The Chemistry of Tobacco Trichomes[1]

M. K. Chakraborty[2] and J. A. Weybrew[3]

North Carolina State College, Raleigh, North Carolina, U.S.A.

The chemistry of tobacco aroma continues to challenge the research scientist and intensive programs are underway both in this country and abroad. While many compounds have been identified as constituents of tobacco or its smoke, not one can be clearly designated as the principal contributor to flavor or aroma.

Several methods for the collection of analytical specimens of the "aromatic oils" of tobacco have been used. Onishi and his associates published a series of reports on the constituents contained in the steam-volatile (Onishi, *et al.*, 1955) and ether-extractible (Onishi, *et al.*, 1956) essential oils from American and Japanese flue-cured tobaccos.

Weybrew and Stephens (1962) and Weybrew and Jones (1962) separated the volatile carbonyls from tobaccos by steam distillation. Onishi and Nagasawa (1957) observed that furfural was generated from carbohydrates during the steam distillation process. Recently, Jones and Weybrew (1962) described a low-temperature, reduced-pressure, moisture-entrainment distillation procedure for the collection of the natural oils from tobacco.

The postulated role of the trichomes as the major source of tobacco aroma stems from the general observations that leaves at the top of a plant are "gummier" and more aromatic than leaves lower on the stalk, and also that a dry-weather crop is gummier and more flavorful than a crop produced in a wet season. The number of trichomes per unit area was found to be higher on the upper leaves of Turkish tobacco (Wolf, 1944, 1946). Frankenburg (1951) linked the gummy exudate of trichomes with the dynamic equilibrium between volatile oils on the one hand and the polymeric resins on the other.

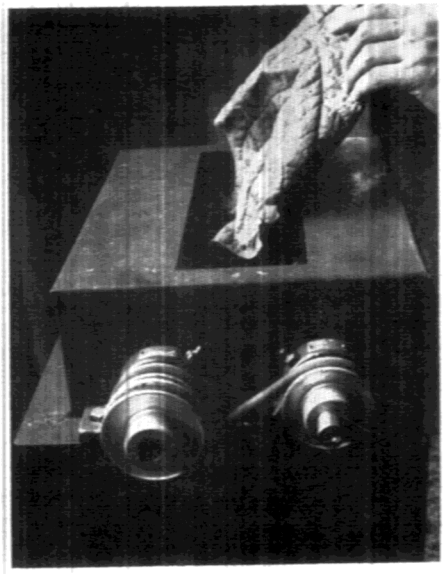

Figure 1. "Hair-Harvester" for collecting tobacco trichomes. (Designed and constructed by the research staff of the Department of Agricultural Engineering, N. C. State College.)

Essential oils obtained from whole-leaf samples, regardless of the degree of analytical sophistication employed, are completely uninformative concerning the site of aroma synthesis. By contrast, a sample made up only of trichomes or trichome exudate would, assuming the correctness of the hypothesis, be definitive with regard to site and also would be much less contaminated with intracellular materials. This paper describes the collection of a sample of tobacco trichomes and the partial characterization of its ether-soluble components.

Experimental Procedures and Results

Collection and Preparation

The "gums" that dirty the hands of tobacco harvesters are the sticky heads of trichomes. This, plus their appearance under the microscope, suggest that trichomes are frail and delicate structures. On the contrary, trichomes prove to be quite resilient in that attempts to harvest significant numbers of leaf-hairs by means of vacuum, or by a jet of compressed air, or by centrifugation were unsuccessful. The Hair Harvester **(Figure 1)** that was finally evolved took advantage of the fact that a trichome becomes frangible when frozen.

In use, mature green tobacco leaves were flattened and frozen overnight in a home freezer at about −16°C. Next morning, leaves were removed individually and, while still frozen, were dipped in and out repeatedly between the counter-rotating, Tynex-bristled, cylindrical brushes of the Harvester. The spacing of the brushes (about ¾ in. between the tips of opposing bristles) was such that a portion of the brittle trichomes would be broken off and, at the same time, abrasion of the cuticle would be minimal. Microscopic examination of the material collected on the plate beneath the brushes revealed a considerable proportion of sand grains, as was expected. The greater part of the organic matter, however, was clearly recognizable as trichome fragments.

