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Quantification of phenylurea herbicides and their free and humic acid-associated metabolites in natural waters

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Abstract

There is increasing interest in and demand for simultaneously monitoring pesticides as well as related degradation products (DPs) in natural waters, as the latter compounds can be even more toxic than the former ones. A method for determining parts per trillion levels of phenylurea herbicides and their DPs, that is their dealkylated forms and aromatic amines, is described. This method is based on solid-phase extraction with a Carbograph 4 cartridge followed by liquid chromatography (LC) with electrospray (ES) mass spectrometric detection. A study aimed at optimizing the response of the ES-MS detector for very weakly basic chloroanilines was conducted. Results showed that ion signal intensities of the above species were dependent on the composition of the LC mobile phase to an astonishing degree. At concentration levels of a few hundred ng/l, laboratory experiments showed that the aromatic amines considered here were mostly associated to dissolved humic acids (HAs) by both reversible and irreversible bindings. The addition of a reducing agent, i.e., $NaBH_4$, succeeded in liberating that fraction of aromatic amines, which being reversibly bound to quinoidal structures of HAs are bioavailable. Analyte recoveries were better than 85% on extraction from 4 l of drinking water (spike level, 25 ng/l), 2 l of ground water (spike level, 50 ng/l) and 0.5 l of river water (spike level, 200 ng/l). Relative standard deviations ranged between 4.6 and 20% for drinking water, 4.3 and 15% for ground water, 5.9 and 13% for river water. Method detection limits calculated for drinking water, groundwater and surface water were between 3 and 11, 6 and 21, 36 and 75 ng/l, respectively.

Keywords: Water analysis; Environmental analysis; Phenylureas; Pesticides

1. Introduction

Phenylurea herbicides (PUHs) are largely used in field applications for pre- and post-emergence weed control in a wide variety of crops. PUHs in the environment are gradually biodegraded by stepwise demethylation or demethoxylation of the urea moiety followed by generation of aromatic amines. While little is known on the toxicity of dealkylated PUHs, both PUHs and aromatic amines are known to be toxic and some of the latter class of compounds can induce cancer [1–4].

Laboratory studies [5–9] have demonstrated that considerable fractions of aromatic amines in aqueous solutions can be bound to dissolved humic acids (HAs) by reversible and irreversible reaction mechanisms. Aromatic amines reversibly bound to HAs are

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of environmental concern as they are still bioavailable.

For every pesticide and any related toxic metabolite, the European Union has set a maximum admissible concentration of 100 ng/l in drinking water.

Therefore, modern analytical methods of PUH residues in water have to meet the following criteria: (1) simultaneous determination of the parent compounds and their metabolites, (2) low quantification limits, and (3) quantification of free and HA-associated aromatic amines. Such methods can be used for monitoring, fate and behavior studies and are the basis for proper ecotoxicological investigation.

Only one gas chromatography (GC)-based method has been elaborated in the past for the simultaneous determination of PUHs and their related aromatic amines [10]. However, the extensive processing requirements of the quantitative determination obviates use of this method for environmental monitoring of PUHs and aromatic amines in water. The problem of simultaneously analyzing PUHs and their two classes of metabolites in natural waters has not yet been addressed. Moreover, no published analytical method devoted to monitoring aromatic amines in aqueous environmental matrices describes any device enabling determination of that fraction of aromatic amines which are reversibly bound to HAs.

Thermolability of many PUHs and their dealkylated forms precludes their direct determination by the use of the highly selective GC–mass spectrometry (MS) technique and makes liquid chromatography (LC) the technique of choice. PUHs as well as their degradation products (DPs) are UV-absorbing species. However, a LC method devoted to detecting ng/l levels of these analytes in aqueous environmental matrices cannot rely on the insufficient selectivity of UV-based detectors and must involve MS detection to be sound and reliable.

Solid-phase extraction (SPE) cartridges filled with graphitized carbon black (GCB) adsorbents have proved to be valuable tools for extracting highly polar organic contaminants even from large volumes of water [11–13].

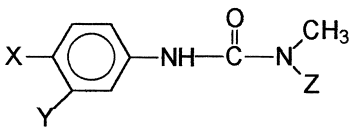
The purpose of this work has been that of elaborating a sensitive and very selective method based on SPE with a recently introduced example of GCB, that is Carbograph 4, followed by LC–MS with an electrospray (ES) interface for assessing trace levels of PUHs and their major DPs in natural waters. Nine of the PUHs most widely used in western countries [14] and 13 DPs (among aromatic amines and dealkylated PUHs) were selected for this study. Particular attention was devoted to finding a simple and effective device enabling determination of even aromatic amines reversibly bound to HAs.

