Development of Enzyme-Linked Immunosorbent Assays for the Insecticide Chlorpyrifos. 2. Assay Optimization and Application to Environmental Waters

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Monoclonal antibody-based enzyme-linked immunosorbent assays (ELISA) to chlorpyrifos were optimized for the analysis of this insecticide in environmental water samples. By optimizing the immunoreagent conditions and the composition of the competition buffer, assays with up to 1 order of magnitude better sensitivities were obtained. Reduction of the detergent (Tween 20) concentration provided most of the sensitivity improvement. Other factors studied, such as pH and ionic strength, behaved consistently with the nonpolar nature of the analyte and contributed in lesser extent to improve assay characteristics. Under optimized conditions, the chlorpyrifos concentration giving 50% reduction of maximum ELISA signal (I_{50}) was in the 0.8−1.0 nM range, and the assays showed acceptable specificity for both chlorpyrifos and chlorpyrifos-methyl. Preliminary evaluation of assay performance in several water samples showed the absence of significant matrix effects for both formats evaluated. ELISAs provided a limit of detection of 0.10−0.14 μg/L and a working range in the 0.21−2.50 μg/L range and allowed the direct determination of submicrograms per liter chlorpyrifos in environmental water samples with acceptable analytical parameters.

Keywords: *Chlorpyrifos; monoclonal antibody; ELISA; optimization; environmental waters*

INTRODUCTION

Due to the widespread use of pesticides, there is a growing concern over the environmental contamination caused by their residues, which demands adequate monitoring. The analysis of pesticides and their derivatives using immunochemical methods is gaining acceptance as a simple, cost-effective screening of many samples prior to confirmatory chromatographic techniques (Brady *et al*., 1995a,b).

Immunoassays rely on the availability of suitable immunochemical tools. In the past decade, antibodies to a great variety of pesticides have been obtained, and subsequently incorporated into several ELISA formats. Antibodies determine primarily the assay affinity and specificity to the analyte, although appropriate immunoassay design can modify these characteristics. It is a common practice to examine several assay haptens and formats to find the highest affinity assay. Once the optimum immunoassay configurations have been selected, the study of other factors, related to the environment where the immunodetection takes place, is advisable to improve the quality of the assay. However, the optimization of these nonspecific factors is not usually undertaken (Hammock *et al*., 1990; Sherry, 1992; Meulenberg *et al*., 1995).

Immunoassays of small organic molecules such as pesticides are based on the competition established between the analyte and a suitable conjugate derivative for binding to a macromolecule (antibody) in an aqueous environment. Hence, variations of the physicochemical properties of the competition medium can modify the assay characteristics by affecting analyte− and hapten−antibody interactions (Tijssen, 1985; Jefferis and Deverill, 1991).

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Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate], an intensively used organophosphorus insecticide, presents important environmental relevance (Odenkirchen and Eisler, 1988; Newman, 1995). Consequently, this insecticide is an appropriate candidate for immunochemical analysis. As reported in the preceding paper, immunoreagents for the sensitive and specific detection of chlorpyrifos were obtained and incorporated into several ELISA systems. As a continuation, herein the optimization of the conditions (concentration, hapten loading of conjugates) of the immunoassay components is described, and the influence of relevant physicochemical factors (detergent, pH, ionic strength) of the competition assay buffer on the assay characteristics is examined, in order to select the optimum conditions to perform the immunochemical determination of chlorpyrifos. Additionally, the optimized ELISAs are applied to spiked water samples from different origins to study matrix effects and assay performance in real world samples.

MATERIALS AND METHODS

Reagents. Chlorpyrifos, chlorpyrifos-methyl, and 3,5,6-trichloro-2-pyridinol (TCP) standards were generously provided by DowElanco (Midland, MI). Other pesticide standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The structures of haptens used in this study are shown in Figure 1. Hapten **TR1** [[(3,5,6-trichloro-2-pyridyl)oxy]acetic acid] is the herbicide triclopyr and was purchased from Riedel-de Haën (Germany). The synthesis of hapten **PNC** [*O*-ethyl *O*-(2,4,5-trichlorophenyl) *N*-(2-carboxyethyl)phosphoramidothioate], preparation of the ovalbumin (OVA)−**TR1** conjugates and the horseradish peroxidase (HRP)−**PNC** enzyme tracer, and production of LIB−**PO** MAb have been described in the preceding paper (Manclús *et al*., 1996).

Preparation of Standard Solutions. Standard solutions were prepared on borosilicate glass tubes instead of the usual plastic ones, since we found that the latter material adsorbs chlorpyrifos. Although chlorpyrifos adsorption may also occur

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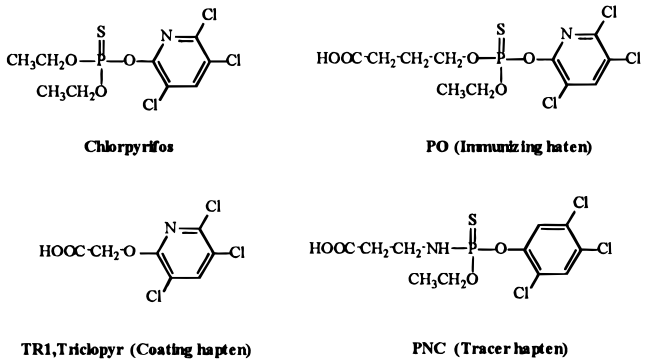

Figure 1. Structures of chlorpyrifos and of the haptens used in this study.

on polystyrene ELISA wells during the competition step of the assay, this phenomenon would equally affect standards and samples. From a working stock of 100 mM chlorpyrifos in 1,4-dioxane (Aldrich Química S.A., Madrid, Spain), serial dilutions in this solvent from 160 μM to 0.4 nM were prepared. From these concentrations, standards from 2 pM to 800 nM were prepared by 1/200 dilution in distilled water. As the assay procedure involved the addition of the same volume of the appropriate immunoreagent concentration, chlorpyrifos standards in the final assay ranged from 1 pM to 400 nM (0.4 ng/L to 140.4 μg/L).

