Anal. Chem. **1999,** *71,* 3538−3543

Immunoassay Techniques for Detection of the Herbicide Simazine Based on Use of Oppositely Charged Water-Soluble Polyelectrolytes

Elena V. Yazynina,[†] Anatoliy V. Zherdev,*[,†] Boris B. Dzantiev,[†] Vladimir A. Izumrudov,[‡] Shirley J. Gee,[§] and Bruce D. Hammock[§]

Immunobiochemistry Laboratory, Institute of Biochemistry, Russian Academy of Sciences, Leninsky prospect 33, 117071 Moscow, Russia, Department of Chemistry, Moscow State University, Vorob'evi gori V-234, 119899 Moscow, Russia, and Department of Entomology, University of California, Davis, California 95616

Linear water-soluble polyelectrolytes, i.e., poly(methacrylate) polyanion and poly(*N*-ethyl-4-vinylpyridinium) polycation, were used as carriers for the reactants in immunoassay. The strength of ionic forces through distance and the cooperative binding of oppositely charged chains, the carriers interact with each other at an extremely high rate and affinity. These properties of the polyelectrolytes made it possible to carry out the immunochemical steps of the assay in true solution and then to quickly separate the resulting products from the reaction mixtures. The above approach was applied to an assay for the herbicide simazine. Both enzyme-linked immunosorbent assay (ELISA) and dot blot formats of the immunoassay were evaluated. In the ELISA format, the polycation was adsorbed on the surface of a microtiter plate. A tracer antigen (simazine) was allowed to interact in solution with components of the reaction mixture containing simazine−peroxidase conjugate, specific antibodies, and staphylococcal protein A conjugated with the polyanion, and then the mixture was added to the immobilized polycation. Quick separation of the immunoreactants was achieved due to formation of interpolyelectrolyte complexes between polycation and polyanion molecules. After washing, the microplate wells were filled with a solution of substrate, and the optical density of the reaction products was measured. In the second format, a solution of the same reaction mixture (after incubation) was filtered through a porous membrane, with the polycation adsorbed. The subsequent addition of substrate led to the development of colored spots. Sensitivity of the dot blot format was close to that of the traditional ELISA format using the same reactants, i.e., 0.5 ng/mL. However, the assay was much faster (assay time decreased from 100−120 to 45 min). Sensitivities of the dot immunoassay were 1 ng/mL for densitometric detection and 10 ng/mL for visual detection with a duration of 20 min. The techniques developed here were used for simazine determination in water, milk, and juices.

Currently, immunoassay techniques are widely applied for detection of compounds having different chemical and biological characteristics.[1,2] ELISA (enzyme-linked immunosorbent assay) and other traditional immunoassay formats are based on the interaction between immobilized and solved components of the immune reaction. This process demands relatively prolonged incubation time in order to reach chemical equilibrium,[3] and therefore, the assay cannot be very rapid. Extremely sensitive assays dependent upon very low concentrations of high-affinity antibodies are very slow to reach equilibrium. Nonequilibrium methods do not lend themselves to high-throughput assays and the current techniques used for ELISA. Homogeneous immunoassays such as EMIT can be very rapid; however, complex procedures may be needed to ensure that the activity of an enzyme or other tracer is modified reproducibly upon binding to antibody.[3,4] As a rule, the sensitivities of these homogeneous formats is not as high as those reported from ELISAs. Therefore, techniques that allow the immune interactions to occur rapidly in homogeneous solution, followed by rapid heterogeneous separation of the reactants, could offer the advantages of both techniques.

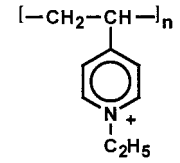
Different approaches have been proposed to solve this task.[5,6] Among them are immobilization of immunoreactants on carriers with temperature-dependent solubility, use of finely ground suspended particles, precipitation of antibody-contained complexes by poly(ethylene glycol), etc. However, such assays can only be conducted in combination with additional devices for separation of the insoluble phase. Therefore, the sphere of their application is highly limited.

