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Volatile Components of Alfalfa Flowers and Pods

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The volatile components of alfalfa flowers and seed pods, isolated both by a Tenax trapping method and by vacuum steam distillation continuous extraction, were analyzed by the capillary gas–liquid chromatography–mass spectrometry combination. A total of 33 compounds was identified in the flowers and 31 compounds in the seed pods. The major component found associated with the flowers (Tenax trapping) was the previously identified (*E*)-β-ocimene. Other major flower volatiles identified included 2- and 3-methylbutanol, (*Z*)-3-hexenyl acetate, decanyl acetate, and dodecanyl acetate. Unusual flower components include neryl 2-methylbutyrate, α-copaene, and octan-3-one. Unusual pod components identified include γ-muurolene and an unidentified long-chain (ca. C_{16}–C_{18}) aliphatic methyl ketone. By use of the Tenax trapping method, the volatiles found associated with alfalfa flowers and pods were compared with those found associated with the leaves and stems.

Alfalfa is used as a food by a complex of insect pests and polinators. Individual parts of the plant are often selected to the exclusion of others. For example, the alfalfa seed chalcid (*Bruchophagus roddi* Guss.) oviposits only in the seed pods (Urbahns, 1920). The plant bug, *Lygus hesperius* Knight, sucks plant sap from the flowers and developing pods which prevents formation of seeds (Schull et al., 1934). Host plant volatiles often mediate the various behavior modes of the insects such as feeding, host finding, and oviposition (Dethier, 1953). We therefore decided to investigate whether alfalfa also contains volatile components that mediate the behavior of alfalfa pests.

Previously, we identified some volatiles from the alfalfa leaves and stems (Buttery and Kamm, 1980). In the present paper we set out to identify the volatiles specifically associated with the alfalfa flowers and seed pods and to compare these with those found in the leaves and stems.

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EXPERIMENTAL SECTION

Materials. Alfalfa (*Medicago sativa*) flowers, seed pods, and leaves plus stems were obtained from experimental fields in Albany, CA. Also, seed pods of known maturity (12 days) were obtained by pollination of alfalfa grown in a greenhouse at Corvallis, OR. Three different varieties, Lahontan, Narrangansett, and Caliverde, were examined. The mature flowers were picked fresh and used the same day. Green seed pods (exact maturity unknown) were picked from field plots and examined the same day. Samples of pods shipped by air from Oregon were kept cool by ice during shipping and overnight storage.

Most authentic chemical compounds were obtained from commercial sources (e.g., Aldrich Chemical Co. or synthesized by established methods and repurified by GLC separation, and their identity was checked by spectral means. Authentic sesquiterpenes (identity verified by

Table I. Volatile Constituents of Alfalfa Flowers

compound[a]	characteristic MS ions, m/e[b]	Kovats GLC index[c]	rel % Tenax trap	rel % vac steam distill.
	Aliphatic Aldehydes			
(*E*)-2-hexenal	*d*	1230	0.5	
(*E*)-2-nonenal	*d*	1530		0.1
(*E*,*E*)-2,4-decadienal	*d*	1790		0.3
	Aliphatic Ketones			
octan-3-one	43, 57, 72, 99, 85, *128*	1240	0-2.0	2.0
1-octen-3-one	55, 70, 43, 97, 83, 111	1290		0.6
	Aliphatic Alcohols			
2-methylbutanol	*d*	1180	4-10	
3-methylbutanol	55, 42, 43, 31, 70	1180		
hexanol	*d*	1330		0.4
(*Z*)-3-hexenol	*d*	1370	4.5-6	1.9
octan-3-ol	59, 55, 83, 41, 101, 112	1390		1.1
1-octen-3-ol	*d*	1460	0.1-0.5	57
octanol	56, 42, 70, 31, 84, 112	1530		0.3
(*E*)-2-octenol	57, 41, 44, 68, 81, 110	1590		0.9
nonanol	56, 70, 42, 31, 98, 126	1630		0.3
decanol	56, 70, 83, 31, 97, 112	1740		0.8
dodecanol	56, 69, 83, 31, 97, 140	1940		0.3
	Aliphatic Esters			
(*Z*)-3-hexenyl acetate	43, 67, 82, 69, 73, 61	1310	8.1	
decanyl acetate	43, 55, 70, 61, 83, 140	1670	7.1	1.9
dodecanyl acetate	43, 55, 69, 61, 140, 168	1880	4.3	1.3
	Terpenoids			
myrcene	*d*	1160	3.0	
limonene	*d*	1180	0.5	
(*E*)-β-ocimene	*d*	1250	25	0.2
α-copaene	*d*	1460	1-1.7	
caryophyllene	*d*	1570	0.1-0.2	0.1
β-farnesene	*d*	1650	0-0.2	0.4
γ-muurolene	*d*	1655	0.7	
neryl 2-methylbutyrate	69, 57, 68, 93, 85, 121	1807	1.0	0.7
	Aromatic Compounds			
benzaldehyde	*d*	1520	0.5	
phenylacetaldehyde	*d*	1650		0.1
methyl salicylate	120, 121, *152*, 92, 39, 65	1730	2-2.8	0.7
benzyl alcohol	79, *108*, 107, 77, 51, 39	1830	0.4-0.7	1.3
2-phenylethanol	91, 92, *122*, 65, 39, 51	1890	1.0	2.1
methyl cinnamate	131, 103, *162*, 77, 51, 63	2050		0.2

