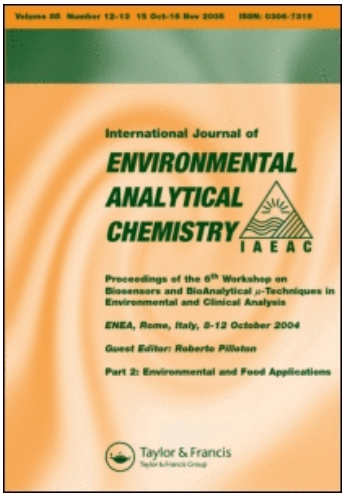

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Development and Validation of an Indirect Enzyme Immunoassay for the Detection of the Herbicide Isoproturon in Water Matrices

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DEVELOPMENT AND VALIDATION OF AN INDIRECT ENZYME IMMUNOASSAY FOR THE DETECTION OF THE HERBICIDE ISOPROTURON IN WATER MATRICES

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An indirect enzyme immunoassay (EIA) for the detection of the phenylurea herbicide isoproturon is described. The specific antibodies did not cross-react with other structurally related compounds. The concentration of isoproturon that inhibits 50% of antibody-antigen binding (IC_{50}) was 0.64 ng/mL. The sensitivities were 0.07 ng/mL (IC_{80}) and 0.02 ng/mL (IC_{90}) respectively, when the crude serum was used in the assay. Matrix effects were observed when river water samples were analyzed showing recoveries as high as 150 %. The IC_{50} was increased to 0.81 ng/mL. To overcome these difficulties, a novel method of antibody purification was developed to reduce the heterogeneity of the medium when the test was performed with complex surface water matrices. This technique involved the extraction of the specific anti-isoproturon antibodies from the crude anti-serum. The refined fraction gave an IC_{50} not higher than 0.29 ng/mL and an IC_{90} of 0.01 ng/mL, when assayed with river water samples. The method was validated using a HPLC procedure with a clean up step involving an immuno-affinity column using the same antibodies. Excellent correlation ($r = 0.998$) was obtained between HPLC and the EIA results when the refined antibody was used in the assay. The use of affinity purified antibodies as an effective procedure in reducing matrix effects was demonstrated.

Keywords: Isoproturon; enzyme immunoassay; antibody; purification; immunoaffinity chromatography.

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INTRODUCTION

Isoproturon [3-(4-isopropylphenyl)-1,1-dimethyl urea] is the most commonly used arylurea herbicide in Europe. It is mainly used in pre- and post-emergence control of annual grasses and many annual broad leaved weeds in spring and winter wheat, barley, rye and triticale. It acts as a selective systemic herbicide by inhibiting the photosynthetic electron transport system, once absorbed by the roots and leaves[1]. Although acute toxicity of arylureas to mammals is assumed to be low[2], their persistence in soil, ground and surface water has been detected in several drinking water supplies in France at concentrations higher than 0.1 μ/L, which is the European Union maximum allowable concentration for a single pesticide[3]. In some regions, such as Brittany, and depending on the season, the level of surface water contamination can exceed 2 ng/mL, the World Health Organization (WHO) guideline. For these reasons, a regular monitoring of such xenobiotic substances becomes critically important for all drinking water supplies. Isoproturon is traditionally analysed by high performance liquid chromatography (HPLC). Extraction and purification are usually needed for sample preparation so as to attain the required detection limit and to eliminate interferences.

Our laboratories have been involved in developing selective solid phase extraction (SPE) procedures using immunoaffinity chromatography for sample preparation.[4–6] The development of rapid and reliable screening methods to monitor environmental exposure is another area we have been investigating, primarily using enzyme immunoassay (EIA) approaches. EIA provides fast, simple and cost-effective screening methods of a large number of samples for environmental exposure monitoring[7]. EIA for phenylurea herbicides has focused on diuron analysis (Table I) since it is most commonly used in the United States[8,9]. Liegeois et al[10,11] described an Enzyme-Linked Immunosorbent Assay (ELISA) technique suitable for the detection of 1 μg/L of isoproturon in water and soil samples. Most recently, Kathmeh and coworkers[12] developed a direct competitive ELISA which was reported to have a sensitivity of 0.03 μg/L for isoproturon monitoring in drinking water supplies. However, matrix effects were obvious, particularly when measurments were made on surface water samples. Interferences with organic matter present in river water samples led to enhanced recoveries (200%).

The indirect EIA described in this paper uses our newly developed anti-isoproturon serum. Cross-reactivity with other related compounds of the phenylurea family was investigated. Affinity purified serum was employed to overcome the difficulties encountered when crude anti-serum was used in a complex matrix such as river water samples. The effect of the purification procedure on the antibody properties was investigated and the assay results were compared to HPLC analyses using an immunoaffinity clean-up procedure for the same river water samples.

