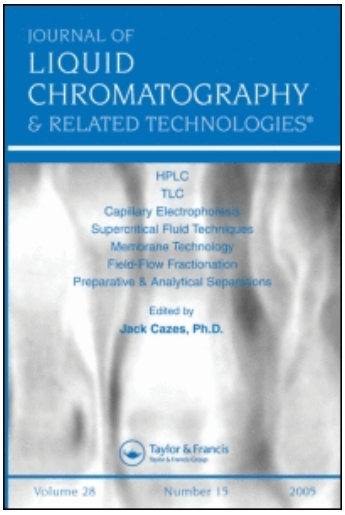

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Abstract: Eight phenylurea herbicides (chlorotoluron, isoproturon, diuron, monolinuron, linuron, metobromuron, chlorbromuron, methabenzthiazuron) were isolated from soil by extraction. The extract was cleaned up using an SPE module. Elution with a methanol-dichloromethane mixture resulted in total recoveries using this method, which included all losses during sample preparation, ranging from 79 to 97% recovery of the herbicides from the activated silica gel sorbent column. Separation of the eight herbicides was easily achieved working under liquid chromatographic conditions. The validation of the analytical processes was described and the parameters of the LC-DAD method are as follows: limits of detection from 0.07 to 0.13 μg/g; precision (RSD) from 2.9 to 10.4%. The proposed LC-DAD method offers good resolution and selectivity.

Keywords: Environmental analysis, Urea herbicides, Liquid chromatography, Solid-phase extraction

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INTRODUCTION

Among all the various kinds of plant protection agents, derivative urea herbicides (N,N-dimethyl and N-methoxy-N-methyl, phenylurea derivatives) are one of the more important groups of these compounds. They are widely used as selective pre- and post-emergence herbicides for the control of most broad leaved weeds and annual grasses in many agricultural crops.[1,2] In recent years, urea herbicides have become very popular worldwide because of their low application rates, low toxicity to mammals, and unprecedented herbicidal activity. Compared to other older herbicides, ureas are degraded in soil more rapidly. Therefore, very low concentrations (part-per-billion, ppb) of these herbicides are to be expected in the environment, especially in the water supply. There is increasing demand for accurate detection and characterization of herbicides, in order to evaluate their levels in the environment. Therefore, the establishment of an accurate multicomponent analysis method is of practical importance.

In spite of the well recognized structure, proprieties, and mode of action, they are still difficult to identify and quantify. This is especially the case in soil for the matrix with complicated composition and specific physical and chemical proprieties. Analysis of herbicide residues involves different steps, such as extraction, clean-up or interference removal, determination of herbicide residues and confirmation of their identity, and these analyses are performed by using various techniques.[3–6] Monitoring of urea herbicides requires analytical methodologies capable of performing determinations at trace levels, such as chromatographic techniques; however, when dealing with real samples, some pre-treatment steps are necessary because the analytes are too dilute or the matrix is too complex.

In the present work, we have developed a method employing liquid chromatography in combination with diode array (LC-DAD) for the simultaneous detection of selected phenylurea herbicides in soil samples.

EXPERIMENTAL

Chemicals and Apparatus

Acetonitrile (ACN) and methanol (MeOH) were of HPLC gradient grade from Alltech (Zwijndrecht, The Netherlands) and were used as received. Dichloromethane, acetone, methanol, ethanol, *n*-hexane, isopropanol, and hydrochloric acid (HCl) were of analytical reagent grade and purchased from Alltech. Double distilled water (Milli-Q, Millipore, Waters Milford, MA, USA) was used for preparation of solutions. Silica gel sorbent, Kieselgel Si-60, 70–230 Mesh ASTM, was used to accomplish the solid-phase extraction (SPE) and was from Merck (Darmstadt, Germany). SPE was carried out in

a pressure chamber, SPE-12G, made of borosilicate glass (J.T. Baker, Gross-Gerau, Germany).

Chlorotoluron, isoproturon, diuron, monolinuron, linuron, metobromuron, chlorbromuron, methabenzthiazuron herbicides were reference materials for residue analysis purchased from Janssen Pharmaceuticalaan (Geel, Belgium). Concentrated analyte solutions (1 mg/mL) were prepared in ACN and stored in glass-stoppered bottles, in the dark, at 4°C. Appropriate volumes of these stock solutions were diluted in ACN-water (10:90, v/v). Figure 1 shows the structures of the phenylurea herbicides.