Chlorpyrifos Immunoassays. All incubations and all assay buffers and solutions were kept at room temperature to avoid undesirable effects of temperature changes on assay precision. ELISA plates were sealed with precut acetate tapes (Dynatech, Chantilly, VA) throughout all assay steps. After each step, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Conditions of the two ELISA formats used were as follows.

Conjugate-Coated Format (CC Format). Flat-bottom polystyrene ELISA plates (Costar High Binding No. 3590, Cambridge, MA) were coated overnight with 100 μL/well of the appropriate concentration of OVA−**TR1** (4 μg/mL of the conjugate with an average hapten to protein molar ratio of 3, in the optimized competitive immunoassay) in coating buffer (50 mM carbonate−bicarbonate buffer, pH 9.6). Then, 50 μL/well of standards or samples was added, followed by 50 μL/well of the appropriate LIB−**PO** MAb concentration (0.10 μg/mL in the final optimized ELISA) in assay buffer [4× PBS, pH 6.5, containing 0.002% Tween 20, as the optimized buffer (PBS = 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl)], and the mixture was incubated for 1 h. Next, plates were incubated with peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako, Denmark) diluted 1/2000 in PBS, pH 7.4, containing 0.05% Tween 20. After 1 h, the HRP activity bound to the wells was determined by adding 100 μL/well of the substrate solution (2 mg/mL *o*-phenylenediamine and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate buffer, pH 5.4). After 10 min, the reaction was stopped by adding 100 μL/well of 2.5 M sulfuric acid, and absorbance was read in dual-wavelength mode (490−630 nm) using an ELISA plate reader (Dynatech Model MR-700).

Antibody-Coated Format (AC Format). ELISA plates were coated overnight with 100 μL/well of the appropriate concentration of LIB−**PO** MAb (10 μg/mL in the optimized competitive immunoassay) in coating buffer. Then, 50 μL/well of standards or samples was added, followed by 50 μL/well of the appropriate HRP−**PNC** concentration (0.25 μg/mL in the final optimized ELISA) in assay buffer (4× PBS, pH 6.5, containing 0.002% Tween 20, as the optimized buffer). After 1 h, the HRP activity bound to the wells was determined as above.

Data Analysis. Standards and samples were run in triplicate wells, and the mean absorbance values were processed. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration. Using Sigmaplot software package (Jandel Scientific, Germany), sigmoidal competitive curves were fit to the four-parameter logistic equation

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the asymptotic maximum (maximum absorbance in absence of analyte, A_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration giving 50% inhibition of A_{max}, I_{50}), and D is the asymptotic minimum (background signal). Determination of fortified samples was performed by interpolating their mean absorbance values in the standard curve run in the same plate.

Waters Analyzed. Water from different sources was used to evaluate potential matrix effects and to be fortified with chlorpyrifos. Waters tested were from Tavernes de la Valldigna Valley (Valencia, Spain), a region with intensive agricultural activities; samples were obtained from a well and from a pond, both used for irrigation, and from the stagnant water of a small river crossing the valley. All water samples showed a pH in the range 6.8−7.5. The river water and pond water were turbid and were filtered prior to use. These water samples were fortified with several chlorpyrifos concentrations covering the assay working range and analyzed by ELISA without any further treatment.

RESULTS AND DISCUSSION

In the preceding paper the production of immunoreagents for the highly sensitive detection of chlorpyrifos was described. LIB−**PO** MAb, derived from hapten **PO** [*O*-ethyl *O*-(3,5,6-trichloro-2-pyridyl) *O*-(3-carboxypropyl) phosphorothioate] (Figure 1), showed the highest affinity to chlorpyrifos. Both the conjugate-coated format and the direct or indirect antibody-coated format afforded similar sensitivities, although different heterologous haptens were used for each format (**TR1** as coating hapten and **PNC** as tracer hapten; Figure 1). These immunoreagents were therefore selected as specific components of the immunoassay to chlorpyrifos. Next, the optimization of assay conditions was carried out for both formats to compare their behavior and performance. For the antibody-coated format, since both the direct and the indirect antibody immobilizations used the same immunoreagents and provided similar assay characteristics, the direct approach requiring fewer steps was chosen for this study.

