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Talanta 65 (2005) 324–330
Talanta
www.elsevier.com/locate/talanta

Comparison of two express immunotechniques with polyelectrolyte carriers, ELISA and FIIAA, for the analysis of atrazine

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Received 28 November 2003; received in revised form 11 February 2004; accepted 14 April 2004
Available online 8 August 2004

Abstract

Conventional immunoassays on microtiterplates are very useful analytical tools in environmental analysis, but the long assay times, usually in the range of hours, are a drawback. To overcome this disadvantage, the development of fast (express) assay formats is described, which use polyelectrolytes as carriers. Two semi-homogeneous immunochemical methods, namely the polyelectrolyte-ELISA (enzyme-linked immunosorbent assay) and the express-FIIAA (flow injection immunoaffinity analysis) for the analysis of the herbicide atrazine were set-up. Using polyclonal antisera for atrazine, the following results were obtained. Standard curves for atrazine showed a linear range from 3 to 100 $\mu g\,l^{-1}$ in polyelectrolyte-ELISA and 0.3–100 $\mu g\,l^{-1}$ in express-FIIAA. The test midpoints in polyelectrolyte-ELISA and express-FIIAA were 12 and 5 $\mu g\,l^{-1}$, respectively. The duration time of the immunochemical reaction was in both assays 15 min, but the total assay time differed (30 min (polyelectrolyte-ELISA) and 18 min (express-FIIAA)). A significant difference between the formats could be observed in the number of samples that can be determined per day. The polyelectrolyte-ELISA can handle samples in parallel on a microtiterplate (usually 20/plate), whereas in the express-FIIAA the samples are automatically analysed one after another. This first demonstration of these techniques shows the potential of these methods, but also their limitations.

Keywords: Atrazine; Polyelectrolytes; ELISA; FIIAA; Screening of water samples

1. Introduction

Immunochemical methods are very powerful tools for environmental analysis, because: (a) they need no or minimum sample preparation, (b) they are very sensitive and selective, (c) they are inexpensive, (d) they are easy to use, and (e) they use only a few microliters of organic solvents, thus causing much less environmentally hazardous waste during analysis [1–3]. One of the drawbacks of conventional immunoassays though is the long assay time, because incubation times are usually in the range of hours. To overcome this disadvantage, the development of fast (express) assay formats is important. Here, the usage of polyelectrolytes proved to be a powerful tool to speed up the assays [4]. This paper shows two semi-homogeneous, fast immunochemical methods, namely the polyelectrolyte-ELISA (enzyme-linked immunosorbent assay), which has been described earlier [5], and the new express-FIIAA (flow injection immunoaffinity analysis), which was transformed from the formerly developed FIIAA [6]. A comparison of both methods is shown and the different features are discussed. As an example, the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine ($C_8H_{14}ClN_5$)) was

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doi:10.1016/j.talanta.2004.07.010

chosen. Although there are restrictions put on the usage of atrazine within the EU since the early 1990s, this herbicide is worldwide still very important, and in addition is on the list of pesticides of endocrine disruptors and possible carcinogens [7]. Its major applications are in maize cultivations and also in total weed control on non-crop land, such as railways and roadsides [8]. In addition, atrazine is a pervasive environmental contaminant. It is strongly persistent and is one of the most significant water pollutants in different waters [8–10]. Therefore fast screening of different water sources for residues of this pesticide is important.

Nowadays, pesticide analysis is mainly performed by conventional methods, such as LC and/or GC. Although these methods give excellent and quantitative analytical results, their drawbacks are the need of sample preparation, the usage of organic solvents, and the requirement of expensive equipment. Fortunately, pesticide residues are exceeding the maximum admissible concentrations only in a minority of water samples in many European countries. Therefore, it would be very appropriate to use environmentally friendly methods, such as immunochemical methods, for routine screening.

