amount (>50%) of [$^{14}$C]permethrin was evolved as $^{14}CO_2$ in unflooded soil. They and other workers (Gaughan et al., 1977; Shono et al., 1978; Glickman et al., 1979) showed that the trans isomer was less stable than the cis. The possibility that permethrin will be degraded to DCVA and $CO_2$ in the upper aerobic layer of natural aquatic systems should be investigated. Most of the applied permethrin will be associated with the bottom sediment and will be degraded to DCVA and other metabolites in the anaerobic environment of bottom sediment. The more insecticidal *cis*-permethrin will remain longer than the trans isomer, and its effect on aqueous systems should be further investigated.

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# Isolation and Identification of a New Conjugated Carbofuran Metabolite in Carrots: Angelic Acid Ester of 3-Hydroxycarbofuran

Hiromi Sonobe, LaVerne R. Kamps,* Eugene P. Mazzola, and John A. G. Roach

A new conjugated carbofuran metabolite was isolated from carrots that had been treated with [$^{14}$C]-carbofuran. The chemical structure was determined to be 2,3-dihydro-2,2-dimethyl-7-[[(methylamino)carbonyl]oxy]-3-benzofuranyl (*Z*)-2-methyl-2-butenoate. Biosynthesis of this compound apparently involves conjugation of 3-hydroxycarbofuran with (*Z*)-2-methyl-2-butenoic acid (angelic acid). This compound is the major carbofuran residue in carrots. Conjugation of xenobiotics with angelic acid has not been previously reported as a metabolic pathway for pesticides in plants.

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a broad-spectrum insecticide–nematocide. When the efficiency of acetonitrile and methanol for the extraction of [$^{14}$C]carbofuran residues in root crops (potatoes, carrots, and radishes) was evaluated, the separation and characterization of carbofuran residues were performed by high-performance liquid chromatography (HPLC) using a $C_{18}$ column (Sonobe et al., 1981). During the determination of carbofuran residues in the extracts of carrots with this HPLC system, a significant quantity of an unidentified carbofuran metabolite was found. This paper reports the isolation and identification of a new conjugated carbofuran metabolite.

## EXPERIMENTAL SECTION

**Reagents and Apparatus.** $^{14}$C Aromatic ring labeled carbofuran with a specific activity of 8.2 mCi/mmol was purchased from New England Nuclear, Boston, MA. The radiochemical purity was 99%. 3-Hydroxycarbofuran and Furadan-4 Flowable were obtained from FMC Corp., Middleport, NY. Aliquots of radioactive extracts were mixed with 10 mL of Insta-gel cocktail (Packard, Downers Grove, IL) and counted by using a Mark III liquid scintillation spectrometer (Searle, Des Plains, IL). Analyses of the unidentified compound by thin-layer chromatography (TLC) were performed on either silica gel TLC plates or $KC_{18}$ reversed-phase TLC plates (Kontes, Vineland, NJ) developed in ethyl ether–*n*-hexane (3:1) and methanol–water (4:1), respectively. Dried TLC plates were exposed to No-Screen X-ray film (Eastman Kodak, Rochester, NY), and the film was developed as usual after 1 week of exposure. HPLC-quality *n*-hexane and 2-propanol were purchased from Fisher Scientific Co., Fair Lawn, NJ. Other reagents were analytical grade.

**Treatment and Collection of Crop Samples.** Carrots (Nantes half long) were grown in pots (21.6 cm diameter × 22.9 cm deep) with silt loam. When roots were ca. 1.5 cm in diameter, $^{14}$C aromatic ring labeled carbofuran mixed with the commercial formulation Furadan-4 Flowable (2.56 mCi/mmol) was applied to the roots and the nearby soil at a rate of 3 lb (6.6 kg) of active ingredient/acre. The carrots were harvested at 5, 10, and 15 days postapplication. Tops were removed and discarded, the adhering soil was removed with a water rinse, and the roots were chopped in a Hobart food chopper (Model 84141, Hobart

Kirin Brewery Co., Ltd., Tokyo, Japan (H.S.), and the Divisions of Chemical Technology and Chemistry and Physics, Food and Drug Administration, Washington, DC 20204 (L.R.K., E.P.M., and J.A.G.R.).

