Volatile Components of Green Walnut Husks

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Volatiles were isolated from whole green mature walnuts (Hartley variety) with husks still intact using dynamic headspace sweeping with trapping on Tenax. A total of 45 volatile compounds were identified by GC−MS. Major volatiles identified included (*E*)-4,8-dimethyl-1,3,7-nonatriene, pinocarvone, pinocarveol, myrtenal, myrtenol, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, caryophyllene epoxide, verbenol, verbenone, and terpinolene. Green walnuts that had been infested with codling moth showed appreciably higher amounts emitted for (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, α- and *β*-pinenes, sabinene, (*E*)-*β*-ocimene, (*E*,*E*)-α-farnesene, and linalool. The infested nuts also emitted benzyl methyl ether, isobutyl cyanide, and 1-nitro-3-methylbutane, compounds not found with the healthy nuts. Volatiles from uninfested green walnuts at the maturity stage where the husk was just beginning to split were also analyzed and compared.

Keywords: *Walnuts; husks; volatiles; GC−MS; identification; codling moth; aflatoxin*

INTRODUCTION

The presence of aflatoxins and other mycotoxins (potent hepatotoxins and hepatocarcinogens) produced by several species of the fungus *Aspergillus* can sometimes be a problem in nut tree crops. It is known that the fungal pathogens are spread to the nut tree crops by insects. There is considerable evidence that insects locate nut tree fruit by detecting the characteristic volatiles that are associated with the fruit. The present study was carried out to identify and get some idea of the amounts of volatiles emitted by the intact green walnuts because this is the stage when most insect infestation occurs. Such knowledge is needed by entomologists investigating the relationship between pest insect behavior and plant volatiles.

Some studies have already been carried out on the identification of the volatiles present in walnut tree leaves (cf. Buttery et al., 1986; Campbell et al., 1999).

MATERIALS AND METHODS

Materials. The walnuts were Hartley variety, grown in Dixon, CA, in the 1998 season and had not been sprayed with insecticide. Three different forms of nuts were obtained. Form 1 consisted of healthy nuts that were at a stage where they were approximately full size, still completely covered with the smooth green husk, and free from insect infestation. Form 2 consisted of nuts that were similar to form 1 but had been infested with codling moth (*Cydia pomonella* (L.)). Form 3 nuts were healthy, noninfested, but at a stage where the green husk had just began to split. The nuts were picked in the early morning, wrapped in aluminum foil, and transported to the laboratory (at room temperatures) within a few hours. Isolation of volatiles was begun within 2 h after the nuts were received at the laboratory.

Diethyl ether (99+%, anhydrous, ACS reagent) was freshly distilled through a 60 cm long Pyrex column packed with glass helices and was protected by adding 1−2 ppm of Ethyl Corp. antioxidant-330.

Authentic samples were obtained from reliable commercial sources, synthesized by established methods, or in the case of some sesquiterpenes, isolated from essential oils. Their purities and identities were verified by capillary gas chromatography (GC) and mass (MS) or infrared (IR) spectrometry.

Isolation of Volatiles. The whole intact nuts (2900 g) were placed in a large, clean modified Pyrex glass desiccator container. The lid of the desiccator contained a suitable standard taper joint into which was fitted a Pyrex head that allowed entry of sweep gas through a Teflon tube, positioned under the walnuts, and exit through a Tenax trap (ca. 10 g of Tenax, 14 cm length, 2.2 cm i.d.). The sweep gas used was purified air at a flow rate of 6 L/min attained by applying reduced pressure (using a Teflon diaphragm pump) to the end of the Tenax trap. This flow was maintained for 20 h. The volatiles were then eluted from the trap with freshly distilled diethyl ether (50−100 mL). The ether extract was concentrated to ca. 20 *μ*L with a warm water bath and micro Vigreux distillation column.

Capillary GC−MS Analysis. These were carried out with a 60 m long × 0.25 mm i.d. fused silica capillary GC column (J & W) coated with DB-Wax (0.25 micron film) using a HP 5890 gas chromatograph that was connected to a HP5971 mass spectrometer (EI mode). The MS interface temperature was 180° C. The column oven was kept at 30 °C for the first 4 min after injection (injector temperature 170 °C) then raised at 2°/min until it reached 170 °C and was held at this temperature for another 30 min.

