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Isolation and Analysis of Carrot Constituents: Myristicin, Falcarinol, and Falcarindiol

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Carrot roots are blended with dichloromethane in the presence of an antioxidant, and the resulting suspension is centrifuged. An aliquot of the clear extract is removed, concentrated, dissolved in hexane, and fractionated by gravity-flow adsorption chromatography on silica gel (70–325 mesh). Carotenoid pigments are removed with hexane, and myristicin (1-allyl-3,4-methylenedioxy-5-methoxybenzene), falcarinol (3-hydroxyheptadeca-1,9-diene-4,6-diyne), and falcarindiol (3,8-dihydroxyheptadeca-1,9-diene-4,6-diyne) are eluted with 45:55 ethyl ether–hexane. After a 20-fold concentration, these compounds are analyzed directly by gas–liquid chromatography (GLC) on SE-52 and OV-17 columns (80–250 °C at 4 °C/min) with methyl palmitate as an internal standard. Recoveries of added myristicin, falcarinol, or falcarindiol (15 ppm) were about 95%. Analyses by this method support previous estimates of falcarinol levels in carrots (40 ppm). Falcarindiol, however, was found in higher concentrations (80 ppm) than reported previously (6 ppm) for whole carrots (Bentley et al., 1969).

In 1967, Crosby and Aharonson (1967) reported that extracts of carrot root were toxic to an indicator organism, *Daphnia magna* Straus. The purified toxicant that they isolated produced "neurotoxic symptoms upon injection into mice"; the LD_{50} was estimated to be 100 mg/kg. In addition to this toxicant (falcarinol; 3-hydroxyheptadeca-1,9-diene-4,6-diyne), other related acetylenes have been reported [falcarindiol, acetylfalcarindiol, and falcarinolone (Bentley et al., 1969)], as well as a hallucinogen, myristicin (Wulf et al., 1978).

Carrot root is believed to contribute 13.9% of our national intake of provitamin A, 0.6% of vitamin B_6, and 0.6% of magnesium (Senti and Rizek, 1974). Thus, it is worthy of close attention to properly assess the impact of deleterious constituents that may attend genetic or environmentally induced changes in the carrot.

If the components to be assayed are volatile, they can be removed from the plant material by steam distillation and purified in the same step by extracting the distillate with an organic solvent (Wulf et al., 1978). Unfortunately, carrot roots have many volatile components that complicate analysis of minor toxicants. The analytical method described herein employs a solvent, dichloromethane, to extract myristicin, falcarinol, and falcarindiol from carrots, along with other plant constituents. The toxicants are then purified by column chromatography and measured by gas–liquid chromatography. These components also can be isolated by scaling up the extraction procedure and isolating the desired compounds by gravity-flow column chromatography.

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EXPERIMENTAL SECTION

Plant Materials. Carrot roots, *Daucus carota*, were bought in commercial markets, grown at NRRC, or grown at various field locations directed by Department of Horticulture, University of Wisconsin, Madison.

Thin-Layer Chromatography of Carrot Components. Commercial precoated, silica gel 60, 0.25-mm plates (E. M. Reagents, MC/B Manufacturing Chemists, Inc., Cincinnati, OH 45212) were developed with (1) ether–hexane (20:80) or with (2) ether–benzene–chloroform (10:30:60). Individual spots were detected by spraying with sulfuric acid and heating at 130 °C for about 3 min. Polyacetylenic compounds such as falcarinol and falcarindiol react immediately after spraying to form chocolate brown spots. Carotenoid pigments also react immediately at room temperature to form a dark spot.

Chromatography Equipment and Conditions. Gravity-flow chromatography was accomplished with 70–325-mesh silica gel (E. M. Reagents) for analytical separations and for initial preparative separations; for final purification steps, 70–230-mesh silica gel 60 (E. M. Reagents) was used. Gas–liquid chromatography was performed with a Bendix 2600 instrument (flame ionization detectors); injector temperature was 240 °C and detector temperature was 260 °C, with helium carrier gas at 10–20 mL/min, air at 500–600 mL/min, and hydrogen at

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50 mL/min. Columns were programmed from 80 to 250 °C at 4 °C/min with a 5-min final hold. Glass columns 6 ft by $^1/_8$ in. were used, one packed with 3% SE-52 (Applied Science Laboratories, Inc.) on 80–100-mesh Gas-Chrom Q (nonpolar) and one packed with 3% OV-17 (Supelco Inc.) on 100–120-mesh Chromosorb W HP (intermediate polarity).

