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Multi-residue analysis of pharmaceutical compounds in wastewaters by dual solid-phase microextraction coupled to liquid chromatography electrospray ionization ion trap mass spectrometry

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ABSTRACT

The aim of this article is to present a new procedure based on dual solid-phase microextraction (dSPME) for the simultaneous extraction of 16 pharmaceutical compounds with acidic and basic characteristics in urban wastewaters. Water samples are divided into two aliquots of 2 mL each extracted by two CW-TPR fibers at different pH values (pH 3 and 11) and with a NaCl concentration of 300 g L^{-1} at 75 °C for 30 min. The analytes in both fibers are desorbed one after the other in the desorption chamber in static mode with mobile phase for 10 min. The extracts are injected into an LC system coupled to an ion trap mass spectrometer, leading to the accurate quantification of 16 pharmaceutical compounds in wastewaters, in MS^2 mode. All the target compounds found in wastewaters provide good signals corresponding to the protonated precursor ion $[M+H]^+$. The parameters influencing adsorption and desorption of the analytes on fiber were optimized. The assessment of the analytical method was performed by studying the linearity (LOQ to 10 ng mL^{-1}) and the intra- and interday accuracy (89.2–109.7%) and precision (RSD <13.6%). The quantification limits obtained ranged between 0.005 and 0.05 μg L^{-1}. The application of the method to real samples proves its effectiveness in identifying and detecting naproxen, valsartan, bezafibrate, torasemide, diclofenac, carbamazepine, citalopram, lorazepan, fluoxetine, imipramine and amitriptyline in influent and effluent wastewater treatment plant samples.

1. Introduction

Emerging pollutants are defined as newly identified or previously unrecognized contaminants that are thought to threaten the environmental ecosystems and human health and safety [1]. This group includes pharmaceuticals, drugs of abuse, personal-care products (PCPs), steroids and hormones, nanomaterials, 1,4-dioxane, swimming pool disinfection by-products (DBPs), surfactants, perfluorinated compounds (PFCs), flame retardants, industrial additives and agents, gasoline additives and their transformation products (TPs). The European Commission established a Water Framework Directive [2] to reduce chemical pollution of surface waters and defined a list of substances presenting a significant risk to aquatic ecosystems [3], which has been reviewed recently [4]. Nevertheless, there is a diverse group of unregulated pollutants as well as pharmaceuticals, drugs of abuse and personal-care products.

Regarding the group of pharmaceuticals, many different compounds used in human health care and in veterinary applications have been found in surface and wastewaters within the ng L^{-1} to low μg L^{-1} levels [5,6]. It is thought that the continuous exposure to these compounds, even at low levels, might affect the health of wildlife and humans [7]. The presence of pharmaceutical products in environmental water results from human excretion in urine and faeces of metabolized and unmetabolized drugs in high percentages and their subsequent discharge into domestic wastewaters [8]. However, most of these drugs and metabolites are able to pass through the wastewater treatment plants. Consequently, these compounds have been found in surface and groundwater.

The spectrum of pharmaceuticals that can be found in environmental waters is very wide. These compounds are present at low levels and in a matrix where many different molecules are present. All this has led to the increased use of gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS). Due to the less volatile character of the compounds, GC–MS requires a derivatization prior to analysis [9–13]. However, LC–MS is the main choice when polar and less volatile compounds such as pharmaceuticals need to be determined [14].

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The ultra trace levels of pharmaceuticals require an analyte preconcentration procedure in order to obtain the required sensitivity. This procedure has usually been carried out by solid-phase extraction (SPE) in the off-line [15–19] and in the on-line mode [20]. Nevertheless, this technique requires large sample volumes and many steps before reaching a concentrated extract suitable for the analysis. An alternative to SPE is the solid-phase microextraction (SPME). Amongst the observed benefits of SPME are its minimal sample volume requirement and that it is easily automated, which allows the preconcentration of the analytes [21–23].

For the determination of pharmaceuticals in waters, SPME has been applied, coupled to GC with mass spectrometry [9–13] and flame ionization detection [24] and to LC with diode array detector [25–27]. In the developed methods, SPME was applied for the extraction of compounds with similar polarity, structures or activities as well as anti-inflammatory drugs [10,12,13,25,26] and sulfonamide antibiotics [28,29]. For the extraction of compounds with different polarities, Suchara et al. [24] proposed the change of the pH value of the sample without interruption of the extraction process. However, there is a lack of SPME methods for the multi-residue determination of compounds with different characteristics. Nowadays, in order to provide wider knowledge about the presence of pharmaceuticals in environmental waters, multi-residue analytical methods are required. The objective of the study was to develop a sensitive and specific multi-residue method for the quantitative determination of the following pharmaceuticals considered as emerging pollutants and frequently detected in environmental waters [1,8,30–32]: analgesic and antiinflamatory drugs (naproxen, diclofenac), selective serotonine reuptake inhibitor (fluoxetine, citalopram, venlafaxine) and serotonine and norepinephrine reuptake inhibitior antidepressants (imipramine and amitriptyline), antibiotic (trimethoprim), lipid regulator (bezafibrate), β-blockers (metoprolol), diuretics (valsartan, torasemide), ansiolitics (lorazepam, diazepam), antiepileptic drug (carbamazepine) and antipsychotic (risperidone). The proposed method involves a modified SPME procedure, The proposed method involves a modified SPME procedure (from now on denoted dual-SPME), which enables the rapid extraction of these substances which have different chemical properties. The optimization of the factors affecting the SPME efficiency as well as the most adequate fiber coating and extraction parameters influencing adsorption (extraction pH, ion strength, organic modifier addition, temperature and time) and desorption (desorption mode, time and desorption solvent mixture composition) of the analytes is discussed. The coupling to LC–ESI-ITMS allows the identification/confirmation and quantitation in a single analysis with the necessary guarantees of sensitivity to be able to determine these substances in wastewaters.

