Development and Application of an Automated Quasi-Continuous Immunoflow Injection System to the Analysis of Pesticide Residues in Water and Soil

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This paper presents the development and evaluation of a modified immunoassay format, applying the technique of flow injection immunoanalysis (FIIA) to the quantitative determination of pesticide traces using atrazine as the representative analyte. A novel antibody column reactor allows for an effective regeneration of the immobilized antibody activity for a minimum of 500 measuring cycles, enabling an automatic control of the status of water contamination by special pesticides. Detection limits for atrazine of about 1 ng/L with a polyclonal antibody and approximately 30 ng/L with a monoclonal antibody could be reached, well below the maximum pesticide concentration permitted by the European Community Directives for drinking water of 100 ng/L. A series of synthetic and environmental water samples as well as soil extracts was analyzed with the optimized FIIA and GC for validation. A close correspondence was found between the results of the FIIA and GC measurements.

INTRODUCTION

Increasing public concern for environmental toxic substances and their potential movement in the ecosystem demands more effective documentation of pollutant residues in the environment. The location of Germany in central Europe, crossed by many large international rivers, which provide drinking water to millions of people, has resulted in considerable emphasis on water purification and water purity control.

Intensive agriculture with the associated use of a large number of different pesticides and the growing concern about the potential contamination of ground water necessitate the availability of fast screening methods.

The protection of drinking water in Europe has led to pertinent regulation, in particular the European Drinking Water Act which sets limits for the amount of pesticide residues in drinking water. The upper limit for pesticide concentrations in drinking water is prescribed to be 100 ng/L for a single substance and 500 ng/L for the total of all pesticides including metabolites.

The methods generally used in water supplies to monitor pesticides are high-performance liquid chromatography (HPLC) and gas chromatography (GC). Unfortunately, these classical analytical procedures that use solvent partitions are time-consuming and costly because often preconcentration of the sample is necessary and they require sophisticated laboratory equipment. For this reason, there is a need to develop less costly alternative procedures as a useful supplement to increase the basic data set. Serological methods such as immunoassays are easy to handle, there is no need of expensive equipment, and they allow for the direct measurement of water samples.

Flow injection analysis (FIA) presents a potentially useful measuring device to continuously monitor for the presence of pollutants, in our case pesticides, especially in drinking water supplies. We have transferred the principle of a competitive enzyme immunoassay to a FIA system, enabling the automation of the control of water contamination by pesticides. Regular surveys of natural water and drinking water by various research laboratories in Germany revealed that many raw waters are polluted with *s*-triazine herbicides in concentrations above the upper limit of the European Community (EC) guidelines (personal communications from Dr. C. Schlett, Central Laboratory of Gelsenwasser AG, Gelsenkirchen, and Dr. U. Oehmichen, Wasserverband Hessisches Ried, Biebesheim). The *s*-triazine atrazine in particular belongs to the most critical compounds because of its persistence. It is still frequently detected in water samples although its use has been forbidden in Germany since spring 1991. For this reason, we focused on the development and optimization of a FIA system for the analysis of atrazine as representative analyte. For this purpose, we worked with a polyclonal anti-atrazine antibody (Wittmann and Hock, 1989) and for comparison with a monoclonal anti-atrazine antibody (Giersch, 1993).

EXPERIMENTAL PROCEDURES

1. **Materials.** *1.1. Chemicals.* The production of the polyclonal anti-atrazine antibody C193 and the preparation of the atrazine–horseradish peroxidase tracer were described previously by Wittmann and Hock (1989). The monoclonal anti-atrazine antibody K4E7 was obtained as a generous gift from Dr. Thomas Giersch, Department of Botany, Technical University of München at Weihenstephan. The triazine standards (especially atrazine) were provided by Riedel de Haen AG, Seelze, Germany. In addition, the following reagents were used: [^{14}C]-atrazine (925 MBq/mmol; Amersham, Braunschweig, Germany), avidin (Sigma Chemie GmbH, Deisenhofen, Germany), *d*-[8,9-^{3}H]biotin (1.1–2.2 TBq/mmol; Amersham), ethanol absolute, p. a. (Merck, Darmstadt, Germany), (3-glycidoxypropyl)trimethoxysilane (GOPS, Janssen Chimica, Geel, Belgium), hydrogen peroxide, 30% (Merck, Darmstadt), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES, Aldrich Chemical Co. Ltd., Gillingham, Dorset, England), 3-(*p*-hydroxyphenyl)propionic acid (HPPA, Sigma), poly(oxyethylensorbitan monolaurate) (Tween 20, Merck), and sulfosuccinimidyl 6-(biotinamido)-hexanoate (Pierce, Rockford, IL). All other chemicals used were of analytical grade.

1.2. Buffers and Solutions. As substrate buffer for peroxidase (carrier buffer) 40 mmol/L phosphate-buffered saline (PBS), pH

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7.2 (containing 8.5 g/L NaCl), was employed. The following substrates for peroxidase were separately dissolved 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 atrazine–peroxidase tracer 40 mmol/L PBS buffer, pH 7.2, containing 0.5 mL/L Tween 20 was used. For regeneration of the immobilized antibodies 10 mmol/L glycine/HCl buffer, pH 2.0, was used.

1.3. Preparation of Atrazine Standards. Atrazine (5 mg) was dissolved in 50 mL of 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 atrazine (=excess, i.e., the analyte concentration where the signal reaches its lowest value). A standard series was prepared by making several dilutions of the stock solution containing the following atrazine concentrations: 0.01, 0.03, 0.1, 0.3, 1, and 10 μg/L. The stock solution and the standard series were made up in distilled water.

