THE HIGHLY UNSATURATED ACIDS IN SARDINE OIL

XXII. AN EXAMINATION FOR THE OCCURRENCE OF DIENOIC ACIDS OF C_{16}-C_{22} AND TRIENOIC ACID OF C_{20} IN SARDINE OIL

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So far as unsaturated acids of C_{16}–C_{22} in sardine oil are concerned, the following members have hitherto been separated and identified: monoenoic acids of C_{16}, C_{18}, C_{20} and C_{22}, trienoic acids of C_{16} and C_{18}, tetraenoic acid of C_{16},[1)2)] C_{18} and C_{20}, pentaenoic acids of C_{20} and C_{22} and hexaenoic acid of C_{22}. Among these unsaturated acids, polyethenoid acids contain C_{20}- and C_{22}-members as their chief components together with a lesser amount of C_{18}-members and a still lesser amount of C_{16}-members. Comparing polyethenoid acid fractions of different carbon numbers in sardine oil, the fractions of a larger carbon number is generally more highly unsaturated than the fraction of a smaller carbon number. Besides these unsaturated acids of C_{16}–C_{22}, a tetradecenoic acid[3)] and a tetracosahexaenoic acid[4)] have been found in sardine oil. A tetracosenoic acid also appears to occur in sardine oil. In spite of the occurrence of such many members of unsaturated acids in sardine oil, none of dienoic acids and neither eicosatrienoic acid nor docosatrienoic acid have been convincibly identified in sardine oil. On the other hand, turning to aquatic animal oils other than sardine oil, the presence of some dienoic acids and eicosatrienoic acid in some aquatic animal oils has been reported in the literature, as is the case with linoleic acid in the oil of a Formosan fish *Chanos chanos*,[5)] 11,14-eicosadienoic and 11,14-docosadienoic acids in the oil of a shark *Carcharodon carcharias*,[6)] 11,14-eicosadienoic and 8,11,14-eicosatrienoic acids in a Cambodian fish oil,[7)] 17,20-hexacosadienoic acid in the oil of a sponge *Spheciospongia vesparia*,[8)] and an octadecadienoic acid recognized as linoleic acid in the oil of fresh water crustaceans, *Palaemon nipponensis* and *Cambarus clarkii*.[9)]

This paper records the results of our examination for the occurrence of dienoic acids of C_{16}–C_{22} and eicosatrienoic acid in sardine oil. As described in a previous report of this series,[10)] the sardine oil fatty acids were segregated by the urea adduct method to give three fractions from the first crop of crystalline urea adduct, the seccond crop of crystalline urea adduct and the filtrate (I). The fatty acid fraction from the second crop of crystalline urea adduct was segregated further into a fraction from the crystalline urea adduct (*A*) and a fraction from the filtrate (II). The fatty acids from the filtrates I and II were converted to methyl esters, and the latter were fractionated further until eventually the following members of the methyl esters of polyethenoid acids were separated: docosahexaenoate,

docosapentaenoate, eicosapentaenoate, eicosatetraenoate, octadecatetraenoate, octadecatrienoate and haxadecatrienoate. It was found in these fractionations that while all methyl ester fractions of C_{22}- and C_{20}-acids had an iodine value which was too high to leave scarcely any room for the occurrence of less unsaturated trienoate and dienoate in these fractions, some of the methyl ester fractions of C_{18}- and C_{16}-acids had a lower iodine value than octadecatrienoate or hexadecatrienoate. Prior to the present study, such methyl ester fractions of lower iodine values were preliminarily examined for dienoate with the results that methyl octadecadienoate was separated and identified while methyl hexadecadienoate was absent or present in a too small amount to permit its separation. In the following, the description of these preliminary experiments are omitted, and the experiments with the fatty acid fraction from the crystalline urea adduct (*A*) are described.

Experimental

As described in a previous report,[10] urea fractionation of the sardine oil fatty acids (15 kg.) of neutralization value 196.9 and iodine value (Wijs) 179.3 gave a fatty acid fraction (1,243 g.) from the crystalline urea adduct (*A*). This was converted to methyl ester, and the latter was fractionally distilled to give five fractions shown in Table 1.

