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Spectrophotometric Determination of Total Gossypol in Cottonseed Meal and Cottonseed Meats[1]

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THE NEED for a simple expeditious method for the estimation of total gossypol (bound and free) in cottonseed meal and cottonseed meats has not been adequately fulfilled by previous methods. Such a method would be of considerable benefit to the cottonseed processing industry, to feed manufacturers, and to nutritionists.

Bound gossypol (4), originally designated as D-gossypol (3), was first determined gravimetrically as dianilinogossypol (3, 5). It was extracted by means of hot aniline from cottonseed meal, which had been extracted previously with ether to remove the free gossypol. The extract was concentrated and allowed to stand several days so that crystallization might occur. A modification of this method (8) permitted the extraction of the free and the bound gossypol by a mixture of hot alcohol and aniline. The gossypol in the dianilinogossypol precipitated from the extract was designated as total gossypol. The bound gossypol was determined by subtracting the free gossypol, found by chemical analysis, from the total. More recently total gossypol has been determined spectrophotometrically as the p-anisidine derivative (7). The details of extraction by the p-anisidine method have been modified, and aniline has been substituted for p-anisidine as the coloring agent (6). Aniline has been substituted for p-anisidine in the revised A.O.C.S. Tentative Method Ba 8-55 (1).

The spectrophotometric method presented herein utilizes the reaction of gossypol with aniline and reduces the manipulative operation and time per determination. In this method the sample is treated with 72% alcohol (aqueous) to soften the particles of either cottonseed meats or cottonseed meal and to rupture any residual resin glands containing gossypol (2, 3). Then the sample is heated with aniline, and the dianilinogossypol thus formed is extracted with chloroform and measured spectrophotometrically. This method may be used for cottonseed meals which either contain acidulated cottonseed oil foots or have been aniline-treated.

Reagents, Materials, and Equipment

Glacial acetic acid: reagent grade.

Ethanol 72% (by weight): dilute 830 ml. of 95% ethanol to 1,000 ml. with distilled water after adding 0.2 ml. of glacial acetic acid (9).

Aniline: freshly distilled, water-white.

Chloroform: U.S.P. grade.

Either pure gossypol or pure dianilinogossypol for standardization: dianilinogossypol may be obtained by extracting cottonseed meats with ether, removing the ether and precipitating dianilinogossypol with aniline. Dianilinogossypol is purified by recrystallization from either boiling benzene or chloroform containing a small amount of aniline. The purified crystals are washed with ether, air-dried, and then dried 12 to 16 hrs. in a vacuum oven at 50°C.

Asbestos: medium fiber.

Glass beads: 6 mm. in diameter.

Hyflo Super-Cel: remove traces of iron, which react with gossypol, by boiling 100 g. of Hyflo Super-Cel with 600 ml. of distilled water and 50 ml. of concentrated hydrochloric acid for 10–15 min. and filter on a large Buchner funnel. Wash well with distilled water. Repeat the process, dry, and pulverize to a powder.

Filter tube: Corning 9480 or similar.

Bell jar: a jar with an aperture in top, Corning 95470, or similar; perforated porcelain disk or plates, diameter 22 mm.

Wash bottle: equipped with a back-pressure check-valve for dispersing chloroform.

Mechanical shaker: conventional type.

A Waring Blendor may be used in place of the shaker for the extraction, small jar with screw cap. Place an aluminum foil liner inside the screw-cap over the regular washer by pulling the foil over the top of the jar and screwing the wet top over it, then fold the edges of the foil over the outside of the lid. Remove the screw-cap containing the liner, and punch a hole approximately 3 mm. in diameter through the center of the cap from the inside to serve as an air vent. The bearing in the jar assembly should be lubricated after about 15 determinations.

Spectrophotometer: A Beckman Model DU was used in this study. Any other good spectrophotometer or photoelectric colorimeter should be satisfactory, after establishing a standard curve for the instrument.

