Development of a simple analytical method for the simultaneous determination of paracetamol, paracetamol-glucuronide and *p*-aminophenol in river water

Lúcia H.M.L.M. Santos, Paula Paíga, Alberto N. Araújo, Angelina Pena,
Cristina Delerue-Matos, M. Conceição B.S.M. Montenegro

ABSTRACT

Paracetamol is among the most worldwide consumed pharmaceuticals. Although its occurrence in the environment is well documented, data about the presence of its metabolites and transformation products is very scarce. The present work describes the development of an analytical method for the simultaneous determination of paracetamol, its principal metabolite (paracetamol-glucuronide) and its main trans- formation product (*p*-aminophenol) based on solid phase extraction (SPE) and high performance liquid chromatography coupled to diode array detection (HPLC-DAD). The method was applied to analysis of river waters, showing to be suitable to be used in routine analysis. Different SPE sorbents were compared and the use of two Oasis WAX cartridges in tandem proved to be the most adequate approach for sample clean up and pre-concentration. Under optimized conditions, limits of detection in the range 40–67 ng/L were obtained, as well as mean recoveries between 60 and 110% with relative standard deviations (RSD) below 6%. Finally, the developed SPE-HPLC/DAD method was successfully applied to the analysis of the selected compounds in samples from seven rivers located in the north of Portugal. Nevertheless all the compounds were detected, it was the first time that paracetamol-glucuronide was found in river water at concentrations up to 3.57 μg/L.

Keywords:
Paracetamol Paracetamol-glucuronide *p*-Aminophenol, Solid phase extraction HPLC, River water

1. Introduction

Paracetamol (acetaminophen or *N*-acetyl-4-aminophenol) is one of the most popular and widely used medicines for the treatment of pain and fever, both as an over-the-counter (OTC) and as a prescribed medicine. It can be used in a wide range of patients, including children, pregnant women or the elderly. Following oral administration, approximately 90% of paracetamol is metabolized, being conjugated with glucuronide (40–67%) and, in a less extent, with sulphate (20–46%), to form inactive metabolites, which are eliminated in urine together with a small fraction of unchanged paracetamol (<5%) [1]. Although paracetamol presents a high removal efficiency (approximately 99%) in WWTPs [2,3], it has been detected in their effluents at concentrations up to low microgram per litre [4–6], contributing to its entrance into surface waters [7–9]. Once in the environment, paracetamol is mainly degraded by microorganisms, which are capable of using it as carbon and energy sources [10].

Although paracetamol is not highly persistent in the environment, continuous input overrules its high transformation rate [11], thus, it can adversely affect aquatic organisms. Acute toxicity effects in the invertebrate *Daphnia magna* (EC_{50} ranging from 26.6 to 50 mg/L) [12–14], the marine bacterium *Vibrio fischeri* (EC_{50} = 549.7 mg/L) and the fish *Oryzias latipes* (EC_{50} = >160 mg/L) [12] have been reported. Effects on cell cultures with EC_{50} values of 19 mg/L have also been described [13].

Nowadays analytical methodologies described in literature are mainly focused in multi-residues methods that allow the simultaneously determination of paracetamol together with a large number of pharmaceuticals from several therapeutic groups [15–17]. Most of them are principally focused in parent compounds and rarely analyze metabolites and/or transformation products. At present, methods reported for the determination of paracetamol metabolites are focused in biological matrices [18–20] rather than in environmental ones [21]. On the other hand, paracetamol may also be degraded, both during wastewater treatment and in the environment, giving different transformation products [22,23]. *p*-Aminophenol was identified as its main transformation product, and its presence in wastewater samples was reported [23]. However the origin of *p*-aminophenol cannot only be attributed to the degradation of paracetamol, since it is also widely used in industrial applications and is known as a transformation product from pesticides. Furthermore, *p*-aminophenol was also described as the primary degradation product of paracetamol during the storage of its medicinal formulations [24].

High-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) has been designated as technique of choice for the determination and quantification of pharmaceuticals in environmental samples [25]. However, these equipments are still very expensive and they are not available in many laboratories for routine analysis. On the other hand, almost all laboratories have HPLC systems with diode array UV absorbance and/or fluorescence detection that may effectively be used for the analysis of pharmaceuticals in environmental samples [26–29].

Due to the complexity of environmental samples, analysis of pharmaceuticals has to be preceded by a pre-concentration step, which allows the detection of low concentrations and simultaneously removes the interferences. This is often performed by solid phase extraction (SPE). Generally, polymeric sorbents, like Oasis HLB, are the most used for pre-concentration of pharmaceuticals from aqueous matrices [16,17,30–32], though mixed-mode ion-exchange sorbents have also been described [33,34].

