Chapter 20

Saffron adulteration

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Chapter Outline

20.1 Introduction

The spice trade has one of the longest and richest histories of any industry. Among spices saffron is considered one of the most valuable due to its ability to add color, taste, and aroma to various foods. Global consumption of saffron is 300 tons and the world trade of saffron is worth 1 billion dollars, which accounts for 3%−4% of the total spices sold internationally (Islamic Republic News Agency). Fraud in the food industry has a long history and dates back thousands of years. Saffron is considered as one of the most common target of fraud (Negbi, 1999). This is mainly due to the high price of the spice and to the lack of technological methodologies available to detect fraud.

Fraudulent practices are most common when the spice is in its powdered form. The history of fraudulence in saffron has been reported as far back as 600 years ago. In those days, serious punishment was imposed for fraud. In some European countries such as Italy, special armed police were established to defend the authenticity of this spice.

The addition of a variety of less expensive and more readily available plant materials, mostly of inferior quality but similar in appearance, has been a common fraudulent practice throughout history. The term adulteration refers to the addition of mineral substances, oils, or molasses for increasing weight; it can also refer to the addition of various dye material to improve its appearance. Addition of artificial colorants is a common way of adulteration with the aim of improving the appearance of dried stigmas or other extraneous materials to misealder consumers. As a general rule, artificial colorants should be absent from saffron according to the ISO 3632 standards (ISO, 2011, 2010). The most commonly occurring adulterations in saffron are listed in Table 20.1 (Sforza, 2013).

Besides synthetic dyes, saffron powder can also be adulterated by addition of natural colorants from other plant tissues. Unlike the stigmas, saffron flower petals are not used commercially and are often used for adulteration of saffron powder. Red table beet, which is rich in betanine, and safflower, which contains carthamidin and carthamin are also used as adulterants. Finally, saffron can also be adulterated by the addition of madder, which is characterized by reddish hydroxyanthraquinones.

20.2 Detecting adulteration

Saffron quality is of major importance to most consumers. Therefore, for the purposes of saffron trading, evaluation of the purity and authenticity of a given product or grading its quality is important. The amount of crocin, picrocrocin, and safranal, which are responsible for saffron color, flavor, and aroma are used to determine the quality of saffron (Fernandez, 2004). The higher the amounts of these compounds, the higher the quality of the saffron.

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TABLE 20.1 Most common forms of adulteration in saffron and saffron powder.

Forms of adulteration	Adulteration consisting of
Without the addition of foreign substances	Mixing with condensed or older saffron
Adding various parts of the saffron plant	Adding stamens or cut and colored perigone
Adding substances that increase weight	1. Increase humidity percentage 2. Soaking in sirup, honey, glycerin or olive oil 3. Adding chemicals such as barium sulphate, sodium, calcium, calcium carbonate, potassium hydroxide, potassium nitrate, monopotassium tartrate, sodium borate, lactose, starch, or glucose to the above sirups
Adding parts from other plants	1. *Carthamus tinctorius* flowers 2. *Calendula officinalis* flowers 3. Stigmas from other saffron types that are shorter and have no dye properties (*Crocus vernus*, *Crocus speciosus*, etc.) 4. *Papaver rhoeas* L., *Punica granatum*, *Arnica montana*, *Scolimus hispanicus* flowers cut in slices. 5. Perianths from certain spices such as carnations 6. Ground red pepper 7. Herbaceous plants cut in pieces and colored in azoic dye substances 8. Small allium porrum roots 9. Sandalwood and campeche wood powder 10. Curcuma
Adding animal substances	Salted and dried meat fibers
Adding artificial substances	Colored gelatin fibers
Adding organic dye substances	Martins yellow, tropeolina, fucsina, picric acid, tartrazine, erythrocine, azorubine, Cochineal A red, orange yellow, naphtanol yellow, rocelline, red, etc.

Source: From Sforza, S. (Ed.), 2013. Food Authentication Using Bioorganic Molecules. DEStech Publications, Inc., Lancaster, Pennsylvania, USA.

Increasing concern of producers, retailers, and consumers for authenticity of valuable products such as saffron underlines the need for high-throughput analytical methods that allow, from a practical point of view, for the rapid screening of potential adulterations. Once the adulteration is detected and the adulterant identified, quantification is also required to estimate the level of adulteration (Petrakis et al., 2017).

