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Determination of Four- and Five-Ring Condensed Hydrocarbons. I. Analysis of Polynuclear Aromatic Hydrocarbons in Yeast Produced by Growth on Both *n*-Hydrocarbon and Dextrose Feeds

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A method has been developed and tested for the analysis of polynuclear aromatic hydrocarbons in the biomass produced by growth of a pure *Candida* strain on both *n*-hydrocarbon and dextrose feeds. The method involves digestion of the yeast and the polynuclear aromatic material is concentrated by extraction, chromatography on alumina, partition, and chromatography on Florisil. Polynuclear aromatic compounds are separated and isolated by two successive thin-layer chromatographic plates, and measured by ultraviolet spectrophotometric procedures. Recovery studies of four components, benzo[*a*]pyrene, benzo[*e*]pyrene, benz[*a*]anthracene, and benzo[*g,h,i*]perylene each at the 5-ppb level averaged 63 to 80% for both *n*-hydrocarbon and dextrose grown yeast. Recovery of benzo[*a*]pyrene added to yeast from the 3- to 250-ppb level ranged from 84 to 88%. Recovery of polynuclear aromatics added individually to yeast was generally greater than that for a multicomponent system. Analysis of dextrose-grown yeast did not yield any of these compounds, whereas analysis of *n*-hydrocarbon-grown yeast showed some of these compounds in a range of 1–11 ppb. Other polynuclear aromatic compounds were detected in each of these yeast materials.

This work describes the first part of a two-part series dealing with the analysis of polynuclear aromatic hydrocarbons in a petroleum fermentation process. Polynuclear aromatic determinations of the biomass product will be considered initially.

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Pryor (1969) considers synthetic foods to be a possible solution to the population–food problem as a means of meeting the demand for new sources of protein. The culture of microorganisms, especially *Candida* yeast strains which grow actively on hydrocarbon substrates, appears feasible for the production of protein. Although there are a number of producers (Bennett *et al.*, 1969; Decerle *et al.*, 1969) employing various organisms and hydrocarbon feeds for production of this "petroprotein" outside the

United States, the production here has been slow to develop. A chief reason for this is the stringent regulations which apply to the food industry especially with regard to any traces of polynuclear aromatic materials carcinogenic in nature. Although Takata (1969) indicates that animal feeding studies show the absence of carcinogenic properties in petroleum-grown yeast, investigation of residual polynuclear aromatic hydrocarbons is of considerable importance.

Gulf Research & Development Company initiated research on the growth of microorganisms using hydrocarbon substrates in 1963 and development of a process for petroprotein based on a *Candida* yeast strain began in 1967. The aim of this work was to carry out fermentation using highly purified *n*-paraffin feeds. Much of the work including fermentation, medium development, yield, and demonstration of the process has been published by Silver and Cooper (1972a,b) and Cooper *et al.* (1973). Therefore, details concerning yeast composition and quality using either pure *n*-hydrocarbons or dextrose as feeds will not be elaborated. Details of the analysis of this hydrocarbon feed and method for the isolation and determination of polynuclear aromatic material will follow in a subsequent work.

Our interest centered on a method for the analysis and recovery of polynuclear aromatics from yeast grown on both dextrose and *n*-hydrocarbons. The growth of yeast using feedstocks of either pure *n*-hydrocarbon or carbohydrate substrates, such as dextrose, plus essential minerals produces single-cell protein with essentially the same composition. The difference between the two mediums is that *n*-hydrocarbon furnishes only carbon and hydrogen whereas carbohydrate furnishes carbon, hydrogen, and some oxygen. The principal difference involves the large volume of oxygen as air supplied to the hydrocarbon process which is about 2.5 times that involved in carbohydrate fermentations. Apart from this, the fermentations appear equivalent. Therefore, the analysis of fermentation product using dextrose feed acts as a suitable blank for evaluation of products employing hydrocarbon feed.

