TABLE IV

Effect of Temperature of Wash Solvent on Yield and Quality of Fractions

Crystallization temperature: 23°F.
Solvent: Methyl ethyl ketone
Washing procedure: Three ½-lb. washes

Test No.	Wash Temp., °F.	Solid Fraction		Liquid Fraction + Washes				
				Yield Filtr. + Washes	Iodine Value			
		Yield	Iodine Value		Filtr.	+ Wash No. 1	+ Washes Nos. 1, 2	+ Washes Nos. 1, 2, 3
G	+15 to +17	32.0	28.3	68.0	66.4	65.8	65.5	65.2
H	−20 to −10	32.0	28.7	68.0	67.7	67.7	67.6	67.5

Summary and Conclusions

The work thus far completed establishes some of the conditions required for fractionation of white grease by crystallization from five selected solvents. It has been shown that satisfactory fractionation is possible with any of these solvents by controlled crystallization from solutions containing one part of white grease to five parts of solvent by weight. For each solvent the effect of crystallization temperature on the yield and quality of the liquid fraction (as indicated by titer, pour point, and iodine value) has been determined. Preliminary information has been obtained regarding the effects of wash solvent volume, wash solvent temperature, and holding-time at crystallization temperature. These data apply only to water-free solvents. The presence of relatively small amounts of water in acetone, of the order of 2%, reduces its solubility for white grease to such an extent that the grease is separated from the solution as a liquid phase at temperatures above the point where fractionation occurs. The other solvents, methyl ethyl ketone, methyl isobutyl ketone, isopropyl acetate, and ethyl acetate, offer the advantage of separating water in excess of about 2 to 10% while retaining the grease in solution.

Before a choice of solvent can be made for pilot-plant investigation, it is evident that additional research will be required. It is planned to extend the present work in the prepilot-size crystallizer to evaluate the fractionating efficiency, in the white grease process, of these solvents saturated with water. Operations on a larger continuous pilot-plant scale will be required subsequently to determine the final choice of solvent, to obtain data for cost estimates and design, and to prepare quantities of various liquid fractions for commercial evaluation.

Acknowledgment

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An Improved Procedure for the Purification of Gossypol

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WORKERS in this laboratory, needing to prepare samples of gossypol for use by collaborating laboratories in pharmaceutical and nutritional studies, found the previous methods to be time-consuming, requiring as many as 17 recrystallizations to obtain gossypol of high purity (2-9, 10-13, 15-18, 20-24). An investigation was undertaken therefore to develop a more rapid procedure that would yield gossypol of high purity.

There resulted the improved procedure described herein, which is based on the formation of the gossypol-acetic acid complex, dissociation of the complex in alkaline solution, and recrystallization of gossypol from a mixture of diethyl ether and xylene. This procedure is rapid and yields gossypol of high purity as indicated by the tests of purity whose application is described.

Preparation of Gossypol-Acetic Acid

It was pointed out by Carruth (9) that gossypol in solution in organic solvents combines with acetic acid in the presence of an excess of the acid to form a crystalline compound consisting of one molecule of acid and one of gossypol.

Gossypol was recovered as gossypol-acetic acid from portions of the butanone extract of cottonseed flakes which were used in preparing 3,000 lb. of cottonseed meal for nutritional investigations.[2] The concentrated extracts, obtained from 85-lb. batches of cottonseed flakes, were filtered and the volume reduced to 1,750 ml. by evaporation under vacuum in the laboratory. The crude acetic acid complex, precipitated by the addition of 850 ml. of glacial acetic acid, was allowed

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[2] Oil-free (hexane-extracted) flakes were re-extracted with butanone in a batch extractor by the Engineering and Development Division (19). When the system was operated under atmospheric pressure, much of the gossypol was decomposed; but at low temperatures, under reduced pressure, recovery was satisfactory. Approximately 39% of the gossypol in the flakes was extracted in 4 hours.

to stand for at least 24 hours, was collected on a Büchner funnel, and was thoroughly washed with petroleum ether. One portion was purified by recrystallizing 5 times from acetone and acetic acid; the crystals were thoroughly washed with petroleum ether and were dried in a vacuum desiccator over soda-lime and paraffin. This portion of the gossypol-acetic acid was used in the following studies to develop a method of determining the extent of dissociation.

