Determination of Polycyclic Aromatic Hydrocarbons in Liquid Smoke Flavors

Richard H. White,* John W. Howard, and Charles J. Barnes

Procedures have been developed for the isolation and determination of polycyclic aromatic hydrocarbons in liquid smoke flavors and resinous condensates. The hydrocarbons are isolated by liquid-liquid partition and thin-layer chromatography, and measured by ultraviolet and spectrophotofluorometric techniques. Recoveries of benzo[*a*]pyrene added to 200 g of liquid smoke flavor samples at levels of 2 ppb ranged from 70 to 89%. Trace quantities of some three- and four-ring type hydrocarbons were isolated from three of the seven flavors studied. A compound identified as 4-methylbenzo[*a*]pyrene was found at a level of 1.7 ppb in one aqueous smoke flavor. Various concentrations of benzo[*a*]pyrene ranging from 25 to 3800 ppb were found in resinous condensates which settle out of the liquid smoke flavors on storage. The identity of the compound was confirmed by fluorescence and mass spectrometry.

Various smoke flavors are in use in the United States to impart a "smoked flavor" to foodstuffs. At the present time there is little available information on the extent to which these flavors have replaced the traditional "chimney" or direct smoking methods. However, the variety of "smoked flavored" products now available to the consumer is evidence of their increased usage in recent years.

The possible advantages of the use of the aqueous flavors over conventional or direct smoking methods have been considered by Draudt (1963) and Hollenbeck (1964). According to the latter author, the major benefit to be derived is flavor reproducibility, which can be rigidly controlled. At least one of the manufacturing processes used in the preparation of the aqueous flavors has been described in detail in the literature (Hollenbeck, 1963, 1964). In brief, it consists of burning dampened hardwood sawdust or chips with a limited amount of air to form smoke. The smoke is conducted upward through a column of inert material countercurrent to a stream of water usually maintained at a temperature of 40° to 140° F. The water removes the resulting smoke extract, which is then recycled repeatedly through the column until a titratable acidity of at least 3% is obtained. The aqueous smoke extract is then filtered into a storage tank through cellulose pulp to remove tars and resins, and the solution is allowed to age, during which time more resinous materials may settle out. The chemical composition of these latter products has not been established; however, the major components are believed to be condensates of phenols and carbonyl compounds.

The demonstrated presence of the carcinogen, benzo[*a*]pyrene, and of other polycyclic hydrocarbons in smoked foods in this country has stimulated interest in the analysis of liquid smoke flavors. Lijinsky and Shubik (1965) isolated various hydrocarbons including pyrene, fluoranthene, benzo[*g,h,i*]-perylene, chrysene, benz[*a*]anthracene, and carbazole from two samples of aqueous flavoring materials. Analyses of fish (salmon and haddock) smoked in the traditional manner revealed trace quantities of benzo[*a*]pyrene, as well as many of the aforementioned hydrocarbons. On the basis of these findings, the authors suggested that the presence of the carcinogen could be avoided with the use of the liquid smoke rather than direct smoking.

Studies on water-soluble flavors were initiated in this laboratory as a part of our continuing methods development program for the analysis of carcinogenic polycyclic hydrocarbons in smoked products. In prior publications a general procedure for polycyclic aromatic compounds and a specific method for benzo[*a*]pyrene in smoked foods with demonstrated sensitivities of 2 parts per billion (ppb) were described (Howard *et al.*, 1966a,c). Analyses of various samples of smoked fish and ham showed the presence of trace quantities of benzo[*a*]pyrene as well as other hydrocarbon types. The objective of the present investigation was to develop analytical methods for the determination of such hydrocarbons in the water-soluble products and to investigate the incidence of these compounds in the various products now commercially available. Since no information was available on the polycyclic aromatic content of the resinous condensates which settle out on storage, samples of these materials were also obtained for analysis.

Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204

EXPERIMENTAL

Materials and Methods. The apparatus and reagents used in this study have been described in detail in previous publications (Howard *et al.*, 1966a,b).

The ultraviolet spectra were recorded with a Cary 11 spectrophotometer using 10 ± 0.05-mm optical pathlength cells (1.5-ml capacity, Optical Cell Co., Inc., Brentwood, Md., or equivalent). An Aminco-Bowman spectrophotofluorometer equipped with a 1P28 photomultiplier tube and slit arrangement No. 2 was used to record fluorescence spectra.

