Capillary Enzyme Immunoassay with Electrochemical Detection for the Determination of Atrazine in Water

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A capillary enzyme immunoassay with electrochemical detection has been developed for the determination of atrazine in water. The assay is based on competitive binding between atrazine and alkaline phosphatase-labeled atrazine for a limited number of antibody binding sites. The antibody is attached covalently to a modified capillary surface. The enzymatic product (*p*-aminophenol) is detected by amperometric flow injection analysis. The calibration curve for atrazine has a linear range of 0.10–10.0 μg/L and a detection limit of 0.10 μg/L. An assay including six standards can be done in less than 60 min. Intra- and inter-assay precisions at 0.1 and 5.0 μg/L are 9 and 8% and 6 and 10%, respectively. Recoveries of added atrazine from commercial bottled water, tap water, and Ohio River water at 0.50 and 5.0 μg/L range from 100 to 115%. Simple filtration is the only step needed for sample cleanup.

Keywords: *Capillary enzyme immunoassay; atrazine; electrochemical detection; water*

INTRODUCTION

The wide application of pesticides and the growing concern about adverse health effects make it important to monitor human exposure to pesticides. Herbicides represent 70–80% of the total amount of pesticides used on crops in developed countries (Sherma, 1993). *s*-Triazine herbicides were introduced by Geigy in 1955 and are used commonly as pre- and post-emergent weed-control agents on corn, sorghum, pineapple, and sugarcane. Because of their extensive use (Burkart et al., 1988), moderate persistence (Erickson and Lee, 1989), and possible carcinogenic risk (Engler and Levy, 1991), a sensitive and fast method is needed to monitor *s*-triazine herbicides in water and soil. In 1992, the U.S. Environmental Protection Agency began to regulate the maximum contaminant level at 3 μg/L in drinking water (U.S. Environmental Protection Agency, 1991). The European Community drinking water ordinance sets an upper limit of 0.1 μg/L for a single species and 0.5 μg/L for the total (European Community, 1980).

Conventionally, the determination of *s*-triazine herbicides uses chromatographic methods. These methods are selective and sensitive, with detection limits of 10^{-3}–10^{-2} μg/L (AOAC, 1990; U.S. Environmental Protection Agency, 1992). With solid-phase extraction, the detection limit can be as low as 10^{-4}–10^{-3} μg/L (Ahel et al., 1992; Cai et al., 1993). The major drawback of these techniques, however, is the need for an extensive cleanup. The sample pretreatment costs time and money, which becomes a major barrier with increasing numbers of samples to be analyzed.

From the beginning of the 1980s, immunochemical methods started to find their application in this area (Van Emon and Lopez-Avila, 1992; Bernett and Clower, 1991; Van Vunakis, 1990; Hammock et al., 1990; Seiber et al., 1990). Initially designed for the determination of biological molecules, immunoassay provides a specific, sensitive, fast, and inexpensive analytical methodology. Using the same strategy of generating an antibody, synthesizing a labeled hapten or antibody, as well as choosing an assay format (Hammock and Mumma, 1980; Harrison et al., 1991), a number of research groups worldwide have successfully applied immunoassay to the determination of *s*-triazines (Huber and Hock, 1985; Wittman and Hock, 1989; Giersch and Hock, 1990; Giersch, 1993; Wust and Hock, 1992; Schlaeppi et al., 1989; Dunbar et al., 1990; Schneider and Hammock, 1992) and other environmentally important compounds. Furthermore, several commercial test kits have been introduced in recent years, with field testing being a particular target (Karu et al., 1991; Bushway et al., 1988, 1991; Bushway and Perkins, 1988; Thurman et al., 1990; Fleeker and Cook, 1991; Goolsby et al., 1991; Rubio et al., 1991).

Capillary immunoassay with electrochemical detection introduces a number of advantages in comparison with conventional immunoassay methodology. The covalent attachment of antibody to a modified capillary can be controlled so that the antibody binding sites are distal to the attachment site. This decreases the possibility of losing antibody activity due to unfavorable orientation as a result of the immobilization process. The small sample size of the capillary (22 μL) greatly reduces the amount of reagent required. More importantly, the time it takes for molecules to reach the surface in a narrow capillary (i.d. 530 μm) is short, which results in a faster assay. Compared to UV spectroscopy, electrochemistry provides a more sensitive detection method (Thompson et al., 1991), which is crucial to trace chemical analysis. For example, a sandwich immunoassay for mouse IgG (Halsall et al., 1988) and a sequential saturation immunoassay for digoxin (Kaneki et al., 1994) have been developed in capillaries with electrochemical detection. These had

