

ACTIVITY-DIRECTED FRACTIONATION  
OF PETROLEUM SAMPLES

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## SUMMARY

A fractionation and bioassay scheme was developed that can be applied to oil samples to assess the potential biological hazards of the various components that remain after an oil spill. The fractionation procedure involves solvent partitioning between heptane and **acetonitrile**, gel permeation chromatography using Bio-Beads S-X8, and silica gel chromatography. The first two steps are effective in removing intractable components that otherwise interfere with bioassay studies. Two in vitro bioassay tests and an in vivo test were studied to assess toxicity and mutagenicity of oil fractions. These were (1) the Ames bacterial mutagenicity test, (2) a mammalian-cell toxicity test, and (3) a mysids toxicity test. All three tests can be run using no more than a total of 30 mg of material.

In the course of developing the fractionation and bioassay protocol, samples of fresh **Prudhoe** Bay crude oil, weathered **Prudhoe** Bay crude oil, and shale oil were fractionated and bioassayed. The bioassay data obtained indicated that the aromatic hydrocarbon fractions are the most toxic fractions and probably represent the greatest biological hazard of any fraction in an oil spill situation. Some of the aromatic hydrocarbon fractions had toxicities comparable to those of phenanthrene and **1-methylpyrene** used as reference materials. Some of the polar oil fractions were also shown to be toxic and slightly mutagenic. The shale oil fractions were often more toxic or mutagenic than the crude oil fractions. The weathered crude oil contained less **volatiles** and somewhat more polar components than the fresh crude oil. The fractionation and bioassay protocol that finally evolved from the study needs to be applied to the three oils to obtain more definitive information on the potential biological hazard of the polar fractions.

## INTRODUCTION

### A. General Nature and Scope of Study

Studies on the biological effects of petroleum and the associated chemical analyses have generally concentrated on the hydrocarbon components that can be readily analyzed by gas chromatography. The aromatic hydrocarbons have been of major interest because many of them, e.g. benzenes, **naphthalenes**, and **phenanthrenes**, are quite toxic while others, e.g. benzo(a)pyrene, are mutagenic and carcinogenic. Petroleum, however, is comprised of many other components including nonvolatile and polar components that can not be analyzed for by the usual **GC** procedures.

In a highly weathered crude **oil** major amounts of the hydrocarbons have been lost by dissolution, evaporation, microbiological degradation, or **photochemical** degradation. **Many** of the components that remain are not amenable to analysis by gas chromatography because they are too nonvolatile or too polar. Nevertheless, any program concerned with the long-term biological effects of oil spills ought to consider such components. Perhaps some of the nonvolatile or polar components are toxic or mutagenic and **should** be studied in monitoring programs. On the other hand such components may have no significant biological effects and should be ignored. However they should not be ignored until there is experimental evidence indicating that they are indeed inactive. They shouldn't be ignored simply because they are not detectable by the usual **analytical** methods.

This research program was undertaken as an effort to determine **whether** any of the nonvolatile and polar **nonhydrocarbon** components are toxic or mutagenic and thus represent a biological hazard in an oil **spill** situation. The program plan involved fractionation of oil into various hydrocarbon fractions and **nonhydrocarbon** fractions followed by **bioassays** to detect any toxicity or mutagenicity. Those fractions exhibiting activity would be **sub-fractionated** and the subfractions checked by **bioassays**. More extensive bioassay and chemical characterization studies would be directed to the active subfractions, hence, the term "activity-directed" fractionation. The program involved primarily a study of fresh and weathered Prudhoe Bay crude oil.

### B. Specific Objectives

1. Develop procedures for fractionating and isolating the **nonhydrocarbon** components of crude oil.
2. Determine the toxicity and mutagenicity of nonhydrocarbon oil fractions using bioassay screening procedures amenable to small amounts of sample.
3. Characterize the active **nonhydrocarbon** oil fractions.

### C. Relevance

The program involved the use of Prudhoe Bay crude oil, the oil most apt to be involved in any oil spill in the Alaskan environment. The weathering was performed in an Alaskan environment to simulate the weathering processes that would occur in an Alaskan oil **spill**. Since the possible biological activity of **nonhydrocarbon** components in oil has been largely ignored in the OCSEAP program, demonstration of significant toxicity or mutagenicity in **nonhydrocarbon** fractions associated with Alaskan-weathered Prudhoe Bay crude oil would need to be considered in the design of any continuing OCSEAP monitoring programs.

## EXPERIMENTAL APPROACH

The original design of the program was based on the concept of activity-directed fractionation. Oil was to be fractionated into a dozen fractions or so, bioassay tests would be run to determine which fractions were toxic or mutagenic, those fractions that showed activity would be **sub-fractionated**, bioassay tests would be run on the subfractions, and the process of **subfractionation** of active fractions followed by bioassay would be continued until **subfractionation** no longer increased the activity per unit weight. The inactive fractions and hydrocarbon fractions obtained would be ignored and the final active nonhydrocarbon fractions would be characterized chemically as well as for biological activity. An effort would be made to account for all of the oil, i.e. obtain a mass balance, so that no potentially **active** fractions would be overlooked.

Three major inherent **problems** seriously interfered with the success of the original program design. One problem involved volubility – the problem of how to get highly water-insoluble components of oil into an aqueous medium required for bioassay. A second problem was the partial volatility of the oil which made it difficult to obtain reliable residue weights and therefore prevented a good mass balance from being achieved. The third problem was the insensitivity of the bioassay screening methods, especially the Ames mutagenicity assay, which meant that in the initial fractionation even those fractions containing active components would be so diluted by inactive components that no activity would be detected. This meant that all of the fractions would appear inactive and no direction would be given to the subfractionation.

### Volubility Problem

The volubility problem was not an unexpected problem but one which had to be **dealt** with somehow. The problem had three facets. A medium was needed that would (1) dissolve or disperse the oil fraction (2) keep the oil fraction dispersed in the aqueous bioassay media, and (3) be nontoxic to the bioassay organisms.

Organic solvents such as tetrahydrofuran, benzene, pyridine, and **methylene chloride** were found to be good solvents for the oil fractions and were sufficiently water soluble but were too toxic. **Dimethyl sulfoxide** and acetone were not toxic and therefore were satisfactory for the bioassay but would not dissolve many of the oil fractions. Numerous other solvents, mainly alcohols and ketones, were investigated and the best ones found were **cyclohexanone** and **cyclohexanol**. These two were good oil solvents, sufficiently water-soluble, and less toxic than tetrahydrofuran. However, they were **still** too toxic and although the oil fractions dissolved in the solvents as soon as the solutions were mixed with aqueous bioassay media the oil fractions separated out.

Several **dispersants** were studied in an effort to keep the oil fractions dispersed in the bioassay media. The dispersants were selected for low toxicity and included **glyceryl monooleate**, lecithin, nonylphenoxypolyethoxyethanol, and **Corexit**. None was effective in the in vitro bioassays. In addition to being ineffective, the **dispersants** interfered with the mammalian cell toxicity assay by causing the cells to become detached from the assay plate.

Because of the problem of at least partial insolubility of some of the oil fractions and the inability to disperse them completely, the plan of attempting to account for the potential biological activity of all fractions was changed to a plan for studying only those fractions that would dissolve in **dimethyl sulfoxide (DMSO)**. This change was justified on the basis that practically all known biologically-active organic compounds are at least slightly soluble in **DMSO**, or, from a more fundamental standpoint, if a component does not dissolve at least slightly in **DMSO** it is unlikely that it can pass into a cellular system to exert a biological effect.

### Volatility Problem

The partial volatility of crude oil affects the accuracy of residue weights and therefore interferes with efforts to achieve a mass balance. In evaporating oil solutions, the oily residue acts as a keeper for the solvent as well as a keeper for the more volatile oil components. The ideal case would be one in which the amount of solvent remaining after evaporation were equal to the amount of volatile oil components lost so that the residue weight observed would fortuitously be equal to what might be called the true residue weight. However as the viscosity and volatility of the oil components vary from fraction to fraction the errors in residue weight determinations will vary considerably. In most cases the residue weight observed was less than the expected true residue weight.

In an effort to decrease the amount of volatile oil components lost during residue weight determinations and thereby improve the mass balance, some of the more volatile components were removed by reduced pressure distillation. The percent of total oil removed in this manner was quite arbitrary and depended upon the distillation conditions. This approach helped considerably in obtaining residue weights that were **closer** to the expected **values** but even so they could only be considered as approximations. In most of the fractionation steps prior removal of **volatiles** was not used and recoveries based on residue weights were frequently about 85 percent. The 15% discrepancy could represent material that was not recovered or could simply represent errors in the residue weight determinations.

### Insensitivity of Ames Test

The problem of the insensitivity of the Ames test can be evaluated on the basis of its theoretical ability to detect **benz(a)pyrene (BaP)** in crude oil. The detection **limit** for **BaP** in the Ames test is about **1  $\mu$ g**. The amount of **BaP** that can be expected in crude oil is about **1  $\mu$ g/g**. If **BaP** were the only mutagen present in the crude oil, all of the **BaP** in one gram of oil would need to be added to an Ames test plate in order for any mutagenicity to be detected. However, the maximum amount of sample that can be accommodated by an Ames plate is about **1 mg** (0.1 ml of a 1% solution in **DMSO**). This means that the **1  $\mu$ g** of **BaP** in 1 g of oil has to be concentrated by a factor of 1000 before it can be detected by the Ames test. If **all** fractions contained equal amounts of material, the crude oil would have to be fractionated into at least 1000 fractions and all of the **BaP** would have to be in just one of those fractions before any mutagenicity would be detected. These quantitative considerations are summarized in Table 1.

TABLE 1. QUANTITATIVE CONSIDERATIONS OF THE AMES TEST

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PREMISE 1:	<b>BaP</b> Concentration In Crude Oil = 1 $\mu\text{g/g}$
PREMISE 2:	<b>BaP</b> Is Only Mutagen In Crude Oil
PREMISE 3:	Limit Of Detection of <b>BaP</b> In Ames Test = 1 $\mu\text{g}$
CONCLUSION 1:	All Of The <b>BaP</b> In 1 g Of Crude Oil Must Be Added To Ames Test To Be Detectable
PREMISE 4:	Maximum Amount Of Sample Accommodated By Ames Test = 100 $\mu\text{l}$ Of 1% Solution = 1 mg
CONCLUSION 2:	Crude Oil Must Be Fractionated In A Manner That Gives A 1000-Fold Concentration Factor for <b>BaP</b>
PREMISE 5:	All Fractions Contain <u>Equal Amounts</u> Of Material
CONCLUSION 3:	Crude Oil Must Be Fractionated Into At Least <u>1000 Fractions</u> And All Of The <b>BaP</b> Must Be In One Of Those Fractions Before Any Mutagenicity Will Be Detected

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The above considerations demonstrated that the concept of initially fractionating the oil into only a dozen or so fractions and using bioassay **activity** to direct subsequent **subfractionations** could not be applied successfully. Therefore, the approach was changed to provide for a number of fractionation before applying bioassay tests. Most of the final fractions contained only 1-2 mg of residual material per gram of oil. Much of the total oil was discarded in **DMSO-insoluble** fractions, high-molecular-weight fractions, or hydrocarbon fractions. A few fractions contained 5-25 mg of residual material per gram of oil. Because of the fractions discarded and the few fractions containing relatively large amounts of material, the total number of fractions obtained was only about 45 instead of 1000.

A **problem** associated with the insensitivity of the bioassay tests was that of scaling up fractionation procedures to provide enough of a fraction for replicate bioassays under different conditions. The scaled up procedures used permitted 10 g of oil to be processed. Fractions which contained only 10-20 mg of material could only be assayed once. More attention was therefore given to fractions containing 50-250 mg of total residual material.

#### Fractionation Scheme

Several different fractionation schemes were investigated during the course of the program. Initial fractionation schemes involved vacuum-stripping of **volatiles** as discussed above, separation of pentane-insolubles (**asphaltenes**), and silica gel chromatography of the pentane solubles. Subsequent **subfractionation** was to be achieved by gel permeation chromatography and HPLC using

reverse-phase systems. The **eluting** solvents used for successive **elutions** in the silica gel chromatography were petroleum ether, 20 percent methylene chloride in petroleum ether, **methylene** chloride, and **acetonitrile**. This **elution** scheme suffered from the fact that **acetonitrile**, like DMSO, is not a very good solvent for many of the oil fractions and thus retarded rather than accelerated the **elution** of components from silica gel.

Tetrahydrofuran (**THF**), a highly polar solvent and an excellent solvent for all oil fractions, was substituted for acetonitrile. Ideally, the fractions **eluted** with THF could be concentrated and assayed directly. However, as described above, THF was too toxic to the assay organisms. It was also found that concentration of fractions and blanks to an oil for solvent exchange resulted in relatively large amounts of residual material that could be attributed to the THF. This material contained peroxides and peroxide decomposition products. Its presence could be avoided by the use of THF containing an antioxidant, usually a **phenolic** compound, or by redistillation immediately before use. The addition of an antioxidant to the system was not at all desirable. Redistillation would help; however the **chromatographic** process took several days and significant peroxide formation can occur during that length of time. Because of these various problems, THF was discontinued as an **eluting** solvent.

The fractionation scheme that **finally** evolved from the program is summarized in Figure 1. The scheme involved solvent partitioning to remove the bulk of the nonpolar material that is not soluble in **DMSO**, gel permeation chromatography to remove polymeric material, and silica gel chromatography to fractionate on the basis of polarity.

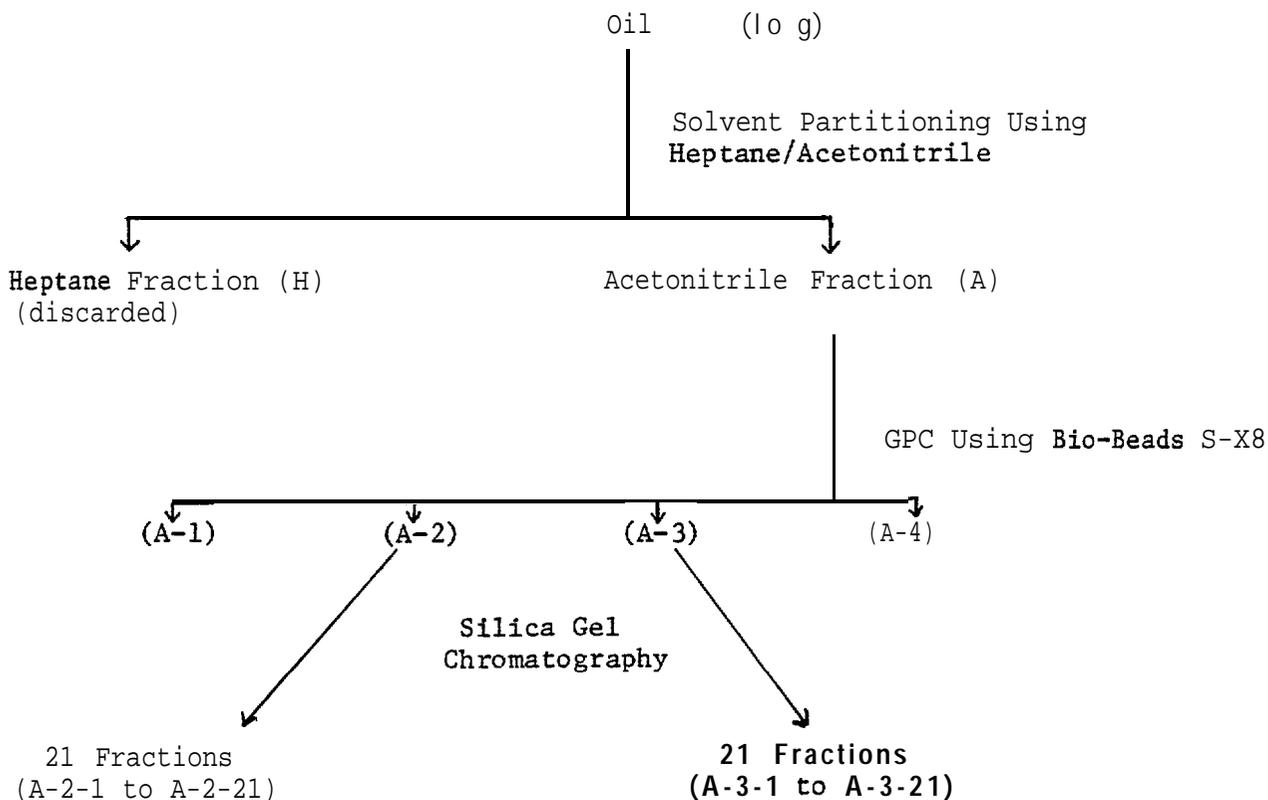


FIGURE 1. OIL FRACTIONATION SCHEME

**Heptane/acetonitrile** partitioning was found to simulate heptane/DMSO partitioning very well and had the added advantage of giving an extract (**acetonitrile**) that could be readily concentrated directly. The partitioning step removed about 75% of the oil as **acetonitrile-** insoluble material and thus reduced considerably the amount of material to be processed by the next step. Of even greater importance, the partitioning removed **most** of the **DMSO-insoluble** material that could not be accommodated by the bioassays.

The gel permeation chromatography (GPC), also referred to as size exclusion chromatography, 'separates components 'primarily on the basis of molecular size. Sephadex **LH-20**, a modified dextran material, was tried initially for this step with various solvent systems, e.g. **isopropanol** and **methylene chloride**, but so much irreversible adsorption resulted that the column could not be reused. **Bio-Beads S-X8**, a **styrene-divinylbenzene** copolymer, worked very well with **methylene chloride** as the **eluting** solvent. The oil extracts were fractionated into four fractions. The first fraction contained the long-chain and polymeric material which accounted for most of the dark color. The last fraction contained small compact **polynuclear** compounds. The bulk of the material was in the two center fractions which accounts for the molecular-size range of most biologically-active organic compounds.

The final fractionation step, adsorption chromatography, used silica gel that was partially deactivated by 10% methanol in ethylene dichloride. This solvent in addition to deactivating the column served as a highly polar wash solvent for removing contaminants from the silica gel. Prior to use the column was partially reactivated by stepwise **elution** with ethylene dichloride followed by hexane. The **elution** scheme used is shown in Table 2.

TABLE 2. ELUTION SCHEME FOR SILICA GEL FRACTIONATION

Fraction No.	Eluting Solvent
<b>1-7</b>	Hexane
8-12	10% Ethylene Dichloride in Hexane
13-16	Ethylene Dichloride
17-21	10% Methanol in Ethylene Dichloride

25 mm ID x 1200 mm upward flow column  
 350 g silica gel, Davison Grade 923  
 10 **ml/min elution** rate  
 250 ml fractions

The column could be regenerated and used a number of times for fractions that had been cleaned up by solvent partitioning and GPC. The **elution** solvents used in the scheme are stable materials that can be readily obtained in high purity and readily concentrated.

