contained in impure bluish fluorescent compound having the same R_f (0.7) by TLC as 4′-*O*-methylcoumestrol. The contents of the tubes were combined, and the solvent was removed under vacuum. The solids were further purified by column chromatography on silica gel (Davidson). Washing of the column with 8% acetone in Skellysolve B gave pure 4′-*O*-methylcoumestrol. Recrystallization from methanol–acetone (1–1) gave colorless crystals (61 mg.), m.p. 331–32° C., no depression with an authentic sample of 4′-*O*-methylcoumestrol. The PMR, UV, and IR spectra of the isolated and authentic samples were also identical.

Proportion of Medicagol and 4′-*O*-Methylcoumestrol in Mixture. The per cent methoxyl calculated for 4′-*O*-methylcoumestrol is 11.0; the per cent found in the mixture is 8.29. The percentage found can be accounted for if the mixture contains 75.4% of 4′-*O*-methylcoumestrol. Similar calculations on the mixture of the acetates of the two compounds indicate 77.8% 4′-*O*-methylcoumestrol based on 7.45% methoxyl in the mixture. Therefore, the alfalfa plant apparently contains about three times as much 4′-*O*-methylcoumestrol as medicagol.

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ISOLATION AND IDENTIFICATION

Constituents of the Cotton Bud. Terpene Hydrocarbons

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The terpene hydrocarbons from the buds (squares) of Deltapine Smoothleaf cotton were obtained by steam distillation and column chromatography. Components identified and their percentages of the essential oil were: *l*-α-pinene (8.88), *l*-camphene (0.41), β-pinene (1.03), myrcene (8.16), α-phellandrene (0.07), α-terpinene (0.02), *l*-limonene (1.21), β-phellandrene (0.09), *trans*-β-ocimene (3.90), γ-terpinene (trace), and terpinolene (0.21). Various gas chromatographic techniques were utilized for preliminary identifications. Positive identifications were made by infrared, proton resonance, and ultraviolet spectroscopy.

PLANT chemical constitution is of fundamental importance in understanding host plant–insect interrelationships. Compounds elaborated by plants as wastes, metabolic intermediates, defense mechanisms, or insect attractants influence the development of highly specific plant-insect ecologies (*3*). Such a specialized relationship exists between the boll weevil, *Anthonomous grandis* Boheman, and its preferred host, the cotton plant, *Gossypium*. Keller and coworkers (*4*) have presented strong evidence for the existence in cotton of a plant attractant for the boll weevil. A previous claim (*16*) of weevil attractancy by a cotton plant distillate has been made, but was not backed by experimental evidence.

Little is known of the volatile components of the cotton plant, one or more of which presumably are responsible for such attractancy. Power and Chesnut (*10*) steam distilled 3.5 tons of cotton plants, isolated the essential oils by extraction, and ultimately identified trimethylamine, ammonia, acetaldehyde, methanol, and amyl alcohol after extended distillation and extraction procedures. They reported the presence of several unidentified sesquiterpenes. In another examination of the plant for nonvolatile constituents, Power and Chesnut (*11*) isolated dipentene and an unidentified sesquiterpene. Isolation procedures utilized in both of these studies were so severe that considerable isomerization and degradation of the labile terpenes must have occurred. No other reports of work on cotton plant volatiles are known to us, although there are many reports on the nonvolatiles. This work is one of a series of investigations by this laboratory into the nature of the chemical components in the cotton flower bud (*15*). All components are being assayed by entomologists to determine whether they are attractive or repellent to insects.

Experimental

Apparatus. Analytical gas chromatograph: Aerograph HiFi Model 600-C equipped with hydrogen flame detector. Preparative gas chromatograph: Aerograph Autoprep Model A-700 equipped with thermal conductivity detector. Columns, packings, and conditions are listed in Table I.

Beckman - IR5A infrared spectrophotometer.

Beckman DK-2A ratio recording spectrophotometer; matched silica cells of 1.0-cm. path length.

Varian Model A-60 Analytical NMR spectrometer; all spectra run in spectrograde CCl_4.

