

# Isolation of Pure Natural Linoleic and Linolenic Acids as Their Methyl Esters by Adsorption Fractionation on Silicic Acid<sup>a</sup>

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POLYUNSATURATED fatty acids, which have theoretical iodine numbers within the tolerance of analytical methods for determining unsaturation, are conveniently prepared by well-known bromination-debromination methods. On the basis of work reported prior to 1941 (1, 2, 3, 4), debromination linoleic acid and even possibly linolenic acid were considered essentially identical with the natural acids of vegetable oils.

In 1941 however Matthews, Brode, and Brown (5) showed that this view was not strictly correct. They found that debromination linoleic and linolenic acids contain about 12 and 15%, respectively, of isomeric acids which give nearly theoretical iodine numbers for 18 C-atom dienic and trienic acids. These isomers have lower melting points and much lower polybromide numbers than the original debromination acids and were believed to be formed during the debromination step. It was logically assumed that these isomers are of the cis-trans type and therefore different from the natural form, which, according to the work of McCutcheon (6), is of the all-cis variety.

Unfortunately, owing to lack of satisfactory development of physical procedures for obtaining pure polyunsaturated acids in their natural form, nearly all research on the chemistry of these compounds has been conducted on debromination acids or on impure concentrates of the natural products. In some instances their use could lead to somewhat incorrect results or conclusions. For example, debromination acids (alkali-isomerized to conjugated forms) have been used as reference standards in developing the spectrophotometric method of analysis for polyunsaturated fatty acids in oils and fats. Yet it is known that geometric forms of conjugated acids may differ in their ultraviolet absorption characteristics. Dingwall and Thomson (7) have shown significant differences in ultraviolet absorption curves for  $\alpha$ - and  $\beta$ -eleostearic acids. Furthermore in a recent investigation at this laboratory (8) it was shown by kinetic studies that cis-cis  $\Delta$  9,12-linoleic acid isomerizes with alkali about 20 times as fast as the trans-trans isomer and gives rise to a mixture of conjugated isomers that differ in a number of important respects from that obtained from the trans-trans form. After 30 minutes at 180°C. in glycerol-KOH, the specific absorption coefficient at the maximum (233  $m\mu$ .) was 89.0 for the former whereas for the latter at the maximum (231  $m\mu$ .) it was only 16.5.

In another study (9) of the conjugated isomers formed in alkali isomerization of linoleic acid, a fraction of constant melting point (22-23°C.) and specific absorption coefficient ( $k_{233} = 94-95$ ) was obtained by systematic crystallization. By mild treatment of this fraction with traces of iodine it was converted to 10,12-trans-trans linoleic acid, identical

in melting point and x-ray diffraction patterns with 10,12-trans-trans linoleic acid prepared according to von Mikusch's procedure (10). It was concluded that this fraction before iodine treatment was relatively pure 10,12-trans-cis linoleic acid and that, owing to its different configuration, its absorption coefficient was lower than the trans-trans form. Similarly, another fraction, m. p. -6 to 3°C.,  $k_{233} = 87.0$ , obtained in the systematic crystallization of alkali-isomerized cis-cis linoleic acid, was predominantly 9,11-cis-trans linoleic acid. In view of these observations it seemed probable that if debromination acids contain 12-15% of trans-isomers and 85-88% of the natural cis-forms, their absorption coefficients after alkali isomerization would be significantly different from those of alkali-isomerized natural acids (100%) cis-forms. If so, the constants now in use in the spectrophotometric method of analysis would be in error. Accordingly it was considered desirable to isolate pure natural polyunsaturated acids for the purpose of investigating their absorption characteristics after isomerization with alkali under the conditions used in the analytical spectrophotometric method.

Natural linoleic acid has previously been isolated by physical means in pure form as judged by iodine number and refractive index. Frankel, Stoneburner, and Brown (11) isolated this acid from a number of vegetable oils by low-temperature fractional crystallization of the mixed acids. Swift, Rose, and Jamieson (12) isolated it by carefully controlled adsorption fractionation on alumina. In both instances however the work was done prior to the development of the analytical spectrophotometric method. In another instance (unpublished) the present authors isolated pure methyl linoleate by low-temperature fractional crystallization and found the specific absorption coefficient (after alkali isomerization) to be 90.6, calculated as the free acid. Although it was noted at the time that this value was significantly higher than the value established on debromination acid, further work on the ultraviolet absorption studies was postponed until means for isolating natural linolenic acid also could be developed.

Low-temperature crystallization methods have not proved successful for isolating pure linolenic acid although concentrates containing about 88% linolenic acid have been obtained (13). So far as we have been able to ascertain, this acid has not been prepared in pure form from a natural source by other than bromination-debromination procedures.

