Quantitation of the Major Cuticular Components from Green Leaf of Different Tobacco Types

Ray. F. Severson,* Richard F. Arrendale, Orestes T. Chortyk, Albert W. Johnson, D. Michael Jackson, G. Richard Gwynn, James F. Chaplin, and Michael G. Stephenson

Surface chemicals of green tobacco leaf were obtained by dipping the leaf into methylene chloride. Leaf wash procedures were developed and evaluated for both small and large numbers of samples. Cuticular constituents containing alcohol functions were derivatized with trimethylsilyl reagents and analyzed by glass capillary gas chromatography on SE-54 columns. Quantitative data were obtained for the major cuticular diterpenoids α- and β-4,8,13-duvatriene-1-ols, α- and β-4,8,13-duvatriene-1,3-diols, *cis*-abienol, labda-13-ene-8,15-diol, and docosanol, hydrocarbons, and sucrose esters. The application of this method allowed the characterization of the cuticular constituents of numerous tobacco varieties and introductions. The relationships between cuticular composition and insect resistance are presented and discussed.

The green leaves of commercial tobacco varieties are covered with a gummy exudate, and the composition of these cuticular waxes may be of significant importance. Dietrich and Demole (1977) and Enzell (1977) have reported that the degradation of the cuticular diterpenoids of green tobacco, during curing and aging, results in the production of numerous volatile terpenes, which may be important tobacco flavor components. Reid (1975) reported that a diterpene fraction, isolated from green leaf cuticular waxes, contained precursors of aroma constituents of commercial tobacco. Cutler et al. (1977) have found that the cuticular diterpenes of green tobaccos have plant growth inhibiting properties. The levels of specific cuticular components are believed to be responsible for the observed resistances of some types of tobacco to green peach aphids [*Myzus persicae* (Sulzer)], tobacco budworm [*Heliothis virescens* (F.)], and tobacco hornworm [*Maduca sexta* (L.)] (Johnson and Severson, 1982, 1984; Severson et al., 1983). Polar cuticular components were found to inhibit the germination of conidia of blue mold [*Peronospora tabacina* (Adam)] (Shepherd and Mandryk, 1962). These observations indicate that green leaf cuticular components may play an important role in the development of pest-resistant high-flavor tobaccos.

Previous work has indicated that the majority of cuticular waxes are diterpenes and C_{25}–C_{36} normal and branched chain aliphatic hydrocarbons (Figure 1) (Severson et al., 1983; Chang and Grunwald, 1976; Reid, 1974). These components accounted for about 95% of the gas chromatographic volatile material of the surface components of young flue-cured tobaccos (Severson et al., 1983).

Depending on the genetic background of a particular tobacco, it may produce duvane and/or labdane diterpenes (Reid, 1974; Colledge et al., 1975). The duvane-producing tobaccos yield a mixture of α- and β-4,8,13-duvatriene-1,3-diols (α- and β-I) (Roberts and Rowland, 1962) and α- and β-4,8,13-duvatriene-1-ols (α- and β-II) (Wahlberg et al., 1981) (Figure 1). The α- and β-I's are the major duvanes found on the young leaves of commercial American tobaccos. Generally, they occur in at least 100 times the concentration of the α- and β-II. I and II readily undergo biodegradation to form the numerous duvanes, which have been isolated from green and cured tobaccos (Enzell and Wahlberg, 1980). The major diterpenes found on the young leaves of most labdane-producing tobaccos are (12*Z*)-labda-12,14-diene-8α-ol (*cis*-abienol, III) and (13*E*)-labda-13-ene-8α,15-diol (IV) (Reid, 1974). These diterpenes have been postulated to be the precursors of the numerous minor labdanoic components identified in the cuticular waxes of labdane-producing varieties (Enzell and Wahlberg, 1980; Wahlberg et al., 1982).

