JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2009,** *57,* 375–384 **375**

Development of an Enzyme-Linked Immunosorbent Assay for Determination of the Miticide Bromopropylate

JAVIER RAMÓN-AZCÓN, FRANCISCO SÁNCHEZ-BAEZA, NURIA SANVICENS, AND M.-PILAR MARCO*

Applied Molecular Receptors Group (AMRg), CIBER of Bioengineering, Biomaterials and Nanomedicine IQAC-CSIC, Jorge Girona 18-26, 08034 Barcelona, Spain

This paper reports for the first time the development of an immunoassay for the analysis of the miticide bromopropylate (BP). The chemical structure of the immunizing haptens was designed to maximize the recognition of the bis-bromophenyl group of BP. Thus, the assay uses polyclonal antibodies raised against 2,2-bis(4-bromophenyl)-*N*-2-hydroxyacetamide-butanoic acid (hapten **2**) conjugated to keyhole limpet hemocyanin from horseshoe crab. A heterologous indirect competitive enzyme-linked immunosorbent assay (ELISA) has been developed that can detect BP down to 0.14 μg L^{-1}. The assay has been proven to tolerate a wide range of ionic strengths and pH values. Studies on the selectivity of this immunoassay have demonstrated a high recognition of related pesticides that contain a bis-halophenyl group in their structure. Other pesticides do not interfere in the analysis of BP using this immunochemical technique. Preliminary experiments have shown that BP can be directly analyzed in white wine samples down to 0.16 μg L^{-1} without the necessity of a cleanup procedure prior to the ELISA.

KEYWORDS: Pesticide; miticide; bromopropylate; antibody; hapten; immunoassay

INTRODUCTION

The occurrence of pesticide residues in fruits and vegetables has drawn the attention of the scientific community, due to the potential hazardous effects on humans and the environment. In this context, bromopropylate (BP) is a contact miticide that has been extensively used in agriculture against all stages of mites (*1*). BP is a nonsystemic and nonpenetrating compound that remains on the peel of fruits and does not migrate into the pulp. Moreover, BP is fairly persistent in the environment in acid and neutral media (50 day and >3 year half-lives, respectively) (*2*). BP has shown low acute toxicity in rats and rabbits (*3, 4*). However, BP effects on humans are unknown. Therefore, the INCHEM recommends an acceptable BP daily intake of 0−0.03 mg/kg of body weight (bw)/day in humans.

BP was first tested in the field for control of mites in 1966. Nowadays, the use of BP has been banned in the United Kingdom, and it has been generally withdrawn across the European Union. However, despite the European legislation, several member states have negotiated essential uses of the compound; therefore, some countries in Europe enable authorized BP use through 2008 while alternatives are sought (Regulation 2076/2002/EC). As a result, temporary maximum residue levels (MRLs) in marketed products such as wine grapes, citrus fruit, and pome fruit (MRL 2 mg kg^{1-}) have been established to accommodate an essential use in accordance with the amended directive 90/642/EEC. Nevertheless, data of the European commission show that there is still a continuous use of BP in vineyards and that BP has been found in Norway, Iceland, Denmark, Italy, and Lichtenstein in table grapes with residue levels exceeding the MRLs (*5*).

To guarantee the quality of fruits and vegetables, monitoring and analysis of BP in alimentary samples is currently conducted using chromatographic techniques such as high-performance liquid chromatography with photodiode array detection (*6*), gas chromatography with electron capture detection (*7*), or gas chromatography−mass spectrometry (*8*−*11*). These techniques have limits of detection around the MRLs. Nevertheless, an important drawback of these procedures is that they require an extraction method such as liquid−liquid or solid-phase extraction or solid-phase microextraction prior to the analysis. As a result, chromatographic techniques are time-consuming, plus they require high-cost equipment and trained personnel. In this context, immunochemical techniques are rapid, reliable, low-cost, and easy-to-use. They can be adapted to the simultaneous analysis of many samples, constituting an excellent high-throughput screening method. Moreover, they can afford the necessary detectability and selectivity for the target analyte with little sample treatment (*7, 12, 13*). In the present paper, we report for the first time the production of selective antibodies against

* Address correspondence to this author at the Department of Chemical and Biomolecular Nanotechnology, IQAC-CSIC, Jorge Girona 18-26, 08034 Barcelona, Spain (telephone 93 4006100; fax 93 2045904; e-mail mpmqob@iiqab.csic.es).

10.1021/jf802821n CCC: $40.75
Published on Web 12/29/2008

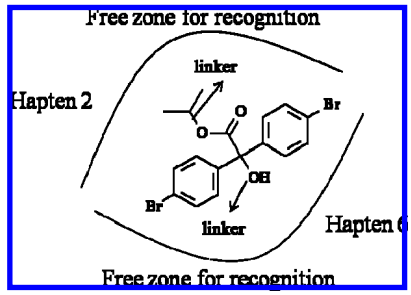


Figure 1. Hapten design.

BP. These antibodies have been used to develop an immunoassay to determine BP.

MATERIALS AND METHODS

Chemistry. *General Methods and Instruments.* Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). Unless otherwise indicated, purification of the reaction mixtures was accomplished by "flash" chromatography using silica gel as the stationary phase. ^{1}H and ^{13}C NMR spectra were obtained with a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) or with a Varian Inova-500 spectrometer (500 MHz ^{1}H and 125 MHz for ^{13}C). Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Formulated BP was obtained from a commercial supplier (Sygenta). Log P_{ow} was calculated using ACD/LogP DB version 3.00.

Synthesis of the Haptens. Spectroscopic and spectrometric data are given as Supporting Information (see **Figures 1**−**3**).

*2,2-Bis(4-bromophenyl)-N-2-hydroxyacetamide-butanoic acid (Hapten **2**).* A 1 M solution of NaOH (5 mL) was added dropwise to a solution of purified BP (1 g, 2 mmol, 1 equiv) in MeOH (5 mL). The mixture was vigorously stirred for 1 h at room temperature until the disappearance of the starting material by TLC. The crude product was washed with hexane, acidified with HCl, and extracted with AcOEt. The organic layer was then dried with $MgSO_4$, filtered, and evaporated under reduced pressure to obtain 2,2-bis(4-bromophenyl)-2-hydroxyacetic acid (930 mg, 92% yield). Subsequently, the acid (660 mg, 1.72 mmol, 1 equiv) and 1,1′-carbonyldiimidazole (CDI) (337 mg, 2.07 mmol, 1.2 equiv) were placed in a two-neck round-bottom flask provided with a magnetic stirring bar and diluted in anhydrous CH_2Cl_2 (10 mL). Then, a solution of methyl 3-aminoproanoate chlorhydrate (265 mg, 1.72 mmol, 1 equiv) in anhydrous CH_2Cl_2 (2 mL) was slowly added under Ar atmosphere to the reaction mixture. The reaction was left for 24 h at room temperature. The CH_2Cl_2 was evaporated, and the remaining crude was dissolved in EtO_2 and washed with saturated $NaHCO_3$. The organic layer was dried with $MgSO_4$, filtered, and evaporated. Finally, the crude product was purified by silica gel flash chromatography using hexane/EtOAc (1:1) as mobile phase to obtain the ester methyl 2,2-bis(4-bromophenyl)-*N*-2-hydroxyacetamide-butanoate (**1**) (380 mg, 42% yield). Subsequently, ester **1** (140 mg, 0.41 mmol, 1 equiv) was hydrolyzed with a 1 M aqueous solution of NaOH (5 mL) in MeOH (5 mL) at room temperature until the complete disappearance of the ester by TLC. The crude product was washed with hexane, acidified with HCl, and extracted with AcOEt. The organic layer was dried with $MgSO_4$, filtered, and evaporated under reduced pressure to obtain hapten **2** (126 mg, 95% yield).

