GOSSYPOL CONTENT OF COTTON SEEDS AS AFFECTED BY HEAT AND MOISTURE

by

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INTRODUCTION

Among the pigments located in certain glands of the cottonseed, is a highly substituted, binapthyl compound, commonly known as gossypol (1). Great interest developed in gossypol in 1915, when Withers and Carruth (2) attributed to this compound the toxic properties of raw cottonseeds. They found that the toxicity of gossypol varied with different species of animals and with level of gossypol fed. An article which reviews completely all facets of gossypol research has recently been published by Adams, Geissman and Edwards (3).

If the meal made from the seeds is to be used as a major source of feed for certain farm animals, the destruction, inactivation, or removal of gossypol must be brought about by processing, and the effectiveness of this processing determined by relatively rapid and accurate analytical methods for residual toxic gossypol.

One of the simplest methods of rendering cottonseeds non-toxic is autoclaving. Moist heat seems to be an important factor in destroying gossypol. Studies of heat and moisture effects have been conducted by Gallup (4, 27); however, it has since been shown that the analytical procedures employed were unsatisfactory for the quantitative determination of free and bound[1] gossypol (5). New methods have been devised which appear to be more reliable (6, 7, 8, 9).

[1]In the early studies, bound or residual gossypol which could not be extracted from seeds or meal with diethyl ether was termed d-gossypol.

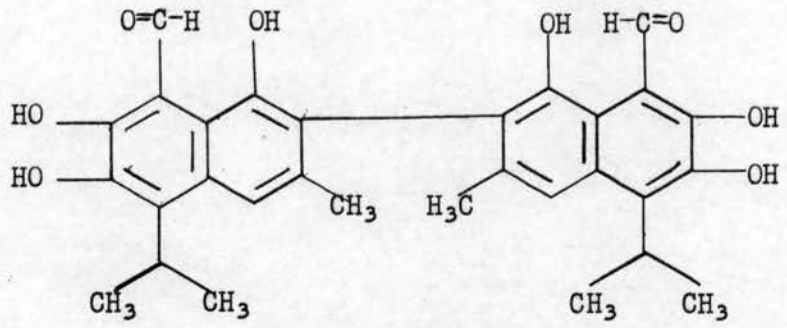
This study, then, was initiated to re-investigate the effect of heat and moisture as factors in the destruction of gossypol in cotton seeds, and to compare newly developed analytical procedures recommended (5) for the estimation of free and bound gossypol. To this end, ground cotton seeds were (a) heated in a drying oven (105°C) for different lengths of time from 2 to 120 hours, (b) allowed to stand at room temperature for 72 and 168 hours, (c) increased in moisture content to about 20%, and allowed to stand at room temperature for different lengths of time from 24 to 168 hours, (d) decreased in moisture content to about 2% and heated for different lengths of time up to 120 hours, (e) increased in moisture content (20%) and heated for different lengths of time up to 120 hours, and (f) autoclaved for different lengths of time from 1 to 24 hours.

REVIEW OF LITERATURE

1. Physical and Chemical Properties of Gossypol.

To understand more fully the nature of gossypol, it is necessary to examine the physical and chemical properties of this compound. It was first isolated by Longmore (10) in 1886 and later purified by Marchlewski (1). Gossypol is found only in the genus Gossypium, and since it displays a polyphenolic nature, it was named by Marchlewski "Gossyp(ium phen)ol" to indicate both its origin in cottonseed and its chemical nature.

Its structure remained unknown until 1938, when Adams et al. (11), proposed the following:



Structural work culminated in 1958, when Edwards (12) presented the formal total synthesis and confirmed the structure as hypothesized by Adams.

Gossypol is a yellow solid, insoluble in water but soluble in most fat solvents. Some of its compounds, e.g., gossypol-acetic acid

and dianilinogossypol, crystallize readily. It is insoluble in low boiling petroleum naptha (13). When heated, gossypol melts with decomposition. Various melting points have been reported, ranging from 180-214°C. These discrepencies have been attributed to polymorphism (13) and to changes effected by variations in temperature during recrystallization (14).

That gossypol is of a complex structure is shown by its variety of reactions. Ethanol solutions make it extremely sensitive to oxidizing reagents (15). It will react with carbonyl reagents such as 2,4-dinitrophenylhydrazine to form the corresponding hydrazone (16). As a polyphenol, gossypol will readily form esters (16) and ethers (17). One of the most widely known reactions of gossypol is that with a primary amine to form a Schiff base (18). This reaction is the basis for the concept of bound gossypol (15, 19, 20). According to this concept, gossypol under favorable conditions combines with the free amino groups of cottonseed protein and becomes insoluble in the usual extraction solvents, e.g., aqueous acetone. "Free gossypol" is that gossypol which is extracted with aqueous acetone and "total gossypol" is that which is obtained only by acid treatment of the cottonseed product, or by some other treatment harsh enough to release all the gossypol (8, 9). Bound gossypol is usually estimated as the difference between total and free gossypol.

II. Physiological and Pathological Effects of Gossypol.

Since the early part of the 19th century, cottonseed meal has been utilized as protein feed for livestock. Success as a feed, however,

was not immediate, for as early as 1845 a report by Voelker (21) indicated that cottonseed meal had toxic properties. That gossypol was the toxic component was reported in 1915, by Withers and Carruth (2), when they separated it from the cotton seed and found it to be toxic to rabbits. A positive correlation between the toxicity of raw cotton seeds and their gossypol content was reported by Schwartze and Alsberg (22, 23) in 1923.

Occurrence of toxic symptoms vary with the type of animal and level of gossypol fed (24). Although cattle can consume large quantities of raw cotton seeds continuously for a long period of time without the slightest indications of any ill effect, animals with simple stomachs will die within a short time if they eat large amounts. The early reports of toxicity in cattle from eating cottonseed meal were probably erroneous in that the symptoms observed were not due to gossypol but rather to the low vitamin A content of the total reaction.

In rats, symptoms are an emaciated condition, diarrhea, and a loss of hair around the head and neck (25, 26). Chronic gossypol intoxication in cats, pigs, and rabbits results in a loss of appetite, paralysis with nerve degeneration, shortness of breath, cardiac hypertrophy, edema of the lungs, and an effusion into the serous cavities (23, 28).

Inhibition of growth is noted in chicks fed gossypol-containing meals (29, 30). Rigdon et al. (31, 32) found that such chicks have a hemolytic anemia, and that a ceroid-like pigment appears in the intestines, spleen, and liver. This pigment then passes to muscular tissue, where it causes necrosis of the striated muscle and cardiac tissue.

Eggs from hens fed cottonseed meal are affected in that hatchability is impaired and egg weight is lowered (33, 34). Also, upon storage, eggs develop discoloration in the yolk and albumin, which lowers sale value. The extent of discoloration is dependent upon the amount of free gossypol present in the diet (35, 36, 37, 38). The extent of discoloration due to bound gossypol is a controversial issue.

III. Detoxification of Gossypol and Cottonseed Meal.

Results of the physiological action of gossypol indicate that to improve cottonseed meal as an animal feed, the gossypol must be destroyed or converted to a non-toxic compound or completely removed.

Many methods of gossypol detoxification have been tried with varying degrees of success. Extraction of gossypol from the meal with suitable solvents has been used since 1918, when Carruth (15) used ether. Other solvents including acetone, petroleum ether, butanone, and chloroform have been used by later workers (39, 40, 41). Addition of calcium (42) and sodium salts (42) to the diets of experimental animals, has been reported to lower the toxicity of the meal. Other methods suggested, have been the addition of soluble iron salts which combine with gossypol (43, 44, 45), and the addition of amines (42), and phloroglucinol (46).

