Journal of Chromatography, 139 (1977) 269–282

CHROM. 10,056

GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF FREE AND TOTAL SOLANESOL IN TOBACCO

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(Received March 4th, 1977)

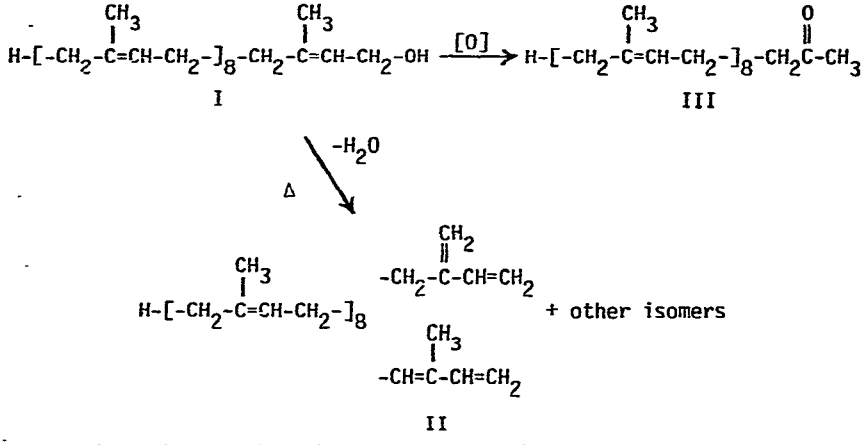
SUMMARY

Solanesol, a trisesquiterpenoid alcohol in tobacco leaf, has been shown to be an important precursor of the tumorigenic polynuclear aromatic hydrocarbons of smoke. Thus, a rapid, reproducible method for the determination of leaf solanesol levels is desirable. We developed procedures based on high-temperature gas chromatography for the analyses of free and total solanesol. The alcohol, as its trimethylsilyl derivative, was separated and quantified on a short Dexsil 300 GC column, with 1,3-dimyristin as an internal standard. The free alcohol was determined by direct derivatization of ground tobacco and its hexane extract with N,O-bis(trimethylsilyl)-acetamide–dimethylformamide reagents. For total solanesol, the ground tobacco or its hexane extract was saponified with ethanolic potassium hydroxide. Acidification and hexane extraction yielded samples suitable for silylation and gas chromatographic analysis. Evaluation of the various methods indicated that free solanesol was best determined in the hexane extract of tobacco, and total solanesol after saponification of ground tobacco. Total solanesol levels in commercially important tobacco types were determined.

INTRODUCTION

The identification of polynuclear aromatic hydrocarbons (PAH) as major contributors to the tumorigenicity of cigarette smoke condensate[1–3] has raised the question of their origin. To determine the PAH precursors in tobacco leaf, several pyrolytic or thermal decomposition studies of tobacco leaf extracts have been conducted[4]. The results indicated that hexane extracts produce disproportionately large yields of PAH and benzo[α]pyrene, a most potent member of the group. The results of a subsequent study strongly suggested[5] that terpenoid components of the hexane extract are the potent precursors of smoke PAH and that the reduced tumorigenicity of the smoke from reconstituted tobacco sheet[6] is due to the partial removal of terpenoid compounds during the sheet manufacture. More recently, a study of the pyrolytic formation of PAH from the light petroleum-extractable constituents of

flue-cured tobacco leaf[7] found that the terpene, solanesol (I; 3,7,11,15,19,23,27,31,35-nonamethyl-2,6,10,14,18,22,26,30,34-hexatriacontanonaen-1-ol; Fig. 1), may produce more than 30% of the total PAH in the pyrolyzate of the total extract. This indicated that solanesol is a major precursor of the tumorigenic PAH of tobacco smoke and that utilization of tobacco with low solanesol content would lead to safer smoking products.



I III II

Fig. 1. Thermal conversion of solanesol (I) to solanesenes (II).

Solanesol was first isolated from flue-cured tobacco by Rowland *et al.*[8] in 1956 in quantities corresponding to 0.4% by dry weight of leaf. Other researchers[9,10] reported solanesol levels to be between 1 and 2% of dry weight of tobacco leaf. Thus, this C_{45} terpenoid is the most abundant component in the lipid fraction of tobacco and because of its co-relation to smoke PAH solanesol levels will have to be determined in all tobacco varieties.

To evaluate solanesol contents of different tobacco varieties, we required a rapid, quantitative method. A review of methods for the gravimetric determination of solanesol by column chromatography on silicic acid (SA), alumina, or Florisil showed that, generally, low and variable values were obtained[9,10]. The thin-layer chromatographic (TLC) densitometric method of Woolen and Jones[10] was considered undesirable since we had observed considerable decomposition on the plates. However, a report by Welburn and Hemming[11] on the gas chromatographic analysis of acetates and trimethylsilyl (TMS) derivatives of long-chain isoprenoid alcohols suggested that short-column, high-temperature gas chromatography (GC) could be used for analysis of solanesol. We adopted this approach and now describe the GC methods we developed for analyzing both free and total solanesol in tobacco leaf.

