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Development of Liquid Chromatography Separation and a Solid-Phase Extraction Method for Phenoxy Alkanoic Acid Herbicides in Water

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Abstract

A sensitive solid phase extraction enhancement reversed-phase high-performance liquid chromatography (RP-HPLC) determination was adopted for the separation and the determination of phenoxy alkanoic acids herbicides in water. The analytes that have been concentrated were eluted and separated on a C^{18} analytical column at a flow of 1 mL min^{-1} and were determinate by measuring their UV absorption at 220 nm. A solid phase extraction (SPE) method was developed for the analysis of seven phenoxy alkanoic acid herbicides. The effects of the sample volume, sorbent mass and pH on the recoveries of these herbicides were investigated. Sample volume under to 1000 mL could be handled without any loss of analytes. The recoveries were dependent of sample pH in the range >80% and were achieved with pH=3 using 5 mL of methanol as desorption solvent. These acid herbicides were determined simultaneously using this method with good linearity (R^2>0.998). The developed method SPE/RP-HPLC-UV permitted the determination of phenoxy alkanoic aids in distilled water down to a level of 0.17 ng L^{-1} showing satisfactory recovery (>85%) for 1 L samples. The developed method was evaluated by analysis of surface water spiked at 1 µg L^{-1}, so this method is suitable for detecting herbicide residues of sub-parts-per-billion levels in surface water.

Keywords: Phenoxy alkanoic acids; Solid-phase extraction; High performance liquid chromatography; UV detection; Aqueous samples

Introduction

Phenoxy alkanoic acids herbicides have been the first class of herbicides in continuous use since 1947 [1]. These herbicides are of significant importance because of their wide distribution and extensive use as plant growth regulators. When applied, they are easily transferred to surface and ground waters due to their polar nature and high solubility [2]. The main interest for these substances is due to their evident chronic toxicity and carcinogenic effect. On the other hand, they can cause acute toxicity and have significant role in suicidal attempts [3].

This group of herbicides is among the classes of chemical pollutants more heavily monitored by water authorities. The European laws limit the amount of total pesticides in drinking waters to 0.5 µg L^{-1} (0.1 µgL^{-1} for each pesticide) [4]. Five of this phenoxy alkanoic acids are designated as priority pollutants: 2-(2-Methyl-4-chlorophenoxy) propionic acid 2,4- MCPP, 2-(2,4-dichlorophenoxy) propionic acid 2,4-DP, (2-methyl-4-chlorophenoxy) acetic acid 2,4-MCPA, (2,4-dichlorophenoxy acetic) acid 2,4-D and (2,4,5-trichlorophenoxy) acetic acid 2,4,5-T. No limit is explicitly declared for surface water, but amounts not more than 30 µgL^{-1} are generally accepted, whereas for fruits and crops even higher quantities are allowed [5,6].

Many analytical methods for the determination of phenoxy alkanoic acids in water samples have been reported. Most developed methods for these compounds use high performance liquid chromatography (HPLC) or gas chromatography for analytical determination [7-15]. Traditional procedures use gas chromatography with nitrogen-phosphorus detection (NPD), electron capture detection (ECD), or mass spectrometry detection (MS) [16-21].

However, the determination of these compounds by gas chromatography is complicated due to their low volatility caused by the hydrogen bonding of their carboxylic acid and phenol functionalities. Furthermore, because of their polar nature, these compounds are adsorbed on the GC stationary phase which causes peak asymmetry. Therefore, the transformation of these polar groups by derivatization to their corresponding esters is needed in order to yield products which can undergo analysis by GC. Methylation by means of trimethylphenyl ammonium hydroxide (TMPH) seems to be a convenient approach [22-26].

Consequently, reversed-phase liquid chromatography (RPLC) with ultraviolet (UV) or mass spectrometry (MS) detection has been, in recent years preferred as an alternative chromatography technique for the determination of phenoxy alkanoic acids herbicides in environmental water samples [27-32]. Solid-phase extraction (SPE), solid phase micro-extraction (SPME) and liquid-phase extraction (LPE) have been used for concentrating some of these herbicides from water samples [25,33-39].

