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Automated sample preparation with extraction columns by means of anti-isoproturon immunosorbents for the determination of phenylurea herbicides in water followed by liquid chromatography–diode array detection and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

The retention of five phenylurea herbicides (chlorotoluron, isoproturon, diuron, linuron and diflubenzuron) was evaluated by solid-phase extraction with an automated sample preparation (ASPEC) system using anti-isoproturon immunosorbents. The extraction was carried out after the percolation of 50 ml of LC-grade water and groundwater samples spiked with a mixture of the five pesticides at the ppb level and then elution with 4 ml of a mixture of methanol–water (70:30, v/v) and 1 ml of LC-grade water. The recoveries obtained ranged from 16 to 97% indicating a good affinity of the polyclonal antibodies of the immunosorbent for compounds with similar structures to the antigen pesticide isoproturon. An inter-laboratory study using Aquacheck certified samples was performed in order to validate the use of the immunosorbent for the analysis of environmental water samples. For the groundwater samples the calibration curves were linear in the range between 1 and 3 μg/l for each compound using liquid chromatography with diode array detection (LC–DAD). The overall mean difference comparing the values obtained by this method and the real values given by Aquacheck varied between 1 and 22%. All samples were analyzed simultaneously by LC–DAD and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. © 1997 Elsevier Science B.V.

Keywords: Immunoaffinity columns; Water analysis; Sample handling; Pesticides; Phenylurea pesticides

1. Introduction

The analysis of pesticides in water samples is generally performed by solid-phase extraction (SPE) followed by gas chromatographic (GC) or liquid chromatographic (LC) techniques. There are a wide variety of solid-phase extraction sorbents such as C_{18} or polymeric ones that are suitable for the determination of pesticides at the 0.1 μg/l level in drinking water (required by the Drinking Water Directive of the Commission of European Community, DWD-CEC) [1]. However, the main problem encountered with SPE is the lack of selective sorbents when analyzing surface waters. In this sense, the matrix of the surface water is difficult to eliminate and it produces a noisy baseline and a large peak at the beginning of the chromatogram, thus making the determination of the most polar analytes laborious.

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Immunoassays have been shown to be an useful tool for screening purposes in order to select samples to be further analyzed by GC and/or LC [2–4]. Furthermore, immunoassays have been involved in monitoring of environmental waters [5,6] showing similar results to those reported by SPE–LC–diode array detection (DAD). Nevertheless, the problem of the cross-reactivity of the antibody is still a difficult problem when quantitative results are needed. On the other hand, there is an increasing need of extracting as many compounds as possible within a given group of pesticides. It is advantageous to combine the selective interactions obtained by affinity chromatography based on antigen–antibody interactions together with the cross-reactivity generated by the antibody. Selective interactions are achieved with immunoaffinity sorbents (ISs) which are constituted of polyclonal antibodies covalently bound to a silica sorbent.

In previous work [7] the development and the evaluation of two immunosorbents for the selective trace SPE of phenylurea and triazine herbicides has been presented. In this study, polyclonal anti-isoproturon and anti-atrazine antibodies were prepared and immobilized in different sorbents. The best conditions for the elution of the immunosorbent were also studied. The use of immunosorbents has been widely applied for the clean-up of river water samples [8–11]. Studies of breakthroughs, capacities and calibration curves obtained for several phenylureas and triazines upon four different immunosorbents were carried out using either off-line or on-line preconcentration cartridges [12]. Taking advantage of the cross-reactivity of a specific antibody it is possible to extract many compounds of the same family. The preconcentration of different compounds having the same chemical group is possible due to the cross-reactivity of the polyclonal antibodies that can recognize the antigen and other compounds with similar structures. This selective preconcentration should not require an additional cleanup step. Most of the papers published up until now, used LC–UV or DAD after the preconcentration step with immunosorbents. To our knowledge only one previous work used an immunoaffinity sorbent for the preconcentration of carbofuran in complex matrices followed by LC–MS detection [9].