Among the earliest and most successful methods of gossypol inactivation or destruction, have been autoclaving and steaming either the raw cotton seeds or the resultant meal after pressing out the oil. It was first noted by Withers and Carruth (47), that the toxic property of cottonseeds is lessened by steam cooking. Later Dowell and Menaul (48) showed that autoclaving the commercial meal destroyed the toxicity. Gallup (4) studied the effect of heat and moisture as factors in the

destruction of gossypol, and noted that heating the seeds in a dry condition effected a change in the form of gossypol (as determined by solubility in ether), but only slowly reduced the toxicity. Heating moist seeds in an autoclave rapidly destroyed the gossypol and produced a nontoxic product. In experiments with pigs, Lyman, Holland, and Hale (49) showed that autoclaving for short periods of time rendered the meal safe if its level in the total diet was kept below 25 per cent. The accepted explanation for detoxification by autoclaving was that the gossypol became bound to the cottonseed protein. Some of it, very likely, was destroyed. Recent research (50, 51, 52) has provided good evidence that gossypol combines with the free amino groups in cottonseed protein, in particular, the epsilon groups of lysine (51, 52).

IV. Levels of Gossypol Tolerated.

The amount of gossypol required to produce undesirable conditions or symptoms of toxicity in different species of animals remains in question. Whether or not free gossypol accounts for all the toxicity of cottonseed meal also remains unknown.

Many of the early workers (2, 16, 23, 25, 43, 47) reported a direct relationship between the free gossypol content and toxicity of cottonseed meal. Recently, Lillie and Bird (30) reported that growth depression of chicks was directly proportional to free gossypol intake. The gossypol in their experiments was given by capsule, either in pure form or as that contained in cottonseed pigment glands. Eagle and Bialek (54, 55) studied the effects of feeding various levels (50-2,400 mg. gossypol per kg. body weight) of pure gossypol in the diets of rats,

and concluded that the body weight losses are proportional to the amount of gossypol added to the diet. Clawson et al. (56), in 1961, noted that as the free gossypol content was increased in the diet of pigs, the average weight gain decreased and the number of deaths increased. Addition of free gossypol at levels of 0.03% and 0.036% to both corn-cottonseed meal and corn-soybean diets resulted in depressed growth and death.

In contrast to the results obtained with free gossypol added to the diet, Boatner (57) in 1947 reported that three different samples of untreated cottonseed pigment glands containing 40.0, 37.6, and 33.7% gossypol were more toxic to the rat than similar amounts of pure gossypol itself. Ambrose and Robbins (58) also noted that when they fed two different samples of cottonseed pigment glands to rats at a level to provide 0.096% gossypol in the diet, only one of the samples caused inhibition of growth. They attributed this result to a difference in toxicity of the glands themselves since the two diets had the same free gossypol content as determined by chemical analysis.

Eagle and Davies (59) reported a study in which the gossypol content of rat diets was kept constant (0.1%), the sources of gossypol being different samples of pigment glands and free gossypol. The pigment-gland diets caused varying body weight depressions, which in all cases were greater than those produced by any of the diets containing added free gossypol.

That there is considerable discrepancy in the reports of level of tolerance of gossypol is evidenced by the following data: Boatner et al. (60) reported that a level of 0.13% gossypol in a chick diet led to relatively little retardation of growth, whereas a level of 0.65% cottonseed pigment glands (0.244% gossypol) led to marked growth

retardation. Moreover, she found a poor correlation between the nutritive value of various cottonseed products and their content of total gossypol. Couch, Chang, and Lyman (29) concluded that when the free gossypol content of the total ration was 0.06% or less, there was no detrimental effect on growth rate of chicks, while Heywang and Bird (61) concluded from their studies that the free gossypol content of the growth ration should not be greater than 0.01% when fed to White Leghorns or greater than 0.02% when fed to New Hampshire chicks. Instability of gossypol preparations, with varying toxicity, and inadequacy of analytical methods for the determination of physiologically active gossypol, free and bound, may very well account for some of these discordant results.

V. Available Analytical Methods.

Results of studies on the physiological activity of gossypol must be evaluated and interpreted with care, as there are a number of factors which will modify its toxicity. Among these are the level and quality of the protein in the diet, the physical state of the gossypol administered, the possible presence of associated toxic material, and dietary products which react with gossypol either before or after administration. Of particular importance are the analytical procedures employed to determine the free and total gossypol of a diet. Most of the so-called free gossypol of the cotton seed, not removed with the oil, becomes bound in the meal during heat processing of the seed for oil.

Free Gossypol in Cottonseed Meals. All methods proposed for the determination of free gossypol in cottonseed products provide for

the extraction of the gossypol from the sample; they differ in the solvents used, the extraction procedure, and in the final measurement (colorimetric, titrametric and gravimetric) (5). Hydrolysis of the bound gossypol appears to be the critical factor in evaluating methods of free gossypol determination.

The first method, proposed by Carruth (18) in 1917, consists of extraction of gossypol with diethyl ether followed by its determination gravimetrically as dianilinogossypol. This method was modified by various workers (22, 62, 63, 64) but all modifications had, to varying degrees, the same undesirable characteristics. Among them is the long extraction time (48-72 hours) during which gossypol may be broken down. Small amounts of ethanol and water in the ether facilitate extraction. The ether should be free of peroxides. Slowness of precipitation of dianilinogossypol from the extracts and frequent contamination of the precipitate are other disadvantages (5).

Podol'skaya (65) proposed a method based on the reduction of Fehlings solution by gossypol, the precipitated cuprous oxide being determined by titration with $KMnO_4$. This method is subject to some of the limitations mentioned above; also, reducing substances other than gossypol are extracted and react with the Fehlings solution.

Several colorimetric procedures have been developed. The first such method, proposed by Lyman, Holland, and Hale (66) in 1943, used ether, water, and alcohol as the extraction solvent, and aniline as the color development reagent. A number of modifications of this method have been proposed, such as utilization of a Waring blendor and various solvents to hasten extraction time. p-Anisidine has been proposed in

place of aniline for color development (8, 67, 68, 69).

Boatner et al. (70) developed a method in which they extracted the cottonseed product with chloroform for 24 hours, then treated the filtered extract containing the gossypol with antimony trichloride. Light absorption by the red product formed was then determined photometrically at 520 mu. Sensitivity of the reagent to moisture, inability to apply the method directly to a crude extract, and the critical reaction time, limit the applicability of this method.

The use of 70% aqueous acetone for extraction of free gossypol was advocated by Pons and Guthrie (67). In this method, an aliquot of the extract containing gossypol is heated with aniline for color development. This method, although adopted by the American Oil Chemists' Society, has certain limitations; for example, the 70% acetone fails to extract all of the gossypol in mixed feeds.

Storherr and Holley (6) have developed a method in which a 2-butanone-water-azeotrope containing aniline is used in extraction, and phloroglucinol is used as the color developing reagent. Although the method is not widely used, results are repeatable.

The most recent procedure, as proposed by Pons and Hoffpauir (71), employs 3-amino-1-propanol as a complexing agent in the removal of free gossypol from the cottonseed meats or meal prior to reaction with aniline. Although it has a high degree of accuracy, it is somewhat time consuming.

Total Gossypol in Cottonseed Meals. Methods for total gossypol determination must recover from the material not only free gossypol but also the bound gossypol. Release of the bound gossypol so that it can be determined as free, presents a problem.

Early methods (62, 72) employed ether to remove free gossypol followed by hot aniline to remove bound gossypol from cotton seeds and cottonseed meal. Gossypol was determined gravimetrically in the extracts by precipitation as dianilinogossypol. These procedures were time consuming and the method for bound gossypol of questionable accuracy.

A method which requires less manipulation was developed by Pons, Hoffpauir, and O'Connor (73). In this method, the bound gossypol is hydrolyzed by 0.1M oxalic acid in an azeotrope of 2-butanone and water. The oxalic acid is then removed by precipitation with barium acetate. The solution is filtered and the gossypol in the filtrate after reaction with p-anisidine determined colorimetrically. This procedure requires high temperatures (75°C) for long periods of time (6 hours or longer), and as a result some gossypol is destroyed.

A method which reduces the time of analysis and utilizes aniline as the color development agent, has recently been developed by Smith (8). The total gossypol is extracted in 72% ethanol and reacted with hot aniline. A large volume of chloroform is added and the solution filtered, and total gossypol determined colorimetrically as dianilinogossypol.

