Avermectin B_{1a} in Celery: Acetone-Unextractable Residues

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Stalk and leaf samples of celery treated with radiolabeled avermectin B_{1a} were serially extracted with acetone, MeOH/H_2O (40/60), and hot DMSO. Characterization of the residues extractable with acetone has previously been reported. Characterization of the residues extractable by aqueous MeOH and hot DMSO, which solubilizes lignin, demonstrated that the residues were mostly unknown polar degradates of avermectin B_{1a}. In leaves from plants treated with [^{14}C]- or [^{3}H]avermectin B_{1a} 18.61 and 15.19%, respectively, of the residue were unextractable after treatment with hot DMSO. In the corresponding stalks 8.78 and 9.82%, respectively, of the residue were unextractable. The ^{14}C- and ^{3}H-labeled nonextractable residue in celery leaves was further hydrolyzed to release glucose. In celery, 4.59 (^{3}H)–14.54% (^{14}C) of the acetone-nonextractable label present at the normal harvest interval was incorporated into glucose.

I. INTRODUCTION

The avermectins are a new class of pesticide produced by the actinomycete *Streptomyces avermitilis* (Burg et al., 1979). Molecular structures and some biological activities have been reported (Albers-Schonberg et al., 1981; Campbell et al., 1983; Dybas, 1989). Abamectin (≥80% avermectin B_{1a}, ≤20% avermectin B_{1b}; Figure 1) is the commercial product that is being developed for various uses, including control of leaf miners and spider mites on celery.

Therefore, an avermectin B_{1a} (B_{1A}) metabolism study was performed on celery, and the acetone-extractable residues were characterized (Moye et al., 1990). The experiments reported here extend these studies to the residues in celery that are unextractable with acetone. These residues, depending on their nature and amount, could be of toxicological concern.

The general systematic approach of Kovacs (1986) was employed. This approach divides the pesticide residues into four types: (1) free degradates; (2) conjugated degradates; (3) bound residues; (4) natural constituents. Free degradates are usually removed from the plants by extraction with cold solvents. Conjugated degradates are removed with water or a water/solvent mixture. Both of these types of residues are, by definition, extractable. The next two types of residues, bound residues and residues incorporated into natural products, are considered unextractable, and distinguishing between them is difficult. Bound residues are usually chemically bound to cellular components. One problem encountered with bound residues is that many methods used in isolating them employ harsh conditions so that the chemical identity of the residue is changed. The last type of residue occurs after the compound is degraded to small fragments which can be incorporated into natural products (e.g., lignin, glucose, proteins).

By use of this outline, B_{1A} residues in celery were investigated. The focus of these investigations was to characterize and, if possible, identify the residues remaining after extraction with acetone.

II. MATERIALS AND METHODS

Chemicals. Radiolabeled and nonradiolabeled B_{1A} and emulsifiable concentrate formulation (EC) were supplied by Merck, Sharp and Dohme Research Laboratories. [^{3}H]B_{1A} (>99+% radiochemical purity, specific activity 1.64 mCi/mg), in which the ^{3}H label was located at the C5 position, was added to unlabeled B_{1A} to give a stock solution of 18 g of B_{1A}/L of EC (300 μCi of ^{3}H/mL of EC). [^{14}C]B_{1A} (>99+% radiochemical purity, specific activity 16.3 μCi/mg), in which the ^{14}C label was located at the C3, C7, C11, C13, or C23 position, was added to unlabeled B_{1A} to give a stock solution of 2.7 g of B_{1A}/L of EC (24.4 μCi/mL of EC; Moye et al., 1990).

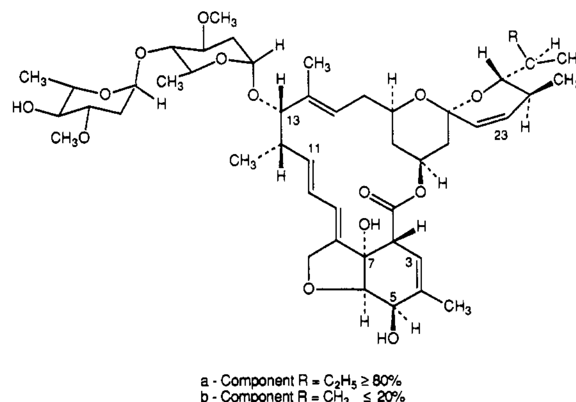

Figure 1. Structure of abamectin.

