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On-line coupling of immunoaffinity-based solid-phase extraction and gas chromatography for the determination of *s*-triazines in aqueous samples

Jens Dallüge*, Thomas Hankemeier, René J.J. Vreuls, Udo A.Th. Brinkman

Vrije Universiteit, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

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Abstract

The potential of immunoaffinity-based solid-phase extraction (IASPE) coupled on-line to gas chromatography (GC) for the determination of micropollutants was studied with emphasis on the interfacing of the immunoaffinity-based SPE and GC parts of the system. The cartridge containing the immobilized antibodies was coupled to the gas chromatograph via a reversed-phase cartridge (copolymer sorbent). After trace enrichment of the analytes on the immunoaffinity cartridge, they were desorbed and recollected on the reversed-phase cartridge by means of an acidic buffer. After clean-up and drying with nitrogen, desorption and transfer to the GC was done with ethyl acetate via an on-column interface in the partially concurrent solvent evaporation mode. The antibodies used in the immunoaffinity cartridge were raised against atrazine; several *s*-triazines were used as test compounds. Triazines that were structurally similar to atrazine, showed quantitative recovery. As an application, immunoaffinity SPE–GC was used for the analysis of river and waste water and orange juice. The selectivity of the system was such that non-selective flame ionization detection (FID) could be used to detect the analytes of interest in these complex matrices. The detection limits for 10-ml water samples were 15–25 ng/l for FID and about 1.5 ng/l for the nitrogen–phosphorus detection.

Keywords: Immunoaffinity precolumns; Sample handling; Water analysis; Large-volume injections; Environmental analysis; Fruit juices; Food analysis; Pesticides; Triazines

1. Introduction

Sample preparation prior to gas chromatography (GC) analysis is often necessary to remove interferences present in the matrix, to achieve preconcentration of the analytes of interest and/or to transfer them into a GC-amenable solvent. Solid-phase extraction (SPE) on alkyl-bonded silicas or copolymer sorbents is often used for this purpose [1]. Unfortunately, because of the limited selectivity of these materials, many matrix constituents are also enriched and can disturb the chromatographic separation and detection. In target analysis, these problems can be solved by using a more selective enrichment, for example, on metal-loaded [2] or enzyme-loaded sorbents [3]. Even better selectivity can be obtained with immobilized antibodies.

In recent years, the on-line combination of immunoaffinity-based sample enrichment and column liquid chromatography (LC) for the determination of drugs or micropollutants in aqueous samples has been reported by several groups [4–8]. The selectivi-

*Corresponding author.

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ty of the immunoaffinity sorbent can be influenced by using antibodies with a high cross-reactivity or a mixture of different antibodies. Immunoaffinity sorbents with a high cross-reactivity can be used for group-selective enrichment for a whole class of analytes, e.g. for all *s*-triazines [8].

If the analytes are amenable to GC, this should be one's first choice as a separation technique because of its excellent separation efficiency and the selective and sensitive detectors available. However, the on-line coupling of immunoaffinity-based enrichment and GC is rather difficult and there is only one publication in the literature which deals with this topic [9].

The main goal of this paper was the development of a system for the on-line coupling of immunoaffinity-based sample preparation and GC and its application to environmental analysis. To this end, an immunoaffinity system based on the use of antibodies raised against atrazine was selected.

2. Experimental

2.1. Materials

All solvents (HPLC-grade water, methanol and ethyl acetate) were purchased from J.T. Baker (Deventer, The Netherlands); methanol and ethyl acetate were distilled before use. Glycine ('for molecular biology', from J.T. Baker) was Soxhlet-extracted with ethyl acetate before use. Hydrochloric acid (37%, reagent grade) was purchased from Riedel-de Haën (Seelze, Germany). Glycine buffer (0.2 mol/l glycine, 0.2 mol/l NaCl) was prepared by dissolving 0.1 mole of glycine and 0.1 mole NaCl in 500 ml of HPLC-grade water; the pH was adjusted to 2.2 with hydrochloric acid.

Phosphate-buffered saline ('PBS buffer', 0.1 mol/l, pH 7.2) was prepared by titrating a solution containing 0.3 mol/l Na_2HPO_4 with 0.3 mol/l NaH_2PO_4 to pH 7.2. Both solutions contained 0.15 mol/l NaCl. About 70 ml of NaH_2PO_4 solution had to be added to 250 ml of Na_2HPO_4 solution to adjust the pH to 7.2.

