Composition Studies on Tobacco V. Free and Combined 3-β-Sterols of Freshly Harvested, Aged or Fermented Tobaccos

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Introduction

Part III of this series concerned a study of total 3-β-sterol levels in unaged, flue-cured tobacco (Stedman *et al*, 1958). The present report is an extension of this work and describes the levels of glycosidated, esterified and free sterols found in a wide variety of tobaccos using a semi-micro analytical procedure.

Description of Method

The method employed in Part III was a macro technique devised for two purposes: To obtain quantitative information and to isolate significant amounts of phytosterols for composition studies[2]. Since this method was too laborious for use in the analysis of a number of tobacco samples, a semi-micro method was sought. A well-known technic previously developed at this laboratory (Wall and Kelley, 1947) for the analysis of green vegetable meals was ultimately modified for use with tobacco. Details of the modification are as follows:

Fifteen to thirty-five g of tobacco ground to pass a 50-mesh screen are extracted for 24 hours with 100 ml acetone in a Soxhlet apparatus. The acetone extract is then cooled, made up accurately to 100 ml with acetone and three 25 ml aliquots are removed. One of these is evaporated to dryness in a 50 ml round-bottom flask[3]. Twenty-five ml of 95 per cent ethanol containing 250 mg sulfuric acid are added to the residue, and the mixture refluxed for fifteen hours to split the glycosides. Fifteen ml of 10 per cent potassium hydroxide (in 95 per cent ethanol) are added to this flask, the contents are refluxed for thirty minutes to hydrolyze the esters and the sterols are extracted as indicated below. This flask gives a value for total sterols since both glycosides and esters of sterols have been hydrolyzed. The second 25 ml aliquot is evaporated to dryness, the residue dissolved in 25 ml 95 per cent ethanol, the contents saponified by adding 15 ml of 10 per cent potassium hydroxide (in 95 per cent ethanol) and after refluxing for thirty minutes the sterols are extracted as indicated below. This flask gives a value for free and esterified sterols. The third aliquot is evaporated to dryness, 25 ml of ethanol are added, the flask contents are boiled and cooled, and the free sterols extracted as described below. Thus, values for free, and total sterols are obtained directly and levels of steroidal esters and glycosides can be calculated from the three aliquots.

The sterols are extracted from the hydrolyzed and unhydrolyzed solutions by adding 25 ml Skellysolve B[4] to each of the solutions and water just sufficient to obtain two layers. The Skellysolve layer is separated and the aqueous layer extracted three more times with Skellysolve. Then 7 ml of water are added to the aqueous layer and four successive extractions with Skellysolve are again made. The eight Skellysolve extracts for each sample are combined and washed four times with methanol containing 10 per cent water to remove xanthophylls and other substances. The Skellysolve layer is evaporated to dryness and the residue dissolved in 25 ml 95 per cent alcohol. Ten ml of a 2 per cent solution of digitonin in 80 per cent ethanol are added to the boiling alcoholic solution and the solution is boiled one minute; then 6.5 ml water are added, the boiling continued one minute and the solution allowed to cool to room temperature. After standing overnight, the precipitate is collected on a tared sintered glass Buchner funnel and washed with small amounts of 80 per cent ethanol followed by diethyl ether. The funnel is dried at 100° C for one hour in a forced-draft oven.

1. *Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.*

2. *Part IV described these studies (Dymicky and Stedman, 1959.)*

3. *A larger flask cannot be employed due to sterol destruction during acid-hydrolysis. Apparently, when the level of the solution is significantly below the waist of the flask, evaporation of solvent at the glass-solution interface during refluxing causes a concentration of acid with concurrent destruction of sterol.*

4. *Use of a specific commercial product does not constitute endorsement by the United States Department of Agriculture.*

cooled to room temperature in a desiccator and weighed. Since the digitonides are quite hygroscopic, exposure to the atmosphere must be reduced to a minimum at all times. The weight of sterol is obtained by multiplying the weight of digitonides by the factor, 0.253 (Wall and Kelley, 1947).

Preliminary Methodological Studies

Considerable preliminary work was performed before adoption of the above method. Some of the information obtained in this work may be of interest to those concerned with the methodology of plant composition studies. Therefore, a summary of the more important findings will be given.

Spectrophotometric vs. gravimetric determination. Wall and Kelley investigated the spectral characteristics of sterol digitonides prepared from green plant material and developed a spectrophotometric determination for sterols based on measurement of light absorption produced by the reaction of the digitonides with the Liebermann-Burchard reagent. Good agreement was reported between spectrophotometric and gravimetric values for such digitonides. In the present study a similar approach was made with tobacco sterol digitonides.

The reaction product obtained with purified tobacco sterol digitonides and the Liebermann-Burchard reagent gave an absorption spectrum from 400-1000 mu which had the same general shape as those of the 15 vegetable and other sterol digitonides reported by Wall and Kelley. However, the spectrum of the tobacco digitonides showed a distinct bathochromic shift of the maximum at the longer wavelength compared to the spectra of these other plant sterols.

