 nm
250 nm
280 nm
300 nm
a
b
c
d
e
f

Figure 2

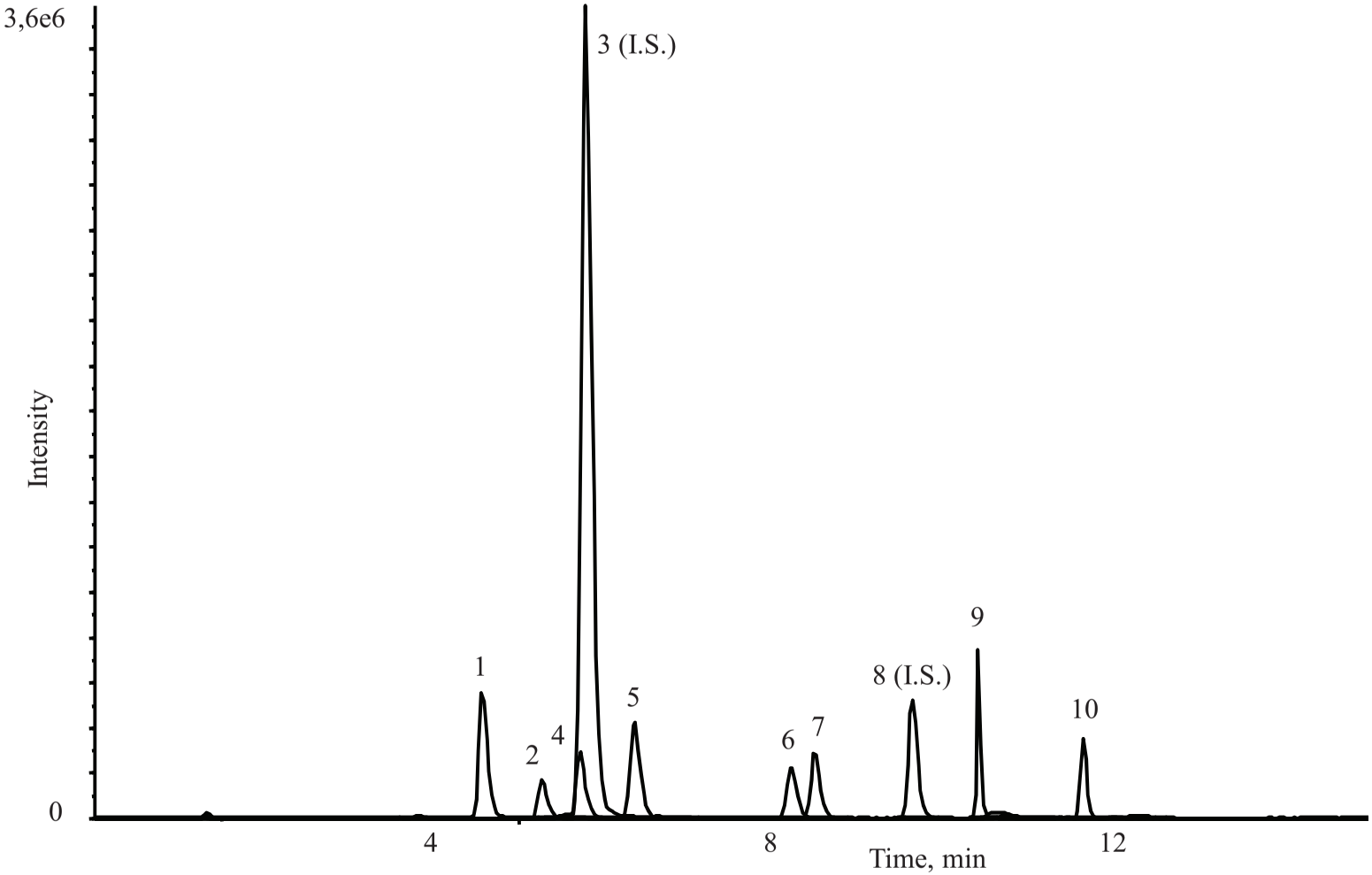
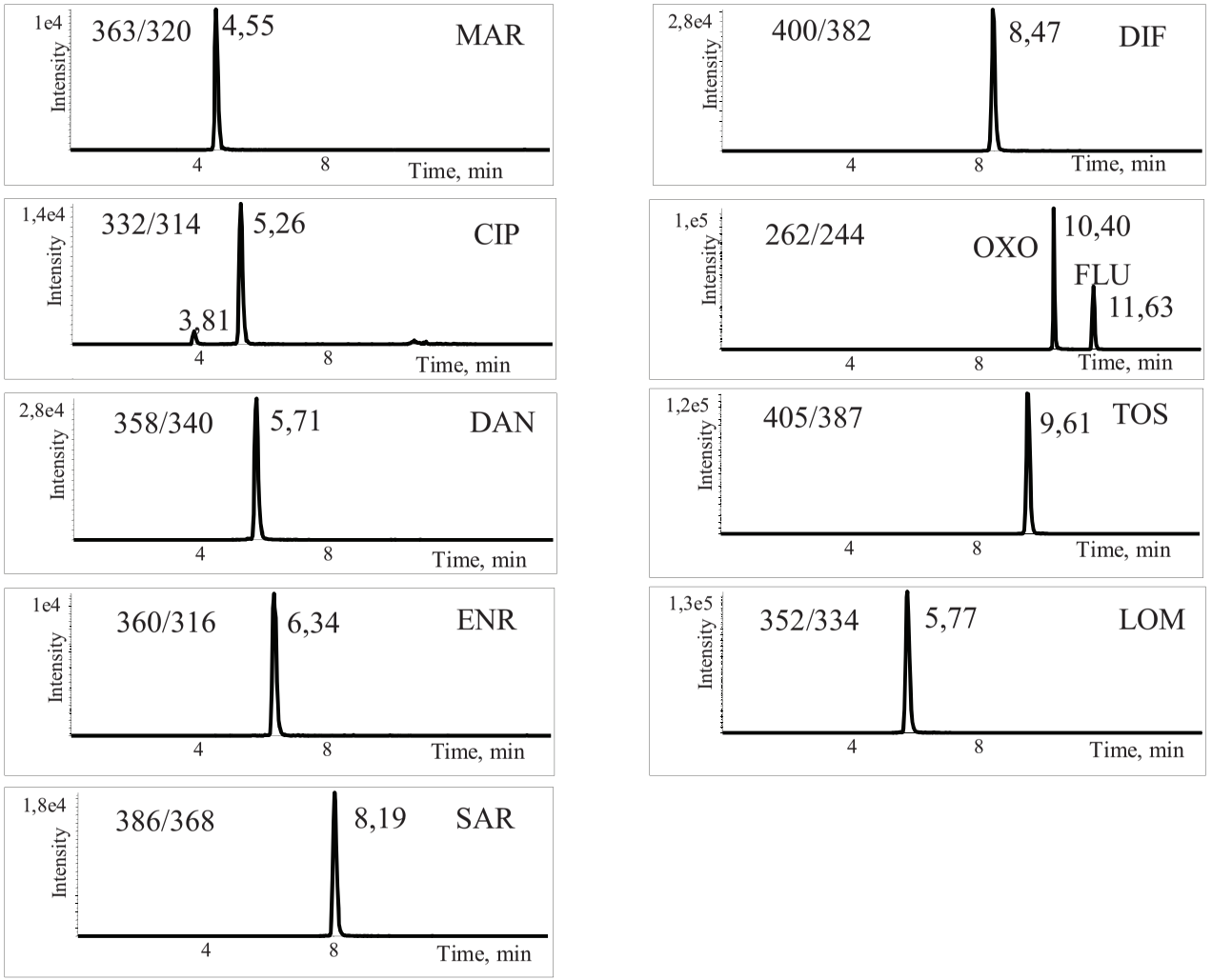


