Determination of the Phenolic Metabolites of Carbofuran in Peppermint Hay and Peppermint Oil by Multiple Ion Detection Mass Spectrometry

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The analysis of the phenolic metabolites of carbofuran, 2,3-dihydro-2,2-dimethyl-7-benzofuranol (7-phenol), 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol (3-keto-7-phenol), and 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol (3-hydroxy-7-phenol), in peppermint oil and spent hay was achieved by using gas chromatography–mass spectrometry. The hay is treated with hydrochloric acid and extracted with dichloromethane–ethanol, the extract is treated with hydrochloric acid–ethanol to convert the 3-hydroxyl group of 3-hydroxy-7-phenol to the ethyl ether, and the phenols are converted to their heptyl ether derivatives. Peppermint oil is diluted with hexane–toluene and extracted with dilute sodium hydroxide solution. After acidification the phenols are extracted with dichloromethane–ethanol, cleaned up on a silica gel column, and derivatized as before. The mixture of heptyl ether derivatives is analyzed by multiple ion detection using ions with m/z 262 and 164 for 7-phenol, 276 and 178 for 3-keto-7-phenol, and 306 and 162 for 3-hydroxy-7-phenol. The sensitivity of the method for the three phenolic metabolites is 0.1 ppm.

Carbofuran has been shown to control strawberry root weevil (*Fumibotys fumalis*) larvae and mint root borer (*Otiorhynchus ovatus*) larvae is peppermint (Berry, 1971). The determination of carbofuran and its carbamate metabolite, 3-hydroxycarbofuran, in both peppermint hay and oil was accomplished by mofidications of an existing method (Inman et al., 1983). Investigations of plant metabolism of carbofuran (Metcalf et al., 1968; Knaak et al., 1970) have shown the phenolic metabolites of carbofuran to be 2,3-dihydro-2,2-dimethyl-7-benzofuranol (7-phenol), 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol (3-keto-7-phenol), and 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol (3-hydroxy-7-phenol). These phenolic metabolites are produced by plant systems as oxidation and/or hydrolysis products of the parent carbofuran moiety or its carbamate metabolites.

Analytical methods employing gas–liquid chromatography of derivatives of the carbofuran phenols have been developed for several different detection systems. When we applied a method using 1-fluoro-2,4-dinitrobenzene (Cook et al., 1977) to peppermint oil and employed a nitrogen–phosphorus-specific thermionic detector, peaks with retention times similar to those of carbofuran phenols were present in untreated oils. We have also attempted to synthesize phosphorus esters of these phenols for this detector using the procedure of Zwierzak (1976). While it was possible to synthesize the esters, application of the procedure to peppermint oil resulted in large interfering peaks. A number of other derivatives have also been reported applicable to determination of carbofuran phenols, including pentafluorobenzyl bromide (Archer et al., 1977), trifluoroacetic anhydride (Archer et al., 1977), and heptafluorobutyric anhydride (Lawrence et al., 1977), but enhanced electron capture sensitivity has not proven to be a worthwhile line of research for this separation problem.

Because of the specificity required for the detection of these residues in agricultural products, some investigators have used mass spectrometry to detect carbofuran phenols (Chapman and Robinson, 1977; Gruenauer and Nelsen, 1979; Nelsen and Gruenauer, 1981). Direct gas chromatography–mass spectrometry of phenolic compounds has been demonstrated (Nelsen and Gruenauer, 1981), and Gruenauer and Nelsen (1979) had success with a residue analysis method combining gas chromatography of the phenols on a column packed with Tenax and multiple ion detection mass spectrometry. We found, however, that a major component of the phenolic metabolite extract of peppermint oil, eugenol, is not separated from the 7-phenol on this column. The mass spectra of this essential oil component and that of 7-phenol are very similar. These two chemicals can be sufficiently separated on a nonpolar methyl silicon column, but separation of all three phenolic metabolites from interferences was not achieved under these GLC conditions and nonlinear response was observed at levels expected in residue analysis.

Chapman and Robinson (1977) reported another approach to multiple ion detection of these residues, but when applied to extracts of peppermint oil, neither chemical ionization nor electron impact ionization of the heptafluorobutyrylated (HFB) residues proved to provide adequate specificity. Furthermore, the HFB derivative of the 3-keto-7-phenol was unstable in samples treated with water as noted by Robinson and Chapman (1980).

