Development of Monoclonal Antibody-Based Immunoassays to the *N*-Methylcarbamate Pesticide Carbofuran

Antonio Abad, María José Moreno, and Angel Montoya*

Laboratorio Integrado de Bioingeniería, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain

To produce monoclonal antibodies (MAbs) to the pesticide carbofuran, three compounds with carboxylic spacer arms of different lengths introduced at the carbamate group of the analyte structure were synthesized, conjugated to proteins, and used as immunizing haptens in mice. MAbs were subsequently characterized for affinity and specificity in the conjugate-coated format and in the antibody-coated format using newly synthesized compounds as heterologous assay haptens. Depending on the immunoreagent combination and assay format, competitive assays with I_{50} values in the 1.2−10.2 nM (0.27−2.27 ng/mL) range were obtained. LIB-BFNB67 MAb in combination with the hapten BFNH, coupled either to horseradish peroxidase or to ovalbumin, was used to develop a direct and an indirect enzyme-linked immunosorbent assay, respectively. Optimized immunoassays displayed very similar analytical characteristics, with an I_{50} value around 0.7 ng/mL and a limit of detection around 0.08 ng/mL. Both immunoassays were able to tolerate the presence of methanol up to a 15% concentration. Compounds very similar in structure to carbofuran (benfuracarb, furathiocarb, bendiocarb, and carbofuran-hydroxy) exhibited cross-reactivity values in the 18−37% range, but major *N*-methylcarbamate pesticides were not recognized by the MAb. These immunoassays should reasonably allow the rapid, low-cost, and sensitive determination of carbofuran in food, in soils, and in the environment at levels of regulatory and practical importance.

Keywords: *ELISA; immunoassay; insecticide; monoclonal antibodies; hapten design; hapten heterology; ELISA format; carbamate*

INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), which is most commonly sold under the trade name Furadan, is a systemic *N*-methylcarbamate pesticide with predominantly contact and stomach action. It is mainly used as a soil-applied chemical to control soil-dwelling and foliar-feeding insects and nematodes on a variety of agricultural crops, including maize, corn, rice, potatoes, alfalfa, and grapes (Tomlin, 1994). Carbofuran undergoes rapid chemical hydrolysis under alkaline conditions to give the corresponding phenolic derivative, whereas the main carbofuran metabolites in animals, plants, and soils are 3-hydroxycarbofuran and 3-ketocarbofuran. Carbofuran is a potent cholinesterase inhibitor [IC_{50} in rats is $(1.2-3.3) \times 10^{-8}$ M], so it is highly toxic to humans and wildlife through the oral and inhalation routes of exposure (acute oral LD_{50} in rats is 8 mg kg^{-1}) (Gupta, 1994). In fact, carbofuran has been involved in recent years in numerous cases of bird poisoning, which prompted the U.S. Environmental Protection Agency and Agriculture Canada to review all registered uses of granular carbofuran in their respective countries (James, 1995; Augspurger et al., 1996). As a result of its widespread use worldwide, with ∼300000 pounds of active ingredient applied per year in the period 1992−1995 in only California (*Farm Chemicals Handbook*, 1995; CDPR, 1995), residues of carbofuran may be present in air, food, soil, and surface water and groundwater.

Carbofuran, like most *N*-methylcarbamates, is thermally unstable and decomposes to the phenol under the usual gas chromatography conditions (Liška and Slobodník, 1996). Although this problem can be overcome by varying the operating conditions (Mueller and Stan, 1990) or by derivatization of the pesticide (Ballesteros et al., 1993; Färber and Schöler, 1993; Bakowski et al., 1994), the preferred technique for the sensitive and selective determination of *N*-methylcarbamates is currently high-performance liquid chromatography (HPLC) with postcolumn derivatization and fluorescence detection (EPA method 531.1) (McGarvey, 1993; Yang et al., 1996). However, this method presents some limitations derived from the fact that laborious cleanup, concentration, and derivatization steps are needed to obtain the desired sensitivity. Thus, the method requires sophisticated equipment that is not available in most analytical laboratories, and it is not very well suited for the analysis of a large number of samples. In fact, most pesticide regulatory programs analyze for *N*-methylcarbamates only a part of the total number of food samples analyzed for other active ingredients, such as organophosphorus pesticides (Gunderson, 1995).

Immunoassays are analytical methods based on the interaction of an analyte with an antibody that recognizes it with high affinity and specificity. They are simple, cost-effective, and field-portable, do not require sophisticated instrumentation, and are able to analyze a large number of samples simultaneously. All of these features, along with the large number of pesticides for

* Author to whom correspondence should be addressed (telephone 34-96-3877093; fax 34-96-3877093; e-mail amontoya@eln.upv.es).

10.1021/jf981184s CCC: $18.00
Published on Web 05/08/1999

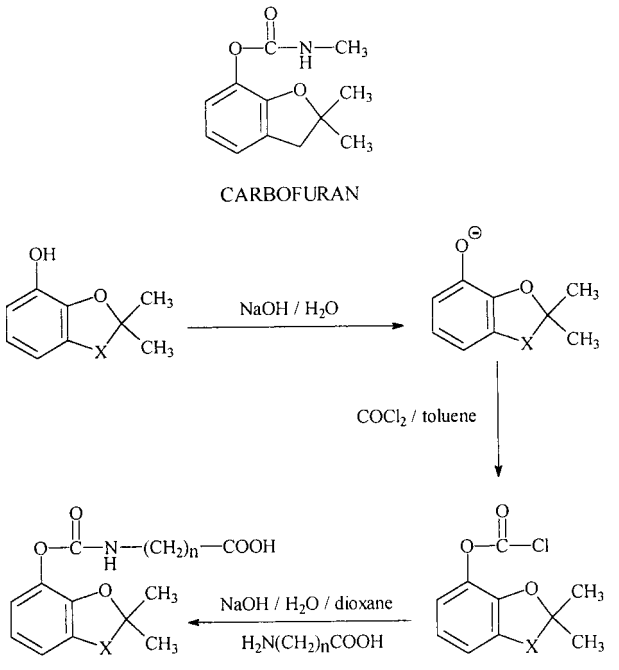
which antibodies have been produced over the past few years, have promoted the acceptance of immunochemical techniques among analytical chemists as alternative and/or complementary methods for the analysis of agrochemicals (Hammock and Gee, 1995; Meulenberg et al., 1995; Dankwardt and Hock, 1997). This is particularly true for pesticides that are difficult and/or costly to determine due to their physicochemical characteristics or for large monitoring programs. In these situations the use of immunoassays as a screening technique could provide a great saving of resources, effort, and toxic solvents. Therefore, although immunoassays are not a panacea, there is nowadays little doubt that they constitute a sensitive, selective, and inexpensive method for the rapid analysis of pesticide residues in a variety of matrices.

Commercial enzyme-linked immunosorbent assay (ELISA) kits for detecting residues of carbofuran are available from Strategic Diagnostics Inc. (Newark, DE). These immunoassays use polyclonal antibodies as the specific immunoreagent and have been applied to the determination of carbofuran in water and soil (Jourdan et al., 1995) and in meat and liver (Lehotay and Argauer, 1993; Nam and King, 1994). Moreover, immunoaffinity columns to purify the pesticide from complex matrices such as potatoes have been prepared from a commercial rabbit polyclonal antiserum (Rule et al., 1994). Nevertheless, the amount of antiserum produced by even a group of animals is limited, and its characteristics vary from animal to animal and also between different bleedings from the same animal. Monoclonal antibodies (MAbs) offer an advantage over polyclonal antisera that an essentially unlimited supply of a single and homogeneous type of antibody is guaranteed. The availability of large quantities of such standardized immunoreagent may facilitate acceptance of immunochemical methods in the analytical laboratory by ensuring a long-term supply of kits with a defined performance, which is a prerequisite for method evaluation, interlaboratory studies, and worldwide marketing (Kaufman and Clower, 1991; Sherry, 1992; Van Emon and López-Ávila, 1992; Meulenberg et al., 1995). The aim of this work was to obtain high-affinity MAbs to carbofuran and to develop enzyme immunoassays as an alternative to liquid chromatographic techniques for the screening of large numbers of agricultural and environmental samples.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. Pesticide and metabolite standards were from Riedel-de Haën (Seelze, Germany) and Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were prepared in dry *N,N*-dimethylformamide (DMF) and stored at 4 °C. Starting products for the synthesis of haptens and hapten−protein coupling reagents were obtained from Fluka-Aldrich Química (Madrid, Spain). 2,2-Dimethyl-1,3-benzodioxol-4-ol was kindly provided by AgrEvo (Cambridge, England).

