With respect to the quantitation of the total toxicity of the pigment glands and the various constituents, it should perhaps be pointed out that gossyverdurin is a very unstable compound and it may be that a significant amount of the gossyverdurin decomposed and was lost during the isolation process.

ACKNOWLEDGMENT

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The Separation of Glycerides by Liquid-Liquid Column Partition Chromatography[1]

B. C. BLACK and E. G. HAMMOND, Department of Dairy and Food Industry, Iowa State University of Science and Technology, Ames, Iowa

Abstract

A liquid-liquid partition chromatography method was developed to separate triglycerides. The solvent was a two phase mixture of acetone, heptane, and water supported on silane treated celite. A study was made of the best means for equilibrating the solvents and support, packing the column, and introducing the sample. The effect of various operating variables such as flow rate, sample size, column length, and solvent compositions was studied using trilaurin and trimyristin as model glycerides. Under the best conditions achieved, it was calculated that glycerides differing by two carbon atoms or one double bond would not separate completely, but glycerides differing by two double bonds or four carbon atoms would be separated. Cocoa butter, a relatively simple triglyceride, was fractionated, and the fatty acid composition of each fraction was determined by gas chromatography. The glyceride composition was calculated and compared with theoretical compositions. The results indicate that useful glyceride separation can be obtained with this system. Probably even more useful separations could be obtained if a more sensitive device were used to detect the triglycerides in the effluent. This would allow the use of solvent compositions which give larger retention volumes and more plate efficiency.

Introduction

LIQUID-LIQUID PARTITION CHROMATOGRAPHY has demonstrated its ability to give analytically useful separations of fatty acids and triglycerides according to chain length and unsaturation. The reversed-phase system based on siliconized celite, developed by Howard and Martin (1), has been particularly useful for separating long chain fatty acids. Various paper and thin-layer partition chromatography methods for separating glycerides have been reported, but they are seriously limited in the amount of glyceride they will separate (2,3,4). This has left quantitative estimation of the glycerides subject to considerable error. Recently Hirsch (5) has reported a liquid-liquid column chromatography method for triglycerides and other lipids using factice as a support.

This paper reports a study of factors affecting the separation of the synthetic simple triglycerides, trilaurin and trimyristin, by liquid-liquid partition chromatography with silane treated celite as a support. This information was then applied to the fractionation of cocoa butter.

Experimental

The apparatus used to fractionate the triglycerides is shown in Figure 1. Columns 1.8 cm inside diameter of various lengths were used. The stationary phase was silane treated celite prepared according to Howard and Martin (1). The solvent system was a two-phase mixture of acetone, heptane, and water. The percentage of each component depended on the triglycerides to be fractionated. The packing was prepared by equilibrating the celite with the solvent in a large separatory funnel in a 30C water bath. The celite and the lower (or mobile) phase were allowed to run into the column, and the celite was packed frequently with a plunger. The remaining lower (mobile) phase was put into a jacketed reservoir and used to elute the glycerides. The sample was introduced into the column (10 mg each of trilaurin and trimyristin or 17 mg of cocoa butter dissolved in pure heptane, 100 mg/ml), and the reservoir of eluting solvent was placed at the top of the column. The fractions eluted from the column were collected by an automatic fraction collector. The flow rate of column was controlled by a teflon plug stopcock. The solvent in each fraction was evaporated, and the elution of the triglycerides from the column was monitored by the colorimetric ester method of Hack (6).

When cocoa butter was analyzed, duplicate runs were made. One run was analyzed colorimetrically to construct a weight curve. In the duplicate run, the solvent in each fraction was evaporated under a stream of purified nitrogen. The fractions were com-