Determination of Concentrations in Trap Extract. Measured amounts (10 *μ*g each) of internal standards 2-heptanone and 4-phenylbutan-2-one, in hexane solution, were added to the Tenax trap extract. The concentrate from this was analyzed using a flame-ionization detector equipped HP-5890 gas chromatograph containing a 60 m long × 0.32 mm i.d. DB-WAX (0.25 micron film) coated fused silica capillary GC column using the same oven programming conditions as outlined above.

Isolation of Juglone from Blended Husks. Green husks (30 g) were peeled from healthy walnuts with a sharp knife and placed in a Pyrex blending jar and ground to fine particles. This was then blended with 100 g of anhydrous sodium sulfate (99.9%, certified ACS) and then mixed thoroughly with an additional 140 g of sodium sulfate. The sodium sulfate-blended husk mixture was then packed into a Pyrex column (35 cm long by 35 mm o.d.) and connected to a large Tenax trap (described above) in a closed loop system similar to that previously described (Buttery and Ling, 1996). The air in the

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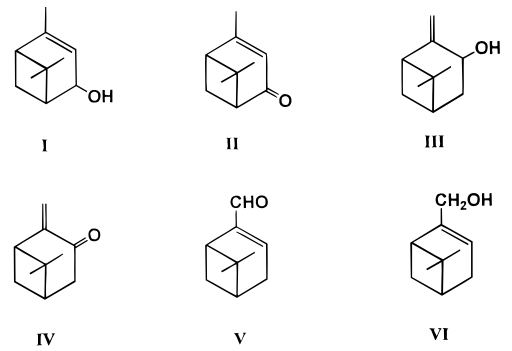

Figure 1. Structures of some of the oxygenated terpenes: verbenol (I), verbenone (II), pinocarveol (III), pinocarvone (IV), myrtenal (V), and myrtenol (VI).

closed loop system was displaced with nitrogen and the nitrogen pumped around the loop at a flow rate of 3−6 L/min for 15 h. The Tenax trap was then removed and eluted with ether and concentrated as described above.

RESULTS AND DISCUSSION

Studies were carried out with three main forms of freshly obtained, intact, green walnuts. These included (1) healthy, whole green walnuts; (2) freshly obtained, whole green walnuts that had become infested with the codling moth; and (3) noninfested green walnuts where the husk had just started to split from the nut. The choice of (2) and (3) was to get some information on the variations in volatiles due to the infestation or a different stage of nut maturity. Such changes in volatiles might effect the behavior of the infesting (and other) insects.

Table 1 lists the compounds identified together with Kovats' GC retention index (K.I.) found and the amounts isolated in terms of ng of compound per hour and per kilogram of nuts at the flow conditions (6 L/ min) used. Compounds were considered identified if their mass spectra and K.I. were consistent with that of authentic samples. For a few compounds, authentic samples were not available, but the mass spectra and K.I. were consistent with published data. These are listed as tentatively identified in Table 1. In general, the volatiles in the intact healthy green walnut husks are very similar qualitatively to those that have been identified in the intact leaves (Campbell et al., 1999).

Data for the healthy nuts are compared relative to those for the infected nuts and to those for the nuts where the husks are just starting to split. Major components in all three forms of nuts include (*E*)-4,8-dimethyl-1,3,7-nonatriene, pinocarvone, pinocarveol, myrtenal, myrtenol, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, caryophyllene epoxide, verbenol, verbenone, and terpinoline. Many of the oxygenated monoterpenes have structures related to α- and *β*-pinene (Figure 1). No authentic sample was available for (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, but the mass spectra and GC retention index were consistent with published data (Maurer et al., 1986).

The mass spectra of many compounds in Table 1 are well enough known. For some of the lesser known compounds, major mass spectral ions found (one each 14 mass units with more intense ions listed first; molecular ion in italics) are listed as follows: 1-nitro-3-methylbutane 43, 55, 71, 81, 97; benzyl methyl ether 91, *122*, 77, 65, 51, 39; (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene 69, 41, 81, 53, 95, 136; pinocamphone, 55, 69, 83, 41, 95, *152*; pinocarvone, 53, 81, 41, 108, 135, 150; myrtenal, 79, 107, 39, 91, 53, 135; pinocarveol, 92, 41, 55, 70, 83, 119.

