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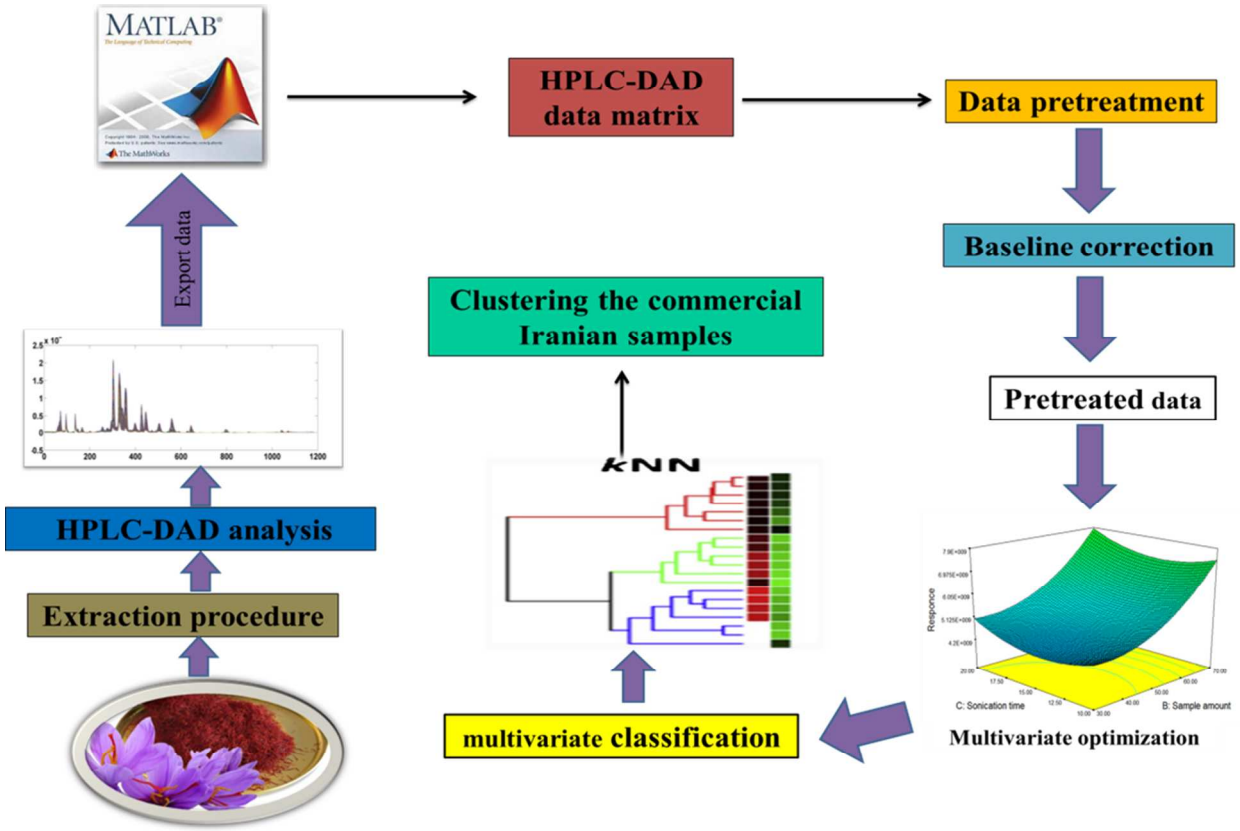

Analysis of bioactive constituents of saffron using ultrasonic assisted emulsification microextraction combined to high-performance liquid chromatography with diode array detector: A chemometric study

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Abstract

In recent years, there is an increasing interest in the analysis of major active components of saffron owing to its significant role in various industries, such as food, medicine and perfume. In other words, major active components of saffron can give a complete picture of its chemical composition which can be used as a reliable index for quality control of different saffron samples (i.e., natural and commercial). The aim of the present work was development of a simple, low cost, efficient and comprehensive strategy for extraction and analysis of bioactive components of saffron. In this regard, ultrasonic-assisted solvent extraction (UASE) combined with ultrasonic-assisted emulsification microextraction (USAEME) is proposed for extraction and preconcentration of bioactive constituents of saffron. The extracted components are then analyzed using reversed-phase high-performance liquid chromatography with diode array detector (RP-HPLC-DAD) technique. The effective parameters on the efficiency of extraction procedure are optimized using multivariate chemometric techniques. As a consequence, the optimum extraction parameters were 79.6 mg saffron sample, 1.1 mL extraction solvent (water), 62.7μL preconcentration solvent (chloroform) and 18.6 min sonication time. In optimum extraction conditions, the relative standard deviations (RSDs) were below 1.0% (n = 3) for all components. Also, the enrichment factors were higher than 10 for most components. Finally, the developed analytical method is used as a reliable method for quality control of fifteen commercial saffron samples prepared from different markets. To do this, multivariate clustering methods of principal component analysis (PCA) and k-means are used for finding similarities and dissimilarities between standard and commercial saffron samples according to their HPLC fingerprints. It is concluded that the proposed method is a fast, simple, accurate and unbiased method for analyzing bioactive components of saffron and fingerprinting of commercial saffron samples which extracts a complete set of information from data compared to conventional methods.

Keywords: Saffron; Chemometrics; High-performance liquid chromatography; Central composite design; Multivariate clustering; Ultrasonic-assisted emulsification microextraction.

1. Introduction

Saffron as the most expensive spice in the world is the dried stigmas of *Crocus sativus L.* flowers. *Crocus* is a genus of Iridaceae family[1] and it is widely cultivated in Iran and other countries such as Spain, India, China, Greece, Morocco, Turkey, Italy and Azerbaijan[2]. The saffron stigmas contain essential compounds, such as vitamins, carbohydrates, minerals and different pigments like flavonoids and carotenes[3]. It is appreciated by consumers as a colorant for foodstuffs as well as for its aromatic and flavoring properties [4,5]. More recently, there has been increasing interest in the biological effects of the saffron constituents and their possible medical applications, particularly those based on their cytotoxic, anti-carcinogenic and anti-tumour properties[6,7]. The value of saffron is determined by the existence of three main secondary metabolites[8]; crocin and its derivatives as hydrophilic carotenoids which are responsible for the characteristic golden yellow color of saffron[9,10], picrocrocin as a monoterpene glycoside which is responsible for the bitter taste of saffron[11, 12] and safranal as a monoterpene aldehyde which is responsible for saffron aroma. However, from medicinal point of view, the major biologically active components of saffron are crocin analogues. The amount of these compounds in dried stigma tissues is the most important indicator of the quality of saffron[1].

