Validation of a Monoclonal Enzyme Immunoassay for the Determination of Carbofuran in Fruits and Vegetables

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The *N*-methylcarbamate pesticide carbofuran is a very important insecticide used worldwide. In the present work, the validation of a monoclonal antibody-based enzyme immunoassay (ELISA) to determine 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 740 ng/L and with a dynamic range between 200 and 3100 ng/L. For recovery studies, peppers, cucumbers, strawberries, tomatoes, potatoes, oranges, and apples were spiked with carbofuran at 10, 50, and 200 ppb. After liquid extraction, analyses were performed by ELISA on extracts purified on solid-phase extraction (SPE) columns and crude, nonpurified extracts. Depending on the crop, mean recoveries in the 43.9−90.7% range were obtained for purified samples and in the 90.1−121.6% range for crude extracts. The carbofuran 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 carbofuran at several concentrations and analyzed as blind samples by ELISA and HPLC after SPE cleanup. The correlation between methods was very good ($y = 0.90x + 2.66$, $r^2 = 0.958$, $n = 25$), with HPLC being more precise than ELISA (mean coefficients of variation of 4.1 and 11.5%, 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 ($y = 1.02x + 10.44$, $r^2 = 0.933$, $n = 29$). Therefore, the developed immunoassay is a suitable method for the quantitative and reliable determination of carbofuran in fruits and vegetables even without sample cleanup, which saves time and money and considerably increases the sample throughput.

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

INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a widely used systemic *N*-methylcarbamate pesticide with predominantly contact and stomach action. It is mainly employed as a soil-applied chemical to control soil-dwelling and foliar-feeding insects and nematodes on a variety of agricultural crops (*1*). Carbofuran is a potent cholinesterase inhibitor (IC_{50} in rats of $1.2-3.3 \times 10^{-8}$ M), so it is highly toxic to humans and wildlife through the oral and inhalation routes of exposure (acute oral LD_{50} in rats of 8 mg/kg) (*2*). In fact, carbofuran has been involved in recent years in numerous cases of bird poisoning, which prompted the U.S. Environmental Protection Agency and Agriculture Canada to review all registered uses of granular carbofuran in their respective countries (*3*, *4*). As a result of its widespread use worldwide—for example, in California alone ~300000 lb of active ingredient was applied per year in the period 1992−1995 (*5*, *6*)—residues of carbofuran may be present in air, soil, water, and food.

Due to the polarity and thermal unstability of carbofuran, the prevalent analytical method for carbofuran 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) (*7*, *8*). 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 by 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 (*9*−*11*). 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

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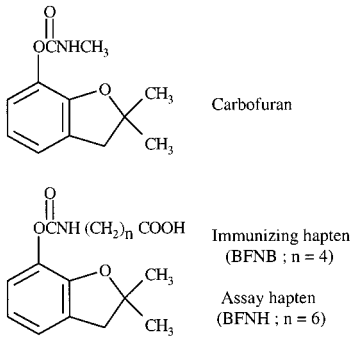


Figure 1. Chemical structures of carbofuran and of the haptens used to develop the immunoassay.

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 complementary 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 enyme-linked immunosorbent assays (ELISAs) are adequate for the determination of pesticide residues in water but not in food samples is still a widespread belief.

We have recently developed a panel of monoclonal antibodies (MAbs) to carbofuran (*12*). The present work describes the validation of a competitive inhibition ELISA based on one of these MAbs for the determination of carbofuran residues in fruits and vegetables. A number of matrices spiked with carbofuran 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-carbofuran monoclonal antibody (LIB-BFNB67 MAb), as well as the synthesis and preparation of the HRP−BFNH conjugate, was carried out in our laboratory as previously reported (*12*). Briefly, six haptens mimicking the carbofuran structure were synthesized. Three haptens differing in the spacer arm length were conjugated to bovine serum albumin (BSA) and used as immunogens for MAb production, whereas all of the synthesized haptens were conjugated to HRP and ovalbumin (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, LIB-BFNB67 MAb and HRP−BFNH assay conjugate were chosen as the most suitable immunoreagents for the development of a competitive inhibition ELISA in the antibody-coated format. Figure 1 shows the structures of the immunogenic hapten and the assay hapten (carbofuran 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 OPA 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.

