FREE PHENOLIC ACIDS IN CIGARETTE SMOKE AND TOBACCO
PAPER CHROMATOGRAPHY: SEPARATION AND IDENTIFICATION

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JOHNSTONE AND PLIMMER[1] reported no previous literature on the identification of polyphenolic acids in cigarette smoke. The recent discovery by YANG *et al.*[2] of caffeic acid (3,4-dihydroxycinnamic acid) in cigarette smoke gave impetus to our belief that the smoke likely contained a number of other related phenolic acids. Paper chromatographic analyses of smoke from regular, market cigarettes commonly used in the U.S. have indeed revealed the presence of more than twelve free phenolic acids in the smoke condensate. All of these phenolic acids have also been found in the smoke from cigarettes especially prepared for us by a reputable cigarette manufacturer so as to contain no flavorings or other common additives. Thus, the phenolic acids did not arise solely from the additives usually present in cigarettes bought on the regular market.

Similar studies on the cigarette tobacco itself proved that the same free phenolic acids found in cigarette smoke were also present in the tobacco of the cigarettes before smoking, with the possible exception of syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid). The phenolic acids which have been identified in the smoke and tobacco of all cigarettes tested thus far include *m*-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic (3,4-dihydroxybenzoic), vanillic (3-methoxy-4-hydroxybenzoic), syringic, *m*-hydroxyphenyl-acetic, *p*-hydroxyphenyl-acetic, *o*-hydroxyphenyl-acetic, *p*-coumaric (4-hydroxycinnamic), ferulic (3-methoxy-4-hydroxy-cinnamic), *m*-hydroxyphenyl-propionic, and *p*-hydroxyphenyl-propionic acids. Tentative identification of sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) has been made on paper chromatograms of smoke.

EXPERIMENTAL

Preparation of the "Acid Fraction-S" from the smoke of regular cigarettes

Four brands of non-filter, regular-size cigarettes (Camel, Lucky Strike, Philip Morris, and Chesterfield) were purchased on the open, retail market. The smoking of these cigarettes for phenolic acid analysis was performed by a procedure similar to the one previously reported by YANG *et al.*[3] The puff duration, puff interval, and puff volume for all cigarettes smoked were kept constant at 2.5 and 60 sec and 35 $\pm$ 1 ml, respectively. Solutions of the trapped smoke obtained from 20 packs of cigarettes (5 packs of each brand) were combined and then taken to dryness in a rotary evaporator under

reduced pressure. The residue was extracted several times with ether. After filtration, the ether filtrate was shaken with 5 % sodium bicarbonate solution, and the bicarbonate extracts were chilled. After acidification with concentrated hydrochloric acid, the water layer was saturated with sodium chloride, and shaken several times with ether to re-extract the phenolic acids. Ether extraction was continued for another 6 h in a continuous extractor. The ether extracts were combined, dried with anhydrous sodium sulfate, and concentrated to a small volume in a rotary evaporator. This fraction prepared from smoke condensate of regular market cigarettes was called "Acid Fraction-S".

Preparation of the "Acid Fraction-RS" from the smoke of cigarettes with no additives

Forty packs of Raleigh cigarettes especially prepared by the Brown and Williamson Corp., Louisville, Ky., to contain no flavoring or other common additives were smoked under the same conditions as were the regular market cigarettes. The "Acid Fraction-RS" was obtained from the combined smoke condensate from the cigarettes with no additives by a procedure similar to the one just described above.

Preparation of the "Acid Fraction-E" from the tobacco of regular cigarettes

This fraction was obtained by ether extraction of ground tobacco powder (about 127 g), representing a mixture of two packs of each brand: Camel, Lucky Strike, Philip Morris and Chesterfield. Prior to the ether extraction, the tobacco powder was extracted with *n*-pentane to remove coloring material. Ether extractions, followed by ethyl acetate extractions, were carried out using dry, fresh solvent in Soxhlet extractors. The bicarbonate extractions of the ether and ethyl acetate solutions were made in a separatory funnel. Re-extraction of phenolic acids from the acidified bicarbonate layer with ether and ethyl acetate gave the "Acid Fraction-E" of the tobacco from regular cigarettes.

