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# Pharmaceutical trace analysis in aqueous environmental matrices by liquid chromatography–ion trap tandem mass spectrometry

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ABSTRACT

An analytical method based on solid-phase extraction followed by liquid chromatography tandem mass spectrometry with an ion trap analyser was developed and validated for the quantification of a series of pharmaceutical compounds with distinct physical–chemical characteristics in estuarine water samples. Method detection limits were between 0.03 and 16.4 ng/L. The sensitivity and the accuracy obtained associated with the inherent confirmatory potential of ion trap tandem mass spectrometry (IT-MS/MS) validates its success as an environmental analysis tool. Two MS/MS transitions were used to confirm compound identity. Almost all pharmaceuticals were detected at ng/L level in at least one sampling site of the Douro River estuary, Portugal.



## 1. Introduction

Nowadays, the worldwide presence of a large variety of environmental micropollutants, particularly pharmaceutical compounds, at trace levels of concentration (μg to ng/L) in various aqueous media is generally well documented [1–5]. The widespread presence of pharmaceuticals in aquatic environments has led to the establishment of monitoring programs [6] and specific surveys based, for example, on spatial, seasonal and different hydrological conditions which have allowed a better understanding of the sources, fates and distribution of these compounds [7–9]. The quantification of pharmaceuticals in aquatic environments represents a growing investigation area since there is a prime concern for the ecotoxicological risks of aquatic organisms and humans caused by active pharmacological compounds detected in the environment [10]. The major sources of pharmaceuticals released into natural aquatic environments are the final effluents from wastewater treatment plants (WWTPs) due to partial removal in the treatment process [11]. Thus, surface and wastewaters represent the two most studied aqueous compartments in which the majority of pharmaceutical compounds have been detected [3,12]. Some publications have reported also the presence of pharmaceutical residues in groundwater and even in drinking water [13,14].

In recent years the advances in analytical techniques, especially the progress of liquid chromatography mass spectrometry (LC–MS) and LC–tandem MS (LC–MS/MS) have played a crucial role in aquatic environment analysis [15,16] mainly due to their versatility, sensitivity and selectivity [17]. Recent trends in environmental mass spectrometry methods have been emerging with special focus on hybrid mass spectrometers such as quadrupole-time of flight (Qq-TOF) [18] and quadrupole-linear ion trap (Qq-LIT) [19]. However, triple quadrupole (QqQ) mass analysers still represent the most used analytical tool in the environment application area [12,20,21]. Only a few publications have employed ion trap (IT) mass spectrometers for environmental determinations, mainly for quantification of antibiotic residues, resulting in sensitive methods and reliable confirmation of positive samples [22–25]. The application of IT mass spectrometers in the structure elucidation analysis and for the identification of unknowns in complex matrices is well established [16]. However, the environmental data aforementioned show that IT mass spectrometers can also be very useful for quantitative analysis [22–25].

This work presents a validated analytical method based on a single solid-phase extraction (SPE) step, with HLB cartridges, followed by ion trap LC–MS/MS with electrospray ionization (ESI) for the quantification of seven pharmaceuticals belonging to six different pharmacological classes. The list of compounds comprised

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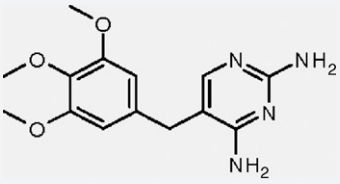
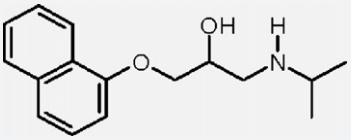
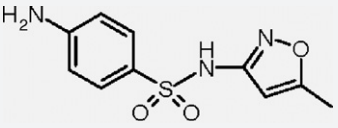
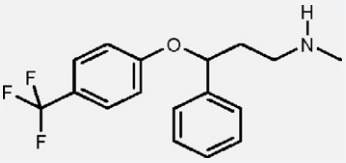
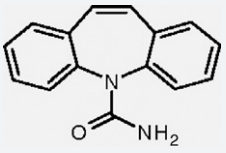
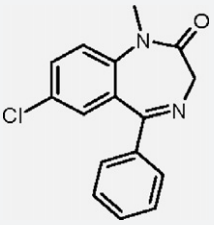
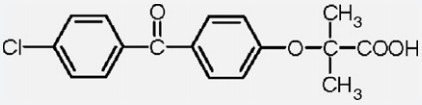
**Table 1**
Chemical structures of the pharmaceutical compounds.

| Compound (abbreviation) | Chemical structure | $M_w$ (g mol$^{-1}$) |
|---|---|---|
| Trimethoprim (TMP) |  | 290.32 |
| Propranolol (PHO) |  | 259.35 |
| Sulfamethoxazole (SMX) |  | 253.28 |
| Fluoxetine (FX) |  | 309.33 |
| Carbamazepine (CBZ) |  | 236.26 |
| Diazepam (DZ) |  | 284.76 |
| Fenofibric acid (FA) |  | 318.76 |

trimethoprim (TMP), propranolol (PHO), sulfamethoxazole (SMX), fluoxetine (FX), carbamazepine (CBZ), diazepam (DZ) and the metabolite of fenofibrate (F), fenofibric acid (FA), as shown in Table 1. The challenge to analyse the selected series of pharmaceuticals is related to the simultaneous extraction and chromatographic analysis of distinct chemical classes of compounds with a wide range of polarities, $pK_a$, $\log K_{ow}$ and different stabilities under acidic, basic and neutral conditions. Furthermore, the selected compounds are frequently detected in distinct aquatic environments and, in some cases, have shown to be persistent and generate bioaccumulative effects [26].

Two different approaches based on qualitative (post-column infusion) [27] and quantitative (post-extraction addition) [28] techniques were used to assess the matrix effects on the IT-MS/MS signal. The importance of evaluating matrix effects as an integral part of any LC–ESI-MS/MS method development and validation is generally well recognized [29].

The confirmation of the compounds identity was performed in compliance with European regulations—EU Commission Decision 2002/657/EC [30]. Recently, in the environmental field, some authors have stressed the need for adopting confirmation rules [31,32].

The applicability of the developed method was demonstrated by the quantification of the series of target pharmaceuticals at several points in the Douro River estuary, Portugal. In order to assess the risk caused by the selected compounds to aquatic organisms, seasonal monitoring studies in this river are under investigation by our group.

## 2. Experimental

### 2.1. Chemicals and materials

Analytical grade formic acid (98%) was obtained from Fluka (Steinheim, Germany) and water used for the mobile phase was purified through a Milli-Q system (Millipore, São Paulo, Brazil). All the other solvents used were of HPLC grade and supplied by J.T. Baker (Philipsburg, PA, USA).

The cartridges used for solid-phase extraction were Oasis® HLB (Hydrophilic–Lipophilic Balance), 500 mg, 12 cc from Waters

Corporation (Milford, MA, USA) and 0.45 μm glass fibre filters were purchased from Millipore (Ireland).

