by exposure to sunlight were observed, but judging from the areas of peaks A and B, beer exposing to sunlight was much more markable than the control.

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Phenolic Components in Cigarette Smoke*

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The isolation and identification of phenolic components contained in cigarette smoke, produced in constant-volume continuous smoking by means of an artificial smoking device, have been studied. Phenol, *m*-cresol and guaiacol were isolated as 3,5-dinitrobenzoates and identified by elementary analysis, mixed examination and infrared spectroscopy. Salicylaldehyde, 2,5-dimethylphenol, *m*-ethylphenol and *p*-cresol were identified by paper chromatography of the phenylazobenzenesulfonic acid-dye derivatives. From the crude phenolic fraction, lauric acid was paper-chromatographically identified and a paraffin (m.p. 64–66°C) was isolated.

In previous papers^{1,2,3)}, it has been reported that the chemical components of cigarette smoke, produced in constant-volume continuous smoking (smoking-volume, 20 ml/sec./1 cigt.; length of butts, 23 mm) of regular-size cigarettes (weight of filled cut-leaves, $1.078 \pm 5\%$; moisture content, 10.0%) by an artificial smoking device, were systematically fractionated and the isolation and identification of low-boiling nitrogenous compounds, neutral compounds, volatile acids and esters were carried out. The present communication deals with the isolation and identification of individual phenolic components contained in the phenolic fraction.

By application of a liquid chromatographic procedure with alumina columns, the purified phenolic fraction was separated into five parts by successive development and elution with petroleum ether, ether, ethyl acetate, ethyl alcohol and sodium hydroxide aqueous solution. After 3,5-dinitrobenzoates of phenolic compounds were prepared from each eluate, the derivatives were chromatographed on silicic acid-celite columns with *n*-hexane containing 1% ether. The isolated individual derivatives were subjected to elementary analysis, mixed examination and infrared spectroscopy, and, phenol, *m*-cresol and guaiacol were identified.

The phenolic compounds which were not isolated as 3,5-dinitrobenzoates owing to their very small quantities, were allowed to couple with *p*-diazobenzenesulfonic acid and the resulting mixture of phenylazobenzenesulfonic acid-dyes

* Part VIII. of a series of papers entitled "Studies on Tobacco Smoke".

1) M. Izawa and Y. Kobashi, This Bulletin, **21**, 357 (1957).

2) M. Izawa, Y. Kobashi and S. Sakaguchi, *ibid.*, **21**, 364 (1957).

3) M. Izawa and Y. Kobashi, *ibid.*, **22**, 47 (1958).

was subjected to paper chromatography. Then, salicylaldehyde, 2,5-dimethylphenol, *m*-ethylphenol, and *p*-cresol were qualitatively identified by their R_F -values and spot colors.

From admixed components in the crude phenolic fraction, lauric acid was paper-chromatographically identified and a paraffin (m.p. 64–66°C) was isolated.

EXPERIMENTAL

I. Purification of Crude Phenolic Fraction

The crude phenolic fraction (88.21 g), separated from smoke-components of 22,000 cigarettes described in a previous paper¹⁾, was divided into halves. One half (42.0 g) was dissolved in ether, treated with 10% sodium hydrogen sulfite aq. to remove admixed carbonyl compounds, and then extracted with 5% sodium hydrogen carbonate aq. to remove included fatty acids. The residual ethereal solution was extracted with 2N sodium hydroxide aq.. The sodium hydroxide extract was then acidified with 30% sulfuric acid aq., saturated with sodium chloride, and the solution was extracted with ether. A purified phenolic fraction (25.5 g) was obtained after evaporation of the solvent.