Figure 3


1e4
Intensity
363/320
4,55
MAR
4
8
Time, min
2,8e4
Intensity
400/382
8,47
DIF
4
8
Time, min
1,4e4
Intensity
332/314
5,26
CIP
3,81
4
8
Time, min
1,e5
Intensity
262/244
OXO
10,40
FLU
11,63
4
8
Time, min
2,8e4
Intensity
358/340
5,71
DAN
4
8
Time, min
1,2e5
Intensity
405/387
9,61
TOS
4
8
Time, min
1e4
Intensity
360/316
6,34
ENR
4
8
Time, min
1,3e5
Intensity
352/334
5,77
LOM
4
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Time, min
1,8e4
Intensity
386/368
8,19
SAR
4
8
Time, min

Figure 4

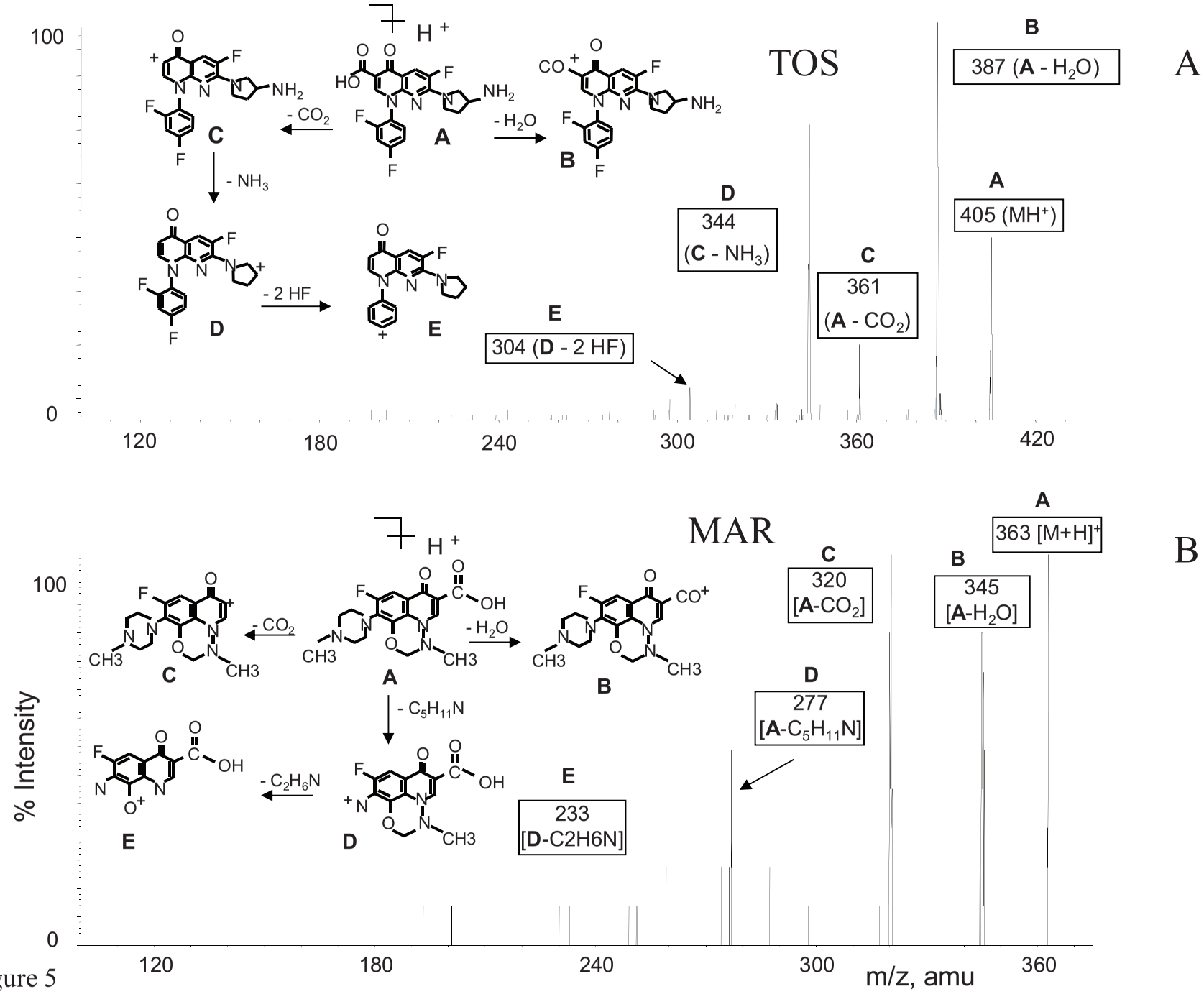
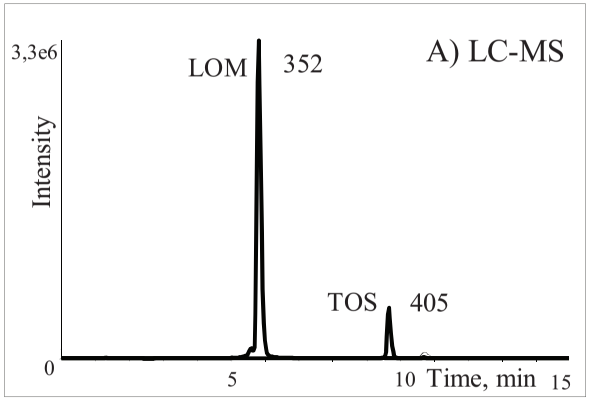

