THE ESTIMATION OF THE AMOUNT OF EMPIRE MIX CRUDE OIL IN MULLET, SHRIMP, AND OYSTERS BY LIQUID CHROMATOGRAPHY

D. Howard Miles and Mary Jane Coign
Department of Chemistry
and
Lewis R. Brown
Department of Microbiology
Mississippi State University
Mississippi State, Mississippi

ABSTRACT

The total quantitative hydrocarbon uptake varies considerably among individual biological specimens. Moreover, total uptake varies between samples of the same type of tissue taken from specimens that have undergone identical exposure conditions. Thus, the problem of determining the uptake of oil from the environment by biological organisms under controlled conditions presents a problem that, utilizing present techniques of analysis, requires a large number of specimens for time-consuming analyses. When several different types of analyses (for enzymes, fatty acids, etc.) are required to be conducted on the same tissue sample, obviously even larger amounts of material are required, and thus, pooled samples are required.

This paper presents a method for rapidly estimating the amount of oil taken up by small (0.1 g) samples of specific tissues from mullet, shrimp, and oysters that have been subjected to exposure to Empire Mix crude oil under controlled laboratory conditions. The results obtained by utilizing the liquid chromatographic (LC) method described in this paper are compared to results obtained using conventional gas chromatographic (GC) techniques. This comparison demonstrates that either method is valid for relatively large samples of tissue from mullet, shrimp, or oysters. Data from routine bioassay experiments have shown that 50% of the samples of shrimp tissue, 23% of the mullet tissue samples, and 49% of the oyster tissue samples showed the presence of oil only by the LC method. Further, this LC method for estimating oil in these organisms affords a greater amount of replications for a given quantity of biological material and affords the opportunity to conduct multiple analyses on a given tissue.

INTRODUCTION

The requirement for a rapid and efficient method for quantitation of hydrocarbons in very small quantities of biological tissue arose in the pursuit of a long-term multidisciplinary study on the fate and effect of Empire Mix crude oil on the aquatic environment of the Gulf Coast. Even in the laboratory aspects of this study, the practical constraint of the number of organisms that can be removed for sampling without either significantly altering the test system or totally depleting the test population made it desirable that enzymatic, fatty acid, and hydrocarbon analyses be performed on the same sample; usually, the latter two analyses are run on 0.1 g samples. Furthermore, the necessity of making large numbers of analyses (100 or more) per week dictates the need for a rapid method of analysis.

A sizeable number of reports [1-18] on the estimation of hydrocarbons in the water column and in marine organisms deal with largely qualitative methods developed primarily for source identification and involve "fingerprinting." Clark [19] has noted that methods based partially (or wholly) on hydrocarbon ratios are not particularly valuable for baseline studies (or in our case, organisms obtained directly from the environment) since these ratios show wide variation. A recent report [20] of baseline studies wherein hydrocarbon concentrations have been measured in the parts per trillion range is based on methods which are not applicable to any laboratory study, since the authors report that "a single trace analysis of macoma clams requires that more than 10,000 of these clams be collected," a sampling requirement that negates any possibility of laboratory bioassays. Similarly, two methods recently presented by Clark [19] and Blaylock [21] for quantitative determination of certain hydrocarbons in marine organisms are not applicable to our program because of the sample size requirement (5-100 g), and the complexity of the method which would not allow for 100 determinations per week.

While total hydrocarbons present in the individual specimen can be determined, variance of feeding habits, physiology, etc., argues against any of the known methods accurately determining either the total hydrocarbon taken up by a specimen, or the origin of this hydrocarbon. Enzymological analyses of test and control specimens consistently have shown wide variations among individuals, which is further evidence of the inherent limitations of any previously described method directed toward determining the impact of crude oil on the biological system.

This paper presents a method for the rapid estimation of the relative amount of oil contained in small samples (0.1 g) of various tissues of certain estuarine organisms (mullet, shrimp, and oyster) that have been subjected to Empire Mix crude oil under controlled conditions. Further, it is believed that this method can lead to the development of similar methods with other oils in a variety of organisms.