2. Experimental

2.1. Chemical and solutions

The reagents used were of analytical grade of the highest purity available. The ammonium formate (99%) used in the mobile phase was supplied by Acros Organics (New Jersey, USA) and the HPLC grade acetonitrile was obtained from Scharlab Chemie (Barcelona, Spain). All solutions were prepared with ultra high purity water (UHP) prepared from tap water pre-treated by Elix reverse osmosis cartridges prior to filtration by a Milli-Q system all from Millipore (Bedford, MA, USA). For the optimization of SPME, sodium chloride, disodium sulfate, disodium hydrogen phosphate, sodium phosphate, citric acid, sodium hydroxide, ammonium hidroxyde and ammonium chloride were obtained from Merck (Darmstadt, Germany) and acetonitrile and methanol were purchased from Scharlab Chemie (Barcelona, Spain).

Naproxen, diclofenac sodium salt, fluoxetine hydrochloride, citalopram hydrobromide, venlafaxine hydrochloride, imipramine hydrochloride, amitriptyline hydrochloride, trimethoprim, bezafibrate, metoprolol tartrate salt, lorazepam, diazepam and carbamazepine were purchased from Sigma (St. Louis, USA), valsartan was obtained from Novartis International Pharmaceutical Ltd. (Cork, Ireland), torasemide from Roche Diagnostics (Mannheim, Germany) and risperidone from LGC standards (East Greenwich, USA). [^{2}H6]fenitrothion (FNT-6d) was purchased from Cambridge Isotope laboratories (Andover, MA, USA) with a chemical purity specification higher than 97.0%.

Stock solutions containing 1 mg mL^{-1} of individual analytes were prepared in methanol from Scharlab Chemie (Barcelona, Spain). These solutions were kept in the dark at −42 °C in a freezer. An aqueous reference solution containing the mixture of all these compounds to a final concentration of 10 mg L^{-1} was prepared from the standard stock solution of each analyte. All working standard solutions were prepared by diluting the appropriate volume of the 10 mg L^{-1} reference solution with up to 10 mL with water.

2.2. Instrumentation

The LC/MS system consisted of an Agilent 1100-series binary pump provided by a vacuum degasser and an autosampler and coupled to an MS/MS system consisting of an MSD Trap XCT Plus spectrometer equipped with a G1948A ESI source. System control and data analysis were provided by the Agilent LC Chemstation and by the Brucker Daltonics Trap Control and QuantAnalysis.

Chromatographic separation was performed on a Mediterranea Sea18 (Teknokroma, Barcelona, Spain) C18 reversed phase column of 100 mm × 2.1 mm id and filled with 3 μm particles. The mobile phase consisted of a mixture of eluent A composed of 20 mM ammonium formate and eluent B was acetonitrile, operating at room temperature and with a flow-rate of 0.3 mL min^{-1}. Prior to use, the eluents were filtered through a 0.22 μm Millipore membrane filter type GVWP using a glass vacuum solvent filtration apparatus obtained from Millipore (Molsheim, France) and degassed by a Selecta Ultrasound System (Selecta, Barcelona, Spain). To achieve the optimal separation of the different pharmaceuticals, the following elution program was used: the elution started with 90:10 (A:B, v/v) followed by a 30 min linear gradient to 30:70 (A:B, v/v); this proportion was maintained for 2 min; then a 1 min linear gradient to 90:10 (A:B, v/v) was applied and this was maintained for 8 min to equilibrate the column. Under these conditions all the analytes were eluted in less than 35 min.

The operating conditions of the ESI interface in the positive-ion mode were as follows: drying gas (N_2) temperature of 350 °C, drying gas (N_2) flow of 9.0 L min^{-1}, nebulizer gas (N_2) pressure of 40 psi and capillary voltage of −3500 V. Full-scan MS spectra were obtained by scanning from 50 to 500 m/z.

For the development of the SPME procedure, commercially available polymeric coated fibers housed in the appropriated manual holder (Supelco, Bellefonte, PA, USA) were used. The SPME/LC interface (Supelco, Bellefonte, PA, USA) consisted of a standard six-port Rheodyne valve and was equipped with a fiber desorption chamber (total volume: 60 μL) which was installed in place of the sample loop. In order to select the most suitable fiber, three commercially available coatings, polyacrylate 85 μm (PA), polydimethylsiloxane/divinylbenzene 60 μm (PDMS-DVB) and carbowax/templated resin 50 μm (CW/TPR), were purchased also from Supelco (Bellefonte, PA, USA). 4 mL screw-cap vials supplied with a PTFE-lined septum (Kimble Glass, Vineland, NJ, USA), a 0.7 cm stir bar and a magnetic stirrer from IKA (Staufen, Germany) were used for magnetic stirring of the solutions in the extraction