To reduce the time required for cleavage of the bound gossypol, Pons, Pittman, and Hoffpauir (9) investigated the use of gossypol complexing agents. Amino alcohols form complexes with gossypol and they do not interfere with the aniline color reaction as do aromatic amines. Of several amino alcohols, the authors selected 3-amino-1-propanol, which is stable and readily available. Their procedure satisfies the requirement for a rapid and simplified method for the analysis of all cottonseed products for total gossypol, and, most importantly, its precision is maximum for that attainable in photometric analysis.

EXPERIMENTAL

I. Reagents.

The gossypol acetic acid was prepared by adding 7 ml. of glacial acetic acid to a filtered extract of 3 gm. of gossypol dissolved in 20 ml. of acetone. The solution was allowed to stand one hour, and then filtered through paper on a Buchner funnel and washed with Skelly Solve F. The product was recrystallized three times from acetone, and after the final crystallization it was transferred to a watch glass and the lumps crushed and dried under vacuum at 50°C for 24 hours. It was then stored protected from light in a bottle in a refrigerator at 10°C (74).

The aniline was distilled under reducing conditions produced by addition of zinc dust and acidification with HCl. It was redistilled until the distillate was water white, then stored under refrigeration.

Iron-free Hyflo Super-Cel was prepared by boiling 100 gm. of the Hyflo Super-Cel with 600 ml. of distilled water and 50 ml. of concentrated HCl for 15 minutes and then filtering on a large Buchner funnel. It was washed with distilled water, and after repeating the process the material was dried, and pulverized.

The butanone-water-azeotrope containing aniline was prepared by distilling a mixture of 2-butanone and water (10:1, v/v), and collecting the azeotrope boiling at 73°C. Five ml. of distilled aniline was then diluted to 1000 ml. with the azeotrope.

The phloroglucinol solution was prepared by dissolving 0.4 gm. in 100 ml. of ethanol.

Chloroform - U.S.P. grade.

Ethanol - 95%

Isopropyl Alcohol - Reagent grade.

Hexane - Commercial grade.

3-amino-1-propanol - Reagent grade.

N,N-Dimethylformamide - Commercial grade redistilled and that portion collected which boiled at 152-153°C.

Acetone - Reagent grade.

Hydrochloric acid - Concentrated. Reagent grade.

Cary 14 Recording Model Spectrophotometer.

Beckman DB Spectrophotometer.

II. Methods of Analysis.

Cottonseeds of the Parrot variety, obtained from the Oklahoma Foundation Seed Stocks, Inc., were delinted with sulfuric acid, washed free of acid, air dried, and then ground as needed in a Micro-Wiley mill to pass through a 20 mesh (1-mm.) or a 30 mesh (0.75-mm.) screen. In different experiments the ground seeds were treated with heat and moisture as desired, and samples were removed for gossypol determination. Duplicate portions were analyzed and the average was used to determine the percentage of gossypol in the sample. The standard curves for all methods were prepared with use of the Carey Recording Spectrophotometer and results confirmed by readings made with the Beckman DB.

Free Gossypol. A comparison was made of two methods for the

determination of free gossypol. Method I, which was that of Storherr and Holley (6) was as follows: A sample of the cotton seeds (0.2 to 0.5 gm.) was weighed and placed in a 250-ml. Erlenmeyer flask fitted with a ground glass stopper. Fifty ml. of a butanone-water azeotrope containing 0.25 ml. of aniline was added as the extracting solvent. (This solvent yields a clear filtrate when gossypol is extracted from samples containing other cereal grains.) For complete comminution of the sample, glass beads were added until the bottom of the flask was covered. The flask was stoppered and mechanically shaken for one hour at room temperature. The extract was filtered through Whatman no. 3 filter paper into a 50-ml. volumetric flask. Evaporation was avoided by placing a watch glass on the funnel. An aliquot (1 to 4 ml.) of the filtrate was transferred to a 25-ml. volumetric flask and one ml. of the phloroglucinol reagent was added. The solution was acidified with 2 ml. of concentrated hydrochloric acid and left at room temperature for 20 minutes. The reaction of gossypol and phloroglucinol produced a red color. After 20 minutes the reaction was stopped by diluting to volume with 95% ethanol. If at this point a white precipitate appeared, it was sedimented by centrifugation at 3,000 x G for 10 minutes. The solution was assayed within the hour, light absorption at 550 mu. being determined in a Beckman DB spectrophotometer. A solution of the reagents served as a blank. The reaction and assay times are important because the free gossypol increases with time, due to a breakdown of soluble bound gossypol. The per cent of gossypol in the sample was determined with the aid of a standard curve. The standard curve was constructed from light absorption readings made on solutions of known amounts of gossypol prepared by dissolving 42.4 mg. of

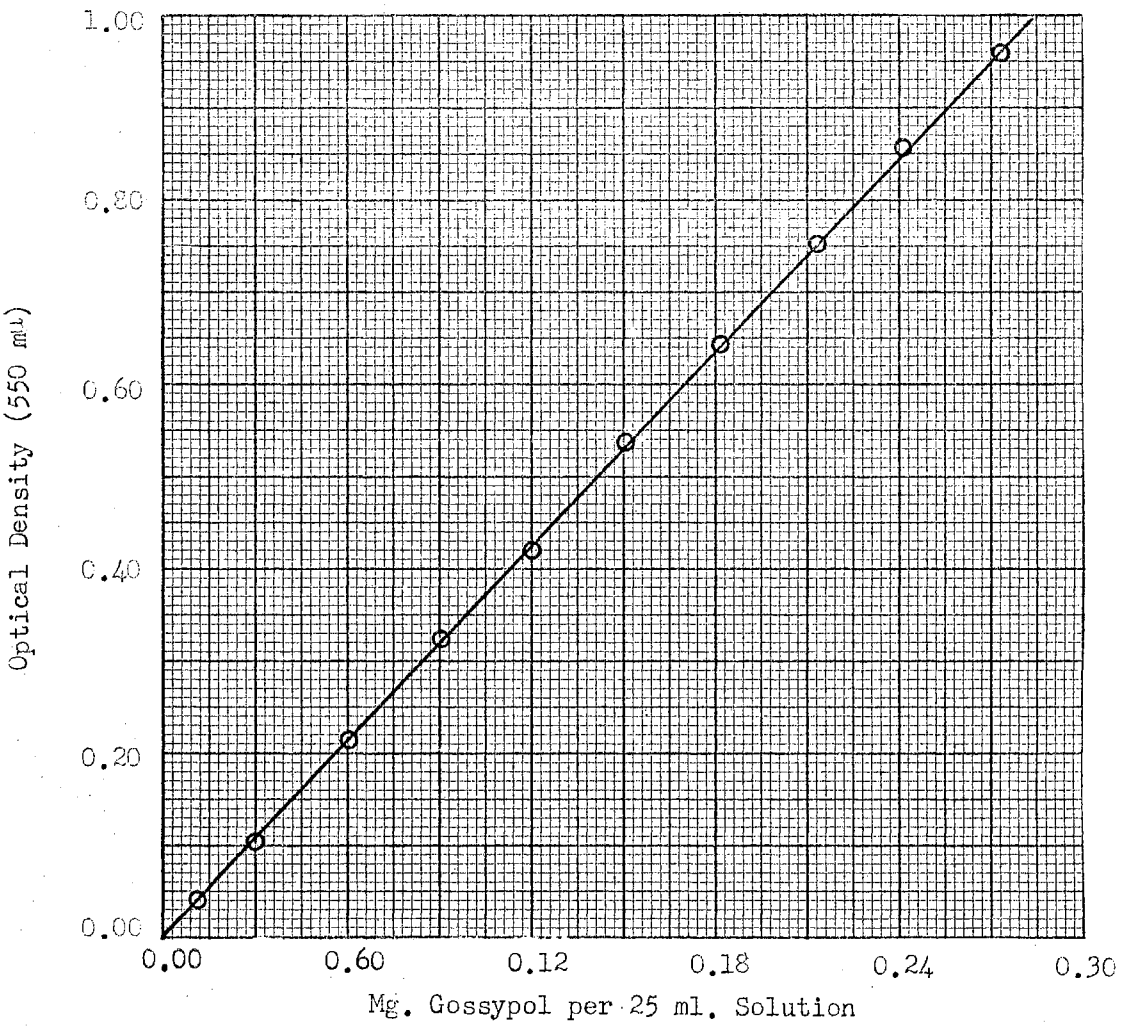


Fig. 1. Standard Curve for Method I

gossypol acetic acid in 250 ml. of the butanone-water-azeotrope containing aniline. Various sized aliquots from 1 to 22.5 ml., equivalent to 0.012 mg. to 0.240 mg. gossypol, respectively, were diluted to 25 ml. Two-ml. aliquots were transferred to 25 ml. volumetric flasks and the color developed and readings taken as described. The blank was made by using one ml. of 95% ethanol in place of the phloroglucinol reagent.

