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Evaluation of Enzyme-Linked Immunoassays for the Determination of Chloroacetanilides in Water and Soils

PATRICIA CASINO, SERGI MORAIS, ROSA PUCHADES, AND ÁNGEL MAQUIEIRA*

Departamento de Química, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

Reliable and sensitive indirect ELISAs for the quantitative determination of metolachlor, alachlor, and acetochlor were developed. Each herbicide was conjugated to a carrier protein via thioether linkage, and the product was used either as an immunogen or to prepare coating conjugates. The suitability of using the same chemical strategy to raise polyclonal antibodies against chloroacetanilides structurally related compounds and their metabolites is discussed. Under best conditions, detection limits of 0.06, 0.3, and 0.4 μg/L for metolachlor, alachlor, and acetochlor were reached, respectively. The optimized ELISAs were also highly specific, showing little or no cross-reactivity to other similar compounds. Immunoassays were used as a tool to determine critical chloroacetanilide herbicides in water and soil samples without purification steps. The excellent recoveries obtained (mean value ranging between 90% and 98%) confirm the potential of this approach to control these herbicides in the environment being applied as a "screening" method either for field monitoring or laboratory.

Introduction

Herbicides are the pesticides whose use has been increased the most (*1*, *2*). Chloroacetanilides can be found between the regularly employed herbicides. Metolachlor, alachlor, and acetochlor are widely used in the United States, particularly in the Midwest (*3*). Both alachlor and acetochlor are manufactured by the Agricultural Group of Monsanto Co. and sold under the trade name of Lasso and Harness, respectively, while metolachlor is being produced by Syngenta and sold by the trade name of Dual. All are selective preemergence herbicides for annual grass and broadleaf weed control on soybean, corn, cotton, and other crops (*4*, *5*). Alachlor and metolachlor have been used for more than 20 years with an estimated annual consumption of 23.6 and 26.8 million kg applied (*6*). Currently, alachlor use has been reduced increasing the applications of acetochlor (*3*). Furthermore, alachlor has been classified by the U.S. Environmental Protection Agency (U.S. EPA) as a class B2 or probable human carcinogenic. On the other hand, metolachlor belongs to class C compounds characterized as a possible carcinogen to humans. On the basis of long-term animal studies, the U.S. EPA has established a maximum contaminant level (MCL) of 2.0 μg/L for alachlor and a Lifetime Health Advisory (HAL) of 100 μg/L for metolachlor in drinking water (*7*).

* Corresponding author phone: +34-96-387 73 42; fax: +34-96-387 93 49; e-mail: amaquieira@qim.upv.es.

In March 1994, acetochlor was conditionally registered by the U.S. EPA as a partial replacement of alachlor, atrazine, butylate, EPTC, 2,4-D, and metolachlor in order to control broadleaf weeds and annual grasses in corn. Acetochlor has been classified by the U.S. EPA as a B2 carcinogen compound making its registration cancelable if the concentration exceeds 0.1 μg/L in groundwater or 2.0 μg/L—as an annual average—in surface water. In this sense, the sale of acetochlor was restricted in areas that may be vulnerable to contamination, such as sandy soils or shallow water tables. Also the registration conditions require that the total use of the abovementioned herbicides should decline by 3.0×10^6 kg in March 1999 based on 1992 data (*8*). But total consumption of the three herbicides increases 1.66 million kg from 1992 to 1997 in U.S.A. (*3*).

In plants, the enzyme glutathione S-transferase contributes to the action of chloroacetanilide herbicides in crops and weeds (*9*). This process rapidly yields, in soil, approximately 12 metabolites, the major one found is the ethane sulfonic acid (ESA) (*10*, *11*). In particular, alachlor-ESA was detected in rural private wells in the Midwest at concentrations ranging from 1.2 to 74 μg/L (*12*). It has also been proven in animals that these herbicides follow the same metabolic pathway as reported in plants producing mercapturate and thioether compounds (*13*).

In general, metolachlor, alachlor, and acetochlor are moderately to very mobile in soil thus quickly transformed, showing half-lives between 15 and 30 days (*14*). Both they and their soil metabolites are water-soluble and can easily reach ground and surface waters. Since water treatment does not usually remove these soluble herbicides and metabolites, their concentrations increase in large rivers and wells, affecting the quality of drinking water supplies (*15*). According to Thurman et al. (*16*), samples collected at 149 reconnaissance sites of the Corn and Soybean Belt in 122 hydrological basins showed concentrations of alachlor and metolachlor ranging from 0.05 to 51 μg/L and from 0.05 to 40 μg/L, respectively, during preplanting, postplanting, and harvesting.

Currently, the reference U.S. EPA multiresidue Methods 507 and 525 are used (*17*) to determine pesticide residues by liquid or solid-phase extraction followed by gas chromatography with nitrogen phosphorus (GC-NPD) or mass spectrometric detection (GC-MS). The limit of detection (LOD) achieved for chloroacetanilide residues are variable and frequently lower than 10 μg/L in soil samples and 0.1 μg/L in water for GC-MS and GC-NPD, respectively (*18*).

These EPA methods, modified in different ways (increasing the sample volume, using other extraction modes, etc.), have shown for alachlor a LOD of 0.05 μg/L and 0.01 μg/L for acetochlor in water (*19*) and 0.5 μg/L in soil for metolachlor and alachlor (*20*).

Using the Association of Analytical Chemists (AOAC) recommended GC-NPD method to detect alachlor and metolachlor in drinking water, a LOD of 0.38 μg/L and 0.75 μg/L, respectively, was reached (*21*). The determination of these herbicides by liquid chromatography presents difficulties, as they are polar compounds and coelute with the peak matrix (*22*). Therefore, although these conventional methods are the reference procedures, they have the typical limitations (time consumption, expensiveness, requirement of qualified staff, and low throughput) of the multiresidue chromatographic methods when applied in a manner different than that they were developed for (*23*).