Soxhlet and similar extraction methods such as those of Howard *et al.* (1966a), Rhee and Bratzler (1970), and Soos and Creleszky (1969) are available for analysis of polynuclear aromatics in smoked foods, meat products, and other diet composites, but these methods do not bring about solubilization of the material being analyzed and, therefore, were not appropriate for the analysis of yeast. Additionally, the general method for polynuclear aromatic hydrocarbons by Howard *et al.* (1968) did not effect complete solubilization of our yeast product, and also had other serious limitations when applied to yeast. Considerable coextracted interfering material was present prior to tlc separation. The significance of obtaining complete solution of yeast was fundamental in this study because there is the distinct possibility of polynuclear aromatic material within the cell wall. Experiments involving cell rupture followed by analysis employing Soxhlet extraction resulted in serious losses of polynuclear aromatic compounds. Kuznetsov *et al.* (1970) report hydrocarbon analysis of petroleum-fed yeast using phenol and aniline as solvents for extraction, but little information is presented on compounds found and pertinent recovery data are not included.

Our investigation has led to the development of a method for the determination of trace polynuclear aromatic constituents based on extraction of a solubilized yeast sample. The recovery of polynuclear aromatic compounds added to yeast at the 5-ppb level was established although the method is sensitive to polynuclear aromatic compounds below this level. The method described involves digestion of the yeast in an alcoholic–aqueous potassium hydroxide system. Polycyclic aromatics are extracted into isooctane which is washed, concentrated, and filtered through a deactivated alumina column. The effluent is partitioned using dimethyl sulfoxide and, followed by separation of dimethyl sulfoxide, polynuclear aromatics are released by the addition of water and extracted into isooctane. Column chromatography on Florisil yields a polynuclear aromatic concentrate which is separated into individual components by two successive thin-layer chromatographic plates. The isolated hydrocarbons are finally identified and measured by ultraviolet spectrophotometric techniques.

MATERIALS AND METHODS

Apparatus. All glassware employed in this method was purchased for and dedicated to the procedure so that possible contamination and the use of acid and detergents in cleaning could be avoided. Glassware was cleaned with solvents and distilled water. The method calls for some specialized glassware, chromatographic columns, and pressure filters as detailed: (a) chromatographic column, 33 mm i.d. × 30 cm length, with coarse fritted glass disk and Teflon stopcock; chromatographic column, 23 mm i.d. × 50 cm length, 500-ml reservoir, with coarse fritted glass disk and Teflon stopcock; (b) pressure filters, 20-ml capacity, fitted with coarse fritted glass disk and 24/40 joint.

Reagents. Isooctane, benzene, and methanol were purchased from Burdick and Jackson Laboratories, Inc. (Muskegan, Mich.) and met the requirements for polynuclear aromatic analysis described by Howard *et al.* (1966a). *n*-Hexadecane was purified by percolation through activated silica gel. Spectrophotometric grade dimethyl sulfoxide, USP grade ethanol, and analytical reagent grade diethyl ether, potassium hydroxide, and anhydrous sodium sulfate were used. Reagent grade *N,N*-dimethylformamide and toluene were redistilled before use. Florisil was prepared by shaking 300 g of this adsorbent with 500 ml of methanol in a glass-stoppered flask. The slurry was transferred to a coarse porosity Büchner funnel using three 35-ml portions of methanol and washed with 100 ml of additional methanol. After the methanol had drained, vacuum was applied and then the Florisil was dried in a vacuum oven at 50° and 24–26 in. of vacuum for 6 hr. After being dried, the Florisil was transferred to a brown bottle and shaken for 1 hr on a mechanical shaker to ensure homogeneity.

It is important that the Florisil be tested for recovery of benzo[*a*]pyrene before use. Also, if Florisil is stored for long time periods it should be rechecked periodically. The test is carried out in the following manner. The addition of 60 g of Florisil is made to a 33 mm i.d. × 30 cm column, and to this is added 15 g of anhydrous sodium sulfate atop of the Florisil. A 100-ml portion of isooctane is used to wash the adsorbent. Benzo[*a*]pyrene (1 ml of 1 μg/ml solution in isooctane) is added to the column and an additional 100-ml portion of isooctane is passed through the column. Benzo[*a*]pyrene is eluted by the addition of 125 ml of benzene to the column and this effluent is collected separately. A 1-ml portion of *n*-hexadecane is added to this effluent and the solution evaporated on a steam bath under a stream of nitrogen to 1 ml. The hexadecane solution is examined spectrophotometrically for recovery of 97–100% benzo[*a*]pyrene.