Carbofuran 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 carbofuran standard in *N*,*N*-dimethylformamide (DMF). The stock solution was stored in an amber flask at −20 °C and used every day to prepare fresh working standard solutions for ELISA and HPLC determinations, as well as carbofuran solutions for fortification studies.

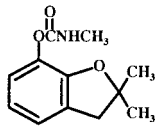
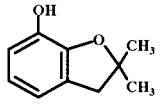
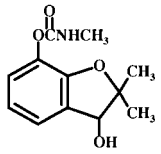
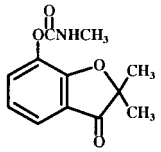
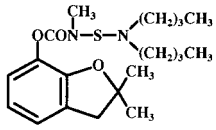
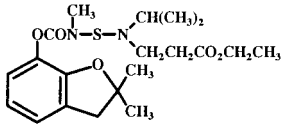
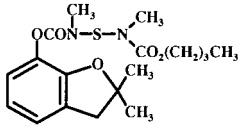
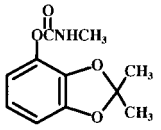
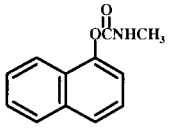
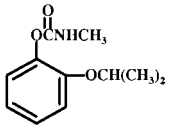
ELISA Instrumentation. Ninety-six-well ELISA polystyrene plates (High Binding Plates, catalog no. 3590) were from Costar (Cambridge, MA). Plates were washed with a 96PW microplate washer from SLT (Salzburg, Austria). Absorbance in the ELISA wells was read in dual-wavelength mode (490−650 nm) with an 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 samples did not contain residues of carbofuran, they were used for recovery studies. Carbofuran 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, the organic solvent was evaporated at reduced pressure in a water bath kept at 30 °C. To prepare blind samples, the same procedure was followed, but the volume of the carbofuran 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 (*13*). 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 light petroleum 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. SPE cartridges were conditioned with 10 mL of dichloromethane. After application of the extract, carbofuran was eluted from the column with 5 mL of dichloromethane and 10 mL of dichloromethane/

Table 1. Recognition of Several Compounds by the Carbofuran Immunoassay

chemical structure	compound	CR (%) [a]
	carbofuran	100.0
	carbofuran-phenol	0.02
	carbofuran-hydroxy	17.0
	carbofuran-keto	0.3
	carbosulfan	2.8
	benfuracarb	16.3
	furathiocarb	38.7
	bendiocarb	17.0
	carbaryl	0.2
	propoxur	1.0

[a] Percentage of cross-reactivity = (I_{50} of carbofuran/I_{50} of other compound) × 100. I_{50} is the analyte concentration that reduces the assay signal to 50% of the maximum value. Cross-reactivity values for the carbamate insecticides methiocarb, aldicarb, and methomyl were <0.01%.

methanol (99:1). To reduce pesticide losses in this step, the collection of the eluent was started at the same time as the sample was applied to the column. Finally, the eluent was evaporated to dryness, redissolved in 10 mL of 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.

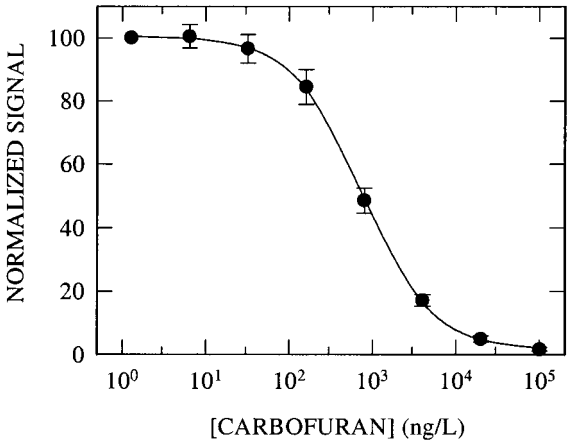


Figure 2. Normalized average standard curve for carbofuran ELISA. Each point represents the mean of 24 values ± standard deviation. Immunoassay conditions are described under Materials and Methods.

