Constituents of the Cotton Bud

Compounds Attractive to the Boll Weevil

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Essential oil from buds of the cotton plant (*Gossypium hirsutum* L. CV Deltapine Smoothleaf) was fractionated by several procedures. Mixtures and single compounds thus obtained were bioassayed for plant attractancy to the boll weevil, *Anthonomus grandis* Boheman. Compounds present in cotton and active as components of the plant attractant complex are (−)-α-pinene, (−)-limonene, (−)-β-caryophyllene, (+)-β-bisabolol (a sesquiterpene alcohol reported only in cotton thus far), caryophyllene oxide, and two as yet unidentified sesquiterpenoids. The boll weevil-cotton plant attractant complex thus appears to be a mixture of many compounds. An aqueous mixture of 10, 3, 100, 100, and 30 p.p.b., respectively, of commercial (+)-α-pinene and (+)-limonene, cotton bud oil (−)-β-caryophyllene and (+)-β-bisabolol, and synthetic caryophyllene oxide from cotton (−)-β-caryophyllene, is 124% as attractive in laboratory assays as the best available attractant mixture from cotton buds. This synthetic mixture remains highly attractive to boll weevils at concentrations 30-fold higher and 10-fold lower.

Within the past several years, our laboratory has systematically isolated and identified compounds from the essential oil of the flower bud (square) of the cotton plant, *Gossypium hirsutum* L. CV Deltapine Smoothleaf (Minyard *et al.*, 1965, 1966, 1967, 1968). The purpose of these investigations has been to gain fundamental knowledge about the plant volatiles and to identify any component(s) that are attractive to the major cotton pest, the boll weevil, *Anthonomus grandis* Boheman. These past reports have been confined to the identification of single compounds or groups of related compounds. This paper describes attractancy bioassays of these isolates, several of which are active, and a variety of direct isolation attempts that demonstrate the presence in the oil of at least two more compounds attractive to the boll weevil.

EXPERIMENTAL

Assay Technique. All bioassays were performed according to the method of Hardee *et al.* (1966b). Samples were normally aqueous solutions or suspensions with concentrations at or close to plant levels (1X strength). When exact analytical data were unavailable, plant concentrations were estimated and samples were assayed at that and bracketing levels (0.1X), 0.5X, and 2X). In some instances half-log dilution series (0.1X, 0.3X, 1X, 3X) of the type used to evaluate flavor were prepared. Occasionally, higher nominal concentrations were evaluated to compensate for losses of active material during separation sequences.

Standards used in the bioassays varied during the several months of experimentation, but were principally *A*, Mexico square extract (Hardee *et al.*, 1966b); *B*, active cottonseed oil (Daum *et al.*, 1967); or *C*, State College square extract. Standards *A* and *C* were hot water extracts of macerated squares prepared from cotton grown in Mexico and at State College, Miss., respectively. Activities of subfractions were compared both with the activity of the composite fraction from which they were obtained and with the most active primary standard then available.

Assay results are reported here as T/S, the ratio of net test to net standard values. The net value used to compute this ratio was the difference between the number of weevils responding to the test sample (T) or standard (S) and the water check. Typically, 50 five-day-old laboratory reared boll weevils (Gast, 1966) preconditioned for optimum response (Hardee *et al.*, 1966a) were used per 15-minute test, and four to six replications of each test were performed with new sample and weevils. The net standard (S) used to compute T/S was the highest value obtained that day with either the water extracted standard, the cottonseed oil, or the fraction from which the test sample had been isolated. This mode of calculation normally limited the T/S value of 1.0 or less.

A typical response to the standard consisted of the movement of about 35 weevils to the standard or control (water) containers, with 6 going to the water and 29 to the standard. Such a response compares favorably with that obtained with other insects but cannot really be compared with moth responses in which only wing movement or body curling is involved. With boll weevils, we are limited to a quantitative measurement of attractancy such as $T\,S$ because we have no reliable behavioral response to use for assay.