2. Experimental

2.1. Reagents

Poly(methacrylic) acid (PMA), M_w 260 kDa, referred to as polyanion, and poly(*N*-ethyl-4-vinylpyridinium) bromide (PEVP), M_w 4400 kDa, referred to as polycation, were synthesized in-house [5]. Material for FIIAA column was Toyopearl AF-Tresyl-650M (TOSOH BIOSEP GmbH, Stuttgart, Germany), bead diameter: 40–90 μm. Microtiterplates used were NUNC MaxiSorp (NUNC GmbH, Wiesbaden, Germany). Protein A, Tris–HCl buffer, pH 8.0, Triton X-100, sodium azide, *N*-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiiimide (DCC), anhydrous dimethylformamide (DMF), 3-(*p*-hydroxyphenyl) propionic acid (HPPA), H_2O_2, and 3,3′,5,5′-tetramethyl benzidine (TMB) were purchased from Sigma–Aldrich, Taufkirchen, Germany. Micr-O-protect (Boehringer Mannheim, now available from Roche Diagnostics GmbH, Penzberg, Germany) was used in solutions during storage of beads with Protein A. NaCl, K_2HPO_4, KH_2PO_4, sodium citrate were from Merck, Darmstadt, Germany. PBS buffer was 0.05 M potassium phosphate buffer, including 0.1 M NaCl, pH 7.5. PBST buffer was 0.05 M potassium phosphate buffer with 0.05% (v/v) Triton X-100. The 0.1 M sodium citrate buffer, pH 3.6, including 0.02% sodium azide, was used for regeneration of the Protein A column in express-FIIAA. Anti-atrazine antiserum and atrazine-HRP were in-house developments [5].

2.2. Conjugations of Protein A to poly(methacrylic) acid and polymethacrylate beads

Protein A was covalently conjugated to polymethacrylic acid using an *N*-hydroxysuccinimide activated ester [11].

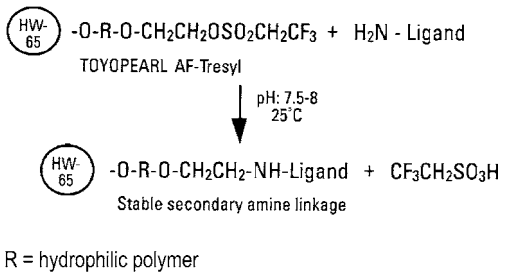

Fig. 1. Conjugation of Protein A to Toyopearl AF-Tresyl-650M (ligand density: 80 μmol/g (dry)). According to TOSOH BIOSEP company (catalogue, p. 161).

Briefly, 10 mg of PMA (polyanion, M_w 260 kDa), 1.03 mg of *N*-hydroxysuccinimide, and 1.97 mg of 1,3-dicyclohexylcarbodiimide were dissolved in 200 μl DMF. After 2 h activation at RT, this solution was centrifuged (10 min, 1500 rpm, Heraeus Sepatech Biofuge 15, Heraeus, Hanau, Germany), and the active ester solution was slowly added to Protein A (2 mg ml^{-1} in 200 mM PBS buffer). The solution was stirred further at 4 °C (refrigerator) over night. The next day, the solution was dialyzed (Slide-A-Lyzer® cassettes, Perbio Science Deutschland GmbH, Bonn, Germany) against 200 mM PBS. After the dialysis, the solution was ready to use and stored at 4 °C. Protein A was also covalently conjugated to Toyopearl AF-Tresyl-650M beads according to the description of the company (Fig. 1). 0.8 mg of Protein A was dissolved in 1 ml 0.05 M Tris–HCl buffer, pH 8, and pipetted to 100 mg of bead material. The solution was incubated for 2 h at 25 °C. After the conjugation, the bead material was washed with 0.1 M Tris–HCl, pH 8.5, and incubated for 1 h with 0.1 M Tris–HCl. After another final washing step with 0.1 M Tris–HCl, the beads were put in 0.04 M PBS, pH 7.5, with 0.5% (v/v) Micr-O-protect, and stored at 4 °C (refrigerator).

