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Abstract

In this work the electrochemical behavior of substances of environmental concern [bentazone, atrazine, carbamazepine, phenytoin and its metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin, HPPH] on a glassy carbon working electrode (Ag/AgCl reference electrode) was studied with the aim to develop a HPLC method coupled with amperometric detection. Constant potential (DC), pulsed amperometric detection modes were studied. For the pulsed mode, several waveforms were set and investigated. Detection conditions were optimized as a function of eluent pH.

In order to reduce the limits of detection and to analyze natural water samples, a SPE protocol was optimized to be coupled to the developed procedure. For this aim, five sorbents of different physico-chemical characteristics were tested optimizing a recovery procedure for each of the cartridge evaluated.

At the optimized SPE conditions, recoveries were included in the range (R = 90.2-100.5% for all the analytes, with excellent reproducibility (< 3%, n = 3). The method detection limits obtained by pulsed amperometry after the SPE protocol (preconcentration factor 100) were 113 ng L^{-1} (0.47 nmol L^{-1}), 67 ng L^{-1} (0.25 nmol L^{-1}), 234 ng L^{-1} (1.1 nmol L^{-1}), for bentazone, HPPH and carbamazepine, respectively. Robustness of the method was assessed for each analyte at a concentration level corresponding to about three times the limit of detection, through the evaluation of intra-day (n=13) and inter-day tests (4 days, n=52). Finally the method was successfully applied for the analysis of a river sample (Po River, Turin, Italy).

Keywords: amperometric detection, glassy carbon electrode, RP-HPLC, emerging contaminants, waters

1. Introduction

Contaminants of emerging concern in water sources have been of particular interest in the last decade and could pose a risk to human health as well as to the environment as a function of their presence, frequency and occurrence; their impact on aquatic wildlife populations has been demonstrated to occur at very low concentrations. Among them, herbicides and pharmaceutical compounds are among the most widely occurring pollutants [1] due to their extensive use.

Many herbicides are potentially dangerous not only to human health but also to other organisms in the environment. Among them, bentazone and atrazine are of environmental concern. Both herbicides inhibit, in chloroplast, the Hill reaction, necessary for the oxygen evolution in the photosynthetic process [2, 3]. Health effects of atrazine as human carcinogen still remain controversial [4].

The widespread contamination of drinking water by atrazine was associated with birth defects and hormonal disturbance effects [5, 6]. Even though banned in the EU, atrazine is the most widely used herbicide in the US.

Bentazone belongs to the thiadiazine group and is widely used as post-emergence herbicide. It exhibits little sorption in soil and due to its relatively high mobility [7], the potential risk of leaching and ground water contamination is very high. Bentazone is commonly detected in ground and surface waters [8]. Toxicologic studies show that bentazone has acute and chronic toxicity (inflammation of the mucous membranes, tachycardia, renal failure).

Pharmaceuticals, like antiepileptic drugs, are found regularly in surface waters, phenytoin and carbamazepine are anti-epileptic and anticonvulsant substances and just like many active drugs, if overdosed are toxic. Recent studies show their presence in finished drinking waters [9]. In addition it was proved that the presence of phenytoin and carbamazepine in wastewater decreases the efficiency of wastewater treatment plants [10, 11].

The monitoring of these contaminants in the aquatic environment is progressively becoming a priority for government agencies, regulatory agencies and the general public.

As regards the analytical determination, the mainly used techniques are based on chromatographic mechanisms (gas chromatographic and HPLC) coupled to specific mass spectrometric and spectrophotometric detectors. Although gas chromatographic methods can successfully be employed for the determination of some compounds like carbamazepine and bentazone at ng L^{-1} levels after solid-phase extraction, GC requires the choice of the correct derivatizing agent that avoids decomposition of the analytes. This in turn implies that the same derivatizing agent cannot be used for the simultaneous analysis of different classes of compounds of interest [12].

As regards HPLC methods, this approach has been chosen for the determination of bentazone, atrazine, phenytoin, carbamazepine in environmental matrices like natural waters, soils, and in foodstuff like rice and cereals. Limits of detection (LODs) depend mainly upon the detection mode and the extraction technique used. For example, LODs ranging from 5-15 μg L^{-1} for bentazone [13, 14] have been obtained by SPE extraction-HPLC with UV detection after a 500-fold preconcentration. Lower detection limits have been

achieved with higher preconcentration ratios (e.g. for carbamazepine: 50 ng L^{-1} [15]). Less satisfying LOD values have been obtained with liquid-liquid extraction techniques (e.g. about 100 μg L^{-1} for carbamazepine with UV detection [16]). Techniques such as LC-MS and LC-MS/MS have been used to determine herbicides and pharmaceutical compounds in natural waters at generally lower detection limits [17-19], but with the main drawback of requiring expensive equipments with high maintenance costs and skilled technical staff.

Electroanalytical techniques have been shown to be useful in the study of toxic substances of environmental concerns such as pesticides [20] and they have been recently used in pulsed amperometric mode also on glassy carbon electrodes for the determination of chlorophenols [21]. When coupled to chromatography, the specificity of the electrochemistry techniques is increased significantly. In recent years, the number of published papers dealing with the use of chromatographic methods with amperometric detection is growing thanks to selectivity, sensitivity and low cost of the detection technique [22]. Amperometric detection proved to be successful for the determination of compounds of environmental concern [23-27].

According to the characteristics of the molecules to be determined, the amperometric detection can be considered for identification and quantification of target compounds.

The aim of this work was to study the chromatographic separation and the electrochemical behavior of bentazone, atrazine, phenytoin, and carbamazepine. Since phenytoin is metabolized by cytochrome P450 enzymes primarily to 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), and is mainly excreted as 5-(4'-hydroxyphenyl)-5-phenylhydantoin O-glucuronide in humans [28], HPPH was included in our study, too. Constant potential (DC) amperometry, pulsed amperometric detection were compared to assess the best electrochemical response for the analytes tested. The chromatographic method optimized was coupled to a solid-phase extraction step which provided reduced detection limits with a simultaneous clean-up of the matrix analyzed. The study performed allowed us to develop a sensitive, affordable analytical procedure based on pulsed amperometry which is of simple application.

This work is the first study devoted to the analysis of bentazone, atrazine, carbamazepine, phenytoin and HPPH by HPLC with amperometric detection.

2. Material and Methods

2.1. Chemicals and standard solutions

All reagents used throughout this work were of analytical grade. Acetonitrile (99.9 %), bentazone, HPPH, phenytoin, carbamazepine, atrazine, nitric acid (65% w/w, d = 1.40 g/mL) and acetic acid (99.8% w/w, d = 1.052 g/mL) were from Sigma-Aldrich (Chemie, Steinheim, DE). Methanol and NaOH (purity > 98%) were from Carlo Erba (Milano IT).

