Chitosan biofilms: insights for the selective electromembrane extraction of fluoroquinolones from biological samples

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Abstract

A selective electromembrane extraction procedure for the extraction of three fluoroquinolones, usually employed as antibiotic in veterinarian use, is proposed by using a chitosan biofilm, composed by 60% (w/w) chitosan and 40% (w/w) Aliquat®336, as biopolymeric support. The interaction mechanism occurring between the target drugs and the biopolymer has been deeply studied since a theoretical point of view. The obtained results show the interaction between the fluoroquinolones and the biomembrane is stabilized by two hydrogen bonds when the Quantum Theory of Atoms in Molecules (QTAIM) is employed. The double hydrogen bond is formed between both the carboxyl and keto groups of the drugs with both the amine and hydroxyl groups of glucosamine in the biopolymer. The energetic results agree with the experimental trend in which Marbofloxacin, Enrofloxacin and Flumequine present high extraction efficiency in terms of enrichment factors.

The chitosan membrane has been proved to play an active role in the selective extraction of the fluoroquinoles mentioned above in presence of Grepafloxacin, Danofloxacin, Gatifloxacin, Norfloxacin and Ciprofloxacin, from animal urine samples.

Therefore, this biopolymeric material seems to be an attractive and alternative support for drug delivery compared to other materials traditionally used as supports in electromembrane extraction of pharmaceuticals.

Keywords: chitosan; biopolymeric support; electromembrane extraction; fluoroquinolones; molecular modelization; quantum theory of atoms in molecules

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1. Introduction

In the last years, biomembranes development has been one of the aims which scientific community has been focused on. Several polymeric and nanoscale materials have been studied for preparing films with different objectives such us contaminant removal from environmental samples or drugs controlled transport in human organism [1-2]. Furthermore, in biomedicine, the use of membranes based on biomaterials has become the key for artificial organs design, as well as different kind of medical devices for exchange process (hemodialysis and plasmapheresis between others) of diverse compounds [3-4].

Within this realm, chitosan is an advantageous biopolymer due to its biological and physiochemical properties like gelation and chelating ability, non-toxicity, antimicrobial activity, bio absorption, biocompatibility or biodegradability which make it an excellent material for numerous and variable applications in the field of the green chemistry [5-7]. As derived from chitin (N-acetyl-D-glucosamine units) in variable degree of deacetylation (70-95%), its polymeric chains present free hydroxyl and amine groups which lead to possible interactions through hydrogen bonding (Fig. 1a) [8-9]. Specifically, the amine groups with pKa value close to 6.5, favor the biopolymer to be positively charged in acidic or neutral solutions. This fact facilitates the use of this biomaterial for the selective extraction of polar active compounds. Indeed, in the last years, chitosan membranes have been prepared in different ways for diverse clinical or environmental applications [2,10-12]. Its excellent mechanical properties and thermal stability specially enable to prepare chitosan-based membranes with different shapes such as microspheres, fibers or films, favoring the design of packaging structures requested for each demanded application [13-14]. Indeed, in previous studies of our research group, chitosan nanocapsules were obtained using gold nanoparticles as crosslinking agents. These nanocapsules may be used as the base for building complex structures with biological applications in the frame of drug delivery and genetic therapy [15].

On the other hand, in the last years many pharmaceuticals like anti-inflammatories or antibiotics have frequently appeared in wastewaters of urban areas consequence of the widespread consumption of drug products by population. This fact has led to consider these compounds as emerging contaminants. Due to the high negative impact of these kind of pollutants have on the environment, analytical

methodologies able to detect and quantify little amounts of these compounds in different types of samples (biological and/or environmental) have been studied. Microextraction methods as sample pretreatments prior instrumental analysis have been usually applied focused on preconcentration as well as sample purification [16-18]. Taking into account the low concentration of many compounds of interest (emerging contaminants) in the samples, as well as the presence of other interfering compounds which may affect the subsequent determination of the target analytes, the application of extraction techniques before instrumental quantification is mandatory. In this context, membrane-based extraction techniques have become crucially important due to their efficiency in removing interfering compounds from the matrix. Also, the excellent enrichment factors obtained in the extractions lead to reach detectable levels for the emerging contaminants [19-20].

In this area, our research group have a wide experience in developing new analytical methodologies based on electromembrane extraction (EME) to be able of detecting and quantifying little amounts of several groups of pharmaceuticals considered emerging contaminants (non-steroidal anti-inflammatory drugs (NSAIDs), polar drugs or antibiotics). Membranes of different nature have been synthesized, characterized and implemented as support for the EME extraction procedures such as agar films containing silver nanoparticles [21], acrylic nanofiber membranes [22], polymer inclusion membranes [23] or chitosan membranes, among others [24]. In fact, in this last work, chitosan tailor-made membranes were successfully synthesized and used as support for the extraction of NSAIDs and polar acidic drugs from biological samples and their posterior chromatographic determination in biological samples. Novel and home-made EME systems were developed for these purpose. Within this realm, chitosan membrane has been proposed, in this case, for the selective EME of fluoroquinolones (FQs).

FQs are a very important group of synthetic heterocyclic drugs structurally derived from quinolones, which present a fluorine substituent in position 7 of the quinolone ring (Fig. 1b-i). They have antibacterial properties and have been widely used as antibiotics. These pharmaceuticals are usually administrated to both humans and animals for treating different kinds of infections, especially digestive, pulmonary and urinary ones. They are active against both gram-positive as well as gram-negative organisms. FQs are absorbed adequately after ingesting them and widely distributed in body tissues. Serum concentrations reached after oral administration are comparable to

intravenous administration, allowing for an early transition between both pathways (intravenous to oral) and the potential cost reduction. FQs are excreted by urine as unchanged compounds [25].

In the last years, their consumption and dosage has increased, affecting negatively to the biota because of their presence as pollutants in urban wastewater (mainly due to its urine excretion by treated patients). This fact leads to the appearance of bacterial resistance to these antibiotics. For this reason, these drugs have been recently included in the list of emerging pollutants [26-28] as well as in the WHO 2019 Watch list as antibiotic classes that have higher resistance potential [29].

Sample preparation techniques based on different extraction procedures have been used for determination of FQs in biological, food or environmental matrices prior instrumental quantification. On-line and off-line solid phase extraction (SPE) [30-31], solid phase microextraction (SPME) [32], molecular imprinted polymer extraction (MISPE) [33], ultrasound solid liquid extraction (USLE) [34], hollow-fiber-liquid phase microextraction (HF-LPME) [35-36] or ultrasound assisted extraction (UAE) [26] have been successfully employed. Electromembrane extraction using commercial polypropylene hollow fiber as support of the liquid membrane has also been developed in our research group for chromatographic determination of seven FQs: Enrofloxacin (ENR), Grepafloxacin (GRP), Danofloxacin (DNF), Gatifloxacin (GTF), Marbofloxacin (MRB), Norfloxacin (NRF) and Ciprofloxacin (CPR) in wastewater samples with high enrichment factors [37].

