Development of an Enzyme-Linked Immunosorbent Assay to Carbaryl. 2. Assay Optimization and Application to the Analysis of Water Samples

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The effect of several physicochemical factors on the analytical characteristics of an immunoassay to carbaryl has been studied and this information used to optimize the assay. The immunoassay is based on the LIB-CNH36 monoclonal antibody (MAb) and employs a heterologous coating conjugate. The optimized enzyme-linked immunosorbent assay (ELISA), performed in a high salt concentration buffer with bovine serum albumin instead of Tween 20, has a carbaryl I_{50} of 0.058 ppb and a detection limit of 0.010 ppb, which means a 4-fold improvement in the assay sensitivity with respect to the nonoptimized conditions. The assay proved to be very specific for carbaryl since not even 1-naphthol, the main carbaryl metabolite, was recognized. Water samples with very different conductivities were spiked with carbaryl at 0.05, 0.1, and 0.5 ppb and directly analyzed by the developed ELISA. The mean recovery was 112%, and the mean coefficient of variation was 10.7%, which proves the suitability of the method to determine carbaryl in waters at such low levels.

Keywords: *Carbaryl; immunoassay; ELISA; optimization; Tween 20; pH; ionic strength; solvent tolerance; water analysis*

INTRODUCTION

Immunoassays for pesticides are usually carried out under physiological conditions, and frequently little effort is made to optimize them for factors such as pH, ionic strength, and presence of detergent. However, these factors 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). Thus, optimization can play a very important role in the development of immunoassays for pesticides. Additionally, optimization provides information on the immunoassay performance that may be of great usefulness in solving problems arising during validation studies with real samples.

In the preceding paper, a very sensitive ELISA for carbaryl based on the LIB-CNH36 monoclonal antibody has been reported (I_{50} = 1.1 nM ≡ 0.2 ppb; Abad et al., 1997). The characterization of this immunoassay with respect to the influence of several physicochemical factors on its performance is described herein. Likewise, the immunoassay was characterized with respect to organic solvent tolerance, and the assay specificity was evaluated by testing a panel of compounds as potential competitors. The developed ELISA was finally applied to the quantification of carbaryl in a variety of water samples at levels even lower than those permitted by European legislation (European Community, 1980).

MATERIALS AND METHODS

Immunoreagents, Chemicals, and Instruments. The preparation of the OVA−1NAH conjugate and the production of LIB-CNH36 MAb have been described in the preceding paper (Abad et al., 1997). BSA, OVA, Tween 20, and OPD were purchased from Sigma-Aldrich Química (Madrid, Spain). HRP-labeled rabbit anti-mouse Igs were from Dako (Glostrup, Denmark). All other chemicals and organic solvents were of reagent grade or better.

Carbaryl, 1-naphthol, carbofuran, propoxur, methiocarb, aldicarb, and methomyl were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 1-Naphthylacetic acid, 1-naphthylacetamide, 1-naphthylacetic acid methyl ester, monuron, propham, and carbendazim were obtained from Riedel-de Häen (Seelze, Germany). Naphthalene, 1-naphthoxyacetic acid, 2-naphthoxyacetic acid, and 1,5-dihydroxynaphthalene were from Sigma-Aldrich Química (Madrid, Spain). *N*-Hydroxymethylcarbaryl, desmethylcarbaryl, and 3-, 4-, and 5-hydroxymethylcarbaryl were generously provided by Rhône-Poulenc Ag Company, Reseach Triangle Park, NC. Stock solutions of all these compounds were prepared in *N*,*N*-dimethylformamide (DMF) and stored at 4 °C.

Polystyrene ELISA plates were from Costar, Cambridge, MA. (High Binding Plates, catalog no. 3590). Plates were washed with an Ultrawash II apparatus, and absorbances were read in dual-wavelength mode (460−630 nm) with an MR 700 microplate reader, both from Dynatech (Sussex, U.K.).

