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Highly Sensitive Enzyme-Linked Immunosorbent Assay for Chlorpyrifos. Application to Olive Oil Analysis

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Highly sensitive enzyme-linked immunoassays for chlorpyrifos, one of the most applied insecticides, are presented. Several haptens were synthesized for immunoreagent production and ELISA development. The best immunoassays obtained are based on an indirect coated-plate immunoassay format. Two assays were optimized; one shows a limit of detection of 0.3 ng L^{-1}, an I_{50} of 271 ng L^{-1}, and a dynamic range between 4 and 16 474 ng L^{-1}. The other one has a limit of detection of 0.07 ng L^{-1}, an I_{50} of 7 ng L^{-1}, and a dynamic range between 0.4 and 302 ng L^{-1}. The assays were used to quantify chlorpyrifos in olive oil using a simple and rapid sample extraction procedure. The good recoveries achieved in both assays (109% mean value) and the agreement with values given by the GC reference method (110% mean value) indicate their potential for either screening or laboratory quantification.

KEYWORDS: Organophosphorus pesticides; chlorpyrifos; ELISA; olive oil

INTRODUCTION

One of the most used organophosphorus pesticides is chlorpyrifos, a nonsystemic chlorinated organophosphate insecticide, developed by Dow Chemical Co. in 1962. It is applied worldwide for agricultural crops protection and livestock healthcare (*1*). The U.S. Environmental Protection Agency (USEPA) estimates that 10 million pounds of chlorpyrifos are applied in United States agriculture every year, half of which is used on corn crops (*2*). It is also widely used in extensive and intensive European agriculture, including crop fruits such as citrus, where it is applied at a rate of over 50 000 kg per year (*3*). Until very recently it was utilized worldwide in homes for pest control, but some uses of this organophosphorus have been restricted or eliminated (*4*).

Residues of chlorpyrifos in soils occur by direct application or through spray drift/foliar washoff. This organophosphorus is highly toxic to fish (acute LC_{50} values range from less than 1 mg L^{-1} to more than 200 mg L^{-1}) and wildlife (*5*, *6*). There are no data suggesting that chlorpyrifos is a human carcinogen (*7*), although it is a suspected endocrine disruptor (*8*). According to these data, chlorpyrifos has been identified as a candidate for priority review under the National Registration Authority's Existing Chemical Review Program by the U.S. National Drugs and Poisons Schedule Committee in 2000 (*9*). Moreover, it is in the Global Monitoring for Environment and Security/Food Europe Comprehensive list of priority contaminants and commodity combinations.

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Chlorpyrifos is one of the insecticides used to treat one of the most serious pests of olives in the Mediterranean basin (*Prays oleae*, the olive moth), and the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have established maximum pesticide residue limits in olives and olive oil (*10*). Olive Oil Pesticide Residue Regulatory Programs are being carried out to update and set new and more restrictive regulations concerning the maximum residue levels in these commodities. Analytical problems associated with the determination of pesticide residues in olive oil are due to its hydrophobic nature (*11*), so they are analyzed by chromatographic techniques after tedious extraction and cleanup steps (*12*). Sample preparation is a crucial stage in the analytical procedure, since even small amount of lipids can harm the gas chromatographic injectors and capillary columns and detectors, which lead to losses in efficiency and sensitivity.

Different chromatographic methods for the determination of chlorpyrifos residues and its main metabolites in food and related matrixes have been developed (*13*−*18*). Although chromatographic techniques provide low limits of detection (0.001−0.1 μg L^{-1} in water), preliminary treatment (extraction, cleanup, preconcentration, etc.) of the sample typically remains a mandatory requirement, which makes the procedure laborious.

Immunoassay-based techniques, such as enzyme-linked immunosorbent assay (ELISA), can be simpler than conventional chromatographic analysis, especially if a rapid extraction procedure is used. Immunoassay is also a good alternative to chromatographic methods for monitoring a single defined target when the multiresidue application is not justified.

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Several sensitive and specific ELISAs for chlorpyrifos have been reported to detect it in primary matrixes (*19*−*24*). Using different haptens, polyclonal (*19*, *20*) and monoclonal antibodies (*21*−*24*) have been obtained to develop immunoassays. The most sensitive assay (*19*) showed a limit of detection of 0.03 ng mL^{-1} (minimal value), with 50% inhibition of antibody binding at 0.2 ng mL^{-1} of chlorpyrifos.

Currently, commercial ELISA-based microtiter plate kits for detecting residues of chlorpyrifos are available from Strategic Diagnostics Inc. (Newark, DE) (RaPID assay and EnviroGard) and EnviroLogix Inc. (Portland, ME). RaPID assay is the most sensitive (I_{50} 0.3 ng mL^{-1}) but is the least selective, with a high cross-reactivity to chlorpyrifos-methyl (600%). EnviroLogix kit is the least sensitive (I_{50} 1.5 ng mL^{-1}) but it is more selective, since cross-reactivity for chlorpyrifos-methyl is only 6.5%.

Determination of chlorpyrifos in environmental and food samples by immunoassay is difficult due to its hydrophobicity. Some authors have reported plastic adsorption of chlorpyrifos, resulting in low recoveries and inaccurate results (*19*, *22*). Therefore, a careful use of the equipment (pipets, tips, ELISA plates) is necessary to minimize analyte losses.

