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# Detection of Drilling Mud-Base Oil in the Bile of Trout, *Salmo gairdneri*

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Crude and refined petroleum oils contain saturated as well as polyaromatic hydrocarbons, and the latter exist in minor amounts (15 to 40%, Korte and Boedefeld, 1978). However, drilling mud-base oils have predominantly (>95%, Payne et al. 1985), and in the present case, almost exclusively saturated components (>99%, in Safver oil 4).

Studies on the fate and effects of petroleum oil in fish have concentrated on the aromatics. The epoxides, quinones and dihydrodiol metabolites formed from these polyaromatic molecules have been implicated in carcinogenicity- mutagenicity related problems (Sims et al. 1974). A recent investigation of the mutagenicity vs toxicity of the various water soluble fractions of Kuwait crude oil suggests that much of the toxicity of that fractionated oil is due to alkanes, alkenes, aliphatics and one-ring cyclic compounds (Vandermeulen et al. 1985). This observation indicates that the fate of naphthenic type hydrocarbons should not be neglected.

Aliphatic molecules such as dodecane and docosane have been shown to bioaccumulate in fish (Freitag et al. 1985), with a bioaccumulation factor of 50 and 10, which is close to that found for aromatic molecules such as toluene (90), naphthalene (30), dibenz(a,h)anthracene (10) and benzene (10). Experiments with vertebrates and particularly fish have shown that the gall bladder concentrates terpenoid type xenobiotics and metabolites of aromatic hydrocarbons prior to excretion. This organ represents a good indicator when trying to assess contamination or exposure of fish to such pollutants (Stratham et al. 1976). Only a few studies have focused on the metabolic fate of non-aromatic molecules in fish (Cravedi and Tulliez 1981, 1982a, 1982b, 1986a and b; Cravedi et al. 1985).

In view of all these observations and since $^{13}C$ NMR analysis of the metabolites (Hellou et al. 1986) found in the gall bladder bile of cunners exposed to No. 2 fuel oil indicated the presence of an aliphatic moiety in the bile mixture, it was deemed of interest to determine if the gall bladder bile would act as a bioconcentrator of an oil quasi-free of aromatics.

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## MATERIALS AND METHODS

Salmo gairdneri were acclimated to 8°C water for at least 1 week prior to exposure and were not fed during that period. Trout varying in weight from 100 to 160 g were intubed through the mouth with 0.3 ml of a drilling mud-based oil. Fish were killed 48, 96, 144 and 192 hours after the beginning of the experiment. The weight and sex of each fish were determined, the gall bladder removed, the bile weighed and stored at -78°C. Twelve fish were used for the exposure and three fish were killed each day. Another twelve fish were starved for the same period and used as control.

Solvents used were HPLC grade or spectral grade. A Varian 3700 GLC equipped with a DB-1 capillary column (30 m x 0.25 mm I.D.) was used with the following temperature programming: initial temperature of 100°C, maintained for 1 minute, increasing to 280°C at a rate of 5°C/minute and maintained at the final temperature for 10 minutes. Combined GLC-EI mass spectrometry was performed on a Finnigan 4021 instrument equipped with an INCOS Data System. An ionization voltage of 70 eV was used and the same capillary column and temperature programming as described above. A Varian CFT-20 NMR spectrometer was used to obtain the $^{13}C$ and $^{1}H$ NMR spectra, using deuterated chloroform as solvent.

After termination of the experiment, the bile samples were thawed and diluted with three equal volumes of water. A 200 µl aliquot was removed from each sample and the bile of fish from the same day and group pooled. Water (2.4 ml) and a mixture of $CH_3OH$: $CH_2Cl_2$ (3 ml:3 ml) were added to each sample and the organic layer separated by centrifugation. Extraction was repeated two more times with an equal volume of $CH_2Cl_2$ (3 ml). The pooled organic layers were evaporated to dryness and redissolved in $CH_2Cl_2$ (30µl). The above samples contain the free bile metabolites and lipids. The fatty acids present in these samples were identified by GLC-MS, by comparing the retention times of peaks observed by GLC to commercial products and by referring to published data (Cravedi and Tulliez, 1982). The aqueous methanol layer was evaporated and redissolved in $H_2O$ (3 ml). A solution containing 4 mg of β-glucuronidase (from Limpets 1,480,000 units/g, from Sigma) per milliliter of acetate buffer (0.3 M, pH 4.8) was added to each fraction (3 ml). Enzymatic hydrolysis took place at 37°C for 24 hours. The samples were then acidified with 10% HCl and extracted three times with $CH_2Cl_2$. The pooled organic layers evaporated and redissolved in $CH_2Cl_2$ (30µl). These samples contain the metabolites which had been conjugated to β-glucuronic acid. Samples obtained after hydrolysis were analyzed by GLC, using n-pentadecane as an internal standard. The samples obtained from the four different days were pooled, treated with diazomethane and analyzed by GLC-MS.