Thus, the present work describes the development and validation of an analytical method based on off-line SPE, using a mixed mode reversed phase/anionic exchange sorbent, followed by LC-DAD for the determination of paracetamol, its main metabolite (paracetamol-glucuronide) and its principal transformation product (*p*-aminophenol) in river waters. The performance and application of this method is important, since allows the simultaneous monitoring of parent compound, metabolite and transformation product as well as the evaluation of their environmental interdependence, using one of the most worldwide consumed pharmaceuticals (paracetamol) as example.

Finally, the developed methodology was successfully applied to the analysis of the selected compounds in seven rivers from north of Portugal. To our knowledge this is the first time that paracetamol-glucuronide was found in surface waters.

2. Materials and methods

2.1. Chemicals and reagents

Paracetamol (PCT) (acetaminophen), *p*-aminophenol (PAP) (4-aminophenol) and paracetamol-glucuronide (PCT-G) (p-acetamidophenyl �d-glucoronide) sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). All standards were of high purity grade (>93%). HPLC-grade methanol, HPLC-grade acetonitrile and HPLC-grade acetone, n-hexane and formic acid (purity ≥98%) were obtained from Merck (Darmstadt, Germany), hydrochloric acid 37% and glacial acetic acid (purity ≥99.7%) were purchased from Carlo Erba (Rodano, Italy), ammonia 25% was obtained from Panreac (Barcelona, Spain), ammonium hydroxide solution, ammonium acetate (purity ≥98%), ethyl acetate and dichloromethane were purchased from Sigma-Aldrich (Steinhein, Germany). HPLC-grade water (18.2 MQ cm) was obtained by purifying deionised water in a Milli-Q Simplicity 185 system (Millipore, Molsheim, France).

Individual stock standard solutions were prepared for each compound by dissolving 10 mg of powder in 10 mL of methanol, obtaining a final concentration of 1000 mg/L, and stored at −20 °C. Stock standard solutions were renewed every week. An intermediate standard solution was daily prepared by mixing the three individual stock solutions and diluting with a mixture methanol–water (10:90, v/v) to give a final concentration of 10 mg/L and kept at 4 °C. Working standard solutions were also prepared in a mixture methanol-water (10:90, v/v) by dilution of appropriate amounts of the intermediate solution. Amber glassware was used to prevent light degradation. These working standard solutions were used for preparation of the calibration curve and for spiking samples in the validation study.

All standard solutions and sample extracts were filtered through a 0.20 μm PTFE syringe filter (Teknokroma, Barcelona, Spain) and homogenized using a vortex mixer (VWR, Radnor, Delaware, USA). All chromatographic solvents were filtered through a 0.20 μm nylon membrane filter (Supelco, Bellefonte, PA, USA) using a vacuum pump (Dinko D-95, Barcelona, Spain) and degassed for 15 min in an ultrasonic bath (Raypa® Trade, Terrassa, Spain).

SPE cartridges used were Oasis® MAX (60 mg, 3 mL), Oasis® WAX (150 mg, 6 mL), Oasis® MCX (150 mg, 6 mL) and Oasis® HLB (200 mg, 6 mL) from Waters (Mildford, MA, USA), LiChrolut® EN/RP-18 (EN 40–120 μm, 100 mg (bottom) and RP-18 40–63 μm, 200 mg (top), 6 ml) from Merck (Poland), Strata$^{\text{TM}}$-SDB-L (500 mg, 6 mL) and Strata$^{\text{TM}}$-X (200 mg, 3 mL) from Phenomenex (USA), and Enviro-clean® (C_8 and quaternary amine, 1000 mg, 6 mL) from Unit Chemical Technologies (UCT), Inc. (Bristol, PA, USA).

2.2. Sample collection

River water (2.5 L) was collected from seven rivers located in the north of Portugal, which is one of the most densely populated areas of the country. Sample collection, preservation and storage were done according to the USEPA Method Guideline [35]. River samples were collected on the river side in amber glass bottles and kept refrigerated (±4 °C) during the transport to the laboratory. Samples were collected along one week in September 2011.

2.3. Sample pre-treatment and extraction

River water samples were vacuum filtered through 1.2 μm glass microfiber filters (GF/C, Whatman, UK), followed by 0.20 μm nylon membrane filters (Supelco, Bellefonte, PA, USA) and stored at −20 °C, until extraction.

For the SPE procedure a vacuum manifold system (Phenomenex, USA) was used. Two Oasis WAX cartridges were initially conditioned, in separate, with 2 mL of methanol, 2 mL of HPLC-grade water, and 2 mL of HPLC-grade water pH 7 (pH adjusted with ammonia) at a flow rate of 1 mL/min. After that, the SPE cartridges were connected in tandem and 50 mL of river water (pH adjusted to 7 with ammonia) were loaded onto the cartridges at a flow rate of 1 mL/min. Finally, analytes were eluted with 5 mL of methanol and 5 mL of 5% ammonium hydroxide in methanol at a flow rate of 1 mL/min, and the eluates were pooled in one single collection vial. Extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted in 250 μL of a mixture methanol–water (10:90, v/v), allowing a pre-concentration factor of 200.