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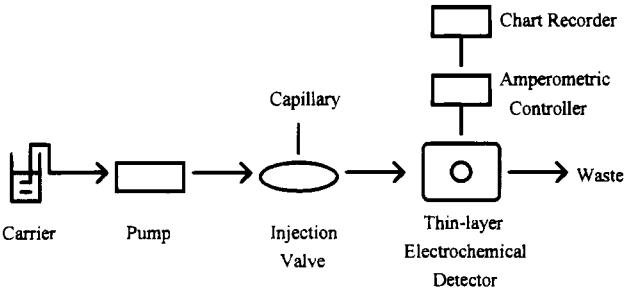

Figure 1. FIAEC system.

detection limits of 6.7×10^{-17} and 3.8×10^{-12} M, respectively.

In this paper we describe the coupling of capillary enzyme immunoassay to electrochemical detection for pesticide analysis. Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine], which is the most widely used triazine herbicide in the United States (Burkart et al., 1988), was chosen as a model compound.

EXPERIMENTAL PROCEDURES

Apparatus. The flow injection analysis with electrochemical detection (FIAEC) system (Figure 1) consisted of a syringe pump (infusion pump 22, Harvard Apparatus, South Natick, MA), an LC-4B amperometric controller, and a CC-4 thin-layer flow cell (Bioanalytical Systems, West Lafayette, IN). The cell (3.5 μL volume) had a glassy carbon working electrode, a Ag/AgCl reference electrode, and a platinum auxiliary electrode. The six-port injection valve (Rheodyne Model 7010, Rainin Instrument Co., Inc., Woburn, MA) had a sample loop of 5 μL and was controlled by a pneumatic actuator (Rheodyne Model 5701) and a 120 VAC solenoid valve (Rheodyne Model 7163). The flow rate was set at 0.4 mL/min. The data were collected by a D-5000 strip chart recorder (Houston Instrument, Austin, TX).

The UV detector and flow cell used in size exclusion chromatography were a Beckman Model 153 detector and a Beckman analytical optical unit (Beckman Instruments, Fullerton, CA). The UV spectrophotometer was a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard Co., Naperville, IL).

Reagents. Protein A-purified monoclonal antibody and alkaline phosphatase-labeled atrazine were produced according to standard procedures (Giersch and Hock, 1990; Giersch, 1993). Ametryn, atrazine, prometon, prometryn, propazine, simazine, and terbutryn were purchased from Sulpelco, Inc. (Bellefonte, PA); didealkylatrazine, deethylatrazine, and deisopropylatrazine were gifts from Ciba-Geigy Corp. (Greensboro, NC). Stock solutions were prepared in ethanol. *p*-Nitrophenyl phosphate (PNPP) was obtained from Boehringer Mannheim (Indianapolis, IN), and *p*-aminophenyl phosphate (PAPP) was synthesized as reported (Tang et al., 1988) and stored at −20 °C. Acetone, 1,4-dioxane, ethanol, magnesium chloride, sodium acetate, sodium chloride, and Tween 20 were obtained from Fisher Scientific (Cincinnati, OH). Adipic acid dihydrazide, boron trifluoride etherate (redistilled), polyethylene glycol 3350, and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, MO). 1,1′-Carbonyldiimidazole, (3-glycidoxypropyl)trimethoxysilane, sodium azide, sodium periodate, and tris(hydroxymethyl)aminomethane (Tris) were from Aldrich Chemical Co. (Milwaukee, WI). Undeactivated fused-silica capillary (catalog no. 605600) was from Alltech Associates (Deerfield, IL). After modification (Kumari, 1991), it was stored at 4 °C until used. A Barnstead organipure water purification system was used (Barnstead Co., Newton, WA).

Buffers. Antibody immobilization buffer was 0.1 M sodium acetate, 0.15 M sodium chloride, and 0.02% sodium azide, pH 4.5. Hapten/AP-labeled hapten incubation buffer was 0.1 M Tris, 0.02% sodium azide, and 0.05% Tween 20, pH 7.8. Substrate development buffer was 0.1 M Tris, 1 g/L magnesium chloride, and 0.02% sodium azide, pH 9.0.