A derivative of the phenols was desired which would be easy to prepare and be chemically and thermally stable. Previous work in our laboratory (Deinzer et al., 1979) indicated that heptyl ether derivatives should be ideally suited for GC–MS detection. Heptyl ether derivatives of the carbofuran phenols proved to have the desired stability and could be adequately separated from interferences when chromatographed and detected as described in this paper.

Our method for the determination of phenolic metabolites of carbofuran in peppermint hay and oil involves preparation of heptyl ethers of these compounds and analysis by multiple ion detection mass spectrometry.

EXPERIMENTAL SECTION

Reagents. Standard laboratory glassware was used throughout the procedure. Solvents employed were distilled in glass. Silica gel, Grade 950, 60–200 mesh, Davidson Chemical Division, W. R. Grace and Co., Baltimore, MD, was stored in a glass container at 130 °C. Tetramethylammonium hydroxide (24% in methanol) was obtained from Aldrich Chemical Co., Milwaukee, WI, and was diluted to 2% in dry acetone and maintained in contact with a molecular sieve. *n*-Iodoheptane was purchased

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from Eastman Organic Chemicals, Rochester, NY, and was used as received. Analytical-grade standards of the three phenols of carbofuran were supplied by FMC Corp., Middleport, NY.

Gas Chromatography–Mass Spectrometry. A Finnigan Model 4023 quadrupole mass spectrometer and data system was used. The gas chromatographic column was 170 × 0.2 cm i.d. glass packed with 6% DC-200 on 100/120 Chromosorb 750. The helium carrier gas was diverted from the mass spectrometer for 30 s after each standard and sample injection. Injection port and column temperatures were 200 and 165 °C, respectively, and the transfer and separator oven was at 200 °C. The ion source was operated at 70 eV and 300 °C.

Procedure: Carbofuran Phenols in Spent Peppermint Hay. *Hydrolysis and Extraction.* A 20-g subsample of chopped and thoroughly mixed spent mint hay was blended with 250 mL of doubly distilled water and the mascerate transferred to a 1000-mL round-bottom flask. Samples for recovery studies were fortified in the blender cup. Hydrolysis to free the conjugated phenols was accomplished by adding 10 mL of concentrated hydrochloric acid to the macerate and refluxing for 1 h. The hydrolyzed samples were allowed to stand overnight. The condensers were then rinsed with 25 mL of 0.25 N hydrochloric acid and the suspension was filtered with vacuum through Whatman No. 1 filter paper by using a Büchner funnel. After recovery of the filtrate, the filter cake was resuspended in 50 mL of 0.25 N HCl and allowed to stand for 5 min before refiltering. This soaking step was then repeated. The macerate was discarded and the filtrate refiltered through Whatman No. 42 filter paper.

After the volume of the extract was measured, an aliquot equivalent to 5 g of the sample was transferred to a 500-mL separatory funnel, 10 g of sodium chloride added, and the aqueous phase extracted with four 50-mL portions of a solvent consisting of 15% diethyl ether and 10% ethanol in dichloromethane. The extract was dried by passing it through anhydrous sodium sulfate, and the glassware and sodium sulfate were rinsed with two 10-mL portions of 15% diethyl ether in dichloromethane. The dried extract was collected in a 500-mL round-bottom flask and concentrated to about 1 mL on a rotary evaporator at 30 °C. Five milliliters of absolute ethanol was added and the extract again concentrated to 1 mL.

Ethoxylation and Liquid–Liquid Partitioning Cleanup. Twenty-five milliliters of absolute ethanol, 7 drops of concentrated hydrochloric acid, and boiling chips were added to the residue, and the sample was refluxed for 0.5 h. The solution was allowed to cool, and 100 mL of ice-cold water containing 5 g of dissolved sodium chloride was added through the condenser. The solution was transferred to a 500-mL separatory funnel and extracted 3 times with 50 mL of dichloromethane. The aqueous phase was discarded, and the combined dichloromethane extracts were partitioned twice with 50 mL of 0.25 N sodium hydroxide. The organic phase was discarded and the combined aqueous phase acidified with 5 mL of concentrated hydrochloric acid, 1 g of sodium chloride was added, and the aqueous phase was extracted with three 50-mL portions of dichloromethane. The organic extract was dried with anhydrous sodium sulfate and concentrated to near dryness on a rotary evaporator at 30 °C.

