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Extraction of polyphenolic compound gossypol from defatted cottonseed using butanol-ethanol-water mixed solvent

Surinder Singh*[a,b], S. K. Sharma[b] and S. K. Kansal[a]

[a]Dr. S. S. Bhatnagar University Institute of Chemical Engineering & Technology, Panjab University, Chandigarh-160 014, Punjab, India

[b]University School of Chemical Technology, Guru Gobind Singh Indraprastha University, Sector-16, Dwarka, Delhi-110 078, India

E-mail: sonubhinder@gmail.com

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Herein a method for extracting gossypol from cottonseed defatted at controlled temperature under acidic conditions to prevent gossypol binding with protein was described. The temperature of extraction was contained up to 348 K and binding of gossypol with protein was avoided. The solvent extraction was carried in acidic medium to allow hydrolysis of bound gossypol from defatted cottonseed meal. The high boiling solvent system utilized was butanol-ethanol-water (80:15:5 v/v) and 0.5 *M* citric acid for providing the acidic medium. The mixed solvent was capable of extracting 91.22% gossypol from defatted cottonseed meal at optimum conditions (348 K, solvent/seed ratio 15 and 180 min). The kinetics of extraction was observed to follow pseudo-second order rate law. Evaluated thermodynamic parameters justified the extraction to be endothermic and irreversible.

Keywords: Solvent extraction, gossypol, kinetics, solvent, acidic medium.

Introduction

Gossypol is a polyphenolic compound found in cotton plant and basically a terpenoid aldehyde and having yellow color. Gossypol belongs to the genus of Gossypium and family of *Malvaceae*[1]. Cottonseed (Gossypium sp.) is composed of 80 genera and around 1000 species. The major species are *Gossypium arboreum, Gossypium herbaceum, Gossypium barbadense* and *Gossypium hirsutum* which are cultivated all around the world. Cotton is mainly harvested for the production of fiber along with other byproducts such as seeds, hulls and roughage. The yield of oil from cottonseeds is affected by the species, climatic factors, pretreatment of the seeds, method of extraction and post-extraction treatments[2]. Gossypol and other terpenoids are present throughout the cotton plant in the glands of foliage, floral organs, and bolls, as well as in the roots. Gossypol acts as a defense compound in cotton plant but is anti-nutritional and toxic compound. Gossypol acts as phytoalexin and protects the plant from insects, pests and pathogens[3]. Gossypol possesses unique biological activities like antifertility agent, anti-tumoral activity, anti-malarial and anti-viral agent[4–6]. The amount of gossypol varies from 0.02 to 6.64% in different parts of cotton plant[7,8]. Although gossypol is non-steroidal compound but it curbs sperm production in men and motility in animals. Gossypol showcases the contraceptive effect by restraining enzyme systems which effect energy metabolism in human sperms[9,10]. Gossypol is also classified as dimeric-sesquiterpenoid. Sesquiterpenoids are the type of terpenes which possess three isoprene units and these are useful in saving the cotton plant from the attack of pathogens and insects. These act as anti-microbial agents and prevent the plant from bacteria and fungi etc. by damaging their cell walls. These are also an active ingredients in many drugs for treatment of diarrhea, burns, influenza, neural damage, migraine and cancer treatment[11]. Owing to its highly useful anti-cancer and industrial application like anti-oxidant properties, gossypol is a compound of utmost interest and have been investigated by the researchers worldwide. But is also act as a toxic material in cottonseed meal when it binds with the

lysine of the protein and gets converted from free to bound gossypol. This bound form denatures the protein and imparts a dark brownish-black color to the extracted oil which further needs refining and detoxification[12,13].

The global production of cottonseed in 2017-18 and 2018-19 was around 44.98 and 43.45 million metric tons respectively and India, China, USA, Brazil and Pakistan are the world's largest cotton producers. The available cottonseed protein around 11.5 million metric tons can generate the protein requirements of around half a billion people at the rate of 50 g per person[14]. During the processing of cottonseed, 62.5% of the weight of the product is actually obtained as the seed. About 20–25% oil is produced from cottonseeds along with 26–30% hulls, 8.5% linters and 45% meal[15]. Cottonseed meal contains about 30–44% good quality protein which can be utilized for animal and aquaculture feeds in place of soybean. However due to the presence of the toxin gossypol the use of cottonseed meal as rich protein source diet is limited in monogastric animals and fish. Gossypol is however a useful byproduct and an effective therapeutic agent[16–19].