During our investigation of the cuticular waxes of a budworm-resistant tobacco, TI-165 (Johnson and Chaplin, 1982), we isolated a series of sucrose esters (Figure 1) in which each glucose moiety was esterified with a mixture of four molecules of C_2–C_7 fatty acids (Severson et al., 1981a). These sucrose esters are the green leaf precursors of [6-*O*-acetyl-2,3,4-tri-*O*-[(+)-3-methylvaleryl]-β-D-glucopyranoside] (Schumacher, 1970) and other tetraacylglucopyranosides (Rivers, 1981) isolated from cured Turkish tobacco. They are the precursors of three important Turkish tobacco smoke flavor components, isobutyric, isovaleric, and β-methylvaleric acids.

A series of free fatty alcohols (C_{16}–C_{30}) and high molecular weight wax esters have been identified as minor components in the cuticular waxes of green tobaccos (Severson et al., 1981b). The major free alcohol is docosanol, and the wax esters consisted of alcohols, identical in compostion to those in the free alcohol fractions, bound to C_{12}–C_{30} saturated fatty acids.

The procedures reported for analyses of cuticular waxes were not acceptable for the analysis of the broad spectrum of components found on tobacco germ plasm. The analytical TLC method of Reid (1974) produced only relative estimates of diterpene levels, while the GC methods of Chang and Grunwald (1976) and Court (1982) yielded only quantitative data on total I. Consequently, we developed procedures to quantitatively extract and analyze the major cuticular constituents, duvanes, labdanes, hydrocarbons, docosanol, and sucrose esters.

EXPERIMENTAL SECTION

Materials. The tobaccos used in this study were grown under conditions normally used for the production of flue-cured tobacco at the Clemson University Pee Dee Research and Education Center, Fluorence, SC, the Tobacco Research Station, Oxford, NC, and the University of Georgia Coastal Plains Experiment Station, Tifton, GA.

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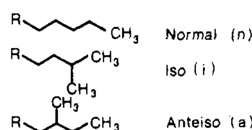
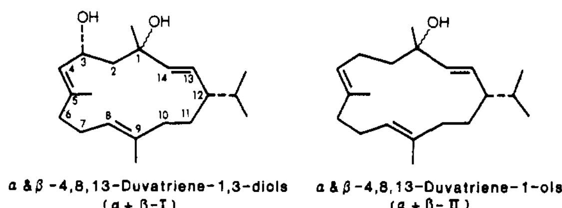
1. Hydrocarbons (C_{25}-C_{36})

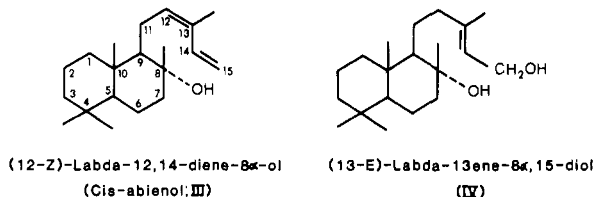


2. Diterpenes

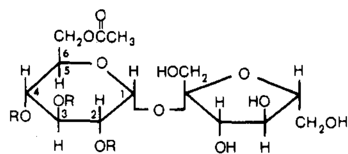
a) Duvanes (Cembranes)



b) Labdanoids



3. Sucrose Esters



(6-O-acetyl-2,3,4-tri-O-acyl)-α-D-Glycopyranosyl-α-D-Fructofuranoside

R = Acyl = β-methylvaleryl, isovaleryl, isobutyryl, methylcaproyl, butyryl, valeryl, caproyl, propionyl

Figure 1. Major cuticular components of green tobacco.

Methylene chloride (CH_2Cl_2) was distilled in glass (Burdick and Jackson, Richmond, CA) and was used as received. Dimethylformamide (DMF) and *N,O*-bis(trimethyl)trifluoroacetamide (BSTFA) were silylation grade (Pierce Chemical Co., Rockford, IL). The tobacco diterpenes I, II, III, and IV and sucrose esters were isolated from cuticular waxes of green tobaccos as previously described (Severson et al., 1981a,b, 1982b). Heptadecanol, docosanol, and *n*-hydrocarbons (Analabs, North Haven, CT) were used as received.