TABLE 1. Fractional Distillation of the Methyl Ester Mixture

Fraction	B.p. (°C/ca. 1 mmHg)	Yield (g.)	Saponif. V.	Iodine V.
1	–165	177	223.3	52.5
2	165–180	373	212.3	105.3
3	180–190	395	188.2	167.5
4	190–205	110	175.5	215.3
5	205–215	150	164.4	243.2
Residue (Diff.)	—	75	—	—

1. Examination for docosadienoic, eicosadienoic and eicosatrienoic acids

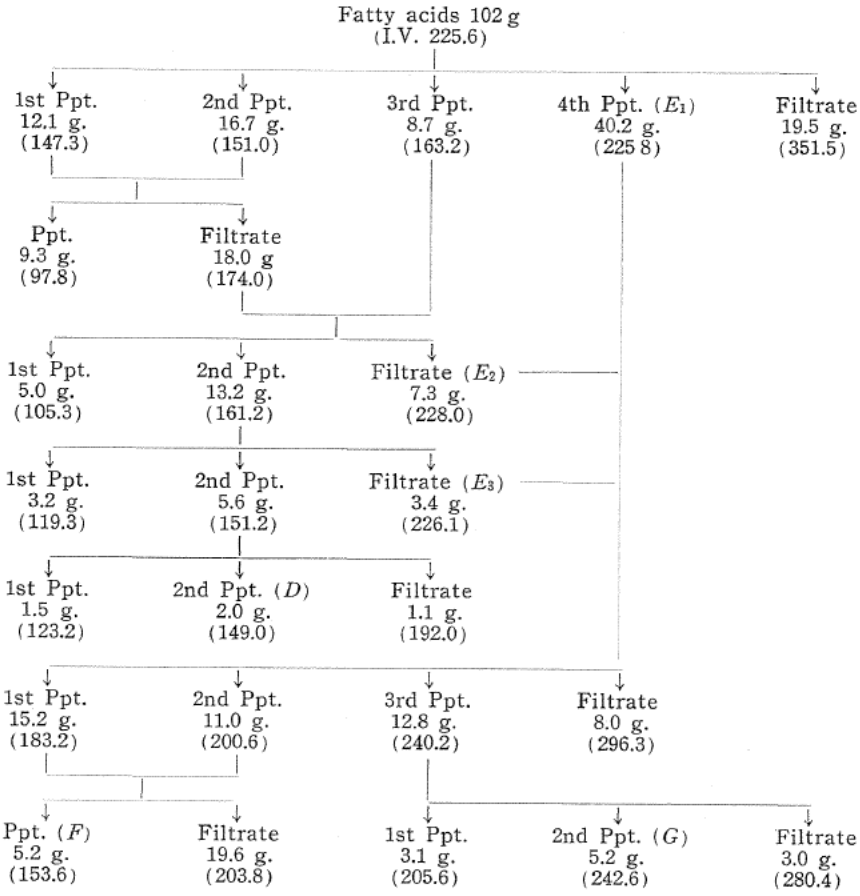
The fatty acids (140 g.) from the fraction 5 in Table 1 were dissolved in ten times weight of acetone, and an approximately 5 N-sodium hydroxide solution was added in small portions to effect fractional precipitations of the fatty acids as their sodium salts from the acetone solution. Six fractions of fatty acids, five from the precipitated sodium salts and one from the filtrate, were obtained. Fractions of a lower iodine value were found to have an increasingly higher neutralization value. Thus the fraction (*B*), 9.4 g., from the second precipitate of sodium salts had N.V. 180.5 and I.V. 176.9 which approximated the corresponding values calculated for eicosatrienoic acid (N.V. 181.9 and I.V. 164.6), and it appeared to leave scarcely any room for the occurrence of docosadienoic acid. The fraction (*B*) was converted to methyl ester, and the latter was subjected to chromatography using silica gel as adsorbent and hexane as eluant. Six eluate fractions were obtained. The first eluate fraction had the lowest iodine value followed by succeeding fractions of an increasingly higher iodine value. The fourth eluate fraction (*C*), 0.4 g., had S.V. 174.0 and I.V. 155.0 (Calcd. for methyl eicosadienoate, S.V. 174.0 and I.V. 157.4). In spite of the coincidence of observed and theoretical iodine values and the proximity of observed saponification value to the theoretical value, the ultraviolet ab-

