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RESEARCH ARTICLE

SEPARATION OF GOSSYPOL FROM COTTONSEED AND PREPARATION OF GOSSYPOL-FREE COTTONSEED CAKE

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ABSTRACT

Gossypol is a yellow pigment poly-phenolic compound found in the cotton seed and it is toxic component in cottonseed. After extraction of oil, cottonseed cake is used only for cattle feed and cannot be used as poultry feed or for human being because of gossypol toxicity. A process for producing gossypol-free cottonseed cake from cottonseed meals the process is characterized by an integrated sequence of drying, flaking, disintegrating, screen separating and solvent extraction separating steps. The process accomplishes the substantially complete removal of gossypol from cottonseed cake. Gossypol-free protein contained in gossypol-free de-oiled cake is 60-62 percent by weight on a de-oiled cake and moisture-free basis. Considerable attention has been given to methods for the isolation of gossypol from cottonseed. These procedures all involve a preliminary solvent extraction of gossypol from defatted meats, by use of solvents such as diethyl ether or acetone. While satisfactory for small-scale preparations, the excessive costs of solvent extraction systems for the removal of a constituent present to the extent of about 1% in the cottonseed kernel mitigate against large scale use of such systems. Additionally, in order to support further studies into the medical activity of gossypol, methods are needed to isolate large quantities of the compound in high purity. A process is described to recover gossypol from cottonseed.

INTRODUCTION

Gossypol pigments, present in cottonseed in amounts ranging from about 0.4 to 1.5 % of the weight of the kernel have long presented unique problems in the processing of this oilseed [1]. These native gossypol pigments have adverse physiological effects on nonruminants [2]. The reduction of free gossypol in cottonseed meal to "poultry grade" levels (a maximum of 450 ppm total, or 10 ppm per protein percentage) is expected to broaden its use and improve the economic value. Worldwide interest in oilseeds as protein-rich supplements in human diets has recently focused attention on cottonseed as a potentially valuable protein supplement in areas where serious protein and caloric deficiencies exist [3].The use of cottonseed-cake and protein in various ways has been studied have been carried out on cottonseed-meal for animal nutrition at the Southern Regional Research Laboratory, USDA. The shortage of the protein in the rations of the animals has been felt all over the world. The USDA organized several conferences to strengthen the research on cottonseed processing to minimize the risk for animals like ruminants such as poultries, swine's, etc. It will be rewarding if the toxic effect of gossypol and related pigments is eliminated from the cottonseed protein which can serve the purpose in solving the protein hunger all over the world, particularly in developing countries.

An ideal solution to the preparation of cottonseed meal concentrates and oils of optimum quality involves selective extraction of such undesirable constituents as gossypol, under conditions favorable to retention of maximum protein value and oil content, followed by extraction of oil under equally favorable conditions. This approach utilizes selective extraction of gossypol with aqueous acetone, a solvent in which glycerides are essentially insoluble, followed by removal either by hexane extraction or mechanical pressing. Although the utility of acetone-water mixtures for the selective extraction of gossypol from cottonseed has been demonstrated [3],the present communication is a report on the condition required for the proportion of meals and oils of optimum quality.Gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxaldehyde] is a poly-phenolic yellow compound found in pigment glands distributed throughout the cotton plant(gossypium sp.). The compound has been associated with a wide range of biological and medicinal activity, including antitumor [5, 6], anti-fertility [7,8], and antiviral effects [9]. Gossypol is also responsible for toxic effects associated with the overuse of cottonseed products in animal feeds [10].Gossypol exists in a number of tautomericand isomeric forms. Because of the placement of methyl and hydroxyl groups on the carbon atoms adjacent to gossypol's binapthalene bridge(fig.1), rotation about this bond is severely restricted. As a consequence, gossypol exhibits atropisomerism and canbe found in twoenantiomer forms. The cotton plant produces both forms in a ratio that varies from approximately 60:40 (+/-) to 45:55(+/-).Several studies indicate that the gossypol isomers contribute disproportionally

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to the compound's biological activity, e.g. (-) gossypol appears tobe responsible for most of the contraceptive effects [11, 12]. Because of these differences in activity, there has been interest in developing techniques to separate the (+) and (-) gossypol forms. Recently, we reported a crystalline form of gossypol that resolves the enantiomers[13],and currently trying to scale up this process to produce the enantiomers in useful amounts. This paper describes a new procedure to prepare the large amounts of gossypol needed to support this work. The earliest isolations of gossypol were from cottonseed soap stock, a by-product of oil refining [14].