II. Isolation and Identification of Phenolic Compounds

Liquid-Chromatographic Separation of Phenolic Compounds: Phenylbenzenesulfonic acid-dye derivatives were prepared from the purified phenolic fraction, and paper-chromatographed according to a method essentially identical with that described by Wen-Hua Chang et al.⁴⁾ (Details of this procedure will be mentioned in a following paragraph). Eight spots, of which four spots were estimated as guaiacol, phenol, *m*- or *o*-cresol, and *p*-cresol, were detected on the paper chromatogram. Then, the isolation of individual phenolic compounds was conducted by liquid-chromatography according to the procedure described by T. Yokotsuka⁵⁾. After 10 ml of petroleum ether (b.p., 40–70°C) was added to 500 mg of the sample in 20 ml of ether, the sample solution was placed on the top of an alumina column (4ϕ × 20 cm, Merck's alumina), successively developed and eluted with 300 ml petroleum ether (b.p. 40–70°C), 400 ml ether, 600 ml ethyl acetate, 600 ml ethyl alcohol, and 500 ml 0.5% sodium hydroxide aq. under a nitrogen gas pressure of 50–350 mm.Hg. After two grams of the sample was developed by means

of four trials with 500 mg, individual eluates obtained from four different organic solvents were combined respectively, and the solvents were distilled off in vacuo under passage of nitrogen gas. The ethyl acetate-eluate gave ca. 400 mg of residual liquor, and the ethyl alcohol-eluate ca. 500 mg. However, nothing was obtained from the petroleum ether- and ether-eluates. The sodium hydroxide-eluate was acidified with 10% sulfuric acid aq., extracted with ether, and dried with anhydrous sodium sulfate. After distilling-off the ether in vacuo under the passage of nitrogen gas, it yielded ca. 500 mg of residual liquor.

Diazo-, ferric chloride-, and fuchsin sulfite-reactions were positive in the ethyl acetate-, ethyl alcohol-, and sodium hydroxide-eluates. On the paper chromatograms of phenylazobenzenesulfonic acid-dye derivatives of each eluate, seven spot (R_F : 0.25, 0.38, 0.41, 0.55, 0.64, 0.70 and 0.80) were detected in the ethyl acetate-eluate, three spots (R_F : 0.12, 0.28 and 0.41) in the ethyl alcohol-eluate, and one spot (R_F : 0.28) in the sodium hydroxide one.

Isolation and Identification of *m*-Cresol and Phenol: The concentrated ethyl acetate-eluate (400 mg) was dissolved in 10 ml of anhydrous pyridine, added with 1.2 g of 3,5-dinitrobenzoyl chloride, and heated at 100°C on a water bath for 1.5 hrs. After cooling, the resulting mixture was poured into 200 ml of chilled 5% sulfuric acid aq., and the deposited crystallines were filtered, washed with water, shaken with 100 ml of 2% sodium hydroxide aq., filtered and washed with water. These treatments yielded 400 mg of a pale yellow massy crystal, which was divided into halves. Each half (200 mg) in a small quantity of ether was kneaded with 3 g of a mixture of silicic acid and celite (by wt., 1:1). After distilling-off the ether, the slurry was placed on the top of a column (2ϕ × 20 cm) packed with a mixture of silicic acid and celite (by wt., 1:1) containing a very small quantity of rhodamine 6G, and developed with *n*-hexane containing 1% ether under a nitrogen gas pressure of 250 mm.Hg. The first and latter half of a broad dark brown band, visible on a yellow fluorescent background under ultra violet irradiation, were eluted separately. The chromatography of 400 mg of the massy crystal gave 160 mg of a pale yellow needle crystal from the eluate of the first-half of the band and 145 mg of the crystal was obtained from the latter one.

From the first crystal, 40 mg of a pale yellow needle crystal (m.p. 159–162°C) was obtained after successive recrystallizations with 99.5% ethyl alcohol. The values

4) Wen-Hua Chang, R. L. Hossfeld and Wm. M. Sandstrom, *J. Am. Chem. Soc.*, **74**, 5766 (1952).

