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Effect of different extraction methods on saffron antioxidant activity, total phenolic and crocin contents and the protective effect of saffron extract on the oxidative stability of common vegetable oils

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SUMMARY: Saffron consists of bioactive compounds with health-promoting properties and is mainly used in medicine, flavoring and coloring. In this study, we aimed to investigate the effect of extraction methods on the antioxidant activity of saffron (Crocus sativus L.) extracts (SE) and to evaluate the antioxidant performance of SE in vegetable oils. Saffron stigmas were extracted in water, ethanol, methanol, and their combinations using maceration extraction (ME), ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), and the combination of UAE with MAE. The results showed that the sample extracted by methanol/water (50:50) using the combination of UAE with MAE methods had the highest amount of total phenolic content (31.56 mg/g GAE) and antioxidant activity (83.24% inhibition). The extract with the highest antioxidant activity was freeze-dried before incorporation into oil samples. Freeze-dried SE contained trans-crocin-4 and trans-crocin-3 (most abundant constituents), kaempferol, and picrocrocin. Moreover, the addition of SE at 1000 ppm resulted in a significant increase in the oxidative stability of canola (CAO), sunflower (SO), and corn oil (COO).

KEYWORDS: *Antioxidant activity; Microwave-assisted extraction; Oxidative stability; Saffron; Ultrasound-assisted extraction.*

RESUMEN: ***Efecto de diferentes métodos de extracción sobre la actividad antioxidante del azafrán, contenido total de fenoles y crocina y efecto protector del extracto de azafrán sobre la estabilidad oxidativa de los aceites vegetales comunes.*** El azafrán contiene compuestos bioactivos con propiedades promotoras de la salud de uso destacado en medicina, saborizante y colorante. En este estudio, nuestro objetivo fue investigar el efecto de los métodos de extracción sobre la actividad antioxidante de los extractos (EA) de azafrán (Crocus sativus L.) y evaluar el rendimiento antioxidante de EA en aceites vegetales. Los estigmas de azafrán se extrajeron en agua, etanol, metanol y sus combinaciones, mediante extracción por maceración (EM), extracción asistida por ultrasonidos (EAU), extracción asistida por microondas (EAM) y la combinación de EAU con EAM. Los resultados mostraron que la muestra extraída con metanol/agua (50:50) usando la combinación de métodos EAU con EAM tuvo la mayor cantidad de fenoles totales (31.56 mg/g GAE) y actividad antioxidante (83.24 % de inhibición). El extracto que incluía la mayor actividad antioxidante se liofilizó antes de incorporarlo a las muestras de aceite. El SE liofilizado contenía *trans*-crocina-4 y *trans*-crocina-3 (los constituyentes más abundantes), kaempferol y picrocrocina. Además, la adición de 1000 ppm de EA dio como resultado un aumento significativo en la estabilidad oxidativa del aceite de canola (C), girasol (G) y maíz (M).

PALABRAS CLAVE: *Actividad antioxidante; Azafrán; Estabilidad oxidativa; Extracción asistida por microondas; Extracción asistida por ultrasonido*

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1. INTRODUCTION

The detection of natural antioxidants has recently become an attractive area of research for both food and pharmaceutical applications. Natural antioxidants can be proposed as substitutes for synthetic antioxidants which have restricted applications due to the harmful health problems like cancerogenic effects which probably occur due to their long-term consumption. Antioxidants cover a broad range of compounds that can retard the degradation of lipids during oxidation and consequently prevent diseases caused by free radicals and enhance the quality and nutritional values of food products (Ahmadian-Kouchaksaraie and Niazmand, 2017).

Saffron (*Crocus sativus L.*) is a triploid sterile plant belonging to the Iridaceae family. This plant is mainly cultivated in Iran (90% of the total annual production of saffron). The dried stigma of the flower of this plant (known as saffron) is considered to be the most expensive spice in the world. Moreover, the main by-product obtained during the harvesting of saffron is its petals. Bioactive components obtained from several parts of saffron (stigmas, petals, and corns), have shown health-promoting properties like cancer prevention, antitumor activities, neuroprotective effects against Alzheimer and Parkinson's disorders, memory enhancement, decrease in anxiety and insomnia, and antidepressant effects (Lambrianidou *et al.*, 2021).

These health-promoting effects result from the valuable nutraceuticals present in saffron, including crocins, safranal, picrocrocin, crocetin, kaempferol, quercetin, α-carotene, β-carotene, and zeaxanthin. The three main bioactive compounds in saffron stigmas are crocins, picrocrocin, and safranal. Crocins (mainly crocin-4) are the water-soluble mono- and di-glycosyl esters of crocetin (a dicarboxylic acid named, $C_{20}H_{24}O_4$). They are derived from zeaxanthin and have the ability to provide the outstanding golden-red color. Picrocrocin ($C_{16}H_{26}O_7$), the second main component of saffron, is a monoterpene glycoside which is responsible for the bitter taste of saffron resulting from the thermal and enzymatic dissociation of zeaxanthin. Safranal ($C_{10}H_{14}O$), the volatile oil that contributes to saffron's unique aroma is a product of the thermal or enzymatic degradation of picrocrocin (Ahmadian-Kouchaksaraie and Niazmand, 2017; Sarfarazi *et al*., 2019).

The extraction methods influence extraction efficiency and the quality of the bioactive constituents obtained from saffron. Maceration extraction (ME), steam distillation and Soxhlet extraction have been traditionally used for the extraction of different bioactive compounds from saffron. Furthermore, several modern procedures like microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), ohmic-assisted extraction (OHAE), supercritical fluid extraction (SFE), subcritical water extraction (SWE) and pulsed electric field have been used to obtain various bioactive constituents from saffron (Ahmadian-Kouchaksaraie and Niazmand, 2017; Hashemi Gahruie *et al.*, 2020; Manouchehri *et al.*, 2018; Pourzaki *et al.*, 2013; Sarfarazi *et al.*, 2019).

