ABSTRACT

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IDENTIFICATION OF MAJOR ORGANIC CONSTITUENTS OF SAFFRON
ISOLATED BY SOLID PHASE EXTRACTION AND COLUMN
CHROMATOGRAPHY

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Water extracts of saffron, a spice derived from the plant Crocus Sativus L. obtained from India and Iran, were analyzed by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). The components in the extracts were separated on four different solid phase extraction cartridges with four different solvents and by silica gel column. Analysis was done by GC-MS, LC-MS and UV-Vis spectroscopy. The extracts separated into three distinct bands (two bright yellow and one orange) on the silica gel column. Based on GC-MS, the extracted compounds show many structural similarities. Using both extraction techniques, several compounds were identified that were not previously reported to be in saffron. Picrocrocin, safranal, and crocins presence in the extracts were evidenced by absorbance bands at wavelengths of 250 nm, 310 nm and 440 nm, respectively, in their UV-Vis spectra. The LC-MS analysis revealed several high molecular weight compounds that were not observed by GC-MS.
IDENTIFICATION OF MAJOR ORGANIC CONSTITUENTS OF SAFFRON
ISOLATED BY SOLID PHASE EXTRACTION AND COLUMN
CHROMATOGRAPHY

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CHAPTER 1
INTRODUCTION

Saffron, the dried and reddest stigma of Crocus Sativus L., is considered the most expensive spice in the world.\textsuperscript{1,2} It is also among the oldest spices widely adopted as a coloring and seasoning agent in food, fabric color, and scent as well as to make a vast range of cosmetic products in various cuisines across the world.\textsuperscript{3} This spice is liked for its aroma, color, taste, and medicinal properties.\textsuperscript{4} Saffron is a French term, whose origin dates back to 12\textsuperscript{th} century. Saffron is a derivative of Latin word safranum, and it is closely linked to Spanish and Italian Azafran and Zafferano, respectively. Safranum is derived from Asfar, an Arabic term which stands for yellow.\textsuperscript{5,6} Crocus Sativus is the scientific name of the plant from which it is extracted,\textsuperscript{7} and it grows in various regions around the world, including Africa, Asia and Europe.\textsuperscript{8} Saffron flower has three major parts, anther (three yellow anthers), stigma, and petal. The stigma part consists of three branches and it weighs about 7.4\% (w/w) of the whole saffron flowers (see Figure 1). This part serves as food and medicine for various health conditions.\textsuperscript{9,10} When harvesting or drying saffron plants, prudence should be observed to avoid harming its taste, color, and aroma of the stigma.\textsuperscript{11} The product has been used for approximately 3000 years in different countries and across various civilizations around the world.\textsuperscript{12}
The saffron plant grows exceptionally well in arid and semi-arid areas because of it does not require much water; it has a unique growing cycle which occurs during winter, and has unique morphological featured leaves which are narrow in shape. Although typically cultivated in temperate climates, 89% - 93% of saffron is grown and produced in Iran since 2005, followed by India, Greece, Spain, Morocco, and Italy.\textsuperscript{13} In 2016, about 105,000 hectares of Iran land was under the plant farming which resulted in the production of about 336 ton. This translates to 3.2 kg per hectare.\textsuperscript{14,15}

The quality of the spice, as determined by its chemical contents, differs based on its place of origin and drying process.\textsuperscript{17} Scientists reported that more than 150 volatile and nonvolatile compounds are present in saffron spice. The distinctive spice has both volatile and nonvolatile components such as lycopene, carotenoids, and zeaxanthin.\textsuperscript{18} Saffron contains four main compounds that play an important role in its properties,
namely, crocins (C_{44}H_{64}O_{24}) the mono-glycosyl and di-glycosyl esters of a polyene dicarboxylic acid, crocetin (C_{20}H_{24}O_{4}) a natural pigment known as carotenoid dicarboxylic acid, picrocrocin a monoterpenic glycoside forerunner of safranal and produce of zeaxanthin, and safranal (2,6,6-trimethyl-1,3-cyclohexadien-1-carboxaldehyde) (see Figure 2).^{19}

The color of the spice is dependent on its crocins content. Crocins are esters of crocetin and are water-soluble compounds that exist in both the trans and cis forms.^{20} Picrocrocin (C_{16}H_{26}O_{7}) is the compound that is responsible for the taste of the spice, specifically the bitter taste that it possesses. Picrocrocin is a precursor of safranal (C_{10}H_{14}O) another important component of the spice.^{21}

![Chemical structures of crocin, crocetin, picrocrocin, and safranal](image-url)

Figure 2. Chemical structures of crocin, crocetin, picrocrocin, and safranal.^{19}
Safranal, the compound that is responsible for the aroma of the spice, is a volatile oil whose quantity depends on the conversion from its precursor, picrocrocin. The process of its formation is pegged to the action of B-glucosidase on picrocrocin, which leads to the liberation of another compound, 4-hydroxy-2,6,6-trimethyl-1-cyclohexane-1-carboxaldehyde (HTCC) with molecular weight of 168 g/mol and formula C_{10}H_{16}O_{2} (see Figure 3). This compound is then converted into safranal through drying. This is what makes the drying process extremely critical to the quality of the saffron that is produced. Different mechanisms of drying are likely to lead to variation in the concentration of the individual contents of the spice, and hence a difference in its taste, aroma and color properties.

**Figure 3.** Scheme showing formation HTCC and safranal from picrocrocin.
Saffron farming is a tedious and expensive task, particularly during the harvesting period. This product is harvested manually on a daily basis because each plant yields only three flowers on different days. In most cases, this plant is harvested manually by cutting and collecting the flowers every day early in the dawn before the sunrises.24 Each kilogram of the newly harvested saffron plant contains 2170 flowers and one kilo of dried saffron can be processed using 78 kilograms of fresh flowers of saffron. While manual harvesting is mostly employed, it negatively affects the quality of the harvested flower, as it decreases its moisture content.25,26 Furthermore; mechanized harvesting operations ensure that flowers are not contaminated as it usually happens in manual harvesting and post-harvesting operations. Notably, harvesting and separation of stigma from the entire flower is arguably the most tedious and expensive task undertaken by farmers of saffron plant.27 After harvesting the plant, post-harvesting operations are performed manually. This encompasses separating stigma from the whole flower immediately after harvest, then proceeded by drying up of the flowers.28 When harvesting manually, a farmer spends between 45 and 55 minutes to pick 1000 flowers and another 100 to 130 minutes to separate stigmas before drying. Therefore, between 370 and 470 hours are spent when producing 1 kilogram of dried saffron.29 It can take up to 75,000 crocus blossoms and 225,000 dried stigmas to yield one pound of saffron spice.30 The very laborious harvesting in conjunction its processing drives up the cost of saffron to between $40 and $50 per gram.31 It has managed to stay in demand and popular regardless.

The uses of saffron in contemporary society are broad. While the spice is still widely used as coloring and seasoning agent in food, its therapeutic and medicinal
purposes seem to be more profound in the modern days. There is adequate evidence which indicates that consumption of saffron offers plenty of therapeutic significance and helps mediate a broad range of health problems in the society.\textsuperscript{19,32} These benefits are attributed to the chemical components of the dried stigmas of saffron. Analysis of its chemical composition shows that this spice is composed of 10\% moisture, 5\% minerals, 12\% protein, 5\% crude fiber, 5\% - 8\% fat and 63\% sugars such as gums, pectin and reducing sugars. Additionally, saffron contains riboflavin and small amounts of thiamine vitamins.\textsuperscript{33,34} The volatile and non-volatile compounds in the stigma bear desirable benefits to the consumers. It has been proven by scientists that saffron components, particularly the carotenoids, which constitute the saffron are important in inhibiting the growth of tumors\textsuperscript{35} in human beings, useful in common gastric diseases,\textsuperscript{36} Alzheimer,\textsuperscript{37} insulin resistance,\textsuperscript{38} and heart problems\textsuperscript{39} as well as depressions.\textsuperscript{40} It is also useful in enhancing individual vision as it hinders fast degeneration of the human retina.\textsuperscript{41} In recent days, many scientists have shown saffron to be as an effective treatment for many diseases, thus making saffron a spice of major importance in the development of treatment in medicine.

