

Tobacco Chemistry 10

Volatile Neutral Constituents of Greek Tobacco

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The volatile constituents of Greek tobacco have been further examined and about 50 more compounds identified using gas chromatography-mass spectrometry as the main analytical tool. Almost all of these, mainly comprising aromatic hydrocarbons and methyl esters of higher saturated fatty acids, have not hitherto been reported to occur in tobacco. Many of the new constituents are important flavour compounds.

Several earlier publications in this series have dealt with the volatile constituents of Greek tobacco and have documented the presence of a large number of compounds not previously encountered in tobacco.¹⁻³ These studies have also demonstrated the high complexity of the material and the occurrence of a vast number of potentially significant constituents which, however, were present in amounts insufficient for characterisation at that time. A more detailed study, using larger quantities of Greek tobacco, has therefore been undertaken and the results obtained constitute the subject of the present paper.

The neutral volatile material of sun-cured tobacco grown in Serres, Greece, was isolated as indicated in Scheme 1 with stringent precautions against contamination.¹ It was separated into a hydrocarbon fraction, A 1, and three more polar fractions, A 2-A 4, by chromatography on silica gel. Fraction A 2, being of most interest from the point of view of flavour, was similarly divided into ten subfractions, B 1-B 10, which were examined by gas chromatography-mass spectrometry using capillary columns. The gas chromatograms of A 2 and B 1, given in Fig. 1, demonstrate the complexity of these materials.

Twelve compounds, not identified in our earlier studies, were encountered at this level of separation and are listed in Table 1. One of these was a new tobacco constituent and its subsequent isolation by liquid chromatography followed by structure determination and synthesis has revealed that the com-