Experimental

Materials and precautions to minimize contamination. All inorganic reagents (ACS reagent grade obtained from Fisher Scientific Company) and all organic solvents (pesticide grade obtained from Burdick and Jackson, Inc.) were redistilled and checked for purity by known procedures [21]. All inorganic reagents were ground and extracted with hexane. Prior to use, all glassware was cleansed in alconox, bathed in a sulfuric acid-dichromate bath, and baked in an

oven for several hours. Extreme care was taken to prevent contamination from handling. Only glass containers were used. All samples were chilled immediately and stored in a commercial freezer until needed.

Procedure. Initially all tissues were homogenized in an 0.2 M sodium phosphate and 0.25 M sucrose buffer solution for use in the enzyme analyses. All shrimp and mullet tissues were 10% homogenates while oysters were 50% homogenates due to the greater water content of the tissue. One ml of the homogenate containing 0.1 g of tissue was placed in a round bottom boiling flask with 25 ml of 0.75 M KOH in 2% aqueous MeOH and a boiling chip and refluxed overnight (15 hours). (The saponification of a sample this size takes place in 1-2 hours, but for convenience in a working lab where 100 samples a week are analyzed, the samples are refluxed overnight.)

The samples were removed from the heat and allowed to cool for a few minutes. Approximately 5 ml of hexane was carefully poured through the condenser into the receiving flask to wash the interior of the condenser. The mixture in the flask was then transferred to a Teflon-stoppered separatory funnel and extracted 4 times with 15-20 ml of hexane to remove the hydrocarbons. (The amount of hexane used in larger samples is 1/4 to 1/3 of the volume of the KOH-MeOH solution to be extracted.) The KOH-MeOH layer contains the fatty acids as potassium salts. This solution was acidified to a pH of 3 and extracted with $CHCl_3$ for analyses of the fatty acids.

The combined hexane extracts then were washed with distilled water and evaporated in vacuo to approximately 2 ml. The concentrated extract was placed on a glass column (20 cm × 1.0 cm ID) with a 70-100-μ-fritted disc packed with 4.5 g 60-200 mesh Activity I silica gel and topped with 2.5 g 80-200 mesh Activity I neutral alumina. The column was packed in hexane and washed with 30 ml of hexane before the extract was placed on the column. The concentrate was washed onto the column with approximately 5 ml of hexane and eluted with 75 ml of hexane. (This eluate contains the aliphatic hydrocarbons.) The column was eluted with 85 ml of benzene to collect the aromatic hydrocarbons. Both fractions were evaporated in vacuo to approximately 1 ml and transferred to small vials where the remaining solvent was removed under nitrogen.

The aliphatic hydrocarbon fraction was analyzed using a Beckman GC-45 with a flame ionization detector and a 6' × 1/8" OD stainless steel 3% SE-30 on 80-100 mesh Chromasorb W column. The injector and detector temperatures were 300°C, and the column temperature was programmed from 100 to 300°C at a rate of 3° per min. Qualitative identification of the components was achieved by comparison of retention times to known standards. The quantitative measurement of the total oil in the sample was calculated from the area of the n-C_{16} peak. Testing with Empire Mix crude oil yielded a ratio of 7.69 μg of total oil per 1 cm^2 area of n-C_{16}.

Therefore the calculation of total oil in the unknown sample would be: (area of n-C_{16} peak in cm^2) (7.69 μg oil/1 cm^2) = μg oil.

The aromatic hydrocarbon fraction was analyzed using a Waters Associates Model 202/402 liquid chromatograph with a Model 6000 solvent delivery system and a UV detector. A 4' × 1/8" OD Bondapak C_{18}/Corasil column was used with a MeOH/H_2O (70:30) solvent system at a flow rate of 2.0 ml/min. A solution of 0.895 μg/μl of hexachlorobenzene in CCl_1 was used as in internal standard. Hexachlorobenzene is not known to occur in oil and has a retention time greater than the oil components. The sample was redissolved in 10 μl of solution and 5 μl was injected into the LC.

