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Study on the liver oil of marine crustacea. I. Sterols and glyceryl ethers in the liver oils of Chionoecetes opilio and Lithodes turritus

by Hajime Seino, Shoichiro Watanabe, Kazuo Kawada and Yoshiro Abe

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STUDY ON THE LIVER OIL OF MARINE CRUSTACEA (THE FIRST REPORT)

STEROLS AND GLYCERYL ETHERS IN THE LIVER OILS OF CHIONOECETES OPILIO AND LITHODES TURRITUS

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The authors are working on the liver oils of edible crabs in the Northern Ocean of Japan. In the present work α-alkyl glyceryl ethers and cholesterol were found in the solid from the unsaponifiables of the liver-oils of *Chionoecetes opilio* (Zuwai-kani) and *Lithodes turritus* (Ibara-kani).

Two kinds of crystalline materials were isolated from the solid part of unsaponifiables by the fractional crystallization from methanol. One of them was identified as cholesterol by means of IR, NMR and mass spectroscopies. The other was confirmed to be the mixture of α-alkyl glyceryl ethers from the results of IR and NMR analyses. Then they were separated through GLC into C_{8}~C_{20} α-alkyl glyceryl ethers. The major components were α-*n*-hexadecyl and α-*n*-octadecyl glyceryl ethers. Their contents were respectively 69.0 %(*C. opilio*), 59.0 % (*L. turritus*) and 19.7 %(*C. opilio*), 35.2 %. (*L. turritus*).

I Preface

There are only several reports available on the study of components of unsaponifiables of marine crustacea. A long time ago, Tsujimoto [1), 2)] made a report on the presence of alcohol in the unsaponifiables of Tarabakani (Paralithodes camtschaticus), and Nakamiya [3)] isolated d-n-hexadecyl glyceryl ether (chimyl alcohol) and d-n-octadecyl glyceryl ether (batyl alcohol). Subsequently one of the writers [4)] also isolated d-n-tetradecyl glyceryl ether and d-n-dodecyl glyceryl ether in addition to the two afore-mentioned glyceryl ethers, and most recently, Tejima et al [5)] reported on sterols, one of components contained in several species of marine crustacea. No other extensive study has been conducted in this field.

The writers have been engaged in the study of the components of the liver oil of marine crustacea. This report is concerned with the study of the solid part of unsaponifiables in the liver oil of Chioneocetes opilio and Lithodes turritus. The research revealed that two kinds of the solid component obtained from the liver oil of Chionoecetes opilio were cholesterin and a mixture of d-alkyl glyceryl ether. Furthermore, the results of the respective GLC analyses on these mixtures of glyceryl ether revealed them to be the mixture of d-alkyl (C_8 - C_{19}) glyceryl ether with major components, d-n-hexadecyl glycery ether (69.0%) and d-n-octadecyl glyceryl ether (19.7%).

Likewise, the writers have found in the liver oil of Lithodes turritus the presence of cholestrin, and d-alkyl (C_{10} - C_{20}) glyceryl ether consisting mainly of d-n-hexadecyl glyceryl ether (59.0%)

and d-n-octadecyl glyceryl ether (35.2%).

2 Experiments and Results

2.1 Extraction of the Liver Oil

2.1.1 Extraction of the Liver Oil of Chionoecetes opilio

On the crab-canning boat the livers were taken out of the Chionoecetes opilio which were captured on the Okhotsk Sea during June and July, 1969. These were landed at Wakkanai and the liver oil was extracted from the livers there. Extraction was performed by the autolysis method. This means that approximately 20 liters of water were added to approximately 40 kilograms of liver, which was heated to 55° C. It was then stirred for three hours in the area of pH 6 for autolysis. Secondly, 37 kilograms of sodium chloride and 70 liters of water were added to the substance, and this was heated to 80° - 90° C, and stirred for three hours. The temperature was maintained at 80° C for six hours. It was then left overnight so as to let the oil float. The water was removed as much as possible by a separating funnel. It was then filtered. Approximately 7.5 kilograms of liver oil was obtained by the method of centrifugalization of the filtrate.