2.3. Polyelectrolyte-ELISA

The principle of the semi-homogeneous polyelectrolyte-ELISA was described earlier [4,5], and was slightly modified as follows. The 50 μl polyanion–Protein A conjugate (3 μg ml^{-1}), 50 μl standard (or water sample), 50 μl enzyme-tracer (atrazine-HRP, Lot 13/5, 1:14,000), and 50 μl anti-atrazine antiserum (Lot 25/6-3, 1:14,000) were all set-up in 0.05 M PBST, pH 7.5, mixed together on a microtiterplate, and incubated for 5 min (with shaking). The 100 μl of this mixture were then transferred to a second plate and incubated for 8–10 min. The latter was coated with polycation (optimised concentration: 5 μg ml^{-1} in 0.05 M PBS, pH 7.5; over night, 4 °C) and washed before. Standard curves in polyelectrolyte-ELISAs for atrazine used standards ranging from zero (0.04 M PBST buffer, pH 7.5) to 10,000 μg l^{-1}. The 100 μl of substrate for the enzyme HRP were added (H_2O_2 (1.3 mM) with the chromogen TMB (0.4 mM)). Reaction was stopped with 50 μl 2 M H_2SO_4, and absorbance was measured at 450 nm (reference 650 nm), Thermomax ELISA-Reader (Molecular Devices, Palo Alto, CA, USA) (Fig. 2).

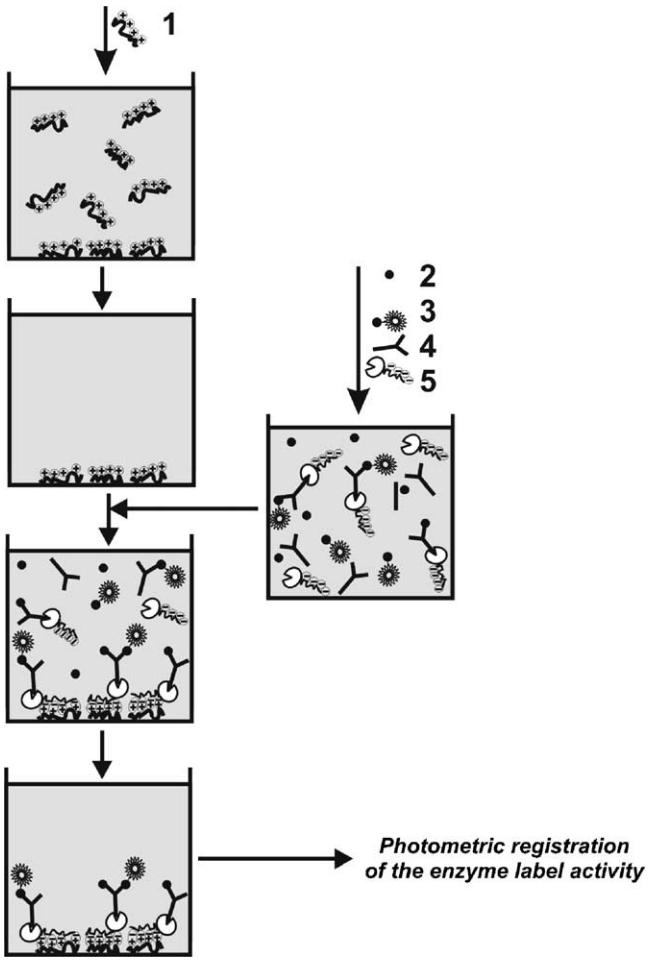

Fig. 2. Polyelectrolyte-ELISA: scheme of a semi-homogeneous polyelectrolyte immunoassay on a microtiterplate: (1) polycation; (2) analyte; (3) enzyme-tracer; (4) antibody; (5) polyanion–Protein A conjugate (for details see text).

2.4. Express-FIIAA

Solutions of atrazine standard (2 ml), enzyme-tracer (2 ml; atrazine-HRP, Lot 13/5, 1:50,000, in 0.05 M PBS, pH 7.5) and anti-atrazine antiserum (2 ml; Lot 25/6-3, 1:50,000, in 0.05 M PBS, pH 7.5) were mixed in glass vials and then incubated for at least 10 min before the measurement with the express-FIIAA was started. For stability reasons, these mixtures were kept at about 4 °C. Atrazine standards were set-up from 0.1 to 10,000 $\mu g\,l^{-1}$ (in 0.05 M PBS, pH 7.5). In the case of zero concentration 10 ml of 0.05 M PBS, pH 7.5, were used, together with 10 ml of enzyme-tracer and 10 ml of antiserum, respectively. The 250 μl of these mixtures (with either zero standard or standard) were pumped onto the affinity column, which consisted of polymethacrylate beads (Fig. 3).