Optimization of the Immunoreagent Conditions. *CC Format.* OVA, the carrier protein used to prepare the coating conjugates, has 20 lysine residues, most of which are available for hapten coupling (Harlow and Lane, 1988). Hence, OVA conjugates with diverse hapten to protein molar ratios (MR) can be prepared simply by varying the initial MR of both species in the conjugation procedure. On the other hand, it has been reported that the degree of hapten loading in coating conjugates can affect the immunoassay sensitivity (Fránek *et al.*, 1994). Therefore, to study the influence of the coating conjugate MR on the assay characteristics, OVA−**TR1** conjugates with MR of 3 and 15 were prepared. Concentrations of these coating conjugates along with concentrations of LIB−**PO** MAb were optimized to provide the inhibition curve with the highest affinity (lowest I_{50}), giving adequate maximum absorbance around 1.0 in the absence of analyte. First, saturating conditions were determined by a noncompetitive two-dimensional titration covering a wide range of concentrations. Next, under subsaturating conditions, concentrations in a narrower range were combined and examined by competitive assays. The parameters of the competitive inhibition curves for each combination tested are shown in Table 1. Several features are worthy of remark. First, as expected, higher concentrations (about 8-fold) of the coating

Table 1. Characteristics of Chlorpyrifos Competitive Curves Obtained with Different Subsaturating Concentrations of the Specific Immunoreagents[a]

CC Format

OVA−**TR1**		[LIB−**PO** MAb]	parameters[c]	
MR[b]	[μg/mL]	(μg/mL)	A_{max}	I_{50} (nM)
15	1.0	0.10	1.11	17.8
	0.7	0.15	0.92	11.5
	0.5	0.20	1.06	9.4
	0.3	0.65	0.98	16.6
3	7.5	0.11	1.01	9.0
	5.0	0.13	1.04	8.2
	4.0	0.18	1.18	7.5
	3.0	0.26	1.09	9.2
	2.0	0.48	1.21	13.1

AC Format

[LIB−**PO** MAb]	[HRP−**PNC**]	parameters[c]	
(μg/mL)	(μg/mL)	A_{max}	I_{50} (nM)
20	0.25	0.98	12.2
15	0.33	1.12	11.8
10	0.45	0.96	10.5
5	1.50	1.01	17.4

[a] For the competition step, standards in distilled water and the immunoreagent (LIB−**PO** MAb or HRP−**PNC**) in 2× PBS containing 0.1% Tween 20, pH 7.5, were incubated for 1 h at room temperaure. [b] Hapten to protein molar ratio. [c] Data obtained from the four-parameter sigmoidal fitting, average of four curves with three replicates at each point.

conjugate of lower MR were required to give appropriate maximum absorbance using similar MAb concentrations. Second, competition curves obtained with the higher MR conjugate [OVA−**TR1** (15)] showed greater variations among their I_{50} values than those obtained with the lower MR conjugate [OVA−**TR1** (3)]. Third, and more important, the highest affinity was achieved by the less loaded conjugate. Coating with 4 μg/mL of OVA−**TR1** (MR = 3) and using the corresponding MAb concentration (0.18 μg/mL) were the optimum conditions to perform the competitive CC immunoassay, as indicated by the lowest I_{50} for chlorpyrifos (7.5 nM).

AC Format. HRP, the enzyme used to prepare the tracer, has six lysine residues. Only two of them are available for hapten conjugation under mild coupling conditions. The remaining residues can react under stronger coupling conditions, *i.e.* using the mixed anhydride procedure, but this may produce loss of enzyme activity (Paek *et al.*, 1993). Hence, for haptens coupled to HRP as amide bonds of lysine residues, only tracers with low MR can be prepared. This was the case of the HRP−**PNC** tracer, prepared by the *N*-hydroxysuccinimide-active ester method, with an estimated MR around 2. Similar to the CC format, competitive curves with several concentrations of immunoreagents (coating MAb and tracer) were obtained and their respective parameters estimated. Results presented in Table 1 indicate that coating with LIB−**PO** MAb at 10 μg/mL and adding the corresponding HRP−**PNC** tracer concentration (0.45 μg/mL) provided competitive curves with adequate signal and with the lowest I_{50} for chlorpyrifos (10.5 nM).

Influence of External Factors on the Immunoassays. Once the optimum concentrations of the specific components of each assay system were selected, time-related effects as well as the influence on assay characteristics of several physicochemical properties of the medium in which these specific components interact were examined.

Time-Related Effects. For the two ELISA formats contemplated in this study, increasing the incubation time of the competition step afforded a certain reduction of immunoreagent concentrations, producing only minor improvements of assay sensitivity. Therefore, the usual incubation time of 1 h was maintained. Nevertheless, assay time can be shortened if desired, but in this case higher immunoreagent concentrations are required to maintain adequate signals, which leads to a slight loss of sensitivity.

On the other hand, and specifically for the AC format, it has been suggested that a time-dependent drift of the results of an ELISA plate may occur as a consequence of different incubation times of analyte solutions (Jung *et al.*, 1989). To study this phenomenon, competitive curves were prepared by dispensing standards and incubating them with the coated antibody for 0, 5, 10, 20, 30, or 60 min prior to the addition of the enzyme tracer. Significant assay drifts were obtained at the highest intervals (30, 60 min), but surprisingly this behavior was also observed in the CC format. Afterward, and throughout the optimization process, it became noticeable that these effects could be related to a nonappropriate ELISA plate covering, considering that volatilization is the major dissipative route of chlorpyrifos from water. In fact, when the study in which each plate was covered with a sealing tape was conducted, no significant differences among the competitive curves performed with different preincubation times were found. Consequently, time-dependent drifts were not caused by differences in the dispensation time of immunoreagents, but they were inherent to volatilization of chlorpyrifos from aqueous buffers, whatever the assay format applied. To avoid these effects, special care should be taken with adequate plate taping.