The volatility of the attractive materials was assessed by steam distilling portions of the Mexican extract *A* for 1, 2, and 3 hours and assaying the distillate as well as dichloromethane extracts of the distillate.

Major Fractionation of Square Oil. Cotton essential oil isolated as described by Minyard *et al.* (1965, 1967) was fractionated by column chromatography on silica gel, the Girard T procedure, solvent partitioning, and vacuum distillation. Small eluate fractions were taken at the interface between the β and γ solvents in one column separation after insect attractant activity had been shown to be eluted there. Details of this separation scheme, outlined in Figure 1, have been reported (Minyard, 1967).

In a simplified version of this procedure, hydrocarbons were removed from the crude oil on a Carbowax 20M coated silica gel column with pentane as a solvent. The polar constituents were eluted from the top of the extruded adsorbant with methanol or other solvents to give a hydrocarbon free oil (HFO) for assay or subfractionation. HFO yielded a carbonyl (T_2)

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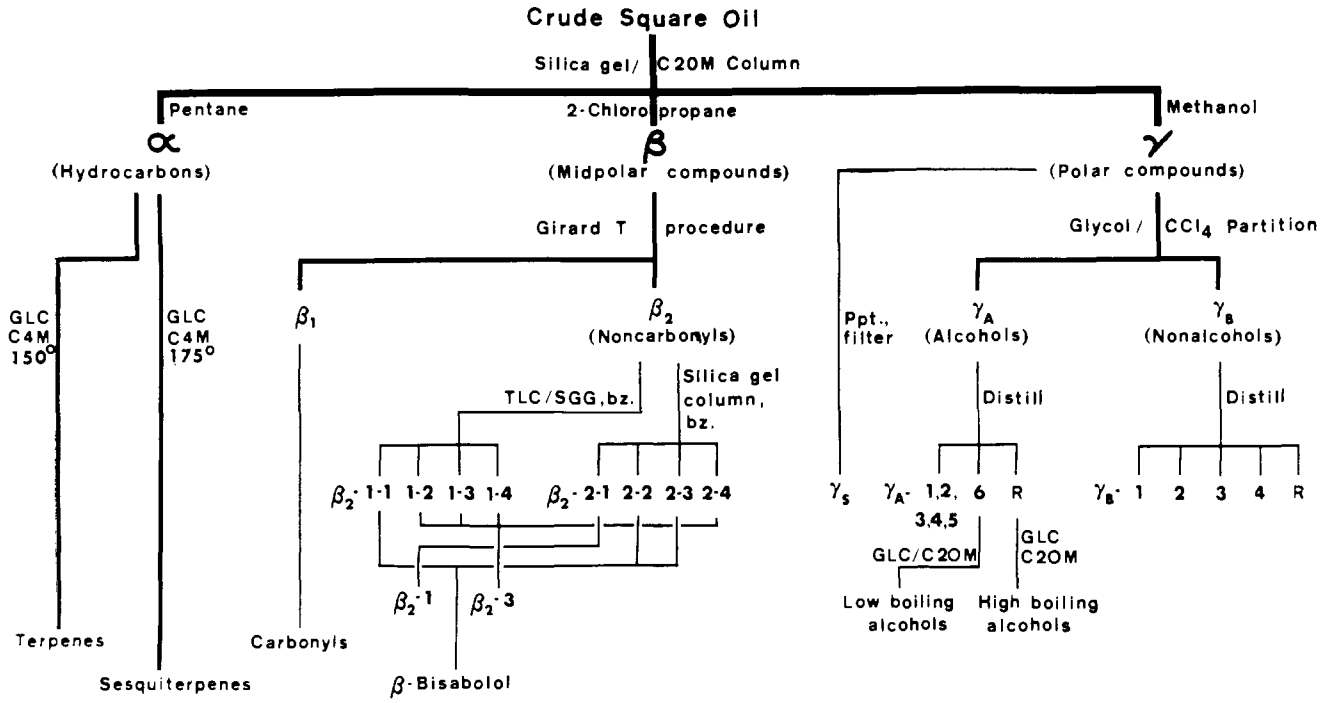

Figure 1. Scheme for separation of crude cotton square oil into groups

and polar noncarbonyl (T_1) fraction when treated by the Girard T procedure (Minyard *et al.*, 1967).