All solvents were of HPLC grade and were obtained from EM Science. Water was of HPLC grade and was procured from a Waters Milli-Q water-purifying system. Sulfuric and hydrochloric acids were of reagent grade and were bought from Fisher Scientific.

Carbo-Sorb and Permafluor V for ^{14}C liquid scintillation counting (LSC) and Monophase 40 for ^{3}H LSC following oxidative combustion and Insta-gel for LSC following HPLC were obtained from Packard Instruments.

Tritium-labeled (3-^{3}H; 13.5 Ci/mmol) or ^{14}C-labeled (1,2,3,4,5,6-^{14}C; 14.4 mCi/mmol) glucose was purchased from New England Nuclear.

Instrumentation. High-performance liquid chromatography (HPLC) and UV detection were accomplished, respectively, with a Spectra Physics 8700 ternary pump and a Hewlett-Packard (HP) 1040 M photodiode array detector. The detector was linked to an HP printer and an HP plotter. An IBM ODS column (250 mm × 4.6 mm) was eluted with 85% MeOH/15% H_2O at a flow rate of 1 mL/min. A guard column filled with Whatman Co:Pell ODS packing was used to protect the analytical column. Fractions (1 mL) of eluate were collected in 7-mL plastic vials by using a Pharmacia Fractomette fraction collector. Radioactivity in the samples was determined by LSC.

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The conditions used for flow and gradient programming during HPLC, when necessary, were as follows: 0 min, 50/50 MeOH/H_2O, 0.5 mL/min flow; 2 min, 50/50 MeOH/H_2O, 0.5 mL/min flow; 3 min, 85/15 MeOH/H_2O, 0.5 mL/min flow; 4 min, 85/15 MeOH/H_2O, 1.0 mL/min flow. Samples were dissolved in 1 mL of MeOH and injected onto this system.

Oxidative combustion was performed with a Tri-Carb, Model B 306 (Packard), instrument. Duplicate samples (approximately 10–45 mg) of each mat were combusted. Radioactivity was determined by LSC.

LSC was performed with either a Packard 4530 or 460 instrument with appropriate quench curves for conversion of cpm to dpm.

Plant Treatment, Harvest, and Extraction. In the celery study performed by Moye et al. (1990), plants were treated with radiolabeled B_{1A} by application of the stock solutions, after they were diluted 1:75 with water (10 applications, each application a week apart). Plants were treated with $[^3H]B_{1A}$ at a rate of 0.10 lb (5 times the maximum proposed use rate) of active ingredient/acre (24 μCi and 1.44 mg/plant per application). Other plants were treated with $[^{14}C]B_{1A}$ at a rate of 0.015 lb (0.75 times the maximum proposed use rate) of active ingredient/acre (1.9 μCi and 216 μg/plant per application). The stems and leaves of treated plants were homogenized and exhaustively extracted with acetone. The resulting mats were air-dried.

In the study reported here, the residues remaining in these mats from plants harvested 7 days after the last application to mature plants were investigated, since this is the projected postharvest interval.

In a preliminary experiment, mats containing ^3H residues were serially extracted with MeOH/H_2O (40/60), $CHCl_3$, CH_2Cl_2, toluene, and cyclohexane. Almost all (83%) of the radioactivity removed was associated with the MeOH/H_2O fraction. Therefore, this solvent mixture was first used to extract the mats.

MeOH/H_2O Extraction. Leaf or stalk mats were mixed with MeOH/H_2O (40/60; approximately 60 mL of solvent/g of mat), extracted for 1 h in Nalgene tissue bottles on a wrist shaker (Burrell Model 75), and centrifuged, and the supernatant was removed. This procedure was repeated four times for all samples; a fifth extraction was performed overnight. All supernatants from each extracted mat were pooled and assayed for radioactivity by LSC. The extracted mats were dried under a heat lamp, placed in a desiccator overnight, and weighed. Total radioactivity remaining in the mats was determined by oxidative combustion of mat subsamples.

Solubilization of Lignin with DMSO. After MeOH/H_2O extraction, the remaining dried mats were further extracted by stirring for 22 h at 80 °C with approximately 400 (^3H-labeled mats) or 600 mL (^{14}C-labeled mats) of DMSO to remove lignin (Haque et al., 1976; Kovacs, 1986; Chin et al., 1973). The samples were filtered or decanted and washed with an additional 100 mL of DMSO. Pooled DMSO extracts for each mat were assayed for radioactivity by LSC. These mats, after extraction with MeOH/H_2O and DMSO, were dried under a heat lamp, placed in a desiccator overnight, and weighed. Total radioactivity in the mats was determined by oxidative combustion of mat subsamples.