Analysis of Water-Soluble Flavors. A well-mixed 200 g sample of the water-soluble flavor was weighed in a 300-ml beaker and transferred to a 1 l. separatory funnel. The transfer was completed by washing the beaker with portions of isooctane, using a total volume of 100 ml. A 450-ml portion of 5.6% KOH solution (425 ml of distilled water + 25 g KOH) was added to the funnel, which was shaken by hand for 3 min. The layers were allowed to separate. (Note: Separations in the initial and subsequent extractions may be facilitated by the addition of 5 to 10 ml of methanol.) The lower layer was drawn off into a second separatory funnel and the extraction was repeated with 100 ml of isooctane. After separation, the lower layer was drawn off into a third separatory funnel and again extracted with 100 ml of isooctane. After separation of layers, the aqueous layer was drawn off and discarded. Each of the isooctane extracts was then washed twice with 50-ml portions of 5.6% KOH. Shaking time was 1 min and, after separation, the aqueous layers were drawn off and discarded. The isooctane extracts were then washed twice with 50-ml portions of distilled water and, after separation, the aqueous layers were drawn off and discarded.

Each isooctane extract was washed three times by shaking with 50-ml portions of 85% H_3PO_4 for 1 min. After separation, the acid layers were drawn off and discarded. Each isooctane extract was washed three times with 100-ml portions of distilled water and the aqueous layers were discarded after each wash.

The isooctane extracts in the first funnel were passed through a chromatographic column (prewashed with 75 ml of isooctane) consisting of 60 g of pre-treated Florisil and 50 g of anhydrous Na_2SO_4 into a 1 l. evaporation flask (Howard *et al.*, 1966a). The first separatory funnel was washed with the extract contained in the second funnel and filtered through the column. The second and first separatory funnels were washed in that order with the extract in the third, and the isooctane was passed through the adsorbent. The third, second, and first funnels were washed in tandem with two successive 50-ml portions of benzene and the washings filtered individually through the column. A 75-ml portion of benzene was then passed through the adsorbent. The collected eluate was then evaporated on a steam bath under nitrogen and vacuum to a volume of approximately 5 ml. The solution was transferred quantitatively with benzene to a 50-ml glass-stoppered Erlenmeyer flask and concentrated carefully on a steam bath under nitrogen to a volume of 0.2–0.3 ml. (CAUTION: The solution should not be evaporated to dryness, since prolonged heating of dry polycyclic aromatic hydrocarbons will cause losses.) The solution was reserved for thin-layer chromatography on cellulose and cellulose acetate.

Analysis of Resinous Condensates. A 25-g sample of the well-mixed condensate was weighed in a 150-ml beaker. The sample was dissolved in small portions of 20% KOH on the steam bath and the washings were transferred quantitatively to a 2 l. separatory funnel, using a total volume of 250 ml of the alkali. The beaker was washed four times with 50-ml portions of ethanol, which were also transferred to the funnel. A 400-ml portion of ethanol was added and the contents of the funnel were mixed. A 250-ml portion of isooctane was added to the funnel, which was then shaken for 3 min. After layer separation, the lower aqueous layer was drawn off into a second separatory funnel. Any residual aqueous phase in the first separatory funnel was allowed to settle and separate and then carefully drawn off into the second funnel. The extraction operation on the aqueous phase was repeated with 200 ml of isooctane as described above. The lower layer was then transferred to a third funnel and the extraction was again repeated. After the third extraction, the aqueous layers and residual materials were drawn off and discarded. Each of the isooctane extracts was washed three times with 200-ml portions of 5% KOH. Shaking time was 1 min and, after separation, the aqueous layer was discarded. Each isooctane extract was then washed three times with 200-ml portions of distilled water. Shaking time was 30 sec and the aqueous layers were discarded after each wash. The isooctane extracts were then passed individually through a Florisil column as described above, and the effluent was discarded. A 250-ml evaporation flask was placed under the column. The separatory funnels were washed with two 50-ml portions of benzene, which were passed through the column as indicated above. An additional 75 ml of benzene was then filtered through the adsorbent. Two milliliters of *n*-hexadecane were added and the solvent was evaporated on the steam bath with the aid of nitrogen and vacuum (Howard *et al.*, 1966a). A 10-ml portion of isooctane was added to the 2-ml hexadecane residue; the solvent was evaporated and the operation was repeated to ensure complete removal of benzene.

The 2 ml *n*-hexadecane concentrate was transferred quantitatively to a 500-ml separatory funnel with portions of isooctane (73 ml total). The solution was washed three times with 100-ml portions of 85% H_3PO_4. The shaking time was 1 min and, after each wash, the layers were allowed to separate and the lower acid layer was discarded. After the third discard, the funnel was swirled and allowed to stand for a few minutes. Any residual acid which settled out was drawn off and discarded.

