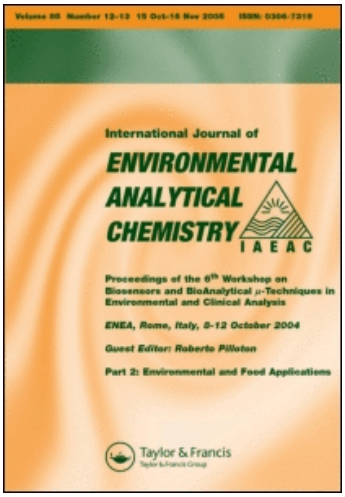

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Gyöngyvér Hegedűs[a]; Vitalia Krikunova[ab]; Iván Bélai[a]; Sergei Eremin[b]; András Székács[a]
[a] Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary [b] M.V. Lomonosov Moscow State University, Moscow, Russia

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AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ACETOCHLOR

GYÖNGYVÉR HEGEDŰS[a], VITALIA KRIKUNOVA[a,b], IVÁN BÉLAI[a], SERGEI EREMIN[b] and ANDRÁS SZÉKÁCS[a,*]

[a]*Plant Protection Institute, Hungarian Academy of Sciences, POB 102, H-1525 Budapest, Hungary;* [b]*M.V. Lomonosov Moscow State University, 119899 Moscow, Russia*

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Competitive enzyme-linked immunosorbent assays (ELISAs) were developed in hapten-homologous and hapten-heterologous formats for the detection of the chloroacetanilide herbicide acetochlor. ELISA systems were devised using antibodies generated against acetochlor conjugated to carrier proteins through a thioether moiety replacing the chlorine atom in the parent structure, while haptens modified both on the chloroacetyl moiety and on the ethoxymethyl group of acetochlor have been used for coating antigens. The optimized ELISA systems allowed the detection of acetochlor 0.2–65 μg/L, and cross-reactivity studies revealed high specificity of the immunoassay: only four (propisochlor, butachlor, alachlor and metolachlor) among 18 structurally related acetanilide herbicides, fungicides and intermediates showed significant (> 1%) cross-reactivity, with even the highest value (propisochlor) being below 10%. Assay performance was not affected detrimentally by methanol up to 10% (v/v) and ethanol up to 5% (v/v). Assay performance was tested by measuring acetochlor concentration in water samples and compared favorably ($r^2 = 0.976$) with those detected by gas chromatographic method coupled with mass spectrometry (GC-MS) using solid-phase microextraction (SPME) for sample preparation.

Keywords: Acetochlor; Enyzme immunoassay; Optimization; Sensitivity

INTRODUCTION

Acetochlor (**1**) is a preemergent or early postemergent herbicide of selective action in maize. Applied to the soil or to plant surfaces, this systemic herbicide is translocated within the plant. Similarly to other chloroacetanilide herbicides, acetochlor also was found to be decomposed to a wide range of metabolites [1] depending on metabolizing organisms and conditions. The compound is stable to both acidic or alkaline hydrolysis (pH 3.6 and 9) [1] and although it is slowly decomposed by photolysis, its metabolites show higher photochemical stability than the parent herbicide [2]. Its half-life is fairly short in soil under aerobic conditions (8–12 days) [1], yet it was claimed to be a leacher in soil and a potential ground-water contaminant [3].

*Corresponding author. Fax: +36-1-4877555. E-mail: aszek@nki.hu

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Although manufacturer studies repeatedly claimed that acetochlor is non-mutagenic on rat germ cells [4,5], it has been shown to be oncogenic and mutagenic (along with alachlor and metholachlor) through quinonimine metabolites [6–8]. Studies indicated its capability to induce thyroid and liver tumors [9]. Nonetheless, it has been mentioned as an example for non-genotoxic carcinogens [10]. An additional concern is its action on certain aquatic animals: it interacts with thyroid hormone and corticosterone receptors, thereby disturbing natural metamorphosis in frogs [11]. The compound has lately become a suspected endocrine disruptor interacting with the estrogen receptor in rats [12].

Acetochlor and its metabolites are required to be analyzed in commodity and in environmental samples. Like other chloroacetanilide herbicides, the compound has lately been analyzed in water by high-performance liquid chromatography coupled with mass-spectrometry (HPLC-MS) [13–15] or by gas chromatography with mass spectrometric detection (GC-MS) using solid-phase extraction (SPE) or solid-phase microextraction (SPME) [16,17]. Nonetheless, less sophisticated GC methods can also be applied as used for the detection of other chloroacetanilides [18–21].

With the spread of immunoanalytical methods in environmental analysis, as early examples of the use of enzyme-linked immunosorbent assay (ELISA) systems for monitoring pesticide active ingredients and metabolites, ELISA systems were developed for alachlor and other chloroacetanilide herbicides (metolachlor and metazachlor) [22–27] and their metabolites [28,29] allowing detection of these target analytes in the low ppb or high ppt range. Although concerns have been expressed regarding false positive results due to cross-reactivity of the antibodies, several commercial microtiter plate ELISA systems have been developed and used in combination with SPE for detection of alachlor in various water samples [30–34]. Among commercial immunoassays of various formats, magnetic particle-based ELISAs for alachlor and metolachlor [35–37], as well as an immunomigration device [38] and a liposome immunomigration strip assay [39] have been developed for the detection of alachlor.