Saffron is an expensive spice due to ever growing demand and limited supply, as it is not grown in most parts of the world. There has been tendency by some producers to undertake adulterations of the spice in an attempt to increase supply for more financial gains. In most cases, the color of the saffron has been a subject of adulteration through artificial means or by using other plants similar to saffron, the most common of which is called safflower (with scientific name \textit{Carthamus tinctorius} L). In effect, the quality of
the product is compromised by adulteration of its color or its weight by adding similar looking substances, such as like beet, pomegranate fibers and marigold.\textsuperscript{42} Other than artificial adulterations, geographical location and drying process influence attributes of saffron such as aroma, color and taste. It influence chemical composition of the stigmas mainly safranal, crocins and picrocrocin.\textsuperscript{43}

In saffron analysis it is important to choose the most efficient and effective methods that are suitable for its components and sensitivity. A variety of methods have been investigated for analytical separation and qualitative studies of saffron. Among these are solid phase extraction (SPE) coupled with gas chromatography–mass spectrometry (GC-MS), UV-Visible spectrophotometry, and liquid chromatography–mass spectrometry (LC-MS). A determination of the most effective analysis method is important because it has implications for the best approach to determine the volatile and non-volatile content of the spice in the future.

GC-MS is the most universally agreed method for determining the volatile content of the spices from various parts of the world.\textsuperscript{44} For saffron analysis, GC-MS is used for qualitative analysis. It is most efficient and requires the least time. However, GC-MS cannot retain the sugar moiety of a compound with a high molecular weight, so the mass spectrum of the compound with the sugar moiety attached may not be directly observed. However, the mass spectrum of the fragment with the missing sugar moiety may be observed.

LC-MS is considered the most efficient analytical technique for the analysis of sensitive compounds in complex extracts of natural products.\textsuperscript{54} The technique allows for
the analysis of both volatile and non-volatile compounds of higher molecular weight, such as sugar containing components in saffron. Moreover, in the LC-MS a fraction collector can be used to collect the target analytes, which will allow for additional identification by other techniques, such as IR and NMR spectroscopy.

Solid phase extraction (SPE) is one of the popular methods of extraction of environmental and food samples. It can be used in many applications, such as purification, trace enrichment, desalting, and class fractionation. There is a convergence between SPE and liquid-liquid extraction (LLE) techniques in general, but the efficiency of LLE is less than SPE due to incomplete phase separations, use of expensive apparatus, and the need for disposal of large quantities of used organic solvents.

The procedure for SPE is based on the adsorption of the analytes on the surface of a sorbent, such as aminopropyl or octadecyl stationary phases that are bonded to silica gel, then using solvents to elute the components. SPE is available in glass or polypropylene columns or on extraction disks (see Figure 4).
In order to achieve effective extraction with SPE it is important to condition the tube with a solvent to activate the sorbent. The sorbent absorbs the analytes and some impurities (if present). The impurities are then flushed with a solvent. This is followed by passing the selected solvent over the cartridge to elute the target analytes (see Figure 5).\textsuperscript{47}

\textbf{Figure 5.} Solid phase extraction steps.\textsuperscript{47}
CHAPTER 2
LITERATURE REVIEW

Sánchez et al.\textsuperscript{48} developed the SPE technique that allowed him to determine compounds by using UV-Vis spectrophotometry. In his work, picrocrocin was extracted from saffron and separated by C18 SPE. The aim was to separate picrocrocin without interfering with crocetin ester, and to investigate the factors such as, concentration of the extract, sample volume and eluents, that may affect separation using SPE. The effectiveness of SPE for the isolation of picrocrocin from saffron from Italy, Iran, Spain and Greece was investigated. Concentration plays a significant role in picrocrocin separation in SPE. A 5\% solution of acetonitrile in water was able to elute picrocrocin, but most of the picrocrocin remained in the SPE cartridge due to its low percentage. In contrast, a 15\% solution of acetonitrile in water was sufficient to elute the crocetin ester with picrocrocin causing impurity. However, the picrocrocin was obtained through selectivity method. In Figure 6, the UV–vis spectra (C) illustrate the two eluted fractions of the picrocrocin and crocetin esters observed at wavelength of 257 nm, while chromatographs (A and B) show the picrocrocin and crocin esters peaks that were isolated successfully.
Figure 6. Chromatograms (A & B) and UV–vis spectra (C) showing crocetin esters and picrocrocin fractions in saffron after SPE extraction.\textsuperscript{48}

Chryssanthi et al.\textsuperscript{49} developed an analytical SPE-HPLC method to determine the total crocetin in human plasma. The method displays good sensitivity, selectivity, precision, linearity and accuracy. The method was adopted for the determination of crocetin in blood plasma of the healthy humans before and even after the intake of a cup of saffron tea. Crocetin was not detected before the consumption of the tea, but its concentration was established to have increased within 2 hours and the trace still detected after 24 hours.

Mohajeri et al.\textsuperscript{50} designed a new molecularly imprinted polymer as a sorbent on SPE to extract the crocin from saffron. Gentiobiose (a glycoside moiety in crocin structure shown in Figure 2) was used to make the imprinted polymer. They linked the crocin to gentobiose-imprinted polymer and compared it with non-imprinted polymer in
aqueous solution. The result showed that the crocin affinity of the imprinted polymer was higher in all concentrations than non-imprinted polymer. The gentiobiose imprinted polymer was then used as a sorbent to isolate and purify the crocin from the saffron flower. This method also proved the greater affinity of gentiobiose imprinted polymer for crocin over other compounds, such as picrocrocin and safranal. The method shows good selectivity for crocin, achieving 84% recovery from a mixture of compounds.

Tarantilis et al.\textsuperscript{51} tried to separate the components that are responsible for the aroma in saffron, using ultrasound-assisted extraction (USE) and microsimultaneous hydro distillation-extraction (MSDE) techniques for isolation. GC-MS was used for the determination of the aroma compounds. They confirmed, through this experiment, that several compounds are likely responsible for the smell of saffron (mainly due to the presence of carotenoids). The chromatogram (see Figure 7) illustrates the presence of the main compounds that possibly account for the aroma, namely; safranal (peak 7) and isophorarone (peak 6), 3,5,5-trimethyl-3-cyclohexen-1-one (peak 8), 2,6,6-trimethyl-1,4 cyclohexadiene-1-carboxaldehyde (peak 5), and 2,6,6-trimethyl-2- cyclohexen-1,4-dione (peak 9).
Figure 7. GC chromatogram displaying the aroma compounds in saffron.\textsuperscript{51}

Caballero et al.\textsuperscript{52} establish an HPLC (UV) analytical protocol for identifying and quantifying the major components found in 11 commercial saffron samples obtained from various sources. The aim was to quantify the concentration of the 10 major saffron compounds in each sample using three different detector wavelengths. Figure 8 presents three representative chromatograms of saffron extracts from three different sources, namely Greece (A), Sigma Product (B), and Tibet (C). The components were identified as follows: Peak numbers 1 to 3 (detected $\lambda = 250$ nm) are picrocrocin, HTCC and 3-gentiobiosyl-kaempferol, respectively; Peak number 7 (detected at $\lambda = 310$ nm) is safranal and peak numbers 4 to 6 ($\lambda = 440$ nm) were trans-crocin 4, trans-crocin 3, and trans-crocin 20, and peaks 8 to 10 (detected at 440 nm) are cis-crocin 4, trans-crocin 2, and cis-crocin 2, respectively.
Figure 8. HPLC chromatograms showing components in extracts of saffron samples from Greece [A], Sigma Product [B] and Tibet [C].

