



Two-step process for gossypol reduction and protein enhancement in cottonseed kernel powder

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Abstract

Cottonseed is the major by-product of cotton and it contains about 33% protein which shows its potential to be utilized for food applications. However, the presence of gossypol restricts its use for edible purpose. The previous attempts made for gossypol reduction in cottonseed meal resulted in poor extraction efficiency and loss in protein quality. The present study aimed at efficient removal of gossypol from the cottonseed kernel powder to produce the high-protein flour with an excellent amino acid profile. The process was divided into two stages; in the first part, gossypol was extracted from the kernel powder at optimized conditions (acetone-to-water ratio 90:10, sample-to-solution ratio (1:10), 30-min shaken time, and number of extractions 4). Further, the oil was removed from the acetone-treated sample that yielded 15.7% oil. In the second-stage processing, the de-oiled sample was treated at optimized conditions (ethanol-to-water ratio 50:50, citric acid 0.2 M, sample-to-solvent ratio 1:15, and incubation time 30 min) in a water bath at 100 °C temperature. The cottonseed flour produced in the present study had 64% protein, 23% carbohydrates, 0.24% total gossypol, and all essential amino acids except tryptophan.

Keywords Cottonseed · Kernel powder · Flour · Protein · Gossypol · Solvent

1 Introduction

Cotton is primarily cultivated for harvesting of its valuable fiber. The principal by-product of cotton is cottonseed, which is produced in enormous quantities at ginning factories

following fiber separation. Cottonseed contains valuable by-products such as oil, linters, hulls, and cottonseed meal, which account for 27%, 8%, 20%, and 45%, respectively [1]. Cottonseed oil is praised for its heart-health benefits. Common uses for cottonseed oil include cooking, salad dressing,

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shortening, and margarine. The cake/meal is generally fed to animals as feed. The terms meal and cake are referred as the residue obtained after oil extraction by solvent and pressing methods, respectively, while the term “flour” is referred as the high protein obtained after value addition. Cottonseed is a rich source of high-quality protein, essential amino acids, carbohydrates, and other minerals like calcium and potassium [2, 3]. Cottonseed hence has the potential to be employed in food-related applications. However, because it contains the poisonous substance “gossypol,” its applications are severely constrained [4]. Gossypol ($C_{30}H_{30}O_8$), a polyphenol, may have harmful effects on non-ruminants and people if taken for an extended period of time [5]. Infertility and low sperm count; pregnancy problems, including early embryo development; liver damage; respiratory distress; anorexia; and other conditions have all been associated to gossypol [6, 7]. Gossypol has been found in two states, free and bound form. Free gossypol is the gossypol and gossypol derivatives that can be extracted with 70% aqueous acetone [8]. The browning reaction at higher temperature creates covalent bond between gossypol and the free epsilon-amino groups from lysine and arginine, which results in the bound form [9]. However, this process decreases the amount of amino acids that the animal can absorb, with lysine being the most negatively impacted [10]. Thus, it is easier to remove the gossypol in “free” form than in “bound” form. The cottonseed meal produced in the industry after oil extraction contains higher level of bound gossypol. Thus, the present study aims for efficient extraction of gossypol from cottonseed kernel powder. In cottonseed, gossypol level may vary from 0.02 to 6.64% [11]. The US Food and Drug Administration, 1974 (FDA) imposed a limit of 450 ppm for free gossypol in human food items [12]. While the UN Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have set limits of 600 ppm and 12,000 ppm for free and total gossypol, respectively [13]. Gossypol is a crucial raw material in the treatment of many diseases. Gossypol can inhibit the growth of many cell lines, including leukemia, breast, colon, and prostate cells. It also exhibits antiviral properties against HSV-II, influenza, and parainfluenza viruses [14, 15].

An average Indian adult is advised to ingest 0.8 to 1 g of protein per kilogram of body weight. However, the usual consumption is around 0.6 g/kg of body weight. In India, a considerable percentage of people follow a vegetarian diet. These people can fulfill their protein needs with cereal. Cereal-based protein was said to be unable to compete with the proteins found in animal products [16]. Protein deficiency is more common in rural areas due to their low socioeconomic status. More options must be found in order to provide these groups, especially the underprivileged and malnourished, with affordable protein-rich dietary sources. Cottonseed-based products have the potential to play an

important role in addressing India’s malnutrition crisis [17]. Cottonseed kernels contain 45% crude protein; therefore, lowering gossypol to a healthy level may improve cottonseed’s value in the food business [18]. The different methods of gossypol detoxification for food and feed applications are pre-processing (glandless cotton) and post-processing (physical, chemical, and biological) while each of these methods has its own advantages and disadvantages [19]. Although several attempts (alkali/solvent methods) have been undertaken to extract gossypol from cottonseed meal [17, 19, 20] and produced cottonseed powder with low gossypol for feed purposes [13, 18, 21], however, there is no standard method available that can be followed to produce cottonseed flour with high protein suitable for human application. Hence, the main aim of the present study was to identify an efficient solvent method for reducing free and total gossypol from cottonseed kernel powder and create a protocol for producing protein-rich cottonseed flour with minimal gossypol for food applications.

2 Materials and method

Experiments were carried out with an aim of gossypol separation, oil extraction, and cottonseed flour production for utilization as an ingredient for foodstuffs. In order to produce high-protein flour, the present study used cottonseed kernel powder as initial material instead of de-oiled cottonseed meal from industry. The cottonseed meal from industry contains residual hulls and linters as impurities. The cottonseed was first delinted followed by dehulled using the scientific oil processing plant, at GTC, ICAR-CIRCOT, Nagpur, India. The kernel was collected, sun-dried, and ground using an electric grinder. The sun-drying was done to bring down the moisture content of kernel from 12–15% to <5%. The sun-drying did not have the effect on free and bound gossypol (results not shown). The ground kernel powder with particle size 0.8 mm was further used for carrying out experiments. The protein content, free gossypol content, total gossypol content, and oil content in the kernel powder at the initial stage were as 33%, 1.06%, 3%, and 31% respectively. In this research work, the process of gossypol removal was divided into two stages. In the first stage, the maximum amount of gossypol removal was attempted from the kernel powder using aqueous acetone at room temperature. After completion of the first stage, cottonseed oil was extracted from the sample using the solvent extraction method by n-hexane. Then, the sample was subjected to the second-stage processing, by giving heat treatment. In this stage, gossypol was reduced to a very low level by treating the sample with an ethanol-citric acid–water mixture in a water bath.

The process optimization was done by single-factor analysis. During the optimization of one parameter, the

other parameters were kept constant. In this manner, one by one, the effect of each parameter on free and total gossypol removal [8, 22] was determined and the best combination of parameters for gossypol removal was optimized. The optimized parameters were used to produce cottonseed flour that had a low level of gossypol. The final flour was further characterized for various quality parameters such as crude protein content, amino acid profile, oil content, carbohydrates, crude fiber, total ash, energy value, calcium, iron, total microbial count (including coliform and *Salmonella* bacteria), FTIR, and SEM analysis following the standard methods [13, 18, 23–25].