Detergent (Tween 20). Tween 20 is a nonionic surfactant commonly used in ELISA to reduce nonspecific interactions. The influence of its concentration on the analytical characteristics of the chlorpyrifos immunoassay was examined. Competitive curves with decreasing concentration of Tween 20 in the buffer of the competition step, from the usual 0.05 to 0%, were obtained in the CC format. The curve parameters A_{max} and I_{50} were estimated and plotted as a function of the Tween 20 proportion. As shown in Figure 2, decreasing the Tween 20 concentration resulted in increased A_{max}, whereas the I_{50} decreased significantly. Consequently, both effects resulted in an increase of assay sensitivity for chlorpyrifos, so that an almost 1 order of magnitude lower I_{50} was obtained when Tween 20 was not added to the competition buffer (Table 2). However, in the last condition, an adverse effect on well-to-well variability was observed. Thus, when the Tween 20 concentration decreased from 0.05 to 0%, the CV of the signals increased from 5% to about 16% (Figure 2). As shown in Table 2, the same influence of the Tween 20 concentration on the analytical parameters of competitive curves was observed in the AC format. For both formats, additional effort was devoted to study whether the higher CV observed in the absence of detergent could be overcome by the addition of proteins (BSA, OVA) to the competition buffer. After protein concentrations providing suitable signals were selected, no significant improvement was found (Table 2). Therefore, the presence of Tween 20 seemed to be necessary to ensure good reproducibility. In these circumstances, a compromise had to be achieved between the improvement of sensitivity and the poor reproducibility afforded

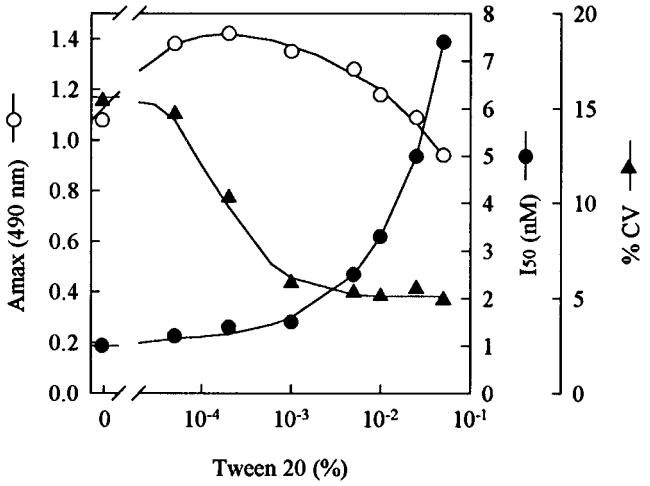

Figure 2. Influence of the Tween 20 concentration of the competition buffer on the analytical characteristics of chlorpyrifos standard curve obtained with format CC: (○) absorbance in absence of analyte (A_{max}); (●) value of I_{50} for chlorpyrifos; (▲) coefficient of variation of the signals corresponding to the standard concentration closest to the I_{50} value. The assay conditions were those previously selected as optimum for the coating conjugate (OVA−**TR1**, MR = 3, 4 μg/mL) and the MAb (LIB−**PO**, 0.18 μg/mL). For the competition step, standards were prepared in distilled water, and the MAb was diluted in 2× PBS, pH 7.5, containing 2 times the concentration of Tween 20 assayed. Each point represents the average value of two plates with eight replicate wells per plate.

Table 2. Characteristics of Chlorpyrifos Immunoassays as a Function of Tween 20 Concentration

CC Format[a]

[LIB−**PO**] (μg/mL)	conditions[b]	parameters[c] A_{max}	I_{50} (nM)	CV[d] (%)
0.18	0.05% Tween 20	0.94	7.4	5.0
0.10	0.001% Tween 20	1.01	1.3	5.9
0.18	−[e]	1.08	0.9	15.8
0.30	0.01% BSA	0.81	1.2	13.1
0.25	0.1% OVA	0.79	1.5	10.6

AC Format[f]

[HRP−**PNC**] (μg/mL)	conditions[b]	parameters[c] A_{max}	I_{50} (nM)	CV[d] (%)
0.45	0.05% Tween 20	1.14	11.2	5.3
0.25	0.001% Tween 20	1.11	1.3	6.2
0.30	−[e]	1.22	1.3	14.7
0.15	0.1% BSA	1.01	1.4	11.0
0.15	0.1% OVA	0.90	1.3	9.6

[a] Plates were coated with 4 μg/mL OVA−**TR1** (MR = 3). [b] Concentration of detergent or protein added to the final assay buffer of the competition step (PBS, pH 7.5). [c] Average values estimated from four plates (addition of Tween 20, or no addition) or two plates (addition of protein). The standard curve was run in octaplicate on each plate. [d] Average of the signals provided by the standard concentration closest to the I_{50} value. [e] Without adding Tween 20 or protein. [f] Plates were coated with 10 μg/mL LIB−**PO** MAb.

by decreasing the Tween 20 concentration. Since the I_{50} values did not vary appreciably at concentrations <0.001%, while still maintaining good CV, this Tween 20 concentration was considered as the optimum to be added to the final competition buffer to perform the chlorpyrifos immunochemical detection. Moreover, this optimum detergent concentration allowed an additional reduction of the immunoreagent (LIB−**PO** MAb for the CC format, HRP−**PNC** for the AC format) concentrations to give A_{max} around 1.0 (Table 2).