Deactivated alumina is prepared in the following way using Woelm neutral Grade 1 alumina. The apparent water content of the alumina is established by determination of the weight loss when 10 g of alumina is heated in a platinum dish at red heat (Bunsen burner) for 15 min. Note that weighings should be made rapidly as the alumina readily adsorbs atmospheric water. Alumina which has been heated is discarded. Sufficient distilled water is added to fresh alumina so that apparent inherent water plus that added accounts for 10.0% by weight. The alumina is shaken vigorously 15 min and stored in a brown glass

bottle for at least 4 hr before use. A test of the water content (as described) should not vary more than ±0.1 wt %.

Extraction and Concentration Procedures. A mortar and pestle is used to grind 200 g of yeast until a fine powder is produced. A 600-ml portion of distilled water, 120 g of potassium hydroxide, and 100 ml of ethanol are placed in a 2-l. flat-bottomed flask equipped with a 7-cm stirring bar, and the mixture is stirred and heated on a hot plate until the solution reaches 45–50°. If recovery studies are to be conducted, add 1 ml of each polynuclear aromatic hydrocarbon solution (1 μg/ml in isooctane) desired. Yeast is added slowly in 50-g aliquots over a period of 30 min to a rapidly stirred solution while increasing the temperature to 80°. Too rapid an addition of yeast will produce excessive foaming. It will be necessary to vary the rate of stirring to control foaming. An adapter is then placed on the flask and an air condenser fitted. The temperature of the solution is maintained between 80 and 85° while stirring for 2 hr. The conditions for yeast solubilization–saponification are critical, especially the rate of addition of yeast and temperature.

The solution is poured while hot into a 2-l. separatory funnel and the flask washed separately with 100-ml portions of both distilled water and ethanol, and these washings added to the solution. The solution is then extracted while warm (this is essential) four times with 200-ml portions of isooctane. Each extraction is shaken 3 min. These extractions are carried out by alternating the extractions between two 2-l. funnels, and collecting the isooctane extracts in a 2-l. separatory funnel. Occasionally emulsions form after shaking the solution with isooctane, especially after the first such extraction. These emulsions are readily broken by the slow addition of 35–50 ml of ethanol. After the fourth isooctane extraction the aqueous phase is discarded and each 2-l. separatory funnel is washed with two 25-ml portions of isooctane in tandem, and these washings are added to the isooctane extracts. The collected isooctane extracts are washed with four 125-ml portions of warm (50–55°) distilled water. A gentle swirling action should be employed for these washings as vigorous shaking will result in emulsions. The aqueous washings are discarded after each wash, and the isooctane transferred to a flask and evaporated on a steam bath under a stream of nitrogen until a volume of 300 ml is reached.

A 60-g portion of 10% water deactivated neutral alumina is placed in the 23 mm i.d. × 50 cm column while tapping the column to facilitate settling. The column is wrapped with aluminum foil to exclude light. Losses of polynuclear aromatics were experienced when light was not excluded. The column is not prewet. The 300-ml isooctane extract is filtered through the column while warm and allowed to drain into a 1-l. separatory funnel. The flask is washed with two 10-ml portions of isooctane and the washings filtered through the column. Finally, an additional 50 ml of isooctane is added to the column. The total effluent is extracted three times with 100 ml of dimethyl sulfoxide (DMSO). DMSO is preequilibrated with isooctane before use. On each extraction, the flask is shaken 3 min and the extract collected in a 2-l. separatory funnel. A 600-ml portion of distilled water is added to the DMSO, and when the mixture has cooled, extracted four times with 50 ml of isooctane. The isooctane extracts are collected and reserved for chromatography on Florisil.

