Determination of Four- and Five-Ring Condensed Hydrocarbons. II. Analysis of Polynuclear Aromatic Compounds in *n*-Paraffin Feed Oil for Yeast Fermentation

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A method has been developed for the quantitative determination of polynuclear aromatics in high quality *n*-paraffin feedstocks used for yeast fermentation. This method incorporates improvements and refinements over the methods for similar material which are necessary, especially when polycyclic thiophene-type compounds are present, and involves extraction with dimethyl sulfoxide, followed by chromatography on Florisil and alumina to concentrate polynuclear aromatics. Isolation of these compounds is accomplished by two successive thin-layer chromatographic separations and measurement is made by ultraviolet spectrophotometric procedures. Recovery studies of benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, benz[*a*]anthracene and 3-methylcholanthrene at the 2-ppb level in 500 g of oil averaged from 81 to 91%. Pyrene and fluoranthene were found in all oils examined, including medical grade white mineral oil; however, phenanthrene and substituted pyrene and phenanthrene were also found in certain oils. Carcinogenic polynuclear aromatics were not found in silica gel treated *n*-paraffins.

The production of single-cell protein by fermentation using pure *n*-hydrocarbon as a substrate has been of keen interest. The development of a method for polynuclear aromatics in yeast was described in part I of this investigation (McGinnis and Norris, 1975). The current work involves the analysis for polynuclear aromatics in *n*-hydrocarbon feedstocks for fermentation, for which a suitable method was developed and is described in this report.

The quality of oils and solvents used in the food processing industry has been the subject of a number of examinations for trace constituents of polynuclear aromatics. Medical grade white mineral oils must meet the regulations set by the U.S. Food and Drug Administration (*Fed. Regist.*, 1964), and the present test method originally based on the works of Haenni *et al.* (1962) and Haenni and Hall (1960) is described by ASTM D 2008-65 (American Society for Testing and Materials, 1965), which was reapproved in 1970. However, this method is unsuitable and inadequate for assessment of the presence of trace quantities (parts per billion) of individual polynuclear aromatic hydrocarbons. Howard et al. (1966b) describe a procedure for polycyclic aromatic hydrocarbons in vegetable oils and include recovery studies for these compounds. However, this method involves phosphoric acid washes and, later, extraction using dimethyl sulfoxide (DMSO) containing small quantities of phosphoric acid. DMSO–phosphoric acid extraction is used to reduce interference from coextracted material and to reduce inherent impurities in DMSO (Howard *et al.*, 1965), but this mixture also reduced recoveries in our investigation. The loss of certain polynuclear aromatics may conceivably be due to water in the phosphoric acid which will partition with DMSO, or oxidation of aromatics in this system. In our work phosphoric acid washes were not found to appreciably affect ultraviolet absorbance due to background material. Additionally, if phosphoric acid is avoided, washing with aqueous sodium hydroxide is not necessary. These basic washes often produce filmy emulsions.

Later work on the analysis of polycyclic aromatic hydrocarbons in hexanes used in the extraction of edible oils (Howard *et al.*, 1968) involves a method which was not found applicable for the oils presently under study. The method for edible oils also involves phosphoric acid wash although DMSO–phosphoric acid extraction mixtures were not employed. The analysis of these hexanes involves evaporation of the sample which is considerably more volatile than feedstock oil composed mainly of C_{13}–C_{17} normal hydrocarbons. However, employing elements of this method and that for vegetable oils, *i.e.*, washing with phosphoric acid prior to extraction with DMSO, was also found unsatisfactory for certain feed oils. This was due to traces of polycyclic thiophenes which were found to concentrate with the "benzpyrene" fraction. Certain feed oils contained a predominance of these sulfur compounds compared to polynuclear hydrocarbons, and further separation of polynuclear aromatics by thin-layer chromatography was not possible without further prior concentration. Although modifications of a method for polynuclear aromatics in paraffin waxes which employs a paper chromatographic step (Howard and Haenni, 1963) may be plausible for the present analysis, it was desirable to eliminate this cumbersome procedure.