2.1 First-stage processing

In the first stage, process parameters were optimized to extract gossypol from cottonseed kernel powder using aqueous acetone. Five-gram sample was taken for analysis and each experiment was triplicated, and the average value was recorded. In this method, initially, the sample was taken in a 100-ml conical flask and an acetone water mixture was added and mixed with a glass rod. The flask was placed in a shaker at 150 rpm for a given period of time and then filtered using a filter paper (Whatman no. 1). In this process, most of the gossypol gets dissolved in acetone that was filtered out and the acetone was separated by means of the distillation method. Four most effective parameters were selected, viz., the acetone water mixture, shaking time (10–50 min), sample-to-solution ratio (R_{ss}), and number of extractions (1–5) as suggested by Satankar et al. [18]. Each experiment was repeated three times and average values were taken into consideration. Table 1 shows the parameters optimized in the first-stage processing.

Before proceeding to the second-stage processing, cottonseed oil was extracted from the flour using n-hexane by standard Soxhlet extraction method [24].

2.2 Second-stage processing

The de-oiled meal was further treated to minimize the remaining gossypol to a negligible level and improve the protein content in the final product. In this stage, the de-oiled meal was treated with a mixture of ethanol–water in

the presence of heat. To obtain maximum recovery, the process parameters were optimized. The parameter and the levels are shown in Table 2. Under this process, the sample size chosen was 5 g and each experiment was replicated three times, and the average values of free gossypol, total gossypol, and protein content were recorded. In the process, the sample was taken in an airtight glass bottle and mixed with a mixture of ethanol, citric acid, and water. The bottle was placed in a water bath at 100 °C for a given period of time. After heat treatment, the bottle was cooled at room temperature and the sample was filtered, washed, and dried using a hot air oven (80 °C). The process flow chart for the second-stage processing is shown in Fig. 1.

The gossypol and protein content in cottonseed flour was estimated as described earlier [8, 22, 24]. The process parameters and their levels are shown in Table 2.

2.3 Yield estimation

The material balance was worked out based on the gossypol recovery, oil recovery, flour production, and losses during the first-stage processing and second-stage processing. During the first-stage processing, gossypol was recovered from the acetone using the distillation method, dried in hot air oven (80 °C) and then, the amount of crude gossypol was determined on weight basis. The oil extraction was made using n-hexane by Soxhlet apparatus and measured on weight basis. The losses during the whole process were also taken into the account. Finally, the end product de-gossypolized and defatted cottonseed flour was dried and the amount was measured.

2.4 Statistical analysis

The obtained data were analyzed in the completely randomized design using one-way analysis of variance (ANOVA) (WASP 1; ICAR Research Complex Goa). For all analysis, the differences were considered to be significant at $P < 0.05$. Microsoft Excel was used for regression analysis to evaluate the effects of independent variables (such as solvent-to-water ratio, sample-to-solvent ratio, no. of extractions, and time) on dependent variables such as free gossypol, total gossypol, and protein at $P < 0.05$ [26].

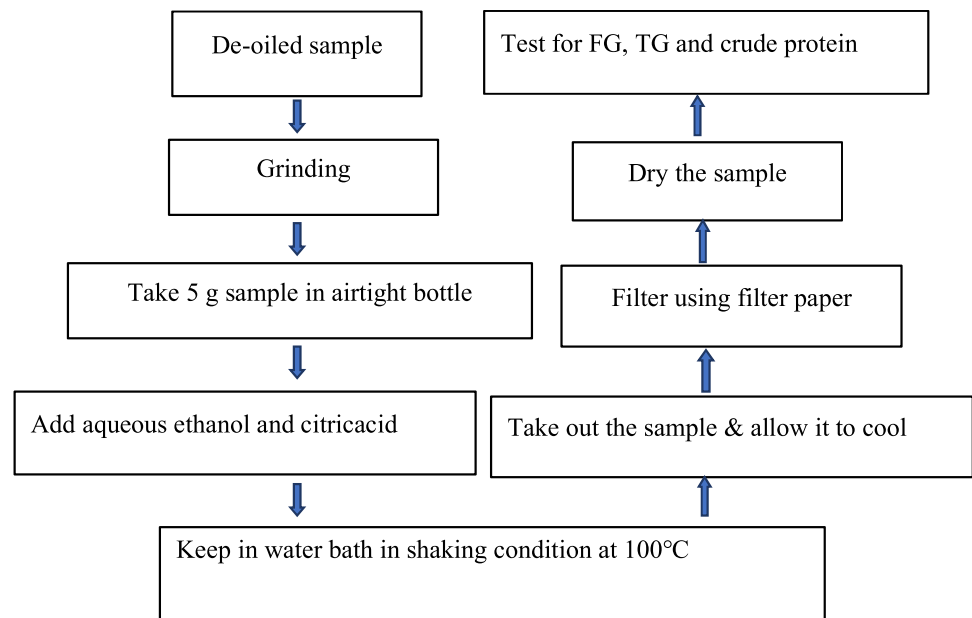
Table 1 Parameters to study the effect on gossypol extractions

Parameters	Level 1	Level 2	Level 3	Level 4	Level 5
Acetone to water	75:25	80:20	85:15	90:10	95:05
Sample-to-solution ratio (R_{ss})	1:2.5	1:5	1:7.5	1:10	1:12.5
Shaking time (min)	10	20	30	40	50
Number of extractions	1	2	3	4	5

Table 2 Variables used in second-stage processing treatments

Parameters	Level 1	Level 2	Level 3	Level 4
Ethanol to water	25:75	50:50	75:25	100:0
Sample-to-solvent ratio	1:5	1:10	1:15	1:20
Incubation time	10	20	30	40
Citric acid	0.1 M	0.2 M	0.3 M	0.4 M

Fig. 1 The process flow chart for the second-stage processing



3 Results and discussion

3.1 First-stage processing

In the first-stage processing, first of all, cottonseed kernel powder was treated with aqueous acetone at room temperature. As acetone is considered the food grade solvent [27, 28] and is considered very effective for gossypol reduction [29]. Hence, five different concentrations of acetone and water were taken to study the effect on total and free gossypol content while keeping the other parameters such as sample-to-solution ratio R_{ss} , shaking time, and no. of extraction (RE) at constant 1:5, 20 min, and 1, respectively. The results are shown in Table 3.