As mentioned above, excellent results were obtained in a previous work by using chitosan membrane in the EME procedure of polar acidic drugs [24]. In this research, an active role of the biopolymer in the extraction process of the target compounds was observed. Taking into account the polar characteristics of FQs due to the presence of fluorine and carboxylic groups in their chemical structure, in addition to the amphoteric properties conferred by carbonyl and nitrile functional groups depending on the pH media; a novel EME procedure has been proposed using chitosan membrane as active support for the selective extraction of three FQs (MRB, ENR and Flumequine (FLM)), in presence of the other FQs mentioned above (GRP, DNF, GTF, NRF and CPR). Posterior determination by means of High Performance Liquid Chromatography (HPLC) combined with UV detection was carried out.

Besides, theoretical calculations have been performed for understanding the most favored interaction between fluoroquinolones and the biopolymeric membrane

during EME procedure. Theoretical calculations reveal the existence of two hydrogen bonds between FQs and chitosan that stabilizes the system and are able to explain the selective extraction of MRB, ENR and FLM. Quantum Theory of Atoms in Molecules (QTAIM) has been used for analyzing the hydrogen bond interactions [38-39].

The agreement of the experimental and theoretical results helps to offer a clear view of the most favorable interaction sites of FQs molecules and the biopolymer during the extraction process, being chitosan a promising support in EME procedures due to its active role during the extraction process.

Also, a Box-Behnken experimental design has been carried out to determinate the influence of most important parameters affecting to EME procedure. The enrichment factors for each extracted FQs are chosen as the response factor in order to obtain the better experimental conditions for performing the extractions.

2. Experimental

2.1. Chemicals and reagents

All reagents and chemicals used were of analytical grade. Chitosan of 310000-375000 Da molecular weight, Enrofloxacin (ENR), Grepafloxacin (GRP), Danofloxacin (DNF), Gatifloxacin (GTF), Marbofloxacin (MRB), Norfloxacin (NRF), Ciprofloxacin (CPR), Flumequine (FLM), methanol, acetonitrile, 1-octanol, formic acid and Aliquat®336 were obtained from Fluka-Sigma-Aldrich (Madrid, Spain). Hydrochloric acid, acetic acid and sodium hydroxide, were obtained from Merck (Darmstadt, Germany). Working solutions were daily prepared by adequate dilutions from stock methanolic solutions (100 mg L^{-1}) of ENR, GRP, DNF, GTF, MRB, NRF and FLM. For CPR, 100 mg L^{-1} stock solution was prepared in 80:20 (v/v) methanol:water. Ultrapure water from Milli-Q Plus water purification system (Millipore, Billerica, MA, USA) was used for dilutions and solutions.

2.2. Chitosan membrane synthesis

Synthetized chitosan membrane had a composition of 60% (w/w) chitosan and 40% (w/w) Aliquat®336 with 30-35 μm thickness, measured using Scanning Electron Microscopy (SEM). The synthesis of the biopolymeric membrane was done as described in a previous paper of Román-Hidalgo et al. [24]. Chitosan (310000-

375000Da) (0.1 g) was solved in acetic acid 1% (v/v) (25 mL), adjusting to pH 5.0 by adding sodium hydroxide 1M. This solution (25 mL) was added to Aliquat®336 (0.066 g) in continuous stirring. The resulting homogenized solution was poured onto a 90 mm diameter glass Petri dish and introduced into a vacuum stove at 35ºC for complete evaporation of the solvent. Afterwards, a washing process of the chitosan membrane, with sodium hydroxide 0.1M and water, was necessary before using it for the EME procedure. Once washed, the membrane was dried again in the vacuum stove and then, peeled off the dishes.

2.3. EME procedure

For carrying out the electromembrane extraction of fluoroquinolones, a self-made device previously developed in our research group was used [21, 24]. 5 x 5 mm pieces of synthetized chitosan membrane were cut and glued to a 2.5 cm length x 4 mm external diameter and 2 mm internal diameter bore glass tube. Once glued, the biopolymer membrane was impregnated with 5.0 µL of 1-octanol and the excess of organic solvent was removed using a wipe. Then, 50 µL of ultrapure water pH 9 (aqueous NaOH solution) were introduced inside the tube, serving as the acceptor phase. The glass tube with the acceptor phase was placed into a vial containing 10 mL of the donor phase (aqueous NaOH solution pH 10 of target analytes). For carrying out the EME procedure, two platinum electrodes (0.25 mm diameter), at 2 mm distance, were introduced into the acceptor and donor phases, respectively. Both electrodes were connected using a three channels Laboratory DC Power Supply (Benchtop Instrument, Pennsylvania, USA) with programmable voltage in the range 1-120 V. Electric current during the extraction process was registered with a digital multimeter (3430 4 ½-digit PeakTech®, Ahrensburg, Germany). An automatized system controlled by a personal computer was also used for data acquisition during the extraction time. Best extraction efficiency was obtained applying a DC potential of 80 V during 20 minutes with constant stirring at 600 rpm.

Average currents in the range 200-300 µA were registered during the extraction time. Once the extraction was finished, 20 µL of the acceptor phase were injected in the HPLC system. An illustrative figure of the device can be seen in Fig. S1.

2.4. HPLC-DAD determination

A LabChrom® VWR-Hitachi (Barcelona, Spain) liquid chromatograph, equipped with a quaternary L-7100 pump, a L-2200 autosampler (20 µL) and a L-7455 diode array detector (DAD) was used for the HPLC separation of the target analytes. An Eclipse® XDB-C18 3.5 µm (150 mm×3.0 mm i.d) (Agilent Technologies, Little Falls, DE, USA) chromatographic column was used with a Kromasil® 100 Å, C18, 5 µm (15 mm×4.6 mm i.d.) (Schrarlab S.L., Barcelona, Spain) guard column. During elution, column was thermostated at 10^{o}C.

Chromatographic separation was achieved with a mobile phase consisted of a mixture of 0.1% (v/v) formic acid aqueous solution (component A) and acetonitrile (component B) at 0.5 mL min^{-1} flow rate. A gradient elution was required, described in TableS1. For fluoroquinolones quantitation, diode array detection (DAD) was employed being the following monitoring wavelengths for each compound: 300 nm for MRB, 280 nm for NRF, CPR, DNF, ENR and GRP, 290 nm for GTF and 320 nm for FLM.

2.5. Statistical Data Processing

Taking into account previous studies, the most significant factors affecting to the extraction procedure were selected and a Box-Behnken experimental design was carried out to improve the extraction efficiency of the target fluoroquinolones. The selected four most important experimental variables of EME were: donor phase pH, acceptor phase pH, applied voltage and extraction time. MODDE statistical software, version 12.1 (MKS Umetrics AB, Sweden), was used for performing graphical and numerical analyses of the results.

