Development of a SPE–UHPLC–MS/MS methodology for the determination of non-steroidal anti-inflammatory and analgesic pharmaceuticals in seawater

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ABSTRACT

An analytical methodology for the simultaneous determination of seven pharmaceuticals and two metabolites belonging to the non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics therapeutic groups was developed based on off-line solid-phase extraction and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (SPE–UHPLC–MS/MS). Extraction conditions were optimized taking into account parameters like sorbent material, sample volume and sample pH. Method detection limits (MDLs) ranging from 0.02 to 8.18 ng/L were obtained. This methodology was successfully applied to the determination of the selected pharmaceuticals in seawater samples of Atlantic Ocean in the Northern Portuguese coast. All the pharmaceuticals have been detected in the seawater samples, with pharmaceuticals like ibuprofen, acetaminophen, ketoprofen and the metabolite hydroxyibuprofen being the most frequently detected at concentrations that can reach some hundreds of ng/L.

1. Introduction

Pharmaceuticals have been recognized as important emerging environmental contaminants [1], being their occurrence reported in different environmental compartments, including surface waters [2], wastewaters [3], groundwater [4], soils [5] and, even drinking water [6], at trace levels (nanograms to few micrograms per liter). Pharmaceuticals can be released into the environment either as parent compounds or as metabolites; therefore monitoring studies should include not only the parent compound but also their metabolites and transformation products [7].

Non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics are among the pharmaceuticals most consumed worldwide, either as prescribed or over-the-counter medicines. Thus, nowadays, they are frequently detected in the environment, posing a risk to aquatic organisms [8]. For instance, cytological changes in liver, kidney and gills of fish species were described when exposed to diclofenac [9,10], while ibuprofen affected the spawning process of Japanese killifish [11]. Notwithstanding that regulatory guidance to assess the presence of pharmaceuticals in the aquatic environment is giving the first steps with the Directive 2013/39/EU [12], amending the Water Framework Directive (2000/60/EC) and the directive 2008/105/EC, establishing the creation of a Watch list, which will include three pharmaceuticals, namely the sex hormones 17alpha-ethinylestradiol and 17beta-estradiol, and the NSAID diclofenac, in order to gather monitoring data that will facilitate the determination of appropriate measures to establish the risk posed by these kind of contaminants.

Advances in analytical technology have been a key factor for the detection of pharmaceuticals, their metabolites, and transformation products in environmental matrices [13]. Presently, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is the analytical technique of choice for the determination of pharmaceuticals in environmental samples, due to its high selectivity and sensitivity, allowing the detection of compounds at levels of just a few ng/L or less [14]. At the same time, improvements in HPLC technology have been made, and nowadays the trend goes toward the use of ultra-high performance liquid chromatography (UHPLC), which uses short, narrow bore columns, packed with particles with a lower diameter (sub-2 μm). UHPLC allows a faster

analysis, better resolution and narrower peaks comparatively to conventional HPLC methodologies [15].

Since pharmaceuticals are usually present in the aquatic environment at very low concentrations, sample preparation is a very important step in order to improve detection sensitivity and eliminate matrix effects. Solid phase extraction (SPE) is still the most commonly used extraction technique, because it allows simultaneous pre-concentration of analytes and extract clean-up in aqueous matrices [16,17].

Although data on the presence of pharmaceuticals in the aquatic environment is growing, the knowledge of their occurrence in coastal areas is still very sparse, since most of research in marine analytical chemistry focuses on classical lipophilic target compounds like organochlorine pesticides and polycyclic aromatic hydrocarbons [18]. Nevertheless, a large uncertainty exists relatively to the presence of a broad variety of pollutants in seawater, including pharmaceuticals. In fact, there are only a few works focusing the study of these emerging contaminants in seawater, showing principally the presence of antibiotics [19–21], NSAIDs [20,22–24], psychiatric drugs [19,20,22,23], lipid regulators [20,22,24], X-ray contrast agents [19] and β-blockers [20,22,23] in seawater.

The main objective of the present work was to develop an analytical method for the quantification of pharmaceuticals in seawater. Twelve relevant compounds were selected, embracing two therapeutic classes (NSAIDs and analgesics), taking into account their consumption among Portuguese population [25] as well as data obtained in our previous studies [26,27] that pointed out pharmaceuticals like ibuprofen, diclofenac and acetaminophen, as being detected in Portuguese environment at levels able to pose an ecotoxicological risk for aquatic organisms (Table 1). The developed method was successfully applied to seawater samples of Atlantic Ocean collected in the Northern Portuguese coast.

2. Experimental

2.1. Reagents, solvents and materials

Ibuprofen (purity ≥98%), hydroxyibuprofen (VETRANAL[TM] grade), carboxyibuprofen (VETRANAL[TM] grade), acetaminophen (purity ≥98%), *p*-aminophenol (purity ≥99%), acetaminophen glucuronide sodium salt (purity ≥98%), acetylsalicylic acid (purity ≥99%), naproxen (VETRANAL[TM] grade), nimesulide, ketoprofen (purity ≥98%), diclofenac sodium salt and dipyrone (grade: analytical standard) were purchased from Sigma–Aldrich (Spain). The isotopically labeled compound ibuprofen-d3 used as internal standard was also purchased from Sigma–Aldrich.