Manufacturing Co., Troy, OH). The chopped sample was thoroughly mixed, and 70-g portions were taken for the extraction study.

**Extraction Procedure.** Each sample portion was extracted with 200 mL of acetonitrile, by using a Polytron blender, and the extracts were collected by filtration. The crop marc was washed twice with 100 mL of acetonitrile and was then washed slowly with 300 mL of acetonitrile at a rate of 5–10 mL/min. The filtrates were combined and concentrated to ca. 90 mL. Fifty milliliters of water was added to the concentrated filtrate to replace water lost by azeotroping. The filtrate was extracted 3 times with 40-mL portions of methylene chloride.

**Isolation and Purification of New Carbofuran Metabolite.** The methylene chloride extracts from eight sample portions were combined and evaporated to dryness. The resulting oily residue was dissolved in *n*-hexane before Florisil column chromatography (Association of Official Analytical Chemists, 1980). The 6 and 15% ethyl ether in petroleum ether eluates (200 mL each) contained negligible radioactivity. The 50% ethyl ether in petroleum ether eluate contained 55% of the total radioactivity determined in the combined methylene chloride extracts. This eluate was evaporated to dryness, and the residue was dissolved in *n*-hexane for chromatography on a silicic acid column (3% water deactivated). The column was consecutively eluted with 20 mL each of 5, 10, and 50% ethyl acetate in *n*-hexane. The 50% eluate was collected, evaporated to dryness, and dissolved in 1 mL of methylene chloride. This cleaned-up eluate was subjected to HPLC. The recovery of the new metabolite in the column chromatography was determined by using the pure metabolite after it was isolated by HPLC. The new metabolite was isolated by semipreparative collection of HPLC fractions. A Model 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA) equipped with a 4.6 mm i.d. × 25 cm Zorbax-SIL column (Du Pont, Wilmington, DE) was operated at a solvent flow rate of 1.0 mL/min, with 10% 2-propanol in *n*-hexane (v/v). Chromatograms were obtained with a UV detector at 220 nm.

**Mass Spectrometric Analysis.** Low-resolution 70-eV mass spectra were obtained with a Finnigan 1015 S/L quadrupole mass spectrometer equipped with a Model 6000 data system. The sample was introduced via a direct insertion probe ballistically heated to 200 °C. The metabolite vaporized between 100 and 150 °C. High-resolution mass spectra were obtained with a Varian CH5/DF mass spectrometer equipped with a Finnigan 2300 INCOS data system. The sample was introduced via direct insertion probe. The metabolite vaporized at 105 °C. The accelerating voltage was 3 kV, the electron energy was 70 eV, and the instrument resolution was set at 8000.

**Fourier Transform NMR Analysis.** $^1$H FT-NMR spectra, described by 4096 data points, were obtained at 80 MHz on a Varian FT-80A NMR spectrometer. Pulse widths of 34 μs were employed, which correspond to tip angles of 67° with 5-mm sample tubes. Samples were dissolved in $CDCl_3$. Chemical shifts are reported relative to internal tetramethylsilane at 0.0 ppm.

**FT-IR Analysis.** FT-IR spectra were obtained by using the micromethod of Chen and Dority (1970) on a DigiLab Model 10M Fourier transform IR spectrometer (DigiLab, Cambridge, MA) equipped with a Wilks Model 45 beam-condensing accessory (Wilks Infrared Accessories, Analabs, North Haven, CT).