A spectrophotometric technique essentially similar to the Wall and Kelley method was developed using the above spectrum. The results obtained with the spectrophotometric method and with the above gravimetric technique were compared, and the former was found to give erratic values which were substantially higher (5-80 per cent) than the gravimetric method. The nature and time-intensity relationship of the absorption produced with the tobacco digitonides suggested the presence of trace amounts of non-steroidal substances which reacted with the Liebermann-Burchard reagent. Therefore, the gravimetric method was employed in the analytical studies. The precision of this method is essentially the same as that reported for the Wall and Kelley gravimetric method (± 5 per cent).

Table 1. Extraction of sterols from flue-cured tobacco (50 mesh) under various conditions

Solvents and Extraction Times* (Hours)			% Sterols			Total Sterols Extracted (%)
A	B	C	A	B	C	
Skellysolve (4)	Acetone (2.5)	None	0.27†	0.10†	...	0.37†
Acetone (4)	Skellysolve (3)	None	0.46† (0.26)	0.01†	...	0.47†
Acetone (4)	Ethanol (12)	Acetic acid (16)	0.26	0.02	0.02	0.30
Acetone (8)	Ethanol (8)	None	0.32	0.03	...	0.35
Acetone (28)	None	None	0.41	...	...	0.41

* *A, B and C refer to successive extractions of the same tobacco sample with the indicated sequence of solvents.*
† *By spectrophotometric determination. All other values are gravimetric.*

Extracting solvents. Preliminary experiments showed that free and combined tobacco sterols are tightly bound within the plant cells. As expected, solvents vary widely in their ability to extract these compounds from tobacco. Table 1 illustrates the amounts of total sterols extracted from a 50 mesh sample of flue-cured tobacco using different periods of time, different solvents and a succession of three solvents. Data based on spectrophotometric measurement are included for comparative purposes subject to the limitations discussed above. On the basis of such findings the extracting solvent eventually selected was acetone and the final extracting period, 24 hours. Although the sterols may not be completely extracted, in the absolute sense, by these conditions, this approach provides a practical means of rapid quantitative determination with primary emphasis on comparative values.

Mesh size of tobacco sample. Preliminary work was conducted on the same sample of unaged, flue-cured Type 12 tobacco used in the isolation study of Part III. This tobacco had been obtained by grinding leaf strips in a Wiley mill and was used without further screening. The degree of pulverization was such that all particles passed a 10 mesh screen and most of them passed a 20 mesh screen. In the present study it was found that reducing the particle size to 50 mesh increased the rate of sterol extraction by approximately 50 per cent.

Relationship with the past study. In Part III it was reported that an unaged, flue-cured sample of Type 12 tobacco contained 0.15 per cent total 3-β-sterols. In the present study, 0.43 per cent total sterols was obtained on the same sample. It is obvious that either more sterols were extracted or that extraneous material was being weighed by the present procedure. Undoubtedly, more sterols were extracted by decreasing particle size. Perhaps of more importance was the overall effect of changing the scale of extracting operation. In the large-scale extraction, the chamber containing the tobacco filled slowly but the tobacco soaked in solvent longer. In the small Soxhlets, a very rapid filling and siphoning occurred and the temperature of the solvent exposed to the tobacco was generally higher.

The probability that extraneous matter was being weighed in the semimicro method is slight. The small amount of precipitated digitonides could be washed much more thoroughly than the large amounts precipitated in Part III; in general, the former were much cleaner on initial precipitation[5]. No indication of significant contamination of the digitonides by hydrocarbons was observed in the current study; extensive Skellysolve rinsings of the digitonides removed no solids. The digitonides contained no substances insoluble in glacial acetic acid.

Sterol Levels in Various Tobaccos

Twenty-three samples of leaf webs and midribs were analyzed, including freshly harvested, aged or fermented flue-cured, burley, Maryland, Turkish, fire-cured, cigar binder and cigar filler types. A few commercial blends used in the manufacture of cigarettes were also evaluated. The analytical

5. *Turkish tobacco yielded digitonides (from free sterols only) that required extensive washing.*

Table 2. Free, esterified and glycosidated sterols in various green, aged or fermented tobaccos.

