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A column-switching method for quantification of the enantiomers of omeprazole in native matrices of waste and estuarine water samples

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ABSTRACT

This work reports the use of a two-dimensional liquid chromatography (2D-LC) system for quantification of the enantiomers of omeprazole in distinct native aqueous matrices. An octyl restricted-access media bovine serum albumin column (RAM-BSA C_8) was used in the first dimension, while a polysaccharide-based chiral column was used in the second dimension with either ultraviolet (UV–vis) or ion-trap tandem mass spectrometry (IT-MS/MS) detection. An in-line configuration was employed to assess the exclusion capacity of the RAM-BSA columns to humic substances. The excluded macromolecules had a molecular mass in the order of 18 kDa. Good selectivity, extraction efficiency, accuracy, and precision were achieved employing a very small amount (500 μL or 1.00 mL) of native water sample per injection, with detection limits of 5.00 $\mu g\,L^{-1}$, using UV–vis, and 0.0250 $\mu g\,L^{-1}$, using IT-MS/MS. The total analysis time was only 35 min, with no time spent on sample preparation. The methods were successfully applied to analyze a series of waste and estuarine water samples. The enantiomers were detected in an estuarine water sample collected from the Douro River estuary (Portugal) and in an influent sample from the wastewater treatment plant (WWTP) of São Carlos (Brazil). As far as we are concerned, this is the first report of the occurrence of (+)-omeprazole and (−)-omeprazole in native aqueous matrices.

1. Introduction

Sample preparation previous to instrumental analysis is a crucial step for establishing a selective and sensitive chromatographic method for trace analysis in complex matrices. Different sample extraction methods and preparation techniques are often involved in the pre-treatment of complex matrices, such as biological and environmental samples [1–3]. In order to improve this type of analysis, faster analytical methods have been developed with concomitant higher sensitivity and selectivity. A large number of extraction techniques for enhancing sensitivity, selectivity, and sample cleanup have also been developed mainly in the field of solid-phase extraction (SPE), such as multifunctionalized sorbents [4,5]. However, SPE is generally employed in an off-line mode and presents some drawbacks, such as long analysis time, use of high amounts of organic solvents, and the generation of waste cartridges; furthermore, it leads to the use of large volumes of samples, especially in environmental analyses [6,7].

In order to achieve automatization, a large number of different restricted-access media (RAM) supports, such as alkyl-diol-silica (ADS), internal surface reversed phase (ISRP), semi-permeable surface (SPS), shielded hydrophobic phase (SHP), mixed-function phase (MFP), and protein-coated silica, have been developed to allow the direct injection of biological fluids and food samples into liquid chromatography systems (LC) [8–10]. However, only few works reported the use of RAM supports for environmental samples [11–13]. In a recent work, a RAM molecularly imprinted polymer (MIP) column with a large injection volume (50 mL) was used for the analysis of pharmaceuticals in river water [14], while in a previous work developed by Chico et al. [15] a pre-concentration step using SPE was employed before the injection into the RAM column. Ding et al. [16] achieved high sensitivity in a LC–MS method for the analysis of macrolide antibiotics using a RAM column in the backflush mode, leading to an injection volume of only 1 mL.

RAM supports allow the extraction/concentration of the analytes through a combination of size exclusion and conventional hydrophobic or ion-exchange interactions, promoting the exclusion of macromolecules while retaining micromolecules [2]. Thus, compounds of low molecular mass are extracted and enriched, into the pore phase, whereas the outer surface of the particles has a

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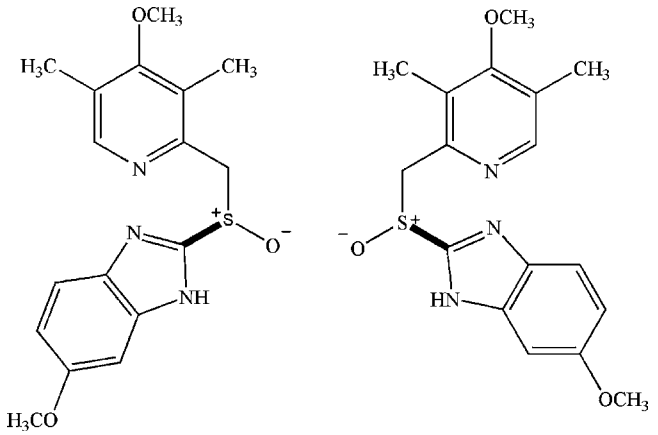

Fig. 1. Chemical structures of the enantiomers of omeprazole.

special topochemistry to prevent adsorption of large molecules, such as humic substances from environmental water matrices, thus allowing their exclusion in the void volume [15].

In this work we evaluated the exclusion capacity of RAM-BSA C_8 and C_{18} columns. For this, environmental water samples and certified standards of aquatic humic substances were analyzed using RAM columns; the results were compared to the ones obtained by the use of a high-performance size-exclusion chromatography column (HPSEC). The RAM-BSA C_8 column was used in the first dimension of a 2D-LC system for sample cleanup, while a polysaccharide-based chiral column was used in the second dimension for the enantioselective separation of omeprazole (OME) (Fig. 1).