During the summer of 1961, nearly 20,000 mature leaves[4] from successive harvests of experimental tobaccos grown at the Central Crops Research Station, Clayton, N.C., were processed in this manner. About 40 gm of the sand-trichome material accumulated and was stored over $CaCl_2$ in a desiccator at 4°C.

Much of the sticky exudate of the trichomes adhered to the bristles themselves. At the end of the harvest

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[2] *Present address: Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India.*

[3] *Professor of Crop Science and Chemistry.*

[4] *Composite of several flue-cured varieties, including Coker 187, DB 224, DB 102, SC 58, Coker 139, Vesta 30, Coker 316, and Oxford 1-181, in approximately equal proportions.*

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period, the bristles were plucked from the brushes and extracted with redistilled diethyl ether in a Soxhlet apparatus. A control sample of new Tynex bristles was similarly extracted and was carried along through the subsequent characterization procedures. Likewise, the sand-trichome sample was also extracted exhaustively with ether. This extract was combined with the extract from the brush bristles and yielded, after removal of the solvent, 7.2 gm of greenish wax. This was the analytical sample representing the ether-soluble constituents of tobacco trichomes.

The crude sample was redissolved in ether and partitioned into seven fractions: (I) Precipitated Wax; (II) Bases; (III) Acids; (IV) Phenols; (V) Hydrolyzed Acids; (VI) Carbonyls; and (VII) Neutral Compounds, by sequential washings with particular reagents. This fractionation scheme was patterned closely after that of Onishi and Yamasaki (1955) and is diagrammed in **Figure 2.** The yields and physical descriptions of the various fractions are summarized in **Table 1.**

Chemical Examination of Fractions

Each fraction was further subdivided by paper-, column-, or vapor-phase chromatography, by fractional or steam distillation, or by other appropriate techniques. Because of the very limited quantity of material in any sub-fraction, attempts at characterization were restricted only to the major components in any fraction. Identifications were based principally on color tests, melting points or other physical data, Rf's and retention times (as compared with authentic standards), ultraviolet and infrared absorption spectrophotometry. Only those compounds whose identifications are reasonably certain are discussed in detail.

