Intercalibration of Analyses of Recently Biosynthesized Hydrocarbons and Petroleum Hydrocarbons in Marine Lipids

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Introduction

The evaluation of the extent and seriousness of petroleum contamination in the marine environment requires the efforts of many investigators. There are no adequate standardized methods for determining the levels of petroleum contamination in marine samples. Thus, it will be necessary, initially, to compare results of analyses in different laboratories often obtained with different analytical procedures. We have conducted an intercalibration exercise to determine the accuracy and precision of hydrocarbon analyses.

BLUMER et al. (1972) have discussed the analytical procedures used or of potential use in determining the presence of petroleum contamination in the marine environment. We have employed column chromatography or thin layer chromatography to isolate hydrocarbons from other lipids. Gas chromatographic (GC)[3]. analysis of the hydrocarbon extracts is then used as a screening method to select hydrocarbon extracts, which will be further analyzed by GC - mass spectrometry and other combinations of spectrophotometric methods and wet chemistry methods to determine the concentration of petroleum hydrocarbons in the samples.

The screening method is based on the following principles:

1) GC columns do not completely resolve all of the many thousands of hydrocarbons present in petroleum. The result is the elution from the GC column of many overlapping and/or superimposed peaks with a wide boiling range which collectively produce a detector signal designated as an unresolved complex mixture signal (Figure 1a). This unresolved complex mixture signal is a characteristic of GC's of crude oils, fuel oils and lubricating oils.

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3. Gas chromatograph, gas chromatogram, gas chromatographic will be designated by GC hereafter.

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II) The results of hydrocarbon analyses of organisms cultured in laboratories or obtained in areas thought to be free of detectable levels of petroleum contamination have shown that the hydrocarbons synthesized by organisms are usually of limited number and are dominated by a few hydrocarbons such as pristane, heneicosahexaene, squalene, or a few of the n-alkanes and n-alkenes with chain lengths of 15-25 carbon atoms (BLUMER, 1967; BLUMER, et al., 1969; HAN, et al., 1969; WINTERS, et al., 1969; BLUMER, et al., 1971; BLUMER, et al., 1970; ALBRO, et al., 1970). N-alkanes with an odd number of carbon atoms usually predominate over n-alkanes with an even number of carbon atoms. These analyses also show that it is unlikely that marine organisms could produce a complex mixture of hydrocarbons similar to that present in petroleum. However, this possibility cannot be ruled out for marine bacteria, yeasts and fungi. Analyses of cultures of these micro-organisms with precautions taken to eliminate influence from petroleum contamination of the culture and during analyses are needed to evaluate this possibility.

Thus one of the criteria of the GC screening method used to identify petroleum contamination in marine samples is the presence of the unresolved complex mixture signal.

The other characteristics of the GC's of most crude oils and fuel oils are the presence of the pristane, n-hexadecane; phytane, n-octadecane pairs; and the presence of a homologous series of resolved peaks of n-alkanes and branched alkanes with little if any predominance of n-alkanes with an odd number of carbon atoms. (Figure 1a). These characteristics are useful when present. However, organisms synthesize n-alkanes and branched alkanes making it difficult to determine the level of petroleum contamination if such contamination is small relative to the concentration of hydrocarbons biosynthesized by organisms. There is also a limitation imposed by the preferential biochemical oxidation of these compounds by micro-organisms in comparison to the naphthenes and aromatic hydrocarbons (ZOBELL, 1969) which are the constituents giving rise to the unresolved complex mixture signal. Thus the absence of a homologous series of n-alkanes and branched alkanes peaks in a GC may indicate that the sample is not contaminated by petroleum or that the n-alkanes and branched alkanes which would have given the homologous series of peaks, have been biochemically oxidized prior to, or after, the incorporation of the petroleum hydrocarbons into the sample.

We use the presence or the absence of the unresolved complex mixture signal in the hydrocarbon gas chromatograms as an ititial screening criterion for the presence or absence of petroleum contamination in marine samples. The presence of a homologous series of peaks in the GC's is used as supplemental evidence for the

presence of petroleum contamination only when reliable information is available concerning the recently biosynthesized hydrocarbons normally present in the sample type in question. The presence of petroleum contamination would be confirmed by further analyses as previously discussed.

