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Rapid trace analysis of alachlor in water and vegetable samples

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Abstract

The use of a rapid and specific (cross-reactivity<4%) enzyme-linked immunosorbent assay (ELISA) for the determination of alachlor residues in water and vegetable samples is addressed. The analytical method consists of a fast extraction procedure followed by an optimised ELISA. The detection limit was 0.44 μg l^{-1}, with a linear range from 0.89 to 143.2 μg l^{-1}. For alachlor extraction from water samples, different solid-phase cartridges (C_{18}, Ph, C_8 and C_2) were assayed using MeOH as eluent. Extracts were diluted (1:4) with distilled water before ELISA. This procedure gave recoveries close to 100% with RSDs<14%. For vegetable samples, alachlor was extracted directly with MeOH and the extracts diluted 1:40 (v/v) with saline buffer prior to ELISA. The results obtained by the proposed procedure correlate well with the reference method (multiresidue extraction–GC–MS) for vegetable samples (r>0.85).

Keywords: Water analysis; Environmental analysis; Vegetables; Food analysis; Immunoassays; Alachlor; Pesticides

1. Introduction

Herbicides account for approximately 65% of all pesticide use worldwide [1]. Alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide] is one of the most widely used chloroacetanilide herbicides and is the active ingredient of Lasso herbicide. It is a pre-emergence herbicide which is applied to the soil before cultivation [2]. Chloroacetanilides have a widespread use in the USA, particularly in the Midwest, with an annual consumption of 50.2 Mkg in 1992; this amount increased to ca. 1.66×10^6 kg between 1992 and 1997 in the USA [3].

Even though pesticides are indispensable in modern civilisation, society is aware of their potential toxicity for humans and animals, so the presence of their residues in commodities shows a potential hazard for consumers. Therefore, maximum residue levels (MRLs) of pesticides in water and food samples to protect both the environment and the consumers have been established.

The US Environment Protection Agency (EPA) has established a maximum contaminant level of 2.0 μg l^{-1} for alachlor while in the European Union (EU) the MRL is 0.1 μg l^{-1} [4], both in drinking water.

In 1990, the Directive of the Council 90/642/ECC, modified later on by 93/58/ECC and 94/30/ECC, established a new legal level in the EU for the fixation of MRLs in products of vegetable origin and for the control of pesticide residues. As a result, the use of alachlor was banned for vegetables (analytical

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detection limit for MRL=0.05 μg kg^{-1}) being only authorised for broccoli, cauliflower and onions (MRL=0.1 μg kg^{-1}).

Since typical water treatments do not usually remove water soluble herbicides, levels of alachlor residues ranging from 0.05 to 51.0 μg l^{-1} have been found in surface and ground waters [5].

EPA multiresidue methods 507 and 525 are used for alachlor determination [6]. Other chromatographic GC–MS or GC with nitrogen–phosphorus detection (NPD) methods are in use, with limits of detection (LODs) lower than 0.1 μg l^{-1} in water samples and 10 μg l^{-1} in soils, respectively [7].

These methods, although robust and well established, are time consuming, expensive, require specialised technicians and instrumentation, and the number of samples that can be processed daily is small. In addition, the amounts of chemicals and toxic solvents that are used often have greater risks than that of the pesticide residue to be determined [8].

These disadvantages clearly show the need for developing fast, easy-to-use, robust, sensitive and cost-effective techniques suitable for field analysis. Immunoassays (IAs) meet these requirements, and many pesticides can be analysed and monitored at regulatory levels without any or minimal sample preparation [9].

The strength of IAs lies in the screening of a large number of samples within a short time being a valuable supplement to conventional analytical methods. Due to the low cost of one analysis, more replicates from one site can be measured or special places can be sampled more often to obtain information about variations of pesticide concentrations in a sample, such as development of surveillance programs (SPs), contamination maps, etc.

In 1990, Feng et al. [10] reported the use of enzyme-linked immunosorbent assay (ELISA) to detect alachlor in water with a detection range of 0.2 to 8.0 μg l^{-1} using a thioether linkage for the synthesis of alachlor protein conjugates.

Presently, different commercial kits are available for alachlor with an inhibition coefficient (I_{50}) ranging from 0.6 to 5.0 μg l^{-1} applicable for water but no references are found for vegetable samples [11]. Whereas water samples do not require hard sample treatment, identification and removal of matrix effects are the major obstacles for the application of pesticide immunoassay in food. In this sense, methods need to be developed to provide reliable data, but they need to use minimum sample processing, or otherwise many of the potential advantages of the immunoassays, such as low cost, speed, and simplicity, would be lost [12].

This paper reports the development of a sensitive methodology based on rapid extraction and ELISA determination of alachlor in water and vegetable samples.

The ELISA optimisation includes a set of steps emphasising assay formats, immunoreagent selection and/or optimisation, assay time, cross-reactivity studies and tolerance of antibodies to organic solvents. The characterisation of this immunoassay with respect to the influence of several physicochemical factors on its performance is described.

Solvent partition or water-miscible solvent are often efficient extraction methods and are directly compatible with immunoassay methods [13,14]. However, the efficiency of the residue extraction must also be studied before a particular solvent is decided on. In this case, more work is needed for the development of a simple extraction method for alachlor in vegetable samples. Thus, the amount of sample, type and volume of organic solvent, extraction and contact time, the MRL for alachlor in the target sample, study of possible matrix interference and dilution or concentration of the extract were examined. Finally, recovery, reproducibility and comparative studies with data obtained by reference methods were performed.

