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Journal of Chromatography A, 1059 (2004) 25–31
JOURNAL OF CHROMATOGRAPHY A
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Continuous microwave-assisted extraction coupled with derivatization and fluorimetric monitoring for the determination of fluoroquinolone antibacterial agents from soil samples

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Received 7 May 2004; received in revised form 22 September 2004; accepted 23 September 2004

Abstract

An automated screening approach for fluoroquinolone (FQ) antibiotics (norfloxacin and ciprofloxacin) in soil samples has been developed. The proposed approach consists on dynamic microwave-assisted extraction and subsequent real-time on-line monitoring of the analytes extracted; thus, the extraction is halted when complete leaching of the analytes has been reached (independently of the sample matrix), avoiding extraction times in excess. The end of the extraction allowed quantifying the total content of the analytes. The extraction was carried out using pure water as extractant and consisted of a number of extraction cycles (depending on the sample matrix) in which the sample was subjected to microwave irradiation while the direction of the extractant was changed in an iterative manner. The target analytes were fluorometrically monitored after derivatization with a terbium (Tb^{3+})/tri-*n*-octylphosphine oxide (TOPO)/cetylpyridinium chloride (CPCl)/acetate buffer solution. Optimum conditions for analytes extraction and formation of FQ–Tb^{3+}–TOPO ternary complexes have been obtained using the experimental design methodology. The mean recoveries from soil samples spiked with 5 and 0.5 μg/g of each analyte were (95.2 ± 4.16%) and (98 ± 5.21%), respectively. The within-laboratory reproducibility and repeatability, expressed as relative standard deviation, were 7.29 and 5.80%, respectively. The approach only allows monitoring of the overall content of the species that yield fluorescent complexes with the derivatizing reagent, so the use of chromatography is mandatory for individual separation/quantification of the target compounds.
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Keywords: Focused microwaves; Fluoroquinolone antibiotics; Derivatization, LC

1. Introduction

In recent years, public and scientific concern about the relevance of pharmaceutical substances that occur in the environment has increased [1,2] due to their potential risk for aquatic and soil organisms [3–8].

Fluoroquinolone (FQ) antibacterial agents are probably among the most important class of synthetic antibiotics in human and veterinary medicines because of their broad activity spectrum and good oral absorption [9,10]. The amount of fluoroquinolones introduced into the environment is probably low. However, their continuous input into the environment, together with their relative persistence [11,12], may lead to a significant concentration.

Several authors have tried to predict the potential environmental fate and risk of some FQs [13–21]. The major source of environmental contamination is human and animal excretion. As FQs are usually administrated orally and they are very poorly absorbed through the digestive tract, about 60–85% of FQs are excreted and largely non-metabolised [22]. The sewage sludge and manure containing FQs are usually applied to agricultural field as fertilizers, so the non-metabolised drugs can accumulate in the soil [23,24] and

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doi:10.1016/j.chroma.2004.09.086

affect terrestrial organisms. The effect of such contamination on terrestrial biota has been investigated under laboratory conditions and on soils where different plant species were grown [3,4]. These studies demonstrated that the drugs altered the normal postgerminative development of the plants and the growth of roots, hypocotyls and leaves. Moreover, FQs can pass into surface and ground water after rain, depending on their mobility in the soil system, and affect aquatic organisms. Among the possible effects, a drug-resistant bacterium is increasingly observed [6,8]. For this reason, it is necessary to monitor their presence in the environment.

A number of methods have so far been proposed for the analysis of FQs in biological matrixes [25–27], while the methods to analyze these compounds in environmental samples have been scarce, and mainly devoted to aqueous samples [28,29]. The monitoring of these drugs in solid environmental samples is based on time-consuming methodologies, as solids require more complex pre-treatments than liquids. Extraction of these analytes from the solid matrix has been performed by sonication or by simple blending or stirring of the sample with either pure or mixture of polar organic solvents and even with aqueous solutions [12,30,31]. The use for these compounds of more advanced extraction techniques, such as pressurized liquid extraction (PLE), has only been reported once, using an organic solvent as extractant [32]. Most determination steps of these compounds in solid environmental samples are based on the use of high performance liquid chromatography (HPLC) with fluorimetric [30,32] or photometric detection [31]. UV detection is more frequently used in pharmaceutical analysis than fluorimetric detection despite the highest sensitivity and better selectivity of the latter, maybe owing to the need for a derivatizing reagent.

