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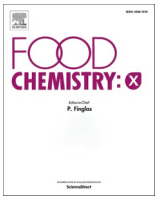

An overview of analytical methods employed for quality assessment of *Crocus sativus* (saffron)

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ABSTRACT

This paper reviews qualitative and quantitative analytical methodologies used for the appraisal of saffron quality, as the most expensive spice. Due to the chemical diversity of biologically active compounds of the *Crocus* genus, analytical methods with different features are required for their complete analysis. However, screening of the main components, such as carotenoids and flavonoids, appears to be sufficient for quality control, a more precise examination needs evaluation of minor compounds, including anthocyanins and fatty acids. High-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), ultraviolet–visible spectroscopy (UV), nuclear magnetic resonance spectroscopy (NMR), and thin-layer chromatography (TLC), are elementary and applicable methods in quality control analysis, whereas HPLC provides metabolite fingerprint and monitoring multi-compound instances at preparative and analytical levels. Combination approaches like metabolomics using different methods could classify saffron types, identify its adulterations, contaminants and provide a comprehensive metabolite map for quality control of selected compounds.

1. Introduction

Iridaceae family is usually well known due to the presence of the genus *Crocus* with 85-100 different species, especially *Crocus sativus* L., known as Saffron (or "Za'afran") for its yellow flowers, which is commercially cultivated and available for cuisine purposes (Abu-Izneid et al., 2022). In saffron, 150 phytochemical compounds, including volatile and non-volatile constituents, have been characterized. Volatile components are mostly terpenoids and terpene alcohols, and non-volatile elements consist of picrocrocin, crocetin, and crocins (Anjum, Pal, & Tripathi, 2015). Moreover, saffron is well-known for its intense yellow color due to the presence of crocin's derivatives, bitter taste related to picrocrocin, odor and aroma corresponding to safranal (a volatile compound) (Guijarro-Díez, Nozal, Marina, & Crego, 2015; Hashemzaei, Mamoulakis, et al., 2020; Hashemzaei, Rezaee, et al., 2020).

The stigma is the most usually used plant part of *C. sativus* that introduced as saffron. In traditional medicine, saffron has been used to treat dysmenorrhea, premature ejaculation, heart disease, and gastric ulcers and is recognized as a nerve tonic, aphrodisiac, and emmenagogue in Islamic Traditional Medicine. The stigma of saffron is known as a nerve sedative, immunity enhancer, stimulant, and aphrodisiac by Indian Traditional Medicine and is used against nervous system disorders, asthma, pertussis, and inflammations by Traditional Chinese Medicine (Mohtashami, Amiri, Ramezani, Emami, & Simal-Gandara, 2021). The stigma and style of saffron have been applied to treat insomnia and migraine and relieve toothache In Iraq and Spain, respectively (Günbatan, Gürbüz, & Özkan, 2016). Saffron has several pharmacological applications and used for treating blood pressure, dysmenorrhea, depression, inflammations, pain, gastric ulcers, cancer, age-related macular degeneration, premature ejaculation (Mohtashami et al., 2021), neurodegenerative disorders, bronchial asthma, anaphylaxis, urological infections, enlargement of the liver and pertussis (Abu-Izneid et al., 2022). It could be used as appetizer, stomach stimulant,

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expectorant, stimulant, adaptogenic agent and as a general tonic for increasing immunity (González, García-Barriuso, & Amich, 2010).

Saffron is commonly introduced as the most expensive medicinal spice due to its high request and low source. Approximately 225,000 handpicked stigmas or 75,000 crocus blossoms are essential for production of one pound of this single spice. In 2023, the market price for 1 kg of quality saffron spice exceeded between US$ 15.3 and US$ 37.5. The worldwide market for saffron is nearly $1 billion. Although the request for saffron in the Middle East and West is growing, the production has even reduced rapidly in numerous countries that have traditionally produced saffron and the quantity of globally produced dried saffron has continued constant at approximately 400 ton per year (Jiang, Cao, Yuan, Chen, Jin, & Huang, 2014; Torelli, Marieschi, & Bruni, 2014). Furthermore, due to its high market value, demanding production, apparent value, and premium price, attempts have been made to adulterate saffron with numerous substances with similar synthetic color and plants. Increasing the weight with foreign matter, and enhancing the color with natural or synthetic colorants, have turned saffron into the most adulterated spice. In adulterated saffron, various plant substances like safflower (*Carthamus tinctorius* L.), turmeric (*Curcuma longa* L.), *Crocus vernus* L. stigmas, and *Calendula officinalis* L., gardenia fruits extract despite the difference in pigments, it is added to saffron because of the similarity in color and morphology. Even safflower petals (as an additive in saffron) become redder and brighter by adding quinochalcone C-glycosides (Kilic Buyukkurt, Guclu, Barutcular, Selli, & Kelebek, 2021). Various analytical methods introduced for detection and quantification of target compounds and adulterants in saffron include UV–Vis (García-Rodríguez, López-Córcoles, Alonso, Pappas, Polissiou, & Tarantilis, 2017) NMR (Dowlatabadi et al., 2017; Petrakis, Cagliani, Polissiou, & Consonni, 2015), HPLC (Suchareau, A. Bordes, & L. Lemée, 2021; Vahedi, Kabiri, Salami, Rezadoost, Mirzaie, & Kanani, 2018) and Liquid chromatography–mass spectrometry (LC–MS) (Pittenauer, Rados, Tsarbopoulos, & Allmaier, 2019).

Due to the high added value of saffron as a spice and its health benefits, the review of applicable analytical methods that are used to evaluate the quality control of saffron considering their geographical origin is important.

2. Methodology

To collection relevant studies, databases PubMed, Scopus, and Google Scholar were searched for the terms “saffron”, “*Crocus sativus*”, “analytical method”, “metabolomics”, “HPLC”, “LC-MS”, “NMR”, “GC”, “GC–MS”, “UV”, “TLC”, “Gas chromatography-mass spectrometry”, “Thin layer chromatography”, “High-performance layer chromatography”, “Nuclear magnetic resonance”, and “Liquid chromatography-mass spectrometry” for reports published from 2014 till June 2023.

3. Chemical compositions

Phytochemical investigations have discovered that the stigmas of the saffron flower comprise a number of chemical constituents counting carbohydrates musilage, pigments, minerals, vitamins B1 & B2 and, crocin, crocetin, carotene, lycopene, α-, and β-carotene, zeaxanthin. Main flavonoids and their derivatives present in *C. sativus* are kaempferol, dihydrokaempferol, astragalin, vitexin, isoorientin, naringenin, orientin, populin, myricetin, quercetin, and rhamnetin that responsible for its antinociceptive, anti-inflammatory and blood pressure lowering effects (Wani, Hamza, & Mohiddin, 2011). Moreover, hydroxycinnamic acids specifically gallic acid, caffeic acid, methylparaben, chlorogenic acid, and pyrogallol have been isolated from saffron (Abu-Izneid et al., 2022).

