positions of the triglycerides. For some fats at least, the free fatty acids do not, therefore, provide a satisfactory measure of the composition of the acids occupying the 1 and 3 positions of the triglycerides.

It must of course be remembered when using pancreatic lipase for the investigation of the fatty acid distribution of natural fats, that rates of hydrolysis for individual fatty acids are only comparable for the higher members of the series. Savary and Desnuelle (20) have reported that similar rates are obtained for lauric, and acids of greater molecular weights, whether saturated or unsaturated. Entressangles et al. (21) and Clément et al. (22) have clearly shown that the presence of short chain acids, particularly butyric, obscure the results of fatty acid distribution deduced from hydrolysis data. Entressangles has drawn attention (23) to the unsuitability of pancreatic hydrolysis for the investigation of such fats as milk fat, which contain appreciable amounts of butyric acid.

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Gossyverdurin: A Newly Isolated Pigment from Cottonseed Pigment Glands

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Abstract

The constituents of cottonseed pigment glands were fractionated by the use of column chromatography with DEAE cellulose ion exchanger and silicic acid, and a new green pigment was isolated. The acute oral toxicity of the new pigment was determined using rats as experimental animals. The LD-50 value obtained was 0.66 g/kg of body weight indicating that the new pigment which was named gossyverdurin is the most toxic of any cottonseed pigment so far reported. Gossyverdurin showed absorption maxima at 250, 370, and 560 mμ. Reaction with para-anisidine under the conditions used for the determination of gossypol gave an absorption peak similar to that obtained with gossypol indicating that the new compound is structurally related to gossypol. In addition a second peak at 342 mμ appears on reaction with para-anisidine indicating important structural differences between gossypol and gossyverdurin.

Introduction

IT HAS BEEN reported that cottonseed pigment glands, which are separated in an essentially unaltered condition from cottonseed kernels by a flotation process (1,2) retard the growth of chicks when the glands or products containing them are included in the diet (3,4). Oral administration of these glands in relatively large doses resulted in the death of rats, mice, guinea pigs, and rabbits (5).

Gossypol, a polyphenolic yellow pigment, is the principal component and gossypurpurin, a gossypol derivative, has been reported to be the most abundant secondary component of cottonseed pigment glands (6).

Eagle et al. (5) studied the relative toxicity of pure gossypol and a number of preparations of pigment glands using rats as experimental animals and reported that the glands were more toxic than an equivalent amount of gossypol, that the toxicity of different gland preparations was not proportional to their content of gossypol, and that toxicity decreased with an increasing content of gossypurpurin. They concluded: "The toxicity of cottonseed pigment glands is attributable to some component or components of the glands other than, or in addition to, gossypol and gossypurpurin." In these studies an acetone-soluble, water-soluble fraction was obtained from the glands which had an LD-50 value of approximately 700 mg/kg body weight, using rats as experimental animals.

Recently the reports of Eagle et al. to the effect that cottonseed pigment glands are more toxic than an equivalent amount of gossypol have been confirmed by El-Nockrashy, Lyman, and Dollahite (7). The purpose of the present communication is to report the isolation of a bright green colored pigment from cottonseed glands which is more toxic than gossypol. This new pigment, which is a gossypol related compound, has been named gossyverdurin.

Experimental

Fractionation of Cottonseed Pigment Glands. Cottonseed pigment glands were separated from rolled decorticated kernels by a flotation technique (8). Twenty-five grams of the pigment glands were extracted with 200 ml of acetone in a Waring Blendor for 5 min, and the mixture was filtered through a Buchner funnel. The residue was re-extracted with 200 ml, and finally with 50 ml of acetone. The com-

PIGMENT GLANDS
Acetone Extraction
Acetone Soluble
Acetone Insoluble
Water Extraction
Water Extract (Colloidal)
Residue
DEAE Column
Water Eluate
60% Acetone Eluate
75% Acetone Eluate
100% Acetone Eluate
Chloroform Eluate
+ 15% Acetic Acid + Chloroform
Chloroform Phase
Acetic Acid
SILICIC ACID
Chloroform Eluate
(Almost entirely free gossypol)
Absolute Methanol Eluate
Recrystallization
GOSSYVERDURIN
(2% of weight of glands)

FIG. 1. Diagram of fractionation of cottonseed pigment glands and isolation of gossyverdurin.