A Milli-Q Plus ultra-pure water system from Millipore (Milford, MA, USA) was used for standard and eluent preparation.

2.2. Instruments

For the chromatographic separations, a Dionex ICS-3000 chromatograph (Thermo Scientific, Sunnyvale, CA, USA), equipped with a reversed-phase C-18 pre-column and analytical column (LiChroCart PuroSphere RP-18, 125 mm x 3.0 mm, 5 μm, Merck) was used. The mobile phases were sodium acetate or sodium formate buffers (50 mM) at different pH values. CH_3CN was used as organic modifier. The analysis were performed in isocratic mode (eluent flow rate 0.5 mL min^{-1}). A 10 μL-injection loop was used throughout this work. Pre-column and analytical column were periodically washed by isopranol and reconditioned with eluent for 35 min.

Two detectors coupled in series were used, namely AD25 Absorbance Detector (λ = 252 nm) and AD40 Electrochemical Detector (both by Thermo Scientific, Dionex), with a Ag/AgCl reference electrode and a glassy carbon (GC) working electrode. The parameters of the electrochemical detector were optimized as described in the section "*Optimization of amperometric detection*". Chromatographic and amperometric data were collected and elaborated by the software Chromeleon 6.80 (Thermo Scientific, Dionex).

2.3. Solid Phase Extraction (SPE)

For the extraction of the analytes, the performance of five SPE supports of different composition were compared. In detail, two C18 based adsorbents: Bond Elut C18 Jr (Agilent), ENVI 18 (Supelco), two polymeric sorbents: LiChroLut EN (Merck), BakerBond SDB (JT Baker) and one carbon based substrate: ENVI Carb (Supelco) cartridges were activated according to manufacturer's indications before use.

For each cartridge, aliquots of 5 mL containing 1 mg L^{-1} each of bentazone, atrazine, phenytoin, HPPH and carbamazepine were loaded at 2 ml min^{-1} flow rate. For each cartridge, the eluent solution was optimized according to the expected interactions analyte-sorbent. Before elution, each cartridge was washed with 2 mL H_2O to remove unretained compounds. In order to check the retention of the analytes in each step of the SPE protocol, the following three fractions were collected and injected in the HPLC system: (i) the solution after loading; (ii) the washing solution and (iii) the eluate.

Recoveries are expressed as average of three independent extractions. In parallel, a blank was processed for all the cartridges tested. For the optimization of the SPE protocol, the UV detection was used.

2.4. Real sample analysis

A river sample (Po river, Turin, Italy) was collected at 24/07/2013 from between km 104 and 105, stored in a Pyrex bottle at 4°C, protected from light until the analysis. Analysis was performed within 24h from the sampling.

Analytes were SPE extracted and analyzed according to the procedure optimized throughout this work which is summarized in Fig. 1 of Supplementary Material section, where all the steps of the protocol are detailed.

3. Results and discussion

The analytes considered in this study are shown in Fig. 2 of Supplementary Material section. Bentazone displays keto-enol tautomerism [29] whereas phenytoin displays tautomerism of the imine–imide type [30], as shown in figure. A similar behavior should be expected even for the metabolite HPPH.The experimental work consisted in a preliminary optimization of the chromatographic separation, followed by a voltammetric study before optimizing the experimental conditions for the electrochemical detection.

3.1. Chromatographic optimization

In order to optimize the separation of the analytes, four mobile phases (50 mM CH_3COOH/CH_3COONa, pH 5.0) with CH_3CN content ranging from 28%-35% were tested (Fig. 3 of Supplementary Material section).

At all the eluent compositions investigated, bentazone is the species less retained by the column since according to its pK_a value bentazone is present in the ionized form (see equilibrium depicted in Fig. 1 of Supplementary Material section). The presence of the –OH group in the phenyl substituent dramatically reduce retention for HPPH in respect to phenytoin. It should be remarked that phenytoin is not ionized at the elution pH condition.

As shown in Fig. 3 of Supplementary Material section, capacity factors for all analytes except bentazone and HPPH are significantly affected by the CH_3CN content, due to the decreased hydrophobic interactions that in turn reduce the retention in the column.

Additionally, the effect of pH on separation was also investigated. The decrease of pH caused an increase in the retention of bentazone (see Fig. 4 of Supplementary Material section), since at this condition the undissociated form of bentazone is also present. The chromatographic behavior for both HPPH and carbamazepine is affected by pH, observing a decreased retention with more acidic pH values.

According to the results obtained, an eluent containing 28% CH_3CN buffered at pH 5 was chosen for the further step of optimization of electrochemical detection conditions.

3.2. Optimization of a SPE protocol

In order to extract the analytes from environmental water samples and to reduce the LOD values, a SPE step was optimized comparing the performance of five sorbents of different chemical composition (see Table 1).

According to the expected analyte-substrate interactions for the octadecyl-silanized silica phases (*Bond Elut C18, ENVI 18)*, the analytes were eluted by 100% CH_3CN. Very satisfactory recoveries were obtained for the *Bond Elut C18* phase for all the analytes, whereas a milky eluate was obtained with the *ENVI 18* sorbent. The *LiChroLut EN* phase required sample acidification to pH 3 in order to enhance the retention of bentazone in the sorbent. For this cartridge, several eluents were tested for the recovery of the analytes (100% CH_3CN, 80:20 $CH_3CN:H_2O$, 70:20:10 $CH_3CN:H_2O$:isopropanol). An increase of eluent polarity was beneficial to increase the recovery for all the analytes except for carbamazepine, whereas the recovery of this last species was favored by the presence of isopropanol. It should be mentioned that in order to avoid peak broadening caused by isopropanol, the eluate was evaporated and reconstituted with H_2O before injection. For this cartridge, the best recoveries (Table 1) were obtained with 70:20:10 $CH_3CN:H_2O$:isopropanol. Due

to the physico-chemical similarity of the *BakerBond SDB* and *LiChroLut EN* phases, the same eluent solution was also used for *BakerBond SDB* cartridge (Table 1). As regards the *ENVI Carb* phase, several elution mixtures at different polarity were tested: CH_3CN; 60:40 CH_3CN:CH_3OH; 50:50 CH_3CN:HNO_3 pH 2; CH_3COCH_3; 80:20 isopropanol:H_2O. Partial recovery (Table 1) was obtained only by the last eluent mixture.

To summarize, the SPE cartridges which provided the best recoveries were the Bond Elut Jr. C18, LiChrolut EN and Bakerbond SDB. For further method performance evaluation and real sample analysis, Bond Elut Jr. C18 was chosen for the faster and simpler procedure involved.

