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## Abstract

The authors attempted to determine if the organic sulfur compounds usually contained in a crude oil could serve as a marker of oil pollution in fish. Eels (*Anguilla Japonica* Temminck et Schlegel) were maintained in a controlled laboratory environment of water with a suspension of crude oil. Gas chromatography-mass spectrometry and mass chromatography of eel flesh extract showed the presence of organic sulfur compounds of alkyl-(from mono- to pentametyl) benzothiophenes, dibenzothiophene and alkyl-(from mono- to trimethyl) dibenzothiophenes, and other organic sulfur compounds of alkyl-(from mono- to pentamethyl) naphthalenes.

**KEYWORDS:** organic sulfur compounds, alkyl benzothiophenes, alkyl dibenzothiophenes, oil pollution, fish, mass chromatography

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## IDENTIFICATION OF ORGANIC SULFUR COMPOUNDS TRANSFERRED TO FISH FROM PETROLEUM SUSPENSION

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**Abstract.** The authors attempted to determine if the organic sulfur compounds usually contained in a crude oil could serve as a marker of oil pollution in fish. Eels (*Anguilla Japonica* Temminck et Schlegel) were maintained in a controlled laboratory environment of water with a suspension of crude oil. Gas chromatography-mass spectrometry and mass chromatography of eel flesh extract showed the presence of organic sulfur compounds of alkyl-(from mono- to pentamethyl) benzothiophenes, dibenzothiophene and alkyl-(from mono- to trimethyl) dibenzothiophenes, and other organic sulfur compounds of alkyl-(from mono- to pentamethyl) naphthalenes.

**Key words :** organic sulfur compounds, alkyl benzothiophenes, alkyl dibenzothiophenes, oil pollution, fish, mass chromatography

Heavy pollution and offensive odors exist in waters near petroleum industrial districts and in the sea after oil spillage. In a previous report, we demonstrated the existence of toluene and other aromatic hydrocarbons in fish caught along the coast of petroleum industrial areas (1). Conditions representing petroleum spillage at sea were simulated in a controlled laboratory aquarium. This was used to raise eels. Aromatic hydrocarbons have been found in such eel flesh (2). In fish reared in fuel oil A, the repugnant odors were due to unsaturated and aliphatic hydrocarbons, and some aromatic hydrocarbons (3).

The authors have demonstrated the presence of organic sulfur compounds possibly due to petroleum in the flesh of one of several turbot fish, caught in the Seto inland sea (4). Subsequently, eels were maintained in a controlled laboratory environment of water with a suspension of crude oil. Gas chromatography of the eel flesh revealed the presence of paraffins and organic sulfur compounds whose concentration increased with increase in rearing time (5). In the present study, organic sulfur compounds in the flesh of eels exposed to this crude oil suspension in the laboratory were analyzed by gas chromatography-mass spectrometry-computer system.

## MATERIALS AND METHODS

The petroleum used was a mixture of Arabian Light Crude oil and Zubea oil (4:1). Ten eels weighing 130–180 g were reared in sea water containing 1000 ppm of crude oil suspension at 20°C and were taken for examination on the seventh day. Eels for the control group were reared and taken under the same conditions. For gas chromatography, eel flesh homogenates were treated as described in the previous paper (5). Compounds eluted from a silica gel alumina (1:1) column with n-hexane were washed with water, then concentrated with a Kuderna-Danish evaporative concentrator (KD). The concentrate was extracted with acetonitrile. The acetonitrile solution was concentrated with KD (25 g ww of eel flesh to 1 ml).

Gas chromatography was as follows: The apparatus employed was a Hitachi 163 with a FPD and FID detector. Analytical conditions: The glass column was 0.3 cm×2 m with 3% Silicon SE 52 on Chromosorb W (AW-DMCS) mesh 80–100. The chromatograph was programmed from 80 to 260°C, 10°C min<sup>-1</sup>. The carrier gas was nitrogen, flow rate, 80 ml min<sup>-1</sup>.

