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Determination of pharmaceuticals in river water by column switching of large sample volumes and liquid chromatography–diode array detection, assisted by chemometrics: An integrated approach to green analytical methodologies

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ABSTRACT

An analytical method for the simultaneous determination of nine β-blockers (sotalol atenolol, nadolol, pindolol, metoprolol, timolol, bisoprolol, propanolol and betaxolol) and two analgesics (paracetamol and phenazone) in river water by liquid chromatography and diode array detection is reported. The method involves a modified precolumn switching methodology replacing the small precolumn with a short C18 liquid chromatography column (50 mm × 4.6 mm, 5 μm particle size), thus allowing the preconcentration of large water sample volumes whereas interferences eluting at the first of the chromatogram were discarded to waste. This approach allowed to preconcentrate 30 mL river water samples, modified with 0.4% MeOH, achieving univariate method detection and determination limits ranged between 0.03 and 0.16 $\mu g\,L^{-1}$ and between 0.2 and 0.5 $\mu g\,L^{-1}$, respectively, with precision values lower than 9.4% for spiking levels at the quantitation limits of each analyte and lower than 4.0%, except bisoprolol (8.3%), for higher spiking levels (1.0 $\mu g\,L^{-1}$ of all analytes). Matrix background was reduced in three way data by a baseline correction following the Eilers methodology, whereas multivariate curve resolution and alternating least squares in combination with the standard addition calibration method, applied to these data, coped with overlapping peak, systematic (additive) and proportional (matrix effect) errors. The method was successfully applied for the determination of the target pharmaceuticals in river water from three places in a river stream with acceptable recoveries and precision values, taking into account the complexity of the analytical problem. The joint statistical test for the slope and the intercept of the linear regression between the nominal concentration values versus those predicted, showed that the region computed contained the theoretically expected values (0) for the intercept and (1) for the slope (at a confidence level of 95%), which indicates the absence of both constant and proportional errors in the predicted concentrations.

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1. Introduction

β-Blockers are pharmaceuticals extensively used for the treatment of cardiovascular disorders such as hypertension, arrhythmia and heart failure, and are among the most prescribed medications worldwide and most frequently detected in the environment [1]. Most of these compounds are basic in nature and at neutral pH they are highly water soluble leading to enhanced availability in the environment. Thus, several β-blockers were detected at high levels in surface water [2], propanolol, bisoprolol and metoprolol appearing at 0.59, 2.9 and 2.2 $\mu g\,L^{-1}$, respectively, whereas lower levels of nadolol and betaxolol (0.028 $\mu g\,L^{-1}$) were also found.

β-Blockers act on specific receptors but can also act as non-selective blocking receptors in humans and many of these receptors might also be present in other mammals, vertebrates and some invertebrates. Thus, these pharmaceuticals are of concern due to their acute and chronic toxicity towards aquatic organisms [3,4].

Paracetamol is a common analgesic and antipyretic drug that is used for the relief of fever, headaches and other minor aches and pains [5]. The mode of action of paracetamol is not yet fully elucidated but it is known that its adverse effects are mainly due to the formation of hepatotoxic metabolites, primarily N-acetyl-*p*-benzoquinone imine, when the availability of glutathione is diminished in liver cells [3]. Its acute effects on algae, invertebrates

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and fish were reported [6] as well as the evidence of ecotoxicological risk [7].

Phenazone, also named antypirine, is a non-steroidal anti-inflammatory drug included in the sub-class of pyrazole derivatives (pyrazolones), with analgesic, antipyretic and anti-rheumatic properties, being among the most frequently detected compounds in aquatic systems [8]. In a recent study [9] it was proved that separately phenazone exhibited weak estrogenic activity, but when mixed with other pharmaceuticals (cimetidine, fenofibrate and furosemide) at extremely low-effect concentrations, it leads to a significant response.

Surface waters are complex samples, often containing substances which can interfere with the compounds of interest, in addition to the low concentration level at which pharmaceuticals are present in these matrices. Therefore, analytical procedures for determining pharmaceuticals in surface water samples include an initial sample preparation step involving purification and concentration of the analytes.

Trends in extraction of liquid samples, which are gaining ground, are solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), and miniaturized solid-phase extraction using well-plates or minicolumns or a syringe or pipette tip packed with solid-phase material (MEPS), along with liquid-phase microextraction (LPME) and membrane-assisted solvent extraction (MASE) techniques [10,11].

These miniaturized techniques are typically solvent-free and are more sustainable than conventional extraction techniques, also reducing exposure of the analyst to solvents, in compliance with the Green Analytical Chemistry concept [12]. Trends focused on reducing the adverse environmental impact of analytical methodologies are (i) reduction of the amount of solvents required in sample pretreatment, (ii) reduction in the amount and the toxicity of solvents and reagents employed in the measurement step, especially by automation and miniaturization and (iii) development of alternative direct analytical methodologies not requiring solvents or reagents.

Among green analytical methodologies, de la Guardia and co-workers [13] cited the use of chemometrics based on mathematical treatment of signals obtained by direct measurements on untreated solid or liquid samples and they concluded that combining miniaturization in analytical systems with advances in chemometrics is of great importance.

