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FRACTIONATION OF POLAR ORGANIC CONSTITUENTS IN ENVIRONMENTAL SAMPLES USING THE LIPOPHILIC DEXTRAN GELS SEPHADEX LH-20 AND SEPHASORB HP ULTRAFINE

APPLICATION TO A WEATHERED EKOFISK CRUDE OIL

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SUMMARY

A method has been developed for the enrichment and fractionation of trace amounts of polar organic constituents in environmental samples. The procedure involves two steps using the lipophilic gel filtration gels Sephadex LH-20 and Sephasorb HP Ultrafine in two different modes: (1) separation and isolation of alcohols, phenols, hydroperoxides, acids and highly polar components as a result of retardation of compounds containing hydroxyl groups on Sephadex LH-20 using stepwise gradients of chloroform, methanol and tetrahydrofuran as the mobile phase; (2) liphophilic–hydrophilic partitioning using Sephasorb HP Ultrafine swollen in methanol–water and eluted with stepwise gradients of *n*-hexane and benzene, which separates non-hydroxyl-containing constituents.

The method leads to essentially quantitative recoveries of the different compounds, and is sufficiently rapid and convenient for use in routine analysis. Minimal degradation of the sample components occurs as this is a pure chromatographic procedure on highly inert gel matrices with no catalytic activity, avoiding the acid/-alkali extractions and chromatography on more labile gel matrices that often accompany other procedures and usually alter the sample to some extent.

The application of the method to a weathered Ekofisk crude oil is described.

INTRODUCTION

Organic constituents from environmental samples, air, water, biota, petroleum and sediments are characteristically complex mixtures of hydrocarbons, alcohols, aldehydes, acids, phenols and pigments. The many mechanisms that act on such constituents in the biosphere result in environmental samples that are so complex that the extracts must be simplified into subfractions prior to instrumental analysis.

Some basic requirements have to be met when developing a method for the fractionation of natural samples into classes. The method should be: quantitative and require a minimal number of operations to avoid manipulative losses[1]; equally efficient over wide molecular weight ranges for a variety of multi-component mixtures

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of different polarities[2]; direct and time-efficient[3]; and free of potential contamination because many ubiquitous contaminants may be the compound of interest[4].

The classical adsorbents silica and alumina are often disadvantageous in the separation of complex mixtures[5]. The fractionation is insufficient, leading to a large unresolved complex mixture (UCM) in high-resolution gas chromatography, their adsorptivity often results in losses of trace constituents, and the reproducibility is often impaired by the modification of the adsorbents with trace amounts of water or other compounds present in the samples. In addition, catalytic activity is often a serious problem in the analysis of oxidized aromatic compounds[6].

This paper describes our observations on the utility of Sephadex LH-20 and Sephasorb HP Ultrafine gel chromatography for the class fractionation of polar constituents in environmental samples, using a weathered Ekofisk crude oil as an example.

EXPERIMENTAL

Chemicals

The following commercial standard compounds were used without further purification: benzene, 1-ethylbenzene, naphthalene, anthracene, tetralin, phenanthrene, triphenylene, dibenz(*a,h*)anthracene, 1-indanone, 9-fluorenone, tetralone, acetone, cyclohexanone, benzophenone, 1,4-naphthoquinone, 9,10-phenanthraquinone, 9-hydroxyfluorene, tetralol, 1-acenaphthol, *p*-hydroxybiphenyl, phenol, 2-naphthol, 1,4-dihydroxybenzene, ethyl acetate, methyl benzoate, 9,10-epoxy-9,10-dihydrophenanthrene, benzyl sulphoxide, dimethyl sulphoxide, 1-naphthoic acid, benzene-1,2-dicarboxylic acid, lauric acid, carbazole, acridine, 4-benzylpyridine, 4-azafluorene, benzothiophene and dibenzothiophene.

Tetralin hydroperoxide, fluorene hydroperoxide, 9,10-dimethylanthracene endoperoxide and 9,10-epoxy-9,10-dihydrophenanthrene were synthesized by known methods[7–10].