2.6. Computational framework

Full geometry optimization of the molecules was performed with Gaussian 09 [40] using Density Functional Theory (DFT) with B3LYP functionals and cc-pVDZ basis set [41-43]. Integral Equation Formalism (IEF) of the Polarisable Continuum Model (PCM) was considered for solvent effect being water as solvent [44-45]. Wave function analysis using the atoms in molecule theory was carried out as it is implemented in AIMAll software package [46]. Chemcraft software was used for the visualization of the molecules [47].

3. Results and discussion

3.1. Molecular interaction modelization

The selectivity of the EME procedure for the studied fluoroquinolones is mainly governed by the specific chemical interaction between the analytes and the biopolymeric substrate. From this point of view, a theoretical analysis of these interactions, which may play a crucial role, can lead to a better understanding of the physical phenomena that are controlling the separation process. For this purpose, a theoretical study of the interaction between chitosan and fluoroquinolones has been carried out by using the single monomer structure of the D-glucosamine (D-GLU) as a representative unit of biopolymer, assuming an adequate compromise between optimal results and an efficient computational time cost.

The interaction of MRB, ENR, FLM, DNF, GRP, GTF, NRF and CPR with a molecule of D-glucosamine in presence of water as solvent was studied. Three different geometrical disposals (type A, B and C) for investigating the chemical interactions have been proposed:

A) Direct approximation of the carboxyl group of the FQs to the amine terminal group of glucosamine.
B) Direct approximation of the carboxyl and keto groups of the FQs to the amine terminal group of glucosamine.
C) Direct approximation of the carboxyl and keto groups of FQs with both the amine and hydroxyl groups of glucosamine.

Fig. 2 shows the three different optimized geometries (type A, B and C, from left to right) for the particular case of the MRB. Dotted lines represent specific interactions between MRB and D-GLU for each case: H···N interaction in type A geometry, O···H and H···N in type B and C geometries. At this point, the obtained results suggest an intermolecular attraction, showing a molecular distance compatible with the typical one corresponding to a bridging hydrogen bond. The different optimized geometries for the interactions of all other fluoroquinolones (24) are shown in the supplementary material (Fig. S2-S4).

Furthermore, the interaction energies (enthalpy) have been calculated for the investigated drugs in presence of glucosamine for the geometries type A, B and C, which are gathered in Table 1. The obtained values for all the FQs indicate that the most favored interaction corresponds to the geometries type C (more stabilizing energies), followed by type A and B (less stabilizing energies) in order of importance. These

results are of interest because can indicate that the hydrogen bond interaction acts as the driving force in stabilizing the analyzed system.

According to the energy values showed in Table 1, type C geometries follow this order of stability: GRP > ENR ~ CPR > DNF > NRF > GTF > FLM > MRB. In this case, the existence of two hydrogen bonds located between the amine and hydroxyl group of glucosamine with carboxyl and keto groups from FQs, respectively, play a major role in the stabilization of the system. These results will be corroborated later by the QTAIM analysis. As energy values indicate, the direct interaction of the amine group in type A geometry is favored over the interaction of amine group with both the carboxyl and keto groups (type B). The values of the interaction energies obtained for type A geometries are very similar and all obtained values are close to -34 kcal/mol with the following order of stability: GRP ~ DNF > NRF > ENR ~ CPR > GTF > MRB > FLM. For the interaction in type B geometries, lower energies are found from -28 to -31 kcal/mol, showing the following stability order is: GTF ~ ENR > FLM > GRP > DNF > NRF > CPR > MRB.

The relevance of the energetic order obtained from this molecular analysis can help to understand two important issues from the analytical point of view: how fluoroquinolones keep retained by the chitosan membrane as well as its retention order during the EME procedure. The preferred interaction site for the FQs with glucosamine corresponds mainly to type C geometry, as it was stated before, which agrees with the poor extraction of GRP, DNF, GTF, NRF and CPR obtained in the analytical experiments when using a complex chitosan biomembrane. Thus, the most probable situation is a membrane with preferential occupation of molecules with type C spatial disposition (the most stable energetically), as well as a lower population of less stabilized interaction sites with type A and B geometries, respectively. This mixed site occupation with different kind of interactions in the real chitosan membrane may be the explanation of the obtained enrichment factors.

The QTAIM theory [38-39] is based on the topological analysis of the electron density (ρ), investigating how the electronic charge is locally concentrated in a spatial region, through the analysis of its Laplacian values ($\nabla^2\rho$). Thus, it is possible to obtain the total energy density (H) at the Bond Critical Point (BCP), which disclose the nature of characteristic interactions under investigation. Hydrogen bonds are a kind of closed-

shell interactions that typically present low values for the electron density (ρ) and positive values of the Laplacian values ($\nabla^2\rho > 0$).

With this purpose, QTAIM theory has been applied to the previously obtained type C geometries in order to analyze the nature of the two hydrogen bonds interaction between fluoroquinolones and glucosamine. Table 2 gathers the obtained results for the interaction between both the hydrogen atom from hydroxyl group of glucosamine (D-GLU) with the oxygen atom from the keto group of the fluoroquinolones (H···O) and the hydrogen atom from the oxygen of the carboxyl group and the nitrogen atom from D-GLU (H···N). The values observed in Table 2 for both H···O and H···N interactions at the BCP reveal typical values which are characteristic of a hydrogen bond. However, for the case of the H···O interaction, the obtained values for total energy density are positive (H > 0), which is a typical result for a weak hydrogen bond as stated elsewhere [48-50]. Nevertheless, for the case of the H···N interaction, the opposite tendency is found for the total energy density (H), presenting negative values for all compounds. These results are usually found in shared interactions for polar or covalent hydrogen bonds with medium strength [48-50]. Furthermore, the nature of these hydrogen bonds interactions can be confirmed through the correlation of the total energy density (H) and the electron density (ρ) obtained values [48,51-53]. Fig. 3 shows the existence of a linear relationship of both variables for the case of the H···O (panel A) and H···N (panel B) investigated hydrogen bonds at the BCP. The inset figures represents the linear dependence of the electron density (ρ) with the bond length, corroborating the nature of the investigated hydrogen bonds in type C geometries. Therefore, the combination of two hydrogen bond interactions of different strength: weak for the H···O and medium for the H···N interactions, act as the driving force that stabilizes the type C geometries from the analysis of the Fig. 3 (panel C).

Depending on the solution properties and the polar characteristics applied to the chitosan membrane, the nitrogen atom from the amine group may exert a higher attraction toward the hydrogen atom from the carboxyl group of the FQs. This fact in combination of a weak hydrogen bond interaction with the oxygen atom from the keto group of the drugs, play a key role in the selective extraction of polar active compounds. This result agrees with the experimental outcome in which MRB, ENR and FLM present the highest enrichment factors of extraction, respectively.