Immunoassay techniques are specially recommended for environmental analysis (*24*, *25*), because they are well suited

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TABLE 1. Chemical Analysis of the Composition of the Soil Samples[a]

soil	A	B	C	D	E	F	G	H
town	Valencia	Altea	Villalonga	Ribarroja I	Salinas	Museros	Puzol	Ribarroja II
crop	market garden	orange tree	orange tree	orange tree	vine	orange tree	orange tree	orange tree
Dap (g/cm^3)		1.32	1.27	1.11	1.27	1.45	1.12	1.18
Fh	0.970	0.984	0.936	0.986	0.986	0.986	0.974	0.958
clay (%)	50	–	15	18	17		30	20
slime (%)	43.5	–	60.0	34.5	25.0		27.5	39.0
sand (%)	6.5	28.0	25.0	47.5	58.0		42.5	41.0
pH in water	8.5	8.2	8.2	7.8	8.5	8.0	8.0	8.0
pH in $CaCl_2$	7.9	7.6	7.6	7.5	7.8	7.7	7.8	7.7
CE 1:2,5 (dS/m)	1.71	0.43	0.27	0.96	0.29	0.51	0.73	0.93
HS (g/g)	–	–	0.47	0.36	0.26	0.30	0.34	0.30
CEes 25 (dS/m)	–	1.7	0.6	4.3	0.9	2.4	4.3	4.0
Na^+ (mequiv/L)	–	4.0	0.8	10.0	0.6	5.6	13.8	7.0
$Ca^{2+} + Mg^{2+}$ (meq/L)	–	13.7	5.7	34.0	6.6	29.4	32.2	39.0
Cl^- (mequiv/L)	–	4.3	0.4	10.2	–	7.6	8.7	10.3
HCO_3^- (mequiv/L)	–	4.7	7.0	8.5	10.0	6.4	4.7	6.0
SO_4^{2-} (mequiv/L)	–	10.7	4.5	10.4	2.7	17.5	15.4	10.9
ClC (ceq/Kg)	–	7.8	21.5	6.7	1.5	4.5	8.8	3.7
$N-NH_4^+$ (mg/Kg)	–	2.9	6.3	6.7	4.2	2.9	4.2	4.2
$N-NO_3^-$ (mg/Kg)	–	29	13	120	60	37	45	9
Polsen (mg/Kg)	–	82	109	162	15.5	89	160	167
CA (g/Kg)	91	133	39	36	71	6	28	61
K^+ (mg/Kg)	661	309	534	467	176	314	514	522

[a] Dap (apparent density); Fh (humidity factor); CE 1:2,5 (conductivity in the extract 1:2,5); HS (humidity of saturation); CEes 25 (conductivity in the extract of saturation a 25 °C); ClC (capacity of ionic exchange); Polsen (P, method Olsen); CA (active limestone).

for intensive and screening studies, such as field plot works and farming practices, where a large number of sample analyses are required and the chromatographic conventional methods would be more expensive and time-consuming. Also, when single analyte studies are developed (e.g. alachlor mapping) the immunochemical methods are really competitive. In this way, ELISA (enzyme-linked immunosorbent assay) has been applied to the detection of herbicide residues in either soil or water (*26*).

In 1990, Feng et al. (*27*) reported the use of ELISA to detect alachlor in water with a detection range of 0.2 and 8.0 μg/L using a thioether linkage for the synthesis of alachlor-protein conjugates. Two years later, they reported another immunoassay developed for metolachlor with an inhibition coefficient (IC_{50}) of 6 μg/L (*28*). On the other hand, Schlaeppi et al. (*29*) developed another ELISA for detection of metolachlor in soil obtaining an IC_{50} value of 1.0 μg/L using a conjugate synthesized by the ester active method. The mixed anhydride ester method has also been used to prepare conjugates for metolachlor. Although requiring the synthesis of a hapten, the ELISA gave a IC_{50} value of 0.85 μg/L. However, each immunoassay has shown different LOD and IC_{50} values depending on the sensitivity of the antibodies. In addition, no papers describing specific immunoassays for acetochlor have been published up until now, but there are commercialised kits to acetanilides able to analyze acetochlor, not specifically.

Different firms market ELISA kits with selectivity for metolachlor, alachlor, and acetanilides with IC_{50} ranging from 0.8 to 1.3 μg/L, 0.6−5.0 μg/L, and 0.3−4.9 μg/L, respectively (*30*). These kits, except for the Idetek/Quantix test for metolachlor, show cross-reactivities to other chloroacetanilide herbicides, making the assays of limited use for specific detection of metolachlor, alachlor, or acetochlor, individually. Seemingly, a major metabolite, ESA, cross-reacts with anti-alachlor antibodies (*12*).

This paper reports the development and application of enzyme-linked immunoassays by raising rabbit polyclonal antibodies using the thioether conjugate method and the same chemical strategy for rapid, simple, selective, and sensitive measurements of metolachlor, alachlor, and acetochlor in water and soil samples. Furthermore, a cross-reactivity study is carried out with other herbicides including chloroacetanilides and their major ethane sulfonic and oxanilic acids metabolites. Such a type of study will allow the application of immunological techniques to surveillance programs.

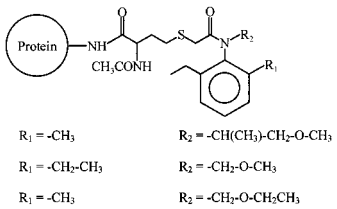

FIGURE 1. Structures of alachlor, metolachlor, and acetochlor haptens.

Experimental Section

Chemicals, Immunoreagents, and Instruments. Metolachlor, alachlor, and acetochlor were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stocks solutions of these compounds were prepared in *N*,*N*′-dimethylformamide (DMF) and stored at 4 °C. Freund's complete and incomplete adjuvants, bovine serum albumin (BSA), ovalbumin (OVA), Keyhole limpet hemocyanin (KLH), *N*-acetylhomocysteine thiolactone (AHT), and Tween 20 and *o*-phenylenediamine (OPD) were purchased from Sigma-Aldrich Química (Madrid, Spain). Peroxidase-labeled goat anti-rabbit immunoglobulins (GAR-HRP) were acquired from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

Polystyrene ELISA plates were from Costar (Cambridge, MA). ELISA plates were washed with a Nunc Immuno Wash 12 (Nunc MaxiSarp, Roskilde, Denmark) and absorbance was read in dual-wavelength mode (490−650 nm) with microplate reader Wallac, Victor model 1420 Multilabel Counter (Turku, Finland).