The interest in developing methods for the determination of chiral pharmaceuticals in the environment is due to the fact that these compounds are now an important issue in the design, discovery, and development of new drugs. Although stereochemistry plays an important role in pharmacology, a large number of chiral drugs under clinical use are still racemic mixtures. The advances in environmental chiral analysis led to a new awareness of the importance of stereoselective behaviors and the fate of chiral drugs [17–20].

In this paper we report the development and validation of, as far as we know, the first procedure for the quantification of the enantiomers of omeprazole in environmental native water matrices by LC with an achiral–chiral column-switching approach, using either UV–vis or IT-MS/MS detection.

2. Experimental

2.1. Chemicals and equipments

All the organic solvents were LC grade from Mallinckrodt Baker (St. Louis, MO, USA). The water used for the mobile phase was purified through a Milli-Q system (Millipore, São Paulo, Brazil). Bovine serum albumin (fraction V powder, minimum 98%) was purchased from Sigma (St. Louis, MO, USA). Sodium polystyrene sulfonates (Polymer Laboratories, Amherst, MA, USA) were used as molecular-mass calibration standards (8, 18, 46 and 100 kDa). Nylon membranes (47 mm i.d. × 0.45 μm, Millipore, São Paulo, Brazil) were used to filter all the mobile phases and water samples. Glutaraldehyde, potassium dihydrogen phosphate, and sodium borohydride were from Merck (Darmstadt, Germany). Omeprazole was generously donated by LIBBS (São Paulo, SP, Brazil). All other reagents were of analytical grade. The mobile phases were prepared in a volume/volume ratio.

Two LC systems were used. The first equipment consisted of two Shimadzu LC-10 ATVP pumps (Kyoto, Japan), with one of the pumps having a FCV-10AL valve for selecting solvent, a SIL-10ADVP autosampler with a 500 μL loop, a DGU-14A degasser, a SPD-10A UV-vis detector, and a SCL-10AVP interface. A LC 7000 Nitronic EA (Sulpelco, St. Louis, MO, USA) six-port valve was used for the automated column-switching. Data acquisition was done using a Shimadzu CLASS-VP software. The second LC system had two Shimadzu LC-20AD pumps (Kyoto, Japan), a SIL-20A autosampler with a 2.0 mL loop, a DGU-20A5 degasser and a CBM-20A interface. The automated column-switching system was also a LC 7000 Nitronic EA six-port valve, and an Esquire 6000 IT mass spectrometer (Bruker Daltonics, Germany) equipped with an ESI source, operating in a positive mode. Data acquisition was carried out using the Data Analysis software (Bruker Daltonics, Germany).

A Total Organic Carbon Analyzer – TOC VCPH Shimadzu was used in the TOC analyses.

2.2. Chromatographic columns

The chiral phase tris-(3,5-dimethylphenylcarbamate) of amylose coated onto APS-Nucleosil (500 Å, 7 μm, 20%, w/w, 150 mm × 4.6 mm i.d.) (CSP) was prepared as described elsewhere [21,22]. The RAM-BSA columns (50 mm × 4.6 mm i.d.) using silica octyl and octadecyl Luna® (10 μm particle size and 100 Å pore size) were prepared as before [23], based on the protocol previously described by Menezes and Felix [24].

An analytical Tsk-Gel® column (Tosoh Bioscience, G3000PW$_{XL}$, 300 mm × 7.8 mm i.d., and 6.0 μm particle size) was used to evaluate the exclusion of aquatic humic substances from the collected water samples.

2.3. Standard solution and spiked sample preparation

A 200 mg L^{-1} stock solution of omeprazole (OME) (±)-(6-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl)-1*H*-benzo[*d*]imidazole] (200 mg L^{-1}) was prepared and diluted to 20 μg mL^{-1} in methanol for the LC–UV–vis method; from which a stock solution of 1000 μg L^{-1} was also prepared for the LC–IT-MS/MS method. Using the appropriate stock solution, two sets of standard working solutions for calibration and two sets for quality controls (QC) were prepared with the following concentrations: 12,800, 6,400, 3,200, 1,600, 800, 400, 300 μg L^{-1}, and 360, 6,000, 10,000 μg L^{-1}, respectively (LC–UV–vis); 8.00, 6.00, 4.00, 3.00, 2.00, 1.50, 1.00 μg L^{-1}, and 1.20, 4.80, 6.40 μg L^{-1} (LC–IT-MS/MS). All stock and working solutions were stable during 2 months when stored at 4 °C in amber bottles; no evidence of degradation of the analytes was observed in the chromatograms.

To prepare the calibration standards and quality control samples, either 100 or 200 μL aliquots of the appropriate standard working solutions were placed in a series of test tubes and the solvent was evaporated to dryness under a nitrogen stream. The dried analytes were reconstituted using either 1.00 mL or 2.00 mL of spring water from the Monjolinho River (São Carlos, Brazil). The solutions were vortex-mixed during 20 s and aliquots of 700 μL or 1500 μL were transferred to autosampler vials from which 500 μL (LC–UV–vis) or 1,000 μL (LC–IT-MS/MS) were injected into the column-switching LC systems.