ELISA Determinations. Ninety-six-well microtiter plates were coated by adding 100 μL per well of a 1.0 μg/mL solution of the LIB-BFNB67 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 carbofuran analysis. From the carbofuran stock solution (20 ppm in methanol), a 2.0×10^5 ng/L solution was prepared in assay buffer (100 mM sodium phosphate and 137 mM sodium chloride, pH 7.2). This solution was then used to prepare working standard solutions in the 2.0×10^5−2.56 ng/L range by serial dilution (1/5) in assay buffer. Fifty microliters of standards or sample extracts adequately diluted in assay buffer was added to triplicate wells, followed by 50 μL per well of a 100 ng/mL solution of the HRP−BFNH conjugate in assay buffer containing 0.1% 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, and 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 linear portion of the standard curve.

HPLC Determinations. Analysis of carbofuran 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. Mobile phase flow rate was kept at 1.5 mL/min. Chromatographic runs were performed using a water/methanol/acetonitrile ternary gradient. The 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 carbofuran 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 carbofuran 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.

Table 2. Recovery Studies by ELISA of Purified and Nonpurified Sample Extracts of Fruits and Vegetables Spiked with Carbofuran 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	7.6 ± 0.9	76.0	11.8	11.4 ± 1.2	114.0	10.5
	cucumber	8.7 ± 0.9	87.0	10.3	21.9 ± 3.5	219.0	16.0
	strawberry	6.8 ± 1.3	68.0	19.1	12.1 ± 0.9	121.0	7.4
	tomato	5.7 ± 1.0	57.0	17.5	10.3 ± 2.2	103.0	21.4
	potato	5.3 ± 0.5	53.0	9.4	15.8 ± 1.0	158.0	6.3
	orange	7.1 ± 0.5	71.0	7.0	13.4 ± 0.8	134.0	6.0
	apple	2.5 ± 1.1	25.0	44.0	9.8 ± 1.5	98.0	15.3
	mean		**62.4**			**135.3**	
50	pepper	42.4 ± 4.8	84.8	11.3	48.6 ± 8.6	97.2	17.7
	cucumber	46.6 ± 1.8	93.2	3.9	61.6 ± 4.8	123.2	7.8
	strawberry	40.6 ± 3.8	81.2	9.4	46.9 ± 2.6	93.8	5.5
	tomato	38.7 ± 3.7	77.4	9.6	44.8 ± 1.4	89.6	3.1
	potato	36.0 ± 1.3	72.0	3.6	54.0 ± 3.7	108.0	6.9
	orange	39.1 ± 5.9	78.2	15.1	46.7 ± 3.6	93.4	7.7
	apple	26.5 ± 2.0	53.0	7.5	41.1 ± 2.5	82.2	6.1
	mean		**77.1**			**98.2**	
200	pepper	167.5 ± 22.0	83.8	13.1	185.1 ± 10.9	92.6	5.9
	cucumber	183.5 ± 14.7	91.8	8.0	180.4 ± 8.7	90.2	4.8
	strawberry	183.9 ± 14.7	92.0	8.0	198.5 ± 11.0	99.3	5.5
	tomato	151.6 ± 25.3	75.8	16.7	204.7 ± 15.1	102.4	7.4
	potato	155.9 ± 15.6	78.0	10.0	197.5 ± 11.6	98.8	5.9
	orange	180.2 ± 18.5	90.1	10.3	200.8 ± 22.5	100.4	11.2
	apple	107.5 ± 19.7	53.8	18.3	180.0 ± 7.9	90.0	4.4
	mean		**80.8**			**96.2**	

RESULTS AND DISCUSSION

Analytical Characteristics of the Carbofuran ELISA. Characterization studies showed that the proposed immunoassay is quite specific for carbofuran, because cross-reactivity values for other important carbamate compounds such as carbaryl, methiocarb, propoxur, aldicarb, and methomyl were <1.0%. Only compounds very closely structurally related to carbofuran were moderately recognized by the antibody (Table 1). Thus, carbofuran-hydroxy (the main carbofuran metabolite in plants), benfuracarb, and furathiocarb (two carbamate pesticides of which the main degradation product is just carbofuran) and bendiocarb (a scarcely used pesticide in the agrochemical field) exhibited cross-reactivity values in the 16−39% range.