sorption spectrum of this fraction after alkali-isomerization* exhibited k_{233} 30.6, k_{269} 22.8, k_{316} 10.2 and k_{348} 2.0, indicating that pentaenoic acid, though in a small amount, as well as tetraenoic acid are contained in the fatty acids of this fraction while dienoic acid may be contained only in a minor amount, if any.

The fatty acids (102 g., N.V. 185.2 and I.V. 225.6) from the fraction 4 in Table 1 were fractionated by means of the fractional precipitation of their sodium salts from acetone in a similar way as described for the fatty acids from the fraction 5. The results are shown in Table 2.

The fatty acid fraction from the precipitate *D* in Table 2 had N.V. 183.3, and the united fatty acid fraction from the precipitate E_1 and the filtrates E_2 and E_3

TABLE 2. Fractionation of the Fatty Acids from the Fraction 4 in Table 1 by Means of the Fractional Precipitation of Their Sodium Salts from Acetone



Notes: Yield (g.) denotes the yield of the fatty acid fraction from each precipitate and filtrate. Figures in parentheses denote the iodine value of the fatty acid fraction.

* Unless otherwise stated, the alkali-isomerization was conducted under the condition of 6.5% KOH-ethylene glycol, 180°C and 45 min.

had N.V. 180.5. These neutralization values approximate the value calculated for C_{20}-acid. The fatty acid fraction from the precipitate *D* was converted to methyl ester, and the latter was fractionated by chromatography on silica gel, giving six eluate fractions of I.V. 82.6–200.3. The third eluate fraction (0.2 g.) had I.V. 146.2, but it showed absorption values of k_{233} 33.5, k_{269} 18.1 and k_{316} 9.1 after alkali-isomerization, indicating that a considerable amount of tetraenoate is present in this fraction in spite of the proximity of its iodine value to the value (157.4) calculated for methyl eicosadienoate. The fatty acid fraction from the precipitate *F* in Table 2 was converted to methyl ester, and the latter was fractionated into three eluate fractions of I.V. 110.0, 156.2 and 290.3 by chromatography on silica gel. The eluate fraction (0.5 g.) of I.V. 156.2 showed absorption values of k_{233} 29.5, k_{269} 24.2, k_{316} 11.0 and k_{348} 1.6 after alkali-isomerization, suggesting the presence of pentaenoate, though in a small amount, together with a considerable amount of tetraenoate in this fraction, while dienoate may occur in this fraction only in a minor amount, if any.

