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By Yaichiro Shimma and Hisako Taguchi

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Separation of Unsaponifiable Compounds of Deep-sea Shark Oils by Chromatography on Florisil

By Yaichiro Shimma and Hisako Toguchi

It was first found by Mr. Tsujimoto[1] and his colleagues that there was squalene and glyceryl ether contained in the unsaponifiable compounds of deep-sea shark oils.

A detailed investigation was then conducted by Mr. Azuma[2] and his colleagues concerning the content of squalene and Vitamin A contained in various Japanese deep-sea shark oils.

The research on the possible content of other unsaponifiable compounds, however, has not yet been undertaken.

A very important fact was reported as a result of an experiment conducted by Mr. Karnovsky on the separation of unsaponifiable compounds in various kinds of shark oils in South Africa by using a column chromatographic procedure[4] with alumina.

In this research, the content of squalene and glyceryl ether in the unsaponifiable matter of Chimaera monstrosa and Echinorhinus spinosus

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1 For example, Tsujimoto, Mitsumaru, Kanyu no Kenkyu (The Study of Oils), Maruzen Co. Ltd., Tokyo, 1936, P. 104

2 For example, Azuma, Hideo and his colleagues, Nissuishi (Japan Fishery Journal), 1953, vol. 19, P. 836

3 Karnovsky, M. L., et al, J. Soc. Chem. Ind., 1947, vol. 66, P. 124

4 Fitelson, J., J. Assoc. Off. Agric. Chem., 1943, vol. 26, P. 499

showed 97.2 and 98.5 percent respectively of the total content of the unsaponifiable matter.

Mr. Carroll[5], in a recent experiment with Florisil involving the separation of cholesterol, triglycerides, and phosphatide, found that Florisil had an excellent nature for producing the separation of fats.

Prior to determining quantitatively the content of unsaponifiable compounds of Japanese deep-sea shark oils, the writers first attempted to investigate various conditions in the separation of squalene, cetyl alcohol, cholesterol, and glyceryl ether by using model compounds of these which might be contained in the unsaponifiable matter.

Some deep-sea shark oils, as is well known, contain a great quantity of Vitamin A. A highly active Florisil which has a great adsorption strength tends to reduce considerably the recovery rate of Vitamin A since this Vitamin is dissolved by Florisil.

This was confirmed in an experiment using different concentrations of Florisil in order to examine the most suitable conditions for recovery rate and for the separation of the model compounds.

The writers describe the experiment and the result involving the

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[5] Carroll, K. K., J. Lipid. Res., 1961, vol. 2, P. 135

separation of unsaponifiable matter in Chimaera barbouri (Japanese - Usagi-Zame) and Centrosymnus owstoni (Japanese - Yonaichi-Zame) by using a concentrated Florisil and a diluted Florisil respectively.

According to an investigation in the Suruga Sea, conducted in 1964 by Mr. Somei Abe, a Technical Officer, it is estimated that the Centrosymnus owstoni (Japanese - Yonaichi-Zame or Yume-Zame) is greater in number than any of the other deep-sea sharks. Mr. Azuma[2] and his colleagues made a detailed examination of the content of squalene contained in the Centrosymnus owstoni oils.

Chimaera barbouri (Japanese - Usagi-Zame or Kokonohoshigin-Zame) is known to contain a great quantity of glyceryl ether in unsaponifiable matter[6].

Methods

Adsorbent; Florisil (60 - 100 mesh), manufacture of Floridin Company which was obtained from Wako Junyaku Kogyo K.K.

Solvent; 35 - 60°C fraction obtained from distillation of petroleum ether (reagent grade), ethyl ether and methyl alcohol (reagent grade) are used.

Model compounds obtained as follows

Squalene; N_D^{40} 1.4893 obtained from unsaponifiable matter of Squalus mitsukurii oil (Japanese - Ai-Zame).

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[2] Azuma, Hideo and his colleagues, Nissuishi (Japan Fishery Journal), 1953, vol. 19, P. 836

[6] Toyama, Yoshiyuki, Tōkōshi (Tokyo Industrial Laboratory), 1925, vol. 20, P. 158

Cetyl alcohol; A commercial preparation which is distilled and chromatographed. According to the gas chromatography, the composition of the substance is C_{14} 0.9%, C_{15} 0.4%, C_{16} 93.2% and C_{18} 5.5%.

Cholesterol; product of Kanto Kagaku K.K. The best quality is used.