An advantage of these immunoassays is their excellent sensitivity and specificity for their target analytes, yet as a drawback, they can rarely be used for the detection of other chloroacetanilide herbicides including acetochlor. Acetochlor has traditionally been heavily applied in Central and Eastern Europe, and with the limitations in the use of alachlor it reached even higher volume applications. Moreover, because chloroacetanilide herbicides are often used in combination, highly selective analytical methods allowing simple and rapid detection of acetochlor among other active ingredients are sought. The present work describes the development and optimization of an ELISA system for the detection of acetochlor.

EXPERIMENTAL

Chemicals

Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) or from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise. 2-Ethyl-6-methylaniline was obtained from Nitrokémia Rt, Hungary (Fűzfőgyártelep, Hungary), chloroacetanilide herbicide and anilide fungicide active ingredients were extracted from respective commercial pesticide preparations in our laboratory [40]. The purity and structures

of haptens and related compounds were confirmed by melting points (uncorrected), analytical thin-layer chromatography (TLC), as well as [^{1}H]- and [^{13}C]-NMR spectroscopy. Analytical TLC was performed on 250 μm silica gel F_{254} plates (Merck KGaA, Darmstadt, Germany), using hexane–acetone (9 : 1 and 8 : 2 v/v) for elution of 2–4, or hexane–acetone–acetic acid (6 : 4 : 0.1 v/v) for 5. Compounds were detected by UV adsorption at 254 nm and by exposure in an iodine vapor chamber. Spectra/Por membrane from Spectrum (Houston, TX, USA) was used for dialysis (MWCO: 12–14 kD). Solid phase microextraction (SPME) fibers and holder assembly were purchased from Supelco (Bellefonte, PA, USA). The following buffers and solutions were used: (1) carbonate (coating) buffer: 100 mM sodium hydrogencarbonate (pH 9.6) in distilled water; (2) phosphate buffer: saline (PBS) 150 mM sodium chloride and 15 mM potassium dihydrogenphosphate (pH 7.4) in distilled water; (3) assay buffer: PBS buffer containing 0.05% (v/v) Tween 20 (PBST 0.05); (4) washing buffer: PBS buffer containing 0.2% (v/v) Tween (PBST 0.2). Substrate, chromophore and other reagents for ELISA are listed in the detailed protocol description below, was carried out on a Varian Saturn 2000 GC-MS instrument (Varian Walnut Creek, CA, USA), ELISAs were carried out in high capacity 96-well microplates (Nunc, Roskilde, DK, #442404), and were read in an iEMS microplate reader (LabSystems, Helsinki, Finland).

Hapten Synthesis

The chemical structures of acetochlor (**1**) and haptens are shown in Fig. 1. The mercaptopropionyl derivative of acetochlor, 2-(2-carboxyethylthio)-6′-ethyl-*N*-(ethoxymethyl)-2′-methylacetanilide (**2**) was prepared from **1** and 3-mercaptopropionic acid in basic ethanolic solution according to a method described in the literature [25,41,42]. Other haptens were synthesized by maintaining the chloroacetyl moiety in them.

Ethyl *N*-(2-ethyl-6-methyphenyl)-4-aminobutyrate (**3a**): A solution of 2-ethyl-6-methylaniline (4.05 g, 30 mmol), ethyl 4-bromobutyrate (7.49 g, 38.4 mmol), anhydrous sodium acetate (3.20 g, 39.0 mmol) in dry ethanol (12 mL) was refluxed with stirring for 30 h. The solvent was removed under reduced pressure, then the residue was dissolved in 1 N aqueous hydrochloric acid. This aqueous solution was washed with diethyl ether, neutralized with sodium hydrogen carbonate, and the isolated oil was extracted with diethyl ether. The organic phase was washed with water and brine, then dried over magnesium sulphate. The product was purified by column chromatography on silica gel using hexane/acetone (12 : 1 v/v) to yield pure **3a** (3.58 g, 48.4%) as oil.: [^{1}H]-NMR(ppm): δ 1.23 t (3H, $J = 7.5$ Hz, CH_3–CH_2Ar), 1.26 t (3H, $J = 7.1$ Hz, CH_3–CH_2O), 1.89–1.96 m (3-CH_2), 2.45 t (2H, $J = 7.3$ Hz, CH_2–CO_2H), 2.63 q (2H, CH_2–Ar), 2.97 t (2H, $J = 7.1$ Hz, CH_2–N), 4.14 q (2H, CH_2–O), 6.86–6.89 m (1H, ArH-4), 6.99–7.03 m (2H, ArH-3 and -5).

Ethyl *N*-(2-ethyl-6-methylphenyl)-4-aminohexanoate (**3b**) was synthesized similarly using ethyl 6-bromohexanoate. In the work-up procedure prior to column chromatography the solution in diethyl ether was washed with 1 N aqueous hydrochloric acid to remove the bulk of the unreacted aniline. Yield: 3.37 g, 41.7%, [^{1}H]-NMR (ppm): δ 1.23 t (3H, $J = 7.2$ Hz), 1.24 t (3H, $J = 7.2$ Hz), 1.39–1.45 m (2H, 4-CH_2), 1.55–1.66 m (4H, 3- and 5-CH_2), 2.27 s (3H, CH_3–Ar), 2.31 t (2H, $J = 7.4$ Hz, CH_2–CO_2H), 2.69 q (2H, $J = 7.5$ Hz, CH_2–Ar), 2.97 td (2H, CH_2–N), 4.10 q (2H, $J = 7.2$ Hz, CH_2–O), 6.97 dd (1H, $J = 7.3$, 2.0 and 2.3 Hz, 3- or 5-ArH), 7.00 t (1H, 4-ArH), 7.05 dd (1H, $J = 7.4$, 2.1 and 2.3 Hz, 3- or 5-ArH).

