Determination of Carbofuran in Water and Soil by a Rapid Magnetic Particle-Based ELISA

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A competitive enzyme-linked immunosorbent assay (ELISA) for the quantitation of carbofuran in water and soil was developed using a magnetic particle-based solid phase. This assay utilizes magnetic particles as the solid phase to attach polyclonal rabbit anti-carbofuran antibodies. The ELISA has an estimated detection limit of 0.056 parts per billion (ppb, ng/mL) of carbofuran in water and 5.6 ppb in soil. The standard curve allows quantitation up to 5.0 ppb in water and 500 ppb in soil. The ELISA compares favorably with HPLC measurements in the analysis of water samples ($r = 0.967$). Recoveries from soils fortified with 0.05–1 ppm of carbofuran averaged 98.6% using 1-h extractions with methanol/water (75:25 v/v).

Keywords: *Carbofuran; carbamate; immunoassay; ELISA; water; soil*

INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a soil-applied insecticide and nematicide, which controls soil-dwelling and foliar-feeding insects, spider mites, and nematodes in vegetables, ornamentals, and agronomic crops such as maize, sorghum, alfalfa, peanuts, and potatoes. Carbofuran, the active ingredient of Furadan (FMC Corp.), has been one of the most widely used pesticides in the United States over the past 25 years (U.S. EPA, 1984). Recently, environmental concern has arisen about carbofuran's toxicity to wildlife. Carbofuran has high acute toxicity to bees, birds, and fish (Briggs, 1992; Eislerl, 1985; U.S. EPA, 1989b; Hudson et al., 1984; Madhun and Freed, 1990). The U.S. EPA proposed cancellation or reduction in use of the granular formulation of Furadan, which has been mistaken by birds as food (*Pesticide and Toxic Chemical News*, 1991, 1994).

Carbofuran may contaminate ground water through runoff and leaching (U.S. EPA, 1984) and is being detected in ground waters of the United States (Parson and Witt, 1988; Cohen et al., 1986; Williams et al., 1988). The maximum contamination level for carbofuran in drinking water is 40 ppb (U.S. EPA, 1990).

The interest in pesticide testing in water, soil, and food has increased dramatically over the past few years. Current testing methods involving gas (GC) and liquid (HPLC) chromatography are expensive and time-consuming and require specialized instrumentation. Growth in pesticide residue testing throughout the world has increased the demand for faster, easier methods that permit the screening of large numbers of samples. The emergence of enzyme immunoassays, which are rapid, sensitive, accurate, and cost-effective, has provided the analytical chemist with an alternative to traditional methods (Van Emon and Lopez-Avila, 1992). The ELISA reported here utilizes magnetic particles as the solid phase to which the antibody is covalently attached. This format eliminates imprecision problems associated with microtiter plates and polystyrene tubes (Howell et al., 1981; Engvall, 1980; Lehtonen and Viljanen, 1980). The benefits and use of magnetic particle-based immunoassays for pesticide detection in water and soil have been described previously (Lawruk et al., 1992; Itak et al. 1992, Rubio et al., 1991).

The assay presented takes less than 1 h to perform and requires no sample preparation for water samples. A simple sample preparation procedure is described to adapt the assay to the analysis of soil samples.

MATERIALS AND METHODS

Immunochemicals. Amine-terminated superparamagnetic particles of approximately 1 μm diameter were obtained from PerSeptive Diagnostics (Cambridge, MA). The procedure for coupling antibodies to paramagnetic particles was previously described (Itak et al., 1992; Rubio et al., 1991). The resulting particle stocks were diluted 1:2000 in buffer for use in the assay. The carbofuran antibodies coupled to paramagnetic particles are available commercially from Ohmicron Corp.

The carbofuran–HRP conjugate was prepared by coupling the ligand, 2,3-dihydro-2,2-dimethylbenzofuran-7-ylbutanoic carbamate, to horseradish peroxidase (HRP). The carbofuran–HRP conjugate is available commercially from Ohmicron Corp. Glutaraldehyde and HRP were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide and tetramethylbenzidine (TMB) were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Additional Chemicals. 3-Ketocarbofuran, 3-hydroxycarbofuran, carbofuranphenol, 3-ketocarbofuranphenol, and 3-hydroxycarbofuranphenol were obtained from FMC Corp. (Princeton, NJ). Carbofuran was purchased from Chem Service (West Chester, PA). Other pesticide standards were purchased from Crescent Chemical Co., Inc. (Hauppauge, NY).