Ovalbumin (OVA), Freund's adjuvants, and *o*-phenylenediamine (OPD) were obtained from Sigma Química (Madrid, Spain). Bovine serum albumin fraction V (BSA), enzyme immunoassay grade horseradish peroxidase (HRP), hybridoma fusion and cloning supplement (HFCS), and polyethylene glycol (PEG) 1500 were purchased from Boehringer Mannheim (Barcelona, Spain). Peroxidase-labeled rabbit anti-mouse immunoglobulins were obtained from Dako (Glostrup, Denmark). Culture media (high-glucose Dulbecco's Modified Eagle's medium with GLUTAMAX I and sodium pyruvate, DMEM), fetal calf serum (Myoclone Super Plus), and supplements were from GibcoBRL (Paisley, Scotland). Culture plasticware was from Bibby Sterilin Ltd. (Stone, U.K.). P3-X63-Ag8.653 mouse plasmacytoma line was from American Tissue Type Culture Collection (Rockville, MD).



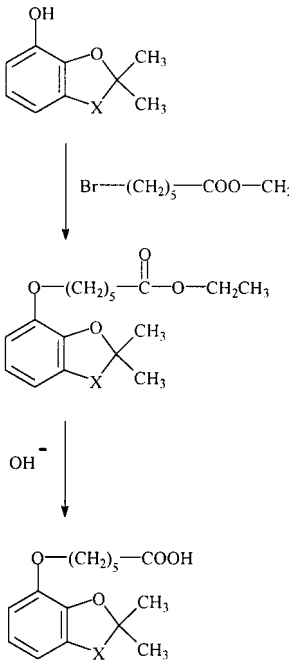
hapten	n	X
BFNP	2	CH_2
BFNB	3	CH_2
BFNH	5	CH_2
BDNH	5	O

Figure 1. Reaction scheme of the synthesis of BFNP, BFNB, BFNH, and BDNH haptens.

Polystyrene culture plates (high-binding plates, catalog no. 3590) were from Costar (Cambridge, MA). ELISA plates were washed with a 96PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria), and absorbances were read in dual-wavelength mode (490−650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 300 spectrometer (Sunnyvale, CA), operating at 300 MHz for ^{1}H. Chemical shifts are reported relative to tetramethylsilane. Ultraviolet−visible (UV−vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan).

Hapten Synthesis. Carbofuran haptens used in this work were prepared by introduction of alkyl chain spacers, ending in a carboxylic acid, at the hydroxyl group of the phenolic precursor, either after being reacted with phosgene to form the carbamate group (Figure 1) or directly by O-alkylation (Figure 2).

3-[[(2,3-Dihydro-2,2-dimethyl-7-benzofuranyloxy)carbonyl]-amino]propanoic Acid (BFNP, Figure 1). 2,3-Dihydro-2,2-dimethyl-7-benzofuranol (4.54 mL, 30.4 mmol) was added to 26 mL of 2.5 M sodium hydroxide. Thereafter, a slight excess of phosgene (25 mL of a 20% phosgene solution in toluene, 48.3 mmol) was slowly added, and the reaction was incubated at room temperature for 4 h with magnetic stirring. (WARNING: phosgene is a highly toxic gas. Work in a well ventilated fume hood and handle carefully.) After water and methylene chloride were added, the organic phase was dried over anhydrous sodium sulfate and evaporated to dryness at reduced pressure. On the basis of its behavior on gas chromatography (GC), the 88.9% of the resulting brown solid was assumed to be 2,3-dihydro-2,2-dimethyl-7-benzofuranyl chloroformate (5.14 g) and subsequently used without further purification. Ami-



hapten	X
BFOH	CH_2
BDOH	O

Figure 2. Reaction scheme of the synthesis of BFOH and BDOH haptens.

nopropanoic acid (2.0 g, 22.6 mmol) was dissolved in 4 mL of 4 M sodium hydroxide, and the solution was cooled at 4 °C. The crude chloroformate (2.57 g, 11.3 mmol) was dissolved in 4 mL of cold 1,4-dioxane, and this solution, along with 6 mL of cold 4 M sodium hydroxide, was added to the amino acid solution in five equal portions, with at least 5 min being allowed between additions. The reaction mixture was stirred in an ice bath for 1.5 h. After acidification to pH 4.0 with concentrated hydrochloric acid, the carboxylic derivative, which separated as an oil, was extracted with ethyl acetate (three 35 mL portions). The ethyl acetate phase was washed several times with diluted hydrochloric acid and extracted with 1 M bicarbonate solution (three 50 mL portions). After the solution had been cooled in an ice bath, it was acidified again with concentrated hydrochloric acid. The precipitated product was collected, washed with water, and dried to yield 1.4 g of crude BFNP. It was then crystallized from hexane/ethyl acetate (70:30) to yield 313.6 mg of pure hapten: ^{1}H NMR (acetone-d_6) δ 1.43 (s, 6 H, 2 CH_3), 2.62 (t, 2 H, CH_2COOH), 3.05 (s, 2 H, CH_2), 3.45 (q, 2 H, CH_2NH), 6.72−6.99 (m, 3 H, aromatic).

4-[[(2,3-Dihydro-2,2-dimethyl-7-benzofuranyloxy)carbonyl]-amino]butanoic Acid (BFNB, Figure 1). This compound was synthesized essentially as described for BFNP, except that 4-aminobutanoic acid was used as spacer arm instead of 3-aminopropanoic acid. After crystallization from hexane/ethyl acetate (70:30), 1.59 g of the pure hapten was obtained: ^{1}H NMR (acetone-d_6) δ 1.43 (s, 6 H, 2 CH_3), 1.86 (m, 2 H, CH_2), 2.42 (t, 2 H, CH_2COOH), 3.05 (s, 2 H, CH_2), 3.26 (q, 2 H, CH_2-NH), 6.72−6.99 (m, 3 H, aromatic).

6-[[(2,3-Dihydro-2,2-dimethyl-7-benzofuranyloxy)carbonyl]-amino]hexanoic Acid (BFNH, Figure 1). This compound was synthesized essentially as described for BFNP, except that 6-aminohexanoic acid was used as spacer arm instead of 3-aminopropanoic acid: ^{1}H NMR (acetone-d_6) δ 1.43 (s, 6 H, 2 CH_3), 1.41−1.68 (m, 6 H, $CH_2CH_2CH_2$), 2.32 (t, 2 H, CH_2-COOH), 3.05 (s, 2 H, CH_2), 3.20 (q, 2 H, CH_2NH), 6.72−6.99 (m, 3 H, aromatic).

6-[[(2,2-Dimethyl-1,3-benzodioxol-4-oxy)carbonyl]amino]hexanoic Acid (BDNH, Figure 1). This compound was synthesized essentially as described for BFNP, except that 2,2-dimethyl-1,3-benzodioxol-4-ol was used as the starting phenol and 6-aminohexanoic acid was used as spacer arm instead of 3-aminopropanoic acid. After crystallization from hexane/ethyl acetate (40:60), 590 mg of the pure hapten was obtained: ^{1}H NMR (acetone-d_6) δ 1.44 (m, 2 H, CH_2), 1.61 (m, 4 H, 2 CH_2), 1.63 (s, 6 H, 2 CH_3), 2.30 (t, 2 H, CH_2COOH), 3.20 (m, 2 H, CH_2NH), 6.58−6.76 (m, 3 H, aromatic).