The retention time and height of peak 3 and the standard hexachlorobenzene peak are measured. Total oil is then calculated by the following method. (1) Multiply the retention time by the height. (2) Divide the product from peak 3 by the product from the hexachlorobenzene peak. (3) In order to calculate the amount of oil in the sample it is first necessary to establish the ratio of the tricyclic aromatics to the standard (hexachlorobenzene) when a known amount of the crude oil is added to a control sample. (For example, this factor for the Empire Mix crude oil employed in this study was 505/1.41 or 358.16 as shown in table 1.) This factor would vary from oil to oil. (4) Therefore, the calculation of total oil in the unknown sample would be

(ratio from step 2) (factor from step 3) = μg of oil

Table 1. Standardization of gas and liquid chromatography methods for determination of oil in biological samples

		LC		GC	
OIL ADDED	SAMPLE	Peak 3 / STD* RATIO	AVERAGE RATIO	Area of n-C_{16} Peak (cm^2)	Average Area (cm^2)
202 ug	Shrimp muscle	0.75		7.65	
202 ug	Shrimp Guts	0.39	0.58	9.90	9.15
202 ug	Shrimp Guts	0.60		9.90	
505 ug	Shrimp Muscle	1.55		22.95	
505 ug	Shrimp Guts	1.13	1.41	28.35	23.34
505 ug	Mullet Guts	1.52		22.68	
505 ug	Shrimp Guts	1.41		18.99	
1010 ug	Shrimp Muscle	2.78		40.05	
1010 ug	Shrimp Guts	2.92	2.82	44.55	48.15
1010 ug	Mullet Guts	2.64		57.60	
1010 ug	Shrimp Guts	2.92		50.40	

*Hexachlorobenzene

Methodological validation. It was noted by GC analyses that mullet and shrimp which had been exposed to oil contained several hydrocarbons such as n-C_{16} that normally were not present in control organisms. For this reason the GC method for determining total oil is based on quantitizing the n-C_{16} peak followed by a comparison with standards (see table 1). The GC method for total oil determination is given to afford a comparison with the LC technique presented in this paper.

Biological samples were divided into four equal portions with one portion to serve as control while known quantities (202, 505, 1010 μg) of oil were added to the other portions as described in table 1. The aliphatic fractions obtained by the normal extraction procedures were analyzed by the GC method and the aromatic fractions were analyzed by the LC method. The results obtained in table 1 show that either method is valid for relatively large samples (~5 g) to which fresh Empire Mix crude oil has been added.

Results and Discussion

When living mullet and shrimp were exposed to low levels of oil in laboratory bioassay tests, the amount of aliphatic hydrocarbons remaining in test organisms after 96 hours of exposure was below the level of detectability of the gas chromatograph on samples derived from a single test organism. It should be noted that the sample size was extremely small with one shrimp gut or one mullet brain weighing approximately 0.1 g. (The mullet ranged in length from ~12 to 18 cm.) In contrast, the LC method is more sensitive because of the utilization of a UV detector at a wavelength of 254 nm. The aromatic hydrocarbons have a high extinction coefficient and can be detected at levels as low as one nanogram [22]. An additional advantage is that since only those materials that absorb UV light are detected, interference by aliphatic hydrocarbons is not a factor. This is important since most contaminants from the solvent, vacuum lines, fingerprints, lubricating oil, and organisms are aliphatic hydrocarbons.

Figures 1-5 are illustrative of data obtained by the LC method. Figure 1 shows oil alone (1a), shrimp gut (1b), and shrimp gut spiked with oil (1c). It is obvious that the shrimp gut without added oil does not have peaks characteristic of oil. Also the profile of the spiked sample is essentially that of oil alone. Since injection of the sample into the LC currently being employed requires injection against pressure, it is difficult to place the same amount of sample on the LC column each time. Also, it is impossible to obtain the same retention time even from consecutive injections. Therefore, an internal standard was employed to obviate this problem. Figure 2 shows that the standard does not camouflage the normal peaks in the oil or organism chromatograms. Of particular significance in the chromatograms is the oil peak number 3.