These initial preparations,however, were quite crude, and the methods were quickly overshadowed by other approaches. Subsequent procedures employed cottonseed or defatted cottonseed [15], the root bark of the cotton plant[16], or isolated pigment glands [17]. Pons et al. proposed using cottonseed gums, a by-product of crude oil refining [18]. At the time, this method was advantageous because gums were highly concentrated in gossypol(4-6%) and the use of gums did not interfere with the utilization of the valuable oil or meal. Unfortunately, the cottonseed industry has subsequently converted from crude oil refining to miscella refining (refining before solvent stripping), and the production of gums has eliminated. Therefore, an alternative procedure is needed to produce large amounts of research-grade gossypol.The general strategy of most recovery methods is to extract gossypol into an organic solvent, concentrate the resulting solution, and precipitate gossypol by adding acetic acid. The product is a crystalline inclusion complex containing an equimolar ratio of gossypol and acetic acid (gossypol acetic acid) [19]. This form of gossypol is relatively stable. It contains the enantiomers in an analytical standard for measuring gossypol in animal tissues and cottonseed products [20, 21]. Methods have been reported to recover pure gossypol from gossypol acetic acid [22, 23]; however, these procedures are tedious and prone to unwanted side reactions.

Protein quality and nutritive value of cottonseed proteins, i.e. gossypol toxicity was investigated [24]. It was observed that when gossypol is combined with the protein, the nutritive value decreased and, when it is removed, nutritive value is restored.When bound gossypol was removed under very mild conditions form cottonseed-meal of poor nutritive value, the quality of the meal improved. The lysine availability increased with the removal of bound gossypol. Extraction of cottonseed flakes with acetone containing 25-30% water removes essentially. In this process recovers a 99% gossypol acetic acid product from cottonseed.

Experimental

Apparatus:The Spectrophotometer Model UV-160 U Shimadzu Scientific Instrument, is operated at 440nm.

The HPLC system consisted of pumping system (Model-PU-2080) and photo-diode array detector (Model-MD-2010) with C18 reverse-phase, Hypersil, ODS, 10 µm column having dimentions 250 x 4.6 mm. The mobile phase was 0.01 M KH_2PO_4 buffer adjusted to pH 3 with H_3PO_4 (20%) in acetonitrile (80%) pumped at a flow rate of 1 ml/min. Injections were 20 µl, and the gossypol complex was detected at 254nm.Rotary vacuum evaporator Heidolph Germany make

Materials

Materials as cotton seed has been collected from Central Institute of Research for Cotton (CIRCOT), Matunga, Mumbai. (C.I.R.C.O.T.). The pure gossypol was procured from SIGMA chemical company, USA. Solvents like hexane, acetone, diethyl ether, chloroform, ethanol, and cyclohexane were obtained from Merck Chem. Ltd. The reagents like aniline, hydrochloric acid, thiourea, sodium hydroxide were obtained from Merck Chem. Ltd.

Methods

Free gossypol contents of the flakes were determined by methods described by the American Oil Chemists' Society (1993), AOCS official method, Ba 7-58, (1993)

Determination of purity: Gossypol purification test by UV-Spectrophotometer absorbance

For determination of purity, accurate weighted 2 mg of gossypol and gossypol acetic acid, using a micro balance, into a 100 mL volumetric flask. 40 mL of spectral grade cyclohexane was added, and warmed on a steam bath, with swirling, to dissolve the compound. Cooled to room temperature, and diluted to volume with cyclohexane. Using a calibrated spectrophotometer and matched 1.000-cm standard, or far ultraviolet, silica cells, determined the absorbance of the gossypol solution against the cyclohexane solvent at 358 nm. Calculated the absorptivity as follows:

$A = A/(c)$ (1)

Where a = absorptivity

c = concentration, in g/L

A = absorbance

1 = light path, 1.000cm

The absorptivity of highest purity primary standard gossypol should be 39.9 ± 0.2, and that of highest purity primary standard gossypol acetic acid 35.8 ± 0.2. Absorptivity values in the range of 39.1-39.9 for gossypol, and 35.1-35.8 for gossypol acetic acid, (98-100% purity), denoted primary standards satisfactory for calibration.

