

COMPOSITION STUDIES ON TOBACCO. XXXV. MOIETIES IN LEAF AND SMOKE CONDENSATE PIGMENTS: ACIDS

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Tobacco leaf and smoke condensate contain a series of high molecular weight brown pigments that may be of importance in tobacco quality and other pragmatic problems. In general, most of these pigments contain polyphenols, alkaloids and volatile bases, a silicone, amino acids and iron. Details of the composition of these pigments have been reviewed elsewhere (8).

Much of the more recent information on these substances has been obtained from a series of compositional studies on subfractions of the pigments. In the main, the alkaline-extractable, chloroform-insoluble, nondialyzable fraction (LP) of Turkish leaf pigment and the weakly acidic, nondialyzable subfraction (SP) of the smoke condensate pigment from domestic cigarettes have been investigated. Among the alkaline fusion products found previously in the condensate pigment were a series of aliphatic fatty acids containing predominately formic and acetic acids and two simple phenols, p-cresol and unsubstituted phenol (4, 5). Since all of these components might have been artifacts produced in the alkaline fusion (4), the existence of fatty acids and simple phenols as moieties in the pigments was not established by these findings. If present in the pigment, such moieties might be released by a less drastic reaction, such as saponification.

The yields of saponification products of LP and SP are shown in Table 1. Actually, each pigment was saponified twice; the "unbroken" pigment from the first saponification was used as starting material for the second hydrolysis. The table gives the total yields. The "unaccounted for" substances were probably gaseous degradation products that escaped during saponification. The amounts of hydrolytic products in the second saponification were less than the yields from the first except in the case of certain bases in SP (nicotine, nornicotine, metanicotone and myosmine). The degrees of fragmentation of LP and SP during saponification roughly parallel the findings obtained on alkaline fusion (2) with SP being more refractory.

Components in the acidic and basic fractions for which identifications were made are given in Table 2. The identified bases represent only a small amount of the total basic material released during hydrolysis. With two exceptions all of the bases listed for LP or SP are also found in the alkaline fusion products of the respective pigments: nicotine is obtained from the fusion of LP and myosmine is not found in the products from either pigment. In general, the composition of bases in the fusion products of both pigments is more complex than that of the saponification products.

The yield of weak and strong acids from SP is higher than LP (Table 1). However, the bulk of the acids in the saponification products of LP can be accounted for by formic and acetic acids, which is not the case with SP although the C₁ and C₂ components are the major

Table 1. Yields from saponification of nondialyzable leaf pigment (LP) and nondialyzable weakly acidic smoke condensate pigment (SP)

Fraction	Yields* (g)	
	SP	LP
Unsaponified pigment	6.1	2.7
Bases	0.09	0.16
Neutrals	0.13	0.09
Weak acids	0.60	0.21
Strong acids	0.36	0.40
Water-solubles	1.8	4.3
Unaccounted for	0.92	2.1

*From 10.0 g of each pigment.

Table 2. Some saponification products of nondialyzable leaf pigment (LP) and nondialyzable weakly acidic smoke condensate pigment (SP)

Product	Percentage*	
	SP	LP
N-Methylprole	.03	.46
Pyrrolidine	<0.1	.10
N-Methylpyrrolidine	<0.1	.10
Nicotine	.22	0
Nornicotine	.04	0
Metanicotone	.02	0
Myosmine	.02	0
Formic and acetic acids	.85	6.6
Propionic acid	.32	1.3
Butyric acid	<0.1	<0.1
Isobutyric	.13	.31
Valeric acid	.04	<0.1
β -Methylvaleric acid	0.8	<0.1
Isocaproic acid	<0.1	<0.1

*Based on weights of pigments degraded in the saponification.

identified acids. Except for isobutyric and β -methylvaleric acids, the other acids in Table 2 have been found in the nondialyzable strongly acidic fraction of condensate pigment which is closely related to SP (4). The presence of the lower fatty acids in LP has not been previously reported. In addition to the fatty acids, a small amount of a white, crystalline solid, was isolated from the water-solubles of LP. The infrared spectrum and m.p. (204°-206.5°C) were similar to authentic p-coumaric acid, m.p. 206.8°C. No depression was obtained in mixed melting point determination with authentic p-coumaric acid. No evidence was obtained for the presence of phenol, cresols or other simple phenols in the saponification products of either pigment.