Saturated glyceryl ether; the glyceryl ether obtained from the unsaponifiable matter of Etmopterus lucifer (Japanese - Fuji-Kujira) and recrystallized from petroleum ether. It contains 281.5 acetyl value. The substance produced is chiefly composed of $C_{21}H_{44}O_3$ which was probably reduced from selachyl alcohol and it is likely to be a mixture which contains C_{19} and C_{23}.

Chromatographic Methods

Chromatography tube, 1.2 cm. in internal diameter and 58 cm. in length was used in this experiment.

10 gm. of Florisil was packed in the tube and the tube was connected to the apparatus reducing pressure. 30 cc. of petroleum ether was added from the top and the solvent was eluted until it reached just above the surface of the adsorbent. The solvent was then discarded. 0.2 to 0.25 gm. of the material to be eluted was accurately weighed and added to the column with the aid of a small amount of petroleum P.50

ether. The column was developed by using various solvents. After the addition of the material, the eluting solvent was collected at 10 cc. intervals, the solvent was evaporated, and the compound was purified. Under these conditions, the column capacity was approxmately 11 cc.

II. Experiment and Result

Chimaera barbouri ↓

1. Chromatography on a highly active Florisil

Table 1. Elution of unsaponifiable components from highly active Florisil

Fraction number	Eluting solvent		Standard materials (253.4 mg)				Unsaponifiables of "Usagi-zame" (205.4 mg)	
			Name	Added	Eluted	Recovery	Eluted	Cont.
		cc		mg	mg	%	mg	%
1—6	1% E·PE*	60	Squalene	122.5	118.3	96.6	3.0	1.4
7—10	10% E·PE	40	—	—	1.4	—	1.4	—
11—18	50% E·PE	80	Cetylalcohol Cholesterol	36.6 34.7	73.0 (32.1)**	102.4 (92.5)	4.1 (1.74)	1.95
19—22	65% E·PE	40	—	—	1.3	—	6.3	—
23—30	5% MeOH·E	80	Sat. glyceryl-ether	59.6	58.1	97.5	174.6	85.1
31—35	20% MeOH·E	50	—	—	0.3	—	6.5	—

As shown in Table 1 and Figure 1, a fresh supply of a highly active Florisil was used with a mixture of the model compounds and unsaponifiable matter of a sample of Chimaera barbouri (Japanese - Usagi-Zame).

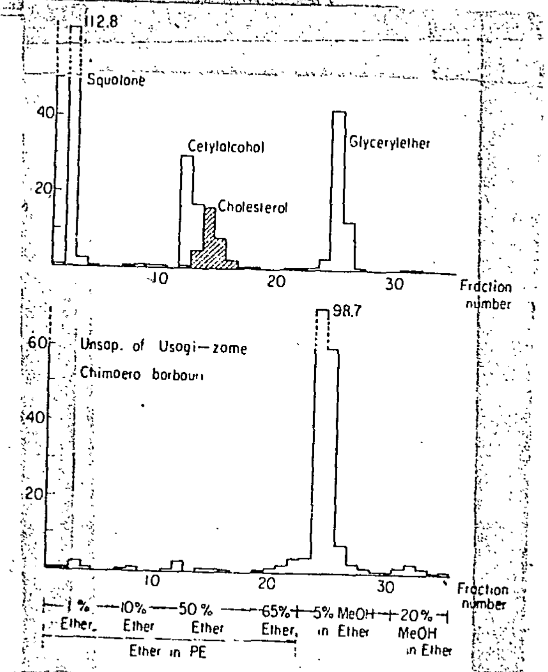

Fig. 1. Separation of unsaponifiable matters on 10 g column of highly active Florisil freshly supplied

Using the materials squalene, cetyl alcohol, cholesterol of model compounds and saturated glycdryl ether, 772(6)

Florisil chromatography was carried out with the addition of a mixture of the following six different eluting solvents; 1) 1% E•PE 60 cc. (1% ether in petroleum 60 cc), 11) 10% E•PE 40 cc., 111) 50% E•PE 80 cc., 1V) 65% E•PE 40 cc., V) 5% MeOH•E 80 cc., V1) 20% MeOH•E 50 cc.

In the chromatographic result, as shown in Fig. 1, the sequences of eluting solvents are indicated in Fractions 1, 111 and V. The amount of materials which was eluted from the column is shown in Table 1. In effecting the recovery was 96.6 percent, cetyl alcohol and cholesterol, 102.4 percent and saturated glyceryl ether, 97.5 percent.

