the diene. Control experiments with an oil containing about 5% linolenate and 50% linoleate showed similar curves. The proportions of diene and triene estimated from the ultraviolet absorption data are in good agreement with those obtained by GLC. The spectra of unisomerized castor oils and methyl esters indicated only traces of conjugated diene and triene.

In addition to the components listed in Table III, traces of myristic, palmitoleic, heptadecanoic, and nonadecanoic acids were found. Altogether, these minor components constitute only about 0.1% of the total fatty acids.

The combination of chromatographic techniques used offers several advantages over the usual analytical methods. The analyses are relatively rapid and simple. Only a small sample is needed, most of which can be recovered. The incompleteness of reaction and the side reactions in previously used procedures (iodine and thiocyanogen absorption, alkali isomerization, acetylation and oxidation) were avoided. Here alkali isomerization, thin layer chromatography, and ultraviolet and infrared spectrophotometry were used to provide qualitative substantiating evidence for the results obtained by quantitative chromatographic procedures.

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Isolation of Pure Linolenate as Its Mercuric Acetate Adduct[1]

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Abstract

After addition of mercuric acetate to the unsaturated methyl esters obtained from the methanolysis of linseed oil, linolenate of 99% minimum purity was isolated by means of a liquid-liquid continuous extraction technique and subsequent decomposition of the mercurial adduct. The methyl linolenate-mercuric acetate addition compound was extracted with 10% methanol in water from an ether solution of the reaction mixture. Infrared analysis of the regenerated methyl linolenate showed the complete absence of *trans*-linkages. The yield, at least 60% of the linolenic acid present in linseed oil, was considerably higher than that obtained by bromination-debromination procedures.

The solubility in aqueous solutions of fatty acid derivatives having 3 or more acetoxymercuri-groups/molecule provides an approach to the fractionation of highly unsaturated oils. For example, a fraction having an iodine value of 395 was isolated readily from the methyl esters of pilchard oil by this technique.

Introduction

THE ISOLATION of highly pure polyunsaturated fatty acids from natural oils always has been a difficult task. A number of techniques have been developed to meet the problem, some of which are effective whereas others yield only concentrates of the desired product.

Although the purest isolates probably are realized from the use of chromatographic techniques, a serious defect usually encountered is the small amount of sample that can be charged on the column. Isolation with this technique of pure linoleate and linolenate (1) as well as fatty esters with 5 or 6 double bonds (2,3) has been described.

The urea segregation and the low temp crystallization procedures (4,5) are of much value in the recovery of linoleate or the free acid, respectively, on a preparative scale. Neither of these methods, however, has proved successful in the isolation in pure form of fatty acid components having more than two double bonds.

Countercurrent distribution is an inherently mild procedure by which a good yield of pure linoleate and linolenate (6) can be obtained. However, its use as a preparative method is limited to the capacity of the complex instrument.

Modification of the fatty acid components by addition to the double bonds, especially of bromine, is used extensively in large scale preparations (7). However, bromination-debromination linoleic and linolenic acids contain an appreciable quantity of isomeric acids that differ from their natural form in vegetable oils (8). This difficulty is not observed with the use of mercuric acetate addition in methanol.

The present investigation describes a procedure for the isolation of 99+% pure methyl linolenate as its

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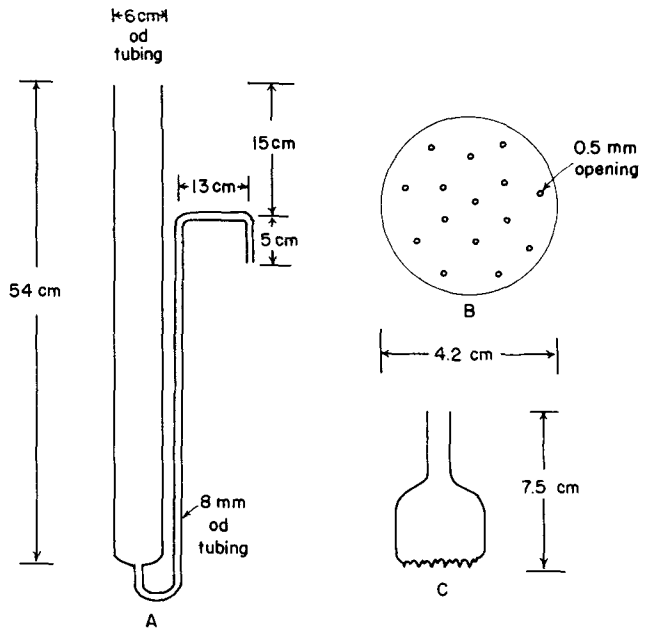

FIG. 1. All-glass apparatus used in the liquid-liquid continuous extraction technique: A, self-leveling extraction tube; B, end view; and C, side view of shower head.

mercuric acetate addition compound from linseed oil methyl esters in decagram and potentially greater quantities.

Experimental

Methanolysis of commercial linseed and pilchard oils was performed at room temp using the Kurz procedure and subsequent handling described earlier (9). The yield of methyl esters averaged about 94% of theory.

