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Identification of Germacrene D in Walnut and Fig Leaf Volatiles

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By capillary GLC–MS and packed-column GLC with batch IR, germacrene D was identified as a major volatile component of both walnut (ca. 0.5 ppm) and fig (ca. 0.4 ppm) tree leaves. Other major volatiles also identified include caryophyllene, (*E*)-β-ocimene, β-pinene, and limonene in walnut leaves and (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, methyl salicylate, and β-cyclocitral in fig leaves.

INTRODUCTION

Some of us have been carrying out studies to identify the volatile constituents of leaves and other parts of important crops (cf. Buttery and Ling, 1984). Such volatile compounds might be involved in the attraction of some insect pests to these crops. Two important tree crops in California are walnuts and figs. The walnut husk fly (*Rhagoletis completa* Cresson) is a pest of walnuts. Fig pests include various Nitidulids and *Drosophila* species. Although the insects attack the fruits, the major volatiles of the plant are probably given off by the considerably greater quantity (and area) of leaves. These may initially attract the insects to the tree.

Some previous studies have been reported on the nonvolatile constituents of fig leaves (e.g., Innocenti et al., 1982), but none were found on the volatiles of fig leaves. Some identification of volatile components of walnut leaves had been reported (Popescu and Ciupe, 1972; Kolesnikova, 1980) from thin-layer chromatography and evidence based on R_f values. These previous workers had found such evidence for the presence of juglone, α- and β-pinene, limonene, δ-3-carene, 1,8-cineole, bornyl acetate, α-terpineol, and cadinenes.

As identification using thin-layer R_f values alone is of doubtful value, we felt that it was necessary to reinvestigate the volatiles of the leaves by the more certain method of the capillary GLC–mass spectrometry combination and, where possible, infrared absorption spectra.

EXPERIMENTAL SECTION

Materials. Walnut tree leaves were obtained from the English walnut (*Juglans regia*) growing in El Cerrito, CA, in the spring and summer of 1984. Fig leaves were obtained from the black Mission fig (*Ficus caprica*) growing in El Cerrito, CA, during the spring and summer of 1983. The intact leaves (kept at 20–25 °C) were used within 4 h after picking. Care was taken that the leaves were not crushed or otherwise damaged.

Isolation of Volatile Oil from Walnut Leaves. The method used was essentially the same as described previously by us for other crops such as corn leaves (Buttery and Ling, 1984). The intact walnut leaves (500 g) were placed in a 12-L flask. A Tenax trap (14-cm length × 2.2 cm diameter; 10 g of Tenax) was attached to the neck of the flask. Air drawn from outside the laboratory (and purified by passage through activated charcoal) was led into the flask through a Teflon tube and out through the Tenax trap. The flow of air was 1 L/min and was continued for 24 h. The trapped volatiles were eluted from the trap with freshly distilled diethyl ether (containing ca. 0.001% of ethyl antioxidant 330). The ether extract was then concentrated to a small volume (20 μL) on a warm-water bath and low-hold-up micro Vigreux type distillation columns.

The particular type of trapping method used was developed on the basis of theoretical calculations involving the volatilities of sesquiterpene and other common plant volatile compounds for the largely aqueous tissue media and for the surface wax layer. Experiments carried out with standard sesquiterpenes, aliphatic aldehydes, and alcohols showed good recovery of all compounds with no noticeable oxidation despite the large volume of air used. The Tenax traps were reactivated in the normal way by heating at 220 °C in a nitrogen stream. A trace of the nonvolatile ethyl antioxidant 330 is left on the Tenax from the elution process. No background was found in blank tests, and the trace antioxidant probably acts as an extra protection to the plant volatiles.

Isolation from Fig Leaves Using Tenax Trapping. This was carried out by exactly the same procedure as used with walnut leaves.