[a] The mass spectrum (complete spectrum) and the Kovats GLC retention index of all compounds listed are consistent with that of authentic samples. [b] Not necessarily the most intense ions but six of those considered the most characteristic for that compound. Ions are listed in descending order of intensity, and the molecular ion (if found) is shown in italic type. Common ions (e.g., 43) may not be listed. [c] The Kovats GLC index for the Carbowax 20M Pyrex capillary is described under Experimental Section. [d] Essentially the same mass spectra as previously reported by some of the authors (Buttery et al., 1981).

infrared spectra) were obtained from hop oil [cf. Buttery et al. (1967) and Naya and Kotake (1970)].

Isolation by Vacuum Steam Distillation Continous Extraction. This was carried out by using a Likens-Nickerson type steam distillation continuous extraction apparatus under reduced pressure (100 mmHg) in essentially the same way as described previously for the whole alfalfa leaves and stems (Buttery and Kamm, 1980) except that ca. 100-g quantities of the whole flower or pods were used. The hexane extracts were concentrated as described previously.

Isolation Using Tenax Traps. Several traps were made from Pyrex glass tubes 1.8 cm o.d. by 16 cm long (including 14/35 standard taper joints at each end) packed with 1.7 g (1.3-cm diameter, 7 cm long) of Tenax GC adsorbent (60–80 mesh, Applied Science Laboratory). The support was held in with Pyrex glass wool plugs. The trap was activated by first eluting with freshly distilled diethyl ether (200 mL), followed by heating at 250 °C for 3 h with a purified nitrogen flow of 200 mL/min. The alfalfa flower, pod or leaf sample (100 g) was enclosed in a 5-L flask and the trap attached to the neck of the flask by standard taper joints. Purified air (500 mL/min) was passed into the flask (via a Teflon tube) and out through the trap by applying suction to the outlet of the trap. The isolation was continued for 24 h at room temperature. The trap was then removed and the trapped material eluted with freshly distilled diethyl ether (100 mL). The ether extract was concentrated by using a warm water bath and low hold up distillation columns.

Capillary Gas–Liquid Chromatography–Mass Spectrometry Analysis (GLC–MS). This was carried out on the concentrates from the Tenax trap and the vacuum steam distillation continuous extraction. The GLC column wa a 150 m long by 0.64 mm i.d. Pyrex glass capillary coated with Carbowax 20M. The GLC temperature program was isothermal at 50 °C for 30 min, 1 °C/min from 50 to 170 °C, and then isothermal at 170 °C. The column inlet pressure was 16 psi He. A single-stage Lewellyn-Littlejohn silicone rubber membrane molecular separator was used to couple the end of the capillary column to the mass spectrometer (a modified Consolated 21-620 cycloidal type). Electron ionization was 70 eV. Separate GLC–MS studies were carried out on four sam-