In this work, a novel indirect enzyme-linked immunoassay for chlorpyrifos has been developed in order to improve both sensitivity and selectivity. With this aim, a collection of haptens was synthesized to produce polyclonal antibodies against chlorpyrifos. Also, the application of the optimized ELISA to the determination of this compound in olive oil with a simple and fast sample pretreatment was another goal.

MATERIALS AND METHODS

Reagents. Analytical grade solvents were from Scharlab (Barcelona, Spain). Pesticide standards used for cross-reactivity studies were purchased from Sigma-Aldrich Química (Madrid, Spain) and Dr. Ehrenstorfer (Augsburg, Germany). Bovine serum albumin (BSA), ovalbumin (OVA), complete and incomplete Freund's adjuvant, *o*-phenylenediamine (OPD), Tween 20, horseradish peroxidase (HRP), and peroxidase labeled goat anti-rabbit immunoglobulins (GAR−HRP) were from Sigma. Chemical reagents for hapten synthesis and protein conjugation purposes were from Aldrich (Madrid, Spain). Keyhole limpet hemocyanin (KLH) was from Pierce (Rockford, IL). Technical quality chlorpyrifos (98%) was generously provided by Laboratorios Alcotan (Sevilla, Spain).

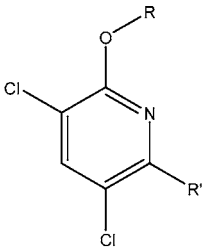
Apparatus. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a 300 Varian spectrometer (300 MHz, Sunnyvale, CA).

UV−vis spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer (Palo Alto, CA). Polystyrene 96-well microtiter plates were from Costar (Cambridge, MA), and the ELISA plate washer was from Nunc MaxiSorp (Roskilde, Denmark). Well absorbances were measured in a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Turku, Finland).

For GC analysis, a 5980 series II Hewlett-Packard automatic sampler equipped with a DB-1701 capillary column (30 m length × 250 μm diameter × 0.25 μm film thickness; J & W Scientific, Folsom, CA) and a flame photometric detector was used.

Gel permeation chromatography (GPC) was carried out with an automated system (GPC VARIO, LCTech, Germany) equipped with a 5-mL loop and a chromatographic column (500 × 25 mm id) filled with 50 g of BioBeads S-X3-resin (200−400 mesh), with a 32-cm gel bed length.

Hapten Synthesis. In general, the synthesis of chlorpyrifos haptens has been carried out from its metabolite, 3,5,6-trichloro-2-pyridinol (TCP), as the starting material. This compound is commercially available only as standard quality, which implies a high cost for synthetic purposes. For this reason, the synthesis of TCP was carried out by hydrolysis of chlorpyrifos, as follows:



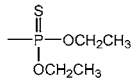
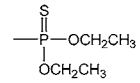
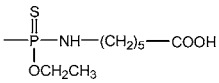
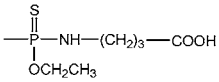
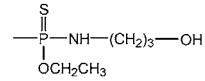
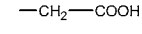
Compound	R	R'
Chlorpyrifos		
Hapten		
C1		
C2		
C3		
C4		
C5		

Figure 1. Chemical structure of chlorpyrifos and the haptens synthesized in this work.

To a solution of chlorpyrifos (3.5 g, 10 mmol) in absolute ethanol (25 mL) was added 25 mL of a solution of KOH (1.42 g, 20 mmol) in absolute ethanol. After reflux for 1 h, the reaction mixture was filtered quickly and the solvent evaporated. A solution of $NaHCO_3$ (5%, 25 mL) was added to the white solid obtained, which was extracted with hexane (3 × 10 mL). The aqueous layer was acidified with phosphoric acid until the formation of a precipitate. Then, the mixture was extracted with dichloromethane (3 × 10 mL), dried, and concentrated to give 3,5,6-trichloro-2-pyridinol as a pure, white solid (1.79 g, 90%). ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.81 (1H, s, CH). ^{13}C NMR ($CDCl_3$, 300 MHz) δ (ppm): 158.2 (COH), 141.5 (C−N), 141.4 (CH), 120.5 (CCl), 117.8 (CCl).

Five haptens (named **C1**, **C2**, **C3**, **C4**, **C5**) have been used in this work. The structures are shown in **Figure 1**.

The hapten **C1**, 3-[3,5-dichloro-6-(diethoxythiophosphoryloxy)-pyridin-2-ylsulfanyl]propionic acid, was prepared following the procedure of Manclús et al. (*22*).

Haptens **C2**, 6-[ethoxy-3,5,6-trichloropyridin-2-yloxy)thiophosphorylamino]hexanoic acid, and **C3**, 4-[ethoxy-3,5,6-trichloropyridin-2-yloxy)thiophosphorylamino]butyric acid, were synthesized following the procedure described by Cho et al. (*20*).

Hapten **C4**, (3-hydroxypropyl)thiophosphoramidic acid *O*-ethyl ester *O*′-(3,5,6-trichloropyridin-2-yl) ester, is a novel chlorpyrifos hapten that contains a hydroxyl group for protein conjugation. This compound was obtained following a strategy similar to those applied by Beasley et al.

