



Optimization of extraction yield of carthamine and safflower yellow pigments from safflower (*Carthamus tinctorious* L.) under different treatments and solvent systems

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Abstract

Background and objectives: Safflower (*Carthamus tinctorious* L.) florets contain valuable red and yellow pigments and are used in food, textile dyeing, and pharmaceutical industries. Red carthamine pigment is formed from a yellow precursor, precarthamine, through an oxidation reaction at the end of flowers' maturation stage. The present study was conducted to find out the most effective procedure for extraction and purification of yellow and red pigments from safflower florets. **Methods:** Two different experiments were designed to assess the suitability of oxidant reagents (KMnO₄ and H₂O₂) on the reddening of florets and different alkaline and organic solutions were used as the solvents (KNO₃, Na₂CO₃, ethanol/hexane, methanol, and NaOH) in order to extract and purify the pigments. All obtained pigments were characterized chromatographically and spectrophotometrically for calculating the yield and evaluating purity of the extracted materials. **Results:** The results showed that KMnO₄ and H₂O₂ treatments were significantly effective for increasing carthamine yield from safflower; but KMnO₄ decreased the amount of yellow pigment extraction during the washing process of treated petals before extraction. Ethanol/hexane demonstrated to be effective in the extraction of yellow pigments, but it was not a good solvent for carthamine extraction. Except for the degassed bicarbonate which showed a high pigment yield, the other three solvents especially KNO₃ did not appear to be of practical use for red pigment preparation. **Conclusion:** The present results showed that pretreatment of petals with hydrogen peroxide and then extracting by degassed solvents resulted in higher yield of red carthamine pigment, and also using reconditioned cellulose or cotton, instead of fresh cellulose, would reduce the cost.

Keywords: alkaline solutions, carthamine, oxidation reaction, pigment yield, safflower

Introduction

In recent years, public concerns about the use of synthetic chemical dyes in food and cosmetics is rapidly increasing and most people prefer to use foods with natural dyes and additives due to their non-allergic and non-carcinogenic properties [1].

Safflower (*Carthamus tinctorius* L.), an annual herb of Asteraceae is an important industrial crop containing yellow and red colorants from C-glucosylquinochalcone flavonoids in the petals [2]. These colorants are applicable as dyeing

agents in processed foods, soft drinks, cosmetics, textile industry, and medicine [3]. The pigments have some medicinal values such as curative effects on coronary heart diseases, rheumatism, diabetes [4], high blood pressure [5], and they have shown antioxidant and hepatoprotective activities [6].

Major components of water soluble yellow pigments are hydroxysafflor yellow A, safflor yellow B, safflomin A, safflor yellow A, safflomin C, isosafflomin C, pre-carthamine, and anhydrosafflor yellow B, which are present at the rate of about 20-30% (w/w) in petals [7]. The main component of safflower red pigments is carthamine which is composed of two chalcon moieties with conjugated bonds. It is insoluble in water and is derived from the yellow colored pre-carthamine through an oxidation reaction [8]. An oxidative enzyme (β -glucose oxidase) contributes in yellow to red color transition of safflower petals at the maturation stage of florets. This enzyme is distributed within vegetative tissues and is active at pH of ~ 4.8 [9]. The enzyme activity is enhanced in the presence of certain oxidizing agents such as hydrogen peroxide [10] and metal ions like manganese [11]; however, such enzymatic conversion requires atmospheric oxygen but the absence of gasses and ions through the use of degassed bicarbonate is proved to be more effective for colorant extraction [12]. The low content of carthamine in safflower petals is a limiting economical factor in terms of its use for industrial purposes; on the other hand, carthamine and safflower yellow pigments are very susceptible to external factors such as temperature, UV-light, pH, metal ions, and certain chemicals during extraction and also afterwards in the free state [13,14]. In plant tissues and before extraction, carthamine and safflower yellow pigments are in the bound form, special precautions should always be taken during extraction, isolation, and subsequent purification and preservation of the dye through a speedy, simple, and mild condition. As a part of the efforts for improving the content and extraction rate of red pigments in the flowers of safflower, effects of KMnO_4 and H_2O_2 on

reddening the florets have been investigated using degassed bicarbonate for extraction. Also, the binding capacity of red carthamine on different cellulose sources and their recovery rates were examined. Different solvent systems were tested for improving the extraction rate of safflower red and yellow colorants.