Traditional extraction approaches suffer from several disadvantages such as prolonged extraction time, use of large volumes of solvents that are not safe for the environment, high energy consumption, low selectivity, and low extraction efficiency (Garavand *et al.*, 2019; Heydari and Haghayegh, 2014).

Conventional ME, includes several steps: firstly, samples are ground to increase the surface area exposed to solvent. Then, they are placed in closed vessels and the appropriate solvent is added. Finally, the solvent containing bioactive compounds is filtered. Time, temperature, and added solvents are defined as important factors for ME (Deng *et al.*, 2017; Ozkan *et al.*, 2021)

Ultrasonication extraction involves special kinds of sound waves with high-frequency (20 kHz and 100 MHz), passing through a medium which causes the formation, developing, and collapsing of bubbles based on the cavitation phenomenon. The collapse of bubbles close to the plant cell wall leads to disruption of the cell. Then, the solvent washes out the cell contents including bioactive components. This method has some advantages such as the possibility of extraction at ambient temperature, enhancing the mass transfer, being simple and rapid, high extraction rate, and high purity of the extract (Altemimi *et al.*, 2016; Garavand *et al.*, 2019).

In the Maceration extraction process, the transfer of mass and heat occurs in opposite directions; while in MAE, it happens in the same direction from inside plant material to the solvent medium. Consequently, during microwave extraction, solute transfer is accelerated as a result of the one-pot heat-mass transfer, and the extraction rate of bioactive compounds increases. On the other hand, conventional extraction methods lead to the collection of high amounts of undesirable components in the extracted solution which cause the quality and purity of the extract to decrease (Sarfarazi *et al.*, 2020). High quality and pure extracts are obtained by using novel methods like MAE and the consumption of

solvent and energy is also optimized. Numerous studies have confirmed the superiority of MAE over ME, UAE, and supercritical CO_2 in the extraction of bioactive compounds from saffron (Garavand *et al.*, 2019).

Vegetable oils are sensitive to oxidation due to containing high levels of polyunsaturated fatty acids, and therefore the use of antioxidants is necessary to retard their oxidation (Oliveira *et al*., 2018).

Several studies have been conducted to evaluate the antioxidant properties of food products supplemented with saffron extract (SE). For example, the incorporation of saffron into wheat flour pasta and fresh ovine cheese enhanced their antioxidant activity and their sensory properties (Aktypis *et al.*, 2018; Armellini *et al.*, 2018). There are no studies regarding the implementation of saffron in vegetable oils to enhance their oxidative stability. The present study was aimed to evaluate the effect of different extraction techniques and solvents on the extraction of bioactive compounds from saffron (ME, UAE, MAE, combinations of UAE and MAE). Moreover, it was aimed to study the influence of SE on the oxidative stability of several common vegetable oils (CAO, SO and COO) containing highly unsaturated fatty acids (UFA).

2. MATERIALS AND METHODS

2.1. Materials

Dried saffron stigmas were purchased from Jamshidi Marandi producer (Khorasan-e-Razavi, Iran). Refined, bleached and deodorized (RBD), canola oil (CAO), sunflower oil (SO) and corn oil (COO), without added antioxidant, were supplied from Cargill Co., Istanbul, Turkey. Crocin-4 with 98% purity was purchased from Biopurify Phytochemicals Ltd (Sichuan, China). Gallic acid, Folin–Ciocalteu's phenol reagent, and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)) reagent were purchased from Merck (Darmstadt, Germany). All solvents were obtained from Merck and were of HPLC-grade.

2.2. Preparation of saffron extract

2.2.1. Maceration extraction (ME), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE)

Five grams of dried saffron stigmas were completely ground using a porcelain mortar and screened with a sieve with mesh size of 0.5 mm. Then, the dried powder from saffron stigmas (0.2 g) was extracted using 15 mL of solvents (water, ethanol, methanol, the mixture of ethanol: water, 50:50 v/v and methanol: water, 50:50 v/v) with different extraction methods including ME, UAE, MAE and combinations of UAE and MAE. Then, the extracts were filtered using a stainless-steel Buchner funnel (Sartorius AG 3400 Gottingen, Germany). Finally, the extracts were completed to 25 mL with extraction solvent, and kept at -20 °C until further analysis.

ME was carried out using a shaker incubator (IKA® KS 4000 I control, Germany). Ground saffron stigmas (0.2 g) were extracted with different previously described solvents for 24 h, at a speed of 100 rpm at room temperature (25 ±1.0 ºC).

UAE was performed using an ultrasonic probe with automatic control of time, cycles, and power (Bandelin Sonoplus HD 3100, 20 kHz frequency with an MS 73 probe). The same amount of saffron was extracted with the same extraction solvents for 3 min using 4 cycles at a power between 50-60% of the maximum power (Jalili *et al.,* 2018).

For MAE, the prepared samples were located in a conventional microwave oven (Arçelik MD 565 S, Turkey). Ground saffron stigmas (0.2 g) were extracted with extraction solvents (as mentioned above) using a microwave power level of 30% for 2 min (Sarfarazi *et al.,* 2020).

2.2.2. Combination of ultrasound-assisted extraction (UAE) with microwave-assisted extraction (MAE)

Ground saffron stigmas (0.2 g) were weighed and the extraction solvents were added. The extraction was performed using combined methods of UAE and MAE as described in the previous section. Firstly, UAE was performed, followed by MAE.