Considerable work has been published which indicates that chromatographic adsorption techniques offer considerable promise as means for obtaining polyunsaturated acids. Dutton and Reinhold (14) recently studied the performance of alumina columns for their effectiveness in fractionating synthetic binary mixtures of ethyl esters of fatty acids. They showed that the efficiency of separation appeared to depend on the difference in degree of unsaturation of the components. Thus greater fractionation was

<sup>a</sup> Presented at the 22nd Fall Meeting of the American Oil Chemists' Society, November 15-17, 1948, in New York City.

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achieved in the binary systems in the following order: linolenate-stearate > linolenate-oleate > linolenate-linoleate. In the latter system fractionation appeared to be relatively poor, but the results were erratic because of the presence of peroxides. No results were published however on the effectiveness of fractionation of esters of total mixed fatty acids from natural oils.

The present paper describes an adsorption fractionating procedure for isolating pure methyl linoleate and linolenate from the fatty acid methyl esters of oils rich in these components.

### Experimental

Oil from tobacco was used as a convenient source of linoleic acid. The oil was freshly extracted in the laboratory from crushed seed by means of cold percolation with peroxide-free ethyl ether. The iodine value was 140.1.

Linseed and perilla oils were selected as convenient sources of natural linolenic acid. These oils were obtained as authentic specimens and were alkali-refined. The iodine values were 184.0 and 204.5, respectively.

The methyl esters were prepared by methanolysis; sodium methylate was used as catalyst (0.3% sodium based on weight of oil plus amount calculated to neutralize any free acidity).

Practically complete conversion to methyl esters took place in 40 minutes of reflux. The esters were distilled at less than 1-mm. pressure through a 30-inch electrically heated Vigreux column. After about 10% of the charge was fractionally distilled to remove esters of acids having less than 18 C-atoms, the remainder was distilled without fractionation, leaving a residue of about 5%. The main distilled portion from tobacco seed oil esters had an iodine value of 144.6; from linseed oil esters, 200.4; from perilla oil esters, 217.8; these were used in experiments on fractionation by adsorption treatment.

**Adsorption Procedure for Fractionation of Fatty Acid Esters.** Based on results of preliminary tests with alumina and silicic acid columns, the latter was selected because the esters could be eluted quantitatively with good fractionation. General procedure used in the fractionation is as follows: Silicic acid<sup>1,2</sup> is mixed intimately with filter aid<sup>3</sup> (80 parts silicic acid, 20 parts filter aid) to facilitate passage of the solvent. A conventional type Pyrex glass column is packed with silicic acid-filter aid mixture under mild vacuum and tamping. Provision is made to flush the entire system with inert gas and to maintain a uniform positive pressure at the top of the column with this gas during the entire fractionation (Fig. 1). The dry packed column is heated electrically to 70-75°C. for 3 hours, with slow passage of CO<sub>2</sub> or N<sub>2</sub> gas, then allowed to cool to room temperature in the stream of gas. The adsorbent is wetted with petroleum ether before the sample is introduced. When the solution containing the sample is added to the separatory funnel or when fresh solvent is added, care should be taken to exclude air. It is apparent that this can be done by closing stopcock A, opening stopcock B slightly, and loosening the stopper at the top of the separatory funnel, thus permitting the gas

to bubble up through the solvent and flush out the head space. This flushing is continued for 10 minutes before the stopper is seated tightly and stopcock A is opened for normal operation.

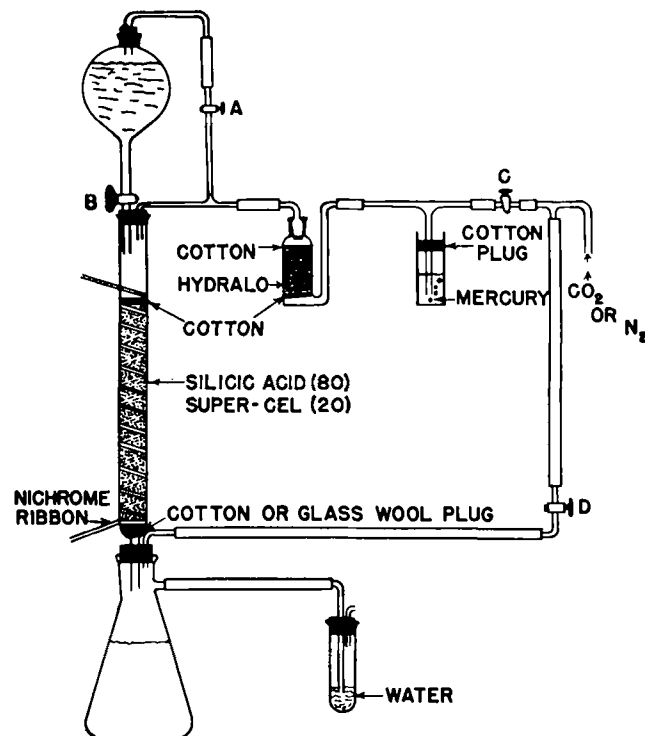


Fig. 1. Apparatus used in adsorption fractionation.