Peak number 3 was chosen as the basis of the quantitation method because it is the largest, most persistent (in oysters), and never detected in control specimens. Furthermore, compounds making up this peak have been shown to be the most resistant to microbial attack (see figure 6). It is obvious from comparison with oil alone (figure 1a) that peak 1 has disappeared, peak 2 has been significantly diminished, and that peak 3 has remained essentially unchanged.

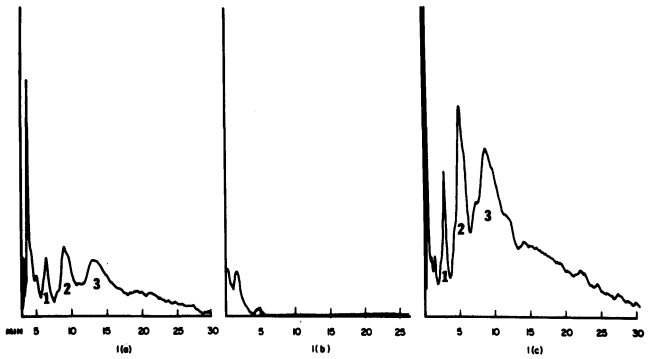

Figure 1. Typical liquid chromatograms of Empire Mix crude oil, normal shrimp gut sample, and normal shrimp gut sample with Empire Mix crude oil added. (1a) Empire Mix crude oil. (1b) Normal shrimp gut sample. (1c) Normal shrimp gut sample with Empire Mix crude oil added

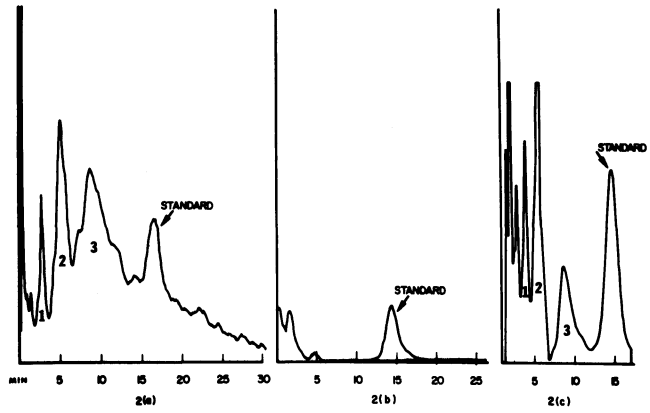

Figure 2. Typical liquid chromatograms with internal standard added of Empire Mix crude oil, normal shrimp gut, and normal shrimp gut with Empire Mix crude oil added. (2a) Empire Mix crude oil. (2b) Normal shrimp gut sample (2c) Normal shrimp gut sample with Empire Mix crude oil added

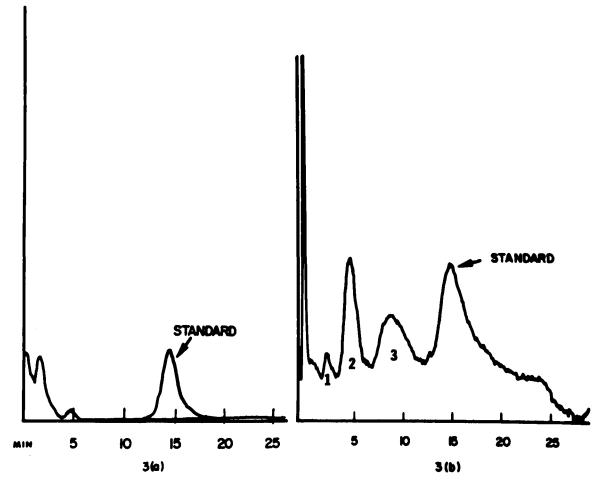

Figure 3. Typical liquid chromatograms of shrimp gut samples obtained from laboratory bioassay tests. (3a) Shrimp gut sample obtained from nontreated shrimp. (3b) Shrimp gut sample obtained from shrimp subjected to 8 ppm Empire Mix crude oil for 18 hours

