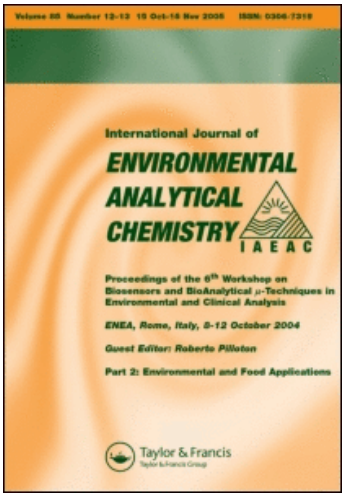

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Immunochemical Techniques and Immunosensors for the Analysis of Dealkylated Degradation Products of Atrazine

C. Wittmann[ab]
[a] University of Stuttgart, Institute for Technical Biochemistry, Stuttgart, Germany [b] Fachhochschule Neubrandenburg, Studiengang Lebensmitteltechnologie, Neubrandenburg, Germany

To cite this Article Wittmann, C.(1996) 'Immunochemical Techniques and Immunosensors for the Analysis of Dealkylated Degradation Products of Atrazine', International Journal of Environmental Analytical Chemistry, 65: 1, 113 — 126
To link to this Article: DOI: 10.1080/03067319608045547
URL: http://dx.doi.org/10.1080/03067319608045547

Intern. J. Environ. Anal. Chem., Vol. 65, pp. 113–126
Reprints available directly from the publisher
Photocopying permitted by license only

IMMUNOCHEMICAL TECHNIQUES AND IMMUNOSENSORS FOR THE ANALYSIS OF DEALKYLATED DEGRADATION PRODUCTS OF ATRAZINE

C. WITTMANN*

University of Stuttgart, Institute for Technical Biochemistry, Allmandring 31, D-70569 Stuttgart, Germany

(Received, 10 October 1995; in final form, 18 February 1996)

Two different immunoassay (IA) formats for pesticide residue analysis, a field format (dipstick IA) and an automated version (flow injection immunoanalysis, FIIA), are described with regard to method development and validation performed by measuring water, soil, and liquid food samples and comparing the results with the respective GC data obtained. As representative analyte a pesticide metabolite, deethylatrazine as the major atrazine metabolite, was selected. The measuring range for deethylatrazine was from 0.1–10 µg/L with the dipstick IA and from 0.01–10 µg/L with the FIIA. With both formats, water and liquid food samples can be measured directly without the need for any prior enrichment or clean-up steps. In the case of the soil samples, two extraction steps can be omitted as compared to extract preparation for GC analysis which usually takes further 3–4 h. A close correspondence was found between the results of the two immunoassay formats and GC measurements.

KEY WORDS: Deethylatrazine, dipstick immunoassay, flow injection immunoanalysis, immunoassay, atrazine metabolites.

INTRODUCTION

The methods generally used for trace analysis of pesticides in water, soil, plant and food samples are GC with NPD, ECD or MS detection or HPLC connected with UV, diode array, fluorescence or mass spectrometric detection. These methods must be in line with pertinent regulations such as the EC Guidelines for Drinking Water and the legislation concerning the amount of pesticide traces in food. Compared to these classic methods in residue analysis serological methods as immunoassays and immunosensors have some distinct advantages such as allowing a more rapid screening of samples at lower costs with no need for a sophisticated laboratory equipment. IAs used in residue analysis are mostly based on microtiter plates as the solid support. They allow the quantification of analytes using sophisticated photometers, often combined with computer-automated calculation. However, these methods are normally restricted to laboratories. Therefore, one objective was to provide a comparable field test system for the screening of environmental samples towards a potential pesticide contamination on-site. This can be achieved by a dipstick IA format. On the other hand, fully automated alarm stations are

* Present address: Fachhochschule Neubrandenburg, Studiengang Lebensmitteltechnologie, Brodaer Straße 2, D-17033 Neubrandenburg, Germany.

requested allowing a continuous monitoring of an aquifer, e. g. a river. For this purpose, automation via a flow injection analysis (FIA) system is most suitable. Recently, a pesticide-specific immunosensor based on the principle of flow injection immunoanalysis (FIIA), using a special antibody column reactor, was developed and successfully applied to the rapid and sensitive determination of atrazine in water and soil samples[1]. Most often it is forgotten that the environment is not only to be protected from a contamination with the parent pesticide compound but that via degradation processes the parent compound is metabolized and several degradation products can occur. Little or much less information is available about the toxicity of the relevant metabolites as compared to the parent pesticide compound although legislation, e. g. the European Guideline for Drinking Water, regulates via the upper permissible limit in drinking water the presence of both, the pesticide parent compound and the pesticide metabolite. Therefore, it was decided to investigate deethylatrazine as representative major atrazine metabolite.

The main objective was to establish two different IA formats, one suitable for an analysis on-site and the other method to provide a continuous monitoring, not only for pesticide analysis but also for the quantitative determination of pesticide metabolites with deethylatrazine as the representative analyte.