All fractions thus obtained were assayed individually and in combinations at concentrations ranging from 0.1X to 10X plant strength.

Thin Layer Chromatographic Separations. Two thin-layer chromatographic (TLC) systems were used for separations of the polar cotton volatiles; A, unactivated silica gel G, chloroform/ether, 9/1; and B, activated silica gel G, pentane/-methylal, 9/1.

Figure 2 shows the separation of 100 μl. of the attractive fraction T_1 on system *A*. Visualization of one plate by iodine vapor, ultraviolet radiation, and concentrated sulfuric acid plus heat guided the removal for methanol elution and assay of 10 bands from a similar untreated plate.

Figure 3, *a,b,c* outlines the sequential separation on systems

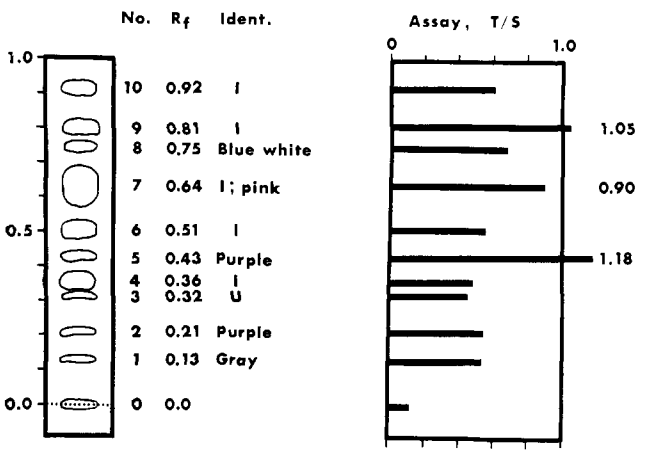

Figure 2. TLC Separation (system A) of fraction T_1 from cotton square oil and results of attractancy assays to boll weevils

Band identification: I, visible after iodine exposure ; U, visible under UV light. Colors are those visible after spraying with concentrated sulfuric acid.

A, B, and again on B of 1 ml. of HFO. Eluates of various bands removed at intermediate stages of separation were assayed at 2X plant level on the first plate and at 3X on the two succeeding plates. Bands were located for scraping and elution by edge-streaking with vanillin/sulfuric acid reagent (Stahl *et al.*, 1965) and allowing the plate to stand 1 hour.

Gas Chromatographic Separations of Active Fractions. All active fractions obtained by column or TLC separations were monitored by gas-liquid chromatography (GLC) on a 12-foot Carbowax 20M column at 175° C. Peaks of interest were trapped into pentane or other solvent as they emerged from the chromatograph.

Peaks 14 and 16 (Figure 4*a*), with Kováts indices of 2065 and 2200 at 175° C., appeared repeatedly in GLC separations of active fractions obtained by column and thin-layer chromatography of plant oil. Both peaks were trapped separately from the gas chromatograph and assayed for insect attractancy at several concentrations, including that which had previously given maximum response from the entire fraction.

TLC on system B of peak 14 showed two principal components, 14a, R_f 0.4, and 14b, R_f 0.8, were present along with several minor compounds. The two major materials were separated by preparative TLC for assay, and their NMR, infrared, and mass spectra were determined. Both compounds were rechromatographed by GLC under conditions identical to those of the original trapping separations to determine if they were degraded by the operating conditions used. Subsequent investigations of GLC separations showed that 14*a* and 14*b* could be separated completely at 160° C. on a 20% Carbowax 20M column (Figure 4*b*). Peaks 14 a and 14b had Kováts indices of 2022 and 2000, respectively, on this column.

Mass spectra of 14a and 14b were obtained on Perkin-Elmer Model 270 and Hitachi RMU-6D gas chromatograph-mass spectrometers. Capillary (200 ft.) and support coated open tubular (50 ft.) Carbowax 20M columns were used for separations ahead of the spectrometer inlets.