Experimental

Materials

Dried petals of safflower (*Cathamus tinctorius* cv. Goldasht) were obtained from Agriculture and Natural Resources Research Center, Golestan Province, Iran. Degassed bicarbonate was prepared through sonication for 1 h. Extraction solutions, i.e. solvents for TLC and HPLC, were purchased from Merck (Darmstadt, Germany). Carthamine and safflower yellow standards were obtained through alkaline extraction, acidification, and cellulose adsorption. Purity of the extracted pigments was confirmed using TLC. After scratching the spots from TLC plates, they were freeze-dried for obtaining pigment powders. To prepare the pigment solutions, these powders were dissolved in certain solvents and then they were examined by spectrophotometry at 520 and 405 nm as specific wavelength of absorption spectrum for carthamine and safflower yellow, respectively [1].

In order to recondition cellulose, first the previously used cellulose polymers were suspended in 0.2 M NaOH and stirred continuously for 1 h at room temperature. Then, they were washed with sufficient amount of deionized water until getting neutral. Afterward, the polymers were re-suspended in 0.2mol HCL, stirred at room temperature for 1 h, and washed with distilled water until pH \sim 7.0. The reconditioned cellulose polymers were dried at 70 °C and kept at room temperature until use [15].

Reddening the florets through oxidative pretreatment

Fine powder of dried florets (1g) was treated with two different oxidative reagents [H_2O_2 (10 μM) and KMnO_4 (10 μM)], stirred at room

temperature for 1 h and then filtered through filter papers. The KMnO_4 treated florets were washed very well with distilled water after enough time for oxidation reaction and removing metal ions from the solution completely had passed. However, the H_2O_2 treated florets were washed once because there were no metal ions and no need to remove H_2O_2 which hydrolyzed immediately to water. Finally, the paste was used for further extraction with Na_2CO_3 .

Extraction solutions

Six treatments including organic and alkaline solutions [Na_2CO_3 (0.5% w/v) as control and regular solvent, NaOH (10 μM), MeOH (80%), ethanol/hexane (50:50 v/v; Eth/Hex), KNO_3 (0.5%), and degassed Na_2CO_3] were examined to find out the best extraction solution for red and yellow pigments from *Carthamus tinctorius*. The extraction process was performed according to Fatahi *et al.* with some modification [1]. One gram of fine dried floret powder was extracted with 20 mL of extraction solution after stirring at room temperature for 30 min; the suspension was centrifuged at 6000 rpm for 8 min at room temperature. The supernatant was retained at 5 ± 1 °C and the residue was used for further 2 more time extraction. The pooled extracts were acidified with citric acid (1 %w/v) to attain the pH of 3.5-5 and were used for adsorption of carthamine. One gram of fine powder of cellulose was added to the acidified solution and was shaken for 30 min at 10 °C (Shaking Incubator, NB-205VL, N-BIOTEK, INC). The suspension was centrifuged at 6000 rpm for 8 min. The supernatant was collected as yellow pigments and the pellet was washed with distilled water until the yellow pigments were removed. The red pigments were extracted with 10 mL acetone (60%), stirred 30 min, and centrifuged at 6000 rpm for 8 min. The supernatant was retained as red pigment and the extraction was repeated until the pellet was bleached.

Adsorption and purification of red carthamine

In order to compare the efficiency of different adsorbents for purification of red carthamine, One gram of fine dried florets of safflower was

extracted using Na_2CO_3 according to the above mentioned method, up to the step for adding cellulose. Then each extract was treated with reconditioned cellulose (1 g) and cotton (1 g).

Determination of pigment content

The red and yellow extracts from each experiment were used for estimating pigment content. Spectroscopic assay was performed at 520 nm and 405 nm for carthamine and safflower yellow, respectively by double beam UV-Visible spectrophotometer. The pigment content was expressed in terms of milligram per gram dry weight (mg/g) of safflower florets.

TLC-check of carthamine and safflower yellow purity

Thin layer chromatographic identification was performed according to Saito and Fukushima [16]. An aliquot of red and yellow concentrated pigments was loaded on the Silica gel type G plate and developed with the chromatographic solution consisting *n*-butanol/acetone/water/acetic acid (30:10:10:0.5). The resulting spots were compared with carthamine and safflower yellow standards.

Statistical analysis

All the determination analyses were replicated three times. Analysis of Variance (ANOVA) was performed and differences in mean values were performed using Duncan's multiple test at $p<0.05$.

Results and Discussion

Reddening the florets

Effect of oxidizing agents on red color formation was quite obvious and these compounds were proved to catalyze the oxidation process efficiently (figure 1); consequently, they naturally decreased the amount of yellow pigment extraction during the washing process of treated petals before extraction. As given in figure 1, the H_2O_2 reagent was more effective for inducing red pigments compared to other reagents. H_2O_2 as an oxidizing agent contains no metal cations which could be very important for obtaining a healthy and safe product.