2.4. Evaluation of RAM-BSA C_8 and C_{18} columns for the exclusion of humic substances

The exclusion was evaluated using certified standards of aquatic humic (HAs) and fulvic (FAs) acids (IHSS, International Humic Substance Society, donated by Embrapa-CNPDIA – São Carlos). Ultrapure water was used to prepare 1.00 mg L^{-1} solutions of HAs and FAs; the pH was adjusted to 8.2 with NaOH or HNO_3. A phosphate buffer (pH 6.8, 0.1 M NaCl) was used as mobile phase at a

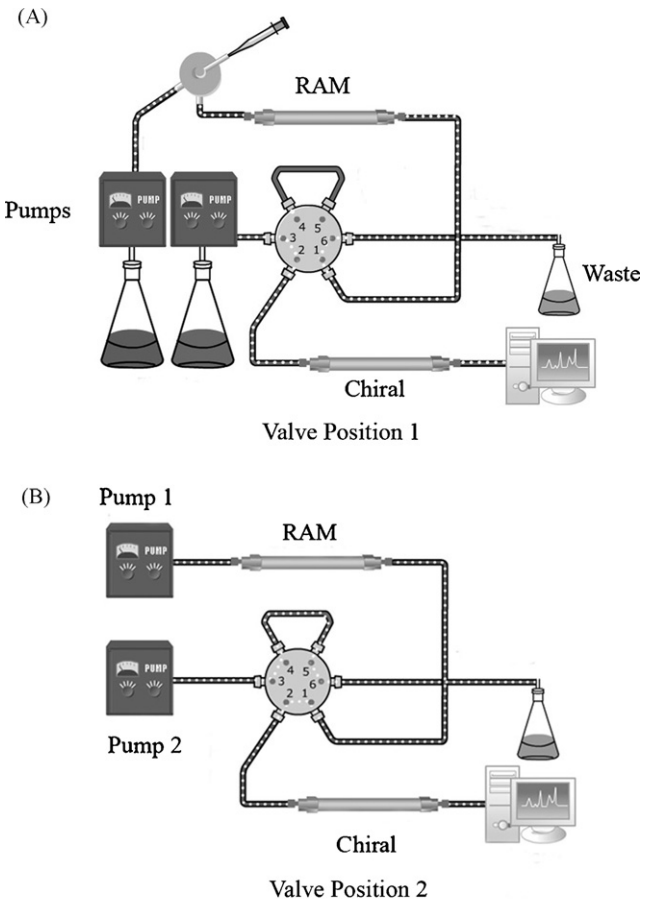

Fig. 2. Schematic diagram of the column-switching system: (A) valve position 1 and (B) valve position 2.

flow rate of 0.5 mL min^{-1}. Sample aliquots of 500 μL were injected and all analyses were done in triplicate; the areas obtained after the direct injection of standards and water samples in the HPSEC column (Tsk-gel® G3000PW$_{XL}$) were compared to those obtained from the RAM-BSA (C_8 or C_{18}) in-line with the HPSEC column.

2.5. Column-switching procedure and analysis conditions

The column-switching systems used for coupling the RAM and the chiral columns are illustrated in Fig. 2. The time sequence used is listed in Table 1. For the IT-MS/MS detection, the flow rate of the mobile phase was split into the source at 100 μL min^{-1} by means of a T-piece. The optimization of the ionization source, voltages on the lenses, and trap conditions was achieved with the expert tune mode of the Daltonics Esquire control software, as described by Madureira et al. [25]. The IT-MS/MS parameters for the analysis were the following: nebulizer pressure, 30 psi; drying gas flow, 8.0 L min^{-1}; temperature, 325 °C; capillary voltage, 3.5 kV; fragmentation amplitude, 0.27 V. The enantiomeric elution order was determined at the established chromatographic conditions using a JASCO CD-2095 plus chiral detector at λ_{max} = 302 nm. For that, 20 μL of a 200 mg mL^{-1} OME solution in ultrapure water were injected into the chiral column. All LC analyses were done at room temperature (±25 °C). The total time of analysis was 35 min, without any additional step for sample preparation.

2.6. Method validation

The two methods were validated in accordance with internationally accepted criteria [26]. The linearity was evaluated using external calibration curves with calibration levels for each enantiomer prepared in triplicate.

The intra- and inter-day precisions of the method were determined by the analysis of three QC samples. Five samples of each concentration were prepared in Monjolinho spring water. The accuracy was evaluated by back-calculation and expressed as the percentage of deviation between the amount found and the amount added at the three concentrations examined.

The extraction transfer efficiency of each enantiomer was measured using the three QC samples. The percentage of recovery was obtained comparing the peak-area ratios of QC samples to the ones prepared at the same concentration in ultrapure water.

The LOD and LOQ values were determined from spiked water samples and were assumed as the minimum detectable amount of OME, with a signal-to-noise (S/N) ratio of 3 for the LOD. The LOQ was the lowest calibration level, and the accepted criterion for the limit of quantification was that the precision and accuracy for the three samples should have a coefficient of variation (CV) ≤ 20%.

The chemical stability of OME was evaluated using three QC samples, at room temperature, as freshly prepared samples, and after 24, 48 and 72 h (autosampler stability). A CV of less than 15% was the criterion for the stability evaluation [26].