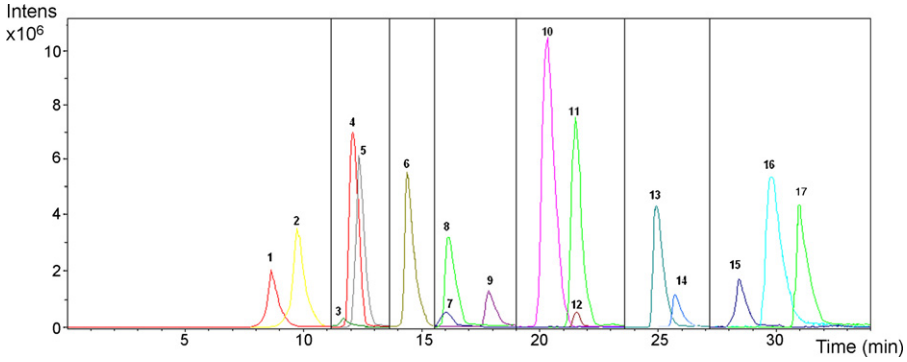

Fig. 1. dSPME–LC/MS/MS chromatogram obtained for a 2 ng mL^{-1} standard. (1) NAP, (2) VAL, (3) TRIM, (4) BEZA, (5) TORA, (6) DICLO, (7) MTL, (8) CBZ, (9) LRZ, (10) VLF, (11) RISP, (12) DZP, (13) CIT, (14) FNT-d6, (15) FLX, (16) IMIP, and (17) AMIT.

step. For the application of temperature during the extraction step a heating block form Selecta (Barcelona, Spain) was used.

2.3. Sample collection and preparation

Wastewater treatment plants (WWTPs) have been identified as a major point source of pharmaceuticals entering the environment since they receive continuous inputs of these compounds either as the parent compound or as a metabolite [7]. However, the efficiencies in WWTPs are often low and compounds which are not removed are released to the environmental waters [18]. Composite (24 h) influent (untreated) and final effluent (treated) urban wastewater samples for method evaluation and quantification were collected on five days in October 2009, from Vitoria-Gasteiz WWTP providing tertiary treatment for approximately 230,000 inhabitants and with an estimated flow of treated water of 32.4 Hm^3/year.

Samples were collected in glass amber bottles (250 mL) supplied by Scharlab (Barcelona, Spain), transported to the laboratory under refrigeration and stored at −42 °C until the analysis. To remove particulates, centrifugation of the wastewater at 420 rad s^{-1} was performed at the same day as sampling, using a centrifuge Orto Alresa Model Unicen (Madrid, Spain).

2.4. SPME procedures

In order to ensure good selectivity and sensitivity results, each day before analysis the fiber was conditioned in the interface with mobile phase for approximately 20 min. Then, the fiber was immersed in ultra high purity water for 5 min with magnetic stirring and dried for 5 min. After conditioning, the fiber could be used for extraction.

Preliminary experiments were performed in order to select the most adequate fiber coating of the three examined. Stock solutions containing 10 μg L^{-1} of each analyte in water were used for this aim. Taking into account the polarities of the analytes and in order to get the best extraction recoveries, in this study a provisional extraction procedure was used: 2 mL of standard solution containing 10 μg L^{-1} of each compound was put in a 4 mL screw-cap vial adding 300 g L^{-1} NaCl and 100 μL of phosphate buffer or 100 μL of citrate buffer. Then, the solution was stirred with a stir bar at a speed of 94 rad s^{-1} for 30 min. The desorption step was carried out in static mode for 10 min. The efficiency of the desorption step was confirmed by performing a second desorption in static mode. As the fiber had been introduced in organic solvents and buffers during the extraction process, after desorption it was immersed in water with magnetic stirring for 5 min and dried for 5 min.

Table 1
Data acquisition parameters for smart parameter setting (SPS) used in LC/MS ion trap for the detection of pharmaceutical compounds. Compound stability and trap drive level are fixed at 100% for all compounds and the fragmentation width was 10.0 (*m*/*z*).

Time segments (min)	Compound name	Surrogate standard	Retention time (min)	LOQ (direct injection) (ng mL^{-1})	Precursor ion $[M+H]^+$	MRM transition (*m*/*z*)	Fragmetation amplitude (V)	Cut-off (*m*/*z*)
1 0–11.2	Naproxen (NAP)	LRZ	8.7	1.0	231	231 → 185	0.40	62
	Valsartan (VAL)	LRZ	10.0	1.0	436	436 → 418	0.33	118
2 11.2–13.6	Trimethoprim (TRIM)	LRZ	11.8	1.0	291	291 → 123	0.70	79
	Bezafibrate (BEZA)	LRZ	12.1	0.5	362	362 → 316	0.45	98
	Torasemide (TORA)	LRZ	12.3	0.1	349	349 → 264	0.44	94
3 13.6–15.4	Diclofenac (DICLO)	LRZ	14.4	0.5	296	296 → 278	0.48	80
4 15.4–19	Metoprolol (MTL)	LRZ	16.0	0.5	268	268 → 116	0.60	72
	Carbamazepine (CBZ)	LRZ	16.1	0.2	237	237 → 194	0.52	64
	Lorazepam (LRZ)	–	17.8	1.0	321	321 → 303	0.47	87
5 19–23.6	Venlafaxine (VLF)	DZP	20.4	0.2	278	278 → 260	0.48	75
	Risperidone (RISP)	DZP	21.4	0.5	411	411 → 191	0.65	111
	Diazepam (DZP)	–	21.6	2.0	285	285 → 257	0.72	77
6 23.6–27.2	Citalopram (CIT)	FNT-d6	24.8	0.5	325	325 → 262	0.57	88
	6D-Fenitrothion (FNT-d6)	–	25.6	1.2	284	284 → 133	0.71	77
7 27.2–35	Fluoxetine (FLX)	FNT-d6	28.4	2.0	310	310 → 148	0.58	86
	Imipramine (IMIP)	FNT-d6	29.8	0.5	281	281 → 234	0.57	75
	Amitriptyline (AMIT)	FNT-d6	31.1	0.5	278	278 → 233	0.60	75