2.4. Liquid chromatography

Chromatographic analysis was performed on a Nexera Ultra-High Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan) equipped with two solvent delivery modules LC-30 AD, a column oven CTO-20 AC, an autosampler SIL-30 AC and an UV/vis photodiode array detector SPD-M20A. The system was controlled by a system controller CBM-20A. Two chromatographic systems were used in this work in order to evaluate the performance of the method for HPLC and fast-HPLC analysis. On the former, separation was carried out using a Luna C18(2) column (150 × 4.6 mm i.d., 5 μm particle size) (Phenomenex, USA) that was kept at 25 °C, while sample vials were kept at 4 °C. An injection volume of 40 μL was used. The optimized mobile phase consisted of 10 mM ammonium acetate/acetic acid (pH 6) as solvent A and acetonitrile as solvent B, using a flow rate of 1 mL/min. The gradient elution was performed as follow: 0–9.0 min, 97–74.8% A; 9.0–10.0 min, return to initial conditions; 10.0–15.0 min equilibration of the column. For fast-HPLC analysis, a Shim-pack XR-ODS (100 × 4.6 mm i.d., 2.2 μm particle size) (Shimadzu, Kyoto, Japan) column was used and it was kept at 25 °C. The separation was performed using the same mobile phase composition (10 mM ammonium acetate/acetic acid (pH 6) as solvent A and acetonitrile as solvent B) at a flow rate of 2 mL/min. The gradient elution was: 0–1.0 min, 96–91.6% A; 1.0–1.5 min, 91.6–80% A; 1.5–3.0 min, 80% A; 3.0–3.5 min, returned to initial conditions; 3.5–4.0 min, equilibration of the column. An injection volume of 10 μL was used and sample vials were kept at 4 °C. The analytes were monitored at their maximum UV wavelengths, namely 243, 232 and 245 nm for paracetamol-glucuronide, *p*-aminophenol and paracetamol, respectively. Lab Solutions software (Shimadzu Corporation, Kyoto, Japan) was used for control and data processing.

2.5. Method validation

Method validation was performed for both HPLC and fast-HPLC analysis, in order to evaluate which chromatographic technique might be more suitable for application to real samples.

Target analytes were identified in the chromatograms by comparison of the retention time of the peaks obtained with these ones of a standard solution. Simultaneously the identification of the analytes was also confirmed comparing the corresponding UV spectra of the peaks of the sample and of standard solution chromatograms. For the quantification of the analytes in real samples the standard addition method was used.

The linearity of the method was established by setting calibration curves using linear regression analysis over the concentration range 50–1500 μg/L; however final concentrations tested depend of the sensitivity reached for each analyte. Method detection limit (MDL) and method quantification limit (MQL) were determined as the minimum amount detectable of analyte with a signal-to-noise ratio of 3 and 10, respectively.

Method accuracy (expressed as recovery percentage) and precision (expressed in terms of relative standard deviation (RSD)) were evaluated by recovery studies of the analytes in river water, spiked at different final concentration levels (0.75, 2.50, 3.75 and 5.00 μg/L). Experiments were performed in triplicate ($n = 3$). Recoveries were determined comparing the concentrations obtained with the initial spiking levels. As river water may contain the target analytes, blanks (samples without standard solution addition) were analyzed in order to determine their concentrations, which were then subtracted to the spiked river water.

Method precision was determined by repeated intra- and inter-day analysis, through five successive injections of a river water sample spiked with a standard mixture containing all the analytes at a final concentration of 5.00 μg/L in one day and its injection in five consecutive days, respectively.

The influence of the matrix in the UV signal was evaluated by preparing a calibration curve in river extract, and comparing it with one achieved for the standards prepared in a mixture of methanol–water (10:90, v/v). A blank (sample with no addition of the standards) was simultaneously assayed in order to subtract the levels of the target analytes present in the sample.

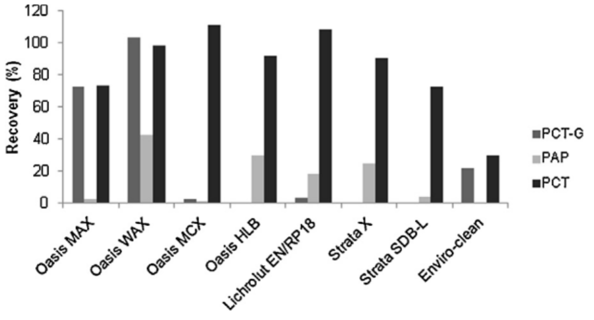


Fig. 1. Recoveries obtained for the target analytes in ultra-pure water with different SPE cartridges. PCT-G – Paracetamol-glucuronide; PAP – *p*-Aminophenol; PCT – Paracetamol.