Method II for the determination of free gossypol was that of the American Oil Chemists' Association (7). Accurately weighed samples (0.2 to 0.5 gm.) of cotton seeds were transferred to 250-ml. glass-stoppered, Erlenmeyer flasks, containing sufficient glass beads (3 mm. dia.) to cover the bottom of the flask. Fifty ml. of a 70% aqueous acetone solution was added and the flask stoppered and shaken on a mechanical shaker for one hour at room temperature. Gossypol is readily soluble in this solvent and forms a stable acetone-gossypol addition compound. As a result, extraction time can vary widely without altering results. The presence of water helps rupture any unbroken pigment glands. After extraction, the solution was filtered through Whatman no. 3 filter paper into a 50-ml. glass-stoppered flask, care being taken to prevent evaporation. Duplicate aliquots (1 to 4 ml.) were pipetted into 25-ml. volumetric flasks. One of the aliquots which served as a correction for the background absorption of the extract, was diluted to volume with 80% aqueous isopropanol. To the other aliquot, 2 ml. of aniline was added, and the solution heated in a boiling water bath for 30 minutes. A reagent blank of 2 ml. of aniline and a volume of aqueous acetone solution equal to the sample aliquot was heated in the same manner. After removal from the water bath, the flasks were cooled and diluted to volume

with the aqueous isopropanol. Eighty per cent aqueous isopropanol was used as this concentration eliminated the turbidity of some extracts. Light absorbance of the solutions was determined at 440 mu. The sample aliquot absorbance was corrected by subtracting from it, the background absorbancy of the sample. The milligrams of free gossypol present in the sample was determined by reference to a standard curve. The standard curve was constructed from readings taken on known solutions of gossypol prepared by dissolving 27.9 mg. gossypol acetic acid in 100 ml. acetone, adding 75 ml. of distilled water and diluting to 250 ml. with acetone. After mixing, 50 ml. of this solution was pipetted into a 250 ml. volumetric flask, 100 ml. of acetone was added, then 60 ml. of distilled water, and the solution then diluted to volume with acetone. Duplicate aliquots from 1 to 10 ml., in 1-ml. steps (0.02 mg. gossypol/ml.) were pipetted into 25-ml. volumetric flasks, and the color developed and read as previously described. The reagent blank was 2 ml. of aniline dissolved in 10 ml. of aqueous acetone and heated in a boiling water bath for 30 minutes.

Total Gossypol. The method of Smith (8) was one selected for total gossypol determination. It is simple, reproducible, and expeditious. This procedure is designated as Method III.

A sample (0.2 to 0.5 gm.) of the cotton seeds was weighed and placed in a 250-ml. Erlenmeyer flask. Two ml. of 72% aqueous ethanol was added to soften the particles and rupture any residual pigment glands containing gossypol. Ten minutes later, 2 ml. of aniline was added, the solution mixed and placed on a steam hot plate for 45 minutes. The steam was regulated to keep the temperature slightly under 100°C. The flask

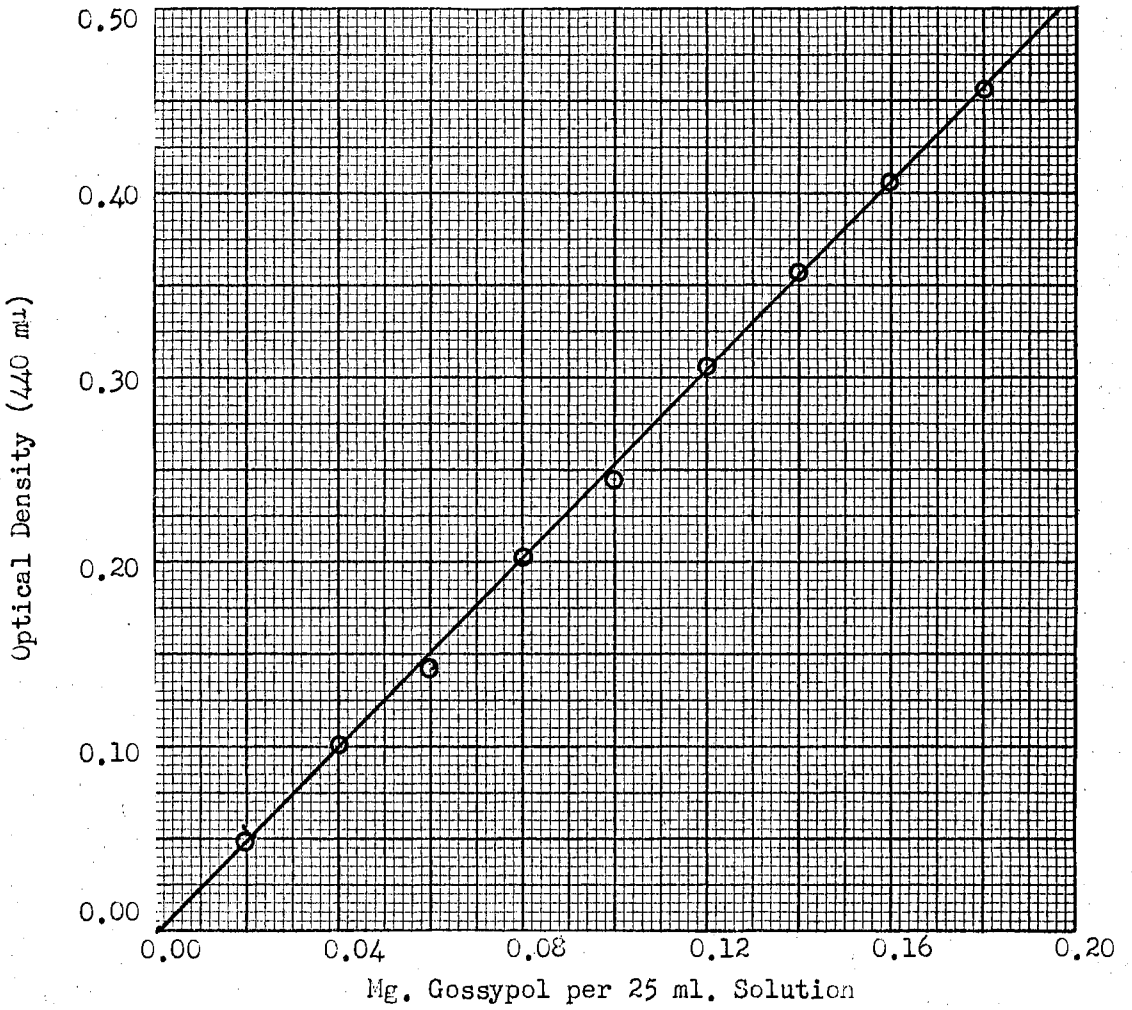


Fig. 2 Standard Curve for Method II

Note: In both Methods II and IV, aniline oxidation occurred during heating unless volumetric flasks with necks of small diameter were used.

was then removed from the hot plate, 60 ml. of chloroform was added, and the flask mechanically shaken for one hour. The solution was then filtered through a specially constructed, rapid flow filter. This consisted of a no. 1 Buchner funnel inserted through a rubber stopper in the aperture of a bell jar. With vacuum applied, a water suspension of asbestos was poured onto the filter to form a thin layer. This was followed by sufficient iron-free Hyflo Super-Cel suspended in 95% ethanol to form a 2-mm. layer of the latter. The ethanol removed the water in the filter and the ethanol was then removed by washing with chloroform. By removing sample residue with a spatula, the filter could be reused. After filtration into a 100-ml. volumetric flask, the solution was made to volume with chloroform and mixed. An aliquot of 1 to 5 ml. diluted to 25 ml. was used in reading the light absorption with a Beckman DB spectrophotometer. This was read against a reagent blank of chloroform. Gossypol (mg./25 ml.) was determined from a calibration curve prepared by dissolving 27.9 mg. of gossypol acetic acid in 100 ml. of chloroform, and mixing. A 10-ml. aliquot was transferred to a 100-ml volumetric 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 plus 0.5 ml. of aniline were heated for 40 minutes on a water bath held at a temperature slightly below boiling. The solutions were cooled, diluted to volume with chloroform, and mixed. The light absorbance was read at 440 mu. on a Beckman DB spectrophotometer, chloroform being used as a blank.