**Synthesis of 2,3-Dihydro-2,2-dimethyl-7-[[(methylamino)carbonyl]oxy]-3-benzofuranyl (*E*)-2-Methyl-2-butenoate.** (*E*)-2-Methyl-2-butenoyl chloride (tigloyl chloride) was prepared by a modification of the method of Adams and Ulich (1920). (*E*)-2-Methyl-2-butenoic acid (tiglic acid; 500 mg, 0.5 mmol) was dissolved in 5 mL of benzene, and oxalyl chloride (0.5 mL, 5.9 mmol) was added dropwise. The solution was allowed to stand 15 min without heating and was then refluxed 1 h. The solution was diluted with 10 mL of toluene and concentrated to 2–3 mL. 3-Hydroxycarbofuran (50 mg, 0.21 mmol) was dissolved in 2 mL of dry pyridine, and the tigloyl chloride solution was added dropwise to this solution. The resulting solution was allowed to stand 1 h at room temperature with occasional mixing. This reaction mixture was poured into a separatory funnel containing 200 mL of water, and this mixture was extracted twice with 40 mL of methylene chloride. The combined methylene chloride extracts were washed twice with 100 mL of water. The methylene chloride extracts were dried over anhydrous sodium sulfate and evaporated to dryness, and the residual material was dissolved in warm *n*-hexane. This solution was chromatographed on the Florisil and silicic acid columns described earlier. A yield of 46 mg (69%) of colorless oil was obtained.

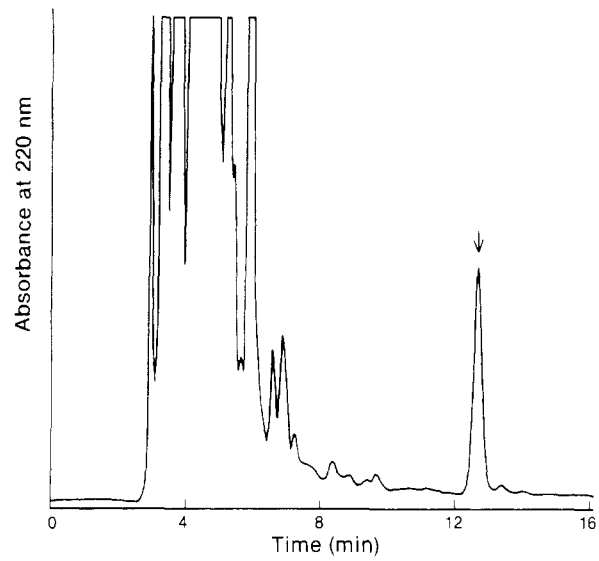



**Figure 1.** HPLC chromatogram of the unidentified carbofuran metabolite. The arrow shows the new metabolite with retention time of 12.51 min. Retention time of carbofuran was 13.30 min (flow rate = 1.0 mL/min).

## RESULTS

Acetonitrile extraction of [$^{14}$C]carbofuran residues from 15-day postapplication carrot samples was efficient (90.0% of the total radioactivity present was extracted). Of the total radioactivity extracted by acetonitrile, 73.7% was methylene chloride extractable while 26.3% was water soluble.

Florisil and silicic acid column chromatography recovered the unidentified metabolite and isolated it from other carbofuran metabolites and carrot coextractives. The recovery of unidentified metabolites in the 50% ethyl ether in petroleum ether fraction from the Florisil column chromatography was 84%, while the recovery in the 50% ethyl acetate in *n*-hexane fraction of silicic acid column chromatography was 89%. The recoveries of 3-hydroxycarbofuran and carbofuran, which were present in carrot extracts, were 0 and 16%, respectively, in the 50% ethyl ether in petroleum ether eluate of Florisil column chromatography.