3.2. Optimization of EME experimental conditions using a Box-Behnken design

The performance of an electromembrane extraction procedure usually depends on several experimental parameters that control the overall process. In order to optimize the extraction efficiency of the analytes, experimental design is commonly carried out considering the main experimental factors that govern the experiment, as well as the interaction between them [54-55]. In this case, pH and volumes of donor and acceptor phases, organic solvent for supported liquid membrane, stirring speed, applied voltage and time of extraction are the variables that can influence the extraction procedure.

In a first step and taking into account previous research, volumes of donor and acceptor phases were studied for EME developed using the same extraction device [21, 24], obtaining that volumes of 10 mL and 50 □L for donor and acceptor phase, respectively, led to better recoveries. Additionally, different organic solvents to be used as supported liquid membrane (SLM) have also been studied in previous works [21-24], being 1-octanol the one that provided better extraction efficiency. Moreover, the extraction efficiency also increased with the stirring speed, which decreased the double layer generated around the SLM, favoring the mass transfer of the analytes through it. Consequently, stirring speed was fixed at the maximum value that does not produce vortex (600 rpm) in the donor solution [56].

According to preliminary studies a Box-Behnken design was carried out considering the following key factors at three different levels: donor phase pH (6-9-12), acceptor phase pH (6-9-12), applied voltage (30-75-120 V) and extraction time (5-17,5-30 minutes). Range for each factor was selected after a screening study which clearly indicated the advantages of keeping pH values between 6-12 both in acceptor and donor phases, and voltages lower than 120 V. Table 3 shows the design matrix of Box-Behnken optimization for 27 experiments, including three central points. EFs obtained for each fluoroquinolone were selected as the response objective for the study. In order to minimize possible effects of uncontrolled factors, all experiments were carried out in randomized order.

Determination coefficient ($R_{squared}$) for the polynomial regression was higher than 0.8, as expected for a signification regression. Additionally, ANOVA test demonstrated the model was significant. Three-dimensional surface plots for MRB as example are shown in Fig. 4a-d, plotting EF versus: a) voltage and extraction time maintaining donor and acceptor phases at pH 9 (central value), b) donor phase pH and

acceptor phase pH maintaining voltage and time at 75 V and 17.5 min, respectively, c) acceptor phase pH versus voltage (pH 9 donor phase and 17.5 min extraction time) and d) acceptor phase pH versus extraction time (pH 9 donor phase and a voltage of 75 V). Three-dimensional surface plots for ENR and FLM are disposal as supplementary material (Fig. S5a-d).

By increasing the voltage, an increase in the extraction efficiency could be expected because it generates an electric field that favors the migration of the analytes through the membrane to the acceptor phase. However, high electric fields make EME non-reproducible due to an unstable electric current and the subsequent generation of bubbles [57-58]. Because of this, voltages higher than 120 V are unable to be applied in this EME system. On the other hand, extraction time can enhance the EME efficiency until equilibrium in the transference process is achieved, followed by a decrease in extraction efficiency due to changes in pH because of electrolysis phenomena [59]. Therefore, extraction time is limited to 30 minutes in this system. Regarding acceptor and donor phase pH, high EFs were obtained when basic pH are used in both donor and acceptor phases, probably due to the ionization of the analytes as a consequence of their pKa values. This fact favors an easily migration of the drugs across the membrane to the acceptor phase.

Best extraction conditions, based on the results of the experimental design were: pH 9 and pH 10 for acceptor and donor phases, respectively, a voltage of 80 V and 20 minutes of extraction time. Under these conditions, EFs of 58, 82 and 83 for FLM, ENR and MRB, respectively, were achieved.

3.3. Validation of the proposed EME method

Once optimized the EME experimental conditions, the extraction of the FQs was carried out. As shown in Table 4, high enrichment factors were obtained for MRB, ENR and FLM, respectively. DNF, GRP and GTF showed poor enrichment factors, whereas NRF and CPR were not extracted. At a glance, it can be assessed that the electromembrane system using chitosan membrane as support leads to a selective extraction of MRB, ENR and FLM. In Section 3.1., a detailed explanation of the interaction between the biopolymer and FQs during the extraction procedure is provided. The computational calculations unravel chemical insights of the active role of

chitosan membrane in the process, conducting to the selective extraction of the three fluoroquinolones.

Quality parameters such as linearity, sensitivity and precision were evaluated in order to complete the suitability of the selective EME procedure since an analytical point of view. Figures of merit of the method are shown in Table 4, including linear range, linearity, Detection and Quantitation Limits (LOD, LOQ) and Enrichment Factors (EF) for the three FQs. LOD and LOQ calculations were done as three and ten times signal to noise ratio, respectively [60].

It can be observed a good linearity in all cases obtaining values for linearity range higher than 97.5%. LODs were 1.3 □g L^{-1} for MRB, 1.7 □g L^{-1} for ENR and 1.5 □g L^{-1} for FLM. Values between 4.2 □g L^{-1} for MRB and 5.6 □g L^{-1} for ENR were obtained for LOQs.

In order to evaluate the repeatability and intermediate precision, the EME proposed method was applied to urine samples spiked at three concentration levels. Measurements were done in one single day and two days per week during three weeks, respectively. The corresponding values for %RSD were 4-7% for repeatability and 5-12% for intermediate precision.

3.4. Analysis of real samples

MRB, ENR and FLM are fluoroquinolones often used as veterinary antibiotics. Therefore, the proposed EME method was applied to four different dog urine samples in order to check its suitability in biological samples. Hence, urine samples were collected from healthy dog pets (with previous consent of their owners), in order to assess the total absence of pharmaceuticals in the animal urine. This way, these urines were used as blank samples (Fig. 5a). Once collected, samples were spiked at three different concentration levels of each FQs (Table 5) and microfiltered (0.22 □m). For MRB, ENR and FLM, the fortification levels were chosen taking into account the dosage usually administrated to animals as well as the amount of drug excreted in unaltered form [61-62]. For the rest of FQs, the spiked levels of concentration were chosen to cover the full range of linearity. Then, samples were diluted (1:1000, v/v) with ultrapure water and submitted to the electromembrane extraction procedure. Found amounts according to the effective recovery of the EME method are shown in Table 5. It can be noticed that only MRB, ENR and FLM have been extracted from the spiked urine

samples. Besides, good agreement can be observed between the spiked amount of these three drugs and the determined amount. Also, as expected, the rest of FQs were not extracted. Chromatogram corresponding to a dog urine sample spiked at a concentration level of 100 mg L^{-1} of MRB, 50 mg L^{-1} of ENR and 100 mg L^{-1} of FLM is shown in Fig. 5b. Once studied the obtained results, it is clear that the proposed EME procedure is suitable for the selective extraction of marbofloxacin, enrofloxacin and flumequine in urine samples.

4. Conclusions

Chitosan biofilm has been used as active support in a novel EME procedure for the selective extraction of marbofloxacin, enrofloxacin and flumequine from animal urine samples.

