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Monoclonal Antibody-Based Flow-Through Immunosensor for Analysis of Carbaryl

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Immunosensor systems have been developed for the rapid and sensitive determination of pesticides, using the insecticide carbaryl as a model analyte. The systems are based on the principle of heterogeneous competitive enzyme immunoassay and used mouse monoclonal anti-carbaryl antibodies either in solution (indirect format) or immobilized (direct format). In both formats, enzyme label (horseradish peroxidase, HRP) and fluorometric detection were employed. In the direct format, antibodies were immobilized on a hydrazide-derivatized agarose gel, and 6-[((1-naphthyloxy)carbonyl)amino]hexanoic acid (CNH hapten) conjugated to HRP was used as the enzyme tracer. The limit of detection was 26 ng L^{-1} (11 min/assay), and the useful life of the sensor was 60−70 cycles. In the indirect format, CNH conjugated to bovine serum albumin was immobilized on an *N*-hydroxysuccinimide-derivatized agarose gel, and a rabbit anti-mouse antibody labeled with HRP was used as a secondary immunoreagent. The limit of detection was 284 ng L^{-1} (17 min/assay), and the useful life of the sensor was 160−200 cycles. The developed methods were applied to the analysis of commercial drinking water and apple juice spiked with carbaryl. Interassay RSD ranged from 9 to 39% in the direct format and from 4 to 28% in the indirect format. Recoveries were between 62 and 109% in the direct format and between 78 and 124% in the indirect format. The results were compared with those obtained by enzyme-linked immunosorbent assay as reference method and indicated the suitability of the immunosensor for quality control in water and food analysis.

The extensive use of pesticide formulations in agriculture, together with the increasingly rigorous regulations of the presence of contaminants in the environment and food products, has brought about the need to develop sensitive and accurate methods for the detection and quantitation of pesticide residues. Pesticide analysis methods have been widely reviewed by Sherma.[1]

As an alternative to the commonly used chromatographic methods, immunoassay techniques for the detection of pollutants have grown steadily in recent years.[2] Immunoassays can be performed with different formats, most of them being enzyme immunoassays.

To facilitate sample handling and analysis automation, on-line methods based on the use of immunosensors are being developed.[3] Immunosensors are devices that use immunochemical principles to carry out analysis in a rapid and automated way. Flow-based heterogeneous immunosensors (flow-through sensors) combine the sensitivity and selectivity of immunoassays with the precision and ability to be automated of flow techniques.[4]

Stability and reusability are key factors in the performance of sensors based on heterogeneous immunoassays. The ideal situation would be to integrate a reversible antibody−antigen reaction in a sensor that is able to maintain the same activity through a high number of assays. However, most immunosensors can be used only for a limited number of assays. In flow immunosensors, antibodies or antigens are immobilized on a suitable support, which must be changed or regenerated by disrupting the antigen−antibody complex. The sensitivity of enzyme immunoassays is primarily determined by the affinity of the antibody used. However, high-affinity antibodies need harsh chemical conditions to accomplish an effective immunosurface regeneration, with the subsequent loss of activity. Hence, a compromise between sensitivity and sensor regeneration is necessary. In this sense, the use of specific monoclonal antibodies (MAbs) is advisable, since it allows the selection of the immunoreagent with the most suitable properties.

In the development of flow-based immunosensors, two different alternatives should be considered, i.e., antibody or antigen/hapten immobilization, the first one being the most common approach found in the literature.[4] The main advantage of this option is the economy of expensive antibodies and the reduction of assay steps. The technique of immobilization of the antigen or hapten, although less employed, has the advantage that the regeneration process can be performed without loss of activity of the immobilized reagent.

Another important issue to be addressed in immunosensor development is the choice of immobilization support.[5] To enhance sensitivity, packed bed reactors offer good performance since they minimize sample dispersion.[6] The most used particulate supports for immobilization are glass, silica, and agarose-based gels, and for all of them several methods have been proposed to solve the critical problem of protein immobilization.[7]

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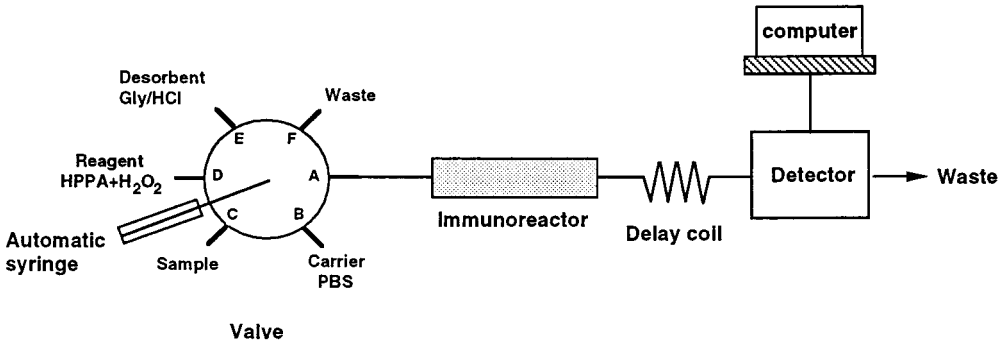

Figure 1. Scheme of the automated flow injection system.