Carbaryl ELISA. The immunoassay to be optimized was an indirect ELISA based on the LIB-CNH36 MAb in combination with the heterologous coating conjugate OVA−1NAH. All incubations were carried out at room temperature. Ninety-six-well microtiter plates were coated with the conjugate (100 μL/well, 1 μg/mL in 50 mM carbonate−bicarbonate buffer, pH 9.6) by overnight incubation. Plates were washed four times with 0.15 M NaCl containing 0.05% Tween 20. Amounts of 50 μL/well of carbaryl standards or samples were added in quadruplicate, followed by 50 μL/well of LIB-CNH36 MAb (0.06 μg/mL, 0.03 μg/mL in the assay). After incubation for 2 h, the plates were washed as before, and 100 μL of a 1/1000 dilution of HRP-labeled rabbit anti-mouse Igs in PBST (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4, containing 0.05% Tween 20) was added to the wells. Plates were incubated for 1 h and washed again, and 100 μL/well of 2 mg/mL OPD and 0.012% H_2O_2 in 25 mM sodium citrate, 62 mM sodium phosphate, pH 5.4, was added. After 10 min, the enzymatic reaction was stopped by adding 100 μL/well of 2.5 M sulfuric acid, and the absorbance at 490 nm was read.

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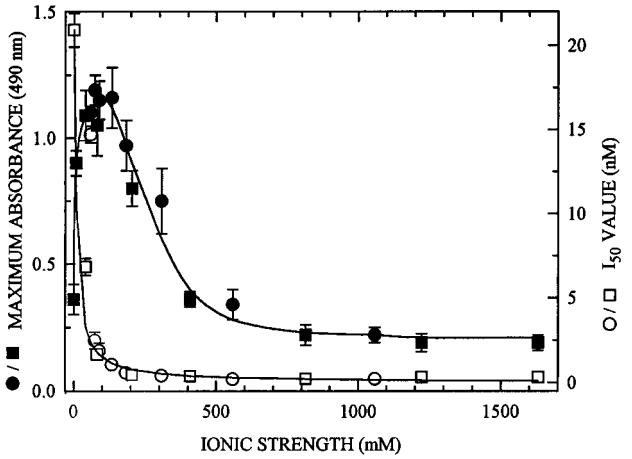


Figure 1. Effect of the ionic strength on the maximum absorbance (solid symbols) and I_{50} (open symbols) of the carbaryl ELISA. Data were obtained from standard curves performed in quadruplicate in buffers of different ionic strengths, which were prepared either by increasing the NaCl concentration of PBS (squares) or by diluting 20× PBS with water (circles). All buffers had a pH between 7.3 and 7.5. Error bars correspond to standard deviations.

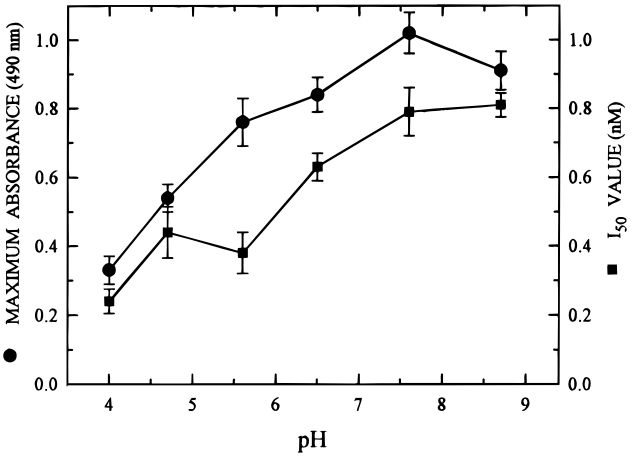


Figure 2. Influence of pH on the ELISA for carbaryl. Data were obtained from standard curves performed in quadruplicate in sodium phosphate/citrate/Tris buffers of different pH values. Ionic strength was kept constant at 220 mM. Error bars correspond to standard deviations.

Ionic Strength Studies. To perform competitive curves, carbaryl standards were dissolved in water and the MAb was dissolved in buffers of different ionic strength, which were prepared either by adding NaCl (from 0 to 2.0 M) to PBS (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) or by diluting 20× PBS with water. The pH of all buffers was kept between 7.3 and 7.5. After the concentration of the ionic species present in each buffer was determined, ionic strengths were calculated according to the formula

$$I = {}^1/_2 \sum c_i z_i^2$$

where I is the ionic strength, c is the concentration of each ion at equilibrium, and z is its charge.

pH Studies. To perform competitive curves, carbaryl standards were dissolved in water and the MAb was dissolved in buffers of different pH values, which were prepared as follows. The pH of a 100 mM sodium phosphate, 100 mM sodium citrate, 100 mM Tris solution was brought up to 8.7 by adding 5 M NaOH (2.0 mL to 1.0 L). Aliquots of this buffer were diluted 1/2 with water and adjusted to different pH values with concentrated hydrochloric acid. Since the ionic species present at equilibrium depend on the pH, different ionic strengths were calculated for the different buffers. To avoid an effect of the salt concentration in the pH study, the ionic strength was adjusted to approximately the same value for all buffers (I = 450 mM) by adding 4 M NaCl, and the pH was further confirmed.

