Characterization of Catechols, Resorcinols, and Hydroquinones in an Acidic Fraction of Cigarette Smoke Condensate

William S. Schlotzhauer,* Douglas B. Walters,[1] Maurice E. Snook, and Howard C. Higman

A biologically active, catechol-containing subfraction of a weakly acidic fraction of cigarette smoke condensate was further separated by gel filtration chromatography. Individual constituents of the gel fractions were identified by gas chromatography–mass spectrometry and ultraviolet spectroscopy. The principal dihydroxybenzene components were catechol, resorcinol, hydroquinone, and their alkyl-substituted derivatives. Other components of this fraction were 4-pyrone, vanillins, dimethoxyphenols, phenylphenols, and naphthols.

The weakly acidic fraction of cigarette smoke condensate (CSC) has been shown to possess tumor-promoting activity (Wynder and Hoffmann, 1964; Bock et al., 1969, 1971), but the identities of the active constituents remain in doubt. Catechol (1,2-dihydroxybenzene), the most abundant phenol in cigarette smoke, has been identified as a major constituent of the weakly acidic fraction (Hecht et al., 1975), but is considered an active co-carcinogen (Van Duuren et al., 1973) rather than a tumor promoter. Resorcinol (1,3-dihydroxybenzene) and hydroquinone (1,4-dihydroxybenzene) have been identified in cigarette smoke (Herrmann, 1964), and the presence of some alkyldihydroxybenzenes in smoke has been confirmed (Brunnemann et al., 1976; Ishiguro et al., 1976). However, because of its complexity, the weakly acidic fraction of CSC requires further resolution before its dihydroxybenzene constituents can be identified unequivocally. Walters et al. (1978) obtained from this fraction a subfraction in which catechol was concentrated and which was both tumorigenic and synergistically tumorigenic with other CSC fractions. In the present paper, we report our identifications of the dihydroxybenzenes (catechol, resorcinol, and hydroquinone, and their alkyl and methoxy derivatives) and other constituents of that subfraction, using gel filtration chromatography, gas chromatography–mass spectrometry (GC–MS), and ultraviolet spectroscopy.

MATERIALS AND METHODS

Fractionation of Cigarette Smoke Condensate. One-half kilogram of CSC, obtained from approximately 25 000 commercial nonfilter cigarettes, was prepared at Roswell Park Memorial Institute as previously described (Swain et al., 1969). CSC was fractionated by the method of Walters et al. (1978) (Figure 1). After removal of 100 g of CSC for control, the remaining material was partitioned (separatory funnel) between diethyl ether and aqueous 1 N sodium hydroxide. All solvents used in this study were redistilled "distilled in glass" grade. The aqueous layer, containing sodium salts of acidic components, was separated and acidified to pH 6.1 with concentrated hydrochloric acid. Diethyl ether extraction of the acidified material gave a weakly acidic fraction (F-8) of CSC (Bock et al., 1969), amounting to 33.54 g (8.38% of CSC). F-8 was further separated on a column (8.5 × 107 cm, glass) containing 500 g of activated (methanol washed; heated at 150 °C for 16 h) 100 mesh silicic acid. Elution of 5.2 L of 4% diethyl ether–95% petroleum ether (bp 30–60 °C) yielded subfraction F-61 (0.95% of CSC); similarly, 4.0 L of 10% diethyl ether–90% petroleum ether yielded subfraction F-62 (1.39% of CSC), 4.0 L of 50% diethyl ether–50% petroleum ether yielded subfraction F-63 (2.97% of CSC), and 1.6 L of methanol yielded subfraction F-64 (2.54% of CSC).