2. Experimental

2.1. Chemicals and immunoreagents

Analytical standards of acetochlor, alachlor, butachlor, metolachlor, propachlor and their oxanilic and sulphonic acids were purchased from Ciba-Geigy (Barcelona, Spain), Dr Ehrenstorfer (Augsburg, Germany) and Riedel-de Häen (Seelze-Hannover, Germany). Stock solutions of pesticides were prepared in methanol (MeOH) and kept at −20 °C. *o*-Phenyl-

enediamine (OPD), poly(oxyethylensorbitan)monolaurate (Tween 20), bovine serum albumin, fraction V (BSA) ovalbumin (OVA) and *N*-acetylhomocysteine thiolactone (AHT) and sea salts were purchased from Sigma (St Louis, MO, USA). Horseradish peroxidase (HRP) was purchased from Boehringer (Mannheim, Germany). All other reagents used were analytical or biochemical grade.

Anti-alachlor polyclonal serum S3 was obtained from female New Zealand white rabbits immunised with alachlor derivative covalently attached to KLH. The serum was obtained and characterised as described in a previous work by Casino et al. [15]

Peroxidase-labelled goat anti-rabbit immunoglobulins (GAR-HRP) were acquired from Sigma.

2.2. Buffers and solutions

The coating buffer (CB) was 50 m*M* sodium carbonate–hydrogen bicarbonate buffer, pH 9.6. Phosphate-buffered saline (PBS) was a solution of 10 m*M* phosphate, 137 m*M* NaCl, 2.7 m*M* KCl, and unless otherwise stated the pH was 7.4. PBS-T was PBS with 0.05% Tween-20. Citrate–phosphate buffer was 25 m*M* sodium citrate, 62 m*M* sodium phosphate buffer, pH 5.4. The enzymatic substrate solution was OPD (2 mg ml^{-1}) and H_2O_2 (0.012%) in citrate–phosphate buffer. Enzymatic reaction was stopped by adding 2.5 *M* sulphuric acid (0.1 ml).

2.3. Herbicide–protein conjugates

The use of a thioether linkage for the synthesis of alachlor protein conjugates has an advantage over both the active ester and the mixed anhydride methods, since it is performed in one step without the necessity of synthesizing haptens. In this way, the herbicide metolachlor was attached to OVA as the carrier protein [16]. Approximately 2.8 mg of metolachlor was conjugated to OVA (20 mg) in a one-step reaction via thioether linkage using AHT (1.7 mg). The conjugate was purified by gel exclusion chromatography on Sephadex G-25, using 10 m*M* PBS pH 7.4 as eluent, and stored at −20 °C. The OVA–metolachlor was used as coating conjugate.

2.4. Instrumentation

2.4.1. ELISA

Polystyrene microtiter plates were purchased from Costar (Cambridge, MA, USA). Washing steps were carried out using a 12-channel microplate washer from Nunc (Roskilde, Denmark). Absorbances were read with a Multilabel Counter 1420 microplate reader from Wallac (Turku, Finland) at dual wavelength mode at 490–650 nm. Data processing was carried out using Sigmaplot software package (Jandel Scientific, Erkrath, Germany).

2.4.2. GC–MS instrumentation

For GC analysis a 6890 Hewlett-Packard device—automatic sampler—provided with a 5% phenyl–methylsiloxane capillary column (HP-5MS) model 19091S-433 (30 m×250 μm diameter, 0.25 μm film thickness) and a flame-ionization detection (FID) system was used. Also, a 5973 mass-selective detector operating in selected ion monitoring (SIM) mode was employed for peak identification.

2.5. ELISA development

2.5.1. Screening of the immunoreagents

Working dilutions were determined by titration assays. Optimal concentrations for pAb S3, OVA–metolachlor and GAR-HRP were chosen to produce absorbances around 0.7–1 U of absorbance in 1 h.

2.5.2. ELISA optimisation

The sensitivity and detection range of the immunoassay (antigen–antibody interaction) would be influenced by a set of experimental parameters. So, the influence of pH, detergent concentration, ionic strength and BSA were studied sequentially. Criteria used to evaluate immunoassay conditions were the I_{50} parameter, maximal and minimal absorbance, and slope of the calibration curves obtained. These experiments were carried out using the optimal concentration of immunoreagents. Several alachlor calibration curves (12 concentrations from 0.1 to 500 μg l^{-1}) were run on the same plate (eight points per concentration). The best experimental conditions such as ionic strength, pH, surfactant concentration and BSA effect were evaluated.

2.5.3. Effect of the ionic strength and pH

For this study, S3 serum was dissolved in different concentrations of PBS (2.5, 5, 10, 15, 20 and 40 m*M*) prepared by diluting 100 m*M* PBS with distilled water, and the best one was assayed at different pH (4–9) values.

2.5.4. Effect of Tween-20

Different concentrations of Tween-20 (0, 0.005, 0.025, 0.05, 0.25 and 0.5%) were added to PBS in order to prepare the alachlor standard curves and the S3 solutions. A new set of competitive curves was then developed with the described conditions.

2.5.5. Effect of BSA

The addition of BSA protein (0.5%, w/v) was checked in order to improve inter-assay reproducibility.

