Gas-Liquid Chromatographic Quantification of Solanesol in Chlorophyll Mutants of Tobacco

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A gas–liquid chromatographic method was developed to analyze solanesol content in plants. The data from four tobacco cultivars representing several chlorophyll genotypes showed that solanesol increased in quantity during leaf growth and reached a maximal level at leaf maturation. Flue-curing resulted in an increase of free solanesol, which is attributed to the degradation of solanesyl esters. Solanesol was not present in leaf cutin but accumulated in chloroplasts. Chlorophyll genes *Py* (pale yellow) and *yg* (yellow green) that regulate the growth and development of chloroplasts also affect the accumulation of solanesol and solanesyl esters in green and cured tobacco leaves. The present results demonstrated varietal differences in solanesol content and loss of solanesol during curing. Since solanesol is the possible precursor of carcinogenic compounds in cigarette smoke, its quantity in tobacco leaf may be lowered through breeding and curing processes.

Solanesol ($C_{45}H_{74}O$) is a trisesquiterpene which was first isolated from tobacco (Rowland et al., 1956). This unsaturated alcohol appears to be a common leaf constituent in plants and is believed to be synthesized within chloroplasts (Griffiths, 1965). Solanesol and derivatives, such as solanesyl esters, account for 1.9 to 2.5% of the aged, cured leaves of different tobacco types; hence solanesol is the most abundant terpenoid in tobacco (Stedman, 1968). The physiological function of solanesol in green plants remains undefined; however, in cured tobacco this compound together with other terpenes contribute to leaf aroma and smoke flavor (Davis, 1976). Tobacco terpenes are also precursors of polynuclear aromatic hydrocarbons in cigarette smoke, some of which exhibit carcinogenic property in experimental animals (Wynder and Hoffmann, 1967).

The production of tobacco products with balanced smoke quality and safety so far as the levels of terpenoid are concerned is an intriguing problem. This may be achieved by selection of varieties with a desirable quantity of solanesol. Solanesol quantification in the past utilized a gravimetric procedure, which requires a large sample of leaf, is tedious, and is low in sensitivity (Rowland et al., 1956, Woolen et al., 1972). We report in this paper a gas–liquid chromatographic (GLC) method by which less than 1 g of plant tissue is needed for solanesol quantification. The accumulation of solanesol during leaf development and after flue-curing of tobacco cultivars having different chlorophyll genotypes was compared using this method. In addition, the cellular distribution of solanesol and the conversion of solanesyl esters to free solanesol during flue-curing were studied to determine the possible alteration of solanesol level in tobacco leaves.

MATERIALS AND METHODS

Tobacco Samples. A randomized block experiment employing tobacco, *Nicotiana tabacum* L., cultivars NC95, SC58, and the respective isogenic lines NC95-Py and SC58-yg was conducted with two replications at the Oxford Tobacco Research Station, Oxford, N.C. in 1975. The seed of SC58-yg was obtained from Dr. T. J. Mann, North Carolina State University. In addition to the duplicate dominant factors for chlorophyll content common for the above cultivars, the recessive factor yellow green (*yg*) in SC58-yg limits chlorophyll accumulation in the early stage of leaf growth (Nolla, 1934), whereas the dominant factor pale yellow (*Py*) in NC95-Py characterizes rapid leaf yellowing with the approach of maturity (Chaplin, 1969). Leaf samples were taken from ten plants in each replicate for solanesol analysis. At transplanting, the samples were the three largest leaves and the entire leaves were used. Beginning 4 weeks after transplanting, leaf discs (13 mm in diameter) were taken at 2-week intervals from the three largest leaves at mid-stalk positions. When the normal cultivars were judged to be ripe, tobacco was harvested and green samples were taken from three stalk positions (bottom, middle, and top). All of the green samples were immediately freeze-dried, pulverized to pass through a 40-mesh screen and stored in a freezer until chemically analyzed. Tobacco from the three stalk position was also flue-cured, and samples were taken and processed as was done with green tissues. The data presented are averages of the two replications.