1.4. Equipment. The laboratory equipment used comprised an enzyme immunoassay photometer for 96-well microtiter plates (Molecular Devices), a microtiter plate washer with 8 channels (Nunc Intermed GmbH, Roskilde, Denmark), and an ultrasonic bath (RK 514, Sonorex Bandelin).

For flow injection immunoassay (FIIA) measurement, the instrumentation setup (cf. Figure 1) consisted of the following apparatus: five peristaltic pumps (Meredos GmbH, Bovenden, Germany), two 3/2-way valves (Lee, Westbrook, CT), two injection valves (Fiastar 5102-002 injector V-100, Tecator, Höganäs, Sweden), a fluorometer with a flow-through cell (Merck Hitachi, Darmstadt, Germany), an integrator (Shimadzu C-R6A Chromatopack), a relay station (GBF, Braunschweig, Germany), and a special column reactor (GBF).

1.5. Further Materials. Further materials used were microtiter plates (96-well, type F-form, high binding capacity, MaxiSorp, Nunc Intermed) for the performance of the enzyme immunoassays (random tests for comparison with FIIA). As support material for the immobilization of the antibodies polystyrene beads (Biosilon microcarriers for cultivation of anchorage-dependent cells in suspension) with a diameter of 160–300 μm (Nunc) and glass beads, acid washed (for disintegrating yeast cells and microorganisms by grinding or blending) with a diameter of 150–212 μm (Sigma, Deisenhofen), were applied.

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

For GC analysis, 1 L of the water sample was enriched by solid-phase extraction (on Bakerbond octadecyl C_{18}, 40 μm, 7025-00 Baker) eluted with acetone and dichloromethane and determined via GC (Grandet et al., 1988). The GC conditions were as follows: column, 30-m quartz capillary column covered with DB-5; detector, nitrogen–phosphorus selective detector (Hewlett-Packard); internal standard, desmetryn.

1.6.1. To check the accuracy of the test and to detect for matrix effects which could lead to false positive results, samples of atrazine-polluted tap water in concentrations between 0.10 and 2.00 μg/L were analyzed with FIIA. In addition, the neat sample was assayed by GC.

1.6.2. In a second step, several environmental water samples were analyzed with FIIA and in another laboratory with GC or HPLC. From the analyzed water samples we chose as representative example environmental water samples provided by Dr. M. Wiegand-Rosinus, Stadtwerke Mainz AG, Mainz. The samples consisted of a raw water sample and water from the Rhine river in the natural state and fortified with the *s*-triazines atrazine and terbuthylazine in three different concentrations. The raw water sample was obtained by bank filtration from the Rhine river. For comparison, the samples not spiked with atrazine were measured with GC.

1.7. Soil Samples. To get an impression of how to minimize the extraction procedure of soil samples for FIIA measurement, altogether six soil extracts at three different extraction stages of two different soil types, a sand and a clay soil, with two different amounts of atrazine added to each soil sample were obtained by Mrs. Heike Dieckmann, group of Prof. Dr. A. M. Bahadir, Institute of Ecological Chemistry and Waste Analysis, Technical University of Braunschweig. The preparation of soil extracts is described under point 2.4.a.

2. Methods. Details of polyclonal and monoclonal antibody production and enzyme tracer synthesis for the development of the atrazine enzyme immunoassay have been described previously for the polyclonal antibody test (Wittmann and Hock, 1989) and the assay using the monoclonal antibody (Giersch, 1993).

2.1. Development of a FIA System with a Reusable Antibody Column Reactor. Starting from the appropriate enzyme immunoassay, the format of a competitive enzyme immunoassay was transferred to a flow injection analysis system. On the basis of a FIA system which was described earlier by Krämer and Schmid (1991a,b), a novel antibody column reactor was developed and the FIA system was further automated introducing a computer control. Several support materials (e.g., diverse glasses partly exhibiting active sites as, for example, amino groups for cross-linking, nylon, latex, polyacrylamide, polystyrene; with different particle diameters ranging from 5 to 10 μm over 160–300 μm up to a diameter of 1000 μm on average) and various immobilization methods (adsorptive, covalent, and via the system avidin/biotin) were studied. Determination of protein on solid supports was achieved using the bicinchoninic acid (BCA) assay. The immunospecific binding activity of immobilized atrazine-specific antibodies on the different support particles was determined by use of [^{14}C]atrazine and additionally [^{3}H]biotin for the measurement of avidin binding activity.

With a polyclonal and a monoclonal anti-atrazine antibody as representative examples, the FIIA system was optimized according to column design with respect to column length, material, and inner diameter. In addition, the incubation times in FIIA, i.e., the incubation with the atrazine–peroxidase tracer and the substrate incubation in the antibody column reactor, were optimized especially according to a maximum possible signal height or area combined with a high displacement of the atrazine–peroxidase tracer by very low atrazine concentrations.

The stability of the antibody column was checked studying especially the number of measuring cycles (each assay takes 15 min to complete including antibody regeneration) which can be performed with a newly packed antibody column reactor. Different methods for antibody regeneration, e.g., high salt concentrations (for example, 2 M NaCl), the use of chaotropic salts (NaSCN, for example), 8 M urea, buffers with a low pH (especially glycine/HCl buffer, pH 2.0), the use of several detergents or buffers at an alkaline pH (for example, diethanolamine buffer, pH 9.0), were studied with respect to a fast and complete regeneration of the immobilized antibodies retaining their full activity and amount of protein bound on the support. In addition, the antibody columns were stored at 4 °C in the refrigerator, inserted every 4 weeks into the FIA system, and checked according to their binding properties as well as their amount of protein still immobilized on the support.

