

FRACTIONATION OF TOBACCO SMOKE CONDENSATE FOR CHEMICAL COMPOSITION
STUDIES

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The evolution of the scheme presently used at the Russell Research Center for the fractionation of smoke condensate will be outlined. This work was carried out for the most part at the Eastern Marketing and Nutrition Laboratory under the direction of Dr. R. L. Stedman. The fractions obtained from the separation procedures are used in both chemical and biological investigations. The results obtained in the biological studies on these fractions will be discussed in another paper by Dr. Bock. Some fractions which were found to be active will be pointed out as a justification for studying the chemistry of the particular fraction and developing further fractionation schemes. This paper is also not intended to be a complete discussion of chemical composition but rather a means of isolating various fractions for chemical studies. Compounds identified in our laboratories will be presented in order to give a general idea of the composition of various fractions.

In our studies we generally start with one kilogram of cigarette smoke condensate (CSC), which is purchased from Roswell Park Memorial Institute in Buffalo, N. Y. The smoke condensate is shipped to us packed in dry ice and is kept frozen until just prior to use. Figure 1 shows the preliminary separation of the condensate into acidic, basic, and neutral fractions. This part of the procedure has not been changed over the years. In our first large-scale fractionation procedure the bases were further separated by adjusting the pH to 11.0 with 12N NaOH and extracting with Et₂O. Components were identified in the Et₂O soluble portion by a GLC technique. These included, several collidines, picolines, and lutidines, 3-ethyl pyridine, pyridine and several alkaloids.

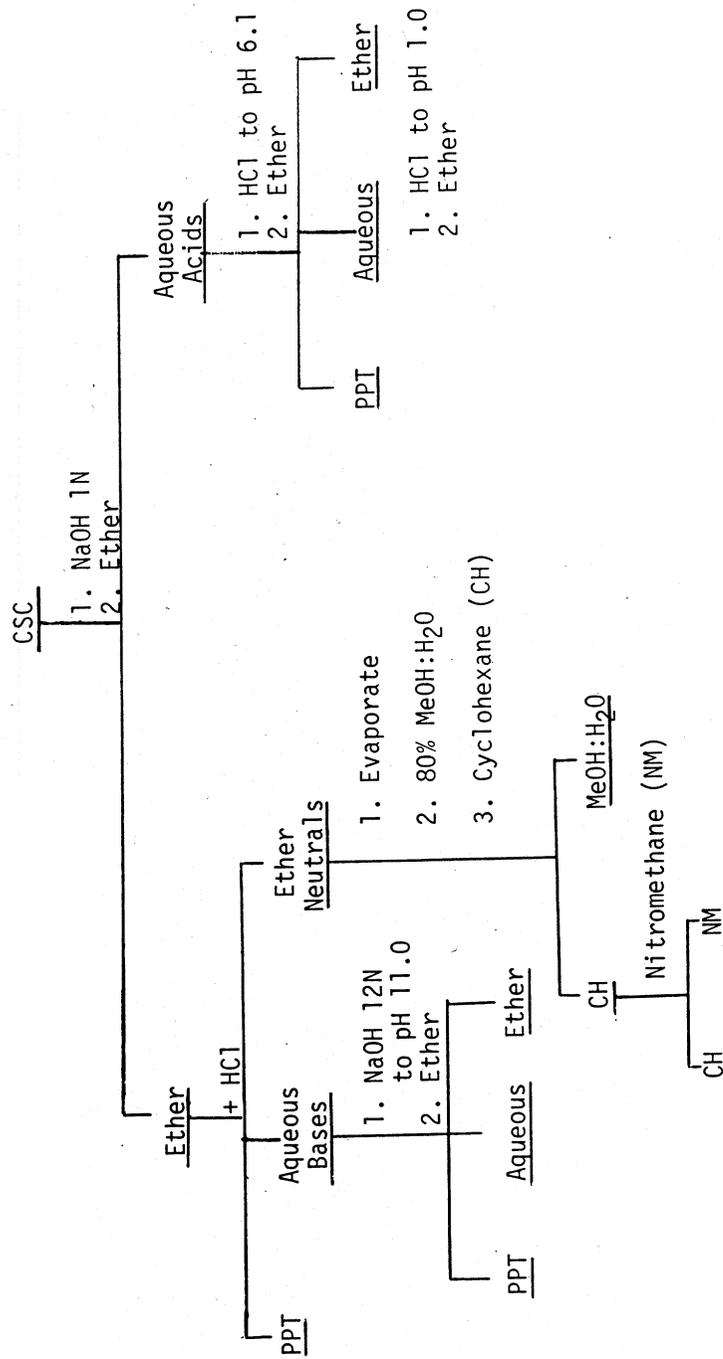


Figure 1. Scheme 1 - Fractionation of smoke condensate.

The acid fraction was also further separated as follows. The pH is adjusted to 6.1 and extracted with ether to remove the weak acids. The pH is further lowered to 1.0 and the ether-soluble strong acids are removed. This fraction will be discussed later in the paper.