Solanesol Extraction. Solanesol was purified from tobacco leaves according to a procedure previously described (Davis, 1976). Briefly, the hexane extract of macerated tobacco leaves was mixed with Florisil, which was subsequently washed with hexanes to remove long-chained hydrocarbons. The residue containing solanesol was eluted with methanol–hexane (1:9, v/v). The eluate was applied to silica gel thin-layer plates developed with benzene–petroleum ether (1:1, v/v) on a preparative scale. Solanesol was detected with the anisaldehyde–sulfuric acid reagent. Solanesol recrystallized in cold methanol and stored under nitrogen was used as a standard in column and GLC procedures developed. The purity of recrystallized solanesol was confirmed by the NMR spectra and by the GLC analysis of hydrogenated samples with a temperature program of 100–320 °C.

Tobacco powder (0.5 g, desicated over $CaSO_4$) was extracted with 100 mL of cold acetone in a Waring blender for 2 min and then filtered through a Buchner funnel washed with several volumes of acetone. The filtrate was evaporated to dryness in vacuo at ambient temperatures. Hydrocarbons together with plastid pigments were taken up into 2–3 mL of heptane which was passed through a silica gel 60 prepacked column (size B; EM Laboratories, Inc.) with a solvent of heptane–diethyl ether–acetone (6:3:2, v/v/v) as described by DeJong (1975). All solvents

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were reagent grade. Solanesol and nonpolar solanesyl esters were eluted from the column in the first 150 mL which was prior to the elution of chlorophyll a.

Leaf surface waxes and cellular organelles were isolated for studies of cellular distribution of solanesol. Immature and mature NC95 leaves from a field plot were dipped into chloroform and/or 50% acetone for 30 s or less to wash off the surface waxes. The washing solvents were reduced in volume or partitioned with chloroform prior to the GLC analysis. The same leaf samples were used for isolation of chloroplasts and mitochondria by differential and sucrose gradient centrifugation with the procedures previously described (Sheen, 1970). These organelle fractions as well as the whole leaves were freeze-dried and compared for the content of free solanesol.

GLC Quantification of Solanesol. The solanesol fraction obtained from column chromatography was evaporated to dryness in vacuo and hydrogenated for 2 h under 2 psi pressure in 30 mL of distilled chloroform, using 40 mg of platinum dioxide as catalyst according to the method of Taylor and Davies (1975). A 300-μg portion of cholesteryl laurate in chloroform, an internal standard, was added to the sample prior to hydrogenation. After hydrogenation, platinum dioxide was removed by filtration and the solution was reduced to approximately 1 mL in vacuo. GLC analysis was carried out on a Varian Aerograph 1400 equipped with flame ionization detector. A coiled glass column (1 m × 4 mm i.d.) packed with 1% OV-101 on Gas-Chrom Q of 80–100 mesh was used throughout the analysis. Other GLC conditions were: column temperature programmed from 290–320 °C with 3 min holding, followed by 8 °C/min, and nitrogen flow rate of 60 mL/min.

Solanesol, in the form of solanesyl esters, was quantified after hydrolysis of leaf powder (0.5 g) or acetone leaf extracts with 50 mL of 2 N KOH in 20% methanolic solution for 1 h with vigorous shaking. Alkaline hydrolysis of leaf powder liberated solanesol from all forms of solanesyl esters, whereas the acetone extract contained mainly those esters involving fatty acids. After acidification to pH 6 with 2 N HCl, the hydrolysate was partitioned in a separatory funnel immediately with 3 × 25 mL volumes of distilled chloroform. The subsequent hydrogenation and GLC analysis were the aforementioned methods.

RESULTS AND DISCUSSION

GLC Analysis of Solanesol. The solanesol fraction eluted from the silica gel column contains solanesol, solanesyl esters, β-carotene, and many hydrocarbons as visualized on thin-layer silica gel F-254 plates with or without a treatment of anisaldehyde–sulfuric acid reagent. When the plates were developed with hexane–diethyl ether–acetic acid (90:10:1, v/v/v), solanesol and its hydrogenated derivative differentiated each other with R_f values 0.58 and 0.98, respectively. Hydrogenation under the conditions described above rendered a complete conversion of solanesol to the reduced form as evidenced by a total loss of solanesol on the thin-layer plate.