The specific objectives of this work were: (i) to study the extraction efficiency of several phenylureas upon anti-isoproturon immunosorbents after the preconcentration of LC-grade and groundwater samples; (ii) to carry out the analysis of the samples by LC–atmospheric pressure chemical ionization mass spectrometry (APCI-MS) in order to identify all the analytes and possible interferences encountered in the ISs and (iii) to evaluate the performance of the ISs for the determination of traces of herbicides in certified Aquacheck samples.

2. Experimental

2.1. Immunosorbent columns

Preconcentration of the water samples was carried out through experimental cartridges prepacked with 0.5 g of silica and 10 mg of anti-isoproturon antibodies. Polyclonal antibodies immobilized on this adsorbent were supplied by Professor Le Goffic (ENSCP, Paris, France). Polyclonal antibodies were synthesized against isoproturon according to the procedure described in a previous study [7]. The hapten preparation and immobilization of the antibodies for the preparation of a immunosorbent for the selective SPE of phenylurea herbicides has been presented in the same work. Pesticides were modified by the introduction of a carboxylic group so that they could be linked to bovine serum albumin (BSA) before injection into rabbits. The antibodies were then covalently bound to a silica matrix in order to obtain a pressure-resistant sorbent.

2.2. Chemicals

HPLC grade solvents acetonitrile, methanol and water were purchased from Merck (Darmstadt, Germany). Pesticide standards: chlorotoluron, isoproturon, diuron, linuron and diflubenzuron were obtained from Promochem (Wesel, Germany). Chemical structures are indicated in Fig. 1. Sodium phosphate, sodium chloride and azide was obtained from Merck. Acetic acid was purchased from Panreac (Barcelona, Spain).

The phosphate-buffered solution (PBS) consists of a 0.01 *M* sodium phosphate buffer containing 0.15 *M* NaCl (pH=7.4) and 0.2% azide.

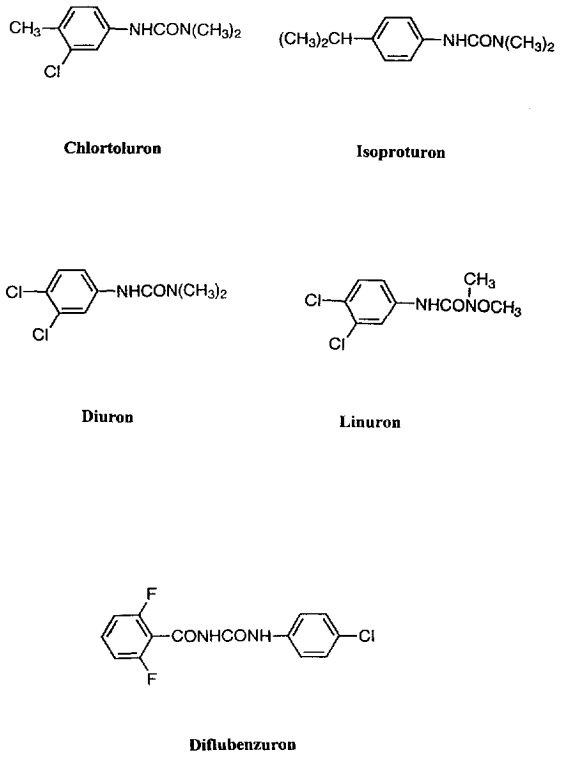

Fig. 1. Structures of the phenylureas studied.

2.3. Apparatus

LC–DAD analyses were performed with a Waters 600-MS solvent delivery unit with a 20 μl injection loop and a Waters 996 photodiode array detector (Waters, Milford, MA, USA). The analytical column used was a 25 cm×4.6 mm I.D. packed with 5 μm octylsilica gel from Shandon (Cheshire, UK). The gradient elution was performed as follows: from 25% A (acetonitrile) and 75% B (LC-grade water) to 100% A and 0% B in 30 min. Quantification was carried out with UV detection at 245 nm for all the compounds under study.

