

Composition Studies on Tobacco

III. Total 3- β -Sterols in Flue-cured Tobacco Leaves

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Introduction

Past studies in this series have shown the presence of stigmasterol (Grossman and Stedman, 1958) and β -sitosteryl monoglucoside (Dymicky and Stedman, 1958) in the leaves of domestic flue-cured tobacco. More recently, a steroidal glyco-

side having an aglycone similar to the structure originally proposed for chalinasterol (Bergmann *et al*, 1945) has also been isolated in this laboratory.² Thus, information on the structure of tobacco phytosterols and phytosterolins is becoming more complete; this is in contrast to

knowledge of the total amounts of steroidal substances in flue-cured tobacco, about which little is known. The present study was undertaken to elucidate this latter point and to determine the presence of other unidentified sterols in tobacco leaves.

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²Complete characterization of this compound has not yet been achieved.

Experimental and Results

Primary extraction. Figure 1 outlines the separation scheme followed. For primary extraction of the leaves, acetone, alcohol and glacial acetic acid were successively used; the last solvent was employed since previous work had established that the unidentified steroidal glycoside is significantly soluble only in hot acetic acid. The initial phases of the extraction were performed in seven Soxhlet extractors, each containing 2500 ml. of acetone and 500 g. of cured (but not aged) Type 12 tobacco of mixed grades. The extraction was continued for fifty hours after which the acetone was replaced by absolute ethanol and the extraction carried on for another fifty hours.

The seven batches of tobacco residues were then removed from the extractors, combined and refluxed with two successive 10 l. portions of fresh acetone for one hour each. This procedure was repeated with ethanol. Subsequently, the tobacco residue was dried and then refluxed with two successive 14 l. portions of glacial acetic acid for eight hours each. All acetone and alcohol extracts were combined and evaporated to dryness (A). The two acetic acid extracts were also mixed, four l. of water were added

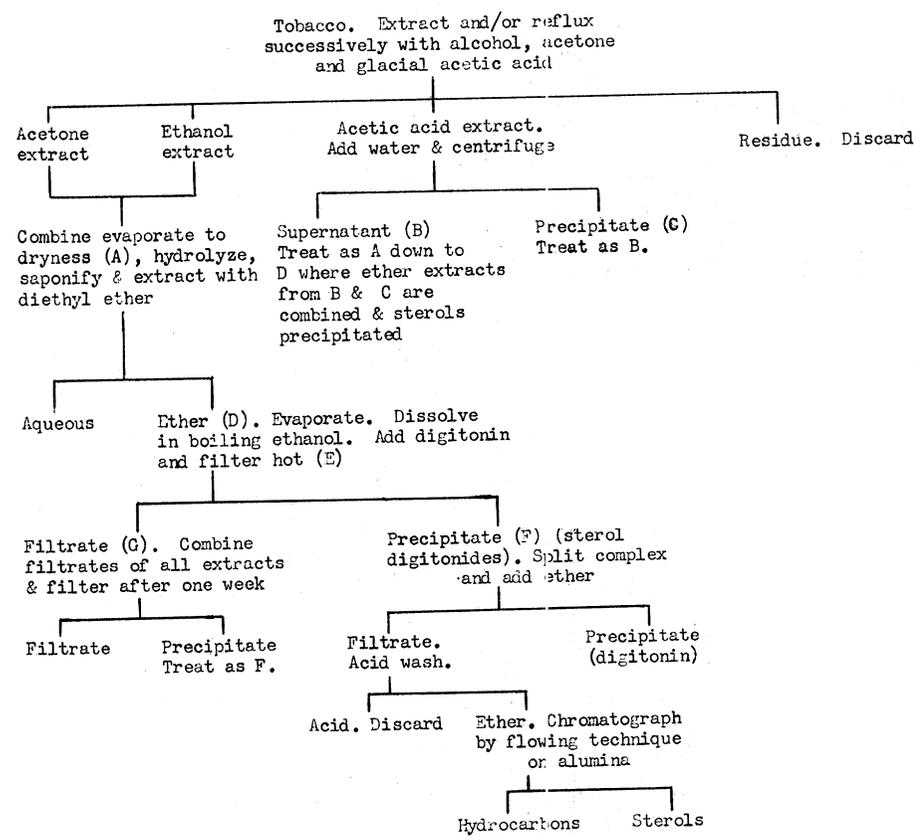


Figure 1. Isolation and purification of total free and bound phytosterols from flue-cured tobacco leaves.

and the mixture was centrifuged. The precipitate (C) was set aside for further treatment, and the supernatant was evaporated to a syrupy mass (B).

Hydrolysis and saponification. Bound phytosterols exist either as glycosides or esters. Glycosides are hydrolyzed by acid, but are not significantly cleaved by saponification; the reverse is true for esters. To insure complete recovery of sterols existing in bound form, both treatments are required.

Residues A and B were hydrolyzed independently by refluxing for 27 hours in five l. absolute ethanol containing 1N sulfuric acid. Residue C was similarly treated with one l. acidic ethanol. At the end of this period, each of the hydrolysis mixtures was saponified by adding potassium hydroxide to a concentration of 10 per cent after neutralization and refluxing for two hours. Subsequently, water was added to each mixture and each was extracted with diethyl ether. The ether extracts from B and C were pooled after preliminary work showed that C contained only small amounts of residue on evaporation of the solvent. The two ether extracts, one from A and the other from pooled B and C, were then subjected to digitonin precipitation.