The GLC conditions suitable for solanesol quantification excluded all low molecular weight hydrocarbons which appeared as numerous peaks at or near the solvent front of the gas chromatogram (Figure 1). β-Carotene as carotane had a retention time of about 5 min, whereas the reduced solanesol (octadecahydrosolanesol) and cholesteryl laurate (cholestanyl laurate) had retention time of about 8 and 12 min, respectively. The absence of cholesteryl laurate and other compounds with retention time identical with that of the reduced cholesteryl laurate in tobacco

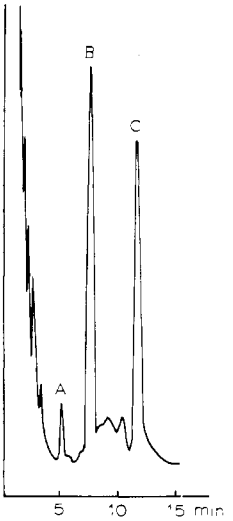

Figure 1. Separation of hydrogenated derivatives of (A) β-carotene and (B) solanesol extracted from tobacco leaves by gas–liquid chromatography. Internal standard is (C) cholesteryl laurate.

assures its suitability as internal standard. Three minor unidentified peaks were present between solanesol and cholesteryl laurate in all tobacco samples analyzed. During the period of experiments, a same relative weight ratio between hydrogenated solanesol and cholesteryl laurate in a standard was obtained, which indicates that the reduced solanesol is stable. The present GLC conditions using appropriate attenuation are also applicable for quantitation of β-carotene.

Precision of Solanesol Quantification. Ten samples (0.5 g each) of the flue-cured NC95 leaves from mid-stalk positions were analyzed for the content of free solanesol by the procedures developed. The mean quantity and standard deviation were 10.98 ± 0.06 mg/g of dry weight, indicating that the present methods of quantification yields reproducible data. To assure that the hydrogenated solanesol is the sole component of the corresponding GLC peak, the hydrogenated fraction of leaf extract containing 2–5 mg of solanesol was streaked onto silica gel F-254 plates which were developed separately with the following solvent systems: benzene–methanol–acetic acid (45:8:4, v/v/v); hexane–benzene (4:1, v/v); hexane–methanol (49:1, v/v); and hexane–chloroform (49:1, v/v). With the exception of the last solvent system whereby the reduced solanesol had a R_f value of 0.88, the others gave a R_f value greater than 0.96. The identity of the hydrogenated solanesol on the plates was confirmed by the GLC analysis. The eluates of the plates devoid of the reduced solanesol were subjected to the same GLC analysis and showed no peak with a retention time identical with that of the hydrogenated solanesol.

Quantitative Change of Solanesol during Leaf Growth and Maturation. On a dry leaf weight basis, solanesol quantity increased with growth and development of the leaf (Figure 2). In comparing the parental line to the isogenic chlorophyll mutant, the expression of *yg* factor during early leaf growth coincided with low solanesol content; conversely, the action of *Py* gene on rapid yellowing of mature leaves was in parallel with the accumulation of solanesol. The latter may be related to leaf senescence which is associated with a rupture of cellular membranes and the destruction of organelles including chloroplasts. The increase of free solanesol in the senescent leaf can be attributed to the degradation of solanesyl esters.

