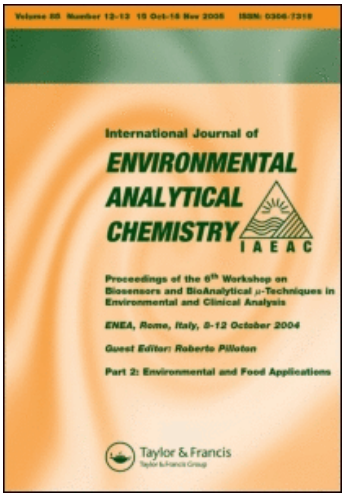

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PRODUCTION OF ANTIBODIES AND DEVELOPMENT OF SPECIFIC POLARIZATION FLUOROIMMUNOASSAY FOR ACETOCHLOR

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Specific polyclonal antibodies towards acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)-acetamide) were obtained from rabbits immunized against a 3-mercaptopropionic acid derivative of acetochlor, covalently attached to bovine serum albumin. A polarization fluoroimmuoassay (PFIA) based on these antibodies was developed and optimized to detect acetochlor in water samples. The optimized PFIA had a detection limit of 9 μg/L, linear working range from 50 to 5500 μg/L and within-assay coefficient of variation less than 4%. Cross-reactivity studies demonstrated that these antibodies are capable of specific detection of acetochlor amongst structurally related chloroacetanilide herbicides. Assay cross-reactivity values were: alachlor 0%, metolachlor 2.4%, propachlor 0%, butachlor 0.2% and dimethachlor 0.5%. Five organic solvents commonly used in sample extraction were evaluated for their effect on acetochlor PFIA performance, and methanol and ethanol were found to be compatible with the assay up to 10% v/v.

Keywords: Polarization fluoroimmunoassay; Acetochlor; Antibody production; Cross-reactivity

INTRODUCTION

Acetochlor (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenylacetamide) is a selective pre-emergent herbicide of the chloroacetanilide family used to control broad-leaf weeds and annual grasses in corn. Acetochlor has been reported to be moderately persistent in the environment and mobile in soil [1], reaching, therefore, ground and surface water. Being a structural analog of the chloroacetanilide herbicides alachlor and metolachlor, acetochlor could be expected to demonstrate similar analytical behavior. In recent years, acetochlor and other chloroacetanilide herbicides have been effectively determined by gas chromatography/mass spectrometry (GC/MS) [2–6] and liquid chromatography [7]. Nevertheless, a rapid, sensitive and low-cost

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technique suitable for both laboratory and in-field detection is needed to monitor chloroacetanilide herbicides in numerous environmental water samples.

Over the last 10 years highly efficient and cost-effective immunochemical methods like enzyme-linked immunosorbent assay (ELISA) have been increasingly used for detection of pesticides [8]. With regard to chloroacetanilide herbicides, ELISAs have been reported for alachlor [9–12] and metolachlor [13,14] and their metabolites [15,16]. Alachlor and metolachlor ELISA kits are commercially available from Millipore Corporation; however, they have been shown in some cases to cross-react with acetochlor. A class-specific acetanilide immunoassay kit is also available, but to date, no spcific immunoassay directed towards acetochlor has been described.

Though ELISA has many advantages over the conventional analytical techniques and allows direct analysis of a large number of samples, this method requires separation of immunoreagents and multiple washing steps. A simpler and faster screening method developed over recent years is polarization fluoroimmunoassay (PFIA). PFIA is a competitive homogeneous technique that utilizes polarized light to detect the speed of molecular rotation in aqueous solution [17]. This method is based on the increase of the fluorescence polarization of a small fluorescein-labeled hapten (tracer) when bound by a specific antibody. PFIA is particularly suitable for the assay of low-molecular weight antigens, because a large increment of analytical signal is achieved upon binding to high-molecular weight antibody. Because of their simplicity, precision and possible automation, PFIAs are widely used in clinical chemistry, and commercial kits for hormones and drugs are available from Abbott Laboratories. The first PFIA application to pesticide analysis was reported by Colbert and Coxon in 1988 [18], and a number of assays have been developed since then [19–23].

Herein we report on the production of polyclonal rabbit antibodies and development of a PFIA for detecting acetochlor. Taking into account that no specific immunoassay was available for acetochlor, the objective of the research described in this paper was to design the immunogen enabling the development of a PFIA specific towards acetochlor. To evaluate assay performance, five structurally related chloroacetanilide herbicides were tested for cross-reaction with the anti-acetochlor antibodies. To investigate which organic solvents are compatible with acetochlor PFIA, several solvents most frequently used for extraction were investigated for their influence on the assay.

EXPERIMENTAL

Chemicals and Instrumentation

Bovine serum albumin (BSA), *N*,*N*′-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide and 3-mercaptopropionic acid were purchased through Sigma Chemical Co. (St. Louis, MO, USA). The analytical standards of acetochlor, alachlor, propachlor, metolachlor and dimethachlor used for cross-reactivity studies were obtained from Riedel-de-Haen (Seelze, Germany).

The buffer PBS is 10 mM phosphate buffer (if not indicated otherwise) containing 0.8% w/v of saline, pH 7.5. Borate buffer is 25 mM sodium borate with 1% w/v NaN_3, pH 8.0.

Pre-coated silica gel 60 F_{254} (0.25 mm) aluminium sheets for thin-layer chromatography (TLC) were acquired from Merck (Darmstadt, Germany).