Method of Determining Extent of Dissociation

In the development of methods of dissociating this complex to obtain pure gossypol the problem arises of developing also a method of judging the extent of dissociation that may be effected by a particular procedure.

In the present work titration of combined aqueous extracts from an ether solution of gossypol-acetic acid with standard alkali, using phenolphthalein as indicator, was found to have sufficient precision for use. Other methods investigated, which were not so satisfactory, included titration of the aqueous layer of a mixture of ether, water, and gossypol-acetic acid with standard alkali, using a glass-calomel electrode system to indicate the equivalence point; and back-titration with standard hydrochloric acid of an aqueous solution of gossypol-sodium and sodium acetate formed by solution of gossypol-acetic acid in a slight excess of dilute sodium hydroxide.

The selected method is carried out as follows. Approximately 1 g. of the preparation to be tested is accurately weighed and transferred to a separatory funnel with 100 ml. of washed ether. This solution is then extracted with 25-ml. portions of boiled distilled water until the pH of the final extract registers 5.5 to 7.0. Thirteen to 15 extractions may be necessary if a large proportion of undissociated acetic acid is in the sample. Each extract is filtered successively through a small portion of cotton. The extracts are combined, and any acidity present is titrated with N/10 sodium hydroxide, using phenolphthalein as the indicator. The acidity is calculated as percentage of acetic acid by weight, and this value is multiplied by 9.62 to obtain the percentage of undissociated gossypol-acetic acid in the sample.

Methods of Dissociation of Gossypol-Acetic Acid

Five different procedures for dissociation of gossypol-acetic acid were investigated. These included 3 reported in the literature (6, 9, 16), crystallization from water-free xylene, and crystallization from a mixture of diethyl ether and petroleum ether.

In the fourth method the crystals which separated upon cooling a solution of the complex in hot, water-free xylene were free from acetic acid. This breakdown of the complex without water was the basis for use of the term dissociation, rather than hydrolysis, in reporting the work.

A sixth method was developed in which the dissociation of the complex and the purification of gossypol were carried out as one procedure. In this rapid procedure gossypol-acetic acid is dissociated with aqueous sodium hydroxide, and the precipitated gossypol is then recrystallized from a mixture of diethyl ether and xylene.

Table I shows the comparative effectiveness of the six methods in dissociating gossypol-acetic acid. The method of evaluating the effectiveness was that described above.

TABLE I

Effectiveness of Different Methods of Dissociation of Gossypol-Acetic Acid

Method and Reagents	Undissociated Gossypol-Acetic Acid
	% by wt.
Ether solution evaporated once over water (9)[a]	13.8 0.0
Ether solution evaporated twice over water (6)[a]	0.0
Suspension in water and heating with stirring 1 hr. at 65°C.	78.4
Heating in water at 100°C. for 15 min. (16)[a]	68.9
Crystallization from xylene	0.0
Crystallization from diethyl ether and petroleum ether of partly dissociated gossypol-acetic acid (No. 3 above)	
1st crystals	0.9
2nd crystals	2.1
3rd crystals	0.0
Residue	17.6
Twice dissolved in N/1 NaOH containing 50 mg. of sodium hydrosulfite per g. of gossypol-acetic acid, ppt. with HCl, dissolved in ether and crystallized from mixture of ether and xylene	0.0

[a] Reference No. at end.

The table shows that when gossypol-acetic acid was dissolved in aqueous sodium hydroxide, containing sodium hydrosulfite, then precipitated with hydrochloric acid, extracted with ether, and recrystallized from a mixture of ether and xylene, the gossypol-acetic acid complex was completely dissociated. This rapid procedure was adopted for use in the purification of additional lots of gossypol.