Chromatographic Analysis

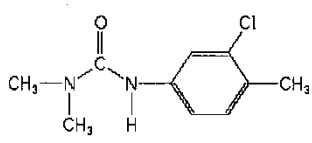
A 1050 HP apparatus (Hewlett Packard, Waldbronn, Germany) consisting of a gradient pump, DAD detector at $\lambda = 250$ nm, and the Vectra QS/HP computer with ChemStation-2 for data collection and instrument control, were selected for chromatographic measurements. A Rheodyne 7125 injection valve with a 10 μL injection sample loop, a C_{18} analytical column (250 mm × 4.6 mm i.d.), and a C_{18} precolumn (25 mm × 4.6 mm i.d.) from Alltech were used. Chromatographic separation was carried out in isocratic mode using ACN-water and MeOH-water in different concentration as mobile phase, and in gradient mode at a flow rate of 1 mL/min. The gradient elution was performed as follows: from ACN-water (39:61, v/v) to ACN-water (57:43, v/v) in 20 min and returning to initial conditions at 10 min.

Calibration Procedure

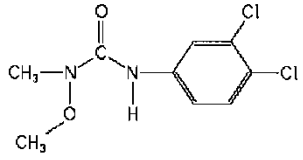
The external standard method of calibration was used for this analysis. At least five standard solutions containing all the eight compounds were analyzed, and calibration plots of the peak areas as a function of the concentrations of analytes injected were linear over the range of 0.1 to 100 ppm. The injection was performed three times for each standard solution to test reproducibility.

Characterization of the Soil Samples

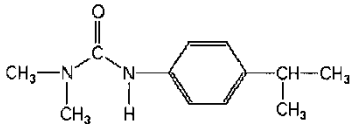
The soil samples were from tillages of berry shrubs and could be classified as the sandy kind with little contents of organic matter. Before analysis, the soil was homogenized, and then sieved by a 2 mm eye strainer to remove solid pollutions. Characteristic of the soil is shown in Table 1.



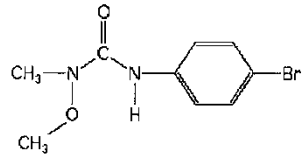
CHLOROTOLURON
N'-(3-chloro-4-methylphenyl)-*N,N*-dimethylurea



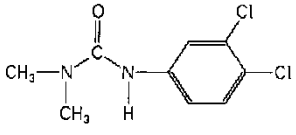
LINURON
N'-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea



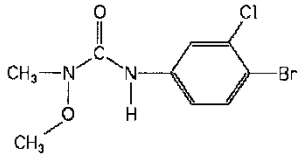
ISOPROTURON
N,N-dimethyl-*N'*-[4-(1-methylethyl)phenyl]urea



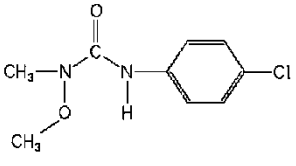
METOBROMURON
N'-(4-bromophenyl)-*N*-methoxy-*N*-methylurea



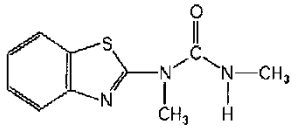
DIURON
N'-(3,4-dichlorophenyl)-*N,N*-dimethylurea



CHLORBROMURON
N'-(4-bromo-3-chlorophenyl)-*N*-methoxy-*N*-methylurea



MONOLINURON
N'-(4-chlorophenyl)-*N*-methoxy-*N*-methylurea



METHABENZTHIAZURON
N-2-benzothiazolyl-*N,N'*-dimethylurea

Figure 1. Common, systematic names and structures of the eight herbicides.

Extraction and Clean-up Procedure

Extraction was optimized to perform with the best efficiency the investigated ureas and some of their metabolites from the soil. The soil samples (100 g) were fortified with 1 mL of the composite standard solution (10 ppm) and were left in contact for one hour before starting the procedure. Extractions were carried out in duplicate by mixing soil samples with 140 mL of methanol, dichloromethane, ethanol, and acetone using a

Table 1. Soil characteristic

Parameter	Value
pH	6.50
Organic carbon (%)	2.79
$CaCO_3$ (%)	0.24
Clays (%)	5.40
Silts (%)	10.50
Sand (%)	84.10

mechanical shaker, fast speeding mixer, and sonicator. Extracts were evaporated at 40°C and reconstituted in 1 mL of *n*-hexane, the former being cleansed by SPE. The SPE was performed with 50 cm × 1 cm i.d. cartridges that were pre-packed with 10 g of activated silica gel. The activation was done by treating the silica gel portion with 3M HCl in 60°C for 6 h, after that adsorbent was washed with deionized water to neutral pH and with MeOH. The material was dried in vacuum. Solid-phase cartridges were equilibrated with 20 mL *n*-hexane. After passing the sample through the cartridges, the cartridges were washed with 100 mL isopropanol/*n*-hexane (40:60, v/v). The analytes were eluted from the cartridges with 120 mL methanol/dichloromethane (1:5, v/v). After evaporating the samples using rotary evaporator and next to near dryness under a gentle nitrogen stream, the compounds were transferred into a final volume of 1 mL of a mixture of ACN-water (10:90, v/v).