The aim of this work was the development of an immunosensor for the determination of pesticides. As a model system, the insecticide carbaryl was chosen. Carbaryl was the first *N*-methylcarbamate pesticide introduced in the market, and it is still one of the most employed insecticides. Its main degradation product is 1-naphthol. The recommended method for carbaryl determination is high-performance liquid chromatography with postcolumn derivatization and fluorescence detection,[8] and it usually requires sample extraction, cleanup, and preconcentration steps.

For immunosensor development, previously selected anti-carbaryl monoclonal antibodies were used, and the performance of different assay formats was studied, with special attention to assay sensitivity and sensor regeneration properties. Bottled water and fruit juice samples spiked with carbaryl were analyzed to evaluate the applicability of the developed flow-through immunosensors.

EXPERIMENTAL SECTION

Chemicals and Biochemicals. Analytical standards of carbaryl, 1-naphthol, carbofuran, aldicarb, methomyl, methiocarb, and propoxur were purchased from Dr. Ehrenstorfer (Augsburg, Germany). **WARNING:** *Insecticides are toxic chemicals. Working in a well-ventilated fume hood and handling them carefully is recommended. Wastes should be discarded following the security recommendations for hazardous reagents.* Tween-20 and 3-(*p*-hydroxyphenyl)propanoic acid (HPPA) were from Sigma (St. Louis, MO), and Affi-Gel Hz hydrazide and Affi-Gel 15 were from Bio-Rad Laboratories (Richmond, CA). Horseradish peroxidase (HRP) was from Boehringer (Manheim, Germany), and *N*,*N*′-dimethylformamide (DMF) was purchased from Sigma-Aldrich (Madrid, Spain). HRP-labeled rabbit anti-mouse IgG was from Dako (Glostrup, Denmark). All other reagents used were analytical or biochemical grade. Mouse monoclonal anti-carbaryl antibodies were obtained in our laboratories from the synthetic haptens CNH (6-[((1-naphthyloxy)carbonyl)amino]hexanoic acid) and CNA (3-[((1-naphthyloxy)carbonyl)amino]propanoic acid).[9] After characterization by enzyme-linked immunosorbent assay (ELISA), the antibodies with the highest affinity and protein conjugates of the haptens were immobilized on Affi-Gel Hz and Affi-Gel 15, respectively. The immunosorbents were subsequently tested using a screening batch method developed for this purpose.[10] This allowed us to select the most suitable MAb to be used in the flow system and the best working conditions to achieve maximum sensitivity and reusability of the immunosensors.

Synthesis of the Enzyme−Hapten Conjugate. In the preparation of the CNH−HRP enzyme tracer, a variation of the mixed anhydride method was used.[9] Basically, 132.7 μL of a 0.1 M CNH solution in DMF was mixed with 62.8 μL of DMF, 2.9 μL of tri-*n*-butylamine, and 1.6 μL of isobutyl chloroformate, and the mixture was stirred for 1 h at room temperature. An aliquot of the product (100 μL) was diluted with 900 μL of DMF, and 136 μL of the resulting solution was added to 3 mg of HRP dissolved in 1.4 mL of 0.05 M carbonate buffer, pH 9.6. The mixture was stirred for 5 h at room temperature. The final product was purified by gel filtration on Sephadex G-25 using 0.1 M phosphate buffer, pH 7.4, as eluant. From absorbance measurements of the conjugate at 280 and 403 nm, a CNH−HRP concentration of 429 mg L^{-1} and a CNH-to-HRP molar ratio of 3.2 were estimated.

The conjugate solution was fractioned in 0.5 mL aliquots and stored at −20 °C until use. To prepare working solutions, each aliquot was mixed with 0.5 mL of a $(NH_4)_2SO_4$ saturated solution. The resulting suspension was stored at 4 °C and used for not more than 2 weeks.

System Design. The scheme of the manifold used is shown in Figure 1. This system consists of a syringe pump connected to a six-way distribution valve (Kloehn Ltd., Las Vegas, NV). The flow-through immunosensor is connected between a valve port and a Perkin-Elmer LS50 spectrofluorometer (Sussex, U.K.), equipped with a Perkin-Elmer flow cell (15 μL inner volume, λ_{ex} = 320 nm, λ_{em} = 405 nm). Liquid handling and fluorometric signals were managed by means of Kloehn Ltd. Winpump and FL Data Manager (Perkin-Elmer) software packages, respectively. Sigmaplot software package (Jandel Scientific, Erkrath, Germany) was used for data treatment.