Procedures. *Antibody Oxidation.* The monoclonal antibody for atrazine was oxidized (Kaneki et al., 1994; Hoffman and O'Shannessy, 1988) as the first step in the procedure for coupling it to the inner walls of the capillary. The following procedure was used: Dissolve 1−2 mg of antibody dry powder in 400 μL of acetate buffer (0.1 M NaAc, 0.15 M NaCl, 0.02% NaN_3, pH 5.5). Add 10 μL of 0.4 M $NaIO_4$. Allow the reaction to proceed in the dark at room temperature for 20 min. Apply the reaction mixture to a Sephadex G-25 column that has been previously equilibrated in acetate buffer (0.1 M NaAc, 0.15 M NaCl, 0.02% NaN_3, pH 4.5). The oxidized antibody passes through the column first and then the salts. Collect the oxidized antibody fraction by monitoring the UV absorbance at 278 nm. Determine the concentration of the oxidized antibody from UV absorbance. Divide the oxidized antibody into aliquots, and store these in a deep freezer (−70 °C) for later use.

Capillary Modification. The capillary was modified to generate a polyethylene glycol linker as well as an amine group to which the oxidized antibody could be covalently attached via the IgG glycan chains. The procedure for modification of a 10 m capillary is as follows: Introduce the solutions into the capillary with a syringe pump (flow rate 2.5 mL/min). Pretreat the fused-silica capillary with 1 M NaOH overnight. Rinse the capillary with 1 M HCl and then organic-free water until neutral pH. Fill the capillary with (3-glycidoxypropyl)-trimethoxysilane (GPTMS) (100 μL of GPTMS + 35 mL of acetate buffer, 0.1 M, pH 5.5). Heat the capillary at 90 °C for 5 h. Rinse the capillary with water and 1,4-dioxane. Fill the capillary with polyethylene glycol (PEG 3350) (0.502 g of PEG + 30 mL of 1,4-dioxane + 600 μL of boron trifluoride etherate as catalyst). Leave the capillary at room temperature for 5 min, and then heat it at 90 °C for 0.5 h. Rinse the capillary with 1,4-dioxane. Fill the capillary with 1,1′-carbonyldiimidazole (CDI) (0.1622 g of CDI + 6 mL of 1,4-dioxane). Leave the capillary at room temperature for 15 min. Rinse the capillary with 1,4-dioxane and water. Fill the capillary with adipic acid dihydrazide (AADH) (0.5226 g of AADH + 8 mL of carbonate buffer, 0.1 M, pH 10.0). Store the capillary at 4 °C for later use.

Assay Procedure. An appropriate length of modified capillary is cut from the original 10 m length and washed sequentially with organic-free water, 1 M sodium chloride, and organic-free water by injecting these solutions through the capillary with a 10 or 20 mL syringe that is connected to the capillary with an adapter consisting of a female luer with a septum inside joined to a male luer. The capillary is filled with oxidized antibody solution by injection with a 1 mL syringe and refrigerated at 4 °C overnight. (The concentration of the stock antibody solution was determined to be 10^{-7} M from a UV absorption spectrum.) The next day the capillary is washed (by sequential injection with a 10 mL syringe) with 1 M sodium chloride, organic-free water, and 1 M sodium chloride to remove noncovalently attached antibody. The capillary is cut into 20 cm long pieces. A separate piece of capillary was used for each standard and sample of atrazine. Standards and unknown samples of atrazine with AP-labeled atrazine are prepared in the hapten/AP-labeled hapten incubation buffer. The following steps are performed for each capillary in a timed sequence such that a new capillary is started every minute: 22 μL of an atrazine/AP-labeled atrazine solution is injected into the capillary by syringe and incubated for 20 min. The capillary is rinsed with the substrate development buffer and then cut cut into two 10 cm segments. Each capillary is filled with 22 μL of the 4 mM PAPP substrate solution. After 20 min, the capillary is connected to the loading loop of the six-port injector, and the contents of the capillary, which contain the enzymatic product PAP, are drawn into the loading loop of the injector for the FIAEC system by suction provided by a syringe used in the withdrawing mode. The sample is then injected into the FIAEC, and PAP is detected amperometrically at +300 mV vs Ag/AgCl reference electrode. Quantitation is made by measuring the peak height of the