From the results, it was established that Greek saffron recorded the highest levels of the components. From the investigation, the results indicated that the differences might have been as a result of the origin of the samples, the drying process as well as the storage conditions of the samples. Such conditions could have affected the concentration of glycosidic carotenoids as they are thermally and photochemically sensitive.

Sereshti et al. sought to evaluate the effect of storage time on the quality of saffron. The quality of saffron is usually gauged mainly by its aroma, color and taste. They conducted the experiment by comparing freshly dried saffron against that stored for 2 years using UV–Vis absorption and GC-FID techniques. The results were analyzed to single out its time biomarkers. Interestingly, whereas the color tends to fade out, its aroma however increases. Saffron coloring agents include crocins, b-isophorone, 4-
hydroxy-3, 5, 5-trimethylcyclohex-2-enone and picrocrocin. Freshly dried saffron has higher levels of these agents. UV-V spectra of samples obtained according to ISO 3632-2 method (see Figure 9) showed that saffron has three absorbance bands assigned to picrocrocin (\(\lambda_{\text{max}} = 250\)), safranal (\(\lambda_{\text{max}} = 310\)) and crocins (\(\lambda_{\text{max}} = 440\)). From Figure 9, it is evident that the amount of safranal was higher in the freshly dried as well as the stored samples, while its crocins content was lower in the stored saffron. Storage time has a significant impact on saffron chemical profile and quality.

**Figure 9.** UV–Vis spectra showing absorbance bands in saffron assigned to picrocrocin (\(\lambda_{\text{max}} = 250\)), safranal (\(\lambda_{\text{max}} = 310\)) and crocins (\(\lambda_{\text{max}} = 440\)) and their variation from freshly dried (green) to 2-year stored (red).\(^{54}\)
The chromatographic and spectroscopic profiles were analyzed through Chemometric gas analysis with the results showing a great difference in profiles between the freshly dried and the stored samples. Moreover, D’Auria et al.\textsuperscript{55} did a similar study on the effects of storage on six samples of saffron from various places in Italy, using SPME–GC–MS technique. They proved that the safranal concentration increases during the first 3 years of storage and then decreases after the first 5 years.

Zarghami et al.\textsuperscript{56} successfully separated the main volatile components in saffron by gas chromatography. These volatiles were identified by NMR, UV, IR and mass spectrometry. Figure 10 shows the compounds are 3,5,5-trimethyl-4-hydroxy-1-cyclohexanon-2-ene (I), 3,5,5-trimethyl-1,4-cyclohexadione (II), 3,5,5-trimethyl-1,4-cyclohexadion-2-ene (III), 3,5,5-trimethyl-2-hydroxy 1-cyclohexadion-2-ene (IV), 2,6,6-trimethyl,4-hydroxy-1-cyclohexene-1-carboxaldehyde (V), and 2,4,4-trimethyl-3-formyl-6-hydroxy-2,5-cyclohexadien-l-one (VI). Moreover, D’Auria et al.\textsuperscript{57} also discovered some additional compounds in saffron from the samples that were derived from different places from Italy as well as Iran. Their SPME-GC–MS analysis technique on four saffron samples shows 18 compounds that were not previously reported for saffron. The compounds were found in all four samples, and were namely; 3,5,5-trimethyl-2-cyclohexen-1-one, 3,5,5-trimethyl-2-cyclohexen-1,4-dione, 2,4,4-trimethyl-6 hydroxy-3-carboxaldehyde-2,5-cyclohexadien- 1-one, 3,5,5-trimethyl-1,4 cyclohexandione, 5,5-dimethyl-2-methylene-1- carboxaldehyde-3-cyclohexene, β-ionone, nonanal and 2,6-dit-butylphenol.
Escribano et al.\textsuperscript{58} put forth a study on the saffron’s inhibiting role in the growth of human cancer cells by isolated crocetin, safranal, crocin, and picrocrocin from the spice extracts for analysis. The isolated compounds summed to a dosage of 50\% of the cell growth inhibition (LD\textsubscript{50}) on HeLa cells. The cells that had been exposed to crocin depicted a wide range of cytoplasmic vacuole-like areas. The cells had pyknotic nuclei, reduced cytoplasm and reduced size. The findings ascertain that crocin has a cytotoxic effect on tumor cells that make it most appropriate in studying cancer treatment.\textsuperscript{58}

2.1 Motivation for Thesis Work

The main objective of this project is to identify new major organic compounds in saffron. The complexity of the saffron is that more than 150 volatile and non-volatile
compounds are reported as present. However, there is no simple method to separate and identify the components. The separation and identification of saffron compounds have become a challenge, and therefore modern separation techniques can be used to reduce the number of compounds been separated before the final analysis by GC-MS and LC-MS. Therefore, in this work two techniques were investigated to separate compounds in saffron, namely, solid phase extraction and silica gel chromatography. Four different SPE cartridges and four different eluting solvents going from polar to non-polar were investigated. The extracted volatile and non-volatile compounds of saffron by SPE and silica gel column were analyzed by GC-MS, LC-MS, UV-Vis spectroscopy.
CHAPTER 3

EXPERIMENTAL SECTION

3.1 Plant Materials

Stigmata of pure red Indian Kashmiri Saffron were purchased from the original source (al Jabri Saffron, KSA) and it was reported as harvested on November 2016 as fresh dried Saffron. Stigmata of pure red saffron were purchased as pure Pushali Iranian saffron.

3.2 Extraction Materials

3.2.1 Silica Gel Column Chromatography

A neutral silica gel stationary phase with methanol mobile phase (the extraction solvent) was one of the techniques that were used to separate saffron compounds. The neutral silica gel had low levels of iron and heavy metals impurities.

3.2.2 Solid Phase Extraction Cartridges

The separation of saffron compounds was also conducted using four different Discovery SPE cartridges (i.e., DSC-NH₂, DSC-CN, DSC-MCAX, and DSC-18) that were purchased from Sigma Aldrich Chemical Company. Each cartridge has a bed wt. of 1 g and a volume of 6 mL. The bed materials are described as follows.
A. **Discovery DSC-18**: Octadecyl silane (Si-(CH$_2$)$_{17}$CH$_3$) is a bonded polymeric phase. The retention mechanism of this cartridge is reversed phase.$^{59}$

B. **Discovery DSC-CN**: Cyanopropyl silane (Si-(CH$_2$)$_3$CN) is a bonded monomeric phase. The retention mechanism of this cartridge can be either normal phase or reversed phase and the cartridge is suitable for absorption of very hydrophobic analytes.$^{59}$

C. **Discovery DSC-MCAX**: Octyl silane (Si-(CH$_2$)$_7$CH$_3$) is bonded with benzene sulfonic acid. The retention mechanism of this cartridge is a mixed between reversed-phase and cation exchange.$^{50}$

D. **Discovery DSC-NH$_2$**: Aminopropyl silane (Si-(CH$_2$)$_3$NH$_2$) is a bonded polymeric phase. The retention mechanism of this cartridge is either normal phase or anion exchange.$^{61}$

### 3.3 Solvents

The following chemicals were purchased from Sigma Aldrich Company and used as received without further purification: HPLC grade water, HPLC grade methanol, 1,2-dichloromethane (DCM), 99% grade hexane, ACS grade acetone, acetonitrile, and formic acid. All glassware were cleaned and dried in the oven before use.

### 3.4 Instrumentations

#### 3.4.1 Ultraviolet-Visible Spectroscopy

UV-Vis absorption spectra were measured with a DU 800 UV-Vis-NIR spectrophotometer. Liquid extracts were placed in 1cm quartz cuvette.
3.4.2 Gas Chromatography–Mass Spectrometry

GC-MS analyses were carried out with an Agilent 7820A GC that was equipped with an HP-5MS capillary column (30 m × 0.250 mm i.d. 0.25 μm film thickness), helium carrier gas at 1.2 mL min\(^{-1}\) and MSD 5977B mass selective detector. The ionization energy was set to 70 eV and the specific ions are analyzed in the m/z-range 50 to 550 at 3.7 scans per second. The detector interface was kept at 280°C. The oven temperature was maintained at 70 °C for 2 min, increased to 300 °C at a rate of 15 °C min\(^{-1}\), and maintained at 300 °C for 6 min. The temperatures for the detector and injector were set at 230 °C and 250 °C, respectively. Sample injection was done with the instrument in splitless injection mode. The injection volume was one microliter; the Indian saffron was auto-injected, and the Iranian saffron was injected manually. The run time of each sample was 23.33 mins. Compounds identification was carried out using the NIST98 mass spectra library.

