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# Large-Scale Fractionation of Cigarette Smoke Condensate for Chemical and Biologic Investigations[1]

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## SUMMARY

Cigarette smoke condensate was separated into acidic, basic, and neutral fractions which were further divided into 12 subfractions by procedures designed to yield adequate amounts for bioassay of all fractions for tumorigenicity. Repetitive runs with 1000 gm of smoke condensate each were made over a period of about 12 months. From each 1000 gm a portion (28%) was removed for control bioassay, and the remainder (720 gm) was fractionated. In addition, a reconstituted sample for control bioassay was prepared by pooling $\frac{1}{3}$ portions of each fraction. An overall average recovery of 90.8% was obtained in nine successive runs for the sum of the 12 subfractions including the reconstituted sample. A study of the variation in yields of all fractions from the successive runs is included.

## INTRODUCTION

At present, the tumorigenic activity of cigarette smoke condensate in animals cannot be explained on the basis of known chemical composition. Current knowledge of the chemical composition of tobacco smoke and the relationships between composition and tumorigenic activity have been reviewed recently (13, 18). In general, the polynuclear aromatic hydrocarbons appear to be the major tumor-initiating compounds in smoke condensate, although other components which have been studied incompletely may play some role in this effect, e.g., alkylating agents (including nitrosamines) (15, 18), free radicals (18), certain radionuclides (18), and enzyme inhibitors (13). The simple phenols appear to be major tumor-promoting agents, but paraffinic hydrocarbons, terpenes, higher fatty acids, and higher alcohols may also contribute to the activity through effects on absorption of tumorigenic agents or by cocarcinogenicity. In addition, other unidentified components in smoke may play a significant role in tumor promotion, and known and unidentified components may act antagonistically and/or synergistically to give tumor rates which are quantitatively unexpected, e.g., PAH[2] (7).

Most of the tumorigenicity of CSC for animals can be concentrated in the neutral and acidic fractions. In an early study (19), roughly quantitative results showed that, although the major tumor-initiating activity was concentrated in the carbon tetrachloride eluate from silicic acid chromatography of the neutrals, some activity was found in the hexane eluate from this column, and additional activity occurred in the acidic, basic, and dichloromethane-insoluble fractions. Most of the activity was lost when the carbon tetrachloride eluate was removed and all the other fractions were recombined. In another publication, the major tumor-initiating activity was found exclusively in hexane eluates from silicic acid columns (17). In further work (16) it was shown that the processes of fractionating and recombining did not result in a loss of activity, since a reconstituted condensate prepared by recombining the separated acidic, basic, and neutral fractions was as active as the original condensate. However, subsequent findings indicated that these results could not be confirmed, and some loss on recombination was evident (17). Recently, a more precise determination showed that a neutral fraction (prepared separately from fresh CSC) had about 80% as much tumorigenicity for mouse skin as thoroughly dried and stored condensate (3). The relative contribution of the traces of carcinogenic PAH presumably present in the neutral fraction was not determined, nor was there any attempt at a direct comparison with a neutral fraction actually isolated from the stored condensate. On the other hand, in the same study a fresh whole CSC which was not dried was twice as active as the same neutral fraction. The substances responsible for the additional tumorigenicity for mouse skin of the fresh *versus* stored condensate were not identified.

All of the studies published to date suffer from several disadvantages. In all cases, fractionation has been relatively superficial. With one possible exception in which unpublished findings were summarized (17), the recently isolated brown pigments of CSC (4–6, 14) have not been separated and tested biologically. In no case have the precise details of fractionation been described. Also, in no instance has the variability of yields obtained in the fractionation been revealed.

The present study is the first stage of a large-scale joint effort to overcome these disadvantages. The present report describes details of the fractionation of CSC, including the variability of yields over a period of 12 months. During this time, a concurrent chemical study of the composition of selected fractions has been underway, and a number of previ-

[1]Part 31 of the series, Composition Studies on Tobacco.

[2]The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; CSC, cigarette smoke condensate.

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ously unidentified constituents has been found and reported, including benzyl esters (12), myristicin (12), several aromatic amines (11), a substituted 1,4-naphthoquinone (2), and a series of high molecular weight pigments of complex structure (4–6, 14). A concurrent report details the results of biologic testing of the fractions obtained in the present study (1).