Chromatography in the Florisil column should be carried out without interruption because loss of polynuclear aromatics will result if contact with Florisil is lengthy. The 33 mm i.d. × 30 cm column is wrapped with aluminum foil to exclude light and packed with 60 g of Florisil. The column is tapped to settle the Florisil and then 15 g of anhydrous sodium sulfate is added to the top of the column. The column is prewet with 100 ml of isooctane and then the 200 ml of isooctane used to extract the aqueous DMSO is passed down the column. The flask is washed with three 10-ml portions of isooctane and this is passed down the column. After the column has drained, 10 ml of benzene is added to the column and allowed to drain. Highly condensed ring compounds, including the "benzpyrene fraction," are retained by the column. This material is collected separately by the addition of 125 ml of benzene to the column in portions of 50 and 75 ml. The column is allowed to drain between the addition of these portions of benzene. The benzene 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 separations with cellulose and acetylated cellulose are used to obtain maximum resolution among the polynuclear aromatics. The technique is essentially that used by White and Howard (1967) with some modification.

The cellulose and acetylated cellulose were prewashed with methanol in a Waring Blendor for 5 min, transferred to a Büchner funnel, washed twice with methanol, and air dried. This treatment removes interfering ultraviolet absorption from the adsorbents.

Plates 20 × 20 cm for cellulose and 20 × 10 cm for acetylated cellulose are prepared in 1-mm thickness and developed as follows. Cellulose, 26 g, and 170 ml of water are mixed in a Waring Blendor for 3 min and the plates prepared immediately from this mixture. After air drying, the plates are washed in the development tank with isooctane in a direction perpendicular to the direction of application of the adsorbent. When the solvent front has reached 0.5 cm of the top, the plate is removed and the top and bottom 1 cm of adsorbent is scraped from the plate. The plates are stored in a dessicator. Acetylated cellulose, 40 g, and 150 ml of ethyl alcohol are mixed in a Waring Blendor for 3 min. The plates are prepared immediately from this mixture and when air dry, stored in a desiccator.

The aromatic concentrate in about 100 μl of benzene is streaked on the cellulose plate with a 50-μl syringe. The sample is applied about 2 cm from the bottom and edges of the plate. The immobile phase, 20% *N,N*-dimethylformamide in diethyl ether, is applied by dipping the plate in the solution to within 0.5 cm of the applied sample. After air drying for about 15 sec, the plate is inverted and placed in a chromatographic tank and developed with isooctane. The acetylated cellulose plate is developed with ethanol–toluene–water, 17:4:4 (v/v/v).

The polynuclear aromatics are detected by their fluorescence and the bands outlined with a pencil. The cellulose bands are removed into separate 50-ml beakers, extracted three times with hot methanol, filtered separately using the pressure filter funnels (about 4 lb of nitrogen is applied to assist filtration), and collected in 50-ml erlenmeyer flasks. After adding 1 ml of *n*-hexadecane to each flask, the solvent is evaporated on a steam bath. Two successive additions of 10 ml of isooctane are added and evaporation repeated. Finally, the hexadecane solutions are ready for quantitization by ultraviolet spectrophotometry. The acetylated cellulose bands are processed the same as the cellulose, except the methanol extractions are done directly in the pressure filters with hot methanol.

Ultraviolet spectra were recorded with a Cary Model 17 recording spectrophotometer using optical cells of 1-cm path length having a capacity of 1 ml.

Notes on Method. The proportion of water, alcohol, and potassium hydroxide is critical for the solution of yeast, as is the temperature (50–85°), and length of time the yeast is heated. Excess foaming can also occur if yeast is added too rapidly. Overheating or heating longer than 2 hr will cause severe emulsions during extraction with isooctane. Unresolvable emulsions will also result during this extraction if the yeast solution is not extracted immediately while warm. Even in this case some emulsions may tend to form but may be easily broken by the addition of

Table I. Recovery of Polynuclear Aromatic Hydrocarbons Added to Yeast

Polynuclear hydrocarbon	Level added, ppb	Recovery, %		Av, %
		Dextrose grown	*n*-Hydrocarbon grown	
	Mixtures Added to Yeast			
Benz[*a*]anthracene	5	71, 75	80, 95	80
Benzo[*e*]pyrene	5	69, 60	61, 63	63
Benzo[*a*]pyrene	5	85, 90	61, 78	79
Benzo[*g*, *h*, *i*]perylene	5	67, 57	97	74
	Individual Compounds Added to Yeast			
Benzo[*a*]pyrene	3.3	88	88	88
Benzo[*a*]pyrene	250	84		84
Benz[*a*]anthracene	3.3	90	92	91
Dibenz[*a*, *h*]anthracene	3.3	100		100

ethanol, as noted. Although the addition of ethanol may cause a fine precipitation of white material in the aqueous phase, this is not harmful to the recovery of polynuclear aromatics.