Protein A was covalently conjugated to this column material (see Section 2.2).

Washing buffer in express-FIIAA was 0.05 M PBS or PBST, pH 7.5. Flow rate was 1 ml min^{-1}. For the enzyme reaction, 200 μl substrate H_2O_2 (2 mM) together with the fluorogen HPPA (5 mM) in 0.05 M PBS, pH 7.5, was used. Fluorescence of the product was determined in a flow through cell of a fluorimeter (λ_{ex} 320 nm; λ_{em} 417 nm; Hitachi F1000, Merck/Hitachi, Darmstadt, Germany) and recorded in mV. In the final step, the polymethacrylate–Protein A column was regenerated with 0.1 M sodium citrate buffer, pH 3.6, and the next assay was started.

2.5. Water samples

Water samples from different origins were taken and analysed by both methods. Water samples A and B are surface waters, drawn in February 2003 from streams in Munich, Germany. Other samples were provided by CSIC, Barcelona, Spain (M. Petrovic, D. Barceló) and were influents or effluents of waste water treatment plants (WWTP). Conductivity and pH of all water samples were determined, and the samples were taken without further treatment. For spiking, samples were diluted 1:2 with PBS (one part water sample plus one part PBS containing the given concentration of atrazine). For these measurements, also the standards for the standard curves had been set-up in one part PBS plus one part PBS with the corresponding atrazine standard.

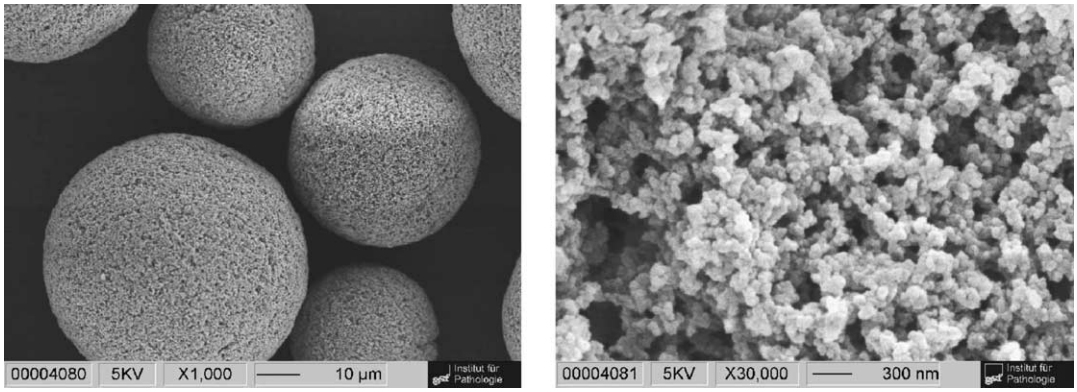

Fig. 3. Scanning electron microscope presentation of the TOSOH polymethacrylate beads (Toyopearl AF-Tresyl-650M (left); TOSOH BIOSEP GmbH, Stuttgart, Germany) and their surface (right). Left: 1000×; right: 30,000×.