Theoretical analysis carried out for the deep study of the extraction mechanism, provides clear evidences of the biopolymer active role during the process. The existence of two hydrogen bonds between the fluoroquinolones and the biopolymer that stabilizes the systems has been analyzed by QTAIM. This analysis reveals H···O and H··N interactions between the hydroxyl and amine group of glucosamine in the biopolymer with keto and carboxyl groups of the FQs. The energetic order of interaction between fluoroquinolones and glucosamine agrees with the experimental trend in which the extraction of MRB, ENR and FLM is favored. In the experimental system, the media and the polarity applied to the chitosan membrane may facilitate a higher H-N and H-O attraction between the biopolymer and FQs that promotes the selective extraction of the polar active compounds.

Unlike previously described methods, the proposed one is able to selectively extract MRB, ENR and FLM in presence of other FQs. Besides, the method has been successfully applied to the selective extraction of the target compounds from animal urine samples.

Acknowledgments: This work has been supported by the Project PGC2018-096608-B-C22 from FEDER/Ministerio de Ciencia e Innovación – Agencia Estatal de Investigación and the project RED2018-102522-T from Plan Estatal de Investigación Científica y Técnica y de Innovación/ Ministerio de Ciencia, Innovación y Universidades. Authors thanks funds from Junta de Andalucía (2019/FQM-106, FQM-291 and FQM-

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Captions of figures

Figure 1. Chemical structures a) Chitosan; b) Marbofloxacin; c) Enrofloxacin; d) Flumequine; e) Norfloxacin; f) Ciprofloxacin; g) Danofloxacin; h) Gatifloxacin; i) Grepafloxacin.

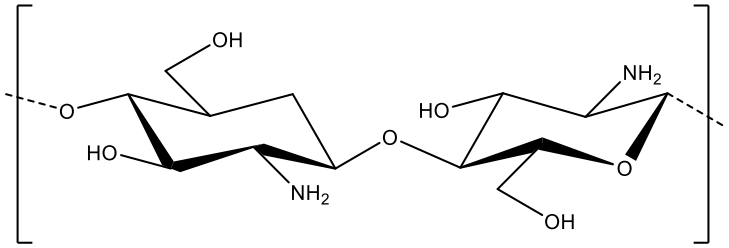
Figure 2. Optimized structures with water as solvent of the interaction between MRB with a glucosamine molecule through the geometries **A**, **B** and **C**. Dotted lines represent the H···O and H···N interactions.

Figure 3. Plots of the total energy density versus density of H···O (A) and H···N (B) hydrogen bonds at the BCP. In the inset is included the relationship between density with bond length. The QTAIM molecular graph of MRB with glucosamine system is shown (C) for clarifying purposes with bond paths in red lines and BCP as green spheres.

Figure 4. Three-dimensional response surface profiles for Marbofloxacine (MRB). Plots of Enrichment Factor (EF, z-axis) as function of: a) voltage and extraction time; b) donor phase pH and acceptor phase pH; c) acceptor phase pH and voltage and d) acceptor phase pH and extraction time. All other factors were set to their central value.

Figure 5. HPLC chromatograms of animal urine samples submitted to the EME procedure. (a) blank urine sample (b) animal urine sample spiked at mid-level concentration of FQs.

Figure 1



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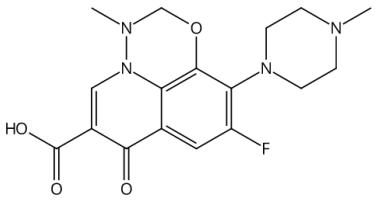

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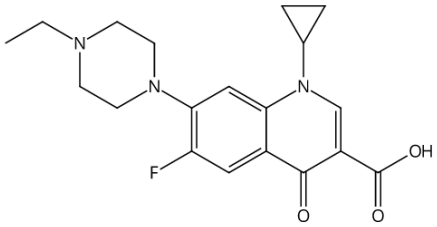

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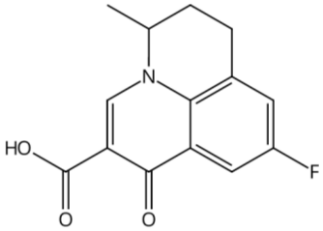

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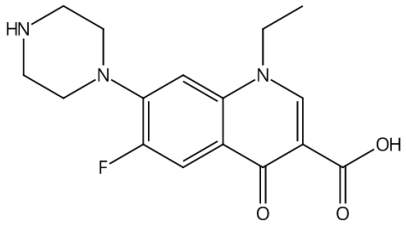

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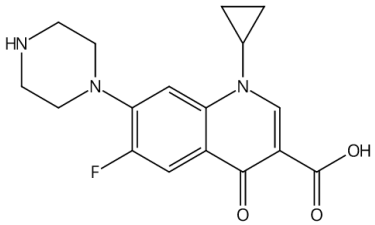

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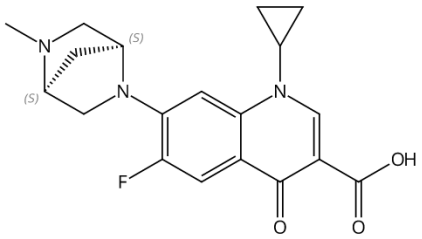

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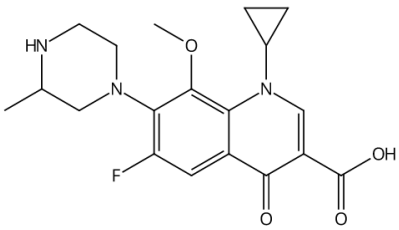

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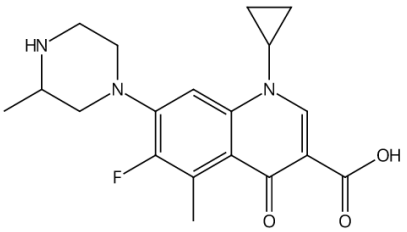


(i)