Specific aroma, flavor, and color are three attractive characteristics of saffron that have made it a valuable food-grade flavoring and coloring agent in the food industry. The major bioactive compounds in saffron are crocin, crocetin, picrocrocin, and safranal. Crocetin and crocin as main apocarotenoids are present in stigmas of in *C. sativus*. Both compounds produce the unique color (golden yellow-orange color) of saffron. This metabolite has two esterified gentiobioses which making it the favorite and ideal coloring agent for non-fatty foods. Crocin ($C_{44}H_{64}O_{24}$, MW: 976.96 g/mol) is a unique hydrophilic carotenoid existing in saffron with high solubility in water related to these sugar moieties that responsible for golden yellow to red color of saffron. This water-soluble compound has five analogs including crocin-1, crocin-2, crocin-3, crocin-4, and crocin-5. Among them, crocin-1 (a-crocin) with abundancy of 10 %, is frequently compound in dry saffron. This compound could be found up to 30 % depending on kind of cultivar, origin, harvesting and processing states. Also, crocin acts as an antioxidant and protected cells and tissues against oxidation by scavenging free radicals (Melnyk, Wang, & Marcone, 2010; Samarghandian & Borji, 2014). Crocetin ($C_{20}H_{24}O_4$, MW: 328.4 g/mol) having the structure of olefin acid, and approximately finding in form of glycoside molecules (crocin: 94 %) while the free crocetin is 6 %. The special therapeutic effects of this compound are anti-inflammatory, cardio-protective, neuro-protective, anti-tumour, and anti-oxidative properties (Bhandari, 2015; Ohba et al., 2016). Aqueous methanol, ethanol, or water is commonly used for the extraction of many bioactive constituents that crocin and crocetin esters are separated with water.

There are about more than 160 volatile compounds have been identified in saffron, however, nothing emphasize about which volatiles are responsible for saffron aroma. Based on the results of GC-olfactometry method, flavor and bitter taste of saffron related to picrocrocin while safranal responsible for specific aroma of saffron. The diversity of presented compounds related to aroma, except of condition and geographical properties, could be associated to sugar and lipid units’ degradation. For example, safranal and D-glucose produce during harvesting and drying of stigmas’ saffron, from picrocrocin degradation. The neuropsychological effects of safranal have been specially investigated and it is considered as an important therapeutic agent (Rezaee & Hosseinzadeh, 2013). Picrocrocin is a colorless glycoside (β-D-glucoside of hydroxysafranal) which is produced from zeaxanthin degradation and in addition, it is converted to safranal compound due to high temperature during drying process. This compound is the second most predominant compound in scale of weight. Picrocrocin can be effectively extracted from saffron stigma by Soxhlet extraction through light petroleum, diethyl ether, and methanol to yield three fractions that the diethyl ether phase containing picrocrocin (Lage et al., 2015). Furthermore, its high-water solubility due to presence of crocin is one of the reasons for widely application of saffron as a colorant in food and medicine. Saffron is applied as a colorant and flavoring agent in sausages, margarine, butter, cheese in the food industry.

4. Extraction methods

Various extraction procedures have been employed for saffron analysis based on the chosen analytical method. Among these procedures, releasing the desired components from samples through disrupting tissue followed by extracting the desired compounds by liquid–solid or liquid–liquid extraction is common. Also, steam distillation, simultaneous distillation–extraction (SDE), simultaneous distillation–extraction (SDE), solvent-assisted flavor evaporation (SAFE), micro simultaneous hydrodistillation extraction (MSDE), solid phase microextraction (SPME), and vacuum headspace (VHS) followed by GC and GC–MS are used to extract volatile components (Farhadi, Iranshahi, Taghizadeh, & Asili, 2020; M. Jiang, Kulsing, Nolvachai, & Marriott, 2015; Kosar, Demirci, Goger, Kara, & Baser, 2017; Mohan, Negi, Melkani, & Dev, 2011). In addition, among non-volatile compounds, safranal, and *cis/trans* crocin are the main components of saffron which have been studied by different extraction and analytical methods. However, it is well known that some bio-active compounds (such as apocarotenoids) may be decomposed by rapid degradation and isomeric conversion during analysis. In this regard, multiple solvent systems for extraction have

Table 1
The main components of Saffron.

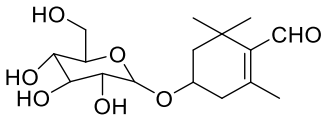
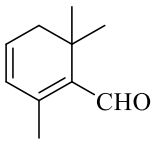
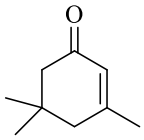
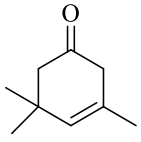
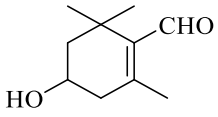
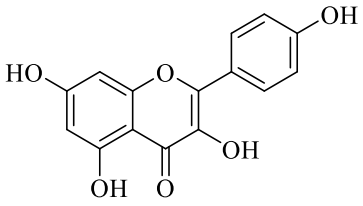
No	Compound	Structure	Class	Plant parts	Ref
1	Picrocrocin		monoterpenoids	stigma	(Sabatino et al., 2011b)
2	Safranal		monoterpenoids	corn	(Sabatino et al., 2011b)
3	α-isophorone		monoterpenoids	flower	(Griggs, Kursinski, & Akos, 2014)
4	β-isophorone		monoterpenoids	flower	(Griggs et al., 2014)
5	HTCC: 2,6,6-trimethyl-4-hydroxy-1-carbox-aldehyde-1-cyclohexene		monoterpenoids	flower	(Corti, Mazzei, Ferri, Franchi, & Dreassi, 1996)
6	Kaempferol		flavonoids	petal	(Giordano, Coletta, Rapisarda, Donati, & Rotilio, 2007; Adil Farooq Wali et al., 2020)

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Table 1 (*continued*)

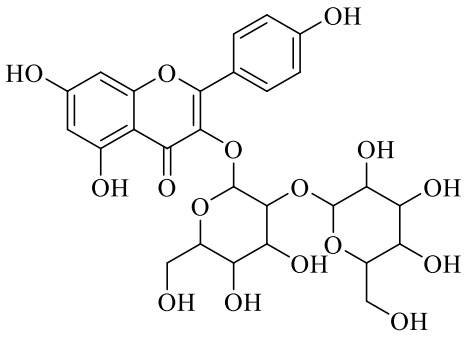
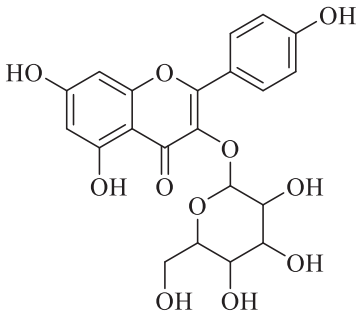
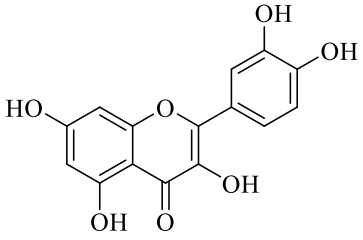
No	Compound	Structure	Class	Plant parts	Ref
7	Kaempferol-3-Osophoroside		flavonoids	tepal	(Luo, Zhao, Tang, Wang, Liu, Ma, 2018a)
8	Kaempferol-3-O-glucoside		flavonoids	stamen, tepal	(Reddy et al., 2020)
9	Quercetin		flavonoids	tepal	(Giordano et al., 2007; Adil Farooq Wali et al., 2020)