(*25*) to diazinon, in two steps. First, thiophosphorochloridic acid *O*-ethyl ester *O′*-(3,5,6-trichloropyridin-2-yl) ester was obtained as follows:

To a stirred mixture of ethyldichlorothiophosphate (1.9 mL, 13.3 mmol) in acetonitrile (24 mL) were added K_2CO_3 (6.8 g) and 3,5,6-trichloro-2-pyridinol (2 g, 10 mmol) dissolved in acetonitrile (6 mL). After stirring for 1 h at room temperature, the mixture was filtered and the solvent was removed under reduced pressure. The residue was column chromatographed (silica gel, hexane/diethyl ether 95/5), giving the product as a colorless oil (1.47 g, 43%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.91 (1H, s, CH), 4.57−4.46 (4H, dq, J = 11.0 and 7.1 Hz, CH_2CH$_3$), 1.53−1.48 (6H, dt, J = 7.1 and 1.1 Hz, CH$_2$CH_3).

In a second step, 0.5 g (1.47 mmol) of the previously obtained compound was dissolved in acetonitrile (11 mL) and the solution cooled in an ice bath. Then, $NaHCO_3$ (327 mg) and 3-amino-1-propanol (223 mg, 2.97 mmol) were added and the mixture stirred overnight at 4 °C. Next, the reaction mixture was filtered and the solvent evaporated. The residue was purified by chromatographic column (hexane/ethyl acetate 7/3) to give a white oil (300 mg, 54%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.86 (1H, s, CH), 4.40−4.29 (2H, dq, J = 7.2 and 6.9 Hz, CH_2CH$_3$), 3.83 (2H, t, J = 5.7 Hz, CH$_2$OH), 3.85−3.70 (1H, m, NH), 3.40−3.25 (2H, m, CH$_2$N), 1.81 (2H, m, CH$_2$), 1.41 (3H, t, J = 6.9 Hz, CH$_2$CH_3). ^{13}C NMR ($CDCl_3$, 300 MHz) δ (ppm): 151.3 (CO), 143.9 (CN), 141.1 (CH), 126.5 (CCl), 121.0 (CCl), 64.3 (CH$_2$OH), 60.4 (*C*H$_2$CH$_3$), 39.7 (CH$_2$N), 33.0 (CH$_2$), 15.9 (CH$_3$).

Hapten **C5**, 3,5,6-trichloro-2-pyridyloxyacetic acid (triclopyr), was generously provided by DowElanco (Indianapolis, IN) and used only for coating purposes.

Immunoreagents Preparation. For immunization purposes, haptens were covalently attached through their carboxylic acid moieties to the lysine groups of BSA and KLH by the active ester method (*26*). Additionally, the set of haptens was covalently attached to HRP to prepare enzyme tracers and to OVA to obtain coating conjugates, both by the same method.

Hapten **C4** was conjugated through its hydroxyl group to BSA, OVA, and HRP following the method described by Beasley et al. (*25*).

Finally, immunogens, tracers, and coating conjugates were purified by gel-exclusion chromatography on D-Salt desalting columns (Pierce, Rockford, IL) using PBS (10 mM, pH 7.4) for elution. The conjugates were stored at −20 °C until use.

The immunogens (0.20 mg in 0.5 mL PBS) were suspended in 0.5 mL of Freund's adjuvant and injected intramuscularly into two female New Zealand rabbits (I and II). After several boosts, whole blood was collected and coagulated overnight at 4 °C. Then, serum was separated by centrifugation and stored at −80 °C. Fourteen sera, from immunogens BSA−**C1**, BSA−**C2**, BSA−**C3**, BSA−**C4**, KLH−**C2**, KLH−**C3**, and KLH−**C4**, were obtained.

To test sera recognition, optimal concentrations of coating conjugates, serum dilution, and enzyme tracers were chosen by checkboard titration (*27*). For this purpose, two assay formats were studied: (a) indirect (coating conjugate) and (b) direct (antibody-coated).

ELISA Optimization. Assay optimization was performed with chlorpyrifos as the competitor analyte following the Tijssen's protocols (*28*). Standards were prepared in distilled water from a stock solution in methanol. Borosilicate glass tubes were used to minimize chlorpyrifos loss.

Antibodies and tracers working solutions were prepared in buffer and mixed with an equivalent volume of standards in the plate.

In brief, flat-bottomed polystyrene ELISA plates were coated with 100 μL/well of the appropriate concentration of OVA−triclopyr conjugate solution in coating buffer (50 mM carbonate−bicarbonate buffer pH 9.6). The plates were then sealed and incubated overnight at 4 °C. The following day, plates were washed six times with PBS-T (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.5 containing 0.05% Tween 20). After that, a volume of 50 μL of the appropriate sera dilution in the tested buffer and 50 μL of standards in deionized water were added to the coated plates and incubated for 1 h at room temperature. After washing as earlier, plates were incubated for 1 h with peroxidase-labeled goat anti-rabbit immunoglobulins (GAR−HRP) diluted 1:4000 in PBST (100 μL/well). Once washed, peroxidase activity was determined by adding 100 μL/well of substrate solution (2 mg mL^{-1} OPD and 0.012% H_2O_2 in 25 mM sodium citrate, 62 mM sodium phosphate, pH 5.5). After 10 min, the enzymatic reaction was stopped by adding 2.5 M H_2SO_4 (50 μL/well) and the absorbance was read.

The influence of different organic solvents normally used in extraction procedures was also evaluated. For this purpose, several percentages of acetone, acetonitrile, ethyl lactate, 2-propanol, methanol, and methyl sulfoxide in the optimized buffer were tested.

Analysis of Oil Samples. Extra virgin olive oil commercial samples were collected from representative Spanish producing areas.

