Monoclonal Enzyme Immunoassay for the Analysis of Carbaryl in Fruits and Vegetables without Sample Cleanup

Antonio Abad,[†] María-José Moreno,[†] Rosa Pelegrí,[‡] María-Isabel Martínez,[‡] Adolfo Sáez,[‡] Miguel Gamón,[‡] and Angel Montoya*,[†]

Laboratorio Integrado de Bioingeniería, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain, and Laboratorio Agroalimentario de la Generalitat Valenciana, C/Pintor Goya 8, 46100 Burjassot (Valencia), Spain

The *N*-methylcarbamate pesticide carbaryl is one of the most important insecticides used worldwide. In the present work, the validation of a monoclonal antibody-based enzyme immunoassay (ELISA) for the determination of this compound in fruits and vegetables is described. The immunoassay is a competitive heterologous ELISA in the antibody-coated format, with an I_{50} value for standards in buffer of 101.0 ± 26.9 ng/L and with a dynamic range between 31.6 and 364.0 ng/L. For recovery studies, peppers, cucumbers, strawberries, tomatoes, potatoes, oranges, and apples were spiked with carbaryl at 10, 50, and 200 ppb. After liquid extraction, analyses were performed by ELISA on both extracts purified on solid-phase extraction (SPE) columns and crude, nonpurified extracts. Depending on the crop and the fortification level, recoveries in the 59.0−120.0% range were obtained for purified samples and in the 70.0−137.7% range for crude extracts. The carbaryl immunoassay performance was further validated with respect to high-performance liquid chromatography (HPLC) with postcolumn derivatization and fluorescence detection (EPA Method 531.1). Samples were spiked with carbaryl at several concentrations and analyzed as blind samples by ELISA and HPLC after SPE cleanup. The correlation between methods was excellent ($y = 1.04x + 0.71$, $r^2 = 0.992$, $n = 33$), with HPLC being more precise than ELISA (mean coefficients of variation of 5.2 and 12.0%, respectively). The immunoassay was then applied to the analysis of nonpurified extracts of the same samples. Results also compared very well with those obtained by HPLC on purified samples ($y = 1.28x - 0.59$, $r^2 = 0.987$, $n = 33$) while maintaining similar precision. Therefore, the developed immunoassay is a suitable method for the quantitative and reliable determination of carbaryl in fruits and vegetables even without sample cleanup, which saves time and money and considerably increases sample throughput.

Keywords: *Immunoassay; ELISA; pesticide; N-methylcarbamates; HPLC; analysis; validation*

INTRODUCTION

Carbaryl (1-naphthyl *N*-methylcarbamate) is an insecticide widely used in agriculture to combat a large number of pests in a great variety of crops because of its effectiveness and low acute toxicity in mammals (*1*). In fact, carbaryl is one of the most frequently detected pesticide residues in food analysis worldwide, and although violative levels are fortunately only exceptionally found (*2*), the presence of traces of carbaryl in fruits and vegetables poses a potential hazard for consumers. In this respect, carbaryl toxicity is being scrutinized more now, because of concerns about long-term effects on fish and aquatic organisms and the unresolved mechanism by which carbaryl induces expression of cytochrome P450 1A1 (*3*−*5*).

Due to the polarity and thermal instability of carbaryl, the prevalent analytical method for carbaryl determination is high-performance liquid chromatography (HPLC), which is used in combination with postcolumn derivatization and fluorescence detection to obtain the desired sensitivity (U.S. EPA Method 531.1) (*6*, *7*). This procedure requires complex and expensive instrumentation that has to be managed by highly qualified personnel, and samples need to be carefully cleaned up prior to analysis. Therefore, although sensitive and well-established, the method is not very well suited for the analysis of the large number of samples required in comprehensive monitoring programs.

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 and do not require sophisticated instrumentation. In addition, they are reputed to be able to simultaneously analyze a large number of samples without sample cleanup and with accuracy and precision comparable to those reached by chromatographic methods (*8*−*10*). All of these features make immunoassays very promising analytical tools in pesticide monitoring programs, particularly for those chemicals that are difficult and/or costly to determine by conventional chromatographic techniques or for specific pesticides that deserve special attention because of their toxicity, extensive use, or high frequency of appearance in foodstuffs. Nevertheless, immunoassays have not currently reached a wide acceptance among analytical chemists as alternative and/or complemen-

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

[†] Universidad Politécnica de Valencia.

