Structure of Unsaturated Glycerides. Analysis by Countercurrent Distribution and Lipase Hydrolysis[1]

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Abstract

The structure of highly unsaturated triglycerides isolated from soybean, linseed and safflower oils by countercurrent distribution has been determined by the pancreatic lipase hydrolysis technique. Compositions of positionally distinguishable glyceride isomers for the 5-double bond fraction obtained from safflower oil, the 7-double bond fraction from soybean oil and the 8-double bond fraction from linseed oil agreed with the theoretical values calculated according to the VanderWal-Coleman theory of 1,3-random-2-random distribution. The fatty acid analyses for individual isomers of the unsaturated glycerides demonstrated that positional isomers of glycerides may be present in nonrandom amounts even though the compositionally distinguishable glycerides approximate a random pattern.

Introduction

Unsaturated fats are not readily fractionated into component glycerides by classical crystallization procedures; consequently, early glyceride structural studies were confined to the more saturated fats where the separations of trisaturated, disaturated, monosaturated and triunsaturated were obtained. Inadequate methods of fractionation and analysis led to the postulation and experimental support of many theories on glyceride structure and, as methods improved, to numerous modifications of these theories. Critical reviews on the glyceride structure of natural fats have been published by Coleman (3), Dutton and Scholfield (7), Kartha (13) and Vander Wal (25).

Fractionation and resolution of the various glycerides and their isomers remain the critical problem in fat structure work. Dutton and co-workers (6, 8,19–21) were the first to investigate glyceride structure of highly unsaturated vegetable oils by countercurrent distribution (CCD). The appearance of micro and simplified methods of glyceride fractionation through column (10) and thin-layer chromatography (11,12) for the resolution of unsaturated fractions provides other techniques. Gas chromatographic analysis and the lipase hydrolysis procedures (15,18) also provide the necessary analytical tools for rapid and accurate positional isomer studies. With these modern techniques it is now feasible to answer the question of whether a randomly constructed fat can be composed of specific positional isomers.

Compositionally distinguishable glycerides of vegetable oils were shown by CCD to approximate a random distribution for the fatty acids, except for cocoa butter (8) which was found to distribute according to a 1,3-random palmito-stearo-2-olein structure. These discordant observations supported the possibility of a distribution proposed by Vander Wal that "in many natural fats long-chain saturated and unsaturated groups become associated by chance in the glyceride molecules formed but are not distributed within each molecule at random (23)."

The present investigations cover this hypothesis of nonrandom position isomers occurring within random glycerides for the highly unsaturated triglycerides of vegetable oils. For the first time the pancreatic lipase approach has been applied to determining positionally distinguishable isomers upon the compositionally homogenous glycerides isolated by CCD.

Materials and Methods

Soybean oil and two different lots each of safflower and linseed oils were obtained from reliable commercial sources. The soybean and safflower were salad oils and the linseed oils were alkali-refined. The sample of soybean hypocotyl oil was prepared in the laboratory by repeated extractions and decantations of the germ with distilled petroleum ether. Hypocotyls, from Lincoln variety soybeans, were sorted and cleaned by hand after cracking and air aspiration of the beans. The germs were essentially undamaged and were free of cracked cotyledons and hulls. Markley (14) reports that hypocotyl makes up 2% of the bean and contains about 10.5% oil. The yield of oil from our sample was 13%. Fatty acid composition of the oils and the lipase hydrolysis data used to calculate a 1,3-random-2-random structure of the whole oil are shown in Table I.