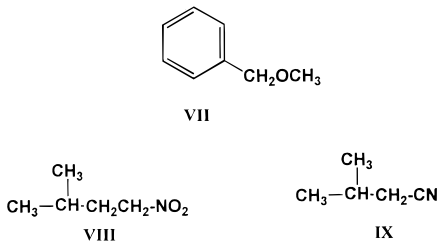

Figure 2. Structures of additional compounds occurring only in the infested husks: benzyl methyl ether (VII), 1-nitro-3-methylbutane (VIII), and isobutyl cyanide (IX).

Determination of Amounts Emitted. The main aim of the study was to identify the volatiles. GC peak area measurements, compared to that of internal standards, were carried out to get some idea of the relative concentrations in the Tenax trap extract. From these data, the trapping time, and weight of walnuts used it was possible to calculate the amounts of each compound emitted in terms of ng h^{-1} kg^{-1} for the conditions used. The calculations are based on GC peak areas and the assumption that all components have the same response in the flame ionization detector, which is a reasonable approximation for most compounds. The isolation of volatiles from these three forms of nuts were all carried out within 1 week using exactly the same isolation conditions. However, no studies were carried out to determine the normal variations occurring in the three different forms.

Major Differences Found between Healthy, Infested, and Split. Three compounds were identified in the isolate from the infested samples that were not detected in the healthy or split husk samples. These compounds were benzyl methyl ether, isobutyl cyanide (3-methylbutylnitrile), and 1-nitro-3-methylbutane (Figure 2). Benzyl alcohol was present in the healthy husks, and the formation of its methyl ether may occur during infestation by the insect. Isobutyl cyanide and 1-nitro-3-methylbutane both occur in some night-blooming flowers (Kaiser and Lamparsky, 1987). As pointed out by Kaiser and Lamparsky, both compounds can be formed from the amino acid leucine following a similar biosynthetic pathway to that suggested for cyanogenic glycosides (Cohn, 1979). It is difficult to determine whether these compounds are produced by the insect (e.g., insect plant digestion products) or by the husk in response to the insects tissue damage. Another possibility is that they are produced by some fungi or bacteria carried by the insect.

In addition to these compounds the infested walnuts emitted higher amounts of the (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, α- and *β*-pinene, sabinene, (*E*)-*β*-ocimene, (*E*,*E*)-α-farnesene, and linalool. Increases in the above C11 and C16 homoterpenoid hydrocarbons, other terpene and sesquiterpene hydrocarbons, and linalool have been reported from apples (Boeve et al., 1996), corn, and cotton plant parts following insect herbivore attack (Turlings and Tumlinson,1992; Pare and Tumlinson, 1997; and other papers cited therein). These authors attributed this to an induced delayed response, by the plant, to the attack.

C6 Green Leaf Compounds and Split Husk Nuts. The expected commonly occurring C6 compounds such

Table 1. Compounds Identified and Amounts of Compounds Isolated[a]