6-(2,3-Dihydro-2,2-dimethyl-7-benzofuranyloxy)hexanoic Acid (BFOH, Figure 2). To 60 mL of dry acetone were added stoichiometric amounts (20 mmol) of 2,3-dihydro-2,2-dimethyl-7-benzofuranol, potassium carbonate, and ethyl-6-bromohexanoate. After reflux for 12 h, the mixture was filtered and the solvent was removed under reduced pressure. The residue was dissolved in 50 mL of ethyl acetate, washed with water (2 × 50 mL), 1 M NaOH (4 × 50 mL), and 4 M NaCl (2 × 50 mL), and finally dried over Na_2SO_4. GC analysis confirmed that 87% of the crude product was ethyl 6-(2,3-dihydro-2,2-dimethyl-7-benzofuranyloxy)hexanoate. After evaporation of the solvent, 50 mL of 1 M NaOH was added to the residue (4.32 g), and the solution was stirred while heated under reflux for 1.5 h. The solution was then acidified with concentrated hydrochloric acid and extracted with ethyl acetate (2 × 50 mL). The organic phase was extracted with 1 M NaOH, and the aqueous solution was acidified with concentrated hydrochloric acid to afford a white solid (1.81 g), which was subsequently crystallized from hexane to obtain 1.19 g of the pure hapten: ^{1}H NMR (acetone-d_6) δ 1.46 (s, 6 H, 2 CH_3), 1.54−1.79 (m, 6 H, $CH_2CH_2CH_2$), 2.36 (t, 2 H, CH_2COOH), 3.02 (s, 3 H, CH_2), 4.04 (t, 2 H, OCH_2), 6.72−6.81 (m, 3 H, aromatic).

6-(2,2-Dimethyl-1,3-benzodioxol-4-oxy)hexanoic Acid (BDOH, Figure 2). This compound was synthesized essentially as described for BFOH, except that 2,2-dimethyl-1,3-benzodioxol-4-ol was used as the starting phenol. After crystallization from hexane/ethyl acetate (30:70), 130 mg of the pure hapten was obtained: ^{1}H NMR (acetone-d_6) δ 1.45−1.55 (m, 2 H, CH_2), 1.60−1.80 (m, 4 H, 2 CH_2), 1.63 (s, 6 H, 2 CH_3), 2.32 (t, 2 H, CH_2−COOH), 4.06 (t, 2 H, OCH_2), 6.39−6.72 (m, 3 H, aromatic).

Preparation of Immunizing Conjugates. Haptens BFNP, BFNB, and BFNH were covalently attached to BSA using the modified active ester method (Langone and Van Vunakis, 1982). Twenty-five micromoles of the hapten was incubated overnight at room temperature with stoichiometric amounts of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in 0.5 mL of DMF. After centrifugation, 400 μL of the clear supernatant containing the active ester was slowly added to 2 mL of a 15 mg/mL BSA solution in 50 mM carbonate buffer, pH 9.6. The mixture was allowed to react at room temperature for 4 h with stirring, and finally the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer, pH 7.4, as eluant. Conjugate formation was confirmed spectrophotometrically. UV−vis spectra showed qualitative differences between the carrier protein and conjugates in the region of maximum absorbance of haptens. The hapten to protein molar ratio of conjugates was then estimated from the spectral data of the hapten, the protein, and the corresponding conjugate. By assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, apparent molar ratios were estimated as 18, 13, and 14 for haptens BFNP, BFNB, and BFNH, respectively.

Preparation of Coating Conjugates. All of the haptens were covalently attached to OVA using the mixed-anhydride method (Rajkowski et al., 1977). Eighteen micromoles of the hapten was allowed to react at room temperature for 1 h with stoichiometric amounts of tri-*n*-butylamine and isobutyl chloroformate in 200 μL of DMF. One hundred microliters of the resulting activated hapten was added to 30 mg of OVA in 2 mL of 50 mM carbonate buffer, pH 9.6. The coupling reaction was incubated at room temperature for 2−3 h with stirring, and the conjugates obtained were purified as described for the immunogens. The extent of coupling of each hapten to OVA was determined by UV spectrophotometry. By assuming

additive absorbance values, hapten to protein molar ratios were evaluated as 4, 5, 5, 4, 6, and 3 for haptens BFNP, BFNB, BFNH, BFOH, BDNH, and BDOH, respectively.

Preparation of Enzyme Conjugates. The mixed-anhydride method was also used for covalent coupling of haptens to HRP. Typically, 2.9 μL of tributylamine and 1.6 μL of isobutyl chloroformate were added to 13.3 μmol of the hapten in 200 μL of DMF. The mixture was stirred for 1 h at room temperature. After the addition of 1.8 mL of DMF, 100 μL of this diluted solution of activated hapten was incubated for 2 h at room temperature with 1 mL of a 2.2 mg/mL solution of HRP in 50 mM carbonate buffer, pH 9.6. HRP−hapten conjugates were purified as described for the immunogens. HRP conjugate concentrations and molar ratios were estimated spectrophotometrically. With the same assumptions as before, the estimated molar ratios were 1.5, 1.2, 2.0, 1.9, 2.1, and 4.2 for haptens BFNP, BFNB, BFNH, BFOH, BDNH, and BDOH, respectively.

Production of MAbs to Carbofuran. *Immunization.* BALB/c female mice (8−10 weeks old) were immunized with BSA-BFNP, -BFNB, and -BFNH conjugates. First dose consisted of 30 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. Two subsequent injections were given at three-week intervals emulsified in incomplete Freund's adjuvant. After a resting period of at least three weeks from the last injection in adjuvant, mice received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion.

Cell Fusion. P3-X63/Ag 8.653 murine myeloma cells (ATCC, Rockville, MD) were cultured in DMEM supplemented with 1 mM nonessential amino acids, 25 μg/mL gentamicin, and 15% fetal bovine serum (referred to as s-DMEM). Cell fusion procedures were carried out essentially as described by Nowinski et al. (1979). Mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximate density of 2×10^5 cells/well in 100 μL of s-DMEM. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) containing 2% HFCS (v/v) was added to each well. Half the medium of the wells was replaced by fresh HAT medium on day 4 post-fusion and by HT medium (HAT medium without aminopterine) on day 8 post-fusion.

Hybridoma Selection and Cloning. Eight to 11 days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized carbofuran. The screening consisted of the simultaneous performance of noncompetitive and competitive indirect ELISAs, to test the ability of antibodies to bind the OVA conjugate of the immunizing hapten and to recognize carbofuran, respectively. For each culture supernatant, the signal obtained in noncompetitive conditions was compared with the competitive one, and the ratio of both absorbances was used as the criterion for selecting high-affinity antibody-secreting clones. Selected hybridomas were cloned by limiting dilution using HT medium supplemented with 2% HFCS (v/v) as a cell growth promoter instead of the classical feeder layer of thymocytes and peritoneal macrophages. Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. Antibodies were purified directly from late stationary phase culture supernatants by saline precipitation with saturated ammonium sulfate followed by affinity chromatography on protein G Sepharose 4 Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden). Most culture supernatants were able to provide enough MAb (5−15 mg/100 mL) for characterization studies and further work. Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

ELISAs. Flat-bottom polystyrene ELISA plates were coated overnight with conjugate or antibody solutions in 50 mM carbonate buffer, pH 9.6. Standards were prepared in PBS (8.03 mM Na_2HPO_4, 1.97 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, pH 7.4) by serial dilutions from a stock solution in DMF, using borosilicate glass tubes. A volume of 100 μL per well was used throughout all assay steps, and all incubations were carried out at room temperature. After each incubation, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Two formats differing in the assay component immobilized into the ELISA plates, namely, conjugate-coated (CC) format and antibody-coated (AC) format, were used to determine antibody sensitivity and specificity to carbofuran. Furthermore, the CC format was also used for the screening of culture supernatants. For competition assays, optimum concentrations of antibodies, hapten conjugates, or enzyme tracers were previously determined. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation (Raab, 1983), using Molecular Devices (Sunnyvale, CA) and Sigmaplot (Jandel Scientific, Germany) software packages.

CC Format. Plates were coated with OVA−hapten conjugates at the concentration previously determined as optimal by checkerboard titration (typically 0.01−0.1 μg/mL). Then, different antibody concentrations in PBS containing 0.1% BSA (PBSB) were added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins diluted 1:2000 in PBST (PBS containing 0.05% Tween 20). Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate, pH 5.4). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and the absorbance at 490 nm was read and recorded. For competitive assays, the procedure was the same except that after coating a competition step was introduced by adding 50 μL of carbofuran standards followed by 50 μL of the appropriate concentration of antibody.