Matrix effects were evaluated by on-line post-column infusion and by on-line extraction using the column-switching system with samples prepared with ultrapure water (Milli-Q) and Monjolinho spring water. In the post-column infusion, 1.0 mL of each water sample was injected into the RAM-BSA column at the established chromatographic conditions (Table 1). A 100 ng mL^{-1} OME solution was infused using a syringe pump at a flow rate of 10 μL min^{-1}, after the chiral column and before the mass spectrometer ionization source [27]. For the on-line extraction procedure, Monjolinho spring water and ultrapure water were spiked with OME (100 ng mL^{-1}). The comparison of the peak areas obtained from the spring water and the ultrapure water samples were used to evaluate the enhancement or ion suppression effect.

Table 1
Time events for the switching of columns and of mobile phases for both methods.

Event	Valve position	Pump	UV–vis Time (min)	IT Time (min)
Humic substances are excluded by RAM column	1	Pump 1 (eluent A)	0.00–3.00	0.00–4.00
Conditioning of the chiral column	1	Pump 2 (eluent D)		
Elution of retained components on the RAM	1	Pump 1 (eluent B)	3.01–7.60	4.00–11.2
Analytes are transferred to the chiral column	2	Pump 1 (eluent B)	6.70–7.60	10.0–11.2
Analysis of the OME enantiomers	1	Pump 2 (eluent D)	7.61–35.0	11.2–35.0
Washing of RAM column	1	Pump 1 (eluent C)	7.61–13.0	11.2–16.2
Conditioning of RAM column	1	Pump 1 (eluent A)	13.0–35.0	16.2–35.0

Pump 1: eluents (A) H_2O; (B) CH_3CN:H_2O (35:65, v/v); (C) CH_3CN:H_2O (80:20, v/v), flow rate: 1.0 mL min^{-1}. Pump 2: eluent (D) CH_3CN:H_2O (35:65, v/v), flow rate: 1.0 mL min^{-1}. λ: 302 nm.

2.7. Site selection and sampling

Water samples were collected along the Monjolinho River in the region of São Carlos, SP, Brazil, in September 2008, at the end of the Winter season, and also in October 2009, in the beginning of the Spring season. Site (1), with latitude 22°00′33″S and longitude 47°50′07″W, refers to the spring water of the Monjolinho River used as the blank matrix. The other sampling sites (2–4) are located in areas considered to be susceptible to human and industrial contamination: latitude 22°01′19.5″S, longitude 47°54′50.3″W (2); and agricultural run-off: latitude 22°00′33″S, longitude 47°50′07″ W (3), and latitude 21°59′25.2″S, longitude: 47°53′29.4″W (4). Regarding the samples collected in October 2009, two additional samples were included, collected at the influent and the effluent of the wastewater treatment plant (WWTP) of São Carlos: latitude 22°3′41.99″S, longitude: 47°55′29.22″W, and latitude 22°3′30.69″S, longitude 47°55′51.66″W, respectively.

Estuarine surface waters were collected along the Douro River estuary (Portugal), in June 2008, in six sampling sites located within the most urbanized estuarine area. The sampling sites 1 (latitude 41°08′44.64″N, longitude 8°38′37.02″W), 2 (latitude 41°08′18.54″N, longitude 8°37′14.34″W), and 3 (latitude 41°08′17.88″N, longitude 8°36′45.84″W) were located bordering Gaia city, an industrialized and densely inhabited district, while sampling sites 4 (latitude 41°08′24.66″N, longitude 8°36′43.08″W), 5 (latitude 41°08′45.96″N, longitude 8°37′55.80″W), and 6 (latitude 41°08′47.22″N, longitude 8°39′30.96″W) were located at the opposite side, on the northern bank of the river.

The wastewater samples, collected directly from the discharges along the Monjolinho River (2–4) and from the influent and effluent samples from the WWTP, were stored in 1 L amber glass bottles pre-rinsed with ultrapure water. Upon collection, samples were kept in ice, transported to the laboratory, and then vacuum filtered through 0.45 μm nylon membrane glass fiber filters, to remove suspended particles, before being stored at 4 °C. The surface estuarine waters (500 mL) were sampled from a depth of approximately 1 m using a peristaltic sampler pump (Global Water, Model: WS300, California, USA) into 500 mL pre-rinsed amber glass bottles.

3. Results and discussion

3.1. Evaluation of RAM-BSA C_8 and C_{18} columns for the exclusion of humic substances

Although, a series of works have described the use of RAM-based column for sample cleanup of native water samples, none of them reports a method to evaluate in depth the efficiency of humic substances exclusion by the used RAM columns. Based on the work of Wu et al. [28] that fractionated humic substances using a HPSEC column, the exclusion capacity of the RAM-BSA columns for the analysis of native water matrices was evaluated in-line with a Tsk-gel® column. The samples used had TOC values of 90.7 mg L^{-1} for the wastewater (site 3) and of 27.5 mg L^{-1} for the estuarine water (site 1). Fig. 3(A) shows similar chromatographic profiles of wastewater, estuarine water, and a mixture of the humic standards (HAs and FAs in the concentration of 1.00 mg L^{-1} each one) in the HPSEC column. This fact indicates that the selected Tsk-gel® column was appropriate to evaluate the exclusion of humic substances by the RAM columns (C_8 and C_{18}). The percentage of exclusion by the RAM column used (Fig. 3(A)) was determined from the comparison between the areas obtained after direct injection of wastewater and estuarine water in the RAM-BSA columns in-line with the HPSEC column (Fig. 3(B)) and the areas obtained from the injections in the HPSEC column alone.

