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Rapid HPLC Method for Monitoring Relevant Residues of Pharmaceuticals Products in Environmental Samples

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Abstract

This work presents a multi-residue analytical method based on solid phase extraction (SPE) followed by high-performance liquid chromatographic (HPLC) with diode array (DAD) detection for the simultaneous determination of a group of pharmaceutical products that include ten antidepressants and three anticancerigenic in environmental samples (water and soil). Baseline separation of the studied compounds was obtained on an ultrabase C_{18} (4.6 mm i.d. × 150 mm, 5 μm particle) column using acetonitrile:phosphate buffer pH 2.5 (35:65, v/v) as mobile phase with a flow rate of 1.5 mL/min. Different aspects including linearity, accuracy, precision and detection and quantification limits were examined in order to validate the proposed method. Detection limits between 1 and 50 ng/mL were obtained for all the target compounds. This method was applied to the analysis of environmental samples as waters and soils of different precedence. Prior, the HPLC determination the samples were purified and enriched using SPE or liquid-liquid extraction (LLE) of the target compounds.

Keywords: High-Performance Liquid Chromatographic (HPLC), Breast Cancer, Antidepressant, Environmental Samples

1. Introduction

The occurrence of residues of pharmaceuticals in the aquatic environment has attracted considerable interest in recent years [1]. The disposal of unused medication via the toilet seems to be of minor importance but many of the pharmaceuticals applied in human medical care are not completely eliminated in the human body. Often they are excreted only slightly transformed or even unchanged mostly conjugated to polar molecules. These conjugates can easily be cleaved during sewage treatment and the original pharmaceutically active compounds (PhACs) will then be released into the aquatic environment mostly by effluents from municipal sewage treatment plants (STPs). Several investigations have shown some evidence that substances of pharmaceutical origin are often not eliminated during wastewater treatment and also not biodegraded in the environment [2]. Under recharge conditions, residues of pharmaceutically active compounds may also leach into groundwater aquifers. Thus, they have already been reported to occur in ground and drinking water samples from water works using bank filtration or artificial groundwater recharge downstream from municipal STPs.

The presence of PhACs from human medical care in groundwater may, however, also be caused by other sources such as landfill leachates [3-5] or manufacturing residues [6]. Nowadays, and especially in the industrialized countries, strong regulations and advanced manufacturing practices shall prevent such spills. In the past, regulations were not as strong and in several cases the release of production residues was either tolerated or even accepted. But the occurrence of pharmaceutical residues in the environment may also be caused by agriculture applying large amounts of PhACs as veterinary drugs and feed additives in livestock breeding. **Figure 1** shows possible sources and pathways for the occurrence of PhAc residues in the environment.

The elimination efficiency of conventional wastewater treatment does not provide a significant reduction of the concentration of pharmaceuticals and their metabolites before they are to re-enter the environment as literature data prove. With the knowledge that wastewater treatment plants discharge pharmaceuticals into surface wa-