Fluorescence polarization standard curves were recorded using a Beacon 2000 fluorescence polarization system (Pan Vera, USA). The inhibition curves were analyzed using a four-parameter equation in Origin 6.0 for Windows.

Hapten density of protein conjugate was determined by matrix-assisted desorption ionization (MALDI) with a time-of-flight mass spectrometer (MS) using a Kratos Kompact MALDI III instrument equipped with 337 nm nitrogen laser. Mass spectra of hapten and tracer were obtained with an API 3000 MS/MS instrument operated in Turbo Ion Spray mode.

Synthesis of the Protein Conjugate

The hapten acetochlor-3-mercaptopropionic acid (AMPA) was obtained from the reaction of acetochlor with 3-mercaptopropionic acid in a similar way as described for s-triazines [24]. Briefly, a mixture of 0.26 g (1 mmol) of acetochlor, 0.11 g (1 mmol) of 3-mercaptopropionic acid and 0.11 g (2 mmol) of potassium hydroxide in 20 mL of ethanol was boiled for 4 h. The reaction mixture was filtered and evaporated under vacuum. The solid was dissolved in 10 mL of 5% $NaHCO_3$ and filtered. The solution was acidified to pH 3.0 with 6 N HCl and the powder was dried under vacuum to give 0.78 g of AMPA (yield 25%). API-MS: [M − H] 338.3 (100%), 243.1 (62%), 266.1 (48%). ApI-MS-MS: 219.9 (100%), 265.8 (71%).

The resulting hapten was covalently attached through its carboxylic group to the lysine groups of BSA by the *N*-hydroxysuccinimide method. A mixture of 70 mg (0.2 mmol) of AMPA, 125 mg (1.2 mmol) of *N*-hydroxysuccinimide and 250 mg (1.2 mmol) of *N*,*N*′-dicyclohexylcarbodiimide in 1 mL of dimethylformamide, stirred under room temperature for 3 h, was added dropwise to a solution of 133 mg (2 μmol) of BSA in 10 mL of distilled water under vigorous stirring. After 3 h incubation at 4°C, 1M $NaHCO_3$ was added to adjust to pH 11. The solution was kept overnight at 4°C, after which the mixture was dialyzed for 48 h in PBS at 4°C and the conjugate AMPA-BSA was lyophilized.

Hapten densities of the protein conjugates were determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) by comparing the molecular weight obtained for the standard BSA with that of conjugates. MALDI-MS spectra were obtained by mixing 0.5 μL of the matrix solution (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg/mL in CH_3CN/H_2O 70 : 30, 0.1% TFA) with 0.5 μL of the conjugate, a solution of the protein or the conjugate (3.5 mg/mL in CH_3CN/H_2O 70 : 30, 0.1% TFA).

Immunization and Polyclonal Antisera Preparation

The acetochlor–BSA conjugate was used to immunize three female New Zealand white rabbits weighing 2.95–3.25 kg according to the following protocol. Routinely, 1 mg of immunizing antigen dissolved in PBS buffer was emulsified with Freund's complete adjuvant (1 : 2 volume ratio). Animals were injected intradermally with 1 mL of the immunogen at five sites on the back from scapula to sacrum and boosted at seven day intervals for five weeks. The next month, rabbits were boosted with an additional 0.5 mg of antigen (emulsified with Freund's incomplete adjuvant) intramuscularly. To estimate antibody titer, small blood samples were taken during the process of immunization. The scheme was repeated three times, and subsequent boostings and bleedings

continued until no increase in the antibody titer was observed. Evolution of antibody titer was analysed by measuring the binding of the antisera to fluorescein-labeled antigen by PFIA. Whole blood (35–40 mL) was obtained six months after the start of immunization by bleeding from the ear vein, allowed to coagulate for 30 min at 37°C and 2 h at 4°C and centrifuged to obtain the serum. Aliquots of the sera were stored at −30°C.

The immunoglobulin G fraction was isolated by precipitation with saturated ammonium sulphate solution. The pellet was resuspended in PBS buffer and precipitated a second time before further clean-up was performed using dialysis for 48 h in PBS buffer (four changes of buffer). The protein concentration was determined by calculating the difference in absorbance at 280 nm against PBS buffer as a blank, assuming that the lgG concetration of 1 mg/mL corresponds to 1.35 absorbance units [25].

Tracer Synthesis

Fluorescein thiocarbamyl ethylene diamine (EDF) was synthesized as previously described from fluorescein isothiocyanate isomer I [26]. The tracer was synthesized using the *N*-hydroxysuccinimide ester method [27]. An amount of 8 mg (80 μmol) *N*-hydroxysuccinimide and 8 mg (40 μmol) of *N*,*N*′-dicyclohexylcarbodiimide was added to a solution of 6 mg (20 μmol) AMPA in 0.2 mL of dimethylformamide. After 2 h stirring at room temperature this solution was added to 5 mg (10 μmol) of EDF. Then the reaction mixture was stirred at toom temperature for 3 h. Small portions of reaction mixture (50 μL) were separated by TLC using methylene chloride/methanol (4 : 1 v/v) as the eluent. The main yellow band at Rf 0.9 was isolated and stored in methanol at 4°C. Concentration of the tracer was estimated spectrophotometrically at 492 nm, assuming absorbance in borate buffer (2.5 mM, pH 8) to be the same as for fluorescein ($\varepsilon = 8.78 \times 10^4\ M^{-1}\ cm^{-1}$). The tracer solution was further diluted in 2.5 mM borate buffer and used for PFIA measurements. API-MS: [M + H] 771 (100%), 640.3 (11.7%), 449.3 (92%).