Extraction of Cuticular Components. *Method A.* Five fully extended bud leaves (1 leaf/plant, 15–18 cm in length) were collected, placed on a tared 20-cm aluminum pie pan and weighed. Methylene chloride was poured into two 400-mL beakers (200 mL in each). Each of the five leaves was dipped 4 times into the first beaker, remaining submerged in the solvent for about 2 s/dip. After the fourth dip, the excess CH_2Cl_2 was permitted to run off and the procedure was repeated in the second beaker. The washings were filtered through folded filter paper (containing about 50 g of anhydrous Na_2SO_4) into a 473-mL (16-oz) amber glass bottle. The beakers and filter paper were washed with 100 mL of CH_2Cl_2, and the bottle was capped (Teflon liner) and placed in dry ice for transport to the laboratory. Beakers and funnel were rinsed with acetone and wiped dry with Kimwipe tissues before extraction of next sample. The washed leaves were placed between two sheets of Hewlett-Packard thermal writing recorder paper and an outline of each leaf was traced. The leaf outlines were cut out, and leaf areas were determined by using a LI-COR Model LI-3000 portable area meter. The samples were stored in the dark at 0 °C until preparation for analysis. Even after 6 months of storage, no changes in sample composition were observed. After warming to room temperature, samples were transferred to a 1000-mL round-bottom flask, and the solvent volume was reduced to about 25 mL on a rotoevaporator (40 °C and about 100 mmHg). The sample was transferred to a 50-mL volumetric flask, mixed, and poured into a 60-mL (2-oz) amber bottle. Samples were stored as above until analyzed.

Method B. Methylene chloride (170 mL) was poured into a 240-mL (8-oz) widemouthed bottle. Five leaves, as above, were individually dipped 8 times and the excess solvent was permitted to run off. The bottle was capped with a Teflon-lined cap. Samples were cooled, transferred to the laboratory, and stored as above. After warming, an appropriate volume of internal standard (ISTD) solution (2.0 mg of heptadecanol/mL of benzene) was added (1.0 mL for tobaccos with normal cuticular chemical levels and 0.5 mL for tobaccos with low levels of diterpenes and sucrose esters). The bottle was capped and shaken, and the contents were filtered through a folded filter paper containing 25 mL of anhydrous Na_2SO_4 into a clean widemouthed bottle. The bottle and filter paper were rinsed with 50 mL of CH_2Cl_2. The bottle was capped as above, shaken gently, and stored for future analysis. Green weight and areas were obtained as above.

Glass Capillary Gas Chromatographic (GC-2) Analyses. A portion of the cuticular washes (equivalent to 0.3–0.6 g green weight or 10–20 cm^2 of leaf area) was transferred to a tapered test tube. For method A, heptadecanol (50 μg) was added to the test tube. The solvent was removed on a nitrogen blow-down apparatus at 40 °C. A 50-μL portion of 1:1 BSTFA–DMF was added to the residue, and the tapered test tube was capped with a Teflon-lined cap and heated for 30 min at 76 °C. After cooling a 50-μL portion of a 1:1 mixture of BSA–pyridine was added to prevent precipitation of the hydrocarbons. After mixing, the derivatized sample was transferred to a microautosampler vial and placed into an autosampler.

The samples were analyzed on a Hewlett-Packard 5840 reporting gas chromatograph [equipped with a 7672A auto sampler and modified for GC-2 as described by Severson et al. (1982a)] by using a 0.3 mm i.d. × 25 m thin film (about 0.1 μm) SE-54 fused silica glass WCOT column (temperature program 150–280 °C at 4 °C/min, 20 min hold at 280 °C, 35 cm/s H_2 flow rate, 100 cm^3/min H_2 split, injection port temperature 250 °C, and flame ionization detector temperature 300 °C). Columns were prepared from Hewlett-Packard fused silica glass tubing according to the procedure of Arrendale et al. (1983).