The above fractionation scheme was applied to both fresh and weathered Prudhoe Bay crude oil. Shale oil\* from a simulated in situ process was generously supplied by Dr. Richard Poulson of the **Laramie** Energy Technology Center and was

\* The designation by LETC was ES-77-209, Run 16, dry.

fractionated for comparison. The major fractions obtained were *evaluated* by bioassay tests, namely the Ames mutagenicity test, a mammalian cell toxicity test, and/or a mysids toxicity test.

## METHODS

### Weathering

The weathered crude oil used in the **program** was prepared by Dr. Stanley Rice of Auke Bay Fisheries Laboratories in Auke Bay, Alaska. A 9.5-liter sample of Prudhoe Bay crude oil was poured into a 1.2 m x 1.2 m pine board open retainer moored in Auke Bay from July 12, 1976, to September 14, 1976. The exposure permitted weathering by evaporation, dissolution, **photo-**chemical oxidation, and microbiological degradation and thus simulated oil spill conditions in an Alaskan environment.

The depth of the oil slick was initially 6 mm. A 1-liter sample was taken on August 16 and used for **all** fractionation studies involving weathered oil. Small samples, 100 ml each, were also collected on August 2, August 13, August 31, and September 14. All samples were stored at **-20°C**.

### Pentane Volubility

Twelve grams of crude oil was mixed with 600 ml of **n-pentane** and the resulting suspension was centrifuged at 1500 rpm. The supernatant, pentane soluble fraction, was saved and the precipitate was resuspended in 50 ml of **n-pentane** and centrifuged. The supernatant from this wash was combined with the previous supernatant. The precipitate, pentane insoluble, was dried in a vacuum desiccator and weighed. Fresh crude oil gave 3.0% pentane insolubles and the weathered oil gave 4.6% pentane insoluble.

### Vacuum Stripping

#### Rotating Evaporator Procedure

Twenty grams of fresh crude oil was placed in a 100-ml round-bottom flask and attached to a rotating evaporator that was fitted with a receiving trap cooled in a Dry Ice-acetone bath. Stripping of **volatiles** was achieved by heating the rotating flask in a water bath at 90°C for a 10-hour period while applying a reduced pressure of about 25 mm using a water aspirator. The **volatiles** collected amounted to 17% of the starting oil. GC analysis of the **volatiles** and the residual oil indicated that most of the components with boiling points up to the **C<sub>3</sub>-benzenes** had been stripped off but most of the **naphthalene** and **alkylnaphthalenes** remained in the residue oil.

#### Distillation Column Procedure

Fifty grams of fresh crude oil was placed in a 100-ml distillation flask fitted with a thermometer, a magnetic stirrer, and a 150 mm x 9 mm O.D. Vigreux column having a distilling head that led to a receiving trap cooled in a Dry Ice-acetone bath. The flask was heated with a heating mantle and reduced pressure was applied using a **vacuum** pump. Distillation was achieved at 0.06 mm by heating to give a pot temperature of up to **150°C**. The head

temperature rose to 100° C under these conditions and then dropped when no more distillate was obtained. The distillate collected amounted to 32% of the starting oil. GC analysis indicated that although the majority of the methylnaphthalenes appeared in the distillate, a significant amount still remained in the residual oil.

#### Acid Extraction

One hundred grams of fresh crude oil in 400 ml of hexane was stirred with 500 ml of 2N H<sub>2</sub>SO<sub>4</sub> for 20 hours. The mixture was then transferred to a separator funnel and allowed to settle for 3 hours. The aqueous layer was withdrawn, washed with 100 ml of hexane, neutralized to pH 7.2 with 4N NaOH and back extracted three times with 200-ml portions of methylene chloride. The combined methylene chloride extracts were dried with anhydrous sodium sulfate and concentrated to 5 ml on a rotating evaporator. Residue weights obtained on 100- $\mu$ l aliquots of the concentrated extract indicated that the total acid extractable components amounted to about 0.04% of the starting oil.

#### Solvent Partitioning

Ten grams of oil was placed in a 1.8 l glass jug with 100 ml of heptane and 500 ml acetonitrile. The heptane was equilibrated with acetonitrile and the acetonitrile equilibrated with heptane prior to use. The mixture was shaken vigorously on a mechanical for one hour and transferred to a 1-liter separator funnel with a Teflon stopcock for separation of the phases. The acetonitrile layer was withdrawn and the heptane layer was reequilibrated with another 500 ml. of acetonitrile. This was repeated three more times to give five successive acetonitrile extracts. Each extract was concentrated to an oil using a rotating evaporator and taken up in 10 ml of methylene chloride. A 0.05 ml aliquot of each methylene chloride solution was evaporated to determine the residue weight. The solutions were then combined and concentrated to 5-10 ml for subsequent GPC fractionation.

Partitioning using other solvents was performed in a similar manner.

#### Solvent Exchange

Solvent exchange with a higher-boiling volatile solvent was accomplished using a vortex evaporator by simply concentrating to a small volume, adding a 5-fold volume of the higher boiling solvent and reconcentrating to a small volume. This work well, for example, for replacing ethylene dichloride with heptane prior to silica gel fractionation.

Solvent exchange of a volatile solvent with a nonvolatile solvent was not very complete because the nonvolatile solvent did not distil to permit removal of the last traces of the volatile solvent by codistillation. However, the complete removal of the relatively toxic volatile solvent, ethylene dichloride, from solutions in a nonvolatile solvent, DMSO, was very important in preparing solutions for bioassay. To avoid the presence of ethylene dichloride in DMSO solutions, the ethylene dichloride was exchanged with heptane, a much less toxic solvent, prior to the addition of DMSO. The heptane was then stripped from the DMSO on a vortex evaporator at 60°C with the full vacuum of an aspirator. GC analysis showed that the resulting DMSO solutions contained less than 10 ppm of ethylene dichloride.

### GPC Fractionation

Gel permeation chromatography (GPC) was used to fractionate oil primarily on the basis of molecular size. The GPC column was prepared by packing a 750 mm x 25 mm I.D. upward flow chromatography column with 150g of Bio-Beads s-x8 that was **preswelled** in **methylene chloride**. The inlet end (bottom) of the column was connected to a Rheodyne Model 7105 injector valve with a 1-ml sample loop and to an Altex Model 110 pump that maintained the solvent flow at 2 ml/min. The column inlet pressure was 15 psi at that flow rate. The void volume of the column was 116 ml. The efficiency of the column was shown to be about 2500 theoretical plates by injecting 0.15 mg of **di-n-octyl phthalate** and monitoring with a UV detector at 254 nm.

Up to 500 mg of oil in 1 ml of ethylene dichloride solution was fractionated in a single run. All components were **eluted** within 175 minutes (350 ml). In most cases four fractions were collected, 0-70 rein, 70-110 rein, 110-130 rein, and 130-175 min. Most of the color eluted in the first fraction. The column was used repeatedly and no significant change in column efficiency or retention time for **di-n-octyl phthalate** was observed.

### Silica Gel Fractionation

A 1.2 m x 25 mm I.D. chromatography column set up for upward flow operation was slurry-packed with 350g of Davison Grade 923 silica gel that was activated by heating at 150°C overnight and deactivated by shaking with 1000 ml of 1:9 **methanol:ethylene dichloride**. The column was fitted with a Rheodyne Model 7105 injection valve that had a 5-ml sample loop. A constant solvent flow rate was achieved by using an Altex Model 110 pump.

The column was rinsed and equilibrated with 1:9 methanol:ethylene dichloride at a flow rate of 1 ml/min for 20 hours. It was then equilibrated with ethylene dichloride for two hours at 5 ml/min, with 1:9 ethylene dichloride:hexane for two hours at 5 ml/min, and finally with hexane for 20 hours at 1 ml/min.

The **sample** to be **fractionated**, up to 1.4 g, was dissolved in heptane and centrifuged to remove any insoluble components. The heptane solution was injected onto the column in two 5-ml injections with approximately one minute between injections. The injector loop outlet flow and sample container rinse combined, approximately 4 ml, was injected as a third injection. The heptane insoluble were dissolved in ethylene dichloride and injected later when **elution** with ethylene dichloride was begun.

The column was **eluted** at a flow rate of 10 ml/min with 1400 ml of **hexane**, followed by 1400 ml of 1:9 ethylene dichloride :**hexane**, followed by 900 ml of ethylene dichloride, and finally with 1400 ml of 1:9 methanol:ethylene dichloride. Fractions were collected every 25 minutes (250 ml each).

Each fraction was concentrated to an **oil** in a rotating evaporator at 30°C and taken up in several portions of **methylene chloride** to a volume of 10 ml. A 50- $\mu$ l **aliquot** of each fraction was used for residue weight determinations.

## Salmonella Mutagenicity Assay

Mutagenicity was determined by the Ames test in which the sample was incubated with a **histidine-dependent** strain of Salmonella bacteria in a **histidine-deficient** culture medium. Mammalian (rat) liver **microsomes** containing **hydroxylase** systems were added in an effort to convert certain inactive compounds to active mutagens. Benz(a)pyrene is an example of a known carcinogen that requires such **microsomal** activation in order for it to exert a mutagenic affect. The Ames test can be run with an without **microsomal** activation in order to differentiate between compounds that are mutagenic as such and those that require activation. However compounds which are mutagenic in the absence of **microsomes** are generally also active in the presence of **microsomes** and therefore, in using the test as a screening procedure, **microsomes** were added to **all** of the plates in this program.

Solvent control plates treated with solvent alone and positive control plates treated with known carcinogens, e.g. **benz(a)pyrene** and 2-aminoanthracene, were used to provide reference data against which activity of test substances could be compared. Sterility control checks were also run for each test sample using nutrient agar.

Whenever a compound or fraction is toxic to the bacteria and kills all of the organisms at the **level** being tested it will not give any mutagenic activity even though it might be mutagenic at a lower dosage level. Therefore, when using the Ames test as a screening procedure at one dosage level, it is necessary to assess the toxicity of the sample in order to avoid false negatives. Salmonella toxicity tests were run on **all** samples screened on the program.

Three different Salmonella typhimurium tester strains were used for the program, TA-98, TA-100, and TA-1537. These are all strains that have been developed for their ability to be reverted back to the histidine-producing wild type by particular mutagens, however they differ in their sensitivity and specificity to different mutagens.

### Liver Microsome Preparations

The activation system for the mutagenesis screening consisted of **Arochlor** 1254-induced microsomes derived from rat livers. Induction was accomplished by a single interperitoneal injection of **Arochlor** (200 mg/ml of corn oil) into adult male rats weighing about 200 g each, at a dosage of 0.5 mg/g of body weight, 5 days before sacrifice. The rats were deprived of food 24 hours before sacrifice. They were stunned by a blow on the head and decapitated,

The livers were aseptically removed from the rats and placed into a cold preweighed beaker containing 10 ml of 0.15M KCl. After the livers were swirled in this beaker they were removed with forceps to a second beaker containing 3 ml of the KCl solution per gram of wet liver weight. The livers were then minced with sterile scissors, transferred to a chilled glass homogenizing tube and homogenized by passing a low-speed motor driven pestle through the livers a maximum of four times. The homogenates were then placed in cold centrifuge tubes and centrifuged for 10 minutes at 9,000 G at 4°C. The

resulting supernatant **microsomes** were decanted, **aliquoted** in 3 ml amounts to **small** culture tubes, quickly frozen in Dry Ice, and stored at -80°C in a **Revco** freezer. Sufficient **microsomes** for use each day were thawed at room temperature and kept on ice before and during use.

### Microsomal Mix

The **microsomal** mix was prepared according to the recommendations of Ames by mixing 2 ml of **microsomes**, 0.4 ml of 0.4 **M** **MgCl<sub>2</sub>**, 0.4 ml of 1.65 **M** **KCl**, 0.1 ml of 1 **M** glucose-6-phosphate, 0.8 ml of 0.1 **M** **NADP**, 10 ml of 0.2 **M** sodium phosphate buffer (pH 7.4), and sterile distilled water to bring the total volume to 20 ml. Stock solutions of **NADP** (0.1 M) and **glucose-6-phosphate** were prepared with sterile water, **aliquoted** in appropriate amounts, and maintained in a **Revco** freezer. The stock salt solutions were prepared, autoclave, and refrigerated. The **microsomal** mix was prepared fresh daily and maintained on ice before and during use.

### Bacteria

The Salmonella tester strains, TA-98, TA-100, and TA-1537, were obtained directly from Ames and stock solutions of the strains were stored at -80°C. At monthly intervals, new bacterial isolates were obtained from this stock supply. Each clonal culture was checked for confirmation of biochemical activity and spontaneous reversion rate. The cultures which conformed to the specifications of Ames were streak isolated and used as master cultures. These master cultures were used as the origin of weekly preparations of working broth cultures. All broth cultures were nutrient broth (**Difco**) supplemented with 0.5 percent **NaCl**. The broth cultures were prepared by inoculating 0.1 ml of master culture into 10 ml of nutrient broth and incubating the culture in a water bath shaker for 16-20 hours. This gave a stock culture containing approximately 10<sup>9</sup> cells per ml. The incubation was performed the night before the assay and the cultures were kept on ice during the process of preparing the assay plates.

### Bacteriological Media

The selective basal medium used in the mutagenicity assays was a 1.5 percent **Bacto-Difco** agar in **Vogel-Bonner** Medium E with 2 percent glucose. The basal medium used in the toxicity assays was nutrient **agar**.

The top agar for both mutagenicity and toxicity assays was 0.6 percent **Difco** agar in 0.5 percent **NaCl**. It was prepared in 100 ml **aliquots**, autoclave, and stored at room temperature. Before use the top agar was melted and mixed thoroughly with 10 ml of a sterile solution of 0.5 **mM** **L-histidine·HCl** and 0.5 **mM** **biotin**. It was then **aliquoted** in 2-ml amounts in sterile culture tubes and maintained at 45°C in a water bath before use.

### Plate Test Procedure

The toxicity determinations were made in duplicate for each sample using each of the three tester strains. The determinations were made as follows. The stock culture of each tester organism was diluted in physiological **saline** to give approximately 300 cells per ml. The molten top agar tubes were treated with 0.1 ml of test material, 0.1 ml of the diluted tester strain, and 0.2 ml

of the **microsomal** mix. It **was** immediately mixed for a few seconds by **vortexing** and poured onto a nutrient agar plate. After solidification of the top agar the plates were incubated at 37°C for 72 hours and the resulting colonies counted.

The plate test procedure for mutagenicity determination was the same as that for toxicity determination except that the stock culture containing approximately 10<sup>9</sup> cells per ml was used instead of the diluted culture and histidine-deficient **Vogel-Bonner** glucose agar was used instead of nutrient agar for the basal medium.

With each set of toxicity and mutagenicity determinations solvent controls were run in which pure solvent was used instead of the test material solution, positive controls (mutagenicity determination only) were run in which solutions containing 10 µg/ml of **benz(a)pyrene** and 50 µg/ml of 2-aminoanthracene were used instead of the test material solution, and sterility checks were run in which no tester organisms were added. In order for a set of tests to be considered valid the following criteria had to be met: (1) the sterility checks of the test materials must give no more than two colonies per plate, (2) the solvent controls in the toxicity determination must give 100 to 300 colonies per plate, (3) the positive controls must give at least a three-fold increase in the number of revertant colonies over the average value of the respective negative control (solvent control), (4) the average number of spontaneous revertant colonies (solvent control) must be 15 to 60 for TA-98, 70 to 250 for TA-100, and 5 to 20 for **TA-1537**. In order for a test material to be considered mutagenic it had to give at least twice the number of revertant colonies as the respective negative control. Test materials that exhibited toxicity were assayed again at lower levels whenever possible.

#### Mammalian Cell Toxicity Assay

An indication of the mammalian **cell** toxicity of test fractions was determined by using a prescreen confluence assay. This is a single plate cell culture assay that permits a rapid determination of the toxicity of test materials in wide ranges of concentrations.

#### Procedure

**Subconfluent** monolayer of cells (embryonic **fibroblasts** obtained from Dr. Charles **Heidelberger** of the University of Southern California) were established in the individual wells of Falcon No. 3008 **multiwell** tissue culture plate by seeding 5 x 10<sup>3</sup> cells in each well and allowing them to grow for 24 hours at 37°C in a humidified 5% CO<sub>2</sub> incubator. The growth medium was Eagle's basal medium with Earl's salt containing 10% heat inactivated **fetal** calf serum with no antibiotics. At the end of the incubation period the growth medium was removed from all of the wells by aspiration. Fresh growth medium containing a desired known concentration of the test material was replaced on the cells. Three of the four wells in each of the six rows of the plates were used for triplicate samples of the test material. The last well in each row was used as a control. Thus each plate was used for six test samples or dosages in triplicate and provided six control replicates. Usually at least three dose levels were run for each fraction evaluated.

The plates were incubated again for 24 hours after which the growth medium was removed, the cells washed with phosphate buffered saline, treated with fresh growth medium, and reincubated. When all the monolayer in the control wells reached 90-100 percent confluence (generally 6 days), all of the wells in the plates were washed, fixed with methanol and the cells stained with Giemsa. After the plates were dry they were scored on a basis of percentage of the surface area covered by the cell monolayer.

### Evaluation of Results

The percentage of surface area coverage (confluence) was averaged for each set of three wells treated with a specific concentration of test material and for the six control wells. The average percent confluence in the test wells was adjusted slightly by dividing by the average percent confluence for the control wells times 100. The dose level of test material that would have given 50 percent confluence, CD<sub>50</sub>, was determined by extrapolation of the adjusted percent confluences found for various levels of the test material. A comparison of CD<sub>50</sub> values provide meaningful information on the relative toxicities of different test materials in the assay system.

### In Vivo Toxicity Assays

Static bioassays were conducted using brine shrimp (Artemia salina L.) and mysids (Neomysis awatschensis). Brine shrimp were obtained by hatching them in the laboratory. Mysids were collected by trawl from Sequim Bay, Washington. After initial-hatching the brine shrimp were maintained in two 5-gallon aquaria at room temperature with gentle aeration. The algae Monochrysis was used as a food source. Adult animals approximately 5mm in length were selected for use in the assay. The mysids were maintained in large flow-through tanks. They were acclimatized for at least one day prior to testing and fed a diet of Artemia nauplii.

All bioassays ran for 48 hours. Twenty animals were placed in each 6-liter static aquaria containing four liters of filtered seawater with gentle aeration. Ambient temperature (10°C) was maintained by water bath and the salinity and temperature of each aquarium was monitored daily.

Brine shrimp were transferred from culture tank to test aquaria using a fine mesh net. This allowed for slow draining of algal culture water with a minimum of animal stress. Mysids were captured by the use of hollow glass tubes. These tubes utilized suction to draw the animal and seawater into their chambers without direct handling. Mysids were placed in an intermediate beaker in groups of twenty and fed for one hour just prior to testing. The seawater, and remaining nauplii were drained and the beaker contents submerged in the bioassay aquarium.