EXPERIMENTAL SECTION

Apparatus

HPLC analyses were performed using a Millenium 2010 workstation (version 2.10) including a Waters 600 controller solvent delivery system, a Waters 712 autosampler and a Waters 486 absorbance detector set to 244 nm. The analytical column was a Waters Symmetry C18, 5 μ, 100 Å, HPLC cartridge (15cm × 0.39cm i.d). The effluent from the affinity columns used for antibody purification was monitored by measuring the absorbance 280 nm with UVICON 860 spectrophotometer. A BioRad microplate washer was used for microtiter plate washing. Optical densities of microtiter wells were measured on a dual beam Titertek multiscan MCC with a 492 nm sample filter and 620 nm reference filter. Data were transmitted to a spread sheet program for analysis. The instrument was checked periodically by a spectrocheck plate and software (QC Technology, New York). Melting points were determined with a Mettler FP 62 apparatus. NMR spectra were measured on a Bruker 200 MHz spectrometer. Chemical shift values are given in ppm downfield from internal tetramethylsilane.

Materials

Sepharose® CL-4B was supplied by Pharmacia. Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Holland). Bovine serum albumin (BSA), ovalbumin, bovine β-lactoglobulin (βLG), goat anti-rabbit IgG peroxidase conjugate (second antibody), Tween 20 and O-phenylenediamine dihydrochloride (OPD) were purchased from Sigma. Dot blots were performed on nitrocellulose membranes supplied by Schleicher & Schuell (product number 439 194). LC grade water was prepared by purifying demineralized water in a Milli-Q (MQ) filtration system (Millipore, Bedford, MA). Antibody solutions were concentrated using Amicon Centriplus 30 (exclusion size of 30kD) ultrafiltration units. Dialysis tubing (10 mm in diameter with a 12 000–14 000 molecular weight cut off) was purchased from Spectrum Medical Industries Inc. (Los-Angeles, CA). Flat bottom polystyrene microtiter plates were obtained from Dynatech laboratories, Inc. (Chantilly, VA). The sorbent used to prepare immunoaffinity columns for river water sample pre-concentration consisted of 40 μm glutaraldehyde activated silica beads, with a pore size of 275 Å and was a kind gift of JT-Baker B.V. (Netherlands). Pesticide standards (isoproturon and other related compounds) were obtained from the pesticide repository of the Food Research Division and were stated to be at least 99% pure by the respective manufacturers. Other chemicals were from Prolabo, Merck, or Fluka.

Buffers

Phosphate-Buffered Saline (PBS, adjusted to pH 7.4) contained 20 mmol of NaH_2PO_4 and 140 mmol of NaCl per liter of deionized water. PBS-BR was obtained by adding 1.5 % (w/v) of skimmed milk as a blocking reagent in PBS. Washing buffer (PBS-T) consisted of 0.1 % Tween 20 (v/v) in PBS. Tris Buffered Saline (TBS, pH 7.5) contained 50 mmol of Tris, 150 mmol of NaCl per liter of deionized water. TBS-T consisted of 0.1 % Tween 20 (v/v) in TBS. Citrate buffer (pH 5.0) consisted of 51 mmol of Na_2HPO_4 and 24 mmol of citric acid per liter of deionized water. The substrate used for the dot blot test consisted of 5 mg of 4 chloro-naphthol dissolved in 30 mL of MeOH/TBS (10/90 v/v) and 60 µL of 30 % H_2O_2. The substrate for the titer determination consisted of 17.5 mg of o-phenylenediamine dihydrochloride (OPD) and 10 µL of 30 % H_2O_2 in 25 mL of citrate buffer. Coating buffer (pH 9.6) contained 13 mmol of Na_2CO_3 and 35 mmol of $NaHCO_3$ per liter of deionized water.

Hapten synthesis procedure

Haptens of isoproturon and chlortoluron (Table I) were prepared by the same procedure and conjugated to carrier proteins to obtain immunizing and screening agents. Figure 1 shows the synthesis strategy for isoproturon. One of the N-methyl groups was substituted by a carboxymethylene moiety according to the following description. A solution of 7 g (51 mmol) of 4-isopropylaniline or 5.6 g (40 mmol) of 3-chloro-4-methyl aniline in dry toluene (40 mL for 5 mL of aniline derivative) was cooled in an ice and water mixture before the addition of an excess of phosgene $COCl_2$ (80 mmol for 40 mmole of aniline derivative), with stirring. Thirty milliliters of toluene were added to the mixture which was stirred at room temperature overnight, then gently refluxed for two hours. The white solid (the remaining aniline hydrochloride) was eliminated by filtration and toluene was evaporated. The viscous isocyanate intermediate was dissolved in pyrid-

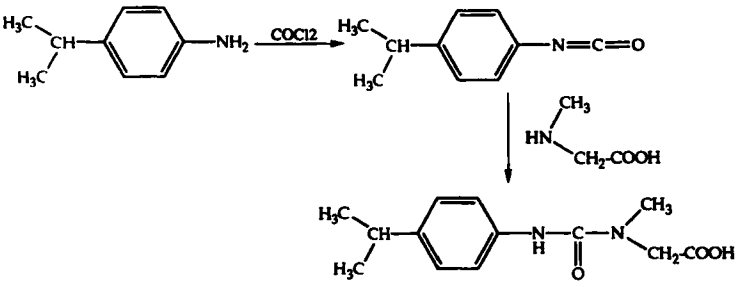


FIGURE 1 Isoproturon hapten synthesis scheme

TABLE I Cross reactivity features of the anti-isoproturon antibodies for both the crude anti-serum and the refined fraction with phenylurea herbicides and other non related compounds

