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# PHYTOSTEROLS IN TOBACCO LEAVES AT VARIOUS STAGES OF PHYSIOLOGICAL MATURITY

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**Key Word Index**—*Nicotiana tabacum*; Solanaceae; tobacco, sterol; steryl ester; steryl glycoside; variation with maturity.

**Abstract**—Leaves of varying maturity from 84-day-old tobacco plants were harvested and analyzed for total sterol and their individual sterol components. The mature leaves had a significant higher sterol content than the immature leaves. Separation into free sterols, steryl esters, steryl glycosides, and acylated steryl glycosides showed that the free sterols accounted for most of the sterol increase, and stimgasterol was principally responsible for this increase.

## INTRODUCTION

As plants mature the total sterol content in leaves increases [1–5]. It has been suggested that the increase in sterol content is due to senescence and the disorganization of intracellular organelles [1,6]. This hypothesis is supported by a study in which tobacco leaves from upper (immature), middle (intermediate maturity) and lower (mature) stalk positions were sampled, and it was found that the mature leaves had significantly higher sterol levels than the immature leaves [7]. However, in one study the leaves of intermediate age had the highest sterol level, but in this study air-dried leaves were used [8]. In only a few experiments on plant ageing have the free sterols, steryl esters, steryl glycosides and acylated steryl glycosides been investigated and in none has all four sterol forms been studied at the same time. In *Solanum andigena* the free sterols increased as the plants matured [2], while in *Solanum tuberosum* the steryl glycosides and acylated steryl glycosides accounted for most of the increase [1].

The work reported here was undertaken to determine whether, with physiological age, quantitative and qualitative changes occur in the free sterols, steryl esters, steryl glycosides and acylated steryl glycosides. Field-grown *Nicotiana tabacum* plants were used since plants grown under artificial conditions may not show a change in sterols [5,9]. To minimize changes due to environmental factors over the growing season, leaves from different stalk positions of the same plants were analyzed. The assumption was made that leaves from the upper stalk position were leaves of youngest physiological age (immature), while leaves from the lowest stalk position were of oldest physiological age (mature).

## RESULTS AND DISCUSSION

The total sterol content of mature leaves of field-grown tobacco had a significant higher sterol content than immature leaves (Table 1). Leaves of intermediate maturity had intermediate sterol values. These data agree with previous reports [3,4] which showed that in field-grown tobacco the total sterol content increased as the leaves matured. An increase in sterol content with leaf age has also been observed in other species [1,2]. If the absolute sterol values are compared to previous reports [3,4] a somewhat smaller increase in sterol content was found in the present experiment (Table 1). A possible reason for the differences in sterol content may be environmental variations.

The four major sterols in tobacco are sitosterol, stigmasterol, campesterol and cholesterol [3,7,8]. Stigmasterol was the only sterol which increased in absolute value with leaf maturity (Table 1). The content of sitosterol, campesterol and cholesterol did not change and, therefore, decreased in relative

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Table 1. Individual sterols of the total sterol fraction from *Nicotiana tabacum* leaves of different maturity

| Leaf maturity | Sitosterol (mg/g) | Sitosterol (%) | Stigmasterol (mg/g) | Stigmasterol (%) | Campesterol (mg/g) | Campesterol (%) | Cholesterol (mg/g) | Cholesterol (%) | Summation (mg/g) |
|---|---|---|---|---|---|---|---|---|---|
| Immature | 0·75 | 44·4 | 0·50 | 30·0 | 0·27 | 16·2 | 0·16 | 9·4 | 1·68 |
| Intermediate | 0·73 | 40·8 | 0·62 | 34·9 | 0·26 | 14·8 | 0·17 | 9·5 | 1·78 |
| Mature | 0·72 | 37·8 | 0·75 | 39·4 | 0·27 | 13·9 | 0·17 | 8·9 | 1·91 |
| LSD | 0·05 | | 0·04 | | 0·01 | | 0·01 | | 0·06 |

terms if expressed as a percentage of total sterols. It has been reported that in field-grown *N. tabacum* the relative concentration of stigmasterol increased while that of sitosterol decreased over an 84-day growing season [3]. Analysis of upper and lower leaves of field grown *N. tabacum* plants showed that the immature, upper leaves had less stigmasterol than the mature leaves [4]. However, under greenhouse conditions the increase in stigmasterol with leaf age was not observed [5]. In developing seedlings an increase in stigmasterol occurred with age [10–13] and the increase was greatest in the older stem tissue [12]. In *S. andigena* leaves, which contain only small quantities of stigmasterol, no increase in the content of this sterol was observed, but instead an increase in sitosterol was found [2].