Experimental value

a = 35.6 for gossypol acetic acid.

a = 39.8 for gossypol

Analysis of gossypol by High performance liquid chromatography (HPLC)

The HPLC system consisted of pumping system (Model-PU-2080) and photo-diode array detector (Model-MD-2010) with C-18 reverse-phase, Hypersil, ODS, 10 µm column having dimensions 250 x 4.6 mm. The mobile phase was 0.01 M KH_2PO_4 buffer adjusted to pH 3 with H_3PO_4 (20%) in acetonitrile (80%) pumped at a flow rate of 1 ml/min. Injections were 20 µl, and the gossypol complex was detected at 254nm. The purity of crude gossypol products was determined by HPLC against a gossypol standard [23]. 100mg was weighed into a 100ml volumetric flask and filled to the mark with reagent grade acetonitrile. A 0.5 ml aliquot was transferred to a capped test tube, mixed with 1.0ml of complex reagent, and heated at 95-100^{o}C for 30 min tube. The complex reagent was 3-amino-1-propanol (2ml), glacial acetic acid (10ml), and dimethylformamide added to a final volume of

100ml. After cooling, the reprivatized sample was diluted with 9 ml acetonitrile.

Standardization: Standard gossypol (Sigma Aldrich Chem.Ltd.) solutions of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml were pipetted into 15x125mm round bottom glass tubes fitted with stopper. 1.0 ml complex reagent was added in to each tube. The tubes were tightly capped and heated at 100^{o}C for 30 min. After cooling, the derivatized sample was diluted up to 10 ml. The 20 µl injector loop was flushed and filled with standard. The standard was injected into the column and the total run was 15 min. (fig.2)

Experimental

Preparation of cottonseed kernels powder

Prime cottonseed was hulled in conventional laboratory scale disc dehulling equipment. The meats were separated from most of the hulls by screening. Kernels were ground in a domestic grinder and pass through a 20 mesh sieve.

Extraction of Gossypol and Precipitation of gossypol acetic acid

Cottonseed crude gossypol glands were separated from crushed kernels by extraction process.The gossypol pigment glands were extracted with 200ml acetone and water mixture of ratio 7:3 in a closed flask with stirrer for 20min. The residue was re-extracted with 200ml mixture of acetone and water, and finally with 50ml of mixture. The mixture was filtered through Buchner funnel. The filtered solution was concentrated with rotary evaporator using vacuum. The combined extract which was yellow, and appeared to be a colloidal suspension transferred in to a graduated cylinder and required amount of glacial acetic acid was added to induce crystallization. During the crystallization process, the solution was stored in the dark to prevent photo-oxidation. Crude product Gossypol acetic acid precipitate was recovered by centrifugation and vacuum filtration through preweighed # 4 Whatman paper

Recrystallization of Gossypol-Acetic Acid

The purity of the initial gossypol product was increased by repeatedly re-crystallization by dissolving in acetone (1:6 w/v), filtering with # 4 Whatman paper, and adding one-third volume of acetic acid. The initially dark solutions were shaken until yellow particles were noticeable for 3-4 min, and the mixture was stored in the dark for 2 hrs. Precipitated gossypol acetic acid was isolated by vacuum filter through # 4 Whatman paper, washing the precipitate with hexane until the filtrate was colorless, and drying under vacuum. The color of the product (bright yellow) improved slightly with each re-crystallization, and the third and the final re-crystallized sample was observed as pure product (Table 1). Optimum conditions of ground seed to 30% aqueous acetone ratio and stirring time for the extraction were established.A concentrated aqueous acetone extract of cottonseed is treated with 80% aqueous acetic acid in amounts equal to one-third of the volume of the extract. Upon warming the mixture, and then allowing it to stand for some time, gossypol acetic acid precipitates, leaving most of the other extract components in the solution. Optimum conditions of concentrated aqueous acetone extracts to acetic acid were studied. (fig.4)

Purified gossypol acetic acid can be converted to gossypol by dissociation in dilute sodium carbonate, under anaerobic conditions, and that relatively pure gossypol can be precipitated by acidification of this alkaline solution with mineral acid. About 10ml of 0.2 molar carbonate solutions was used per gram of purified gossypol acetic acid. Sodium hydrosulfite, 0.1% was added to the carbonate solution to insure reduction of gossypol products. A thin layer of hexane was floated on the surface of the carbonate solution, prior to acidification, to minimize further atmospheric oxidation. The gossypol produced in this manner was filtered and water washed to remove adsorbed salts. The vacuum-dried product was almost equivalent in purity to the gossypol acetic acid used.