A 0.2 mol/l solution of formic acid was prepared using formic acid (reagent grade, 98–100%, Riedel-de Haën), a 0.014 mol/l solution of hydrochloric acid was prepared from hydrochloric acid 36–38%, (J.T.Baker) and a 0.05 mol/l phosphate buffer of pH 2.5 was prepared by titrating a solution of NaH_2PO_4 with hydrochloric acid.

To avoid bacterial and fungal growth, the PBS buffer contained 0.1% NaN_3 as fungicide; 2–3% (v/v) of acetonitrile were added to HPLC-grade water and the glycine buffer for the same reason.

Two mixtures, with five and seven *s*-triazines, were used as test compounds. The triazines atrazine, cyanazine, terbuthylazine, sebuthylazine, simetryn, prometryn, terbutryn and dipropetryn were purchased from Riedel-de Haën.

2.2. Immunoaffinity material

Monoclonal antibodies K4E7 raised against atrazine were used as the affinity ligand. These antibodies, which were immobilized on beaded cellulose material (ONB carbonate; Eurochrom, Dr. Ing. Herbert Knauer, Berlin, Germany) were a gift of Professor B. Hock (Technische Universität München, Department of Botany, Freising, Germany). The preparation of the immunoaffinity material is described in detail in Refs. [12,13].

2.3. Instrumentation

Sample preparation system. The fully automated on-line system (Fig. 1) consisted of a Kontron valve-switching unit MCS 670 'Tracer' (Kontron Analytical, Zürich, Switzerland) with a solvent selection valve and four six-port valves. The solvents, except for ethyl acetate, were pumped with two HPLC pumps (Kratos Spectroflow 400 pumps; Kratos, Ramsey, NJ, USA). Ethyl acetate was delivered by a Phoenix 20 pump (Carlo Erba, Milan, Italy).

All solvents except the water samples were cleaned by passing them through a 10×4.6-mm I.D. stainless-steel cartridge packed with 20 μm, 100 Å styrene-divinyl benzene copolymer (PLRP-S; Polymer Labs., Church Stretton, UK). This step was necessary to remove contaminants, especially from the glycine buffer.

An immunoaffinity cartridge, a 10×3-mm I.D. stainless-steel cartridge was manually slurry-packed with the immunoaffinity sorbent. Analytes were desorbed from the immunoaffinity cartridge and

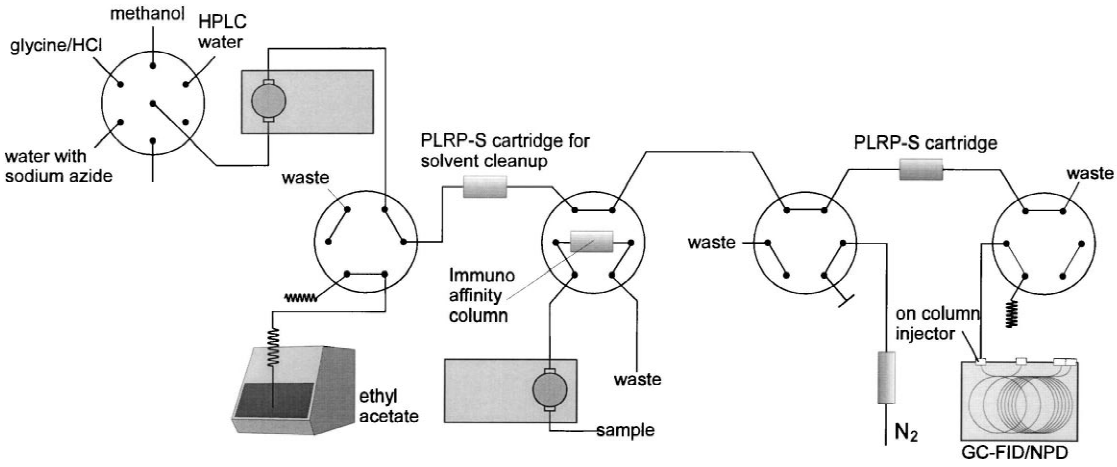

Fig. 1. Set-up of the automated on-line system.

recollected on-line on a 10×2-mm I.D. cartridge packed with 20-μm, 100 Å PLRP-S copolymer. The nitrogen used for drying of the PLRP-S cartridge was cleaned by passing it through a 60×2.8-mm I.D. stainless-steel cartridge packed with Carbotrap 20–40 mesh (Supelco, Bellefonte, PA, USA).

The analytes were desorbed from the PLRP-S cartridge with ethyl acetate and led directly into the GC. The transfer of the extract to the GC was done through a 20 cm×110 μm I.D. metal capillary that penetrated the septum of the on-column injector.