EXPERIMENTAL

Materials

All solvents (Burdick and Jackson*, Muskegon, Mich., U.S.A.; distilled-in-

* Reference to a company or product name does not imply approval or recommendation by the USDA.

glass) were redistilled according to the procedure described by Schepartz *et al.*[12]. Dimethylformamide (DMF) and N,O-bis(trimethylsilyl)acetamide (BSA) were silylation grade (Analabs, North Haven, Conn. U.S.A.). Stock solanesol was obtained from Hoffmann-LaRoche (Nutley, N.J., U.S.A.). The dimyristin internal standard was used as obtained from Analabs. The following tobacco samples were analyzed:

Flue-Cured (1968)—commercial Eastern Carolina type, flue-cured, redried, aged.

Burley I (1975)—United States Department of Agriculture, Agricultural Research Service (USDA-ARS), experimental, air-cured.

Burley II (1971)—commercial, air-cured, redried.

Cigar Filler (1972)—commercial, Pennsylvania, air-cured.

Maryland (1971)—commercial, air-cured, redried.

Turkish (1967)—commercial, Samsum, sun-cured.

Tobacco sample preparation

Tobacco samples were equilibrated at laboratory conditions for two days and then ground in a Wiley Mill to pass through a 32-mesh screen.

Determination of dry tobacco weight

For moisture determination, 200 mg of ground sample was heated for 3 h at $95 \pm 0.5°$ in a vented oven[13]. Moisture was determined before each extraction or hydrolysis.

Gas chromatography

GC analyses were performed on a Hewlett Packard Model 5750 gas chromatograph equipped with a 18 in. × 1/8 in. stainless-steel column containing 5% Dexsil 300 GC on 100–120 mesh Chromosorb W-AW (temperature program was 210° for 4 min, 210–330° at 6°/min, and 330° for 6 min; helium flow-rate, 50 ml/min; injector temperature, 300°; and flame ionization detector temperature, 350°). Peak areas were measured with an Autolab Systems IV integrator. (The above temperature program was required to obtain satisfactory baseline tracking of the integration system. During developmental work, other GC conditions were used and they are listed in the text as discussed.)

For preparative GC, the columns were switched from the flame ionization detector to a thermal conductivity detector (maintained at 330°). The components were collected in melting point capillary tubes under conditions identical to those used for analytical GC.

To obtain several columns with essentially identical resolution and retention characteristics, the following procedure for column preparation was used. A 15 ft. × 1/8 in. portion of stainless-steel tubing was washed consecutively with about 300 ml each of benzene, acetone, chloroform and acetone. The column was dried by air pulled through it for about 45 min and filled with 5% Dexsil 300 GC on 100–120 mesh Chromosorb W-AW by the gravity-vertical drop method[14]. The column was conditioned under helium flow (50 ml/min at room temperature) by repeated (about 10 times) heating from 90 to 330° at 2°/min, with a 1-h hold at 330°. To extend column life, the carrier gas was passed through a molecular sieve trap, followed by an oxygen trap. The ends of the column (about 6 in.) were discarded and the remainder

was cut into 18-in. sections. About 0.5 in. of the packing from each end was carefully removed and replaced with silanized glass wool. A glass liner was placed in the injection port and the 18-in. column was conditioned by two injections of about 25 μl of BSA–DMF (1:1) followed by temperature programming under conditions listed above. The glass liners were changed periodically to prevent excessive build-up of non-volatile material.

Column chromatography

About 0.5 g of hexane extract or an equivalent amount of hydrolyzed hexane extract as described below was deposited on 20 g of pre-washed, activated SA and placed on a 100-g SA column as described by Severson *et al.*[15]. The column was eluted with 1-l portions of light petroleum, benzene–light petroleum (1:3, v/v), benzene, diethyl ether and methanol. Eluate was collected in 100-ml fractions and reduced in volume prior to GC analysis. Fractions containing similar components were combined and subjected to preparative and analytical GC analyses.