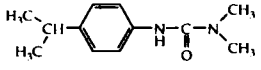
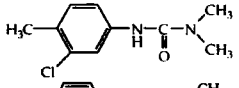
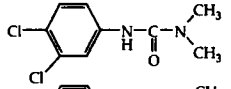
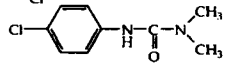
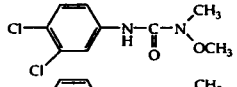
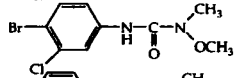
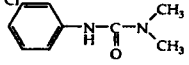
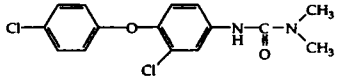
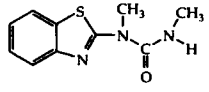
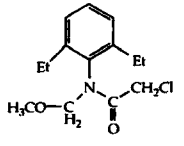
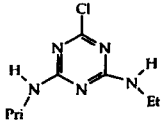
Compound	Name	IC50 Cr.[1] (ng/mL)	IC50 Ref.[2] (ng/mL)	%C.R.[3] Cr/**Ref.**
	**Isoproturon**	0,64	0,37	100/**100**
	**Chlortoluron**	186	223	0,34/**0,16**
	**Diuron**	208	208	0,31/**0,18**
	**Monuron**	331	233	0,19/**0,16**
	**Linuron**	1477	1955	0,04/**0,02**
	**Chlorbromuron**	657	1012	0,097/**0,04**
	**Fenuron**	N.R.	N.R.	< 0,001 %
	**Chloroxuron**	712	1280	0,09/**0,03**
	**Metabenzthiazuron**	N.R.[4]	N.R.[4]	< 0,001 %

TABLE I Cross reactivity features of the anti-isoproturon antibodies for both the crude anti-serum and the refined fraction with phenylurea herbicides and other non related compounds (continued)

Compound	Name	IC50 Cr.[1] (ng/mL)	IC50 Ref.[2] (ng/mL)	%C.R.[3] Cr/**Ref.**
	**Alachlor (NRC)**[5]	N.R.[4]	N.R.[4]	< 0,001 %
	**Atrazine (NRC)**[5]	N.R.[4]	N.R.[4]	< 0,001 %

1 Cr.: Crude serum
2 Ref. : Refined
3 %C.R.: % Cross reactivity = (100*IC50 (isoproturon))/(IC 50 compound)
4 N.R. : Non recognized compound
5 NRC : Non related compound

ine. N-(methyl-amino)ethanoic acid hydrochloride (N-methyl glycine hydrochloride) (3.7 g, 30 mmol) in pyridine was added dropwise to the solution (total volume 150 mL). The resulting solution was stirred overnight at room temperature, then evaporated to driness. The residue was dissolved in 100 mL of a 0.1 M sodium carbonate and ethyl acetate mixture (30/70). The aqueous solution was extracted three times with 30 mL aliquots of ethyl acetate then acidified with 1M HCl to pH 2. The resulting precipitate was filtered and washed with water until the filtrate was pH 5. The obtained urea was dried under vacuum over $CaCl_2$. The crude product was recrystallized from ethyl acetate/methanol (90/10) to give white crystals. The respective yields were 39% (3.15 g) of isoproturon derivative and 40% (3 g) of chlortoluron derivative. Isoproturon derivative : mp 153.4° C ; 1H NMR (DMSO-d_6) δ 12.58 (s, 1 H, COOH), 8.30 (s, 1 H, NH), 7.34 (d, J = 2.1 Hz, 2 H, Ar-$H_{3,5}$), 7.1 (d, J = 2.0 Hz, 2 H, Ar-$H_{2,6}$), 4.01 (s, 2 H, NCH_2CO_2), 2.84 (s, 3 H, NCH_3), 2.73 (m, 1 H, $C\underline{H}(CH_3)_2$), 1.18 (d, J = 7.5 Hz, 6 H, $(C\underline{H}_3)_2CH$); Chlortoluron derivative: mp 151.4° C 1H NMR (CD_3OD) δ 7.47 (s, 1 H, Ar-H_2), 7.22 (d, J = 3.31 Hz, 2 H, Ar-$H_{5,6}$), 4.1 (s, 2 H, NCH_2CO_2), 3.07 (s, 3 H, NCH_3), 2.28 (s, 3 H, CH_3-Ar); ^{13}C NMR (CD_3OD) δ 173.38 (acid C=O), 158 (amide C=O), 139.88, 134.79, 131.66, 131.34, 122.57, 120.69 (Ar-$C_{1,2,3,4,5,6}$), 51.20 (CH_3N), 36.16 (NCH_2CO_2), 19.31 (CH_3-Ar).

Protein conjugate preparation and epitopic density determination

In order to prepare the immunogen, the carboxylic acid derivative of isoproturon (IPCOOH) was conjugated to BSA via the mixed anhydride method[13] using 105 molar equivalents of the hapten. IPCOOH was also conjugated to βLG to prepare the plate coating protein using 45 molar equivalents of the hapten according to the same conjugation technique. Another βLG conjugate was made with the carboxylic derivative of chlortoluron (βLG-CLT) in order to prepare the affinity chromatography ligand for antibody purification. The choice of this ligand is based on a previous study on isoproturon antibody purification[14] and the procedure developed by Assil et al.[15] who used a protein conjugate as an affinity ligand. To leave enough free amine functions for the further binding to CNBr activated Sepharose® beads, only 27 molar equivalents of the chlortoluron anhydride activated derivative were used.