Gas chromatographic measurements of the "benzpyrene" concentrate were investigated briefly for the determination of individual polynuclear aromatics. A number of reports have detailed the analysis of high-boiling polycyclic aromatic mixtures, but in these studies, complete separation of certain significant compounds, such as resolution of benzo[*a*]pyrene from benzo[*e*]pyrene, was not effected (Liberti *et al.*, 1964; De Maio and Corn, 1966; Beeson and Pecsar, 1969; Gump, 1969). However, Bhatia (1971) demonstrated a gas chromatographic separation of a number of polycyclic aromatics using columns packed with OV-7 (20% phenylmethylsilicone on glass beads). Our investigation of this technique using a dual-flame ionization detector system and a linear temperature programmer yielded sporadic results. Additionally, it became evident during the examination of samples that unequivocal identification required spectral characterization. For these reasons, gas chromatographic techniques were abandoned in preference to separation employing two successive thin-layer chromatographs, followed by ultraviolet spectroscopy.

The purpose of this work was the development of a method for polynuclear aromatics in *n*-paraffins used for hydrocarbon fermentation. This report describes a method for the isolation and determination of polynuclear aromatics such as benzo[*a*]pyrene and similar highly condensed compounds at the 2-ppb level from 500 g of *n*-paraffins (*ca.* C_{13}–C_{17}). The method involves extraction with DMSO, partition, and concentration of the polynuclear aromatics by chromatography on Florisil and alumina. Isolation is made by two successive thin-layer chromatograms and the hydrocarbons are measured by ultraviolet spectrophotometric techniques. A number of variously treated feed oils have been analyzed and the results com-

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pared with Ultraviolet Absorbance Test CFR 121.1146 (*Fed. Regist.*, 1967).

MATERIALS AND METHODS

Materials and Apparatus. The apparatus and reagents employed in this study are described in part I of this work (McGinnis and Norris, 1975). Solvents met rigid ultraviolet absorbance specifications described by Howard *et al.* (1966a). Florisil was pretreated with methanol, dried in a vacuum oven at 50° and 24–26 in. of vacuum for 6 hr, and standardized with benzo[*a*]pyrene. This treated Florisil may alter in storage; additional details are provided in part I (McGinnis and Norris, 1975). Woelm neutral Grade 1 alumina deactivated by 10% water was prepared by initially establishing the water content of a 10-g sample. The weight loss occurring when the alumina is heated in a platinum dish at red heat for 15 min is considered the apparent water content. Sufficient distilled water is added to a fresh batch of alumina so that the total water content is 10.0%. The alumina is shaken for 15 min and stored for 4 hr before use. A test of the water content should not vary by more than ±0.1 wt %. This deactivated alumina is used in a chromatographic column 6 mm i.d. × 20 cm having a 20-ml glass reservoir at the top. A plug of spun glass is used at the bottom of the column.

Ultraviolet spectra were recorded with a Cary Model 11 recording spectrophotometer using optical cells of 1-cm path length having a capacity of 1 ml.

Procedures. A 500-g sample of *n*-paraffin is weighed and placed in a 2-l. separatory funnel. To this is added 500 ml of isooctane, of which portions are used to transfer the sample from a tared flask used to weigh the sample to the separatory funnel. This solution is extracted three times with 250-ml portions of preequilibrated dimethyl sulfoxide (DMSO), shaking 3 min for each extraction. The DMSO extracts are collected in a separatory funnel, 1200 ml of water is added and mixed with it, and the mixture is permitted to cool. This aqueous solution is extracted three times with 250-ml portions of isooctane (shake 1.5 min for each extraction), and the isooctane extracts are collected in a 1-l. erlenmeyer flask. Two separatory funnels are used for the extraction of the aqueous–DMSO mixture. The lower aqueous phase is transferred to the second funnel after the first extraction, and then transferred back to the first funnel after the second extraction; the same procedure is followed for the third extraction. The aqueous–DMSO is discarded after the third extraction and each funnel is washed with 10 ml of isooctane and added to the extracts. The total isooctane extract is evaporated on a steam bath under a nitrogen stream until a volume of 50 ml is reached. This solution is reserved for chromatography on Florisil.