	Percent*			
	Glycosides	Esters	Free	Total
Flue-cured				
Green leaves, Hicks, overmature	0.11	0.06	0.10	0.27
Green midribs, Hicks, overmature	0.03*	0.05*	0.05*	0.13*
Aged leaves, Type 11, 1955 crop	0.10	0.14	0.11	0.35
Aged leaves, blend of flue-cured types	0.16	0.16	0.13	0.45
Aged midribs, blend of flue-cured types	0.05	0.04	0.06	0.15
Burley				
Green leaves, Ky. 41A, overmature	0.05	0.11	0.18	0.34
Green midribs, Ky. 41A, overmature	0.03*	0.05*	0.08*	0.16*
Aged leaves, sample X	0.17	0.06	0.16	0.39
Aged midribs, sample X	0.03	0.01	0.07	0.11
Aged leaves, B_3F grade	0.12	0.07	0.15	0.34
Maryland				
Green leaves, Wilson strain, under mature	0.10	0.05	0.09	0.24
Green midribs, Wilson strain, under mature	0.06*	0.01*	0.06*	0.13*
Green leaves, Wilson strain, mature	0.06	0.09	0.11	0.26
Green midribs, Wilson strain, mature	0.03*	0.04*	0.06*	0.13*
Aged leaves, 1954 crop	0.18	0.09	0.11	0.38
Aged midribs, 1954 crop	0.01	0.03	0.08	0.12
Aged blend, Burley and Maryland leaves	0.15	0.08	0.13	0.36
Turkish				
Aged leaves, Smyrna	0.11	0.06	0.09	0.26
Aged leaves, Turkish blend	0.09	0.10	0.08	0.27
Fire-cured				
Fermented leaves	0.03	0.08	0.09	0.20
Fermented midribs	0.03	0.03	0.05	0.11
Cigar types				
Fermented filler leaves	0.06	0.02	0.08	0.16
Fermented binder leaves	0.05	0.01	0.08	0.14

** All values are on a moisture-free basis except the green midribs. These midribs were partially dehydrated as described in text and values calculated on the basis of total weight without moisture correction.*

results are given in **Table 2:** the values are for single determinations in most cases.

The freshly harvested leaves were pooled samples collected late in the 1958 growing season from top, middle and bottom portions of plants on experimental plots at Department installations in Beltsville.

The aged and fermented tobaccos were representative commercial samples. Available descriptive data are given in the table. "Leaves" refer to leaf webs; the term "green" includes all freshly harvested leaves regardless of degree of maturity. All values have been corrected for moisture content except those for midribs of green leaves. These midribs were obtained from the intact leaves and diced into small fragments and dried at room temperature and humidity for two weeks before analysis; the sterol values were calculated on the weight of this partially dried material.

All inferences drawn from these data are presented with the usual reservations regarding biological variation, sample size and other factors which limit broad generalizations.

Among the aged and fermented tobaccos, total sterol levels varied from approximately 0.1 to almost 0.5 per cent. Some indication of a correlation between total sterol content and tobacco leaf type was noted. Aged bright, burley and Maryland had approximately the same sterol levels and showed higher concentrations than the other types. Turkish, fire-cured and cigar types had progressively less total sterols. The various commercial samples of the same general tobacco type gave values quite similar except in the case of flue-cured leaves. In almost all instances, the midribs of aged or fermented leaves showed from one-third to one-half the total sterol levels of the leaf tissue associated with the midribs.

The sterol contents of the green and aged leaves cannot be compared directly since corrections for losses in dry matter during curing and aging are necessary. Experimental data on this point were unavailable. However, theoretical corrections based on reported average losses (Frankenburg, 1946 and 1950) could be calculated. Corrected values obtained in this way[6] showed that the sterol contents of green leaves were either slightly less (—10 per cent) than aged leaves (bright and Maryland) or, in the case of burley, significantly more (+30 per cent) than aged samples. Perhaps a logical conclusion would be that curing and aging do not drastically alter the sterol levels of green tobacco.

Although corrections for moisture were not made for green midribs, it would seem that the relative sterol contents of green leaves and midribs have essentially the same pattern as those of the aged or fermented tobaccos. This conclusion is based on the fact that the midribs would have had to contain 50 per cent moisture to give sterol contents comparable to the leaves. By inspection, the moisture contents of the midribs were considerably less than this figure.

No consistent relationship was observed between the proportions of free, esterified and glycosidated sterols, and tobacco type. Some tendency toward leaves having a higher content of free and glycosidated sterols compared to esterified forms was noted among the aged and fermented samples. This tendency could be interpreted as a reflection of differential solubilities of the various steroidal forms in acetone. On the basis of chemical structure, Skellysolve B would be expected to be a better solvent for esters than acetone. However, superficial experiments showed that the greater extractive properties of acetone are evident with all three forms of tobacco sterols.

Summary

A semi-micro gravimetric method

[6] The following average values reported by Frankenburg (1946, 1950) were used in the calculations: Losses in dry weight due to curing, flue-cured, 11%; burley and Maryland (not primed), 25%; losses on aging, flue-cured, 2%; burley and Maryland, 4%.

for the determination of 3-β sterols in free and combined forms is described. Preliminary methodological studies are also summarized.

A number of green, aged or fermented samples of tobacco leaves and midribs was analyzed with the developed procedure. The approximate total sterol levels of all samples varied between 0.1 and 0.5 per cent. Aged samples of flue-cured, burley and Maryland leaves showed the highest sterol concentrations. These three types gave approximately similar levels. Turkish, fire-cured and cigar types contained progressively less total sterols. The midribs of commercial samples of aged or fermented tobaccos showed significantly less sterols than the leaf tissue. Although an accurate comparison between the sterol levels of green and aged leaves was difficult to make, it appears that curing and aging do not drastically alter the sterol contents of green leaves.

All samples contained free, esterified and glycosidated sterols but no rigid correlation between the proportions of each and tobacco type was observed.

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