Samples were analyzed for chlorpyrifos residues by optimized ELISA after being fortified and extracted following a previously developed protocol (*29*). Briefly, 0.5 mL of the olive oil was mixed with 0.5 mL of methanol by vortex. The mixture was frozen at −80 °C for 1 h and then the methanolic extract was collected. Samples were quantified using standards in olive oil extracted as described.

Prior to GC analysis, 5 mL of each sample was mixed thoroughly with 25 mL of ethyl acetate−cyclohexane (1/1, v/v) in 100-mL Erlenmeyer flasks, and the mixture was stirred at 300 rpm for 24 h at room temperature. Afterward, 7 mL of supernatant was filtered through a nylon syringe filter (0.45 μm pore size, 25 mm diameter; Whatman Inc., NJ). Subsequently, a 5-mL aliquot of the sample extract was cleaned up by gel permeation chromatography (GPC). The mobile phase was ethyl acetate−cyclohexane (1/1, v/v) at a flow rate of 5 mL min^{-1}. The first 85 mL (17 min) of the oil extracts was discarded; the following 100 mL was collected as the pesticide fraction (20 min) and reduced to dryness in a rotary evaporator. Finally, the residue was redissolved in 1 mL ethyl acetate−cyclohexane (1/1, v/v) and analyzed by GC with flame photometric detection (FPD). The chromatographic determination was based on the method described by Jongenotter et al (*30*), with modifications.

The GC conditions were as follows: column temperature, 60 °C (1 min), from 60 to 120 °C at 30 °C min^{-1}, from 120 to 220 °C at 5 °C min^{-1} (held for 3 min), 15 °C min^{-1} to 280 °C (held for 24 min); carrier gas, helium; injection temperature, 250 °C; injection volume, 2 μL with HP 7673 autosampler; injection mode, splitless; detector temperature, 300 °C.

RESULTS

Hapten Design and Synthesis. To design specific haptens for a compound, it is desirable to obtain a mimic of its structure and electronic and hydrophobic properties. Taking this into account, two types of haptens were synthesized by attaching the spacer arm on different sites of the structure: the pyridyl ring (hapten **C1**) and the thiophosphate moiety (haptens **C2**−**C4**) (**Figure 1**).

The election of haptens was based on the results obtained by several researchers (*19*−*24*), who have developed sensitive ELISAs for chlorpyrifos.

Hapten **C1** was prepared by chlorine substitution using 3-mercaptopropionic acid as nucleophile. This structure maintains the thiophosphate moiety, having the spacer arm as an aromatic ring substituent.

Haptens **C2** and **C3** were synthesized, respectively, by introduction of 6-aminopropionic acid and 4-aminobutanoic acid as amide linkage to the thiophosphate ester. This structure has provided highly specific antibodies for other organophosphorus compounds (*31*).

Previous studies carried out by the authors for diazinon and fenthion (*32*, *33*) showed that haptens with a spacer arm attached through the phosphate ester and ended with a hydroxyl group were the most successful for immunizing purposes. Accordingly, a new hapten (**C4**) was designed. Briefly, the synthesis involved the reaction of ethyl dichlorothiophosphate with the sodium salt of 3,5,6-trichloro-2-pyridinol and the subsequent displacement of the chlorine by the amino substituent of 3-amino-1-propanol.

Serum Screening. All sera were tested against homologous and heterologous coating conjugate formats to determine the

Table 1. Titration: Comparison of Coating Conjugates and Antibodies[a]

serum	OVA–**C1**	OVA–**C2**	OVA–**C3**	OVA–**C4**	OVA–**C5**
BSA–**C1**-I	M	L	L	L	N
BSA–**C1**-II	M	L	L	L	L
BSA–**C2**-I	N	H	M	M	M
BSA–**C2**-II	M	H	H	H	H
BSA–**C3**-I	N	M	H	M	N
BSA–**C3**-II	N	M	H	M	M
BSA–**C4**-I	N	N	N	N	N
BSA–**C4**-II	N	L	L	L	N
KLH–**C2**-I	N	H	H	H	M
KLH–**C2**-II	N	H	H	H	H
KLH–**C3**-I	N	H	H	H	H
KLH–**C3**-II	N	M	H	H	L
KLH–**C4**-I	N	N	N	N	N
KLH–**C4**-II	N	L	L	L	N

serum	BSA–**C1**	BSA–**C2**	BSA–**C3**	BSA–**C4**	BSA–**C5**
KLH–**C2**-I	L	H	H	M	M
KLH–**C2**-II	M	H	H	M	H
KLH–**C3**-I	L	H	H	H	H
KLH–**C3**-II	L	H	H	L	M
KLH–**C4**-I	N	N	N	N	N
KLH–**C4**-II	N	L	L	N	N

[a] L (low), M (medium), and H (high) correspond to the dilution factor applied to the sera for coating conjugate. Coating conjugate concentration ranges from 0.001 to 1.0 mg L^{-1} to obtain an absorbance signal between 0.5 and 1.2. L < $^1/_{10\,000}$, M = $^1/_{10\,000}$ to $^1/_{30\,000}$, and H > $^1/_{30\,000}$. N means not detected. I and II are sera from different rabbits.

titers. First, the behavior of the sera using an antibody-coated format with all HRP conjugates was investigated. None of the sera showed enough titer to carry out competitive assays.

All haptens were conjugated to OVA and BSA and used as coating conjugates. The results of the screening of the antibodies in indirect immunoassay format are presented in **Table 1**.