During the commercial solvent extraction of cottonseed oil, gossypol along with other pigments gets co-extracted along with the oil which imparts dark color to the oil. The crude cottonseed oil holds around 0.21% gossypol depending on the nature and extent of heat treatment of the seed prior to extracting and expelling[20]. Besides imparting color to the oil, these pigments are toxic, which makes it necessary to impart intensive refining, clarification, re-refining, strong bleaching or a combination of these processes. This adds additional costs to the cottonseed processing. The presence of gossypol makes the cottonseed meal and protein unfit for animals and humans.

Binaphthalenic polyphenolic chemical structure of gossypol ($C_{30}H_{30}O_8$) is shown in Fig. 1. In gossypol two aromatic naphthalene groups and six hydroxyl groups are present at *ortho* positions, two of which are at *peri* positions (1,1′) in conjunction with the two aldehyde groups; which provide gossypol the high reactivity and capacity for tautomeric transformation as well to exhibit enantiomers[21].

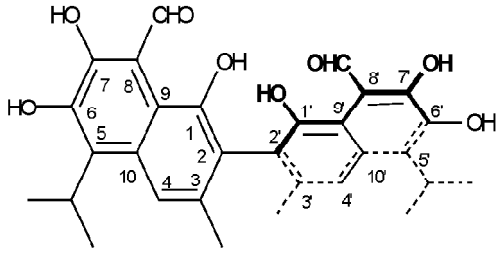

Fig. 1. Chemical structure of gossypol (Mol. wt. 518.56) IUPAC name 1,1′,6,6′,7,7′-hexahydroxy-5,5′-di-isopropyl-3,3′-di-methyl-(2,2′-binaphthalene)-8,8′-dicarboxaldehyde.

The binding of gossypol to lysine part of the meal protein occurs due to covalent bonds between the gossypol molecule and amine groups as shown in Fig. 2 as shown below.

Due to the binding the free gossypol which is otherwise non-toxic gets converted into the bound form which is toxic and denatures the meal protein[12]. The gossypol gets bound through covalent bonds between the free epsilon-amino groups from lysine and gossypol through Maillard or brown-

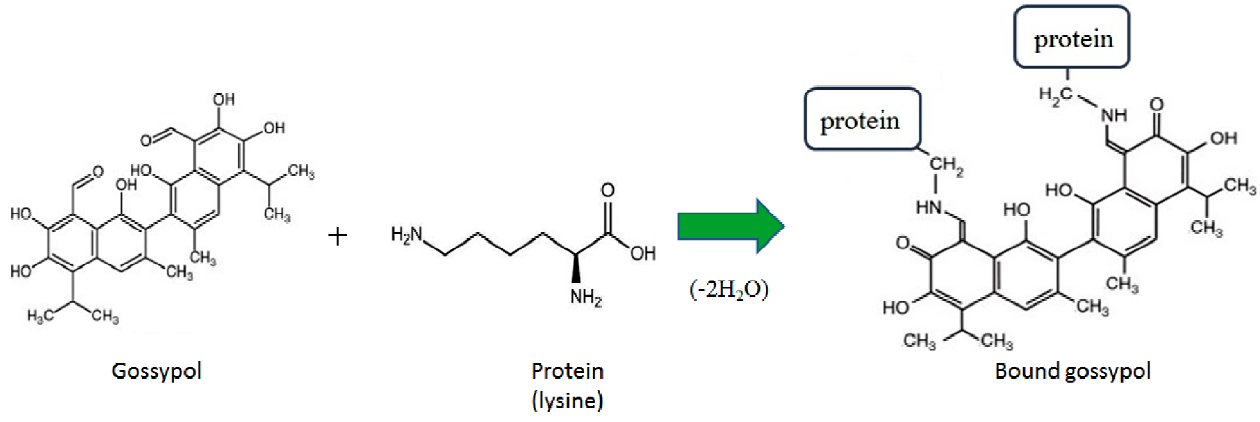

Fig. 2. Binding of gossypol with lysine of protein.

ing reaction[22]. Due to this binding effect, availability of amino acids especially lysine for absorption by the animals and poultry gets reduced and digestion problems arise[23].