2.3. Characterization of saffron extract

2.3.1. Total phenolic content (TPC)

The TPC of all extracted solutions was determined calorimetrically at 725 nm using the Folin–Ciocalteau reagent according to the method described by Mohamed *et al.* (2018) with slight modifications. Each solution of saffron extract (100 µL) was taken in a separate test tube and completed to 3 mL with deionized water. After that, they were mixed completely by vortex with 0.5 mL Folin-Ciocalteau for 3 min. Finally, 2 mL of 20% Na_2CO_3

solution were added and left for 1 h at room temperature in the dark. Then, the absorbance of the samples was measured at 725 nm against a blank using UV-Vis spectrophotometer SP-3000 plus, OPTIMA INC. Japan. Gallic acid was used as a standard for establishing the calibration curve. A series of standard solutions with concentrations of 12.5 -75 µg/mL were prepared and their absorbance measured. TPC was calculated using the linear equation based on the calibration curve of gallic acid (y = 0.0161 x + 0.092; $R^2 = 0.98$).

2.3.2. ABTS radical scavenging activity

The antioxidant property of the extract was analyzed according to the ABTS method (Bhatt *et al.,* 2012). A mixture (1:1, v/v) of ABTS (7.0 mM) and potassium persulfate (2.45 mM) was made and allowed to stand for 16 h. A working solution was diluted with ethanol to the absorbance value of 0.7 at 734 nm. An aliquot of 100 µL of each sample was mixed with the working solution (2.9 mL) and the decrease in absorbance was measured at 734 nm after standing for 6 min at room temperature in the dark. The percentage ABTS inhibition was calculated using the following formula (1):

$$ABTS \text{ inhibition } (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100 \quad (1)$$

2.3.3. Determination of the main characteristics of saffron using the UV–Vis spectrophotometric method

Saffron samples were analyzed according to the ISO 3632 trade standard (ISO/TS 3632, 2003). This method allows the determination of the main characteristics of saffron related to picrocrocin, safranal, and crocin contents. The higher amount of these components means a higher quality of saffron. As explained before, a total of 0.2 g of saffron sample were extracted using different methods and solvents, filtrated and finally, total volume was completed to 25 mL. Then, 0.3 mL of each extract were transferred to 50-mL volumetric flask and completed to 50 mL with each solvent. The final concentration of powder saffron in the measured samples was 0.005% (w/v). The absorbance was measured for SE on a UV-Vis spectrophotometer (SP-3000 plus) at 257, 330, and 440 nm, where picrocrocin, safranal, and crocin had the maximum absorbance values, respectively. The results were obtained by direct reading of the absorbance, D, at three wavelengths, as follows:

$E^{1\%}_{1\,cm}$ 257 nm: absorbance at about 257 nm (maximum absorbance of picrocrocin); $E^{1\%}_{1\,cm}$ 330 nm: absorbance at about 330 nm (maximum absorbance of safranal); $E^{1\%}_{1\,cm}$ 440 nm**:** absorbance at about 440 nm (maximum absorbance of crocins), where:

$$E^{1\%}_{1\,cm} = (D * 10000) / [m * (100 - H)] \quad (2)$$

Where D is the specific absorbance; m is the mass of the saffron sample in grams; H is the moisture and volatile content of the sample, expressed as a mass fraction. For our sample, the H value was about 5%.

2.4. Preparation of freeze-dried saffron extract

After the characterization of the extracts obtained by different solvents and extraction methods, the extract which had the highest levels of bioactive components was selected for the second part of the study. This extract was freeze-dried to be inserted into the oils for the evaluation of their oxidative stability.

This extract was obtained using methanol/water (50:50) and combinations of UAE and MAE. Then, the obtained extract was concentrated by a rotary evaporator, filtered and kept at -20 °C for 24 h. Finally, the extract was freeze-dried using a LyoAlfa 6-50 freeze-dryer (Telstar, Terrassa, Spain) for 16 h.

2.5. HPLC and LC-MS analysis of the freeze-dried saffron extract

The Shimadzo HPLC system (Kyoto, Japan) with two pumps (LC-20AD) was applied for the detection of SE bioactive components. This system was equipped with a photodiode array detector (UV-Vis PDA, SPD-M20A). The freeze-dried SE in the concentration of 100 µg/mL was re-dissolved in 50:50 methanol/water, then filtered through a 0.2 µm (Millipore) filter and injected into the system at a volume of 20 µL at 30 °C. The column was Alltima (C18 zorbax, 250 mm × 5 m; 4.6 mm id). The mobile phase consisted of solvent A (Water containing 0.1% formic acid) and solvent B (Acetonitrile containing 0.1% formic acid). A gradient program was performed to analyse the SE components: 20% B for 5 min, then increased linearly until 80% B in 30 min, then adjusted to 98 % B and kept for 5 min and then decreased to 20 % B and kept for 15 min at a constant flow rate of 0.8 $mL \cdot min^{-1}$. The detection wavelengths were set at 440

nm and 250 nm, i.e., the maximum absorbance for crocins and picrocrocin, respectively.

Standard solutions of crocin-4 were prepared in 50% methanol/water (v/v) at concentrations of 1-20 µg/mL. (Y=156331X, R^2 = 0.999). Picrocrocin was identified by LC-MS, while its quantification was performed using the regression equation from the literature, Y = 1952830X - 3808.1 (Cossignani *et al*., 2014). The identification of other crocin compounds was carried out by the LC-MS method and the quantity of each crocin was expressed as mg crocin-4 equivalent per gram of extract.

The identification of each compound was performed with the LC system including solvent delivery pump and PDA detector. It was coupled to a LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, USA). The HPLC conditions were the same as the conditions described above for HPLC-UV. The peaks were detected at wavelengths of 279.5-280.5 nm. Mass spectra were obtained in positive (ESI^+) and negative ion modes (ESI^-) in scan ranges of 120-2000 m/z (Larbat *et al.,* 2014).

2.6. Characterization of oils

2.6.1. Physiochemical characteristics

Peroxide value (PV) and acid value (AV) for the oil samples were determined according to the AOCS methods (Cd–8–53 and AOCS Cd–3a–63) (AOCS, 1988). The PV and AV values of the oil samples under investigation were at acceptable levels (Table 4).