3.3 Preliminary characterization of the electrochemical behavior of the target compounds

In order to set proper electrochemical conditions for the detection of the analytes, cyclic voltammetries (CV) were initially performed from -1.00 V to +2.00 V, back again to -1.00 V in 60 seconds (scan rate 0.1 V s^{-1}) directly inside the detection cell of the ICS-3000 system, under static conditions. Cyclic voltammetries were performed on a blank (eluent solution: 72 % of a 50 mM CH_3COOH/CH_3COONa, pH 5.0 solution and 28% CH_3CN), and on solutions of individual analytes prepared in the eluent. The CV profiles shown in Fig. 1 point out that some capacitative current is present in the system. As shown, both atrazine and HPPH do not present any current peak in the range of potentials investigated. The other three analytes show oxidation peaks (E^{ox}_p) at 1.30 V (bentazone), 1.55 V (carbamazepine) and 1.10 V (phenytoin) proving their electroactivity at the GC electrode. Their voltammograms show that they are oxidized in single irreversible processes. In order to better understand the electrochemical behavior of the molecules and in order to define an operative pH range for the following amperometric detection optimization, experiments were repeated at pH 8 (72 % of a 50 mM NaH_2PO_4, pH 8.0 solution and 28% CH_3CN).

This value was chosen accordingly to the pK_a values of the tested analytes in order to explain the electrochemical behavior with the chemical form of the species.

For bentazone, the oxidation potential decreased of 0.18 V in agreement with the shift of approximately -60 mV pH^{-1}. Peak current values (i^{ox}_p) decreased from (0.225 μA to 0.04 μA), suggesting that the –OH group is unlikely to be involved in the oxidation process. The oxidation peak observed at 1.1 V at pH 5 suggests the fouling of the electrode and a restored electrode activity for the amount of species remained in solution. The fouling of the glassy carbon electrode in presence of bentazone was also evidenced by Garrido et al. [31] while studying the electrochemical oxidation of bentazone, who hypothesized a dimerization after the oxidation reaction (corresponding to an electron transfer) and the adsorption of the product on the electrode surface.

For phenytoin, voltammograms at pH 8 do not reveal oxidation peaks, confirming also for this molecule the fact that the hydroxyl group (see Fig. 2 of Supplementary Material section) should be probably not involved in the electrochemical process.

According to carbamazepine's pKa values, dissociation for this species is not affected by pH variations. According to the voltammetry performed at pH 8, the oxidation potential decrease of 0.3 V. Peaks observed at 0.2 V and -0.4 V confirm the irreversibility of the oxidation process and suggest that the reduction of species different from the oxidation product of carbamazepine is occurring.

3.4. Optimization of amperometric detection

This part of the work was accomplished by coupling an amperometric detection, both in the DC and pulsed amperometry modes, to the chromatographic separation previously optimized. We tested the capabilities of DC detection at six potential values (1.0, 1.1, 1.2, 1.3, 1.4, 1.5 V) around the oxidation potentials observed in the CV study. As a consequence of the reversible fouling behavior observed for the electrode when performing the cyclic voltammetries, in addition to DC mode, pulsed amperometry mode was also studied for the determination of the target analytes, comparing four waveforms and setting the oxidation potential at 1.2 V.

Four types of waveforms were studied (see Table 2); time of delay, time of integration, detection potential and cleaning potential were set through the software Chromeleon 6.80.

Waveforms 1 and 4 differ in the step for electrode cleaning: for waveform 1 a reduction/oxidation cycle is performed, whereas for waveform 4 a oxidation/reduction cycle is set. It should be remarked that in waveform 4 it was not possible to set the same negative potential as for waveform 1 (-2.0 V), since the baseline was not stable. Therefore, the most negative potential value that still ensured baseline stability was chosen (-1.1 V).

Analytes were injected at a concentration of 4 μmol L^{-1}. As a further optimization, in order to evaluate the response of analytes, three different pH values in the weak acidic range (pH 3-5) were evaluated. Since the pK_a for bentazone is included within the weak acidity pH range, it is reasonable to expect also a change in the chromatographic behavior for this compound, as shown in Fig 4 of Supplementary Material section.

For each pH condition tested and for each analyte, the performance of the DC and pulsed amperometry detection modes was evaluated calculating both peak areas and the signal/noise (S/N) ratio. During this study, a UV detector (252 nm) was coupled before the electrochemical detector in order to univocally identify the compounds.

3.4.1. Eluent pH 5.0

First analyses were performed by the DC detection mode. Differently from what expected by the previous CV studies, phenytoin which showed E^{ox}_p= 1.1 V, could not be detected at none of the potential values investigated (see hydrodynamic voltamograms in Fig. 2), probably for the slow kinetics of the oxidation reaction, preventing detection under flow conditions. The opposite behavior was observed for phenytoin's metabolite (HPPH) for which a chromatographic peak in hydrodynamic conditions is observed. It can be hypothesized that during CV analysis, performed in static conditions, HPPH adsorption on the electrode hampered the electrochemical process. In agreement with CV results, atrazine was not detected even in

hydrodynamic conditions. As expected by the CV studies, carbamazepine is not detectable for *E* values lower than 1.1 V. According to the data obtained, the highest S/N values were obtained at 1.2 V. This value was subsequently set as the oxidation potential in the pulsed amperometry waveforms (see Table 2).

As shown in Fig. 3, for pulsed amperometry, the best detection conditions can be achieved by waveform 1, where the highest S/N ratios (Figure 3b) are obtained. It is interesting to note that the inversion of the order between oxidation and reduction processes in the electrode cleaning cycle (waveforms 1 and 4) greatly affects the sensitivity of the detection, especially for bentazone and HPPH. Typical chromatograms obtained by the waveforms investigated are shown in Figure 3c. Owing to the low sensitivity exhibited by waveforms 2-4, they were not further included in the electrochemical study performed at pH 4 and 3 (see following sections).

The best results in terms of S/N values and peak area obtained at pH 5 (namely DC: 1.2 V and pulsed amperometry: waveform 1) which are compared in Fig. 5 of Supplementary Material Section, show that waveform 1 provided the best S/N ratio values for all the species, although the highest areas were obtained by the DC mode. This situation is clearly depicted in Fig. 4 where a far less noisy baseline is obtained for pulsed amperometry detection, resulting in a better S/N.