The precipitates shown in Figure 1 are the pigments. There are three subfractions of these: the basic, weakly acidic, and strongly acidic pigments. These have been shown to be similar to the brown pigments of tobacco leaf. The weakly acidic pigment is the largest fraction by weight and represents between 6 and 9 per cent of the condensate weight. Most of the weakly acidic subfraction was found to be nondialyzable and to contain a component having a molecular weight $\geq 100,000$, which yielded a silicone, nicotine, and a series of bases on alkaline fusion (1).

We now come to the neutrals. These are divided into three fractions by solvent partitioning between methanol-water, cyclohexane, and nitromethane. This type of separation was first used by Drs. Wynder and Hoffmann (2) to concentrate the polynuclear hydrocarbons, particularly benzo(a)pyrene in the nitromethane fraction. A considerable amount of work has been done in our laboratory to identify some of the components in addition to the polynuclear hydrocarbons in this fraction.

The nitromethane fraction was further separated by silicic acid and alumina column chromatography. These columns are developed by gradually increasing the polarity of the eluting solvent. Some of the compounds which have been identified in the nitromethane fraction were indoles, carbazoles, benzyl benzoate, and myristicin along with polynuclear aromatic hydrocarbons (3). Also in a separate study carried out by R. L. Miller et al., several interesting aryl amines were identified in this fraction (4).

In the large-scale fractionation work done by Dr. Ansel Swain and Mr. Joseph Cooper, an overall recovery average of 90.8% was obtained following nine fractionations of cigarette smoke condensate in 1 kg quantities (5). These fractions were tested at Roswell Park by Dr. Bock and it was found that the weak acid ether-soluble and the three neutral fractions contained the major tumor promoting substances (6). This led us to the development of our second fractionation scheme. Separation of the acids, bases, and neutrals was carried out in the same manner as scheme 1. The basic fraction was not tested in this run. The acids were worked up as shown in Figure 2. In a recent study of the chemical composition of these fractions, Miss Elizabeth Strange has developed a method of separating the long chain fatty acids from the ether-soluble portion by urea occlusion and gas chromatography. By this method she has identified the fatty acids: palmitic, stearic, oleic,

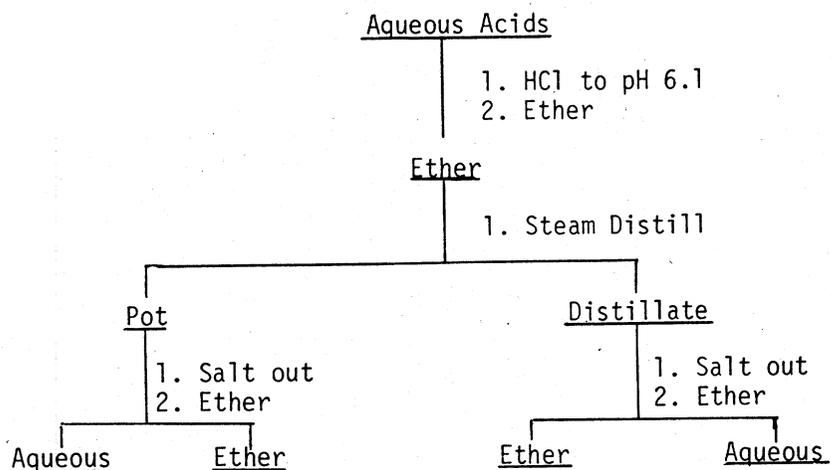


Figure 2. Scheme 2 - Fractionation of acids

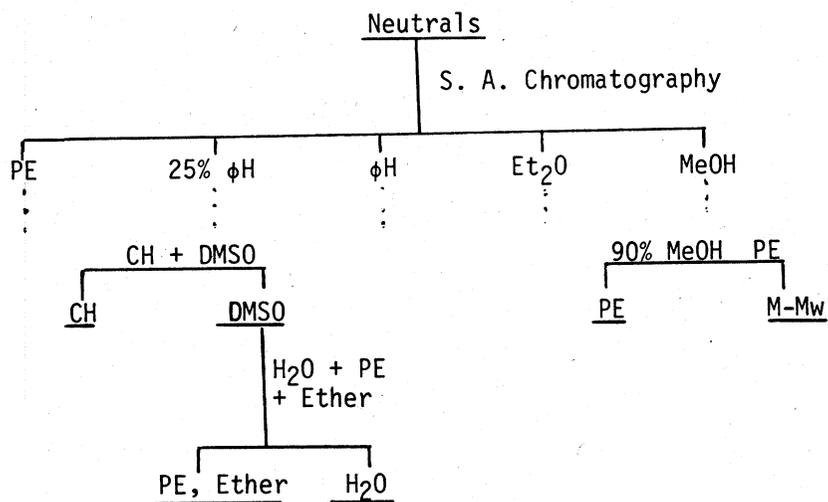


Figure 3. Scheme 2 - Fractionation of neutrals

linoleic, and linolenic acids as their methyl esters. Also in the same fraction she has identified hydroquinone and catechol by GLC.