Detailed Procedure for Preparation of Pure Gossypol

For each gram of crude gossypol-acetic acid, use 7.5 ml. of normal sodium hydroxide solution, 50 mg. of a good grade of sodium hydrosulfite ($Na_2S_2O_4$), and 15 ml. of solvent-grade ether. Dissolve the hydrosulfite in the sodium hydroxide solution, add ether, and stir the mixture to destroy peroxide. Then add the solid gossypol-acetic acid and continue stirring until the solid is dissolved in the aqueous layer. Alkaline solutions of gossypol oxidize readily on exposure to the air. To minimize oxidation the rate of stirring should be regulated to avoid the formation of a film of gossypol-sodium on the side of the container above the ether layer. Add normal hydrochloric acid slowly, with mild agitation, until all of the gossypol is precipitated, as indicated by the change in color of the aqueous layer. Continue the stirring for a few minutes and transfer the mixture to a separatory funnel; shake until nearly all of the gossypol is dissolved in the ether layer. Then draw off and retain the aqueous layer. To a suitable filtering flask add 9 ml. of xylene, ACS grade (b.p. 137°-140°C.), for each gram of the starting material. Decant the ether layer into the flask through a folded filter paper (S&S No. 588 or its equivalent)[3] which had previously been half filled with anhydrous sodium sulfate. Use glass beads to prevent bumping and close the flask with a stopper carrying a thermometer extending into the liquid but not touching the bottom of the flask. Evaporate the ether under reduced pressure (not more than 25 mm.) on a steam bath, shake the flask occasionally during the distillation and frequently toward the end. Do not allow the temperature of the liquid to rise above 40°C. When crystals begin to form in the liquid, remove the flask from the steam bath and shake continuously during the crystallization. Now allow the

TABLE II

Tests of Purity of Gossypol Preparations

Sample No.	Volatile matter	Residual solvent	Extinction coefficients in cyclohexane[a]			Percentage of gossypol based on	
			358 mμ	268 mμ	236 mμ	Extinction coefficient at 358 mμ[a]	Alkalimetric titration[a]
	%					%	%
1	0.8	None	40.2	71.5	216.1	100.7	98.4
2	0.5	None	40.2	72.2	212.3	100.7	100.7
3	0.8	Faint odor xylene	39.3	69.9	206.3	98.5	98.7
4	0.8	None	39.5	70.7	203.2	99.0	100.9
5	1.2	None	39.8	70.9	216.3	99.7	101.4
6	1.3	None	39.9	71.3	211.5	100.0	101.5
7	1.9	None	39.9	71.3	213.5	100.0	100.3
8	5.3	Turbid odor xylene	39.3	70.5	213.5	98.5	102.0
9	0.3	None	39.8	70.4	211.0	99.7	102.4
Average	1.4	....	39.8	71.0	211.5	99.7	100.7

[a] On dry basis.

flask and contents to stand for about 10 minutes. Rinse the crystals from the flask into a Büchner funnel, using Whatman No. 2[3] paper or its equivalent, with two very small portions of cold (about 5°C.) xylene; and, finally, wash the flask and filter with several portions of petroleum ether until the washings are colorless. For further purification a second crystallization should be conducted in which the following modifications are made. Add 4 instead of 9 ml. of xylene for each gram of the original gossypol-acetic acid and rinse the crystals out of the flask with petroleum ether instead of xylene. Wash the crystals 3 times by transferring them to a beaker and stirring them into a paste with petroleum ether. Filter and wash with the same solvent.

Break the cake of final crystals into pieces about 3-5 mm. across, spread them on a watch glass, and dry them overnight in a vacuum oven at 50°C. The second set of crystals is usually quite pure, and an additional recrystallization is usually not required. The crystals may be brought into equilibrium with the air by allowing them to stand in an open container out of contact with light for 12 hours. Store the crystals under refrigeration in a closed container out of contact with light.

The yield of the purified gossypol, based on the amount of gossypol in the starting material, is approximately 56%.

If it is desired to recover the waste gossypol, rinse the containers used in the purification with ether and add the rinsings to the aqueous extracts. Warm this mixture on the steam bath to evaporate the ether and filter to recover the solid crust of impure gossypol on the surface of the water. Extract gossypol from the xylene filtrates with normal sodium hydroxide solution containing sodium hydrosulfite. Add solid gossypol from the aqueous extracts to the sodium hydroxide solution, reprecipitate the gossypol with hydrochloric acid, and recrystallize it by the procedure used in the second crystallization. A maximum recovery is obtained by adding approximately 2 volumes of petroleum ether to the xylene after the ether has been evaporated on the steam bath. Pure gossypol may be obtained by additional recrystallizations.