Methanol and ethyl acetate (Merck, Darmstadt, G.F.R.) were purified by distillation in a 1-m glass column packed with wire-mesh rings to give a high efficiency with a maximum of 53 theoretical plates.

n-Hexane and chloroform (technical grade) were subjected to clean-up procedures[11] and then distilled as above.

Fractionation procedure

Gel chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) with chloroform and stepwise gradients between chloroform, methanol and tetrahydrofuran (THF) was performed as follows. Sephadex LH-20 was allowed to swell overnight in chloroform and packed in a 109 × 1.27 cm I.D. glass Cheminert LC column (Laboratory Data Control, Riviera Beach, FL, U.S.A.) as a slurry. The gel was settled and packed by pumping the solvent downwards through the bed at a flow-rate of 10 ml/min using a Constametric I solvent-delivery system. The system was equipped with an LDC Model 1203 filter photometer detector operating at 254 nm.

The elution of the different polar constituents was performed by stepwise gradients using chloroform to elute hydrocarbons, alcohols*, hydroperoxides and all

* The term "alcohols" in this paper refers to all hydroxyl-containing compounds where the hydroxyl group is not directly linked to an aromatic moiety, *e.g.*, tetralol, *n*-pentanol and 9-fluorenol.

other non-hydroxyl-containing polar components, 5% methanol–chloroform to elute phenols, 10% methanol–chloroform to elute diols and dihydroxy compounds, 20% THF–chloroform to elute fatty acids and other monoacids and 30% THF–methanol to remove the remainder of the polar material sticking to the gel bed.

Liquid partition chromatography on Sephasorb HP Ultrafine (Pharmacia) was performed as follows. The gel was allowed to swell overnight in methanol–water (70:30), packed as a slurry in a stainless-steel column (50 × 0.8 cm I.D.) and then run under a pressure of 1400 p.s.i. with methanol–water (70:30) for several hours. *n*-Hexane equilibrated with methanol–water (70:30) was then pumped through the column at a flow-rate of 2.0 ml/min until the eluate became clear. The optimal flow-rate was found to be 0.25 ml/min. The elution of the components that were not eluted with *n*-hexane was performed by a gradient between 20% and 30% benzene–*n*-hexane.

Fractions of 10 ml each were collected from the weathered Ekofisk crude oil using an LKB Ultrarac fraction collector equipped with an event marker. Several of these fractions were analysed by high-resolution gas chromatography using a Fractovap 2920 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a 20-m glass capillary column containing SE-54 and a flame-ionization detector.

Sampling procedure and extraction of surface film of weathered Ekofisk crude oil

Samples of the oil surface layer were collected by immersing a screen made of four 20 × 5 cm PTFE plates attached to a frame of stainless steel down to the surface and withdrawing the screen with oil adsorbed on the plates. The plates were removed from the frame with clean tweezers, placed in a clean glass container and extracted with two 50-ml volumes of ethyl acetate and one 50-ml volume of chloroform. The water was removed by adding anhydrous sodium sulphate.

A more elaborate description of the technique and equipment was given by Larsson *et al.*[12].

RESULTS AND DISCUSSION

The fractionation scheme shown in Fig. 1 takes advantage of the lipophilic and hydrophilic characters of the Sephadex LH-20 and Sephasorb HP Ultrafine gels, respectively.

The first step very effectively isolates compounds containing hydroxyl groups, probably by a mechanism involving hydrogen-bonded interactions between the glucose hydroxyl groups in the Sephadex LH-20 gel and the polar moiety of the solute molecules. In contrast, gel filtration seems to be the dominating mechanism in determining the order of elution of the hydrocarbons without the typical adsorption effects observed with alcoholic solvents. Polar organic constituents such as ketones, quinones, endoperoxides, ethers, sulphoxides and sulphur- and nitrogen-containing compounds, which do not possess any hydroxyl groups, show virtually no retardation on the Sephadex LH-20 gel with chloroform as the mobile phase. Previous results even indicated some kind of an keto exclusion effect of steroid ketones in halogenated solvents, and the same effect may well account for the observed elution order in our experiments[13].