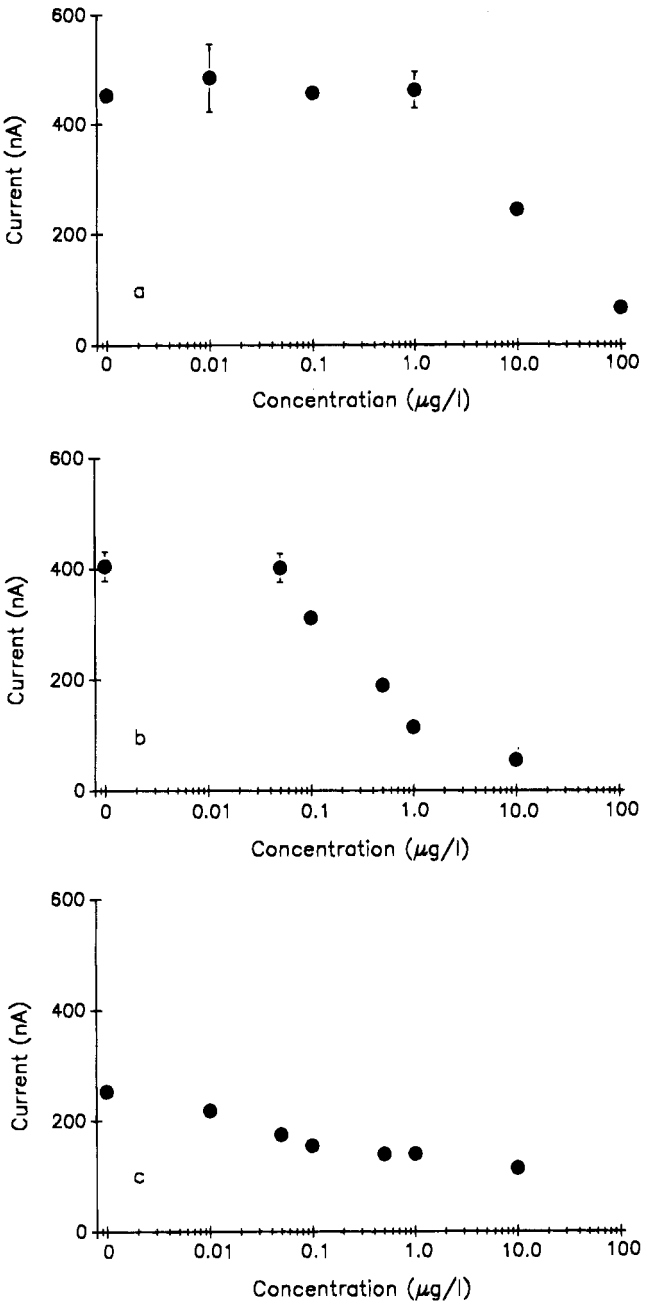

Figure 2. Calibration curves obtained at different antibody concentrations: antibody immobilization overnight (a) 10-fold dilution, (b) 600-fold dilution, (c) 1000-fold dilution; atrazine/AP-labeled atrazine incubation, 10 min, 50000-fold dilution; substrate incubation, 30 min.

FIAEC signal. Except for some steps in coating the capillary, all operations are carried out at room temperature.

The total time required to run a set of standards (duplicate measurements of six concentrations) is less than 1 h, which includes 20 min each for the atrazine/AP-labeled atrazine and substrate incubations, approximately 2 min for washing, and 12 min of offset time for the 1 min interval between starting times for each tube. With increasing number of samples to be analyzed, the assay time required per sample would be decreased.

RESULTS AND DISCUSSION

Optimization. For a competitive binding assay used for hapten analysis, the detection limit is ultimately determined by the binding constant of antibody to hapten. The higher the binding constant, the more hapten is bound by the antibody, and hence the lower the detection limit that is obtained. Because there is a competition between the hapten and the enzyme-labeled hapten for a limited number of antibody binding sites, the amount of the antibody immobilized in the tube and the concentration of enzyme-labeled hapten added to each standard and sample are also important with respect to the assay performance.

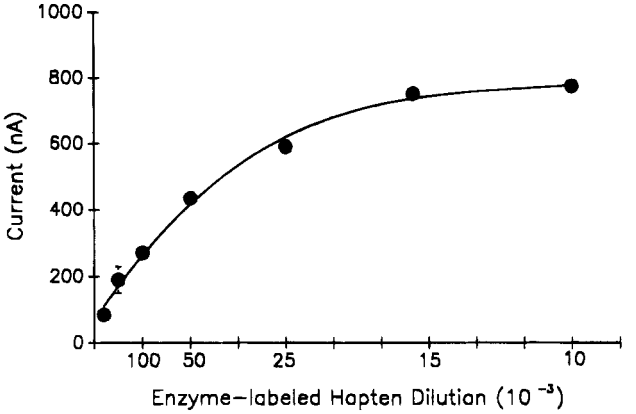

Figure 3. Antibody titration curve: antibody immobilization, 600-fold dilution, overnight; atrazine/AP-labeled atrazine incubation, 10 min; substrate incubation, 30 min.