Cottonseed contains 30–40% of good quality protein but during conventional processing the lysine residue of meal protein gets attached (bound) to gossypol and its nutritive value gets decreased and it causes toxicity to the ruminants and other animals if the free gossypol is greater than 450 ppm in the animal rations[24,25]. Hence there is a dire need to remove gossypol from cottonseed meal by solvent extraction or other techniques such as supercritical CO_2 extraction or adsorptive removal. Gossypol after extraction can be used as a useful byproduct having medicinal value and other applications which can be marketed.

Several techniques such as mechanical fractionation, gland flotation, hydraulic pressing, screw pressing, pressure cooking, liquid cyclone process, air classification, solvent extraction, adsorption, ultrasound assisted extraction, membrane separation, super critical CO_2 extraction and azeotropic solvent extraction have been applied by various researchers for extraction of gossypol[4]. Solvent extraction is the most commonly used method to extract gossypol from cottonseed meal. It was used first time in America in 1934 as an industrial process[26]. The efficiency of the solvent extraction process depends on the pre-treatment of the seeds and cleaning, process temperature, pressure, continuous/batch technique and design of extraction equipment. The selection of solvent depends upon the solubility of gossypol in desired solvent, effectiveness of the process, non-toxicity, reusability and low cost[27–30].

In solvent extraction, gossypol is dissolved in the liquid solvent by vigorous contact of the cottonseed with the liquid solvent. This is a two-step procedure in which first the solvent reaches the solid surface, then permeates into the gossypol glands. In second step the dissolved gossypol in the solvent diffuses back to the bulk solution[31]. Guangfeng Jia performed the gossypol acetic acid extraction from cottonseed soap stock using ultrasound-assisted extraction and crystallization method[32]. Kuk *et al.* performed solvent extraction using mixtures of isohexane and alcohols and acetone and hexane[33,34]. Different solvents have been employed for solvent extraction which include hexane, isohexane, ethanol, 2-propanol, 1-butanol, 1-butanol hydrochloride, cyclohexane, methylene chloride, trichloroethylene, acetone and mixed solvents like ethanol-methylene chloride, ethyl alcohol-hexane and isohexane and 2-propanol or ethanol[35–46]. Gossypol is insoluble in water.

From the literature it was found that mixed solvents have good capacity to extract gossypol and it was suggested to perform gossypol extraction with higher boiling solvents like isopropanol and butanol[3,4,43–46]. It was also pointed out in the literature that acidic extraction of gossypol is highly useful and if contained at low temperatures, the binding of gossypol with lysine of protein can be minimized[31,42,47,48]. Based on these considerations mixed solvent system i.e. butanol-ethanol-water was utilized to extract gossypol from cottonseed in acidic medium, using 0.5 *M* citric acid. The aim of the present work was to study the gossypol extraction and find the effect of process parameters affecting the extraction i.e. acid concentration, solvent to seed ratio, temperature and extraction time. The process parameters were optimized and kinetics of extarction was evaluated along with the thermodynamic parameters to study the feasibility of gossypol extraction. It was found that butanol-ethanol-water (80:15:5 v/v) was capable of extracting 91.22% gossypol from defatted cottonseed meal at optimum conditions i.e. at 0.5 *M* acid concentration, 348 K, solvent to seed ratio of 15 and extraction time of 180 min.