Rapid Gas Chromatographic Determination of Catechol. F-8 and subfractions F-61 through F-64 were initially screened for catechol. A 50-mg portion of the fraction was dissolved in 5.0 mL of acetonitrile. An aliquot of the solution was transferred to a 1.0-mL "Reacti-vial" (Pierce Chemical Company), and a fourfold excess of "Tri-Sil BSA" [*N,O*-bis(trimethylsilyl)acetamide] was added. The reaction mixture was placed in a heater flock at 75 °C for 30 min. Aliquots were withdrawn by syringe and analyzed by gas chromatography (GC) in a Varian Aerograph Model 2700, equipped with dual thermal conductivity detectors and dual 0.25 in. × 10 ft stainless steel columns containing 10% OV-11 on 80/100 mesh Chromosorb W [carrier (helium) flow, 85 mL/min; injector and detector temperature, 240 °C; oven temperature held at 75 °C for 4 min postinjection, then programmed to 235 °C at 4 °C/min]. Under these conditions, the bis(trimethylsilyl)catechol eluted in 22.0 min. Catechol was quantified by comparison of the catechol peak area, integrated by an Infotronics CRS-204 digital integrator, with a standard curve. Results showed that 97% of the catechol content of F-8 was concentrated in subfraction F-63. Me_3Si derivatives were used in this screening process only; all subsequent analyses were performed using underivatized samples.

Gel Filtration Chromatography. The gel filtration chromatographic procedure of Snook et al. (1976), previously described for the isolation and identification of polynuclear aromatic hydrocarbons of CSC, was modified for the separation of dihydroxybenzenes. Bio-Beads SX-8 (Bio-Rad Laboratories) in benzene were packed into four glass columns (1.25 × 109 cm) connected in series. A 250-mg aliquot of F-63 was placed on the lead column with a 1.0-mL injection loop. Benzene flow was maintained at 120 mL/h by a constant-flow pumping system, and 8-mL gel fractions were collected. Six successive separations of F-63 were performed and corresponding gel fractions were pooled. The elution pattern of the dihydroxybenzenes in F-63 were predetermined by use of a measured dose of ^{14}C-labeled catechol (New England Nuclear Corp.) added to a separate sample. Distribution of the recovered radioactivity in the gel fractions indicated that catechol was eluted in gel fractions (GF) 41 through 55, with a maximum elution in GF-47. GF 41 through 48 were selected for analyses as representative of the dihydroxybenzene content of F-63.

GC Analyses of Gel Fractions. The gel fractions selected for analysis were concentrated to 1.0 mL under

Tobacco Laboratory, Science and Education Administration—Federal Research, U.S. Department of Agriculture, Athens, Georgia 30604.

[1]Present address: National Institute Environmental Health Service, Research Triangle Park, N.C. 27709.

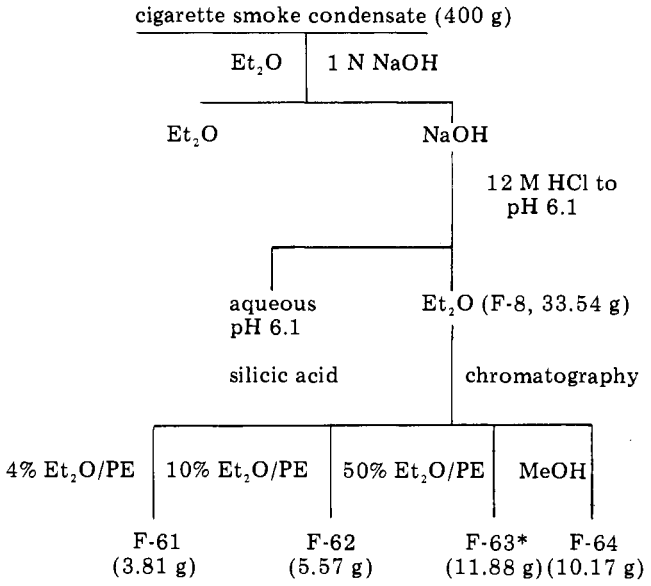

Figure 1. Fractionation of CSC to yield dihydroxybenzene-containing subfraction F-63*.

a stream of dry nitrogen and maintained in solution whenever necessary by addition of a minimum of acetone. Gel fractions were analyzed directly, without derivatization, on a 2 mm × 2 m glass "U" column containing 6% OV-17 on 80/100 Chromosorb G-HP in a Varian 2100 gas chromatograph, equipped with flame ionization detector (helium flow, 32 mL/min; hydrogen flow, 32 mL/min; air flow, 300 mL/min; injector, 240 °C; detector, 310 °C; column temperature, 100 °C to 290 °C at 2 °C/min.) Under these conditions, underivatized catechol eluted in 13 min. The amounts of dihydroxybenzenes and other constituents were determined from peak areas (Hewlett Packard 3380A integrator) and use of response data obtained for authentic compounds. Whenever standard compound was not available, the unknown was quantified on the basis of response data obtained for the standard compound closest in structural and GC retention characteristics.

