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Determination of solanesol in tobacco by capillary gas chromatography

W. J. CHAMBERLAIN*, R. F. SEVERSON and O. T. CHORTYK
United States Department of Agriculture, Agricultural Research Service, R. B. Russell Agricultural Research Center, Tobacco Quality & Safety Research Unit, P. O. Box 5677, Athens, GA 30613 (U.S.A.)
and
V. E. SISSON
United States Department of Agriculture, Agricultural Research Service, Crops Research Laboratory, P. O. Box 1555, Oxford, NC 27565 (U.S.A.)
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ABSTRACT

A gas chromatographic (GC) method has been developed for solanesol, a long-chain (C_{45}) terpenoid alcohol of tobacco. The method separates and quantitates solanesol as its volatile trimethylsilyl derivative. After saponification of the tobacco sample with methanolic potassium hydroxide to liberate bound solanesol, total solanesol was determined on a wide-bore fused-silica SE-54 capillary column. The reproducibilities of both the extraction and GC methods were found to be excellent. As an example of application, six tobacco varieties, used in a low-solanesol tobacco breeding study, were analyzed. In these tobaccos, free solanesol content varied from 70 to 90% of the total solanesol, which ranged from 1.9 to 2.8% of dry weight.

INTRODUCTION

Solanesol [$H(CH_2–C(CH_3)=CH–CH_2)_9–OH$] is the major trisequiterpenoid (C_{45}) alcohol of tobacco. Solanesol (3,7,11,15,19,23,27,31,35-nonamethyl-2,6,10,14,18,26,30,34-hexatriacontanonaene-1-ol) was first isolated from tobacco by Rowland *et al.*[1] in 1956 and occurs both in the free and bound (esterified) form in tobacco. It is the major terpene component of the lipid fraction (hexane extractables) of tobacco and represents up to 5% of dried leaf lamina[2,3]. Solanesol has been shown to be a major precursor of the tumorigenic polynuclear aromatic hydrocarbons (PAHs) of tobacco smoke[4]. It produces more than 30% of the total PAHs that are formed on pyrolyzing the hexane-extractable fraction of tobacco[5].

Reduction of solanesol would lead to a safer smoking product, due to reduced PAH levels in cigarette smoke. Therefore, a breeding program has been undertaken to genetically develop tobacco with a lower solanesol content. In order to accomplish this goal, a rapid method to analyze thousands of tobacco samples was needed.

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Gravimetric determinations of solanesol by column chromatography[6,7] have produced low and variable results and were not suitable for routine analyses. A thin-layer chromatography–densitometry method presented difficulties, due to decomposition of solanesol[7]. A packed column gas chromatography (GC) method reported by Sheen *et al.*[8] involved a lengthy extraction procedure and hydrogenation of solanesol. Court and Hendel[9] have recently published a determination of solanesol by high-performance liquid chromatography. Consequently, it was decided to improve a GC method developed in this laboratory[10]. This method determined solanesol by GC of its trimethylsilyl (TMS) derivative, on a short, packed Dexsil 300 GC column. However, since substantial amounts of leaf solanesol may be bound as esters, an improved method for total solanesol was needed. Our previously described method for total solanesol[10] required hydrolysis of the ground tobacco in a saponification flask, followed by solvent extractions of the solanesol. Both steps were time consuming for routine analyses of large numbers of samples. In this manuscript, we describe a micro test tube hydrolysis–extraction method for ground tobacco to yield total solanesol, which was quantitated by wide-bore capillary gas chromatography on SE-54 coated columns. These GC columns have been employed for the last six years to successfully analyze over 1500 tobacco varieties and introductions in a low solanesol tobacco breeding program[11]. Recently, a similar GC method for solanesol in environmental tobacco smoke has been reported[12].

MATERIALS AND METHODS[a]

The tobaccos used in this study were grown at the Crops Research Laboratory, Oxford, NC, U.S.A., under conditions normally used for the production of flue-cured tobacco. Cured tobacco leaf lamina samples were dried over anhydrous silica in a desiccator for two days and then ground in a Wiley Mill to pass through a 20-mesh screen. All solvents were Burdick & Jackson (Muskegon, MI, U.S.A.) distilled-in-glass grade.

Solanesol purification

Crude solanesol (Hoffmann-La Roche, 80+%) was purified by repetitive recrystallizations from hexane to yield a 98+% pure compound (by GC).

Preparation of butyl triacontanoate internal standard

About 0.6 g of triacontanoic acid (Fluka, purum grade, *ca.* 98% by GC), 0.5 g *p*-toluene sulfonic acid monohydrate and 8 ml of butanol (Aldrich, 99+%, redistilled from KOH) were added to a test tube. The tube was capped and heated at 80°C for 4 h. After cooling, the mixture was transferred to a separatory funnel with hexane (50 ml) and extracted with saturated Na_2CO_3 (2 × 20 ml) and water (3 × 20 ml). The hexane fraction was dried over Na_2SO_4, filtered, and taken to dryness on a rotary evaporator. The residue was transferred with hexane to a 40-g silicic acid column. Elution with methylene chloride–hexane (1:3) yielded the C_{30} butyl ester, which was recrystallized from hexane (m.p. 65–66°C, 98%+ by GC).