Preparation of the "Acid Fraction-RE" from the tobacco of cigarettes with no additives

Ten packs of Raleigh cigarettes with no additives (216 g of ground tobacco powder) were used in preparation of "Acid Fraction-RE" by the same extraction procedures as have been described for "Acid Fraction-E" above.

Chromatography of the "Acid Fractions"

Descending chromatography with Whatman No. 1 paper was used throughout this investigation. Details of the sewing techniques have been reported previously[3]. Solvent systems employed in this study are benzene–acetic acid–water (6:7:3, v/v/v upper layer)[4], henceforth called BzAW; chloroform–acetic acid–water (2:1:1, v/v/v, bottom layer)[5], called CAW; aqueous sodium chloride (8 %)–glacial acetic acid (100:1, v/v)[6], called 8% NaCl; *n*-butyl alcohol–ethyl alcohol–ammonia–ammonium carbonate (1.5 *N*) buffer (40:11:19, v/v/v, upper layer)[7], called BEB; *n*-butyl alcohol–acetic acid–water[2], called BAW; and 2 % aqueous acetic acid solution, called 2 % HOAc.

An aliquot of each "Acid Fraction" was first subjected to two-dimensional chromatography, using the BzAW system for the first direction and the 8% NaCl system for the second direction on large sheets of chromatography paper ($18^1/_4 \times 22^1/_2$ in.). Each dried chromatogram was inspected under long wavelength (3660 Å)

and short wavelength (2537 Å) ultraviolet light, with and without exposure to ammonia vapor. The phenolic acid spots thus located were marked on the chromatograms. Since chromatograms were prepared in duplicate for each sample, one was sprayed with diazotized sulfanilic acid[8], called DzS, followed by 10 % sodium carbonate solution. The other was sprayed with the stable salt of diazotized *p*-nitroaniline (Fast Red Salt GG)[9], called FRS-GG, followed by 10% sodium carbonate solution.

Tentative identification of phenolic acids present in each "Acid Fraction" was made by comparing the compounds thereon (Figs. 1 and 2 and Table I) with those on "Reference Map" chromatograms prepared by using the same procedure as described above on a mixture containing authentic samples of all suspected phenolic acids. Although some differences in detail were found on the chromatograms of the "Acid Fraction" of tobacco extracts as compared with chromatograms of this fraction from smoke, the qualitative pattern of free phenolic acids on chromatograms of both was somewhat similar. The acids that could be recognized in common on these first two-dimensional chromatograms of tobacco and of smoke include caffeic, protocatechuic, *p*-coumaric, *p*-hydroxyphenyl-acetic, *p*-hydroxyphenyl-propionic, vanillic, sinapic, and ferulic acids. As will be described in later paragraphs, the "Acid Fraction" from the smoke contained a relatively higher amount of *m*-hydroxybenzoic acid than of *p*-hydroxybenzoic acid, whereas the reverse was the case in extracts of the tobacco. The "Acid Fraction" from tobacco extracts also contained on the first chromatograms, detectable amounts of *o*-hydroxyphenyl-acetic acid and melilotic acid (2-hydroxyphenyl-propionic acid). The occurrence of melilotic acid in tobacco leaf has been cited by GEISSMAN AND HINREINER[10]. Syringic acid was not detected in the tobacco extract.

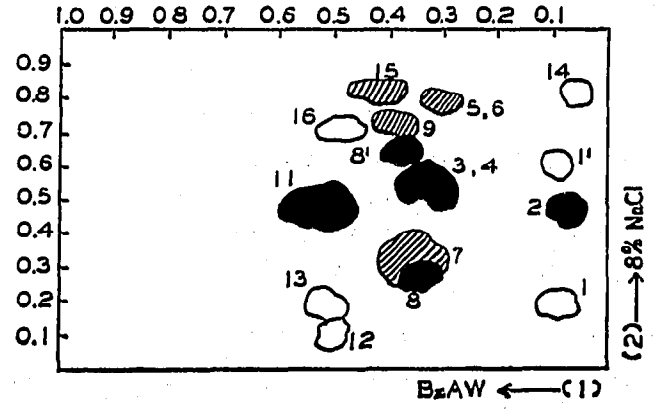

Fig. 1. Two-dimensional chromatogram of the "Acid Fraction" from the extracts of cigarettes.