Precipitation and purification of sterols. In these procedures, the two residues resulting after evaporation of the ether extracts were treated independently and in an identical manner during most of the fractionating procedure. Each residue was dissolved in boiling ethanol and a 3.0 per cent digitonin solution (in 80 per cent ethanol) was added in increments until precipitation was complete. After the further addition of excess digitonin and a small amount of water, the crude digitonides were filtered from the hot solution and the precipitate was thoroughly washed with boiling absolute ethanol, 80 per cent ethanol and water. The digitonides were split in the conventional manner: the precipitate was boiled in pyridine and the released digitonin precipitated by ether. The filtrate containing sterols was shaken with acid to remove the pyridine, and the ether layer evaporated to give a residue of crude sterols. The residue was dissolved in petroleum ether containing five per cent benzene and purified on acid-washed alumina using a flowing chromatographic technique. The sterols were eluted by a succession of solvents (25 per cent diethyl ether in benzene, 50

Table 1. Weights (g/kg tobacco*) of various fractions obtained in the determination of total 3- β -sterols in flue-cured leaves.

Fraction	Precipitated Hot†		Precipitated Cold†	
	Alcohol-acetone extract	Acetic acid extract	Combined extracts	Total Yields
Digitonides	4.6	0.55	2.5	7.7
Crude sterols	1.6	0.19	0.61	2.4
Purified sterols	0.91	0.14	0.43	1.5

*Moisture-free basis. The tobacco contained 6.25% moisture.

†See text for designations.

per cent diethyl ether in benzene, diethyl ether, one per cent methanol in diethyl ether and methanol). The crude sterol mixture derived from A contained some white waxy solid which was not absorbed on the column and was collected in the first fractions; this material gave a melting point (62-64°C) in the range of a C₂₈-C₂₉ saturated hydrocarbon and an x-ray diffraction pattern characteristic of a mixture of long-chain unbranched compounds. The crude sterols derived from the glacial acetic acid extracts did not contain this waxy substance. In all cases the purified sterols were white crystalline solids which were substantially free of extraneous matter.

The above procedure provided information on the relative proportions of phytosterols extractable with alcohol-acetone and glacial acetic acid. However, the method did not give quantitative values for total phytosterols since the digitonides possess partial solubility in hot ethanol and the above crude digitonides were obtained by filtration of the hot ethanolic solutions (E). To recover the digitonides found in the filtrates (G) derived from both the original alcohol-acetone and glacial acetic acid extracts, the filtrates and washings from the digitonin precipitation of all extracts were pooled, stored at room temperature for one week and the precipitated digitonides then worked up in a manner similar to the above procedure.

Total phytosterols and their nature. Table 1 lists the weights obtained in the various isolated fractions. The sterols and digitonides obtained from filtration of the hot ethanolic solutions or on standing at room temperature for one week are designated "Precipitated Hot" and "Precipitated Cold", respectively. The overall phytosterol (3- β -ol) content of the flue-cured tobacco leaves was 0.15 per cent (moisture-free basis). The com-

bined acetone-alcohol extraction removed about seven times as much sterol as acetic acid.

Since various color tests and other procedures are available for detecting certain classes of phytosterols, the purified sterols were examined for saturated, conjugated and other types of components.

Using the Anderson and Nabenhauer method for the separation of saturated and unsaturated sterols (1924), approximately 80 mg. of sterol were obtained in the saturated fraction resulting from one g. of sterol mixture. This material showed m. p. 134-135°C and $[\alpha]_D^{20} = -20$ (chloroform). This optical rotation is more positive than that of known Δ^5 phytosterols and is, of course, more negative than the saturated sterols. In addition, the material obtained from the saturated fraction gave a weak Liebermann-Buchard reaction indicating the presence of an unsaturated compound. It was concluded that the sterols in the saturated fraction were probably a mixture of saturated and unsaturated compounds.

Two additional color reactions were performed on the tobacco sterols: the Rosenheim test (1929) for conjugated structures and the Tortelli-Jaffe test (Fieser and Fieser, 1949) for compounds having a ditertiary double bond or a bond capable of migration to such positions. The Rosenheim reaction of the isolated tobacco phytosterols was negative. However, a positive Tortelli-Jaffe test was obtained. This finding would indicate the presence of a Δ^8 (9) or Δ^8 (14) compound, such as zygmasterol (Δ^8 (9)), or of a structure which can rearrange to such a configuration, such as α -spinasterol (Δ^7). An authentic sample of the latter compound was run as a control and found to give a positive Tortelli-Jaffe reaction.

Although column chromatography is generally believed to be ineffective in separating mixtures of closely related phytosterols except under special conditions (Bergmann, 1953), examination of the sterol fractions eluted from alumina showed that a degree of separation was achieved in one instance. On chromatographing the sterols derived from digitonides that precipitated from the filtrates (G), 44 fractions of 50 ml each were collected, of which the last eight contained sterols. After recrystallization from hot ethanol, fraction 42 was found to be pure β -sitosterol, m. p. 135-136.5°C, $[\alpha]_D^{20}$ —36.9 (chloroform) with an infrared spectrum identical with authentic material. However, none of the remaining fractions appeared to consist of pure compounds.

Summary

The total 3- β -sterol content of mixed grades of American Type 12 tobacco was determined by exhaustive extraction of the leaves with acetone, alcohol and glacial acetic acid followed by precipitation with digitonin. The total sterol content was 0.15 per cent (moisture-free basis). It was shown that tobacco leaves contain types of sterols other than the Δ^5 compounds known to be present. The occurrence of saturated and Δ^7 or Δ^8 components was demonstrated, but no evidence for the presence of conjugated sterols was obtained.

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