2. Experimental

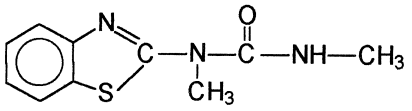
2.1. Reagents and chemicals

Structures and commercial names of the PUHs considered in this study are visualized in Fig. 1. Six commercially available dealkylated forms of PUHs were used (abbreviations are reported in parentheses): 4-chlorophenylurea (4-ClPhurea); 3-chloro-4-methylphenylurea (3-Cl-4-MePhurea); 4-(1-methylethyl)phenylurea (4-*i*-PrPhurea); 3,4-dichlorophenylurea (3,4-diClPhurea); *N*-methyl-*N′*-[4-(1-methylethyl)phenyl]urea (4-*i*-PrPhMeurea); *N′*-(3,4-dichlorophenyl)-*N*-methylurea (3,4-diClPhMeurea). *N′*-(4-Chlorophenyl)-*N*,*N*-dimethylurea (monuron), which is a superseded PUH, was used as internal standard (I.S.). All the above compounds were purchased from Alltech, Sedriano, Italy. Except for 4-chloroaniline (4-Claniline) and 3,4-dichloroaniline (3,4-diClaniline) (Aldrich, Steinheim, Germany), the other aromatic amines of interest are not commercially available and were synthesized from the corresponding PUHs by catalytic hydrolysis on silica [10]. They were as follows: 3-chloro,4-methoxyaniline (3-Cl-4-methoxyaniline); *N′*-2-benzothiazolyl-*N*-methylamine (benzothiazolylMeamine); 4-bromoaniline (4-Braniline); 4-isopropylaniline (4-*i*-Praniline); 3-chloro,4-methylaniline (3-Cl-4-Meaniline). Individual standard solutions were prepared by dissolving 20 mg of them in 200 ml of methanol. Composite working standard solutions were prepared by suitably mixing the standard solutions mentioned above. The solution containing the I.S. was further diluted with methanol to obtain a concentration of 1 μg/ml.

Trifluoroacetic acid (TFA), HA sodium salts and sodium borohydrate ($NaBH_4$) were purchased from Aldrich. A basified HA solution with a dissolved



	X	Y	Z
Metoxuron	OCH_3	Cl	CH_3
Chlorotoluron	Cl	Cl	CH_3
Isoproturon	i-C_3H_7	H	CH_3
Diuron	Cl	Cl	CH_3
Neburon	Cl	Cl	C_4H_9
Linuron	Cl	Cl	OCH_3
Metobromuron	Br	H	OCH_3
Monilinuron	Cl	H	OCH_3



Methabenzthiazuron

Fig. 1. Structures and common names of the most widely used phenylurea herbicides.

organic carbon (DOC) concentration of 250 mg/l was prepared as reported elsewhere [15].

For LC, distilled water was further purified by passing it through the Milli-Q Plus apparatus (Millipore, Bedford, MA, USA). Methanol ''Plus'' and acetonitrile ''Plus'' of gradient grade were obtained from Carlo Erba, Milan, Italy. All other solvents were of analytical grade (Carlo Erba) and they were used as supplied.

2.2. Apparatus

Half-gram Carbograph 4 extraction cartridges and the tool for performing back-flushing elution of the analytes were from Alltech. Back-flushing elution was found necessary to achieve quantitative re-extraction with moderate extractant volumes of some of the analytes strongly adsorbed on the GCB surface. The Carbograph 4 cartridge was fitted into a side-arm filtration flask and liquids were forced to pass through the cartridge by vacuum (water pump). Before processing water samples, the cartridge was washed with 10 ml of the eluent phase for the analytes (see below), followed by 3 ml methanol and 10 ml distilled water.

2.3. Aqueous samples

Grab samples of river (Tiber) water (11 mg/l DOC) and ground water (0.8 mg/l DOC) from a well located near Rome were collected in brown bottles. Bottles were kept in the dark at 4°C until analysis. Before spiking with the analytes, carbonyl groups of HA in the river water sample were reduced by adding 0.5 g/l $NaBH_4$ and then agitating the sample for 10 min. This addition was made to inhibit covalent binding between HA and aromatic amines (see below). Drinking water samples were collected from the tap in the laboratory. Hypochlorite in drinking water was eliminated by adding 0.5 g/l $Na_2S_2O_3$. For recovery studies of aromatic amines reversibly bound to HA, a simulated river water (SRW) sample was also used. This sample was prepared by adding to distilled water HAs from the standard solution to create a DOC concentration of

20 mg/l and then decreasing the pH of this solution to about 8 with addition of diluted HCl.

