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# Glyceride Structure of Vegetable Oils by Countercurrent Distribution. III. Safflower Oil[1]

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WIDELY DIFFERENT VALUES for the glyceride composition of safflower oil have been obtained by various workers. While Vidyarthi (9) found by a bromination procedure very little trilinolein in an oil containing 61% linoleic acid, by the same fractionation method Lagawonkar *et al.* (6) found 45% trilinolein in an oil which contained 56.7% linoleic acid. Barker and Hilditch (2) by crystallization from ether solution calculated 31% trilinolein and 67% dilinolein for an oil containing 76% linoleic acid.

Attempts to fit these values to various theoretical patterns of glyceride distribution give conflicting conclusions. In the oils fractionated by Vidyarthi and Lagawonkar about 20% trilinolein would be allowed under a random distribution, and none under an even distribution. The values calculated by Barker and Hilditch are in agreement with an even distribution pattern.

In this laboratory countercurrent distribution has been found to have a higher resolving power than methods previously available for triglyceride fractionation, particularly when the more highly unsaturated oils are used. Such studies on linseed and soybean oil, described in previous papers of this series (5, 7), have shown them to follow a random pattern. By contrast the study on cocoa butter showed that it follows neither the strict even nor the strict random pattern (8). The present work on safflower oil indicates that it follows a random distribution like the two liquid oils examined.

## Experimental Procedure

Two samples of safflower seed, varieties N-10 and Pacific No. 2, were obtained from the U.S.D.A. Field Crops Research Branch. Both were grown in California in 1956. These samples, after grinding, were extracted with pentane-hexane in a Soxhlet extractor. Because crude safflower oil has little tendency to emulsify in a countercurrent distribution apparatus, it was used without degumming. The iodine value and fatty acid composition of the two oils are shown in Table I.

Iodine values were determined by the Wijs method (1). This procedure was scaled down as required to allow for the small samples available from countercurrent distribution. Polyunsaturated acids were measured using the 45-min. isomerization of Brice *et al.* (3). Triene conjugation was observed but may possibly be the result of minor oxidative change.

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TABLE I

Analyses of Safflower Oils

| Sample | Iodine value | Linolenic acid | Linoleic acid | Oleic acid | Saturated acids | Palmitic acid |
|---|---|---|---|---|---|---|
| | | % | % | % | % | % |
| N-10 | 143.8 | 0.1 | 76.6 | 12.6 | 10.7 | 7.3 |
| Pacific No. 2 | 146.6 | 0.3 | 79.2 | 9.3 | 11.2 | 5.8 |

Because the apparent linolenic acid content was too low to affect glyceride composition appreciably, only linoleic acid was measured in the fractions obtained by countercurrent distribution.

Palmitic acid content of the oils was determined by gas chromatography of the methyl esters on an Apiezon L column at 240°C.

Countercurrent distribution was carried out in a 200-tube automatic instrument by a procedure similar to that previously described (7). The solvent system was prepared by mixing 10 l. of pentane-hexane, 4 l. of furfural, and 4 l. of nitroethane. Forty-ml. portions of the lower phase were introduced into each of the tubes with the exception of the first five. A 10-g. sample of oil dissolved in the equilibrated solvents was placed in the first five tubes, and the solvent pump was adjusted to deliver the desired amount of upper layer to tube 0 at each transfer. After 200 transfers had been completed in the instrument, the fraction collector was started and was set to combine two effluent fractions in each tube. Another 800 transfers were then applied so that 400 fractions were obtained.

In a preliminary experiment 5-ml. portions of upper layer were used as was done in the soybean oil distributions. All the material emerged from the safflower oil distribution after 660 transfers, that is, in the first 220 fractions, and no glycerides containing more than 6 double bonds were found. Therefore the volumes of upper layer were reduced to 3 ml. in order to apply more transfer stages so that better resolution was obtained.