Peak 16 (Figure 4, *a,b*) was shown to be a single compound

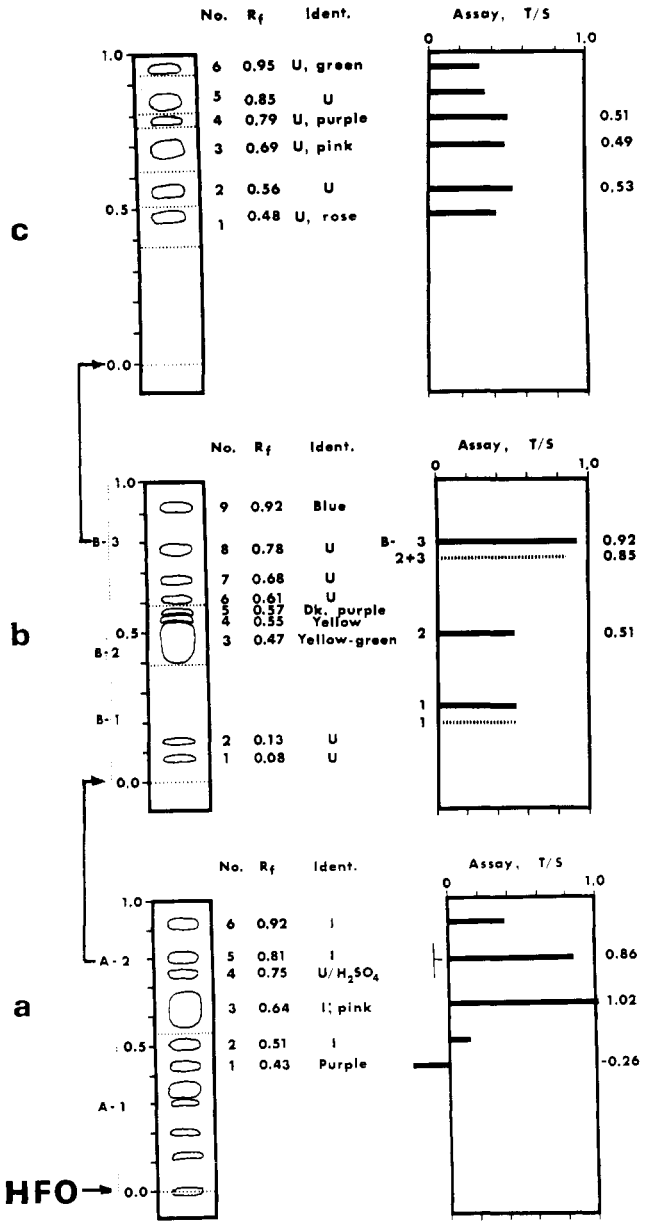

Figure 3. Sequential TLC separations of hydrocarbon free oil and results of attractancy assays to boll weevils

(*a*) system A, (*b*) system B, (*c*) system B. Band identification: I, visible after iodine exposure; U, visible under UV radiation. Colors are those visible 1 hour after spraying with vanillin-sulfuric acid reagent

by GLC and TLC separations. This compound was trapped for assay and spectral data were obtained in a fashion similar to 14a and 14b. In a like manner its thermal stability under the GLC separation conditions was determined.

RESULTS AND DISCUSSION

The essential oils obtained by steam distillation of leaves, stems, or flower buds of the cotton plant, as well as cottonseed oil (Daum *et al.*, 1967) are attractive to the boll weevil. Attractiveness of all fractions assayed appeared to increase with concentration to a maximum and reach a plateau at or about plant concentration of the test substance. Only modest increases or even slight decreases in attractiveness accompanied further increases in concentration of the test fraction in the aqueous medium.