RESULTS AND DISCUSSION

Cuticular Wax Extraction. Chang and Grunwald (1976), Reid (1974), and Court (1982) employed chloroform to extract the cuticular waxes of green tobacco. However, data on the quantitative efficiency of the extraction procedures were not given. We found that the ethanol preservative in chloroform was difficult to remove and degradation of the silylation reagents occurred. The efficiency of other solvents for cuticular wax extraction were investigated. The polar diterpenes and the sucrose esters had low solubilities in hydrocarbon solvents, such as hexane. The volatile, water-miscible solvents, such as acetone and methyl alcohol, rapidly dehydrated the leaf

Table I. Efficiency of Methylene Chloride Cuticular Wax Extraction Procedures

tobacco	wash no.	% recovered[a]						
		α-I	β-I	III	IV	docosanol	hydrocarbons	sucrose esters
				Method A[b]				
TI-165[c]	1	98.7	98.7	–[d]	–	96.4	98.8	99.4
	2	1.3	1.3	–	–	3.6	1.2	0.6
	3	–	–	–	–	–	–	–
NC 2326[c]	1	97.7	97.6	–	–	97.8	98.6	–
	2	2.3	2.4	–	–	2.2	1.4	–
	3	–	–	–	–	–	–	–
NFT[c]	1	–	–	98.5	96.8	97.1	97.3	99.3
	2	–	–	1.5	3.2	2.9	2.7	0.7
	3	–	–	–	–	–	–	–
				Method B[e]				
TI-165[f]	1	97.0	97.1	–	–	97.0	95.4	98.1
	2	2.9	2.9	–	–	3.0	3.5	1.9
	3	0.1	–	–	–	–	1.6	–
NC 2326[c]	1	96.9	96.9	–	–	96.8	96.4	–
	2	3.0	3.0	–	–	3.2	3.1	–
	3	0.1	0.1	–	–	–	0.4	–
NFT[f]	1	–	–	98.7	97.7	96.3	95.9	98.8
	2	–	–	1.3	2.3	3.7	3.6	1.2
	3	–	–	–	–	–	0.4	–

[a] Based on total amount of material recovered from five 13–18-cm leaves, 6 weeks after transplanting, Tifton, GA, 1981. [b] Two-beaker wash procedure. [c] Average of three determinations. [d] Absent or below detection limits. [e] Widemouthed bottle wash procedure. [f] One determination.

and extracted internal leaf components. The nonpolar cuticular components also had low solubility in these solvents. We found that methylene chloride very efficiently removed all the cuticular components being analyzed.

We first employed the method A wash procedure, where young leaves were sequentially dipped into two beakers. The efficiency of the wash procedure, for tobaccos with different levels of cuticular chemicals, is given in Table I. For the major components listed, 96+% of the cuticular components recovered were found in the first wash. Additional material was not removed by a third wash step. On the basis of total material recovered, the two washes resulted in quantitative extraction of the wide range of cuticular components.

For large numbers of samples, material costs and time required for sampling and workup made the method A extraction procedure undesirable. However, we found that acceptable data could be obtained by using a simplified widemouthed bottle wash procedure (method B). The extraction efficiency of this procedure is given in the bottom portion of Table I. For the tobaccos tested, 95+% of the major cuticular components were obtained. Method B was utilized when analyses of a large number of samples were required.

Separation and Quantitation of Cuticular Components. It was found that, after conversion of the diterpene alcohols and sucrose esters to trimethylsilyl (Me_3Si) derivatives, the major cuticular components could be readily separated and quantitated by GC-2. Without derivatization, considerable degradation of the diterpene alcohols occurred, and the nonvolatile sucrose esters were not detected. The GC-2 chromatograms of the derivatized cuticular constituents of a Turkish tobacco (Samsun 15) and a typical flue-cured tobacco (NC 2326) are shown in Figures 2 and 3, respectively. These profiles illustrate the differences in cuticular compositions as well as the separation of components possible on an SE-54 column. An open retention window permitted the use of heptadecanol as an internal standard (ISTD) for quantitation. Peak integrity was confirmed by GC–mass spectrometry analyses. The numerous peaks eluting between α- and β-II and