The foregoing method for the determination of total gossypol was compared with the procedure of Pons, Pittman, and Hoffpauir (9). In the

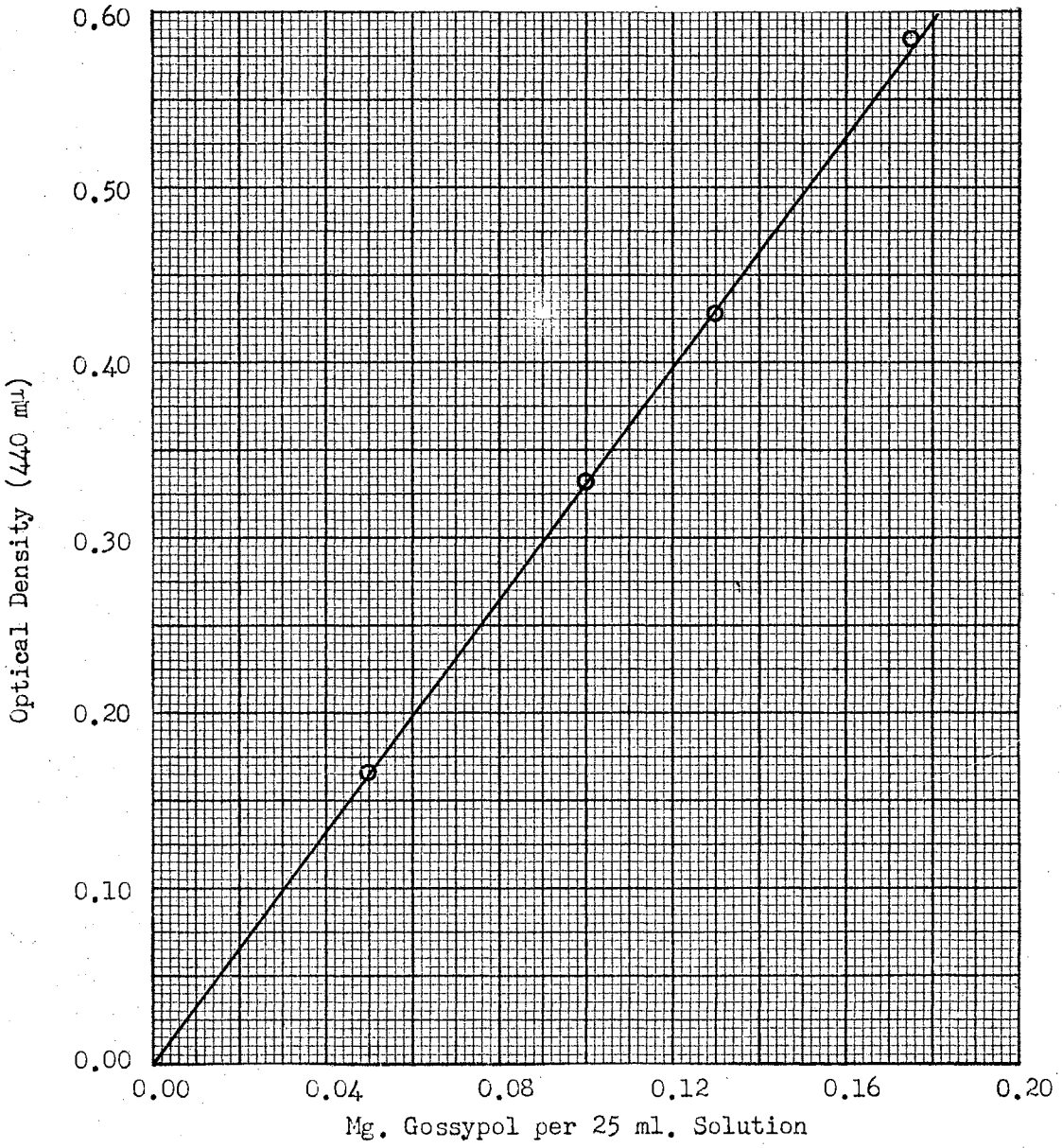


Fig. 3. Standard Curve for Method III

procedure of Pons et al., designated herein as Method IV, a sample of the cotton seeds (0.2 to 0.5 gm.) was placed in a 50-ml. volumetric flask. Ten ml. of a complexing reagent was added, and the solution heated for 30 minutes in a boiling water bath. The complexing reagent consisted of 3-amino-1-propanol in a medium of N,N-dimethylformamide. The 3-amino-1-propanol was selected as it is stable, is readily available, and does not interfere with the aniline colorimetric reaction. The N,N-dimethylformamide was chosen as the reaction medium, as it is a stable, high-boiling solvent in which both aminopropanol and the gossypol complex are readily soluble. It permits the extraction to be conducted at 100°C and minimizes the time required for cleavage of bound gossypol.

After heating, the flask was cooled to room temperature, diluted to volume with an isopropanol-hexane (60:40 v/v) solution, mixed, and filtered through Whatman no. 3 filter paper into a glass-stoppered flask. Duplicate aliquots (1 to 5 ml.) of the filtered extract were pipetted into 25-ml. volumetric flasks. One aliquot was made to volume with the isopropanol-hexane mixture and reserved as a reference solution. To the other aliquot, two ml. of aniline was added, and the color developed by heating in a water bath for 30 minutes. It was then removed, cooled, and made to volume with the isopropanol-hexane mixture. A reagent reference solution and an aniline reagent blank were prepared according to the above procedure but with the omission of the cottonseed sample. With a Beckman DB spectrophotometer, the corrected absorbance at 440 mu. of the sample aliquot was obtained by substracting from the sample absorbance the absorbance of the reference solution. The amount of gossypol in the sample aliquot was determined by reference to a calibration curve, which was prepared by dissolving 27.9 mg. of gossypol acetic acid in 50 ml. of the

