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EFFECTS OF FLUE-CURING AND AGEING ON THE VOLATILE, NEUTRAL AND ACIDIC CONSTITUENTS OF VIRGINIA TOBACCO*

Dedicated to Professor H. Erdtman on his 75th birthday

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Key Word Index—*Nicotiana tabacum*; Solanaceae; Virginia tobacco; volatile neutral and acidic compounds; flue-curing and ageing.

Abstract—Several volatile compounds are generated and the concentrations of many others increase on flue-curing and ageing of Virginia tobacco. Oxidative degradation of isoprenoids, Maillard reactions and phenylalanine metabolism are the major processes accounting for the formation of the majority of these compounds. A number of alcohols, aldehydes and ketones, which are apparently products of fatty acid degradation, are lost during the post-harvest handling.

INTRODUCTION

Recent examinations have disclosed the presence in processed tobacco of a great number of flavour components, presumably generated by oxidative degradation of tobacco carotenoids, diterpenoids and alkaloids [1–3]. These degradation reactions are likely to occur, or to be accelerated, during the aerobic post-harvest treatment of the tobacco leaf, i.e. during the curing and ageing processes. Consonant with this, curing, which involves air, heat, fire or sun drying and leads to a fixation of the gross chemical composition of the tobacco leaf, is known to reduce the concentrations of carotenoids [4], diterpenoids [5, 6] and nicotine and to create the typical tobacco flavour. Furthermore, an inverse relationship between flavour and carotenoid content of tobacco has been found and explained as being due to a more extensive degradation of carotenoids in the leaves which are richer in flavour [4].

However, little is known about the actual compositions of the volatile flavour fractions derived from tobacco at various stages of leaf processing. In view of this we found it desirable to study the effect of flue-curing (heat drying) and ageing on the volatile substances isolable from a Virginia tobacco. The present paper describes the effects on the neutral and acidic constituents, whereas the results for the bases will be reported separately [7].

RESULTS AND DISCUSSION

During the 1971 season, tobacco of the most commonly-produced variety in North Carolina, U.S.A., (*Nicotiana tabacum* var. Coker 319) was grown under normal field practice. Three harvests per plant were taken during July and August and a sample of fresh green leaves was taken from each harvest. These three samples were frozen, crushed, freeze-dried and combined prior to analysis (GN). The remaining tobacco from each of the three harvests was cured separately in an experimental curing chamber. Following the completion of the yellowing phase during each of the three curings, a sample of yellowed tobacco was removed from the chamber and freeze-dried (YE). The flue-cured tobacco obtained from the three harvests was uniformly combined and divided into four identical batches, one of which was taken as a sample of unaged tobacco (FC). The remaining three batches were packed in wooden drums for ageing for six, twelve and twenty-four months respectively (6 M, 12 M, 24 M).

The headspace vapours over each of the six tobacco samples were collected using a charcoal-trapping system [8]. The charcoal filters were extracted with ether and the extracts obtained were separated into fractions containing neutral (N), acidic (A) and basic (B) compounds. These fractions, the acidic fractions after methylation with diazomethane, were examined in detail by GLC and GC–MS. To allow for quantitative comparisons within a series of fractions, GLC peak areas were normalized on an added internal standard and were recalculated on a weight per dry weight of tobacco basis. Efforts were made to achieve reproducibility in each experimental step. Identification of individual components was accomplished by comparison of MS and, where possible, of retention times with those of authentic samples.

The total amounts of neutrals obtained by the headspace technique increase on curing of the tobacco and reach maximum values in the 6 M tobacco, where they

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Table 1. Tobacco samples and their volatile fractions

Tobacco sample	Weight of tobacco sample (kg)	Humidity (%)	Weight of neutral fraction (g)	Weight of acidic fraction (g)	Weight of basic fraction (g)
GN	27.5	11.4	4.9	1.4	12.3
YE	26.4	12.6	6.2	1.6	11.0
FC	25.5	12.4	18.2	1.8	12.0
6 M	24.1	11.8	19.7	2.1	10.5
12 M	24.4	11.0	16.4	2.0	8.1
24 M	23.5	10.6	13.7	2.1	5.4*

* This value is uncertain due to an experimental error.

are roughly four times greater than in GN tobacco (Table 1). A certain increase, although less striking, is observed for the total amounts of acids on curing and ageing of tobacco. The results obtained from the quantitative comparisons are summarized in Tables 2 and 3.* Although the GN tobacco had been subjected to tissue damage, which may have initiated or modified some of the post-harvest catabolic processes, the quantitative results show that curing and ageing affect the compositions of the volatile fractions considerably. A fair number of components are generated, several are lost, and the concentrations of many are altered significantly during the various stages of curing and ageing.

A few compounds, e.g. α-pinene (**19**), camphene (**24**), β-pinene (**30**), limonene (**40**), *p*-cymene (**49**) and estragol (**89**) are evidently derived from the pine boxes in which the tobacco samples were stored, since they are not previously known tobacco constituents and were detected only in the fractions obtained from aged tobacco. Although encountered in tobacco before, this may also be true for myrcene (**37**) and α-terpineol (**94**) which were found only in aged tobacco.

Since the present study deals with more than two hundred tobacco constituents, it has been necessary to simplify the presentation of the results. The tobacco constituents have therefore been grouped according to their assumed biogenetic origins and the results obtained are discussed for each group separately. It is evident, however, that in the absence of appropriate tracer experiments, such a classification basis is necessarily uncertain.

Nor-compounds derived from cyclic carotenoids

A fair number of volatile constituents structurally reminiscent of carotenoids has recently been encountered in flavour extracts from various plant products such as tomatoes [9], tea [10, 11] and tobacco [1]. Concurrent *in vivo* and *in vitro* studies have given support to the view that these compounds are generated by oxidative cleavage of the polyene side-chain of cyclic carotenoids. Enzymatic oxidation is most certainly

* Since the amounts of compounds released during the headspace collection period do not represent those actually present in the tobacco and since the observed relative abundance within a fraction is biased by differences in volatility, a quantitative comparison can only be made with respect to a given compound between fractions. Rather than presenting more than one thousand quantitative values, we have choosen to indicate the observed changes for the individual compounds by +, − and = signs in Tables 2 and 3.

Table 2. Results obtained for the neutral compounds

	Compound	Detected in	Effect on concentration*
1	2-Methylpropanal	GN, YE	−
2	Methylfuran	GN, YE	−
3	Butanal	GN	−
4	Methyl acetate	FC—24 M	+
5	Ethyl acetate	GN—24 M	+
6	3-Methylbutanal	GN—24 M	=
7	Ethanol	GN—24 M	−
8	Benzene	GN—24 M	+
9	Ethylfuran	GN, YE	−
10	2-Methyl-1-buten-3-one	GN, YE	−
11	Pentanal	GN, YE	−
12	Thiophene	GN—24 M	+
13	1-Penten-3-one	GN, YE	−
14	4-Methyl-2-pentanone	GN, YE	−
15	Toluene	GN—24 M	+
16	Propylfuran	GN, YE	−
17	Dimethyldisulphide	GN—24 M	=
18	2-Methyl-2-buten-1-al	GN	−
19	α-Pinene	GN—24 M	+†
20	Butylfuran	GN—6 M	−
21	Hexanal	GN—FC	−
22	*n*-Decane	FC—24 M	+
23	3-Penten-1-al	GN, YE	−
24	Camphene	6 M—24 M	+†
25	Xylene	YE—24 M	+
26	1-Butanol	GN—FC	−
27	Xylene	YE—24 M	+
28	1-Penten-3-ol	GN—6 M	−
29	Pentyl acetate	GN—24 M	=
30	β-Pinene	6 M—24 M	+†
31	3-Penten-1-al	GN—6 M	−
32	Xylene	GN—24 M	+
33	2-Methyl-1-butanol	GN—24 M	−
34	2-Hexenal	GN—24 M	−
35	Dimethyl-4-hydroxy-butanoic acid lactone	GN—24 M	−
36	*n*-Undecane	6 M—24 M	+
37	Myrcene	6 M—24 M	+†
38	C_3H_7-Benzene	GN, YE	−
39	C_3H_7-Benzene	GN—24 M	=
40	Limonene	6 M—24 M	+†
41	1-Pentanol	GN—24 M	−
42	C_3H_7-Benzene	6 M—24 M	+
43	6-Methyl-2-heptanone	GN—24 M	=
44	Pentylfuran	GN—24 M	−
45	Methyl 3-hexenoate	GN	−
46	3-Hexenyl formate	GN, YE	−
47	3-Octanone	GN	−
48	C_3H_7-Benzene	6 M—24 M	+
49	*p*-Cymene	6 M—24 M	+†
50	3-Methyl-2-buten-1-ol	GN—24 M	−
51	Hexyl acetate	6 M—24 M	+