LC–APCI-MS with positive mode of operation was used for the determination of the phenylureas herbicides. The eluent was delivered by a gradient system from Waters 616 pumps coupled to a Model Waters 600S controller. A VG Platform mass spectrometer from Fisons Instruments (Manchester, UK) equipped with an atmospheric pressure chemical ionization source (APCI) interface was used. The VG Platform APCI interface consists of a heated nebuliser probe and the standard atmospheric pressure source configured with a corona discharge needle [13]. The different operating parameters included a drying gas (N_2) flow-rate of 250–300 l/h and a nebulizing gas flow-rate of 10 l/h. The cone voltage were set at 20 V and the corona voltage at 3.5 kV. The ion source was set at 180°C and the probe temperature was 400°C. The instrument control and data processing utilities included the use of the MassLynx application software installed in a Digital DEC PC 466. The gradient elution was performed in the same way as in LC–DAD with the only difference that in both mobile phases acetic acid was added, at 0.5%, in order to enhance the ionization of some of the compounds studied such as diuron and linuron.

2.4. Sample preparation

Stock standard solutions of 500 μg/ml were prepared by weighing the solutes and dissolving them in methanol. A stock solution of 1 μg/ml was used to spike LC-grade and groundwater at the μg/l level for the preconcentration through the cartridge and further determination of recoveries and construction of the calibration graphs.

Preconcentration of the samples was performed off-line with an automated sample preparation system. The (ASPEC) XL system, fitted with an external 306 LC pump for the dispensing of samples through the immunosorbent cartridge and with a 817 switching valve for the selection of samples, was a gift from Gilson (Villiviers-le-Bel, France).

The first step of the SPE consisted of conditioning the immunosorbent (0.5 g of bonded silica) with 10 ml of PBS and then with 5 ml of LC-grade water. 50 ml of the sample was percolated through the immunosorbent at a flow-rate of 2 ml/min followed by 5 ml of LC-grade water. The sample volume of 50 ml was chosen according to the results obtained in the previous work [8]. The compounds trapped on the immunosorbent were eluted first with 4 ml of a mixture containing 70% methanol and 30% LC-grade water and then with 1 ml of LC-grade water. These 5 ml extracts were rotaevaporated until 500 μl and then evaporated carefully until dryness with a gentle stream of nitrogen. Afterwards methanol was

added up to a volume of 200 μl and 20 μl were injected into the LC–DAD and LC–APCI-MS systems.

For the recovery studies 50 ml of LC-grade and groundwater sample spiked at 3 μg/l were percolated through the immunosorbent. This experiment was performed in triplicate and the extracts were analyzed simultaneously by the LC–DAD and LC–APCI-MS systems.

Blanks of LC-grade water and groundwater were percolated through the immunosorbent and then analyzed by LC–APCI-MS in SCAN conditions in order to evaluate the possible interferences present in the cartridge and eluted after.

The calibration curves were obtained by percolating 50 ml of Aquacheck water sample spiked in the trace level range of 1–3 μg/l in order to have the same matrix as in the intercalibration water sample.

Validation of the immunosorbent was carried out by performing an inter-laboratory calibration study for herbicide compounds organized by Aquacheck (WRC, Medmenham, UK). A certified standard solution containing an unknown concentration of pesticides and a 2 l bottle of groundwater was provided by the organization. The aim was to spike the groundwater with the solution provided in order to determine the levels of these pesticides in water. Normally, it is established to spike 500 ml of groundwater sample with 50 μl of the certified standard solution. However, in this case, 200 ml of the groundwater sample was spiked with 500 μl of the certified standard solution in order to have a high spiking level and then a better sensitivity by preconcentrating only 50 ml of water sample. These adjustments in the spiked waters from Aquacheck are not allowed in the inter-laboratory studies. However, in this case, the study was carried out in order to validate the use of the immunosorbent. On the other hand, the change in the spiking of water does not influence either the results or the maximum acceptable errors. The reason for why only 50 ml of the water samples were preconcentrated is that breakthrough occurs early for some compounds such as linuron and diflubenzuron [8]

When the immunosorbent was not in use, it was stored at 4°C in a solution of PBS containing 0.2% azide after a washing step using 70% methanol (5 ml).