The sample preparation system was controlled by a PC/XT Commodore via an I/O card 'Multi I/O' (Graf Elektronik Systeme, Kempten, Germany) and a laboratory-written computer programme written in C.

2.3.1. GC system

The GC system consisted of a Hewlett-Packard gas chromatograph HP 5890 Series II equipped with an on-column and a split/splitless injector, a flame ionization detection (FID) system and a nitrogen–phosphorus detection (NPD) system. The GC system was operated in the 'constant-flow on' mode at a flow-rate of 1.1 ml/min. Helium with a purity of 99.999% (Praxair, Oevel, Belgium) was used as carrier gas.

The on-column injector was connected to a 3 m×0.53 mm I.D. retention gap, deactivated with diphenyltetramethyl disilazane (DPTMDS; BGB Analytik, Basel, Switzerland), a 1.5 m×0.25 mm I.D. retaining precolumn and a 25 m×0.25 mm I.D. analytical column, both containing HP-5MS with a film thickness of 0.25 μm (Hewlett-Packard, Waldbronn, Germany). A solvent vapour exit (SVE) was installed between the retaining precolumn and the analytical column. All columns were connected with glass press-fit connectors (BGB Analytik). The solvent vapour exit was opened and closed with a six-port HPLC valve (Valco Instruments, Schenkon, Switzerland).

An effluent splitter (split ratio 1:1) was installed at the end of the analytical column. It was constructed from a press-fit splitter and two pieces of 20 cm×0.1 mm I.D. DPTMDS-deactivated retention gap in order to allow the simultaneous use of both detectors.

2.4. Analytical procedure

The system was used for two different enrichment procedures: (i) an immunoaffinity-type sample enrichment using both an immunoaffinity sorbent and a non-selective PLRP-S cartridge (referred to as IASPE) and (ii) a procedure using only the non-selective PLRP-S cartridge for a 'hydrophobic' enrichment (referred to as SPE).

2.4.1. Immunoaffinity sample enrichment (IASPE)

Before use, the immunoaffinity cartridge was cleaned with 10 ml of the glycine buffer and

conditioned with 5 ml of HPLC water. Then the sample was pumped through the immunoaffinity cartridge to waste, followed by 5 ml of HPLC water as a clean-up step to remove the sample from the on-line system.

Direct desorption of the analytes from the IASPE cartridge into the GC system was not possible as the packing material was not compatible with an organic solvent which would be suitable for injection into GC. However, on-line coupling was possible via a PLRP-S cartridge [10]. This PLRP-S cartridge was conditioned with 2 ml of ethyl acetate and 5 ml of water during the sampling step on the IASPE cartridge.

The analytes were desorbed from the immunoaffinity cartridge using 20 ml of glycine buffer and transferred from the immunoaffinity cartridge to the PLRP-S cartridge. After clean-up of the SPE cartridge with 10 ml of HPLC-grade water to remove the buffer, the copolymer cartridge was dried for 30 min with nitrogen at a flow-rate of 30 ml/min. The PLRP-S trapping cartridge was desorbed with 100 μl of ethyl acetate at a flow-rate of 70 μl/min and the entire extract was transferred via the transfer capillary directly into the GC column.

2.4.2. Reversed-phase sample enrichment (SPE)

First, the PLRP-S trapping cartridge (which now acted as a trace-enrichment cartridge) was conditioned with 2 ml of ethyl acetate and 5 ml of water. Then the sample was pumped through the cartridge (i.e., bypassing the immunoaffinity cartridge) which was subsequently flushed with 5 ml of HPLC water to achieve some clean-up. Next, the copolymer cartridge was dried with nitrogen for 30 min and desorbed with 100 μl of ethyl acetate as described above for the immunoaffinity sample enrichment.

2.4.3. GC analysis

The complete 100 μl of the ethyl acetate extract were injected under partially concurrent solvent evaporation conditions. The solvent vapour exit was opened before the start of the transfer and closed a few seconds after the evaporation of the solvent was complete. The rather late closure of the SVE, which will result in a loss of volatile compounds, was preferred in order to protect the NPD bead. The bead consists of rubidium chloride that is easily damaged when large volumes of a polar solvent pass through the detector. This approach caused no problems for the analytes of interest in the present study which have sufficiently high boiling points.

The injection was performed at an oven temperature of 70°C. After 5 min the oven temperature was programmed to 280°C at 10°C/min.