Table 2—*continued.*

	Compound	Detected in	Effect on concentration*
52	4-Methyl-1-pentanol	GN—24 M	=
53	Dodecane	6 M—24 M	+
54	3-Hexenyl acetate	GN—24 M	+
55	6-Methyl-5-hepten-2-one	GN—24 M	−
56	3*E*-Hexen-1-ol	GN—24 M	−
57	2,4-Hexadienal	GN, YE	−
58	1-Hexanol	GN—24 M	− +
59	3*Z*-Hexen-1-ol	GN—24 M	−
60	Furfural	GN—24 M	=
61	Isophorone	GN—24 M	=
62	2-Acetylfuran	GN—24 M	+
63	Methyl octanoate	GN—24 M	+
64	1-Heptanol	GN—24 M	=
65	Benzaldehyde	GN—24 M	=
66	1-Octen-3-ol	GN—24 M	=
67	2,4-Heptadienal	GN—24 M	+
68	2-Methyl-2-hepten-6-ol	GN—24 M	=
69	Furfuryl acetate	YE—24 M	+
70	5-Isopropyl-3-hepten-2-one	GN—24 M	=
71	Methylfurfural	GN—24 M	+
72	3,5-Octadien-2-one	GN, YE	−
73	Benzonitrile	12 M—24 M	+
74	2-Propylpyrrole	GN—24 M	+
75	2-Methyl-3,6-heptadione	GN—24 M	+
76	3,5-Octadien-2-one	GN, YE	−
77	Methyl nonanoate	6 M—24 M	+
78	Linalool	GN—24 M	+
79	6-Methyl-3,5-heptadien-2-one	GN—24 M	+
80	Furfuryl alcohol	GN—24 M	+ −
81	Methyl benzoate	GN—24 M	+
82	1-Octanol	GN—24 M	=
83	6-Hydroxy-2,2,6-trimethylcyclohexanone	GN, YE	−
84	Acetophenone	6 M—24 M	+
85	4-Methyl-4-hydroxy-5-hexenoic acid lactone	GN—24 M	+
86	Benzyl formate	GN	−
87	β-Cyclocitral	GN—24 M	+
88	3-Cyanopyridine	6 M—24 M	+
89	Estragole	6 M—24 M	+†
90	Methylfurfuryl alcohol	GN—24 M	+ −
91	Methyl decanoate	GN—24 M	=
92	Benzyl acetate	GN—24 M	+
93	1-Nonanol	GN—FC	−
94	α-Terpineol	6 M—24 M	+†
95	Methyl phenylacetate	GN—24 M	+
96	2-Phenylethyl formate	GN—24 M	=
97	Solanone	GN—24 M	+
98	2-Phenylethyl acetate	GN—24 M	+
99	Methyl undecanoate	GN—24 M	=
100	Benzyl alcohol	GN—24 M	+ −
101	2-Phenylethanol	GN—24 M	+ −
102	Damascenone	6 M—24 M	+
103	Damascone	GN—24 M	+
104	2-Hydroxy-5-isopropyl-2-methyl-3-nonen-8-one	12 M—24 M	+
105	2-Acetylpyrrole	GN—24 M	+
106	1-Decanol	GN—24 M	=
107	Methyl dodecanoate	GN—24 M	=
108	Geranylacetone	GN—24 M	+
109	2-Formylpyrrole	YE—24 M	+
110	Solanofuran	GN—24 M	=
111	8-Hydroxy-5-isopropyl-2-methyl-1,3-nonadiene	GN—24 M	+
112	β-Ionone	GN—24 M	=
113	2-Formyl-5-methylpyrrole	FC—24 M	+
114	β-Ionon-5,6-epoxide	GN—24 M	+
115	1,3,7,7-Tetramethyl-9-oxo-2-oxabicyclo-[4.4.0]-dec-5-ene	GN—24 M	+
116	Methyl tridecanoate	GN—24 M	=
117	Megastigma-4,7,9-trien-3-one	FC—24 M	+
118	1,3,7,7-Tetramethyl-9-oxo-2-oxabicyclo-[4.4.0]-decane	6 M—24 M	+
119	1,1,3-Trimethyl-5-hydroxy-3-cyclohexen-2-one	GN—24 M	=
120	2,3-Dimethyl-4-hydroxy-2,4-nonadienoic acid lactone	GN—24 M	+
121	Neophytadiene	GN—24 M	+ −
122	Methyl tetradecanoate	GN—24 M	=
123	Megastigma-4,6,8-trien-3-one	GN—24 M	+
124	Megastigma-4,6,8-trien-3-one	GN—24 M	=
125	Nicotyrine	GN, YE	−
126	Methyl pentadecanoate	GN—24 M	+
127	Megastigma-4,6,8-trien-3-one	YE—24 M	+
128	Hexahydrofarnesyl-acetone	GN—24 M	+
129	Dihydroactinidiolide	GN—24 M	+
130	Megastigma-4,6,8-trien-3-one	GN—24 M	+
131	Methyl hexadecanoate	GN—24 M	+
132	Phytofuran	GN—24 M	+
133	Ethyl hexadecanoate	GN—24 M	+
134	Phytol	24 M	+
135	Farnesylacetone	GN—24 M	+
136	3-Hydroxydamascone	YE—24 M	+
137	3-Oxo-α-ionol	YE—24 M	+

* +/− means an increase/decrease of the concentration of a compound (weight/dry weight of tobacco) on curing and/or ageing; = means that the concentration remained unchanged.
† Probably derived from pine storage box.

Table 3. Results obtained for the acidic compounds

	Acidic compound	Detected in	Effect on concentration*
138	Acetic acid	GN—24 M	†
139	Propanoic acid	GN—24 M	†
140	2-Methylpropanoic acid	GN—24 M	+ −
141	Pivalic acid	GN—24 M	=
142	Butanoic acid	GN—24 M	+
143	Methacrylic acid	12 M	
144	2-Methylbutanoic acid	GN—24 M	=
145	3-Methylbutanoic acid	GN—24 M	+
146	3,3-Dimethylbutanoic acid	GN—24 M	=
147	2-Butenoic acid	GN—24 M	+
148	Pentanoic acid	GN—24 M	=
149	2,3-Dimethylbutanoic acid	GN—24 M	=
150	2-Ethylbutanoic acid	GN—24 M	+
151	2-Methyl-2-butenoic acid	GN—24 M	=
152	4-Pentenoic acid	GN—24 M	=