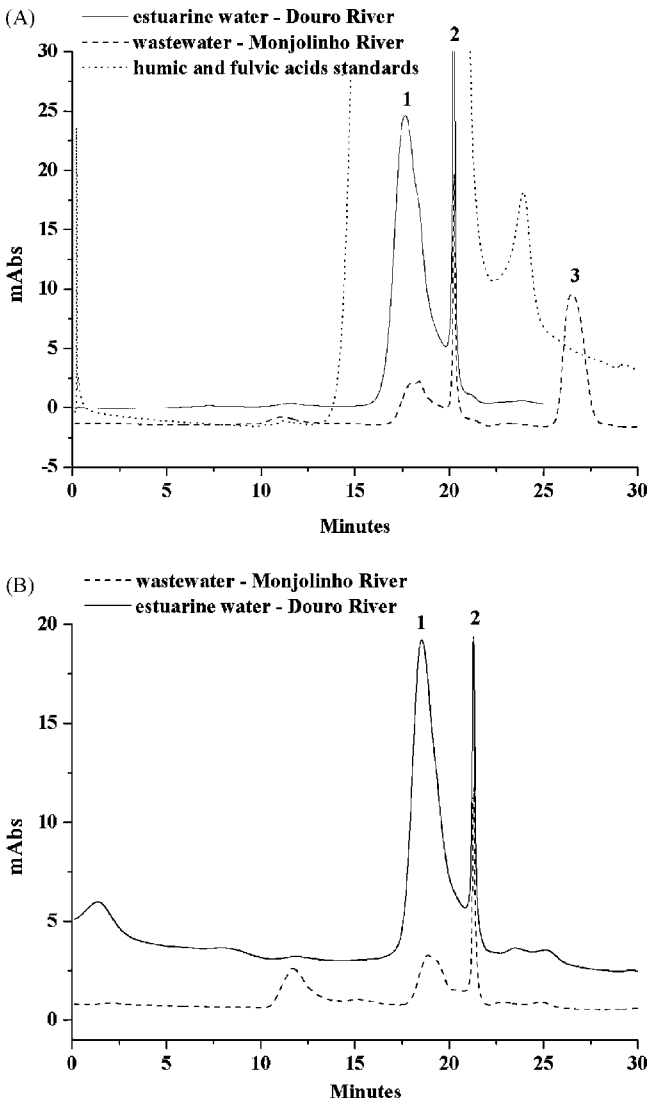

Fig. 3. Representative chromatograms (A) of estuarine and wastewater samples and humic and fulvic acids standards in the HPSEC; (B) obtained after direct injection of estuarine and wastewater in a RAM-BSA C_8 column in-line with HPSEC. Chromatographic conditions: phosphate buffer (0.1 mol L^{-1}; pH 6.8) as a mobile phase at a flow-rate of 0.5 mL min^{-1} with detection at 250 nm, and injection volume of 500 μL.

Higher percentages of exclusion were obtained for the estuarine water with both RAM columns (76.6%—RAM-BSA C_8 and 82.1%—RAM-BSA C_{18}), compared to the results obtained for the wastewater samples (64.0%—RAM-BSA C_8 and 61.4%—RAM-BSA C_{18}). This might be related to the compositions of the used waste and estuarine water. This statement is supported by the observation of a chromatographic band, with a high retention time (band 3), corresponding to organic matter with lower molecular mass, which was observed only in the chromatogram for the wastewater sample, Fig. 3 (A).

The molecular masses of the humic substances, excluded by the RAM columns from both natural water samples, were estimated in the order of 18 kDa using sodium polystyrene sulfonates as calibrators.

3.2. Chiral analysis

Polysaccharide-based chiral phases can be used in normal-phase, reverse-phase, and polar organic mode [29–31]. Previous studies in our group proved that these chiral stationary phases are

efficient for the resolution of a series of chiral suphoxides, including the benzoimidazoles (omeprazole, pantoprazole, and lanzoprazole) commonly used as proton-pump inhibitors (PPIs) [30,32,33].

Based on these results, an amylose CSP was selected for the separation of the enantiomers of omeprazole in aqueous samples under reversed-phase mode by multidimensional liquid chromatography. Thus, the retention factor (k), enantioselectivity (α), and enantioresolution (Rs) of omeprazole were evaluated in reversed-phase elution mode using the CSP column. High selectivity ($\alpha = 2.37$) and resolution (Rs = 1.95) were obtained with low retention times when CH_3CN:H_2O (35:65, v/v) was used as organic modifier.