In the present work, we propose new immunoassay systems based on the formation of a polycation−polyanion pair, i.e., poly(*N*-ethyl-4-vinylpyridinium) polycation and polymethacrylate polyanion (Figure 1). These linear, water-soluble polyelectrolytes were used as carriers for immobilization of immunoreactants. Coopera-

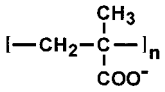
* Corresponding author. (fax) 7(095) 954-2732. (e-mail) bdzan@glasnet.ru.
[†] Russian Academy of Sciences.
[‡] Moscow State University.
[§] University of California.

(1) Tijssen, P. *Practice and Theory of Enzyme Immunoassay*; Elsevier: New York, 1985.
(2) Wild, D., Ed. *The Immunoassay Handbook*; Stockton Press: New York, 1994.
(3) Sciutto, E.; Garat, B.; Ortega, A.; Larralde, C. *Mol. Immunol.* **1987**, *24*, 577−85.
(4) Ngo, T.; Lenhoff, H. M. *FEBS Lett.* **1980**, *116*, 285−8.
(5) Ngo, T.; Lenhoff, H. M. *Enzyme-Mediated Immunoassay*; Plenum Press: New York, 1985.
(6) Auditore-Hargreaves, K.; Houghton, R. L.; Monji, N.; Priest, J. H.; Hoffman, A. S.; Novinski, R. C. *Clin. Chem.* **1987**, *33*, 1509−16.

10.1021/ac990072c CCC: $18.00
Published on Web 07/10/1999



Poly-N-ethyl-4-vinylpyridinium



Polymethacrylate

Figure 1. Structural formulas of the polyelectrolytes used.

tive interaction between the resulting conjugates was driven by the high density of opposite charges. The extremely high rate of interaction was facilitated by having both antigen and analyte in true solution. Ionic interactions are very rapid, and the interpolyelectrolyte complexes provided abrupt and complete separation of the components. Changing the ethyl derivative of poly-(4-vinylpyridine), in this pair, to polymers with longer alkyl radicals (propyl, butyl, isopropyl, and so on) is not reasonable, since it leads to an increase in hydrophobicity and an increase in the distance between ion-pairs and, hence, weakening of electrostatic interaction. Clearly, however, a variety of polyanion and -cation structures can be used in these formats.

In previous work,[7,8] we described techniques for preparation of polyelectrolyte−protein conjugates and developed a system for detection of antigens based on separation of the interpolyelectrolyte complexes by centrifugation. In this work, the polyelectrolytes were applied in both modified ELISA (quantitative test) and dot blot immunoassay (qualitative test) formats. The proposed techniques do not need additional equipment for assays, and they result in a pronounced reduction in the time of the assay.

In this study, the well-known herbicide simazine (2-chloro-4,6-di(*N*-ethylamino)-1,3,5-triazine) is used as a model antigen. This choice was based on the large number of pesticide analyses performed for this compound for environmental monitoring and food quality control.[9,10] Recently we developed a traditional ELISA for simazine detection.[11] This facilitates comparison of the standard ELISA and polyelectrolyte assay techniques on the basis of the same immunoreactants (especially comparison of duration and sensitivity for these assays).

EXPERIMENTAL SECTION

Materials. 1-Cyclohexyl-3(2-morpholinoethyl)carbodiimide (Cal-Biochem), 2,4,6-trinitrobenzensulfonic acid (Chemapol), 3,3′-diaminobenzidine tetrahydrochloride, *N*-hydroxysuccinimide, sodium dodecyl sulfate (all from Sigma), poly(ethylene glycol) (M_w, 6000), Tween-20, Triton X-100, dimethylformamide, ethylenediaminetetraacetic acid (all from Serva), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Boehringer Mannheim), and protein A from Staphylococcus aureus (Vostok, Novosibirsk, Russia) were used in the experiments.

Purified preparations of simazine and its 2-ethylcarboxy and 2-propylcarboxy derivatives were generously provided by Dr. S. A. Eremin, Moscow State University, Russia.