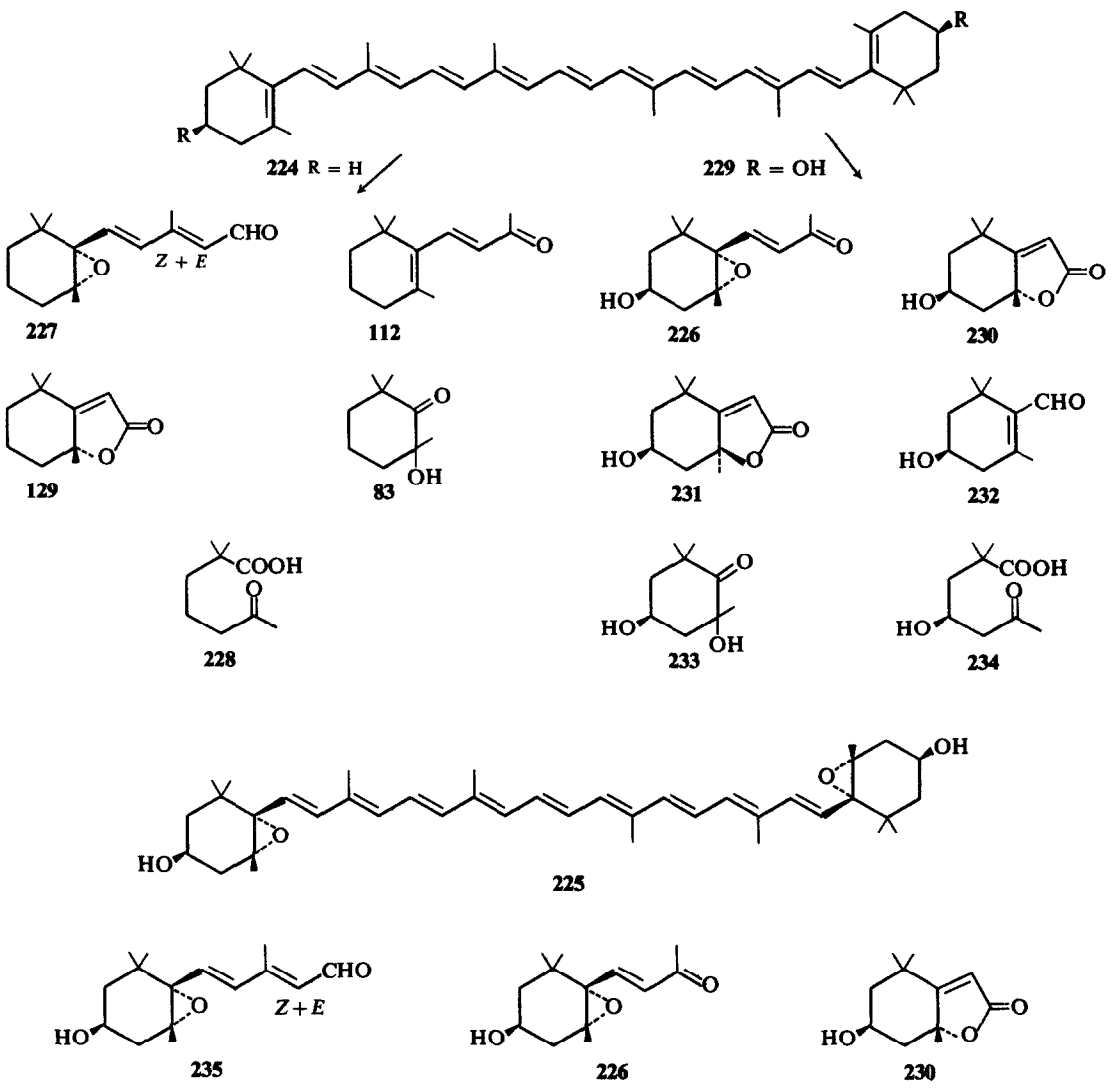
Table 3—*continued.*

	Acidic compound	Detected in	Effect on concentration*
153	2-Methylpentanoic acid	GN—24 M	=
154	4-Methyl-3-pentenoic acid	GN—6 M	−
155	3-Methylpentanoic acid	GN—24 M	+
156	3-Methyl-2-butenoic acid	GN—24 M	+
157	4-Methylpentanoic acid	GN—24 M	+
158	2-Ethylpentanoic acid	GN—24 M	=
159	2-Methyl-2-butenoic acid	GN—24 M	+
160	2-Pentenoic acid	GN—24 M	+
161	Hexanoic acid	GN—24 M	=
162	3-Methyl-3-pentenoic acid	GN—24 M	=
163	2-Methylhexanoic acid	GN—24 M	=
164	5-Hexenoic acid	GN—24 M	=
165	3-Methylhexanoic acid	GN—24 M	+
166	3-Methyl-3-pentenoic acid	GN—24 M	=
167	3-Hexenoic acid	GN—24 M	−
168	5-Methylhexanoic acid	GN—24 M	=
169	4-Methylhexanoic acid	GN—24 M	=
170	2-Hexenoic acid	GN—24 M	+
171	Phenol (as anisole)	GN—6 M	−
172	2-Ethylhexanoic acid	GN—24 M	=
173	Heptanoic acid	GN—24 M	+
174	4-Heptenoic acid	GN—6 M	−
175	5-Methyl-2-hexenoic acid	GN—24 M	+
176	5-Methylheptanoic acid	GN—6 M	−
177	2-Hydroxy-3-methyl-butanoic acid	GN—24 M	=
178	Benzyl alcohol (as the methyl ether)	GN—24 M	=
179	Furoic acid	GN—24 M	=
180	*o*-Cresol (as *o*-methyl-anisole)	GN	−
181	2-Methyloctanoic acid	GN—24 M	=
182	2-Heptenoic acid	6 M—24 M	+
183	*p*-Cresol (as *p*-methyl-anisole)	GN—6 M	−
184	*m*-Cresol (as *m*-methyl-anisole)	GN—6 M	−
185	Octanoic acid	GN—24 M	+
186	5-Octenoic acid	GN—24 M	+
187	7-Octenoic acid	GN—24 M	=
188	2-Hydroxy-3-methyl-pentanoic acid	YE—24 M	+
189	7-Methyloctanoic acid	GN—FC	−
190	Furoic acid	FC—24 M	+
191	2-Octenoic acid	6 M—24 M	+
192	3,3-Dimethyloctanoic acid	GN—6 M	−
193	Nonanoic acid	GN—24 M	=
194	Benzoic acid	GN—24 M	+−
195	4-Oxo-5-methyl-hexanoic acid	GN—24 M	=
196	7-Methylnonanoic acid	6 M—24 M	+
197	2-Nonenoic acid	6 M—24 M	+
198	Decanoic acid	GN—24 M	=
199	2,6-Nonadienoic acid	12 M—24 M	+
200	2-Ethyl-3-methyl-maleic anhydride	GN—24 M	+
201	*m*- or *p*-Toluic acid	GN—24 M	−
202	Phenylacetic acid	GN—24 M	+−
203	*m*- or *p*-Toluic acid	GN—24 M	=
204	Salicylic acid	GN—YE	−
205	Geranic acid	GN—24 M	=
206	2-Isopropyl-5-oxo-hexanoic acid	GN—24 M	−
207	Undecanoic acid	GN—24 M	=
208	2-Ethyl-3-methylmaleic acid	GN—6 M	−
209	4-Isopropyl-7-methyl-5,7-octadienoic acid	GN—24 M	=
210	Phenylpropanoic acid	YE—24 M	+−
211	4-Hydroxy-1,1,3-trimethyl-3-cyclohexen-2,5-dione	YE—24 M	+
212	Phenol	GN—24 M	+
213	Dodecanoic acid	GN—24 M	=
214	3-Isopropyl-6-oxo-2-heptenoic acid	GN—24 M	
215	Pyrrole-2-carboxylic acid	FC—24 M	+
216	3-Isopropyl-5-hydroxy-pentanoic acid lactone	GN—24 M	+
217	*p*-Cresol	GN—24 M	+
218	*m*-Cresol	GN—24 M	=
219	*o*-Methoxybenzoic acid	GN—YE	−
220	Tridecanoic acid	GN—24 M	−
221	Tetradecanoic acid	GN—24 M	+
222	Pentadecanoic acid	GN—24 M	+
223	Hexadecanoic acid	GN—24 M	+

* +/− means increase/decrease of the concentration of a compound (weight/dry weight of tobacco) on curing and/or ageing; = means that it remained unchanged. † Quantitative data was not obtained for this acid due to the high volatility of the corresponding methyl ester.

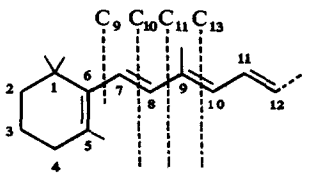
the major route for carotenoid degradation in intact plants. Thus, soy bean lipoxygenase is known to catalyze the conversion of β-carotene (**224**) to β-ionone (**112**) [9], and of violaxanthin (**225**) to 3-hydroxy-β-ionon-5,6-epoxide (**226**) [12], reactions which are coupled with the oxidation of a fatty acid such as linolenic acid. Furthermore, a tea enzyme preparation in the presence of oxidized tea flavanols degrades ^{14}C-labelled β-carotene (**224**) into β-ionone (**112**) and a number of minor, unidentified volatile products [10]. Stevens [9] has demonstrated that the carotenoids in tomatoes are rapidly broken down enzymatically after tissue damage and that there is a correlation between the concentration of certain degradation products and the content of their presumed carotenoid precursors.

In non-photosynthetic plant tissues, degradation of carotenoids is likely to occur by photo-oxygenation. It is thus of interest to note (Scheme 1) that exhaustive photo-oxygenation of β-carotene (**224**) yields desoxyxanthin (**227**), β-ionone (**112**), dihydroactinidiolide (**129**), 6-hydroxy-2,2,6-trimethylcyclohexanone (**83**) and geronic acid (**228**), while the corresponding hydroxy derivative, zeaxanthin (**229**), a tobacco carotenoid, affords 3-hydroxy-β-ionon-5,6-epoxide (**226**), loliolide (**230**), isololiolide (**231**), 3-hydroxy-β-cyclocitral (**232**), 2,4-dihydroxy-2,6,6-trimethylcyclohexanone (**233**) and hydroxygeronic acid (**234**) [13]. Violaxanthin (**225**), another tobacco carotenoid, has been reported to give rise to xanthoxin (**235**), 3-hydroxy-β-ionon-5,6-epoxide (**226**) and loliolide (**230**) on photo-oxygenation [14].

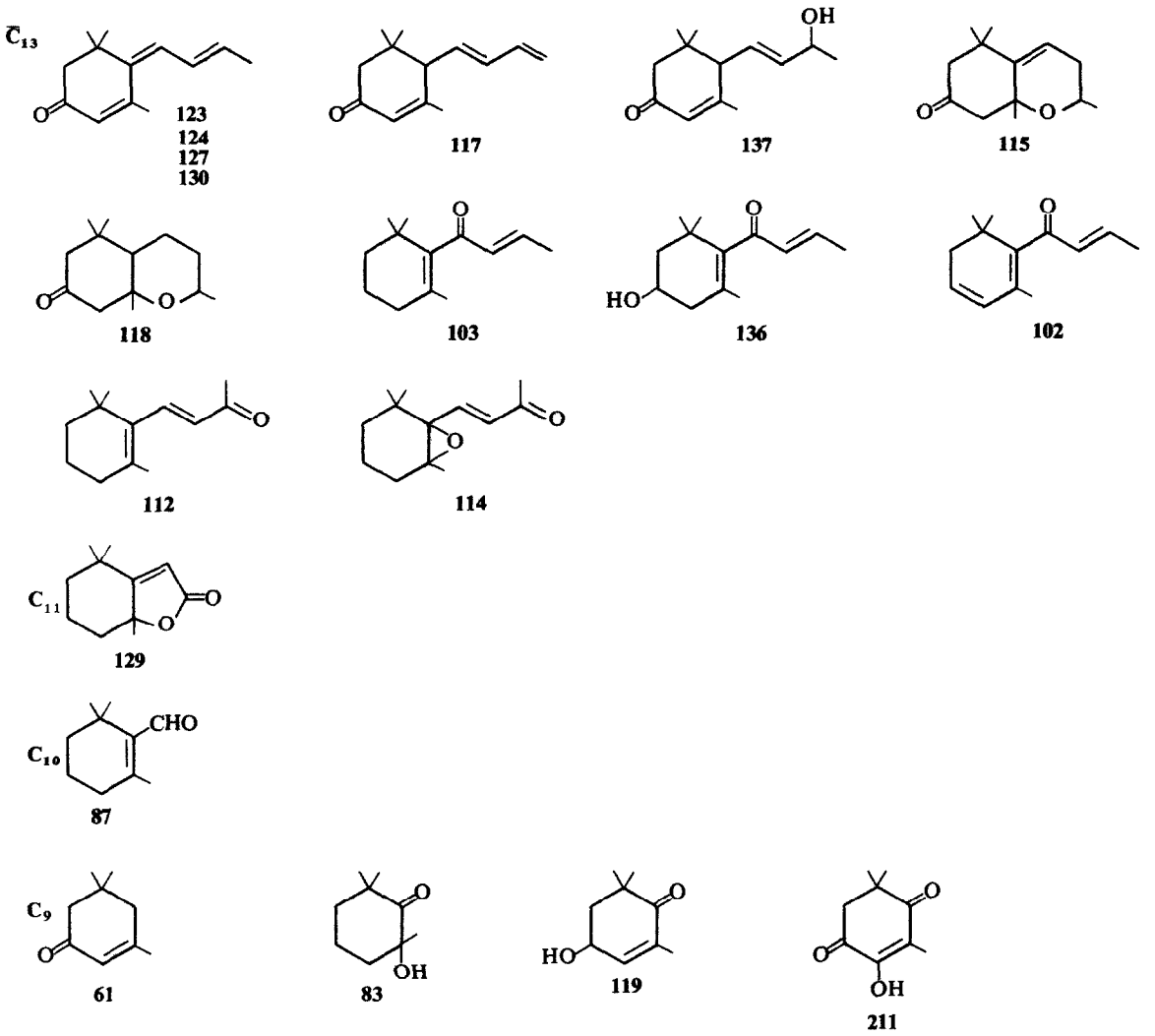


Scheme 1

Nineteen compounds, which may formally arise by oxidative cleavage of the 6–7, 7–8, 8–9 or 9–10 bonds of appropriate cyclic tobacco carotenoids (Scheme 2) were identified in our tobacco-volatile concentrates. Although the majority of these constituents were found to be present in the fractions derived from GN tobacco (*vide supra*), curing and ageing normally increased their concentrations.