Conversion of gossypol acetic acid to gossypol

Purified gossypol acetic acid, 1 gm was dissolved with slow stirring in 12ml of 0.2molar sodium hydrosulfite. To insure against air oxidation a thin layer of hexane was floated on the surface of the carbonate solution before addition of the gossypol acetic acid. Sulfuric acid 4% was added with stirring until the evolution of carbon dioxide ceased and the solution became acidic. The hexane layer was decanted, and the precipitated gossypol was filtered under reduced pressure by using a coarse paper. It was washed free of acid with hot water and then vacuum-dried at 50^{o}C. The yield of gossypol was quantitative, amounting to a recovery of 89.6% by weight of the gossypol acetic acid used.

RESULT AND DISCUSSION

Extracting cottonseed with 70-75% acetone removed 96-98% of essentially all the free gossypol, most free fatty acids and much of the sugars, but negligible quantities of neutral oil.The amount of water in the acetone was the major variable that influenced the extraction of flake constituents. When the water content of the acetone varied from 10 to 40% (other variables remains constant), minimum extraction of flake constituents occurred with acetone containing 20-30% of water (Fig. 4). Decreasing the water content below 20% markedly increased the extraction of flake components, primarily because of greater extraction of neutral oil.

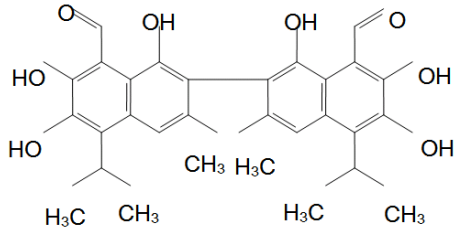

Fig. 1 Structure of gossypol

Conversely, increasing the water content of acetone above 20% also increased the total amount of extracted material; the increase was primarily attributable to the removal of water-soluble carbohydrates. Maximum extraction of gossypol, on the order of 96%, occurred with 25-30% water in acetone. It is observed that the extraction of free fatty acid from the input flakes was high, 80-85% at all water: acetone a ratio whereas the extraction of neutral oil markedly decreased as water in acetone was increased in the range of 25-40 %.