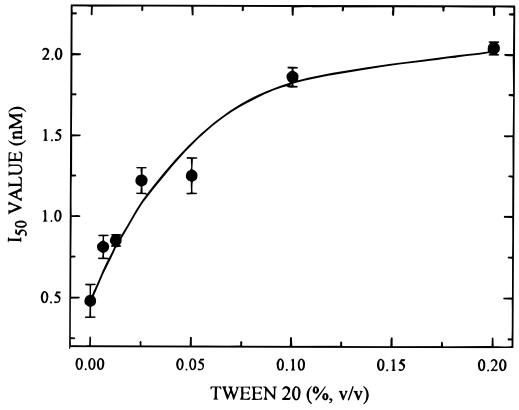


Figure 3. Effect of Tween 20 on the sensitivity of the carbaryl ELISA. Data were obtained from standard curves performed in quadruplicate in C buffers containing different concentrations of the detergent. Error bars correspond to standard deviations.

Tween 20 Concentration Studies. Carbaryl standards were prepared in concentrated phosphate buffer (C buffer; 100 mM sodium phosphate, 137 mM NaCl, pH 6.3), and the MAb was dissolved in the same buffer containing different concentrations of Tween 20 (from 0 to 0.8%). The C buffer has an ionic strength of 270 mM.

Since indirect evidence seemed to indicate that the influence of the Tween 20 concentration on the immunoassay maximum absorbance depends in turn on the ionic strength, saline buffers (137 mM NaCl) with different phosphate concentrations (from 10 to 400 mM), and containing or not 0.05% Tween 20, were also prepared. The pH values of all these buffers were kept between 6.3 and 6.5. A new set of competitive curves was then performed with the MAb dissolved in the described buffers.

Solvent Effect. Competitive curves were performed from carbaryl standards in C buffer and the MAb dissolved in CB buffer (C buffer with 0.1% BSA) containing different proportions of ethanol, methanol, acetone, tetrahydrofurane, acetonitrile, and 2-propanol (from 0 to 40%).

Determination of Cross-Reactivities. The ability of LIB-CNH36 MAb to recognize several related compounds was tested by performing competitive assays and determining their respective I_{50} (nanomolar) values (analyte concentration that reduces the maximum signal of the competitive ELISA to 50%). Inhibition curves were analyzed by mathematically fitting quadruplicate experimental points to a four-parameter logistic equation using a commercial software package (Sigmaplot, Jandel Scientific). Cross-reactivity was calculated as $[I_{50}(\text{carbaryl})/I_{50}(\text{compound})] \times 100$.

Spiked Water Samples. The optimized ELISA was applied to carbaryl determination in different water samples. Privado-1, Casablanca, and Cosario are well waters from Almería, Spain, and they were kindly provided by Dr. A. R. Fernández-Alba. To prevent pesticide hydrolysis, water samples were first acidified with glacial acetic acid until pH 4.0. Then, they were spiked with carbaryl at 0.05, 0.1, and 0.5 ppb and stored at 4 °C. Prior to the ELISA determination, the ionic strength of water samples was roughly brought up to that of the standard curve buffer by adding 50 μL of 10× C buffer to 450 μL of the sample.

RESULTS AND DISCUSSION

Assay Optimization. *Reagent Concentrations and Assay Time.* Unlike noncompetitive ELISAs, in which an excess of reagents is used to increase detectability, limiting concentrations of immunoreagents are required in competitive ELISAs to obtain good sensitivities. The

effect of monoclonal antibody, coating conjugate, and HRP-labeled antibody concentrations on the analytical parameters of the competitive curve was studied. On the one hand, increasing concentrations of either coating conjugate or antibody produced higher maximum signals and I_{50}. On the other hand, HRP-labeled antibody concentration has no significant effect on the ELISA I_{50}, while maximum absorbance increased as lower labeled antibody dilutions were used. The highest maximum absorbance to I_{50} ratio was obtained by using 1 μg/mL of OVA−1NAH as coating conjugate, 0.04 μg/mL of LIB-CNH36 MAb, and a 1/1000 dilution of HRP-labeled antibody.