Polarization Fluoroimmunoassay (PFIA)

Dilution curves were constructed by incubating (1 min at room temperature) a fixed amount of tracer (1.4 nM) with different dilutions of the IgG fraction covering the range from 1 : 20 to 1 : 20,480 in a total volume of 0.5 mL, followed by measurement of fluorescence polarization. The ability of acetochlor analyte to compete with fluorescein-labeled antigen for antibody binding was investigated by measuring inhibition curves. Inhibition curves were constructed using acetochlor stock solution (1 g/L in methanol) diluted with borate buffer to give 5, 10, 100, 500, 1000, 10,000 and 100,000 μg/L. These standards (50 μL) were vortex mixed with fluorescein-labeled antigen (50 μL) and an appropriate dilution of antiserum (50 μL) and buffer to make up the 500 μL incubation volume. After 1 min incubation, fluorescence polarization was measured. Criteria to evaluate the PFIAs were the IC_{50} concentration value (the concentration of an analyte producing a 50% inhibition in fluorescence polarization), the maximal fluorescence polarization signal, the minimal fluorescence polarization signal and the slope of the curve. The limit of detection was determined from the inhibition curve using the blank value and the threefold confidence interval and converted into the corresponding concentration value [28].

Assay Optimization

Cross-reactivity to related compounds was determined by running the assay with standards of the test compounds over the concentration range from 10 to 100,000 μg/L and the IC_{50} for each compound was defined. The IC_{50} value in μg/L of acetochlor was divided by the corresponding value from the analyte and multiplied by 100 to produce the percentage cross-reactivity values. The percentage cross-reactivity of the antibodies to acetochlor calculated in this way was 100%.

The effect of organic solvents was examined by recording acetochlor PFIA inhibition curves with the addition of solvent (methanol, ethanol, dimethylsulfoxide, acetonitrile and ethyl acetate) to give the solvent content of 1, 5, 10 or 15% v/v in the incubation volume. Reduction in maximal fluorescence polarization (i.e. at maximal antibody binding) and increase of IC_{50} of the inhibition curve, as affected by the solvents, were calculated and expressed as percentage of the values for the inhibition curve in borate buffer.

RESULTS AND DISCUSSION

Hapten Conjugation to Carrier Protein and Generation of Antibodies

Because of their small molecular weight, pesticides such as acetochlor are not generally immunogenic and therefore require conjugation to protein carrier molecules in order to elicit an antibody response [29]. The key step in developing an antiserum is, therefore, the design of the hapten, and its covalent linkage to carrier protein.

In designing the hapten, we tried to present as much as possible the chemical structure, electronic distribution and spatial conformation of acetochlor [30,31]. To ensure specific antibody production, it was necessary to preserve unique acetochlor features, dissimilar to other chloroacetanilides, in a hapten molecule. Since chloroacetanilide herbicides differ by their aromatic ring radicals and chloroacetamide moiety, these groups of acetochlor should be left free and exposed to the immune system, providing maximum specificity towards the target compound.

In considering the functional groups of the acetochlor molecule for hapten–protein conjugation, we thought it desirable to utilize the chloroacetamide group, the single functional group that is common to all chloroacetanilide pesticides. This approach has been successfully applied to synthesis of hapten–carrier protein conjugates for a variety of chloroacetanilide compounds [10,15]. Direct conjugation to protein thiol groups, however, was shown to result in insufficient hapten density of the conjugate [32]. To achieve the required rate of conjugation, Feng *et al.* suggest the thiolating cross-linking agent *S*-acetylmercaptosuccinicc acid (AMSA) that reacts with ε-amino groups of lysine residues. The AMSA thiolation of free amine groups on lysine residues leads to spontaneous formation of covalent linkage with the chloroacetamide moiety under basic conditions.

To ensure conjugation to carrier, the chloroacetamide moiety can be derivatized by incorporation of a terminal functional group capable of reaction with ε-amino groups of lysine residues of carrier protein. This strategy was reported for another class of chlorine-containing pesticides – triazines [24,32,33]. The derivatizing agent, 3-mercaptopropionic acid, is a heterobifunctional compound that could on one side react with the chloroacetamide moiety and, on the other, provide a terminal carboxyl

group – an appropriate linker for covalent attachment to the protein. The derivatization of acetochlor molecule with 3-mercaptopropionic acid leads to the substitution of chlorine atom with sulfur. The modification could be considered as "soft" one because of the following factors [24]: (1) sulfur resembles chlorine in electronic structure better than other atoms, except perhaps oxygen, (2) the atomic size of sulfur is closer to that of chlorine than other possible atoms, (3) sulfur will not allow hydrogen bonding as will oxygen and nitrogen.