Dibenzylaniline was added as internal standard to the ethyl acetate (10 pg/μl) to compensate for changes in the detector response.

2.4.4. Sample pretreatment

All water samples were filtered through a 40-μm cellulose membrane filter. The orange juice was neutralized using sodium hydroxide [11] and filtered over a glass fiber filter and, next, a 40-μm cellulose membrane filter.

3. Results

3.1. Optimization of desorption of the immunoaffinity cartridge

The analytes are retained on the immunoaffinity cartridge in an aqueous environment with a neutral pH [12]. The most common techniques for desorption of analytes from immunoaffinity cartridges are by changing the pH or by using an organic solvent. A change in pH is achieved with an acidic buffer such as, e.g. a phosphate or a glycine buffer, or formic or hydrochloric acid [13,6]. However, the use of these acidic solutions is incompatible with the final GC separation. Therefore, an additional step had to be included in the procedure. After desorption of the analytes with the acidic buffer, they were trapped again on a cartridge packed with the highly hydrophobic PLRP-S copolymer.

First, the volume for the complete desorption of five *s*-triazines (atrazine, terbuthylazine, prometryn, terbutryn and cyanazine) from the immunoaffinity cartridge was determined. After loading the cartridge with 16 ml of a mixture of 100 ng/ml of each of the *s*-triazines in HPLC water, desorption was carried out with volumes of 0.5–20 ml of an acidic buffer. Fig. 2 shows the desorption profiles for all *s*-triazines tested using different desorption solvents.

The elution profiles of Fig. 2 show that desorption

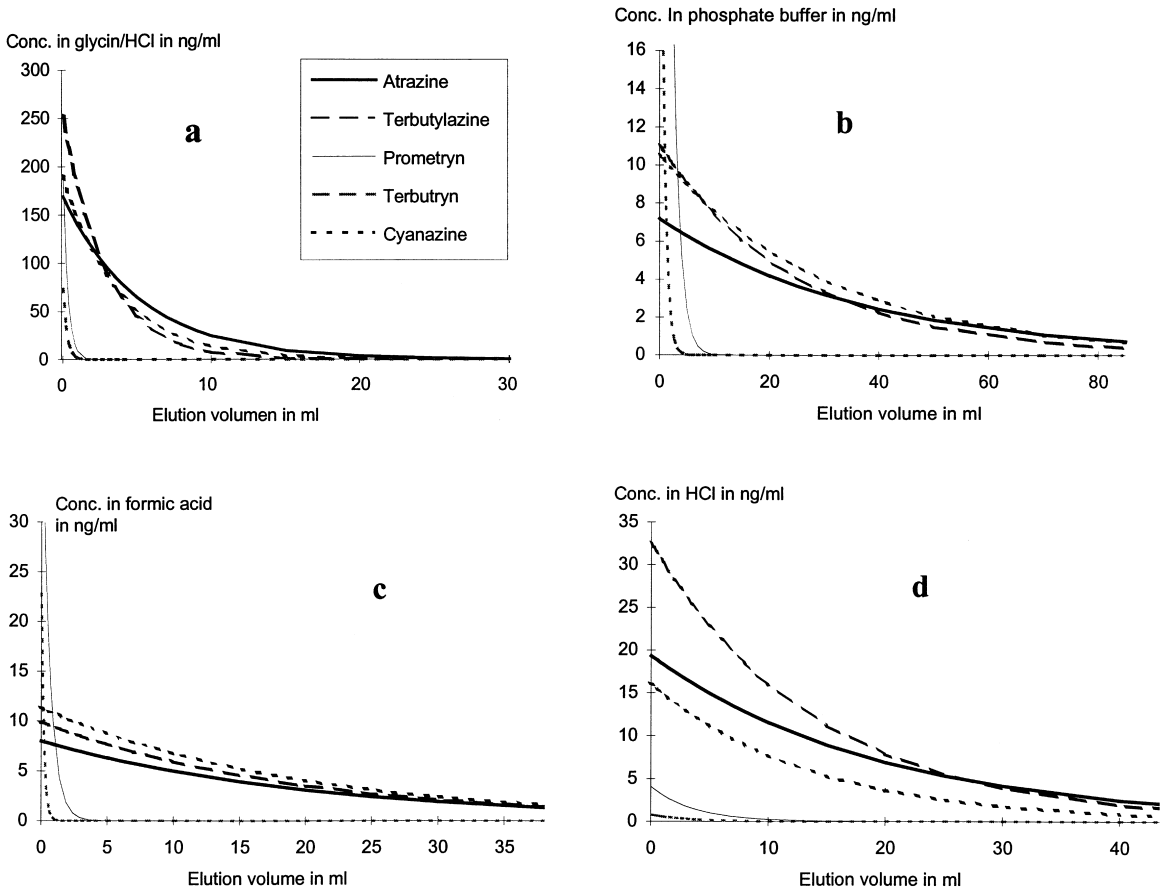

Fig. 2. Elution profile for the desorption of five *s*-triazines from the immunoaffinity cartridge with (a) glycine buffer, (b) phosphate buffer, (c) formic acid, (d) hydrochloric acid.