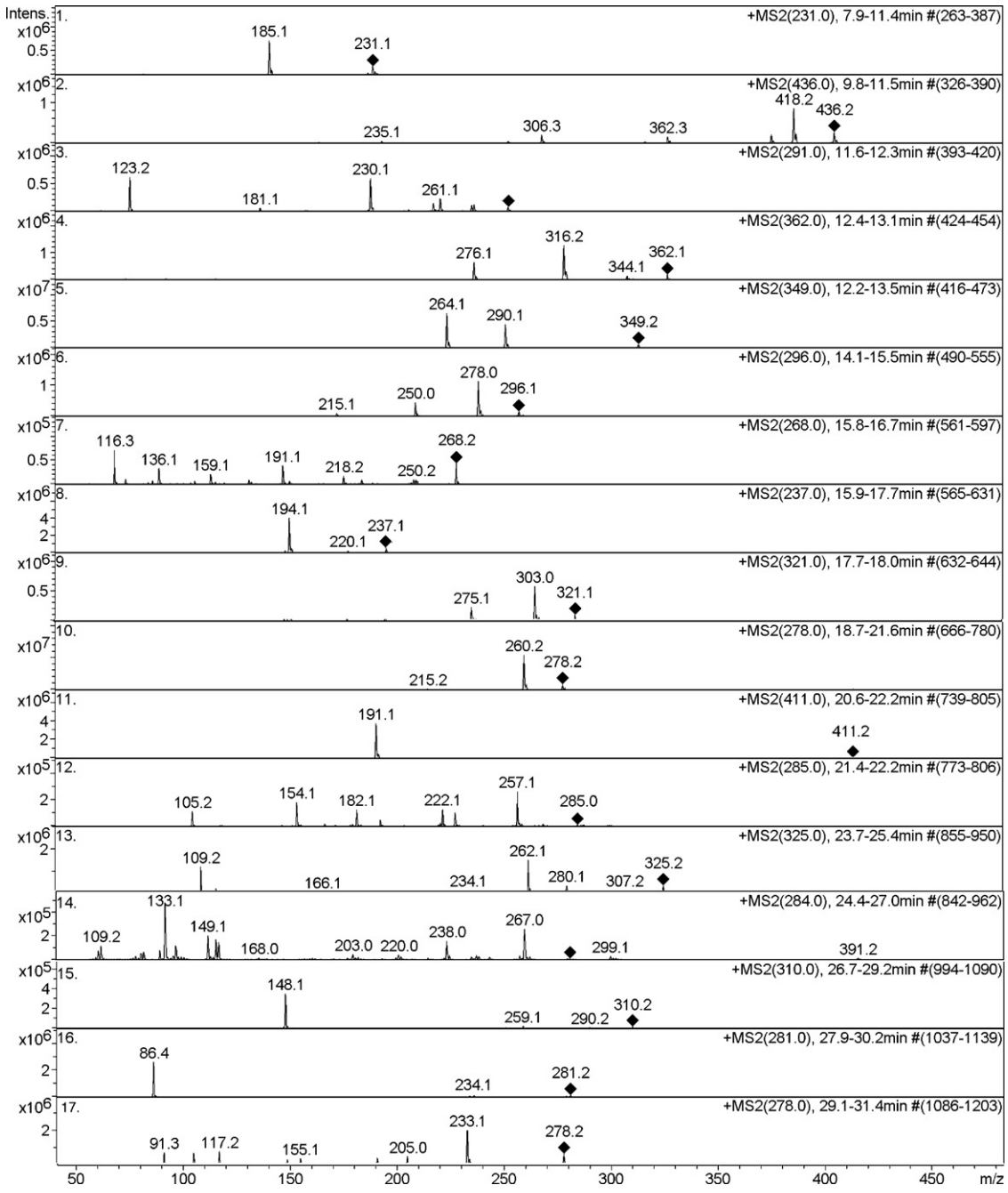

Fig. 2. MS^2 spectra of (1) NAP, (2) VAL, (3) TRIM, (4) BEZA, (5) TORA, (6) DICLO, (7) MTL, (8) CBZ, (9) LRZ, (10) VLF, (11) RISP, (12) DZP, (13) CIT, (14) FNT-6d, (15) FLX, (16) IMIP, and (17) AMIT.

In the study with real samples surrogate standards were used, so that 100 μL of a 0.1 μg mL^{-1} solution of the standards mixture were added to the samples prior to extraction and mixed thoroughly.

The conditions of the desorption step were established to ensure the total removal of the extracted analytes from the fiber. For this aim, solvent composition (acetonitrile, methanol and mobile phase), desorption time (5, 8, 10, 20 and 30 min), and desorption mode (static or dynamic mode) were studied.