Column chromatography is carried out by the addition of 40 g of Florisil to a column 33 mm i.d. × 30 cm fitted with a coarse fritted glass disk, to which is added 15 g of anhydrous sodium sulfate atop of the Florisil. The column is wrapped with aluminum foil so that light is excluded. This column is prewet with 60 ml of isooctane and then the contents of the erlenmeyer flask are quantitatively transferred and filtered through the column. The erlenmeyer flask is rinsed with five 10-ml portions of isooctane which are added to the column. A 45-ml portion of isooctane is added to the column and allowed to drain. The isooctane eluent contains two- and three-ring aromatic compounds and other material (see part I of this work; McGinnis and Norris, 1975), but does not contain benzo[*a*]pyrene, benz[*a*]anthracene, or other more highly condensed ring-system aromatics. These compounds are eluted with benzene. A 25-ml portion of benzene is added to the column, permitted to drain, and retained for further study. This eluent should not contain benzo[*a*]pyrene, benz[*a*]anthracene, and other polynuclear aromatics which are more strongly held by the adsorbent. Polynuclear aromatics are eluted with three 45-ml portions of benzene, allowing the column to drain after each addition. This effluent is collected in a separate flask and concentrated to about 0.2 ml on a steam bath under a stream of nitrogen, and 10-ml of isooctane is added and again concentrated to 0.2 ml. This procedure is repeated three times and the final volume adjusted to 2 ml in isooctane.

Table I. Recoveries of Polynuclear Aromatic Hydrocarbons Added to 500 g of *n*-Paraffin Oil at the 2-ppb Level

Polynuclear aromatic hydrocarbon	Recoveries, %				Av
Benzo[*a*]pyrene	80,	88,	90,	99	89
Benz[*a*]anthracene	79,	83,	87,	92	85
3-Methylcholanthrene	70,		81,	92	81
Dibenz[*a*,*h*]anthracene	80,	90,	95,	97	91

A 20 cm × 6 mm i.d. column containing a glass fiber plug is prepared for chromatography by the addition of 4.25 g of alumina deactivated by 10% water. The column is tapped to settle the adsorbent. The column is washed with 10 ml of isooctane and the sample is quantitatively transferred to the column using three 2-ml portions of isooctane. The addition of 20 ml of isooctane is made to the column and permitted to drain to the top of the adsorbent bed and then 20 ml of benzene is added and allowed to drain in the same manner. The receivers are changed and the polynuclear aromatics eluted with two successive 10-ml portions of 1:1 benzene–diethyl ether. This operation should be carried out in subdued light by wrapping the column with aluminum foil and out of direct light. Solvent flow can be assisted by a slight positive pressure of nitrogen but only until the solvent reaches the top of the adsorbed bed. The 1:1 benzene–diethyl ether solution is evaporated on a steam bath under a stream of nitrogen to *ca.* 1 ml and reserved for thin-layer chromatography.

Thin-layer Chromatography. Two successive thin-layer chromatographs are employed to separate polynuclear aromatics. The first plate employs cellulose as adsorbent and an immobile phase of *N*,*N*-dimethylformamide and is developed with isooctane. The second plate employs acetylated cellulose as adsorbent and is developed with a mixture of ethanol–toluene–water in a ratio of 17:4:4 (v/v/v). Benzo[*a*]pyrene is used as a reference compound in each case. Details for the preparation, washing the plates before use, and development appear in part I of this work (McGinnis and Norris, 1975). After development, the position of each fluorescent band is marked by examination of the plate in a Chromato-Vue cabinet. R_f values are determined and the fluorescent areas are removed from the plates and polynuclear aromatics removed from the adsorbent with methanol as detailed in part I. The addition of 1 ml of *n*-hexadecane is made to each methanol solution and the volume evaporated to 1 ml on a steam bath under a stream of nitrogen.

Determination of polynuclear aromatics is made by recording the ultraviolet spectrum using a Cary Model 11 ultraviolet spectrophotometer. The procedure is detailed in part I.

RECOVERY STUDIES

Solutions of 1 ml of benzo[*a*]pyrene, benz[*a*]anthracene, 3-methylcholanthrene, and dibenz[*a*,*h*]anthracene containing 1 μg/ml in isooctane were added to 500 g of oil. The addition of 1 μg to 500 g is equivalent to 2 ppb. In three cases, each of these compounds was added to the oil, and in one case, all but 3-methylcholanthrene were added. The results of this study are shown in Table I. The analysis for these compounds was carried out as described after deter-