FQs present weak native fluorescence; so, their ability to form highly fluorescent complexes with lanthanide ions has been exploited. Terbium ions (Tb^{3+}) show unique fluorescence properties when forming complexes with organic ligands. The strong ion emission of these complexes originates from an intrachelate energy transfer from the triplet state of the ligand to the excited energy levels of the lanthanide ion. The formation of terbium chelates with FQs has been the basis for the development of spectrofluorometric [33] and HPLC–fluorimetric [34,35] methods for the analysis of these compounds in biological samples.

Here, we report an extraction-monitoring approach for two FQs (namely, norfloxacin (NOR) and ciprofloxacin (CIP)) in soil samples. The use of a dynamic microwave-assisted extraction system (using pure water as leaching agent) coupled with a flow injection (FI) manifold that acts as interface between the extractor and the detector provides a fully automated screening approach. The removal of the analytes by microwave-assisted extraction allows the acceleration of the sample preparation step. Moreover, the dynamic extraction system facilitates coupling the extraction with the other steps of the analytical process. The analytes were monitored after derivatization based on the energy transfer from FQs to Tb^{3+} in the presence of tri-*n*-octylphosphine oxide (TOPO) in weakly acidic (pH 5.5) micellar solution of cetylpyridinium chloride (CPCl).

2. Experimental

2.1. Instrument and apparatus

Microwave extraction was performed with a Soxwave-100 focused microwave digestor (Prolabo, Fontenay-sous-Bois, France) with a maximum irradiation power of 300 W. A TX 32 device (Prolabo) was used for the control of the microwave unit. A Gilson Minipuls-3 low-pressure peristaltic pump (Gilson, Worthington, OH, USA) programmed for changing the rotation direction at preset intervals, two Rheodyne low-pressure selection valves (SV) (Rheodyne, Cotati, CA, USA), a laboratory-made Teflon chamber (7 cm × 7.5 mm i.d.) and Teflon tubing of 0.8 mm i.d. were used to build the leaching system.

The dynamic on-line derivatization/detection system was performed by a flow injection manifold constructed with a Gilson minipuls-3 low-pressure peristaltic pump (Gilson, Worthington, OH, USA), a Rheodyne 5041 low-pressure injection valve (IV) (Rheodyne, Cotati, CA, USA) and PTFE tubing of 0.5 mm i.d. (Scharlau, Barcelona, Spain), connected to a Kontron, model SFM 25 fluorimeter (Kontron, Zurich, Switzerland) equipped with an 18 μl flow cell from Hellma (Jamaica, NY).

The coupling of the dynamic extraction system with the FI manifold and the fluorescence detector was as shown in Fig. 1.

The individual separation of the analytes in the extract was performed by an HP1100 liquid chromatograph (Hewlett Packard, Avondale, PA, USA) consisting of a G1311A high-pressure quaternary pump, a G1322A vacuum degasser, a Rheodyne 7725 high pressure manual injection valve (20 μl injection loop) and a chromatographic diode array detector (DAD) (Hewlett Packard). An Ultrabase C_{18} (250 mm × 4.6 mm; 5 μm particle size from Scharlau) was used as analytical column.

2.2. Reagents

Fluoroquinolone antibiotics, norfloxacin and ciprofloxacin, were obtained from Sigma-Aldrich (Steinheim, Germany). Stock standard solutions of each compound (300 μg/l each) were prepared in ultrapure water and stored in the dark under refrigeration. Working standard solutions of quinolones were prepared daily by appropriate dilution with ultrapure water. Ultrapure water was used as extractant. Acetate stock buffer solution (pH 5.5; 1 M), aqueous stock solutions of Tb^{3+} (20 mM) and CPCl (20 mM) and ethanolic solution of TOPO (10 mM) were also prepared. The derivatizing reagent solution of Tb^{3+} (4 mM)–TOPO (2.6 mM)–CPCl (6 mM)–acetate buffer (pH 5.5; 0.15 M) was prepared by mixing the appropriate volumes of the stock solutions. HPLC