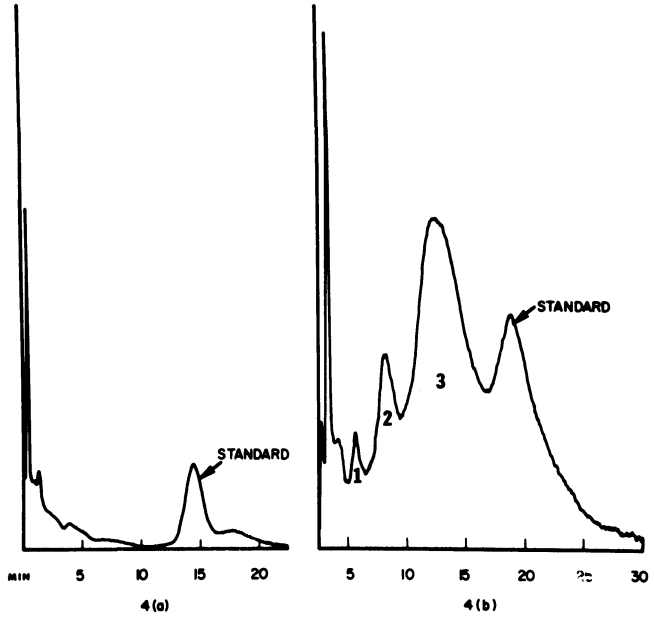

Figure 4. Typical liquid chromatograms of shrimp muscle samples obtained from laboratory bioassay tests. (4a) Shrimp muscle sample obtained from nontreated shrimp. (4b) Shrimp muscle sample obtained from shrimp subjected to 4 ppm Empire Mix crude oil for 18 hours

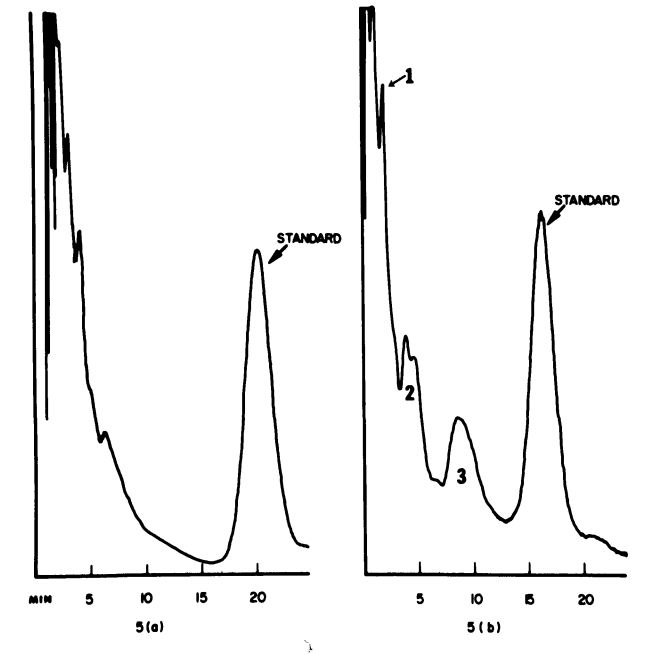

Figure 5. Typical liquid chromatograms of mullet muscle samples obtained from laboratory bioassay tests. (5a) Mullet muscle sample obtained from nontreated mullet. (5b) Mullet muscle obtained from mullet subjected to 75 ppm Empire Mix crude oil for 18 hours