Scheme 2



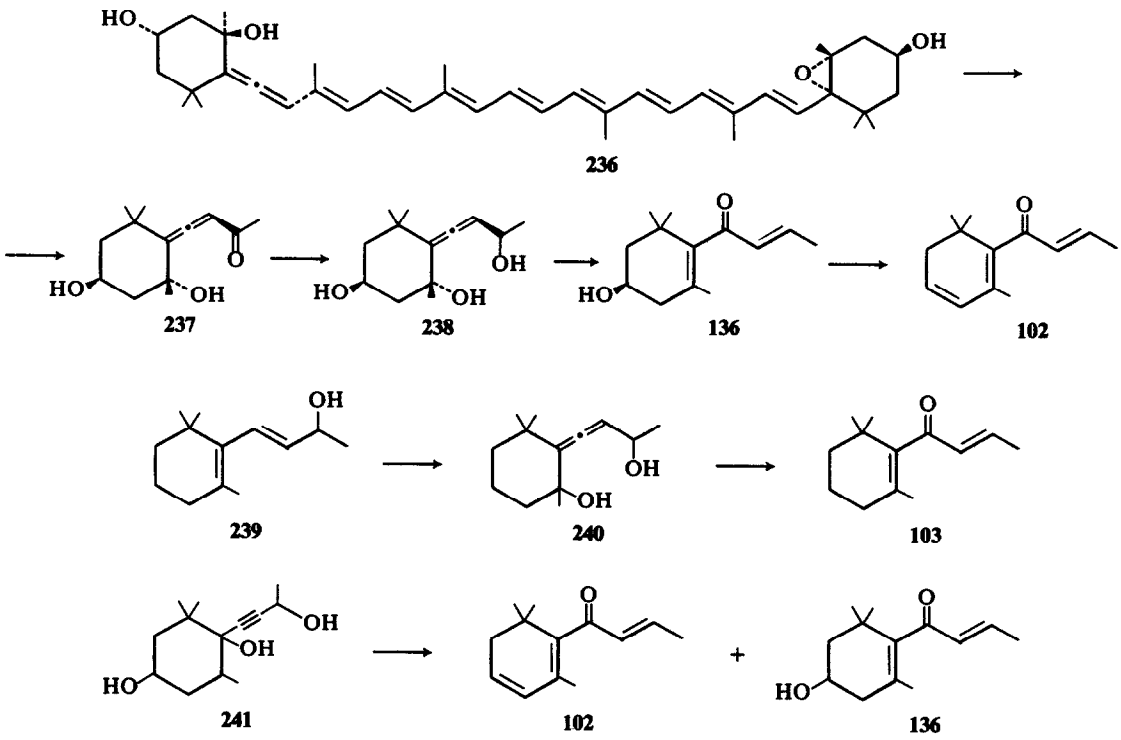
Thus of the four isomeric 4,6,8-megastigmatrien-3-ones **123**, **124**, **130** and **127**, the first three were minor components of the fraction from GN tobacco, while **127** was generated during the yellowing stage. Curing and ageing greatly increased the concentrations of **123**, **127** and **130** whereas the fourth isomer **124**, remained a fairly minor constituent in all tobacco samples. 4,7,9-Megastigmatrien-3-one (**117**) is generated during the curing treatment and its concentration increases on ageing of the tobacco.

All these compounds are easily obtained synthetically [15] from 3-oxo-α-ionol (**137**), another tobacco nor-cartoenoid, and it seems likely that their formation in tobacco proceeds via this alcohol. 3-Oxo-α-ionol (**137**) and the corresponding 7,8-dihydro derivative are also synthetically verified and biologically plausible precursors of the pyran derivatives **115** and **118** [16]. While **118** is generated during the ageing procedure, **115** is present already in GN tobacco, but its concentration is increased roughly thirty times as a result of curing and ageing.

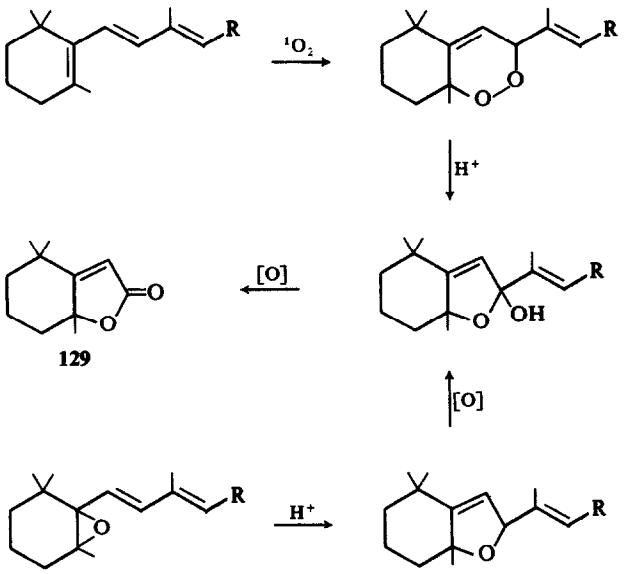
The levels of damascone (**103**) and the corresponding 3-hydroxy derivative (**136**) rise during the post-harvest treatment, whereas damascenone (**102**), an important flavour component first isolated from Bulgarian rose oil [17], is generated during the ageing period. It has been suggested [18, 19] that these compounds may be formed from neoxanthin (**236**) via the grasshopper ketone (**237**) and the allenic triol (**238**). Although none of these conversions have been carried out experimentally, an analogous set of reactions is involved in the $^1O_2$ oxidation of β-ionol (**239**) to the allenic diol (**240**), which is converted to damascone (**103**) [20]. The biomimetic synthesis comprising acid catalysed conversion of the trihydroxyacetylene (**241**) to damascenone (**102**) and 3-hydroxydamascone (**136**) constitutes an alternative route (Scheme 3) [18].

Of the remaining C_{13} nor-carotenoids, β-ionone (**112**) and β-ionon-5,6-epoxide (**114**) can be regarded as derived from β-carotene (**224**). The level of β-ionone (**112**) remains fairly constant whereas the content of the epoxide (**114**) undergoes a slight increase during the tobacco processing.

Dihydroactinidiolide (**129**) is a fairly abundant flavour component, whose concentration is roughly doubled as a result of curing and ageing. It may be formed in tobacco from a C_{13} precursor such as β-ionone (**112**) or via 5,8-epidioxides or 5,6-epoxides, which can rearrange to 5,8-epoxides. These may yield dihydroactinidiolide (**129**) on further oxidation (cf. Scheme 4) [21–23].



Scheme 3



Scheme 4

β-Cyclocitral (**87**), whose concentration is slightly increased during the post-harvest treatment, may arise by simple cleavage of the 7,8 bond of β-carotene (**224**).

The C_9 constituents **61**, **119** and **211** are minor components, which remain at virtually constant levels during curing and ageing. The C_9 ketol (**83**) was detected only in GN and YE tobacco.