3. Results and discussion

3.1. Chromatography and mass spectrometry considerations

The mobile phase composition was optimized with respect to the shorter analysis time and maximum chromatographic resolution of the analytes. Isocratic and gradient methods were studied using different reagents, buffer and ion-pair reagents, which were added to the aqueous phase and different organic modifiers. The use of a volatile buffer, compatible with an LC–MS system, increased the retention of the analytes on the column and provided a good separation and resolution of the compounds in less than 35 min. A gradient method using a mobile phase containing an aqueous phase of 20 mM ammonium formate and acetonitrile as organic modifier was found to give the best separation and good chromatographic shapes. Nevertheless, these conditions did not enable a suitable separation of some of the studied compounds which practically co-elute at the same time: peaks 3, 4 and 5, 7 and 8, 11 and 12 (Fig. 1). For this, the chromatographic separation was divided into seven time segments as shown in Table 1 and an ESI-LC–ITMS method was optimized in order to obtain the best sensitivity, analyte iden-

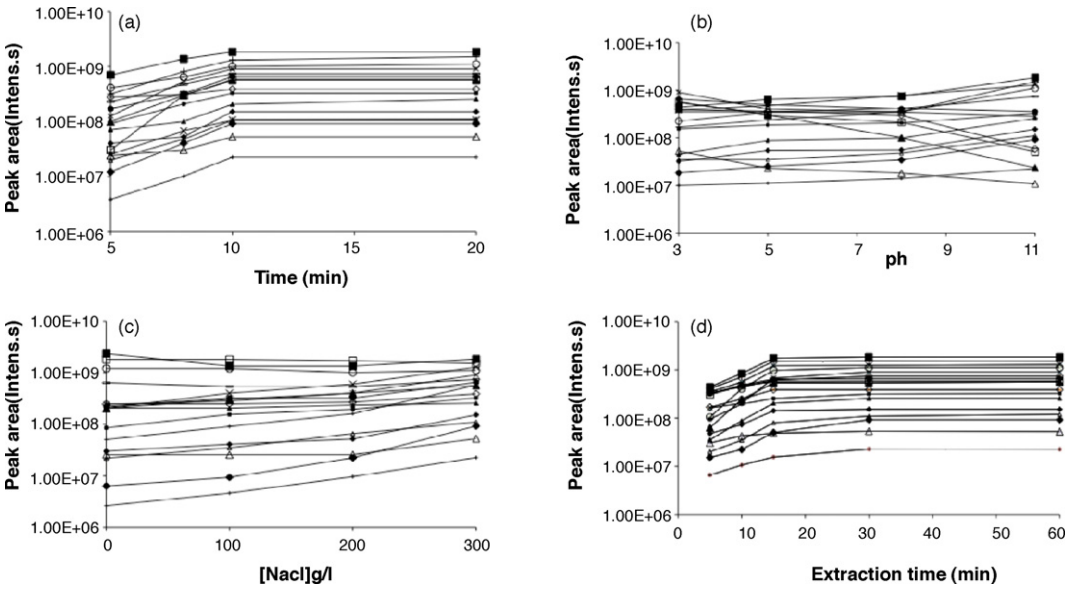

Fig. 3. Effect of (a) desorption time, (b) extraction pH, (c) extraction ionic strength, and (d) extraction time on the response of the analytes. SPME procedure: 2 mL of standard containing 10 μg L^{-1} of each compound in water. (◊) VAL, (▲) NAP, (+) TRIM, (□) BEZA, (●) TORA, (×) DICLO, (♦) MTL, (■) CBZ, (♦) LRZ, (■) VLF, (△) DZP, (×) RISP, (△) FNT-6d, (−) CIT, (▲) FLX, (+) IMI, (◯) AMIT.

tification and quantitative measurement. This also avoided long cycle times and insufficient data points per second when configuring the multiple-reaction monitoring (MRM) to repetitively step through the precursor ions and MS/MS scans for all the analytes. These segments allowed the reduction of the precursor ions to be analyzed in each segment and sensitivity to be enhanced. Multiple-reaction monitoring (MRM) transitions, using as precursor ion the protonated molecular ions $[M+H]^+$, and MRM parameters are summarized in Table 1.

Direct infusion experiments, where the analyte solution is flowed to the electrospray source using a syringe pump, were carried out to select the optimum MS and MS/MS parameters and to examine ionization and fragmentation patterns of the analytes. The MS spectra of analytes shown as precursor ion the protonated molecular ions $[M+H]^+$, which were selected to generate MS/MS spectra. The optimum fragmentation amplitude for each analyte was determined by infusing a 10 mg L^{-1} solution of a compound or the group of compounds that eluted at the same segment and increasing the fragmentation amplitude until the precursor ion intensity was between 5 and 15% of its major product ion response. The cut-off values (the minimal value of m/z ratio, for the ions with smaller values than these quantities not to be trapped by the IT) were set to the default value (27%) from the precursor ions m/z ratio.

MRM transitions and the product ion spectra generated from the precursor ions (Fig. 2) were recorded for each analyte at the retention time expected, in order to get a sufficient number of identification points for suitable confirmation according the EU guidelines [33] for LC–MSn.

The highest intensity product ions were chosen for the LC–MS/MS quantitative analysis. The data acquisition parameters and MS/MS transitions selected for identification and quantification of analytes are summarized in Table 1. LOQs were estimated as minimal concentrations that could be quantified with RSD at 20% and those in the table refer to the ones obtained without the use of SPME devices.

3.2. Dual-SPME (dSPME)

As previously stated, the first experiments performed permitted the selection of adequate fiber coating. With the less polar PA and PDMS/DVB fiber coatings there was no signal for most of the analytes, only some of the less polar compounds as well as venlafaxine, risperidone, fluoxetine and citalopram were extracted with the PDMS/DVB fiber. However, the most polar CW/TPR fiber permitted *a priori* a good extraction of the analytes so this fiber was selected as the most suitable fiber coating.