The rate of addition of solvent to the top of the column is carefully adjusted to the rate of percolation so that a fairly constant level of liquid is kept above the top of the adsorbent. It is obvious that the receiver can easily be flushed out by opening stopcock D. During these flushing operations it usually is necessary to open stopcock C a little more in order to maintain a slow rate of bubbling through the head of mercury and uniform pressure on the column. The rate of percolation can be decreased or increased within limits by raising or lowering the mercury level. It is convenient to adjust the rate of percolation and to use a receiver and separatory funnel of such size that the entire fractionation can be continued on a 24-hour schedule without requiring personal attention during the night. This schedule was not strictly adhered to in the experiments reported here, but in more recent experiments it was most satisfactory. Owing to lack of visible color bands to serve as a guide the progress of fractionation is followed by analysis of fractions as soon as they are obtained. The solvent is removed from eluted fractions by distilling to a low volume on a steam bath, the remaining solvent removed under water-pump vacuum. For this purpose it is convenient to use flasks with interchangeable standard taper ground glass joints and, when water-pump vacuum is applied, a connecting tube having an appropriate ground joint and a stopcock is attached to the flask.

After constant pressure is attained on the water pump, the stopcock is closed, and the flask under vacuum is transferred to an assembly where the vacuum can be released to a slight positive pressure with inert gas. The residue in the flask, consisting

<sup>1</sup> Silicic acid (precipitated), analytical grade, from Mallinckrodt Chemical Works.

<sup>2</sup> The mention of any product in this paper does not constitute a recommendation or endorsement by the Department of Agriculture over similar products not mentioned.

<sup>3</sup> Hyflo Super-Cel filter aid.

of eluted material, is then quickly transferred with low boiling petroleum ether to a small weighed round-bottom flask (100 ml.  $\text{F}$ ). This solvent is removed under water-pump vacuum with gentle warming; the flask is then connected to a high vacuum oil pump, and the last traces of solvent are removed. The vacuum is released with inert gas to a slight positive pressure. The residue is now ready for weighing and analysis. It can be stored to advantage at low temperatures in this flask, leaving the connecting tube attached, the stopcock being closed after vacuum is released to positive pressure with inert gas.

**Isolation of Methyl Linoleate.** The adsorption column used to fractionate the esters of tobacco seed oil was 6.3 cm. in diameter and 65 cm. in height. It was packed with silicic acid-filter aid mixture to a depth of 35 cm., requiring 455 g. After the dry adsorbent was heated as described, and the column was wetted with 1,000 ml. of petroleum ether, 16.1 g. of distilled methyl esters, iodine value 144.6 dissolved in 250 ml. of petroleum ether, was added to the column. All elutions were made with petroleum ether. The rate of percolation was about 700 ml. per hour. Table I

TABLE I  
Fractionation of Distilled Methyl Esters of Tobacco Seed Oil by Adsorption Technique

Fraction Number	Volume of Eluate	Eluant	Weight of Fraction	Iodine Value (Wijs)
Original	.....	.....	16.1	144.6
1	8,000	Petroleum ether (30-43°)	0.37	3.4
2	2,000	Petroleum ether (50-60°)	0.84	12.6
3	1,000	Petroleum ether (50-60°)	3.52	112.4
4	1,000	Petroleum ether (50-60°)	7.19	162.4
5	1,000	Petroleum ether (50-60°)	3.36	172.8
6	1,000	Petroleum ether (50-60°)	0.66	177.8
7	1,000	Petroleum ether (50-60°)	0.15	199.4

gives the data on the fractions obtained in successive elutions together with other descriptive and analytical data. Fraction 5<sup>4</sup> was pure methyl linoleate, iodine value 172.8 (theory, 172.5); melting point  $-35$  to  $-34^\circ\text{C}$ .;  $n_D^{25} = 1.45932$ . After isomerization with glycerol-KOH at  $180^\circ\text{C}$ . for 30 minutes, this ester had a specific absorption coefficient of 86.5 at 232  $m\mu$ .; calculated as the acid, it was 91.0. This fraction contained no triene or tetraene and no conjugation before alkali treatment. The tetrabromide number of linoleic acid obtained from this ester, determined by the method of Brown and Frankel (1), was 101.5. This value compares favorably with 100.6, reported for crystallization linoleic acid (11) and with 102.9, reported for debromination  $\alpha$ -linoleic acid recrystallized many times (5), but is in contrast with 90.6, the average tetrabromide number for debromination  $\alpha$ -linoleic acid (without crystallization).

