THE HIGHLY UNSATURATED ACIDS IN SARDINE OIL. VII. THE SEPARATION OF HIGHLY UNSATURATED C_{22}-ACIDS.

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As stated in the first report[1] of this series, the highly unsaturated acids of sardine oil contain clupanodonic acid $C_{22}H_{34}O_2$ as a most important constituent. It was first separated by Tsujimoto. Regarding its constitution there have been appeared the studies of the same author and also of Inoue and Sahashi, which will be mentioned in a succeeding paper. This acid appears to be of common occurrence in marine animal oils as an important constituent of highly unsaturated acids, since it has hitherto been found in various kinds of marine animal oils besides sardine oil. Tsujimoto and his co-workers[2] found the same acid in giebel oil, in the liver oil from *Stereolepis ischinagi,* in eel oil, cod liver oil and herring roe oil. According to Ueno and Iwai,[3] the menuke oil and the liver oil from *Scoliodon laticaudes* contain clupanodonic acid. We[4] found clupanodonic acid in several kinds of whale oils (oils from the whales belonging to Mysticete), cod liver oil, sperm oil, pilot-whale oil, and in several kinds of the liver oils from Elasmobranch fish. The majority of the oils examined by us seemed to contain, in addition to clupanodonic acid, an acid $C_{22}H_{36}O_2$, whilst there were indications of the presence of a more highly unsaturated acid $C_{22}H_{32}O_2$ in the liver oils from Elasmobranch fish. Suzuki and his co-workers[5] stated, in their studies on glycerides constituents, the separation of some glycerides containing clupanodonic acid as a fatty acid component from a number of marine animal oils, and also a glyceride containing an acid $C_{22}H_{36}O_2$ from

(1) This Bulletin, **4** (1929), 83.

(2) Tsujimoto, *Report of the Tokyo Imperial Industrial Research Laboratory,* **18** (1923), No. 2; *ibid.,* **24**(1929), No. 4; *ibid.,* **26** (1931), No. 10; Tsujimoto and Koyanagi, *ibid.,* **25** (1930), No. 4; Tsujimoto and Kimura, *J. Soc. Chem. Ind., Japan,* **26** (1923), 1162.

(3) *J. Soc. Chem. Ind., Japan,* **37** (1934), 121, 562.

(4) Toyama, *J. Soc. Chem. Ind., Japan,* **28** (1925), 95, 104; *ibid.,* **29** (1926), 531, 538, 624; *ibid.,* **30** (1927), 519; Toyama and Tsuchiya, *ibid.,* **28** (1925), 966; *ibid.,* **30** (1927), 63, 116, 207; Toyama, *Report of the Tokyo Imperial Industrial Research Laboratory,* **27** (1932), No. 2.

(5) *Proc. Imp. Acad. Japan,* **5** (1929), 265, 269.

sardine and herring oils. Brown and Beal[6] found clupanodonic acid and an acid $C_{22}H_{32}O_2$ among the highly unsaturated C_{22}-acids in menhaden, cod liver, herring, salmon and sardine oils. Recent investigations by Ono,[7] Okada,[8] and Tomiyama[9] showed clupanodonic acid to be a constituent of the oil obtained from the egg of *Squalus sucklii* and the tunny body and liver oils. Although the conclusions set forth by some of the above-mentioned authors appear not to have been supported by a sufficient experimental evidence, and although the constituents of the highly unsaturated acids are not alike, depending on the kinds of marine animal oils, it seems as a whole that sardine oil and other marine animal oils contain clupanodonic acid and some other acids, such as $C_{22}H_{32}O_2$ and $C_{22}H_{36}O_2$, as highly unsaturated C_{22}-acids. The acid $C_{22}H_{36}O_2$ has been reported to occur also in alligator oil,[10] badger fat,[11] ox liver fat,[12] and algae oil,[13] besides the marine animal oils.

The following two methods have hitherto been employed for the separation of clupanodonic acid from the marine animal oils:

(1) Bromination of the mixed fatty acids of marine animal oils and the separation of the ether-insoluble bromides give the highly unsaturated acids on subsequent debromination. Methyl esters of these acids are then fractionally distilled and a fraction corresponding to methyl clupanodonate is collected separately.

