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Scientific paper

ISSN 0351-9465, E-ISSN 2466-2585

<https://doi.org/10.5937/zasmat2303300F>



Zastita Materijala 64 (3)  
300 - 307 (2023)

## Bio-refining of cottonseed meal from oil industry for the isolation of eco-friendly natural protein polymer

### ABSTRACT

Cottonseed meal (CSM) is a by-product of the oil industry. Globulins (salt soluble, vicilin, and legumin families) are the major dominant storage proteins in cottonseed and account for 60%–70% of seed proteins. However, these are unsuitable for human consumption due to their gossypol content. In this work, protein was extracted from defatted cottonseed flour with alkaline solution and its precipitation at different pH value was investigated. The crude protein was quantified using standard micro-Kjeldahl nitrogen method. Central composite design expert was conducted and the effect of variables in the extraction of protein were studied, namely, extraction solvent concentration, temperature and time. The optimum scheme of extraction was obtained at 0.1M NaOH with material to liquor ratio (MLR) 1:20 at pH 12 using temperature of 67°C and a time of 90 min. Under these conditions, the response gives a flour dissolution percentage of 80.52% and protein purity percentage 80.40%. In addition, protein solubility and water absorption capacity were also determined and the chemical structure of protein was characterized by means of Fourier Transform Infrared Spectroscopy.

**Keywords:** Cottonseed meal, Cottonseed protein, Cottonseed protein flour, Extraction, Protein characteristics, Precipitation

### 1. INTRODUCTION

The growing challenges of greenhouse gas emissions, climate change, the increasing gap between economic growth and environmental sustainability, dwindling fossil resources have enforced attention towards the development of sustainable bio-based platforms [1]. However, the ranking of the agricultural sector as the second-highest contributor to greenhouse gas emissions has focused interest towards the optimal valorization of agricultural waste residues as an alternative to their burning or dumping in landfills [2].

Cotton (from the Arabic *quothn*) is a an agricultural crop which uses to produce spinnable fibers (lint) on the seed coats [3]. According to the world count report in 2020, around 27 million tons of cotton is produced globally in countries such as USA, India, China and others in a year. Cottonseed

contains, on an average 45 percent hulls and linters and 55 percent meats [4]. Nearly 45-55% of this meal is a protein [5].

Cottonseed meal (CSM) is a by-product of the oil industry. Globulins (salt soluble, vicilin, and legumin families) are the major dominant storage proteins in cottonseed and account for 60%–70% of seed proteins. Albumins (water soluble) and gliadins (alkali soluble) are in low concentrations. It is a rich source of protein characterized by a high concentration of amino acids. However, a relatively low concentration of lysine restricts the quality of protein in CSM. It's use as feed material in poultry nutrition is limited mainly due to the presence of free gossypol in cotton, as well as high variability in nutrient concentration [6]. 80% of defatted cottonseed is used as fertilizer or substrates for edible mushroom, while only 5-10% is used as feed materials, which results in a substantial wastage of protein.

The presence of amino and carboxylic acid imparts protein hydrophilicity, which is recognized as a surface-active component. Recently, a protein used as non-petroleum, safe, and biodegradable

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Paper received: 27.03. 2023.

Paper accepted: 06. 05. 2023.

Paper is available on the website: [www.idk.org.rs/journal](http://www.idk.org.rs/journal)

and renewable resource was focused by many researchers. Carboxyl, amido, amide, hydroxy, sulfhydryl groups exist in the main chain of the protein molecule. Chemical modification such as grafting, copolymerization, and crosslinking could result in biomaterial production like hydrogel of proteins [7].

Based on the above considerations, an attempt has been made in this research mainly to extract protein from the cottonseed flour. The effect of different extraction parameters were investigated and the resulting protein extraction would be taken for further investigation like modification, such as graft copolymerization with other monomers, in order to prepare different functional cottonseed protein derivatives or composite materials.

## 2. MATERIALS AND METHODS

### 2.1. Materials

**Cottonseed meal:** Dry cottonseed meal was provided by Teshome Eshetu Edible Oil Factory, Adama, Ethiopia.

**Chemicals:** Sodium hydroxide (NaOH), Ethanol, Hydrochloric acid (HCl), and other auxiliary chemical were used for protein extraction.