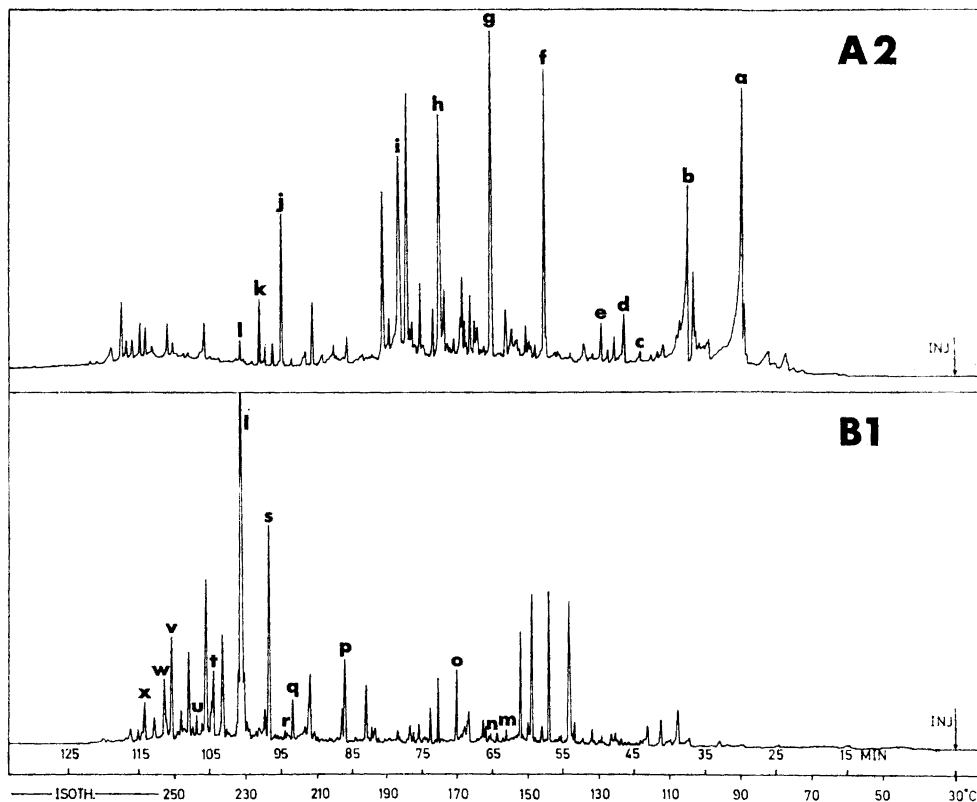


Fig. 1. Gas chromatograms of fractions A 2 and B 1. a. Benzyl alcohol. b. β -Phenylethanol. c. Safranal. d. Decanal. e. β -Phenylethyl acetate. f. Solanone. g. Geranylacetone. h. Dihydroactinidiolide. i. 3-Oxo- α -ionol. j. Hexahydrofarnesylacetone. k. Farnesylacetone. l. Methyl hexadecanoate. m. 1,1,6-Trimethyl-1,2-dihydronaphthalene. n. Isoamyl benzoate. o. 5-Methoxy-6,7-dimethylbenzofuran. p. Methyl tetradecanoate. q. Methyl pentadecanoate. r. 1-Methylfluorene. s. Neophytadiene. t. Ethyl hexadecanoate. u. Methyl heptadecanoate. v. Methyl octadecadienoate and octadecatrienoate. w. Methyl octadecenoate. x. Methyl octadecanoate.

pound is 3-oxo- α -ionol.^{4,5} This compound (12) as well as several of the other constituents (7–10, 25) are nor-isoprenoids, and, as pointed out previously,² are probably derived from carotenoids or other higher isoprenoids. Several of the compounds encountered (4, 5, 7–11) are established flavour constituents.^{6,12} Four of them (5, 7, 9, 10) have previously been detected in tobacco and three (3, 4, 11) in tobacco smoke.

It rapidly became clear that many more constituents might be identifiable after further fractionation on silica gel prior to the gas chromatographic examination. Accordingly, separation of fraction B 1 into fractions C 1–C 10 and separate examination of these revealed the presence of a large number of compounds, of which those not described in the earlier publications are shown

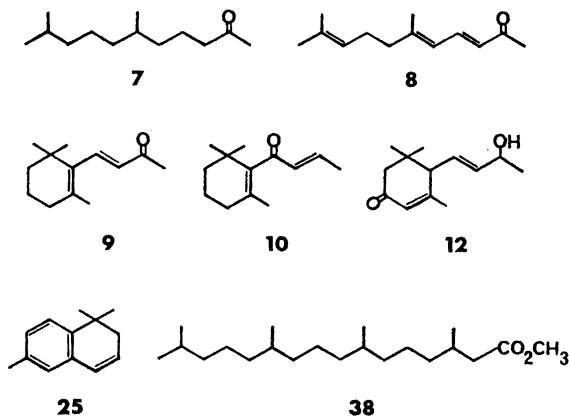
Table 1. New constituents of Greek tobacco.

Compounds	Previously detected T/S ^c	Ref.	Method of identification	Ref.	Amount ppm	Fraction
1. Benzyl isobutanoate			GC, MS	24	0.01	B 2
2. β -Phenylethyl formate			GC, MS	24	0.02	B 2
3. 2-Acetyl-5-methylfuran ^a	S	7	MS	8	0.2	B 4, B 5
4. Hexanal	S	9	GC, MS	35	0.2	B 2
5. <i>o</i> - or <i>m</i> -Methylbenzaldehyde	T	10, 11	GC, MS	35	0.01	B 2
6. 2-Pentadecanone			GC, MS		0.02	B 2, B 3
7. Tetrahydrogeranylacetone	T	12	GC, MS	8	0.03	B 3
8. <i>trans</i> - ψ -Ionone			GC, MS	36	0.2	B 4
9. β -Ionone	T	12	GC, MS	36	1.3	B 4
10. <i>trans</i> - β -Damascone	T	12	GC, MS		0.5	B 3
11. Indole	S	13	GC, MS	24	0.2	B 4
12. 3-Oxo- α -ionol ^b				4,5	14	B 9

^a The positions of the substituents uncertain.

^b Structure determination, synthesis.^{4,5}

^c T=tobacco, S=tobacco smoke.



in Table 2. Some of the unidentified constituents were present in amounts sufficient to permit their isolation by preparative gas chromatography and further spectroscopic studies. The structure of one of them, 5-methoxy-6,7-dimethylbenzofuran (54), has been determined. The identification and synthesis of this new natural product is discussed elsewhere.^{14,15}

Although the hydrocarbons occur almost exclusively in fraction A 1 and only represent a very small portion of fraction B 1, a number of such constituents not previously detected in Greek tobacco were encountered. The majority of these have molecular weights higher than those reported earlier¹ and it seems probable that this may be due to the larger amount being processed and to more efficient distillation. Thus, four monomethyl and seven dimethyl

Table 2. New constituents of Greek tobacco.

