

Isolation of Eicosapentaenoic and Docosapentaenoic Acids From Natural Sources as Their Methyl Esters by Adsorption and Distillation Techniques¹

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THE isolation of pure linoleic, linolenic, and arachidonic acids from natural sources by physical rather than by chemical methods has been described in recent publications (1, 2) from this laboratory. The importance of geometrical configuration of the polyunsaturated acids in relation to the alkali-isomerization spectrophotometric method of analysis has also been discussed. New constants determined on these pure "natural" acids were proposed (3) for use in this method.

In the course of the work on isolation of arachidonic acid (as its methyl ester) from adrenal lipids, fractions of greater unsaturation than that of methyl arachidonate were obtained (2). Preliminary examination of this material indicated the presence of methyl eicosapentaenoate. After alkali-isomerization the product had characteristic absorption maxima at 346 and 328 m μ in addition to the principal maxima at 315, 301, 268, and 233 millimicrons.

This highly unsaturated material has now been subjected to further fractionation on silicic acid adsorption columns and by high-vacuum distillations. Eicosapentaenoic and docosapentaenoic acids as their methyl esters were isolated and their specific extinction coefficients determined after isomerization with alkali. Further evidence of their identity was obtained by iodine values, refractive indices, and by melting points, saponification equivalents, and X-ray diffraction measurements on hydrogenated portions. The results permit an extension of the spectrophotometric method to the analysis of highly unsaturated lipids, such as are found in nearly all glandular fats and specialized tissues.

Experimental and Discussion

Starting Materials. All fractions of methyl esters having iodine values greater than the theoretical value (318.8) for methyl arachidonate, which were obtained in the adsorption fractionation of adrenal lipid methyl esters (2), served as starting material. In all 11.6 g. were obtained from this previous work. The average iodine value was 359.0. The specific extinction³ coefficients of this concentrate after isomerization in 11% KOH-glycerol (4) for 45 minutes at 180°C. under nitrogen were: $k_{233} = 49.8$; $k_{268} = 35.0$; $k_{315} = 21.8$; $k_{346} = 8.7$. No absorption maximum was found above 346 m μ .

Adsorption Fractionation on Silicic Acid. Essentially the same technique as described in previous work (1, 2) was followed except that the glass columns were packed by introducing a pre-heated slurry of the silicic acid-filter aid mixture in petroleum ether.

In general, for samples up to 5 g. in weight, a glass column 1.9 cm. in diameter was used; for larger samples, the diameter was 3.2 cm. The lengths of the columns were varied to accommodate a ratio of about 30 g. of silicic acid-filter aid mixture per gram of sample. Redistilled petroleum ether, boiling range 30-50°C., was used as eluant although it was usually found desirable to add 0.25% ethyl ether to this solvent in the latter stages of the column treatment.

The methyl esters used as a source of pentaene were subject to this adsorption treatment resulting in the fractions shown in Table I.

TABLE I
Adsorption Fractionation on Silicic Acid of Pentaene-Rich Methyl Esters

Fraction Number	Weight of Fraction	Iodine Value
	g.	Wtgs
A-1.....	0.42	20.3
2.....	0.48	300.5
3.....	0.74	334.1
4.....	0.71	357.4
5.....	0.61	363.4
6.....	0.54	364.9
7.....	1.91	372.8
8.....	0.99	373.3
9.....	0.90	377.9
10.....	0.67	380.1
11.....	1.76	380.7

The presence in the starting material of minor quantities of esters of low unsaturation and probably small amounts of methyl arachidonate is indicated by the iodine values of A-1, 2, and 3. There is a suggestion of a "plateau" in the iodine values for A-4, 5, and 6 in the range of the theoretical iodine value for methyl docosapentaenoate (368.4). Suspecting the presence of this compound, these fractions were combined and a small portion hydrogenated. The hydrogenated product was crystallized from acetone at -15°C. The first crop of crystals was then crystallized twice, after which the purified fraction represented a yield of 35% and melted at 51.5-52.0°C.; mixed melting point with known pure methyl docosanoate (m.p. 54°C.) was 53.5-53.8°C. The X-ray diffraction pattern (5) and the saponification equivalent 349.3 further established methyl docosanoate as the principal component. A second crop of crystals obtained from the filtrates of the above crystallization represented a yield of about 55% and melted at 46-48°C.; saponification equivalent, 342.5. Its X-ray diffraction pattern corresponded well with that of a test mixture made with equal parts of known methyl eicosanoate and methyl docosanoate. Consideration of all these data and the extinction coefficients shown in Table II led to the tentative conclusion that the combined fractions A-4, 5, and 6 contained about 60-65% methyl docosapentaenoate; the remaining 35% probably consisted of methyl eicosatetraenoate and possibly some methyl eicosapentaenoate.