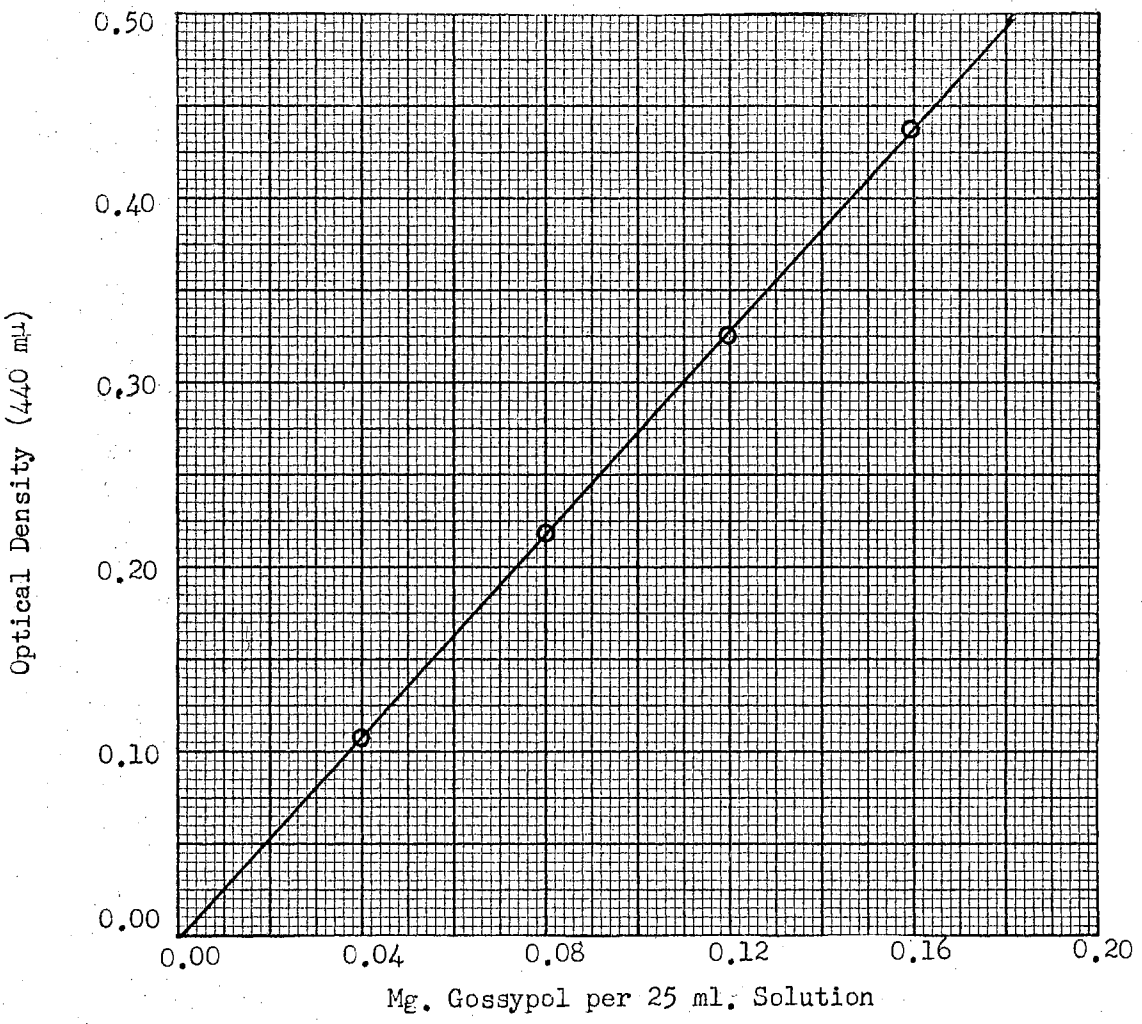


Fig. 4. Standard Curve for Method IV

complexing reagent. Aliquots from 1 to 5 ml. were pipetted into 25-ml. volumetric flasks and complexing reagent added to make the total volume 5 ml. Five ml. of the complexing reagent was used as a blank. The flasks were heated for 30 minutes in a boiling water bath, cooled, and diluted to volume with the isopropanol-hexane mixture. Duplicate, 2-ml. aliquots of each standard and the blank were pipetted into 25-ml. volumetric flasks and the color developed and corrected absorbance determined as described for the sample portion. The above volumes used in preparing the standards were one-half the recommended volumes in the original procedure (9). The larger volumes failed to yield a straight line relationship between the light absorbance and gossypol concentration.

III. Calculations.

To determine the per cent gossypol in the sample for all methods used, the following equation was used:

$$\text{Gossypol } (\%) = \frac{5 \times G}{W \times V}$$

G = Mg. gossypol in sample aliquot

W = Weight of sample in grams

V = Volume of aliquot used for analysis

In Method III, the correction factor 10 was used instead of 5.

IV. Experiments.

Storage time of cotton seeds, their moisture content previous to and during processing, and the heat developed in processing are variable factors in the commercial preparation of cottonseed meal. These factors

account largely for the variation in toxicity and gossypol content of the meal.

Experiment 1. This experiment compared two recommended methods for the determination of free gossypol and two methods for the determination of total gossypol. In all methods bound gossypol was determined by difference. The experiment also determined the effect of fineness of grinding on the recovery of gossypol from raw and heated seeds. Delinted cotton seeds were ground in a Micro-Wiley mill to 20-mesh fineness and thoroughly mixed. A thin layer, not exceeding 3 mm. in depth, was then spread in a preheated Pyrex dish and placed in an electric oven at 105°C. A portion of the heated cotton seeds was taken for analysis at initial time and at intervals thereafter until a period of 120 hours had elapsed. These samples were cooled to room temperature and aliquot samples weighed for determination of free and total gossypol. Methods I and II were used to determine free gossypol, and total gossypol was determined by Methods III and IV. The experiment was then repeated with cotton seeds ground to 30-mesh fineness instead of 20 mesh. Moisture was determined on all samples by drying at 105°C for 4 hours.

Experiment 2. Effect of storage of the air-dry ground seeds on recovery of gossypol was studied in this experiment. Cotton seeds (20 gm.) were ground to 20-mesh fineness, mixed, placed in an 8-ounce sample bottle, and stored in the light at room temperature. The bottle (screw-cap) was opened only to remove sample aliquots at initial time, at 72 hours, and at 168 hours. Free and total gossypol were determined by Methods II and IV, respectively.

Experiment 3. Effect of storage in presence of moisture, without heat, on gossypol was determined in this experiment. Water was added to

ground cotton seeds (20-mesh) until the moisture content was approximately 20%. The sample was mixed until it appeared homogeneous, then stored in the light at room temperature in a closed 8-ounce sample bottle. Sample aliquots were removed at initial time, and at 24-hour intervals thereafter until 168 hours had elapsed. Free and total gossypol were determined by Methods II and IV, respectively. Moisture content of the sample was determined at initial time and at the completion of the experiment.

Experiment 4. This experiment was conducted to determine the change in form and amount of gossypol in heated cotton seeds as affected by their initial moisture content. It consisted of three parts. The first part consisted of grinding cotton seeds to 20-mesh fineness, spreading them in a thin layer in a Petri dish in a desiccator and drying them at room temperature, in vacuo, over P_2O_5 and anhydrous $CaSO_4$ for 96 hours. The Petri dish with sample was removed from the desiccator and placed immediately in an electric oven (105°C). Samples for analysis were removed at various time intervals up to 120 hours. The samples were cooled to room temperature and portions weighed for determination of free and total gossypol content.

Part 2 consisted of grinding cotton seeds to 20-mesh fineness, spreading them in a thin layer in a preheated Pyrex dish, and placing them in an oven at 105°C. Sample removal and gossypol determinations were conducted the same as in the first part of this experiment.

Part 3 of the experiment consisted of grinding cottonseeds to 20-mesh fineness and adding water to increase their moisture content to approximately 20%. They were then spread in a thin layer in a preheated Pyrex dish and placed in an oven at 105°C. Sample removal and gossypol

determinations were conducted in the same manner as in the first part of the experiment.

Moisture determinations in all three parts of the experiment were conducted at initial time. Methods II and IV were used for the determination of free and total gossypol, respectively.

Experiment 5. This experiment was conducted to determine the change in form and amount of gossypol in cotton seeds subjected to moist heat (autoclave). Ground cotton seeds (20 mesh) were placed in a Petri dish and water added to bring the moisture content to approximately 20%. They were then placed in an autocalve and heated at 121°C and 15 lb. pressure. Samples were removed at time intervals up to 24 hours and placed in 8-ounce sample jars which were immediately closed. The samples were allowed to cool to room temperature. Portions of each sample were weighed and analyzed for moisture in the usual manner, and for free and total gossypol by Methods II and IV, respectively.

RESULTS AND DISCUSSION

I. Experiment 1.

In Experiment 1, total and free gossypol determinations were made by four methods on all samples of cotton seeds treated with dry heat. This replication of methods seemed essential in view of the conflicting reports in relating gossypol content to toxicity of cotton seeds and cottonseed meal. The results obtained with seeds ground to different degrees of fineness and subjected to dry heat for periods of time extending from 0 to 120 hours, are shown in detail in Table I.