**Isolation of Methyl Linolenate.** The column used in fractionating methyl esters of linseed oil was 3.8 cm. in diameter and 55 cm. in height. It was packed to a depth of 40 cm. with the silicic acid-filter aid mixture requiring 158 g. The column was treated in the manner described and wetted with 200 ml. of petroleum ether. Distilled linseed oil methyl esters (8.1 g.) iodine value 200.4, dissolved in 100 ml. of petroleum ether, was added to the column. Successive portions of petroleum ether, and finally petroleum ether containing 1% ethyl ether, were used in elut-

ing the fractions. Data on the fractionation are shown in Table II. The rate of percolation was ap-

TABLE II  
Fractionation of Distilled Methyl Esters of Linseed Oil by Adsorption Technique

Fraction Number	Volume of Eluate	Eluant	Weight of Fraction	Iodine Value (Wijs)
	ml.		g.	
1	3,500	Petroleum ether (30-43°)	0.34	35.9
2	600	Petroleum ether (60-68°)	0.67	82.4
3	600	Petroleum ether (60-68°)	0.56	85.6
4	600	Petroleum ether (60-68°)	0.40	91.8
5	500	Petroleum ether (60-68°)	0.32	130.2
6	700	Petroleum ether (60-68°)	0.36	163.3
7	600	Petroleum ether (60-68°)	0.32	168.8
8	1,500	Petroleum ether (60-68°)	0.49	182.5
9	1,100	Petroleum ether (60-68°)	0.42	239.0
10	1,000	Petroleum ether (60-68°)	0.77	253.9
		(1% ethyl ether)		
11	700	Petroleum ether (60-68°)	1.80	255.2
		(1% ethyl ether)		
12	550	Petroleum ether (60-68°)	1.35	258.1
		(1% ethyl ether)		
13	500	Petroleum ether (60-68°)	0.32	258.0
		(1% ethyl ether)		

proximately 250 ml. per hour. It is evident that the least unsaturated components are removed in the earlier fractions whereas the most unsaturated, which are more strongly absorbed, are the last to be removed. From iodine number considerations it is estimated that fractions 10, 11, 12, and 13 combined represent approximately 70-75% of the linolenate in the sample (65% of 8.1 g.). The average iodine number of these fractions, if combined, would be 256.2, a value 1.6% lower than the theoretical value, 260.4. Ethyl ether, 1% in petroleum ether, was added after fraction 9 was removed because it was thought that the addition of a little ethyl ether at this stage would hasten the removal of the material remaining on the column and still give sufficient fractionation to yield pure linolenate. The highest iodine number obtained, 258.1 (fraction 12), however, was about 1% lower than the theoretical value. It seemed likely that had petroleum ether alone been continued, still further fractionation might have occurred. Accordingly fractions 11, 12, and 13, and nearly identical fractions from a preceding experiment, totaling 5.2 g., were treated exhaustively with petroleum ether on a fresh column of the same size as described. The results, summarized in Table III, show that further fractionation was achieved, yielding in fraction 3 pure methyl linolenate; iodine value, 260.2. This ester melted at  $-46.5$  to  $-45.5^\circ\text{C}$ . and had  $n_D^{25} = 1.47074$ .

TABLE III  
Summary of Results Obtained on Further Fractionation of Composite of Fractions 11, 12, and 13 (Table II)

Fraction Number	Volume of Eluate	Eluant	Weight of Fraction	Iodine Value (Wijs)
	ml.		g.	
1	8,000	Petroleum ether	0.3	256.0
2	2,500	Petroleum ether	0.6	258.2
3	2,500	Petroleum ether	1.68	260.2
4	1,000	Petroleum ether	Trace	.....
5	3,000	(Petroleum ether containing 1% ethyl ether)	2.54	259.2

Further evidence of effective fractionation as well as of purity of methyl linolenate is presented by specific extinction coefficients determined on fractions after isomerization in glycerol-KOH at  $180^\circ$  for 30 minutes. In some instances, as shown in Table IV, several fractions were combined and iodine values were redetermined for comparison with spectroscopic

<sup>4</sup>This fraction was reserved for comprehensive studies on alkali isomerization under the various conditions proposed for the spectrophotometric method of analysis. The results are being published in another article.

