TABLE II

Triglyceride Types and Isomers (% Wt.) in Butter Fat and Milk Fat

	Butter fat				Milk fat[a]			
	Sample 1		Sample 2		Calculated from data on		Average	Random
	Calc.	Random	Calc.	Random	Diglycerides	Monoglycerides		
SSS	24.5	24.5	30.9	31.2	28.1	28.0	28.1	28.2
SUS	13.4	14.7	9.9	14.8	17.3	10.1	13.7	14.8
SSU	30.6	29.4	35.0	29.6	27.3	34.7	31.0	29.6
USU	9.5	8.8	9.9	7.0	6.6	10.8	8.7	7.8
UUS	16.8	17.6	11.2	14.1	16.7	12.5	14.6	15.6
UUU	5.2	5.2	3.1	3.3	4.0	3.9	4.0	4.1
S_3	24.5	24.5	30.9	31.2	28.1	28.0	28.1	28.2
S_2U	44.0	44.0	44.9	44.4	44.6	44.8	44.7	44.4
SU_2	26.3	26.3	21.1	21.1	23.3	23.3	23.3	23.3
U_3	5.2	5.2	3.1	3.3	4.0	3.9	4.0	4.1

[a] Calculated from data by Patton *et al.* (3). The initial, rough separation of the fat from the aqueous phase was as butter fat. The samples are therefore comparable.

tribution. Those for Sample 1 are closer to the random pattern than those for Sample 2.

The values for the triglyceride types in the lower portion of Table II are all close indeed to those which would be found if distribution of S and U were completely at random. Those of Bhalerao and co-workers, which are not included in the table, are not quite as close to the random pattern but not far distant.

The data in Table II show clearly that in butter fat the fatty acyl groups, classified only as saturated (S) and unsaturated (U), have been brought together in groups of three at random, or nearly so; these triplets constitute triglycerides. It is equally obvious from the data in the first part of Table II that they do not assume positions within the molecules completely at random but to some degree become segregated in the 2-, and 1-, 3-positions. In this they behave like the individual fatty acids comprising each group, as recorded in Table I.

Butter fat is, then, another of the group of fats in which S and U become associated as S_3, S_2U, SU_2, and U_3 in proportions which can be specified, at least approximately, by application of the laws of probability operating freely or with some restriction. Kartha's mathematical formulae (4,5,6) comprise one method of arriving at this end. In butter fat this circumstance persists in spite of considerable variation in the fatty acid composition and in the distribution of the individual fatty acyl groups.

Acknowledgment

The authors are indebted to August Armattoe for technical assistance, and to Edward G. Perkins for the synthesis of known compounds.

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Analysis of the Glyceride Structure of Cocoa Butter by Thermal Gradient Crystallization[1]

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Cocoa butter was separated into 43 fractions by crystallization in a thermal gradient. Similar fractions were pooled and converted into methyl esters which were analyzed by gas-liquid chromatography. The amount of cocoa butter separated into a pure glyceride type was 85%. No significant difference was found in the ratio of palmitic to stearic acid in the GSU_2 and GS_2U. In the GS_2U, ternary and binary eutectic mixtures are predicted by the ideal solution theory. When the eutectics are taken into consideration, the individual glyceride composition of cocoa butter agrees well with the composition predicted by restricted random distribution. To test the ability of thermal gradient crystallization to separate GU_3 and GS_2U a sample of cocoa butter plus 10% triolein was analyzed. The apparent composition of the cocoa butter and triolein indicated that the GU_3 and the GS_2U separation was incomplete.

CONSIDERABLE WORK has been done on the glyceride structure of cocoa butter because of its valuable physical properties. Hilditch and Stainsby (1) separated cocoa butter into three fractions by crystallization and attempted to deduce its glyceride structure from the fatty acid composition of the fractions. Meara (2) separated cocoa butter into 11 fractions by "exhaustive" crystallization and computed the glyceride structure in a like manner. In making these calculations, it was necessary to assume that each fraction contained only two glycerides. While the present work was in progress, Scholfield and Dutton (3) published a paper describing the analysis of cocoa butter by using countercurrent dis-

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tribution. From the fatty acid composition of the fractions and certain assumptions they deduced values for the monounsaturated triglycerides.