Figure 2

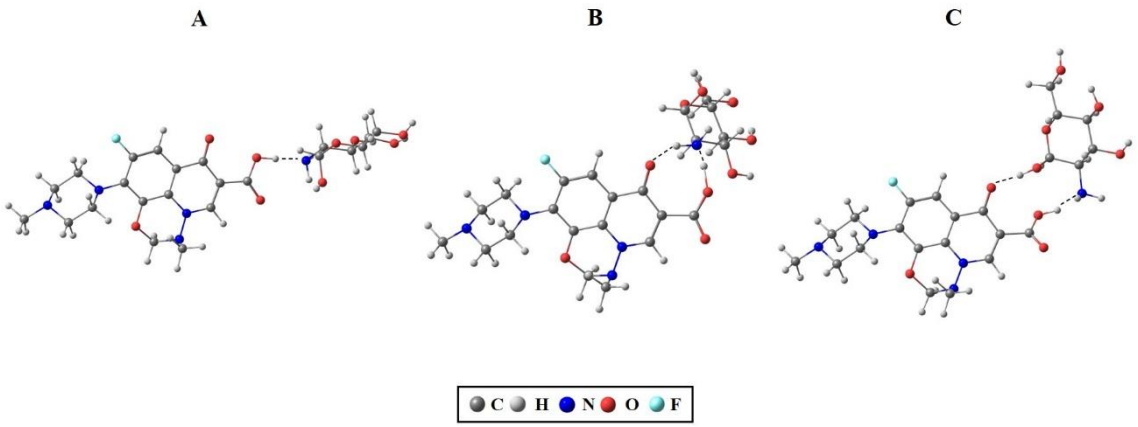


Figure 3

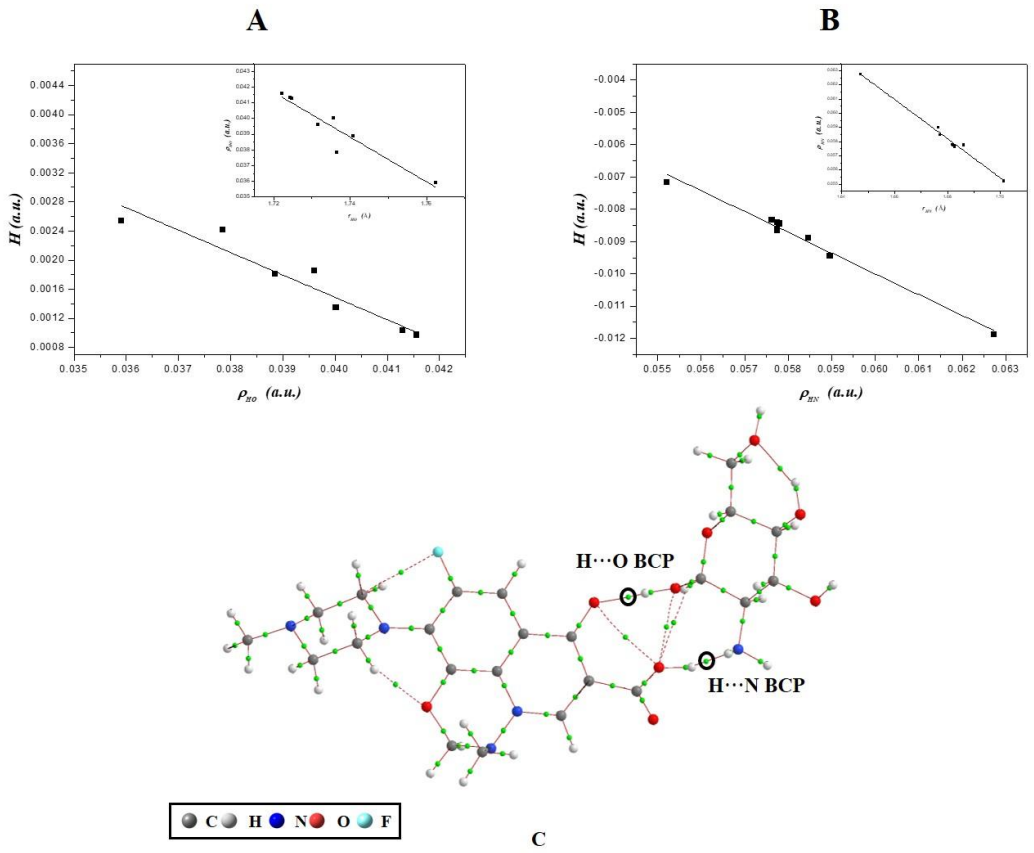
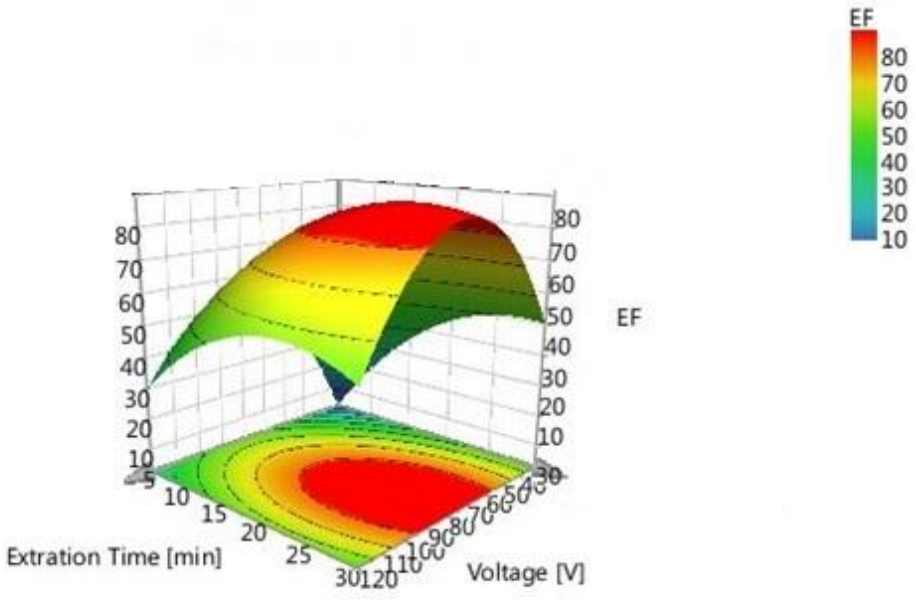
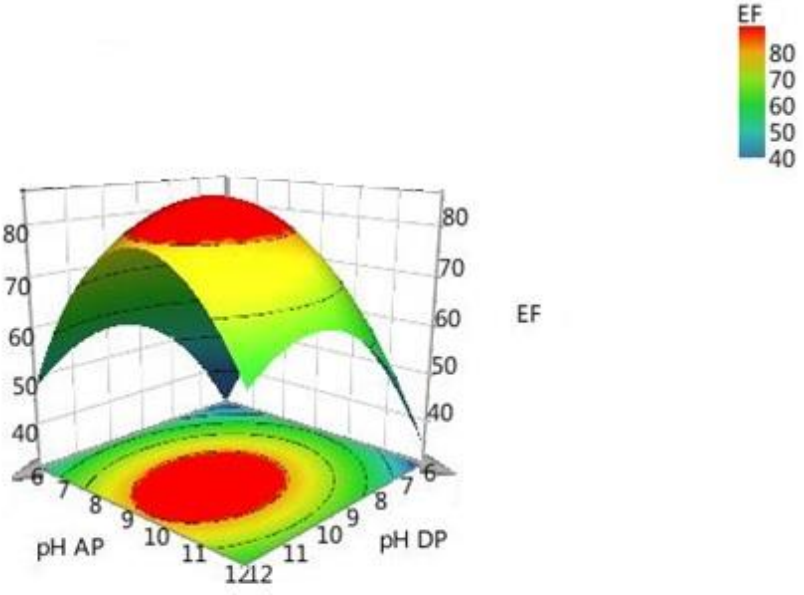