The 10% water deactivated neutral alumina column was found to retain considerable quantities of colored and polar material while permitting polynuclear aromatics to elute with isooctane. The concentrated highly yellow isooctane extract must be filtered warm to prevent material from precipitating. The effluent isooctane is water white. This step was found more efficient in the removal of colored and other interfering material than the use of phosphoric acid, which Fazio *et al.* (1973) and Howard *et al.* (1966a) use in a method for polycyclic aromatics in foods.

The Florisil column is especially critical. It was found that improved recoveries of polynuclear aromatics are obtained when light is excluded from the column. This radiation apparently alters polynuclear aromatics adsorbed on Florisil. Further, the Florisil, even while stored in a tightly capped brown glass bottle, has been found to be unstable when stored over long periods. A batch of Florisil 1 year old, which when originally prepared yielded quantitative recovery of benzo[*a*]pyrene, yielded only 57.1% benzo[*a*]pyrene in the benzene eluent. This finding, and the fact that some recent preparations of Florisil failed to yield satisfactory recoveries of benzo[*a*]pyrene, prompted a study of Florisil. The results suggest that excessive loss of methanol causes the Florisil to become too active and thus to retain polynuclear aromatics, which may only be eluted by solvents of greater polarity. For example, additional recovery of 35.5% benzo[*a*]pyrene was obtained by elution of the aged Florisil with diethyl ether. Pyrolysis gas chromatography was used to determine the difference between the aged Florisil and a fresh batch which yielded 100.0% recovery of benzo[*a*]pyrene. The quantity of methanol in the fresh batch was found to be four times that in the old batch. This finding indicates that the loss of methanol causes poor yields of benzo[*a*]pyrene and leads to instability of the adsorbent on long storage.

RECOVERY STUDIES

Recovery studies were conducted on both *n*-hydrocarbon and dextrose grown yeast. The yeast fermentations were carried out under aseptic conditions, all organisms being excluded except the specific one inoculated into the system, and the air used in the process was filtered as described in the references. Essential nutrients were examined and found to be free of polynuclear aromatic contamination. Only highly purified *n*-hydrocarbons ranging between C_{14} and C_{18} were used as feed. This feed oil had an absorptivity of not more than 0.01 in the 260–350-nm range and so met the requirements of food additive regulation test CFR 121.1146 (*Fed. Regist.*, 1967) for white oils. The dextrose feed was also examined and found to contain no polynuclear aromatic compounds. Preliminary analysis for polynuclear aromatic hydrocarbon was conducted before recovery studies.

Solutions of benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*e*]pyrene, dibenz[*a,h*]anthracene, and benzo[*g,h,i*]perylene were prepared at concentrations of 1 μg/ml in isooctane and 1 ml of these solutions was added to either 200 or 300 g of yeast. The additions give individual compound concentrations of 5 and 3.3 ppb, respectively. In one case, 5 ml of a 10 μg/ml solution of benzo[*a*]pyrene in isooctane was added to 200 g of yeast to produce a concentration of 250 ppb of this compound.

Usually each of four compounds at concentrations of 1 μg/ml was added to yeast. However, in the *n*-hydrocarbon grown yeast, traces of some of these compounds were detected in yeast analyses in which polynuclear aromatics were not added. Suitable adjustments were made for compounds found in this yeast for determination of recovery studies.