[‡] Laboratorio Agroalimentario de la Generalitat Valenciana.

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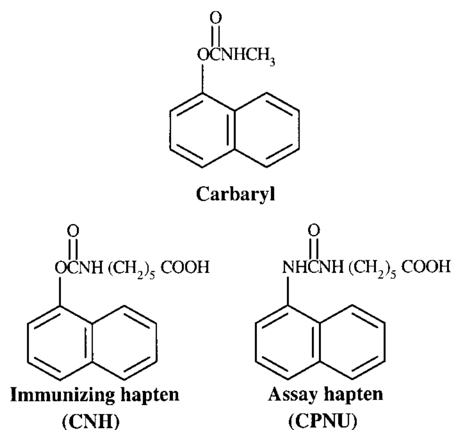

Figure 1. Chemical structures of carbaryl and the haptens used to develop the immunoassay.

tary methods for the analysis of agrochemicals in fruits and vegetables. This situation may be ascribed to the lack of rigorous and comprehensive validation studies in foodstuffs, so the misconception that enzyme-linked immunosorbent assays (ELISAs) are suitable for the determination of pesticide residues in water but not in food samples is still a widespread belief.

The first described immunoassays for carbaryl were based on polyclonal antibodies, and they were applied to the analysis of carbaryl in waters (*11*) and in vegetable and fruit extracts (*12*). We have recently developed a monoclonal antibody-based ELISA for carbaryl (*13*), which was first applied to the analysis of this pesticide in water (*14*) and fruit juices (*15*). The present work describes the validation of this ELISA for the determination of carbaryl residues in fruits and vegetables. A number of matrices spiked with carbaryl at several levels were extracted and processed as required for HPLC analysis and further analyzed by both ELISA and HPLC. ELISA results were compared in terms of precision and accuracy with those generated by HPLC as the reference method. Moreover, the possibility of eliminating the sample cleanup step for ELISA determination was assessed. To this purpose, spiked samples were also analyzed by immunoassay as crude extracts without purification.

MATERIALS AND METHODS

Chemicals and Immunoreagents. Enzyme immunoassay grade horseradish peroxidase (HRP) for enzyme tracer preparation was purchased from Boehringer Mannheim (Barcelona, Spain), and *o*-phenylenediamine (OPD) for ELISA color development was obtained from Sigma Química (Madrid, Spain). The production of the anti-carbaryl monoclonal antibody (LIB-CNH45 MAb), as well as the synthesis and preparation of the HRP−CPNU assay conjugate, was carried out in our laboratory as previously reported (*13*). Figure 1 shows the structures of the immunogenic hapten [CNH, 6-[[1-naphthyloxy)carbonyl]-amino]hexanoic acid] and the assay hapten [CPNU, 1-(5-carboxypentyl)-3-(1-naphthyl) urea]. Carbaryl structure is also included for comparison.

Dichloromethane, acetone, and petroleum ether for pesticide residue analysis and HPLC grade methanol and acetonitrile were obtained from Scharlau (Barcelona, Spain). Water for HPLC analysis was produced using a Barnstead Nanopure II system (Dubuque, IA). Reagent grade *o*-phthaldialdehyde (OPA), 2-mercaptoethanol, sodium hydroxide, and sodium tetraborate decahydrate were from Merck (Darmstadt, Germany).

OPA reagent was prepared fresh daily by dissolving 50 mg of *o*-phthaldialdehyde in 5 mL of methanol. This solution was transferred to a 500-mL volumetric flask and diluted to mark with 0.05 M sodium borate. After filtering and degassing, 25 μL of 2-mercaptoethanol was added.

