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Studies on the lipids of the Japanese littleneck, *Tapes japonica* (Deshayes)
I. Composition of acetone-soluble lipids

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Studies on the Lipids of Japanese Littleneck, (Tapes japonica)(Deshayes). I.

Composition of Acetone-soluble Lipids

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1. Preface

Studies on lipids of *Tapes japonica* (Deshayes) have already been done by Tsujimoto and Koyanagi[1] in connection with extraction of the lipids and determination of properties as well. Although Matsumoto and Tamura[2] made study on the sterols by which the presence of 7-dehydrostigmasterol was confirmed, no research seems to be made on the compositions of the lipids. *Tapes japonica* being one of the most popular edible shellfishes in Japan, the author has furthered the research on this line.

Since it has already been reported by Tsujimoto *et al* concerning the outstanding quantity of phosopholipids (62%) contained in lipids of *Tapes japonica*, the author intends to report on the results of study on the composition of acetone-soluble lipids other than phosopholipids in this paper particlularly from the viewpoint of determining whether there exist significant seasonal variation or not.

2. Experiments and Results

2-1. Samples

Samples were purchased from the same retail store where they were sold stripped in April, August and December. These were cultured at Fukukawa, Yamaguchi Prefecture. The average weight of twenty samples selected from among them and by weighing of individual smaples indicated considerable intra-seasonal variations as were shown in the instances of the two experiments conducted in April.

Samples were weighed after washing lightly in water, removing foreign matters and then boiling for five minutes with some more water added and drying by wrapping them in the cotton cloth so that water would be removed as much as possible.

2-2. Extraction of Lipids

Extraction of total lipids from the samples was conducted with the use of ethyl-alcohol-ether or methyl-alcohol-chloroform.

Samples were first homogenized with added ethyl-alcohol of the equal volume and running it for two minutes by a mixer which was then put to the suction filtering treatment after adding ethyl- alcohol, a half of the sample volume. The remainder was soaked overnight in the same volume of ether, and after drying, solvents were removed in carbonic acid gas flow under the

reduced pressure; thus total lipids were obtained by putting these treated samples together. The remainder of these ether soaking treatments that were repeated twice by then was subjected to further treatment with chloroform-methanol (1:1) for two minute homogenizing by a mixer and although the extracts were sought in the similar manner, they being very small in volume, it was considered that the lipids had been extracted adequately by two soakings in ether.

The extraction with methanol-chloroform was carried out for the samples purchased in April. The samples were first put in the mixer for two minutes with the same volume of chloroform and the double quantity of methanol added to them. Then, adding a half volume of chloroform, they were suction filtered after homogenizing for thirty seconds. The residue was again homogenized with chloroform of the equal quantity and suction filtered after adding the half volume of chloroform. The similar treatment to that for ether extraction was repeated for the filtered solution.

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The total extracts thus obtained were dissolved in the double volume of ether and were placed in the refrigerator for one night for the inclined separation of the unsolubles after pouring them into the quintuple quantity of acetone. Acetone then was removed from the solution under the reduced pressure,

and the residue of which was treated by Folch's washing[3] thus obtaining the acetone-soluble lipids. In other words, lipids that had been obtained from the acetone solution were first dissolved in chloroform five times larger in volume than the solution (2:1) and, by using a separating funnel, were quietly poured into a beaker of 200 ml capacity and it in turn was sank in a larger one with one litre capacity which was filled with distilled water. After leaving it overnight in the refrigerator, we took out the chloroform layer with a pipette which we dried and on removing of the chloroform, obtained the acetone-soluble lipids.

Folch's washing was done to keep the formation of emulsions at the lowest possible level in the counter-current distribution.

The acetone-soluble lipids that were thus obtained had dark brown colour with seaweed like smell and were partially crystalized even during the summer season and was*

The results of extraction and its chemical properties are shown in Table 1.

Table-1. I ... .cal Properties

Table-1 Extraction of lipids and its chemical properties.