TOS
A
100
0
120
180
240
300
360
420
B
387 (A - H2O)
A
405 (MH+)
D
344
(C - NH3)
C
361
(A - CO2)
E
304 (D - 2 HF)
- CO2
- NH3
- H2O
- 2 HF
H +
MAR
B
% Intensity
m/z, amu
A
363 [M+H]+
C
320
[A-CO2]
B
345
[A-H2O]
D
277
[A-C5H11N]
E
233
[D-C2H6N]
- C5H11N
- C2H6N

Figure 5


3,3e6
Intensity
0
LOM
352
A) LC-MS
TOS
405
5
10
Time, min
15

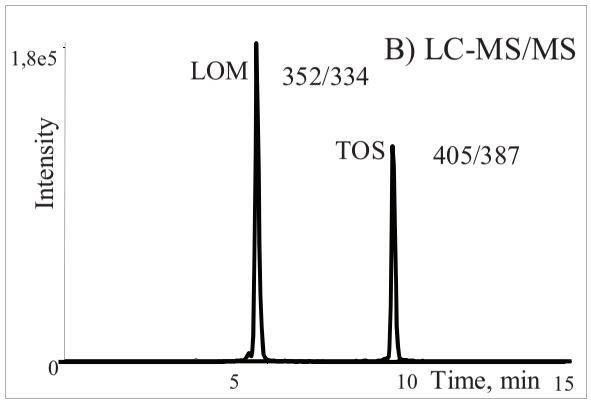

1,8e5
Intensity
0
LOM
352/334
B) LC-MS/MS
TOS
405/387
5
10
Time, min
15

Figure 6

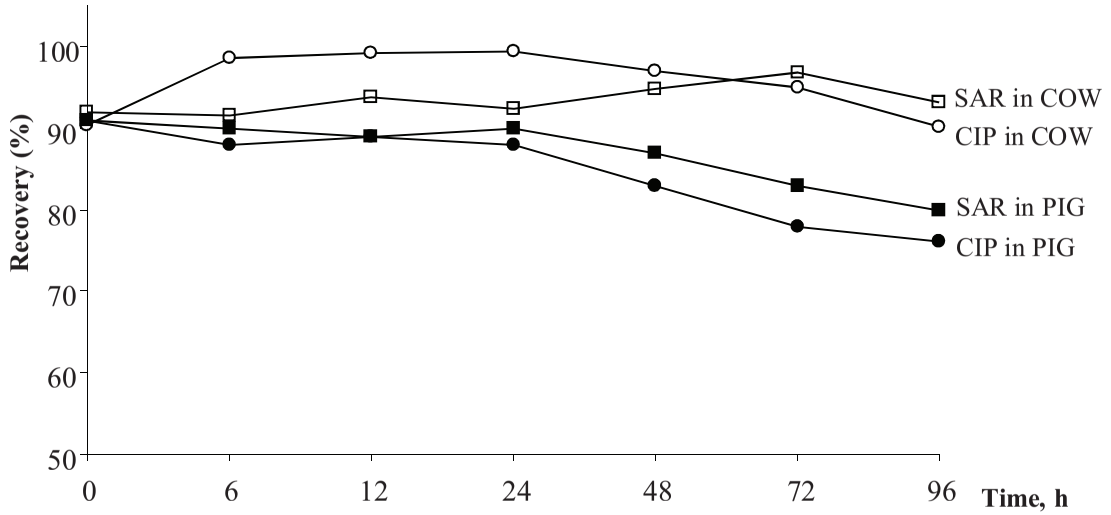

100
90
80
70
60
50
Recovery (%)
0
6
12
24
48
72
96
Time, h
SAR in COW
CIP in COW
SAR in PIG
CIP in PIG