GC–MS of Gel Fractions. Gel fractions were subjected to GC–MS in a Hewlett Packard 5710A gas chromatograph, equipped with a 2 mm × 1.83 m coiled glass column containing 6% OV-17 on 80/100 mesh Chromosorb G-HP and a flame ionization detector, and interfaced with a DuPont 21-492 mass spectrometer. Gas chromatographic conditions were as described above for the underivatized compounds. GC column effluent was split 1:8, one part of the effluent being directed to the flame ionization detector, and eight parts, enriched by use of a modified Ryhage jet separator, being directed into the spectrometer source. The interface system and the source area of the spectrometer were maintained at 300 °C. With electron energy at 70 eV, the spectrometer was adjusted to scan at 2 s/mass range as often as possible during recorded elution of each GC peak to determine homogeneity of the effluent peak. MS data were converted and stored by an AEI DS-30 data system equipped with a 256 K-word random access disc.

Ultraviolet (UV) Spectroscopy. The gel fractions were also separated by a Varian 2700 gas chromatograph equipped with thermal conductivity detector and operated under conditions described for underivatized samples. Effluents corresponding to the MS-analyzed peaks were collected in glass capillaries fitted into the exit port of the gas chromatograph. The collected materials were removed from the capillaries with a minimum of spectral grade cyclohexane and UV spectra of the solutions were obtained with a Beckman Acta CIII UV-visible recording spectrophotometer in double-beam operating mode. Spectra were compared with those obtained for authentic compounds and with those published in the literature.

RESULTS AND DISCUSSION

F-63, a major catechol-containing subfraction of CSC, was separated by gel filtration chromatography into 70 gel fractions. The dihydroxybenzenes eluted in GF 41 through 55 but identification analyses were conducted on the most concentrated fractions, GF 41 through 48. The elution order from gel columns was similar to that reported for polynuclear aromatic hydrocarbons (PAH) (Snook, 1976). Elution volumes for PAH were found to depend upon an adsorptive mechanism, possibly involving the formation of π-complexes between PAH and the polystyrene gel. Elution volumes increased with the number of rings in the PAH. Addition of alkyl side chains to the aromatic rings interfered with the adsorptive mechanism; hence, elution volumes decreased with increasing extent of alkyl substitution. The elution patterns for the weakly acidic components identified in F-63 generally reflected the interference effect of substituent groups on the aromatic ring.

Gas chromatograms of selected gel fractions are shown in Figures 2–6. These chromatograms were obtained using the conditions previously described for GC analysis of gel fractions. Peak identifications were based on GC retention times, MH fragmentation patterns, and UV spectra. Catechol (relative retention time, or RRT, assigned 1.00 for convenience) eluted somewhat earlier than resorcinol and hydroquinone, which were unresolved under the GC conditions used. Alkyl derivatives of these dihydroxybenzenes eluted in the order: catechols, hydroquinones, and resorcinols. MS fragmentation patterns allowed assignment of molecular structures to isomers of alkyldihydroxybenzenes (McLafferty, 1963). Thus, for example, an ethyldihydroxybenzene lost a CH_3 fragment more readily and yielded a proportionally higher M-15 ion than a dimethyldihydroxybenzene. GC retention data confirmed the MS-based structure assignments. Some isomers, such as *n*-propyl- and isopropyldihydroxybenzenes, were not resolvable by GC or by MS; however, retention characteristics and UV spectra allowed us to distinguish between the C_3-catechols, C_3-resorcinols, and C_3-hydroquinones. Characterization of positional isomers depended on GC resolution. Thus, the 3-methyl- and 4-methylcatechols (RRT, 1.23 and 1.34, respectively) were characterized, whereas the corresponding ethyl-substituted catechols were not (RRT of 1.70 for both isomers).