2.6.2. Fatty acid profile of oils

The fatty acid composition of the oils was determined by the conversion of oil to fatty acid methyl esters (FAMEs) according to the modified method of Zahran and Tawfeuk (2019). FAMEs were analyzed on an Agilent 7890B Series gas chromatography (GC-FID) using a polar capillary column SP®-2560 (100m, 0.25mm id, 0.2µm film thickness). Helium was used as carrier gas at a flow rate of 20 $cm \cdot sec^{-1}$, and split ratio of 100:1. The column temperature was held at 100 °C for 5 min, increased to 240 °C at 4 $°C \cdot min^{-1}$; held at 240 °C for 30 min. A sample volume of 1.0 µL was injected into the GC-FID system. FAMEs were identified by comparing their relative and absolute retention times to the authentic standards of FAMEs (from C4:0 to C24:0).

2.6.3. Oxidative stability index (OSI)

Before measuring the OSI of the oil samples, the saffron extract was blended homogenously with the oil samples. At first the freeze-dried saffron extract was dissolved in an appropriate volume of 1,2-propanediol (20% w/w). Then, the solution was placed in the test tubes containing oil samples (10 mL) and subjected to the sonication (4 cycles, power of 50-60% maximum power) for 2 min using an ultrasonic probe.

The SE at concentrations of 1000 and 1500 ppm (w/w) were examined for the stabilization of oil samples. Since no significant differences were observed in the OSI values of the oil samples enriched with both concentrations of SE, 1000 ppm level was selected to be inserted into the oil samples. The OSI of control oils and oils treated with SE and BHT (200 ppm) was measured using the Metrohm Rancimat model 743 (Herisau, Switzerland) according to (AOCS, 1998). All measurements were performed at 100 °C with an air flow rate of 20 $L \cdot h^{-1}$.

2.7. Statistical analysis

All experiments were conducted in triplicate. The values of the means were statistically analyzed by IBM SPSS statistics software package (version 17.0). The results were analyzed by one-way ANOVA and followed by the TUKEY test. The cluster analysis was used to classify objects into relative groups according to Minitab® 16 Statistical Software, 2010. Data were presented as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

Extraction techniques can be used alone or in combination with other methods for the separation and purification of different bioactive ingredients from various parts of saffron. The quality of the extracted bioactive ingredients is important for further applications in the formulation of nutraceutical and functional food products (Ozkan *et al.*, 2021). In this study, the effects of extraction methods such as ME, UAE, MAE, and both UAE and MAE on the recovery of the bioactive compounds from saffron were evaluated. Moreover, the influence of SE on the oxidative stability of common vegetable oils (CAO, SO and COO) was assessed.

3.1. Effect of different extraction methods on TPC and antioxidant activity

As can be seen from Table 1, the type of solvent and extraction method had significant effects on TPC ($p < 0.05$). In the case of ME, among the pure solvents, extraction with methanol showed the highest recovery of phenolic compounds (29.54 mg GAE /g in saffron dry basis) followed by ethanol and water (18.83 and 13.08 mg GAE/g, respectively). Similar findings were reported by other authors, who studied the extraction of phenolic compounds from rice bran and mango waste. They explained that methanol was more efficient than ethanol to extract polyphenols (Dorta *et al*., 2012; Zhou and Yu, 2004).

The TPC of the ethanol/water (50:50, v/v) extract was higher than that obtained with absolute ethanol. According to the previous studies, the blending of organic solvents with water increases the polarity of the extraction medium and may allow easier extraction of the components which are soluble in water or in organic solvents (Socaci *et al.,* 2018) .

In the case of UAE with pure solvents, the TPC of the extracts was in the range of 4.43 to 28.03 mg GAE /g in the following decreasing order: water > methanol > ethanol. Using solvent and water mixtures (50:50, v/v) for extraction led to a significant increase in TPC. The most prominent increase was observed for ethanol; the TPC of 4.43 mg GAE /g obtained with pure ethanol increased to 29.27 mg GAE/g when 50:50 (v/v)

Table **1.** Total phenolic content and ABTS radical scavenging activities of saffron extract obtained with different solvents and protocols

Treatments	Sample code	Total phenolic content (TPC) (mg GAE/g of DW*)			Antioxidant activity using ABTS+ (% inhibition)		
Maceration for 24 h							
Water	1	13.08	±	0.82^{j}	63.28	±	3.31^{f}
Ethanol	2	18.83	±	0.16^{h}	57.37	±	1.51^{i}
Methanol	3	29.54	±	0.38^{bc}	75.50	±	3.31^{bc}
Ethanol/Water (50:50)	4	24.15	±	0.22^{fg}	68.32	±	0.40^{ef}
Methanol/Water (50:50)	5	29.46	±	0.71^{bc}	78.76	±	1.91^{ab}
Ultrasonication for 3 min							
Water	6	28.03	±	0.88^{cd}	72.16	±	0.20^{cde}
Ethanol	7	4.43	±	0.44^{l}	47.03	±	1.71^{hi}
Methanol	8	27.25	±	0.44^{de}	71.66	±	0.10^{cde}
Ethanol/Water (50:50)	9	29.27	±	0.33^{bc}	72.16	±	0.20^{cde}
Methanol/Water (50:50)	10	30.86	±	0.49^{ab}	79.83	±	0.60^{ab}
Microwave at 30% / 2 min							
Water	11	11.22	±	0.82^{j}	75.64	±	0.30^{bc}
Ethanol	12	16.65	±	0.05^{i}	50.71	±	1.41^{g}
Methanol	13	27.29	±	0.49^{de}	68.75	±	1.81^{def}
Ethanol/Water (50:50)	14	25.62	±	0.22^{ef}	74.50	±	1.10^{bcd}
Methanol/water (50:50)	15	30.05	±	0.44^{ab}	79.62	±	0.30^{ab}
Ultra sonication for 3 min + Microwave at 30% / 2 min							
Water	16	22.98	±	0.33^{g}	80.54	±	1.00^{ab}
Ethanol	17	8.66	±	0.05^{k}	56.46	±	1.51^{gh}
Methanol	18	25.93	±	0.11^{ef}	74.57	±	1.41^{bcd}
Ethanol/Water (50:50)	19	24.18	±	0.16^{fg}	69.96	±	1.51^{cde}
Methanol/water (50:50)	20	31.56	±	0.38^{a}	83.24	±	0.60^{a}