After the method for the preparation of pure gossypol was developed, it was found that gossypol-acetic acid of a high degree of purity can be prepared by following the rapid procedure for the purification of gossypol to the second crystallization stage. At this point a volume of glacial acetic acid equal to that of the xylene used is added with the xylene. The mass of crystals formed after evaporation of the ether under reduced pressure on the steam bath is washed first with 1:1 xylene-acetic acid mixture and finally with petroleum ether. Traces of solvent are removed by drying the sample under vacuum at 50°C.

Tests of Purity of Gossypol

The complex nature of the gossypol molecule, the variety of its crystalline forms, and its instability under certain conditions require that standards of purity and uniformity be set up for preparations which are intended for studies of chemical, physical, and pharmaceutical properties.

To establish such standards, 9 lots of gossypol (Table II) were prepared by the improved procedure. After microscopic examination each sample was tested for residual solvents, volatile matter, color in ether solution, water-soluble acids, and percentage of gossypol. The percentage of gossypol was determined by alkalimetric titration (Fig. 1) and by spectrophotometric extinction coefficients at a wave length of 358 mμ.

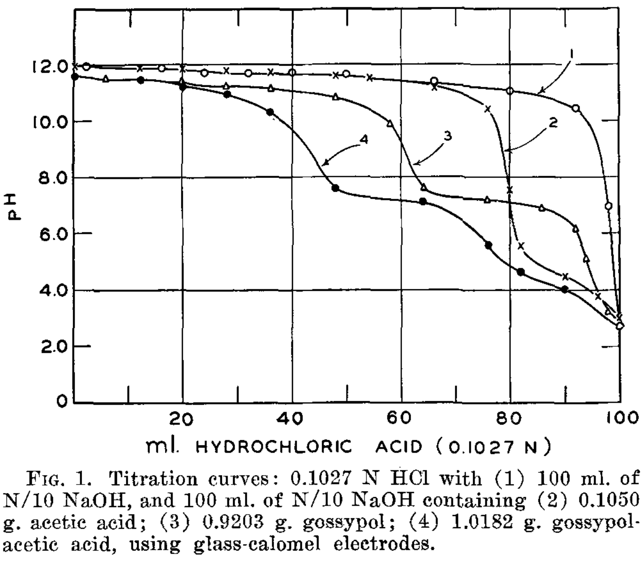

FIG. 1. Titration curves: 0.1027 N HCl with (1) 100 ml. of N/10 NaOH, and 100 ml. of N/10 NaOH containing (2) 0.1050 g. acetic acid; (3) 0.9203 g. gossypol; (4) 1.0182 g. gossypol-acetic acid, using glass-calomel electrodes.

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[3] The mention of firm names or trade products does not imply their endorsement by the Department of Agriculture over other firms or products not mentioned.

In addition to the tests on all 9 lots, one lot (No. 6) was selected for elementary analyses, the determination of characteristic spectral data (Fig. 2), and for molecular weight determinations.

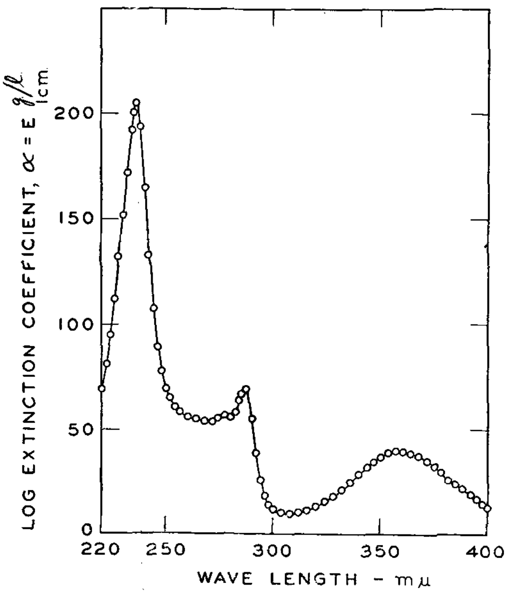

FIG. 2. Spectrophotometric curve for gossypol purified by the rapid procedure described in this paper.