The most conspicuous observation in this first step is the marked retardation of

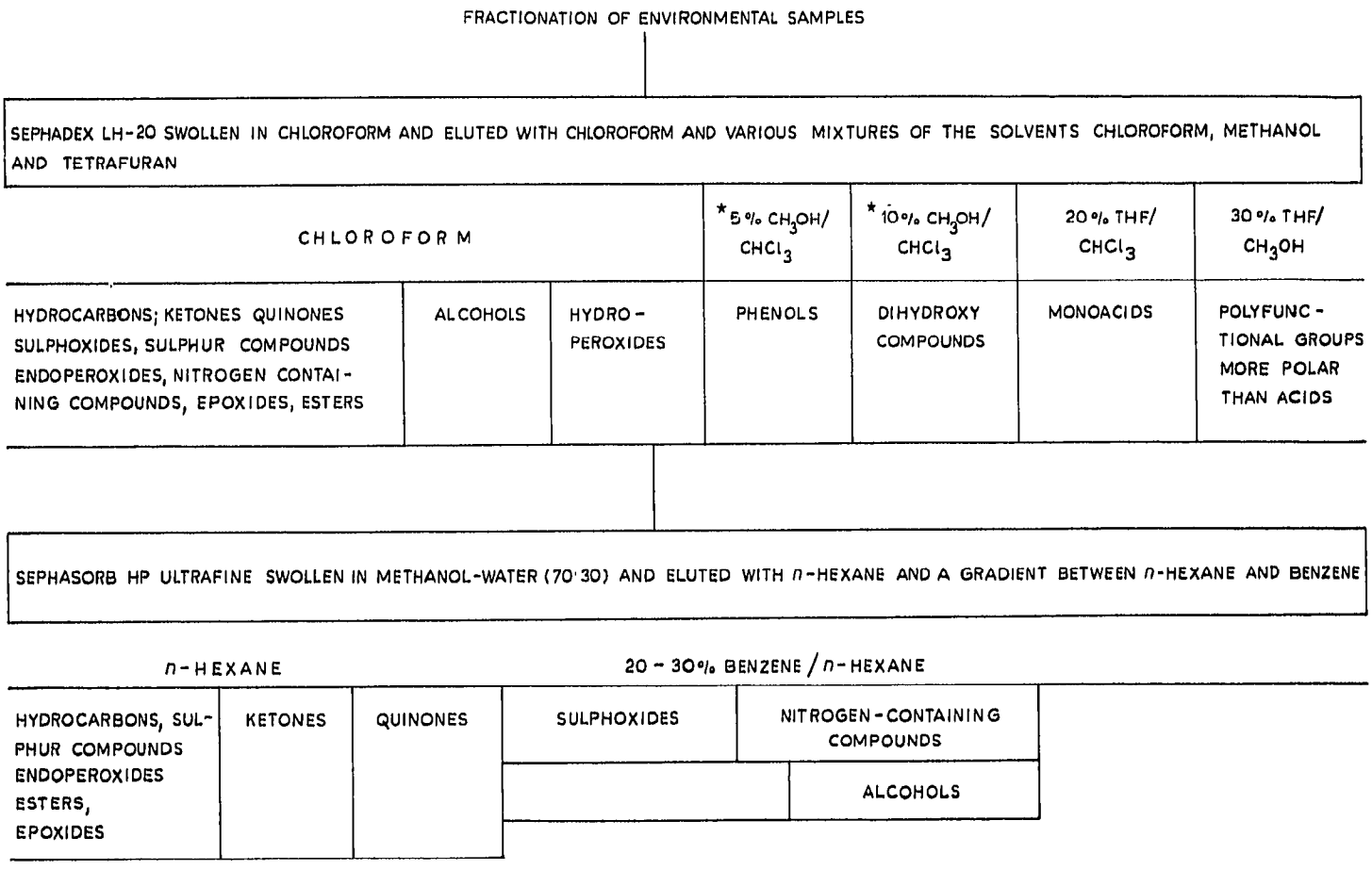

Fig. 1. Fractionation scheme for isolation and trace enrichment of polar organic constituents in environmental samples. * From a practical point of view we replaced pure methanol with methanol–ethanol (1:1) in order to keep the swelling of the gel bed to a minimum when changing from chloroform to a solvent mixture containing methanol.