The effect of the incubation time of each ELISA step on signal and sensitivity was also studied. In general terms, an increase of the total assay time resulted in higher maximum absorbances and slightly lower carbaryl I_{50}. Therefore, incubation times of 2 h for the competition step, 1 h for the labeled antibody interaction, and 10 min for the enzymatic reaction were selected and used through this work.

Ionic Strength. The sensitivity of the carbaryl immunoassay clearly improved (lower I_{50}) as the ionic strength of the competition buffer increased (Figure 1). Interestingly, data followed the same tendency irrespective of how the buffers were prepared, which proved that ionic strength was the real cause of the observed effects. Maximum absorbance initially increased with the ionic strength, but from 100 mM it was gradually lost. Since the beneficial effect of ionic strength on the assay sensitivity was counterbalanced by the negative influence on the signal, a compromise was adopted ($I = 270$ mM).

Similar results concerning the influence of the salt concentration have been previously reported for other antibodies and analytes (Harrison et al., 1989; Li et al., 1991; Marco et al., 1993; Lee et al., 1995). An improvement in maximum signal and sensitivity due to the increase of salt concentration has been also obtained in our laboratory with a MAb to chlorpyrifos (Manclús and Montoya, 1996b), whereas the opposite effect on both parameters was observed with a MAb to TCP (3,5,6-trichloro-2-pyridinol), a chlorpyrifos metabolite negatively charged at neutral pH (Manclús and Montoya, 1996a). All of these findings, along with the fact that changes in substrate and inhibitor specificity of enzymes have been correlated with the hydrophobicity of the reaction medium (Klibanov, 1989), suggest that the interaction between antibodies and hydrophobic analytes is favored by polar environments.

pH Effect. The pH dependence of both signal and sensitivity of ELISAs for pesticides has been previously reported (Jung et al., 1991; Li et al., 1991; Krämer et al., 1994; Lee et al., 1995). Therefore, the influence of this physicochemical parameter on the carbaryl ELISA was also evaluated (Figure 2). On the one hand, maximum absorbance increased with pH to reach an optimum at pH 8. On the other hand, carbaryl I_{50} also increased from pH 4 to pH 9, so more sensitive immunoassays can be performed at low pH values. On the basis of these results, and since carbaryl hydrolyzes in alkaline media, slightly acidic buffers (pH 6.0−6.5) seem to be more adequate for this carbaryl immunoassay.

Influence of Tween 20. The inclusion of detergents in buffers to reduce nonspecific interactions is a very common practice in immunoassays, Tween 20 being perhaps the most extensively employed detergent. To study if this reagent had some effect on the carbaryl assay, standard curves with different Tween 20 concentrations were performed. As shown in Figure 3, I_{50} increased with Tween 20 concentration, whereas the reduction or even the absence of Tween 20 did not have any significant effect on the background signal (data not shown). The influence on the maximum absorbance was surprising, since it in turn depended on the ionic strength of the buffer. Thus, at low phosphate concentrations, maximum signal was higher in the presence of 0.025% Tween 20 than in its absence, but from around 50 mM phosphate the opposite trend was found (Figure 4a). In both cases, signal gradually decreased as salt concentration increased, in agreement with the results reported above on the influence of ionic strength on the maximum absorbance, but absorbance dropped more rapidly when the buffer contained Tween 20 than when it did not. An effect of the Tween 20 concentration on the maximum absorbance and sensitivity was also found in immunoassays for pyrethroids and polychlorinated biphenyls (Stanker et al., 1989; Chiu et al., 1995). Concerning carbaryl I_{50}, it decreased as salt concentration increased, both in the presence and in the absence of Tween 20 (Figure 4b). At a given phosphate concentration, I_{50} was always lower when Tween 20 was not present in the buffer. Therefore, at the ionic strength previously determined as optimal, the presence of Tween 20 is detrimental to both the sensitivity and the maximum signal of the assay.