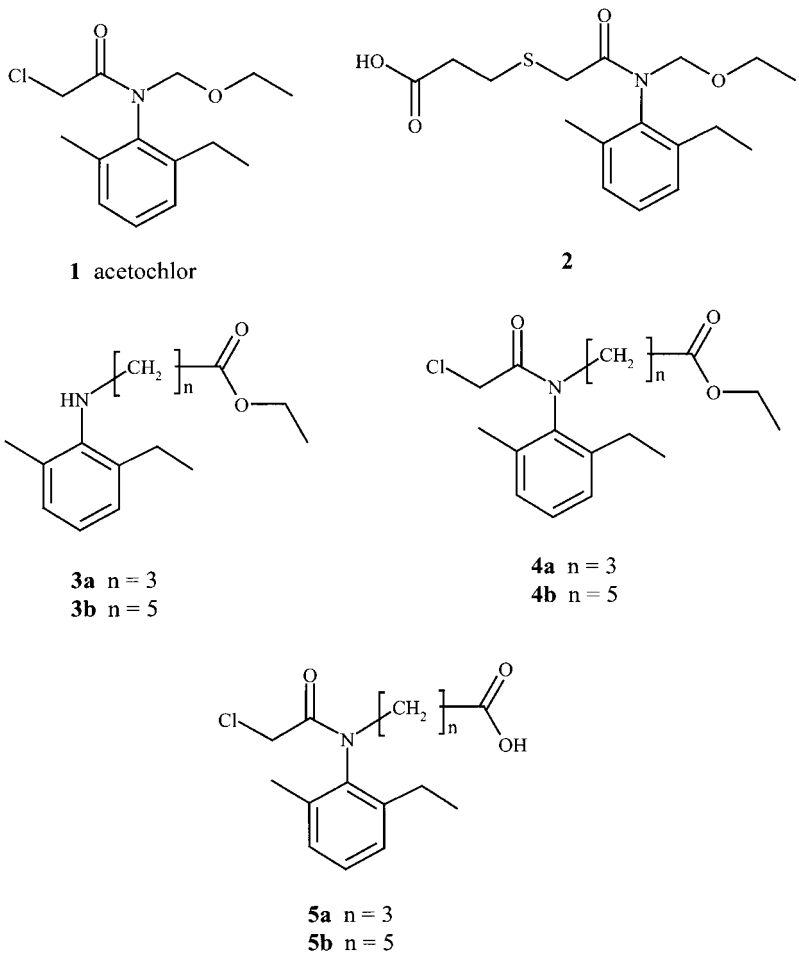

FIGURE 1 Chemical structure of acetochlor (**1**), haptenic compounds and intermediates.

Ethyl *N*-chloroacetyl-*N*-(2-ethyl-6-methylphenyl)-4-aminobutyrate (**4a**): To a cooled and stirred solution of **3a** (1.3 g, 5.2 mmol) in dry toluene (15 mL) containing pyridine (0.71 mL, 7.3 mmol) was dropped chloroacetyl chloride (0.62 mL, 7.8 mmol) dissolved in dry toluene (3 mL) within 10 min. After stirring for 0.5 h with cooling and then for 1 h at ambient temperature the reaction mixture was washed successively with water, with 1 N aqueous hydrochloric acid three times and again with water, dried over magnesium sulphate then evaporated to obtain **4a** (1.5 g, 92.5%) as oil, which was hydrolyzed to **5a** without further purification.

Ethyl *N*-chloroacetyl-*N*-(2-ethyl-6-methylphenyl)-4-aminohexanoate (**4b**) was similarly prepared from **3b** (2.91 g, 10.5 mmol). At the end of the work-up procedure, upon dissolving the oily material in hexane (30 mL), a solid by-product (precipitated after a one-day standing) was filtered out.

N-Chloroacetyl-*N*-(2-ethyl-6-methylphenyl)-4-aminobutyric acid (**5a**): A solution of **4a** (1.4 g, 4.3 mmol) in AcOH (6 mL), water (8 mL) and methanesulfonic acid (2 mL) was refluxed for 3 h. The reaction mixture was poured into water (25 mL) and extracted with diethyl ether. The organic layer was washed with water and the product was