of terbutryn and prometryn occurs much more readily than that of the other triazines, i.e. in a volume between 2 and 10 ml depending on the acidic solution. Obviously the interactions between these two analytes and the antibodies are much weaker (cf. below). The data in Table 1 show that a desorption volume of at least 40 ml is needed for the complete desorption of all analytes for all buffers except glycine buffer. For obvious reasons, 20 ml of glycine buffer was the option preferred for all further work. An additional 10 ml of glycine buffer were used for clean-up before each run.

Table 1
Comparison of different solvents for the desorption of triazines from an immunoaffinity cartridge

Desorption solvent	Volume (ml) needed for complete desorption of:		
	Atrazine	Prometryn	Terbutryn
Glycine buffer	20	2	1.5
Phosphate buffer	>85	10	5
Formic acid	>40	4	2
Hydrochloric acid	>40	9	4

3.2. Selectivity of the immunoaffinity sorbent

The cross-reactivity of the antibodies was studied in the IASPE mode of the system by analysing 10 ml of river water spiked with 1 μg/l of seven triazines. The results were compared with a reversed-phase-type sample enrichment (SPE) using only the PLRP-S cartridge. For quantification, NPD rather than FID

Table 2
Percent recoveries of triazines from river water using either reversed-phase SPE or immuno-affinity sample preparation (IASPE)[a]

Analyte		Recovery (%) with:	
Name	No.	SPE	IASPE
Atrazine	1	68	88
Terbuthylazine	2	68	64
Sebuthylazine	3	86	81
Simetryn	4	87	<1
Prometryn	5	101	1
Terbutryn	6	97	1
Dipropetryn	7	94	<1

[a] GC–NPD; 10-ml river water sample; spiking level, 1 μg/l.

was used because of the better signal-to-noise ratio. The FID chromatograms were recorded to show the differences in selectivity between the two types of enrichment.

In Table 2 the analyte recoveries for both extraction procedures are compared. As was to be expected on the basis of the desorption data reported above, the antibodies, although raised against atrazine, also show a significant affinity to terbuthylazine, and to sebuthylazine. Fig. 3 shows that, indeed, the structures of these three triazines are very similar: they only differ with respect to an isopropyl vs. *tert.*-butyl vs. isobutyl group. In marked contrast, retention of the other four triazines, simetryn, prometryn, terbutryn and dipropetryn on the immunoaffinity cartridge is rather poor. The chlorine group obviously plays a key role in the retention of the triazines by the antibodies. Similar results were obtained by Giersch and co-workers [12,13] with an enzyme-linked immunosorbent assay and an immunosorbent, respectively, using the same antibody, viz. K4E7.

The cross-reactivities determined with immunoassays and the recoveries obtained with IASPE are related: analytes with higher cross-reactivities in immunoassays will be more strongly retained on the immunoaffity column in IASPE. However, in immunoassays a competition exists between the analyte and the tracer. In IASPE, due to the high number of antibody binding sites (almost) no such competition will occur and analytes with a lower affinity are still retained.