Besides the fractionation of the N-10 and Pacific No. 2 safflower oils, two additional distributions are also described. In order to compare the glyceride structure of these native oils with a safflower oil known to be of random structure, the N-10 safflower oil was interesterified by using sodium methoxide as the catalyst. To provide a convenient method of analysis for palmitic acid and to demonstrate the effectiveness of the interesterification catalyst, 37.9 mg. of $C^{14}$-labelled methyl palmitate containing 1 $\mu$c of radioactivity were added to 12.51 g. of safflower oil during

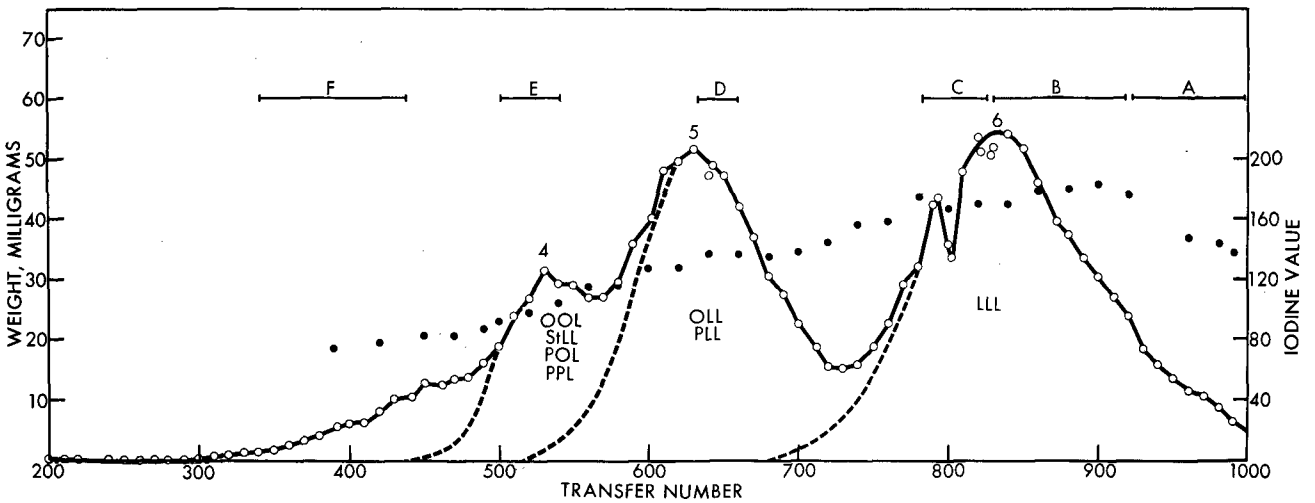


FIG. 1. Countercurrent fractionation of N-10 safflower glycerides with a pentane-hexane, furfural-nitroethane solvent system. Open circles—weight curve. Closed circles—iodine value curve. Samples analyzed in Table II are indicated by straight lines at top.

the randomization. Subsequent to its countercurrent distribution, radioactivity was measured in every fifth fraction, using an automatic TRICARB Liquid Scintillation Spectrometer.[2] The weight of palmitic acid in each fraction was calculated by comparing its radioactivity with that of the original interesterified oil.

The second additional distribution was of a mixture of 50.5% N-10 safflower oil and 49.5% olive oil. This distribution was run to investigate the possibility of interesterification of triglycerides during countercurrent distribution.

## Results and Discussion

Weight and iodine value data for the fractions obtained by distribution of N-10 safflower oil are plotted against the transfer number in Figure 1. In accord with results on linseed and soybean oils it appears from these data that the safflower oil was fractionated according to the number of double bonds in the glyceride molecule. The glycerides containing 6, 5, and 4 double bonds are sufficiently well separated so that it is possible to estimate the area under the curve which should be assigned to each class of glyceride unsaturation.

To ascertain the most accurate way to divide the areas between the curves, trilinolein obtained by countercurrent distribution was passed through the instrument again. It was then possible to compare the weight curve of this pure triglyceride with a calculated theoretical curve (4). The two curves are quite similar on the left side of the peak, but on the right side the actual weight curve is skewed to include a greater area than calculated. Accordingly the areas assigned to the different glyceride classes were determined by drawing the theoretical curves down the left side from the peaks. The extension of these theoretical curves is shown as dotted lines under the weight curves.

Fractions were combined to give six samples, which were analyzed for linoleic acid and iodine value as shown in Table II. Samples A, B, and C in the region of curve 6 show that material under this curve is the 6 double bond triglyceride trilinolein. This material accounts for 46.5% of the area under the weight curve. Therefore the oil contains 46.5% trilinolein.