In each case, the anhydride activated hapten was prepared in dioxan and added to the protein in a 1:1 water dioxan mixture. The alkaline pH of the solution was adjusted to maintain protein solubility. After a 5-h incubation at 4°C, the mixture was dialyzed against water for 36 hours. The recovered protein was then freeze-dried and stored at 4°C. The yield approximated 90 %. The number of pesticide derivatives per carrier protein was calculated by determining the free

amino groups of lysine side chains with 2,4,6-trinitro-benzenesulfonic acid (TNBS) reagent[16]. The differential absorption of the native and the conjugated proteins at 420 nm allowed the determination of an average conjugation ratio.

Immunization and test for the presence of anti-isoproturon specific antibodies

Two New Zealand white rabbits were intradermally inoculated with the BSA conjugate (500 µg/rabbit) in complete Freund's adjuvant. A first boost was given 14 days later. Other boosts were performed every two months. Incomplete adjuvant, rather than complete Freund's adjuvant, was used for all the boosting doses. For long term storage, the harvested anti-serum was kept frozen at –20° C. A qualitative test was performed to check for the presence of anti-isoproturon antibodies by dot blot test. Briefly, isoproturon-conjugated βLG (βLG-IP) was dissolved in PBS (5 mg/ mL) and 2 µL were blotted on a nitrocellulose membrane. The strips were left for one hour in PBS-BR then incubated for two hours in the antibody solution (25 µg of proteins). After 3 washing steps with 10 mL of PBS-T, 1/2000 diluted peroxidase conjugated goat anti-rabbit antibodies in PBS-BR were added to the strips for a 2 hour incubation. The membranes were then washed 3 times with 10 mL of TBS-T and finally put in the substrate solution (4-chloro-naphthol). The intensity of the colour that appeared on the strip was representative of the amount of anti-isoproturon antibodies in the test solution.

Plate sensitizing and titer determination

βLG-IP was used as the coating protein at 1 µg/mL and 10 µg/mL ovalbumin was added to sensitize the 96 well plates[18]. Serial dilutions of antisera were made in PBS-BR and 200 µL were added to the wells of the sensitized plate in triplicate. After a further 30 min incubation at 4 °C and washing (5 wash and soak cycles of 8 seconds each), a second antibody horseradish peroxidase conjugate was added followed by another 30 min incubation at room temperature and washing. Subsequently, the substrate (OPD) and H_2O_2 were added. The colour development was stopped after 30 min by addition of 2.5 M H_2SO_4 and the optical densities were read at 492 nm.

Competitive Immunoassay

The indirect competitive ELISA procedure was similar to that reported by Newsome et al[18]. A 1 mL aliquot of anti-serum diluted 1/15,625 in PBS-BR was added

to 25 µL of spiked MQ water or river water sample. After mixing and incubation at 4°C for an hour, 200 µL was added to the wells of the sensitized plate in triplicate. The addition of the second antibody and the plate reading was achieved exactly as described previously (titer determination). The standard curves consisted of 16 concentrations of isoproturon (0, 0.015–150 ng/mL) in MQ water and filtered river water. The antibody cross-reactivity was also determined using the same procedure with 16 concentrations of each tested pesticide. Results are featured in % B/B_0 vs log (concentration of isoproturon) or another related compound, where B is the OD (492 nm) of a spiked sample and B_0 that of a blank.

Purification of antibodies on βLG-chlortoluron conjugated columns

Sepharose® CL–4B (10 mL gel suspension) was activated by cyanogen bromide according to March[19]. The amount of unbound conjugated protein was determined by performing a BCA protein assay. The modified Sepharose® CL–4B (7 mL of swollen gel in 0.1 M phosphate buffer saline, pH 7.2) incorporated an average of 1.1 mg of ligand per mL of wet gel and was placed in a column (1 cm diameter) then washed with 5 volumes of 0.1 M PBS. One fifth diluted crude anti-serum was percolated through the column at a rate of 0.5 mL/min. A washing step with PBS (50 mL) ensured that all unbound antibodies were removed. The most efficient eluent consisted of 3.10^{-4} M isoproturon or its carboxylic acid derivative dissolved in PBS and 2 % MeOH[14]. The eluted fractions were concentrated on Centriplus ultrafiltration modules and dialyzed against 0.9 % NaCl. The titer of the purified fractions was determined as described earlier and the antibody solutions were stored at 4°C if used shortly after, or freezed at –20°C for longer periods.

Immunosorbent preparation and HPLC analyses

The aldehyde functions on the activated silica matrix were covalently linked to the amine function of the anti-isoproturon IgG by a reductive amination reaction[4]. The resulting immunosorbent (0.5 g packed into a 3 mL plastic syringe barrel) was conditioned following the manufacturer instructions to eliminate all unbound antibodies. Briefly, three washing steps were performed using successively solutions of PBS, NaCl 1M and 10% ethylene glycol in PBS. A solution of 0.3 ethanolamine (pH adjusted to 8.2) was passed through the column for 4 hours, to saturate all unreacted aldehyde functions. A river water sample of 20 mL, spiked with a known concentration of isoproturon was then percolated through the cartridge. The immunosorbent was washed with 8 mL of LC-grade water. The elution was performed with 8 mL of a 70% MeOH / 30% LC-grade

water solution. The eluate was evaporated to 250–500 µL and an aliquot of 50 µL was injected into the L.C. system. Isoproturon was separated using the following linear gradient: 20% to 30% acetonitrile in water from 0 to 30 min, 30% to 45% from 30 to 36 min, 45% to 47% from 36 to 46 min, 47% to 100% from 46 to 66 min. Between each injection the C18 column was reconditioned using the following gradient: 100% acetonitrile from 0 to 20 min, 100% to 20% acetonitrile in water from 20 to 30 min. This composition was maintained for 20 min before another injection.