Purification of solanesol

About 1 g of stock solanesol (80% purity by GC), dissolved in benzene, was placed on a 100-g SA column. The column was eluted with 1-l portions of benzene and diethyl ether. The ether fraction was evaporated to dryness. GC analysis of its TMS derivative showed that the ether fraction consisted of low-molecular-weight impurities and solanesol at a purity of about 89%. The ether fraction was subjected to preparative high-pressure liquid chromatography (HPLC) on a Varian 8500 liquid chromatograph on a 50-cm, 10-μm silica column. The eluate was monitored with a Varian Aerograph refractive index detector. The center cut of the major component, which was eluted after about 11 min with methylene chloride–hexane (1:4) at a flow-rate of 90 ml/h, was collected in a screw top test tube. The solvent was removed by a stream of nitrogen. After repeated collections and solvent removal, the test tube was placed in a vacuum desiccator under nitrogen and the residue dried under reduced pressure for several hours.

Free solanesol determination via hexane extract

A 80 × 25 mm cellulose extraction thimble containing 7–9 g of ground tobacco and a glass wool plug was placed in a small Soxhlet extractor fitted with an Allihn condenser and a 300-ml flat-bottom boiling flask containing boiling stones and 250 ml of hexane. (The hexane was distilled[12] from potassium hydroxide and purged with nitrogen before use.) The tobacco was extracted under a blanket of nitrogen for 16–18 h with rapid recycling of the hot hexane[16]. After the extract had cooled, the hexane was removed on a roto-evaporator. The residue was dried by azeotropic distillation *in vacuo* with benzene (thrice 10 ml) and quantitatively transferred with hexane to a 10-ml volumetric flask. From 0.1–0.2 ml of the extract solution and exactly 1.0 ml of the 1,3-dimyristin internal standard solution (1 mg/ml in benzene) were quantitatively transferred to a tapered test tube. After removal of the solvent under a stream of nitrogen, 35 μl each of BSA and DMF were added to the residue. The test tube was sealed with a PTFE-lined cap and heated for 10 min at 76°. An aliquot (1 to 5 μl) was analyzed by GC.

Total solanesol determination via hydrolyzed hexane extract

An aliquot (1 to 2 ml) of the volumetrically diluted hexane extract solution, described above, was quantitatively transferred to a 300-ml saponification flask, containing 40 ml of 2 *N* ethanolic potassium hydroxide and fitted with a 24/40 reflux condenser. The mixture was refluxed under nitrogen for 2.5 h and then cooled. Saturated potassium chloride solution (5 ml) was added and the solution was acidified to pH 2 with concentrated hydrochloric acid. Then, 10 ml of hexane was added, and the mixture was shaken. If necessary, small portions of water were added until a clear meniscus was obtained. The mixture was quantitatively transferred to a 125-ml separatory funnel and extracted with 10-ml portions of hexane until two successive hexane extracts were colorless. The combined hexane extracts were quantitatively diluted to 100 ml. This solution (1 to 2 ml) and 1.0 ml of the internal standard solution were quantitatively placed in a tapered test tube and the solvent removed. The residue was treated with BSA and DMF as described above and the silylated mixture was analyzed by GC.

Total solanesol determination via direct hydrolysis of ground tobacco

About 2 g of ground tobacco and 40 ml of 2 *N* ethanolic potassium hydroxide were placed in a saponification flask and the sample was hydrolyzed and processed for GC analysis for its solanesol content by the procedures described above.

Free solanesol via direct BSA–DMF extraction of tobacco

About 50 mg of ground tobacco and 1.0 ml of internal standard solution were placed in a tapered test tube or Reacti-Vial. After removal of the solvent under a stream of nitrogen, 100-μl portions of BSA and DMF were added. The vial was sealed with a PTFE-lined cap and vigorously agitated on a Super-Mixer (Lab-Line Instruments). The sample was heated for 30 min at 76° with frequent agitation. The tobacco was allowed to settle and 1 to 5 μl of the solution were analyzed by GC.

Spectral analysis

The components collected in melting point capillary tubes by preparative GC were removed with hexane and slowly deposited as thin films on potassium bromide plates. After the solvent was allowed to evaporate, IR analysis was done using a Beckman IR 4230 spectrophotometer. The samples were washed from the potassium bromide plates with cyclohexane into cuvettes and UV data were obtained using a Beckman Acta C-III spectrophotometer. Small portions of the capillary tubes containing GC preparative material were placed in the direct insertion probe and were analyzed using a DuPont 21-492 spectrometer.