The benzoimidazoles are extensively metabolized in the liver via the cytochrome P450 enzyme system, which exhibits polymorphic metabolism in humans and thus justifies the great interest for quantification of the separated enantiomers in the biota. Furthermore, AstraZeneca has carried out the chiral switch of omeprazole to its (*S*)-(−)-enantiomer under the trade name of Nexium®, launched in the beginning of this decade [34–36].

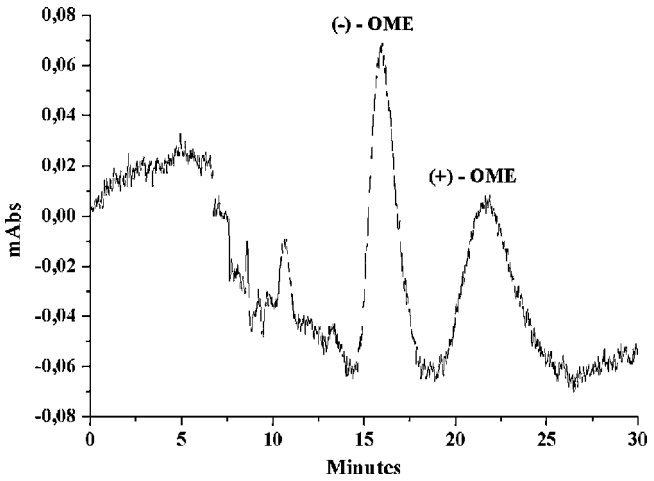

Fig. 4. Representative chromatograms of a sample collected at site 6 of the Douro River estuary.

3.3. Method development

The insight for the application of a 2D chromatographic system in the analysis of environmental native water samples was brought on by the results of previous works on the analyses of enantiomers of PPIs in human plasma [34–36]. Furthermore, RAM-BSA coupled to polysaccharide-based chiral columns has been successfully employed in methods for the quantification of a diverse number of racemic mixtures [34–39].

The use of large sample volumes is usually required to achieve the necessary sensitivity in environmental water analyses. This was the main concern while transferring the methods developed for the analyses of these PPIs from biological fluids to waste and estuarine water samples.

The large content of organic matter associated with the low concentrations (ng L^{-1} to μg L^{-1}) of pharmaceutical compounds in aquatic environments makes pre-concentration a crucial step. The high capability of the RAM-BSA columns to exclude humic substances, as demonstrated by the in-line approach described, indicates that small volumes of native water samples can be used. Thus, different injection volumes were investigated and thus the amount of 500 μL was selected. The selection of the appropriated band transfer time from the first to the second dimension was also

Table 2
Linearity parameters, detection and quantification limits for the method.

Compound	UV–vis					IT-MS/MS				
	Range (μg L^{-1})	Calibration equation	r	LOD (μg L^{-1})	LOQ (μg L^{-1})	Range	Calibration equation	r	LOD (μg L^{-1})	LOQ (μg L^{-1})
(+) OME	15.0–640	$y = 1.30E6x + 2.72E3$	0.999	5.00	15.0	0.0500–0.400	$y = 1.95E7x - 2.34E4$	0.998	0.0250	0.0500
(−) OME		$y = 1.39E6x + 1.96E3$					$y = 2.13E7x - 2.14E5$			

Table 3
Accuracy, average of intra-day ($n = 5$), inter-day ($n = 5$), variability and extraction efficiency.

Compound (μg L^{-1}) UV–vis	1st Day[a]		2nd Day[a]		3rd Day[a]		Average (3 days)[b]		Extraction efficiency
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	(%)
(−) OME									
18.0	105	3.05	104	1.70	103	5.21	98.8	5.21	105
300	106	1.25	105	1.30	98.5	4.29	99.5	4.29	106
500	96.9	1.29	90.2	2.00	111	1.31	101	1.31	96.9
(+) OME									
18.0	100	6.45	103	2.80	104	13.8	94.5	13.8	100
300	110	1.34	111	1.30	100	10.5	98.8	10.5	110
500	99.2	1.64	95.7	3.42	112	1.82	96.2	1.82	99.2

Compound (μg L^{-1}) IT-MS/MS	1st Day[a]		2nd Day[a]		3rd Day[a]		Average (3 days)[b]		Extraction efficiency
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	(%)
(−) OME									
0.0600	110	1.27	98.5	2.59	88.2	9.01	98.9	4.29	94.6
0.240	102	9.88	94.2	4.77	77.9	16.1	91.4	10.2	112
0.320	75.6	4.60	84.8	6.09	77.3	3.22	79.2	4.64	92.2
(+) OME									
0.0600	104	6.04	92.6	2.18	84.3	13.2	93.6	7.14	104
0.240	97.9	12.6	91.9	7.84	77.3	17.6	89.0	12.7	91.0
0.320	77.6	4.01	83.5	6.65	76.6	5.12	79.2	5.26	113

[a] $n = 5$.
[b] $n = 15$.

