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An Enzyme-Linked Immunosorbent Assay for Metalaxyl in Foods

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An enzyme-linked immunosorbent assay was developed for residues of the fungicide metalaxyl in foods. The procedure is applicable to the analysis of methanol extracts without prior cleanup and will quantitate from 0.1 to 2.0 ppm of the parent compound in various commodities. Accuracy and precision compare favorably to a gas chromatographic method involving a solvent partitioning and adsorption column cleanup, although specificity is less with cross reactivity being observed with the herbicides metolachlor and diethatyl ethyl and to a lesser extent the fungicide furalaxyl. The simplicity of the method permits 4.5 times more samples to be analyzed per day than a conventional gas chromatographic procedure.

Metalaxyl (methyl *N*-(2-methoxyacetyl)-*N*-(2,6-xylyl)-alaninate) is a fungicide registered for use in the United States on a variety of fruit and vegetable crops. Several methods have been published for the determination of crop residues by gas chromatography with nitrogen-selective detectors (e.g., Caverly and Unwin, 1981; Tafuri et al., 1981; Speck and Dirr, 1980). Cleanup techniques have ranged from simple water partitioning for tomato extracts (Waliszewski and Szymczynski, 1983) to sweep codistillation followed by silica gel chromatography for soil and sunflower foliage extracts (Tafuri et al., 1981). It has been our experience that a cleanup step involving adsorption chromatography was necessary when analyzing certain commodities on which metalaxyl is registered to prevent anomalous quantitative results arising from accumulation of coextractives on the gas chromatographic column.

An alternative approach to residue analysis with the potential of more efficient processing of samples is immunochemical determination based on competitive binding to an antibody (Hammock and Mumma, 1980). Radioimmunoassay procedures have been applied successfully to the determination of such pesticides as paraquat (Fatori and Hunter, 1980), 2,4-dichlorophenoxyacetic acid and (Rinder and Fleeker, 1981) parathion (Ercegovich et al., 1981), and benomyl (Newsome and Shields, 1981). More recently, enzyme-linked immunosorbent assay (ELISA) has been employed for the analysis of diflubenzuron (Wie and Hammock, 1982) and paraquat (Niewda et al., 1983) and has the advantage of not requiring a radioligand and associated counting equipment. The following report describes development of an ELISA procedure for metalaxyl residues in foods and compares it to a gas chromatographic method.

EXPERIMENTAL SECTION

Materials. Analytical standards of metalaxyl, its free acid, CGA 109097, and furalaxyl were gifts from Ciba-Geigy Ltd., Mississauga, Ont., while those of metolachlor, alachlor, propachlor, and benzoylprop-ethyl were obtained from Agriculture Canada, Ottawa, Ont. Diethatyl ethyl was obtained from Hercules Inc., Wilmington, DE. Stock solutions consisting of 0.5 mg mL^{-1} of each compound were prepared by dissolving in HPLC grade methanol (Caledon Laboratories, Georgetown, Ont.). Working standards of metalaxyl were prepared by serial dilution in methanol to give concentrations of 2.5, 5.0, 10, 20, and 40 ng mL^{-1}. Freund's complete adjuvant was purchased from Difco Laboratories, Detroit, MI. Bovine serum albumin (RIA grade), human serum albumin, ovalbumin, antirabbit IgG peroxidase conjugate, *o*-phenylenediamine dihydrochloride, and Tween 20 (polyoxyethylene sorbitan monolaurate) were obtained from Sigma Chemical Co., St. Louis, MO. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride was supplied by Aldrich Chemical Co., Inc., Milwaukee, WI, while tri-*n*-butylamine and isobutyl chloroformate were obtained from Eastman Kodak Co., Rochester, NY.

Instruments. The optical density of microtiter plate well content was read on a Dynatech MR 600 dual beam plate reader. Gas chromatography was performed on a Varian 1400 fitted with a N-P detector, a 0.25 μm DB-5 capillary column (0.25 mm × 10 m, J:W Scientific, Inc., Rancho Cordova, CA), and a J:W oncolumn injector. Helium carrier gas was supplied at a linear velocity of 33 cm s^{-1}. Flows of nitrogen makeup gas, air, and hydrogen to the detector were 30, 175, and 4.5 mL^{-1}, respectively. The detector operating temperature was 250 °C. Injections of 1.0 μL were cold trapped and after a 1-min delay temperature programmed at 50 °C min^{-1} to 210 °C. Under these conditions, approximately 50% full scale deflection was obtained from the injection of 1.1 ng of metalaxyl.