Herbicide-Protein Conjugates. The use of the thioether linkage has an advantage against both the ester active and the mixed anhydride methods as it is performed in one step without being necessary to synthesize haptens. In this way, the herbicides metolachlor, alachlor, and acetochlor were attached to carrier proteins (KLH, BSA, or OVA) using the

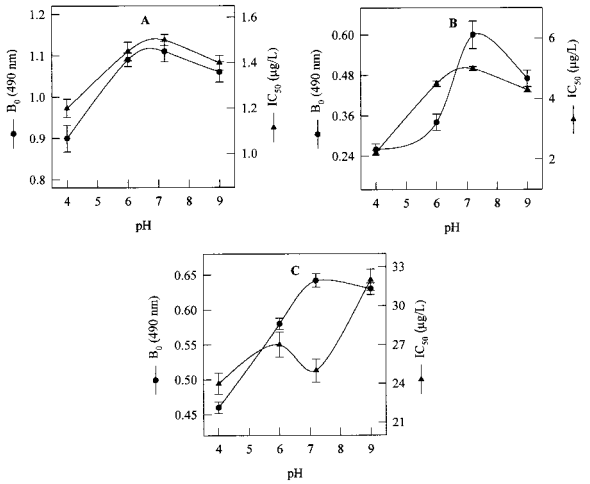

FIGURE 2. Influence of pH of the media on the performance of the immunoassays: (A) metolachlor, (B) alachlor, and (C) acetochlor. Several standard curves were prepared in PBST at 4.0, 6.0, 7.2, and 9.0. Each point represents the average of three determinations.

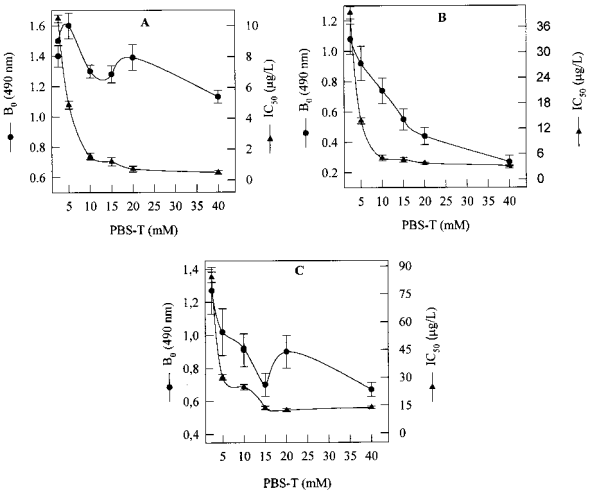

FIGURE 3. Influence of salt concentration of the media on the performance of the immunoassays: (A) metolachlor, (B) alachlor, and (C) acetochlor. Several standard curves were prepared at different PBS concentration (0.2−4.0 ×). Each point represents the average of three determinations.

same chemical synthesis strategy (*28*). Each herbicide (approximately 2.8 mg) was conjugated to protein (20 mg) in a one-step reaction via thioether linkage using AHT (1.7 mg). The conjugates were purified by gel exclusion chromatography on Sephadex G-25 using 10 mM sodium phosphate buffer pH 7.4 for the elution and stored at −20 °C. KLH-alachlor, BSA-metolachlor, and BSA-acetochlor conjugates were used as immunogens, whereas BSA-alachlor, OVA-metolachlor, and OVA-acetochlor were used as coating conjugates (Figure 1).

Production of Polyclonal Antibodies. Each immunizing conjugate (0.25 mg) was suspended in 1.0 mL Freund's complete adjuvant and injected intradermally into three female New Zealand white rabbits. Animals were boosted at 2−3 week cycles with 0.25 mg of the same immunogen suspended in 1.0 mL of Freund's incomplete adjuvant and bled 7−10 days after each boost. Finally, rabbits were bled when appropriate titer was reached, and the antisera was aliquoted and stored at −20 °C in 50% ammonium sulfate.

Immunoassay Development. (i) Screening of the Sera. Concentrations of coating conjugates and sera dilution were chosen to produce absorbance values ranging from 0.5 to 1.5 units in 1 h. For this purpose, checkerboard titration assays were run to determine the avidity of the antibodies by measuring the binding of serial dilutions of the sera ranging from 1/500 to 1/512 000 for eight different concentrations of coating conjugates (0.001−1000 µg protein/L). The screening of the competitive assays was performed as reported by Marco et al. (*31*).

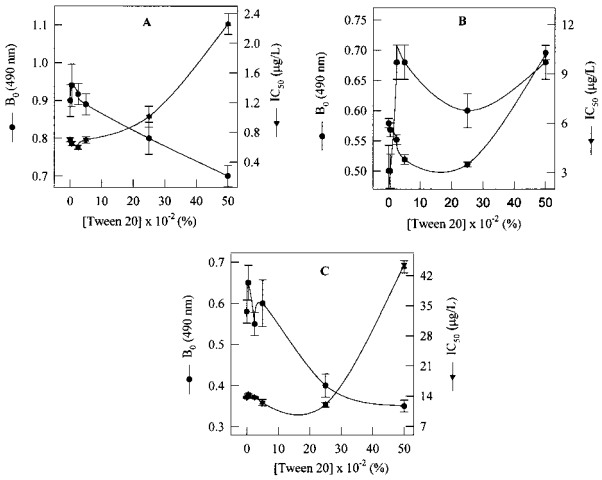

FIGURE 4. Influence of Tween 20 of the media on the performance of the immunoassays; (A) metolachlor, (B) alachlor, and (C) acetochlor. Several standard curves were prepared in PBST with different concentration of Tween 20 ranging from 0 to 0.5%. Each point represents the average of three determinations.