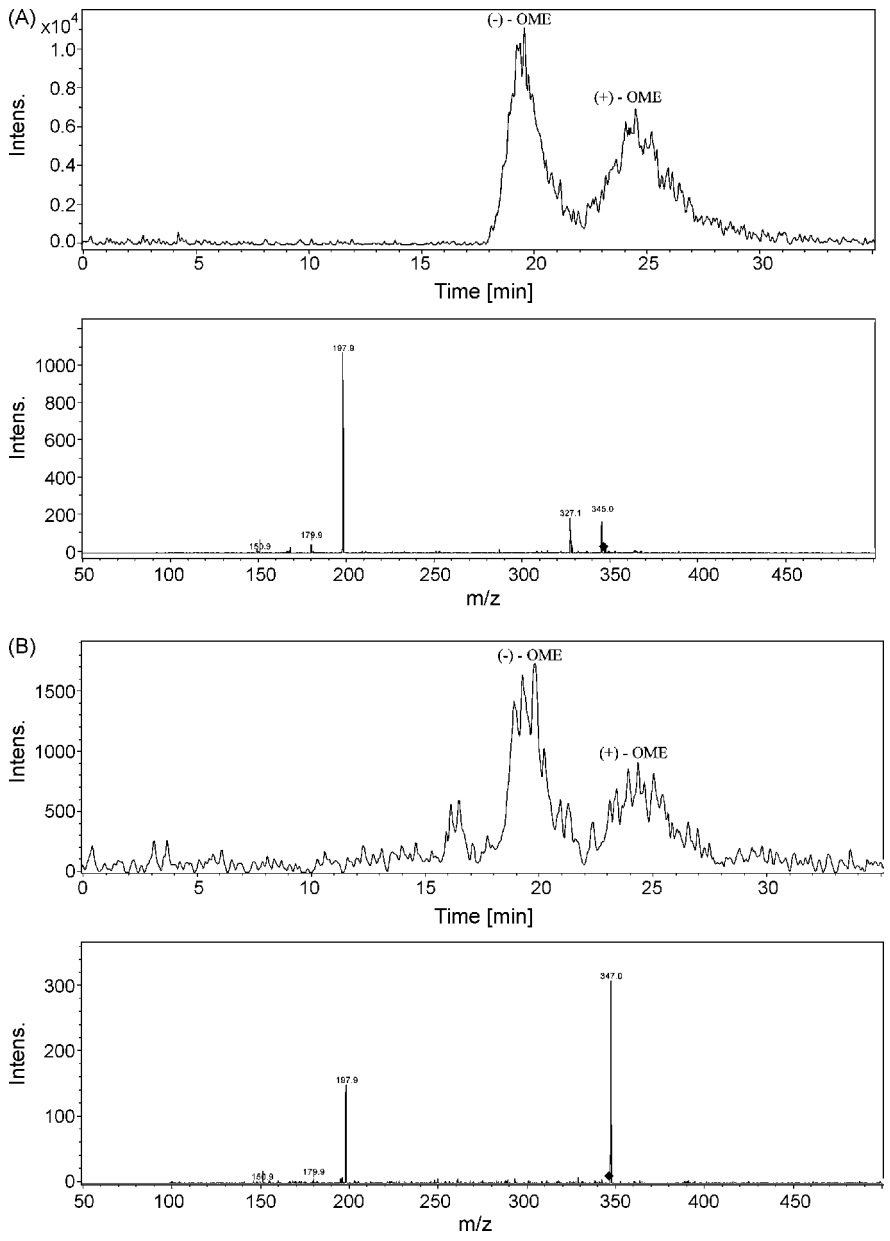

Fig. 5. Extracted ion chromatograms and mass spectra for (A) low QC sample and (B) influent sample.

carefully examined. The direct-flush mode was again maintained to prevent clogging of the chiral column [34–39].

The column-switching system used is schematically illustrated in Fig. 2. First, the chromatographic exclusion profile of humic substances for the blank matrix was evaluated using the selected RAM-BSA C_8 column connected directly to the UV detector (Fig. 4). The chromatographic conditions were optimized with the macromolecules' exclusion time of 3 min, at the flow rate of 1 mL min^{-1}, and a 500 μL sample injection using ultrapure water as the mobile phase (Fig. 2; Table 1—eluent A). Under this condition, OME was retained as a racemic mixture by the RAM column. To estimate the transfer time, a blank matrix was spiked with a high concentration of OME (20.0 mg L^{-1}). The analyte was transferred to the chiral column using CH_3CN:H_2O (35:65, v/v) as solvent. For the cleanup of the RAM column, CH_3CN:H_2O (80:20, v/v) was used as solvent in order to avoid undesired adsorption of humic substances and/or other substances on the column.

The elution order of the enantiomers was determined, under the developed chromatographic conditions, using a circular dichroism detector coupled to an LC multidimensional system. The first enantiomer to elute was (−)-OME and the second one was (+)-OME.

3.4. Method validation

Under the described operating conditions for the LC–UV–vis system, the validation parameters were assessed. The obtained results (see Tables 2 and 3) are all within the accepted criteria of validation and demonstrate the high extraction capability of the RAM-BSA column for sample cleanup procedures. Calibration standards were run sequentially from low to high concentrations, with accuracy and CV for the replicates in the ranges 96.4–105% and 0.130–10.8%, respectively, indicating that no carry over has happened between injections. Moreover, the comparison of the chromatograms for non-spiked and calibration samples showed that no compounds were interfering with the detection of the enantiomers of omeprazol. The LOQ of a method is the lowest amount of the targeted analyte in a sample that can be quantified with a well-defined accuracy [26]. Within the accepted criteria, the LOQ value was

15.0 μg L^{-1} while the LOD value was 5.00 μg L^{-1} for each the enantiomers of omeprazole. These concentration levels are quite high for monitoring drugs in environmental water samples. In addition, the lack of an unambiguous assignment of the LC signals prompted us to fully revalidated the developed method, but now using an LC–IT-MS/MS system and 1.0 mL sample injections.

