Ultrasound-assisted extraction of saffron bioactive compounds; separation of crocins, picrocrocin, and safranal optimized by artificial bee colony

Messiah Sarfarazi a, Qadir Rajabzadeh b, Razieh Tavakoli c, Salam A. Ibrahim f, Seid Mahdi Jafari d, e, *

a Faculty of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
b Research Institute of Food Science and Technology (RIFST), Mashhad, Iran
c Department of Electrical and Medical Engineering, Khayyam University, Mashhad, Iran
d Universidade de Vigo, Nutrition and Bromatology Group, Department of Analytical Chemistry and Food Science, Faculty of Science, E-32004 Ourense, Spain
e Department of Food Materials and Process Design Engineering, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
f Food and Nutritional Sciences Program, North Carolina Agricultural and Technical State University, E. Market Street, 1601, Greensboro, NC 27111, USA

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A B S T R A C T

In this work, a four-factor five-level full factorial central composite design (CCD) was used to optimize the ultrasound-assisted extraction (UAE) of saffron major components, namely picrocrocin, safranal and crocin. The process parameters included ethanol concentration (0–100%), extraction time (2–10 min), duty cycle (0.2–1.0) and ultrasonic amplitude (20–100%). The extracted compounds were measured both by spectrophotometry and chromatography techniques. The results revealed that the middle concentrations of ethanol and relatively long process durations along with high duty cycles and ultrasonic amplitudes had the most profound impact on the yields of the extracted bioactives. UAE was optimized using response surface methodology (RSM) and artificial bee colony (ABC); a comparison between these methods indicated their optimization power was approximately the same. According to the RSM analysis, an ethanol concentration of 58.58%, extraction time of 6.85 min, duty cycle of 0.82 and amplitude of 91.11% were the optimum extraction conditions, while the optimal conditions resulting from ABC were 53.07%, 7.32 min, 0.93 and 100% for the UAE variables respectively. Finally, HPLC analysis was carried out on the UAE optimum extract resulting from RSM. Four crocetin esters were detected in the optimal extract.

1. Introduction

Iran is by far the major saffron producer (nearly 95%) throughout the world with 47,000 ha under cultivation and annual production of 160,000 kg. [1]. Both lipophilic and hydrophilic carotenoids occur in saffron. Lycopene, tetra-hydrolycopene, α-, β- and γ-carotene, zeaxanthin, phytoene and phytofluene are the lipophilic ones [2,3]. The hydrophilic carotenoids which are much more frequent, provoke the charming red-yellowish color of saffron. These apocarotenoid compounds are comprised of a C_{20} di-carboxylic acid named crocetin, as well as its mono/di glycosyl esters with the comprehensive name of crocin, including digentiobioside (α-crocin), diglucoside, gentiogluco-side, gentiobioside, glucoside and etc. α-crocin is the most abundant crocin in saffron. Picrocrocin, the major appealing bitter taste of saffron, is a colorless glycoside composed of a monoterpenic aldehyde (4-hydroxy-2,6,6-trimethyl-1-carboxaldehyde-1-cyclohexene (HTCC)) and glucose as the aglycon and glycon moieties respectively [4]. However, other compounds such as flavonoids also contribute to saffron bitterness [1]. Safranal (dehydro-β-cyclocitrinal), is the product of thermal or enzymatic dissociation of picrocrocin. This monoterpene aldehyde is responsible for the adorable aroma of saffron. The content of the other aromatic components of saffron varies from 2 to 29% relative to the safranal content [5,6].

Ultrasound waves are classified into two distinct groups, namely, high-intensity (typically 10–1000 W/cm²) or low-frequency (20 – 100 kHz), and low-intensity (typically < 1 W/cm²) or high-frequency (100 kHz – 10 MHz). High-intensity or power ultrasound is used in food processes such as homogenization, sterilization, cleaning, cell disruption
and emulsification [7,8]. With the evolution of “Green Chemistry” concept in recent years, the eco-friendly technologies have drawn many researchers’ attention. Power ultrasound is one of these methods which is a rapid, inexpensive and simple technique compared with conventional extraction methods. In addition, it increases the purity and yield of the component to be extracted via cavitation (9,10,11). This phenomenon is defined as the generation, growth, extremely quick oscillation and even violent collapse of micro-bubbles in a liquid [12,13]. The principal advantage of cavitation is concerned with its capability of concentrating the acoustic energy in tiny volumes, leading to an increase in temperature and pressure [14]. During the collapse of the bubbles, the localized temperature and pressure reach up to 5000 K and 5000 atm respectively [15]; that is why they are also called hot spots. However, since the bubbles formation and collapse take place during few microseconds, there is not enough time for heat transfer from the bubbles to the surrounding medium; as a consequence, the liquid temperature is raised slowly [16]. At the same time, the asymmetrical collapse of the bubbles is so fast and leads to the development of shock waves and micro-jets with a velocity of as high as 110 m/s, resulting in turbulent micro-streams and shear forces in the liquid [17,18,19]. Cavitation also induces light emission which is called sonoluminescence [20].