20.2.1 Chromatographic techniques

Different methods are used to detect adulteration. Chromatographic techniques are used to detect what is today the most common type of fraud—adulteration with water-soluble acid colorants. Saffron powder can often be adulterated by addition of synthetic dyes such as tartrazine, methyl orange, or ponceau-4R, which can be easily detected by LC-DAD or LC-MS/MS. The thin layer chromatographic method allows the detection of artificial water-soluble dye acid substances. It applies to saffron threads as well as to saffron powder. The detected dye substances are yn oline yellow, S−napthol yellow, tartrazine, amaranth, A−cochineal red, azorubine, orange II, erythrocine, and rocceline.

High Performance Liquid Chromatography (HPLC) method is used for the determination of three different elements: identification of dye substances responsible for saffron coloring intensity (crocines); identification of artificial dye substances, water-soluble acid, pursuant to the ISO/TS 3632, 2003; and identification of fat-soluble dye substances.

Although usually accurate and reliable, a main drawback of chromatographic methods are that they are often time-consuming and require expensive instrumentation. For this reason, in the last few years efforts have been focused on the development of quick and cheap assays for spice authentication, mainly based on UV-vis spectroscopy (ISO 3632-2) (Fig. 20.1).

The adulteration can also be detected by chromatographic analysis coupled with a UV detector. As an example, Haghighi et al. (2007) applied a HPLC-UV analysis for the determination of the main saffron dyes in order to assess the possible addition of exogenous natural colorants (Fig. 20.2). The authors analyzed pure saffron before and after addition of known amounts of selected adulterants (saffron petals, madder, safflower, and red beet). The method obtained correct classification of pure saffron against those colored by addition of safflower, madder, and red beet. However, the method failed to recognize saffron colored by saffron petals.

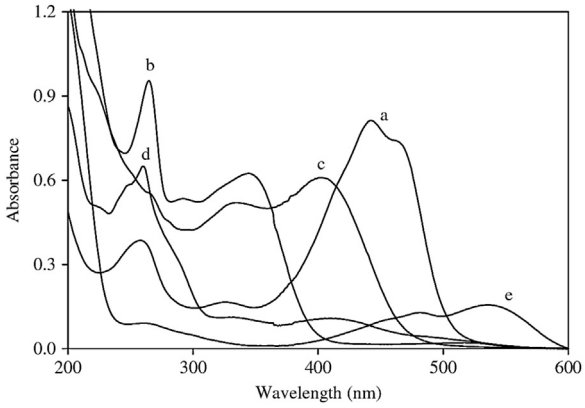

FIGURE 20.1 UV-visible spectra of saffron (a, 0.01 mg mL^{-1}) and saffron petals (b, 0.25 mg mL^{-1}), safflower (c, 0.15 mg mL^{-1}), madder (d, 0.17 mg mL^{-1}), and red beet (e, 2 mg mL^{-1}) colorants. *From Haghighi, B., Feizy, J., Kakhki, A.H., 2007. LC determination of adulterated saffron prepared by adding styles colored with some natural colorants. Chromatographia 66, 325−332.*