MATERIALS AND METHODS

Materials

Chemicals

The triazine derivative for the preparation of the immunoconjugate (for antibody production) and for tracer synthesis, 4-chloro-6-amino-2-aminohexanecarboxylic acid-1,3,5-triazine, was synthesized by Dr. U. Doht, Riedel de Haen AG (Seelze, Germany). The triazine and triazine metabolite standards were also provided by Riedel de Haen AG. In addition, the following reagents were used: albumin from chicken egg (Sigma Chemie GmbH, Deisenhofen, Germany), avidin (Sigma), d-[8,9-^{3}H] biotin (1.1–2.2 TBq/mmol; Amersham, Braunschweig, Germany), bovine serum albumin (BSA, Sigma), dicyclohexylcarbodiimide (DCC, Sigma), dioctylsulfosuccinate, sodium salt (Sigma), ethanol absolute, p. a. (Merck, Darmstadt, Germany), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC, Sigma), goat anti-rabbit IgG (Sigma), horseradish peroxidase (HRP, 1350 U/mg = 22,505 nKat; Serva, Heidelberg, Germany), hydrogen peroxide, 30% (Merck), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES; Aldrich Chemical Co. Ltd., Gillingham, Dorset, England), 3-(p-hydroxyphenyl)propionic acid (HPPA, sigma), N-hydroxysuccinimide (NHS, Aldrich), poly(oxyethylenesorbitan)monolaurate (TWEEN 20, Merck), sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce, Rockford, IL), and 3,3',5,5'-tetramethylbenzidine (TMB, Sigma).

Buffers and solutions

(a) for dipstick immunoassay performance
The following were used: (1) carbonate buffer, 50 mM, pH 9.6, for coating; (2)

phosphate-buffered saline (PBS), 40 mM, pH 7.2 (containing 8.5 g/L NaCl), for the dilution of the peroxidase tracer; (3) PBS washing buffer, 4 mM, pH 7.2 (containing 0.85 g/L NaCl and 0.5 mL/L TWEEN 20), for washing the test strips; (4) tetramethylbenzidine (TMB) substrate, 2 mM, for dipstick assay: 400 µL of TMB stock solution (6 mg TMB were dissolved in 1 mL dimethylsulfoxide) + 1 mL dioctylsulfosuccinate (DSS) solution (8 mg DSS/mL ethanol) + 3.6 mL 0.1 M acetate solution, pH 5.5 (pH adjusted by addition of citric acid) + 50 µL 1% (v/v) hydrogen peroxide solution.

(b) for FIIA measurement
40 mM phosphate buffered saline (PBS), pH 7.2 (containing 8.5 g/L NaCl), was employed as the substrate buffer for peroxidase (carrier buffer). The following substrates for peroxidase were each dissolved separately in this buffer: (1) 5 mM 3-(p-hydroxyphenyl) propionic acid (HPPA) and (2) 2 mM hydrogen peroxide. For the 1/5000 dilution of the deethylatrazine peroxidase tracer 40 mM PBS buffer, pH 7.2 (containing 0.5 mL/L TWEEN 20), was used. For the regeneration of the immobilized antibodies 0.01 M glycine/HCl buffer, pH 2.0, was used.

Preparation of standards

5 Milligrams of deethylatrazine or related s-triazine compounds were dissolved in 50 mL absolute ethanol with the aid of an ultrasonic bath (20 min). Starting with this solution, a stock solution was prepared consisting of 1 mg/L deethylatrazine (= excess). A standard series was prepared by making several dilutions of the stock solution containing the following deethylatrazine concentrations: 0.01, 0.03, 0.1, 0.3, 1, 10, and 100 µg/L. The stock solution and the standard series were made up in distilled water.

Equipment

The following laboratory equipment was used:

(a) for dipstick immunoassay measurement.
A reflectometer RQflex (Merck, Darmstadt, Germany), including the barcode "testroutine" was used. This barcode enabled the output of remission values in % measured at a wave-length of 657 nm.

(b) for FIIA measurement.
Five peristaltic pumps (Meredos GmbH, Bovenden, Germany), two 3/2-way valves (Lee, Westbrook, CT, USA), two injection valves (Fiastar 5102–002 Injector V-100, Tecator, Höganäs, Sweden), a fluorimeter with a flow-through-cell (Merck Hitachi, Darmstadt, Germany), an integrator (Shimadzu C-R6A, Chromatopack), a relay station (Keithey, Metrabyte), and a special column reactor (GBF, Braunschweig, Germany); the system was controlled using a personal computer and the Q-FIA programm (GBF, Braunschweig, Germany).

Further materials

For the dipstick IA the antibodies were immobilized onto a nylon membrane (Biodyne B, pore size 0.45 µm, PALL). As the inert plastic stripes PVC sheets were used onto which

the membrane pieces were fixed with the aid of double-sided adhesive tape (3 M, Neuss, Germany). For the immobilization of the antibodies for FIIA measurement as support material porous microglass beads surface modified with carboxylic groups for covalent coupling were used which were obtained as a generous gift from Schuller GmbH (Wertheim/Main, Germany). The beads had a diameter of 50–100 μm, a pore size of 31.6 nm and exhibited a high density of carboxylic acid groups for covalent coupling, according to the manufacturer.

Liquid food samples, environmental water and soil samples

An important step for any validation study is the analysis of samples spiked with the analyte and the measurement of real environmental samples with the comparison of the results with data of an established analysis method, i. e. in our case GC measurements.

With the dipstick IA, several environmental water samples were measured. Four water samples were provided from the Lake Constance (Germany) together with the respective GC-MS data for s-triazines and metabolites by Mr. Petri, Bodensee Wasserversorgung, Sipplingen, Germany. The water samples were measured in the original state and after spiking with two different deethylatrazine concentrations (0.1 and 0.5 μg/L) with the dipstick IA. For the dipstick IA the analyses were carried out in six replicates. In addition, liquid foods such as fruit juices, milk, vegetable juices and black tea were analyzed in the original state and after spiking with deethylatrazine.