Similar marked influence of the Tween 20 concentration on the assay characteristics has been reported for immunoassays to permethrin (Stanker *et al.*, 1989), endosulfan (Lee *et al.*, 1995), and carbaryl (Abad and Montoya, 1996), while no influence was found for an immunoassay to TCP, a polar degradation product of chlorpyrifos (Manclús and Montoya, 1996). These results suggest that the phenomenon may be related to nonspecific hydrophobic interactions between the detergent and nonpolar small organic molecules in an aqueous environment, competing thereby with the specific analyte−antibody interaction.

pH. To examine the influence of pH on the assay systems, competitive curves for chlorpyrifos were obtained by preparing standards in distilled water and adding the adequate concentration of LIB−**PO** MAb or HRP−**PNC** tracer in 2× PBS containing 0.002% Tween 20, at different pH values covering the range 4.5−10.5. The variation of the curve parameters as a function of pH in the CC and AC formats is depicted in parts A and B of Figure 3, respectively. For both formats, a similar pH influence on assay parameters was found. Thus, the recognition of conjugated haptens (OVA−**TR1**, HRP−**PNC**), represented by the response in the absence of analyte (A_{max} values), did not change significantly in the pH range 5.5−10.5 but dropped drastically at pH 4.5. On the other hand, the recognition of chlorpyrifos (I_{50} values) was similar throughout the pH range tested. This behavior can be explained by considering the effect of varying the pH on each component participating in the interaction. The structure of chlorpyrifos is invariable within the pH range, and, due to its nonpolar nature, it is very unlikely that charged, pH-dependent, amino acid residues are present in the antibody binding site. Hence, the observed pH effects may be caused by conformational changes of the macromolecules participating in the interaction.

To estimate the pH range providing the highest sensitivity to chlorpyrifos, the variations of both parameters were considered together. The A_{max}/I_{50} ratio would be an appropriate estimate of the assay sensitivity, the higher ratio indicating the higher sensitivity assay. This ratio was then estimated for both formats and plotted as a function of pH. As shown in Figure 3C, these plots roughly follow those of their respective A_{max} values, since the I_{50} variation is small in both formats. As the influence of pH can be considered not important in the ranges 5.5−8.5 and 5.5−7.5 for the CC and AC formats, respectively, any pH within these ranges would be appropriate. Nevertheless, considering that the hydrolysis rate of chlorpyrifos increases under alkaline conditions, a slightly acidic pH (6.5) was preferred for the buffer of the competition step of the immunoassays.

Salt Concentration. The influence of the ionic strength on the assay characteristics was also studied. For both immunoassay formats, competitive curves were obtained using several dilutions of 10-fold concentrated PBS as assay buffer of the competition step. The plots of assay parameters (A_{max}, I_{50}) as a function of salt concentration are depicted in Figure 4 (part A, CC format; part B, AC format). As shown, the variation of the parameters with the ionic strength followed different patterns for each format. Thus, by increasing the salt concentration, the recognition of the corresponding conjugated hapten (A_{max}) diminished or increased for the CC or AC format, respectively. Furthermore, the recognition of chlorpyrifos (I_{50}) did not change markedly as a consequence of the salt concentration increase in

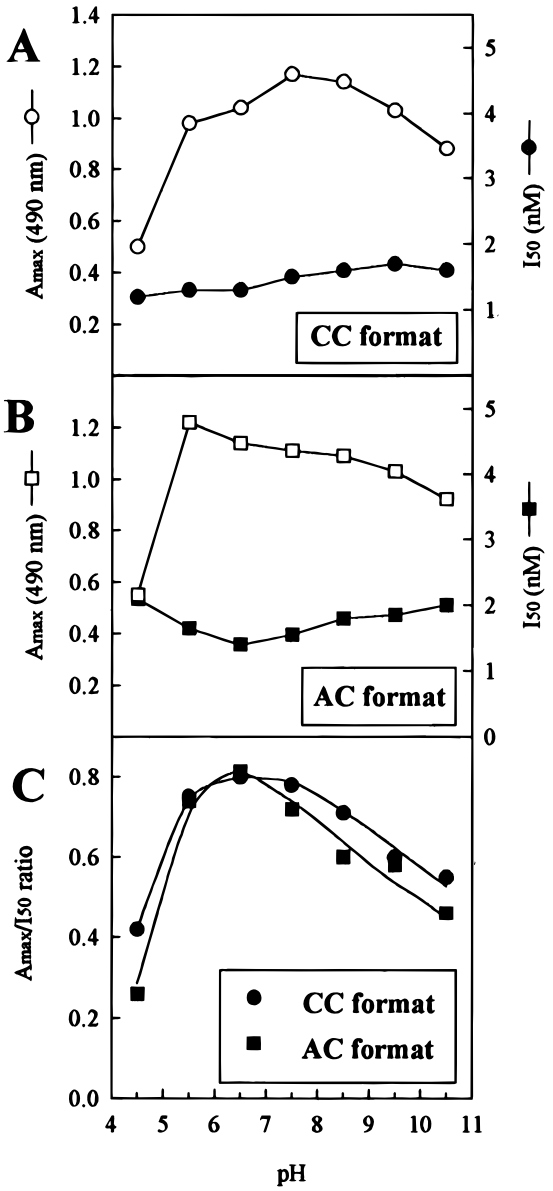

Figure 3. Influence of the pH of the assay buffer on the analytical characteristics of chlorpyrifos competitive standard curves: (A) CC format (OVA−**TR1**, MR = 3, 4 μg/mL; LIB−**PO** MAb, 0.1 μg/mL); (B) AC format (LIB−**PO** MAb, 10 μg/mL; HRP−**PNC**, 0.25 μg/mL). For the competition step, standards were prepared in distilled water, and the MAb or the tracer was diluted in 2× PBS containing 0.002% Tween 20, at the pH assayed: (open symbols) absorbance in absence of analyte (A_{max}); (solid symbols) value of I_{50} for chlorpyrifos. (C) Representation of the A_{max}/I_{50} ratio for each format. Each point represents the average value of four plates with three replicate wells per plate.

