Quantitation of Fatty Acids from Flue-Cured Tobacco by Combined Preparative Thin Layer Chromatography-Gas Chromatography

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ABSTRACT

Flue-cured tobacco was subjected to alkaline hydrolysis, and, after acidification, the fatty acids and nonsaponifiables were extracted into hexane. Treatment of the hexane extract with diazomethane yielded fatty acid methyl esters. The methyl esters were separated from interfering hydrocarbons and sterols by preparative thin layer chromatography (PTLC). After addition of an internal standard, the esters were quantitated by gas chromatography on the column packing, Silar 10C. Quantitation of the C_{14}-C_{32} fatty acid esters was possible by means of temperature programming.

INTRODUCTION

Thin layer chromatography (TLC), column chromatography, and gas chromatography (GC) are routinely used in the analysis of plant and animal lipids. When used in combination, the separation of complex lipid mixtures into individual compounds is possible. Lipids can be separated into neutral and polar fractions equally well by TLC and by column chromatography; the choice is usually determined by sample size (1). Neutral lipid fractions are mixtures of hydrocarbons, fatty alcohols, fatty acids, sterols, esters, carotenoids, and any other neutral compounds. TLC can effectively separate such mixtures into classes of neutral lipids (2,3). Further resolution into individual components is then most readily performed by GC.

The resolution of lipid mixtures from tobacco leaf is a challenge to all available chromatographic tools. We recently reported a TLC procedure for class separation of plant neutral lipids (3). Use of this procedure on a preparative scale (PTLC) with tobacco lipids yielded a fatty acid methyl ester fraction, the components of which were then identified by gas chromatography-mass spectrometry (4).

In the present work, we attempted to use a method typical of those procedures commonly employed in lipid analyses (1,9) when it is desirable to separate hydrolyzates into neutral and acid fractions. The acid fraction is normally analyzed by GC either directly, as the free acids, or after derivatization. However, quantitation of the higher molecular weight fatty acids, C_{22}-C_{32}, was not possible by GC because of interference from non-fatty acid components that had been extracted into the acid fraction.

Because of the difficulties encountered with reported procedures, we employed the method outlined in the experimental and obtained a highly purified methyl ester fraction by PTLC (3). This allowed the quantitative analysis of tobacco fatty acids ranging from C_{14} to C_{32}.

TABLE I

Fatty Acid Methyl Esters

Fatty acid[a]	Initial extraction (μg/g)	Overnight extraction (μg/g)
14:0	149.6 ± 6.9[b,c]	tr
Anteiso 15:0	25.2 ± 1.1	tr
15:0 (Iso 15:0)[d]	51.5 ± 2.1	tr
15:1	184.2 ± 6.9	tr
16:0	1737 ± 32.6	43.7 ± 17.2
Iso 17:0	98 ± 3.8	tr
17:0	95 ± 9.2	tr
Iso 18:0	48 ± 4.9	tr
18:0	321 ± 11.1	10.6 ± 5.2
18:1	570 ± 12.3	16.4 ± 4.6
18:2, 19:0	1150 ± 20.9	31.6 ± 13
20:0	127.7 ± 13.3	tr
18:3 (21:0)	1515 ± 84.9	42.7 ± 16.4
22:0	65.5 ± 5.5	tr
23:0	36.1 ± 2.1	tr
24:0	46.9 ± 6.2	tr
25:0	9.8 ± 1.1	tr
26:0	34.3 ± 2.9	tr
27:0	12.9 ± 3.5	tr
28:0	93.8 ± 8.8	tr
29:0	20 ± 5.6	tr
30:0	48.3 ± 4.1	tr
31:0	17.9 ± 3.5	tr
32:0	64 ± 6.3	tr

[a]Listed in order of elution from Silar 10C, Ref. 4. Values are μg of fatty acid per g of tobacco.

[b]All standard deviations calculated from four replicates of total procedure.

[c]To convert to percentage basis move decimal point four places to left.

[d]Parentheses indicate shoulder included in quantitation of major peak.