Figure 7

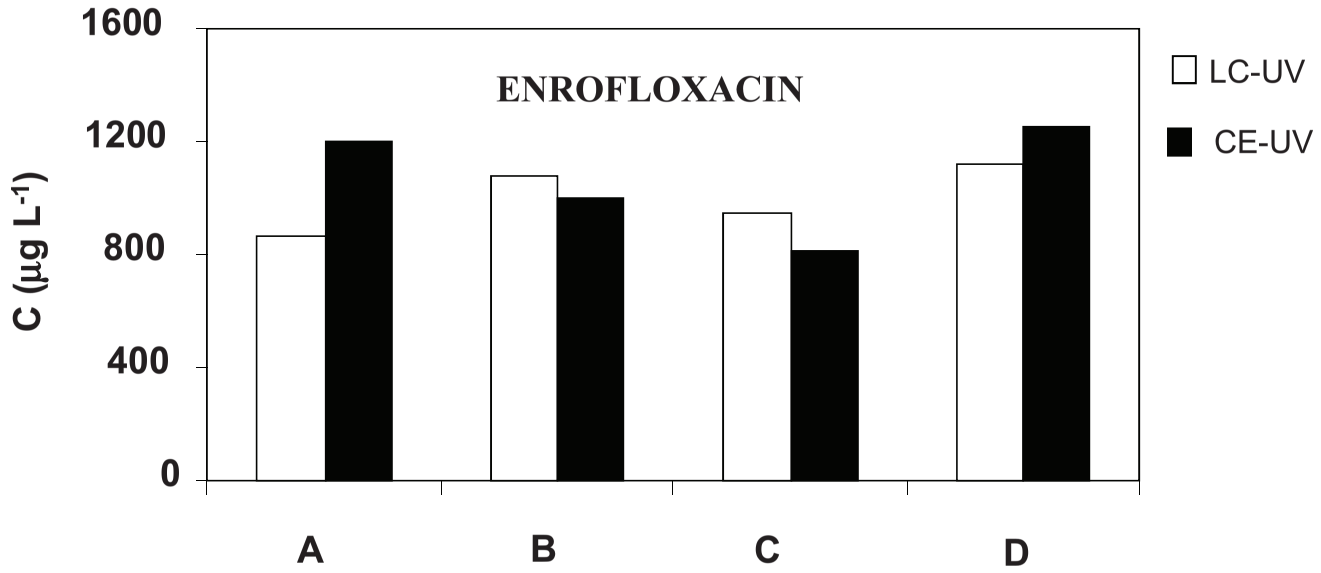
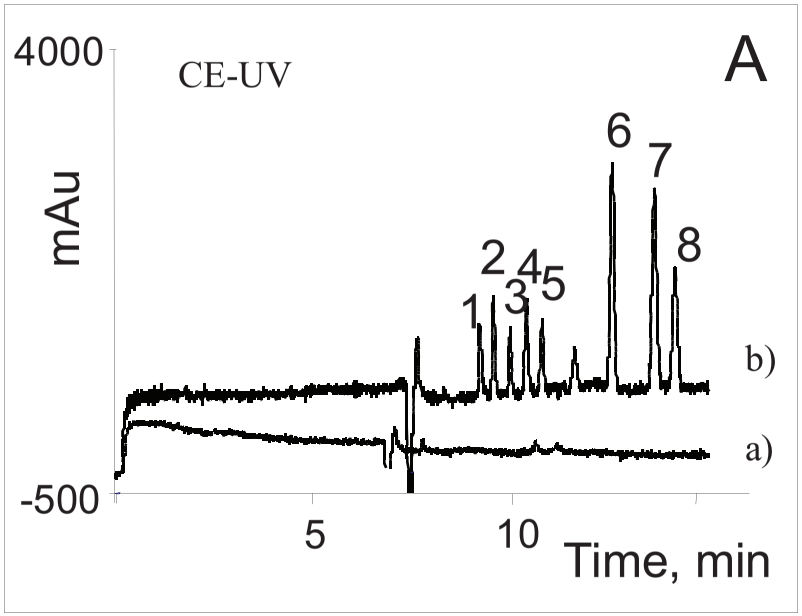
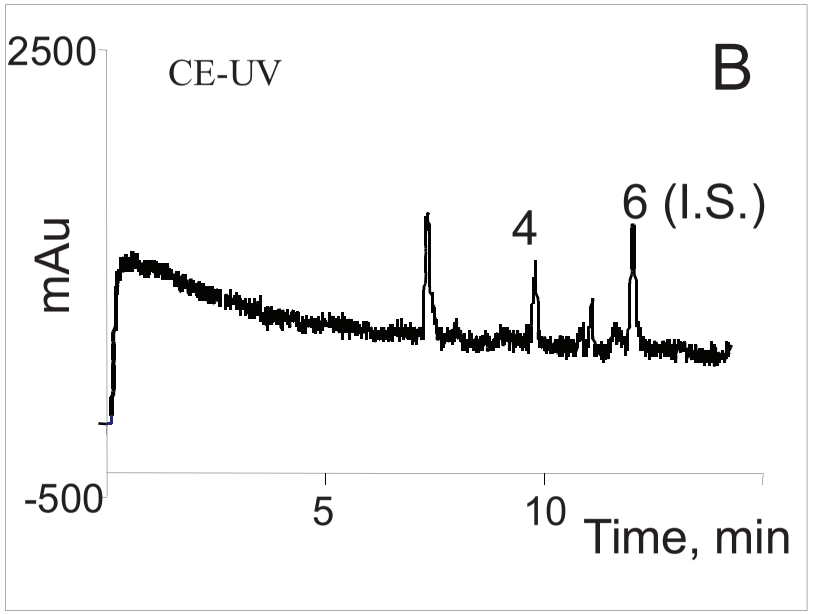