Most publications on extraction of phenoxy alkanoic acids herbicides and cleanup of environmental samples with SPE describe the use of bonded silica sorbents, especially C^{18} or C^8 [11,40]. Styrene divinylbenzene (SDB) sorbent has been reported to give good recoveries for acidic herbicides from water [41,42].

In this paper we have investigated analytical approaches that could be used for rapid identification and determination of phenoxy alkanoic acids herbicides for the environmental survey in water. The optimization of separation stages provided by RP-HPLC is performed by controlling the pH and nature of the mobile phase. Moreover, the

influence of different parameters affecting the pre-concentration are studied to offer maximum recoveries for acidic herbicides from water.

Materials and Methods

Standards and reagent

All chemicals and solvents were of analytical-reagent grade. Methanol, acetonitrile and acetone were obtained from Baker (Deventer, The Netherlands). The orthophosphoric acid 85% (H_3PO_4) and dihydrogenphosphate salt of potassium (KH_2PO_4) were purchased form Fluka (Buchs, Switzerland). Standards phenoxy alkanoic acids with purities exceeding 90% were products commercialized by Supelco. Individual stock standards solutions (1 mg mL^{-1}) of phenoxy alkanoic acids were prepared in acetone. These solutions were stored at -20°C, and diluted standard mixtures were prepared weekly in acetonitrile and stored at 4°C. The 10 mM aqueous phosphate buffers prepared at different pH were filtered through a Whatman filter (0.45 μm × 47 mm).

Apparatus

Experiment were performed using a (Thermo Finnigan) high-performance liquid chromatograph equipped with two quaternaries model Spectra System P 1000XR Pumps, a model Spectra system UV2000 variable-wavelength UV detector and the injection valve was a Rheodyne model 7125 (20 μl sample loop). The signal processing is performed using AZUR software chromatography.

For pH measurements, PCE-PH-22 pH-meter (glass electrode and a reference electrode with a 3.0 M KCl solution in water as a salt bridge) was used.

HPLC procedure

A separation column, Thermo Hypersil C^{18} (25 cm × 4.6 mm I.D. with a particle size of 5 μm), was used with a SUPELCOSIL LC-18 guard pre-column (2 × 4 mm id) pre-packed with 5 μm C^{18}. The mobiles phase was hydro-organic: 10 mM phosphate buffer pH=3/acetonitrile/methanol (for details, see Table 1). Dead times were evaluated for all the experimental conditions by injection NaNO$_3$ (20 mg L^{-1}) solutions.

N° of gradient	Solvent	Time (min)				Resolution (RS)	
		0	20	40	50		
		Proportion %				2,4-DP/2,4-MCPP	2,4-DB-2,4/MCPB
I	A[a]	7.5	25	30	30	0.8	0.76
	B[b]	7.5	25	30	30		
	C[c]	85	50	40	40		
II	A	7.5	25	25	25	1.27	0.65
	B	7.5	25	35	35		
	C	85	50	40	40		
III	A	7.5	25	20	20	0.66	0.82
	B	7.5	25	40	40		
	C	85	50	40	40		
IV	A	7.5	25	0	0	1.23	0.98
	B	7.5	25	60	60		
	C	85	50	40	40		

[a]:A= Acetonitrile;[b]:B=MeOH;[c]:C=Tampon phosphate pH=3

Table 1: The composition of the mobile phase for tested elution and resolution factor of two pairs of solutes (2,4DP; 2,4-MCPP) et (2,4-DB; 2,4-MCPB).

SPE procedure

Samples were prepared by spiking 500 mL of distilled water with mixed standards solutions. During method development the sample pH was adjusted to 1 with 6 M HCl. The extraction cartridge was rinsed by passing 5 mL of acetone and 5 mL of methanol through the cartridge followed by 10 mL of water adjusted to the same pH as the sample. Teflon tubes were connected between sample reservoir and cartridges. Sample loading was performed at a flow rate of 5-10 mL min^{-1} under vacuum. The sorbent was never allowed to dry during the rinsing and sample loading procedures. After extraction, the cartridges were dried with a gentle stream of nitrogen. Elution was performed at a flow rate of 2-5 mL min^{-1} under vacuum. Different elution solvents

and elution volumes were tested. The eluate was evaporated to 1 mL and analyzed by HPLC.