3.4.3 Liquid Chromatography–Mass Spectrometry (LC-MS)

LC-MS analyses were carried out with an Agilent G 6125B LC/MSD equipped with a Zorbax SB-C18 (2.1 × 0.50 mm 1.8- Micron, 600 Bar) column, G711B Quat Pump, G7129A vial sampler and a UV–Vis photodiode array detector operating at two different wavelengths. The mobile phase was a linear 45 min gradient of 10% to 100% methanol in HPLC water containing 1% formic acid, representing a slight modification of published procedure. The flow rate was 0.4 mL min\(^{-1}\) and the sample injection volume was 1μl. UV absorption was measured at wavelengths of 250-310 nm and 310-440 nm.
for detection of picrocrocin, safranal, and crocins compounds. The analyses were done in duplicate for each sample at the assigned wavelengths.

3.5 Experimental Preparation and Procedures

3.5.1 Silica Gel Column Chromatography

The experimental procedure that was used to prepare the saffron sample for silica gel column separation is as follows. Approximately 0.1g of Iranian saffron was transferred to a conical flask and diluted with 25 ml HPLC grade. The sample was then heated to 75 °C for 30 min, left stirred for 30 min at room temperature and then placed in a refrigerator for 24 hours. The silica gel column (about 7.4 inch long) was conditioned with 150 ml of HPLC grade water. Six mL of sample was immediately added to the column which was then flushed with methanol as a mobile phase. The extract fractions were collected in vials (1.5 mL volume). The Indian saffron was prepared and extracted under the same procedure as the Iranian saffron.

3.5.2 Solid Phase Extraction

A 0.2 g portion of Indian saffron was transferred to a conical flask with 50 ml HPLC grade water. The sample was then heated between 60-70 °C for 30 min, further stirred for 30 min at room temperature and then placed in a refrigerator for 24 hours.

The SPE cartridges were conditioned before use with HPLC grade water (3 mL), methanol (3 mL), hexane (3 mL), and again with HPLC grade water (3 mL). Three mL of the sample was passed through SPE cartridges that were placed on the SPE vacuum
The cartridge was washed with 5 mL of the solvent (methanol, 1,2-dichloromethane (DCM), hexane, or acetone) and the extract was collected.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Introduction

There is continued interest in determining what compounds are present and can be extracted from saffron. This interest is what drives this research to further identify what compounds or groups of compounds are extracted by the solid phase extraction (SPE) cartridge substrate and the solvent used for eluting from the SPE cartridge. In this research, gas chromatography-mass spectrometry was used to identify the compounds extracted. In later experiments it was determined that liquid chromatography-mass spectrometry would provide evidence of high molecular compounds not detected via GC-MS. In addition to those compounds identified by SPE extraction followed by GC-MS, a comparison of “total compounds extraction” with the saffron extracts separated by a neutral silica column and eluted with methanol was conducted.

The GC-MS results of the saffron extract based on SPE cartridges and the eluting solvent (eluent) clearly showed similarities and differences among the cartridges, in the compounds eluted. Differences observed in relative total ion intensities in the GCMS of the compounds extracted show that certain cartridges had a greater affinity for those compounds extracted. These differences will be discussed later. One of the surprising findings is the neutral silica column elution results of the saffron extract. Elution of the compounds with methanol after the saffron extract was applied to neutral silica gel
The mass spectral data shows structural similarities among the compounds that eluted in the GC chromatograms, based on fragmentation patterns, though they had different retention times.

In addition to the mass spectral analysis, ultraviolet-visible (UV-Vis) spectroscopy was used to identify the compounds in the methanol extract based on the knowledge that the saffron compounds, picocrocins, safranal, crocins and, shows UV-Vis absorption at wavelengths of 250 nm, 310 nm and 440 nm, respectively. The knowledge of the absorption wavelengths for these three groups of compounds was extended to the LC-UV-Vis-MS analysis of the “total compounds” in saffron extract from the neutral silica column.

4.2 Characterization

4.2.1 Gas Chromatography–Mass Spectrometry

4.2.1.1 Solid Phase Extraction

Gas chromatography-mass spectrometry was used for analytical separation and qualitative ways to determine compounds in saffron that were extracted by the solvents. The GC-MS results for the SPE showed similar results of compounds were eluted the same in some cartridges and different compounds in others cartridges, as well as identifying compounds in saffron never been reported.
Table 1 lists the compounds in saffron from India that were separated by SPE and identified by GC-MS analysis. Figures 11 and 12 show DSC-18 and DSC-CN cartridges. It can be said that the same compounds eluted on both cartridges, whether methanol or acetone was the eluting solvent. The identity of the components based on mass spectra are as follows: 3,5,5-trimethyl-3-cyclohexen-1-one (RT 4.896 min) (1), 3,5,5-trimethyl-2-cyclohexen-1-one (RT 5.840 min) (5), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (RT 6.075 min) (6), 2,2,6-trimethyl-1,4-cyclohexanediione (RT 6.315 min) (7). Two peaks in the chromatogram at RT of 6.676 min (11) and 16.918 min (23) showed similar mass spectra and were both identified as 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal) by the GC-MS analysis (see Table 1). The mass spectrum of peak at RT of 6.676 min (11) matches that of safranal more closely than that at RT of 16.918 min (23). The first peak was therefore assigned to saffranol while the second is considered to be closely related or in the in the family of safranal. Moreover, 4-hydroxy-3,5,5-trimethylcyclohex-2-enone (14), 2,4,5-trimethyl-benzaldehyde(17), and 2-isopropylidene-3-methylhexa-3,5-dienal(18) show a peaks at (RT 7.700, 7.850 and 8.782 min), respectively (see Table 1 and Figures 11 and 12).

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>Quality %</th>
<th>SPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 3,5,5-Trimethyl-3-cyclohexen-1-one</td>
<td>4.896</td>
<td>93</td>
<td>1</td>
</tr>
<tr>
<td>2 - Benzoic acid-methyl ester</td>
<td>5.503</td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td>3 - 4-(1-Methylethyl)-benzaldehyde</td>
<td>5.411</td>
<td>90</td>
<td>3,1</td>
</tr>
<tr>
<td>4 - 1-(2,5-Dimethylphenyl)-ethanone</td>
<td>5.497</td>
<td>92</td>
<td>2</td>
</tr>
<tr>
<td>5 - 3,5,5-Trimethyl-2-cyclohexen-1-one</td>
<td>5.749</td>
<td>90</td>
<td>ALL</td>
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</table>

