A Comparison of Methods for Hydrocarbon Analysis of Marine Biota

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Introduction

The analysis of biological materials for hydrocarbons requires the use of sample preparation procedures for the isolation of hydrocarbons from other matter and for the fractionation of hydrocarbons according to chemical type. Isolation procedures more commonly in use include Soxhlet extraction (Blumer et al., 1970), manual extraction (Stegeman and Teal, 1973), and alkaline digestion followed by manual extraction (Farrington et al., 1973). Various modifications of these and other techniques have been reviewed by Clark (1974). Fractionation methods include column chromatography (Blumer et al., 1970), thin layer chromatography (Hunter et al., 1974) and post-analysis computer manipulation of mass spectral data (Chesler et al., 1976).

As a preliminary to measurements of hydrocarbons in marine biota of Alaskan waters, we carried out a comparison of several of these procedures to determine which is most satisfactory for use in our laboratory. We do not wish to imply that the procedures which we have selected as a result of this study should be adopted as a standard method. While we recognize the need for interlaboratory calibration and comparison, we also believe that flexibility and continued method development are necessary. Furthermore we believe that environmental studies of which hydrocarbon measurements are part, are seeking knowledge that is in essence ecological, not chemical in nature. Highly precise and accurate descriptions of concentrations and molecular constitutions of environmental hydrocarbons are only of academic interest unless that information can be related to biological effects. In our opinion the lack of uniform methodology in chemical analysis at present is by no means the weakest link in the chain of knowledge needed to establish a causal connection between hydrocarbon pollution and its ecological effects. Therefore we present our results in the hope that they will guide others to analytical procedures best suited to their particular needs.

We have compared the efficiency of Soxhlet extraction followed by saponification with extraction by alkaline digestion (direct saponification). For the latter extraction procedure, we compared the effectiveness of two hour and 24 hour reaction times. Each of the procedures was performed on approximately 60 g of soft parts of *Saxidomus gigantea* (butter clam) and repeated with a similar weight of tissue spiked with a mixture of aliphatic and aromatic hydrocarbons as shown in Table 1. Each of the extracts was split and subjected to column chromatography on alumina packed over silica using both fully activated and partially deactivated sorbents. This experimental scheme is shown in Table 2.

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TABLE 1: Concentrations of hydrocarbons in hexane spike solution.

COMPOUND	CONCENTRATION mg/ml
hexadecane	0.046
docontane	0.038
dotriacontane	0.028
naphthalene	0.044
anthracene	0.034
chrysene	0.048
perylene	0.042
TOTAL HYDROCARBONS	0.280

Methods

For each analysis, approximately 60 g of tissue (3 clams) was cut into small pieces and macerated in 100 ml methanol with a Virtis homogenizer. To spiked samples 1.0 ml of the hydrocarbon solution described in Table 1 was added. Before Soxhlet extraction of a sample, cellulose thimbles were extracted for 24 hours with 300 ml of an equal parts mixture of methanol and benzene. This solvent was discarded and replaced with fresh solvent. After another 24 hour extraction, the solvent was concentrated on a rotary evaporator to less than 1.0 ml. This concentrate was checked by gas chromatography (GC) to establish freedom from contamination. The macerated samples were added to the clean extraction thimbles with 50 ml methanol and 150 ml benzene (total solvent volume 300 ml). The Soxhlet extraction was run for 48 hours (approximately 150 extraction cycles).

Either directly after maceration, or after Soxhlet extraction, 100 ml 1N KOH in methanol and 50 ml H_2O were added. This mixture was saponified for either two or 24 hours, after reflux was attained. The pH of the saponified sample was checked to be sure it was greater than 10. In other samples run by this procedure, the Soxhlet extract had been concentrated to approximately 100 ml before saponification. An oily residue formed when the *S. gigantea* samples were concentrated, so nearly the entire 300 ml was saponified.

After saponification the samples were extracted three times with 100 ml hexane. The combined hexane extracts were washed with 150 ml saturated aqueous NaCl. The hexane extract was dried overnight with Na_2SO_4 and then concentrated to 10 ml. A 100 µl aliquot was air dried and weighed on an electrobalance to determine total weight of nonsaponifiable lipid.

Columns of 10 ml Al_2O_3 (Harshaw Chemical Company AL-0102P) packed over 20 ml SiO_2 (Davidson Chemical grade 922, 200-325 mesh) were used. The columns were 11 mm in diameter by 35 cm. The alumina and silica were prepared according to Farrington et al., (1972) for partially deactivated sorbents. For fully active columns the deactivation step was omitted. An adsorbent to lipid ratio of approximately 1000:1 by weight was used. For these samples that was 20 mg, 20% of the lipid extract. The final volume of samples charged to the column was adjusted

TABLE 2: Experimental scheme for biological methods comparison

Extraction Method	Extraction Time	Spiked?	Column Activation
Alkaline Extraction	2 hours	yes	full
			partial
		no	full
			partial
	24 hours	yes	full
			partial
		no	full
			partial
Soxhlet Extraction	48 hours followed by 2 hour saponification	yes	full
			partial
		no	full
			partial

to less than 1.0 ml. A 40 ml hexane elution volume and a 40 ml benzene elution volume were collected (four pore volumes of each solvent). The eluates were concentrated to approximately 1.0 ml and the volume precisely measured. For these analyses the concentration of total hydrocarbons was approximately 20 mg g^{-1} based on wet weight of tissue extracted.