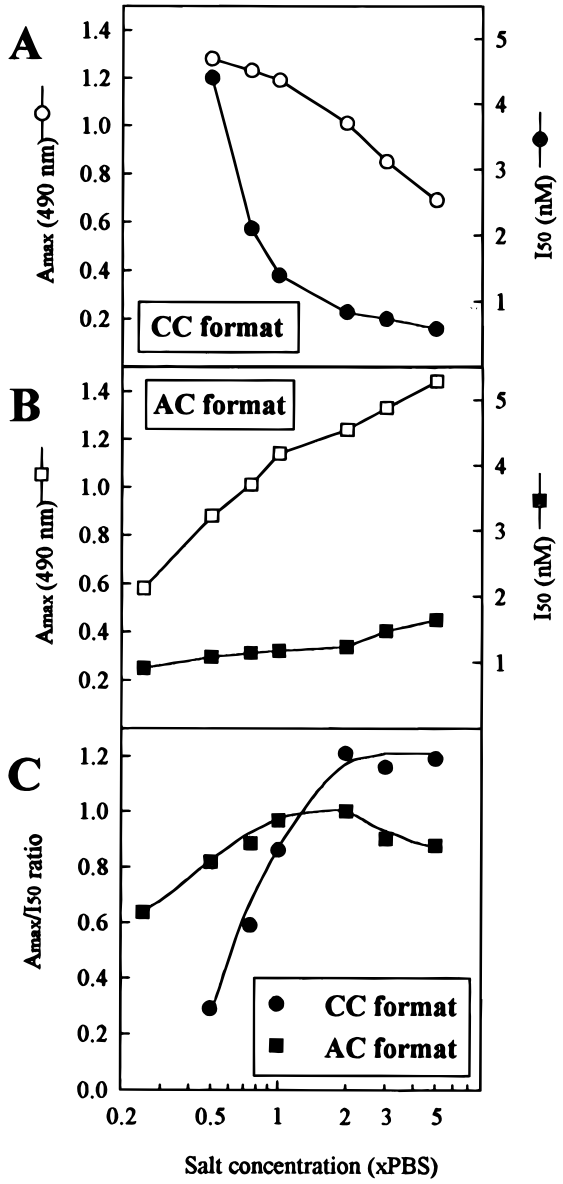

Figure 4. Influence of the salt concentration of the assay buffer on the analytical characteristics of chlorpyrifos competitive standard curves: (A) CC format (OVA−**TR1**, MR = 3, 4 μg/mL; LIB−**PO** MAb, 0.1 μg/mL); (B) AC format (LIB−**PO** MAb, 10 μg/mL; HRP−**PNC**, 0.25 μg/mL). For the competition step, standards were prepared in distilled water, and the MAb or the tracer was diluted in 2-fold concentrated PBS assayed concentration, pH 6.5, containing 0.002% Tween 20: (open symbols) absorbance in absence of analyte (A_{max}); (solid symbols) value of I_{50} for chlorpyrifos. (C) Representation of the A_{max}/I_{50} ratio for each format. Each point represents the average value of four plates with three replicate wells per plate.

the AC format, whereas a large improvement was seen (lower I_{50}) in the CC format.

Similar to the pH study, the representation of the A_{max}/I_{50} ratio against the salt concentration was helpful in the selection of the optimum buffer concentration. As shown in Figure 4C, there is an appreciable variation of the ratio in the AC format, increasing as the salt concentration increased. In the CC format, the affinity to chlorpyrifos improved much more markedly when the salt concentration was increased up to 2× PBS. Hence, this PBS concentration was selected for the buffer of the competition step of the immunoassays of chlorpyrifos performed in the two formats. By comparing the influence on both formats, the characteristics of the CC format seemed to be more affected than those of the AC format, which may be related to the antibody being in solution or immobilized, respectively. Thus, a certain rigidity provided by antibody immobilization could involve a higher tolerance to variations of the ionic strength of the buffer.