The cleaned-up silicic acid eluate was subjected to

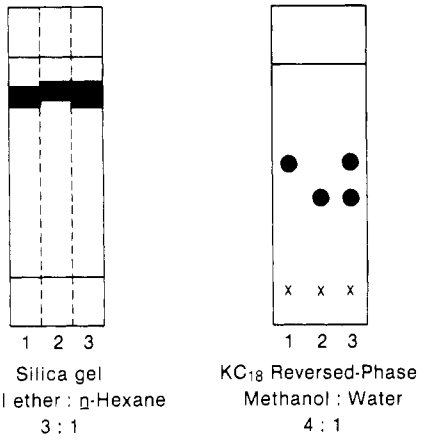



**Figure 2.** TLC radioautograms of unidentified carbofuran metabolites and carbofuran. (1) Carbofuran; (2) unidentified; (3) carbofuran plus unidentified.

HPLC analysis. The new metabolite was detected, and the fraction containing it was collected as shown in Figure 1. The unknown peak indicated in Figure 1 contained 84% of the total radioactivity injected, and the fractions collected were shown to be radiochemically pure by radioautography using both silica gel and $KC_{18}$ reversed-phase TLC (Figure 2).

The low-resolution mass spectrum of the isolated metabolite is shown in Figure 3A. Comparison of the mass spectrum of the unknown with that of 3-hydroxycarbofuran suggested that the unknown and 3-hydroxycarbofuran were closely related. The mass spectrum of the unknown compound is distinguished by characteristic ions at $m/z$ 83, 262, and 319. Elimination of *N*-methyl isocyanate from the molecular ions at $m/z$ 319 ($C_{17}H_{21}NO_5$) gives rise to $m/z$ 262 ($C_{15}H_{18}O_4$). This fragmentation is typical for *N*-methylcarbamates (Safe and Hutzinger, 1973). The difference of 82 in molecular weight between 3-hydroxycarbofuran and the unknown can be most readily explained by the cleavage of an ester linkage at the 3-hydroxy position. The compound would be an ester of a C-5 carboxylic acid containing one double bond and 3-hydroxycarbofuran. Cleavage of the ester linkage with a hydrogen rearrangement would account for the similarity of the unknown spectrum with the spectrum of 3-hydroxycarbofuran and would also explain the abundant fragment at $m/z$ 83 in the unknown spectrum. In the high-resolution mass spectrometric analysis of the unknown compound, the observed elemental compositions for the significant fragment ions were all within 2 mm$\mu$ of their calculated values.

On the basis of the partial elucidation of the structure of the unknown metabolite, an ester was synthesized from 3-hydroxycarbofuran and (*E*)-2-methyl-2-butenoic acid (tiglic acid), a known constituent of higher plants. The mass spectrum of this ester (Figure 3B) was identical with that of the unknown metabolite. But the identification of the compound was not complete since electron impact mass spectrometry cannot distinguish between all isomeric forms. The synthesized tiglic acid ester was eliminated from among the possible structures for the unknown metabolite because the HPLC retention volume (12.77 mL) of the synthesized ester did not coincide with that of the unknown compound (11.62 mL).

$^1$H FT-NMR analysis readily led to the final determination of the structure of the new metabolite. Some of the chemical shifts and spin coupling patterns of the unidentified compound were identical with those of the synthesized tiglic acid ester. The spectrum of the unknown compound could, however, only be interpreted as the ester of 3-hydroxycarbofuran and (*Z*)-2-methyl-2-butenoic acid (angelic acid), the geometrical isomer of the synthesized tiglic acid ester. The proton assignments are shown in Table I. Assignments of the $\beta$-$CH_3$ and $\beta$-H were made by means of spin decoupling. When the tiglic acid derivative was irradiated at 1.77 ppm (3 H, d, $J$ = 7 Hz), the signal at 7.0 ppm (1 H, q, $J$ = 7 Hz) collapsed. When the new metabolite was irradiated at 6.1 ppm (1 H, qq, $J$ = 1, 7 Hz), the signal at 1.95 ppm (3 H, dq, $J$ = 1.5, 7 Hz) collapsed, and a broadened three-proton singlet appeared. As shown in Table I, the reported chemical shifts and coupling constants of (*E*)-2-methyl-2-butenoic acid and (*Z*)-2-methyl-2-butenoic acid (Bovey, 1967) were consistent with the results obtained. The unknown metabolite was