RESULTS AND DISCUSSION

The analysis of pesticide residues in environmental samples requires the availability of high efficiency separation methods able to determine the compounds of interest in complex samples free from matrix interferences. LC-DAD has shown to be an effective technique for the separation and detection of phenylurea pesticides. Efficient elution is usually performed on an analytical C_{18} column with a mixture of ACN and/or MeOH, with water as the mobile phase providing adequate retention and resolution (selectivity) by adjusting the eluotropic strength and/or (partially) the type of modifier. This method encouraged us to investigate its potential in this field of analysis of great interest for the assessment of human safety. The analytes included in this study were selected based on their extensive use as herbicides in agricultural areas.

Because of the difference in polarity between phenylurea compounds, their separation is usually performed with a isocratic elution. However, in trace analysis involving uncleaned soil extracts, gradient elution conditions

are more favorable in order to reduce baseline disturbances by matrix interferences. Therefore, we investigated the possibility of the two modes of elution, isocratic and gradient, for the separation of the selected herbicides (Fig. 1).

Selection of HPLC Determination Conditions

The chromatographic process in HPLC depends on interactions of sample analytes with the stationary phase and the mobile phase to effect the separation. The hydrophobic patches of the phenylurea molecules interact with the apolar surface of the C_{18} adsorbent causing the ureas to be retained. Molecules remain there until the organic content of the eluent rises high enough to weaken the interactions between the ureas and the column matrix. As the concentration of the organic solvent increases, the adsorbed ureas are eluted in order of least (most polar) to most strongly bound molecules (least polar).[7]

To develop a partition equilibrium between phases in the RPLC system, constant and reproducible conditions are presupposed. Consequently, for the separation of the target compounds, UV spectrophotometric detection at $\lambda = 250$ nm was chosen. The injection of a standard solution was prepared in ACN-water medium at 10:90 (v/v). The stationary phase used in the investigations was the silica gel with the octadecyl ligands (C_{18}) chemically bonded to the surface. The selectivities of the separation processes were optimized as a function of changes in the composition of hydroorganic binary mobile phases, ACN-water and MeOH-water.

Isocratic Mode-Selection of the Composition of the Mobile Phase

Based on the separation goals,[7] adequate retention of the analytes, $1 < k < 10$, and resolution between two neighboring peaks of the analytes, $R_s \geq 1.4$, should be obtained. Using ACN-water mixture in concentration 90:10 (v/v), compounds of interest were eluted with minimum differences between retention times, so the retention factor k was nearly the same for all the investigated phenylureas. Column dead time was quantified using the method described by Poole.[8] The following step in the investigations was to change the strength of mobile phase. As a "rule of three," it can be taken that decreasing the volumetric concentration of the modifier by 10% increases the retention time about three-fold.[9,10] Addition of water in quantities of 40% changed the elution conditions and separation of only four compounds. Lowering the ACN concentration the satisfactory separation was reached, but the retention time was too high, $t_R = 174$ min for the last eluting compound. In practice, it is desirable that the time of analysis of such compounds should not be greater than $t_R = 20$ min.

Originally the basic foundation of the optimizing procedure is the following: retention factor logarithms (log k) of analyzed compounds are linearly changed, together with change of composition of the mobile phase. Graphical interpretation of this foundation is presented in Figure 2. If the straight line representations of the separated compounds are parallel in relation to itself, e.g., chlorbromuron and monolinuron, it means that separation is still the same in the all area of changes of the organic component. Instead, points in which straight are crossed means that the separation is not achieved at the given composition of the mobile phase.[11]