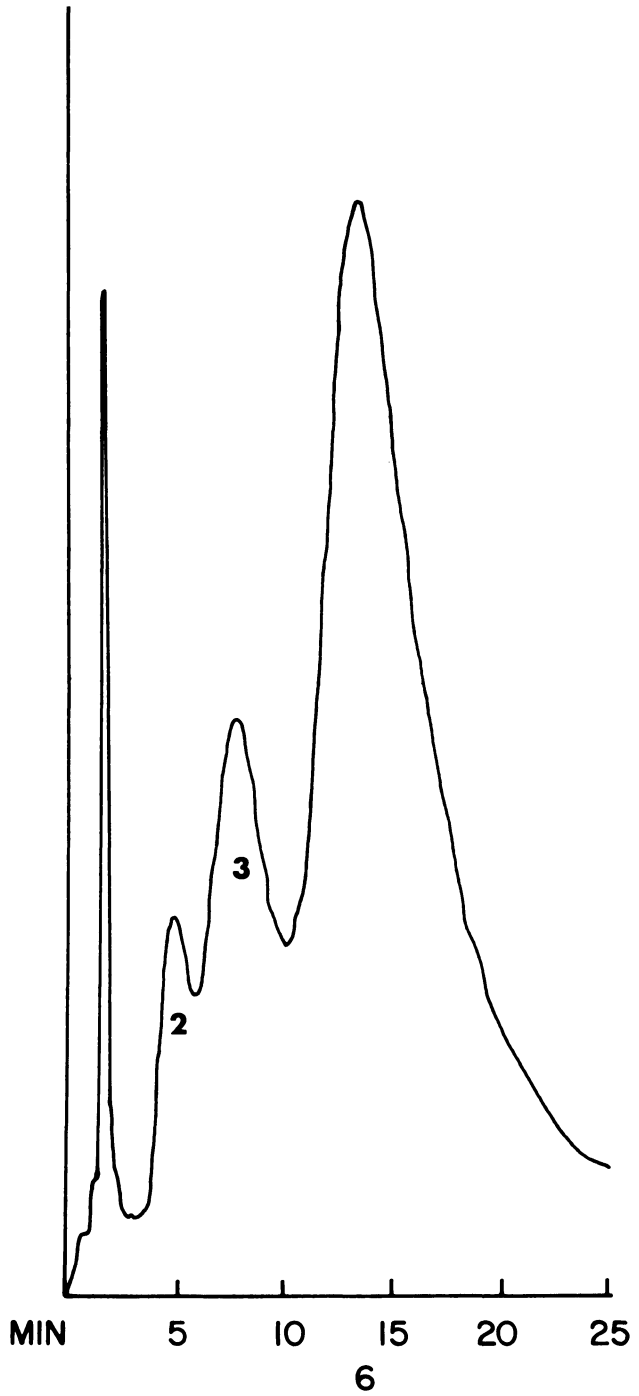

Figure 6. Liquid chromatogram of Empire Mix crude oil after microbial exposure

The composition of the aromatic hydrocarbon fraction was determined by the utilization of an integrated GC-mass spectrometer in a manner previously described [23]. Structural assignments were made with the aid of the atlas of mass spectra data [24] and subsequent comparison of possible structures with compounds known to be present in crude oil [25]. On this basis, the Empire Mix crude oil contains the aromatics listed in table 2 and the potential carcinogens listed in table 3. As many of these compounds as possible were obtained as standards for the purpose of determining their LC retention time. This allowed the obtainment of the data presented in table 4 which indicates some of the components present in the various peaks in the LC trace. These data demonstrate that we are quantitating total oil based on a peak which is composed of a number of compounds including several potential carcinogens [26].

Table 2. Aromatic constituents of Empire Mix crude oil as determined by mass spectrophotometry