2.4. Extraction procedure

The extraction procedure was performed as previously reported [13] except that, after reversing the cartridge, analytes were re-extracted by passing through the cartridge 1.5 ml of methanol followed by 6 ml CH_2Cl_2–CH_3OH (80:20, v/v). Both eluent phases were acidified with 10 mmol/l HCl. Eluates were collected in a silanized glass vial. The silanization procedure was carried out as reported elsewhere [13]. This precaution avoided significant loss of aromatic amines, when present in the vial at quantities lower than 100 ng. Before concentrating the extract, 100 μl of the I.S.-containing solution was added. Solvents were then removed at 40°C in a water bath under a gentle flow of nitrogen, until the extract reached a volume of about 50 μl. Thereafter, the extract was partially neutralized by adding 70 μl of 0.5 mol/l ammonia and 40 μl of the final extract was injected into the LC column.

2.5. LC–ES-MS analysis

LC was carried out with a Varian (Walnut Creek, CA, USA) Model 9010 chromatography system. The analytes were chromatographed on an ''Alltima'' 25 cm×4.6 mm I.D. column filled with 5-μm C_{18} reversed-phase packing (Alltech). For fractionating the analytes, the phase A was acetonitrile and the phase B was water. Both solvents contained 20 μmol/l TFA. The initial composition of the mobile phase was 0% A that was first increased linearly to 25% in 10 min, then to 50% in 25 min, and finally to 100% in 20 min. The flow-rate of the mobile phase was 1 ml/min and 50 μl/min of the column effluent was diverted to the ES source. A Micromass ''Platform'' benchtop mass spectrometer (Manchester, UK) consisting of a pneumatically-assisted ES interface and a single quadrupole was used for detecting and quantifying target compounds in the LC column effluent. The MS was operated in the positive-ion mode by applying to the capillary a voltage of 4.0 kV. The source temperature was maintained at 65°C. Structurally significant fragment ions were obtained by the collision-induced decomposition (CID) process, after setting the cone voltage at 30 V (Table 1). Full-scan LC–MS chromatograms were obtained by scanning the quadrupole from 70 to 310 m/z with 2-s scan.

2.6. Quantification

The concentration of each analyte in water was calculated by measuring the total peak area obtained by selecting ion signals for both parent and fragment ions, relating this area to that of the I.S. and comparing this relative peak area with that obtained from standard solutions. These solutions were prepared by dissolving known and appropriate volumes of the working standard solution in the acidic solvent system used for eluting analytes from the Carbograph 4 cartridge and then following the last part of the procedure reported above. The mass spectrometry data handling system used was the ''Mass Lynx'' software from Micromass.

Table 1
Mass-to-charge ratios and relative abundances of protonated adduct ions of the analytes and related fragment ions obtained by setting the sample cone voltage at 30 V

Compound	m/z (relative abundance)
4-ClPhurea	93 (30), 128 (80), **171**[a] (100)
3-Cl-4-methoxyaniline	108 (10), 123 (40), **158** (100)
Metoxuron	72 (100), **229** (60)
3-Cl-4-MePhurea	107 (20), 142(40), **185** (100)
benzothiazolylMeamine	150 (10), **165** (100)
Monuron (I.S.)	72 (100), **199** (30)
4-*i*-PrPhurea	94 (20), 137 (100), **179** (70)
3,4-diClPhurea	127 (20), 162 (50), **205** (100)
4-Claniline	93 (30), **128** (100)
4-*i*-PrPhMeurea	94 (30), 136 (30), 151 (50), **193** (100)
Methabenzthiazuron	165 (100), **222** (20)
4-Braniline	93 (15), **172** (100)
3,4-diClPhMeurea	127 (20), 162 (80), **219** (100)
Chlorotoluron	72 (100), **213** (100)
Isoproturon	72 (100), 165 (25), **207** (20)
Monolinuron	126 (100), 148 (70), **215** (30)
Diuron	72 (100), **233** (70)
3-Cl-4-Meaniline	107 (20), **142** (100)
4-*i*-Praniline	94 (20), **136** (100)
Metobromuron	88 (60), 148 (100), 170 (80), **259** (45)
3,4-diClaniline	127 (20), **162** (100)
Linuron	88 (60), 160 (100), 182 (90), **249** (60)
Neburon	88 (55), 114 (25), **275** (100)

[a] Intact protonated adduct ions are reported in boldface.