AC Format. In this format, plates were coated with antibodies at 1 μg/mL. Next, the competition was established for 1 h between carbofuran standards and the selected concentrations of enzyme tracers. Peroxidase activity was measured as above.

RESULTS AND DISCUSSION

Hapten Design. The synthesis of the immunizing haptens, namely BFNP, BFNB, and BFNH, essentially consisted of the introduction of spacer arms of different lengths through the carbamate group characteristic of carbofuran (Figure 1). In this way, that is, by elongation of an aliphatic chain already present in the analyte, both the structure and the electronic distribution of the carbofuran molecule are hardly modified, as demonstrated by the high-affinity MAbs previously obtained following this chemical approach for carbaryl (Abad and Montoya, 1994), methiocarb (Abad et al.., 1998), and bendiocarb (unpublished results).

Apart from the immunizing haptens required for antibody production, several heterologous haptens were also synthesized to try to improve the sensitivity of immunoassays in further steps of ELISA development. One hapten (BDNH) differs from the immunizing haptens only in the nucleus structure, because it was obtained following the same chemical approach but starting with 2,2-dimethyl-1,3-benzodioxol-4-ol, the precursor in the industrial synthesis of the pesticide bendiocarb, instead of 2,3-dihydro-2,2-dimethyl-7-benzofuranol (Figure 1). BFOH and BDOH haptens were obtained by a different chemical approach consisting of direct O-alkylation with ethylbromohexanoic acid of 2,3-dihydro-2,2-dimethyl-7-benzofuranol and 2,2-dimethyl-1,3-benzodioxol-4-ol, respectively (Figure 2).

MAb Production. In the screening of fusion supernatants only wells showing a signal inhibition in the presence of 1 μM carbofuran >80% with respect to that obtained in the absence of analyte were considered as derived from high-affinity antibody-secreting clones.

According to this criterion, initial fusion experiments led only to the obtention of antibodies with low to moderate affinity for carbofuran, so they were not selected for further cloning and expansion. Because excellent MAbs had been previously obtained for carbaryl and methiocarb using the same experimental strategy, the possibility of an inappropriate hapten design was considered doubtful. A very high affinity of these antibodies to the immobilized conjugate was instead thought to be the cause of these unsuccessful attempts to obtain antibodies with the desired affinity to carbofuran. We reasoned that an increase in the apparent affinity of these antibodies to carbofuran could be obtained by using a lower coating concentration in the assay of fusion supernatants. Accordingly, in the screening of later cell fusions, supernatants were assayed into ELISA plates coated with two conjugate concentrations (0.1 and 1.0 μg/mL, the usual coating concentration). Hybridomas giving a strong positive response in the absence of the analyte ($A_{490} \geq 3.0$) were reassayed by diluting the culture supernatant to ensure that antibodies were not in excess. Although most of the hybridomas did not behave competitively under these new screening conditions, a low but significant number of them were able to recognize carbofuran with high affinity when assayed in ELISA wells coated with 0.1 μg/mL of conjugate, whereas the inhibitory effect was negligible for the same hybridomas when assayed in plates coated with 1.0 μg/mL.

From three fusions with each one of the three immunizing haptens assayed using this approach, 10 hybridomas secreting antibodies with high affinity to carbofuran were obtained: 4 hybridomas came from the BFNP hapten, and 6 were derived from the BFNB hapten. The fact that no hybridomas were obtained from the BFNH hapten should not be necessarily attributed to the spacer arm length but rather to the high intrinsic variability of the cell fusion process. In fact, the use of immunizing haptens with long spacer arms has previously resulted in excellent MAbs for carbaryl and methiocarb.

Characterization of the MAbs. From preliminary characterization of culture supernatants, the four MAbs with the highest affinity to carbofuran were characterized in the CC and AC formats, using both homologous and heterologous haptens. One of these antibodies (LIB-BFNP21) was derived from mice immunized with the hapten BFNP, whereas the other three antibodies (LIB-BFNB52, LIB-BFNB62, and LIB-BFNB67) were obtained using BFNB as the immunizing hapten.

Evaluation of the CC Format. Bidimensional titration experiments were performed to determine optimal immunoreagent concentrations. As shown in Figure 3 for a representative experiment carried out using two antibody concentrations, coating concentrations >0.05 μg/mL did not result in a higher maximum signal, whereas the I_{50} of the assay rose sharply as the coating concentration increased. Therefore, very low coating concentrations (0.01 μg/mL) were required to obtain maximum sensitivity, as expected from the information provided by fusion supernatants.

Once subsaturating conditions were determined for each pair of immunoreagents, competitive ELISAs were performed with the four MAbs in combination with the collection of hapten−protein conjugates previously prepared. I_{50} values for all of these combinations are shown in Table 1. Attending to the values in homologous

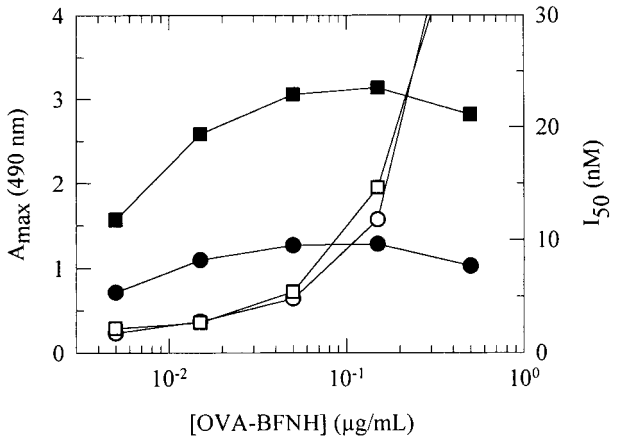


Figure 3. Effect of the coating concentration of OVA-BFNH conjugate on the A_{max} (solid symbols) and I_{50} (open symbols) of competitive assays using LIB-BFNB67 MAb at 0.01 μg/mL (circles) and at 0.03 μg/mL (squares).

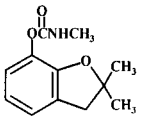
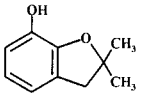
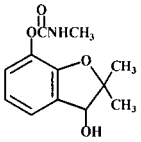
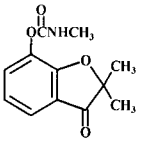
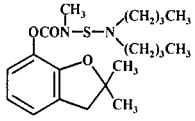
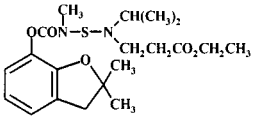
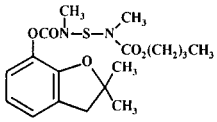
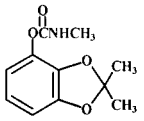
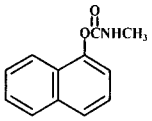
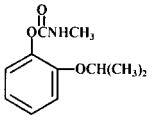
Table 1. Carbofuran I_{50} Values (Nanomolar) of the Best MAbs Obtained, Determined in the CC and AC ELISA Formats

	LIB-BFNP21	LIB-BFNB52	LIB-BFNB62	LIB-BFNB67
Conjugate-Coated ELISA Format				
coating conjugate				
OVA-BFNP	1.75	5.50	1.39	1.19
OVA-BFNB	1.98	10.20	1.56	1.39
OVA-BFNH	1.90	5.77	1.41	1.68
OVA-BDNH	1.65	2.76	1.52	1.59
OVA-BFOH	2.02	3.19	2.08	1.48
OVA-BDOH	1.95	4.35	1.59	1.15
Antibody-Coated ELISA Format				
enzyme tracer				
HRP-BFNH	2.37	5.76	2.43	1.56

combinations, the less sensitive antibody was LIB-BFNB52 (I_{50} = 10.2 nM), whereas the other three antibodies displayed very similar affinity values for carbofuran (I_{50} = 1.4−1.8 nM). Nevertheless, it is interesting to point out that LIB-BFNB52 was the only MAb that experienced a significant improvement in sensitivity by the use of heterologous haptens, so very similar I_{50} values were obtained with the four antibodies by selecting the proper conjugate.