The effect of various concentrations of acetone–water mixture on total gossypol and free gossypol content is presented in Fig. 2a and b. It can be seen in Fig. 2a that the total gossypol content reduces by increasing the amount of acetone in the acetone water mixture. The

total gossypol was found to be 1.18% in kernel powder at concentration 90:10 of acetone to water which was the highest reduction of total gossypol among all concentrations. The reason may be that if the volume of acetone is increased in an acetone–water mixture, it affects the solubility of gossypol. A proper combination of acetone and water enhances the solubility of gossypol, and in our study, the combination of 90:10 of acetone and water mixture showed higher removal of gossypol. By increasing the volume of acetone in the mixture, more gossypol can dissolve in the acetone phase. The results agree with a previous study of a similar kind carried out by [30]. It was also observed that at this level, free gossypol content was found 0.18%.

The free gossypol extraction was higher (0.06%) at 75:25 acetone to water mixture, and the results are in agreement with previous reports where 70% aqueous acetone was found better for free gossypol extraction [18]. In this study, acetone to water mixture of 90:10 was taken for further experiment.

After optimizing the first variable, i.e., acetone to water, the next variable to be optimized was sample-to-solution ratio (R_{ss}). In this treatment, the amount of aqueous solvent was varied from 12.5 to 62.5 ml and the other parameters were kept constant as given in Table 4. The results showed that the total gossypol and free gossypol content decreases by increasing the amount of aqueous solvent for treatment. The reason may be that a large amount of solvent would be available to the sample to extract maximum gossypol. A similar trend was observed in the previous study carried out by Satankar et al. [18]. The maximum gossypol reduction was obtained at R_{ss} 1:10 (Fig. 3).

Table 3 Effect of acetone to water on gossypol content

Acetone to water	R_{ss}	Time	RE	TG (%)	FG (%)
75:25	1:5	20	1	1.85 ^a	0.06 ^b
80:20	1:5	20	1	1.65 ^b	0.11 ^a
85:15	1:5	20	1	1.42 ^c	0.16 ^a
90:10	1:5	20	1	1.18 ^d	0.18 ^a
95:05	1:5	20	1	1.2 ^d	0.21 ^c

R_{ss} sample-to-solution ratio, RE number of extractions, TG total gossypol, FG free gossypol. Treatment values followed by the same alphabet do not differ significantly at $P < 0.05$

Fig. 2 Effect of acetone to water on **a** total gossypol and **b** free gossypol

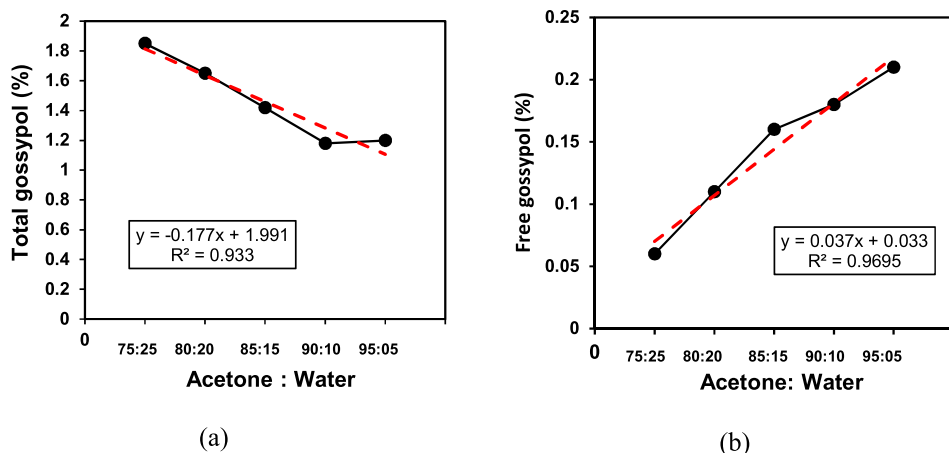


Table 4 Effect of solvent-to-sample ratio on gossypol content

R_{ss}	Acetone to water	Time (min)	RE	TG (%)	FG (%)
1:2.5	90:10	20	1	1.4 ^a	0.2 ^a
1:5	90:10	20	1	1.18 ^b	0.18 ^a
1:7.5	90:10	20	1	1.02 ^c	0.12 ^a
1:10	90:10	20	1	0.79 ^d	0.05 ^b
1:12.5	90:10	20	1	0.79 ^d	0.04 ^b

R_{ss} sample-to-solution ratio, RE number of extractions, TG total gossypol, FG free gossypol. Treatment values followed by the same alphabet do not differ significantly at $P < 0.05$

The effect of shaken time on free and total gossypol content is presented in Fig. 4a and b. In this treatment, the shaken time was varied from 10 to 50 min by keeping the other parameters at constant such as acetone to water mixture 90:10, R_{ss} 1:10, and RE 1 to study the effect of shaken time on gossypol reduction. It was observed that gossypol content was reduced with increasing the shaken time. The reason may be that gossypol is soluble in aqueous acetone to some extent, allowing it to be leached out of the treated

material over time. As the treatment duration increases, more gossypol molecules may leach out, resulting in a reduction of gossypol levels. Results showed that 40 min time was found to be optimum for gossypol reduction (Table 5).

In this treatment, the no. of extraction was varied from 1 to 5 while other parameters like acetone-to-water ratio, amount of aqueous solvent, and shaken time were kept constant as shown in Table 6. The result of this treatment indicates that total gossypol content decreases by increasing the no. of extractions (Fig. 5). This is primarily due to the fact that gossypol is not completely soluble in the extraction solvent, and residual amounts of gossypol can remain in the extracted material after each extraction cycle. The process can be repeated multiple times to further extract the gossypol content in the remaining material, while free gossypol content slightly increases. The reason may be that with each subsequent extraction, more cellular structures are disrupted, leading to the release of additional gossypol, including free gossypol, into the extracted powder. The result showed that four extractions give higher reduction in total gossypol content (0.5%) (Table 6). There was no effect on total gossypol content by further increasing the no. of extractions.