bined extracts were evaporated by passing a stream of nitrogen over the surface. The deep red viscous acetone-soluble residue was then re-extracted with distilled water in a Waring Blendor until the extract became clear. The combined water extract (500 ml) which was yellow, and appeared to be a colloidal suspension, was then passed over a column of DEAE cellulose ion exchanger 1 in. diam and 12 in. long, and the column was washed with water until a clear effluent was obtained. The combined water effluent was lyophilized and a pale yellow sticky material was obtained (ca. 0.40 g). The DEAE column was then eluted using the following eluants in succession: 60% acetone, 75% acetone, 100% acetone, and chloroform. The eluted fractions were collected, and evaporated under vacuum on a warm water bath (40C). The 60% and 75% acetone eluates (0.60 and 0.85 g, respectively) were yellowish brown oily solids. The 100% acetone and the chloroform eluates (1.58 and 0.45 g, respectively) were brown oily liquids. The DEAE was then transferred to a separatory funnel with two volumes of 15% acetic acid solution, and two volumes of chloroform and the mixture was shaken vigorously. This treatment resulted in complete elution of all colored material adsorbed on the DEAE. The eluted material was found in the chloroform layer. The DEAE was washed twice with chloroform, and the chloroform eluate and wash was then concentrated to 100 ml by evaporation under vacuum using a warm water bath.

The chloroform eluate, yellowish green in color, was then passed over a silicic acid column, 1 in. diam and 12 in. long. Two bands separated on the column, a green band at the top and a yellow band below. The yellow band, which proved to be gossypol, rapidly passed through the column and was collected in the chloroform eluate. The column was then exhaustively washed with chloroform. The chloroform eluate and washing was evaporated under vacuum at low temperature (40C). The green colored band was then eluted readily and completely with absolute methanol. Evaporation of the methanol eluate at 40C, under vacuum, left a dark green crystalline material. The green colored pigment was recrystallized from absolute methanol solution, and then dried in a vacuum desiccator containing phosphorous pentoxide. A diagram of the fractionation procedure is given in Figure 1.

TABLE I
Characterization of Fractions from Cottonseed Pigment Glands

Description of fraction	% of original pigment glands contained in the fraction	Total gossypol content[a]	Free gossypol content[a]
		%	%
Original pigment glands	100.00	43.00	38.10
Acetone-soluble fraction	60.40	70.10	64.70
Water extract of acetone soluble fraction	40.30	60.00	43.20
Eluates from DEAE column			
Water effluent	1.20	0.10	0.00
60% Acetone	2.40	0.35	0.25
75% Acetone	3.40	0.23	0.15
100% Acetone	6.70	0.09	0.04
Chloroform	1.40	0.11	0.00
Eluates from silicic acid column			
Chloroform	......	92.01	88.39
Methanol	......	32.50	25.00

[a] As determined colorimetrically after reaction with para anisidine (9).

Characterization of the Fractionation Products. The various fractions were analyzed for free and total gossypol using the method of Pons et al. (9,10). Phosphorous was determined by the method of Fiske and SubbaRow (11). The absorption spectra of the green pigment was obtained by the use of the Beckman D.U. Spectrophotometer.

Physiological Studies. Rats were used as experimental animals for the study of the physiological significance of the isolated fractions. The care of the animals, the method of administering the doses, and the procedures for evaluating the results have been described previously (8). LD-50 values were calculated according to the method of Bliss (12).

Results and Discussion

Fractionation of Pigment Glands. The percentages by weight of the original pigment glands obtained in the various fractions and the content of free and total gossypol as determined by the colorimetric procedure after reaction with para-anisidine are given in Table I.

The acetone-soluble fraction showed high total gossypol content mostly as free gossypol. The five fractions collected from the DEAE column constituted 15.1% of the pigment glands. These five fractions were oily or waxy in texture. It was thought that these might be phospholipid-bound gossypol, but analysis did not show any phosphorous, and the peculiar thing is that the total gossypol content of the five fractions was very small, ranging from 0.10–0.35%. The chloroform eluate, a yellowish green solid obtained by treating the DEAE with acetic acid and chloroform, constituted 23.5% of the original pigment glands weight. The chloroform effluent collected from the silicic acid column, a bright yellow, showed as high as 92.01% total gossypol, almost all as free gossypol. The green colored pigment, gossyverdurin, on analysis by the standard methods for the determination of free and total gossypol, showed an apparent content of 25.00% free gossypol and 32.50% total gossypol. The yield of gossyverdurin was ca. 2.0% of the weight of the original pigment glands.