Based on the above factors, the scheme of synthesis was chosen to produce specific acetochlor antibodies. Chloroacetamide fragment of acetochlor molecule was derivatized by a one-step reaction with 3-mercaptopropionic acid (Fig. 1) to generate a hapten with an elongated 'handle'. A spacer of three to six carbon atoms was reported [34,35] to be optimal for favoring recognition of attached hapten by the immune system and avoiding shielding effects of the carrier. Hapten derivatization with 3-mercaptopropionic acid is expected to result in acetochlor specific antibodies, since this way of conjugation allows maximal exposure of the hapten to the immune system. Important antigenic determinants in the acetochlor chemical structure such as substituted

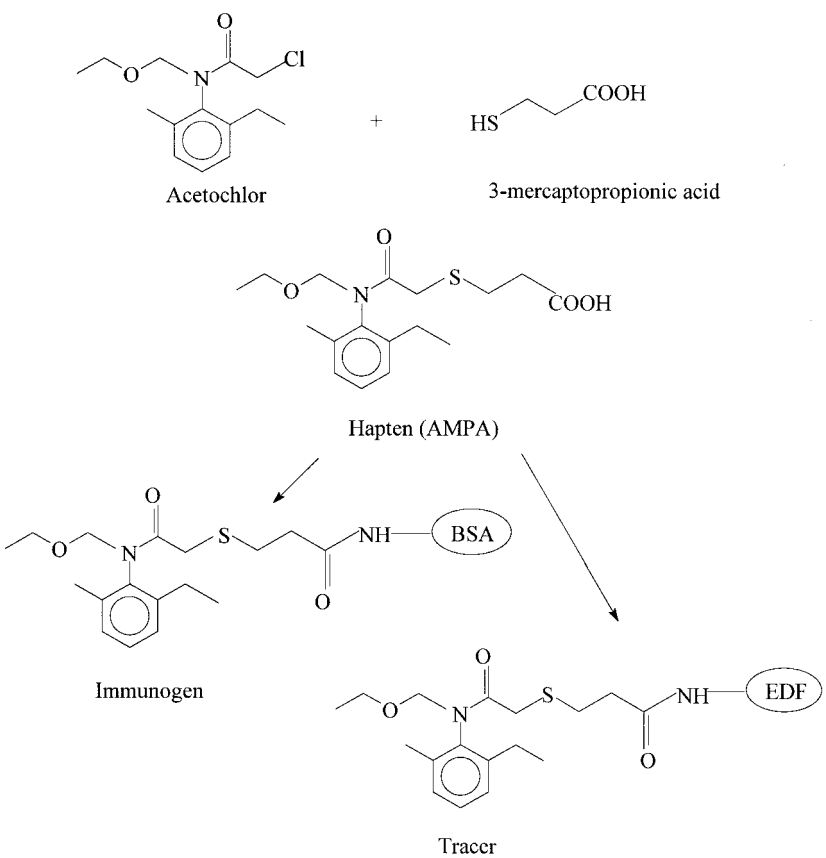

FIGURE 1 Scheme of acetochlor covalent conjugation to carrier protein (BSA) and fluorescent dye (EDF). The protein conjugate was used to induce the production of polyclonal antibodies in rabbits. The fluorescein conjugate was used as a tracer in PFIA.

aromatic ring and chloroacetamide side chain radical remain far away from the shielding effect caused by the carrier protein.

A conjugate of 3-mercaptopropionic acid derivative of acetochlor (AMPA) with BSA as a carrier was synthesized and used for immunization of three rabbits. Characterization of this conjugate by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) showed its hapten density to be 1 : 7 (ratio was calculated by comparing M + H peaks for the immunogen and with that of intact BSA). It is evident from literature data [36] that about 10–30 haptenic groups per 100 kDa of carrier protein are required to generate good production of antibodies. As molecular weight of BSA is about 66.5 kDa, sufficient extent of conjugation was reached.

The Polarization Fluoroimmunoassay

PFIA is a homogeneous competitive assay technique [17] based on the difference in rotational motion between bound and free fluorescein-labeled molecules (tracer). Signal given by free tracer is low due to its rapid Brownian movement while the bound tracer's signal is much higher. The fluorescence polarization can be expressed according to Eq. (1):

$$P = \frac{I_v - I_h}{I_v + I_h} \tag{1}$$

where P equals the polarization and I_v and I_h are the vertical and horizontal components of the emitted fluorescence intensity. The measured polarization depends on the amount of free and bound tracer in the sample [37].

Tracer and Antibody Conditions

Since PFIA is a competitive assay, concentration of labeled competitor (tracer) is an important parameter to be optimized. The quantity of tracer used determines the competition degree between acetochlor and labeled antigen for the antibodies binding sites and it also sets the intensities of polarized light emission. The lowest possible tracer concentration, which allows the reliable detection of label and does not affect the competition, should therefore be used to reach precise and sensitive assay. For acetochlor PFIA, the lowest concentration of homologous tracer, a fluorescein-labeled derivative of AMPA, was determined to be 1.4 nM. This value corresponds to a signal approximately 10 times higher than the background signal from buffer. Higher tracer concentration reduces sensitivity while lower concentrations lead to loss of precision.

The optimum antibody concentration was determined by recording dilution curves. Dilution curves were constructed for antisera, obtained from each animal (namely antisera 5, 6 and 7) and the antibody dilution corresponding to 50% binding (titer) were determined. The serum was assessed by PFIA after every bleeding until no increase in the titer was observed. The bleeds reached acceptable titer value after the fifth immunization step yielding a titer of 1/1000 (Fig. 2). Antiserum from rabbit 6 showed the best results and was chosen for further experiments. The optimum concentration of antibody was chosen when about 70% of tracer was bound, which was equal to 9×10^{-7} M rabbit IgG.