Sera from hapten **C1**, the only hapten with the spacer arm attached to the aromatic ring, presented low titer when hapten **C2**, **C3**, or **C4** was used for coating purposes.

Haptens **C2** and **C3**, which differ only in the length of the spacer arm, rendered sera with higher titers than the other haptens, especially **C2**, which has the larger spacer arm. Nevertheless, sera obtained with hapten **C4** showed lower titers, although its structure is similar to that of **C3**, differing only in the attaching group to proteins (−OH).

For coating, the use of haptens without the thiophosphate group and with spacer attachment by O-alkylation has provided the best results in other works. Also, studies about spacer arm length indicated that the shorter the arm, the higher the sensitivity (*32*, *33*). Applying this strategy to chlorpyrifos, hapten **C5** was tested as coating hapten, to introduce a major degree of heterology. As can be seen in **Table 1**, OVA−**C5** conjugate was highly recognized by sera obtained from haptens **C2** and **C3**.

All the combinations of serum/coating conjugates that showed specific recognition were used to carry out competitive assays in order to set up the most sensitive assay for chlorpyrifos. Results for more sensitive combinations are given in **Table 2**. The pairs KLH−**C3**-I/OVA−**C5** (1:6000/1 mg L^{-1}) (assay A) and BSA−**C2**-II/OVA−**C5** (1:4000/0.01 mg L^{-1}) (assay B) were selected for immunoassay optimization purposes, due to its wide dynamic range and its low I_{50} value, respectively.

ELISA Optimization. The effect of pH, ionic strength, surfactant concentration, and incubation time on assay performances (A_0 and I_{50}) was studied at room temperature for the indirect ELISA format. Results are shown in **Table 3**.

Table 2. I_{50} Values for the Best Serum-Coating Conjugate Combinations

serum	coating conjugate	I_{50} (ng mL^{-1})
BSA–**C1**-II	OVA–**C4**	1600
BSA–**C2**-I	OVA–**C5**	32
BSA–**C2**-II	OVA–**C5**	0.04
BSA–**C3**-II	OVA–**C5**	3
KLH–**C2**-I	OVA–**C5**	10
KLH–**C2**-II	OVA–**C5**	7
KLH–**C3**-I	OVA–**C5**	2
KLH–**C3**-II	OVA–**C5**	7
KLH–**C2**-I	BSA–**C5**	7
KLH–**C2**-II	BSA–**C5**	4
KLH–**C3**-II	BSA–**C5**	3

Table 3. Influence of pH, Buffer Concentration, Tween 20, and Incubation Time on I_{50} and Assay Binding[a]

	assay A[b]		assay B[c]	
variable	I_{50} (ng L^{-1})	A_0	I_{50} (ng L^{-1})	A_0
pH				
4	1348	0.871	3.5	0.514
5	1798	1.065	5.8	0.709
6	1348	1.000	19.8	0.847
7.5	1809	0.831	39.6	0.944
8	2023	0.995	59.3	0.969
9	2472	1.029	79.1	1.009
PBS (mM)				
0	19551	0.783	1137.6	1.120
5	12412	0.869	741.9	0.870
10	10943	0.953	79.1	0.936
20	1348	1.000	19.8	0.847
40	974	0.864	9.9	0.647
80	824	0.834	4.7	0.468
Tween 20 (%)				
0	75688	0.744	10.8	1.217
0.005	6404	0.928	17.9	1.144
0.01	1742	0.841	21.8	1.059
0.05	1381	1.003	18.1	0.908
0.1	1348	1.000	19.8	0.848
0.5	365	0.989	146.3	0.562
time (min)				
15	202	0.649	4.3	0.849
30	270	0.802	6.9	1.023
45	271	0.838	8.6	1.123
60	365	0.989	10.8	1.217

[a] RSD ranges from 1% to 8%. [b] Assay A, pair KLH–**C3-I**/OVA–**C5** (1:6000/1 mg L^{-1}). [c] Assay B, pair BSA–**C2-II**/OVA–**C5** (1:4000/0.01 mg L^{-1}).

First, the effect of pH between 4.0 and 9.0, using PBS-T (20 mM 0.1% v/v Tween 20) and 1 h of incubation time was tested. I_{50} values and maximum signal (A_0) do not change significantly in this pH range for the immunoassay A and both parameters increase when pH does for assay B. The best combinations of I_{50}/signal were obtained at pH 6.0. So it was selected to continue the assays optimization.

To estimate the influence of salt concentration, PBS-T solutions at 0, 5, 10, 20, 40, and 80 mM, all containing 0.1% v/v Tween 20 at the optimized pH, were tested. In all cases, I_{50} values decreased gradually as concentration increased, as had been previously observed for other organophosphorus immunoassays, probably due to similar biochemical interactions between analyte and antibody. Also, the maximum signal decreased for both immunoassays when the concentration is higher than 20 mM. In this study, a concentration of 20 mM PBS was selected as a compromise between maximum A_0 signal and minimum I_{50}.