There have been a few studies carried out on the extraction of saffron bioactive compounds using the concept of “Green Chemistry”, Kadkhodae & Hemmati-Kakhki, [21] employed high-intensity ultrasound to extract saffron active compounds. Jalali-Heravi, Parastar, & Ebrahimi-Najafabadi, [22] optimized the UAE of the volatile compounds of Iranian saffron. Kyriakoudi, Chrysanthou, Mantzouridou, & Tsimi-dou, [23] conducted UAE to recover saffron active compounds. They optimized the process independent variables using RSM. Sereshiti, Heidar, & Samadi, [24] exploited a combined extraction technique, including UAE allied with dispersive liquid-liquid microextraction for the isolation and enrichment of saffron volatile compounds. Esmaelian, Jafari, Feizy, & Einafshar, [25] optimized UAE through RSM to determine the total phenolic content and antioxidant activity of saffron corn. Sarfarazi, Jafari, Rajabzadeh, & Feizi, [26] and Sarfarazi, Jafari, Rajabzadeh, & Galanakis, [27] respectively employed subcritical water and microwave to enhance the extraction efficiency of saffron major compounds.

The objective of the present study was to optimize UAE of saffron bioactives, including crocin, picrocrocin and safranal using RSM and ABC. To that end, ethanol concentration, extraction time, duty cycle and sonication amplitude were selected as independent variables. We also aimed at comparing the afore-mentioned optimizing techniques by the empirical and theoretical results.

### 2. Materials and methods

#### 2.1. Preparation of saffron samples

Saffron was supplied from a farm in Torbat Heidarieh, Khorasan, Iran. Providing the entire sample from an individual plantation enabled us to neglect the impacts of the climatic conditions, the type and extent of irrigation as well as the soil type. The stigmas from saffron were ground with a pestle and mortar and sieved using a 0.5 mm mesh screen. The stock sample was kept in the dark inside a desiccator during experiments.

#### 2.2. Measurement of the moisture and volatile matter content

About 2.5 g of the samples were weighed using a digital balance and kept in a hot-air oven (U-15, Memmert, Germany) at 103 ± 2 °C for 16 h. After that, the remaining matter was weighed and the moisture and volatile matter content was computed by the following equation [28]:

$$H = \frac{W_t - W_f}{W_t} \times 100$$  

(1)

### Table 1

Coded and uncoded levels of UAE independent variables.

| Independent variables | Symbols | Coded levels |
|-----------------------|---------|--------------|
| Ethanol concentration (v/v %) | X<sub>1</sub> | 0 25 50 75 100 |
| Extraction time (min) | X<sub>2</sub> | 2 4 6 8 10 |
| Duty cycle | X<sub>3</sub> | 0.2 0.4 0.6 0.8 1.0 |
| Ultrasound amplitude (%) | X<sub>4</sub> | 20 40 60 80 100 |

### Table 2

Central composite design matrix and response variables.