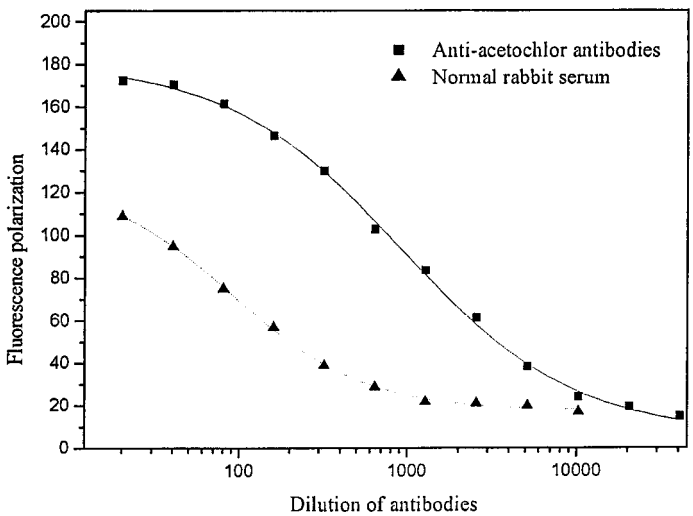

FIGURE 2 PFIA dilution curves of IgG fractions from anti-acetochlor antiserum (squares) and normal rabbit serum (triangles) using AMPA-EDF tracer. Subsequent antibody dilutions were incubated with fixed amount of tracer (1.4 nM) in a total volume of 0.5 mL for 1 min. Initial antibody concentration (rabbit 6, IgG fraction) was 2.3×10^{-5} M. Fluorescence polarization units are plotted against antibody dilution.

Calibration and Sensitivities

Inhibition curves were constructed at analyte concentration range of 5–100,000 μg/L. The calibration graph using acetochlor as analyte and anti-acetochlor antibodies at 70% binding is presented in Fig. 3. Curve fitting was performed using a four parameter logistic model [38]. To normalize fluorescence polarization signal, relative units B/B_0 (where B_0 is the maximum fluorescence polarization value of inhibition curve and B is the current value) were used [39].

Under optimal conditions, the PFIA of acetochlor reported here has an IC_{50} of 540 μg/L, a working range (80–20% of blank polarization value) between 50 and 5500 μg/L and a detection limit (concentration corresponding to blank signal minus threefold confidence interval) of 9 μg/L. Measurement of samples in five replicates gave within-assay CV values of 1.03–3.7%. Assay run on 3 different days gave between-assay CV of 1.24–8.63%.

The sensitivity reached by this immunoassay is comparable with other pesticide PFIAs [18–20,23]. Homogeneous immunoassays are known to be less sensitive than heterogeneous immunoassays like ELISA, but they provide rapid and simple methods for screening of a large number of samples. The most of reported PFIAs allow the detection of pesticides at high μg/L or low mg/L level, which exceeds the maximal admissible concentration of single pesticide in drinking and surface water, recommended by European Commission.

When sensitivity of direct PFIA measurement is not sufficient to detect target analyte in environmental water samples, coupling with solid-phase extraction (SPE) could improve the overall detection limit by several orders of magnitude. The combination of SPE and ELISA as was reported for alachlor [11], provided a selective and sensitive analytical method. To apply SPE as sample pre-treatment technique for

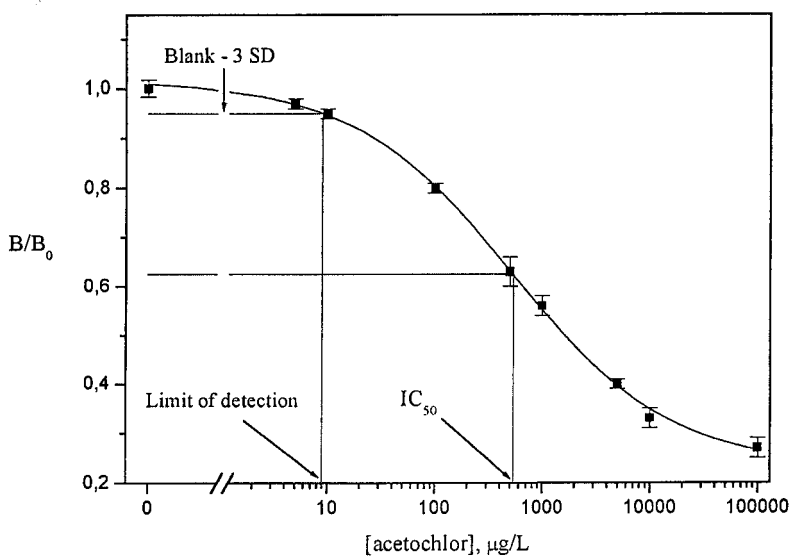

FIGURE 3 Optimized PFIA inhibition curve of acetochlor using rabbit 6 IgG fraction at concentration 9×10^{-7} M and AMPA-EDF tracer. Normalized fluorescence polarization signals (B/B_0), where B_0 is fluorescence polarization at zero acetochlor dose, were plotted against acetochlor concentration. Limit of detection was determined as analyte concentration corresponding to blank signal minus threefold blank standard deviation. IC_{50} was determined as analyte concentration giving 50% displacement of bound tracer.

enhancement of acetochlor PFIA sensitivity is an objective of ongoing research in our group.