Values are means ± standard deviation (n = 3); Different superscript letters in the same column represent significant difference ($p < 0.05$, one-way ANOVA with Tukey test) *DW, dry weight of saffron stigmas.

ethanol/water mixture was used. The solvent properties can affect the extraction of bioactive components from plant cells. Since ethanol has higher viscosity compared to other solvents, the mass transfer rate of these compounds reduces and moreover, the very short extraction time of the UAE method (3 min) can also result in less extraction of phenolic compounds with ethanol. Extraction with a mixture of water and ethanol enhances mass transfer and therefore accelerates the extraction of TPC due to its lower viscosity (Esmaeilzadeh Kenari *et al.,* 2014).

Kyriakoudi *et al.* (2012) used aqueous methanol to recover crocins and picrocrocin from dried saffron stigma. They concluded that the recovery of apocarotenoids was enhanced by using ultrasonic extraction.

For MAE, a similar tendency as for ME was observed. The TPC increased significantly ($p < 0.05$) when the combination of UAE and MAE was used, which ranged from 8.66 to 31.56 mg GAE /g for ethanol and methanol/water, respectively. Moreover, the combination of UAE and MAE was more efficient especially in the case of using methanol/water as the solvent.

The results of the Pearson correlation analysis shown in Figure 1 reveals that antioxidant activity is associated with TPC. The highest TPC (31.56 mg GAE/g) and the corresponding highest antioxidant activity (83.24% inhibition) were obtained by using methanol/water mixture and combined UAE and MAE techniques for extraction. Karimi *et al.* (2010) explained that saffron's bioactive components, such as phenolics, safranal, crocin, crocetin, and carotenoids contribute to its antioxidant activity. Muzaffar *et al.* (2016) evaluated the percent inhibition for two methanolic saffron extracts belonging to different ecogeographical zones (Jammu and Kashmir, India) using the ABTS radical scavenging activity method, and reported that percent inhibition of methanolic extracts of the stigma samples increased in the range of 41.34 to 92.20 % by increasing the concentrations of extract in aqueous methanol from 100 to 300 µg/mL. The percent inhibition for the methanolic extract in our study obtained by ME was 75.50, which was in the reported ranges.

3.2. Effect of extraction methods on crocin, safranal and picrocrocin contents

The saffron samples, extracted with different techniques and solvents, were analyzed by spectrophotometric analysis in order to evaluate the absorbance val-

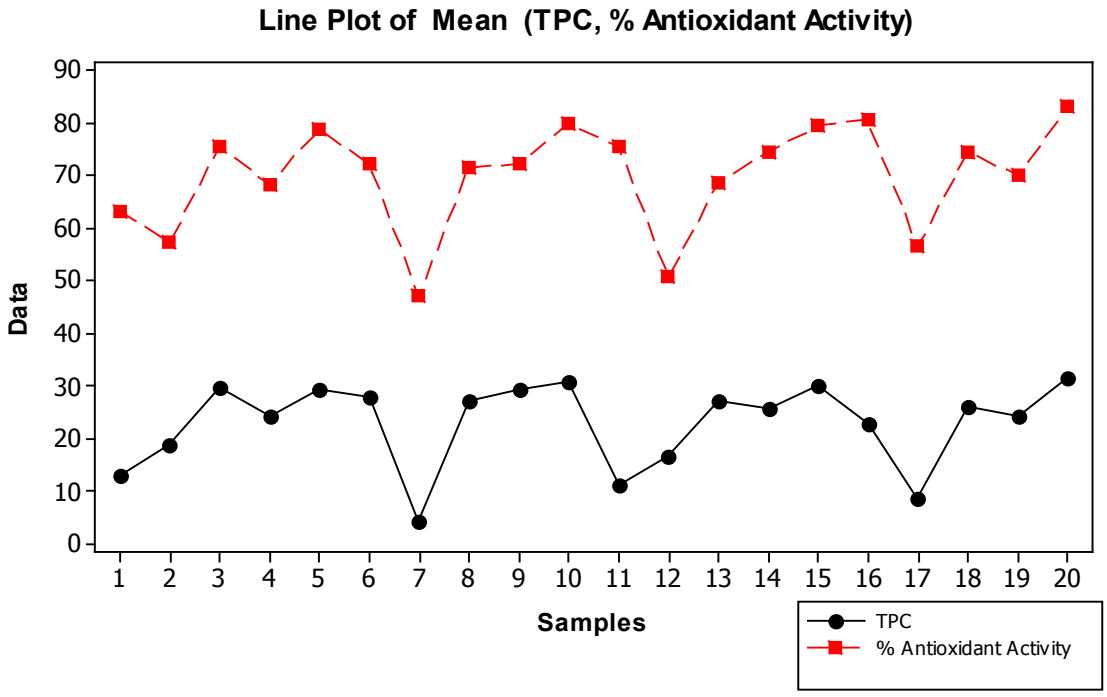

Figure 1. The correlation between antioxidant activity and total phenolic content in saffron extracts *(Pearson correlation* of TPC and antioxidant activity (%) = 0.678, p-value = 0.001