Gas chromatography-mass spectrometry was as follows: A Shimadzu LKB 9000 gas chromatograph-mass spectrometer was used in combination with a gas chromatograph-mass spectrometer PAC 300 DG computer system containing, an OKITAC 4300 mini-computer. Gas chromatographic conditions: The glass column was 0.3 cm×2 m with 3% Silicon SE 52 on Chromosorb W (AW-DMCS) mesh 80–100. The chromatograph was programmed from 80 to 280°C, 10°C min<sup>-1</sup>. The carrier gas was helium, flow rate, 35 ml min<sup>-1</sup>. A total ion collector was used as the detector for gas chromatography mass spectrography. The mass spectrometric conditions were as follows: The ion source temperature was held at 250°C. The mass spectra were obtained at 20 eV of electron energy, 3.5 kV acceleration voltage, and 60  $\mu$ A of trap current. The repeating scan speed was at 6 sec of mass chromatography.

## RESULTS

*Gas chromatograms.* Eel flesh was analysed by gas chromatograms (FPD and FID) of acetonitrile extracts of crude oil. Gas chromatograms (Fig. 1, B) showed many peaks for the crude oil (Fig. 1, A). These were not seen in control eel flesh (Fig. 1, C). The appearance of many peaks on FPD gas chromatogram of oil polluted fish flesh indicated that organic sulfur compounds in crude oil suspension had transferred from the water to the fish. The differences in peak height in Fig. 1, B and Fig. 1, C are probably individual differences in concentration in eel flesh.

*Gas chromatography-mass spectrometry and Mass chromatography.* Mass spectra (Fig. 2) and mass chromatograms (Fig. 3) were taken over almost the entire range of peaks in the gas chromatograms. The compounds of the gas chromatograph-mass spectra in crude oil and eel flesh were identified by a comparison of the gas chromatograph-mass spectra of specimens with the mass spectra of

authentic substances (6), and by mass chromatograms of the specimens. The compounds found in crude oil and eel flesh are summarized in Table 1. This indicates the presence of methyl derivatives of naphthalene, benzothiophene and dibenzothiophene.

Peaks 2', 3' and 4' are major peaks of the TIC gas chromatogram of eel flesh extract. The mass spectra of these peaks indicated that methyl derivatives of benzothiophene and naphthalene occurred in the same peak. Peak 6' showed dibenzothiophene. Peaks 7'-9' contained methyl derivatives of dibenzothiophene. Other compounds were only present in minor amounts and correlated well with the patterns of authentic controls (6). These peaks, therefore, are omitted.

Dimethyl benzothiophene and dimethyl naphthalene were not separated on the gas chromatographic column under the conditions studied, but appeared