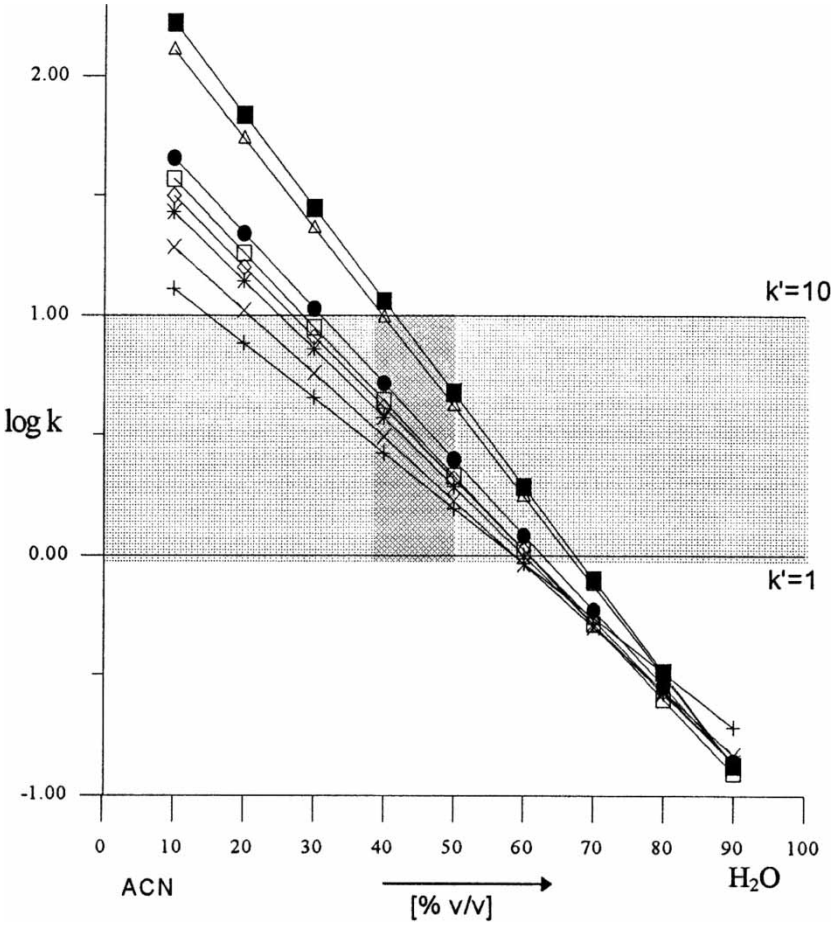


Figure 2. Retention factor k versus percent of organic modifier, ACN, in mobile phase. +-methabenzthiazuron; ×-chlorotoluron; ∗-isoproturon; ◇-diuron; □-linuron; ●-metobromuron; △-monolinuron; ■-chlorbromuron.

As a result, the composition of the binary mobile phase ACN-water (45:55, v/v) was defined at which the resolution between eluting analytes was the greatest ($R_s \approx 1.2$) and in a respectively short time. For this mobile phase the solvent strength is equal:

$$S_T = \sum S_i \psi_i \tag{1}$$

where: S_T–solvent strength for mobile phase, S_i–strength for i solvent, and ψ_i–the volume fraction of i solvent in mobile phase.

$$S_T = S_{org}\psi_{org} + S_{H_2O}\psi_{H_2O} \tag{2}$$

$$S_T = 3.2 \cdot 0.45 + 0 \cdot 0.55 = 1.44 \tag{3}$$

The next step in process optimization was qualification of the composition of mobile phase MeOH-water, for which the solvent strength was the same as for the mobile phase ACN-water. From this equation we received:

$$\psi_{org} = 1.44/2.6 = 0.55 \quad 55\% \text{ MeOH} \tag{4}$$

Theoretically, at this composition of mobile phase, MeOH-water (55:45, v/v), the retention times of the eluting compounds should not be different from those obtained for mobile phase ACN-water. Instead, the selectivity of the system should change due to use of the other modifier. In this case, the retention times are twice lowered for each analyte and the R_s value between the worst separated compounds (methabenzthiazuron and metobromuron) was 0.3. It follows that the selected composition of mobile phase, MeOH-water, did not cause improvement of separation between investigated phenylureas. Further steps in procedure were similar as described for ACN-water. From given data presented in Figure 3, it is seen that using as a mobile phase a mixture of MeOH and water, obtaining the satisfactory separation between eluting compounds during $t_R = 20$ min was impossible.

Using ACN as a modifier of the mobile phase, the worst separated compounds was diuron and linuron (peaks #4 and #5 on Figure 4), for which the R_s value was 0.8 during $t_R = 20$ min of analysis time. Using MeOH, it was possible to obtain a comparable separation only after 30 min. It could be observed that the change in order of the eluting analytes explained the different mechanism of interaction between compounds, mobile, and stationary phases.