Acetonitrile and methanol (LC–MS grade) were supplied by J.T. Baker (Deventer, The Netherlands), hydrochloric acid 37% was obtained from Carlo Erba (Rodano, Italy), formic acid (purity ≥98%) was obtained from Merck (Darmstadt, Germany), ammonia 25% was obtained from Panreac (Barcelona, Spain), and ammonium hydroxide solution was purchased from Sigma–Aldrich (Steinhein, Germany). Deionised water was produced using an Elix apparatus (Millipore, Molsheim, France) and ultra-pure water (resistivity of 18.2 MΩ cm) using a Simplicity 185 system (Millipore, Molsheim, France). All chromatographic solvents were filtered through a 0.22 μm nylon membrane filter (Supelco, Bellefonte, PA, USA) and degassed for 15 min in an ultrasonic bath (Sonorex Digital 10P, Bandelin DK 255P, Germany).

Both individual stock standard and isotopically labeled internal standard solutions (at a concentration of 1000 mg/L) were prepared on a weight basis in acetonitrile, except for *p*-aminophenol, acetaminophen glucuronide, naproxen, diclofenac and dipyrone, which were prepared in acetonitrile–methanol (50:50, v/v), since these substances are very slightly soluble in pure acetonitrile and

freely soluble in methanol [30]. For all pharmaceuticals stock standard solutions were stored at −20 °C and renewed every six months. In the case of *p*-aminophenol, a fresh stock standard solution was prepared monthly due to its limited stability.

Working standard solutions, containing all pharmaceuticals, were prepared in acetonitrile–ultra-pure water (30:70, v/v) by mixing appropriate amounts of the stock solutions. These solutions were prepared before each analytical run.

The cartridges used for solid phase extraction (SPE) were Strata-X (200 mg, 3 mL), Strata X-AW (200 mg, 3 mL), Strata-X-A (200 mg, 3 mL) and Strata X-C (200 mg, 3 mL) from Phenomenex (USA).

2.2. Sample collection and pre-treatment

Seawater samples were collected in three beaches (Leça da Palmeira (41°11′24.38″ N, 8°41′40.86″ W), Matosinhos (41°10′34.09″ N, 8°41′40.86″ W) and Azul (Conchinha) (41°12′14.82″ N, 8°42′55.06″ W)) located near Oporto in the Northern Portuguese coast during the bathing season in July 2013. All the beaches belong to Matosinhos municipality, where it is located the second largest artificial Portuguese harbor (Leixões harbor), an oil refinery, and a WWTP that is designed to serve 80,000 population equivalents and has a primary and secondary treatment operating by activated sludge. The treated WWTP effluents are directly discharged into the Atlantic Ocean. Leça River, a river that suffers a strong anthropogenic pressure, also reaches the Atlantic Ocean in Matosinhos coastal area.

Beaches were selected according to the classification of bathing water quality, being beaches A (Leça da Palmeira), B (Matosinhos), and C (Azul (Conchinha)) classified as excellent, good, and sufficient bathing water quality, respectively [31]. Seawater samples were collected in bottles previously rinsed with ultra-pure water and at a depth of at least one meter as grab samples. Samples were kept at 4 °C during the transport to the laboratory. Sampling frequency was determined taking into account the bathing water quality, thus the excellent samples were collected monthly; for good every two weeks, and for sufficient bathing water quality every week. Upon reception in the laboratory, samples were vacuum filtered through 1.2 μm glass microfiber filters (GF/C, Whatman, UK), followed by 0.22 μm nylon membrane filters and stored at −20 °C, until extraction.

2.3. Solid phase extraction

The optimized SPE procedure used Strata-X cartridges (200 mg, 3 mL) from Phenomenex (USA) conditioned with 5 mL of methanol followed by 5 mL of ultra-pure water and 5 mL of ultra-pure water at pH 2. 500 mL of seawater, with pH adjusted to 2 with concentrated HCl, was percolated through the cartridge. Afterwards the cartridge was rinsed with 5 mL of ultra-pure water, and then dried under vacuum for 1 h to remove the excess of water. Elution was performed with 10 mL of methanol. Extracts were evaporated under a gentle stream of nitrogen and reconstituted with 1 mL of acetonitrile–ultra-pure water (30:70, v/v). Finally, 10 μL of an ibuprofen-d3 standard solution was added to obtain a final concentration of 50 mg/L in the extract of the internal standard.

2.4. Liquid chromatography

Chromatographic separation was carried out with a UHPLC–MS/MS system consisting of a Shimadzu LCMS-8030 triple-quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) operated in the electrospray ionization (ESI) mode, and a Shimadzu Nexera UHPLC system (Shimadzu Corporation, Kyoto, Japan) which consisted of two solvent delivery modules LC-30 AD, a column oven CTO-20 AC, and an autosampler SIL-30

Table 1

Chemical structures and physicochemical properties of the selected pharmaceuticals.

Compound	Chemical structure	Formula	Molecular weight (g/mol)	pKa[c]	Log P[c]
Dipyrone		$C_{13}H_{16}N_3NaO_4S$	333.37	4.85	−0.82
Acetaminophen glucuronide[a]		$C_{14}H_{17}NO_8$	327.29	3.17; 12.22	−1.04
Acetylsalicylic acid		$C_9H_8O_4$	180.16	3.41	1.24
Acetaminophen		$C_8H_9NO_2$	151.16	9.46	0.91
Carboxyibuprofen[a]		$C_{13}H_{16}O_4$	236.26	3.97[d]	2.78[d]
Hydroxyibuprofen[a]		$C_{13}H_{18}O_3$	222.28	4.63[d]	2.37[d]
p-Aminophenol[b]		C_6H_7NO	109.13	5.43; 10.40	0.84
Ketoprofen		$C_{16}H_{14}O_3$	254.28	3.88	3.61
Naproxen		$C_{14}H_{14}O_3$	230.26	4.19	2.99
Nimesulide		$C_{13}H_{12}N_2O_5S$	308.31	6.86	1.79
Diclofenac		$C_{14}H_{11}Cl_2NO_2$	296.15	4.00	4.26
Ibuprofen		$C_{13}H_{18}O_2$	206.28	4.85	3.84

[a] Metabolite.
[b] Degradation product.
[c] Adapted from Ref. [28].
[d] Ref. [29].