extracted into 5% sodium hydrogen carbonate solution (3×20 mL). The aqueous solution was washed with diethyl ether, neutralized with 4 N aqueous hydrochloric acid and the product was extracted with diethyl ether. The organic phase was dried over magnesium sulphate and concentrated in vacuum to afford **5a** (0.71 g, 55.5%) which solidified on standing, m.p. 108–109°C, [^{1}H]-NMR (ppm): δ 1.25 t (3H, $J = 7.6$ Hz), 1.88–1.96 m (2H, 3-CH_2), 2.25 s (CH_3–Ar), 2.39 t (CH_2–CO_2H), 2.55 qd (2H, $J = 7.6$ Hz as well as 2.46 and 2.31 Hz, CH_2–Ar), 3.55–3.72 m (2H, CH_2–N), 3.67 s (2H, CH_2–C1), 7.15 dd (1H, $J = 7.6$ and 1.1 Hz, 3- or 5-Arh), 7.22 dd (1H, 3- or 5-ArH), 7.26–7.29 m (1H, 4-ArH), [^{13}C]-NMR (ppm): δ 14.29 (CH_3–CH_2), 18.44 (CH_3–Ar), 22.03 (CH_2–Ar), 23.51 (CH_2–CH_2–CH_2), 31.62 (CH_2–CO_2H), 41.52 (CH_2–C1), 49.50 (CH_2–N), 127.30 (ArCH), 129.04 (ArCH), 129.25 (ArCH), 135.77 (C–Ar), 138.26 (C–Ar), 141.54 (Ar–C–N), 167.11 (CO–N), 177.82 (CO_2H).

N-Chloroacetyl-*N*-(2-ethyl-6-methylphenyl)-4-aminohexanoic acid (**5b**) was synthesized similarly from **4b** (2.82 g, 8.0 mmol) to yield **5b** (1.66 g, 63.8%) as oil, [^{1}H]-NMR (ppm): δ: 1.24 t (3H, CH_3), 1.32–1.38 m (2H, 4-CH_2), 1.54–1.67 m (4H, 3-, 5-CH_2), 2.25 s (3H, CH_3–Ar), 2.33 t (2H, $J = 7.4$ Hz, CH_2–CO_2H), 2.55 qd (2H, $J = 7.6$ and 1.7 Hz, CH_2–CH_3), 3.48–3.64 m (2H, CH_2–N), 3.66 s (3H, CH_2–C1), 7.15 dd (1H, $J = 7.6$ and 1.8 Hz, 3- or 5-ArH), 7.22 dd (1H, $J = 7.9$ and 1.8 Hz, 3- or 5-ArH), 7.25–7.29 m (1H, 4-ArH), [^{13}C]-NMR (ppm): δ 14.40 (CH_3–CH_2), 18.49 (CH_3–Ar), 23.53 (4-C), 24.31 (CH_2–CH_3), 26.64 (3-C), 27.10 (5-C), 33.82 (CH_2–CO_2H), 41.70 (CH_2–C1), 50.19 (CH_2–N), 127.26 (ArCH), 128.93 (ArCH), 129.20 (ArCH). 135.82 (C–Ar), 138.45 (C–Ar), 141.58 (Ar–C–N), 166.75 (CO–N), 179.18 (CO_2H).

Preparation of Protein Conjugates of Acetochlor

Protein conjugates were prepared either by coupling acetochlor to thiolated proteins [25] or by conjugating haptens **2**, **5a** or **5b** to carrier proteins by the active ester method [43] using *N*-hydroxysuccinimide (NHS) and dicyclohexyl carbodiimide (DCC) as a dehydrating agent. In the direct coupling of acetochlor, carrier proteins, bovine serum albumin (BSA) or ovalbumin (OVA) were first thiolated with *S*-acetylmercaptosuccinic anhydride (AMSA) [22], and acetochlor was *in situ* conjugated without isolating the thiolated proteins as described by Yakovleva *et al*. [42].

In coupling haptens **2**, **5a** or **5b** to carrier proteins 0.15 mmol of the appropriate hapten (40.5 mg, 44.7 mg or 48.9 mg of **2**, **5a** or **5b**, respectively) and NHS (12.3 mg, 0.18 mmol) were dissolved in dry dimethyl formamide (DMF) (3.1 mL), DCC (36.8 mg, 0.18 mmol) was added, and the reaction mixture was stirred for 2 h at room temperature, and was filtered. In the meantime, OVA (150 mg) was dissolved in the mixture of water (16 mL) and DMF (0.9 mL) in a separate reaction vessel, and the above activated hapten solution was added dropwise. The mixture was stirred for 24 h at room temperature, and was dialyzed against water for 2 days (water exchange in every 4 h). Hapten 2 was similarly conjugated to BSA as well. The resultant conjugates were stored in a refrigerator until use. Hapten densities of the BSA conjugate were determined by MALDI-MS by comparing the molecular weight obtained for the standard BSA with that of conjugates. MALDI spectra were recorded using the conjugate **2**-BSA (30 pmol, 0.6 µL of 50 µM solution) mixed with sinapinic acid (22.5 nmol, 0.5 µL of 45 mM solution). The conjugates were stored at −20°C until use.

Immunizations were carried out using the conjugate **2**-BSA as described by Yakovleva *et al.* [42].