Bovine serum albumin (BSA, Minsk Institute of Epidemiology and Microbiology, Belorussia), hemocyanin from *Paralithodes camtschatica* (HC, generously provided by Dr. I. Yu. Sakharov, Moscow State University, Russia), soybean trypsin inhibitor (STI, Reanal), ovalbumin (OA, Serva), and horseradish peroxidase (HRP, RZ = A_{403}/A_{280} = 3.0, Biolar, Latvia) were used for syntheses of protein−pesticide conjugates.

The ELISA was carried out in Dynatech and Costar optically transparent polystyrene microplates. Nitrocellulose membranes Hybond-N (Amersham, pore size 0.45 μm) were used in dot blot immunofiltration assays.

Methods. *Preparation of Water-Soluble Polyelectrolytes.* Poly(methacrylic acid) (PMA) and poly-(4-vinylpyridine) (PVP) were synthesized and fractionated according to previously described techniques.[12,13] PMA was prepared by radical polymerization of methacrylic acid (Fluka) in benzene at 60 °C under a nitrogen atmosphere using 2,2′-azobisisobutyronitrile as the polymerization initiator. PVP was prepared by radical polymerization of vinylpyridine (Fluka) in methanol at 60 °C in a nitrogen atmosphere using 2,2′-azobisisobutyronitrile as the polymerization initiator. Samples of PMA and PVP were fractionated with ethyl acetate−methanol. Weight-average molecular weight, M_w, measured by light-scattering, was determined in methanol at 25 °C. A narrow ethyl acetate−methanol fraction of PMA with an average molecular mass of 260 kDa was used. The PVP fraction (M_w = 1000) was quaternized exhaustively by ethyl bromide to prepare poly(*N*-ethyl-4-vinylpyridinium) bromide (PEVP). The quaternization degree was more than 90% according to spectral data.[7] The PEVP preparation with M_w = 2000 was used.

Differences of the polyelectrolyte molecules by polymerization degree were characterized by their value of M_w/M_n, where M_n was a number-average molecular mass. This ratio was equal to 1.2, 1.3, and 1.3 for PMA, PVP, and PEVP, respectively.

Conjugation of PMA with Protein A.[14] 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (6 mg) was added to 1 mL of PMA sodium salt solution (6 mg/mL), and the pH was adjusted from 5.0 to 7.7−7.8 by addition of 1 M KOH. The mixture was incubated for 5 min under rigorous stirring at room temperature; then a solution of 6 mg of *N*-hydroxysuccinimide and 6 mg of protein A in 1 mL of 0.1 M potassium phosphate buffer, pH 7.8, was added. The mixture was incubated for 2 h at constant pH and room temperature. The reaction products were separated from low molecular weight compounds by gel filtration on Toyopearl HW-55 (Toyosoda, 1.6 × 100 cm column) in 0.05 M potassium phosphate buffer, pH 7.4, with 0.1 M NaCl (hereafter named PBS) and/or by dialysis.

(7) Dzantiev, B. B.; Blintsov, A. N.; Bobkova, A. F.; Izumrudov, V. A.; Zezin, A. B. *Doklady Biochemistry* **1995**, *34*, 77−80.

(8) Blintsov, A. N.; Dzantiev, B. B.; Bobkova, A. F.; Izumrudov, V. A.; Zezin, A. B.; Atabekov, I. G. *Dokl. Biochem.* (*Transl. of Dokl. Akad. Nauk*) **1995**, *34*, 175−8.

(9) Aga, D. S.; Thurman, E. M. ACS Symposium Series 657; American Chemical Society: Washington, DC, 1997; pp 1−20.

(10) Nam, K. S.; King, J. W. *J. Agric. Food Chem.* **1994**, *42*, 1469−74.

(11) Dzantiev, B. B.; Zherdev, A. V.; Romanenko, O. G.; Sapegova, L. A. *Int. J. Environ. Anal. Chem.* **1996**, *65*, 95−111.

(12) Fuoss, R. M.; Strauss, U. P. *J. Polym. Sci.* **1948**, *3*, 246−51.

(13) Leyte, J. C.; Mandel, M. *J. Polym. Sci., Part A: Gen. Pap.* **1964**, *2*, 1879−87.