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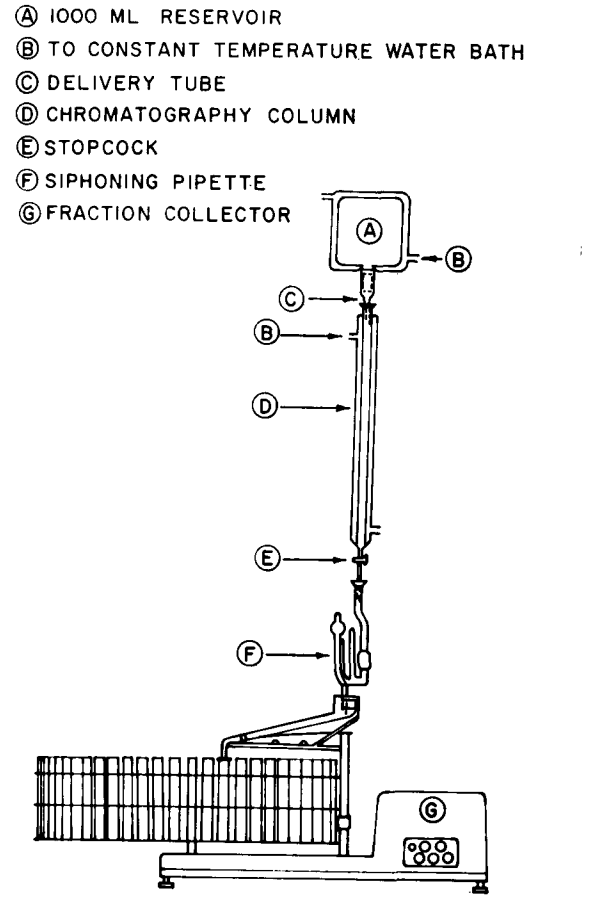

FIG. 1. Schematic diagram of liquid-liquid partition chromatography apparatus.

bined into 12 groups of sufficient size for fatty acid analysis, using the weight curve as a guide. The combined fractions were weighed and converted to methyl esters by refluxing the sample in methanolic sodium hydroxide for 1.5 hr (7). Then the samples were acidified with concentrated hydrochloric acid, diluted with five volumes of water, and extracted with Skellysolve B. The solution of methyl esters in Skellysolve B was neutralized and dried by shaking with a 1:1 mixture of sodium sulfate and sodium bicarbonate (8). The methyl esters were analyzed by gas-liquid chromatography (GLC) using a thermal conductivity detector. The column was 8 ft long and packed with Craig's (9) butandiol succinate polymer on chromosorb. The peaks were identified by comparison with known methyl esters. From the areas under the peaks, determined by triangulation, the weight percentages of the methyl esters were calculated. The number of theoretical plates and the separation factor were calculated by the methods used in GLC (10).

The phase diagram of acetone-heptane-water was constructed by measuring the amounts of water that must be added to get two phases at various ratios of acetone and heptane. The plait point was found by starting with a definite amount of acetone and adding heptane and water until two phases just formed and the two phases had equal volumes.

Results and Discussion

Factors Affecting the Separation of Trilaurin and Trimyristin. Figure 2 is a phase diagram for acetone-heptane-water at 30C. The phase diagram was an aid in choosing a suitable solvent. A solvent composition near the plait point (the point where the two phases

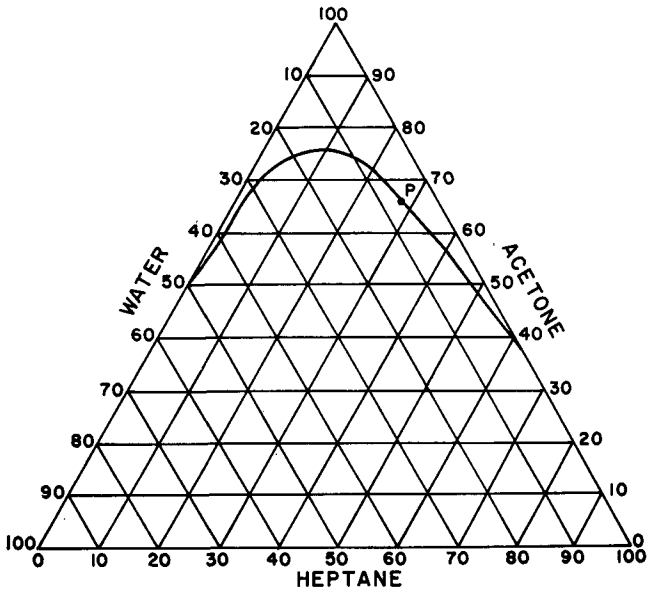

FIG. 2. Phase diagram for the acetone-heptane-water system at 30C.

are most similar) should be selected for a favorable partition coefficient. Tie lines were assumed to be parallel to a tangent through the plait point in the region near the plait point.