Evaluation of the AC Format. As in the CC format, bidimensional titration experiments were performed prior to competitive assays to determine subsaturating concentrations of the coating antibody. For all of the MAbs, this concentration was 1 μg/mL. The most remarkable aspect of this experiment was that only one of the six synthesized enzyme tracers was recognized at a sufficient extent as to develop an immunoassay in the AC format. These results agree with previous authors' experience in this field, which indicates that the number of the HRP−hapten conjugates recognized by immobilized antibodies is always lower that the number of OVA−hapten conjugates recognized in the CC format by the same antibodies (Manclús and Montoya, 1996a; Manclús et al., 1996; Abad et al., 1997a,b, 1998). Moreover, the only useful tracer was that based on one hapten with a large spacer arm, which also agrees with previous results by other authors indicating that haptens with short linking groups are less suitable for enzyme conjugates, probably due to steric hindrances (Skerrit et al., 1992; Bekheit et al., 1993; Schneider et al., 1994).

Table 2. Recognition of Several Compounds by Carbofuran Antibodies in the CC ELISA Format[a]

		monoclonal antibody cross-reactivity[b] (%)			
chemical structure	compound	LIB-**BFNP**21	LIB-**BFNB**52	LIB-**BFNB**62	LIB-**BFNB**67
	carbofuran	100.0	100.0	100.0	100.0
	carbofuran-phenol	0.02	5.2	0.01	0.02
	carbofuran-hydroxy	18.5	0.2	16.8	25.2
	carbofuran-keto	0.6	3.1	0.3	0.4
	carbosulfan	2.9	4.5	2.7	3.1
	benfuracarb	13.2	14.4	17.1	18.6
	furathiocarb	37.0	43.1	31.8	36.5
	bendiocarb	21.2	10.5	19.8	25.0
	carbaryl	0.1	0.1	0.2	0.2
	propoxur	0.7	0.1	0.8	1.0

[a] ELISA plates were coated with the OVA-BFNH conjugate at 0.01 μg/mL, except for the LIB-BFNB52 MAb, for which a coating conjugate concentration of 0.05 μg/mL was used. [b] Percentage of cross-reactivity = (I_{50} of carbofuran/I_{50} of other compound) × 100. Cross-reactivity values for the carbamate insecticides methiocarb, aldicarb, and methomyl were <0.01%.

With respect to the sensitivity of these assays, the four MAbs exhibited I_{50} values in the AC format very similar to those found in the CC format (Table 1).

Cross-Reactivity Studies. The specificity of the MAbs was evaluated by performing competitive assays using several compounds as competitors, and the obtained I_{50} values were used to calculate cross-reactivities. Assayed compounds included *N*-methylcarbamates (carbaryl, methiocarb, propoxur, and bendiocarb), *N*-methylcarbamoyloximes (aldicarb and methomyl), carbofuran metabolites (carbofuran-phenol, carbofuran-hydroxy, and carbofuran-keto), and pesticides structurally related to carbofuran (carbosulfan, benfuracarb, and furathiocarb). The study was performed with the four MAbs and using both the CC format and the AC format. No differences in the specificity pattern of the antibodies as a function of the assay format were found. Data in Table 2 are the mean cross-reactivity values for the CC

format calculated from four experiments performed in different days.

MAbs may be divided into two groups according to the recognition of carbofuran metabolites. LIB-BFNB52 MAb showed a slight recognition of carbofuran-phenol and carbofuran-keto (5.2 and 3.1%, respectively), whereas the cross-reactivity to carbofuran-hydroxy was negligible. The behavior of the three other antibodies was essentially the opposite: they did not significantly recognize carbofuran-phenol and carbofuran-keto, but they did exhibit a moderate recognition of carbofuran-hydroxy (~20%, depending on the antibody).

The recognition of the rest of the assayed compounds was very similar for all of the MAbs. Thus, cross-reactivity values for benfuracarb, furathiocarb, and carbosulfan were around 15, 37, and 3%, respectively. These three compounds have in common that they are carbamate pesticides having carbofuran as the main degradation product. In fact, the transformation of these products to carbofuran occurs quite easily in acid medium (Rouchaud et al., 1990), a characteristic that has sometimes been used to analyze these carbamates as carbofuran equivalents. *N*-Methylcarbamates and *N*-methylcarbamoyloximes were not significantly recognized by any of the MAbs. The only exception was bendiocarb (cross-reactivity ~ 20%), a pesticide closely structurally related with carbofuran but scarcely used in the agrochemical field.

Influence of Physicochemical Factors on Immunoassays. From the affinity and specificity data presented above, in combination with culture characteristics of hybridomas such as growth rate and antibody production, we decided to use the MAb LIB-BFNB67 for the development of immunoassays for carbofuran. The antibody was used with OVA-BFNH in the CC format and with HRP-BFNH in the AC format.

Factors such as the pH and the ionic strength of the assay buffer, as well as the presence of organic solvents, can directly affect the assay sensitivity by modifying the presentation of the soluble analyte to the antibody or by changing the interaction of the antibody with the conjugated hapten used in the assay (Hammock et al., 1990). Accordingly, the influence of these factors on the characteristics of carbofuran immunoassays was studied.

Salt Concentration Effect. To examine the influence of the ionic strength on the assay systems, competitive curves for carbofuran were obtained using assay buffers prepared by diluting 16-fold-concentrated PBS to cover the range of salt concentrations indicated in Figure 4. As shown, the variation of the assay parameters A_{max} and I_{50} with the ionic strength followed different patterns for each format. Thus, by increasing the salt concentration, the recognition of the corresponding conjugated hapten (A_{max}) diminished or increased for the CC or AC format, respectively. Furthermore, the recognition of carbofuran (I_{50}) did not change markedly as a consequence of the salt concentration increase in the AC format, whereas a large improvement was seen in the CC format (lower I_{50}).

The representation of the A_{max}/I_{50} ratio against the salt concentration was helpful in the selection of the optimum buffer concentration. As shown in the lower graph of Figure 4, for both assay formats the ratio appreciably increases with the salt concentration up to 2 × PBS. Hence, this ionic strength was selected for the buffer of the competition step in the two formats.

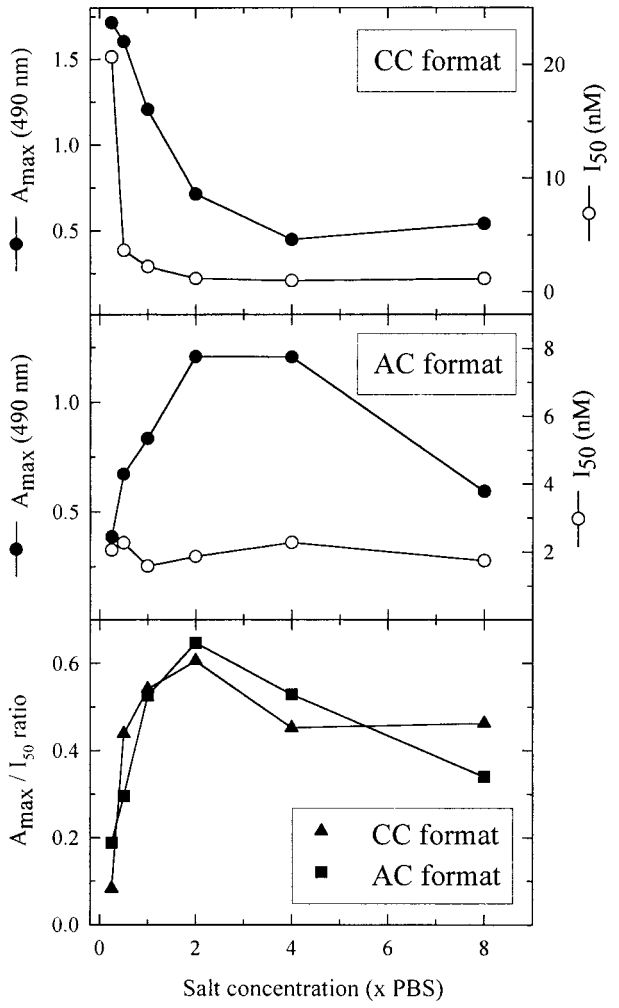


Figure 4. Influence of the salt concentration of the assay buffer on the A_{max} (●) and I_{50} (○) of carbofuran competitive standard curves: (top graph) conjugate-coated format; (middle graph) antibody-coated format; (bottom graph) representation of the A_{max}/I_{50} ratio for each format. Data were obtained from standard curves performed in triplicate.