Materials and methods:

Cottonseed hybrid variety RCH-776 [BT cotton (*G. hirsutum*)] was obtained from local market. Commercial hexane was procured from S. D. Fine-Chem Ltd., India. 1-Butanol, ethanol, 3-amino-1-propanol, glacial acetic acid, citric acid and N,N-dimethyl formamide were purchased from Merck Specialities Private Ltd., India. Double distilled water was utilized for making the required stock solutions. Standard solutions of gossypol were made using gossypol standard purchased from Sigma Aldrich, India. All the reagents, solvents and chemicals used were of analytical grade. Cottonseed was defatted using n-hexane as per method described in the literature[31].

Analysis of total gossypol

Analysis of total gossypol was done using Bureau of Indian Standard method IS: 4876-1986[31,49]. 3-Amino-1-propanol along with glacial acetic acid and dimethyl-formamide in ratio 2:10:88 v/v was used as complexing reagent. Total gossypol present in cottonseed sample reacts with aniline

and a colored complex of gossypol is formed.

The total gossypol amount is determined by evaluating the difference between the absorption values of sample which has been reacted with aniline and absorption of blank using a UV-Visible spectrophotometer (Systronic, model 2202) as per the standard method. The absorbance of sample and blank was measured at wavelength of 440 nm using UV-Visible spectrophotometer[31,49].

Extraction of gossypol

The extraction experiments of gossypol from defatted cottonseed were performed by taking cottonseed samples kept at required temperatures with known solvent to seed ratio of butanol-ethanol-water (80:15:5 v/v) solvent and put in a flat bottom flasks. The seed-solvent mixtures were extracted at desired temperatures (318 to 348 K) using temperature controlled hot plates kept in glass enclosures. A stir bar was employed to impart thorough mixing and proper contact with the solvent. The samples were taken out for analysis after known periods of extraction time (i.e. 5, 10, 15, 30, 60, 120, 180 min). The samples were filtered using a buchner funnel and dried at temperature of 50°C by using a convection oven. All the extraction experiments were performed in duplicate and graphs with error bars have been shown.

Results and discussion

Effect of acid concentration:

The effect of acid concentration on gossypol extraction was studied for the mixed solvent butanol-ethanol-water (80:15:5 v/v) acidified with citric acid at different molar concentrations (0.3 *M* to 0.6 *M*). The experimental design used was one factor at a time (OFAT). One factor or parameter was varied at a time while other parameters were kept fixed. So when acid concentration was varied, the other process parameters viz. temperature, solvent to seed ratio and extraction time were fixed at 348 K, 15 and 180 min. The results of variation of acid concentration from 0.3 *M* to 0.6 *M* are shown in Fig. 3. It shows that the percentage gossypol extraction increased with increase in concentration of the citric acid used for acidifying butanol-ethanol-water solvent from 0.3 *M* (79.29%) up to 0.5 *M* (91.22%) but after this level no increase in extraction was noticed. This was attributed due to the fact that by increasing the molar concentration of the organic acid, the acidic effect of the solvent increases and acidic medium facilitates extraction of gossypol[47]. By

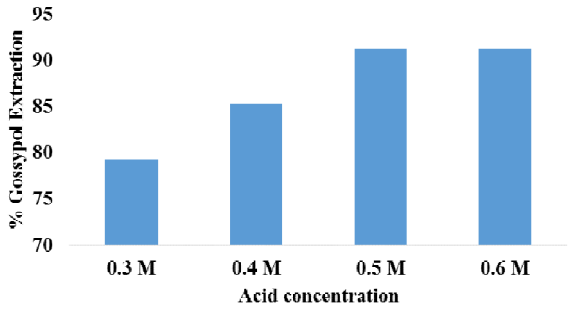

Fig. 3. The effect of acid concentration on gossypol extraction at 348 K and SR 15.

increasing the molar concentration of the acid, hydrolysis of gossypol takes place thereby increasing gossypol extraction up to optimum value. After optimum value of molar concentration (i.e. 0.5 *M*) no significant increase was observed in the gossypol extraction which is attributed to the maximum ion dissociation effect and thus after further increase in acid concentration no significant extraction is obtained.