| Independent variables | Time (min) | Duty cycle | Amplitude (%) | Dependent variables E<sub>257</sub> | E<sub>330</sub> | E<sub>440</sub> |
|-----------------------|------------|------------|---------------|---------------------------------|----------------|----------------|
| Ethanol concentration (%) |            |            |               |                                 |                |                |
| 25                    | 4          | 0.4        | 40            | 60.10 ± 1.52                    | 23.78 ± 0.30   | 80.10 ± 5.96   |
| 50                    | 2          | 0.6        | 60            | 68.54 ± 1.22                    | 30.70 ± 0.00   | 158.59 ± 1.07  |
| 75                    | 8          | 0.8        | 80            | 83.35 ± 1.98                    | 37.83 ± 0.91   | 207.89 ± 3.82  |
| 25                    | 8          | 0.8        | 40            | 69.62 ± 0.00                    | 29.94 ± 0.15   | 89.63 ± 7.18   |
| 50                    | 6          | 1          | 60            | 80.10 ± 1.68                    | 37.18 ± 0.61   | 195.35 ± 1.68  |
| 75                    | 4          | 0.4        | 80            | 57.62 ± 1.68                    | 24.97 ± 0.76   | 135.02 ± 5.65  |
| 25                    | 4          | 0.8        | 80            | 70.48 ± 2.14                    | 30.37 ± 1.37   | 108.54 ± 7.33  |
| 50                    | 6          | 0.6        | 60            | 79.63 ± 5.36                    | 36.75 ± 1.98   | 192.72 ± 10.36 |
| 75                    | 4          | 0.4        | 40            | 58.37 ± 3.05                    | 25.29 ± 1.22   | 137.40 ± 5.65  |
| 25                    | 4          | 0.8        | 40            | 61.72 ± 0.45                    | 26.81 ± 0.30   | 146.05 ± 1.07  |
| 75                    | 8          | 0.4        | 100           | 70.12 ± 1.52                    | 31.45 ± 0.76   | 171.13 ± 5.96  |
| 0                     | 6          | 0.6        | 60            | 71.02 ± 3.82                    | 29.40 ± 2.44   | 95.13 ± 0.30   |
| 75                    | 8          | 0.8        | 40            | 72.32 ± 0.76                    | 31.89 ± 0.76   | 176.21 ± 3.36  |
| 50                    | 6          | 0.6        | 60            | 78.37 ± 1.68                    | 35.78 ± 0.76   | 187.35 ± 5.04  |
| 25                    | 8          | 0.4        | 80            | 71.45 ± 2.90                    | 30.70 ± 1.83   | 106.59 ± 6.42  |
| 25                    | 4          | 0.8        | 80            | 72.10 ± 1.98                    | 31.78 ± 1.22   | 103.45 ± 1.98  |
| 50                    | 6          | 0.2        | 60            | 70.91 ± 3.05                    | 32.21 ± 1.83   | 170.91 ± 4.73  |
| 25                    | 4          | 0.8        | 40            | 68.00 ± 0.76                    | 28.86 ± 0.45   | 97.29 ± 4.28   |
| 50                    | 6          | 0.6        | 60            | 80.32 ± 2.29                    | 37.08 ± 1.68   | 197.83 ± 5.80  |
| 75                    | 4          | 0.8        | 80            | 69.18 ± 1.83                    | 30.37 ± 1.07   | 165.62 ± 5.80  |
| 25                    | 8          | 0.4        | 40            | 74.27 ± 0.45                    | 33.83 ± 0.76   | 128.54 ± 9.60  |
| 50                    | 10         | 0.6        | 60            | 82.70 ± 0.76                    | 38.05 ± 0.30   | 197.51 ± 5.96  |
| 100                   | 6          | 0.6        | 60            | 10.37 ± 2.75                    | 4.21 ± 1.37    | 162.21 ± 2.75  |
| 75                    | 8          | 0.4        | 40            | 70.37 ± 1.68                    | 30.91 ± 0.91   | 168.86 ± 6.11  |
| 50                    | 6          | 0.6        | 20            | 76.10 ± 1.83                    | 34.70 ± 0.76   | 179.78 ± 5.04  |
| 25                    | 8          | 0.8        | 80            | 74.05 ± 0.15                    | 35.67 ± 0.30   | 102.81 ± 1.07  |
| 50                    | 6          | 0.6        | 100           | 83.78 ± 1.07                    | 39.89 ± 0.15   | 202.43 ± 3.51  |
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Table 3

Analysis of variance for the three response variables.

| Source                  | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-------------------------|----|---------|---------|---------|---------|
| E                        |    |         |         |         |         |
| Total                   | 63 | 41809.8 | 662.42  | 11.67   | 0.000   |
| Lack-of-Fit             | 39 | 2776.2  | 72.00   | 2.32    | 0.112   |
| Time (min)*Amplitude (%)| 1  | 1.69    | 1.69    | 0.15    | 0.697   |
| Time (min)*Duty cycle   | 1  | 0.58    | 0.58    | 0.05    | 0.819   |
| Ethanol concentration (%)| 2 | 421.5   | 210.75  | 7.02    | 0.004   |
| Total                   | 63 | 5379.8  | 857.92  | 10.49   | 0.000   |
| Lack-of-Fit             | 39 | 509.80  | 13.07   | 4.22    | 0.010   |
| Error                   | 49 | 140275  | 287.70  | 15.35   | 0.000   |

Table 3 (continued)

| Source                  | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-------------------------|----|---------|---------|---------|---------|
| 2-Way Interaction       | 6  | 254.1   | 42.35   | 0.81    | 0.564   |
| Ethanol concentration (%)| 1 | 121.2   | 121.17  | 2.33    | 0.133   |
| Time (min)*Ethanol conc | 1  | 80.0    | 79.99   | 1.54    | 0.221   |
| *Ethanol conc (%)       | 1  | 0.5     | 0.47    | 0.01    | 0.924   |
| *Amplitude (%)          | 1  | 9.3     | 9.35    | 0.18    | 0.673   |
| Time (min)*Amplitude (%)| 1  | 8.1     | 8.44    | 0.16    | 0.689   |
| Duty cycle*Amplitude (%)| 1  | 34.6    | 34.65   | 0.67    | 0.418   |
| Error                   | 49 | 2547.2  | 51.98   | 1.03    | 0.015   |
| Lack-of-Fit             | 39 | 2446.1  | 62.72   | 1.21    | 0.002   |
| Pure Error              | 10 | 101.1   | 10.11   | 0.08    | 0.776   |
| Total                   | 63 | 11037.0 | 175.5   | 1.08    | 0.000   |

where $H$ stands for the moisture and volatile matter content, $W_1$ and $W_2$ respectively represent the weight of the powdered stigmas and the glass plate before and after heating. $H = 7.5\%$ in this study.