Compounds	Previously detected T/S ^e	Ref.	Method of identification	Ref.	Fraction
13. Docosane	T, S	16, 17	GC, MS		C 1
14. Monomethyl anthracenes or phenanthrenes ^a	S	18	MS	19	C 4
15. Dimethyl anthracenes or phenanthrenes ^a	S	18	MS	19	C 4
16. 1-Methylfluorene	S	20	GC, MS	19	C 4
17. 2-Methylfluorene			GC, MS		C 4
18. 3(or 4)-Methylfluorene			MS ^b		C 4
19. 4(or 3)-Methylfluorene			MS ^b		C 4
20. 9-Methylfluorene	S	21	GC, MS		C 4
21. Pyrene	T, S	22,23	MS	19	C 4
22. Diphenylmethane			GC, MS	19	C 4
23. 1,2-Diphenylethane			GC, MS	19	C 4
24. Ethylbiphenyls ^c			MS	24	C 4
25. 1,1,6-Trimethyl-1,2-dihydronaphthalene			MS	37	C 2, C 3
26. Methyl octanoate			GC, MS	34	C 10
27. Methyl nonanoate			GC, MS	34	C 9, C 10
28. Methyl decanoate			GC, MS	34	C 10
29. Methyl undecanoate			GC, MS	34	C 10
30. Methyl dodecanoate	T	25	GC, MS	34	C 9
31. Methyl tridecanoate			GC, MS	34	C 10
32. Methyl pentadecanoate			GC, MS	34	C 8, C 9
33. Methyl heptadecanoate			GC, MS	34	C 8, C 9
34. Methyl octadecanoate			GC, MS	34	C 8, C 9
35. Methyl eicosanoate			GC, MS	34	C 8, C 9
36. Ethyl tetradecanoate			GC, MS	34	C 9
37. Ethyl octadecanoate			GC, MS	34	C 8
38. Methyl phytanate			GC, MS		C 8
39. C ₁₄ H ₂₈ COOMe, branched			MS		C 8, C 10
40. C ₁₅ H ₃₁ COOMe, branched			MS		C 9
41. Methyl 5-methylhexadecanoate			MS	34	C 8, C 9
42. C ₁₆ H ₃₃ COOMe, branched			MS		C 8, C 9
43. C ₁₇ H ₃₅ COOMe, branched			MS		C 8
44. C ₁₈ H ₃₇ COOMe, branched			MS		C 9
45. Hexyl benzoate			GC, MS		C 7, C 8
46. Isoamyl benzoate			GC, MS		C 8
47. Benzyl hexanoate			GC, MS		C 10
48. Diphenylether			GC, MS	35	C 5
49. 2-Methoxynaphthalene			GC, MS	19	C 6
50. 2-Ethoxynaphthalene			GC, MS	35	C 6
51. Thymol/carvacrol methyl ether			MS		C 5
52. 3-Phenylfuran			MS	8	C 4
53. Dibenzofuran	S	26	GC, MS	35	C 4
54. 5-Methoxy-6,7-dimethylbenzofuran ^d				14, 15	C 6

^a Four monomethyl and seven dimethyl derivatives detected. Substitution patterns unknown.

^b MS very similar to 1- and 2-isomers.

^c Two isomers, substitution pattern unknown.

^d Structure determination, synthesis.^{14,15}

^e T=tobacco, S=tobacco smoke.

anthracenes or phenanthrenes were detected, whereas previously only the corresponding unsubstituted compounds were encountered. The fact that there are eight monomethyl and 40 dimethyl isomers theoretically possible and that these exhibit similar fragmentation patterns and retention times has precluded location of the substituents on the basis of the available data. The monomethyl fluorenes represent a more favourable case and, in fact, all of the five possible isomers were found. One tetracyclic hydrocarbon, pyrene, was detected in addition to five bicyclic derivatives, diphenylmethane, 1,2-diphenylethane, two ethylbiphenyls, and 1,1,6-trimethyl-1,2-dihydronaphthalene. Diphenylmethane and 1,2-diphenylethane are both known flavour components and the latter has recently been found to occur in tolu balsam.²⁷ Only two of the hydrocarbons given in Table 2, docosane and pyrene, have previously been encountered in tobacco,^{16,22} and the present findings, as well as those published^{1,3} earlier, suggest the desirability of further studies of tobacco hydrocarbons, particularly those of higher molecular weight.