**Equipment:** Different size beakers, digital balance, thermometer, litmus paper, oven dryer, centrifuge, non-woven heat-sealable pouch, petri dish, porous filter plate, filter paper, Infrared Analysis (FT-IR), Freeze dryer.

### 2.2. Methods

The general flow for the extraction of protein from cottonseed flour is presented in Figure 1.

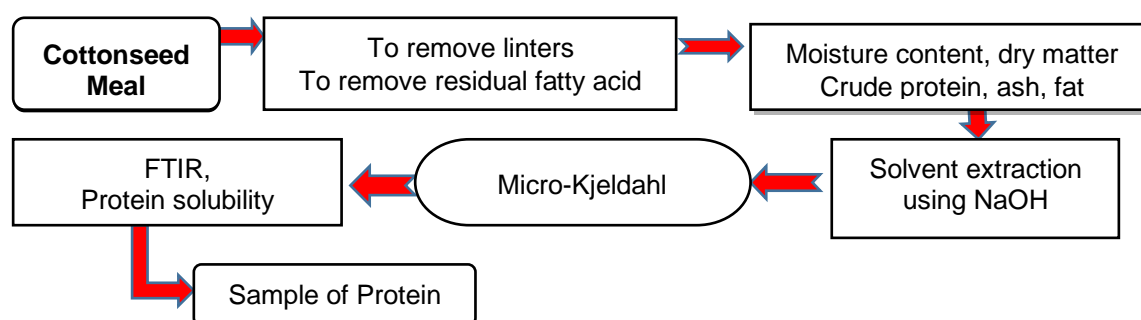


Figure 1. Extraction of Protein from Cottonseed Meal

Slika 1. Ekstrakcija proteina iz brašna pamuka

#### 2.2.1. Pretreatment & proximate analysis of defatted cottonseed meal

##### 2.2.1.1. Defatting

The cotton seed meal was defatted using the method described by [8]. Meal (1 kg) was added to diethyl ether and stirred at room temperature for 60 min, and then left standing, until natural sedimentation of the meal and organic solvent separation occurred. Recovery of organic solvents was then completed, and the precipitated cottonseed meal was added to diethyl ether, and this procedure was repeated three times. The fat percentage was then calculated using equation 1:

$$\text{Fat \%} = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

where  $W_1$  &  $W_2$  are weights (g) of sample before and after defatting respectively.

##### 2.2.1.2. Moisture Content

Moisture content was determined after drying 5 grams of cottonseed meal in an oven at 105°C for 3 hours according to AOAC (2000) using equation 2:

$$\text{Moisture \%} = \frac{W_1 - W_2}{W_1} \times 10 \quad (2)$$

where  $W_1$  &  $W_2$  are weights (g) of sample before and after drying respectively.

##### 2.2.1.3. Crude Protein

Crude protein was quantified by the standard micro-Kjeldahl nitrogen method. 0.5 g of each sample was measured and placed in the digestion flasks. Then 1 g of Kjeldahl catalyst and 6 ml of sulfuric acid were added and the sample was allowed to digest. The sample was digested at 350°C for 180 min. After the digestion, it was distilled with water, 40% sodium hydroxide, 4% of boric acid and reagents followed by acid - base titration with hydrochloric acid. According to AOAC (2000) crude protein was estimated by multiplying the nitrogen content with a factor of 6.25 using equation 3:

$$\text{Protein \%} = ((A - B) \times M \times 1.4007 \times 6.25) / W \quad (3)$$

where,

A = Volume (ml) of 0.2 M HCl used in sample titration

B = Volume (ml) of 0.2 M HCl used in blank titration

$M$  = Molarity of HCl

$W$  = Weight (g) of sample

14.007 = Atomic weight of nitrogen

6.25 = Protein-nitrogen conversion factor for fish and its by-products

#### 2.2.1.4. Ash Content

Ash content was determined as the weight of the residue after 2 grams of the sample with pre weighted crucible which was ashed at 575°C in a muffle furnace overnight as per Association of Official Analytical Chemists [AOAC (2000)] procedure using equation 4:

$$\text{Ash \%} = \frac{\text{weight of crucible with ash}}{\text{weight of crucible with sample}} \times 100 \quad (4)$$