The silane treated celite did not act as an inert support for the stationary phase, but disturbed the equilibrium between the two phases. The amount of stationary phase and, presumably, the composition of the absorbed phase varied with the ratio of stationary phase to mobile phase to weight of celite. Table I shows the effect when acetone-heptane-water (7.5:1.5:1 by volume) was used. To overcome this, the solvent mixture and support were equilibrated in a large separatory funnel placed in a water bath at 30C. This mixture was used to pack the column and to elute the sample.

Several methods of packing were tried to get a uniformly packed column. Bubbles often formed in the column after it was packed. These could be removed by the stirring of the column contents, but this led to a reduced plate efficiency. Howard and Martin (1) recommended stirring similar columns with a perforated plunger which was moved up and down in the column during packing. This method removed the bubbles, but the plate efficiency was not improved over simple gravity packing. Therefore, when short columns (30.5 or 61 cm) were used, the packing was allowed to settle by gravity. When long columns (150 cm) were used, the celite was tamped down frequently during packing with a glass tamper which just fit into the column.

The sample should be put in solution in a small segment at the top of the column (ideally the volume represented by one theoretical plate). Since long chain fats with low degrees of unsaturation are not

TABLE I

The Amounts of Stationary Phase Absorbed by 1 g of Silane-Treated Celite Under Various Conditions

Tubes	I	II	III	IV
Ml of stationary phase added	0.2	0.5	1.0	2.0
Ml of mobile phase added	5.0	5.0	5.0	5.0
Ml of stationary phase after equilibration	0.0	0.1	0.5	1.3
Ml of stationary phase absorbed	0.2	0.4	0.5	0.7

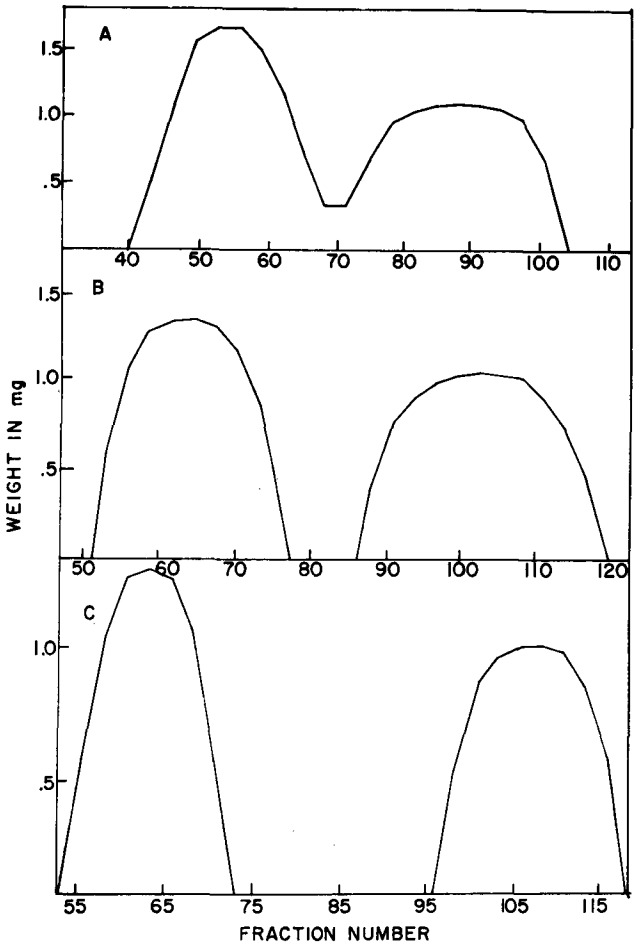

FIG. 3. Separation of trilaurin and trimyristin achieved on silane treated celite columns 30.5 cm (A), 61 cm (B), and 150 cm long (C).

very soluble in most solvents, this meant that the sample size had to be restricted. If all the sample did not dissolve, the sample was eluted as a peak followed by a long tail. The sample size was reduced until no tail could be seen. This was about 17 mg for cocoa butter and 10–12 mg for trilaurin and trimyristin. Further reduction in sample size did not appreciably improve plate efficiency. Since solution of the sample in the mobile or stationary phase required large solvent volumes or caused phase separation, the sample was introduced in about 0.1 ml of heptane.