Effect of solvent to seed ratio (SR):

To study the effect of solvent to seed ratio (SR) on percentage gossypol extraction using butanol-ethanol-water (80:15:5 v/v) solvent acidified by 0.5 *M* citric acid the experiments were performed by taking different solvent to seed ratios i.e. 5 to 20 and keeping at other parameters constant. The temperature was fixed at 348 K and acid concentration and time were fixed at 0.5 *M* and 180 min respectively. The results as shown in Fig. 4 show that % gossypol extraction increased from 78.23% at SR 5, to 85.61% at SR 10 and then to maximum value of 91.22% at SR 15 in 180 min. Afterwards increasing the SR to 20, produced no significant increase in the percentage gossypol extraction. The increase

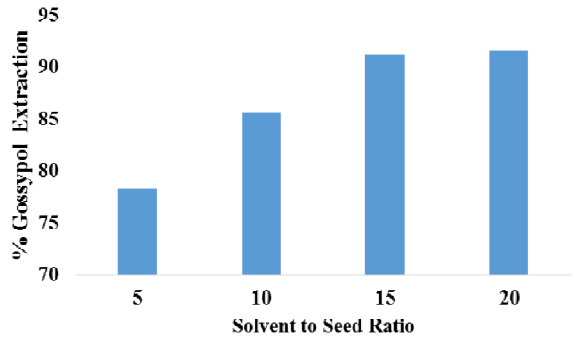

Fig. 4. The effect of solvent to seed ratio on gossypol extraction at 348 K and 180 min.

in gossypol extraction with SR was attributed to the effect of strong driving force of large quantity of fresh solvent available for gossypol extraction in the beginning with high contact of gossypol with the solvent. The increase in gossypol extraction by increasing the SR may be attributed to the solubility of the gossypol in the solvent. After reaching optimum SR the solubility reaches saturation point and no further extraction takes place.

On further increasing the SR to 20, no significant diffusion of the remainder gossypol in glands took place due to max solubility of solute in solvent, resistance of solids and liquid film present at solute-solid interface. Hence the SR of 15 was taken as optimum for further study.

Effect of temperature:

The effect of temperature on gossypol extraction was determined at optimized SR of 15 and extraction time of 180 min by varying the temperature values from 318 to 338 K. From the results of extraction at different temperatures values as shown in Fig. 5(a), it was found that the gossypol extraction increased with increase in temperature from 318 to 348 K and maximum extraction was observed at temperature of 348 K. After increasing the temperature to further higher values i.e. 358 K, no significant increase in % extraction was obtained as shown in Fig. 5 (b), so 348 K was chosen as the optimum temperature.

The enhancement in the gossypol extraction by increase in temperature could be due to the fact that at higher temperatures, solubility of gossypol increases in the solvent and also the reduction in viscosity of the solvent takes place. Also temperature helps in overcoming the energy required to break the solute-solid barrier at increased temperatures. The results of percentage gossypol extraction at SR 15 and 30 to 180 min are shown in Fig. 5(a) with error bars. The experimental results indicates that percentage extraction of gossypol increased from 73.67% at 318 K to 91.22% with increase in temperature from 318 K to 348 K respectively in 180 min.

Effect of extraction time:

To study the effect of time on solvent extraction of gossypol using defatted meal with butanol-ethanol-water (80:15:5 v/v) acidified with 0.5 *M* citric acid, extraction experiments were carried out at extraction times of 5 to 180 min keeping all other parameters fixed. The results of variation of gossypol extraction with time at temperatures of 338 K and 348 K are shown in Fig. 6. The results indicate that there is fast increase in the gossypol extraction initially from 0 to 60 min (80.69% at 338 K and 83.5% at 348 K), thereafter the extraction becomes slow and reaches equilibrium in about 180 min (86.66% at 338 K, 91.22% at 348 K). The % increase in extraction is 4.56% between temperature 338 K and 348 K (Fig. 6) which is a significant increase in gossypol extraction. The extraction after 180 min is negligible due to the effect of saturation of the extracted solute. The initial high extraction (first one hour) is due to the available strong driving force of fresh solvent while later on the extraction rate lowers due to high resistance of solute and low diffusion rate of gossypol from solid surrounding the gossypol glands and solubility limit of gossypol at 338 and 348 K.