Direct Competitive Method. (1) Antibody Immobilization. LIB-CNA36 MAb was immobilized on Affi-Gel Hz according to the manufacturer's instructions.[10] Then, 150 μg of antibody suspended in 50% saturated $(NH_4)_2SO_4$ was dialyzed against 0.05 M sodium acetate buffer, pH 5.5. The dialysate (∼0.5 mL) was mixed with 60 μL of 0.1 M $NaIO_4$ solution in acetate buffer, and the mixture was kept for 1 h at room temperature in the dark with gentle stirring. The product was purified by gel filtration on Sephadex G-25, using acetate buffer as eluant. The first fraction

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Table 1. Scheme of the Flow Sensor Assay Protocol in the Competitive Direct Format

step	event	time (min)
1	syringe washing with 0.55 mL of sample (mixture of enzyme tracer and problem)	0.27
2	injection of 0.55 mL of sample at 0.18 mL min^{-1} flow rate	3.50
3	syringe washing with 1 mL of PBS.	3.88
4	reactor washing (×3) with 1 mL of PBS at 2 mL min^{-1} flow rate	6.30
5	syringe washing with 0.3 mL of substrate (HPPA 0.4 g L^{-1}, H_2O_2 0.006%, in PBS)	6.48
6	injection of 0.15 mL of substrate at 2 mL min^{-1}	6.65
7	delay time of 30 s for incubation	7.15
8	syringe washing with 1 mL of PBS	7.55
9	reactor washing with 1 mL of PBS at 2 mL min^{-1}; peak registration	8.32
10	syringe washing with 1 mL of desorbent solution (0.1 M Gly/HCl, pH 2.00)	8.67
11	reactor desorption with 0.35 mL of desorbent solution at 0.5 mL min^{-1} flow rate	9.03
12	syringe washing with 1 mL of PBS	9.45
13	reactor regeneration (×2) with 1 mL of PBS at 2 mL min^{-1} flow rate; end of cycle	11.0

(3.3 mL) corresponded to the oxidized antibody. The protein content in this fraction determined by Bradford assay[11] was 37 μg mL^{-1}. The oxidized antibody was incubated with 1 mL of Affi-Gel Hz for 24 h at room temperature with gentle stirring. After the coupling reaction, the gel was washed with acetate buffer and then with phosphate-buffered saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4; PBS). The estimated immobilization yield was 69%, and the MAb concentration in the gel was about 82 μg mL^{-1} of gel. The immobilized antibody was kept at 4 °C in 4 mL of PBS containing 0.02% sodium azide as preservative. No detectable loss of antibody activity was observed for more than 9 months.

(2) Standard and Sample Preparation. Stock solutions of pesticides at concentrations ranging from 10 to 20 mg mL^{-1} were prepared in DMF, except carbaryl, which was dissolved at 100 mg L^{-1} in methanol. All stock solutions were kept at −20 °C until use. Standard aqueous solutions were prepared daily by dilution of the stock solutions with water. To test the proposed system, commercially available bottled water and apple juice were used. The water samples were spiked with carbaryl in the range 0.1−0.5 μg L^{-1}. Apple juice samples were spiked with carbaryl in the range 0.1−0.5 mg L^{-1} and diluted 1:1000 (v/v) with water. A 1 mg L^{-1} solution of CNH−HRP conjugate was prepared by diluting a $(NH_4)_2SO_4$ suspension of the enzyme tracer in 10-fold concentrated PBS. Aliquots of these solution were diluted 1:10 with aqueous standards of carbaryl, related compounds, or spiked samples, so that the final saline concentration corresponded to that of PBS. This mixture was kept in ice and protected from light.

(3) Assay Protocol. A description of the events in the assay is given in Table 1. All injections were performed automatically. The total assay time was 11 min.

Indirect Competitive Method. (1) Hapten Immobilization. CNH hapten was immobilized on Affi-Gel 15 through the CNH−BSA conjugate prepared as previously described.[12] Then, 2.5 mL of CNH−BSA (1.2 mg L^{-1}, molar ratio 1:45) in 0.05 M carbonate buffer, pH 9.6, was added to 1 mL of activated gel. The suspension was allowed to react for 4.5 h with gentle stirring at 4 °C. After the coupling reaction, the remaining active sites were blocked by adding 1 mL of 1 M ethanolamine/HCl, pH 8.0, and keeping the mixture for 1 h at room temperature. The gel was then washed with water and stored at 4 °C in PBS containing 0.02% sodium azide. The immobilization yield was estimated from the measurement of the absorbance at 280 nm of the conjugate solution before and after coupling, and it was around 100%. The concentration of immobilized conjugate was estimated to be 3 mg of CNH−BSA/mL of gel. The immobilized conjugate was stored at 4 °C in PBS−NaN_3 and used for more than 5 months without any detectable loss of activity.