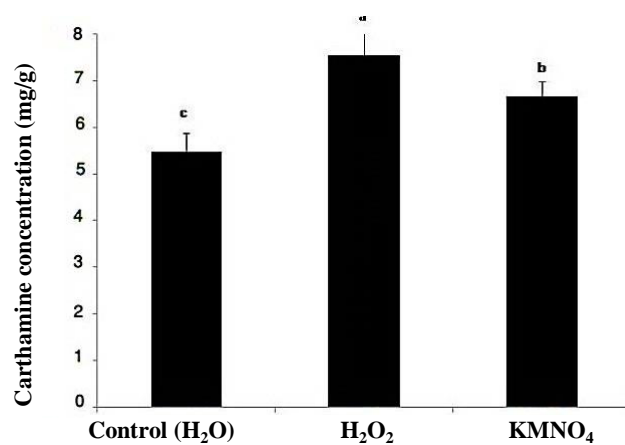


Figure 1. Effects of oxidizing agents H₂O₂, and KMnO₄ on red carthamine color formation of safflower florets. Means in each column followed by similar letters are not significantly different at $p \leq 0.05$ using DMRT test.

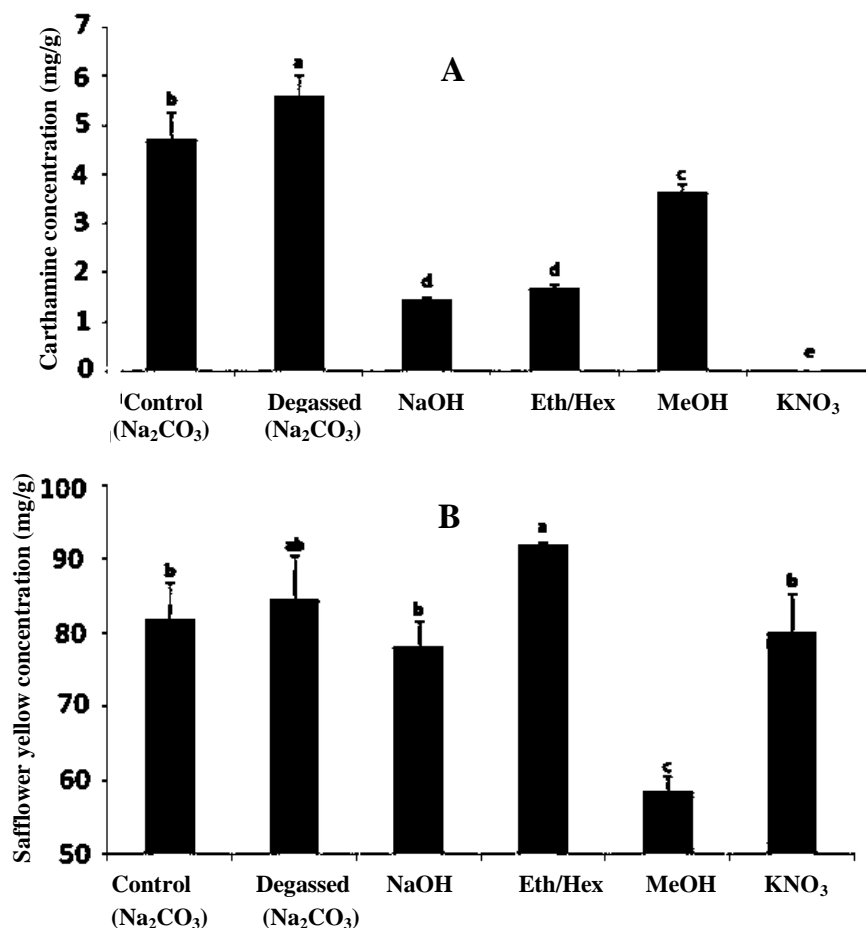


Figure 2. Efficiency of solvent systems on carthamine (A) and safflower yellow (B) extraction in safflower florets. Means in each column followed by similar letters are not significantly different at $p \leq 0.05$ using DMRT test.

It is readily transformed into harmless O_2 and H_2O during the course of oxidation reaction [11]. Saito (1993) studied an incubation model in which hydrogen peroxide and precarthamine were co-existing, instead of exposing the florets directly to β -glucose oxidase enzyme, and suggested that glucose oxidase may participate indirectly in carthamine formation via the process of generating its by-product, hydrogen peroxide, which could react directly with precarthamine [9].