2.1.2 Extraction of the Liver Oil of Lithodes turritus

The livers of the Lithodes turritus which were captured in the vicinity of Kunasir Island (N 44°, E 146° 8') in the Okhotsk Sea on November 30, 1969, were removed on the crab-canning boat. They were shipped to the Laboratory of the University of Hokkaido

for separation of the liver oil. The method of separation was similar to that of the Chionoecetes opilio. However, because of the slow process of autolysis, the aqueous solution of saturated sodium carbonate was added to the material from time to time. While the pH 8 - 9 was maintained, it was stirred at 60° C for about one hour. It was then left in the room to cool down to the room temperature. The oil-like substance which floated was extracted with ethyl ether. The extracted fluid was filtered and ether was removed. Again, it was dissolved in petroleum ether (bp 30-60°) in order to remove protein and other components which had been mixed in the extracts. After the solution was filtered, it was rinsed in water until it became neutral, and was dehydrated with anhydrous sodium sulphate to eliminate petroleum ether. Thus 9.5 kilograms of liver oil were obtained from approximately 60 kilograms of liver.

The liver oils obtained in the above methods were dark reddish brown and half-sold at room temperature in both cases. The constants of these substances are shown in Table 1.

Table 1: Characteristics of Liver Oils of Chionoecetes opilio and Lithodes turritus

Liver oils	n_D^{15}	A.V.	S.V.	I.V.	Unsaponifiable matter(%)
Chionoecetes opilio	1.4788	15.3	178.7	123.5	6.9
Lithodes turritus	1.4760	56.9	172.4	137.0	5.5

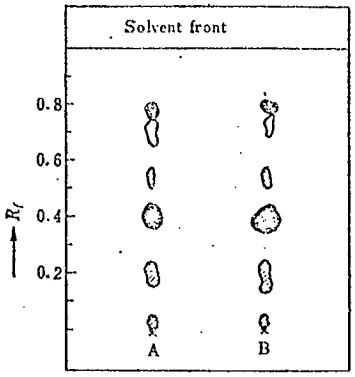
2.2 Extraction of Unsaponifiables

Five hundred millilitres of 1 N-ethanol potassium were added to a 50-gram liver oil which had been extracted in 2.1. It was saponified through convection in the nitrogen air flow for two hours. Then, 1,500 millilitres of water were added to the reaction fluid. The unsaponifiables were extracted with ethyl ether for eight hours by the use of Asahina Fluid Extraction Apparatus. The water left after the extraction was poured into the separating funnel and the remaining unsaponifiable matters were further extracted with 500 millilitres of ethyl ether. The ether solvent was added. After rinsing and dehydration, ether was removed and the unsaponifiables were obtained.

2.3 TLC of the Unsaponifiables

In order to separate the component in the unsaponifiables obtained in 2.2, the writers examinted them to determine developing conditions, using chloroform, benzene, n-hexane ether, ethyl ether and the solvent mixture of all these. As a result, the best possible separation was achieved when the combined solvent of n-hexane ether-ethyl ether = 30:70 (vol/vol) was used. The results of this experiment were shown in Figure 1. Kieselgel G (0.5 mm) was used as an absorbent. Colouration was made by spraying 30% of the sulphate and heating the plate.

Figure 1: TLC of Unsaponifiables from the Liver Oils of Chionoecetes opilio and Lithodes turritus



A: unsaponifiable matter of liver oil of *Chionoecetes opilio*
B: unsaponifiable matter of liver oil of *Lithodes turritus*
Adsorbent: Kieselgel G, 0.5mm
Solvent: *n*-hexane-ether 30: 70 (vol/vol)
Indicator: 30% H_2SO_4