Table I. Quantity of Free and Esterified Solanesol in Mature Leaves from Middle Stalk Position of Four Tobacco Chlorophyll Genotypes before and after Flue-Curing

Genotype	Solanesol in acetone extract, mg/g of dry wt	Solanesol in alkaline hydrolysate of acetone extract, mg/g of dry wt	Solanesol in alkaline hydrolysate of tissue, mg/g of dry wt	Percent of solanesol in solanesol esters
		Leaf before curing		
NC 95	8.77	9.25	19.19	54
NC 95-Py	6.16	9.06	26.53	77
SC 58	4.54	4.68	10.21	56
SC 58-yg	4.62	5.28	19.67	77
LSD 0.05	2.38		5.68	
LSD 0.01	3.41		8.16	
		Leaf after flue-curing		
NC 95	10.74	14.00	15.75	32
NC 95-Py	11.08	15.19	15.01	26
SC 58	8.49	12.66	12.08	30
SC 58-yg	14.94	19.24	19.70	24
LSD 0.05	3.78		3.66	
LSD 0.01	5.43		4.68	

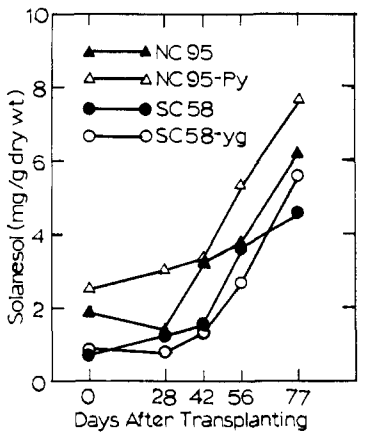

Figure 2. Accumulation of free solanesol during leaf growth and development in four chlorophyll genotypes of tobacco.

Cellular Distribution of Solanesol. During leaf growth, hydrocarbons as waxy substances are deposited on the leaf surface. The possible deposition of solanesol on leaf surface was examined by the GLC method. Solanesol was not detected, suggesting that the accumulation of solanesol during leaf growth occurred inside the cell. In view of the evidence that chloroplasts are the sites of solanesol biosynthesis and accumulation (Stevenson et al., 1963; Griffiths, 1965), free solanesol was quantified in chloroplasts, mitochondria, as well as in mature leaves. On a dry weight basis, the amount of solanesol was 25.15, 8.85, and 5.25 mg/g in chloroplasts, mature leaves, and mitochondria, respectively. The mitochondrial fraction usually contains chloroplast fragments; therefore, the association of solanesol with mitochondria is questionable. Chloroplasts from seedling leaves of NC95 contained free solanesol less than 10% of that found in chloroplasts of mature leaves. The present results not only substantiate the fact that solanesol is probably synthesized and accumulated in chloroplasts but also shown its association with chloroplast growth and development. This is consistent with the role of solanesol as the precursor of plastoquinone in chloroplasts (Goodwin, 1971). However, the significance of the greater solanesol content in tobacco chloroplasts than in those of other plant species (Stevenson et al., 1963) remains to be elucidated.

Effect of Stalk Positions and Flue-Curing. In most cases, free solanesol increased in quantity from lower to upper leaf stalk position (Figure 3). This pattern held true for both mature leaves at time of harvest and after flue-curing. Flue-curing increased free solanesol. Therefore, free solanesol accumulation seems to be related to chloroplast disintegration which takes place during leaf senescence and curing. Leaf senescence does not occur uniformly throughout the leaf, as evidenced by a random distribution of green and yellow areas in mature tobacco leaves that are ready to be primed and cured. Such variation within a leaf may determine the quantitative distribution of solanesol; hence leaf disc samples composed of predominantly the yellowing laminae of primed leaf would be expected to have high levels of free solanesol. This is a possible explanation for the great amount of free solanesol in the NC95 samples from middle stalk leaves. Within a tobacco plant the quantity of chlorophyll and carotenoids were the greatest in top leaves and declined in those toward the lower stalk positions (Grunwald et al., 1977). This is in keeping with the distribution of solanesol and supports the premise that solanesol is associated with chloroplasts in the leaf.