Continued
<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>Quality %</th>
<th>SPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 - 2,6,6-Trimethyl-2-cyclohexene-1,4-dione</td>
<td>6.075</td>
<td>91</td>
<td>ALL</td>
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<tr>
<td>7 - 2,2,6-Trimethyl-1,4-cyclohexanedione</td>
<td>6.315</td>
<td>87</td>
<td>1.2</td>
</tr>
<tr>
<td>8 - 2-Isopropyl-5-methylhex-2-enal</td>
<td>6.372</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>9 - 3,5-Dimethyl-benzaldehyde</td>
<td>6.515</td>
<td>93</td>
<td>3</td>
</tr>
<tr>
<td>10 - Dodecane</td>
<td>6.556</td>
<td>94</td>
<td>3</td>
</tr>
<tr>
<td>11 - 2,6,6-Trimethyl-1,3-cyclohexadiene-1-Carboxaldehyde (safranal)</td>
<td>6.676</td>
<td>97</td>
<td>ALL</td>
</tr>
<tr>
<td>12 - 2,6,6-Trimethyl-2,4-cycloheptadien-1-one</td>
<td>6.905</td>
<td>87</td>
<td>3</td>
</tr>
<tr>
<td>13 - (10-Methylnonadecane)</td>
<td>7.391</td>
<td>89</td>
<td>4</td>
</tr>
<tr>
<td>14 - 4-Hydroxy-3,5,5-trimethylcyclohex-2-enone</td>
<td>7.700</td>
<td>83</td>
<td>1,4</td>
</tr>
<tr>
<td>15 - 2,6,10-Trimethyl-tetradecane</td>
<td>7.820</td>
<td>83</td>
<td>1</td>
</tr>
<tr>
<td>16 - Tetradecane</td>
<td>8.512</td>
<td>97</td>
<td>3,4</td>
</tr>
<tr>
<td>17 - 2,4,5-Trimethyl-benzaldehyde</td>
<td>7.850</td>
<td>94</td>
<td>ALL</td>
</tr>
<tr>
<td>18 - 2-Isopropylidene-3-methylhexa-3,5-dienal</td>
<td>8.782</td>
<td>83</td>
<td>ALL</td>
</tr>
<tr>
<td>19 - 9-Octyl-heptadecane</td>
<td>11.694</td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td>20 - N-butyl-benzenesulfonamide</td>
<td>11.688</td>
<td>97</td>
<td>2</td>
</tr>
<tr>
<td>21 - Octadecane</td>
<td>11.688</td>
<td>98</td>
<td>3,1,4</td>
</tr>
<tr>
<td>22 - Octacosane</td>
<td>23.050</td>
<td>94</td>
<td>3,4</td>
</tr>
<tr>
<td>23 - Unknown*</td>
<td>16.918</td>
<td>70</td>
<td>1,2,4</td>
</tr>
</tbody>
</table>

*The mass spectrum of this compound in the region below m/z = 168 is similar to that of compound 26*
Figure 11. GC chromatograms of Indian saffron extracts eluted by methanol from DSC-18 and DSC-CN cartridges.
The relative intensities in DSC-18 in major compounds of (11), (18), and (23) were higher than that one in DSC-CN. In contrast, the relative intensities for the same major compounds (11), (18), and (23) that were eluted by acetone were almost same in relative intensities (see Table 1 and Figures 11 and 12).
Table 1 and Figure 13 show the GC-MS chromatograms of Indian saffron extract that was eluted separately from DSC-NH$_2$ by methanol and acetone. Two major compounds were extracted by the acetone namely; 3,5,5-trimethyl-2-cyclohexen-1-one (RT 5.749 min) (5), and 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.676 min) (11). Methanol was able to extract these two compounds in addition to benzoic acid-methyl ester (RT 5.503 min) (2), 1,3,5,5-tetramethyl-1,3-cyclohexadiene (RT 5.651 min), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (RT 6.058 min) (6), 2-isopropyl-5-methylhex-2-enal (RT 6.372 min) (8), 3,5-dimethyl-benzaldehyde (RT 6.515 min) (9), 2,6,6-trimethyl-2,4-cycloheptadien-1-one (RT 6.905 min) (12), 4-hydroxy-3,5,5-trimethylcyclohex-2-enone (RT 7.700 min) (14), 2,4,5-trimethyl-benzaldehyde (RT 7.850 min) (17), and 2-isopropylidene-3-methylhexa-3,5-dienal (RT 8.782 min) (18). Noteworthy, is that methanol and acetone did not elute the major component that is expected at RT 16.918 min as was observed using the DSC-18 and DSC-CN cartridges. Components 2, 8, 9, and 12 were not eluted in previous results (see Figure 13 and Table 1).
Figure 13. GC chromatograms of Indian saffron extracts that were eluted by methanol and acetone using cartridges of DSC-NH₂.

Table 1 and Figure 14 show the GC-MS chromatograms of Indian saffron extract that was eluted by non-polar solvent, hexane on all the four cartridges. The first two cartridges were DSC-NH₂ and DSC-CN. The following compounds were eluted from both cartridges: 4-(1-methylethyl)-benzaldehyde (RT 5.411 min) (3), 3,5,5-trimethyl-2-cyclohexen-1-one (RT 5.749 min) (5), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (RT 6.072 min) (6), 2,2,6-trimethyl-1,4-cyclohexanedione (RT 6.315 min) (7), 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.676 min) (11), 2,4,5-trimethyl-benzaldehyde (RT 7.850 min) (17), tetradecane (RT 8.512 min) (16).
Figure 14. GC chromatograms of Indian saffron extracts that were eluted by hexane from DSC-NH$_2$ and DSC-CN cartridges.

Noteworthy is that except two compounds dodecane (RT 6.556 min) (10) and 2-isopropylidene-3-methylhexa-3,5-dienal (RT 8.782 min) (18) were eluted from DSC-NH$_2$ by hexane but not from DSC-CN. Moreover, the relative intensities of the major compounds from DSC-CN cartridge were higher than those from DSC-NH$_2$, while the relative intensities for 2-isopropylidene-3-methylhexa-3,5-dienal was reversed for DSC-
NH₂ (see Figure 14). As shown in Figure 15, the following set of compounds were eluted from DSC-18 by hexane; 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.676 min) (11), 10-methylnonadecane (RT 7.391 min) (13), 2,6,10-trimethyl-tetradecane (RT 7.820 min) (15), 9-octyl-heptadecane (RT 11.694 min) (19), and octacosane (RT 13.050 min) (22), compare to others cartridges, major compounds expected in the chromatogram at (RT 16.918) (23) were not observed, and hence did not elute from both of the cartridges by hexane. Components 3, 10, 13, 15, 16, 19 and 22 were not mentioned in previous cartridges (see Table 1 and Figure 15).

![Figure 15. GC chromatograms of Indian saffron extracts that were eluted by hexane from DSC-18 cartridges.](image-url)
Figures 16 and 17 show GC-MS chromatograms of components in saffron extract that were eluted from cartridges DSC-18, DSC-CN, and DSC-NH₂ by dichloromethane. The following set of compounds were eluted from these cartridges by dichloromethane; 4-(1-methylethyl)-benzaldehyde a peak (RT 5.411 min) (3), 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.670 min) (11), 2-isopropylidene-3-methylhexa-3,5-dienal (RT 8.753 min) (18) and 2,4,6-trimethyl-benzaldehyde (RT 7.855 min) (17), 3,5,5-trimethyl-2-cyclohexen-1-one (RT 5.749 min) (5), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (RT 6.058 min) (6), 2,2,6-trimethyl-1,4-cyclohexanedione (RT 6.321 min) (7), 4-hydroxy-3,5,5-trimethylcyclohex-2-enone (RT 7.712 min) (14), and n-butyl-benzenesulfonamide (RT 11.688 min) (20). However, an additional compound, 1-(2,5-dimethylphenyl)-ethanone eluted from cartridge DSC-CN (RT 5.497 min) (4). It is clear that there was no major component observed at (RT 16.918) (23) in this chromatogram compare to chromatograms of dichloromethane eluants from other cartridges, indicating that this non polar solvent could not elute this compound. In addition, the relative intensity for major components at (RT 6.670 min) (11), and (RT 8.753 min) (18) on DSC-18 is higher than DSC-CN and DSC-NH₂ and that may be due to the interactions between the solvent and these compounds. Components 4 and 20 were not eluted in previous results.
Figure 16. GC chromatograms of Indian saffron extracts that were eluted by dichloromethane from DSC-18 and DSC-CN cartridges.
Figure 17. GC chromatograms of Indian saffron extracts that were eluted by dichloromethane from cartridge DSC-NH$_2$ cartridges.
Figure 18 shows GC-MS chromatograms of components in saffron extract that were eluted from the DSC-MCAX cartridge by four solvents. With methanol and acetone solvents eluted the following compounds; 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.676 min) (11), 2-isopropylidene-3-methylhexa-3,5-dienal (RT 8.782 min) (18), and unknown compound (RT 16.918 min) (23). With hexane and dichloromethane solvents the first two components listed above (11 and 18) were eluted by these solvents. Additional components not listed above were also eluted, namely; (10-methylnonadecane) (RT 7.391 min) (13), 2,4,5-trimethyl-benzaldehyde (RT 7.873 min) (17), and tetradecane (RT 8.512 min) (16). Clearly, with both hexane and dichloromethane solvents, there were no major component in observed in the chromatogram of the extracts at (RT 16.918) as was the case with acetone and methanol solvents (see Table 1 and Figure 18).
Figure 18. GC chromatograms illustrates the compounds that were eluted by four solvents from DSC-MCAX cartridge. 1 - methanol, 2 - acetone, 3 - hexane, 4 - dichloromethane.
SPE results of Indian saffron that were analysed by GC-MS show compounds that were never before reported in this plant as follow; benzoic acid-methyl ester (2), 1-(2,5-dimethylphenyl)-ethanone (4), 2-isopropyl-5-methylhex-2-enal (8), (10-methylnonadecane) (12), 2,6,10-trimethyl-tetradecane (15), 9-octyl-heptadecane (18), n-butyl-benzenesulfonamide (19) (see Table 1). In addition, SPE results show that several compounds eluted from the cartridges by more than one solvent, while some compounds did not elute by certain solvent. Nevertheless, based on the result of the SPE cartridges, it was decided to conduct separation using a silica gel column and to investigate how the result would compare with the SPE cartridges.