The silane treated celite was usually screened to 50–70 mesh (U.S. standard). This allowed a rapid flow of solvent. Better separation and plate efficiency were obtained at slow flow rates. The slowest flow rate practiced in these experiments was 60–70 ml/hr. Slower flow rates required the stopcock to be throttled so much that it rapidly became blocked with fine celite particles. Hirsch (5) has explained better separation resulting from slower flow rates in terms of a steric effect; i.e., the large glyceride molecules might have difficulty penetrating the pores of the packing. A slower flow rate or more open packing would facilitate this exchange. Theoretically, there should be an optimum flow rate balance between the lag in equilibrium of the solute between stationary and mobile phases and diffusion of the solute just as in GLC.

Three lengths of column were used: 30.5, 61, and 150 cm. There was an increase in the number of theoretical plates with the increase in the length of column while the separation factor remained essentially constant. However, the height of a theoretical

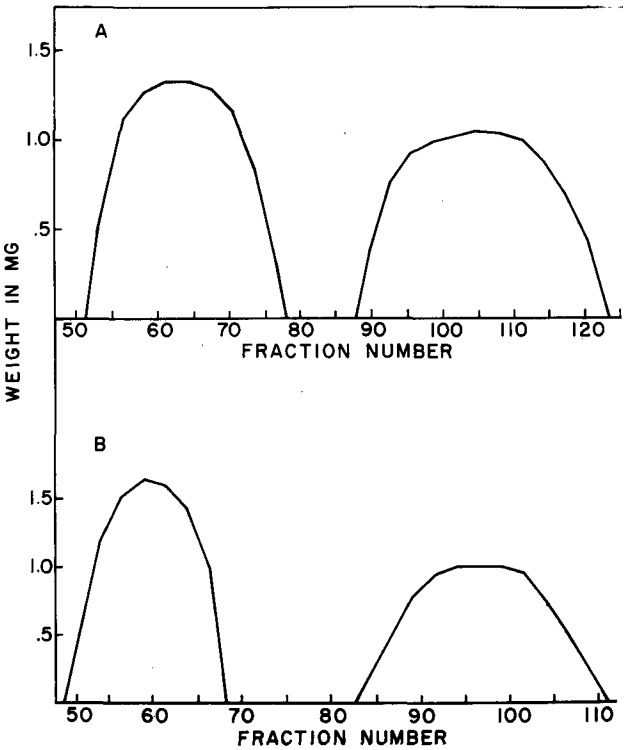

FIG. 4. Separation of trilaurin and trimyristin achieved on the 61 cm columns using silane treated celite (A) and factice (B).

plate increased with increasing column length. Probably the long columns could not be packed as uniformly as the short ones. Figure 3 shows the increase in separation of triluarin and trimyristin with increasing column length at constant solvent composition. Curve A is from a 30.5 cm column with 90 plates; Curve B is from a 61 cm column with 144 plates; and Curve C is from a 150 cm column with 269 plates.

While this work was in progress, Hirsch (5) published a paper describing the separation of glycerides which he obtained with columns of "factice," a polymerized vegetable oil, as a stationary phase and support. It was hard to calculate the plate efficiency and separation factors from his data; however, in general, he seemed to obtain less separation factor but better plate efficiency than our columns gave. The higher plate efficiency which he obtained may be partially because he used a column of smaller diameter which could be packed more uniformly.

A comparison between the silane-treated celite and the factice column was made. A 61 cm factice column was prepared according to the procedure of Hirsch, and the trilaurin and trimyristin standards were introduced into the column and eluted with an acetone-water solvent (95% acetone, 5% water). Figure 4, Curve A shows the results using the silane treated celite column. Curve B shows the results using the factice column. With silane treated celite (Curve A), a separation factor of 1.80 and a plate efficiency of 73 plates/ft were obtained from the trilaurin and trimyristin peaks. With the factice column (Curve B), a separation factor of 1.56 and a plate efficiency of 94 plates/ft were obtained from trilaurin and trimyristin peaks. This difference in plate efficiency in these experiments may be a reflection of the difference in "dead" volume of the celite and factice matrices. The actual separation achieved was slightly better with the factice support.