The nonpolar nature of carbofuran could explain the observed behavior, because wherein hydrophobic interactions are important driving forces of ligand−receptor biochemical interactions, these are favored by increasing the ionic strength of the reaction medium (Jefferis and Deverill, 1991). In fact, a nearly identical tendency has been found in similar studies carried out in our laboratory with several other hydrophobic pesticides, such as chlorpyrifos and carbaryl (Manclús and Montoya, 1996c; Abad and Montoya, 1997), whereas the opposite effect was noticed with 3,5,6-trichloro-2-pyridinol, a polar compound that is the major degradation product of chlorpyrifos (Manclús and Montoya, 1996b).

pH Effect. To study the influence of pH on the assay characteristics, competitive curves were obtained by preparing standards in distilled water and adding the adequate concentration of LIB-BFNB67 MAb or HRP-BFNH tracer in 4 × PBS at different pH values covering the range 4.0−10.0. The variation of the curve parameters as a function of pH in the CC and AC formats is depicted in Figure 5 (top and middle graphs, respectively). For both formats, a similar pH influence on assay parameters was found. Thus, the recognition of conjugated haptens (OVA-BFNH and HRP-BFNH), represented by the response in the absence of analyte

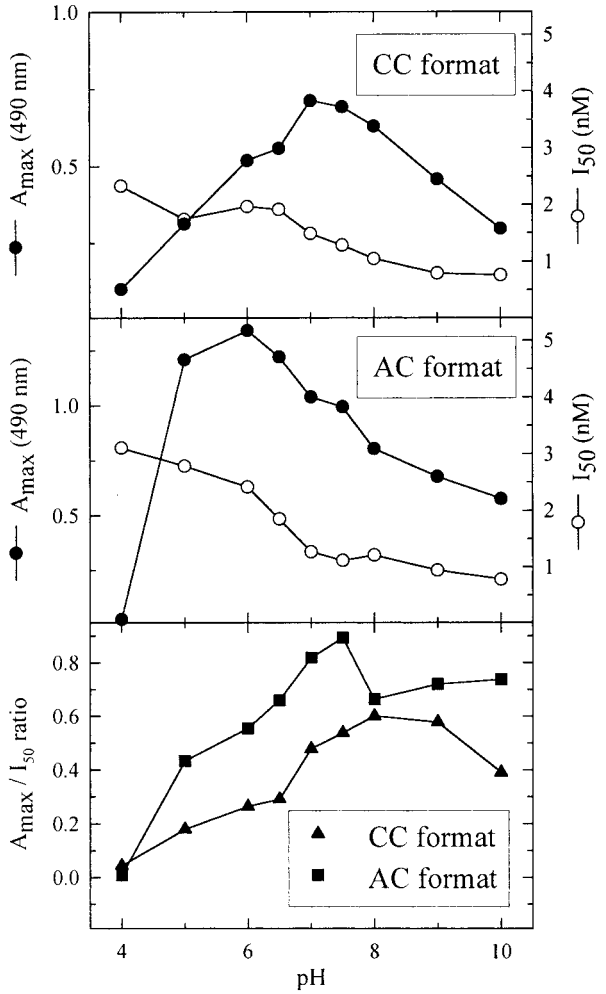


Figure 5. Influence of the pH of the assay buffer on the A_{max} (●) and I_{50} (○) of carbofuran competitive standard curves: (top graph) conjugate-coated format; (middle graph) antibody-coated format; (bottom graph) representation of the A_{max}/I_{50} ratio for each format. Data were obtained from standard curves performed in triplicate.

(A_{max} values), showed a maximum at pH 6.0−7.0. With respect to the recognition of carbofuran, only minor variations in the I_{50} values with pH were noticed, although both immunoassays showed a slight tendency to increase the affinity to carbofuran (lower I_{50} values) as pH increased. Similar to the salt concentration study, the variation of both parameters was considered together (A_{max}/I_{50} ratio) as a method to estimate the optimum pH range for these carbofuran immunoassays. From the curves plotted in the lower graph of Figure 5, it seems that a slightly basic pH (7.4) could be a reasonable choice for the buffer of the competition step.

Solvent Tolerance. The effect of several concentrations of methanol, acetone, and 2-propanol on the performance of the carbofuran ELISAs was studied (Figure 6). The CC format is more tolerant to the presence of acetone and 2-propanol in the assay buffer than the AC format, as evidenced by the sharper decrease in the A_{max} of this format as the solvent proportion increased. Nevertheless, although a sufficient maximum signal remained in the CC format even at 15% acetone and 2-propanol, the loss of sensitivity was so important that made this format also unsuitable for carbofuran analysis. On the contrary, A_{max} was not adversely affected by methanol in the range of concentrations tested, and the sensitivity of the assay did not change (AC format) or it experienced only a slight increase (CC format). Therefore, methanol was very well tolerated by both assay formats up to a 15% concentration, which may be of great importance for the application of these immunoassays to the analysis of carbofuran in foodstuffs and soils.

Table 3. Summary of the Characteristics of the Carbofuran Immunoassays

	format	
	CC	AC
immunoreagent concentrations		
[OVA-BFNH] (μg/mL)	0.01	na[a]
[LIB-BFNB67 MAb] (μg/mL)	0.025	1.0
[HRP-BFNH] (μg/mL)	na	0.05
buffer conditions		
% BSA	0.1	
salt concentration	100 mM phosphate, 137 mM NaCl	
pH	7.4	
preservatives	0.01% thimerosal	
solvent tolerance	15% methanol	
analytical characteristics of standard curve[b]		
A_{max}	1.14	1.05
A_{min}	0.06	0.02
I_{50} (ng/mL)	0.66	0.77
slope	1.14	0.98
concentration giving		
20−80% inhibition (ng/mL)	0.20−2.74	0.18−3.34
10% inhibition (ng/mL)	0.092	0.078
assay time		
coating	overnight	
primary immunoreaction	1 h	
secondary immunoreaction	1 h	na
color development	10 min	

[a] Not applicable. [b] Data averaged from 40 standard curves performed over 5 months. Curves were fitted to the four-parameter equation.

CONCLUSIONS

The goal of obtaining high-affinity MAbs to the *N*-methylcarbamate pesticide carbofuran was achieved from mice immunized with BSA conjugates of two haptens differing in the spacer arm length. To our knowledge, these MAbs are the first reported for this insecticide. This result, along with previous experience with other carbamates, confirms the excellence of this hapten synthesis strategy as a general and simple approach for the obtention of antibodies to this family of pesticides. Nevertheless, unlike our previous experience with other compounds, a more careful design of the procedure to test fusion supernatants was required to obtain antibodies of the desired affinity to carbofuran, because these MAbs exhibit an affinity unusually high to the coating conjugate.

Four MAbs were characterized with respect to carbofuran affinity in two assay formats and using several assay haptens. No noticeable improvement in the assay sensitivity was obtained by the use of heterologous haptens, which may indicate that these haptens are not sufficiently dissimilar in structure from the immunizing haptens. Very similar sensitivities were obtained in the CC and AC formats for the four MAbs studied. The main difference between ELISA formats is the number of conjugated haptens suitable for the development of immunoassays, which is clearly higher in the CC format.