RESULTS AND DISCUSSION

Table I summarizes the recoveries of polynuclear hydrocarbons for both dextrose and *n*-hydrocarbon grown yeast. At the level of 1 μg/ml added to 200 g of yeast (5 ppb) recoveries averaged 63 to 80% in the analysis of four component systems. The recovery of benzo[*a*]pyrene at the 3.3-ppb level was little changed from its recovery at the 250-ppb level, indicating that the advantage of higher concentration does not yield a significantly greater percentage recovery using the analytical method described. Recovery studies involving individual compounds were generally higher than those for multicomponent systems. This is probably due to separations involving tlc procedures. Although recovery studies shown were carried out at a minimum of 3.3 ppb, it would be possible to determine quantities much lower than this based on the intensity of the electronic spectra. Howard *et al.* (1966b) indicate that 0.5 ppb (0.05 'g recovered from 100 g of smoked foods) of this hydrocarbon is detectable by the procedure he described. The procedure presently described produces less background in the 360–400-nm range than the Howard *et al.* (1966b) method applied to yeast. Thus, detectability of benzo[*a*]pyrene at levels below 3.3 ppb is plausible.

A large number of yeast samples have been analyzed by the described method and a number of polynuclear aromatic hydrocarbons have been found. The identification of these compounds was made by comparing their ultraviolet spectra and R_f values with those of known compounds. A representative selection of yeasts analyzed, substrate indication, and analytical data for polycyclic aromatics are shown in Table II. These yeast samples were the product of other manufacturers (no.'s 10, 87, and 91) as well as trial run fermentations of our own produced

Table II. Polynuclear Aromatic Hydrocarbons Found in Yeast[a]

		ppb										
Yeast ident.	Substrate	Benzo-[g,h,i]-perylene	Benzo-[a]-pyrene	Benz-[a]anthracene	Di-benzo-[a,h]pyrene	Coronene	Di-benz-[a,h]-anthracene	Benzo-[e]-pyrene	Pyrene	Fluoranthene	Chrysene	4-Methylpyrene
10	Dextrose	ND[b]	ND	1	ND	ND	ND	ND	1	6	ND	ND
12	Dextrose	ND	ND	ND	ND	ND	ND	ND	0.4	2	ND	ND
21	Dextrose	ND	ND	ND	ND	ND	ND	ND	1.8	1.4	ND	ND
71	*n*-Hydrocarbon	ND	ND	ND	ND	0.9	ND	ND	1.5	3.3	ND	ND
73	*n*-Hydrocarbon	ND	1.1	0.8	ND	2.0	ND	1.0	1.4	2.5	1.0	ND
87	*n*-Hydrocarbon	ND	5.9	11.0	1.5	3.8	ND	2.2	12.0	1.0	4.4	1.5
91	*n*-Hydrocarbon	ND	2.3	0.5	ND	1.0	0.2	1.3	2.0	3.1	0.7	ND

[a] Values not corrected for recovery percentages. [b] ND, not detected.

during optimization of conditions. Fermentation conditions were the same as those for the recovery study, and inorganic nutrients were screened for polycyclic aromatics in the same manner as for that study. Hydrocarbon feed was checked by CFR 121.1146 test in the case of our own fermentations. Silica gel treated feed oil used for fermentation no. 71 measured less than 0.01 absorbance unit in the 260–350-nm region, whereas feed oil used for fermentation no. 73 measured not more than 0.25 absorbance unit over this region.

Pyrene and fluoranthene were found in all samples examined. However, as discussed by Howard *et al.* (1966a), about 35% of pyrene and fluoranthene will be lost if the isooctane eluent from the Florisil column is not examined. However, the "benzpyrene fraction" including benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*g,h,i*]perylene, and other compounds is quantitatively recovered from the benzene eluent from this column.

Dextrose grown yeast was not found to contain any highly condensed aromatics apart from pyrene and fluoranthene, whereas silica gel treated *n*-paraffin feed produced yeast containing these same materials in addition to a trace of coronene. Benzo[*g,h,i*]perylene was not found in any of the samples studied. However, whenever benzo[*a*]pyrene was found in a sample a considerable number of other carcinogenic polynuclear aromatics were also detected. Pyrene, fluoranthene, and coronene are not considered carcinogenic. It would seem reasonable that an analysis for benzo[*a*]pyrene alone would provide evidence for the presence or absence of harmful polynuclear aromatics pharmacologically significant.

The source of contamination by benzo[*a*]pyrene and other harmful polynuclear compounds may be due in part to the air and certain additives used in the process. For example, our analysis of an anti-foam food grade additive was shown to contain these materials. However, no reasonable explanation is available for the omnipresence of pyrene and fluoranthene in yeast samples.

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