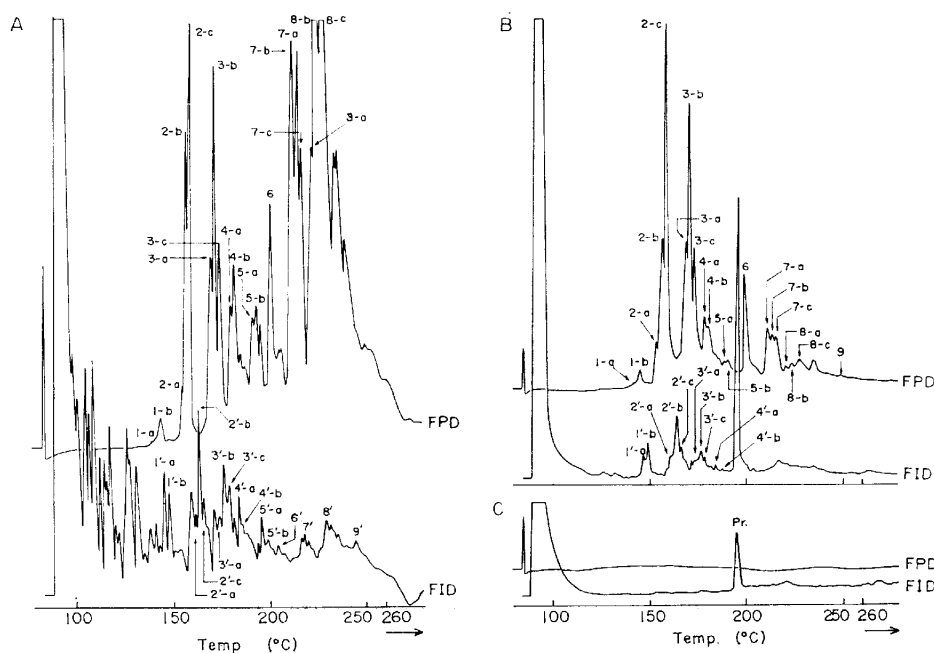


Fig. 1. Gas chromatograms of the extract from the acetonitrile fraction obtained from cleaned up crude oil and the flesh of eels reared in crude oil suspension. A: crude oil, (125 mg/ml) B: eel reared in crude oil suspension, C: control eel flesh. Symbols of the peak number correspond to the numbers in Table 1. Conditions of FPD, (Wave length of filter; 394 nm,  $H_2$  80 ml  $min^{-1}$ ,  $O_2$  20 ml  $min^{-1}$ ). FPD and FID curves were drawn by 2 pen recorders simultaneously. The analytical sensitivity of the FID curve in Fig. 1, B is 1.6 times as high as that of Fig. 1, C. Pr in Fig. 1, C; pristane originally contained in eel flesh. The analytical sensitivity of peaks in FPD gas chromatograms (of 2-c and of more number 2-c) having longer retention times, was one half that of peaks having shorter retention times (Fig. 1, A).

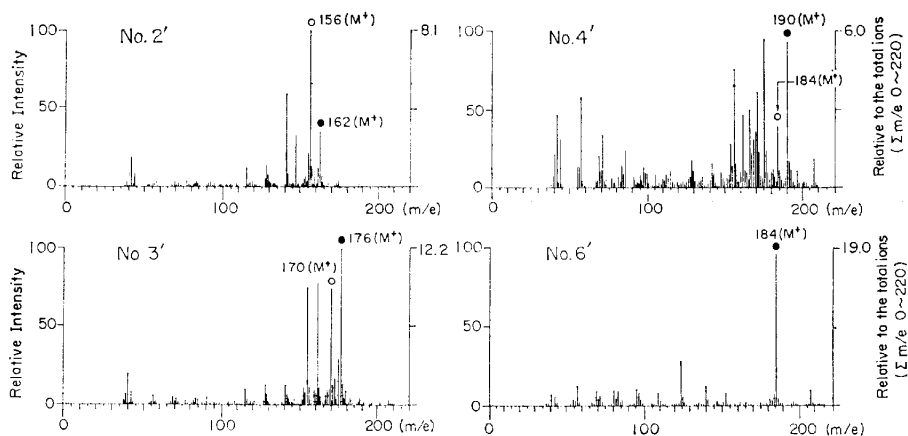


Fig. 2. Mass spectra of peaks No. 2', 3', 4' and 6' in gas chromatograms of eel flesh. (○ indicates the base peak of methyl derivatives of naphthalene and ● indicates methyl derivatives of benzothiophene in No. 2'-No. 4', and dibenzothiophene in No. 6'). The right ordinate indicates ratio (%) of ion current of the having maximum peak height marked as symbols ○ to the total ion current. And the peak numbers of TIC (total ion current) corresponded to the peak numbers of FID in Fig. 1.

together in a single peak as shown in the TIC chromatogram in Fig. 3, A. These two compounds, however, were obtained as separate peaks in mass chromatograms (Fig. 3, A). Fig. 3 also shows that: (1) benzothiophene derivatives with smaller numbers of methyl groups were more frequent in eel flesh than those having greater numbers of methyl groups. The benzothiophene derivatives with two to four methyl groups, however, occurred at almost the same rate in crude oil; and that (2) benzothiophene derivatives which have alkyl group(s) composed of more than seven carbons did not occur in the eel flesh extract although they were present in small amounts in crude oil as shown in A of Fig. 3. These minute components are probably benzothiophene derivatives with methyl and larger alkyl groups. These facts indicate that with more numerous and larger alkyl groups, benzothiophene becomes less transferable to eel flesh from the water. The similar phenomenon to benzothiophene was observed in mass chromatograms of dibenzothiophene derivatives (Fig. 3), the concentrations of which in the eel flesh are in the order of dibenzothiophene ( $n=0$ ) methyl dibenzothiophene (MD) ( $n=1$ )  $>$  MD ( $n=2$ )  $>$  MD ( $n=3$ ), though the concentration of methyl dibenzothiophenes in crude oil are similar each other (Fig. 3).

