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Validation of an Immunoassay for Methomyl in Water and Dislodgeable Residues on Grape Leaves

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Methomyl, trade name Lannate® is a systemic, broad spectrum insecticide registered for use on more than 100 crops worldwide for control of pests on vegetables, soybeans, cotton, other field crops, some fruit crops, and ornamentals (Du Pont Co. 1993; Royal Society of Chemistry 1991; Baron 1991). Since it only weakly adsorbs to soil and so is freely available for leaching (Bromilow et al. 1986) monitoring for methomyl in water can be an important indicator of contamination. Loosely bound residues on treated foliage represent another possible source of exposure for workers who contact plants too soon after pesticide application (Gunther et al. 1973). Measurement of residues under field conditions is necessary in evaluating hazards that workers encounter with treated crops and in determining safe waiting intervals for worker reentry.

Several investigators have evaluated methods for quantifying levels of pesticide residues available on treated foliage (Blewett and Krieger 1990; Iwata et al. 1977; Gunther et al. 1973). The conventional procedures involve agitating leaf discs of known surface area in a surfactant solution, followed by the use of standard procedures to extract residues from the rinsate (Dong et al. 1991; Blewett and Krieger 1990; Iwata et al. 1977). The amount of foliar pesticide residues recovered from this analytical procedure is commonly referred to as the dislodgeable foliar residue (DFR), normally expressed in weight of pesticide per unit area of leaf surface (Dong et al. 1991; Iwata et al. 1977).

This paper describes a quick and easy immunochemical method for the determination of methomyl residues in water and an easy technique for quantifying the levels of pesticide available on treated foliage. The procedure uses an enzyme-linked immunosorbent assay (ELISA) based on magnetic particles as the solid support and means of separation. A simple surface wash procedure is used to adapt the magnetic particle-based immunoassay described to the determination of dislodgeable foliar residue.

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MATERIALS AND METHODS

Methomyl RaPID Assay®, Methomyl Sample Diluent and RaPID Prep™ DFR Wash were obtained from Ohmicron Environmental Diagnostics, Newtown, PA. Methomyl oxime (99.75%) and oxamyl oxime (99.2%) were supplied by E.I. Du Pont de Nemours and Co., Wilmington, DE. Methomyl (≥ 98% pure), oxamyl (≥98% pure) and other pesticides and pesticide metabolites, all ≥97% pure, were purchased from ChemService, West Chester, PA. All other chemicals were reagent grade or chemically pure.

The RPA-I™ Photometric Analyzer and RaPID Magnetic Separation Unit™ were obtained from Ohmicron Environmental Diagnostics, Newtown, PA. The 2.54 cm (1 inch) diameter leaf punch and collecting jar were purchased from Rabbit Tool U.S.A., Rock Island, IL.

All samples were assayed according to the RaPID Assay package insert. The procedure required adding 200 µL of the methomyl standards, control or sample to a disposable test tube, along with 250 µL of enzyme labeled methomyl and 500 µL of rabbit anti-methomyl linked to paramagnetic particles. After a 30 minute incubation at room temperature, the particles were drawn to the sides of the tubes using the magnetic separation unit and the supernatant was decanted. The particles were washed twice with 1 mL of washing solution. Chromogen solution (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine), 500 µL per tube, was added and allowed to develop for 20 minutes . The color reaction was stopped with 500 µL of stopping solution (0.5% sulfuric acid). Photometric analysis of the colored product was made using the RPA-I Analyzer set at 450 nm which compares the observed sample absorbances to a regression line using a log-linear standard curve derived from the calibrator absorbances and reports parts per billion (ng/mL) methomyl in the sample.

The relative sensitivity of the immunoassay was determined by assaying a dilution series of each compound in sample diluent (sodium acetate buffered saline with 0.1% BSA) and comparing the IC_{so} values (concentration of analyte producing a 50% decrease in the maximum normalized response) to the IC_{50} value of methomyl.

For the method comparison study, tap and well water collected in Pennsylvania were fortified with methomyl from 1 to 40 ppb. Samples were analyzed by EPA method 531.1 at ABC Research Corporation (Gainsville, FL).