Also, the effect of surfactant concentration (0, 0.005, 0.01, 0.05, 0.1, and 0.5% Tween, v/v) on immunoassay performance

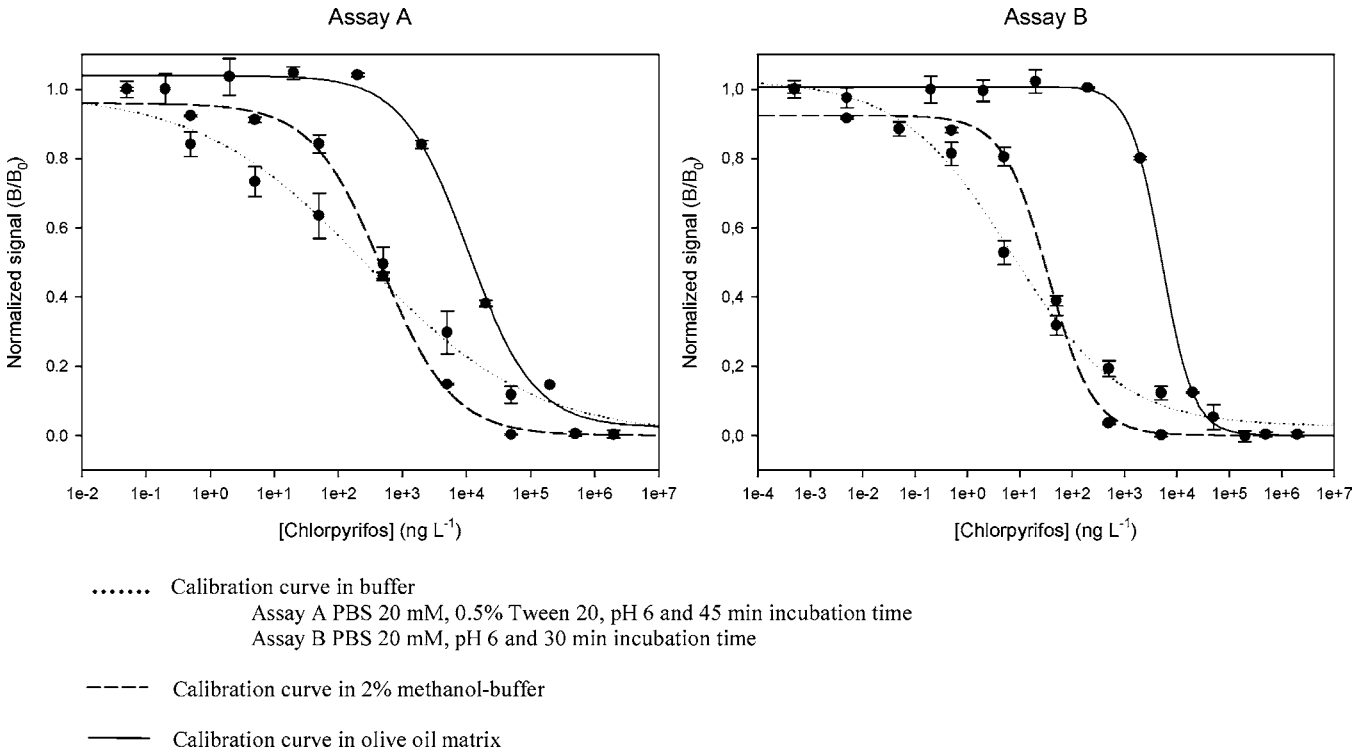


Figure 2. Calibration curve for the optimized chlorpyrifos immunoassays in different media. Mean values ± standard deviation ($n = 3$).

was studied, using the optimal pH and PBS concentration. For assay A, lower I_{50} values were observed when Tween 20 concentration increased. For this reason, 0.5% Tween 20 was selected.

On the other hand, the lowest I_{50} value was obtained for assay B when no surfactant was added to the buffer, and this condition was selected to carry out the assay.

Finally, the influence of the incubation time on the competition step (15, 30, 45, and 60 min) was investigated. I_{50} and A_0 values increased gradually as time increased. So, competition times of 30 min for assay B and 45 min for A were selected in order to obtain sensitive assays with an appropriate signal, in the minimum time. Shorter times can be also used, but the assay reproducibility is lower.

After optimization, the assay A exhibited an I_{50} of 271 ng L^{-1}, a working range between 4 and 16 474 ng L^{-1}, and a LOD of 0.3 ng L^{-1}. Although assay A has an I_{50} similar to the previously best reported chlorpyrifos' ELISAs (*19*, *23*), the limit of detection is considerably low and the competitive curve shows a wide dynamic range. The immunoassay B exhibits an I_{50} of 7 ng L^{-1}, a working range between 0.4 and 302 ng L^{-1}, and a LOD of 0.07 ng L^{-1}. This is, to our knowledge, the most sensitive immunoassay reported for chlorpyrifos. **Figure 2** illustrates the competition curve of each assay, showing a good repeatability for results from assay B (average RSD = 3%), but a worse one for assay A (average RSD = 4%) (dotted lines).

Cross-Reactivity Studies. Assay selectivity was evaluated using a set of organophosphorus insecticides and metabolites—because of their similar structure to chlorpyrifos—and several nonchemically related pesticides, due to their widespread agricultural and domestic use. I_{50} and CR data for each compound are given in **Table 4**.

In both ELISA's, there were interferences with chlorpyrifos-methyl, as it had been previously reported in other works (*19*, *20*, *23*). Only the immunoassay described by Lawruk et al. (*24*) showed a lower CR (26%) for this compound, probably because the hapten used for immunization maintains both ethoxyl groups in the phosphate ester. Chlorpyrifos-methyl is not a troublesome interferent, as it is present in many commercial chlorpyrifos formulations (*18*).

For chlorpyrifos-oxon, the cross-reactivity values were higher than those given by other authors, but similar (30%) to that reported by Hill et al. (*19*).