Due to the nonpolar nature of chlorpyrifos, it seems reasonable to assume that hydrophobic interactions are predominant in the antibody−analyte binding. Generally, wherein hydrophobic interactions are important driving forces of ligand−receptor biochemical recognitions, these are favored by increasing the ionic strength of the reaction medium (Jefferis and Deverill, 1991), which could explain the behavior observed. The same tendency has been found in a study of the ionic strength

Table 3. Summary of Characteristics of the Chlorpyrifos Immunoassays

	format	
	CC	AC
immunoreagent concentrations		
[OVA−**TR1**, MR = 3] (μg/mL)	4	na[a]
[LIB−**PO** MAb] (μg/mL)	0.10	10
[HRP−**PNC**] (μg/mL)	na	0.25
buffer conditions		
% Tween 20	0.001	0.001
pH	6.5	6.5
salt concentration	2× PBS	2× PBS
analytical characteristics of standard curve[b]		
A_{max}	1.15	1.10
A_{min}	0.01	0.01
slope	1.65	1.18
I_{50} (nM)	0.80	1.00
concentration giving		
20−80% inhibition (nM)	0.30−2.00	0.30−3.50
10% inhibition (nM)	0.20	0.15
analytical characteristics in water[c]		
I_{50} (μg/L)	0.56	0.70
working range (μg/L)	0.21−1.40	0.21−2.50
LOD (μg/L)	0.14	0.10

[a] Not applicable. [b] Data averaged from five standard curves performed on different days. Curves were fit to the four-parameter equation. [c] Data were calculated by converting nM into μg/mL units and considering the 1:2 dilution of water samples in the assay.

influence on the sensitivity of ELISAs to carbaryl, a relatively nonpolar pesticide (Abad and Montoya, 1996). On the contrary, ELISAs to the polar compound TCP, the major degradation product of chlorpyrifos, behaved in the opposite manner, *i.e.*, the assay sensitivity was adversely affected by increasing ionic strength (Manclús and Montoya, 1996).

Characteristics of the Optimized Chlorpyrifos Immunoassays. Assay characteristics were estimated from standard curves run in triplicate, which was also the usual procedure followed to carry out the determinations. Analytical data are summarized in Table 3. Representative standard curves of chlorpyrifos obtained with the two optimized ELISA formats are shown in Figure 5. The competitive curve obtained with the CC format allowed the detection of chlorpyrifos (20−80% inhibition) from 0.3 to 2 nM, with an average I_{50} value of 0.8 nM. Using the AC format, which provided a lower slope standard curve, the analyte could be determined in the 0.3−3.5 nM range, with an I_{50} of 1.0 nM. The zero dose was assayed four times in triplicate, and the mean absorbance and standard deviation (SD) were estimated. The mean absorbance minus 2 SD corresponded to 0.20 and 0.15 nM chlorpyrifos, for the CC and AC formats, respectively. These data were similar to the analyte concentrations giving 10% inhibition of the maximum absorbance. Both methods are frequently used to estimate the limit of detection (LOD) of pesticide immunoassays (Sherry, 1992).

Assay specificity was evaluated using several structurally related compounds, metabolites, and other organothiophosphates as competitors. I_{50} and cross-reactivity (CR) data for each compound are given in Table 4. For both formats, the CR pattern was similar to that obtained in nonoptimized conditions and reported in the preceding paper. Both chlorpyrifos and chlorpyrifos-methyl were almost equally recognized. The assay showed lower affinity for other halogenated organothiophosphates being phenyl-type instead of pyridyl-type compounds (CR ≤ 10%). The oxygen analogue of chlorpyrifos was weakly recognized (chlorpyrifos-oxon, CR < 1.3%), and the CR of the major metabolite

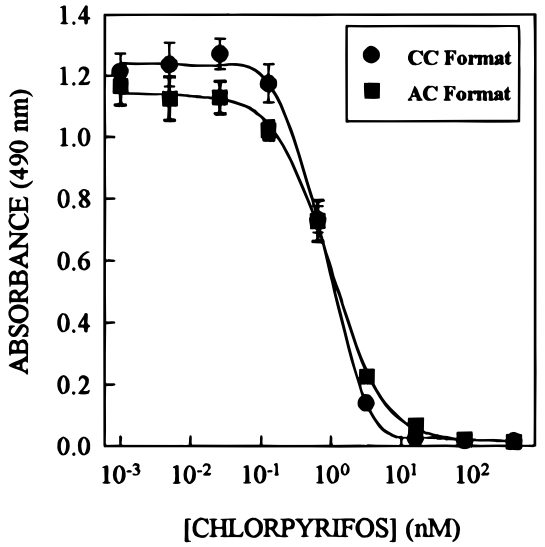

Figure 5. Representative standard curves for chlorpyrifos obtained under the optimized assay conditions with (●) CC format (OVA−**TR1**, MR = 3, 4 μg/mL; LIB−**PO** MAb, 0.1 μg/mL) or (■) AC format (LIB−**PO** MAb, 10 μg/mL; HRP−**PNC**, 0.25 μg/mL). For the competition step, standards were prepared in distilled water, and the MAb or the tracer was diluted in 4× PBS, pH 6.5, containing 0.002% Tween 20. Each point represents the mean ± SD of three plates with three replicate wells per plate. Competitive curves are fit to experimental points by the four-parameter logistic equation.

Table 4. Specificity of the Optimized Chlorpyrifos Immunoassays

	format			
	CC		AC	
compound	I_{50}[a] (nM)	CR[b] (%)	I_{50}[a] (nM)	CR[b] (%)
chlorpyrifos	0.8	100	1.0	100
chlorpyrifos-methyl	0.7	114	0.8	125
chlorpyrifos-oxon	67	1.2	90	1.1
fenchlorphos	8	10	11	9.1
bromophos	18	4.4	24	4.2
trichloronate	15	5.3	17	5.9
trichlofenthion	308	0.26	409	0.24
3,5,6-trichloro-2-pyridinol (TCP)	16000	<0.01	30000	<0.01

[a] Average values from two curves with three replicates at each point. [b] Percentage of cross-reactivity = (I_{50} of chlorpyrifos/I_{50} of compound) × 100.