2.3. UAE apparatus and procedure

UAE was carried out using SONOPULS HD 3200 ultrasonic homogenizer (Bandelin, Germany) operating at 20 kHz $\pm$ 500 Hz with the maximum HF-power of 200 W. The apparatus was equipped with GM 3200 HF-generator, UW 3200 ultrasonic convertor and SH 213 G booster horn. The probe used in this work was VS 70 T (13 mm in diameter and 170 $\mu$m in amplitude). The process variables included ethanol concentration (0–100%), extraction time (2–10 min), duty cycle (0.2–1 s) and ultrasonic amplitude (20–100%). Duty cycle is defined as the duration of “ON” pulsation divided by that of the entire cycle which was considered 1 s; for example, a duty cycle of 0.2 was equal to 0.2 s “ON” pulsation and 0.8 s “OFF” pulsation. Preliminary experiments demonstrated that the solute concentration (0.1–0.5% w/v) did not have a significant effect on the responses at $p < 0.05$. Therefore, the constant solute concentration of 0.1% (w/v) was chosen for sample preparation. 0.05 g of the sample was dissolved in 50 mL of the solvent inside a stainless steel vessel. In order to prevent solvent vaporization, the vessel was screwed to the booster horn and placed in an ice bath. The solutions were subsequently sonicated under the pre-determined conditions. All the experiments were duplicated.

2.4. Spectrophotometric measurements

After the extraction, the solution was passed through a Whatman filter paper No. 42. Then, 2.5 mL of the filtrate was transferred to a 50-mL volumetric flask and made to the mark with distilled water. The final concentration of the powdered saffron in water was 0.005% (w/v). Then, the absorbance was read using the DR-5000 spectrophotometer (Hach-Lange, Germany), equipped with a 1-cm path quartz cell, at 257, 370, and 390 nm, respectively. The apparatus was equipped with GM 3200 and 2280 ultrasonic convertors and R 2020 booster horn. The probe used in this work was VS 70 T (13 mm in diameter and 170 $\mu$m in amplitude). The process variables included ethanol concentration (0–100%), extraction time (2–10 min), duty cycle (0.2–1 s) and ultrasonic amplitude (20–100%). Duty cycle is defined as the duration of “ON” pulsation divided by that of the entire cycle which was considered 1 s; for example, a duty cycle of 0.2 was equal to 0.2 s “ON” pulsation and 0.8 s “OFF” pulsation. Preliminary experiments demonstrated that the solute concentration (0.1–0.5% w/v) did not have a significant effect on the responses at $p < 0.05$. Therefore, the constant solute concentration of 0.1% (w/v) was chosen for sample preparation. 0.05 g of the sample was dissolved in 50 mL of the solvent inside a stainless steel vessel. In order to prevent solvent vaporization, the vessel was screwed to the booster horn and placed in an ice bath. The solutions were subsequently sonicated under the pre-determined conditions. All the experiments were duplicated.

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$$E_{\text{max}} = \frac{D \times 1000}{m(100 - H)}$$

where $E_{\text{max}}$ represents the molecular extinction coefficient of the saffron solution at a concentration of 1% (w/v) in a 1-cm path length; $D$ is the absorbance value, $m$ denotes the sample weight (g) and $H$ shows the moisture and volatile matter content [29].

2.5. HPLC and GC–MS analyses

A Knauer apparatus (Berlin, Germany) was utilized to conduct the HPLC analyses. The apparatus was equipped with a Wellchrom K-2600...
UV–Visible detector, a Wellchrom K-1001 pump and a Nucleosil RP C18 column (150 mm length, 4.6 mm internal diameter, 5 µm particle size and 100 Å pore size, Nucleosil). A linear gradient elution was done starting with methanol:water (20:80% v/v) as the mobile phase. The methanol concentration was increased at 1%/min and a constant flow rate of 1.0 mL/min during 60 min at 25 °C. The sample size was 20 µL, and the measurements were performed by integrating the areas under the peak of the standard crocin (Sigma-Aldrich) and the crocin of the samples at 440 nm [26,27].