*4-(2,2-Bis(4-bromophenyl)-2-chloroacetamido)butanoic Acid (Hapten **4**).* Ester **1** (112 mg, 0.23 mmol, 1 equiv) was placed in a round-bottom flask provided with a magnetic stirring bar and Dimroth refrigerant under Ar atmosphere. $SOCl_2$ (165 μL, 2.2 mmol, 10 equiv) was added dropwise, and the mixture was heated until reflux for 1 h. The crude product was diluted with hexane, washed with 1 M NaOH, and extracted with AcOEt. The organic layer was dried with $MgSO_4$, filtered, and evaporated under reduced pressure to obtain the ester methyl 4-(2,2-bis(4-bromophenyl)-2-chloroacetamido)butanoate (**3**) (59 mg, 50% yield). Next, ester **3** (59 mg, 0.11 mmol, 1 equiv) was hydrolyzed with a 1 M aqueous solution of NaOH, following the same protocol as described above for hapten **2** preparation, to obtain hapten **4** (50 mg, 90% yield).

*3-((Isopropoxycarbonyl)bis(4-bromophenyl)methoxy)propyl 3-Iodopropionate (Hapten **6**).* Purified BP (1 g, 2.3 mmol, 1 equiv) was placed in a round-bottom flask provided with a magnetic stirring bar and a condenser. $SOCl_2$ (700 μL, 9.3 mmol, 4 equiv) was added dropwise under Ar atmosphere and the mixture left to react for 30 min until the disappearance of the starting material by TLC. The crude product was diluted with hexane, washed with 1 M NaOH, and extracted with AcOEt. The organic layer was then dried with anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure to obtain the ester isopropyl 2,2-bis(4-bromophenyl)-2-chloroacetate (0.93 g, 90% yield). Next, a solution of this ester (900 mg, 2 mmol, 1 equiv) in 1,3-propanediol (2 mL) was gently heated under Ar atmosphere. Then, anhydrous Cs_2CO_3 (167 mg, 0.51 mmol, 0.2 equiv) was added to the solution, and the mixture was left to react for 4 h at 120 °C. The crude product was diluted in AcOEt, washed with a saturated solution of NaCl, dried with $MgSO_4$, filtered, and evaporated under reduced pressure to obtain the ester isopropyl 2-(3-hydroxypropoxy)-2,2-bis(4-bromophenyl)acetate (**5**) (506 mg, 41% yield). Subsequently, a solution of **5** in anhydrous CH_2Cl_2 (2 mL) was added under Ar atmosphere to iodoacetic acid (338 mg, 1.8 mmol, 3 equiv) The mixture was left to react for 24 h at 50 °C. Then, the crude product was diluted in AcOEt, washed with a saturated solution of NaCl, dried with $MgSO_4$, filtered, and evaporated. The crude was purified silica gel flash chromatography using hexane/AcOEt (4:1) to obtain hapten **6** (204 mg, 62% yield).

*Isopropyl 2-(2-Formylethoxy)-2,2-bis(4-bromophenyl)acetate (Hapten **7**).* A solution of pyridinium chlorochromate (PCC) (414 mg, 1.9 mmol, 2 equiv) in anhydrous CH_2Cl_2 (4 mL) was added to **5** (467 mg, 0.96 mmol, 1 equiv) under Ar atmosphere. The mixture was left for 2 h at room temperature, then diluted in AcOEt, filtered with zeolite, and concentrated to dryness. The crude was purified by silica gel flash chromatography using hexane/AcOEt (4:1) as mobile phase to obtain hapten **7** (278 mg, 60% yield).

*Methyl 2-(2-Formylethoxy)-2,2-bis(4-bromophenyl)acetate (Hapten **9**).* A solution of isopropyl 2-(3-hydroxypropoxy)-2,2-bis(4-bromophenyl)acetate (**5**) (500 mg, 1.03 mmol, 1 equiv) in MeOH (2 mL) was hydrolyzed with 1 M NaOH (2 mL) as described above to obtain 2-(3-hydroxypropoxy)-2,2-bis(4-bromophenyl)acetic acid (324 mg, 71% yield). To a solution of this acid (100 mg, 0.96 mmol) in anhydrous MeOH (2 mL) was added dropwise $SOCl_2$ (200 μL, 2.6 mmol, 4 equiv) under Ar atmosphere. The mixture was heated for 30 min, then diluted with hexane, washed with 1 M NaOH, and extracted with AcOEt. The organic layer was dried with $MgSO_4$, filtered, and evaporated. The crude product was purified by silica gel flash chromatography using hexane/AcOEt (4:1) as mobile phase to obtain methyl 2-(3-hydroxypropoxy)-2,2-bis(4-bromophenyl)acetate (**8**) (105 mg, 32% yield). Following the same protocol as described above for hapten **7** preparation, hapten **9** (96 mg, >95% yield) was obtained from PCC (97 mg, 0.45 mmol, 2 equiv) from product **8** (105 mg, 0.22 mmol, 1 equiv).

Immunochemistry. *Instrumentation.* The matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with the software Voyager-DE-RP (version 4.03) developed by Perspective Biosystems Inc. (Framingham, MA) and Grams/386 (for Microsoft Windows, version 3.04, level III) developed by Galactic Industries Corp. (Salem, NH). The pH and conductivity of all buffers and solutions were measured with a pH-meter (pH 540 GLP) and a conductimeter (LF 340), respectively (both from WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). The vacutainer blood collection set was acquired from Becton Dickinson (Meylon Cédex, France). Washing steps were performed on an SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read using a SpectramaxPlus microplate reader (Molecular Devices, Sunnyvale, CA) at a single wavelength of 450 nm. The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Sofware Inc., San Diego, CA). Unless otherwise indicated, the data presented correspond to the average of at least two well replicates.

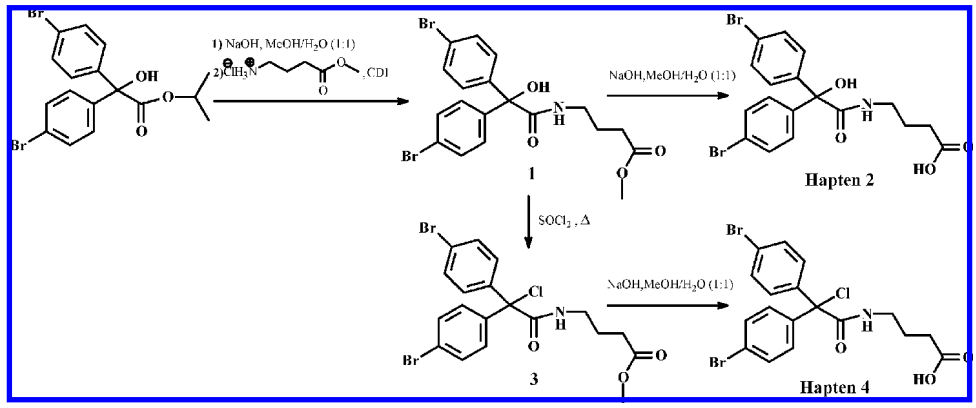


Figure 2. Synthetic pathways used for the preparation of haptens **2** and **4**.

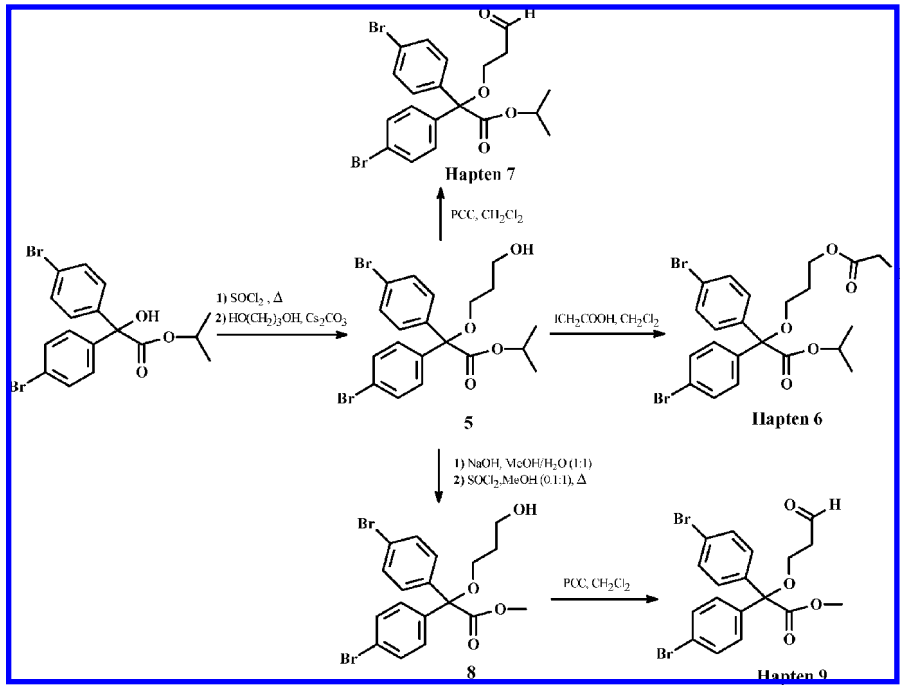


Figure 3. Synthetic pathways used for the preparation of haptens **6**, **7**, and **9**.