Figure 8


4000
CE-UV
A
mAu
1
2
3
4
5
6
7
8
b)
a)
-500
5
10
Time, min


2500
CE-UV
B
mAu
4
6 (I.S.)
-500
5
10
Time, min

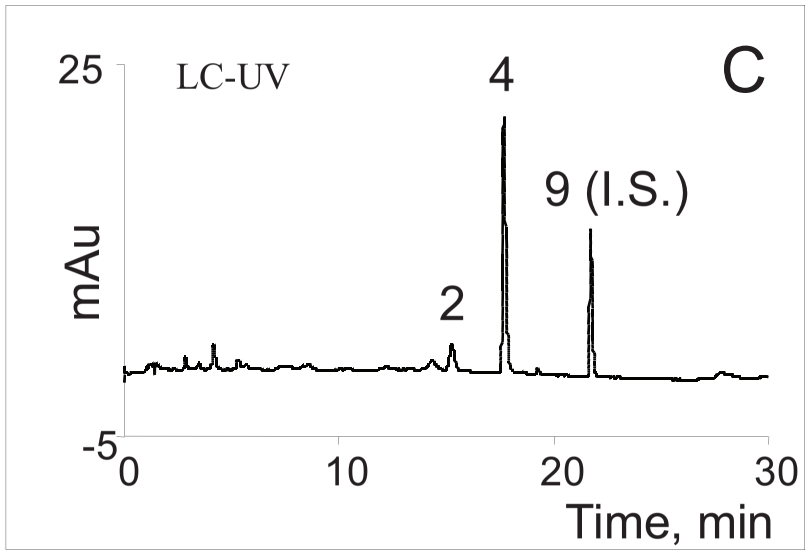

25
LC-UV
C
mAu
2
4
9 (I.S.)
-5
0
10
20
30
Time, min

Figure 9

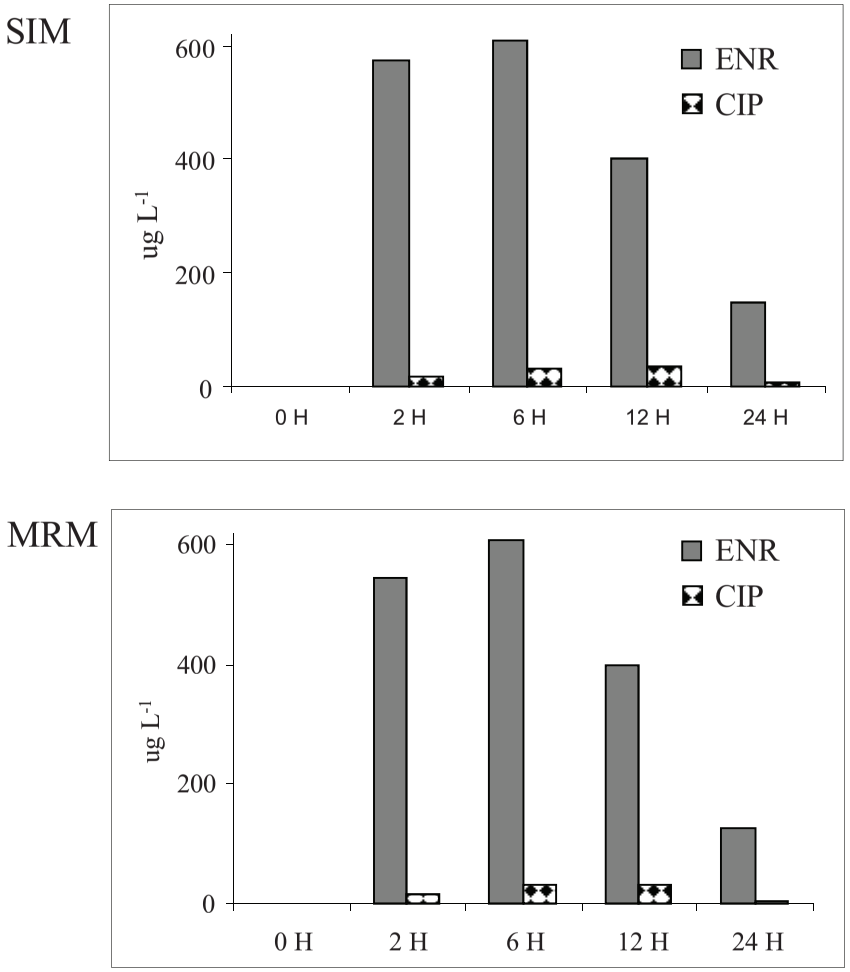

SIM
600
400
200
0
ug L-1
ENR
CIP
0 H
2 H
6 H
12 H
24 H
MRM
600
400
200
0
ug L-1
ENR
CIP
0 H
2 H
6 H
12 H
24 H

Figure 10

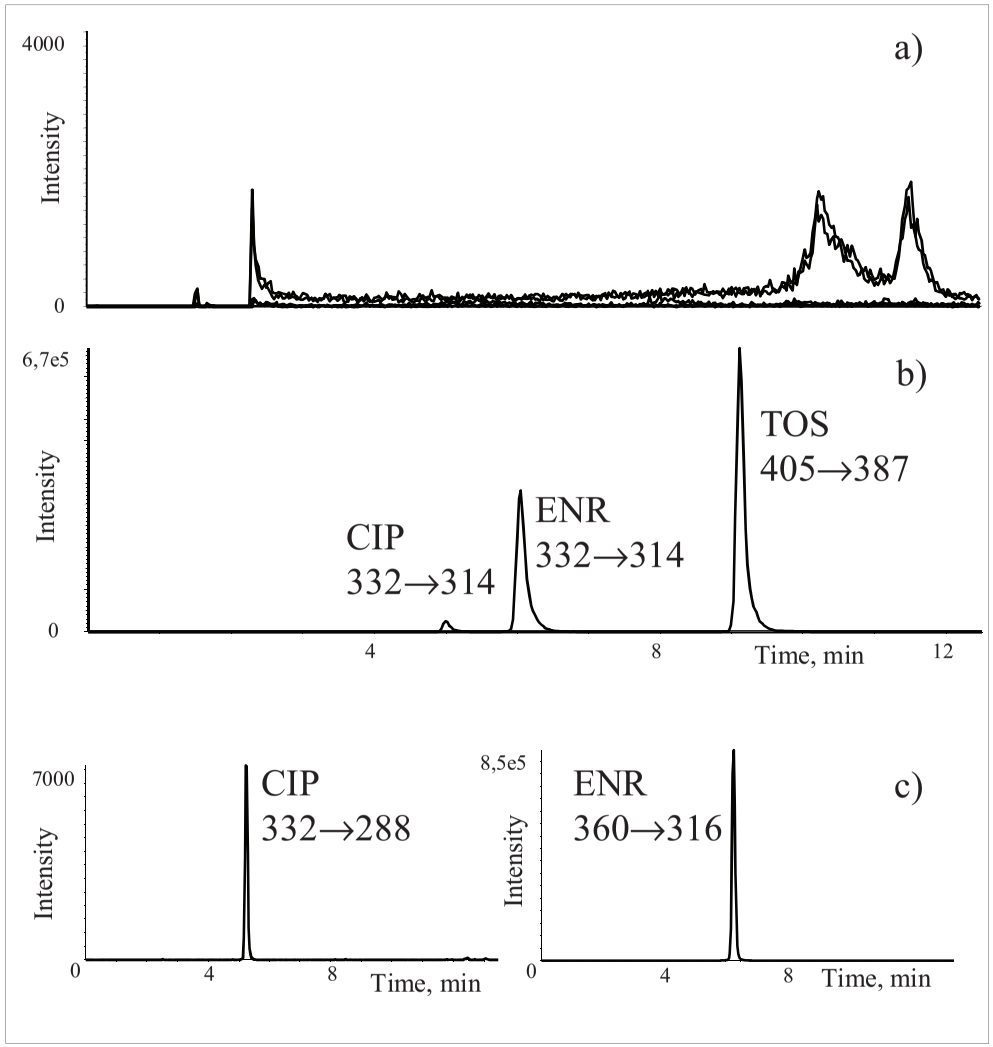

4000
Intensity
0
a)
6,7e5
Intensity
0
b)
TOS
405→387
ENR
332→314
CIP
332→314
4
8
12
Time, min
7000
Intensity
0
CIP
332→288
4
8
Time, min
8,5e5
Intensity
0
ENR
360→316
c)
4
8
Time, min

Figure 11

Table 1. SPE conditions for the different sorbents used in this work.

Sorbent	Preconditioning	Washing	Elution
Strata X Poly (benzylpiperidone)	1 mL MeOH 1 mL Milli-Q water	2 mL Milli-Q water	2 mL 1% TFA:MeCN (25:75, v/v)
Oasis HLB Poly (divinylbenzene-co-N-vinylpyrrolidone)	1 mL MeOH 1 mL Milli-Q water	2 mL Milli-Q water 1 mL 0.5% TFA: MeCN (90:10, v/v) 2 mL Milli-Q water	2 mL 1% TFA:MeCN (25:75, v/v) 1 ml MeCN
ENV+ Isolute (Hydroxylated polystyrene-divinylbenzene)	2 mL MeOH 2 mL Milli-Q water 2 mL 50 mM Phosphate pH 3	2 mL Milli-Q water 7.5 mL hexane	5 mL 2 % TFA:MeCN (25:75, v/v) 1 ml MeCN