(2) Standard and Sample Preparation. Bottled water samples were spiked with carbaryl in the range 0.4−2.0 μg L^{-1}. Apple juice samples were spiked with carbaryl in the range 0.20−1.00 mg L^{-1} and diluted 1:1000 (v/v) with water. A solution of LIB-CNH45 MAb (0.6 mg L^{-1}) was prepared by diluting a 50% saturated $(NH_4)_2SO_4$ suspension of the antibody in 10-fold concentrated PBS. This solution was diluted 1:10 with the pesticide aqueous standard, water, or juice sample.

(3) Assay Protocol. A description of the assay protocol is given in Table 2. The total assay time was 17 min.

Reference Method. An ELISA to carbaryl was chosen and applied as reference analytical method.[13] The suitability of this ELISA for the analysis of carbaryl in fruit juice and water samples had been previously demonstrated.[14,15]

RESULTS AND DISCUSSION

Direct Format. (1) Assay Optimization. Chemical and hydrodynamic variables were optimized for maximal sensitivity, sensor stability, and speed of analysis. Some variables such as the most suitable MAb and desorption reagent were assayed with the batch technique,[10] while sample volume, flow rate, reactor design, and enzyme tracer concentration were tested directly with the flow injection system. For antibody screening, eight monoclonal anti-carbaryl antibodies were immobilized and tested with the batch method for sensitivity and immunosorbent reusability. Although most MAbs gave high assay sensitivity, the only one which could be reused for at least 15 times was LIB-CNA36. The optimum antibody density in the immunosorbent was between 75 and 100 μg/mL of gel. Higher antibody densities diminished assay sensitivity, and lower antibody densities led to excessive loss of activity. With respect to the sensor material and dimensions, the results were obtained with a poly(methyl methacrylate) tube of 5 mm × 2 mm i.d. (inner volume 17 μL). Glass tubes also used to confine the immunosorbent produced nonspecific adsorption of the enzyme tracer, so their use is not recommended.

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Table 2. Scheme of the Flow Sensor Assay Protocol in the Competitive Indirect Format

step	event	time (min)
1	syringe washing with 1 mL of sample (mixture of antibody and problem)	0.30
2	injection of 1 mL of sample at 0.25 mL min^{-1} flow rate	4.47
3	syringe washing with 1 mL of PBS containing 0.05% Tween-20 (PBST)	4.78
4	reactor washing (×2) with 1 mL of PBST at 2 mL min^{-1} flow rate	6.23
5	syringe washing with 0.3 mL of 1:10 000 labeled secondary antibody solution	6.38
6	injection of 0.2 mL of secondary antibody solution at 0.25 mL min^{-1} flow rate	7.28
7	syringe washing with 1 mL of PBST	7.58
8	reactor washing (×4) with 1 mL of PBST at 2 mL min^{-1} flow rate	10.48
9	syringe washing with 0.3 mL of substrate (HPPA 0.4 g L^{-1}, H_2O_2 0.006%, in PBS)	10.63
10	injection of 0.15 mL of substrate at 2 mL min^{-1} flow rate	10.82
11	delay time of 60 s for incubation	11.82
12	syringe washing with 1 mL of PBST	12.13
13	reactor washing with 1 mL of PBST at 2 mL min^{-1}; peak registration	12.82
14	syringe washing with 1 mL of desorbent solution (0.1 M Gly/HCl, pH 2.00)	13.12
15	reactor desorption (×3) with 1 mL of desorbent solution at 2 mL min^{-1} flow rate	15.25
16	syringe washing with 1 mL of PBST	15.53
17	reactor regeneration (×2) with 1 mL of PBST at 2 mL min^{-1}; end of cycle	17

All reagents were diluted in PBS. Different sample volumes were assayed, ranging between 150 and 750 μL. The best result was found for a sample volume of 550 μL at 0.18 mL min^{-1}. Higher flow rates and lower sample volumes diminished assay sensitivity, while lower flow rates and higher samples volumes increased the assay time without improving sensitivity. Enzyme tracer concentrations between 0.025 and 0.25 mg L^{-1} were assayed, and 0.1 mg L^{-1} was chosen as optimum. Lower concentrations did not improve sensitivity, and higher concentrations resulted in poorer sensitivity. Freshly prepared 0.4 g L^{-1} HPPA and 0.006% H_2O_2 were previously selected as optimum concentrations for the enzymatic substrate solution. An incubation time of 30 s was enough to obtain good fluorescence signals.

The most critical parameters in immunosensor development were the desorption solution and its time of application. Using the batch technique, different desorbent solutions were first assayed for all MAbs tested. These solutions were 3 M NaSCN; 0.1 M glycine/HCl, pH 1−3; 0.1 M acetic acid, pH 2.8; 0.1 M phosphate buffer, pH 2.2; and ethanol/water (80:20 v/v). The best results were obtained with 0.1 M glycine/HCl, pH 2.0. In flow assays, the desorption was effectively accomplished by the injection of 0.35 mL of 0.1 M glycine/HCl, pH 2.0 at 0.5 mL min^{-1} flow rate. When glycine solutions at higher pH were tested, the enzyme tracer was desorbed but the carbaryl analyte was not, as evidenced by the reduction of peak heights when new carbaryl solutions were injected. On the other hand, pHs lower than 2.0 produced a rapid decrease of the sensor life.