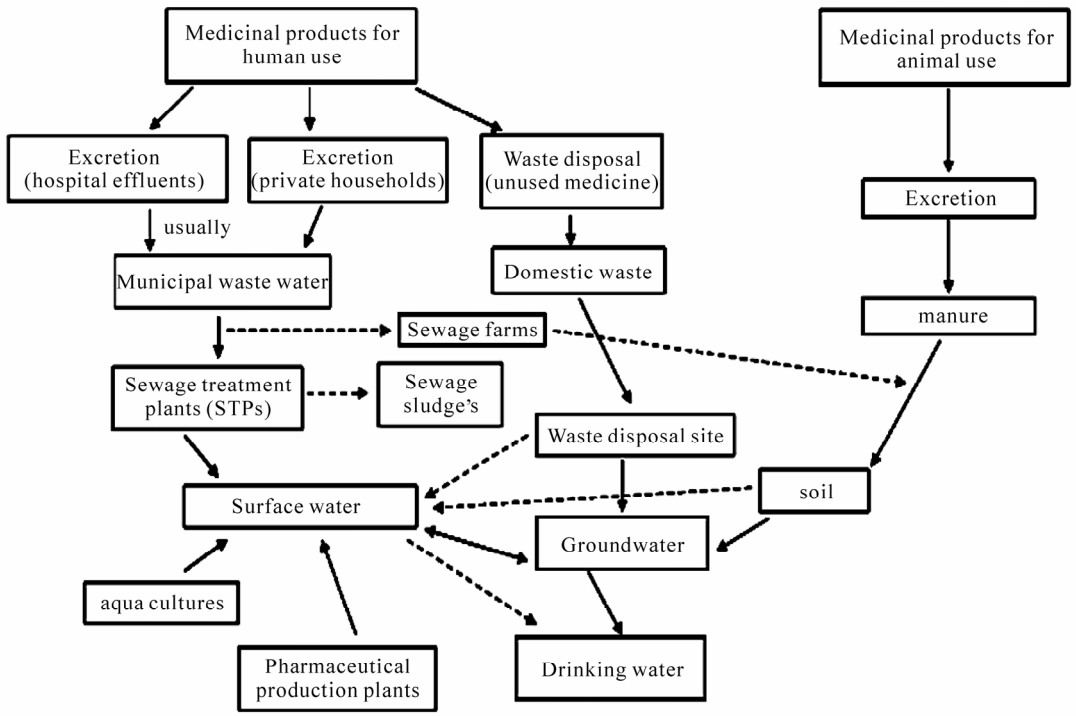

Figure 1. Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment.

ters like rivers and lakes [7,8] as well as into the sea [9,10], strategies for an advanced elimination of these pollutants became overdue.

However effluents from wastewater treatment plants (WWTPs) can be considered one of the most important sources of pharmaceuticals in the environment.

In the recent years, the presence of pharmaceuticals in WWTPs effluents have been reported [11-16]. Concentration of pharmaceuticals in the environment, their temporary evolution and their possible synergic and antagonist effects depend not only in the amount discharged from WWTPs but also on the geographical area and climate conditions. Because of that the study of the concentration evolution of these pharmaceuticals, related to climate conditions in the studied area, is necessary to evaluate the risk of negative environment effects.

Accurate and sensitive methods are necessary for the determination of pharmaceuticals in environmental samples, in order to, evaluate the amount of these compounds that are being discharged into the aquatic environment. HPLC-MS [12,13,17-22] and GC-MS [14,15,23] have been used to determine pharmaceutical in water samples. However, when analyzing highly contaminated samples, such as wastewater, a suppression of the electrospray ionization is likely to occur. Besides, these instruments are still very expensive and consequently not widely distributed. On the contrary, almost, every laboratory has a common HPLC system, thus our alternative proposed in to apply HPLC with diode array detector to determine pharmaceutical residues in environmental samples.

Antidepressants and anticancerigenic are class of pharmaceuticals that is extensively used in industrialized countries and they can be too environmental pollutants and cause adverse effects on ecological systems.

So, in this paper, a method for routine determination of ten antidepressants (fluvoxamine, fluoxetine, citalopram, trazodone, paroxetine, sertraline, clomipramine, imipramine, doxepine, venlafaxine) and three anticancerigenic used for breast cancer (letrozole, anastrozole, exemestane) in environmental samples is proposed.

The method involves sample pre-treatment by solid phase extraction (SPE) for water samples and analytical determination by HPLC with diode array (DAD) detector.

The aim of this work was to provide an accurate, sensitive and inexpensive alternative to the use of HPLC-MS in the routine determination of these pharmaceuticals. The proposed method has been conveniently validated in waters of several precedence and soils.

2. Experimental

2.1. Chemicals and Reagents

All chemicals and solvents used were of analytical reagent grade. All reagents were from Panreac (Barcelona,

Spain).

Clomipramine (CLO), citalopram (CIT) and fluvoxamine (FLV) were supplied by Tocris. Imipramine (IMI) and letrozole (LE) were kindly supplied by Novartis Laboratories. Fluoxetine (FLX), paroxetine (PAR), trazodone (TRA) and anastrazole (ANA) were purchased from Sigma-Aldrich, Glaxosmithkline, Farma-Leporl and Astrazeneca laboratories respectively. Exemestane (EXE) and sertraline (SER) were supplied by Pfizer and doxepine (DOX) was purchased from Farmasierra S.A.

