Identification and Determination of Some Metalaxyl Degradation Products in Lettuce and Sunflower

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The presence of four compounds obtained by chemical hydrolysis of metalaxyl into metalaxyl-treated lettuce and sunflower plants was examined. The identity of these products was confirmed by gas chromatography and IR, NMR, and GS–MS spectra. There was evidence of the presence of two metabolites, one of them nonconjugated and the other conjugated to give water-soluble compound, in the treated vegetables. No conjugated metabolites were found in the water-insoluble residue.

The common objective in all biological organisms appears to be the conversion of xenobiotics into innocuous forms that may be excreted or stored as inactive materials in the organism. In the animals, pesticides and other xenobiotics would seem to be metabolized in two phases. Phase I metabolism involves hydrolysis, oxidation, or reduction reactions followed by phase II, in which products from phase I are conjugated and detoxified before excretion. As the excretion of the conjugated pesticide metabolites from plants is insignificant, these metabolites must be compartmentalized within plant cells, and this is accomplished by a phase III type metabolism that is peculiar to plants (Matsumura and Krishna Murti, 1982). Phase I reactions modify the pesticide, which exposes free hydroxyl, carboxyl, or amino groups, and so makes the compound available for phase II conjugation reactions, which yield simple and complex glucosides, glutathione conjugates and related products, and amino acid conjugates, all water-soluble metabolites that are relatively immobile in the plants. Secondary conjugation or incorporation of pesticide residues into water-insoluble compounds, nontoxic substances with limited bioavailability, are common in phase III reactions (Matsumura and Krishna Murti, 1982).

In this paper we present the results of our attempt to establish the presence of some degradation products of metalaxyl (DL-*N*-(2,6-dimethylphenyl)-*N*-(2-methoxyacetyl)alanine methyl ester) in treated vegetables. Because hydrolysis is one of the most common pathways in the phase I mechanism of pesticide degradation, we wanted to see whether free or conjugated compounds obtained by chemical hydrolysis of metalaxyl were present in metalaxyl-treated lettuce and sunflower plants.

EXPERIMENTAL SECTION

Apparatus (Tafuri et al., 1981). Gas–liquid chromatography (GLC) analyses were performed isothermally by using a Perkin-Elmer Model 900 Chromatograph equipped with a nitrogen-selective detector and a Hitachi Perkin-Elmer 196 5-mV recorder. A glass column, 2 m × 6 mm, packed with 1.5% cyclohexane dimethanol–succinate on 80–100-mesh Gas-Chrom Q was used at 180 °C. General operating conditions were as follows: carrier gas, helium; flow rate, 25 mL/min; hydrogen and air flow rates, 5 and 90 mL/min; injector temperature, 240 °C; chart speed, 5 mm/min. Mass spectra were carried out by a Varian MAT 44 gas chromatograph–quadrupole mass spectrometer equipped with a multiple-ion detector. The instrument was operated in the electron-impact mode. NMR spectra were obtained with a Perkin-Elmer R 24 B instrument. Samples were run in [^{2}H] chloroform by using 1% tetramethylsilane (Me_4Si) as the internal standard. Infrared spectra were determined on a Perkin-Elmer 21 spectrometer and the samples were run as KBr pellets.

Reagents. All organic solvents were of reagent grade and were distilled before use. Silica gel, RS (0.05 × 0.2 mm), was from C. Erba, Milan, Italy; alumina oxide was from Merck and Celite from Hyflo-Super-Cel.

Hydrolysis of Metalaxyl. One gram of metalaxyl was added of 100 mL of 10% sulfuric acid solution and hydrolyzed by boiling for 2–3 h. The solution was then neutralized with NaOH solution, saturated with NaCl, extracted with ethyl acetate (3 × 100 mL), and evaporated until just dry in a rotary evaporator at 50 °C. The cleanup of the extract was performed as described under Metalaxyl and Its Nonconjugated Metabolites. Cleanup.