Amount of Antibody. When the amount of antibody is large, competition between the hapten and the enzyme-labeled hapten occurs only at high concentrations, which could result in a high detection limit. In this sense, the amount of antibody to be used should be as low as possible. On the other hand, the sensitivity of the assay will drop with decreasing amount of antibody. Therefore, the amount of antibody is a trade-off between the sensitivity and the detection limit of the assay. By comparing the calibration curves obtained with a wide range of concentrations of the antibody, we chose a 600-fold dilution of oxidized antibody with the antibody immobilization buffer as the best compromise between sensitivity and detection limit (Figure 2). Because there is a loss of activity of the stored oxidized antibody with time, the antibody concentration was increased to compensate for this loss during the course of experimentation (about 1 year).

Concentration of Enzyme-Labeled Hapten. The concentration of the enzyme-labeled hapten should be compatible with both the optimized amount of antibody and the concentration of hapten present. An excess concentration of the enzyme-labeled hapten would result in an unbalanced competition where little hapten would be bound. When this happens, no calibration curve would be obtained. From the antibody titration curve (Figure 3), a 50000-fold dilution of AP-labeled atrazine with the hapten/enzyme-labeled buffer resulted in close to half-saturation of the antibody binding sites. Consequently, this dilution of the AP-labeled atrazine stock solution was chosen.

Antibody Immobilization. For the antibody immobilization procedure, a maximum signal in the assay was observed for capillaries in which oxidized antibody solution had been sitting for 12 h (Figure 4).

Time Study. Each incubation step was optimized to keep the total assay time to a minimum. A substrate development time of 20 min was chosen because it gave a high signal, and the progress curve up to this point was linear, indicating that the enzyme–substrate reaction was zero kinetic order with respect to the substrate (Figure 5). For the hapten/AP-labeled hapten incubation, 20 min was chosen because it gave a high signal

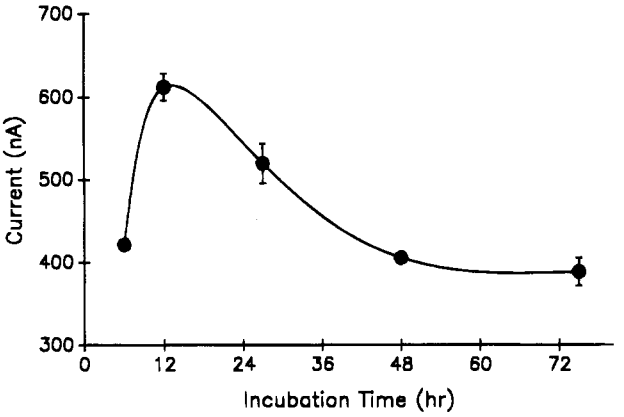

Figure 4. Effect of antibody immobilization time: antibody immobilization, 600-fold dilution; atrazine/AP-labeled atrazine incubation, 50000-fold dilution, 10 min; substrate incubation, 30 min.

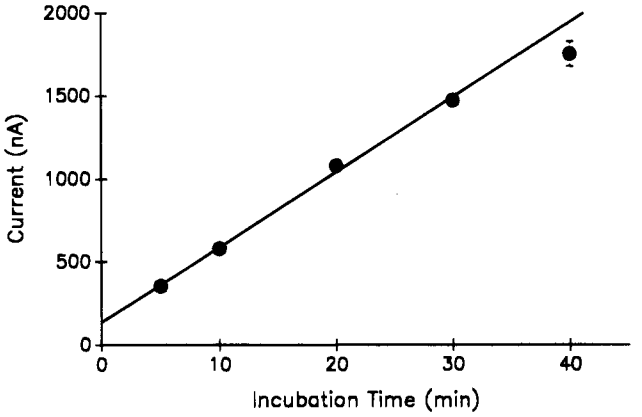

Figure 5. Effect of substrate incubation time: antibody immobilization, 600-fold dilution, overnight; atrazine/AP-labeled atrazine incubation, 50000-fold dilution, 30 min.