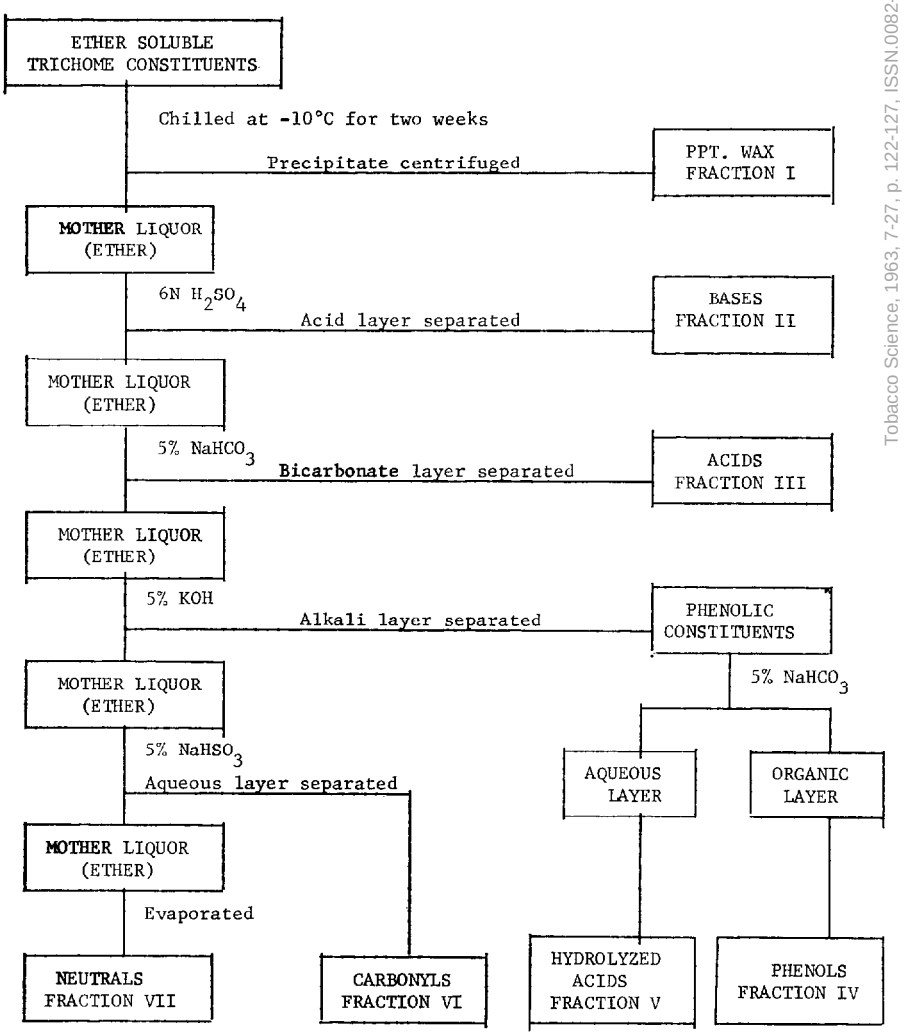

Figure 2. Fractionation of tobacco trichomes.

Precipitated Wax (I). During storage of the ether solution of the crude sample at −10°C, a greenish wax precipitated (Fraction I). This was separated and further purified chromatographically through silicic acid-celite (2:1 w/w; hexane), and by two reprecipitations from cold acetone. The white crystalline solid

Table 1. The yield and odor characteristics of different fractions of trichome constituents

Fraction No.	Description	Yield in mgm.	Per cent of total trichome extract	Characteristics
I	Precipitated wax	208	2.9	odorless
II	Bases	114	1.6	pungent odor
III	Acids	86	1.2	sweet, heavy odor
IV	Phenols	800	11.1	non-descriptive odor
V	Hydrolized acids	90	1.2	strong smell of butyric acid
VI	Carbonyls	32	0.4	sharp odor
VII	Neutrals	5,870	81.6	light, aromatic odor

Total Number of Tobacco Leaves: Approximately 20,000
Total Yield of Trichome Exudates: 7,200 mgm.

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melted at 68°C and gave an infrared spectrum characteristic of a straight-chain, saturated hydrocarbon. Its identification as n-hentriacontane, $C_{31}H_{64}$, a compound first identified as a constituent of tobacco by Thorpe and Holmes in 1901 and since verified by others, is suggested.

Bases (II). The basic fraction, extracted from the main bulk of the ether solution by dilute H_2SO_4, had a sharp odor, a pink color, and fluoresced green in ultraviolet light. Since the bristle extract (control sample) was also pink, contamination of the basic fraction by bristle components was suspected. Removal of these impurities was effected by chromatography on silicic acid-celite (2:1 w/w, hexane followed by ether). The pink contaminants passed through the column while the basic constituents were retained.

The packing material was extruded, made alkaline (pH 12), and extracted with ether. After drying over anhydrous Na_2SO_4, an aliquot of the concentrated extract was chromatographed on paper according to the method of Griffith *et al.*, (1955) and developed with cyanogen bromide. The distinctive color of the single spot and its position (Rf) on the chromatogram was clearly indicative that nicotine was the major constituent in the basic fraction.

Acids (III). Following the removal of the bases, the ethereal mother liquor was washed repeatedly with bicarbonate to separate acids (III). This fraction had a heavy, sweet odor, and fluoresced blue under ultraviolet irradiation.

The blue fluorescence suggested the presence of polyphenols in this fraction. The Rf's of this compound on paper chromatograms using several different solvent systems failed to agree with any of the available polyphenol standards. Identification was not accomplished.