Chemicals and Immunochemicals. Chemicals were acquired from Aldrich Chemical Co. (Milwaukee, WI). Aminodextran (AD) (MW 70000) with an amino functionalization ratio of 21.5 amino groups/mol of dextran was purchased from Molecular Probes (Leiden, The Netherlands). Standards for cross-reactivity studies were kindly supplied by Prof. Damià Barceló from the Department of Environmental Chemistry, IIQAB-CSIC (Barcelona, Spain). Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mM. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The preparation of the protein conjugates and the antisera is described below.

Buffers and Solutions. Unless otherwise indicated, phosphate-buffered saline (PBS) is 0.01 M phosphate buffer, 0.8% saline solution, pH 7.5. PBST is PBS with 0.05% Tween 20. Borate buffer is 0.2 M boric acid−sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate−bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. Enzymatic reactions were stopped by adding 4 N H_2SO_4.

*Preparation of Hapten **2**−HCH and Hapten **6**−HCH (Immunogens) and Other Immunoreagents. Mixed Anhydride (MA) Method.* Following described procedures (*14, 15*), hapten **2** (15 μmol) was reacted with tributylamine (4 μL, 16.5 μmol) and isobutyl chloroformate (3 μL, 18 μmol) in anhydrous dimethylformamide (DMF; 160 μL). The activated hapten was then divided in two equivalent fractions and added dropwise to a solution of hemocyanin from horseshoe crab (HCH) and to a solution of bovine serum albumin (BSA) (30 mg/each) in 0.2 M borate buffer (1.8 mL). *Active Ester (AE) Method.* According to previously described procedures (*16*), haptens **2** and **4** (60 μmol each) were activated with freshly prepared solutions of *N*-hydroxysuccinimide (NHS; 8.62 mg, 75 μmol) and dicyclohexylcarbodiimide (DCC; 30.90 mg, 150 μmol) in anhydrous DMF (200 μL) for 2 h at room temperature and reacted with the albumins BSA, CONA, and OVA (10 mg each) in 0.2 M borate buffer (1.8 mL). *Aldehyde Conjugation (AC) Method.* As described elsewhere (*17*), haptens **7** and **9** (10 μmol) in anhydrous DMF (100 μL) were mixed with BSA, CONA, and OVA (10 mg/each) and AD (16.4 mg, 5 μmol of amino groups) solutions in 0.2 M borate buffer (800 μL) (*18, 19*). Subsequently, $NaCNBH_3$ (100 μmol) in 10 mM PBS buffer (100 μL) was added. Reactions were left overnight at 4 °C. Then, an additional 100 μmol of $NaCNBH_3$ was added, and reactions were left for 30 min more at room temperature. Protein conjugates (AD and BSA) that were not completely soluble after reconstitution were centrifuged and the pellet was discarded. Recoveries were 20 and 55% for AD and BSA, respectively. *Halogen Conjugation (HC) Method.* As previously described (*20*), hapten **6** (10 μmol) in anhydrous DMF (100 μL) was added dropwise to HCH and BSA solutions (30 mg each) in 0.2 M borate buffer (1.8 mL). Proteins had been previously modified with 2-iminothiolane hydrochloride and purified by column Hi-Trap desalting with Sephadex G-25. The reaction was left for 24 h at room temperature. Protein conjugates were purified by column Hi-Trap desalting with Sephadex G-25. All protein conjugates were purified by dialysis against PBS (0.5 mM, 4 × 5 L)

Table 1. Hapten Densities of the BSA Conjugates[a]

conjugation method	hapten	δ-hapten[b] BSA	δ-hapten[b] CONA
mixed anhydride	**2**	4	*c*
active ester	**2**	3	2
	4	24	14
halogen conjugation	**6**	4	*c*
aldehyde conjugation	**7**	11	5
	9	*d*	*d*

[a] Hapten densities were calculated by MALDI-TOF-MS. [b] Moles of hapten per mole of protein. [c] Conjugates **2**−CONA and **6**−CONA were not prepared. [d] Analysis of **9**−BSA and **9**−CONA conjugates was not possible by MALDI-TOF-MS. Conjugation was verified by ELISA.

and Milli-Q water (1 × 5 L) and stored freeze-dried at −40 °C. Unless otherwise indicated, working aliquots were stored at 4 °C in 0.01 M PBS at 1 mg mL^{-1}.

Hapten Density Analysis. Hapten densities of the BSA and the CONA conjugates were determined by MALDI-TOF-MS by comparing the molecular weights of the native proteins to those of the conjugates (see **Table 1**). MALDI spectra were obtained by mixing 2 μL of freshly prepared matrix (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in CH_3CN/H_2O 70:30 (v/v), 0.1% TFA) with 2 μL of a solution of the proteins (10 mg mL^{-1} in CH_3CN/H_2O 70:30 (v/v), 0.1% TFA). Hapten density (δ hapten) was calculated according to the following equation: [MW(conjugate) − MW(protein)]/MW(hapten).

Polyclonal Antisera. The immunization protocol was performed on female New Zealand white rabbits weighing 1−2 kg, as previously described (*16*). Rabbits 174, 175, and 176 were immunized with **2**−HCH and rabbits 177, 178, and 179 were immunized with **6**−HCH using 100 μg of the corresponding hemocyanin conjugate and boosted each month for 6 months until no significant increase in the antibody titer was observed. The corresponding antisera (As) obtained were named with the rabbit numbers. Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the different antisera to microtiter plates coated with hapten **2**−BSA (AE) for As174−176 and with hapten **6**−BSA (HC) for As177−179. After an acceptable antibody titer was observed, the animals were exsanguinated, and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −40 °C in the presence of 0.02% NaN_3.

Antibody Characterization. Noncompetitive indirect ELISA was used for the screening of the avidity of the 6 antisera obtained versus the 12 coating antigens. Microtiter plates were coated with a 1 μg mL^{-1} concentration of each of the BSA, CONA, and OVA conjugates overnight at 4 °C, covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 μL/well), and antisera (1/2000; 100 μL/well in PBST) were added and incubated for 30 min at room temperature. Plates were then processed as described below.

Noncompetitive ELISA. The plates were coated with the coating antigen dilutions (10 μg mL^{-1}−9 ng mL^{-1};100 μL/well in 0.05 M carbonate buffer) overnight at 4 °C, covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 μL/well), and antisera serial dilutions (1/1000 to 1/64000; 100 μL/well in PBST) were added and incubated for 30 min at room temperature. The plates were washed again as before, and a solution of anti-IgG−HRP (1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution (0.004% H_2O_2 and 0.01% tetramethylbenzidine in 0.04 M citrate buffer) was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. From these experiments, optimum concentrations for coating antigens and antisera dilutions were chosen to produce around 0.5−1 unit of absorbance in 30 min.

Competitive Indirect ELISAs. The avidity of BP to compete with the different coating antigens for the antibody binding was investigated by adding 12 serial dilutions of the analyte (10000 nM−1 pM, in PBST, 50 μL/well) to the coated plates followed by the appropriately diluted As (50 μL/well). The mixture was incubated for 30 min, and the plates were then processed as described above. The standard curve was fitted to a four-parameter equation according to the following formula: $Y = [(A - B)/1 - (x/C^D)] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

Optimization of ELISA. Different experimental parameters, length of the competitive step, preincubation, Tween 20, pH effect, and ionic strength were studied sequentially in this order using the above-described protocol. However, each time an evaluated parameter was changed, this new value was used for the evaluation of the next condition.