3. Results and discussion

3.1. Selection of the LC mobile phase

In ES-MS, a short survey was conducted by flow injection analysis to evaluate the influence of the carrier stream composition on the production of gas-phase ions from several anilines [16]. As expected on the basis of the solution chemistry, abundant amounts of gas-phase protonated molecules was achieved from the test compounds by adding 1% (v/v) formic acid to a water–methanol (50:50) carrier stream. When performing LC–ES-MS analysis, however, this condition can provoke severe deformation of the chromatographic band for a sufficiently basic compound due to its partial protonation in the LC mobile phase. A study aimed at finding the most convenient LC mobile phase able to give a good response of the ES-MS detector for all the analytes, while minimizing peak tailing for the most basic aromatic amines considered, i.e., 4-*i*-Praniline and benzothiazolylMeamine, was conducted. For this purpose, the composition of the LC mobile phase was varied by adding various additives as well as changing the nature of the organic modifier, namely methanol and acetonitrile. In any case, 50 ng of each analyte was injected from a composite standard solution into the LC column. Under each chromatographic condition and for each analyte, the response of the ES-MS detector was evaluated by calculating the signal-to-noise ratio (S/N). This parameter was obtained by comparing the analyte peak height to one standard deviation of the mean baseline signal. For conciseness, S/N for only some selected analytes are reported in Table 2. Regardless of the nature of the organic modifier, sharp peaks for all the analytes were observed when ammonium acetate (NH_4OAc) was added to the mobile phase. However, this condition gave unacceptably weak ion signals, especially for the least basic aromatic amines. The very low ion signals experienced when buffering the mobile phase with ammonium acetate can be explained considering that the very low proton concentration in the electrosprayed solution does not favor a shift of the equilibrium towards formation of protonated species for very weakly basic compounds. As a matter of fact, the production rate of gas-phase MH^+ ions from the aromatic amines decreased by passing from the most basic amine, i.e., 4-*i*-Praniline to the least basic one, i.e., 3,4-diClaniline.

Increasing the proton concentration by adding formic acid to the ammonium acetate-buffered mobile phase enhanced amine detectabilities. This improvement, however, was still inadequate to the purpose of measuring ng/l levels of 3,4-diClaniline in water. Replacing methanol–NH_4OAc with acetonitrile–NH_4OAc achieved the negative effect of

Table 2
Signal-to-noise ratios (S/N) of selected analytes by varying the LC mobile phase composition (injected amount of each analyte: 50 ng)

Compound	S/N					
	Organic modifiers and additives in the LC mobile phase					
	MeOH, 50 μ*M* $AcNH_4$, pH 6.6[a]	MeOH, 50 μ*M* $AcNH_4$, pH 4.0	CH_3CN, 50 μ*M* $AcNH_4$, pH 4.0	MeOH, 200 μ*M* HCOOH, pH 4.1	CH_3CN, 200 μ*M* HCOOH, pH 4.0	CH_3CN, 20 μ*M* TFA, pH 4.6
4-*i*-Praniline	80	330	615	930	270	410
4-Claniline	3	40	35	180	280	210
3,4-diClaniline	n.d.[b]	3	19	6	380	240
4-*i*-PrPhurea	160	73	95	120	260	170
3,4-diClPhurea	60	39	45	51	80	90
3,4-diClPhMeurea	80	48	60	75	450	150
Isoproturon	140	66	380	250	560	430
Diuron	160	70	60	210	350	250
Linuron	85	40	10	160	190	170

[a] Actual pH values in a water–organic solvent (50:50) mixture measured by a glass-electrode pH meter.
[b] n.d.=Not detected.

depressing signals for some of the PUHs. Elimination of the salt and acidification of the water–methanol mobile phase with 200 μmol/l formic acid resulted in an increase of the performance of the ES-MS detector, except for 3,4-diClaniline. An about 60-times increase of the ion signal intensity for this compound was surprisingly achieved by simply substituting methanol with acetonitrile to the water–organic solvent–formic acid solution. No reasonable hypothesis accounting for this astonishing effect was found by us. From an analytical point of view, a drawback of the above mobile phase was that it produced strongly tailed peaks for the most basic aromatic amines. The best compromise between detectability and peak symmetry was reached by substituting HCOOH with one order of magnitude lower amount of a strong acid, such as TFA. Probably, this result was due to the fact that the small amount of TFA added to the mobile phase was insufficient to protonate substantial fractions of the most basic amines in the LC column, but it sufficed to shift equilibrium towards formation of protonated species of the least basic amines in a solution which is greatly concentrated in the ion source by the electrospray process.

3.2. Release of bioavailable amines bound to humic acids

Quinone groups in the framework of HAs are mainly responsible for binding aromatic amines. Two reaction mechanisms by which anilines are incorporated into HAs have been proposed [17,18]: (A) 1,2 Nucleophilic addition on the carbonyl group of quinone-type moieties to form imines (Schiff bases). This process is rapid and reversible. (B) 1,4-Addition (Michael addition) to quinone structures to form sequentially aminohydroquinones, aminoquinones and, finally, a variety of nitrogen heterocycles. This process is slow and substantially irreversible.