Solvent systems

The solubilizing power of the solvents for extracting carthamine and safflower yellow from dry florets is shown in figure 2. Degassed Na_2CO_3 and Eth/Hex seemed to be the most promising solvents for safflower yellow followed by Na_2CO_3 and KNO_3 . Although MeOH is the frequently used for elevating the extraction yield of yellow pigments from safflower florets, it is not as efficient as Na_2CO_3 or Eth/Hex (figure 2B).

Degassed bicarbonate significantly extracted more carthamine than Na_2CO_3 (control), which has often been applied as the most common solvent (figure 2A). Among other applied solvents, methanol was found to be more effective. Eth/Hex and NaOH did not prove to be effective (figure 2A). These results were in agreement with those by Saito and Katsukura (1992), who concluded that organic solvents could not be as effective as alkaline solutions in mixture with acidic solutions for red dye extraction from safflower. Since pH of NaOH (10 μ M) is the same as Na_2CO_3 (0.5%), they were expected to have the same carthamine yield; but, sodium bicarbonate was not only much more efficient, but also a green and safe solvent with low toxicity and cost. Extraction efficiency of degassed bicarbonate could be due to the enhancement effect on swelling of hydrophilic polymers in the substrate, resulting in the optimum extraction of oxygen-labile carthamine product [12].

Adsorption and purification of red carthamine

Purification efficiency of cotton for carthamine was less than fresh and reconditioned cellulose; however, it did not show significant differences (figure 3). This method could have the economic advantage of using cellulose for several times (after reconditioning), or even using high quality cotton as a very cheap alternative adsorbent, besides obtaining colored cotton as a low-cost by-product.

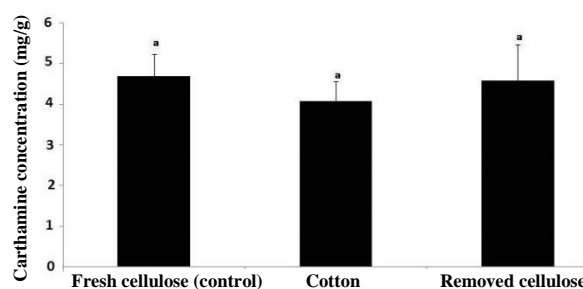


Figure 3. Efficiency of cellulose, cotton, and recovered cellulose in the adsorption of carthamine in safflower florets. Means in each column followed by similar letters are not significantly different at $p \leq 0.05$ using DMRT test.

Purity of extracted pigments

TLC results proved that, in all treatments, red extracted pigments were identical to carthamine and all yellow pigments were identical to safflower yellow. One specific bond for each pigment was detected at the wavelength of 366 nm. The desired bonds were shown by white arrows in figures 4A and B.

In conclusion, the present results led to some practical hints on simple, low-cost, green, and efficient methods which could be applied for extracting and isolating natural products from biomaterials. In this study, the results demonstrated that pretreatment of petals with hydrogen peroxide and then extracting by degassed solvents resulted in higher yield of red carthamine pigment and also using reconditioned cellulose or cotton, instead of fresh cellulose, would reduce the cost. These findings could be promising for further applications of valuable pigments from safflower in pharmaceutical, cosmetic, and food industries.

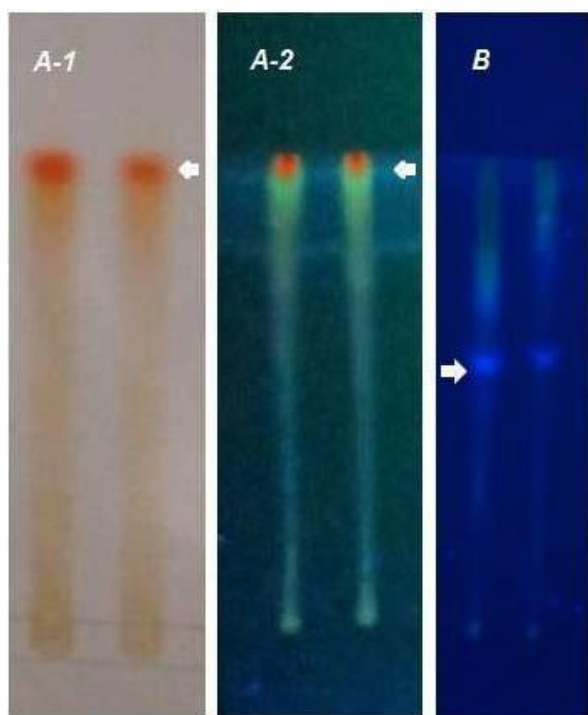


Figure 4. TLC results for confirming the purity of red (A) and yellow (B) pigments from safflower florets. A-1: Red carthamine on TLC plate, A-2: Red carthamine at the wavelength of 366 nm, B: Safflower yellow using UV detector at the wavelength of 366 nm. In all images, the left sample belongs to the standard agent and the right one is related to the extracted pigment.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