3.4.2. Eluent pH 4.0

The same study was repeated at pH 4.. As regards the analysis by DC mode, *E*=1.2 V still represents the compromise value for the highest S/N ratio for all the analytes. At this value, S/N ratios are lower than those obtained at pH 5 (carbamazepine: 42, bentazone: 62, HPPH: 92). For E>1.3 V, S/N for bentazone highly increases (120) at the expense of sensitivity for carbamazepine and HPPH whose S/N value is dramatically low (below 10). Pulsed amperometry mode provides S/N values included within 68-72 for the three analytes and hence it can be considered preferable in respect to the DC mode.

3.4.3. Eluent pH 3.0

According to the chromatographic behavior shown in Fig. 4 of the Supplementary Material section, in order to solve the coelution between carbamazepine and bentazone observed at pH 3, the eluent composition was slightly changed increasing CH_3CN content to 32%.

The data obtained for DC mode analysis indicate that for E=1.3 V, S/N ratios close to those obtained at pH 5 can be obtained for bentazone (50) and carbamazepine (150) but not for HPPH (20). E= 1.4 V is the compromise potential for the S/N ratios for HPPH (102), bentazone and carbamazepine.

The pulsed amperometry mode was unsatisfactory for carbamazepine (S/N=5) .

The best detection conditions achieved for each pH value are summarized in Table 3. Despite carbamazepine has a better S/N ratio value at pH 3 with DC detection, the other two substances are poorly detectable at this pH value. The overall best S/N values are obtained at pH 5 by waveform 1, therefore the figures of merit of the method were evaluated at these experimental conditions.

3.5 Glassy carbon electrode activation

During this experimental work, a decreased electrode response and/or a non-reproducible response was noticed. This decreased reactivity could not even be restored by the polishing procedure with alumina, as recommended by the manufacturer.

It is now generally recognized [32-35] that the presence of carbonaceous material microstructures, the purity of the electrode surface and the presence of functional groups on the surface are crucial to determine the reactivity of the glassy carbon electrode. Reports involving the use of carbon electrodes often describe pretreatment procedures that were found necessary to observe reproducible and well-defined electrochemical behavior.

In order to overcome the lack of reproducibility observed, a pretreatment step of the electrode which consisted in a 24h-storage of the electrode in the eluent solution 28%: CH_3CN, 72%: 50 mM CH_3COOH/CH_3COONa, pH 5, was adopted. The explanation for the restored electrode activity can be found in the presence of functional groups on the surface of glassy carbon (typically carboxylic and hydroxyl groups), which, depending on pH conditions, could increase the density of active sites at the electrode surface and improve the electron transfer of the reaction [36]. Electrostatic and/or hydrogen bonds between the electrode surface and analytes can also be established, promoting analytes' oxidation.

3.6. Figures of merit

Linearity of the method was verified over two order of magnitude (see Table 4). Limits of detection (LODss) and limits of quantification (LOQs) were evaluated as the concentration referred to a signal S_m defined as $S_m = S_b + 3s_b$ for LOD and $S_m = S_b + 10\ s_b$ for LOQ, where S_b and s_b are the average signal for blank and its standard deviation, respectively. LODs and LOQs were calculated performing subsequent analysis of the blank (n=14; any "outlier" was highlighted by the *Q* test). The signal was integrated in the "elution window" of each species.

The LOD values obtained by pulsed amperometry and reported in Table 3 are significantly better than those obtained by UV detection [16, 37], expecially for HPPH for which LOD is about two order of magnitude lower [38].

Inter-day and intra-day reproducibility of the method (expressed as relative standard deviation, % RSD) were determined both for retention times and peak areas by repeatedly running a mixture of 125 µg L^{-1} bentazone, 67 µg L^{-1} HPPH, 240 µg L^{-1} carbamazepine for 4 days (n=52).

For intra-day (within day, n=13) measurements, % RSD for retention times is included within 0.2% and 0.7% whereas for peak areas, % RSD was below 5.3% for bentazone, 4.1% for HPPH and 6.2% for carbamazepine, with the exception of day 3 (6.4% for bentazone, 11.3% for HPPH and 17.3% carbamazepine). For inter-day measurements, % RSD for retention times is lower than 1.0 % for all the analytes. The peak area % RSD was lower than 13.1%. The values obtained are satisfactory in respect to the concentration tested that are only slightly higher than LOQ values.

At the optimized chromatographic and SPE conditions (preconcentration factor 100), the method detection limits calculated for the amperometric detection of bentazone, HPPH and carbamazepine are 113±3 ng L^{-1}, 67±1 ng L^{-1}, 234±1 ng L^{-1} respectively, whereas the method quantitation limits were 377±10 ng L^{-1}, 222±4 ng L^{-1}, 782±2 ng L^{-1} respectively. These limits can be improved by increasing the preconcentration factor.

It is interesting to note that our method quantitation limits for bentazone and carbamazepine are in the same range of those reported by Loos et al. [39] and by Pedrouzo et al. [40] who applied a solid-phase extraction followed by triple-quadrupole liquid chromatography mass spectrometry (LC-MS(2)) and by ESI-MS respectively. To the best of our knowledge no reports on HPPH determination in environmental samples by HPLC-MS techniques are available for a comparison.

The optimized detection and extraction conditions (summarized in Fig. 1 of the Supplementary Material section) were used for the analysis of a river sample (Po river, Turin, Italy). According to the chromatograms obtained, bentazone was present in the sample analyzed. Bentazone was identified by the standard addition method, by injection of four aliquots of the sample spiked with known amounts of bentazone up to 40 µg L^{-1} (R^2= 0.9970). According to the data obtained, a final concentration of 0.216 µg L^{-1} in the river sample was found.

4. Conclusions

This work presents the first report on the study and optimization of the amperometric detection on a glassy carbon electrode of compounds of current environmental concern belonging to different classes (herbicides, pharmaceutical compounds and metabolites).

After optimization of chemical and electrochemical parameters through the evaluation of S/N ratios, it was shown that pulsed amperometry showed better sensitivity than the DC mode. Satisfactory results were achieved for bentazone, HPPH and carbamazepine in terms of sensitivity and method robustness.

The coupling of a properly optimized SPE technique allowed to enhance sensitivity and the application of the method developed to a natural water sample.

The monitoring of the peculiar behavior of glassy carbon electrode, due to its surface structure, allowed us to provide a robust protocol to maintain the performance of the electrode.

The use of amperometric techniques can offer a valid, simple and not expensive analytical approach for the determination of compounds of current environmental concern.

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Table 1. Recovery yields optimized for each tested substrates. Concentration of analytes: 1 mg L^{-1} each; sample volume: 5 mL; elution volume: 5 mL. For activation and recovery procedures see text.