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were carried out in 96-well microplates coated by incubating overnight at 4°C with 100 μL per well of the coating antigen (e.g., 5 μg/mL of **2**-OVA or **1**-AMSA-OVA, or 1 μg/mL of **5b**-OVA) in 0.1 M carbonate buffer (pH = 9.6). After washing, plates were blocked by incubation for 1 h at room temperature with 150 μL per well of blocking agent (1% gelatin in PBS (pH 7.4)). After a washing step, standards or samples (50 μL per well) and diluted antiserum (50 μL per well) were added, and plates were incubated for 90 min at 37°C. After an additional wash, 100 μL per well of goat anti-rabbit IgG-HRP conjugate (BioRad Laboratories, Hercules, CA, USA) at a dilution of 1 : 12,000 was added, and incubated for 60 min as before. After a final washing step, 200 μL per well of the substrate solution (1.2 mM H_2O_2 with 3 mM 1,2-phenylenediamine in 0.5 M citrate buffer (pH 5.0) was added, and after sufficient color development (after 10–60 min) the enzymatic reaction was stopped by the addition of 50 μL per well of 4 N H_2SO_4. Color intensities in the wells (endpoint mode) were read at 492 nm.

Detection of Acetochlor by GC-MS Using SPME

Acetochlor was detected in distilled water using gas chromatography with a mass spectrometric detector (GC-MS). Distilled water was spiked with acetochlor at concentrations between 0.5 and 50 μg/L, and samples were subjected to solid-phase microextraction (SPME) prior to GC-MS. Thus, 4-mL portions of each water sample were directly extracted by SPME using a carbowax/divinylbenzene (CW/DVB) fiber of 65 μm thickness. Extraction time was 20 min at room temperature with stirring on a magnetic stirrer. After extraction, sample desorption from the fiber was carried out at 250°C by direct injection into the GC system.

GC-MS conditions were as follows: fused-silica column CP-Sil 8 CB, 0.25 μm film thickness, 30 m length × 0.25 mm internal diameter (Chrompack BV, Middelburg, the Netherlands); column temperature programmed from 90°C (held for 1 min) to 300°C at a rate of 25°C/min; injector temperature, 250°C; injection mode, splitless; helium was used as carrier gas, pressure, 0.097 MPa; ionization current, 350 μA; electron energy, 70 eV. The ion trap was scanning in EI-mode from 45 to 400 amu. Solvent delay was 3 min. The selected ions for quantitation of acetochlor were 162 and 174.

Sample Preparation

River water samples were collected from the Danube river (Budapest, Hungary). Samples of river water and distilled water were spiked at concentrations of acetochlor of 0.5, 2, 5, 20 and 50 μg/L. Spiking with acetochlor was also carried out in apple juice (100% fruit content, 11.5% dry weight, Rauch Hungária Kft., Budapest, Hungary) from commercial source, undiluted and diluted to various rates (1 : 5, 1 : 10, 1 : 20, 1 : 50 and 1 : 100) with PBST 0.05. Corn samples were ground in a ball mill, and were extracted for analysis. Corn flour (0.5 g) was extracted with a mixture of 1.5 mL of methanol and 28.5 mL of PBST 0.05 with vigorous shaking overnight. The filtered extract was spiked with acetochlor at three different spiking concentrations i.e., at 1000, 100 and 20 mg/L. Fish samples were taken from animals collected from the

Don river (Rostov-na-Donu, Russia). The extracts were prepared as follows. 10 g of fish tissue was milled and extracted with 30 mL of ethanol with vigorous shaking for 30 min. Then filtered extracts were stored at 4°C. For ELISA, the extracts were diluted 1 : 20 in PBST 0.05 before measurements.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugation

In most previous attempts for immunoassay development for chloroacetanilides, a bioisosteric replacement of the chlorine atom in the chloroacetyl moiety has been used for the synthesis of both the immunogen and the coating antigens. Such replacement has been successfully applied in other ELISA systems including those for triazine herbicides. Yet, in the case of chloroacetanilides, the chlorine atom is a characteristic reactive functional moiety of the molecule. In the case of the immunogen, the replacement of a reactive moiety with a more stable one (i.e. the mercaptoalkyl group) is considered an advantage. In consequence, the conjugate **2**-BSA was used in the present study as the immunogen. To obtain haptens for coating antigens, however, both the chloroacetyl moiety or the *N*-alkoxyalkyl chain of acetochlor was modified in order to broaden chemical diversity of the haptenic compounds used. For the ease of chemical synthesis, the ethoxymethyl moiety was replaced by carboxyalkyl groups omitting the oxygen from the chains in these haptens. In this set of compounds, a butyric acid derivative (**5a**) and a hexanoic acid derivative (**5b**) were prepared. These haptens, *N*-chloroacetyl-*N*-(2-ethyl-6-methylphenyl)-4-aminobutyric acid (**5a**) and its 4-aminohexanoic acid analog (**5b**) were synthesized in three steps. First, 2-ethyl-6-methylaniline was reacted with ethyl 4-bromobutyrate or ethyl 6-bromohexanoate, respectively, in ethanol using sodium acetate as acid acceptor to afford ethyl *N*-(2-ethyl-6-methylphenyl)-4-aminobutyrate (**3a**) or ethyl *N*-(2-ethyl-6-aminohexanoate (**3b**). Compound **3a** has been mentioned, but not characterized in a patent application [44]. *N*-chloroacetylation of **3a** or **3b** in toluene in the presence of pyridine gave **4a** or **4b**, respectively, which were hydrolyzed in acidic medium (acetic acid/water/methanesulfonic acid) to obtain **5a** or **5b**, respectively.