Table 2. Optimized MS and MS-MS conditions and ions selected for quantification in SIM mode and MRM transitions selected for quantification and identification of the quinolones in bovine plasma.

	MAR	CIP	DAN	ENR	SAR	DIF	OXO	FLU	LOM	TOS
Parent ion (m/z)	363	332	358	360	386	400	262	262	352	405
Cone voltage (V)	45	45	45	45	47	47	38	38	54	55
Fragment ion (m/z)	320	314	340	316	368	382	224	244	334	387
	345	288	283	342	342	356	216	202	308	344
		245	96	245	299	399				
MS (SIM)										
Ion monitoring	363	332	358	360	386	400	262	262	352	405
MS/MS (MRM)[a]										
Transition quantification	363→ 320	332→ 314	358→340	360→ 316	386→ 368	400→ 382	262→244	262→244	352→334	405→387
Collision energy (V)	22	32	31	29	35	30	26	26	30	30
Transition identification	363→ 345	332→ 288	358→ 283	360→ 342	386→342	400→356	262→216	262→202	352→308	405→344
Collision energy (V)	30	27	31	29	29	30	45	45	25	32

[a] Experiments performed using a collision-activated dissociation (CAD) at 4.

Table 3. Comparison of the results obtained by LC-UV in calibration curves and recovery for both IS (LOM and TOS) used, in cow plasma.