Fraction V, the hydrolyzed acids (see **Figure 2**) was combined with Fraction III for the investigation of organic acids. Steam distillation was employed for the separation of the volatile acids (IIIa) from the non-volatile acids (IIIb). The volatile acids in the distillate were converted to their hydroxamic acid derivatives by a modification of the method of Bergmann and Segal (1956) and chromatographed on paper (BuOH: HAc:HOH, 4:1:5 v/v) as described by Shaw (1960). The close correspondence of the three red-purple spots, when sprayed with ethanolic $FeCl_3$, to a parallel chromatogram of authentic standards served for the presumptive identification of acetic, propionic, and butyric acids (or possibly isobutyric acid) as the major constituents in this subfraction. These acids had been reported by Onishi, *et al.* (1955) among the components in the essential oils from flue-cured tobacco.

The non-volatile acids (IIIb) were esterified with diazomethane at 0-5°C, then separated by vapor-phase chromatography (F & M Model 300; 10% neopentylglycolsuccinate on acid-washed Chromosorb W; 200° C; 120 ml He per minute). Only two ester peaks were evident on the chromatogram. Their retention times agreed with those of authentic methyl myristate and methyl palmitate under identical conditions. Onishi, *et al.* (1957) attribute part of the "aroma and taste of excellent Virginia tobacco leaf' to the presence of these and other acids.

Phenols (IV). Phenols (IV) were extracted from the main bulk of the original ether solution with alkali. Steam distillation was used to separate simple phenols (IVa) from the resin acids (IVb).

The phenolic subfraction had the distinctive pungent smell of phenols. Substances in this fraction reacted