The acidic hydrolysis of acetylated LP under special conditions was examined superficially. After hydrolysis with acetic acid containing a small amount of hydrochloric acid (0.25 percent) a yellowish-white solid was isolated that had an infrared spectrum of a higher fatty acid. The material was identified as a mixture of lauric, myristic and palmitic acids by cochromatography of the methylated acids and spectral comparisons with authentic compounds. The occurrence of these acids as saponification products of the non-acetylated pigment could not be verified.

Assuming mean molecular weights of 100,000 and 30,000 for SP and LP (1,3,8), respectively, the numbers of hydrolyzable ester (and amide) groups per mole for SP and LP are about 390 and 300, respectively, based on the saponification equivalents (Table 3). Thus, the molecular fragment containing one ester (or amide) group in LP is smaller than the comparable fragment in SP. Also, assuming that SP is derived from LP as previously postulated (8), the increase in molecular weight of SP over LP does not result from reactions involving the formation of additional ester (or amide) linkages. Based on a value of 2.5 percent hydrolyzable chlorogenic acid in Turkish LP (1) the number of ester linkages in LP due to chlorogenic acid would be about 5 groups per mole if the $-\text{COOH}$ group of the quinic acid moiety is esterified. The additional ester groups in LP are made up in part of esters of formic and acetic acids. Based on yields in Tables 1 and 2 (calculated as acetic acid), about 24 acetate groups per mole exist in LP. Thus, the bulk of the hydrolyzable ester groups in LP are not due to esterified chlorogenic, formic and acetic acid linkages if the bonds split in the saponification are not amides.

The quantitative aspects of methylation and acetylation are also of interest in structural considerations of the pigments. Based on the data in Table 3 and assuming the above molecular weights, the calculated $-\text{OCH}_3$ groups per mole are about 60 and 10 for SP and LP, respectively. After methylation, values of about 440 and 110 are obtained for SP and LP, using the decrease in nitrogen values as an index of the increase in molecular weight. Thus, about 380 and 100 $-\text{OH}$ groups per mole can be methylated in SP and LP, respectively. Based on the above level of hydrolyzable chlorogenic acid in LP, this polyphenol would contribute about 12 $-\text{OH}$ groups per mole, assuming that all $-\text{OH}$ groups are unsubstituted. Thus, the majority of hydroxyl groups in the pigments are not derived from chlorogenic acid.

Based on the "Hydroxyl" values of Table 3, SP and LP contain about 410 and 110 free hydroxyl groups, respectively, which can react with acetylating agents. These values closely approximate the numbers of $-\text{OH}$ groups capable of methylation in the pigments. These data indicate that most of the groups which can be acetylated are $-\text{OH}$ groups rather than $-\text{NH}_2$ or $-\text{NH}-$ groups.

EXPERIMENTAL

Pigments. The nondialyzable subfraction of Turkish tobacco leaf pigment and nondialyzable weakly acidic pigment from cigarette smoke condensate were isolated as previously described (2,3). Essentially, the isolation of leaf pigment consisted of extracting leaf with water at pH 10.0, removing hexane and chloroform solubles from the extract, precipitating the pigment by adjustment of the aqueous extract to pH 1.0, dialyzing the pigment against distilled water and removing the chloroform-soluble material by continuous extraction which yielded the chloroform-insoluble pigment used herein.