Apart of milestones related to green sample treatment, which appear cited in the above mentioned review, considerable attention should be paid to column switching techniques in both precolumn switching (PC-LC) and coupled column (LC–LC) liquid chromatography implementations, which interest consist in enhancement of sensitivity, selectivity and potential for automation [14]. In preconcentration with PC-LC, a small size precolumn is loaded with a large volume of untreated sample and then desorbed using a small volume of a proper eluent, directly to the analytical column. For clean-up purposes, after loading with a sample, the precolumn is subsequently flushed with small volumes of solvents with increasing elutropic strength in order to remove interfering compounds. The fraction containing the solutes of interest is then transferred, in a small volume of eluent, to the analytical column ("heart cutting" procedure) [15]. This approach allows for the direct sampling of a considerable volume of water sample (up to 50 mL), but a drawback is its lack of selectivity [14].

In LC–LC, the first column consists in an analytical column, which offers the possibility of removing a large excess of early-eluting polar interferences combining the preconcentration and clean up in a single procedure. However, its inconvenience consists in that the volume of sample to be analyzed is lower than in PC-LC, offering limited sample enrichment. Both approaches are focused on the reduction of the amount of solvents required in sample pretreatment and automation of this step.

Recently, we have developed a methodology involving a combination of both above-mentioned column switching methodologies, which have been successfully applied to the preconcentration of pesticides [16,17] and β-blockers [18] in surface water. This approach preconcentrated large volumes of sample (between 10 and 50 mL) as in PC-LC, but using a short analytical column instead of the small precolumn, which allowed most polar interferences to be discarded whereas the enrichment step was performed.

As for the target pharmaceuticals, apart of the conventional SPE extraction procedures, some advanced extraction techniques have been reported in the literature. Thus, Bones et al. [19] reported a simplified preconcentration method for a range of pharmaceuticals (atenolol and propanolol among them) in river and tap water by on-line SPE using a micro-reversed-phase monolithic silica column. This approach allowed for fast trace enrichment from large volume samples (500 mL) with minimal sample handling. Recoveries were about 70% for propanolol and 0% for atenolol due to insufficient retention on the sorbent. Pitarch et al. [20] investigated the potential of capillary-column-switching liquid chromatography coupled to tandem mass spectrometry (cLC·MS2) for the trace determination of five pharmaceuticals including metoprolol and bisoprolol, in surface, ground and drinking water. This methodology allowed the fully automated analysis of 50 $\mu g\,L^{-1}$ of these drugs consuming only 25 μL of sample and a total time of 20 min. Recoveries were in the range 70–80% for bisoprolol, and in the range 10–20% for metoprolol due to the presence of matrix effect.

On the other hand, Moeder et al. [21] studied the extraction performance of different SPME fibre coatings for the enrichment of polar biologically active substances, paracetamol and phenazone among them. Polyacrylate proved to be the best suited coating for all substances but recoveries for paracetamol and phenazone were rather low, due to their extremely low octanol–water partition coefficient (k_{ow} = 0.46 and k_{ow} = 0.38 for paracetamol and phenazone, respectively) in combination with their high water solubility. Einsle et al. [22] extracted some polycyclic musk compounds and drugs, including phenazone, by using a two phase extraction system consisting of polyethylene membrane bags filled with an organic solvent. In general, chlorophorm proved to be most suited as acceptor phase for all analytes except the highly polar phenzone, whose recoveries were 15 and 18% in bi-distilled and waste-water, respectively. Müller et al. [8] developed an automated hollow fibre membrane technique to extract several pharmaceutical and endocrine disrupting compounds in water samples. Enrichment was carried out inside a porous polypropylene hollow fibre membrane, which separated the aqueous and organic phases and regulated the transfer of analytes, and using *n*-octanol placed inside the hollow fibre as acceptor solution. Phenazone, with the lowest $\log k_{ow}$ value and the largest water solubility, yielded a low enrichment factor, but its favourable signal-to-noise ratio in GC–MS analysis allowed achieve satisfactory detection limits. Stir-bar sorptive extraction (SBSE) followed by liquid desorption in combination with large volume injection (LVI)-in port silylation and gas chromatography–mass spectrometry was applied for the determination of a broad range of 46 acidic and polar organic pollutants in water samples. Again, phenazone provided an enrichment factor lower than 1 [23].

In this work, we propose the combination of coupled column switching and the use of large sample volumes linked to multivariate curve resolution and alternating least squares (MCR–ALS) in combination with standard addition, to solve coelution peaks and effect matrix problems, as an integrated strategy in the preconcentration and quantitation of nine β-blockers (sotalol, atenolol, nadolol, pindolol, metoprolol, timolol, bisoprolol, propanolol and

betaxolol) and two analgesics (paracetamol and phenazone) in environmental surface water.

2. Experimental

2.1. Chemicals and solvents

Analytical standards (pestanal quality) of sotalol (SOT), atenolol (ATE), paracetamol (PARA), nadolol (NAD), pindolol (PIN), phenazone (PHEN), metoprolol tartrate salt (MET), timolol maleate salt (TIM), bisoprolol (BIS), propanolol hydrochloride (PRO) and betaxolol (BEX) were purchased from Sigma–Aldrich (Germany).

Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were obtained from J.T. Baker (Holland). Ortho phosphoric acid (H_3PO_4, 85%), potassium dihydrogenphosphate (KH_2PO_4) of analytical grade were purchased from Merck (Darmstadt, Germany) and sodium hydroxide (NaOH) was obtained from Panreac (Spain).

Buffer solution at 0.025 mol L^{-1} concentration was prepared by dissolving appropriate amount KH_2PO_4 and adjusted to pH 3.0 with H_3PO_4 0.1 mol L^{-1}. Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Mobile phase solvents were filtered through a 0.45 μm cellulose acetate (KH_2PO_4 0.025 mol L^{-1} buffer adjusted at pH 3.0 and Milli-Q water) or polytetrafluoroethylene (PTFE) (MeOH and ACN) and degassed with helium prior and during use.