Table 4
Prerequisites of UAE optimization using RSM and ABC, in addition to the predicted and experimental results of the responses.

| Response | Goal | Lower Target | Upper | Predicted results by RSM 95% confidence interval | Predicted results by ABC 95% prediction interval | Predicted results by ABC | Experimental results |
|----------|------|--------------|-------|--------------------------------------------------|--------------------------------------------------|----------------------------|----------------------|
| E₄₅₀    | Maximum | 14.27 | 211.45 | 203.95 (175.28–232.62) | 197.66 (143.13–264.77) | 205.02 ± 4.52 |
| E₃₃₀    | Maximum | 3.24 | 40.00 | 40.29 (36.72–43.86) | 42.94 (32.72–47.86) | 42.59 ± 0.83 |
| E₂₅₇    | Maximum | 8.43 | 85.40 | 85.43 (77.68–93.17) | 90.23 (69.00–101.86) | 88.75 ± 4.12 |

Fig. 1. Convergence of ABC algorithm for multi-objective optimization of UAE.

Table 5
Optimal conditions predicted by RSM and ABC algorithms.

| Variables            | RSM  | ABC |
|----------------------|------|-----|
| Ethanol concentration (%) | 58.58 | 53.07 |
| Time (min)           | 6.85 | 7.32 |
| Duty cycle           | 0.82 | 0.93 |
| Amplitude (%)        | 91.11 | 100 |

Fig. 2. Effects of ethanol concentration and time on E₂₅₇, with duty cycle and ultrasonic amplitude being considered constant at their central levels.
A GC–MS apparatus (Agilent Technologies, Santa Clara, California, The USA) was applied comprising of a 7890B GC system with a HP-5 capillary column (30 m, 0.25 mm, 0.25 µm) along with a 5977A MS detector. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The sample size was 1.0 µL/min. The injector and detector temperatures were adjusted to 230 and 270 °C, and the injector split ratio was set at 1:15. Heating was begun at 80 °C. The temperature was kept constant for 1.0 min and then raised to 140 °C at a rate of 10.0 °C/min. It was subsequently kept constant for 20 min and then elevated to 270 °C at 8 °C/min. The mass spectra were recorded at an ionizing energy of 70 eV and a solvent delay of 3 min. The mass range was scanned at m/z 50–500 amu [26,27].

2.6. Experimental design and statistical analysis

Using the Minitab® software version 18.1 (Minitab Inc. the USA., 2017), a four-factor five-level full factorial CCD was created to verify the impacts of the process variables on the extinction coefficient of the saffron major bioactives (Table 1). Screening experiments were carried out to determine the ranges of variables (data not shown). A total of 32 experiments were performed in duplicate, including 12 replicates of the center point.

To investigate the relationship between the independent and dependent variables, the data were subjected to regression analysis using the method of least squares (MLS). MLS is a multiple regression technique used to fit a mathematical model to an experimental dataset producing residuals as low as possible (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). Residual is the difference between the observed and
fitted values. In order to do so, the full quadratic model (Equation (3)) was fitted to the experimental data:

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_i X_j \]  

(3)

where \( Y \) denotes the response, \( \beta_0 \) is the model intercept, \( \beta_i, \beta_{ii}, \beta_{ij} \) represent the regression coefficients for the linear, quadratic and interactive effects respectively, \( X_i \) and \( X_j \) stand for the independent variables, and \( k \) is the number of them. The regression coefficients are determined according to the fact that they should minimize the residual sum of squares (Baş & Boyacı, 2007a). Analysis of variance (ANOVA) was applied to evaluate the significance of the regression coefficients at 95% confidence level. Eventually, the experimental data were optimized regarding the maximization of all the responses with the same weight (\( w = 1 \)). The optimal conditions were triplicated, and the theoretical and empirical results were compared.

For the multi-objective optimization via ABC, the method previously suggested by Das, Kumar, Barman, & Sahoo, [30] was used with some modifications. The second-order full quadratic models of the three response variables were applied to the following equation:

\[ Z = \sum_{i=1}^{3} W_i Y_i \]

(4)

where \( Z \) denotes the optimal solution, \( Y_i \) is the second-order polynomial for \( E_{257}, E_{330} \) and \( E_{440} \), and \( \max (Y_i) \) and \( W_i \) stand for the maximum value and weight of each response respectively. Similar to the RSM optimization, all the weights were considered to be the same (\( w = 1 \)). The number of populations, onlooker and recruited bees were all considered to be equal to the number of observations (\( n\text{-Pop} = n\text{-OB} = n\text{RB} = 64 \)). The abandonment limit was equal to 1000, and the number of iterations was regarded as 50. At the same time, a lot of trial and error

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Fig. 5. Effects of time and duty cycle on \( E_{330} \), with ethanol concentration and ultrasonic amplitude being considered constant at their central levels.