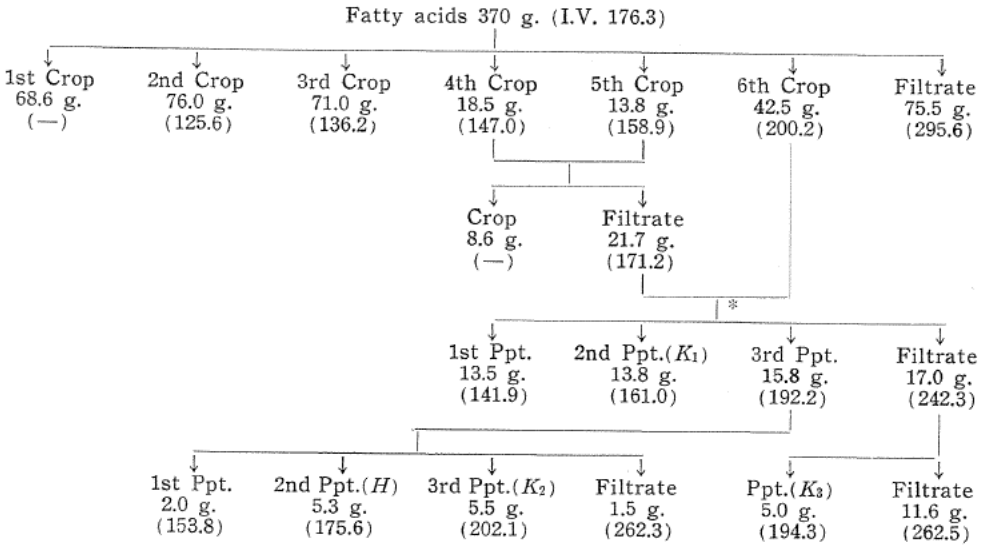
The iodine value (242.6) of the fatty acid fraction from the precipitate *G* in Table 2 is close to the value (248.5) calculated for eicosatrienoic acid. Yet, the methyl ester from this fatty acid fraction could be fractionated into five eluate fractions of I.V. 152.3–312.3 by chromatography on silica gel. The third eluate fraction (1.2 g.) had S.V. 174.6 and I.V. 223.9 (Calcd. for methyl eicosatrienoate, S.V. 175.1 and I.V. 237.6) and the ultraviolet absorption spectrum after alkali-isomerization showed k_{233} 32.8, k_{269} 29.3, k_{316} 13.8, k_{348} 6.8 and k_{374} 1.2. Accordingly, this fraction is considered to contain tetraenoate, pentaenoate and even hexaenoate among others, while any considerable amount of dienoate scarcely appears to be present in this fraction.

2. Separation of octadecadienoic acid

The fatty acids of N.V. 197.1 and I.V. 176.3 from the fraction 3 in Table 1 were fractionated by the urea fractionation followed by the fractional precipitation of lithium salt from acetone in the following way. The fatty acids (370 g.) were dissolved in 3 *l.* of methanol and 1,100 g. of urea was added to the solution. The mixture was refluxed until all urea was dissolved, and the solution was allowed to cool at room temperature. The crystalline urea adduct (1st crop in Table 3) formed was separated by filtration. To the filtrate was added urea succesively twice in each 500 g. portion, and the 2nd and 3rd crops of crystalline urea adduct formed were separated. The filtrate from the 3rd crop of crystalline urea adduct was concentrated to give the 4th and 5th crops of crystalline urea adduct. After removal of methanol from the filtrate of the 5th crop of crystalline urea adduct, the residue was treated with dilute hydrochloric acid to recover free fatty acid mixture which was again subjected to urea fractionation, giving the 6th crop of crystalline urea adduct and the final filtrate. After repeating such fractionation procedure by way of urea adduct, some of the fatty acid fractions obtained were fractionated further by dissolving the fatty acids in ten times weight of acetone and adding an approximately 5 N-lithium hydroxide solution in small portions so as to effect fractional precipitations of the fatty acids as their lithium salts. Results obtained by these procedures are shown in Table 3.

The fatty acid fraction from the precipitate *H* in Table 3 had N.V. 200.2 and I.V. 175.6, which approximated the values calculated for octadecadienoic acid, N.V. 200.1 and I.V. 181.0. This fraction was converted to methyl ester, and the latter

TABLE 3. Fractionation of the Fatty Acids from the Fraction 3 in Table 1



Notes: * The preceding fractionations were conducted by way of urea adduct, while the succeeding fractionations were conducted by the fractional precipitation of lithium salt from acetone. Yield (g.) denotes the yield of the free fatty acid from each fraction. Figures in parentheses denote the iodine value of the fatty acid fraction.