*Optimized Competitive ELISA (Hapten **9**−BSA/As 174).* Microtiter plates were coated with hapten **9**−BSA (0.5 μg mL^{-1} in coating buffer, 100 μL/well) overnight at 4 °C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL/well). BP standards were prepared by serial dilutions in DMSO (1075 × 10^3 to 34 nM), diluted 200 times (5375 to 0.17 nM) in 10 mM PBS, 0.5% DMSO, and added to the microtiter plates (50 μL/well), followed by the antisera As174 (1/1000 in PBS, 50 μL/well). After 15 min of incubation at room temperature, the plates were washed again as described before and a solution of anti-IgG−HRP (1/6000 in PBST) was added (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm.

Cross-Reactivity Determinations. Stock solutions of different long-chain fungicides, herbicides, and acaricides were prepared in DMSO at a concentration of 1 mM. Standard curves were prepared by serial dilutions in DMSO (1075 × 10^3 to 34 nM), diluted 200 times (5375 to 0.17 nM) in 10 mM PBS, 0.5% DMSO, and measured with the optimized competitive ELISA protocol describe above. The cross-reactivity values were calculated according to the equation (IC_{50} BP/IC_{50} compound) × 100.

Accuracy. This parameter was assessed by preparing nine different blind spiked samples in Milli-Q water. Samples were buffered with PBS prior to analysis with the optimized ELISA to adjust the ionic strength and pH. Analyses were made in triplicate.

Effect of Ethanol. PBS solutions containing different concentrations of EtOH (v/v) (0, 1, 2, 5, 10, and 20%) were used to prepare standard curves and were run simultaneously in the competitive ELISA according to the procedure described above.

Matrix Effect Studies. White and red wine samples (commercially available) were used to assess the performance of the competitive ELISA method. Samples were buffered to adjust pH and conductivity and used to prepare standard curves of BP. The curves were run according to the conditions described above for the optimized assay hapten **9**−BSA/As 174, and the absorbances obtained were adjusted to the four-parameter equation to compare their parallelism to the curve prepared in the absence of wine.

RESULTS AND DISCUSSION

Hapten Synthesis. We considered two different chemical structures as immunizing haptens to produce antibodies against BP (see **Figure 1**). In both haptens the spacer arm was placed to maximize the exposure to the immune system of the bis-bromophenyl group, which appeared as one of the most important epitopes. In hapten **2**, the ester group was replaced by the spacer arm, preserving the tertiary hydroxyl group. In contrast, the chemical structure of hapten **6** had substituted the hydroxyl group, leaving the isopropyl ester untouched. Hapten **2** (4-(2,2-bis(4-bromophenyl)-2-hydroxyacetanamido)butanoic acid) was prepared using BP as starting material (see **Figure**

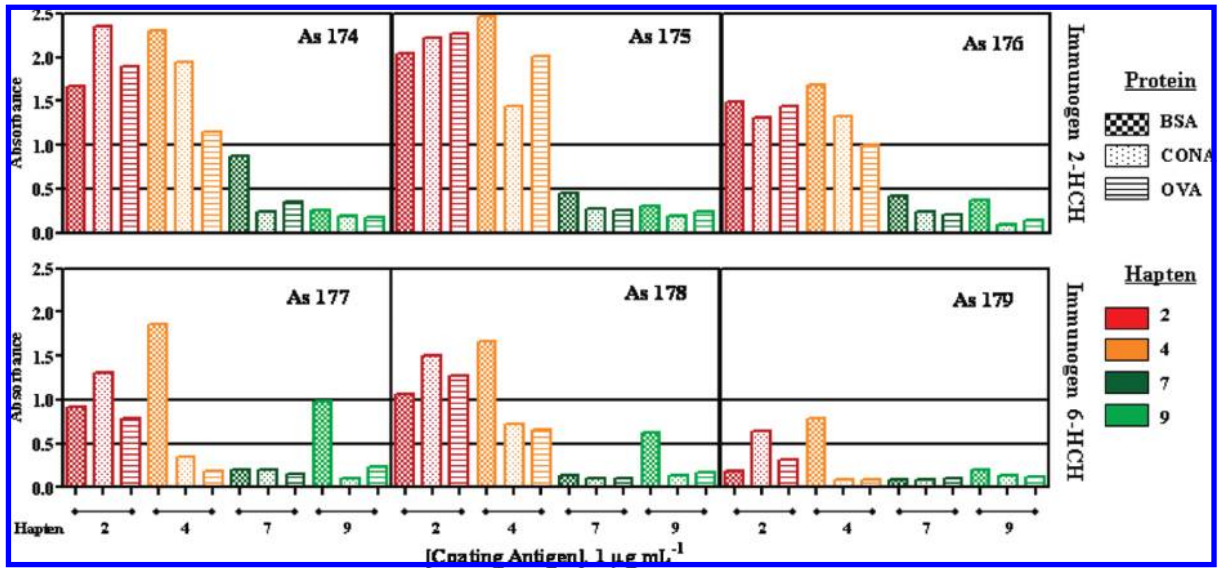


Figure 4. Graph showing the avidities of the antisera raised against BP: As174−179 versus the battery of competitor haptens (haptens **2**, **4**, **7**, and **9** coupled to BSA, CONA, or OVA). The titration experiments were done using a noncompetitive indirect ELISA format (see Antibody Characterization under Materials and Methods) with an As dilution of 1:2000 and a coating antigen concentration of 1 μg mL^{-1}. The straight lines indicate the absorbance corresponding to 0.5 and 1.

2). Formulated BP was obtained from a commercial supplier, and the active principle was isolated and the purity checked by ^{1}H NMR. The isopropyl ester was hydrolyzed affording the corresponding carboxylic acid, which was reacted with methyl 4-aminobutanoate hydrochloride using CDI as activator to obtain the corresponding ester derivative **1** (*21*, *22*). The hydrolysis of the ester **1** afforded the desired hapten **2** with a 37% global yield. Hapten **6** (3-((isopropoxycarbonyl)bis(4-bromophenyl-)methoxy)propyl 3-iodopropionate) synthesis was approached by exchanging the tertiary hydroxyl group with a chlorine atom using $SOCl_2$ as a chlorinating agent (see **Figure 3**). As a result, we obtained the ester isopropyl 2,2-(bis(4-bromophenyl)-2-chloroacetate, which was reacted with 1,3-propanediol to obtain the ester **5** isopropyl 2-(3-hydroxypropoxy)-2,2-bis(4-bromophenyl)acetate. The last step of the synthetic route involved the esterification reaction of the hydroxyl group of compound **5** with iodoacetic acid. The desired hapten **6** was obtained with a global yield of 25%. All products were purified and characterized by spectroscopy (^{1}H and ^{13}C NMR) and spectrometric (MS) methods.

In addition to the immunizing haptens **2** and **6**, three different haptens, **4**, **7**, and **9**, were prepared to be used as competitors (see **Figures 2** and **3**). It has been described that some competitive immunoassays work better under heterologous conditions (*12*). The idea is that a chemical structure of the hapten used as competitor that varies slightly from that of the immunizing hapten and, therefore, from that of the analyte favors the binding of the antibody to the analyte by diminishing the affinity of the antibody versus the competitor. In this manner, haptens **4**, **7**, and **9** preserved the bis-brophenyl group in their structure, but they contained small modifications compared to haptens **2** and **6**. Hapten **4** had a chlorine atom instead of the tertiary hydroxyl group of hapten **2**. Hapten **6** contained an iodoacetic group in its structure for the conjugation to the carrier proteins that could influence antibody avidity. To minimize this influence, haptens **7** and **9** were prepared with a different spacer arm from that in hapten **6**. Moreover, hapten **9** contained a methyl ester moiety instead of the isopropyl ester of hapten **6**.

Immunoreagent Preparation. Hapten **2** was conjugated to HCH and BSA following the MA method by covalently coupling the hapten through its carboxylic group to the lysine residues of the proteins. Hapten **6**−HCH and hapten **6**−BSA were prepared using the HC method, which requires a previous modification of the proteins. Thus, HCH and BSA were functionalized with 2-iminothiolane hydrochloride to maximize the number of accessible thiol groups. Subsequently, conjugates **6**−HCH and **6**−BSA were obtained by covalent coupling of hapten **6** through its halogen group to the thiol residues of HCH and BSA. The coupling reactions were verified by MALDI-TOF-MS analysis of the BSA derivatives by comparing the observed molecular weight to that of the intact protein. An average of four haptens per molecule of BSA was estimated for both conjugates. The **2**−HCH conjugate was used as immunogen to raise polyclonal antibodies named As174, As175, and As176, whereas antisera As177, As178, and As179 were obtained from the three rabbits immunized with the **6**−HCH conjugate.