Derivatization. The residue was transferred to a 15-mL screw-cap test tube with several small rinses of dichloromethane and the solvent was evaporated under a jet of dry air, care being used when removing the last traces of solvent. The residue was redissolved in 1 mL of dry acetone, and 0.25 mL of 2% tetramethylammonium hydroxide in acetone was added and thoroughly mixed, followed by addition of 0.65 mL of *n*-iodoheptane. A Teflon-lined screw cap was installed, the reaction mixture mixed again and allowed to stand overnight. Excess reagents were removed by transferring the reaction mixture to a 125-mL separatory funnel with 25 mL of dichloromethane and 50 mL of water which was then agitated. After separation, the dichloromethane layer was collected and concentrated to 1.0 mL for analysis.

Carbofuran Phenols in Peppermint Oil. *Extraction.* Five grams of peppermint oil was dissolved in 100 mL of 1:1 hexane–toluene (v/v) and extracted 2 times with 75 mL of 0.25 N sodium hydroxide in a separatory funnel. The aqueous base extracts were combined and the organic fraction was discarded. The aqueous phase was then back-washed 2 times with 25 mL of chloroform and the chloroform discarded. Seven milliliters of concentrated hydrochloric acid and 10 g of sodium chloride were added to the aqueous phase which was then extracted 4 times with 50 mL of 15% diethyl ether and 10% ethanol in dichloromethane. Anhydrous sodium sulfate was used to dry the combined extract, and two 10-mL portions of 15% diethyl ether in dichloromethane were used to rinse the glassware and the sodium sulfate. The dried extract was collected in a 500-mL round-bottom flask and concentrated to near dryness on a rotary evaporator at 30 °C. Ten milliliters of toluene was added and the residue again concentrated to near dryness. The residue was then redissolved in 4 mL of toluene.

Silica Gel Column Cleanup. An 8 mm i.d. glass chromatographic column with a 50-mL reservoir and a Teflon stopcock was used. The column was prepared by plugging the bottom with glass wool, adding 5 mm of anhydrous sodium sulfate, 5 g of Davidson 950 silica gel, and another 5 mm of anhydrous sodium sulfate. The adsorbent bed was then saturated with toluene. After the sample residue was transferred to the column it was eluted first with 25 mL of toluene, then with 25 mL of 50% toluene in dichloromethane, and then with 20 mL of dichloromethane which was discarded. The 7-phenol and the 3-keto-7-phenol were eluted with 40 mL of 10% ethyl acetate in dichloromethane and the 3-hydroxy-7-phenol was eluted with an additional 15 mL of ethyl acetate. The ethyl acetate eluate was collected in a 500-mL round-bottom flask and concentrated for conversion to the 3-ethoxy-7-phenol identically with the phenolic metabolite residues in the hay samples.

Derivatization. After extraction of the ethoxylated metabolite residues, both the dried extract and the 10% ethyl acetate in dichloromethane eluate from the silica gel column were concentrated and reacted with tetramethylammonium hydroxide and *n*-iodoheptane reagents to yield the heptyl ether derivatives for analysis as described before.

Quantitation. Quantitation of both fortified samples and unknowns was made by direct comparison with external standards. Data were collected for paired sample and standard injections. The standards were injected approximately 8.5 min after the sample, and the selected ions were monitored for a total of approximately 15 min per sample–standard pair.

Peak area integrations for quantitation were manually obtained from the data system by displaying the reconstructed chromatograms for the masses of interest on a CRT display and specifying the beginning and ending points for the integration with cursors. A minimum of three pairs of sample and standard injections were made