RESULTS AND DISCUSSION

To develop a successful GC method for solanesol, we have applied the short GC column methodology of Parkin and Schuller[17]. Stock solanesol was analyzed by GC on the short column using a temperature program from 160 to 330° for 6°/min. Its chromatogram showed four major GC peaks (Fig. 2). Comparison of the IR and UV data of the major components, isolated by preparative GC, with data reported by Rodgman *et al.*[18] showed that the first three peaks in Fig. 2 were a mixture of

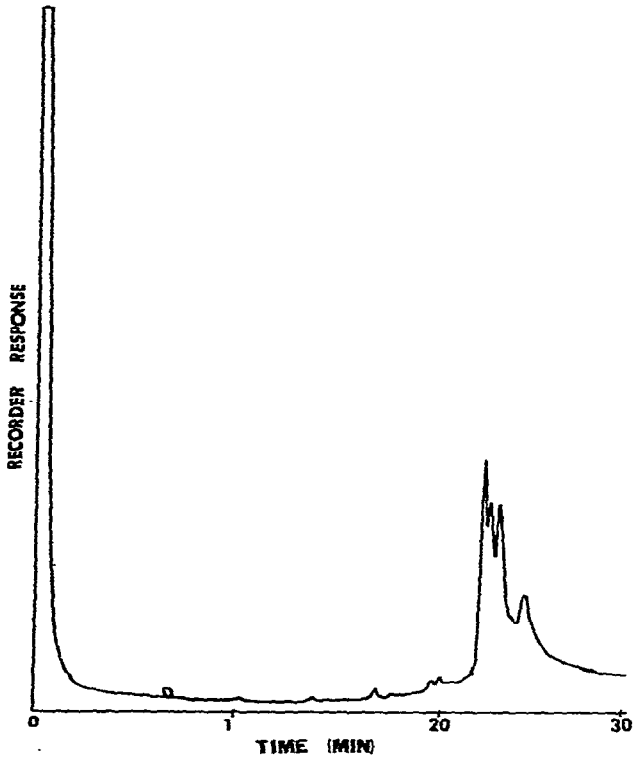


Fig. 2. Direct GC of solanesol on Dexsil 300 GC.

solanesenes (II, Fig. 1), formed by the thermal dehydration of solanesol. Therefore, direct GC analysis of the C_{45} alcohol was not feasible.

Because Welburn and Hemming[11] reported successful chromatography of the trifluoroacetate of solanesol, we reacted solanesol with excess trifluoroacetic anhydride in a sealed vial at 60° for 15 min. IR analysis of the product after removal of the excess anhydride showed that the alcohol had been quantitatively converted to the acetate. However, the solanesyl trifluoroacetate appeared to decompose almost quantitatively to II during GC analysis (Fig. 3). The formation of II during GC was confirmed by IR analysis after preparative GC. Pyrolysis of solanesol acetate has been shown to produce a hydrocarbon fraction (II) identical to that obtained from the dehydration of the parent alcohol[18].

The next attempt to volatilize solanesol was the preparation of the TMS derivative by reaction of the alcohol with BSA reagent in DMF. This attempt was successful and yielded only one GC peak, eluting in 23 min (320°) using an oven temperature program of isothermal hold at 210° for 5 min followed by a 6°/min increase to 330°. For confirmation of its identity the peak was collected by preparative GC and analyzed by IR (Fig. 4). Characteristic silyl ether absorption bands at 1250, 1065, 845 and 750 cm^{-1} (ref. 19) confirmed it to be the trimethylsilyl ether of solanesol (TMS-I).

This derivatization–GC approach was next applied to the determination of

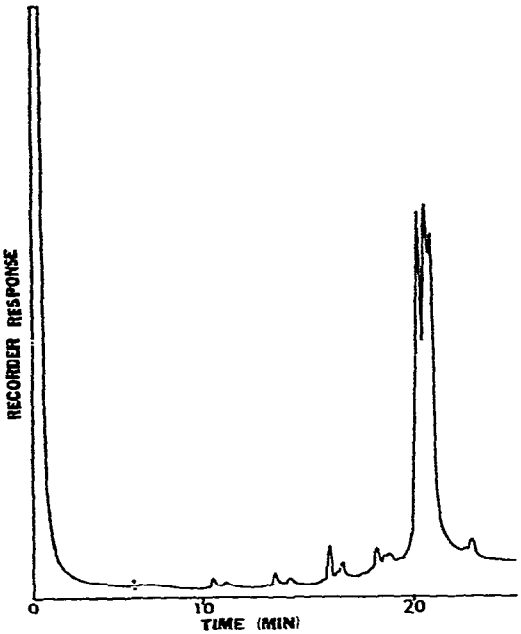


Fig. 3. Gas chromatogram of solanesol trifluoroacetate, showing thermal decomposition to solanesenes.