The leaf sampling and surface wash procedures used for the analysis of methomyl residues on grape leaves were modifications of Gunther et al. (1973). To determine an appropriate dilution of the surface extract to overcome matrix interferences in the immunoassay, the DFR Wash (0.04% Aerosol-OT) was diluted in sample diluent and the neat response and spike recovery at two concentrations of methomyl(2 and 10 ppb) was evaluated. To prepare spiked grape leaf samples,

methomyl(100 µg/mL) was applied to one-inch punched leaf discs of organically grown Thompson seedless grape leaves from Maricopa County, Arizona. The residues were allowed to dry overnight. Each sample set, consisting of forty (40) leaf punches, was placed into a glass jar with 50 mL of DFR Wash and the jar was vigorously shaken by hand for 1 minute. A sample of this dislodged foliar residue solution was then diluted in sample diluent and assayed as the sample in the assay. Leaf sets spiked at 0.1 and 0.05 µg/cm^2 were diluted 1:100 (100 µL + 1.9 mL sample diluent); the unspiked and 0.025 µg/cm^2 samples were diluted 1:20 (20 µL + 1.98 mL sample diluent) for analysis.

The surface area of each leaf disk (for a 1 inch or 2.54 cm diameter punch), taking into account both sides of the leaf, is equivalent to approximately 10.13 cm^2, or 202.68 cm^2 for the forty samples tested. To convert the immunoassay values to final sample concentrations, the following formula was used:

$$\text{assay result (ng/mL)} \times \frac{\text{50 mL wash}}{\text{40 leaves}} \times \frac{\text{leaf}}{\text{10.13 cm}^2} \times \text{dilution} = \text{concentration (ng/cm}^2\text{)}$$

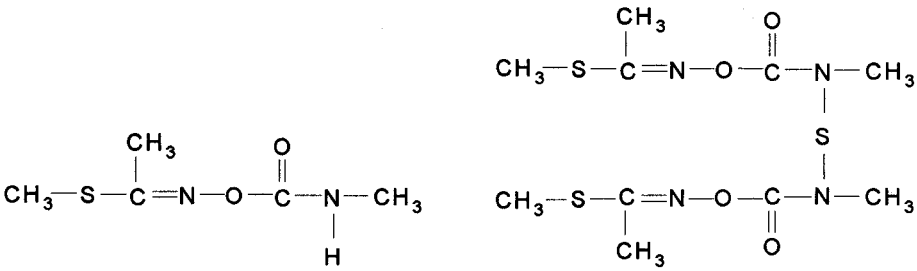
Results are reported here as µg of pesticide per cm^2 leaf.

RESULTS AND DISCUSSION

In the immunoassay described, the enzyme-labeled methomyl competes with unlabeled (sample) methomyl for the antibody binding sites so the color developed is inversely proportional to the concentration of methomyl in the sample. It is common to describe color inhibition in terms of a *B/Bo* measurement. *B/Bo* is defined as the absorbance observed for a sample or standard divided by the absorbance at a zero analyte concentration.

The range of the immunoassay calibration curve is 1 to 15 ppb methomyl with an estimated least detectable dose (LDD) of 0.45 ppb. The assay LDD, defined as the lowest concentration that can be distinguished from zero, was based on an average 90% *B/Bo* estimation from 50 assays (Midgley et al. 1969). The 0.45 ppb LDD estimate is conservative compared to a 4 SD estimate from the "true" zero, determined by calculating the standard deviation and mean absorbance value for three sets of twenty replicates of the zero standard.

Table 1 summarizes the immunoassay cross-reactivity with methomyl and other related and unrelated pesticides and metabolites. The LDD was determined as the amount of each compound necessary to achieve 90% *B/Bo*. Results show the assay to be very specific for methomyl with <0.01% cross-reactivity to methomyl oxime, the closely related compound, oxamyl, and the oxamyl oxime. Thiodicarb, which metabolizes to methomyl, was the only compound showing significant reactivity in the assay. The structures of both methomyl and thiodicarb are shown in Figure 1.



Methomyl **Thiodicarb**

Figure 1. Structures of methomyl and thiodicarb.

Table 2 summarizes a precision study conducted with four concentrations of methomyl in four water samples: creek, tap and two well waters. Methomyl was added at 2, 4, 6 and 10 ppb. Each level was assayed five times per day in singlicate over 5 days. The within- and between-assay and total variations were determined according to Bookbinder and Panosian (1986) using Statistical Analysis Software (SAS Institute 1988).