EXPERIMENTAL

Preparation of IDOE-5 Reference Sample

Cod liver lipids extracted from a Cod sampled in the East Greenland Current were spiked with 371.8 ppm of a distillate cut n-C_{16} (n-hexadecane-287° C, 760 mm Hg) to n-C_{28} (n-octacosane-432°C, 760 mm Hg) of South Louisiana Crude Oil. The polarity of the distillate cut was such that 95% was eluted from an Al_2O_3:SiO_2 column as described in Method 1. below.

Methods of Analysis

Farrington. The lipid sample was saponified under reflux for two hours in 0.5 N KOH in 95% Methanol:Benzene (4:1). The non-saponifiable lipids were partioned into n-pentane. The n-pentane was evaporated under reduced pressure until 0.5 to 1.0 ml of extract remained. This was charged to a column of Al_2O_3 (5% H_2O) packed over SiO_2 (5% H_2O) -1:1 with a sample-to-absorbent ratio of 1:50. The hydrocarbons were eluted by one of the following procedures:

1. One column volume each of n-pentane, 10% benzene in pentane, and 20% benzene in pentane, to give a total of three fractions. The latter two fractions were combined prior to further analysis.

2. Three column volumes of 5% benzene in pentane. In each case the solvent was removed by evaporation at reduced pressure and the residue redissolved in 100 ul of CS_2. The hydrocarbons in the samples were determined by GC using either or both of the following procedures.

A. Comparison of the total integrated detector signal of a known amount of an n-alkane internal standard, e.g., n-C_{20} (n-eicosane) added to the sample at the time of saponification.

B. Comparison of a known amount of a series of n-alkanes analyzed separately from the sample on the same day using the same conditions of analysis.

In both of the above cases the detector signal is expressed in terms of area under the peak or area under the unresolved complex mixture signal. The peak areas were determined using the peak height X peak width at half height, while the unresolved complex mixture signal area was determined by planimetery.

The GC columns employed were a 2.3 m 3% Apiezon L on Chromasorb W 80/100 mesh and a 3.1 m 12% FFAP on Chromasorb W 80/100 mesh. Both columns were 2.2 mm i.d. stainless steel and were operated with an N_2 carrier gas flow rate of 10-15 ml/min. The Apiezon L column was programmed from 80° C to 290° C and the FFAP column was programmed from 120° C to 270° C, both at 6°/min. Varian Aerograph model 650D, 1200, and 1700 GC's, equipped with flame ionization detectors, were used for these analyses.

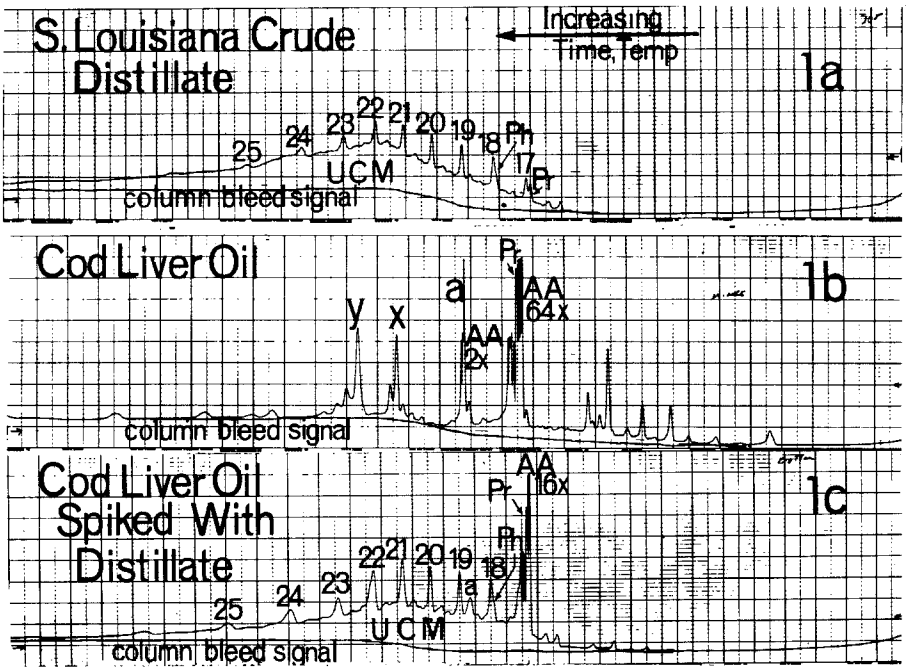
Pristane and squalene were identified by co-injection with authentic standards on the Apiezon L and FFAP GC columns, and by their retention indices before and after hydrogenation on the same GC columns.