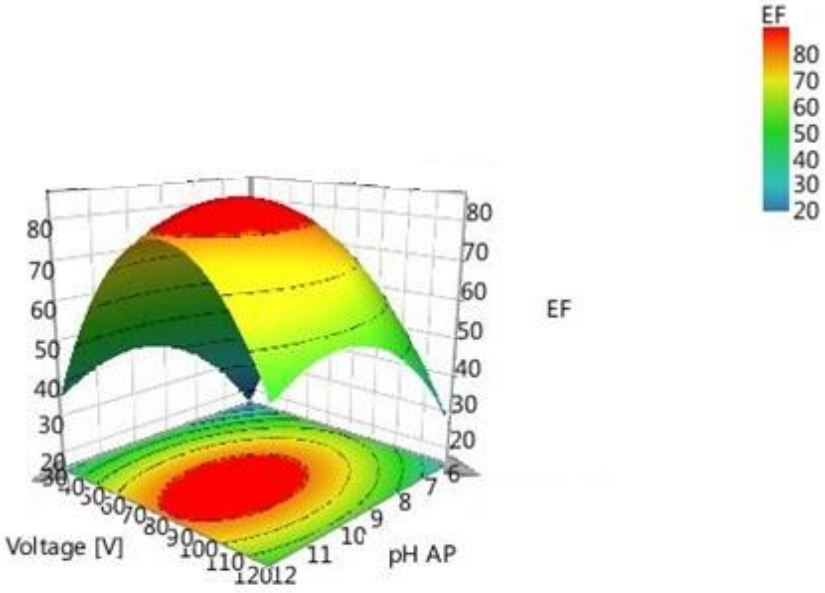
Figure 4


EF
80
70
60
50
40
30
20
10
Extration Time [min]
Voltage [V]
EF

(a)


EF
80
70
60
50
40
pH AP
pH DP
EF

(b)


EF
80
70
60
50
40
30
20
EF
Voltage [V]
pH AP

(c)

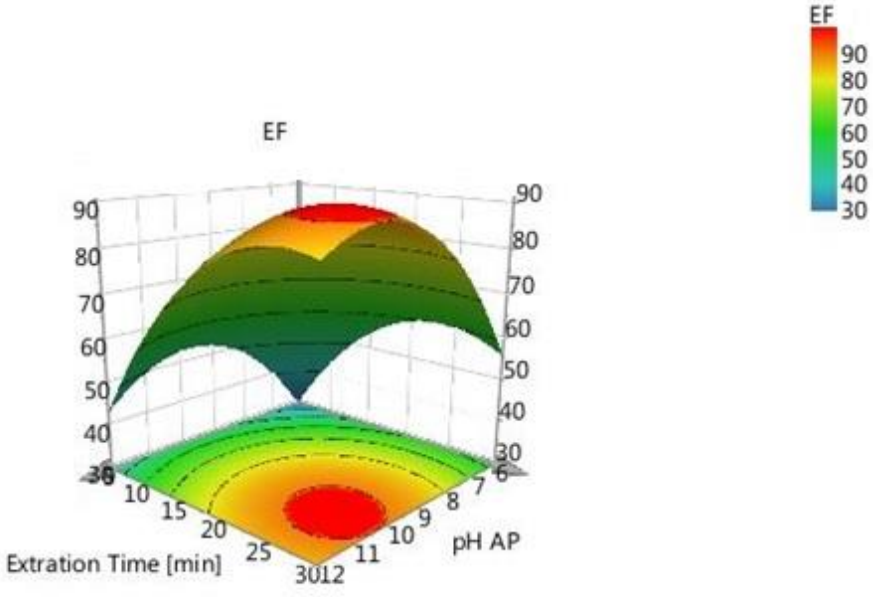
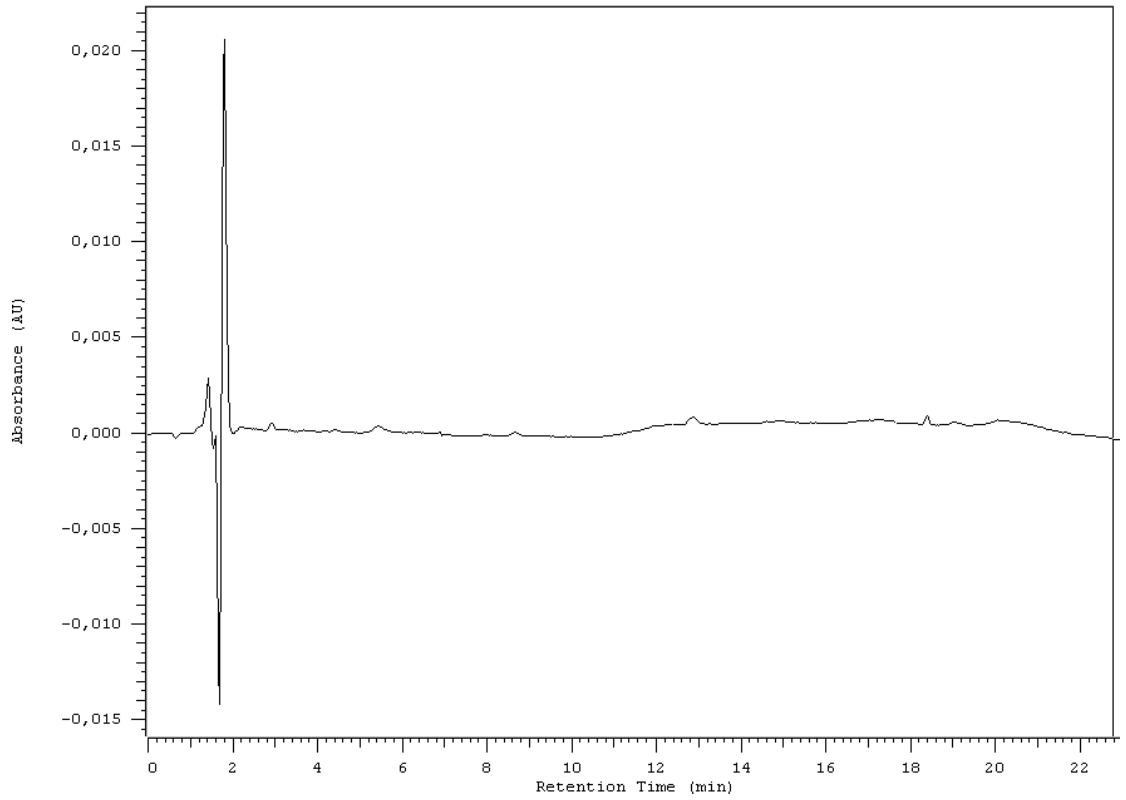

EF
90
80
70
60
50
40
30
EF
Extration Time [min]
pH AP

Figure 5


Absorbance (AU)
0,020
0,015
0,010
0,005
0,000
-0,005
-0,010
-0,015
0
2
4
6
8
10
12
14
16
18
20
22
Retention Time (min)