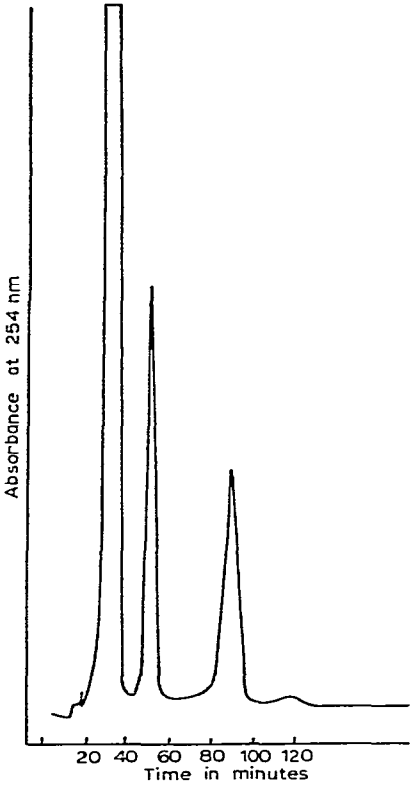

Fig. 2. Gel chromatographic separation of tetralin, tetralol and tetralin hydroperoxide on Sephadex LH-20 with chloroform as the mobile phase.

hyperoxides relative to their respective alcohols (Fig. 2), together with a very pronounced difference in elution volumes between the hydroperoxides studied, which can be partly ascribed to the difference in acidity between the peroxides.

As yet we have not arrived at any conclusive theoretical explanation for the retardation of hydroperoxides on Sephadex LH-20, but it seems likely that the active polar moieties of these compounds are being lined up with the Sephadex gel in such a way that the interaction is stronger than would be the case with the corresponding alcohols. However, we cannot ignore the possibility that some hydroperoxides, because of steric or electronic effects, have been rendered unreactive and accordingly are not subjected to the same retardation effects on Sephadex LH-20[14].

The affinity for hydroperoxides in adsorption chromatography is greatly diminished relative to the corresponding alcohols and in many instances a separation between hydrocarbons and hydroperoxides is a serious problem in adsorption chromatography because of the very weak acidic nature of hydroperoxides. Sephadex LH-20 is the only gel, to our knowledge, with which such a pronounced retardation of hydroperoxides has been observed.

Another striking illustration of the resolving power of Sephadex LH-20 gel in this mode of operation is the pronounced difference in retardation between phenols and alcohols. In fact, phenol itself could be eluted only with great difficulty with

chloroform, and naphthols and higher phenols could be eluted from the gel matrix only after modifying the mobile phase by adding 5% of methanol. Further, the alcohols have also been shown to exhibit an increasing elution volume with increasing acidity, which might lead to some overlap between the alcohol and hydroperoxide regions. The latter compounds can be recognized by their characteristic reaction with starch–iodide paper[15].

Hydroperoxides can also be conveniently reduced to alcohols by the method of Barnard and Wong[16] using an excess of triphenylphosphine or by adding tin(II) chloride[17,18]. The reduced alcohols are then re-chromatographed with Sephadex LH-20 and chloroform as the mobile phase to separate the reduced alcohols from unreacted triphenylphosphine and triphenylphosphine oxide. These latter compounds show virtually no retardation under the given conditions.