Results and Discussion

Analytical liquid chromatography separation

The trace-level determination of phenoxy alkanoic acids by reverse-phase liquid chromatography is a very complex process that depends on particular: Characteristics of the stationary phase of the chromatographic column, the nature and composition of organic modifiers, the pH of the mobile phase and the molecular properties of the solutes to be separated [43,44].

Detection wavelength: The UV absorption is an applicable technique to the detection of phenoxy alkanoic acids herbicides, since they all have chromophore groups. The UV spectra show that the detection is much less sensitive at 280 nm, approximately six times smaller than at 220 nm. Several authors recommend a wavelength 220 nm for detection. Optionally, some authors have chosen a wavelength of 280 nm when using a gradient mobile phase with which absorbs at 230 nm [2].

Variation of the retention with mobile phase pH: The variation of the retention time with mobile phase pH was examined for the studied herbicide. The measurements were carried out by working according to an isocratic mode (60% MeOH, 40% aqueous buffer solution H_3PO_4/KH_2PO_4) in a pH range of 2.5 to 4.5. The retention times of the different compounds for tested pH are reported in Figure 1.

These results show that:

- Regardless of the pH used the retention of all solutes increases with the number of the methylene group (-CH2-) on the carbon chain of the acid and with increasing the number of substituent of methyl and/or chlorine on the benzene ring. Example, the retention of 2,4-DB is greater than 2,4-D and the retention of 2,4,5-TP is higher than 2,4-MCPP.

- For all solutes, the retention decreases as the pH increases and the trailing peak (asymmetry) increases, this significantly reduces the chromatographic resolution.
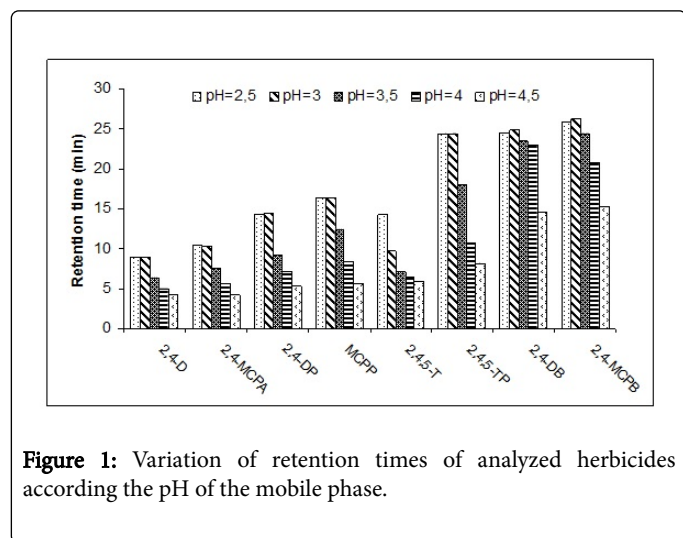

Figure 1: Variation of retention times of analyzed herbicides according the pH of the mobile phase.

Variation of capacity factor K': The curves represent the variations of the capacity factor K' as a function of pH for 8 studied herbicides are given in Figure 2.

We note that, the obtained curves are in agreement with theoretical predictions.

- The variation of K' as a function of pH is shown by a double-log curve (sigmoid), which has an inflection point whose coordinates are:

$$\left(pK_{A,M}; \frac{K'_{A^-} + K'_{AH}}{2} \right).$$

Therefore, the pH of the mobile phase must be below its pKa. For pH values above the pK_a of the acid, the retention decreases as the concentration of undissociated molecules significantly decreases.

- In the pH range tested, only two curves relating to herbicides MCPP and 2,4,5-T have intersections. In this case, the intersection point corresponds to a pH resulting in a co-elution of both solutes. These are not eluted in the same order for pH set before and after the intersection curves.

- The majority of the inflection point of the curves corresponds to the pH of about 3.5.

To select a range of pH of the mobile phase ensuring better separation of 8 phenoxy alkanoic acids, we examined the variation of the selectivity factor (α) as a function of this parameter.