3. Results and discussion

3.1. Solid phase extraction optimization

The SPE method was optimized using ultra-pure and river water.

The selection of the most adequate SPE sorbent is a critical point in the development of an extraction procedure; therefore the performance of different SPE cartridges was evaluated and the recoveries in ultra-pure water, pH adjusted to 7, are shown in Fig. 1. As can be seen, paracetamol could be efficiently recovered by almost all SPE cartridges (with the exception of Enviro-clean®), while for its metabolite higher recoveries (72–103%) were obtained with the mixed mode reversed phase/anionic exchange sorbents Oasis MAX and WAX, since at pH 7, paracetamol-glucuronide is negatively charged (Fig. S1, Supplementary data), establishing ionic interactions with those sorbents. Therefore, polymeric sorbents such as Oasis HLB, Strata-X and Strata SDB-L, which extract analytes by reversed phase mechanisms, were not able to recover this compound. Furthermore, *p*-aminophenol showed the lowest recovery, which did not exceed 43%, using the Oasis WAX cartridges, and it was not recovered at all with Enviro-clean®, given that at pH 7, *p*-aminophenol is in a neutral form (Fig. S1, Supplementary data), and this mixed mode sorbent (cationic exchange and C_8) is not able to interact with *p*-aminophenol by reversed phase mechanisms. As it is depicted in Fig. 1, the hydrophilic polymeric sorbents Oasis HLB and Strata X yielded similar recoveries for all analytes, which is in agreement with their similar physico-chemical properties [36].

Comparing the different SPE sorbents tested, it can be seen that highest average recoveries were achieved for all the analytes with Oasis WAX (Fig. 1).

Mixed-mode ion-exchange sorbents allow two types of interaction mechanisms, namely reversed phase and ionic-exchange. Thus, taking special attention to pH and solvents employed in each step of SPE protocol, analytes can be selectively eluted [37]. In this context, the effect of sample's pH was studied for the mixed-mode ion-exchange sorbents (Oasis MAX, WAX and MCX) in order to enhance the *p*-aminophenol recovery. Fig. 2 shows the recoveries obtained in ultra-pure water. For paracetamol-glucuronide, better recoveries were achieved using reversed phase/anionic exchange sorbents (Oasis MAX and WAX) at a pH range between 3 and 7, which is in agreement with its p*K*a (Table 1). At this pH range paracetamol-glucuronide is negatively charged (Fig. S1, Supplementary data), being able to bind to the sorbent by ionic interactions, since Oasis MAX and WAX are a strong and a weak anionic-exchange polymeric sorbent, respectively, that are based on Oasis HLB (poly(*N*-vinylpyrrolidone-divinylbenzene) copolymer) chemically modified with quaternary amine groups (dimethylbutylamine) and piperazine groups, respectively [37]. On the other hand, Oasis MCX is a strong cation-exchange polymeric sorbent that has an Oasis HLB skeleton chemically modified with