#### 2.2.2. Protein Extraction

Protein extraction was carried out using sodium hydroxide (NaOH) alkaline solution. Central composite designs (CCD) with three factors (concentration, temperature, and time) and a

$$\text{Extraction rate \%} = \frac{\text{Total protein after extraction}}{\text{Total original cottonseed protein}} \times 100\% \quad (5)$$

#### 2.2.3. Protein Characterization

##### 2.2.3.1. Solubility

The solubility of extracted protein was determined according to the method described by [9]. In brief, 0.5 g of protein was dispersed in 50 ml of deionized water which was maintained at different pH values (pH 3.0, 5.0, 7.0, 9.0, and 11.0) (solutions prepared using water and sodium hydroxide). The mixture was then stirred for 1h and centrifuged at 1700×g for 30 min. The protein content of the supernatants was determined using the Kjeldahl method. Protein solubility was expressed as percentage ratio of supernatant protein content to the total protein content.

##### 2.2.3.2. Water Absorbency

Water absorbency was determined using the method described by [10]. Protein sample (0.5 g) was dispersed in distilled water in a 10 ml pre-weighed centrifuge tube. The dispersion was shaken for 1 min, allowed to stand for 30 min, and centrifuged at 1700×g for 30 min at room

constant solvent to flour ratio were used to determine the number of run and combination. Protein purity and flour dissolution percentage has been taken as response.

Defatted cottonseed meal protein was extracted with a solvent-to-flour ratio of 20:1 using 0.1-0.5 M NaOH in water bath using AUTOWASH 2762 after the desirable temperature, pH and time were adjusted. After extraction, the solution was filtered immediately. Then the extract was acidified with 1M hydrochloric acid and then kept steady at 5°C for 24 hours until the isoelectric value was attained (precipitation). Generally, isoelectric point ( $pI$ ) of plant oil proteins ranges from pH 4 to 5. Due to different techniques of oil extraction, the highest precipitation for cottonseed protein from various source usually occurs at pH between 3.5 to 5.5. Later, the precipitated protein was washed with 50% 2-propanol and then freeze-dried. The following equation was used to determine extraction rate % (equation 5):

temperature. The supernatant was decanted, excess water in the upper phase was drained for 10 min, and the tube containing the protein residue was weighed again to determine the amount of water retained per gram of sample.

##### 2.2.3.3. FTIR Analysis

The functional groups of sample protein were characterized using FTIR spectrophotometer (JASCO FT/IR - 6600). KBr pellets were prepared using 1-2 mg of protein powder which was observed under IR. Transmittance values were recorded at wave numbers between 4,000 and 400  $\text{cm}^{-1}$ .

## 3. RESULTS AND DISCUSSION

### 3.1. Composition of Cottonseed Meal

The proximate composition of cottonseed meal was determined in terms of moisture content, fat content, residual oil content, crude protein content and total ash %. Analytical data on the cottonseed flours before protein extraction are shown in Table 1.

Table 1. Standard and obtained values of CSM

Tabela 1. Standardne i dobijene vrednosti CSM

Flour source	Moisture (%)	Total fat (%)	Residual oil (%)	Crude protein (%)	Ash, (%)
Standard as per Food Safety and Standard Regulations (FSSR, 2011)	< 8% dry wt.	< 3% dry wt.	-	> 45% dry wt.	< 7% dry wt.
Cottonseed flour in current study	4.68%	2.83	6.23	35.47	5.90

The cottonseed meal cake for this study was obtained from one round mechanical press technique. Table 1 shows that it yields lower oil yields (6.23%) than scientific solvent oil extraction, which has low oil residual, i.e., the remaining oil in mechanical press meal is high. The meal can leave the press with up to 20% of the original oil still present in it [11]. As a result, there is residual oil in the cake. Similarly, a lower crude protein content of the current study CSM than the standard may be due to factors like varieties and location differences, varied agronomic practices (fertilizer), and differences in degree of cortication, proportion of husk, and processing methods during oil

extraction [6] and the lower fat content which is in standard might be due to the defatting process.