The two immobilization procedures yielding the best results with regard to the long-term stability of the protein amount immobilized and the remaining antibody activity are described under point 2.2.a. In our case, long-term stability means that the immunoreactor column can be regenerated for a minimum of 500 measuring cycles (i.e., 5 days) at room temperature or that the antibody-immobilized support can be stored in the refrigerator for at least 3 months, respectively.

2.2. Performance of the Optimized FIIA. 1. Preparation of Immunoreactor Column. The antibodies were immobilized to glass or polystyrene beads via the system avidin/biotin using a procedure modified from a method performed by Locascio-Brown et al. (1990).

2.2.1.1. Immobilization of Antibodies to Polystyrene Beads. Polystyrene beads (20 g) were incubated on a horizontal shaker with a 0.1 mg/mL solution of avidin in 0.01 M HEPES buffer, pH 8.0, with 0.01% thimerosal (a bacteriostat) for 15 h at room temperature. The beads were then washed three times with the same buffer. The polystyrene beads to which avidin was adsorbed were further derivatized with either polyclonal or monoclonal anti-atrazine antibodies which were covalently modified with biotin. Biotinylation of anti-atrazine antibody was achieved by incubation of antibody on 0.02 M HEPES buffer, pH 8.2, with a 5-fold molar excess of sulfosuccinimidyl 6-(biotinamido)-hexanoate at room temperature for 3 h. The reaction mixture

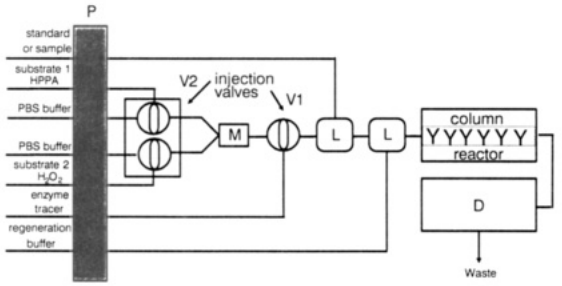

Figure 1. Instrumentation setup of FIIA for atrazine monitoring. Five pumps with different reagents work in a time-controlled sequence. All reagents have to pass the antibody reactor where the specific antibodies are located. These antibodies are immobilized after biotinylation on avidin-derivatized polystyrene or glass beads, and the antibody-supported beads are filled in a specially constructed column reactor which is regenerated within each measuring cycle. The fluorescence of the enzyme reaction product is measured with a fluorimeter and the peak height and area are registered with an integrator or by computer (Q-FIA program). P, pump; M, mixing chamber; L, Lee valve (3/2-way valve); D, detector (fluorimeter combined with an integrator and/or computer).

was dialyzed extensively (for 48 h with a 2-fold exchange of buffer) against 0.01 M phosphate-buffered saline (PBS), pH 7.4. Biotinyl–antibody was added to avidin-derivatized beads at a 2-fold molar excess of antibody binding sites (as determined with [^{3}H]-biotin). 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 7.4, alternated with 4 volumes of a citrate buffer containing NaCl, pH 3, followed by a final rinse with PBS, pH 7.4.

2.2.1.2. Immobilization of Antibodies to Glass Beads. The glass beads (50 g) were first refluxed in 5% nitric acid for 45 min, rinsed in water, and then reacted with 250 mL of 10% aqueous (v/v) solution of (3-glycidoxypropyl)trimethoxysilane (GOPS) at 90 °C for 1 h. The pH of the reaction mixture was adjusted to 3 by additions of 1 N H_2SO_4. Beads were rinsed in approximately 200 mL of water and cured at 110 °C overnight. The glycidoxy groups were oxidized to aldehydes with periodic acid. Beads were incubated for 1.5 h with twice their volume of 0.5% periodic acid (w/v) in 80% glacial acetic acid. Beads were then coupled to the amino functional groups of avidin via formation of a Schiff base by incubation in an equal volume of 0.1 mg/mL protein in 0.1 M carbonate buffer, pH 9.6, at 4 °C for 20 h. $NaBH_4$ (0.5 mg/g of beads) was then added and allowed to react for 1 h at room temperature. Derivatized beads were washed three times with 4 volumes of PBS, pH 7.4, alternated with 4 volumes of a citrate buffer containing NaCl, pH 3, and last with PBS, pH 7.4. The biotinylated antibodies were then coupled to the avidin-derivatized beads according to the same procedure as described for the polystyrene beads (cf. above).

The antibody-immobilized beads were either filled into the column reactor for direct use or stored at 4 °C in solution after the addition of 2 mL of 0.01% thimerosal or 2 mL of 2% NaN_3. The particles were packed into a 3 mm (i.d.) × 6 cm plexiglass column. The total amount of active antibody binding sites per column was about 10^{-13} mol for both the glass and the polystyrene particles with the polyclonal or the monoclonal antibodies immobilized. Nylon mesh frits (60 μm) were used to retain the particles in the column.