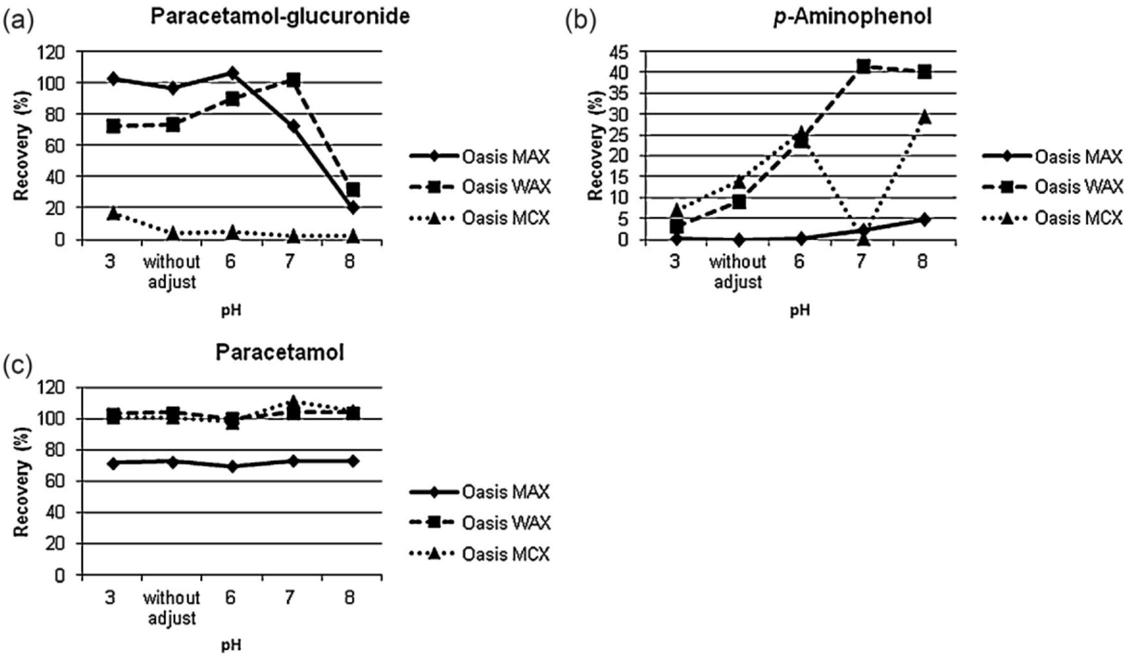


Fig. 2. Recoveries obtained in ultra-pure water at different sample's pH. (a) Paracetamol-glucuronide; (b) *p*-Aminophenol; and (c) Paracetamol.

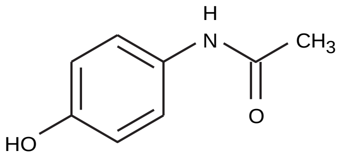
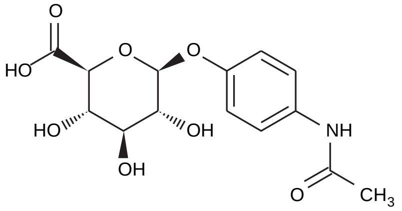
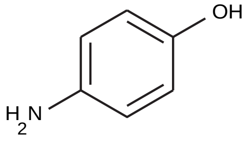
sulfonic groups [37], therefore is not able to interact with a negatively charged compound, and for that reason the recoveries for Oasis MCX are very low (<20%).

Like paracetamol-glucuronide, the highest recoveries of *p*-aminophenol were also achieved with a reversed-phase/anionic exchange sorbent, namely Oasis WAX, at a pH range between 6 and 8 (Fig. 2b). According to *p*-aminophenol p*K*a, at these pH values the molecule is in a neutral form (Fig. S1, Supplementary data), being extracted through reversed phase mechanisms. However, recoveries of *p*-aminophenol using Oasis MCX were similar to that one obtained with Oasis WAX for a pH up to 6. At this pH range *p*-aminophenol presents the amine group positively charged (Fig. S1, Supplementary data) and, therefore, is able to establish ionic interactions with the sulfonic groups of the sorbent.

Relatively to paracetamol, all the mixed-mode ion-exchange sorbents tested provided good recoveries (higher than 70%) in all the studied pH range (Fig. 2c), since it is in a neutral form (Fig. S1, Supplementary data), being extracted through reversed phase mechanisms. Based on the results obtained, a sample's pH of 7 was chosen for further studies.

In contrast to the excellent recoveries of paracetamol and its metabolite obtained with Oasis WAX, the recovery of *p*-aminophenol may be improved using two SPE cartridges in tandem. Different possible combinations were tested using Oasis WAX, MCX and HLB. Oasis HLB was used in this study due to have been proved that, at pH 7, *p*-aminophenol is extracted by reversed phase mechanisms, so an increase in the mass sorbent of the lipophilic/hydrophilic balance Oasis HLB sorbent might improve

Table 1
Physico-chemical properties of the selected compounds.

	Chemical structure	Formula	CAS no.	M_w	Solubility (g L^{-1})	p*K*a[a]	log K_{OW}[b]
Paracetamol (PCT)		$C_8H_9NO_2$	130-90-2	151.16	12.8	9.46	0.46
Paracetamol-glucuronide (PCT-G)		$C_{14}H_{17}NO_8$	120595-80-4	327.29	27.7	3.17; 12.22	−1.23
p-Aminophenol (PAP)		C_6H_7NO	123-30-8	109.13	15.0	5.43; 10.40	0.24

[a] Adapted from reference [38].
[b] Adapted from reference [39].