Specificity studies revealed that the only tested pesticides that may interfere with the determination of

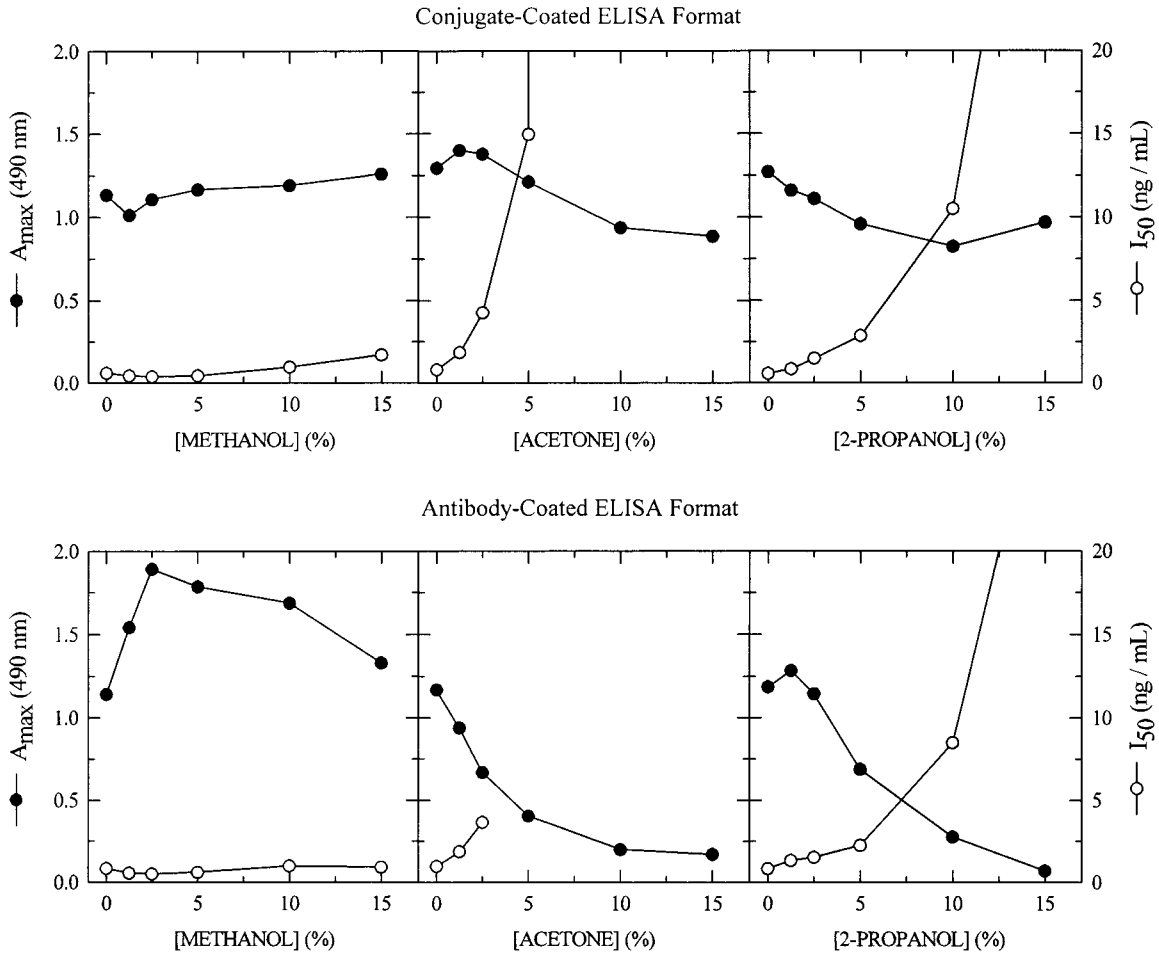


Figure 6. Effect of the organic solvent concentration in the assay on the A_{max} (●) and I_{50} (○) of carbofuran competitive standard curves. Data were obtained from standard curves performed in triplicate. Some I_{50} values are absent from the graphs because absorbance values of their corresponding standard curves did not properly fit the four-parameter logistic equation.

carbofuran are bendiocarb, carbosulfan, furathiocarb, and benfuracarb, which is not surprising due to the close structural similarity among all of these compounds. However, this circumstance should not be a practical problem, because it is unlikely that two or more of these compounds are used together to combat pests in the same crop. On the contrary, this cross-reactivity could allow the use of the immunoassays herein developed to monitor the presence of these pesticides in treated crops, even further taking into account that carbofuran is the main metabolite of carbosulfan, furathiocarb, and benfuracarb. In fact, these ELISAs have already been applied to study the time-dependent degradation of carbosulfan in orange trees, and conclusions were identical to those derived from chromatographic analysis, but at a fraction of the cost (unpublished results).

On the basis of the use of the LIB-BFNB67 MAb together with OVA-BFNH and HRP-BFNH conjugates, two immunoassays were developed. As a result of the optimization process, which included the study of the influence of important parameters such as pH, ionic strength, and solvent tolerance, optimum conditions for the two ELISAs were established (Table 3). The most prominent findings of this study are the low coating concentration of conjugate required in the CC format to get a highly sensitive assay (0.01 μg/mL) and the high tolerance of both formats to methanol (up to 15% in the assay, 30% in the sample). The analytical characteristics of the immunoassays listed in Table 3 were estimated from the standard curves depicted in Figure 7, which

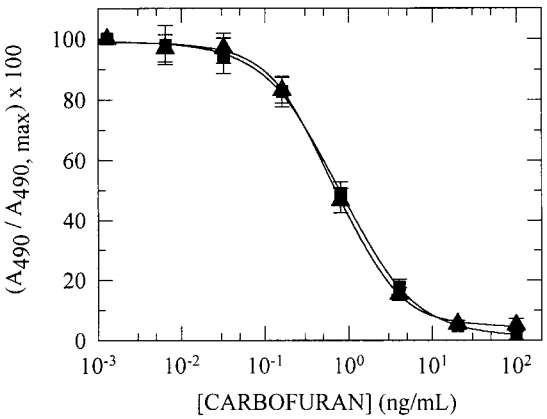


Figure 7. Normalized standard curves for carbofuran obtained under the optimized assay conditions with the AC format (■) and with the CC format (▲). Each plot represents the mean of 40 carbofuran standard curves performed in triplicate. Error bars correspond to standard deviations.

were obtained by averaging 40 normalized curves, each one run in triplicate, performed over 5 months. The limit of detection, estimated as the analyte concentration giving a 10% inhibition of the maximum absorbance, and the assay working range, calculated as the analyte concentrations providing a 20−80% inhibition of the maximum signal, were very similar for both ELISA formats. This fact is clearly shown in Figure 7, where curves in the CC and AC formats are nearly indistin-

guishable. I_{50} values for carbofuran were 0.66 and 0.77 ng/mL for the CC and AC formats, respectively, which favorably compare with the sensitivity of the only carbofuran immunoassay developed so far (I_{50} = 0.82 ng/mL; Jourdan et al., 1995). Therefore, the monoclonal immunoassays herein described seem to be very promising analytical techniques for cost- and labor-effective agricultural and environmental monitoring of carbofuran.

ABBREVIATIONS USED

AC, antibody-coated; A_{max}, maximum absorbance; BSA, bovine serum albumin; CC, conjugate-coated; CR, cross-reactivity; DMEM, Dulbecco's Modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HFCS, hybridoma fusion and cloning supplement; HRP, horseradish peroxidase; I_{50}, concentration giving 50% inhibition of maximum response; Ig, immunoglobulin; MAb, monoclonal antibody; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; PBSB, phosphate-buffered saline containing 0.1% BSA; PBST, phosphate-buffered saline containing 0.05% Tween 20; PEG, polyethylene glycol; UV−vis, ultraviolet−visible.

LITERATURE CITED

Abad, A.; Montoya, A. Production of monoclonal antibodies for carbaryl from a hapten preserving the carbamate group. *J. Agric. Food Chem.* **1994**, *42*, 1818−1823.

Abad, A.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 2. Assay optimization and application to the analysis of water samples. *J. Agric. Food Chem.* **1997**, *45*, 1495−1501.

Abad, A.; Manclús, J. J.; Mojarrad, F.; Mercader, J. V.; Miranda, M. A.; Primo, J.; Guardiola, V.; Montoya, A. Hapten synthesis and production of monoclonal antibodies to DDT and related compounds. *J. Agric. Food Chem.* **1997a**, *45*, 3694−3702.

Abad, A.; Primo, J.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 1. Antibody production from several haptens and characterization in different immunoassay formats. *J. Agric. Food Chem.* **1997b**, *45*, 1486−1494.

Abad, A.; Moreno, M. J.; Montoya, A. Hapten synthesis and production of monoclonal antibodies to the *N*-methylcarbamate pesticide methiocarb. *J. Agric. Food Chem.* **1998**, *46*, 2417−2426.

Augspurger, T.; Smith, M. R.; Meteyer, C. U.; Converse, K. A. Mortality of passerines adjacent to a North Caroline corn field treated with granular carbofuran. *J. Wildlife Dis.* **1996**, *32*, 113−116.