	April I	April II	August	December
Mean weight of one sample (g)	2.4	3.2	3.9	2.9
Sample weight, total (g)	570.0	620.0	590.0	830.0
Lipids (g)	10.24	13.02	10.81	25.73
(%)	1.8	2.1	1.8	3.1
Acetone-soluble lipids (%)	43.0	36.2	38.3	29.5
A.V.	9.0	8.8	9.7	8.6
S.V.	147.0	140.5	133.2	164.0
Unsaponifiables (%)	27.3	31.3	32.0	36.6
Fatty acids N.V.	191.9	203.5	193.1	209.9
I.V. (Wijs)	184.2	176.0	172.5	162.6

* Translator's note: not legible till the end of the paragraph.

Besides the components of lipids mentioned in Table-1, the acetone-soluble lipids contained phospholipids as indicated in the thin-layer chromatogram (2~4, Fig.-2) which was the result of the experiment conducted by the method explained in the later section of this report, and although in fact the treatment for separation of acetone still seemed somewhat inadequate, the samples were regarded as the acetone-soluble lipids without further repetition of the treatment.

2-3. Counter-Current Distribution

There are many reports already published on the counter-current distribution of lipids.[4] In this experiment, separation of the acetone-soluble lipids was conducted by repeating transfers for 100 times with such solvents as petroleum ether (bp 40-60°C) and 95% methanol. The counter-current device used for this purpose was Type CDA-100 manufactured by Shibata Kagaku.

Counter-current distribution curves were drawn on the basis of weights of the residues which were collected by removal of solvents under the reduced pressure separately from the upper and the lower layers after 100 transfers. One of the examples is shown below as Figure 1.

Figure-1. Counter-Current Distribution of Acetone-Soluble Lipids of Japanese Littleneck

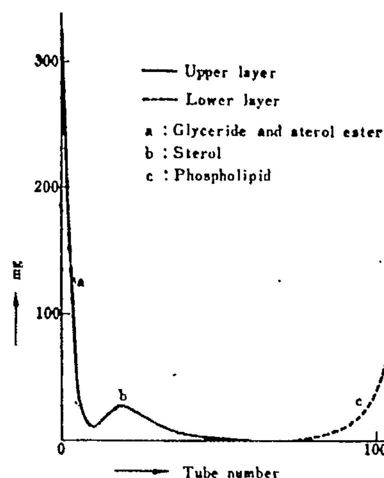

Fig.-1 Countercurrent distribution of acetone-soluble lipids of Japanese Littleneck.

Each peak in the Figure-1 was identified by the infrared absroption spectrum and the thin-layer chromatography. As is known from the above figure, the separation of the free sterol in the upper layer and phospholipids in the lower layer was possible while glyceride and sterol ester could not be separated.

Under these circumstances, the ratio of sterol ester and glyceride was calculated through determination of the weight of the unsaponifiables in the mixture of these two substances which was collected from the samples. However, for calculation, the average molecular weight which was computed on the basis of the neutralization value of mixed fatty acids was used as the molecular.

weight of fatty acids, and in the case of sterol, 7-dehydrostigma-sterol, $C_{29}H_{46}O$ (molecular weight 410.7), to which reference is made in the later section of the present report, was used. Composition of the acetone-soluble lipids was sought, using the weight of free sterol separated by counter-current distribution, and determining the quantity of phospholipids from the difference of the total weights of sterol ester and glyceride as well as free sterol, and the weight of the sample used for counter-current distribution.

The results are shown below in Table-2.

Table-2 Composition of Acetone-Soluble Lipids of Japanese Littleneck

Table-2 Composition of acetone-soluble lipids of Japanese Littleneck.