Different extraction techniques have been proposed for extraction and preconcentration of volatile and non-volatile components of saffron. Hydrodistillation (HD), vacuum headspace (VHS) [12], supercritical fluid extraction (SFE) [13, 14], thermal desorption (TD) [15], extraction with organic solvents [16, 17], solid-phase microextraction (SPME)[18] and ultrasonic-assisted solvent extraction (UASE) [19] have been used for the extraction of chemical components of saffron. Amongst the above mentioned methods, UASE has some advantages due to the application of ultrasonic waves for extraction. Ultrasound waves pass through a medium by creating

compression and expansion which create bubbles in a liquid and produce negative pressure. The bubbles form, grow and finally collapse. This process produces a phenomenon called cavitation, which means production, growth and collapse of bubbles. Therefore, a large amount of energy can produce from the conversion of kinetic energy of motion into heating the contents of the bubble. As a consequence, ultrasound in extraction can disrupt biological cell walls, facilitating the release of contents. Thus, efficient cell disruption and effective mass transfer are cited as two major factors leading to the enhancement of extraction with ultrasonic power. Coupling the extraction process with a preconcentration method, such as ultrasound-assisted emulsification microextraction (USAEME) [20] enhances the efficiency of the method. The USAEME method is a fast and simple method with high efficiency, recovery and enrichment factor[20, 21].

In recent years, most of the studies on the chemical composition of saffron have been focused on the non-volatile compounds of saffron[1, 3, 8] owing to their medical applications. Optimization of extraction procedures are usually performed using one-variable-at-a-time (OVAT) approach, which facilitate the interpretation of the obtained results, but interactions between variables are not taken into account[22, 23]. Therefore, a false minimum or maximum may be attained which is not the best analytical response. Experimental design methods (e.g., factorial designs and response surface methodology) have been frequently applied to optimize the extraction procedures [22, 24]. In this approach, the main effects of the factors, their interactions and curvatures are estimated. The curvature in the response surface means curvature in the relationship between factors as laid out in the model. These terms are quadratic terms in the developed model. This is one of the greatest advantages of multivariate optimization compared to OVAT optimization. Another advantage is that the number of experiments is considerably reduced particularly in the case with many factors [22, 24].

High-performance liquid chromatography with diode array detector (HPLC-DAD) is one of the best techniques for separation and identification of the non-volatile and thermally labile components of saffron. In other words, HPLC coupled with tandem mass spectrometry (LC-MS/MS) is a better technique for separation and identification of bioactive components of saffron. However, it is more expensive than HPLC-DAD and providing access to it is not as simple as HPLC-DAD. Nowadays, there is an increasing interest in identification of chemical composition of complex samples by their chromatographic signals that is called chromatographic fingerprints. A chromatographic fingerprint is a unique pattern that indicates the presence of chemical components in the analyzed sample. Chromatographic fingerprinting becomes one of the most powerful approaches for quality control of complex natural samples, such as herbal medicines, and represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality and ensuring the consistency and stability of the chemical constituents observed by chromatography[25, 26].

The aim of the present work was offering a simple, low cost, efficient and environment-friendly technique for extraction, preconcentration and chromatographic analysis of bioactive components of saffron with the aid of chemometric techniques. For this purpose, a two-step extraction process consisted of UASE followed by USAEME is proposed. The first step includes direct extraction of saffron components from solid stigmas into water as a suitable solvent accelerated by ultrasound. In the second step, USAEME method is used for preconcentration of the isolated components. The important parameters of UASE–USAEME method including saffron sample, extraction solvent, preconcentration solvent and sonication time are optimized using response surface methodology (RSM). Finally, the developed method is used for analysis of commercial saffron samples and to obtain their LC fingerprints to control the quality of

different saffron samples. The similarities and dissimilarities among samples are determined using multivariate clustering techniques of principal component analysis (PCA) [27,28] and k-means[29].

2. Experimental

2.1. Saffron samples and chemicals

Fresh stigmas of standard saffron sample were obtained from cultivation in the area of Qaen in South Khorasan province of Iran. The stigmas have been dried at temperature between 20 and 30 ◦C in the absence of light for 24 h. The dried stigmas were kept at 4◦C in the absence of light until their analysis.

HPLC grade methanol and acetonitrile and analytical grade chloroform were purchased from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system from Millipore.

2.2. Extraction procedure

The procedure of extraction and preconcentration of saffron components was as follow: grounded saffron sample was precisely weighted and placed in a round end test tube and water as extraction solvent was added to it. The mixture was subsequently exposed to ultrasonic waves. During this time the temperature of the ultrasonic bath was maintained at 25 °C. To separate the solid remains of the saffron sample, the mixture was centrifuged. Then, chloroform as preconcentration solvent was added to the upper phase solution and the mixture was sonicated. Accordingly, the solution became cloudy containing tiny drops of chloroform distributed in the sample solution. In this situation, the extraction process was accomplished. Then, the cloudy

solution was centrifuged to separate the chloroform as the lower phase. The extraction was performed at ambient temperature and in the absence of direct light to protect the light-sensitive components. The chloroform was dried using gentle flow of nitrogen and then methanol was added to it. Finally, 20 µL of extract was analyzed using reversed-phase high performance liquid chromatography with diode array detector (RP-HPLC-DAD). In general, 30 experiments were performed with 6 replicates. The relative standard deviations (RSD, %) of the replicates were below 1.0 % which was acceptable.

2.3 Chromatographic conditions and instrumentation

Chromatographic analyses were performed using a Knauer HPLC system, equipped with a Smartline pump 1000 and a diode array absorbance detector (DAD) system. Analyses were carried out on a C18 column (150 mm × 4.6 mm i.d., 5 µm particle diameter, MN, Germany) with following conditions: flow rate 1 $mLmin^{-1}$and injection volume 20 µL with mobile phase consisting of water (A) and acetonitrile (B). The gradient elution program was: 20–40% B at 0–3 min, 40–50% B at 3–8 min, 50–50% B at 8–12 min, 50-80 B% at 12–15 min. The UV spectra were recorded between 200 and 499 nm. It should be mentioned that each extract was filtered through a syringe filter (0.22 µm) prior to injection to HPLC-DAD. It should be pointed out that no significant effects of the adsorption of the extracted components of saffron on the filter were observed in this work.