2.2. Instrumentation and software

On-line sample preconcentration and separation was performed using a PC-LC–DAD system consisted of a high-flow isocratic Model 510 LC pump (P-1), a low-flow gradient Model 600E LC quaternary pump (P-2) both from Waters (Milford, MA, USA), a Type 7000 high-pressure column-switching valve (HP) from Rheodyne (Berkeley, CA, USA) and a 2996 diode array detector (DAD) from Waters.

Sample preconcentration was performed directly on a Hypersil Gold C18 (50 mm × 4.6 mm, 5 μm particle size, 175 A pore size) from ThermoQuest (Waltham, MA, USA) as first column (C-1). LC separation of drugs was carried out using a second analytical column (C-2) Gemini C18 (150 mm × 4.6 mm, 5 μm particle size, 110 A pore size) from Phenomenex (USA). A Digital Venturis FP 575 pentium personal computer using Empower™ 2 (Chromatography Manager, Waters) software was used for acquisition and treatment of data.

Routines for data pretreatment and processing were written in MATLAB (MATLAB 6.0, The MathWorks, Natick, MA, USA, 2000). Routines for MCR–ALS were available on the Internet (http://www.ub.edu/mcr/welcome.htm). Baseline routines are an adaptation of those described in Ref. [24] for second order data and were kindly provided by J. Braga (Unicamp, Brazil). PDS was implemented with PLS Toolbox routines.

2.3. Preparation of standards, spiked samples and standard additions for calibration purposes

Stock standard solutions of each individual compound with concentrations ranged between 300 and 400 mg L^{-1} were prepared by exact weighing of the powder analytical standard and dissolution in 25 mL of MeOH. They were stored at −18 °C in the dark and in these conditions they were stable for at least 3 months. Intermediate working standard solutions of each compound containing 10 mg L^{-1} were prepared by appropriate dilutions of the stock solutions with MeOH and stored under refrigeration at 4 °C. These solutions were replaced by new fresh solutions after 3 weeks.

Working standard solutions of the analytes were daily prepared in MeOH:water (20:80, v/v) and were filtered through Millipore

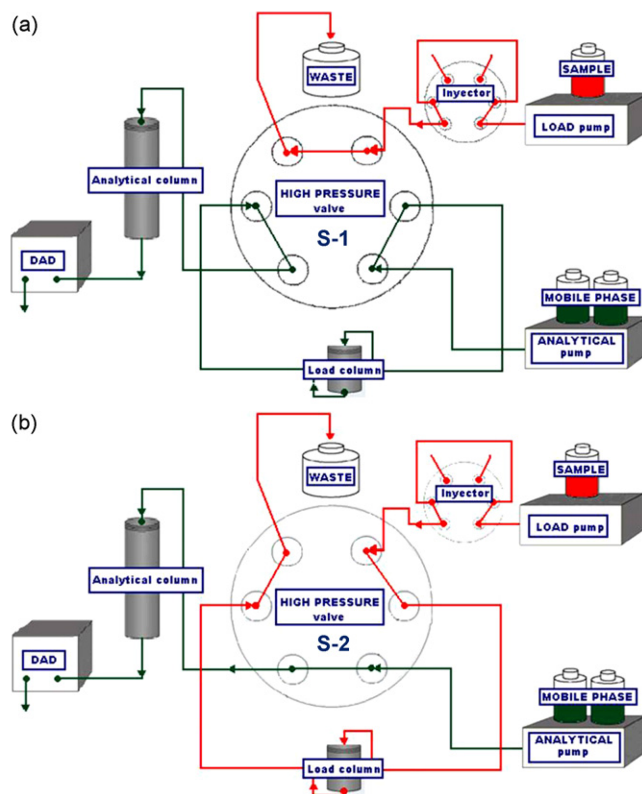

Fig. 1. Schematic diagram of the PC-LC–DAD system used for the on-line preconcentration of pharmaceuticals in river water. (A) HP valve in position S-1 and (B) HP valve in position S-2.

membrane PTFE filters (0.45 μm particle size) before injection into the chromatographic system.

Five river water samples (R1, R2-1, R2-2, R3-1 and R3-2) from three different points along the stream of Nacimiento river (Almería, Spain) (R1, R2 and R3) were used for recovery studies. R2-1 and R2-2 were sampled at the same place with a week interval, as well as R3-1 and R3-2, whereas only a sample was obtained for the point R1, located at the higher part of the stream.

The river water samples were spiked at different concentration levels of β-blockers (see Table 2), thus simulating real samples containing the analytes and then, 0.0, 1.0, 2.0, 3.0 and 4.0 μg L^{-1} of each analyte were added to five aliquots of each previously spiked sample for calibration by the standard addition method. The sample R3-2 was prepared in triplicate with the aim of checking repeatability.

For on-line preconcentration of drugs, after spiking, river or Milli-Q water samples were filtered through a 0.45 μm cellulose acetate membrane and then 400 μL of MeOH were added to each 100 mL of water sample (corresponding to 0.4% of organic modifier in the water sample) just before preconcentration in the PC-LC system.

2.4. On-line procedure and LC separation

The on-line procedure to determine the drugs by PC-LC–DAD methodology is based on a column-switching technique using a HP valve to connect the two LC columns. The HP valve has two positions (S-1 and S-2) that are shown in Fig. 1. The full automated on-line procedure to determine analytes in water sample includes the following three main steps.