Dichloromethane Extraction–Column Chromatographic Purification–Gas–Liquid Chromatographic (DE–CCP–GLC) Analysis of Carrots. Fifty grams of carrot pieces (1 × 1 × 0.2 cm) was placed in a blender cup along with 20 mg of antioxidant (Antioxidant 330, Ethyl Corp., Ionox 330, Shell Chemicals) and 100 mL of dichloromethane. The mixture was blended in a commercial Waring Blendor at moderate speeds for 12 min. Alternate cycles of blending and cooling (4 min each) reduce evaporation of dichloromethane. Any solvent losses were corrected for by weighing the capped blender cup and contents before and after blending and replacing the lost solvent. Teflon seals were inserted in the blender cup blade assembly to replace the standard seals. After dichloromethane lost by evaporation was replaced, the mixture was blended for an additional 30 s and transferred to a 250-mL centrifuge bottle capped with a screw cap or Saran Wrap. The mixture was centrifuged for 15 min at 1500*g*, and then 50 mL of the clear extract was removed with a large syringe fitted with a long 17-gauge needle passed through the pulp. This aliquot was concentrated under vacuum on a rotating evaporator at 25 °C to about 3 mL; 7 mL of hexane was added and the solution was reconcentrated to 3 mL; 4 mL of hexane was added and the solution was reconcentrated to 1 mL. The resultant hexane solution was chromatographed on 2 g of silica gel. Glass tubing (8-mm o.d.) pulled to a fine point and cut to a 23-cm length made an acceptable column. Yellow pigments were eluted with hexane until the hexane eluate was colorless (40–60 mL). The fraction of interest was eluted with 20 mL of ether–hexane (45:55). Methyl palmitate (1.5 mg) was added and the eluate was concentrated to 0.75 mL. Analysis by GLC (3–6-μL injection) gave symmetrical peaks, which were integrated electronically.

Quantities of individual toxins were calculated by using the formula

$$\frac{\text{mg of toxin}}{\text{kg of sample}} = F_t \frac{A_t}{A_p} \frac{W_p}{W_c} \frac{V_a}{V_r} \times 10^3$$

where F_t is the response factor of the toxin in relation to methyl palmitate, A_t and A_p are the areas of the toxin and methyl palmitate gas chromatographic peaks, respectively, W_p is quantity of methyl palmitate added in milligrams, W_c is the initial weight of carrots used, V_a is the initial volume of solvent used for extraction, and V_r is the volume of solvent taken after centrifuging as the analyses aliquot.

The following response factors (F_t) were determined by chromatographing mixtures of the toxicants with methyl palmitate in ratios of 0.25, 0.33, 0.50, 0.67, 1.00, 1.50, 2.00, 3.00, and 4.00; small, medium, and large injections of each mixture were analyzed:

	OV-17	SE-52
myristicin	1.11	1.14
falcarinol	0.99	0.98
falcarindiol	1.55	1.33

Isolation of Milligram Quantities of Falcarinol. Falcarinol was isolated from carrot root by scaling up the analytical procedure to 350-g samples of carrot (1 mg of antioxidant/20 g of sample). After extraction and centrifugation, the clear extract was removed by using an evacuated filter flask connected to a glass tube. This solution was concentrated to 5 mL. The process was repeated with additional plant material until 2.1 kg of carrots had been processed (final volume, 30 mL). An equal volume of hexane was added and the solution was concentrated to $^1/_3$ volume (final volume, 20 mL). The concentrate was centrifuged and the solvent layer was removed; the precipitate was washed once with 10 mL of hexane and the suspension was centrifuged. The wash was removed and combined with the concentrate (final volume, 30 mL). This solution was further concentrated to 2 mL. A silica gel column (50 g of silica gel/g of residue) was prepared, and the concentrated solution was chromatographed by using the following elution schedule at a flow rate of $^1/_{20}$ of the silica gel volume/min: (a) elute with hexane until a brown band (first colored band) is removed (approximately 100 mL); (b) elute with ether–hexane (5:95) until a red band is at the bottom of the column (approximately 200 mL); (c) elute with ether–hexane (10:90) and collect fractions (1 mL/3 g of silica gel) until an orange band is eluted (two to three fractions after the red band is eluted); (d) elute with ether–hexane (20:80) and collect about six fractions; (e) elute with ether (2 mL/g of silica gel) and collect in one fraction.