- [1] Fatahi N, Carapetian J, Heidari R. Spectrophotometric measurement of valuable pigments from petals of safflower (*Carthamus tinctorius* L.) and their identification by TLC method. *Res J Biol Sci.* 2008; 3(7): 761-763.
- [2] Kazuma K, Takahashi T, Sato K, Takeuchi H, Matsumoto T, Okuno T. Quinochalcones and flavonoids from fresh florets in different cultivars of *Carthamus tinctorius* L. *Biosci Biotechnol Biochem.* 2000; 64(8): 1588-1599.
- [3] Meselhy M, Kadota R, Momose S, Hatakeyama Y, Kusai N, Hattori A, Namba T. Two new quinochalcone yellow pigments from *Carthamus tinctorius* and Ca^{2+} antagonistic activity of tinctormine. *Chem Pharm Bull.* 1993; 41(10): 1796-1802.
- [4] Asgary S, Rahimi P, Madani H, Mahzoni P, Kabiri N. Effects of hydroalcoholic extract of *Carthamus tinctorius* on activity of hepatic transaminases in alloxan-induced diabetic rats. *J Shahrekord Univ Med Sci.* 2010; 12(1): 46-52.
- [5] Bai Y, Ping L, Han C, Yu C, Chen M, He F, Yi D. Hydroxysafflor yellow A (HSYA) from flowers of *Carthamus tinctorius* L. and its vasodilatation effects on pulmonary artery. *Molecules.* 2012; 17: 14918-14927.
- [6] Wu S, Yu Y, Tian H, Li Z, Li X, He W, Ding H. *Carthamus* red from *Carthamus tinctorius* L. exerts antioxidant and hepatoprotective effect against CCl_4 -induced liver damage in rats via the Nrf2 pathway. *J Ethnopharmacol.* 2013; 148(2): 570-578.
- [7] Kazuma K, Takahashi T, Sato K, Takeuchi H, Matsumoto T, Okuno T. Chalcones and flavonoids from fresh florets in different cultivars of *Carthamus tinctorius* L. *Biosci Biotechnol Biochem.* 2000; 64(8): 1588-1599.
- [8] Cho MH, Paik YS, Hahn TR. Enzymatic conversion of precarthamin to carthamin by a purified enzyme from the yellow petals of safflower. *J Agric Food Chem.* 2000; 48(9): 3917-3921.
- [9] Saito K. Glucose oxidase, a potential contributor towards flower colour modification in the capitula of *Carthamus tinctorius* L. *Biochem Physiol Pfl.* 1993; 188(6): 405-417.
- [10] Saito K, Takahashi M. A new technique for the chemical processing of reddened florets from dyer's saffron capitula. *Food Chem.* 1993; 48(4): 387-389.
- [11] Saito K. A new method for reddening dyer's saffron florets: evaluation of carthamine productivity. *Z Lebensm Unters For.* 1991;

- 192(4): 343-347.
- [12] Ogawa I, Yamano H, Miyagawa K. Application of deaerated water in extraction of colorants from dyer's saffron florets. *J Appl Polym Sci.* 1999; 74(7): 1701-1704.
- [13] Fatahi N, Carapetian J, Heidari R. Comparing stability of carthamine and safflower yellow pigments at pH, temperature and light, from safflower (*Carthamus tinctorius* L.) florets. *Res J Biol Sci.* 2009; 4(3): 250-253.
- [14] Saito K, Matsuhisa Y, Naruse A, Kanehira T. Successive entrapping of red and yellow quinoid chalcones from aqueous extracts of dyer's saffron florets: a newly established and practically approved technique. *Z Lebensm Unters For.* 1989; 189(5): 418-421.
- [15] Saito K, Yamamoto T, Miyamoto KI. Isolation and partial purification of carthamine: an instrumentation manual. *Z Lebensm Unters For.* 1992; 195(6): 550-554.
- [16] Saito A, Fukushima A. An improved method for large-scale isolation of a water-soluble safflor pigment from dyer's saffron flowers. *Food Chem.* 1989; 32(4): 297-306.