Results in Table I show definite trends in gossypol content in relation to method of determination. When the seeds were coarsely ground (20-mesh) and heated for periods up to and including 4 hours, Method IV tended to give higher results for total gossypol than Method III; for example, a range of 1.16%-1.24% versus 1.13%-1.17%. When heat treatment was extended to 8, 12, and 24 hours, the two methods yielded similar results, for example, ranges of 0.92%-1.09% and 0.92%-1.08%, but when extended to 48, 72, and 120 hours, Method III gave higher results than Method IV, 0.64%-0.84% compared with 0.52%-0.76%. When the seeds were finely ground (30-mesh), Method III yielded the higher results over all periods of dry heating time; e.g., 1.70% *vs.* 1.53% initially, and 0.65% *vs.* 0.44% at 120 hours. The high results obtained by Method III as compared to Method IV with coarse ground seeds heated for periods of 48,

TABLE I

GOSSYPOL CONTENT OF GROUND COTTONSEEDS AS DETERMINED BY DIFFERENT METHODS AND AS AFFECTED BY PARTICLE SIZE AND DRY HEAT

Particle Size[a]	Heating Time at 105°C	Gossypol Content[b]			
		Total		Free	
		Method III	Method IV	Method I	Method II
	Hours	%	%	%	%
20	0	1.15	1.24	1.21	1.20
20	2	1.17	1.23	0.96	0.89
20	4	1.13	1.16	0.92	0.82
20	8	1.08	1.09	0.85	0.71
20	12	1.04	1.04	0.82	0.65
20	24	0.92	0.93	0.69	0.58
20	48	0.84	0.76	0.49	0.50
20	72	0.78	0.68	0.46	0.43
20	120	0.64	0.52	0.34	0.29
30	0	1.70	1.53	1.55	1.54
30	2	1.23	1.21	1.13	1.03
30	4	1.14	1.10	1.02	0.96
30	8	1.07	1.01	0.85	0.82
30	12	1.02	0.93	0.80	0.75
30	24	0.91	0.81	0.72	0.64
30	48	0.80	0.66	0.57	0.50
30	72	0.72	0.58	0.48	0.39
30	120	0.65	0.44	0.36	0.30

[a]Numbers 20 and 30 represent sieve mesh size used in grinding the seeds.
[b]Dry matter basis. Original moisture content of seeds, 5.41%.

72, and 120 hours may be reasonably explained by the fact that at these longer heating periods seeds yielded dark extracts, and no correction for sample absorption was made in Method III as in Method IV. This difference in color of extracts associated with higher results obtained with Method III increased as the fineness of grinding increased. Because of this experience, Method IV was used throughout the subsequent experiments for determination of total gossypol.

In the determinations of free gossypol, the general trend with both coarse and fine ground seeds was for Method I to give higher results than Method II. This may be accounted for in two ways, namely, the more effective action of the solvent used in Method I and the heat applied to the extract in Method II. In Method I, the solvent was composed of acidified ethanol which may release soluble bound gossypol and make it appear in the final extract as free gossypol. In Method II the use of heat possibly destroys some free gossypol or renders it insoluble. A troublesome white precipitate was formed in the colored sample solution in Method I when samples of both coarse and fine ground seeds were heated for periods of 8 hours or longer. The precipitate was readily sedimented by centrifugation at 3000 x G for 10 minutes. Method II, because of its greater expediency, was used for determination of free gossypol in all succeeding experiments.

These data show that cotton seeds ground to a fine particle size yield a larger amount of both free and total gossypol. They also show that for raw cotton seeds, differences between the comparative procedures for free and total gossypol are not so great as differences within each procedure as produced by particle size.

Effect of particle size on average total, bound, and free gossypol content of cotton seeds heated for different periods of time is shown in Table II.

TABLE II

AVERAGE[a] TOTAL, BOUND AND FREE GOSSYPOL CONTENT OF GROUND DRY-HEATED COTTON SEEDS

Heating Time at 105°C	Gossypol in Seeds Ground to Pass 20 Mesh			Gossypol in Seeds Ground to Pass 30 Mesh		
	Total	Bound[b]	Free	Total	Bound[b]	Free
Hours	%	%	%	%	%	%
0	1.20	0.00	1.20	1.62	0.08	1.54
2	1.20	0.27	0.93	1.22	0.14	1.08
4	1.14	0.27	0.87	1.12	0.13	0.99
8	1.09	0.31	0.78	1.04	0.20	0.84
12	1.04	0.30	0.74	0.98	0.20	0.78
24	0.93	0.29	0.64	0.86	0.17	0.69
48	0.80	0.30	0.50	0.73	0.19	0.54
72	0.73	0.28	0.45	0.65	0.21	0.44
120	0.58	0.27	0.31	0.55	0.22	0.33

[a]Average of Methods III and IV for total, and of Methods I and II for free, gossypol.
[b]Calculated by difference.

It is obvious from inspection of the results in Table II that seeds ground to pass 30-mesh sieve as compared to 20-mesh yield a higher percentage of total gossypol which is almost entirely in the free state. After heating for periods of 2 hours and longer, however, the coarse

ground seeds (20-mesh) decreased in average total gossypol content from 1.20% at 0 time, to 0.93% at 24 hours, a decrease of about 22%, whereas, the finer ground seeds (30-mesh) decreased in total gossypol from 1.53% to 0.86% at 24 hours, or about 44%. The effect of fineness of grinding on free gossypol determinations was similar although not so marked. For example, after 24 hours of heating, free gossypol in coarse ground seeds decreased from 1.20% to 0.64%, a change of 47%, and that in fine ground seeds decreased from 1.54% to 0.69%, a change of 55%. That is to say, the difference in percentage of both total and free gossypol destroyed by heating for 24 hours was related to the difference in the amounts found in the raw seeds ground to different degrees of fineness. The absolute amounts of total gossypol in fine and coarse ground seeds were similar at 24 hours, 0.93 and 0.86%; the amounts of free gossypol were 0.64 and 0.69%. This greater percentage destruction of both total and free gossypol in the fine ground seeds is believed due to a greater release of gossypol, as a greater percentage of the pigment glands are ruptured by mechanical means.

Upon heating, bound gossypol, calculated by difference between total and free gossypol, was consistently higher in the coarse ground seeds than in the fine ground. These differences, however, were too small to be related positively to differences in fineness of grinding or heating time. The amounts found over all heating periods ranged from 0.13% to 0.31%. It appears therefore, that dry heat, even over extended periods, produces relatively small amounts of bound gossypol but destroys free gossypol.

II. Experiment 2.

Since ground cotton seeds show discoloration on standing, determinations were made of gossypol content of ground seeds stored at room temperature for 0, 72, and 168 hours. Results are presented in Table III.

TABLE III

CHANGE IN GOSSYPOL CONTENT OF GROUND COTTONSEEDS ON STANDING AT ROOM TEMPERATURE[a]

Time	Gossypol Content[b]		
	Total[c]	Bound	Free[d]
Hours	%	%	%
0	1.24	0.04	1.20
72	1.25	0.06	1.19
168	1.20	0.00	1.20

[a]Ground to pass 20-mesh sieve.
[b]Dry matter basis. Original moisture content of seeds, 5.41%.
[c]Method IV.
[d]Method II.

Results in Table III indicate very little, if any, breakdown of gossypol in ground, air-dry seeds stored for as long as a week at room temperature. Further, the total gossypol content of the seeds, 1.24%, essentially all of which was in the free state at 0 hours, showed essentially no change in distribution between the free and bound forms.

III. Experiment 3.

The effects of moisture on total and free gossypol content of ground cotton seeds during storage at room temperature are shown in Table IV.

TABLE IV

CHANGE IN GOSSYPOL CONTENT OF WET, GROUND COTTONSEEDS ON STANDING AT ROOM TEMPERATURE

Time	Moisture Content	Gossypol Content[a]		
		Total[b]	Bound	Free[c]
Hours	%	%	%	%
0	20.6	1.22	0.13	1.09
24	----	1.22	0.24	0.99
48	----	1.16	0.38	0.78
72	----	1.13	0.40	0.73
120	----	1.12	0.43	0.69
168	20.3	1.11	0.44	0.67

[a]Dry matter basis.
[b]Method IV.
[c]Method II.