The desired pH range was determined using the window diagrams. The retention times in Figure 2(A) suggest that there are many pH values for some possible separation. To find the optimum separation, we plot α for each pair of solutes as a function of pH. The window diagrams in Figure 2(B) show the variation in selectivity factor with pH for the pairs of solutes that are hardest to separate: 2,4-DP with 2,4-MCPP and 2,4-DB with 2,4-MCPB (for all other pairs of solutes, α>2 at all pH levels). The changes in the selectivity of these two pairs of solutes according to pH are shown in Figure 2(B). Thus, we can choose here the mobile phase pH below 3 how provide the greatest selectivity. According to our results, the optimum pH giving the better selectivity is between 2.8 and 3.2. This pH range is relatively wide; we have the assurance of a good reproducibility.

The composition of the mobile phase: Optimization of k' by fixing the optimum pH using isocratic elution was not enough to achieve an acceptable resolution for both pairs of herbicides previously discussed. We searched for a way to improve this parameter while keeping the mobile phase pH constant.

The chromatographic parameters governing the resolution between two peaks are linked by the following relationship of Purnell [33]:

$$R_S = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K'_2}{1 + K'_2}\right)(N_2)^{\frac{1}{2}}$$

- N_2: is the number of theoretical plates on the most retained solute and which measures the efficiency of the column. Changing this parameter is related to the characteristics of the column and the flow rate of the mobile phase;

- K'_2: is the capacity factor of the solute of the great retention. This parameter depends on both the properties of the solute and the column.

-α: is the selectivity factor (size thermodynamics) and measuring differences distributions of two substances between two phases.

In our situation, it remains to optimize the selectivity factor α.
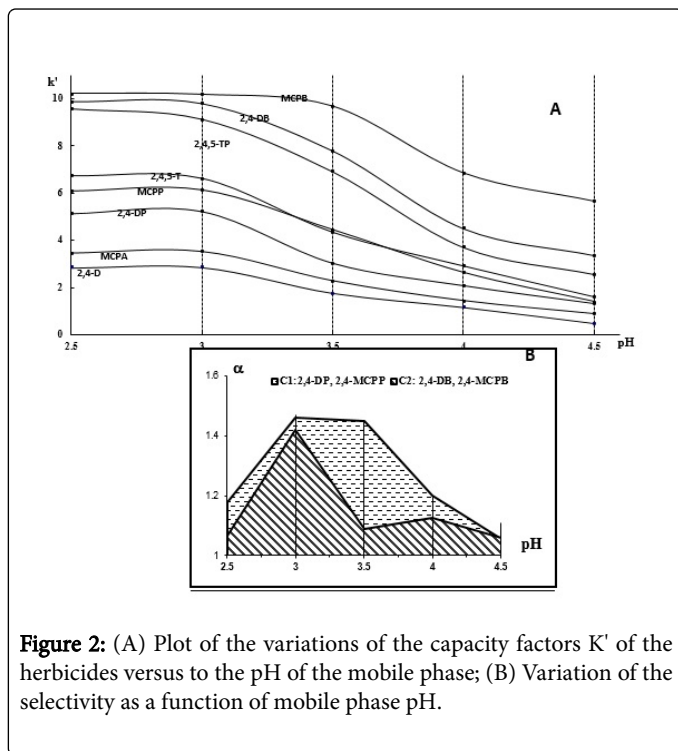
Figure 2: (A) Plot of the variations of the capacity factors K' of the herbicides versus to the pH of the mobile phase; (B) Variation of the selectivity as a function of mobile phase pH.