5) T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **27**, 276 (1953).

of elementary analyses of this compound were identical with those of the 3,5-dinitrobenzoate of an authentic *m*-cresol. Found: C, 55.23; H, 3.07; N, 9.40. Calcd. for $C_{14}H_{10}N_2O_6$: C, 55.63; H, 3.34; N, 9.27.

Mixed examination with the 3,5-dinitrobenzoates of an authentic *o*-, *m*-, and *p*-cresol (m.p.'s; 138°C, 165°C, and 189°C, respectively) showed that this derivative was that of *m*-resol. Its infrared spectrum was identical with that of an authentic *m*-cresol. The paper chromatography of phenylazobenzenesulfonic acid-dye derivatives, prepared after the esterification of this derivative, gave only one spot (R_F : 0.41) equivalent to *m*-cresol. The presence of *m*-cresol was confirmed by the above-mentioned facts.

On the other hand, recrystallizations of the second crystal gave 40 mg of a white needle crystal (m.p. 143–145°C), of which values of elementary analyses were identical with those of 3,5-dinitrobenzoate of an authentic phenol. Found: C, 54.26; H, 2.85; N, 9.31. Calcd. for $C_{13}H_8N_2O_6$: C, 54.17; H, 2.80; N, 9.72.

The m.p. of this crystal was not depressed upon admixture with an authentic specimen and the two crystals had the same infrared spectrum. Consequently, phenol was identified.

Isolation and Identification of Guaiacol: Pale yellow massy crystals of 3,5-dinitrobenzoate (150 mg) were prepared from the concentrated ethyl alcohol-eluante (300 mg). The derivatives were separated into three bands by the same liquid-chromatography as

described above. These bands were eluted and distilled off the solvent. A yellow viscous substance obtained from the first band, was repeatedly recrystallized from 99.5% ethyl alcohol, and identified as the 3,5-dinitrobenzoate of *m*-cresol by infrared spectroscopy.

From the second band, a white needle crystal (35 mg) of m.p. 143–145°C was obtained. The m.p. of this crystal was not depressed upon admixture with the 3,5-dinitrobenzoate of an authentic phenol (m.p. 145°C), and infrared spectra of the two specimens were found to be identical. Therefore, this compound was proved as phenol.

The eluate from the third band gave a pale yellow crystal (40 mg), which was recrystallized from 99.5% ethyl alcohol until 10 mg of a pale yellow needle crystal (m.p. 140–141°C) was obtained. The values of elementary analyses of this crystal were identical with those of the 3,5-dinitrobenzoate of an authentic guaiacol (m.p. 141°C), and the m.p. of the former crystal was not depressed upon admixture with the latter crystal; the infrared spectra of the two specimens were also identical. From these evidences, guaiacol was identified. Found: C, 53.10; H, 3.34; N, 8.49. Calcd. for $C_{14}H_{10}N_2O_7$: C, 52.84; H, 3.17; N, 8.80.

III. Paper-chromatographic Identification of Salicylaldehyde, 2,5-Dimethylphenol, *m*-Ethylphenol, and *p*-Cresol

As it was estimated that several phenolic compounds other than *m*-cresol, phenol and guaiacol may be present

TABLE I. PAPER CHROMATOGRAPHY OF PHENYLAZOBENZENESULFONIC ACID-DYE DERIVATIVES OF PHENOLIC COMPOUNDS IN CIGARETTE SMOKE

Compound	Authentic Specimen		Specimen from Smoke	
	R_F	Color	R_F	Color
Guaiacol*	0.12	Orange	0.12	Orange
Phenol*	0.25	Yellow	0.25	Yellow
Salicylaldehyde	0.38	Yellow	0.38	Yellow
<i>m</i> -Cresol*	0.41	Yellow	0.41	Yellow
<i>o</i> -Cresol	0.41	Yellow	—	—
2, 5-Dimethylphenol	0.55	Orange Yellow	0.55	Orange Yellow
?	—	—	0.64	Orange Yellow
<i>m</i> -Ethylphenol	0.71	Yellow	0.70	Yellow
3, 5-Dimethylphenol	0.72	Yellow	—	—
<i>o</i> -Phenylphenol	0.74	Orange Yellow	—	—
<i>p</i> -Cresol	0.81	Pink	0.80	Pink

Remarks:

Paper...Whatman No. 1 paper impregnated with 4% $NaCO_3$ aq.