Kern Full-Circle polarimeter equipped with a 1.0-dm. cell and sodium emission lamp (SLA-5C; George W. Gates and Co.).

Isolation of Cotton Square Extract. *Gossypium hirsutum*, Deltapine Smoothleaf cotton squares (flower buds) were weighed, ground in a SerVall Omnimixer in a minimum of water, and steam distilled in an all-glass system for 1 hour. The distillate was collected in a trap consisting of two flasks connected in series, the first cooled in ice water and the second in an acetone–dry ice bath.

The combined distillate was extracted with three portions of dichloromethane, each portion consisting of 50 ml. of the solvent per 1000 ml. of distillate. The solvent was removed under vacuum at room temperature, and the extract stored in a freezer at 0° C. until used. A total of 444.6 kg. of cotton squares yielded 83.2 grams (0.019%) of the cotton square essential oil.

Additionally, 5 kg. of cotton squares were ground and cold pressed in water. The aqueous solution was extracted with pentane, the extract concentrated by evaporation under vacuum at room temperature, and the hydrocarbons were separated by column chromatography. The hydrocarbons were stored in pentane solution at 0° C. until used.

Column Study and Preliminary Fractionation. It has been widely reported (*7*, *12*) that the terpene hydrocarbons undergo degradation and isomerization on solid adsorbent materials. Accordingly a column study of three recommended adsorbent materials was undertaken.

A mixture of commercial standards of known composition was employed and three columns were compared to determine the degree of isomerization and degradation of terpene hydrocarbons on each. The mixture was chromatographed on 2% (w./w.) Carbowax 20M coated silica gel (see below), as described by Kugler and Kovats (*7*), on alumina and on untreated silica gel columns. Pentane was used as the solvent in each case. A sample of each eluate was gas chromatographed on an analytical Carbowax 4000 column (Table I).

Comparison of the chromatograms of the eluates and the standard mixture indicated that the Carbowax 20M coated silica gel column produced no significant isomerization whereas the other two were active in this respect.

Two grams of Carbowax 20M were dissolved in CH_2Cl_2 and the solution poured over 98 grams of Baker Analyzed silica gel in a round-bottomed flask, together with enough CH_2Cl_2 to form a slurry. The slurry was mixed by rotation and the CH_2Cl_2 simultaneously removed by vacuum at 35° C. The coated silica gel was dried in a large pan in an oven at 110° C. for 12 hours.

A 2-cm. i.d. glass column equipped with a fritted filter was packed to a height of 12 cm. with a pentane slurry of the Carbowax 20M coated silica gel. Cotton square extract, 2 ml., was placed on the column, and the hydrocarbons were eluted with 200 ml. of pentane. The pentane was removed under vacuum at 31° C. until approximately 1 ml. of solution remained.

Gas Chromatographic Analyses. Several standard terpene hydrocarbons were chromatographed on two columns. Conditions of use and packing for each column are listed in Table I. α-Pinene and limonene retention ratios were calculated for each standard as follows:

$$R_\alpha = \frac{T_x'}{T_\alpha'} \qquad (1)$$

Table I. Gas Chromatographic Operating Conditions

	Preparative Carbowax 4000	*Analytical Carbowax 4000*	*Analytical Apiezon L*
Detector	Therm. cond.	H_2 flame	H_2 flame
Column length, ft.	20	20	20
Column diam., inch	1/4	1/8	1/8
Column material	Al	Cu	Cu
Per cent stationary phase, w./w.	28.5	28.5	20
Solid support	60- to 80-mesh Gas-Chrom P[a]	60- to 80-mesh Gas-Chrom P[a]	60- to 80-mesh Gas-Chrom P[a]
Carrier gas flow rate ml./min.	He: 100	N_2: 14	N_2: 15
Inlet pressure, p.s.i.g.	57	39	40
Outlet pressure, p.s.i.g.	0	0	0
Temperatures:			
Column	130° C.	130° C.	130° C.
Injector	173° C.	...	...
Detector	200° C.	130° C.	130° C.

[a] Hexamethyldisilazane-treated.