(a)

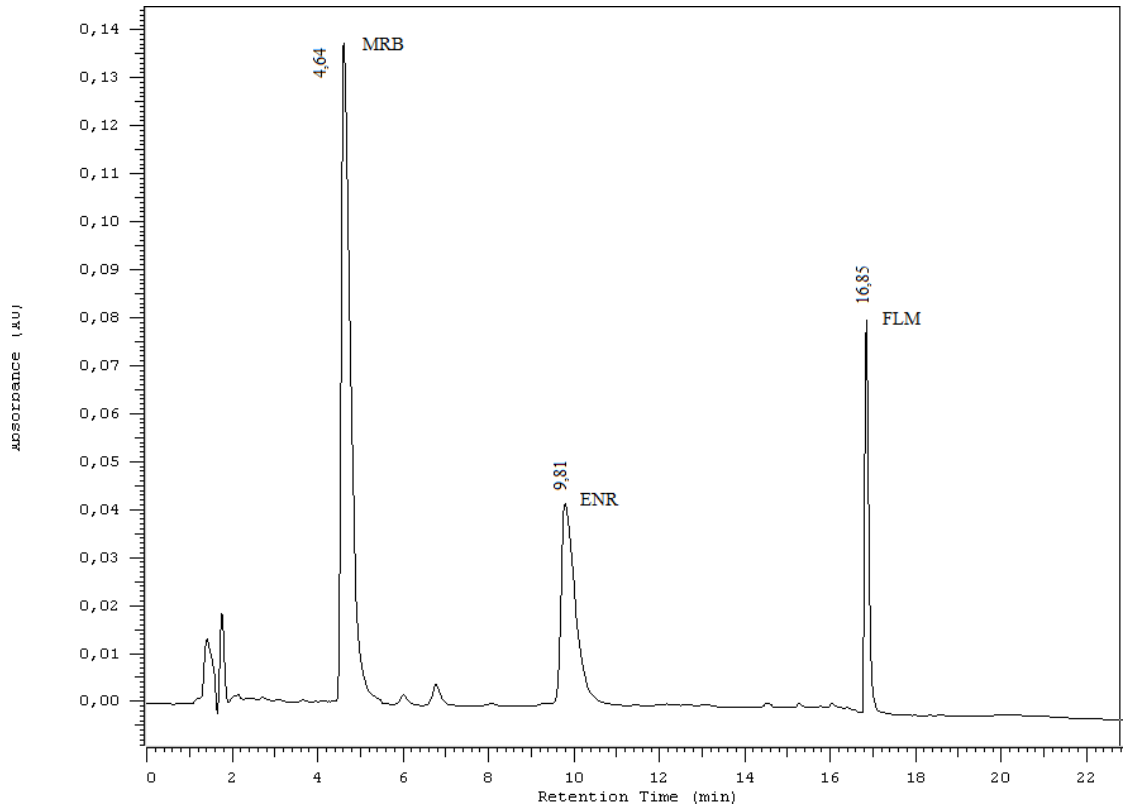

Absorbance (AU)
0,14
0,13
0,12
0,11
0,10
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
4,64
MRB
9,81
ENR
16,85
FLM
0
2
4
6
8
10
12
14
16
18
20
22
Retention Time (min)

Table 1. Interaction enthalpy associated with the geometries **A**, **B** and **C** shown in Fig. S3-S5.

	ΔH_i (kcal/mol)		
	A	B	C
MRB	-34.029	-27.798	-34.626
ENR	-34.235	-30.852	-37.182
FLM	-33.968	-30.433	-34.703
DNF	-34.614	-28.436	-37.046
GRP	-34.692	-28.791	-38.058
GTF	-34.073	-30.877	-34.917
CPR	-34.199	-28.005	-37.173
NRF	-34.494	-28.161	-35.477

Table 2. Electron density (ρ), its Laplacian ($\nabla^2\rho$) and total energy density (H) at the H···O and H···N bond critical point associated with the interaction shown in Figures ???.

	H···O			H···N		
	ρ	$\nabla^2\rho$	H	ρ	$\nabla^2\rho$	H
GRP_	0.04156	0.14006	9.70E-04	0.057631	0.119471	-0.008345
ENR	0.0413	0.1391	0.00103	0.057797	0.119459	-0.008449
CPR	0.04129	0.1389	0.00103	0.057746	0.11948	-0.008414
DNF	0.03885	0.13579	0.00181	0.062733	0.118253	-0.011877
NRF	0.03961	0.14413	0.00185	0.057752	0.116372	-0.008651
GTF	0.03591	0.13537	0.00254	0.055215	0.115538	-0.007165
FLM	0.03785	0.14431	0.00242	0.058966	0.116749	-0.009442
MRB	0.04002	0.13614	0.00135	0.058464	0.11913	-0.008899

Table 3. Design matrix of Box-Behnken

Exp No	Run Order	pH AP	pH DP	Voltage	Extration Time
1	10	6	6	75	17.5
2	1	12	6	75	17.5
3	23	6	12	75	17.5
4	16	12	12	75	17.5
5	26	9	9	30	5
6	6	9	9	120	5
7	2	9	9	30	30
8	27	9	9	120	30
9	9	6	9	75	5
10	3	12	9	75	5
11	21	6	9	75	30
12	4	12	9	75	30
13	22	9	6	30	17.5
14	12	9	12	30	17.5
15	24	9	6	120	17.5
16	17	9	12	120	17.5
17	25	6	9	30	17.5
18	15	12	9	30	17.5
19	7	6	9	120	17.5
20	18	12	9	120	17.5
21	13	9	6	75	5
22	11	9	12	75	5
23	14	9	6	75	30
24	19	9	12	75	30
25	20	9	9	75	17.5
26	8	9	9	75	17.5
27	5	9	9	75	17.5

Table 4. Calibration data. Limit of Detection (LOD). Limit of Quantitation (LOQ) and enrichment factor (EF) for the analytes.

Fluoroquinolone	**Linear range** ($\mu g\ L^{-1}$)	**Linearity**		**LOD** ($\mu g\ L^{-1}$)	**LOQ** ($\mu g\ L^{-1}$)	**EF**
		(%)	**(R^2)**			
MRB	4.2-200	98.1	0.9934	1.3	4.2	83
ENR	5.6-200	97.5	0.9934	1.7	5.6	82
FLM	5.1-200	97.6	0.9950	1.5	5.1	58

Table 5. Application of EME proposed procedure to animal urine samples.

	Urine concentration (mg L^{-1})					
	Low level		**Mid-level**		**High level**	
Fluoroquinolone	**Spiked**	**Found**	**Spiked**	**Found**	**Spiked**	**Found**
MRB	60	58±3	100	112±6	200	190±4
ENR	20	19±2	50	54±3	100	98±4
FLM	70	68±2	100	98±3	200	201±2