In the course of carbofuran 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 absorbance was 1.17 ± 0.34. 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 740 ± 110 ng/L. The central section of the standard curve with a nearly linear response ($I_{80}-I_{20}$) was assumed as the operative working range of the assay (200−3100 ng/L).

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 carbofuran in vegetables and fruits. Six replicates of each matrix were spiked at 10, 50, and 200 ppb. Maximum residue limits for carbofuran are ∼100 ppb, depending on the crop and the country.

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 (1/5, 1/15, and 1/60 for samples fortified at 10, 50, and 200 ppb, respectively) to bring them 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 62.4, 77.1, and 80.7% 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 43.9% in apples to 90.7% in cucumbers.

Low recoveries observed in samples spiked at 10 ppb, especially for tomatoes, potatoes, and apples, were probably due to matrix effects caused by an insufficient dilution of these sample extracts in the assay buffer. Unfortunately, the use of a higher dilution was not feasible, because this approach had brought the 10 ppb samples out of the working range of the standard curve. Recoveries for samples fortified at 50 and 200 ppb, although clearly <100%, may be considered as acceptable, with the only exception of the apple samples. Furthermore, recoveries did not significantly differ between samples fortified at 50 and 200 ppb, which indicates than a 1/15 dilution of the sample extracts can be enough to minimize matrix effects.

Recovery Studies in Nonpurified Sample Extracts. Although the above-described results proved that this immunoassay was able to quantitatively analyze carbofuran down to 50 ppb in a variety of matrices, the inclusion of the sample cleanup step has an evident detrimental effect on sample throughput and on method simplicity, two of the main advantages of immunoassays over chromatographic methods. There-

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

		purified samples				nonpurified samples	
		ELISA		HPLC		ELISA	
matrix	sample	mean (ppb)	CV (%)	mean (ppb)	CV (%)	mean (ppb)	CV (%)
strawberry	1	<LQ		5.4	5.4	<LQ	
	2	<LQ		15.9	1.2	26.4	26.8
	3	28.5	19.1	31.5	1.5	40.0	22.7
	4	27.2	16.6	30.7	7.0	34.6	5.3
	5	40.0	12.5	42.4	9.5	56.8	7.5
	6	0.0		0.0		0.0	
	7	0.0		0.0		0.0	
	8	99.7	0.7	90.1	6.0	125.1	6.2
	9	134.4	9.2	123.3	3.8	154.3	1.8
	10	0.0		0.0		0.0	
	11	<LQ		9.6	2.6	<LQ	
	12	25.2	11.5	19.2	12.9	40.3	4.1
	13	23.1	13.6	22.2	4.6	33.3	13.0
	14	81.4	10.9	68.7	8.3	74.9	1.9
apple	1	0.0		0.0		0.0	
	2	<LQ		5.1	5.0	<LQ	
	3	<LQ		14.5	5.7	17.6	19.0
	4	58.8	13.5	62.2	2.5	95.1	4.2
	5	122.7	5.8	148.0	1.4	139.3	5.6
	6	48.9	6.1	57.0	4.2	57.2	5.5
	7	<LQ		15.3	4.9	17.3	13.1
	8	24.3	11.3	24.0	1.7	24.8	13.3
	9	<LQ		6.4	2.7	<LQ	
	10	23.6	18.4	27.9	0.6	27.7	9.8
	11	50.6	11.9	49.5	3.7	93.8	10.1
	12	32.9	9.2	36.3	4.2	45.0	9.0
	13	<LQ		12.6	2.0	20.1	13.8
	14	<LQ		9.7	1.2	<LQ	
pepper	1	16.4	18.0	17.4	13.3	26.7	9.7
	2	80.4	4.0	84.5	1.4	85.9	12.8
	3	87.7	13.7	100.6	1.6	104.0	9.2
	4	87.7	5.7	108.7	1.4	105.2	12.0
	5	125.3	9.1	154.0	1.1	152.8	6.4
	6	111.1	8.3	120.3	5.3	136.2	5.1
	7	0.0		0.0		0.0	
	8	<LQ		12.8	5.5	<LQ	
	9	<LQ		7.7	0.0	<LQ	
	10	24.0	11.1	26.1	2.5	42.8	10.9
	11	165.6	11.2	170.3	1.2	200.9	11.7
	12	43.2	21.4	51.3	2.8	70.7	8.5
	13	0.0		0.0		0.0	
	14	30.8	14.4	32.9	10.1	47.2	6.4
mean			**11.5**		**4.1**		**9.8**