Standard Calibration Curve

A standard optical-density-concentration curve for pure gossypol as the aniline derivative in chloroform was prepared as follows. First, 25 mg. of pure gossypol were dissolved in chloroform, diluted to 100 ml. with chloroform, and mixed. A 10-ml. aliquot of the standard was transferred to a 100-ml. volumetric

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flask, diluted to volume with chloroform, and mixed. Aliquots of 2, 4, 5, and 7 ml. of this solution, equivalent to 0.050, 0.100, 0.125, and 0.175 mg. gossypol, respectively, were transferred to 25-ml. volumetric flasks. The aliquots were heated on a water bath regulated to a temperature slightly below boiling for 40 min. after adding 0.5 ml. of freshly distilled (water-white) aniline in order to convert the gossypol to dianilinogossypol. The solutions were allowed to cool, diluted to 25 ml. with chloroform, and mixed. The optical density was determined at 440 mμ on the Beckman Model DU spectrophotometer, using chloroform as a reference solution. Optical density plotted against concentration expressed as mg. of gossypol/25 ml. of solution gave a straight line (Figure 1), indicating that the standards conformed to Beers' law. The $E^{1\%}_{1cm.}$ value for gossypol as the dianilino derivative determined on the Beckman spectrophotometer at 440 mμ is 821.2.

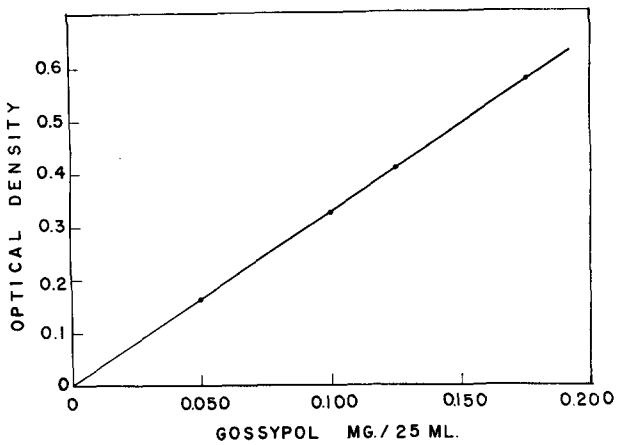

FIG. 1. Calibration curve of gossypol as the aniline derivative in chloroform.

Inasmuch as pure gossypol may not be readily available, an alternate method for the preparation of the standard optical-density-concentration curve is to dissolve 32.3 mg. of purified dianilinogossypol, which is equivalent to 25 mg. of gossypol, in chloroform and dilute to 100 ml., then mix. Transfer 10 ml. of the solution to a 100-ml. volumetric flask, add 2 ml. of freshly distilled aniline, dilute to volume with chloroform, and mix. Transfer aliquots, as previously indicated, to 25-ml. volumetric flasks, dilute to volume with chloroform, mix, and read the optical density, using chloroform as a reference solution. This optical-density-concentration curve is equivalent to the one prepared from pure gossypol, as evidenced by an $E^{1\%}_{1cm.}$ value of 826.0 at 440 mμ.

Analytical Procedure

Cottonseed meal: place 0.5000 g. of cottonseed meal or cottonseed meats, ground to 40 mesh, in a 250-ml. glass-stoppered flask, add 2 ml. of the prepared 72% ethanol, and allow to stand for 10 min. at room temperature. Thoroughly mix 2 ml. of freshly distilled aniline with the sample and, with stopper removed, place on the metal top or porcelain covers of a water bath for 45 min. with the steam regulated to keep the water in the bath slightly under boiling. This operation, which results in the formation of dianilinogossypol, should be carried out under a hood.

Meanwhile prepare the filter by placing the perforated porcelain disk in the filter tube inserted through a rubber stopper in the aperture at the top of the bell jar. With vacuum applied, pour a suspension of asbestos in water through the filter tube to form a layer over the porcelain disk. Follow with Hyflo Super-Cel suspended in 95% alcohol to form a layer of Hyflo Super-Cel about 2 mm. thick and to remove the water from the filter. Wash the filter with a few ml. of chloroform to remove the alcohol. It is necessary to have the filter free of water to prevent turbidity in the filtrate. The filter may be used repeatedly by removing the residue with a small spatula.

Add 60 ml. of chloroform to the flask containing the aniline-treated sample, swirl to mix, and insert the stopper tightly with a twisting motion to prevent it from being expelled by internal pressure. Place the flask on a mechanical shaker, and shake with sufficient vigor to keep the sample washed down from the walls of the flask for 15 min. Remove the stopper, and rinse it with chloroform dispensed from a fine-tipped washed bottle. Filter the extracted mixture under 10-to-15-in. vacuum through the prepared filter, receiving the filtrate in a 100-ml. volumetric flask placed under the bell jar. Rinse the flask with chloroform dispensed from a wash bottle, and pour the washings through the filter. Wash the filter with several small portions of chloroform, then dilute to 100 ml. with chloroform, and mix.