### 2.2. Reference standards

Trimethoprim (TMP), propranolol hydrochloride (PHO), sulfamethoxazole (SMX), fluoxetine hydrochloride (FX), carbamazepine (CBZ), diazepam (DZ) and fenofibrate (F) were purchased from Sigma–Aldrich (Steinheim, Germany). Fenofibric acid (FA) was prepared by hydrolysis of fenofibrate with subsequent purification and re-crystallisation in ethanol, as described elsewhere [33,34]. The product was characterised by IR, NMR $^{1}H$, $^{13}C$ and elementary analysis. All reference standards were of >98% purity. Isotopically labelled compounds, used as internal standards (IS) were [$^{13}C_1$, $^{15}N_1$]-carbamazepine and [$d_4$]-sulfamethoxazole obtained from Sigma–Aldrich (Steinheim, Germany) and Toronto Research Chemicals Inc. (North York, Canada), respectively. Individual stock solutions (1 mg/mL) were prepared by weighing out approximately 10 mg of each standard and dissolving it in 10 mL of ethanol. The solutions were stored in the dark at −20 °C in amber bottles to avoid degradation. No evidence of degradation was observed during the study period. For IS, solutions of 10 μg/mL were prepared in ethanol. Through an appropriate combination of stock solutions, six working calibration standards were prepared in ethanol at the following range of concentrations: 16.2–230 ng/mL (TMP), 0.60–8.10 ng/mL (PHO), 33.0–446 ng/mL (SMX), 185–2498 ng/mL (FX), 0.54–7.30 ng/mL (CBZ), 13.0–176 ng/mL (DZ) and 6.00–81.0 ng/mL (FA). Three quality control (QC) standard solutions of each pharmaceutical were also prepared: 19.0, 112, 205 ng/mL for TMP, 0.72, 3.96, 7.20 ng/mL for PHO, 39.0, 220, 400 ng/mL for SMX, 222, 1211, 2200 ng/mL for FX, 0.64, 3.57, 6.50 ng/mL for CBZ, 15.6, 82.8, 150 ng/mL for DZ and 7.20, 39.6, 72.0 ng/mL for FA.

### 2.3. Site description and sample collection

Water samples were collected along the Douro River estuary in the region of Porto, Portugal, from the outfalls of treated and untreated wastewater discharges in July 2008. The selection of the sampling sites was based on a recent study which had identified the presence of several endocrine disrupting chemicals in the Douro River estuary [35]. The four sampling sites are located in the first 9 km of the estuary, corresponding to the most urbanized area. An extra sampling site was collected in a non-polluted area of the estuary (20 km from the estuary mouth) in order to study the matrix effects. Surface waters (2 L) were sampled from a depth of approximately 1 m using a peristaltic sampler pump (Global Water, Model: WS300, CA, USA) into 2.5 L pre-rinsed amber glass bottles. Upon collection, samples were kept on ice, transported to the laboratory and then vacuum filtered through 0.45 μm glass fibre filters to remove suspended particles that may interfere with the extraction procedure. The filter was washed with approximately 5 mL of methanol and added to the samples. Samples were kept refrigerated at 4 °C in the dark and extracted within a maximum of 72 h after collection.

Spring water samples from the Monjolinho River (São Carlos, SP, Brazil) were used for method validation. This water matrix was also sampled and prepared as described above.

### 2.4. Solid-phase extraction

Off-line SPE was performed on Oasis® HLB 500 mg sorbent cartridges using a Varian vacuum extraction device. Conditioning step was carried out with 32 mL of dichloromethane, 32 mL of methanol and then the cartridge was equilibrated with 32 mL of ultrapure water, at a flow rate of 1 mL/min. Surface water samples (2 L) were loaded at a constant flow rate of 10 mL/min followed by a washing step with 32 mL of water. Cartridges were dried under vacuum during 30 min and then eluted with 32 mL methanol/dichloromethane (70:30, v/v) at 1 mL/min into a test tube containing 4 μL of each IS (10 μg/mL). The resulting eluates were evaporated to dryness in a thermostatic bath at 40 °C under a gentle nitrogen stream and reconstituted with 400 μL of ethanol.

### 2.5. Liquid chromatography

A Shimadzu HPLC system (Kyoto, Japan) equipped with two LC-20AD pumps, a SIL-20A auto-sampler, a DGU-20A5 degasser, a UV/Vis SPD-20A detector and a CBM-20A interface was used for the LC analyses. Chromatographic separation was achieved with a Shimadzu $C_{18}$ endcapped column (150 mm × 2.1 mm, 5 μm) with a 0.1% aqueous solution of formic acid (v/v) (solvent A) and acetonitrile with 0.1% formic acid (v/v) (solvent B) as mobile phases. The gradient elution was linear from 10 to 65% of B in 20 min and then from 65 to 85% of B in 4 min with a flow rate of 0.2 mL/min. The equilibration time was 5 min and the injection volume was set to 30 μL. For preliminary studies the absorbance was monitored at 270 nm.

### 2.6. Mass spectrometry

An Esquire 6000 IT mass spectrometer (Bruker Daltonics, Germany) equipped with an ESI source was used. The analyses were performed in positive mode and the mobile phase flow was split into the source at 100 μL/min. The optimization of the ionization source, voltages on the lenses and trap conditions were achieved with the expert tune mode of Daltonics Esquire control software. For the establishment of these parameters, individual standard solutions of each compound (100 ng/mL) were directly infused in continuous flow mode by a syringe pump (Cole Parmer, EUA) at a flow rate of 10 μL/min into the mobile phase stream. This was done by means of a T-piece after the chromatographic column and before the mass spectrometer ionization source. The mobile phases used were set at isocratic mode with the percentages of the organic modifier employed at gradient elution for each compound. This allowed the analytes to reach the ESI source under similar conditions as those that would be encountered during a typical sample analysis. A nebuliser pressure of 30 or 40 psi (for TMP), a drying gas flow of 8 L/min and a temperature of 325 °C were selected. Nitrogen was used as the nebulising and drying gas and helium gas was used to induce dissociation for MS/MS acquisition data. The mass spectrometer was run in a multiple reaction monitoring (MRM) mode in which the protonated molecular ion was isolated and the fragment ions were monitored. MRM transitions were monitored in five different elution time windows. The selection of fragmentation products for each compound was based on the most specific transitions that produced the highest signal.

### 2.7. Matrix effect

The post-column infusion and the post-extraction addition methods were carried out on water samples from both the Douro and Monjolinho rivers. In the post-column infusion method, all compounds were infused separately (100 ng/mL) with a syringe pump at a flow rate of 10 μL/min into the LC stream as described before, according to the published procedures [27,36]. Ethanol and blank water samples from Douro and Monjolinho rivers, which were extracted by SPE, were injected onto the analytical column using the optimized analytical conditions. Effluent from the HPLC column combined with the infused analytes entered the electrospray interface. Under these conditions, the eluates from SPE may enhance or suppress the ESI signals of the infused compounds when compared with the solvent responses.

For the post-extraction addition method the peak areas of two extracted samples from Douro and Monjolinho rivers, spiked with all compounds (100 ng/mL) after extraction ($A$) were compared with the areas obtained for a solvent solution at the same concentration level ($B$). The experimental procedure was based on published works [28,37]. Two non-spiked water samples were also extracted and analysed. The matrix effect (ME) ratio obtained was expressed as: ME (%) $= \frac{B}{A} \times 100$ in accordance with the equation proposed by Matuszewski et al. [28]. The absence of absolute matrix effect is indicated by a value of 100%. A value >100% indicates an ionization enhancement and a value <100% corresponds to ionization suppression.