MATERIALS AND METHODS

Sample Preparation

Eastern Carolina flue-cured tobacco (1968 crop) that had been cured, redried, and aged was obtained from Universal Leaf Tobacco Company, Richmond, VA. The leaves were ground to pass a 32-mesh screen, and a 119-g sample of known moisture content (5) was extracted with hexane for 16 hr in a Soxhlet apparatus. The hexane was removed in vacuo to leave 11.26 g of extract (yield, dry weight basis, 9.95%). The extract was redissolved in 50 ml of redistilled hexane, and aliquots were removed for the analyses.

Hydrolysis

A 0.3-ml aliquot of the stock solution of extract, equivalent to 69.3 mg of extract, was transferred to a Teflon-lined screw-cap test tube, and the hexane evaporated under a stream of nitrogen. The residue was mixed with 1.5 ml of 1N KOH in 95% ethanol, and the tube was capped and placed in a boiling water bath for 2 hr. The tube and contents were cooled by immersion in a water bath, water (4 ml) was added to the hydrolyzate, the pH adjusted to 2 with conc. HCl, and the solution saturated with solid KCl. Lipids were extracted with five 10-ml portions of hexane. The hexane layers were combined, hexane was evaporated in vacuo, and the esters were generated by treatment with ethereal diazomethane (6,7). After extraction of the acids, 10 ml of hexane was added to the aqueous-ethanol layer

with mixing and the biphase system left overnight. The hexane layer was then removed, and the aqueous-ethanol layer washed with a second 10-ml portion of hexane. The hexane layers were combined, the solvent was removed, and the residue treated with diazomethane as above.

Thin Layer Chromatography

The fatty acid methyl esters were separated from the other neutral lipids by PTLC. The PTLC method (3) involved a two-step development in one dimension with the solvent system: (a) hexane-diethyl ether (98:2, v/v) and (b) hexane-diethyl ether-acetic acid (50:50:1, v/v). Silica Gel H (Brinkmann Instruments, Inc., Westbury, NY) at a thickness of 2000 μ was used on 20 x 20 cm glass plates. Samples in hexane were applied as streaks. After development, the plates were air dried and treated with Rhodamine 6G for visualization in the UV. The fatty acid methyl ester bands were scraped off, and components eluted (8).

Quantitative Gas Chromatography

The methyl esters were quantitated by use of a Hewlett-Packard 7610A gas chromatograph equipped with a flame ionization detector (FID) and connected to an Infotronics automatic digital integrator. Glass "U" columns (1.8 m x 2 mm ID) were used, packed with 10% Silar 10C (Applied Science Labs, Inc., State College, PA) on 100/120 mesh Gas Chrom Q. Column temperature was programmed from 100 to 250 C at 2°/min. Injector temperature was 275 C, FID 300 C, and helium flow 40 ml/min. Methyl undecanoate (300 μl of a 2.482 μg/μl stock solution) was added as internal standard to the methyl ester samples just prior to sample injections.

RESULTS AND DISCUSSION

A highly pure fraction of fatty acid methyl esters was obtained (by PTLC) from lipids extracted from flue-cured tobacco leaf. Data are presented in Table I which show that more than 97% of the acids are recovered after five 10-ml washes of the acidified hydrolyzate. The fatty acid composition of these lipids is presented and include the C_{22}-C_{32} acids, which are not easily obtained quantitatively by other GC procedures. Standard deviations were obtained and are within the limits suggested by Iverson and Sheppard (10): major components (>10%), ±5%; minor components (2-10%), ±10%; and trace components (<2%), ±25%. Only the relative standard deviations for $C_{18:3}$, C_{27}, and C_{29} acids exceeded the suggested limits.

PTLC yielded a highly purified fatty acid methyl ester fraction which allowed quantitation of high molecular weight acids (C_{22}-C_{32}) by GC. The combined PTLC-GC method also avoided losses which occurred when the hydrolyzate was divided into neutral and acidic fractions.

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