(2) A concentrated fraction of highly unsaturated acids is first prepared by means of the lithium-soap-acetone method or the sodium-soap-acetone method. This is then converted into methyl esters and the latter subjected to a fractional distillation, by which a fraction corresponding to methyl clupanodonate is collected separately.

These methods will give a good result, indeed, provided that the highly unsaturated C_{22}-acids consist almost entirely of clupanodonic acid, but when clupanodonic acid is present in association with some other highly unsaturated C_{22}-acids, such as $C_{22}H_{32}O_2$ and $C_{22}H_{36}O_2$, it seems quite difficult to separate them into individual constituents by a single application of the above methods; consequently the highly unsaturated

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(6) *J. Am. Chem. Soc.*, **45** (1923), 1289.
(7) *J. Agr. Chem. Soc. Japan*, **8** (1932), 788.
(8) *Journal of the Imperial Fisheries Institute*, **28** (1932), 105.
(9) *Bulletin of the Japanese Society of Scientific Fisheries*, **2** (1933), No. 1.
(10) Kobayashi, *J. Soc. Chem. Ind., Japan*, **25** (1922), 691.
(11) Ueno and Kuzei, *J. Soc. Chem. Ind., Japan*, **29** (1926), 525.
(12) Kimura, *J. Soc. Chem. Ind., Japan*, **28** (1925), 1366.
(13) Tsujimoto, *J. Soc. Chem. Ind., Japan*, **28** (1925), 386.

C_{22}-acid which was prepared by the above methods as a clupanodonic acid can not be taken as proved to be a chemical individual. The analytical constants of additive character, such as elementary composition, neutralisation value, iodine value, alone do not furnish a criterion for the purity of the clupanodonic acid prepared by the above methods, since there may be a possibility that a mixture containing the acids $C_{22}H_{32}O_2$ and $C_{22}H_{36}O_2$ together with clupanodonic acid may show the analytical constants which agree with or, at any rate, do not deviate widely from the calculated values for clupanodonic acid. Under these considerations, we have first separated in these experiments the highly unsaturated C_{22}-acids by means of the sodium-soap-acetone method and the fractional distillation of the methyl esters, then we have subjected the highly unsaturated C_{22}-acids to a further separative method in order to effect a separation into the portions having different degrees of unsaturation. For this purpose the sodium-soap-acetone method was modified; i.e., the highly unsaturated acids were separated into more highly and less highly unsaturated portions by dissolving in acetone, partially neutralising with sodium hydroxide solution, and fractionally precipitating the sodium soaps, the amount of water and alcohol in the solvent (acetone) being made extremely small. Each portion was treated again in a similar way to effect a further separation, and after repeating this separative operation many times, we have succeeded in separating clupanodonic acid in a much purer state than was hitherto prepared. There was obtained also a fraction consisting mainly of an acid $C_{22}H_{32}O_2$ on the one hand, and a fraction having a lesser degree of unsaturation than clupanodonic acid on the other. The latter was, however, found to be contaminated with a small portion of cetoleic acid $C_{22}H_{42}O_2$ belonging to mono-ethylenic acid series. The presence of an acid $C_{22}H_{36}O_2$ could not be ascertained.

Experimental.

1. **Separation of Highly Unsaturated C_{22}-acids from Sardine Oil.** In the 4th report,[14] the methyl esters (8 kg.) of crude highly unsaturated acids were subjected to fractional distillation in order to separate the fraction consisting of the methyl esters of highly unsaturated C_{20}-acids. The higher fractions obtained thereby contained the methyl esters of highly unsaturated C_{22}-acids. At first, these fractions were worked up for the separation of highly unsaturated C_{22}-acids, but since they were found to contain some esters formed by intramolecular rearrangement, or intramolecular polymerisation, of highly unsaturated esters, further experiments on these fractions were discontinued, and another specimen of sardine oil was used as

(14) This Bulletin, **10** (1935), 241.

a starting material for the separation of highly unsaturated C_{22}-acids. Such intramolecular rearrangement, together with intermolecular polymerisation, is considered to take place during the fractional distillation when the temperature of the bath is too high.