### 2.8. Method validation

Linearity was evaluated using calibration curves with six calibrators, each one prepared in triplicate as described above. Accuracy, intra- and inter-batch precision were determined by analysing three replicates of each QC sample. Precision was expressed as the relative standard deviation (RSD) of the replicate measurements and the accuracy of the method was evaluated as the percentage of agreement between the method results and the nominal amount of compound added [38]. Blank matrixes fortified at the three QC concentrations were used for the recovery assays and the efficiency of the extraction was calculated by comparing the peak areas of the compounds with those of similar concentrations in ethanolic standard solutions. Method detection limits (MDL) and method quantification limits (MQL) were determined from spiked Monjolinho water samples, prior to the extraction procedure, and were assumed as the minimum detectable amount of compound with a signal-to-noise (S/N) ratio of 3 for MDL and 10, with a RSD $\leq$20% ($n = 3$), for MQL. The stability of the compounds was evaluated by comparing assay results in fortified samples at three different concentrations (QC samples) and by analysing aliquots of the same samples after 24 and 48 h at room temperature ($\pm$22 °C).

### 2.9. Samples confirmation and quantification

According to the EU Commission Decision 2002/657/EC for the confirmation and identification of pharmaceuticals, when using LC–tandem MS as an instrumental technique, a minimum of three identification points (IPs) are required [30]. In this study, two MRM transitions were monitored for almost all the compounds and the MRM ratio were calculated as the relation between the abundances of both transitions. In addition, the retention times ($t_R$) were used to confirm the presence of the pharmaceutical compounds in the samples.

The first transition was used for quantification and the second one for confirmatory purposes. Quantification was performed by plotting the peak area against the concentration of each compound with the exception of SMX, CBZ and FX. In these cases the calibration curves were a plot of the peak area ratio of the compound signal to the respective IS *versus* nominal concentration. For FX quantification the [$d_4$]-SMX was used. Calibration standards were analysed at the beginning and at the end of a sample sequence. The variations in signal intensity were monitored by the analyses of three QC samples after approximately ten injections. All samples that were quantified above the highest concentration of the calibration curves were diluted with ethanol and re-analysed. In order to guarantee the accuracy and precision of the procedure the dilutions were validated with the use of a standard solution.

## 3. Results and discussion

### 3.1. Solid-phase extraction

The development and optimization of the sample preconcentration for the selected series of pharmaceutical compounds was carried out as described in a previous work [39]. However, in the present work 400 μL of ethanol was used to reconstitute the samples resulting in a 5000-fold pre-concentration.

### 3.2. Liquid chromatography and ion trap mass spectrometry

LC separation was achieved by gradient elution using an aqueous solution with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) as mobile phase. The selection of the mobile phase solvents was based on previous chromatographic separations published for multi-class compounds [40,41]. Different isocratic and gradient elution conditions were evaluated

**Table 2**
Selected MS/MS conditions for all compounds.

| Compound | $t_R$ (min) | Percursor ion ($m/z$) $[M+H]^+$ | MRM transitions | Transitions ratios | Capillary voltage ($V$) | Fragmentation amplitude ($V$) | Segment period |
|---|---|---|---|---|---|---|---|
| Trimethoprim | 6.7 | 291 | 291 > 230[a]<br>291 > 123[b] | 1.66 | 4033 | 0.40 | I (0–9 min) |
| Propranolol | 11.3 | 260 | 260 > 183[a]<br>260 > 183[b] | 1.60 | 3664 | 0.40 | II (9–13 min) |
| Sulfamethoxazole | 12.3 | 254 | 254 > 156[a]<br>254 > 108[b] | 8.69 | 3664 | 0.45 | II (9–13 min) |
| [$d_4$]-Sulfamethoxazole (IS) | 12.3 | 258 | 258 > 160[a]<br>258 > 112[b] | | 3664 | 0.40 | II (9–13 min) |
| Fluoxetine | 14.4 | 310 | 310 > 148[a] | | 3836 | 0.37 | III (13–17 min) |
| Carbamazepine | 15.2 | 237 | 237 > 194[a]<br>237 > 192[b] | 14.89 | 3836 | 0.35 | III (13–17 min) |
| [$^{13}C_1$, $^{15}N_1$]-Carbamazepine (IS) | 15.2 | 239 | 239 > 194[a]<br>239 > 192[b] | | 3836 | 0.40 | III (13–17 min) |
| Diazepam | 19.0 | 285 | 285 > 257[a]<br>285 > 154[b] | 1.01 | 3541 | 0.50 | IV (17–21 min) |
| Fenofibric acid | 22.8 | 319 | 319 > 233[a]<br>319 > 139[b] | 32.5 | 4426 | 0.38 | V (21–24 min) |

[a] Transition used for quantification.
[b] Transition used for confirmation.