Buffers. Phosphate buffered saline (PBS) contained 2.42 g of NaH_2PO_4 and 8.26 g of NaCl per L of distilled water. The pH was adjusted to 7.2 with NaOH before making to volume. PBS-Tween washing solution was prepared by adding 0.5 mL of Tween 20 per L of PBS. Antiserum diluent contained 0.1% bovine serium albumin in PBS. Coating buffer, pH 9.6, contained 1.59 g of

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Na_2CO_3 and 2.93 g of $NaHCO_3$ per L of distilled water. Citrate–phosphate buffer, pH 5.0, was prepared from 0.2 M NaH_2PO_4 (257 mL), and 0.1 M citric acid (243 mL) diluted to 1 L with distilled water. Peroxidase substrate, prepared immediately before use, consisted of *o*-phenylenediamine dihydrochloride (70 mg) in 100 mL of citrate–phosphate buffer to which was added 30% H_2O_2 (40 μL).

Preparation of Immunogen. Metalaxyl (558 mg, 2 mequiv) was hydrolyzed to the acid by refluxing in 1 N NaOH (5 mL) for 2 h. The hydrolyzate was acidified with 1 N HCl and extracted with dichloromethane. After drying and removal of the solvent, 441 mg of an oil which later crystallized (mp 144–145 °C) was obtained. The acid (5.59 mg, 21 μmol) was coupled to human serum albumin (14 mg, 0.2 μmol) in 0.2 M phosphate buffer pH 7.2 (2 mL) with 1-[3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (95.5 mg, 500 μmol). After reaction at room temperature overnight, the protein was dialyzed for 3 days against frequent changes of distilled water, then lyophilized.

Preparation of Coating Antigen. The mixed anhydride of metalaxyl acid was prepared by treating the acid (13.3 mg, 50 μmol) with isobutyl chloroformate (8 μL) and tri-*n*-butylamine (15 μL) in dry dioxane (0.5 mL) as described by Wie and Hammock (1982). After 30 min the reaction mixture was added dropwise to a stirred solution of ovalbumin (44 mg, 1 μmol) in 0.2 M $NaHCO_3$, pH 9.3 (3 mL). After standing for 6 h, the protein was dialyzed for 3 days against disilled water. The resulting solution was diluted to 10 mL and frozen in 200-μL aliquots.

Antisera. Four New Zealand rabbits were each injected subcutaneously with 0.5 mL of an emulsion consisting of 1 mg mL^{-1} of metalaxyl–human serum albumin conjugate in a 1:1 mixture of 0.14 N NaCl and Freund's complete adjuvant. Booster injections were administered at monthly intervals for 5 months and test bleedings for titer determination were conducted one week after each booster. Serum for immunoassay development was prepared from blood collected 6 months after the initial injection.

Microtiter Plate Coating. Polystyrene "Cooke Microtiter" plates (Fisher Scientific Ltd., Ottawa, Ont.) were washed before use by filling and emptying twice with 10% diethyl ether in ethanol. After air drying they were immersed in distilled water and sonicated for 30 min. A solution of metalaxyl–ovalbumin conjugate (8 μg mL^{-1}, 180 μL) in pH 9.6 coating buffer was added to each well and the plate placed in a refrigerator at 4 °C overnight. Coated plates were washed 4 times with PBS-Tween then 3 times with distilled water and stored at −20 °C.

Sample Preparation. Crop material was composited by homogenization in a Waring blender. Subsamples (5 g) of each commodity were spiked with various concentrations of metalaxyl in methanol (0.10 mL) and extracted by homogenization with methanol (40 mL) in a Sorvall Omni-Mixer. After filtration through Whatman No. 1 paper on a Buchner funnel, the extracts were transferred to 50-mL volumetrics and brought to volume, and aliquots were taken for immunoassay or further cleanup prior to gas chromatographic determination.