[2] The mention of products does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

TABLE II

Analyses of Fractions from Countercurrent Distribution of N-10 Safflower Oil Illustrated in Figure 1

| Sample | Transfer number | Iodine value | Linoleic acid | Oleic acid | Saturated acids |
|---|---|---|---|---|---|
| | | | % | % | % |
| A | 922–998 | 171.3 | 97.1 | 3.6 | −0.7 |
| B | 830–918 | 174.9 | 100.5 | 0.9 | −1.4 |
| C | 782–826 | 171.1 | 96.9 | 3.8 | −0.7 |
| D | 632–658 | 137.1 | 66.2 | 25.7 | 8.1 |
| E | 502–542 | 101.7 | 45.3 | 25.8 | 28.9 |
| F | 340–438 | 73.2 | 36.8 | 11.0 | 52.2 |

The area under curve 5 accounts for 34.1% of the area under the weight curve. Sample D in this region contains 64% linoleic acid corresponding to the 5 double bond triglyceride oleodilinolein. However the iodine value is lower than the 144 required for this glyceride, and there is 8% of saturated acids present. The occurrence of saturated acids in these fractions is caused by palmitoglycerides which move through the distribution apparatus at a rate comparable to that of the corresponding oleoglycerides and more rapid than the corresponding stearoglycerides. Thus palmitodilinolein appears to have a partition coefficient approximating oleodilinolein. This interference of palmitic acid is more serious in safflower oil than in linseed or soybean oils because in safflower oil the ratio of palmitic acid to oleic acid is greater. Sample D is calculated to consist of 76% oleodilinolein and 24% palmitodilinolein. If Sample D is representative of the material under curve 5, then the oil contains 25.9% oleodilinolein and 8.2% palmitodilinolein.

The area under curve 4 accounts for 13.1% of the area under the weight curve. The material in this region would be expected to contain the 4 double bond glycerides, dioleolinolein and stearodilinolein, as well as palmitooleolinolein and dipalmitolinolein. Because there are four glycerides and because four independent linear equations can be set up involving iodine value and fatty acid composition, it should be possible to calculate the glyceride composition corresponding to Sample E in this region. In practice the roots of these equations change rapidly with small changes in analytical values so that small errors produce combinations of negative and unreasonably large glyceride percentages, and such calculations are not useful. However the composition of Sample E is quite consistent with that expected for a random distribution.

Sample F consists of unresolved, more saturated glycerides. No calculations were made in this region, but it may be noted that the saturated acid content is much too high for an even distribution.

The measured glyceride composition is summarized in Table III and is compared with that calculated for random and even distributions.

TABLE III

Composition of Safflower Glycerides Compared with Compositions Under Random and Even Distribution Patterns

| Glyceride | Experimental | Random | | Even |
|---|---|---|---|---|
| | % | % | | % |
| Variety N-10 | | | | |
| LLL | 46.5 | 44.95 | | 31.2 |
| OLL | 25.9 | 22.18 | | 38.7 |
| PLL | 8.2 | 13.20 | | 19.6 |
| OOL | 13.1 | 3.65 | 15.15 | 10.5 |
| StLL | | 5.64 | | |
| POL | | 4.34 | | |
| PPL | | 1.52 | | |
| Variety Pacific No.2 | | | | |
| LLL | 50.9 | 49.68 | | 39.2 |
| OLL | 15.1 | 17.50 | | 28.6 |
| PLL | 12.3 | 10.91 | | 15.6 |
| OOL | 14.5 | 2.06 | 15.58 | 16.6 |
| StLL | | 10.16 | | |
| POL | | 2.56 | | |
| PPL | | 0.80 | | |

The distribution of the Pacific No. 2 oil was carried out in a similar manner to that of the N-10 oil, and it yielded quite similar results. The glyceride composition of this oil is also compared in Table III with that calculated for random and even distributions. The experimental composition of both oils agrees rather well with that predicted for a random distribution.

The distribution of the interesterified N-10 oil serves two purposes. First, it provides a comparison of a true random distribution with that of the natural oil. Second, the radioactive palmitate locates the palmitoglycerides and shows their position on the distribution curve with respect to the oleoglycerides. Linoleic acid content and iodine value of the interesterified oil were slightly lower than those of the natural oil. The interesterified oil contained 74.5% linoleic acid and had an iodine value of 141.3. In Figure 2 the weight, iodine value, and palmitic acid content of the fractions obtained from the interesterified oil are plotted against the transfer number. The shape of the weight curve is quite similar to that obtained with the natural oil in Figure 1. The slight difference in location of the peaks is caused by a small difference in the volumes of upper layer used since the adjustment of the pump may vary slightly from run to run. The area under the 6 double bond peak corresponds to 44.6% trilinolein and is comparable to 41.35% trilinolein calculated under a random distribution.