2.2.2. Assay Format of the Optimized FIIA. Figure 1 shows the instrumentation setup for FIIA. Flow injection immunoanalysis is a sequential saturation assay in which the hapten (atrazine) and the corresponding enzyme-labeled hapten (an atrazine derivative–peroxidase conjugate) compete for a limited number of antibody binding sites. All reagents were moved in a cross-flow over the column reactor in a time-controlled cycle of pumping and injection. In a first step the FIIA is rinsed for 1 min with carrier buffer (40 mmol/L PBS, pH 7.2, flow rate 0.74 mL/min) to equilibrate the immobilized antibodies located in the column reactor. Atrazine standard or sample is then pumped for 3 min over the column reactor (flow rate 0.78 mL/min). After the injection loop is filled with the atrazine–peroxidase tracer, a volume of 40 μL of enzyme tracer is injected and pumped in a "stop and go" cycle in five intervals of 20 s over the antibody column reactor. After a short rinsing step with carrier buffer for 2 min, the enzyme substrates 3-(*p*-hydroxyphenyl)propionic acid (HPPA) and hydrogen peroxide, 40 μL each, are filled in another double-injection loop, injected, and pumped through a mixing chamber in the antibody column reactor where the flow stops for an incubation time of 2 min. The fluorescence of the enzyme-generated product is measured downstream in a fluorometer flow-through cell (excitation wavelength 320 nm; emission wavelength 404 nm). Fluorescence intensity was registered as peak height or peak area alternatively by an integrator or a special computer program (Q-FIA, GBF). The peak height or peak area is inversely proportional to the atrazine concentration in the sample. Each assay took 15 min to complete including the regeneration step. The regeneration step was performed by alternative rinses with 0.01 mol/L glycine/HCl buffer, pH 2.0, for 1.5 min (flow rate 0.72 mL/min) followed by a subsequent rinsing step with carrier buffer for 1.5 min to readjust the pH to 7.2. Background signals, caused by unspecific binding, were determined using polystyrene or glass beads on which only avidin was immobilized.

For the calculation of atrazine concentrations in unknown samples, the background signal was subtracted and the data were converted to % B/B_0 values according to the formula

$$\% \; B/B_0 = (\text{rel } F - \text{rel } F_{\text{excess}})/(\text{rel } F_0 - F_{\text{excess}}) \times 100$$

where rel F is the relative fluorescence minus background, rel F_{excess} is the relative fluorescence at the excess concentration of atrazine (=1 mg/L), and rel F_0 is the relative fluorescence at the zero concentration of atrazine.

The detection limits were calculated according to the method of Funk et al. (1985) from 20 calibration curves.

2.3. Measurement of Water Samples. If the pH of a sample was lower than 4.0 or exceeded pH 9.0, the sample was adjusted to a pH between 7.0 and 7.5 [i.e., usually 1 part PBS buffer, pH 7.2, plus 9 parts sample (v/v)] for the measurement of the synthetic and environmental water samples with FIIA. If the atrazine concentration of a sample exceeded 5 or 1 μg/L, respectively, dilutions of the samples were carried out until the atrazine concentration was between 0.001 and 1 μg/L with the polyclonal antibody and between 0.01 and 1 μg/L with the monoclonal antibody.

2.4. Measurement of Soil Samples. 2.4.1. Preparation of Soil Extracts. The soil was mixed and sieved to a particle size <2 mm. The soil samples obtained were either extracted directly or stored frozen at −15 °C. To prepare the atrazine stock solution (which was used to spike the soil samples), 10 mg of atrazine was dissolved in 10 mL of methanol. The atrazine concentration was verified by a GC analysis. The soil extracts were prepared according to the following procedure modified from Steinwandter (1991): Fifty grams of a sand soil spiked with 0.1 mg/kg atrazine and 50 g of a clay soil fortified with 12.5 μg/kg atrazine 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, 70 mL was taken, the organic phase was 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 (extract A). Another 75 mL of the 200-mL organic phase was evaporated to dryness and taken up in 10 mL of a 50/50 (v/v) mixture of 10 mL of cyclohexane/ethyl acetate (an aliquot of this 10 mL represents extract B). From this 10-mL solution, 4.97 mL was given on a gel permeation chromatography column packed with Bio-Beads St_8 for purification and enrichment and eluted with 1 mL of methanol (extract C). Only the methanolic extract could be analyzed with GC.

2.4.2. Measurement of Soil Extracts by FIIA. For the analysis of atrazine in soil extracts, the pH was adjusted to 7.0–7.5 (if the pH of the extract was not between pH 4.0 and 9.0) and the samples were accordingly diluted, as required. When the samples

contained organic solvents, they were diluted to a final concentration of 1% of the organic solvent. Higher solvent concentrations can disturb the test.

For better comparison of FIIA data with the appropriate atrazine enzyme immunoassay, spot checks were performed with some water and soil samples. The performance of the atrazine enzyme immunoassay was described previously on microtiter plates for the polyclonal antibody assay (Wittmann and Hock, 1989) and the monoclonal antibody test (Giersch, 1993).

RESULTS

Our main goal was to develop an automated analysis system for the quantitative determination of pesticides that can be used as an alarm station to routinely monitor for the presence of herbicides, in our case atrazine, in water and soil samples. Legislative requirements such as the EC Directives on drinking water and the German Drinking Water Ordinance prescribe an upper limit for pesticide contamination in drinking water of 0.1 μg/L and have to be considered for method development.

A flow injection system for the analysis of pesticide residues which was based on an antibody-supported membrane reactor was described earlier by Krämer and Schmid (1991a,b). Because of problems with the membrane-exchange mechanism, the higher detection limit of the membrane FIIA (compared to enzyme immunoassay), the higher coefficients of variation, and the higher antibody amount needed per assay, an antibody column reactor was developed to overcome the described disadvantages. Another reason was that monoclonal anti-atrazine antibodies and other antibodies could not be used in the FIA system based on the membrane reactor because the immobilization of these antibodies onto the membrane resulted in a very high background signal caused by a very high amount of unspecific binding. For this reason, we focused on developing an antibody column reactor packed with suitable antibody-supported carriers. A plexiglass column of 6 cm in length with an inner diameter of 3 mm turned out to be the ideal reactor device in the FIIA system.