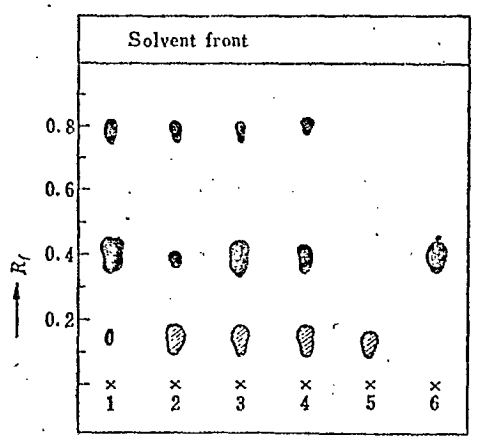
0.4 Separation of the Solid Components

The unsaponifiable matter of the liver oil of Chionoecetes opilio was dissolved in five times its volume of methanol (in the proportion of 1 gram of the unsaponifiable matter to 5 ml. of methanol). This matter was maintained at 0° C to separate crystals through filtration. White plate-formed crystals (Z-1) were obtained at the rate of 24.3% of the unsaponifiable matter. Next, after methanol was removed once from the filtrate, the remaining matter was again dissolved in the previously stated volume of methanol. The portion to be separated was filtered at the temperature of -20° C. A yellow wax-like solid component (Z-2) was obtained at the rate of 19.0% of the unsaponifiable matter.

The unsaponifiable matter of the liver oil of Lithodes turritus was likewise treated. As a result, white crystals (I-1) and a yellow solid component (I-2) were obtained at the rate of 37.2% at 0° C, and 12.4% at -20° C respectively.

TLC was applied to these Z-1 and -2 and I-1, and -2 using such developing solvents as the standard samples of d-n-hexadecyl glyceryl ether and cholesterol coupled with n-hexane-ether-ethyl ether (30:70). The results of TLC are shown in Figure 2.

Figure 2: TLC of the Solid Parts Obtained from the Liver Oils of Chionoecetes opilio and Lithodes turritus



1: Z-1 (obtained from *Chionoecetes opilio*)
2: Z-2 (obtained from *Chionoecetes opilio*)
3: I-1 (obtained from *Lithodes turritus*)
4: I-2 (obtained from *Lithodes turritus*)
5: α-n-hexadecyl glyceryl ether
6: cholesterol
Adsorbent: Kieselgel G, 0.5mm
Solvent : n-hexane-ethyl ether 30: 70 (vol/vol)
Indicator : 30%-H_2SO_4

On the TLC, were located three spots for each of the Z-1 and -2, and the I-1 and -2. It was assumed that the spots closest to the solvent front would be hydrocarbon because of the weakness of the polarity and the light purple colouration caused by sulphate.

The central spots were red-purple owing to the sulphate. The Rf value of the samples was almost the same as that of cholesterol. Accordingly, they were presumed to be the sterol group. Hereinafter this component shall be called the sterol group. The component which remained in the area of the starting point shall be called the glyceryl ether group since the Rf value was the same as that of the standard sample of glyceryl ether. The sterol group was mainly contained in Z-1. A great deal of the glyceryl ether group was contained in Z-2. A considerable amount of each of both groups was contained in I-1 and -2.

2.5 Refining of the Sterol and the Glyceryl Ether Groups

The sterol and the glyceryl ether groups which were obtained in 2.4 were refined as follows:

The substance of Z-1 was dissolved in 10 times as much n-hexane ether and cooled at -20° C. The sterol was separated. Recrystalization of the sterol is repeated by use of ethanol as a solvent to produce crystals at a melting point 143° - 149° C.

When the substance of Z-2 was dissolved in 7 times as much ethyl ether and cooled off at 0° C, only glyceryl ether was separated. Furthermore, this substance was recrystalized in ethyl ether so as to remove the sterol group.

Furthermore, glyceryl ether was collected from the filtrate of Z-1 and a sterol was obtained from the filtrate of Z-2 in the same method as mentioned before. These were also refined by the same

method. Coupled with the crystals obtained before, the total volume obtained was as follows: the sterol was obtained from the material of approximately 18% and glyceryl ether from the material of approximately 58% of the total volume of Z-1 and -2.