With the FIIA ground water and surface water samples in the original state and fortified with atrazine and deethylatrazine were measured in quadruplicates. The water samples were provided (already fortified where the spiked amounts were unknown prior to FIIA measurements) together with the respective GC-MS data for s-triazines and metabolites by Dr. Wiegand-Rosinus, Wasserforschung Mainz GmbH, Mainz, Germany. In addition, soil samples were collected in the Northern part of Germany (Braunschweig) and the soil extracts prepared. The soil extracts were obtained from Dr. H. Dieckmann (group Prof. Bahadir, TU Braunschweig, Braunschweig, Germany).

Methods

The production of the polyclonal deethylatrazine-specific antibody C10 and the preparation of the deethylatrazine-horseradish peroxidase tracer were reported previously[2]. In addition, details about the development of a highly sensitive enzyme immunoassay for deethylatrazine determination and the successful application of the IAs for the measurement of environmental water samples have been described previously[3].

Development and performance of dipstick IA

(a) Preparation of dipsticks.

To prepare the test strips, the nylon membrane Biodyne B was in a first step coated with a primary antibody (goat anti-rabbit IgG). For this purpose, a square membrane piece (10.6 cm × 10.6 cm = 110 cm^2) is cut (using a scalpel, tweezers and gloves) and incubated in the goat anti-rabbit IgG solution (1 $\mu L/cm^2$) for 2 h at room temperature on a horizontal shaker moving gently. After being washed two times with PBS washing buffer, the membrane piece was incubated with the specific polyclonal antibody C10

(produced in a rabbit) in an antibody concentration of 0.025 μL/mL. Another washing step (see above) followed. In subsequence, to prevent unspecific binding to the membrane surface a blocking step with 0.5% ovalbumin solution was performed which could be omitted in most cases. After a last washing step the membrane is dried on air at room temperature for about 30 min. In the last step, the outer 0.5 cm of the membrane edges were removed and 0.8 cm-squares were cut which were then mounted onto the inert plastic support using double-sided adhesive tape. At this stage the test strips (144 dipsticks were obtained) were ready for use in the assay and could be stored at 4°C for several months.

(b) Assay protocol
To perform the dipstick assay all incubation steps were performed in 2 mL plastic tubes. Prior to the assay the plastic tubes were rinsed with 2 mL of PBS washing buffer for 10 min and the washing buffer thoroughly removed. The test strips were then dipped into the solutions and incubated as long as required. For the immunoreaction the test strips were incubated with a mixture of 800 μL standard or sample and 200 μL of the enzyme tracer (dilution 1/50,000) for 10 min. After a washing step, three times with PBS washing buffer, 800 μL of TMB enzyme substrate was added and incubated for another 10 min. Total assay time was ca. 25 min. The dipsticks were taken out of the tubes and the remission of the coloured product was measured in-between a time period of 30 min with the RQflex reflectometer at 657 nm (red light emitting diode, LED) as the absorption maximum of the coloured product lies at 650 nm. After 30 min the colour intensity of the test strips will decrease rapidly.

(c) Calculation of dipstick IA results
The measured remissions were normalized according to the following formula:

$$R_{NORM}\,(\%) = [(R - R_0)/(RE - R_0)] \times 100,$$

where R_{NORM} = normalized remission, R_0 = remission of the zero control, R = measured remission of the standard or sample, R_E = remission of the analyte excess concentration (excess = concentration where the signal almost reaches its maximum).

Development and performance of the FIIA

(a) Preparation of the immunoreactor column.
The antibodies were immobilized via the system avidin/biotin on porous microglass beads with activated surfaces containing carboxylic groups via carbodiimide activation.

Avidin was coupled via its amino groups to the carboxyl-containing support at pH 6.3 in the presence of the water-soluble carbodiimide 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC). One gram of the beads was centrifuged and the supernatants discarded. Two mL of a 1 mg/mL avidin solution (= 15 μM) and 50 mL 0.003 M phosphate buffer (coupling buffer), pH 6.3, were added to the bead pellet. After resuspension, the mixture was incubated at 4°C for 1 h. Then 400 mg EDC were added and after vigorous mixing the solution was maintained at 4°C for at least 4 h. Then several washing steps followed to completely remove non-covalently bound avidin. The beads were rinsed two times with 0.01 M PBS, pH 7.2, once with 1.4 M NaCI-PBS, pH 7.2, and another two times with PBS, after which the beads were allowed to stand on ice (4°C) for at least 4 h to allow complete regeneration of the bound avidin. This was

followed by two washing steps with PBS. The centrifuged beads were resuspended in 0.005 M phosphate buffer, pH 7.2 (containing 1% (v/v) BSA). The beads could then be stored in the refrigerator after the addition of 5 mL of 2% (w/v) sodium azide solution as a preservative. Under these conditions the beads were generally stable for at least 90 days at 4°C.

Biotinylation of the deethylatrazine-specific antibodies was achieved by incubating the antibody in PBS buffer, pH 7.2, with a five-fold molar excess of sulfosuccinimidyl-6-(biotinamido)-hexanoate at room temperature for 3 h. The reaction mixture was dialyzed extensively (for 48 h with a two-fold exchange of buffer) against 0.01 M PBS, pH 7.2. Biotinyl-antibody was added to avidin-derivatized beads at a two-fold molar excess of antibody binding sites (as determined with [^{3}H] biotin). The beads were incubated while shaking with biotinyl-antibody for 30 min at room temperature and then washed three times with 4 volumes of PBS, pH 7.2, alternated with 4 volumes of a citrate buffer containing NaCl, pH 3, followed by a final rinse with PBS, pH 7.2.