CCD of the oils was conducted in the 200-tube apparatus described previously by Dutton, Scholfield and co-workers (6,21). Freshly distilled solvents were used and each solvent of the immiscible pair was saturated with the companion (nonmiscible) phase before introduction into the apparatus. For fractionation of soybean, soybean hypocotyl and safflower oils, the lower phase (stationary—40 ml) of the solvent system was composed of a 50:50 mixture of furfural and nitroethane. Hexane constituted the upper phase (mobile—5 ml). With linseed oil the solvent system was furfural and hexane. A sample (8–10 g) was introduced into the hexane layer of the first 5 tubes. When the desired number of transfers had been completed under a single-withdrawal operation (5), as determined by the refractive index curve (1), the remaining glycerides were recycled

TABLE I

Fatty Acid Composition of Vegetable Oils (Mole %)

Oil	Pal[a]	St	Ol	Lo	Ln	IV[b]
Safflower I	7.1	2.7	12.0	78.3	....	146.5
Monoglyceride	0.2	0.0	11.4	88.3	....	....
Safflower II	6.8	2.0	10.7	80.5	....	148.5
Monoglyceride	0.0	0.0	9.2	90.8	....	....
Soybean	10.9	4.1	22.5	54.3	8.2	135.9
Monoglyceride	0.7	0.0	20.3	71.2	7.8	....
Soybean hypocotyl	11.3	3.0	11.4	57.0	17.2	154.6
Monoglyceride	1.2	0.0	10.8	74.3	13.8	....
Linseed I	6.3	3.6	17.9	16.2	56.0	190.3
Monoglyceride	0.0	0.0	21.3	21.9	56.8	....
Linseed II	6.0	3.9	19.3	14.5	56.3	189.4
Monoglyceride	0.0	0.0	22.5	20.6	56.9	....

[a] Pal, palmitic; St, stearic; Ol, oleic; Lo, linoleic; and Ln, linolenic acid contents.
[b] Iodine values calculated from GLC data.

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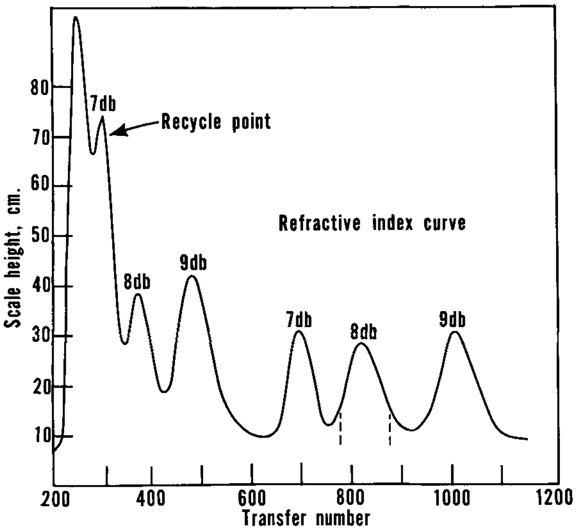

FIG. 1. Countercurrent fractionation of linseed oil glycerides with a hexane-furfural solvent system, showing the refractometric curve and the recycle point where the lower unsaturated fractions were removed from the apparatus and the higher unsaturated 7-, 8- and 9-double bond (db) fractions allowed to recycle.

through the machine and a greater resolution of the unsaturated glycerides was obtained (Fig. 1). The fractions containing the 5-double bond safflower triglycerides were collected directly by single withdrawal in the appropriate tubes of the collector as indicated by the refractive index curve of the distribution. Similarly, fractions containing the 7-double bond peak were obtained from the soybean and soybean hypocotyl oil distributions. The hexane solutions of the appropriate tubes were combined and thoroughly washed with 75% aqueous ethanol to remove traces of furfural and nitroethane. Then the solutions were dried, the hexane was evaporated from a tared flask and the weight of sample was determined. In the fractionation of soybean hypocotyl oil, the contents of each individual tube were washed and then evaporated, and a weight distribution curve was established over the area of the 6- to 8-double bond triglycerides (Fig. 2). Yields of the respective glycerides are in essential agreement with those reported previously from this Laboratory (6,19–21). Part of the sample was used to determine the fatty acid composition of the fractionated triglycerides and the remaining sample reserved for lipase hydrolysis. All determinations on either composition or structure were run in duplicate. Any analysis in which duplicates did not agree within the expected accuracy of GLC ester analysis (±2) was repeated. Methyl esters were prepared from the glycerides by using sodium methoxide as the transesterification catalyst.