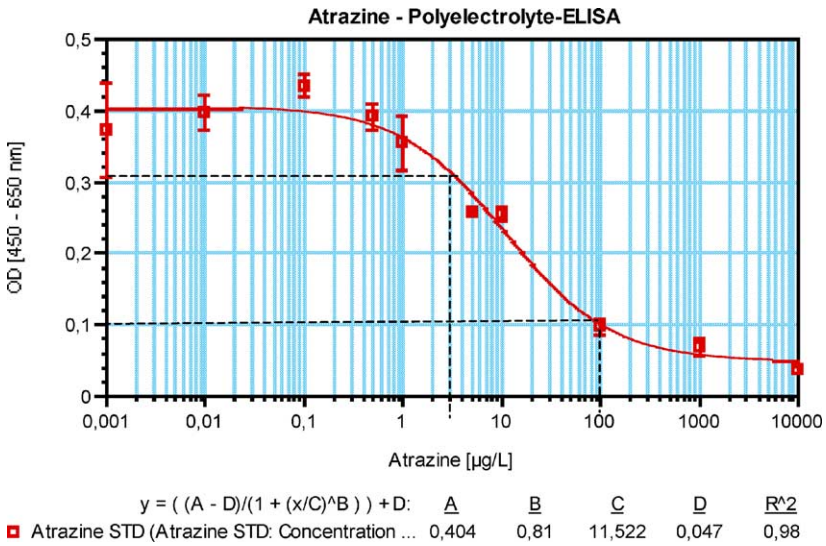

Fig. 4. Polyelectrolyte-ELISA: standard curve for atrazine. Zero (=buffer) in this graph is identical with 0.001 $\mu g\,l^{-1}$ (logarithmic scale). The linear range was from 3 to 100 $\mu g\,l^{-1}$ (which refers to 80 and 20% of zero signal). Medium percentage standard deviation of standards: 9.1 (n = 3).

3. Results and discussion

Using the polyelectrolyte material described earlier [4,5], a standard curve for atrazine with the semi-homogeneous polyelectrolyte-ELISA was carried out. With this polyclonal antiserum and an atrazine-HRP as enzyme-tracer [5], the linear range of the standard curve ranged from 3 to 100 $\mu g\,l^{-1}$ with a test midpoint of about 12 $\mu g\,l^{-1}$ (Fig. 4).

These reagents were transferred to the newly developed express immunotechnique, named express-FIIAA, which is based on the formerly demonstrated FIIAA [6]. This is an automated assay, therefore it is necessary to regenerate the surface material located in the column. A search was performed for the best suitable solid polymer, to which Protein A (or G) could be immobilized. After a search and check of several surface materials (e.g., Biosilon, Nunc, 220 µm; UltraLink™ Protein A/G, Pierce), the best material appeared to be the Toyo-pearl AF-Tresyl-650M material (TOSOH BIOSEP), which consists of polymethacrylate beads in the range of 40–90 µm (Fig. 3). The covalent immobilization of Protein A (or G) was done according to a protocol provided by the company (Fig. 1). This enabled the regeneration of the Protein A immunoaffinity column material after each assay, which was executed several hundred times.

Prior to each measurement of standard or sample, a zero control value was measured, which was PBS buffer. This zero value (maximum signal) was stable over time and showed a standard deviation of 9.7% (n = 28; Fig. 5). A background value, which was determined without antibody, but with the addition of enzyme-tracer, was also determined and was on average 18% of the maximum signal (Fig. 5). If this background was subtracted from the mV values of the standards, the final %control values were slightly negative when high concentrations of atrazine standards were determined (for example, see Fig. 6). The basis of this was that the mV values of the high standard concentrations were lower than the background mV values. An explanation for this is that there is a slightly higher unspecific binding of the enzyme-tracer to the column, when no antibody is present. These background values were therefore not always subtracted (e.g., Fig. 7).

An additional requirement of this automated analysis was that the results with mixtures of standards, enzyme-tracer and antiserum did not change over time. A standard curve with three replicates was carried out, which took about 24 h (Fig. 6). On each column of this figure the time is written at which the measurement of the corresponding standard was done. No major changes in the %control values were observed (detailed numbers, see Fig. 6).

After all optimisations, the standard curve for atrazine in express-FIIAA showed a linear range from 0.3 to 100 $\mu g\,l^{-1}$ and a test midpoint (50 %control) of ca. 5 $\mu g\,l^{-1}$ (Fig. 7).