Fig. 3 Effect of sample-to-solution ratio (R_{ss}) on **a** total gossypol and **b** free gossypol

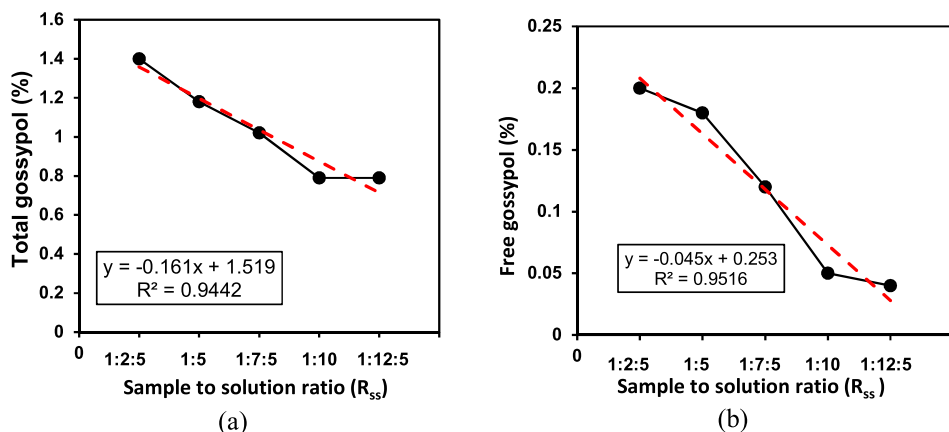


Fig. 4 Effect of shaken time on **a** total gossypol and **b** free gossypol

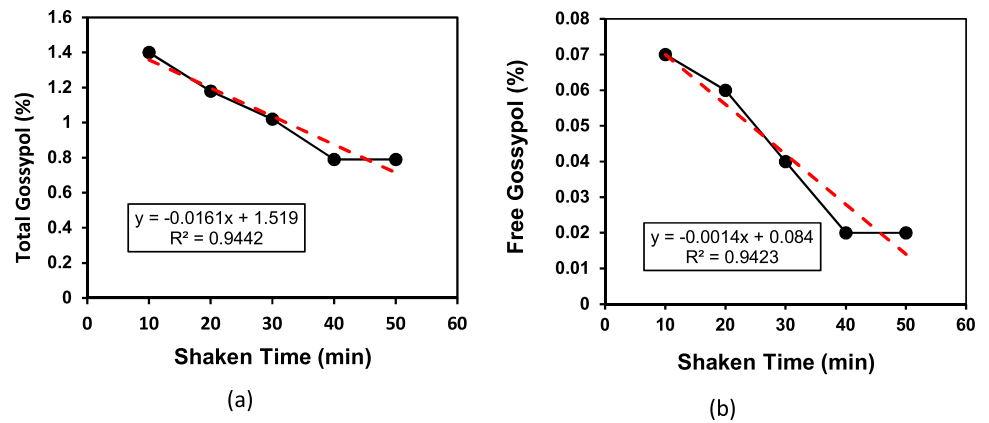


Table 5 Effect of shaking time on gossypol content

Time (min)	Acetone to water	R_{ss}	RE	TG (%)	FG (%)
10	90:10	1:10	1	1.4 ^a	0.07 ^a
20	90:10	1:10	1	1.18 ^b	0.06 ^a
30	90:10	1:10	1	1.02 ^c	0.04 ^a
40	90:10	1:10	1	0.79 ^d	0.02 ^b
50	90:10	1:10	1	0.79 ^d	0.02 ^b

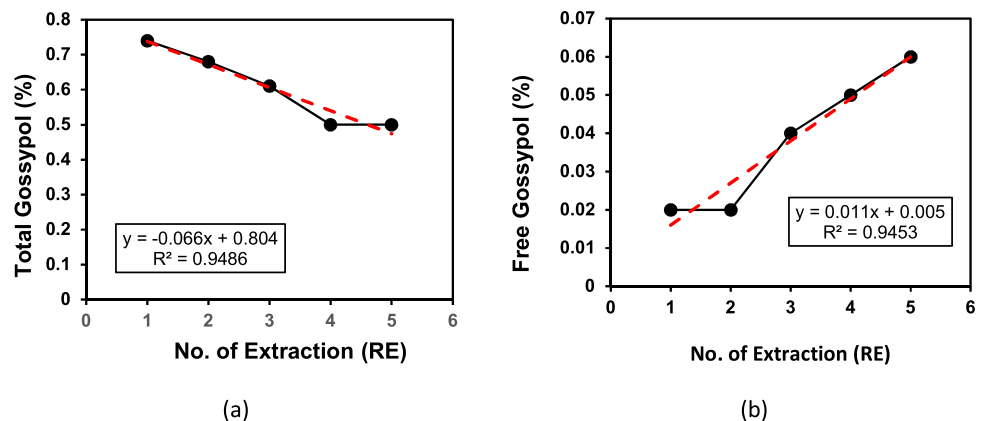
R_{ss} sample-to-solution ratio, RE number of extractions, TG total gossypol, FG free gossypol. Treatment values followed by the same alphabet do not differ significantly at $P < 0.05$

Table 6 Effect of repetitive extraction on total gossypol and free gossypol content

RE	Acetone: Water	R_{ss}	Time (min)	TG (%)	FG (%)
1	90:10	1:10	40	0.74 ^a	0.02 ^a
2	90:10	1:10	40	0.68 ^a	0.02 ^a
3	90:10	1:10	40	0.61 ^a	0.04 ^b
4	90:10	1:10	40	0.5 ^b	0.05 ^b
5	90:10	1:10	40	0.5 ^b	0.06 ^b

R_{ss} sample-to-solution ratio, RE number of extractions, TG total gossypol, FG free gossypol. Treatment values followed by the same alphabet do not differ significantly at $P < 0.05$

Fig. 5 Effect of no. of extraction on **a** total gossypol and **b** free gossypol



The product (kernel powder) of first-stage processing was characterized for various quality parameters such as protein content, oil content, carbohydrate, crude fiber, total gossypol, energy value, calcium, and iron content. The results are shown in Table 7.

It was found that the free gossypol and total gossypol content in the product was 0.05% and 0.5% respectively while the crude protein content was 46.5%. The product of first-stage processing was also tested for amino acid profile and found that the sample had all the essential amino acids except tryptophan. The lysine content was found as 2.67 g per 100 g of sample.

3.2 Second-stage processing

Following first-stage processing, the sample underwent second-stage processing with the goal of increasing the protein content and lowering the total gossypol to the lowest possible level. In many past studies, it was found that ethanol at high temperature can be very effective for gossypol reduction in meal [27, 31, 32]. Hence, in the second-stage processing, ethanol was chosen for gossypol reduction. Before proceeding to second-stage processing, the oil was extracted from the sample to obtain the de-oiled meal. With a fixed sample size of 5 g, the de-oil meal was further treated with

Table 7 Quality parameters of optimized cottonseed kernel powder in the first-stage processing

Test parameter	Measurement unit	Test result
Protein	g/100 g	46.5
Oil content	g/100 g	15.7
Carbohydrate	g/100 g	19
Crude fiber	g/100 g	1.3
Total ash	g/100 g	3.6
Energy value	kcal/100 g	466.55
Calcium	mg/kg	1752.67
Iron	mg/kg	216.72
Free gossypol	%	0.05
Total gossypol	%	0.5
Moisture content	%	3.4

the mixture of ethanol, water, and citric acid at a heating condition (100 °C). In this view, four parameters were varied for four different levels; viz., ethanol to water (EW) varied from 25:75, 50:50, 75:25, 100:0; citric acid concentration (0.1 M, 0.2 M, 0.3 M, and 0.4 M); sample-to-solvent ratio (SSR) varied from 1:5, 1:10, 1:15, and 1:20; and incubation time varied from 10 to 40 min to optimize the best levels for producing cottonseed flour with high protein content and negligible gossypol content. In this method, each of the four parameters was chosen individually and varied for each of its four levels while the other three remained at constant levels to optimize the best results of the particular parameters. Each process was replicated three times and average value was considered for calculation.