As a rule of thumb, the reaction mechanism A should be largely inhibited in aqueous solution, because the overall equilibrium should favor hydrolysis. However, when aromatic amines are reacted with sterically hindered quinones, the dominant mode of attack can in some instances occur through the mechanism A [8]. Experiments were conducted to: (1) assess the extent at which aromatic amines here considered were incorporated into HAs and (2) find a simple and effective device to liberate readily that fraction of aromatic amines which, being reversibly associated to HAs, are of ecotoxicological importance. For these purposes, two SRW samples (see Experimental) were spiked with the seven aromatic amines at two individual concentrations, i.e., 0.2 and 2 μg/l. Thereafter, these solutions were kept under continuous agitation and 1-l aliquots were taken from the two solutions at intervals of 1, 8 and 24 h. Each aliquot was analyzed in duplicate. Long-term effects leading eventually to formation of stable aromatic amine–HA complexes were not considered in this study. After 24 h from the beginning of the experiment, 0.5 g/l $NaBH_4$ was added to the two solutions. This salt is a commonly used carbonyl group reducing agent and has been already used for inhibiting ''a priori'' the reaction of aniline with a fulvic acid isolate [8]. The rationale behind the use ''a posteriori'' of $NaBH_4$ was that of converting quinones to hydroquinones, so shifting the equilibrium of the reaction A towards free amines. Following this addition, the solutions were vigorously agitated for 10 min and then reanalyzed. Finally, the effectiveness of the SPE cartridge to extract free amines from water samples containing relatively large amounts of HAs was checked by analyzing two control samples. These samples were prepared as reported above, with the difference that $NaBH_4$ was added before spiking with amines at the two different concentrations reported above, in order to block any reaction between quinones and amines which could take place during the extraction step. Results of these experiments are reported in Table 3.

From the observation of the experimental results, two general considerations can be made. One is that, provided no opportunity was allowed for targeted aromatic amines to react with quinone moieties of HAs, their extraction from water with the SPE cartridge was not affected by the presence of relevant amounts of HAs. The second consideration is that binding of amines to Aldrich HAs occurred in a relatively short time, as the free amine concentrations in the two test solutions did not decrease significantly by prolonging the reaction time from 8 to 24 h. This finding contrasts with previous observations [5].

Table 3
Binding kinetics of aromatic amines to humic acids in water and percentages of free aromatic amines after adding sodium borohydride

Compound	Percentages of free amines in water									
	Nominal analyte concentration in water (μg/l)									
	0.2					2				
	Reaction time (h)				Time passed after $NaBH_4$ addition (min): 10	Reaction time (h)				Time passed after $NaBH_4$ addition (min): 10
	0[a]	1	8	24		0[a]	1	8	24	
3-Cl-4-Methoxyaniline	93	44	18	16	70	99	63	59	58	92
BenzothiazolylMeamine	95	86	68	64	62	95	100	92	93	95
4-Claniline	99	65	37	37	77	95	87	88	88	98
4-Braniline	101	67	38	39	78	98	93	89	89	97
3-Cl-4-Meaniline	96	64	39	34	74	93	90	86	87	96
3,4-diClaniline	98	58	35	36	75	99	75	76	75	95
4-*i*-Praniline	92	53	35	25	67	95	67	68	69	95

[a] Analytes added after pretreatment of the aqueous sample with $NaBH_4$.

Table 4
Accuracy, precision and method detection limits for phenylureas and their metabolites added (spike levels in parentheses) to three types of natural waters