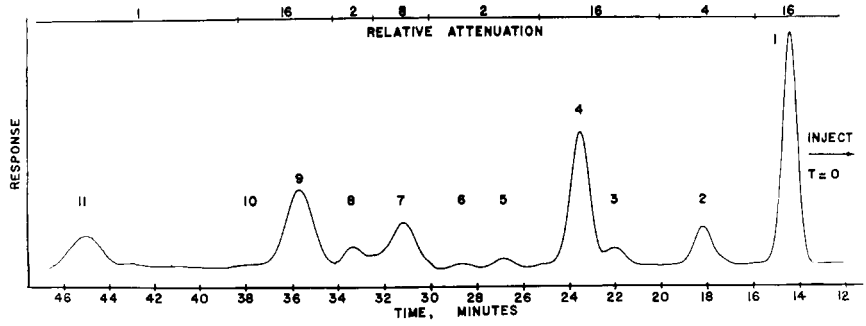

Figure 1. Analytical chromatogram of cotton square terpene hydrocarbons on Carbowax 4000

Maxima: 1. *l*-α-Pinene. 2. *l*-Camphene. 3. β-Pinene. 4. Myrcene. 5. α-Phellandrene. 6. α-Terpinene. 7 *l*-Limonene. 8. β-Phellandrene. 9. *trans*-β-Ocimene. 10. γ-Terpinene. 11. Terpinolene

where R_α = relative retention ratio based on α-pinene
T_x' = retention time of a peak corrected for air volume
T_α' = retention time of α-pinene corrected for air volume

$$R_L = \frac{T_x'}{T_L'} \qquad (2)$$

where R_L = relative retention ratio based on limonene. Kovats' indices (I_k) (*6*) were also calculated for each standard.

The hydrocarbon sample from cotton square oil was chromatographed on each column. A chromatogram of the hydrocarbon sample on Carbowax 4000 is shown in Figure 1. After cumene had been added as an internal standard to the hydrocarbon sample, the mixture was rechromatographed. Values for R_α and R_L were calculated for each peak based on previously determined R_α and R_L values for cumene. Kovats' indices were calculated from a chromatogram of a mixture of sample and n-C_{10}–C_{14} paraffins. Sample maxima obscured by the paraffin maxima were subsequently computed from a sample chromatogram using sample maxima I_k values as secondary standards. Retention values are listed in Table II.

In order to have a greater variety of standards for comparison, calibration curves were prepared based on reported retention times in the literature. Zubyk and Conner (*18*) have listed 31 terpene hydrocarbons with their R_α values on a Carbowax 4000 column. By plotting the authors' R_α values against those of Zubyk and Conner for standards common to both, a straight line was obtained. R_α values for standards unavailable to the authors could thus be found by interpolation. A similar calibration curve was obtained by using values reported for Apiezon L (*5*).

Limonene retention ratios obtained by chromatography of the authors' standards and interpolated values described previously were used to construct a plot of log ($R_L \times 10^3$) on Apiezon L *vs.* log ($R_L \times 10^3$) on Carbowax 4000 (Figure 2). The plots obtained agreed with those of Klouwen and ter Heide (*5*).

The sample peaks were plotted in the same manner. Peaks 1, 2, and 3 were found to lie on the bicyclic curve; peaks 5, 6, 7, 8, 10, and 11 on the monocyclic curve; and peaks 4 and 9 on the acyclic curve.

Preparative Gas Chromatography. The hydrocarbon fraction, 5 ml. in 200-μl. samples, was injected onto a preparative Carbowax 4000 column (Table I). Peaks 1, 4, 7, and 9, which were present in large amounts, were collected in Aerograph 5-ml. collection flasks immersed in liquid nitrogen. The smaller peaks were collected by bubbling through CCl_4.

Each fraction was rechromatographed on Carbowax 4000 and Apiezon L analytical columns to determine purity. Those peaks which were incompletely resolved as 3 and 4 and 7 and 8 showed considerable mutual contamination in the infrared and proton resonance spectra. No significant amount of isomerization or degradation was apparent in any of the compounds.

Infrared, proton resonance, and in some cases ultraviolet spectra were run on each material in its pure state or in solution in the case of the minor components.