Teal. The saponification procedure was the same as described above. Column chromatography procedure No. 1 (above) was used with the exception that the Al_2O_3 and SiO_2 were not deactivated with 5% H_2O. The quantities of hydrocarbons were determined by the GC method B (above). The GC column employed was a 3.1 m 2.2 mm i.d. 3% Apiezon L on Chromasorb W 80/100 mesh. The column was temperature programmed from 80° C to 290° C at 5°/min. Nitrogen carrier gas flow was 10-15 ml/minute. A Hewlett Packard Model 700 GC, equipped with a flame ionization detector, was used for the analyses.

Quinn. The lipid sample was placed in a 25 ml centrifuge tube equipped with a Teflon lined screw cap and saponified under nitrogen at 100°C for 30 minutes in 0.5N KOH in 80% methanol:benzene (3:1). After cooling, 5 ml of distilled water was added and the contents of the tube shaken and the phases separated by centrifugation. The benzene layer, containing the non-saponifiable lipids, was removed and the aqueous-methanol phase was twice extracted with 5 ml portions of benzene. The combined benzene extracts were evaporated under reduced pressure.

The residue was dissolved in a small volume of chloroform and applied to predeveloped (in chloroform) 0.37 mm thickness silica gel G (Merck) thin layer chromatography plates (20cm X 20 cm). The developing system employed was petroleum ether:ethyl ether:acetic acid (95:5:1). The hydrocarbon band was visualized by brief exposure

Figure 1. Gas Chromatograms of Hydrocarbons in Pentane Eluate of Column Chromatography.*



* Note that squalene does not elute in this fraction.
AA. Automatic attentunuation of detector signal
Pr- pristane; Ph-phytane; numbers -n-alkanes; a-phytadienes; x,y unknown hydrocarbons; UCM- Unresolved complex mixture. Conditions of analysis were those described for Farrington. GC column was 3% Apiezon L. Detector sensitivity set at 10^{-11} x 16.

TABLE 1

INTERCALIBRATION OF HYDROCARBON ANALYSES, IDOE - 5
(ug hydrocarbons/g cod liver lipid)

	QUINN	TEAL	FARRINGTON	AVERAGE	(s.d.	(r.s.d.))*
Peaks + Complex Mixture**	373.3	437.7	406.9	406	26.3	(6.5%)
Peaks	85.5	87.7	64.4	79.2	10.5	(13.3%)
Complex Mixture	287.8	350.0	342.5	326.8	27.7	(8.5%)
Pristane	263.5	276.0	271.8	270.4	5.2	(1.9%)
Squalene	251.7	271.0	292.1	271.6	16.5	(6.1%)
TOTAL	888.5	984.7	970.8	948.0	42.5	(4.5%)

*s.d.--standard deviation; r.s.d.--relative standard deviation
** This was the estimate of petroleum hydrocarbons. The sample was spiked with 371.8 µg petroleum hydrocarbons/g cod liver lipid. Includes peaks other than pristane and squalene.

to iodine vapors, scraped from the plate and extracted from the silica gel with chloroform:methanol (9:1). (Our experience has shown that brief exposure to iodine does not interfere with the analyses). The extract was evaporated under reduced pressure and the hydrocarbons redissolved in a small amount of CS_2. They were then analyzed on a Hewlett Packard Model 5750 GC equipped with a flame ionization detector. The column used was 1.8 m X 2.2 mm i.d. stainless steel containing 3% Apiezon L on Chromasorb W, 80/100 mesh. It was programmed from 150°C to 280°C at 6°C/min. with a nitrogen carrier gas flow rate of 10 ml/min. Quantitation of hydrocarbons was by GC method A (above).