Magnusson and Hammond (4) have described the separation of some synthetic glycerides by thermal gradient crystallization. Since cocoa butter is a relatively simple fat and since its glyceride structure has been studied rather thoroughly, it seemed to be an ideal fat to test the ability of thermal gradient crystallization to elucidate the glyceride structure of natural fats.

There has been considerable controversy about the ability of the crystallization method to give reliable information about the glyceride structure of fats (5,6, 7,8,9). This has centered around the ability of the crystallization method to separate completely the triunsaturated and monounsaturated glycerides of a fat. In order to test this point a known amount of triolein was added to cocoa butter, and the error in the recovery of the triolein was determined.

Experimental

The apparatus used to fractionate the cocoa butter has been described previously (4).

The cocoa butter was furnished by the Walter Baker Division of General Foods, Dorchester, Mass. The acetone was purified by refluxing with sodium hydroxide and potassium permanganate for 3 hrs., followed by distillation. The Skellysolve B was distilled over potassium carbonate. The methanol was reagent grade.

Approximately 0.5 g. of cocoa butter was used as a sample. The starting solvent was 200 ml. of acetone, and the eluting solvent was Skellysolve B. Fractions of 12 ml. were collected by an automatic fraction collector, and the flow rate was approximately 200 min. per 12 ml. At the beginning the temperature at the bottom of the column was −29°C. and +2°C. at the top. After it was estimated that enough fractions had been collected so that the GSU_2[2] and other very soluble components had been eluted from the column, the temperature at the bottom was raised in steps to increase the solubility of the remaining glycerides and to speed their elution from the column.

The elution of the cocoa butter from the column was followed gravimetrically. The fractions were transferred to weighed test tubes, and the solvent was evaporated in a stream of nitrogen. The test tubes were dried and weighed to obtain the weight of cocoa butter eluted from the column. Where preliminary work had shown the contents of adjacent tubes to be of similar composition, they were pooled into larger fractions. When mixed glyceride types were being eluted from the column, as many tubes as possible were analyzed separately; however 10 mg. were considered the minimum size for a reliable analysis.

The combined fractions were converted to methyl esters by refluxing the sample in methanolic sodium hydroxide (10). After a refluxing period of 1½ hrs. the samples were acidified with concentrated hydrochloric acid, diluted with 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 (11).

The methyl esters were analyzed by gas-liquid chromatography on a Podbielniak Series 9475V instrument. The column was 8 ft. long and packed with Craig polyester succinate on Chromosorb (12). The column was maintained at 187°C., and the sample injector at 300°C. The carrier gas was dried helium at a flow rate of approximately 40 ml. per min. The peaks were identified by comparison to known methyl esters, and the areas under the peaks were determined by their weights. From the areas under the peaks the weight percentages of the methyl esters were calculated and converted to mole percentages.

[2] The abbreviations GS_2, GS_2U, GSU_2, and GU_3 are used for the four glyceride types.

Results and Discussion

Figure 1 shows the weight of cocoa butter eluted from the column in each tube collected. A weight recovery of 98.0% of the 0.6604 g. of added cocoa butter was attained. No special significance could be attributed to each peak although the general shape of the curve is reproducible. The peak observed in Tube 4 was pure GSU_2 while the peak at Tube 13 was composed of a mixture of GSU_2 and GS_2U. Preliminary work had shown that all of the GSU_2 was eluted from the column in the first 18 tubes; therefore the temperature at the bottom of the column was raised after 23 tubes had been collected to hasten the elution of the remaining glycerides. The temperature was raised again for the same reason after Tubes 30 and

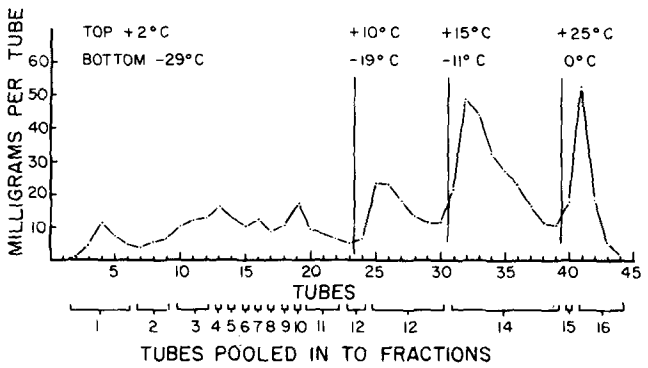

FIG. 1. Temperature of elution and the weight of cocoa butter eluted from the thermal gradient column per tube, and a schematic diagram showing the tubes pooled into fractions.