The higher sterol content in mature tobacco leaves is mainly due to an increase in free sterols (Table 2). Physiologically young tobacco leaves contained 1·03 mg of free sterol per g dry leaf tissue while old leaves had 1·23 mg/g. Similarly in leaves of *S. andigena* the increase in free sterol accounted for most of the increase in total sterols [2]. The free sterols are structural components of membranes [14], and since older tissue contains less membrane [15] it is surprising to find the higher free sterol level in this tissue. A possible explanation is that in mature leaf tissue the free sterols are not only structural components of membranes but are also part of a pool which is non-metabolic in nature. In a previous study it was observed that dark grown plants had more free sterol than light grown plants [13]. In the present experiment the lower (mature) leaves received less light and this may have influenced the sterol level. However, tobacco grown over the growing season also showed an increase in sterol level with plant age [3]. In tobacco the acylated steryl glycosides and steryl glycosides accounted for only a minor part of the total sterols and these two sterol conjugates increased slightly with leaf maturity (Table 2). Similar results have been reported for *S. andigena* [2]. However, in potato, which has relatively large quantities of steryl glycoside and acylated steryl glycoside, a significant increase in these sterol forms occurred [1]. The steryl esters were highest in immature leaf tissue of tobacco and lowest in tissue of intermediate age (Table 2). The mature leaves had an intermediate steryl ester content. In *S. andigena*, there was an increase in steryl esters with age but this was not consistent [2].

The individual sterol compositions of the free sterols, steryl esters, steryl glycosides and acylated steryl glycosides were similar (Table 3). Sitosterol was the major sterol in the ester, glycoside and acylated glycoside fraction at all physiological ages and it decreased with maturity. In immature leaves, and leaves of intermediate maturity free stigmasterol was of equal concentration with that of sitosterol but in mature leaves stigmasterol was the major sterol. The decrease in sitosterol and in-

Table 2. Free sterol, steryl ester, steryl glycoside and acylated steryl glycoside content in *Nicotiana tabacum* leaves of different maturity

| Leaf maturity | Free | Ester | Glycoside | Acylated glycoside | Summation |
|---|---|---|---|---|---|
| Immature | 1·03 | 0·30 | 0·29 | 0·04 | 1·66 |
| Intermediate | 1·10 | 0·23 | 0·31 | 0·07 | 1·71 |
| Mature | 1·23 | 0·27 | 0·30 | 0·09 | 1·89 |

Units expressed as mg sterol/g dry wt.

Table 3. Sterol composition of the free sterols, steryl esters, steryl glycosides and acylated steryl glycosides from *Nicotiana tabacum* leaves of different maturity

| Sterol form | Leaf maturity | % Composition by weight Sitosterol | Stigmasterol | Campesterol | Cholesterol |
|---|---|---|---|---|---|
| Free | Immature | 36·9 | 36·6 | 15·7 | 10·8 |
| | Intermediate | 36·9 | 37·0 | 14·2 | 11·9 |
| | Mature | 33·7 | 44·1 | 12·2 | 10·0 |
| Ester | Immature | 49·0 | 16·6 | 20·3 | 14·1 |
| | Intermediate | 46·6 | 21·0 | 18·8 | 13·6 |
| | Mature | 46·7 | 23·6 | 15·8 | 13·9 |
| Glycoside | Immature | 52·3 | 27·5 | 14·0 | 6·2 |
| | Intermediate | 49·1 | 33·0 | 12·7 | 5·2 |
| | Mature | 46·7 | 36·0 | 12·2 | 5·1 |
| Acylated glycoside | Immature | 44·9 | 22·9 | 15·6 | 16·6 |
| | Intermediate | 42·0 | 24·4 | 18·2 | 15·4 |
| | Mature | 39·5 | 28·4 | 15·7 | 16·4 |

crease in stigmasterol with leaf maturity was minor but consistent. In ageing *S. andigena* a general increase was found [2] in free sitosterol and sitosteryl esters but essentially no change was observed in sitosteryl glycoside. Stigmasterol in tobacco (Table 3) was the second most important sterol in the ester, glycoside, and acylated glycoside fractions and it was the only sterol which increased with physiological leaf age in all sterol forms. No comparable increase in stigmasterol with age was observed in *S. andigena* [2]. In the various sterol fractions campesterol accounted for 12–20% (Table 3). The campesterol component decreased with leaf maturity in all sterol forms except in the acylated steryl glycosides. Cholesterol occurred at different levels in the free sterols, steryl esters, steryl glycosides and acylated steryl glycosides, and no quantitative change with maturity was observed (Table 3). The above changes in sterol composition with leaf maturity are not very large. Possibly only minor modifications are required to influence the biochemistry and physiology of the leaf, since it has been shown that slight changes in sterol concentration influence membrane permeability [14].