The safver oil 4 (commercial name) was analyzed by $^{13}C$ NMR spectroscopy using the gated proton decoupling technique and a non-interacting paramagnetic reagent, chromium III acetyl acetonate. The oil was also analyzed by TLC using precoated silica gel plates with fluorescent indicator (Eastman Kodak, silica gel

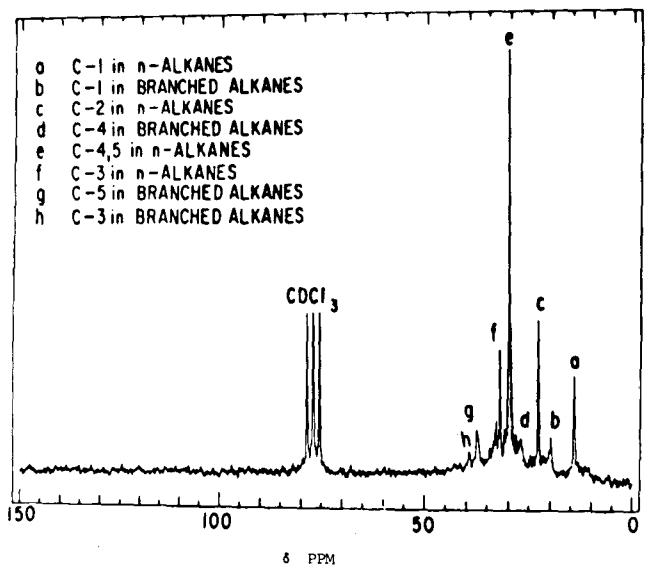


Figure 1. Proton decoupled 13C NMR spectrum of the drilling mud-base oil used in this experiment. $CDCl_3$: deuterated chloroform. Assignment of the peaks is indicated by letters a to h (top left).

13181). The UV absorbing band was separated by column chromatography using silica (Aldrich, 240-400 mesh) and represented less than 1% (by weight) of the oil. The composition of the oil was also examined by GLC, using the same temperature programming described above.

## RESULTS AND DISCUSSION

Analysis of the drilling mud-base oil by $^{13}C$ NMR spectroscopy indicated the absence of aromatic carbons and the same saturated carbons composition expected for crude or refined oils (Figure 1). The GLC profile showed a hydrocarbon distribution typical of petroleum products, with n-hydrocarbons ranging from C-13 to C-22, where C-17 and C-18 predominate (Figure 2). The expected branched aliphatic chains were also detected, such as norpristane, pristane, and phytane. A UV absorbing band could be observed on TLC, in hexane (Rf=0.33), slightly more polar than the sulfuric acid positive band (Rf=0.45) due to the saturated hydrocarbons. Separation of this band by column chromatography indicated that the aromatics represent less than 1% by weight, of the oil. The aromatics in this oil have been previously analyzed by HPLC and shown to be benzenoid type molecules only (Payne et al. 1985).

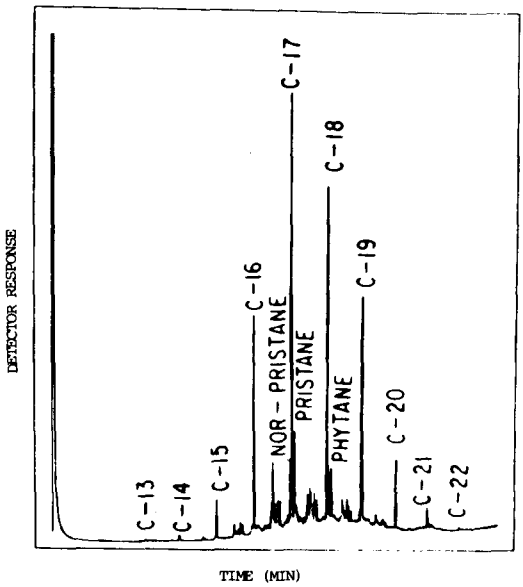


Figure 2. GLC spectrum obtained on the drilling mud-base oil used in this experiment. (C-13,14,. . . represent n-alkanes.)

The bile extracts containing the metabolites conjugated to β-glucuronic acid have been analyzed by GLC, after hydrolysis. The results obtained from the integration of the area of the unresolved complex mixture indicate that the excretion of metabolites into the bile reached a maximum around the sixth day of the experiment. This is similar to the results obtained after exposure of trout to No. 2 fuel oil (Hellou and Payne 1986) and analysis of the bile by fluorescence and GLC.

There are three major components in the bile extract containing free metabolites: a C16, a C18:1 fatty acid and cholesterol. The relative percentage of these three lipids has been examined and compared to the lipids in control trout and in trout injected intraperitoneally with No. 2 fuel oil (Hellou and Payne 1986). Although a difference was observed by Cravedi and Tulliez (1982) in a long-term experiment, our data do not show any significant difference between the relative ratio of these lipids in exposed vs control fish, during the short term experiment.