(2) Sensitivity. Sensitivity was estimated from the calibration data. The analytical signal used in the calibration curves was the normalized signal $100 \times B/B_0$, where B_0 is the peak height of the blank (maximum peak height), and B is the peak height of each standard or sample solution. B/B_0 values were plotted vs carbaryl concentration and the experimental points fitted to a four-parameter logistic equation. Seven calibration curves were performed in quadruplicate in different days (Figure 2). Curves showed I_{50} values ranging from 0.32 to 0.60 μg L^{-1}. The limit of detection (LOD, defined as the analyte concentration for which B/B_0 is 90%) was in the range 0.011−0.034 μg L^{-1} (mean value 0.026 μg L^{-1}). These results indicate that the sensor could be used for carbaryl monitoring in drinking water without sample preconcentration, since its LOD is below the maximum level allowed in the EU.[8] The sensitivity of the immunosensor using the direct competitive format was comparable to that of the competitive indirect ELISA method using MAbs of the same family.[9]

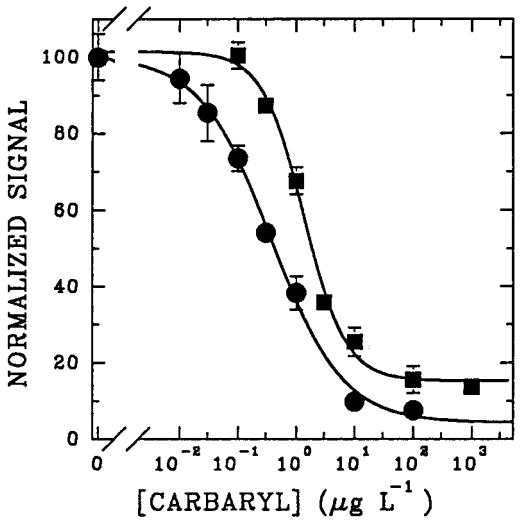

Figure 2. Representative calibration curves for the carbaryl immunosensor, using both direct (●) and indirect (■) formats. Each point represents the mean ± SD of four measurements.

The precision of the method was determined from 11 measurements of standards at 0.30 μg L^{-1} carbaryl. RSD value for B/B_0 was 5.4%.

(3) Sensor Life and Reusability. Reusability of immunosurfaces is the main problem found in immunosensor development. To estimate sensor mean life, repeated analyses of blanks and samples with a carbaryl concentration near I_{50} were carried out. The sensor was considered useful while the normalized B/B_0 signal remained constant, and B_0 did not diminish more than 15% of the initial value.

In a continuous work, B/B_0 remained constant for 60−70 assay cycles. Along these assays, B_0 disminished progressively to 85% of the initial value. More than 70 assay cycles resulted in a rapid decrease of the B_0 value and in significant variations of B/B_0. Therefore, the useful life of the sensor was established as 60−70 assay cycles. Calibration curves performed at the begining and at the end of this period showed very similar I_{50} values, around 0.49 μg L^{-1} (2.44 nM). After 70 assay cycles, the sensor had to

Table 3. Cross-Reactivity of 1-Naphthol and Some *N*-Methylcarbamate Pesticides

	direct format				indirect format			
			LOD				LOD	
compound	I_{50} (nM)	cross-reactivity (%)	in nM	in μg L^{-1}	I_{50} (nM)	cross-reactivity (%)	in nM	in μg L^{-1}
carbaryl	2.13	100	0.13	0.026	5.08	100	1.41	0.284
1-naphthol	200.47	1.06	10.52	1.51	15 450	0.03	1908	275
carbofuran	28.93	7.36	2.88	0.637	$>4 \times 10^4$	<0.013	$>4 \times 10^4$	>9300
methiocarb	36.21	5.88	4.03	0.907	8767	0.06	941	208
propoxur	139.93	1.52	7.46	1.56	$>9 \times 10^4$	<0.005	$>9 \times 10^4$	>19 800
aldicarb	$>4 \times 10^4$	$<4 \times 10^{-3}$	$>5 \times 10^4$	>9000	$>4 \times 10^4$	<0.013	$>4 \times 10^4$	>9000
methomyl	$>10^5$	$<3 \times 10^{-3}$	$>6 \times 10^4$	>19 800	$>10^5$	<0.005	$>10^5$	>25 900

be discarded, since I_{50} drastically increased to 4.7 μg L^{-1}, which was in agreement with the variations found in B_0 and B/B_0 values. No physical damage or loss of hydrodynamic properties of the support was observed.