Fractions were examined by TLC (solvent 1), and those fractions containing falcarinol were combined (add back original quantity of antioxidant 330). The falcarinol solution was rechromatographed by using silica gel 60 and eluted with ether–benzene–chloroform (10:30:60). Fractions (1 mL/2 g of silica gel) were collected until the eluate was colorless, and then two additional fractions were collected. Fractions were again examined by TLC (solvent 2); those containing falcarinol were combined (add back original amount of antioxidant 330). The falcarinol solution was rechromatographed by using the latter procedure, taking fractions of 1 mL/4 g of silica gel. Fractions containing falcarinol were combined and evaporated to constant weight at 25 °C with nitrogen directed against the surface. One milligram of antioxidant was added per 5 mg of falcarinol, and the solution was stored in the dark at −18 °C.

Falcarinol. From 2 kg of carrots, 20 mg of 95% pure falcarinol (GLC analysis) was isolated. The structure of falcarinol isolated was confirmed by mass spectrometry (MS), infrared spectrometry (IR), proton nuclear magnetic spectrometry (^{1}H NMR), and ultraviolet spectrometry (UV). Carbon-13 NMR is as follows:

ppm	carbon character	relative strength
136.4	CH=	7.8
133.1	CH=CH	19.8
122.1	CH=CH	20.8
116.9	$H_2C=$	17.2
96.2	unknown	7.2
80.3	—C≡C—	2.4
74.3	—C≡C—	1.0
71.4	—C≡C—	1.0
64.2	—CH,OH	1.8
63.6	—CH,OH	14.0
31.8	CH_2	17.4
31.6	CH_2CH=CH—	11.0
29.2	unknown	68.7
27.2	unknown	23.1
26.9	unknown	2.9
22.7	CH_2	28.1
17.7	—CH_2C≡	24.0
14.1	unknown	26.9

Falcarindiol. The structure of falcarindiol, isolated in a similar manner from carrots, was confirmed by comparison of its MS, IR, ^{1}H NMR, and UV spectral data to

that of authentic falcarindiol supplied by B. Garrod.

Myristicin. Myristicin was not isolated but was identified by comparing its chromatographic behavior and MS to that of authentic myristicin (Saber Laboratory, Morton Grove, IL 60053).

RESULTS AND DISCUSSION

The DE–CCP–GLC method was designed for analysis of falcarinol, with the capability for analysis of additional compounds as necessary. The method has been extended to myristicin, a hallocinogen, and falcarindiol, an antifungal compound and perhaps a phytoalexin (Garrod et al., 1978).

The carrot root, if freshly harvested, is brushed lightly with a bristle brush to remove soil. Some myristicin and falcarinol are lost on prolonged contact with water (30 h); therefore, carrots are not washed. Because falcarindiol is located mainly in the outer layer of the carrot (Garrod and Lewis, 1979), a portion of it also may be lost if carrots are washed.

Dichloromethane removes essentially 100% of falcarinol in a single extraction. Analysis of a 74.2-mL aliquot of a 100-mL extract showed that 2.20 mg of falcarinol was present in this aliquot; there should be 0.76 mg of falcarinol in the remaining 25.8 mL of extract and associated pulp. Two more extractions—made by twice adding 100 mL of dichloromethane to the remaining extract and associated pulp and removing 100-mL portions—yielded 0.56 mg of additional falcarinol. The remaining 0.20 mg of falcarinol was in the dichloromethane associated with the pulp. Had there been incomplete extraction initially, the two final extracts would have contained a larger than expected amount of falcarinol.