To minimize interferences due to the potential side reactions that may have occurred during the preparation of the immunogens, the coating antigens were obtained using different coupling methods. Haptens **2** and **4** were coupled to BSA, CONA, and OVA using the AE method. Haptens **7** and **9** were conjugated to BSA, CONA, and OVA using the AC method by covalent coupling of the aldehyde groups of the haptens to the lysine residues of the proteins. **Table 1** shows the hapten densities of the BSA and CONA coating antigens estimated by MALDI-TOF-MS.

Development of a Competitive ELISA. To test whether the antisera recognized the battery of competitors, the avidity of all antisera versus the 12 competitors was tested by noncompetitive ELISAs (see **Figure 4**). Antisera raised against **2**−HCH showed a higher avidity than those raised against **6**−HCH. As176 and As179 had the lowest titers among antisera obtained from immunogens **2**−HCH and **6**−HCH, respectively. In general, both immunogens **2**−HCH and **6**−HCH rendered similar patterns of recognition of the coating antigens. Antibody titers were significantly higher for the coating antigens prepared from haptens **2** and **4**. In particular, the coating antigen **4**−BSA was the most recognized by all the antisera tested. In contrast, coating antigens obtained from haptens **7** and **9** were only slightly recognized with some antisera. The coating antigen

Table 2. Features of the Best Competitive Assays Obtained with the Antisera As174−176 and As177−179 Raised against **2**−HCH and **6**−HCH, Respectively[a,b]

immunogen	antigen/As	A_{max}	A_{min}	IC_{50}[c]	slope	R^2
2−HCH	**9**−BSA/As174	1.20	0.28	0.68	−0.52	0.98
	7−BSA/As174	1.30	0.31	5.3	−0.55	0.99
	4−BSA/As 174	0.72	0.07	0.67	−0.9	0.90
	2−BSA/As176	0.99	0.01	17.3	−0.62	0.99
	2−CONA/As176	1.35	0.02	51.1	−0.72	0.99
	2−OVA/As176	1.53	0.01	33.6	−0.54	0.99
	4−BSA/As176	1.19	0.06	24.0	−0.87	0.99
6−HCH	**4**−BSA/As178	1.34	0.03	165.3	−0.73	0.99
	4−OVA/As178	0.97	0.05	89.7	−0.57	0.99

[a] Only those assays showing acceptable features and IC_{50} values below 200 $\mu g\ L^{-1}$ are indicated. [b] The parameters are extracted from the four-parameter equation used to fit the standard curve according to the following formula: $Y = [(A - B)/1 - (x/C)D] + B$, where A is the maximum absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. [c] IC_{50} values are expressed in $\mu g\ L^{-1}$.

7−BSA was significantly recognized by As174, whereas the coating antigen **9**−BSA was recognized by As177 and As178. Altogether, these results indicate that the recognition of the two bis-bromophenyl groups is maximized when the spacer arm is introduced through the ester group instead of through the hydroxyl moiety.

Those antisera/coating antigen combinations showing acceptable titers were assessed in two-dimensional titration experiments to establish the appropriate concentrations of the immunoreagents for the competitive assays. The antiserum/coating antigen combinations that rendered absorbance values higher than 0.5 were tested on a competitive ELISA format to determine the antiserum's ability to recognize the analyte BP. **Table 2** shows the features of the best competitive ELISAs obtained. Immunoassay **9**−BSA/As174 was selected for further optimization and evaluation.

ELISA Evaluation. *Reproducibility.* Experiments done on consecutive days showed a remarkable variability in the assay features (data not shown). We questioned whether the particular features of BP could be the reason for the insufficient day-to-day reproducibility. BP is highly lipophilic (log $P = 5.43$) and has low solubility in water (0.1 mg L^{-1}). Previous studies performed in our laboratory with the compound 2,4,6-trichloroanisole (log $P = 4.11$; water solubility = 10 mg L^{-1}) had demonstrated that lipophilic substances tend to absorb to surfaces and that the use of plasticware should be minimized (*23*). Moreover, contact with detergents derived from nonionic surfactants was completely avoided. Due to the low water solubility of BP, we also contemplated the preparation of the standard curve in DMSO instead of the usual assay buffer. This strategy had already been shown to improve the reproducibility of an immunoassay developed for the nonpolar insecticide chlorpyrifos (log $P = 4.96$; water solubility = 1.12 mg L^{-1}) (*24*). Additionally, a fresh standard curve for BP was prepared every day, because it has been reported that freeze−thaw cycles affected the reproducibility of immunoassays developed for lipophilic compounds such as chlorpyrifos (*25*) and the fungicide fenpropimorph (log $P = 4.88$) (*26*). By taking all of these precautions it was possible to obtain a good reproducibility for the assay **9**−BSA/As174 and to proceed to a reliable evaluation of other physicochemical parameters that would help to improve the immunoassay performance.

A reduction in the *length of the competitive step* translated into a decrease of the assay signal and a simultaneous increase in the immunoassay detectability (*16*, *23*, *27*). Therefore, we chose an incubation period of 15 min as a compromise between detectability and maximal absorbance. *Preincubation* of BP with As174 for 30 min considerably improved the detectability of the assay (see **Figure 5A**). Preincubation periods of longer than 30 min did not affect the detectability. We also found that the assay was strongly influenced by the concentration of the nonionic detergent *Tween 20* (see **Figure 5B**). Thus, the assay

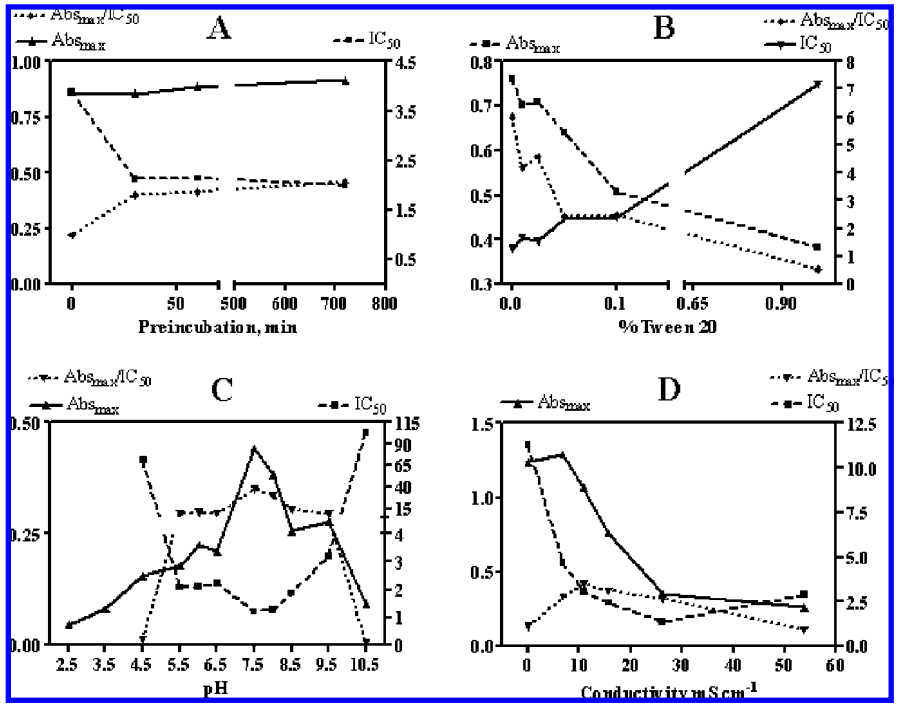


Figure 5. Graphs showing the influence of several parameters on the performance of the immunoassay **9**−BSA/As174: (**A**) effect of preincubation time; (**B**) effect of detergent concentration; (**C**) effect of pH; (**D**) effect of ionic strength. The data presented are extracted from the four-parameter equation used to fit the standard curves. Standard curves were prepared using two well replicates.

