Effect of time and ultrasonic amplitude on extraction carotenoid compounds from saffron stamen

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Abstract: Saffron flowers by products including sepals, petals and stamens have no application in Iran's food industries; nevertheless stigmas contain a considerable amount of carotenoids with high antioxidant properties. In this study carotenoid compounds were extracted by an organic solvent (mixture of N-hexane, ethanol and acetone; 2:1:1 v: v) and ultrasound waves. Ultrasound waves were applied at a frequency of 24 kHz and amplitude of 20%, 60% and 100% for 5, 10 and 15 min. All treatments were tested in triplicates. The extraction efficiency, total carotenoid and phenolic compounds, free radical scavenging and reducing power of Fe III were analyzed. The results showed that ultrasound waves had significant effects on the extraction carotenoid compounds from saffron stamen so that ultrasonic treatment at 100% amplitude for 15 min caused the highest extraction efficiency (8.882%), whereas ultrasound waves with amplitude of 100% imposed for 10 min showed the highest carotenoid extraction (485.2 mg/ml), total poly phenol compounds (551.7 mg/ml), reduction power of FeIII (443.3 mmolFe2+/Mass) and the IC50 (23.59 mg/ml). Totally it was observed that the most efficient conditions for anthocyanin content and antioxidants ability were ultrasound amplitude of 100% for 15 min and 10 min.

Keywords: antioxidant, carotenoid, extraction efficiency, saffron stamen, ultrasonic

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1 Introduction

Fresh saffron flower is composed of sepal and petal (86.42 %), stamen (5.93 %) and stigma and style (7.64 %) (Hematy Kakhaki, 2010). Currently, Iran accounts for 65% of saffron production and is the greatest producer and exporter in the world. Area cultivation and saffron production in Khorasan province of Iran has been estimated about 59,000 ha and 210,000 t, respectively. In addition, this province has produced 210 t dried saffron during 2009-2010 (Anon, 2014). Sepals, petals and stamens of saffron flower are produced as by products in saffron production in Iran which are normally discarded meanwhile contain considerable amount of pigments with high antioxidant activity, from which, the extraction of anthocyanins, carotenoids and antioxidants might be possible. The color of food is considered as one the most important factors affecting food's markets, the inappropriate color of food the reduce demand of it. Synthesized food colorants have been applied widely in different foods for enhancing food acceptance. On the other hand, these colorants have harmful effects on human health, therefore, extraction and purification of natural food colorants out of natural cheap sources has been taken into account. Antioxidants play an important role in protecting tissues from oxidizing effects of free oxygen and other active radicals so that prevent inflammatory diseases, cancer, diabetes, heart attack, Alzheimer and Parkinson(Khanipour et al , 2008).

In a study, Negri et al. (2011) investigated phenol compounds and antioxidant activity of pollen samples using HPLC (High Performance Liquide Cromatography) method in detecting antioxidants compounds and the

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Folin–Ciocalteu reagent (FCR) in determination of anti-oxidative activities. Similarly, Arráez-Román and co-workers (2007) detected phenol compounds by electrophoresis methods.

Extracting using ultrasound waves is a simple and cheap method (Albu et al., 2004). The main mechanism related to cavitation phenomenon creating tiny bubbles, growing to critical size quickly then explode, hence in the extraction of plant extracts ultrasound waves can be increased the speed and the efficiency of extraction meanwhile reduced the amount of applied solvent (Basiri, 2011). Regarding the lack of information about the antioxidant potential of saffron stigma, the aim of this study was to determine the time and frequency of the ultrasonic waves on the extraction efficiency, and quantify the carotenoids content, phenolic compounds and antioxidant capacity of saffron stigmas.

2 Materials and methods

2.1 Plant material

Saffron (*Crocus sativus* L.) flowers (15 kg) were prepared from Khorasan Razavi Agricultural and Natural Resources Research and Education Center Iran. The stamens were separated and dried at room temperature and then grounded into a fine powder. Powdered samples were packed and kept in dark place at -18 ℃.

2.2 Methods

2.2.1 Ultrasonic extraction

Extraction of vegetables' compounds diversity with different structures depends on various factors including solvent type, extraction way, particle size and sample/solvent ratio. In order to extract carotenoids and antioxidant compounds from saffron stamens an organic solvent (mixture of N-hexane, ethanol and acetone; 2:1:1 v: v) was applied. Powdered samples were mixed into the solvent (10:1) and placed in the ultrasound assisted extractor and sonicated for 5, 10 and 15 min, one time at 20%, another time at 60% and finally at 100% amplitude of out put power, 3 times for each sample, without stirring. Extraction was performed using flooding methods exposed to ultrasound waves, in a 24 kHz constant waves produced by ultrasound apparatus Heilscher, Germany-UP400S, 400 W power, probe H7 titanium type, 7mm diameter, length 100 m and one cycle (Farhoosh, 2006).