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³Specific extinction coefficient $k = D/bc$, where D = spectral density of the solution (compared with solvent), b = length of cell in cm., and c = conc. in grams/liter.

TABLE II

Specific Extinction Coefficients of Selected Fractions Obtained in the Fractionation of Methyl Esters of Pentaene Acids

Specific Extinction Coefficients ^a						
Fraction Number	Iodine Value	k ₂₃₂	k ₂₆₈	k ₃₁₅	k ₃₄₆	k ₃₇₄
A-4,5,6.....	363.0	45.5	33.4	21.2	9.21	1.09
B-1.....	380.0	51.2	36.6	25.7	12.43	0.0
B-2.....	375.6	51.7	38.4	23.6	10.14	0.60
C-2.....	375.4	48.9	36.6	25.5	11.87	0.0
C-3.....	389.0	49.2	33.9	25.4	13.35	0.0
E-2.....	393.3	45.5	30.7	25.6	14.53	0.0
F-3.....	376.4	51.5	36.6	24.0	10.87	0.706
F-4.....	371.8	50.5	36.4	22.8	9.99	0.736
G-1.....	368.7	44.5	31.7	22.2	10.81	0.0

^a Isomerization in 11% KOH-glycerol for 45 minutes at 180°C. under nitrogen.

Isolation of Methyl Eicosapentaenoate. Since Fractions A-7, 8, 9, 10, and 11 had iodine values higher than the theoretical for methyl docosapentaenoate, it was presumed that these represented mixtures of methyl esters of C₂₀ and C₂₂ pentaene acids and accordingly were combined for more extensive attempts to separate them. From previous experience (2) in distilling methyl eicosatetraenoate (arachidonate), it was found necessary to restrict the size of the column and to use high vacuum to avoid heat bodying effects. Consequently the short column used was relatively inefficient although significant fractionation was achieved. The same still was also used in the present instance. Two principal fractions and an undistilled residue were obtained:

Fraction Number	Weight of Fraction	Iodine Value
	<i>g.</i>	<i>W_{ijs}</i>
B-1.....	3.04	380.0
B-2.....	1.53	375.6
B-Residue.....	0.68	359.6

The fractionation was slight, as expected, but offers support to our presumption concerning the composition. The C₂₀ pentaene ester, being the more volatile, tended to concentrate in the first distilled portion as indicated by the higher iodine value.

Fraction B-1 was subjected to further silicic acid column treatment yielding the following fractions:

Fraction Number	Weight of Fraction	Iodine Value
	<i>g.</i>	<i>W_{ijs}</i>
C-1.....	0.12	229.4
C-2.....	0.68	375.4
C-3.....	1.74	389.0

The adsorption treatment also resulted in significant fractionation. The tendency is for the C₂₂ pentaene to be removed from the column at a faster rate than the C₂₀ pentaene owing to the greater unsaturation of the latter. The iodine value for methyl eicosapentaenoate is 401.1; for methyl docosapentaenoate, 368.4.

The remainder of C-3, after analysis, was distilled under high vacuum in the same still as previously described. The following fractions were obtained:

Fraction Number	Weight of Fraction	Iodine Value
	<i>g.</i>	<i>W_{ijs}</i>
D-1.....	0.91	390.5
D-2.....	0.33	382.5
D-Residue.....	0.36

The remainder of D-1 was given a final silicic acid column treatment yielding the following fractions:

Fraction Number	Weight of Fraction	Iodine Value	Refractive Index 25°
	<i>g.</i>	<i>W_{ijs}</i>	
E-1.....	0.26	388.2
E-2.....	0.42	393.3	1.48770
E-3.....	0.07	389.0