2.5 Software requirements

ChromGate v. 3.3.2 was used for HPLC-DAD data collection, exportation and conversion to ASCII format. The statistical computer package "Design-Expert v. 7.1.3" (Stat-Ease Inc., Minneapolis) was used for design of experiments, model development and optimization. The PLS Toolbox version 3.5was used for multivariate cluster analysis. All calculations were performed in MATLAB v. 8.3 (Mathworks, Natick, MA, USA). The MATLAB codes for baseline correction and elution time shift correction were downloaded from the internet (*http://www.models.kvl.dk/algorithms*).

3. Results and discussion

3.1. Multivariate optimization of UASE-USAEME procedure

In the first step of the present study, effective parameters on the UASE–USAEME method including type and volume of extraction solvent, type and volume of preconcentration solvent, amount of sample and sonication time was optimized using multivariate strategy. In this regard, the sum of peak areas of all detectable constituents in HPLC-DAD was chosen as the response for modeling. Due to the qualitative nature of the type of extraction and preconcentration solvents, their optimum solvents were chosen using univariate strategy.

The extraction solvents for UASE were chosen based on the number of components that can be extracted from saffron and the peak area of these components in HPLC-DAD chromatogram. In this regard, solvent polarity, molecular weight and viscosity were considered as important solvent parameters in the extraction. According to these properties, several solvents such as methanol as a protic solvent (MW= 32.04 g mol^{-1}, d=0.7918 g cm^{-3}, dipole moment= 1.69 D), water (MW= 18.01 g mol^{-1}, d= 0.9999 g cm^{-3}, dipole moment= 1.85 D) and acetonitrile as an aprotic solvent (MW=41.05 g mol^{-1}, d=0.7860 g cm^{-3}, dipole moment= 3.92D) were tested.

Among these solvents, water showed the highest efficiency in terms of peak areas and number of peaks (i.e., 15 peaks). Therefore, it was selected as extraction solvent.

On the other side, USAEME requires high –density preconcentration solvent which was chosen based on immiscibility in water and good solubility of the target analytes. Therefore, 100 µL of aprotic organic solvents, such as ethyl acetate ($C_4H_8O_2$) (d= 0.897 g cm^{-3}), carbon tetrachloride (CCl_4) (d=1.587 g cm^{-3}), chloroform ($CHCl_3$) (d=1.489 g cm^{-3}) and dichloromethane (CH_2Cl_2) (d=1.330 g cm^{-3}) were individually tested. Inspection of the results showed that chloroform is the most effective solvent in preconcentration of the analytes (enrichment factor were higher than 10); therefore, it was chosen as USAEME solvent in this work.

Figure 1 goes here

Fig. 1 depicts the HPLC chromatogram of the extracted saffron components after UASE (a) (60 mg saffron sample, 2.0 mL H_2O as extraction solvent and 20 min sonication time) and after preconcentration by UASE-USAEME (b) (60 mg saffron sample, 2.0 mL H_2O as extraction solvent, 20 min sonication time and 100 µL chloroform as preconcentration solvent). It should be pointed out that HPLC analyses were carried out on a C18 column (150 mm × 4.6 mm i.d., 5 µm with the following conditions: flow rate 1 $mLmin^{-1}$ and injection volume 20 µL with mobile phase consisting of water (A) and acetonitrile (B). The gradient elution program was: 20–40% B at 0– 1403 min, 40–50% B at 3–8 min, 50–50% B at 8–12 min, 50-80 B% at 12–15 min. It is clear that USAEME can enrich most of the extracted components in UASE step.

After finding proper solvents for extraction and preconcentration of saffron components, optimization of other effective parameters on UASE-USAEME procedure including extraction solvent volume, preconcentration solvent volume, sonication time and sample amount was performed using response surface methodology (RSM).

In order to achieve the highest practical method performance and to obtain the conditions that the procedure generates the best response, a rotatable central composite design (CCD)[30] was used. In this study, a rotatable CCD with α= 2.00 was used for the optimization of the effective factors on UASE-USAEME for the characterization of non- volatile components in saffron. Table 1 shows the effective factors, their abbreviations and their levels for the rotatable CCD. Also, Table S1 (supporting information) demonstrates the CCD design matrix and obtained response for each run. It is important to note that the low and high levels of each factor were determined according to literature data and preliminary studies [1,3,31].

Table 1 goes here

A step-wise multiple linear regression (MLR) was used to select a suitable response surface model. To evaluate the model and the significance of the effects, analysis of variance (ANOVA) was used. Table 2 shows the ANOVA table for CCD design matrix. The F-values implies that the proposed model is important and the lack of fit is not significant relative to the pure error.

Table 2 goes here

After analyzing the data, a quadratic response surface model based on a higher *F*- and *R*-value and lower lack of fit (LOF) to fit the experimental data was selected. This model which consists of four main effects, a couple of two factor interactions and four curvature effects are shown in Eq. (1) in a coded form:

$$Y = 4.7\times10^{9} - 1.8\times10^{8}A + 1.4\times10^{9}B + 1.9\times10^{8}C - 2.7\times10^{9}D + 8.6\times10^{8}AD - 6.8\times10^{8}BD + 4.9\times10^{8}A^{2} + 8.2\times10^{8}B^{2} + 5.5\times10^{8}C^{2} + 8.3\times10^{8}D^{2} \quad \text{(Eq. 1)}$$

The *p*-value of 0.077 for LOF implies that it is not significant relative to the pure experimental error and confirms the validity of the model. Other statistical parameters of the model are shown in Table 3.