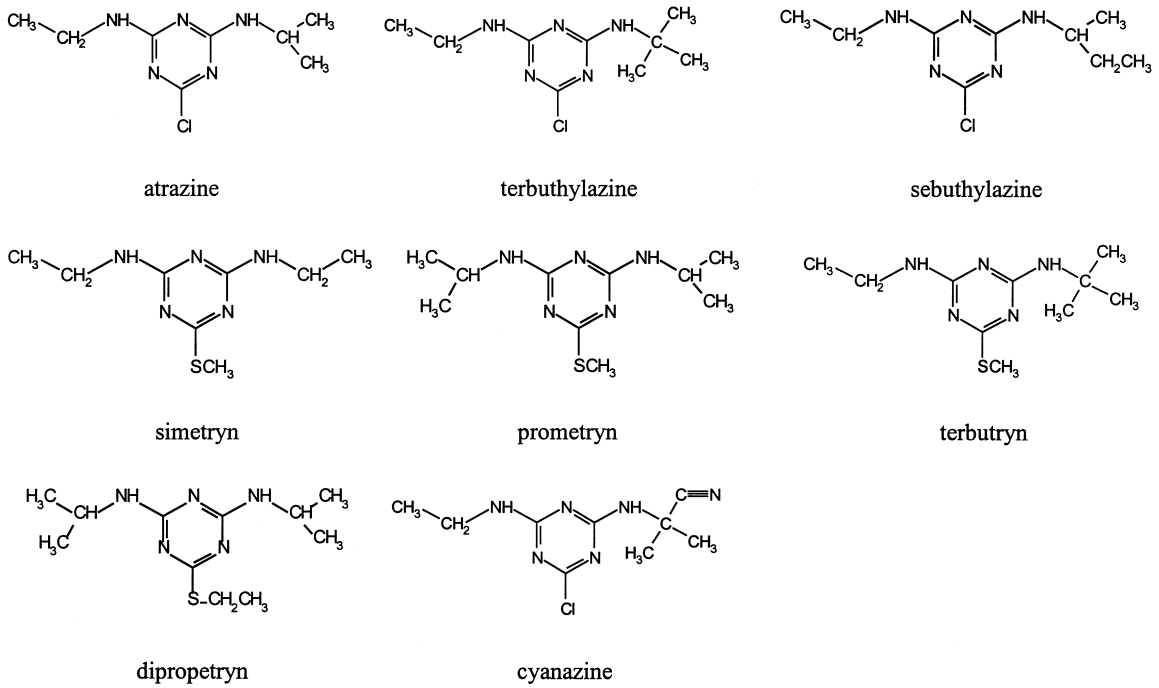

Fig. 3. Structures of the *s*-triazines used as test compounds.

Deethylatrazine, the most important degradation product of atrazine, could not be determined with the present set-up. As expected from the cross-reactivity data in [13] it should be possible to retain this compound on the immunocolumn; however, it could not be transferred to the PLRP-S column. Because of the large volume of glycine buffer needed for the transfer of the analytes from the immunoaffinity column to the PLRP-S column (20 ml) plus the clean-up step (10 ml water), this relatively polar analyte broke through the PLRP-S column.

The results obtained with a conventional copolymer sorbent show that there is little non-specific binding in the case of the immunoaffinity procedure; good recoveries were obtained with all analytes, viz. 68–100%. As regards the difference in selectivity that is achieved for real-life samples with IASPE as compared to SPE, this will be discussed with Fig. 4 below.

3.2.1. Capacity of the immunoaffinity cartridge

The capacity of the 10×3 mm I.D. immunoaffinity cartridge was measured by determining the amount of atrazine trapped by the immunosorbent when using 10 ml of HPLC water containing 2 μg atrazine per ml for enrichment. When freshly prepared, the immunoaffinity cartridge packed with 71 μl immunosorbent had a capacity of 1.5 μg or about 7 nmol of atrazine. This value corresponds with 21 μg atrazine/ml gel, which is close to the capacity determined by Marx et al. (20 μg/ml gel) [12]. The theoretical binding capacity of the present immunosorbent, which can be calculated from the amount of immobilized antibodies, using the assumption that both binding sites of the antibodies are accessible to the analyte [14], is 34 μg atrazine/ml gel. The lower experimental capacity indicates that not all binding sites are available to the analytes, e.g. due to the fact that antibodies which are immobilized in such way that their active sites are oriented towards the stationary phase will have a strongly reduced ability to bind triazine molecules.

The immunoaffinity cartridge was used for more than 40 analyses over three months using different kinds of aqueous samples which included river water, waste water, and orange juice. The remaining capacity after these analyses was about 1.4 μg atrazine/ml gel, which was still amply sufficient for the immunoaffinity extractions. In other words, the above experiments were performed under such conditions that the capacity of the immunoaffinity column was sufficient for the intended analyte extractions.