(4) Cross-Reactivity. For cross-reactivity studies, calibration curves for different *N*-methylcarbamate pesticides and 1-naphthol were performed. Subsequently, their respective I_{50} and LOD were calculated and compared to those of carbaryl. Cross-reactivity was estimated as 100 $\times$ I_{50} carbaryl (nM)/I_{50} related compound (nM). The value of LOD indicates the concentration of each compound below which it is not recognized. As shown in Table 3, methiocarb and carbofuran are the best recognized compounds besides carbaryl, so eventual interferences must be taken into account when applying the method to unknown samples which may contain these insecticides.

In this format, cross-reactivity values were similar to those obtained with the same antibody immobilized on controlled-pore glass.[16] The chemical nature of the compounds assayed with LIB-CNA36 MAb is probably the basis of the recognition pattern found. Thus, *N*-methylcarbamate pesticides that do not possess the aromatic ring (aldicarb and methomyl) were not recognized at all (cross-reactivity below 10^{-4}%), but compounds containing the aromatic rings (carbofuran, methiocarb, propoxur, and 1-naphthol) cross-react to a greater or lesser extent (7.40−1.06 cross-reactivity).

Figure 3a shows the comparative calibration curves for carbaryl and 1-naphthol. The displacement to the right of the 1-naphthol curve indicates that much higher concentrations of the metabolite (100 times) are required to produce the same inhibition as carbaryl.

(5) Analysis of Spiked Samples. Commercial bottled water and apple juice samples were spiked with carbaryl and analyzed by the developed flow-through immunosensor without any sample pretreatment other than a 1:1000 (v/v) dilution for juice samples. Results are shown in Table 4. With the exception of the highest carbaryl concentration assayed in juice samples, good recoveries were obtained, ranging from 77 to 90% in water samples and from 62 to 109% in juice samples. RSDs were also acceptable in both matrices (9−24%), although values higher than 25% were found for the lowest carbaryl concentrations.

Indirect Format. (1) Assay Optimization. In the indirect format, the amount of antibody that binds to the conjugate immobilized on the immunosurface must be measured and correlated to the analyte concentration. This can be accomplished by labeling the primary antibody or by using a labeled secondary antibody raised against the first antibody. The last option was preferred because two or more molecules of secondary antibody can bind to a molecule of the first, thus providing extra amplification. Furthermore, the labeled secondary antibody can be chosen to serve as a universal reagent to mouse immunoglobulins. As a drawback, the use of the secondary antibody implies an additional analysis step, which results in a longer analysis time.

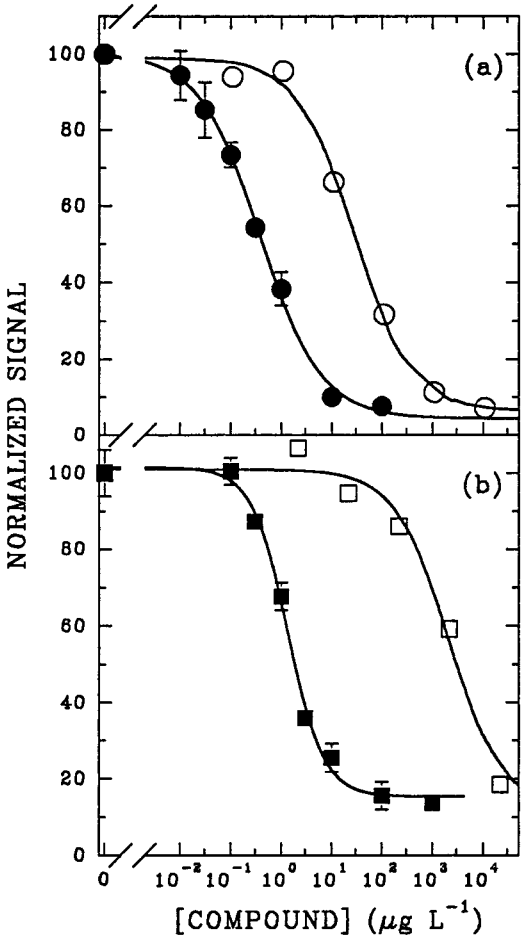

Figure 3. Calibration graphs for carbaryl (full symbols) and 1-naphthol (open symbols) using both direct format (a) and indirect format (b). Each point represents the mean ± SD of four measurements.