### 3.2. Protein Extraction

To optimize better combination of factors for a maximum protein yield and flour dissolution percentage i.e. protein in the form of solution before precipitation and maximum protein purity percentage, a central composition design was used. The maximum protein dissolution percentage was calculated using the residual undissolved impurities and the maximum protein purity percentage was quantified by the standard micro-Kjeldahl nitrogen method using the conversion factor of 6.25. The results are presented in Table 2.

Table 2. Protein Purity and flour Dissolution Percentage

Tabela 2. Čistoća proteina i procenat rastvorljivosti brašna

Run	Factor 1 A: Concentration (M)	Factor 2 B: Time (Min)	Factor 3 C: Temperature (°C)	Response 1 Dissolution (%)	Response 2 Protein purity (%)
1	0.3	37.5	45	55.44	57.77
2	0.3	37.5	45	57.59	56.90
3	0.1	60	60	64.77	67.15
4	0.3	37.5	19.77	54.49	53.60
5	0.5	15	30	60.45	64.86
6	0.3	37.5	45	52.81	54.23
7	0.63	37.5	45	62.19	59.89
8	0.3	37.5	45	56.02	54.86
9	0.1	15	30	41.48	50.86
10	0.5	60	30	63.72	59.23
11	0.5	60	60	67.55	53.98
12	0.5	15	60	58.65	56.27
13	0.1	60	30	48.49	60.93
14	0.3	75.340	45	60.05	59.90
15	0.1	15	60	53.80	64.90
16	0.3	37.5	70.23	74.17	56.49
17	0.3	37.5	45	51.32	54.27
18	0.3	37.5	45	57.12	54.18

#### 3.2.1. Effect of concentration on dissolution percentage

The effect of concentration on the dissolution percentage is clearly shown in Fig. 2a. The lines in the Figure from top to bottom describe the relation between predicted, calculated and actual value of dissolution percentage respectively. Concentration has a significant effect on the dissolution percentage and as it increases the dissolution percentage also increases up to the optimum value. The degree of protein solubility in an aqueous medium is the result of electrostatic and hydrophobic interactions between protein molecules. At pH above or below isoelectric pH

(pI), the proteins become negatively or positively charged depending on the pH, resulting in electrostatic repulsions between molecules and hydration of charged residues, contributing to the solubility of proteins [12]. However, cottonseed protein is prone to degradation and denaturation and difficult to be kept in strong alkaline condition [7].

#### 3.2.2. Effect of time on flour dissolution percentage

Figure 2b shows the significant effect of time on the dissolution percentage. As time increases the dissolution percentage also increases. After 15 minutes, extraction rate amounted to 50%. While after 40 minutes the extraction rate was seen slowly decreasing.