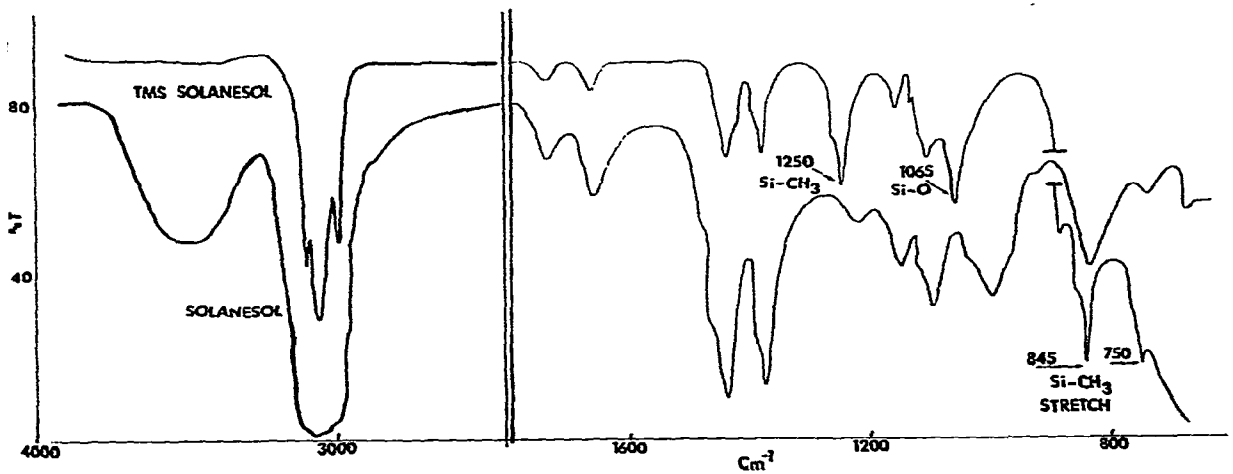


Fig. 4. IR spectrum of TMS derivative of solanesol.

solanesol in the hexane extract of flue-cured tobacco. The resulting gas chromatogram is shown in Fig. 5. Preparative GC cuts that corresponded to peaks 1, 2, 3, and 4 were obtained. The IR spectrum of peak 4 was identical to TMS-I. Spectral analysis of the triplet preceding TMS-I showed that 1 and 3 were identical to solanesenes. Peak 2 yielded an IR spectrum identical to solanesenes, except for a carbonyl ab-

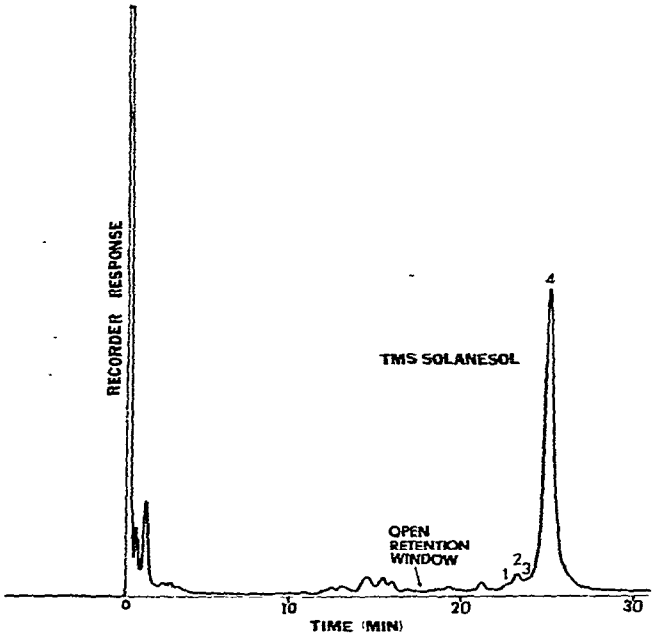


Fig. 5. Gas chromatogram of the TMS derivatives of the hexane extract of flue-cured tobacco.

sorption at 1720 cm^{-1}. The spectrum of the material in the tailing portion of peak 4 contained ester absorption bands. Comparison of GC retention data of known compounds in the hexane extract of tobacco[8] indicated that these bands were due to very low levels of steryl esters.

Since solanesyl esters have been reported in flue-cured tobacco[8,20], it was necessary to determine the quantity of bound solanesol, in addition to the free solanesol analyzed by the above procedure. Accordingly, the hexane extract was saponified with ethanolic potassium hydroxide, and the recovered organics were derivatized. The gas chromatogram of the TMS derivatives is shown in Fig. 6. Preparative GC cuts were obtained for peaks 1–4. The IR spectrum of the material corresponding to the back portion of peak 4 showed no ester absorption, indicating the absence of steryl esters. IR and UV spectra showed that peaks 1, 2, and 3 were identical to those labeled correspondingly in the hexane extract.