Physical and Chemical Characteristics of Gossyverdurin. Gossyverdurin is very soluble in chloroform,

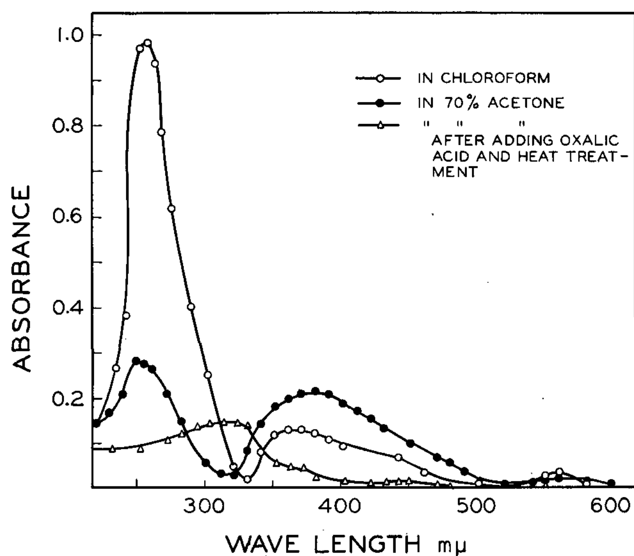

FIG. 2. Absorption spectra of gossyverdurin, concn 2 mg/100 ml.

methanol, acetone, diethylether, and ethanol, and insoluble in petroleum ether. It became brown in color at 210C indicating decomposition, but did not show any melting point when heated to a temperature as high as 310C.

Microanalytical results of the pigment showed: C, 62.93; H, 6.19; N, 1.90; O, 21.09; ash, 8.20%.

Several qualitative tests (13) were applied to gossyverdurin, in an effort to determine to what extent it was related to gossypol and gossypurpurin. Positive reactions were obtained with the same reagents which were found to give positive results with gossypol, viz., those indicating the presence of one or more carbonyl groups such as Tollen's reagent and Fehling's solution, and the presence of two ortho-phenolic hydroxyls, and a hydroxy para or ortho to a carbonyl (using ferric chloride). Concentrated sulphuric acid reacts with the green pigment to produce a permanent green color, in contrast to the dark red color developed immediately with gossypol, and the yellow-green color which becomes orange after ten minutes with gossypurpurin. A deep blue color is obtained when the green pigment is treated with glacial acetic acid, in contrast to the yellow precipitate obtained with gossypol, and the green precipitate obtained with gossypurpurin.

The absorption spectrum of gossyverdurin in chloroform solution has maxima at 250, 370, and 560 mμ (Fig. 2). Gossypol in chloroform has maxima at 288 and 363 mμ (Fig. 3).

Considerable difference in the spectra of gossypol and gossyverdurin in chloroform at the lower wave lengths will be noted. While the sharp decrease in the case of gossyverdurin, below 250 mμ might suggest the absence of a maxima at 236 mμ and hence the absence of a diene conjugated system, chloroform is not the ideal solvent for examination in this range of the spectrum; further work would be required to establish such a conclusion.

The shift in the absorption band of gossypol at 363 mμ in chloroform to somewhat longer wave lengths in acetone (Fig. 3) can be accounted for, we believe, by the formation of the gossypol acetone complex. This bigger complex molecule will vibrate at a somewhat lower frequency or longer wave length.

Figure 4 shows the absorption spectra of the reaction products of gossypol and gossyverdurin with para anisidine at the same concentrations under the conditions used in the determination of gossypol. The similarity of the shape of the peaks at 447 mμ suggests a structural relationship between gossypol and gossyverdurin. The apparent free gossypol content of gossyverdurin as determined colorimetrically after reaction with para anisidine was 25.00%. The apparent total gossypol content obtained after hydrolysis with oxalic acid was 32.50%.