Table 3. Selected analytical values for nondialyzable leaf pigment (LP) and nondialyzable weakly acidic smoke condensate pigment (SP)

Pigment	N	Percentage		Saponification equivalent
		Methoxyl	Hydroxyl	
LP	5.74	1.07	6.5	98.5
SP	5.09	1.90	7.0	250.8
Methylated LP	5.40	11.11	—	—
Methylated SP	4.17	11.35	—	—

This pigment is heterogeneous, having major subfractions with molecular weights of about 4,000-50,000. The condensate pigment was isolated by removal of alkaline-soluble material from smoke condensate, precipitation at pH 6.1 of the pigment in the alkaline extract, removal of ether-solubles from the precipitated pigment by continuous extraction and extensive dialysis of the ether-insoluble pigment against phosphate buffer at pH 10.0. This nondialyzable pigment is heterogeneous with major and minor subfractions of $> 100,000$ and about 4,000, respectively.

Saponification. Ten g of each pigment was refluxed for 6 hrs in 2 N aqueous sodium hydroxide. The saponification mixture was separated into bases, acids and neutrals as detailed previously (3,4). The partially degraded pigment was recovered and the above saponification was repeated. The aqueous solutions remaining after removal of ether-soluble bases, acids and neutrals from all saponification mixtures were pooled and evaporated to a residue. The residue was extracted with hot ethanol which removed the "water-soluble" fraction from the salts.

Identification of acids and bases. These components were isolated and identified by the methods employed previously (2,3).

Methylation. About 5.0 g of pigment was dissolved in 100 ml 0.1 N sodium hydroxide. Fifty ml dimethyl sulfate were added with stirring, the temperature was maintained under 50°C by cooling and 10 percent aqueous sodium hydroxide was added dropwise to maintain the pH at 10 over a 30 minute period. After standing overnight the reaction was adjusted to pH 1.0 and filtered. The precipitate was washed with water until the washings gave no precipitate with aqueous barium hydroxide. The precipitate was dried at $100^\circ\text{C}/0.2$ mm Hg. Methoxyl determinations were performed on the original and methylated pigments by the method of Smith and Shriner (6).

Acetylation. About 5.0g of pigment were mixed with 100 ml acetic anhydride and 10 g sodium acetate. Twenty-five ml of acetyl chloride were added dropwise. After standing overnight, the mixture was heated under reflux at a bath temperature of 100°C for 4 hrs and then at 150°C for 2 hrs. The reaction mixture was distilled to a residue which was suspended in 200 ml water and stirred 15 mins. The mixture was filtered and the precipitate dried as described above.

Other determinations. Saponification equivalent and hydroxyl values were determined by the methods of Smith and Shriner (6), using alkaline diethylene glycol as the solvent in the former determination.

Acidic hydrolysis. 12.5g of acetylated leaf pigment was suspended in 200 ml acetic acid and 0.25 percent hydrochloric acid and the mixture was boiled for 1-2 mins and filtered. The filtrate was evaporated to dryness and the residue was dissolved in 400 ml ether. After decolorization (5.0 g charcoal added and the mixture stirred for 2 hrs), the charcoal was filtered off and a white precipitate separated on concentration of the filtrate. Infrared spectral examinations and gas chromatographic separa-

tions of the precipitate showed the material to contain about 0.43 g lauric, myristic and palmitic acids with myristic being the major component.

Isolation of p-coumaric acid. The water-soluble fraction of the saponification mixture from LP (10.0 g) was evaporated to a residue which was extracted with hot ethanol. The solvent was evaporated from the extract, and the residue was dissolved in methanol and chromatographed on activated silicic acid (373 g). The column was developed with mixtures of petroleum ether-ether and ether-methanol. p-Coumaric acid (about 5 mg) was eluted with 15 percent methanol in ether.

SUMMARY

Saponification of certain nondialyzable fractions of the acidic brown pigments from leaf and smoke condensate shows the presence of lower fatty acids with formic and acetic predominating. A few volatile bases and alkaloids are also released in small amounts by saponification of both pigments. Evidence for the presence of p-coumaric acid in the leaf pigment was also obtained. Lauric, myristic and palmitic acids were found in the products from leaf pigment using a special acidic hydrolysis method. In both pigments, most of the groups reacting with acetylating agents are hydroxyl rather

than amino or imino groups.

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