(14) Dzantiev, B. B.; Choi, M. J.; Park, J.; Choi, J.; Romanenko, O. G.; Zherdev, A. V.; Eremin, S. A.; Izumrudov, V. A. *Immunol. Lett.* **1994**, *41*, 205−11.

Syntheses of Simazine−Protein Conjugates.[15,16] The simazine carboxylate derivative (0.05 mmol) was diluted in 0.5 mL of dimethylformamide; then 0.1 mmol *N*-hydroxysuccinimide and 0.1 mmol 1-ethyl-3(3-dimethylaminopropyl)carbodiimide were added, and the mixture was stirred for 2 h at room temperature. Next, the solution of the activated hapten was cooled to +4 °C and added to a cooled protein solution (4 mg of BSA, 2 mg of HC, 4 mg of STI, 4 mg of OA, or 4 mg of HRP in 0.5 mL of 0.02 M sodium carbonate buffer, pH 9.5) containing the same volume of dimethylformamide. The initial hapten/protein molar ratio was varied from 3:1 to 50:1 (for the preparation of tracer conjugates for immunoassays) and to as much as 500:1 (for immunogens). The mixture was incubated with stirring for 1 h at room temperature and for 16 h at +4 °C. The resulting conjugates were separated from low molecular weight compounds in the reaction mixture by gel filtration on Sephadex G-25 (Pharmacia, 1 × 20 cm column, in PBS) and/or by dialysis.

Determination of the Composition of Simazine−Protein Conjugates. To calculate hapten/protein ratios of the conjugates, the number of surface amino groups in the original protein molecule was compared with protein conjugated to the hapten. These groups were estimated by a spectral technique using 2,4,6-trinitrobenzensulfonic acid.[17,18] Completeness of the dissociation of the carbodiimide−protein complexes during the synthesis of the conjugates was shown by titration of surface amino groups in the protein preparations that had been exposed to activation and dialysis but were not incubated with the derivatives of simazine.

Immunization. To raise antibodies, Chinchilla rabbits weighing 3−4 kg were immunized according to the following procedure. An immunogen (simazine-BSA or simazine-HC) dissolved in PBS was emulsified with an equal volume of Freund's complete adjuvant (Difco) to a final concentration of 0.5 mg/mL (by protein). For the first stage of the immunization, 1.0 mL of this mixture was injected four times (days 1, 15, 29, 43) intradermally at multiple sites on the back from scapula to sacrum. After two months (day 103), the first boost was carried out: the rabbits were injected intravenously and intradermally with 0.3 mL and 0.2 mL of the immunogen, respectively, dissolved in PBS at the same concentration as initially used. Seven days later (day 110) the rabbits were bled. This boosting/bleeding procedure was repeated on a monthly basis (boosting − days 133, 163, and 193; bleeding − days 140, 170, and 200).

Antiserum Separation.[19] Antisera were extracted by settling blood samples for 16 h at +4 °C. Upper layers were carefully collected, divided into aliquots, and stored at −20 °C.

Simazine ELISA (Traditional Technique). The protein A solution (100 μL, 2.5 μg/mL) in PBS was added into wells of the microtiter plate and incubated for 1.5 h at 37 °C (or for 16 h at 4 °C). Subsequently, the wells were washed four times with PBS containing 0.05% Tween-20 or Triton X-100 (hereafter named PBST). The specific antiserum was added (100 μL, dilution 1:1000 in PBST) and incubated for 1 h at 37 °C, and the microplate was washed repeatedly. Then, 50 μL of simazine solutions (row of dilutions from 0.1 ng/mL to 20 μg/mL, in PBST) and 50 μL of simazine-HRP conjugate (60 ng/mL by HRP, in PBST) were added into the wells. The microplate was incubated for 1 h at 37 °C and then washed. Finally, peroxidase activity for immune complexes formed on the surface of the wells was measured as described below.

Peroxidase Activity Measurement in ELISA Systems. The substrate solution was prepared using ABTS (0.7 mM) and H_2O_2 (1.8 mM) in 30 mM sodium acetate buffer, pH 4.5. This solution (100 μL) was added into each microplate well and incubated for 15−30 min at room temperature. The optical density of the product was measured with an MR-600 vertical photometer (Dynatech) at 405 nm.