Haptens **2**, **5a** and **5b** were covalently attached through its carboxylic group to the lysine groups of BSA or OVA by the active ester method using NHS [43]. The BSA-conjugate of **2** was prepared to be used as an immunogen, OVA conjugates were prepared for purposes of being used as coating antigens. Characterization of **2**-BSA by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) indicated a molar protein/hapten density of 1 : 6.5 (the ratio was calculated by comparing M + H peaks for the conjugate and with that of intact BSA). Conjugation of **5b** to proteins resulted in conjugates of too high lipophilicity to prepare coating antigens of proper water solubility. Therefore, OVA-conjugate of a shorter chain analogue, **5a** was only used in the indirect ELISA systems.

Immunization and Antiserum Characterization

Crude antisera were characterized by their titers (serum dilution causing 50% binding) in ELISA, and the sensitivity of the immunoassays was evaluated by their IC_{50} value

TABLE I Titration of acetochlor antiserum using different coating antigens at various concentrations

Coating antigen		*Serum titer*
Type	*Concentration* [μg/mL]	
1-AMSA-OVA	0.01	1 : 2000
	0.025	1 : 4000
	0.1	1 : 5000
	0.5	1 : 8000
	1	1 : 12,000
	5	1 : 40,000
	10	1 : 80,000
2-OVA	0.1	1 : 2000
	0.2	1 : 3000
	1	1 : 4000
	5	1 : 39,000
	10	1 : 46,000
5a-OVA	0.1	1 : 270
	1	1 : 2300
	5	1 : 15,000
	10	1 : 28,000

TABLE II Detection sensitivity (characterized by the IC_{50} value) and assay conditions of the optimized ELISA systems

Coating antigen		*Serum dilution*	IC_{50}[a] [ng/mL]	LOD[b] [ng/mL]
Type	*Concentration* [μg/mL]			
1-AMSA-OVA	5	1 : 60,000	2.64 ± 1.14 (0.62)	0.3
2-OVA	5	1 : 40,000	4.05 ± 1.53 (0.64)	0.2
5b-OVA	1	1 : 3000	2.15 ± 0.77 (0.51)	0.2

[a]IC_{50} value defined as the analyte concentration causing a 50% decrease in the assay signal as compared to the uninhibited signal. Values in parentheses indicate the slope of the sigmoid standard curve at the IC_{50} value.
[b]Limit of detection defined as the analyte concentration, causing a decrease of three standard deviations from the blank standard absorbance (a mean of at least three replicates).

(the analyte concentration causing a 50% decrease in the assay signal as compared to the uninhibited signal), curve slope at the IC_{50}, and the limit of detection (the analyte concentration, causing a decrease of three standard deviations from the blank standard absorbance). Coating antigens were applied at concentrations between 0.01 and 10 μg/mL. As seen in Table I, titer values were strongly dependent on the type and concentration of the coating antigen applied.

Assay parameters of the optimized immunoassays are listed in Table II. As seen from the IC_{50} values, coating antigens **1**-AMSA-OVA and **2**-OVA allowed titers of 1 : 60,000 and 1 : 40,000, and resulted in statistically not significantly different IC_{50} values of 2.67 and 4.05 μg/L, respectively ($n = 5$, $LSD_{5\%} = 1.74$, $t = 2.09$, $t_{5\%} = 2.78$). The data do not indicate significant difference between these hapten-homologous and hapten-heterologous indirect ELISA systems. The hapten-heterologous ELISA based on coating antigen **5a**-OVA allowed a comparable IC_{50} value to those in the

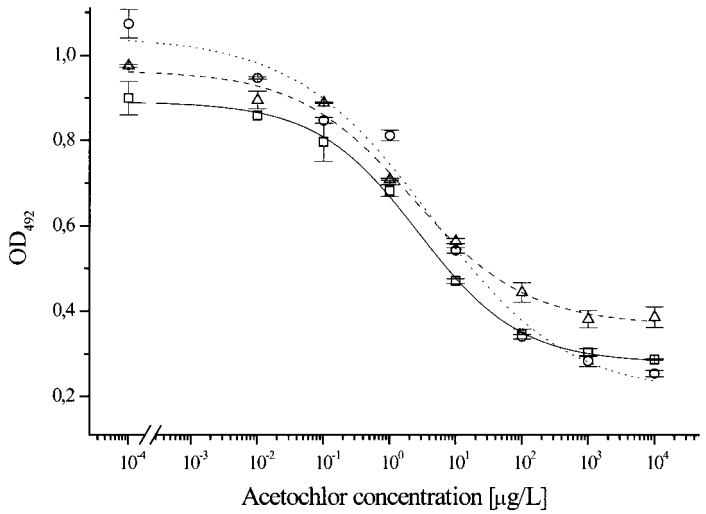

FIGURE 2 Standard inhibition curves, under optimized assay conditions, in the ELISA systems for acetochlor. Assay conditions: coating antigen 5µg/mL **1**-AMSA-OVA, serum dilution 1 : 60,000 (□–□); coating antigen 5 µg/mL 2-OVA, serum dilution 1 : 40,000 (○ · · · ○); coating antigen 1 µg/mL **5a**-OVA, serum dilution 1 : 1000 (Δ– –Δ). Corresponding IC_{50} values were 2.67 ± 1.14, 4.05 ± 1.53 and 2.15 ± 0.77 µg/L, respectively. Assays were carried out in a single microtiter using spiked concentrations of acetochlor of 10,000, 1000, 100, 10, 1, 0, 1 and 0 µg/L.

first two ELISA systems, but the antiserum titer was significantly poorer with this coating antigen. The limits of detection (LOD) also appeared similar to all three optimized immunoassays. Optimal standard curves for the immunoassays with the highest sensitivity are seen in Fig. 2. It has to be noted that lower IC_{50} values may be reached at lower coating-antigen concentrations or at higher serum dilutions, but such modifications resulted in worse recoveries.