Table I presents the yields of the identified components in GF 41 through 48 of F-63. The principal components in GF-41 were a mixture of *n*-propyl- and isopropyldihydroxybenzenes, *o*-phenylphenol, and 2-hydroxy-3-methyl-4-pyrone. The latter compound has been identified in cigarette smoke (Elmenhorst, 1971) and in a tumor-promoting subfraction of CSC weak acids (Hecht et al., 1975). Other components of GF-41 included the methoxycatechols, vanillin, isomeric dimethoxyphenols, and *p*-phenylphenol. The elution of these constituents was almost complete before the constituents of GF-43, namely, catechol, 3-methyl-, and 4-methylcatechol began to elute. Additional components identified through GF-43 included ethylvanillin, coumarin, 2-naphthol, and methyl-2-naphthols. The presence of vanillins in CSC is possibly due to oxidative degradation of eugenol and isoeugenol, which have been identified in tobacco leaf and smoke (Herrmann, 1964; Rodgeman and Cook, 1964), or may

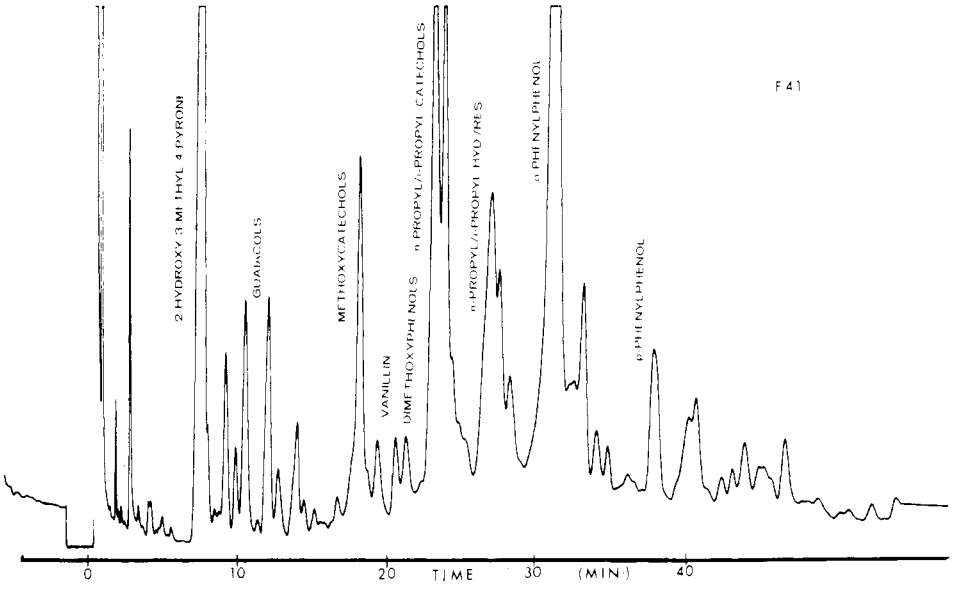

Figure 2. Gas chromatogram of gel fraction 41 of F-63.

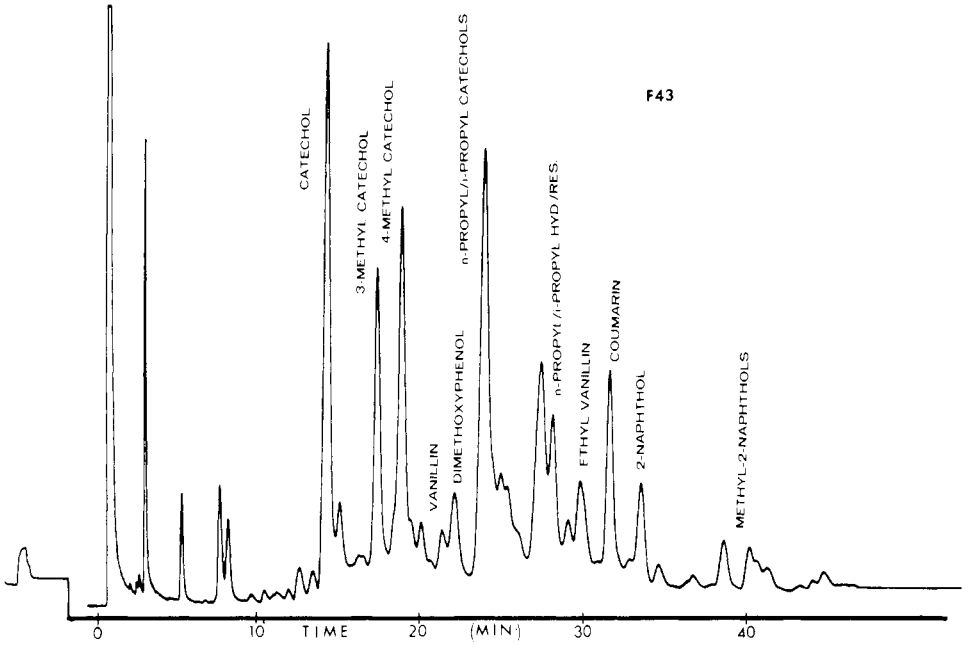

Figure 3. Gas chromatogram of gel fraction 43 of F-63.