Isolation from Fig Leaves Using Vacuum Steam Distillation. The intact fig leaves (1 kg) were placed in a 12-L flask and covered with water (6 L). A Likens-Nickerson type steam distillation continuous-extraction head (cf. Nickerson and Likens, 1966) was connected to the neck of the flask. A 250-mL flask containing ca. 120 mL of purified hexane (with a trace of ethyl antioxidant 330) was attached to the solvent arm of the head. Vacuum

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Table I. Compounds Identified in the Tenax-Trapped Volatiles of Walnut Leaves

peak[a] no.	compd[b]	major MS ions[c] (one ea 14 MS units)	Kovat's GLC index[e]	rel %
		Monoterpenoids		
16	α-pinene[d]	93, 77, 121, *136*, 105, 67	940	5
20	sabinene	93, 77, 41, *136*, 69, 121	975	7
21	β-pinene[d]	93, 41, 69, 77, *136*, 121	980	11
22	myrcene	93, 41, 69, 79, 53, 121	990	5
25	limonene[d]	68, 93, 41, *136*, 53, 79	1030	10
27	(*E*)-β-Ocimene	93, 41, 79, 53, 121, 67	1050	12
32	linalool	93, 71, 41, 55, 80, 121	1120	1
		Sesquiterpenes		
58	caryophyllene[d]	41, 69, 93, 79, 133, 55	1435	15
60	(*E*)-β-farnesene	69, 41, 93, 79, 55, 133	1450	2
63	germacrene D[d]	161, 105, 91, 119, 81, 133	1495	13
66	α-Farnesene	41, 93, 69, 55, 107, 79	1520	4
		Other		
24	(*Z*)-3-hexenyl acetate	43, 67, 82, 69, 73	1020	2
		Unidentified Compounds		
19	MW 134	91, 119, 41, 77, 105, *134*	965	1
23	MW 134	91, 41, 119, 77, *134*, 105	1000	2
33	MW 150	69, 41, 81, 53, 107, *150*	1130	4

[a] Peak numbers in Figure 1. [b] Complete mass spectrum and GLC KI are consistent with that of authentic samples. [c] The most intense ion each 14 mass units above m/z 34. Ions in descending order of intensity with molecular ion (if listed) in italic type. [d] Infrared absorption spectrum also consistent with that of an authentic sample in addition to (*b*). [e] Kovat's GLC index (KI) for the Silicone OV-3 capillary GLC column.

steam distillation continuous extraction was carried out at 100 mmHg with the leaves at 50–55 °C for 3 h with ice water to cool the condensor. The hexane extract was dried by freezing out the water and concentrated to ca. 10 μL.

Capillary Gas Chromatography–Mass Spectrometry (GLC–MS). Two types of laboratory-constructed capillary GLC columns were used. One column was a 150-m length by 0.66-mm i.d. Pyrex glass capillary, wall coated with Silicone OV-3. The other column was also Pyrex of the same dimensions but was wall coated with Carbowax 20-M. The Silicone column was programmed from 30–170 °C at 1°/min and held at 170 °C. The Carbowax column was programmed at 2°/minute from 50 to 170 °C and then held at 170 °C. The carrier gas flow velocity for both columns was 28 cm/s of helium. The injector temperature was 170 °C. A flame ionization detector was used for monitoring and peak area measurements. A single-stage Llewellyn-Littlejohn Silicone rubber membrane molecular separator was used to couple the end of the column to the mass spectrometer (a modified Consolidated 21-620 instrument). Electron ionization voltage was 70 MeV.

Packed-Column Gas Chromatography–Batch Infrared Spectrometry. Components were separated from the walnut and fig leaf volatile oils on a 3 m × 0.64 cm o.d. stainless-steel column packed with 80–100-mesh Chromasorb G-DMCS coated with 2% Silicone SF96(100). The column was linearly programmed from 50 to 170 °C at 2°/min. Samples were collected in 3-mm o.d. × 14-cm length Pyrex tubes. The infrared (IR) spectra were measured as thin films between ultramicro salt plates on a Perkin-Elmer Model 197 instrument.