TABLE IV  
Specific Extinction Coefficients of Fractions Obtained in Chromatographic  
Separation of Methyl Esters of Linseed Oil  
(Specific Extinction Coefficients)

Fraction	Iodine Value (Wijs)	K <sub>232</sub>		K <sub>262</sub>		K <sub>268</sub>		K <sub>274</sub>	
		Methyl Ester	Acid	Methyl Ester	Acid	Methyl Ester	Acid	Methyl Ester	Acid
(Table II)									
4+5.....	104.4	19.3	20.3	.....	.....	.....	.....	.....	.....
6+7.....	164.9	79.7	83.7	.....	.....	.....	.....	.....	.....
8.....	182.5	76.5	80.3	5.96	6.26	7.43	7.80	5.51	5.79
9.....	239.0	61.6	64.6	28.8	30.5	37.3	39.2	27.6	29.0
10+11.....	254.5	56.7	59.5	35.2	37.0	45.4	47.7	33.6	35.3
12+13.....	258.1	56.1	58.9	35.9	37.7	46.4	48.7	34.4	36.1
(Table III) <sup>1</sup>									
3.....	260.2	56.4	59.2	36.1	37.9	47.0	49.3	34.7	36.4

<sup>1</sup>The coefficients were determined on this fraction by Dr. M. L. Swain; they will be discussed in a separate paper dealing with revision of constants for use in the spectrophotometric method of analysis.

data. These data show that as greater purity of linolenate was approached, as indicated by iodine values, constant and uniform absorption coefficients were attained. These coefficients are significantly different from those obtained on debromination linolenic acid.

Fractionation of distilled esters of perilla oil was carried out by the same procedure used for linseed oil esters except that elution with petroleum ether was continued until pure methyl linolenate, iodine value 259.6, was obtained. The melting point, refractive index and ultraviolet absorption characteristics of this specimen were identical with those obtained on methyl linolenate from linseed oil esters.

### Discussion

When it is borne in mind that the isolation of pure methyl linoleate and linolenate from these natural sources involves the separation of colorless compounds of similar structure and equal chain length, differing only by one double bond, the fractionation achieved by adsorption technique is remarkable. This is particularly true in view of the fact that no extensive studies were made to determine optimum conditions. Consequently it seems probable that further investigation of the variables that influence fractionation may result in improved procedure.

Methyl linoleate prepared by this adsorption technique was identical with that obtained previously (9) by low temperature crystallization methods. Both preparations had  $K_{232} = 90.5-91.0$ , calculated to acid basis after isomerization in glycerol-KOH at 180°C. for 30 minutes. The finding that methyl linoleate and linolenate isolated from natural sources by adsorption technique differ significantly from corresponding debromination acids in ultraviolet absorption characteristics is considered independent evidence in support of the contention of Matthews, Brode, and Brown that debromination acids contain significant amounts of isomers other than the natural pure cis-forms.

This finding may be regarded also as further substantiation of the authors' earlier observation that alkali isomerization of linoleic acids of different geometrical configuration produces different conjugated isomers, which in turn have significantly different ultraviolet absorption characteristics.

The possibility that there may be more than one configuration of linoleic or linolenic acid present in the natural oils and that these may have been fractionated to some extent in the adsorption treatment is considered unlikely. Methyl linoleate prepared by crystallization and that prepared by adsorption had

the same ultraviolet absorption properties after alkali isomerization. Likewise, the tetrabromide number of linoleic acid prepared by adsorption was in good agreement with that of crystallization acid and with that reported for debromination  $\alpha$ -linoleic acid, which had been recrystallized many times to remove isomeric acids. Furthermore closer agreement of analytical data on the composition of natural fats and oils obtained by the use of the specific absorption coefficients determined on these preparations would render unlikely the possibility that these preparations contained more than one geometrical entity or that any significant change had taken place in these compounds during their isolation. This closer agreement of analytical data and proposed revision of constants for use in the spectrophotometric method will be discussed in a separate manuscript dealing with a more comprehensive study of the behavior of these natural acids under various conditions of alkali isomerization.

### Summary

An effective procedure is described for fractionating methyl esters of oils rich in linoleic and linolenic acids by adsorption on silicic acid columns. Pure methyl linoleate from methyl esters of tobacco seed oil and pure methyl linolenate from methyl esters of linseed and perilla oils were isolated by this procedure. These compounds were characterized by the usual physical and chemical constants and by spectrophotometric examination. These natural acid esters differed significantly from corresponding debromination acid esters in the intensity of ultraviolet absorption at their maxima under the conditions of the alkali-isomerization spectrophotometric method of analysis.

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