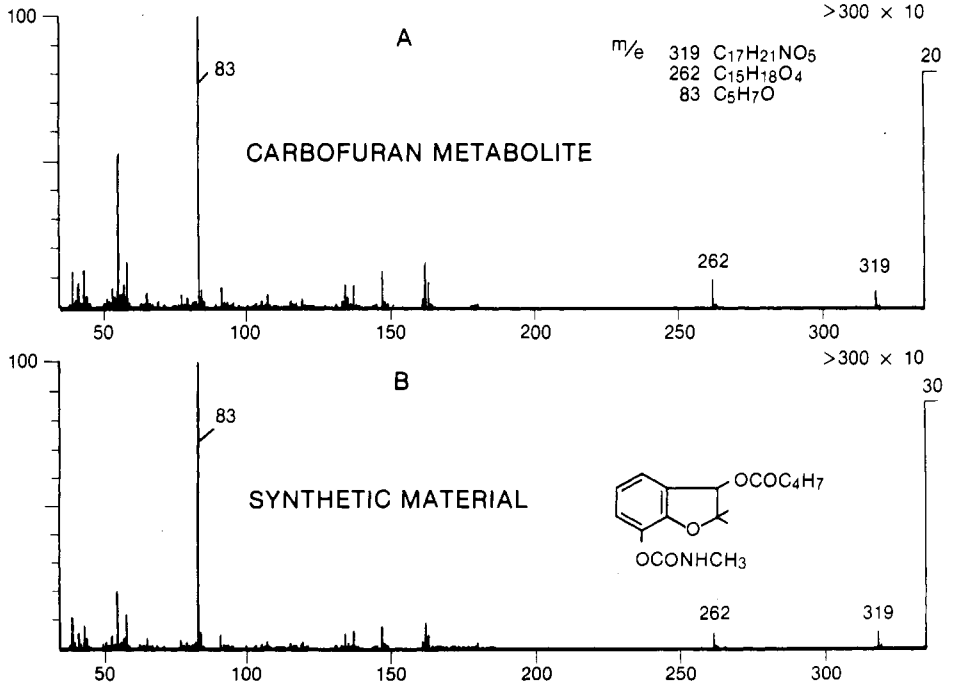
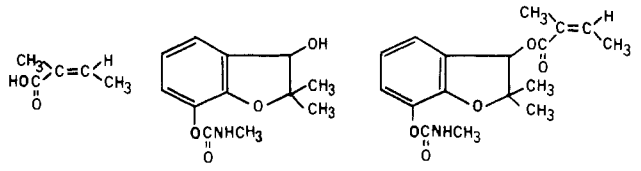



**Figure 3.** Mass spectra of the new carbofuran metabolite and synthesized material.

Table I. Assignment of Protons by $^1$H FT-NMR[a]

| proton | tiglic acid ester | tiglic acid | angelic acid | unknown compd |
|---|---|---|---|---|
| *gem*-$CH_3$ | 1.46, 1.48 | | | 1.48 |
| $\alpha$-$CH_3$ | 1.81 | not reported | not reported | 1.87 m |
| $\beta$-$CH_3$ | 1.77 d (7) | 1.86 d (7) | 2.06 d (7) | 1.95 dq (1.5, 7) |
| N-$CH_3$ | 2.9 d (5) | | | 2.9 d (5) |
| N-H | 5.2 br | | | 5.2 br |
| H-3 | 6.0 | | | 6.0 |
| aromatic H | 6.7-7.2 | | | 6.7-7.2 |
| $\beta$-H | 7.0 q (7) | 6.97 q (7) | 6.18 q (7) | 6.1 qq (1, 7) |

[a] Spin coupling patterns are indicated as follows: no notation, singlet; br, broad; d, doublet; m, multiplet; q, quartet. Values in parentheses are coupling constants, in hertz.