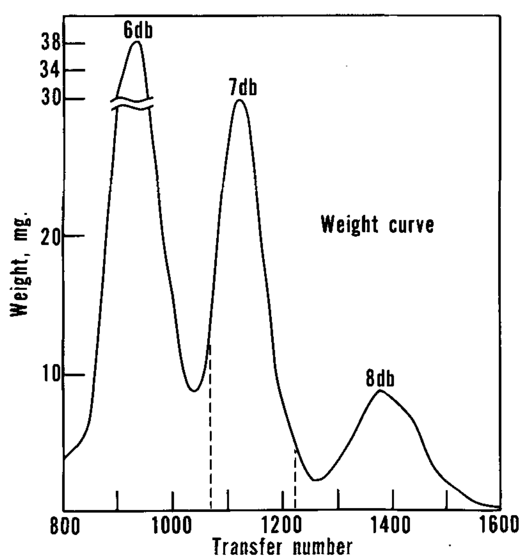

FIG. 2. Countercurrent distribution of soybean hypocotyl oil with a hexane-furfural-nitroethane system, showing the weight distribution of the highly unsaturated 6-, 7- and 8-double bond (db) glycerides.

Lipase hydrolysis was carried out as described by Mattson and Volpenhein (16). The pure monglyceride fraction was obtained by a single liquid-liquid chromatographic separation on silicic acid, by the method of Evans et al. (9). Analysis for fatty acid composition of the 2-position was obtained from the isolated pure monoglycerides, and fatty acid composition of the 1- and 3-positions was obtained by calculation. Previous studies in our Laboratory have shown that rapid isomerization of the 2-monoglyceride to the 1-monoglyceride would introduce errors if analyses were based on acids freed during lipase hydrolysis.

Data have been reported in the form used by Mattson and Volpenhein (17), and glyceride composition was calculated by the method of Coleman and Fulton (4).

Results and Discussion

Safflower Glycerides

Safflower oil is unique for glyceride structure studies in that it may contain more than 80% linoleic acid. Thus, a high percentage of trilinolein and dilinolein triglycerides can be expected and the combinations of fatty acids that make up 5-double bond glycerides are limited to those of linoleic and oleic acid. The only contaminate glyceride in the 5-double bond fraction will be the dilinoleio-palmitate. This contamination arises because both palmitate and oleate exhibit almost the same polarity (distribution coefficient) in CCD systems. The presence of palmitate does not, however, complicate the calculations of glyceride structure since its content can be accurately determined and since there can be only one palmitate fatty residue per glyceride molecule. Lipase analysis shows that more than 98% of the palmitic acid present in the 5-double bond fraction occurs in the 1- or 3-position (Table II). Identical results are shown for the two different lots (I and II) of safflower oil. According to the random theory of glyceride structure the nonsymmetrical isomer of any glyceride will be present at twice the concentration of symmetrical isomer. The small percentages of 0.4 and 0.7 for the symmetrical isomers indicate that the statistical rules of random distribution do not apply to the palmitate-containing glycerides but that the rule which states saturated fatty acids of the vegetable oils are found exclusively in the 1- and 3-positions predominates and is followed reliably.