Cultural Conditions. Sunflower plants were grown in a controlled environment: light intensity = 14 000 lx; photoperiod = 12 h; temperature = 20 °C; relative humidity = 80%. Seeds were planted in 35 cm × 60 cm boxes, each containing 36 kg of a Mollic psammaquent soil (pH_{H2O} = 7.5; organic matter 11.1%; sand, silt, and clay 818, 64, and 118 g/kg, respectively). Metalaxyl was applied by incorporation into the soil at the rate of 2 g/m^2 as granular Ridomil (5% of a.i.). Twenty days later the leaves were harvested, homogenized, and stored at −24 °C to await analysis (Marucchini et al., 1983).

Lettuce was grown in hydroponics in a controlled environment at the same conditions as the sunflower. The nutrient solution employed was the same used in a previous work (Tafuri et al., 1973). When the plants had two to four leaves, the nutrient solution was replaced with another one having the same concentration of nutritives but containing 100 ppm of metalaxyl. This treatment was performed for 2 days and then the first nutrient solution was used until the end of the experiment. Plants were harvested 20 days after the metalaxyl treatment was stopped and stored at −24 °C to await analysis.

Metalaxyl and Its Nonconjugated Metabolites. *Extraction.* Chopped vegetable matter corresponding to 100 g of fresh material was blended with 200 mL of ethyl acetate–methanol solution (90:10) for 10 min. The mixture was centrifuged and supernatant filtered into a suitable volume flask. The filtrate was collected in a separating funnel to separate the organic from aqueous phase. After phases separation, the organic layer was collected and the aqueous phase was extracted with ethyl acetate (3 × 30 mL). Organic phases were collected and the aqueous phase was stored. The extraction procedure with ethyl acetate–methanol (90:10) was repeated with additional (3 × 100 mL) ethyl acetate–methanol solution. The residues resulting from the centrifugation was further used for aqueous extraction as described below. All the combined organic extracts were evaporated until just dry by using rotary evaporator at 50 °C.

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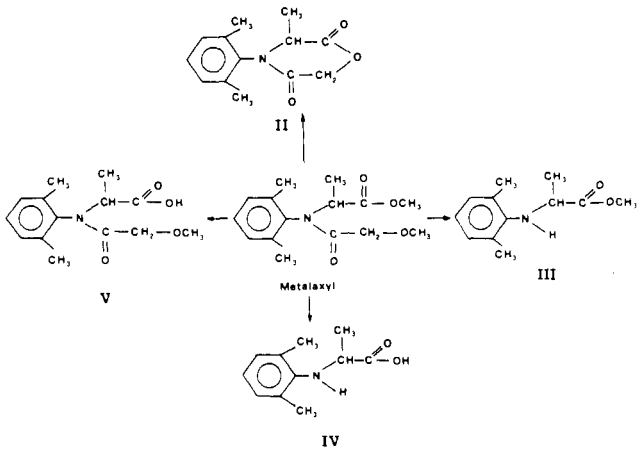

Figure 1. Metalaxyl and its degradation products after acid hydrolysis.

Cleanup. A chromatographic column (2.5-cm diameter) with a fritted disk (G1) was filled with a slurry of 7 g of silica gel in chloroform–ethyl acetate (2:1) and the slurry allowed to settle. The extracted residue from the previous section was taken up on three 2-mL portions of chloroform–ethyl acetate (2:1) and transferred to the top of the column, allowing the first portion to be absorbed into the column before adding the next. The column was eluted with 150 mL of chloroform–ethyl acetate (2:1) in order to collect metalaxyl and its degradation products (Figure 1), II and III (fraction A). The column was then eluted with 150 mL of methanol to collect the products IV and V (fraction B). The A and B fractions were evaporated in rotary evaporators at 50 °C until just dry. The residue from fraction B was dissolved in a mixture of methanol (0.5 mL) and diethyl ether (3 mL) and transferred to the tube in a methylation apparatus. Methylation was carried out using diazomethane under the conditions previously reported (Tafuri et al., 1972, 1977). Thirty minutes after the start of reaction, the content of the test tube was evaporated in a gentle stream of dry nitrogen. The residues from fractions A and B obtained after the methylation procedure were taken up separately in three (3-mL) portions of benzene, and each was transferred to the top of columns (1.0-cm diameter) containing a slurry prepared by shaking 5 g of Al_2O_3 (Merck, activity II) with benzene. The columns were eluted with 60 mL of benzene and the eluate was discarded. The columns were then eluted with 50 mL of chloroform–ethyl ether (90:10), and the eluate was collected and evaporated under vacuum at 50 °C until just dry. The residues were dissolved in 0.2 mL of ethyl acetate, and aliquots (1–2 μL) were injected into the gas chromatograph and into the gas chromatograph–mass spectrometer apparatus. Figure 2 is a schematic diagram of the method described.