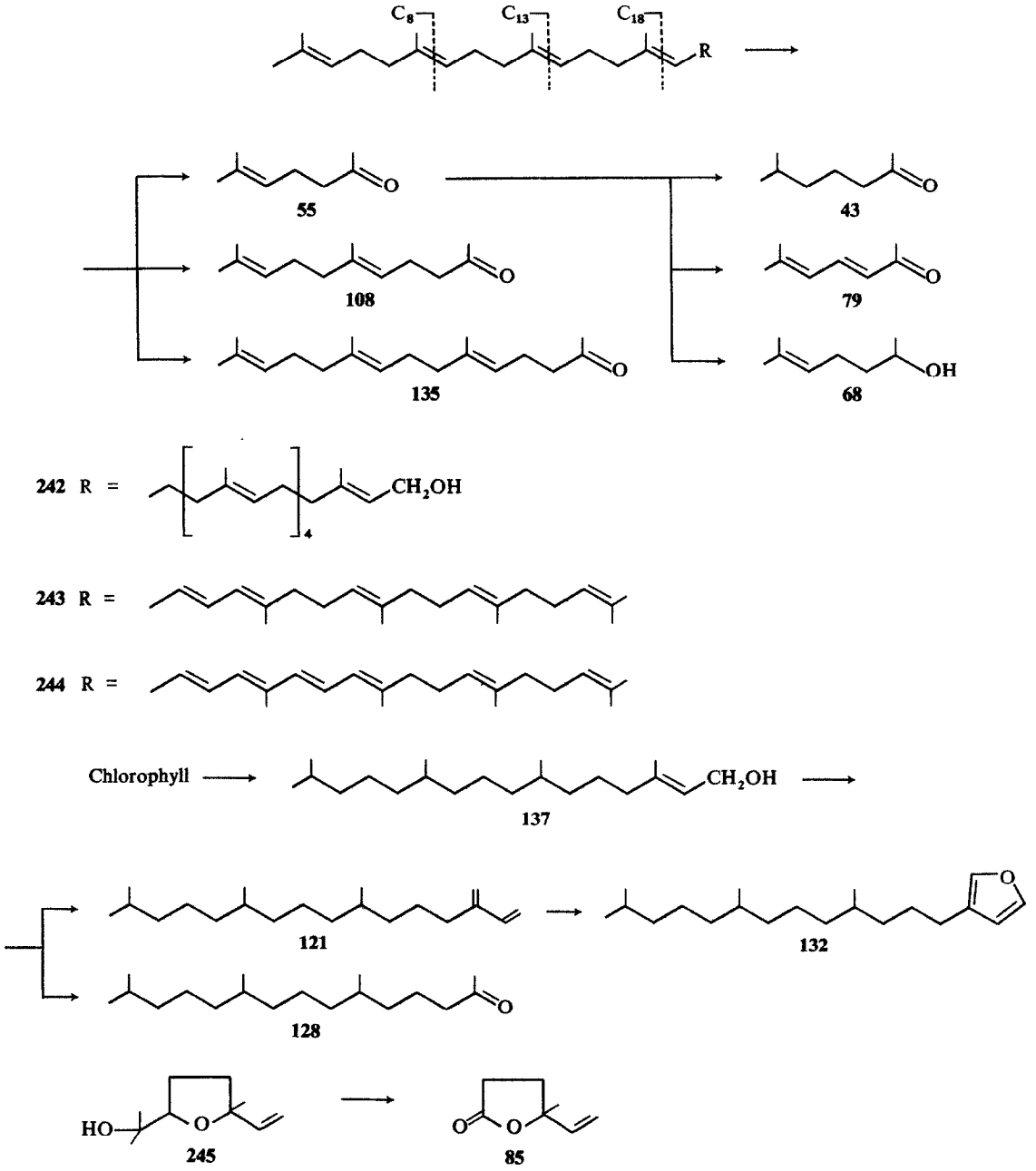
Nor-compounds derived from acyclic isoprenoids

A number of tobacco constituents may be regarded as carboacyclic nor-terpenoids generated from acyclic isoprenoids by oxidative fragmentation and subsequent chemical alterations (Scheme 5). Four C_8 (**43**, **55**, **68**, **79**), one C_{13} (**108**) and two C_{18} (**128**, **135**) constituents

conforming to this pattern were identified in the present examination. Solanesol (**242**), phytoene (**243**) and phytofluene (**244**) and some meroterpenoids, also encountered in tobacco, are appropriate precursors of 6-methyl-5-hepten-2-one (**55**), geranylacetone (**108**) and farnesylacetone (**135**), whereas the saturated hexahydrofarnesylacetone (**128**) may be derived from other tobacco constituents such as phytol (**134**).

The post-harvest treatment was found to increase the relative amounts of the C_{13} (**108**) and C_{18} (**128**, **135**) constituents and to leave those of 6-methylheptan-2-one (**43**) and 2-methyl-2-hepten-6-ol (**68**) at fairly constant levels. The concentration of 6-methyl-5-hepten-2-one (**55**) decreased during the ageing period and since a corresponding increase of the content of 6-methyl-3,5-heptadien-2-one (**79**) was found, it seems reasonable to assume that a conversion of **55** to **79** occurs during the ageing process.

The lactone **85** is a minor constituent, whose concentration is doubled on curing and ageing. It is probably formed from the monoterpenoid precursor linalool oxide (**245**) by oxidation.



Scheme 5

Terpenoids

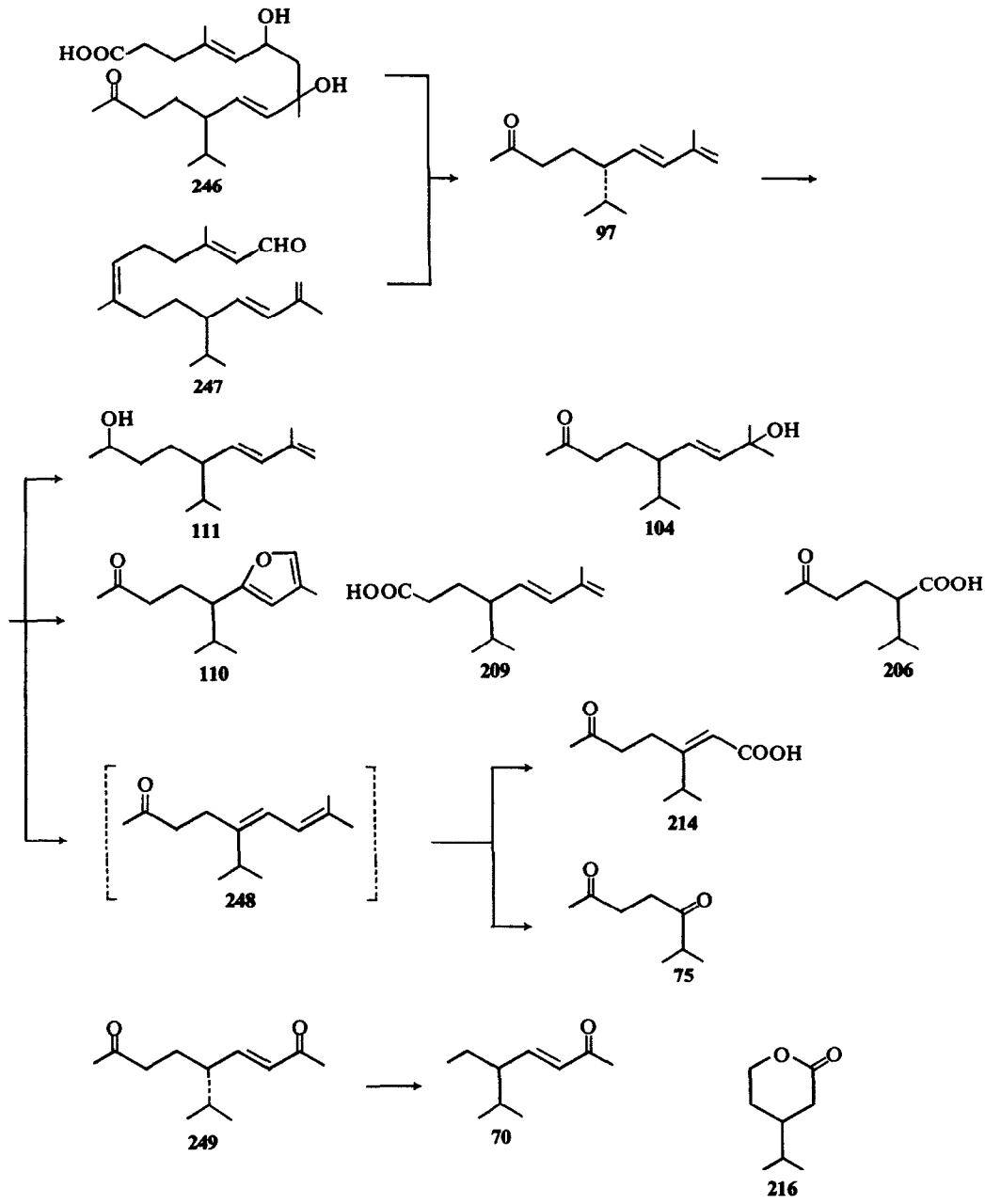
The acyclic diterpene neophytadiene (**121**) is by far the major volatile neutral constituent of Virginia tobacco. Its concentration increases considerably on flue-curing [24] and reaches a maximum in 6 M tobacco, where the content is almost ten times higher than in GN tobacco. Prolonged ageing lowers the concentration somewhat, which indicates that neophytadiene (**121**) is metabolised further.

It has been proposed that neophytadiene (**121**) originates from phytol (**134**), which is generated on degradation of chlorophyll (Scheme 5) [25]. Neophytadiene (**121**) is a probable biological precursor of phytofuran (**132**), an assumption supported by the facts that the corresponding conversion has been carried out synthetically by dye-sensitized oxygenation [26] and that the level of phytofuran rises during ageing.

Linalool (**78**) and geranic acid (**205**) were the only acyclic monoterpenoid constituents encountered. While the concentration of the former (**78**) increases slightly during the processing of the tobacco, that of geranic acid (**205**) remains constant.

Nor-thunberganoids

Recent phytochemical studies have revealed that cultivars of *N. tabacum* synthesize normally either diterpenoids of the labdane or thunbergane types [5, 27], a result which is consistent with the fact that the putative



Scheme 6

progenitors of *N. tabacum* are *N. sylvestris* and *N. tomentosiformis* [28], thunbergane and labdane producing species respectively. Maturation and curing [5, 6] have been reported to drastically reduce the content of diterpenoids, which is understandable in view of the fact that these compounds are present in the cuticular wax of the tobacco leaves and are hence highly susceptible to photochemical alteration and/or oxidation. Although experimental evidence is as yet not available, it has been postulated that a series of volatile constituents having adequate irregular isoprenoid skeletons are products of the catabolism of thunberganes [1, 29–31].