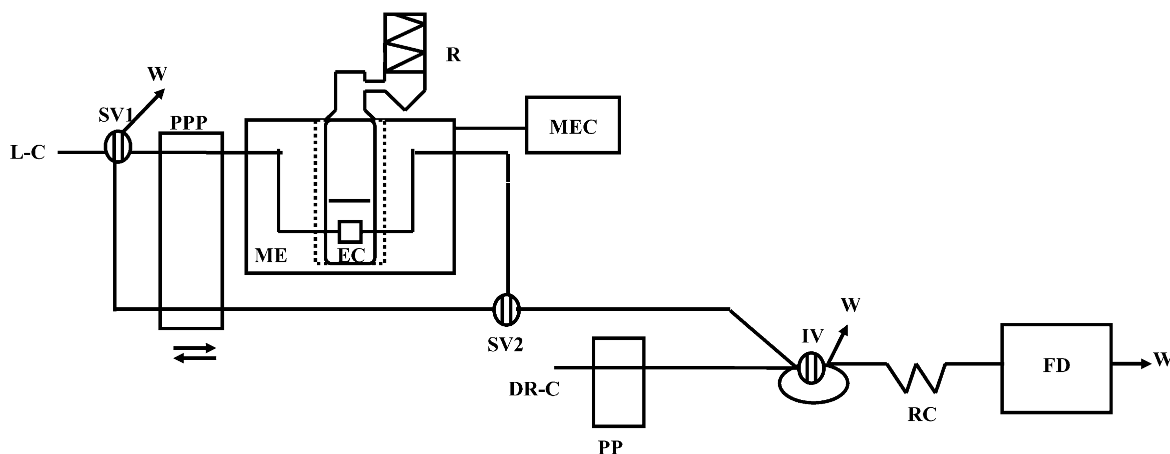

Fig. 1. Experimental set-up. L-C, leacher-carrier; SV, selection valve; PPP, programmable peristaltic pump; W, waste; ME, microwave extractor; EC, extraction chamber; R, refrigerant; MEC, microwave extractor controller; DR-C, derivatization reagent-carrier; PP, peristaltic pump; IV, injection valve; RC, reaction coil; FD, fluorimetric detector.

grade methanol and acetonitrile were used as mobile phase in the chromatographic analysis.

2.3. Sample preparation

The optimization of the extraction step was carried out using a soil spiked with the target analytes to obtain a total concentration of 10 μg/g (5 μg/g of each analyte, related to dry soil mass). A soil spiked with 1 μg/g (0.5 μg/g of each analyte, related to dry soil mass) was used for the application of the method to a sample with environmentally representative concentration. Spiked samples were prepared as follows: (i) two portions of 300 or 100 g of air-dried soil were sieved to a size smaller than 1 mm; (ii) 150 or 75 ml of ethyl ether, containing the necessary volume of stock standard solution of the fluoroquinolones, were added to the soil portions in order to obtain a total concentration of 10 and 1 μg/g, respectively; (iii) the slurries were shaken for 72 h and, after evaporation of the solvent, the soils were completely dried under an N_2 stream and then stored at −20 °C for three months until use, in order to allow sorption equilibrium to be established.

Two natural contaminated soils were collected from two different places near to a farm in Córdoba, Spain. These soils were subjected to the application of the manure of this farm. The soil samples were dried under an N_2 stream, sieved to a size smaller than 1 mm and stored at room temperature until use.

2.4. Procedure

2.4.1. Extraction step

An amount of either 0.2 g of spiked soil or 0.1 g of natural soil was located into the sample chamber, which was placed in the microwave vessel (which contained ≅50 ml of water). Then, an extraction program was applied, which consisted of a number of cycles (which depended on the extraction kinetics of the target sample). Each cycle involved the following steps: (1) filling the closed system (with a total volume of 2.5 ml) with water by a programmable peristaltic pump (PPP) by selecting the leacher-carrier (LC) position of valve SV1 with valve SV2 in the closed-circuit position; (2) microwave irradiation of the sample at 120 W for 5 min. During microwave irradiation, the direction of the extractant (at 1.2 ml/min) was changed each 120 s in an iterative manner (with the help of PPP), thus minimizing increased compactness of the sample in the extraction chamber, and avoiding overpressure in the system as a result; (3) unloading the closed system by switching the selection valve SV2 and driving the extract to the on-line derivatization/detection system.