The test material solutions were added via a syringe to the seawater at levels of 1 to 1000 µl/l. In the initial tests the solutions were added to 250 ml of seawater in a stainless steel Waring blender, blended for 30 seconds and stirred into the bioassay aquaria containing the animals and 3250 ml of seawater. The blender was rinsed twice by blending two 250-ml portions of seawater which were added to the aquaria to give a total volume of 4 liters. In later studies the test material solutions were added to a volumetric flask

containing two liters of seawater and 2 l of Corexit dispersant. This was mixed by inversion 15 times and stirred into aquaria containing the animals and another two liters of seawater.

The number of live animals remaining **after** 24 hours and 48 hours was recorded and the corresponding **LC<sub>50</sub>** values determined by extrapolation.

## RESULTS

### Fractionation Studies

#### Solvent Partitioning

Solvent partitioning in **heptane/acetonitrile** was used to simulate **heptane/DMSO** partitioning. In order to assess the performance of **acetonitrile** relative to that of DMSO and other polar solvents in such a system, a series of reference compounds ranging from very nonpolar compounds to moderately polar compounds was used in equilibration studies. Gas **chromatographic** analysis was used for the quantitation. The results, given in Table 3, show that **acetonitrile** does perform similarly to DMSO. In these studies in which the heptane was partitioned with an equal volume of **acetonitrile**, moderately **polar** compounds such as **acetylnaphthalene** and **quinoline** are almost quantitatively partitioned into the **acetonitrile** in one equilibration. For the partitioning step used in fractionation scheme applied to oil a 5:1 ratio of **acetonitrile** to heptane was used instead of 1:1. Also the partitioning was performed five times with fresh solvent. Any compounds that would remain in the heptane under such conditions would very probably not be biologically active and could be justifiably discarded.

TABLE 3. SOLVENT PARTITIONING STUDIES

Compound	% Extracted Into Given Solvent When Partitioned With Equal Volume of <b>Heptane</b>			
	<b>Acetonitrile</b>	DMSO	Nitromethane	Methanol
<b>C<sub>16</sub></b>	2	2	2	2
<b>C<sub>26</sub></b>	2	2	2	2
<b>Androstane</b>	2	2	2	2
<b>Hexaethylbenzene</b>	17	16	20	20
2,3,6-Trimethylnaphthalene	43	48	48	49
<b>Naphthalene</b>	61	71	62	66
Phenanthrene	71	90	69	73
Pyrene	71	92	66	72
Dibenzothiophene	68	91	69	74
Dibenzofuran	67	82	69	72
<b>Acetylnaphthalene</b>	94	98	96	98
<b>Quinoline</b>	98	>98	>98	98

The amounts of oil extracted by each individual partitioning using 5:1 acetonitrile:heptane are shown in Figure 2. The first partitioning accounted for the major amount of the extractable material. The **total** amount extracted into the **acetonitrile** was about 23 percent of fresh and weathered Prudhoe Bay crude oil and about 45 percent of the shale oil.

### GPC Fractionation

The second step in the fractionation scheme was GPC fractionation using Bio-Beads S-X8 with **methylene** chloride as the **eluting** solvent. A representative **elution** profile was obtained using a small amount of an **acetonitrile** extract of weathered oil. The amount present in each five-minute (10-ml) fraction was determined by residue weight measurements. The **elution** profile obtained is shown in Figure 3. For comparison the **elution** ranges found for various reference compounds are indicated. Long-chain compounds **eluted** early and the compact aromatic compounds **eluted** considerably later. Polar aromatics **eluted** earlier than less-polar aromatics.

The **acetonitrile** extracts of 10-gram samples of the three oils were **fractioned** into four fractions designated as A-1 through A-4 in Figure 3. The **elution** profiles obtained for the oils in this manner are shown in Figure 4. The shale oil **eluted** somewhat earlier than the crude oils possibly because of a higher content of **heterocyclic** more polar components.

The effectiveness of the GPC fractionation system is indicated by the gas **chromatograms** of weathered oil fractions shown in Figures 5 and 6. The main individual peaks in A-2 (Figure 5) are trace amounts of normal paraffins; most of the material does not **elute** from the GC column. Fraction A-3 (Figure 6) on the other hand gives the usual pattern of aromatic hydrocarbons, the methylnaphthalenes (MN), dimethylnaphthalenes (DMN), and trimethylnaphthalenes (TMN), etc. No significant overlap of the two fractions is noted.

### Silica Gel Fractionation

Silica gel fractionation was applied to the A-2 and A-3 fractions from each of the three oil samples. The **elution** profiles obtained are given in Figure 7. The largest amounts of material were in the first six fractions, the hydrocarbon fractions. However at about fractions 9, 13, and 18 when **elution** with progressively more polar solvents was begun, significant amounts of more polar components were obtained, especially in the larger-molecule or more polar A-2 fractions. The shale oil and to a lesser extent the weathered oil had more polar material than the fresh oil.

The efficiency of the silica gel fractionation, which used silica gel that had been previously deactivated with 10% methanol in ethylene dichloride, is indicated by the gas chromatograms of hydrocarbon fractions shown in Figures 8 and 9. Fraction 4 from A-3 from fresh crude oil (Figure 8) gives a GC pattern indicative primarily of naphthalene (N), methylnaphthalenes (MN), dimethylnaphthalenes (DMN), and trimethylnaphthalenes (TMN). The next fraction, fraction 5 (Figure 9) contains very few naphthalenes but mainly the phenanthrenes, fluorenes, pyrenes, and fluoranthenes.

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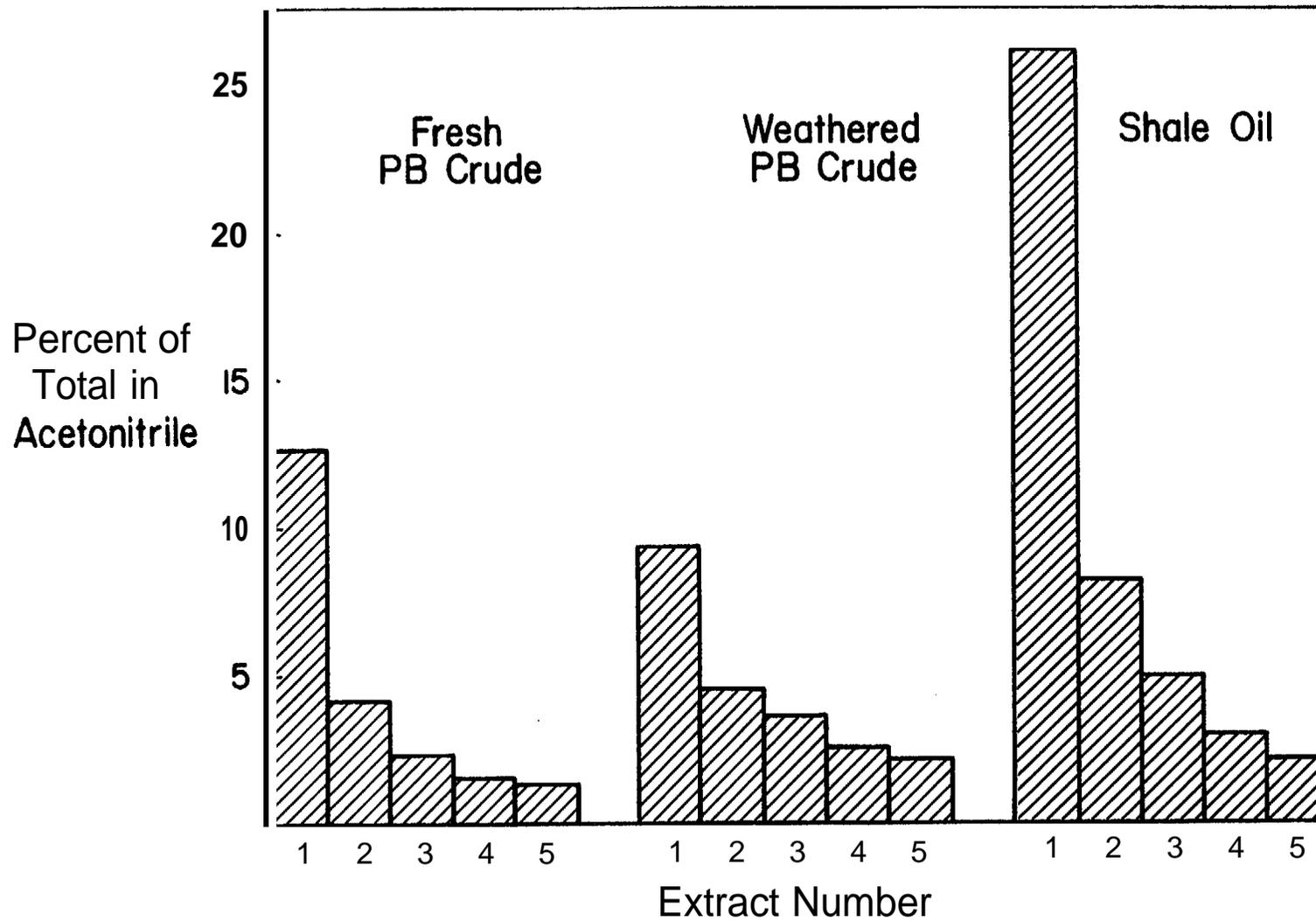