Cross-reactivity to Related Compounds

Selectivity of the acetochlor PFIA was evaluated by using five structurally related compounds. The results are presented in Fig. 4 and Table I. Competitive PFIA calibration curves were obtained using optimized antibody and tracer concentrations with analyte

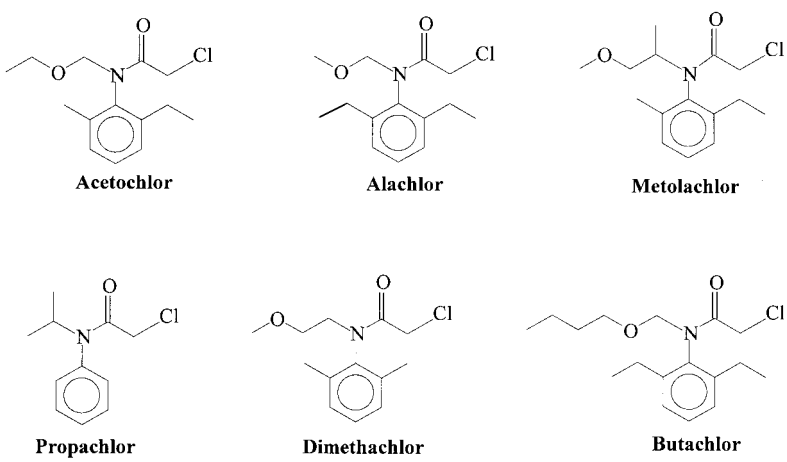

FIGURE 4 Structures of chloroacetanilide herbicides tested for cross-reactivity in the acetochlor PFIA.

TABLE I Assay cross-reactivities (CR) reported for chloroacetanilide herbicides

Compound	*CR, % PFIA*[a]	*CR,%*[b] [10,31]	*CR,%*[c] [12]	*CR,%,*[d] [13]
Acetochlor	100.0	4.4	7.3	–
Alachlor	< 0.1	100	1.7	23
Propachlor	< 0.1	0	< 0.1	0
Metolachlor	2.4	1.8	100	100
Butachlor	0.2	1.2	3.4	–
Dimethachlor	0.5	–	–	–
Metalaxyl	–	–	0.7	5

[a]Data described in this paper. [b–d]Data from reported ELISAs for alachlor and metolachlor.

(acetochlor or cross-reactant) over 10–100,000 μg/L concentration range in borate buffer. Calibration curves were compared using a four-parameter plot [40]; cross-reactivity value was obtained from these plots according to Eq. (2)

$$\text{Cross-reactivity (\%)} = \frac{\text{Standard IC}_{50}}{\text{Cross-reactant IC}_{50}} \times 100\% \quad (2)$$

All cross-reactants tested contain common structural elements – a 2,6-substituted phenyl ring linked with chloroacetamide radical. It can be observed that, in spite of the high extent of similarity of the chloroacetanilide compounds, the specificity of the PFIA towards one of them – acetochlor – is very high. Other analogs with different structures of aromatic ring-side radicals and chloroacetamide moiety are recognized only to a minor degree.

Generally, it can be shown that the more the steric structure of a cross-reactant differs from that of acetochlor, the lower is the cross-reactivity. Little or no binding is observed if structures of both the aromatic ring and the chloroacetamide moiety are disturbed compared to the acetochlor molecule (alachlor, propachlor, dimethachlor and butachlor). Metolachlor, bearing the same aromatic ring radicals, but altered ethoxymethyl side chain compared to acetochlor, demonstrates much higher binding. Notwithstanding that the phenyl ring fragment of metolachlor is identical to that of acetochlor, the affinity of the antibodies towards metolachlor is low, as reflected by the cross-reactivity of only 2.4%.

Compared with ELISAs reported for alachlor and metolachlor (Table I), the assay described herein demonstrates enhanced specificity. While an ELISA developed for alachlor [10,32] exhibits about 4% cross-reactivity towards acetochlor, the PFIA for acetochlor exhibits negligible recognition of alachlor. Amongst chloroacetamide compounds tested for interference in the ELISA for metolachlor [12], acetochlor showed the highest binding. We observed the same tendency, which could be explained by structural identity of aromatic ring substitution patterns of these two compounds. Both anti-metolachlor and anti-acetochlor antibodies are sensitive to chloroacetamide side chain alterations resulting in low absolute value of cross-reactivity (7.3 and 2.4%, respectively). Another assay [13] reported for metolachlor showed significant cross-reactivity towards alachlor; data for acetochlor were not indicated.

By analogy with descriptions of immunoassays for related compounds, the PFIA for acetochlor we present in this paper could be used for specific detection of acetochlor in environmental samples.

Solvent Interference

The effect of five water miscible solvents, commonly used in sample extraction, on the performance of acetochlor PFIA was studied. The results obtained at four different concentrations of solvents are presented in Fig. 5. Observed effects are dependent on