Table 2
UHPLC–ESI–MS/MS conditions for the selected pharmaceuticals.

Compound	Rt (min)	Precursor ion (*m/z*)	Product ion (quantifier)				Product ion (qualifier)				Ion ratio (± SD) (*n* = 6)
			Q3	Q1 Pre bias (V)	CE (V)	Q3 Pre bias (V)	Q3	Q1 Pre bias (V)	CE (V)	Q3 Pre bias (V)	
Dipyrone	1.05	310.30	191.05	16	15	21	80.05	16	35	16	2.78 ± 0.03
Acetaminophen glucuronide[a]	1.07	326.30	112.95	16	15	24	149.90	16	27	16	2.18 ± 0.01
Acetylsalicylic acid	1.51	179.20	136.85	22	10	29	93.15	22	22	19	1.87 ± 0.03
Acetaminophen	1.56	150.20	107.15	14	18	21	–	–	–	–	–
Carboxyibuprofen[a]	2.68	235.30	191.20	12	8	14	72.90	12	17	15	1.39 ± 0.02
Hydroxyibuprofen[a]	2.72	221.30	177.15	11	8	21	–	–	–	–	–
p-Aminophenol[b]	3.30	151.00	109.85	−16	−10	−23	64.95	−16	−32	−24	3.98 ± 0.11
Ketoprofen	3.90	253.30	208.90	13	8	23	–	–	–	–	–
Naproxen	3.96	229.10	169.90	10	17	19	169.05	10	35	18	1.01 ± 0.01
Nimesulide	4.11	307.00	229.10	10	18	16	79.10	10	29	16	7.63 ± 0.05
Diclofenac	4.34	294.20	249.85	14	12	18	34.90	14	24	13	13.04 ± 0.04
Ibuprofen-d3	4.46	207.50	164.25	13	10	18	–	–	–	–	–
Ibuprofen	4.48	205.30	161.20	13	10	18	–	–	–	–	–

CE, collision energy.

[a] Metabolite.

[b] Degradation product.

AC. The system was controlled by a system controller CBM-20A. Lab Solutions software (Shimadzu Corporation, Kyoto, Japan) was used for control and data processing. A Kinetex C18 column (2.6 mm × 150 mm i.d.; 1.7 µm particle size) (Phenomenex, USA) was used. The optimized separation conditions were achieved using ultra-pure water as solvent A and acetonitrile as solvent B at a flow rate of 0.22 mL/min. The gradient elution was performed as follows: initial conditions: 30% B; 0–1.0 min, 30–35.6% B; 1.0–2.0 min, 35.6–100% B; 2.0–6.0 min, 100% B; 6.0–6.5 min, return to initial conditions; 6.5–10.5 min, equilibration of the column. The injection volume was 5 µL and column oven was set at 30 °C. The autosampler was operated at 4 °C and the autosampler needle was rinsed before and after aspiration of the sample using acetonitrile.

MS settings were analyte-specific and were optimized by direct injection of individual standard solutions of each compound at 10 mg/L. Pharmaceuticals were analyzed in the negative ESI mode, with the exception of *p*-aminophenol that was analyzed in the positive mode. Mass spectrometer was operated in multiple reaction monitoring mode (MRM) and two MRM transitions were monitored for each compound, being the most intense used as quantifier and the second one as qualifier. In the case of acetaminophen, hydroxyibuprofen, ketoprofen, ibuprofen and ibuprofen-d3 only one transition could be recorded due to their poor fragmentation [32]. A summary of individual MS/MS parameters is shown in Table 2. A dwell time of 25 ms was used for all compounds.

Source-dependent parameters were also optimized by direct injection, using a standard mixture solution at 10 mg/L, and are as follows: nebulizing gas (nitrogen) and drying gas (nitrogen) at a flow rate of 2.6 and 12.5 L/min, respectively; interface voltage was set at 5.0 kV; desolvation temperature was 250 °C, and heat block temperature was 300 °C. Argon was used as the collision induced dissociation gas (CID) at a pressure of 230 kPa.

2.5. Method validation

The performance of the method was evaluated through the estimation of the linearity, extraction recoveries, method detection (MDL) and quantification limits (MQL), precision (intra- and inter-day), and matrix effects.

Recoveries were determined by comparing the concentrations obtained, calculated by internal standard calibration, with the initial spiking levels. For each recovery test, blanks (no-spiked samples) were analyzed in order to determine their concentrations, which were afterwards subtracted to the spiked seawater samples. Matrix effects were also evaluated.

The precision of the method was determined by repeated intra-day and inter-day analysis (six successive injections of a 100 µg/L standard solution with all the analytes in one day and in five successive days, respectively), expressing it as the relative standard deviation (RSD) of these replicate measurements.

Method detection limits (MDL) and method quantification limits (MQL) were determined as the minimum detectable amount of analyte with a signal-to-noise of 3.3 and 10, respectively. MDLs and MQLs have been calculated as the average of those estimated in real samples and in spiked samples.

Calibration curves were generated using linear regression analysis and over the established concentration points ranging from 0.1 to 1000 µg/L, depending on the compounds. Quantification of target analytes was performed by the internal standard approach. Calibration standards were measured at the beginning and at the end of each sequence, and one calibration standard was measured repeatedly throughout the sequence to check the signal stability.