Table 8 Effect of independent parameters on gossypol and protein content

S.No	EW	CA	SSR	Time	TG	R ²	Protein %	R ²
1	25:75	0.1	1:10	20	0.4 ^a	0.956	48 ^a	0.966
2	50:50	0.1	1:10	20	0.41 ^a		50 ^b	
3	75:25	0.1	1:10	20	0.45 ^b		51 ^b	
4	100:0	0.1	1:10	20	0.48 ^b		52 ^b	
5	50:50	0.1	1:10	20	0.41 ^a	0.975	51 ^a	0.9
6	50:50	0.2	1:10	20	0.32 ^b		51 ^a	
7	50:50	0.3	1:10	20	0.28 ^c		48 ^b	
8	50:50	0.4	1:10	20	0.22 ^d		46 ^b	
9	50:50	0.2	1:5	20	0.37 ^a	0.9	44 ^a	0.899
10	50:50	0.2	1:10	20	0.33 ^b		51 ^b	
11	50:50	0.2	1:15	20	0.27 ^c		63 ^c	
12	50:50	0.2	1:20	20	0.27 ^c		63 ^c	
13	50:50	0.2	1:15	10	0.29 ^a	0.9	62 ^{NS}	0.891
14	50:50	0.2	1:15	20	0.27 ^a		63 ^{NS}	
15	50:50	0.2	1:15	30	0.24 ^b		64 ^{NS}	
16	50:50	0.2	1:15	40	0.24 ^b		64 ^{NS}	

EW ethanol-to-water ratio, CA citric acid, SSR sample-to-solvent ratio, TG total gossypol. Treatment values followed by the same alphabet do not differ significantly at $P < 0.05$. NS not significant

3.2.1 Effect of ethanol-to-water ratio on gossypol and protein content

In this treatment the ratio of ethanol to water was varied from 25 to 100% keeping the other parameters at constant level such as citric acid concentration at 0.1 M, sample-to-solvent ratio 1:10, and incubation time 20 min, and the effect of this parameter on total gossypol content (TG) and protein content was analyzed. The result of this treatment showed that lowest gossypol content (0.41%) found with 50:50. At this level, the protein content was observed 50%. Further increase in the ratio increases the gossypol content in the sample as shown in Table 8 serial no. 1–4.

3.2.2 Effect of citric acid concentration on gossypol and protein content

Pelitire et al. [27] revealed that acidic solvent extraction is quite effective to reduce gossypol and improve protein in cottonseed meal. Hence, in the present study, citric acid was involved for gossypol extraction. The concentration of citric acid was varied from 0.1 to 0.4 M to study the effect on gossypol content and protein content. Level of citric acid was based on a previous study related to the preparation of gossypol from cottonseed gums [32]. During this study, the other parameters such as ethanol water ratio, sample-to-solvent ratio, and incubation time were kept constant at 50:50, 1:10, and 20 min respectively as shown in Table 8 serial no. 5–8. The results of this study showed that 0.2 M citric acid gives the highest protein,

51%, and total gossypol, 0.32%. It was also found that further increase in the citric acid concentration reduces the gossypol content to lower level but the protein content was also reduced which was not desirable. Hence, the 0.2 M citric acid was found optimal for the treatment. Citric acid, being a weak organic acid, can enhance the solubility of gossypol in aqueous solutions and also cause protein denaturation to some extent at lower pH values, due to the exposure of hydrophobic regions of proteins, promoting their aggregation and precipitation from solution [33].

3.2.3 Effect of sample-to-solvent ratio on gossypol and protein content

The experiment was carried out to optimize the ratio of sample to solvent in second-stage processing. During this treatment, other parameters such as ethanol-to-water ratio, citric acid, and incubation time were kept as 50:50, 0.2 M, and 20 min, respectively, while the sample-to-solvent ratio was varied from 1:5 to 1:20. The result of this study showed that 1:15 performed better among all which gave 63% crude protein and 0.27% total gossypol as shown in Table 8 serial no. 9–12.

3.2.4 Effect of incubation time on gossypol content and protein content

Incubation time was the period of heat treatment in which the sample was kept into the water bath at 100 °C. To optimize the incubation time, four different levels of incubation time (IT) were selected as 10, 20, 30, and 40 min while the other parameters were kept constant such as ethanol-to-water ratio at 50:50, citric acid concentration 0.2 M, and sample-to-solvent ratio 1:15. The results of the study revealed that 30-min incubation time showed better results in terms of total gossypol reduction to 0.24% and higher protein content (64%) as shown in Table 8 serial no. 13–16.

Hence, in the second-stage processing, the optimized parameters were found as ethanol-to-water ratio 50:50, citric acid concentration 0.2 M, sample-to-ethanol ratio 1:15, and incubation time 30 min. When de-oiled meal was given heat treatment at the above optimized conditions, the flour was produced with 64% protein and negligible amount of total gossypol (0.24%).

3.2.5 Characterization of final optimized sample (cottonseed flour)

The final product (cottonseed flour) was produced at optimized conditions, i.e., 50:50 ethanol-to-water ratio, 0.2 M citric acid, sample-to-solvent ratio 1:15, and 30-min

Table 9 The test results of processed cottonseed flour

Test parameter	Measurement unit	Test result	[32]	[28]
Protein	g/100 g	64	48.54	72
Total fat	g/100 g	0.98	-	0.9
Carbohydrate	g/100 g	23	-	-
Crude fiber	g/100 g	4.7	17.08	-
Total ash	g/100 g	4.3	6.39	8.13
Energy value	kcal/100 g	356.54	-	-
Calcium	mg/kg	4294.68	2300	-
Iron	mg/kg	287.54	-	-
Free gossypol	%	Nil	0.038	0.0331
Total gossypol	%	0.24	-	-
Total bacterial count	CFU/g	2200	-	-
Coliform bacteria	CFU/g	Nil	-	-
<i>Salmonella</i> bacteria	CFU/g	Nil	-	-
Moisture content	%	2.6	-	-

Table 10 Amino acid profile of the cottonseed flour

Amino acid profile	Measurement unit	Test results	[32]	[33]
Lysine	g/100 g	3.65	1.97	1.56
Histidine	g/100 g	2.01	1.20	1.54
Arginine	g/100 g	7.95	5.35	4.53
Tryptophan	g/100 g	Absent	0.58	-
Aspartic acid	g/100 g	6.50	4.18	3.81
Threonine	g/100 g	2.23	1.46	1.21
Serine	g/100 g	3.21	1.99	1.71
Glutamic acid	g/100 g	13.81	8.55	7.49
Proline	g/100 g	2.64	1.27	-
Glycine	g/100 g	2.91	1.85	1.82
Alanine	g/100 g	2.80	1.72	1.82
Valine	g/100 g	3.23	2.01	1.87
Methionine	g/100 g	1.19	0.67	0.50
Isoleucine	g/100 g	2.30	1.38	1.21
Leucine	g/100 g	4.76	2.76	2.20
Tyrosine	g/100 g	2.08	1.07	1.13
Phenylalanine	g/100 g	3.74	2.90	2.03

incubation time, and evaluated for various quality parameters such as crude protein content, amino acid profile, oil content, carbohydrates, crude fiber, total ash, energy value, calcium, iron, FTIR, and SEM analysis following the standard methods. The results are presented in Tables 9 and 10.