Furthermore, the sterol was separately obtained with n-hexane ethyl ether 30:70 (vol/vol) as a developing solvent by the use of the 1 mm-thick Kieselgel H plate, and refined with TLC to obtain crystals with a melting point 146.5° - 148.0° C. These crystals were used for analyses which will be described below. As for glyceryl ether, re-crystalization was repeated with ethyl ether and n-hexane ether until the melting point became almost stabilized. As a result, crystals with a melting point of 59.0° - 61.0° C were obtained and used as samples for analysis.

2.6 Analysis

2.6.1 The Analysis of Sterol

An analysis was made in the following way of the sterol which was isolated from the liver oils of Chiroscyllium opilio and Lithodes turritus.

First of all, the Rosenheim Reaction, Tortelli-Jaffe Reaction and Liebermann Reaction were applied to each of the samples. The tests revealed that both sterols showed a colouration of deep green with the Liebermann Reaction, which was identified as Δ^5-sterols.

Secondly, the melting point and rotatory power for each sample were measured. These results together with the result of the elementary analysis and the measure value or theoretical value of cholesterol are shown in Table 2.

Table 2: Characteristics of Sterols Obtained from the Liver Oils of Chionoecetes opilio and Lithodes turritus.

Sterols	mp (°C)	$[\alpha]_D^{19}$	Elementary analysis	
			C(%)	H(%)
Sterol A	146.5~148.0	−40.8°	82.57	11.78
Sterol B	145.4~147.5	−40.1°	82.09	11.47
Cholesterol	146.5~147.5	−39.5°	83.80*	11.99*

Sterol A : obtained from the liver oil of *Chionoecetes opilio*
Sterol B : obtained from the liver oil of *Lithodes turritus*
* : calculated for $C_{27}H_{46}O$

Thirdly, IR, NMR and mass spectra of each sample were also measured. These results were almost the same as the results of the measurement of cholesterol. A Hitachi EPI-G2 model Diffraction Grating Infrared Spectrometer was used for IR spectrum and measurement was made by the tablet method. Nichiden Valian (phonetics) make A-60 model apparatus was used for NMR spectrum. A sample was made into a solution of carbon tetrachloride for measurement. Mass spectrum was measured by the use of Nihon Denshi make JMS-OIS model apparatus.

The afore-mentioned results indicate that sterols of both the liver oils of Chionoecetes opilio and Lithodes turritus were cholesterol.

2.6.2 The Analysis of Glyceryl Ether

Figures 3 and 4 show the results of IR spectra and NMR spectra of the glyceryl ethers obtained from the liver oils of Chionoecetes opilio and Lithodes turritus.

Figure 3: IR Spectra of Glyceryl Ethers Obtained from the Liver Oils of Chionoecetes opilio (top) and Lithodes turritus (bottom).

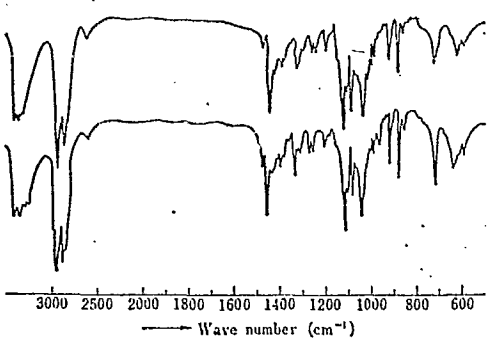
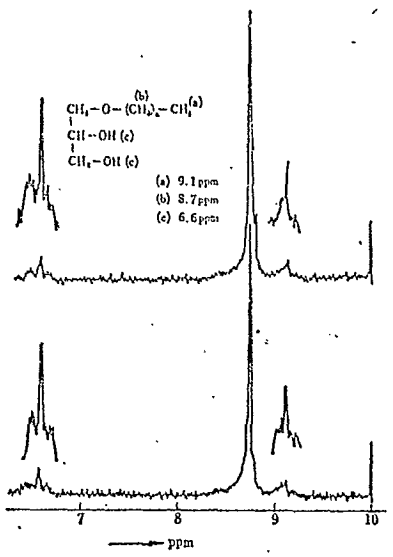


Figure 4: NMR Spectra of Glyceryl Ethers Obtained from the Liver Oils of Chionoecetes opilio (top) and Lithodes turritus (bottom).