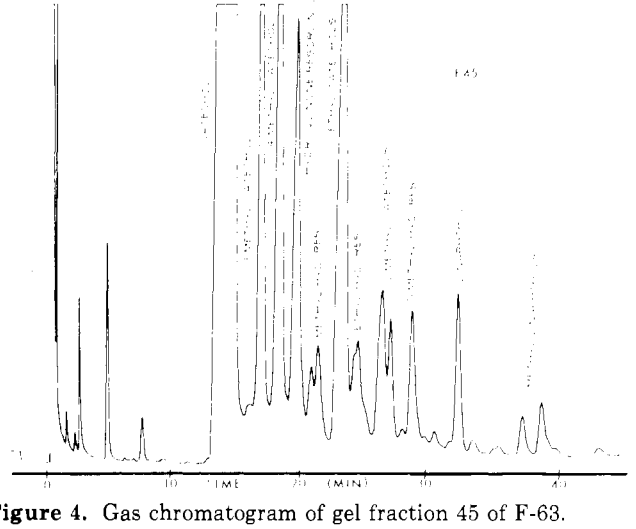

Figure 4. Gas chromatogram of gel fraction 45 of F-63.

derive from added flavorants. The identification of coumarin in this weakly acidic subfraction of CSC was not unexpected, as this lactone is known to undergo ring opening upon alkali treatment and ring closure upon subsequent acidification. The majority of coumarin of CSC apparently remained in the ether-extractable neutral portion (Chamberlain et al., 1977). Coumarin (1,2-benzopyrone) is a toxic component (Dickens and Jones, 1965) previously identified in CSC. Whether it derives from the degradation of naturally occurring tobacco leaf components or from tobacco flavor additives is still unknown. The fractions that eluted after GF-45 contained increasing concentrations of the parent dihydroxybenzenes and correspondingly decreased amounts of the alkyl-substituted (propyl, dimethyl, ethyl, and methyl) components. Vinylcatechols were identified in minor amounts in GF-46 and vinylresorcinols and vinylhydroquinones in GF46 and 47. A vinylcatechol has been identified as a tobacco smoke constituent (Ishiguro et al., 1976). Our

Table I. Yields (mg) of Constituents Identified per Gram of Acid Fraction F-63

components	RRT[a]	41	42	43	44	45	46	47	48	total
2-hydroxy-3-methyl-4-pyrone	0.53	12.62	2.02							14.64
catechol	1.00		1.66	5.09	12.02	13.09	16.04	19.23	14.34	81.02
3-methylcatechol	1.23			2.99	3.14	1.63	1.11	0.69	0.26	9.82
4-methylcatechol	1.34			3.63	3.47	1.77	1.29	0.93	0.41	11.50
methoxycatechols	1.37	5.54	3.37							8.91
hydroquinone/resorcinol	1.41				0.57	1.07	2.06	4.15	4.68	12.53
vanillin	1.46	1.60	0.57	0.30						2.47
methylhydroquinone	1.51				0.26	0.13	0.08	0.03		0.50
dimethyoxyphenols	1.56	1.65	0.68	0.41						2.47
methylresorcinols	1.57				0.60	0.20	0.10			0.90
dimethoxyphenols	1.61	1.72	0.99	0.86						3.57
ethylcatechols	1.70				4.30	2.17	1.64	1.39	0.75	10.25
propylcatechols	1.74-1.79	20.11	6.86	10.86						37.83
ethylhydroquinone/ ethylresorcinols	1.80				0.80	0.31	0.18	0.10		0.67
dimethylcatechols	1.98				3.74	1.53				5.27
propylhydroquinones/ propylresorcinols[b]	2.00-2.07	12.22	5.66							17.88
vinylcatechols	2.04						0.64			0.64
dimethylhydroquinones/ dimethylresorcinols[b]	2.14				0.64	0.25				0.89
ethylvanillin	2.18		0.99	0.82						1.81
vinylhydroquinone/ vinylresorcinols[b]	2.20						0.19	0.12		0.31
coumarin	2.32			1.21	0.23					1.44
o-phenylphenol	2.35	19.09	4.30							23.39
2-naphthol	2.47			0.64	0.57	0.29	0.22	0.16	0.06	1.94
p-phenylphenol	2.50	2.59	0.63							3.22
methyl-2-naphthols	2.82			0.34	0.18	0.10	0.04			0.66
methyl-2-naphthols	2.99			0.32	0.40	0.12	0.08			0.92
total identified		77.14	27.23	28.47	30.20	22.66	23.67	26.80	20.55	255.82