RESULTS AND DISCUSSION

The predominant peaks in the GC's of the unspiked cod liver hydrocarbons and the spiked cod liver extract hydrocarbons are pristane and squalene. Figure 1b. shows the hydrocarbons in the pentane fractions from column chromatography for the unspiked cod liver extract. The unresolved complex mixture of the crude oil distillate present in the GC chromatogram of Figure 1a. is also present in the GC of the hydrocarbons in the spiked cod liver extract. This indicates the presence of petroleum hydrocarbons in the spiked cod liver extracts. Two further indications of the presence of petroleum hydrocarbons in this extract are the equal distribution of the n-alkanes with odd numbered carbon chains and even numbered carbon chains, and the presence of phytane. Most organisms produce a predominance of odd numbered n-alkanes while crude oil and distillate products such as fuel oil contain a fairly equal distribution of odd and even numbered n-alkanes. Phytane has not been found in shark liver and zooplankton extracts in detectable quantities (BLUMER, 1967). Phytane is present in crude oils and most distillate products.

The hydrocarbons giving resolved peaks in Figure 1b. are most likely all recently biosynthesized. Our current data on this sample does not allow a more definite statement.

The results of the analyses from three laboratories of the IDOE-5 reference sample (Table 1) show that the participants were fairly accurate in their assessment of the level of petroleum contamination and agreed very well with one another for both the petroleum hydrocarbon concentration and the concentration of the recently biosynthesized hydrocarbons pristane and squalene. The use of gas chromatography as an initial screening method to determine if samples were contaminated by petroleum hydrocarbons has been shown to be fairly accurate and precise for hydrocarbons in the boiling range 287°C - 450°C with a polarity such that they would be isolated from column or thin layer chromatography using the procedures we have set forth.

We have concentrated on gas chromatography as an initial screening method of analysis since it provides us with an estimate of both the concentration and relative amounts of recently biosynthesized and petroleum hydrocarbons in a sample. Once samples have been shown to contain the unresolved complex mixture, the sample can be analyzed further by additional column chromatography and/or thin layer chromatography to obtain several fractions of hydrocarbons--e.g. n-alkanes; naphthenes, mono-, di-, and polyaromatic hydrocarbons. U.V. spectra of selected fractions and low voltage mass spectrometry of aromatic hydrocarbon fractions gives the analyst further information with which to confirm or reject the indication of petroleum contamination provided by the initial gas chromatography screening. The employment of these methods in the analysis of an actual field sample is discussed in full by EHRHARDT (1972).

Our analytical methods, as employed, would not have detected the presence of asphaltenes; O,N,S containing aliphatic and cyclic compounds, nor would these methods have accurately determined the concentration of hydrocarbons much below or above the boiling range we specified. Thus our methods would provide only a minimum estimate of the extent of petroleum contamination of a sample unless the petroleum contamination consisted of only hydrocarbons having a boiling range and polarity range within the specified ranges. A search of the literature shows that this is the state of the art. No one method reported in the literature provides a precise and accurate determination of the petroleum contamination of a sample when such contamination extends throughout the boiling range and polarity range of the compounds present in crude oils. We suggest that reports of the petroleum contamination of marine samples include both the boiling range and polarity range within which the analytical methods employed are precise and accurate; and the boiling range and polarity range of the reported contaminants.

Further reference samples containing the several types of petroleum products spilled to the marine environment in several different matrices likely to be contaminated by these products--e.g. water, sediment, organisms with high lipid content and organisms with low lipid content--need to be prepared and then analyzed by several different laboratories. We have enough of the IDOE-5 reference sample available for at least 80 more analysts to participate in the intercalibration exercise using this sample, and we ask that interested investigators contact the senior author.

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