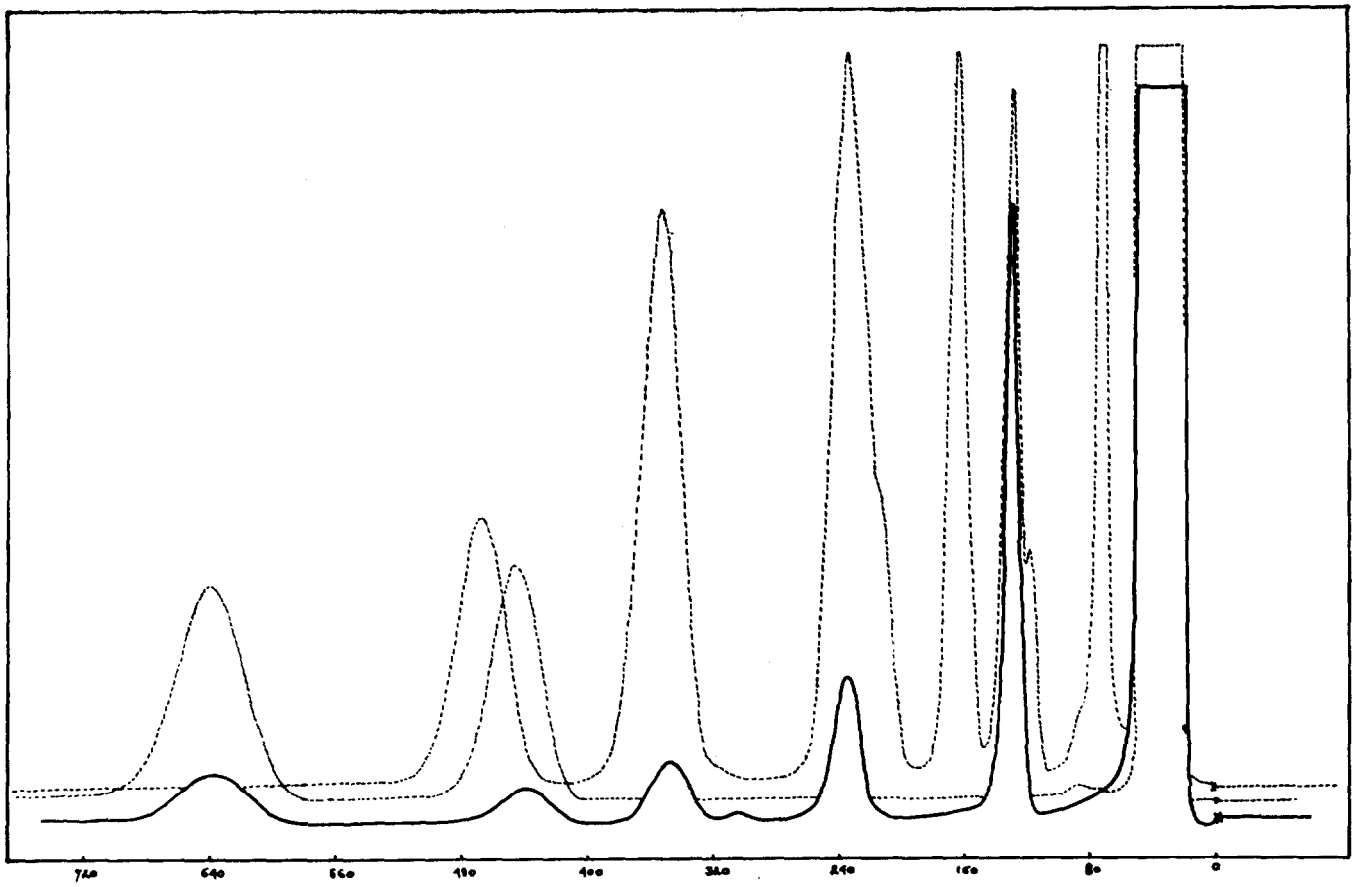

Figure 3. A vapor phase chromatogram of carbonyls isolated from tobacco trichomes (solid line). Dotted line: authentic standards.

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with diazotized sulphanilic acid, but attempts to isolate individual oxy-azo derivatives were unsuccessful. Thus the evidence for the occurrence of simple phenols among the constituents of trichomes is limited to the characteristic odor and the positive color reaction with sulphanilic acid.

The residue from the steam distillation was presumed to be "resin acids" (IVb), an ill-defined and complex mixture of highly labile substances. Some further subdivision was accomplished by chromatography on silicic acid-celite (2:1 w/w) using n-hexane followed by increasing proportions of ether in hexane. Saponification equivalents of the four eluate subfractions ranged between 650 and 1500. Although individual isolations were not effected, the infrared spectra of the eluate subfractions were quite complex with bands suggestive of branched chains, unsaturation, aromatic nuclei, and various oxygenated functions.

Hydrolized Acids (V). In the process of the alkali extraction of phenols (IV), some hydrolysis occurred. The liberated acids were recovered by extraction with bicarbonate. The odor of this fraction was suggestive of butyric acid. As described earlier, these hydrolyzed acids (V) were combined with the acids (III) for further examination and, in fact, butyric (or isobutyric) acid was identified as one of the constituents.

Carbonyls (VI). Carbonyls were removed from the primary solution as their bisulfite addition complexes. These substances had a sharp, penetrating odor. The carbonyls were converted to their 2,4-dinitrophenylhydrazone derivatives, extracted with CCl_4, and separated by vapor-phase chromatography (F & M Model 119; 4-ft dinonyl phthalate column; 87°C; 100 ml He per minute) using the α-ketoglutaric acid exchange reaction as modified by Stephens and Teszler (1960). **Figure 3** shows a reproduction of a vapor-phase chromatogram superposed over chromatograms of authentic carbonyls. Acetone, methyl ethyl ketone, and isovaleraldehyde had been reported previously by Onishi, *et al.* (1957) or by Weybrew and Stephens (1962) as constituents of whole leaves. Methyl propyl ketone and methyl isobutyl ketone are reported for the first time and may be constituents unique to trichomes.

Neutral Compounds (VII). Quantitatively, the greater portion of the trichome constituents were chemically neutral and remained in the primary solution through all of the previous partitionings. Waxes (VIIa) were precipitated from the residual solution by flooding with cold acetone, then separated, to leave the dewaxed neutrals (VIIb).

The crude waxes were further separated by column chromatography (silicic acid-celite, 2:1 w/w), eluting with n-hexane and ether-hexane mixtures. A white waxy solid that was eluted from the column in hexane, melted sharply at 60°C and gave an infrared spectrum characteristic of paraffins. On this basis it was identified as n-heptacosane. Gladding and Wright (1959) found n-heptacosane to be a constituent of burley tobacco.