STEP 1. The HP valve was at S-1 position and the two LC columns (C-1 and C-2) were coupled in-line whereas the mobile phase of the P-2 pump in the initial conditions (KH_2PO_4 buffer:MeOH 85:15,

v/v) passed through both columns at a flow rate of 1.5 mL min^{-1} for conditioning before processing the first water sample.

STEP 2. After 10 min, the HP valve was switched to the S-2 position in such a way that the mobile phase pumped by P-2 passed only through the C-2 column, while the water sample is loaded on C-1 using the P-1 pump at a flow rate of 1.5 mL min^{-1} during 20 min. In this step the analytes were retained in C-1 while most non-retained interferences were eliminated to the waste.

STEP 3. Next, the HP valve was automatically switched to the S-1 position and the retained analytes were transferred onto the C-2 column in which they were separated and detected in the DAD system. Both transference and separation were carried out by using a programmed gradient with KH_2PO_4 buffer solution (0.025 mol L^{-1} at pH 3.0) as solvent A, MeOH as solvent B and ACN as solvent C at a flow rate of 1.5 mL min^{-1}. The solvent program consisted in a linear gradient from A:B (85:15, v/v) as initial conditions to A:B:C (55:25:20, v/v/v) in 8 min, next 4 min in isocratic conditions and finally a linear gradient to the initial conditions in 2 min, remaining in this conditions for 4 min.

Under the above-described chromatographic conditions, all the analytes were simultaneously analyzed by DAD using a wavelength range between 200 and 350 nm. The total time for the entire PC-LC methodology was 38 min. Univariate analytical figures of merit for the determination of the eleven pharmaceuticals in river water were calculated using PC-LC–DAD signals selected at 222.5 nm, except for PARA and TIM, which were determined at 245.0 and 294.4 nm, respectively.

In order to avoid carryover, during the conventional LC analysis of target drugs in C-2, the P-1 pump was washed with Milli-Q water for 10 min to clean the system before loading the next sample.

3. Results and discussion

3.1. LC optimization

According to the basic properties of β-blockers and PARA, with pK_as around 9 [25,26], their LC analysis requires to control the pH of the mobile phase by adding buffers in order to obtain an adequate and reproducible separation. Thus, several analytical methodologies are available in the literature for the determination of these compounds by LC using phosphate buffer at different pH in the mobile phase [27,28].

Although PHEN does not show the same basic properties than the other analytes, the behaviour of its signal in the selected conditions allowed their determination. The eleven pharmaceuticals were separated on the chromatographic column following a method previously developed by us for the determination of seven β-blockers in river water [18], which was modified to make it suitable for a higher number of analytes. The best results were found using the above described gradient program with KH_2PO_4 buffer solution (0.025 mol L^{-1} at pH 3.00), MeOH and ACN as mobile phase, but it was impossible for the complete separation of the eleven analytes.

3.2. On-line preconcentration

As for the preconcentration step, we proceeded as in the above cited paper [18], including some improvements. Thus, the short chromatographic column was changed from a Hypersil Elite C18 (50 mm × 4.6 mm, 5 μm particle size, 175 A pore size) to a Hypersil Gold C18 (50 mm × 4.6 mm, 5 μm particle size, 175 A pore size) which allowed working without adjusting the sample pH and using only a 0.4% of MeOH as organic modifier, whereas in the previous work it was necessary to adjust the pH of the sample at 3.0 and to use 5% MeOH as organic modifier.

A flow rate of 1.5 mL min^{-1} and a preconcentration time of 20 min were selected as optimal enrichment parameters, corresponding to 30 mL of modified water sample containing 29.88 mL of water sample and 120 μL of MeOH.

However, the use of only 0.4% of MeOH as organic modifier in the aqueous sample resulted in the retention of some non-polar interferences eluting along with the less retained analytes, involving interference problems which were further on resolved by application of chemometric strategies.

During the conventional LC analysis of target drugs in C-2, a washing step was performed for 10 min by pumping Milli-Q water with P-1 through the part of the chromatographic devices which were not involved in the separation task. This was enough to clean the system before loading the next sample.

It must be emphasized that, with respect to the firstly developed methodology, the above-described modifications involved the reduction in the use of organic solvent, also avoiding the need of adjusting the pH of the aqueous phase.

3.3. Analytical figures of merit

Analytical figures of merit were estimated separately for the target analytes by spiking blanks of river water from the lower part of Nacimiento River in Almería (Spain), where the matrix background would be higher, i.e. in the less favourable conditions, and applying the entire PC-LC–DAD method (Table 1).

Method detection limits (MDLs) for the overall PC-LC–DAD method were calculated as proposed by the U.S. EPA [29] in such a way that this parameter takes into account the possible effect of the sample matrix and the variability introduced by all the sample processing steps. The results obtained in this way for the target analytes ranged between 0.03 and 0.16 μg L^{-1}.

Quantitation limits (LOQs), calculated according to the EURACHEM Guidance [30] for $n = 3$ and a relative standard deviation fixed as 10%, were ranged between 0.2 and 0.5 μg L^{-1}.

The linear range was established for each pharmaceutical, according to the criterion defined by Massart et al. [31] and the calibration curves were obtained with eight standards covering the whole linear range (each point in triplicate) and processed through the entire analytical method. They showed good linear relationship ($r^2 > 0.98$) between 0.2 and 10.0 μg L^{-1} according each analyte.