The stability of the antibody column was verified by studying especially the number of measuring cycles that can be performed with a newly packed antibody column reactor. The antibody column filled with polystyrene or glass beads with the antibodies immobilized via the avidin/biotin system turned out to be stable in antibody activity and amount of immobilized protein for a minimum of 500 measuring cycles and can be stored for at least 4 months at 4 °C in the refrigerator without any significant loss of antibody activity or protein amount bound to the support. For both antibodies, the protein amount immobilized turned out to be 1.3×10^{-13} mol/mm^2 of the beads at the beginning and 1.0×10^{-13} mol/mm^2 after 500 measuring cycles with a standard deviation of $\pm 1 \times 10^{-14}$ mol/mm^2. The binding capacities of both antibodies to [^{14}C]atrazine amounted to 1.2×10^{-13} mol/mm^2 at the start and to 1.1×10^{-13} mol/mm^2 after 500 measuring cycles combined with a standard deviation of $\pm 1 \times 10^{-15}$ mol/mm^2.

The ideal antibody regeneration method was the one rinsing the FIIA system for 1.5 min with 0.01 M glycine/HCl buffer, pH 2.0, followed by a subsequent re-equilibration with carrier buffer to readjust the pH to 7.2. A low pH for a short time turned out to be superior to the other methods studied (as the use of buffer at an alkaline pH, buffer with a high salt concentration, chaotropic salts, a detergent, and 8 M urea) especially concerning a considerable loss in antibody activity and the immobilized protein amount over a longer time period as observed with all methods except the use of glycine/HCl buffer, pH 2.0.

In a first step, the method developed, a flow injection immunoanalysis, has to be characterized according to detection limit, sensitivity, accuracy, and precision. Figure 2 shows the calibration curves obtained with the optimized FIA systems using the polyclonal antibody C193 and the monoclonal antibody K4E7 for atrazine determination. The midpoint of the test is located on the curve where 50% of the antibody binding sites are occupied by atrazine and 50% are bound by the atrazine–enzyme tracer. The midpoint of the C193 test was found at ca. 6 ng/L and of the K4E7 test at ca. 180 ng/L, i.e., a factor of 30 higher than that of the C193 test. With the C193 FIA a detection limit of about 1 ng/L could be reached; the range of measurement lies between 0.001 and 1 μg/L atrazine. A detection limit for atrazine of approximately 30 ng/L could be reached with the monoclonal antibody K4E7 FIA, i.e., as with the polyclonal antibody FIA well below the maximum pesticide concentration permitted by the EC guidelines for drinking water. The range of measurement was from 0.03 to 5 μg/L atrazine. An atrazine standard series consisting of a minimum of three concentrations between zero and the pesticide excess concentration (=1 mg/L) has to be run before measurement in sequence of a maximum of 20 samples prior to new calibration. Each standard concentration and sample was measured in quadruplicate. In addition, with respect to the concentration result a coefficient of variation of 4% on average was achieved with both the polyclonal and the monoclonal antibodies. Some important assay parameters of the FIA system such as the midpoint of the assay, the lower detection limit, and the coefficients of variation are similar compared to those of the appropriate enzyme immunoassays, although the measuring range of FIA is always narrower than that of the respective enzyme immunoassay. This may be due to the fact that in contrast to enzyme immunoassay the FIA system does not work under equilibrium conditions.

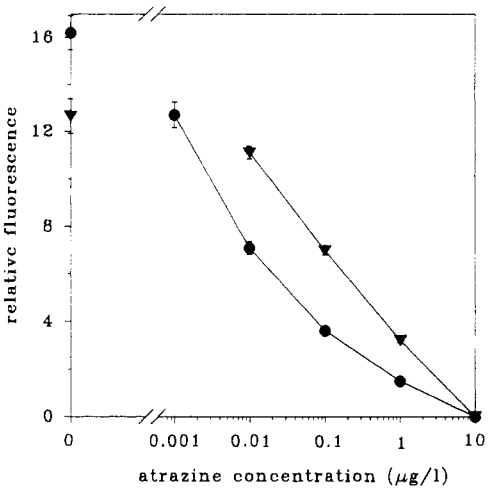

Figure 2. Representative standard curves obtained with the optimized FIIA using the polyclonal antibody C193 (●) and the monoclonal antibody K4E7 (▼) for atrazine determination. The tests were run in quadruplicate with FIIA. The standard deviations are indicated as error bars.

An important step for any validation study is the analysis and verification of samples supplemented with the analyte and comparison of the results with data of an established analysis method, i.e., in our case GC. Table 1 shows that atrazine concentrations of fortified tap water samples can be precisely determined with FIA. The level of atrazine in the untreated tap water was confirmed by GC analysis. With an atrazine concentration of 30 ng/L determined with FIA, there is a good correlation with the result of a GC analysis (of 30 ng/L atrazine). It is obvious that the results yielded acceptable values after subtraction of the

Table 1. Synthetic Samples of Tap Water[a]

sample no.	atrazine added (μg/L)	atrazine concn determined with FIIA (μg/L ± SD)[b]	variation coeff CV (%)	internal standard determined with FIIA (μg/L)
1		0.03[c] ± 0.002	8.3	
2	0.10	0.13 ± 0.008	6.1	0.10
3	0.20	0.24 ± 0.017	6.9	0.21
4	0.50	0.53 ± 0.019	3.6	0.50
5	1.00	1.01 ± 0.038	3.7	0.98
6	2.00	2.25 ± 0.065	2.9	2.22

[a] Tap water was analyzed for its atrazine concentration. Then, atrazine was added in concentrations of 0.10, 0.20, 0.50, 1.00, and 2.00 μg/L. The resulting synthetic water samples were measured with FIIA. The tests were run in quadruplicate. [b] SD, standard deviation. [c] The contamination of the tap water sample was confirmed by a GC analysis which yielded an atrazine amount of 0.03 μg/L.