The methyl esters of the C_8 – C_{20} straight chain fatty acids represent the largest group of the oxygenated constituents now found in Greek tobacco. (In an earlier paper we have reported the presence of methyl tetradecanoate and methyl and ethyl hexadecanoate in Greek tobacco.²) Several of these esters (26–30) as well as the co-occurring ethyl tetradecanoate and octadecanoate are established flavour compounds.⁶ In addition to these, methyl phytanate and methyl esters of several branched fatty acids were encountered, but only one of the latter compounds, methyl 5-methylhexadecanoate, could be unambiguously identified. In contrast, the three aromatic esters benzyl hexanoate, hexyl benzoate, and isoamyl benzoate, which are all commercial flavour compounds, were readily identified. Surprisingly, only one of the above esters, methyl dodecanoate, has previously been found in tobacco²⁵ and none has been found in smoke.

The presence of diphenylether (48), 2-methoxynaphthalene (49), 2-ethoxynaphthalene (50) and thymol/carvacrol methyl ether (51), of which two (48, 50) are recognised flavour substances, was also readily demonstrated. None of these ethers, nor the three furans encountered (52–54), have previously been detected in tobacco and only one of them, dibenzofuran, has been reported to occur in tobacco smoke.³⁵

Further studies on the volatile constituents of Greek tobacco are in progress and will be discussed later. Some of the constituents found during the present work occur predominantly in the more polar fractions and those analyses will be described elsewhere.