The isooctane layer was extracted three times with 100 ml of the 4:1 dimethyl sulfoxide-phosphoric acid mixture according to Howard *et al.* (1965). The extracts were washed in tandem with a 30-ml portion of isooctane and transferred to a 2-l. separatory funnel containing 300 ml of water. The solution was extracted twice with 100-ml portions of isooctane. Shaking time for each extraction was 2 min. The aqueous layer was drawn off and discarded after the final extraction. The isooctane extracts were then washed twice with 200-ml portions of distilled water. After phase separation, the aqueous layers were discarded.

The isooctane extracts were passed individually through a Florisil column as described above, and the effluent was discarded. The hydrocarbons were then eluted from the column with benzene and collected in the 250-ml evaporation flask. The eluate was evaporated on the steam bath to approximately 5 ml, then quantitatively transferred with benzene to a 50-ml flask, and evaporated to 0.2 to 0.3 ml as described for the water-soluble flavors. The concentrate was reserved for thin-layer chromatography on cellulose and cellulose acetate.

Thin-Layer Chromatography. The thin-layer chromatographic systems were described in detail in previous reports (Howard *et al.*, 1966b,c; White and Howard, 1967).

SYSTEM 1. The entire concentrate with washings was streaked on cellulose layers 500 μ thick. The plates were developed in a pre-equilibrated chamber for 1.25 hr (25° C), using 20% *N,N*-dimethylformamide in ethyl ether as the stationary phase and isooctane as the mobile solvent. The fluorescent bands were outlined in a Chromato-Vue Cabinet, collected in a beaker, and the polycyclic aromatic hydrocarbons eluted by extraction four times with 5 to 10-ml portions of hot methanol. The individual extracts of each band were filtered successively through a pressure filter under nitrogen and collected together in a 50-ml glass-stoppered Erlenmeyer flask. The extract from each band was concentrated on the steam bath under nitrogen to a volume of 0.2 to 0.3 ml and reserved for chromatography on cellulose acetate.

SYSTEM 2. Each concentrated extract with washings was spotted on cellulose acetate layers 1000 μ thick. The plates were developed in a preequilibrated chamber for 1.5 hr (25° C), using ethanol-toluene-water (17:4:4 v/v/v) as the developer. The fluorescent spots were collected and eluted with methanol as described above. One milliliter of *n*-hexadecane was added to each extract and the methanol was evaporated on the steam bath under nitrogen. Residual methanol was removed by two successive additions of 5 ml of isooctane and reevaporation. Each 1.0 ml hexadecane residue was transferred into a 10-mm path length cell (1.5 ml capacity) and the ultraviolet spectrum was recorded against isooctane in the reference cell. The observed maxima were compared with those in the spectra of known polycyclic aromatic hydrocarbons obtained under the same instrumental conditions. Estimation of the quantity of the identified hydrocarbon was made by the baseline technique in conjunction with spectra of these hydrocarbons. The identification was confirmed by spectrophotofluorometry.

Recovery Studies. A solution of benzo[*a*]pyrene was prepared at a concentration of 0.4 μg/ml in isooctane. An appropriate aliquot was then placed in the separatory funnel containing the water-soluble flavor and the analysis was carried out as previously described. Prior to conducting the recovery studies, the aqueous flavors were analyzed for benzo[*a*]pyrene to establish its absence.

RESULTS AND DISCUSSION

The recoveries of benzo[*a*]pyrene added at a level of 2 ppb to 200 g of water-soluble flavor ranged from 70 to 89%. No recoveries of benzo[*a*]pyrene from the resinous condensate samples were conducted, since all of these products were found to contain relatively high levels of the hydrocarbon, as discussed below. Because of their enormous background content, it was necessary to rechromatograph the isolated hydrocarbon several times on the thin-layer plates in order to obtain satisfactory ultraviolet and fluorescence spectra.

The procedures described above have been applied to seven water-soluble flavors obtained from four different manufacturers in the United States. Four resinous condensate materials were also procured during the survey. The results of the analysis are summarized in Table I. The hydrocarbons were identified by comparing their R_f values with those of known compounds, and by their ultraviolet and fluorescence spectra. Trace quantities of anthracene, phenanthrene, pyrene, fluoranthene, and triphenylene were isolated from three of the aqueous flavors analyzed in this study, whereas no detectable amounts of polycyclic compounds were found in the remaining four products. Levels of the hydrocarbons ranged from 2.4 ppb for pyrene to 35 ppb for phenanthrene. An unidentified compound with ultraviolet and fluorescence spectra similar to those of benzo[*a*]pyrene was reported by Lijinsky and Shubik (1965) in one liquid smoke sample. We also found a like substance in one liquid smoke. This compound was clearly separated from benzo[*a*]pyrene and benzo[*g,h,i*]perylene by thin-layer chromatography. For example, the following R_f values for the three compounds were obtained on cellulose and cellulose acetate, respectively: benzo[*a*]pyrene, 0.40 and 0.23; benzo[*g,h,i*]perylene, 0.33 and 0.51; and the unknown hydrocarbon, 0.57 and 0.42.