To determine whether solanesenes (peaks 1, 2, and 3) and the carbonyl compound in peak 2 were formed during the derivatization and/or GC, we separated portions of the starting and hydrolyzed hexane extracts by SA column chromatography. Continuous monitoring of the eluant by GC revealed the majority of solanesenes (characterized after preparative GC by IR, UV) eluted from the column with light petroleum–benzene (1:3). Mass spectral data of this preparative material revealed that co-eluting with the solanesenes (*m/e* 612) by both SA chromatography and GC were other similar hydrocarbons with masses of 614, 616 and 618 a.m.u. The steryl esters, present only in the starting hexane extract, also eluted in this fraction. In good agreement with our previous assignments, these esters began to

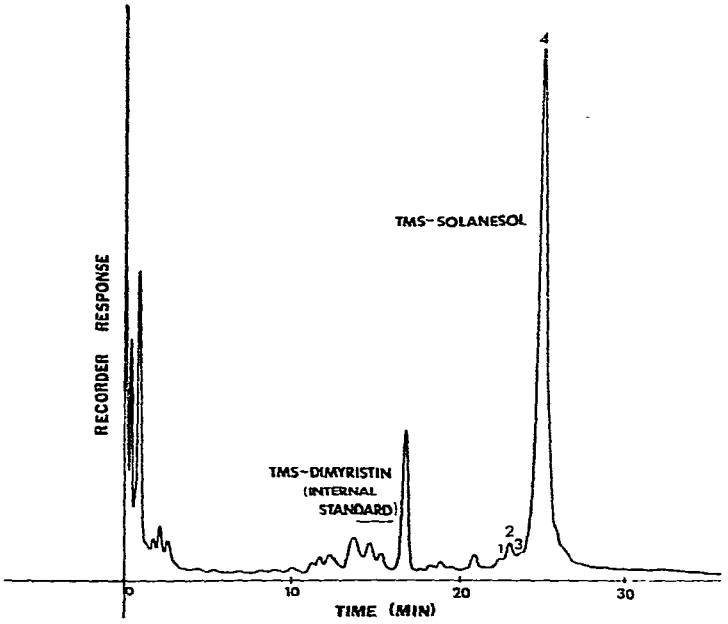


Fig. 6. Gas chromatogram of the TMS derivatives of base hydrolyzed hexane extract of flue-cured tobacco.

elute from the GC column on the backside of the TMS-I peak. Benzene eluted a carbonyl component whose GC retention time was identical to that of peak 2 (Figs. 5 and 6). After preparative GC, the UV, IR and mass (*m/e* 602) spectra of this compound were identical to the lipid component, bombiprenone (III; 6,10,14,18,22,26,30,34-octamethyl-5,9,13,17,21,25,29,33-pentatriacontaen-2-one), isolated from flue-cured tobacco by Irwine *et al.*[21]. They postulated that bombiprenone is formed during the biochemical breakdown of plastoquinone-A in the ripe leaf. Since bombiprenone likely derives from a C_{45} isoprenoid moiety, we included it in quantitating the C_{45} terpenes. Diethyl ether eluted residual traces of bombiprenone with solanesol from the SA column. Mass spectrometry of TMS-I obtained by preparative GC showed a molecular ion at *m/e* 702 and the usual fragmentation patterns of silyl ethers[19]. The levels of solanesenes and bombiprenone determined in this manner were in good agreement with those in total extract. These findings showed that little, if any, solanesol was decomposed during hydrolysis, derivatization, and/or GC. Analyses of the SA fractions showed that with the hydrolyzate the GC retention window for TMS-I was essentially free of other GC volatile material and that in the GC of the hexane extract less than 2% of the apparent peak area assigned to TMS-I was due to steryl esters. Thus, solanesol, solanesenes and bombiprenone can be quantitated by direct GC analyses of both the total and hydrolyzed tobacco hexane extract.

The success of the potassium hydroxide hydrolysis of the hexane extract in determining the total solanesol content spurred us to abbreviate the procedure by direct potassium hydroxide hydrolysis of ground tobacco. The resulting gas chromatogram of the TMS derivatives of the tobacco hydrolyzate products was identical

to that of the hexane extract hydrolyzate. In this way, extraction with hexane could be eliminated and total solanesol analyzed by a rapid, two-step procedure—the first step being direct ethanolic potassium hydroxide extraction hydrolysis of a tobacco sample and the second step GC analysis.

Because the presence of glucosidated sterols has been confirmed in both tobacco leaf and smoke[22], it appeared possible that a small amount of solanesol also exists in leaf as a glucoside. Since base hydrolysis would not cleave glycosidic linkages, the hexane extract was hydrolyzed by both sulfuric acid and potassium hydroxide[23]. Fig. 7 shows the disappointing results of this treatment. Solanesol was almost completely destroyed by the acid. Thus, in the following discussion, "bound solanesol" refers to that obtained by base hydrolysis of solanesyl esters.