The attractive compounds in hot water extracts of cotton

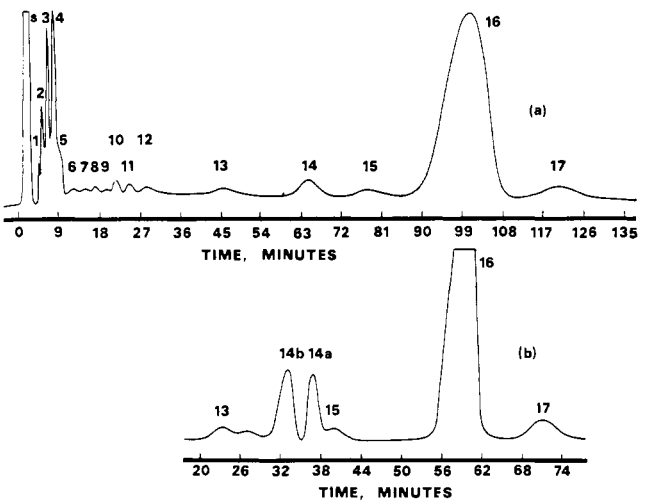

Figure 4. Gas chromatographic separations of β-γ interface fractions of cotton square oil

(*a*) Barber-Colman 5000 GC, FID, 12-foot × 5/16 in., 20% Carbowax 20M column, 175° C, N_2 carrier, 70 ml/min. (*b*) Aerograph 95-P-3 GC, FID, 6-foot × 1/8 in. 20% Carbowax 20M column, 160° C, He carrier, 68 ml/min.

buds are completely volatilized by steam in one hour, although only 30% of the activity of the aqueous distillate can be demonstrated in dichloromethane extracts of the distillate. This may reflect inefficient extraction, masking by the solvent, or both. Masking due to traces of dichloromethane left after reduced pressure removal may well be important in view of similar observations that were made during isolation of the sex attractant for the American cockroach, *Periplaneta americana* (L.) (Jacobson and Smalls, 1966). Furthermore, crude bud oil that contained traces of dichloromethane was not fully active until that solvent was removed by column chromatography in the production of HFO. Assays of crude oil were improved by more than two-fold by removal of the hydrocarbons and residual dichloromethane (Table I).

Table I shows the assay results for the major fractions isolated from crude square oil (Figure 1). Consistent activity

Table I. Attractiveness to the Boll Weevil of Cotton Square Oil Fractions

Fraction[a,b]		Attractiveness, T/S[c]
Crude square oil (1.5X)		0.50
Hydrocarbon free oil (0.5X)		0.92
(HFO)	(1.0X)	1.15
	(1.5X)	1.04
	(2.0X)	1.21[d]
	(2.5X)	0.85
T_1	(1X)	1.07
T_2	(1X)	0.38
α	(1X)	−0.04
β_1	(1X)	0.31
β_2	(1X)	0.78
	(2X)	0.59
γ	(1X)	1.06
γ_A	(1X)	0.83
γ_B	(1X)	0.33

[a] See Figure 1 for explanations of fraction designations.
[b] Concentration of fraction in water compared with that occurring naturally in cotton plant bud is given in parentheses.
[c] Ratio of net number (insects to test sample minus insects to water control) of insects responding to test fraction T and to standard S. Hardee *et al.* (1966a) give more details of the assay.
[d] Average of four experiments prepared separately and at different times from the other four assay levels of HFO.

Table II. Attractiveness to the Boll Weevil of Compounds Found in Cotton Square Oil

Test Substance[a]	Concentration, P.P.M.[b]	Attractiveness, T/S[c]
(+)-β-Bisabolol	1	0.38
	3	0.46
	10	0.54
	100	0.37
	1000	0.12
β-Caryophyllene oxide[d]	1	0.41
	3	1.03
	10	0.62
	30	0.54
	100	0.11
Compound 14a	1	0.37
	2	0.64
	6	0.44
	10	0.30
	20	0.08
Compound 14b	1	0.47
	2	0.43
	6	0.58
	10	0.49
	20	0.23
	100	0.14
(−)-β-Caryophyllene	0.1	0.17
	1	0.36
	10	0.70
	100	0.36
(+)-Limonene[e]	0.3	0.86
	1	0.57
	3	0.66
	10	0.66
	30	0.60
	100	0.51
(+)-α-Pinene[e]	1	1.13
	3	0.54
	10	0.46
	30	0.42
	100	0.35