3. Results and discussion

3.1. Analytical performance

Fig. 2 shows the comparison between the chromatogram of the extract obtained after the percolation of 50 ml of groundwater sample spiked at 3 μg/l with a mixture of phenylureas through the immunosorbent and the chromatogram obtained after the on-line percolation of the same sample through a PLRP-s cartridge. As can be seen in the second chromatogram (b), the peak corresponding to the matrix of the water is lower than in the first one (a),

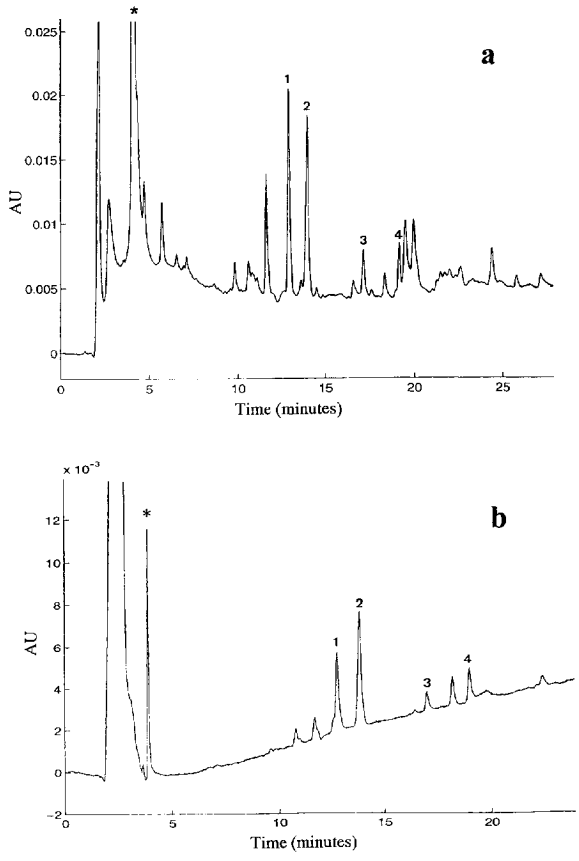

Fig. 2. LC–DAD chromatogram at 220 nm obtained after preconcentration of: (a) 50 ml of ground water sample spiked at 3 μg/l through a PLRP-s cartridge; (b) 50 ml of ground water sample spiked at 3 μg/l through an anti-isoproturon cartridge. Peaks: 1=chlortoluron, 2=isoproturon+diuron, 3=linuron, 4=diflubenzuron, *=water matrix. Gradient conditions as described in Section 2.3.

thus indicating a good selectivity of the anti-isoproturon immunosorbent for the selected compounds. Moreover, as it is observed in this figure, the interferences encountered with the analysis using immunosorbents are smaller than that obtained with polymeric cartridges. This fact indicates that the use of immunosorbents for the selective SPE of phenylureas from environmental waters is an advantageous technique as compared with the conventional ones that use C_{18} or PLRP-s cartridges.

Using LC–DAD, it was impossible to quantify isoproturon and diuron as both of them coelute at the same retention time due to their similar polarity. Therefore, the use of LC–APCI-MS was essential for the identification of these two compounds. The confirmation of all the compounds studied was carried out by analyzing all the extracts in the LC–APCI-MS system in the positive mode of operation and under SIM conditions. The main ions and typical fragments corresponding to each pesticide are shown in Table 1. Almost all compounds gave only the protonated molecule in positive ion mode except diflubenzuron that gave as a major ion the $m/z=158$ corresponding to the fragmentation between the C–N bond of the two amide groups present in the molecule. Fig. 3 shows the chromatograms obtained under SIM conditions ($m/z=158$, 207, 213, 233 and 249) after the analysis of the same extract as shown in Fig. 2. Isoproturon and diuron have different molecular ions, so that, it is possible to quantify them independently as is shown in this figure. No interferences were encountered in the extracts analyzed in SCAN conditions after the preconcentration with blank samples.