[a] Mention of a commercial instrument or product does not constitute Agricultural Research Service endorsement.

Total solanesol determination by test tube hydrolysis methods

In method A, dried ground tobacco (50 ± 3 mg) was weighed into an 8-ml screw-cap culture test tube and 3 ml of a 1 *M* KOH in methanol–water (95:5) solution were added. The tube was sealed with a PTFE-lined cap. The solvent level was marked on the outside of the test tube and the mixture was heated at 80°C, in a heat block for 4 h, to insure complete hydrolysis of the bound solanesol. After cooling, and if necessary, methanol was added to bring solvent level to the initial volume and 3 ml of isooctane, containing the internal standard (IS, 0.5 mg of butyl triacontanoate) were added. After sonification for 15 min, 1 ml of water was added and the mixture was allowed to stand in the dark, at room temperature for two or more hours. A 250-μl aliquot of the isooctane layer was transferred to a 1-ml Reacti-vial, the solvent was removed by a stream of nitrogen at 40°C, and 100 μl of N,O-bis(trimethylsilyl)-acetamide (BSA) were added. The vial was sealed with a PTFE-lined cap and heated at 80°C for 30 min. After cooling, the sample was transferred to a micro auto-sampler vial and 1 μl was analyzed by GC.

In an alternative procedure (method B), the sample was treated as above, except that the IS was not added to the isooctane in the test tube. Instead, after standing overnight at room temperature, 250 μl of the isooctane layer (containing the saponified compounds) were added to a Reacti-vial, together with the IS (80 μg). The sample was then treated as above.

Total solanesol by saponification flask hydrolysis method

About 1 g of dried ground tobacco and 40 ml of methanolic potassium hydroxide were placed into a 250-ml saponification flask, with a 24/40 joint, and fitted with a reflux condensor. The mixture was refluxed for 2 h under nitrogen. After cooling, the mixture was filtered through fluted filter paper into a separatory funnel and the flask and filter were washed with 50 ml of a 1:1 mixture of benzene and ethanol–water (85:15). Hexane (50 ml), 25 ml of aqueous saturated KCl solution and 50 ml of water were added to the funnel and the funnel was vigorously shaken to effect solvent partitioning. The hexane layer was removed and the aqueous layer was extracted with hexane (2 × 25 ml). The hexane extracts were combined, washed with water (3 × 25 ml), reduced in volume on a rotary evaporator, and transferred to a 25-ml volumetric flask. A 300-μl aliquot was removed for GC analyses and concentrated BSA reagent was added and the sample was treated as above.

Gas chromatography

Samples were analyzed on a HP 5710A gas chromatograph, modified for capillary GC and containing an flame ionization detector[13]. The column was a 10 m × 0.53 mm I.D. fused-silica capillary column, coated with SE-54 (4 mg/ml). The detector temperature was 350°C and the injector temperature was 250°C. For total solanesol determination, the oven was heated from 220 to 300°C at 8°C/min and held 8 min at 300°C. For free solanesol determination[10], the oven temperature conditions were: hold for 2 min at 100°C, then program from 100 to 300°C at 8°C/min. The lower starting temperature was needed to allow determination of other, major leaf constituents, including malic and citric acid, fructose, glucose, sucrose and chlorogenic acid.

RESULTS AND DISCUSSION

As stated earlier, we have for a number of years routinely analyzed solanesol in cured tobacco leaf, using 45-cm packed Dexsil 300 GC columns[10]. One major disadvantage was unpredictable column life, which varied from 100 to 500 analyses per column. When wide bore (0.53 mm I.D.) glass capillary columns became available, we investigated bonded SE-54 columns as replacements for the packed columns and found that short (10 m or less) and thin-film columns lasted for 1000+ analyses and gave very reproducible results. An example of a wide-bore capillary GC separation is shown in Fig. 1.

Another problem with the packed column analyses was the cost and stability of the 1,3-dimyristin internal standard employed at that time. Consequently, we investigated a number of substitutes and found that the butyl ester of triacontanoic (C_{30}) acid was a suitable replacement.

The next step in modifying the total solanesol method was to streamline the labor-intensive flask saponification and extraction procedures. By changing the hydrolysis solvent from aqueous ethanolic KOH to methanolic KOH, we were able to hydrolyze bound solanesol and then extract the free solanesol into isooctane in the same reaction vessel, an 8-ml capped test tube. Briefly, the procedure consists of (1) methanolic KOH hydrolysis of a small sample of ground tobacco, (2) ultrasonic extraction of the hydrolysis mixture with iso-octane and addition of water to assist in phase separation, and (3) derivatization of the free solanesol with a silylating reagent (BSA) for GC analysis.