The 5-double bond fraction of safflower oil is composed of 36% palmito-dilinolein, three times the palmitic acid content of 12%, and approximately 60% oleo-dilinolein. Since there is only one oleic acid group in oleo-dilinolein, the amount present in Lot I can be no more than 56.4% or (3 × 18.8) or 60.6% or (3 × 20.2) for oil from Lot II. These figures on safflower oil glyceride distributions are more complete and somewhat different than those reported earlier (19) which were based on calculations made

TABLE II

Lipase Hydrolysis of Isolated Safflower Glycerides with 5-Double Bonds

Sample	Composition, per cent fatty acid (mole %)							
	Pal[a]		St		Ol		Lo	
	Lot I	Lot II	Lot I	Lot II	Lot I	Lot II	Lot I	Lot II
Triglyceride	12.9[b]	14.3	0	0.3	18.8	20.2	68.2	65.3
2-Monoglyceride	0.4	0.7	0	....	14.3	15.7	85.3	83.6
Proportion[c]	1.0	1.6	0	0.0	25.3	25.9	41.3	42.7

Glyceride structure	Determined, %				Calculated, %			
	Lot I		Lot II		1,3-Random-2-random Lot I		Random Lot I	
Lo-Pal-Lo	0.4		0.7		0.3		12.9	
Pal-Lo-Lo	38.3		42.2		46.5		25.8	
	(3 × 12.9)	38.7	(3 × 14.3)	42.9		46.8		38.7
Lo-Ol-Lo	14.3		15.7		14.8		20.4	
Ol-Lo-Lo	42.1		44.9		38.4		40.9	
	(3 × 18.8)	56.4	(3 × 20.2)	60.6		53.2		61.3
Total		95.1		103.5		100.0		100.0

[a] See footnote a in Table I. Palmitic, stearic, oleic and linoleic acid contents of Lots I and II, respectively.
[b] Value based on at least two duplicate determinations.
[c] Percentage of fatty acid in 2-position.

from iodine values and alkali isomerization data. The difference of 95.1 and 103.5 from 100 is the error involved in the various analytical procedures. The absence of stearic acid lends further evidence that the fraction is composed of only the two types of glycerides and their isomers.

The ratio of symmetrical to the nonsymmetrical isomer of oleo-dilinolein is approximately 1 to 3, i.e., 14.3 to 42.1 for Lot I and 15.7 to 44.9 for Lot II. These values do not agree with the statistical random calculation (1:2), but have fair agreement with the ratio calculated for the "1,3-random-2-random" theory 14.8 to 38.4.

Good agreement is attained between compositionally distinguishable glyceride ratios for the amounts isolated and for the amounts calculated as present from the known composition of the starting oils. Values determined for the types of glycerides lie between the values calculated by strict random distribution and the 1,3-random-2-random distribution, indicating that either theory approximates actual data. However, the percentages of the individual positionally distinguishable isomers are approximated only by the 1,3-random-2-random theory.

Soybean Oil Glycerides

Analysis of several hypocotyl oils (unfractionated) indicates that 2 to 3 times more linolenic acid is present than is found in the natural composite soybean oil. The linoleic acid and the saturated acid contents are about the same as in soybean oil. The increased content of linolenic acid is balanced by an equal decrease in the content of oleic acid. Unsaturated glyceride structure and compositional data on the 7-double bond fractions obtained from soybean oil and soybean hypocotyl oil are shown in Table III. Because of similarities in fraction composition and glyceride structure, these two oils will be discussed together.

Possible glyceride structures containing 7-double bonds can be composed of oleo-dilinolenin or linoleno-dilinolein and their position isomers. Since oleic acid is a component, we can also expect some palmitic acid-containing triglycerides in this fraction as previously discussed. The amounts of palmito glycerides present will be in proportion to their respective contents. No estimate is made of the composition of the palmito glycerides, but it would be assumed to be mostly dilinolenin. Because of the small percentage present, they have been included only in the total weight balance.