The most common way to optimize the selectivity is to vary the nature and composition of the eluent phase by making use of the specific properties of the organic solvents used in reverse phase liquid chromatography. We used as an eluting phase, the organic fraction consists of methanol and acetonitrile, therefore the mobile phase is a ternary mixture. We have conducted more separations in a linear gradient elution and the tested Programs are resumed in Table 1. For each case, the elution time was set to 50 min at the initial instant and the organic fraction, composed of ACN and MeOH in the same proportions is 15% of the eluting phase. For all tested gradients, the aqueous fraction from 85% to 50% during the first 20 minutes and reaches 40% at the end of program. The difference between the four tested gradients lies in the composition of organic fraction eluting after 20 min. In this fraction the proportions of MeOH and ACN are either equal; gradient I or different; gradients II, III and IV. The better separation, in terms of resolution is obtained with the gradient No IV (Table 1). The chromatograph of Figure 3 shows the quality of separation obtained. Recent chromatographic conditions were used for the rest of our study.
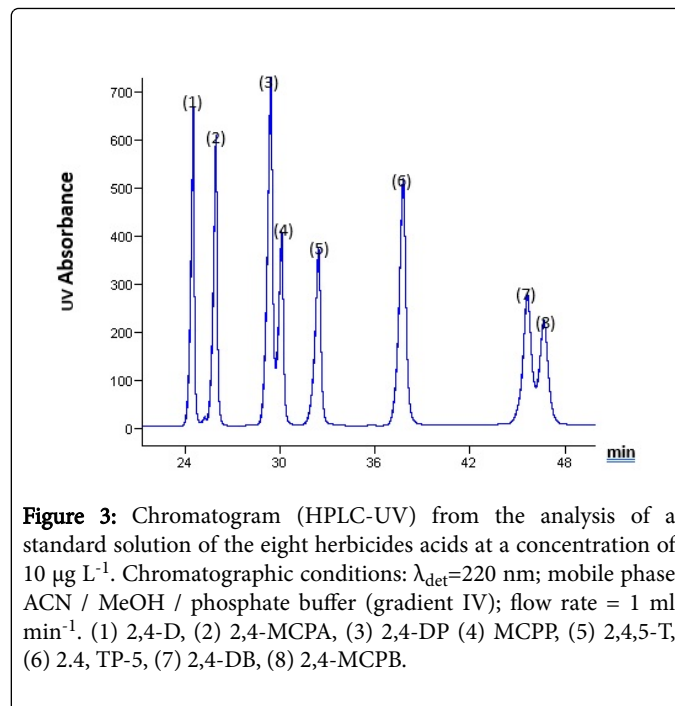
Figure 3: Chromatogram (HPLC-UV) from the analysis of a standard solution of the eight herbicides acids at a concentration of 10 µg L^{-1}. Chromatographic conditions: λ_{det}=220 nm; mobile phase ACN / MeOH / phosphate buffer (gradient IV); flow rate = 1 ml min^{-1}. (1) 2,4-D, (2) 2,4-MCPA, (3) 2,4-DP (4) MCPP, (5) 2,4,5-T, (6) 2,4, TP-5, (7) 2,4-DB, (8) 2,4-MCPB.

Preconcentration parameters

Eluent solvent: Methanol and acetonitrile are commonly used eluents for SPE, and they were tested for their feasibilities as eluents in this experiment. The recoveries of herbicides were above 80% and 90% (n=5) for acetonitrile and methanol respectively for all the solvent when each eluent was spiked with the herbicides to 200 ppb each. However, methanol showed slightly higher solubility for the acidic herbicides than acetonitrile. On the other hand, we did not find the obvious elimination of the humic and fulvic acids observed by other authors [25].

Effect of pH in the recoveries of SPE extraction: To set the pH of the sample before the treatment, we examined the variation of the extraction efficiency of herbicides to analyze eight herbicides acids, depending on the pH. The experiments were carried out with extraction cartridges type bond Elut containing 1 g of adsorbent. The sample is processed in each case a volume of 1 liter with distilled water spiked with herbicides at a concentration of 10 µg L^{-1}. The three pH values tested are pH=1, pH=3 and pH=5. Whatever the pH of the sample, the most results present, in terms of relative standard deviation (RSD), a reproducibility of less than 10% (Table 2). These results show that a significant decrease affect the extraction yield of 4 herbicides: 2,4- D, 2,4-MCPA, 2,4-DP and 2,4-MCPP (these herbicides have a pKa ≤ 3.7) when the pH increases from 1 to 5 (Table 2). For all herbicide to be analyzed, the lowest pH=1 is most appropriate.