Virginia tobacco produces diterpenoids of the thunbergane type [2] and six nor-thunberganoids were identified in the neutral and four in the acidic fractions. The C_{13} compound solanone (**97**) is an abundant constituent, whose concentration is doubled during the two-year ageing period. Two acyclic diterpenoid precursors, the keto acid (**246**) and the aldehyde (**247**), each having undergone one of the two most commonly encountered ring cleavage reactions, have been proposed [29, 30] as precursors of solanone (**97**) (Scheme 6).

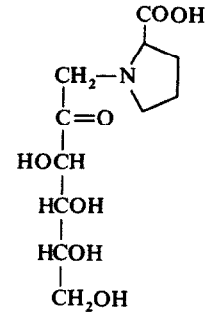
Solanone (**97**) is, in turn, a likely precursor of the majority of the nor-thunberganoids encountered here. Thus, the alcohol **111**, whose concentration increases sixfold on curing and ageing, can be viewed as a reduction product, while the ketol **104**, only detected in the 12 M and 24 M neutral fractions, may be formed on hydration of solanone (**97**). The formation of solanofuran (**110**) probably involves oxidative conversion of the diene group of solanone (**97**) to a furan moiety, a reaction which has been carried out synthetically [32]. Solanone (**97**) may also undergo oxidative degradation yielding the C_{12} acid **209**, the C_9 acid **206** and via rearrangement to isosolanone (**248**) the C_{10} acid **214** and the C_8 diketone **75**. The relative amounts of solanofuran (**110**) and the acid **209** remain fairly constant during the post-harvest handling, that of the C_9 acid **206** is reduced on storage implying further metabolism, whereas the keto acid **214** was only detected in the GN and 24 M acidic fractions.

The C_{10} enone **70**, which is a minor constituent and whose content is not significantly altered in the various tobacco samples, is possibly formed from a C_{12} precursor such as norsolanadione (**249**). Although there are clearly several alternative precursors of the lactone **216**, it is worth mentioning that curing and ageing increase its concentrations.

Compounds derived from sugar–amino acid reactions

The Maillard reaction between sugar and amino acids is known to produce brown pigments as well as volatile compounds, the latter often responsible for the cooked or roasted aroma of heat-treated foodstuffs. The process appears to be initiated by the formation of sugar amino acid compounds via an Amadori rearrangement. The labile Amadori compounds undergo subsequent decomposition by complex pathways, hitherto not fully elucidated [33]. As demonstrated by a number of model experiments, pyrroles, furans and pyrazines are products of such degradation reactions [34–39]. Furans, however, can also be formed in carbohydrate decomposition not involving Maillard reactions [40] and also as shown for pentylfuran (**44**) by oxidation of linoleic acid [41].

Flue-cured tobacco contains up to two per cent of Amadori compounds, the most abundant one being 1-deoxy-1-prolino-fructose (**250**) derived from L-proline and D-glucose [42–44]. Although the rate of formation of Amadori compounds has been reported to exceed that of their degradation during flue-curing and the initial period of ageing of tobacco [45], it is reasonable to assume that some degradation products are generated during these stages. This is supported by the fact that twenty-one compounds, all plausible products of Maillard type reactions, and including furans, pyrazines and a novel series of *N*-substituted-2-formylpyrroles, have recently been detected in flue-cured tobacco [46].



250

Our examination demonstrates that curing and ageing have a considerable effect on the concentrations of such compounds. Thus, furfuryl alcohol (**80**) and a corresponding methyl substituted derivative (**90**) show maximum concentration in 6 M tobacco, where they are of medium abundance. This indicates that *de novo* synthesis is predominant during flue-curing and the first ageing period, while prolonged ageing converts these compounds to other products. Although the identities of these products remain unclear it is worth mentioning that 2-acetylfuran (**62**), furfuryl acetate (**69**), methylfurfural (**71**) and a furoic acid (**190**), all of which are minor components, accumulate, the relative contents of furfural (**60**) and another furoic acid (**179**) undergo no major alterations, while a series of alkylfurans (**2**, **9**, **16**, **20** and **44**) are lost during the post-harvest handling.

Four pyrroles, **74**, **105**, **109** and **113**, were encountered in the neutral and one, pyrrole-2-carboxylic acid (**215**), in the acidic fractions examined here. Of these, the acid (**215**), 2-formylpyrrole (**109**) and 2-formyl-5-methylpyrrole (**113**) are generated on flue-curing and accumulate on storage, while a certain increase of the content of propylpyrrole (**74**) is found throughout the post-harvest treatment. The most dramatic effect is observed for 2-acetylpyrrole (**105**), whose concentration is increased roughly eighty times on flue-curing and then remains at this high level on storage of the tobacco.

Compounds related to phenylalanine and lignin metabolism

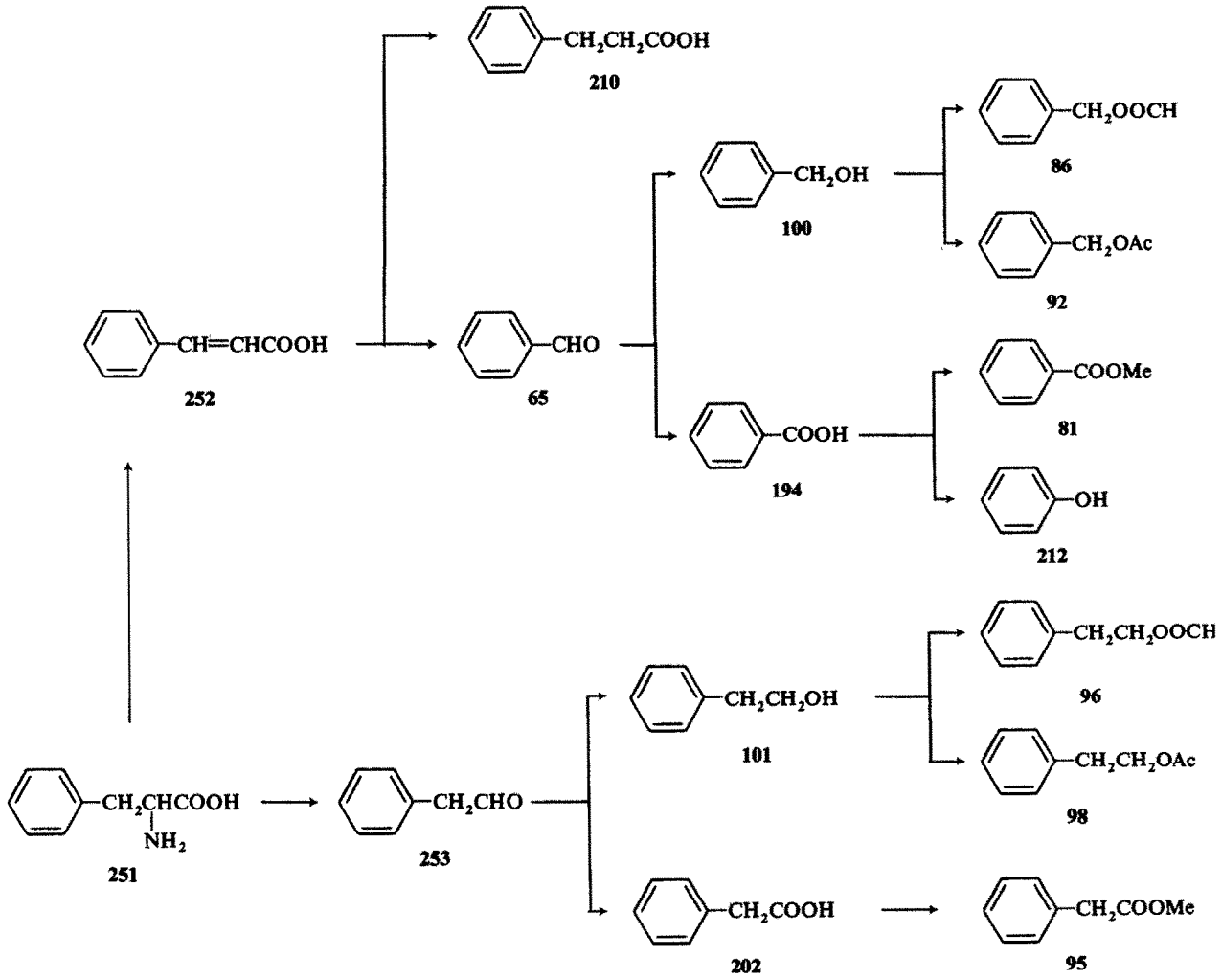
Virginia tobacco contains a fair number of abundant volatile aromatic constituents, the majority of which are mono-substituted and probably related to a compound

such as phenylalanine (**251**) and accordingly to lignin.* Although most of these compounds are present in GN tobacco, curing and ageing were found to affect their concentrations, a result suggesting active phenylalanine and cinnamic acid metabolism after harvest of the tobacco.

The reactions assumed to take place are summarized in Scheme 7. Thus, a phenylalanine ammonia lyase catalyses the conversion of phenylalanine (**251**) to cinnamic acid (**252**), which is a probable precursor of benzaldehyde (**65**) and phenylpropanoic acid (**210**). Benzaldehyde (**65**) may be oxidized to benzoic acid (**194**), which is converted to methyl benzoate (**81**) and phenol (**212**), while reduction of benzaldehyde (**65**) affords benzyl alcohol (**100**), a precursor of benzyl acetate (**92**) and benzyl formate (**86**).

Alternatively, phenylalanine (**251**) may undergo Strecker degradation to form phenylacetaldehyde (**253**), a compound, which although not present in detectable quantities in the tobacco samples examined here, may serve as a direct precursor of 2-phenylethanol (**101**) and phenylacetic acid (**202**). 2-Phenylethanol (**101**) is converted to the corresponding formate (**96**) and acetate (**98**) and phenylacetic acid (**202**) to methyl phenylacetate (**95**).