Cross-reactivity Studies in the Optimized ELISA System

Recognition of the haptenic compounds and intermediates, as well as six chloroacetanilide herbicides and five anilide fungicides was tested in the optimized ELISA system using **1**-OVA as coating antigen. Relative cross-reactivity (CR) values, defined as the percentage ratio between the IC_{50} value of the reference compound (acetochlor) and that of the given compound, are listed Table III. It has to be mentioned that CR values are better interpreted stoichiometrically when molar IC_{50} are compared, yet values in the table are based on IC_{50} values expressed in µg/L units. The reason for this is to compare absolute sensitivities. Moreover, the distinction between weight and molar concentrations does not cause a great difference because the molecular weights of the cross-reacting analytes were in the same range (between 255.75 and 311.86).

The assay proved to be specific to acetochlor: only four among 17 structurally related compounds showed noticeable (above 1%) cross-reactivity, and even the highest cross-reactivity remained below 10%. All four compounds were chloroacetanilides, showing that key haptenic epitopes recognized by the antibodies are (in the order of importance): (1) the presence of the chloroacetyl moiety, (2) alkylation pattern of the aromatic ring, (3) the composition of the *N*-alkoxyalkyl substituent. Thus, alkoxyacetyl-, arylacetyl- and heteroarylcarboxylanilide fungicides did not show

TABLE III Cross-reactivities of various chloroacetanilide herbicides, acetanilide fungicides and other aniline derivatives in the optimized ELISA system for acetochlor

Cross-reagent[a]	IC_{50}[b] [ng/mL]	$\%CR$[c]
Acetochlor	2.6	100
Propisochlor	29.8	8.7
Butachlor	36	7.2
Alachlor	130	2.0
Metolachlor	185	1.4
Dimetachlor	326	0.79
4b	2364	0.11
5b	> 50,000	< 0.01
2-Ethyl-6-methylaniline	> 50,000	< 0.01
Propachlor	> 50,000	< 0.01
Metalaxyl	> 50,000	< 0.01
Benalaxyl	> 50,000	< 0.01
Furalaxyl	> 50,000	< 0.01
Oxadixyl	> 50,000	< 0.01
CGA29212	> 50,000	< 0.01
NKI42478	> 50,000	< 0.01
NKI42479	> 50,000	< 0.01
4a	> 50,000	< 0.01
5a	> 50,000	< 0.01

[a]For the chemical structures of the chloroacetanilide herbicides and acetanilide fungicides, see pesticide handbooks, e.g. [1]. Chemical structures of related compounds tested: CGA29212, *N*-(2,6-dimethylphenyl)-*N*-(2-chloroacetyl)-*DL*-alanine methyl ester; NKI42478, *N*-(2,6-dimethylphenyl)-*DL*-alanine methyl ester; NKI42479, *N*-(2,6-dimethylphenyl)-*DL*-alanine ethyl ester.
[b]IC_{50} value defined as the analyte concentration causing a 50% decrease in the assay signal as compared to the uninhibited signal. Assay conditions: coating antigen 5 μg/mL 2-OVA, serum dilution 1 : 40,000; IC_{50} 2.6 μg/L.
[c]Relative cross-reactivity defined as a percentage obtained by calculating the ratio of the IC_{50} value of the reference compound (acetochlor) to that of the given compound in the ELISA system.

detectable cross-reactivity. Nonetheless, the presence of the chloroacetyl moiety is no guarantee for recognition, as neither the chloroacetanilide containing no alkyl groups on the aromatic ring (propachlor), nor the experimental compound (CGA2912) containing both chloroacetyl moiety and methyl groups on the aryl ring, but an alkoxycarbonyl moiety on its *N*-alkyl substituent was recognized by the assay.

The expressed specificity of the optimized ELISA system is not unusual as most p ELISA systems appeared to show similar specificity to their target analytes alachlor [23,24] or metolachlor [35]. It was rather surprising, however, that only compound **4b** was recognized among the haptenic compounds tested, and that the cross-reactivity even for that compound was as low as 0.11%.

Solvent and Matrix Effects on Assay Performance

The two most common solvents used in pesticide residue analyses to extract soil and produce samples, methanol and ethanol were tested for their effect on assay performance. Results (listed in Table IV) indicate that the assay well tolerates the presence of methanol and ethanol up to 10% and 5% (v/v), respectively. In the case of ethanol, the IC_{50} value of the assay increased from 2.4 at 5% (v/v) ethanol content to 4.9 at 10% (v/v) ethanol content.