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Table 1 (*continued*)

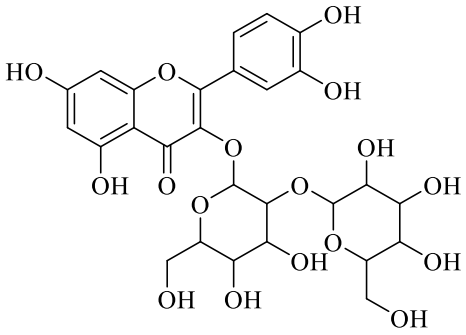
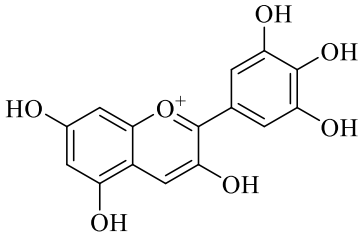
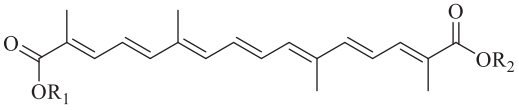
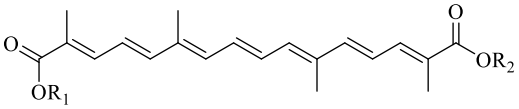
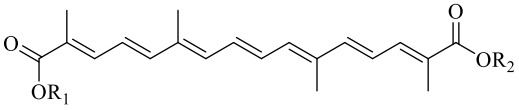
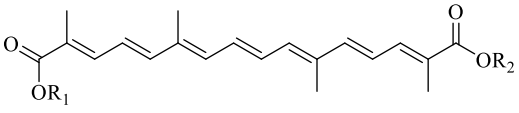
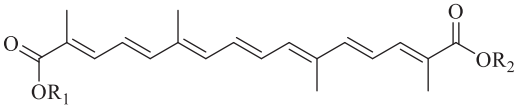
No	Compound	Structure	Class	Plant parts	Ref
10	Quercetin-3-O-sophoroside		flavonoids	stamen, tepal	(Reddy et al., 2020)
11	Delphinidin		anthocyanins	tepal	(Giordano et al., 2007; Adil Farooq Wali et al., 2020)
12	Crocetin		carotenoids	corn, stigma	(Verma & Middha, 2010b)
13	Crocin-1	$R_1 = R_2 = H$ 	carotenoids	tepal, stigma	(Chryssanthi, Lamari, Georgakopoulos, & Cordopatis, 2011)
14	Crocin-2	$R_1 = R_2 =$ β-D-glycosyl 	carotenoids	tepal, stigma	(Carmona, Zalacain, Sánchez, Novella, & Alonso, 2006)
15	Crocin-3	$R_1 =$ β-D-glucopyranosyl, $R_2 =$ β-gentiobiosyl 	carotenoids	stigma, flower	(Chryssanthi et al., 2011)
16	Crocin-4	$R_1 =$ β-D-glycosyl, $R_2 =$ β-gentiobiosyl 	carotenoids	stigma	(Chryssanthi et al., 2011)
		$R_1, R_2 =$ β-gentiobiosyl			

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Table 1 (*continued*)

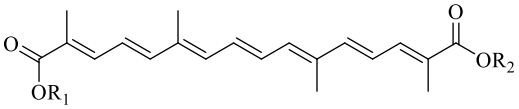
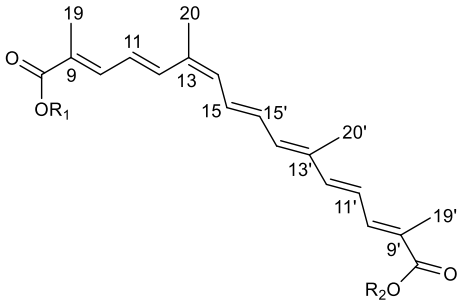
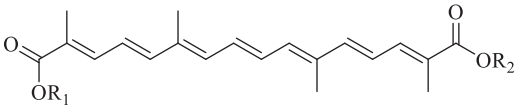
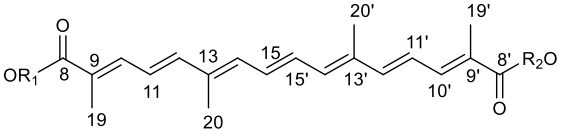
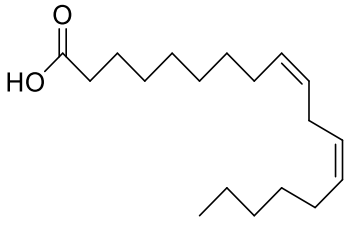
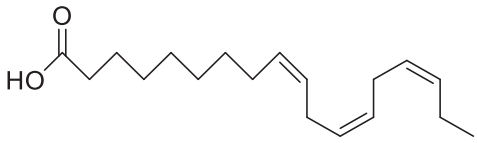
No	Compound	Structure	Class	Plant parts	Ref
17	Crocin-5		carotenoids	stigma, tepal	(Chryssanthi et al., 2011)
18	*cis*-digentiobiosycrocetin	R_1 = β-D-neapolitanose, R_2 = β-D-glucosyl, 	carotenoids	flower	(Van Calsteren et al., 1997)
19	Dimethyl crocetin	R_1 = β-D-gentobiosyl, R_2 = β-D-glucosyl 	carotenoids	stigma	(Roberto Consonni, Stella A Ordoudi, Laura R Cagliani, Maria Tsiangali, & Maria Z Tsimidou, 2016)
20	*trans*-digentiobiosyl crocetin	R_1 = R_2 = methyl 	carotenoids	stigma	(Assimiadis, Tarantilis, & Polissiou, 1998)
21	Linoleic acid	R_1, R_2 = β-D-gentobiosyl, β-D-glucosyl R_1,R_2 = β-D-gentobiosyl 	fatty acid	leaves	(Sobolev et al., 2014)
22	linolenic acid		fatty acid	leaves	(Sobolev et al., 2014)

Table 2
Analytical method described for detecting metabolites in Saffron.