Definitive evidence on the partition coefficients of palmitoglycerides was obtained from the use of $C^{14}$-labelled palmitate. As expected, the palmitoglycerides behave similarly to the oleoglycerides. Because they appear in the same areas in the weight curve but are displaced slightly in the direction of lower transfer numbers, the palmitoglycerides must move through the instrument slightly more rapidly than do the oleoglycerides. The Gaussian shape of these curves demonstrates the independent behavior of individual triglycerides.

During the course of our studies on the countercurrent distribution of linseed, soybean, and cocoa butter oils the question has been raised whether any interesterification of the triglycerides occurred. Although we have never had any evidence that such a reaction takes place, it was deemed advisable by actual experimentation to remove any doubts that interesterification might alter the observed glyceride structures and account for our finding random patterns.

To answer this question properly a mixture of safflower oil and olive oil was fractionated by countercurrent distribution. Because the safflower oil contained 46.5% trilinolein, as shown in Table III, it would contribute 23.5% trilinolein. Actually the mixture was found to contain 25.2% trilinolein. The olive oil contained 13.9% linoleic acid and would be expected to have very little trilinolein. Because the mixture contained 45.6% linoleic acid, it would contain only 9.5% trilinolein if complete interesterification took place. The results confirm the assumption we have made in all our glyceride structure work that interesterification does not occur. In further support we may also add that similar results have been obtained upon measuring the 8 and 9 double bond glycerides in a mixture of linseed and olive oil.

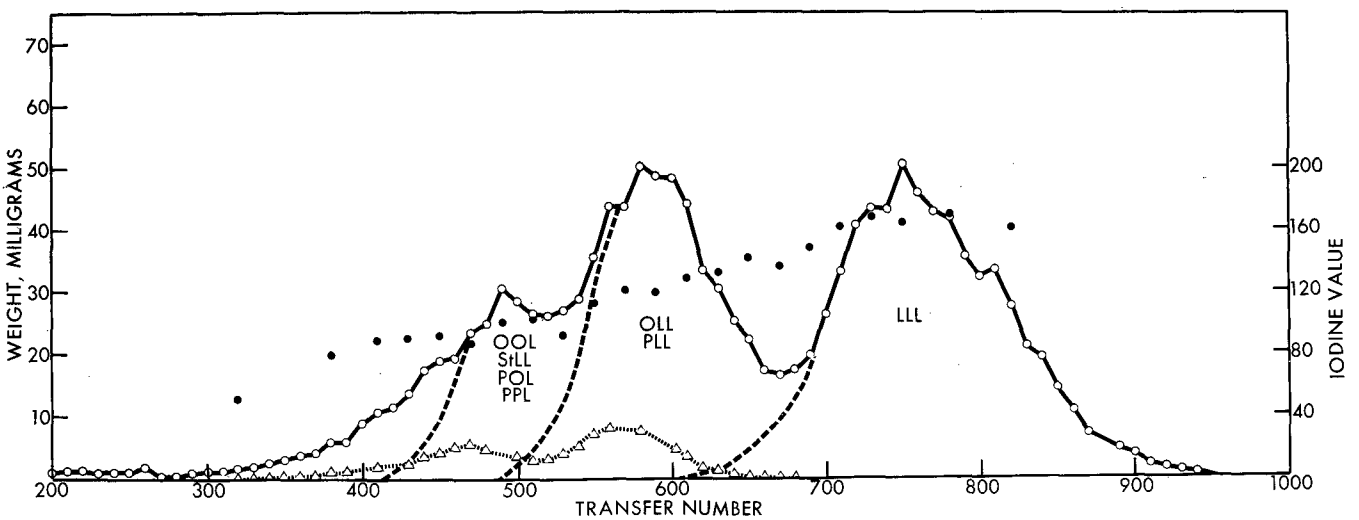


FIG. 2. Countercurrent fractionation of interesterified N-10 safflower oil glycerides and $C^{14}$ palmitic acid. Open circles—weight curve. Closed circles—iodine value curve. Triangles—palmitic acid weight curve.