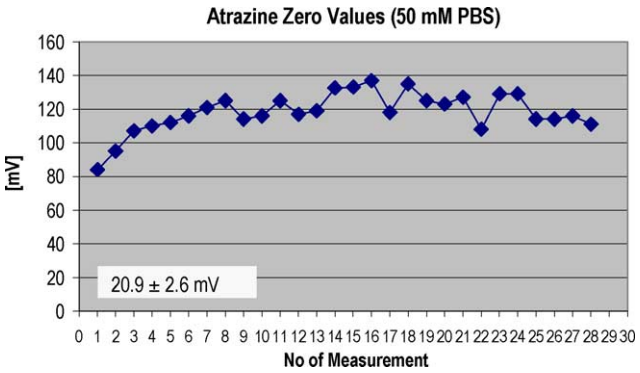

Fig. 5. Express-FIIAA: zero measurements (PBS buffer). Average is 118.3 ± 11.5 mV (S.D.: 9.7%; n = 28). Background value: 20.9 ± 2.6 mV (S.D.: 12.4%; n = 14). Anti-atrazine AS, Lot 25/6-3, 1:50,000 in 0.05 M PBS; atrazine-HRP, Lot 13/5, 1:50,000 in 0.05 M PBS.

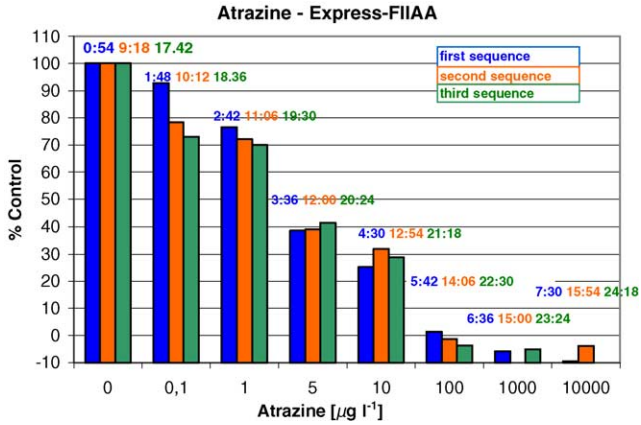

Fig. 6. Express-FIIAA: standard curve for atrazine (three sequences). The time above each column shows the time of the measurement. Anti-atrazine antiserum, Lot 25/6-3, 1:50,000 in 50 mM PBS, pH 7.5. Atrazine-HRP, Lot 13/5, 1:50,000 in 50 mM PBS, pH 7.5. Zero value (PBS) is 134 ± 7.5 mV (=5.6% standard deviation). %Control values (average ± S.D.) are as follows: 0.1 µg l^{-1}: 77.3 ± 9.8; 1 µg l^{-1}: 72.9 ± 2.8; 5 µg l^{-1}: 39.8 ± 1.2; 10 µg l^{-1}: 28.7 ± 2.7; 100 µg l^{-1}: −1.2 ± 2.1; 1000 µg l^{-1}: −7.0 ± 2.2; 10,000 µg l^{-1}: −7.9 ± 3.0. Minus values for the higher concentrations were observed, when a background value (incubation of enzyme-tracer without antibody) was subtracted.

In order to demonstrate advantages and limitation of these express immunotechniques, a few water samples were drawn and analysed in parallel by both methods (Tables 1 and 2).

As shown in the tables, the variation within the pH of the samples was not much, but a clear difference in conductivity was determined. The latter varied by a factor of 4 between the samples.

The samples were analysed with both methods in a different manner. In polyelectrolyte-ELISA, they were measured against the standard curve in buffer. None of the samples contained atrazine in the range of quantitation of the standard