Once the fiber coating was selected, several parameters affecting desorption and extraction steps were evaluated. The first step was the evaluation of desorption parameters such as desorption mode, solvent and time. Two modes of desorption, dynamic and static modes, were evaluated. In dynamic mode, the fiber was placed into the desorption chamber and the mobile phase passed through the chamber for a time ranging from 2 to 7 min. Although the recoveries were satisfactory, very broad chromatographic peaks were obtained. In the static mode, acetonitrile, methanol and mobile phase were evaluated as desorption solvents by varying the desorption time from 5 to 20 min (Fig. 3a). It was found that the most suitable solvent was the mobile phase (20 mM ammonium formate:acetonitrile; 50:50, v/v) for 10 min since it achieved the best recoveries. Once selected, it was proved that at the selected desorption conditions there was no evidence of carry over, ensuring the effectiveness of the procedure.

The next step was the establishment of extraction parameters. pH values ranging between 3 and 11 were studied by adding citrate or phosphate buffer solutions adjusted to yield the desired pH (Fig. 3b). For the compounds with a basic character, whose non-ionic form predominates at high pH values, the best recoveries were obtained at pH 11. However, the compounds with an acidic character were extracted at pH 3. The simultaneous extraction of these wide spectrum of pharmaceuticals with different physicochemical properties requires a compromise that would not result in obtaining the best conditions for all analytes. Some assays were done beginning the extraction at pH 3 to promote the extraction of acidic analytes and after 30 min changing to pH 11 as proposed by Suchara et al. [24]. However, acidic pharmaceuticals did not show good recoveries since they seemed to be desorbed when the pH was adjusted to the basic value.

For these reasons, a simultaneous dual-SPME (dSPME) procedure was developed. In this procedure, two aliquots of 2 mL of the samples were used, one of them adjusted to pH 11 and the other one to pH 3. A CW/TPR fiber (Fiber 1) was introduced in

Table 2
Summary of validation results: linearity, quantification limits, precision and accuracy of the dSPME–LC–ITMS analytical method for three concentration levels.

Analyte	*R*	LOQ (ng mL^{-1})	Intraday (n = 10)						Interday (n = 10)					
			0.1 ng mL^{-1}		1 ng mL^{-1}		10 ng mL^{-1}		0.1 ng mL^{-1}		1 ng mL^{-1}		10 ng mL^{-1}	
			RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
NAP	0.998	0.010	7.2	96.3	9.2	97.8	6.8	105.6	13.6	103.4	9.3	105.4	9.2	97.5
VAL	0.999	0.005	9.1	98.2	8.2	107.5	8.2	94.5	11.9	105.6	10.3	98.5	8.4	104.6
TRIM	0.999	0.050	6.7	94.3	10.2	91.0	8.3	98.4	8.4	101.8	12.7	107.4	10.3	94.3
BEZA	0.999	0.010	6.9	103.2	7.3	96.4	7.5	89.4	7.6	97.4	10.6	96.5	10.2	105.4
TORA	0.993	0.010	7.7	102.5	8.2	99.3	8.8	95.6	8.4	99.6	10.7	106.4	9.5	95.6
DICLO	0.995	0.005	6.2	97.3	9.5	101.3	10.6	105.3	12.3	103.5	9.6	96.7	12.8	106.5
MTL	0.999	0.050	9.2	96.2	6.9	99.4	9.3	100.5	8.6	107.4	8.1	97.6	11.0	98.5
CBZ	0.998	0.010	7.9	99.1	8.1	104.7	7.9	107.3	7.9	95.6	10.2	94.5	10.3	94.7
LRZ	0.998	0.010	10.3	101.3	10.4	94.6	9.2	94.5	7.4	106.3	9.5	104.5	9.7	103.6
VLF	0.994	0.010	10.2	94.5	9.8	104.3	8.6	105.0	13.3	97.4	10.2	107.9	11.4	96.8
RISP	0.999	0.005	8.5	100.5	11.6	105.7	9.3	103.9	9.0	94.5	11.0	96.3	10.6	104.8
DZP	0.998	0.010	8.4	104.2	8.2	100.5	10.1	93.7	10.6	96.3	8.7	99.6	9.5	96.4
CIT	0.995	0.010	7.9	95.2	7.8	95.2	9.5	96.4	9.1	97.4	8.9	98.5	9.0	97.3
FLX	0.997	0.010	6.6	89.5	6.2	109.7	8.0	98.2	8.4	104.9	10.5	94.1	8.6	105.1
IMIP	0.992	0.010	7.0	93.5	7.7	92.6	8.1	92.7	7.7	94.5	12.5	102.1	8.2	93.4
AMIT	0.994	0.010	9.9	106.9	9.4	89.2	9.9	104.3	12.1	106.4	8.9	106.5	9.4	105.3

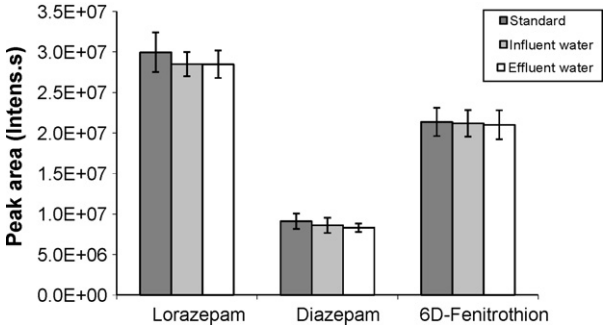

Fig. 4. Study of the matrix effects for the selected surrogate standards at 2 ng mL^{-1}: lorazepan, diazepam and 6D-fenitrothion. Analytical signals obtained using dSPME and LC–ITMS procedure proposed (n = 3).