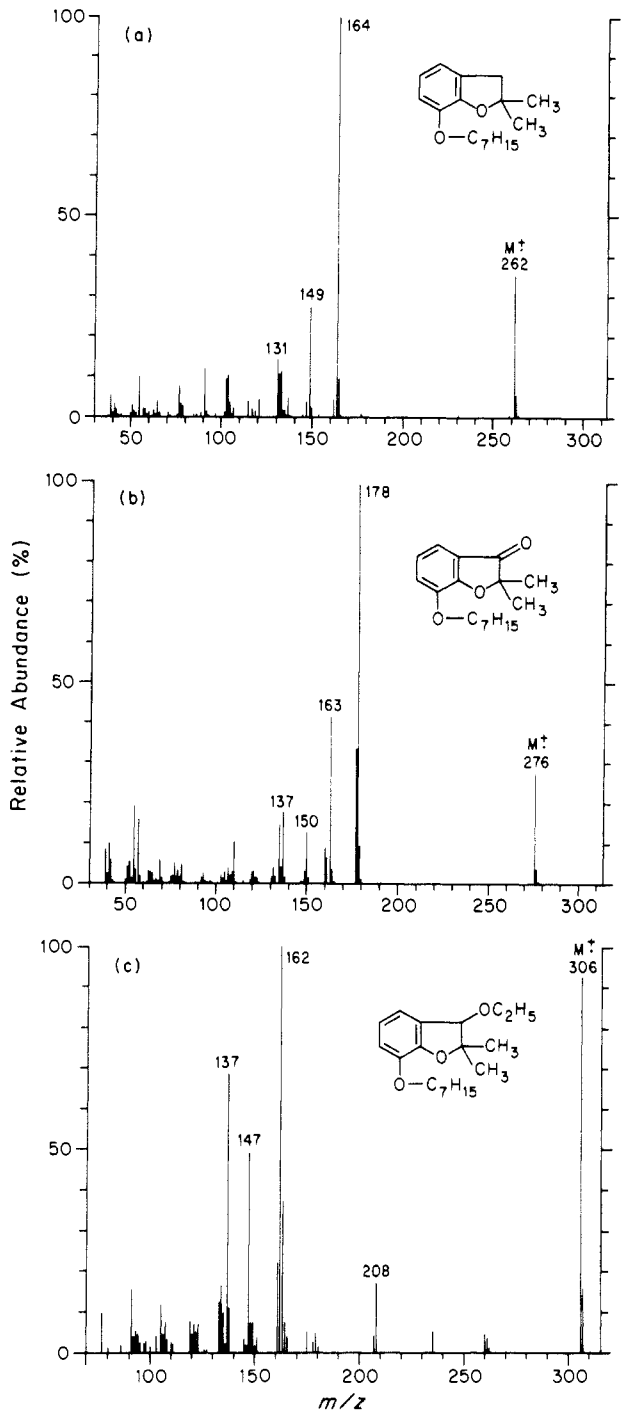

Figure 1. Electron impact mass spectra of the heptyl ether derivatives of the carbofuran phenols: (a) 7-phenol; (b) 3-keto-7-phenol; (c) 3-ethoxy-7-phenol. Spectra obtained on a Finnigan 4023 instrument. Ionization voltage was 70 eV.

for each recovery, and the injection volumes of standards were adjusted to approximate or bracket the fortification level.

RESULTS AND DISCUSSION

The analytical method described here overcomes the considerable separation problems encountered in the analysis of peppermint hay and oil for residues of carbofuran phenols by employing a derivatization technique not previously reported for analysis of these residues in conjunction with multiple ion detection gas chromatography–mass spectrometry.

Spectra obtained from the GC–MS system (Figure 1) indicated that both the molecular ion and the base peak ion could be monitored for each derivative. The molecular

Table I. Electron Impact Ionization Masses Monitored for MID Determination of Heptyl Ether Derivatives of Phenolic Metabolites of Carbofuran

m/z	phenol metabolite[a]	fragment ion[b]
137	3-ethoxy-7-phenol	unidentified
147	3-ethoxy-7-phenol	unidentified
162	3-ethoxy-7-phenol	$(M - C_7H_{14} - C_2H_5O - H)^{+\cdot}$
164	7-phenol	$(M - C_7H_{14})^{+\cdot}$
178	3-keto-7-phenol	$(M - C_7H_{14})^{+\cdot}$
262	7-phenol	$M^{+\cdot}$
276	3-keto-7-phenol	$M^{+\cdot}$
306	3-ethoxy-7-phenol	$M^{+\cdot}$

[a] As heptyl ether derivatives. [b] M = molecular ion.