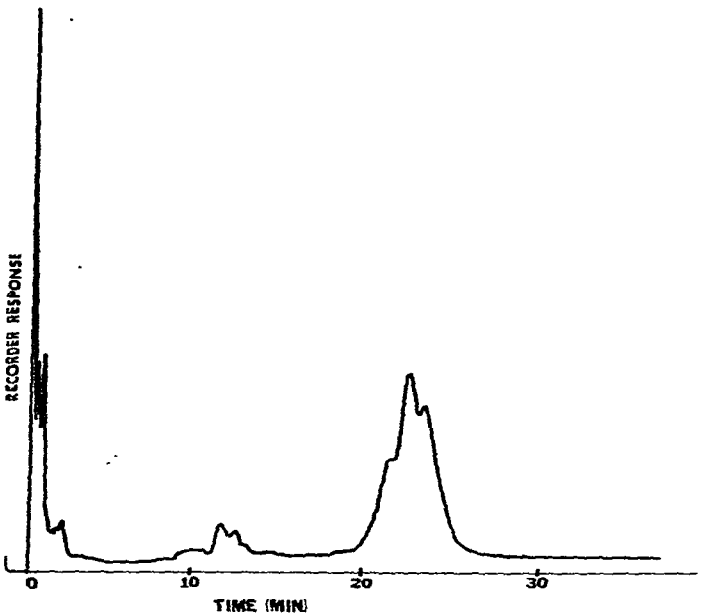


Fig. 7. Gas chromatogram of the TMS derivatives from acid and base hydrolyzed hexane extract of flue-cured tobacco.

A one-step analysis for free solanesol was also attempted by direct derivatization of ground tobacco. GC of the derivatives yielded a chromatogram identical to that obtained from the tobacco hexane extract.

To obtain a detector response factor for solanesol, we needed pure solanesol. However, solanesol purified by TLC, recrystallization, or column chromatography, still showed carbonyl impurities when analyzed by IR or the presence of solanesenes and/or bombiprenone when analyzed by GC. Column chromatography on silicic acid and then HPLC with 10-μm silica, yielded acceptably pure solanesol (94% by GC).

We were able to quantitate the method when we found that the TMS derivative of 1,3-dimyristin fitted perfectly into an open retention window in the chromatogram of both the hexane extract and saponified extract (Fig. 5 and 6). Solanesol levels could now be quantitated with this internal standard. The gas chro-

matogram of the HPLC purified solanesol and dimyristin is shown in Fig. 8. The other peaks surrounding the TMS-I peak were assumed to be related isoprenoids; consequently, the total area was used for the calculation of response data.

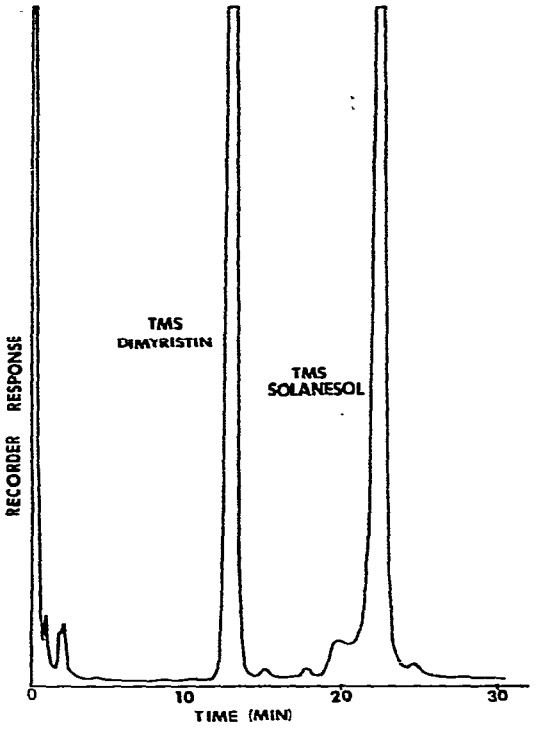
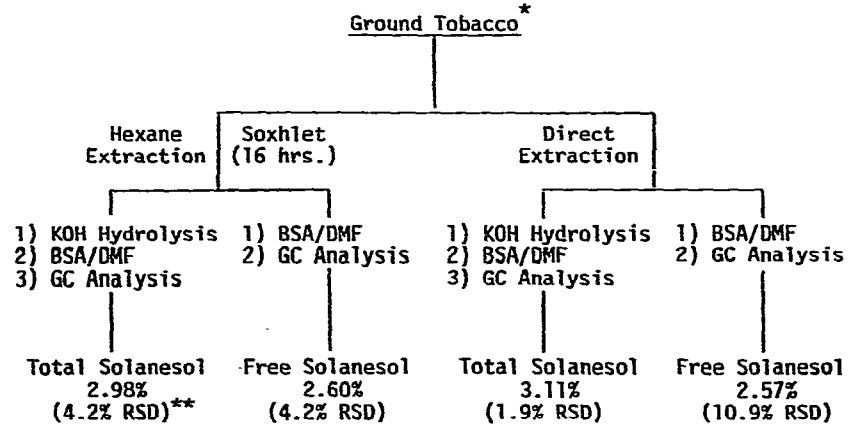


Fig. 8. Gas chromatogram of the TMS derivative of "pure" solanesol.