RESULTS AND DISCUSSION

Most published immunoassays were developed using the competitive indirect format[20]. Figure 2 shows the steps of such EIA. This format has certain advantages. The assay is simple to handle and there is no need to an efficient enzyme-pesticide conjugate with the required activity. Choices of commercially available enzyme labelled secondary antibodies can be used.

However, the sensitivity of such a test format has been reported to be lower than that of the immobilized antibody format[20,21]. We pre-incubate the antibody with the pesticide or sample extract prior to exposure to the solid phase. Such procedure has been reported to increase test sensitivity[20].

Hapten choice and immunogen synthesis

Newsome[22] and Karu et al[8] developed synthesis schemes which were applicable to most phenylurea herbicides. This strategy aims at not altering the electronic and steric properties of the internal nitrogen linked to the phenyl group. Better sensitivity was obtained when a spacer arm of three methylene units was introduced. However, a shorter spacer arm of one methylene unit was chosen in our case so as to limit the spacer arm recognition while keeping the substituted phenyl ring as the most exposed group. BSA was chosen as the carrier protein for immunogen preparation and was covalently coupled to the hapten using the mixed anhydride method. As reported previously[4], the epitopic density of the immunizing conjugates ranged from 20 to 30. This was determined using the comparative spectrophotometric absorption of TNBS-modified conjugates so as to measure the remaining free lysine side residues[16] and confirmed by electro-spray ionization mass spectroscopy (ESI-MS). These conjugates were considered suitable for antibody production since about 10–30 haptenic moieties/100 kDa of carrier protein are required for this procedure[23].

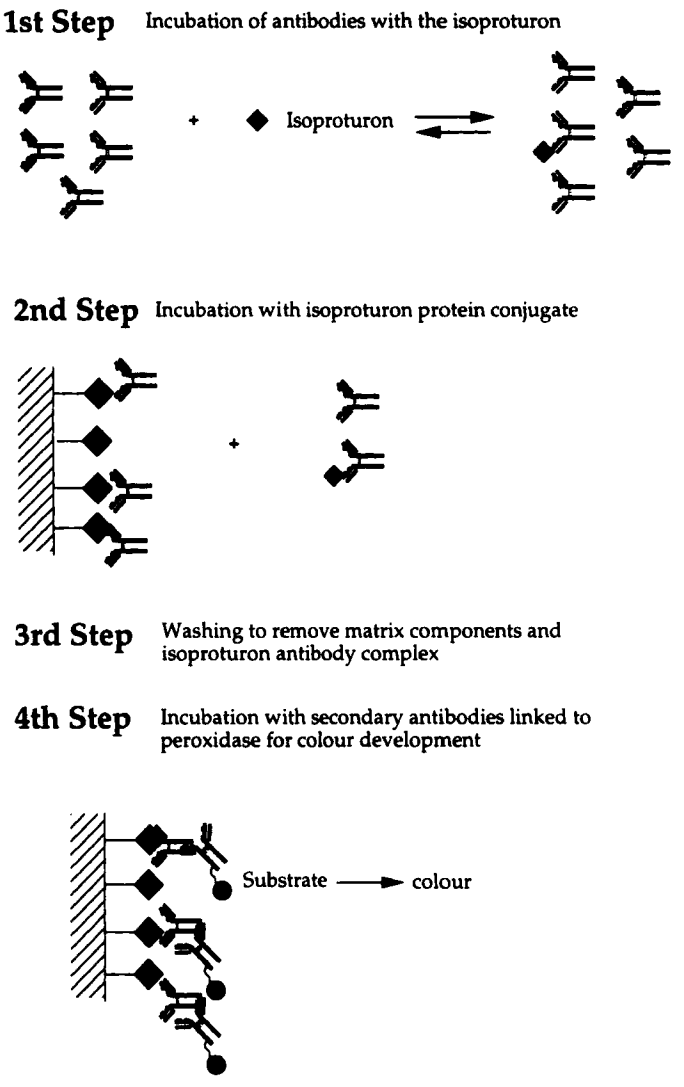


FIGURE 2 Indirect competitive EIA procedure for determining isoproturon in water samples

Antibody characterization and optimization

Pre-immunized sera showed no specific antibody response. βLG-IP was used for dot blot assay for titer determination, for βLG, itself, showed no cross reactivity with the anti-serum and a βLG-conjugated Sepharose® column retained less than 0.7 % (w/w) of the percolated anti-isoproturon serum.

The dot blot assay performed with anti-serum collected after 6 months of immunization showed high titer at a dilution as much as 1:20,000. Although immunized under the same conditions, one rabbit gave a titer twice as high as the other one. Figure 3 shows the titer obtained with the anti-serum collected from rabbit 1 after a year of immunization. A dilution of 15,625 was used for the ELISA, for absorbance readings at 492 nm equal to 1–1.5. The reproducibility of these results was checked by performing the experiments over 3 consecutive days.