According to these results, the ideal situation would be to omit Tween 20 from the buffer used in the competition step. However, signal coefficients of variation were too high if a minimal proportion of Tween 20 (0.01%) was not present in the buffer. BSA has been often included in buffers to reduce well to well variability. In preliminary experiments, suitable standard curves were obtained with 0.1% BSA, so this BSA concentration was evaluated to eventually replace Tween 20 in the competition step. Twenty carbaryl standard curves were then performed along 5 days either in CT buffer (C buffer containing 0.01% Tween 20) or in CB buffer (C buffer containing 0.1% BSA), and the results were statistically analyzed by means of the Student t test. Relevant assay parameters improved very significantly when BSA instead of Tween 20 was included in the buffer (Figure 5). Thus, maximum absorbance was always higher (mean 1.32 vs 0.96, $p < 0.001$) and I_{50} was always lower (mean 0.058 vs 0.091 ppb, $p < 0.001$), while maintaining very similar slopes (mean 1.21 vs 1.22), background absorbances (mean 0.010 vs 0.011), and signal coefficients of variation (mean 6.1% vs 6.2%). An additional advantage of using CB instead of CT buffer was the possibility of lowering the optimal MAb concentration from 0.04 to 0.03 μg/mL.

In summary, the optimal conditions for the carbaryl ELISA were as follows: a monoclonal antibody (LIB-CNH36) concentration of 0.03 μg/mL; a coating conjugate (OVA−1NAH) concentration of 1 μg/mL; a 1/1000 dilution of the HRP-labeled rabbit anti-mouse immunoglobulins; incubation times of 2 h for the competitive step, 1 h for the auxiliary step, and 10 min for the enzymatic reaction; and a saline phosphate buffer for the competition step with an ionic strength of 270 mM, pH 6.5, with 0.1% BSA.

Organic Solvent Tolerance. The effect of several concentrations of methanol, ethanol, acetone, acetonitrile, tetrahydrofuran, and 2-propanol on the performance of the carbaryl ELISA was studied (Figure 6). A clear decrease in maximum signal and sensitivity was

Table 1. Cross-Reactivity of the Carbaryl ELISA for Selected Compounds

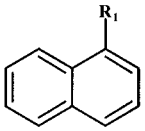
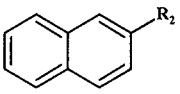
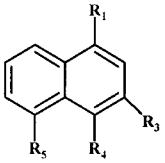
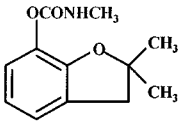
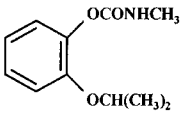
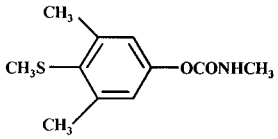
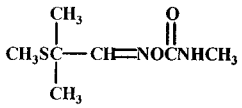
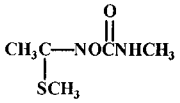
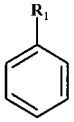
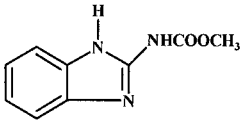
Chemical Structure	Compound name	CR (%)
		
$R_1 = OCONHCH_3$	Carbaryl	100
$R_1 = OH$	1-Naphthol	<0.1
$R_1 = H$	Naphthalene	<0.1
$R_1 = OCONHCH_2OH$	N-Hydroxymethylcarbaryl	21.3
$R_1 = OCONH_2$	Desmethylcarbaryl	2.0
$R_1 = CH_2CONH_2$	1-Naphthaleneacetamide	<0.1
$R_1 = CH_2COOCH_3$	1-Naphthylacetic acid methyl ester	0.2
$R_1 = CH_2COOH$	1-Napthylacetic acid	<0.1
$R_1 = OCH_2COOH$	1-Naphthoxyacetic acid	<0.1
$R_1 = OCONH(CH_2)_2COOH$	CNA	8.8
$R_1 = OCONH(CH_2)_5COOH$	CNH	173.1
$R_1 = NHCONH(CH_2)_5COOH$	CPNU	12.3
$R_1 = CONH(CH_2)_5COOH$	1NAH	<0.1
		
$R_2 = OCH_2COOH$	2-Naphthoxyacetic acid	<0.1
$R_2 = CONH(CH_2)_5COOH$	2NAH	<0.1
		
$R_1 = OCONHCH_3$ $R_3 = OH$ $R_4 = H$ $R_5 = H$	3-Hydroxycarbaryl	16.1
$R_1 = OCONHCH_3$ $R_3 = H$ $R_4 = OH$ $R_5 = H$	4-Hydroxycarbaryl	28.6
$R_1 = OCONHCH_3$ $R_3 = H$ $R_4 = H$ $R_5 = OH$	5-Hydroxycarbaryl	39.3
$R_1 = OH$ $R_3 = H$ $R_4 = H$ $R_5 = OH$	1,5-Dihydroxynaphthalene	<0.1
	Carbofuran	<0.1
	Propoxur	<0.1
	Methiocarb	0.1
	Aldicarb	<0.1
	Methomyl	<0.1
		