[a] See text and Figure 1 for designations and identities of test substances.
[b] Assayed as aqueous solutions or suspensions.
[c] Ratio of net number (insects to test sample minus insects to water control) of insects responding to test fraction T and to standard S. Hardee *et al.* (1966a) give more details of the assay. Values given are averages of two to five different preparations.
[d] Present in cotton square oil, but assays conducted on synthetic compound prepared (Nigam and Levi, 1965) from (−)-β-caryophyllene purified by GLC from cotton square oil.
[e] Assays on commercial, unpurified material; D-α-pinene, K & K Laboratories; *d*-limonene, MC & B. (−) Isomers present in cotton.

was localized in the polar compound fractions HFO, T_2, β, and γ, particularly at or near the solvent interface between the β and γ column fractions. Consistent with this was the finding that methanol, the γ fraction solvent, was much better for eluting the active compounds from silica gel column or TLC adsorbent than was acetone, methylal, diethyl ether, dichloromethane, or benzene.

Although the α fraction was unattractive as a mixture (Table I), several individual hydrocarbons in it are very active. α-Pinene, limonene, and (−)-β-caryophyllene were moderately to quite attractive (Table II) at levels approximately equal to their natural abundance in cotton square oil.

None of the attractants appear to be aldehydes or ketones. The T_1 fraction, being HFO less the carbonyl compounds, was active, but the carbonyl compounds in T_2 showed little attractiveness.

TLC separations and assays on T_1 (Figure 2) and HFO (Figure 3), as well as assays of GLC peaks 14a, 14b, and 16 (Figure 4*b*, Table II) clearly show that several oxygenated compounds contribute to the insect attractancy of the polar oil fractions. Correlations and identities of specific oxygenated attractant compounds isolated are as follows.

(+)-β-Bisabolol. This sesquiterpene alcohol (Peak 16, Figure 4) has been found only in the cotton plant, and its structure has been elucidated by Minyard *et al.* (1968). It corresponds to band 7, Figure 2 and band 3, Figure 3*a*. It is the most abundant polar compound (5.6% by weight) in cotton bud essential oil and is one of the major components of the boll weevil-cotton plant attractant complex (Table II). Its activity as an attractant is enhanced by addition of caryophyllene oxide.

Caryophyllene Oxide. Peak 14b, Figure 4*b*, contains two compounds, one of which corresponds to band 4 in Figure 3*b*. After isolation by preparative TLC on silica gel G, pentane/methylal 95/5, this compound was identified as β-caryophyllene oxide by comparison of its infrared and NMR spectra with standard spectra (Freemen, 1968; Plíva *et al.*, 1960). Its GLC and TCL behavior, mass, infrared, and NMR spectra were identical with those of the oxide synthesized from (−)-β-caryophyllene which was isolated from cotton oil by GLC (Minyard *et al.*, 1966; Nigam and Levi, 1965). Further, the synthetic caryophyllene oxide, although a mixture of geometrical isomers, is attractive to boll weevils (Table II).

Compound 14b. A second major compound in peak 14b, Figure 4*b*, corresponds to band 9, Figure 2, and band 8, Figure 3*b*. This material was one of the two principal compounds separated from GLC peak 14 by TLC, the other being compound 14a. Compound 14b has an *Rf* of 0.8 in System B and was lilac-purple upon spraying with vanillin-sulfuric acid and heating. This sesquiterpenoid ($C_{15}H_{24}O$) is unidentified as yet, but appears to be an ether, perhaps an epoxide like caryophyllene oxide. It is attractive by itself, but adds little to the attractiveness of β-bisabolol (Table II).