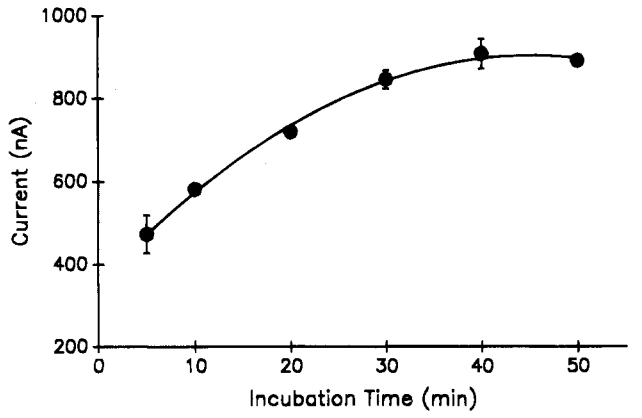

Figure 6. Effect of AP-labeled atrazine incubation time: antibody immobilization, 600-fold dilution, overnight; atrazine/AP-labeled atrazine incubation, 50000-fold dilution; substrate incubation, 20 min.

within a relatively short time (Figure 6). Incubations of longer than 20 min introduced relatively large errors to the measurement.

Calibration Curve. Under the optimized conditions, the calibration curve of atrazine typically had a linear range of 0.10–10.0 μg/L and a detection limit of 0.10 μg/L (Figure 7). A detection limit as low as 0.05 μg/L was reached with some batches of capillary. An assay consisting of six standards could be done in less than 1 h.

Cross-Reactivity. The cross-reactivity of the antibody was scanned with nine triazine compounds (ametryn, prometon, prometryn, propazine, simazine, terbutryn, didealkylatrazine, deethylatrazine, and deisopropylatrazine), of which simazine, propazine, deethylatrazine, and deisopropylatrazine were found to cross-react. The common structure of these four compounds, and atrazine, suggests that the antibody was capable of recognizing most of the triazines that possess a chloro group at position 2. Didealkylatrazine was an exception because of the important structural change in the side groups. It is therefore possible that the antibody could be used to detect a group of triazine compounds containing such a chloro group.

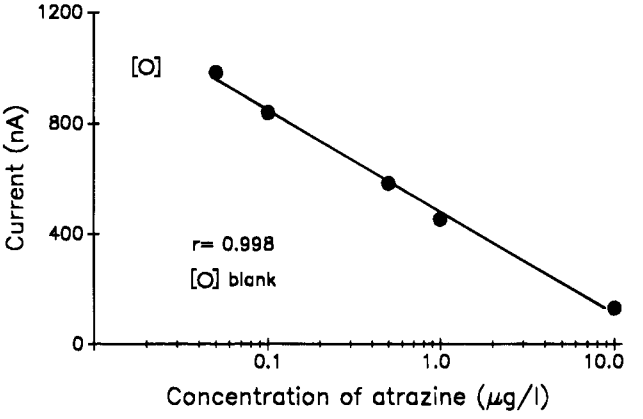

Figure 7. Calibration curve for atrazine: antibody immobilization, 600-fold dilution, overnight; atrazine/AP-labeled atrazine incubation, 50000-fold dilution, 20 min; substrate incubation, 20 min.

Table 1. Precision[a]

	intra-assay		inter-assay	
[atrazine]	0.10 μg/L	5.0 μg/L	0.10 μg/L	5.0 μg/L
mean (i/i_0)	0.90	0.32	0.90	0.33
RSD (%)	9	8	6	10

[a] Antibody immobilization, 200-fold dilution, overnight; atrazine/AP-labeled atrazine incubation, 50000-fold dilution, 20 min; substrate incubation, 20 min; sample number = 10; i, current obtained at different concentrations of atrazine; i_0, current obtained at zero dose of atrazine.