The antibody-coated beads were either filled into the column reactor for direct use or stored at 4°C in solution after the addition of 2 mL 0.01% thimerosal or 2 mL 2% NaN_3. The beads were packed into a 3 mm (inner diameter) × 6 cm plexiglass column. The total number of active antibody binding sites per column was about 10^{-11} mol. Nylon nets (10 μm) were used to retain the beads in the column.

(b) Assay protocol

Figure 1 shows the instrumentation setup for FIIA. Details of FIIA development and optimization were described previously[4]. Flow injection immunoanalysis is a sequential saturation assay where the analyte (deethylatrazine) and the corresponding enzyme-labelled analyte (deethylatrazine-peroxidase conjugate) compete for a limited number of antibody binding sites. All reagents were moved in a cross-flow over the antibody column reactor in a time-controlled cycle of pumping and injection. The fluorescence of

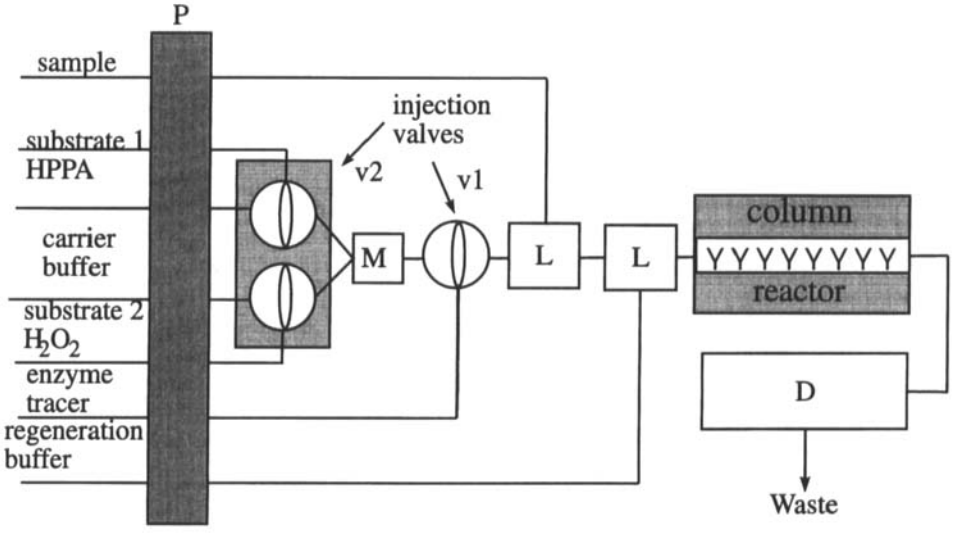

Figure 1 Instrumentation setup of flow injection immunoanalysis (FIIA), P: pump, M: mixing chamber, L: Lee valve, D: detector (fluorimeter combined with a PC).

the enzyme-generated product is measured downstream in a fluorimeter flow-through-cell (excitation wavelength: 320 nm, emission wave length: 404 nm). Fluorescence intensity was registered as peak height or peak area either by an integrator or a special computer program (Q-FIA). The peak height or peak area is inversely proportional to the analyte concentration in the sample. Each assay took 15 min to complete including the regeneration step. Regeneration was carried out by alternating rinses with 0.01 M glycine/HCI buffer, pH 2.0, for 1.5 min and rinsing steps with carrier buffer for 1.5 min to readjust the pH to 7.2. Background signals caused by unspecific binding were determined using glass beads on which only avidin was immobilized.

(c) Data calculation of FIIA results
To calculate deethylatrazine concentrations in unknown samples, the background signal was subtracted and the data were converted to $\%B/B_0$, values according to the formula:

$$\% B/B_0 = [(\text{rel. F} - \text{rel. F}_{\text{excess}})/(\text{rel. F}_0 - \text{rel. F}_{\text{excess}})] \times 100,$$

where rel. F = relative fluorescence minus background, rel. F_{excess} = relative fluorescence at the excess concentration of deethylatrazine (= 1 mg/L; excess = concentration where the signal almost reaches its lowest value), and rel. F_0 = relative fluorescence at the zero concentration of deethylatrazine.

Assay characteristics of dipstick IA and FIIA

The parameters describing the optimized IA formats (accuracy, precision, detection limit, sensitivity or slope) were determined. The lower detection limit is defined as the smallest amount of a substance that can be quantitatively assayed with the required statistical certainty at a single analysis with a statistical error of < 5%. The detection limits lor deethylatrazine with both IA formats were calculated according to Funk *et al.*[5] from 20 calibration curves. In addition, the results for the detection limits were confirmed with the help of a software program (AssayZap, Biosoft, Cambridge, England) by setting the statistical confidence interval to 99%.

The cross-reactivities of the antibody were related to deethylatrazine (= 100%). The data were obtained from standard curves with different s-triazines and further herbicides (measured with the FIIA). The midpoints of the FIIA tests were compared to calculate the cross-reactivities according to the following formula:

$\%$ cross-reactivity $= (H/C_r) \times 100$
H: hapten concentration at $50\% B/B_0$
C_r: concentration of the cross-reacting compound at $50\% B/B_0$.