Table 2. Crocin, safranal and picocrocin contents in saffron extracts

Treatment	Sample Code	Crocin content $E^{1\%}_{1\,cm}$ 440			Safranal content $E^{1\%}_{1\,cm}$ 330			Picrocrocin content $E^{1\%}_{1\,cm}$ 257		
Maceration for 24 h										
Water	1	331.2	±	3.0^{i}	127.4	±	9.9^{def}	273.1	±	12.2^{fg}
Ethanol	2	513.4	±	41.8^{h}	89.8	±	2.3^{fg}	227.4	±	11.4^{g}
Methanol	3	1084.9	±	9.1b^{cd}	148.4	±	4.6^{de}	366.1	±	9.9^{de}
Ethanol/Water (50:50)	4	994.1	±	12.9^{def}	152.2	±	3.8^{de}	354.8	±	4.6^{de}
Methanol/water (50:50)	5	1019.4	±	21.3^{cdef}	224.2	±	6.8^{a}	467.7	±	10.6^{bc}
Ultra sonication for 3 min										
Water	6	924.7	±	53.2^{f}	199.5	±	6.8^{abc}	415.6	±	23.6^{cd}
Ethanol	7	331.2	±	9.1^{i}	39.8	±	3.0^{h}	346.8	±	29.7^{e}
Methanol	8	1191.4	±	39.5^{ab}	147.3	±	15.2^{de}	381.2	±	23.6^{de}
Ethanol/Water (50:50)	9	1178.0	±	34.2^{ab}	162.9	±	11.4^{bcde}	459.1	±	15.2^{c}
Methanol/water (50:50)	10	995.7	±	10.6^{def}	168.8	±	15.2^{bcd}	372.0	±	18.2^{de}
Microwave at 30% / 2 min										
Water	11	1031.2	±	45.6^{cdef}	204.3	±	19.8^{ab}	463.4	±	33.5^{bc}
Ethanol	12	674.7	±	9.9^{g}	72.0	±	3.0^{gh}	249.5	±	3.0^{g}
Methanol	13	1055.4	±	2.3^{cde}	125.8	±	6.1^{ef}	326.9	±	3.0^{ef}
Ethanol/Water (50:50)	14	1054.3	±	2.3^{cde}	150.5	±	7.6^{de}	525.8	±	15.2^{ab}
Methanol/water (50:50)	15	1050.5	±	1.5^{cde}	159.1	±	7.6^{cde}	369.4	±	8.4^{de}
Ultra sonication for 3 min + Microwave at 30% / 2 min										
Water	16	1124.2	±	14.4^{abc}	194.6	±	15.2^{abc}	470.4	±	16.0^{bc}
Ethanol	17	636.6	±	4.6^{g}	59.7	±	9.9^{gh}	361.8	±	34.2^{e}
Methanol	18	1137.6	±	85.2^{abc}	140.3	±	17.5^{de}	334.4	±	9.1^{ef}
Ethanol/Water (50:50)	19	959.1	±	13.7^{ef}	141.9	±	12.2^{de}	526.3	±	3.8^{ab}
Methanol/water (50:50)	20	1226.3	±	9.9^{a}	226.3	±	5.3^{a}	536.0	±	3.8^{a}

Values are means ± standard deviation (n = 3); Different superscript letters in the same column represent significant difference ($p < 0.05$, one-way ANOVA with Tukey test).

ues due to the presence of their secondary metabolites, crocins, picrocrocin, and safranal according to the ISO 3632 trade standard (ISO/TS 3632-1/2, 2003). In general, the content of these components is related to the quality of the saffron. In fact, the contents of these components differ greatly from country to country, based on several factors such as climatic conditions, drying process, harvesting and storage (Carmona *et al*., 2006) as well as extraction conditions. In particular, the techniques and solvents used in extraction directly affect the crocins, picrocrocin, and safranal contents. Table 2 shows the contents of the twenty analyzed samples under different conditions.

The absorbance values for the crocin component of the extracts were higher than picrocrocin and safranal. They ranged from 331.2 for water extract (ME) to 1226.3 for methanol/water extract using UAE in combination with MAE. The absorbance values for the picrocrocin component at 257 nm ranged from 227.4 for ethanolic extract by ME to 536.0 for methanol/water extract using combinations of UAE and MAE (Table 2). However, the absorbance values for safranal at 330 nm ranged from 39.8 for the ethanolic extract using UAE to 226.3 for the methanol/water extract using UAE together with MAE techniques.

Ethanol-water solvent mixtures were found to be the best media for the extraction of crocin and polyphenols from saffron with traditional extraction methods (Garavand *et al.*, 2019)

From the obtained data, it could be concluded that the MAE method, as well as UAE affected cro-