Blending carrot pieces with dichloromethane is a crucial step in the DE–CCP–GLC assay. Under the conditions of analysis, falcarinol may decompose and, therefore, was protected with an antioxidant. Antioxidant 330 has been used successfully in this method and by other workers (Buttery et al., 1979). Losses of dichloromethane can occur by both evaporation and leakage. Evaporation losses are minimal, about 2–3 g, if the blending is sepd. into 4-min cycles of blending and cooling. Losses of extract due to leakage cannot be replaced. Complete recovery of extract is not necessary; analysis is based on using an aliquot of the total. Blender cups may have to be modified to accommodate dichloromethane. If rubberlike seals are present they should be replaced with Teflon or another inert material. Due to their softness, Teflon seals have to be replaced after a month or two of use.

Concentrating samples at high temperatures or allowing samples to go to dryness could result in loss of myristicin (the most volatile of the three toxins analyzed), falcarinol, or falcarindiol (the least volatile) due to evaporation. However, the conditions herein described gave good recoveries (Table I).

Gravity-flow silica gel column chromatography proved an excellent tool in separating the components of carrot root extracts. These columns, though heavily loaded, provide the resolving power needed to separate both the less polar carotenoid pigments and the highly polar molecules from the toxic fraction (myristicin, falcarinol, and falcarindiol). When carotenoid pigments are not separated from falcarinol, much lower recoveries (70%) of falcarinol resulted. The carotenoid pigments are eluted from the column with hexane, but other components are retained. If, however, dichloromethane is not completely removed in the evaporation step, the hexane–dichloromethane mixture becomes a more polar eluent and may result in loss of some of the components to be analyzed. Highly polar compounds, if not eliminated by column chromatography, will be retained on the GLC column and may bleed off during subsequent analyses.

Table I. Recoveries of Myristicin, Falcarinol, and Falcarindiol by Dichloromethane Extraction–Column Chromatographic Purification–Gas-Liquid Chromatographic Analysis of Carrots

sample	endogenous, mg	added, mg	analyzed, mg	recovery of toxicant, %: added[a]	recovery of toxicant, %: total[b]
		Myristicin			
1[c]	0.025	0.430	0.434	95.1	95.4
2[c]	0.016	0.428	0.406	91.1	91.4
3[c]	0.000	0.858	0.826	96.3	96.3
		Falcarinol			
1[c]	1.690	0.233	1.915	96.6	99.6
2[c]	0.955	0.466	1.412	98.1	99.4
3[d]	1.555	0.608	2.138	95.9	98.8
4[e]	1.282	0.608	1.802	93.4	95.3
		Falcarindiol			
1[c]	1.652	1.274	2.914	99.1	99.6
2[c]	2.030	0.637	2.628	93.8	98.5

[a] [(Milligrams analyzed − milligrams endogenous)/milligrams added] × 100. [b] [Milligrams analyzed/(milligrams endogenous + milligrams added)] × 100. [c] Carrots purchased from local supermarket. [d] Spartan bonus. [e] Imperator.

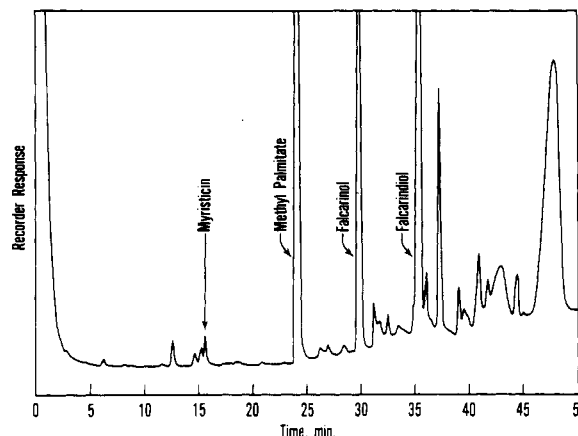

Figure 1. GLC recording of separation of carrot (commercial) fraction on 3% OV-17: myristicin, 0.6 mg/kg; falcarinol, 18.2 mg/kg; falcarindiol, 41.6 mg/kg.

Falcarindiol is much less soluble in hexane than in dichloromethane and comes out of hexane solution at −18 °C. Therefore, samples to be analyzed should be warmed to room temperature and examined to ensure complete solution.