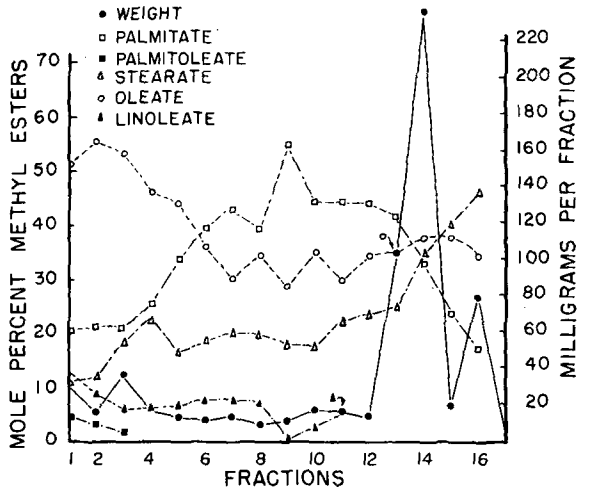

FIG. 2. Methyl ester analysis of fractions of cocoa butter and weight of cocoa butter per fraction.

39 had been collected. The tubes were combined into fractions as indicated in Figure 1.

Figure 2 shows the weights of the combined fractions and the composition of these fractions analyzed as methyl esters by gas-liquid chromatography. The sum of the mole percentages of unsaturated methyl esters in Fractions 1 and 2 is approximately 66⅔%. This indicated that Fractions 1 and 2 are predominately GSU_2. Fractions 3 through 8 are of mixed glyceride types, and Fractions 9 through 16 are predominately GS_2U. In Fractions 9 through 16 the variation of the mole percentage of unsaturated methyl esters from 33⅓% is attributed to the inaccuracy of the analysis of the methyl esters. The high concentration of linoleate in the early fractions and the increase in stearate with the decrease in palmitate in the late fractions indicate some separation of glycerides by chain length and number of double bonds as well as by number of unsaturated fatty acids.

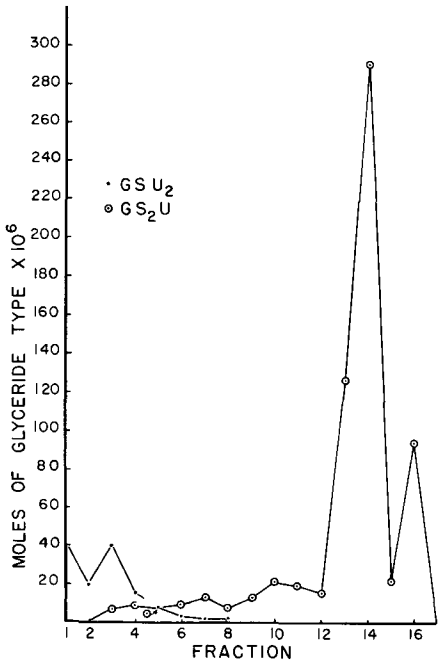

FIG. 3. Separation of GSU_2 and GS_2U achieved on one pass through the thermal gradient column.

Figure 3 shows the moles of glyceride type per combined fraction. About 46.9% of the total GSU_2 was obtained pure, 91.8% of the total GS_2U was pure, and 84.9 mole % of the total fat was obtained as a pure glyceride type. The analysis of the methyl esters by gas-liquid chromatography was not accurate enough to detect any GS_3 or GU_3.

The analysis of the methyl esters by gas chromatography was a major source of error in these experiments. Separation by fatty acids of the same chain length was not complete, and the ratio of the components appeared to vary with the size of the sample injected. Analysis of the unfractionated cocoa butter by gas chromatography indicated that the mole percentages were 29.6 palmitate, 1.0 palmitoleate, 24.7 stearate, 39.5 oleate, and 5.1 linoleate. When a very large sample was injected, trace constituents were found that corresponded to the retention times of saturated C_{13} and C_{17} and monounsaturated C_{20} methyl esters. Analysis by lipoxidase (13) showed 3 mole % of linoleate. The iodine value was 37.2, indicating about 34.2 mole % of monoene. The molar composition calculated from the summation of all the fractions was 35.6% palmitate, 1.43% palmitoleate, 27.0% stearate, 33.9% oleate, and 2.1% linoleate.