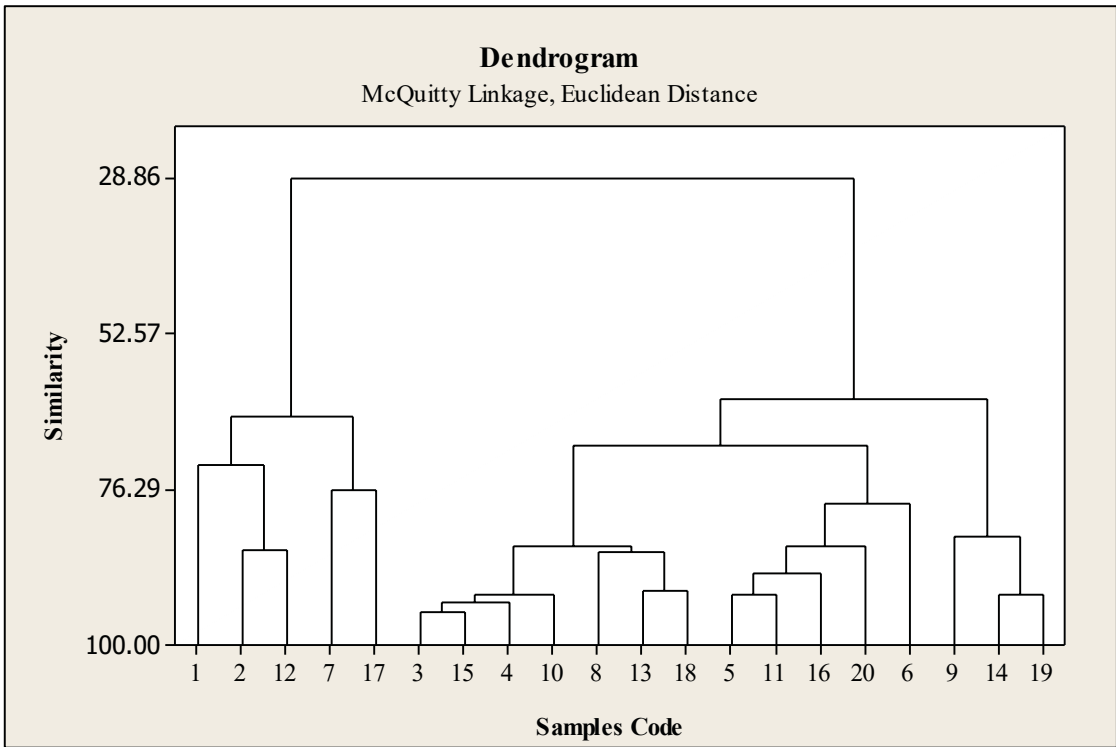

Figure 2. Dendrogram of crocin, safranal and picrocrocin content extracted by different solvents and techniques

cins, picrocrocin, and safranal contents. In addition, the extraction solvent was the most important factor in the efficiency of the extraction process. Sarfarazi *et al.* (2015) studied the effect of temperature, extraction time and ethanol concentration during maceration on the contents of crocins, picrocrocin, and safranal of SE. Based on the response surface methodology analysis, they concluded that ethanol concentration and temperature had more influence on the responses than extraction time.

A cluster analysis was carried out to classify the results of the three extraction methods and five extraction model systems into relative groups. The Dendrogram classified various crocins, picrocrocin, and safranal contents in this study into four main groups as shown in Figure 2. The cluster analysis verified the results of the model systems in which ethanol extraction showed low levels for crocins, picrocrocin, and safranal contents (First group of samples with codes of 2,7,12 and 17). the methanol (3,10 and 15) and methanol/water (8,13 and 18) showed high similarity in component contents, while the other extracts contained moderate amounts of crocins, picrocrocin, and safranal contents.

3.3. Identification of saffron extract compounds by HPLC and LC-MS analysis

Table 3 displays the quantity of main bioactive molecules of freeze-dried SE detected by LC-MS. The LC-MS analysis results were carefully discussed according to (Lech *et al.*, 2009; Cossignani *et al.*, 2014; Carmona *et al.*, 2006; Kabiri *et al*., 2017) and detailed information about retention times, fragmentation patterns and molecular weight of the mol-

Table 3. Results of LC-MS analysis of freeze-dried saffron extract

Compound	Quantity (mg/g of Extract)
Picrocrocin	3.28±0.00
Campherol diglucoside	nd[a]
Trans-crocine-4 (2 gen)	168.17±0.28
Trans-crocin-3(gen, glu)	60.55±1.99
Cis-crocin-4	10.47±0.06
Cis -crocin-3	29.49±0.47
Cis-crocin-2' (2 glu)	nd

[a] nd: not determined, value = means ± standard deviation (n = 3)

Table 4. Chemical characteristics of corn (COO), canola (CAO) and sunflower (SO) oil samples

Parameter	Oil samples		
	Corn oil	Canola oil	Sunflower oil
Acid value (mg/g)	0.12 ±0.01	0.23 ±0.02	0.17 ±0.01
Peroxide value ($meqO_2$/kg)	0.82 ±0.03	0.85 ±0.03	0.78 ±0.07
Fatty acids		Area %	
Myristic acid ($C_{14:0}$)	0.04	0.05	0.09
Palmitic acid ($C_{16:0}$)	11.46	5.1	7.9
Palmitoleic acid ($C_{16:1}$)	0.53	0.21	0.14
Margaric acid ($C_{17:0}$)	nd*	nd	0.04
Heptadecanoic acid ($C_{17:1}$)	nd	nd	0.03
Stearic acid ($C_{18:0}$)	2.14	1.7	2.7
Oleic acid ($C_{18:1}$)	33.4	60.2	35.33
Linoleic acid ($C_{18:2}$)	50.5	23.7	52.3
Linolenic acid ($C_{18:3}$)	0.93	6.6	0.16
Arachidic acid ($C_{20:0}$)	0.44	0.52	0.26
Arachidonic acid ($C_{20:1}$)	0.41	1.2	0.27
Behenic acid ($C_{22:0}$)	0.15	0.6	0.6
Erucic acid ($C_{22:1}$)	nd	0.13	nd
Lignoceric acid ($C_{24:0}$)	nd	nd	0.21
∑ SFA	14.23	7.97	11.55
∑ UFA	85.77	91.91	88.2
∑ MUFA	34.34	61.60	35.74
∑ PUFA	51.43	30.3	52.46

*nd= not detected; value = mean ± SD (n=3)

ecules can be found in our previous work (Najafi *et al.*, 2021).

The total crocin content in our freeze-dried saffron extract was 268.7 mg of crocin-4 eq/g of extract which was similar to the findings of Lahmass *et al.* (2017) in which they obtained a total crocin amount of 298 mg of crocin-4 eq/g DM of extract.

3.4. Fatty acid composition

Vegetable oils are rich in unsaturated fatty acids, particularly monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The fatty acid composition of COO, CAO and SO, expressed as saturated and unsaturated (mono and polyunsaturated) fatty acids are summarized in Table 4. The content of saturated fatty acids (SFA) amounted to 14.23 and 11.55% in COO and SO, respectively, which were both higher than CAO (7.97%). However, the unsaturated fatty acid (UFA) contents were 85.77, 88.2 and 91.91% for COO, SO and CAO, respectively. It can also be noted that the content of MUFA in canola oil (61.6%) was significantly higher than SO and COO (35.74 and 34.34%, respectively). On the contrary, the PUFA content in CAO was lower (30.3%) in comparison with COO and SO (51.43 and 52.46%, respectively). These values are in agreement with the data obtained by Kozłowska and Gruczyńska, (2018) for SO and by Carrillo *et al.* (2017) for COO.