The evidence obtained by these countercurrent distributions indicates that safflower oil, like soybean and linseed oils, has essentially a random glyceride distribution pattern. The amounts of the different triglycerides which could be isolated and measured approximate those calculated for a random distribution. In addition, no significant change in the weight curve or composition of the oil was found after interesterification.

### Summary

Safflower oil was fractionated in a 200-tube countercurrent distribution apparatus, and the oil was also fractionated after interesterification with $C^{14}$-labelled palmitic acid. The glyceride composition of the interesterified oil was similar to that of the natural oil. The glycerides were separated on the basis of both unsaturation and chain length of the constituent fatty acids, and the palmitoglycerides had only slightly higher partition coefficients than the oleoglycerides. The amounts of trilinolein, oleodilinolein, and palmitodilinolein found were similar to those calculated for a random distribution. Distribution of a mixture of safflower oil and olive oil showed that no mixing or randomization of triglycerides occurred during countercurrent distribution. It is concluded that fatty acids in safflower triglycerides are distributed in an essentially random pattern.

### Acknowledgment

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# Phenolic Antioxidants and the Stability of Perirenal Rat Fat[1]

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PREVIOUS investigators have attempted to improve the resistance of extracted animal fats to the onset of rancidity by alteration in the antioxidant composition of the diet. The natural antioxidants, the tocopherols, can be deposited in the fat of the rat (2, 19, 10, 3, 20), swine (4, 5, 33), rabbit (22), turkey (6, 23, 24, 25), and chicken (24, 25, 11). Providing the fatty acid composition is constant, the stability of the extracted fat depends upon the tocopherol content of the diet. Only a very small percentage of the large doses of tocopherol fed however are actually stored in the carcass.

The effect of the ingestion of other antioxidants has been investigated. Ascorbic acid, hydroquinone, α-naphthol, nordihydroguaiaretic acid, lecithin, and tocopherols were fed to rats. Of these only the tocopherols were effective in increasing the stability of the extracted fat (27, 10).

Siedler *et al.* (30) studied the effect on the stability of depot fat extracted from broilers fed a diet supplemented with 6% animal fat stabilized with 0.02% Santoquin (6-ethoxy-2,2,4, -trimethyl-1, 2-dihydroxyquinoline), 0.02% BHT (ditertiary-butyl-p-cresol), or 0.02% DPPD (diphenyl-p-phenylenediamine) for nine weeks. They have shown that the depot fat of birds fed these antioxidants showed little or no increase in stability over the controls.

Pudelkiewiez *et al.* (28) have shown that the depot fat and egg yolk of chickens fed diphenyl-p-phenylenediamine contain this antioxidant in amounts sufficient for estimation.

The use of specified synthetic antioxidants in fats intended for human consumption has been legislatively approved in many countries. The number of compounds so approved is increasing (15). Of those approved, two of the most important are butylated hydroxyanisole (BHA) (16) and butylated hydroxytoluene (BHT) (8). The experiments described in this paper were designed to assess the effect of ingested BHA and BHT upon the stability of extracted perirenal rat fat.

### Experimental

*Diets.* The stock diet (7) consisted of rolled oats 300 g., crushed wheat 300 g., B mixture 300 g., cooked beef mince 80 g., water 200 ml., milk 80 ml., and "Potantol"[3] 0.5 ml. The B mixture contained pollard (shorts) 10 parts, wheat germ 10 parts, lactic casein 7 parts, full cream powdered milk 6 parts, sodium chloride 1.25 parts, and calcium carbonate 0.5 parts.

The stock diet was supplemented by lard to the extent of either 100 or 200 g. per 900 g. of the dry materials of the diet. The total fat content of the supplemented diets was 16.6, or 24.5%, respectively, on a dry weight basis as calculated from standard tables (1).

The freshly rendered lard, guaranteed free of added antioxidants, was purchased at three-month intervals and stored under nitrogen in sealed containers at −15°C. until required. At weekly intervals sufficient lard was removed from nitrogen storage for one week's supply. The antioxidants were added to the

[1] Presented in part at the meeting of the Australian Biochemical Society, Melbourne, June 5–8, 1957.
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[3] "Potantol," a commercial vitamin concentrate containing not less than 16,000 I.U. vitamin A and 2,000 I.U. vitamin D per ml.