Area	Analytes	Analytical method			Ref.
		Methods	Extraction protocol	Detection system	
Ukraine	Crocin, picrocrocin, safranal	HPLC-DAD	Stigma samples ground in liquid nitrogen and extracted with 50 % methanol	ACE C18 column (250 × 4.6 mm, 5.0 μm), UV: 250, 310, and 440 nm	(Mykhailenko et al., 2021b)
Italia	Crocetin, safranal, picrocrocin, and kaempferol	HPLC-DAD	Ground sample extracted with a water-methanol 1:1 v/v and stirring for 1 h	250 × 4.6 mm Kinetex C18 water (A) and acetonitrile (B) flowing rate 1.0 mL/min. 95 % A to 5 % A (30 min); 5 % A to the initial composition (5 min)	(Biancolillo, Maggi, De Martino, Marini, Ruggieri, & D'Archivio, 2020)
Markets in Spain	Crocin, crocetins and picrocrocin	HPLC	A 100 mg + 5 mL of cold ethanol 80 % (v/v). Centrifuged at 5000 g for 10 min and cleaned with 5 mL of ethanol.	Sep-Pak C_{18} Mobile Phase: 20 to 80 % (v/v) acetonitrile: water	(Moratalla-LÃ3pez, Bouhadida, Bagur, GarcÃa-RodrÃguez, Oueslati, & Alonso, 2016; Sujata, Ravishankar, & Venkataraman, 1992)
Ukrainian	Picrocrocin, safranal, and crocin	HPLC	0.01 g of water and hydroethanolic sample was extracted with 10 mL of methanol for 30 min. 10 μL was inserted into the HPLC system.	ACE C18 column (250 mm × 4.6 mm, 5.0 μm) Mobile phase: 0.1 % acetic acid in water and acetonitrile	(Olha Mykhailenko, Ivan Bezruk, Liudas Ivanauskas, & Victoriya Georgiyants, 2021; Mykhailenko, Petrikaitė, et al., 2021)
Lebanon	Picrocrocin, safranal, crocin, and crocetin	HPLC	The saffron extracted by methanol and water (50:50, v/v) over 24 h. The solvent evaporated at 40 °C.	ACE C18 column Mobile phase: water/methanol (50:50, v/v)	(Nassar et al., 2020b)
China	Picrocrocin, kaempferol-3-Osophoroside, and safranal	HPLC	The powdered sample (10.0 mg) was added to 5.0 mL of 50 % ethanol. The sample provided in an ultrasonic bath for 20 min (100 W, 40 kHz) at 25 °C in the dark.	Agilent C18 (250 mm × 4.6 mm i.d., 4 μm) Mobile phase: water and acetonitrile	(Liu et al., 2018; Tong et al., 2015)
Iran, Himalayan region	Trans-4-GG-crocin, *trans*-3-Ggcrocin, *cis*-4-GG-crocin, *trans*-2-gg-crocin, and *trans*-crocetin	HPLC	The sample extracted with ethanol–water at 40 °C for 3 h. The extract was kept in an amber-colored bottle below −4 °C after distillation.	Spherisorb RP C18 column Mobile phase: 0.1 % formic acid in water and acetonitrile	(Girme et al., 2021)
Italy	Crocetin (β-D-triglucoside)-(β-D-gentibiosyl) ester, trans and *cis* isomers of crocetin (β-D-neapolitanose,)-(β-D-glucosyl,) ester, and *cis* crocetin (β-D-neapolitanose,)-(β-D-gentibiosyl)	HPLC	A 100 mg + 20 mL of methanol (v/v). Centrifuged at 5000 g for 10 min and washed with 5 mL of methanol and directly analyzed by HPLC.	XDB-C18 and Phenomenex Luna C18 column Mobile phase: water (or acidified with 0.25 % formic acid) and acetonitrile	(Cusano et al., 2018)
Italy	Trans-4-GG, trans-3-Gg and trans-2-G	HPLC	20 mg of powdered plant + 10 mL of water (H_2O) and motivated in the shade for 60 min, filtered through a 0.45 μm, 25 mm PTFE syringe filter.	Luna C18 column Mobile phase: acetonitrile and 0.25 % formic acid in water	(Cusano et al., 2018)
Italy, Iran	Trans-crocin 4 and *trans*-crocin 3	HPLC	Saffron stigmas (50 mg) + 10 mL of 70 % ethanol, attuned to pH 2.0 with formic acid.	Luna C18 column Mobile phase: 90 % H_2O up to 100 % CH_3CN	(Alessandra Biancolillo, Maggi, Martino, Marini, Ruggieri, & D'Archivio, 2019)
Italia	Crocin, picrocrocin, crocetin, safranal, kaempferol-3-O-sophor oside, kaempferol-3-O-glucoside, and quercetin-3-O-sophoroside	HPLC	A 50 mg of sample extracted by methanol and water. 700 μL of the extract (methanol/water = 3:1, precooled at −40 °C) comprising an internal standard (2-chloro-DL-phenylalanine, 1 μg/mL) was added.	RP Kinetex® C18 column Mobile phase: MeOH in H_2O	(Mottaghipisheh et al., 2020)
USA	Crocetins and safranal	HPLC/ UHPLC-PDA-MS (QToF)	5 mg of powder extracted with 1 mL of methanol: water (1:1), then sonicated for 30 and centrifugation (10 min, 5000 rpm)	Gemini C18 column (150, 4.6-mm I.D.; 5-μm) Mobile phase: water (A) and acetonitrile (B), (0.1 % formic acid in both solvents): 0 min, 90 % A: 10 % B (15 min) to 15 % A:85 % B to 100 % B in (2 min); flow rate: 1.0 mL/min.	(Avula, Katragunta, Wang, Upton, & Khan, 2022b
Italy	773.21: 771.65 (Kaempferol 3-O-sophoroside-7-O-glucoside), 627.15: 625.14 (Quercetin 3-O-sophoroside) 627.15: (Delphinine 3,5-di-O-glucoside)	LC–ESI (HR) MS^n	0.2 M phosphoric acid (A), and acetonitrile (B) flow rate: 1.0 mL/min. 100 % (A) to 85 % in 20 min; to 65 % in 40 min; to 10 % in 50 min.	Gemini C18 column (150 × 4.60 mm, 3 μm, Phenomenex)	(Tuberoso, Rosa, Montoro, Fenu, & Pizza, 2016)
Greece	287 (Kaempherol- tri-hexoside), and 303(Quercetin −3,4-di-O-glucosid)	LC-DAD/MS	water acetic acid (0.2 %) (A), and acetonitrile (B): 5–30 % B (0–74 min), 30–60 % B (74–84 min), 60–90 % B (84–94 min).	Supelco Discovery HS C18 column (25 cm × 4.6 mm × 5 μm) operated (35°C)	(Kakouri, Daferera, Paramithiotis, Astraka, Drosinos, & Polissiou, 2017)
Iran	311, 473, 491, 675 (Trans-4-GG crocin)	HPLC-DAD-MS	Water 0.1 % formic acid acetonitrile and formic acid(A) acetonitrile (B)	Sunfire C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) at 30 ∘C.	(Marlène Suchareau, Alexandra Bordes, & Laurent Lemée, 2021)
Spain	[M-163-H] - kaempferol 3-O-glucoside, [M-173-H] $^-$ kaempferol 3-O-sophoroside	LC/MS QTOF	Water and 0.1 % formic acid (A) ammonium formate in acetonitrile (B),5%B to 95 %B in 33 min and backed to starting conditions in 1 min	100 × 2.1 mm, 0.5 μm thick porous shell particle size of 2.7 μmon stationary phases (C18, Cyano	(Miguel Guijarro-Díez, Leonor Nozal, María Luisa Marina, & Antonio Luis Crego, 2015)

been optimized with supercritical carbon dioxide and ultrasonication-assisted extraction at room temperature to prevent structure disturbing (Girme et al., 2021). However, common solvent extraction methods in dark conditions with common solvents, including distilled water, cold ethanol, methanol–water, and hexane (García-Rodríguez, Moratalla-López, López-Córcoles, & Alonso, 2021; Mykhailenko, Ivanauskas, Bezruk, Marksa, Borodina, & Georgiyants, 2022; Mykhailenko, Petrikaitė, et al., 2021).