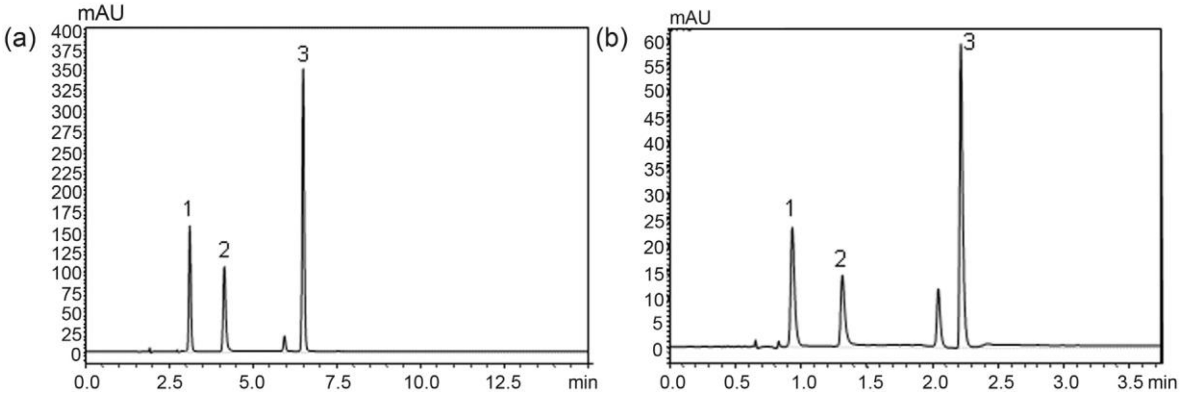


Fig. 3. Example of a chromatogram of a standard mixture 10 µg/mL for the selected analytes analyzed by (a) HPLC and (b) fast-HPLC. 1 – Paracetamol-glucuronide; 2 – *p*-Aminophenol; and 3 – Paracetamol.

the recovery of *p*-aminophenol. Results showed that two Oasis WAX columns in tandem allowed increasing the recovery of *p*-aminophenol (64%), maintaining good recoveries of the others analytes (Table S1, Supplementary data).

Moreover, different organic solvents were tested namely methanol, acetonitrile, acetone, ethyl acetate, *n*-hexane and dichloromethane, as well as different proportions of ammonium hydroxide in methanol. No improvement on *p*-aminophenol recovery was observed comparatively to the elution protocol suggested by the manufacturer that is methanol, for the extraction of neutral compounds extracted by reversed phase mechanisms, and 5% ammonium hydroxide in methanol, to revert the ionic interactions and release the compounds extracted trough anionic exchange mechanisms.

After the SPE protocol has been settle, different sample volumes were studied using two Oasis WAX cartridges in tandem. For paracetamol and paracetamol-glucuronide, recoveries around 100% were achieved for sample volumes up to 100 mL, although the recovery of *p*-aminophenol decreased with increasing sample volume (Fig. S2, Supplementary data). Thus, attending that the main goal of this work was to develop an extraction procedure for the determination of paracetamol, its main metabolite (paracetamol-glucuronide) and its main transformation product (*p*-aminophenol) in river waters, a sample volume of 50 mL was chosen as a commitment between the recovery of the analytes and the intended application of the developed method.

3.2. Optimization of chromatographic conditions

In order to optimize the chromatographic separation, different mobile phases were tested, using methanol, acetonitrile or a mixture of methanol and acetonitrile as organic solvent and water with different additives, such as ammonium acetate and acetic acid at different concentrations as aqueous phase. The best separation was achieved using 10 mM ammonium acetate/acetic acid (pH 6) and acetonitrile. This mobile phase was used for both developed chromatographic procedures.

3.2.1. HPLC analysis

After the mobile phase composition had been established, the elution gradient and flow rate were adjusted in order to improve the chromatographic resolution as well as the peaks shape and to get the shorter analysis time. The optimum flow rate was set at 1.0 mL/min and the elution was performed on a 15 minutes gradient. Different column temperatures were also studied (23 °C, 25 °C and 30 °C) and for all the analytes the best peak shape and resolution were obtained using a temperature of 25 °C. Finally, the injection volume was tested (20, 30 and 40 µL) and the

Table 2
(a) Linearity, detection and quantification limits of the method (MDL, MQL), precision intra- and inter-day of the developed SPE-HPLC/DAD and SPE-fast-HPLC/DAD methods.
(b) Recoveries obtained, expressed in percentage (%), for the selected analytes in river water for the developed methods.

(a)

Compound	SPE-HPLC/DAD						SPE-fast-HPLC/DAD					
	Linear range (µg/L)	Correlation coefficient (r^2)	MDL (µg/L)	MQL (µg/L)	Precision intra-day (% RSD)	Precision inter-day (% RSD)	Linear range (µg/L)	Correlation coefficient (r^2)	MDL (µg/L)	MQL (µg/L)	Precision intra-day (% RSD)	Precision inter-day (% RSD)
PCT-G	50–1500	0.9999	0.040	0.134	0.547	0.836	50–1500	0.9997	0.199	0.664	2.75	5.06
PAP	50–1500	0.9999	0.067	0.225	0.368	0.632	500–1250	0.9990	0.275	0.905	2.14	4.39
PCT	50–1500	0.9999	0.042	0.141	0.097	0.958	50–1500	0.9996	0.210	0.701	0.913	3.56

(b)

Compound	SPE-HPLC/DAD				SPE-fast-HPLC/DAD		
	Recoveries (RSD %)						
	Spiking level (µg/L)				Spiking level (µg/L)		
	0.75	2.50	3.75	5.00	2.50	3.75	5.00
PCT-G	100(2.4)	117 (2.1)	110 (0.7)	112 (3.0)	108(2.5)	99 (0.7)	112 (1.2)
PAP	53(5.5)	62 (4.3)	64 (0.5)	60 (0.2)	62(3.7)	65 (4.0)	59 (2.7)
PCT	99(1.5)	103 (2.3)	107 (3.9)	109 (0.02)	106(3.5)	105 (4.0)	108 (0.9)