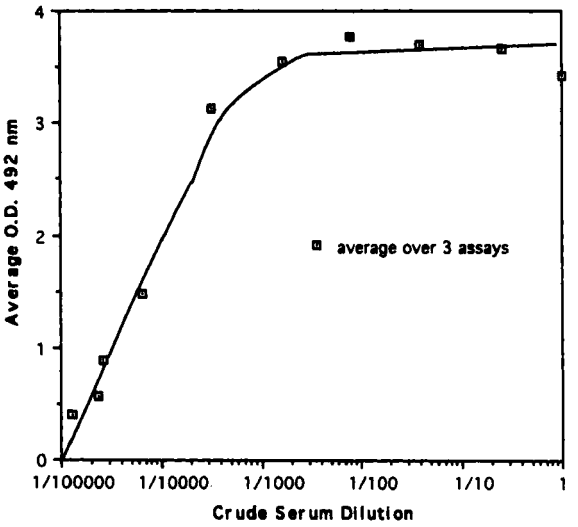


FIGURE 3 Determination of crude serum titer

Antibody cross-reactivities were studied by performing the competitive ELISA test using other related phenylurea herbicides and some other common agrochemicals, that are likely to appear in field treatment mixtures. Neither atrazine, nor alachlor gave an inhibition of binding and were therefore not recognized. Also, it is shown in Table I that compounds with no substitution of the phenyl ring such as fenuron or with an important structural difference with isoproturon such as methabenzthiazuron were not recognized at all. A chlorinated phenyl ring showed certain cross-reactivity (CR < 0.2%) such as that observed with chlortoluron, diuron and monuron. Such compounds would not be recognized by our sensitive ELISA procedure. Linuron however displayed a very weak %CR. This was probably related to the methoxy subtituted nitrogen which induced quite different electronic effects from an alkyl substituent and therefore altered the antibody recognition[5, 8].

Calibration curves with spiked water

Calibration curves were performed using concentrations of isoproturon ranging from 0.015 ng/mL to 150 ng/mL in MQ-water. Figure 4 shows the $\%B/B_0$ curve and its logit-log transformation for 9 standard calibration experiments. When the crude serum was used, the concentration of isoproturon that inhibits 50% of antibody-antigen binding (IC_{50}) reached 0.64 ng/mL. The IC_{80} and IC_{90} were found to be 0.07 and 0.02 ng/mL respectively. These values are often considered as the detection limit of the assay and satisfy therefore the European guideline. The inter-day standard deviation of the assays did not exceed 6%. Applied to river water samples, the IC_{50} (Figure 5) was raised to 0.81 ng/mL. The negative blank river water samples produced a $\% B/B_0$ as high as 10 %. This value is similar to those observed for fumonisins[24] and Cyanazine[25] that was not subjected to extraction and clean-up steps. The inhibition may be due to the presence of organic interferents occuring in the river water matrix which occupy nonspecifically some binding sites of the antibodies. HPLC analysis of a river water sample after prior clean up using an isoproturon selective immunoaffinity cartridge confirmed the absence of any phenylurea in the river water at a concentration higher than 0.1 ng/mL which was the detection limit of this method[4]. To overcome this difficulty and to try to ameliorate the sensitivity of the assay, we evaluated affinity purified antibodies. This type of purification would reduce the heterogeneity of the medium when the test is performed in real samples. This one-step procedure was preferred over purification steps necessary for each sample.

Influence of antibody purification on the sensitivity of the ELISA test

The selected antibodies were obtained using an affinity chromatography procedure described elsewhere[14]. The affinity gel used a chlortoluron conjugate linked to a flexible linker protein (βLG) and immobilized on Sepharose. This ligand was found to be the most suitable giving a compromise between the selection of the target IgG and the easiness of elution. The effectiveness of the purification procedure was checked using a dot blot test and comparative FPLC chromatograms. Recovery did not exceed 50 % even with optimized eluting conditions. However, the titer of the purified fraction showed a six fold increase in activity over the IgG extracted by a commercial gel (AvidChrom gel, Unisyn Technologies, MA, USA). A concentration of no more than 110 ng/mL of refined antibodies was necessary to obtain a blank with an OD (492 nm) of 1. The cross-reactivity features of the refined fraction are reported in Table I. The antibody characteristics were similar to those obtained with the crude antisera as the methoxy substituted ureas gave the least % CR. The cross reactivity appeared to