Metalaxyl Conjugated Metabolites. *Extraction.* The vegetable residue from centrifugation was treated with 100 mL of distilled water and homogenized in blendor for 10 min. The suspension was centrifuged at 5000 rpm for 15 min and the supernatant filtered. This solution was collected with the aqueous phase partitioned in ethyl acetate as described under Metalaxyl and Its Nonconjugated Metabolites. Extraction.

Hydrolysis. The aqueous solution from previous section was acidified adding 20 mL of concentrated HCl (12 N) and boiled for 2 h. The mixture was brought to pH 7 with 10 N and 0.1 N NaOH, saturated with KCl, and extracted first with ethyl acetate (3 × 50 mL) and then with 50 mL of ethyl acetate–tetrahydrofurane (THF) (30:20). The organic extracts were collected, passed through anhydrous

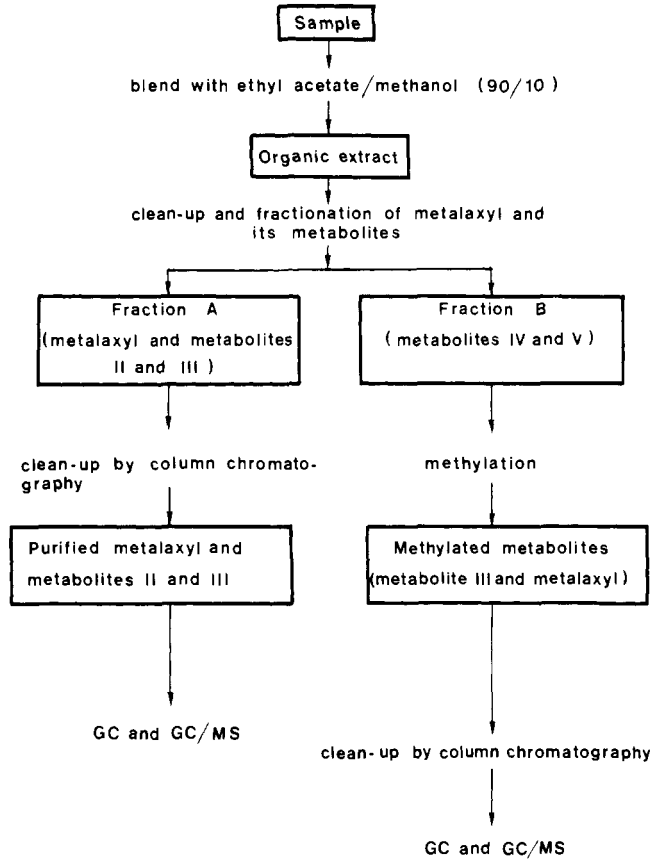

Figure 2. Schematic diagram for the analysis of metalaxyl and its nonconjugated metabolites.

sodium sulfate, and evaporated just to dryness in a rotary evaporator at 50 °C (fraction C). The solid residue left after aqueous extraction was dispersed in 100 mL of 2 N HCl and boiled for 2 h. The mixture was then neutralized, extracted, and evaporated as described for the aqueous solution (fraction D).