Method precision was evaluated during the same day (intraday) using six river water samples spiked at the LOQ concentration levels of each analyte and at 1 μg L^{-1}. It can be observed that the RSD was lower than or equal to 9.4% for the LOQ level, whereas for 1 μg L^{-1} the RSD was 8.3% for BIS and considerably lower for all the other analytes.

Comparing the above results of validation with those obtained in the previous work, it was concluded that the use of a different short column for preconcentration allowed an improvement in the sensitivity by achieving lower MDLs and LOQs, also with acceptable precision.

3.4. Complexity of the matrix samples. Application of background correction

The high complexity of the analytical problem under study can be appreciated in Fig. 2A, which shows a chromatogram ($\lambda = 210$ nm) of a river water sample, obtained after spiking with the eleven analytes at different concentration levels (sample R2-1). Table 3 shows the elution order for all the compounds and the regions in which the total chromatographic data were divided in order to simplify the analysis, including the corresponding sensors and time intervals. As can be seen, only PARA (peak number 3) and BIS (peak number 9) were completely separated from the other ana-

Table 1
Univariate analytical figures of merit for the determination of the eleven pharmaceuticals in river water using PC-LC–DAD signals selected at λ = 222.5 nm, except for PARA (λ = 245.0 nm) and TIM (λ = 294.5 nm).

Analyte	Linear range ($\mu g L^{-1}$)	r^2	Repeatability RSD (%)[a]		MDL ($\mu g L^{-1}$)	LOQ ($\mu g L^{-1}$)
			LOQ concentration level	1 $\mu g L^{-1}$		
SOT	0.5–10.0	0.9997	5.2	3.5	0.11	0.5
ATE	0.2–10.0	0.9990	9.4	4.0	0.09	0.2
PARA[b]	0.5–10.0	0.9972	2.6	2.4	0.16	0.5
NAD	0.5–10.0	0.9968	7.5	4.2	0.16	0.5
PIN	0.5–10.0	0.9998	4.0	1.2	0.10	0.5
PHEN	0.5–10.0	0.9999	7.5	2.6	0.12	0.5
TIM[c]	0.2–10.0	0.9969	8.0	3.0	0.14	0.2
MET	0.5–10.0	0.9977	8.1	2.1	0.13	0.5
BIS	0.2–10.0	0.9904	6.9	8.3	0.11	0.2
PRO	0.2–10.0	0.9975	8.0	4.1	0.03	0.2
BEX	0.5–10.0	0.9987	5.3	4.4	0.15	0.5

[a] $n = 6$.
[b] λ = 245.0 nm.
[c] λ = 294.5 nm.

lytes by the chromatographic procedure. In addition, a considerable number of matrix compounds were also retained in the preconcentration procedure, which coeluted with the analytes leading to strong overlapping (see peak number 9) and a considerable baseline drift. Owing to these drawbacks, neither identification of the analytes nor application of classical univariate calibration to quantitate them was possible, being necessary the use of chemometric strategies for identification and quantitation.

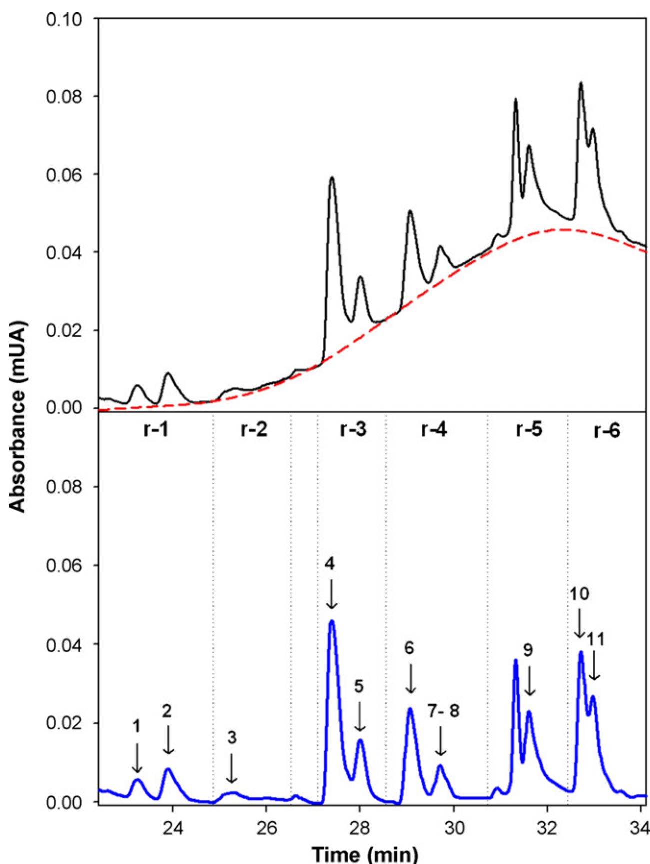

Fig. 2. (A) Chromatogram (λ = 210 nm) of a water river sample obtained after spiking the river water sample with different concentrations of the eleven analytes (sample R1-1) (black solid line) and the base line calculated at the same wavelength (red dashed line). (B) Corrected chromatogram (λ = 210 nm) obtained by substraction of the base line to the original chromatogram (blue solid line). The numbers correspond to analytes (see Table 3), and r-1 to r-6 correspond to regions in which the data were split. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Reducing the complexity of the matrix sample through the elimination of the chromatogram baseline has been shown to be a critical step to improve the quality of the analytical results [32–34]. With this aim, among the different strategies for baseline correction being proposed in the literature we chose the asymmetric least-squares method, a methodology proposed by Eilers, which is the multidimensional extension of the spline-based approach, a novel algorithm taking advantage of the special structure of both the data as an array and the model matrix as a tensor product [24,35].