Simazine ELISA Using Polyelectrolytes. PEVP solution (100 μL, 5 μg/mL, in PBS) was added into each microplate well and incubated for 16 h at +4 °C (or for 2 h at 37 °C). The wells were washed with PBST. Solutions of simazine (25 μL, concentrations from 0.1 ng/mL to 20 μg/mL), simazine−HRP conjugate (25 μL, 4 μg/mL by HRP), antisimazine antiserum (50 μL, dilution 1:500), and protein A-PMA conjugate (50 μL, 60 μg/mL by protein A) in PBST were successively added into a second incubation microplate. The resulting mixture was incubated for 15 min at room temperature with stirring. Then, aliquots of the mixture (100 μL) were put into the PEVP treated plate and incubated for 10 min at room temperature. (During kinetic studies, the duration of this step was varied from 15 s to 15 min.) The wells were washed, and peroxidase activity was measured as described above.

Simazine Detection by Dot Blot Immunofiltration Technique. The reaction mixture was prepared in microplates by successive addition of simazine, simazine−HRP conjugate, antisimazine antiserum, and protein A-PMA conjugate solutions (in PBST, concentrations and volumes were the same as described above for the ELISA). The mixture was incubated for 15 min at room temperature with stirring. A special membrane holder was used to separate the mixture of compounds. It consisted of two plain acrylic panels (each of them 1.0 cm in thickness) that could be assembled together or taken apart by thumb screws. The top panel had 32 holes through it (conical wells with a 0.5-cm top diameter and a 0.3-cm bottom diameter, each with a volume of 125 μL). The bottom panel was a solid acrylic sheet. Sixteen or 32 layers of filter paper were placed on the bottom panel. The Hybond-N membrane (Amersham, pore diameter, 0.45 μm) was placed over the filter paper. After tightly assembling the two panels with special clamping screws, 50 μL of the polycation solution (40 μg/mL, in PBS) was added into each hole. After complete filtration of this solution, 50 μL of PBST was added into the holes for washing. Then 100 μL of the above reaction mixture was added. After its complete filtration, the membrane was washed with 100 μL of PBST. The holder was taken apart, and the membrane was placed in the substrate solution (see below).

Peroxidase Activity Detection in Dot Blot Immunoassays. 3,3′-Diaminobenzidine tetrahydrochloride (2.5 mg) was diluted in 5 mL of PBS and then combined with 100 μL of 40 mM $CoCl_2$ and 50 μL of 1 M H_2O_2 aqueous solutions. (*Note that diaminobenzidine is carcinogenic and should be handled using suitable precautions.)* The resulting mixture was incubated with the membrane for 1 min. The intensity of the spots formed was detected visually, with a ScanJet 5p scanner (Hewlett-Packard) or with a CS-9000 flying-spot scanner (Shimadzu, Japan) at 530 nm.

(15) Wittmann, C.; Hock B. *Food Agric. Immunol.* **1989**, *1*, 211−24.
(16) Wittmann, C.; Hock B. *J. Agric. Food Chem.* **1993**, *41*, 1421−5.
(17) Habeeb, A. F. S. *Anal. Biochem.* **1966**, *14*, 328−33.
(18) Fields, R. *Biochem. J.* **1971**, *124*, 581−90.
(19) Ishikawa, E.; Imagawa, M.; Hashida, S.; Yoshitaki, S.; Hamaguchi, Y.; Ueno, T. *J. Immunoassay* **1983**, *4*, 209−327.

RESULTS AND DISCUSSION

To compare the proposed immunoassay techniques, all their steps should be optimized. First we have analyzed the ELISA format. All specific interactions in this assay (antibody + antigen, antibody + labeled antigen, antibody + polyanion−protein A conjugate) were carried out in solution. After formation, the immune complexes were separated by the interpolyelectrolyte reaction between immobilized polycation and polyanion-containing complexes. Hence, the following parameters of the assay were chosen: (1) optimum regime of polycation immobilization; (2) polycation concentration during immobilization; (3) optimum regime of immune interactions; (4) concentrations of the immunoreactants used (labeled antigen, antibodies, and polyanion−protein A conjugate).