Validation experiments by measuring water liquid food and soil samples

For an evaluation of the two lA formats elaborated different samples matrices (water, liquid foods, and soil) were measured with the immunochemical methods and data were compared with the results of GC measurements. The water and liquid food samples were measured directly without any prior sample pretreatment required with the dipstick assay and FIIA (only in case of the water samples). If the pH of a water sample was lower than

4.0 or exceeded 9.0, the sample was adjusted to a pH between 7.0 and 7.5 (usually with one part PBS buffer, pH 7.2, to nine parts sample (v/v)) especially for the measurement of the fruit and vegetable juices.

In the case of the soil samples a single extraction step had to be performed for FIIA in contrast to 2 further extraction steps required for extract preparation for GC analysis.

(a) Soil extraction procedure.

The soil was mixed and sieved to a particle size < 2 mm. The soil extracts were prepared according to the following procedure modified from Steinwandter[6]: Fifty grams of the soil sample (unspiked or fortified) were extracted with 50 mL of distilled water and 100 mL of acetone by mixing on a mechanical shaker overnight at ca. 220 rpm. After the addition of 15 g of NaCl and 100 mL of dichloromethane, the flasks were shaken for an additional hour. The sum of organic phase was 200 mL. The organic phase was dried on a magnetic stirrer after the addition of anhydrous Na_2SO_4 for 15 min. From the 200-mL organic phase 50 mL was taken, the organic phase evaporated under reduced pressure to a volume of ca. 0.5 mL, and the residual organic phase was removed under nitrogen. The residue was then suspended in 2 mL of distilled water and represented the extract for FIIA determination. Another 100 mL of the 200 mL organic phase was further prepared for GC analysis and for this purpose first evaporated to dryness and taken up in 10 mL of 50/50 (v/v) mixture of cyclohexane/ethyl acetate. From this 10-mL solution, 4.97 mL was given on a gel permeation chromatography column packed with BioBeads St_8 for purification and enrichment and eluted with 1 mL of methanol. Only this methanolic extract could be analyzed with GC.

RESULTS AND DISCUSSION

Two different IA formats, one suitable as a field test and the other an automatic alarm station, for the routine monitoring of deethylatrazine were established and applied for measuring a variety of different sample matrices, e. g. water, soil and liquid food samples. Figures 2 and 3 show the representative standard curves for deethylatrazine obtained by FIIA and the dipstick IA, respectively. As calculated by the software AssayZap (with a confidence interval of 99%) a lower detection limit of 0.1 µg/L with the dipstick IA and 0.01 µg/L with the FIIA could be reached, i. e. only in the latter case suitable for a routine monitoring of drinking water for pesticide contamination in accordance with European legislative requirements. For the dipstick IA the measuring range was between 0.1 and 10 µg/L, for FIIA analysis it ranged from 0.01–10 µg/L with relative standard deviations of 6 and 4%, respectively. It can be clearly seen that the FIIA reaches a lower detection limit than the dipstick IA using the same immunoreagents but different detection principles. With the FIIA the same measuring range as with the respective immunoassay can be reached as the absorption and the relative fluoresence are more sensitive detection parameters as the remission due to physical reasons. In addition, it is shown in Figure 3 that the antibody cross-reacted with another atrazine metabolite, deisopropylatrazine, to nearly the same extent yielding a sum parameter for both metabolites. As it is obvious from Table 1 the antibody can be advantageously used to screen for the sum of the two atrazine metabolites deethylatrazine and desisopropylatrazine. Together with another extreme, the monospecific IA format, these are both the only assay types which are suitable in terms of analytics. So the major importance is on the antibody compound (especially the

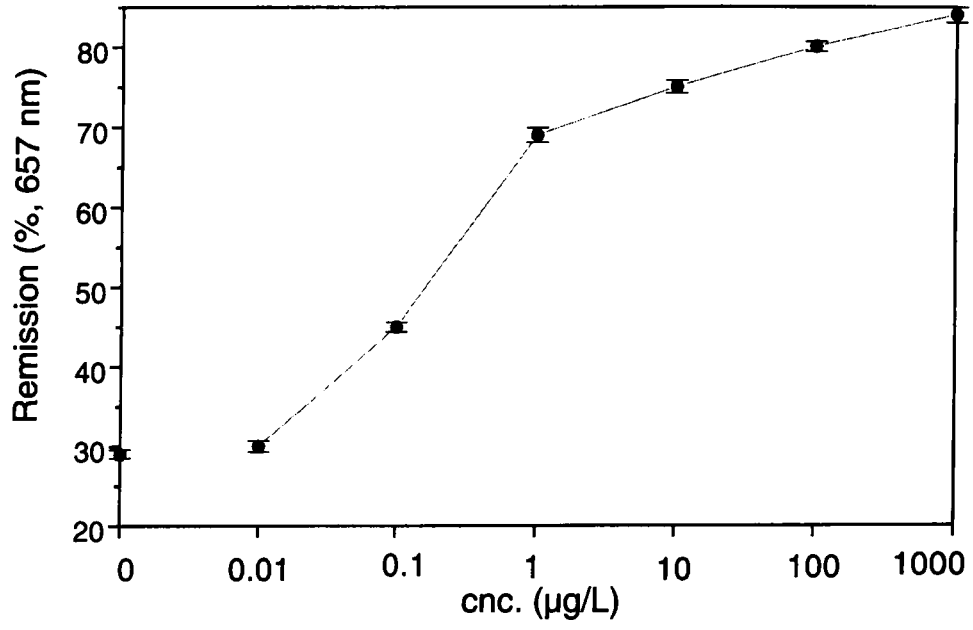

Figure 2 Representative standard curve obtained with the optimized dipstick IA for deethylatrazine determination. The tests were run in quadruplicates. Relative standard deviations (RSD) are indicated as error bars.