Table 3 goes here

R-squared that is a measure of the amount of variation around the mean explained by the model was 0.873 for this model. Another important parameter for evaluating the model is adjusted R-squared (R_{Adj}^2). This parameter is considered as a measure of the amount of variation around the mean explained by the model adjusted for the number of terms in the model. In other words, the R_{Adj}^2 decreases as the number of terms in the model increases. In addition, predicted R-square (R_{Pred}^2) which is a measure of the amount of variation in new data explained by the model can be applied for the evaluation of the model. The R_{Pred}^2 and the R_{Adj}^2 values for the above model were 0.806 and 0.641, respectively. The term "adequate precision" in Table 3 represents the signal-to-noise (S/N) ratio. Ratio greater than 4.0 indicates that the model is adequate[32]. For the proposed model, this value is 12.27 and indicates a very good signal-to-noise ratio. All of these statistical parameters show the reliability of the model. After obtaining the desired model and statistical evaluation of it, confirming the absence of outlier in the data is very important. In this regard, two frequently used methods of leverage and Cook's distance were used[24, 33].

Figure 2 goes here

Fig. 2(a) shows the leverage plot for quadratic model obtained from central composite design. It can be seen from this figure that all of the leverage values are lower than 0.75 (threshold value) and it can be concluded that there is no outliers or unexpected errors in the model. This result has been confirmed with the Cook's distance plot in Fig. 2(b) where all runs are in the confidence interval and there no outlier in the model.

Figure 3 goes here

Fig. 3(a) and (b) depicts the response surface and contour plot showing the effect of sample amount (B) and extraction solvent volume (D) on the response at the fixed values of preconcentration solvent volume (A) and sonication time (C) in their center values (see Table 1). This figure clearly shows that the extraction solvent (water) volume has a negative effect on response but sample amount has a positive effect on the response. Presence of curvature in the model shows that interaction between sample amount and extraction time is significant.

Figure 4 goes here

Fig. 4(a) and (b) demonstrates the response surface and contour plot showing the effect of preconcentration solvent volume (A) and extraction solvent volume (D) on the response at the fixed values of sample amount (B) and sonication time (C) in their center values (see Table 1). This figure also clearly shows interaction between preconcentration solvent volume (A) and extraction solvent volume (D) is significant.

In general, 30 experiments have been performed using experimental design to find optimum conditions for four effective extraction parameters in five levels with corresponding replicates. In case of OVAT strategy, at least 60 experiments (3 replicates for each run) were needed to find optimum conditions. However, the interaction effects between factors and their quadratic terms showing the curvature in the response surface cannot be studied.

The validated response surface model was finally optimized using Nelder-Mead simplex optimization method (also known as variable-size simplex method) to get the optimum values of the effective factors on UASE-USAEME. In this regard, the optimization space of the significant factors in the obtained model was constrained in their initial range (shown in Table 1) and the goal of optimization was obtaining maximum sum of peak areas. The simplex algorithm found

the maximum peak area of 1.89×10^{10} for the model in Eq. (1) where the optimum extraction parameters were as follows: 79.6 mg sample amount, 1.1 mL water as extraction solvent, 62.7μL chloroform as preconcentration solvent, and 18.6 sonication time.

Finally, for evaluation of the developed model and corresponding optimum extraction parameters, the UASE-USAEME procedure and HPLC-DAD analysis were repeated three times (n = 3) at optimum conditions and the experimental response of 1.80×10^{10} was obtained. The experimental response was in agreement with the calculated one by model according to the confidence interval in the data which was in the range 1.6×10^{10} to 2.1×10^{10}.

3.2. Characterization of the Non-volatile components of saffron

The HPLC-DAD Chromatogram of non-volatile components of saffron in the optimized extraction conditions is shown in Fig. 5.

Figure 5 goes here

As it can be seen, a lot of number of components is extracted from saffron and separated with reasonable chromatographic resolution. Identification of the isolated components from saffron was carried out by comparing their spectral profiles and retention times with those of standards and identified components in the literature[34,35,36].

Table 4 goes here

The main identified components and their retention times and their maximum absorption wavelengths are presented in Table 4. These main components are safranal, picrocrocin[34], crocetin derivatives[10] such as crocin, crocetin-mono-(β-D-glucosyl)-ester, crocetin-di-(β-D-glucsyl)-ester and carotenoids derivatives such as kaempferol[35,36] and kaempferol-3,7,40-triglucoside[35].

The obtained HPLC-DAD chromatograms of standard saffron sample can be considered as a reference chromatographic fingerprint for the quality control of different commercial saffron samples. Additionally, the proposed analytical method can be used as an alternative method to ISO3632 for quality control of saffron. However, the proposed method has many advantages prior to the ISO3632. These advantages are faster extraction (18.6 min instead of 60 min for ISO method), lower solvent (1.1 mL instead of 5.0 mL for ISO method), lower sample amount (79.6 mg instead of 500 mg for ISO method), more efficient extraction of components (twenty extracted chemical components for current method instead of three components for ISO method) with higher relative concentrations), and considering more number of components in the quality evaluation of saffron.

3.3 Multivariate clustering of commercial saffron samples

To show the potential of our method for saffron quality control, fifteen commercial samples are chosen and they extracted and analyzed using optimized UASE-USAEME-HPLC-DAD method in triplicate.

Figure 6 goes here

Figure 6 shows the overlaid HPLC-DAD fingerprints of fifteen saffron samples from five different commercial brands listed in Table 5.

Table 5 goes here

Shifts of elution times for the same chemical components in different samples were an important issue that can be clearly seen from this Figure. It should be pointed out that the amounts of elution time shift were different among various runs and for different peaks. In addition, other common chromatographic problems, such as baseline/background contribution, low S/N peaks,

noise and peak overlap existed in the chromatographic fingerprints of saffron samples. Therefore, effects of baseline/background contribution and elution time shifts were corrected using asymmetric least squares (AsLS)[37] and correlation optimized warping (COW)[38], respectively before cluster analysis.

For multivariate clustering of chromatographic fingerprints of commercial saffron samples and comparing their fingerprints with the standard saffron sample, the corrected data matrix was analyzed using PCA. Autoscaling was chosen as a preprocessing step before PCA analysis.