2.4.2. On-line derivatization/fluorimetric monitoring of the extract

When an extraction cycle had finished, the extract was driven to the 450-μl loop of the injection valve IV. At the same time, the peristaltic pump PP was activated and the derivatization reagent-carrier was circulated through the FI manifold at 0.5 ml/min for establishing the baseline of the detector. Once the sample loop was filled, the injection valve IV was switched and the content of the loop was injected into the FI manifold where the derivatizing reagent-carrier dispersed the extract and the derivatization product was formed in the reaction coil (2 m length) before reaching the detector. The detector wavelengths were set at 333 and 546 nm for excitation and emission, respectively.

For introduction of the standards in the system, the procedure is the same as in the previous case, with the only difference that the loop of valve IV was manually filled with the help of a syringe.

2.4.3. Chromatographic determination

The HPLC separation of the analytes was performed at a flow rate of 1 ml/min using the following gradient elution program: the initial mobile phase was 100% acetonitrile and a linear gradient was established in order to reach a 50:50 acetonitrile:methanol composition in 15 min. Finally, 5 min

Table 1
Optimization of the method

Step	Variables	Tested range	Optimum value
Derivatization	Tb^{3+} concentration (mM)	1.3–4	4
	CPCl concentration (mM)	3–6	6
	TOPO concentration (mM)	0.26–2.6	2.6
	Buffer concentration (M)	0.05–0.15	0.15
	Sample volume (μl)	50–450	450
	Flow rate (ml/min)	0.5–5	0.5
Extraction	Irradiation power (W)	120–300	120
	Irradiation time (min)	1–5	5
	Extractant volume (ml)	1.5–2.5	2.5
	Extractant flow rate (ml/min)	0.2–1.2	1.2
	Number of cycles	1–5	–*

* Depending on the sample matrix (see text).

was necessary for re-establishing the initial conditions. The injection volume was 20 μl. Photometric detection was performed at 277 nm. Quantification of the analytes was carried out by running two calibration curves (one for each analyte) using standard solutions between 0.01 and 1 μg/ml.

3. Results and discussion

The method here proposed involves removal of the target analytes from the solid sample and subsequent on-line derivatization and fluorescence monitoring of the extracts. Quantification of each analyte requires individual chromatographic separation/detection. The experimental design methodology was used to carry out the optimization study of the method. The ranges over which the variables were studied and the optimum values found are listed in Table 1.

3.1. Optimization of the chromatographic step

The experimental variables optimized in order to obtain an appropriate separation of the analytes were the composition of the mobile phase, the flow rate and the injection volume. Different acetononitrile:water and acetonitrile:methanol mixtures and different gradients were used for separation of the target analytes by the Ultrabase C_{18} column. The influence of the mobile phase flow rate was studied in the range 0.5–1.5 ml/min, and the best separation was obtained for 1 ml/min. An injection volume of 20 μl was selected in order to obtain quantifiable photometric signals. Complete separation of the analytes was achieved within 15 min using the gradient elution program given in Section 2.

3.2. Optimization of the on-line derivatization/detection

In preliminary experiments, it was checked that weakly acidic (pH 5.5) aqueous solutions of NOR and CIP show an intrinsic fluorescence ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 440$ nm), which effectively decreases in the presence of Tb^{3+} and TOPO. At the same time, a new emission band characteristic for terbium ion fluorescence in the range 450–610 nm appears due to the energy transfer from the fluoroquinolone ligand to the emitting energy level of Tb^{3+}. The fluorescence signal of the ternary complex of fluoroquinolones is higher than the native fluorescence signal. Moreover, the derivatization reaction is selective, so interferences from extracted species prone to form fluorescent compounds is improbable.

The variables optimized were the concentrations of Tb^{3+}, CPCl, TOPO and buffer, the flow rate of the derivatizing reagent and the sample volume.