## MATERIALS AND METHODS

**Cigarette Smoke Condensate.** This material was prepared in 1000-gm batches at the Roswell Park Memorial Institute. For each kilogram lot about 50,000 cigarettes were smoked. They were purchased on the open market, were domestic, blended, 85-mm nonfilter cigarettes, and were smoked on an automatic machine using a 2-sec puff of 35-ml volume once a minute to a butt length of not less than 20 mm. The smoke was condensed in traps cooled in a Dry Ice-acetone mixture, and the condensate was removed from the traps with acetone. The solvent was removed *in vacuo* at a low temperature and shipped under nitrogen at –20°C or lower to our laboratories. On receipt, the condensate was stored in a deep-freeze box until the fractionation was begun, usually within two days after receipt.

**Solvents.** Ether was analytical reagent grade, redistilled from glass, and stored in 5-pound metal cans; tests showed that this effectively prevented the formation of peroxides. Ordinary distilled water was redistilled from glass before use in extractions or for making up dilute solutions of other reagents. Analytical reagent grade sodium hydroxide was dissolved in redistilled water at a concentration sufficient to precipitate any carbonate before dilution to other concentrations. Hydrochloric acid, reagent grade, was redistilled from glass before use. Cyclohexane was spectrophotometric grade, specified to contain no more than 0.0003% residue upon evaporation. Nitromethane was also of spectrophotometric quality. All other solvents and reagents were of analytical quality and were used without further purification.

In all solvent partitioning steps, the solvent pairs were shaken together before use to saturate each solvent with the other. Except for ethanolic solutions, all fractions containing organic solvents were dried over anhydrous sodium sulfate and were evaporated *in vacuo* to residues at 30–35°C.

**Fractionation of CSC.** The general plan followed in repetitive fractionations is indicated by the flow diagram in Chart 1. The kilogram of CSC was brought to room temperature before removal of 280 gm for use as a control sample. This fraction was immediately bottled under nitrogen gas and returned to cold storage until the fractionation was completed.

**Separation of Acids from Bases Plus Neutrals.** The condensate remaining after removal of the control sample (720 gm) was transferred quantitatively to a 6.0-liter separatory funnel with the aid of several small portions of ether and 1.0 N sodium hydroxide until transference was completed. Subsequently, 250 ml of ether and 250 ml of aqueous sodium hydroxide were added to the funnel. After shaking, a portion of the mixture, arbitrarily set at 200 ml, was withdrawn, and the processes of adding ether and sodium hydroxide (250 ml each) and withdrawing (200 ml) were repeated until a line of demarcation between layers became visible. To see the boundary layer a bright light momentarily placed behind the funnel stem is helpful. This procedure is necessary in the initial stages because of the extremely dark, opaque color of both layers. Use of larger volumes of solvents initially would not reduce the opacity appreciably but would lead to unmanageable quantities of both layers for subsequent steps. When the line of demarcation was plainly visible in the original funnel, the bottom layer was completely removed, and the ether and aqueous extracts were each cross-washed with the opposite solvent until the washes were transparent and had a light yellow or amber color. All ether layers and all aqueous layers were combined, giving a total of 3200–3400 ml for each layer.

**Separation of Bases from Neutrals.** The above ether solution was transferred to a 4000-ml beaker, and 100 ml of 2 N hydrochloric acid were added while the contents were stirred magnetically, resulting in the appearance of a viscous, black precipitate. This precipitate and the lower layer were separated individually. The addition of HCl to the ether layer and removal of the formed precipitate was repeated twice more with stirring. Then the ether solution was transferred to a 6000-ml separatory funnel and extracted repeatedly with 200-ml portions of HCl; the combined HCl layers were cross-washed with repeated additions of ether. Additional small amounts of the precipitate continued to form and were collected. The combined precipitate was triturated with small quantities of ether and 2 N HCl which finally converted it to a more or less