2.5.6. Effect of organic solvents

The use of organic solvents has to be adequately optimised since ELISA antibodies generally are not tolerant to solvent concentrations greater than 10%. Therefore, using the optimised conditions described above, competitive curves were carried out for alachlor standards containing percentages from 0 to 20% of acetone, acetonitrile, ethanol and methanol, respectively.

2.6. Optimised competitive ELISA

ELISAs were performed as follows: 100 μl of coating antigen OVA-metolachlor (10 μg ml^{-1}) in coating buffer were passively adsorbed to microtiter wells by incubation overnight at 4 °C. After washing the plates four times with assay buffer (PBS-T), 50 μl of standards, controls or samples in PBS 2× (pH 8.7) were added to the appropriate wells in triplicate. After the addition of 50 μl rabbit pAb S3 (1:10 000) in PBS 2× (pH 8.7), the plates were incubated at room temperature for 1 h. Once the plates were washed four times with PBST, 100 μl of goat anti-rabbit IgG-HRP conjugated (1:4000) in PBST were added, incubated at room temperature for 1 h and washed again four times with PBS-T. Afterwards, 100 μl of substrate solution (2 mg ml^{-1} *o*-phenylenediamine in 25 m*M* sodium citrate, 62 m*M* sodium phosphate buffer, pH 5.4, containing 0.012% H_2O_2) were added and incubated at room temperature for 10 min. Finally, the plates were read in a spectrophotometer at 490–650 nm and the absorbance values from standards mathematically fitted to a four-parameter logistic equation. The analyte concentration of samples was then determined by interpolation of the mean absorbance on the resulting standard curve.

2.7. Cross-reactivity determinations

The ability of S3 serum to recognize several related compounds was considered. Stock solutions of 1000 mg l^{-1} of acetochlor, butachlor, metolachlor, propachlor and their oxanilic and sulphonic acids were prepared in MeOH. The cross-reactivity (CR) values of each compound were established performing competitive assays and determining their respective I_{50} values (analyte concentration that reduces the maximum signal of the competitive ELISA to 50%) according to the following equation: $\%CR = [I_{50}/I_{50}\ (\text{derivative})] \times 100$.

2.8. GC–MS determinations

Prior to GC–MS analysis, vegetable extracts were conditioned with ethylacetate–hexane (1:1). Helium was used as carrier gas with a flow-rate of 1.2 ml min^{-1} and samples were injected into the splitless mode by an autoinjector. The column temperature was held at 60 °C for 1 min, then increased 30 °C/min to 110 °C, 10 °C/min to 240 °C, 30 °C/min to 285 °C and held at this temperature for 10 min. Injector temperature was 250 °C. Alachlor was detected by selected ion monitoring of three characteristic fragment ions (*m*/*z* 160, 188, 237).

2.9. Water samples

The optimised ELISA was applied to alachlor determination in five water samples from different sources: commercial tap water, surface water samples from Albufera lake, Vera irrigation ditch and Júcar river (Valencia, Spain) and seawater samples collected from a bathing zone on Cullera beach (Valencia, Spain).

For alachlor extraction from these water samples, different solid-phase extraction (SPE) cartridges

(C_{18}, Ph-phenyl, C_8 and C_2) were assayed using MeOH as eluent. The cartridges (3-ml capacity) containing 500 mg of bonded silica gel were from Varian (Harbor, CA, USA). Twenty-eight samples of distilled water (160 ml of each) were fortified with alachlor at two levels: 0 (as control), 0.1 and 0.5 μg l^{-1} and were extracted using SPE. The cartridges were activated with MeOH (3 ml) and washed with water (3 ml). After 160 ml of sample was passed through the cartridges at a flow-rate of 1.5 ml min^{-1}, they were briefly dried under vacuum and alachlor was eluted with 1 ml of MeOH. Finally, the extracts were diluted (1:4, v/v) with distilled water and added (50 μl) to the appropriate wells by triplicate.

2.10. Vegetable samples

Fresh and processed vegetables (asparagus, bean, broccoli, bonnet pepper, celery, cucumber, lettuce, melon, pea, red pepper, tomato and watermelon) were collected from a local market in Valencia (Spain) and checked for alachlor presence in residues by GC–MS. Before the extraction procedure, samples were chopped and homogenised in an Osterizer blender (Milwaukee, WI). After this, alachlor-free samples were fortified at different levels [0 (as control), 50, and 500 μg l^{-1}] with a 1000 mg l^{-1} alachlor standard, mixed, homogenised overnight and extracted according to the following procedure (Fig. 1). Vegetable samples (5 g) and 10 ml of MeOH were blended for 10 min, and the supernatant vacuum filtered through 0.45-μm nylon filters (Durapore membrane filters) using a Millipore extraction device. The extracts were then conditioned by diluting 1:20 in PBS 2×. Finally, food samples were checked for alachlor with both ELISA and GC–MS methods. In order to assess assay reproducibility, triplicates of each fortification level were performed.