The sardine oil used for this experiment was procured from Hokkaido and had d_4^{15} 0.9293, n_D^{15} 1.4818, acid value 2.2, saponification value 189.7, iodine value[15] 176.3, unsaponifiable matter 0.59%. The mixed fatty acids yielded 61.49% of ether-insoluble bromides.

This oil was first treated by the sodium-soap-acetone method in the following manner: 50 g. of oil was saponified with 75 c.c. of sodium hydroxide solution prepared by dissolving 50 g. of sodium hydroxide in 100 c.c. of water and 200 c.c. of 95% alcohol. After saponification, the excess of sodium hydroxide was neutralised with an acetic acid solution prepared by mixing equal volumes of glacial acetic acid and water. Denoting the number of c.c. of acetic acid solution required for neutralisation by A, 880 c.c. of acetone and $(45-A)$ c.c. of water were added to the soap solution, and the precipitate of insoluble sodium soaps was filtered. The sodium soaps remaining in the filtrate were decomposed with hydrochloric acid to obtain the free fatty acids, which consisted mainly of highly unsaturated acids and had neutralisation value 181.9 and iodine value 311.5. The yield of these fatty acids was about 4 kg. from 10 kg. of sardine oil. These were converted into methyl esters (4 kg.) which on fractionation yielded the results given in Table 1.

Table 1.

Fraction	B.p./2 mm.	Saponif. value	Iodine value	Yield (g.)
(1)	Below 170°	191.6	190.2	610
(2)	170–180°	186.4	236.8	660
(3)	180–190°	177.6	283.3	653
(4)	190–200°	170.4	325.1	744
(5)	200–214°	163.4	365.4	1180
Residue and loss	—	—	—	153

The fraction (4) yielded, on refractionation, appreciable amounts of a fraction boiling over 200°/2 mm. The latter was united with the fraction (5) and subjected to a repeated fractionation, by which a fraction (394 g.) boiling at 207–212°/2 mm. was collected separately. It had d_4^{15} 0.9247, n_D^{15} 1.4959, saponif. value 162.4, iodine value 370.8. The free acids liberated from this methyl ester fraction had d_4^{15} 0.9397, n_D^{15} 1.5039, neutr. value 170.1, iodine value 386.2, and gave 129% of ether-insoluble bromides having Br-content 71.40%. Hydrogenation of the free acids yielded behenic acid which, after recrystallisation from 95% alcohol, had neutr. value 164.5 and melted at 80.5–81° alone or admixed with a pure specimen of behenic acid. Accordingly the free acids obtained above were proved to consist of C_{22}-acids. Their