Large volumes of non-sterile samples or samples with a high concentration of high-molecular-weight compounds will probably accelerate the deterioration

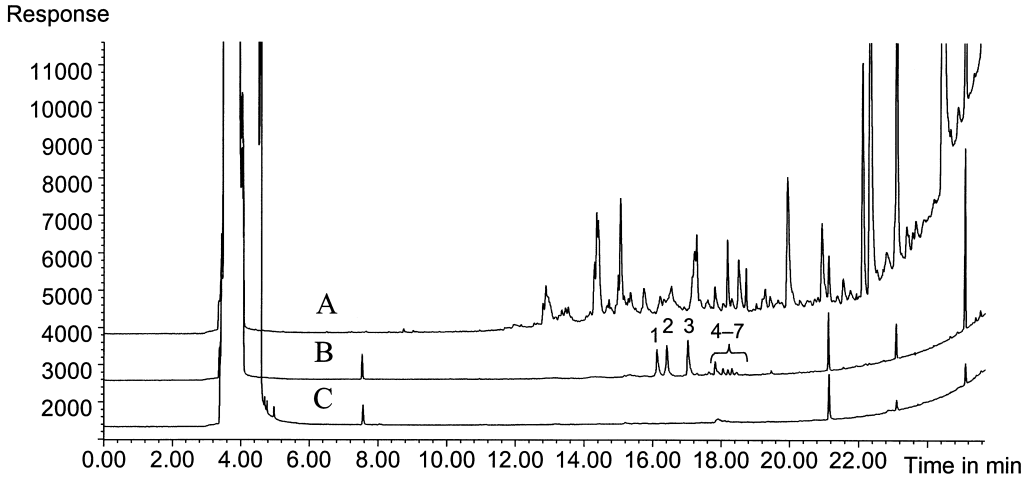

Fig. 4. GC–FID chromatograms of an extract obtained by (A) SPE and (B) an IASPE of 10 ml of municipal waste water, spiked with 1 μg/l of seven *s*-triazines (for peak numbers cf. Table 4). (C) Blank run of IASPE–GC–NPD of 10 ml of HPLC water.

of the immunoaffinity column. The antibodies should be kept under physiological conditions as much as possible to maintain their integrity.

3.3. Analytical data

The performance of the system was evaluated by using 10-ml tap water samples spiked with 100 ng/l of each of the *s*-triazines. The system showed high recoveries, i.e. 96% for atrazine, 84% for terbuthylazine and 98% for sebuthylazine. The relative standard deviation for the entire analysis was about 7% ($n=6$) for these analytes.

The detection limits were 120–170 pg for FID, and about 15 pg for NPD. For all experiments an effluent splitter was used, with 50% of the effluent going to the FID system and 50% to the NPD system.

If even lower detection limits than those reported above are required, one way to go is to use larger sample volumes, e.g. 100 ml or more. Apart from the fact that the analytical procedure now becomes more time-consuming (longer loading time), there is also an increasing risk of breakthrough on the immunoaffinity column [8]. To study this aspect, different sample volumes containing 1 ng of each analyte were analysed by IASPE–GC–NPD. The recoveries are included in Table 3. Obviously, the breakthrough for terbuthylazine already starts to occur much below 100 ml, while for atrazine and sebuthylazine, with their higher affinities for the antibodies, ca. 250 ml is a safe upper limit. For the rest, the data show that detection limits of ca. 1 ng/l can be obtained if this is really necessary. Here, one should keep in mind that large volumes of highly contaminated samples may adversely affect the quality of the immunoaffinity column.

3.4. Immunoaffinity sample preparation for complex matrices

The present procedure was applied to the determination of triazines in aqueous samples with a high concentration of organic matter. As stated already in the introduction, with such samples many matrix compounds are also retained when using conventional SPE techniques and their number and relatively high concentrations will adversely affect the identification and quantification of the analytes of interest. Furthermore, the high concentration of organic compounds may cause early breakthrough of these analytes and the large amounts of high-boiling compounds in the extract will deteriorate the GC system, especially when using on-column injections.

Municipal waste water was used as an example of a complex matrix. The sample was spiked with 1 μg/l of each of seven triazines.

Fig. 4A shows that when using the SPE procedure, many non-polar organic compounds are indeed retained and, subsequently, detected. The interfering matrix compounds make recognition and quantification of the triazines on the basis of their FID signal impossible. Even in combination with selective detection (NPD), it is very difficult to quantify the analytes because of their bad peak shapes (Fig. 5). In addition, after only one injection of an extract of a 10-ml waste water sample, the performance of the GC system had deteriorated to such an extent that the retention gap had to be exchanged.

Table 3
Analytical data for IASPE–GC of selected triazines

Analyte	Recovery[a] (%) for:			R.S.D. ($n=6$)[b] (%)	LOD (pg, $S/N=3$)	
	10 ml, 100 ng/l	100 ml, 10 ng/l	250 ml, 4 ng/l		FID	NPD
Atrazine	96	87	70	6.6	170	15
Terbuthylazine	84	57	47	7.0	120	15
Sebuthylazine	98	104	67	7.1	120	15

[a] All recovery data recorded with GC–NPD; sample, tap water spiked at indicated level.
[b] R.S.D. data for 100 ng/l spikes.
LOD=Limit of detection.