Table 2 presents the limits of detection in LC–DAD and LC–APCI-MS of all the compounds under study. The SIMs limits of detection were calculated using a signal-to-noise ratio of 3 (the ratio between the peak intensity in SIM conditions and the noise) and spiked levels of 0.5 μg/l.

3.2. Recoveries

In previous studies [8] it has been shown that a strong affinity for the antigen pesticide is obtained in anti-isoproturon immunosorbents. Nevertheless, after a rather long period of immunization, the affinity for compounds other than the antigen is also achieved due to the similarity in the chemicals structures of compounds between the same family (see Fig. 1).

The recoveries of extraction of several phenylureas from LC-grade water on the anti-isoproturon immunosorbent are presented in Table 3. These recoveries were obtained after the percolation of 50 ml of LC-grade water spiked at 3 μg/l with a mixture of phenylureas and they were calculated using two different methods of detection: diode array and mass spectrometry. High recoveries are obtained for chlorotoluron, isoproturon and diuron. Nevertheless, it has been shown [8] that anti-chlortoluron immunosorbent is most appropriate for a screening purpose since the recoveries obtained with this immunosorbent are higher than 75% for all the compounds studied here. Chlortoluron contains a disubstituted phenyl ring and therefore anti-chlortoluron immunosorbent is more suitable for trapping those phenylureas containing disubstituted phenyl rings in the chemical structure than the anti-isoproturon one. In spite of isoproturon being a monosubstituted phenyl ring, the anti-isoproturon immunosorbent is capable of retaining four of the five phenylureas studied and can even trap diuron with a high recovery of 91%. Table 3 shows also the recoveries obtained after the percolation of 50 ml of groundwater through the anti-isoproturon immunosorbent. Slightly differences are observed in the retention of the analytes from the two sorts of water. This result confirms again the high selectivity of the immunosorbent for the compounds present in any type of water. Selectivity is already achieved by the

Table 1
Typical fragment ions and relative abundances (RAs) of chlortoluron, isoproturon, diuron, linuron and diflubenzuron in LC–APCI-MS in PI mode of operation

Compound	M_n	m/z of main ions	RA
Chlortoluron	212	213 $[M+H]^+$	100
Isoproturon	206	207 $[M+H]^+$	100
Diuron	232	233 $[M+H]^+$	100
Linuron	248	249 $[M+H]^+$	100
Diflubenzuron	310	158 $[M-(C_6H_4NHCOCl+H]^+$	100
		311 $[M+H]^+$	45

Cone set at 20 V and corona at 3.5 kV. Carrier stream: acetonitrile–water containing 1% of acetic acid at a flow-rate of 1 ml/min.
M_n=Nominal mass.