FIGURE 2. HEPTANE / ACETONITRILE PARTITIONING

460

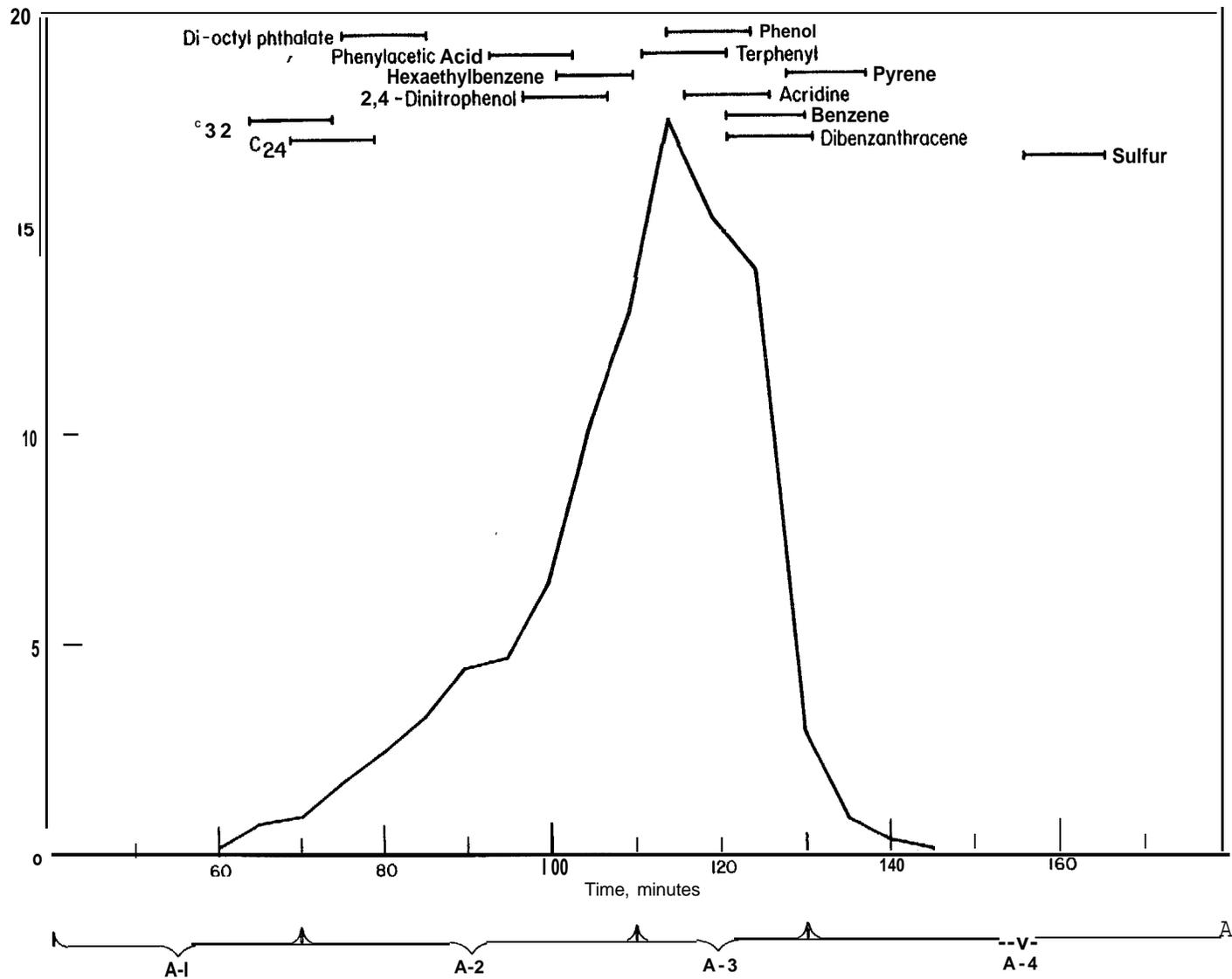


FIGURE 3. ELUTION PROFILE OF ACETONITRILE EXTRACT OF WEATHERED PB CRUDE OIL USING BIO-BEADS S-X8

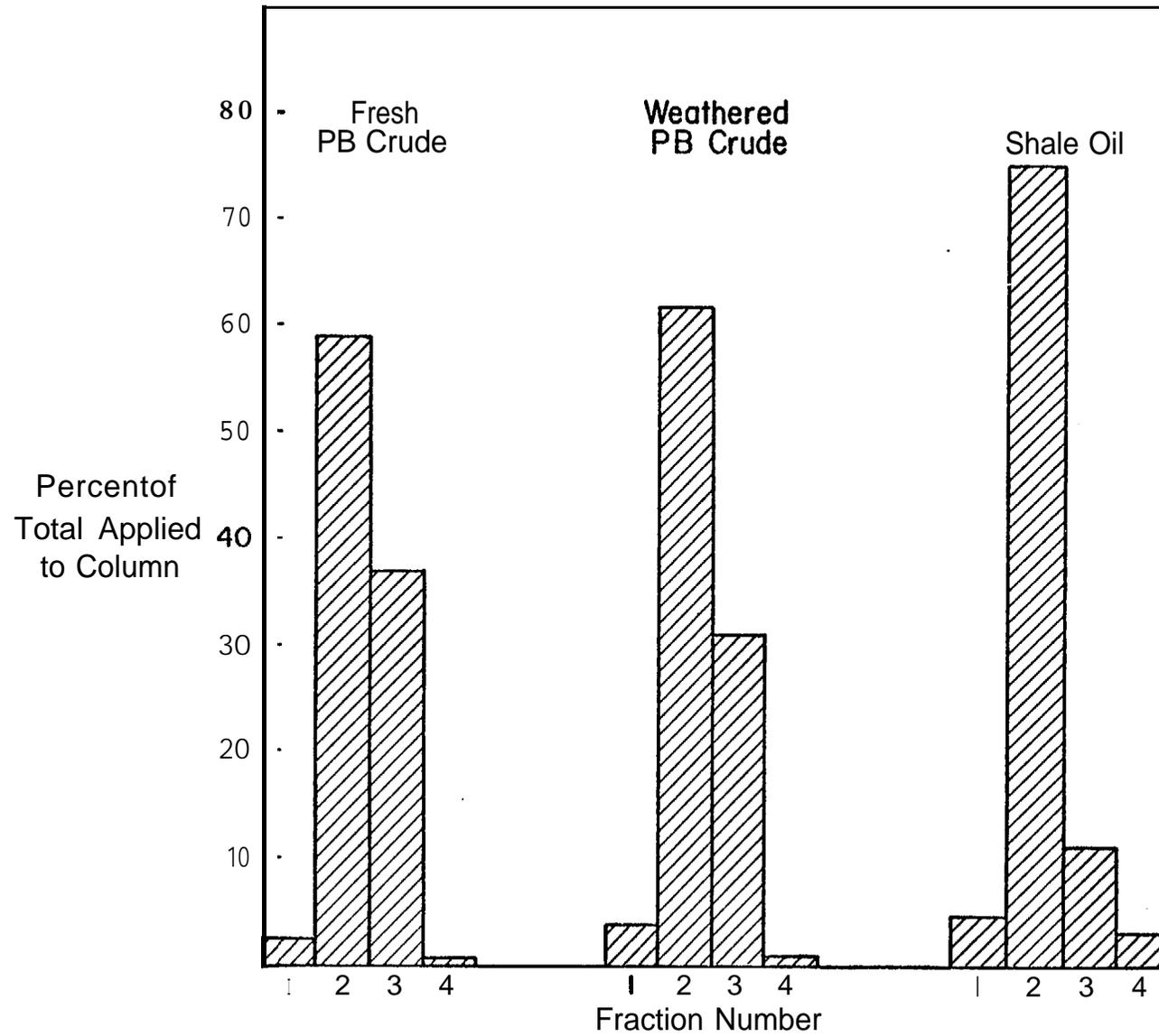


FIGURE 4. ELUTION PROFILES FROM FRACTIONATION USING BIO - BEADS S-X8

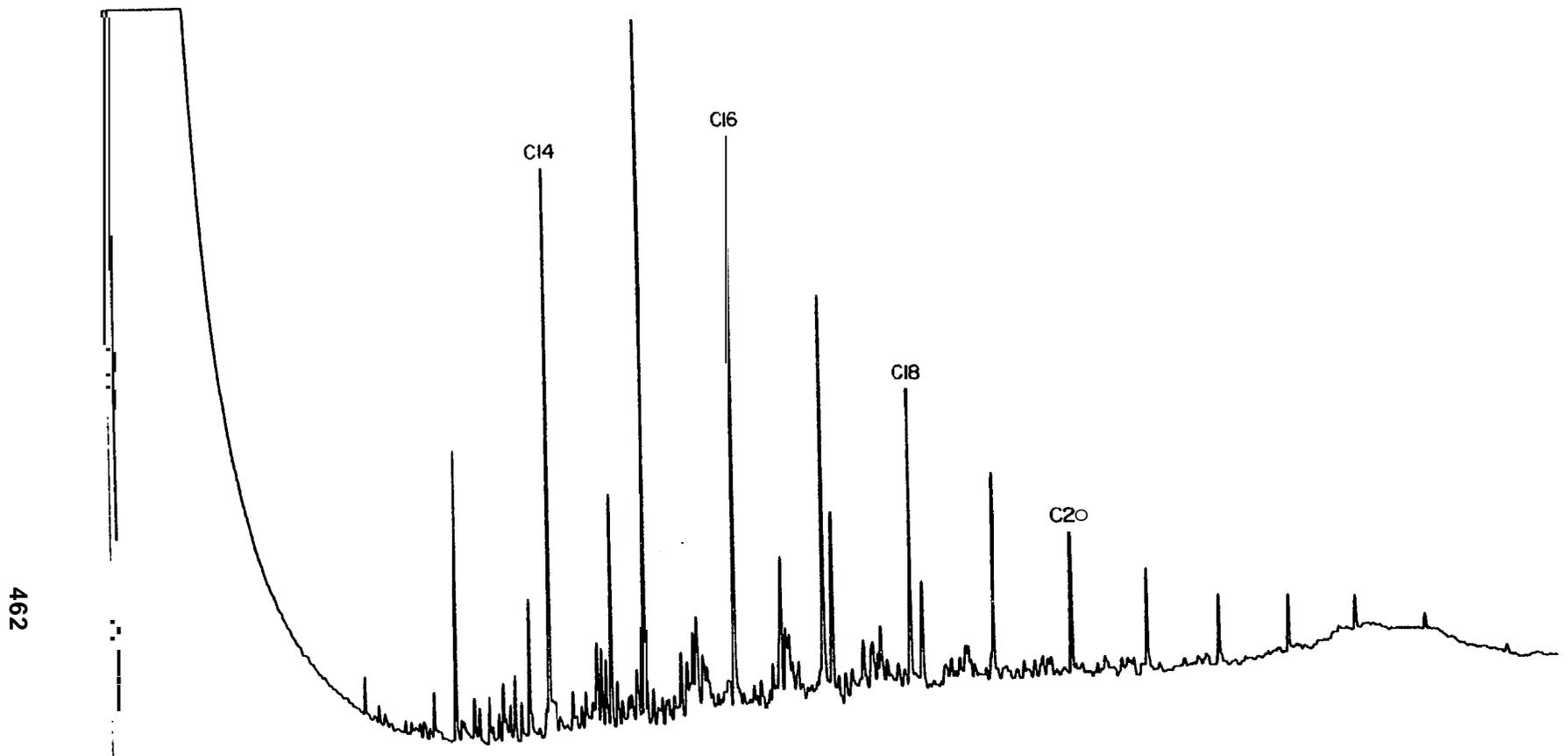


FIGURE 5. GAS CHROMATOGRAM OF FRACTION A-2 FROM WEATHERED PB CRUDE OIL

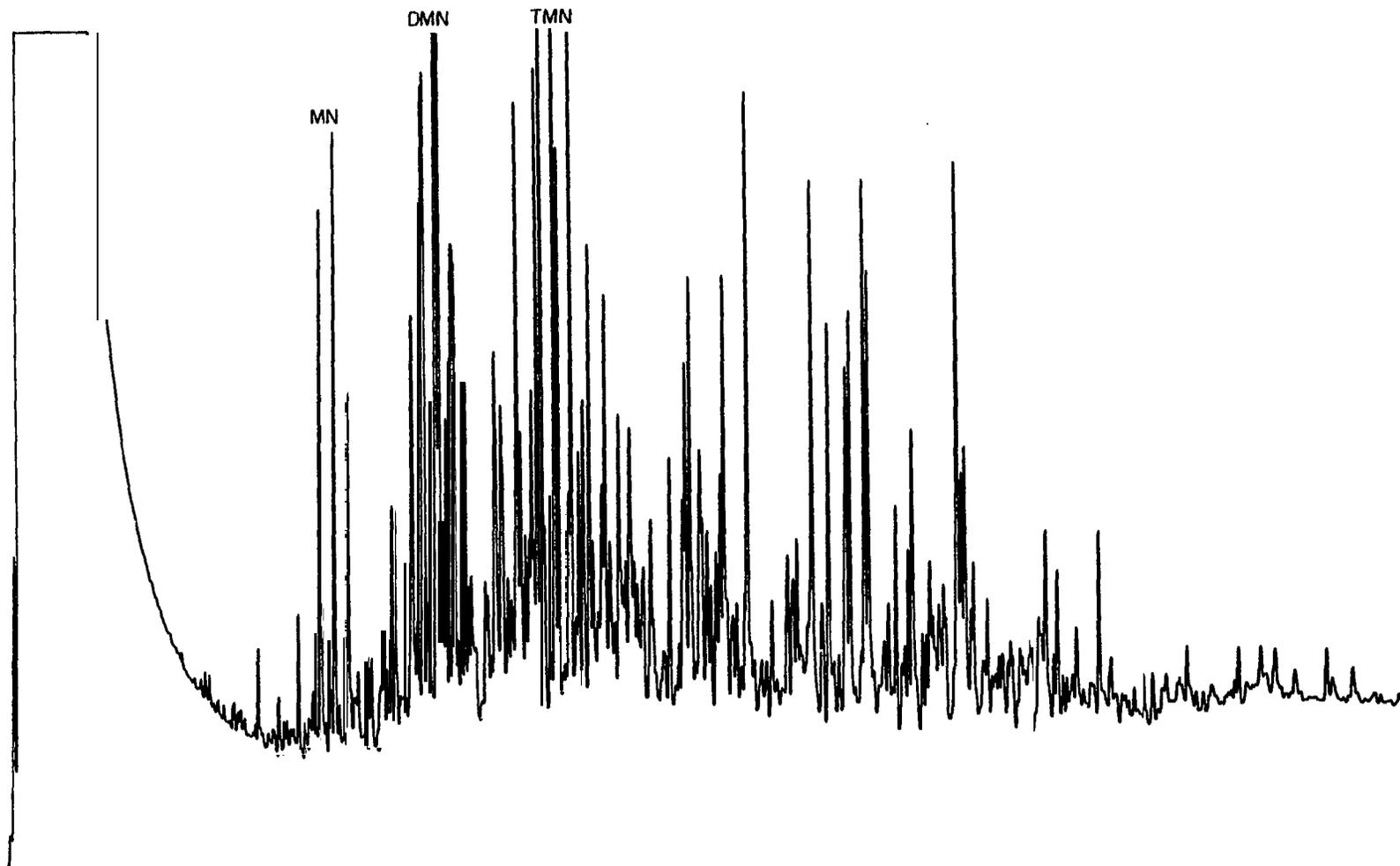


FIGURE 6. GAS CHROMATOGRAM OF FRACTION A-3 FROM WEATHERED PB CRUDE OIL

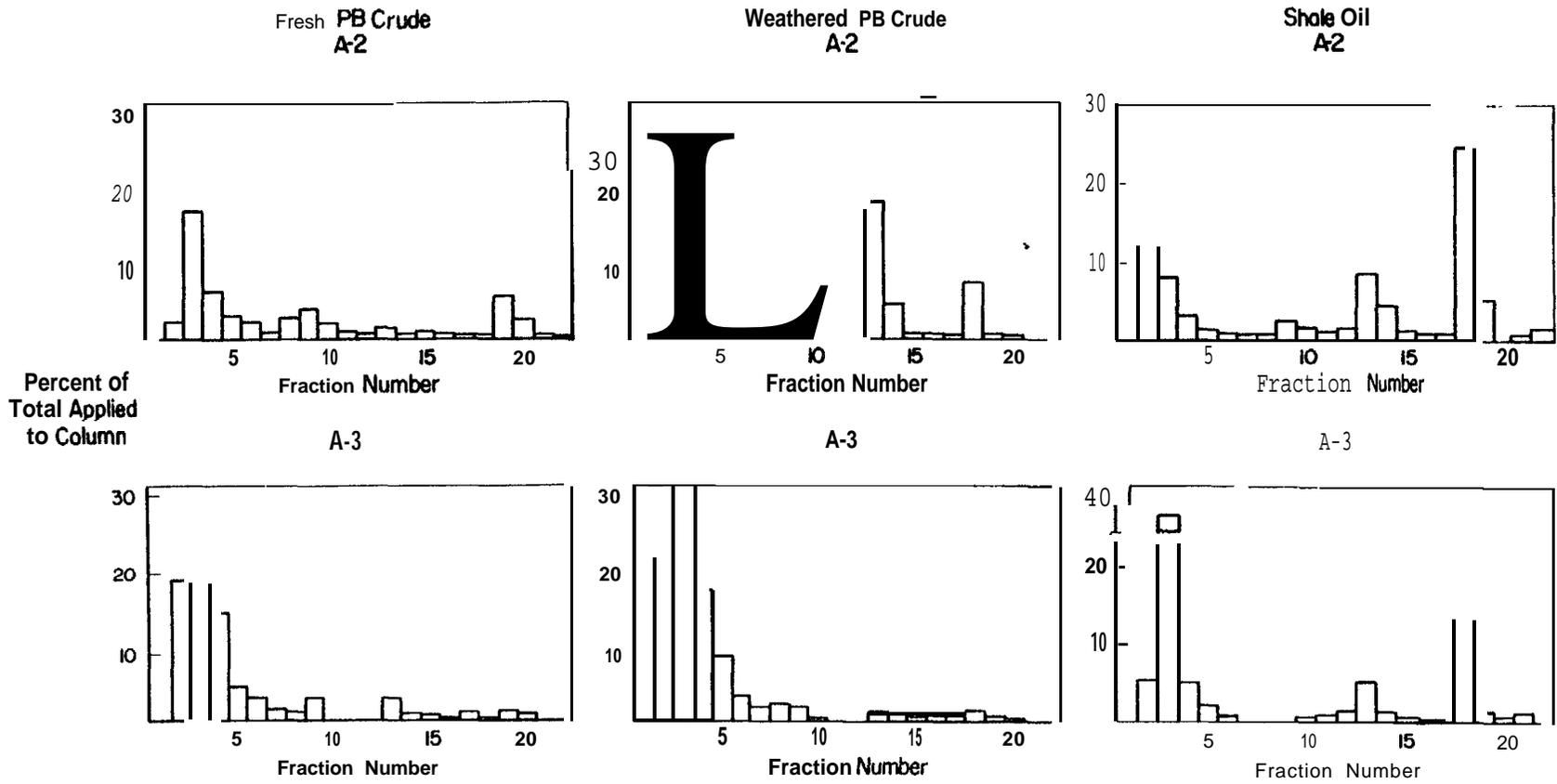


FIGURE 7. ELUTION PROFILES FROM FRACTIONATION USING SILICA GEL

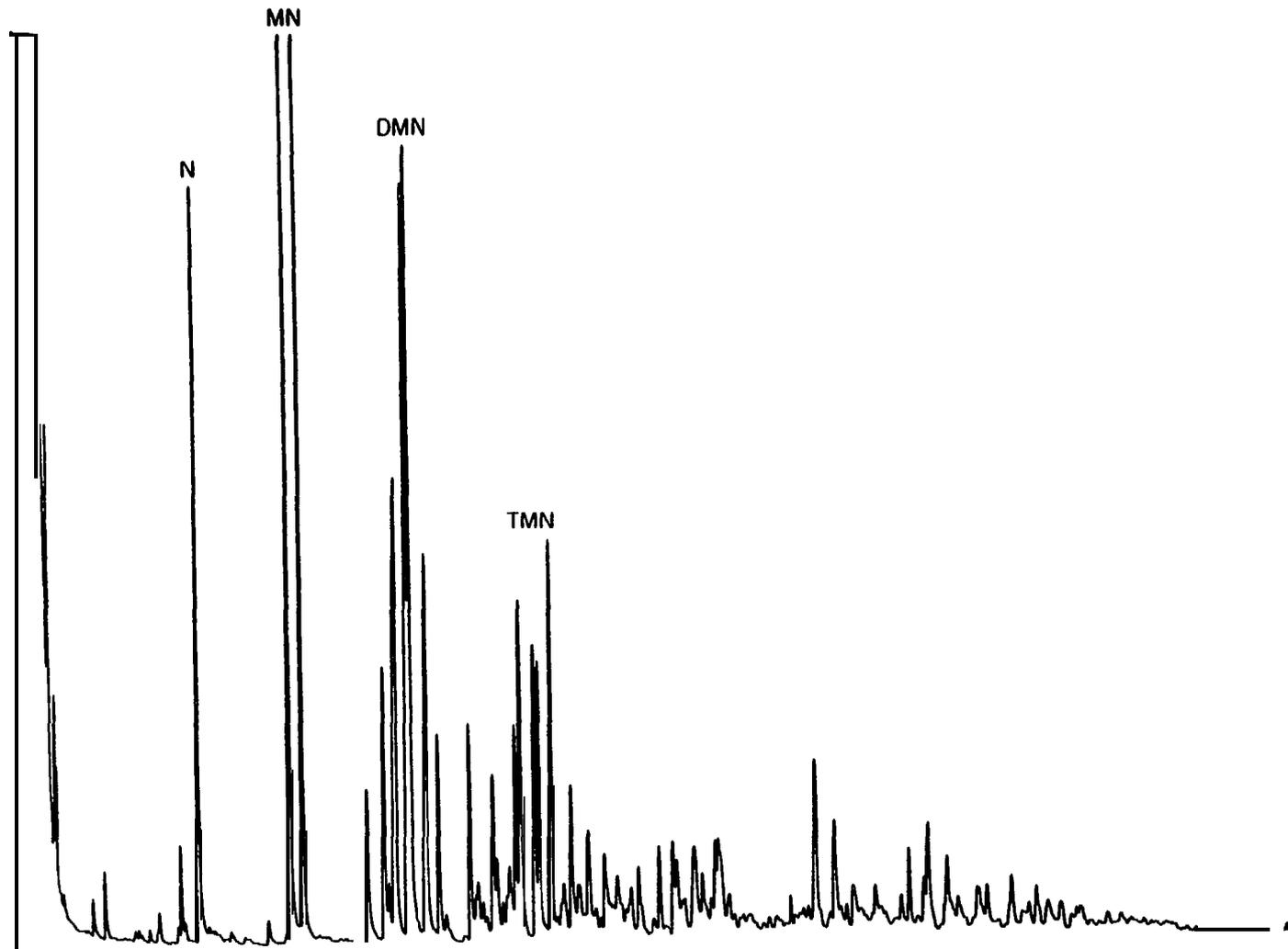


FIGURE 8. GAS CHROMATOGRAM OF FRACTION A-3-4 FROM FRESH PB CRUDE OIL

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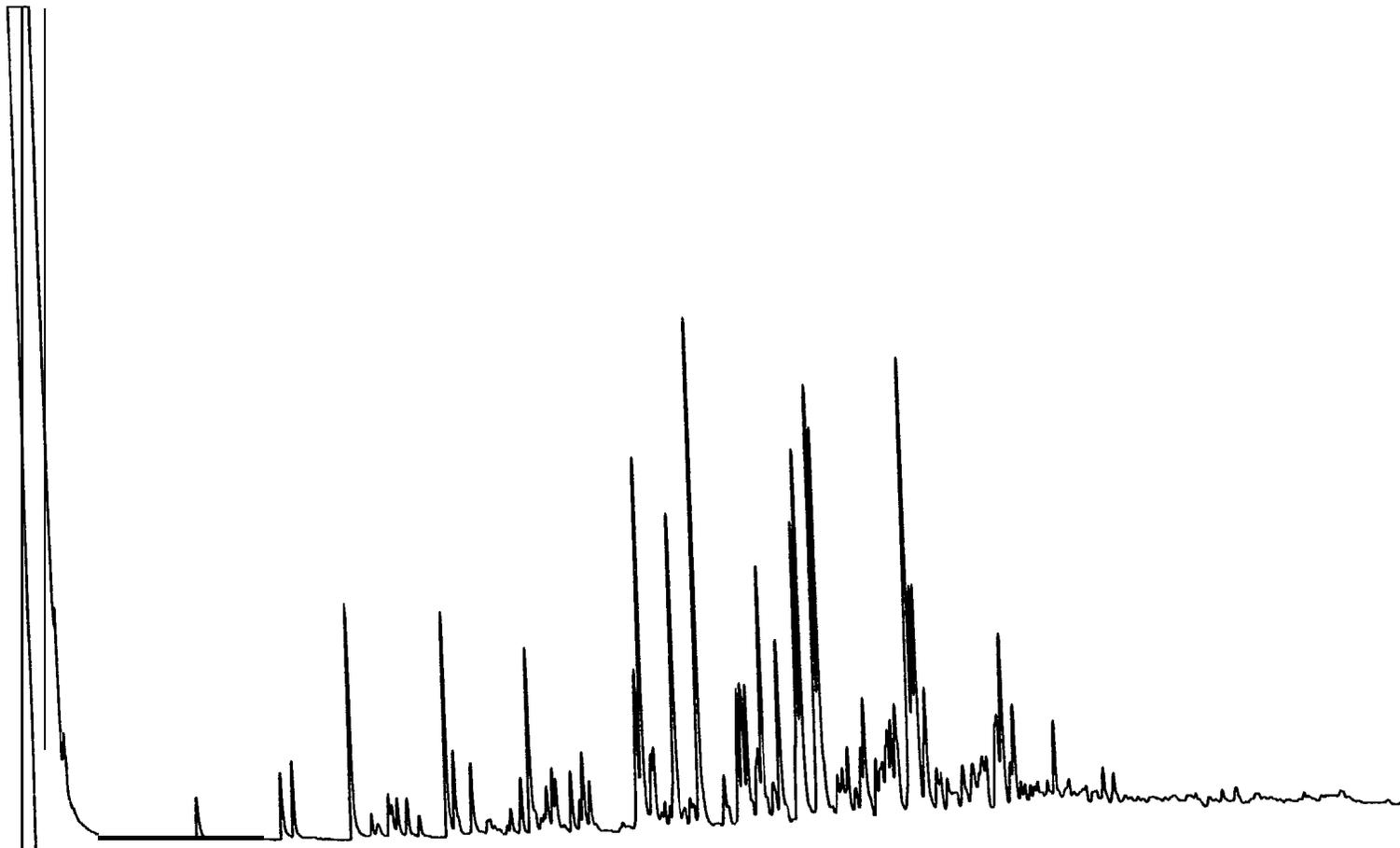


FIGURE 9. GAS CHROMATOGRAM OF FRACTION A-3-5 FROM FRESH PB CRUDE OIL

## Bioassay Studies

### In Vitro Studies

Various fractions of crude oil and shale **oil** as well as reference compounds were subjected to in vitro biological screening tests, namely the Ames Salmonella mutagenicity test and a prescreen confluence mammalian cell toxicity assay. The results obtained are summarized in Table 4.

Many of the oil fractions were as toxic or somewhat more toxic than reference compounds such as **naphthalene, carbazole, 4-methylphenol, and 2-naphthol**. The shale oil fractions were more consistently toxic than the crude oil fractions. The A-2 and A-3 series of silica **gel** fractions, which had the benefit of cleanup by solvent partitioning and GPC, were more toxic than silica gel fractions from whole oil.

No mutagenicity was exhibited by the various fractions except for a slight mutagenicity from some of the shale oil fractions. Very likely significantly greater mutagenicity would have resulted from modifying the bioassay protocol.