Sample / Compositon (%)	April I	April II	August	December
Glycerides	52.3	53.3	51.6	54.2
Sterol, ester	8.5	8.8	8.1	7.8
Sterol, free	14.2	16.1	16.9	19.8
Phospholipids	25.0	21.8	23.4	18.2

2-4. The Thin-Layer Chromatography

In order to identify the separation of these fractions, the thin-layer chromatography was carried out. The plates were made first using Wako gel B-5 (manufactured by Wako Jun Yaku) which is a kind of silica gel used for the thin-layer chromatography, to form 0.22 mm thick layer of silica gel and then placed for the analysis after activation at 110°C for 30 minutes. Such

solvent as were used by Malins, Mangold[5], namely, petroleum ether(bp 60-70°C) - ethyl ether - glacial acetic acid(90:10:1vol/vol), and diisobutylketone - glacial acetic acid - water (40:25:3vol/vol) by Kaneko, Kawanishi[6] were used. Spots were detected after devloping for 10 cm at room temperatures and subsequent dehydration at 110°C. For this purpose, either method of spraying 50% sulfuric acid and heating at 110°C or spray of 15% antimony trichloride - chloroform solution and heating at 90°C was employed.

Figure-2 illustrates the thin-layer chromatograms of acetone-soluble lipids by two different solvents. Whereas spots a, b and d showed brown colour by spray of both sulfuric acid and antimony trichloride solution, spot c showed reddish purple by the former and pinkish red by the latter. And spot c' turned grayish blue by both. Identification of the respective spots was conducted by simultaneous development with that of standard substances. Those standard substances are 7-dehydrostigmasterol separated from the unsaponifiables as sterol and olive oil as glyceride, furthermore, as sterol ester, cholestrol palmitate separated by the column chromatography using alminum oxide after having made cholesterol and palmitic acid react to each other. For phospholipids, the portion of acetone-unsolble that had previously been separated was used.

Figure-2 <u>Thin-Layer Chromatograms of Acetone-Soluble Lipids</u>

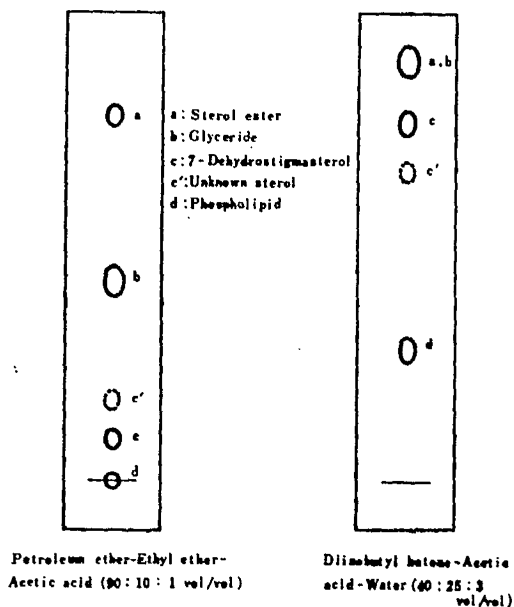

Fig.-2 Thin-layer chromatograms of acetone-soluble lipids.

Consequently, spots a, b, c and d coincided with respective spots of sterol ester, glyceride, 7-dehydrostigmasterol and phospholipids. However, it is considered c' is a different kind of sterol from c, judging from its colour and <u>Rf</u> value.

The separation of these fractions by counter-current distribution was indentified with the thin-layer chromatography.

2-5. <u>The Unsaponifiables</u>

The unsaponifiables separated by the standard method were crystals with orange colour, of which 0.1208 g formed 0.2475 g of unsoluble <u>digitonin</u> and indicated 51.2% sterol content in 1% <u>digitonin</u> - ethyl alcohol(90%) solution.

Through the column chromatography of the unsaponifiables with silicic acid (100 mesh, manufactured by Mallinckrodt), crystals effluent from petroleum ether (bp 40-60°C) - ether (70: 30vol/vol) were recrystalized with acetone and methanol thus obtaining crystals of mp138-140°C, $[\alpha]^{26}_{D}$ - 39° (chloroform). These were again recrystalized with methanol upon acetylation of the same by pyridine-glacial acetic acid and, as a result, crystals of mp127-128°C were obtained. In Figures 3 and 4 are shown respective infrared absorption spectra. (by KBr pill method).