Fig. 6. Effects of ethanol concentration and duty cycle on \( E_{440} \), with time and ultrasonic amplitude being considered constant at their central levels.
3. Results and discussion

3.1. Model fitting and optimization

The CCD matrix and the responses are presented in Table 2. As mentioned earlier, the second-order polynomial model was fitted to the empirical data which are as follows:

\[ E_{257} = 37.4 + 0.859X_1 + 4.09X_2 + 12.7X_3 - 0.068X_4 - 0.01527X_1^2 - 20.3X_2^2 + 0.00067X_3^2 + 0.0389X_1X_2 + 0.316X_3X_4 + 0.00024X_4^2 + 1.35X_2X_3 - 0.0128X_2X_4 + 0.260X_3X_4 \]

\[ E_{330} = 5.6 + 0.592X_1 + 3.99X_2 + 8.5X_3 - 0.014X_4 - 0.008083X_1^2 - 0.165X_2^2 - 14.5X_3^2 + 0.0017X_1^3 + 0.0116X_2X_3 + 0.119X_3X_4 - 0.00030X_4X_2 - 0.34X_2X_1 + 0.0057X_3X_4 + 0.182X_4X_1 \]

\[ E_{440} = -110.4 + 4.82X_1 + 27.8X_2 + 114X_3 + 0.67X_4 - 0.05998X_1^2 - 1.723X_2^2 - 140.6X_3^2 - 0.00845X_1^3 + 0.1270X_2X_3 + 1.295X_3X_4 + 0.00319X_4X_2 - 9.4X_2X_1 - 0.042X_2X_4 + 1.16X_3X_4 \]

The determination coefficient \( R^2 \) and adjusted determination coefficient \( R^2 \text{adj} \) of the three models were respectively equal to 76.92\% and 70.33\% for \( E_{257} \), 80.52\% and 74.96\% for \( E_{330} \), and 75.11\% and 68.00\% for \( E_{440} \). A model is considered adequate when its \( R^2 \) and \( R^2 \text{adj} \) are high enough and close to each other [31]. The relatively high determination coefficients of the models, which were also near each other, revealed that the full quadratic model could acceptably, although not excellently, explain the variance within the obtained data.

ANOVA (Table 3) revealed that the quadratic model as well as its linear and square segments were significant for all the responses at 95\% confidence level, as a model segment is considered significant if at least one of its terms has a significant effect on the independent variable [31]. At the same time, none of the interactive effects of the model significantly affected the response variables (\( p \) greater than 0.05); therefore, the two-way interaction segment of the model became significant for none of them. It can be seen in Table 3 that the linear effects of ethanol concentration and time in addition to the second-order effect of ethanol concentration had significant effects on all the responses at 95\% confidence level. Furthermore, duty cycle linearly influenced \( E_{257} \) and \( E_{330} \) (\( p < 0.05 \)), and ultrasonic amplitude only had a significant linear effect on \( E_{330} \) at \( p < 0.05 \); even though its linear effect on \( E_{257} \) could also be regarded as significant with some connivance, because its \( p \)-value is slightly greater than 0.05.

RSM optimization and ABC algorithms were applied to explore the optimal conditions of the UAE process. The optimization goal was to maximize all the responses, and the prerequisites as well as the predicted and experimental results are summarized in Table 4. In the case of ABC, the program was run for 50 iterations, and all the times, it converged to the optimal solution (\( Z = 3.06 \)) after about 15 iterations (Fig. 1), revealing the robustness of the algorithm. The optimum conditions of the two methods are demonstrated in Table 5. The conditions and results predicted by both techniques were so near each other, indicating their approximately same power of optimization. In the case of \( E_{257} \) and \( E_{330} \), the results predicted by ABC were closer to the empirical ones, while it was reversed for \( E_{440} \). At the same time, since both the ABC-predicted and experimental results were within the confidence and prediction intervals of the RSM-predicted ones at 95\%, it could be deduced that no significant differences existed among the empirical and predicted results.