Oleo-dilinolenin content of the two different soybean oils is almost identical, and the isomer distribution, as well as the amounts present, agrees well with that calculated by assuming the 1,3-random-2-

TABLE III

Lipase Hydrolysis of Isolated Soybean Glycerides (A) and Soybean Hypocotyl Glycerides (B) Each with 7-Double Bonds

Sample	Composition, per cent fatty acid (mole %)							
	Pal		Ol		Lo		Ln	
	A	B	A	B	A	B	A	B
Triglyceride	1.1	1.3	2.0	1.8	63.8	62.9	33.0	34.0
2-Monoglycerides	0.4	0.3	2.4	2.1	75.3	77.0	21.8	20.6
Proportion	12.1	7.7	40.0	38.9	39.3	40.8	22.0	20.2

Glyceride structure	Determined, %				Calculated, % 1,3-Random-2-random			
	A		B		A		B	
Pal-?-?	3.3		3.9					
	(3 × 1.1)	3.3	(3 × 1.3)	3.9				
Ln-Ol-Ln	2.4		2.1		1.9		2.2	
Ol-Ln-Ln	3.6		3.3		4.1		3.4	
	(3 × 2.0)	6.0	(3 × 1.8)	5.4		6.0		5.6
Lo-Ln-Lo	19.6		18.4		21.7		18.1	
Lo-Lo-Ln	67.4		72.8		72.3		76.3	
	(3 × 29.0)	87.0	(3 × 30.4)	91.2		94.0		94.4
Total		96.3		100.5		100.0		100.0

See footnotes a in Table I and c in Table II.

random theory of distribution. The statistical random relationship of 1:2 per symmetrical to nonsymmetrical isomers is not followed. Although the ratios of symmetrical to nonsymmetrical isomers in both oils are in excellent agreement and close to 2:3, it is doubtful if any significance can be attached to such figures in view of the small amounts present.

The major triglyceride in soybean oil having 7-double bonds is linoleno-dilinolein. This glyceride makes up 90% of the fraction, and theoretical calculations based on a 1,3-random-2-random distribution indicate a content of 94% linoleno-dilinolein. Positional isomer distribution again favors the nonsymmetrical component and by a large ratio, approximately 4:1. This is the highest ratio we have observed and there is good agreement between that calculated by the 1,3-random-2-random theory and that observed for soybean oil. Hypocotyl oil positional isomer distributions are also in good agreement with theory.

Linseed Oil Triglycerides

Two different linseed oils were fractionated and the glyceride composition of fractions of 96–97% purity studied (Table IV). The fatty acid analysis for the 8-double bond fractions indicates immediately that the glyceride is linoleo-dilinolenin, i.e., one-third linoleic acid and two-thirds linolenic. Agreement between samples is remarkably good for both the composition of the fractions and positional isomer distribution. Agreement between experimental values on composition, for the two linseed oil lots selected at random, is somewhat better than the agreement to the theory based on calculations of a 1,3-random-2-random distribution.

The only combination of fatty acids in linseed oil to give an 8-double bond fraction is linoleo-dilinolenin. The presence of a small amount of oleic acid indicates a contaminate glyceride, obtained from an overlapping tail in CCD of the 7- and 8-double bond peaks. The amount of contaminate is small, totaling only 3%, and its presence is easily accounted for. The positional isomer distribution for this small contaminate fraction is reported but, again, no significance can be attached to these figures. The percentage 0.6 of linoleno-dilinolein is arrived at by calculation on the basis of random distribution, since there is no way to determine an experimental value. The two isomers of linoleo-dilinolenin are present in almost equal amounts, which is considerably different from the highly unsaturated glycerides obtained from either safflower or soybean oils. Positional isomer distribution of 43:54 is indicated by the composition of linseed oil Lot I, when calculated by the 1,3-random-2-random distribution.

Support for the 1,3-Random-2-Random Theory

Compositional glycerides isolated from the highly unsaturated safflower, soybean and linseed oils follow closely the distribution predicted by calculation from the fatty acid composition according to the rules of the 1,3-random-2-random theory (2,25). On the basis of lipase hydrolysis, individual positionally distinguishable isomers within the various unsaturated triglycerides showed a distribution in good agreement with the 1,3-random-2-random theory. The distribution ratio between symmetrical and unsymmetrical isomers varied from 1:1 for 8-double bond linoleo-dilinolenin from linseed oil to 1:4 for linoleno-dilinolein from soybean oil. These results indicate that the formation of individual isomers is restrictive since statistical random calculations would result in a constant ratio of 1:2 for symmetrical to nonsymmetrical isomers. These distributions offer proof for the Vander Wal-Coleman theory that the individual positional isomers are distributed in the predicted manner.