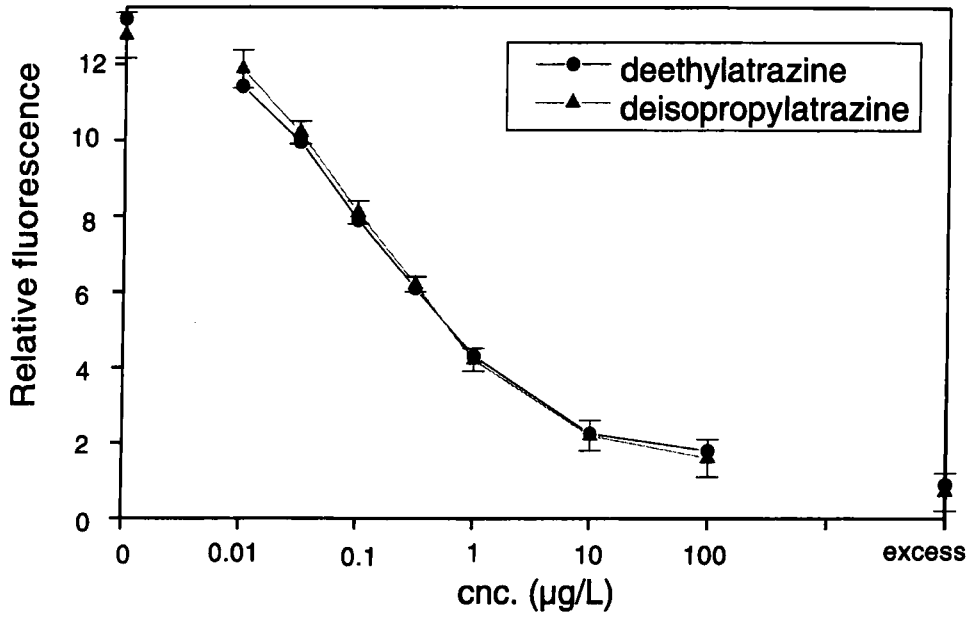

Figure 3 Representative standard curves obtained with the optimized FIIA for deethylatrazine and deisopropylatrazine determination. The tests were run in quadruplicates. RSD are indicated as error bars.

Table 1 Cross-reactivities of antiserum C10 (blood collection, February 27, 1990)[a]

Compound	*Lower detection limit with FIIA, µg/L*	*50% B/B_0 with FIIA, µg/L*	*Cross-reactivity, % based on deethylatrazine*
Deethylatrazine	0.010	0.200	100
Deisopropylatrazine	0.010	0.280	70

[a]The following s-triazines could not be detected at concentrations of 100 µg/L: atrazine, deethyldeisopropylatrazine, hydroxyatrazine, simazine, propazine, terbuthylazine, ametryn, aziprotryn, simetryn, prometryn, terbutryn, 2-ethylamino-4-thiomethyl-6-amino-triazine, 2-amino-4-thiomethyl-6-isopropylamino-triazine, 2-amino-4-methoxy-6-isopropylamino-triazine.
The following other herbicides were not detectable at concentrations of 10 mg/L: acetochlor, alachlor, butachlor, diethyl-ethyl, dimetachlor, metazachlor, metolachlor, propachlor, pretilachlor, methabenzthiazuron.

affinity constant of the antibody for its analyte) rather than on the sensitivity of the detection system. However, even in case of the dipstick IA valuable measurements are possible giving a first indication about a potential pollution with deethylatrazine as it is obvious from Table 2. Even in the case of liquid food samples (cf. Figure 4) a direct measurement (only after pH adjustment) with the dipstick IA was possible with the

Table 2 Analysis of natural water samples from the Lake Constance (Germany) by dipstick IA and GC[a].

Sample	*Origin*	*Deethylatrazine cnc. added, µg/L*	*Deethylatrazine cnc. determined with dipstick IA, µg/L ± SD[b]*	*Deethylatrazine cnc. determined ed with GC[c], µg/L*
1a	Lake (surface,	–	0.1 ± 0.07	0.05
1b	docks)	0.1	0.2 ± 0.02	
1c		0.5	0.7 ± 0.03	
2a	Raw water	–	0.1 ± 0.01	0.07
2b	(60 m depth,	0.1	0.3 ± 0.05	
2c	collected 4.12.1995)	0.5	0.6 ± 0.03	
3a	Sandfilter influx	–	0.1 ± 0.08	0.06
3b	(collected	0.1	0.2 ± 0.05	
3c	4.12.1995)	0.5	0.5 ± 0.09	
4a	Sandfilter outflux	–	< 0.1	0.03
4b	(collected	0.1	0.1 ± 0.01	
4c	4.12.1995)	0.5	0.4 ± 0.02	

[a]The environmental water samples were obtained by Mr. Petri, Bodensee Wasserversorgung (BWV), Betriebs- und Forschungslabor Süssenmühle, 78354 Sipplingen, Germany, together with the respective GC/MS data for their s-triazine contents.
[b]SD, standard deviation
[c]The detection limit for GC analysis was 0.01 µg/L for all s-triazines detected. The recovery rate for atrazine amounted to 103% (69% for deethylatrazine) with coefficients of variation of 10% for atrazine and 20% for deethylatrazine. The GC data were projected to 100% (personal communication by Mr. Petri.)