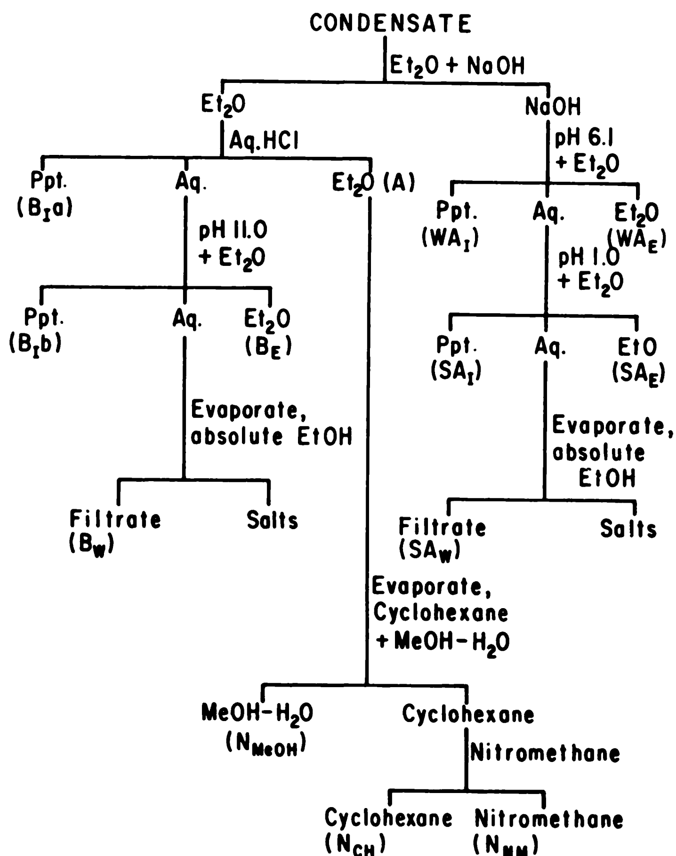


Chart 1. Fractionation of cigarette smoke condensate. For details see text (Materials and Methods).



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granular form. The material was filtered, washed with water to remove excess HCl, and air dried ("Bases, insoluble, a") ($B_I$a, Chart 1). The ether and aqueous HCl washes used in triturating the precipitate were separated in a funnel and pooled with the respective layers from the main bulk of the fractionation. The ether and aqueous layers were each cross-washed until the last wash was light yellow or amber in color. All washes were combined with the main ether and HCl layers, and final solutions were filtered. The filtered ether solution was washed with several small portions (50 ml) of water until no more free HCl was being removed. Its volume was about 5500 ml and it contained the ether-soluble neutral compounds (A, Chart 1) to be further fractionated by procedures described below. The volume of aqueous HCl solution was generally 2800 ml (range 2400–3000 ml) and contained the bases soluble in 2 N HCl under the conditions used.

**Further Fractionation of Bases.** The aqueous HCl solution was treated with 12 N sodium hydroxide solution in a beaker with magnetic stirring. As the neutral point was approached, a viscous black precipitate began to appear which agglomerated into sticky, viscous masses clinging tenaciously to all surfaces. The addition and stirring was continued until the pH reached 11.0. The insoluble material was separated, triturated in ether, washed with water, filtered, and air dried ("Bases, insoluble, b") ($B_I$b, Chart 1). The ether and water washes were added to the main extraction funnel. The alkalinized HCl solution was separated from the ether layer and was extracted repeatedly with ether. The combined ether layers were cross-washed with dilute sodium hydroxide (1 N) and then washed with several portions of water. These washes were added to the aqueous solution. The final ether solution (volume about 1600 ml) contained "Bases, ether-soluble" ($B_E$, Chart 1). The alkalinized HCl solution was adjusted to pH 7.0 with strong HCl solution and then readjusted to approximately pH 9 by the addition of concentrated ammonium hydroxide (final volume, 5500 ml). The water and ammonia were completely removed *in vacuo*, and the resulting residue was treated with sufficient absolute ethanol to dissolve the organic matter. The salts were filtered off and washed thoroughly by resuspending in fresh absolute alcohol. The final filtrate volume of about 1500 ml contained "Bases, water-soluble," ($B_W$, Chart 1).