Salmonella toxicity tests were run concurrently with the mutagenicity tests to determine whether the number of revertants might be influenced by a toxicity effect. In many cases very significant toxicity, 90% kill or greater, occurred in the Salmonella toxicity tests while no toxicity was observed in the mutagenicity tests as indicated by a heavy background Lawn. Observation of a background lawn was therefore chosen as a more reliable indicator of no significant toxicity in the mutagenicity assay.

### In Vivo Studies

Initial in vivo studies were conducted using both brine shrimp and mysids. Phenanthrene was used as a standard **toxicant** to provide an index of the sensitivity of the animals. **Cyclopentanone** and **tetrahydrofuran**, initially considered for use as solvents for oil fractions, as well as fresh crude oil in **cyclopentanone** were also bioassayed using **mysids**. The results are given in Table 5. **Mysids** were found to be much more sensitive than brine shrimp to exposure of phenanthrene. **Mysids** are also a much more realistic indicator species than brine shrimp for determining the ecological damage of an oil spill. For these reasons, **mysids** were selected for all subsequent studies.

**Cyclopentanone** was less toxic than THF by a factor of about two. The results indicated that it could be used at a concentration of 100  $\mu$ l/l with little effect on **mysids**. A solution of 1 ml of Prudhoe Bay crude oil in 9 ml of **cyclopentanone** could therefore be used in the bioassay to give crude oil levels up to 10  $\mu$ l/l without interference by the **cyclopentanone**. Bioassay at this maximum level resulted in no significant toxic effects from the crude oil. Phenanthrene was at least 100 times more toxic than the crude oil. This indicated that all of the different components in the crude oil that have toxicities as great as phenanthrene comprise altogether no more than one percent of the total unless there are also protective components present. Therefore, in order to provide toxic fractions, the fractionation process used must concentrate

TABLE 4. IN VITRO BIOLOGICAL SCREENING STUDIES

No.	Sample	Relative Mutagenicity <sup>d</sup> at Given Dosage, <u>µg/plate</u>			Mammalian Cell Toxicity, CD <sub>50</sub> <sup>b</sup> , µg/ml
		1000	500	200	
1.	Benzo(a)pyrene		7 at 1 µg/plate		--
2.	2-Aminoanthracene		20 at 5 µg/plate		--
3.	Benzene	1			>100
4.	1,2,4-Trimethylbenzene	1			80
5.	Naphthalene	1			100
6.	<b>2-Methylnaphthalene</b>	1			70
7.	Phenanthrene	c			>100
8.	<b>2-Naphthol</b>	c			75
9.	4,4'-Methylbiphenyl	1			>100
10.	<b>1-Methylpyrene</b>	1			40
11.	<b>4-Methylphenol</b>	1			>100"
12.	<b>Carbazole</b>	1			>100
13.	Fresh PB Fraction SG-21 <sup>d</sup>	1			>100
14.	Fresh PB Fraction SG-22	1			>100
15.	Fresh PB Fraction SG-23	1			>100
16.	Fresh PB Fraction SG-24	1			>100
17.	Fresh PB Fraction SG-25	1			>100
18.	Fresh PB Fraction SG-26	1			>100
19.	Weathered PB Fraction SG-51	1			>100
20.	Weathered PB Fraction SG-52	1			>100
21.	Weathered PB Fraction SG-53	1			>100
22*	Weathered PB Fraction SG-54	1			>100
23.	Weathered PB Fraction SG-55	1			>100"
24.	Weathered PB Fraction SG-56	1			>100
25.	Fresh PB Acid Extractable		1	1	--
26.	Fresh PB DMSO Extractable		1		--
27.	Fresh PB Extract AN-1 <sup>e</sup>		1	1	35
28.	Fresh PB Extract AN-3			1	35
29.	Fresh PB Extract AN-5			1	>40
30.	Weathered PB Extract AN-1			1	>40
31.	Weathered PB Extract AN-3			1	>40
32.	Weathered PB Extract AN-5			1	>40
33.	Fresh PB Extract M-5 <sup>f</sup>			1	>40
34.	Fresh PB Extract M-6			1	>40
35.	Fresh PB Fraction A-2-6 <sup>g</sup>	1	1		>100
36.	Fresh PB Fraction A-2-9	1	1		>100
37.	Fresh PB Fraction A-2-13	1	1		75
38.	Fresh PB Fraction A-2-19	1	1		60
39.	Fresh PB Fraction A-3-6 <sup>h</sup>	1	1		30
40.	Fresh PB Fraction A-3-9	2	2		>100
41.	Fresh PB Fraction A-3-13	1	1		50
42.	Weathered PB Fraction A-2-3	1	1		60
43.	Weathered PB Fraction A-2-9	1	1		>100
44.	Weathered PB Fraction A-2-13	1	1		70
45.	Weathered PB Fraction A-2-18	1	1		60
46.	Weathered PB Fraction A-3-4	1	1		30

TABLE 4. (Continued)

No.	Sample	Relative Mutagenicity <sup>a</sup> at Given Dosage, $\mu\text{g}/\text{plate}$			Mammalian Cell Toxicity, $\text{CD}_{50}^{\text{b}}$ , $\mu\text{g}/\text{ml}$
		1000	500	200	
47.	Weathered PB Fraction A-3-6	1	1		35
48.	Weathered PB Fraction A-3-8	1	1		>100
49.	Shale Oil Fraction A-2-4	1	1		70
50.	Shale Oil Fraction A-2-6	1	1		35
51.	Shale Oil Fraction A-2-9	1	1		30
52.	Shale Oil Fraction A-2-11	1	1		30
53.	Shale Oil Fraction A-2-13	1	1		40
54.	Shale Oil Fraction A-2-15	1	2		45
55.	Shale Oil Fraction A-2-18	1	2		40
56.	Shale Oil Fraction A-2-21	G	1		20
57.	Shale Oil Fraction A-3-3	1	1		30
58.	Shale Oil Fraction A-3-4	2	1		10
59.	Shale Oil Fraction A-3-13	c	1		40
60.	Shale Oil Fraction A-3-18	c	2		>100

- a.  $\frac{\text{Revertants in Sample Plate}}{\text{Revertants in Control Plate}}$
- b. The dosage level that would result in only 50% confluence.
- c. Toxic at this dosage.
- d. PB refers to Prudhoe Bay crude oil  
SG refers to silica gel chromatographic fractions from whole oil.
- e. AN indicates an acetonitrile extract of oil in heptane.
- f. M indicates a methanol extract.
- g. A-2 fractions are silica gel chromatographic fractions from the second GPC fraction.
- h. A-3 fractions are silica gel chromatographic fractions from the third GPC fraction.

TABLE 5. IN VIVO BIOASSAY EVALUATION STUDIES

Concn, mg/l	Animals	No. of Test Animals Surviving After Given Exposure Time				LC50, mg/l, for Given Exposure Time	
		24 hr		48 hr		24 hr	48 hr
Phenanthrene/Mysids							
0.050	30	25	83%	23	77%	0.096	0.076
0.100	30	14	47%	8	27%		
0.200	30	1	3%	0	0%		
0.500	30	1	3%	0	0%		
Control	30	27	90%	18	60%		
Phenanthrene/Brine Shrimp							
0.025	20	20	100%	18	90%	>0.200	>0.200
0.050	20	19	95%	17	85%		
0.100	20	20	100%	17	85%		
0.200	20	20	100%	18	90%		
Control	20	20	100%	19	95%		
Cyclopentanone/Mysids							
10	20	20	100%	20	100%	800	420
100	20	20	100%	17	85%		
500	20	14	70	9	45%		
1000	20	9	45%	2	10%		
Control	0	19	95%	19	95%		
THF/Mysids							
10	20	20	100%	20	100%	570	240
100	20	20	100%	18	90%		
500	20	12	60%	3	15%		
1000	20	1	5%	0	0%		
Control	20	20	100%	19	95%		
Prudhoe Bay Crude Oil in Cyclopentanone/Mysids							
0.5	20	20	100%	20	100%	>10	>10
1.0	20	20	100%	19	95%		
5.0	20	19	95%	19	95%		
10.0	20	19	95%	16	80%		
Control	20	19	95%	18	90%		

the toxic components of oil at least 100-fold.

The mysids toxicity assay was applied to various reference compounds and a number of oil fractions. The results are given in Table 6. The majority of the oil fractions were not toxic at the levels assayed. The shale oil fractions in general were more toxic than the crude oil fractions. The most toxic fractions were A-3-4 and A-3-5 from weathered crude oil. Fraction A-3-4 is the fraction that contains the **naphthalenes** and A-3-5 is the fraction that contains the phenanthrenes and pyrenes, etc. All of the components present in A-3-5 are apparently as toxic as the phenanthrene and **1-methylpyrene** included as reference compounds. The fairly large polar compounds, fraction A-2-18 from both weathered PB and shale oil, are also significantly more toxic than whole crude oil.

**Dimethyl sulfoxide** was shown to be less toxic than oil solvents such as THF or **cyclopentanone** by a factor of 20 or more. A concentration of at least 0.1% can be used with very little chance of toxic effects appearing. This means that a 1% solution of an oil fraction in DMSO can be used to administer the oil at levels up to 10 mg/l without solvent effects.

**Corexit**, a dispersant that is highly effective in oil spill situations, was also included in the assay. It was relatively nontoxic in that the 48 hour LC<sub>50</sub> was 100 mg/l. This shows that the level of 1 mg/l that is commonly used in practice would have no biological effect detectable by the mysids test.

TABLE 6. IN VIVO BIOLOGICAL SCREENING STUDIES USING MYSIDS

No.	Sample	LC50 For Given Exposure Time, mg/l	
		24 Hr	48 Hr
1.	Phemnthrene	0.2	0.2
2.	1,2,4-Trimethylbenzene	8	7
3.	2-Methylnaphthalene	0.6	0.7
4.	<b>1-Methylpyrene</b>	0.4	0.2
5.	<b>4-Methylphenol</b>	9	4
6.	Dibenzothiophene	2	2
7.	Fresh PB <b>Volatiles</b> <sup>a</sup>	>10	>10
8.	Fresh PB Residuals	>10	>10
9.	Fresh PB Fraction <b>SG-16</b> <sup>b</sup>	>10	>10
10.	<b>SG-21</b>	>10	>10
11.	<b>SG-22</b>	>10	>10
12.	<b>SG-23</b>	>10	>10
13.	<b>SG-24</b>	>10	>10
14.	<b>SG-25</b>	>10	>10
15.	<b>SG-26</b>	>10	>10
16.	Weathered PB Fraction <b>SG-44</b>	>10	>10
17.	<b>SG-46</b>	>10	>10
18.	<b>SG-51</b>	>10	>10
19.	<b>SG-54</b>	>10	>10
20.	<b>SG-56</b>	>10	>10
21.	Fresh PB Fraction <b>A-2-9</b> <sup>c</sup>	>20	>20
22.	A-2-19	>10	10
23.	Weathered PB Fraction A-2-3	>10	>10
24.	A-2-13	>40	<b>29</b>
25.	<b>A-2-18</b>	15	5
26.	<b>A-3-4</b> <sup>d</sup>	2	<b>1</b>
27.	A-3-5	0.8	<b>0.5</b>
28.	Shale Oil Fraction A-2-4	5	2
29.	A-2-9	>10	3
30.	<b>A-2-13</b>	4	1
31.	A-2-18	5	2
32.	A-3-18	4	1
33.	<b>Dimethyl sulfoxide</b>	>10,000	10,000
34.	Corexit	500	100
35.	Fresh PB Crude Oil <sup>e</sup>	>10	>10

a. PB refers to Prudhoe Bay crude oil.

b. SG refers to silica gel **chromatographic** fractions from whole oil.

c. A-2 fractions are silica gel chromatographic fractions from the second GPC fraction.

d. A-3 fractions are silica gel **chromatographic** fractions from the third GPC fraction.

e. 1 mg/l of Corexit was used to aid dispersion.

## DISCUSSION

Many of the concerns and problems associated with developing a sound program have already been discussed in the Experimental Approach section. A key aspect in the development of the program was accepting the premise that major portions of petroleum are intractable in terms of being available to biological systems and such intractable nonavailable portions need not be accounted for in the bioassay program. The fractionation scheme that evolved, using solvent partitioning, GPC fractionation, and silica gel fractionation, worked very well and was quite effective in removing intractable components. Most of the intractable components stayed in the heptane layer during the solvent partitioning. These components were probably comprised mainly of the paraffin waxes and other highly paraffinic material. The GPC fractionation removed most of the colored and polymeric intractable components that remained.

Many of the final fractions did not contain enough material for bioassaying. The fractions that were studied indicated that most of the toxicity was associated with the aromatic hydrocarbon fractions. Some of the more polar fractions exhibited toxicity and also a hint of mutagenicity. Subfractionation and mutagenicity assays employing a more sensitive system would be needed to demonstrate any very significant mutagenic or potential carcinogenic effects.

Because of the strong parallel between mutagens and carcinogens, the mutagenicity assay is of major interest for detecting potential long-range biological effects of spilled oil. The insensitivity of the Ames mutagenicity assay as used was a serious deficiency in the program. One of the main causes of the insensitivity is the fact that an attempt was made to use the test as a screening method for all fractions using only one or two concentration levels. The number of plates per test were therefore kept to a minimum. The Ames test in a complete form can involve 5 bacterial strains, 5 levels of test material, with and without microsomal activation, in triplicate, with one set for mutagenicity and another complete set for toxicity. This complete form of the test entails the preparation and counting of 300 agar plates. Our procedure consisted of screening 3 strains, with only one level of test material in most cases, with microsomal activation using one set for mutagenicity and another complete set for toxicity. This procedure entailed the preparation and counting of only 12 plates instead of 300.

The 12-plate protocol was fully justifiable as a substitution for a 300-plate protocol as an initial screening protocol. Unfortunately, in retrospect with additional understanding and data obtained, neither protocol suffices for determining potential mutagenicity of oil fractions. Both of the above protocols work well for detecting the mutagenicity of 1 µg of BaP but they do not serve well for detecting mutagenicity in oil fractions. They are not optimized for handling a 1000-µg sample of oily matrix. Two major deficiencies were found. The first deficiency involves the separate toxicity assay which is designed to determine whether or not a low value for the number of revertants results from partial toxicity instead of from a lack of mutagenicity. The deficiency stems from the fact that about  $10^8$  cells are used in the mutagenicity assay but only about  $10^3$  cells are used in the toxicity assay. When applied to 1 to 10 µg of a pure compound the toxicity test results can be considered relevant to the mutagenicity test. However when 1000 µg of an oil fraction is used, considerable toxicity may be observed in the toxicity test when

in reality there may be no significant toxicity in the corresponding mutagenicity test as indicated by a normal background lawn. This inconsistency between the toxicity test and mutagenicity test can be explained on the basis of physical adsorption effects related to the number of cells. The 1000- $\mu\text{g}$  oil sample can nearly overwhelm the  $10^8$  cells in the toxicity test by simple physical adsorption rather than by actual cellular toxicity. When the 1000  $\mu\text{g}$  is added to  $10^8$  cells in the mutagenicity test however, the cells are in great excess and the number of cells that become inactivated by physical adsorption of the oil is an insignificantly small proportion of the total. The toxicity of an oil fraction in the mutagenicity test can therefore best be evaluated by observing the background lawn. The use of a range of test dosages would also help factor out the toxicity effect. The effectiveness of the background lawn observation approach was demonstrated by assaying an oil fraction at levels of 500, 1000, and 2000  $\mu\text{g}$ . The corresponding numbers of revertants found were 114, 18, and 5, respectively. There was a partial reduction in the background lawn at the 1000  $\mu\text{g}$  level and almost complete absence of background lawn at the 2000  $\mu\text{g}$  level. The separate toxicity test should therefore be deleted from any future protocols.

The second deficiency, which is a much more serious deficiency, involves optimization of the mutagenicity assay. The assay is quite sensitive to changes in the amount of microsomes added to each plate. The mutagenicity assay used for this program was optimized for use with 1  $\mu\text{g}$  of BaP, a representative petroleum mutagen. The optimization involves optimizing the concentration of the microsomal fraction in the microsomal mix as well as optimizing the amount of microsomal mix added to each plate. Representative data from such an optimization study are given in Table 7. On the basis of these data, 0.15 ml of 10% microsomal mix were considered optimal in using this particular microsomal mix and TA-98 culture.

TABLE 7. OPTIMIZATION OF MUTAGENICITY TEST FOR BaP

Amount of Microsomal Mix, ml/plate	Number of Revertants Per Plate When Using Given Percent of Microsomal <i>Fraction</i> in the Microsomal Mix <sup>a</sup>		
	7.5	10	12.5
0.1	136-126 <sup>b</sup>	136-145	247-223
0.2	179-193	231-236	54-56
0.3	73-75	62-42	49-54
0.4	73-62	38-46	45-41
0.5	54-57	36-44	39-42

a. All tests were conducted using strain TA-98 and 1  $\mu\text{g}$  of BaP.

b. Individual results from duplicate plates are given. The values obtained for spontaneous reversions in the absence of BaP in triplicate plates were 28, 29, and 32.

The microsomal activation is used to convert compounds such as BaP, which are inactive as such, into hydroxylated metabolites that are mutagenic. In this manner the test simulates what happens in an actual mammalian system.

The amount of **microsomes** present, however, is quite critical. An insufficient amount of microsomes will result in much of the **BaP** staying in its original inactive form while an excess of microsomes will metabolize the active **hydroxylated** metabolizes further to yield inactive species.

When a test is optimized for 1  $\mu\text{g}$  of **BaP** it will not be optimized for an oil fraction. This was shown by using various different amounts of **microsomal** mix when assaying 1000  $\mu\text{g}$  of one of the oil fractions. With 0.1, 0.2, 0.3, 0.4, and 0.5 ml of 10% **microsomal** mix the numbers of revertants were 36, 54, 71, 125, and 205, respectively. Perhaps with a greater amount of **microsomal** mix an even greater number of revertants would have been obtained. The average number of spontaneous revertants in this test was 30. Therefore by using 0.15 or 0.2 ml of **microsomal** mix as dictated by optimization studies with **BaP**, the number of revertants is **less** than two times background and not considered significant. By using greater amounts of **microsomes** a number of revertants of at least 7 times background can be achieved which indicates very definite mutagenicity.

The need for greater amounts of microsomes when assaying 100 to 1000  $\mu\text{g}$  of an oil fraction can be readily understood by considering that there are many more molecules competing for **microsomal** action than when only 1  $\mu\text{g}$  of **BaP** is present. Therefore in order for traces of **BaP**-type molecules to be optimally converted to active metabolizes many more **microsomes** are required. This effect can also affect the amount of oil fraction that is optimal for the assay. **For** a given amount of **microsomal** mix, the mutagenicity of 200  $\mu\text{g}$  of a given oil fraction may be much greater than that of 500  $\mu\text{g}$  or 1000  $\mu\text{g}$  simply because at the higher levels the ratio of microsomes to oil molecules is not great enough to give optimal conversion of **BaP**-type molecules. There may be many components that preferentially interact with the **microsomes** without being converted to active metabolizes.