Matrix effects were assessed in order to evaluate the degree of ion suppression or enhancement, and to what extent target compounds were sensitive to them. Matrix effects were calculated according to Eq. (1) by dividing the slopes of the matrix-matched calibration curves prepared in seawater (slope$_{\text{matrix-matched}}$) and the slopes of the calibration curves prepared in solvent (slope$_{\text{solvent}}$). A value of 100% indicates that there is no matrix effect, while for values higher than 100% there is ion enhancement and for lower values (<100%) there is ion suppression.

$$\text{Matrix effect}(\%) = \left(\frac{\text{slope}_{\text{matrix-matched}}}{\text{slope}_{\text{solvent}}} \right) \times 100 \qquad (1)$$

3. Results and discussion

3.1. Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS)

3.1.1. Chromatographic separation

In order to optimize the chromatographic separation and sensitivity of the studied analytes, different mobile phases and additives were tested. Acetonitrile, methanol or mixtures of both were tested as organic phase while non-buffer ultra-pure water and buffers such ammonium formate, ammonium acetate and addition of 0.1% formic acid were evaluated as mobile phase for the aqueous phase.

The best separation was achieved using acetonitrile and ultra-pure water, since this mobile phase provided better resolution, peak shape and sensitivity for the studied pharmaceuticals.

Once the mobile phase composition was established, the mode of elution (isocratic or gradient elution), as well as the flow rate were optimized with the aim to improve chromatographic resolution and peak shapes and simultaneously to reduce total analysis time. The optimum flow rate was set to 0.22 mL/min. Furthermore, different oven temperatures (25, 30, 32, 35 and 38 °C) were tested. Peak shape and chromatographic response improved when 30 °C was used. A chromatogram of a standard solution under the optimized conditions is presented in Fig. 1. As can be seen, the separation of analytes is not complete, what is not necessary for the selective MS/MS detection, which allows an improved detectability and a reduction in ion suppression effect [33].

3.1.2. Mass spectrometry (MS/MS)

The precursor ion for each analyte was selected by recording chromatograms in full scan mode. From the 12 pharmaceuticals studied in this work, all showed higher response in negative electrospray ionization mode, with the exception of p-aminophenol for which positive electrospray ionization was more sensitive. In general, $[M-H]^-$ was chosen for precursor ion for negative ionization with the exception of dipyrone, which precursor ion was $[M-Na]^-$. p-Aminophenol was the only compound analyzed under positive ionization and the adduct formed with acetonitrile present in the mobile phase ($[M+CH_3CN+H]^+$) was chosen as precursor ion. For most of pharmaceuticals, two MRM were monitored between the precursor ion and the most abundant product ions for each compound, being the first transition used for quantification and the second one for identification purposes. In the case of acetaminophen, ibuprofen and hydroxyibuprofen only one MRM could be monitored due to their poor fragmentation. Since the isotopically labeled internal standard (ibuprofen-d3) is not likely to be found in the environment, only one MRM was also monitored (Table 2).

Ionization parameters like drying and nebulizing gas flow rates, heat block and desolvation temperatures and interface voltage are key parameters to obtain higher sensitivity; therefore these parameters were also optimized. The nebulizing gas flow rate was studied between 0.5 and 3.0 L/min and the drying gas flow rate from 10.0 to 20.0 L/min. Higher sensitivity was observed using 2.6 and 12.5 L/min for nebulizing gas and drying gas flow rate, respectively. The study of the desolvation temperature was made for values between 200 and 300 °C, while for the heat block temperature values ranged from 200 to 500 °C. The signal of the studied analytes was increased when 250 and 300 °C were used for desolvation and heat block temperatures, correspondingly. Finally, the interface voltage was tested between 0.5 and 5.0 kV, showing that the higher the interface voltage the greater was the sensitivity for all analytes.

3.2. Solid phase extraction optimization

Usually SPE is the technique of choice for the extraction of pharmaceuticals from water samples [17]. Therefore, a SPE procedure was developed for the analysis of the selected pharmaceuticals in seawater. Different parameters were studied, namely type of sorbent, sample's pH and sample's volume, in order to evaluate which conditions yielded higher recoveries of the target pharmaceuticals. The SPE method was optimized using ultra-pure water and seawater.