(ii) Protocol Conditions. First, flat bottom polystyrene ELISA plates were coated with conjugate solution in 50 mM carbonate-bicarbonate buffer, pH 9.6 (100 µL/well). Then, plates were sealed and incubated overnight at 4 °C. The following day, plates were washed four times with PBST (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4, containing 0.05% Tween 20), and 100 µL of the optimized dilution of sera was added in each well followed by 1 h incubation at room temperature. For competitive assays, a mixture of appropriate sera dilution (50 µL/well in 2-fold concentrated PBST) and different herbicide concentration (50 µL/well in deionized water) was added to previously coated plates. After 1 h incubation, the plates were washed four times with PBST, and GAR-HRP (1/4000 in PBST) solution was added (100 µL/well) and incubated again for 1 h. The peroxidase activity was measured by adding 100 µL/well of the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM sodium citrate, 62 mM sodium phosphate, pH 5.4). After 10 min, the enzymatic reaction was stopped by adding 100 µL/well of 2.5 M H_2SO_4 (100 µL/well). The absorbance was read in dual-wavelength mode (490−650 nm).

(iii) ELISA Optimization. Under the best competitive assay conditions—for metolachlor (0.1 µg/L OVA-metolachlor and serum R48 at 1/50 000 dilution), for alachlor (10 µg/L OVA-metolachlor as coating conjugate and serum S3 at 1/10 000 dilution), and for acetochlor (0.1 µg/L OVA-acetochlor and serum S8 at 1/40 000)—the effect of a set of chemical parameters (pH, salt, and surfactant concentration) on the technique performance was studied sequentially with the aim of improving immunoassay sensitivity. Criteria used to evaluate immunoassay performances were IC_{50}, maximal absorbance (B_0), dynamic range (RD), and limit of detection (LOD). In this sense, the effect of pH was evaluated using different PBST solutions ranging from pH 4.0 to 9.0. Then, several concentrations of PBS at 0.2; 0.5; 1.0; 1.5; 2.0; and 4.0 × [PBS 10 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH 7.2], always at 0.05% (v/v) Tween 20, were used for the estimation of the salt concentration effect. Finally, the influence of surfactant Tween 20 concentration (0, 0.01, 0.05,

TABLE 2. Characteristics of ELISAs for Alachlor, Metolachlor, and Acetochlor

analyte	combination (antisera/coating conjugate)	$A_{max.}$	$A_{min.}$	IC_{50} (μg/L)	LOD (μg/L)	DR (μg/L)	slope
metolachlor	R48/metolachlor-OVA	0.9	0.02	0.7	0.06	0.1−4.2	2.0
alachlor	S3/metolachlor-OVA	0.6	0.03	3.8	0.30	0.7−27.9	1.9
acetochlor	S8/acetochlor-OVA	0.7	0.12	12.5	0.40	1.4−135.0	1.5

TABLE 3. Cross-Reactivity of Chloroacetanilides Structurally Related Compounds and Main Metabolites

	assay					
	metolachlor[a]		alachlor[b]		acetochlor[c]	
compound	IC_{50} (μg/L)	CR (%)	IC_{50} (μg/L)	CR (%)	IC_{50} (μg/L)	CR (%)
metolachlor	0.7	100	>1000	<0.4	>1000	<1.25
alachlor	>1000	<0.07	3.8	100	442	2.85
acetochlor	195	0.40	175	2.2	12.5	100
butachlor	300	0.20	200	1.9	>1000	<1.25
propachlor	>1000	<0.07	>1000	<0.4	>1000	<1.25
AOA	>1000	<0.07	27.5	13.8	>1000	<1.25
AOM	>1000	<0.07	>1000	<0.4	>1000	<1.25
AOAcet	>1000	<0.07	>1000	<0.4	>1000	<1.25
Acet-ESA	>1000	<0.07	>1000	<0.4	>1000	<1.25
A-ESA	>1000	<0.07	>1000	<0.4	>1000	<1.25
M-ESA	94.1	0.80	>1000	<0.4	>1000	<1.25

[a] R48/metolachlor-OVA. [b] S3/metolachlor-OVA. [c] S8/acetochlor-OVA.

0.1, 0.5% v/v) on immunoassay performance was also studied.

(iv) Cross-Reactivity Determinations. The specificity of the ELISA assays was determined using the chloroacetanilide herbicides alachlor, metolachlor, acetochlor, butachlor, propachlor, and the metabolites—alachlor oxanilic acid (AOA), metolachlor oxanilic acid (AOM), acetochlor oxanilic acid (AOAcet), sulfonic acid metabolite of alachlor (A-ESA), metolachlor (M-ESA), and acetochlor (Acet-ESA)—which were dissolved in methanol and prepared and the cross-reactivity (CR) was calculated by the following equation: [IC_{50} (analyte)/IC_{50} (interferent)] $\times$ 100.

Water and Soil Sample Analysis. Eight water samples from different sources, tap water, commercial bottled water, lake water (the Albufera of Valencia) and well water from the Valencia region, Spain, were used for analytical purposes. Samples showed a pH between 7.0 and 8.4 and were used as blank. All waters were filtered and stored at 4 °C prior to use. Each sample was spiked with either metolachlor, alachlor, or acetochlor covering the analytical working ranges and analyzed directly without previous extraction.

On the other hand, eight soil samples from different locations (Table 1 shows the chemical composition of each soil) from the Valencia region, Spain, were treated as usual and extracted before analysis according to Mills and Thurman procedure (*32*), using only 10-g of soil. Prior to use, the extracts were diluted at least 1:25 in deionized water in order to avoid solvent interference. Then, the extracts were spiked with metolachlor, alachlor, and acetochlor at different concentrations covering the appropriate working range.