Because of its marked similarity in both ultraviolet and fluorescence spectra to benzo[*a*]pyrene and its greater relative movement on the cellulose thin-layer plate, the unknown was suspected to be a methyl derivative of benzo[*a*]pyrene. In order to identify this hydrocarbon, various methyl derivatives of benzo[*a*]pyrene were obtained, including the 1-, 2-, 3-, 4-, 5-, 6-, and 7-methylbenzo[*a*]pyrenes. On the basis of the R_f values, all of the aforementioned derivatives could be eliminated except the 1- and 4-methylbenzo[*a*]pyrenes. The movement of 1-methylbenzo[*a*]pyrene was slightly greater than that of the other two compounds; however, the difference was not sufficient to rule out this hydrocarbon. The ultraviolet and

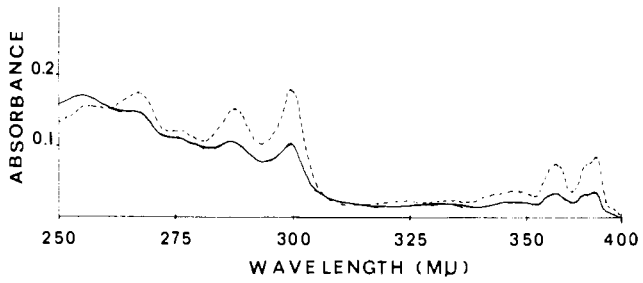

Figure 1. Ultraviolet absorption spectrum of 4-methylbenzo[*a*]pyrene isolated from 200 g of liquid smoke flavor. Solid line, unknown; broken line, reference (reference standard 0.8 mg/l.)

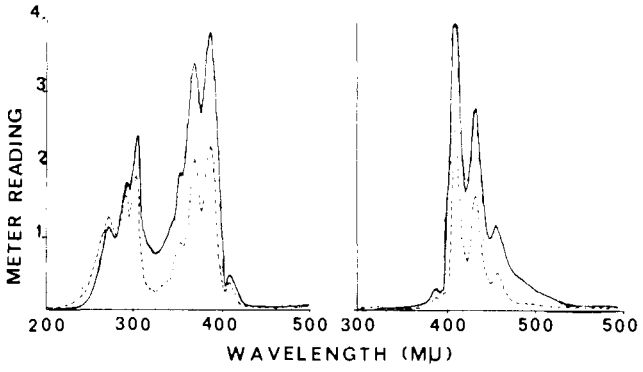

Figure 2. Fluorescence spectra of 4-methylbenzo[*a*]pyrene isolated from 200 g of liquid smoke flavor. Solid line, unknown; broken line, reference (reference standard 0.8 mg/l.). Left: Excitation spectra at emission 415 mμ, 0.001 mm, slit arrangement 2. Right: Emission spectra at excitation 390 mμ

fluorescence spectra of 4-methylbenzo[*a*]pyrene and the unknown compound were identical (Figures 1 and 2), whereas the spectrum of the 1-methylbenzo[*a*]pyrene, compared to the aforementioned hydrocarbons, showed a bathochromic shift in the near ultraviolet and visible regions. For example, the two major peaks in this region were at 368 and 389 mμ for the 1-methyl derivative in contrast to 363 and 383 mμ for 4-methylbenzo[*a*]pyrene and the unknown compound.

Lijinsky and Shubik (1965) compared the fluorescence emission spectra of benzo[*a*]pyrene, benzo[*g,h,i*]perylene, and the unknown compound, and noted that there were slight differences. These findings have been confirmed in our laboratory. At an excitation setting of 390 mμ, fluorescence emission maxima of 410, 415, and 425 mμ were observed for benzo[*a*]pyrene, the unknown derivative, and benzo[*g,h,i*]perylene, respectively.

On the basis of the R_f values with two different adsorbents and solvent systems and the ultraviolet and fluorescence spectra, the authors concluded that this compound is 4-methylbenzo[*a*]pyrene.