The fractionation scheme also makes possible the complete isolation of dihydroxy compounds from a complex mixture in a single chromatographic run. This may prove useful as an initial isolation procedure for metabolites formed both as a result of microbial degradation and metabolism of xenobiotics by higher invertebrates[19,20].

Successively larger amounts of methanol had to be incorporated into the mobile phase in order to elute acids and more polar compounds. Unfortunately, large amounts of methanol in the mobile phase cause the gel to swell, which leads to pressure problems in the column. In order to avoid this, methanol was replaced with THF for the elution of compounds with polarities similar to those of fatty acids and other organic monoacids. In order to elute even more polar material from the column, the flow direction was reversed and the gel was cleaned with 30% THF–methanol. The column was then equilibrated overnight with the chloroform solution at a flow-rate of 0.5 ml/min and the eluate was monitored by measuring its refractive index.

The next step involves partition chromatography using Sephasorb HP Ultrafine swollen in methanol–water as the stationary phase and *n*-hexane as the mobile

TABLE I

ELUTION CHARACTERISTICS OF SOME STANDARD COMPOUNDS ON SEPHASORB HP ULTRAFINE WITH *n*-HEXANE AS THE MOBILE PHASE

Flow-rate, 0.25 ml/min. Compounds indicated by a dash could not be eluted with *n*-hexane after 3 h.

Compound	*Elution volume (ml)*	*Compound*	*Elution volume (ml)*
Benzene	10.1	Cyclohexanone	22.6
Naphthalene	10.3	9-Fluorenone	23.0
Anthracene	10.7	2,5-Dimethylbenzoquinone	24.5
Triphenylene	12.6	1,4-Naphthoquinone	36.5
Dibenz(*a*,*h*)anthracene	14.2	9,10-Phenanthraquinone	46.0
Thioanisole	10.7	Dimethyl sulphoxide	–
9,10-Dimethylanthracene endoperoxide	7.5	Dibenzyl sulphoxide	–
9,10-Epoxy-9,10-dihydrophenanthrene	9.3	4-Azafluorene	–
Ethyl acetate	10.3	Carbazole	–
Methyl benzoate	13.3	1-Acenaphthol	–
Acetone	22.5	Tetralin hydroperoxide	–

phase. Operating in this mode the gel effectively isolates and separates ketones, quinones, sulphoxides, nitrogen-containing compounds and alcohols, whereas epoxides, esters and endoperoxides overlap with the non-polar hydrocarbons to some extent.

Sephasorb HP Ultrafine has a structure similar to that of Sephadex LH-20, but has a smaller and more uniform particle distribution and consequently a higher matrix density. This gel can also withstand some pressure and the denser packing possible with this gel might explain the more pronounced partition effects observed with this gel compared with Sephadex LH-20. The separation mechanism can be envisioned as a partition between the methanol–water retained on the gel matrix and the *n*-hexane in the mobile phase as it passes through the column according to their solubilities in the two-solvent system. The gel matrix in this way acts as an inert support for the methanol–water such that the separation occurs essentially by a continuous liquid–liquid mechanism. Lipophilic constituents are eluted fairly quickly by *n*-hexane while hydrophilic components are retained by the methanol–water containing gel illustrated in Fig. 3 where benzene, 9-fluorenone and 1,4-naphthoquinone are being retained according to their polarity. As can be seen from Table I, only small differences in elution volume between benzene and three-ring aromatic compounds can be observed, while some four- and five-membered ring compounds are more retained. Ketones have been shown to be retarded, but only minor differences in retention time have been observed for different ketones. Quinones are even more retained than ketones, different quinones leading to more pronounced differences in the elution profile than are observed for hydrocarbons and ketones.