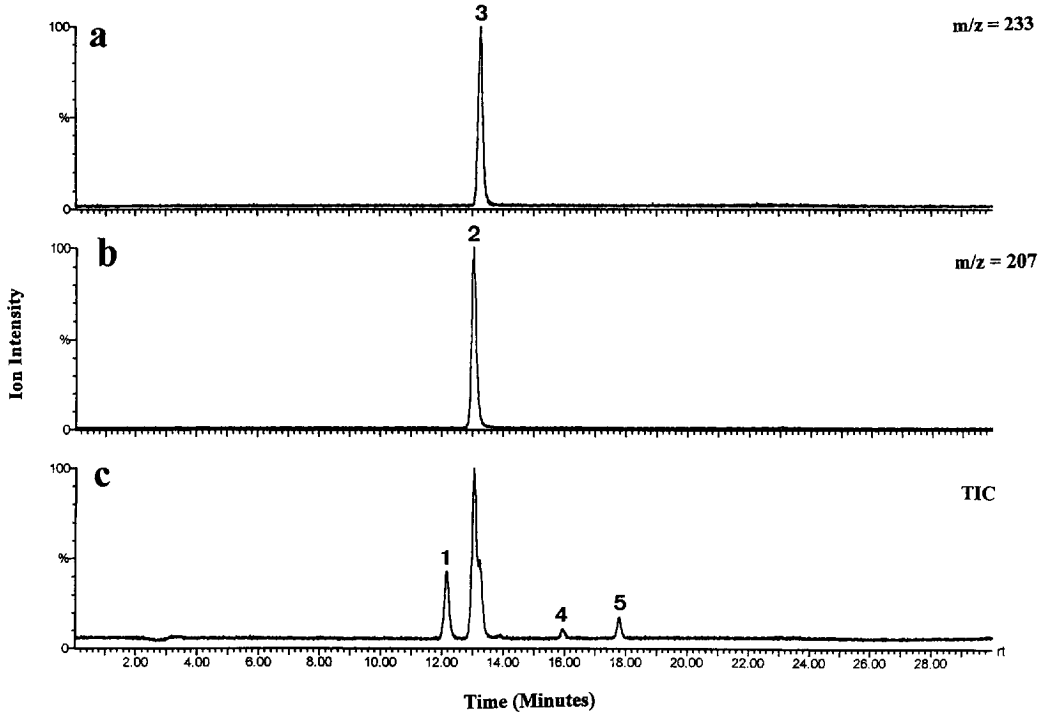

Fig. 3. LC–APCI-MS chromatogram under SIM conditions after preconcentration of 50 ml of ground water sample spiked at 3 μg/l through anti-isoproturon cartridge: (a) extracted ion chromatogram at m/z=233; (b) extracted ion chromatogram at m/z=207; (c) total ion current (TIC) under SIM conditions. Peaks: 1=chlortoluron, 2=isoproturon, 3=diuron, 4=linuron, 5=diflubenzuron.

sorbents. The matrix of the water is not retained at all in the immunosorbent, so that the interaction between the matrix and the antibodies is low, thus leading to a higher selectivity with the analytes studied. The only phenomena taking place in the immunosorbent is the competition between the compounds for their binding to the recognition sites of the antibodies [7]. Only the use of LC–APCI-MS permitted us to calculate the recovery values for isoproturon and diuron since these two compounds

Table 2
Limits of detection (LOD) (ng/l) obtained with LC–DAD and LC–APCI-MS in SIM conditions for the studied pesticides after preconcentration of 50 ml of LC-grade and groundwater through the anti-isoproturon immunosorbent

Compound	LOD			
	LC–DAD		LC–APCI-MS	
	LC-grade	GW	LC-grade	GW
Chlortoluron	151	154	86	125
Isoproturon	n.q.	n.q.	57	76
Diuron	n.q.	n.q.	115	145
Linuron	586	605	825	1003
Diflubenzuron	464	444	743	923

n.q.=not quantified due to coelution.

Table 3
Recoveries of extraction obtained after the percolation of 50 ml of LC-grade and groundwater spiked at 3 μg/l with a mixture of phenylureas through the anti-isoproturon immunosorbent

Compound	Recoveries (%)			
	LC–DAD		LC–APCI-MS	
	LC-grade	GW	LC-grade	GW
Chlortoluron	58	60	63	68
Isoproturon	n.q.	n.q.	97	114
Diuron	n.q.	n.q.	91	86
Linuron	17	16	20	19
Diflubenzuron	44	44	58	56

The relative standard deviation varied between 3 and 15% (n=3).
n.q.=not quantified due to coelution.

coelute in LC–DAD. Nevertheless, in many cases quantitation is difficult in this kind of system, as reported in a previous work [13].