Carbaryl standard (≥99.9%) was from Riedel-de Haën (Seelze, Germany). A 20 ppm standard stock solution in methanol was made by dilution of a 100 mM solution prepared by dissolving 30−40 mg of carbaryl standard in *N,N*-dimethylformamide (DMF). The stock solution was stored in an amber flask at −20 °C and used to daily prepare fresh working standard solutions for ELISA and HPLC determinations, as well as carbaryl solutions for fortification studies.

ELISA Instrumentation. Ninety-six-well ELISA polystyrene plates (High Binding Plates, catalog no. 3590) were from Costar (Cambridge, MA). Plates were washed with a 96PW microplate washer from SLT (Salzburg, Austria). Absorbance in the ELISA wells was read in dual-wavelength mode (490−650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA). Data processing and analysis were performed using SOFTmax PRO software from Molecular Devices and Sigmaplot software from Jandel Scientific (San Rafael, CA).

HPLC Instrumentation. All of the equipment for HPLC analysis was supplied by Waters (Milford, MA). The instrument consisted of a so-called carbamate analysis system (which includes a quaternary analytical pump and the reaction coils and oven required for postcolumn *N*-methylcarbamate hydrolysis and derivatization) equipped with a 600E system controller, a 715 variable-volume injector and autosampler, two pumps for delivering the OPA and NaOH solutions to the postcolumn reaction coils, an in-line degassing system, and a 474 fluorescence detector for signal monitoring. Data acquisition and processing were performed on a Digital Venturis 486 computer using Waters Millenium software, version 2.15.2.

Sample Fortification and Extraction. Fruits and vegetables were bought from a local market. Once confirmed by HPLC analysis that the samples did not contain residues of carbaryl, they were used for recovery studies. Carbaryl solutions at 2, 10, and 40 ppb in dichloromethane were prepared from the 20 ppm stock solution, and 75 mL of these fortification solutions was added to 15 g of well-mixed, chopped crop sample, so fruits and vegetables were spiked at 10, 50, and 200 ppb. After 10 min, organic solvent was evaporated at reduced pressure in a water bath kept at 30 °C. For method comparison the same procedure was followed, but the volume of the carbaryl solutions in dichloromethane added to the samples was variable in order to fortify samples at several levels.

Sample extraction and purification were performed according to the method of De Kok and Hiemstra (*16*). Fortified samples were homogenized with 30 mL of acetone in a centrifuge bottle for 30 s with an Ultra-turrax T-25 apparatus from Hanke and Junkle. Thirty milliliters of dichloromethane and 30 mL of petroleum ether were subsequently added, and the mixture was homogenized for another 60 s. After centrifugation for 5 min at 4000 rpm, the organic layer was transferred to a graduated cylinder to determine the recovered volume. Two portions of 20 mL were transferred to conical-bottom flasks, and the solvent was evaporated to dryness at reduced pressure in a water bath at 30−35 °C. One of the aliquots was redissolved in 10 mL of water, pH 3, while the other one was redissolved in 10 mL of dichloromethane and purified on a 1-g Bond-Elut aminopropyl-bonded silica extraction column (Varian/Analytichem, Harbor City, CA). Extracts in water were determined only by ELISA, whereas cleaned up (column purified) extracts were analyzed by both HPLC and ELISA for direct comparison of method performance. Solid-phase extraction (SPE) cartridges were conditioned with 10 mL of dichloromethane. After application of the extract, carbaryl was eluted from the column with 5 mL of dichloromethane and 10 mL of dichloromethane/methanol (99:1). To minimize pesticide losses in this step, the collection of the eluant was started at the same time as the sample was applied to the column. Finally, the eluant was evaporated to dryness, redissolved in 10% methanol in water, pH 3, and filtered through Gelman Acrodisc GHP filters (13 mm, 0.45 μm). Both purified and

nonpurified sample extracts were stored at −20 °C in the dark until analysis (typically 1 week). To evaluate method reproducibility, six replicates of each matrix were spiked at each of the fortification levels mentioned above.