the aliquot with pH 11, while simultaneously another CW/TPR fiber (Fiber 2) was introduced in the aliquot with pH 3. Then, the analytes were extracted following the provisional conditions previously described. It is important to point out that this dual extraction process using two fibers, can be carried out simultaneously, thereby saving considerable analysis time. For the desorption, first of all Fiber 1 was introduced in the desorption chamber previously filled with mobile phase and static desorption was carried out for 10 min. After this period of time, Fiber 1 was removed from the chamber and Fiber 2 was introduced and the analytes were desorbed in the static mode for 10 min. At this moment, and not before, the valve was switched from the load to the inject position and the mobile phase passed through the chamber dragging all the analytes desorbed from Fiber 1 and 2. Since the extraction conditions have changed regarding to the provisional conditions selected for the optimization of the desorption process, and with the aim of ensuring that the selected desorption conditions were still adequate, the absence of carry over was checked.

As regards the ionic strength effect, different concentrations between 0 and 300 g L^{-1} of NaCl and Na_2SO_4 were assayed. As shown in Fig. 3c, it could be said that most of the analytes undergo an increase in extractability with the increase of the salt concentration, while the extraction efficiencies observed with NaCl and Na_2SO_4 were negligible. Since the comparative study between NaCl and Na_2SO_4 showed that the addition of NaCl caused less problems in the fiber cleaning process than Na_2SO_4, 300 g L^{-1} of NaCl was chosen.

The effect of the organic solvent content of the sample was also evaluated and showed a fall in the extractability of the analytes as the concentration of acetonitrile or methanol increased (0, 5, 10 and 20%). As a consequence, no addition of organic solvent was made to the sample.

Temperature plays an important role in the extraction of the analytes influencing their mass transfer rates and the partition coefficients, and so the extraction efficiency was studied at room temperature (20 ± 1), 40 and 75 °C. For all the compounds, the best recoveries were observed at the higher temperature.

Finally, the effect of the equilibrium time was estimated for a period of time ranging from 5 to 60 min (Fig. 3d). The results showed that some compounds reached the equilibrium time in 15 min while other compounds reached it in 30 min. Although it is possible to obtain good extraction yields and reliable analysis also in non-equilibrium conditions, in order to obtain the best quantification limits, an extraction time of 30 min was chosen for further experiments.

Based on these data, the experimental conditions of the dSPME procedure were as follows: two aliquots of 2 mL of sample were put into two 4 mL screw-cap vials. To the first aliquot 0.1 mL of phosphate (2 M, pH 11) and 0.6 g of NaCl were added. Then, a CW/TPR

fiber, Fiber 1, was immersed in the solution. To the second aliquot 0.1 mL of citrate buffer (2 M, pH 3) and 0.6 g of NaCl were added. Then, another CW/TPR fiber, Fiber 2, was immersed in the solution. Both aliquots were stirred at a controlled speed of 94 rad s^{-1} for 30 min at 75 °C. Once the extraction was performed, the desorption was carried out as previously outlined: Fiber 1 was introduced in the desorption chamber which had previously been filled with mobile phase consisting of 20 mM ammonium formate: AcN (50:50, v/v) and static desorption was carried out for 10 min. After this period of time, Fiber 1 was removed from the chamber, without injecting the sample, and Fiber 2 was introduced. Then, the analytes were desorbed in the static mode for 10 min. At this moment, and not before, the valve was switched from the load to the inject position. A representative dSPME–LC–ITMS segmented chromatogram in MRM mode is shown in Fig. 1.

3.3. Analytical assessment of the method

The analytical assessment of the developed dSPME/LC–MS methodology was evaluated in terms of linearity, quantification limits, precision and accuracy.

For quantification, surrogate standards were used. But since isotopically labelled compounds were not available for all the analyzed compounds, three compounds were selected as the surrogate standards. In previous studies, two of the compounds used as surrogate standards (lorazepam and diazepam), were not detected in the analyzed samples. Also, a deuterated substance, FNT-6d, was included, which, under experimental conditions, eluted at t_R close to the compounds under study. The surrogate standard for each analyte is showed in Table 1. On the other hand and as can be observed in Fig. 4 there was no significant statistical difference between the signal of surrogate standards in the pure water standard and in the urban wastewater samples. Therefore, it could be assumed that there is no matrix effect in these three compounds.

The calibration curves using analyte/surrogates relations and six-point of calibration over the range of LOQ to 10 μg L^{-1} were generated using linear regression analysis. All the correlation coefficients were better than 0.992 confirming that the responses were linear in the concentration range studied. The estimated LOQ values ranged from 0.005 to 0.05 μg L^{-1} (Table 2). As can be seen, the obtained LOQ are between 10 and 200 times lower, depending on the substances, than those reached without the microextraction stage, using a direct injection of water samples.

Intra- and interday precision and accuracy were also tested for all the analytes and for the three concentrations levels: 0.1, 1 and 10 μg L^{-1}. For each level, ten repetitive extractions were made in the same day and also at intervals over a 2-week period (n = 10). As shown in Table 2, the intra- and interday precision presents coefficients of variation below 13.6%. The accuracy of the assay, based on the deviation of the mean measured value from the theoretical (doped) value, ranged from 89.2 to 109.7%.