Immunoassay Procedure. Aliquots (25 μL) of sample extract, extract diluted 1:5 in methanol, or of 0–40 ng mL^{-1} metalaxyl standard in methanol were added to 12 × 75 mm glass tubes containing 1.0 mL of antiserum diluted 1:6000 in antiserum diluent. After equilibration for 30 min at room temperature, quadruplicate aliquots (200 μL) were added to wells of a previously coated microtiter plate and the plate refrigerated at 4 °C for 30 min. The plate was then emptied by inversion and washed 4 times with PBS-Tween. Horseradish peroxidase coupled to antirabbit IgG was diluted 1:1000 in antiserum diluent and aliquots (225 μL) added to the plate which was equilibrated 30 min at room temperature. After a 4-fold wash with PBS-Tween, substrate (200 μL) was added and the enzyme reaction permitted to proceed for 20 min in the dark. The reaction was terminated by the addition of 2.5 M H_2SO_4 (50 μL) and the optical density read at 490 nm. Concentrations of metalaxyl were determined by reference to a semilog plot of the concentration against optical density of the standards run concurrently.

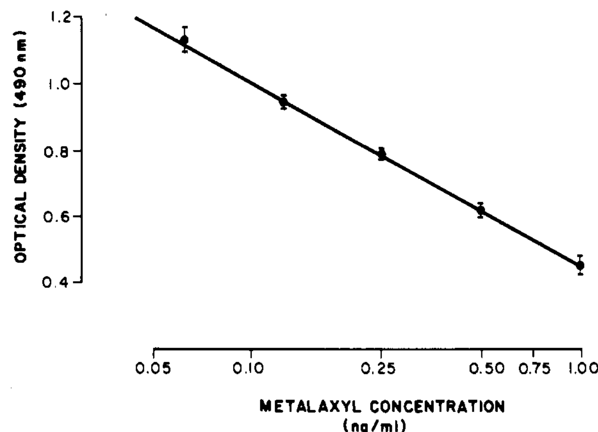

Figure 1. Standard cuve for the determination of metalaxyl concentration by ELISA. Values are the means of quadruplicate determinations. Vertical bars represent the within run standard deviations.

Gas Chromatographic Procedure. An aliquot (40 mL) of methanol extract was transferred to 125-mL separatory funnel and diluted with water (40 mL). Five percent Na_2SO_4 (10 mL) was added and the aqueous phase extracted with chloroform (3 × 10 mL). The combined extracts were dried with anhydrous Na_2SO_4 and the solvent removed on a rotary evaporator. The residue was dissolved in toluene (1 mL) and added to a 2-g bed of silica prepared in toluene in an 8 mm id × 20 cm glass chromatographic column. The column was rinsed with a further volume of toluene (1 mL) and eluted first with 50% ethyl acetate in toluene (6 mL) and then with ethyl acetate (6 mL). The latter fraction was evaporated to dryness on a rotary evaporator and the residue taken up in toluene (1.0 mL) for quantitation by gas chromatography.

RESULTS AND DISCUSSION

A linear relation was found between the log of the metalaxyl concentration and the optical density as shown in Figure 1. The coefficient of variation between replicates was 5% or less and the minumum detectable amount, defined as that concentration required to produce a decrease in binding equal to two coefficients of variation was 63 pg mL^{-1}, equivalent to 0.025 ppm in a commodity. Since the maximum concentration obtainable from the standard curve was approximately 0.4 ppm, sample extracts were also run at a 1:5 dilution to span a range of residue levels from 0.10 to 2.0 ppm. The addition of dilute sample extract did not alter the standard curve.