On the other hand, the cross-reactivity to TCP is negligible. Since this is the main chlorpyrifos metabolite in water, it is possible to determine chlorpyrifos in aqueous media without TCP interference.

The optimized ELISAs showed interference with bromophos-methyl, but slight cross-reactivity with bromophosethyl. Bromophos-methyl had lower CR (4.6%) for the assay described by Cho et al. (*20*), and no data were reported by other authors.

A cross-reactivity of 15% for fenchlorphos was displayed for assay B, but it was negligible with assay A. Reported immunoassays for chlorpyrifos showed variable cross-reactivity for this compound (6 and 75%) (*19*, *21*).

It is remarkable that 15 of the 18 compounds tested did not have appreciable cross-reactivity for both chlorpyrifos immunoassays developed in this work.

Tolerance to Organic Solvents. The effect of different organic solvents, which are necessary for pesticide extraction in complex matrixes, was studied in the optimized immunoassays. As is shown in **Table 5**, the assay A tolerated the tested PBS−organic solvents mixtures worse than assay B. Methanol was one of the best tolerated solvents by assay A (up to 20%). Acetonitrile and acetone were well-tolerated up to 10%, 2-propanol and methyl sulfoxide up to 4%, but ethyl lactate only up to 2%. In some cases, 2-propanol at 1% and 2% showed better I_{50} than pure PBS-T.

Assay B tolerated well all tested percentages of 2-propanol. In fact, the assay is more sensitive if this solvent is used

Table 4. Cross-Reactivity of Chlorpyrifos-Related Compounds, Metabolites, and Nonrelated Pesticides[a]

Compound \ Assay	A		B	
	I_{50} (ng L^{-1})	CR (%)	I_{50} (ng L^{-1})	CR(%)
Chlorpyrifos	271	100	7	100
Chlorpyrifos-methyl	194	140	14	50
Chlorpyrifos-oxon	661	41	15	46
TCP	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Atrazine	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Azinphos-methyl	$2\cdot10^{+6}$	0.014	$2\cdot10^{+6}$	$3.5\cdot10^{-4}$
Bromophos-ethyl	$2.5\cdot10^{+6}$	0.011	1700	0.4
Bromophos-methyl	319	85	28	25
Diazinon	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Dichlofenthion	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	53000	0.01
Fenchlorphos	$2\cdot10^{+6}$	0.014	47	15
Fenitrothion	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$1.3\cdot10^{+6}$	$5\cdot10^{-4}$
Fenthion	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Glyphosate	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Malathion	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Parathion-methyl	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$1.5\cdot10^{+6}$	$5\cdot10^{-4}$
Pirimiphos-ethyl	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Pirimiphos-methyl	$>5\cdot10^{+6}$	$< 6\cdot10^{-3}$	$>5\cdot10^{+6}$	$<2\cdot10^{-4}$
Tetrachlorvinphos	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$5\cdot10^{+5}$	$1.4\cdot10^{-3}$
Triclopyr	$>5\cdot10^{+6}$	$<6\cdot10^{-3}$	$5.6\cdot10^{+5}$	$1.3\cdot10^{-3}$

[a] Assay A, pair KLH–**C3-I**/OVA–**C5** (1:6000/1 mg L^{-1}), PBS 20 mM, 0.5% v/v Tween 20, pH 6. Assay B, pair BSA–**C2-II**/OVA–**C5** (1:4000/0.01 mg L^{-1}), PBS 20 mM, pH 6. Average RSD = 5%.

compared with PBS alone, although the signal decreases at percentages higher than 4% of 2-propanol. Up to 10% of methyl sulfoxide can be used as well with the same performances and methanol, acetonitrile, and acetone up to 4%. Ethyl lactate was the least favorable solvent (tolerated up to 2%). As can be seen, even under the worst conditions the I_{50} values are good, maintaining the ng mL^{-1} sensitivity level.

Methanol and 2-propanol were the best tolerated solvents for the developed immunoassays. 2-Propanol partially dissolves the olive oil, making difficult the subsequent extraction and analysis. Therefore, methanol was the selected solvent for extraction purposes. Moreover, previous studies carried out by the authors on pesticide extraction in olive oil samples led to a simple and effective protocol using methanol (*29*).

Table 5. Effect of Organic Solvents on Chlorpyrifos Immunoassays Performance[a]

	assay A[a]		assay B[b]	
medium	I_{50} (ng L^{-1})	A_0	I_{50} (ng L^{-1})	A_0
buffer	271	0.838	7	1.023
methanol				
1	298	0.846	7	0.998
2	244	0.612	7	0.972
4	352	0.797	7	0.945
10	325	0.817	81	0.950
20	678	0.835	6500	0.886
acetonitrile				
1	379	0.955	3	1.119
2	285	0.916	6	1.099
4	222	0.857	11	1.037
10	214	0.717	nc[c]	2.161
20	nc	0.586	nc	0.323
acetone				
1	339	0.885	4	1.052
2	298	0.711	4	1.032
4	314	0.798	9	0.930
10	599	0.768	3806	0.717
20	886	0.519	19956	0.222
2-propanol				
1	173	0.859	5	1.043
2	152	0.845	6	0.977
4	369	0.760	3	1.026
10	139901	0.585	2	0.898
20	510044	0.184	2	0.759
methyl sulfoxide				
1	270	0.867	4	1.056
2	291	0.698	8	0.993
4	428	0.781	7	0.957
10	8282	0.714	9	0.852
20	339834	0.526	1235	0.567
ethyl lactate				
1	325	0.863	21	1.048
2	407	0.729	58	0.920
4	7046	0.664	3134	0.736
10	nc	0.325	nc	0.253
20	nc	1.279	nc	0.889

[a] Assay A, solvent percentage in PBS 20 mM, 0.5% v/v Tween 20, pH 6. [b] Assay B, solvent percentage in PBS 20 mM, pH 6. [c] nc means no competitive curve.