**Separation of Phenols and Other Weak Acids from Strong Acids.** Hydrochloric acid (6 N) was added to the alkaline solution resulting from the partitioning of the crude condensate in an amount sufficient to bring the pH almost to neutral. A black tarry precipitate began to form and increased in amount with continued stirring. The pH was adjusted to 6.1 using 2 N HCl, and the liquid was decanted into a 6.0-liter separatory funnel. The precipitate was washed and triturated using ether and water in portions. The air-dried precipitate was designated "Weak acids, insoluble" ($WA_1$, Chart 1). The washes were added to the funnel, and the combined aqueous layers were extracted repeatedly with ether as in the other separations already described. The combined ether layers and the aqueous layer were cross-washed with the opposite solvent until the last washes removed only insignificant amounts of color. Final volumes of the aqueous and ether layers were about 4500 ml and 1800 ml respectively. The ether solution contained "Weak acid, ether-soluble" ($WA_E$, Chart 1).

**Fractionation of Strong Acids.** The above aqueous layer was treated with HCl sufficient to bring the pH to 1.0. A black, tarry precipitate formed which was handled in the usual way. The air-dried precipitate is designated "Strong acids, insoluble" ($SA_I$, Chart 1). The aqueous layer and washes were extracted repeatedly with ether in a funnel. The combined ether extracts and the aqueous layer were cross-washed with 2 N HCl and ether respectively until the last washes removed only insignificant amounts of color. The aqueous layer was evaporated *in vacuo*, and the organic material in the residue was separated from salts by the use of absolute ethanol as discussed above. The alcohol-soluble substances are designated "Strong Acids, Water-soluble," ($SA_W$, Chart 1). The combined ether layers contained "Strong acids, ether-soluble" ($SA_E$, Chart 1).

**Further Fractionation of Neutral Compounds.** The washed and dried ether solution (A, Chart 1) containing the ether-soluble neutral compounds was evaporated *in vacuo* until no more ether was being removed as judged by insignificant weight losses upon further treatment. The residue was then fractionated by a procedure designed to concentrate benzo[a]-pyrene and other PAH neutral compounds (8, 9). The residue was dissolved in cyclohexane (200 ml) and transferred to a funnel using 3 additional portions (100 ml each) of cyclohexane to rinse. This solution was shaken with 200 ml of methanol:water (80:20 v/v). After a suitable settling interval, a volume of 190 ml was withdrawn and another 200-ml portion of methanol:water was added. This procedure was continued until a dividing line between layers was visible. Then the bottom layer was withdrawn completely, and the combined bottom layers were extracted once with 200 ml of cyclohexane in another funnel, adding the top layer to the first funnel. Extraction of the combined cyclohexane solution with 100-ml portions of methanol:water was continued until successive extracts did not differ appreciably in color. Then the combined methanol:water layers (volume about 2400 ml) were extracted with 100-ml portions of cyclohexane, and these extracts were added to the cyclohexane solution in the first funnel. Since 12 to 15 cross-washes were required, the final volume of the cyclohexane solution was 2000 to 2400 ml. The combined cyclohexane solution was next extracted with nitromethane in 100-ml portions, until the last extract was light yellow in color. The combined nitromethane solutions (volume about 1200 ml) were cross-extracted with cyclohexane (5 portions of 100 ml each) and the extracts were returned to the major cyclohexane extract. These three fractions are designated "Neutrals soluble in methanol-water," ($N_{MeOH}$, Chart 1), "Neutrals, cyclohexane-soluble" ($N_{CH}$, Chart 1), and "Neutrals, nitromethane-soluble" ($N_{NM}$, Chart 1).

**Reconstituted CSC.** As each of the above fractions were obtained, $\frac{1}{3}$ was weighed or measured for preparation of the reconstituted sample, and the remaining $\frac{2}{3}$ of each fraction was evaporated as described below. The 12 aliquots for the reconstituted sample were mixed in a 6.0-liter Erlenmeyer flask. Layers were not seen in the opaque mixture, but the insoluble fractions and precipitates did not entirely redissolve in the mixed solvents. The insoluble substances were suspended in freshly mixed solvents, in which dissolution occurred slowly, and the solution was simply rinsed into the evaporation flask

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near the end of the solvent removal procedure. As the final portions of solvent evaporated, the insoluble materials dispersed evenly in the tarry residue.

**Weights of Fractions.** The four insoluble fractions were sucked dry on a Buchner funnel and then spread out to dry further in air before weighing.