Compound	4 l Tap water (20 ng/l)				2 l Well water (50 ng/l)				0.5 l River water (200 ng/l)			
	$\bar{x}$[a]	S.D[b]	t[c]	MDL[d]	$\bar{x}$	SD	t	MDL	$\bar{x}$	SD	t	MDL
4-ClPhurea	19	1.7	2.0	5	46	4.8	2.0	14	198	17	0.29	52
3-Cl-4-methoxyaniline	19	2.0	0.84	6	50	4.4	0.16	13	178	22	2.4	66
Metoxuron	21	2.2	0.98	7	51	5.2	0.64	16	202	18	0.27	54
3-Cl-4-MePhurea	19	1.1	2.2	3	45	6.3	1.9	19	192	15	1.3	45
benzothiazolylMeamine	19	3.2	0.45	10	49	5.1	0.47	15	175	18	3.3	54
4-*i*-PrPhurea	20	2.1	0.46	6	49	6.5	0.30	20	193	17	0.99	51
3,4-diClPhurea	18	2.3	1.7	7	46	6.6	1.4	20	191	22	0.98	66
4-Claniline	20	3.4	0.07	10	49	7.1	0.37	21	182	18	2.4	54
4-*i*-PrPhMeurea	20	1.4	0.34	4	49	5.1	0.71	15	202	22	0.22	66
Methabenzthiazuron	16	1.5	5.8	4	44	4.3	3.4	13	199	13	0.18	39
4-Braniline	19	3.1	0.46	9	51	6.3	0.30	19	186	23	1.5	69
3,4-diClPhMeurea	19	1.4	1.7	4	51	5.3	0.32	16	208	21	0.92	63
Chlorotoluron	20	1.0	0.72	3	51	3.1	1.0	9	204	12	0.83	36
Isoproturon	20	1.9	0.13	6	49	5.0	0.29	15	188	13	2.2	39
Monolinuron	20	1.8	0.26	6	52	4.5	0.91	14	205	25	0.48	75
Diuron	20	1.8	0.26	6	51	4.2	0.51	13	199	19	0.13	57
4-*i*-Praniline	19	3.6	0.40	11	51	5.1	0.24	15	172	20	3.4	60
3-Cl-4-Meaniline	19	3.0	1.1	9	46	5.0	1.9	15	182	19	2.3	57
Metobromuron	19.5	0.9	1.3	3	49	2.1	1.7	6	193	12	1.4	36
3,4-diClaniline	18	3.6	1.4	11	50	6.0	0.0	18	176	23	2.5	69
Linuron	21	1.8	1.3	5	51	4.9	0.44	15	202	25	0.19	75
Neburon	19	1.3	1.5	4	47	3.6	2.0	11	201	17	0.14	51

[a] $\bar{x}$=Mean values from six determinations.
[b] SD=Standard deviation.
[c] Critical t value (P=0.05)=2.6.
[d] MDL=Method detection limit.

At the 0.2 μg/l spike level, covalent binding to HAs decreased dramatically the fraction of free amines in water. At amine concentrations 10-times larger, this effect was much less remarkable and only the concentrations of three amines out of seven decreased significantly. This latter result is somewhat surprising as, from the estimation made by Parris [5], Aldrich HAs should have a number of sites able to sequester aromatic amine quantities far exceeding those present in the most concentrated solution. Weber et al. [8] anticipated this effect could occur in an environmental scenario where aromatic amines are present in water at quite lower concentrations than those used in the past for laboratory binding experiments.

Treatment of the SRW sample having the largest amine concentrations with $NaBH_4$ was effective in rapidly and completely displacing that fraction of amines reversibly bound to HAs. Conversely, when this treatment was applied to the most diluted solution, not all of the amines sequestered by HAs were liberated. Prolonging the reaction time between the reducing agent and HAs from 10 to 60 min did not liberate additional quantities of aromatic amines. This result could be due to some incapability of $NaBH_4$ to reduce any type of quinone structures or to the fact that both mechanisms A and B concurred to sequester aromatic amines. The second hypothesis should be true considering that: (1) benzothiazolylMeamine is a secondary amine and, as