A Hewlett Packard model 5710A with flame ionization detector was used for GC analysis. The columns were 1/8" x 12' stainless steel packed with 3% OV 101 on Chromosorb W 100-120 mesh AW-DMCS. The temperature program was 80° to 280° at 8° per minute. The carrier gas was helium at 50 ml per minute. Mass spectral analyses of several of the extracts were performed with a Hewlett Packard model 5930/5933 gas chromatograph-mass spectrometer-data system (GC-MS).

Results and Discussion

From these experiments we have obtained information about the efficiency of the various procedures, about the completeness with which esters are removed by two hour and 24 hour saponification, about the separation characteristics of fully activated and partially deactivated column sorbents, about the extent of contamination introduced by the various procedures and about the relative convenience of performing the procedures.

The percentage recovery of hydrocarbons added to samples of clam meats is shown in Table 3. These spiked samples were subjected to the complete analytical procedures and the recoveries determined by measuring peak heights in the resulting gas chromatograms. The recoveries of the added aromatic hydrocarbons could not be determined because, even using chromatograms of unspiked samples for comparison, the size of the added peaks was obscured by superposition of peaks from the sample. The bottom two lines of data in Table 3 labeled "Chromatography of Spike Solution" give the recoveries of the spike solution from column chromatography alone.

Table 3 indicates that similar recoveries of added alkanes are obtained by the three extraction methods. The table also shows that fully activated columns give slightly better recoveries of both alkanes and aromatics than do the partially deactivated columns. It is noteworthy that the recoveries of alkanes from column chromatography alone are no better than the recoveries from the full extraction procedures. We reject the explanation that all other steps of the extraction procedures are quantitative; instead we suspect that in the presence of clam lipids the recoveries from column chromatography are higher. This higher recovery could be the result of binding of polar lipids to retentive sorbent sites. In the absence of non-hydrocarbon lipids, such sites would irreversibly remove hydrocarbons.

Table 2 shows that a total of eight samples were subjected to saponification for two hours (either directly or following Soxhlet extraction). The benzene eluates from column chromatography of five of the eight were analysed by GC-MS. Of these five, three showed the presence of methyl esters. Among the four samples subject to saponification for 24 hours, the benzene eluates of two were given GC-MS analysis. Neither show evidence of esters. The absence of esters has generally been confirmed in subsequent analyses of extracts that have been saponified for 24 hours. We have not investigated the monitoring of the completeness of saponification by infrared spectroscopy; however, it is clear from

TABLE 3: Recovery of added hydrocarbons

		Percentage Recovery						
Extraction Method	Column[a] Type	Hexa-decane	Doco-sane	Dotria-contane	Naphth-alene	Anthra-cene	Chry-sene	Pery-lene
2 hour saponification	PD	53	55	68	---	---	---	---
	FA	13[b]	80	79	---	---	---	---
24 hour saponification	PD	72	100+[c]	65	---	---	---	---
	FA	92	81	120[d]	---	---	---	---
Soxhlet	PD	79	64	69	---	---	---	---
	FA	109	104	124[d]	---	---	---	---
Chromatography of spike solution	PD	77	80	81	04	45	40	44
	FA	93	92	96	26	53	51	63

[a] FA=fully active, PD=partially deactivated

[b] Sample evaporated to dryness after column chomatography

[c] Peak appears as shoulder, baseline is uncertain

[d] Spike peak superimposed on sample peak, baseline is uncertain

this study that following pH is not a reliable guide in this respect.

Comparison of gas chromatograms of samples which had been liquid chromatographed on fully active and partially deactivated columns showe that several peaks which appeared in the hexane eluate using partially deactivated columns appeared completely or in part in the benzene eluat if fully active columns were used. Mass spectral analysis of some of these peaks indicated that they were hydrocarbons with 25 to 30 carbon atoms and six to eight units of unsaturation. Table 4, which shows the concentrations of hydrocarbons eluting in the column chromatography usi fully active and partially deactivated sorbents, indicates that these variable alkenes are the major hydrocarbon constituents of *Saxidomus gigantea*.

TABLE 4: Concentration of Hydrocarbons mg g^{-1}

Distribution of hydrocarbons between the first and second column chromatographic eluate fractions. Data shown are for extract prepared by 24 hour saponification. Other extraction procedures gave similar results.

	Fully Active	Partially Deactivated
First Fraction	0.5	16
Second Fraction	22.0	3.3
TOTAL	22.5	19.3

Contamination introduced during analysis was satisfactorily low foı all procedures. Blank analysis indicated that background from all sources was always at concentrations less than one percent of the sampl total hydrocarbon concentrations.