The infrared spectrum of a light-blue fluorescent fraction eluted in 10% ether-hexane was suggestive of carbonyls or esters. With further purification, a melting point in the range 53-54°C was observed. The material was saponified with ethanolic KOH and gave a saponification equivalent of about 900. The similarity of its infrared spectrum to that of solanesyl palmitate (Rowland and Latimer, 1959) led to the supposition that this subfraction was a mixture of solanesyl esters.

A third eluate fraction yielded, with further purification, a colorless viscous oil that gave an infrared spectrum suggestive of phthalic acid esters of reasonable purity. The ester was saponified with ethanolic KOH. The acid, recovered from the saponification mixture, melted at 205-206°C and was not depressed when mixed with authentic phthalic acid. Comparative infrared spectra verified the identification of phthalic acid.

The alcohols from the saponification were esterified with acetic anhydride and separated by vapor-phase chromatography (10% neopentylglycolsuccinate on Chromosorb W; 50-245°C at 9°/min.; 120 ml He per minute). Two major and eight minor peaks were observed. The retention time of the first and by far the largest single peak fell midway between those of authentic n-hexyl and n-octyl acetate. It was therefore interpolated to be either iso-octyl or n-heptyl acetate. Stedman and Dymicky (1959) reported the principal phthalate of aged flue-cured tobacco to be an ester of a branched alcohol containing more than six C-atoms.

The semisolid, dewaxed neutral subfraction (VIIb) proved to be a complex mixture. Even though chromatographed and rechromatographed extensively eluting with a progression of hexane, benzene, ether, chloroform, and methanol, and followed by recrystallizations, clean isolations were not accomplished. For the most part, the chemical character of these impure eluate subfractions could only be inferred from the interpretation of their infrared spectra.

One eluate fraction yielded, after repeated recrystallizations, a relatively pure compound whose infrared

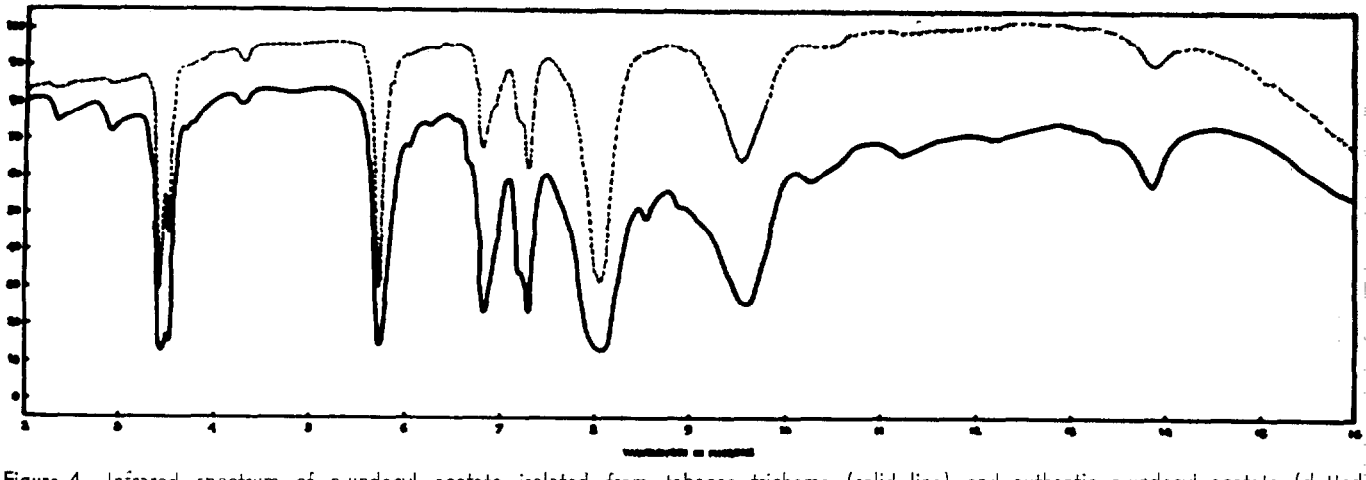

Figure 4. Infrared spectrum of n-undecyl acetate isolated from tobacco trichome (solid line) and authentic n-undecyl acetate (dotted line).