3.4. Applicability of the dSPME procedure to urban wastewater samples

The dSPME–LC–ITMS method was applied to determine these pharmaceuticals compounds in wastewater samples. The effectiveness of the proposed dSPME/LC–MS method was tested by analyzing influent and effluent urban wastewater samples.

The presence of ionizable substances coming from the matrix can interfere with the ESI ionization processes and on the other hand, these and other substances also can affect the SPME extraction efficacy. Nevertheless, since SPME is an equilibrium method that uses small volumes of solvent in desorbing the analytes from the fiber, there are likely to be fewer co-extracting contaminants than would be seen in other extraction techniques such as SPE. In

Table 3
Concentration (ng mL^{-1}) of the detected target pharmaceutical compounds in influent and effluent waters (n = 3).

Compound	14/10/2009		15/10/2009		18/10/2009		19/10/2009		20/10/2009	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
NAP	0.200 ± 0.021	n.d.[a]	0.262 ± 0.021	n.d.	0.299 ± 0.025	n.d.	0.199 ± 0.017	n.d.	0.234 ± 0.021	n.d.
VAL	0.141 ± 0.013	<LOQ[b]	0.129 ± 0.010	<LOQ	0.138 ± 0.014	<LOQ	0.153 ± 0.012	<LOQ	0.186 ± 0.016	<LOQ
TRIM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BEZA	< LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
TORA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DICLO	0.156 ± 0.013	0.097 ± 0.006	0.146 ± 0.012	0.088 ± 0.008	0.087 ± 0.010	0.081 ± 0.006	0.188 ± 0.021	0.070 ± 0.007	0.070 ± 0.006	0.094 ± 0.010
MTL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CBZ	0.031 ± 0.003	0.043 ± 0.003	0.036 ± 0.002	0.051 ± 0.005	0.022 ± 0.002	0.019 ± 0.002	0.021 ± 0.002	0.010 ± 0.001	0.016 ± 0.001	0.016 ± 0.002
LRZ	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.
VLF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
RISP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DZP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CIT	0.062 ± 0.006	0.059 ± 0.003	0.048 ± 0.04	0.052 ± 0.004	0.051 ± 0.005	0.050 ± 0.005	0.047 ± 0.005	0.051 ± 0.004	0.048 ± 0.003	0.055 ± 0.005
FLX	0.177 ± 0.020	0.127 ± 0.010	0.069 ± 0.005	0.085 ± 0.007	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IMIP	0.041 ± 0.005	0.043 ± 0.004	0.039 ± 0.003	0.039 ± 0.003	0.048 ± 0.003	0.030 ± 0.002	0.029 ± 0.003	0.033 ± 0.004	0.034 ± 0.003	0.055 ± 0.006
AMIT	0.214 ± 0.019	0.122 ± 0.009	0.096 ± 0.009	0.087 ± 0.008	0.089 ± 0.007	0.065 ± 0.007	0.069 ± 0.005	0.077 ± 0.006	0.065 ± 0.006	0.076 ± 0.008

[a] n.d.: non-detected.
[b] <LOQ: detected but not quantified.

effect, the extracted sample obtained after SPME contains less of the original matrix than the extract arising from SPE, thus reducing the observed matrix effects [28]. For all these reasons, the matrix effect related to the electrospray ionization processes can be considerably compensated using dSPME and the surrogate standards [34]. Furthermore, the use of the surrogate standards minimizes any error that may occur during the dSPME procedure.

The method was applied for the quantification of the pharmaceuticals in influent and effluent urban wastewaters from a WWTP. In Table 3, the results obtained for these real samples can be seen. The concentrations in which these compounds appear in the influent ranged between 0.010 and 0.299 ng mL^{-1}. In spite of these low level of concentrations, it is noticeable that the elimination of these compounds in the WWTP is effective in some cases, while in others it was quite low and discharges to the environment through effluents can be higher that 0.1 ng mL^{-1} for some of them (FLX, AMIT). Thus, five of the analytes were not detected in any of the analyzed samples: TRIM, MTL, VLF, RISP, DZP. A further two, BZF and TORA, were detected in all the samples, but were not able to be quantified as their levels were lower than the LOQ. Among the analytes found it is worth mentioning that NAP and VAL were removed during the treatment process in the WWTP. On the other hand, it may be assumed that CBZ, CIT and IMIP were not removed in the treatment. The rest of the compounds, AMIT, FLX, DICLO were partially removed but, depending on the day of sampling, were still present in different concentrations and a definitive conclusion cannot be reached.

4. Conclusions

For the first time, a dSPME–LC–MS method for the quantification of 16 pharmaceutical compounds considered as emerging pollutants in wastewaters was developed. This method enables the simultaneous extraction and quantification of emerging pharmaceutical compounds with acidic and basic characteristics. The developed procedure minimizes laborious and complicated sample preparation procedures. The selectivity of the dSPME procedure together with the selectivity of the MS detector guarantees the identification of the analytes. The quantification limits obtained with this method allowed the quantification of the analytes in the samples in an accurate and precise way. The low sample volume requirements, the ease of the extraction and the minimization of the electrospray matrix effect make this method useful for measuring these analytes in wastewater samples. The application of the method to real samples proves its effectiveness in identifying and detecting ten of these compounds in influent and effluent urban wastewater samples.

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