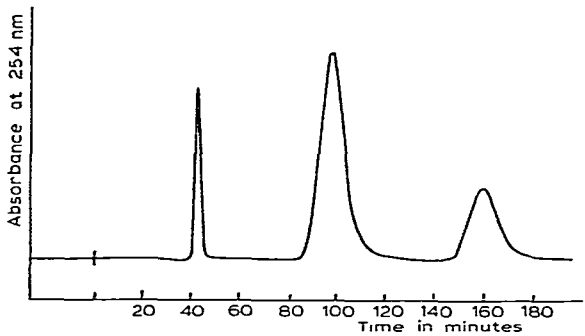

Fig. 3. Elution behaviour on Sephasorb HP Ultrafine of benzene, 9-fluorenone and 1,4-naphthoquinone.

No noticeable retention of sulphur compounds has been observed, in contrast to a marked retention of nitrogen-containing constituents, the basic heterocyclic nitrogen compounds being the most retained. In general, nitrogen-containing compounds can be eluted only after modifying the mobile phase with 25–30% of benzene. Sulphoxides also show a pronounced retention on Sephasorb HP Ultrafine and a 20–25% benzene–*n*-hexane solution is required in order to elute these constituents. A careful gradient between 20 and 30% benzene–*n*-hexane should make possible the separation of most of the nitrogen-containing components from the sulphoxides. Aliphatic alcohols are even more retained than heterocyclic nitrogen compounds, but some degree of overlap between these two classes of compounds is likely. This should not pose any problems in the further analysis as they can easily be distinguished by mass spectrometry.

The ether oxygen in epoxides, endoperoxides and esters shows virtually no interaction with the stationary phase in this mode of operation, resulting in strong size exclusion effects of these compounds because of the appreciable size of oxygen-containing functional groups. Endoperoxides are excluded the most, whereas their esters even show some retardation as a result of the active carbonyl group, aromatic esters being more retarded than aliphatic esters. It can be seen from Table I that the isolation of these compound classes from non-polar hydrocarbons in environmental samples by this procedure will be diffucult in most instances.

Epoxides are highly reactive and toxic and many of them become very unstable when purified. It is therefore highly desirable to isolate and characterize epoxides by trapping them from dilute solutions with a suitable nucleophile[21]. The resulting adducts are stable and can easily be purified by gel filtration chromatography using Sephadex LH-20 and chloroform as the mobile phase.

To test for the presence of endoperoxides, an aliquot of the sample is added to tin(II) chloride[17,18]. The resulting diols can then easily be isolated and purified using the described Sephadex LH-20 procedure with 10% methanol–chloroform as the mobile phase.

Esters can easily be monitored in complex mixtures with hydrocarbons using an infrared detector in tandem with a UV detector[22]. After having removed all other polar components by the described procedure, esters can be separated from hydrocarbons by adsorption chromatography. Adsorption chromatography has proved useful in the analysis of many hydrocarbon samples, and should not pose any problems in the isolation of esters.

The general elution mechanism appears to be a combination of several effects, gel filtration and adsorption effects attributed to hydrogen bonding of the polar moiety of the solute molecules to the gel matrix probably being the most important. Many similarities between the partition effects observed with Sephasorb HP Ultrafine can also be found with Sephadex LH-20 operating in this mode. However, the analysis time, amount of solvents used and the regeneration time of the column between each run are much reduced. In addition, we have also found a more pronounced group separation with Sephasorb HP Ultrafine, possibly owing to the fine particle size and denser packing of the gel.

APPLICATION OF THE METHOD

A very thin film of Ekofisk crude oil subjected to photochemical transformations under natural sunlight in late March at a latitude of 60° will serve as an example to illustrate how this fractionation procedure can be used to solve biogeochemical problems.

Fig. 4 shows the gel chromatographic run needed to isolate the polar constituents from the surface film. The first, sharp peak eluting very early has been shown to correspond to polymeric material, either resinous material being leached out from adjacent tar balls or other polymerization products being formed as a result of photooxidation.

