Preparation of Gossypol From Cottonseed Pigment Glands[1]

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GOSSYPOL is recognized (1-6) as an excellent antioxidant, and its polyphenolic nature suggests (7) many applications of this compound. More recent investigations (8) have indicated possible pharmaceutical uses for this product of cottonseed. However, interest in the practical uses for gossypol has been limited because of the difficulty of obtaining the pigment in pure form. Attempts (9, 10) to synthesize gossypol or closely related derivatives have not yet been successful; consequently it can only be obtained by isolation from cottonseed or its derived products.

Gossypol was first prepared (11, 12) from cold-pressed cottonseed oil, but the isolation of gossypol from this source is difficult, and cold-pressing of cottonseed has largely been abandoned, particularly in the United States. Hydraulic- and screw-pressed cottonseed oils, prepared by pre-cooking the seed, contain so little gossypol (3, 13, 14, 15) that they do not represent a satisfactory source of this pigment. Direct extraction of gossypol from cottonseed or from defatted cottonseed meal (5, 14-29) is not practical for the production of gossypol on a large scale.

Cottonseed pigment glands are obtained as a by-product of oil and meal by the recently developed gland flotation process (30, 31). The gland flotation method is also applicable for the separation of pigment glands from solvent-extracted cottonseed meals when solvents such as hydrocarbons and chlorinated hydrocarbons of low moisture content have been used for extraction of the oil (30-34).

The content of pigment glands in seed of different species of cotton has been reported (35) to vary over a wide range and to be a characteristic of each species. Seed of different varieties of upland cotton, *G. hirsutum*, is intermediate between that of different varieties of *G. herbaceum*, which have a lower content of pigment glands, and that of different varieties of *G. barbadense*, which have a higher content of pigment glands. Seed of the four varieties of *G. hirsutum* most commonly grown in this country were found (34) to contain pigment glands varying in amount from 2 to 5% of the weight of the kernels.

During semi-pilot plant development (31) of the gland flotation process (30), 75 to 83% of the theoretical yields of pigment glands (7) were recovered in a relatively pure state. Gossypol was found (34) to constitute from 35 to 50% and gossypurpurin from 1 to 3% of the weight of the separated glands. Commercial application of the gland flotation process will make available relatively large quantities of cottonseed pigment glands containing most of the cottonseed pigments. For example, if the 3.2 million tons of cottonseed processed in the United States in 1945 by expression methods (36) had all been processed by the gland flotation method, there would have been produced approximately 65,000 tons of pigment glands containing approximately 25,000 tons of gossypol and 1,000 tons of gossypurpurin.

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Laboratory Preparation of Gossypol from Cottonseed Pigment Glands

The investigation being reported here was undertaken with the object of developing practical methods for extraction of gossypol from cottonseed pigment glands, and for isolation of gossypol from pigment gland extracts. For this purpose two lots of pigment glands produced during semi-pilot plant development (31) of the gland flotation process (30) were used. The pigment glands used in all of the experiments summarized in Table 1 were obtained by processing flaked cottonseed meats. These glands contained 34.9% of extractable gossypol as determined by the antimony trichloride-spectrophotometric method (37) using chloroform extracts or chloroform solutions of aqueous ethanol extracts (33, 34) of the pigment glands. The data listed in Table 2 present the results of independent preparations of gossypol from two lots of cottonseed pigment glands. It was found, as shown in Table 2, that the absorption spectrum of the antimony trichloride reaction product (37) furnished as reliable a criterion of the purity of various gossypol preparations as elementary analysis (7, 29). Consequently, the more readily applicable antimony trichloride reaction product was adopted in preference to elementary analysis as the criterion of purity for gossypol. The purity of the gossypol obtained by different procedures was calculated on the basis of the specific extinction coefficient of its reaction product with antimony trichloride (28, 37).