Authentic Samples. Most authentic samples of monoterpenes were obtained from commercial sources (K & K, Aldrich, Fluka) except for (*E*)-β-ocimene, which was obtained from Opopanax oil. Caryophyllene was obtained commercially. (*E*)-β-Farnesene was obtained from hop oil, and α-farnesene was obtained from ylang-ylang oil by silica gel liquid–solid chromatography and preparative GLC separation methods. Germacrene D was obtained from *Pinus edulis* oleoresin (cf. Snajberk and Zavarin, 1975; Flath et al., 1985) and ylang-ylang oil (cf. Saga et al., 1979) with silver nitrate to form a crystalline complex. All compounds were repurified by GLC separation and their identities verified by spectral (MS and IR) and GLC retention means.

RESULTS AND DISCUSSION

Walnut Leaves. The amount of volatile oil obtained from the walnut leaves by the Tenax trapping method was of the order of 4 ppm of the leaves. Figure 1 shows the capillary GLC analysis of this oil. Table I lists the compounds identified together with some idea of their relative concentrations from GLC peak area measurements. It can be seen that the major components are terpene and sesquiterpene hydrocarbons. At 15% the commonly occurring caryophyllene (peak 58) was the major component of the walnut leaf oil. A second component of almost as high a concentration (peak 63, 13%) was more unusual. The mass spectrum of peak 63 showed the following ions (two most intense ions each 14 mass units above m/z 34, relative intensities in parentheses): 41 (40), 43 (18); 53 (14), 55 (23); 67 (14), 69 (14); 79 (28), 81 (37); 91 (39), 93 (28); 105 (48), 107 (14); 119 (32), 120 (27); 133 (16); 147 (4); 161 (100); 189 (0.6); 204 (13) (cf. Herz et al., 1971). This was consistent with that of an authentic sample of germacrene D isolated from *P. edulis* oleoresin (cf. Snajberk and Zavarin, 1975; Flath et al., 1985). Peak 63 was also isolated by packed-column GLC for batch infrared (IR) spectrometry. The IR spectrum was also consistent with that of an authentic sample of germacrene D, showing the following absorption maxima in the region 5–12 μm: strong, 6.13, 6.90, 7.21, 7.30, 10.2, 10.3, 11.3; medium, 6.08, 6.23, 8.16, 8.42, 10.7, 11.8; weak, 5.9, 7.58, 7.87, 8.62, 9.53, 9.95, 12.1, 12.3. the Kovat's GLC index (KI) of peak 63 on the Silicone OV-3 column was 1495 (KI = 1430 for caryophyllene on the same column). With Carbowax 20-M the KI was 1680 (KI = 1570 for caryophyllene on this column). Both KI figures were consistent with those of an authentic sample of germacrene D measured on the same columns. Both spectral and GLC data were also in agreement with data published by Maarse and Van Oss (1973) for germacrene D.

Fig Leaves. Volatile oils from fig leaves were obtained both by Tenax trapping (ca. 2 ppm oil obtained) and by vacuum steam distillation–continuous extraction (ca. 10 ppm oil obtained). These oils were analyzed by capillary