3.2. Effects of UAE variables on responses

In the ethanol concentration range of 0–20\% (Fig. 2), the increase in time did not affect \( E_{257} \). It seems that the solvent was not that able to extract picrocrocicn in this range and was soon saturated. Thus, the picrocrocicn content did not increase as the time was elevated. Considering the use of ice bath in all the experiments and prevention of temperature increase, \( E_{257} \) did not decrease with time. On the other hand, due to the low temperature of the process, \( \beta \)-glucosidase was not inactivated and picrocrocicn might have converted to HTCC [32]. However, since this compound, similar to picrocrocicn, absorbs light at 257 nm, spectrophotometry cannot distinguish between them [33]. At 20–100\% ethanol concentration, \( E_{257} \) rose as the extraction time was raised, showing the mass transfer rate increased over time, and picrocrocicn was not dissociated [21]. The negative quadratic effect of ethanol concentration can also be observed in this graph. With an increase in this factor, the stigma cell membranes were destructed to a higher extent, which may be the reason behind the rise in the picrocrocicn content up to medium concentrations. However, higher concentrations of ethanol denatured the cell membrane proteins and consequently lowered the diffusion rate of picrocrocicn into the solvent [34].
The positive linear effects of duty cycle and ultrasonic amplitude can be observed in Fig. 3. At low duty cycles, amplitude increase did not have a noteworthy effect on picrocrocin content, which could be ascribed to the short “ON” pulsation time at low duty cycles. However, the significant positive effect of amplitude can be observed at high duty cycles, leading to more intense cavitation, higher mass transfer rate and enhanced extraction efficiency [35]. Similarly, duty cycle did not remarkably affect the response at low and medium amplitudes. Nevertheless, its significant (p < 0.05) linear positive effect can be observed thereafter, which could be due to the synergistic effect of high amplitude

![HPLC chromatograms](image)

**Fig. 8.** HPLC chromatograms of a) Sigma-Aldrich reference crocin and b) RSM-optimized UAE extract. Peaks 1–6 denote crocins 1–6.

| Table 6 |
|---|
| HPLC quantitative analyses of Sigma-Aldrich reference crocin and RSM-optimized UAE extract. |

| Sigma-Aldrich reference crocin | Optimum sample |
|---|---|
| Crocin ester | Retention time (min) | Area | % Area<sup>a</sup> | Concentration (ppm) | Retention time (min) | Area | % Area | Concentration (ppm) |
| Crocin 1 | 28.100 | 5,009,696 | 1.80 | 20,000 | – | – | – | – |
| Crocin 2 | 33.017 | 40,448,349 | 14.56 | 20,000 | 33.017 | 1,077,481 | 7.55 | 532,7688 |
| Crocin 3 | 38.033 | 111,926,701 | 40.30 | 20,000 | 38.083 | 8,648,478 | 60.58 | 1545.382 |
| Crocin 4 | 41.417 | 20,082,496 | 7.23 | 20,000 | 41.583 | 3,266,627 | 22.88 | 3253.208 |
| Crocin 5 | 42.517 | 12,662,798 | 4.56 | 20,000 | – | – | – | – |
| Crocin 6 | 49.650 | 28,285,698 | 10.18 | 20,000 | 49.817 | 852,786 | 5.97 | 602.9803 |

<sup>a</sup> % Area implies the area under the curve of each peak divided by the total area under the curve, multiplied by 100.

The positive linear effects of duty cycle and ultrasonic amplitude can be observed in Fig. 3. At low duty cycles, amplitude increase did not have a noteworthy effect on picrocrocin content, which could be ascribed to the short “ON” pulsation time at low duty cycles. However, the significant positive effect of amplitude can be observed at high duty cycles, leading to more intense cavitation, higher mass transfer rate and enhanced extraction efficiency [35]. Similarly, duty cycle did not remarkably affect the response at low and medium amplitudes. Nevertheless, its significant (p < 0.05) linear positive effect can be observed thereafter, which could be due to the synergistic effect of high amplitude
and long “ON” pulsation time on picrocrocin extraction efficiency. This is in contrast with the findings presented by Kyriakoudi et al., [23] who reported that elevating the duty cycle up to a middle level increased $E_{257}$, but this response was not favored by high duty cycles.

The negative quadratic effect of ethanol concentration and the positive linear effect of the ultrasonic amplitude on $E_{300}$ can be seen in Fig. 4. Similar to picrocrocin, safranal content also increased as the amplitude was raised, and the high temperatures generated through the collapse of the cavitation bubbles at high amplitudes did not seem to have destroyed this component, because of the application of ice bath during all the experiments. In the case of ethanol concentration, it was again proved that medium concentrations were more efficient in
extracting safranal, conforming to the results previously found out by [34,27]. The reason for this is similar to that of picrocrocin.

From Fig. 5, it can be understood that both sonication duration and duty cycle positively influenced $E_{330}$, which is reflected in the positive significant (P < 0.05) linear effects of both variables. Safranal content increased until approximately the 7th minute of the UAE and then remained constant. The higher the duty cycle was, the sooner the response reached a maximum owing to the longer “ON” pulsation time of the sonication. Since there was a steep concentration gradient of the solutes between the plant cells and the solvent at the beginning of the process, the extraction efficiency was high at the early minutes of the UAE, whereas it was subsequently lowered when the solvent was saturated by the constituents [21]. At the same time, the semi-circular shape of the contour plot along with the negative quadratic coefficients of the variables implies that $E_{330}$ would have decreased if the UAE had continued beyond 10 min. This suggests that the UAE time of more than 10 min could be detrimental to safranal, which might be caused by temperature increase and/or sonochemical reactions.