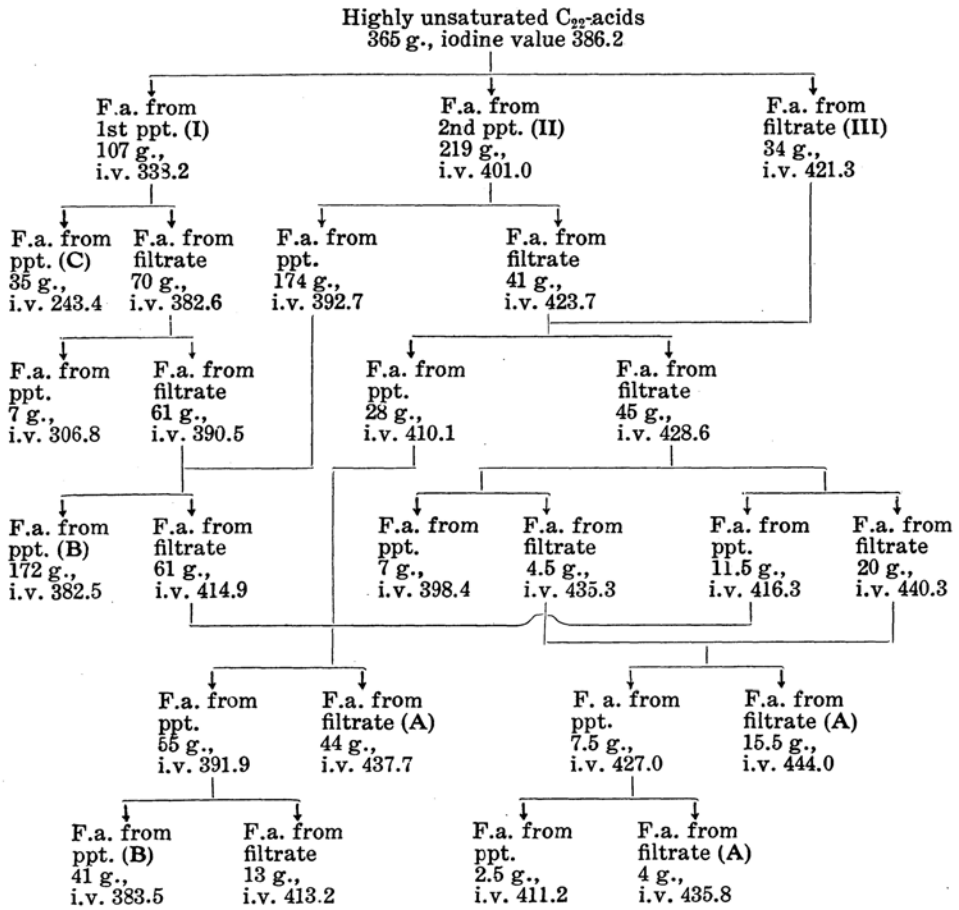
(15) Unless otherwise stated, the iodine values recorded in this paper were determined by the Wijs method.

iodine value is close to the calculated value (384.2) for clupanodonic acid $C_{22}H_{34}O_2$, but a further examination described below revealed that they contained, in addition to clupanodonic acid, an acid $C_{22}H_{32}O_2$ and some less unsaturated acids.

2. **Further Separation of Highly Unsaturated C_{22}-Acids by Means of Fractional Precipitation of their Sodium Soaps.** The highly unsaturated C_{22}-acids obtained above were separated into the fractions having different degrees of unsaturation in the following manner: 50 g. of the acids were dissolved in 1 l. of acetone and partially neutralised by using a portion of the sodium hydroxide solution prepared by dissolving 50 g. of sodium hydroxide in 100 c.c. of water and 200 c.c. of 95% alcohol. After the precipitate of sodium soaps was once brought into the solution by warming on the water-bath, the solution was allowed to cool to reprecipitate the insoluble sodium soaps which were collected, and on acidification there were obtained the fatty acids (I). The fatty acids remaining in the filtrate were once recovered and dissolved again in acetone, partially neutralised as before and the precipitated sodium soaps on acidification gave the fatty acids (II). The filtrate contained the fatty acids (III) of highest unsaturation which were recovered in the usual way. The fatty acids thus obtained (I, II, and III) were subjected to a further separation by dissolving in a little acetone, partially neutralising by using a portion of the sodium hydroxide solution prepared by dissolving 50 g. of sodium hydroxide in 50 c.c. of water and 100 c.c. of 95% alcohol, and adding more acetone so as to precipitate the insoluble sodium soaps. On decomposing the latter with hydrochloric acid, the fatty acids of comparatively low unsaturation were obtained, whereas the filtrate yielded the remaining fatty acids of highest unsaturation. For every 100 g. of the fatty acids, about 2 l. of acetone was used in most cases; but the quantity of acetone was somewhat changed depending upon the quantity of sodium hydroxide solution used for the partial neutralisation. In short, this method of separation is based upon the fact that the solubility of the sodium soaps of highly unsaturated acids in acetone containing water or alcohol increases with the degree of unsaturation of the highly unsaturated acids. Since the solubility of the sodium soap of a highly unsaturated acid decreases with the decrease in water or alcohol content in acetone and this effect is remarkably high, caution must be taken to determine the purity of acetone when the recovered acetone is used for this purpose. After repeating this separative operation, there were obtained the results given in Table 2.