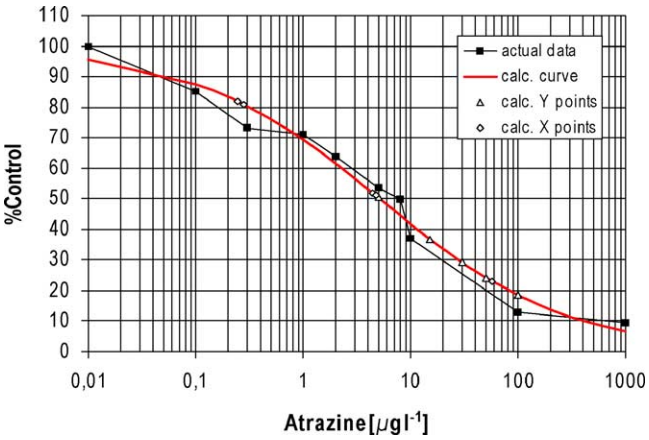

Fig. 7. Express-FIIAA: standard curve for atrazine. Original data and curve fitting (four-parameter values of the standard curve: A = 99.8; B = 0.5; C = 5.2 µg l^{-1}; D = 0.0. Calculation of curve fitting provided by R.O. Harrison, Cape Technologies, MA, USA. Zero (=buffer) in this graph is identical with 0.01 µg l^{-1} (logarithmic scale). Considering the %S.D. of the standard measurement, the linear range was set at 20–80 %control, which corresponds to 0.3–100 µg l^{-1}.

Table 1
Measurement of samples for atrazine with polyelectrolyte-ELISA

Sample, source of water	pH	Conductivity (µS cm^{-1})	Polyelectrolyte-ELISA atrazine (µg l^{-1})
Water A, surface water, Munich	7.35	375	Range[a]
Water B, surface water, Munich	7.64	344	0.05[a]
P-3, WWTP influent	7.69	1286	0.9[a]
P-5, WWTP effluent	7.60	1601	0.1[a]

WWTP: waste water treatment plant.
[a] Out of range of quantitation.

curve (Table 1). This corresponded with the result obtained by the reference analysis. The latter showed that atrazine was below the limit of detection, which was 0.05 µg l^{-1} (M. Petrovic, personal communication).

Using express-FIIAA, these water samples were determined in another way. First, they were measured against the zero standard (PBS) and expressed as %control. Here, sample P-5 lowered significantly the signal, which—as a result—would be judged as a positive sample (Fig. 8). Taking the reference analysis into account, this would then be a false positive result.

In addition, we set the measurement signal [mV], generated with the water sample, as 100% control, and then expressed the spiked concentrations as %control in relation to the original sample (Table 2). It is seen that the same amount spiked does not correspond to the same decrease in %control. On the other hand, though, increasing spiked concentrations always show a decrease in signal. Some authors show the same result in running standard curves in the matrix, which results in a shift of the standard curve to the right or also lowering the signal (e.g., [12,13]).

This significant influence of the matrix, particularly in sample P-5 (WWTP effluent), which contained no atrazine by reference analysis, is also seen in Fig. 8.

In principle, this difference in results can be caused by two reasons: (1) atrazine is either present in the sample at a concentration >10 µg l^{-1}, therefore the addition of atrazine in

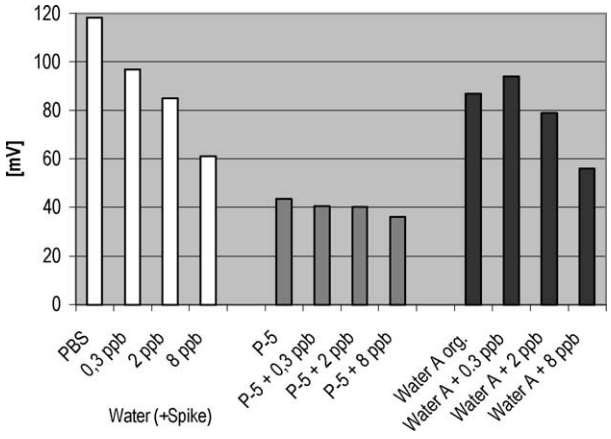

Fig. 8. Express-FIIAA for atrazine: screening of water samples. Expression of results (fluorescence signal) as mV. For details, see text.

Table 2
Measurement of samples for atrazine with express-FIIAA

Sample, source of water	pH	Conductivity ($\mu S\,cm^{-1}$)	Express-FIIAA, atrazine spike ($\mu g\,l^{-1}$)	Express-FIIAA, atrazine (%control)
Water A, surface water, Munich	7.35	375	Water sample, +0.3, +2, +8	100, 108, 91, 67
Water B, surface water, Munich	7.64	344	Water sample, +3.3, +16.7, +100	100, 83, 51, 37
P-3, WWTP influent	7.69	1286	Water sample, +2	100, 70
P-5, WWTP effluent	7.60	1601	Water sample, +0.3, +2, +8	100, 92, 91, 82

WWTP: waste water treatment plant.