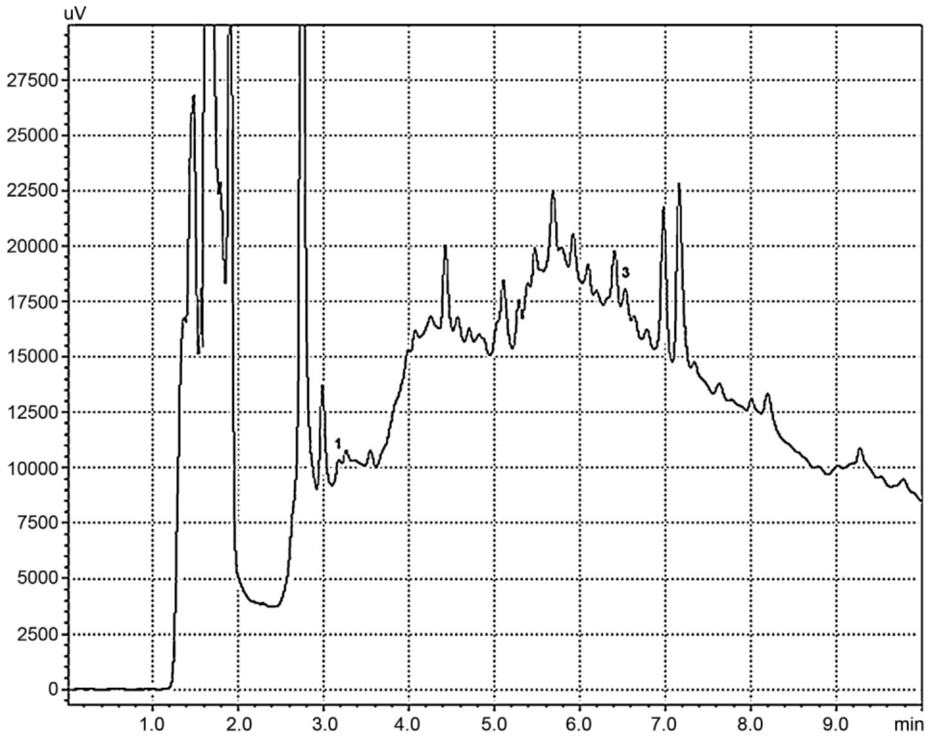


Fig. 4. Chromatogram of Douro river sample. 1 – Paracetamol-glucuronide; 3 – Paracetamol.

chromatographic response was improved, without loss of resolution or peak shape, when an injection volume of 40 µL was used. A representative chromatogram of a 10 µg/mL standard mixture of the compounds analyzed is presented in Fig. 3a.

3.2.2. Fast-HPLC analysis

Upon reducing the particle size from chromatographic column from 5 µm to 2.2 µm, it was possible to use higher flow rates, obtaining a faster separation. Parameters such as elution gradient and flow rate were optimized in order to find the best chromatographic resolution and to obtain narrower peaks in the shortest analysis time. This was achieved using a gradient elution performed on 4 minutes with an optimal flow rate of 2.0 mL/min. The influence of different temperatures in the range 25–35 °C was also studied and the best peak shapes was obtained for a temperature of 25 °C. Lastly, the injection volume was varied from 5 to 10 µL in order to improve the chromatographic response without loss of the peak shapes. This was achieved for an injection volume of 10 µL. An example of a chromatogram obtained with the optimized chromatographic conditions is depicted in Fig. 3b.

3.3. Method validation

The performance of the developed methods was validated in terms of sensitivity, linearity, recoveries, precision (intra- and inter-day) and interference of the matrix, using river water. Detailed analytical quality assurance data is shown in Table 2.

As it is depicted in Table 2a, HPLC and fast-HPLC methods showed differences in what concern to sensitivity, being the lowest MDL and MQL obtained for the former.

Linearity was studied in the range 50–1500 µg/L, setting calibration curves using linear regression analysis. Depending on the sensitivity reached by the chromatographic system employed, each analyte presents different linear responses. Both methods gave correlation coefficients (r^2) higher than 0.999 for all analytes (Table 2a). A six point calibration curve for each compound was daily performed and the possible fluctuation in signal intensity was checked by injecting a standard solution at two concentration levels after each eight injections.