Standard stock solutions were prepared by dissolving the appropriate amount of the pure substance in 100 mL to give a final concentration of 100 mg/L. LE and EXE were dissolved with ethanol-water 50/50 (v/v); TRA, CIT, FLV, IMI, VEN, ANA and FLX were dissolved with Milli-Q water and DOX, CLO, PAR and SER with methanol. All the solutions were stored under refrigeration at 4˚C.

Working standard solutions were prepared daily by dilution of the stock standard solution with Milli-Q water.

Mobile phases and buffer solution were prepared from analytical-grade-reagent Na_2HPO_4, NaH_2PO_4 and H_3PO_4 from Panreac (Barcelona, Spain) and HPLC-grade acetonitrile from Panreac. The buffers and acetonitrile solutions were filtered through 0.45 μm filters (HNWP membrane filters). This type of membrane filter was purchased from Millipore.

2.2. Chromatographic Conditions

A Thermo Finnigan™ Surveyor® Plus HPLC system with diode-array detector was utilized. The system was monitored by means of a computer equipped with Chrom Quest 5.0 software, which was used for all measurements and data treatment. Compounds were separated on a 4.6 mm i.d. × 150 mm, 5 μm particle, ultrabase C_{18} reversed phase column (Análisis Vínicos, Ciudad Real, Spain) with acetonitrile and 70 mM phosphate buffer, pH 2.5 (35:65, v/v), as mobile phase. Isocratic elution was performed at a flow rate of 1.5 mL/min. The volume injected was 20 μL. Use of diode-array detection enabled extraction of chromatograms at different wavelengths. The optimization process was made monitoring the analytes at 230 nm and the validation procedure at 215 and 230 nm.

All the analyses were made by duplicate and peak areas were used for the quantification.

2.3. Treatment of the Samples

The environmental samples objects of study were water samples from different origin (tap, sea and wastewater) and ground samples.

Water samples were collected using glass bottles prerinsed with ultra-pure water.

Extraction phase-solid (SPE) was used. The SPE cartridges (Sep-pack Plus tC_{18}, waters) were conditioned using 5 mL of methanol and 5 mL of 10 mM phosphate buffer solution (pH 7.0). The water samples were transferred to the SPE cartridges through a Teflon tube using a vacuum manifold system (Supelco Visiprep™ Sep-pack system, Madrid, Spain) coupled to a vacuum pump (Millipore XF 54 23050).

The wastewater samples, prior to extraction were filtered through 0.45 μm of size pore filters. Concerning tap and sea water samples the pH was adjusted to 6.5 with HCl and only tap water samples was necessary to fit the ionic strength to 50 mM by addition of NaCl.

After the conditioning step, water samples (50 mL) were percolated through the cartridges at a flow rate of 10 mL/min. Only for wastewater samples, the loaded cartridges were washed with 8 mL of 10 mM phosphate buffer (pH 7.0) and 2 mL of methanol:water (30:70, v/v). Finally, the elution was performed with 2 mL of methanol.

The soil samples belong to the province of Ciudad Real (Spain) and were collected using bottles of polyethylene. First, it was come to the breakage of aggregates with a wood mortar. Later, the soil samples were introduced in a furnace to dry them and afterwards were extended until the humidity balances with the one of the atmosphere, removing from time to time. Finally these samples were sifted with a mortar until reduce the size of soil particles.

Next, 0.5 g of the soil samples was placed in a conical bottom glass tube and 5 mL of methanol was added. After, 10 min of vertical agitation the samples were centrifugated (5000 rpm, 10 min) and the supernatant was transferred into another conical glass tube, this process was repeated three times. Finally the extract was evaporated under nitrogen stream and reconstituted with 5 mL of methanol.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions

To optimize the chromatographic separation of the thirteen analytes studied, several of preliminary experiments was performed testing different mobile phases consisting of methanol, acetonitrile or mixture of both as organic phase and different buffer solution at various concentration.

The optimal separation of 13 compounds studied was achieved using an isocratic elution with 70 mM phos-

phate buffer (pH 2.5) and acetonitrile (65:35, v/v).