Mercuration of the saturated product (50 g) was affected as described (9). The reaction mixture was cooled to room temp and diluted with 400 ml of ether, the minimum amount necessary to maintain a homogenous epiphase. Excess mercuric acetate which precipitated from solution was removed by filtration through glass wool, and the ether solution allowed to flow into a self-leveling glass extraction tube (Figure 1) which contained 400 ml of water. The ether layer was extracted with 10% methanol in deionized water (v:v, 10:90) which had been previously equilibrated with ether. A spray from a glass shower head (Figure 1) allowed fine streams of the aqueous extraction liquid to fall through the ether layer containing the organic derivatives. Each stream upon striking the ether surface broke into droplets which fell through the ether layer. The size of the droplets was inversely proportional to the extraction rate. The usual flow rate was 50–75 ml/min. When faster rates (e.g. 100 ml or more/min) were used, an emulsion tended to form at the ether-water interface and carry over into the collection vessel. This problem could be avoided by stopping the extraction and allowing the emulsion to clear or by decreasing the extraction rate.

The hypophase flowed into a 6-liter Erlenmeyer flask which was continuously flushed with nitrogen and into which 750 ml of concentrated hydrochloric acid was placed. The solution was occasionally swirled to insure good mixing with the affluent stream. When the total volume reached five liters (final concentration of acid, about 5%), another flask was placed under the overflow tube of the extractor.

Further decomposition of the addition products, if not already complete, was achieved as described previously (9). The isolated fractions were stored under nitrogen at −10C.

Prior to analysis each fraction was dissolved in purified hexane and percolated through a MgO: supercel (w:w, 1:1) column to remove autoxidation products that might be present.

The hydrogenation-iodine value was calculated (10) after determining the hydrogen uptake of a warmed mixture of 25 mg of 5% Rh on alumina powder as catalyst, 10 mg of glacial acetic acid, and 250 mg of sample. AOCS method Cd 1-25 (11) also was used for the evaluation of iodine value with and without the addition of mercuric acetate as catalyst (12). All infrared measurements were made on undiluted samples 0.025 mm in width between sodium chloride plates. Alkali isomerization was effected by the techniques described in AOCS method Cd 7-58 (11). Equations from the latter method were also used to determine the amounts of conjugated polyunsaturated constituents in non-alkali treated samples. Gas liquid partition chromatographic analyses were made in the manner already described (9).

TABLE I

Properties of the Methyl Linolenate Isolated with the Mercuric Actate Procedure

Measurement	Value found	Literature value and reference
Unsaturation		
Hydrogen iodine value	260.2	260.4 (theory)
Iodine value—Wijs—1 hr	258.7	.....
Iodine value—Wijs—mercuric acetate catalyst—3 min	259.4	.....
Refractive index (n_D^{40})	1.4633	1.4637[a]
Infrared absorption spectrum		
Trans linkages	Nil	.....
Conjugation		
Diene	0.09%	.....
Triene	Nil	.....
Alkali-isomerization		
Absorptivity value, 233	60.5	61.6 (13)
Absorptivity value, 268	49.8	50.7 (13)
Gas liquid partition chromatography		
Linolenate	99.3%	.....
Linoleate	0.6%	.....
Oleate	0.1%	.....
Stearate	Nil	.....
Palmitate	Nil	.....

[a] The n_D^{30} of methyl linolenate isolated by countercurrent distribution was 1.4675 (6). The value of 1.4637, shown above, was corrected to 40C by subtracting 0.00038 unit/degree centigrade rise in temp (14).

Results and Discussion

Isolation of Linolenate. A high purity linolenate was isolated as its mercuric acetate adduct from an ether solution of 50 g mixed methyl esters of linseed oil with 12 liters of 10% methanol. The yield was 18.3 g, which represented a recovery of 73% of the linolenic acid initially present. From the data in Table I it is evident that the isolated fraction consisted almost wholly of *cis*-methyl linolenate. The iodine value measured by three methods approached the theoretical value (260.4).

No measurable *cis-trans* isomerization occurred in the reaction, as evidenced by the lack of an absorption band in the 10–11 micron region of the infrared spectrum. Similarly, migration of unsaturated linkages was absent, as shown by the close agreement of the absorptivity values of the isolated product with literature values after alkali isomerization together with the negligible amount of conjugated diene in the product.

Gas chromatographic analysis showed that the contaminants of the linolenate preparation were linoleate and oleate in a combined total of less than 1%, whereas stearate and palmitate were absent.

Rate of Linolenate Extraction. Collection and analysis of the lipid fractions progressively isolated/

liter of falling hypophase revealed that the early fractions were highest in linolenate content (Table II). No contaminant was detectable in the first five samples. However, it may be that means of detection more sensitive than those employed would yield evidence of trace impurities. The upper limit of solubility of linolenate in the aqueous medium was near 0.3% (1.3% on the basis of derivative wt). A product (15.81 g) of purity above 99% methyl linolenate which represented 70% of the total amount isolated in the experiment was recovered in the first 6 liters; of the linolenate originally present 22.47 g or 89.8% was removed in 12 liters. In a typical bromination-debromination procedure (7) about 10% of the linolenic acid in the starting linseed oil is isolated.