After establishing the saponification and extraction conditions, it was necessary to evaluate the reproducibility of the method. The reproducibility of the GC procedure was first examined (Table I). Two representative tobacco varieties were analyzed for solanesol by test tube method B. A sample from each tobacco was hydrolyzed and the hydrolyzate was analyzed four times to give the results in Table I. From the relative

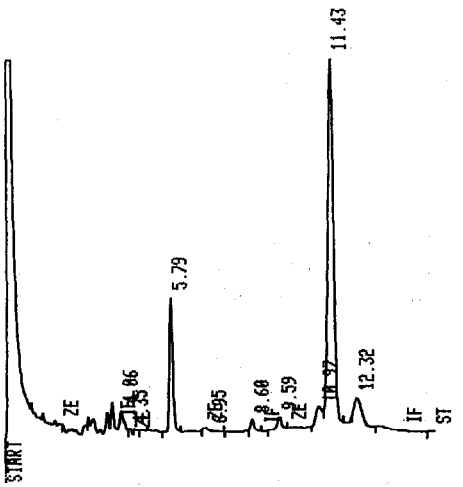

Fig. 1. Gas chromatogram for analysis of total solanesol in tobacco leaf lamina. IS at 5.79 min and solanesol at 11.43 min.

TABLE I

REPRODUCIBILITY OF GC ANALYSES FOR SOLANESOL

Tobacco[a]	*%Dry weight*				
	Run number				*Mean ± R.S.D.*
	1	*2*	*3*	*4*	
KY-14	2.75	2.77	2.83	2.81	2.78 ± .03
OX 259	2.88	2.99	2.86	2.97	2.92 ± .06

[a] See Table II.

standard deviation (R.S.D.), it was apparent that the GC part of the method yielded very reproducible and acceptable results.

In Table II, the reproducibilities of micro test tube methods A and B (for the hydrolysis and extraction of total solanesol) are compared to that of our standard saponification flask procedure. This saponification flask method contains several improvements over the previously reported[10] method. It was apparent that the more rapid test tube methods yielded data of equal validity and reproducibility compared to the original flask saponification method. Thus, the addition of internal standard to the isooctane extraction solvent (method A) or to the separated isooctane prior to GC (method B) produced identical results. Consequently, the analyses of a large number of field samples could be performed rapidly and confidently by either test tube method.

In our program to breed tobacco with a lower solanesol content, we have examined many tobacco varieties. As an example of solanesol variation and method applicability, the results for six tobaccos are shown in Table III. As each tobacco was grown in four separate field plots and samples were taken from each plot, there was

TABLE II

REPRODUCIBILITY OF METHODS FOR THE DETERMINATION OF TOTAL SOLANESOL

Tobacco[a]	*Method*	*%Dry weight*				
		Run number				*Mean ± R.S.D.*
		1	*2*	*3*	*4*	
KY-14	Saponification flask	2.86	2.76	2.82	–	2.81 ± .04
	Test tube A	2.79	2.77	2.83	2.86	2.81 ± .03
	Test tube B	2.86	2.77	2.76	2.78	2.79 ± .04
Sp G-28	Saponification flask	2.40	2.46	2.50	–	2.45 ± .04
	Test tube B	2.43	2.40	2.36	2.34	2.38 ± .04
OX 259	Test tube A	2.93	2.88	2.94	2.86	2.90 ± .03
	Test tube B	2.83	2.86	2.95	2.89	2.88 ± .04

[a] Kentucky 14 (KY-14) is a representative burley tobacco, Speight G-28 (Sp G-28) is a representative flue-cured tobacco and Oxford 259 (OX 259) is a recent flue-cured variety. Analyses data represent repetitive runs on the same sample.

TABLE III

FREE AND TOTAL SOLANESOL LEVELS OF SELECTED FLUE-CURED TOBACCOS

Values represent averages of four or more samples collected in the field.

Tobacco	*Total solanesol*[a] *% leaf (dry wt.) ± R.S.D.*	*Free solanesol*[b] *% leaf (dry wt.) ± R.S.D.*	*% Free solanesol*
Harrison Pryor	2.18 ± 0.02	1.68 ± 0.36	77
Oxford 3	1.90 ± 0.10	1.34 ± 0.23	70
Sp G-70	2.06 ± 0.08	1.83 ± 0.26	89
Coker 48	2.07 ± 0.37	2.05 ± 0.34	99
NC 95	2.81 ± 0.30	2.49 ± 0.48	89
NC 82	2.43 ± 0.20	2.31 ± 0.16	95

[a] Method A.
[b] Ref. 8.

much more variability in results between plots and this resulted in higher R.S.D., as compared to the data in Table II. The percent free solanesol (determined by the method in ref. 10) varied from 70 to 99% of the total solanesol values. Therefore free solanesol values cannot be used to calculate total solanesol in tobacco. This methodology should be applicable to analyses of other plant alcohols, such as free and bound sterols. With a pH adjustment step after saponification, free and bound fatty acids could also be determined.

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