Elution with the same isocratic conditions at different flow rates were made and optimal performance based on a compromise between the speed, separation, efficiency, peak width and column backpressure was obtained using 1.5 mL/min. In the same way, the effect of temperature of the chromatographic column on the separation was studied varying this parameter between 18 - 30˚C. A tem- perature of column of 20˚C was found advantageous over the others temperatures essayed in terms of better resolu- tion between peak of the target compounds and analysis time not too long. An example of a chromatograms corresponding to a standard solution containing 3 mg/L of each studied compound is shown in **Figure 2**. As can be seen very good separation was achieved in an analysis time of 25 min.

3.2. Optimization of Extraction and Preconcentration Procedure

Generally, the methodologies developed for the drugs residue analysis in environmental samples includes extraction and enrichment steps followed by chromatographic determination of target analytes. In our cases, SPE has been employed in order to eliminate possible matrix interferences and enrich the studied compounds in water samples. SPE was optimized by spiking 50 mL aliquots of each water samples (tap, sea or wastewater) with 3 mg/L of the drugs studied. In the case of wastewater the samples were filtered before that the SPE was optimized. So, preliminary experiments demonstrate that this step not affect to analytes. Extraction of thirteen drugs studied from water samples was performed in reversed-phase C_{18} cartridges, which were conditioned prior to use with 5 mL of methanol followed by 5 mL of 10 mM phosphate buffer solution (pH 7.0). Then, 50 mL of water sample at several pH and ionic strength were slowly loaded into the conditioned cartridge. Once the retention had been completed, the cartridge was submitted to several washing steps depending the precedence of water samples.

The best results were obtained when the pH water was adjusted to 6.5 and only the loaded cartridges with wastewater were necessary washed with 8 mL of 10 mM phosphate buffer (pH 7.0) and 2 mL of methanol:water (30:70, v/v). Finally, different solvents (acetone, methanol and acetonitrile) were tested in order to elute the analytes. Methanol was chosen because allows the best extraction recoveries of most of the analytes studied.

A chromatogram corresponding to an extract of a wastewater sample spiked with 500 ng/mL of the analytes studied is show in **Figure 3**.

Respect to the soil samples, the extraction procedure of analytes was optimized using a sample obtained by spiking 2 mg/L of the drugs studied over 0.5 g of soil Several extraction procedures using different solvents (acetone, methanol, water, 2-propanol, carbon tetrachloride) at several percentages were essayed and the recoveries them were calculated. The best results were obtained using a volume of 5 mL of methanol for the extraction of the analytes from the soil samples; this experiment was repeated three times.

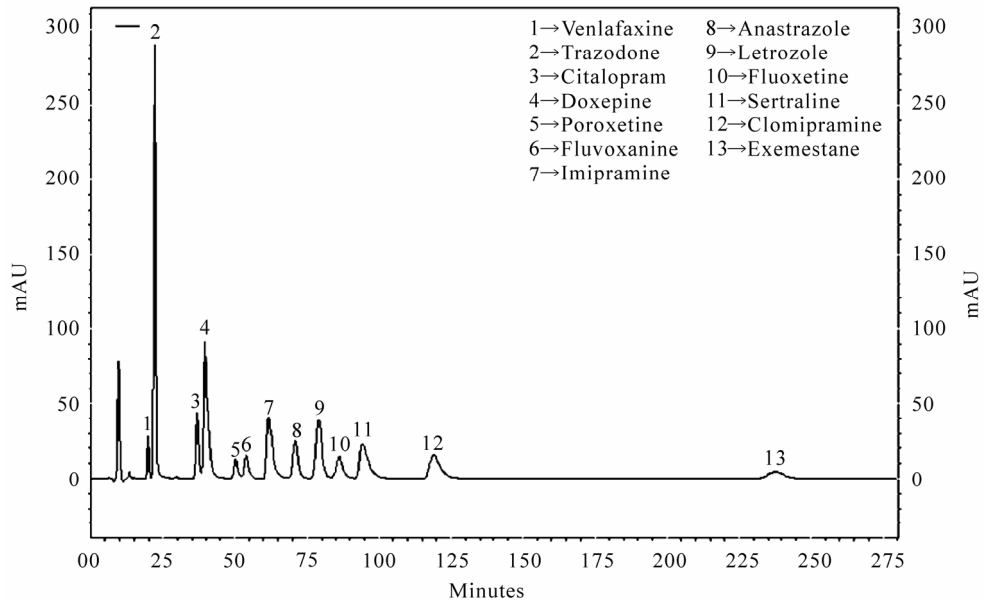

Figure 2. Chromatogram of a standard solution containing 3 mg/L of each compound, under the optimized conditions, recorded at 215 nm.