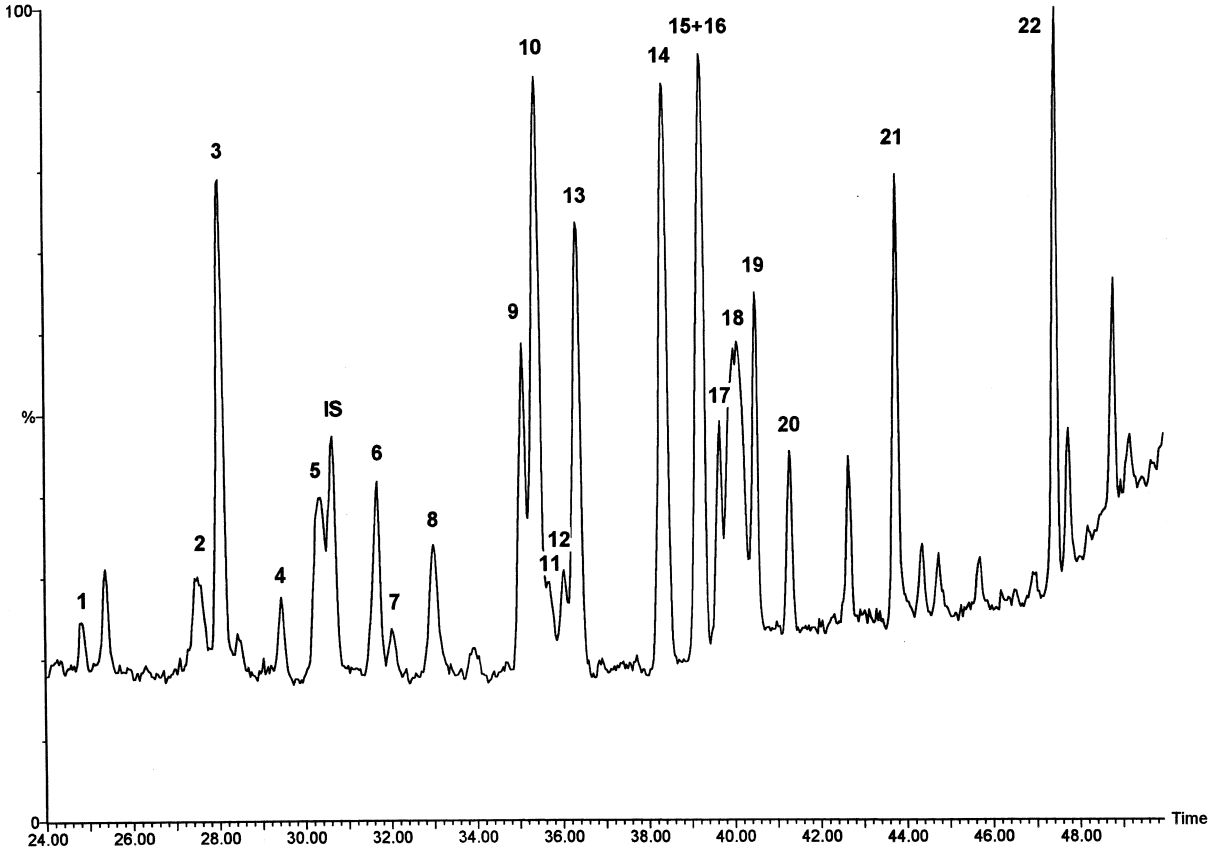


Fig. 2. Full-scan LC–ES-MS chromatograms obtained by analyzing 4 l drinking water spiked with 20 ng/l each of the analytes. Peak numbering: 1=4-ClPhurea; 2=3-Cl-4-methoxyaniline; 3=metoxuron; 4=3-Cl-4-MePhurea; 5=benzothiazolylMeamine; I.S.=monuron; 6=4-*i*-PrPhurea; 7=3,4-diClPhurea; 8=4-Claniline; 9=4-*i*-PrPhMeurea; 10=methabenzthiazuron; 11=4-Braniline; 12=3,4-diClPhMeurea; 13=chlorotoluron; 14=isoproturon; 15=monolinuron; 16=diuron; 17=4-*i*-Praniline; 18=3-Cl-4-Meaniline; 19=metobromuron; 20=3,4-diClaniline; 21=linuron; 22=neburon. Time scale in min.

such, it can react with HAs only by the irreversible mechanism B, and (2) no significant amount of this amine was displaced from HAs by treatment with $NaBH_4$.

Conceding that the artificial river water sample prepared by us mimics real river waters, our experiments indicate that, when present in water at a few hundred ng/l levels, aromatic amines are preferentially associated to dissolved HAs. In this situation, treating a surface water sample with $NaBH_4$ before analysis is a simple rapid and effective device which avoids large underestimation of the concentrations of bioavailable aromatic amines.

3.3. Accuracy, precision and method detection limits (MDLs)

The accuracy and precision of the method was evaluated by analyzing samples of drinking water, ground water and river water amended with the analytes. Before adding analytes to the river water, this medium was pretreated with $NaBH_4$. To simulate real samples at quite low contamination levels, appropriate analyte amounts were added to each type of aqueous matrix. Each of these samples was analyzed six times and results are reported in Table 4. A typical LC–MS chromatogram resulting from analyzing the analyte-amended drinking water sample is presented in Fig. 2. Relative standard deviations ranged between 4.6 and 20% for drinking water, 4.3 and 15% for ground water, 6.2 and 12% for river water. In order to check if the method was affected by systematic errors, the *t*-test ($P=0.05$) was applied. Except for methabenzthiazuron in drinking water and ground water, benzothiazolylMeamine and 4-*i*-Praniline in river water,