fore, experimental work 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 135.3, 98.2, and 96.2% for samples spiked at 10, 50, and 200 ppb, respectively, whereas with regard to the matrix mean recoveries ranged from 90.1% in apples to 121.6% in potatoes (Table 1).

As for the cleaned up sample extracts, matrix effects due to the inadequate dilution of the extract were also recorded for the nonpurified extracts of samples spiked at 10 ppb. However, in this case the effect was the opposite; that is, the ELISA showed a tendency to overestimate carbofuran concentrations, especially for cucumbers, potatoes, and oranges. Mean recovery values at 50 and 200 ppb were higher than those found with cleaned up samples and, more importantly, they were much closer to 100%.

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 similar precision. Taking into account the procedure applied for sample extraction, the dilution requirements of samples for the immunoassay (minimum 1/15), and the operative working range of the ELISA standard curve, the limit of quantitation (LQ) of the whole method for fruits and vegetables can be established at 16 ppb.

Comparison between Immunoassay and HPLC Analysis. Correlation studies between methods were performed on 14 samples each of strawberries, apples, and peppers spiked with carbofuran. 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 carbofuran concentration was unknown to the analysts. Further-

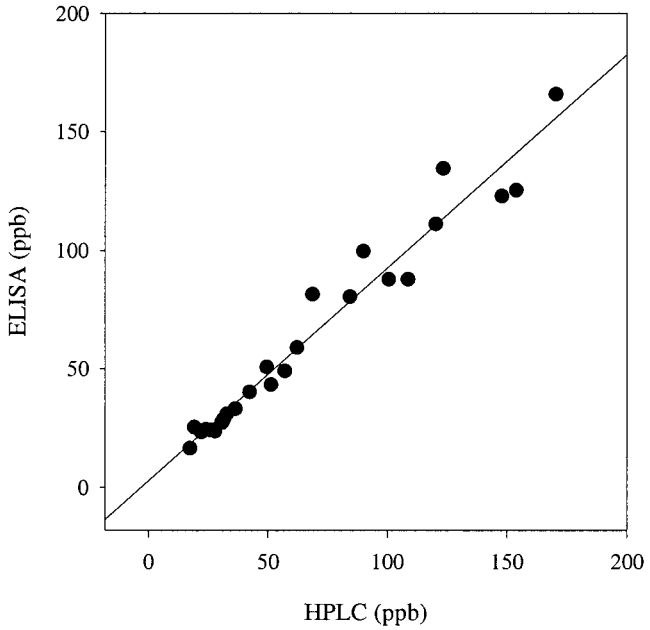


Figure 3. Correlation between ELISA and HPLC results for purified samples spiked with carbofuran. $y = 0.90x + 2.66$, $r^2 = 0.958$, $n = 25$.