Since activity was shown to be present in all three of the neutral fractions from scheme 1, it was decided to try silicic acid (S.A.) column chromatography in order to concentrate the major tumor promoting activity into one fraction or at least find out a little more about the type of compounds causing this activity. As can be seen in Figure 3, the first three fractions from the silicic acid column were partitioned between cyclohexane (CH) and dimethyl sulfoxide (DMSO). Water was then added to the DMSO fraction and this was extracted with successive portions of petroleum ether (PE) and ether (Et₂O) which were combined. In using this scheme it was found that more than 97% of the benzo(a)pyrene (BaP) content of smoke was found in one fraction, ØH-PE-DMSO, with small amounts occurring in each of the adjacent DMSO solutions. By this method, it is possible to obtain an enriched fraction of BaP from smoke condensate containing 105 ppm BaP rather than the 1 ppm usually encountered. In additional studies, the ØH-PE-DMSO fraction was further separated on cross-linked polystyrene by lipophilic gel filtration using acetone as developing solvent. The elution curves showed that the polynuclear hydrocarbons could be separated easily from the bulk of the sample weight. Based on the elution pattern obtained, concentrations of at least 10,000 ppm BaP can be obtained by collecting just the fraction containing BaP. This fraction was screened and found to contain principally aromatic hydrocarbons along with the pesticide degradation product TDEE [1-chloro-2,2-bis-(4'-chlorophenyl)ethylene], N-phenyl-4-isopropylamine, 9,9-dimethylacridan, and diphenylamine (8).

In the subsequent DMSO fraction, i.e., ØH-DMSO, R. L. Miller has identified 2-methyl, 2,5-dimethyl, 2,3-dimethyl, 2,6-dimethyl, and 2-, 3-, and 4-ethyl benzonitrile along with several alkylphenols (9). The occurrence of alkylphenols in the neutral fraction may seem unexpected. Closely related compounds have been reported as constituents of the weakly acidic fraction of CSC, such as 2-ethylphenol and 2,6-dimethylphenol. In phenols, the ionization of the hydroxyl group is influenced usually by inductive, resonance, and steric effects of substituents. Alkyl substitution in the 2- and 6-positions may reduce the acidity or produce other effects so that the phenol is insoluble in aqueous alkali. To determine whether the bulk of the isolated alkylphenols would be expected in the neutral or weakly acidic fractions of CSC, partition coefficients of the isolated compounds in ether-aqueous sodium hydroxide were determined. The results indicated that most of the 2-butylphenol, 2-isobutylphenol, and possibly 2-ethyl-5,6-dimethylphenyl should be in the neutral fraction but substantial amounts of the 2-ethyl-6-methylphenol might be in the weakly acidic fraction depending on the number of successive partition steps employed.

In analyzing the results of the biological testing of fractions from scheme 2 it was found that severe losses in activity were observed when all eluates were combined to obtain a reconstituted neutral fraction. In addition to other factors, it appeared that such losses may have been due at least in part to a failure to elute the columns completely. Even after elution with methanol, all columns showed a tan color indicating that some material remained thereon. To study this problem further, activated and non-activated silicic acid and florasil were employed to separate the neutrals of CSC. Biological data on the strongly adsorbed material indicated that the major loss in activity on recombining chromatographic eluates probably does not arise from this source. In order to avoid this problem it was decided to develop a scheme of separation which eliminated the use of silicic acid column chromatography. We returned to partitioning of the neutrals between 80% methanol:water and cyclohexane as in scheme 1. The cyclohexane fraction was then separated by counter current distribution using a 200 tube instrument. After two hundred partitions between cyclohexane and nitromethane, the tubes were divided into five fractions for bioassay analysis. It was found that the BaP content was in tubes 46 - 140 (this was done by the addition of carbon 14 labeled BaP) while the major portion of the weight was found at either end of the distribution.

By this time we had received the results of the fractionation in scheme 2 and had found that the M-Mw fraction as well as the ØH-PE-DMSO fraction contained a significant amount of activity. This dictated going back to silicic acid chromatography and working further on the M-Mw fraction about which very little is known. In order to do this scheme 4 was developed. The silicic acid column was eluted in the usual manner and M-Mw fraction obtained. This was subjected to lipophilic gel filtration using benzene as the eluting solvent. The gel was a polystyrene gel with a molecular exclusion limit of 2700. A series of compounds of known molecular weights were run on the column for calibration purposes. The major portion of the weight (34%) appears to lay in a molecular weight range between 800 and 450. Molecular weights of these fractions were confirmed by vapor pressure osmometry. Preliminary investigations of these fractions indicate a large number of components of complex structure differing only slightly (length of side chain) and therefore making isolation extremely difficult. The i.r. spectra of the fraction molecular weight 450 to 800 are rather nondescript indicating the presence of an OH group and possibly an ester linkage.

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