Buffers. Tris-buffered saline (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% BSA was used for diluting the rabbit anti-carbofuran magnetic particles and carbofuran hapten–HRP enzyme conjugate. Sodium acetate buffer (pH 4.0) with 1 mM EDTA and 0.1% gelatin was used to dilute the carbofuran standards.

Equipment. The magnetic separation rack and RPA-I Analyzer were supplied by Ohmicron Corp.

Competitive Immunoassay Procedure. One hundred microliters of the carbofuran standards, control, and samples to be tested was added to a disposable test tube along with

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Table 1. Composition of Soils Fortified with Carbofuran

soil	% sand	% silt	% clay	% humus	CEC[a]	pH
Sassafras sandy loam	60	29	11	2.02	6	7.0
Plano loam	38	48	26	4.5	12	6.1

[a] Cation-exchange capacity (mequiv/100 g of soil).

250 μL of carbofuran–HRP conjugate and 500 μL of solution containing the paramagnetic particles with carbofuran-specific antibodies attached. Tubes were vortexed and incubated for 20 min at room temperature. The reaction mixture was separated in the magnetic separation rack, and the particles were washed twice with water. Five hundred microliters of color reagent (TMB and hydrogen peroxide mixed 1:1) was added per tube. The tubes were vortexed to resuspend particles and incubated for another 20 min at room temperature to allow color development. The color reaction was then stopped with 500 μL of 2 M sulfuric acid solution. The final concentrations of carbofuran for each sample were determined using the RPA-I photometric analyzer, which records the absorbance at 450 nm and compares the observed sample results to a linear regression line using a log/logit standard curve prepared from calibrators containing 0, 0.1, 1, and 5 ppb of carbofuran. The functions of the RPA-I photometric analyzer have previously been described in detail (Rubio et al., 1991). Samples greater than 5 ppb were diluted in the zero standard for analysis.

HPLC Method. Environmental water samples were analyzed by FMC Corp. (Princeton, NJ) by HPLC with postcolumn derivatization and fluorescence detection using a Perkin-Elmer HPLC equipped with a 250 mm by 1.4 i.d. Pickering column, specific for carbamates. Carbofuran was extracted from water samples and concentrated using C_{18} solid phase extraction (Nash, 1990). The detection limit of the method was 0.1 ppb of carbofuran.

Determination of Cross-Reactivity. The relative sensitivity of the immunoassay to various compounds was determined by assaying a series dilution of each compound in sodium acetate buffer and comparing the IC_{50} values (concentrations of analyte that produce a 50% decrease in the maximum normalized response).

Soil Extraction and Analysis. Spiking was performed as follows: 100-g batches of air-dried soils (Table 1) were mixed for 2 h with 100 mL of carbofuran-spiked solutions prepared in water to yield concentrations in soil of 0.05–1 ppm. Soils were then air-dried for 3 days and ground with a mortar and pestle. Ten grams of soil was extracted for 1 h by agitating in 20 mL of methanol/water (75:25 v/v). After settling for approximately 15 min, the extract supernatant was diluted at least 1:50 (20 μL in 950 μL) in zero standard to eliminate solvent interferences. The diluted soil extract was assayed as described above, and the results were multiplied by the appropriate dilution factor to determine the soil carbofuran concentration (i.e., multiply by 100 to correct for a 1:50 dilution to correct for the initial 1:2 dilution of soil with methanol/water).