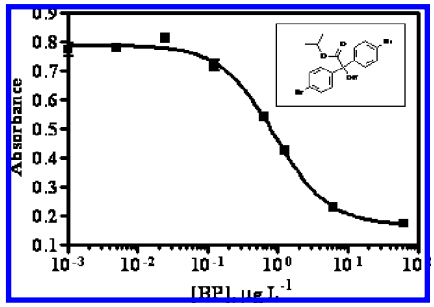


Figure 6. Calibration curve of the optimized BP immunoassay **9**−BSA/As174. The data presented correspond to the average and standard deviation of eight assays runs on five different days. The curves were prepared using two well duplicates. See **Table 3** for the features of the optimized immunoassay.

Table 3. Features of the Optimized Immunoassays **9**−BSA/As174 and **9**−AD/As174[a]

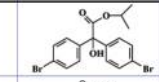
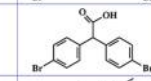
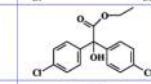
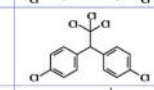
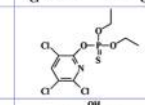
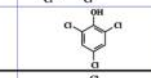
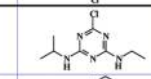
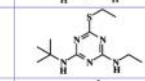
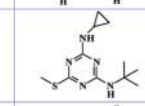
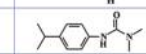
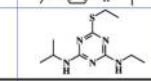
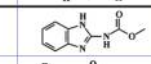
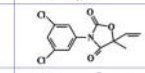
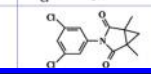
	9−BSA/As174[b]	**9**−AD/As174[b]
A_{min}	0.21 ± 0.05	0.10 ± 0.03
A_{max}	0.79 ± 0.13	1.61 ± 0.03
slope	−1.51 ± 0.27	−1.51 ± 0.18
IC_{50} (μg L^{-1})	1.22 ± 0.14	1.52 ± 0.19
LOD (μg L^{-1})	0.14 ± 0.08	0.21 ± 0.04
dynamic range (μg L^{-1})	0.40 ± 0.16 to 4.92 ± 1.20	0.49 ± 0.03 to 3.64 ± 0.19
R^2	0.99	0.99

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve according to the following formula: $Y = [(A - B)/1 - (x/C)D] + B$, where A is the maximum absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve. [b] The data presented correspond to the average of eight calibration curves run on five different days.

performed much better in the total absence or at very low concentration of Tween 20 (<0.01%). This result is in agreement with those published for chlorpyrifos and 2,4,6-trichloroanisole immunoassays, respectively (*23, 24*). The remarkable influence of Tween 20 on the immunoassay features could be explained by the nonspecific hydrophobic interactions that nonpolar analytes establish with detergent molecules, which compete with the specific interaction analyte−antibody (*28, 29*). Therefore, a decrease in Tween 20 concentrations enhances the interaction between BP and As174. Studies of the *pH effect* showed that the assay performed well in the range between 5.0 and 9.5, although a pH of 7.5 rendered the best detectability (see **Figure 5C**). Finally, we observed that the maximum absorbance was significantly affected when the *ionic strength* was increased. Conductivity values of >10 mS cm^{-1} produced a considerable decrease in the maximum absorbance value (see **Figure 5D**). Therefore, a conductivity value of 15 mS cm^{-1} (10 mM in terms of PBS) was chosen as a compromise between maximum absorbance and detectability. **Figure 6** shows the curve corresponding to the optimized immunoassay **9**−BSA/As174, and **Table 3** summarizes the parameters defining the calibration graph of the ELISA. The limit of detection accomplished was 0.14 ± 0.08 μg L^{-1}. A 4% CV within assay and a 11% CV between assays performed on different days demonstrate immunoassay reproducibility.

Immunoassay specificity was evaluated by preparing standard curves of fungicides such as vinclozolin, carbendazim, and procymidione, herbicides such as atrazine, isoproturon, Irgarol 1051, and ametryne, and insecticides/miticides such as chlorobenzilate, 2,4,6-trichorophenol, chlorpyrifos, and DDT plus BP main metabolite 4,4′-bromobenzylic acid. The results of the cross-reactivity study (**Table 4**) demonstrated that the interference caused by the coexistence of these compounds was negligible except for those that, similar to BP, had a bis-halophenyl group in their structure. This is the case of chlorobenzilate, a miticide structurally close to BP but with two chlorophenyl groups instead of bromophenyls. BP metabolite 4,4′-bromobenzylic acid was recognized, as well. Those molecules containing a halophenyl moiety in a different geometry from the bridged bis-halogenphenyl did not cross-react. DDT, despite containing a bis-chlorophenyl moiety, was not recognized.

Table 4. Interference Caused by Structurally Related Chemicals on the Immunoassay **9**−BSA/As174, Expressed by Their IC_{50} Values and the Percentage of Cross-Reactivity (% CR)[a]

Group	Compound	Formula	IC_{50}[b]	% CR.
Miticides, Insecticides	**Bromopropylate**		3.46	100
	4,4′-bromobenzilic acid		5.16	67
	Chlorobenzilate		5.43	64
	DDT		>1000	0
	Chlorpyrifos		>1000	0
	2,4,6-TCP		>1000	0
Herbicides	Atrazine		>1000	0
	Tertbutryn		>1000	0
	Irgarol 1051		>1000	0
	Isoproturon		>1000	0
	Ametryne		>1000	0
Fungicides	Carbendazim		>1000	0
	Vinclozolin		300	1
	Procymidione		249	1

[a] Cross-reactivity is expressed as a percent of the IC_{50} of BP divided by the IC_{50} of the compound. [b] IC_{50} values are expressed in nM.

Immunoassay accuracy was evaluated by measuring a set of blind samples with the optimized immunoassay **9**−BSA/As174. As shown in **Figure 7**, the correlation between the spiked and measured values was good, with a regression coefficient of $R^2 = 0.96$ and a slope value of 1.027. Due to the frequent use of BP as a miticide in vineyards in Europe (*5*) and its stability—enough to reach the must and wine, one of our ultimate objectives was to measure BP in wine samples. With this purpose, we first evaluated the *effect of EtOH* on the assay performance. An increase in the content of EtOH affected the detectability, which decreased when the percentage of EtOH in the buffer was increased(see **Figure 8**). Next, we performed some preliminary studies of the *matrix effect* caused by white and red wines in

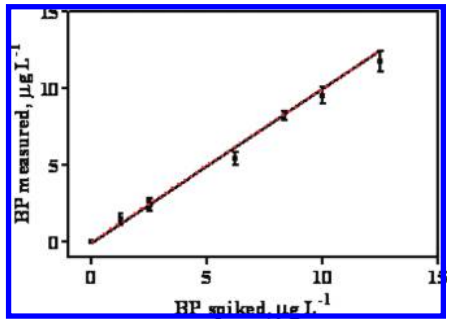


Figure 7. Graph showing the correlation between the spiked concentrations and the values measured by ELISA. Blind samples were prepared in Milli-Q water. Samples were buffered with PBS to adjust the pH and the ionic strength before ELISA measurements. The data shown correspond to average and standard deviation of analyses made on three different days. The curves were prepared using three well duplicates. The dotted line corresponds to a perfect correlation (slope = 1).

the **9**−BSA/As174 assay (see **Figure 9**). The pH and the conductivity of both wines were adjusted to the working conditions of the assay by buffering the samples, and then standard curves were prepared in raw and PBS-diluted wine. Red wine samples could not be directly analyzed under the present conditions. The undesired matrix effect was eliminated by diluting the red wine sample 40 times in PBS. Under these conditions, the detectability was obviously compromised and the LOD value would be set at about 5.60 μg L^{-1}. On the other hand, despite the presence of 11% EtOH in the white wine, this matrix did not produce a significant interference in the assay. One explanation could be that the presence of certain compounds in the white wine matrix compensates for the effect of the EtOH content. This unexpected result was verified by analysis three different types of white wine (see **Figure 10**). Finally, to evaluate the applicability of the method, blind spiked samples were measured directly in white wine. The results shown in **Table 5** indicate a good accuracy of the ELISA to analyze white wine samples, because all of the recovery values were close to 100%.