3.5. Oxidative stability index (OSI)

The induction time for the control CAO was 17.38 h, and it increased significantly ($p < 0.05$) to 20.82 h in the case of samples treated with BHT (200 ppm), and samples treated with SE at 1000 ppm (19.41 h) (Figure 3).

There were no significant differences ($p < 0.05$) between the induction times of SO treated with BHT and SE (12.50 and 12.12 h, respectively). The difference was clear with the control (9.61 h). On the other hand, the results for COO showed a protective effect against oxidation for the samples containing BHT (21.43 h) and SE at 1000 ppm (17.55 h) compared to the control (15.28 h). The protection factor (PF) was calculated (Bandonien *et al*., 2000) and SE was found to be more effective in the protection of SO (PF=1.26) against oxidation than CAO (PF=1.12).

Some studies investigated the antioxidant activity of SE against synthetic antioxidants by the DPPH and ABTS methods (Baba *et al*., 2015; Jadouali *et al.,* 2019), but no published data were found regarding the use of SE to protect vegetable oils.

Merrill *et al*. (2008) assessed the oxidative stability of some vegetable oils with the OSI test, and they used high oleic canola oil (HOCAO), corn oil and sunflower oil purchased from Cargill. They evaluated the effect of several natural antioxidants and TBHQ (200 ppm) on the oxidative stability of some stable high-oleic vegetable oils. The Rancimat test was performed at 110 °C, and the OSI values were increased for HOCAO from 12.9 h (control) to 16 h and 36 h in oil fortified with rosemary extract and TBHQ (200 ppm), respectively (Merrill *et al.*, 2008). The difference between the OSI value for the control in our study (17.38 h) and previous study (8.4 h) can be attributed to the lower analysis temperature in the

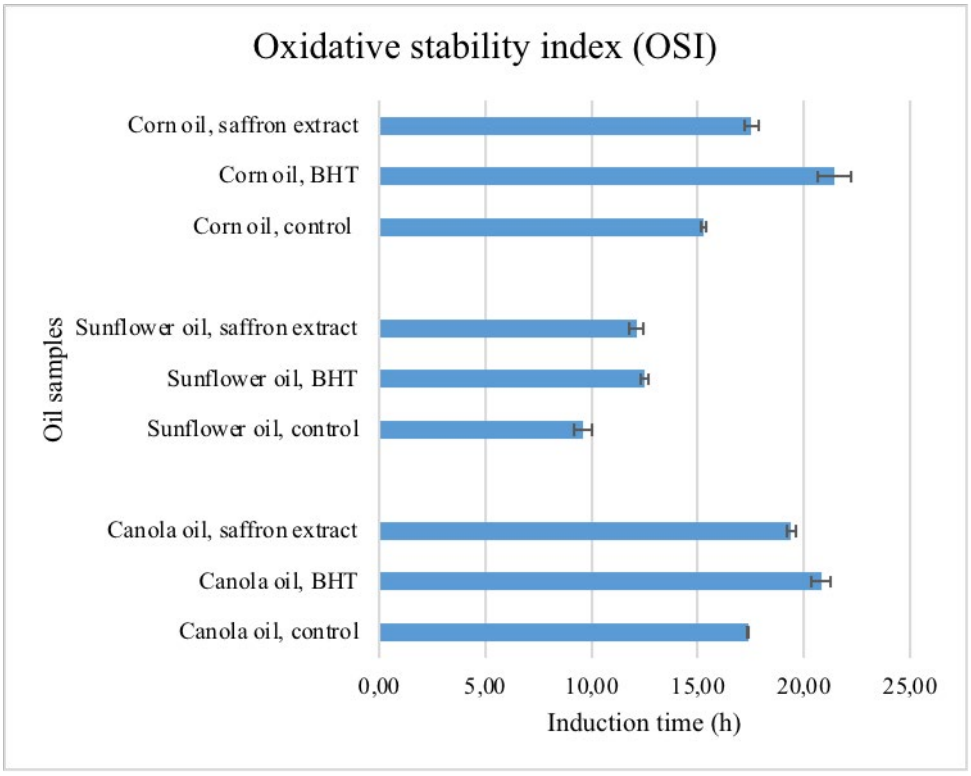

Figure 3. Oxidative stability index (OSI) of oil samples treated with 1000 ppm saffron extract, control oil and oils treated with 200 ppm BHT. The values provided in the figures are the mean values of triplicate analyses with standard deviation.

Rancimat test (100 °C) and also the difference in fatty acid profile of the oils tested. The α- linolenic content of CAO was 10% in the mentioned previous study; while the sample we used had less α-linolenic acid (6.6%).

For corn oil without antioxidant, the OSI value was reported as 9.8 h by Merrill *et al*. (2008). The difference with our result (15.28 h) might be due to the different temperature setting during OSI analysis since the fatty acid profiles were similar.

4. CONCLUSIONS

According to the results, among the different extraction methods used in this study, UAE combined with the MAE method revealed the highest TPC as well as antioxidant activity. The type of extraction solvent was found to be important to enhance the efficiency of extraction of bioactive compounds from saffron stigmas. The LC-MS analysis showed trans-crocin-4 and trans-crocin-3 as the main constituents of freeze-dried SE and this extract contained high a level of total crocin content (268 mg/g). Therefore, it exhibited significant potential in the inhibition of CAO, SO and COO oxidation in comparison with BHT as a synthetic antioxidant. Based on the oxidative stability analysis, SE (1000 ppm) had the same effect ($p < 0.05$) as BHT (200 ppm), in preventing the oxidation of sunflower oil used in the study. In the future, further studies could be conducted with different concentrations of SE on the stability of vegetable oils during accelerated storage and frying.

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