It can be concluded from the above considerations that the amount of **microsomes** used in the mutagenicity assay must be optimized for each individual oil fraction. This conclusion, of course, suggests quite profound changes in **any** protocol intended as a screening test. Variations in both the amount of microsomes per plate and the amount of test material per plate must be studied. One approach would be to use four different amounts of microsomes, e.g. 0.2, 0.4, 0.8, and 1.6 ml., with each of four different levels of oil fraction, e.g. 800, 400, 200, and 100  $\mu\text{g}$ . If run in duplicate, this would require 32 plates per sample per test strain used. Our experience and that of other workers, however has indicated that the use of TA-98 alone is satisfactory for detecting all mutagenic components of petroleum materials. Therefore a satisfactory screening protocol could involve 32 plates per sample which is considerably more than the 12 plates per sample used in this program but is still a reasonable number. Most important, of course, is the fact that such a protocol would have a much greater chance of detecting the mutagenicity of an oil fraction.

Because of the very limited amount of material available in many of the oil fractions, the amount required by the mutagenicity protocol is an important consideration. It is of interest to note that the 32-plate protocol described above requires 12 mg, exactly the same amount as the 12-plate protocol used at the 1000  $\mu\text{g}$ /plate level. If the 800  $\mu\text{g}$  level were deleted, the 32-plate protocol would become a 24-plate protocol and only 5.6 mg of test material would be required. This is about the same as required for the 12-plate protocol at the

500 µg/plate level. Thus the protocol described above is a reasonable screening protocol in terms of sample requirements as well as effectiveness.

It was indicated earlier that either further subfractionation or a more sensitive mutagenicity assay system would be required to demonstrate significant biological effects of oil fractions. It is very likely that the screening protocol described above would be at least 10 times more sensitive than the 12-plate protocol used and thus would achieve the requirements of an activity-directed fractionation program.

One of the problems associated with using the mysids toxicity test has been the relatively large amount of sample required. In order to test an oil fraction at levels of 10, 4, 1, 0.4, and 0.1 mg/l in duplicate at least 62 mg is needed. Most of the fractions obtained didn't contain that much material. However if the highest dose were deleted from the assay only 11.2 mg would be needed. Since the aromatic hydrocarbon fractions gave LC50s in the range of 0.4 to 2 mg/l and since they represent a major fraction of the oil, it is reasonable to consider that any of the minor fractions that is not toxic at a level of 4 mg/l will not be significant in an oil spill situation and can justifiably be ignored. On this basis, the mysids test could be used to assay even minor fractions that show toxicity in in vitro assays.

#### CONCLUSIONS

Major components of petroleum *are so* intractable that they can not be evaluated in bioassay tests. However such components are so water insoluble and even insoluble in dimethyl sulfoxide that it is unlikely that they would ever exert any biological effect. Fractionation of oil using solvent partitioning between heptane and acetonitrile in conjunction with gel permeation chromatography can be used effectively to remove the intractable components that otherwise interfere with bioassay studies. Subsequent subfractionation using silica gel chromatography serves very well for obtaining a series of 20 fractions having increasing polarities.

An in vivo test involving toxicity to mysids, an in vitro mammalian-cell toxicity test based on confluency of growth, and the Ames bacterial mutagenicity test all work well to assess the biological activity of oil fractions that have had intractable components removed. The total sample requirement for running all three tests is less than 30 mg. In applying the Ames mutagenicity test to an oil fraction, varying amounts of microsomes need to be added to each of several concentrations of the oil fraction in order to optimize the test for that particular fraction. By using such an optimization protocol the test should be much more sensitive than the protocol actually used for this program.

On the basis of the limited bioassay results obtained on the program, the main petroleum fractions from an oil spill, that would represent a biological hazard are those that contain the aromatic hydrocarbons. However some of the polar fractions are toxic and mutagenic to a lesser extent and may also be significant biological hazards. The polar fractions from shale oil are more toxic and mutagenic than those from Prudhoe Bay crude oil.

The final bioassay scheme that evolved from the program has not been

applied to the oil samples. The complete fractionation and bioassay scheme is based on experimental evidence that indicates that it will work very **well**. It should be applied in a somewhat scaled-up mode to fresh crude oil, weathered crude oil, and shale oil to obtain more complete and more definitive data on the potential biological hazards of polar petroleum components.

#### NEEDS FOR FUTURE STUDY

The knowledge gained in this research program now permits a **proven** fractionation-bioassay scheme to be applied to the determination of **biologically-**active nonhydrocarbon fractions of petroleum or petroleum-like materials, e.g. shale oil. It is unfortunate that some of the key factors in the scheme were not obtained **until** the end of the program. Therefore the total data obtained give a very **incomplete** picture of what are the potential biological hazards **of, the** nonhydrocarbon components of weathered **oil** remaining after an oil spill.

The limited data that were obtained confirm **the** fact that the aromatic hydrocarbons represent the greatest environmental hazard of any of the petroleum components. At the same time, however, there were indications that some of the more polar fractions **may** be biologically active. It would be well worthwhile at this point to repeat the fractionation with a modest scale up of 5 to 10 times and assay all of the nonhydrocarbon fractions using all three of the bioassay tests with the modifications discussed above. This would entail considerably less time and effort than has been devoted to the program so far but would result in much more definitive data that would be very important to the OCSEAP program.

#### ACKNOWLEDGEMENTS

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APPENDIX 1:  
FRACTIONATION AND BIOASSAY OF WEATHERED  
PRUDHOE BAY CRUDE OIL

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## ABSTRACT

The less **volatile** and nonhydrocarbon components of weathered petroleum that remain after an oil spill are generally overlooked in **biological** studies and associated gas chromatographic analyses. In an effort to determine whether such components present a potential environmental hazard weathered Prudhoe Bay crude oil was fractionated by solvent partitioning, gel permeation chromatography, and adsorption chromatography. Fractions were assayed for mutagenicity and toxicity by using the Ames Salmonella mutagenicity assay and a mammalian-cell prescreen confluence assay. The toxicity of selected fractions was also determined 'by in vivo studies using mysids as the test organism. Fresh Prudhoe Bay crude oil and shale oil were included in the study for comparison purposes.



FRACTIONATION AND BIOASSAY OF WEATHERED PRUDHOE BAY CRUDE OIL

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The program that I'll discuss here can be considered as stemming from a concern about oil spills -- a concern about what environmentally harmful components may be left by an oil spill.

As we are all well aware, a great deal of effort has been devoted in recent years to studying the biological effects of oil spills, i.e., the biological effects of petroleum. The biological studies are frequently supported by chemical analyses in an effort to determine which petroleum components are *causing* the effects observed.

We've been involved in the chemical analysis end of programs, including methods development, for the past 6 or 7 years. Generally chemical analyses are limited to those components that can be detected most easily, the volatile and semivolatile hydrocarbons that are easily determined by gas chromatography. These include the saturated hydrocarbons n-paraffins, isoprenoids, and naphthenes which are essentially nontoxic, but which are sometimes considered as indicators of petroleum, as well as aromatic hydrocarbons such as benzenes, naphthalenes, and phenanthrenes which are of interest because of their toxicity, and polynuclear aromatics, such as benzo[a]pyrene and dibenzanthracene which are of interest because of their carcinogenicity.

Petroleum however is comprised of many other components **including** nonvolatile and polar components. In a highly weathered oil • 80 or 90 percent of the oil may be "nonGC-able" components. This is because many of the GC-able hydrocarbons found in a fresh oil are degraded by microorganisms,

photochemically degraded, or *lost* from an oil slick by dissolution and evaporation. It therefore seemed that any program concerned with the long-term biological effects of an oil spill ought to consider components other than the GC-able hydrocarbons. Perhaps some of the nonvolatile and polar components are toxic or mutagenic and should be studied in monitoring programs. On the other hand such components may have no significant biological activity and we should continue to ignore them. However we should not ignore them until we have experimental evidence indicating that.. they are indeed not important. We **should'nt** ignore them simply because they're not detectable by the analytical methods usually employed.

We therefore initiated a program, sponsored by NOAA as part of its Alaskan Outer Continental Shelf Environmental Assessment Program, to study nonhydrocarbon components of weather oil. The plan called for what we termed activity-directed fractionation. This meant that we would fractionate the oil into a dozen fractions or so, run bioassay tests to determine which ones were toxic or mutagenic, subfractionate t-nose active fractions, run bioassay tests on the subfractions, and continue in that manner until subfractionation no longer increased the activity per unit weight. We would ignore the inactive fractions, ignore the hydrocarbon fractions, and chemically characterize the active nonhydrocarbon fractions. We hoped to end up with all of the oil accounted for, i.e., a mass balance, so we wouldn't miss any potentially active fraction.

In discussing the program here I'll describe some of the things we did that didn't seem to work out very well; I'll describe some of the approaches that did seem to work and put us on the *right* track; and I'll discuss some of the problems involved. I won't be giving the answers, in terms of what are the nonhydrocarbon toxic components, not because I'm holding out but because we just haven't gotten that far along with the program.

There were several problems that seriously interfered with the success of the plan. One major problem was the basic phenomenon that oil and water don't mix. This presented the problem of how to get a highly water-insoluble component into an aqueous ~~time~~ <sup>medium</sup> required for bioassay. A second problem was the partial volatility of the oil which made it difficult to obtain very reliable residue weights and therefore **prevented a** good mass balance from being achieved. A third problem was the insensitivity of the

bioassay methods which meant that in the initial fractionation all of the fractions might appear inactive and no direction would be given to the subfractionation.

The volatility problem was one that hit us right off the bat. I don't mean to imply that we didn't expect it but we just had to deal with it somehow. There were really two problems -- one was finding a water-soluble or water-dispersible organic solvent that would dissolve the oil fraction but not be toxic in the bioassay; and the other was how to keep the oil fraction dispersed in the aqueous bioassay medium. Solvents such as tetrahydrofuran, benzene, and methylene chloride are good solvents for the oil fractions and are sufficiently water soluble, but are too toxic in the bioassay. Dimethyl sulfoxide and acetone are not toxic in the bioassay but are not satisfactory solvents for many of the oil fractions. We investigated various other solvents and found cyclohexanone and cyclohexanol to be good oil solvents, sufficiently water-soluble and less toxic than THF; but even so they really weren't satisfactory. They were still too toxic and although the oil fractions were soluble in the solvents, as soon as the solutions were added to the aqueous bioassay medium the oil fraction separated out. We tried several dispersants but they didn't work very well either.

At about that point we decided to take the sour grapes approach. We decided to go ahead and use dimethyl sulfoxide and if an oil component didn't dissolve in the DMSO we just wouldn't be interested in it after all. We rationalized the decision on the basis that just about every biologically-active organic compound we could think of was at least slightly soluble in DMSO -- or to put it more basically, if a component wouldn't dissolve at least slightly in DMSO it was unlikely that it could get into a cellular system to exert a biological effect. So, despite the sour grapes aspect of the decision we felt we actually were on pretty firm ground.

The partial volatility problem affected residue weights and therefore the mass balance. In evaporating oil solutions, the oily residue acts as a keeper for the solvent as well as a keeper for the more volatile oil components. The ideal case would be one in which the amount of solvent remaining after evaporation is equal to the amount of volatile oil components lost so that the residue weight observed is equal to what might be called the

true residue weight. However, as the viscosity and volatility of the oil components vary from fraction to fraction true errors in residue weight determinations will vary. In most cases the residue weight observed is probably less than the true residue weight. Working with oil that had the more volatile components removed by reduced pressure distillation helped considerably but even so the residue weights obtained could only be considered approximations. In most of our fractionation steps, recoveries based on residue weights varied between 85 and 95 percent. However, when we got a recovery of 85% we could never be sure whether the 15% discrepancy actually represented material that was not recovered or simply represented errors in residue weight determinations.

The problem of insensitivity of the bioassay methods was particularly true for the Ames mutagenicity assay. Fractionation into a large number of fractions prior to bioassay was necessary. I'll discuss that some more a little later.

At this point I think it would be helpful if I would summarize (see Figure 1) the scope of our program as it evolved after studying the problems that I've described so far. We studied 3 different oils. Although we were primarily concerned with weathered Prudhoe Bay crude oil, we used fresh crude oil and shale oil for comparison. The shale oil was from a simulated in situ process of the Laramie Energy Research Center. The weathered crude oil was provided by Dr. Stan Rice of the National Marine Fisheries Auke Bay Laboratory in Alaska. He exposed the oil during the summer for 2 months in a pine frame attached to a floating dock. The oil was therefore subjected to dissolution into the water, evaporation into the air, photochemical degradation, and microbiologic degradation, thus simulating in many respects an oil spill situation in an Alaskan environment.

The fractionation involved solvent partitioning, gel permeation or size-exclusion chromatography, and adsorption chromatography.

The bioassays included two in vitro tests, namely the Ames bacterial mutagenicity test and a mammalian-cell toxicity test, and an in vivo assay using mysids. The in vivo studies are being directed by Dr. Jack Anderson at Battelle Northwest's Sequim Marine Biology Laboratory.

### STARTING OIL SAMPLES

Fresh PB Crude Oil  
Weathered PB Crude Oil  
Shale Oil

### FRACTIONATION METHODS

Heptane/Acetonitrile Equilibration  
Gel Permeation Chromatography – Bio-Beads S-X8  
Adsorption Chromatography – Silica Gel

### BIOASSAY METHODS

Ames Salmonella Mutagenicity Test  
Mammalian-cell Prescreen Confluence Test  
Mysid Toxicity Test

FIGURE 1. PROGRAM SCOPE

To give an appreciation for the problems associated with the sensitivity of the Ames mutagenicity test, let's look at some of the quantitative considerations (see Figure 2) in applying the Ames test to the detection of mutagenicity of BaP in crude oil. If we consider that the BaP concentration in a crude oil is 2 µg/g, and the detection limit for BaP in the Ames test is 2 µg, and assume that BaP is the only mutagen present, then we can conclude that all of the BaP in 1g of crude oil has to be added to the Ames test plate in order for any mutagenicity to be detected. Furthermore, since the maximum amount of sample that can be accommodated by an Ames test plate is about 1 mg, the crude oil must be fractionated in a manner that gives a 1000-fold concentration factor for BaP. If all fractions contained equal amounts of material, the crude oil would have to be fractionated into at least 1000 fractions and all of the BaP would have to be *in just one* of those fractions before any mutagenicity would be detected. Fortunately, the situation is probably not quite that dismal. In the first place fractions that contain BaP undoubtedly contain other mutagens and secondly a major inactive portion of the oil can be removed by solvent partitioning.

The fractionation scheme that we used is shown in Figure 3. The scheme involved solvent partitioning to remove the bulk of the nonpolar material, size exclusion chromatography using Bio Beads S-X8 to remove polymeric material, and silica gel chromatography to fractionate on the basis of polarity.

The heptane/acetonitrile partitioning was used to simulate a heptane/DMSO partitioning. Acetonitrile has a big advantage over DMSO in that it is quite volatile and extracts can be readily concentrated directly. When DMSO is used, the extract has to be diluted with water and the oil components back extracted into a solvent such as cyclopentane. In order to assess how well acetonitrile might work as a DMSO substitute we ran solvent partitioning studies with various reference compounds. The results are shown in Table 1. The values given are the percent of the compound extracted into the polar solvent when partitioned with an equal volume of heptane. The very nonpolar saturated hydrocarbons stayed almost entirely in the heptane, significant or major amounts of aromatic hydrocarbons went into the polar solvents, and essentially all of the more polar compounds went into the polar solvents. In the heptane/acetonitrile partitioning that we used for the oil fractionation we used a 5:1 ratio of acetonitrile

PREMISE 1: BaP Concentration In Crude Oil = 2  $\mu\text{g/g}$

PREMISE 2: BaP Is Only Mutagen In Crude Oil

PREMISE 3: Limit Of Detection Of BaP In Ames Test = 2  $\mu\text{g}$

**CONCLUSION 1:** All Of The BaP In 1 g Of Crude Oil Must Be Added To Ames Test To Be Detectable

PREMISE 4: Maximum Amount of Sample Accommodated By Ames Test = 100  $\mu\text{l}$  Of 1% Solution = 1 mg

**CONCLUSION 2:** Crude Oil Must Be Fractionated In A Manner That Gives A 1000-Fold Concentration Factor For BaP

PREMISE 5: All Fractions Contain Equal Amounts Of Material

**CONCLUSION 3:** Crude Oil Must Be Fractionated Into At Least 1 000 Fractions And All Of The BaP Must Be In One Of Those Fractions Before Any Mutagenicity Will Be Detected

FIGURE 2. QUANTITATIVE CONSIDERATIONS OF AMES TEST

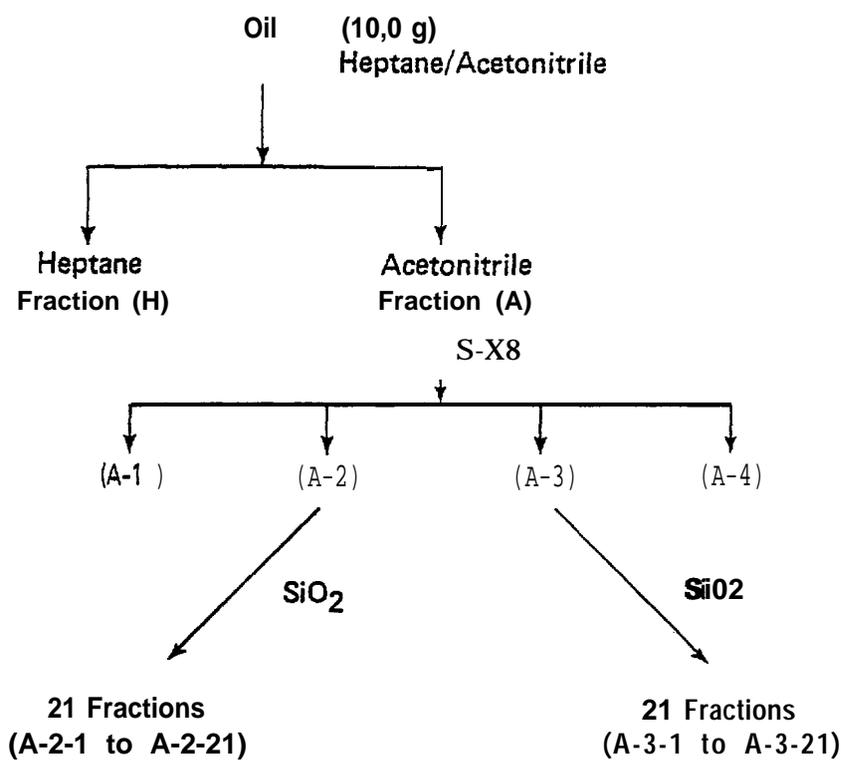


FIGURE 3. FRACTIONATION SCHEME

TABLE 1. SOLVENT PARTITIONING

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<u>Compound</u>	% Extracted into Given Solvent When <u>Partitioned With Equal Volume of Heptane</u>			
	<u>Acetonitrile</u>	<u>DMSO</u>	<u>Nitromethane</u>	<u>Methanol</u>
C16	< 2	< 2	< 2	< 2
C26	< 2	< 2	< 2	< 2
Androstane	< 2	< 2	< 2	< 2
Hexaethylbenzene	17	16	20	20
2,3,6-Trimethylnaphthalene	43	48	49	49
Naphthalene	61	71	62	66
Phenanthrene	71	90	69	73
Pyrene	71	92	66	72
Dibenzothiophene	68	91	69	74
Dibenzofuran	67	82	69	72
Acetylnaphthalene	94	98	96	98
Quinoline	98	> 98	> 98	98

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to heptane instead of 1:1 and repeated the extraction 5 times. Any components that would remain in the heptane under such conditions would probably not be biologically active and were therefore discarded. The amounts extracted by each individual partitioning are shown in Figure 4. The first partitioning, of course, accounted for a major amount of the extractable material. The total amount extracted into the acetonitrile was about 23% of fresh and weathered Prudhoe Bay crude oil and about 45% of the shale oil. This step thus reduced considerably the amount of material to be processed by the next step, the gel permeation chromatography, and, of even greater importance it removed most of the DMSO-insoluble material that could not be accommodated by the bioassay methods anyway.