Microscopical examination of all 9 lots showed that gossypol obtained by the improved procedure consisted of small, pale-yellow, rod-like crystals free from visible extraneous material. The results of the tests for volatile matter, residual solvent, and percentage of gossypol by spectrophotometric determination are given in Table II.

To test for xylene and other residual solvents, 0.1 g. of the sample was dissolved in 10 ml. of normal sodium hydroxide solution. The odor of traces of xylene can be detected, and the solution becomes turbid when an appreciable amount is present. Xylene was found in lots 3 and 8.

To determine volatile matter, 1 g. of the sample was dried for 16 hours in a vacuum oven at 50°C. Volatile matter did not exceed 1.9% except in lot No. 8, which also contained xylene and may not have been recrystallized or dried properly.

In 1.0% ether solution the preparations were pale yellow in color and were free from ether-insoluble matter and water-soluble acid.

The determination of water-soluble acids was made by dissolving the 1-g. samples from the volatile matter determinations in 100 ml. of peroxide- and acid-free ether, and extracting the solution with 10-ml. portions of boiled, distilled water until the last extract ranged from pH 5.5 to 7.0. Only one drop of N/10 sodium hydroxide was required to neutralize the combined extracts to the phenolphthalein end-point.

The extracted ether solutions were used in determining gossypol content of the 9 samples by alkalimetric titration.

In Figure 1, curves 1 to 4 show changes in pH occurring when sodium hydroxide alone (curve 1), sodium hydroxide containing acetic acid (curve 2), gossypol (curve 3), and gossypol-acetic acid (curve 4) are titrated with hydrochloric acid. A comparison of the curves shows that gossypol and acetic acid are being titrated between pH 8.2 and 3.0. (pH 8.2 is the accepted stoichiometric equivalence point for the titration of organic acids such as acetic acid.)

The amount of gossypol in the ether solutions from the water-soluble acid determinations was determined by extracting the gossypol with N/10 sodium hydroxide and then determining the amount of N/10 hydrochloric acid required to lower the pH from 8.2 to 3.0 as measured by a glass-calomel electrode system. In carrying out the determinations the ether and sodium hydroxide solutions were agitated in a separatory funnel, the aqueous layer was filtered into a 250-ml. beaker, and the extraction continued with 10-ml. portions of distilled water containing 2 drops of N/10 sodium hydroxide until the aqueous layer remained colorless. All the extracts were combined and titrated. After subtracting the titration blank, the number of ml. of N/10 hydrochloric acid required in titrating 1 g. of gossypol (No. 6, extinction coefficient of 39.9) was 38.6. This is a measure of gossypol as well as of any other acidic pigments in the sample. The number of ml. of N/10 acid required per gram in titrating the 9 samples varied from 37.8 to 39.4. The percentage of purity was calculated by dividing the number of ml. of acid used by the number used in titrating the standard sample, that is, 38.6. The percentage of purity varied from 98.4 to 102.4% (Table II).

In measuring the degree of purity by the spectrophotometric procedure, specific extinction coefficients were determined in cyclohexane at wavelengths of 358, 286, and 236 mμ. A Beckman model DU quartz spectrophotometer was used. Pons and coworkers (20) considered the absorption at 358 mμ to be a measure of the binaphthyl group and to be satisfactory for the determination of gossypol. They obtained an average extinction coefficient of 39.9 on purified gossypol which they prepared. The percentage of purity is determined by dividing the extinction coefficient of the unknown by 39.9 and multiplying by 100. The values obtained on the 9 lots are shown in Table II and varied from 98.5 to 100.7%. The average extinction coefficient at 286 mμ was 71.0 and at 236 mμ was 211.5. The latter values are somewhat higher than those reported by Pons and others (20).