Recovery test in water samples from the Danube river (Budapest, Hungary) was carried out in comparison with distilled water. While recoveries in distilled water varied between 80% and 118%, in river water samples the matrix effect was more

TABLE IV The effect of methanol and ethanol on assay performance

Solvent content [%]	IC_{50}[a] [ng/mL]	
	Methanol	*Ethanol*
0	2.27 ± 0.83	2.27 ± 0.83
0.5	1.56 ± 0.91	1.06 ± 0.44
1	1.67 ± 1.02	1.33 ± 0.20
2	2.28 ± 0.95	2.61 ± 0.94
5	2.50 ± 0.66	2.39 ± 0.97
10	1.84 ± 0.45	4.92 ± 1.61

[a]IC_{50} value defined as the analyte concentration causing a 50% decrease in the assay signal as compared to the uninhibited signal. Assay conditions: coating antigen 5 μg/mL **2**-OVA, serum dilution 1 : 40,000. To exclude plate-to-plate variability, IC_{50} values were corrected to 2.27 ± 0.83 μg/L in PBS containing no organic solvent.

rather perceptible – recoveries ranged between 59% and 145%. As a general tendency, recoveries in river water matrix appeared to be unrealistic above 20 μg/L, but were acceptable between 0.5 and 5 μg/L of acetochlor concentration. Because acetochlor is often used for agricultural treatment in apple orchards, matrix influence on ELISA was also tested in apple juice, and a significant matrix effect was found. This required dilution of the juice: several dilution factors were investigated from 1 : 5 to 1 : 100 and low recovery rates (24–90%) were recorded even at 1 : 100 dilution. This fact can be explained by the absorbance of acetochlor to the apple pulp.

The main agricultural usage of acetochlor is treating maize fields. For this reason, recovery tests were also performed in corn extracts. Acetochlor was spiked directly into corn extract (corn flour was extracted with mixture of methanol and PBST 0.05 as described above). Under such sample extraction procedure no significant matrix effects were observed, and recoveries ranged between 88% and 99%.

Because pesticides often leach from the application site to nearby surface and ground waters, and because fish are natural accumulators of lipophilic pesticides in aquatic environments, fish extract was also investigated as a possible sample matrix. Since the ELISA system tolerated ethanol at the level of 5%, ethanolic fish extracts in ethanol were diluted 1 : 20 with PBST 0.05 before usage. The IC_{50} values from the ELISA calibration curves in fish extract (4.40 ± 1.43 μg/L) displayed a minor shift relative to a corresponding standard curve in ethanol.

GC-MS/SPME

To compare the accuracy of the detection of acetochlor content by the ELISA methods, the concentration of acetochlor was detected in parallel by ELISA and GC-MS in distilled water samples spiked with the target analyte. The measurement of acetochlor concentration in water by GC-MS was carried out using a SPME procedure for sample preparation. The quantitative determination of acetochlor was established based on peak areas of standard water samples (0.5 μg/L, 1 μg/L, 5 μg/L, 10 μg/L, 25 μg/L and 50 μg/L). The chosen GC column and temperature program allowed a peak retention time (R_t) of 8.43 min. The calibration offered excellent regression characteristics ($r^2 = 0.999$).

Comparative measurement of ELISA and GC-MS/SPME was carried out using five different spiking concentrations (0.5 μg/L. 2 μg/L, 5 μg/L, 20 μg/L and 50 μg/L)

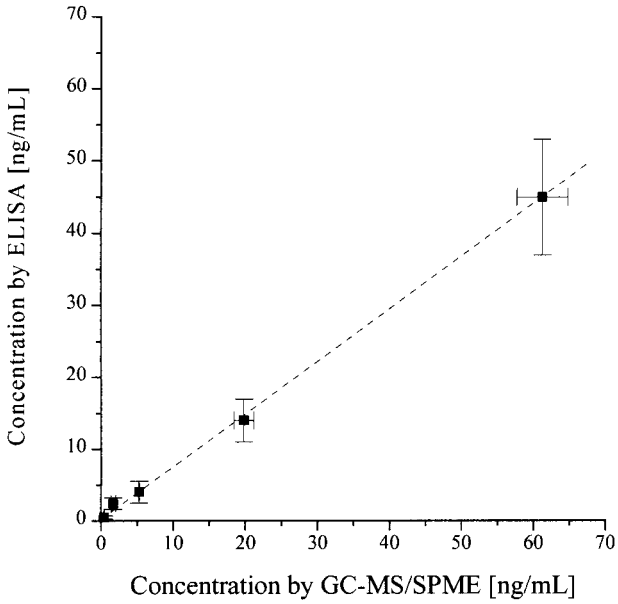

FIGURE 3 Correlation between acetochlor concentrations detected in water by ELISA and GC-MS/SPME ($n = 5$, $r^2 = 0.969$, $c_{ELISA} = 0.835\, c_{GC\text{-}MS} + 0.187$). ELISA conditions: coating antigen 5 μg/mL **2**-OVA, serum dilution 1 : 40,000.

in water. Acetochlor concentrations detected by GC-MS and ELISA and the correlation of these two analytical methods are depicted in Fig. 3. The two methods show a good correlation, although concentrations measured by ELISA appeared to be underestimated, and therefore were systematically lower than those detected by GC-MS. This phenomenon was reflected in the relatively low slope (0.835) but good regression coefficient ($r^2 = 0.969$) of the regression line and an intercept below the LOD of the ELISA system (0.187 μg/L).

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