5. Identification methods

HPLC instrument was done on a KNAUER System equipped with a binary wellchrome K1001, a multiple wavelength UV–Vis (DAD)-2800 model for determination of crocetin in saffron. The column used for isolation was a KNAUER Eurospher RP C18 (16 × 250 144 mm × 10 μm), and detection at 427 nm using PDA detector with flow rate 1 mL/min and injection volume of 10 μL. The mobile phase is (i) acetonitrile (A) and 0.1 % v/v formic acid in water (B) and (ii) MeOH in H_2O (Kabiri, Rezadoost, & Ghassempour, 2017; Reddy, Bharate, Vishwakarma, & Bharate, 2020). Moreover, the separation of crocetin ester extract was performed on a Merck Hitachi Elite LaChrom HPLC (Merck Hitachi, Tokyo, Japan) using a solvent system with gradient elution under the following conditions: solvent A, ultrapure water/0.05 % formic acid; solvent B, methanol/0.05 % formic acid; separation column, polar endcapped Waters XTerra MS C8 (Waters, Milford, MA, USA); particle size 3.5 μm, column dimension 2.1 mm × 150 mm; column oven temperature, 40 °C; flow rate, 200 μL/min and gradient elution from 30 to 95 % solvent B in 45 min and then additional 10 min isocratically at 95 % solvent B before returning to the start conditions (Pittenauer et al., 2019; Suchareau et al., 2021). In another study, crocetin and its isomer was detected by XDB-C18 analytical column (4.6 × 250 mm, 5 lm particle size; Agilent Technologies, Santa Clara, CA) operating at 25 °C. Separation was carried out by using gradient elution with a mixture of water(A): acetonitrile (B) (30–70 % B in 20 min) at a flow rate of 0.9 mL/min (Nescatelli et al., 2017).

Crocin and isomers including *trans*-4-GG-crocin, *trans*-3-Ggcrocin, *cis*-4-GG-crocin, *trans*-2-gg-crocin, and *trans*-crocetin detected by HPLC-DAD analysis that carried out on a Shimadzu SPD-M20A HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with a Shimadzu SPD-M20A photodiode array detector, an on-line degasser unit (Shimadzu DGU-20A5R), a column oven (Shimadzu CTO-20AC column oven) and autosampler (Shimadzu SIL-20ACHT) using a RP Kinetex® C8 column (5 μm, 100 Å, 150 × 4.6 mm, Phenomenex, Torrance, USA) at 30 °C. Chromatographic elution of the samples was accomplished with a gradient solvent system by changing the ratio of MeOH in H_2O (containing 0.066 % of H_3PO_4) and the mobile phase comprising 0.1 % formic acid in water and acetonitrile. The samples were monitored in the UVmax of the standards 440 nm (Girme et al., 2021; Mottaghipisheh et al., 2020). In another study, for detection of crocin using the HPLC analyses that performed on an Agilent 1260 instrument equipped with a DAD and an autosampler with a Zorbax Sb C18 column (250 × 4.6 mm i.d., 5 μm) (Agilent Technologies, USA) at 30 °C. The mobile phase consisted of water (A) and acetonitrile (B) and the injection volume was 10 μL, the flow rate was 1.0 mL/min (Liu et al., 2018; Y. Tong et al., 2015).

For detection of safranal and picrocrocin, HPLC-DAD were done using a Shimadzu Nexera X2 LC-30 AD HPLC system that chromatographic separation was performed using an ACE C18 column (250 × 4.6 mm, 5.0 μm). The separation of safranal was attempted using the following two methods: (i) a gradient run from 20 to 80 % (v/v) acetonitrile in water in 20 min at a flow-rate of 0.5 mL/min (ii) an isocratic run with 76 % (v/v) acetonitrile in water at a flow-rate of 0.5 mL/min, detection at 308 nm. The isolation of picrocrocin was performed by (i) 0.1 % acetic acid in water and acetonitrile (ii) water/methanol (50:50, v/v) at 250 nm (Mykhailenko et al., 2021a; Mykhailenko, Petrikaitė, et al., 2021; Nassar et al., 2020a). Recently, electrospray ionization mass spectrometry (ESI-MS) and time-of-flight mass spectrometry (QTOF- MS) detectors among mass hyphenation procedures, have been applied for saffron analytical assays and MS^n analyzers are considered a useful tool due to MS^n capabilities for high throughput of structural analysis (Avula, Katragunta, Wang, Upton, & Khan, 2022a; Luo, Zhao, Tang, Wang, Liu, & Ma, 2018) and it has been used as a quantitative approach for saffron compounds (Suchareau et al., 2021). This method based on [M-H] signals, consists of two protocols for flavonoids and glycosides (e.g., kaempferol, delphinidin, quercetin, and their glycoside derivatives) and for apocarotenoid compounds (e.g., crocetin esterified with their glycoside derivatives) identification in saffron (Wali et al., 2020; Zhang et al., 2010). As shown in Table 2, the MS and MS^n fragmentations are performed in negative and positive modes to evaluate the synthesis pathway of the main compounds of saffron (Al-Madhagy, Mostafa, Youssef, Awad, Eldahshan, & Singab, 2019; Felipe, Brambilla, Porto, Pilau, & Cortez, 2014). In the MS spectrum, the primary recorded fragmentations for kaempferol -triglucoside derivatives in negative mode are MS: [771.1811] [M-H], [609.1387] [M-162-H], MS^3: [609.1386] [M-162-H], [447.0854] [M-324-H], [285.0320] [M-486-H], and [284.0263] [M-487-H], but [773.2076] $[M+H]^+$, [611.1546] $[M-162+H]^+$, [449.1063] $[M-324+H]^+$ and M^3 [611.1543] $[M-162+H]^+$, [449.1049] $[M-324+H]^+$, and [287.0531] $[M-486+H]^+$ in positive mode (Guijarro-Díez, Castro-Puyana, Crego, & Marina, 2017; Guijarro-Díez et al., 2015). Crocetin has been found as two isomers (*cis* and *trans*) with different glycoside linkage molecules, creating orange-to-yellow colors. So, identification and quantification of this compound seem to be essential for saffron quality control. Among the analytical method, LC-MS and LC-MS/MS have provided accurate mass for protonated or deprotonated corocetin and derivatives with high resolution in a single run. Based on LC-MS studies before 2015, the main fragmentations for corocetin in negative mode are MS: [327 327] [M-H], and M^3: [283] [M-COOH], [239] [M-COOH-COO], and [165] [M-$C10H11O_2$]. Likewise, these fragmentations for crocin E are MS: [489] and M^3: [489] (M-H), [327] [M-C6H11O5], [323] [M-C6H11O5-3H], [283] [M-C6H11O5-COO], and [324] [M-C6H11O5-2H] (Verma & Middha, 2010a).