All solutions were evaporated *in vacuo* in tared flasks in rotating evaporators at a bath temperature of 30°C or less until loss in weight upon further exposure to the evaporation conditions became insignificant. Since all solutions were dried over sodium sulfate before evaporation, variability due to retained water was much less than in the case of the air-dried solids. More drastic drying was avoided because of the possibility of altering constituents.

## RESULTS AND DISCUSSION

Although an effort was made to reproduce the fractionation exactly from run to run, the data presented in Table 1 show that significant differences in yields were obtained. In general, the greatest variability occurred with those fractions obtained in smaller amounts (less than 40 gm), as shown in Table 2, which lists the yields in order of decreasing coefficients of variability. Perhaps the greatest source of variability was in the removal and recovery of the four insoluble fractions, all of which tenaciously absorbed the solutions from which they precipitated. An additional source of variability was undoubtedly introduced in the drying of these precipitates. Weights reported for the insoluble fractions may include highly variable amounts of water, although all were air dried before being bottled. The drying procedure for the insoluble fractions was adopted since more drastic drying conditions might have altered constituents and less drastic conditions required too much time under the imperatively tight bioassay schedule. To a lesser extent, variability in drying contributed to variation in yields of other fractions. This variability was partially compensated for in the bioassay procedure, since for this purpose all fractions were dissolved or suspended and diluted to a standard volume for the particular assay level, i.e., the volume used for the original crude CSC at that level. This was done so that each fraction would be administered in the same concentration at which it existed in the CSC before fractionation. At the same time, this procedure automatically eliminated all differences due solely to variability in drying technic, leaving only those components of variability arising from other factors in the separation procedure and from variation in the original CSC from run to run. Unfortunately, the relative contributions of these several factors to overall variability is not known. As might be expected, the differences in yields of individual fractions tended to cancel out in the reconstituted fraction, and this is also reflected in the low coefficient of variability recorded for the sum of Fractions 2–14 (Table 2).

An overall average recovery of 90.8% was obtained for the sum of the twelve subfractions and the reconstituted condensate. Probably, the major loss occurs during solvent removal by evaporation under vacuum, since the more volatile components might distill at least partially under these conditions. Considering the chemical properties of substances in condensate which may or do contribute to the tumorigenic activity (17), it is probable that lost volatile compounds contribute little to the tumorigenicity of smoke condensate using the mouse back-painting technic. Of course, some of the variation shown in the tables may be a function of the variability in the composition of condensate itself and not a reflection of analytical error. Relatively wide variations in the levels of phenols (1.7–9.4 mg/gm of condensate) and benzo[a]pyrene (0.4–2.2 μg/gm condensate) have been reported in a series of condensates employed in short-term tests related to carcinogenicity (10).

Table 1

| Fraction | | Run no. | | | | | | | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Name | No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | ± S.E. |
| Reconstituted CSC | 2 | 219.6[a] | 208.1 | 212.0 | 184.8 | 208.1 | 227.8 | 192.2 | 199.4 | 227.9 | 208.9 ± 5.0 |
| $B_I a$ | 3 | 8.3 | 6.8 | 6.6 | 4.9 | 4.6 | 3.0 | 5.5 | 6.0 | 5.5 | 5.7 ± 0.5 |
| $B_I b$ | 4 | 4.0 | 3.2 | 1.6 | 2.1 | 3.8 | 2.4 | 6.3 | 2.7 | 3.5 | 3.3 ± 0.5 |
| $B_E$ | 5 | 28.9 | 28.2 | 23.0 | 16.8 | 43.8 | 25.3 | 33.4 | 33.9 | 31.5 | 29.4 ± 2.5 |
| $B_W$ | 6 | 15.3 | 11.9 | 8.7 | 11.8 | 15.9 | 6.9 | 8.2 | 13.0 | 13.8 | 11.7 ± 1.1 |
| $WA_I$ | 7 | 28.8 | 40.4 | 64.1 | 31.9 | 26.6 | 44.7 | 27.7 | 33.5 | 43.0 | 37.9 ± 4.0 |
| $WA_E$ | 8 | 46.6 | 52.8 | 33.4 | 45.9 | 57.6 | 48.5 | 45.4 | 57.0 | 45.5 | 48.1 ± 2.4 |
| $SA_I$ | 9 | 4.5 | 8.3 | 10.1 | 10.9 | 7.5 | 6.1 | 11.8 | 7.2 | 8.3 | 8.3 ± 0.8 |
| $SA_E$ | 10 | 8.8 | 9.3 | 11.7 | 12.9 | 17.0 | 12.8 | 13.0 | 21.1 | 17.2 | 13.8 ± 1.3 |
| $SA_W$ | 11 | 160.6 | 193.9 | 184.5 | 195.7 | 150.9 | 186.4 | 163.6 | 133.6 | 166.9 | 170.7 ± 7.0 |
| $N_{MeOH}$ | 12 | 29.7 | 26.3 | 31.3 | 26.4 | 15.8 | 21.3 | 20.5 | 22.0 | 24.8 | 24.2 ± 1.6 |
| $N_{CH}$ | 13 | 82.2 | 72.0 | 67.0 | 60.0 | 77.4 | 92.8 | 73.5 | 99.0 | 77.6 | 77.9 ± 4.1 |
| $N_{NM}$ | 14 | 14.3 | 12.6 | 14.6 | 14.2 | 12.4 | 10.5 | 9.3 | 19.0 | 18.4 | 13.9 ± 1.1 |
| Total | 2–14 | 651.6 | 673.8 | 668.6 | 618.3 | 641.4 | 688.5 | 610.4 | 647.4 | 683.9 | 653.8 ± 9.2 |