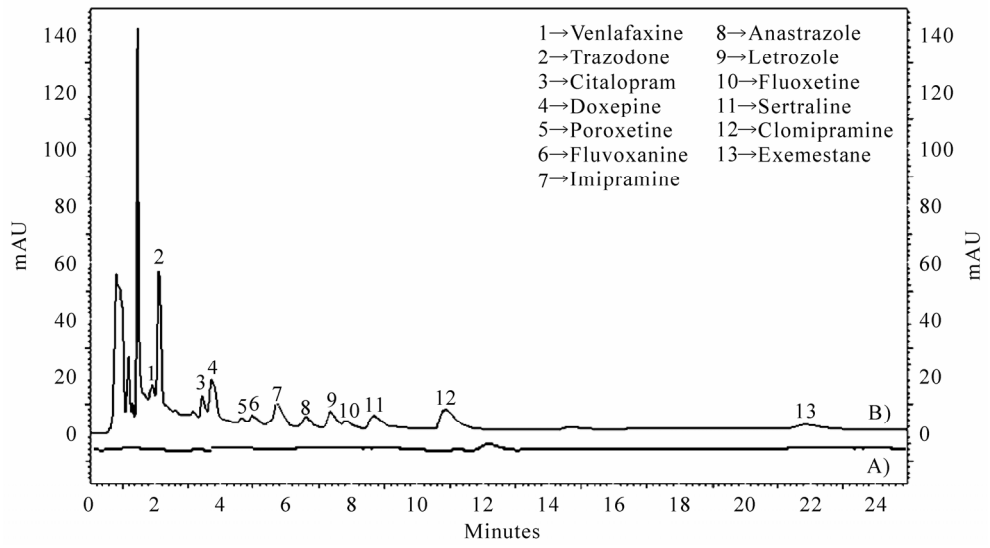

Figure 3. Chromatogram corresponding: A) blank of wastewater B) extracts from wastewater analyzed spiked with 0.5 mg/L for all analytes.

All the extraction procedures are exhaustively described in the Experimental section.

3.3. Validation of the Method

The proposed method was adequately validated in all the environmental samples object of study.

Validation was performed by measuring peak areas at the wavelength of maximum absorbance of each of the analytes, to maximize sensitivity. The wavelength used was 230 nm for trazodone, citalopram, doxepine, paroxetine, fluvoxamine, imipramine, fluoxetine, sertraline and clomipramine, and 215 nm for venlafaxine, anastrozole, letrozole and exemestane.

In order to evaluate the precision of the proposed method within-laboratory repeatability and reproducibility were estimated. To ensure correct quantification of studied analytes, in the environmental samples a spiked extract at 0.12 mg/L of each sample was injected nine times in the same day and nine times in different days. Comparison of the two sets of data was carried out by applying the Snedecor F-test on relative standard deviation (RSD) values obtained for migration times and peak areas.

In terms of repeatability, it is remarkable that all the relative standard deviations were lower than 3.95% for peak areas and 0.89% for retention times. In terms of reproducibility, the comparison of the averages by means of the Snedecor F-test did not provide any significant difference between both series for a signification level of 0.05 (n = 18).

Limits of detection (LOD) and limits of quantification (LOQ) were calculated using the maximal sensitivity allowed by the system and calculating the standard deviation (SD) of this response. LOD and LOQ were estimated by multiplying the SD of blanks by a factor of 3 and 10, respectively. Under these conditions LODs and LOQs obtained were subsequently validated separately by the analysis of six standards prepared at their respectttive concentrations of all the compounds.

The linearity in the response was studied using matrix-matched calibration solutions prepared by spiking environmental samples extracts at six concentration levels for every compound, ranging from 35 to 1500 ng/mL in the samples. The linear regression equations were calculated using the least-squares method and coefficients of correlation values higher than 0.99 were obtained. In **Table 1** analytical parameters obtained with the proposed method for the analysis of studied compounds in wastewater are shown. Similar results were obtained for the other environmental samples studied.