Table 2. Environmental Water Samples Spiked with Atrazine[a]

sample no.	triazine added (μg/L)	atrazine concn determined with C193 FIIA (μg/L ± SD)[b]	atrazine concn determined with K4E7 FIIA (μg/L ± SD)
		A. Raw Water	
1		0.04[c] ± 0.001	0.05[c] ± 0.002
2		0.03[c] ± 0.001	0.05[c] ± 0.001
3	A:[d] 0.05 T:[d] 0.05	0.10 ± 0.004 (0.06)[e]	0.11 ± 0.006 (0.06)
4	A: 0.50 T: 0.50	0.54 ± 0.021 (0.50)	0.69 ± 0.027 (0.64)
5	A: 9.00 T: 0.50	8.93 ± 0.397 (8.89)	8.33 ± 0.373 (8.28)
		B. Rhine River Water	
1		0.14[c] ± 0.003	0.17[c] ± 0.002
2		0.12[c] ± 0.005	0.16[c] ± 0.006
3	A: 0.05 T: 0.05	0.19 ± 0.007 (0.06)	0.24 ± 0.009 (0.07)
4	A: 0.50 T: 0.50	0.65 ± 0.026 (0.52)	1.00 ± 0.040 (0.83)
5	A: 9.00 T: 0.50	8.95 ± 0.398 (8.82)	8.58 ± 0.343 (8.41)

[a] The water samples were provided by Dr. M. Wiegand-Rosinus, Stadtwerke Mainz AG, Mainz. The samples consisted of a raw water sample and water from the Rhine river, which were obtained in the natural state and fortified with the *s*-triazines atrazine and terbuthylazine in three different concentrations. [b] SD, standard deviation. [c] The GC analysis of the raw water yielded 0.03 μg/L atrazine, of the Rhine river water 0.14 μg/L atrazine. [d] A, atrazine; T, terbuthylazine. [e] Amount of atrazine determined by FIIA (μg/L).

atrazine amount of the untreated sample. In consideration, it is remarkable that the German Drinking Water Ordinance allows for a standard error of ±50 ng/L at the 100 ng/L concentration (Deutsche Trinkwasserverordnung—German Drinking Water Ordinance, 1986).

From the natural water samples analyzed we chose as representative examples environmental water samples provided by Dr. M. Wiegand-Rosinus, Stadtwerke Mainz AG, Mainz. Table 2 shows the results. It is shown that the added atrazine concentrations could be precisely determined. No matrix effects causing overestimations or false positive results were observed. With the polyclonal antibody only atrazine from the two different *s*-triazines was detected. This was expected as the polyclonal antibody C193 shows a cross-reactivity of only 3% with terbuthylazine. In contrast, the monoclonal antibody K4E7 exhibits a cross-reactivity of 26% with terbuthylazine, causing slight overestimations of the results. A GC analysis confirmed the atrazine pollution of 30 ng/L for the raw water and 140 ng/L atrazine for the Rhine river water sample.

Table 3. Synthetic Soil Samples[a]

A. Sand Soil Spiked with 0.100 mg/kg Atrazine

soil extract	atrazine concn determined with GC (mg/kg)	atrazine concn determined with FIIA using antibody C193 (mg/kg ± SD)[b]	atrazine concn determined with FIIA using antibody K4E7 (mg/kg ± SD)
A		0.11 ± 0.004	0.11 ± 0.007
B		0.09 ± 0.005	0.09 ± 0.003
C	0.0995	0.10 ± 0.002	0.11 ± 0.004

B. Clay Soil Fortified with 12.5 μg/kg Atrazine

soil extract	atrazine concn determined with GC (μg/kg)	atrazine concn determined with FIIA using antibody C193 (μg/kg ± SD)	atrazine concn determined with FIIA using antibody K4E7 (μg/kg ± SD)
A		10.2 ± 0.40	35.7 ± 1.28
B		11.0 ± 0.36	22.4 ± 0.95
C	8.750	10.3 ± 0.34	15.0 ± 0.50

[a] Six soil extracts of two different soil types, a sand soil and a clay soil, at three different extraction stages (A–C) and fortified with two different concentrations of atrazine were obtained together with the GC results for the two C extracts by Mrs. H. Dieckmann, group of Prof. Dr. A. M. Bahadir, Institute of Ecological Chemistry and Waste Analysis, TU Braunschweig. After appropriate dilution of the soil extracts, they were measured with FIIA in quadruplicate. [b] SD, standard deviation.

Table 3 shows the results for the differently prepared soil extracts with the FIA system using the polyclonal antibody C193 and the monoclonal antibody K4E7. The FIA results for extracts A–C were compared with corresponding GC data obtained for extract C. For the sand soil the recovery rate was nearly 100% for both analysis methods and using both antibodies. For the clay soil extracts, there was a higher recovery rate with the FIA system using the polyclonal antibody (88%) as compared to GC analysis (70%). Using the monoclonal antibody K4E7 in the FIA system, overestimations due to matrix effects were observed which could be reduced with progressive purification of the extract. The matrix effects can be derived from interferences of the monoclonal antibody with humic acids on which atrazine was adsorbed (Kalouskova, 1989). In addition, the soil sample was analyzed according to a potential contamination with terbuthylazine by GC as this compound is a cross-reacting substance of the monoclonal antibody. As terbuthylazine was not detected by GC, a matrix effect due to a cross-reactivity of the antibody could be excluded. As a result, it can be stated that FIA can also be applied to the analysis of soil samples where two extraction steps can be omitted. With an analysis time of the soil extracts of 15 min, FIA is faster than GC analysis for which another 3–4 h is required for two further extraction steps.