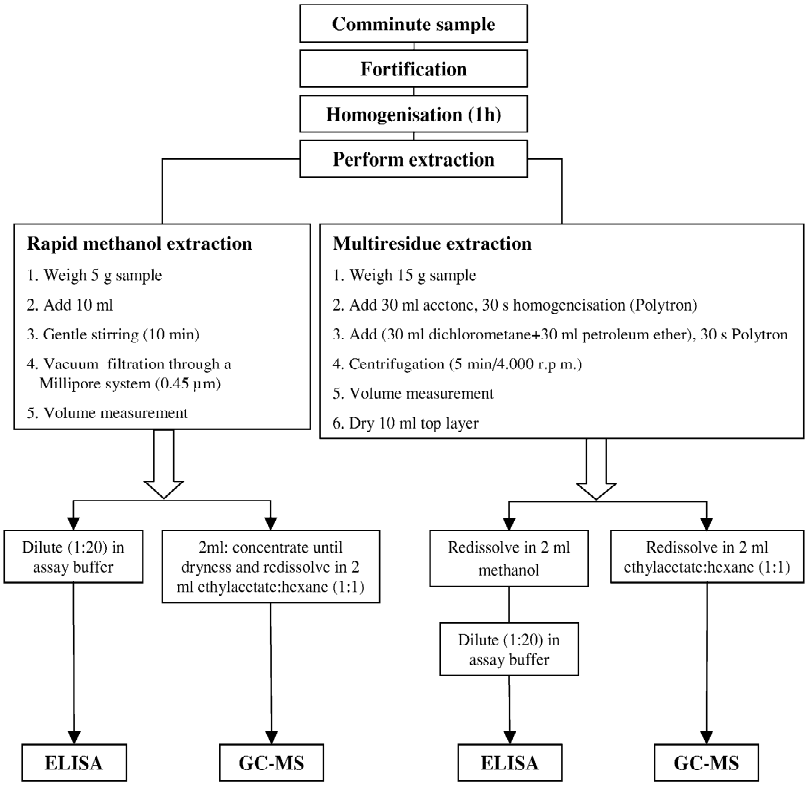

Fig. 1. General protocol used for preparation and analysis of vegetable samples.

The results obtained by rapid extraction (RE) coupled to ELISA assay were compared with the data obtained by GC–MS.

Samples were also extracted using the method described by Luke et al. [17] with slight modifications. Briefly, fortified samples (15 g) were homogenised for 30 s with 30 ml of acetone using an Ultra-turrax T-25 apparatus mixer (Hanke and Junkle, Germany); 30 ml of dichloromethane and 30 ml of light petroleum were subsequently added, and the mixture was homogenised again for another 60 s. After centrifugation for 5 min at 4000 rpm, a portion of 25 ml of the organic layer was transferred to a conical-bottom flask, the solvent evaporated to dryness and reconstituted in 5 ml of an ethyl acetate–hexane mixture (1:1, v/v). Finally, 2 μl of each sample were injected, by pulsed splitless, on the GC column for alachlor quantification. For ELISA determination, the extract was reconstituted in 2 ml of MeOH and conditioned in the assay buffer.

3. Results and discussion

The performances of S3 serum, OVA–metolachlor and GAR-HRP concentrations on the analytical parameters of the competitive ELISA curve were studied. Coating conjugate concentrations varied from 0.1 to 100 μg ml^{-1}. Dilutions factors of 1:100 000–1:10 and 1:20 000–1:100 were assayed for S3 serum and GAR-HRP, respectively.

The higher the concentration of immunoreagents S3, coating conjugate, or tracer, the higher absorbances and I_{50} values achieved (>1 a.u.). The highest maximum absorbance-to-I_{50} ratio was obtained using 10 μg ml^{-1} OVA–metolachlor as coating conjugate, 1:10 000 dilution of rabbit pAb S3 and 1:4000 dilution of goat anti-rabbit IgG tracer.

To improve immunoassay performance, the influence of several parameters on the assay were investigated. Despite the influence that physical parameters like time and temperature have on assay performance, a 1-h step reaction at room temperature is generally the most used condition to carry out indirect ELISA. Therefore, incubation times of 16 h overnight for coating purposes, 1 h for the competition step, 1 h for the labelled antibody interaction and 10 min for the enzymatic reaction were selected and used throughout this work.

Sensitivity of the alachlor immunoassay improved (lower I_{50}) as ionic strength of the competition buffer increased. Since the beneficial effect of ionic strength on the assay sensitivity was counterbalanced by the negative influence on the background signal, a concentration of 20 m*M* PBS was selected as optimum.

Regarding the effect of pH, we found that the immunoassay was much more sensitive at pH values between 8 and 9. A pH value of 8.7 was chosen as optimum to keep an acceptable signal, increasing sensitivity.

The addition of detergents in buffers to reduce non-specific interactions is a very common practice in immunoassays, Tween 20 being perhaps the most extensively employed detergent. Also, BSA has often been included in buffers to reduce well-to-well and inter-assay variability. As described in Section 2.5.4, a set of competitive curves with different concentrations of Tween 20 was developed (data not shown). At the ionic strength previously determined as optimal, the presence of Tween 20 is detrimental to both sensitivity and the maximum signal of the assay. In addition, relevant assay parameters did not improve significantly when BSA was included in the assay buffer. According to these results, the best situation was the absence of Tween 20 and BSA in the buffer used in the competition step.