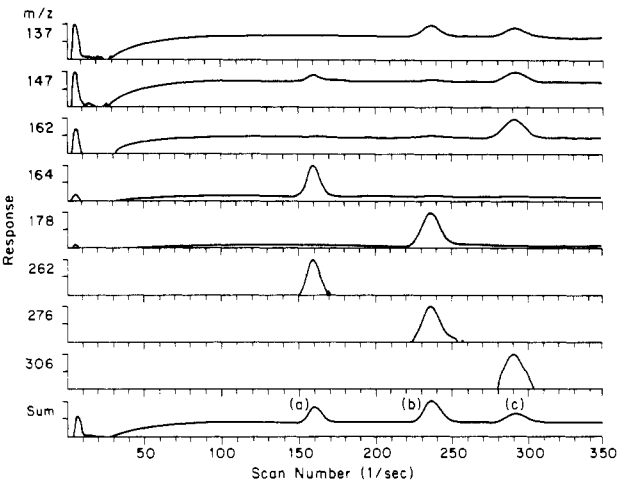

Figure 2. Reconstructed mass chromatograms obtained from an injection containing 10 ng of each carbofuran phenol heptyl ether derivative: (a) 7-phenol (b) 3-keto-7-phenol; (c) 3-ethoxy-7-phenol.

ions for heptyl ether derivatives of the 7-phenol, 3-keto-7-phenol, and the 3-ethoxy-7-phenol were observed at m/z 262, 276, and 306, respectively. Each of the parent ions produce $(M - C_7H_{14})^+$ by cleavage of the alkyl group between the oxygen and α-carbon with concomitant hydrogen atom transfer to the oxygen (Budzikiewicz et al., 1967). For the 7-phenol and 3-keto-7-phenol heptyl ether derivatives these ions are observed at m/z 164 and 178, respectively. They are the base peaks and were also used for quantitation. The equivalent ion at m/z 208 for the 3-ethoxy-7-phenol derivative was only about 20% of the base peak (m/z 162) and was, therefore, not used for quantitation. The mechanism for formation of the fragment ion with m/z 162 is more obscure. The ion responsible for this peak may result from stepwise loss of an ethoxyl radical and hydrogen atom from the $(M - C_7H_{14})^+$ ion. An alternative and perhaps a more favorable pathway to the m/z 162 ion is initial loss of an ethoxyl radical and hydrogen atom to give ions with m/z 261 and 260, respectively, followed by elimination of heptene as before.

In addition to the m/z 306 and 162 ions, there were ions at m/z 137 and 147 which were also monitored. For most analyses these lower masses were plagued with too many interfering peaks to be employed in quantitation. For some samples, however, mass 147 yielded more useful information than did mass 162 for analysis of residues of 3-ethoxy-7-phenyl heptyl ethers. The ions monitored during the sample analyses are listed in Table I. The identity of the fragment ions responsible for the peaks at m/z 137 and 147 has not been established.

When operated in the MID mode, the GC–MS data system samples all eight masses once each second during the GC run. The system can then reconstruct mass

Table II. Residues of Phenolic Metabolites of Carbofuran in Peppermint Oil and Spent Peppermint Hay

location	days after application	residues, ppm, from application of carbofuran: peppermint oil 7-phenol	peppermint oil 3-keto-7-phenol	peppermint oil 3-hydroxy-7-phenol	spent peppermint hay 7-phenol	spent peppermint hay 3-keto-7-phenol	spent peppermint hay 3-hydroxy-7-phenol
Medaryville, IN[a]	140	1.11	<0.1	<0.1	0.17	<0.1	<0.1
Harrah, WA[b]	307	0.70	<0.1	<0.1	na[e]	na	na
Stanfield, OR[c]	339	0.51	<0.1	<0.1	<0.1	<0.1	<0.1
Junction City, OR[d]	342	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a] One application of Furadan 10G granules, 2.2 kg of AI/ha. Applied April 2, 1979; harvested Aug 20, 1979. Furadan is an FMC Corp. formulation containing carbofuran as the active ingredient. [b] One application of Furadan flowable, 0.48 kg/L; applied at 2.2 kg of AI/ha by a ground sprayer. Applied Sept 28, 1978; harvested Aug 1, 1979. [c] One application of Furadan flowable, 0.48 kg/L; applied at 2.2 kg of AI/ha by a ground sprayer. Applied Sept 22, 1977; harvested Aug 30, 1978. [d] One application of Furadan flowable, 0.48 kg/L; applied at 2.2 kg of AI/ha by a ground sprayer. Applied Sept 16, 1977; harvested Aug 27, 1978. [e] Not analyzed.