Competitive curves were mathematically analyzed by fitting experimental points to a four-parameter logistic equation (*33*) using the Sigmaplot software package (Jandel Scientific, Erkrath, Germany). Standards and samples were run in three replicate wells, and the mean absorbance values were recorded.

Results and Discussion

Preparation of the Herbicide-Protein Conjugates. Using thioether synthesis, a set of immunizing and coating conjugates were prepared. For immunizing, KLH and BSA were selected due to their high amount of specific sites for attaching the derivative herbicides. On the other hand, either BSA or OVA was used as proteins for coating conjugates preparation. Because of KLH's high molecular size, it was difficult to calculate the hapten/protein ratio; however, it was expected to be higher than those obtained using other proteins such as BSA and OVA, due to the higher number of free amine groups (*34*). Thus, for hapten density calculation purposes, BSA and OVA conjugates were simultaneously prepared to verify conjugation and to estimate hapten/protein ratio according to UV absorbance. In this sense, a derivative herbicide/protein ratio ranging from 4 to 18 was obtained for coating and immunizing conjugates, respectively. It is felt (*28*) that a hapten to protein molar ratio of 10−20 is optimal for the generation of antibodies.

Sera Screening. Rabbits S1, S2, and S3 were immunized with alachlor derivative covalently attached to KLH, R48, R49, and R50 with metolachlor-BSA and S7, S8, and S9 with acetochlor-BSA conjugate. The serum of each animal was tested against homologous and heterologous coating conjugates on an indirect ELISA format once an acceptable antibody titer was reached. The avidity was calculated in relative units (low, medium, and high) corresponding to the dilution factor applied to the sera. Coating conjugate concentration varying from 0.001 to 1000 μg/L using both

TABLE 4. Sensitivity and Cross-Reactivity Comparison between Commercial Immunoassay Kits and Developed ELISAs for Chloroacetanilides in Water Samples[e]

	anti-metolachlor		anti-alachlor		anti-acetochlor	
assay	LOD (μg/L)	IC_{50} (μg/L)	LOD (μg/L)	IC_{50} (μg/L)	LOD (μg/L)	IC_{50} (μg/L)
metolachlor[a]	0.05	0.85	1.30	84.0	0.06	6.55
metolachlor[b]		0.75		100.0		70.0
metolachlor[d]	0.06	0.70 ± 0.03		>1000		>1000
alachlor[a]	5.60	80.0	0.05	c.a. 1.0		
alachlor[b]		80.0		0.48		23.0
alachlor[c]	0.60	40.0	0.15	0.6		
alachlor[d]		>1000	0.30	3.80 ± 0.20		442
acetanilides[c]	0.02	0.25	0.55	4.9	0.02	1.70
acetochlor[d]		195		175	0.40	12.50 ± 1.00

[a] Ohmicron. [b] Idetek. [c] Millipore. [d] At this work. [e] According to ref *44*.

TABLE 5. Metolachlor Determination in Water Samples

metolachlor added (μg/L)	Valencia mean ± SD[a] (μg/L)	Valencia R (%)[b]	bottled water mean ± SD[a] (μg/L)	bottled water R (%)[b]	Albufera mean ± SD[a] (μg/L)	Albufera R (%)[b]	Yátoba mean ± SD[a] (μg/L)	Yátoba R (%)[b]
0	<LD		<LD		<LD		<LD	
0.5	0.45 ± 0.06	90	0.51 ± 0.05	100	0.45 ± 0.01	90	0.36 ± 0.04	72
0.75	0.80 ± 0.03	107	0.80 ± 0.05	107	0.77 ± 0.03	103	0.61 ± 0.03	80
1.5	1.60 ± 0.07	107	1.35 ± 0.06	90	1.22 ± 0.10	80	1.45 ± 0.20	97
mean		101		99		91		83

metolachlor added (μg/L)	Buñol mean ± SD[a] (μg/L)	Buñol R (%)[b]	Alborache mean ± SD[a] (μg/L)	Alborache R (%)[b]	Macastre mean ± SD[a] (μg/L)	Macastre R (%)[b]	Benisanó mean ± SD[a] (μg/L)	Benisanó R (%)[b]
0	<LD		<LD		<LD		<LD	
0.5	0.42 ± 0.07	84	0.45 ± 0.06	90	0.45 ± 0.06	90	0.44 ± 0.05	88
0.75	0.63 ± 0.03	84	0.70 ± 0.02	93	0.72 ± 0.03	96	0.78 ± 0.03	104
1.5	1.35 ± 0.20	90	1.61 ± 0.50	107	1.56 ± 0.30	104	1.49 ± 0.10	99
mean		86		97		97		97

[a] SD (standard deviation $n = 3$). [b] R (%) = recovery in percentage.

TABLE 6. Alachlor Determination in Water Samples

alachlor added (μg/L)	Valencia mean ± SD[a] (μg/L)	Valencia R (%)[b]	bottled water mean ± SD[a] (μg/L)	bottled water R (%)[b]	Albufera mean ± SD[a] (μg/L)	Albufera R (%)[b]	Yátoba mean ± SD[a] (μg/L)	Yátoba R (%)[b]
0	<LD		<LD		<LD		<LD	
1	1.03 ± 0.02	100	0.79 ± 0.02	79	0.88 ± 0.05	90	0.89 ± 0.08	90
5	4.70 ± 0.24	94	4.57 ± 0.15	91	4.35 ± 0.20	87	4.20 ± 0.09	84
15	14.31 ± 0.45	95	16.10 ± 0.33	107	12.29 ± 0.31	82	14.64 ± 1.33	97
mean		97		92		86		90

alachlor added (μg/L)	Buñol mean ± SD[a] (μg/L)	Buñol R (%)[b]	Alborache mean ± SD[a] (μg/L)	Alborache R (%)[b]	Macastre mean ± SD[a] (μg/L)	Macastre R (%)[b]	Benisanó mean ± SD[a] (μg/L)	Benisanó R (%)[b]
0	<LD		<LD		<LD		<LD	
1	0.84 ± 0.03	84	0.94 ± 0.08	94	0.92 ± 0.06	92	0.93 ± 0.09	93
5	4.71 ± 0.11	94	4.32 ± 0.23	86	5.21 ± 0.14	104	4.53 ± 0.29	90
15	14.02 ± 0.74	93	14.71 ± 0.93	98	15.90 ± 0.92	106	14.90 ± 1.52	99
mean		90		93		101		94

[a] SD (standard deviation $n = 3$). [b] R (%) = recovery in percentage.

homologous and heterologous conjugates was assayed. For metolachlor, antisera R48 and R49 showed medium recognition (dilution factor 1/10K to 1/100K) to homologous and heterologous conjugates, while R50 proved low recognition (dilution factor < 1/10K) in heterology and no recognition in homology conditions.