RESULTS AND DISCUSSION

Dose Response Curve and Sensitivity. The immunoassay described uses a competitive assay format. Since the enzyme-labeled carbofuran competes with unlabeled (sample) carbofuran for the antibody sites, the color developed is inversely proportional to the concentration of carbofuran in the sample. It is common to report displacement in terms of B/B_0 measurement to describe color inhibition. B/B_0 is defined as the absorbance observed for a sample or standard divided by the absorbance at zero analyte concentration. To construct a calibration curve for the immunoassay, the B/B_0 values for the carbofuran calibrators were linearly transformed using a log/logit curve. The reproducibility of the calibrators is presented in Table 2 as mean B/B_0 and SD values collected over 47 runs. The displacement at the 0.1 ppb level is significant, 85.3% B/B_0. The assay sensitivity, the lowest concentration that can be distinguished from zero, based on 90% B/B_0 is 56 parts per trillion (ppt, pg/mL) of carbofuran (Midgley et al., 1969). This sensitivity exceeds the method detection limit reported for U.S. EPA Method 531.1 of 1.5 ppb using direct aqueous injection HPLC with postcolumn derivatization (U.S. EPA, 1989a).

Table 2. Reproducibility of Carbofuran Calibrators

carbofuran (ppb)	mean B/B_0[a] ±SD	no. of assay runs
0.1	0.853 ± 0.023	47
1.0	0.460 ± 0.023	47
5.0	0.205 ± 0.013	47

[a] B/B_0 is the absorbance observed for a sample or standard divided by the absorbance at zero analyte concentration.

Table 3. Precision of Carbofuran Measurement

	sample[a]			
	1	2	3	4
replicates	5	5	5	5
days	5	5	5	5
N	25	25	25	25
mean (ppb)	0.2	0.4	2.1	3.9
%CV (within-assay)	9.4	10.7	8.9	7.4
%CV (between-assay)	11.3	6.1	2.2	4.9
%CV (total assay)	14.0	12.1	8.7	8.7

[a] Carbofuran-spiked surface waters (sample 1 spiked at 0.25 ppb, sample 2 spiked at 0.5 ppb, sample 3 spiked at 2 ppb, and sample 4 spiked at 4 ppb) assayed in five singlicates each over 5 days.

Quantitation with the immunoassay should be limited to within the range of the standard curve, from 0.1 to 5.0 ppb in water. To analyze samples with higher carbofuran concentrations, the water or soil extract samples are diluted in the zero standard for analysis and sample concentrations calculated by multiplying results by the appropriate dilution factor.

Precision. A precision study in which four surface water samples were spiked with carbofuran at 0.25, 0.5, 2, and 4 ppb and each assayed five times in singlicate per assay on five different days is shown in Table 3. The water samples included a sample from a small creek, a sample from the Delaware River, and two samples from the Brandywine River. The within- and between-day variations were determined by analysis of variance (ANOVA) (Bookbinder and Panosian, 1986) using SAS software (SAS Institute, 1988). Coefficients of variation (%CV) within- and between-day were less than 11 and 12%, respectively. The total %CV ($n = 25$) was less than 15% at all concentrations tested.

Method Comparison. Correlation of 10 environmental water samples obtained according to the present ELISA method (y) and an established HPLC method (x) is illustrated in Figure 1. The regression analysis yields a correlation of 0.967 and a slope of 1.18 between methods. The apparent higher carbofuran sample concentrations could result from cross-reactivity of the antibody with metabolites or other carbamates or could be due to the loss of analyte during the sample extraction and concentration steps of the HPLC method.

Accuracy. The accuracy of the ELISA was established by adding known amounts of carbofuran to four water samples obtained locally. The water samples included a municipal drinking water source, a small creek, the Delaware River, and the Brandywine River. The accuracy was evaluated by analyzing the samples before and after the addition of carbofuran and sub-

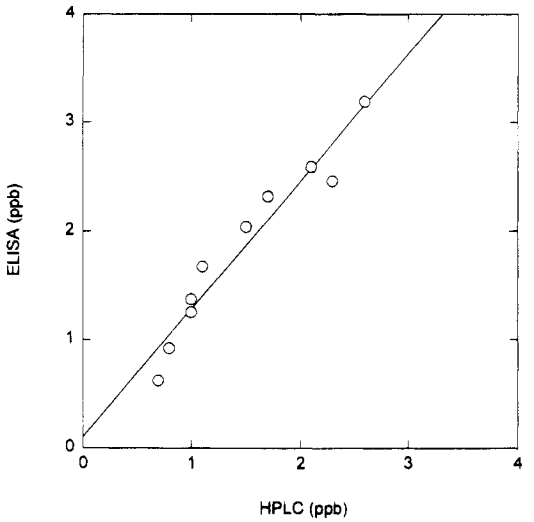


Figure 1. Correlation between carbofuran concentrations as determined by ELISA and HPLC methods. $n = 10$, $r = 0.967$, $y = 1.18x + 0.10$.