$R_1 = NHCON(CH_3)_2$	Monuron	<0.1
$R_1 = NHCOOCH(CH_3)_2$	Propham	<0.1
	Carbendazim	<0.1

Table 2. Analysis by the Optimized ELISA of Water Samples Fortified with Carbaryl

sample[a]	conductivity (μS)	carbaryl added (ppb)	carbaryl recovered[b] (ppb)	SD[c] (ppb)	CV[d] (%)	recovery (%)
distilled water	7	0.050	0.062	0.007	11.3	124
		0.100	0.116	0.012	10.3	116
		0.500	0.600	0.050	8.3	120
bottled water	223	0.050	0.053	0.006	11.3	106
		0.100	0.102	0.006	5.9	102
		0.500	0.570	0.050	8.8	114
Casablanca well water	370	0.050	0.057	0.006	10.5	114
		0.100	0.105	0.007	6.7	105
		0.500	0.590	0.040	6.8	118
Cosario well water	1240	0.050	0.060	0.008	13.3	120
		0.100	0.101	0.010	9.9	101
		0.500	0.490	0.070	14.3	98
tap water	1330	0.050	0.051	0.010	19.6	102
		0.100	0.093	0.010	10.8	93
		0.500	0.520	0.060	11.5	104
Privado-1 well water	5260	0.050	0.060	0.009	15.0	120
		0.100	0.115	0.010	8.7	115
		0.500	0.680	0.060	8.8	136
mean					10.7	112

[a] Casablanca, Cosario, and Privado-1 are water wells located in Almería, Spain. [b] Data are the average of 10 independent determinations. Each determination was run in quadruplicate, and the mean absorbance was interpolated in the standard curve performed in the same microtiter plate. [c] Standard deviation. [d] Interassay coefficient of variation.

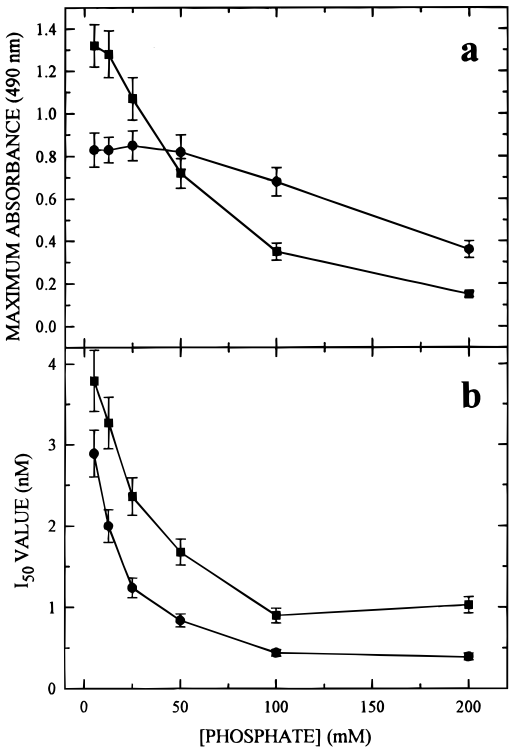


Figure 4. Influence of Tween 20 on the ELISA for carbaryl as a function of the ionic strength of the buffer. Data were obtained from standard curves performed in quadruplicate in saline buffers (137 mM NaCl) of different sodium phosphate concentrations, pH 6.5. Buffers did not contain Tween 20 (●), or they contained 0.025% Tween 20 (■). Error bars correspond to standard deviations.