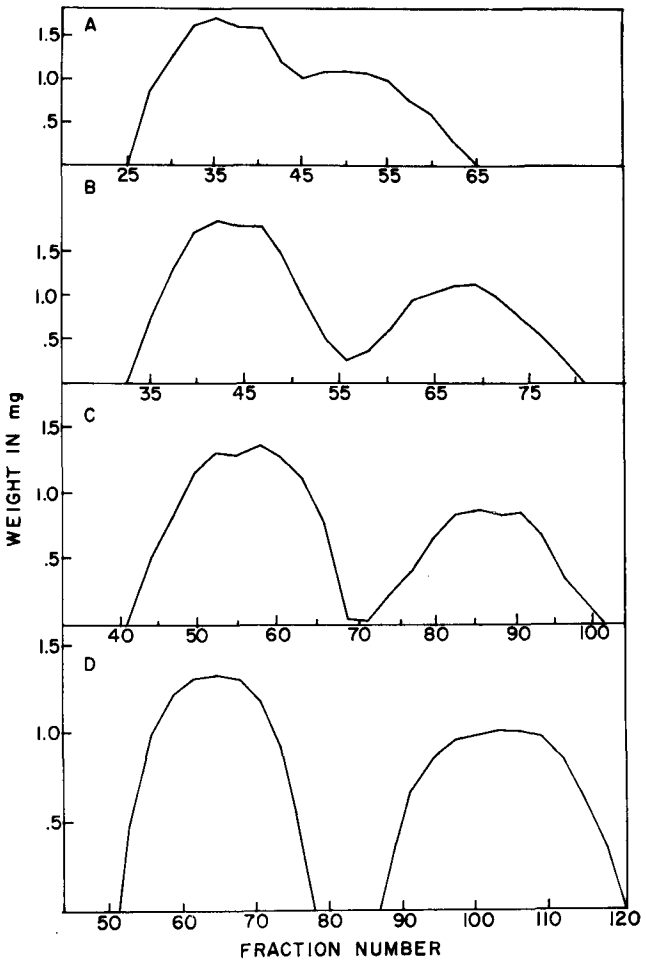

FIG. 5. Separation of trilaurin and trimyristin achieved on a 61 cm silane treated celite column with varying percentages of water in the solvent. The percentage of water was increased from curve A to D.

Figure 5 shows the separation obtained with trilaurin and trimyristin mixtures on a 61 cm column with various solvent compositions. The compositions of acetone-heptane-water were 5:1:0.628 for A, 5:1:0.667 for B, 5:1:0.704 for C, and 5:1:0.740 for D. The number of plates per foot of column and separation factors were calculated. They were: separation factor 1.75 and 35 plates/ft for Curve A; separation factor 1.79 and 56 plates/ft for Curve B; separation factor 1.80 and 73 plates/ft for Curve C; and separation factor 1.81 and 90 plates/ft for Curve D. Thus the separation factor is about the same for the two glycerides at all solvent compositions tried, but the plate efficiency increases as the retention volume increases. Similar effects have been noted in GLC (10). This increase in plate efficiency is not affected by the sample size, since halving the sample size did not increase the efficiency. One cannot increase the retention volume much more than in Curve D. As the retention volume increases, the peaks get flater. The colorimetric measurement used was sensitive only to about 0.1 mg of triglyceride. When the peaks get low and flat, the accuracy of measuring the quantity of glyceride is poor.

An Examination of Cocoa Butter Glycerides. On the 150 cm column packed with silane treated celite, triglycerides differing by six carbons were completely separated. It was estimated that triglycerides differing by four carbons should be barely separated. Instead of testing this on synthetic triglycerides, cocoa butter was used since it represents a relatively simple triglyceride mixture.