Sorbent	Recovery (%)				
	Bentazone	HPPH	Carbamazepine	Phenytoine	Atrazine
Bond Elut C18	90±3	100±2	98±3	100±2	90±2
LiChrolut EN	82±3	88±8	85±1	73±9	85±0
Bakerbond SDB	90±1	103±1	99±3	85±2	90±6
ENVI Carb	2±0	18±0	43±0	45±1	47±0

Table 2. Waveforms set for pulsed amperometry.

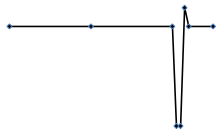
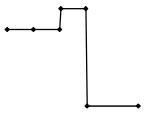
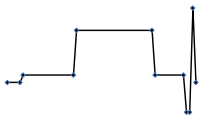
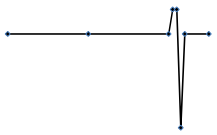
Waveform n°1			Waveform n°2			Waveform n°3			Waveform n°4		
											
Time (s)	Potential (V)	Integration	Time (s)	Potential (V)	Integration	Time (s)	Potential (V)	Integration	Time (s)	Potential (V)	Integration
0.00	1.20		0.00	1.20		0.00	-0.20		0	1.20	
0.20	1.20	Begin	0.20	1.20	Begin	0.04	-0.20		0.20	1.20	Begin
0.40	1.20	End	0.40	1.20	End	0.05	0.00		0.40	1.20	End
0.41	-2.00		0.41	1.80		0.21	0.00	Begin	0.41	1.80	
0.42	-2.00		0.60	1.80		0.22	1.20		0.42	1.80	
0.43	1.80		0.61	-1.00		0.46	1.20		0.43	-1.10	
0.44	1.20		1.00	-1.00		0.47	0.00	End	0.44	1.20	
0.50	1.20					0.56	0.00		0.50	1.20	
						0.57	-1.00				
						0.58	-1.00				
						0.59	1.80				
						0.60	-0.20				

Table 3. Comparison of the S/N ratios at the best detection conditions obtained for each pH value. Detection mode a) DC, *E*=1.4 V; b) pulsed amperometry, waveform 1.

pH	S/N		
	Bentazone	HPPH	Carbamazepine
3 [a]	43	37	121
4 [b]	74	69	69
5 [b]	150	182	70

Table 4 – Linearity, LOD and LOQ values for the optimized method. Chromatographic conditions: column: LiChroCart PuroSphere RP-18; eluent: 72% of a solution containing 50 mM CH_3COOH/CH_3COONa , pH 5.0 and 28% CH_3CN, eluent flow rate 0.5 mL min^{-1}. Injection volume: 10 µL Detection: pulsed amperometry, waveform 1.

Analyte	Equation	Range (µg L^{-1})	R^2	LOD (µg L^{-1})	LOQ (µg L^{-1})
bentazone	$y = 3.5 \cdot 10^{-4}\, x + 9.8 \cdot 10^{-3}$	15-1500	0.9993	12.5	41.7
HPPH	$y = 1.3 \cdot 10^{-3}\, x + 1.4 \cdot 10^{-2}$	10-1000	0.9989	6.6	22.2
carbamazepine	$y = 2.22 \cdot 10^{-4}\, x + 1.31 \cdot 10^{-2}$	25-2500	0.9965	24.0	80.0

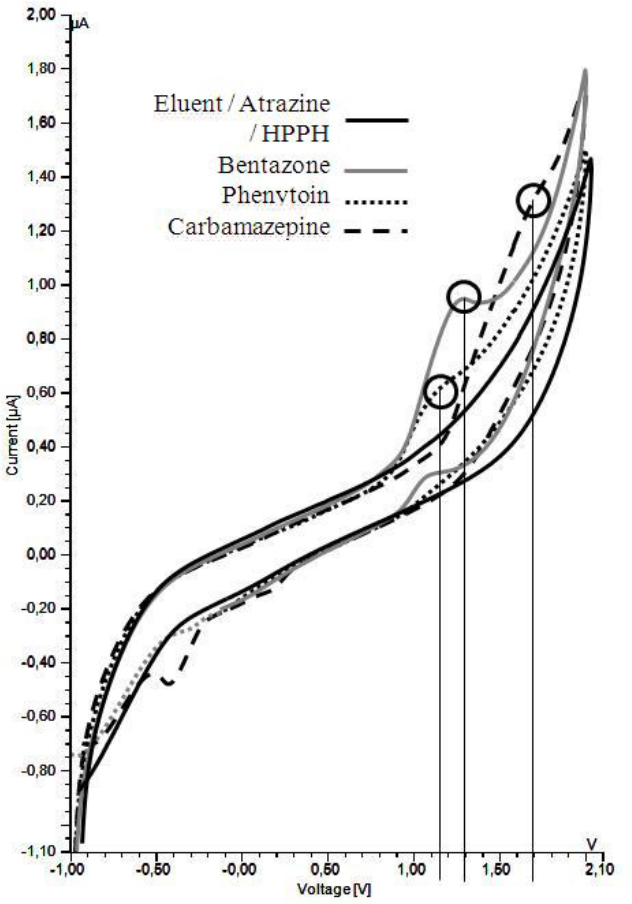


Fig. 1. Cyclic voltamograms for bentazone, HPPH, carbamazepine, phenytoine, atrazine individually prepared at 1 mg/L in eluent solution (72 % of a 50 mM CH_3COOH/CH_3COONa, pH 5.0 solution and 28% CH_3CN) at a glassy carbon electrode, with a potential scan rate of 0.1 V s^{-1}. Cyclic voltammetry for the eluent is also shown. Oxidation potential peaks for each analyte are indicated by circles.

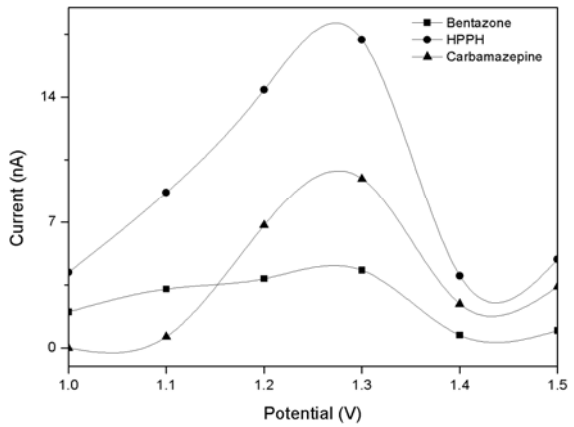


Fig. 2. Hydrodynamic voltamograms for the target analytes. HPLC separation and DC detection mode (glassy carbon electrode). Oxidation potential as shown. Colum: LiChroCart PuroSphere RP-18, 125 mm x 3.0 mm, 5 µm. Mobile phase: 50 mM CH_3COOH/CH_3COONa, pH 5. Eluent flow rate: 0.5 mL min^{-1}. Analytes' concentration: 1 mg L^{-1}.