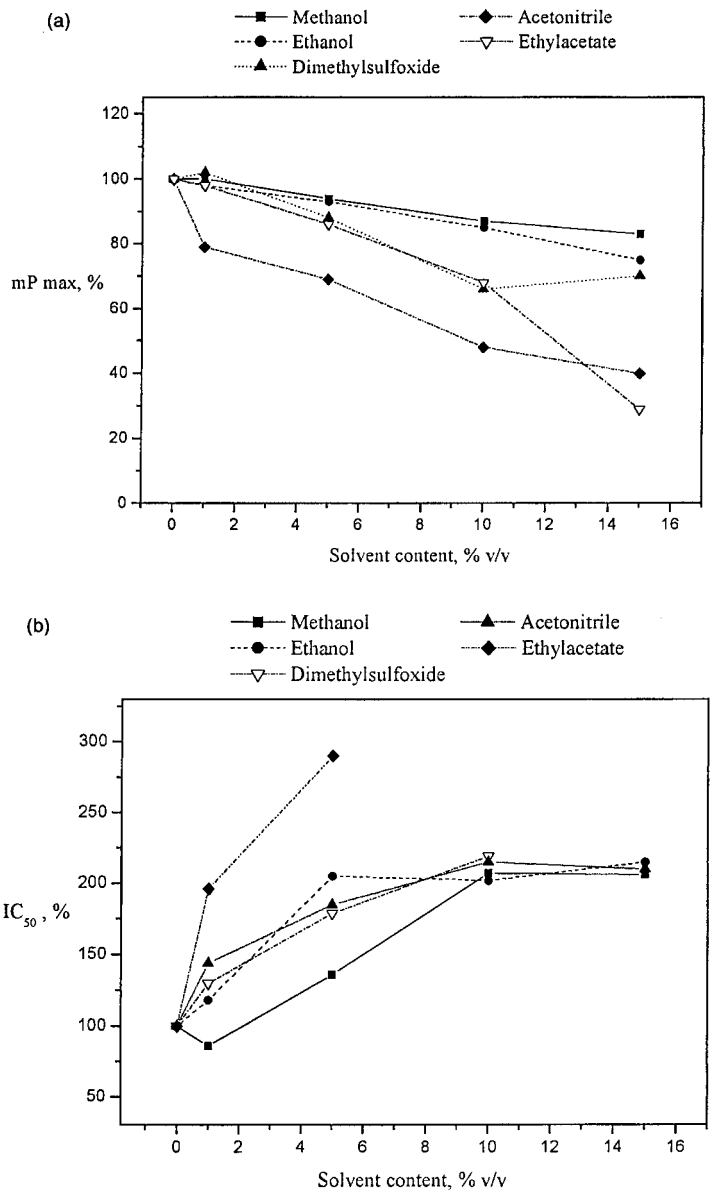

FIGURE 5 Effect of organic solvents on acetochlor PFIA performance: (a) reduction in maximal fluorescence polarization value; (b) increase of IC_{50}. Different concentrations of solvents (final concentration in the incubation volume was 0, 1, 5, 10 and 15% v/v) were used to prepare standards of acetochlor. Assay was run as described in experimental section. The reported results were extracted from four parameter logistic equations used to fit the standard curves.

solvent type and concentration. Methanol and ethanol did not adversely affect assay performance, resulting in 13–15% loss in maximum fluorescence polarization signal at concentrations up to 10% (Fig. 5a). The other solvents, dimethylsulfoxide, ethyl acetate and especially acetonitrile, caused more dramatic decrease in maximum signal. The solvent effect on IC_{50} (Fig. 5b) was not in the same order as that on maximum polarization signal. With almost all solvents, nearly the same IC_{50} deterioration was found, equal to twofold lower sensitivity. Ethyl acetate brings a more dramatic drop of sensitivity, resulting in threefold increase in IC_{50} at concentration 5% v/v; with further increment of ethyl acetate content, the inhibition curve could not be analyzed using four parameter logistic curve fitting. These data do not correlate with any solvent properties such as hydrophobicity, dielectric constant or viscosity. The mechanism of solvent effects is, therefore, of a complex nature.

Summarizing, up to 10% content of methanol and ethanol seem to be compatible with the acetochlor PFIA, owing to the minor interference with assay performance. According to the standard protocol of the PFIA reported in this paper, sample is diluted 10 times in the analyzed probe and, therefore, 100% methanol and ethanol extracts can be directly assayed.

CONCLUSIONS

We report here, for the first time, the production of specific antibodies and development of a polarization fluoroimmunoassay for the chloroacetanilide herbicide acetochlor. To produce enhanced assay specificity towards the target compound, we designed hapten structure to preserve important antigenic determinants of acetochlor such as the substituted aromatic ring and the acetamide moiety side chain. To enable coupling to carrier protein, the chloroacetamide moiety was derivatized with 3-mercaptopropionic acid. As was expected, cross-reactivity studies proved that antibodies raised against this immunogen allow specific of detection of acetochlor in the presence of related compounds. The assay was evaluated for the effects of organic solvents and two solvents were found to be compatible with acetochlor PFIA. Future work will be focused on improvement of acetochlor PFIA performance by careful selection of competitor structure and validation of the assay with real environmental samples.

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References

[1] United States Environmental Protection Agency, *Prevention, Pesticides and Toxic Substances. Questions and Answers*, Conditional Registration of Acetochlor, U.S. EPA, Washington, DC (1994).
[2] C.E. Lindley, J.T. Stewart and M.W. Sandstrom, *J. AOAC Int.*, **79**, 962–966 (1996).
[3] P.D. Capel, L. Ma, B.R. Schroyer, S.J. Larson and T.A. Gilchrist, *Environ. Sci. Technol.*, **29**, 1702–1705 (1995).
[4] L.Q. Huang, *J.AOAC Int.*, **72**, 349–354 (1989).