Before proceeding any further, we have shown that nonspecific adsorption of proteins and conjugates on the microplate surface (measured in the absence of any one compound of the specific complexes) is negligibly small at the assay conditions. To put it otherwise, the detected complexes are formed primarily through the polycation−polyanion interactions.

Generally speaking, the ability to bind polyelectrolytes is not a unique property of the oppositely charged polymers. However, despite the proteins' ability to form electrostatic complexes with the polyelectrolytes, the second polyelectrolyte can break down these complexes effectively. In previous work[8] we have shown that the high affinity of the cooperative polycation−polyanion interaction leads to exclusion of the protein molecules from their complexes with one of the polymers.

We have studied the kinetics of the interpolyelectrolyte interaction on the microplate surface. Ninety percent saturation of binding sites was reached in 8 min, whereas 2 min of incubation proved sufficient for binding of more than 50% of the sites. Thus, an appropriate level of measured signal can be achieved even after rather short incubations. This fast kinetics has made it possible to carry out the microplate immunoassay in a short time and at nearly equilibrium conditions.

Concentrations of the dissolved compounds were also optimized. We examined concentrations of 5−60 μg/mL for the protein A−polyanion conjugate (by protein A), 1:200−1:6400 dilutions for antisimazine antiserum, and 0.05−10 μg/mL for the simazine−peroxidase conjugate (by peroxidase). The objective was to maximize the sensitivity for simazine detection. From the results of these experiments, optimal conditions of simazine immunodetection were determined (see Experimental Section).

The total duration of the technique was 45 min, while the assay time of the traditional ELISA with the same immunoreactants was 100−120 min. Although both assays include steps of heterogeneous interaction, the polycation−polyanion binding is a significantly faster process as compared with the antigen−antibody one. This is related to the lack of need for strong orientation of the interacting molecules. Furthermore, the polyelectrolyte interaction leads to irreversible binding of the polymers due to the high cooperativity of elementary reactions between links of their chains.[7,8]

Sensitivities of the traditional and the modified immunoassay formats were virtually identical. A typical calibration curve for the proposed assay is shown in Figure 2. The limit of reliable detection

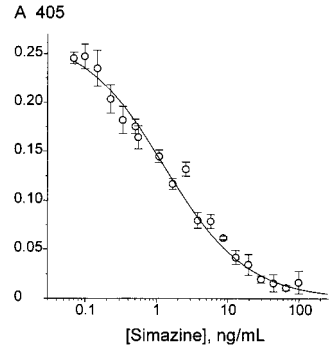


Figure 2. Calibration curve for simazine detection by the developed ELISA technique employing polyelectrolytes. Concentration of reactants and duration of steps are given in the Experimental Section. Measurements were carried out in triplicate. *x*-axis, simazine concentration in the tested sample; *y*-axis, optical density of ABTS oxidation products.

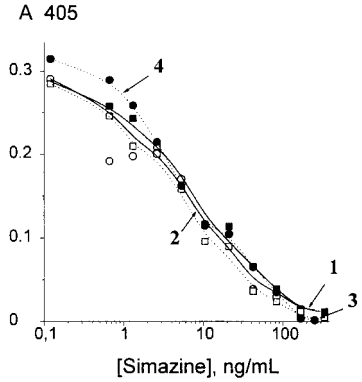


Figure 3. Competitive curves for simazine detection by the polyelectrolyte ELISA in different matrixes: standard PBST solution (1), drinking water (2), orange juice (3), and milk (4). Concentration of reactants and duration of steps are given in the Experimental Section. Measurements were carried out in four repetitions. *x*-axis, simazine concentration; *y*-axis, optical density of ABTS oxidation products. The coefficient of variation of the optical densities varied from 2.7 to 9.8%.

for simazine is 0.5 ng/mL. The coefficient of variation of the measured optical density varied from 3.5 to 9.3% for simazine concentrations in the range 0.1−1 ng/mL. Thus, the proposed polyelectrolyte ELISA is a microplate immunoassay that is conducted in less time than the standard ELISA and does not significantly lose sensitivity.