4.2.1.2 Silica Gel Column Chromatography

GC-MS was also used to identify compounds in saffron that were extracted using silica gel column chromatography with methanol solvent as a mobile phase. Although saffron is a very complex mixture, silica gel extraction shows that there are three possible groups of these compounds. Figure 19 shows the three distinct bands that were separated on the column. There were two bright two bright yellow bands on either side of an orange band.
Figure 19. Silica gel columns showing the three distinct bands for saffron extracts.

The bands were analyzed to determine if the components match the SPE results. A comparing of the result of the silica gel chromatography with those SPE using the same extraction same solvent shows some compounds were not extracted by SPE technique.
Table 2 shows all the compounds that were extracted and identified by the silica gel column in first, second and third bands. The quality of the match of the mass spectrum for each peak with that in the library is also included. Figure 20 shows GC-MS chromatograms for extracts from the first bright yellow band of the Indian and Iranian saffron. Through the GC-MS analysis, the following compounds (see Table 2) in both saffron samples were identified; 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (26), eicosane (30), 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde (31), 1-methyl-4(1-methylethylidene)-cyclohexane (32), hexadecane (33), pentacosane (34), and 1,2-Benzenedicarboxylic acid-heptyl-octyl ester (41), at retention times of 6.674, 9.359, 9.474, 9.914, 10.183, 10.943 and 15.768 min, respectively.

Table 2. List of Compounds in Indian and Iranian Saffron Extracts That Were Separated by Silica Gel Column Chromatography and Analysed by GC-MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>Quality %</th>
<th>Silica Gel G#</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 - 3,5,5-Trimethyl-2-cyclohexen-1-one</td>
<td>5.829</td>
<td>90</td>
<td>1.2</td>
</tr>
<tr>
<td>25 - 2,2,6-Trimethyl-1,4-cyclohexanedione</td>
<td>6.350</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>26 - 2,6,6-Trimethyl-1,3-cyclohexadiene-1-carboxaldehyde</td>
<td>6.647</td>
<td>97</td>
<td>1,2,3</td>
</tr>
<tr>
<td>27 - 4-Hydroxy-3,5,5-trimethylcyclohex-2-enone</td>
<td>7.717</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td>28 - 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde</td>
<td>8.507</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>29 - 2-Isopropylidene-3-methylhexa-3,5-dienal</td>
<td>8.759</td>
<td>86</td>
<td>1</td>
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<tr>
<td>30 - Eicosane</td>
<td>9.359</td>
<td>72</td>
<td>1</td>
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<tr>
<td>31 - 2,6,6-Trimethyl-1-cyclohexene-1-acetaldehyde</td>
<td>9.474</td>
<td>81</td>
<td>2</td>
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<tr>
<td>32 - 1-Methyl-4-(1methylethylidene)cyclohexane</td>
<td>9.914</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>33 - Hexadecane</td>
<td>10.183</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>34 - Pentacosane</td>
<td>10.944</td>
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<tr>
<td>35 - 8-Methyl-1,2,3,4,4a,9,10,10a-octahydro phenanthrene</td>
<td>11.987</td>
<td>90</td>
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<th>Quality %</th>
<th>Silica Gel G#</th>
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<td>36 - 1-Methyl-4-(1,2,2-trimethylcyclopentyl)-,(R)-benzene</td>
<td>12.175</td>
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<td>37 - 9,10-Dimethoxy-1,2,3,4-tetrahydro-1,4-ethanoanthracene</td>
<td>12.369</td>
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<td>38 - Hexadecanoic acid-methyl ester</td>
<td>12.564</td>
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<td>3</td>
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<td>39 - 7-Octadecenoic acid-methyl ester</td>
<td>13.731</td>
<td>98</td>
<td>3</td>
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<td>40 - Eicosanoic acid-methyl ester</td>
<td>15.041</td>
<td>99</td>
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<td>41 - 1,2-Benzenedicarboxylicacid-heptyl-octyl ester</td>
<td>15.768</td>
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<td>42 - Unknown*</td>
<td>16.681</td>
<td>70</td>
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</tr>
<tr>
<td>43 - Corticosterone</td>
<td>19.894</td>
<td>91</td>
<td>3</td>
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*Mass spectra of this compound is similar to compound 26 below m/z 168

Figure 20. GC-MS chromatograms of extracts of Indian and Iranian saffron from the first of the three bands separated on silica gel column.
The compounds, 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde and 1,2-benzenedicarboxylic acid-heptyl-octyl ester, were never before reported in saffron. Furthermore, there was an additional group of compounds between retention times of 11.820 min and 16.20 min. The peaks noted have very similar mass spectra indicating that they are in the same family (see Figure 20). For example, the compound shows that the mass spectra of peaks at retention time of 6.647 min (1), 6.762 min (2), 9.474 min (3), and 16.907 min (4) from the second band are similar (see Figure 21 and Appendix A1).

Figure 21. Mass spectra of peaks at RT (6.647 (1), 6.762 (2), 9.474(3), and 16.907 (4) min) in the chromatogram of Indian saffron extract from the first band.
The GC-MS cannot accommodate high molecular weight molecules with moieties, such as glucose attached to this compound 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde, (safranal) or has a slight difference in the function group causing the similarities in the mass spectra. Additionally, Figure 22 and Appendix A2 show similarity in mass spectra of peaks at retention time of 11.854, 12.404, and 12.665 min. These compounds could be related in structure to 9,10-dimethoxy-1,2,3,4-tetrahydro-1,4-ethanoanthracene. In order to distinguish between them, an instrument that can separate and analyze higher molecular weight compounds will be required.

Figure 22. Mass spectra of peaks at RT of 11.854 (1), 12.404 (2), and 12.667 (3) min in the chromatogram of Indian saffron extract from the first silica gel band.
In this band there are other groups of peaks such as peaks at RT of 13.983, 14.092, and 14.223 min that show similar mass spectra, and may be related to the compound, androstane-3,17-dione (see Figure 23 and Appendix A3).

Figure 23. Mass spectra of peaks at RT of 13.983 (1), 14.092 (2), and 14.223 (3) min in the chromatogram of Indian saffron extract from the first silica gel band.
The last group of peaks in this band having similar mass spectra and fragmentation are peaks at RT of 15.814, 15.883, 15.991, and 16.043 min, these components most likely related to 1,2-benzenedicarboxylic acid-heptyl octyl ester (see Figure 24 and Appendix A4), the difference were in the number of carbons in R1 versus R2 as well as the structure shape.