By "exhaustive" crystallization Meara (2) succeeded in obtaining separation of cocoa butter into 13 fractions. The degree of separation of the glyceride types that he achieved is similar to that in the present experiments. This would indicate that one pass through the thermal gradient column was as good as the best results obtained by repeated fractional crystallizations.

In another experiment the first 20 fractions of cocoa butter from the column were pooled and refractionated on the thermal gradient column. This portion of the glycerides contained all the GSU_2 and amounted to 31.1% by weight of the original sample. When refractionated and analyzed, the separation was almost identical with the early fractions in Figure 2. Thus the separation seems to reach the limits possible with the temperature and solvents employed with one pass through the thermal gradient apparatus. It is doubtful that a lower temperature at the bottom of the column would improve the separation of the GSU_2 and GS_2U. Essentially the same separation was attained at −23°C. in another experiment as was attained at −29°C. in the present experiment. Probably eutectic formation is limiting the separation. It is possible that better separation of the components of the GS_2U could have been attained if the temperature of the column had not been raised as much as it was at Tube 23 and more fractions had been taken before raising it any more.

Recent experiments with pancreatic lipase have revealed that there is a sharp difference in the way saturated and unsaturated fatty acids are distributed in cocoa butter and other fats (14). It is interesting to consider whether there is any evidence for a difference in the distribution of the palmitic and stearic acid in cocoa butter. If there were no discrimination between palmitic and stearic acid in the synthesis of cocoa butter glycerides, the ratio of palmitic to stearic acid in the GSU_2, GS_2U, and the whole fat ought to be the same. These values were determined from the present data and found to be 1.32, 1.12, and 1.20, respectively. These ratios are believed to be the same within experimental error.

If there is no preferential selection of palmitic or stearic acid into the GSU_2 or GS_2U, one may consider whether there is any preferential combination of the palmitic or stearic with the oleic acid in the GS_2U, that is, are the ratios, of GSt_2O, GStPO, and GP_2O[3] the same as random distribution would predict? Hilditch and Stainsby (1) and Meara (2) considered that in any fraction of their GS_2U only two of the glycerides, GSt_2O, GStPO, and GP_2O were present. From this they determined a relatively high concentration of GStPO, which they took to indicate a tendency to even distribution. However the ratio of GStPO to GSt_2O and GP_2O would appear higher than in random distribution if the GSt_2O and GP_2O were not completely separated.

Magnusson and Hammond (4) concluded that eutectic formation limited the separation of trilaurin and trimyristin in thermal gradient crystallization

[3] These symbols stand for glycerides containing stearic (St), palmitic (P), and oleicc (O) acids. When preceded by "G," the symbols indicate only the fatty acid composition. Without the "G" the order indicates the positional isomers in the order *alpha-beta-alpha*.

and that the composition of the eutectic eluted by solvent was essentially the same as that formed by the two glycerides by themselves. Moreover the composition of the eutectic could be calculated with fair accuracy from ideal solution theory.

Since the *beta* position is almost completely occupied by oleate, the major glycerides will be SOS, SOP, and POP. These should form a ternary eutectic, and its composition can be approximated as follows. Let the SOS, SOP, and POP be designated as S_1, S_2, and S_3, respectively, with melting points T_1, T_2, and T_3, and heats of fusion H_1, H_2, and H_3. Let T_4 be the melting point of the ternary eutectic. Then