A half-fractioned 2^{6-1} factorial design involving 32 randomized runs plus three centered points [36] was built for a screening study of these variables.

The conclusions were that the concentrations of Tb^{3+}, CPCl, TOPO and buffer were not influential variables in the ranges under study; however, better signals were obtained with the highest values tested. Thus, a derivatizing solution of Tb^{3+} (4 mM)–CPCl (6 mM)–TOPO (2.6 mM)–acetate buffer (pH 5.5; 0.15 M) was used for subsequent experiments. The other variables – namely, sample volume and derivatizing flow rate – were influential variables. Better signals were obtained with the highest sample volume and the lower flow rate tested; therefore, higher sample volumes and lower flow rates were tested using a full factorial design involving eight randomized runs plus three centered points [36].

The results of this study showed that both variables were non-significant statistically, but the highest values tested (450 μl of sample volume and 1 ml/min of derivatizing solution flow rate) were selected as these values provided higher signal.

3.3. Optimization of the extraction step

In all experiments, an amount of 0.2 g of spiked sample was used, limited by the dimensions of the extraction chamber (7 cm × 7.5 mm i.d.). With these dimensions, the whole extraction chamber was in the irradiation zone of the focused microwave device. Water was selected as extractant.

The variables susceptible of being optimized in the leaching step were the irradiation power, the irradiation time and the extractant flow rate. The volume of extractant (1.5 ml), corresponding to the capacity of the closed circuit, was kept constant. This volume was considered enough, due to the small amount of sample.

The optimization procedure was developed as follows:

A first full factorial design [36] was built for a screening study of the behaviour of the main variables affecting the extraction step.

The study showed that all the variables were not significant statistically, but with a positive effect on the extraction efficiency; therefore, the highest values tested (300 W of power, 1.2 ml/min extractant flow rate and 5 min extraction time) were selected. However, with these working conditions the extraction efficiency was lower than 50%, which shows that the partition equilibrium of the analytes between the extract and the sample matrix is not favourable to extraction. This was due to the zwitterionic character of FQs (pK_a

COOH = 5.9–6.3, pK_a NH_2 = 7.9–10.2). Due to the presence of protonable functional groups in the chemical structure of the FQs, the pH remarkably influences solubility. At extreme pHs the anionic sites of FQs are protonated or deprotonated which supposed high water solubility and better extraction efficiencies. At neutral pHs, like the pH of pure water, the solubility of FQs reaches a minimum.

Thus, phosphoric and ammonia aqueous solutions should be tested as extractant. However, these extractants were not compatible with the derivatization/detection step. The derivatizing reaction takes places at a pH of 5.5 and the buffer capacity did not allow reaching this value when these extractants were used.

In order to improve the extraction efficiency using pure water as extractant, two new variables were considered in the optimization study (namely, extractant volume and the number of extraction cycles). Thus, the next stage of the optimization procedure was to propose a new screening study of the behaviour of all the variables affecting the extraction step (namely, the irradiation power, irradiation time, extractant flow rate, volume of extractant and number of cycles). A half-fractioned 2^{5-1} factorial design involving 16 randomized runs plus three centered points [36] was performed.

The conclusions of the study were that the irradiation power had non-significant and negative effect on the extraction efficiency, so the lowest value tested (120 W) was selected for subsequent experiments. The irradiation time, the extractant flow rate and the extractant volume were also not significant variables; however, better extraction efficiencies were obtained with the highest values tested. Thus, 5 min extraction time, 1.2 ml/min extractant flow rate and 2.5 ml of extractant were selected for further experiments. The number of cycles was the key variable.

To determine the number of cycles necessary for quantitative recovery of the target compounds from soil spiked with 5 μg/g of each analyte, a study of the extraction kinetics was performed. Five extractions from 1 to 5 cycles were carried out. The other extraction variables were fixed at their optimum values. Recovery of the analytes higher than 90% was obtained with three cycles.