Isolation of a Polyunsaturated Fraction from Fish Oil. A (9 g) clear liquid concentrate of highly unsaturated esters (iodine value 395, n_{40}^{D} 1.4821) was obtained from 50 g of the mixed methyl esters of pilchard oil by the mercuric acetate procedure. The first 4 liters of 10% methanol to pass through the ether layer carried more than 90% of this material. After alkali isomerization of a portion of the concentrate for 15 min with 21% KOH-glycol absorptivity values at 346μ and 315μ were 33.0 and 44.5, respectively. Unfortunately, the absorbance of the isomerized sample was not read at 375μ to obtain data on hexene.

TABLE II

Fractionation of Mercurated Methyl Esters of Linseed Oil

Fraction[a]	Weight	Composition[b]		
		Linolenate	Linoleate	Oleate
	g	%	%	%
Original[c]	50.13	51.0	15.7	22.1
1	2.83	100	.....	.....
2	2.43	100	.....	.....
3	2.92	100	.....	.....
4	2.86	100	.....	.....
5	2.41	100	.....	.....
6	2.36	99.1	0.9	.....
7	2.01	97.8	1.6	0.6
8	1.36	99.5	0.5	.....
9	1.20	98.4	1.6	.....
10	.87	98.0	2.0	.....
11	.65	95.7	3.2	1.1
12	.58	95.2	3.9	0.9
Final[c]	24.85	8.7	30.1	43.7

[a] Each of the fractions 1–12 was extracted from the ether epiphase with 1 liter of 10% aqueous methanol which had been equilibrated with ether.

[b] The composition in terms of weight % was calculated from the gas chromatographic peak areas determined by multiplying the peak height × the width at half-height.

[c] The original mixed esters contained 1.4% palmitate and 3.8% stearate; the residual esters contained 11.7% palmitate and 5.8% stearate.

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• *Letters to the Editor*

Evidences for Fat Secretion in the Intestine of the Fish

IN THE COURSE of an experiment concerning the "in vivo" incorporation of C^{14}-labeled acetate into fish fatty acids, the intestinal and body fat from a group of fish kept on a fat free diet was studied. Two fresh water fish (*Pimelodus maculatus*, Bagre) were fed a fat-free ration similar to that described by Kelly et al. (1) except that fish meal (VioBin Corp., Ill.) was substituted for egg albumen. Both the starch and fish meal in the diet were extracted with acetone. The fat content of the entire diet was determined to be 0.2 g/100 g. After 10 weeks on that ration the fish were injected intraperitoneally with 0.18 mc/kg of body wt of sodium 1-C^{14}-acetate. The fish were sacrificed 24 hr later and their muscle and liver lipids were first extracted by the method of Folch et al. (2) and then fractionated on silicic acid into triglycerides and phospholipids. The lipids in the intestinal lumen were washed out according to the procedure of Irwin et al. (3) and Deuel et al. (4). Fatty acid compositions were ascertained by gas liquid chromatography on a column of 10% ethylene glycol adipate polyester on chromosorb P (Johns-Manville, New York) using argon as the carrier gas. Fat samples mounted on frosted glass planchets were tested for radioactivity in a GM counter, Model D-47 (Nuclear Chicago Corp., Ill.) with a "Micromil" window, up to 10,000 counts.

The lipids collected from the intestines amounted to 16 and 25 mg, respectively. The fact that they were radioactive proved unequivocally that, as it has been demonstrated in the rat (5), fat is secreted into the intestinal lumen of the fish. The fatty acid com-

Fatty Acid Composition of Muscle Triglycerides and Intestinal Lipids of Fat Free Diet Fed Fish (in moles %)[a]

Fatty acid[b]	Muscle triglycerides	Intestinal lipids	Fatty acid[b]	Muscle triglycerides	Intestinal lipids
12 to 14	1.8	3.4	20:0	tr.	4.1
15:0 r[c]	0.8	1.5	20:1	1.8	tr.
15:0	0.6	5.4	20:2 ?	0.1	tr.
16:0 r[c]	0.2	0.2	20:3 ?	0.3	0.3
16:0	20.0	15.5	20:3' ?	0.2	0.4
16:1	6.7	3.6	21:0	.....	2.3
16:2 or 17:0 r[c]	0.6	0.7	20:4	0.5	tr.
17:0	0.4	5.3	20:5	0.2	0.4
16:3 or 17:1 or 18:0 n or c[d]	0.4	.....	22:0	tr.	.....
			22:1	tr.	.....
18:0	7.3	5.0	22:4 ?	tr.	0.5
18:1	54.2	43.8	22:5 ?	0.2	0.3
18:2	2.8	1.3	22:5'	tr.	0.3
18:3	0.2	.....	22:6	0.3	0.7
19:0	tr.	4.5			
19:1 ?	0.2	0.3			
18:4 ?	0.2	0.2			

[a] Fatty acid composition of liver triglycerides was very similar to that of muscle triglycerides and consequently not shown.

[b] The number before the colon stands for the number of carbons in the chain. The number after the colon indicates number of double bonds.

[c] r = iso acids.

[d] n or c stands for neo or cyclic acids.