Figure-3 <u>Infrared Absorption Spectrum of Sterol obtained from Unsaponifiables (KBr)</u>

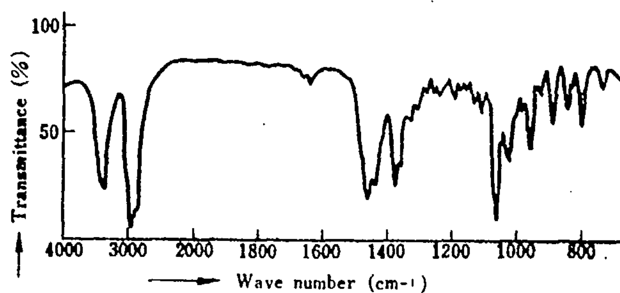

Fig.-3 Infrared absorption spectrum of sterol obtained from unsaponifiables (KBr).

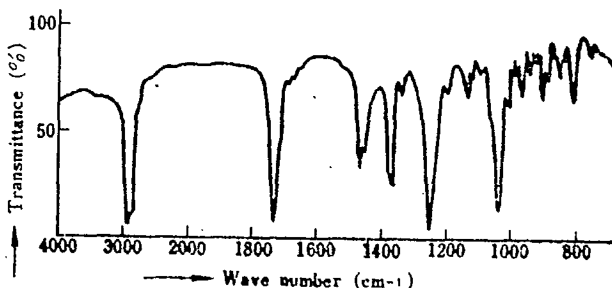

Fig.-4 Infrared absorption spectrum of sterol acetate (KBr).

Figure-4 <u>In</u> ... <u>Acetate</u>
<u>(K</u>

The ultraviolet absorption spectrum of the sterol obtained from the unsaponifiables shown in Figure-5 indicated the same maximum wave number as was noted for the sterol separated from Japanese Littleneck by Matsumoto and Tamura[2] and from Maboya( ) by Takagi *et al*[7] (Table-3).

Figure-5 Ultraviolet Absorption Spectrum of Sterol obtained from Unsaponifiables(EtOH)

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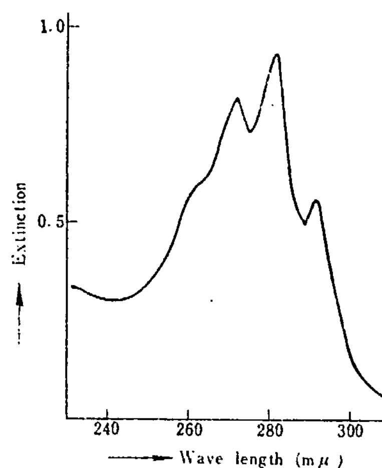


Fig.-5 Ultraviolet absorption spectrum of sterol obtained from unsaponifiables (EtOH).

Table-3 Ultraviolet absorption of 7-dehydrostigmasterol.

Author	λ_{max} (mμ)		
Matsumoto, Tamura[2]	271.5	282	293
Takaki *et al.*[7]	271	282	294
Observed	272	282	292

Table-3 Ultraviolet Absorption of 7-dehydrostigmasterol

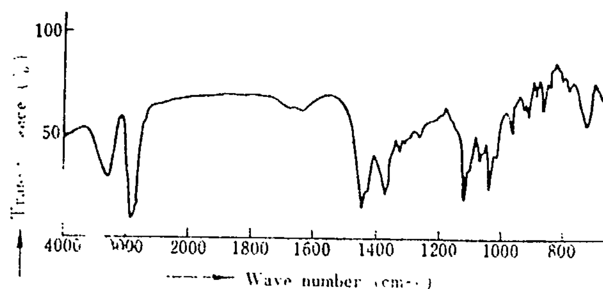
Also, the infrared absorption spectrum of acetate of the present experiment and the result obtained by Takagi et al[7] were identical. Accordingly, it was deduced that the sterol separated from the unsaponifiables in this experiment was 7-dehydrostigmasterol, however, because of insufficient refining, this substance showed unusually low * point and angle of rotation.