The various procedures which were applied for the extraction of gossypol from the pigment glands were based on the previously reported (30, 32, 33, 34) properties of the gland walls. Preliminary experiments were carried out with each solvent and each extraction method investigated in order to determine the minimum period of contact of the solvent with the pigment glands and the minimum ratio of solvent to pigment glands which could be used for complete extraction of gossypol from the glands. The solvents investigated comprised the following: methanol, ethanol, isopropanol, 1,4-dioxane, acetone, and diethyl ether. Preliminary wetting of the glands and violent agitation of the suspensions of glands in solvent were used for accelerating the rate of extraction.

Gossypol was isolated from each of the pigment gland extracts by means of one or more of the following procedures:

1. An organic solvent in which gossypol is only slightly soluble was added directly to the extracts for fractional precipitation of gossypol.
2. Water was added to extracts obtained with water-miscible solvents, and the precipitates of crude gossypol thus obtained were treated with organic solvents in which gossypol is only slightly soluble.
3. Acetic acid was added to the extracts to precipitate the gossypol acetic acid complex which was then hydrolyzed by suspending it in hot water for one hour as recommended by Murty, Murty, and Seshadri (38).

The results shown in Table 1 can be summarized as follows: gossypol separates from alcoholic extracts

of pigment glands in very poor yields, even though methanol, ethanol, and isopropanol have been shown (33, 34) to be effective for rapid rupture of the pigment gland walls. With the use of 1,4-dioxane for extracting gossypol from pigment glands, impure gossypol is obtained, and no method of purification has been found which does not result in poor yields. Pure gossypol is readily obtained in good yields from extracts of pigment glands made with diethyl ether or acetone.

The use of diethyl ether results in complete extraction of gossypol from pigment glands, and it may be applied in several ways. Contact for 24 hours is required for complete extraction of gossypol when the glands are suspended in diethyl ether without preliminary rupture of the gland walls or agitation of the ethereal suspension. The rate of extraction of gossypol by diethyl ether is accelerated by wetting the glands to rupture the walls followed by drying the glands and grinding them to a fine powder. However, it was found that the powdered glands contained considerably less gossypol extractable with diethyl ether than did the original glands. Violent agitation in a Waring Blendor of an ethereal suspension of pigment glands and decolorizing carbon resulted in complete extraction of gossypol within five minutes. The Blendor was cooled periodically during the extraction in order to reduce both evaporation of ether and decomposition of extracted gossypol.

It was found that pure gossypol could be separated in good yields from ethereal extracts of pigment glands prepared in a Waring Blendor by adding four volumes of light petroleum naphtha [3] to the filtered extract and allowing the mixture to stand at 3.3°C.(38°F.) for 48 hours.

The use of acetone gave complete extraction of gossypol when applied in two ways. Suspension of the glands in acetone without agitation yielded complete extraction of gossypol within two hours whereas violent agitation in a Waring Blendor of a suspension of pigment glands and decolorizing carbon in acetone yielded complete extraction within five minutes.

Precipitation of the gossypol acetic acid complex by addition of one-fourth volume of acetic acid to the filtered acetone extract was found to be the best method for separating gossypol from acetone extracts of pigment glands. Precipitation of gossypol acetic acid was essentially complete when the acetone-acetic acid mixture was allowed to stand over night at 3.3°C.(38°F.). Good yields of pure gossypol were obtained by first washing the precipitated gossypol acetic acid with light petroleum naphtha [3] and then suspending it in the form of an aqueous slurry in hot water for one hour to hydrolyze the gossypol acetic acid complex.

[3] Pentane-hexane mixture, boiling range 95°F. to 130°F.