Preliminary studies were made to evaluate the performance of different SPE sorbents according to the variation of sample's pH. For that, the extraction efficiency of the polymeric reversed phase Strata-X (200 mg, 3 mL), the mixed-mode reversed phase/anionic
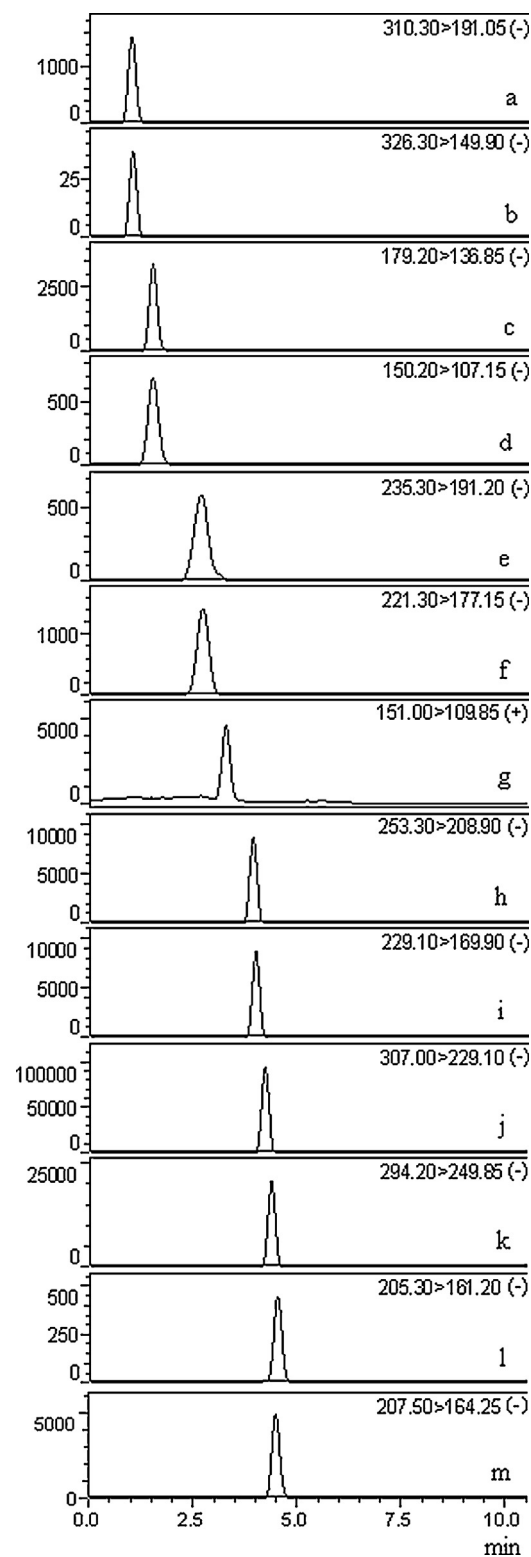

Fig. 1. Representative chromatograms of the MRM transition used for quantification of a 100 μg/L standard mixture of the studied pharmaceuticals. (a) Dipyrone; (b) acetaminophen glucuronide; (c) acetylsalicylic acid; (d) acetaminophen; (e) carboxyibuprofen; (f) hydroxyibuprofen; (g) p-aminophenol; (h) ketoprofen; (i) naproxen; (j) nimesulide; (k) diclofenac; (l) ibuprofen; and (m) ibuprofen-d3.
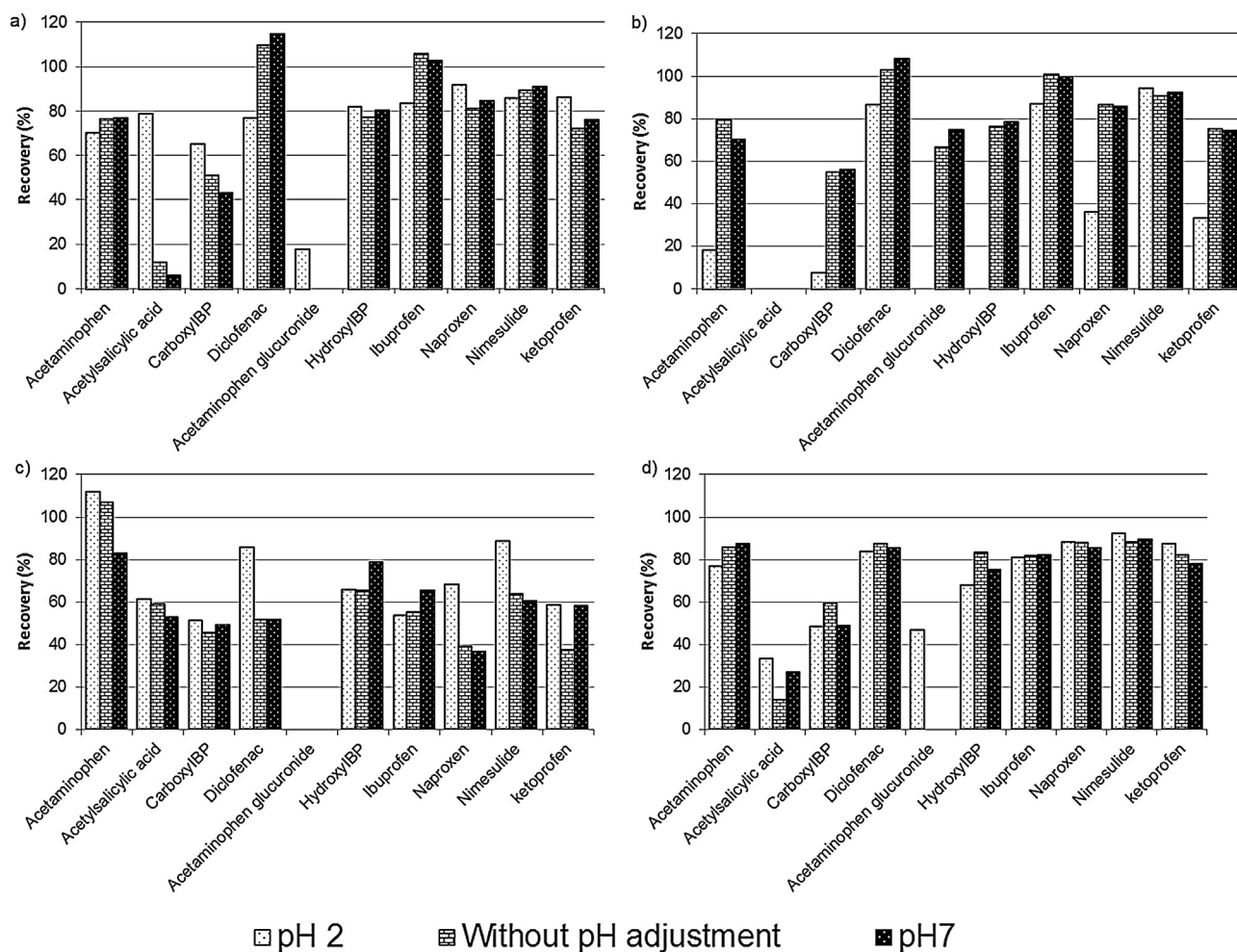
Fig. 2. Recoveries obtained for the target analytes with different SPE cartridges, using different sample's pH. (a) Strata-X; (b) Strata X-AW; (c) Strata X-A; and (d) Strata X-C.