The method consists in obtaining a background correction matrix with the same dimensions as those for sample and spiked samples matrices by using spline basis functions (herein, ten of them were used), with a single regularization parameter whose value was 1. Details about the implementation of the algorithm can be found in the literature [35]. Fig. 2A shows the baseline calculated at λ = 210 nm and Fig. 2B shows the resultant chromatogram (λ = 210 nm), when subtraction of the baseline allowed us to obtain a corrected data matrix. The simplification of the data complexity is evident after this data pretreatment.

3.5. Quantitation in river water samples by MCR–ALS modelling and the standard addition method for calibration

In order to evaluate the presence of matrix effect, slopes and intercepts of univariate calibration curves built with standards prepared in Milli-Q and river water were compared using hypothesis tests (Statgraphics Plus V.4). These comparisons evidenced matrix effect and the presence of systematic constant errors for most analytes (p-values < 0.05 in all cases).

As river water samples contained unexpected interferences and sensitivity changes due to sample matrix were also observed, a strategy involving standard addition calibration in combination with MCR–ALS [36] was implemented. In this way, the standard addition calibration deals with matrix effect and MCR–ALS coped with coeluting interferences, on account of the second order advantage inherent to this algorithm [37], which, in addition, is able to handle data sets deviating from trilinearity, like the HPLC-data analyzed in the present report. In order to exploit the mentioned advantages, in MCR–ALS data are augmented along the mode which is suspected of breaking the trilinear structure, i.e. if a matrix-to-matrix variation of profiles occurs along the column direction, a column-wise augmented matrix is created. The bilinear decomposition of the augmented matrix **D** is performed according to the expression:

$$\mathbf{D} = \mathbf{C} \times \mathbf{S}^T + \mathbf{E} \quad (3)$$

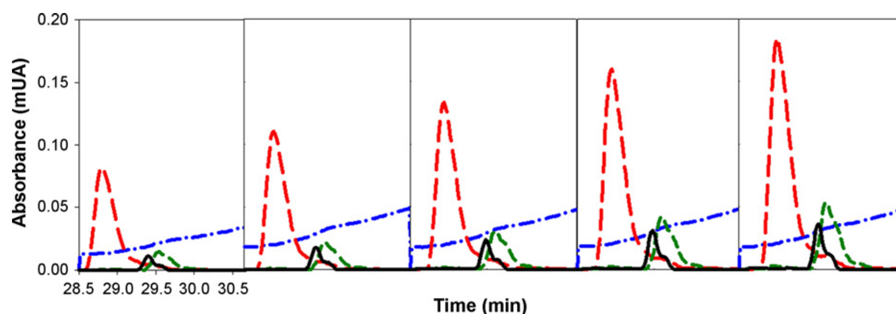

Fig. 3. Successive time profiles corresponding to the MCR–ALS analysis for region 4 which includes peaks number 6 (PHEN) (red long dashed line), 7 (TIM) (black solid line), 8 (MET) (green short dashed line), and the interference (blue dash dotted line) on sample R2-1. The remaining four profiles correspond to four standard addition calibration samples of 1.00, 2.00, 3.00 and 4.00 $\mu g L^{-1}$ of each analyte. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Nominal concentrations of the eleven analytes in validation samples.

Analyte	Spiked concentration in validation samples ($\mu g L^{-1}$)				
	River 1	River 2		River 3	
	R1-1	R2-1	R2-2	R3-1	R3-2[a]
SOT	1.00	1.50	2.50	2.00	1.00
ATE	3.00	1.00	1.00	2.00	1.00
PARA	1.00	1.50	2.50	2.00	1.00
NAD	1.00	1.50	2.50	2.00	1.00
PIN	3.00	1.00	1.00	2.00	1.00
PHEN	2.00	3.00	1.50	2.00	1.00
TIM	1.00	1.50	2.50	2.00	1.00
MET	3.00	1.00	1.00	2.00	1.00
BIS	2.00	3.00	1.50	2.00	1.00
PRO	3.00	1.00	1.00	2.00	1.00
BEX	2.00	3.00	1.50	2.00	1.00

[a] Sample R3-2 was prepared in triplicate.

Table 3
Regions in which chromatographic data were divided.

Region	Analytes (assigned number)	Sensors (data points)	Times (min)
1	SOT (1) ATE (2)	1–145	22.42–24.89
2	PARA (3)	146–250	24.85–26.58
3	NAD (4) PIN (5)	280–370	27.08–28.57
4	PHEN (6) TIM (7) MET (8)	371–500	28.59–30.74
5	BIS (9)	501–600	30.75–32.40
6	PRO (10) BEX (11)	601–701	32.42–34.10

where the rows of **D** contain the absorption spectra measured (J wavelengths) as a function of time (K times), the columns of **C** contain the time profiles of the N compounds involved in the process, the columns of **S** their related spectra, and **E** is a matrix of residuals not fitted by the model. Appropriate dimensions of **D**, **C**, $\mathbf{S}^T$ and **E** are thus $(1+I)K \times J$, $(1+I)K \times N$, $N \times J$ and $(1+I)K \times J$, respectively (I = number of training samples). Decomposition of **D** is achieved by iterative least-squares minimization of $||\mathbf{E}||$ under suitable constraining conditions, i.e. nonnegativity in spectral profiles, and unimodality and nonnegativity in concentration profiles.