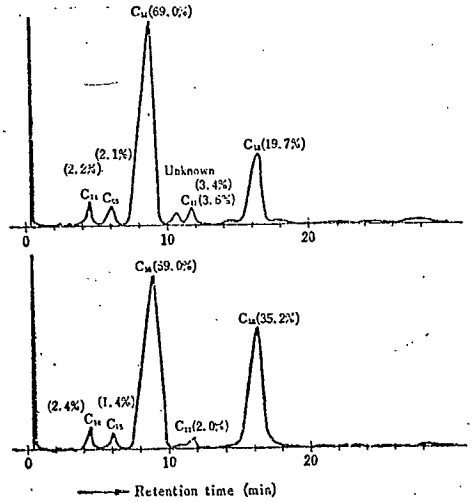


With respect to both components, one can observe on the IR spectrum the absorption of OH expansion and vibration which made a division in the area of 3,400 cm^{-1}, the ether compound at 1,120 cm^{-1}, the absorption of secondary alcohol at 1,100 cm^{-1} and primary alcohol at 1,040 cm^{-1}.

On NMR spectra in both cases, one can observe a signal which belongs to a proton of hydroxyl at τ value 6.6 ppm. The afore-said measurement results almost correspond with the analytical results of synthetic d-alkyl glyceryl ether by R. Wood et al [6), 7)] and C. N. Joo et al [8)] and the analytical results of d-glyceryl ether of the liver oil of Elasmobranchii by B. Hallgren et al [9)]. The components obtained from the liver oils of Chionoecetes opilio and Lithodes turritus were identified as a mixture of d-glyceryl ether.

Then, GLC analysis was conducted on these glyceryl ethers. Many reports have been made on the GLC analysis of the glyceryl ether group, i.e., the method of analysis of making it into isopropylidene inductive [6), 7), 10), 11), 12)], the method of making it into dimethoxy inductive [9), 13)], the method of making it into trimethyl cyrel ether or trifluoroacetate ester [15)], and the method of disconnecting the ether compound with hydroiodic acid [15)]. The writers, following after the method of R. Wood et al [14)], made a GLC analysis, making glyceryl ether into trifluoroacetate ester. Approximately 1 millilitre of anhydrous trifluoroacetate was added to approximately 1 milligram of glyceryl ether, and this mixture was occasionally shaken and left for 15 minutes at room temperature. Then, the produced trifluoroacetate and surplus anhydrous trifluoroacetate were removed under the reduced air pressure to obtain trifluoroacetate ester. Figure 5 shows the results of the GLC analysis of this trifluoroacetate ester. The writers used Hitachi 063-0050 GLC apparatus. Measurement conditions are also shown in Figure 5.

Figure 5: GLC of Trifluoroacetates of d-alkyl Glyceryl Ethers Obtained from the Liver Oils of Chionoecetes opilio (top) and Lithodes turritus (bottom).

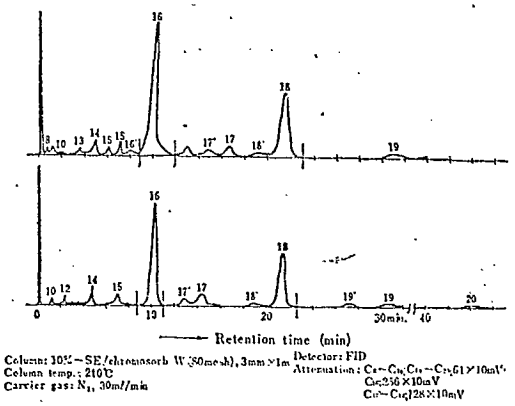


Column: 10%-SE 30/Chromosorb W (80mesh), 3mm×1m
Column temp.: 200℃
Carrier gas: N_2, 30ml/min
Detector: FID
Attenuation: 128×10mV (C_{16} 256×10mV)