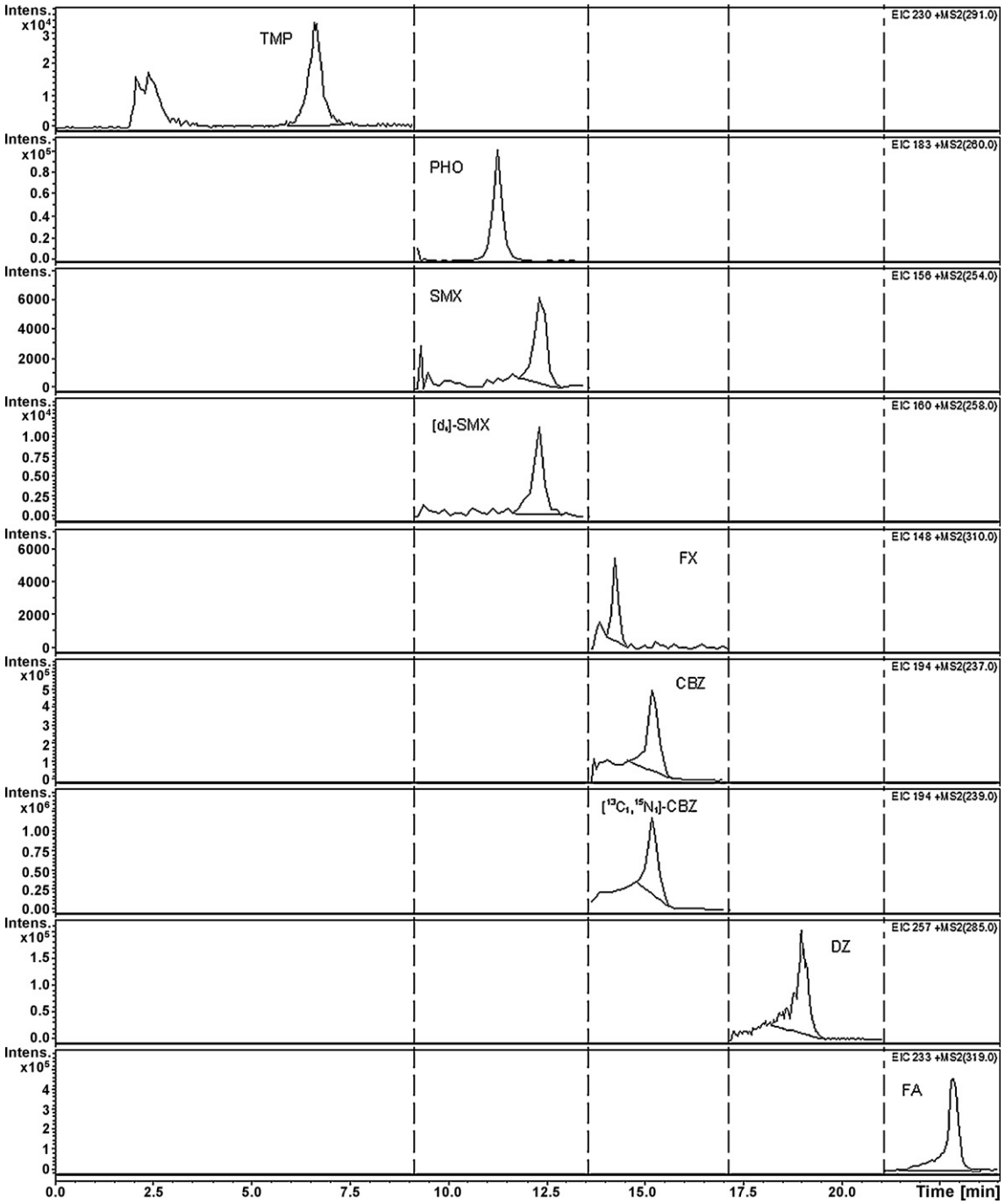



**Fig. 1.** Representative MRM chromatograms of a Monjolinho River water sample spiked with all compounds (100 ng/mL).

but, considering the wide range of retention times, a linear gradient elution was selected for highest selectivity with an analysis time of 24 min (Fig. 1). Formic acid was added to both solvents in order to guarantee a uniform enhancement of the ESI signals during all gradient elution.

Full scan acquisitions were made over specific mass ranges for individual compounds to determinate the mass spectrometry conditions and the optimum mode of ionization (Table 2). For all pharmaceuticals, an intense protonated molecular ion $[M+H]^+$ was obtained in the positive ESI mode. Although for clofibric acid and analogues the negative ESI mode is generally used [42], in this study the deprotonated ion of FA proved to be extremely unstable. Thus, the protonated molecular ions $[M+H]^+$ were used as precursor ions for all compounds. Product ions were determined under MS/MS conditions and the fragment ions are in agreement with published data. The main fragments of TMP correspond to the loss of two molecules of $CH_3O$ $[M-2CH_3O]^+$ ($m/z = 230$) for the first transition and $[M-C_9H_{12}O_3]^+$ ($m/z = 123$) for the second one [43]. For PHO, $m/z = 183$ is identified as $[M-H_2O-C_3H_7NH]^+$ and $m/z = 116$ corresponds to $[M-C_{10}H_7O]^+$ [44]. SMX and CBZ, two of the most studied pharmaceuticals in aquatic environments, have characteristic product ions at $m/z = 156$ $[H_2NPhSO_2]$, $m/z = 108$ $[H_2NPhO]^+$ and at $m/z = 194$ $[M+H_2-CONH_2]^+$, $m/z = 192$ $[M-CONH_2]^+$, respectively [44,45]. For DZ, the first transition corresponds to the loss of CO $[M-CO]^+$ ($m/z = 257$) and the second transition can be attributed to the loss of $[M-C_8H_5ON]^+$ ($m/z = 154$) [46]. The major fragmentation pattern detected for FX was attributed to $[M-F_3C_7H_4O]^+$ ($m/z = 148$) [44]. The main fragments of FA selected in this

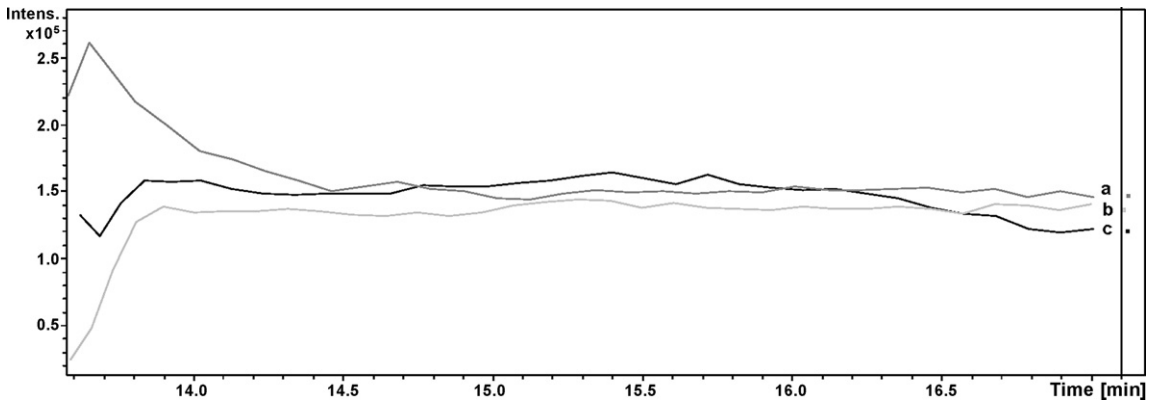



**Fig. 2.** LC–MS/MS extracted ion chromatograms from the post-column infusion of a standard solution of CBZ (100 ng/mL) in (a) ethanol, (b) Monjolinho River extracted water sample and (c) Douro River extracted water sample.

**Table 3**
Matrix effect (%) of the different compounds in water samples from the Douro and Monjolinho rivers.

| Compound | Matrix effect (%) | | | |
|---|---|---|---|---|
| | Monjolinho River extract I | Monjolinho River extract II | Douro River extract I | Douro River extract II |
| Trimethoprim | 41.4 | 47.5 | 44.9 | 45.8 |
| Propranolol | 40.7 | 42.0 | 31.4 | 48.2 |
| Sulfamethoxazole | 13.8 | 9.70 | 14.0 | 16.2 |
| [$d_4$]-Sulfamethoxazole (IS) | 13.8 | 9.80 | 11.5 | 14.3 |
| Fluoxetine | 6.20 | 9.70 | 6.30 | 4.10 |
| Carbamazepine | 94.4 | 92.6 | 94.8 | 92.9 |
| [$^{13}C_1$, $^{15}N_1$]-Carbamazepine (IS) | 93.3 | 94.1 | 93.9 | 94.0 |
| Diazepam | 113 | 99.3 | 94.9 | 101 |
| Fenofibric acid | 46.4 | 53.6 | 49.1 | 38.9 |

study, $m/z = 233$ and $m/z = 139$ correspond to $[C_{13}H_{10}ClO_2]^+$ and $[C_7H_4ClO]^+$, respectively. This MS/MS fragmentation is in accordance with the data previously reported for this compound [47].

### 3.3. Matrix effects

Humic substances (humic and fulvic acids) represent the major components of dissolved organic matter in surface waters responsible for the matrix interferences that generally impair the efficiency of sample extraction and the detection of the target compounds in aquatic environments [19,48]. The quantitative LC–ESI-MS/MS analysis is highly susceptible to co-extracted components that might affect compound ionization resulting in either enhancement [14,49] or more frequently in a suppression of the signals [12,14,50]. Therefore, the matrix effects must be evaluated in order to avoid erroneous quantifications. The ion suppression can be caused by distinct mechanisms. The matrix interferences can mask the compound peaks, reduce ionization efficiency of the chemical compounds or, alternatively, sorb them [51].