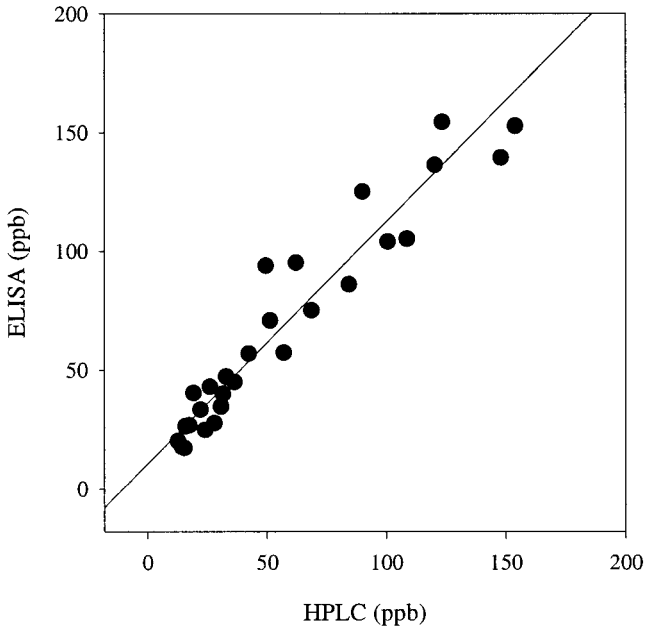


Figure 4. Correlation between ELISA analysis of nonpurified samples and HPLC results for purified samples spiked with carbofuran. $y = 1.02x + 10.44$, $r^2 = 0.933$, $n = 29$.

more, ELISA and HPLC analysis 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 type of samples by the two methods are shown in Table 3. Six samples were scored as negative by both HPLC and ELISA and on both purified and nonpurified sample extracts. All of the remaining 36 samples were perfectly quantifiable by HPLC, whereas 11 purified extracts and 7 nonpurified extracts were under the limit of quantitation of the ELISA. This is in agreement with the lower limit of quantitation of HPLC (5 ppb) as compared with that of ELISA (16 ppb). Linear regression analysis on purified samples yielded a good correlation between methods ($r^2 = 0.958$), although the immunoassay showed

Table 4. Recovery by HPLC (Purified Samples) and ELISA (Nonpurified Samples) with Respect to the Declared Values in Blind Samples

			HPLC		ELISA	
matrix	sample	declared value (ppb)	mean (ppb)	recovery (%)	mean (ppb)	recovery (%)
strawberry	2	20.0	15.9	79.5	26.4	132.0
	3	33.3	31.5	94.6	40.0	120.1
	4	46.6	30.7	65.9	34.6	74.2
	5	60.0	42.4	70.7	56.8	94.7
	8	100.0	90.1	90.1	125.1	125.1
	9	167.0	123.3	73.8	154.3	92.4
	12	26.5	19.2	72.5	40.3	152.1
	13	40.0	22.2	55.5	33.3	83.3
	14	86.6	68.7	79.3	74.9	86.5
apple	3	20.0	14.5	72.5	17.6	88.0
	4	100.0	62.2	62.2	95.1	95.1
	5	167.0	148.0	88.6	139.3	83.4
	6	66.5	57.0	85.7	57.2	86.0
	7	20.0	15.3	76.5	17.3	86.5
	8	26.5	24.0	90.6	24.8	93.6
	10	33.3	27.9	83.8	27.7	83.2
	11	100.0	49.5	49.5	93.8	93.8
	12	46.6	36.3	77.9	45.0	96.6
	13	20.0	12.6	63.0	20.1	100.5
pepper	1	27.0	17.4	64.4	26.7	98.9
	2	93.0	84.5	90.9	85.9	92.4
	3	107.0	100.6	94.0	104.0	97.2
	4	120.0	108.7	90.6	105.2	87.7
	5	173.0	154.0	89.0	152.8	88.3
	6	146.0	120.3	82.4	136.2	93.3
	10	33.0	26.1	79.1	42.8	129.7
	11	213.0	170.3	80.0	200.9	94.3
	12	67.0	51.3	76.6	70.7	105.5
	14	40.0	32.9	82.3	47.2	118.0
mean				**78.0**		**99.0**