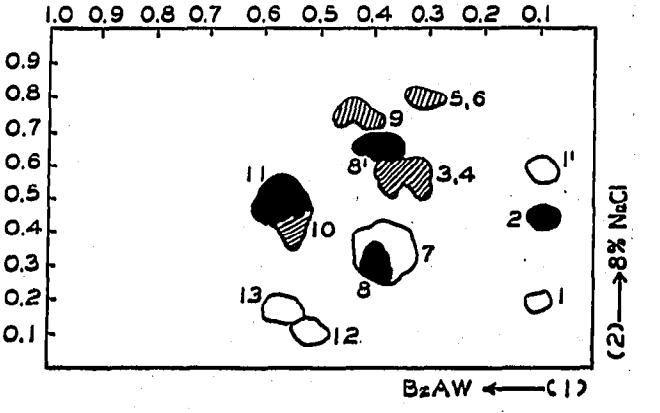

Fig 2. Two-dimensional chromatogram of the "Acid Fraction" from cigarette smoke.

IDENTIFICATION OF SPOTS IN FIGS. 1 AND 2

Spot No.	*Description*	*Spot No.*	*Description*
1	*trans*-Caffeic acid	8'	*cis*-*p*-Coumaric acid
1'	*cis*-Caffeic acid	9	*p*-Hydroxyphenyl-propionic acid
2	Protocatechuic acid	10	Syringic acid
3	*m*-Hydroxybenzoic acid	11	Vanillic acid
4	*p*-Hydroxybenzoic acid	12	*trans*-Sinapic acid
5	*p*-Hydroxyphenyl-acetic acid	13	*trans*-Ferulic acid
6	*m*-Hydroxyphenyl-acetic acid	14	Homoprotocatechuic acid (?)
7	Scopoletin	15	*o*-Hydroxyphenyl-acetic acid
8	*trans*-*p*-Coumaric acid	16	*o*-Hydroxyphenyl-propionic acid

TABLE I

R_F VALUES AND QUALITATIVE COLOR REACTIONS OF REFERENCE PHENOLIC ACIDS

Compound	R_F*					U.V.				DzSA + 10% Na_2CO_3	FRS-GG + 10% Na_2CO_3
	BzAW	CAW	8% NaCl	2% HOAc	BEB	luv + NH_3		suv + NH_3			
3,4-$(OH)_2$BA	0.14	0.13	0.58	0.64	(0.13)	—	PBl	dkBl	dPBl	ltBr	ltGrBr
3,4-$(OH)_2$PAA	0.10	0.14	0.82	0.83	(0.05)	—	—	Dk	Dk	ltBr	ltGrBr
trans-*p*- CA	0.45	0.57	0.44	0.48	0.51	—	dBl	dkP	Dk	ltR	dkBl
cis-*p*- CA	0.45	0.57	0.69	0.75	0.51	—	dBl	dkP	Dk	ltR	dkBl
p-(OH)BA	0.42	0.46	0.66	0.70	0.35	—	—	Dk	Dk	dY	RoR
m-(OH)BA	0.46	0.46	0.70	0.74	0.46	—	dP	dPBl	Dk	dY	RoR
p-(OH)PAA	0.37	0.45	0.84	0.84	0.44	—	—	Dk	Dk	OR	P
m-(OH)PAA	0.45	0.46	0.83	0.83	0.47	—	—	Dk	Dk	Y	RP
o-(OH)PAA	0.50	0.61	0.85	0.84	0.71	—	—	Dk	Dk	dkY	RP
p-(OH)PPA	0.51	0.63	0.78	0.77	0.58	—	—	Dk	Dk	RO	P
m-(OH)PPA	0.54	0.63	0.79	0.78	0.60	—	—	Dk	Dk	Y	RP
o-(OH)PPA	0.63	0.77	0.78	0.78	0.72	—	—	Dk	Dk	dkY	RP
Vanillic acid	0.70	0.79	0.59	0.62	0.25	—	—	dkBl	dkPBl	O	dP
Syringic acid	0.68	0.84	0.55	0.58	0.21	—	—	dkP	P	R	dBl
trans-Ferulic acid	0.69	0.82	0.39	0.43	0.35	dsBl	dsBl	dsBl	dsBl	ltPR	ltBlGr
cis-Ferulic acid	0.69	0.82	0.63	0.64	0.35	dsBl	dsBl	dsBl	dsBl	ltPR	ltBlGr
trans-Sinapic acid	0.66	0.85	0.32	0.36	(0.23)	dBl	dgrY	dsBl	dgrBl	Pk	ltBl
cis-Sinapic acid	0.66	0.85	0.61	0.64	(0.23)	dBl	dgrY	dsBl	dgrBl	Pk	ltBl