Table 1
Cross-reactivity of the alachlor ELISA for structurally related compounds

Compound	I_{50} (μg l^{-1})	CR (%)
Alachlor	1.76	100.0
Acetochlor	45.12	3.91
Butachlor	79.26	2.22
Metolachlor[b]	85.66	2.05
Alachlor[b]	89.85	1.96
Acetochlor[a]	91.74	1.92
Metolachlor[a]	1.0×10^{2}	1.68
Metolachlor	4.0×10^{2}	0.45
Propachlor	5.0×10^{3}	3.0×10^{-2}
Alachlor[a]	1.0×10^{3}	1.0×10^{-2}
Acetochlor[b]	$>2.0\times10^{4}$	7.0×10^{-3}

The values are means of three determinations.
[a] Oxanilic acid.
[b] Sulphonic acid.

Taking into account all these factors, the optimised ELISA for the determination of alachlor has a range between 0.32 and 95.0 μg l^{-1} (80 and 20% of the maximal absorbance, respectively). The midpoint of the test response was found at 1.76 μg l^{-1} of alachlor, with a detection limit of 0.08 μg l^{-1} (90% of the maximal absorbance).

The selectivity of S3 sera was checked using as competitors some structurally related compounds and CR values calculated as percentages. From data shown in Table 1 the reported immunoassay can be considered specific for alachlor since all compounds tested present CR values below 4%.

In order to use the optimised ELISA for alachlor analysis, water samples must be concentrated to achieve the EU MRL in drinking water.

The effect of several concentrations of organic solvents, commonly used to extract pesticide residues from water and foods, on the performance of the alachlor ELISA was studied (Fig. 2). A clear decrease in maximum signal (B_0) and sensitivity was observed when increasing amounts of solvents were added to the assay buffer. MeOH was the best tolerated of the solvents tested (up to 10%), maintaining good sensitivity and absorbance value in absence of pesticide (B_0).

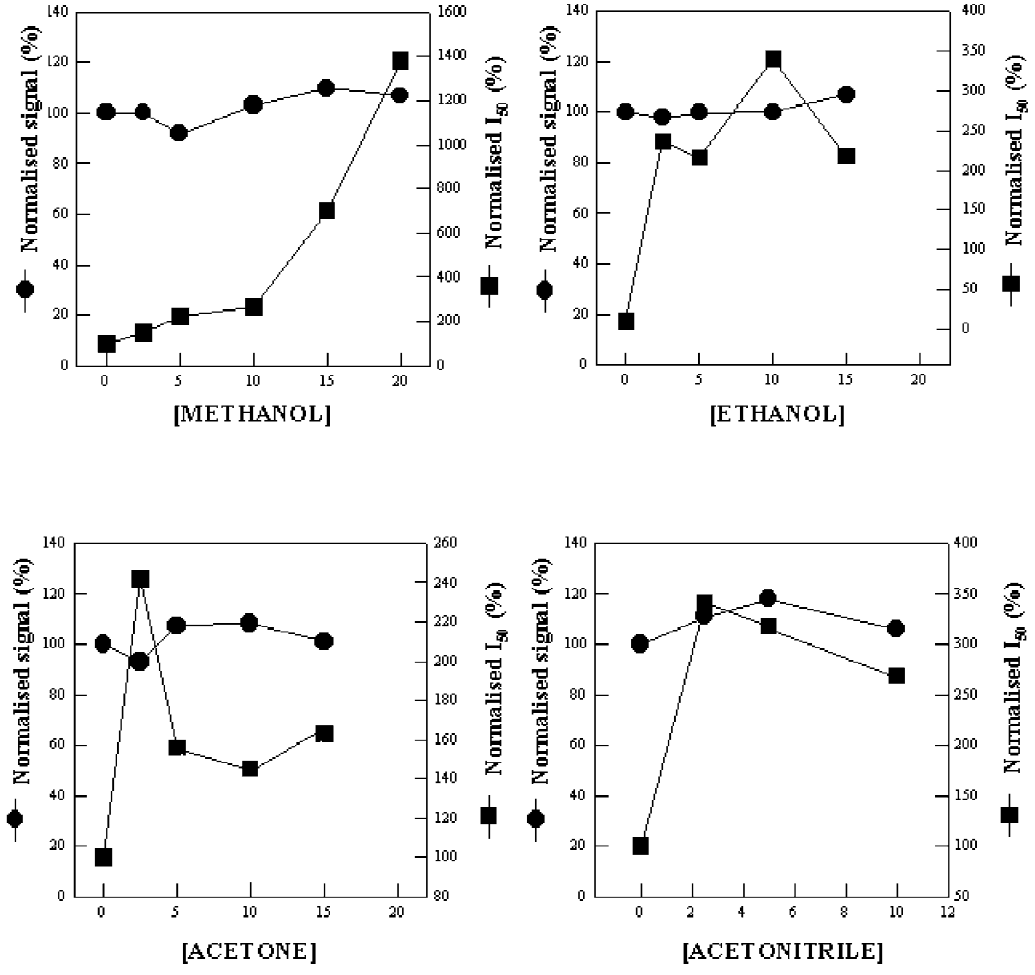

Fig. 2. Organic solvent tolerance of the alachlor immunoassay. Data were obtained from calibration curves carried out in buffer containing different concentrations of the solvents (from 0 to 20%). Maximum absorbance (●) and I_{50} value (■) are expressed relative to the control inhibition curve, performed without organic solvent.