In this work the matrix effects have been evaluated by two means: post-column infusion and post-extraction addition methods. For CBZ, no differences in the signals were observed by post-column infusion analysis (Fig. 2), irrespective of the matrix used (ethanol, Douro or Monjolinho extracted water samples). Meanwhile, using the post-extraction addition technique an ion suppression of 5.22–7.45% was obtained for CBZ in agreement with the qualitative result (Table 3). The post-column infusion analysis demonstrated a high suppression effect for the FX signal using both aqueous extracted samples (Fig. 3). These results were confirmed by the mean value obtained for this compound (93.4%) with the post-extraction addition technique (Table 3). Overall, signal suppressions were measured for all the seven pharmaceuticals. However, CBZ and DZ present low suppression levels when compared with FX and SMX (Table 3). These results demonstrated

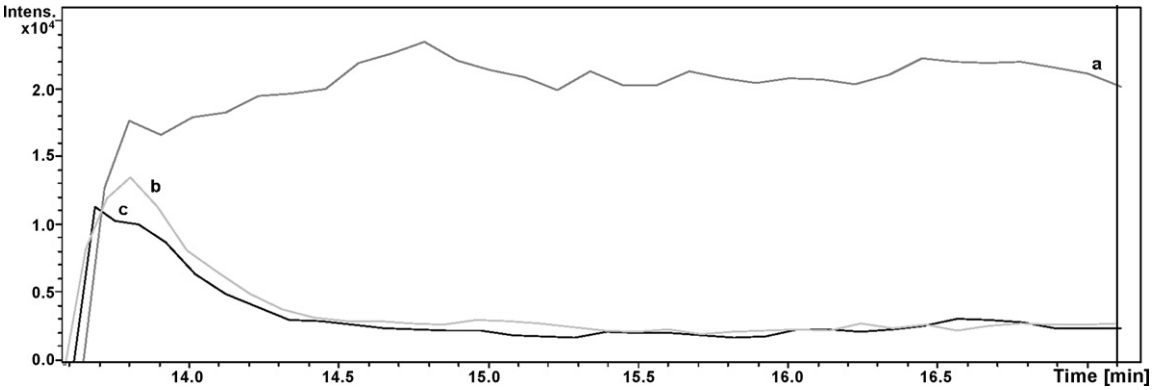



**Fig. 3.** LC–MS/MS extracted ion chromatograms from the post-column infusion of a standard solution of FX (100 ng/mL) in (a) ethanol, (b) Monjolinho River extracted water sample and (c) Douro River extracted water sample.

**Table 4**
Linearity parameters, detection and quantification limits of the method.

| Compound | Linearity parameters | | | MDL (ng/L) | MQL (ng/L) |
|---|---|---|---|---|---|
| | Range (ng/mL) | Calibration equation | $r$ | | |
| Trimethoprim | 16.2–230 | $y = 8590.2x - 9460.9$ | 0.991 | 1.25 | 3.24 |
| Propranolol | 0.60–8.10 | $y = 14552x + 6834.5$ | 0.998 | 0.03 | 0.12 |
| Sulfamethoxazole | 33.0–446 | $y = 0.003x - 0.0759$ | 0.997 | 4.40 | 6.60 |
| Fluoxetine | 185–2498 | $y = 0.002x - 0.3699$ | 0.990 | 16.4 | 37.0 |
| Carbamazepine | 0.54–7.30 | $y = 0.0046x + 0.013$ | 0.995 | 0.03 | 0.11 |
| Diazepam | 13.0–176 | $y = 14107x - 80275$ | 0.998 | 1.30 | 2.60 |
| Fenofibric acid | 6.00–81.0 | $y = 17272x - 44021$ | 0.998 | 0.20 | 1.20 |

that the chemical nature of a compound interferes on the matrix effect which is in accordance with the published work [36]. The matrix composition in the electrospray ion source can also affect the ionization capacity of a compound and, consequently its signal response [52]. It is then extremely relevant to perform comparative matrix effect studies when different aquatic matrices are considered. In the particular case of this study, when analysing compounds behaviour in the presence of estuarine water from the Douro River and Monjolinho River water samples similar matrix effects were obtained. The results are shown in Table 3. In the light of these results, spring water samples from the Monjolinho River were used to prepare the calibrators, QC samples and non-spiked blank samples; while the validated method was used for the analysis of a series of estuarine water samples of the Douro River.

Currently, different strategies have been used to overcome matrix effects, such as the use of standard addition method or isotopic labelled IS to compensate for the signal alterations. The standard addition method is considered laborious and time consuming, and thus, inadequate for monitoring purposes. Meanwhile, the isotopic IS approach is difficult in practice, since it is hard to find suitable IS for each compound in a series of compounds with distinct physical–chemical properties. The high cost of an isotopic IS is another drawback to this approach [21]. Injecting smaller sample volumes, decreasing the flow rate delivered to the ESI interface and dilution of samples have also been explored in published works [44,53]. However, these actions might affect the method's sensitivity [44,53].

In this work the sensitivity obtained in non-split conditions, i.e. delivering the total column effluent to the ESI interface (200 μL/min), were compared with split assays (100 and 50 μL/min) at the same and distinct injection volumes (30, 40 and 50 μL). The highest sensitivity was obtained with a LC effluent split of 100 μL/min into ESI interface with 30 μL of injection volume in the chromatographic system. Two IS ([$d_4$]-SMX and [$^{13}C_1$, $^{15}N_1$]-CBZ) were selected to compensate for matrix effects. However, the two IS proved to be insufficient to correct the ionization of all compounds since the matrix effects were different for each compound. TMP, PHO and FA and IS signal were differently affected by the co-eluted matrix components. As the signal suppression of FX was very similar to [$d_4$]-SMX, this IS was used to correct the signal not only of the SMX but also the FX signal. The [$^{13}C_1$, $^{15}N_1$]-CBZ was used only as IS for CBZ.

As an ongoing project, an alternative sample preparation is under investigation by our group to try to overcome the limitations caused by matrix effects that are observed using SPE for sample clean-up.

### 3.4. Method validation

The calibration curves were linear for the established calibration ranges. The correlation coefficients ($r > 0.990$) and the regression equations for all pharmaceuticals are shown in Table 4. Precision was lower than 19.0% and accuracy values were between 81.8 and 120% for almost all calibration concentrations in the ranges established.

The MDL and MQL obtained were from 0.03 to 16.4 ng/L and from 0.11 to 37.0 ng/L, respectively (Table 4). The limits achieved were satisfactory for environmental analysis.

Intra- and inter-batch precision as well as accuracies of the method were assayed by analysing three replicates of each of the three QC water samples (low, medium and high concentrations) on three consecutive days. Accuracy values were between 76.9 and 122% (Table 5) which were considered appropriate results since the values obtained encompassed the complete sample preparation procedure and not just a consecutive sequence of injections of the same sample. These results are in accordance with the accuracy ranges reported in the literature [49]. Even when using the [$d_4$]-SMX to correct the FX signal the lack of accuracy during the validation process, did not permit the quantification of this pharmaceutical. Table 5 demonstrates the intra- and inter-batch precisions (RSD) obtained for the selected compound series ($\leq$18.9%).