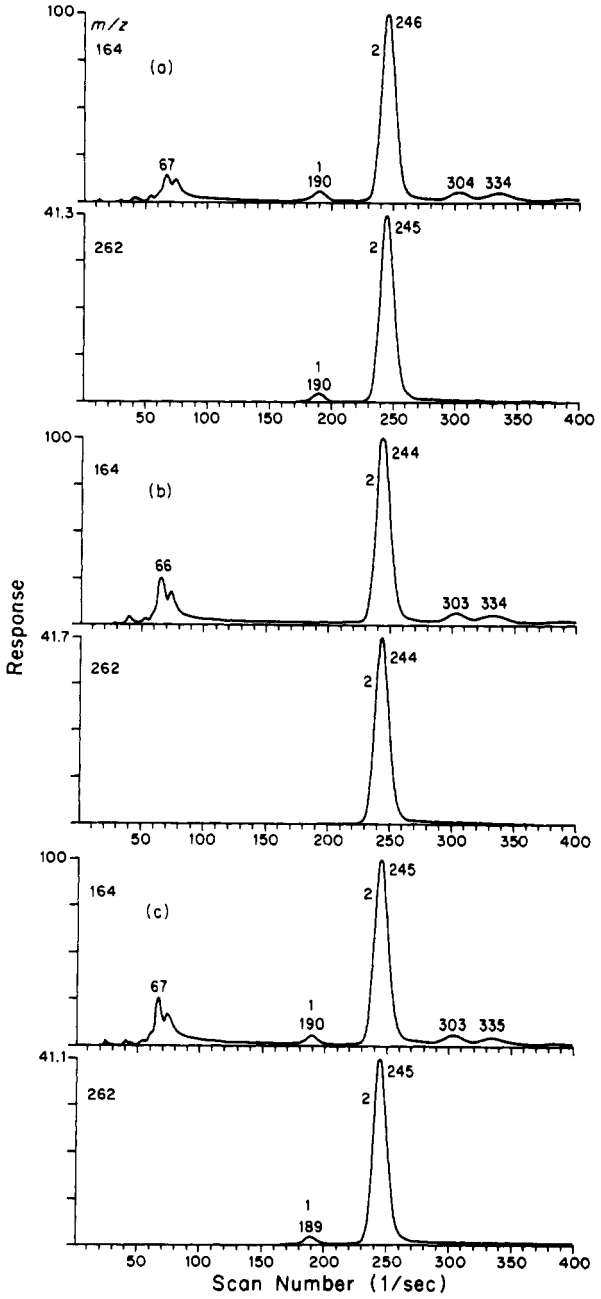

Figure 3. Reconstructed mass chromatograms of selected ions in extracts of peppermint oils: (a) 2.2 kg/ha treated; (b) unfortified control; (c) 1.0 ppm fortified control showing (1) 7-phenol heptyl ether and (2) eugenol heptyl ether.

chromatograms for each of the masses. Reconstructed chromatograms for an injection containing 10 ng of each derivatized phenol are shown in Figure 2. The sum of the eight masses monitored in the MID mode is also shown.

Reconstructed chromatograms showing the masses monitored for determination of the 7-phenol residues in peppermint oil samples are shown in Figure 3. The oil distilled from crops treated 140 days before harvest at 2.2 kg/ha (Figure 3a) was determined to contain a 1.11-ppm residue of the 7-phenol. Analysis of the corresponding control sample yielded chromatograms (Figure 3b) indicating no residues and analyzable to a level of 0.05 ppm. These chromatograms were obtained from a 5.0-μL injection of the control sample at 1.0-mL sample volume. Chromatograms (Figure 3c) show a recovery of 80% of a 5.0-μg fortification added to the control peppermint oil before the analysis. While these chromatograms (Figure 3) have been constructed with the heptyl ether derivative of eugenol as the base peak, chromatograms for quantitative analysis were constructed so as to exclude the eugenol heptyl ether derivative peak. This resulted in a much expanded scale at the 7-phenol derivative retention time.

Typical sample residue data, obtained from crops treated at a single rate but representing different locations and dissipation times, are shown in Table II. Only the spring treatment resulted in residues of the 7-phenol in both oil and spent hay. Residues of the 7-phenol were also detected in oil samples from crops treated at a higher application rate, but no residues of the other phenolic metabolites were detected.

The sensitivity of the analytical method was estimated to be 0.1 ppm with a 5-g sample for both the mint hay and oil. The reliability of the analytical method was tested by adding known amounts of the carbofuran phenols to peppermint oil and hay and analyzing as described above. The following recoveries were obtained with each compound replicated 7 times: 7-phenol, fortification range 0.4–1.5 ppm, average recovery and standard deviation 93 ± 26.9%; 3-keto-7-phenol, fortification range 0.75–1.5 ppm, average recovery and standard deviation 87 ± 30.9%; 3-hydroxy-7-phenol, fortification range 0.75–1.5 ppm, average recovery and standard deviation 78 ± 13.3%.