Retention of urea herbicides increased with increasing the size of alkyl chains and halogen atoms (bromine atom caused greater retention on column than chlorine atom). The methoxy group connected with a

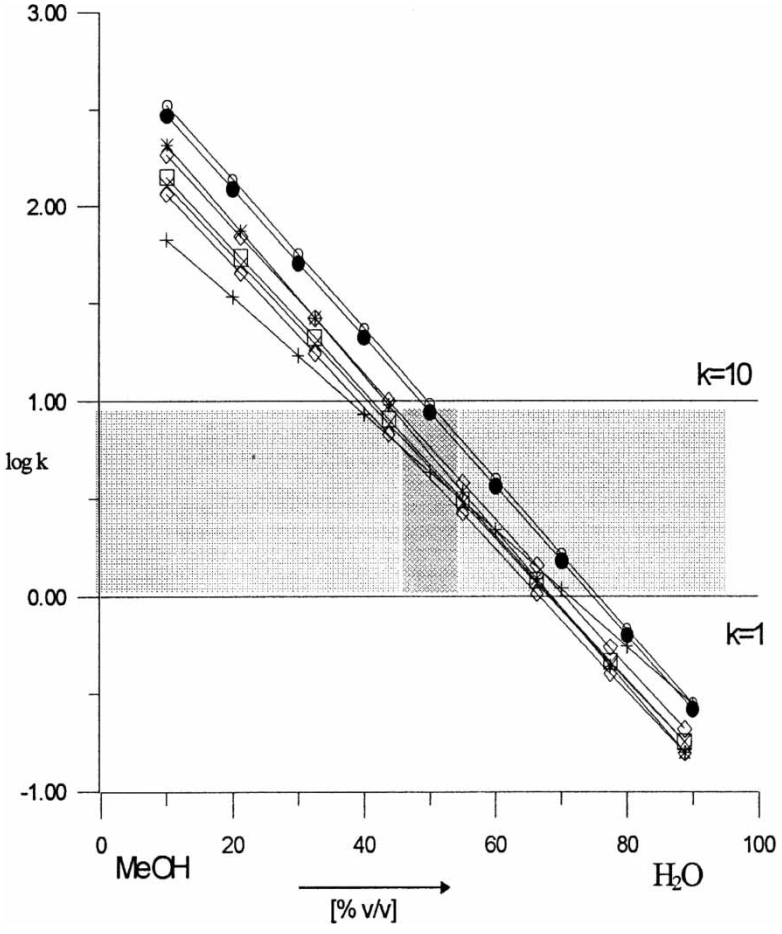


Figure 3. Retention factor k versus percent of organic modifier, MeOH, in mobile phase. +-methabenzthiazuron; ×-chlorotoluron; ∗-isoproturon; ◇-diuron; △-linuron; □-metobromuron; ●-monolinuron; ○-chlorbromuron.

benzene ring caused considerable lowering of the retention time, however, connected with a nitrogen atom it enlarged the retention time more than the methyl group in the same place. This was probably due to mutual influences between neighboring oxygen and nitrogen atoms in lowering the polarity of the molecule.

The final chromatogram obtained in the optimization process of separating urea herbicides is presented on Figure 4. This chromatogram does not fulfill our goals: the resolution between two neighboring peaks of the analytes, $R_s \geq 1.4$, was not obtained in the required time of 20 min using mobile phases ACN and MeOH with water in isocratic mode.

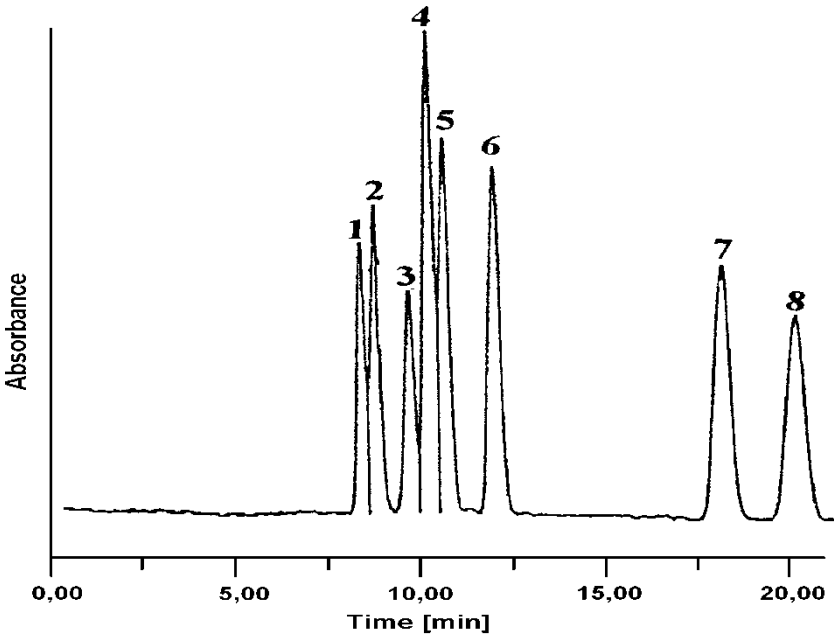


Figure 4. LC-DAD chromatogram, isocraztic elution, ACN-water (45:55, v/v). Peaks: 1-methabenzthiazuron; 2-chlorotoluron; 3-isoproturon; 4-diuron; 5-linuron; 6-metobromuron; 7-monolinuron; 8-chlorbromuron.