Table II. Volatile Constituents Found in Alfalfa Seed Pods

compound[a]	characteristic MS ions, m/e[b]	Kovats GLC index[c]	rel % Tenax trap	rel % vac steam distill.
	Aliphatic Aldehydes			
hexanal	*d*	1108	2	0.2
nonanal	*d*	1390		0.1
(*E*)-2-hexenal	*d*	1230	0.5-2	
(*E*)-2-octenal	*d*	1430	1-7	
(*E*)-2-nonenal	*d*	1530		0.2
(*E*)-2-undecenal	41, 70, 83, 97, 121, 124	1740		0.4
(*E*,*E*)-2,4-heptadienal	*d*	1660		0.4
(*E*,*Z*)-2,4-decadienal	*d*	1740		0.4
(*E*,*E*)-2,4-decadienal	*d*	1790		0.4
	Aliphatic Ketones			
octan-3-one	*e*	1240	1.5	5.5
1-octen-3-one	*e*	1290		0.8
	Aliphatic Alcohols			
2-methylbutanol	*e*	1180	2.5	
3-methylbutanol	*e*	1180		
hexanol	*e*	1330	0.5	2.5
(*Z*)-3-hexenol	*e*	1370	10-40	12.6
(*E*)-2-hexenol	57, 41, 44, 31, 82, *100*	1380	4	0.4
octan-3-ol	*e*	1390		2.5
1-octen-3-ol	*e*	1460	3-9	42
octanol	*e*	1530		0.06
(*E*)-2-octenol	*e*	1590		0.5
	Aliphatic Esters			
(*Z*)-3-hexenyl acetate	*e*	1310	7-22	
	Terpenoids			
(*E*)-β-ocimene	*e*	1250	4	
α-copaene	*e*	1460	1-5	
caryophyllene	*e*	1570	0.5	
β-farnesene	*e*	1650	4.0	
γ-muurolene	*e*	1655	4-7	
	Aromatic Compounds			
benzaldehyde	*e*	1520	1	0.6
methyl salicylate	*e*	1730	1-2	
benzyl alcohol	*e*	1830	4-6	1.5
2-phenylethanol	*e*	1890	3-8	0.7
benzyl cyanide	*117*, 90, 116, 39, 51, 63	1890		0.4

[a-d] As for Table I. [e] See Table I.

ples of flowers, five samples of pods, and five samples of leaves and stems.

RESULTS AND DISCUSSION

Two main methods of isolation of the volatiles from the alfalfa flowers and pods were used. The first involved sweeping purified air over the plant material and trapping the volatiles in the exist air stream on a Tenax adsorbent trap [cf. Cole (1980) and Dressler (1979)]. The volatiles were then eluted from the Tenax with diethyl ether, and the concentrate was used for the GLC–MS analysis. In the second method the volatiles were isolated by vacuum steam distillation continuous extraction using hexane as the solvent, and the hexane concentrate was used for GLC–MS analysis.

Alfalfa Flowers. Table I lists the volatiles identified for the alfalfa flowers by using both isolation methods. Also listed are the relative percentages of the components in the GLC chromatograms obtained, based on the areas of the peaks.

The major component found in the flowers by the Tenax trapping method was (*E*)-β-ocimene, which had been previously reported in alfalfa flowers by Loper et al. (1971). The other monoterpenes, myrcene and limonene, were also identified, occurring in considerably lesser amounts. Sesquiterpenes identified included α-copaene, γ-muurolene, β-farnesene, and caryophyllene. The major oxygenated components included (*Z*)-3-hexenyl acetate, decanyl acetate, and dodecanyl acetate. The alcohols 2- and 3-methylbutanol and (*Z*)-3-hexenol were also prominent. Probably the most unusual compound identified is neryl 2-methylbutyrate. The isomer geranyl 2-methylbutyrate had been identified previously by some of the authors in carrots (Buttery et al., 1979).

Several compounds could not be identified. A major unidentified compound had a Kovats index of 1300 (Carbowax 20M), a molecular ion of m/e 150, and a very strong base ion at m/e 69. It appears to be a terpenoid but did not match any of the published spectra of terpenoids available to the authors.

Alfalfa Pods. Table II lists compounds identified in alfalfa seed pods. There was considerable variation in the relative amounts of the volatile compounds found in different samples of pods, possibly depending on the exact age of the pods. (*Z*)-3-Hexenyl acetate, (*Z*)-3-hexenol, 1-octen-3-ol, benzyl alcohol, and 2-phenylethanol were the most consistent major components found by using the Tenax trap. 1-Octen-3-ol was by far the major component of the vacuum steam volatile oil but was generally a relatively moderate component of the Tenax-trapped material. A number of components present in the flowers were not detected in the pods. These included the higher molecular weight esters. An unusual, probably aliphatic, methyl ketone was detected in both the pods and flowers