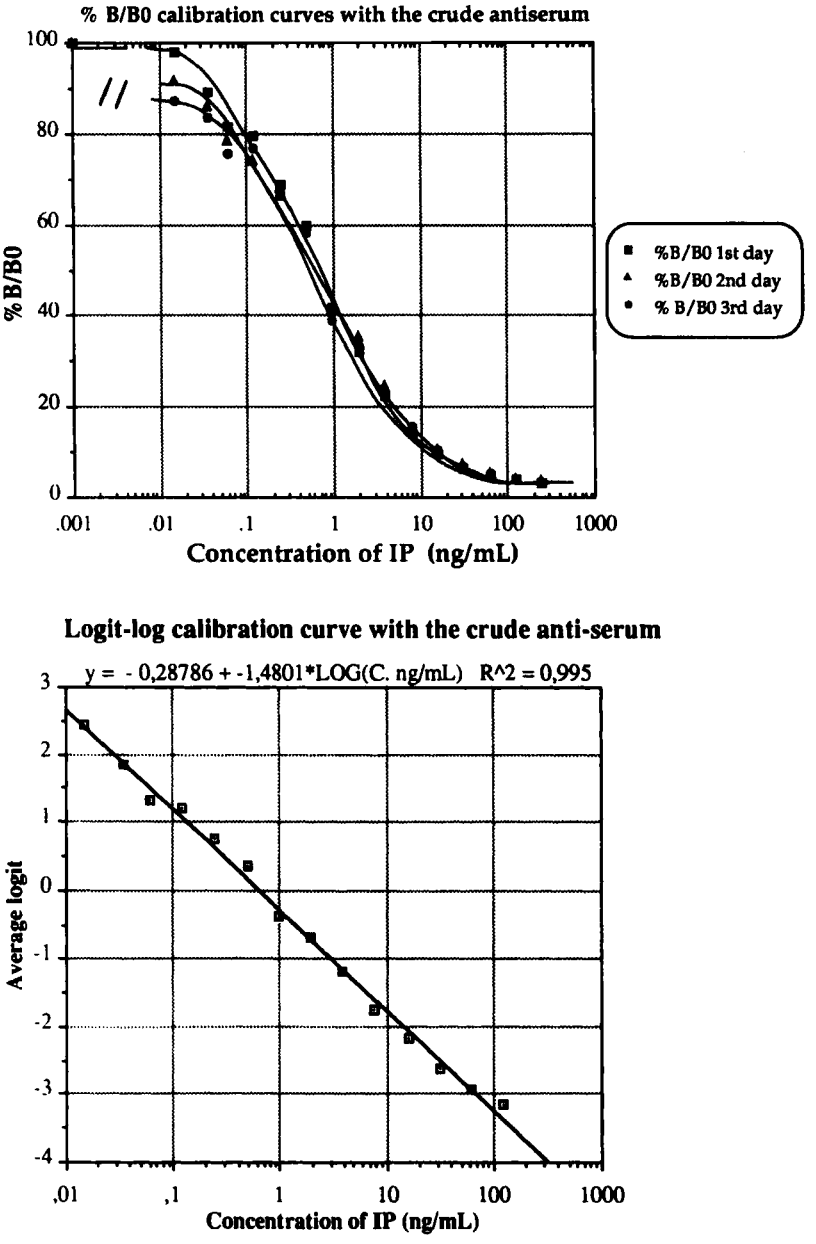


FIGURE 4 Calibration curve and its logit-log transformation for MQ spiked water using the crude anti-serum (IP = Isoproturon)

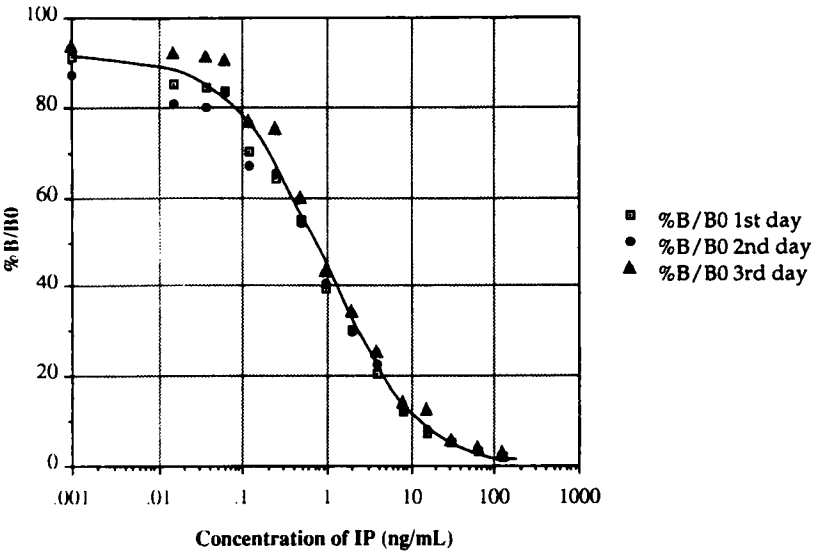


FIGURE 5 Calibration curves using crude anti-serum for Ottawa River water spiked with isoproturon (IP)

decrease for most of the related compounds. Table II summarises the comparison between the characteristics of the assay performed with the crude serum and that with the refined fraction, under similar conditions. Although 7 to 10 % inhibition was still observed with river water blanks, a significant improvement was noticed since the IC_{50} value dropped from 0.81 ng/mL with the crude serum to 0.29 ng/mL with the refined fraction. The IC_{90} was of an order of magnitude lower than the required detection limit of the European Union (0.1 ng/mL). The %SD showed that the test is less variable with the purified antibodies as shown in figure 6. As stated below, recoveries were decreased well below 150% with this refined fraction, giving a better correlation with HPLC analyses. It is clear that the serum purification improved most of the assay characteristics. This conclusion is consistent with what was obtained by Assil et al[15] for their indirect immunoassay of sulfonamides.