Accuracy of the method was estimated from recovery experiments of the target analytes at different concentration levels. Four fortification levels in river water were tested for both methods; however fast-HPLC method could not be validated to the 0.75 µg/L level due to its lower sensitivity comparatively to HPLC method, but it was validated to the other fortification levels (2.50, 3.75 and 5.00 µg/L) as is shown in Table 2b. The recoveries obtained were satisfactory for all the compounds (recovery from 60 to 120%, with RSD values lower than 6%), except for *p*-aminophenol. In this case, a value lower than 60% for 0.75 µg/L spiking level was obtained, which could be related with losses during the pre-concentration step due to the physico-chemical properties of the compound.

The precision of the method was evaluated in terms of repeatability (intra-day) and reproducibility (inter-day), exhibiting RSD values below to 3% and 6%, respectively, for both methods (Table 2a).

The influence of the matrix in the UV signal was evaluated by comparing a calibration curve in river extract with one achieved for the standards prepared in a mixture of methanol–water (10:90, v/v). The calibration curves obtained for all the compounds exhibited similar slopes and higher Y-intercept values for the river extract, indicating that there is a slightly matrix effect, however this is similar within the linear range. In order to correct the matrix effect, the standard addition method was used.

3.4. Application to real samples

In order to demonstrate the applicability of the developed method, samples from seven rivers located in the north of Portugal were analyzed. As SPE-HPLC/DAD showed to be more sensitive, with lower MDL and MQL for all the compounds, samples were only analyzed using this method. Quantification of the river samples was

Table 3

Concentration of the analyzed compounds, expressed in μg/L, in river waters from the north of Portugal. Standard deviation (SD) values are indicated in brackets.

Sample	Paracetamol-glucuronide	*p*-Aminophenol	Paracetamol
Cabrum river	<MQL	n.d.	<MDL
Douro river	<MQL	n.d.	<MQL
Ave river	0.36 (±0.02)	1.63 (±0.05)	0.17 (±0.002)
Leça river	3.57 (±0.06)	1.25 (±0.02)	0.25 (±0.01)
Tâmega river	<MQL	0.40 (±0.01)	<MDL
Lima river	<MQL	<MQL	<MDL
Minho river	0.18 (±0.01)	0.52 (±0.05)	<MDL

n.d. – not detected; <MDL – below method detection limit; <MQL – below method quantification limit.

done using the standard addition method and the confirmation of the positive findings was carried out by comparing the UV spectra of the peaks present in the samples with that one obtained in the standard solution chromatogram. An example of a chromatogram of a river sample obtained with the developed methodology is shown in Fig. 4.

All the compounds were detected in Portuguese rivers (Table 3). As expected, paracetamol reported the lowest concentrations (up to 0.25 μg/L), since it is efficiently removed (approximately 99%) in WWTPs [2]. The results are in agreement with concentrations of paracetamol reported in literature in rivers from Spain [17], Serbia [16] and United Kingdom [9].

On the other hand, the principal metabolite of paracetamol (paracetamol-glucuronide) and its main transformation product (*p*-aminophenol) could be detected in Portuguese rivers at levels up to 3.57 μg/L and 1.63 μg/L, respectively, usually having higher concentrations than paracetamol (Table 3). Given that paracetamol-glucuronide entry in the environment through human excretion and it was previously detected in a WWTP effluent at levels up to 462 μg/L [21], WWTP effluents may be pointed out as the principal source of entrance of this metabolite into surface waters.

p-Aminophenol was identified as a transformation product of paracetamol and its presence reported in wastewaters [23]. Our results showed that *p*-aminophenol could also be found in surface waters, being detected in five out of seven studied rivers, with concentrations from below MQL 1.63 μg/L (Table 3). Although these concentrations are lower than those one reported in two Romanian rivers [40].

4. Conclusions

A rapid, simple and sensitive method for the simultaneous determination of paracetamol, paracetamol-glucuronide and *p*-aminophenol in river water was developed. The extraction procedure allowed the analysis of three related compounds with different physico-chemical properties in a single step using two Oasis WAX cartridges in tandem and a sample pH of 7. Recoveries higher than 60% were obtained for all the compounds. The SPE procedure allows the elimination of interferences and, at the same time, a pre-concentration of the analytes, depicted in MDLs from 40 to 67 ng/L. Comparatively to HPLC-MS/MS, HPLC-DAD has the advantageous of being an inexpensive analytical technique that can be seen as an affordable, useful and cost-effective alternative for routine analysis of pharmaceuticals, their metabolites and transformation products in environmental waters.

The developed method was applied to the determination of the selected analytes in samples from seven Portuguese rivers, showing their occurrence in surface waters with levels up to few micrograms per litre. The results obtained shows that besides pharmaceuticals, scientific community should also focus its attention in the evaluation of the presence of metabolites and transformation products in the environment, seeing that, sometimes, they may be at higher concentrations than the parent compounds. The proposed methodology may be applied to monitoring the behaviour of the selected analytes during wastewater treatment as well as their ability to adsorb to soil and sediments.

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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.jchromb.2013.04.032.

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