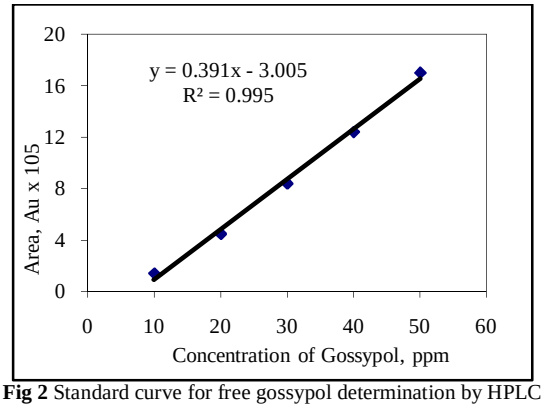

Fig 2 Standard curve for free gossypol determination by HPLC method

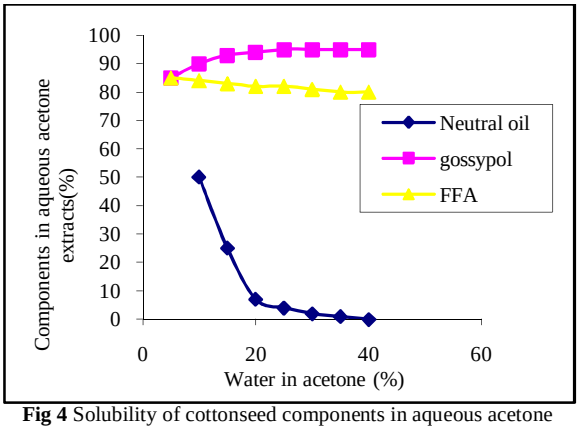

Fig 4 Solubility of cottonseed components in aqueous acetone

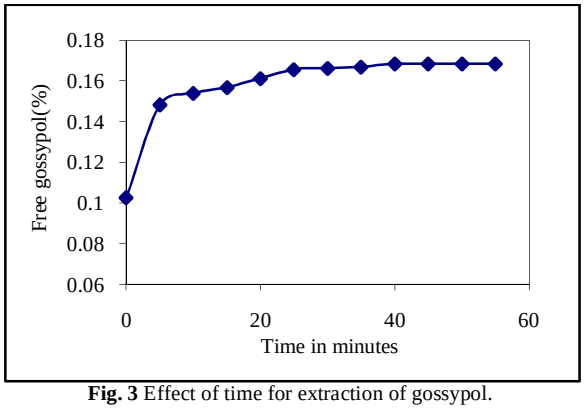

Fig. 3 Effect of time for extraction of gossypol.

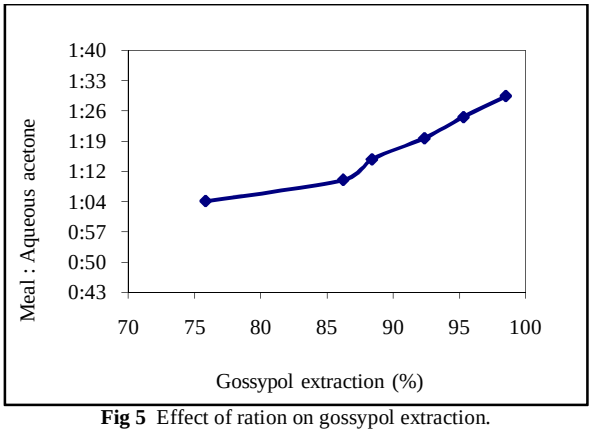

Fig 5 Effect of ration on gossypol extraction.