TABLE II

Weight and Molar Composition of the Twelve Fractions of Cocoa Butter

Fraction	Weight (mg)	Palmitic	Oleic	Stearic	Linoleic	Palmitoleic
1	0.7	59.4	27.0	5.7	4.7	3.2
2	2.1	53.5	32.0	7.8	3.7	3.0
3	2.0	49.1	32.8	12.4	3.6	2.1
4	1.5	45.5	33.5	19.4	1.6	
5	1.4	39.4	33.5	27.1		
6	2.0	34.5	33.9	31.6		
7	2.2	25.4	33.7	40.9		
8	2.0	19.8	34.2	46.0		
9	1.0	16.7	34.3	49.0		
10	1.6	10.2	33.8	54.4		1.6
11	1.1	6.2	33.5	60.3		
12	0.6		33.2	66.8		
Composite	18.2	31.1	33.6	33.3	1.2	0.8
Original fat	16.9	29.1	34.4	33.9	1.8	0.8

The 150 cm column was filled with 130 g of silane treated celite equilibrated with acetone-heptane-water (5:1:0.629). A 16 mg sample of cocoa butter was added in 0.16 ml of heptane. The fractions were collected, evaporated and analyzed spectrophotometrically using Hack's (6) procedure for the color test as described in the experimental section. The results are shown in Figure 6. The weight curve consists of three partially resolved peaks which, presumably, represent the three major types of glycerides in cocoa butter: 1,3-dipalmito-olein (POP), 1-palmito-2-oleostearin (POS), and 1,3-distearo-olein (SOS).

A duplicate chromatographic run was made using the same conditions and sample size. The solvent was completely evaporated under nitrogen. When the fractions were completely evaporated, they were pooled into 12 large fractions, weighed, and transesterified. Each of the 12 large fractions was analyzed by GLC. The weight percentage of each fatty acid in each of the 12 fractions was calculated from the area under the curves. The weight percentage of each fatty acid was converted to mole percentage, and this was used in the calculation of amounts of the various glycerides. The mole percentage of the acids is shown in Table II and Figure 6.

The weight and composition of the calculated composite of the fractions obtained from the column agreed fairly well with that of the original cocoa butter.

Studies on cocoa butter with pancreatic lipase (11) have shown that the beta position of the glyceride is almost 100% occupied by oleic acid. The data of Dutton et al. (12) have also indicated that each cocoa butter glyceride contains at least one oleic acid. The present results were obtained with a sample of cocoa butter relatively low in oleic acid. They indicate that, in the first fractions, if each glyceride is to contain at least one unsaturated fatty acid, the acid must sometimes be linoleic and palmitoleic. If the total unsaturated fatty acid is calculated for all the fractions, it amounts to at least one-third of the total acid within experimental error, thus confirming the observation of Dutton et al. Although there really are small amounts of trisaturated glycerides in cocoa butter (13), they cannot be detected under the present circumstances.

For these reasons the following assumptions were made in calculating the glyceride composition of the individual fractions from the fatty acid analyses: 1) The beta position was completely unsaturated. 2) The presence of a double bond in a fatty acid moiety made it behave in the separation like a saturated acid moiety

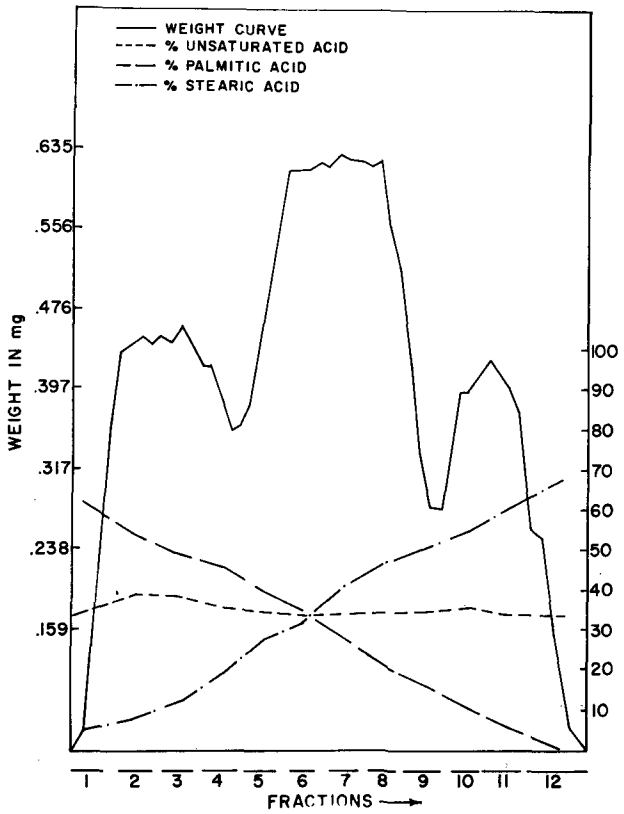

FIG. 6. Weight curve of cocoa butter showing the percentages of fatty acids in each fraction.