	MAR	CIP	DAN	ENR	SAR	DIF	OXO	FLU
LC - UV								
IS (LOM)								
Slope	0.936	1.091	1.162	1.293	0.983	0.954	1.033	0.862
Intercept	0.011	- 0.024	- 0.020	- 0.015	- 0.021	0.004	- 0.007	- 0.009
r	0.9998	0.9996	0.9999	0.9999	0.9996	0.9998	0.9995	0.9998
%Recovery	92 (± 1)	91 (± 1)	92 (± 1)	93 (± 1)	91 (± 1)	92 (± 1)	93 (± 1)	92(± 1)
IS (TOS)								
Slope	1.732	2.018	2.148	2.391	1.818	1.764	1.915	1.594
Intercept	0.009	- 0.054	- 0.048	- 0.042	- 0.049	- 0.003	- 0.038	- 0.026
r	0.9998	0.9995	0.9999	0.9999	0.9996	0.9999	0.9995	0.9996
%Recovery	96 (± 1)	93 (± 1)	95(± 1)	97(± 1)	94 (± 1)	96 (± 1)	99 (± 2)	96 (± 1)

y = Slope*C + Intercept (y=area quinolone/internal standard ratio; C = concentration quinolone/internal standard ratio).

Table 4. LOD, LOQ and calibrations curves for quinolones in cow and pig plasma samples by different techniques.

	MAR	CIP	DAN	ENR	SAR	DIF	OXO	FLU
COW PLASMA								
LC – UV [a]								
LOD (μg L^{-1})	12	10	13	10	14	12	10	12
LOQ (μg L^{-1})	40	35	45	35	47	41	35	41
Slope	1.732	2.018	2.148	2.391	1.818	1.764	2.643	1.594
Intercept	0.009	- 0.054	- 0.048	- 0.042	- 0.049	- 0.003	- 0.033	- 0.026
r	0.9998	0.9995	0.9999	0.9999	0.9996	0.9999	0.9995	0.9996
LC – MS [a]								
LOD (μg L^{-1})	1	2	0.5	0.5	1.5	1	0.5	0.5
LOQ (μg L^{-1})	4	7	2	2	5	4	2	2
Slope	1.945	0.648	1.997	2.206	1.182	1.723	0.967	0.995
Intercept	0.172	0.134	0.273	0.417	0.147	0.280	0.211	0.194
r	0.985	0.920	0.984	0.980	0.978	0.985	0.963	0.974
LC - MS/MS [a]								
LOD (μg L^{-1})	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1
LOQ (μg L^{-1})	0.7	0.7	0.5	0.5	0.7	0.7	0.5	0.5
Slope	0.334	0.376	1.709	0.902	0.730	1.431	1.717	1.704
Intercept	0.027	0.040	0.087	0.041	0.001	0.079	0.238	0.230
r	0.984	0.960	0.997	0.998	0.997	0.997	0.965	0.984
PIG PLASMA								
LC – UV [a]								
LOD (μg L^{-1})	12	12	10	10	7	10	8	10
LOQ (μg L^{-1})	40	40	31	31	23	40	25	40
Slope	1.633	2.150	2.124	2.356	1.679	1.620	1.209	1.504
Intercept	-0.041	-0.037	-0.078	-0.097	-0.020	-0.018	-0.032	-0.033
r	0.999	0.999	0.999	0.999	0.998	0.999	0.998	0.999
CE – UV [b]								
LOD (μg L^{-1})	150	150	150	100	-	100	100	150
LOQ (μg L^{-1})	450	450	450	300	-	300	300	450
Slope	0.930	0.589	0.636	0.670	-	0.564	1.314	1.107
Intercept	-0.044	0.023	-0.023	-0.024	-	-0.035	-0.058	-0.068
r	0.989	0.984	0.989	0.997	-	0.989	0.993	0.995
LC – MS[a]								
LOD (μg L^{-1})	1	2	1	1	2	1	1	1
LOQ (μg L^{-1})	2.5	5	2.5	2.5	5	2.5	2.5	2.5
Slope	1.036	0.674	1.669	1.804	1.003	1.452	0.789	0.906
Intercept	0.385	0.112	0.342	0.593	0.234	0.364	0.248	0.261
r	0.940	0.910	0.974	0.950	0.970	0.970	0.950	0.960
LC - MS/MS[a]								
LOD (μg L^{-1})	0.3	0.4	0.1	0.2	0.3	0.3	0.1	0.1
LOQ (μg L^{-1})	1	1	0.5	0.5	1	1	< 0.5	< 0.5
Slope	0.250	0.313	1.649	0.868	0.637	1.357	1.764	1.707
Intercept	0.040	0.037	0.029	0.017	0.035	0.065	0.099	0.130
r	0.970	0.950	0.998	0.998	0.996	0.997	0.990	0.992

[a] The range of calibration curve was LOQ – 3200 μg L^{-1} for LC-UV, LOQ-2000 for LC-MS and LC-MS/MS; IS (TOS) at a concentration of 1000μg L^{-1}.

[b] The range of calibration curve was LOQ – 10000 μg L^{-1}; IS (PIR) at a concentration of 2500 μg L^{-1}.

Calibration curve: y = Slope*C + Intercept (y=area quinolone/internal standard ratio; C = concentration quinolone/internal standard ratio).

Table 5. Calibration curves for quinolones in water samples by LC-MS and LC-MS/MS.