Validation of the assay using HPLC analyses

Spiked samples of river water were analyzed using the assay with the crude serum and with the purified antibodies. Three analyses of each sample were performed on the same day, and the experiment was repeated over three consecutive days. Each sample was simultaneously analyzed by HPLC on a C18 column using a cleanup procedure with an anti-isoproturon immunosorbent prior to analysis. As shown in figure 7, the recoveries were greater than 100 % when the

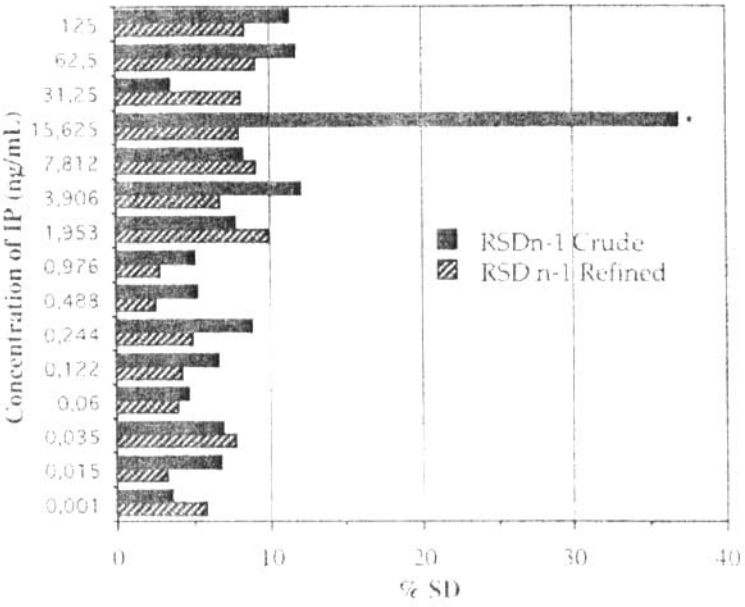


FIGURE 6 Comparison of standard deviations (%SD) for calibration curves obtained when the assay was performed using the crude (n = 3) and the refined (n = 6) serum with spiked Ottawa River water. (IP = isoproturon, * Outlier data)

assay was carried out using the crude serum as it clearly displayed a positive bias relative to the HPLC results. This was particularly evident for the 2 ng/mL and the 4 ng/mL spikes. The use of refined serum gave a lower inter-day standard deviation and allowed recoveries ranging between 83% to 99.7%. The correlation with HPLC analyses was therefore significantly improved using the purified antibodies as shown in figure 7 (r = 99.8%)

TABLE II Summary of the characteristics of the EIA performed on river water samples with crude antiserum and affinity purified antibodies

Parameter	*Assay performed with crude anti-serum*	*Assay performed with refined antibodies*
IC50 (ng/mL IP)	0.81	0.29
IC80 (ng/mL IP)	0.09	0.03
IC90 (ng/mL IP)	0.02	0.01
Logit-log curve	y = −1.4525 log (C IP ng/mL) − 0.131 R^2 = 0.979	y = −1.4439 log (C IP ng/mL) − 0.77 R^2 = 0.985
number of assays	3 assays / day over 3 days	3 assays / day over 3 days
% SD (range of standard deviation between days)	5–13%	2–10%

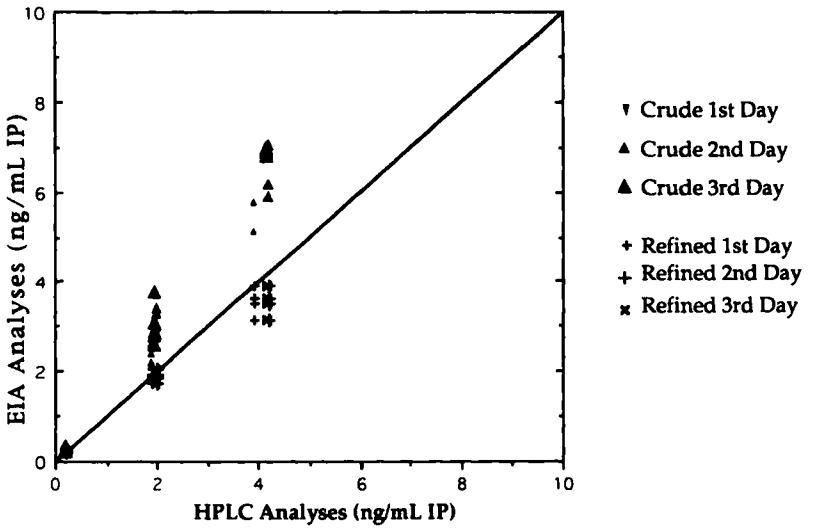


FIGURE 7 Comparison of HPLC and EIA on river water samples

CONCLUSION

We have developed a new indirect enzyme immunoassay for the detection of isoproturon in water matrices using newly developed antibodies. Antibody purification using affinity chromatography was shown to improve the selectivity of the test and the limit of detection when the assay was performed with real matrices such as river water. The IC_{90} value thus obtained was about 0.01 ng/mL and was 10 times lower than the European guideline for such a compound. HPLC analyses of spiked samples tested with the developed assay confirmed the validity of the assay particularly with the refined serum. We also showed that purification of antibodies can be used to overcome matrix effects in some cases, thus avoiding a sample purification step that otherwise is required. The use of affinity purified antibodies as an effective procedure in reducing matrix effect was demonstrated.

Acknowledgements

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