Quantitation. A mixture of 1.8645 grams of cotton square extract and 0.1167 gram of tetradecane was chromatographed on a Carbowax 20M coated silica gel column, and the hydrocarbons were eluted with pentane.

The eluate was evaporated to approximately 1 ml. and a 0.5-μl. sample was gas chromatographed on the Carbowax 4000 analytical column. Peak areas were measured by triangulation, and the percentage of each compound in the hydrocarbon mixture was calculated. Total terpene peak area was normalized to 100%. The percentage of terpenes in the square extract was computed from the ratio of tetradecane area to total terpene hydrocarbon area.

Preparation of α-Fenchene. Thionyl chloride (25 ml.) was slowly added to the same amount of fenchyl alcohol, while mixture was shaken under reflux. The mixture was heated gently and refluxed for 20 minutes, after which excess $SOCl_2$ was distilled off. Distillation was discontinued when the temperature reached 95° C., and the fenchyl chloride dehydrochlorinated with aniline (*14*). After the reaction mixture had been acidified with glacial acetic acid, the mixture was steam distilled and the oil layer separated from the aqueous phase in the distillate.

Crude α - fenchene was chromatographed on the standard Carbowax 20M coated silica gel column with pentane, the pentane removed under vacuum, and the purified product gas-chromatographed on a 20-feet × 3/8 inch 20% SE-30 preparative column. The major component, which had a retention time equal to that of camphene, was collected and rechromatographed on the analytical Carbowax 4000 column. The R_α value of this compound (1.28) agreed with that expected for α-fenchene on the analytical column. Proton resonance and infrared spectra of the purified material were taken and were entirely consistent with the structure of α-fenchene. The spectra were similar to, but not identical with those of camphene, as would be expected.

Results and Discussion

It was suspected that steam distillation might produce rearrangements or degradation of the notoriously labile terpenes. However, gas chromatography on Carbowax 4000 of the hydrocarbons isolated by steam distillation and cold pressing indicated that both samples contained the same compounds in approximately the same percentages. Accordingly, all subsequent isolation was by steam distillation.

Table II. Retention Values and Quantitative Data for Terpenes in Cotton Square Extract

Sample Peak No.	Standard Compounds	Carbowax 4000 R_α	Carbowax 4000 $R_L \times 10^3$	Carbowax 4000 I_k	Apiezon L R_α	Apiezon L $R_L \times 10^3$	Apiezon L I_k	Cotton Extract, %
1		1.01	433	1071	1.00	545	962	8.88
	α-Pinene	1.00	434	1073	1.00	538	962	
2		1.30	559	1124	1.14	621	982	0.41
	Camphene	1.29	559	1124	1.14	614	982	
3		1.59	684	1166	1.38	753	1014	1.03
	β-Pinene	1.58	684	1166	1.38	742	1013	
4		1.73	745	1182	1.14	621	982	8.16
	Myrcene	1.72	745	1182	1.14	614	982	
5		2.00	865	1211	1.56	852	1034	0.07
	α-Phellandrene	1.98	856	1211	1.59	855	1038	
6		2.13	923	1225	1.63	885	1042	0.02
	α-Terpinene	2.10	920	1225	1.66	883	1044	
7		2.31	1000	1242	1.83	1000	1062	1.21
	Limonene	2.31	1000	1242	1.86	1000	1061	
8		2.48	1073	1257	1.91	1045	1068	0.09
	β-Phellandrene	2.48	1068	1257	1.90	1038	1066	
9		2.68	1151	1273	1.67	914	1048	13.90
	trans-β-Ocimene [a]	...	1250	...	...	952	...	
10		2.78	1220	1289	2.14	1160	1092	Trace
	γ-Terpinene	2.74	1220	1286	2.13	1160	1085	
11		3.40	1472	1317	2.52	1378	1113	0.21
	Terpinolene	3.31	1461	1319	2.53	1380	1113	

[a] Values by interpolation from Klouwen and ter Heide (*5*).