ELISA Determinations. Ninety-six-well microtiter plates were coated by adding 100 μL per well of a 1.5 μg/mL solution of the LIB-CNH45 MAb in 50 mM carbonate buffer, pH 9.6. After overnight incubation at room temperature, plates were washed four times with 0.15 M NaCl containing Tween 20 (0.05%, v/v). At this stage ELISA plates were ready for carbaryl analysis. Working standard solutions in the 5×10^{-1}−4×10^{4} ng/L range were prepared from the carbaryl stock solution by serial dilution (1/5) in assay buffer (100 mM sodium phosphate, 137 mM NaCl, pH 7.2). Fifty-microliter of standards or sample extracts adequately diluted in assay buffer were added to triplicate wells, followed by 50 μL per well of a 70 ng/mL solution of the HRP−CPNU conjugate in assay buffer containing 0.1% bovine serum albumin (BSA) and 0.02% thimerosal. Plates were incubated for 1 h and washed as before, and finally the color was developed by adding 100 μL per well of a 2 mg/mL OPD solution in reaction buffer (25 mM sodium citrate, 62 mM sodium phosphate, 0.012% H_2O_2, pH 5.4). After 10 min at room temperature, the enzymatic reaction was stopped by adding 100 μL of 2.5 M sulfuric acid, and the absorbance was read. Absorbance values from standards were mathematically fitted to a four-parameter logistic equation, and the analyte concentration of samples was determined by interpolation of the mean absorbance on the resulting standard curve.

HPLC Determinations. Analysis of carbaryl was performed on a Waters C18 (4 μm particle size) 150 × 3.9 mm i.d. reversed-phase column used in conjunction with Waters Nova-Pak C18 guard-pak inserts. The column oven temperature was kept at 30 °C, and the sample injection volume was 400 μL. The mobile phase flow rate was kept at 1.5 mL/min. Chromatographic runs were performed using a water/methanol/acetonitrile ternary gradient. Postcolumn oven temperature was kept at 80 °C. Both NaOH and OPA solutions were delivered to the hydrolysis and derivatization system at 0.5 mL/min. Detection of carbaryl as the fluorescent isoindole derivative was carried out using 339 and 445 nm as wavelengths for excitation and emission, respectively. Sample concentrations were calculated by external calibration. Calibration standards of carbaryl at 100, 25, and 5 ppb were prepared in water, pH 3, from the stock solution and run at the beginning of the analysis and every 10 samples.

RESULTS AND DISCUSSION

Analytical Characteristics of the Carbaryl ELISA. For the development of an ELISA to carbaryl, five haptens mimicking the carbaryl structure were synthesized. All of them were conjugated to BSA and used as immunogens for MAb production. Haptens were also conjugated to HRP and OVA to be used as assay conjugates. On the basis of a thorough characterization of the panel of MAbs obtained in combination with all of the assay conjugates, a highly sensitive ELISA to carbaryl was developed by using LIB-CNH45 MAb with HRP−CPNU assay conjugate in the antibody-coated ELISA format (*13*).

In the course of carbaryl analysis in fruits and vegetables, an eight-point standard curve was included in each ELISA plate to better estimate analyte concentrations. As it corresponds to competitive assays, the signal was inversely proportional to the analyte concentration and standard points fitted to a sigmoidal equation. The average maximum signal was 1.32 ± 0.41. The ELISA standard curve shown in Figure 2 was obtained by averaging 24 individual standard curves normalized by expressing the absorbance (A_{490}) of each standard point as the percentage of the maximum response [$100 \times (A_{490}/A_{490,max})$]. The sensitivity of the immunoassay for standards in buffer, expressed as the analyte concentration that reduces the assay signal to 50% of the maximum value (I_{50}), is 101.0 ± 26.9 ng/L, and the central section of the curve with a nearly linear response (I_{80}−I_{20}) was assumed as the operative working range of the assay (31.6−364.0 ng/L).