As the limit for free gossypol for human food products set by the U.S. Food and Drug Administration (FDA) in 1974 is 450 ppm [12] and the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) have established limits for free and total gossypol at 600 ppm and 12,000 ppm [13], respectively. As a result, in the current study, the free gossypol content of the final

cottonseed flour was zero while the total gossypol content was 0.24%, which indicates its potential for use in the development of food products.

It can be seen in Table 9 that the free gossypol was absent in the final cottonseed flour while the total gossypol content was found very low (0.24%). Also, the crude protein content in the final flour was obtained as 64%. The amino acid profile of the final sample is shown in Table 10. The nutritive and amino acid profile of processed cottonseed flour obtained in this study was found better as compared to the previous reports of the similar study [28, 34, 35] (Tables 9 and 10). The nutritive properties of cottonseed flour in terms of gossypol, protein, and amino acid content were found better in comparison to previous studies. As per Food Safety and Standards Regulations, 2011, version IV, 2017, published on 9/11/2017 [13], the available lysine content in the cottonseed meal/flour should be more than 3.6 g per 100 g of cottonseed flour. In the present study, the final optimized sample had a lysine content of 3.65 g. Additionally, the amino acid profile revealed that the final flour contained all of the essential amino acids, except tryptophan, indicating its excellent potential for use as a human protein supplement.

3.3 Fourier transform infrared (FTIR) spectroscopy determination

Fourier transform infrared (FTIR) is a powerful technology used to recognize the secondary structure and specific functional groups of a compound. Cottonseed flour is rich in protein and is made up of amino acids; each has a specific chemical structure such as amide, hydroxyl, and aliphatic. Amide can be easily recognized using FTIR compared to other functional groups [36].

The FTIR spectrum of cottonseed flour is presented in Fig. 6a; distinct amide peaks are observed, which are indicative of the presence of peptide bonds and secondary protein structures. The amide I band lies between 1600 and 1700 cm^{-1} . In line with this, the spectrum of cottonseed flour showed a prominent peak centered at 1637.56 cm^{-1} , which primarily arises from the carbonyl group (C=O) stretching vibration in the peptide bond [37, 38]. It also indicates the presence of secondary structures such as α -helices, β -sheets, or random coils in proteins [37]. This is complemented by the amide II band (1480–1575 cm^{-1}) and spectrum of cottonseed flour showed peak at 1543.05 cm^{-1} , which is attributed to N–H bending vibrations coupled with C–N stretching, further confirming the presence of proteinaceous materials [35, 37]. The peak 1240.23 cm^{-1} represents amide III which can range from 1220 to 1300 cm^{-1} , and it represents the primary vibrations of combination of N–H bending and C–N stretching vibrations [35]. Overall, the spectral data robustly support the presence of proteins, with the amide I, II, and

III peaks [37–40]. The FTIR spectrum of acetone-extracted sample presented in Fig. 6b showed the peaks at 1653 cm^{-1} and 1531 cm^{-1} which correspond to the amide I and amide II bands, confirming the presence of proteins or peptides. Additional peaks at 1388 cm^{-1} and 1238 cm^{-1} indicate C–H bending in aliphatic compounds and C–N or C–O stretching, respectively [35–40].

In the spectrum of acetone-extracted and defatted sample in Fig. 6c, a similar trend of amide bands was observed; the peak at 1625 cm^{-1} likely represents the amide I band, indicating the presence of carbonyl stretching in an amide group. The amide II band, generally found near 1500–1600 cm^{-1} , arises from N–H bending and C–N stretching vibrations. Here, the peak at 1529 cm^{-1} can be attributed to the amide II band. In the spectrum, the peak at 1236 cm^{-1} is indicative of the amide III band. Similar results were observed in previous studies on the protein fractions of cottonseed [37–40]. Further, FTIR spectra of ethanol-extracted and defatted sample are shown in Fig. 6d. Specifically, the amide I band at 1653 cm^{-1} corresponds to C=O stretching vibrations, while the amide II band at 1531 cm^{-1} is indicative of N–H bending and C–N stretching vibrations. Additionally, the amide III band, observed in the range of 1236 cm^{-1} , confirms the presence of N–H bending and C–N stretching vibrations. These characteristic peaks confirm the presence of amide functionalities, suggesting that the sample contains proteins with amide linkages. A similar trend in the spectrum was observed by Kumar et al. [13] in the isolation of protein from cottonseed meal. N–H bonds show stretching vibrations at about 3300 cm^{-1} of the characteristic amide A region [37]. All four samples showed significant peak, at 3277–3286 showing the presence of characteristic amide A region.

It was also observed that the FTIR spectrum of cottonseed kernel flour was found close to the major spectral features of soybean protein isolate [41, 42]. Finally, some more differences in banding pattern were observed among the samples. In analyzing the FTIR spectra of samples “a” and “d,” significant differences are observed in the carbohydrate and hydrophobic regions. The single peak around 1050 cm^{-1} is associated with carbohydrates as it is one of the major components in cottonseed-based products [39]. This shows variation in intensity between the samples, indicating differing carbohydrate contents. Notably, the strong peak at 2924 cm^{-1} , attributed to hydrophobic CH_2 asymmetrical and symmetrical stretching vibrations, highlights the presence of oil components within the samples [40]. The pronounced peak at 2924 cm^{-1} in sample “a” indicates a higher oil content because cottonseed kernel powder retains more of its natural oils. In contrast, sample “d” treated with ethanol shows reduced oil content due to ethanol’s effectiveness in extracting and removing oils from the kernel powder.