Peaks by d-n-tetradecyl, d-n-hexadecyl and d-n-octadecyl glyceryl ethers were determined by a comparison of the TLC analytic results of trifluoroacetate ester obtained from their respective standard glyceryl ethers. In order to examine in more detail carbon chains of alkyl radical that was coupled with ether at d-position, the ether compound was disconnected with hydroiodic acid to make a mixture of alkyl iodides. Then GLC analysis was

made on it. Disconnection of the ether compound was performed in accordance with the method of K. E. Guyer et al [15] as stated below. Ten millilitres hydroiodic acid ware added to the glyceryl ether mixture of 0.1 gram. This substance was heated for convection for 24 hours. After that, the reaction mixture was dissolved in 150 millilitres of ether and transferred into the separating funnel. The ether solvent was rinsed in 20 millilitres of water and again rinsed in the 1- millilitre aqueous solution of saturated sodium carbonate to eliminate surplus hydroiodic acid. Then, free iodine was removed from 10 millilitre of 50% sodium thiosulphate. The substance was again rinsed in the aqueous solution of saturated sodium chloride. After the ether solution was dried with anhydrous magnesium sulphate, the ether was removed to obtain alkyl iodides. The GLC results of this mixture of alkyl iodides are shown in Figure 6. A Hitachi 063-0050 GLC Apparatus was used for this analysis and the measurement conditions are indicated in Figure 6.

Figure 6: GLC of Alkyl Iodides Obtained from the Glyceryl Ethers in the Liver Oils of Chlamydoselachus Anguineus and Lithodes Turritus.



Each peak was determined by the standard samples of alkyl iodides which were obtained from saturated alcohol of normal chains of the even number units of the number of carbons of C_8 - C_{18} and also those obtained in the same way from unsaturated mono-alcohol of C_{16} - C_{18}. With respect to those peaks which did not have standard samples, they were estimated from the graphs of logarithms of the retention time and the number of carbons.

The areas of the peaks of trifluoroacetate ester shown in Figure 5 were measured by the method of mid-value counter and the percentage composition of each component was sought. The results are also shown in Figure 5. However, in this composition, a corrective factor was not employed. The calculation was based on the total percentage of the areas of the peaks which indicate percentages in the diagram, as 100.

3 Conclusion

In the unsaponifiable matters of the liver oil of Chionoecetes opilio, the writers recognized the presence of a few kinds of components using TLC. Of these components, two kinds of white crystal components were re-crystalized, separately obtained and isolated through TLC. Between the two components, one with a higher melting point was identified as cholestrol because the analytic results by IR, NMR and mass spectra coincided with those of cholestrol. On the other hand, the other component with the strong polarity was identified as d-alkyl glyceryl ether because of the absorption of the primary and secondary alcohol-ether compound on the IR spectrum and the results of the NMR spectrum. Secondly, as a result of applying GLC to that component which is made into trifluoroacetate ester, it was identified as a mixture of glyceryl ether which consists of mainly d-n-hexadecyl glyceryl ether (69.0%) and d-n-octadecyl glyceryl ether (19.7%). Furthermore, in order to make a close examination of the chain length of the alkyl radical which is connected with ether at d-position, the ether connection was disconnected with hydroiodic acid to obtain a mixture of alkyl iodides. GLC was applied to this mixture. As a result, the writers recognized the presence of C_8 - C_{19} carbon chains including the odd number carbon chains.

By the same methods, the writers detected the presence of cholesterol in the liver oil of Lithodes turritus, and the

presence of d-alkyl glyceryl ether consisting mainly of C_{16} (59.0%) and C_{18} (35.2%) and ranging from C_{10} to C_{20}.

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