Compound 14a. Peak 14a in Figure 4b corresponds to band 5, Figure 2, and presumably to band 1, Figure 3*a*. Improper assay concentration or repellent materials in assay fraction A-1 must account for its negative assay shown in Figure 3*a*. This compound appears to be a sesquiterpene alcohol (*m/e* 222, parent ion) which is unidentified as yet. It is extremely labile to heat and decomposes readily on our GLC columns, giving a variety of sesquiterpene hydrocarbons. This behavior has made purification very difficult, and suggests that the alcohol may be tertiary. Its TLC behavior and blue-purple color with vanillin-sulfuric acid spray are very similar to β-bisabolol, another tertiary alcohol.

Besides these major boll weevil plant attractant compounds, several more may be present. This multiplicity of compounds in the attractant complex is similar to the findings of Silverstein *et al.* (1966) concerning the aggregating pheromone of the beetle *Ips confusus* (LeConte). It is also analogous to our findings concerning the boll weevil feeding stimulant complex in the cotton plant (Hedin *et al.*, 1966; Struck *et al.*, 1968a,b; Temple *et al.*, 1968), as well as to most findings in mammalian food flavor and aroma studies. For the boll weevil, and perhaps for other insects as well, it appears likely that responses to plants as food sources are evoked by multicomponent mixtures rather than by single compounds. Ten or more compounds ultimately may be implicated as significant contributors to the cotton plant-boll weevil attractant aroma complex.

Despite this, the results in Table III clearly demonstrate that we can equal or exceed the attractiveness of our best standards with a mixture of (+)-α-pinene, (+)-limonene, (−)-

Table III. Attractiveness to the Boll Weevil of Mixtures of Compounds Found in Cotton Square Oil

Test Substances[a]	Concentration, P.P.M.[b]	Attractiveness, T/S[c]
(+)-β-Bisabolol, Caryophyllene oxide	10, 1	0.60
	10, 10	0.80
	100, 10	0.54
	100, 100	0.34
(+)-β-Bisabolol, Caryophyllene oxide, limonene, (+)-α-pinene	10, 3, 3, 1	0.89
(+)-β-Bisabolol, (−)-β-caryophyllene, Caryophyllene oxide, limonene, (+)-α-pinene	10, 10, 3, 0.3, 1 (1X)	0.34
	(0.3X)	0.98
	(0.1X)	1.20
	(0.01X)	1.24
	(0.001X)	0.95

[a] See text and Figure 1 for designations and identities. Substances tested from cotton oil except for α-pinene and limonene and synthetic β-caryophyllene oxide obtained from cotton (−)-β-caryophyllene. See footnotes *e*, *f*, Table II.
[b] Assayed as aqueous solutions or suspensions. Concentrations listed in same order as test compounds. Figures in parantheses are concentrations of entire test solution relative to 1X level.
[c] See footnote *c*, Table II.

β-caryophyllene, caryophyllene oxide synthesized from cotton caryophyllene, and (+)-β-bisabolol. The maximum T/S value (1.24) was obtained at a concentration 100-fold below the optimum for each component alone. Other mixtures of two and four components (Table III) also were normally more active than any one of the individual compounds was at the same concentration. Although cotton pinene and limonene were levorotatory isomers, the commercial dextrorotatory materials are also attractive; this anomaly is under further study.

All compounds in these test mixtures except β-bisabolol are commercially available or can be synthesized from commercial materials. This has obviously important implications in the practical use of such an attractant formulation. A second important property of the five-component mixture is the broad concentration range over which it is highly attractive. This contrasts with the behavior of the individual components (Table III), with the exception of limonene. Employment of the mixture as a lure in a trap or other device would be facilitated by using initial concentrations on the high side of the optimum. As the lure evaporated, its attractiveness would actually improve for a time before falling back to its original level of potency.

Identification of 14a, 14b, or other natural attractants in square oil, and their addition to the five compounds would probably increase the attractiveness of the mixture somewhat. Nevertheless, in view of the nonadditivity of attractiveness of the five compounds already known, it is questionable whether any dramatic increase in activity would be observed. The formulation of the five-component attractant thus supports the hypothesis that the plant odor profile attracts the boll weevil, and simultaneously represents a reasonably practical duplication of that attractant complex. Synthetic routes to β-bisabolol are being explored, and field tests of the attractant mixture are planned.

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