Increase of Solanesol by Alkaline Hydrolysis. The increase of free solanesol during flue-curing may be derived from the degradation of solanesyl esters. A number of esters as conjugates of solanesol and fatty acids have been identified in cured tobacco leaves (Rowland and Latimer, 1959). These esters are extracted with acetone and are eluted from a silica gel column in advance of free solanesol. Upon alkaline hydrolysis, the acetone extract of primed leaves from middle stalk positions yielded 3% more solanesol in SC58 and up to 47% in NC95-Py (Table I). However, the increased amount failed to account for total solanesol quantity in the acetone extract of the corresponding flue-cured leaves. Furthermore, the averaged quantity (11.31 mg/g) of four culitvars retained 35% more solanesol in the form of esters extractable in acetone. These results clearly point to the presence of additional solanesyl esters that are not extracted by acetone. Alkaline hydrolysis of the primed leaf resulted in more than onefold increase in free solanesol quantity. The two chlorophyll mutants contained more solanesyl esters than the respective parental lines. On the average of four cultivars, 68% of solanesol existed in the form of solanesyl esters but only 5.5% consisted of the esters of fatty acids. Since solanesol is associated with chloroplasts, it may occur as a component of lipoproteins in chloroplast membrane. Curing processes that cause the rupture of chloroplast membrane would facilitate the liberation of solanesol, owing to protein degradation.

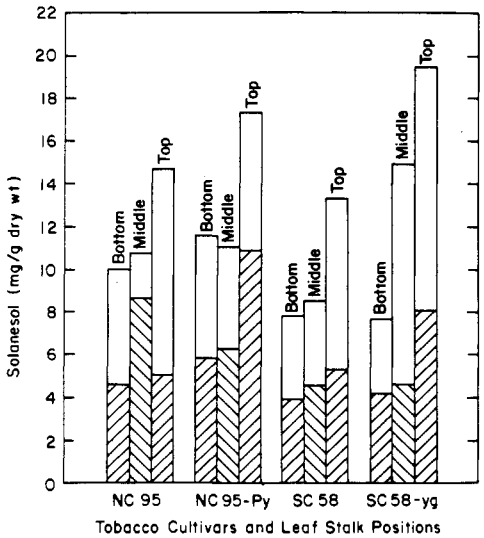

Figure 3. Effect of stalk positions and flue-curing on the amount of free solanesol in the leaf of four chlorophyll genotypes of tobacco. Shaded columns represent the quantity at time of harvest, whereas open columns show the increased amount after flue-curing.

In flue-cured leaf, solanesyl esters in acetone extract increased substantially and their quantity was equal to the amount of esters liberated by alkaline hydrolysis of cured tissue. This suggests the occurrence of transesterification as a consequence of the degradation of nonacetone-extractable solanesyl esters during curing. The amount of acetone extractable solanesyl esters varies from 3.26 to 4.30 mg/g of the four cultivars, which is at least tenfold more than the reported quantity (0.27 mg/g) in flue-cured tobacco (Rowland and Latimer, 1959). The reported solanesyl esters are mainly identified as fatty acid esters. The present results, however, suggest that a higher proportion of solanesol is esterified with low molecular weight acids rather than fatty acids. In flue-cured leaf, approximately one-third of the solanesol remained in esterified form, but total solanesol was 17% less than that of the primed leaf before curing. This change in quantity indicates the degradation of solanesol during flue-curing.

In summary, the present GLC method greatly facilitates the quantification of solanesol not only with small amounts of tobacco leaves but also with cellular fractions such as chloroplast. Solanesol accumulates mainly in chloroplasts, and solanesyl esters are the predominant form of solanesol in green tobacco leaves. The degradation of solanesyl esters during leaf senescence and curing resulted in an increase of free solanesol. Varietal differences in solanesol quantity are evident (Table I), correlations with chlorophyll genotypes were inconclusive. The present results indicate that total solanesol in tobacco leaf may be modified by breeding. This should be initially approached by the investigation of the genetic variability of solanesol within *Nicotiana tabacum* and among *Nicotiana* species. Since total solanesol decreased during flue-curing, the feasibility of altering its quantity through curing processes and effects of various curing methods on minimizing solanesol and solanesyl ester content in cured tobacco leaves are being studied.

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