two carbons shorter. This has been demonstrated repeatedly in paper chromatography and counter current distribution (2,12). From this it follows that the major glycerides of the first peak will be POP and POO,[2] in the second peak SOP and SOO, and in the last peak SOS. 3) The triunsaturated glyceride was negligibly small. 4) In all fractions where Po and/or L occurred with the O, the Po and L were distributed in the same way as O so that the three could be summed as U. However, in fraction 10 it was assumed that the Po present was all in SPoS. 5) The SUU had the same retention volume as the SUP so that the SUU in the last six tubes represented half of the total SUU. To calculate the composition of the first six fractions, the first half of the SUU was distributed among the six fractions in proportion to the SUP in the fractions. Probably none of these assumptions are entirely justified, but they should not introduce much error into the calculation. The results of these calculations are given in Table III.

The theoretical distribution of the glycerides in cocoa butter were calculated according to the assumptions of Dutton et al. (12) and of VanderWal (14). In the former case the beta position of the cocoa butter is assumed to be unsaturated, and the remaining acyl groups are distributed at random in the alpha positions. In calculating the distribution according to VanderWal, the experimental values for trisaturated glyceride found on this sample of cocoa butter (0.9%) (13) and the total saturated fatty acids in the fat were used to calculate the distribution of saturated acid on the alpha and beta positions. The glyceride compositions from these theories is compared with the experimental values in Table IV. The theoretical and experimental values agree quite well. The high experimental value for SUP may indicate that the SOS and POP were not completely separated and gave an apparently high SUP value.

TABLE III

Glyceride Composition of the Pooled Fractions in Mole Percent

Fractions	Glycerides % SUS	SUP	SUU	PUP	PUU	Weight (mg)
1		16.1	1.2	79.3	3.4	0.7
2		21.8	1.6	62.1	14.5	2.1
3		34.6	2.6	49.8	13.0	2.0
4		54.3	4.0	40.5	1.2	1.5
5		80.9	0.5	18.6		1.4
6		93.1	1.6	5.2		2.0
7	22.7	76.3	1.0			2.2
8	37.9	59.4	2.7			2.0
9	47.1	50.1	2.8			1.0
10	58.5	30.6	10.9			1.6
11	80.9	18.7	1.4			1.1
12	99.7		0.3			0.6

[2] Where P,S,O,L,Po, and U stand for palmityl, stearyl, oleyl, palmitoleyl, linoleyl, and unsaturated acyl groups, respectively.

TABLE IV

Comparison of the Calculated and Experimental Mole Percentage of the Triglycerides in Cocoa Butter

	PUU	SUU	PUP	SUP	SUS	UUU	Trisat. gly.
Experimental	3.3	2.7	21.0	50.1	22.9		
Calc. values, Dutton's distribution theory	3.21	3.42	21.76	46.65	24.91	0.05	
Calc. values, VanderWal's distribution theory	3.63	3.88	21.34	45.62	24.40	0.15	0.90

From the colorimetric weight curve it was estimated that the three major peaks should contain 31, 46, and 23% of the total glyceride. This agrees closely with the 25, 50, and 25% that should be in each peak according to Dutton's distribution and the 25, 49, 24% calculated from VanderWal's distribution.

There is some evidence that the effect of a double bond on the migration of glyceride is not the same as that of shortening the chain two carbons. Thus fraction 10 contains some Po which is presumably present as SPoS. However, this compound should migrate with SOP and reach its maximum in tube 6. Likewise, SLS should be present and reach a maximum in tube 6, but no linoleate was detected after tube 4. Kaufmann, Makus, and Khoe (4) noticed similar discrepancies in the migration of triglycerides in thin-layer chromatography.

ACKNOWLEDGMENTS

Synthetic triglycerides prepared by J. R. Magnusson (15). Cocoa butter obtained from Walter Baker Division, General Foods, Dorchester, Mass.

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