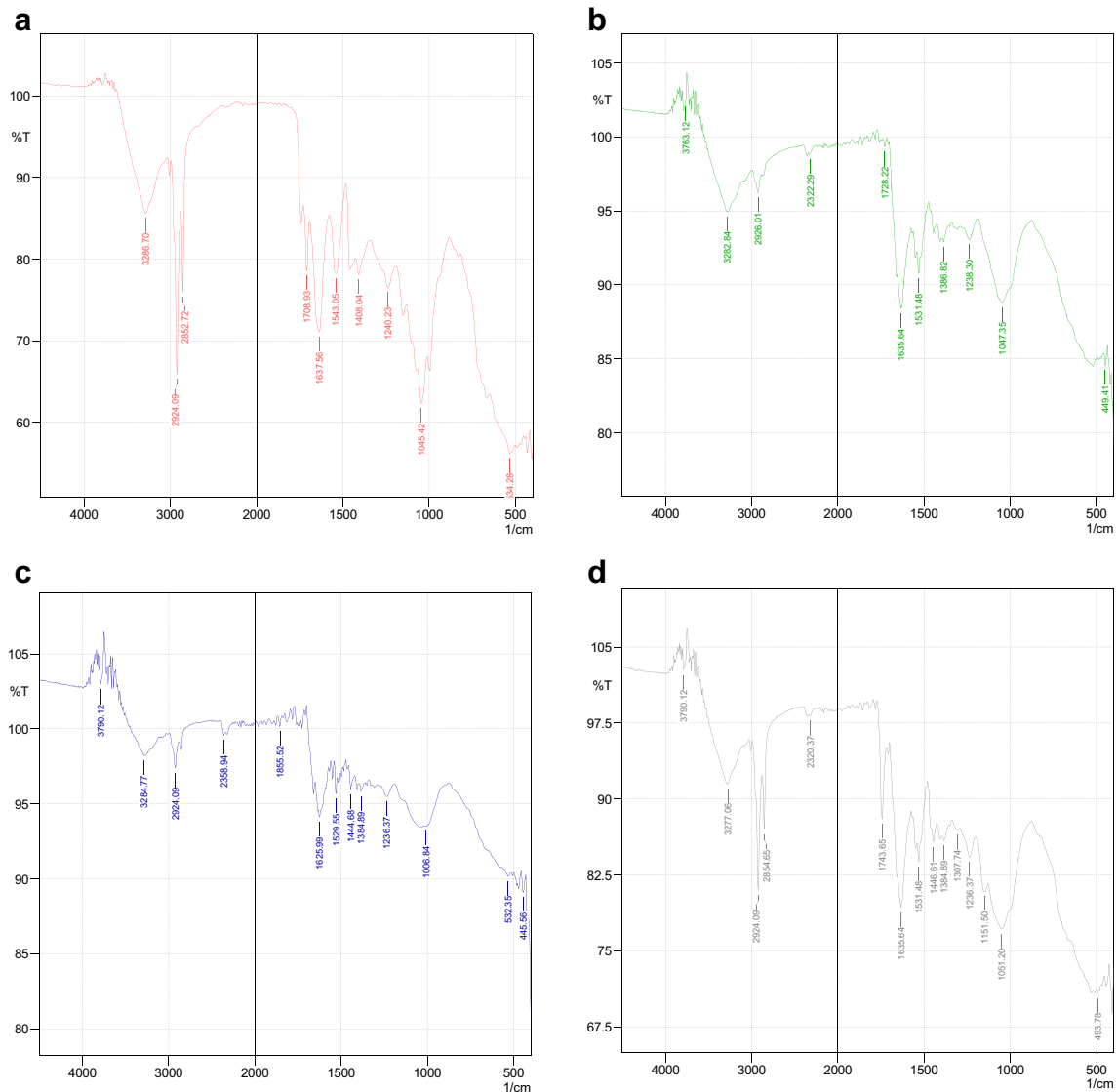


Fig. 6 FTIR analysis of cottonseed kernel powder. **a** Kernel powder (control). **b** Acetone-extracted sample. **c** Acetone-extracted and defatted sample. **d** Ethanol-extracted sample

3.4 Scanning electron microscope micrograph (SEM) analysis

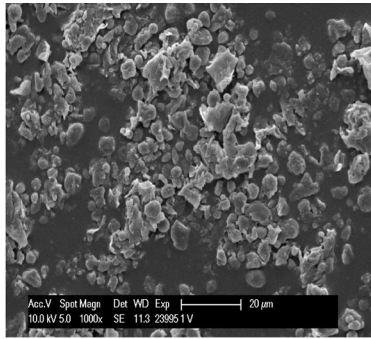
SEM analysis of four samples, namely, control-cottonseed kernel powder (CSKP), acetone-extracted powder (A-EP), acetone-extracted and defatted powder (A-EDP), and ethanol-extracted powder (E-EP) were done. The morphological characteristics of all four samples of cottonseed flour were analyzed under two magnifications $\times 1000$ and $\times 2000$ (Fig. 7a–h). The CSKP (control) has a comparatively more wrinkled, spongy, and porous structure than the extracted samples. On the other hand, the extractions (A-EP, A-EDP, and E-EP) have flattened, tight microstructure and sharp angles than the control. Among the extractions, E-EP has a comparatively more flattened, tight microstructure with sharp angles. The results also showed that there is a

tendency of conglomeration of particles in acetone-extracted and defatted powder. The surface properties of flattened, tight microstructure with sharp angles and less porosity are due to the loss of non-protein components such as cellulose, lignin, hemicellulose, and fiber in the protein extractions. The results obtained in this study are in agreement with previous reports on SEM analysis of cottonseed protein extractions [43, 44].

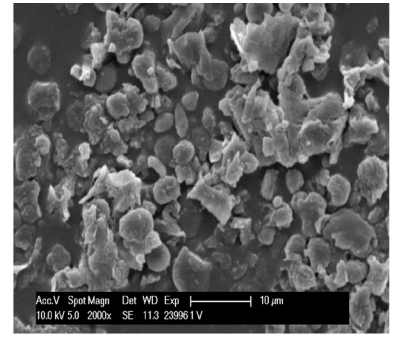
3.5 Material balance

Based on the research work, the material balance was worked out in view of protein-rich flour production, recoverable gossypol, and cottonseed oil production. The material balance is shown in Fig. 8. The yield of cottonseed flour, oil,

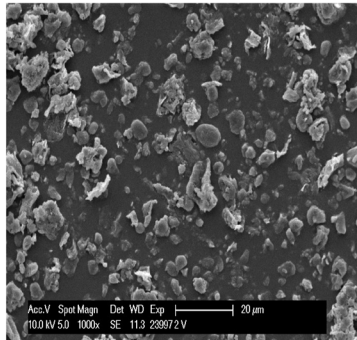
Fig. 7 SEM analysis of cottonseed kernel powder