Bakowski, R. S.; Ali, M. S.; White, J. D.; Phillippo, E. T.; Ellis, R. L. Gas chromatographic/mass spectrometric confirmation of ten *N*-methylcarbamate insecticides in liver. *J. AOAC Int.* **1994**, *77*, 1568−1574.

Ballesteros, E.; Gallego, M.; Valcárcel, M. Automatic gas chromatographic determination of *N*-methylcarbamates in milk with electron capture detection. *Anal. Chem.* **1993**, *65*, 1773−1778.

Bekheit, H. K. M.; Lucas, A. D.; Szurdoki, F.; Gee, S. J.; Hammock, B. D. An enzyme immunoassay for the environmental monitoring of the herbicide bromacil. *J. Agric. Food Chem.* **1993**, *41*, 2220−2227.

CDPR (California Department of Pesticide Regulation). *Pesticide Use Reporting*; Sacramento, CA, 1993; http://www.cdpr.ca.gov/docs/pur/purmenu.htm.

Dankwardt, A.; Hock, B. Enzyme immunoassays for analysis of pesticides in water and food. *Food Technol. Biotechnol.* **1997**, *35*, 164−174.

Färber, H.; Schöler, H. F. Gas chromatographic determination of carbamate pesticides after flash-heater methylation with trimethylsulfonium hydroxide. *J. Agric. Food Chem.* **1993**, *41*, 217−220.

Farm Chemicals Handbook; Meister Publishing: Willoughby, OH, 1995.

Gunderson, E. L. FDA total diet study, July 1986-April 1991, dietary intakes of pesticides, selected elements, and other chemicals. *J. AOAC Int.* **1995**, *78*, 1353−1363.

Gupta, R. C. Carbofuran toxicity. *J. Toxicol. Environ. Health* **1994**, *43*, 383−418.

Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M. H.; Li, Q. X.; Lucas, A. D.; Székács, A.; Sundaram, K. M. Immunological technology in environmental analysis: addressing critical problems. In *Immunological Methods for Environmental Analysis*; Van Emon, J. M., Mumma, R. O., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990; pp 112−139.

Hammock, B. D.; Gee, S. J. Impact of emerging technologies on immunochemical methods for environmental analysis. In *Immunoassays for Agrochemicals. Emerging Technologies*; Nelson, J. O., Karu, A. E., Wong, R. B., Eds.; ACS Symposium Series 586; American Chemical Society: Washington, DC, 1995; pp 1−19.

James, P. C. Internalizing externalities: granular carbofuran used on rapeseed in Canada. *Ecol. Econ.* **1995**, *13*, 181−184.

Jefferis, R.; Deverill, I. The antigen antibody reaction. In *Principles and Practice of Immunoassays*; Price, C. P., Newman, D. J., Eds.; Stockton Press: New York, 1991; Chapter 1.

Jourdan, S. W.; Scutellaro, A. M.; Fleeker, J. R.; Herzog, D. P.; Rubio, F. Determination of carbofuran in water and soil by a rapid magnetic particle-based ELISA. *J. Agric. Food Chem.* **1995**, *43*, 2784−2788.

Kaufman, B. M.; Clower, M., Jr. Immunoassay of pesticides. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 239−247.

Langone, J. J.; Van Vunakis, H. Radioimmunoassay of nicotine, cotinine, and γ-(3-piridyl)-γ-oxo-*N*-methylbutyramide. *Methods Enzymol.* **1982**, *84*, 628−640.

Lehotay, S. J.; Argauer, R. J. Detection of aldicarb sulfone and carbofuran in fortified meat and liver with commercial ELISA kits after rapid extraction. *J. Agric. Food Chem.* **1993**, *41*, 2006−2010.

Liška, I.; Slobodník, J. Comparison of gas and liquid chromatography for analysing polar pesticides in water samples. *J. Chromatogr. A* **1996**, *733*, 235−258.

Manclús, J. J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for 3,5,6-trichloro-2-pyridinol. 1. Production and characterization of monoclonal antibodies. *J. Agric. Food Chem.* **1996a**, *44*, 3703−3709.

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.* **1996b**, *44*, 3710−3716.

Manclús, J. J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for the insecticide chlorpyrifos. 2. Assay optimization and application to environmental water samples. *J. Agric. Food Chem.* **1996c**, *44*, 4063−4070.

Manclús, J. J.; Primo, J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for the insecticide chlorpyrifos. 1. Monoclonal antibody production and immunoassay design. *J. Agric. Food Chem.* **1996**, *44*, 4052−4062.

McGarvey, B. D. High-performance liquid chromatographic methods for the determination of *N*-methylcarbamate pesticides in water, soil, plants and air. *J. Chromatogr.* **1993**, *642*, 89−105.

Meulenberg, E. P.; Mulder, W. H.; Stoks, P. G. Immunoassays for pesticides. *Environ. Sci. Technol.* **1995**, *29*, 553−561.

Mueller, H. M.; Stan, H. J. Thermal degradation observed with different injection techniques: quantitative estimation by the use of thermolabile carbamate pesticides. *J. High Resolut. Chromatogr.* **1990**, *13*, 759−763.

Nam, K. S.; King, J. W. Supercritical fluid extraction of enzyme immunoassay for pesticide detection in meat products. *J. Agric. Food Chem.* **1994**, *42*, 1469−1474.

Nowinski, R. C.; Lostrom, M. E.; Tam, M. R.; Stone, M. R.; Burnette, W. N. The isolation of hybrid cell lynes producing monoclonal antibodies against the p15(E) protein of ecotropic murine leukemia viruses. *Virology* **1979**, *93*, 111−126.

Raab, G. M. Comparison of a logistic and a mass-action curve for radioimmunoassay data. *Clin. Chem.* **1983**, *29*, 1757−1761.

Rajkowski, K. M.; Cittanova, N.; Desfosses, B.; Jayle, M. F. The conjugation of testosterone with horseradish peroxidase and a sensitive enzyme assay for the conjugate. *Steroids* **1977**, *29*, 701−713.

Rouchaud, J.; Gustin, F.; Van de Steene, F.; Pelerents, C.; Vanparys, L.; De Proft, M.; Seutin, E.; Gillet, J.; Benoit, F.; Ceustermans, N. Comparative plant and soil metabolism of carbosulfan, furathiocarb and carbofuran in brussels sprouts, cauliflower, and sugar beet crops. *Toxicol. Environ. Chem.* **1990**, *25*, 109−124.

Rule, G. S.; Mordehai, A. V.; Henion, J. Determination of carbofuran by on-line immunoaffinity chromatography with coupled-column liquid chromatography/mass spectrometry. *Anal. Chem.* **1994**, *66*, 230−235.

Schneider, P.; Goodrow, M. H.; Gee, S. J.; Hammock, B. D. A Highly sensitive and rapid ELISA for the arylurea herbicides diuron, monuron, and linuron. *J. Agric. Food Chem.* **1994**, *42*, 413−422.

Sherry, J. P. Environmental chemistry: the immunoassay option. *Crit. Rev. Anal. Chem.* **1992**, *23*, 217−300.

Skerritt, J. H.; Hill, A. S.; Beasley, H. L.; Edward, S. L.; Mcadam, D. P. Enzyme-linked immunosorbent assay for quantitation of organophosphate pesticides—fenitrothion, chlorpyrifos-methyl, and pirimiphos-methyl in wheat grain and flour-milling fractions. *J. AOAC Int.* **1992**, *75*, 519−528.

Tomlin, C. In *The Pesticide Manual: A World Compendium*, 10th ed.; Crop Protection Publications: Surrey, U.K., 1994.

Van Emon, J. M.; López-Ávila, V. Immunochemical methods for environmental analysis. *Anal. Chem.* **1992**, *64*, 78A−88A.

Yang, S. S.; Goldsmith, A. I.; Smetena, I. Recent advances in the residue analysis of *N*-methylcarbamate pesticides. *J. Chromatogr. A* **1996**, *754*, 3−16.

Received for review October 29, 1998. Revised manuscript received March 20, 1999. Accepted March 31, 1999. This work was supported by a grant (ALI96-1232) from the Comisión Interministerial de Ciencia y Tecnología (Spain). Limited amounts of experimental kits for the determination of carbofuran are available on request.

JF981184S