Using the optimized alachlor ELISA protocol in methanol medium, a set of individual curves ($n=50$) were normalized by expressing the absorbance as the percentage of the maximum response to obtain the alachlor calibration curve (each point represents the mean±standard deviation).

As can be seen in Fig. 3, the main analytical features of the curve are an average I_{50} for alachlor of 2.72 μg l^{-1}, a LOD of 0.44 μg l^{-1} and a working range from 0.89 to 143.2 μg l^{-1}.

To select the optimum SPE, previous experiments were performed using fortified distilled water as described in Section 2.9. A 160-ml volume was selected as optimum in order to bring alachlor concentrations, within the EU MRL, into the working range of the ELISA. Results obtained using C_{18} cartridges were poorer than those obtained using C_8, Ph-phenyl and C_2. As can be see in Table 2, this procedure gave recoveries close to 100% with RSDs<14%. Based on standard deviation values, C_8 was selected as optimum. This cartridge was used to accomplish a surveillance of alachlor residues in surface and ground waters. The results are in accordance with the recommendations made by the manufacturer that advise the use of C_8-Octyl for the analysis of most frequent herbicides in water samples.

To achieve more knowledge about alachlor levels in natural selected waters, five samples at five different points were obtained. Water samples were

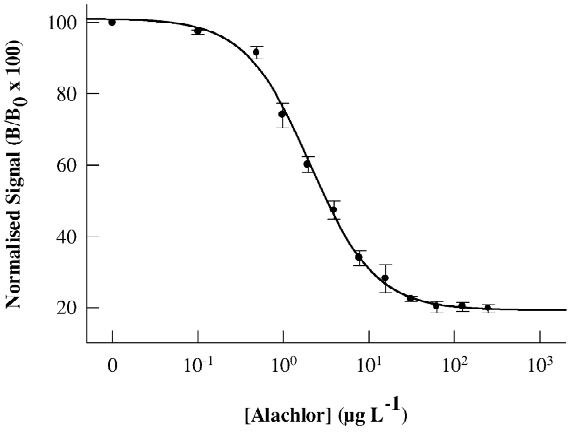

Fig. 3. Calibration curve for alachlor using optimised assay conditions in methanol medium. Each point represents the mean of 50 determinations.

Table 2
Determination of alachlor in water by SPE–ELISA

SPE cartridge bound phase	Alachlor added (μg l^{-1})	Alachlor detected[a] (μg l^{-1})	Recovery (%)
C_8-Octyl	0.0	n.d.	–
	0.1	0.092±0.010	92.5
	0.5	0.054±0.000	108.0
Ph-phenyl	0.0	n.d.	–
	0.1	0.096±0.005	96.5
	0.5	0.390±0.056	78.0
C_2-Ethyl	0.0	n.d.	–
	0.1	0.112±0.010	112.5
	0.5	0.485±0.007	97.0

[a] SD (standard deviation $n=3$).

Table 3
Analysis of alachlor in tap, surface and seawater samples

Sample source		Mean pH	Alachlor found[a] (μg l^{-1})
Tap water	T1	7.98	<LOD
	T2		<LOD
	T3		<LOD
	T4		<LOD
	T5		<LOD
Albufera Lake	A1	7.80	<LOD
	A2		<LOD
	A3		0.061±0.003
	A4		1.190±0.005
	A5		0.0870±0.007
Vera irrigation ditch	V1	7.93	1.200±0.010
	V2		0.690±0.030
	V3		0.091±0.004
	V4		0.052±0.005
	V5		5.000±0.014
Jucar River	J1	7.90	<LOD
	J2		<LOD
	J3		<LOD
	J4		<LOD
	J5		<LOD
Cullera beach	C1	8.20	<LOD
	C2		<LOD
	C3		<LOD
	C4		<LOD
	C5		<LOD

[a] SD (standard deviation $n=3$).

processed, using C_8 as the solid phase, as described in Section 2.9. Prior to the extraction procedure, samples were filtered though a filter paper (Whatman No. 5) to eliminate any solid material. One fraction of each sample was used as control for false positive results.

The level of alachlor in the selected water samples was obtained interpolating the absorbance values in the alachlor calibration curve, whereas for seawater alachlor values were acquired from an artificial calibration curve containing 35 g l^{-1} of sea salts. This was done because the absolute signals obtained when analysing seawater were much higher than in buffered media. As shown in Table 3, from the 25 samples tested, only four gave alachlor levels over the established MRL (0.1 μg l^{-1}). These results are expected since there were some agricultural areas near the sampling points. Alachlor was not detected when river and seawater samples were analysed.

3.1. Vegetables

In order to optimise the efficiency of the extraction method, 30 canned tomato samples were fortified with alachlor at 50 μg kg^{-1}. For this experience, the following parameters were assayed: amount of sample (5, 10, 15 and 20 g), volumes of methanol and acetone from 10 to 40 ml, extraction and contact time between 5 and 20 min, and dilution of the extracts from 1/20 to 1/100 in order to eliminate possible matrix interferences. Finally, the alachlor values were determined by the optimised ELISA.

The best results were achieved using MeOH as extractant (89.2–99.9%) since acetone extracts gave alachlor recoveries from 101 to 141%. Those recovery values could be explained by the lower polarity of acetone solvent, able to extract organic components from the sample such as proteins, tannins and polyphenols. Concerning the amount of sample and volume of organic solvent, 5 g and 10 min as contact time were enough to achieve recoveries close to 100%. In addition, 1:20 dilution of the extracts with buffer (1:40 in the well) was adequate to remove matrix effects.