$$S_1 + S_2 + S_3 = 1$$

and

$$\ln(S_1) = (H_1/R)(1/T_1 - 1/T_4)$$

and similar equations can be set up for S_2 and S_3. These four equations may be solved for the four unknowns if the melting points and heats of fusion are known for each solute. Assuming that all the glycerides are unsaturated on the *beta* position and crystallize in the most stable form, the melting points are 317.5, 311.2, and 311.5°K. (15). The H_1 has been found by Lutton (16) to be 40,000 cal./ mole. Assuming that the effect of chain length is the same in these glycerides as in monoacid saturated glycerides, H_2 and H_3 may be estimated to be 37,900 and 35,800. Using these constants, it is found that the ternary eutectic is 11.6% SOS, 44.7% SOP, and 43.7% POP. The ternary melting point is 33.8°C. Thus at the beginning the GS_2U would be expected to be eluted with a composition similar to that of the ternary eutectic. After all the POP has been eluted in this form, the SOP and SOS should be eluted as an eutectic whose composition and melting point may be computed by a similar procedure to be 78.5% SOP and 21.5% SOS and 36.3°C. The remainder of the SOS should come out pure.

From Figure 2 it may be seen that the separation agrees fairly well with these predictions. From the composition of the ternary eutectic it can be deduced that the first GS_2U to come out pure should have a palmitic-to-stearic ratio of about 1.95. This is found to be true. Then binary eutectic will come out at a palmitic-to-stearic ratio of 0.645, and the palmitic-to-stearic ratio should drop sharply. Evidently the temperature was not low enough to get good separation of the ternary and binary eutectic, which melt rather close together. However the trinary eutectic should separate well from the pure SOS, and if this assumption is made, the amounts of SOS, SOP, and POP can be calculated. In the fractions that were a mixture of GSU_2 and GS_2U it was assumed that the SOS, SOP, and POP were present in the ternary ratio. In some fractions a little additional POP was assumed to be present to account for the observed palmitic-to-stearic ratios. Making these assumptions, one finds that the SOS, SOP, and POP of the cocoa butter are present in nearly random ratios.

The glyceride type of composition of the cocoa butter is compared with that calculated by restricted random distribution in Table I. The value of GS_3 was obtained by a method based on the addition of mercaptoacetic acid to the double bonds of a fat. The neutral GS_3 is separated from the acidic products by extraction of the ammonium soaps and treatment with ion exchange cellulose (17). The restricted random distribution was calculated by a new formula (18), which gives more accurate values. The calculated values are based on the iodometric determination of unsaturated fatty acids. The agreement between restricted random and the experimental values is close. Table II shows that the values of the individual glycerides agree closely with restricted random distribution if the eutectics are taken into consideration.

TABLE I

Glyceride Types of Cocoa Butter in Mole Percentage

Glyceride type	Composition of cocoa butter		Cocoa butter plus 10% triolein	
	Found	Restricted random	Apparent	Calculated
GU_3	.....	0.5	8.5	10.0
GSU_2	15.2	11.5	21.5	13.8
GS_2U	83.9	87.1	70.0	76.2
GS_3	0.9	0.9	.....	.....

TABLE II

Glycerides of Cocoa Butter in Mole Percentage

Glycerides	Restricted random distribution	Found by use of eutectic formulas	Found by assuming two components per fraction
GPO_2	6.2	8.8	8.8
$GStO_2$	5.2	6.6	6.6
GSt_2O	18.0	17.0	6.7
GStPO	43.2	46.7	67.0
GP_2O	25.9	21.0	10.6

It was not possible to decide from the present data if oleic and linoleic were distinguished in the synthesis of the cocoa butter. Youngs and Sallans have presented data (19) which indicate that the unsaturated fatty acids in the different glyceride types have the same composition. Reiser and Reddy (20) have recently claimed that linoleate tends to concentrate in the GU_3 of lard. From the present data it can be seen that linoleate and palmitoleate came out in the earlier fractions while GSU_2 was still coming out. However it is obvious that this is partly caused by the greater solubility of the glycerides containing these fatty acids. There was so much inaccuracy in the determination of linoleate that it was not possible to see if the linoleate could be divided proportionately between the GSU_2 and GS_2U.

In order to test the separation of GU_3 and GS_2U by thermal gradient crystallization 10 mole % of triolein was added to cocoa butter, and the separation was run under the same conditions as the cocoa butter alone. The weight curve was similar to Figure 1, and the compositions of the early fractions are shown in Figure 4. Obviously the triolein tended to come out in the first few fractions, but it did not come out completely before the GS_2U began to come out. The apparent composition is compared to the calculated composition in Table I. The results are complicated by the fact that the gas-phase analysis shows a recovery of more oleate than was added. This makes the apparent triolein and GSU_2 both too high. Thus the error due to the incomplete separation lies somewhere between 1.5 and 7.7%. This could be an important degree of error in the analysis of some fats. Results showing that the glyceride type of determination, based on crystallization, agrees with Kartha's oxidation method (20,21) may indicate that both methods give similar errors rather than that they are both reliable.