Table 3
Comparison of features: polyelectrolyte-ELISA and express-FIIAA

Feature	Polyelectrolyte-ELISA	Express-FIIAA
Support	Water-soluble polymers	Solid polymer beads
Separation step	Polyanion–polycation reaction	Binding of Protein A (or G) to Fc of ab
Preincubation	Preincubation of polyanion–Protein A (or G), analyte, enzyme-tracer and ab: 5 min (shaking)	Preincubation of analyte, enzyme-tracer and ab: 10 min to 24 h
Reactions	One-time reaction	Regeneration of Protein A (or G)
Assay time (min)	30	18
Immunochemical part (including separation step) (min)	15	15
Enzymatic reaction	15 min	1 min incubation/2 min detection
Parallel measurement	Yes	No
Analysis time for 20 samples	About 1 h	About 20 h (including measurement of standards and zero control values before each measurement)
Automated	No	Yes

concentrations of 0.3, 2 and 8 $\mu g\,l^{-1}$ shows only little reduction in %control values, and/or (2) the water matrix (including other interfering substances) is the cause of the reduction in %control (%control values, see Table 2). Comparing sample P-5 and water A, one can clearly see that the latter had a much lower interference within express-FIIAA.

The results demonstrate that this technique gives only qualitative results, especially when waste water samples are screened. This matrix has a lot of interfering substances, which can have an influence either on the antibody-hapten reaction, on the enzyme reaction, or might even interfere with Protein A. Bjarnason et al. [14] observed a similar phenomenon on Protein G.

Using complex matrices, immunochemical techniques are only useful as screening methods, when at least one of the following prerequisites is fulfilled: (1) availability of uncontaminated matrix, (2) analysis of water samples against standards in comparable matrix, (3) possibility of dilution, or (4) information about the analyte distribution by reference analysis before screening.

Other authors used, for example the dilution of samples [12], or got good recovery of analytes from a difficult matrix, when synthetic waste water was used [15].

Comparing polyelectrolyte-ELISA and express-FIIAA, some differences in features should be pointed out and are summarized in Table 3.

Besides the polyelectrolytes, there are other variations, e.g. in the separation step, the automation of the assay, and the times for analysis. The assay times in both formats are shorter than in the corresponding ELISA or FIIAA, ranging from 30 min (polyelectrolyte-ELISA) to about 18 min (express-FIIAA). On the one hand, this seems to be favourable for the express-FIIAA, but on the other hand, here samples have to be run successively, thus increasing the analysis time when many samples have to be screened. In addition, we always ran a zero standard prior to the standard or samples, which also increased the time per standard/sample.

The number of samples in polyelectrolyte-ELISA is up to 20 samples in parallel on one microtiterplate, whereas in express-FIIAA the time to run 20 samples is in the range of 20 h.

Generally, automation is a plus, because samples can also be run during the night and weekends.

4. Conclusions

Although immunochemical screening might not be suitable for all kinds of water matrices, it could be useful for some. Speed of analysis, together with cost effectiveness and environmental safety could provide a higher frequency of sample measurements. The methods should always be conditioned and optimised to the water matrix of interest. Changes in signal obtained with the characterized matrix would then imply a closer look and eventually a detailed examination with reference analysis.

A clear expansion in the immunochemical analysis in express-FIIAA could be the use of a two columns combination: first an immunoaffinity column for selective binding of the analyte of interest with low affinity antibodies, thus excluding the matrix, and second an analytical column with

high affinity antibodies for the same analyte. This combination of columns could be easily put into automated systems. Similar set-ups were already demonstrated for conventional analysis [16].

Acknowledgements

This project was funded in part by INCO-Copernicus grant ICA2-CT-2001-10007 (EXPRESS-IMMUNOTECH). The authors thank Ms. Cristina-M. Weber, IEC, GSF, Germany, for her help in optimisation of the ELISA. Dr. Mira Petrovic and Prof. Damia Barceló, CSIC, Barcelona, Spain, are acknowledged for proving some of the water samples and reference analysis.

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