The next step in the fractionation scheme was gel permeation chromatography also referred to as size exclusion chromatography. We originally tried Sephadex LH-20 for this step but got so much irreversible adsorption that we couldn't reuse the column. Bio-Beads S-X8, a styrene-divinylbenzene copolymer, worked quite well with methylene chloride as the eluting solvent. It's a system that we've used quite successfully for cleaning up extracts of environmental samples. An elution profile obtained for the acetonitrile extract of weathered crude oil is shown in Figure 5. For comparison I've also indicated the elution ranges of various reference compounds. Long-chain compounds elute early and the compact aromatic compounds elute considerably later. Polar aromatics elute earlier than less-polar aromatics. It's also of interest to note that elemental sulfur elutes even later than aromatic compounds and although that's not of concern to this program it provides a very useful technique for removing sulfur from hydrocarbon-containing sediment extracts.

The oil extracts were fractionated into four fractions designated as A-1 to A-4. The first fraction contained a major portion of the dark color, presumably polymeric material. This is material that would be largely irreversibly retained during subsequent silica gel chromatography. The fourth fraction contained the very small molecules especially compact polynuclear aromatics having strong pi-bonding characteristics. The bulk of the material was in the two center fractions A-2 and A-3.

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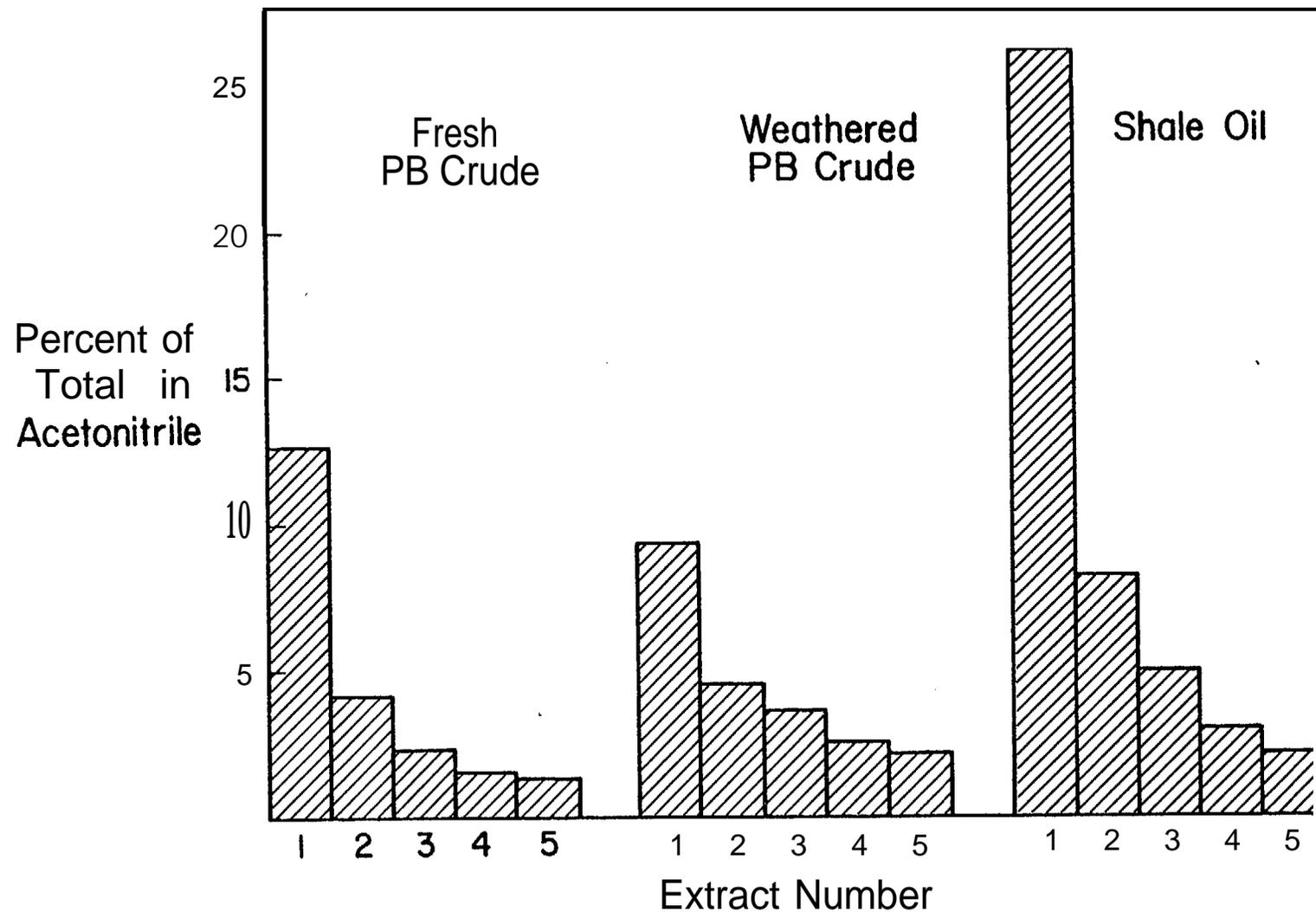


FIGURE 4. HEPTANE / ACETONITRILE PARTITIONING

4.4

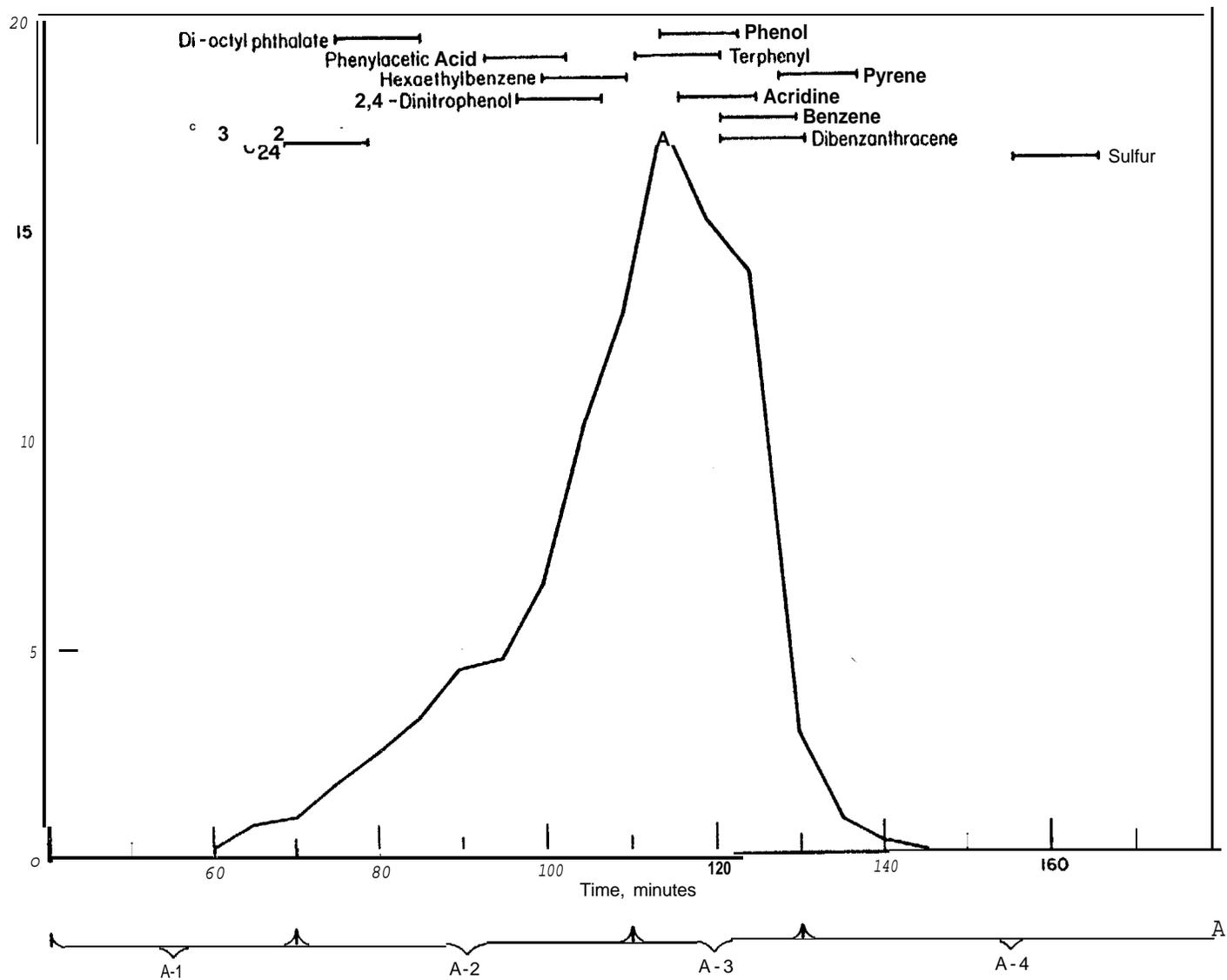


FIGURE 5. ELUTION PROFILE OF ACETONITRILE EXTRACT OF WEATHERED PB CRUDE OIL USING BIO-BEADS S-X8

The elution profiles obtained for the three oils in this manner are shown in Figure 6. The shale oil eluted somewhat earlier than the crude oils possibly because of a higher content of heterocyclic more polar components.

The effectiveness of this Bio-Beads fractionation system is indicated by the gas chromatograms shown in Figures 7 and 8. The main individual peaks in A-2 (see Figure 7), are trace amounts of normal paraffins; most of the material does not elute from the GC. Fraction A-3 (see Figure 8) on the other hand gives the usual pattern of aromatic hydrocarbons, the "methylnaphthalenes, dimethylnaphthalenes, trimethylnaphthalenes, etc.

The next fractionation step used was adsorption chromatography using silica gel. The elution scheme used is given in Table 2. The column was prepared in 10% methanol in ethylene dichloride and was therefore somewhat deactivated. Prior to use it was washed with straight ethylene dichloride and then with hexane. In the elution process the hexane would elute any saturated hydrocarbons first, then the benzenes and naphthalenes, then phenanthrenes and higher polynuclear aromatic hydrocarbons. With the addition of ethylene dichloride, heterocyclic nitrogen- and oxygen-containing compounds would elute and later with the addition of methanol much more polar compounds would elute.

The elution profiles obtained for the A-2 and A-3 Bio-Beads fractions from the three oils are given in Figure 9. The largest amounts of material were in the first six fractions, the hydrocarbon fractions. However around fraction 9 when 10% ethylene dichloride was begun, fraction 13 when straight ethylene dichloride was begun, and fraction 19 when 10% methanol was begun, significant amounts of more polar components were obtained, especially in the larger-molecule or more polar A-2 fractions. The weathered crude oil and shale oil had significantly more polar material than the fresh crude oil, it's this polar material, which represents only about 5% of the starting oil, that we're most interested in.

The efficiency of the silica gel fractionation used is indicated by the gas chromatograms shown in Figures 10 and 11. These are chromatograms of hydrocarbon fractions that we're familiar with. Fraction 4 from A-3 from fresh crude oil (see Figure 10) gives a GC pattern indicative primarily of naphthalenes, methylnaphthalenes, dimethylnaphthalenes, and trimethylnaphthalenes. The next fraction, fraction 5, (see Figure 11) contains very few naphthalenes but mainly the phenanthrenes and fluorenes.