Table II. Paraffin Analyses

	Ultraviolet Absorbance Test CFR 121.1146						
	Acid washed, absorbance for sample no.			Silica gel treated, absorbance for sample no.			
Wavelength, nm	1	2	3	4	5	6	7
260–269	0.23	0.51	0.68	0.44	0.65	0.61	0.01
270–279	0.19	0.54	0.49	0.25	0.48	0.46	0.01
280–289	0.14	0.54	0.48	0.25	0.47	0.46	0.01
290–299	0.08	0.28	0.43	0.25	0.47	0.46	0.01
300–329	0.05	0.12	0.40	0.25	0.44	0.42	<0.01
330–350	0.02	0.02	0.26	0.15	0.26	0.25	<0.01
Sulfur, ppm	4	3	9	5	9	12	3
Nitrogen, ppm	<0.2	<0.2	<0.2	<0.2	<0.2	< 0.2	<0.2

Table III. Polynuclear Aromatic Hydrocarbons Found in *n*-Paraffin Oils Used for Fermentation and Mineral Oil[a]

Sample ident.	Pyrene, ppb	Fluoranthene, ppb	Phenanthrene, ppb	Subst. phenanthrene, ppb	Subst. pyrene, ppb
1	0.5	3.0		6.0	
2	0.8	1.8			1.0
3	0.6	1.0	2.1	4.4	1.1
4	0.5	0.7	2.5	11.5	
5	0.8	7.3	2.9	12.7	
6	0.9	6.5	2.0	6.6	
7	0.5	0.5			
Mineral oil	0.5	3.0			

[a] Values not corrected for recovery percentages.

mining that the *n*-paraffin oil was free of the materials under investigation.

RESULTS AND DISCUSSION

Table I summarizes the recoveries of representative polynuclear aromatic hydrocarbons from *n*-paraffins. Average recoveries at the 2-ppb level in 500 g of oil ranged from 81 to 91%. This oil was silica gel treated and analyzed prior to recovery study procedures.

This procedure for the analysis of polynuclear aromatics has been applied to a considerable number of feed oils. However, we have included data on only seven samples of *n*-paraffins obtained from various suppliers and a medical grade white mineral oil as being representative. Certain oils were treated by percolation through silica gel, at a silica gel–oil ratio of 20:1, whereas others were oleum treated by the manufacturer and were not contacted with the gel. The white mineral oil was not treated. The acid treated oils yielded considerably more background material as would be expected. In fact, in some cases, without the use of the 10% water-deactivated alumina column, thin-layer chromatography was not possible due to the relatively large sample at this point. The composition of the bulk of this background material is aromatic heterocyclic compounds. High-resolution mass spectrometric analysis of the benzene eluent from the Florisil column was carried out, and while absolute concentrations were not obtained, the results indicated the presence of abundant furan, thiophene, and some pyridine polycyclic material. Additionally, gas chromatographic–microcoulometric sulfur analysis of this eluent indicated a number of polycyclic sulfur compounds including isomeric naphthodibenzothiophenes and benzthiophenanthrene. Chromatography on 10% water-deactivated alumina greatly reduces the presence of heteroatomic material in the "benzpyrene" concentrate, and final separations are amenable to thin-layer techniques.

Table II summarizes analytical, absorptivity, and processing information for the oils studied. Tests for sulfur, nitrogen, and carbon number distribution were carried out by standard routine methods, while the absorbance tests were performed according to the procedure CFR 121.1146. The sulfur content of all oils was fairly high, ranging from 3 to 12 ppm, and was relatively unaffected by processing. Polycyclic thiophenes detected probably account for the sulfur values. The bulk (97–99 wt %) of the normal hydrocarbons was from C_{13}–C_{17}, with nonnormals accounting for less than 1.0%, while the remainder was accounted for by traces of normals from C_{10} to C_{19}. Table III gives the results for polynuclear aromatic analysis for these oils, and, for comparison, results are included for a medical grade white mineral oil. Mineral oil had no more than 0.01 absorbance in all wavelength ranges. There is no simple correlation between the absorbance and the level of polynuclear aromatics found even in the 300–350-nm range. Polycyclic heteroatomic material, as noted, contributes to the absorptivity of the oil. Pyrene and fluoranthene were present in all of the oils studied; however, phenanthrene, substituted phenanthrene, and substituted pyrene were found in certain acid treated and silica gel treated oils. The levels of substituted phenanthrenes must be considered approximate and their identification tentative as reference compounds were not available for comparison. Concentrations were determined based on phenanthrene as a reference. Pyrene and fluoranthene were determined by the base-line technique.