Finally, reproducibility, recovery and comparative studies with data obtained by reference method were done.

With any analytical technique, the reproducibility of the results is very important. Six different samples were fortified or not and provided as blind samples. Three subsamples of each one were processed using the optimised method (MeOH extraction and ELISA

Table 4
Analysis of alachlor in blind samples

Sample	Alachlor added (μg l^{-1})	Alachlor found (μg l^{-1})	Mean[a] (μg l^{-1})	Recovery (%)
1A	300	270.0	271.5±11	90.5
2A		283.0		
3A		261.0		
1B	180	189.5	196.7±18	109.3
2B		218.0		
3B		182.8		
1C	0	<LOD		
2C		<LOD		
3C		<LOD		
1D	90	89.6	88.2±2	98.0
2D		89.6		
3D		85.6		
1E	450	477.8	488.6±31	108.5
2E		524.0		
3E		464.2		
1F	1000	1086	1003±94	100.3
2F		1023		
3F		901.0		

[a] SD (standard deviation $n=3$).

determination). Data obtained (Table 4) demonstrated the viability of the proposed method for the determination of alachlor in a wide concentration range. For alachlor values near to 1000 μg kg^{-1}, the accuracy of the assay method decreased. This fact can be explained since those concentrations are not in the working range of the competition curve (minimum asymptote).

In order to determine the recovery of the extraction method and the precision of the assay, 12 subsamples of canned tomato fortified at 200 μg kg^{-1} with alachlor were extracted using MR and RE, and determined by ELISA. The overall results are excellent with RSDs<17%.

On the other hand, comparative studies were carried out in a set of different vegetables. For this purpose, samples were fortified at two levels: 50 and 500 μg kg^{-1} with alachlor and analysed in triplicate, by both the proposed and reference methods, as described in Section 2.10. In all cases a non-fortified sample was checked by GC–MS for incurred alachlor residues and used as negative control.

As can be seen in Table 5, mean recoveries for fresh and processed vegetable samples using the proposed method were 92.5 and 89.5%, respectively and similar to those obtained for reference method (90.2–87.9%). Based on RSD values (<20% in all samples), the suitability of the proposed methodolo-

Table 5
Recovery values obtained for alachlor in vegetables using GC–MS and ELISA techniques; comparison of extraction methods

Matrix	Fortified level (μg kg^{-1})	Found level of alachlor											
		GC–MS determination						ELISA determination					
		Multiresidue extraction			Rapid extraction			Multiresidue extraction			Rapid extraction		
		Mean value	RSD (%)	Recovery (%)	Mean value	RSD (%)	Recovery (%)	Mean value	RSD (%)	Recovery (%)	Mean value	RSD (%)	Recovery (%)
Tomato[1]	50	42.3	6.8	86.4	49.1	7.4	98.3	36.5	11.5	73.0	41.2	8.9	82.4
	500	448.2	4.0	89.6	378.0	7.6	75.6	462.0	2.0	92.4	448.0	10.0	89.6
Bonnet pepper[1]	50	51.6	3.9	103.2	41.1	5.0	82.3	48.8	8.0	97.6	43.7	10.3	87.3
	500	476.8	4.0	95.4	497.2	5.9	99.4	466.7	19.4	93.3	456.3	9.0	91.3
Bean[1]	50	44.7	13.0	89.5	48.7	4.4	97.5	45.2	3.6	90.3	54.1	3.7	108.2
	500	361.7	7.2	72.4	442.0	8.3	88.4	377.2	5.7	75.4	455.4	6.9	91.0
Pea[1]	50	47.7	16.4	95.5	46.0	5.3	92.1	46.4	9.8	92.9	40.6	12.9	81.1
	500	398.4	7.1	79.7	445.0	3.9	89.0	404.4	8.6	80.9	433.3	9.0	86.7
Asparagus[1]	50	51.5	2.7	103.0	36.8	4.6	73.6	46.7	10.7	93.3	46.0	10.8	92.0
	500	495.8	4.4	99.1	468.0	1.3	93.6	442.5	19.0	88.5	421.5	4.5	84.3
Broccoli[2]	50	48.0	11.2	96.1	40.2	0.6	80.5	41.9	14.7	83.8	36.4	5.8	72.9
	500	436.0	2.0	87.2	400.8	10.2	80.2	493.0	1.8	98.6	409.9	7.8	82.0
Watermelon[3]	50	40.8	8.9	81.6	48.2	14.2	96.4	43.6	13.4	87.2	50.4	3.0	100.9
	500	455.3	1.4	91.0	429.0	0.5	85.4	395.2	14.7	79.0	477.84	10.1	95.6
Melon[3]	50	45.6	8.5	91.2	40.6	3.2	81.2	40.2	0.5	80.4	42.6	5.0	85.2
	500	468.7	4.0	93.7	488.0	3.5	97.6	431.4	15.0	86.3	464.2	4.8	92.8
Lettuce[3]	50	42.8	5.5	85.6	41.8	0.3	83.6	46.3	6.6	87.3	44.6	0.3	89.2
	500	525.3	6.0	105.0	430.5	1.6	86.1	483.4	15.6	96.7	406.7	8.4	81.3
Cucumber[3]	50	53.0	0.7	106.0	46.7	2.3	93.5	48.5	12.0	97.0	54.0	8.1	108.0
	500	467.3	2.4	93.5	487.5	0.0	97.5	452.1	10.0	90.4	438.0	6.4	87.6
Celery[3]	50	56.7	3.2	113.5	46.0	3.2	91.9	59.2	3.6	118.4	44.8	11.0	89.6
	500	443.6	2.0	88.7	513.7	5.4	102.7	403.4	2.4	80.7	465.0	14.2	93.0
Red pepper[3]	50	47.2	6.9	94.4	40.0	11.5	80.0	43.2	0.6	86.4	38.7	16.5	77.4
	500	405.9	4.2	81.2	494.7	10.2	98.9	412.5	9.4	82.5	464.0	5.8	92.8