The extraction recovery results for all compounds were determined by comparing the peak areas of ethanol solutions at three different control levels with those of the QC samples. The data presented in Table 5 concerns the mean recovery obtained and ranged from 40.5 to 92.2% with RSD values less than 15.9%, according to the compound. The recovery values obtained for the three concentration levels demonstrated that there is no influence between recovery and the concentration of the QC standards evaluated.

The room temperature stability assays demonstrated that the permanence of QC standards in the auto-sampler tray for 24 and 48 h at room temperature (approximately 22 °C) had no significant effect on the quantitative determination of the pharmaceutical compounds (RSD <14.9%).

Two blind samples of all compounds, prepared in triplicate, containing unknown concentrations to the analyst (80 ng/mL-TMP, 8 ng/mL-PHO, 70 ng/mL-SMX, 6 ng/mL-CBZ, 16 ng/mL-DZ and 32 ng/mL-FA) produced accuracies in the range of 90.4–119% with RSD values from 2.91 to 8.45%.

### 3.5. Confirmation of positive samples

The most common criteria that have been used for positive confirmation of compounds in the environmental field is the one established by European Commission Decision 2002/657/EC, which is based on a system of Identification Points (IPs) [30]. The European Decision stated that the number of IPs required to confirm a positive finding is entirely dependent on the mass spectrometer used, differentiating between MS and $MS^n$ and whether it is low or high resolution equipment. For low resolution mass spectrometers, such as IT, analysing two transitions (3 IPs) for one precursor ion (1 IP) is acceptable for satisfactory confirmation of compound identity, since the European criteria recommends a minimum of three IPs. Additionally, the deviation of the retention time (RSD <2.5%) and the ion intensity ratio have to be monitored and compared with those of reference standards.

**Table 5**
Recovery, accuracy, intra- and inter-batch precision.

| Compound nominal concentration (ng/mL) | 1st day[a] | | 2nd day[a] | | 3rd day[a] | | Recovery (%)[a] (RSD%) |
|---|---|---|---|---|---|---|---|
| | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) | |
| Trimethoprim | | | | | | | |
| 19.0 | 117 | 8.8 | 121 | 7.4 | 108 | 3.0 | 58.9 (7.0) |
| 112 | 90.8 | 4.9 | 84.3 | 12.8 | 96.1 | 13.2 | 60.2 (6.8) |
| 205 | 89.7 | 2.5 | 76.9 | 6.7 | 78.7 | 11.9 | 58.8 (8.7) |
| Propranolol | | | | | | | |
| 0.72 | 89.8 | 15.7 | 89.1 | 18.9 | 83.4 | 12.1 | 83.9 (2.9) |
| 3.96 | 80.8 | 6.8 | 78.2 | 9.2 | 96.1 | 12.7 | 78.6 (9.9) |
| 7.20 | 79.6 | 14.9 | 81.5 | 6.9 | 83.4 | 10.2 | 85.8 (2.2) |
| Sulfamethoxazole | | | | | | | |
| 39.0 | 120 | 13.1 | 120 | 15.0 | 120 | 99 | 42.0 (1.4) |
| 220 | 81.6 | 12.6 | 88.3 | 13.5 | 92.0 | 18.0 | 61.2 (7.4) |
| 400 | 91.2 | 16.1 | 114 | 9.7 | 87.3 | 10.4 | 65.7 (15.9) |
| Carbamazepine | | | | | | | |
| 0.64 | 119 | 12.3 | 116 | 14.7 | 122 | 13.4 | 87.5 (25) |
| 3.57 | 102 | 17.7 | 94.2 | 13.5 | 93.6 | 11.9 | 92.2 (4.9) |
| 6.50 | 113 | 13.0 | 117 | 11.1 | 110 | 6.3 | 83.2 (3.1) |
| Diazepam | | | | | | | |
| 15.6 | 104 | 13.9 | 108 | 8.0 | 96.2 | 9.5 | 64.5 (9.8) |
| 82.8 | 79.4 | 2.9 | 84.5 | 10.0 | 90.3 | 9.8 | 71.6 (7.1) |
| 150 | 80.7 | 2.1 | 85.7 | 2.0 | 79.4 | 3.7 | 75.1 (4.3) |
| Fenofibric acid | | | | | | | |
| 7.20 | 115 | 6.0 | 121 | 5.0 | 101 | 3.0 | 41.4 (8.4) |
| 39.9 | 93.2 | 15.9 | 101 | 12.7 | 120 | 12.2 | 48.9 (13.0) |
| 72.0 | 90.1 | 8.8 | 102 | 6.7 | 104 | 11.9 | 40.5 (7.5) |

[a] $n = 3$.

The tolerances established for the ion ratio are dependent on the relative abundance of the transitions (tolerances range from ±20% for a relative abundance of >50%, to ±50% for <10%) [30].

In this work, the collected water samples were analysed for the selected series of pharmaceuticals using the above-mentioned strategy. Based on that, two specific transitions were selected for each compound avoiding the use of neutral losses, such as $H_2O$, $CO_2$ and HCl, which could result in false positives since there may be interferences that share the same transition [32]. Some authors have recently stressed the importance of this point as a reliable confirmation of compound identity [34,35].

An illustrative example of a positive sample of the Douro River, for TMP, is presented in Fig. 4. The two characteristic ions of TMP were detected in the sample ($m/z = 230$ and $m/z = 123$) and the deviation obtained between the ion ratio and retention time in the sample and in a standard solution are in accordance with the European criteria.

### 3.6. Occurrence of pharmaceuticals in the Douro River, Portugal

The positive quantification findings in the four sampling sites along the Douro River are represented in Fig. 5. With the excep-