Acid Hydrolysis of Celery Extracts. Acid hydrolysis of MeOH/H_2O and DMSO extracts was performed to liberate possible conjugated residues. Hydrolysis conditions had been previously established by using standard $[^{14}C]B_{1A}$. For MeOH/H_2O extracts hydrolysis was performed at a concentration of 0.033 N HCl overnight at room temperature with stirring. Under these acid hydrolysis conditions, 83.13% of added radiolabeled B_{1A} remained intact. B_{1A} (^{14}C or ^3H) was then added as an internal standard to MeOH/H_2O extracts containing residues of the opposite radiolabel. A control (no acid) and an acid-hydrolyzed sample were examined in all cases.

After hydrolysis, MeOH/H_2O extracts were then extracted three times with a volume of CH_2Cl_2 equal to one-third the volume of the extract. The residues extracted by CH_2Cl_2 from the MeOH/H_2O extracts were concentrated, dissolved in 100 μL of MeOH, and analyzed by reversed-phase HPLC (85/15 MeOH/H_2O) and LSC. Calculated percentages for CH_2Cl_2 extracted residue were corrected for standard recovery and normalized for percent of standard hydrolyzed (assuming CH_2Cl_2 extracted residue is similar to B_{1A}).

DMSO extracts were then subjected to acid hydrolysis. Hydrolysis conditions were again established by using standard $[^{14}C]B_{1A}$. Acid hydrolysis was performed at a concentration of 0.035 N HCl overnight at room temperature. After hydrolysis of DMSO extracts, 87.50% of radiolabeled B_{1A} standard remained. $[^{14}C]$- and $[^3H]B_{1A}$ were then added as an internal standard, respectively, to DMSO extracts [diluted 1:1 (v/v) with H_2O prior to acidification] containing ^3H and ^{14}C residues. A control (no acid) and an acid-hydrolyzed sample were examined for each DMSO extract.

After acid hydrolysis, DMSO extracts were extracted with cyclohexane (six times with a volume of cyclohexane equal to half the volume of extract). The residues extracted by cyclohexane from the DMSO extracts of leaves from plants treated with $[^3H]B_{1A}$ were concentrated, dissolved in 100 μL of MeOH, and analyzed by HPLC. However, the concentrated cyclohexane extract of the other three treatments (stalk from $[^3H]B_{1A}$-treated plants; leaf and stalk from $[^{14}C]B_{1A}$-treated plants) caused high back pressures during HPLC. Therefore, to circumvent this problem, a combination of flow and gradient programming was used to concentrate the sample at the head of the column as described above. Calculated percentages for cyclohexane-extracted residue were corrected for standard recovery and normalized for percent of standard hydrolyzed.

Glucose Isolation and Derivatization. After extraction with MeOH/H_2O and DMSO, incorporation of radiolabel into glucose of celery leaf mats was determined.

Two sets of duplicate leaf samples were examined. For the first set, radiolabeled glucose standard was spiked into leaf mats. This sample set was derivatized by using the procedure indicated below and combusted, therefore serving as an external standard for the amount of spiked glucose derivatized. The second set of samples, consisting of unspiked leaf mats, was then derivatized and combusted. Determination of residue incorporated into glucose was made by correcting for the amount of spiked standard derivatized in the first set.

A variation of a previously reported procedure (Honeycutt and Adler, 1975) was used. Briefly, the radiolabeled glucose standard was added to 0.5 g of mat of the opposite radiolabel (i.e., ^{14}C-labeled glucose was added to ^3H-labeled mat and ^3H-labeled glucose was added to ^{14}C-labeled mat), and 5 mL of 70% H_2SO_4 was added. The mixture was shaken for 3 min, left for 1 h at 10 °C, and then shaken again for 3 min. The mixture was left for another 16 h at 10 °C. Then 50 mL of HPLC H_2O was added, and the mixture was refluxed for 4 h. The suspension was filtered, and the pH was adjusted to 7.0 with NH_4OH. For derivatization, 5 g of phenylhydrazine hydrochloride and 7.5 g of sodium acetate were added to the refluxed mixture, and water was added for a final volume of 140 mL. This mixture was heated in a boiling H_2O bath for 7 h with constant stirring; it was then placed in a refrigerator overnight. The precipitate, containing the water-insoluble osazone derivative, was filtered, washed from the filter paper with MeOH, dried on a hot plate, placed in a desiccator, and weighed. Total radioactivity in the precipitated material was determined by oxidative combustion.