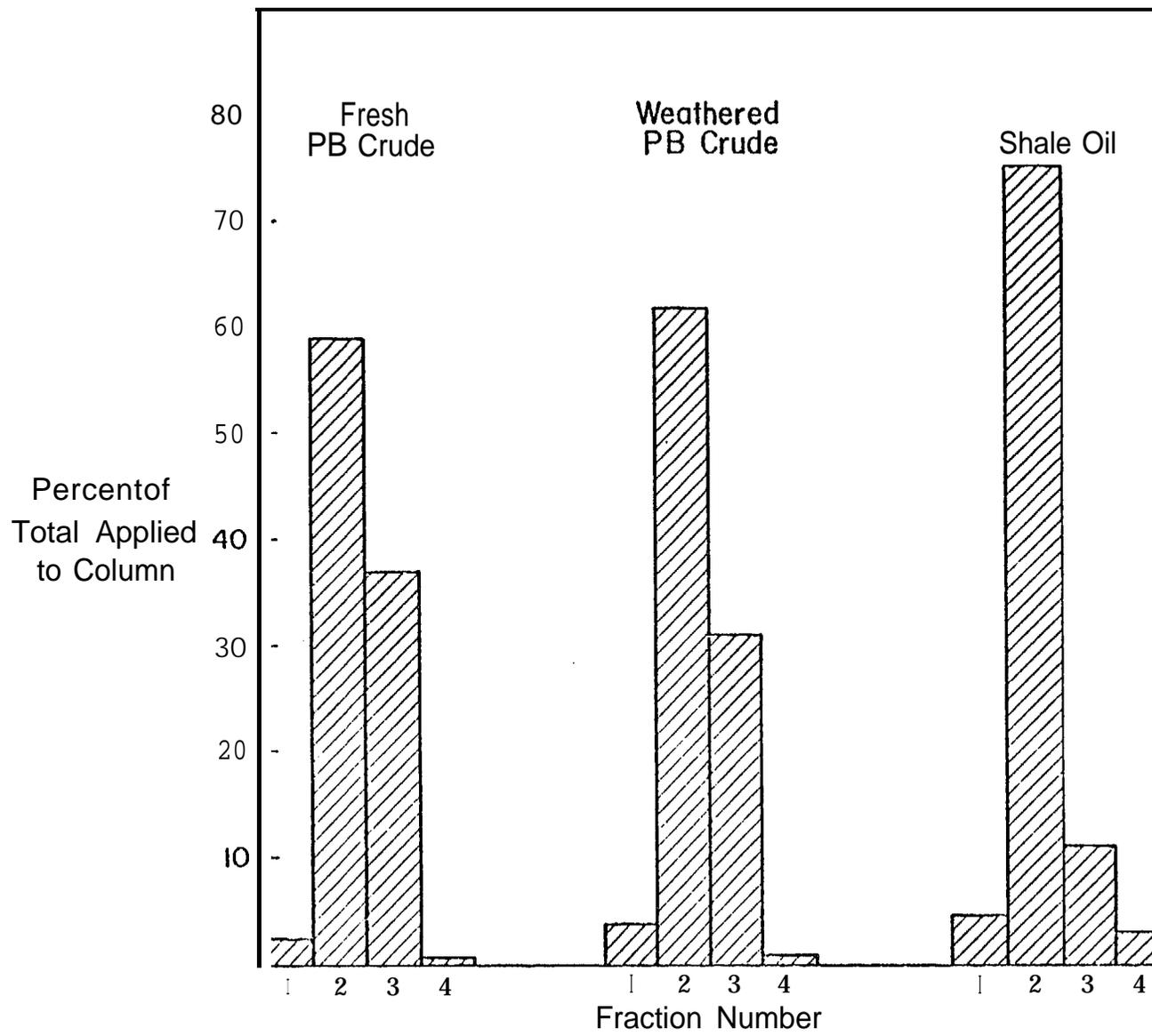


FIGURE 6. ELUTION PROFILES FROM FRACTIONATION USING BIO-BEADS S-X8

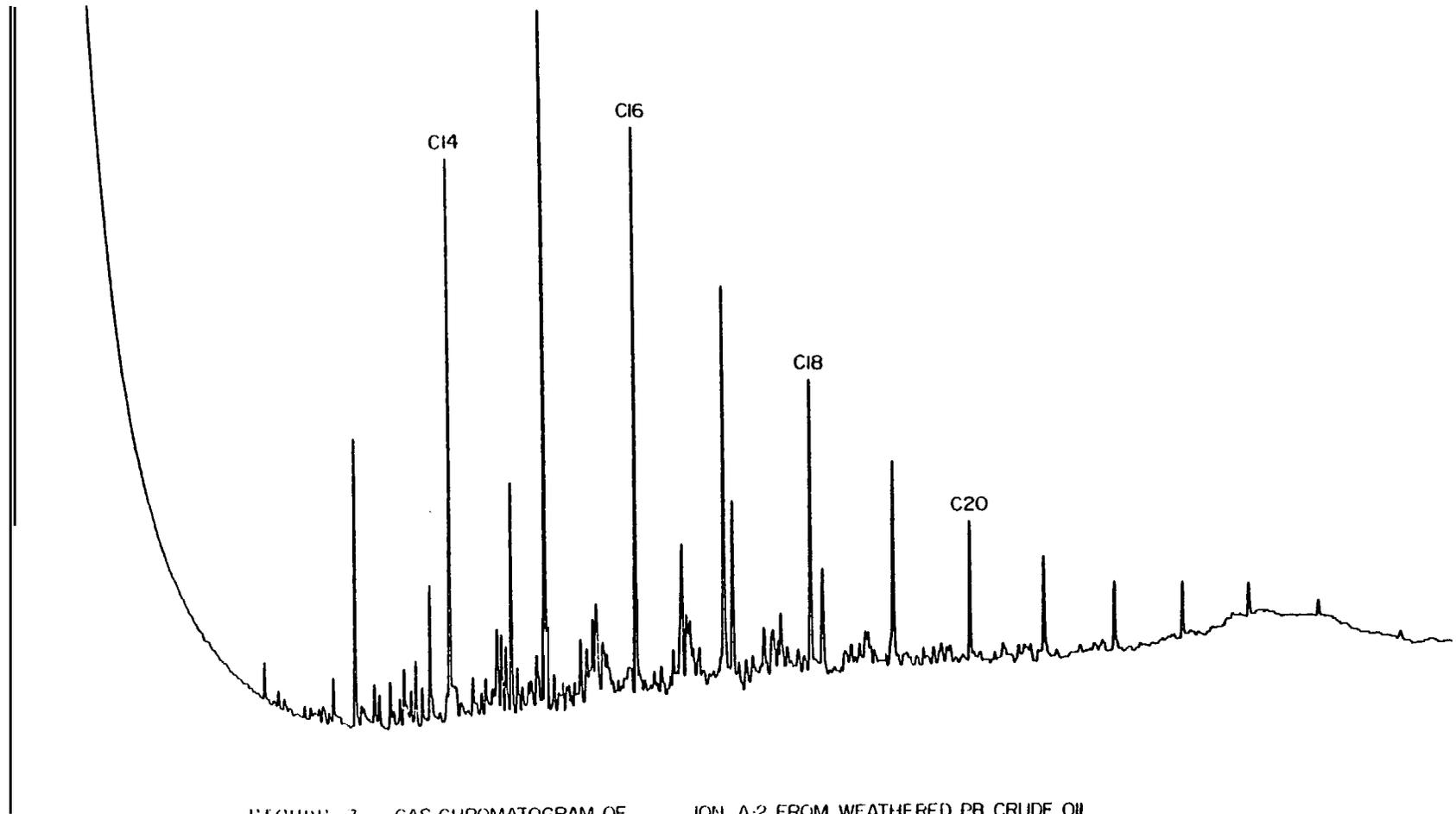


FIGURE 7. GAS CHROMATOGRAM OF ION A-2 FROM WEATHERED PB CRUDE OIL

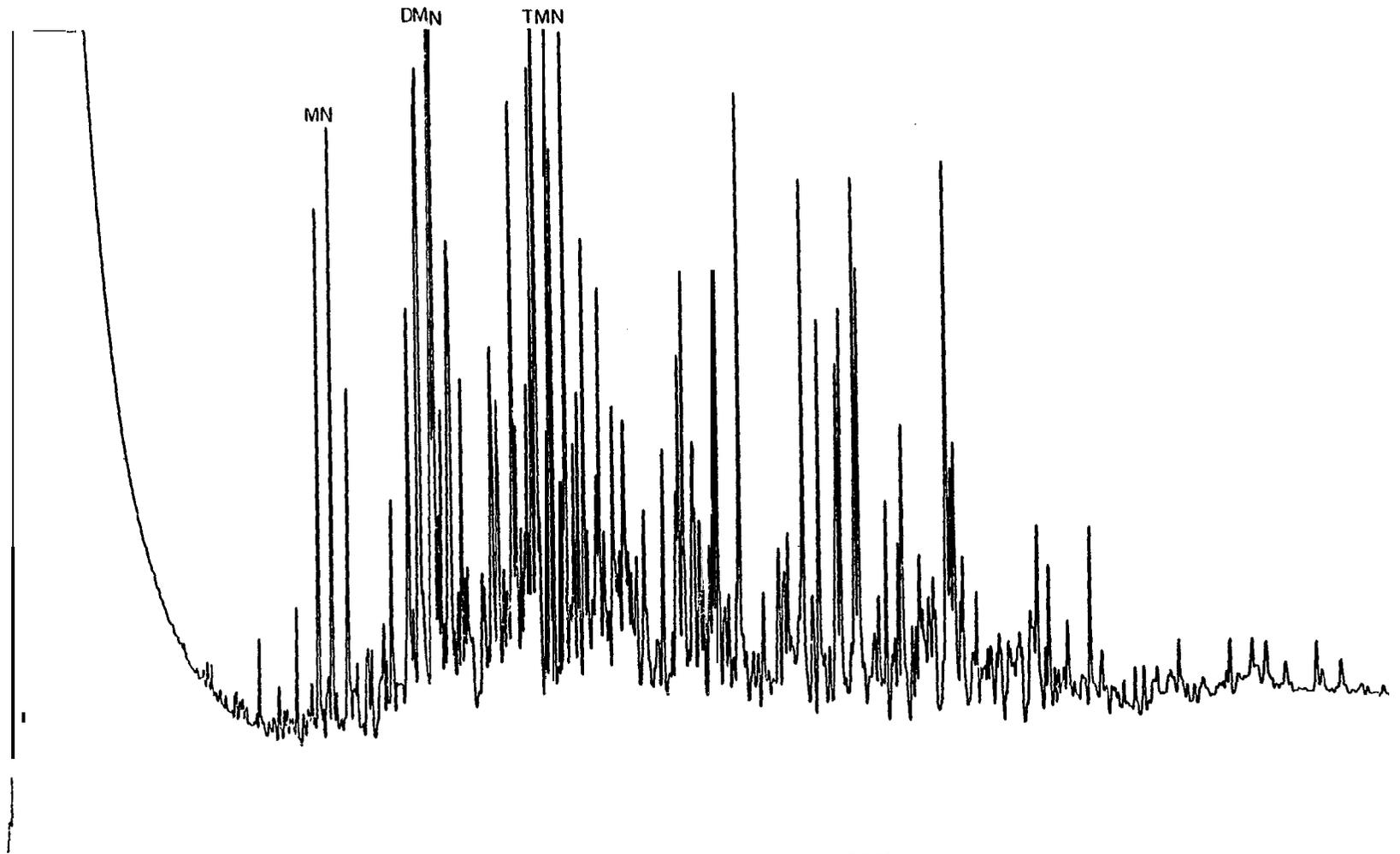


FIGURE 8. GAS CHROMATOGRAM OF FRACTION A-3 FROM WEATHERED PB CRUDE OIL

TABLE 2. **SILICA GEL** FRACTI ONATI ON

<u>Fraction No.</u>	<u>Eluting Sol vent</u>
<b>1 - 7</b>	<b>Hexane</b>
<b>8 - 12</b>	<b>10% Ethylene Dichloride in Hexane</b>
13 - 16	Ethyl ene Di chl ori de
17 - 21	10% <b>Methanol in Ethylene Dichloride</b>

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**25 mm ID x 1200 mm upward flow column**  
**350 g silica gel, Davison Grade 923**  
**10 ml/min elution rate**  
**250 ml fractions**

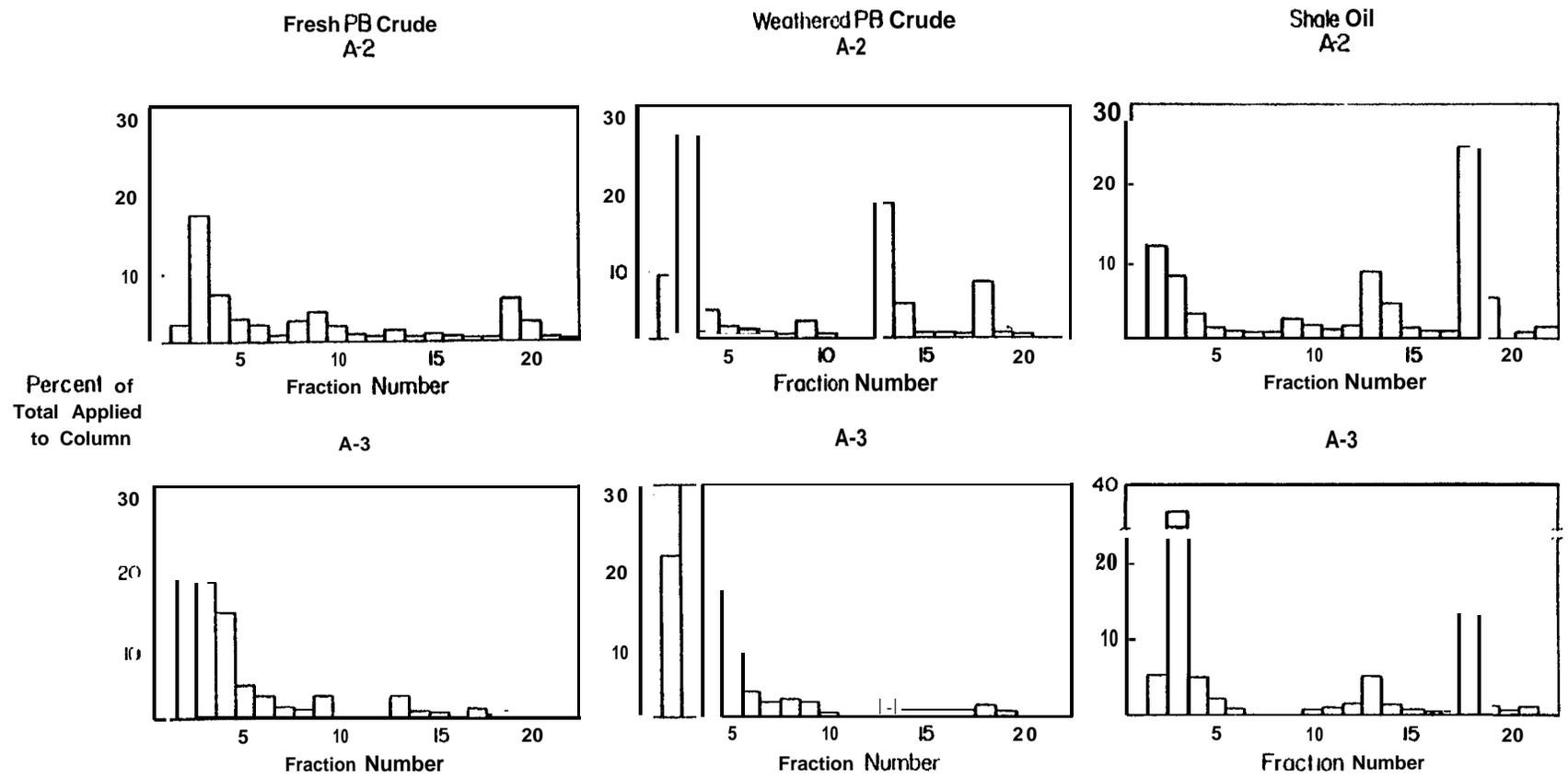


FIGURE 9. ELUTION PROFILES FROM FRACTIONATION USING SILICA GEL

500

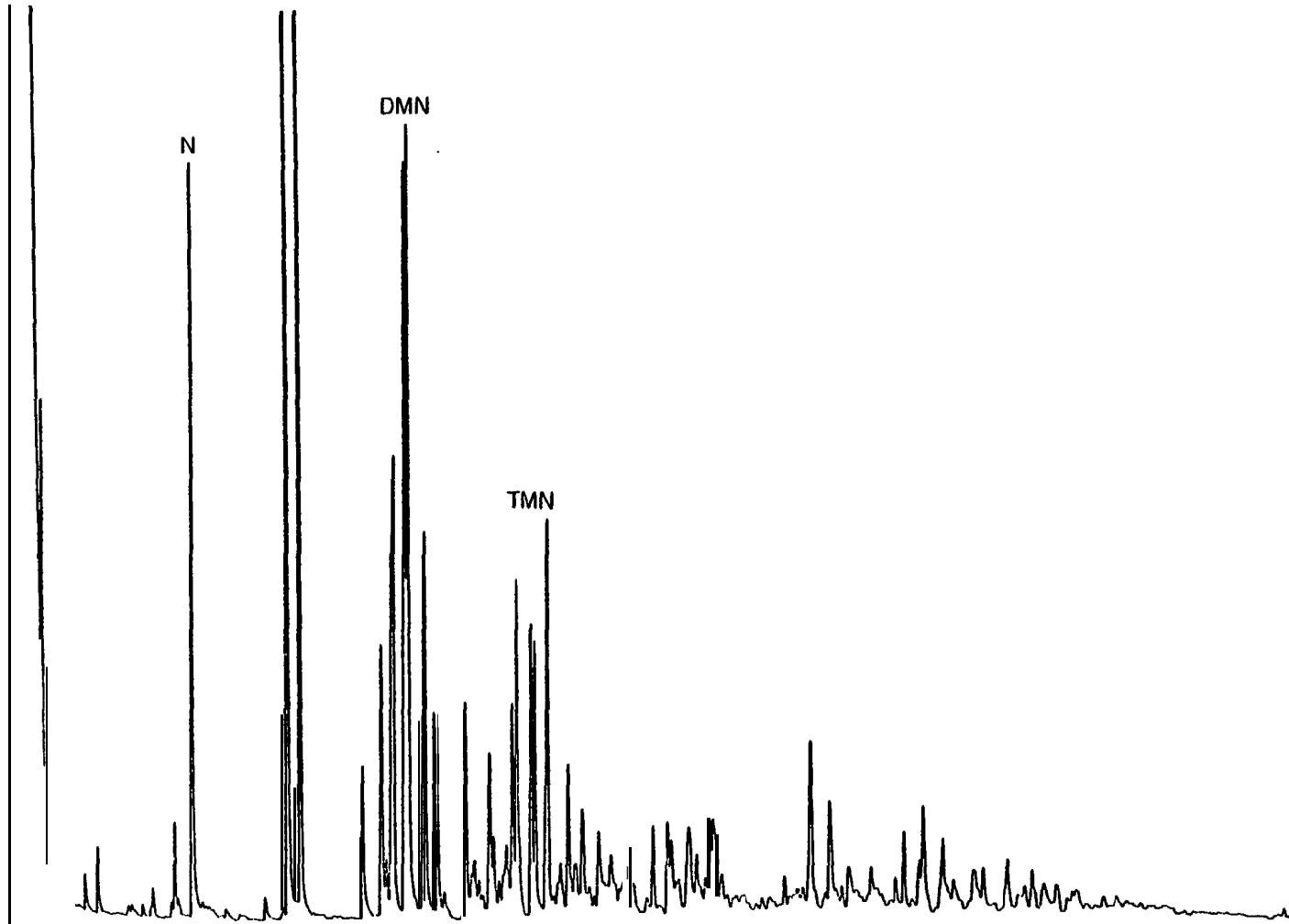


FIGURE 10. GAS CHROMATOGRAM OF FRACTION A-3-4 FROM FRESH PB CRUDE OIL

502

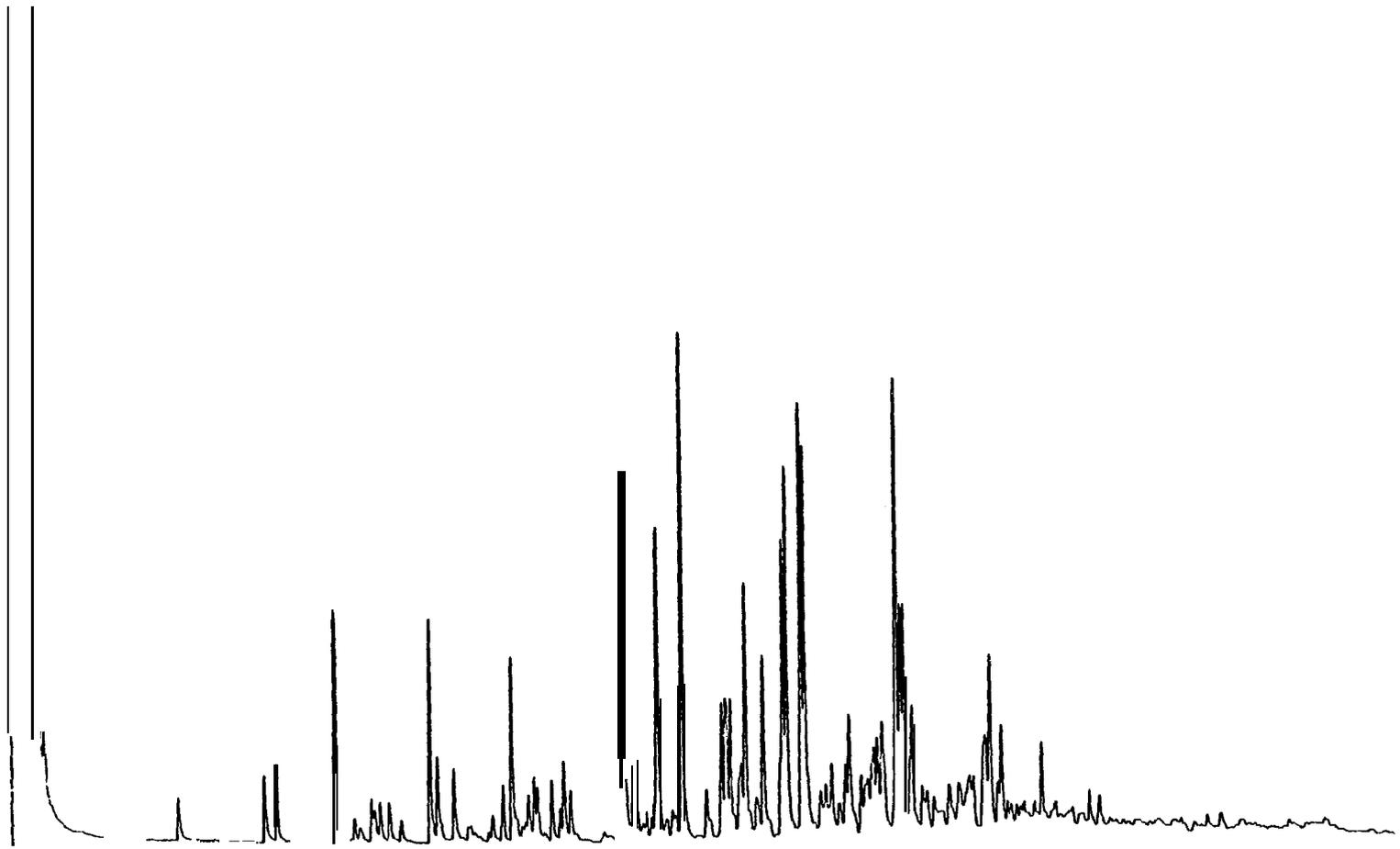


FIGURE GAS CHROMATOGRAM OF FRACTION A3-5 FROM FRESH PB CRUDE OIL

That covers the fractionation studies. I wish I could give bioassay results on all the fractions but that work is not completed. We've used various reference compounds in the toxicity assays, including 2-methylnaphthalene, phenanthrene, 1-methylpyrene, 4-methylphenol, 2-naphthol, dibenzothiophene, and carbazole, and found 1-methylpyrene to be somewhat more toxic than the others. We have also found that some of the polar fractions of oil have toxicities comparable to that of 1-methylpyrene, so it will be interesting to study those fractions in greater detail.

In the *Ames* mutagenicity assay we have found a slight amount of activity in some of the polar fractions but further optimization studies need to be run before we can make any valid conclusions.

Although I've not been able to provide bioassay data, I hope the presentation of the approach and the discussion of the problems involved have provided some useful insights into working on this type of problem.