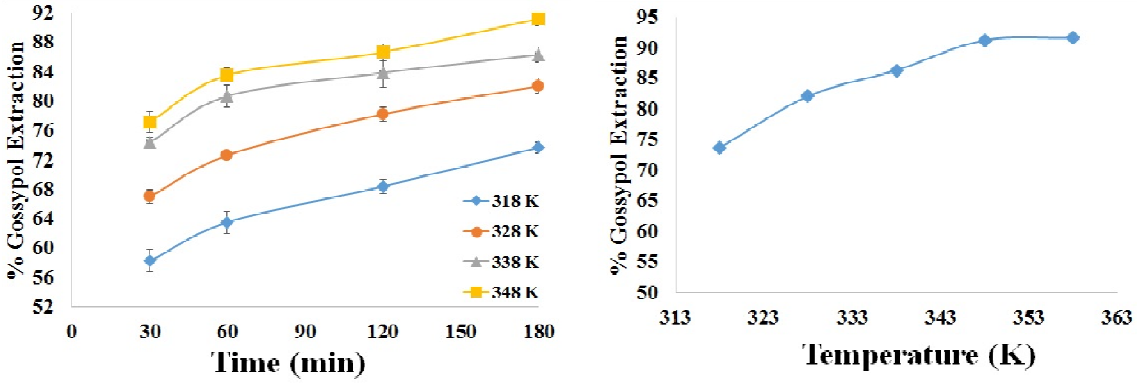

Fig. 5. (a) Effect of temperature with time on extraction of gossypol at SR 15 and 180 min and (b) effect of temperature on extraction of gossypol at SR 15 and 180 min.

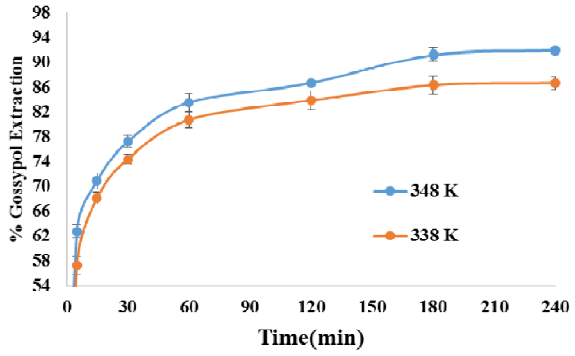

Fig. 6. Effect of extraction time on gossypol extraction at SR 15 and 338 and 348 K.

was equal to $1/E_i$ and rate constant k was found using the relation

$$E_i = kC_e^2 \quad (1)$$

The linear relationship of t/C_t versus time shown in Fig. 7(b) confirmed the pseudo-second order type extraction kinetics[21]. The values of kinetic parameters for pseudo-second order model are given in Table 1. Pseudo-first order model was also analyzed to explain the kinetics of gossypol extraction as shown in Fig. 8[55]. The Fig. 8 shows the plot between log (C_e–C_t) and time't'[54].