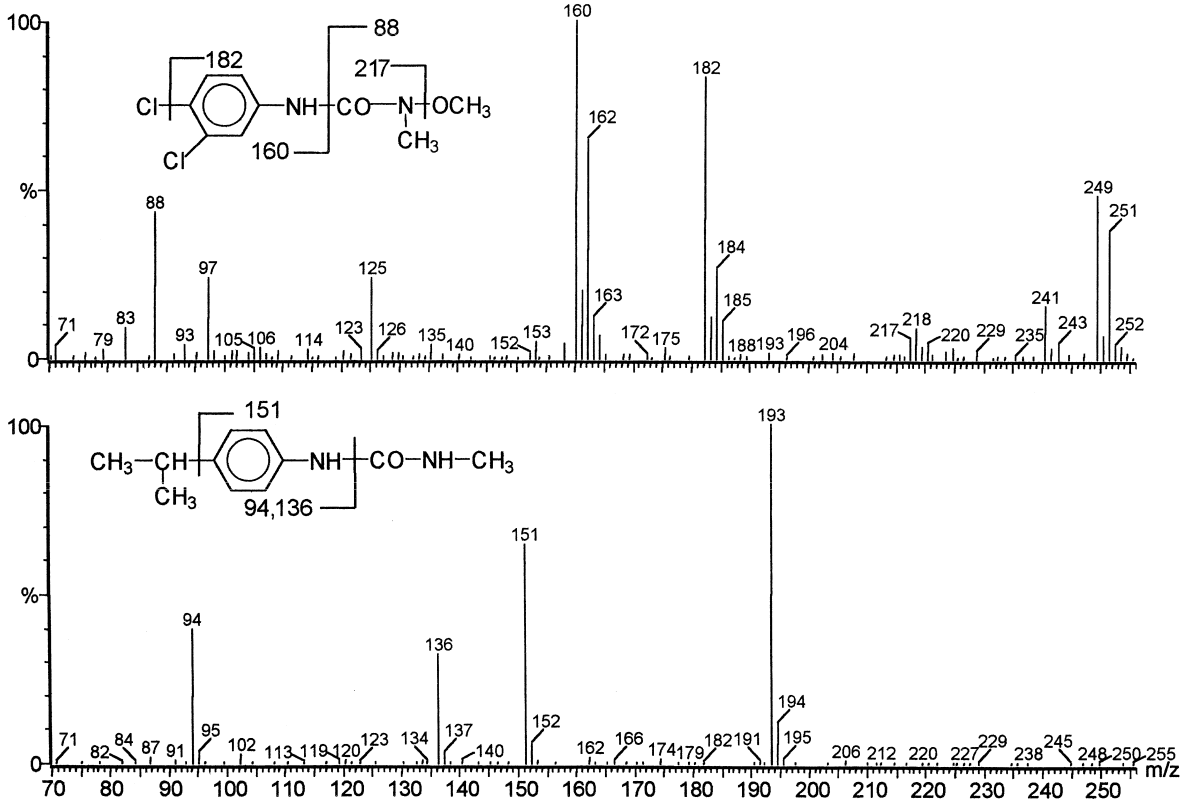


Fig. 3. Background-subtracted CID spectra obtained from injection of about 27 ng of both (upper) linuron and (lower) 4-*i*-PrPhMeurea. The fragment ion at *m*/*z* 182 can be formed with the additional loss of CH_3OH, while the fragment ion at *m*/*z* 94 can be formed with the additional loss of propene.

the calculated *t* values were lower than the critical value, this showing that this method was generally free from systematic errors. Some inaccuracy in analyzing very low amounts of methabenzthiazuron was traced to incomplete re-extraction of this compound from the SPE cartridge by the eluent phase [13]. Very few active sites on the Carbograph 4 surface able to irreversibly adsorb methabenzthiazuron and which are rapidly saturated were presumably responsible for this effect. The slight loss of benzothiazolylMeamine and 4-*i*-Praniline on extracting them from the river water sample was less intuitive. Maybe, the addition of $NaBH_4$ failed in part to reduce any particular carbonyl group of the HAs present in the actual river water sample so that incorporation of the two mostly basic aromatic amines into HAs was not completely inhibited.

The MDL is here defined as three-times the standard deviation obtained by analyzing an analyte at a sufficiently low spike level. MDLs calculated for drinking water and ground water were between 3 and 11 ng/l and between 6 and 21 ng/l, respectively. Therefore, this method could satisfy the stringent requirements imposed by the European Union Directive cited above. Fig. 3 shows background-subtracted in-source CID spectra of two selected analytes taken from the apices of chromatographic peaks obtained by analyzing 4 l of drinking water spiked with 20 ng/l each of the analytes. It appears that all the major signals for parent and daughter ions are present in the spectra of the two analytes. This demonstrates that the high specificity of the method is still retained nearby the calculated MDLs. For surface water, MDLs ranged between 37 and 76 ng/l. Thus, this method could be effectively used for studying the fate and the impact of targeted PUHs and their DPs in the aquatic ecosystem.

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