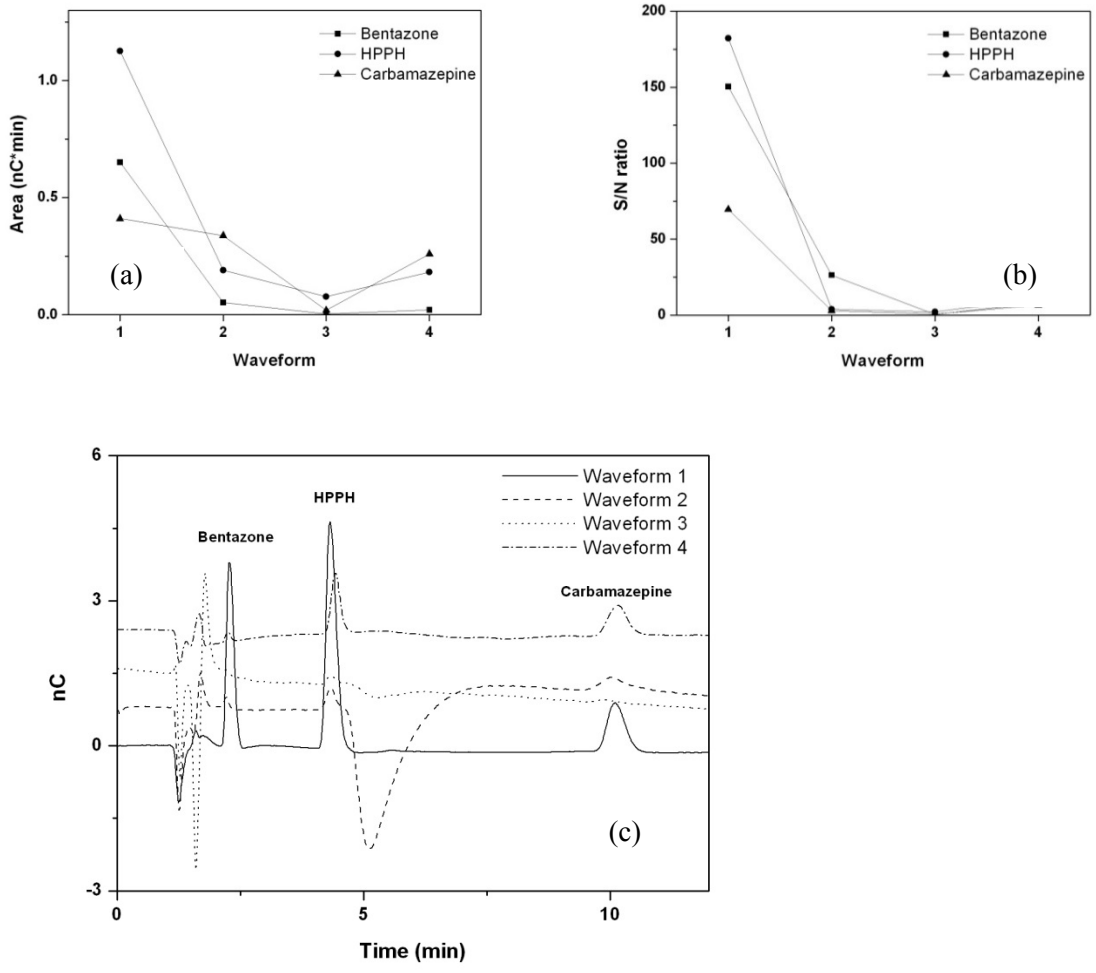


Fig. 3. HPLC separation coupled with pulsed amperometry (glassy carbon electrode). Details of waveforms are shown in Table 2. Colum: LiChroCart PuroSphere RP-18, 125 mm x 3.0 mm, 5 µm. Mobile phase: 50

mM CH_3COOH/CH_3COONa, pH 5. Eluent flow rate: 0.5 mL min^{-1}. Analytes' concentration: 1 mg L^{-1}. For each analyte, peak areas (a), S/N ratios (b) and typical chromatograms obtained (c) are shown.

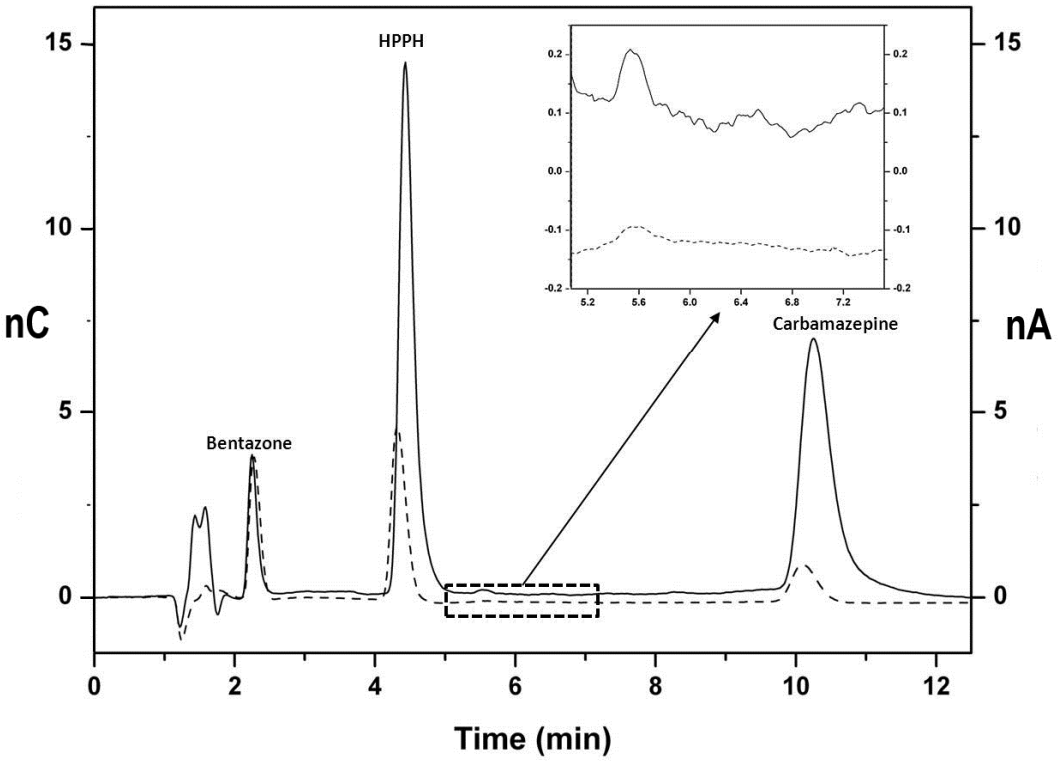


Fig. 4. Comparison of the separation obtained by DC (dashed line) and pulsed amperometry - waveform 1 (continuous line) modes at pH 5. The inset highlights the baseline for each mode. Chromatographic conditions as for Fig. 3.