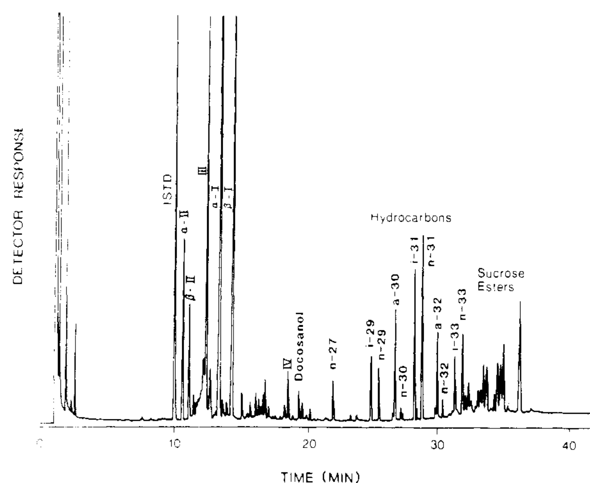


Figure 2. Gas chromatogram of the silylated cuticular chemicals of a Turkish tobacco (Samsun 15).

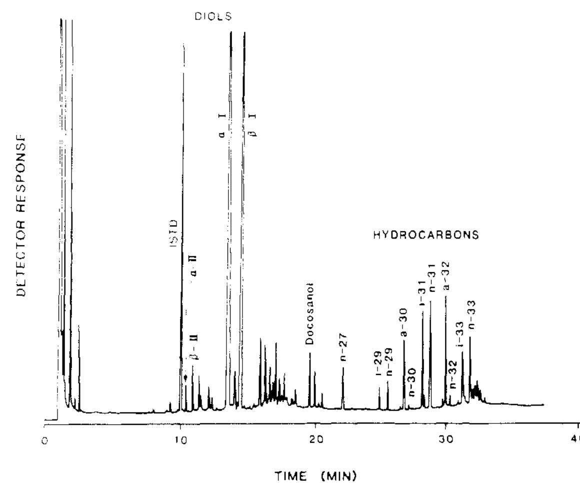


Figure 3. Gas chromatogram of the silylated cuticular chemicals of a U.S. flue-cured tobacco (NC 2326).

α- and β-I and between α- and β-I and docosanol (Figure 2) produced fragmentation patterns characteristic of the

Table II. Reproducibility of GC Retention and Response Data[a]

compound	rel retention time[b]	rel % error[c]	rel response (K)[b]	rel % error[c]
heptadecanol (ISTD)	1.00		1.00	
α-II[d]	1.06	0.01	0.98	3.05
β-II[d]	1.11	0.01		
III	1.24	0.01	0.89	6.66
α-I	1.34	0.01	1.06	2.67
β-I	1.44	0.01	1.04	4.51
IV	1.87	0.01	0.96	4.40
docosanol	1.95	0.01	0.86	2.85
C_{30} hydrocarbon	2.72	0.01	0.85	3.10
sucrose esters[d]	3.30–3.62	0.16	0.62	3.95

[a] Relative to heptadecanol internal standard (ISTD). [b] Average of four analyses; K = area of x/mg of x/(area of ISTD/mg of ISTD. [c] SD/mean. [d] Isolated as a mixture; relative response data obtained on mixture assuming identical response for each component.

duvane system. We assume these components to be hydroxyepoxy, hydroxyoxo, and trihydroxy degradation products of I and II (Dietrich and Demole, 1977; Enzell, 1977; Enzell and Wahlberg, 1980). Note the low levels of docosanol in the chromatograms (Figure 2 and 3). It is the only alcohol quantified in the total wax profile. The wax esters eluted after the hydrocarbons. Due to their low levels and low chromatographic responses, the wax esters were below detection limits, under the employed GC conditions.