III. RESULTS AND DISCUSSION

The total residue in mature celery treated with B_{1A} at 7 days after the last application was 20 (^{14}C, 0.75x) or 238 ppb (^3H, 5x) for stalk and 197 (^{14}C, 0.75x) or 1134 ppb (^3H, 5x) for leaf (Moye et al., 1990). Approximately 60–80% of the residues were extractable with acetone (Table I; Moye et al., 1990), leaving 18.70–42.20% (7–406 ppb) of the terminal residues for the subsequent analyses presented in this paper. Most of the acetone-extractable residues in these plants were characterized as polar degradates of B_{1A} (Moye et al., 1990).

The results of the present study also show that most of the residues unextractable with acetone are more polar than B_{1A} (see the following sections); they do not extract into organic solvents with B_{1A} and remain in the aqueous

Table I. Release of Residues Unextractable with Acetone from Celery Leaves and Stalks

	^3H	
	leaves	stalks
total residue before acetone extraction, ppb	1134	238
total residue after acetone extraction, % (ppb)	35.80 (406)[a]	18.70 (45)
$MeOH/H_2O$ extract, % (ppb)	13.70 (155)	4.92 (12)
DMSO extract, % (ppb)	6.91 (78)	3.96 (10)
glucose, % (ppb)	4.59 (52)	
remaining in mat, % (ppb)	10.60 (120)	9.82 (24)

	^{14}C	
	leaves	stalks
total residue before acetone extraction, ppb	197	20
total residue after acetone extraction, % (ppb)	42.20 (83)	33.00 (7)
$MeOH/H_2O$ extract, % (ppb)	14.59 (29)	14.29 (3)
DMSO extract, % (ppb)	9.00 (18)	9.93 (2)
glucose, % (ppb)	14.54 (29)	
remaining in mat, % (ppb)	4.07 (8)	8.78 (2)

[a] Calculated from Moye et al. (1990).

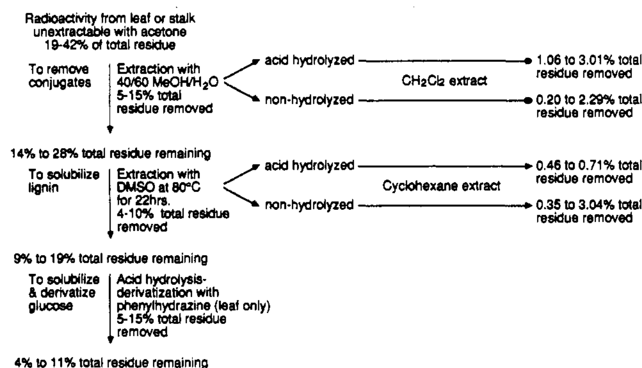

Figure 2. Successive removal of ^{14}C and ^3H acetone-nonextractable residues from celery expressed as percent of total residue.

layers after extraction. The procedure for serial removal of residues unextractable with acetone is summarized in Figure 2.

MeOH/H$_2$O Extraction. The acetone-extracted leaf and stalk mats were extracted with $MeOH/H_2O$ to remove free and/or possibly conjugated degradates of B_{1A}. Extraction with $MeOH/H_2O$ (40/60) removed 4.92–14.59% of the terminal residue, leaving 13.78–27.61% of the total radioactivity (Table I) in the remaining mat.

DMSO Solubilization of Lignin. Extraction of the mats with hot DMSO solubilizes lignin, one of the most common compounds found in plants (Marton, 1966). Lignin is also the most abundant aromatic compound found in cell walls. It is a complex, cross-linked polymeric material which can be a repository for bound residues (Marton, 1966; Huber and Otto, 1983). Hot DMSO extracts another 3.96–9.93% of the terminal B_{1A} residue, indicating that the radioactivity is associated with lignin or possibly incorporated into it (Table I). Of the total radioactivity, 8.78–18.61% remains unextracted.

The amount of residues associated with the extracts is shown in Table I, expressed as percent of total residue and parts per billion of whole plant. Comparable percentages of residue remain in leaves (15.19 and 18.61%) and in stalks (9.82 and 8.78%) for both ^3H and ^{14}C mats after serial extraction with $MeOH/H_2O$ and DMSO.