The optimized method was applied to the detection of simazine in different liquid matrixes, namely, drinking water, orange juice, and milk. The resulting competitive curves are shown in Figure 3. One of the milk preparations analyzed was contaminated with simazine before spiking. This fact was detected by both traditional and polyelectrolyte techniques. The background concentration of simazine was determined as 5 ± 0.5 ng/mL.

Recovery of different simazine concentrations in these matrixes was also studied. Data from the measurements are presented in Table 1. The recovery varied from 95 to 135%, making this a suitably quantitative method.

The second aspect of our investigation was devoted to the evaluation of the dot blot immunofiltration assay (see Figure 4). All immunoreactants were optimized as previously described for the ELISA formats. In addition, by varying the thickness and structure of the filtration layer, the rate of filtration through the

Table 1. Precision of Simazine Measurement by ELISA Using Polyelectrolytes

added concn (ng/mL)	recovery of simazine[a] (%)		
	drinking water	orange juice	milk
2.5	106 ± 10		105 ± 15
5	109 ± 10		102 ± 9
10	113 ± 8	135 ± 12	95 ± 11

[a] Average recovery (n = 4) ± coefficient of variation, defined as (SD/mean) × 100.

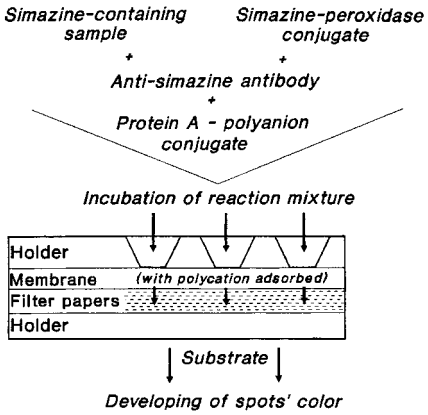


Figure 4. Principle and device for the dot blot immunofiltration assay.

membrane can be changed. The filtration time was varied from 1 to 30 min. A 2−3 min filtration of polycation proved to be sufficient. Increasing the speed of filtration did not influence the intensity of the spots formed by reaction with substrate. However, further acceleration of filtration was not reasonable with the apparatus described, because the time could not be standardized for all wells. By using alternate dot blot systems, it is possible that even higher filtration rates could be used. This approach also offers the advantage of generating very small spots of high density.

Immobilization of the polycation was carried out at different concentrations in the range 5−100 μg/mL. A complete saturation of adsorption sites on the membrane was achieved at a concentration of 40 μg/mL.

After a proper choice of concentration of immunoreactants, the optimal assay regime was proposed (see Experimental Section). Under these conditions, the nonspecific adsorption on the membrane (binding of peroxidase conjugates in the absence of protein A−polymethacrylate conjugate or antiserum) was low and did not influence the visual detection of specific signals.

Figure 5 demonstrates the competitive curve obtained for the dot blot immunofiltration technique. These data were generated on the same membrane. The limit of simazine detection with instrumental measurement of spot density was 1 ng/mL which was close to the detection limit achieved with the ELISA format. This result was anticipated since the optimum conditions selected for the immune interaction in both schemes were nearly identical.

Visual detection in this dot blot immunoassay was typically limited to distinguishing "positive" and "negative" samples by comparison with some cutoff level. The sensitivity of such tests corresponds to a midpoint of the competitive curve and was about 10 ng/mL in our experiments.

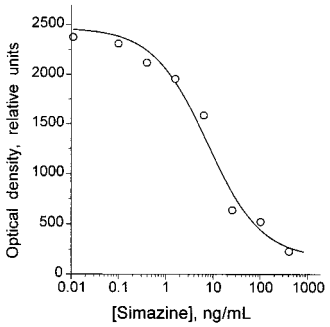


Figure 5. Calibration curve for simazine detection by the developed dot blot immunofiltration assay technique with polyelectrolytes. Concentration of reactants and duration of steps are given in the Experimental Section. *x*-axis, simazine concentration in the tested sample; *y*-axis, integrated optical density of the spots formed during DAB oxidation (in relative units).