Although the concentration of benzaldehyde (**65**) is not altered significantly during the post-harvest processing, the quantitative results obtained for most of the other compounds support the reaction sequence outlined in Scheme 7. Thus, the relative amounts of benzoic (**194**) and phenylacetic (**202**) acids increase on flue-curing and reach maximum values in 6 M tobacco, in which their concentrations are respectively *ca* ten and forty times higher than in GN tobacco. The concentrations of the corresponding alcohols, benzyl (**100**) and 2-phenylethyl (**101**) alcohols, both of which are abundant neutral constituents, also show maximum values in 6 M tobacco. However, the difference in concentration as compared with GN tobacco are smaller, 1.5 and 3 times respectively, for these alcohols. The fact that prolonged



Scheme 7

* Some of the aromatic tobacco constituents may derive from lipids [47] or carbohydrates [48].

ageing reduces the content of these four compounds (**194**, **202**, **100**, **101**) supports the view that they are metabolised further. Moreover, the observed accumulation of the methyl esters **81** and **95**, the acetates **92** and **98** and of phenol (**212**) during the post-harvest treatment indicates that these are likely products. The remaining three conversion products, benzyl (**86**) and 2-phenylethyl (**96**) formate and phenylpropanoic acid (**210**) are minor constituents. A virtually constant level was found for 2-phenylethyl formate (**96**), whereas benzyl formate (**86**), only detected in the fraction derived from GN tobacco, is probably converted to benzyl acetate (**92**) by trans-esterification. Phenylpropanoic acid (**210**) is generated during the flue-curing procedure and reaches its maximum abundance in 6 M tobacco.

Several other aromatic compounds, all present in modest quantities, were encountered in Virginia tobacco. These include benzene (**8**), alkyl benzenes (**15**, **25**, **27**, **32**, **38**, **39**, **42**, **48**, cresols (**180**, **183**, **184**, **217**, **218**) *m*- and *p*-toluic acids (**201**, **203**) and salicylic acid (**204**). It is worth mentioning that benzonitrile (**73**) and acetophenone (**84**) are generated on storage of the tobacco.

Compounds related to lipid metabolism

It is well established that lipids are converted by the action of light, air and/or enzymes to volatile constituents, which often contribute to the aroma of various plant products [47]. There are several pathways of lipid degradation. Thus, fatty acids are released on hydrolysis of triglycerides and other lipid components. The lipoxygenase system, which is present in plants, catalyzes the oxidation by molecular oxygen of the *cis,cis*-1,4-pentadiene system in unsaturated fatty acids such as linoleic, linolenic and arachidonic acid. This reaction, which appears to be coupled with the oxidation of carotenoids (*vide supra*) leads to the formation of hydroperoxides. These decompose, either spontaneously or catalytically in the presence of a lipohydroperoxidase, to give rise to a variety of products including saturated and unsaturated alcohols and aldehydes [47]. Thus, a purified pea lipoxygenase has been found [41] to convert linoleic acid to 1-propanol, 1-pentanol (**41**), 1-hexanol (**58**), *trans*-2-heptenal, *trans*-2-octenal, *trans*-2-nonenal, *trans,trans*-2,4-nonadienal, *trans,cis*- and *trans,trans*-2,4-decadienal and pentylfuran (**44**). Leaf alcohol, 3*Z*-hexen-1-ol (**59**) and 2-hexenal (**34**) are possibly derived from the 13-hydroperoxide of linolenic acid via 3*Z*-hexenal [47, 49]. 2,6-Nonadienal and 1-penen-3-ol (**28**) are related to linolenic acid, whereas linoleic acid may be a precursor of 1-octen-3-ol (**66**). The formation of these compounds can take place enzymatically or non-enzymatically by auto-oxidation or photo-oxygenation [47].

The alcohols and aldehydes generated in this manner may be converted to the corresponding acids, which undergo methylation, oxidation to shorter chain or α,β-unsaturated acids, or partial β-oxidation to methyl ketones [47].

A fair number of structurally simple alcohols, aldehydes, ketones, acids and esters, which may be related to lipids, were encountered in Virginia tobacco. For convenience, we have also included in the discussion below some compounds for which other biogenetic origins are plausible or even more likely, e.g. 4-methyl-2-pentanone (**14**), 4-methyl-1-pentanol (**52**), 4-methylpentanoic acid (**157**), 3-methylbutanoic acid (**145**) and 2-hydroxy-3-methylbutanoic acid (**177**) may arise by oxidative cleavage of isoprenoids. Amino acids may undergo Strecker degradation to form branched chain aliphatic aldehydes, which are converted to the corresponding acids or alcohols. Thus, the tobacco constituents 2-methylpropanal (**1**) and 2-methylpropanoic acid (**140**) may emanate from valine, 3-methylbutanal (**6**) and 3-methylbutanoic acid (**145**) from leucine and 2-methylbutanol (**33**) and 2-methylbutanoic acid (**144**) from isoleucine.

The GC and GC–MS analyses allowed the identification of nine saturated alcohols including *n*-butanol to *n*-decanol (**26**, **41**, **58**, **64**, **82**, **93**, **106**), 2-methylbutanol (**33**) and 4-methylpentanol (**52**). Their concentrations are either reduced or unaffected by curing and ageing. Of the unsaturated alcohols, 3-methyl-2-buten-1-ol (**50**) and 3*Z*-hexen-1-ol (**59**) are fairly abundant components of GN tobacco, whereas 1-penten-3-ol (**28**), 3*E*-hexen-1-ol (**56**) and 1-octen-3-ol (**66**) are present in small quantities. Except for 1-octen-3-ol (**66**), whose concentration is maintained at a fairly constant level, the relative amounts of all these alcohols are reduced considerably as a result of the post-harvest processing.

Eleven acyclic aldehydes (**1**, **3**, **6**, **11**, **18**, **21**, **23**, **31**, **34**, **57**, **67**) and six acyclic ketones (**10**, **13**, **14**, **47**, **72**, **76**) were found as minor constituents of GN tobacco. The majority of these compounds were not retained on curing and ageing.

A variety of acyclic acids were encountered in Virginia tobacco. Of these, *n*-butanoic to *n*-decanoic acid (**142**, **148**, **161**, **173**, **185**, **193**, **198**) are fairly abundant in the acidic fraction derived from GN tobacco,* whereas *n*-undecanoic to *n*-hexadecanoic acid (**207**, **213**, **220–223**) are present in small quantities. The branched-chain acids include *iso*-acids (**140**, **145**, **157**, **168**, **189**), *anteiso*-acids (**144**, **155**, **169**, **176**, **196**), acids having a methyl (**141**, **149**, **153**, **163**, **181**) or ethyl (**150**, **158**, **172**) substituent at C-2 and acids branched at other positions (**146**, **165**, **192**). With a few exceptions (**140**, **144**, **145**, **149**) these compounds are present in modest amounts.

The class of unsaturated acids is exemplified by the α,β-unsaturated 2-butenoic to 2-nonenoic acids (**147**, **160**, **170**, **182**, **191**, **197**), two isomers of 2-methyl-2-butenoic acid (**151**, **159**), 3-methyl-2-butenoic acid (**156**), 5-methyl-2-hexenoic acid (**175**) and 2,6-nonadienoic acid (**199**), the majority of which are quantitatively prominent constituents of 24 M tobacco. Except for 3-hexenoic and 4-methyl-3-pentenoic acid (**167**, **154**), which are fairly abundant, the remaining unsaturated acids, 4-pentenoic, 5-hexenoic, 4-heptenoic, 5- and 7-octenoic acid, and two isomers of 3-methyl-3-pentenoic acid (**152**, **164**, **174**, **186**, **187**, **162**, **166**) are present in small amounts.

A few oxygenated acids, 2-hydroxy-3-methylbutanoic acid (**177**), 2-hydroxy-3-methylpentanoic acid (**188**) and 4-oxo-5-methylhexanoic acid (**195**), were detected as minor constituents.

While the concentrations of many acids are virtually unaffected, the post-harvest procedures were found to raise the relative contents of quite a few, i.e. *n*-butanoic

* Acetic and propanoic acid (**138**, **139**) were also encountered, but due to the high volatility of the corresponding methyl esters quantitative data could not be obtained for these acids.

(142), *n*-heptanoic (173), *n*-octanoic (185), *n*-tetradecanoic to *n*-hexadecanoic acids (221–223), the saturated branched-chain acids 145, 150, 155, 157, 165, and of all but one (151) of the α,β-unsaturated acids. Some of the latter (182, 191, 197, 199) were actually generated on storage of the tobacco. In contrast, the concentration of 3-hexenoic acid (167) was considerably reduced and a few acids (174, 176, 189, 192), present in GN and FC tobacco, could not be detected in the aged material.

Some lipid-related esters including methyl octanoate to methyl hexadecanoate (63, 77, 91, 99, 107, 116, 122, 126, 131), methyl 3-hexenoate (45), 3-hexenyl formate and acetate (46, 54), pentyl and hexyl acetate (29, 51) were encountered as minor components. Methyl 3-hexenoate (45) and 3-hexenyl formate (46) were lost, hexyl and 3-hexenyl acetate (51, 54) and some of the methyl esters accumulate, whereas the concentrations of the remaining esters are virtually unaffected by curing and ageing. A dimethyl-4-hydroxybutanoic acid lactone (35), whose concentration decreases and 2,3-dimethyl-4-hydroxy-2,4-nonadienoic acid lactone (120), whose concentration increases slightly, were detected in the neutral fractions. *n*-Decane to *n*-dodecane (22, 36, 53) are minor components generated during the processing.