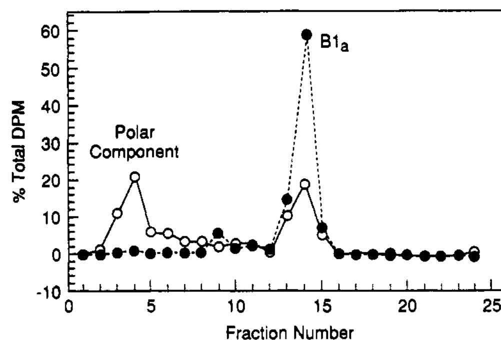

Figure 3. HPLC of ^3H celery stalk, CH_2Cl_2 of $MeOH/H_2O$ extract, spiked with ^{14}C-labeled B_{1A}. (—) ^3H; (- - -) ^{14}C. HPLC conditions: IBM ODS column eluted with 85/15 $MeOH/H_2O$; flow rate 1 mL/min; fractions collected every minute.

Table II. Characterization of $MeOH/H_2O$ Residues That Are Extracted with CH_2Cl_2[a]

	total CH_2Cl_2 extractable,[c] %	HPLC analysis[b]		
		B_{1a}, %	polar component, %	remainder, %
^3H leaf nonhydrolyzed	2.29	=0.31	0.52	1.46
hydrolyzed	3.01	=0.10	1.21	1.70
difference[d]	+0.72	−0.21	+0.69	
^3H stalk nonhydrolyzed	0.75	=0.11	0.16	0.48
hydrolyzed	1.06	=0.19	0.41	0.46
difference	+0.31	+0.08	+0.25	
^{14}C leaf nonhydrolyzed	1.63	=0.0	0.94	0.69
hydrolyzed	2.75	=0.0	2.17	0.58
difference	+1.12	0.0	+1.23	
^{14}C stalk nonhydrolyzed	0.20	=0.0	0.15	0.05
hydrolyzed	1.38	=0.0	1.01	0.37
difference	+1.18	0.0	+0.86	

[a] Expressed as percent of total residue. [b] IBM ODS column eluted with 85/15 $MeOH/H_2O$; flow rate 1 mL/min. [c] Any residue not present as B_{1A} or the polar component is evenly distributed throughout the chromatogram. [d] Hydrolyzed − nonhydrolyzed = difference.

Residue levels in stalks are always less than residue levels in leaves, probably because B_{1A} was applied directly to the leaves in the celery metabolism study (Moye et al., 1990).

Acid Hydrolysis of Celery Extracts. Acid hydrolysis of $MeOH/H_2O$ and DMSO extracts was performed to liberate possible conjugated or bound residues. Base hydrolysis was not performed because of lability of the avermectins to base (Pivnichny et al., 1983, 1988).

Methylene chloride extraction of residues extracted by $MeOH/H_2O$ indicates that the majority of the residues present in $MeOH/H_2O$ are polar in nature since they remain in the aqueous fraction. While 4.92–14.59% of the terminal residue is present in this $MeOH/H_2O$ fraction (Table I), only 0.20–3.01% of the total residue is conjugated to plant material that extracts into CH_2Cl_2 from this fraction. HPLC analysis of the residue liberated by acid hydrolysis of the $MeOH/H_2O$ extracts and extracted into CH_2Cl_2 indicates a polar component and B_{1A}. The remaining radioactivity present in the chromatogram is present as background (Figure 3; Table II). The polar component may contain the monosaccharide derivative of B_{1A}. Previous studies have shown that the monosaccharide is formed during acid hydrolysis (Fisher and Mrozik, 1989). In addition, when this system is used, the polar component is close to the void volume of the analytical column (2 mL) and could, therefore, contain more than one residue. From these data it is apparent that acid hydrolysis typically releases about 1% more

Table III. Characterization of DMSO Residues That Are Extracted with Cyclohexane[a]

	total cyclohexane extractable,[c] %	HPLC analysis[b]		
		B_{1a}, %	polar component, %	remainder, %
^3H leaf nonhydrolyzed	0.75	=0.12	0.06	0.57
hydrolyzed	0.46	=0.0	0.06	0.40
difference[d]	−0.29	−0.12	0.0	
^3H stalk nonhydrolyzed	0.44	=0.22	0.03	0.19
hydrolyzed	0.71	=0.39	0.08	0.24
difference	+0.27	+0.19	+0.05	
^{14}C leaf nonhydrolyzed	0.35	=0.23	0.08	0.04
hydrolyzed	0.57	=0.34	0.11	0.12
difference	+0.22	+0.11	+0.03	
^{14}C stalk nonhydrolyzed	3.04	=0.76	1.50	0.78
hydrolyzed	0.67	=0.21	0.36	0.10
difference	−2.37	−0.55	−1.14	

[a] Expressed as percent of total residue. [b] Conditions same as in Table II for leaf treated with ^3H-labeled B_{1A}; see text for flow and gradient programming conditions for other samples. [c] Any residue not present as B_{1A} or the polar component is evenly distributed throughout the chromatogram. [d] Hydrolyzed − nonhydrolyzed = difference.

residue compared to control samples (no acid), and generally, this 1% is present as the polar component. These data also suggest that trace amounts of B_{1A} (<0.31% of total residue) are still present in $MeOH/H_2O$ extracts.