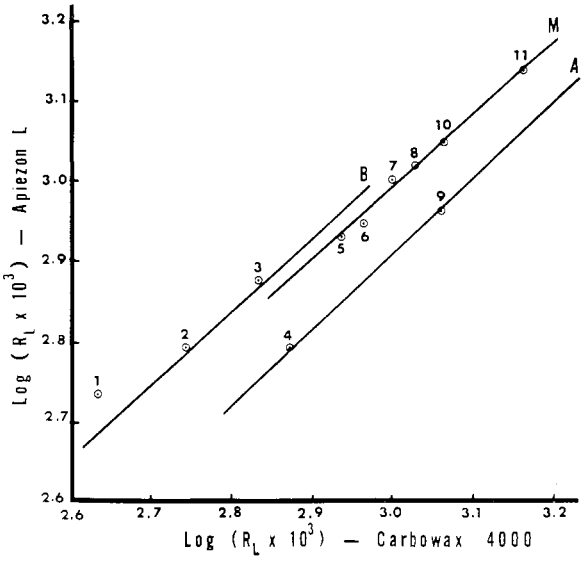

Figure 2. Logarithmic plot of retention data, Carbowax 4000 and Apiezon L columns

A—acyclic, M—monocyclic, B—bicyclic, R_L—limonene retention ratio. Maxima: see Figure 1 for identification of numbers

It was determined gas chromatographically (see experimental) that the terpene hydrocarbons comprise 34.0% of the cotton square extract. The percentages of the individual terpene hydrocarbons in the cotton square extract and retention values on several columns are given in Table II. Future communications will deal with the remaining constituents in the essential oil.

Component 1 was identified as *l*-α-pinene by comparison of infrared and proton resonance spectra to standards. The infrared spectrum of this material is completely superimposable upon that of authentic α-pinene. The same is true of the proton resonance spectrum, with maxima at δ (p.p.m.) = 0.79 (singlet, 3 protons); 1.12 (doublet, J = 8 c.p.s., 1 proton); 1.22 (singlet, 3 protons); 1.63 (quadruplet, J = 1.8 c.p.s., 3 protons); 2.16 (multiplet, broad, 5 protons); and 5.13 (multiplet, broad, 1 proton). Optical rotation measured in heptane on this component collected from 2.3 ml. terpenes averaged −1.43°, indicating the levo isomer.

Component 2 gave an infrared spectrum with 15 maxima agreeing in wavenumber and relative intensity with those of a standard spectrum of camphene.

Inasmuch as α-fenchene and camphene should have similar infrared and

nuclear magnetic resonance spectra and could not be resolved on any of the GLC columns employed, α-fenchene was prepared, purified gas chromatographically, and infrared and nuclear magnetic resonance spectra were taken. Proton resonance maxima appeared at δ (p.p.m.) = 1.00 (triplet, J = 2 c.p.s., 6 protons); 1.61 (multiplet, broad, 7 protons); 2.55 (multiplet, broad, 1 proton); 4.62 (quadruplet, J = 13.5, 6.0 c.p.s., 2 protons).

Camphene can be distinguished from α-fenchene on the basis of the splitting of its signals at δ (p.p.m.) = 1.01 (doublet, J = 2.0 c.p.s., 6 protons) and 4.56 (doublet, J = 13.0 c.p.s., 2 protons). The proton resonance spectrum of component 2 contained both these features, as well as maxima at 0.81, 1.24, 1.61, 1.85, and 2.63 p.p.m., which agree with the spectrum of camphene. Accordingly it was concluded that this material was camphene. Net optical rotation in n-heptane averaged −0.36° on the material trapped from 2.3 ml. of terpenes, indicating the levo isomer.

Component 3 was incompletely separated from component 4 on Carbowax 4000 and was present in only small amounts. Proton resonance and infrared spectra (*13*) of CCl_4 solutions were consistent with β-pinene contaminated with myrcene (component 4, see below). Observed proton resonance maxima correlated with β-pinene signals occurred at δ (p.p.m.) = 0.71, 1.22 (singlets, 3 protons each, endo- and exo- methyls, respectively); 1.32, 1.47, 1.91, 2.33, 2.42, and 4.54 (multiplet, terminal exocyclic methylene). The infrared spectrum contained no maxima unassignable to β-pinene or myrcene, with 20 maxima agreeing in wavenumber with corresponding absorptions in the β-pinene spectrum. Optical rotation in CCl_4 was slightly levo, but was not large enough on the available sample to be considered statistically significant. Behavior on two different GLC columns (Table II) supported the identification as β-pinene.