During thin-layer chromatographic separations, a number of pale blue and yellow fluorescent bands were observed. Ultraviolet identification of these materials could not be made, usually because only a few or no maxima were demonstrated, but occasionally no matching spectra of a known compound could be located. Unidentified material was either below the limit of detectability of the method or inconsistent with voluminous published spectra of polynuclear aromatics. These substances may be aromatic heteroatomic compounds but rigorous study of spectra for identification was not pursued.

The results of this study indicate that high quality *n*-paraffin used for fermentation to produce single-cell protein contains trace quantities of polynuclear aromatics, but no known carcinogenic material was found. Contamination of single-cell protein could not be traced to the hydrocarbon source.

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Carrot-Root Oil Components and Their Dimensional Characterization of Aroma

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Cold hexane–acetone extraction of fresh carrots produced an oil in which 28 components, in decreasing concentration, were tentatively identified as isoprene, β-caryophyllene, linalool, acetaldehyde, *p*-cymene, terpinolene, dipentene, ethanol, camphene, bisabolene, β-ionone, 2-nonenal, nonanal, α-pinene, γ-terpinene, β-pinene, α-terpineol, α-ionone, dodecanal, α-terpinene, nopol, borneol, bornyl acetate, 4-terpineol, biphenyl, myrcene, carotol, and ionene. Multidimensional scaling of the odor of these components against 52 descriptive terms produced a two-dimensional relationship in which distances between points in the geometry correspond to the appropriateness of the terms for the chemical odors.

Compared to fruit, there has been relatively little attention given to the flavor and aroma of vegetables. These are usually tenuous and difficult to describe, and often, in spite of botanical and other differences, difficult to easily distinguish. Strong and distinctive vegetables, like onion or cabbage, or those which are economically significant, such as potatoes, are exceptions. The delicate flavor and aroma of fresh carrots fall in the elusive and more difficult to define category.

The flavor and aroma of cooked carrots have been studied by several authors. Otsuka and Take (1969) attribute the taste of a carrot soup to the presence of three carbohydrates, glutamic acid, and the buffer action of various other amino acids. Isolation by steam distillation produced a carrot-root oil that possessed an aroma similar to that of cooked carrots (Buttery *et al.*, 1968). The nature of the compounds responsible for the aroma remains undefined, however, and the results of Heatherbell and Wrolstad (1971) illustrate the complexity of the problem. Degradation of terpenoid substances is largely responsible for loss of carrot acceptability (Ayers *et al.*, 1964; Farine *et al.*, 1965; Heatherbell *et al.*, 1971) and the flavor and aroma of carrots, whether raw or cooked, probably result from a complex interaction of several of these compounds and the nonvolatile constituents (Alabran and Mabrouk, 1973).

Recent approaches in sensory measurement have employed a new type of data analysis called "multidimensional scaling" (Schiffman, 1974). The aim of this class of procedures is to construct a "map" in a geometrical space. Odors that are similar to each other are located near each other in this geometry, whereas those that are dissimilar are placed far away from each other. To achieve the map, subjective differences between pairs of odors (or between odors and words) are obtained through experimental procedures. Computer programs treat these differences as distances between points in the geometry (Woskow, 1968; Kruskal and Carmone, 1969). Programming procedures have been described in sufficient detail for developing a program on automatic computers, and such a program is available from J. B. Kruskal (Kruskal, 1964). These procedures have been used to study the geometrical configuration of odors (Berglund *et al.*, 1972; Yoshida, 1972).

The first dimension to be uncovered in the analysis is the hedonic dimension of pleasantness–unpleasantness (Yoshida, 1972). Other dimensions can vary, depending upon the selection of starting odors, and the dimensions are named according to the odors that lie at either extreme. The procedures do not provide names to the dimensions, but only the projection of points on each axis.

The present study modified the procedure of multidimensional scaling by attempting to place both stimuli and descriptors in the same geometrical space. The space contains sufficient information to determine what labels best correspond to its dimensions. Also, since both odorants and descriptors are placed in the same space, subtle variation of aroma description becomes evident. This investigation obtains information on a collection of odors of essential oil components of raw carrots and tests a method of quality representation of carrot aroma. Gas chromatography was used to identify and quantify the oil components. Heating during preparation of the oil was avoided to prevent artifact formation, and organic solvents were used to assure complete extraction of all oil components.

EXPERIMENTAL SECTION

Imperator carrot variety was purchased in California and shipped air freight to Natick Laboratories. Upon arrival the carrots were stored at 5° for 2–4 days.

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