exchange Strata X-AW (200 mg, 3 mL) and Strata-X-A (200 mg, 3 mL), and the mixed-mode reversed phase/cationic exchange Strata X-C (200 mg, 3 mL) was studied at different sample's pH (pH 2, without pH adjustment and pH 7). The obtained results are presented in Fig. 2. In general, for most of studied pharmaceuticals, similar recoveries were yielded with the tested SPE sorbents, though Strata X-A showed lower values and it did not allow recovering acetaminophen glucuronide (Fig. 2C). On the other hand, p-aminophenol was never recovered with any of the sorbents, whereas dipyrone was only extracted with Strata X-AW, but with a low efficiency (36%) (Table S1, Supplementary material). Since dipyrone is negatively charged at all pH range, this analyte was able to establish ionic interactions with the sorbent material (a polystyrene–divinylbenze–pyrrolidone polymer skeleton modified with ethylenediamine groups [34]). On the other hand, the mixed-mode reversed phase/weak anionic exchange sorbent Strata X-AW showed higher recovery values for NSAIDs for a sample's pH of 7 (Fig. 2B). This is in agreement with NSAIDs pKa values (pKa between 4 and 4.5) (Table 1), given that for higher pH values they are negatively charged (Table S2, Supplementary material), establishing ionic interactions with the diamine groups present in the sorbent material. At pH 7, acetaminophen glucuronide also presents negative charge, sharing the extraction by ionic interactions with NSAIDs. On the other hand, acetaminophen is in its neutral form (Table S2, Supplementary material) and was extracted by reversed

phase mechanisms. Although Strata X-AW showed a good extraction efficiency, Strata-X achieved a better performance, with RSD values between 1.2% (acetaminophen) and 9.5% (acetaminophen glucuronide), when sample pH was adjusted to 2, together with high recovery values (Fig. 2, Table S1 Supplementary material). Strata-X is a polymeric sorbent with a styrenic skeleton chemically modified with a pyrrolidone group [35] and pharmaceuticals were extracted through reversed phase mechanisms and π–π interactions, since at the selected pH NSAIDs are in their neutral form (Table S2, Supplementary material). Therefore, the protocol based on Strata X cartridges was chosen for further studies, because it showed to be more suitable for the extraction of a greater number of pharmaceuticals with a better performance and higher recoveries.

The sample volume was also studied and the breakthrough SPE volume was determined using 50, 100, 250, 500, and 1000 mL of ultra-pure water. For most of the studied compounds recovery values maintained constant for sample volumes up to 500 mL, being observed a decrease for higher volumes, whereas for acetylsalicylic acid the recovery remained constant for all the studied volumes. However, for acetaminophen and acetaminophen glucuronide, it was observed a decrease in the recovery values with increasing sample volumes. In this way, and attending that pharmaceuticals have been found in seawater at low levels (few ng/L) [20,22], a sample volume of 500 mL was chosen, because it would allow to

better concentrate pharmaceuticals present in real samples and simultaneously to get higher recoveries for most of the studied NSAIDs/analgesics.

3.3. Method validation

Since it was not possible to recover p-aminophenol, dipyrone and acetaminophen glucuronide under the optimized SPE conditions, these compounds were not considered for method validation purposes.

The performance of the developed method was validated in terms of sensitivity, linearity, recoveries, precision (intra- and inter-day) and matrix effects, using seawater. Detailed analytical quality assurance data is shown in Table 3.

Accuracy of the method was estimated from recovery experiments of the target analytes at different concentrations. Thus, three fortification levels (0.2, 0.5, and 1 µg/L) were tested. The recoveries obtained varied from 11.2% for acetaminophen to 101% for naproxen and ketoprofen (Table 3). Linearity was studied in the concentration ranges presented in Table 3 with correlation coefficients (r^2) higher than 0.99. Depending on the sensitivity of each analyte different linear responses were obtained. The precision of the method was evaluated in terms of precision (intra and inter-day), exhibiting RSD values below 4.48% and 8.10%, respectively. MDLs and MQLs ranged from 0.02 (naproxen) to 8.18 ng/L (carboxyibuprofen) and 0.06 (naproxen) to 24.8 ng/L (carboxyibuprofen), respectively. The potential interferences of the matrix in the measurements were evaluated. For the studied NSAIDs, all compounds showed ion enhancement, being the effect more pronounced for pharmaceuticals like ibuprofen and nimesulide. In order to correct the matrix effect and avoid inaccurate quantification, an isotopically labeled standard was used. This approach has been identified as a very powerful tool to compensate adverse matrix effects observed with ESI for the analysis of aqueous matrices [20]. Therefore, in this study, internal standard calibration using the isotopically labeled standard ibuprofen-d3 was used as the strategy to correct matrix effects. It should be kept in mind that the selection of an internal standard has to be based on its similarity with the compounds of interest, for what concerns to mass spectrometric response, chemical structure, chromatographic retention time and matrix effect (ion suppression or enhancement) [20].