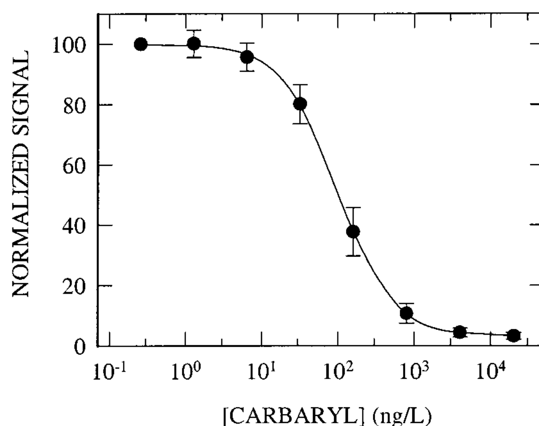

Figure 2. Normalized average standard curve for carbaryl ELISA. Each point represents the mean of 24 values ± standard deviation. Immunoassay conditions are described under Materials and Methods.

To assess whether the immunoassay was prone to suffer from interferences from other compounds, assays were performed with several carbamates as competitors (carbofuran, methiocarb, propoxur, bendiocarb, and aldicarb), and their respective I_{50} values were compared with that of carbaryl. The immunoassay proved to be very specific for carbaryl, because none of the compounds assayed was significantly recognized (cross-reactivity <0.1%). Not even the pesticide metabolite 1-naphthol was in fact recognized.

Recovery Studies in Purified Sample Extracts. Peppers, cucumbers, strawberries, tomatoes, potatoes, oranges, and apples were chosen as model matrices to evaluate the suitability of the immunoassay for the analysis of carbaryl in vegetables and fruits. Six replicates of each matrix were spiked at 10, 50, and 200 ppb. These fortification levels are well below the maximum residue limits (MRLs) for carbaryl in these crops, which are in the 1.0−5.0 ppm range, with the exception of potatoes, for which the MRL is established at 0.1 ppm.

Common procedures for the chromatographic analysis of *N*-methylcarbamate pesticides include a column cleanup step to remove interfering compounds. Accordingly, 20-mL portions of the sample extracts were cleaned up as is done for HPLC analysis. Column-purified samples were then adequately diluted in assay buffer to bring the samples into the working range of the ELISA standard curve and to minimize potential matrix effects. Finally, samples were analyzed by ELISA. Irrespective of the matrix, mean recoveries were 88.1, 87.5, and 87.3% for samples spiked at 10, 50, and 200 ppb, respectively (Table 1). With respect to the crop and irrespective of the fortification level, mean recoveries ranged from 69.8% in apples to 110.2% in oranges.

Recovery Studies in Nonpurified Sample Extracts. Although the above-described results proved that this immunoassay was able to quantitatively analyze carbaryl in a variety of matrices, the inclusion of the sample cleanup step has an evident detrimental effect on sample throughput and method simplicity, two of the main advantages of immunoassays over chromatographic methods. Therefore, experimental work

Table 1. Recovery Studies by ELISA of Purified and Nonpurified Sample Extracts of Fruits and Vegetables Spiked with Carbaryl at 10, 50, and 200 ppb (n = 6 Replicates)

fortification level (ppb)	matrix	purified samples			nonpurified samples		
		mean ± SD (ppb)	recovery (%)	CV (%)	mean ± SD (ppb)	recovery (%)	CV (%)
10	pepper	8.9 ± 1.1	89.0	12.4	7.0 ± 0.6	70.0	8.6
	cucumber	9.4 ± 0.2	94.0	2.1	10.2 ± 0.4	102.0	3.9
	strawberry	8.0 ± 0.4	80.0	5.0	7.2 ± 0.4	72.0	5.6
	tomato	7.9 ± 0.6	79.0	7.6	8.2 ± 0.7	82.0	8.5
	potato	9.6 ± 0.6	96.0	6.3	9.3 ± 0.5	93.0	5.4
	orange	12.0 ± 0.8	120.0	6.7	11.7 ± 0.4	117.0	3.4
	apple	5.9 ± 0.9	59.0	15.3	9.5 ± 0.6	95.0	6.3
mean			**88.1**			**90.1**	
50	pepper	42.2 ± 2.2	84.4	5.2	39.5 ± 5.9	79.0	14.9
	cucumber	43.0 ± 2.8	86.0	6.5	49.2 ± 1.6	98.4	3.3
	strawberry	39.4 ± 5.1	78.8	12.9	46.5 ± 1.9	93.0	4.1
	tomato	44.0 ± 3.3	88.0	7.5	56.1 ± 3.3	112.2	5.9
	potato	49.4 ± 2.6	98.8	5.3	49.5 ± 2.9	99.0	5.9
	orange	49.3 ± 2.2	98.6	4.5	48.3 ± 5.3	96.6	11.0
	apple	39.1 ± 1.7	78.2	4.3	50.6 ± 2.6	101.2	5.1
mean			**87.5**			**97.1**	
200	pepper	141.2 ± 11.5	70.6	8.1	182.4 ± 13.1	91.2	7.2
	cucumber	172.5 ± 13.7	86.3	7.9	215.7 ± 13.1	107.9	6.1
	strawberry	167.3 ± 13.5	83.7	8.1	215.3 ± 7.9	107.7	3.7
	tomato	171.1 ± 27.8	85.6	16.2	275.4 ± 29.0	137.7	10.5
	potato	201.5 ± 11.2	100.8	5.6	218.7 ± 12.0	109.4	5.5
	orange	223.7 ± 52.2	111.9	23.3	253.6 ± 72.8	126.8	28.7
	apple	144.6 ± 19.9	72.3	13.8	236.2 ± 18.1	118.1	7.7
mean			**87.3**			**114.1**	