Gradient Elution–Selection of Conditions

When isocratic elution does not permit quantitative and qualitative determination of eluting compounds, using gradient elution appears to be the answer. It is clear that in gradient elution, separation conditions rely on gradual changing of the concentration of solvent during separation, in such a manner that solvent strength increases from the beginning to the end of the analysis. Retention factor k of analytes are changed during their moving along the column.

The initial and final concentrations of ACN in the mobile phase were varied in order to optimize the procedure. It was done by injecting the mixture of standards on the chromatographic column in concentration of 10 ppm. The resolution was also improved by changing the duration and the gradient range. Taking into account all the above described conditions, finally the gradient elution was performed as follows: from ACN-water (39 : 61, v/v) to ACN-water (57 : 43, v/v) in 5.5 min. An example of the obtained chromatogram is presented in Figure 5 and, is composed from eight eluting ureas during less than than 10 min. The superior performance of LC-DAD in gradient mode is clearly displayed. In comparison to the isocratic mode, it provides higher selectivity. As indicated in the

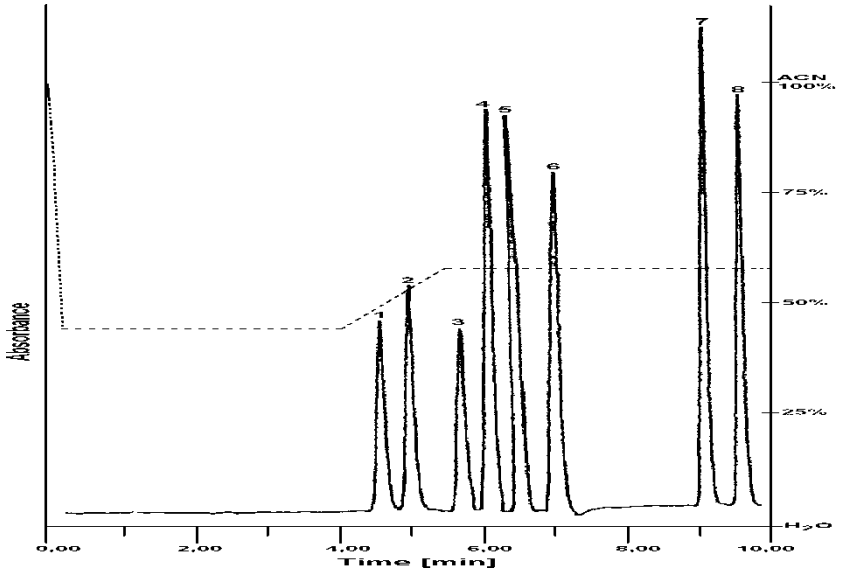


Figure 5. LC-DAD chromatogram, gradient elution, ACN-water, 39% to 57% ACN in 20 min.

chromatogram, improvements in the separation of the individual compounds, especially for: methabenzthiazuron (#1) and chlorotoluron (#2) as well as isoproturon (#3), diuron (#4), and linuron (#5), were reached. After every gradient run, the column was rinsed during 10 min with ACN-water (90 : 10, v/v) to wash remain particles.

Clean-up by Solid-Phase Extraction

Solid phase extraction (SPE) prior to chromatographic determination was used in order to achieve a more sensitive method for the quantification of the investigated phenylurea herbicides. A clean up SPE procedure was carried out to remove co-extracted compounds that may interfere with the chromatographic determination, or to be detrimental to the analytical instrumentation.

For most analytes, and especially for the urea herbicides, the choice of instrumentation and method for the chromatographic separation and detection of components to be isolated by SPE must be made before the SPE procedure can be tested. The choice of SPE sorbents, techniques, and subsequent sample treatment employed for acidic herbicides is dependent on the analytical instrumentation used for final determination.[12,13]

Based on our experience with the extraction of polar and moderately polar pesticides (triazines and metabolites, sulphonyl urea herbicides) from food, soil, and water,[14–16] silica gel activated by 3M HCl was selected. It is the

most commonly used adsorbent. The polar sites of silica (≡Si-OH) adsorb moderately polar compounds dissolved in organic solvents. A polar interaction via hydrogen bonding takes place between a hydrogen of the urea group in the herbicides and the oxygen of a silanol group. The interaction with the silica gel caused the retaining of the compounds by the adsorbent. Elution is accomplished with a polar solvent, such as methanol–dichloromethane mixture, which forms strong hydrogen bonds with the silanol groups, thus, displacing the compounds.