2.2.2 Solvent extraction (maceration method)

In brief, 10 g of dried samples were twice extracted with 100 ml of organic solvent (N-hexane, ethanol and acetone (2:1:1)) with shaking at room temperature for one h. The extracts were filtered through a filter paper and the filtrates were pooled. The residues were re-extracted with 60 ml of the solvent at room temperature for 2 h. Deionized distilled water was added to the pooled filtrates until 20% v: v. Mixing with water results in the production of separate organic and aqueous phases. The upper phase contains non-polar compounds, such as fat soluble carotenoids and the other phase contain polar compounds such as water soluble carotenoids. The phases were condensed with a rotary evaporator under reduced pressure below 40˚C and then dried in vacuum below 40˚C and weighed to determine extraction yield (Farhoosh, 2006).

Extraction efficiency

Extraction efficiency was calculated as the ratio of dried extract weight to the initial weight of the material and expressed as percentage (Khanipour et al., 2007).

Total carotenoid compounds

The extracted samples were diluted by the ratio of 1: 20, and then optical absorption measurements were performed by spectrophotometer (Shimadzu UV-160A, Kyoto, Japan) in the wavelength range from 350 to 500 nm. The recovery percentage was calculated using following

Equation (Khanipour et al., 2007; Scott, 2001)

(Amount recovered/starting amount) × 100 = % recovery

Total phenolic compounds

It was evaluated using a modified colorimetric method. The method involves the reduction of Folin-Ciocalteau reagent by phenolic compounds, with a

concomitant formation of a blue complex according to the method described by Capannesi et al., (2000). The results were reported as mg Gallic acid equivalent per gram. A calibration curve of Gallic acid in methanol was fitted in the concentration range of 0.04–0.40 mg/ml.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH radical-scavenging assay is a commonly used method to evaluate the ability of plant extracts to scavenge free radicals generated from DPPH reagent according to Siger et al., (2007).

Saffron stamen methanolic extracts were mixed with 1 ml of 0.5 mM DPPH methanol solution and 2 ml of 0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture was incubated at room temperature in the dark for 30 min, and then the absorbance was measured at 517 nm using spectrophotometer. The IC_{50} value was determined as the concentration of each sample required to give 50% DPPH radical scavenging activity (Einafshar et al., 2012).

Ferric reducing-antioxidant power (FRAP) test

FRAP test is a simple, reproducible, rapid, and inexpensive procedure that measures the ability of anti−oxidative compounds to reduce the ferric ion Fe^{3+} to ferrous Fe^{2+}, as a measure of total antioxidant capacity. Acetate buffer (0.3 M, pH 3.6) was prepared by dissolving 3.1 g $C_2H_3O_2Na$ -$3H_2O$ and 16 ml of acetic acid in 1 L of distilled water. 2, 4, 6-Tripyridyl-S-triazine (TPTZ) solution was prepared by dissolving 23.4 mg of TPTZ in 7.5 ml of 40 mM HCl solution. Ferric solution (20 mM) was prepared using $FeCl_3$- $6H_2O$. The final working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ, and ferric solutions at a ratio of 10:1:1. In brief, 900 ml FRAP working reagent was mixed with 90 ml distilled water and was warmed to 37˚C in a water bath. The reagent control reading was recorded at 595 nm, followed by adding 30 ml of sample solutions (100 mg in 10 ml of N-hexane). The absorbance was taken at 595 nm, against the control solution. A standard curve was prepared using different concentrations of $FeSO_4$-$7H_2O$ (200-2000 mmol/L). All solutions were freshly prepared. The results were expressed in mM Fe^{+2} per mass (Benzie and Strain, 1996).