was undertaken to assess whether this immunoassay was able to quantitatively analyze nonpurified sample extracts. To this purpose, 20-mL portions of the original extracts in organic solvent were evaporated, redissolved in water, pH 3, and analyzed by ELISA after being properly diluted in assay buffer. Samples were analyzed unfiltered, even with solid particles in suspension. With respect to the fortification level, mean recoveries were 90.1, 97.1, and 114.1% for samples spiked at 10, 50, and 200 ppb, respectively, whereas with regard to the matrix mean recoveries ranged from 80.1% for peppers to 113.5% for oranges (Table 1). Mean recovery values were therefore higher than those found with cleaned up samples, especially for cucumbers, strawberries, tomatoes, and apples.

Overall, these results prove not only that sample cleanup may be omitted for ELISA determinations without deleterious effects but also that this approach can provide even more accurate results while maintaining comparable precision. Taking into account the procedure applied for sample extraction, the sample dilution requirement of the immunoassay (minimum 1/20), and the operative working range of the ELISA standard curve, the limit of quantitation of the whole method for fruits and vegetables can be established at 3.4 ppb.

Comparison between Immunoassay and HPLC Analyses. Correlation studies between methods were performed on 13 samples each of strawberries, apples, and peppers spiked with carbaryl. After liquid extraction, a portion of the extract was evaporated and redissolved in water, whereas another portion was cleaned up on an SPE column. Column-purified sample extracts were analyzed by both ELISA and HPLC with fluorescence detection, whereas crude extracts were analyzed only by ELISA because nonpurified food samples are not amenable to HPLC analysis. Samples were identified by a code number, so the carbaryl concentration was unknown to the analysts. Furthermore, ELISA and HPLC analyses were performed in different laboratories, and the results were put together and compared only after the whole study was finished. The analytical results obtained with the two types of samples by the two methods are shown in Table 2. Six samples were scored as negative by both HPLC and ELISA, and on both purified and nonpurified sample extracts. Linear regression analysis on data obtained with purified samples yielded an excellent correlation between methods (r^2 = 0.992), with a slope of 1.04 (Figure 3). Method precision, based on three determinations of each sample performed on different days, was 12.0% for ELISA and 5.2% for HPLC. Therefore, when applied to purified extracts, HPLC provided more precise determinations than the immunoassay, whereas both methods yielded nearly identical carbaryl concentrations. Linear regression analysis was also applied to data provided by ELISA for nonpurified samples versus those provided by HPLC for purified samples (Figure 4). Despite the fact that in this case the comparison was established on samples subjected to different treatments, ELISA results correlated well with HPLC (r^2 = 0.987) while maintaining similar precision values (mean CV = 9.0%). The only significant discrepancy between results was the slope of the linear regression analysis, which was >1.0 ($y = 1.28x - 0.59$). Therefore, either ELISA analysis of nonpurified samples overestimated carbaryl concentrations or results for purified samples (by both HPLC and ELISA) underestimated the true values. To answer this question, carbaryl concentrations in blind spiked samples were requested from the person who had prepared them, and these data were compared with the three sets of analytical determinations. Linear regression analysis proved that carbaryl concentrations in nonpurified samples determined by ELISA were much more similar to the spiked values ($y = 1.00x - 2.64$, $r^2 = 0.989$) than those in purified samples determined by HPLC ($y = 0.77x - 0.80$, $r^2 = 0.979$) or ELISA ($y = 0.81x - 0.48$, $r^2 = 0.969$). This observation supports the idea that the true carbaryl concentrations are underestimated in samples purified according to HPLC requirements, which is in agreement with results from recovery studies. These findings are probably due to