Herbicide	Sample pH		
	1	3	5
	Recovery (%)*		
2,4-D	85.03 (4)	69.70 (6)	10.22 (4)

2,4-MCPA	87.24 (3)	64.51 (5)	36.16 (5)
2,4-DP	88.83 (8)	75.78 (7)	53.74 (6)
2,4-MCPP	104.91 (6)	95.07 (5)	57.56 (5)
2,4,5-T	81.60 (9)	72.45 (10)	75.72 (5)
2,4,5-TP	85.10 (3)	85.34 (7)	83.50 (13)
2,4-DB	98.27 (8)	75.80 (3)	86.42 (11)
2,4-MCPB	85.43 (10)	88.11 (9)	85.40 (7)
*			

Table 2: Average recovery (n=3) of studied herbicides of spiked one liter distilled water samples (10 µg L^{-1}) at various pH.

Determining the breakthrough volume:

Sample volume: Since there is a volume above which the solutes are eluted from the stationary phase, it is important to determine the maximum volume of sample that can be percolated with no decrease in the efficiency of extraction of studied compounds. This volume depends on the nature, the mass of the solid phase extraction and the characteristics of the solutes to be extracted.

Three spiked sample volumes were tested: 500, 1000, 1500 mL. The spking levels are chosen such that the mass of each extracted solute is 10 micrograms. The results obtained are shown in Table 3.

We note that the observed recoveries for water sample volumes of 500 mL and 1000 mL do not show significant differences. When the volume of treated water reaches 1500 mL, the recovery of all tested solutes submit a rapid fall reaching, for example, 80% for 2,4-D.

These results show that the breakthrough volume of the examined eight herbicides in these operating conditions is less than 1000 mL.

Compounds	Volume (mL)		
	500	**1000**	**1500**
	Recovery (%)*		
2,4-D	85.92 (10)	88.88 (4)	21.68 (8)
2,4-MCPA	85.58 (4)	86.62 (11)	31.55 (10)
2,4-DP	84.37 (9)	88.65 (5)	34.08 (4)
2,4-MCPP	89 ;82 (5)	105.13 (2)	57.45 (4)
2,4,5-T	82.66 (8)	85.15 (5)	51.12 (6)
2,4,5-TP	82.40 (6)	90.78 (5)	69.66 (10)
2,4-DB	86.01 (11)	98.88 (8)	21.64 (8)
2,4-MCPB	82.35 (5)	87.58 (7)	23.97 (4)

*Average of 3 measurements, the value in brackets is the relative standards deviation (RSD)

Table 3: Herbicides recovery level depending on the volume of the sample spiked with 10 µg L^{-1} of each compound.

Masse of the sorbent: Furthermore, to examine the effect of the adsorbent mass on the retention volume, we determined the yield of extraction of studied herbicides from a one liter of distilled water. The adsorbent masses used are 500 and 1000 mg silica C-18 (Bond Elut). The results show that the retention volume is substantially the same for the two tested masses.

Real sample analysis

To evaluate the results obtained with distilled water and cartridges C-18 Bond Elut, we applied the conditions of enrichment on spiked real water samples at 1 µg L^{-1}. It is surface water for irrigation and collected to an agricultural area at 30 km from the Bizerte city. Our objective is to determine the effects of the matrix on the recovery and the separation of the herbicides acids to interfering peaks. In addition, we treated this type of sample with SDB columns extraction and the columns filled with silica C-18 Type ENV-18.

With extraction cartridges Bond Elut, the recovery rates of analyzed herbicides from a surface water undergoing on the whole, a reduction of 10 to 25% (Table 4) by comparison with the results obtained by distilled water (Table 3). This decrease results from the simultaneous extraction of solutes to be analyzed and a polar organic matter in the treated surface water. For a mass of 1 g of extraction phase, the observed recoveries are above 70% for most of the tested herbicides. This recovery, for a volume of 500 mL of treated water, achieves the limits of quantification below 0.1 ng mL^{-1}.

For the other two types of tested extraction columns as described in the literature for preconcentration phenoxy alkanoic acids herbicides [25], the results are comparable to those of cartridge Bond Elut (1 g). The histograms in Figure 4 show this comparison. The Figure 5 shows the quality of an example of chromatograms of SPE / HPLC-UV of unspiked distilled water and doped surface water with studied herbicides at the optimum analytical conditions. Thus, the solid phase employed in this work is suitably adapted to the preconcentration of phenoxy alkanoic acids herbicides.