Selection of proper conditions and reagents to derivatize the diterpene alcohols was critical. It was found that a 1:1 mixture of BSTFA and DMF, with heat treatment, completely derivatized all components. When cuticular component mixtures were treated with a 1:1 BSA–DMF silylation reagent and heated, III was not converted to its Me_3Si ether, and there was incomplete derivatization of the other diterpene alcohols. On the SE-54 column the mono-Me_3Si derivatives of I and IV eluted prior to their di-Me_3Si ethers. Sucrose esters and docosanol were completely derivatized by BSA–DMF. frequently, the hydrocarbons would precipitate on cooling from the BSTFA–DMF system. This precipitate did not interfere with the quantitation of the other more polar components. When hydrocarbon quantitation was desired, a quantity of 1:1 BSA–pyridine was added, after derivatization, to redissolve them.

Table III. Major Cuticular Chemical Levels of Various Tobacco Cultivars and Tobacco Introductions

type/tobacco	leaf levels, μg/cm² [b] α-II	β-II	α-I	β-I	III	IV	docosanol	total hydrocarbons[a]	sucrose esters
flue cured[c]									
NC 2326	0.16	0.32	32.2	11.0	–[d]	–	0.20	10.7	–
Speight G-28	0.11	0.34	32.5	10.9	–	–	0.23	13.3	–
Burley[c]									
KY 14	0.18	0.19	22.5	7.8	–	–	0.07	11.2	–
Maryland[c]									
Sweeney	0.64	0.17	27.9	8.6	–	–	0.22	13.9	–
dark fired[c]									
DF-300	0.45	0.91	58.3	20.0	–	–	0.23	14.1	–
cigar wrapper[c]									
Gen 911	0.49	0.35	34.8	12.5	9.6	0.14	0.10	14.5	16.6
Conn Bd1	0.44	0.84	55.9	19.1	–	–	0.30	15.0	–
Magnolia	0.18	0.21	30.3	11.1	14.3	0.81	0.11	11.6	–
cigar binder[c]	0.18	0.21	30.3	11.1	14.3	0.81	0.11	11.6	–
Havana K-2	0.23	0.28	23.9	9.2	2.6	1.93	0.06	13.6	13.1
Turkish[c]									
Samsun	2.06	1.24	27.7	10.4	8.3	0.17	0.20	8.2	34.8
Gavurkoy I	0.56	0.36	33.6	13.7	–	–	0.40	14.8	19.7
tobacco introductions[e]									
TI-170	0.53	0.54	54.2	20.8	–	–	0.48	13.8	10.2
TI-1112	–	–	1.1	0.4	–	–	0.02	10.5	–
TI-1028	0.03	–	0.16	0.05	–	–	0.45	14.2	–
PD-964[f]	0.02	0.02	1.0	0.4	0.78	2.90	0.10	8.3	3.2
TI-1223	17.2	6.27	0.8	0.8	10.1	0.58	0.51	9.5	7.2
TI-1341	31.9	2.05	11.2	17.3	–	–	0.25	13.0	11.1

[a] Total C_{27}–C_{33} hydrocarbon isomers. [b] Method B at onset of flower development. [c] Oxford, NC, 1981. [d] Absent or below detection limits. [e] Florence, SC, 1981. [f] A selection from TI-1113.

Table IV. Cuticular Diterpene and Sucrose Ester Levels vs. Insect Damage Ratings

tobacco	μg/cm² [b] α- + β-II	α- + β-I	III + IV	sucrose esters	resistance ratings[a] budworm	aphids	hornworm
NC 2326	0.54	37.0	–[c]	–	S	S	S
TI-1024	–	0.2	–	–	R	R	MS
TI-1112	–	0.3	–	–	R	R	R
I-35	–	0.2	–	–	R	R	R
TI-1298	–	0.1	–	–	R	R	MR
NFT	–	0.4	–	–	MR	MS	R
TI-163	1.0	35.0	–	13.0	R	S	S
TI-165	0.8	50.0	–	16.0	R	S	MR
TI-1396	1.2	38.0	16.0	19.0	R	S	MS
TI-1223	18.0	0.9	8.0	4.7	S	R	S

[a] Field ratings relative to NC 2326: R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible. [b] Method B; 6 weeks after transplant, Tifton, 1982. [c] Absent or below detection limit.