The results of these experiments agree with the results of Scholfield and Dutton (3) in that the

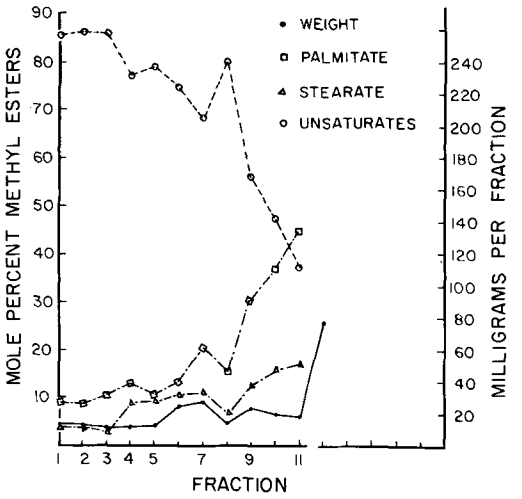

FIG. 4. Methyl ester analysis of fractions of cocoa butter plus 10 mole % triolein and weight per fraction.

glyceride structure of cocoa butter seems to agree with the predictions of restricted random distribution. However restricted random distribution does not take into account the nonrandom *alpha-beta* distribution of the saturated and unsaturated fatty acids. Kartha's explanation of the *alpha-beta* asymmetry (22) is evidently based on a misconception of the implications of random distribution. In more recent work Dutton, Scholfield, and Mounts (23) have extended their work with cocoa butter and have shown that their results can be explained by assuming that the *beta* position is occupied by oleic acid exclusively and that the remaining acids are randomly distributed on the *alpha* positions. These results agree fairly well with the restricted random distribution in predicting the amounts of the various glyceride types.

Vander Wal (24) has recently proposed a scheme that assumes that the fatty acids are distributed randomly except for a nonrandom distribution on the *alpha* and *beta* positions of the glycerol. Calculations based on this scheme agreed with the experimental results reported for a number of fats. If the amount of GS_3 and S in the cocoa butter is used as the known parameters in Vander Wal's equations, values of GS_2U and GSU_2 may be calculated. The values obtained in this way agree closely with restricted random distribution and hence with our experimental results.

It is obvious that a restriction on the amount of saturated fatty acid that can occupy the *alpha* or *beta* position will decrease the amount of GS_3 and give a distribution approaching restricted random distribution. The distribution proposed by Vander Wal agrees closely with the restricted random distribution in the amounts of the glyceride types when various values of GS_3 and S are used as the known parameters. In cocoa butter the distribution proposed by Dutton, Scholfield, and Mounts agrees with both of the above distributions. Thus any of these schemes might explain the amounts of the various glycerides found in the present experiment and by Dutton and co-workers. Vander Wal's scheme has the advantage of taking the *alpha-beta* asymmetry into account and of being applicable to a number of fats. Further studies to improve the analyses of glycerides and to investigate the mechanism of their synthesis are needed for a clearer understanding of the structure of fats.

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[Received April 22, 1960]

Erratum

Through an oversight a correction on the galley proofs for the paper entitled "Color Index for Cottonseed Oils" by Pons, Kuck, and Frampton was not made (*37*, 671, 1960). The correct formula is shown here: "Thus, if the Beer-Lambert law describes the absorption behavior of the solutes in cottonseed oil, the relationship

$$C_t = \frac{R \sum_{\lambda=400}^{\lambda=550} \left(\log \frac{I}{Io}\right)}{b \sum_{\lambda=400}^{\lambda=550} \left(\sum_{p=1}^{p=n} k_p\right)}$$

where C_t is the total concentration of the absorbing solutes, R is a proportionality constant, b is the length of the light path, λ is the wavelength in millimicrons, I and I_o are light intensities, p is the number of solute components, and k_p is the absorptivity for the $_p$th component at wavelength λ, can be used to estimate the total concentration of solutes in the oils."