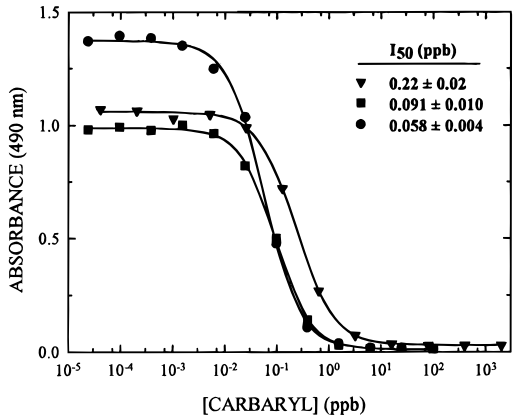


Figure 5. Comparison of carbaryl standard curves performed under nonoptimized conditions (▼, [MAb] = 0.05 μg/mL), in CT buffer (■, [MAb] = 0.04 μg/mL), and in CB buffer (●, [MAb] = 0.03 μg/mL). Microtiter plates were coated with OVA−1NAH at 1 μg/mL, and the HRP-labeled antibody was used at a 1/2000 (▼) or 1/1000 (■, ●) dilution in PBST. The primary immunoreaction was incubated for 2 h, the auxiliary step was incubated for 1 h, and the enzymatic reaction was stopped after 10 min. Each plot represents the mean of 20 carbaryl standard curves performed in quadruplicate.

observed when increasing amounts of the solvents were added to the assay buffer. Furthermore, absorbance values fitted poorly to a sigmoidal curve as the solvent concentration increased. Concentrations in the assay higher than 5% of methanol, the best tolerated of the solvents tested, clearly modified the assay parameters. Therefore, even minimal solvent amounts had a negative effect on the carbaryl immunoassay, so samples containing organic solvents, e.g. food extracts, must be adequately diluted in the assay buffer to be reliably analyzed. Fortunately, the high sensitivity of the assay would allow dilution of the organic extracts as much as 1/1000 without affecting its capability to analyze carbaryl residues in food at levels well below tolerance.

Specificity of the Carbaryl Immunoassay. A number of pesticides and carbaryl metabolites and derivatives were tested for cross-reactivity (Table 1). The immunoassay proved to be very specific for carbaryl, since none of the assayed pesticides was significantly recognized (CR < 0.1%). It is interesting to point out that the *N*-methylcarbamate group of carbaryl is a very important antigenic determinant, as shown by the negligible to low cross-reactivities exhibited by desmethylcarbaryl and 1-naphthol, the main carbaryl me-