Compound	SIM	MRM
	Standard curve (in water)	Standard curve (in water)
MAR	y = 0,434C + 0,207 (r = 0,975)	y = 0,085C + 0,002 (r = 0,988)
CIP	y = 0,298C + 0,144 (r = 0,991)	y = 0,158C + 0,014 (r = 0,996)
DAN	y = 0,572C + 0,266 (r = 0,986)	y = 0,422C + 0,036 (r = 0,995)
ENR	y = 0,922C + 0,160 (r = 0,980)	y = 0,214C + 0,029 (r = 0,991)
SAR	y = 0,369C + 0,164 (r = 0,990)	y = 0,178C + 0,011 (r = 0,997)
DIF	y = 0,574C + 0,215 (r = 0,990)	y = 0,328C + 0,035 (r = 0,997)
OXO	y = 0,739C - 0,236 (r = 0,910)	y = 0,345C + 0,180 (r = 0,930)
FLU	y = 0,422C + 0,119 (r = 0,991)	y = 0,539C + 0,058 (r = 0,988)

y = area quinolone/internal standard radio; C = concentration of quinolone/internal standard radio.

Table 6. Data of recovery and precision of quinolones in cow and pig plasma by different techniques.

	MAR	CIP	DAN	ENR	SAR	DIF	OXO	FLU
BOBINE PLASMA								
LC - UV								
%Recovery[a]	96 (± 1)	93 (± 1)	95 (± 1)	97 (± 1)	94 (± 1)	96 (± 1)	99 (± 2)	96 (± 1)
Precision (%)								
Intra-day (n=15)[b]	1 - 6	2 - 7	2 - 4	2 - 6	2 - 5	2 - 4	3 - 5	2 - 5
Inter-day (n=45)[b]	2 - 7	3 - 8	3 - 6	3 - 8	3 - 5	2 - 9	2 - 6	3 - 9
LC - MS								
%Recovery[a]	91 (± 4)	90 (± 2)	91 (± 2)	91 (± 3)	94 (± 2)	98 (± 4)	93 (± 3)	91 (± 2)
Precision (%)								
Intra-day (n=15)[b]	3 - 7	3 - 6	4 - 7	2 - 7	2 - 4	1 - 3	4 - 9	2 - 8
Inter-day (n=45)[b]	8 - 12	4 - 8	5 - 9	4 - 9	3 - 6	3 - 5	7 - 10	4 - 10
LC - MS/MS								
%Recovery[a]	99 (± 2)	94 (± 1)	95 (± 1)	95 (± 3)	90 (± 2)	90 (± 2)	96 (± 4)	93 (± 1)
Precision (%)								
Intra-day (n=15)[b]	2 - 7	3 - 6	4 - 9	5 - 8	3 - 6	2 - 4	4 - 10	3 - 5
Inter-day (n=45)[b]	5 - 10	3 - 6	5 - 11	6 - 10	4 - 7	4 - 5	6 - 11	4 - 8
PORCINE PLASMA								
LC - UV								
%Recovery[a]	96 (± 0.5)	86 (± 0.5)	96 (± 0.5)	99 (± 0.5)	93 (± 0.5)	100 (± 0.5)	100 (± 1)	94 (± 0.5)
Precision (%)								
Intra-day (n=15)[b]	1 - 2	1 - 8	1 - 4	1 - 4	1 - 6	1 - 3	2 - 5	1 - 5
Inter-day (n=45)[b]	1 - 3	1 - 8	1 - 5	2 - 5	1 - 7	1 - 4	3 - 9	2 - 7
CE – UV								
%Recovery[a]	93 (± 2)	93 (± 3)	83 (± 2)	86 (± 2)	-	97 (± 4)	100 (± 3)	99 (± 2)
Precision (%)								
Intra-day (n=15)[b]	5 - 13	6 - 11	5 - 13	6 - 12	-	3 - 13	2 - 8	4 - 13
Inter-day (n=45)[b]	12 - 13	10 - 13	10 - 14	12 - 14	-	12 - 16	9 - 14	8 - 16
LC - MS								
%Recovery[a]	96 (± 3)	80 (± 1)	96 (± 6)	87 (± 6)	94 (± 1)	81 (± 2)	98 (± 3)	99 (± 6)
Precision (%)								
Intra-day (n=15)[b]	5 - 13	1 - 13	2 - 7	5 - 7	2 - 14	6 - 8	6 - 8	8 - 14
Inter-day (n=45)[b]	9 - 12	9 - 13	4 - 11	7 - 15	4 - 14	5 - 10	8 - 14	11 - 12
LC – MS/MS								
%Recovery[a]	99 (± 3)	87 (± 1)	93 (± 2)	95 (± 2)	82 (± 2)	91 (± 1)	95 (± 4)	92 (± 2)
Precision (%)								
Intra-day (n=15)[b]	5 - 9	4 - 8	5 - 9	6 - 14	2 - 10	8 - 14	7 - 13	6 - 11
Inter-day (n=45)[b]	10 - 13	8 - 15	5 - 12	7 - 15	5 - 14	9 - 15	8 - 14	10 - 15

[a] Numbers in brackets are standard error (%).

[b]The intra- and inter-day data showed are the minimum and maximum RSD values obtained in the corresponding samples, which were prepared at 50, 1000 and 2500 μg L^{-1} for LC-UV; 1000, 4000 and 8000 μg L^{-1} for CE-UV and 50, 1000 and 2000 μg L^{-1} for LC-MS and LC-MS/MS.

Table 7. Results (μg L^{-1}) obtained after the analysis of positive cow plasma samples.

Enrofloxacin	A	B	C
LC-MS	156 (6)	153 (12)	130 (3)
LC-MS/MS	142 (4)	139 (5)	116 (7)
Ciprofloxacin			
LC-MS	93 (9)	111 (8)	99 (3)
LC-MS/MS	86 (6)	95 (10)	83 (5)