The CNH−BSA conjugate was immobilized on Affi-Gel 15. The dimensions of the sensor were the same as in the direct competitive format. A density of immobilization of 3 mg of CNH−BSA conjugate (molar ratio 1:45)/mL of gel was found to be the

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Table 4. Determination of Carbaryl in Bottled Water and Apple Juice Samples Using Direct Format

Bottled Water [carbaryl] (μg L^{-1}) added	found[a]	RSD (%)	recovery (%)
0.10	0.077 ± 0.026	34	77
0.20	0.178 ± 0.053	30	89
0.30	0.285 ± 0.025	9	95
0.40	0.360 ± 0.061	17	90
0.50	0.412 ± 0.037	9	82
Apple Juice[b] [carbaryl] (mg L^{-1}) added	**found[a]**	**RSD (%)**	**recovery (%)**
0.10	0.062 ± 0.024	39	62
0.20	0.200 ± 0.066	33	100
0.30	0.311 ± 0.074	24	104
0.40	0.437 ± 0.084	20	109
0.50	1.399 ± 0.260	19	280

[a] Results expressed as mean ± SD of four measurements. [b] Dilution factor 1:1000 (v/v).

Table 5. Determination of Carbaryl in Bottled Water and Apple Juice Samples Using Indirect Format

Bottled Water [carbaryl] (μg L^{-1}) added	found[a]	RSD (%)	recovery (%)
0.40	0.326 ± 0.077	24	82
0.60	0.437 ± 0.121	28	78
0.80	0.747 ± 0.095	13	93
1.00	1.037 ± 0.056	5	104
2.00	1.960 ± 0.120	6	98
Apple Juice[b] [carbaryl] (mg L^{-1}) added	**found[a]**	**RSD (%)**	**recovery (%)**
0.20	0.248 ± 0.038 (<LOD)	15	124
0.40	0.401 ± 0.026	7	100
0.60	0.940 ± 0.239	25	157
0.80	1.311 ± 0.052	4	164
1.00	1.359 ± 0.286	21	136

[a] Results expressed as mean ± SD of four measurements. [b] Dilution factor 1:1000 (v/v).

optimum and, therefore, was used in all the assays. For MAb selection, data from batch assays indicated that the antibodies LIB-CNH103 and LIB-CNH45 gave the best results in this assay format.[13] These antibodies, together with LIB-CNH36 MAb, which gave the best results in indirect competitive ELISA techniques, were tested in the flow sensor. The antibody LIB-CNH45, at a concentration of 0.6 mg L^{-1}, gave the best assay sensitivity. The optimum sample volume and flow rate were 1 mL and 0.25 mL min^{-1}, respectively. After injection of the mixture of sample and antibody, 0.2 mL of labeled secondary antibody diluted 1:10 000 in PBS containing 0.05% Tween-20 (PBST) was injected at the same flow rate. A washing step with PBST was included to minimize unspecific adsorptions of secondary antibody. In this format, incubation time of the HPPA−H_2O_2 substrate solution was 60 s.

The same desorbent solutions as in the direct format were tested in preliminary batch assays. The best desorbent was also 0.1 M glycine/HCl solution, pH 2.0. In flow assays, complete desorption was accomplished with three cycles of 1 mL of 0.1 M glycine/HCl, pH 2.0, at a flow rate of 2 mL min^{-1}.

(2) Sensitivity. The analytical signal used in the calibration curves for the sensor employing competitive indirect format was also 100 × B/B_0, but in this case the labeled secondary antibody produced a small peak due to unspecific adsorption (typically less than 10% of the full fluorometer scale). This peak was measured every day and substracted in all the measurements.

B/B_0 values were plotted vs carbaryl concentration, fitting the experimental points to the above-mentioned logistic equation (see Figure 2). I_{50} values ranged from 1.02 to 1.39 μg L^{-1}, and LODs were in the range 0.25−0.35 μg L^{-1} (mean value 0.28 μg L^{-1}). These results indicated that this sensor achieved good sensitivity, comparable to other ELISAs for carbaryl employing polyclonal antibodies,[17] and better than a micellar flow injection fluorometric assay for carbaryl.[18]

The precision of the method was also determined from 11 measurements of standards at 1.00 μg L^{-1} carbaryl. The RSD value for B/B_0 was, in this case, 7.8%.

(3) Sensor Life and Reusability. The reactor containing the immobilized haptenic conjugate had very different behavior as compared to the reactor with immobilized antibody. In this case, slight changes in the amount of conjugate immobilized in the reactor did not produce significant variations on the overall performance of the assay. The same immunoreactor was used for five consecutive days, and although the peak heights (B_0 and B) were not exactly the same every day, the calibration curves always showed an I_{50} value around 1 μg L^{-1}. After 160−200 assay cycles, the sensor had to be replaced due to the loss of its hydrodynamic properties. The same batch of immobilized conjugate, stored at 4 °C in PBS−NaN_3, was used for 5 months with no detectable loss of activity.

(4) Cross-Reactivity. Cross-reactivities were determined as described for the direct format. As shown in Table 3, interferences were negligible, since cross-reactivity values were very low for the compounds tested. The values obtained were very similar to those obtained in ELISA with antibodies raised from the CNH hapten.[12] The different behavior of the two immunosensors developed was very likely due to the antibody used in each case, since the selectivity of the assay is primarily determined by the monoclonal antibody used.