Table 2. Analysis by ELISA and HPLC of Nonpurified and Purified Extracts of Fruits and Vegetables Spiked with Carbaryl at Unknown Levels ($n = 3$ Determinations)

		purified samples				nonpurified samples	
		ELISA		HPLC		ELISA	
matrix	sample	mean (ppb)	CV (%)	mean (ppb)	CV (%)	mean (ppb)	CV (%)
straw-berry	1	5.6	24.7	5.0	4.2	4.7	16.4
	2	14.8	10.3	13.9	0.8	18.6	10.0
	3	31.7	10.1	29.4	4.1	34.0	9.0
	4	47.9	24.7	40.7	9.8	51.1	11.2
	5	0.0		0.0		0.0	
	6	0.0		0.0		0.0	
	7	98.3	9.8	86.2	5.4	103.6	4.4
	8	112.3	9.6	104.6	7.4	150.9	4.8
	9	0.0		0.0		0.0	
	10	11.5	12.0	9.4	9.3	12.8	25.9
	11	23.7	14.2	18.7	14.8	24.3	8.1
	12	24.0	12.7	21.5	5.5	25.5	9.0
	13	82.2	10.2	66.8	8.7	81.0	5.4
apple	1	0.0		0.0		0.0	
	2	5.7	12.2	5.2	7.7	8.3	21.5
	3	14.7	12.3	14.7	3.9	17.5	5.4
	4	148.7	13.5	141.7	4.7	187.7	6.9
	5	61.2	16.5	55.8	7.1	68.0	3.5
	6	15.6	18.2	15.7	5.2	15.6	12.5
	7	20.9	12.2	23.2	6.3	23.2	8.5
	8	6.0	13.1	6.3	0.0	11.9	7.2
	9	29.9	10.8	27.9	4.3	33.3	2.7
	10	38.0	11.4	35.8	2.3	45.9	8.3
	11	12.9	11.5	12.5	5.6	16.2	5.7
	12	11.1	16.4	10.0	6.3	10.4	8.4
	13	5.5	8.5	5.0	3.5	4.9	14.7
pepper	1	14.2	16.3	14.8	14.0	17.3	7.6
	2	77.4	5.0	77.2	1.8	85.6	2.7
	3	88.9	5.8	88.8	0.8	105.1	10.3
	4	130.3	6.2	134.3	1.9	167.4	6.9
	5	121.5	10.2	114.3	5.6	139.7	10.2
	6	0.0		0.0		0.0	
	7	11.8	13.4	11.8	2.4	13.4	8.6
	8	6.5	15.0	7.1	4.9	8.3	16.2
	9	21.9	9.8	21.8	2.5	31.1	5.6
	10	46.0	8.1	45.0	2.8	64.9	3.1
	11	0.0		0.0		0.0	
	12	32.8	8.5	29.7	6.4	39.3	5.7
	13	40.7	2.2	39.6	1.5	62.5	9.4
mean			**12.0**		**5.2**		**9.0**

carbaryl losses incurred as a consequence of sample cleanup and evaporation/redissolution steps. Therefore, the proven ability of this immunoassay to accurately analyze carbaryl in nonpurified samples entails an undoubted practical advantage over methods requiring a previous sample cleanup.