* Descending chromatography with Whatman No. 1 paper; distance traveled by solvents: 40–45 cm.

BzAW = benzene–acetic acid–water (6:7:3, v/v/v); CAW = chloroform–acetic acid–water (2:1:1, v/v/v); 8% NaCl = sodium chloride (8%)–acetic acid (100:1, v/v); BEB = *n*-butyl alcohol–ethyl alcohol–ammonia–ammonium carbonate buffer (40:11:19, v/v/v).

luv = long-wave ultraviolet light; suv = short-wave ultraviolet light; DzSA = diazotized sulfanilic acid; FRS-GG = stabilized diazo salt of *p*-nitroaniline (Antara Chemicals Division of General Aniline and Film Corp.).

lt=light; d=deep; dk=dark; s=sky; Bl=blue; Br=brown; Gr=green; O=orange; P=purple; Pk=pink; R=red; Ro=rose; Y=yellow.

A = acid; BA = benzoic acid; PAA = phenylacetic acid; PPA = phenylpropionic acid; CA = coumaric acid.

Further study of phenolic acids in the "Acid Fractions" after preliminary separation

A preliminary separation was carried out by streaking the concentrate of each "Acid Fraction" on 18 × 57 cm sheets of chromatography paper and developing each sheet in the BzAW system. Each chromatogram was cut into five zones (Table II), and each zone was eluted separately with methyl alcohol in an elution chamber at room temperature. This paper covers studies on zones 1, 2, 3, and 4 only. In addition to the compounds identified in these zones, other compounds that probably also are phenolic acids have been found in these areas of the chromatograms, but have not yet been identified. Studies on these unknowns plus those of zone 5 are in progress. Each zone 1 eluate ("S_1", "E_1", "RS_1", and "RE_1") was again subjected to two-dimensional chromatography in CAW and 8 % NaCl as one solvent system combination and in BAW and 2 % HOAc as another combination. All other eluates of zones 2, 3, and 4 were studied similarly, using CAW and 8 % NaCl as one combination, but BEB and 2 % HOAc as the second combination. Results of these studies are summarized in Table II.

TABLE II

TWO-DIMENSIONAL CHROMATOGRAPHY OF PHENOLIC ACIDS AFTER PRELIMINARY SEPARATION

Separation in BzAW		*Identification*
Zone	*R_P values*	
1	0.00–0.20	Caffeic acid, protocatechuic acid, and a homoprotocatechuic acid-like compound*
2	0.20–0.35	*p*-Hydroxybenzoic acid, *m*-hydroxybenzoic acid, and *p*- and *m*-hydroxyphenyl-acetic acids
3	0.35–0.50	Scopoletin[11], *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, *p*-coumaric acid, *p*- and *m*-hydroxyphenyl-acetic acids, *p*- and *m*-hydroxyphenyl-propionic acids, and *o*-hydroxyphenyl-acetic acid
4	0.50–0.75	Vanillic acid, ferulic acid, sinapic acid, and syringic acid**

* Found only in extract of tobacco.
** Found only in smoke.