Methylation and Cleanup. The C and D fractions were dissolved in a mixture of methanol (0.5 mL) and diethyl ether (3 mL), methylated, and evaporated. The residues were taken up in three (3-mL) portions of benzene, and each was trasferred to the top of columns (2.5-cm diameter) containing a slurry prepared by shaking 7 g of silica gel with benzene. Benzene (150 mL) was added to the columns and the eluate was discarded. The columns were then eluted with 150 mL of chloroform–benzene (100:50), and the eluate was collected and concentrated under vacuum at 50 °C until a volume of 2–3 mL was obtained. A further purification was performed by columns (1.0-cm diameter) each containing 5 g of acidic alumina (Merck, activity II) in benzene. The columns were eluted with 50 mL of chloroform–benzene (1:1) by volume and the eluate was collected to detect compound III. The columns were then eluted with 50 mL of chloroform–ethyl acetate (1:1) by volume and the eluate was discarded. Finally, the columns were eluted with 50 mL of ethyl acetate–methanol (1:1) by volume and the eluate was collected for detecting compound II. The eluates were evaporated under vacuum at 50 °C. The residues were each dissolved in 0.2 mL of ethyl acetate, and aliquots (1–2 μL) were injected into the gas chromatograph and into the gas–chromatograph–mass spectrometer apparatus. Figure 3 is a schematic diagram of the method described.

RESULTS AND DISCUSSION

Metalaxyl was degraded after acid hydrolysis to give the products II–V (Figure 1). The identity of these products

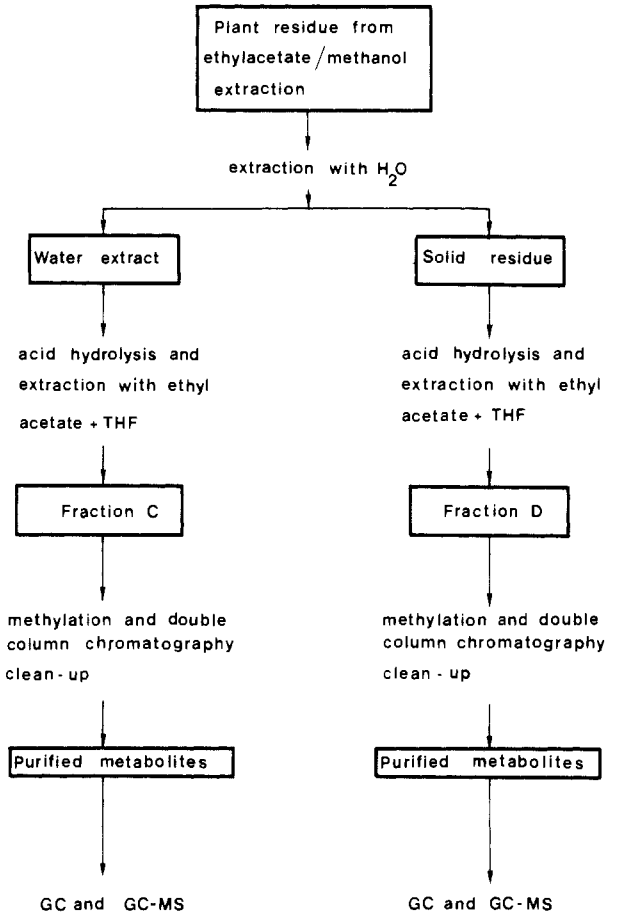

Figure 3. Schematic diagram for the analysis of conjugated metalaxyl metabolites.

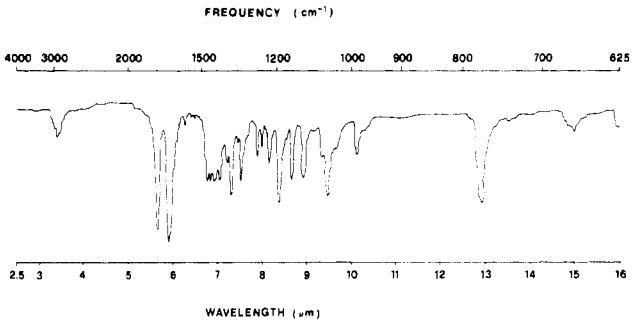

Figure 4. Infrared spectrum of compound II.

was confirmed by gas chromatography and IR, NMR, and GS–MS spectra. The IR spectrum of compound II (Figure 4) shows the presence of CO vibrations at 1770 and 1695 cm^{-1} and the loss of NH and OH vibrations. The mass spectral data indicate the molecular ion M^+ at m/e 233 and significant cleavages at m/e 175 and m/e 105. The comparison of NMR spectra of metalaxyl (Figure 5) and compound II (Figure 6) revealed that compound II had the structure shown in Figure 1. The spectral data of compound III, which indicate molecular ion M^+ at m/e 207 and significant cleavages at m/e 176 and m/e 105, give the structure shown in Figure 1.