3 Results and discussion

3.1 Effect of ultrasound process time on investigated parameters

The effect of time on investigated factors is shown in Table 1. Table 1 shows that phenolic compounds was decreased from 659.6 mg/kg in blank to 625.3 mg/kg in 15 min sonication. According to the available reports (Mason and Petrier, 2004; Paniwnyk et al. 2009) addition of polar solvents like water to a system reduces the antioxidant levels due to poor solubility in the extraction solvent. Also, the generation of free radicals like hydroxyl and peroxyl during sonication process might possibly degrade and react with the antioxidants in the extract. From the results, increase in extraction time from 5 min to 15 min had a significant and positive effect on the extraction efficiency ($p < 0.05$), in other words, extraction efficiency increased from 7.186% to 7.518% due to 10 min more sonication. Extended time led to increase mass transfer, therefore more compounds can be extracted. Before the establishment of equilibrium for the objective constituents in and out of plant cells, the yield of extraction increases with time (Dong et al. 2010). It also displays that IC_{50} ,the amount of anthocyanin and FRAP were significantly increased ($p<0.05$) it can be concluded that both time and ultrasound amplitude effect on total phenolic compounds, IC_{50}, the amount of anthocyanins, extraction efficiency and ferric reducing power; however, latter was more effective on carotenoid extraction, total poly-phenol compounds, free radical inhibitory power and reduction power of Fe^{+3}. The most efficient conditions for anthocyanin content, total poly-phenol compounds, free radical inhibitory power and reduction power of Fe^{+3} were ultrasound amplitude of 100% for 15 min and ultrasound amplitude of 100% for 10 min, respectively. During the first 10 min ultrasonic treatment and then decreased. Time duration can influence

the extraction yield (Galhiane, et al., 2006). It has been reported that long period of extraction time favors the phenolic compounds production and reducing power of Fe^{+3} (Rodrigues, and Pinto, 2007).

Table 1 Effect of ultrasound process time on investigated parameters

Time, min	Total phenolic compounds, mg/kg	IC_{50}, mg/ml	Anthocyanin, mg/ml	Extraction efficiency, %	FRAP, mmol Fe^{2+} mass^{-1}
0	659.6±1.3 a	6.589±0.1 d	314.1±3.2 d	6.285±0.2 d	409.4±5.1 d
5	632±2.1 b	13.37±0.3 c	386.7±2.7c	7.186±0.1 c	422.6±8.2 c
10	614.6±2.5 d	17.44±0.3 a	424.2±7.1 a	7.365±0.2 b	429.1±9.6 a
15	625.3±2.3 c	14.88±0.2 b	419.4±4.2 b	7.518±0.2 a	427.1±8.5 b

Note: Means, in each column, followed by at least one letter in common are not significantly different at the 5% probability level using Duncan's Multiple Rang Test

3.2 Effect of ultrasonic amplitude on investigated parameters

The effect of 20%, 60% and 100% ultrasound amplitude on investigated parameters in 10 min and 24 KHz is given in Table 2. As can be seen, ultrasound amplitudes had significant effect on all investigated parameters so that extraction yield, carotenoid compounds and reducing power of Fe^{+3} increased with increasing amplitude from 0 (control) to 60%. In contrast, total phenolic compounds decreased from 659.6 in control to 621.7 mg/kg at 100% amplitude sonikation. It was also found that at 100% amplitude all parameters were at the maximum level except total phenolic compounds. This phenomenon probably arose from the fact that created shear forces and their high energy amount and their effects in cell wall destruction, cell contents leakage to surrounding area, mass transfer improvement, particle size reduction increasing contact surface, increasing solvent diffusion (Lee et al., 2007). The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations produced in the solvent by the passage of an ultrasound wave (Ghafoor, et al., 2009). Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the tissue, increasing the contact surface area between the solid and liquid phase. As a result, the solute quickly diffuses from the solid phase to the solvent (Rostango et al., 2003).

Table 2 Effect of ultrasonic amplitude on investigated parameters

Amplitude, min	Total phenolic compounds, mg/kg	$IC_{50,}$ mg/ml	Anthocyanin, mg/ml	Extraction efficiency, %	FRAP, mmol Fe^{2+}/mass
0	659.6±21.3 a	6.589±0.3 d	314.1±30.5 d	6.285±0.3d	409.4±12.5 d
20	644.5±19.4 b	14.02±0.7c	391.5±33.7c	6.754±0.5c	419.8±27.2c
60	621.7±5.2 c	18.49±0.8b	458.2±41.9 b	7.783±0.7b	435.4±22.1b
100	570.0±9.7d	21.83±0.2 a	476.7±32.4 a	8.603±0.3 a	440.5±19.9 a

Note: Means, in each column, followed by at least one letter in common are not significantly different at the 5% probability level using Duncan's Multiple Rang Test