The presence of isomers of methyl derivatives of benzothiophene and dibenzothiophene was confirmed by the fact that peak groups on mass chromatogram of the same number of  $M^+$  consisted of several sharp and small peaks; for example, mass chromatogram peaks of  $M^+176$  (peak 3) in the upper part and  $M^+198$  (peak 1) in the lower part of Fig. 3, A consisted of small peaks,

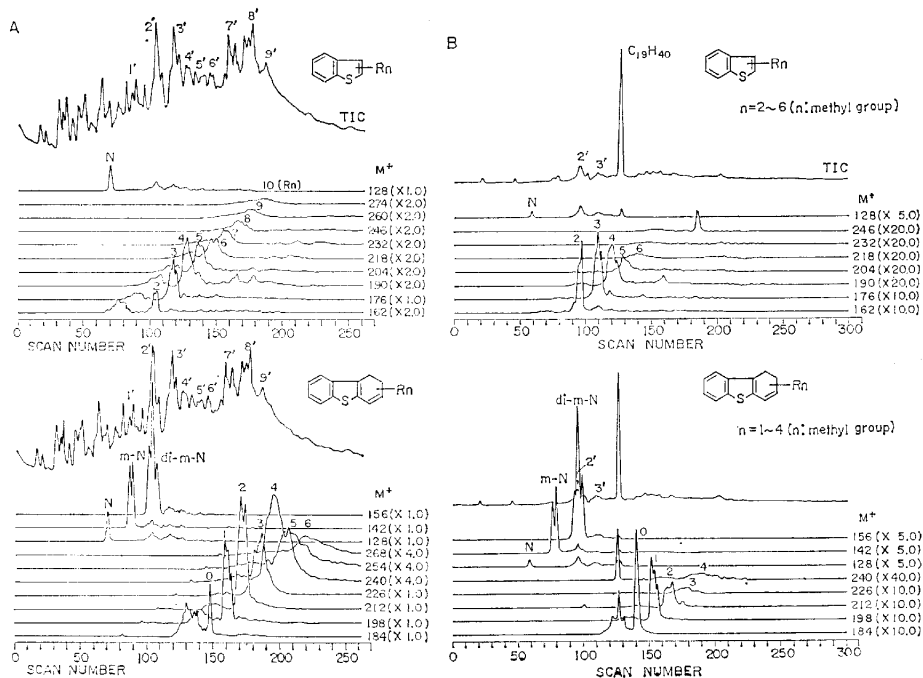


Fig. 3. Mass chromatogram (MC) of alkyl benzothiophene in crude oil and contaminated eel flesh. A; crude oil. B; eel flesh. TIC=total ion current. N=naphthalene. The  $M^+$  number shows molecular ion for each compound. The number of each MC peak indicates the carbon numbers of the alkyl group. R. T. 2.00 min and interval 6.00 sec ( $C_{19}H_{40}$  is pristane in eel flesh). Note; numbers peaks 7, 8' and 9' on the TIC curve of upper part of Fig. A did not correspond to carbon numbers, 7, 8 and 9 of alkyl group of the peaks on the curves of benzothiophenes on mass chromatogram although numbers of 7, 8', and 9' in lower part of Fig. A corresponded to carbon numbers 1, 2 and 3 of alkyl group of dibenzothiophenes.