The pure spectra of the compounds should be the same in all experiments, but the profiles in the different **C** sub-matrices need not share a common shape. This is the reason why chromatographic runs can be analyzed together as long as the spectra of the compounds involved in the process remain invariant.

The five test samples (samples R1-1, R2-1, R2-2, R3-1 and R3-2) were spiked with the concentrations corresponding to the eleven analytes which are displayed in Table 2 and then, 0.00, 1.00, 2.00, 3.00 and 4.00 $\mu g L^{-1}$ of each analyte were added on each of the five aliquots of each test sample and used for calibration by the standard addition method. Each test sample was analyzed in the following way: (a) firstly, in order to simplify the models, the spectral-time matrix for a given test sample was partitioned in six regions (see Table 3), and (b) then, for each region, the resulting data matrix was augmented with the five matrices recorded for the calibration samples.

It is necessary to point out that MCR–ALS requires initialization with system parameters which should not be random numbers. In this case (column-wise augmentation mode), the analyte spectra are required as obtained from either pure analyte standards or from the analysis of the purest spectra based on the so-called SIMPLISMA (simple interactive self-modelling mixture analysis) methodology [38], a multivariate curve resolution algorithm which extracts pure component spectra from a series of spectra of mixtures of varying composition. We have reached excellent fitting results by using the latter methodology. The generation of suitable initial estimations was only possible when the correct noise level of the data was took into account, due to the low signal-to-noise ratio presents in the analyzed samples (it is worthy to note that extremely low concentrations of the analytes are being analyzed). In the present case, the noise level was increased from 0.1 up to reaching a consistent selection of spectra (ca. 10%).

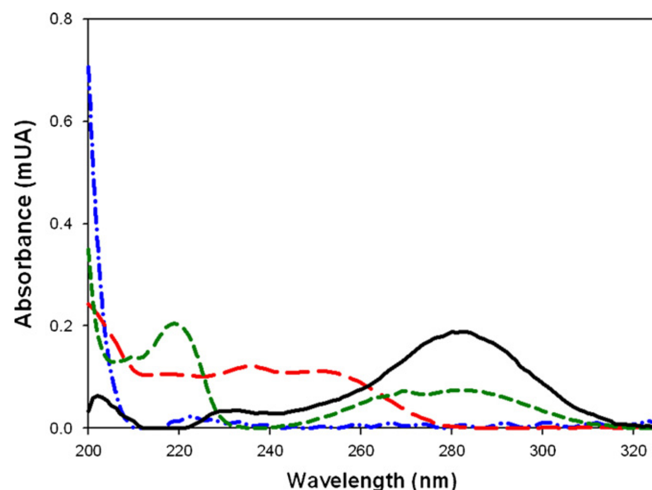

Fig. 4. MCR–ALS spectral profiles for peaks number 6 (PHEN) (red long dashed line), 7 (TIM) (black solid line), 8 (MET) (green short dashed line) and the interference (blue dash dotted line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Finally, decomposition was performed by imposing the restrictions of nonnegativity in spectral profiles and unimodality and nonnegativity in concentration profiles.

As an example of how each sample was analyzed, Fig. 3 shows time profiles extracted when analysing region number 4, which corresponds to PHEN, TIM and MET, in sample number R3-2. As can be seen in the five sub-figures, four profiles were extracted, showing a severe overlapping between them. This fact is especially evident for TIM and MET because their elution times are rather similar. Interestingly, the decomposition of the data into the relevant contributions by using the MCR–ALS algorithm allows using the extracted profiles for quantitative purposes. In this case, the successive parts in Fig. 3 show signals corresponding to the increasing concentration of analytes related to the standard additions and a constant signal. Thus, the isolation of the signals which can be ascribed to every analyte in each studied test sample can be used for accurate analyte quantitation. Consequently, the use of the relative peak areas for the three analytes corresponding to the studied region (number 4) let us build the corresponding pseudo-univariate standard addition curves. It is interesting to point out that the interference profile, which is also observed in Fig. 3, remains constant, indicating that it is present in the original sample in a constant way and, therefore, it may be assigned as a constant interfering matrix compound. Finally, the spectral profiles extracted for PHEN, TIM and MET and the interference in the same sample (R3-2) are shown in Fig. 4.

The presence of interferences in each of the peaks being analyzed was checked through the number of estimated components, which were calculated by applying singular value decomposition (SVD) to each region of each data matrix. This number depends on the time window selected and the origin of the sample, and when it is higher than the number of analytes, confirms that the choice of a second order strategy is the most convenient option for calibration. The number of components for the present system is displayed in Table 4, which also summarized, for comparison, the number of analytes eluting in each region. In general, interferences appear in all regions, in a number that does not significantly changes between regions, days and sampling sites, except for the samples from the lower point at the river stream (R3), due to the presence of more abundant interferences in this place, as would be expected.

Table 4
Number of analytes eluting in each region and number of components obtained when applying singular value decomposition to the matrix corresponding to each sample.