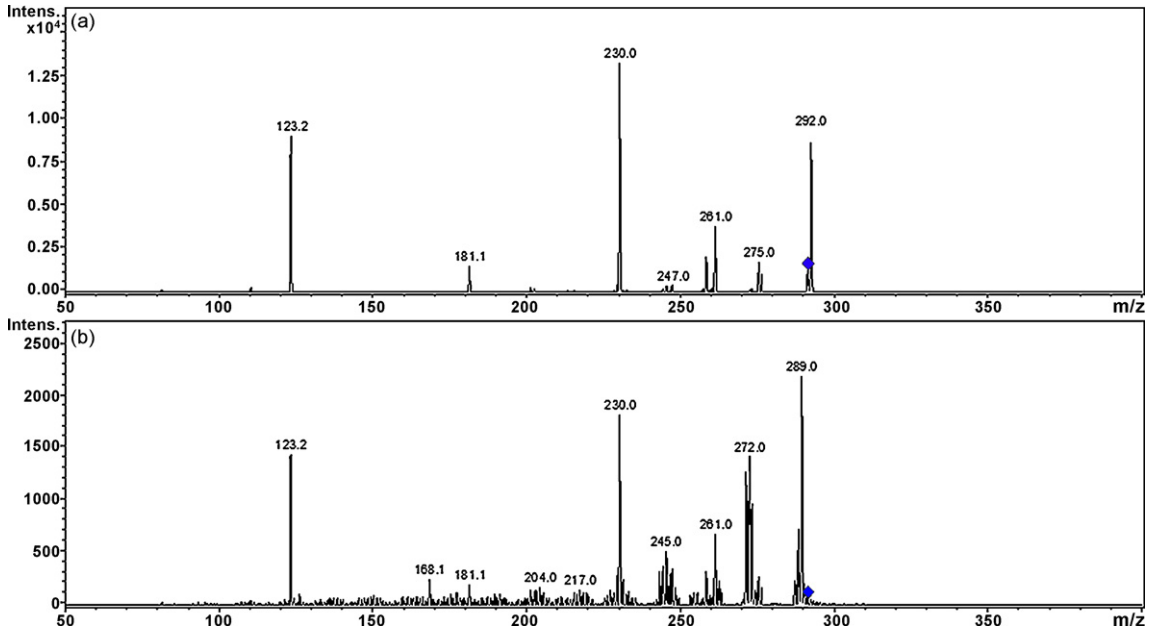



**Fig. 4.** MS/MS spectra for: (a) 100 ng/mL standard solution of TMP and (b) sample confirmed to be positive for TMP.

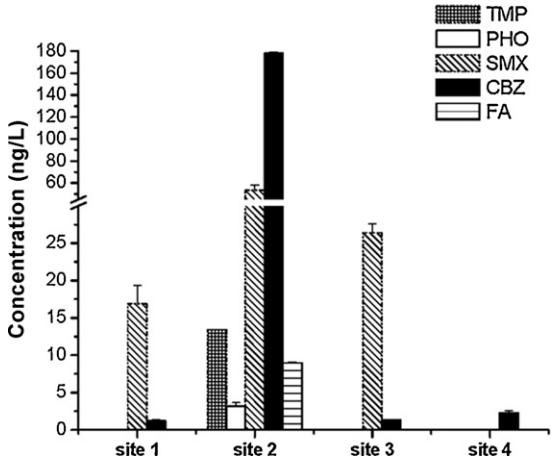



**Fig. 5.** Pharmaceutical concentrations (ng/L) measured in the different sampling sites of the Douro River, Portugal.

tion of DZ, all of the other compounds studied were quantified at concentrations higher than the MQL in samples collected at site 2 located close to the mouth of the river, immediately downstream from the discharge of a municipal WWTP. The highest concentrations were also detected at this sampling site (178 ng/L for CBZ and 53.3 ng/L for SMX) which can be explained by the location of the WWTP (Fig. 6). SMX was detected in three of the selected sample points (16.9–53.3 ng/L) while CBZ was ubiquitous in all other collecting sites. In fact, CBZ has been reported to be highly persistent in the environment and for this reason some authors have suggested the use of CBZ as a potential molecular marker for tracing sewage inputs to aquatic environments [26,54]. The concentrations found for this compound were between 1.25 and 2.32 ng/L with the exception of site 2, as mentioned. CBZ concentration ranges were in line with the ones found in surface waters from Otonabee (0.7 ng/L) and Lambro Rivers (175.3 ng/L) [55,56].

With regard to FA, significantly lower concentrations were detected in the Douro River when compared with the data obtained from the Rhine River (maximum concentrations of 35 ng/L) [47]. According to the sampling sites distinct concentration values were obtained for SMX, TMP and PHO.

## 4. Conclusions

A SPE-LC–MS/MS method with ion trap detection was developed and validated for the quantification of trimethoprim, propranolol, sulfamethoxazole, carbamazepine, diazepam and the active metabolite of fenofibrate, fenofibric acid. Appropriate recoveries were obtained considering the distinct physical–chemical properties of the selected compounds (40.5–92.2%). The method achieved quantitative limits below 37.0 ng/L and was successfully used in trace environmental analyses.

Matrix evaluations were carried out with two different aqueous samples (estuarine water from the Douro River, Porto, Portugal and spring water from the Monjolinho River, São Carlos, SP, Brazil) by two complementary approaches (post-column and post-extraction methods). The results of these experiments have demonstrated that the signals of the compounds can be affected differently by the ion suppression caused by the matrix, demonstrating the need to overcome this drawback, common in many environmental works, by using alternative sample preparation strategies. In this LC-ion trap MS/MS method, two MS/MS transitions were monitored according to European Commission Decision 2002/657/EC, allowing the reliable confirmation of compound identity and avoiding false positive results. This fact represents an advantage when

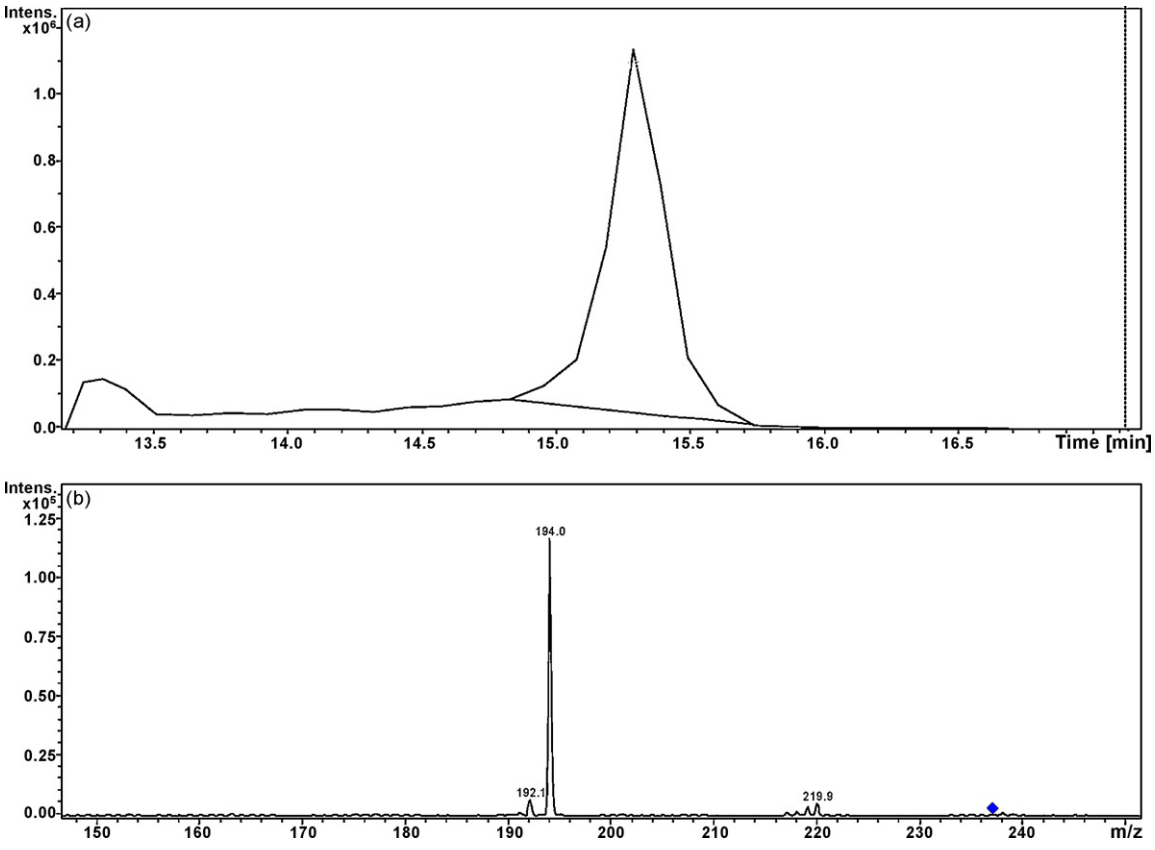



**Fig. 6.** Example of a positive sample for CBZ (178 ng/L): (a) extracted ion chromatogram and (b) MS/MS spectra.

compared with other methods of LC–MS or LC–MS/MS that used only one monitoring transition. Further to the known confirmatory capacity of the IT spectrometer, this work demonstrated its quantitative ability for the analysis of a broad mixture of pharmaceuticals in complex aqueous environmental matrices. The applicability of the developed method was demonstrated through the analysis of several estuarine water samples from the Douro River and the data obtained revealed the occurrence (ng/L) of almost all pharmaceuticals, with CBZ and SMX being the most frequently detected.