In order to check the influence of the sample matrix on the number of cycles needed for quantitative recovery, different samples (namely, a soil spiked with 0.5 μg/g of each analyte and two natural contaminated soils) were also subjected to kinetics studies. Only one cycle was necessary for total removal of the analytes in the soil spiked with 0.5 μg/g of each analyte; meanwhile three cycles were necessary for the natural contaminated soils, where the analytes were more strongly retained. The results from the two spiked soils show that when the concentration in the soil is high, the number of cycles required to complete the extraction is higher than for soils with low concentration levels of FQs (Fig. 2).

As each extraction cycle requires 5 min and the time for the screening of the extract is around 3 min, a maximum time of 24 min is necessary for complete extraction monitoring, with a total extraction volume of 7.5 ml.

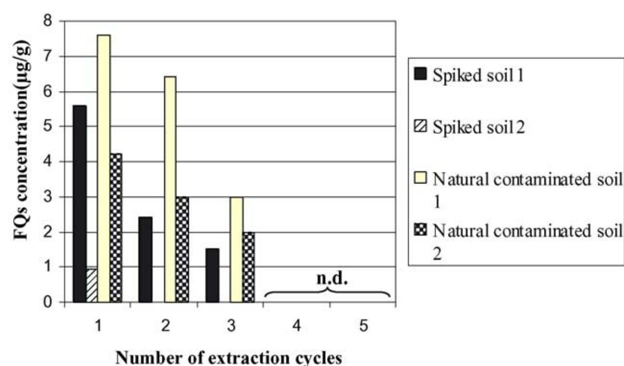

Fig. 2. Kinetics study. Spiked soils 1 = 5 μg/g of each analyte; spiked soil 2 = 0.5 μg/g of each analyte; n.d., not detectable.

3.4. Features of the method for monitoring the extracts

The analytes were jointly quantified due to the fact that the maxima excitation and emission for both analytes appear at the same wavelengths (λ_{ex} = 333 nm; λ_{em} = 546 nm). Also, similar fluorescence intensity was obtained from individual solutions of the analytes with equal concentrations.

A calibration graph was run with equal-concentration mixtures of the two analytes. Solutions of the mixture in water were prepared in the range 0.2–6 μg/ml; that is, ranging between 0.1 and 3 μg/ml for each individual analyte. The calibration graph, which showed a linear shape within the range studied, was used for calculation of the extraction recovery in each cycle.

The relative detection limit, defined as the concentration corresponding to a signal equal to three times the standard deviation of the blank, was 0.02 μg/ml. Taking into account the amount of sample (0.2 g) and the volume of extract (1.5 ml), this corresponds to a detection limit in the soil of 0.15 μg/g. The quantification limit, expressed as the mass of analyte, which gives a signal that is 10σ above the mean blank signal, was 0.05 μg/ml.

Table 2
Results from the fluorimetric monitoring of the samples

	Concentration		Recovery (%)	R.S.D.[a] (%)
	μg/ml[b]	μg/g[c]		
(a) Spiked soils				
Spiked soil at 10 μg/g	0.16	9.5	95	4.2
Spiked soil at 1 μg/g	0.04	0.98	98	5.2

	Concentration found		R.S.D.[a] (%)
	μg/ml[b]	μg/g[c]	
(b) Natural contaminated soils			
Natural contaminated soil 1	0.12	14.3	5.7
Natural contaminated soil 2	0.08	9.3	5

[a] $n = 3$.
[b] Concentration in the extract, the volume of which depends on the number of cycles: 2.5 ml (one cycle) for the spiked soil 2 and 7.5 ml (three cycles) for the other samples.
[c] Total concentration in the soil.

Table 3
Chromatographic results from the extracts obtained by the proposed approach

	Spiked soil at 10 μg/g		Spiked soil at 1 μg/g		Natural contaminated soil 1		Natural contaminated soil 2	
	NOR	CIP	NOR	CIP	NOR	CIP	NOR	CIP
Concentration (μg/g)[b]	4.98	4.93	0.49	0.49	9.8	5.8	6.2	3
Recovery (%)	99.6	98.6	99	99	–	–	–	–
R.S.D.[a] (%)	4	4.8	3.7	4	4.8	4	5	5.5

[a] $n = 3$.
[b] The same as in Table 2.

Analytical recovery was studied by analyzing soil samples spiked with fluoroquinolones at two different concentrations and the results are shown in Table 2a.