When compound IV was methylated and injected into the gas chromatograph, its retention time was the same as that of compound III.

Finally, the gas chromatographic analysis showed that metalaxyl was obtained after methylation of compound V.

Recovery experiments were run on a number of samples by adding known amounts of metabolites at the chloroform–ethyl acetate step at the beginning of the cleanup

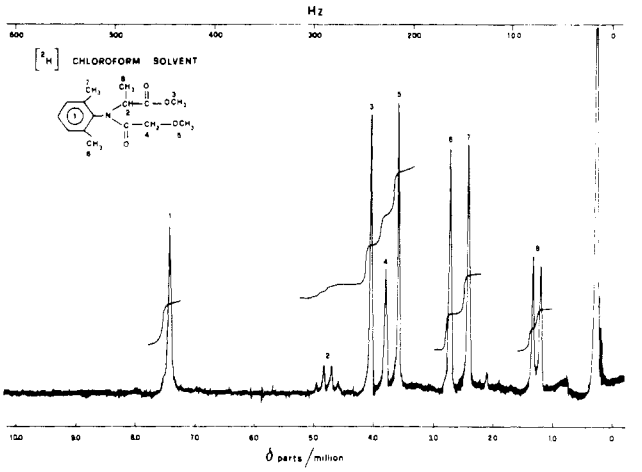

Figure 5. 60-MHz proton magnetic resonance spectrum of metalaxyl.

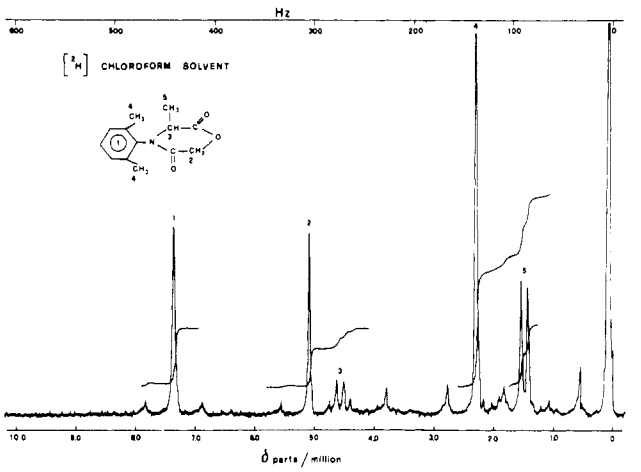

Figure 6. 60-MHz proton magnetic resonance spectrum of compound II.

Table I. Concentration and Distribution of Metalaxyl and Its Identified Metabolites in Lettuce and Sunflower

	concn[a] in lettuce, ppm of fresh wt		concn[a] in sunflower, ppm of fresh wt	
compounds	free compds (in organic extract)	conjugated compds (in aqueous extract)	free compds (in organic extract)	conjugated compds (in aqueous extract)
metalaxyl	4.90		4.94	
II	0.12	0.03[b]	0.23	0.03[b]
III		0.01[b]		0.01[b]
V	0.02	0.03	0.01	0.04

[a] Mean of three determinations. [b] Probably derived from compound V after acid treatment.

procedure. The results for the different compounds in the 0.01–1.0-ppm range were metalaxyl 89–98%, II 94–97%, III 85–96%, IV 79–96%, and V 70–82%. The limit of sensitivity for all compounds using NPD detector was 0.01 ppm.