Tinned[1], Frozen[2] and Natural[3] foods. Values are mean of three determinations.

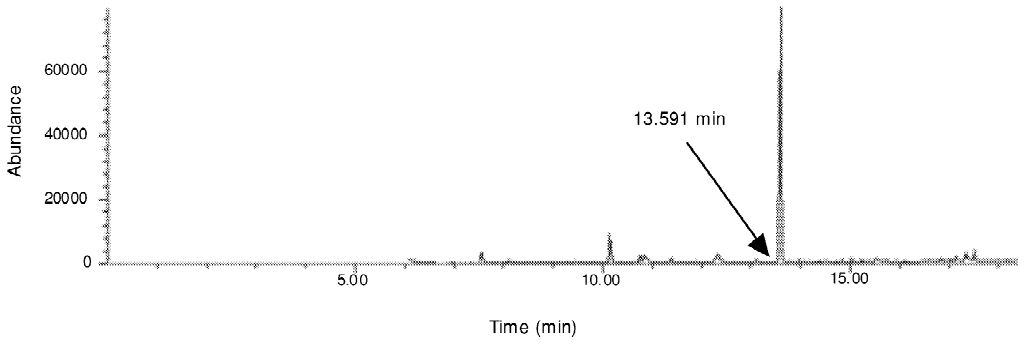

Fig. 4. GC–MS chromatogram of an extract of lettuce sample fortified with alachlor at 0.5 μg kg^{-1}.

gy (RE coupled to ELISA assay) was demonstrated. These matrices do not present interference compounds in the chromatographic area of interest, thus allowing an optimal quantification of alachlor (Fig. 4).

In addition, results obtained by the proposed procedure correlated well with the reference method (multiresidue extraction–GC–MS) in all vegetable samples ($r > 0.85$). A slope of 0.907 demonstrates that there is no bias between the techniques. Fig. 5 shows this correlation at low fortification level (50 μg kg^{-1}).

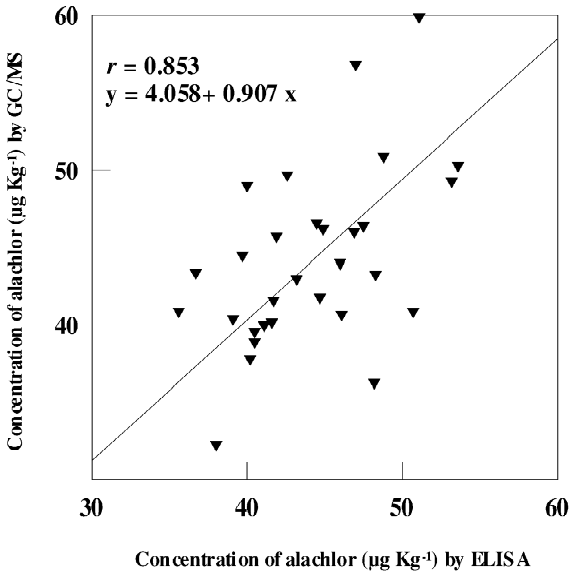

Fig. 5. Comparison between ELISA and GC–MS methods. Samples fortified at 50 μg kg^{-1} level.

4. Conclusions

A highly sensitive and specific ELISA for alachlor has been developed. The demonstrated ability of the antibodies to distinguish alachlor from other chloroacetanilide herbicides allowed the successful application of this assay to the analysis of environmental water samples.

The use of C_8 SPE cartridges to concentrate trace levels of the target analyte resulted in an assay with a low detection limit for alachlor in compliance with the EU MRL for drinking water.

Multiresidue and rapid extraction sample treatment methods show equivalent results in both immunoassay and GC–MS procedures. Both methods give recoveries around the mean values with comparable coefficients of variation. For this reason, rapid extraction could be advantageous as a screening methodology. Also, the rapid procedure uses a low quantity of methanol being a friendly environmental extraction method.

The main advantage of the reported methodology is the possibility to perform direct and accurate measurements of vegetable samples without purification or pre-concentration steps.

For vegetable sample analysis, the choice of a proper dilution factor minimises matrix interference and improves recoveries to achieve quantitative yields. ELISA methods have the advantages of high

throughputs and low price, the analysis of a large number of samples being possible simultaneously. This method can be applied in routine analysis for screening of alachlor residues in large number of vegetable samples.

In addition, the ability of the developed ELISA for the determination of alachlor in crude extracts coming from rapid or multiresidue extraction, implies that immunoassay in organic media can be included as a complementary method in pesticide regulatory programs.

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