Herbicide	Masse de l'adsorbant (mg)	
	500	1000
	Récupération (%) b	
2,4-D	57.60 (15)	85.92 (10)
2,4-MCPA	54.53 (11)	84.58 (4)

2,4-DP	58.19 (8)	80.37 (9)
2,4-MCPP	76.68 (11)	81.82 (5)
2,4,5-T	61.56 (9)	77.66 (8)
2,4,5-TP	62.46 (7)	82.40 (6)
2,4-DB	51.19 (10)	76.01 (11)
2,4-MCPB	65.35 (11)	82.35 (5)

[a]Mass of each herbicide extract is 10 µg.

[b]Average of 3 measurements, the value in brackets is the RSD

Table 4: Recovery of herbicides from surface water treated with Bond Elut extraction columns a.
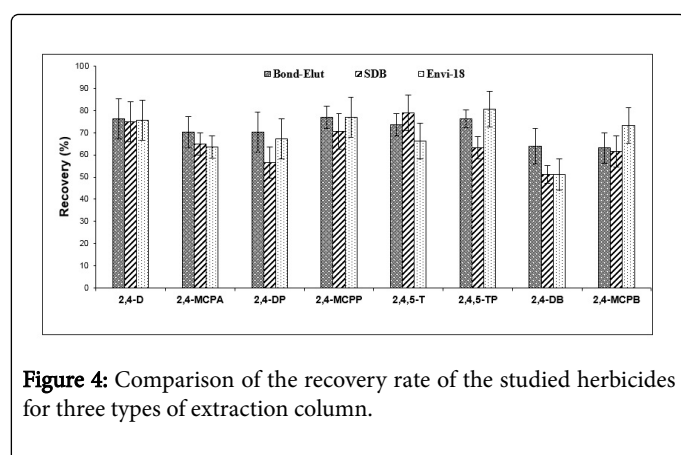

Figure 4: Comparison of the recovery rate of the studied herbicides for three types of extraction column.

Quality assurance

The linearity of response was tested by a series of standard solutions diluted with 4 concentrations of between 5 and 20 ng L^{-1}. The calibration curve of peak area Ap (y) (mV) versus concentration (x) (ppb) for each herbicide was plotted, and their regression equations and correlation coefficients (R^2) were determined and represented in Table 5. In order to evaluate the precision of the developed method, the reproducibility of retention time and response were assessed by the result of a series of 5 injections of 20 µL of a standard solution containing the eight studied herbicides at a concentration of 100 ng L^{-1}. The relative standard 10 deviations observed are less than 0.3% for retention time and between 0.77 and 3% for the response (Table 5). The limits of detection (LOD) were evaluated by proportionally comparing the sensitivity (expressed as peak area for 1.00 ng L^{-1})

obtained in the calibration plot with a peak area in the chromatogram corresponding to a signal-to-noise ratio equal to 3. Detection limits are reported in Table 5 and range between 0.06 ngL^{-1} (2,4-MCPA) and 0.17 ng L^{-1} (2,4-DB).