Figure 7 goes here

Figure 7 shows the results of PCA analysis. The PC1-PC3 plot accounted for 51.86 % explained variance (PC1= 22.91 %, PC2=15.81 % and PC3=13.14 %). The scores plot in Figure 7 shows samples distribution in 3D space of the first, second and third principal components. The chromatographic fingerprint of standard saffron sample is shown in red. As can be seen, most of the samples have similar scores as standard. However, there are some samples with thoroughly different scores on three PCs. PCA can give a clear picture of the similarities and dissimilarities of chromatographic fingerprints of commercial saffron samples with the standard one.

To have a better discrimination between clear-cut clusters distance-based clustering methods, such as hierarchical cluster analysis (HCA) and k-means can be used[39].

Figure 8 goes here

As an instance, Figure 8 shows the cluster analysis results obtained by k-means method. By selecting the linkage of 1.5 as the threshold in this dendrogram, samples belong to three clear-cut clusters. Standard saffron sample is highlighted as red in this figure. Similar to the PCA results, the similarities and dissimilarities between standard and commercial saffron samples can be

clearly seen using this figure. In other words, the samples with similar chemical composition (chromatographic fingerprint) as standard saffron sample are placed in the same cluster (green color) and the other samples are placed in two different clusters (red and blue colors).

In summary, the chemometrics-based strategy in this work provided a complete set of useful information from the chromatographic fingerprints of *saffron* in the presence of different chromatographic problems. Multivariate optimization of UASE-USAEME-HPLC-DAD was performed and then the optimized method combined with multivariate clustering method was used for quality control of commercial saffron samples. Additionally, main chemical components of saffron were tentatively identified.

4. Conclusion

A chemometric-assisted strategy was proposed for extraction of bioactive constituents of saffron using UASE-USAEME combined to HPLC-DAD. Multivariate optimization based on RCCD and MLR was used for optimization of the effective parameters on the efficiency of extraction procedure. Good statistical parameters were obtained for the developed model and the values of RSDs and enrichment factors were below 1.0% (n=3) and higher than 10 for all extracted components, respectively in the optimum extraction conditions. All of these results confirmed the reliability of the proposed method. Finally, the developed analytical method was used as a reliable method for quality control of commercial saffron samples provided from different markets. Additionally, multivariate clustering methods of PCA and k-means are used for finding similarities and dissimilarities between standard and commercial saffron samples according to their HPLC fingerprints. Furthermore, the proposed strategy in this work can be used as an alternative method to ISO3632 for quality control of saffron. Inspection of the results showed

that the proposed strategy in this work is more accurate and unbiased and also extracts a more complete set of information from data, compared to conventional methods.

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Table 1 Factors, their notations, and their levels in central composite design (CCD)

Factor	Symbol	Level				
		-α	-1	0	+1	+α
Preconcentration solvent volume (μL)	A	20	40	60	80	100
Sample amount (mg)	B	10	30	50	70	90
Sonication time (min)	C	5	10	15	20	25
Extraction solvent volume (mL)	D	1	2	3	4	5

Table 2 Analysis of variance (ANOVA) table of the quadratic response surface model.

Source	SS	d.f.	MS	F-Value	Prob > F	
Model	2.844×10^{20}	10	2.844×10^{19}	13.12	< 0.0001	**Significant**
A	7.640×10^{17}	1	7.640×10^{17}	0.35	0.5598	
B	4.575×10^{19}	1	4.575×10^{19}	21.10	0.0002	
C	9.277×10^{17}	1	9.277×10^{17}	0.43	0.5208	
D	1.796×10^{20}	1	1.796×10^{20}	82.86	< 0.0001	
AD	1.189×10^{19}	1	1.189×10^{19}	5.49	0.0302	
BD	7.522×10^{18}	1	7.522×10^{18}	3.47	0.0780	
A^2	6.821×10^{18}	1	6.821×10^{18}	3.15	0.0921	
B^2	1.853×10^{19}	1	1.853×10^{19}	8.55	0.0087	
C^2	8.422×10^{18}	1	8.422×10^{18}	3.88	0.0635	
D^2	1.872×10^{19}	1	1.872×10^{19}	8.63	0.0084	
Residual	4.119×10^{19}	19	2.168×10^{18}			
Lack of fit	3.759×10^{19}	14	2.685×10^{18}	3.73	0.0768	**Not significant**
Pure Error	3.596×10^{18}	5	7.192×10^{17}			
Corrected Total	3.256×10^{20}	29				

Table 3 Statistical parameters for the quadratic model in Eq. (1)

Parameter	Value	Parameter	Value
Mean	6.88×10^{9}	R^2_{Adj}	0.807
C.V. %	21.40	R^2_{Pred}	0.642
R^2	0.874	Adequate Precision	12.27

Table 4 Retention time, maximum wavelength of absorption in the UV/Vis spectra and tentative identities of the most important detected constituents in saffron extract

Peak number	Retention time(min)	λ_{max}(nm)	Estimation of chemical species
1	1.3	250	Picrocrocin
2	1.6	260, 320, 380	kaempferol-3,7,4'-triglucoside
3	3.7	320	Safranal
4	4.4	250, 330, 450	Cis-crocin3
5	5.0	250, 440, 470	Trans-crocin3
6	5.6	255, 425, 450	Trans-crocetin
7	5.9	260, 320, 448	Trans-crocin4
8	6.9	319, 419, 443	Cis-crocetin
9	8.2	260, 353, 450	Cis-crocin4

Table 5 The commercial saffron samples and their codes for multivariate cluster analysis

Number	Brand name	Samples code
1	Bahraman	B1(1), B2(2), B3(3)
2	Abbasszadeh	A1(4), A2(5), A3(6)
3	Standard	Std(7)
4	Naffis	N1(8), N2(9), N3(10)
5	Saharkhiz	S1(11), S2(12), S3(13)
6	Golestan	G1(14), G2(15), G3(16)

Figure captions

Fig. 1. HPLC chromatogram of the extracted saffron components (a) after UASE and (b) after extraction and preconcentration by UASE-USAEME.

Fig. 2. (a) Leverage plot and (b) Cook's distance plot for quadratic model obtained from central composite design. Red lines in (a) and (b) show the threshold level which calculates using statistical tests. An experiment with values greater than limit values is generally regarded as an outlier in the independent variable space.

Fig. 3. (a) 3D response surface and (b) contour plot for extraction solvent volume (D) vs. sample amount (B).

Fig. 4. (a) 3D response surface and (b) contour plot for preconcentration solvent volume (A) vs. extraction solvent volume (D).