Peak Number	Constituents	Mass
1-	Benzene	(78)
2-	Toluene	(92)
3-	Ethyltoluene	(120)
	Isopropylbenzene	"
	Trimethylbenzene	"
4 & 5-	Butylbenzene	(134)
	Propyltoluene	"
	Dimethyl-ethylbenzene	"
	Diethylbenzene	"
	Cymene	"
	Ethyl-xylene	"
6-	Diethyltoluene	(148)
	Ethylcumene	"
	Methylbutylbenzene	"
	Butyltoluene	"
7-	Methylindan	(132)
8-	Methylindan	(132)
9-	Methylindene	(130)
10-	Phenylhexane	(162)
	Phenyl-methylpentane	"
	Dimethyl-butylbenzene	"
	isomers of 3,4 & 5	120 & 134
11-	Hexamethylbenzene	(162)
	Butyl-ethylbenzene	"
	Dimethyl-diethylbenzene	"
	Triethylbenzene	"
	Trimethyl-isopropylbenzene	"
12-	same as #10 & #11	(162)
13-	Naphthalene	(128)
14-	Phenylcyclohexane	(160)
	Trimethylindan	"
15-	1-Methylnaphthalene	(142)
16-	2-Methylnaphthalene	(142)
17-	Tetramethylindan	(174)
	Tetrahydro-trimethylnaphthalene	"
	Trimethyl-tetrahydranaphthalene	"
18-	Dimethylnaphthalene	(156)
	Biphenyl	(154)
19-	Ethylnaphthalene	(156)
20-	Fluorene	(166)
	Diphenylmethane	(168)
	Biphenylmethane	"
	Methylbiphenyl	"
21-	Phenyl ether	(170)
	Diphenyl ether	"
	Diphenylethane	(182)
	Ethylbiphenyl	"
22,23 & 24	Methyl-ethylnaphthalene	(170)
	Dihydro-dimethylthieno-thiophene	"
	Propyl-methylnaphthalene	(184)
25-	Ethyl-ethylnaphthalene	(184)
	Ethyl-dimethylnaphthalene	"
	Propyl-methylnaphthalene	"
26, 27 & 28	Di-butylthiophene	(196)
	Propyl-isoamythiophene	"
	Dihydro-methyl-phenylbenzofuran	(210)
	Allyl-biphenyl ether	"
	Propyl-Hexylthiophene	"
	Dimethylnaphtho-thiophene	(212)
	Diethyl-terephthalate	(222)

Table 3. Potentially carcinogenic compounds present in Empire Mix crude oil as determined by mass spectrophotometry

Compound	Mass
1,2-Benzanthracene 1,2-Benzphenanthrene (Chrysene) 3,4-Benzphenanthrene 9,10-Benzphenanthrene	228
1-Methylchrysene (4 isomers) 1-Methylbenzphenanthrene (5 isomers) 1-Methylbenzanthracene (11 isomers) 10-Methyl-1,2-benzanthracene	242
3,4-Benzpyrene	252
9,10-Dimethyl-1,2-benzanthracene 5,8-Dimethylbenzphenanthrene	256
20-Methylcholanthrene	268
1,2,3,4-Dibenzanthracene 1,2,5,6-Dibenzanthracene 2,3-Dibenzphenanthrene 6,7-Dibenzphenanthrene 3,4,9,10-Dibenzpyrene	278
9,10-Dimethyl-1,2,5,6-Dibenzanthracene	306
9,10-Diphenylanthracene	330

* As reported by ZoBell[26]

The value of this method has been proven in routine bioassay experiments. Figures 3-5 give typical LC chromatograms of tissues from these tests. It is noteworthy to point out that 50% (37 of 74) of the tissue samples obtained from shrimp exposed to oil showed the presence of oil only by the LC method and not by the GC method. Similarly, 23% (103 of 441) of the oil-exposed mullet and 49% (105 of 213) of the oil-exposed oysters showed the presence of oil only by the LC method. The majority of the samples which showed oil only by LC consisted of ~0.1 g of shrimp gut, mullet liver, or mullet brain. Oil can be seen in muscle tissue by either method because of the relatively large amount of sample available. Thus a rapid method for detecting and subsequently estimating oil in small samples of biological materials has been developed. This is especially important in the design and conduct of bioassay experiments because it allows for either (1) a greater amount of replication for a given quantity of biological material or (2) affords the opportunity to conduct a multiplicity of analyses on a given tissue or pool of tissues. For example, approximately 2 grams of shrimp guts requiring pooling of 20 shrimp are normally required for detecting and estimating oil in this tissue by the GC method. In contrast, the newly developed LC method allows the use of only one shrimp gut for the same analysis. Furthermore, a shrimp gut sample of only 0.2 g (pool of 2 shrimp guts) will allow for the three separate analyses which include total oil by LC, fatty acids, and enzymes.

ACKNOWLEDGMENTS

This work as supported by the Environmental Protection Agency Contract No. 68-01-0745, "Fate and Effect of Oil in the Aquatic Environment-Gulf Coast Region." Special thanks go to Beth Cade and Georgia Monnerat for their invaluable assistance.