The accuracy of the assay was assessed by evaluating four water samples each fortified with methomyl at 2, 4, 6 and 10 ppb. Each sample was assayed three

Table 1. Specificity (cross-reactivity) in the immunoassay

Compound	LDD, ppb (90% B/Bo)	IC_{50},[1] ppb	% Cross Reactivity[2]
Methomyl	0.45	4.15	100
Thiodicarb	0.49	11.1	37
Oxamyl	1000	>10,000	<0.01
Thiobencarb	1378	>10,000	<0.01
Dinoseb	2110	>10,000	<0.01
Thiofanox	3160	>10,000	<0.01
Diazinon	5081	>10,000	<0.01
Methomyl Oxime	5961	>10,000	<0.01

[1] Inhibitory concentration estimated at 50% B/Bo (IC_{50}).

[2] Percent cross-reactivity was determined by comparing the IC_{50} for each compound to the IC_{50} for methomyl.

The following compounds demonstrated no reactivity in the Methomyl RaPID Assay at concentrations up to 10 ppm: alachlor, aldicarb, aldicarb sulfone, atrazine, azinphos-methyl, captafol, carbaryl, carbofuran, chlorothalonil, chlorpyrifos, chlorpyrifos-methyl, cyanazine, 2,4-D, dicamba, EPN, folpet, iprodione, malathion, metalaxyl, methamidophos, methiocarb, metolachlor, metribuzin, mevinphos, paraquat, parathion-ethyl, parathion-methyl, phorate, phosmet, procymidone, propaclor, propoxur, simazine, terbufos and triclopyr. Aldrin, benomyl, carbendazim, thiabendazole and vinclozolin were only tested up to 100 ppb due to solvent (DMF and acetone) tolerance limitations.

Table 2. Precision of methomyl measurement in water by immunoassay

Sample	2.0 ppb	4.0 ppb	6.0 ppb	10 ppb
Replicates	5	5	5	5
Days	5	5	5	5
n	25	25	25	25
Mean ppb	1.83	4.11	6.30	10.39
% CV Intra	11.5	8.3	8.2	5.4
% CV Inter	<0.1	<0.1	2.1	3.0
% CV Total	11.1	8.2	8.4	6.1

Table 3. Accuracy of methomyl measurement in water by immunoassay (n= 12)

Methomyl added (ppb)	Mean (ppb)	S.D. (ppb)	% Recovery
2.0	1.85	0.28	93
4.0	4.09	0.38	102
6.0	6.28	0.47	105
10.0	10.14	1.09	101
Average			100

times in duplicate to verify reproducibility. Table 3 summarizes the accuracy of the methomyl immunoassay in environmental water samples. Added amounts of methomyl were recovered quantitatively in all cases with an average assay recovery of 100%, indicating the assay is linear over the entire range defined by the calibrators with no observed matrix interferences.

The following compounds were added to blank and fortified methomyl water samples at 500 parts per million (ppm, µg/mL) and evaluated for possible interference in the immunoassay: nitrate (sodium), nickel (sulfate), thiosulfate (sodium), sulfite (sodium), magnesium (chloride), calcium (chloride), mercury (chloride) and manganese (chloride). No interferences in neat samples or spike recoveries were noted. In addition, sulfate (sodium) up to 5000 ppm, copper (chloride), phosphate (sodium), silicates (sodium meta-) up to 250 ppm, peroxide (hydrogen) and zinc (chloride) up to 100 ppm, iron (chloride) up to 25 ppm and sodium chloride up to 1.0M exhibited no interference in the assay. All concentrations were corrected to the ions of interest. In addition, sample pH had no adverse effect on neat samples or spike recoveries in the assay from pH 2 to 11.

These results suggest that the assay is reliable and free from interferences from commonly found groundwater components. To further examine this, 142 water samples from around the United States were evaluated neat and spiked with 6 ppb