Final purification and identification of each individual phenolic acid

Several phenolic acids found to be present in relatively large quantity were able to be isolated individually in paper chromatographically pure form for further proof of identity by comparison studies with corresponding authentic reference samples. These acids are protocatechuic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, *p*-coumaric, and vanillic. The separation and purification of these acids were accomplished by zone chromatography on paper with mass paper chromatographic techniques involving cutting, sewing, and re-chromatography of each zone in appropriate solvent systems.

Protocatechuic acid. This acid was first isolated from the eluate of zone 1 by using the solvent systems, CAW, BAW, 2 % HOAc, and BAW in the order listed. Final purification was achieved by developing the partially purified acid twice in both the CAW and 2% HOAc systems. The eluate of the pure protocatechuic acid zone checked with the reference acid in all the solvent systems used (Table I) and in its ultraviolet absorption spectrum (Table III). Co-chromatography in the combination solvent systems CAW–8 % NaCl and BAW–2% HOAc also confirmed its identity with the reference

acid. The spray reagents, 1 % ferric chloride solution and a saturated solution of ammonium molybdate, were found to be useful for studying this acid.

p-Hydroxybenzoic, m-hydroxybenzoic, and p-coumaric acids. These acids were isolated in pure form mainly from the eluate of zone 3. Some scopoletin persisting in the "Acid Fraction" was first separated from the phenolic acids of zone 3 by repeated developments in the CAW system. The eluate containing phenolic acids of zone 3 free from scopoletin was then subjected to further purification stepwise, using the BEB and BzAW systems to obtain partially purified zones of *p*-hydroxybenzoic, *m*-hydroxybenzoic, and *p*-coumaric acids. When separated on paper, these three acids can be differentiated from each other by observing them under both short and long wavelength ultraviolet light, with and without exposure to ammonia vapor. *p*-Hydroxybenzoic acid exhibits no fluorescence under long wavelength light, but it can be seen as a dark zone under the short wavelength, with or without exposure to ammonia. When exposed to ammonia, both *m*-hydroxybenzoic and *p*-coumaric acids exhibit deep purple-blue fluorescence under long wavelength light. The fluorescence of *m*-hydroxybenzoic acid remains unchanged under short wavelength light, but that of *p*-coumaric acid is quenched.

The final purification of *p*-hydroxybenzoic acid was completed by repeating the chromatographic developments in the BEB, BAW, and BzAW systems. On co-chromatography with an authentic reference standard, the purified *p*-hydroxybenzoic acid was found to be identical in the various solvent systems of Table I, including two-dimensional chromatography in BEB and 2 % HOAc, and in their ultraviolet absorption spectra (Table III).

Further separation of *m*-hydroxybenzoic acid was continued by starting with the BzAW and 2 % HOAc systems followed by the BEB, BAW, and BzAW systems as was done for the last steps in the purification of *p*-hydroxybenzoic acid. Using two-dimensional chromatography and BEB and 2 % HOAc as solvent systems, the purified *m*-hydroxybenzoic acid co-chromatographed with an authentic sample of reference *m*-hydroxybenzoic acid without separation. Both the isolated and reference compounds gave the same ultraviolet absorption spectra (Table III). The eluate of partially purified *p*-coumaric acid was subjected to further purification in the CAW and 2 % HOAc systems. Due to the separation of *cis* and *trans* isomers, *p*-coumaric acid appeared in two zones on the last chromatogram. Each eluate of *cis*- and *trans*-*p*-coumaric acids was then developed twice in the CAW and BAW systems followed by final purification in the BzAW, BAW, and 2 % HOAc systems. The purified *cis*- and *trans*-*p*-coumaric acids were proved to be identical with authentic reference *p*-coumaric acid by comparison studies in the various solvent systems listed. The absorption spectra of these isolated acids were also identical with the corresponding *cis*- and *trans*-*p*-coumaric acids prepared from the authentic *p*-coumaric acid, using the 2 % HOAc system (Table III).

Vanillic acid. This acid was isolated and purified first from the eluate of zone 4 by stepwise chromatography, using the BEB, BAW, and 2% HOAc systems. Final purification of vanillic acid was completed by cutting off the impurity from its wide zone and by repeated developments in the solvent systems, 2 % HOAc and BzAW. The purified vanillic acid and authentic reference vanillic acid co-chromatographed without separation on two-dimensional chromatography, using BEB–2 % HOAc as one solvent system combination and BzAW–8 % NaCl as another. The isolated and reference

vanillic acids also exhibited the same ultraviolet absorption spectra (Table III).