Supplementary material for

Simple SPE-HPLC determination of some common drugs and herbicides of environmental concern by pulsed amperometry

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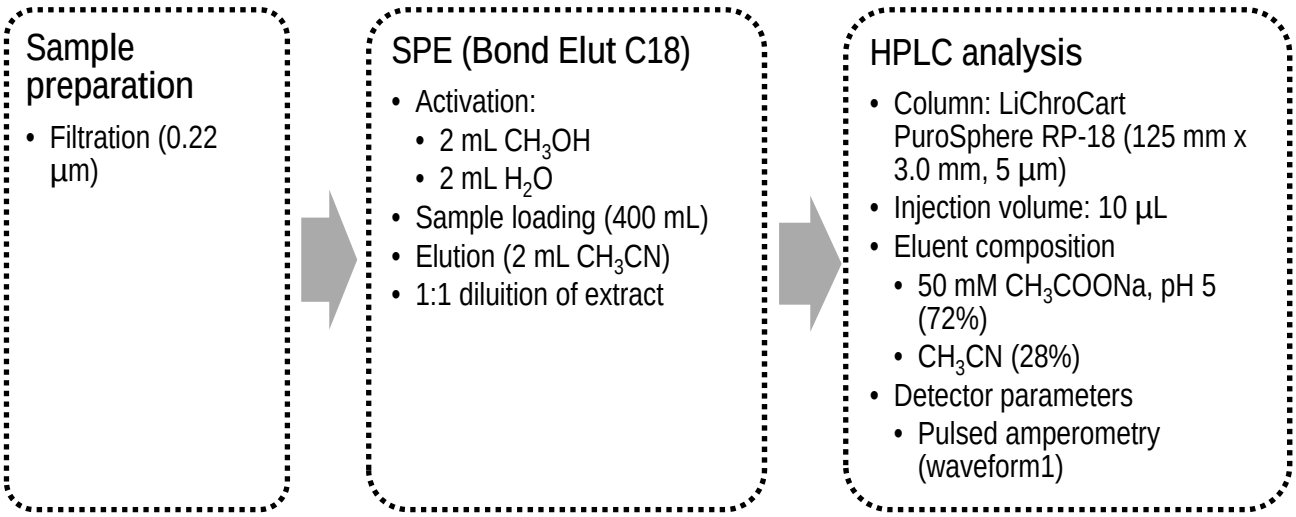

Fig. 1. Scheme of the SPE-HPLC-amperometric detection protocol optimized and applied to real sample analysis

Analytes were SPE extracted and analyzed according to the procedure optimized throughout this work. In this figure, all the steps of the protocol are detailed.

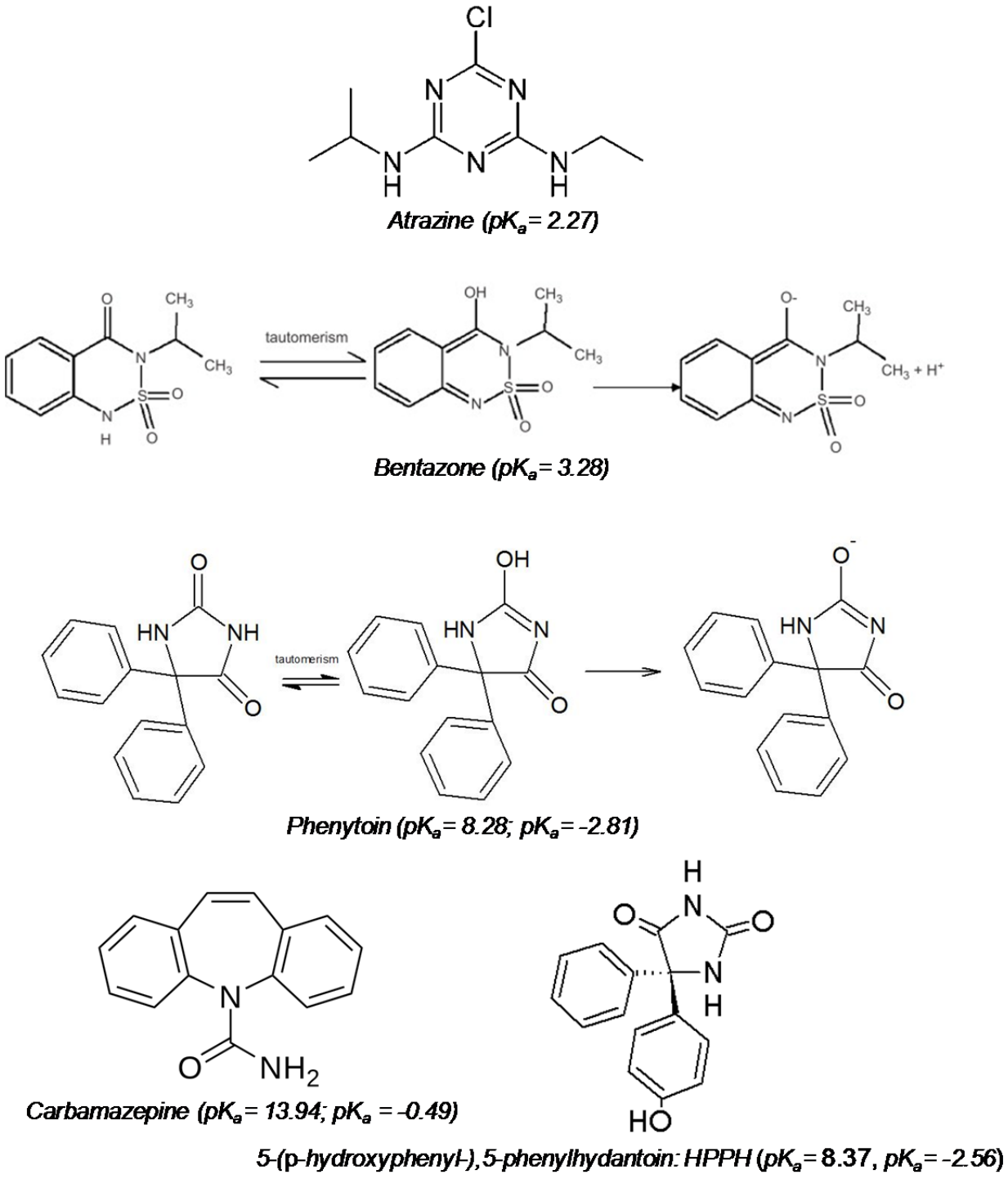

Fig. 2. Chemical structure of the compounds studied. The pK_a values were taken from Scifinder.