In this process, however, a separation of cetyl alcohol and cholesterol was unsuccessful. The isolation of a mixture of cetyl alcohol and cholesterol in Fraction 111 was determined by the use of the coloration method (Momose-Ho)[7] and the recovery rate of cholesterol was 92.5 percent. In this experiment, the rate of flow of cholesterol was a little slower than that of cetyl alcohol. The flow of cholesterol in Fraction number 12 was very small and the peak of the flow was observed in Fraction number 14, while continuosly decreasing the amount by Fraction number 18. (Fig. 1) P.51

Under the same circumstances mentioned above, 205.4 mg. of the unsaponifiable matter of Chimaera barbouri was chromatographed. In this experiment, 95.4 percent of the unsaponifiable matter was eluted

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7. Momose, T., et al, Anal. Chem., 1963, vol. 35, P. 1751

by Fraction V1 weighing 195.9 mg. of the material.

In the sequence of Fractions, a larger amount of glyceryl ether, 85.1 percent was found in the eluted materials in Fraction V.

In a report by Mr. Tsujimoto [8], it was mentioned that squalene is not contained in Chimaera barbouri oil but according to our experiment, 1.4 percent of hydrocarbon was found to be contained in the oil, as shown in Table 1.

In Fraction 111, in the unsaponifiable matter, the content of cetyl alcohol and cholesterol was only 1.95 percent and, out of the content, cholesterol determined by the coloration method was calculated as 1.74 mg. out of 4.1 mg. of the eluted materials. In addition to cholesterol a monovalent alcohol may also be contained in the eluted materials.

When glyceryl ether of the model compounds was used, an elution of glyceryl ether showed the peak in Fraction number 25 (Fig. 1) and where the unsaponifiable matter of Chimaera barbouri was used, the peak of the flow was observed in Fraction number 24.

This result indicates that the unsaponifiable matter of Chimaera barbouri contains more than 85 percent glyceryl ether and such a large amount helps to start an earlier flow than with the model compounds.

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8 Tsujimoto, Mitsumaru, Tōkōshi (Tokyo Industrial Laboratory), 1923, vol. 18, P. 68

The properties of acetyl value, iodin value and refractive index in Fraction numbers 24 and 25 are shown in Table 2.

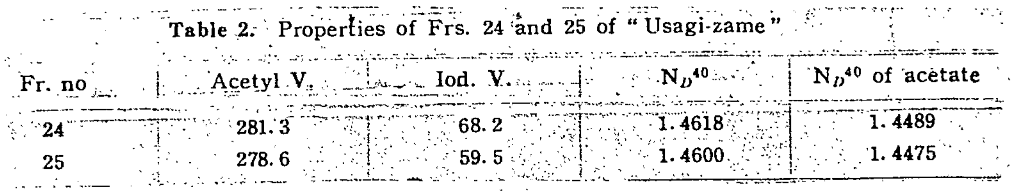

Table 2. Properties of Frs. 24 and 25 of "Usagi-zame"

Fr. no	Acetyl V.	Iod. V.	N_D^{40}	N_D^{40} of acetate
24	281.3	68.2	1.4618	1.4489
25	278.6	59.5	1.4600	1.4475

The content of unsaturated glyceryl ether eluted in Fraction number 24 is higher than in number 25. It is therefore considered that unsaturated glyceryl ether is eluted more easily than saturated glyceryl ether.

In experimenting with the unsaponifiable matter of Chimaera barbouri, the material to be eluted with a mixture of eluting solvent of 65 percent ether in 40 cc. of petroleum ether might possibly contain glyceryl ether. However, because of the very small amount present, examination was not possible. If a quantity of glyceryl ether is to be eluted at an early stage, approximately 3 percent experimental error must be taken into consideration for the content calculated.

2. Chromatography using a diluted Florisil

Chromatography of Vitamin A Concentrate

Freshly supplied Florisil has strong adsorption properties. When petroleum ether is used as the eluting solvent, Vitamin A is greatly adsorbed by the reaction of the Florisil and the recovery rate is less than 40 percent and showing a blue colour. As Florisil is gradually deactivated if stored for several years after use, Vitamin A is not affected by the Florisil.