No solvent system has been found as yet which will completely separate *p*-hydroxyphenyl-acetic acid from *m*-hydroxyphenyl-acetic acid in the mixture or which will completely separate *p*-hydroxyphenyl-propionic acid from *m*-hydroxyphenyl-propionic acid in the mixture. Each pair of these acids, however, can readily be shown as a partially overlapped spot when developed in the BEB–2 % HOAc combination.

The mixture of *p*- and *m*-hydroxyphenyl-acetic acids contained in the eluate of zone 3 was found to move together with the major zone of *m*-hydroxybenzoic acid in the solvent systems employed except one. The 2% HOAc separates the mixture from the *m*-hydroxybenzoic acid. Further purification of the mixture of hydroxyphenyl-acetic acids was carried out by stepwise paper chromatography using the BAW and

TABLE III

ULTRAVIOLET ABSORPTION SPECTRA OF PHENOLIC ACIDS

Acid	*mμ*			
	λ_{max}	λ_{min}	λ_{max}	λ_{min}
Protocatechuic	258	234	291	278
p-Hydroxybenzoic	252	223	—	—
m-Hydroxybenzoic	234	261	293	—
p-Coumaric				
cis-	—	243	289	—
trans-	—	244	300	—
Vanillic	258	234	289	279.5

the 2 % HOAc systems. The R_F values and color reactions of the two acids thus isolated agreed with those from the mixture of the authentic samples of *p*- and *m*-hydroxyphenyl acetic acids prepared under the same conditions in the BEB–2 % HOAc combination. With the solvent systems employed, *m*-hydroxyphenyl-acetic acid always moved slightly ahead of the *p*-hydroxyphenyl-acetic acid. By a procedure similar to that described above, the mixture of *p*- and *m*-hydroxyphenyl-propionic acids plus *o*-hydroxyphenyl-acetic acid was separated from the major zone of *p*-coumaric acid and purified. Comparison studies of the two-dimensional chromatograms of the purified acids with those of the mixture of the three authentic reference compounds in the BEB–2 % HOAc combination proved the presence of *p*- and *m*-hydroxyphenyl-propionic and *o*-hydroxyphenyl-acetic acids in the eluate of zone 3.

Ferulic acid completely free of all impurities was not obtained. The presence of ferulic acid in the eluates of zone 4 from smoke and extract, however, was proved by comparison studies using two-dimensional chromatography in the BEB–2% HOAc and the BzAW–2 % HOAc solvent system combination. Its R_F values and the color reactions produced by the two diazotized reagents agreed with those of authentic reference ferulic acid on the chromatograms prepared under the same conditions. Further identification studies on syringic, sinapic, and melilotic acids are in progress.

Ultraviolet absorption spectra of the phenolic acids

The absorption spectrum of each pure phenolic acid isolated from smoke or extract was compared with the spectrum of the respective, authentic reference acid, $5 \cdot 10^{-5} M$ solution, in the pH 3.5 phosphate buffer[12] over the range of 210–360 mμ on a Beckman

DK-1 recording spectrophotometer, using 1 cm silica cells. The concentration of the isolated acid in the same phosphate buffer solution was adjusted to that of the reference acid solution at the maximum wavelength of the primary band. For every one of the compounds thus studied (Table III), the spectra of the reference and isolated compounds were the same.

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SUMMARY

The following free phenolic acids have been identified in the smoke of regular market cigarettes: *m*-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, *p*-hydroxyphenyl-acetic, *m*-hydroxyphenyl-acetic, *o*-hydroxyphenyl-acetic, *p*-coumaric, ferulic, *m*-hydroxyphenyl-propionic, and *p*-hydroxyphenyl-propionic acids. These acids have also been identified in the smoke from cigarettes prepared without the usual flavorings or other additives, and in the ether and ethyl acetate extracts of the tobacco from both the regular and unflavored cigarettes. Paper chromatography separation and identification procedures have been described.

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