The analytes considered in this study are here shown . Bentazone displays keto-enol tautomerism [29] whereas phenytoin displays tautomerism of the imine–imide type [30]. A similar behavior should be expected even for the metabolite HPPH.

The dissociation equilibrium for bentazone is also shown.

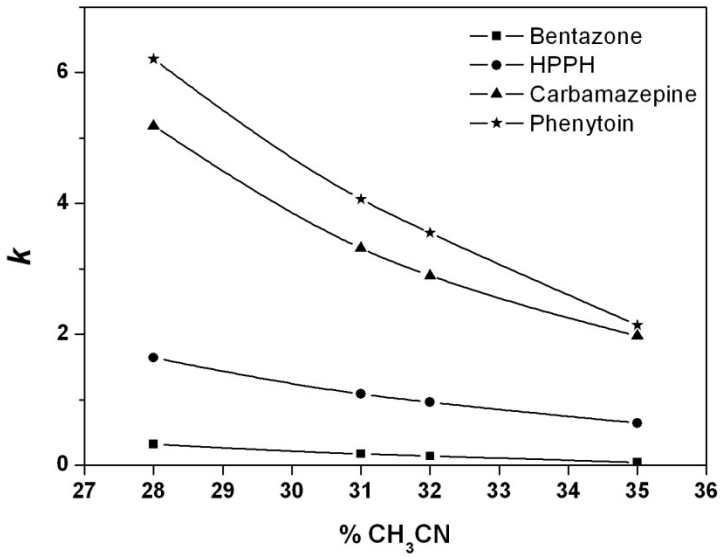

Fig. 3. Effect of CH_3CH on analyte capacity factors (*k*). Column: LiChroCart PuroSphere RP-18, 125 mm x 3.0 mm, 5 µm. Mobile phase: 50 mM CH_3COOH/CH_3COONa, pH 5. Eluent flow rate: 0.5 $mLmin^{-1}$. Detection: UV (252 nm).

At all the eluent compositions investigated, bentazone is the species less retained by the column since according to its pK_a value bentazone is present in the ionized form (see equilibrium depicted in Fig. 1 of Supplementary Material section). The presence of the –OH group in the phenyl substituent dramatically reduce retention for HPPH in respect to phenytoin. It should be remarked that phenytoin is not ionized at the elution pH condition. Capacity factors for all analytes except bentazone and HPPH are significantly affected by the CH_3CN content, due to the decreased hydrophobic interactions that in turn reduce the retention in the column.

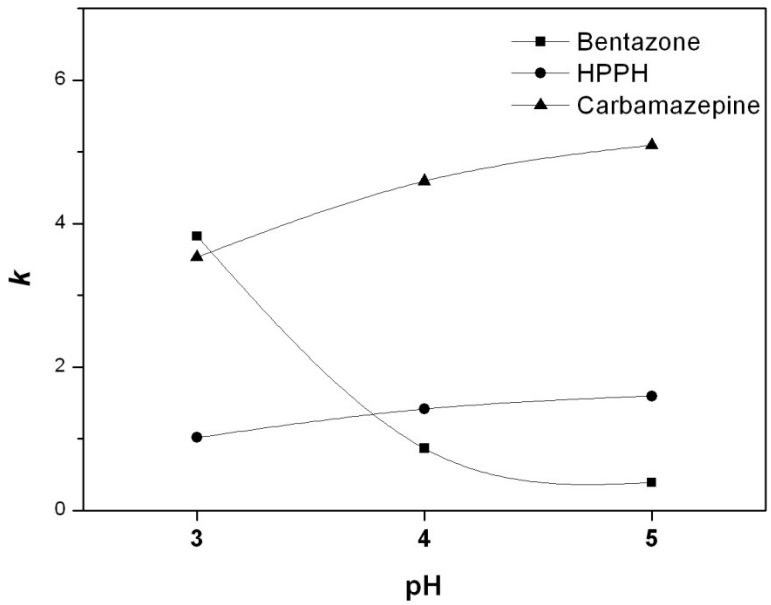

Fig. 4. Effect of pH on analyte capacity factors (*k*). Column: LiChroCart PuroSphere RP-18, 125 mm x 3.0 mm, 5 µm. Mobile phase: 72%: 50 mM CH_3COOH/CH_3COONa (for pH and 5) or 50 mM HCOOH/HCOONa (for pH 3); 28% CH_3CN. Eluent flow rate: 0.5 mL min^{-1}.

The decrease of pH caused an increase in the retention of bentazone, since at this condition the undissociated form of bentazone is also present. The chromatographic behavior for both HPPH and carbamazepine is affected by pH, observing a decreased retention with more acidic pH values.

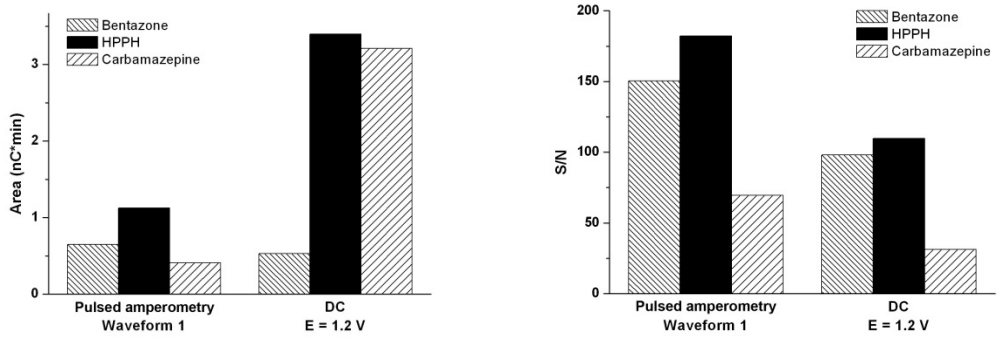

Fig. 5. Comparison of the performance of DC and PAD (waveform 1) modes at pH 5. For each analyte, peak areas (a) and S/N ratios (b) are shown. Chromatographic conditions as for Fig. 3 of manuscript.

The best results in terms of S/N values and peak area obtained at pH 5 (namely DC: 1.2 V and pulsed amperometry: waveform 1), which are here compared, show that waveform 1 provided the best S/N ratio values for all the species, although the highest areas were obtained by the DC mode.