Sample No. 6 showed negative analyses for nitrogen and ash. Analytical values for carbon and hydrogen and a value for oxygen determined by difference were 69.55%, 6.11%, and 24.34%, respectively, as compared to 69.48%, 5.83%, and 24.68% calculated from the molecular formula ($C_{30}H_{30}O_8$). Values for these elements found for a specimen of gossypol-acetic acid, prepared by crystallization from xylene and acetic acid as previously described, were 66.42%, 5.92%, and 27.65%, respectively, as compared to the calculated values of 66.51%, 6.11%, and 27.38%.

In Figure 2 the logs of the extinction coefficients for sample No. 6 were plotted against wavelengths. The curve shows definite maxima at 236, 268, and 358 mμ, which are considered to be characteristic of gossypol (20).

As a final test of the high degree of purity obtained by the rapid method, the molecular weight of sample No. 6 and of gossypol-acetic acid were determined from the data used in plotting the titration curves in Figure 1 and by titrating the acetic acid extracted from gossypol-acetic acid.

In the first procedure the amount of acid required in titrating the sodium hydroxide solution of gossypol to pH 8.2 was subtracted from the amount used in titrating the same amount of alkali under the same conditions without the gossypol. The difference is a measure of the amount of sodium hydroxide reacting with the gossypol. Gossypol reacts as a dibasic acid. The value obtained for the molecular weight of gossypol (520) is in close agreement with the value (518.54) calculated from its molecular formula.

A similar procedure was used in determining the molecular weight of gossypol-acetic acid. Gossypol-acetic acid reacts as a tribasic acid. The molecular weight calculated from the titration data (578) is in close agreement with the theoretical value (578.5) calculated from the molecular formula for gossypol-acetic acid.

In the second procedure acetic acid was extracted from the gossypol-acetic acid by the procedure used for the determination of water-soluble acid. The acetic acid was titrated with sodium hydroxide solution to the phenolphthalein end-point. The molecular weight of gossypol-acetic acid calculated from these data is 577 and that of gossypol 517.

Standards of Purity

From these results the following standards of purity are suggested. Gossypol prepared by the improved procedure should consist of small, pale yellow, rod-like crystals free from extraneous material. Total volatile matter, including moisture, xylene, and other solvents, should not exceed 2% by weight. A 1-g. portion of the sample should dissolve in 20 ml. of ether, and no more than a trace of insoluble material should remain; when diluted to 100 ml., the solution should be pale yellow in color. The water-soluble acid extracted from the ether solution should be neutralized with one drop of N/10 sodium hydroxide solution. Total gossypol determined by alkalimetric titration may vary from 98 to 102% and by spectrophotometric measurement from 98.5 to 100.7%. The molecular weight of gossypol calculated from alkalimetric titration data should be approximately 518 and that of gossypol-acetic acid approximately 578.

Summary

Methods of dissociating the gossypol-acetic acid complex prepared from the butanone extract of defatted cottonseed flakes and methods of recrystallizing gossypol were studied. The study led to the development of an improved rapid procedure for the purification of gossypol.

In this procedure gossypol-acetic acid is dissociated with aqueous sodium hydroxide containing sodium hydrosulfite; the gossypol is precipitated with hydrochloric acid, extracted with ether, and recrystallized from a mixture of ether and xylene. Two crystallizations by this procedure gave gossypol free from acetic acid and of a high degree of purity. Gossypol-acetic acid of a high degree of purity was prepared by a modification of this procedure.

The degree of purity of the gossypol prepared by the improved procedure was determined by a number of tests, which are described. Spectral and titration curves for pure gossypol are presented, and standards of purity are suggested.

The molecular weight of gossypol and gossypol-acetic acid prepared by the procedures described is approximately 518 and 578, respectively.

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Structure and Aggregation in Dilute Solutions of Surface Active Agents[1]

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EXTENSIVE data have been published regarding the effect of structure of surface active agents on various properties such as detergency, bacteriostatic action, etc. These involve very dilute solutions, the concentrations of which are usually well below the critical micelle concentration (CMC). We shall be chiefly concerned in this report with solutions of somewhat higher concentrations and the manner in which structure of the surface active agents affects aggregation, i.e., micelle formation. In addition, these data are discussed in the light of recent concepts of micelle structure. Data presented below are taken in

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