Figure 24. Mass spectra of peaks at RT of 15.814 (1), 15.883 (2), 15.991 (3), and 16.043 (4) min in the chromatogram of Indian saffron extract from the first silica gel band.
Figure 25 shows the compounds that were identified in the second band ("orange band") of Indian and Iranian saffron. The chromatogram of this band is similar to that from SPE cartridges DSC-18 and DSC-CN cartridges that were eluted with methanol solvent (see Figure 11). The compounds identified were: 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.647 min) (Safranal) (26), 4-hydroxy-3,5,5-trimethyl cyclohex-2-enone (RT 7.717 min) (27), 2,6,6-trimethyl-1-carboxaldehyde (RT 8.507 min) (28), 2-isopropylidene-3-methylhexa-3,5-dienal (RT 8.759 min) (29), unknown (RT 16.907 min) (42), except two compounds were extracted in Iranian saffron, 3,5,5-trimethyl-2-cyclohexen-1-one (RT 5.829 min) (24), and 2,2,6-trimethyl-1,4-cyclohexanedione at(RT 6.350 min) (25). Additionally, the relative intensities for major compounds in Indian saffron were higher than Iranian saffron. The peaks at RT 6.647 and 16.907 min are both showing same mass spectra below m/z = 168. They were both identified by the mass spectral library as 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde. The compounds attributed to these to two peaks are obviously different. So similarity in the mass spectra may be due to the fact that the mass range for the GC-MS cannot accommodate higher molecular weight fragments from the mass spectrum of the component at RT of 16.907 min (42) (see Table 2).
Figure 25. GC-MS chromatograms of extracts of Indian and Iranian saffron from second of three bands separated on silica gel column.
Figure 26 shows GC-MS chromatograms of Indian and Iranian saffron extracts that eluted from the third band in the silica gel column. The identity of each peak was assigned as follows (see Table 2): 2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (RT 6.647 min) (26), 2-isopropylidene-3-methylhexa-3,5-dienal (RT 8.759 min) (29), hexadecanoic acid- methyl ester (RT 12.575 min) (38), 7-octadecenoic acid-methyl ester (RT 13.725 min) (39), eicosanoic acid-methyl ester (RT 15.054 min) (40) and, corticosterone (RT 19.894 min) (43).

**Figure 26.** GC-MS chromatograms of the methanol extracts of Indian and Iranian saffron of the third band from the silica gel column.
To the best of our knowledge, this is the first report of these compounds (38, 39, 40, and 43) in saffron. Peaks are observed at RT of 12.575, 13.725, 13.868, and 15.054 min. The similarity in the mass spectra of these peaks suggests that these compounds may belong are related in structure to hexadecanoic acid-methyl ester (see Figure 27 and Appendix A5).

**Figure 27.** Mass spectra of peaks at RT 12.575 (1), 13.725 (2), 13.868 (3), and 15.054 (4) min in the chromatogram of the methanol extracts of Indian saffron from the third silica gel band from the silica gel column.
Figure 28 shows the chemical structures of the new compounds in saffron extracts of saffron from the SPE and silica gel column separation and GC-MS analysis. These following compounds were identified and have not been previously reported to be in saffron: benzoic acid-methyl ester (2), 1-(2,5-dimethylphenyl)-ethanone (4), 2-isopropyl-5-methylhex-2-enal (8), (10-methylnonadecane) (12), 2,6,10-trimethyl-tetradecane (15), 9-octyl-heptadecane (18), n-butyl-benzenesulfonamide (19), 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde (30), 8-methyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthrene (35), hexadecanoic acid-methyl ester (38), 7-octadecenoic acid-methyl ester (39), eicosanoic acid-methyl ester (40), 1,2-benzenedicarboxylic acid-methyl ester (41), corticosterone (43). Different method of analysis may provide a pathway to distinguish between the similar peaks that were observed in the saffron band eluted by the silica gel column. To approach this analysis we choose to use liquid chromatography-mass spectrometry (LC-MS) that can separate, differentiate, and identify high molecular weight compounds. It is improbable to have more than one peak sharing the exact same mass spectra at different retention time; unless the compounds are closely related in structure. This relationship would suggest a higher molecular weight moiety, such as sugars or protein, thus having large molecular weight that is not observed in the GC-MS.
Figure 28. Chemical structures of compounds isolated from Indian and Iranian saffron that were not previously isolated by SPE and silica gel column and identified by GC-MS analysis.
4.2.2 Ultraviolet-Visible Spectroscopy (UV-Vis)

The electronic transitions in molecules may be determined by studying molecular absorption in the ultra-violet and visible (UV-Vis) spectrometral region, where molecules absorb or reflect radiation. It is reported that the UV-Vis absorbance spectra of saffron show three peaks that are assigned as follow: picrocrocin ($\lambda_{\text{max}}$ 250 nm), safranal ($\lambda_{\text{max}}$ 310 nm) and crocins ($\lambda_{\text{max}}$ 440 nm).62,63 The samples of saffron were dissolved in methanol and analyzed by UV-Vis spectroscopy. Figures 29 and 30 show the UV-Vis spectra of the first band “bright yellow” from extracts of Indian and Iranian saffron. Picrocrocin was observed at $\lambda_{\text{max}}$ 250 nm, and safranal was observed as a very small shoulder at $\lambda_{\text{max}}$ 310 nm. However, there was no peak at $\lambda_{\text{max}}$ 440 nm for the crocins.

Figure 29. UV-Vis spectra of the methanol extracts of the first band separated on silica gel column for Indian saffron.
Figure 30. UV-Vis spectrum of the methanol extracts of the first band separated on silica gel column for Iranian saffron.

Figures 31 and 32 illustrate the UV-Vis spectra of the second band from Indian and Iranian saffron extracted by methanol from the silica gel column, respectively. The absorbance band at $\lambda_{\text{max}} \sim 250$ nm is assigned to picrocrocin, while band observed at $\lambda_{\text{max}} \sim 310$ nm is assigned to safranal. In addition, the absorbance at $\lambda_{\text{max}} \sim 440$ nm in from saffron extracts in the second and third bands are assigned to crocins. In addition, the absorbance of picrocrocin and crocins in Indian saffron were much higher than Iranian saffron in the second band.
**Figure 31.** UV-Vis spectrum of the methanol extracts of the second band separated on silica gel column for Indian saffron.

**Figure 32.** UV-Vis spectrum of the methanol extracts of the second band separated on silica gel column for Iranian saffron.
Figures 3 and 4 demonstrate the UV-Vis spectra of the third band for Indian and Iranian saffron extracts that were separated on silica gel column, respectively. The absorbance of picrocrocin is observed at $\lambda_{\text{max}} \sim 250$ nm. The absorbance of safranal which observed at $\lambda_{\text{max}} \sim 310$ nm is decreased in the third band. Moreover, the absorbance observed at a $\lambda_{\text{max}} \sim 440$ nm is assigned to crocins in the third band. The relative absorbance of picrocrocin and crocins in the third band were higher than in the second band. The absorbance in third band of picrocrocin and crocins in Indian saffron was higher than Iranian saffron.