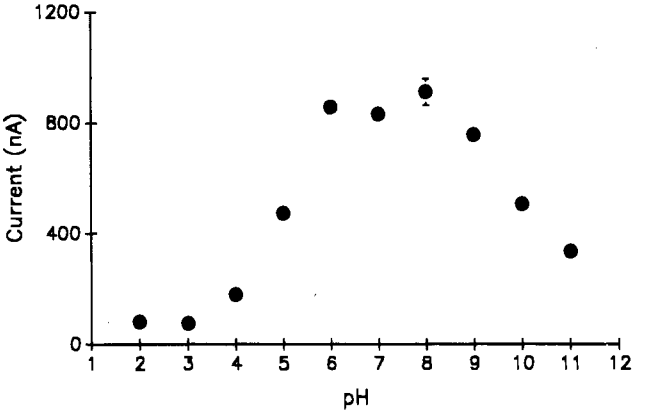

Figure 8. Effect of pH on antibody–atrazine binding: antibody immobilization, 200-fold dilution, overnight; atrazine, 1.0 μg/L, AP-labeled atrazine, 50000-fold dilution; 20 min; pH 2–6, 0.1 M sodium formate, 0.02% sodium azide, 0.05% Tween 20; pH 7–11, 0.1 M tris(hydroxymethyl)aminomethane, 0.02% sodium azide, 0.05% Tween 20; substrate incubation, 20 min.

Validation. To adapt the method to real sample analysis, the assay performance was evaluated with respect to the following characteristics.

Stability. Because the shapes of the calibration curves varied slightly through the experimental period,

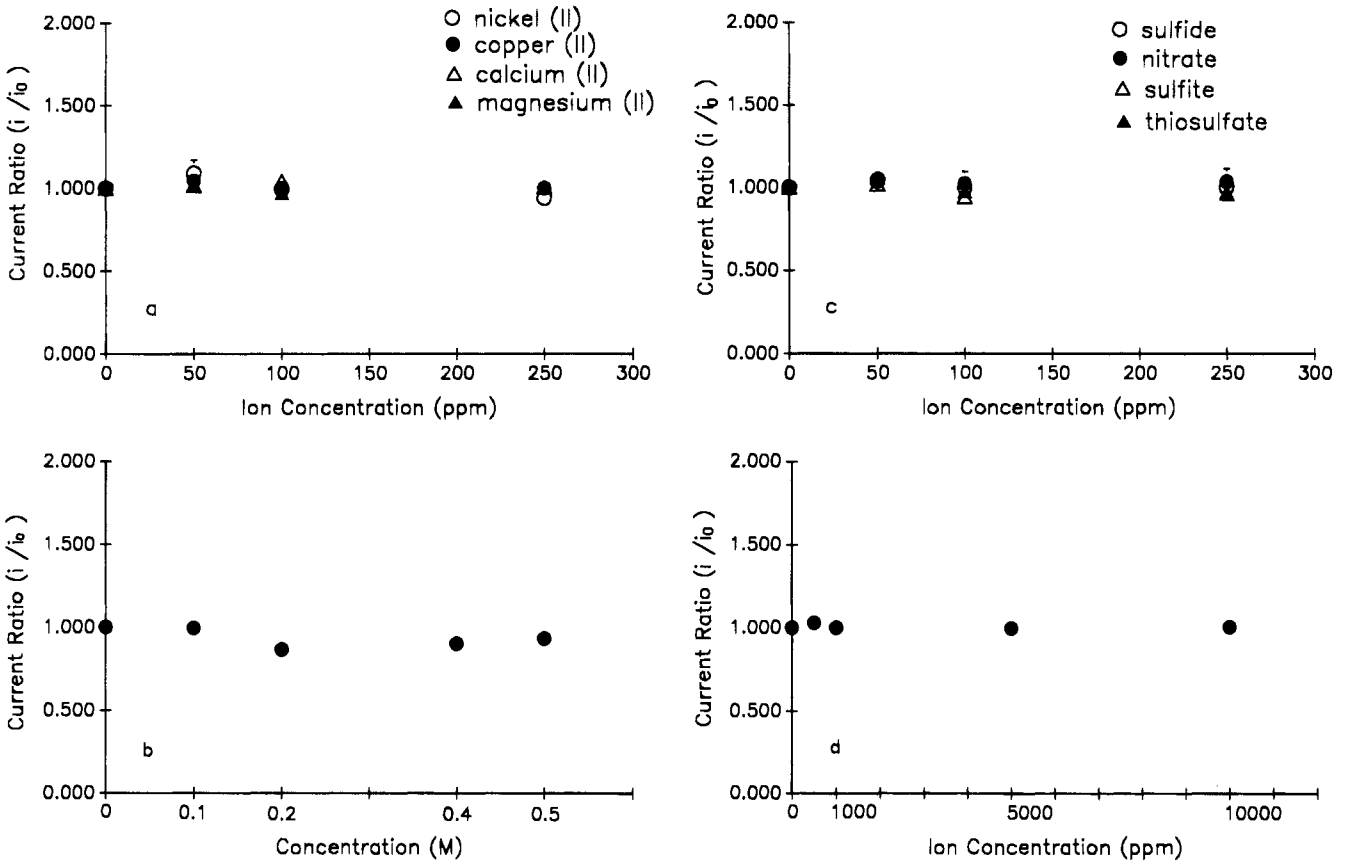

Figure 9. Effect of ions on antibody–atrazine binding: antibody immobilization, 200-fold dilution, overnight; atrazine, 1.0 μg/L, AP-labeled hapten, 50000-fold dilution; 20 min; substrate incubation, 20 min; i, current obtained at different concentrations of ion; i_0, current obtained at zero concentration of ion.