TABLE 1

Data Pertaining to the Isolation of Gossypol

Prep. No.	Conditions of extraction [1]				Conditions of recovery					Gossypol obtained	
	Kind of solvent	Ratio, solvent to glands, ml./g.	Method	Time, min.	Temperature control	Precipitant added	Ratio, precipitant to extract, ml./ml.	Treatment of mixture	Treatment of mother liquor	Yield,[2] % of theory	Purity[3] %
1	Methanol	100/25	Blendor	5	None	Water	25/100	Heated at 105°C., 20 min.[4]	None	14	92
2	Ethanol	100/25	Blendor	5	None	Water	25/100	HCl added, heated 20 min.[4]	None	10	97
3	Isopropanol	100/25	Blendor	5	None	Water	25/100	HCl added, heated 20 min.[4]	None	5.2	94
4	1,4-Dioxane	40/2	Equilibrated	120	None	Water	40/40	Heated 20 min.	None	68	82
5	Diethyl ether	50/5	Blendor	5	Cooling	Petroleum naphtha[5]	100/25[6]	Cooled to 3.3°C., 48 hrs.	Residue[7] in Et_2O–Pet. naphtha, 48 hrs., at 3.3°C.	60[8]	100
6	Diethyl ether	100/25	Blendor	5	Cooling	Petroleum naphtha[5]	400/100	Cooled to 3.3°C., 48 hrs.	Residue[7] in Et_2O–Pet. naphtha, 48 hrs., at 3.3°C.	55[8]	100
7	Diethyl ether	40/10[9]	Equilibrated	30	None	Petroleum naphtha[5]	160/40	Cooled to 3.3°C., 48 hrs.	None	32	100
8	Diethyl ether	40/10[9]	Equilibrated	30	None	None	.....	Evaporated[7, 10]	None	42	100
9	Acetone	100/25	Equilibrated	120	None	Water	100/100	Heated 5 min.[10]	None	63	100
10	Acetone	100/25	Equilibrated	120	None	Water	25/100	Heated 15 min.[10]	None	65	97
11	Acetone	50/12.5	Blendor	5	Cooling	Acetic acid	12.5/50	Room temp., 30 min.[11]	Cooled 24 hrs., 3.3°C.[11]	73[8]	100
12	Acetone	50/12.5	Blendor	5	Cooling	Acetic acid	17/50	Room temp., 30 min.[11]	Cooled 24 hrs., 3.3°C.[11]	58[8]	100
13	Acetone	50/12.5	Blendor	5	Cooling	Acetic acid	12.5/50	Room temp., 1 hr.[11]	Cooled 24 hrs., 3.3°C.[11]	59[8]	100
14	Acetone	50/12.5	Blendor	5	Cooling	Acetic acid	17/50	Room temp., 1 hr.[11]	Cooled 24 hrs., 3.3°C.[11]	65[8]	100
15	Acetone	50/12.5	Blendor	5	Cooling	Acetic acid	12.5/50	Cooled to 3.3°C., 17 hrs.[11]	Room temp., 72 hrs.[12]	59	96
16	Acetone	50/12.5	Blendor	5	Cooling	Acetic acid	17/50	Cooled to 3.3°C., 17 hrs.[11]	Room temp., 72 hrs.[12]	65	94

[1] During rapid extractions, decolorizing carbon was added to the pigment glands (4g./25g. pigment glands) before extraction; for slow extraction, carbon was added to the extract after removal of the gland residue and before precipitation of gossypol or gossypol acetic acid.
[2] Yields were calculated on the basis of the original content of gossypol in the pigment glands, 39.4%, as determined by application of the antimony trichloride-spectrophotometric method to chloroform solutions of aqueous ethanol extracts of the pigment glands.
[3] Purity based on specific extinction coefficient at 520 mμ of the antimony trichloride reaction product.
[4] The product was purified by washing it with pentane-hexane, mixture.[5]
[5] Pentane-hexane mixture, boiling range 95°F. to 130°F.
[6] The combined extract and wash solutions were evaporated at room temperature under vacuum to the volume indicated.
[7] The solvents were removed by evaporation at room temperature under vacuum.
[8] Combined products obtained from treatment of original extract and mother liquor.
[9] Pigment glands were first wetted in order to rupture gland walls; then dried and ground to a fine powder.
[10] The product was purified by three digestions with toluene, 10 ml. per g. of product.
[11] Precipitated gossypol acetic acid was thoroughly washed with light petroleum naphtha and then hydrolyzed by suspending it in boiling water for one hour.
[12] Only a very small additional amount of gossypol acetic acid precipitated from the supernatant.