In order to test the accuracy of the proposed method the environmental samples object of study were spiked with studied compounds at several concentrations levels. These samples were analysed using the extraction and chromatographic procedure optimized in this work. Signals obtained from spiked samples were compared with the peak areas obtained by injecting standard solutions directly. Recoveries obtained from soil and wastewater samples spiked at several concentration levels are shown in **Table 2**. As can be seen, good recoveries ranged between 85 and 100% were obtained for wastewater. Similar results were achieved when tap and sea water were analysed. However, with the extraction procedure optimized in this work, poor extraction recoveries of citalopram were obtained in soil samples. Whereas recoveries

Table 1. Analytical parameters obtained with the proposed method for the analysis of studied compounds in wastewater.

	Linearity				Repeatability %RSD; (n = 9)		Reproducibility %RSD; (n = 18)	
Compounds	**Equation**	**r^2**	**LOD ($ng \cdot mL^{-1}$)**	**LOQ ($ng \cdot mL^{-1}$)**	**Tr***	**PA****	**Tr***	**PA****
VEN	Y = 66.1X + 102.9	0.9992	10.0	30.0	0.47	0.77	1.68	1.11
TRA	Y = 930.0X − 44.1	0.9983	1.0	3.0	0.43	0.36	1.99	0.57
CIT	Y = 214.8X + 80.9	0.9997	10.0	30.0	0.49	1.79	2.72	1.82
DOX	Y = 255.9X + 80.8	0.9993	8.0	24.0	0.74	0.93	2.81	1.04
PAR	Y = 30.8X − 57.1	0.9980	10.0	30.0	0.77	2.02	3.29	2.37
FLV	Y = 109.4X − 9.9	0.9986	10.0	30.0	0.59	0.60	3.20	2.18
IMI	Y = 511.8X + 63.7	0.9984	9.5	28.5	0.85	1.39	3.38	1.41
ANA	Y = 242.1 + 191.7	0.9995	7.0	21.0	0.30	0.75	1.88	1.55
LE	Y = 171.1X + 46.9	0.9994	5.0	15.0	0.30	0.92	1.85	2.82
FLX	Y = 49.6X + 25.9	0.9994	10.0	30.0	0.82	3.95	3.83	3.99
SER	Y = 144.7X − 20.8	0.9996	10.0	30.0	0.89	1.86	3.86	2.68
CLO	Y = 226.1X + 116.9	0.9990	40.0	120.0	0.81	1.15	4.02	2.96
EXE	Y = 127.6X + 187.5	0.9961	50.0	150.0	0.48	1.10	2.31	1.25

Table 2. Recoveries obtained for wastewater and soil.

	Recovery (%)			Recovery (%)			Recovery (%)			Recovery (%)		
Analyte	**Added ($ng \cdot mL^{-1}$)**	**Waste water**	**Soil**	**Added ($ng \cdot mL^{-1}$)**	**Waste water**	**Soil**	**Added ($ng \cdot mL^{-1}$)**	**Waste water**	**Soil**	**Added ($ng \cdot mL^{-1}$)**	**Waste water**	**Soil**
VEN	35.0	94.5	59.1	50.0	101.5	60.8	75.0	91.7	60.2	100.0	93.3	58.3
TRA	35.0	89.5	85.2	50.0	104.1	80.6	75.0	101.2	82.3	100.0	102.4	84.1
CIT	35.0	98.5	8.1	50.0	100.2	7.1	75.0	101.3	8.2	100.0	98.6	7.7
DOX	35.0	98.0	71.2	50.0	105.9	76.1	75.0	103.4	76.9	100.0	105.8	77.6
PAR	35.0	93.5	96.0	50.0	99.5	62.9	75.0	103.1	61.2	100.0	104.4	59.6
FLV	35.0	99.6	60.7	50.0	94.9	61.2	75.0	97.1	59.8	100.0	103.9	55.9
IMI	35.0	92.2	71.3	50.0	98.2	70.4	75.0	101.2	69.9	100.0	103.6	65.5
ANA	35.0	99.0	90.4	50.0	101.5	93.5	75.0	93.7	92.8	100.0	104.2	90.5
LE	35.0	91.1	93.5	50.0	97.6	90.8	75.0	94.1	91.2	100.0	100.8	94.8
FLX	35.0	96.8	78.8	50.0	102.6	80.1	75.0	98.7	79.8	100.0	91.0	80.6
SER	35.0	99.6	96.6	50.0	102.8	97.3	75.0	96.9	85.4	100.0	102.2	88.5
CLO	160.0	103.1	97.5	200.0	102.2	100.2	300.0	92.8	99.1	500.0	100.2	97.5
EXE	160.0	97.4	94.9	200.0	98.5	92.6	300.0	104.6	96.3	500.0	102.9	95.7

of the others studied drugs were in the ranged from 59% to 103%.