Table 5 shows that the SPE/RP-HPLC-UV method is of good repeatability and high sensitivity, and it can be used in analyzing herbicides of sub-ppb levels. The method may be used in detecting herbicides of a lower concentration because the sample volume can be as high as 1000 mL without a significant decrease in the percent recoveries. The method was also assessed for the feasibility of detecting herbicides in a real water sample. Compared with the unspiked real water sample as the control, recoveries >80% were obtained from 1000 mL samples spiked with a 1 ppb herbicide each.
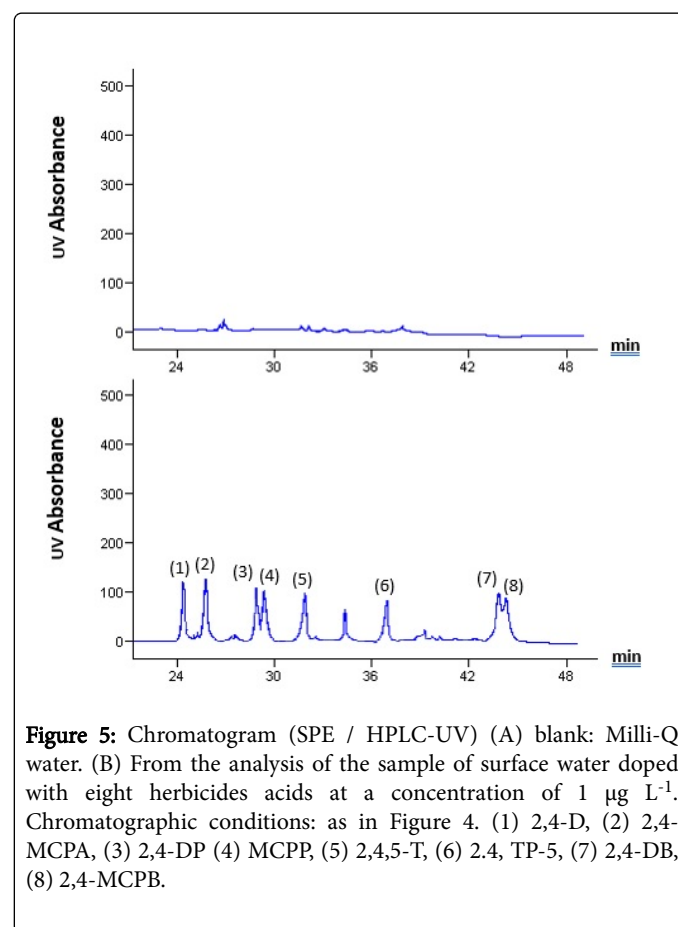
Figure 5: Chromatogram (SPE / HPLC-UV) (A) blank: Milli-Q water. (B) From the analysis of the sample of surface water doped with eight herbicides acids at a concentration of 1 µg L^{-1}. Chromatographic conditions: as in Figure 4. (1) 2,4-D, (2) 2,4-MCPA, (3) 2,4-DP (4) MCPP, (5) 2,4,5-T, (6) 2, TP-5, (7) 2,4-DB, (8) 2,4-MCPB.

Compound	Linear range (ng L^{-1})	Regression functions Y=ax+b	R^2	LOD (µg L^{-1})	Instrument precision Target value (ng L^{-1})	%RSD of tra (n=7)	%RSD of Apb (n=5)
2,4-D	May-20	Y=39.8x+79.9	0.9989	0.08	100	0.15	0.77
2,4-MCPA	May-20	Y=36.2x+39.1	0.9966	0.06	100	0.23	0.97
2,4-DP	May-20	Y=55.1x+53.7	0.9949	0.12	100	0.28	2.29
2,4-MCPP	May-20	Y=33.1x+42.6	0.9968	0.15	100	0.27	2.17

2,4,5-T	May-20	Y=30.8x+30.1	0.9994	0.08	100	0.29	0.75
2,4,5-TP	May-20	Y=74.9x+39.9	0.998	0.11	100	0.28	0.41
2,4-DB	May-20	Y=39.3x+16.3	0.9975	0.17	100	0.26	3.01
2,4-MCPB	May-20	Y=27.5x-32.9	0.9952	0.11	100	0.19	0.49
^{a}tr: Retention time; ^{b}Ap: Peak area							
LOD: Detection limit							
RSD: Relative standard deviation							

Table 5: Assurance Quality.

Conclusion

The determination of herbicides in water (surface water, groundwater, water distribution...) requires selective, reliable and sensitive methodology given the extreme diversity of organic substances in water and very low concentrations of solutes. The liquid chromatography coupled with a preliminary enrichment by solid phase extraction step achieves this objective. This methodology does not require the use of large volumes of solvents and reduces the risk of contamination between sample collection and analysis. In addition, it has the advantage of directly analyzing the polar solutes, particularly ionizable compounds without using a derivatization step.

Indeed, we showed in this study that a judicious choice of pH and composition of the mobile phase makes it possible, in a reasonable time, a good separation of eight phenoxy alkanoic acids herbicides, despite the structural similarity and the ionizable character thereof.

In addition, the operating conditions suitable for preconcentration columns Bond Elut C_{18} extraction allowed extracting herbicide acids in surface water with satisfactory yields. Quantification limits under the proposed protocol is estimated at a sub- ppb value.

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