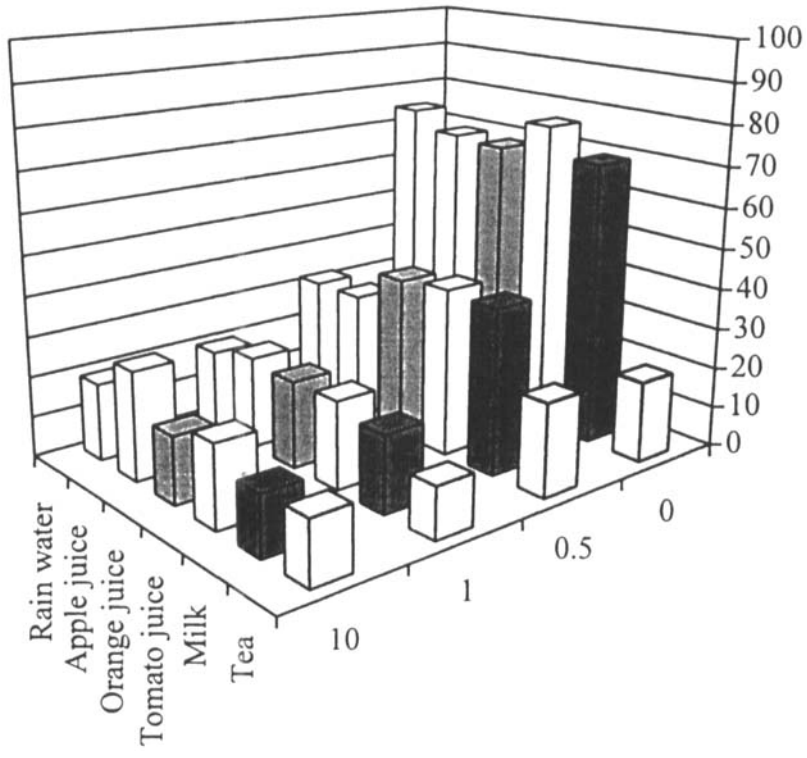

Figure 4 % B/B values of the remission obtained for several liquid foods (as indicated in the graph: Apple juice, orange juice, tomato juice, milk, black tea; with rain water as reference sample) in the original state and after spiking the samples with the following deethylatrazine concentrations: 0.50—1—10 μg/L.

exception of the black tea sample. In the latter case the dipsticks showed a yellow-brownish colour which did not disappear even after the washing step resulting in disturbances of the remission measurement. This could stem from tannin compounds present in the tea sample binding unspecifically to the membrane surface. Table 3 shows that the water samples could be analyzed directly and quasi-continuously by the FIIA giving a good correlation with the related GC data for different water matrices (ranging from ground to surface water). In the case of soil samples, for FIIA measurement two extraction steps could be omitted which would require further 3–4 h time for sample pretreatment prior to GC analysis. The latter could only be conducted with the methanolic extract requiring three extraction and one purification (GPC) step prior to the determination whereas the immunochemical formats could be applied after one single extraction step only (as shown in Table 4). The main reason for this can be derived from the high selectivity of the antibodies toward the analyte. If electroultrafiltration is the procedure carried out for soil sample extraction (applying directly the aqueous soil suspension) then the filtrate can be used directly to conduct the immunochemical determination. In this case one advantage is that only the bioavailable part of the pesticide in the sample is calculated.

Table 3 Environmental water samples from the river Rhine measured with FIIA. The water samples were obtained from Dr. Wiegand-Rosinus, Wasserforschung Mainz GmbH (WFM), Rheinallee 41, 55118 Mainz, Germany, together with the respective GC/MS data for their s-triazine contents.

Sample No.	*Origin*	*Spiking*	*s-triazines by GC/MS (μg/L)*	*Deethylatrazine by FIIA (μg/L ± SD[b])*
1	Drinking water	–	A[a]: < 0.01 DEA: < 0.025 S: 0.028 T: 0.026	< 0.01
2	Surface water (river Rhine)	–	A: 0.14 DEA: 0.051 S: 0.097 T: 0.06	0.06 ± 0.006
3	Surface water (river Rhine, fortified)	A: 0.15	A: 0.3 DEA: 0.045 S: 0.077 T: 0.066	0.07 ± 0.004
4	Surface water (river Rhine, fortified)	A: 0.15	A: 0.29 DEA: 0.052 S: 0.07 T: 0.061	0.05 ± 0.003
5	Surface water (river Rhine, fortified)	A: 0.05 DEA: 0.15	A: 0.21 DEA: 0.19 S: 0.076 T: 0.057	0.22 ± 0.010
6	Drinking water, fortified	A: 0.10 DEA: 0.05	A: 0.1 DEA: 0.054 S: < 0.01 T: < 0.01	0.06 ± 0.003

[a]A: atrazine, DEA: deethylatrazine, S: simazine, T: terbuthylazine
[b]SD, standard deviation

Table 4 Measurement of soil samples (unspiked and fortified) by the FIIA. The soil samples were collected by Dr. H. Dieckmann (group Prof. Bahadir, Institute for Environmental Chemistry, TU Braunschweig, Germany) in Northern Germany. Every soil extract was analyzed in quadruplicates with the FIIA. GC analysis of the methanolic soil extracts was performed with the kind help of Dr. H. Dieckmann.