The selectivity of the method was verified and not interferences were found at the retention times of the 13 analyzed drugs. As can be seen in the **Figures 3**, **4** and **5** where are shown the chromatograms corresponding to two different extracts from blank waters and the last one from soil extract. In these figures is possible to see the

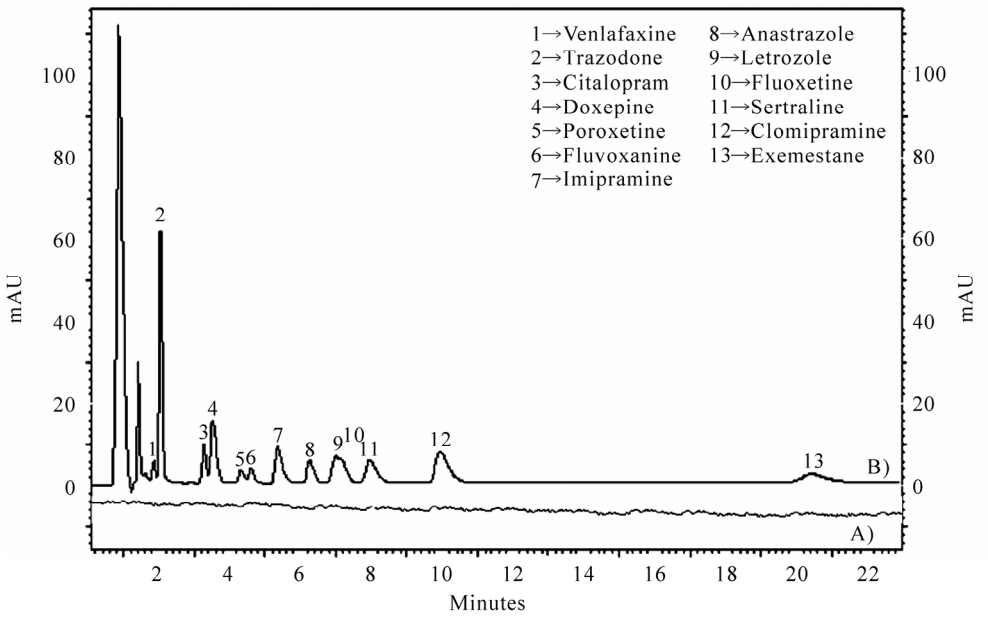

Figure 4. Chromatogram corresponding: A) blank of seawater B) extracts from seawater analyzed spiked with 0.5 mg/L for all analytes.