[5] M. Natangelo, S. Tavazzi, R. Fanelli and E. Benfenati, *J. Chromatogr. A*, **859**, 193–201 (1999).
[6] S. Hong and A.T. Lemley, *J. Chromatogr. A*, **822**, 253–261 (1998).
[7] A. Balinova, *J. Chromatogr. A*, **643**, 203–207 (1993).
[8] M.-C. Hennion and D. Barcelo, *Anal. Chim, Acta.*, **362**, 3–34 (1998).
[9] P.C. Feng, S.J. Wratten, S.R. Horton, C.R. Sharp and E.W. Logussch, *J. Agic. Food Chem.*, **38**, 159–163 (1990).
[10] P.C. Feng, S.R. Horton and R. Sharp, *J. Agric. Food Chem.*, **40**, 211–214 (1992).
[11] D.S Aga, E.M. Thurman and M.L. Pomes, *Anal. Chem.*, **66**, 1495–1499 (1994).
[12] J.C. Hall, L.K. Wilson and R.A. Chapman, *J. Environ. Sci. Health*, **27**, 523–544 (1992).
[13] T.S Lawruk, C.E. Lachman, S.W. Jourdan, J.R. Fleeker, D.P. Herzog and F.M. Rubio, *J. Agric. Food Chem.*, **41**, 1426–1431 (1993).
[14] T.S. Lawruk, C.S. Hottenstein, D.P. Herzog and F.M. Rubio, *Bull. Environ, Cantam. Toxicol.*, **48**, 643–650 (1992).
[15] D.M. Tessier and J.M. Clark, *Anal. Chim. Acta*, **376**, 103–112 (1998).
[16] C.A. Striley, R.E. Biagini, J.P. Mastin, B.A. MacKenzie and S.K Robertson, *Anal. Chim. Acta*, **399**, 109–114 (1999).
[17] W.B. Dandliker, M. Hsu, J. Levin and B.R. Rao, *Meth. Enzymol.*, **74**, 3–28 (1981).
[18] D.L Colbert and R.E. Coxon, *Clin. Chem.*, **34**, 1948–1949 (1988).
[19] S.A. Eremin, In: J.O. Nelson, A.E. Karu and R.B. Wong, (Eds.), *Immunoanalysis of Agrochemicals: Emerging Technologies*, *ACS Symp. Ser.*, Vol. 586, American Chemical Society, Washington DC, pp. 223–234 (1995).
[20] S.A. Eremin and J.V. Samsonova, *Anal. Lett.*, **27**, 3013–3025 (1994).
[21] F.G. Sanchez, A.N. Díaz, F. Alonso and J. Lovillo, *J. Agric. Food Chem.*, **41**, 2215–2219 (1993).
[22] P. Önnerfjord, S. Eremin, J. Emneus and G. Marko-Varga, *J. immunol. Methods*, **213**, 31–39 (1998).
[23] F.G. Sanchez, A.N. Díaz, A.F. González Díaz and J. Lovillo, *Anal. Chim. Acta*, **439**, 131–138 (2001).
[24] M.H. Goodrow, R.O. Harrison and B.D. Hammock, *J. Agric. Food Chem.*, **38**, 990–996 (1990).
[25] E. Harlow and D. Lane, *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York, pp. 673 (1988).
[26] M. Pourfarzaneh, G.W. White, J. London and D.S. Smith, *Clin. Chem.*, **26**, 730–733 (1980).
[27] J. Gascón, A. Qubińa, B. Ballesteros, D. Barceló, F. Camps, M.-P. Marco, M.A. González-Martínez, S. Morais, R. Puchades and A. Maquieira, *Anal. Chim. Acta*, **347**, 149–162 (1997).
[28] D. MacDougall and W.B. Crummett, *Anal. Chem.*, **52**, 2242–2249 (1980).
[29] F. Jung, S.J. Gee, R.O. Harrison, M.H Goodrow, A.E. Karu, A.L. Braun, Q.X. Li and B.D. Hammock, *Pest. Sci.*, **26**, 303–317 (1989).
[30] M.H Goodrow and B.D. Hammock, *Anal. Chim. Acta*, **376**, 83–91 (1998).
[31] M.H. Goodrow, J.R. Sanborn, D.W. Stoutamire, S.J. Gee and B.D. Hammock, In: J.O. Nelson, A.E. Karu and R.B. Wong, (Eds.), *Immunoanalysis of Agrochemicals*, *Emerging Technologies*, *ACS Symp. Ser.*, Vol. 586, American Chemical Society, Washington DC, pp. 119–139 (1995).
[32] P.C. Feng, S.J. Wratten, E.W Logussch, S.R. Horton and C.R, Sharp, In: J.N. Van Emon and R.O. Mumma, (Eds.), *Immunochemical Methods for Environmental Analysis*, *ACS Symp. Ser.*, Vol. 442, American Chemical Society, Washington DC, pp. 180–192 (1990).
[33] Q.-X. Li, M.-S. Zhao, S.J. Gee, M. Kurth, J.N. Seiber and B.D. Hammock, *J. Agric. Food Chem.*, **39**, 1685–1692 (1991).
[34] A. Oubińa, B. Ballesteros, R. Galve, D. Barceló and M.-P. Marco, *Anal. Chim. Acta*, **387**, 255–266 (1999).
[35] M.P. Marco, S. Gee and B.D. Hammock, *Trends Anal. Chem.*, **14**, 415–425 (1995).
[36] M. Worterg, K. Cammann, K. Strupat and F. Hillenkamp, *Fresenius J. Anal. Chem.*, **348**, 240–245 (1994).
[37] S.A. Eremin, *Food Technol. Biotechnol.*, **36**, 235–243 (1998).
[38] M.A. O'Connell, B.A. Belanger and P.A. Haaland, *Chemometr. Intell. Lab.*, **20**, 97–114 (1993).
[39] P. Schneider and B.D. Hammock, *J. Agric. Food Chem.*, **40**, 525–530 (1992).
[40] D. Rodbard and D.M. Hutt, In: Proceedings, Radioimmunoassay and Related Procedures in Medicine, 1, International Atomic Energy Agency, Vienna, Austria, New York, pp. 165–166 (1974).