For this reason, Mr. Carroll[5] recommends the use of a hydrated Florisil, and in his experiment, Mr. Carroll used Florisil hydrated with 7 percent water. Deep-sea shark oil usually contains only a very small amount of Vitamin A[9].

A great quantity of Vitamin A is reported to be contained in <u>Heteroscymnus longus</u> (Japanese - <u>Kaeru-Zame</u>)[10].

Keeping in mind the chromatography of unsaponifiable matter which contains Vitamin A, the writers examined the relationship between the degree of dilutinn of Florisil and the recovery rate of Vitamin A. Vitamin A Concentrate (2,150,000 unit/g) refined from distilled whale oil which was chromatographed by the use of a highly active Florisil, and also by Florisil hydrated with 7, 8, 10, and 12 percent water. The weight of the eluted materials obtained in each Fraction and adsorption at 325 mμ. are shown in Table 3. In using a highly active Florisil, Vitamin A was strongly adsorbed and only a small amount was accumulated in Fraction 111. The flow of the content was larger in Fraction V. Even with Florisil diluted within a range of 7 to 12 percent water, only a small difference was found, and a maximum quantity of Vitamin A flowed

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[5] Carroll, K. K., J. Lipid. Res., 1961, vol. 2, P. 135

[9] Azuma, Hideo and his colleagues, Nissuishi,(Japan Fishery Journal), 1953, vol. 21, P. 448

[10] Azuma, Hideo and his colleagues, Nissuishi (Japan Fishery Journal), 1955, vol. 21, P. 448

in Fraction 111.