The iodine value of Fraction E-2 is about 2% lower than the theoretical value of methyl eicosapentaenoate (401.1) but probably still contains a significant amount of methyl docosapentaenoate. The amount of Fraction E-2 was only sufficient for spectrophotometric examinations, refractive index, and iodine value. The main portion of E-1, a less pure sample of C₂₀ pentaene however, was hydrogenated and the product crystallized from acetone at -15°C. The crystalline material obtained, representing about 90% of the product melted at 45.0-45.3°C.; mixed melting point with known pure methyl eicosanoate 45.0-45.3°C.; saponification equivalent 330.7 (Theory 326.5). The X-ray diffraction pattern corresponded very closely to that of a known mixture of 90% methyl eicosanoate and 10% of methyl docosanoate. The specific extinction coefficients of E-2 after alkali isomerization are included in Table II.

Isolation of Methyl Docosapentaenoate. Efforts were also made to isolate methyl docosapentaenoate. The remainder of those fractions whose iodine values were in the range 360-378 (B-2, B-residue, and C-2) were combined and distilled under high vacuum. The following fractions were obtained:

Fraction Number	Weight of Fraction	Iodine Value
	<i>g.</i>	<i>W_{ijs}</i>
F-1.....	0.07	381.2
F-2.....	0.17	377.8
F-3.....	0.38	376.4
F-4.....	0.46	371.8
F-Residue.....	0.25

The iodine numbers are in accord with what would be expected in the distillation of a mixture of methyl esters of C₂₀ and C₂₂ pentaene acids. The iodine number of Fraction F-4 was fairly close to the theoretical for methyl docosapentaenoate, but examination of its alkali-isomerized product showed a slight maximum at 374 mμ. The absorption in this region may indicate presence of traces of hexaene acids or slight oxidation of pentaene acids. Therefore Fractions F-2, 3, and 4 were combined and given a silicic acid adsorption fractionation in an effort to get a sample of this compound free of the impurity responsible for this absorption. The following fractions were obtained:

Fraction Number	Weight of Fraction	Iodine Value	Refractive Index 25°
	<i>g.</i>	<i>W_{ijs}</i>	
G-1.....	0.45	368.0	1.48642
G-2.....	0.25	368.9
G-3.....	0.13	375.1

Fractions G-1 and G-2 had iodine values almost identical with theoretical for methyl docosapentaenoate (368.4). A hydrogenated small portion after crystallization from acetone melted at 52-53°C. and was identified as methyl docosanoate by X-ray diffraction measurement. Mixed melting point with the known compound showed no depression. Specific extinction coefficients of G-1 after isomerization in 11%

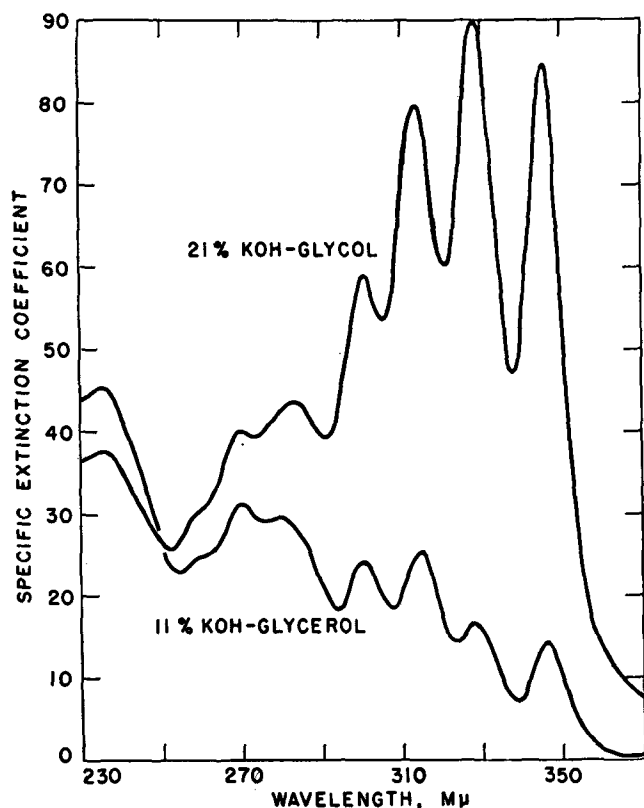


Fig. 1. Ultra-violet absorption spectra of methyl eicosapentaenoate after isomerization in 11% KOH-glycerol at 180°C. for 45 minutes under nitrogen and in 21% KOH-glycol at 180°C. for 15 minutes under nitrogen.