The concentration of the metalaxyl and its derivatives that were detected in the plants are given in Table I. In addition to metalaxyl, nonconjugated compounds II and V were collected from the organic extracts. Compounds II, III, and V were identified after acid hydrolysis of the aqueous extract. As conjugated metabolites II and III may also derive from the action on compound V of the strongly acid medium used to hydrolyze conjugated compounds,

there is no evidence for these in the plants examined and this is supported by the fact that compound II is short of free organic groups that can promote conjugation reactions and compound III was not found among the nonconjugated metabolites. Compound V, other than from hydrolysis of its conjugate, can eventually derive from action, only on metalaxyl, of the strongly acid medium used to hydrolyze conjugated compounds. As nonconjugated metalaxyl was completely extracted in the ethyl acetate–methanol phase and as it is a compound short of free hydroxyl, carboxyl, or amino groups, it could not be present as a conjugate; there is evidence that the conjugated metabolite is compound V. This is supported by the presence of compund V among nonconjugated compounds.

No conjugated metabolites were found in the solid residue.

In conclusion, although metalaxyl is generally regarded as a stable compound, particularly in the cultural conditions used in these experiments, it was found to be subject to a certain decomposition that lead to hydrolysis of the molecule, giving compound V, which would seem to be the only responsible metabolite for the formation of conjugated derivative and, in a cyclization, for giving nonconjugated metabolite II.

Registry No. II, 88945-75-9; III, 52888-49-0; IV, 67617-64-5; V, 87764-37-2; metalaxyl, 57837-19-1.

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Cultivar Differences in Gelling Characteristics of Soybean Glycinin

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The gelation and gel properties of glycinins were examined for five soybean cultivars having different subunit compositions. The gelling characteristics of glycinin differed significantly among cultivars, arising from the differences in the nature of protein itself as well as protein concentration. Glycinins of three of the cultivars studied formed a gel within half the time required for the other two cultivars, where the former cultivars contained acidic subunit AS-IV, which is linked with a basic subunit to form an intermediary subunit through some bonds other than disulfide bonds, and the latter cultivars did not contain acidic subunit AS-IV. Hardness of the gels was different among cultivars, depending on the percentage of AS-III, which is the largest constituent acidic subunit of glycinin. Turbidity of the gels had a tendency to increase with increasing content of sulfhydryl groups of glycinins.

The seed storage proteins of legumes contain legumin that occurs in large amounts and appears to be made up of twelve subunits, of which six subunits are acidic and six subunits are basic in nature (Derbyshire et al., 1976). The diversity of the subunit composition of legumin among cultivars has been shown by Harada (1972) and Kitamura et al. (1980) for soybean, by Tombs (1965) for groundnut, by Blagrove and Gillespie (1978) for lupin, by Thomson and Schroeder (1978) and Casey (1979) for pea, and by Utsumi et al. (1980) for broad bean. We have recently demonstrated that the subunit compositions of glycinins isolated from the seeds of various cultivars of soybean vary among the cultivars and may be classified into five groups according to differing molecular charges of the subunits (Mori et al., 1981).

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On the other hand, it is generally known that the major components of seed storage proteins are responsible for contributing to the quality, particularly the physical properties, of foods made from these seeds, their flour, and protein products. It has been reported that the quality of tofu gel (a traditional Japanese food made from soybeans) differs according to the cultivars used (Smith et al., 1960; Saio et al., 1969; Wang et al., 1983). The diversity of subunit composition of the major components of seed storage proteins among cultivars is most likely to be related to the physical properties of the foods made. 11S globulin (referred to as glycinin), one of the major components of soybean storage protein, has been shown to have intermediary subunits (AB), disulfide-bonded acidic (A) and basic (B) subunits, and the 6 (AB) structure (Badley et al., 1975; Kitamura et al., 1976; Mori et al., 1979). The acidic subunits have been separated by DEAE-Sephadex column chromatography into four fractions designated as AS-I (M_r 34 800), AS-II (M_r 34 800), AS-III (M_r 38 000), and AS-IV (M_r 34 800) in the order of elution from the column (Mori et al., 1982a). Acidic subunits I, II, and III are linked to their basic subunit counterpart by disulfide bridges. However, AS-IV is an exception; the linkage is noncovalent

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