In general, the alachlor antisera revealed medium recognition and only low avidity was shown by S2 and S3 in heterology. Finally, acetochlor antisera showed medium recognition in heterology and high avidity (sera coating dilution factor > 1/100K) in homology. All the combinations serum/coating conjugates that showed specific recognition were used to carry out competitive assays in order to determine the most sensitive assay for each analyte. Although some combinations of serum/coating conjugate provided poor competitive assays (IC_{50} > 1000 μg/L), several of them established sensitivities at low μg/L levels.

Before optimization, the optimum sera/coating conjugate concentrations giving the lowest IC_{50} value were R48/metolachlor-OVA (1:50 000/0.1 μg/L), S3/metolachlor-OVA (1:10 000/10 μg/L), and S8/acetochlor-OVA (1:40 000/0.1 μg/L) for metolachlor, alachlor, and acetochlor determination. The sensitivity (IC_{50}) achieved for metolachlor, alachlor, and acetochlor assays was 1.5, 5.0, and 25.0 μg/L, respectively.

ELISA Optimization. With the aim of improving immunoassay performance, the influence of pH, salt, and surfactant concentration on the signal and sensitivity was investigated. Despite the influence that physical parameters such as time and temperature have on assay performance, 1-h step reaction at room temperature are generally the most used conditions to carry out indirect ELISA. In this sense, the above-mentioned physical conditions (1 h/step and room temperature) were considered as the optimum before ELISA optimization.

The effect of the pH of the media is shown in Figure 2. It was observed that immunoassays for metolachlor, alachlor, and acetochlor are more sensitive (lower IC_{50}) in acidic than in basic conditions. However, below pH 7.2, the signal (B_0) sharply diminished, and, in particular, for alachlor and acetochlor immunoassays it was lower than 0.5 absorbance units, having an influence on the assay performance (standard curves with large slopes and narrow dynamic range). As metolachlor, alachlor, and acetochlor are degraded at strong basic and acid conditions and have a nonionizable nature, the effects observed must be due to conformational changes of the macromolecules participating in the interaction. Similar pH effects on immunoassays performance for polar compounds have been reported (*35*). Therefore, a pH value

TABLE 7. Acetochlor Determination in Water Samples

acetochlor added (μg/L)	Valencia mean ± SD[a] (μg/L)	Valencia R (%)[b]	bottled water mean ± SD[a] (μg/L)	bottled water R (%)[b]	Albufera mean ± SD[a] (μg/L)	Albufera R (%)[b]	Yátoba mean ± SD[a] (μg/L)	Yátoba R (%)[b]
0	<LD		<LD		<LD		<LD	
3	3.30 ± 0.18	110	2.90 ± 0.25	97	2.80 ± 0.29	93	2.60 ± 0.24	87
12	12.82 ± 0.25	98	12.95 ± 0.52	108	12.52 ± 0.76	104	12.39 ± 1.71	103
48	47.04 ± 4.82	98	48.02 ± 1.84	100	44.24 ± 4.03	92	48.49 ± 3.23	101
mean		102		102		96		97

acetochlor qadded (μg/L)	Buñol mean ± SD[a] (μg/L)	Buñol R (%)[b]	Alborache mean ± SD[a] (μg/L)	Alborache R (%)[b]	Macastre mean ± SD[a] (μg/L)	Macastre R (%)[b]	Benisanó mean ± SD[a] (μg/L)	Benisanó R (%)[b]
0	<LD		<LD		<LD		<LD	
3	2.70 ± 0.34	90	2.40 ± 0.53	80	2.51 ± 0.45	83	2.60 ± 0.52	87
12	11.92 ± 1.57	99	12.14 ± 0.70	101	12.39 ± 1.23	103	12.27 ± 1.14	102
48	48.03 ± 5.30	100	49.01 ± 5.20	102	48.22 ± 5.90	100	48.28 ± 4.10	101
mean		96		94		95		97

[a] SD (standard deviation $n = 3$). [b] R (%) = recovery in percentage.

TABLE 8. Metolachlor Determination in Soil Extracts Samples[c]

metolachlor added (μg/L)	A mean ± SD[a] (μg/L)	A R (%)[b]	B mean ± SD[a] (μg/L)	B R (%)[b]	C mean ± SD[a] (μg/L)	C R (%)[b]	D mean ± SD[a] (μg/L)	D R (%)[b]
0	<LD		<LD		<LD		<LD	
0.25	0.22 ± 0.02	88	0.21 ± 0.01	84	0.25 ± 0.03	100	0.23 ± 0.01	92
0.5	0.46 ± 0.04	92	0.42 ± 0.02	84	0.44 ± 0.11	88	0.46 ± 0.02	92
2.5	2.40 ± 0.20	96	2.81 ± 0.10	112	2.20 ± 0.20	88	2.43 ± 0.16	97
mean		92		93		92		94

metolachlor added (μg/L)	E mean ± SD[a] (μg/L)	E R (%)[b]	F mean ± SD[a] (μg/L)	F R (%)[b]	G mean ± SD[a] (μg/L)	G R (%)[b]	H mean ± SD[a] (μg/L)	H R (%)[b]
0	<LD		<LD		<LD		<LD	
0.25	0.24 ± 0.02	96	0.25 ± 0.50	100	0.22 ± 0.01	88	0.24 ± 0.02	96
0.5	0.47 ± 0.08	94	0.46 ± 0.07	92	0.48 ± 0.03	96	0.43 ± 0.02	86
2.5	2.20 ± 0.30	88	2.35 ± 0.15	94	2.10 ± 0.29	84	2.23 ± 0.25	89
mean		93		95		89		90

[a] SD (standard deviation $n = 3$). [b] R (%) = recovery in percentage. [c] A (Valencia); B (Altea); C (Villalonga), D (Ribarroja I); E (Salinas); F (Museros), G (Puzol); H (Ribarroja II).

between 6.0 and 8.0 was chosen as optimum to keep an acceptable signal-to-sensitivity ratio.