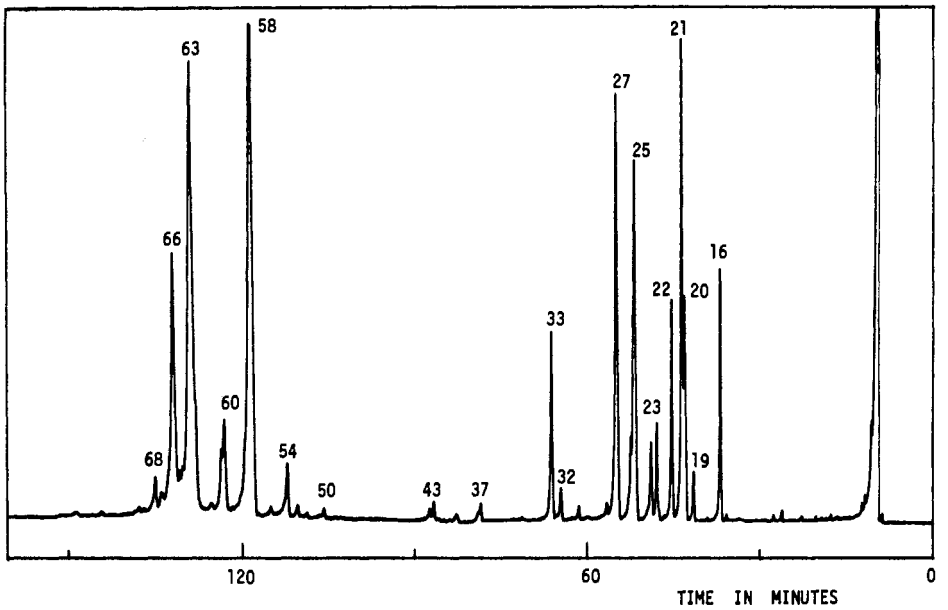

Figure 1. GLC analysis of the Tenax-trapped volatile oil from walnut leaves using the Silicone OV-3 capillary GLC column (fid detection) and GLC conditions described in the text.

GLC–MS using the Carbowax 20-M capillary. Germacrene D was identified as the major component of both oils, forming 20% of the Tenax isolated oil and 56% of the vacuum steam distillation isolated oil. It was also isolated from the vacuum steam volatile oil by packed-column GLC for batch infrared spectrometry. The mass and infrared spectra and GLC Kovat's index (on Carbowax 20-M) were all consistent with that of an authentic sample of germacrene D and with the data described above for the sample isolated from walnut leaves. Other components also identified in the fig leaf volatile oils included (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, methyl salicylate, and β-cyclocitral (only in the vacuum steam distillation isolated oil) whose mass spectra and GLC KI values were consistant with those of authentic samples.

Occurrence of Germacrene D in Other Plants. Germacrene D has been reported to occur in the green leaves of a number of other plants such as *Erigeron annus* (L.) Pers., *Eupatorium chinese* L., *Chrysanthemum morifolium*, and Douglas fir (cf. Tahara et al., 1975; Kepner et al., 1975). Kepner et al. (1975) had shown that germacrene D only occurred in young Douglas fir leaves and not in the more mature leaves. With both fig and walnut leaves, in the present work we found germacrene D as a major volatile component in both young and mature leaves.

Possible Importance to Insect Behavior. In recent years a number of sesquiterpene hydrocarbons have been found to be important to insect behavior. Germacrene D itself is a known pheromone of the cockroach (Tahara et al., 1975). Caryophyllene has been shown to be attractive to the boll weevil (Minyard et al., 1969). (*E*)-β-Farnesene is an alarm pheromone for aphids (Wohlers et al., 1981). Recently (+)-α-copaene was found to be the best known attractant for the medfly (Jacobsen et al., 1984). The large variety and unique structures of the sesquiterpene hydrocarbons as well as their availability on the leaves of plants have apparently made them useful markers for insects. Because germacrene D is a major volatile component of walnut and fig leaves, it seems quite possible that it could be important to the behavior of the insect pests of these crops.

We (in cooperation with an experienced entomologist) hope to test germacrene D with the walnut husk fly in the near future using the electroantenogram technique.

Registry No. α-Pinene, 80-56-8; sabinene, 3387-41-5; β-pinene, 127-91-3; myrcene, 123-35-3; limonene, 138-86-3; (*E*)-β-ocimene, 3779-61-1; linalool, 78-70-6; caryophyllene, 87-44-5; (*E*)-β-farnesene, 18794-84-8; germacrene D, 23986-74-5; α-farnesene, 502-61-4; (*Z*)-3-hexenyl acetate, 3681-71-8; (*Z*)-3-hexenol, 928-96-1; methyl salicylate, 119-36-8; β-cyclocitral, 432-25-7.

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