The precision of the proposed screening approach was evaluated with two measurements of each analyte per day during 7 days [37]. In all experiments, 0.1 g of soil spiked with 5 μg/g of each analyte was used under the optimum working conditions. The repeatability and within-laboratory reproducibility, expressed as relative standard deviation, were 7.29 and 5.80%, respectively.

3.5. Determination of FQs in natural contaminated soils

Several environmental samples were analysed in order to assess the applicability and performance of the method developed. The FQs were monitored in two natural contaminated soils taken from two different zones near a farm in Córdoba, Spain. The results obtained are shown in Table 2b. The presence of NOR and CIP in these soils demonstrated that FQs reach the terrestrial environment via animal excretion. Moreover, the relative high levels in soils indicate the high affinity of FQs toward soils and their persistence in the terrestrial environment.

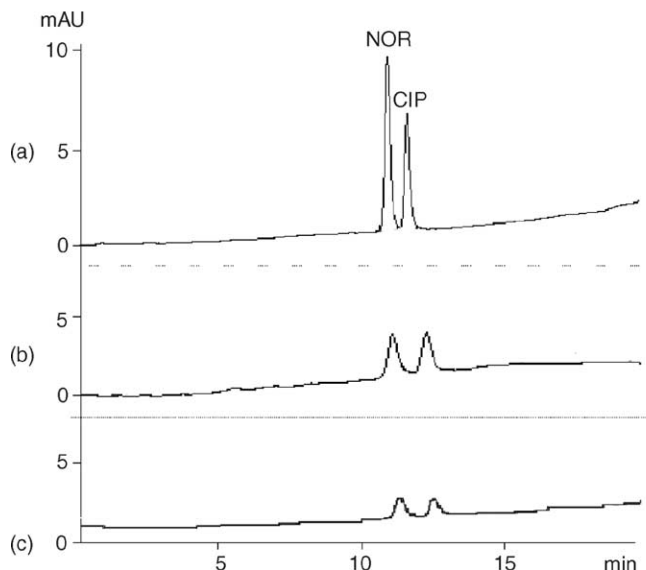

Fig. 3. Chromatograms of the target analytes obtained with a standard solution of the mixture (a), and after extraction from a spiked soil (b) and a natural contaminated soil (c). NOR, retention time, rt = 11.6 min; CIP, rt = 12 min; mAU, milliabsorbance units.

3.6. Individual quantification of the analytes in different soil samples

HPLC separation and photometric quantification of extracts from spiked and natural soils, obtained under the optimal extraction conditions, was performed for each target analyte with the aim of checking both the suitability of the on-line procedure and the reproducibility of the proposed method. The results of this study, shown in Table 3, allow concluding that the method provides satisfactory results in terms of both recovery and reproducibility. The similitude between the results obtained using the chromatographic analysis and those provided by the proposed approach, shows the suitability of the latter as semi-quantitative method.

It is worth to emphasizing that no clean-up was necessary prior to the chromatographic step, as can be seen in the chromatogram in Fig. 3.

4. Conclusions

The construction of the proposed approach has been supported on the coupling of the following devices: (a) a focused microwave-assisted extractor with a dynamic extraction system that allows assembling the extractor with other dynamic devices for on-line derivatization and detection; (b) a simple FI manifold which acts as interface between the extractor and the fluorimetric detector. The use of auxiliary energy for the extraction of the analytes together with the formation of high fluorescent complexes between FQs and Tb^{3+} allow the development of a simple, automated, rapid and sensitive method for the determination of FQs from environmental solid samples.

The present screening approach provides qualitative and semi-quantitative information from natural and spiked samples. It permits to obtain a yes/no answer and the determination of the overall content of the analytes in routine analyses. Moreover, the extraction kinetics is monitored and thus the end of the leaching step determined independently of the sample matrix. Thus, the approach is useful for establishing the level of contamination of any type of sample without using time in excess.

With the proposed approach, the extraction was performed with pure water during 8–24 min as compared with a previous method, which used an acid aqueous solution–organic solvent mixture and required 60–90 min. Thus, the proposed

method reduces costs and provides an environmental friendly method.

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