Table 2. Recoveries of Added Atrazine from Various Water Samples[a]

	bottled water		tap water		Ohio River water	
added (μg/L)	5.0	0.50	5.0	0.50	5.0	0.50
measured (μg/L)	5.7	0.53	5.2	0.51	4.9	0.51
recovery (%)	115 ± 20	110 ± 20	104 ± 8	100 ± 20	100 ± 20	100 ± 20

[a] Antibody immobilization, 200-fold dilution, overnight; atrazine/AP-labeled atrazine incubation, 50000-fold dilution, 20 min; substrate incubation, 20 min; sample number = 10.

a daily calibration was used to ensure the reliability of the assay. This lack of robustness was attributed to the fact that different batches of capillary and antibody behave differently.

Precision. The intra-assay and inter-assay precisions were determined on the basis of the analysis of 10 samples (Table 1). The relative standard deviations (RSD) in most cases were below 10%. The intra-assay error for 10 samples run in the same experiment with the same batch of modified capillary and oxidized antibody stems from electrochemical detection, manual operation of the capillary, enzymatic reaction, and degree of homogeneity of the modified capillary surface. Increasing the capillary length or decreasing the enzymatic reaction time decreased the RSD for each by 1%. Since the inter-assay error for samples run on different days with different batches of capillary is approximately the same as the intra-assay error, it appears that daily calibration takes care of batch to batch variations in the capillary and the oxidized antibody.

Matrix Interference. The strength of the antibody–hapten interactions, being fundamentally electrostatic or hydrophobic in character, is dependent on pH and ionic strength, which in real water samples may differ considerably. For example, the pHs of the commercial bottled water, tap water, and Ohio River water were 6.62, 8.02, and 7.40, respectively. The effects of pH and ionic strength on antibody–hapten binding were assessed accordingly.

1. pH. The hapten and enzyme-labeled hapten mixtures were prepared in buffers with pH of 2–11. The signals from the immunoassays were greatest at pH 6–8 (Figure 8), indicating the necessity of adjusting the water sample to pH 7.8 before assay.

2. Ionic Strength. LiCl, NaCl, $MgCl_2$, $CaCl_2$, $CuCl_2$, Na_2S, Na_2SO_4, NaS_2O_4, $NaNO_2$, and $NaNO_3$ were added to hapten and AP-labeled hapten mixtures prepared in hapten/AP-labeled hapten incubation buffer to test for interference. Up to the concentration tested, no significant change in immunoassay signal was observed within the uncertainty of the assay procedure (Figure 9). Since the concentrations used in the tests of each compound exceed those found in most groundwaters (APHA, 1989), removal of these ions from the water sample is unnecessary.

Recovery. The recovery of atrazine added to samples of commercial bottled water, tap water, and Ohio River water was determined by filtering the water samples through a 0.45 μm nylon membrane, adding incubation buffer chemicals for the hapten/AP-labeled hapten incubation buffer, adding AP-labeled atrazine, adding atrazine, and then carrying out the immunoassay

procedure on each sample. Recoveries of the added atrazine from these water samples were tested at both low and high concentrations (Table 2). The recoveries ranged from 100 to 115%.

SUMMARY

This work demonstrates the feasibility of using capillary enzyme immunoassay with electrochemical detection for the determination of triazine compounds. The detection limit achieved is comparable with those obtained by chromatographic methods using no preconcentration. The present method, however, requires only a simple filtration of the sample prior to analysis. Compared to the reported results where a similar antibody and AP-labeled hapten were used (Giersch and Hock, 1990), our method provides a somewhat lower detection limit (0.10 vs 0.5 μg/L) and a faster assay (less than 60 vs 180 min). The amount detected is 2.2 vs 125 pg. Evaluation of the assay performance suggests that it can be readily adapted to the analysis of real samples.

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