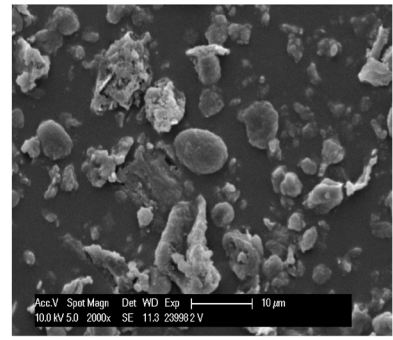
(a) Cottonseed kernel powder (Control) (1000x)



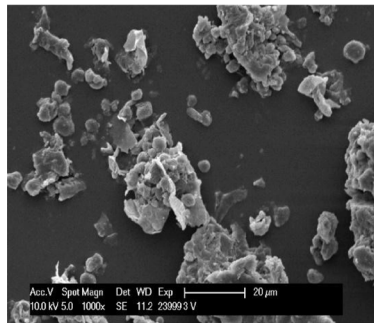
(b) Cottonseed kernel powder (Control) (2000x)



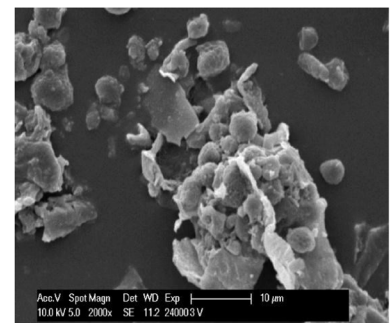
(c) Acetone extracted (1000x)



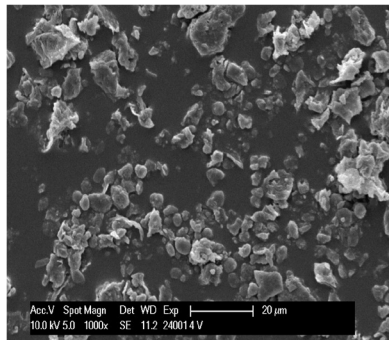
(d) Acetone extracted (2000x)



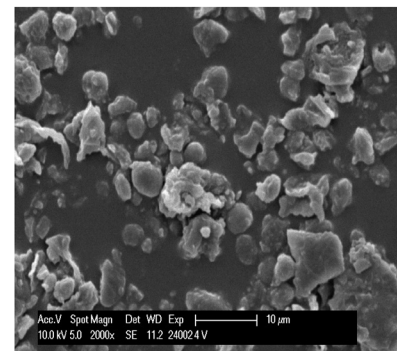
(e) Acetone extracted and defatted (1000x)



(f) Acetone extracted and defatted (2000x)

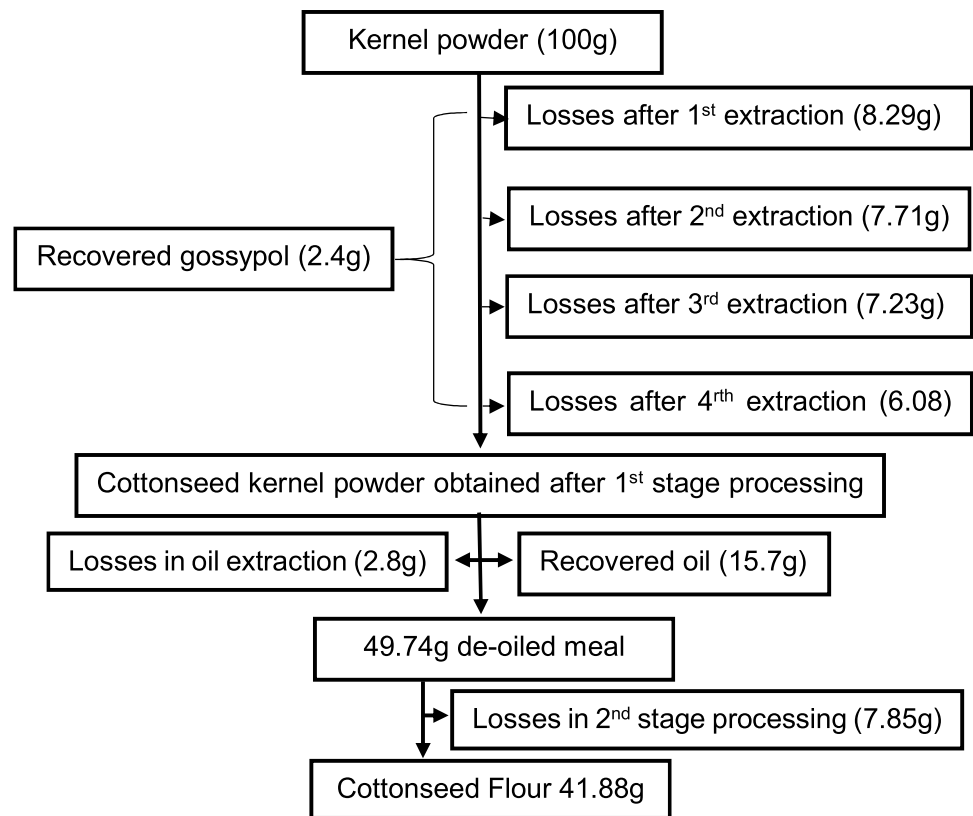


(g) Ethanol treated (1000x)



(h) Ethanol treated (2000x)

Fig. 8 Flowchart for material balance from kernel powder flour



and gossypol obtained in the process was 41.88, 15.7, and 2.4 percentages respectively.

4 Conclusion

The present study highlights the novel two-stage solvent process to reduce gossypol from the cottonseed kernel powder and produce the cottonseed flour with a high protein content. The optimized conditions in the first-stage process are 90:10 acetone–water mixture and sample-to-solution ratio 1:10 for 30 min shaken time, with four number of extractions at room temperature. The optimized conditions of second-stage process are ethanol to water 50:50, citric acid concentration 0.2 M, sample-to-solvent ratio 1:15, and incubation time 30 min at 100 °C. The resultant flour had total gossypol (0.24%), free gossypol (zero), and protein content (64%) with an excellent amino acid profile.

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and Sivasubramanian Palanisamy; project administration: Vellaichamy Mageshwaran, Nadir Ayrilmis, Tabrej Khan, Sivasubramanian Palanisamy, and Varsha Satankar; writing original draft: Varsha Satankar, Durwesh Jhodkar, Mohan Singh, and Manoj Kumar; writing, review, and editing: Nadir Ayrilmis, Sivasubramanian Palanisamy, Vellaichamy Mageshwaran, and Tabrej Khan; validation and visualization: Tabrej Khan, Sivasubramanian Palanisamy, and Durwesh Jhodkar; funding acquisition and supervision: Vellaichamy Mageshwaran, Tabrej Khan, and Nadir Ayrilmis. All authors have read and agreed to the published version of the manuscript.

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Data availability The data pertaining to the study is available with the first author. The data will be shared upon request.

Declarations

Ethics approval and consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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