Yields of cigarette smoke condensate fractions. CSC, cigarette smoke condensate; A, acids; B, bases; N, neutrals; S, strong; W, weak. Subscripts: I, insoluble; E, ether-soluble; W, water-soluble; MeOH, methanol-soluble; CH, cyclohexane-soluble; NM, nitromethane-soluble. S.E. = standard error of the mean, $\sqrt{V/9}$; V, variance, $\Sigma(X_i-\bar{X})^2/8$. One-third of the isolated amounts of Fractions 3–14 were pooled to give Fraction 2. The values for Fractions 3–14 in the table represent the yields obtained from the remaining $\frac{2}{3}$.

[a]gm from 720 gm CSC.

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Table 2

| Fraction Name | No. | Mean (gm) | Average deviation | V | S.D. | Coefficient of variation |
|---|---|---|---|---|---|---|
| $B_Ib$ | 4 | 3.3 | 0.99 | 1.9 | 1.38 | 42.0 |
| $WA_I$ | 7 | 37.9 | 9.05 | 143.1 | 11.95 | 31.0 |
| $SA_E$ | 10 | 13.8 | 3.13 | 15.9 | 3.99 | 29.0 |
| $SA_I$ | 9 | 8.3 | 1.80 | 5.4 | 2.33 | 28.0 |
| $B_W$ | 6 | 11.7 | 2.50 | 10.1 | 3.37 | 28.0 |
| $B_E$ | 5 | 29.4 | 5.50 | 58.3 | 7.63 | 26.0 |
| $B_Ia$ | 3 | 5.7 | 1.10 | 2.3 | 1.50 | 26.0 |
| $N_{NM}$ | 14 | 13.9 | 2.40 | 10.4 | 3.22 | 23.0 |
| $N_{MeOH}$ | 12 | 24.2 | 3.90 | 23.5 | 4.85 | 20.0 |
| $N_{CH}$ | 13 | 77.9 | 8.90 | 147.4 | 12.14 | 15.0 |
| $WA_E$ | 8 | 48.1 | 5.20 | 53.6 | 7.32 | 15.0 |
| $SA_W$ | 11 | 170.7 | 17.30 | 441.9 | 21.02 | 12.0 |
| Reconstituted CSC | 2 | 208.9 | 11.50 | 224.1 | 14.97 | 7.1 |
| Total | 2–14 | 653.8 | 22.20 | 759.2 | 27.55 | 4.2 |

Variability in yields of CSC fractions. Mean, $\Sigma X_i/9$; average deviation, $\Sigma(X_i-\bar{X})/9$; $V = \Sigma(X_i-\bar{X})^2/8$; S.D., standard deviation, $\sqrt{V}$; coefficient of variation, 100 S.D./mean. See Table 1 for abbreviations.

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