Conclusions. Results in this work clearly prove that the immunoassay herein presented is able to analyze carbaryl in a variety of fruits and vegetables at levels of regulatory relevance, with accuracy and precision comparable to those obtained with the reference method. This ELISA requires minimum equipment and is easy to perform even by unskilled people. From precoated plates, the immunoassay takes 2 h to be performed, including proper dilution of the samples, with the possibility of analyzing dozens of samples simultaneously. An additional issue of major importance is the ability of this ELISA to determine carbaryl in crude sample extracts, which has several practical consequences. First, time of analysis significantly decreases, allowing a higher sample throughput. Second, the cost of the analysis is reduced, as is organic solvent consumption. Third, the same extract used for chromatographic multiresidue methods is amenable to immunoassay without further treatment, so this immunoassay may be easily included as a complementary method in pesticide regulatory programs.

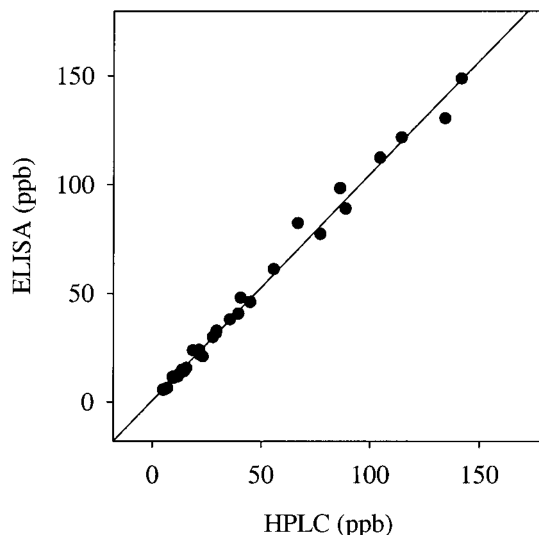

Figure 3. Correlation between ELISA and HPLC results for purified samples spiked with carbaryl. $y = 1.04x + 0.71$, $r^2 = 0.992$, $n = 33$.

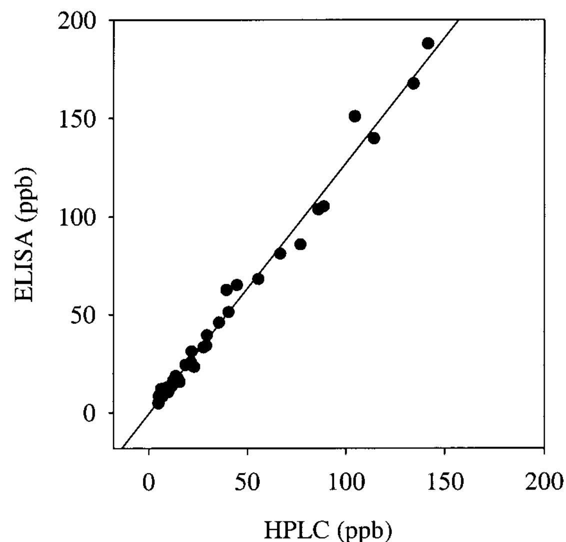

Figure 4. Correlation between ELISA analysis of nonpurified samples and HPLC results for purified samples spiked with carbaryl. $y = 1.28x - 0.59$, $r^2 = 0.987$, $n = 33$.

ELISAs have very often been considered to be valuable methods for screening purposes. Although this is of course an important application of the immunoassay herein described, it is worth emphasizing that the analytical quality of the data, together with the high specificity displayed by this ELISA, also allows its use for confirmation purposes. Overall, these results should reasonably contribute to increase the acceptance of immunological methods among analytical chemists involved in pesticide residue analysis in foods.

ABBREVIATIONS USED

BSA, bovine serum albumin; CNH, 6-[[1-naphthyloxy)carbonyl]amino]hexanoic acid; CPNU, 1-(5-carboxypentyl)-3-(1-naphthyl) urea; DMF, *N,N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; HRP,

horseradish peroxidase; I_x, analyte concentration reducing the assay signal to x% of the maximum value; MAb, monoclonal antibody; OPA, *o*-phthaldialdehyde; MRL, maximum residue limit; OPD, *o*-phenylenediamine; OVA, ovalbumin; SPE, solid-phase extraction.

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