(In lieu of shaking, the samples may be treated with alcohol and aniline in 50-ml. beakers and then transferred to the Waring Blendor jar with 60 ml. of chloroform, blended for 3 min., and filtered.)

Transfer 2 ml. of the filtrate to a 25-ml. volumetric flask, dilute to volume with chloroform, and mix. Read the intensity of the color on a spectrophotometer or photoelectric colorimeter at 440 mμ, using chloroform as a reference solution. The weight of gossypol, in mg., in the aliquot diluted to 25 ml. for reading may be scaled from the standard curve or calculated from the a value derived from the optical-density-concentration relationship. The mg. of gossypol in the 25 ml. of solution times 10 gives the total gossypol, in percentage, when the foregoing sample size and dilutions are used.

Cottonseed meats: extract 0.2500 g. of cottonseed meats (ground to about 30 mesh), as described for cottonseed meal, except that 40 to 50 glass beads are placed in the flask to complete the comminution of the sample while shaking with chloroform for one hour. An aliquot of 2 to 5 ml., diluted to 25 ml. with chloroform, is usually satisfactory for reading the color density.

Gossypol values are slightly higher from a 0.25-g. than from a 0.5-g. sample of cottonseed meats. This does not seem to be the case with cottonseed meal. The uncooked cottonseed meats appear to hold a minute amount of gossypol, as indicated by a slight yellow color which was more marked for the larger sample. The yellow color in the residues could not be removed by shaking again with chloroform. It could be removed however by treating with aniline a second time and then shaking with chloroform. About 0.01 to 0.02% gossypol was obtained by the second extraction. These observations indicate that some component or components of the cottonseed have an affinity for gossypol almost as great as that of aniline. This point warrants further study.

The coarsely-ground cottonseed meats are thor-

oughly comminuted by shaking with beads for one hour. The mean value for 24 samples shaken one hour was only 0.006% higher than that for the same samples shaken 30 min. The difference between values obtained by the proposed method and the A.O.C.S. Method Ba 8-55 were of the order shown for cottonseed meal. The values by the former were higher without exception.

In the development of this method various levels of alcohol and of aniline and different periods of heating were used in the extraction to determine the conditions necessary for the complete extraction of total gossypol from samples of cottonseed meal. The data in Table I indicated that 2 ml. of 72% ethanol and 2 ml. of aniline were adequate to obtain the maximum amount of gossypol. Heating for at least 45 min. on the water bath was required to drive off the alcohol and water to give a clear solution when the meals were extracted with chloroform. Under these conditions uniform and consistent results were obtained.

TABLE I

The Effect of Alcohol, Aniline, and Time of Heating on the Estimation of Total Gossypol in Cottonseed Meal

Cottonseed meal	Determination No.	Treatments			Appearance of solution	Gossypol content
		Alcohol	Aniline	Heat		
		ml.	*ml.*	*min.*		%
A	1	2	1	20	Turbid	1.38
	2	2	1	20	Turbid	1.37
	3	1	1	45	Clear	1.40
	4	1	1	45	Clear	1.30
	5	2	2	45	Clear	1.46
	6	2	2	45	Clear	1.46
	7	2	2	45	Clear	1.43
	8	2	2	45	Clear	1.45
B	1	2	1	30	Turbid	1.50
	2	2	1	30	Turbid	1.38
	3	2	1	45	Clear	1.48
	4	2	1	45	Clear	1.56
	5	1	1	45	Clear	1.53
	6	1	1	45	Clear	1.51
	7	2	2	45	Clear	1.59
	8	2	2	45	Clear	1.59
	9	2	2	45	Clear	1.60
	10	2	2	45	Clear	1.57

It was found that the chloroform-cottonseed meal extract mixture could be filtered through a plug of glass wool, which was readly washed free of dianilinogossypol by small portions of chloroform. The filtrate and washings collected in 100-ml. volumetric flasks were diluted to 100 ml. and mixed. A portion of this solution was filtered through Whatman No. 4 filter paper into a 50-ml. volumetric flask, after discarding the first few milliliters to pass through the filter. The funnels were covered with watch glasses during filtration to prevent evaporation.