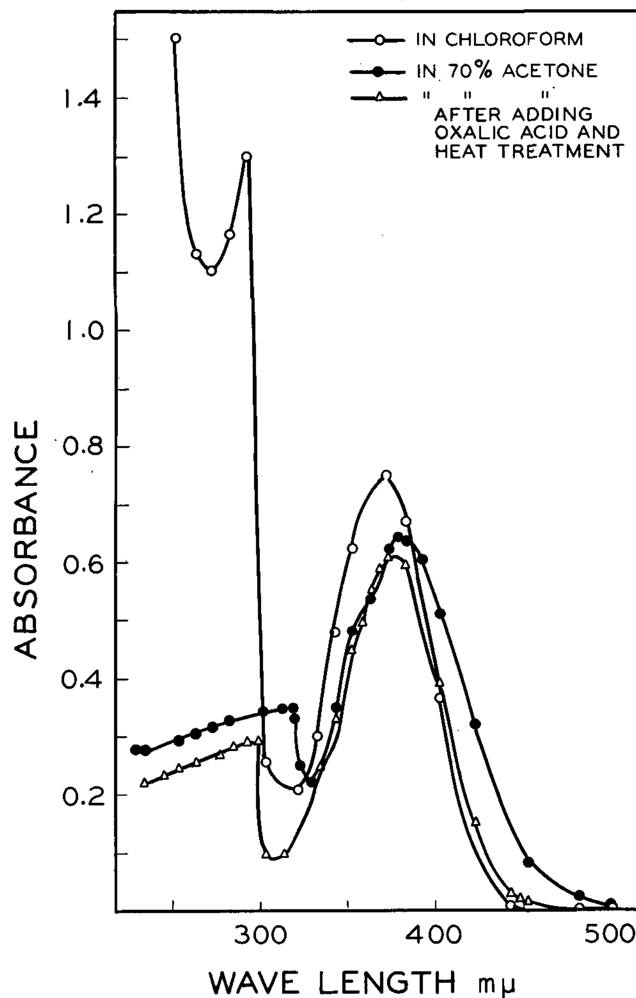

FIG. 3. Absorption spectra of gossypol, concn 2 mg/100 ml.

Figure 5 shows the absorption spectra with concentrations adjusted so as to give approximately the same height of the peak at 447 mμ. These curves were prepared by subtracting the absorption of the unreacted material as is done in the determination of free and total gossypol. Of particular interest is the appearance of a second peak in the curves for gossyverdurin at 342 mμ which does not occur in the curve for gossypol. Since this peak also does not occur in the curve for unreacted gossyverdurin, it may offer a possibility for the quantitative determination of gossyverdurin in the presence of gossypol.

When gossyverdurin is hydrolyzed with oxalic acid in preparation for the standard determination of total gossypol, the green color quickly disappears and the solution becomes brown. Under the same conditions gossypol is not changed.

This difference in behavior of gossypol and gossyverdurin on heating in oxalic acid solution is illustrated in the absorption spectra shown in Figures 2 and 3.

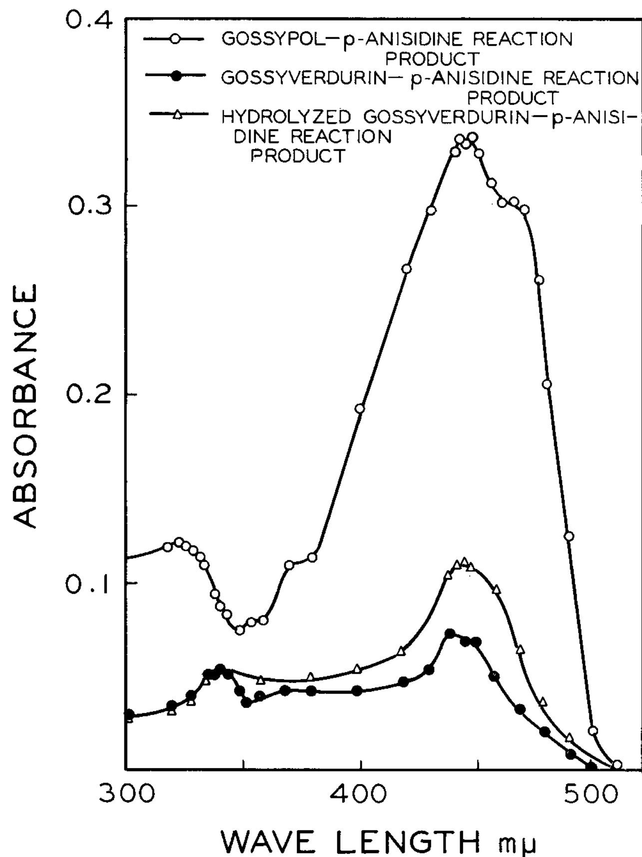

FIG. 4. Absorption spectra of gossypol and gossyverdurin—para-anisidine reaction products, concn 0.1 mg. in 25 ml.