Table 4. Accuracy of Carbofuran ELISA

amt of carbofuran added (ppb)	carbofuran recovery[a]			
	mean (ppb)	n	SD (ppb)	% recovery
0.50	0.51	12	0.05	102
1.00	1.01	12	0.04	101
2.00	2.06	12	0.08	103
4.00	3.82	12	0.14	98
av				101

[a] Four water samples each spiked at the desired concentration and assayed in duplicate in the ELISA.

Table 5. Linearity upon Sample Dilution[a]

	undiluted	1:2	1:4	1:8
sample 1				
obtained (ppb)	1.87	0.97	0.49	0.24
expected[b] (ppb)	1.87	0.94	0.47	0.23
recovery (%)		103	104	104
sample 2				
obtained (ppb)	2.88	1.46	0.74	0.40
expected (ppb)	2.88	1.44	0.72	0.36
recovery (%)		101	103	111
sample 3				
obtained (ppb)	4.95	2.57	1.29	0.72
expected (ppb)	4.95	2.48	1.24	0.62
recovery (%)		104	104	116

[a] Samples diluted in the zero standard. [b] Expected concentrations are derived from the carbofuran concentration obtained from the undiluted sample.

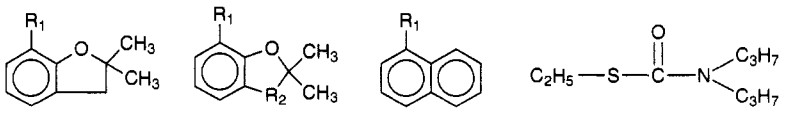
tracting the concentration of carbofuran before spiking. Table 4 summarizes the accuracy of the carbofuran ELISA. Added amounts of carbofuran were recovered quantitatively in all cases with an average assay recovery of 101%. An inaccurate recovery of spiked carbofuran, less than 80% or greater than 120%, would suggest the presence of an interference. The recovery of the spiked samples indicates that no sample matrix problems or interferences were present in the samples tested, and the accuracy of the ELISA is linear across the range of the assay.

Sample Dilution. Samples that apparently contain carbofuran can be diluted with the zero standard and reassayed to determine "parallel" dilution. If the positive result was due to nonspecific interferences, the values of the diluted samples would not assay as expected, i.e., the standard curve should be parallel to the curve obtained by diluting a sample (Jung et al., 1989). If the ELISA were susceptible to interferences, the difference between expected and observed values would increase with increasing dilutions. Values obtained from one spiked ground water and two spiked river water samples diluted in the zero standard showed agreement between the measured and expected values (Table 5). The expected values are derived from the carbofuran concentration in the undiluted (neat) sample.

Specificity. Table 6 summarizes the immunoassay cross-reactivity with carbofuran, carbofuran metabolites, a variety of carbamates, and nonstructurally related agricultural products. The antiserum is most reactive with carbofuran and slightly reactive with the metabolite 3-ketocarbofuran. The antiserum shows a preference for carbofuran metabolites that contain a carbamate chain on the R_1 position. Carbaryl and EPTC were the only agricultural products showing reactivity at less than 10 ppm.

Drift. An optimized ELISA should exhibit little or no variation in sample values from the beginning to the end of a run due to timing. The time needed to complete sample and reagent additions depends upon the number of samples being assayed. The magnetic particle-based ELISA minimizes the drift effect because the immunological reaction is not initiated until the addition of the final reagent, the antibody-coupled magnetic particle, which can be added rapidly to all tubes with a repeating pipet. To evaluate drift, water samples containing 0 and 2.0 ppb of carbofuran were assayed in 48 replicates or 58 tubes total including the standards

Table 6. Specificity (Cross-Reactivity) in the Immunoassay[a]