The gas chromatographic method used as reference involves a partition step similar to that described by Caverly and Unwin (1981) but includes an additional column chromatographic cleanup found necessary for accurate quantitation. As indicated by the data in Table I, recoveries determined by ELISA agree closely with those determined by gas chromatography. The coefficient of variation of six samples of tomato fortified at 0.5 ppm was

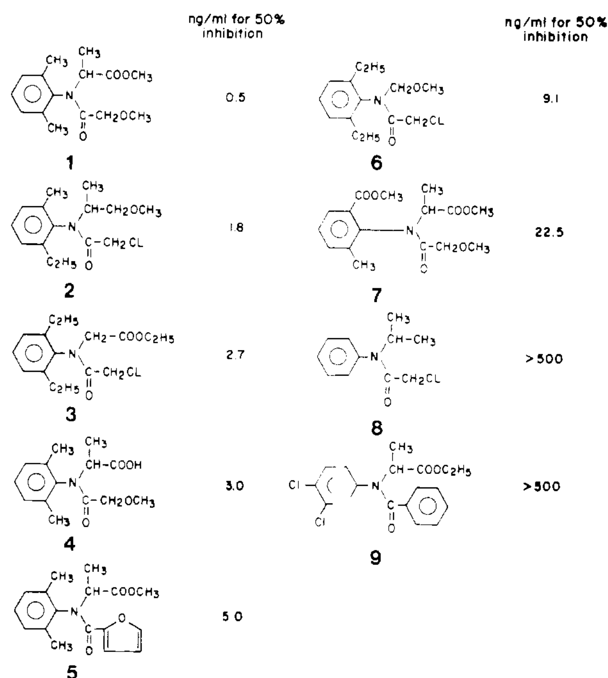

Figure 2. Structures and activities of compounds assayed for cross-reactivity in the determination of metalaxyl by ELISA: (1) metalaxyl; (2) metolachlor; (3) diethatyl ethyl; (4) metalaxyl acid; (5) furalaxyl; (6) alachlor; (7) CGA 109097; (8) propachlor; (9) benzoylprop-ethyl.

4.2% by ELISA and 5.9% by gas chromatography indicating a similar degree of within run reproducibility by the two methods. The overall recovery from five commodities by ELISA was 87% and by GLC was 94% with coefficients of variation of 10.5% and 11.5%, respectively, indicating a somewhat larger between run variation. The lower recovery obtained by ELISA was not significant ($p < 0.01$).

Several compounds structurally similar to metalaxyl were examined for cross-reactivity in the immunoassay system (Figure 2). Cross-reactivity was measured by determining the amount of binding obtained in the absence or presence of varying amounts of test compound. Comparisons were made on the basis of that amount of test compound required to produce 50% inhibition relative to that obtained in the absence of inhibitor. Metolachlor (2) has more readily recognized than the acid form of metalaxyl (4), possibly due to the negative charge on the latter. Replacement of the methoxyacetyl group of metalaxyl with furoyl to give furalaxyl (5) resulted in a 10-fold decrease in response. Comparison of the affinity of the antibody for metolachlor (2) with that of alachlor (6) suggests that the methyl group β to the methoxyl is an important factor in structural recognition, although such would not appear to be the case with diethatyl ethyl (3). Replacement of a ring methyl with a methoxycarbonyl as in 7 resulted in a greatly decreased affinity. Neither of the herbicides propachlor (8) nor benzoylprop-ethyl (9) exhibited measurable activity.

Although immunochemical analysis is less specific than the gas chromatographic method, it is useful for screening large numbers of samples and may be readily automated. In the manual form, a single technician is capable of analyzing 36 samples per day compared to 8 by gas chromatography. The use of monoclonal antibodies may provide somewhat greater specificity than the present antiserum (Hammock and Mumma, 1980). Alternatively, different immunizing haptens or coating antigens could be investigated to gain selectivity.

Table I. Recovery of Metalaxyl from Various Fortified Commodities as Determined by ELISA or GLC

metalaxyl added, ppm	metalaxyl recovered, %			
	Cucumber		Squash	
	ELISA	GLC	ELISA	GLC
0.10	75	94	84	88
0.50	87	88	90	87
1.0	94	95	98	98
2.0	90	112	103	109
	Avocado		Tomato	
	ELISA	GLC	ELISA	GLC
0.10	106	75	80	91
0.50	76	80	86	97
1.0	87	92	97	104
2.0	95	93	97	101
	Potato			
	ELISA	GLC		
0.10	81	107		
0.25	90	111		
0.50	72	78		
1.0	83	84		

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