## EXPERIMENTAL

*Plant material. Nicotiana tabacum* L. variety Burley 21 was grown in the field under conventional methods as described previously [3]. Selected 84-day-old plants were harvested and the leaves taken from the upper one-third, middle one-third and lower one-third of the plant. Each batch of leaves was freeze-dried, the midvein removed, and the remainder ground to pass through a 40-mesh screen. The samples were stored in plastic bags in the dark at room temp. Dry weights of samples were determined at the time of analysis.

*Sterol analysis.* Total sterols were extracted from tobacco (3 g) with acetone (150 ml) in a Soxhlet for 24 hr. The extract was taken to dryness, redissolved in 95% EtOH (50 ml) containing $H_2SO_4$ (0·3 ml) and refluxed for 12 hr. 10% (w/v) KOH in 95% (v/v) EtOH (30 ml) was added and refluxed for 30 min. The mixture was neutralized and extracted 4× with hexane (75 ml) and enough $H_2O$ to obtain two layers. The hexane fractions were pooled, taken to dryness, and the residue redissolved in EtOH. The sterols were precipitated with digitonin as described elsewhere [16]. The quantitative sterol analysis was by GLC. The total sterol values are based on the analysis of six plants.

Two composite samples of the above six plants were produced for the separation of free sterols, steryl esters, steryl glycosides and acylated steryl glycosides. Tobacco (5 g) was extracted with acetone (150 ml) in a Soxhlet apparatus for 24 hr. The dried acetone extract was taken up in hexane and applied onto a 100–300 mesh silica gel column (30 g) packed in hexane [13]. Serial elution was as follows: steryl esters—10% (v/v) benzene in hexane (150 ml) followed by 40% (v/v) benzene in hexane (700 ml); free sterols—benzene (150 ml) followed by $CHCl_3$ (800 ml); acylated steryl glycosides—2% (v/v) MeOH in $CHCl_3$ (700 ml); steryl glycosides—5% (v/v) MeOH in $CHCl_3$ (600 ml). The steryl esters were saponified with 5% (w/v) KOH in 95% EtOH for 30 min and the steryl glycosides and acylated steryl glycosides were hydrolyzed with 0·5% (v/v) $H_2SO_4$ in 95% (v/v) EtOH for 12 hr. The sterols were extracted from the neutralized alcholic mixture with hexane and precipitated with digitonin.

For quantitative and qualitative analysis the sterol digitonide precipitates were cleaved with pyridine containing the internal standard cholestane [17]. The GLC was equipped with a FID, effluent splitter and electronic integrator. A U-shaped glass column (1·80 m × 6 mm) packed with 5% OV-101 on 80/90 mesh Anakrom ABS was used. The column temp. was 262°, flash heater and detector temperatures were 310° and helium was the carrier gas at 95 ml/min. For quantitation corrections were made for differences in detector response.

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## REFERENCES

1. Dupéron, R., Dupéron, P. and Thiersault, M. (1971) *Compt. Rend.* **273D**, 580.
2. Bae, M. and Mercer, E. I. (1970) *Phytochemistry* **9**, 63.
3. Grunwald, C., Bush, L. P. and Keller, C. J. (1971) *J. Agric. Food Chem.* **19**, 216.
4. Davis, D. L. (1972) *Phytochemistry* **11**, 489.
5. Cheng, A. L. S., Kasperbauer, M. J. and Rice, L. G. (1971) *Phytochemistry* **10**, 1481.
6. Dupéron, P. and Dupéron, R. (1969) *Compt. Rend.* **269D**, 157.
7. Davis, D. L., Legg, P. D. and Collins, G. B. (1970) *Crop. Sci.* **10**, 545.
8. Stedman, R. L. and Rusaniwskyj, W. (1960) *Tobacco Sci.* **4**, 17.
9. Ingram, D. S., Knights, B. A., McEvoy, I. J. and McKay, P. (1968) *Phytochemistry* **7**, 1241.
10. Bush, P. B. and Grunwald, C. (1972) *Plant Physiol.* **50**, 69.
11. Kemp, R. J., Goad, L. J. and Mercer, E. I. (1967) *Phytochemistry* **6**, 1609.
12. Genus, J. M. C. (1973) *Phytochemistry* **12**, 103.
13. Bush, P. B., Grunwald, C. and Davis, D. L. (1971) *Plant Physiol.* **47**, 745.
14. Grunwald, C. (1971) *Plant Physiol.* **48**, 653.
15. Hitchcock, C. and Nichols, B. W. (1971) in *Plant Lipid Biochemistry*, pp. 258–259. Academic Press, London.
16. Keller, C. J., Bush, L. P. and Grunwald, C. (1969) *J. Agric. Food Chem.* **17**, 331.
17. Grunwald, C. (1970) *Anal. Biochem.* **34**, 16.