(5) Analysis of Spiked Samples. Commercial water and apple juice samples spiked with carbaryl in the range 0.4−2.0 μg L^{-1} and 0.2−1.0 mg L^{-1}, respectively, were analyzed with the indirect immunosensor. Spiked juice samples were diluted 1:1000 with water and analyzed without any other sample pretreatment. Results are given in Table 5. Good recoveries (78−124%) were obtained for all spiked water samples and for spiked juice samples up to 400 μg L^{-1}. Higher concentrations of carbaryl in juice samples were clearly overestimated. In both matrices, RSD values were acceptable, ranging from 5 to 28%.

Comparison of Flow-Through Immunosensors with ELISA Determinations. To asses the analytical performance of the developed flow-through immunosensors, they were compared with ELISA as a reference method. For this purpose, a set of samples were spiked with carbaryl and analyzed as blind samples by both methods. Bottled water samples were spiked in the range 0.05−1 μg L^{-1} and apple juice samples in the range 50−400 μg L^{-1}. The

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Table 6. Comparative Analysis of Carbaryl in Blind Samples by ELISA and Flow-Through Immunosensors

		flow-through immunosensor[a]	
sample	ELISA	direct format	indirect format
W-1[b]	43	49 ± 20	<LOD
W-2	90	81 ± 19	<LOD
W-3	158	178 ± 33	226 ± 14
W-4	282	287 ± 24	336 ± 13
W-5	305	366 ± 32	366 ± 20
W-6	423	419 ± 20	481 ± 21
W-7	756	758 ± 39	691 ± 15
W-8	866	1222 ± 76	1060 ± 124
J-1[c]	43	62 ± 14	<LOD
J-2	97	70 ± 11	<LOD
J-3	237	185 ± 29	257 ± 22
J-4	224	307 ± 53	349 ± 36
J-5	344	436 ± 31	403 ± 32

[a] Values expressed as mean ± SD of four replicates. [b] Carbaryl concentration in ng L^{-1}. [c] Carbaryl concentration in μg L^{-1}.

results are given in Table 6. The intermethods linear regression analysis corresponding to 13 samples showed good correlation coefficients for each method as compared to ELISA, $r = 0.971$ (slope 1.256) for the direct immunosensor and $r = 0.963$ (slope 1.024) for the indirect one. For the direct immunosensor, RSDs were below 20%, with the exception of samples containing the lowest carbaryl concentrations (<0.1 μg L^{-1}). For the indirect format, RSD values were near or below 10%, but in this case the lowest carbaryl concentrations could not be determined.

All these results indicated the suitability of both immunosensors for the analysis of carbaryl in these matrices. Nevertheless, there are some differences between methods that can allow the selection of one of them. The indirect format has the advantage of higher reactor reusability, as well as the option of using any suitable antibody without the need of preserving its activity after desorption. Also, the indirect method is more specific and precise than the direct one. However, the direct format is recommended due to its lower assay time, highest correlation with ELISA, and minimum antibody consumption. In addition, the main advantage of this format is its low limit of detection, which allows the determination of carbaryl in water at levels below the maximum residue limits established by the European Union for drinking water, without sample preconcentration.

CONCLUSIONS

On the basis of the use of MAbs, two immunosensors have been developed for the analysis of the insecticide carbaryl, using both direct and indirect formats. In immunosensor development, the optimum procedure of immunosurface regeneration was different for each hapten−antibody pair tested, although the acidic elution with glycine/HCl solutions proved to be the most appropriate. As a consequence of the different recognition properties of the MAbs used in each format, the flow-through sensor used in the indirect format is specific for carbaryl, while that based on the direct format also recognizes, to some extent, other *N*-methylcarbamate insecticides.

Sensors based on immobilized conjugates (indirect format) were more stable than those based on immobilized antibodies (direct format). This behavior could be due to the fact that the immobilized conjugate is not the active reagent, but it has only to maintain its integrity under desorption conditions to be recognized by the antibody. On the contrary, the immobilized antibody could be irreversibly denatured by repeated desorption processes, thus losing its recognition properties.

In spite of both developed immunosensors having proven their suitability for carbaryl analysis in water and fruit juice samples, the direct format is recommended for routine applications, mainly due to its higher sensitivity, antibody economy, and lower assay time.

Biosensors based on heterogeneous immunoassays provide a very sensitive, rapid, and automated control test for haptenic molecules such as pesticides, with little sample pretreatment. The high sensitivity of the immunosensor based on the direct format, which is comparable to the sensitivity of the best instrumental methods, makes it appropriate for the analysis of drinking water without preconcentration. For fruit juice analysis, dilution is the only sample treatment required.

To improve the performance of flow-through immunosensors, especially their useful life, new assay formats using alternative immunoreagents are currently under investigation. Provided that MAbs for other analytes are available, the applicability of the developed methodology could be extended by exploring new possibilities, such as the analysis of other target compounds and/or the use of different detection systems.

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