On the other hand, the effect of the salt concentration on the assay performance is shown in Figure 3. Different concentrations of PBS, ranging from 0.2 to 4.0-fold of the original PBS buffer concentration, always 0.05% (v/v) Tween 20, were tested. Essentially, for every immunoassay studied B_0 and IC_{50} decreased gradually as buffer salt concentration increased. It seems that the effect of the salt concentration on the assay is due to biochemical interactions, mainly hydrophobic, between analyte and antibody. These interactions are generally favored by increasing the ionic strength of the reaction medium, while the increase exerts a detrimental effect on interactions where ionic driving forces prevail (*36*). However, some studies suggested that antibody affinity rose for a polar analytes by increasing the buffer concentration (*35*). As metolachlor, alachlor, and acetochlor are relatively polar analytes, the interactions with the antibody are weak type such as dipole−dipole, etc. In this study, the optimum salt concentration of the media (PBS-T) which provided the highest B_0 and lowest IC_{50} was 20 mM for all assays for metolachlor and acetochlor and 10 mM for alachlor.

Using the established pH and buffer concentration conditions, the effect of Tween 20 as surfactant on both the signal and sensitivity was studied. The usual concentration of Tween 20 for most pesticide immunoassay is 0.05%. However, for several pesticides there is a marked effect of detergent concentration on ELISA performance. Actually, higher affinities can be achieved by decreasing or even removing the addition of surfactant in the competition buffer (*37*). In this study (Figure 4), the negative influence observed as surfactant concentration increased (lower signal and slightly lower sensitivity) may be related with nonspecific hydrophobic interactions between the surfactant and interferences with the specific immunoreaction. Therefore, 0.05% Tween 20 was selected as a trade between sensitivity and absence of nonspecific interactions.

Taking into account all these factors, Table 2 summarizes the characteristics of the competitive optimized ELISAs. The LOD was calculated as the concentration corresponding to 10% inhibition of the maximum absorbance. The quantitative working range was established between the concentrations producing 20% and 80% inhibition. As is shown, a LOD below 0.1 μg/L for metolachlor, 0.3 μg/L for alachlor, and 0.4 μg/L for acetochlor was reached. On the other hand, it is worth mentioning that after ELISA optimization, the sensitivity was

TABLE 9. Alachlor Determination in Soil Extracts Samples[c]

alachlor added (μg/L)	A mean ± SD[a] (μg/L)	A R (%)[b]	B mean ± SD[a] (μg/L)	B R (%)[b]	C mean ± SD[a] (μg/L)	C R (%)[b]	D mean ± SD[a] (μg/L)	D R (%)[b]
0	<LD		<LD		<LD		<LD	
0.6	0.58 ± 0.06	97	0.64 ± 0.07	107	0.60 ± 0.08	100	0.63 ± 0.08	105
4	3.81 ± 0.31	95	4.30 ± 0.12	108	3.92 ± 0.22	98	4.31 ± 0.34	108
28	28.61 ± 0.42	102	27.49 ± 1.40	98	27.50 ± 1.10	98	27.30 ± 1.40	98
mean		98		104		99		104

alachlor added (μg/L)	E mean ± SD[a] (μg/L)	E R (%)[b]	F mean ± SD[a] (μg/L)	F R (%)[b]	G mean ± SD[a] (μg/L)	G R (%)[b]	H mean ± SD[a] (μg/L)	H R (%)[b]
0	<LD		<LD		<LD		<LD	
0.6	0.60 ± 0.10	100	0.56 ± 0.05	93	0.53 ± 0.07	88	0.58 ± 0.03	97
4	4.21 ± 0.42	105	4.00 ± 0.32	100	4.10 ± 0.12	102	3.75 ± 0.12	94
28	27.53 ± 1.0	98	27.02 ± 1.20	96	27.71 ± 0.30	99	27.30 ± 0.91	98
mean		101		96		96		96

[a] SD (standard deviation $n = 3$). [b] R (%) = recovery in percentage. [c] A (Valencia); B (Altea); C (Villalonga), D (Ribarroja I); E (Salinas); F (Museros), G (Puzol); H (Ribarroja II).

TABLE 10. Acetochlor Determination in Soil Extracts Samples[c]

acetochlor added (μg/L)	A mean ± SD[a] (μg/L)	A R (%)[b]	B mean ± SD[a] (μg/L)	B R (%)[b]	C mean ± SD[a] (μg/L)	C R (%)[b]	D mean ± SD[a] (μg/L)	D R (%)[b]
0	<LD		<LD		<LD		<LD	
3	2.40 ± 0.41	80	2.80 ± 0.07	93	2.60 ± 0.04	87	2.20 ± 0.20	73
15	13.31 ± 0.52	89	13.62 ± 0.80	91	14.49 ± 1.50	97	13.82 ± 0.82	92
75	76.02 ± 2.20	101	73.50 ± 1.51	98	75.10 ± 2.10	100	73.89 ± 1.51	99
mean		90		94		95		88

acetochlor added (μg/L)	E mean ± SD[a] (μg/L)	E R (%)[b]	F mean ± SD[a] (μg/L)	F R (%)[b]	G mean ± SD[a] (μg/L)	G R (%)[b]	H mean ± SD[a] (μg/L)	H R (%)[b]
0	<LD		<LD		<LD		<LD	
3	2.30 ± 0.41	77	2.60 ± 0.40	87	2.40 ± 0.33	80	2.20 ± 0.40	73
15	13.32 ± 1.40	89	13.70 ± 1.61	91	13.42 ± 2.41	89	13.21 ± 2.43	88
75	74.51 ± 2.40	99	74.80 ± 1.80	100	76.19 ± 2.33	102	74.79 ± 1.90	100
mean		88		93		90		87

[a] SD (standard deviation $n = 3$). [b] R (%) = recovery in percentage. [c] A (Valencia); B (Altea); C (Villalonga), D (Ribarroja I); E (Salinas); F (Museros), G (Puzol); H (Ribarroja II).