KOH-glycerol for 45 minutes under nitrogen are given in Table II. No evidence of an absorption maximum at 374 was found in Fractions G-1 or G-2. Fraction G-3 and traces of material removed later than G-3 showed a maximum in this region.

Specific Extinction Coefficients and Alkali-Isomerization. It is generally known that the extinction coefficients vary considerably under different conditions of alkali-isomerization. The concentration of alkali is very important. Potter and Kummerow (6) showed that k_{316} for arachidonic acid was 27.7 whereas Beadle *et al.* (7) reported a value of 22.6. However the former used 12.5% KOH-glycol, the latter only 6.6% KOH-glycol.

More extensive work on the effect of various concentrations of alkali and time of heating are reported by Holman and Burr (8). They showed that k_{300} for arachidonic acid (tetraene) was as high as 62.2 when isomerization was performed in 18% KOH-glycol for 8 minutes at 178°C., as compared to k_{300} of 25.8 when isomerization was done in 6.6% KOH-glycol for 25 minutes at 180°C.

These results prompted the authors to investigate the effects of different concentrations of alkali and time of heating on the amounts of conjugation produced from these highly unsaturated acids. This work will be reported in detail in another paper. Isomerization in 21% KOH-glycol for 15 minutes at 180°C. under nitrogen seemed to be most effective in producing maximum conjugation of tetraene and pentaene (315 $m\mu$ and 346 $m\mu$), and results by this method are included for comparison with those shown in Table II for E-2 (C_{20} -pentaene) and G-1 (C_{22} -pentaene). The following specific extinction coefficients were obtained:

	k_{235}	k_{298}	k_{315}	k_{346}	k_{374}
E-2.....	37.7	39.4	78.8	83.6	0.0
G-1.....	46.3	53.2	52.9	41.4	0.0

It will be noted that these values are vastly different from those reported in Table II for these fractions. The results suggest that where the determination of tetraene and pentaene acids is of chief concern, isomerization in 21% KOH-glycol for 15 minutes provides a basis for a much more sensitive determination. Greater differences in spectral properties of E-2 and G-1 were observed when they were isomerized by this method than when isomerized in 11% KOH-glycerol for 45 minutes. These differences could possibly be ascribed to a) an unknown impurity in the preparations, b) conditions of alkali isomerization employed may not have been optimum for producing maximum pentaene conjugation in each compound, c) different positions of the double bonds, or d) different geometrical configurations of the C_{20} and C_{22} pentaene acids. More complete comparison of the spectral properties of these compounds after isomerization is shown in Figures 1 and 2.

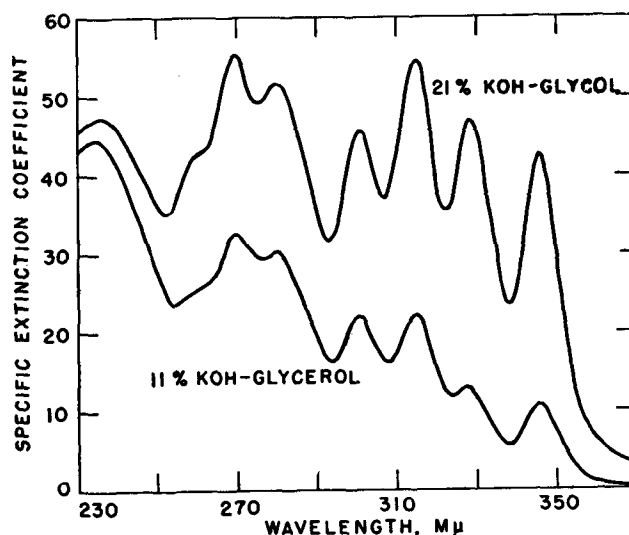


Fig. 2. Ultra-violet absorption spectra of methyl docosapentaenoate after isomerization in 11% KOH-glycerol at 180°C. for 45 minutes under nitrogen and in 21% KOH-glycol at 180°C. for 15 minutes under nitrogen.

Summary and Conclusions

Methyl eicosapentaenoate and docosapentaenoate were isolated by adsorption and distillation techniques from the highly unsaturated esters of beef adrenal lipids. Specific extinction coefficients were determined under two different conditions of alkali isomerization.

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