*Eastern Carolina, flue-cured, redried, and aged tobacco.
**RSD = Relative Standard Deviation from the mean.

Fig. 9. Summary of solanesol methodology.

TABLE I

COMPARISON OF METHODS FOR THE DETERMINATION OF FREE AND TOTAL SOLANESOL IN TOBACCO LEAF (EASTERN CAROLINA FLUE-CURED)

	Method	*Isoprenoid*	*Run number*								*Mean ± S.D.*
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
			% Weight of dry leaf								
Free isoprenoids	Hexane extraction of tobacco	Solanesol	2.74	2.61	2.44						2.60 ± 0.11
		Solanesenes, Bombiprenone	0.18	0.17	0.16						0.17 ± 0.08*
	BSA–DMF extraction of tobacco	Solanesol	2.52	2.46	2.53	2.73	3.10	2.62	2.08	2.54	2.57 ± 0.28
		Solanesenes, Bombiprenone	0.09	0.10	0.14	0.17	0.21*	0.16	0.11	0.16	0.14 ± 0.04*
Total isoprenoids	KOH hydrolysis of tobacco hexane extract	Solanesol	3.00	2.84	2.88	3.14	2.99	3.02			2.98 ± 0.05
		Solanesenes, Bombiprenone	0.28	0.21	0.22	0.26	0.26	0.25			0.24 ± 0.01*
	KOH extraction of tobacco	Solanesol	3.11	3.12	2.93	3.10	3.29				3.11 ± 0.06
		Solanesenes, Bombiprenone	0.30	0.29	0.25	0.24	0.16				0.25 ± 0.03*

* Calculated assuming a detector response identical to that for TMS-Solanesol.

All of the attempted solanesol methods are summarized in Fig. 9, and the quantitative aspects of the determination for free and total solanesol, or more accurately isoprenoids, are given in Table I. The table lists the data for an Eastern Carolina, flue-cured, redried tobacco analyzed by the various methods. Both procedures for the analysis of the free isoprenoids yielded essentially identical values. However, the direct silylation procedure yielded data with a much larger deviation. Comparison of the two methods for total solanesol showed that the average value was about 4% higher by the direct hydrolysis method. However, levels for solanesenes and bombiprenone were the same for both methods. The direct hydrolysis procedure, with only a 1.9% relative standard deviation from the mean, appeared to be the better method for determining total solanesol. By difference, about 14–19% of the C_{45} isoprenoids were bound by base-hydrolyzable linkages.

Subsequently, we analyzed a series of tobacco samples by the two potassium hydroxide hydrolysis methods for total solanesol and compared the results (Table II). Except for the Burley II sample, the direct potassium hydroxide hydrolysis of ground tobacco gave a higher value for total solanesol than the potassium hydroxide hydrolysis of the hexane extract.

TABLE II

DETERMINATION OF TOTAL SOLANESOL

Tobacco type	*% Dry Leaf*					
	KOH hydrolyzed hexane extract method			*KOH hydrolyzed tobacco method*		
	Solanesenes, bombiprenone (%)	*Total solanesol (%)*	*Total isoprenoids (%)*	*Solanesenes, bombiprenone (%)*	*Total solanesol (%)*	*Total isoprenoids (%)*
Flue-Cured*	0.24	2.99	3.23	0.25	3.11	3.36
Burley I**	0.33	2.07	2.40	0.18	2.14	2.32
Maryland***	0.22	2.04	2.26	0.13	2.09	2.22
Turkish§	0.06	0.87	0.93	0.07	1.20	1.27
Burley II§§	0.17	0.88	1.05	0.10	0.81	0.91
Cigar Filler§§§	0.13	0.78	0.91	0.09	0.98	1.18

* 1968, Commercial Eastern Carolina, flue-cured, aged, and redried.
** 1975, ARS Experimental, air-cured.
*** 1971, Commercial, air-cured, and redried.
§ 1967, Samsun.
§§ 1971, Commercial, air-cured, and redried.
§§§ 1972, Commercial, Pennsylvania, air-cured.

ACKNOWLEDGEMENTS

We thank Drs. S. H. Rubin and W. E. Scott, Hoffmann-LaRoche, Inc., for providing us with a generous supply of solanesol and Mr. Howard C. Higman, USDA, ARS, Tobacco Laboratory, Athens, Georgia, for obtaining mass spectral data.

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