AD as Coating Antigen. Although coating antigens had been lyophilized and stored at −20 °C, after several months we observed that the coating antigen **9**−BSA had become partially insoluble in aqueous buffer, which translated into a change in the immunoassay features. Due to BP and derivate hapten lipophilic characteristics, we considered the possibility that the orientation of the hapten **9** molecules on the surface of the protein would have changed with time toward more hydrophobic pockets in the protein's tertiary structure, leading to a diminished antibody recognition. In this regard, it has been previously reported that hydrophobic hapten−protein conjugates are unsuitable as coating antigens due to their poor adsorption properties (*30*). On the other hand, other authors have suggested that using highly hydrophilic carriers to raise antibodies against hydrophobic haptens diminished the potential physicochemical interactions of those with the carrier (*18*, *31*). As a result, we decided to prepare hapten **9** conjugates using AD. The use of dextran to prepare coating antigens for immunoassay has been previously described (*18*, *22*). The coating antigen **9**−AD was assessed using the optimized conditions for the **9**−BSA/As174 immunoassay. **Table 3** shows that the **9**−AD/As174 immunoassay has features similar to the **9**−BSA/As174 immunoassay. Until now (several months after the **9**−AD conjugate was prepared), the coating antigen was still stable and soluble in buffer media. No changes had been observed in the features of the **9**−AD/As174 immunoassay, which confirms the stability of the conjugate **9**−AD.

In summary, the immunochemical method reported here is a promising tool that could be used as an analytical screening

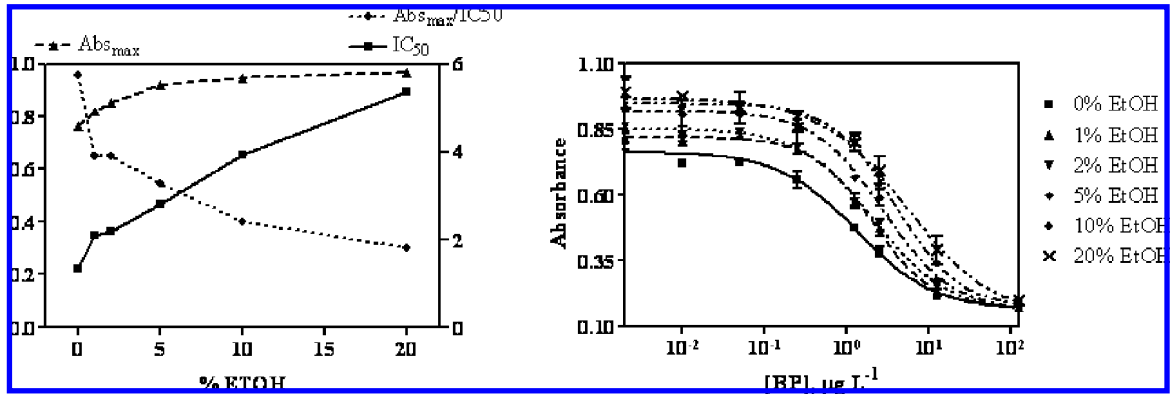


Figure 8. Graphs showing the interference produced by the EtOH concentration on the **9**−BSA/As174 immunoassay features. EtOH percentages range between 0 and 20%. Standard curves were run in duplicate in the same microtiter plate. The data presented are extracted from the four-parameter equation used to fit the standard curve.

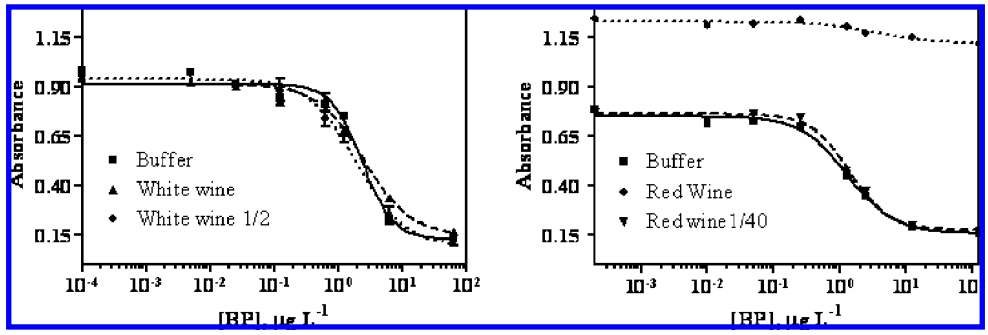
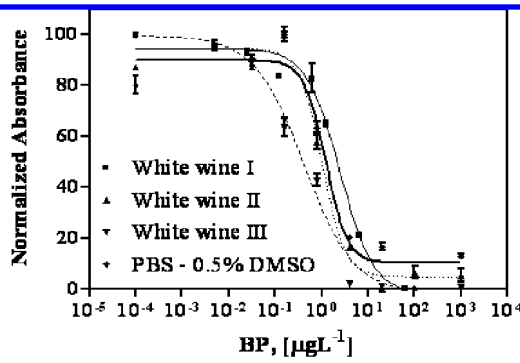


Figure 9. Graphs showing the interference produced by the white and red samples tested in the ELISA **9**−BSA/As174.



	White wine I	White wine II	White wine III	PBS
IC50 (μgL-1)	2.53 ± 0,06	1.33 ± 0,09	1.21 ± 0,10	1.24 ± 0,09
LOD (μgL-1)	0.20 ± 0,07	0.17± 0,11	0.12 ± 0,14	0.24 ± 0,09

Figure 10. Graph showing the analysis of three different types of white wines in the ELISA **9**−BSA/As174. The table indicates the IC_{50} values and the limits of detection (LOD) of the immunoassay in white wine. The data presented are extracted from the four-parameter equation used to fit the standard curve and correspond to average and standard deviation of analyses made on three different days. The curves were prepared using two well duplicates.

Table 5. Results from Preliminary Accuracy Studies Performed in White Wine with the Immunoassay **9**−BSA/As174[a]

spiked concn (μg L^{-1})	measd concn (μg L^{-1})	recovery (%)
1.25	1.28 ± 0.06	102
4.00	3.72 ± 0.15	93
4.00	4.40 ± 0.11	110

[a] The accuracy was evaluated by preparing blind spiked samples in white wine and analyzing them by the optimized immunoassay protocol. Each value corresponds to the mean of three replicates.

method for monitoring the presence of BP and related compounds in alimentary and environmental samples. Knowledge of the physicochemical properties of this analyte has been critical to establishing a reproducible and robust method. The immunizing haptens have been designed to maximize recognition of the bis-bromophenyl group. Therefore, BP and related compounds can be detected using this immunochemical technique without the interference of other pesticides. The immunochemical method allows BP detection down to 0.14 mg L^{-1} in the assay buffer, which is far below the MRL established in Europe. Moreover, this ELISA is rapid (75 min) and easy to use and allows the simultaneous analysis of multiple samples. Preliminary studies indicate that BP can be directly analyzed in white wine samples without any previous extraction or preconcentration steps. Unfortunately, red wine matrices cause interference in the immunoassay. Nevertheless, simple techniques such as immunoaffinity chromatography that are compatible with the immunoassay could be developed to eliminate this undesirable effect.