compd[b]	KI DBWAX[c]	ng h^{-1} kg^{-1} d healthy	infested	hull split
aliphatic aldehydes, alcohols and esters				
hexanal	1077	5	18	6
(*E*)-2-hexenal	1214	2.5	5	1
(*Z*)-3-hexen-1-yl acetate	1312	3.5	2.5	1
1-hexanol	1350	2	3.5	1.5
(*Z*)-3-hexen-1-ol	1380	2.5	2	2
nonanal	1390	5	3.5	4.5
aliphatic acids				
acetic acid	1440	5	–[e]	7.5
hexanoic acid	1825	25	15	3.5
heptanoic acid	1935	15	15	15
octanoic acid	2050	10	10	1.5
nonanoic acid	2175	5	5	5
terpene and sesquiterpene (+homo−) hydrocarbons				
α-pinene	1020	10	40	10
α-thujene	1022	3.5	10	3
β-pinene	1106	25	90	20
sabinene	1117	15	80	10
myrcene	1157	10	35	3
limonene	1197	25	50	25
γ-terpinene	1241	5	15	1.5
(*E*)-*β*-ocimene	1245	15	75	3.5
p-cymene	1264	10	15	4
terpinolene	1278	35	50	1.5
(*E*)-4,8-dimethyl-1,3,7-nonatriene	1302	90	200	30
β-bourbonene	1516	5	10	5
caryophyllene	1594	20	20	40
(*E*)-*β*-farnesene	1662	15	15	23
humulene	1666	1	–	–
germacrene-D	1707	15	15	10
(*E*,*E*)-α-farnesene	1744	15	30	10
AR-curcumene	1769	4	–	–
(*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (tent.)	1808	50	120	13
oxygenated terpenes and sesquiterpenes				
1,8-cineole	1209	20	20	25
sabinene hydrate	1465	5	–	5
linalool oxide B ((*Z*)-furanoid)	1470	5	–	4
campholene aldehyde (tent.)	1485	15	10	20
chrysanthenone (tent.)	1504	10	20	30
linalool	1546	5	55	10
pinocamphone	1548	5	–	10
pinocarvone	1565	75	35	65
myrtenal	1626	70	35	55
pinocarveol	1654	70	40	60
verbenol	1680	45	20	45
neral	1681	0.5	–	–
verbenone	1707	35	15	25
geranyl acetate	1755	3	–	–
myrtenol	1794	55	25	55
p-cymen-8-ol	1846	0.5	–	–
caryophyllene epoxide	1986	45	35	110
others				
isobutyl cyanide	1120	<2.5[f]	31	<2.5[f]
1-nitro-3-methylbutane	1317	<2.5[f]	12	<2.5[f]
benzyl methyl ether	1377	<2.5[f]	17	<2.5[f]
acetophenone	1645	–	–	–
benzyl alcohol	1874	25	–	33

[a] In ng of compound per hour and per kilogram of intact green walnuts using a sweep flow rate of 6 L of purified air per minute. Data are compared for healthy green walnuts, infested green walnuts and healthy green walnuts where the hull was just beginning to split [b] Mass spectra and GC retention index consistent with those of authentic samples except for those listed as (tent.) where the data were consistent with published data but no authentic sample was available. [c] Kovats' index found on DB-Wax capillary column. [d] Nanograms (ng) of compound per hour and per kilogram of nuts. Relative to internal standards and the assumption made that all compounds had the same response in the flame ionization detector. [e] Where a dash is shown the amounts could not be measured due to overlap of adjacent peaks or because the components were in trace amounts. [f] Not detected by MS or GC.

as hexanal, 1-hexanol, (*Z*)-3-hexen-1-ol, etc. were identified but in relatively low amounts. The amounts found were higher with the infested walnuts, probably being produced by plant enzyme action occurring during the initial phase of tissue damage produced by the insect. The volatiles from the nuts with split husks showed similar emission rates for C6 compounds to the healthy intact nuts. Apparently the splitting does not cause the type of tissue damage that occurs with mechanical or insect damage. Many other volatiles in the split husk nuts also showed similar amounts emitted to those for the healthy intact nuts. These included most of the monoterpene and sesquiterpene hydrocarbons and oxygenated forms. However, some including (*E*)-*β*-ocimene, terpinoline, and the homoterpenoids (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-

tridecatetraene were released at considerably lower rates for the split husk nuts. Caryophyllene epoxide showed higher amounts emitted for the split husk nuts.

Juglone from Blended Husks. Juglone, a well-known component of walnut husks (cf. Binder et al., 1989), was not detected in the volatiles obtained by dynamic headspace isolation from the intact green husk covered nuts. It was isolated, however, by blending the husks, mixing them with sodium sulfate to bind water, and carrying out dynamic headspace sampling using a closed loop system. Isolation for 15 h in this way gave 60 mg of juglone/kg of blended husks. Lesser amounts of the related compounds 1,4-naphthaquinone, 2,3-dihydro-5-hydroxynaphthalenedione, and 2,3-dihydro-5-hydroxy-2-methyl-1,4-naphthalenedione were also isolated, their (and juglone's) mass spectra and GC retention indices being consistent with published data (Binder et al., 1989).

The failure to isolate juglone by dynamic headspace from the intact walnuts could be due to its low volatility in aqueous systems, because it is considerably more water soluble than most volatiles. This failure could also be due to the fact that it is bound as a glycoside in the intact walnut husks (Daglish, 1950; Hedin et al., 1980) and only released by enzyme (and chemical) hydrolysis when the husks are damaged such as by blending.

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