The GC trace of the hydrocarbons is shown in Fig. 5a, and surprisingly shows the presence of large amounts of *n*-alkanes even after 20 days on the sea surface. The GC trace of the phenols and possibly dihydroxy compounds after being silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is shown in Fig. 5b. These chro-

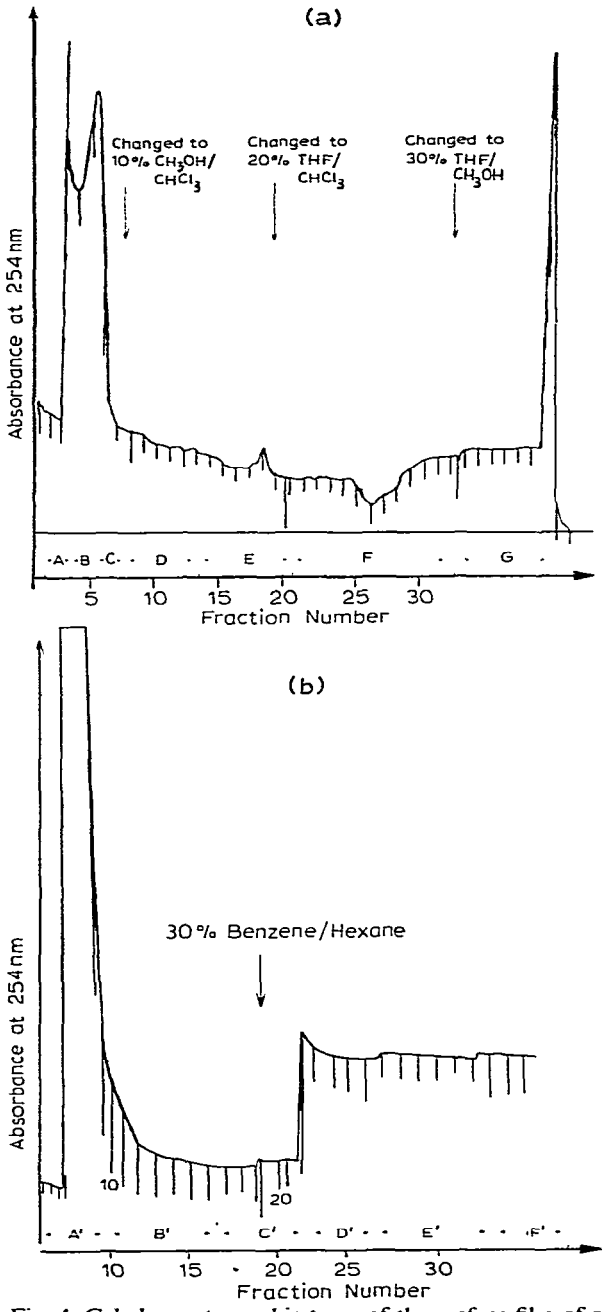

Fig. 4. Gel chromatographic trace of the surface film of a weathered Ekofisk crude oil. (a) Isolation of hydroxyl-containing compounds on Sephadex LH-20. The pooled fractions A, B, C, D, E, F and G, represent the polymeric material, hydrocarbons, alcohols, hydroperoxides, phenols, dihydroxy compounds and acids and strongly polar material, respectively. (b) Isolation of non-hydroxyl-containing polar organic constituents on Sephasorb HP Ultrafine. The pooled fractions A′, B′, C′, D′, E′ and F′ represent hydrocarbons, ketones, quinones, sulphoxides, nitrogen-containing compounds and alcohols, respectively. The reference cell in the UV detector was filled with a 30% benzene–*n*-hexane solution.

matograms illustrate strikingly the effectiveness of Sephadex LH-20 gel in that most of the peaks are well resolved with no apparent unresolved hump. The peaks were then subjected to mass spectral investigation.