In terms of laboratory convenience, the procedure of Soxhlet extrac tion followed by saponification is inherently slightly inferior to dire saponification since the former requires two separate operations. Howe er, this consideration is minor compared to others.

A drawback to the Soxhlet extraction procedure is the lengthy thimk cleaning step. We are aware that some investigators reduce the need fc this step by re-using thimbles without intermediate cleaning. Our limi ted experience with re-using thimbles has indicated that memory effects can be a problem unless only samples of approximately uniform hydrocarbon concentration are extracted. The cleaning requires both time and solvents. Since we have not found a totally reliable commercial suppli of "contamination free" solvents, we re-distill all solvents and analys every one gallon batch. Thus, the use of 600 ml of solvents to clean each thimble requires a significant supporting distillation effort. Tl added time of this cleaning further means that the entire Soxhlet extra tion procedure requires at least one week.

Another important difference between the procedures is the tendency of extract solution to form an emulsion when extracted with hexane. By far the worst emulsions were formed when the two hour saponification was used. These were sometimes stable for days and drastically increased the time and effort required for liquid:liquid extraction. The 24 hour saponification and extraction followed by two hour saponification each gave much less troublesome emulsions than the two hour saponification. The Soxhlet extraction was slightly superior to the 24 hour saponification. Subsequent work has indicated that if the 24 hour saponification is performed on diced by non-macerated tissue, hydrocarbon recovery is not affected but the emulsion problem is further reduced.

Conclusions

We judge that 24 hour saponification and column chromatography on partially deactivated columns constitute the best procedure tested. Soxhlet extraction followed by two hour saponification did not completely remove methyl esters and was the most laborious technique. Direct two hour saponification was also questionable in the removal of esters and led to severe emulsion problems. The recoveries of hydrocarbons by the three procedures were substantially the same. Although the recoveries from fully active columns were slightly superior to those from partially deactivated columns, the latter were selected to avoid the possibility of alkene isomerization (Blumer et al., 1972) which may be a particular problem in the analysis of alkene rich biological materials. Both fully active and parially deactivated columns consistently gave a clean separation of alkanes from aromatics. However, several alkenes which eluted in the first (alkane) fraction when partially deactivated columns were used, appeared partially or totally in the second (aromatic) fraction when fully active columns were employed. This behavior is not surprising in view of intermediate chemical character of alkenes. While this situation will cause little problem in heavily polluted samples in which alkenes are only a small percentage of the total hydrocarbons, in unpolluted biological materials where alkenes may be prominent, classification of molecules as alkanes or aromatics based only on column chromatographic behavior must be done with caution. This is illustrated by the data of Table 4. For this latter case more elaborate chromatographic procedures or further analysis by GC-MS appear necessary.

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REFERENCES

Blumer, M., G. Souza, and J. Sass. 1970. Hydrocarbon Pollution of Edib Shellfish by an Oil Spill. Marine Biology 5:195-202.

Stegeman, J. J. and J. M. Teal. 1973. Accumulation, Release and Retent of Petroleum Hydrocarbons by the Oyster *Crassostrea virginica*. Marii Biology 22:37-44.

Farrington, J. W., J. M. Teal, J. G. Quinn, T. Wade, and K. Burns. 1973 Intercalibration of Analyses of Recently Biosynthesized Hydrocarbons and Petroleum Hydrocarbons in Marine Lipids. Bulletin of Environmental Contamination and Toxicology 10:129-136.

Clark, R. C. 1974. Methods for Establishing Levels of Petroleum Contamination in Organisms and Sediment as Related to Marine Pollution Monitoring. *In* Marine Pollution Monitoring (Petroleum) Proceedings of a Symposium. National Bureau of Standards Special Publication No. 409. p. 189-194.

Hunter, L., H. E. Guard, and L. H. DiSalvo. 1974. Determination of Hydrocarbons in Marine Organisms and Sediments by Thin Layer Chromatography. *In* Marine Pollution Monitoring (Petroleum) Proceedings of a Symposium. National Bureau of Standards Special Publication No. 409. p. 213-216.

Chesler, S. N., B. H. Gump, H. S. Hertz, W. E. May, S. M. Dyszel, and D. Enagonio. 1976. Trace Hydrocarbon Analysis. The National Bureau of Standards. Prince William Sound/Northeast Gulf of Alaska Baseline Study. National Bureau of Standards Technical Note 889. p. 28.

Farrington, J., C. S. Gaim, G. R. Harvey, P. Parker, and J. Teal. 1972. Analytical Techniques for Selected Organic Compounds. *In* E. D. Goldberg (ed.) Marine Pollution Monitoring. Strategies for a National Program. Allan Hancock Foundation, Los Angeles. p. 163.

Blumer, M., P. C. Blokker, E. B. Cowell, and D. F. Duckworth. 1972. Petroleum. *In* Goldberg, E. D., ed. A Guide to Marine Pollution. Gordon and Breach, New York. p. 29.