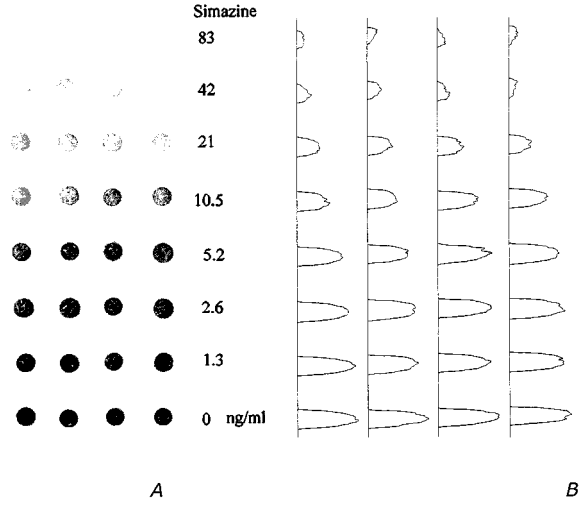


Figure 6. Simazine detection by the dot blot immunofiltration assay technique with polyelectrolytes. Concentration of reactants and duration of steps are given in the Experimental Section. (A) Spots formed in four parallel tests. Increasing concentrations of simazine (right) inhibit color development. (B) Linear distribution of optical density for four rows presented in A. *x*-axis, location on the membrane (in cm); *y*-axis, optical density of the corresponding point (in relative units).

The proposed dot blot immunofiltration assay was characterized by high reproducibility for a few repetitions on the same membrane. Results of four parallel tests are presented in Figure 6. Intensities of the spots (integrated for their area) have the following CVs: 3.2% for 5.2 ng/mL of simazine, 3.4% for 10.5 ng/mL, and 5.1% for 21 ng/mL.

The immunofiltration technique was tested for the same variety of liquid samples as described above in the ELISA format. The presence or absence of simazine was determined without any loss of sensitivity as compared with assays performed in buffer.

The advantage of the proposed visual test is its speed. Total analysis time was 20 min compared with 100−120 min for the ELISA formats. The system developed here permits detection of simazine without any special equipment or pretreatment of liquid samples. In dealing with real-world samples, especially under field conditions, measurement errors are reduced by using volumes of several hundred microliters rather than smaller volumes.

However, small spots give a higher density of signal-to-noise and thus improve sensitivity and reproducibility.[20] This polyelectrolyte technique allows the use of large sample volumes without compromising speed and facilitates the rapid concentration of these volumes to a small spot.

It is noteworthy that the conjugate of polyanion with protein A of *S. aureus* used in both proposed techniques is a universal reagent. By using the polyanion of protein A, the described assays do not require the preparation and purification of specific antibody derivatives. Therefore, the same schemes can be easily adapted for other pesticides.

Because of the high speed of the dot blot format, this application has considerable promise for agricultural and environmental monitoring, and especially for out-of-laboratory field tests.

(20) Chu, F. W.; Edwards, P. R.; Ekins, R. P.; Berger, H.; Finckh, P.; Krause, F. In *Immunochemical Technology for Environmental Applications*; Aga, D. S., Thurman, E. M., Eds.; ACS Symposium Series 657; American Chemical Society: Washington, DC, 1997; pp 170−184.

ACKNOWLEDGMENT

The research described in this paper was made possible in part by Award no. RN 1-426 of the U.S. Civilian Research & Development Foundation for the Independent States of the Former Soviet Union (CRDF). U.S. investigators were funded in part by the National Institute of Environmental Health Sciences Superfund Basic Research Program ES04699, U.S. Environmental Protection Agency Center Grant CR819658, and the National Institute of Environmental Health Sciences Center ES05707. B.D.H. was a McMasters' Fellow of the CSIRO (Canberra, Australia) and an NSF Fellow. Although portions of this work were supported by the U.S. Environmental Protection Agency, no endorsement of the information contained herein is implied.

Received for review January 27, 1999. Accepted May 19, 1999.

AC990072C