3. **Separation of a Concentrated Fraction of Docosahexenoic Acid $C_{22}H_{32}O_2$.** It appears from the iodine values that the fractions (A) recorded in Table 2 contain a large amount of docosahexenoic acid $C_{22}H_{32}O_2$. These were combined, and subjected to a fractional precipitation of sodium soaps in acetone. The less unsaturated portion was removed as the precipitate of insoluble sodium soaps. The more unsaturated portion recovered from the filtrate was treated again as before in order to obtain the still more unsaturated portion by removing a further quantity of less unsaturated portion, and after this separative operation had been repeated several times, there was obtained finally a fatty acid fraction of the highest unsaturation which was deemed to consist largely of docosahexenoic acid together with a little clupanodonic acid. It had the following constants: d_4^{15} 0.9521, d_4^{20} 0.9486, n_D^{15} 1.5129, n_D^{20} 1.5109, neutralisation value 170.1, iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 456.0 and 440.8 respectively (calc. for $C_{22}H_{32}O_2$: neutr. value 170.9, iodine value 464.0), thiocyanogen value 225.7 (calc. for the formation of hexathiocyanate:

Table 2.

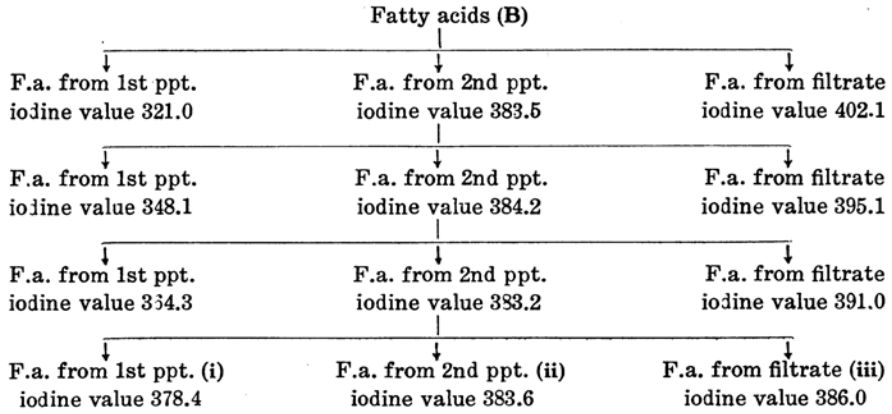


232.0). It yielded 177% of ether-insoluble bromide which turned black at about 240° without melting (Found: Br, 74.00. Calc. for $C_{22}H_{32}O_2Br_{12}$: Br. 74.50%).

4. **Separation of Clupanodonic Acid** $C_{22}H_{34}O_2$. The fatty acid fractions (B) in Table 2 were united and dissolved in acetone, and a small amount of the sodium hydroxide solution was added so as to precipitate a small portion of the fatty acids as insoluble sodium soaps which were filtered. The filtrate was then completely neutralised with the sodium hydroxide solution and the bulk of the fatty acids was precipitated as insoluble sodium soaps. The free fatty acids were isolated from the 1st and 2nd precipitates and also from the final filtrate in the usual way. The fatty acids obtained from the 1st precipitate showed comparatively low iodine value, whilst those obtained from the final filtrate showed the highest iodine value. The fatty acids obtained from the 2nd precipitate were treated again as before in order

to separate into three portions, and after repeating this separative operation, there were obtained the results shown in Table 3.

Table 3.



The iodine values of the fatty acid fractions (i, ii, and iii) obtained by the final separation do not differ much from one another, and the fatty acid fraction (ii) seems to consist largely of clupanodonic acid, the iodine value of which is 384.2.

The fatty acid fraction (ii) was converted into methyl ester and the latter was subjected to the fractional distillation by which a fraction boiling at 207–212°/2 mm. was collected as methyl clupanodonate. It had the following constants: d_4^{15} 0.9240, d_4^{20} 0.9205, n_D^{15} 1.4955, n_D^{20} 1,4934, molecular refraction 108.8 (calc. for $C_{23}H_{36}O_2$ ⌐5: 107.7), saponif. value 163.0 (calc. 163.0), iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 368.0 and 353.9 respectively (calc. 368.6), thiocyanogen value 148.9 (calc. for the formation of tetrathiocyanate: 147.5).