The pseudo-first order model does not fit the data well

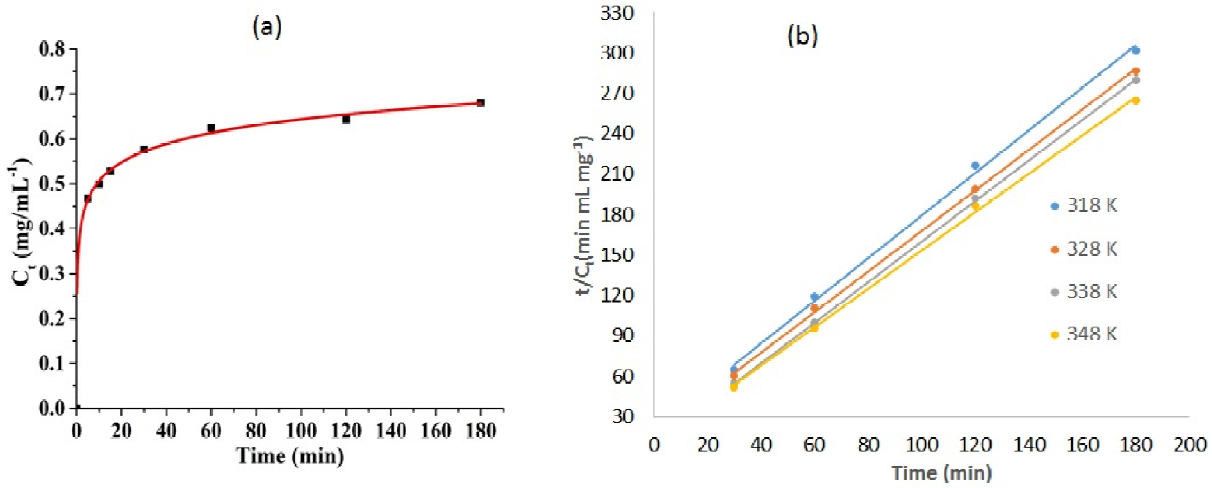

Fig. 7. (a) Plot of extraction of gossypol at 348 K at SR 15 and (b) second order kinetics of gossypol extraction at different temperatures.

Kinetics of extraction:

The kinetics of gossypol extraction was analyzed using a pseudo-second order kinetic model and already described in literature[31,50–53]. The results of gossypol extraction rate versus time (C_t vs t) and a series of plots of t/C_t vs t (linear form of rate equation) are shown in Fig. 7(a) and Fig. 7(b). In the beginning period gossypol extraction is fast due to the effect of strong driving force of the fresh solvent. The extraction rate reduces in the end due to high resistance of solute-solid binding near the equilibrium. The values of kinetic parameters viz. initial extraction rate, E_i pseudo-second order rate constant, k, and the solute concentration at equilibrium, C_e were evaluated by determining the slope and intercept of graphical plot shown in Fig. 7(b). From the plot of t/C_t versus time, the calculated slope was equal to $1/C_e$, the intercept

Table 1. Kinetic parameters of gossypol extraction using pseudo-second order model

SR (mL/g)	Temp. (K)	C_e (mg mL^{-1})	E_i (mg mL^{-1}min^{-1})	k (mL mg^{-1}min^{-1})	R^2
15	318	0.6172	0.0455	0.1193	0.9980
15	328	0.6671	0.0559	0.1257	0.9950
15	338	0.6744	0.0697	0.1532	0.9993
15	348	0.7027	0.0917	0.1856	0.9980

for all points and throughout the extraction process as can be seen from Fig. 8, although it seems to fit the data at some initial points corresponding to about 60 min, so this model was rejected. Hence it was concluded that the kinetics of gossypol extraction was fully described by pseudo-second order model as it fits the data fully over the entire extraction range.

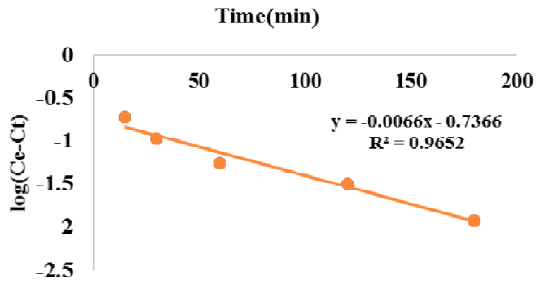

Fig. 8. Pseudo-first order kinetics of gossypol extraction at 348 K.

Mechanism of extraction:

The mechanism of gossypol extraction consists of a two-step procedure:

In first part the gossypol contained in solid is contacted with the solvent during which the solid gets solubilized in the solvent and the easily soluble gossypol gets extracted. During the second part intense scrubbing of solid takes place at the solid-liquid interface and transfer of solute takes place from inside of the solid to liquid phase by molecular diffusion.