3.3 Effect of time and ultrasound amplitude on investigated parameters

The effect of time and ultrasound amplitude on extraction yield, IC_{50}, total poly-phenolic compounds, anthocyanin content and reducing power of Fe^{+3} is presented in Table 3. According to the results, extended time from 5 to 10 min, increased all parameters except for Total phenolic compounds. Ultrasonic treatments in contrast with control, were increased all parameters except for Total phenolic compounds. Applying 10 min ultrasound waves at amplitude of 100% led to the highest carotenoid amount (485.20 mg/ml), IC_{50} (23.52 mg/kg), reducing power of Fe^{+3} I (443.30 μmol/ml) and the lowest Total phenolic compounds (551.10 mg). The highest

extraction yield was achieved in 15 min ultrasound treatment with 100% amplitude. Significant differences between ultrasound treated samples and control samples refers to shear stress of ultrasound waves and broken big molecules consequently, amplitude was more effective than time. It has been shown that ultrasound improves about 40% the extraction rate in contrast of control by disrupting plant cells and hence increasing the diffusion of the cell contents across the cell wall. The beneficial effects of sound waves on extraction are attributed to the formation and asymmetrical collapse of micro-cavities in the vicinity of cell walls leading to the generation of micro-jets rupturing the cells. Ultrasonic treatment extracted more poly-phenolic compounds than blank because of cavitation bubbles destruction near cell wall cause more effective contact between solvent and vegetable materials, in addition a produced rapid flow of waves acted as a micro pump enforcing solvent into cells and dissolved related compounds (Albo et al, 2004). In fact , ultrasound propagation in liquid solid phase creating expansion, contraction cycles (causing to produce growing destructed bubbles), vibration of liquid solid particles, speeding up under ultrasound wave action, as well as emulsification, diffusion and tissue damage that lead to more extraction.

Table 3 Effect of time and ultrasonic amplitude on investigated parameters

Time, (min	Amplitude, %	Total phenolic compounds, mg/kg	IC_{50}, mg/ml	Anthocyanin, mg/ml	Extraction efficiency, %	FRAP, mmol $Fe^{2+.}$/mass
0	0	659.6±31.7 a	6.589±0.1 i	314.1±19.2 i	6.285±0.9 g	409. 4±33.1i
5		654.0±26.4 b	12.83±0.7 f	327.3±18.3h	6.553±0.03I	410.8±29.4h
10	20	636.3±22.5 d	16.91±0.5 e	423.8±22.6 g	6.677±0.4h	426.5±28.6f
15		643.2±21.6 c	12.32±0.6 h	423.5±20.5g	7.031±0.2 g	422.0±24.2g
5		635.5±20.1 d	12/54±0.3 g	440.3±19.8 f	7.598±0.3f	433.1±32.6 e
10	60	611.2±19.9 f	22.68±0.2 b	473.9±19.4 c	7.818±0.1 e	438.0±31.9 c
15		618.4±19.3 e	20.26±0.4 d	460.5±24.1e	7.934±0.1d	435.9±29.7 d
5		579.0±20.1 g	21.53±0.2 c	465.3±26.1d	8.306±0.07 c	437.3±29.4 c
10	100	551.1±20.1 h	23.59±0.9 a	485.2±33.2a	8.682±0.2 b	443.3±25.2 a
15		579.9±18.4 g	20.36±0.5 d	479.6±31.4b	8.822±0.09 a	441.0±28.1 b

Note: Means, in each column, followed by at least one letter in common are not significantly different at the 5% probability level using Duncan's Multiple Rang Test.

4 Conclusion

Saffron's stamens as a carotenoid source provide a high potential of color and antioxidant source. Considering the harmful effects of synthetic antioxidants and food colors and nutritional and medicinal properties of pollen on the other side, applying saffron's stamens to utilize in food and pharmaceutical industries as a new natural antioxidant resource is open to discussion. According to the obtained results, it can be concluded that both time and ultrasound amplitude effect on total phenolic compounds, IC_{50}, the amount of anthocyanins, extraction efficiency and ferric reducing power; however, latter was more effective on carotenoid extraction, total poly-phenol compounds, free radical inhibitory power and reduction power of Fe^{+3}. The most efficient conditions for anthocyanin content, total poly-phenol compounds, free radical inhibitory power and reduction power of Fe^{+3} were ultrasound amplitude of 100% for 15 min and then ultrasound amplitude of 100% for 10 min.

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