TABLE 2

Preparation of gossypol sample[1]	Composition[2]		Antimony trichloride reaction product	
	C %	H %	$E^{1\%}_{1.27\,cm.}$ at 520 mμ (average values)	% Purity
Preparation as in No. 5	69.01 69.03	6.08 6.02	67.7	100
Preparation as in No. 6	68.96 69.10	5.82 5.86	68.3	100
Preparation as in No. 8, then recrystallized from diethyl ether and light petroleum naphtha	66.57 66.72	6.08 6.13	66.5	100
Preparation as in No. 15	67.01 66.82	6.02 6.16	62.8	95.9
Preparation as in No. 15	66.82 66.99	5.84 6.05	64.4	98.3
Preparation as in No. 9, then recrystallized from a mixture of diethyl ether and light petroleum naphtha	68.54 68.62	6.06 6.17	65.9	100
Preparation as in No. 16	66.91 67.10	5.98 6.08	62.1	94.9
Above precipitate of gossypol recrystallized from diethyl ether and light petroleum naphtha	68.72 68.68	6.10 6.08	69.1	100

[1] The numbers refer to the methods of preparation described in Table 1.
[2] Calculated for $C_{30}H_{32}O_8$: C, 67.16; H, 5.97. See reference 29.
[3] The value of $E^{1\%}_{1.27\,cm.}$ at 520 mμ for pure gossypol has been shown (37) to be 65.5 ± 1.9.

Since both the diethyl ether-petroleum naphtha and acetone-acetic acid methods produced equally good yields of pure gossypol, these methods were applied for extraction of gossypol from a second lot of pigment glands in order to test the general applicability of both methods. The second lot of pigment glands was prepared by application of the gland flotation method to hexane-extracted cottonseed meal which had been desolventized in a vacuum oven at a temperature not exceeding 210°F. following extraction of the oil with commercial hexane. The gossypol content of the separated glands was found to be 45.6% (33, 34, 37).

Production of gossypol from these glands by the diethyl ether-petroleum naphtha method was carried out according to the same procedure used with the first lot of pigment glands. A suspension of 25 g. of pigment glands and 4 g. of decolorizing carbon in 100 ml. of commercial grade diethyl ether was agitated for five minutes in a Waring Blendor, with periodic cooling in an ice bath in order to minimize both evaporation of ether and decomposition of extracted gossypol. The residue of gland walls and carbon was removed by filtration and was washed with small volumes of diethyl ether until the combined volume of the extract and wash solutions was 100 ml. Four hundred ml. of light petroleum naphtha[3] was added to the extract, and the mixture was allowed to stand at 3.3°C.(38°F.) for 48 hours. The precipitate of crystalline gossypol which was deposited was washed by decantation with light petroleum naphtha[3] until the wash solution became colorless. Pure gossypol was obtained by this procedure, but the yield was only 28% of the theoretically calculated amount.

Application of the acetone-acetic acid method to the second lot of pigment glands resulted in yields of pure gossypol comparable to those obtained with the first lot of glands. An acetone extract of the pigment glands was prepared by suspending 25 g. of pigment glands and 4 g. of decolorizing carbon in 100 ml. of acetone. The suspension was agitated for five minutes in a Waring Blendor and was then cooled in an ice bath. The residual gland walls and carbon were separated and washed with successive small portions of acetone until the volume of the combined extract and wash solutions was 100 ml. After addition of 25 ml. or one-fourth volume of glacial acetic acid to the extract the mixture was allowed to stand 17 hours at 3.3°C.(38°F.). The crystalline gossypol acetic acid which precipitated was washed with light petroleum naphtha[3] and made into a slurry with a small volume of distilled water. The slurry was then suspended in 400 ml. of hot distilled water on a steam bath for one hour to hydrolyze the gossypol acetic acid complex. A yield of 6 g. of gossypol (52% of the theoretical) of 98% purity was obtained. By allowing the supernatant acetone-acetic acid mixture to stand for 72 hours at 3.3°C.(38°F.), an additional precipitate of gossypol acetic acid was obtained which upon hydrolysis yielded 1 g. of gossypol (8% of the theoretical yield) of 93% purity. In a duplicate preparation from a second sample of the same pigment glands a yield of 6 g. of gossypol (52% of the theoretical) of 97% purity was obtained from the first precipitate of gossypol acetic acid, and an additional 0.9 g. of gossypol (7% of the theoretical yield) of 93% purity was obtained from the precipitate formed in the acetone-acetic acid supernatant.