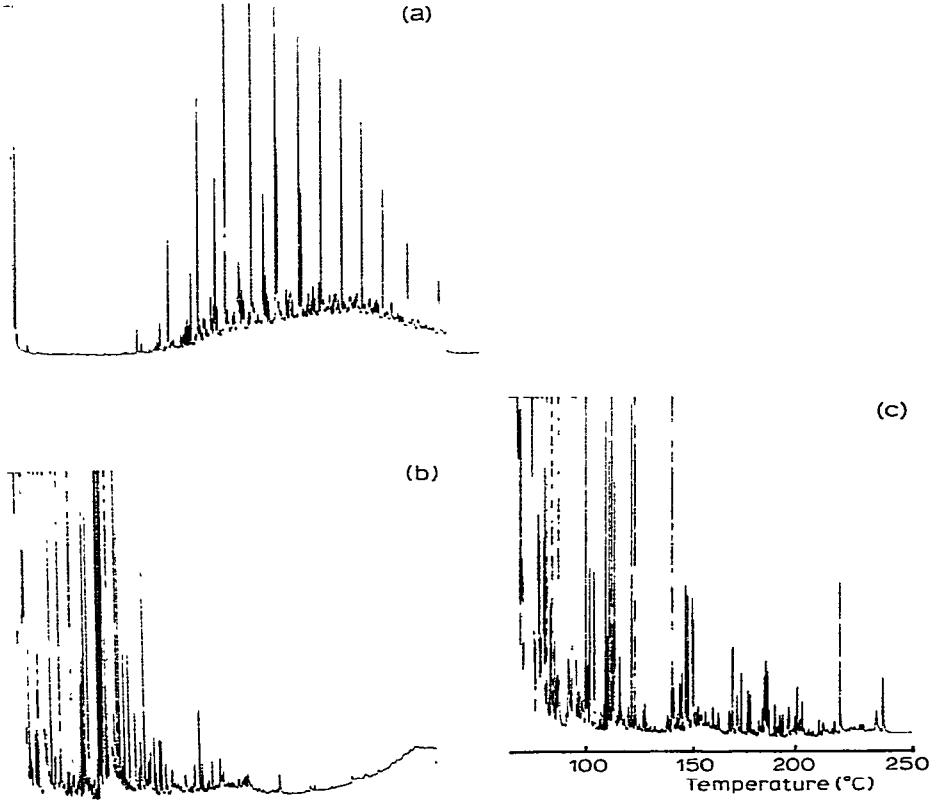

Fig. 5. Gas chromatographic traces of (a) hydrocarbons, (b) phenols and dihydroxy compounds and (c) alcohols and nitrogen-containing compounds from the surface film of weathered Ekofisk crude oil. Phenols, dihydroxy compounds and alcohols were silylated with BSTFA prior to gas chromatographic analysis. A 20 × 0.34 mm I.D. SE-54 WCOT glass capillary column programmed from 70 to 300°C at 4°C/min was used.

Fig. 5c shows the chromatogram of the silylated alcohols together with some nitrogen-containing compounds after being isolated on Sephasorb HP Ultrafine. In many practical applications we observe some overlap between hydrocarbons and alcohols on Sephadex LH-20 with chloroform owing to the large amounts of hydrocarbons present in most of the natural samples. Therefore, in order to give a better isolation of hydrocarbons from alcohols, we re-chromatographed both the hydrocarbons and the alcohols on Sephasorb HP Ultrafine, which led to a complete separation of the two classes of compounds.

Although it is generally agreed that high-resolution liquid chromatography offers the greatest separation efficiency in liquid chromatography today, this technique is seldom used directly as a first step in the clean-up and isolation of polar organic constituents from environmental samples. The main reason is that the

available column types, being either adsorption, gel filtration, partition or ion-exchange types, render the group isolation of polar organic components virtually impossible even if columns with different selectivities and a carefully worked out column-switching technique are used. In addition, the loading capacity is relatively low and adsorption columns are often associated with catalytic effects.

On the other hand, gel filtration gels such as Sephadex LH-20 and Sephasorb HP Ultrafine, which take advantage of the combined effects of absorption, gel filtration and partition, have previously been tested and proved valuable for the fractionation of hydrocarbons in environmental samples[23–25]. We have now shown that these gels are also valuable substitutes for the more commonly used high-pressure gels for the fractionation of polar organic constituents in environmental samples.

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