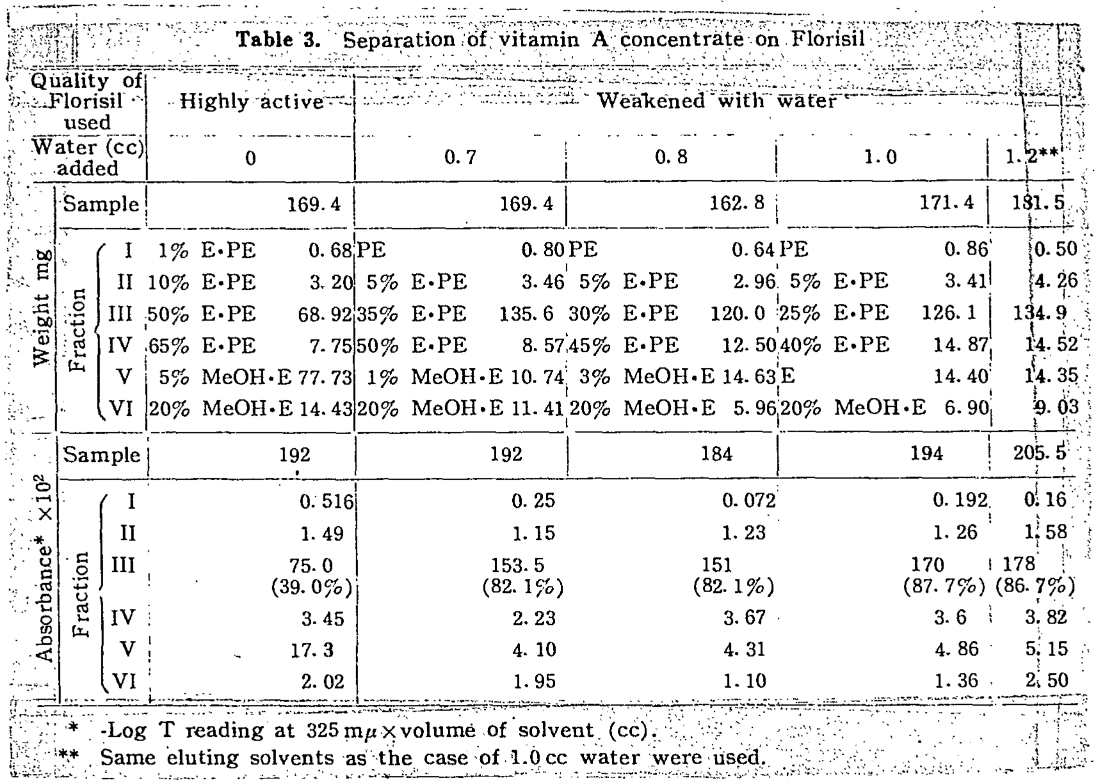

Table 3. Separation of vitamin A concentrate on Florisil

	Quality of Florisil used	Highly active	Weakened with water			
	Water (cc) added	0	0.7	0.8	1.0	1.2**
Weight mg	Sample	169.4	169.4	162.8	171.4	181.5
Fraction	I	1% E·PE 0.68	PE 0.80	PE 0.64	PE 0.86	0.50
	II	10% E·PE 3.20	5% E·PE 3.46	5% E·PE 2.96	5% E·PE 3.41	4.26
	III	50% E·PE 68.92	35% E·PE 135.6	30% E·PE 120.0	25% E·PE 126.1	134.9
	IV	65% E·PE 7.75	50% E·PE 8.57	45% E·PE 12.50	40% E·PE 14.87	14.52
	V	5% MeOH·E 77.73	1% MeOH·E 10.74	3% MeOH·E 14.63	E 14.40	14.35
	VI	20% MeOH·E 14.43	20% MeOH·E 11.41	20% MeOH·E 5.96	20% MeOH·E 6.90	9.03
Absorbance* $\times 10^2$	Sample	192	192	184	194	205.5
Fraction	I	0.516	0.25	0.072	0.192	0.16
	II	1.49	1.15	1.23	1.26	1.58
	III	75.0 (39.0%)	153.5 (82.1%)	151 (82.1%)	170 (87.7%)	178 (86.7%)
	IV	3.45	2.23	3.67	3.6	3.82
	V	17.3	4.10	4.31	4.86	5.15
	VI	2.02	1.95	1.10	1.36	2.50

* -Log T reading at 325 mμ × volume of solvent (cc).

** Same eluting solvents as the case of 1.0 cc water were used.

In order to examine the most suitable condition of Florisil, a diluted Florisil with 10 percent water was used. The eluted material which flowed in Fraction 111 was collected, an antioxidant was added to the fraction, and the fraction was again passed through diluted Florisil containing 8, 10, 12, and 14 percent water. 8% column was washed with 80 cc. of 30% E•PE and the same amount of 25% E•PE added to other columns. The amount eluted was determined at 325 mμ. The recovery rates of Vitamin A in each of four columns were 89, 97, 96, and 94 percent respectively. Chromatography using 10 gm. of Florisil diluted with 1 cc. of water was most successful in the recovery of Vitamin A.

Chromatography of unsaponifiable matter of model compounds and Chimaera barbouri.

Centrosymnus owstoni

Table 4. Elution of unsaponifiable components from weakened Florisil

Fraction number	Eluting solvent		Standard materials (261.1 mg)				Unsaponifiables of "Yonaichi-zame" (224.4 mg)	
			Name	Added	Eluted	Recovery	Eluted	Cont.
		cc		mg	mg	%	mg	%
1—6	PE	60	Squalene	143.5	134.7	93.9	170.3	75.9
7—10	5% E·PE	40	—	—	2.0	—	0.4	—
11—18	25% E·PE	80	Cetylalcohol Cholesterol	50.5 16.7	66.0 (16.3)*	98.2 (97.6)	1.5 (1.3)	0.67
19—22	40% E·PE	40	—	—	1.5	—	0.2	—
23—30	E	80	Sat. glyceryl-ether	50.4	52.1	103.4	32.5	14.5
31—35	20% MeOH·E		—	—	0.2	—	2.2	—

* See footnote of Table 1.

The experiment was carried out with a mixture of model compounds and the unsaponifiable matter of Centrosymnus owstoni oil. The result of the analysis is shown in Table 4 and Figure 2.

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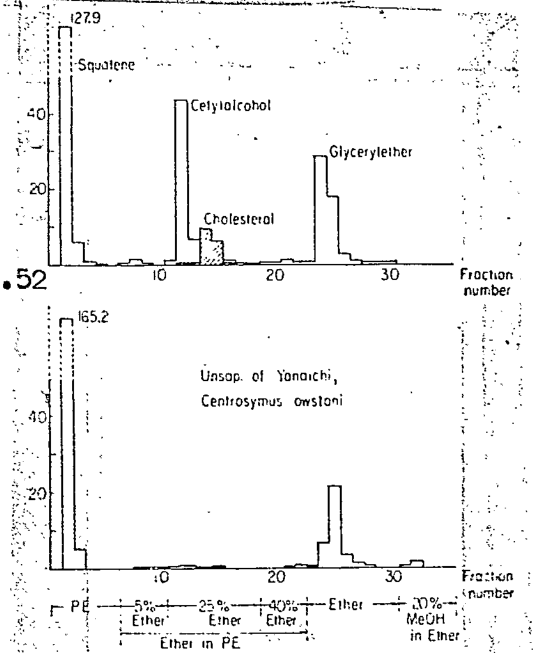

Fig. 2. Separation of unsaponifiable matters on 10 g column of weakened Florisil

The same mixture of model compounds as used in the previous experiment was chromatographed with addition of six different eluting solvents as follows; 1) PE 60 cc., 11) 5% E•PE 40 cc., 111) 25% E•PE 80 cc., 1V) 40% E•PE 40 cc., V) E 80 cc., and V1) 20% MeOH•E 50 cc.