I II III

**Figure 4.** Structure of angelic acid and carbofuran metabolites. (I) (*Z*)-2-Methyl-2-butenoic acid (angelic acid); (II) 2,3-dihydro-2,2-dimethyl-3-hydroxy-7-benzofuranyl methylcarbamate (3-hydroxycarbofuran); (III) 2,3-dihydro-2,2-dimethyl-7-[[(methylamino)carbonyl]oxy]-3-benzofuranyl (*Z*)-2-methyl-2-butenoate.

conclusively identified as 2,3-dihydro-2,2-dimethyl-7-[[(methylamino)carbonyl]oxy]-3-benzofuranyl (*Z*)-2-methyl-2-butenoate, the ester of (*Z*)-2-methyl-2-butenoic acid and 3-hydroxycarbofuran. The structures of these compounds are shown in Figure 4.

The FT-IR spectrum of the new metabolite is consistent with the identification made (data in $cm^{-1}$): 3380 (N—H stretching), 2860 ($CH_3$ asymmetric stretching), 2830 ($CH_3$ symmetric stretching), 1750 (C=O stretching in C=O—CO—O), 1730 (C=O stretching in —CO—N), 1230 (C—O—C stretching in O—CO—N), 1120 (C—O—C stretching in ester), 600 (CH out-of-plane deformation of three adjacent H's).

The synthesis of the angelic acid ester of 3-hydroxycarbofuran was attempted. Angelic acid was synthesized from tiglic acid by the method of Buckles et al. (1955), and the identity and purity were verified by proton NMR spectroscopy. Analogous procedures for the preparation of the angelic acid ester to those used for the tiglic acid ester were carried out using angelic acid. However, investigation of the synthesized product with HPLC using a Zorbax-SIL column showed that the major compound was the tiglic acid ester (more than 95%). The compound having an identical HPLC retention time and an identical mass spectrum to the isolated carbofuran metabolite, apparently the angelic acid ester, was only a minor product of the synthesis. The isomerization of angelic acid to tiglic acid evidently occurred during the formation of the acid chloride, which was not isolated and/or characterized before the subsequent esterification. Several mild esterification procedures, including two-phase base esterification using benzyltriethylammonium chloride and esterification using *N*,*N*′-dicyclohexylcarbodiimide, were not successful.

This angelic acid ester was the major carbofuran residue in carrots. In the 15-day postapplication sample, 61% of the total radioactivity in the combined methylene chloride extracts was associated with this compound, while 2.8% was identified as carbofuran and 22% was identified as 3-hydroxycarbofuran. The angelic acid ester did not occur as a major metabolite in the other root crops studied (potato and radish). Little unidentified radiolabeled metabolite was observed in those crops. The results of the quantitation of residue levels of this new carbofuran metabolite, carbofuran, and 3-hydroxycarbofuran in carrots harvested at 5, 10, and 15 days postapplication will be presented in a future publication.

## DISCUSSION

The metabolism of carbofuran in various plants, animals, and insects has been reported by many workers (Metcalf et al., 1968; Dorough, 1968; Dorough and Ivie, 1968; Knaak et al., 1970; Miles et al., 1971; Ashworth and Sheets, 1972; Pree and Saunders, 1974) and reviewed by Menzie (1969) and in an Environmental Protection Agency (1976) publication. It is well established that the major metabolic pathway involves hydroxylation to 3-hydroxycarbofuran. Subsequent conjugation of 3-hydroxycarbofuran to give glycosides or glucuronides is one of several pathways available. Oxidation to 3-ketocarbofuran is always observed.

Types of conjugates which had been found in animals, plants, and microorganisms were summarized by Geissbuhler (1976). They are classified as glucuronides, glycosides, amino acid conjugates, sulfuric acid esters, glutathione conjugates, and acylated and alkylated metabolites. Angelic acid ester conjugates of xenobiotics have not been previously reported. This type of conjugation is unusual since the metabolite formed is lipid soluble, while most of the known conjugated pesticide residues are water soluble. Conjugates of endogenous alcohols with angelic acid have been found in many plants (Geissman and Crout, 1969) including the family of *Umbelliferacea*, of which the carrot is a member. Angelic acid is found in the essential oil of carrot (Parczewski and Rajkowski, 1963). Therefore, it is not surprising that an ester conjugate could be synthesized biologically from 3-hydroxycarbofuran and angelic acid in carrots.