[a] Relative retention time (catechol = 1.00) under GC conditions described in text. [b] Not previously reported in cigarette smoke condensate.

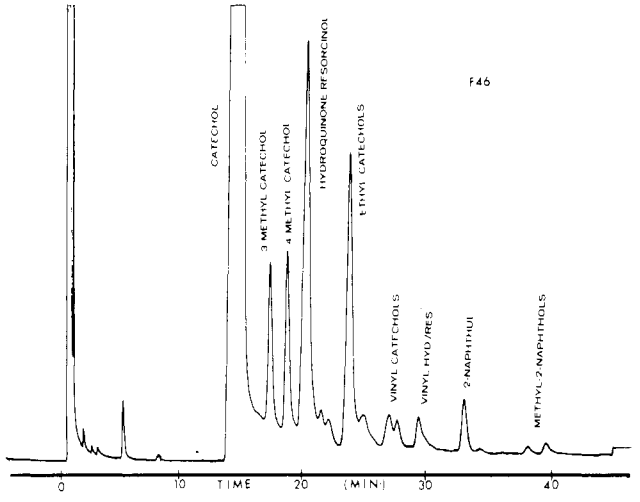

Figure 5. Gas chromatogram of gel fraction 46 of F-63.

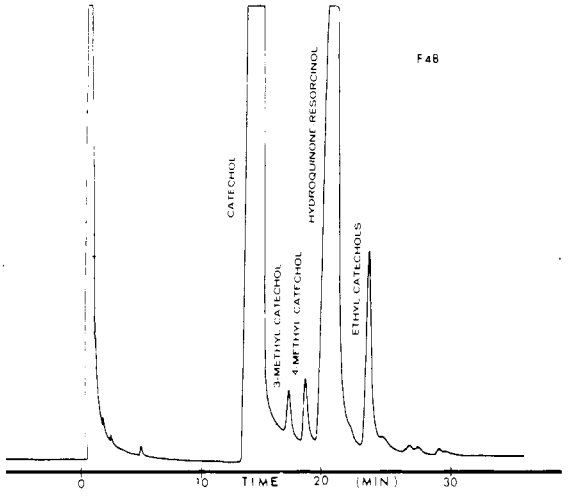

Figure 6. Gas chromatogram of gel fraction 48 of F-63.

calculated quantities for the vinyldihydroxybenzenes may be low, as vinylhydroquinone (and presumably other vinyl derivatives) has been shown to readily undergo oxidative polymerization (Updegraff and Cassidy, 1949), under conditions encountered in most CSC fractionation procedures. The presence in whole cigarette smoke or larger amounts of these compounds cannot be ruled out.

The components identified in this study accounted for approximately 25% of the total weight of F-63. Since the total residue weight of GF 41 through 48 was 346 mg, the components identified (Table I) represent 74% of these gel fractions. Parent dihydroxybenzenes and their alkylated derivatives amounted to 77% of the identified components in the analyzed fractions. The relatively high percentage of catechol among these components was not unexpected, as catechol is the most abundant phenol in cigarette smoke (Brunnemann et al., 1976). The amounts of propyl-substituted dihydroxybenzenes were higher than anticipated and possibly arose from the incomplete thermal degradation of the tobacco leaf caffetannin, chlorogenic acid (3-*O*-caffeoyl-*d*-quinic acid) or from one of the tobacco leaf flavanoids, rutin and quercetin. All of these compounds have been shown to produce dihydroxybenzenes on pyrolysis (Zane and Wender, 1963).