The toxic fraction, after column chromatography, was concentrated carefully and analyzed by GLC. The two column packings selected (SE-52 and OV-17) gave excellent resolution and were well matched to each other with respect to temperature range and column life. Ordinarily, 100–125 samples were analyzed before repacking columns. Figures 1 and 2 show the resolution obtained with the toxic fraction. This is a typical GLC record: myristicin is present in low concentrations in carrot root (0.7 mg/kg); falcarinol is present in intermediate concentrations (19.1 mg/kg); falcarindiol is usually more abundant than falcarinol (43.2 mg/kg). Other peaks are not yet identified. Methyl palmitate is added as an internal standard (2.0 mg/50 g of carrot root).

The precision of this method is shown in Table II. The extract from several carrots was spiked with myristicin and pooled, and individual aliquots were removed and analyzed separately.

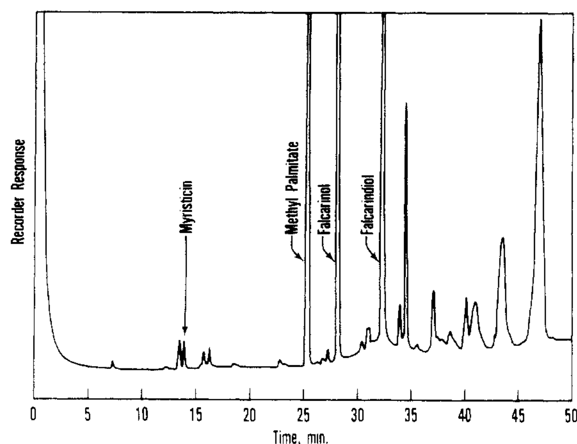

Figure 2. GLC recording of separation of carrot (commercial) fraction on 3% SE-52: myristicin, 0.8 mg/kg; falcarinol, 20.0 mg/kg; falcarindiol, 44.9 mg/kg.

Table II. Precision of GLC Analysis of Aliquots of a Pooled Carrot Root (Red Cored Chantenay) Extract with Added Myristicin

	myristicin, mg/kg	falcarinol, mg/kg	falcarindiol, mg/kg
mean of 6 aliquots	34.4	30.3	83.5
standard deviation	0.6	0.7	2.6

For the most accurate results, the ratio of toxin to internal standard should be 0.5 to 2.0. Ratios of 0.33 to 3.0 are acceptable, but the standard curves were not linear beyond this range. Also, both the OV-17 and SE-52 columns irreversibly absorb the toxins. Therefore, each sample injected should contain at least 10 μg of the toxicant or the analysis may show smaller amounts of the toxins than are actually present.

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Gas Chromatographic Analysis of the Free Amino Acid Pool of the Potato and Gas Chromatography–Mass Spectrometry Identification of γ-Aminobutyric Acid and Ornithine

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The free amino acid pool of the potato has been studied by using the *N*-heptafluorobutyryl isopropyl ester derivatives. Directly coupled gas chromatography–mass spectrometry allowed positive identification of the amino acids, where earlier work has relied solely on GC retention times. The nonprotein amino acids γ-aminobutyric acid and ornithine were shown to be present. γ-Aminobutyric acid was relatively abundant, at 27% of the free amino pool. The analytical procedure described is rapid and simple and, in combination with an established library of mass spectra of amino acid derivatives, provides a routine technique for the identification of free amino acids.

The amino acid composition of the free amino acid (FAA) pool of the potato was reviewed recently by Synge (1977). A few nonprotein amino acids were reported to occur in this fraction: α-aminobutyric acid (α-ABA), γ-aminobutyric acid (GABA), β-alanine, ornithine, L-pipecolic acid, and *S*-methylmethionine. Only GABA among these amino acids was present in significant quantity [10% (Thompson et al., 1953)].

The only other reports available on gas chromatographic analysis of the FAA pool of the potato are those of Hoff et al. (1971) and March (1975). In these studies, the identification of the amino acids was based on retention times.

In this report the *N*-heptafluorobutyryl isopropyl ester derivative of amino acids, as introduced by Golan-Goldhirsh and Wolfe (1979a), was used for the gas chromatographic analysis of the FAA extract of the potato. The

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