DISCUSSION

Our aim was to develop and evaluate a flow injection analysis system that allows for a rapid screening of water and soil for a potential pollution with the herbicide atrazine as representative pesticide.

We focused on the analyte atrazine for reasons that atrazine is still frequently detected in groundwater and surface water samples although its application in Germany has been forbidden since spring 1991. Groundwater contamination with atrazine is frequently reported as a result of its widespread agricultural use. Atrazine pollution is described in many countries of the EC (Buser, 1990; Funari et al., 1989; Leistra and Boesten, 1989), in the United States (Bushway et al., 1992; Pionke and Glotfelty,

1990; Richards and Baker, 1992; Ritter, 1990; Southwick et al., 1990; Spalding et al., 1989), in Canada (Frank et al., 1990a–c), and in South Africa (Pick et al., 1992). McMahon et al. (1992) stated that despite atrazine's apparent biodegradability in soils, atrazine and its metabolites still are found in the deep unsaturated zone and shallow groundwater underlying some agricultural areas. This indicates that atrazine degradation rates in some soils are slower than atrazine transport rates, resulting in atrazine transport to the water table. By other authors as well the leaching effect of atrazine is described as being responsible for its presence in water supplies and soil (Helling et al., 1988; Paya-Perez et al., 1992). Even the environmental water samples we chose as representative examples, the Rhine water and the raw water sample, exhibited atrazine residues, which could be precisely analyzed by the FIIA presented in this paper.

Concerning the method development especially with regard to the regeneration step, an interesting aspect was the observation that the alternated rinses with glycine/HCl buffer, pH 2.0, followed by carrier buffer were sufficient for a complete antibody regeneration. This may be due to the medium-height antibody affinity constants. Both the monoclonal and the polyclonal anti-atrazine antibodies exhibited affinity constants in the range 10^7 L/mol (Giersch, 1993; Wittmann and Hock, 1989). It can be assumed that for this reason the hapten–antibody dissociation is facilitated and that the conditions for a complete regeneration of the antibodies do not have to be as drastic as, for example, the use of 8 M urea.

The FIIA presented in this paper for atrazine analysis as representative example allows for an automated, rapid, and sensitive control of drinking water purity and the detection of atrazine traces in the environment, i.e., in our case in water and soil. Water samples can be measured directly in the FIIA. There is no need for any enrichment or purification prior to the analysis, which is an advantage compared to the conventional methods in trace analysis such as GC and HPLC. For this reason, the FIA system offers a faster screening. In addition, the sample amounts required are rather small (about 2.5 mL per determination) in contrast to GC and HPLC, for which 1–2-L water samples are necessary. Another advantage of the optimized polyclonal antibody FIA is the very low detection limit of 1 ng/L atrazine that can be reached, as often the samples exhibit only small traces of atrazine which are problematic to detect with GC or HPLC. To our knowledge, other flow injection systems based on immunological detection (Kusterbeck et al., 1990; Locascio-Brown et al., 1990; Tang et al., 1991) and even immunosensors developed for the detection of small analytes such as pesticides (Bier et al., 1992) generally show detection limits above 100 ng/L and greater standard deviations. For soil samples two extraction steps can be omitted for the extract preparation for subsequent FIIA as compared to GC analysis which would take further 3–4 h. At the same time the limitations should also be considered. They are mainly seen in the specificity of the antibodies, so that for this reason with one single antibody in most cases only one single compound can be analyzed. An important advantage of the FIA system is the possibility that only by an exchange of the antibody column reactor and the use of another enzyme tracer another pesticide can be analyzed, provided that suitable antibodies are available. In addition, FIA can as well be applied to ensure or confirm the results of GC or HPLC analyses. As a further application besides environmental analyses, checking of blood and urine samples for atrazine or pesticide applicators in general by FIIA is conceivable with regard to potential health risks posed by pesticide application.

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LITERATURE CITED

Bier, F. F.; Stöcklein, W.; Böcher, M.; Bilitewski, U.; Schmid, R. D. Use of a fibre optic immunosensor for the detection of pesticides. *Sens. Actuators B* **1992**, *7*, 509–512.

Buser, H.-R. Atrazine and other s-triazine herbicides in lakes and in rain in Switzerland. *Environ. Sci. Technol.* **1990**, *24*, 1049–1058.

Bushway, R. J.; Hurst, H. L.; Perkins, L. B.; Tian, L.; Cabanillas, C. G.; Young, B. E. S.; Ferguson, B. S.; Jennings, H. S. Atrazine, alachlor, and carbofuran contamination of well water in central Maine. *Bull. Environ. Contam. Toxicol.* **1992**, *49*, 1–9.

Deutsche Trinkwasserverordnung—German Drinking Water Ordinance. *Bundesgesetzblatt* **1986**, *I*, 760.

Frank, R.; Braun, H. E.; Clegg, B. S.; Ripley, B. D.; Johnson, R. Survey of farm wells for pesticides, Ontario, Canada 1986–1987. *Bull. Environ. Contam. Toxicol.* **1990a**, *44*, 410–419.