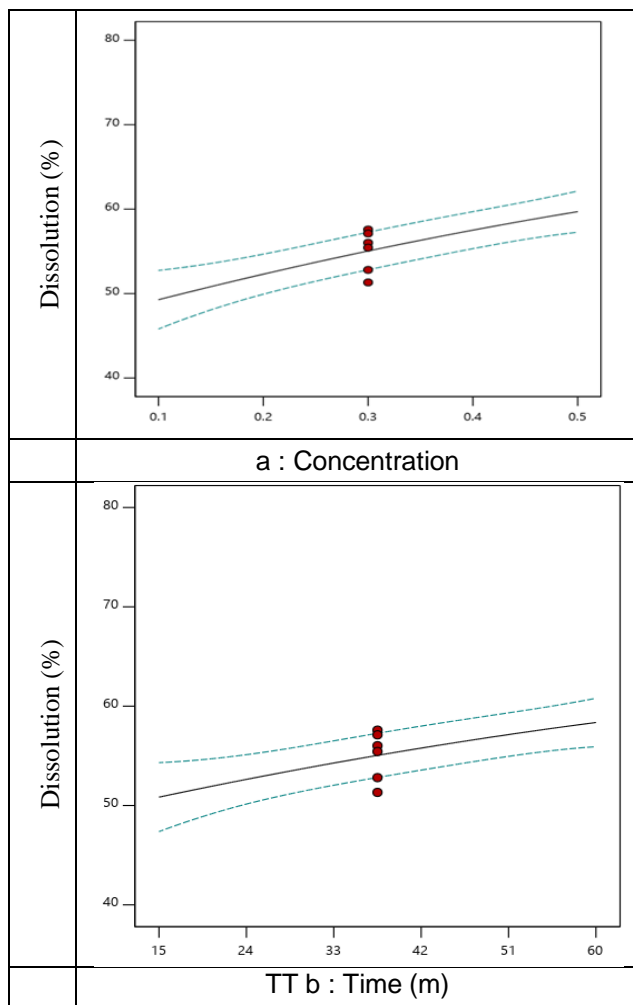


Figure 2. Effect of concentration (a) and time (b) on dissolution percentage

Slika 2. Uticaj koncentracije (a) i vremena (b) na procenat rastvaranja

### 3.2.3. Effect of temperature on dissolution percentage

Temperature has a significant effect on the dissolution percentage of cottonseed flour protein. Fig. 3 shows that, with the increasing temperature, flour protein dissolution percentage increased. Dissolution is directly proportional to increase of temperature. When temperature is increased, molecules move fast and the mass transfer rate of interface between solid and liquid is developed. Therefore, increasing temperature promotes mass transfer and solubility, reduce viscosity of solution and thus increase extraction rate. However, high temperature causes the reduction of protein activity and thermal denaturing of protein. Additionally, with the increase of temperature, color of the protein extract becomes dark.

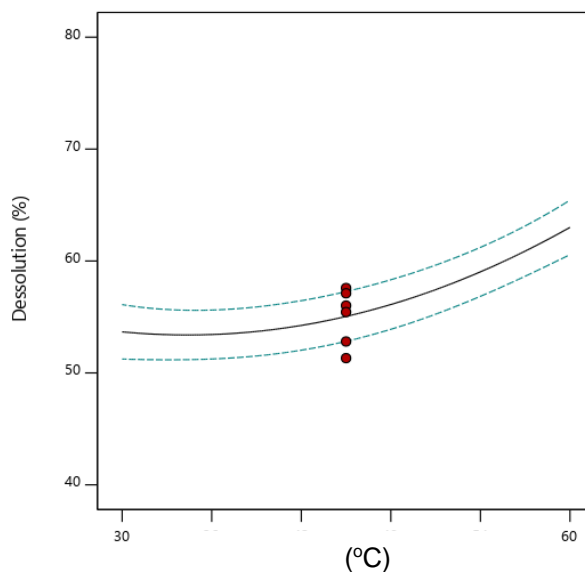


Figure 3. Effect of temperature on dissolution percentage

Slika 3. Uticaj temperature na procenat rastvaranja

### 3.3. Characterization of cottonseed protein

#### 3.3.1. Protein solubility

During an increase or a decrease in pH of the protein solution, the side chains can assume strong positive or negative charges, resulting in strong repulsive forces between proteins that pull proteins apart from each other. These strong positive or negative charges will favor the protein-water interactions thus causing the solubility of proteins. Figure 4 shows the protein solubility at different pH conditions. The maximum protein solubility occurs at alkaline condition i.e. at pH 11, whereas, the least protein solubility occurs at an acidic pH of 5 [13]. This result was due to the reduced interaction between protein and water, and this phenomenon enhances protein-protein interactions, resulting in the protein aggregation and precipitation. The degree of protein solubility in an aqueous medium is the result of electrostatic and hydrophobic interactions between protein molecules. At pH above or below isoelectric pH (pI), the proteins become negatively or positively charged depending on the pH, resulting in electrostatic repulsions between molecules and hydration of charged residues, contributing to the solubility of proteins [13, 14]. Strong electrostatic repulsions among protein molecules and the increased protein-solvent interaction at this pH could have contributed to this increased solubility [12].