Region	Sample					
	Analytes	R1-1	R2-1	R2-2	R3-1	R3-2
r-1	2	5	4	4	7	7
r-2	2	3	3	4	5	4
r-3	2	3	4	4	3	3
r-4	3	4	4	4	4	4
r-5	1	4	4	4	3	4
r-6	2	3	4	4	4	4

Predictions for all the eleven analytes in the five validation samples (Table 2) are displayed in Table 5, together with recoveries which were computed taken into account the nominal concentrations spiked in the water river samples. It is important to note that predictions in most of the samples can be considered acceptable taking into consideration the complexity of the analytical problem and the low concentration of the analytes. However, to assess the accuracy of the predictions, the obtained values were compared with the nominal ones corresponding to the eleven analytes. For this purpose, the joint statistical test for the slope and the intercept of the linear regression between the nominal concentration values versus those predicted was applied. When applying this test, predictions are regarded as being accurate if the theoretical values of intercept and slope (zero and the unity, respectively) are included within the ellipse, which describes the mutual confidence region. As has been previously suggested, when multianalyte analysis is performed, it is highly convenient to include experimental data corresponding to all analytes in order to better estimate the variance corresponding to the regression discussed above. This avoids the oversizing of the joint confidence region

Table 5
MCR–ALS predictions obtained on real river water samples spiked with different amounts of the analytes (see Table 2).

Analyte	Predicted concentrations ($\mu g\,L^{-1}$)[a]				
	River 1	River 2		River 3	
	R1-1	R2-1	R2-2	R3-1	R3-2[b]
SOT	1.10 (110.0)	1.54 (102.7)	2.56 (102.4)	1.93 (96.5)	0.94 [1.0] (94.0)
ATE	3.00 (100.0)	0.98 (98.0)	0.77 (77.0)	2.19 (109.5)	1.00 [10.0] (100.0)
PARA	1.16 (116.1)	1.58 (105.3)	2.82 (112.8)	1.85 (92.5)	0.96 [3.0] (96.0)
NAD	0.91 (91.0)	1.33 (88.7)	2.02 (80.8)	2.11 (105.5)	0.88 [2.0] (88.0)
PIN	3.33 (111.0)	0.96 (96.0)	1.40 (140.0)	2.01 (100.5)	1.20 [14.0] (120.0)
PHEN	2.14 (107.0)	3.29 (109.7)	1.59 (106.0)	2.12 (106.0)	1.07 [3.0] (107.0)
TIM	0.71 (71.0)	1.83 (122.0)	2.54 (101.6)	2.07 (103.5)	1.15 [6.0] (115.0)
MET	3.17 (105.7)	1.32 (132.0)	0.90 (90.0)	2.03 (101.5)	0.95 [7.0] (95.0)
BIS	2.03 (101.5)	2.80 (93.3)	1.54 (102.7)	1.94 (97.0)	0.85 [10.1] (85.0)
PRO	3.07 (102.3)	1.06 (106.0)	0.77 (77.0)	2.20 (110.0)	1.10 [3.0] (110.0)
BEX	1.64 (82.0)	2.72 (90.7)	1.41 (94.0)	1.80 (90.0)	1.22 [13.0] (122.0)

[a] Recoveries in parenthesis.
[b] CV (%) for sample R3-2 in square brackets.

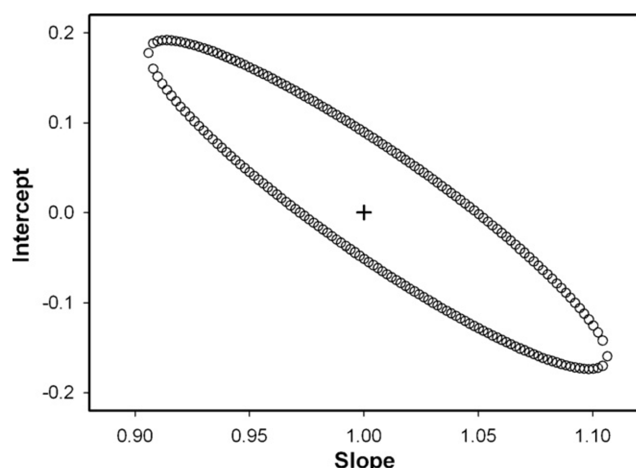

Fig. 5. Elliptical region for the global data set for predictions using MCR–ALS algorithms on the five river water samples (Table 4). The cross-mark corresponds to the theoretical value of zero for intercept and one for slope.

due to large experimental random errors and thus the probability of not detecting the presence of bias [39]. Fig. 5 shows that the region computed for predictions of the global data sets contains both the theoretically expected values (0) for the intercept and (1) for the slope (at a confidence level of 95%). The computed values were slope = 1.01 (s = 0.03) and intercept = 0.01 (s = 0.07). This fact is indicative of the absence of both constant and proportional error.

Finally, the relative standard deviations (RSD%) computed for results obtained when analyzing the three replicates performed for sample R3-2 were lower than or equal to 10%, except for PIN and BEX. Once again, the overall precision could be considered acceptable taking into consideration the complexity of the analytical problem and the low concentration at which the pharmaceuticals were analyzed.

4. Conclusions

A simple, efficient, selective and low cost methodology for the determination of nine β-blockers and two analgesics in environmental surface water was developed by HPLC–DAD assisted with the combination of coupled column switching linked to the use of large sample volumes and chemometric approaches.

In the chromatographic approach, the column switching performed a combined preconcentration and clean-up step, which was partially automated, without modify the pH of the water sample and using minimum volumes of organic solvent, in compliance with the goals of green analytical methodologies.

As for chemometrics, a baseline correction step was implemented, making possible to reduce the large baseline drift caused by interferences which, even though the clean-up step, were present at the retention times of the analytes. Additionally, second order data, generated by recording spectra during the chromatographic time evolution, were successfully handled to assess the content of the analytes, even with overlapped peaks or in the presence of interferences and matrix effect. This was achieved by data modeling with the MCR–ALS algorithm in combination with the standard addition calibration mode.

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