Table I. Polycyclic Aromatic Hydrocarbons[a] Found in Water-Soluble Liquid Smoke Flavors Sample

	Found (ppb)					
	Anthracene	Phenanthrene	Pyrene	Fluoranthene	Triphenylene	4-Methylbenzo[*a*]-pyrene
1	10	35	2	3	7	2
2	9	34	2	3	7	...
3	7	35	11	...	...	...

[a] All values were rounded off.

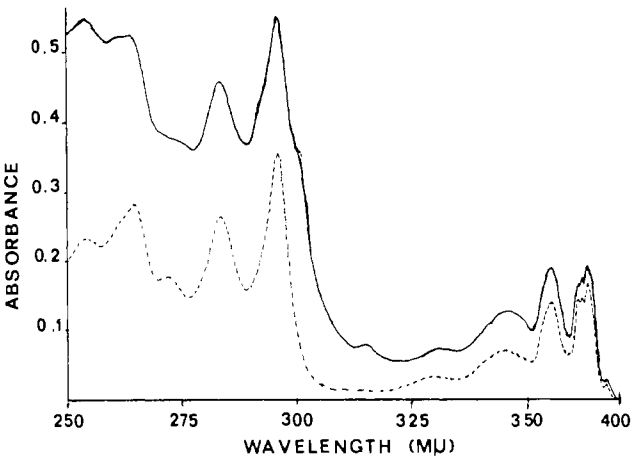

Figure 3. Ultraviolet absorption spectrum of benzo[*a*]pyrene isolated from 5 g of residual smoke condensate. Solid line unknown; broken line, reference (reference standard 1.4 mg/1.)

Benzo[*a*]pyrene was found in all four of the resinous condensates at levels of 25, 340, 3700, and 3800 ppb, respectively. The typical ultraviolet spectrum of this hydrocarbon isolated from sample 3 is presented in Figure 3. Identification of the compound in each of the four samples was also confirmed by fluorescence and by its molecular weight (*m/e* 252) as determined by mass spectrometry. Numerous fluorescent bands other than benzo[*a*]pyrene were observed when the extracts of the resinous materials were chromatographed on the cellulose plates. However, no attempt was made to isolate and identify these compounds, since our main objective was to determine if benzo[*a*]pyrene was present.

Trace quantities of polycyclic aromatic hydrocarbons were found in three of the seven water-soluble flavors analyzed. However, with the exception of one aqueous flavor in which 4-methylbenzo[*a*]pyrene was identified, no carcinogenic types were isolated. Varying concentrations of benzo[*a*]pyrene were found in all of the resinous condensates. The high levels present in the latter materials indicate the importance of their efficient removal from the aqueous flavor prior to its use in foodstuffs.

ACKNOWLEDGMENT

We are most appreciative of the cooperation of the following members of the Additives and Instrumentation Branch, Division of Food Chemistry and Technology, for the indicated assistance: Edward O. Haenni, Branch Chief, for general guidance and advice and for assistance in the preparation of the report; and Joseph N. Damico, for the mass spectrometric studies. We are also indebted to the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, and the Cancer Chemotherapy Center, National Cancer Institute, National Institutes of Health, Bethesda, Md., for supplying some of the polycyclic aromatic hydrocarbons used. Our sincere appreciation is also expressed to Guido H. Daub, University of New Mexico, Albuquerque, N.M., and Louis F. Fieser, Harvard University, Cambridge, Mass., for the various methyl derivatives of benzo[*a*]pyrene used in the study.

LITERATURE CITED

Draudt, H. M., *Food Technol.* **17,** 85 (1963).
Hollenbeck, C. M., *Food Process.* **25,** 136 (1964).
Hollenbeck, C. M., U.S. Patent 3,106,473 (1963).
Howard, J. W., Haenni, E. O., Joe, F. L., Jr., *J. Ass. Offic. Anal. Chem.* **48,** 304 (1965).
Howard, J. W., Teague, R. T., Jr., White, R. H., Fry, B. E., Jr., *J. Ass. Offic. Anal. Chem.* **49,** 595 (1966a).
Howard, J. W., Turicchi, E. W., White, R. H., Fazio, T., *J. Ass. Offic. Anal. Chem.* **49,** 1236 (1966b).
Howard, J. W., White, R. H., Fry, B. E., Jr., Turicchi, E. W., *J. Ass. Offic. Anal. Chem.* **49,** 611 (1966c).
Lijinsky, W., Shubik, P., *Toxicol. Appl. Pharmacol.* **7,** 337 (1965).
White, R. H., Howard, J. W., *J. Chromatogr.* **29,** 108 (1967).

Received for review August 11, 1970. Accepted October 28, 1970.