Fig. 5. The second-order HPLC-DAD chromatogram of extracted saffron constituents in optimum extraction conditions.

Fig. 6. Overlaid HPLC-DAD fingerprints of fifteen saffron samples from five different commercial brands listed in Table 6.

Fig. 7. The score plot of PCA analysis**.** Red circle shows the standard saffron sample.

Fig. 8. Dendrogram obtained by k-means method for standard and commercial saffron samples. Red box demonstrates the standard saffron sample.

Figure 1

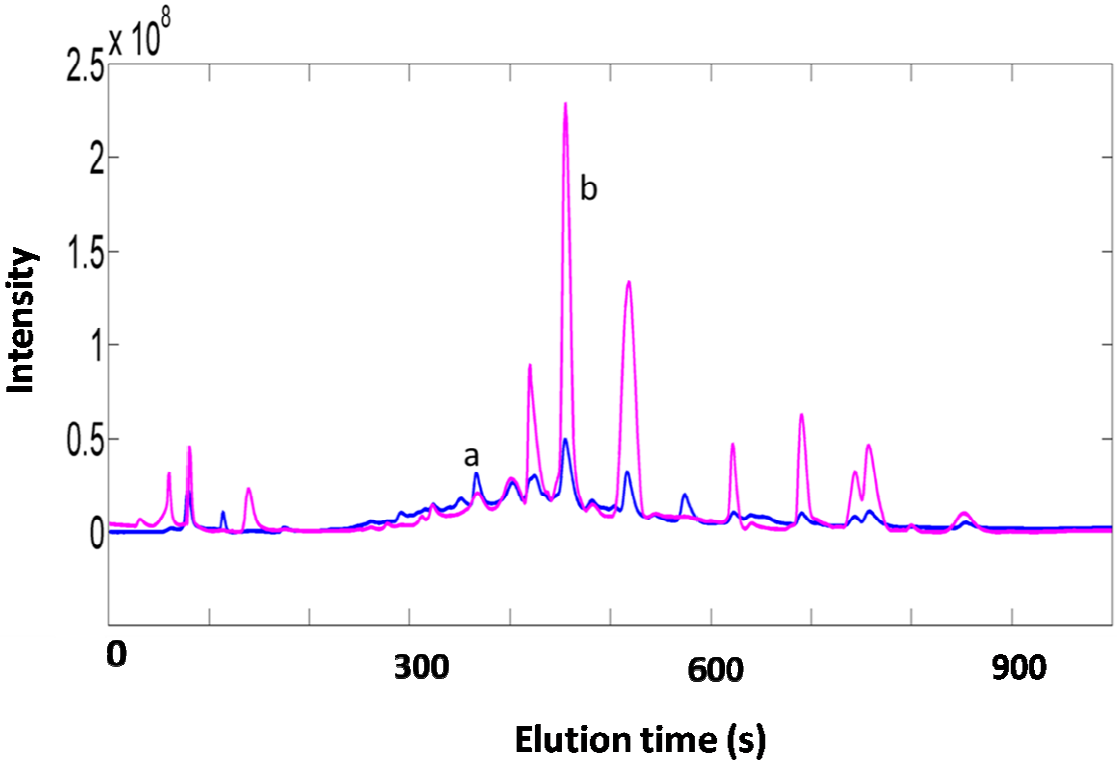


Figure 2

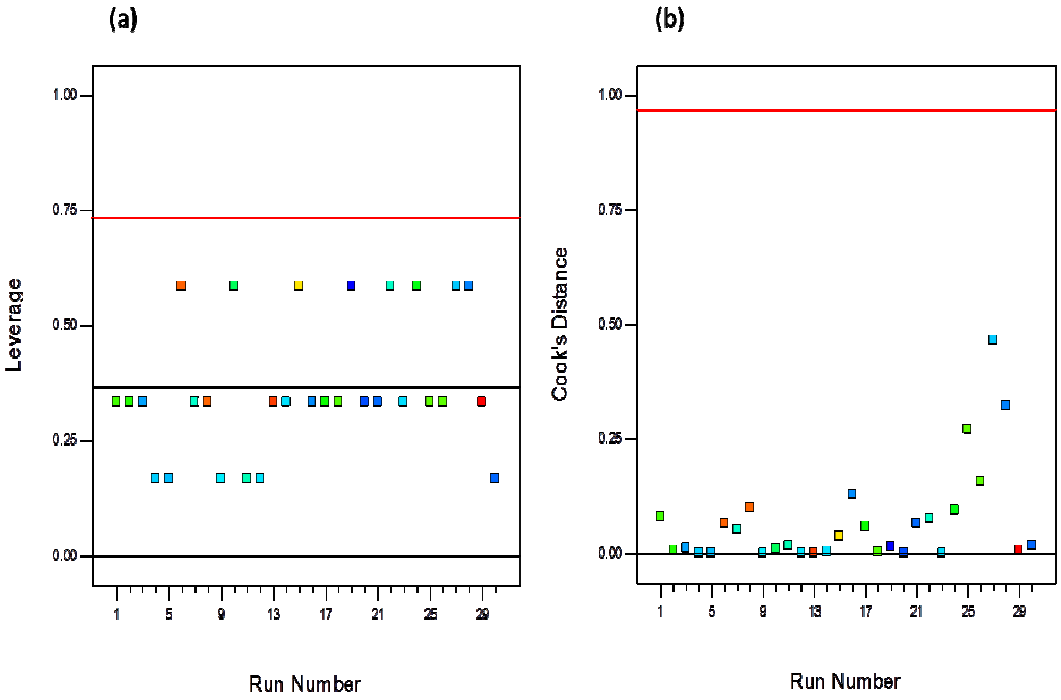


Figure 3

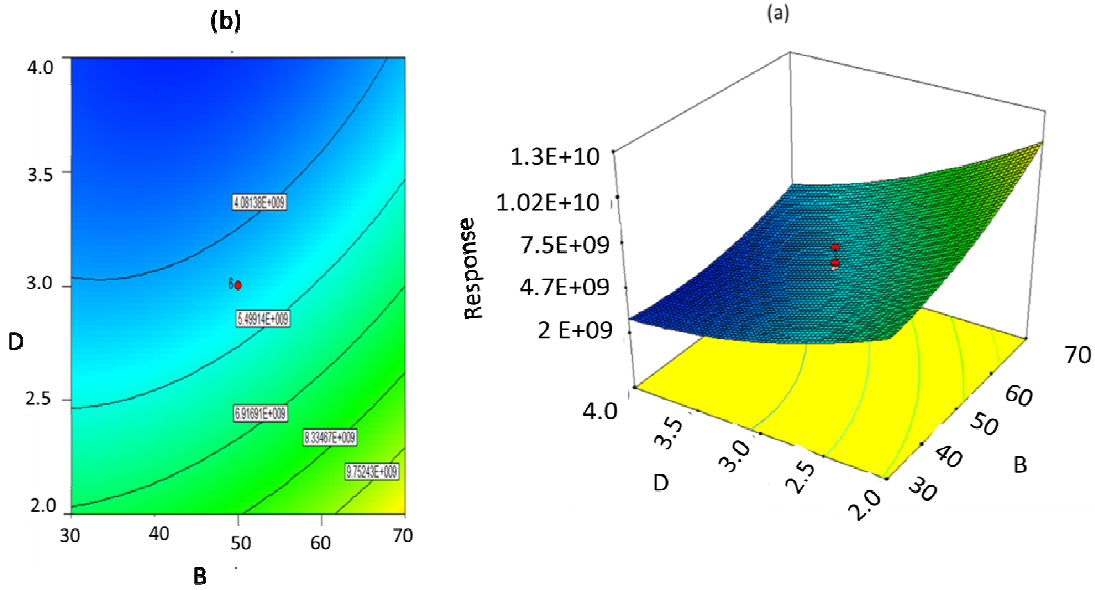
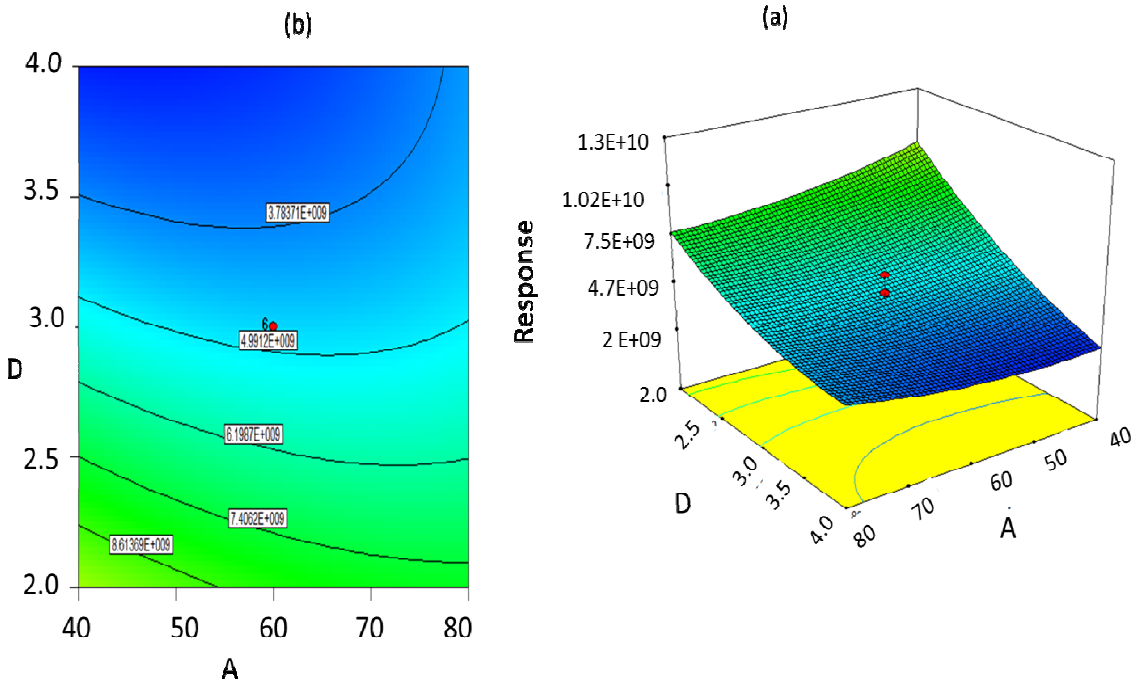


Figure 4



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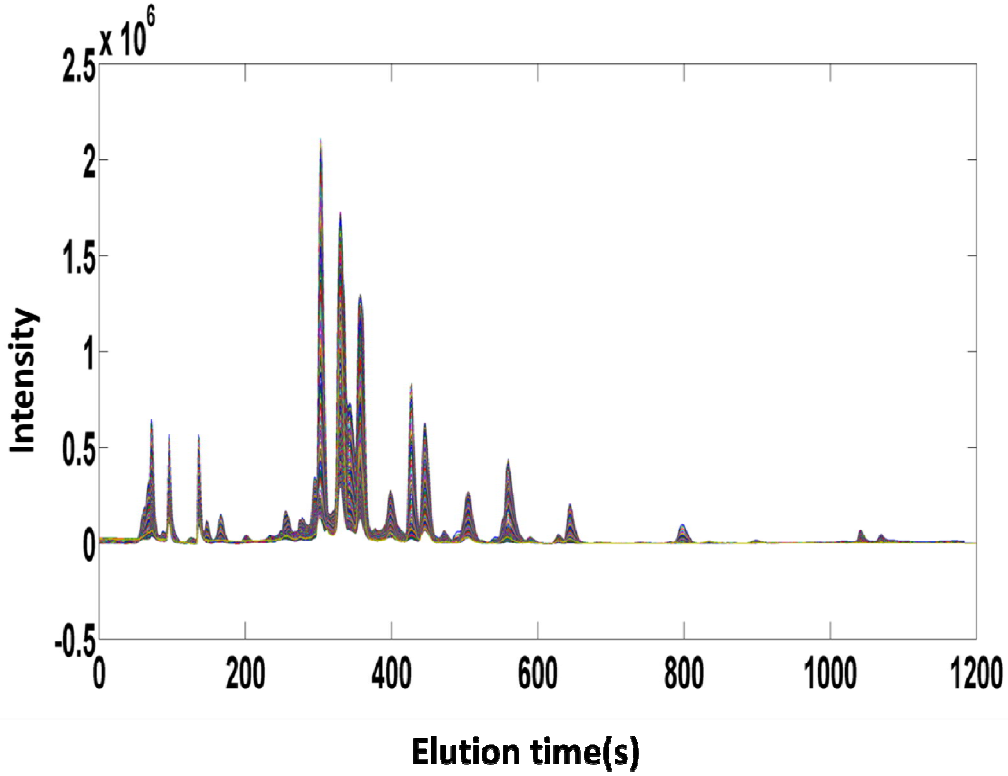