Matrix effects were investigated by post-column infusion and by on-line sample extraction as described in the experimental section. No significant effect was noticed by the post-column infusion assay, while the on-line sample extraction procedure showed 7% of suppression for (−)-OME and 3% for (+)-OME. These results demonstrate that an effective sample treatment was achieved by the use of the RAM-BSA column, confirming the results obtained by the in-line exclusion assay as discussed in this work.

The OME enantiomers were analyzed in the positive-ion mode (ESI+), while the multiple reaction-monitoring (MRM) mode was carried out for the acquisition. The protonated OME molecular ion $[M+H]^+$ (m/z = 346) was used as the precursor ion. Two MRM transitions were monitored and, based on the EU Commission Decision 2002/657/EC, the MRM ratio and the deviation of the retention time were used to confirm the presence of OME in the samples [40]. The first transition of OME was used for quantification and the second one for confirmatory purposes. The first transition corresponds to the loss of $[M–H_3CO–C_7H_4N_2]^+$ (m/z = 198) and the second one to the loss of $[C_9H_{13}NO]^+$ (m/z = 151). This MS/MS fragmentation pattern is in accordance with the data previously reported for this compound [41].

The figures of merits for the LC–IT-MS/MS method are shown in Tables 2 and 3. The calibration curves were linear in the range 0.0500–0.400 μg L^{-1} for each enantiomer. This is a three-hundred-fold increase of the LOQs when compared with those of the UV–vis detection method. Furthermore, it represents a five-fold increase when compared with the results of a recent published work, which reports as the first calibration standard the value of 0.250 μg L^{-1} for the racemate, using an LC–MS/MS method with the SPE off-line treatment sample approach [42]. Accuracy, inter- and intra-day precision and extraction efficiency were in the range of accepted criteria, especially if one considers that the values obtained were for the QCs replicates and not just for a consecutive sequence of injections of a same sample [26].

The stability of the spiked samples was evaluated by the two methods. In both cases the samples were stable for 48 h in the autosampler at room temperature. As for long-term stability, the spiked samples were stable for 7 days, at 4 °C, as evaluated by the UV–vis detection method; whereas when the LC–IT-MS/MS was used the sample could be considered stable for only 2 days.

3.5. Application to distinct water samples

The wastewater samples collected along the Monjolinho River were analyzed by both methods. Due to time schedule, the samples from the Douro River estuary were analyzed only by the LC–UV–vis method, while the influent and effluent samples of the WWTP were analyzed only by the IT-MS/MS method. For the analyses, new calibration curves were obtained for each sample batch and three QC were injected between samples. The obtained results were in accordance with the accepted validation criteria [40].

The OME enantiomers were not detected in the wastewater samples collected along the Monjolinho River. However, they were detected in a sample collected at site 6 in the Douro River estuary (Fig. 4), located at the lower stretch of this estuary, which corresponds to the most urbanized area. As demonstrated in a previous work of our group, the first 9 km of the estuary are generally most affected by pharmaceutical contaminations and all target pharmaceuticals were found at the highest concentrations in a sampling site located closer to site 6 [25].

The OME enantiomers were detected in the influent sample of the WWTP. Fig. 5(A) and (B) shows the extracted ion chromatogram and the respective mass spectra of the enantiomers for the low QC and the influent sample, respectively. The positive confirmation of OME was based on the MRM transition ratio between m/z = 198 and m/z = 151, which is in accordance with European criteria. In addition, the deviations of the retention times for each enantiomer in the sample were below 2.5% when compared with the standard calibration solutions. The OME enantiomers were not found in the WWTP effluent sample.

It is important to call attention to the fact that the occurrence of OME in influent sewage waters has already been reported [41]. Furthermore, Gracia-Lor et al. [42] recently reported that OME was found in 43% of samples collected in WWTP effluents in a maximum concentration of 0.100 μg L^{-1}.

4. Conclusions

A fully automated liquid chromatographic method was developed for the quantification of the enantiomers of omeprazol, using small amounts of native estuarine and wastewater samples. The high efficiency demonstrated by the RAM-BSA columns allowed the use of small volumes (500 μL or 1.00 mL) of native water samples and indicated that the method can be used in routine analyses. This is a great achievement, especially if one considers the transportation/storage of large volumes of water samples required by other methods. The performance of both columns was maintained over 500 injections of native water samples. An in-line method was also developed and it represents a new tool to be used for assessing the exclusion efficiency of RAM-based columns employed in direct injection of environmental samples.

Furthermore, as far as we know this work reports for the first time the occurrence of (+)-OME and (−)-OME in an estuarine aqueous matrix and in an influent WWTP sample.

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