Cyclohexane extraction of the residues extracted with DMSO (after addition of H_2O) also indicates that the majority of residues present in DMSO are polar in nature since they remain in the aqueous fraction. While 3.96–9.93% of the terminal residue is present in this DMSO/H_2O fraction (Table I), only 0.35–3.04% of the total residue is bound to lignin that is extracted with cyclohexane from the DMSO fraction (Table III). Also, upon evaporation of the cyclohexane extract approximately 50% of the tritium label is volatilized (^{14}C not done). This probably indicates that B_{1A} has been degraded into smaller carbon fragments. From the cyclohexane extracts, as with the $MeOH/H_2O$ extracts (Figure 3), only two distinct peaks are present in the radioactivity histograms: B_{1A} and a polar component (data not shown). Data analysis is difficult due to the low amount of radioactivity associated with these residues (in many cases <100 dpm). Generally, these samples also contain small amounts of B_{1A} (0.76% and less; Table III).

Glucose Isolation and Derivatization. After lignin extraction with DMSO, a crude cellulose mat remains. These mats are acid hydrolyzed under severe conditions (70% H_2SO_4) to liberate glucose. The amount of radioactivity incorporated into glucose, isolated as the osazone derivative, is 4.59 and 14.59%, respectively, for ^3H and ^{14}C residues (Table I; Figure 2). Therefore, B_{1A} has been degraded and a significant portion of the terminal residue has been incorporated into a natural product. More ^{14}C residue (14.59%) is incorporated than ^3H residue (4.59%). Approximately 48% of added [^{14}C]glucose standard vs 31% of added [^3H]glucose is derivatized by the osazone procedure. This difference could be due to loss of ^3H during the hydrolysis and derivatization process. Glucose is a six-carbon compound, and since B_{1A} is incorporated into glucose, B_{1A} is probably degraded into fragments that contain six or less carbons. In addition, more [^{14}C]glucose from B_{1A}-treated plants would be expected since [^{14}C]-B_{1A} contains label at C3, C7, C11, C13, and C23 while [^3H]B_{1A} contains label only at C5.

Two earlier investigations (Maynard et al., 1989; Ku and Alvaro, unpublished data) suggest that B_{1A} can be degraded and incorporated into natural products in citrus and cotton. These results along with the results of the present study, provide convincing evidence for the extensive degradation of B_{1A} during normal field use.

The polar degradates of B_{1A} also provide additional evidence for breakdown. Results from the B_{1A} celery metabolism study (Moye et al., 1990) suggest that the majority of radioactive residue present at the postharvest interval is polar in nature. The majority of the radioactive residue nonextractable with acetone, investigated in the present celery study, is much more polar than B_{1A}. Also, results from photolysis studies in water, on soil, and as thin films on glass demonstrate that B_{1A} degrades to multiple components, including a polar fraction (Wislocki et al., 1989). In these studies the polar residues do not possess UV absorbance at 245 nm that is characteristic of the conjugated diene present in B_{1A} (Figure 1). This extensive breakdown of B_{1A} indicates that B_{1A} would not persist in the environment.

The total residue remaining unextractable in celery leaf and stalk, after the experimental procedures presented in this paper, is approximately <10%. A large portion of the unextractable residues are incorporated into the natural product glucose. Less than 1% of the unextractable terminal residues are present as B_{1A}; the majority of the remaining unextractable residues are unknown polar degradates of B_{1A}. Polar degradates of B_{1A}, generated on citrus, were devoid of the toxicity associated with abamectin in the CF_1 mouse, the animal species most sensitive to avermectin toxicity (Gordon and Crouch, unpublished data). Similarly, polar degradates of B_{1A} have been tested for toxicity in Daphnia (a sensitive nontarget organism) and possess an LC_{50} more than 160 times that of B_{1A} (Wislocki et al., 1989). These data indicate that the residues remaining in celery after acetone extraction are not of toxicological significance.

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