A B C D

compd	ring structure or structure	R_1	R_2	LDD[b] (ppb)	IC_{50}[c] (ppb)
carbofuran	A	$OCONHCH_3$		0.056	0.815
3-ketocarbofuran	B	$OCONHCH_3$	O	1.2	17
3-hydrocarbofuran	B	$OCONHCH_3$	OH	16	220
carbofuranphenol	A	OH		3000	4700
3-ketocarbofuranphenol	B	OH	O	380	>10000
3-hydroxycarbofuranphenol	B	OH	OH	1700	>10000
EPTC	D			340	4600
carbaryl	C	$OCONHCH_3$		780	>10000

[a] No reactivity was seen for the following compounds up to 10 ppm: alachlor, aldicarb, ametryn, aminocarb, atrazine, benomyl, butachlor, butylate, captafol, captan, carbendazim, chloropropham, cyanazine, 2,4-D, 1,4-dichloropropene, dinoseb, MCPA, methomyl, metolachlor, metribuzin, pentachlorophenol, picloram, propachlor, prometryn, simazine, terbufos, thiabendazole, and thiophanate-methyl. [b] Least detectable dose (90% B/B_0). [c] 50% inhibition concentration (50% B/B_0).

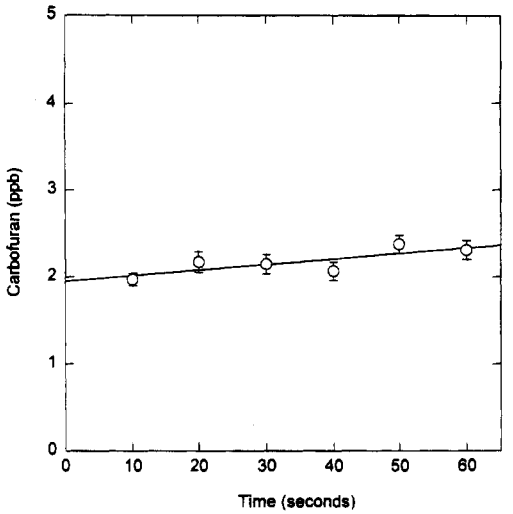


Figure 2. Assay drift: at each time interval, magnetic particles were added to a set of four tubes with a 2 ppb of carbofuran sample. Each data point represents mean values of three runs with four replicates (n = 12). Vertical bars indicate ±SD about the mean.

and controls. At time "zero", particles were added to the first tube of the standard curve. At 10 s, particles were added to the first set (four replicates or tubes) of samples. Particles were then added to additional sets of tubes at 10-s intervals up to 1 min. This experiment was repeated three times. Figure 2 illustrates a drift of sample concentrations in this ELISA. For the 2.0 ppb sample, there was approximately a 12% increase in predicted concentrations at 1 min (50 s between first and last sets). The slope of the regression line (0.00635 ppb/s) suggests that for the 40-tube assay the analyte concentration difference from beginning to end would be minimal, 10% at the 2.0 ppb of carbofuran level. The mean, standard deviation, and %CV for three runs with 2 ppb samples were 2.17 ppb, ±0.27, and 12.37, respectively.

For the water samples with 0 ppb of carbofuran, there was a positive change in predicted concentration, but mean values (N = 12) of three runs at each time interval were less than 0.05 ppb. If 1 min is used to pipet magnetic particles, there would not be enough drift for a "negative" sample to test "positive".

Sample Spike Recovery. Three hundred drinking, surface, and ground water samples obtained from throughout the United States were fortified with a known concentration of carbofuran to evaluate sample matrix effects. The carbofuran concentration of the water samples ranged from less than 0.1 to 3.19 ppb before they were fortified. Recoveries ranged from 80 to 130% when these water samples were spiked with 1.0 ppb of carbofuran, indicating that no sample matrix effects were present in the ELISA. The mean recovery of all samples was 102 (SD = 10%).

Interferences. The following compounds were added to blank and spiked carbofuran water samples at 250 parts per million (ppm, μg/mL) and evaluated for possible interferences in the ELISA: nitrate, copper, nickel, thiosulfate, sulfite, sulfide, iron, calcium, and magnesium. Table 7 demonstrates that no interferences are present up to the tested levels of various common components. In addition, sulfate to 1000 ppm, sulfite to 5 ppm, sodium to 1.0 M, and humic acid to 50 ppm exhibited no interferences. The concentrations of the compounds chosen are those that would most likely exceed levels found in environmental water samples (American Public Health Association, 1989).