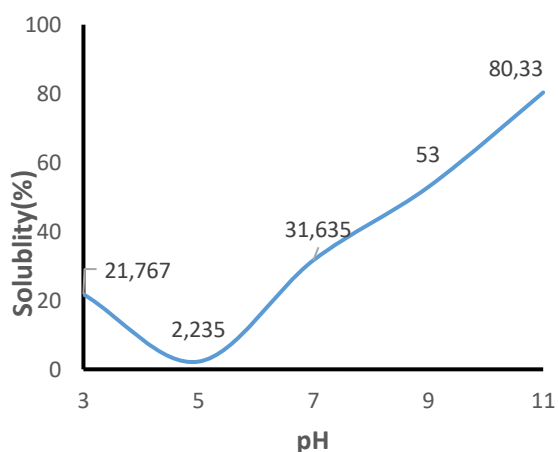


Figure 4. Protein solubility under different pH

Slika 4. Rastvorljivost proteina pri različitim pH

### 3.3.2. Protein water absorbency capacity (WAC)

Protein water absorbency is the index of the ability of proteins to absorb and retain water. It directly influences the water holding capacity of a synthesized material from proteins for various applications. The average water holding capacity of the extracted cottonseed protein was 1.624 g/g (protein flour to water ratio) and this result indicates the presence of more of soluble proteins and higher availability of polar amino acids in the cottonseed protein isolate. It may be concluded based on the results that, the cottonseed protein demonstrates a desirable ability to absorb and retain the water [15].

### 3.3.3. FTIR Analysis

FTIR spectra of raw cottonseed meal and extracted protein are presented in Fig. 5. It may be observed that, both the raw cottonseed meal and protein isolate samples display similar relevant peaks. The characteristic peak at  $1653\text{ cm}^{-1}$  for protein isolate can be attributed to amide I. Amide I corresponds to the C=O stretching vibration with minor contribution from C-N stretching vibration. Similarly, the peak at  $1532\text{ cm}^{-1}$  can be attributed to amide II which arises from N-H bending and C-N stretching vibration. The broad peak observed between  $3300\text{--}3400\text{ cm}^{-1}$  results from the overlapping contributions of the stretching vibrations of both O-H and N-H groups. Furthermore, the area of the peak at  $1385\text{ cm}^{-1}$  is related to the symmetric stretching vibrations of COO- groups due to the carboxyl group vibrations of aspartic and glutamic acids and belongs to the C-N for amide III [16]. Interestingly, the presence of the peak at  $1532\text{ cm}^{-1}$  (amide II) in protein isolate sample which was not present on raw sample indicates that the extraction was highly targeted to the amide groups in proteins or protein-like compounds isolation. The transmission peak at  $1652\text{ cm}^{-1}$  which is related to C=O stretching vibration with minor contribution from C-N stretching vibration in cottonseed protein isolate sample demonstrates that, the extraction was significantly focused on isolating the protein like compounds from the raw sample [17].