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High-Speed, Low-Pressure Liquid Chromatography of Chloroplast Pigments from Tobacco Mutants

Donald W. De Jong* and William G. Woodlief

A chromatographic method was developed for the separation and quantification of chloroplast pigments from tobacco (*Nicotiana tabacum* L.) leaves. The apparatus consisted of a low-pressure pumping system, a sealed silica gel column, and a recording spectrophotometer. Pigments were eluted with a stepped gradient solvent mixture containing heptane, diethyl ether, and acetone. The rapidity and accuracy of the technique provided a convenient means for individually measuring and collecting each of the chlorophyll and carotenoid components in leaf extracts from selected yellow mutant and green tobacco genotypes. The pattern of pheophytin a distribution in leaves harvested from different levels on the stalk showed no correlation with chlorophyll a content. It is suggested that pheophytin a is not necessarily an extraction artifact but could have a physiological role in photosynthesis.

Published methods for column chromatography of chlorophylls and carotenoids are tedious and time-consuming (Strain et al., 1971). They are difficult to replicate and require collection of fractions for quantification. As a consequence, paper and thin-layer methods are commonly used for rapid comparative analyses and column methods are used only for larger scale separations of individual pigments (Holden, 1965; Sěstâk, 1971). Weybrew (1957) reported that plastid pigments extracted from tobacco leaf would not separate on a conventional starch column, presumably because of the "poisoning" effect of nicotine. Whitfield and Rowan (1974) employed thin-layer chromatography to follow the decline of chlorophylls and carotenoids during senescence of tobacco leaves. Their work showed that the rate of chlorophyll loss equalled the rate of carotenoid loss in lower leaves, whereas chlorophyll disappeared faster in upper leaves. Some chlorophyll mutants have low levels of chlorophyll and appear yellowish throughout development; according to Benedict (1972) mutants of this type are widely distributed among crop plants. The existence of yellow and variegated mutants has stimulated research with regard to their mode of inheritance (Burk and Menser, 1964), chloroplast structure (Schmid and Gaffron, 1969; Shumway and Kleinhofs, 1973), and photosynthetic behavior (Highkin et al., 1969; Schmid, 1971). However, except for chlorophyll a/b ratios, which are generally higher in the mutants, little information is available on the range of total pigment levels in green and yellow genotypes. These differences could be important because, for example, the *Su/su* tobacco mutant examined by Homann and Schmid (1967) demonstrated high Hill rates (O_2 evolution) and superior photosynthetic capabilities. Boardman (1971) considered the C3 yellow mutants analogous to C4 type plants in terms of their photosynthetic efficiency.

In a previous paper we attempted to correlate growth vigor with the chloroplast properties of a normal green and a pale-yellow (*Py*) mutant of tobacco (De Jong and Woodlief, 1974). We have since added two genotypes to the study and have now examined the concentrations of chloroplast pigments in all four tobaccos. During preliminary efforts to develop a satisfactory methodology, we became frustrated with published methods of column chromatography. No fundamental improvement in the method of Perkins and Roberts (1962) has appeared in the literature and their technique was totally unsuited for rapid surveying of chlorophylls and carotenoids in comparative samples. After experimenting with a number of different solid phase absorbents and eluting solvents, we succeeded in assembling a reliable and reusable high-speed, low-pressure chromatographic system that totally separated all major leaf pigments during a run of 20-min duration. The eluate stream was monitored by passing it through a recording spectrophotometer. Fractionation of chloroplast pigments graphically displayed on the chromatogram was directly quantified from peak heights. In addition to conducting a comparative survey of pigment levels in leaves from four diverse tobacco genotypes harvested from three different portions of the stalk, we also examined the occurrence of pheophytin in the leaf extracts.

MATERIALS AND METHODS

Plant Types and Growth Conditions. Tobacco plants (*Nicotiana tabacum* L.) of four genotypes were grown in controlled-environment chambers (Sherer-Gillett CEL

Tobacco Research Laboratory, Science and Education Administration, U.S. Department of Agriculture, Oxford, North Carolina 27565 and the Department of Botany, North Carolina State University, Raleigh, North Carolina 27650.

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