The chromatogram in Figure 6 shows a blank soil sample after cleansing on the SPE column. The peaks originating from the soil for which the retention times are close to the analytes retention times, but do not coelute together, could be observed.

Extraction-Choice of Solvent

Sample processing of soils usually requires extraction of the analytes followed by clean-up and concentration steps, in order to increase selectivity and/or sensitivity of the analytical method.

To select the appropriate extraction conditions, different extraction solvents were studied. Acetone, ethanol, methanol, and dichloromethane were selected for the extraction of phenylurea herbicides from soil. Obtained results, including efficiency of extraction, which was measured by recovery rates, depended on the solvent used for each herbicide; they are presented in Table 2. Maximum recoveries were in the case of methanol and dichloromethane solvent extraction, while lower recoveries were obtained for both acetone and ethanol. From this study, only pure methanol

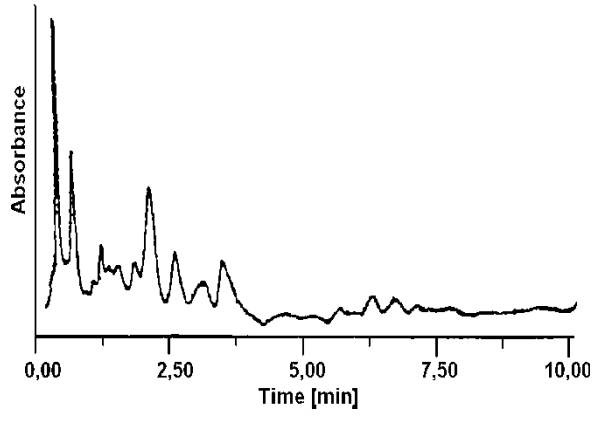


Figure 6. LC-DAD chromatogram of blank soil sample after SPE clean-up.

Table 2. Recovery rates of herbicides for different extraction solvents

Peak No.	Compound	Extraction solvent (% recovery)			
		Acetone	Ethanol	Methanol	Dichloromethane
1	Methabenzthiazuron	42	50	89	87
2	Chlorotoluron	45	45	87	89
3	Isoproturon	55	67	89	90
4	Diuron	54	70	93	87
5	Linuron	56	58	91	88
6	Metobromuron	66	54	79	75
7	Monolinuron	75	40	95	93
8	Chlorbromuron	74	43	88	98

was able to extract the target analytes from soil samples with high efficiency and lower amounts of coextractives, compared to dichloromethane.

The example values for diuron (data not presented), extracted using methanol, proves that the most effective method for extraction of a soil sample is traditional, mechanical shaking. The advantage of extraction using sonication was a comparatively short time of duration, however, obtained results did not classified this method for utilization in routine analysis.

Method Validation

Analytical curves for standards containing between 0.1 to 100 ppm of the seven phenylurea herbicides, prepared according to the procedure described in experimental, were obtained by plotting the analyte peak area against the analyte concentration. The linear range was similar for all herbicides. The R^2 values were at least 0.998 for phenylurea analytes.

A study of the recovery and precision of the extraction method at two different concentration levels, 20 ppb and 200 ppb, was done. The analytical figures of merit for a soil sample used for identification/quantitation are summarized in Table 3. Total recoveries using the method, which included all losses during sample preparation, ranged from 79 to 97% recovery of the herbicides from the silica gel sorbent column. Detection limits (LOD) were calculated as the minimum concentration providing a chromatographic signal three times higher than background noise, and ranged between 0.07 to 0.13 $\mu g/g$. The precision of the method (as RSD) of repeat measurements ($n = 6$) was checked using eight standards containing all herbicides, and were between 2.9–10.4%. No significant differences in validation parameters were observed between these two levels of fortified soil samples. The LC-DAD chromatogram of a fortified soil sample is presented in Figure 7.

Table 3. Correlation coefficients, limits of detection (LOD), average recoveries, relative standard deviations (precision, RSD) and for herbicides from soil samples

Peak No.	Compound	R^2	LOD (μg/g)	20 ppb		200 ppb	
				Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
1	Methabenzthiazuron	0.9984	0.13	88	5.8	87.5	6.4
2	Chlorotoluron	0.9994	0.12	88	2.9	92.5	6.4
3	Isoproturon	0.9983	0.07	89	5.0	92.5	6.4
4	Diuron	0.9988	0.08	92	2.9	92.5	10.4
5	Linuron	0.9990	0.08	93	3.0	93.2	7.3
6	Metobromuron	0.9996	0.10	79	5.8	91	4.8
7	Monolinuron	0.9992	0.10	95	2.9	97	8.7
8	Chlorbromuron	0.9985	0.10	88	10.4	97	8.7