Filtration under vacuum through the filter tube placed in the top of a bell jar has been the system generally used because it is rapid and is not affected by evaporation of the solvent or the presence of the extracted residue since the solution is made to volume after the filtration is completed. Nevertheless values obtained from extracts filtered through glass wool and then through paper have been satisfactory.

The proposed method was applied to meals containing 3% added acidulated cottonseed soap stock. Satisfactory results were obtained by reading against chloroform. At higher levels of foots, or with highly colored products, a chloroform extract of the sample should be used, and in the case of aniline treated meals must be used, for the reference blank.

Comparative Analysis of Cottonseed Meals by Different Procedures

A. *Proposed Method vs. "p-Anisidine" Method.* Data obtained from the analysis of six cottonseed meals are presented in Table II. Results, by the proposed method, from extraction by the Waring Blendor are presented in column under Blendor, and those from extraction by shaking are presented in column under Shaker. The samples, after the alcohol-aniline treatment, were shaken for one hour with 65–70 ml. of chloroform, then filtered and diluted. The optical density was determined as previously described.

Results from the analysis of the same six cottonseed meals by the method of Pons *et al.* (6) are shown under "p-anisidine."

Duplicate samples of each meal, Table II, were extracted by the Blendor, the shaker, and the p-anisidine method, and the extracts of the former two were analyzed by the proposed method and the latter by the p-anisidine method.

Statistical analysis of the data in Table II showed that the difference between the Blendor and shaker extractions with chloroform was not significant at the 5% level, but the mean value for the meals by the p-anisidine method was significantly lower than the value obtained by the proposed method.

B. Proposed Method vs. A.O.C.S. Method Ba 8-55. Six samples of cottonseed meal, Nos. 7–12, were analyzed for total gossypol by the proposed method. These meals were extracted by shaking with approximately 60 ml. of chloroform after they had received the alcohol-aniline treatment. The effects of time of shaking, methods of filtration, acidulated foots, and reading on Evelyn colorimeter *vs.* Beckman DU spectrophotometer were studied. Comparative values were obtained by the A.O.C.S. Tentative Method Ba 8-55 (1) for total gossypol.

The statistical analysis of these data showed that

TABLE II

Total Gossypol in Cottonseed Meal Determined by the Proposed and the p-Anisidine Methods

Cottonseed meal No.	Determination	Effect of extraction method[a]		p-Anisidine
		Blendor	Shaker	
		%	%	%
1	a[b]	1.358	1.361	1.294
		1.358		1.279
	b	1.358	1.370	1.268
		1.358		1.265
2	a	1.306	1.325	1.184
		1.325		1.181
	b	1.318	1.312	1.166
		1.321		1.163
3	a	1.095	1.067	1.039
		1.104		1.033
	b	1.095	1.080	0.969
		1.095		0.969
4	a	1.352	1.312	1.253
		1.349		1.253
	b	1.352	1.331	1.265
		1.349		1.265
5	a	1.355	1.355	1.242
		1.352		1.233
	b	1.382	1.358	1.236
		1.385		1.236
6	a	1.219	1.198	1.143
		1.222		1.143
	b	1.225	1.188	1.143
		1.234		1.152
Mean	....	1.286± 0.013[c]	1.271± 0.008	1.182± 0.029

[a] By proposed method.
[b] The two values for *a* and *b* indicate two aliquots were read from each extract.
[c] Standard deviation per duplicate determination.

there was no significant difference between shaking 15 and 60 min., Table III, or filtering under vacuum or through glass wool, then through paper. There were no significant differences noted when 3% acidulated cottonseed foots were added to the sample, or values obtained by the Beckman DU spectrophotometer or Evelyn colorimeter. Values obtained by the proposed method were higher than those by the A.O.C.S. Tentative Method Ba 8-55 to a highly significant degree.

TABLE III

Factors Affecting the Estimation of Total Gossypol Mean Values

	Mean		L.S.D..05	
	%	%		
Alcohol-aniline-chloroform method				
Shaking time, 15 *vs.* 60 min. (respectively)	1.075	1.078	0.005	N.S.[a]
Glass wool *vs.* vacuum	1.082	1.077	0.014	N.S.
Beckman DU vs. Evelyn colorimeter	1.073	1.079	0.015	N.S.
Foots *vs.* no foots	1.073	1.082	0.012	N.S.
Alcohol-aniline-chloroform *vs.* A.O.C.S. Ba 8-55	1.080	0.921	0.015	H.S.[b]

[a] Nonsignificant.
[b] Highly significant.