The close agreement of the analytical results obtained by CCD and lipase hydrolysis on different vegetable oil samples selected at random is indicative of reliability and reproducibility of the methods employed. Agreement between different lots of oil and between lots and the theory indicates that any correction factor applied to the 1,3-random-2-random theory will be small. The important and the predominating rule that saturated acids are exclusively directed to the 1- and 3-position is exemplified by the data. Small deviations are believed to result from analytical errors, and isomer distribution percentages based on lipase fatty acid analysis of 1% are not significant. It is believed that the true value for palmitic acid in the 2-position is essentially zero in these vegetable oil samples.

Jurriens and co-workers (11,12) have developed a micro-method for glyceride structure studies based on thin-layer chromatographic separations using silica

TABLE IV

Lipase Hydrolysis of Isolated Linseed Glycerides with 8-Double Bonds

Sample	Composition, per cent fatty acid (mole %)					
	Ol		Lo		Ln	
	Lot I	Lot II	Lot I	Lot II	Lot I	Lot II
Triglyceride	0.9	1.1	32.5	33.0	66.6	65.9
2-Monoglyceride	1.3	2.0	48.2	47.0	50.5	51.0
Proportion	48.1	60.6	49.4	47.5	25.3	25.8

Glyceride structure	Determined, %				Calculated, %			
					1,3-Random-2-random			
	Lot I		Lot II		Lot I		Lot II	
Ln-Ol-Ln	1.3		2.0		1.1		1.3	
Ol-Ln-Ln	1.4		1.3		1.7		2.0	
	(3 × 0.9)	2.7	(3 × 1.1)	3.3		2.8		3.3
Ln (Lo)$_2$	0.6		0.7		0.6		0.7	
		0.6		0.7		0.6		0.7
Ln-Lo-Ln	47.6		46.5		42.9		45.0	
Lo-Ln-Ln	48.7		51.3		53.7		51.0	
	(3 × 32.1)	96.3	(3 × 32.6)	97.8		96.6		96.0
Total		99.6		101.8		100.0		100.0

See footnotes a in Table I and c in Table II gel impregnated with silver nitrate. The method is not satisfactory for the highly unsaturated glycerides (those containing linolenic acid) and the assumption is made that each fraction exclusively contains triglycerides having the same number of double bonds. They also report that soybean oil glyceride composition follows the distribution pattern of the Vander Wal-Coleman theory when the all-saturated acids are grouped together and the unsaturated acids are considered individually. Youngs (26) and Subbaram and Youngs (22) included soybean and linseed oils in their glyceride composition studies, which were based on oxidation of unsaturated acids. In this method all the unsaturated acids are grouped together with no distinction, nevertheless Vander Wal (25) has been able to use the six glyceride types of S_3, SUS, SSU, USU, USS, U_3 as substantial proof of the 1,3-random-2-random theory.

Subbaram and Youngs (22) report individual saturated acids. Glycerides containing myristic, palmitic and stearic acids were handled in groups of equal carbon numbers, and good agreement was reported between the determined and calculated compositions for these glycerides in linseed, corn, olive, cottonseed and soybean oils. Coleman (2) established a similar good agreement between the determined and calculated glyceride structure of soybean oil by grouping the saturated acids together and all the unsaturated acids except linoleic.

The validity of the 1,3-random-2-random theory is substantiated by our data, which supplies the proof required (10,24), for the random intrapositional distribution of the individual unsaturated fatty acids ing the glycerides of safflower, soybean and linseed oil. The fractionation results obtained indicate that the glyceride structure of the vegetable oils of commercial interest can be correctly calculated from lipase hydrolysis data by the 1,3-random-2-random theory.

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