Based on GC–MS essential oil studies, safranal, α-isophorone, and β-isophorone are the main constituents of *C. sativus* (Table 1). Furthermore, in other species, (E)-p-menthan-2-one, *ar*-turmerone, hexanol, nonanal, 1-octadecanol acetate, pentadecanoic acid, and 5-methyl-2-furancarboxaldehyde were detected (Mohtashami et al., 2021). Headspace extraction-GC–MS spectrometry (HS-GC/MS) is a valid and new method that was evaluated to determine the saffron aroma in cured ham, by Gómez-Sáez et al. Based on the results safranal was the main compound, and could be of interest to food industries (Gómez-Sáez, Moratalla-López, Lorenzo, Vergara, & Alonso, 2021). Recently, hyphenated GC methods, which yield higher-quality data for 1D GC, have grown. GC × GC method with high-resolution and the ability to enhance the signal-to-noise ratios could provide a new avenue to improve the identification of low-abundance and co-eluting compounds that appear in 1D GC method (Jiang et al., 2014). Furthermore, the new GC–MS-olfactometric technique has demonstrated the aroma constituent in many aromatic samples, especially in saffron, and recognized many compounds, including 2,3-butanedione, hexanal, and (E)-2-nominal (Amanpour, Sonmezdag, Kelebek, & Selli, 2015).

Crocin, picrocrocin, and crocetins were determined on TLC plates in a solvent system of *n*-butanol-acetic acid–water (4:1:1 v/v), and safranal was spotted on TLC plates in hexane–ethyl acetate (9:1 v/v). The four crocetins recognized at RF values of 0, 0.41, 0.75, and 0.98 were totally repotted as crocetin. Crocin and crocetins were observable in white light, while picrocrocin was identified at 254 nm as a dark brown brilliant spot in *n*-butanol-acetic acid–water (4:1:1 v/v). Crocin derivatives, *trans*-Crocin, *trans*-Crocin 3-*trans*-Crocin 4, *cis*-Crocin 4/*trans*-Crocin 2, picrocrocin, *cis*-Crocin 4/*trans*-Crocin 2, HTCC/3-Gentiobiosyl-kaempferol, *cis*-Crocin 2, spotted on TLC plates and saturated with a mobile phase (1-butanol, acetic acid and distilled water (4:1:1 v/v) at room temperature, had Rf values of 0.19, 0.29, 0.43, 0.56, 0.63, 0.80,

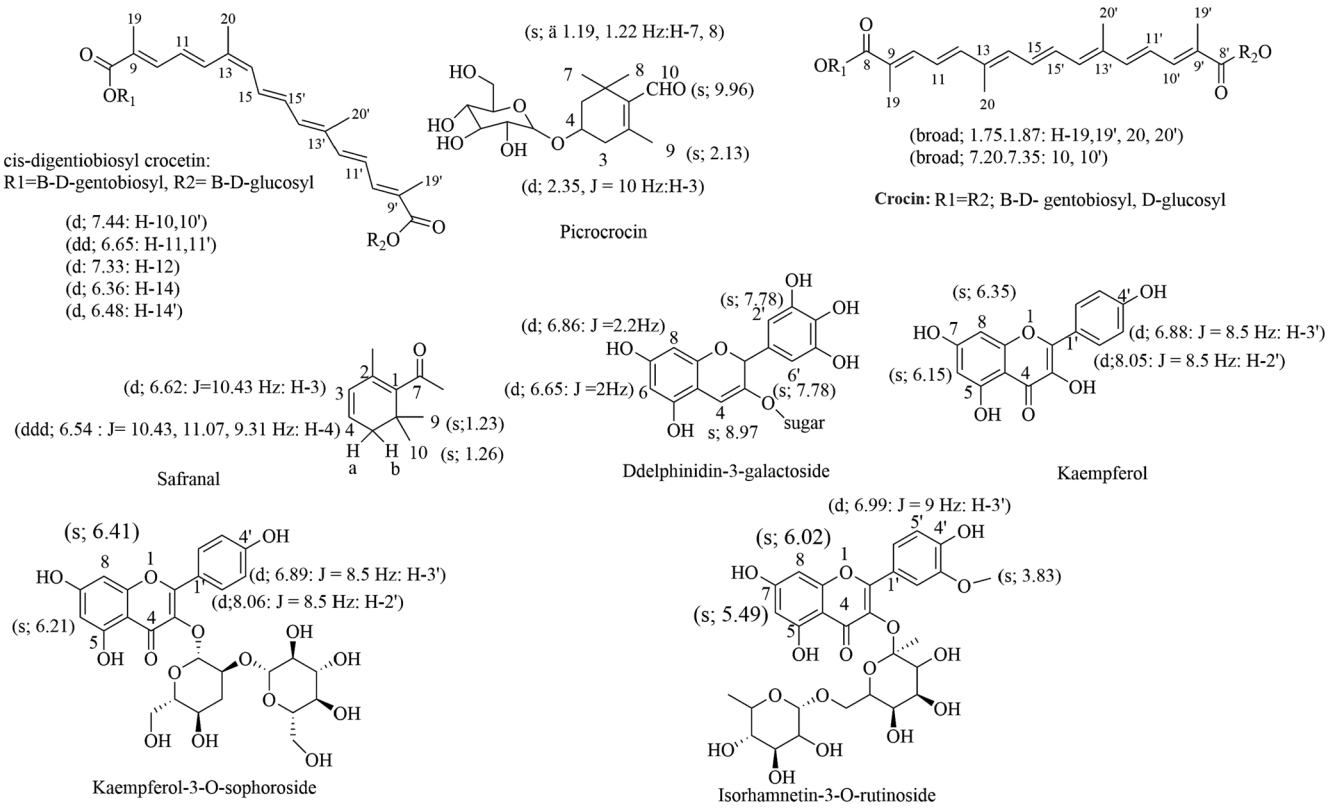


Fig. 1. The structure elucidation of saffron compounds by NMR.

and 0.96, respectively (Sereshti, Poursorkh, Aliakbarzadeh, & Zarre, 2018).

The ISO 3632 which sets standards for dried saffron quality, recommends the use of UV spectrophotometer at 250, 310, and 440 nm respectively for detection of picrocrocin, safranal, and polar dyes and pigments (Sabatino, Scordino, Gargano, Belligno, Traulo, & Gagliano, 2011a). In recent studies, both *trans*- and *cis*-crocins, placed around 440 nm, display a secondary absorption at 260–264 nm, and the *cis*-crocins exhibit an extra peak at 326–327 nm (Cusano, Consonni, Petrakis, Astraka, Cagliani, & Polissiou, 2018; Hegazi, Khattab, Frolov, Wessjohann, & Farag, 2022). Kaempferol derivatives, the main flavonoids of saffron, are observed at 265–330 nm (D'Archivio & Maggi, 2017). Additionally, crocetin esters display absorption at 324–327 nm (only *cis*-crocins) and 250–260 nm (both *cis*- and *trans*-isomers) (A. Biancolillo, Foschi, & D'Archivio, 2020).