The reproducibility of the GC retention times and the response data for selected standards are given in Table II. The use of an autosampler resulted in very reproducible retention data. the α- and β-II and sucrose esters were isolated as mixtures and response data were calculated based on unitary response for each component in the mixture. Low-level degradation (thermal rearrangement) of Me_3Si-III resulted in a higher error for its analysis.

Cuticular Chemistry of Various Cultivars and Tobacco Introductions. The methodology described above was used to determine the major cuticular components of various commercial tobacco cultivars and tobacco introductions (Table III). The U.S. flue-cured, Burley, Maryland, and dark-fired tobacco types produced very similar composition and levels of cuticular components. Unlike the other U.S. tobacco types, a variation in surface chemical composition was observed with cigar wrapper cultivars. Gen 911 produced duvanes, labdanes, and sucrose esters. Connecticut broad leaf (Conn. Bdl.) was similar to dark fired (DF-300), and Magnolia yielded duvanes and labdanes. Turkish and cigar binder tobacco types that we have evaluated generally produced labdanes, duvanes, and sucrose esters. The exception was Gavurkoy I, which yielded only duvanes and sucrose esters.

Variations in the physical characteristics of leaf trichomes and chemical profiles were observed in various tobacco introductions (TIs), that we have investigated. TI-170, with glands on the ends of trichomes similar to commercial tobaccos, produced high levels of duvanes and sucrose esters. In contrast, TI-1112, with simple nonglanded trichomes (Goodspeed, 1957), yielded very low levels of diterpenes and fatty alcohols (based on docosanol levels). Other tobaccos with nonglanded trichomes, such as I-35 (Table IV), also produced very low levels of diterpenes and sucrose esters. Even though TI-1028 and PD-964 had glanded trichomes, they produced low levels of diterpenes. Hydrocarbon levels were not effected by trichome type.

In agreement with the cultivars, when low levels of II were present in the cuticular waxes of the TIs, an approximate 3 to 1 ratio of α-I to β-I was observed. However, in TI-1341, where α-II was the major diterpene compound, the relative level of the α-I decreased. When the II compounds were the major duvanes present, such as with TI-1223, an α-II to β-II ratio approaching 3 to 1 was observed.

Cuticular Chemical Composition and Insect Resistance. During the past few years, we have analyzed the cuticular waxes of susceptible and insect-resistant tobaccos and have found two general types of cuticular chemistry to be involved in insect resistance (Table IV). Tobaccos with very low duvane levels (TI-1024, TI-1112, I-35, TI-1298, and NFT) show resistance to budworm, aphid, and hornworm. In general, tobaccos with low diterpene levels will have resistance to these insects. Controlled cage studies have shown that the major mode of budworm resistance of these tobaccos is oviposition nonpreference (Jackson and Severson, 1982). Tests have shown that the α- and β-I fraction of the NC 2326 leaf wash when applied to TI-1112 increased ovipositional activity (Jackson and Severson, 1982).

A second type of tobacco budworm resistance, observed with TI-163, TI-165, and TI-1396, appears to result from larval antibiosis. Due to high levels of I, these tobaccos were very susceptible to oviposition, but preliminary studies indicate a low budworm larval survival rate on these tobaccos. Work is continuing to determine relationships between cuticular leaf components and budworm larval antibiosis. We are also applying the developed analytical methods to investigate mechanisms of hornworm and aphid resistances.

ACKNOWLEDGMENT

We thank Patricia F. Mason, Katheryn L. McDuffie, and Linda B. Smith of the USDA-ARS, Tobacco Safety Research Unit, Athens, GA, for their fine technical assistance.

Registry No. α-I, 57605-80-8; β-I, 57605-81-9; α-II, 80126-41-6; β-II, 25269-17-4; III, 17990-16-8; IV, 10267-31-9; docosanol, 30303-65-2.

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Received for review August 16, 1983. Revised manuscript received January 6, 1984. Accepted January 24, 1984. Reference to a company or product name does not imply endorsement or recommendation by the U.S. Department of Agriculture.