Large Scale Preparation of Gossypol from Cottonseed Pigment Glands

On the basis of the good yields of gossypol produced by the acetone-acetic acid method from both lots of pigment glands, it was concluded that this method is applicable to pigment glands obtained from either non-defatted or defatted cottonseed, whereas the diethyl ether-petroleum naphtha method is applicable only to glands obtained from non-defatted cottonseed. Since the acetone-acetic acid method is rapid and employs inexpensive and easily handled reagents, it is readily adaptable to production of gossypol on a large scale.

Large scale operation involves relatively little modification of the laboratory procedure. However, a reduction in the ratio of decolorizing carbon and acetone to pigment glands may be possible. Treatment of the original extract with carbon improves both the yield and purity of the gossypol produced. By mixing the carbon with the pigment glands prior to extraction of gossypol, both extraction of gossypol and the adsorption of impurities are accomplished simultaneously, thereby reducing the time of exposure of gossypol to the action of acetone. If it is desirable to use less vigorous agitation than that which occurs in the Waring Blendor, the period of contact of the glands with acetone can be prolonged.

Certain precautions must be observed in order to obtain high yields of gossypol. For example, gossypol should be held in solution only for the shortest possible period, and these solutions should be protected from light and maintained at as low temperatures as are practical. The pigment glands and reagents should be pre-cooled, and all operations should be carried out as rapidly and at as low temperatures as possible. Since gossypol acetic acid is quite unstable, it should be hydrolyzed to gossypol as rapidly as possible, and gossypol should be immediately washed free of acid.

When further purification of gossypol obtained by hydrolysis of gossypol acetic acid is desirable, the two following procedures can be employed either alternatively or successively:

1. In most cases, gossypol can be freed of impurities merely by washing it with light petroleum naphtha or toluene.

2. Gossypol of highest purity can be obtained by recrystallizing it from a mixture of diethyl ether and light petroleum naphtha, but considerable loss occurs by this procedure. Purification can be effected by the latter method by dissolving the gossypol in the minimum volume of diethyl ether.[4] Decolorizing carbon is added to the ethereal solution, and, after a short period of contact, is removed. Four volumes of light petroleum naphtha are added to the ethereal solution, and the mixture is allowed to stand at a low temperature until precipitation of gossypol is complete. This occurs in 48 hours when the temperature is maintained at 3.3°C.(38°F.). The precipitated gossypol, which usually deposits in the form of clusters of large dog-toothed prisms, is freed of solvent by decantation and washing with light petroleum naphtha. Further quantities of relatively pure gossypol can be obtained by evaporating the supernatant solution and recrystallizing the residue from diethyl ether-light petroleum naphtha.

Gossypol has been found to deteriorate rapidly upon storage at room temperature when no special precautions are taken to exclude air. Therefore until more specific information has been obtained concerning the best method for its preservation, it should be stored for as short a time as possible and under conditions which tend to minimize decomposition of normally unstable organic compounds, for example, in an inert atmosphere or a vacuum, at low temperatures, and in the absence of light.

Summary

Cottonseed pigment glands, produced from whole cottonseed meal and defatted cottonseed meal by the gland flotation process, have been investigated as a raw material for the production of gossypol.

Methods based on the previously reported properties of gossypol and cottonseed pigment glands have been developed for the relatively rapid isolation of gossypol from pigment glands.

Extraction of gossypol from pigment glands with acetone followed by precipitation of gossypol acetic acid from the extract was found to be the preferable method for obtaining pure gossypol in good yields.

The precautions which must be applied in order to produce gossypol on a large scale by the acetone-acetic acid method are discussed together with methods for the purification and preservation of gossypol.

[4] The solubility of pure gossypol in diethyl ether is about 10 g./100 ml., but the solubility of crude gossypol is usually greater than this value and depends on the nature and amount of impurities present.

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