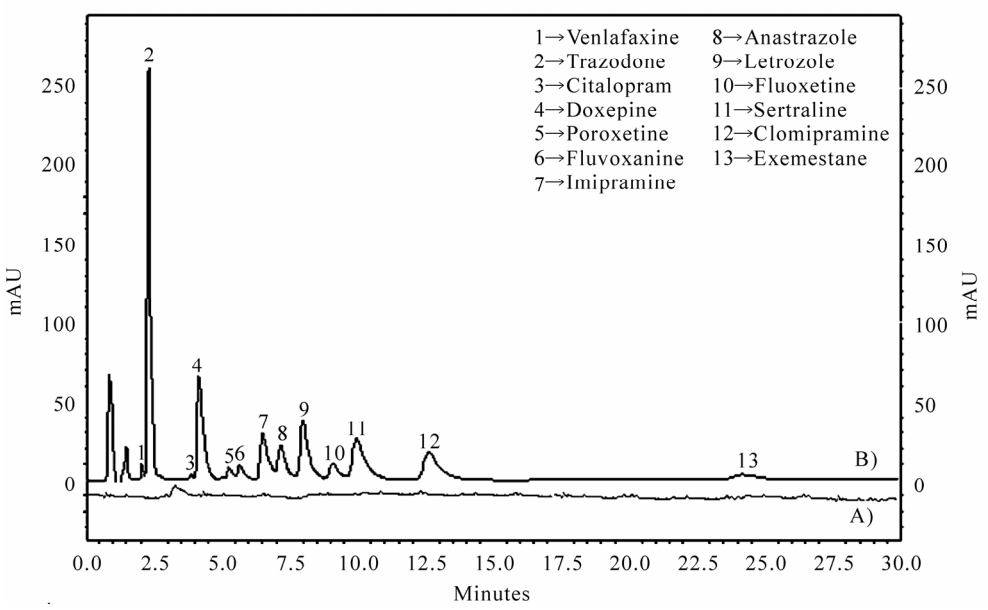

Figure 5. Chromatogram corresponding: A) blank of soil B) extracts from soil analyzed spiked with 2 mg/L for all compounds.

differences after an spiked of different amounts of our drugs in waters (0.5 mg/L) and soil (2 mg/L). The selectivity was also determined, by measurement of peaks homogeneity using the techniques of normalization and comparison of spectra from different peak sections and absorbance measures at two wavelengths [24]. Both techniques proved again to have a high level of purity of the peak corresponding to the studied compounds in all the samples. In conclusion the proposed method showed a good selectivity for the environmental samples chosen for our analysis.

4. Applications

To demonstrate the applicability of the described method in this work, several environmental samples as water of different precedence (tap, sea and waste) and soils belong to different zones from the province of Ciudad Real

were analyzed. The water samples analysed were samples in different weather stations. All the samples were fortified with ranged between 35 and 1000 ng/mL concentrations of studied compounds and submitted to the analytical procedure described for each case in this work. The recoveries obtained in all the water samples analyzed were ranged between 90 and 109% for all compounds studied.

Chromatogram of a sea water sample fortified with 500 ng/mL of drugs studied is shown in **Figure 4** as example.

As can be observed in the figure the high selectivity provided by the proposed method allows a reliable identification of the target compounds in wastewater and obtaining similar results in all the water samples analyzed.

Respect to soil samples analyzed, good recoveries were obtained (ranged between 59 and 103%) for all the target compounds except for citalopram that was around 8%.

A chromatogram corresponding to a spiked soil sample is shown in **Figure 5**. Good selectivity was observed too, in this type of samples.

5. Conclusions

In the present work, a simple and fast multi-residue method based on SPE step followed by an HPLC-DAD determination has been developed for the simultaneous extraction and analysis of ten antidepressants (fluvoxamine, fluoxetine, citalopram, trazodone, venlafaxine, paroxetine, doxepine, sertaline, imipramine and clomipramine) and three aromatase inhibitors (letrozole, anastrazole and exemestane) in environmental samples. It has been shown that this method represents an easy and fast analytical approach, viable for routine analysis, using instrumental very simple, available in almost every laboratory.

The proposed method was exhaustively validated in terms of linearity, accuracy, specificity and precision in environmental samples. Quantitative recoveries were obtained for all the target compounds ranging from 59 and 103% except citalopram determination which is extracted from soil samples with recoveries around of 8%. Linearity with $R^2 > 0.994$ and precision of with the RSD (%) between 0.30% and 3.99% was very satisfactory.

The SPE step optimized allows not only the elimination of hydrophobic interferences but also an important sample preconcentration which results in LOD(s) in water samples between 1 and 50 ng/mL. HPLC-DAD is an inexpensive analytical technique compared to HPLC-MS and, because of this is a useful and affordable alternative to HPLC-MS for routine analysis of pharmaceuticals. It can be a useful tool to known the amount of these compounds discharged from wastewater treatment plants (WWTPs) to the aquatic environment and to evaluate the effect of WWTPs in the elimination of pharmaceutical compounds.

In conclusion, methods are being developed to enhance the capabilities for measuring emerging chemical contaminants and their associated degradation products in the environment. Therefore, prioritization of compounds investigated as the analyzed drugs requires careful evaluation of the potential for their environmental occurrence and persistence, potential health effects, and the appropriate level at which they should be measured.

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