3.4. Application to seawater samples

The developed method was applied to seawater samples of Atlantic Ocean collected in the North of the Portuguese coast during the 2013 bathing season. Ten samples from three beaches with different bathing water quality, namely excellent, good, and sufficient were collected and analyzed.

Results obtained are summarized in Fig. 3. All the pharmaceuticals were detected at least in one seawater sample. The pharmaceuticals ibuprofen, hydroxyibuprofen, acetaminophen and ketoprofen had a frequency of detection of 100%, while for the remaining NSAIDs the frequency of detection varied from 10% (carboxyibuprofen, acetylsalicylic acid and nimesulide) to 30% (naproxen). The concentrations detected for the different pharmaceuticals varied from 0.46 ng/L for nimesulide to 600.5 ng/L for carboxyibuprofen, being the highest concentrations reported for ibuprofen and its metabolites and acetaminophen (Fig. 3), two pharmaceuticals with a high consumption rate among Portuguese population [25]. In general, these highest concentrations were detected in the Azul (Conchinha) beach, whose bathing water quality is classified as sufficient. On the other hand, nimesulide was only detected in the excellent bathing water beach (Leça da Palmeira) (Fig. 3).

Table 3
Recoveries obtained for the different fortification levels, expressed in percentage (%), for the selected pharmaceuticals in seawater, linearity, detection and quantification limits of the method (MDL, MQL), and intra- and inter-day precision of the developed SPE–UHPLC–MS/MS method.

| Pharmaceuticals	Linear range (µg/L)	Fortification I (0.2 µg/L) Recovery (%)	RSD (%)	Fortification II (0.5 µg/L) Recovery (%)	RSD (%)	Fortification III (1.0 µg/L) Recovery (%)	RSD (%)	Correlation coefficient (r^2)	MDL (ng/L)	MQL (ng/L)	Precision intra-day (%RSD)	Precision inter-day (%RSD)
Acetylsalicylic acid	5–75	84.5	3.4	81.7	3.0	85.1	1.8	0.9980	0.10	0.32	2.27	6.60
Acetaminophen	5–75	11.2	5.4	13.8	9.5	14.7	2.3	0.9938	0.30	0.94	3.69	2.70
Carboxyibuprofen	100–1000	68.7	5.8	68.2	3.0	65.8	4.3	0.9925	8.18	24.8	2.47	0.40
Hydroxyibuprofen	5–100	85.2	2.8	80.5	2.5	83.7	2.8	0.9974	3.90	11.8	4.48	2.70
Ketoprofen	5–100	101	1.3	94.5	0.99	91.3	1.7	0.9940	0.30	0.90	1.10	2.70
Naproxen	10–100	95.5	2.8	94.6	0.52	101	3.9	0.9962	0.02	0.06	2.61	5.90
Nimesulide	0.1–1	88.5	4.7	88.8	5.4	84.6	0.37	0.9951	0.06	0.18	2.42	3.90
Diclofenac	1–75	88.6	1.7	85.6	2.8	80.3	3.2	0.9990	0.02	0.06	1.80	4.40
Ibuprofen	10–100	86.5	2.2	89.5	4.3	89.0	4.0	0.9957	0.08	0.26	2.59	8.10

n.d., not detected.
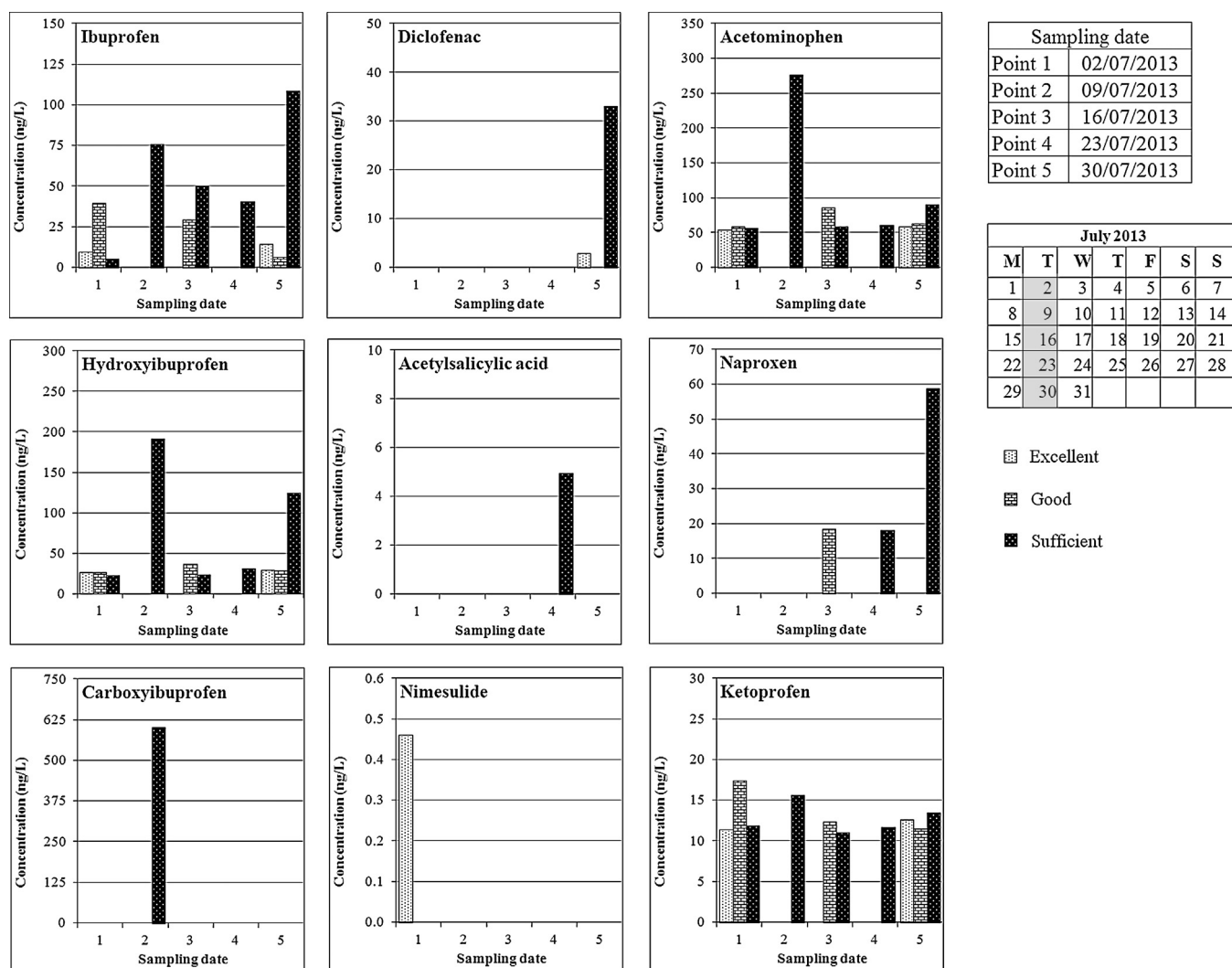
Fig. 3. Concentration of NSAIDs/analgesics, expressed in ng/L, detected in seawater samples.