Results obtained from aniline-treated meals are shown in Table IV. These data show that the amount of gossypol converted to aniline gossypol by the aniline processing treatment of the meal may be obtained from the chloroform extract blank by reading against chloroform as the reference solution. This value subtracted from the value obtained from the alcohol-aniline treated sample is the bound gossypol content. The addition of the 2 ml. of 72% alcohol and heating on the water bath, then extracting with chloroform, gave lower values for the gossypol present as the aniline compound, 0.292 *vs.* 0.331%, respectively. This was probably caused by the hydrolysis of aniline gossypol and the combination of the liberated gossypol with the protein of the meal.

Specificity of the Proposed Method

Specificity of the proposed method was established by comparing the spectral-absorption curves as $E^{1\%}_{1\,cm.}$ values of the dianilinogossypol extracted from the respective meals with that obtained with pure dianilinogossypol dissolved in chloroform. The excess aniline was removed from the cottonseed meal extracts by washing 5 ml. of the solution in a separatory funnel, after adding about 10 ml. of chloroform, successively with 1.0, 0.5, 0.2, and 0.1 ml. of glacial acetic in 25 ml. of water and then four times with 25 ml. of water. The chloroform solutions were dried with anhydrous sodium sulfate and read vs. chloroform. The $E^{1\%}_{1\,cm.}$ values for the various wavelengths were calculated from the amount of gossypol found in the washed and dried chloroform solution by reading the optical density as described by the proposed method. Spectral curve A, Figure 2, is the mean of six individual curves, one each for samples 7 through 12. This curve is very similar to curve B obtained from pure dianilinogossypol. The differences were slight in the ultraviolet region with the maxima and minima occurring at the same wavelengths and the curves coincide above 400 mμ. These data indicate that the material read by the proposed method is dianilinogossypol, with the possibility of the presence of a very small amount of an aniline compound of a gossypol derivative. Consequently the values obtained should be considered as total gossypol.

TABLE IV

Determination of Gossypol in Aniline-treated Cottonseed Meal

Meal No.	Determination No.	As aniline cpd.	As total	As bound
		%	%	%
13	1	0.326	1.187	0.861
	2	0.335	1.211	0.876
14	1	0.366	1.129	0.763
	2	0.356	1.120	0.764

Residues of meals Nos. 7–12 extracted by A.O.C.S. Tentative Method Ba 8-55 (1) were combined, placed on a Buchner funnel, and washed free of liberated gossypol with 70% acetone. Then the residues were treated on the water bath with 5 ml. of 72% alcohol and 2 ml. of aniline, then shaken with chloroform for one hour, filtered, and diluted to 100 ml. Five ml. of this solution were washed free of aniline and dried; the spectral curve, expressed as $E^{1\%}_{1\,cm.}$, was determined on a Beckman DU spectrophotometer. This curve is similar to those of dianilinogossypol, indicating that all of the gossypol or gossypol derivative was not extracted by the Ba 8-55 method.

This observation is substantiated further by extracting the residues from the Ba 8-55 method, washed free of liberated gossypol with 70% acetone, by the alcohol-aniline-chloroform method and by removing the chloroform under reduced pressure to about 0.5 ml. When the residue was taken up in about 20 ml. of hexane and passed through a column packed with equal parts of magnesium oxide and Hyflo Super-Cel, a band formed at the top of the column similar to

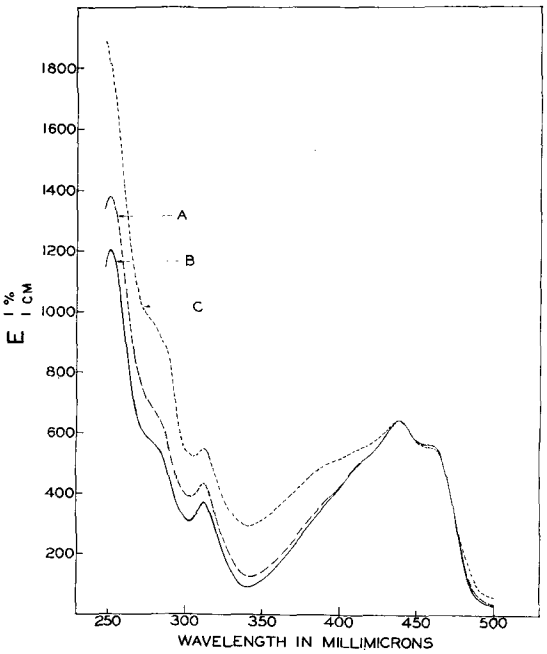

FIG. 2. Spectral absorption curves of dianilinogossypol in chloroform.
A. Mean curve of dianilinogossypol extracted from one sample each of cottonseed meals Nos. 7 to 12 by proposed method.
B. Pure dianilinogossypol.
C. The alcohol-aniline-chloroform extract of residues from the A.O.C.S. Ba 8-55 method after washing out the liberated gossypol with 70% acetone.