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Table 2. Summary of the chemical fractionation of the ether-soluble constituents of tobacco trichomes.

Fraction Number	Designation	Identified Compounds	Approximate Yield, mg.
I	Precipitated Wax	*n*-Hentriacontane	180
II	Bases	Nicotine	50
III	Acids (includes V)		
	a. Volatile Acids	Acetic, Propionic, Butyric Acids	30
	b. Nonvolatile Acids	Myristic, Palmitic Acids	170
IV	Phenols		
	a. Simple phenols		15
	b. Resin acids		750
V	Hydrolized Acids (combined with III)		
VI	Carbonyls	Acetone, Methyl ethyl ketone, Isovaleraldehyde, Methyl propyl ketone, Methyl isobutyl ketone	30
VII	Neutral Compounds		
	a. Waxes		
	Paraffins	n-Heptacosane	270
	Solanesyl esters		95
	Phthalic esters	Iso-octyl phthalate	300
	b. Dewaxed Neutrals		
	Branched hydrocarbons		240
	Aliphatic ethers		340
	Solanesyl esters		60
	Higher alcohols		50
	Higher carbonyls		70
	Terpene alcohols		640
	Esters	n-Undecyl acetate	95
	Resin alcohols		1400
	Unaccounted for (by difference)		2445
			7200

spectrum showed a sharp band at 8.05μ that suggested acetate ester(s). Programmed gas chromatography (10% neopentylglycolsuccinate on Chromosorb W; 50-245°C at 9° per minute) gave a single sharp peak. Authentic acetate esters were synthesized from a series of alcohols. Chromatographed isothermally at 150°C, the retention times of authentic n-undecyl acetate and the unknown were identical at 7.16 minutes. Their infrared spectra are compared in **Figure 4** and establishes the identity of this compound.

At each stage in the entire fractionation sequence, either whole fractions or aliquots thereof were evaporated and weighed to provide estimates of yields. This accounting is summarized in **Table 2.**

Discussion

The fifteen compounds that have been identified in the sample of ether-solubles from tobacco trichomes, collectively account for only 15% of the sample, and probably represent an even smaller fraction of the number of compounds present. The quantity of analytical material necessarily diminished with each fractionation and subfractionation so that, as one approached the isolation of a pure entity, more often than not the sample was inadequate for the characterization. The odors of many of these subfractions were most interesting but could not be described adequately.

The occurrence of the two paraffin hydrocarbons, hentriacontane and heptacosane, in the trichome sample suggests cuticular contamination. While this possibility cannot be eliminated, microscopic examination of trichomes has shown that the cuticle extends at least part way up the stalk and so, depending on how one defines his sample, these compounds may appropriately be part of it.

By comparison with a green leaf, a tobacco leaf after curing has considerably fewer intact trichomes. Undoubtedly some get broken off in the handling and, in fact, stumps can be observed. It seems more reasonable, however, that these structures would be the first to lose water and become flaccid in the curing process. As they collapse onto the surface of the leaf, much of the exudate of trichomes would dissolve in and become part of the waxy cuticle. This concept has been advanced by Frankenburg (1951).

Green tobacco leaves are completely lacking in the aroma that characterizes cured tobacco. It is possible, therefore, that the constituents contained in this particular sample are no more than precursors of true aroma. Plans are being developed to study the chemical changes that take place in the trichome during flue-curing.

Summary

Approximately 20,000 mature green tobacco leaves were processed individually in the frozen state to yield a sample of trichome fragments from which 7.2 grams of ether-soluble material was recovered. This crude mixture was subjected to a long series of fractionations involving solvent partitioning, paper-, column-, and gas chromatography. Only fifteen of the very many compounds contained in the sample have been tentatively identified. Of these, eleven had been previously reported as constituents of whole leaves. Methyl propyl ketone, methyl isobutyl ketone, iso-octyl phthalate, and n-undecyl acetate are newly identified constituents of tobacco and may be present only in trichomes.

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