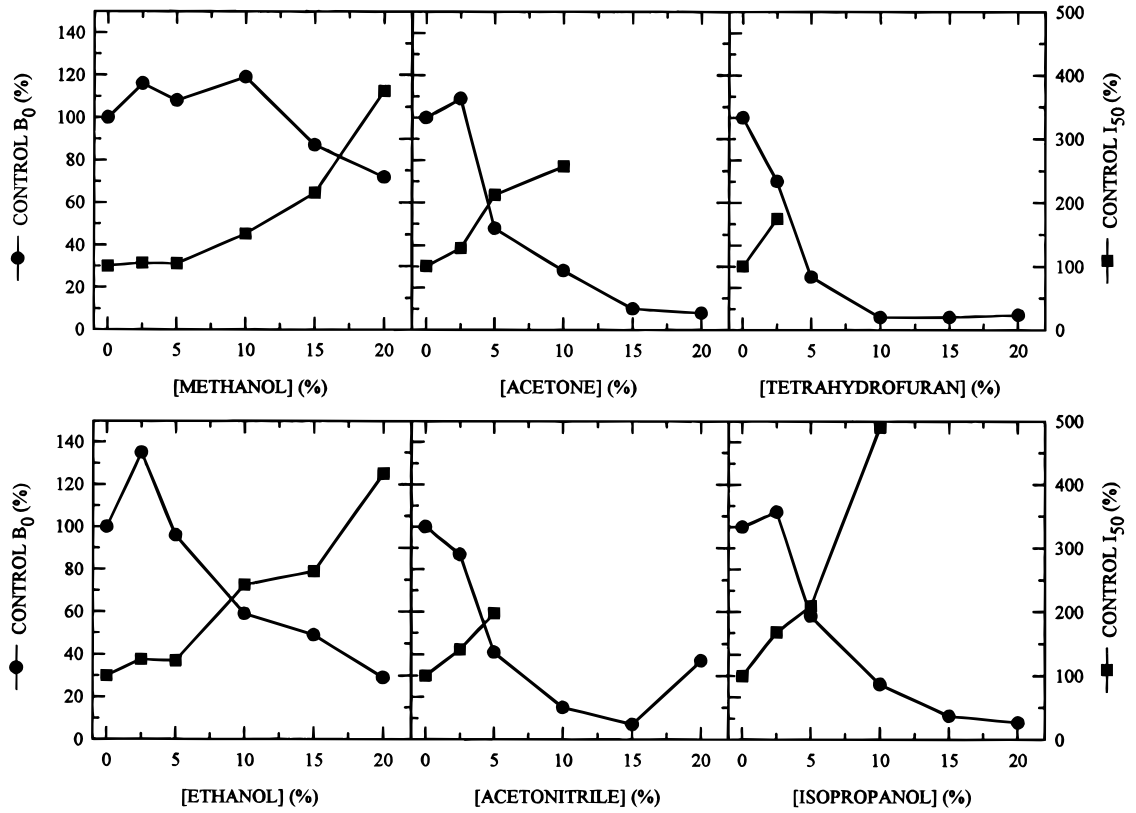


Figure 6. Organic solvent tolerance of the carbaryl ELISA. Data were obtained from standard curves performed in quadruplicate in CB buffers containing different concentrations of the solvents indicated in each graph. Maximum absorbance (●) and I_{50} (■) values are expressed relative to the control inhibition curve, performed without solvent. Some I_{50} data are absent from the graphs because absorbance values of their corresponding standard curves did not properly fit to the four-parameter logistic equation.

tabolite. Apart from some of the haptens synthesized for this work (CNA, CNH, and CPNU), the only compounds recognized to some extent were the hydroxylated metabolites of carbaryl (*N*-hydroxymethylcarbaryl and 3-, 4-, and 5-hydroxycarbaryl, 20−40% CR). The gradual increase of recognition from 3-hydroxycarbaryl to 5-hydroxycarbaryl could be indicating that the interaction of the antibody with carbaryl occurs mainly through the aromatic ring to which the *N*-methylcarbamate group is attached. These results confirmed our preliminary findings indicating that both the naphthalene aromatic ring and the *N*-methylcarbamate group must be present for effective antibody binding and recognition (Abad and Montoya, 1994).

Analysis of Spiked Water Samples. Water samples with different conductivities were fortified with carbaryl at 0.05, 0.1, and 0.5 ppb and analyzed by the optimized immunoassay (Table 2). Determinations were made in quadruplicate, and the mean absorbance was used to estimate carbaryl concentration by interpolation in the standard curve performed in the same plate. Each spiked sample was analyzed 10 times along 5 days. The detection limit of the ELISA, calculated as the carbaryl concentration that reduces absorbance to 90% of the maximum, was 0.022 ppb for water samples (0.010 ppb for standards). Control samples without carbaryl were also systematically included in the analysis, and values lower than the assay detection limit were found in all cases, so no false positives were detected. Irrespective of the fortification level and water type, very similar coefficients of variation were found, ranging from 5.9% to 19.6% (10.7% mean). Although the ELISA showed a slight tendency to overestimation, accuracy was excellent for such a sensitive and simple method (112% mean recovery).

CONCLUSIONS

A highly sensitive and specific ELISA for carbaryl based on the LIB-CNH36 MAb has been developed and characterized. The ELISA employs a heterologous coating conjugate which had previously entailed a 3.5-fold increase in sensitivity with respect to the homologous hapten (an I_{50} decrease from 0.78 to 0.22 ppb; Abad et al., 1997). This initial improvement was further enhanced by studying the influence of physicochemical parameters on the assay performance. Thus, I_{50} for standards was reduced from 0.22 to 0.058 ppb, which confirms the importance of the optimization process to enhance the sensitivity of immunoassays for low molecular weight compounds. Therefore, by combining hapten heterology and assay optimization, a 13-fold improvement in sensitivity was achieved, which enabled us to quantify carbaryl in water at levels down to 0.1 ppb, the maximum concentration for a single pesticide established by European legislation for drinking water. In fact, the detection limit of this assay (0.01 ppb) is 20 times lower than that of the coating conjugate ELISA developed by Marco et al. (1993). Another advantage of the immunoassay herein developed is its higher maximum absorbance, while both ELISAs perform similarly with respect to specificity and assay time. Our immunoassay is also 25 times more sensitive than a commercially available immunoassay for carbaryl (Itak et al., 1993).

ABBREVIATIONS USED

1NAH, *N*-(1-naphthoyl)-6-aminohexanoic acid; BSA, bovine serum albumin; C, concentrated phosphate buffer; CB, C buffer containing 0.1% BSA; CT, C buffer

containing 0.01% Tween 20; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; Igs, immunoglobulins; I_{50}, analyte concentration required for 50% inhibition; MAb, monoclonal antibody; OPD, *o*-phenylenediamine; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20.

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