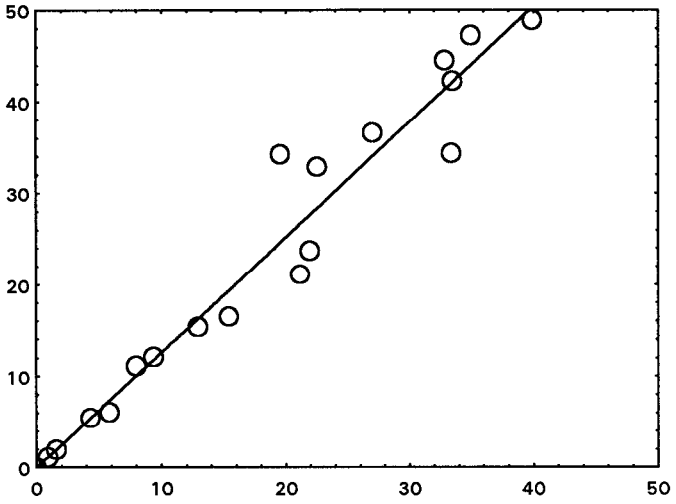


Figure 2. Comparison of methomyl concentration in spiked water samples as determined by ELISA and HPLC methods. $y = 1.26x + 0.043$, r = 0.9767, n = 20.

methomyl to determine recovery and possible matrix interferences. A mean recovery of 106% (SD = 7) was observed for the 142 samples.

Results of a study conducted to compare results of the ELISA method (*y*) with EPA [HPLC] method 531.1 (*x*) are shown in Figure 2. Twenty water samples containing from 1 to 40 ppb methomyl, plus two sample blanks ("0" ppb), were prepared from two water sources (tap and well water) and analyzed by HPLC and the immunoassay. The regression analysis yields a correlation (*r*) of 0.977 and a slope of 1.26 between methods. The immunoassay averaged 116% recovery of target values; HPLC analysis averaged 94%.

The surfactant used in this study and most other DFR studies was dioctyl sodium sulfosuccinate, also known as Sur-Ten or Aerosol-OT (American Cyanamid, NJ). The surfactant solution could not be assayed directly in this immunoassay because it suppressed the signal (color development), resulting in false positive results and falsely elevated recoveries. Therefore, prior to beginning any application work it was necessary to determine an appropriate dilution of the DFR wash solution to avoid matrix interferences. This data is summarized in Table 4. A minimum dilution of 1:20 was required to dilute out matrix effects from the surfactant; a 1:100 dilution was required to quantitate samples at the regulatory cutoff levels.

Methomyl recoveries from grape leaves fortified at three methomyl spike levels are shown in Table 5. Three complete replicate analyses were performed at each

Table 4. Determination of appropriate dilution of DFR wash solution to overcome matrix effects in the immunoassay

Dilution	Assay Result (ppb)	Spike Recovery (%)	
		2 ppb	10 ppb
Neat	1.16	216	91
1:2	1.33	212	120
1:10	0.57	131	112
1:20	nd	109	106
1:50	nd	102	98
1:100	nd	104	105
1:200	nd	97	99

nd = none detected

concentration level. The Limit of Quantitation (LOQ) for this method, based on the concentration of the first assay calibrator, is 2.46 ng/cm^2 (0.0025 µg/cm^2) using a 1:20 dilution of the surface wash solution, or 12.3 ng/cm^2 (0.0123 µg /cm^2) for a 1:100 dilution of the rinsate. Regulatory cutoffs for worker reentry following application of methomyl to grape vines are set at 0.1 µg/cm^2 by the California EPA and 0.05 µg/cm^2 by the US EPA (Register 1992).

Table 5. Recovery of methomyl applied to grape leaf samples

Spike (µg/cm^2)	Methomyl Recovery	
	µg/cm^2	% Recovery
0	<0.001	
0.025	0.025	101
0.050	0.043	86
0.100	0.109	109

The ratio of approximately 1 inch leaf punches to surfactant solution was standard for most investigators at about 40 punches per 50 mL (Blewett et al. 1990; Gunther et al. 1973). The extraction method used for this paper, a modification of previous methods for removing surface residues of pesticides, has been shown to be effective for the removal of dislodgeable residues. The method can be adapted for different foliage sizes by using a smaller die size (1.8 cm size is available) or by using weight/area conversions for very small foliage or grasses. Surface washes of fruit and vegetables, or other crops, could also be adapted for analysis in this assay.

The work presented shows the immunoassay described to be accurate, precise and sensitive for the determination of methomyl residues. The procedure is easy to perform and has a turn around time of approximately one hour which should make it useful for screening large numbers of environmental water samples. The assay compares favorably with traditional HPLC analysis and is free from interferences from pH and compounds commonly found in water. This study indicates that the immunoassay can be used to quantitatively measure methomyl residues well below regulatory levels following application to grape vines.

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