The features of the immunoassays at 2% methanol (final concentration in ELISA plate) were an I_{50} of 244 ng L^{-1}, a working range between 74 and 2688 ng L^{-1}, and a LOD of 22 ng L^{-1} for assay A (**Figure 2**). An I_{50} of 7 ng L^{-1}, a working range between 1.4 and 487 ng L^{-1}, and a LOD of 0.4 ng L^{-1} were reached for assay B. Under these conditions, the assays can be applied for chlorpyrifos determination with similar sensitivity to the calibration curve obtained without methanol.

Analysis of Olive Oil Samples. Ten extra virgin olive oil samples (free of chlorpyrifos, as determined by GC) were analyzed through the developed assays. Samples were spiked at different chlorpyrifos levels and extracted as described in Materials and Methods. The methanolic extracts were diluted 1:25 in distilled water (2% methanol in plate) and quantified by ELISA using sera diluted in the optimized buffer.

Poor chlorpyrifos recoveries were achieved using standard curves in 2% methanol due to the matrix effect (**Figure 2**), as was also observed in previous studies (*29*). To normalize for matrix effects, the standard curves were made using spiked olive oil and extracted in the same way as the samples. As can be seen in **Table 6**, the recovery values obtained using standard curves in oil matrix were satisfactory, between 70% and 130% for assay A and between 80% and 119% for assay B. Assay B exhibited better precision than assay A.

Table 6. Chlorpyrifos Determination by ELISA in Spiked Extra Virgin Olive Oil Samples[a]

		assay A		assay B	
sample	[spiked]	[found]	*R*	[found]	*R*
1	1000	1156 ± 159	116	1027 ± 18	103
2	800	918 ± 249	115	688 ± 36	86
3	500	488 ± 54	98	481 ± 29	96
4	350	409 ± 60	117	367 ± 21	105
5	200	200 ± 20	100	203 ± 6	102
6	100	91 ± 23	91	119 ± 3	119
7	95	98 ± 14	103	76 ± 4	80
8	50	57 ± 4	114	45 ± 3	90
9	20	26 ± 2	130	19 ± 2	95
10	10	7 ± 1	70	10 ± 1	100
mean			103		98

[a] [Spiked] and [found] values are expressed in ng mL^{-1}± standard deviation (n = 3). *R*, recovery, expressed in % respect to the spiking levels.

Table 7. Comparison of Chlorpyrifos Determination by ELISA and GC Methods in Spiked Olive Oil Samples[a]

		assay A		assay B		GC	
sample	[spiked]	[found]	*R*	[found]	*R*	[found]	*R*
1	200	240 ± 42	120	224 ± 31	112	248 ± 38	124
2	500	507 ± 43	101	488 ± 54	98	475 ± 45	95
3	1000	1247 ± 39	125	1137 ± 39	114	1035 ± 154	104
4	200	208 ± 11	104	212 ± 22	106	205 ± 56	103
5	1000	1145 ± 159	115	1143 ± 136	114	1270 ± 153	127
6	500	496 ± 38	99	497 ± 42	99	528 ± 52	106
mean			111		107		110

[a] [Spiked] and [found] values are expressed in ng mL^{-1}± standard deviation (n = 3). *R*, recovery, expressed in % respect to the spiking level.

Finally, six oils were analyzed as blind samples by immunoassays and gas chromatography methods. The obtained results (**Table 7**) showed good correlations with the reference method: for assay A, $y = 0.933x + 29.376$ ng mL^{-1}, $r = 0.969$; for assay B, $y = 1.006x + 6.469$ ng mL^{-1}, $r = 0.985$.

The results show that the developed ELISAs are sufficient to quantify chlorpyrifos levels below the established MRL (50 μg kg^{-1}) in olive fruit for olive oil production by the Spanish legislation. Also, it is worth mentioning the high chlorpyrifos extraction and quantification throughput using both optimized methodologies. Our procedure is competitive, reducing considerably the cost and analysis time compared with the reference chromatographic method. Although immunoassay has demonstrated to be a valuable tool for the screening of a high number of samples, confirmatory analysis by chromatographic techniques should be carried out, as chromatography offers automation, robustness, and high sensibility.

DISCUSSION

A new hapten for chlorpyrifos has been synthesized, although the best sera were obtained with previously described haptens. Nevertheless, important improvement in the sensitivity compared with reported chlorpyrifos immunoassays has been achieved using a different coating conjugate, lacking the thiophosphate ester, due to heterology effects.

ELISAs have been developed to determine chlorpyrifos and applied to the screening of olive oil samples. The methodology offers strong advantages, such as simple extraction, rapidity, sensitivity, and good reproducibility, despite the characteristics of the matrix. These immunochemical techniques offer advan-

tages to chromatographic procedures when applied to one target compound, chlorpyrifos.

One of the developed chlorpyrifos immunoassays (assay B) is, to our knowledge, the most sensitive, exhibiting also a good tolerance to organic solvents, which makes it very valuable for its application in complex matrixes.

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