NMR-based metabolite fingerprinting may recognize alterations by comparing reliable and adulterated products (Eghbaliferiz, Soheili, Tayarani-Najaran, & Asili, 2019; Petrakis et al., 2015; Reyhani, Iranshahi, Taghizadeh, Saberi, & Farhadi, 2022). The NMR fingerprinting of picrocrocin, crocins, all-*trans*-digentiobiosyl crocetin, 13- *cis*-digentiobiosyl crocetin, safranal, Kaempferol, Kaempferol-3-Osophoroside, Delphinidin-3-galactoside is displayed in Fig. 1. The other metabolites identified in ^{1}H spectrum of saffron are linoleic acid: 2.33 (CH_2-2), 1.61 (CH_2-3), 1.34 (CH_2-4), 2.07 t (CH_2-8,14), 5.34 (CH-9,10,12,13), 2.78 t (CH_2-11), 1.32 (CH_2-15,16,17), 0.91 t (CH_3-18) and linolenic acid 1.34 (CH_2-1,4,5,6,7), 2.33 (CH_2-2), 1.61 (CH_2-3), 2.08 t (CH_2-8), 5.34 (CH-9,10,12,13), 2.81 t (CH_2-11,14), 5.30 (CH-15), 5.38 (CH-16), 2.08 t (CH_2-17), and 0.97 t (CH_3-18) (Sobolev et al., 2014).

6. Metabolomics

Nowadays, certain metabolites of commercial natural products are approved by spectrophotometric methods based on ISO3632. In this procedure, the value of saffron is approved based on the quantification of picrocrocin, safranal, and crocins at their λmax of 250, 310, and 440 nm, respectively. However, the detection of adulteration, differentiation among geographical areas, and production and maintenance times of saffron by this procedure face a high potential for error. Analytical techniques, including spectroscopic, chromatographic, and electrophoretic, are able to illustrate the particular patterns of complex metabolites in saffron. Therefore, its fingerprint analysis by spectroscopic procedures such as UV–Vis, NMR, near/mid-infrared spectroscopy (N/MIR), and chromatographic methods like GC–MS, HPLC, and UHPLCESI/QTOF-MS are mighty comprehensive approaches for evaluation, classification, authentication, qualification, and stability assessment of different compounds of saffron except for picrocrocin, safranal, and crocin (M. A. Farag, N. Hegazi, E. Dokhalahy, & A. R. Khattab, 2020; Guijarro-Díez et al., 2017; Samaha, Chahine, Sobolev, Menghini, & Makhlouf, 2021; Senizza et al., 2019). The complexity of the extract(s) of the saffron samples matrix, high level of baseline, and peak overlapping are the main problems of chromatographic fingerprint approaches. Consequently, several statistical and chemometric approaches have been used for the extraction of data from chromatographic spectra (Bansal, Chhabra, Rawal, & Sharma, 2014). In plant metabolite studies, multivariate data examination methods are generally used to achieve reliable results (Farhadi, Asili, Iranshahy, & Iranshahi, 2019). For this, multivariate data analysis has used hierarchical cluster analysis, supervised techniques like linear discriminant investigation, principal constituent study, and hierarchical cluster examination as pattern recognition methods (Amirvaresi, Rashidi, Kamyar, Amirahmadi, Daraei, & Parastar, 2020).

MS is a sensitive method that provides valuable information related to low-abundance compounds and is able to discover new markers and detect unusual adulterations. Therefore, various studies used MS to accurately detect adulteration, especially in rare cases (Xu, Ge, Li, Guo, Dai, & Yang, 2019). For instance, Senizza et al. used the UHPLCESI/QTOF-MS-based metabolomics to discriminate the possibility of mixing styles with stamen and tepals. The results indicated that the

Table 3
Practical methods used for identification of adulterants in Saffron.

Method	Type of adulteration	Detection	Ref.
NMR	1)Arnica, calendula, cayenne and safflower 2) turmeric 3) tartrazine 4) sandalwood	1) 1.23 ppm dominant peak of fatty acids 2) 7.2 ppm 3) 7.3 and 8.2 ppm 4) 6.3 and 9.5 ppm	(Gunning, Davies, & Kemsley, 2023)
TLC ESI-MS	1) Rhodamine B 2) Magenta III	1) *m/z* 443 2) *m/z* 330	(Bhooma, Nagasathiya, Vairamani, & Parani, 2020)
FTIR with Raman spectroscopy	Safflower Marigold Turmeric Calendula	UV–Vis: safflower at 405 nm marigold at 350 nm ATR-FTIR: Marigold 2922 and 2852 cm^{-1}. 1732 cm^{-1} Curcumins:1626 cm^{-1} 1602 and 1511 cm^{-1} Calendula: 1738 and 1631 cm^{-1}	(Varliklioz Er, Eksi-Kocak, Yetim, & Boyaci, 2017)
Computer vision and electronic nose	Styles of saffron safflower	10 % adulteration levels E1 cm1% 330 nm	(Kiani, Minaei, & Ghasemi-Varnamkhasti, 2017, 2018)
NIR spectroscopy coupled to multivariate analysis	Onion (4.4–30.2 w/w%) Calendula (5.2–30.6 w/w %) Pomegranate (4.2–29.8 w/w%) Turmeric (4.9–30.4 w/w %)	Concentration predictions: 10 %	(Castro, Ribeiro, Santos, & Pascoa, 2021)
TLC with image analysis and chemometrics	Sumac Turmeric Safflower Common madder Quinoline Yellow Sunset yellow Tartrazine	100 % prediction	(Sereshti et al., 2018)
UHPLC-DAD-MS	Gardenia iridoids	*m/z*: 1) 207, 225, 427 2) 209, 227, 411 3) 147, 165, 309, 471, 697, 719	(Moras, Loffredo, & Rey, 2018)
TLC	Safflower Red 40 Turmeric Yellow 5	Actual adulteration (w/w%) 49.4 0.75 56.1 25.7	(Dai, Gao, & He, 2020)
UV–Vis	Allura red AC Amaranth Azorubine Naphthol yellow Ponceau 4R Quinoline yellow Sunset yellow Tartrazine	λmax (nm) 570 586 484 584 570 460 533 492	(Zalacain et al., 2005)
Magnetic solid phase extraction	Tartrazine	dynamic range of 0.02 to 10 mg/L	(Fooladi, Ebrahimi, Nakhaei, & Asadi, 2021)
MALDI MS/MS	Curcumin	LOD: 47.63 ppm LOQ: 56.53 ppm	(Aiello, Siciliano, Mazzotti, Di Donna, Athanassopoulos, & Napoli, 2020)
FT-IR	Tartrazine, Sunset, Azorubine, Quinoline	1800–1830 cm^{-1}, 2600 cm^{-1} and 3700–3850 cm^{-1}	(Karimi, Feizy, Mehrjo, & Farrokhnia, 2016)
HPLC	Tartrazine, Sunset, Quinoline	Saffron and synthetic dyes extracted with a methanol and 0.02 M ammonium acetate few drops of 10 % ammonium hydroxide HPLC condition XDB-C18, 5 μm particle size, 250 mm 4.6 mm Mobil phase: Ammonium acetate (A 10 %) and methanol (B 80 %) in 40 min, flow rate was 1.0 mL/min	(Barani & Tajik, 2019)
UV–Vis and TLC	Erythrosine	527 nm ($R_f = 0.89 \pm 0.03$)	(Ordoudi, Tsioga, & Tsimidou, 2009)
LC-MS	Geniposide	LOD: 41.7 μg of geniposide per gram	(Guijarro-Diez, Castro-Puyana, Crego, & Marina, 2017)
NMR	Curcuminoid	H1,7: 7.541 H2,6:6.751 ppm H4: 6.059 ppm . aromatic protons:7.318, 7.147, and 6.819 ppm	(Petrakis, Laura R Cagliani, Moschos G Polissiou, & Roberto Consonni, 2015)