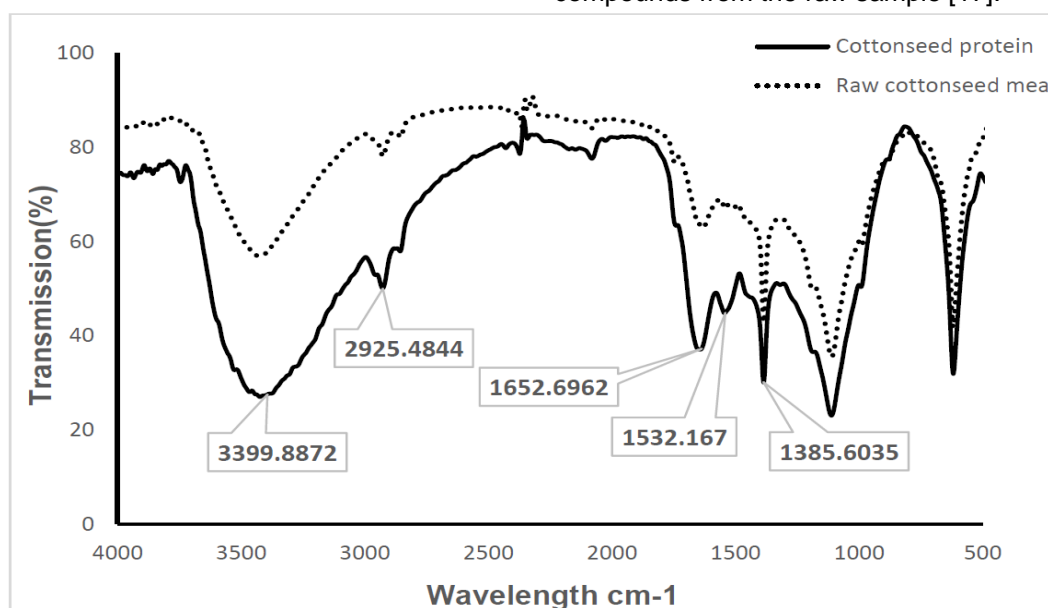


Figure 5. FT-IR spectra of raw cottonseed meal and isolated protein

Slika 5. FT-IR spektri sirovog pamučnog brašna i izolovanog proteina

#### 4. CONCLUSION

This work focused on extraction of protein from cottonseed meal using NaOH as a solvent with a solvent-to-flour ratio of 20:1. Several factors were investigated to determine their effects on the extraction of proteins from cottonseed meal and to optimize the extraction protocol. The optimized parameters used for extraction were a concentration of 0.1M NaOH, temperature 67°C, pH 12 and a time of 90 minutes. Under these optimum conditions, it was possible to extract about 81% of protein from the raw cottonseed meal. The flour dissolution percentage indicates the amount of dissolved flour which can be precipitated for protein isolation. Temperature has a significant effect on flour dissolution since the energy of the molecule increases as the temperature increases which promotes mass transfer and solubility. Furthermore, this study provided basic information on the characteristics of extractable proteins. The micro-Kjeldahl nitrogen method result indicated that the extractable proteins had a better protein purity as this may be useful for the production of protein based bio-products. The protein was also characterized using FTIR and the protein solubility was also determined. It is possible conclude that, the extracted protein could be directly used for any chemical modification without any further treatments. As a result, rather than burning or dumping cottonseed wastes in landfills, it is preferable to valorize them by refining important polymers such as protein from them, thereby reducing their contribution to greenhouse gas emissions for environmental protection.

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## IZVOD

### **Bio-rafiniranje sačme pamučnog semena iz naftne industrije za izolaciju ekološki prihvatljivog prirodnog proteinskog polimera**

Sačma od semena pamuka (CSM) je nusprodukt naftne industrije. Globulini (familije rastvorljivih u soli, vicilina i legumina) su glavni dominantni proteini za skladištenje u semenu pamuka i čine 60-70% proteina semena. Međutim, oni su neprikladni za ljudsku ishranu zbog sadržaja gosipola. U ovom radu, protein je ekstrahovan iz odmašćenog pamučnog brašna alkalnim rastvorom i ispitivano je njegovo taloženje na različitim pH vrednostima. Sirovi protein je kvantifikovan korišćenjem standardne mikro-Kjeldahlove metode azota. Obavljen je centralni ekspert za dizajn kompozita i proučavan je uticaj varijabli na ekstrakciju proteina, odnosno koncentracije rastvarača za ekstrakciju, temperature i vremena. Optimalna šema ekstrakcije je dobijena na 0,1M NaOH sa odnosom materijala i tečnosti (MLR) 1:20 na pH 12 korišćenjem temperature od 67°C i vremena od 90 min. Pod ovim uslovima, odgovor daje procenat rastvorljivosti brašna od 80,52% i procenat čistoće proteina 80,40%. Pored toga, utvrđena je rastvorljivost proteina i kapacitet apsorpcije vode, a hemijska struktura proteina je okarakterisana pomoću infracrvene spektroskopije Furijeove transformacije.

**Ključne reči:** brašno od pamučnog semena, protein semena pamuka, proteinsko brašno semena pamuka, ekstrakcija, karakteristike proteina, precipitacija

Naučni rad

Rad primljen: 27. 03. 2023.

Rad prihvaćen: 06. 05. 2023.

Rad je dostupan na sajtu: [www.idk.org.rs/casopis](http://www.idk.org.rs/casopis)