Due to the size (1-2 mg) of the pooled hydrolyzed metabolites sample, analysis by $^{13}C$ NMR spectroscopy, as described previously, was not possible (Hellou et al. 1986). An $^{1}H$ NMR spectrum was obtained on this sample. No aromatic protons were detected, only multiplets (m) or broad singlets (bs) were observed between 0 and 5 ppm. The predominant peak is due to $(CH_2)_n$ and appears at 1.25 ppm (bs).

GLC-MS was more successful in partially elucidating the structure of this complex mixture. Due to the number of small peaks in the chromatogram, multiple ion monitoring (MIM) by GLC-MS, which is a highly sensitive analytical technique, was attempted.

It has been shown in the field of petroleum chemistry that the ions at m/z 83, 85 and 191 are important indicators of the type of molecules present in the hydrocarbons mixture. Iso and anteiso alkanes fragment to give an ion at m/z 85. The presence of alkylcyclohexyl and alkylcyclopentyl alkanes can be ascertained by screening at m/z 83, while bicyclic, tricyclic and tetracyclic terpenoid type hydrocarbons give rise to a major fragment at m/z 191. These ions were therefore monitored in order to see if they could be helpful in the analysis of the bile of fish exposed to saturated hydrocarbons.

Spectra obtained on the peaks after monitoring at m/z 85 indicated that fatty acids were predominant on this chromatogram (although present in trace amounts). The fatty acids were identified by GLC-MS, by comparing the retention time of observed peaks to that of commercial products and by referring to published data (Cravedi and Tulliez 1982). The fatty acids observed had an even number of carbons (14:0, 16:0, 16:1, 18:0, 18:1, 20:1 and 22:1), and therefore cannot be thought to derive from the petroleum oil. Some minor peaks were detected when screening for cyclohexyl and cyclopentyl derivatives at m/z 83. Analysis of individual spectra and a computer-library search, did not elucidate the exact structure of these metabolites. These cyclic derivatives appeared over a large range of retention times, corresponding to a temperature of 130°C to 270°C. Characteristic fragments appeared at m/z 56, 68, 81, 95 and 109 (probably due to $C_4H_8$, $C_5H_8$, $C_6H_9$, $C_7H_{11}$ and $C_8H_{13}$) in some cases, and at m/z 55, 69, 83, 97 and 111 (probably due to $C_4H_7$, $C_5H_9$, $C_6H_{11}$, $C_7H_{13}$ and $C_8H_{15}$) in others. This pattern would indicate the presence of alkylcyclohexene (or alkylcyclopentene) and non-cyclic alkene fragments in the MS of the hydrolyzed metabolites. The former fragments would possibly arise from metabolites of cyclic alcohols, while the later would result from metabolites of saturated alcohols. In view of the results obtained by Cravedi et al. (1985, 1986b) after exposure of fish to single saturated molecules, oxidation could be expected to have taken place at least at the w position and/or on the ring moiety of the metabolites. The parent ions seem to appear around m/z 200 to 400, agreeing with the presence of molecules containing from 12 to 24 carbons, as would be expected after analysis of the drilling mud-base oil. The fragmentation at higher masses is not as clearly defined, making the position of the hydroxylation difficult to determine.

Three steroidal compounds with relatively long retention times were identified in the reconstructed chromatogram at m/z 191. The computer searchmatched the spectra with 3β-cholest-5-en-3-ol, 3β-ergost-5-en-3-ol, and 3β-stigmast-5-en-ol. The presence of the latter two steroids was not further confirmed.

Other peaks detected in the reconstructed ion chromatogram, with a shorter retention time than cholesterol, appeared to have an

aromatic moiety. This was deduced from the presence of intense fragments at m/z 77 or 79, 91, 105, 119 and 131 or 133 (probably due to $C_6H_5$ or $C_6H_7$, $C_7H_7$, $C_8H_9$, $C_9H_{11}$ and $C_{10}H_{13}$ or $C_{10}H_{15}$), in the spectrum of individual peaks. This fragmentation would be expected from alkylbenzene type metabolites hydroxylated on the alkyl part of the molecule.

The present study has demonstrated that the gall bladder acts as a bioconcentrator of metabolites deriving from an oil quasi-free of aromatics. Although the components present in the hydrolyzed bile extract could not be individually identified, (due to the absence of identical commercial samples), with the help of GLC-MS, the chemical constitution of this mixture was partially revealed. Saturated and unsaturated metabolites have been detected in the bile of trout exposed to a drilling mud-base oil, which were not present in control trout. Further work is needed to find out if the aromatics present in the bile originate from the aromatic fraction of the oil or are due to metabolic transformations of the cyclohexane type molecules present in the oil. The aromatization of cyclohexane carboxylic acid has been observed in other animals (Babior and Bloch 1966; Baldwin et al. 1960) and such a process could conceivably be taking place in the present case. Our results would also indicate that a small fraction of the saturated carbons observed in the $^{13}C$ NMR of cunners exposed to No. 2 fuel oil (Hellou et al. 1986) could be due to naphthenic type metabolites.

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