![Figure 33. UV-Vis spectrum of the methanol extracts of the third band separated on silica gel column for Indian saffron.](image-url)
4.2.3 Liquid Chromatography–UV-Mass Spectrometry (LC-UV-MS)

Liquid chromatography coupled with UV-Vis and mass spectrometry (LC-UV-MS) detectors were used to analyze the volatile and non-volatile compounds in saffron. LC-MS is an essential tool for analysis of compounds that have higher molecular weights, up to 3000 m/z, in saffron extracts. The technique is also useful in identifying peaks and making distinction between compounds that show similarities in their mass spectra. Moreover, LC-MS with a fraction collector attached, offer the advantage of isolating individual compounds, which allows for additional characterization with technique such as NMR and IR spectroscopy. The LC-MS-UV chromatograms of the saffron extracts

Figure 34. UV-Vis spectrum of the methanol extracts of the third band separated on silica gel column for Iranian saffron.
from three silica gel column bands in are shown in Figures 20, 25, and 26. The positive and the negative ions modes of the LC-MS analysis were used to conduct the mass spectral analyses on the three bands of saffron separated on silica gel column. Figures 35 and 36 show the chromatograms of extracts from the first “bright yellow” band of Indian and Iranian saffron separated by silica gel column, using both positive ion mass spectra and UV-Vis detectors (at two wavelengths, $\lambda_{\text{max}} = 250$ and 310 nm). The relative intensities for some peaks in the Indian saffron seem to be higher than Iranian saffron.

**Figure 35.** LC-UV ($\lambda=250$ nm (a), $\lambda = 310$ nm (b)) and LC-MS positive ion (c) chromatograms of extracts from first band in silica gel column of Indian saffron.
Figure 36. LC-UV (\(\lambda=250\) nm (a), \(\lambda=310\) nm (b)) and LC-MS positive ion (c) chromatograms of extracts from first band in silica gel column of Iranian saffron.

Figure 37 shows the mass spectrum of the compound with the peak at RT 23.0 min in the chromatogram of extract from first silica gel band of Indian saffron that was detected simultaneously at wavelengths 250 nm and 310 nm and with the MSD. This chromatogram peak has a mass spectral base peak of 149.0 m/z in addition to other ions with m/z at 189.0, 205.0, 245.1, 279.1, 301.2, 359.3, 409.2 and 429.2 m/z.
These ions are similar to those observed for the peaks in the GC-MS chromatogram of the same extract (see Figure 24). Moreover, no peaks were detected at $\lambda_{\text{max}} 440$ (see Figure 38) suggesting the absence of crocins. Figure 39 shows the overlay of negative ion mode mass spectra. It was observed that more compounds were ionized with the MS in the positive and in comparison to the negative ion mode.
Figure 38. LC-UV ($\lambda=440$ nm) chromatogram of methanol extracts from first band in silica gel column of Iranian (a) and Indian (b) saffron.
Figure 39. LC-MS negative ion chromatogram of extracts from first band in silica gel column of Iranian (a) and Indian (b) saffron.

The methanol extract of the second band from silica gel column was also analyzed by the LC coupled with MS and UV-Vis detector. Figures 40 and 41 show chromatograms of the second band that were measured at wavelength 250-310 nm along with MSD detector for Indian and Iranian saffron extracts.
Figure 40. LC-UV (λ=250 nm (a)-λ=310 nm(b)) and LC-MS positive ion (c) chromatograms of extracts from second band from silica gel column of Indian saffron.
The absorbance at wavelength 250 nm has major peak around 5 min compares with the same wavelength of the first band, while more peaks are absorbed at 310 nm compare to the first band. In addition, third band also measured at wavelength 440 nm.

Whereas, in Iranian saffron additional peaks were absorbed more than the Indian saffron at 440 nm between RT 13.5 min and 19.5 min in the second band, and in comparison
with the first band at the same wavelength 440 nm, no clear peaks were observed in first band (Figure 42). Figure 43 shows LC chromatograms (with MS in negative ion mode) methanol extracts of second band eluted from silica gel column for Indian and Iranian saffron. Clearly, observed in both saffron new peaks where present compared with the negative mode of the extracts from first band, as well as in positive ion mode (Figure 43).

Figure 42. LC-UV (λ=440 nm) chromatogram of methanol extracts from second band in silica gel column of Indian (a) and Iranian (b) saffron.
Figure 43. LC-MS negative ion chromatogram of extracts from second band in silica gel column of Indian (a) and Iranian (b) saffron.

The methanol extract of the third band from silica gel column was also analyzed by the LC coupled with MS and UV-Vis detector. Figures 44 and 45 show chromatograms of the third band that were measured at wavelength of 250-310 nm along with MSD detector for Indian and Iranian saffron extracts.
Figure 44. LC-UV ($\lambda=250$ nm (a)-$\lambda=310$ nm(b)) and LC-MS positive ion (c) chromatograms of extracts from third band from silica gel column of Indian saffron.
Figure 45. LC-UV ($\lambda=250$ nm (a)-$\lambda=310$ nm(b)) and LC-MS positive ion (c) chromatograms of extracts from third band from silica gel column of Iranian saffron.

The absorbance at wavelength of 250 nm decreased for the major peak around RT 5 min compares with the same wavelength of the second band. The absorbance at wavelength 310 nm have decreased in relative intensities for certain peaks are observed compare to the first and the second bands at the same wavelength, while the MSD detector observed some peaks have been ionized. Furthermore, the third band also were measured at wavelength 440 nm, peaks are absorbed in Indian extracts more than the
Iranian extracts. In Iranian saffron, two peaks observed at retention time between 13.5 min and 15.5 min, in comparison with Indian saffron (Figure 46). Figure 47 shows LC chromatograms (with MS and negative ion mode) methanol extracts of third band eluted from silica gel column for Indian and Iranian saffron. Clearly, a sharp peak around 6 min has been ionized along with others after retention time 15 min, in compared with the first band which no peaks show in the first 15 min, while the second band were observed more than one peaks, but not all.

![Figure 46](image)

**Figure 46.** LC-UV (λ=440 nm) chromatogram of methanol extracts from third band in silica gel column of Iranian (a) and Indian (b) saffron.
Figure 47. LC-MS negative ion chromatogram of extracts from third band in silica gel column of Indian (a) and Iranian (b) saffron.
CHAPTER V

CONCLUSION

This work demonstrates modern methods of separation and analysis that allows a number of compounds to be isolated from saffron based on a chosen cartridge as well as solvent. Four different support materials for solid phase extraction cartridges (DSC-NH₂, DSC-CN, DSC-MCAX, and DSC-18) were used with four different solvents to effect the separation of polar and non-polar compounds in saffron. Whereas results from SPE were comparable, yet selective, we decided to pursue the identification of the minor components that were excluded by using a silica gel column. The silica gel extraction results show clearly that there are three possible groups of these compounds that separated as three bands in the silica gel column. The compounds eluted in the second band from the silica gel column were similar to the major compounds found in the SPE samples. There were similarities in mass spectra of certain compounds among the three bands in silica gel column. In the solid phase extraction cartridges and silica gel column, we were able to identified compounds in saffron that were never before reported. These compounds are benzoic acid-methyl ester, 1-(2,5-dimethylphenyl)-ethanone, 2-isopropyl-5-methylhex-2-enal, (10-methylnonadecane), 2,6,10-trimethyl-tetradecane, 9-octyl-heptadecane, n-butyl-benzenesulfonamide, 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde, 8-methyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthrene, hexadecanoic acid-
methyl ester, 7-octadecenoic acid-methyl ester, eicosanoic acid-methyl ester, 1,2
benzenedicarboxylic acid-heptyl octyl ester, corticosterone. It was observed that peaks
for the extracts of all the three bands from the silica gel column in LC-MS in (negative
ions mode) compared to peaks in (positive ions mode). This suggests that more
compounds in saffron extracts can be identified and characterize by LC-MS with the MS
sin the negative ion mode.
Figure A1. Mass spectra of peaks at RT 6.647 min (1) in the chromatogram of Indian saffron extract and the library match (2).
Figure A2. Mass spectra of peaks at RT 11.854 min (1) in the chromatogram of Indian saffron extract and the library match (2).
Figure A3. Mass spectra of peaks at RT 13.983 min (1) in the chromatogram of Indian saffron extract and the library match (2).
Figure A4. Mass spectra of peaks at RT 15.814 min (1) in the chromatogram of Indian saffron extract and the library match (2).
Figure A5. Mass spectra of peaks at RT 13.868 min (1) in the chromatogram of Indian saffron extract and the library match (2).
REFERENCES


60. Sigma-Aldrich Co. 2003.