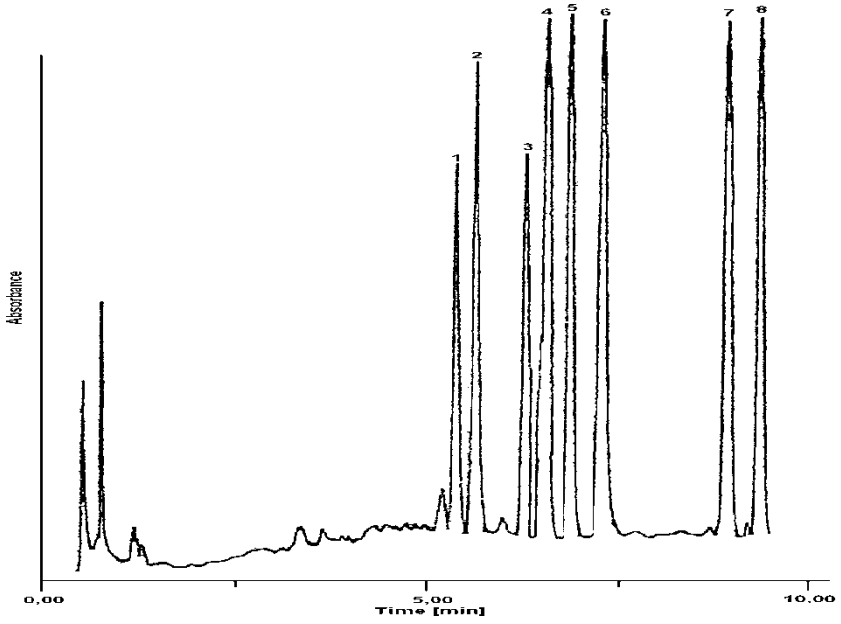


Figure 7. LC-DAD chromatogram of fortified soil sample.

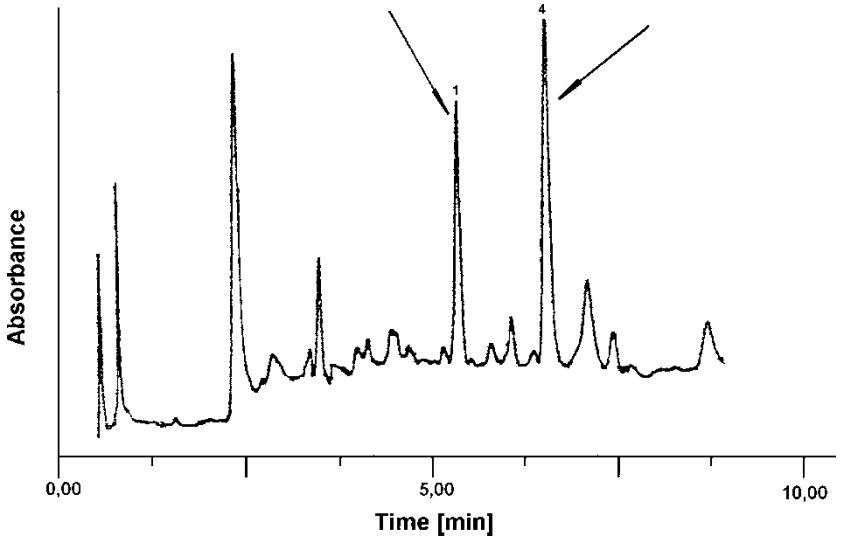


Figure 8. LC-DAD chromatogram of real soil sample containing an incurred residue of methabenzthiazuron (#1) and diuron (#4) (indicated by arrows).

Analysis of Phenylurea Herbicides in Real Soil Samples

The optimized LC-DAD method was applied to the analysis of phenylurea herbicides in real soil samples. Figure 8 presents the chromatogram of the real soil sample received after cleaning the extract. Comparison of retention times and spectrums of herbicide standards allow affirming methabenzthiazuron and diuron presence in the sample. The LC-DAD analysis of an extract of a collected field soil sample containing 15 μg/kg of methabenzthiazuron and 18 μg/kg of diuron displayed in Figure 8, clearly illustrates the ability of the developed technique to determine urea herbicides at this concentration level.

CONCLUSIONS

It was demonstrated that LC-DAD with clean-up using SPE is a sensitive and selective technique for the determination and quantitation of herbicides in environmental soil samples. Very low detection limits can be reached due to the enhanced selectivity and high sensitivity obtained with this methodology. Furthermore, this method has clearly demonstrated good recoveries (79–95%), good precision (2.9% $\leq$ RSD $\leq$ 10.4%), and good sensitivity by SPE using the silica gel cartridge.

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