Frank, R.; Braun, H. E.; Ripley, B. D.; Clegg, B. S. Contamination of rural ponds with pesticide, 1971–85, Ontario, Canada. *Bull. Environ. Contam. Toxicol.* **1990b**, *44*, 401–409.

Frank, R.; Clegg, B. S.; Sherman, C.; Chapman, N. D. Triazine and chloroacetamide herbicides in Sydenham river water and municipal drinking water, Dresden, Ontario, Canada, 1981–1987. *Arch. Environ. Contam. Toxicol.* **1990c**, *19*, 319–324.

Funari, E.; Aquafresca, G.; Arca, F.; Baldi, M.; Bastianetti, I.; Cappelli, A.; Carli, G.; Carniel, A.; Chierici, S.; Fanuzzi, A.; Ferraro, P.; Lopez, A.; Mattioni, R.; Narese, M.; Peretti, A.; Salamana, M.; Zapponi, G. Preliminary report on the atrazine and molinate water supply contamination in Italy. *Chemosphere* **1989**, *18*, 2339–2343.

Funk, W.; Dammann, V.; Vonderheid, C.; Oehlmann, G. (Herausgeber) *Statistische Methoden in der Wasseranalytik—Begriffe, Strategien, Anwendungen*; VCH-Verlagsgesellschaft: Weinheim, 1985.

Giersch, T. A new monoclonal antibody for the sensitive detection of atrazine with immunoassay in microtiter plates and dipstick formate. *J. Agric. Food Chem.* **1993**, *41*, 1006–1011.

Grandet, M.; Weil, L.; Quentin, K.-E. Gas Chromatographic Determination of Triazine Herbicide and its Metabolite in Water. *Z. Wasser Abwasser Forsch.* (*J. Water Wastewater Res.*) **1988**, *21*, 21–24.

Helling, C. S.; Zhuang, W.; Gish, T. J.; Coffman, C. B.; Isensee, A. R.; Kearney, P. C.; Hoagland, D. R.; Woodward, M. D. Persistence and leaching of atrazine, alachlor, and cyanazine under no-tillage practises. *Chemosphere* 1988, *17* (1), 175–187.

Kalouskova, N. Adsorption of atrazine on humic acids. *J. Environ. Sci. Health* **1989**, *24* (6), 599–617.

Krämer, P. M.; Schmid, R. D. Automated quasi-continuous immunoanalysis of pesticides with a flow injection system. *Pestic. Sci.* **1991a**, *32*, 451–462.

Krämer, P.; Schmid, R. Flow injection immunoanalysis (FIIA)—a new immunoassay format for the determination of pesticides in water. *Biosens. Bioelectron.* **1991b**, *6*, 239–243.

Kusterbeck, A. W.; Wemhoff, G. A.; Charles, P. T.; Yeager, D. A.; Bredehorst, R.; Vogel, C.-W.; Ligler, F. S. A continuous flow immunoassay for rapid and sensitive detection of small molecules. *J. Immunol. Methods* **1990**, *135*, 191–197.

Leistra, M.; Boesten, J. J. T. I. Pesticide contamination of groundwater in Western Europe. *Agric. Ecosyst. Environ.* **1989**, *26*, 369–389.

Locascio-Brown, L.; Plant, A. L.; Horvath, V.; Durst, R. A. Liposome flow injection immunoassay: Implications for sensitivity, dynamic range, and antibody regeneration. *Anal. Chem.* **1990**, *62*, 2587–2593.

McMahon, P. B.; Chapelle, F. H.; Jagucki, M. L. Atrazine mineralization potential of alluvial-aquifer sediments under aerobic conditions. *Environ. Sci. Technol.* **1992**, *26* (8), 1556–1559.

Paya-Perez, A. B.; Cortes, A.; Sala, M. N.; Larsen, B. Organic matter fractions controlling the sorption of atrazine in sandy soils. *Chemosphere* **1992**, *25* (6), 887–898.

Pick, F. E.; van Dyk, L. P.; Botha, E. Atrazine in ground and surface water in maize production areas of the Transvaal, South Africa. *Chemosphere* **1992**, *25*, 335–341.

Pionke, H. B.; Glotfelty, D. W. Contamination of groundwater by atrazine and selected metabolites. *Chemosphere* **1990**, *21*, 813–822.

Richards, R. P.; Baker, D. B. Pesticide concentration patterns in agricultural drainage networks in the lake Erie basin. *Environ. Toxicol. Chem.* **1992**, *12*, 13–26.

Ritter, W. F. "Pesticide contamination of ground water in the United States—A review"; Miscellaneous Paper 1186 of the Delaware Agricultural Experiment Station, 1990.

Southwick, L. M.; Willis, G. H.; Bengston, R. L.; Lormand, T. J. Atrazine and metolachlor in subsurface drain water in Louisiana. *J. Irrig. Drain. Eng.* **1990**, *116*, 16–23.

Spalding, R. F.; Burbach, M. E.; Exner, M. E. Pesticides in Nebraska's ground water. *Ground Water Monit. Rev.* **1989**, Fall.

Steinwandter, H. Contributions to residue analysis in soils. II. Miniaturization of the on-line extraction method for the determination of some triazine compounds by RP-HPLC. *Fresenius' J. Anal. Chem.* **1991**, *339*, 30–33.

Tang, H. T.; Halsall, B.; Heineman, W. R. Electrochemical enzyme immunoassay for phenytoin by flow-injection analysis incorporating a redox coupling agent. *Clin. Chem.* **1991**, *37* (2), 245–248.

Wittmann, C.; Hock, B. Improved enzyme immunoassay for the analysis of s-triazines in water samples. *Food Agric. Immunol.* **1989**, *1*, 211–224.

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