3.3. Validation

Calibration graphs were constructed in the range from 1 to 3 μg/l in Aquacheck groundwater. Good linearity was observed for chlortoluron and linuron using the UV detection with coefficients of correlation higher than 0.99. Isoproturon and diuron were not quantified by LC–DAD due to the problem of coelution as reported in Section 3.2. They were quantified by LC–APCI-MS using a single point calibration. The immunosorbent was validated by participating in the Aquacheck inter-laboratory exercise organized by the Water Research Center at Medmenham, UK. Every 2–3 months, certified samples of groundwater containing herbicides (atrazine, simazine, propazine, MCPA, MCPB, mecoprop, chlortoluron, isoproturon, diuron and linuron) were distributed. In Table 4, the results obtained in two of the inter-laboratory exercises are reported. The percentage of error as regards to the target values reported by Aquacheck is also indicated. The results are evaluated according to the limits imposed by the organization: results below 17% are acceptable. Flagged and double flagged results are results exceeding the maximum acceptable error or twice the maximum acceptable error. If we consider the AOAC limits, the maximum acceptable error between laboratories is of 22% [14]. In the present paper, 22% is considered to be the maximum allowable error as previously reported for organophosphorus pesticides in water [15]. Aquacheck inter-laboratory exercises have the objective of improving and controlling the quality of water analysis. The target for bias and for precision set at 17% by Aquacheck is unrealistic as this limit cannot be established for the analysis of water samples spiked with pesticides. The target values for bias and precision can only be established after a sufficient period of expertise of the participating laboratories. So that, owing to the difficulties encountered when polar pesticides are analyzed in water, the coefficient of variation and target values should be higher. As an example, the EEC-BCR certificate exercises for PCBs, an easier inter-laboratory exercise, has established a precision of 12% in standard solutions and 25% in spiked samples. This is a more realistic approach than the 17% set by Aquacheck. Most of the compounds gave acceptable values according to the fact that the generally maximum accepted errors between laboratories is 22%. Somewhat higher standard deviations were encountered in the analysis of the samples by LC–APCI-MS system owing to problems detected in the probe tip after injecting several raw samples into the mass spectrometer. The problems encountered are that the sensitivity decreases as source is plugging from salt present in the samples.

4. Conclusions

The use of anti-isoproturon immunosorbents for

Table 4
Mean concentration (ng/l) and mean difference (%) (n=3) in relation to reference values of herbicide pesticides from two inter-laboratory studies (results are obtained after preconcentrating 50 ml of groundwater sample spiked with the certified solution from Aquacheck)

Compound	April 1996				June 1996			
	LC–DAD		LC–APCI-MS		LC–DAD		LC–APCI-MS	
	Conc.	Error (%)	Conc.	Error (%)	Conc.	Error (%)	Conc.	Error (%)
Chlortoluron	1129	−1.8	1244	8.1	2036	10.0	2045	10.5
Isoproturon	n.q.	n.q.	2377	21.9	n.q.	n.q.	1448	9.3
Diuron	n.q.	n.q.	1128	10.0	n.q.	n.q.	853	21.8
Linuron	1777	−5.2	1787	−4.7	1059	−1.5	1084	1.0

The relative standard deviation varied between 5 and 16% (n=3).
Analytical conditions are described in Section 2.
n.q.=not quantified due to coelution.

the selective multiresidue preconcentration of several phenylureas in water samples was feasible applying an automated off-line sample preparation system (ASPEC) followed by LC–DAD and LC–APCI-MS detection. The use of the SIM in the LC–APCI-MS system allowed the determination of isoproturon and diuron herbicides since both compounds coeluted in LC–DAD detection.

The affinity of the ISs for compounds other than the antigen was achieved due to the similarity in the chemical structures. The matrix of water is not retained at all in the ISs. For this reason, a clear baseline was observed in all the chromatograms, allowing to a better quantification of the analytes. High recoveries were obtained for three of the five compounds under study and LODs were in the high ppt level thus indicating a good selectivity of the immunosorbent for all the compounds. Acceptable results were reported in the inter-laboratory exercise carried out with the certified material provided by Aquacheck. The major drawback of this method is the low breakthrough that show many phenylureas upon anti-isoproturon immunosorbents. With other type of immunosorbents such as anti-chlortoluron ISs it is possible to increase the percolated volume and therefore to improve the LODs.

In future work it will be studied the feasibility of the immunosorbents for on-line methodology and posterior MS detection for a variety of pesticides in soil samples.

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