Results in Table IV show a marked increase in the bound gossypol content of ground cotton seeds as the result of high moisture content during storage. It appears that this bound gossypol was formed at the expense of the free gossypol, with the result that there was little change in total gossypol content. For example, bound gossypol nearly doubled during the first 24 hours, increasing from 0.13% to 0.24% and

then increasing more slowly during the next 48 hours to 0.40%. At the end of 168 hours (7 days), there was a further small increase in bound gossypol to 0.44%. These increases were directly related to decreases in free gossypol. It is believed that these changes result from the rupturing of pigment glands, thus increasing availability of gossypol for reaction with other seed constituents, e.g., protein (51, 52). A phospholipid containing gossypol has been isolated from cottonseed meal (75). Complexes of gossypol with glycine, starch, dextrose, and cephalin have been described by others (78, 79, 80).

IV. Experiment 4.

Since the foregoing experiments demonstrated that heat alone (absence of added moisture) decreased free and total gossypol in the ground seeds, this decrease being related to length of time of heating, and that it increased bound gossypol only during the initial heating periods (2-8 hours); whereas moisture alone, without heat, had little or no effect on total but markedly increased bound gossypol, experiments were conducted in which ground seeds of low (2%), medium (5%), and high (21%) initial moisture contents were heated for varying periods of time. Results are presented in Table V.

The results in Table V show a somewhat regular decrease in free gossypol content in relation to heating time and initial moisture content. Seeds with initial moisture contents of 2, 5, and 21% decreased in their free gossypol content of over 1% to 0.36, 0.29, and 0.16%, respectively, at the end of 120 hours of heating; total gossypol in those seeds, which was 1.24% originally, decreased to 0.56, 0.52 and 0.50%, respectively,

TABLE V

GOSSYPOL CONTENT OF GROUND COTTONSEEDS OF LOW, MEDIUM AND HIGH INITIAL MOISTURE CONTENT HEATED IN A DRYING OVEN AT 105°C FOR DIFFERENT PERIODS OF TIME

Initial Moisture (Approx.)[b]	Heating Time	Gossypol Content[a]		
		Total[c]	Bound	Free[d]
%	Hours	%	%	%
2	0	1.25	0.08	1.17
2	2	1.25	0.30	0.95
2	4	1.25	0.36	0.89
2	8	1.17	0.42	0.75
2	12	1.11	0.40	0.71
2	24	0.98	0.35	0.62
2	48	0.89	0.34	0.55
2	72	0.78	0.32	0.46
2	120	0.56	0.21	0.36
5	0	1.24	0.04	1.20
5	2	1.23	0.33	0.89
5	4	1.16	0.34	0.82
5	8	1.09	0.38	0.71
5	12	1.04	0.39	0.65
5	24	0.93	0.35	0.58
5	48	0.76	0.27	0.50
5	72	0.68	0.25	0.43
5	120	0.52	0.23	0.29
21	0	1.22	0.13	1.09
21	2	1.21	0.48	0.74
21	4	1.08	0.39	0.69
21	8	1.00	0.41	0.60
21	12	0.91	0.34	0.57
21	24	0.79	0.27	0.52
21	48	0.63	0.22	0.40
21	72	0.56	0.22	0.33
21	120	0.40	0.24	0.16

[a] Dry matter basis.
[b] Actual initial moisture content, 1.73, 5.41 and 20.63%.
[c] Method IV.
[d] Method II.

over the same period. Perhaps the most striking change was the rapid formation of bound gossypol early in the experiment and the slow change thereafter, particularly in the seeds of low initial moisture content, 2 and 5%. In fact, in the 2 and 5% moisture seeds it appeared that during the first 8 hours of heating, bound gossypol continued to be formed more rapidly than it was destroyed. At the 2-hour heating period, bound gossypol in the seeds with initial moisture contents of 2, 5, and 21% had increased from a level of 0.13% or less to 0.30, 0.33 and 0.48%, respectively. Thereafter, the bound gossypol in the 2% moisture seeds remained between 0.30 and 0.42%, until after 72 hours of heating, and at 120 hours it was 0.21%. Bound gossypol in the 5% moisture seeds remained between 0.33 and 0.39% until after 12 hours of heating and at 48 and 120 hours it was 0.27 and 0.23%, respectively. In contrast to these results, bound gossypol in the 21% moisture seeds decreased, although irregularly, from 0.48% at 2 hours to 0.22% at 48 hours and remained at this level through the longer heating periods of 72 and 120 hours.

That the bound gossypol found in the seeds after 2 hours of heat treatment was formed at the expense of free gossypol appears very likely from the results of the total gossypol determinations. Total gossypol was essentially unchanged in amount during the first two hours of heat treatment, that period when there was a marked decrease in free gossypol and an increase in bound gossypol. After 4 hours of heating, total gossypol declined at a rate not unlike that of free gossypol.

It is of note also that during the first four hours of heating, when differences in initial moisture content were probably still effective, total gossypol in the seeds previously dried in a vacuum (2%

moisture) remained at 1.25%. In the same period, total gossypol decreased to 1.16% in the 5% moisture seeds and declined from 1.22% to 1.08% in the 20% moisture seeds. These observations give further support to the idea that moisture facilitates the rupturing of pigment glands with subsequent release of free gossypol and its reaction with seed constituents to form bound gossypol.

V. Experiment 5.

Since the results of Experiments 3 and 4 seemed to show a slow development of bound gossypol in ground cottonseeds simply by increasing their moisture content at room temperature (Table IV), and a rapid development by heating for short periods (2 hours) followed by destruction by long heating, particularly in seeds of high initial moisture content (21%, Table V), further tests were made with seeds heated (autoclaved) in the presence of excess moisture. The results are given in Table VI.

Results in Table VI show a very rapid formation of bound gossypol during the first hour of autoclaving. At one hour, 70% of the total gossypol was bound, at two hours, 90%, and at four hours essentially all the gossypol present was in the bound form. From a comparison of these results with those in Table V, it is evident that heat and moisture acting together are more effective than either one alone in bringing about binding and destruction of gossypol. The results obtained with heat alone (Table V) are not in accord with early ones (4, 27) in which free gossypol was apparently destroyed within 30-60 minutes. The results obtained by autoclaving, however, are in general accord with those results (4, 27) and many later ones (53, 76, 77).

TABLE VI

GOSSYPOL CONTENT OF GROUND COTTONSEEDS AS AFFECTED BY AUTOCLAVING[a]

Time	Gossypol Content[b]			Moisture Content
	Total[c]	Bound	Free[d]	
Hours	%	%	%	%
0	1.22	0.13	1.09	21
1	0.88	0.61	0.27	29
2	0.63	0.57	0.08	33
4	0.39	0.38	0.01	38
8	0.21	0.20	0.01	41
12	0.01	0.01	0.00	43
24	0.00	0.00	0.00	44

[a]120°C. 15 lb. pressure.
[b]Dry matter basis.
[c]Method IV.
[d]Method II.

CONCLUSIONS

Cottonseeds were ground to pass either 20- or 30-mesh screen and treated with heat and moisture for different periods of time. From analysis and compilation of data for free and total gossypol content of the raw and treated seeds, the following conclusions are drawn:

1. As cotton seeds are ground to a finer particle size, 30-mesh, as compared with 20-mesh, the finer particle size yields a larger amount of both free and total gossypol.

2. In the analysis of raw cotton seeds, differences between the recommended procedures for free and total gossypol are not so great as differences within each procedure as produced by particle size.

3. Dry heat, even over extended periods, produces relatively small amounts of bound gossypol. It slowly reduces the amount of total and free gossypol as determined by chemical analysis.

4. Gossypol remains relatively stable and inactive in ground, air-dry seeds stored for periods as long as one week.

5. Moisture appears to facilitate the formation of bound gossypol, perhaps by rupturing of cotton seed pigment glands with subsequent release of free gossypol and its reaction with seed constituents.

6. Heat and moisture acting together are more effective than either one alone in bringing about rapid binding and destruction of gossypol in cotton seeds.

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