TCP was negligible (<0.01%). Other organothiophosphates (parathion, azinphos-methyl, diazinon) showed no CR up to 10 μM.

Application of the Chlorpyrifos Immunoassays to Environmental Waters. *Evaluation of Matrix Effects.* Prior to the application of the developed immunoassays to determine chlorpyrifos in water, their susceptibility to nonspecific interferences originated by the different compositions of real water samples was examined. With this aim, standard curves were prepared in water samples from different sources, and in distilled water as control, and used to obtain competitive curves for chlorpyrifos (50% of the water tested in the final assay solution). In either format, there was no noticeable difference among their respective family of curves, and the assay parameters showed the usual interplate variability of chlorpyrifos standard curves obtained in distilled water. Consequently, the chlorpyrifos determination seemed to be directly applicable to environmental waters without any sample treatment, since, at least for the sample tested, matrix effects were not observed. In these circumstances, the practical analytical parameters can be estimated just by taking

Table 5. Analysis of Chlorpyrifos-Spiked Environmental Waters by ELISA

		CC format			AC format		
source	chlorpyrifos added (μg/L)	chlorpyrifos recovered[a] (μg/L)	recovery (%)	CV[a] (%)	chlorpyrifos recovered[a] (μg/L)	recovery (%)	CV[a] (%)
river water	0.30	0.25	83	29	0.28	93	5
	0.60	0.56	93	18	0.51	85	6
	1.20	0.88	73	6	0.97	81	2
well water	0.30	0.27	90	13	0.28	93	9
	0.60	0.39	65	12	0.43	72	10
	1.20	1.52	127	22	1.22	102	2
irrigation water	0.30	0.38	127	16	0.40	133	12
	0.60	0.48	80	15	0.65	108	3
	1.20	0.98	82	16	1.14	95	3
mean			91	16		96	6

[a] Data obtained from four determinations performed in the same ELISA plate.

into account the 1:2 sample dilution of the competition step. Thus, as shown in Table 3, the average I_{50} values are 0.56 μg/L (1.6 nM) and 0.7 μg/L (2.0 nM), the working ranges (chlorpyrifos concentrations giving inhibition between 20 and 80%) 0.21−1.40 and 0.21−2.5 μg/L, and the LOD 0.14 and 0.10 μg/L, for the CC and AC formats, respectively.

Analysis of Fortified Water Samples. Spiking matrix samples with several amounts of analyte is a common practice to perform a preliminary evaluation of analytical assay reliability. Hence, water samples from different sources were spiked at several concentrations of chlorpyrifos covering the established working range. Each fortified sample was analyzed four times in triplicate by the two immunoassay systems herein studied. Results are presented in Table 5. The mean recovery data for three environmental waters at three levels of fortification were 91% (varying from 65 to 127%) and 96% (from 72 to 133%), for the CC and AC formats, respectively. These data proved that acceptable recoveries were obtained for both formats. Concerning the reproducibility, average intra-assay CVs were 6% and 16% for the AC and CC formats, respectively.

CONCLUSIONS

Immunochemical detection of small nonpolar analytes such as chlorpyrifos takes place in aqueous buffers, where their solubility is very low. Nevertheless, the determination can be performed provided that highly sensitive immunological tools are available. In this sense, chlorpyrifos immunoassays with sensitivities in the nanomolar order were developed, as described in the preceding paper. In this study, the importance of optimizing external factors affecting the specific immunochemical interactions has been demonstrated, since up to 10-fold better sensitivity was obtained, which allowed adequate chlorpyrifos determination down to micrograms per liter levels in environmental waters.

After the immunoreagent conditions and the composition of the assay buffer were optimized, the reduction of the Tween 20 concentration in the competition assay buffer provided the major contribution to the sensitivity improvement achieved. Therefore, there is a nonspecific interference of the detergent in the chlorpyrifos−MAb interaction, a phenomenon that could be generalized to the immunochemical detection of other small nonpolar analytes in an aqueous environment. On the other hand, the influence of other factors studied, such as pH and salt concentration, is in agreement with hydrophobic interactions playing a key role in the analyte binding by the MAb.

In the final optimum conditions, the specificity of the chlorpyrifos immunoassays showed a cross-reactivity pattern similar to that reported in the preceding paper. Although antibodies determine primarily the immunoassay sensitivity and specificity, the results of this optimization study point out that sensitivity can be modulated not only by the immunoassay configuration, as often reported, but also by the assay conditions. On the contrary, specificity can be considered an inherent characteristic of each MAb, thus being rather independent of the optimization process.

Performance of the optimized chlorpyrifos immunoassays in the determination of submicrogram per liter fortified real environmental water samples was satisfactory, since acceptable recoveries and precision were obtained. Therefore, these analytical techniques seem to be adequate for cost- and labor-effective environmental monitoring of chlorpyrifos.

ABBREVIATIONS USED

AC, antibody-coated; A_{max}, maximum absorbance in absence of analyte; BSA, bovine serum albumin; CC, conjugate-coated; CR, cross-reactivity; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; I_{50}, concentration giving 50% inhibition of maximum response; LOD, limit of detection; MAb, monoclonal antibody; MR, molar ratio; OVA, ovalbumin; PBS, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl; TCP, 3,5,6-trichloro-2-pyridinol.

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