that formed by a hexane solution of pure dianilinogossypol under similar conditions.

Reagent blanks included with 14 sets of determinations by the proposed method gave an average density of 0.0015 on the Beckman DU spectrophotometer. The O.D. range was −0.002 to 0.004. These values are sufficiently low that it is not necessary to run reagent blanks with each set of determinations.

Samples of 0.5000 g. of meals Nos. 7 to 12 were treated with 2 ml. of alcohol, extracted with chloroform, and diluted as directed, then read on the Beckman DU spectrophotometer. The mean O.D. was 0.0047 with a range of 0.003 to 0.007. These low values are the justification for using chloroform as the reference solution. The extract may be used as a reference solution to correct for the low O.D. obtained from the chloroform extract when the ultimate in accuracy is required.

There were no significant differences in the results obtained with the Beckman DU spectrophotometer and the Evelyn colorimeter on meals Nos. 7 to 12 by the proposed method, Table III. These data indicate that instruments such as the Evelyn colorimeter give accurate results by this method, and any good photoelectric colorimeter or spectrophotometer should give satisfactory results after establishing a calibration curve.

Along with simplicity, greater accuracy, and a high degree of precision the proposed method offers the advantage of a greatly reduced time requirement as compared to methods previously used. An analysis may be completed in about 1.5 hrs.

Summary

An improved method has been developed for the determination of total gossypol in cottonseed and cottonseed meal. The sample is heated with aniline to convert the gossypol to dianilinogossypol, which is extracted with chloroform and measured spectrophotometrically. The values for total gossypol are slightly higher and more accurate and precise as determined by the proposed method because of more complete extraction than by a recent p-anisidine method or the revised A.O.C.S. Tentative Method Ba 8-55. The advantages of the proposed method are its simplicity, accuracy, reproducibility, and expeditiousness.

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Fat Emulsions. Effect of Polyoxyethylene and Alkyl Content of Emulsifiers on Stability to Sterilization[1,2]

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IN DISCUSSIONS of the theoretical and practical aspects of emulsions as found in the extensive literature of the subject, for example, the book by Berkman and Egloff (1), the role of the specific influence of emulsifying agents on certain properties of systems of oil (vegetable or hydrocarbon) and an aqueous phase in constant proportion has received little attention. Sherman (2) reports the influence of the emulsifying agent on the viscosity of water-oil emulsions at room temperature, stating that he found but few previous observations on the specific influence of emulsifiers. Broughton and Squires (3) report a similar study with oil-water systems, in which the effect of the type of stabilizer on the viscosity was determined. These two reports, concerned with tests made at room temperature, constitute the only available information as to the effect of various emulsifiers on the properties of emulsions.

In the development of fat emulsions for intravenous alimentation, one requirement is that such emulsions must be stable under conditions required for sterilization, namely 121°C. for 10–30 min. In the literature reports cited above, no reference is made to the characteristics of fat emulsions at elevated temperatures. Heat, in fact, is a means of demulsification in a standard test for emulsion stability (4). Differences in the characteristics of emulsions at ordinary temperatures and at 121°C. therefore are to be expected and actually were found. These differences most probably are caused by variations in interfacial tension, solubility, or other phenomena involving the emulsifying agents. Emulsifiers of the nonionic type are soluble in water because of association of their hydrophilic groups with the water molecules. As the temperature of such solutions increases, the degree of association decreases until at a critical temperature the point of solubility inversion is reached and turbidity follows. Benerito and Singleton (5), in determining the effect of heat on the solubility of hydrophilic emulsifiers, found that the critical temperature at the point of solubility inversion of nonionic emulsifiers in water is highest for those emulsifiers which are very hydrophilic. As the content of hydrophilic polyoxyethylene groups in

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