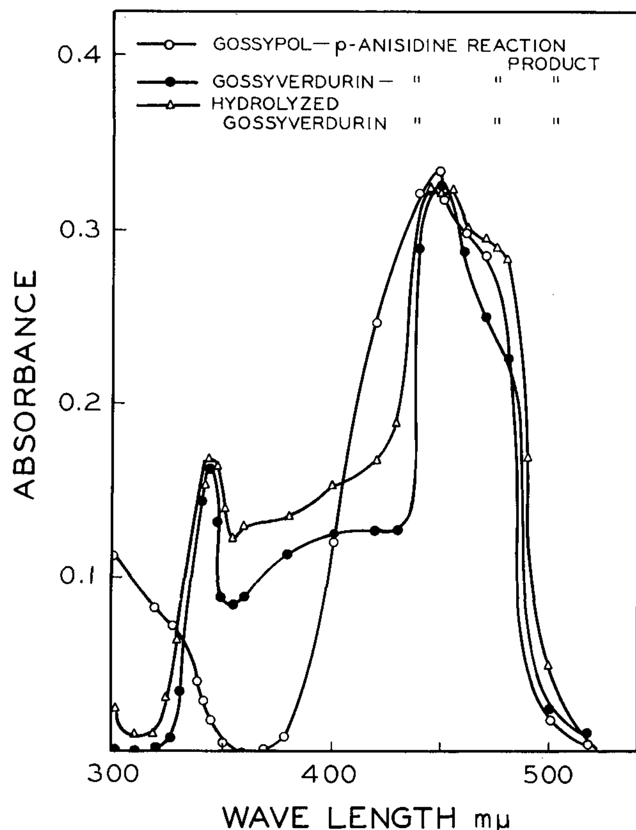

FIG. 5. Absorption spectra of gossypol and gossyverdurin—para-anisidine reaction products, concn gossypol 0.1 mg/25 ml; gossyverdurin 0.4 mg/25 ml; hydrolyzed gossyverdurin 0.3 mg/25 ml. These curves were prepared by subtracting the absorption of the unreacted material as is done in the determination of free and total gossypol.

TABLE II

Nutritional Study of Fractions Obtained from Cottonseed Pigment Glands[a]

Material fed	Dose as g/kg of body wt	No. of rats given the dose	No. of rats which died	% gain or loss in wt among surviving animals (avg)	Calculated LD-50 g/kg
Acetone soluble fraction of pigment glands	2.00	10	10	–f[b]	
	1.25	10	8	19.4	0.79
	0.75	10	6	12.2	
	0.50	10	0	9.4	
Fractions eluted from DEAE column					
Water effluent..........	1.00	5	0	+12.4	......
	2.00	5	0	+ 3.8	......
60% Acetone..........	2.00	5	0	+ 1.4	......
75% Acetone..........	2.00	5	0	+ 0.2	......
100% Acetone..........	1.00	5	0	+10.4	......
	2.00	5	0	+ 5.8	......
	4.00	5	0	− 3.2	......
Chloroform..............	2.00	5	0	− 1.4	......
Gossyverdurin..............	2.00	8	8	–f[b]	
	1.00	8	7	21.2	0.66
	0.75	8	4	18.9	
	0.50	8	4	20.0	

[a] The doses were given in corn oil to male rats weighing 160 (± 10) g, and the rats were kept under observation for 7 days or until death.
[b] Fatal to all rats tested.

Physiological Studies. Table II shows physiological effects of the different fractions obtained from the pigment glands. The LD-50 value for the original glands was 1.12 g/kg body weight. Details of this assay have been reported previously (8). The value obtained for the acetone extract was 0.79 g/kg body weight and the free gossypol content of this extract was 64.70%. If gossypol was assumed to be the only physiologically active compound in the extract then the calculated LD-50 value for gossypol would be 0.55 g/kg body weight. It has been shown previously that this amount of gossypol does not kill rats (8). There was also a direct relationship between the dose given and the per cent loss in weight among the surviving animals. Autopsy of dead rats revealed symptoms similar to those observed in rats fed pigment glands (8) where consistent hyperemia of the gastrointestinal tract was observed. The livers showed prominent lobules. The kidneys and the lungs were congested.

None of the five fractions eluted from the DEAE proved to be fatal to rats at the doses given. The rats given the water effluent, at the two levels of 1.00 and 2.00 g/kg, gained weight. The most interesting point is that these five fractions which were isolated from the acetone-soluble, water-soluble fraction of the pigment glands were not lethal at the levels tested although they constituted ca. 37.5% of the acetone-soluble, water-soluble fraction.

The gossyverdurin, when examined for its physiological effects, proved to be the most toxic material obtained from cottonseed which has so far been reported. While the LD-50 value for the pigment glands, gossypol, and gossypurpurin were 1.12, 2.57, and 6.68 g/kg body weight, respectively (8), the gossyverdurin showed an LD-50 value of 0.66 g/kg body weight. Almost all rats on the four gossyverdurin doses were immobilized, and showed severe diarrhea with blood in the stools. The rats which did not die by the end of the experiment showed considerable losses in weight. Autopsy of the dead animals revealed consistent hyperemia of the gastrointestinal tract. Severe inflammations and hemorrhages were observed in the pyloric region of the stomach. Congestions throughout the intestines, highly inflamed caecum and, in severe cases blood and mucus, were observed. The liver was light in color and the lobules were prominent. The kidneys showed badly congested medulla. The lungs were congested.

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.

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

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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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