The reddish brown resin like matter mixed with some crystals was obtained from the orange coloured solution of the unsaponifiables after repeating separation of crystals twice with hydrate methanol and removing it and then extracting with petroleum ether. This was $[\alpha]_D$* =-8° (chloroform) with sterol contents of 23.7% by digitonin method, of which 0.18g underwent the column chromatography on silicic acid, and while 0.09g eluted by ether - petroleum ether)50:50) was considered to be 7-dehydrostigmasterol on the basis of its infrared absorption spectrum and of the result of the thin-layer chromatographic analysis, 0.04 g eluted by ether- methanol (75:25) appeared to be containing somewhat different sterol. Therefore, it was placed under further analysis of the column chromatography on silicic acid, and as a result, yielded 0.01 g of crystals with very light yellowish colour eluted with ether- petroleum ether (50:50). p.53

Judging from its infrared absorption spectrum (Figure-6), this was considered to be different sterol than 7-dehydrostigmasterol yet unidentified.

* Translator's note: not legible.

Figure-6 Infrared Absorption Spectrum of Unidentified Sterol (KBr)



By Liebermann-Burchard reaction test conducted on this unidentified sterol, it turned from blackish blue to dark green, on the other hand, 7-dehydrostigmasterol showed, first, pinkish red colour and gradually turned from reddish purple to dark green. Further, no indication of the maximum absorption at its ultra-violet portion was noted. It also showed a grayish blue spot at Rf 0.67 as a result of its thin-layer chromatography with diiso-butylketone - glacial acetic acid - water as compared to the red-dish purple spot at Rf0.78 shown by 7-dehydrostigmasterol. This led to a concusion that the spot given by the unidentified sterol corresponded to spot c' as indicated by the thin-layer chromato-gram in Figure-2.

From the above results, it was indicated that whereas the sterol of the unsaponifiables of Japanese Littleneck was mainly 7-dehydrostigmasterol, there was a small quantity of another sterol present although yet unidentified.

3 Observation

Tsujimoto and Koyanagi (through their experiments carried out in June, 1933) reported that of 2.0% extracts extracted from Japanese Littleneck by alcohol - ether, the acetone-soluble lipids make up 35.9%. As are shown in Table-1 of the present report, the results obtained from the author's experiment approximately coincided with the above, except for the samples collected in December in which somewhat higher contents of the total lipids were noted. On seasonal variations in lipids of shellfish, Matsumoto[8] reported that lipids of magaki(*Ostrea gigas*, Thunberg) start increasing in December and show a drastic decline after spawning in August. However, spawning period of Japanese Littleneck is known to extend from spring to autumn and to vary from one habitat to another.[Note 1] Although the results tabulated in Table-1 indicate a decrease in lipids contents as early as in April, a comparison with the lipid properties of another group of the samples collected in April reveal that variations in the contents of the lipids could not be considered only seasonal.

Note 1) : The author's thanks are due Prof. Kobayashi of the faculty of Biology in the University of Hiroshima who so kindly provided the information on which this statement is based.

According to Tsujimoto and Koyanagi, the properties of the acetone-soluble lipids of Japanese Littleneck are: Acid Value

17.2, Saponification Value 103.2, Iodine Number 188.7, Unsaponifiables 45.7%. Some deviations from the above, however, are noted in the results compiled in Table-1 of this report. Moreover, seasonal variation of the composition of the acetone-soluble lipids(Table-2) cannot be said prominent.

In closing, the author wishes to extend his sincere appreciation to Prof. Matsuura of Department of Natural Science in the University of Hiroshima for his guidance.

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