a tendency to provide slightly lower carbofuran concentrations than HPLC ($y = 0.90x + 2.66$; Figure 3). Mean coefficients of variation, based on three determinations of each sample performed on different days, were 11.5% for ELISA and 4.1% for HPLC. Therefore, when applied to purified extracts, HPLC provided more precise measurements than the immunoassay, whereas both methods yielded similar carbofuran 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, both sets of measurements correlated well ($r^2 = 0.933$). However, in 26 of 29 samples on which the comparison could be established, ELISA results were higher than those obtained by HPLC (Table 3), as is also evidenced by the intercept of the linear regression analysis ($y = 1.02x + 10.44$). Therefore, either ELISA analysis of nonpurified samples overestimated carbofuran concentrations or results on purified samples underestimated the true values. To answer this question, the two sets of data were compared with the nominal carbofuran concentrations in the blind spiked samples (Table 4). Nominal values were not available to the analysts performing the HPLC or ELISA determinations. Even though linear regression analysis proved that carbofuran determinations by both methods correlated well with the declared values ($r^2 = 0.970$ for ELISA and $r^2 = 0.967$ for HPLC), ELISA results were much closer to the true values than HPLC ones, as evidenced by the mean recovery values (99.0% for ELISA in nonpurified samples and 78.0% for HPLC in purified samples). These findings support the idea that carbofuran concentrations are underestimated in puri-

fied samples, probably due to analyte losses occurring as a consequence of the sample cleanup and additional evaporation/redissolution steps. Overall, these results prove that this ELISA is able to accurately analyze carbofuran without sample cleanup, which entails an undoubted practical advantage of this immunoassay over methods requiring previous sample purification.

Conclusions. The immunoassay herein presented is able to analyze carbofuran 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 sample extracts, with the possibility of analyzing dozens of samples simultaneously. An additional issue of major importance is the ability of this ELISA to determine carbofuran in crude sample extracts, which has several practical consequences. First, the time of analysis significantly decreases, which entails a higher sample throughput. Second, the cost of the analysis is reduced, as is the organic solvent consumption. Third, the same extract used for GC multiresidue methods is amenable to immunoassay without further treatment, so this ELISA may be easily included as a complementary method in pesticide regulatory programs.

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 specificity displayed by this ELISA, also allows its use for confirmation purposes. Overall, this work 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; 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; LQ, limit of quantitation; MAb, monoclonal antibody; OPA, *o*-phthaldialdehyde; OPD, *o*-phenylenediamine; OVA, ovalbumin; SPE, solid-phase extraction.

LITERATURE CITED

(1) Tomlin, C. D. S. In *The Pesticide Manual: A World Compendium*, 11th ed.; Crop Protection Publications: Surrey, U.K., 1997.

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

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

(4) Augspurger, T.; Smith, M. R.; Meteyer, C. U.; Converse, K. A. Mortalities of passerines adjacent to a North Carolina corn field teated with granular carbofuran. *J. Wildlife Dis.* **1996**, *32*, 113−116.

(5) *Farm Chemicals Handbook*; Meister Publishing: Willoughby, OH, 1995.

(6) CDPR (California Department of Pesticide Regulation). *Pesticide Use Reporting;* Sacramento, CA, 1995; http://www.cdpr.ca.gov/docs/pur/purmenu.htm.

(7) Moye, H. A.; Scherer, S. J.; St. John, P. A. Dynamic fluorogenic labeling of pesticides for high performance liquid chormatography: detection of *N*-methylcarbamates with *o*-phthaldehyde. *Anal. Lett.* **1977**, *10*, 1049−1073.

(8) Krause, R. T. Liquid Chromatographic determination of *N*-methylcarbamate insecticides and metabolites in crops. I. Collaborative study. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 726−733.

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

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

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

(12) Abad, A.; Moreno, M. J.; Montoya, A. Development of monoclonal antibody-based immunoassays to the *N*-methylcarbamate carbofuran. *J. Agric. Food Chem.* **1999**, *47*, 2475−2485.

(13) De Kok, A.; Hiemstra, M. Optimization, automation, and validation of the solid-phase extraction cleanup and online liquid chromatographic determination of *N*-methylcarbamate pesticides in fruits and vegetables. *J. AOAC Int.* **1992**, *75*, 1063−1072.

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