Table 1 shows that the kinetic parameters of gossypol extraction i.e. C_e, E_i, k all increased with increase in temperature, thus temperature had a positive effect on the gossypol extraction kinetics. The another important parameter i.e. activation energy of extraction; E was also calculated using modified Arrhenius equation[31,55] and as given below:

$$\ln(k) = \ln(k_o) + \left(\frac{-E}{R}\right)\frac{1}{T} \quad (2)$$

The values of E and k_0 were calculated from the slope and intercept of graphical plot between ln (k) and 1/T as shown in Fig. 9. The obtained values of E and k_0 were and 13.884 kJ mol^{-1} and 3.0668 mL g^{-1} min^{-1} respectively which shows that gossypol extraction is an endothermic process.

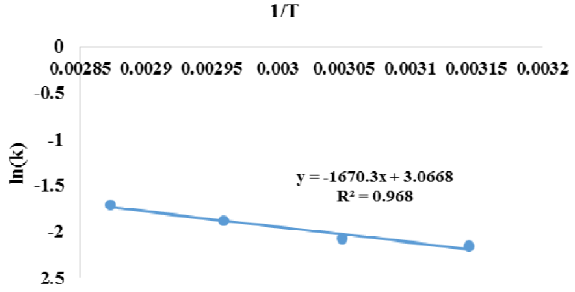

Fig. 9. Plot between second-order extraction rate constant, ln (k) and temperature (1/T).

Thermodynamic parameters:

Thermodynamic parameters i.e. equilibrium constant, (K_e) enthalpy change (ΔH^o) and entropy change (ΔS^o) for the gossypol extraction were evaluated using the following equations[31]:

$$K_e = \frac{C_e}{C_{ss}} \quad (3)$$

$$\Delta G^o = -RT \ln K_s \quad (4)$$

$$\ln K_e = \left(-\frac{\Delta H^0}{R}\right)\frac{1}{T} + \frac{\Delta S^0}{R} \quad (5)$$

Van't Hoff plot as shown in Fig. 10 between ln K_e vs l/T was plotted to calculate the values of ΔH^o and ΔS^o using slope and intercept of the graph. The obtained values of thermodynamic parameters K_e, ΔG^o, ΔH^o and ΔS^o are shown in Table 2. The thermodynamic parameters (ΔG^o, ΔS^o and ΔH^o) confirmed that the gossypol extraction was spontaneous, irreversible and endothermic.

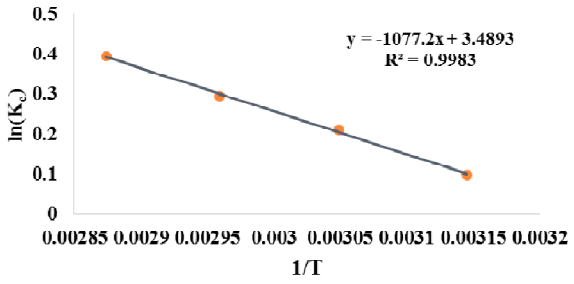

Fig. 10. Vant Hoff's plot of ln (K_e) vs 1/T for gossypol extraction.

Table 2. Thermodynamic parameters for gossypol extraction

SR (mL/g)	Temp. (K)	Equilibrium constant (K_e)	Gibbs free energy (ΔG^o) (J/mol)	ΔH^o (J/mol)	ΔS^o (J/mol K)
15	318	1.1045	–262.802	8955.84	29.01
15	328	1.3108	–574.523		
15	338	1.3440	–830.956		
15	348	1.4849	–1143.71		

Conclusions

(i) Butanol-ethanol-water (80:15:5 v/v) solvent acidified with citric acid was found to be potential solvent for gossypol extraction.

(ii) The solvent was capable of extracting 91.22% gossypol from cottonseed meal at optimum conditions i.e. acid concentration of 0.5 *M*, SR of 15, temperature of 348 K and time of 180 min.

(iii) The kinetics of extraction followed pseudo-second order.

(iv) The initial extraction rate E_i, solute concentration at equilibrium, C_e and second-order extraction rate constant k, were evaluated to be 0.0917 mg mL^{-1} min^{-1}, 0.7027 mg mL^{-1}, and 0.1856 mL mg^{-1} min^{-1} respectively at 348 K and SR 15 using the model.

(v) The extraction of gossypol was positively affected by temperature and activation energy of extraction was found out to be 13.884 kJ mol^{-1}.

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