Component 4 was identified as myrcene on the basis of its infrared and proton resonance spectra, which were superimposable on those of authentic myrcene run in the same manner. Proton resonance maxima occurred at δ (p.p.m.) = 1.49, 1.57 (singlet, 3 protons each, isopropylidene methyls); 2.06 (doublet, J = 0.3 c.p.s., 4 protons); 4.91 (singlet, 2 protons, terminal methylene); 5.00 (multiplet, 3 protons); and 6.30 (quadruplet, J = 11.0, 17.0 c.p.s., 1 proton, conjugated vinyl).

Components 5, 6, 8, and 10 were tentatively identified as α-phellandrene, α-terpinene, α-phellandrene, and γ-terpinene, respectively, on the basis of their gas chromatographic behavior (Table II) on two different columns. Insufficient quantities of these components precluded trapping and definite spectral identification.

Component 7 was identified as *l*-limonene. Its infrared and proton resonance spectra both matched spectra run in the same manner on commercially available limonene. Nineteen maxima in the infrared spectrum matched the standard in wavenumber and relative intensity. Proton resonance maxima occurred at δ (p.p.m.) = 1.63 (multiplet, 8 protons); 1.93 (multiplet, broad, 5 protons); 4.67 (singlet, 2 protons); and 5.35 (multiplet, broad, 1 proton). The latter two signals are presumably due to the terminal isopropylene protons and the ring olefinic proton, respectively. Net optical rotation measured in n-heptane on this material collected from 4.5 ml. of terpenes averaged −0.72°.

Component 9 was identified as *trans*-β-ocimene on the basis of infrared, proton resonance, and ultraviolet spectra. Twenty-two maxima agreed in wavenumber and relative intensity with corresponding maxima of the *trans*-β isomer spectrum reported by Ohloff, Seibl, and Kovats (*9*). This was confirmed by an infrared spectrum obtained from Fisher and Lawrence (*2*).

The proton magnetic resonance spectrum also agreed with data (*9*) for the *trans*-β isomer. The triplet at δ = 2.77 p.p.m. (J = 7.0 c.p.s., 2 protons) assigned to the methylene between two double bonds established the beta configuration, which has a terminal isopropylidene rather than isopropenyl structure. This was substantiated by the presence of an infrared maxima at 830 $cm.^{-1}$, as discussed by Barnard (*7*). The quadruplet at δ = 6.30 p.p.m. (J = 17.0, 10.0 c.p.s., 1 proton) resulting from the interior vinyl proton in a conjugated double bond pair confirmed the trans assignment about the Δ^5 bond, since the cis configuration gave rise to an equivalent quadruplet at 6.73 p.p.m. Presence in the infrared spectrum of a 965-$cm.^{-1}$ maximum associated with trans out-of-plane hydrogen deformation and lack of a maximum around 690 $cm.^{-1}$, similarly associated with the cis configuration, confirmed the trans configuration.

The ultraviolet spectrum in heptane agreed with that reported by O'Conner and Goldblatt (*8*).

Component 11, identified as terpinolene, agreed at 20 maxima in wavenumber and relative intensity with the infrared spectrum of terpinolene reported by Wrolstad and Jennings (*17*). Commercial terpinolene was separated by GLC on Carbowax 4000 and proton resonance and infrared spectra of a component having the proper R_α for terpinolene were run. The infrared spectrum was identical with terpinolene (*17*) and with component 11. The proton resonance spectrum of the purified terpinolene also matched perfectly that of component 11, resonance signals appearing at δ (p.p.m.) = 1.61 (singlet, 9 protons); 2.10 (multiplet, wide, 4 protons); 2.64 (singlet, broad, 2 protons); and 5.29 (multiplet, broad, 1 proton).

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