Clupanodonic acid set free from the methyl ester showed d_4^{15} 0.9390, d_4^{20} 0.9356, n_D^{15} 1.5035, n_D^{20} 1.4934, molecular refraction 104.1 (calc. for $C_{22}H_{34}O_2$ ⌐5: 103.0), neutr. value 170.4 (calc. 169.8), iodine values by the Wijs and the Rosenmund-Kuhnhenn methods 383.2 and 366.3 respectively (calc. 384.2), thiocyanogen value 155.2 (calc. for the formation of tetrathiocyanate 153.7). It yielded 156% of ether-insoluble bromide which turned black at about 240° without melting (Found: Br, 70.88. Calc. for $C_{22}H_{34}O_2Br_{10}$: Br, 70.76%).

5. **Examination of the Comparatively Less Unsaturated Fraction.** The iodine value (243.4) of the fatty acid fraction (C) in Table 2 indicates that this fraction contains some less unsaturated fatty acids than clupanodonic acid. The fraction was separated into two portions by means of fractional precipitation of sodium soaps in acetone, the less unsaturated portion obtained from the precipitate of insoluble sodium soaps was subjected to a further separation, and after repeating this separative operation, there was obtained finally a fatty acid fraction (about 5 g., iodine value 109.9) from the precipitate of insoluble sodium soaps. On cooling the solution of this fraction in 80% alcohol, a crystalline solid separated; it had neutr. value 165.0, iodine value 74.9 and m.p. 32–32.5°, and it was identified as cetoleic acid (neutr. value

165.8, iodine value 75.0, and m.p. 32.5–33°) by the mixed melting point test. It was thus ascertained that the fraction (C) contained cetoleic acid, though in a minor amount. This indicated that when the sardine oil was first treated by the sodium-soap-acetone method, a small amount of cetoleic acid remained in the acetone solution, so that it entered into the concentrated fraction of highly unsaturated acids, and became concentrated in this fraction (C) by subsequent separative operations. The filtrates which came from the separative operations on the fraction (C) yielded the fatty acid fractions having iodine values 350.8, 304.4, and 205.7 respectively, but no individual acid could be isolated from these fractions. Should the sardine oil contain an acid $C_{22}H_{36}O_2$, it would enter into the fatty acid fraction (C), but its presence in the fraction (C) could not be ascertained by this experiment, though the absence of this acid should not be prematurely concluded.[16]

Summary.

A concentrated fraction of highly unsaturated acids has been separated from sardine oil by means of sodium-soap-acetone method. It was converted into methyl esters and the latter subjected to a fractional distillation which yielded a fraction consisting of the methyl esters of C_{22}-acids. This fraction and the free fatty acids liberated from it showed iodine values which were close to those of methyl clupanodonate and clupanodonic acid respectively, but on separating the fatty acids of this fraction by a fractional precipitation of sodium soap in acetone solution, they were found to contain, in addition to clupanodonic acid, some acids of different degrees of unsaturation. After a repeated separation, clupanodonic acid has been separated in much purer state than prepared hitherto, and also a more highly unsaturated portion consisting chiefly of docosahexenoic acid $C_{22}H_{32}O_2$ has been separated. There was obtained also a less unsaturated portion than clupanodonic acid; this was, however, found to be a mixture contaminated with cetoleic acid $C_{22}H_{42}O_2$, though in a minor amount. Docosatetraenoic acid $C_{22}H_{36}O_2$ was not separated, although this acid could not be altogether deemed to be absent.

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(16) In previous studies (*loc. cit.*) on the fatty acids constituents of various kinds of marine animal oils, we have inferred the presence of an acid $C_{22}H_{36}O_2$ from the fact that in the case of the majority of the oils examined, the highly unsaturated C_{22}-acids showed an iodine value somewhat lower than the calculated value for clupanodonic acid. However, taking the results of the present investigation into consideration, the low iodine values of the highly unsaturated C_{22}-acids obtained in the previous studies might have been due to the contamination of some less unsaturated acids, such as cetoleic acid $C_{22}H_{42}O_2$, and not to the presence of the acid $C_{22}H_{36}O_2$.