Figure 6

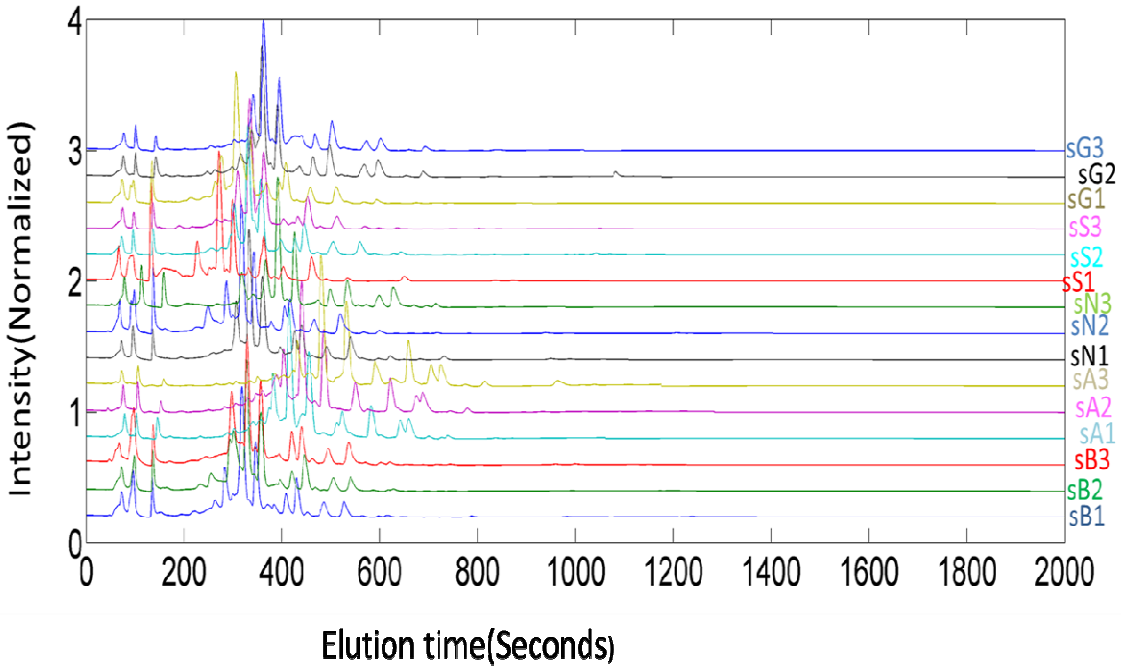

Figure 7

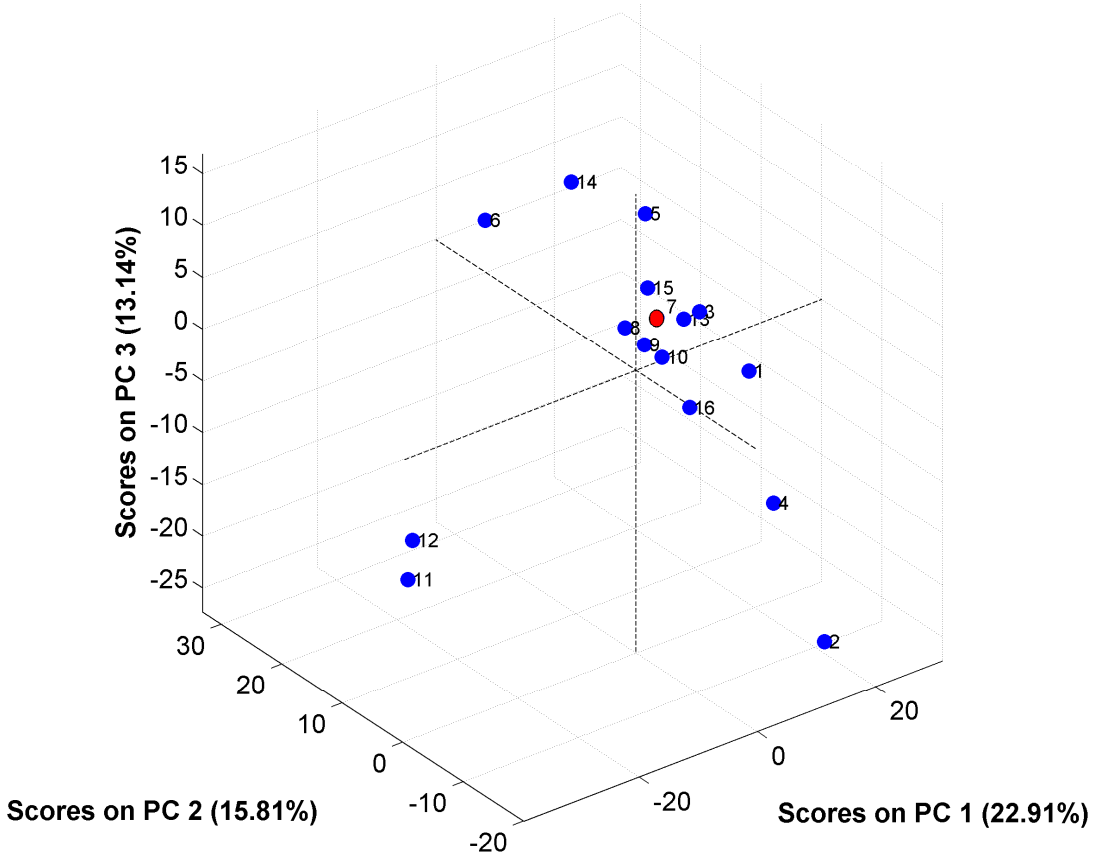

Figure 8