In this experiment, the same result was obtained as in the previous operation, and each of the model compounds was distributed in the same way. (Fig. 2)

Table 4 indicates the weight of the eluted materials obtained in each Fraction. A recovery rate of model compounds in Fraction 1, 111, and V was 93.9 percent squalene, a mixture of cetyl alcohol and cholesterol, 98.2 percent, saturated glyceryl ether, 103.4 percent. A recovery rate of choresterol in Fraction 111 examined by the coloration method was 97.6 percent.

Under the same circumstances, 224.4 mg. of unsaponifiable matter of Centrosymnus owstoni was chromatographed. A total of 207.1 mg. was eluted in Fraction Vl. This was 92.3 percent of the total content. P.53

Hydrocarbon constituted the major part of Fraction 1 and Fraction V followed. The content of hydrocarbon contained in unsaponifiable matter of Centrosymnus owstoni showed 75.9 percent and the total of hydrocarbon and glyceryl ether was more than 90 percent. The result agrees with that obtained by Mr. Karnovsky.

In Fraction 111, choresterol weighed 1.3 mg. of the 1.5 mg. eluted and there was no monovalent alcohol present.

Discussions

The result of this study showed that chromatography on a highly active Florisil and a diluted Florisil produced only a slight difference in the recovery rate of the model compounds.

It may be considered that the difference is due to experimental error rather than to the degree of Florisil activation. With most of the material used, recovery was more than 95 percent, except 93.9 percent of squalene and 92.5 percent of cholesterol as shown in Table 2 and Table 1. In an experiment in which real material is used, there is no necessity to weigh the eluted material at 10 cc. intervals. The experimental errors will be much smaller because the elution of the compounds is classified by eluting solvents into six groups. Freshly supplied Florisil which has a strong activation, is not deactivated for a few months if the cover is kept tightly closed, and therefore, special storage is not necessary. The writers used Florisil in the amount of approximately 100 gm. and were careful not to open the container too frequently. Mr. Carroll used a hydrated Florisil for fear that fats might be decomposed by a highly active Florisil.

However, in the case of the separation of unsaponifiable matter contained in deep-sea shark oils, the compounds are not likely to be affected by a highly active Florisil except in the dissolution of Vitamin A. If unsaponifiable matter of Chimaera barbouri oil containing a great amount of glyceryl ether are then chromatographed by a diluted Florisil under the conditions shown in Table 4, a satisfactory separation cannot be obtained, due to the flow of glyceryl ether. A highly active Florisil seems to be preferable for the purpose of our study.

Vitamin A is easily detected by the adsorption power of ultraviolet rays. Only a few deep-sea shark oils contain more than 5,000 lU/gm of Vitamin A except in the case of *Kaeru Zame*[9]. Considering these facts, a highly active Florisil will produce better results in a chromatographic analysis.

It has been reported that when silicic acid is used for column chromatography, the chromatography tube should be kept at a certain temperature.

This experiment was carried out at a room temperature of 20±50C. No particular attention was paid to the temperature; however, the temperature did not seem to affect the experiment to any degree.

Summary

Column chromatography of unsaponifiable compounds was done by using a highly active Florisil and a diluted Florisil, and conditions were examined on the separation of hydrocarbon, monovalent alcohol and glyceryl ether in the analysis of unsaponifiable matter of *Centrosymnus owstoni* and *Chimaera barbouri* oils.

The authors gratefully acknowledge the assistance of Mr. Somei P.54 Abe, a Technical Officer, in obtaining and examining deep-sea shark oils to be used as material; and Mr. Tokugoro Kaneko, a Technical Officer, in providing the very useful materials for the study.

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[9] Azuma, Hideo and his colleagues, Nissuishi, (Japan Fishery Journal), 1953, vol. 21, P. 448

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