2-fold higher for metolachlor and acetochlor and 1.3 times for alachlor. As it can be observed in Table 2, the precision for the determination of metolachlor and alachlor is better than the one obtained for acetochlor.

Cross-Reactivity Study. In Table 3 are shown the cross-reactivity measurements of the developed immunoassays. For metolachlor assay, acetochlor (0.4%), butachlor (0.2%), and its main soil metabolite ethanesulfonic acid (0.8%) were the principal interfering compounds. A weak cross-reactivity was observed for alachlor assay by structurally related compounds such as acetochlor (2.2%), butachlor (1.9%), and alachlor oxanilic acid (13.8%), which is a major soil metabolite. Finally, for acetochlor assay, alachlor (2.8%) was the only interfering compound. This cross reactivity value can be considered virtually negligible taking into account the sensitivity level for the acetochlor assay. Thus, the developed immunoassays for metolachlor, alachlor, and acetochlor were highly specific against the main chloroacetanilides and their metabolites. Other common herbicides such as glyphosate, atrazine, simazine, and 2,4-D were not recognized at 1000 μg/L level.

When comparing the results obtained in this work with those developed by commercial immunoassay kits (Table 4) it can be seen that the developed ELISA for metolachlor offers more sensitivity. However, the sensitivity of alachlor using the Idetek kit was slightly better. No kit nor immunoassay has been developed specifically for acetochlor. However, Millipore and Abraxis have performed immunoassays for acetanilides that can be used to detect whatever chloroacetanilide with an IC_{50} value for metolachlor, alachlor, and acetochlor of 0.26, 4.9, and 1.7 μg/L, respectively. Therefore, any positive result must be confirmed by reference methods. On the other hand, all the immunoassay kits have shown low cross-reactivity, but our results are much better because they are specific (CR < 10%).

Analysis of Water and Soil Samples. The application of the proposed method for the determination of metolachlor, alachlor, and acetochlor in water samples was evaluated. Eight water samples from different sources were analyzed for each analyte. Table 5 shows the results obtained from water samples spiked with metolachlor at three concentration levels (0.5, 0.75, and 1.5 μg/L). Recoveries between 72 and 107% were found. In Table 6 are shown the recovery results (79−107%) for water samples spiked with alachlor (1, 5, and 15 μg/L).

Other authors (*38*−*41*) have done comparative studies in water samples between the results obtained with ELISA and GC. In general ELISA gives more variable results for metolachlor and alachlor, over- or underestimating herbicide concentrations related to instrumental method of comparison. This indicates that although ELISA is less precise than GC methods, it is very useful to discriminate water samples that could contain acetanilide residues.

Finally, Table 7 shows the results obtained when water samples were spiked with acetochlor at three levels (3, 12, and 48 μg/L). The recovery percentages were between 80 and 110%, with a coefficient of variation (CV) less than 20%. The published results (*30*) indicate that recovery at 1.0 μg/L is good, but inaccurate results are obtained at 0.1 μg/L of acetochlor. Chromatographic methods have shown recoveries between 66 and 72% (*42*).

On the other hand, extracted soil samples were also analyzed by ELISA after being spiked either with metolachlor, alachlor, or acetochlor. As shown in Table 8 the recovery results obtained when extracted soil samples were spiked with metolachlor at 0.25, 0.5, and 2.5 μg/L were between 84 and 112%. Other authors have determined by ELISA the presence of metolachlor in soil samples with recoveries around the 90% (*43*). In Table 9, extracted soil samples spiked with alachlor at 0.6, 4, and 28 μg/L showed recoveries values between 88 and 108%. Finally, Table 10 shows the recovery percentages obtained from extracted soil samples spiked with acetochlor, which were between 73 and 102%. The CV values are lower than 20%.

In summary, the results clearly confirmed the utility of the developed ELISAs in comparison with chromatographic methods for determination of chloroacetanilide residues in environmental samples. Especially in soil, which is a complex matrix, data do not show the important differences between the different samples, considering the heterogeneity in chemical composition (ratios clay/slime/sand).

Also, the immunoassay has a very good sensitivity, especially for metolachlor, because it is possible to achieve a LOD of 0.06 μg/L directly in water samples, when GC/ECD and GC/MS are about 0.7−0.05 μg/L using a preconcentration step (*41*).

In this way, immunoassay has advantages, especially the much shorter time required for developing ELISA assays (the ratio in time ELISA/GC for water samples is 2−3 h to days). Furthermore, ELISA has economic advantages, because equipment and consumables are cheaper. Also, immunoassay is profitable in portability and operates reliably with users not experienced in this technique. Additionally, ELISA could be used not only as a primary screening procedure for chloroacetanilides residues analysis but also, as herein demonstrated, as a reliable and sensitive quantitative method. Therefore, immunoassay and chromatographic techniques may be used in conjunction for positive samples confirmation.

Finally, these results are in agreement with the "*Performance-based measurement systems*" (*44*) adopted by the U.S. EPA that allows facilities to use validated alternative methods for monitoring environmental contamination with the purpose of increasing the quality of the analytical procedures. Since there is a tendency to reduce the use of metolachlor and alachlor or replacing its use by acetochlor, the immunoassays appear as an attractive and reliable methodology for monitoring chloroacetanilide residues.

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