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## References

[1] K.G. Karthikeyan, M.T. Meyer, Sci. Total Environ. 361 (2006) 196.
[2] F. Comeau, C. Surette, G.L. Brun, R. Losier, Sci. Total Environ. 396 (2008) 132.
[3] A. Togola, H. Budzinski, J. Chromatogr. A 1177 (2008) 150.
[4] S.D. Kim, J. Cho, I.S. Kim, B.J. Vanderford, S.A. Snyder, Water Res. 41 (2007) 1013.
[5] J.H. Al-Rifai, C.L. Gabelish, A.I. Schafer, Chemosphere 69 (2007) 803.
[6] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Environ. Sci. Technol. 36 (2002) 1202.
[7] K. Choi, Y. Kim, J. Park, C.K. Park, M. Kim, H.S. Kim, P. Kim, Sci. Total Environ. 405 (2008) 120.
[8] J.M. Conley, S.J. Symes, M.S. Schorr, S.M. Richards, Chemosphere 73 (2008) 1178.
[9] M.J. Benotti, B.J. Brownawell, Environ. Sci. Technol. 41 (2007) 5795.
[10] C.G. Daughton, T.A. Ternes, Environ. Health Perspect. 107 (1999) 907.
[11] T.A. Ternes, Water Res. 32 (1998) 3245.
[12] N.M. Vieno, T. Tuhkanen, L. Kronberg, J. Chromatogr. A 1134 (2006) 101.
[13] F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhornb, J. Chromatogr. A 938 (2001) 199.
[14] Z. Ye, H.S. Weinberg, M.T. Meyer, Anal. Chem. 79 (2007) 1135.
[15] A. Kot-Wasik, J. Debska, J. Namiesnik, TrAC Trends Anal. Chem. 26 (2007) 557.
[16] W.M.A. Niessen, J. Chromatogr. A 1000 (2003) 413.
[17] D. Barceló, M. Petrovic, TrAC Trends Anal. Chem. 26 (2007) 2.
[18] M. Farré, M. Gros, B. Hernández, M. Petrovic, P. Hancock, D. Barceló, Rapid Commun. Mass Spectrom. 22 (2008) 41.
[19] M.S. Díaz-Cruz, M.J. García-Galán, D. Barceló, J. Chromatogr. A 1193 (2008) 50.
[20] Z.L. Zhang, J.L. Zhou, J. Chromatogr. A 1154 (2007) 205.
[21] M. Gros, M. Petrovic, D. Barceló, Talanta 70 (2006) 678.
[22] J. Feitosa-Felizzola, B. Temime, S. Chiron, J. Chromatogr. A 1164 (2007) 95.
[23] S. Yang, K.H. Carlson, J. Chromatogr. A 1038 (2004) 141.
[24] V. Andreu, Y. Pico, Anal. Chem. 76 (2004) 2878.
[25] A.L. Batt, D.S. Aga, Anal. Chem. 77 (2005) 2940.
[26] M. Clara, B. Strenn, N. Kreuzinger, Water Res. 38 (2004) 947.
[27] S. Souverain, S. Rudaz, J.-L. Veuthey, J. Chromatogr. A 1058 (2004) 61.
[28] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[29] T. Benijts, R. Dams, W. Lambert, A. De Leenheer, J. Chromatogr. A 1029 (2004) 153.
[30] Commission Decision (2002/657/EC) of 12 August 2002, Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Communities L221, Brussels, Belgium, pp. 8–36.
[31] F. Hernández, J.V. Sancho, M. Ibáñez, C. Guerrero, TrAC Trends Anal. Chem. 26 (2007) 466.
[32] O.J. Pozo, J.V. Sancho, M. Ibáñez, F. Hernández, W.M.A. Niessen, TrAC Trends Anal. Chem. 25 (2006) 1030.
[33] A. El-Gindy, S. Emara, M.K. Mesbah, G.M. Hadad, Il Farmaco 60 (2005) 425.
[34] N.P. Rath, W. Haq, G.K. Balendiran, Acta Crystallogr., Sect. C: Cryst. Struct. Commun. 61 (2005) o81.
[35] C. Ribeiro, M.E. Tiritan, E. Rocha, M.J. Rocha, Arch. Environ. Contam. Toxicol. 56 (2009) 1.
[36] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[37] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[38] U.S. Food and Drug Administration, Bioanalytical method validation, in: Guidance for Industry, 2001, pp. 1–22.
[39] T.V. Madureira, M.J. Rocha, Q.B. Cass, M.E. Tiritan, J. Chromatogr. Sci., in press.
[40] J.M. Conley, S.J. Symes, S.A. Kindelberger, S.M. Richards, J. Chromatogr. A 1185 (2008) 206.
[41] E. Botitsi, C. Frosyni, D. Tsipi, Anal. Bioanal. Chem. 387 (2007) 1317.
[42] M. Gros, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. 386 (2006) 941.
[43] C. Nebot, S.W. Gibb, K.G. Boyd, Anal. Chim. Acta 598 (2007) 87.
[44] M.J. Gómez, M. Petrovic, A.R. Fernandez-Alba, D. Barceló, J. Chromatogr. A 1114 (2006) 224.
[45] M. Petrovic, M.D. Hernando, M.S. Díaz-Cruz, D. Barceló, J. Chromatogr. A 1067 (2005) 1.
[46] T.L. Laurito, G.D. Mendes, V. Santagada, G. Caliendo, M.E.A.d. Moraes, G.D. Nucci, J. Mass Spectrom. 39 (2004) 168.
[47] F. Sacher, M. Ehmann, S. Gabriel, C. Graf, H.-J. Brauch, J. Environ. Monit. 10 (2008) 664.
[48] F.C. Wu, R.D. Evans, P.J. Dillon, Y.R. Cai, Appl. Geochem. 22 (2007) 1598.
[49] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, J. Chromatogr. A 1161 (2007) 132.
[50] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Talanta 74 (2008) 1299.
[51] J.E. Renew, C.H. Huang, J. Chromatogr. A 1042 (2004) 113.
[52] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[53] A. Kloepfer, J.B. Quintana, T. Reemtsma, J. Chromatogr. A 1067 (2005) 153.
[54] N. Nakada, K. Kiri, H. Shinohara, A. Harada, K. Kuroda, S. Takizawa, H. Takada, Environ. Sci. Technol. 42 (2008) 6347.
[55] X.S. Miao, C.D. Metcalfe, Anal. Chem. 75 (2003) 3731.
[56] E. Zuccato, S. Castiglioni, R. Fanelli, J. Hazard. Mater. 122 (2005) 205.