Table 4. Liquid chromatographic distribution of some of the aromatic components of Empire Mix crude oil

Preliminary Peaks	Peak # 1	Peak # 2	Peak # 3
Benzene	Dimethylnaphthalene	Pyrene	1, 2-Benzanthracene
Ethylbenzene	Anthracene	Phenylcyclo-hexane	2,3-Benzfluorene
Diethylbenzene	Fluorene	Diphenylbenzene	9,10-Dimethyl-anthracene
Cumene	Phenanthrene	Fluoranthene	
Cymene	Benzanthracene	1,2,3,4-Dibenzanthracene	
Diisopropylbenzene	Chrysene	3,4,9,10-Dibenzpyrene	
Trimethylbenzene	Durene		
Tert-butylbenzene	9,10-Diphenyl-anthracene		
Triethylbenzene	20-Methylchlanthene		
Tetrahydrothiophene			
Naphthalene			
p-Terphenyl			
Phenyl Ether			
Tetrahydronaphthalene			
1-Methylnaphthalene			
2-Methylnaphthalene			
Acnenaphthalene			
Dimethylnaphthalene			

*As determined by LC of standard compounds.

REFERENCES

1. Gruenfeld, Michael. 1973. *Environ. Sci. Technol.* 7:636.
2. Mattson, J.S. 1971. *Anal. Chem.* 43:1973.
3. Kawahara, F.G. 1969. *Environ. Sci. Technol.* 3:150.
4. American Petroleum Institute. 1970. *J. Inst. Pet.* 46:548.
5. Adlard, E.R.; Creaser, L.F.; Mathews, P.H.P. 1972. *Anal. Chem.* 44:64.
6. Adlard, E.R. 1972. *J. Inst. Pet.* 58 (560):63.
7. Thruston, A., and Knight, R.W. 1971. *Environ. Sci. Technol.* 5:64.
8. Ehrhardt, M., and Blumer, M. 1972. *Environ. Pollut.* 13:179.
9. McAuliffe, C. 1963. *Nature* 200:1092.
10. McAuliffe, C. 1969. *Science* 158:478.
11. Blumer, M.; Guillard, R.R.; Chase, T. 1971. *Marine Biology* 8:183.
12. Blumer, M.; Sanders, H.; Grassle, T.; and Hamsson, G. 1971. *Environment* 13:2.
13. Blumer, M.; Souza, G.; Sass, J. 1970. *Marine Biology*, 5:195.
14. Cole, R.D. 1971. *Nature*, 233:546.
15. Zitko, V. 1971. *Bull. of Environ. Contam. and Toxi.* 5(6):559.
16. Walker, J.P., and Cooney, J.J. 1973. *Appl. Micro.* 26:705.
17. Farrington, J.W., et al. 1973. *Bull. Environ. Contam. Toxi.* 10:129.
18. Blumer, M.; Myers, J.; and Zafiriou, O. 1972. Woods Hole Oceanographic Institution, July, 1972, ref. therein.
19. Clark, R.C., Jr., and Finley, J.S. 1973. *Proc. of Joint Conf. on Prevention and Cont. of Oil Spills*, p. 161. Washington, D.C.: American Petroleum Institute.
20. Dimension/NBS, February 1974, p. 36-37.
21. Blaylock, J.W.; O'Keefe, P.W.; Roehm, J.N.; and Wildung, R.E. 1973. *Proc. of Joint Conf. on Prevention and Cont. of Oil Spills* p. 1973. Washington, D.C.: American Petroleum Institute.
22. Dark, W.A., and Crossman, L.W. 1973. Waters Associates, Inc., Framingham, Mass.
23. Miles, D.H.; Mody, N.V.; Minyard, J.P. and Hedin, P.H. 1973. *Phytochem* 12:1399.
24. Stehagen, E.; Abrahamsson, S.; McLaFFerty, F.W.; 1969. *Atlas of Mass Spectral Data* New York: Wiley.
25. Mair, D.J. 1964. *The Oil and Gas Journal* Sept. 14, 1964.
26. ZoBell, Claude E. 1971. *Proc. of Joint Conf. on Prevention and Cont. of Oil Spills,* p. 441. Washington, D.C.: American Petroleum Institute.