unsupervised and supervised Orthogonal Plans to Latent Structures Discriminant Analysis (OPLS-DA) discriminated authentic saffron from styles with added floral mechanisms, while anthocyanins and glycosidic flavonols were introduced as the main markers of the styles' adulteration (Senizza et al., 2019). Kaempferol derivatives and geniposide were considered unique authenticity and adulteration indicators in a metabolomics study of saffron. Guijarro-DíezA et al., using HPLC/QTOF/MS, evaluated the least quantifiable adulteration ratio (0.2 %) of these compounds in nineteen commercial saffron samples (Guijarro-Díez et al., 2017). In addition, by UPLC-HR MS, Gikas et al. studied the phytochemical content of *C. sativus* L. from Iran, Morocco, Kashmir, Greece, India, and Italy; they found differences in metabolite profiling of *C. sativus* L. among different countries (Gikas, Koulakiotis, & Tsarbopoulos, 2021).

Chemometrics-based GC–MS can also assay adulterations and differences among geographical areas based on volatile markers of saffron (Aliakbarzadeh, Sereshti, & Parastar, 2016; D'Archivio, Pietro, Maggi, & Rossi, 2018; Morozzi, Zappi, Gottardi, Locatelli, & Melucci, 2019). The effect of the roasting process and adding some plants like calendula as adulteration was studied by Farag et al., based on Principal component analysis (PCA) analysis; authors reported safranal and 2-caren-10-al as the main volatile compounds of saffron and estragole, β-caryophyllene and eugenol as the discriminate compounds of fraudulent admixture. In addition, ketoisophorone in fresh saffron and safranal in long-stored saffron was introduced as the key markers (M. A. Farag et al., 2020) (Farag et al., 2020). Moreover, HPLC, FT-IR, and NMR are used in the targeted metabolomics analysis of saffron (Consonni, Ordoudi, Cagliani, Tsiangali, & Tsimidou, 2016; Samaha et al., 2021; Vahedi et al., 2018).

7. Adulterations in saffron

The rate of adulterations is exponentially growing in the case of saffron. The most common analytical methods used for evaluating saffron adulteration are indicated in Table 3. Saffron adulterations, including synthetic dyes and herbal materials, have been studied by various analytical methods(Dowlatabadi et al., 2017). Using FT-IR fingerprinting technique joined with chemometrics PLS-DA could discover the adulteration with sufficient accuracy of 3–5 % (Foschi, Tozzi, Di Donato, Biancolillo, & D'Archivio, 2022). Headspace flash GC, compelled with flame ionization detection, was used to examine the adulteration of turmeric and marigold in the saffron sample. The results indicated that this method is suitable for adulterant trappings below 33 % w/w (Morozzi et al., 2019). In a study performed by Farag et al., GC–MS was employed to recognize safflower and calendula in the saffron sample. Safranal was introduced as an indicator to separate saffron from safflower, while monoterpene hydrocarbons dominated calendula aroma (Mohamed A. Farag, Nesrine Hegazi, Eric Dokhalahy, & Amira R. Khattab, 2020; M. A. Farag et al., 2020). Adulterations of saffron either nature-based (by adding *C. tinctorius*, maize silk, safflower, marigold, wild carrot, turmeric, etc.) or synthetic-based (by adding liquid glycerine, codeine phosphate, nylon fibers, and various types of dyes), need special consideration (Kumari, Jaiswal, & Tripathy, 2021). It was revealed that the main source of saffron samples in markets was not *C. sativus* L. or *C. tinctorius* L., but they were from another source of saffron with different contents of aroma compounds (Moratalla-López, Bouhadida, Bagur, García-Rodríguez, Oueslati, & Alonso, 2016). In a study by Sabatino et al., HPLC/PDA/ESI-MS was used to examine adulteration in saffron. From marigold, isorhamnetin 3-O-neohesperidoside was detected at m/z 623, and from safflower, hartamin, and anydrosafflor yellow B were detected at m/z 909 and 1044. So, this method could expose the marker molecules at concentrations of up to 5 % w/w (Sabatino et al., 2011a). Moreover, the new ion mobility spectrometry method combined with multivariate analysis could discriminate between saffron samples and synthetic edible dyes (Fattahi, Mani-Varnosfaderani, Barzegar, & Sahari, 2022, 2023). *Nelumbo nucifera* Gaertn and *Zea mays*, with a similar appearance to saffron, are the new adulterations applied. Based on this awareness, Li et al. demonstrated that with the chemometric method, NIR spectroscopy could effectively identify these adulterations in saffron without damaging the samples (Li, Xing, Lin, Yi, & Shao, 2020). Proper techniques for the determination of adulterants in saffron have been reviewed by Kumari et al (Kumari et al., 2021).

8. Conclusion

In general, three groups of secondary metabolites are the main components of saffron, including apocarotenoids (crocin, responsible for the yellow to orange color) and picrocrocin and safranal accountable for the taste and smell of saffron, respectively. But in examining the quality, potential adulterations, impurities and contaminants, evaluating just based on these compounds is not valid. Many derivatives of the mentioned compounds and other categories of components that are often found in smaller amounts can be introduced as specific signs to authenticate saffron samples with high confidence. Several techniques have been applied for qualitative and quantitative examination of these metabolites in saffron. Our literature review showed that HPLC alone and in hyphenated like HPLC-ESI-MS and LC-QTOF-MS could be considered a potent and accurate method for quality control of saffron. Moreover, GC and related techniques, including GC–MS, GC × GC, and olfactometric methods, are potent for assessing volatile compounds in saffron and saffron products. Also, sensitive and selective analytical approaches like metabolomics-based LC-MS, GC–MS, and NMR are used for saffron authentication.

Ethical Statement

This is a review article. Ethical approval is not required for the study.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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