TABLE 1. ESTIMATION OF COMPONENTS IN THE FRACTION OF AROMATIC COMPOUNDS FROM CRUDE OIL BY MASS SPECTROMETRY

Peak No.	m/e	Cal. formula	Estimated compound	Peak No.	m/e	Cal. formula	Estimated compound
1-a. b	148	$C_9H_8S$	Monomethyl-bzth.	4'-a. b. c.	184	$C_{14}H_{16}$	Tetramethyl-naph.
1'-a. b	142	$C_{11}H_{10}$	Monomethyl-naph.	5-a. b. c.	204	$C_{13}H_{16}S$	Pentamethyl-bzth.
2-a. b. c.	162	$C_{10}H_{10}S$	Dimethyl-bzth.	5'-a. b. c.	198	$C_{15}H_{18}$	Pentamethyl-naph.
2'-a. b. c.	156	$C_{12}H_{12}$	Dimethyl-naph.	6	184	$C_{12}H_8S$	Dibzth.
3-a. b. c.	176	$C_{11}H_{12}S$	Trimethyl-bzth.	7	198	$C_{13}H_{10}S$	Monomethyl-dibzth.
3'-a. b. c.	170	$C_{13}H_{14}$	Trimethyl-naph.	8	212	$C_{14}H_{12}S$	Dimethyl-dibzth.
4-a. b. c.	190	$C_{12}H_{14}S$	Tetramethyl-bzth.	9	226	$C_{15}H_{14}S$	Trimethyl-dibzth.

(Crude oil contained Tetra-, Penta-, and Hexa dibenzothiophene) Cal.=Calculated naph.=naphthalene bzth.=benzothiophene dibzth.=dibenzothiophene

a., b. and c. indicate isomers of respective compounds.

Monomethyl, Dimethyl-, Trimethyl-, Tetramethyl- and Pentamethyl-benzothiophene are contained in peak No. 1 and 1', 2 and 2', 3 and 3', 4 and 4', and 5 and 5' respectively.

## DISCUSSION

Coleman *et al.* (7) reported the presence of heterocyclic sulfur compounds in crude oil. The authors, in a previous report (5), reported that organic sulfur compounds in crude oil suspension were transferred from water to fish.

The present experiment indicates that methyl derivatives of benzothiophene, dibenzothiophene and naphthalene were transferred to eels.

Trace amounts of the organic sulfur compounds in both crude oil and eel flesh could be detected by FPD gas chromatography; therefore, these compounds are recommended as a marker for oil pollution in fish.

The presence of alkyl benzothiophenes and alkyl dibenzothiophenes in crude oil and fish was confirmed as follows. (a) Distinct peaks in FPD curves for gas chromatogram using a FPD with 394 nm filter which is specific to sulfur compounds. (b) The ratio 95:4 of the intensity of  $M^+$  and  $M^+ + 2$  mass peaks suggested the presence of  $^{32}\text{S}$ : $^{34}\text{S}$ . In this experiment, alkyl benzothiophenes and alkyl naphthalenes having the same number of methyl groups were recorded as single gas chromatographic peaks (No. 1–No. 5 of Fig. 1) when SE 52 was the liquid phase. This was proved by gas chromatograph-mass spectra and mass chromatograph.

Alkyl benzothiophenes were considered to be mainly composed of methyl derivatives of benzothiophenes because of the following reasons: a) The presence of isomers of alkyl benzothiophene and alkyl dibenzothiophene in peak groups composed of several sharp and narrow peaks of  $M^+$  curves on mass chromatogram of oil and eel. This suggests that more alkyl benzothiophene and alkyl dibenzothiophene composed of isomers of several methyl benzothiophene and methyl dibenzothiophene rather than alkyl benzothiophene and alkyl dibenzothiophene having longer chains, *i.e.* ethyl-, propyl- or butyl-benzothiophenes and dibenzothiophenes. b) In gas chromatograms isomers of methyl benzothiophene have narrow peaks, whereas ethyl-, propyl-, and butyl-benzothiophene have more complex or broader peaks. c) The presence of monomethyl-benzothiophenes in crude oil has been reported by Koga (8).

The presence of groups such as ethyl-, propyl-, butyl groups as minor benzothiophene derivatives was suggested by the peaks having  $M^+ = 232 \sim M^+ = 274$  on mass chromatograms of crude oil (Fig. 3, A). Detailed investigation of these minor components is in progress.

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