Table 7. Effect of Possible Interfering Substances

compd	max concn of compd tested (ppm)	0 ppb of carbofuran sample	1 ppb of carbofuran sample
nitrate	250	ND[a]	0.88
copper	250	ND	1.19
nickel	250	ND	1.00
thiosulfate	250	ND	0.94
sulfite	5	ND	1.04
sulfide	250	ND	0.95
sulfate	1000	ND	0.97
iron	250	ND	0.83
magnesium	250	ND	1.06
calcium	250	ND	0.96
humic acid	50	ND	1.07
sodium chloride	1.0 M	ND	1.04

[a] ND, none detected (<0.056 ppb).

Table 8. Recovery of Carbofuran From Soils[a]

soil	carbofuran added (ppb)	carbofuran recovery: mean (ppb)	n[b]	SD (ppb)	% recovery
Sassafras sandy loam	none	nd	4		
	50	56.3	4	15.4	112.5
	100	112.0	4	15.6	112.0
	250	245.0	4	12.3	98.0
	500	453.8	4	51.4	90.8
	750	738.8	4	43.3	98.5
	1000	908.8	4	105.5	90.9
	av				100.4
Plano loam	none	nd	4		
	50	53.5	4	6.1	107.0
	100	103.3	4	6.7	103.3
	250	228.5	4	9.9	91.4
	500	496.3	4	43.7	99.3
	750	685.0	4	23.5	91.3
	1000	885.0	4	18.7	88.5
	av				96.8

[a] Ten grams of carbofuran-spiked soils was extracted with 20 mL of methanol/water (75:25 v/v). Soil extracts were diluted in the zero standard diluent and analyzed in the ELISA. [b] Two extractions analyzed in duplicate in the ELISA.

Soil Fortification Study. Recovery of carbofuran in sandy loam and loam soils spiked with 0.05–1 ppm was determined using a 1-h extraction (Table 8). All unspiked samples assayed as less than the soil detection limit of 5.6 ppb. Diluting the soil extracts in the zero standard diluent eliminates the need for solvent evaporation and reduces any possible matrix or solvent interferences in the assayed sample, making it unnecessary to prepare standards containing methanol. Using a 1:50 dilution provides a detection range of 5.6–500 ppb of carbofuran in soil. Samples containing greater than 500 ppb must be diluted further. Methanol tolerance in the assay is presented in Table 9.

Conclusions. This work describes a magnetic particle-based ELISA for pesticide residues and its performance characteristics in the quantitation of carbofuran in water and soil. The assay results compare favorably to HPLC determination, with no false negative values for the 10 samples analyzed using a cutoff of 0.1 ppb. The ELISA exhibits within- and between-assay precision of less than 11.3% and average accuracy of 101%, which provide consistent monitoring of environmental samples. The magnetic particle-based system is rapid (results within 1 h) and more sensitive than EPA

Table 9. Assay Response with Carbofuran Extracts Containing Methanol

methanol concn[a] (%)	0 ppb of carbofuran sample	2.5 ppb of carbofuran sample	% recovery[b]
0	0.00	2.68	107
0.2	0.00	2.81	112
0.5	0.00	2.73	109
1	0.00	2.73	109
2	0.00	2.83	113
3	0.00	2.74	109
4	0.00	2.80	112
5	0.00	2.73	109
10	0.00	2.65	106
20	0.00	2.35	94
50	0.08	2.18	87

[a] Methanol concentration v/v. [b] Acceptable range of carbofuran recovery was 100 ± 20%.

Method 531.1 (detection limit of 1.5 ppb) for the determination of carbofuran. Detection of carbofuran in soil at parts per billion levels fulfills the sensitivity requirements for envrionmental monitoring. The specificity of the antibody employed allows for the detection of carbofuran in the presence of other pesticides and commonly found ground water and soil components. The assay is ideally suited for adaptation to on-site monitoring of carbofuran in water and soil.

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