was fractionated into seven eluate fractions by chromatography on silica gel. Three eluate fractions of I.V. 161.2, 168.3 and 174.5 were united, and the united fraction was fractionated further by chromatography, giving eventually 1.2 g. of a fraction (*M*) of methyl octadecadienoate of S.V. 190.2 and I.V. 169.0 (Calcd., S.V. 190.5 and I.V. 172.4). The free acid from this fraction had d_4^{20} 0.8998, n_D^{20} 1.4697, N.V. 199.3 and I.V. 178.0. The absorption spectrum of this fraction in methanol showed specific extinction coefficients of 1.01, 0.46, 0.46, 0.44, 0.11, 0.09 and 0.08 at 233, 262, 268, 274, 310, 316 and 322 mμ, respectively. A calculation[11] from these absorption values indicates the presence of 0.82% of conjugated diene and 0.01% of conjugated triene in this fraction. The absorption spectrum after alkali-isomerization showed k_{233} 89.8 and k_{268} 1.6 when the isomerization was conducted under the condition of 6.5% KOH-ethylne glycol, 180°C and 45 min., and k_{233} 90.7 and k_{268} 4.9 when the isomerization was conducted under the condition of 21% KOH-ethylene glycol, 180°C and 15 min. From these data, it is seen that the fraction *M* is not pure methyl octadecadienoate but is contaminated with some trienoate and monoenoate components.

In another experiment, the fatty acid fractions from the precipitates K_1, K_2 and K_3 were united, and the united material was again fractionated by the fractional precipitation of lithium salt from acetone. The fractions of relatively low iodine values (134.5–174.2) thus obtained were united, and the united fraction (18.8 g.) was brominated in hexane. The bromide insoluble in cold hexane was separated and dissolvod in hot ethanol. The hot ethanol solution, after removal of a small amount of insoluble bromide, was cooled, and the crystalline bromide formed was

separated and debrominated with zinc and 5 N-methanolic sulfuric acid in methanol to give a methyl ester fraction (2.3 g.) which still had a relatively high iodine value of 202.3. This was purified further by chromatography on silica gel, and after removal of eluate fractions of higher iodine values, an eluate fraction (0.3 g.) of S.V. 191.0 and I.V. 181.5 was separated. The fatty acid from this fraction gave a hydrogenation product which had m.p. 69°–70°C and N.V. 196.8 after recrystallization from ethanol and was identified with stearic acid (Calcd., N.V. 197.2).

3. Examination for hexadecadienoic acid

The fraction 2 in Table 1 is comparable with a methyl ester of C_{16}-acids in its saponification value. Its iodine value is remarkably lower than the value calculated for methyl hexadecadienoate and is rather close to the value calculated for hexadecenoate. The fatty acids from the fraction 2 were segregated by way of urea adduct, and after removal of fractions of relatively low iodine values, a fraction (64 g.) of N.V. 210.5 and I.V. 177.3 was obtained. Both neutralization and iodine values of this fraction are lower than the values calculated for hexadecadienoic acid (Calcd., N.V. 222.3 and I.V. 201.2). On fractionally distilling this fraction, a fraction (12.3 g.) of b.p. below 180°C/ca. 1 mmHg, N.V. 219.1 and I.V. 139.0 was separated and then chromatographed on silica gel. After removal of eluate fractions of lower iodine values, an eluate fraction (0.8 g.) of N.V. 219.5 and I.V. 172.6 was separated. This fraction, as compared with hexadecadienoic acid, is a little low in its iodine value, whereas the absorption spectrum of this fraction after alkali-isomerization showed k_{233} 38.2, k_{268} 16.4 and k_{316} 8.7, suggesting the presence of more unsaturated acids than dienoic acid, including even tetraenoic acid, in this fraction. Although the possible presence of hexadecadienoic acid is not excluded, this acid appears not to be present in a sufficient amount to permit its separation.

Summary

A fatty acid fraction of N.V. 200.4 and I.V. 176.3 separated from the sardine oil fatty acids by urea fractionation was subjected to a series of fractionation procedures in order to examine for the occurrence of dienoic acids of C_{16}-C_{22} and trienoic acid of C_{20}. Among these fatty acid members, an octadecadienoic acid fraction was separated, though in a small amount, but no fraction consisting of any one of other members could not be separated. The fatty acid members other than octadecadienoic acid are considered to occur in sardine oil in an extremely minor amount, if any.

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