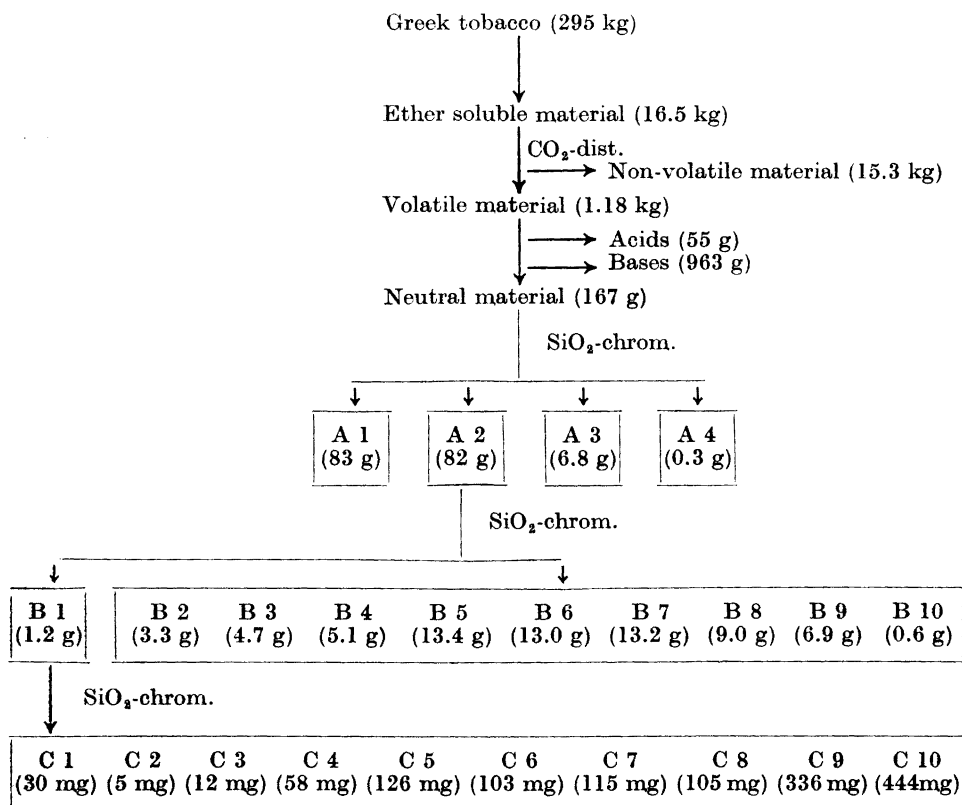
EXPERIMENTAL

Material and methods. The solvents, silica gel, and drying agents were purified as previously described.¹ NMR spectra were recorded in deuteriochloroform on a Varian A 60-A instrument and IR spectra on a Perkin-Elmer 257 instrument. Gas chromatography in combination with mass spectrometry (GC–MS) was carried out on a modified²⁸ LKB 9000 instrument operating at 70 eV. The capillary columns used were made by coating stainless steel tubes (50 m × 0.5 mm, Handy and Harman grade 316–S) with Apiezon L dissolved in hexane (10 %), using the dynamic packing method.²⁹ Analytical (capillary) gas chromatography was performed with a Varian 1700 instrument fitted

with a capillary injector and a flame ionization detector; preparative gas chromatography was performed with the Varian 1700 using a 3 m × 3.2 mm glass column packed with 5 % Carbowax 20 M on Chromosorb G. Fractions were collected at -70° in U-shaped teflon tubes equipped with an electrostatic precipitator.³⁰

Isolation. Nine batches (each ca. 33 kg) of sun-cured Greek tobacco, *Nicotiana tabacum*, grown in Serres (1968), were each extracted with 60 l of ether for 90 h (cf. Scheme 1) in a stainless steel Soxhlet-type apparatus. The ether extracts from a total of 295 kg leaves (7 % water content) were concentrated to a viscous, dark oil by careful distillation of the ether at atmospheric pressure through a column (60 × 3.5 cm) packed with Raschig rings. In the further work, solutions were concentrated using a Vigreux column (1 m) to reduce losses of the most volatile materials. As complete removal of solvents was deliberately avoided, the weights given for the various fractions are necessarily inaccurate.

The combined concentrated extract was distilled batchwise (each ca. 300 g, distilled twice for 2 h) *in vacuo* using carbon dioxide as carrier gas and liquid nitrogen as cooling agent in the previously described system.^{2,31} The volatile material (ca. 7 % of the total extract) was extracted consecutively with aqueous sulphuric acid and sodium hydroxide, eliminating basic and acidic material, and the neutral fraction was chromatographed on a silica gel column using pentane-ether mixtures (1:0→0:1) as eluents. This provided a hydrocarbon fraction (A 1) and two more polar fractions (A 2, A 3). The last fraction taken (A 4) was eluted with 10 % ethanol in ether. The major polar fraction, A 2, was



Scheme 1.

separated into ten subfractions (B 1–B 10) by chromatography on silica gel, eluting with pentane–ether mixtures. Fraction B 1 was subjected to further chromatography on silica gel (eluents: pentane–isopropyl ether mixtures) furnishing in turn ten subfractions (C 1–C 10). Fractions B 1–B 10 and C 1–C 10 were studied by GC–MS. When reference compounds were available, co-chromatography with the natural material and recording of mass spectra under identical conditions were carried out.

Preparation of reference compounds. Tetrahydrogeranylacetone: Geranylacetone (200 mg) (K & K Laboratories) in ethanol (5 ml) was hydrogenated at room temperature and atmospheric pressure using 10 % palladium on carbon (30 mg) as catalyst. Removal of the catalyst by filtration was followed by dilution with water and extraction with pentane. Tetrahydrogeranyl acetone was obtained as a colourless liquid (175 mg), homogeneous according to GLC, after removal of the pentane.

Methyl phytanate: Phytal (365 mg) was obtained by treating phytol (400 mg) (Fluka AG) in acetone (50 ml) with Jones' reagent³² (15 ml) at room temperature for 2 h. The aldehyde was isolated by ether extraction, and converted to the corresponding methyl ester according to Corey's method.³³ Thus, the aldehyde (200 mg), sodium cyanide (500 mg), acetic acid (0.2 ml) and manganese dioxide (4 g) in methanol (50 ml) were stirred for two days at room temperature. The mixture was then diluted with water, extracted with ether and the ether-extractable material chromatographed on a silica gel column. The unsaturated methyl ester (144 mg) thus obtained was hydrogenated in ethanol (10 ml) at room temperature and atmospheric pressure using 10 % palladium on carbon (25 mg) as catalyst to give pure methyl phytanate (115 mg).

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