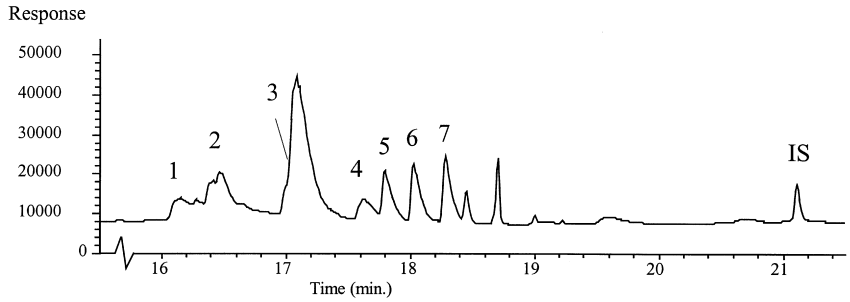

Fig. 5. SPE–GC–NPD chromatogram of 10 ml of municipal waste water, spiked at 1 μg/l with seven *s*-triazines. For peak numbers, see Table 4.

When using, on the other hand, the immunoaffinity enrichment procedure, the interfering matrix compounds are completely removed (Fig. 4B). All other peaks are caused by contamination originating from the system (Fig. 4C).

The recoveries for atrazine, terbuthylazine and sebuthylazine were between 78% and 86%. As was to be expected from the recoveries obtained with river water samples (Table 2), the recoveries for the other analytes in IASPE–GC–NPD were about an order of magnitude lower (Table 4).

3.4.1. Orange juice

Neutralized and filtered orange juice was spiked with the triazine mixture at the 1 μg/l level, and 10 ml of the spiked juice were extracted using both IASPE and SPE.

Table 4
Triazine recoveries for immunoaffinity enrichment of municipal waste water and orange juice compared with SPE using the NPD signal for quantification

Analyte		Recovery (%) from:			
		Municipal waste water		Orange juice	
		IASPE	SPE	IASPE	SPE
Atrazine	1	86	n.q.[a]	105	9
Terbuthylazine	2	78	n.q.	100	10
Sebuthylazine	3	86	57	101	9
Simetryn	4	9	6	<1	14
Prometryn	5	10	13	<1	17
Terbutryn	6	10	14	<1	16
Dipropetryn	7	10	15	<1	15

[a] n.q.: not quantifiable due to interferences, $n=1$.

The data reported in Table 4 show that the analyte recoveries obtained by SPE are rather low in all instances (9–17%), which is due to losses during sorption which are caused by the highly complex matrix. With IASPE–GC–NPD, on the other hand, the recoveries of these triazines which show a higher affinity for the antibody-loaded SPE column, were quantitative.

4. Conclusions

This paper presents, for the first time, the on-line coupling of immunoaffinity enrichment and GC for the determination of pesticides in aqueous samples. The combination provides a useful tool for target analysis in complex sample matrices.

The present immunoaffinity material could be used for the selective sample enrichment of *s*-triazines with a structure similar to that of atrazine. Essentially no other organic compounds present in the sample, even at much higher concentrations were retained on the immunoaffinity cartridge. Therefore, no deterioration of the GC system occurred and the analyses were not disturbed by matrix effects. Even waste water could be analyzed and, generally speaking, the results were distinctly superior to those obtained with conventional (copolymer) SPE with 10-ml samples and NPD, detection limits were as low as 1.5 ng/l.

The major disadvantage of immunoassays – the cross-reactivity to structural similar compounds – can be used in immunoaffinity extraction techniques for the enrichment of a group of analytes. Less

selective antibodies or a mixture of antibodies raised against different analytes enable the extraction of a larger group of analytes, or even several different groups and the simultaneously determination of all individual compounds in a single run [7]. In cases where cross-reactivity can not be avoided, immunoassays only give a sum parameter and can not be used for the quantification of a single compound. Since the immunoaffinity cartridges considerably simplify complicated sample pretreatment and such columns can be re-used frequently, the cost of such analyses is not as high as it is often thought.

The system described in this work represents a general approach that can be used as well for the determination of other compounds in aqueous samples. Admittedly, the production and immobilization of antibodies is still time-consuming and requires special skills. The (easy) availability of suitable antibodies and ready-to-use immunoaffinity columns should, therefore, be a main point of concern in the future.

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