The results presented above demonstrate that several typical lipid degradation products such as 3*Z*-hexen-1-ol (59), 1-penten-3-ol (28), 1-octen-3-ol (66), 1-penten -3-one (13), the 2,4-dienals (57, 67) and the 3,5-octadienones (72, 76) are present in GN tobacco and that curing and ageing tend to reduce their concentrations. This indicates that the oxidative reactions giving rise to these compounds take place possibly during the early stages of curing or as a result of mechanical damage. Such reactions are apparently no longer possible in flue-cured tobacco.

Since the contents of aldehydes, ketones and some of the alcohols and acids decrease, and since some of the acids, esters and hydrocarbons accumulate as a result of curing and ageing, it can be inferred that oxidations to acids, and subsequent esterification and decarboxylation of these are likely to take place in tobacco. However, since the majority of the aldehydes, ketones, alcohols and esters are present in small amounts, the quantitative measurements give clear indications as to the nature of specific transformations in only a few cases. Thus, the loss of butanal (3) and butanol (26) is accompanied by an accumulation of butanoic acid (142). The reduced concentrations of 3*Z*-hexen-1-ol (59) and of 3-methyl-2-buten-1-ol (50) in aged tobacco is partly accounted for by the raised level of 3-hexenyl acetate (54) and of 3-methyl-2-butenoic acid (156) respectively. The generation of the α,β-unsaturated acids may be explained by a dehydrogenation of the corresponding saturated acids and by isomerisation of appropriate unsaturated acids. They may also be formed by oxidation of the corresponding alcohols and aldehydes, even though these were not detected at a significant level in tobacco. The increased concentrations of *n*-tetradecanoic to hexadecanoic acid (221–223) in 24 M as compared with GN tobacco, may be ascribed to hydrolysis of triglycerides.

Compounds formed by degradation of chlorophyll

2-Ethyl-3-methylmaleic acid was encountered both as the dimethyl ester (208) and as the anhydride (200) on GC-analyses of the methylated acidic fractions. This compound, whose net concentration is not altered significantly on curing and ageing, is probably a chlorophyll degradation product [50] (cf. neophytadiene (121) and phytol (134) above).

EXPERIMENTAL

Extreme care was taken to avoid contamination during the collection and separation of the tobacco headspace material. The Et_2O and Na_2SO_4 were purified as described previously [51] and all aq. solns required in the separations were extracted with Et_2O prior to use. The charcoal was prepared for use as detailed before [8]. The previously described headspace collection system was designed to hold a tobacco bulk vol. of *ca* 20 l. [8]. For the present work, a larger all-glass apparatus, holding up to 200 l., was constructed. It consisted of a teflon-lined membrane pump (NK 400 MEDT), connected to a pump protection filter containing 100 g of purified activated charcoal, a glass column (length 200 cm; i.d. 22.5 cm) and a trapping filter holding 125 g of charcoal. This was attached to a second identical unit comprising in turn a glass column, trapping and pump protection filters. The latter filter was connected to the pump thus closing the system. The carrier gas used was N_2 and the flow, from bottom to top of each column, ranged from 50–120 l/min^{-1} depending on the packing characteristics of the tobacco sample in each case. The whole apparatus was enclosed in a foamed polystyrene environmental cabinet and kept at exactly 40° with a thermometer-relay controlled fan heater. Analytical GC was performed on a Varian 2700 instrument equipped with a capillary injector, a flame ionization detector, and an Autolab System I integrator. Samples were injected with a split ratio of 1:30 on a glass capillary column (50 m × 0.35 mm) coated with Ucon oil HB-5100 and using N_2 as a carrier gas (1 ml min^{-1}). Program rate: 2° min^{-1}.

GC–MS was carried out on an LKB 2091 instrument operated at 70 eV, in which the original gas chromatograph had been replaced by a Varian 2700 unit. The MS was connected online to a computer system comprising a 2310-C Raytheon Miniverter A/D converter, an HP (type 2116-B) 16K computer, a teletype and a plotter.

Growth and curing of tobacco. Tobacco (*Nicotiana tabacum* var. Coker 319) was grown under normal field practice at the Oxford Tobacco Research Station, Oxford, North Carolina, during 1971. Three sequential harvests of 6 leaves per plant, constituting bottom, middle and top leaf positions respectively were made and a sample of fresh green leaf (1/6 of total) was taken from each. These three samples were freeze-dried and combined (GN). The three harvests were cured separately in an experimental curing chamber, in which the air was forced circulated from bottom to top of the tobacco. The barn was lined with aluminium foil on the inside and equipped with an inlet air-filter containing charcoal in order to avoid contamination from external sources. The curing schedule was divided into three phases—a yellowing phase lasting for two to three days during which the temp. was raised to *ca* 40°, a leaf-drying phase requiring one day during which the temperature of the chamber was rapidly advanced to 53–59° and a stem-drying phase lasting for about two days and marked by a further increase in temperature to 74–84° with full venting. At the appropriate time during each of the three curings, a sample (1/5 of total) of yellowed tobacco was removed from the chamber and freeze-dried (YE). The dry flue-cured tobacco samples obtained from the three harvests were uniformly combined and divided into four equal randomised batches, one of which was taken as a sample of unaged tobacco (FC). The remaining three batches were moisture-conditioned and were packed separately in paper-lined pine boxes for ageing at ambient temp. and under normal industrial practice for 6, 12 and 24 months respectively (6 M, 12 M and 24 M). Table 1 lists weights as well as humidities of the tobacco samples.

Freeze-drying and rehumidification. The batches of GN and YE tobaccos were separately cut into 7.5 cm squares, frozen to

−75°, then rapidly passed twice through a standard ice-crushing machine. After removal of 2 kg of GN and 1.5 kg of YE tobacco for the small-scale operation, the bulk of each frozen tobacco was spread onto clean stainless steel trays and loaded into the vacuum chamber of a Virtis FFD-100 freeze-drier. During the drying process, which lasted for 4 days, the shelf temp. was kept at below 40° while the internal pressure gradually decreased to *ca* 0.03 mm Hg.

For the small-scale processing of GN and YE tobacco an all-glass freeze-drier was used. It consisted of four substrate flasks, each of 2 l. vol. and a condensor flask, which was cooled with dry ice/Me_2CO. The apparatus was connected via a cold trap to a vacuum pump, which retained the internal pressure below 0.3 mm Hg. Ambient air was blown over the substrate flasks during the drying period, which lasted for *ca* 2 days/sample. The ice condensed in the receiver flask was melted under 1 l. Et_2O and the aq. phase obtained was extracted twice with Et_2O. The Et_2O extracts were dried, concnd to a small vol. using the method of ref. [8] and examined by GC–MS. The analyses demonstrated that the compositions of the small condensed fractions, 146 mg and 205 mg from GN and YE tobaccos respectively, were markedly similar to those of the volatile fractions obtained on headspace collection of the corresponding freeze-dried tobaccos.

Prior to headspace collection the samples of GN, YE and FC tobaccos, 25.5, 23.9 and 23.7 kg respectively, were separately rehumidified by direct spraying of distilled H_2O. This was carried out using a stainless steel recirculator, in which N_2 circulated from bottom to top through the tobacco. After 18 hr, the GN, YE and FC tobaccos were found to be uniformly humidified to 11.4, 12.6 and 12.4% (dry basis) moisture content respectively.

Headspace collection and separation. The 6 samples of Virginia tobacco, GN, YE, FC, 6 M, 12 M and 24 M were separately cut in a Brabender rotary mill to pass a 7 mm grid and loaded into the two columns of the headspace apparatus. The system was purged with N_2, sealed and the pump started with the collection environment at 40°. After 7 days of operation, the charcoal trapping-filters were renewed and the re-purged system operated for a further 7 days. The combined trapping-filter charcoal was Soxhlet-extracted for 4 days with 2 l. of purified Et_2O [52]. The extract was dried over Na_2SO_4, carefully concnd using a vacuum-jacketed Vigreux column packed with nickel Dixon rings [8] to ca 600 ml and separated into fractions containing bases (B), acids (A) and neutrals (N) according to the following procedure. Treatment of the Et_2O concentrate with 5 × 250 ml of 5% aq. H_2SO_4 afforded an aq. phase, which was washed with 2 × 250 ml Et_2O to remove possible neutral components, made alkaline to pH 12 by addition of a concn aq. soln of NaOH, and satd with NaCl. Subsequent extraction with Et_2O gave the basic fraction (B). The Et_2O concentrate, now devoid of bases, was treated with 5 × 250 ml 4% aq. NaOH. The aq. phase thus obtained was washed with 2 × 250 ml Et_2O (N), acidified to pH 1 using conc HCl, saturated with NaCl and extracted with Et_2O which afforded the acidic fraction (A). The remaining Et_2O concentrate was combined with the Et_2O washings (N) obtained during the treatment of the aq. basic and acidic fractions which gave the neutral fraction (N). This as well as the basic and acidic fractions were washed with 5 × 250 ml saturated aq. NaCl, dried over Na_2SO_4 and concnd using the three-stage distillation method [8] to small vols (Table 1). The charcoal of the pump-protector filters was similarly extracted and the extract was concnd to a small vol. GC–MS analyses showed that these concentrates contained nothing but small amounts of AcOH, thus confirming that no oversaturation of the trapping-filters had taken place.

GLC and GC–MS analysis. All fractions, the acidic after methylation using diazomethane at 0°, were examined by GLC and GC–MS. 1-Nonanol was used as internal standard on GLC-integration of the fractions containing neutrals and 2-heptanol for the methyl esters and bases.

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