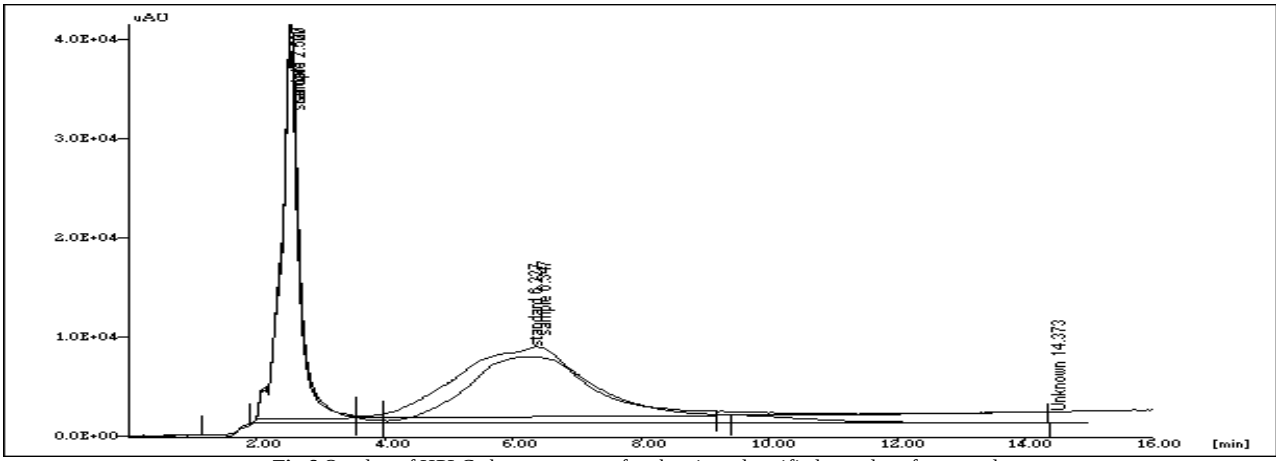

Fig 6 Overlap of HPLC chromatogram of authentic and purified samples of gossypol

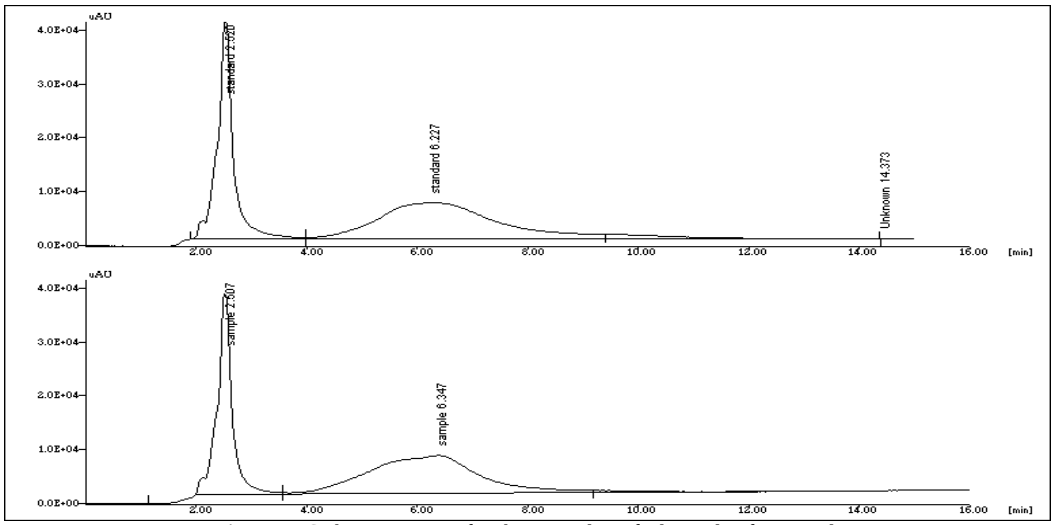

Fig 7 HPLC chromatogram of authentic and purified sample of gossypol

Wet marc from extractions with 30%, or less, water in acetone were readily filtered, but those from higher water concentrations tended to be sticky and difficult to filter. Varying extraction time from 10-60 min (other variable remained constant) had little effect on the extraction of total solids, nitrogen, neutral oil, or free fatty acids. However maximum gossypol removal required a 30-min extraction (Fig 3). A summary of yield data from experiments, utilizing the batch process, is given in table 1. Isolation of gossypol from the kernels ranged from about 40 to 68% of the gossypol present with an average yield of 50 to 75%. The result indicate that gossypol of 98% purity can be produced with an overall product yield of 62% and a product of 99% purity with a yield of 59%. The purity of gossypol was determined on HPLC, and compared with a authentic pure gossypol. Both curves, (Fig. 7), practically coincide. From these spectra, it is concluded that the material extracted from cottonseed kernels is gossypol. The reduction of free gossypol in cottonseed meal was observed below 450 ppm level and meal is expected to broaden its use for poultry grade level as well as for human nutrition.

Table 1 Effect of precipitation conditions on yield of Gossypol acetic acid from aqueous acetone extract Processing variables

Precipitation conditions			Crude gossypol acetic acid	
Ratio Concentrate: Acetic acid	Time Hr	Temp ^{0}C	Purity %	Yield %
1:10	8	30	94.0	50.7
1:5	1	30	94.0	52.0
1:5	8	30	95.1	58.0
1:3	0.5	30	94.0	60.4
1:3	1	30	95.3	62.8
1:3	8	30	95.6	68.5
1:2	1	30	94.3	69.1
1:2	8	30	94.0	75.3
1:5	8	4	94.0	58.5
1:3	8	4	94.0	68.1
1:2	8	4	94.0	73.9

Table 2 Recrystallization of gossypol acetic acid

Number of re-crystallizations	Product yield[b] (%)	Purity (%)	Re-crystallization[c] recovery	Net gossypol[d] recovery
Initial material	-	94.3	-	-
1	86.8	98.4	91.2	84.17
2	77.2	98.7	85.5	78.91
3	68.1	98.6	80.6	74.39

[b]Product yield = (final product weight / weight of initial crude material) x 100
[c]Re-crystallization recovery = (weight of gossypol in re-crystallized sample/weight of gossypol in the initial crude sample) x 100.
[d]Net gossypol recovery = (crude product recovery x re-crystallization recovery)/100

Meals produced by this process should be suitable for unrestricted use in the diets of the most gossypol-sensitive nonruminant animals and would be expected to find applications high-quality protein supplements in human nutrition. Substantiating data were provided by comparison of the HPLC chromatogram of the crystalline material with that of pure gossypol. Almost identical curves, (Fig. 6) indicate positively that the crystalline product obtained from cottonseed kernels is pure gossypol. It is observed that absorptivity for gossypol acetic acid is 35.6 and for gossypol is 39.7, which lies between specified values given by AOCS method, indicates 98-100% purity.

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