Table III. Comparison of Relative Amounts of Tenax-Trapped Volatiles in Flowers, Pods, and Leaves

compounds	rel % of total volatiles[a]		
	flowers	pods	leaves
hexanal		2	
myrcene	3		0.1-2
(*E*)-2-hexenal	0.5	0.5-2	0.2
2- and 3-methylbutanol	4-10	2-5	0.3-0.7
limonene	0.5		
(*E*)-β-ocimene	25	4	0.8-3
octan-3-one	0-2	1.5	<0.1
hexyl acetate			0.2
(*Z*)-3-hexenyl acetate	8.1	7-22	72-85
hexanol		0.5	
(*Z*)-3-hexenol	4.5	10-40	6-8
(*E*)-2-hexenol		4	
1-octen-3-ol	0.5	3-9	0.4
α-copaene	1-1.7	1-5	0.1
caryophyllene	0.1-0.2	0.5	<0.1
β-farnesene	0-0.2	4	0.1
γ-muurolene	0.7	4-7	0.3
decanyl acetate	7		
methyl salicylate	2-3	1-2	0.8
benzyl alcohol	0.4-0.7	4-6	0.4
neryl 2-methylbutyrate	1		
dodecanyl acetate	4		
2-phenylethanol	1	3-8	0.3

[a] Based on relative GLC peak areas.

but could not be identified. It had a Kovats index of 2120, which is reasonable for 2-hexadecanone. However, comparison of the mass spectrum of the unknown with that of an authentic sample of 2-hexadecanone showed that they were somewhat different, indicating that our unknown has a higher molecular weight (probably C_{17} or C_{18}) with possible branching and unsaturation.

Isolation Methods. It is difficult to choose a perfect method for isolating and concentrating the volatile compounds. It can be seen from Tables I and II that the two isolation methods used in this study gave marked differences in the relative amounts of volatiles found. The Tenax trapping method might be expected to give a picture closest to what the insect is exposed to in the atmosphere around the plant material. Disadvantages of the Tenax trapping method include the small amounts of volatiles obtained, the possible adsorption of higher boiling compounds on the walls of the container used for holding the sample, and the possibility of chemical changes occurring on the Tenax surface.

The vacuum steam distillation continuous extraction method has the advantage of providing more of the trace volatile compounds for a given amount of plant sample. The reduced pressure may, however, result in damage to cell walls, thus facilitating enzyme-catalyzed breakdown and the resultant production of extraneous volatiles. This latter effect may explain the much greater amount of 1-octen-3-ol (a lipid oxidative breakdown product) found with the vacuum steam distillation method.

Quantitative Aspects. The present study was mostly concerned with the identification (qualitative analysis) of the major components. It is much more difficult to obtain a reasonable quantitative analysis. This is dependent on a number of complex factors including the exact procedure for sampling and the exact maturity of the flowers and pods. There was some variations between samples, and the figures given in Tables I–III are only meant to give a general idea for a typical sample.

The total amount of volatile oil obtained by vacuum steam distillation continuous extraction was of the order of 10 parts per million (ppm) for the alfalfa flowers and 1 ppm for the pods. It was more difficult to measure the amount from the Tenax traps, but calculations from GLC peak areas indicated that this was of the order of 50–100 parts per billion (10^9) for the flowers, pods, and leaves with the flowers and leaves containing the most material.

Comparison with Leaf Volatiles. The volatiles of the leaves and stem of the alfalfa plant had been previously studied by using vacuum steam distillation continuous extraction to isolate the volatiles (Buttery and Kamm, 1980). In the present work volatiles were also isolated from the whole leaves and stems by the same Tenax trapping procedure used for the flowers and pods. The Tenax trapping method gave quite a different quantitative picture than that obtained by vacuum distillation continuous extraction. Comparison of the major volatiles found by the Tenax trapping method for alfalfa flowers, pods and leaves (plus stems) is shown in Table III. Most volatiles seem to be in all three parts of the alfalfa plant. However, there are some distinct differences. The flowers, as might be expected from previous work (Loper et al., 1971), had the largest proportion of (*E*)-β-ocimene. The flowers also were the only part to contain detectable amounts of decanyl acetate, dodecanyl acetate, and neryl 2-methylbutyrate. The leaves had by far the highest proportion of (*Z*)-3-hexenyl acetate. The pods have the largest proportion of (*Z*)-3-hexenol, (*E*)-2-hexenol, 1-octen-3-ol, caryophyllene, β-farnesene, γ-muurolene, benzyl alcohol, and 2-phenylethanol.

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