Similar to picrocrocin and safranal, the middle concentrations of ethanol resulted in the highest extraction efficiency of crocin (Fig. 6), whose reason has been previously elaborated. It is interesting to note when duty cycle was raised at low concentrations of ethanol, a slight reduction was observed in $E_{440}$, while at high concentrations, a reverse trend was observed and crocin content rose with an increase in duty cycle. The high proportion of water in the solvent mixture at low ethanol trend was observed and crocin content rose with an increase in duty cycle. As mentioned earlier, the rise in the response could be ascribed to the intensified cavitation leading to the rupture of the cell walls and membranes to a higher degree. However, the decline could be attributed to the formation of sonochemicals which produce oxidative effects by generating free radicals and dissociating conjugated double bonds [37].

### 3.3. HPLC and GC–MS analyses

HPLC analysis was carried out on the UAE optimum extract resulting from RSM (Fig. 8). Four crocetin esters were detected in the optimal extract (Table 6). Given the similarity between the polarity of the stationary and mobile phases utilized in this research and those in other studies, the optimal extract crocetin esters were identified through comparison between their detection order and those reported in earlier papers. According to Petros A Tarantilis, Tsouparas, & Polissiou, [38], crocins 2, 3, 4 and 6 could be equivalent to trans-crocin 4, trans-crocin 3, trans-crocin 2’ and cis-crocin 4 respectively. The same detected components could also represent trans-crocin 3, trans-crocin 2’, cis-crocin 5 and cis-crocin 3 respectively [39]. Whilst, based on Caballero-Ortega, Pereda-Miranda, & Abdullaev, [40], trans-crocin 3 (crocin 2), trans-crocin 2 (crocin 3), cis-crocin 4 (crocin 4) and cis-crocin 2 (crocin 6) were detected in the optimal UAE. At the same time, crocins 2, 3, 4 and 6 denote trans-crocin 3, trans-crocin 2, trans-crocin 2 and cis-crocin 3 respectively [41].

By comparing the findings of this research with those previously reported in our earlier studies [26,27], it was deduced that UAE had a much better efficiency than microwave-assisted extraction (MAE) and subcritical water extraction (SWE), as the concentrations of the crocetin esters detected in the UAE optimum extract were many times higher than those in the optimum extracts of MAE and SWE. This revealed that UAE was much more efficient than MAE and SWE in the extraction of crocin.

The total ion chromatogram and mass spectrum of the RSM-optimized extract are depicted in Fig. 9. GC–MS only detected 2,6,6-trimethyl,1,3-cyclohexadiene-1-carboxaldehyde (safranal) with a retention time of 6.75 min and a molecular weight of 150. This finding conforms to those previously reported by Carmona et al., [42] and Petros A. Tarantilis & Polissiou, [43]. Like the HPLC results, the GC–MS results of this study were also compared with those of our earlier works [26–27]. It was realized that the safranal absorbance value was nearly 270,000, while it was approximately 480,000 and 800,000 in the SWE and MAE optimal extracts respectively. In contrast to the crocetin esters, this demonstrates that UAE was less efficient than MAE and SWE in safranal extraction. This could be due to the application of ice bath and inhibition of temperature increase in the present study, as safranal needs high temperatures to be optimally extracted [34,27].

### 4. Conclusions

RSM and ABC were employed to examine the impacts of ethanol concentration, sonication time, duty cycle and ultrasonic amplitude on the extraction efficiency of saffron bioactive compounds during UAE. Ethanol concentration and UAE time more influenced the responses than duty cycle and amplitude did. RSM optimization and ABC algorithms predicted nearly the same values for all the independent and dependent variables, indicating that their optimization capabilities were approximately the same. HPLC showed that UAE was much better than MAE and SWE in extracting the crocetin esters. However, GC–MS demonstrated that MAE followed by SWE were more able to extract safranal compared with UAE.

In conclusion, according to our experience gained during the past decade, we can maintain that ‘Green Chemistry’ technologies, including UAE, can be appropriate alternatives to conventional methods for the extraction of saffron bioactives, as they are faster and simpler techniques compared with the conventional ones. Furthermore, they raise the extraction efficiency of the target bioactive substances. The only drawback of such technologies could be their relatively big capital investment. At the same time, their higher extraction yields and shorter extraction durations could compensate for this issue over time.

**CRediT authorship contribution statement**

**Messiah Sarfarazi:** Data curation, Investigation, Methodology, Writing – original draft. **Qadir Rajabzadeh:** Formal analysis, Validation, Visualization. **Razieh Tavakoli:** Formal analysis, Methodology, Investigation. **Salam A. Ibrahim:** Validation, Resources, Writing – review & editing. **Seid Mahdi Jafari:** Conceptualization, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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