The use of MID has proven essential for the determination of carbofuran phenols in peppermint oil and peppermint hay. It should be useful for the analysis of these residues in other crops as well, in particular, those containing large numbers of essential oils.

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Registry No. Carbofuran, 1563-66-2; 2,3-dihydro-2,2-dimethyl-7-benzofuranol, 1563-38-8; 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol, 17781-16-7; 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol, 17781-15-6.

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Phenolic and Tannin Contents As Related to Anatomical Parameters of Soybean Resistance to Agromyzid Bean Flies

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Leaves and stems of four agromyzid bean fly resistant soybean (*Glycine soja*) varieties and two-susceptible ones (*Glycine max*) were assayed for phenolic, tannin, and condensed tannin contents. Phenolic content and the protein-binding or -precipitating capabilities of tannins both were higher in the leaf than in the stem of most studied soybean varieties, but differences did not correlate to bean fly resistance vs. susceptibility. No condensed tannins were detected in leaves or stems of any soybean variety. Through relating anatomical compartmentalizations of total phenols, tannins, and lignins in soybeans to the species-specific feeding behaviors of bean fly larvae in such plants, an understanding of the functions of such chemicals in the plant's defenses against insects seems to emerge. Thus, phenols and/or tannins are involved in the resistance of undifferentiated tissues, and phenols, tannins, and lignins are involved in the resistance of the differentiated soybean stem to bean fly larvae.

Tannins and other phenols have a broad spectrum of biological activity. They have been reported as being especially allomonic against viruses, bacteria, fungi, insects, and mammals (Feeny, 1970; Swain, 1979). Such phenolic compounds precipitate proteins (Swain, 1979) and have been extensively discussed as digestibility-reducing agents. As such, they may act as quantitative, i.e., dosage-dependent, defenses against herbivores (especially insects) in plants (Levin, 1971; Feeney, 1976; Rhoades and Cates, 1976).

Condensed tannins have been found in the tissues of several legumes including the leaves of some herbaceous species (Sarkar et al., 1976) and the grains of common bean (*Phaseolus vulgaris*) (Ma and Bliss, 1978), horsebean (*Vicia faba*) (Martin-Tanguy et al., 1977), cowpea (*Vigna unguiculata*), pigeon pea (*Cajanus cajan*), black gram (*Phaseolus mungo*), and azuki bean (*Vigna angularis*) (Price et al., 1980). They have been involved in southern pea, *Vigna unguiculata*, as a specific parameter of legume resistance to insects. An increased number of tannin sacs in the pods of such peas has been considered as a resistance factor against the cowpea curculio, *Chalcodermus aenueus* (Gundlach and Chambliss, 1977). In the present study efforts were made to identify any roles which phenolics and especially tannins play in the resistance of soybean plants to agromyzid bean flies.

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Larvae of agromyzid bean flies, *Melanagromyza sojae*, *Ophiomyia centrosematis*, and *Ophiomyia phaseoli*, kill soybean seedlings throughout the year in tropical areas of Asia by feeding inside the leaf and stem. A varietal screening program has identified four wild soybean (*Glycine soja*) varieties as being highly resistant and two commercialized (*Glycine max*) varieties as being susceptible to all three bean fly species (Chiang and Talekar, 1980). Some morphological, anatomical, and physiological parameters which correlate with this soybean resistance were previously established and discussed (Chiang and Norris, 1983a,b). Additional interrelated biochemical and anatomical plant parameters which also correlate positively with such soybean resistance are now presented in this paper. Dynamic interrelationships among the differentiation and development of the secondary plant body, the differentiation and lignification of sclerenchyma, and the total phenolic content in bean fly resistant soybeans are discussed.

EXPERIMENTAL SECTION

Materials. Five plants of each of four bean fly resistant soybean varieties, i.e., Asian Vegetable Research and Development Center (AVRDC) accession no. G3089, G3091, G3104 and G3122, and two bean fly susceptible varieties, i.e., AVRDC accession no. G1935 and G6, were grown from seeds in a University of Wisconsin—Madison greenhouse under the conditions previously detailed by Chiang and Norris (1983b). Leaves and stem portions for each of the experimental plants were collected separately at 8 weeks

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