Sample No.	*Soil type*	*Deethylatrazine spiked (μg/kg)*	*Deethylatrazine cnc. detected with GC (μg/kg)*	*Deethylatrazine cnc. determined with the FIIA (mg/kg)*
1a	Sand	–	8	10.2 ± 1
1b	Sand	+ 10	9.7	22 ± 3
1c	Sand	+ 100	108	125 ± 6
2a	Loamy sand	–	< 2	< 0.8
2b	Loamy sand	+ 25	20	30 ± 5
2c	Loamy sand	+ 200	210	190 ± 9
3a	Clay	–	< 2	< 0.5
3b	Clay	+ 50	65	49 ± 8
3c	Clay	+ 300	294	409 ± 20

CONCLUSION

In conclusion, the major prerequisite for the development of highly sensitive and specific immunochemical methods, is the high quality of the antibodies applied. Therefore, it was possible to reach an acceptable lower detection limit for deethylatrazine even with the dipstick IA although the assay is not yet suitable to control drinking water samples in line with the EU Guidelines for Drinking Water. It can be assumed that the dipstick IA can, in principle, serve as a field test to gain knowledge about a potential contamination with a pesticide or a pesticide metabolite on-site. This holds especially true for the liquid food samples. On the other side, a big advantage of the FIIA is its potential for a quasi-online device to continuously monitor pesticide residues and their relevant metabolites.

The final goal in the IA techniques still is the development of immunosensors for multi-component analysis since biosensors allow, at least in theory, for chemometric assays in a miniature system. From the immunosensor formats described so far[8–15] none is suitable yet for monitoring drinking water samples with regard to the upper limit of 0.1 µg/L or even 0.5 µg/L for the sum of pesticides present in drinking water in accordance with the EU Guidelines for Drinking Water. Although some optical immunosensors[16] and an electrochemical immunosensor[17] are described reaching promising detection limits for single pesticide compounds the relative standard deviations were usually reported to be > 10% and reproducibility was poor. But work is in progress to achieve better accuracy and precision with these immunosensors.

Both formats described in this paper, the dipstick assay with multiple antibodies immobilized on one test strip as a field test device and the FIIA with multiple antibody column reactors connected in parallel for on-line monitoring, are promising approaches for multiresidue screening.

Acknowledgements

The help in GC analysis following an established EPA method for s-triazine analysis by Dr. H. Dieckmann (group Prof. Bahadir, TU Braunschweig, Institute for Environmental Chemistry, Braunschweig, Germany) and in the collection of the soil samples in Northern Germany is gratefully acknowledged. I want to thank Mr. Petri, Bodensee Wasserversorgung (Sipplingen, Germany) for the supply and GC-MS analyses carried out of four environmental water samples from the Lake Constance, Germany. I am grateful to Dr. Wiegand-Rosinus, Wasser-forschung Mainz GmbH, Mainz, Germany, for providing me with natural water samples from the river Rhine together with the corresponding GC-MS data.

References

1. C. Wittmann and R. D. Schmid, *J. Agric, Food Chem.*, **42**, 1041–1047 (1994).
2. C. Wittmann and B. Hock, *J. Agric. Food Chem.*, **39**, 1194–1200 (1991).
3. C. Wittmann and B. Hock, *J. Agric. Food Chem.*, **41**, 1795–1799 (1993).
4. C Wittmann and R. D. Schmid, *J. Agric. Food Chem.*, **42**, 1041–1047 (1994).
5. W. Funk, V. Dammann, C. Vonderheid, and G. Oehlmann, (eds), *Statistische Methoden in der Wasseranalytik—Begriffe, Strategien, Anwendungen*, (VCH-Verlagsgesellschaft, Weinheim, 1985).
6. H. Steinwandter, *Fresenius`J. Anal. Chem*, **339**, 30–33 (1991).
7. C. Wittmann and B. Hock, *J Agric. Food Chem.*, **41**, 1421–1425 (1993).
8. M. A. Roberts and R. A. Durst, *Anal. Chem.*, **67**, 482–491 (1995).

9. S. T. A. Siebert, S. G. Reeves and R. A. Durst, *Anal. Chim. Acta*, **282**, 297–305 (1993).
10. T. Kalab and P. Skladal, *Anal. Chim. Acta*, **304**, 361–368 (1995).
11. K. Yokoyama, K. Ikebukuro, E. Tamiya, I. Karube, N. Ichiki and Y. Arikawa, *Anal. Chim. Acta*, **304**, 139–145 (1995).
12. F. F. Bier, W. Stöcklein and R. D. Schmid, in: *GBF Monographs* (R. D. Schmid and F. Scheller, eds VCH, Weinheim, 1991) pp 205–208.
13. F. F. Bier, Stöcklein, M. Böcher, U. Bilitewski and R. D. Schmid, *Sensors and Actuators B*, **7**, 509–512 (1992).
14. F. F. Bier and R. D. Schmid, *Biosensors & Bioelectronics*, **9**, 125–130 (1994).
15. G. G. Guilbault, B. Hock and R. Schmid, *Biosensors & Bioelectronics*, **7**, 411–419 (1992).
16. F. F. Bier, W. Stöcklein. M. Böcher, U. Bilitewski and R. D. Schmid, *Sensors and Actuators B*, **7**, 509–512 (1992).
17. T. Kalab and P. Skladal, *Anal. Chim. Acta*, **304**, 361–368 (1995).