Solvent...*sec*-butanol: 2% $NaCO_3$ aq. (by vol. 1:1)

Development...Descending technique, room temp., 15 hrs.

*...Identified as 3,5-dinitrobenzoates.

in very small amounts, paper-chromatographic identification according to a procedure similar to that of Wen-Hua Chang et al.⁴⁾ was carried out employing their phenylazobenzenesulfonic acid-dye derivatives.

The purified phenolic fraction (ca. 50 mg) as described in the first paragraph, was dissolved in 2N-sodium hydroxide aq. (0.5 ml), added *p*-diazobenzenesulfonic acid (ca. 10 mg), acidified with 10% hydrochloric acid aq., and diluted with ethyl alcohol. A very small quantity of the thus prepared solution was descendingly developed with a solvent of *sec*-butanol: 2% sodium carbonate aq. (by vol. 1:1) on a Whatman No. 1 paper at room temperature for about 15 hrs. Before use, the paper was air-dried after spraying with 4% sodium carbonate aq..

The results are shown in Table I. Salicylaldehyde, 2,5-dimethylphenol, *m*-ethylphenol, and *p*-cresol were paper-chromatographically identified by the judgment of R_F -values and color of spots. However, a compound, showing an orange yellow color and R_F 0.64 on the paper chromatogram, was not identified.

IV. Paper-Chromatographic Identification of Lauric Acid

In the former paragraph it has already been described that admixed fatty acids in the crude phenolic fraction were extracted with 5% sodium hydrogen carbonate aq.. This extract was acidified with 10% sulfonic acid aq. and extracted with ether. The concentrated ether extract was paper-chromatographed by a reverse phase-technique for higher fatty acids according to the manner of Y. Inoue et al.⁶⁾

A Whatman No. 1 paper (2 × 30 cm) was impregnated with petroleum hydrocarbon (b.p. 140–165°C) and the excess solvent was removed by slight pressing with two sheets of filter paper. A minute portion of petroleum hydrocarbon solution of the sample was quickly spotted on the paper, and ascendingly developed with a solvent of methanol: petroleum hydrocarbon (by vol. 4:1) at 30°C in a closed chamber. After complete development, only one spot (R_F , 0.74) equivalent to that of lauric acid, was detected on the paper sprayed with an alkaline alcoholic solution of 0.2% bromocresol green. From this experiment it was confirmed that lauric acid is present as a higher fatty acid admixed in the crude phenolic fraction.

V. Isolation of Paraffin

The ether-layer, remaining after the extraction of the crude phenolic fraction with 2N-sodium hydroxide aq.

(already described in the paragraph "Purification of Crude Phenolic Fraction"), gave an orange yellow viscous substance after solvent-evaporation. This substance was repeatedly treated with 99.5% ethyl alcohol, and a paraffin (m.p. 64–66°C), insoluble in 99.5% ethyl alcohol, was obtained. This paraffin was estimated to be identical with the paraffin reported in a previous paper²⁾.

DISCUSSION

Recent papers concerning phenolic components in cigarette smoke are presented by C. H. Rayburn et al.⁷⁾ and B. T. Commins et al.^{8,9)}

C. H. Rayburn et al. determined the quantities of phenol, guaiacol, *m*-, and *o*-cresol in the smoke of cigarettes prepared from each of the four types of tobacco leaves. The smoking conditions were two-sec. puffing twice per minute, 35 ml/two sec. volume, and 23-mm butt. In their experiments, coupled derivatives between phenols and diazotized *p*-nitroaniline were paper-chromatographed and each derivative was eluted on a separated strip of paper and spectrophotometrically determined.