The presence of pharmaceuticals belonging to NSAIDs and analgesics had also been reported in the Mediterranean Sea [20,23], though at lower levels than those reported herein, while Wu et al. (2010) [24] found similar concentrations of naproxen, diclofenac and ibuprofen in Marine Bay (Singapore). On the other hand, the NSAIDs and analgesics selected in this study were not detected in the Baltic Sea [19] or in the North Sea [22]. Nödler et al. [22] quantified several pharmaceuticals, such as carbamazepine, caffeine, clarithromycin, and sulfamethoxazole, in a sample collected in the Baltic Sea (Ahlbeck, Usedom). Levels up to 58 ng/L were found for the pharmaceuticals with the maximum level being observed for caffeine. The NSAIDs included in the referred study, namely, diclofenac, ibuprofen, naproxen, acetaminophen, and phenazone were not detected in the analyzed seawater sample despite the low method quantitation limits (MQLs) in the range 2.0–4.8 ng/L.

Wille et al. [22] reported a LC–MS/MS method for the simultaneous quantification of 13 pharmaceuticals, including the NSAIDs salicylic acid, ketoprofen and diclofenac. Salicylic acid was found in eleven out of twelve samples collected in three locations of Belgium coast, in the period 2007–2009, in concentrations up to 660 ng/L. The LOQs for ketoprofen and diclofenac were 50 ng/L, which were significantly higher than the ones obtained in our study (0.90 and 0.06 ng/L, respectively).

Relatively to ibuprofen metabolites, carboxyibuprofen is its human metabolite with the highest percentage of excretion [29,36] therefore it is more probable to be found in the environment than hydroxyibuprofen and ibuprofen. However, contrarily to what was expected, carboxyibuprofen was only detected in one sample from Azul (Conchinha) beach (Fig. 3), being hydroxyibuprofen the metabolite most frequently detected in seawater. Similar findings have been reported in different environmental aqueous matrices [29,37]. Thus, it could be concluded that hydroxyibuprofen might be a more stable metabolite than carboxyibuprofen, which also showed a higher removal rate comparatively to other ibuprofen metabolites in batch experiments [29,37]. However, when ibuprofen and its two main metabolites were detected in the same sample (sampling day 09/07/2013), carboxyibuprofen showed higher concentrations (600.5 ng/L). The same behavior was observed in seawater samples from the Breivika Harbor (Norway) [37] as well as in Ter river (Spain) [29], although while in the former levels did not exceed 5.3 ng/L, in the river samples carboxyibuprofen reach concentrations of 3.95 µg/L.

4. Conclusions

A new analytical method based on off-line SPE–UHPLC–MS/MS was developed to determine the presence of pharmaceuticals

belonging to NSAIDs and analgesics therapeutic groups in seawaters. UHPLC technology was used in order to obtain faster analysis, reduction in solvent consumption and a higher sample throughput. MS/MS detection was applied to get better sensitivity and a more precise identification of the studied pharmaceuticals in a complex matrix as seawater, since two MRM transitions were monitored for most of compounds. The present method allowed achieving MDLs in the low ng/L range, proving to be a robust and reliable tool for monitoring pharmaceuticals in environmental samples.

The developed method was successfully applied to the analysis of seawater samples from the Atlantic Ocean collected in the North of the Portuguese coast. The results revealed that NSAIDs and analgesics are widespread in seawater, being ibuprofen, hydroxyibuprofen, acetaminophen and ketoprofen detected in all the samples. The concentrations found ranged from 0.46 to 600.5 ng/L for nimesulide and carboxyibuprofen, respectively, and it was proved the importance of including human metabolites in analytical methods, given that they might be at higher levels than the parent compound, as was demonstrated by the analysis of ibuprofen and its main human metabolites.

The described method could be a useful tool to be used in monitoring studies in order to evaluate the occurrence and fate of pharmaceuticals (NSAIDs and analgesics) in seawaters as well as to help in the identification of possible sources of contamination of marine environments.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2014.06.017.

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