ABBREVIATIONS USED

AC, aldehyde conjugation; AE, active ester; AD, aminodextran; As, antisera; BP, bromopropylate; BSA, bovine serum albumin; CDI, 1,1′-carbonyldiimidazole; CONA, conalbumin; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; HC, halogen conjugation; HCH, keyhole limpet hemocyanin from horseshoe crab; MA, mixed anhydride; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometer; MRL, maximum residue level; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; TLC, thin-layer chromatography

Supporting Information Available: Spectroscopic and spectrometric data of the compounds and haptens and general ELISA protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Vial, J.; Ostrovsky, V.; Monterrat, G. Un nouveau acaricide: le dibromobenzilate d'isopropyl. *Phytopharm* **1971**, *20*, 31.
(2) Corta, E.; Bakkali, A.; Barranco, A.; Berrueta, L. A.; Gallo, B.; Vicente, F.; Bogdanov, S. Study of the degradation products of bromopropylate, chlordimeform, coumaphos, cymiazole, flumethrin and Tau-fluvalinate in aqueous media. *Talanta* **2000**, *52* (2), 169–180.
(3) Bathe, R.; Sachsse, K. *Acute Dermal LD50 in the Rat*; 790969; GS 19'851 tech: Oct 9, 1979; submitted to WHO by Ciba-Geigy Ltd., Basle, Switzerland.
(4) Kuhn, J. O. *Acute Dermal Toxicity Study in Rabbits*; 6032-89; Stillmeadow, Inc., Houston, TX, April 11, 1989; submitted to WHO by Ciba-Geigy Ltd., Basle, Switzerland.
(5) *COMMUNITIES, C. O. T. E., Monitoring of Pesticide Residues in Products of Plant Origin in the European Union, Norway, Iceland, Denmark, Italy and Liechtenstein*; **2005**.
(6) Martel, A.-C.; Zeggane, S. Determination of acaricides in honey by high-performance liquid chromatography with photodiode array detection. *J.Chromatogr., A* **2002**, *954* (1−2), 173–180.
(7) Lee, N. A.; Kennedy, I. R. Environmental monitoring of pesticides by immunoanalytical techniques: validation, current status, and future perspectives. *J. AOAC Int.* **2001**, *84* (5), 1393–1406.
(8) Liapis, K. S.; Aplada-Sarlis, P.; Kyriakidis, N. V. Rapid multiresidue method for the determination of azinphos methyl, bromopropylate, chlorpyrifos, dimethoate, parathion methyl and phosalone in apricots and peaches by using negative chemical ionization ion trap technology. *J. Chromatogr., A* **2003**, *996* (1−2), 181–187.
(9) Jimenez, J. J.; Bernal, J. L.; del Nozal, M. J.; Alonso, C. Liquid−liquid extraction followed by solid-phase extraction for the determination of lipophilic pesticides in beeswax by gas chromatography−electron-capture detection and matrix-matched calibration. *J.Chromatogr., A* **2004**, *1048* (1), 89–97.
(10) Rial-Otero, R.; Gaspar, E. M.; Moura, I.; Capelo, J. L. Gas chromatography mass spectrometry determination of acaricides from honey after a new fast ultrasonic-based solid phase microextraction sample treatment. *Talanta* **2007**, *71* (5), 1906–1914.
(11) Korta, E.; Bakkali, A.; Berrueta, L. A.; Gallo, B.; Vicente, F.; Bogdanov, S. Determination of amitraz and other acaricide residues in beeswax. *Anal. Chim. Acta* **2003**, *475* (1−2), 97–103.

(12) Oubiña, A.; Ballesteros, B.; Bou, P.; Galve, R.; Gascón, J.; Iglesias, F.; Sanvicens, N.; Marco, M.-P. Inmunoassays for environmental analysis. Sample handling and trace analysis of pollutants. In *Techniques, Applications and Quality Assurance*; Barceló, D., Ed.; Elsevier Sciences: Amsterdam, The Netherlands, 2000; pp 289−331.

(13) Van Emon, J. M. Immunochemical applications in environmental science. *J AOAC Int.* **2001**, *84* (1), 125–133.

(14) Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M. P. Enzyme-linked immunosorbent assay for the determination of the anti-fouling agent Irgarol 1051. *Anal. Chim. Acta* **1997**, *347*, 139–147.

(15) Marco, M.-P.; Hammock, B. D.; Kurth, M. J. Hapten design and development of an ELISA (enzyme-linked immunosorbent assay) for the detection of the mercapturic acid conjugates of naphthalene. *J. Org. Chem.* **1993**, *58*, 7548–7556.

(16) Gascón, J.; Oubiña, A.; Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M.-P.; González-Martinez, M.-A.; Morais, S.; Puchades, R.; Maquiera, A. Development of an highly sensitive enzymelinked immunosorbent assay for Atrazine. Performance evaluation by flow-injection immunoassay. *Anal. Chim. Acta* **1997**, *347*, 149–162.

(17) Somayaji, V. V.; Sykes, T. R.; Naicker, S. S.; Noujaim, A. A. Optimization of protein conjugation using an aldehyde derivatized bifunctional chelating agent. *Appl. Radiat. Isot.* **1996**, *47* (1), 71–77.

(18) Estevez, M.-C.; Kreuzer, M.; Sanchez-Baeza, F.; Marco, M.-P. Analysis of nonylphenol: advances and improvements in the immunochemical determination using antibodies raised against the technical mixture and hydrophilic immunoreagents. *Environ. Sci. Technol.* **2006**, *40*, 559–568.

(19) Tsang, V. C. W.; Greene, R. M.; Pilcher, J. B. Optimization of the covalent conjugating procedure (NAIO4) of horseradish peroxidase to antibodies for use in enzyme-linked immunosorbent assay. *J. Immunoassay* **1995**, *16*, 395–418.

(20) Traut, R. R. Methyl 4-mercaptobutyrimidate as cleavable cross-linking and its application to the *Escherichia coli* 305-ribosome. *Biochemie* **1973**, *12* (17), 3266–3273.

(21) Naya, A.; Sagara, Y.; Ohwaki, K.; Saeki, T.; Ichikawa, D.; Iwasawa, Y.; Noguchi, K.; Ohtake, N. Design, synthesis and discovery of a novel CCR1 antagonist. *J. Med. Chem.* **2001**, *44*, 1429–1435.

(22) Xiao, H.; Clarke, J. R.; Marquardt, R. R.; Frohlich, A. A. Improved methods for conjugating selected mycotoxins to carrier proteins and dextran for immunoassays. *J. Agric. Food Chem.* **1995**, *43*, 2092–2097.

(23) Sanvicens, N.; Varela, B.; Marco, M.-P. Immunochemical determination of 2,4,6-trichloroanisole as the responsible agent for the musty odor in foods. 2. Immunoassay evaluation. *J. Agric. Food Chem.* **2003**, *51*, 3932–3939.

(24) Manclús, J. J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for 3,5,6-trichloro-2-pyridinol. 2. Assay optimization and application to environmental water samples. *J. Agric. Food Chem.* **1996**, *44*, 3710–3716.

(25) Hill, A. S.; Skerritt, J. H.; Bushway, R. J.; Perkin, L. B.; Larkin, K.; Thomas, M.; Korth, W.; Bowmer, K. H. Development and application of laboratory and field immunoassays for chlorpyrifos in water and soil matrices. *J. Agric. Food Chem.* **1994**, *42*, 2051–2058.

(26) Jung, F.; Meyer, H. D.; Hamm, R. T. Development of a sensitive enzyme-linked immunosorbent assay for the fungicide fenpropimorph. *J. Agric. Food Chem.* **1989**, *37*, 1183–1187.

(27) Ballesteros, B.; Barceló, B.; Sanchez-Baeza, F.; Camps, F.; Marco, M.-P. Influence of the hapten design on the development of a high sensitive competitive ELISA for the determination of the antifouling agent Irgarol 1051 at trace levels. *Anal. Chem.* **1998**, *70*, 4004–4014.

(28) Stanker, L. H.; Bigbee, C.; Van Emon, J.; Watkins, B.; Jense, R. H.; Morris, C. An immunoassay for pyrethroids: detection of permethrin in meat. *J. Agric. Food Chem.* **1989**, *37*, 834–839.

(29) Lee, N. J.; Skerritt, J. H.; McAdam, D. P. Hapten synthesis and development of ELISAs for detection of endosulfan in water and soil. *J. Agric. Food Chem.* **1995**, *43*, 1730–1739.

(30) Vyjayanthi, V.; Capoor, A. K.; Sashindhar, R. B. Binding characteristics of bovine serum albumine aflatoxin B1 to polystyrene microtiter plates: importance of hapten to carrier protein molar ratio. *Indian J. Exp. Biol.* **1995**, *33*, 329–332.

(31) Fasciglione, G. F.; Marini, S.; Bannister, J. V.; Giardina, B. Hapten carrier interactions and their role in the production of monoclonal antibodies against hydrophobic haptens. *Hybridoma* **1996**, *15*, 1–9.

Received for review September 11, 2008. Revised manuscript received November 20, 2008. Accepted November 24, 2008. This work has been supported by the European Community Information Society Technology (IST-2003-508774). We acknowledge the support of the Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Barcelona, Spain.

JF802821N