On the other hand, in studies on the smoke of Virginia cigarettes, B. T. Commins et al. spectrophotometrically determined phenol, *o*-, *m*-, *p*-cresol, α -naphthol, β -naphthol, catechol, resorcinol, and hydroquinone by converting them into methylesters with dimethyl sulfate.

In the present work, the isolation and identification of phenolic compounds as crystalline derivatives were attempted and the presence of phenol, *m*-cresol and guaiacol was confirmed in a crystalline state of their 3,5-dinitrobenzoates. Phenols, not isolated as crystalline derivatives owing to their very small quantity, were studied by paper chromatography of their phenylazobenzenesulfonic acid-dye derivatives; and, salicylaldehyde, 2,5-dimethylphenol, *m*-ethylphenol, and *p*-cresol were qualitatively identified. Although the presence of another unidentified substance was observed, spots equivalent to α -

7) C. H. Rayburn, W. R. Harlan and H. R. Hamner, *Anal. Chem.*, **25**, 1419 (1953).

8) B. T. Commins and A. J. Lindsey, *Analytica Chimica Acta*, **15**, 557 (1956).

9) B. T. Commins and A. J. Lindsey, *Brit. J. Cancer*, **10**, 504 (1956).

6) Y. Inoue and M. Noda, *J. Agr. Chem. Soc. Japan*, **26**, 634 (1952).

naphthol, β -naphthol, catechol, resorcinol, and hydroquinone, were not detected in the authors' sample. The presence of *o*-cresol, already reported by other authors, was not confirmed because the amount obtained was very small and the R_F -value was same as that of *m*-cresol.

The phenols, whose presence could not be confirmed in the authors' sample, will be investigated in further studies concerning the

smoke of Japanese cigarettes.

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Free Amino Acids in Cigarette Smoke (I)*

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Free amino acids in the cigarette smoke, produced in constant-volume continuous smoking by the use of an artificial smoking-device, have been paper-chromatographically studied, and twelve amino acids, i.e., α -alanine, β -alanine, glycine, glutamic acid, glutamine, serine, γ -amino butyric acid, valine, leucine, aspartic acid, proline, and ornithine(?) were qualitatively identified. Besides these amino acids, the presence of the other two unidentified ninhydrin-positive substances was observed. The presence of ten amino acids, other than glutamic acid and glutamine, has not yet been reported in the literature concerning tobacco smoke.

Up to the present, a number of papers dealing with amino acids in tobacco leaves have been reported by many authors^{1,2,3,4)}. Concerning tobacco smoke, however, only one paper is available. This is the paper recently published by D. A. Buyske et al.⁵⁾, who paper-chromato-

graphically identified glutamic acid and glutamine, together with the other four unidentified ninhydrin-positive substances, in cigarette smoke.

The present paper deals with the qualitative identification of 12 separate amino acids contained in the smoke of Japanese blended cigarettes by means of paper chromatography.

Regular-size cigarettes were continuously smoked under a condition of constant-volume smoking by an artificial smoking device. The smoke was collected in a train of absorbing bottles and the resulted smoke solution was purified by shaking with ether and by ion-exchange column chromatography. The purified

* Part IX. of a series of papers entitled "Studies on Tobacco Smoke"

1) H. Michl und H. Kuhn, *Fachl. Mitteil. Österr. Tabakregie*, **Jg. 1954**, **Hft. 1**, 10 (1954).

2) F. Habeler, H. Kuhn und H. Michl, *ibid.*, **Jg. 1955**, **Hft. 1**, 12 (1955).

3) S. Ranjan and M. M. Laloraya, *Nature*, **177**, 235 (1956).

4) M. Nagasawa and K. Yamamoto, *Sci. Papers Centr. Res. Inst. Japan Monop. Corp.*, **No. 96**, 50 (1956).

5) D. A. Buyske, J. E. Flowers, Jr., P. Wilder, Jr. and M. E. Hobbs, *Science*, **124**, 1080 (1956).