The angelic acid ester of 3-hydroxycarbofuran is considered to be the original form in carrots. It is quite unlikely that the angelic acid ester was formed by isomerization during the extraction procedure. The isomerization of angelic to tiglic acid has been reported to occur on treatment with acid, on heating with base above 100 °C, or upon heating in water in a sealed tube (Buckles et al., 1955). The opposite isomerization has not been reported.

Since this metabolite was formed after the use of an application technique that is not normally used to apply carbofuran in the field, the occurrence of the angelic acid ester of 3-hydroxycarbofuran by normal application techniques should be investigated. Although carbofuran is not approved for use on carrots, the angelic acid ester may be formed from carbofuran residues which remain in the soil from previous application to other crops. Since the ester is probably lipid soluble, contains the carbamoyl moiety,

and is the major residue in carrots resulting from carbofuran treatment, it appears to be a logical candidate for toxicity testing.

Acid hydrolysis of the angelic acid ester conjugate of 3-hydroxycarbofuran in 0.25 N HCl is expected to produce 3-hydroxycarbofuran. The development of a quantitative procedure for this compound in its unaltered, conjugated form is necessary to determine the amount of this compound which may be present. The angelic acid ester conjugate is the major carbofuran metabolite in carrots, but the extent of its occurrence in other crops is unknown.

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# A Method for Determining Fenamiphos and Its Sulfoxide and Sulfone in Plants and Soil

Marilyn J. Brown

A method was developed for the individual determination of residues of fenamiphos (Nemacur) and its sulfoxide and sulfone in raspberry fruit, carrots, and a sandy loam soil. Separation of the two metabolites was achieved on silica gel, and cleanup was improved on charcoal. Direct gas chromatographic detection was accomplished by using a flame photometric detector. Recoveries were calculated on samples fortified with 0.01, 0.1, and 1.0 ppm of each of the three compounds and ranged from 75.0 to 106.7%.

Fenamiphos (Nemacur, ethyl 3-methyl-4-(methylthio)-phenyl (1-methylethyl)phosphoramidate) is currently under investigation for postplanting control of the lesion nematode, *Pratylenchus penetrans* (Cobb), the dagger nematode, *Xiphinema bakeri* (Williams), common pests in raspberry plantings in British Columbia, and the carrot rust fly, *Psila rosae* (Fab.).

Fenamiphos dissipates very rapidly (Waggoner and Khasawinah, 1974) and its thiooxidation products, fenamiphos sulfoxide and sulfone, have been identified as the major metabolities in both soils and plants (Waggoner, 1972). Published residue methods determine either the parent compound only (Sagredos and Eckert, 1977) or the sulfone as the total residue after permanganate oxidation of both the parent compound and the sulfoxide (Thornton, 1971). So that the degradation of fenamiphos can be followed, a new residue method for the individual determination of the parent compound and its sulfoxide and sulfone in soil, raspberries, and carrots has been developed and is presented in this paper.

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EXPERIMENTAL SECTION

**Apparatus.** A Microtek MT 220 gas chromatograph equipped with a flame photometric detector was used. Peak quantitation was performed by a Hewlett-Packard Model 3385A reporting computing integrator.

**Reagents.** Silica gel (Davidson Chemical Division, W. R. Grace and Co., Grade 923, 100–200 mesh) and Hyflo Super Cel were used as received. Nuchar C was acid-washed as previously described (Brown, 1975) and a 2:5 mixture of charcoal–Whatman CF11 cellulose powder was prepared. All solvents were distilled in glass. Anhydrous

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