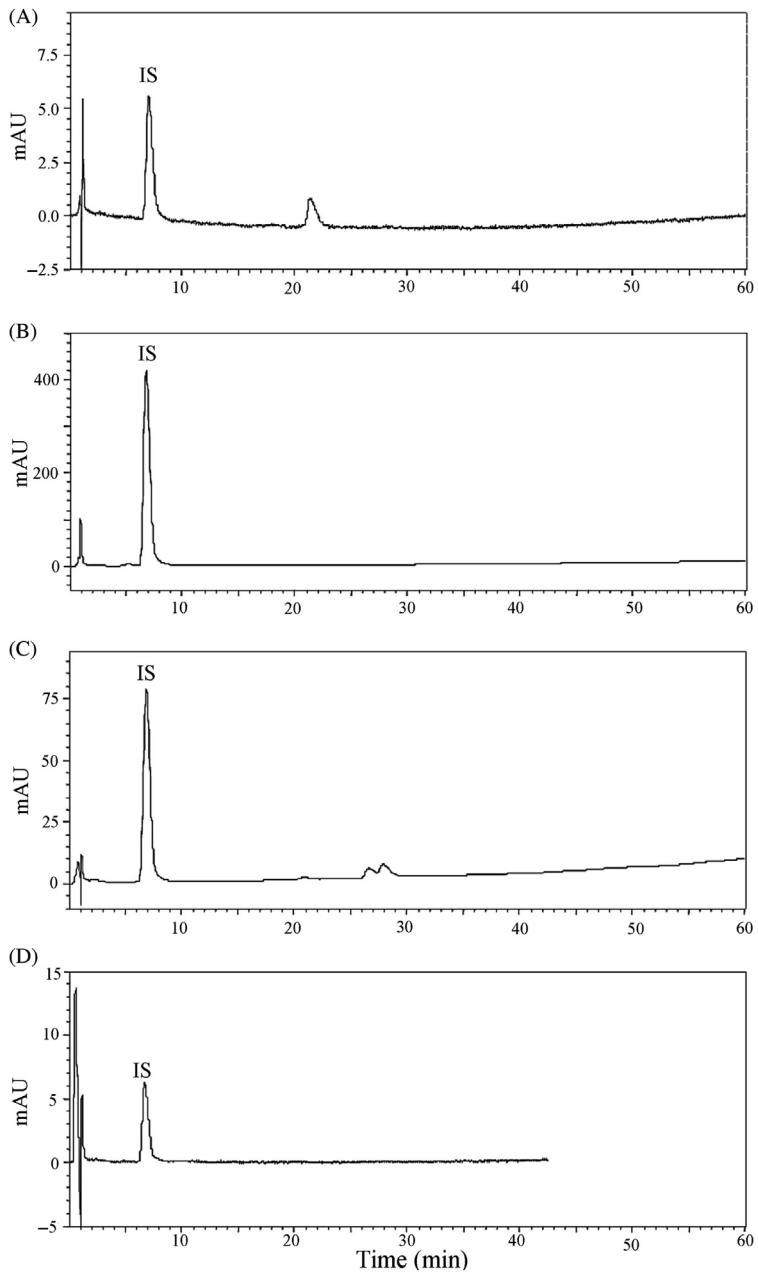

FIGURE 20.2 Chromatograms of the methanol−water (50%, v/v) extract of the styles colored with the colorants of saffron petals (A), safflower (B), madder (C), and red beet (D) recorded at 520 (A), 402 (B), 260 (C), and 535 (D) nm, including 4-nitroaniline as internal standard (IS). Concentrations of IS and colored styles, 0.09 and 0.35 mg mL^{-1}, respectively. *From Haghighi, B., Feizy, J., Kakhki, A.H., 2007. LC determination of adulterated saffron prepared by adding styles colored with some natural colorants. Chromatographia 66, 325−332.*

TABLE 20.2 HPLC/PDA/MS chemical characterization of saffron, safflower, marigold, and turmeric extracts.

Peak	Name	Rt (min)	UV-vis (nm)	$[M-H]^-$ (m/z)
Crocus sativus L. (saffron)				
1	4-(α-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (picrocrocin)	20.2	250	375
2	trans-Crocetin (β-D-neapolitanosyl)-(β-D-gentibiosyl) ester	41.2	260, 440	1137
3	trans-Crocetin di-(β-D-gentibiosyl) ester	46.5	260, 420, 460	975
4	trans-Crocetin (β-D-glucosyl)-(β-D-neapolitanosyl) ester	50.1	260, 440	975
5	cis-Crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester	51.6	260, 330, 435, 460	813
6	cis-Crocetin di-(β-D-gentibiosyl) ester	57.5	260, 320, 435, 460	976
7	cis-Crocetin di-(β-D-glucosyl) ester	58.8	260, 325, 440, 465	813
Calendula officinalis (marigold)				
a	Quercetin 3-*O*-rutinosylrhamnoside	28.4	255, 355	755
b	Quercetin 3-*O*-rutinoside	30.6	255, 355	609
c	Isorhamnetin-3-*O*-rutinosylrhamnoside	32.2	255, 350	769
d	Narcissin	34.7	255, 355	623
e	Isorhamnetin 3-*O*-neohesperidoside	38.7	255, 345	623
f	Isorhamnetin-3-*O*-glucoside	39.9	255, 355	477
Carthamus tinctorius (safflower)				
I	Hydroxysafflor yellow A (safflomin A)	30.1	225, 410	611
II	6-Hydroxykaempferol 3-*O*-β-D-glucoside	30.6	275, 340	464
III	Kaempferol 3-*O*-β-rutinoside	37.7	265, 350	593
IV	Safflor yellow B	40.2	225, 410	1060
V	Anydrosafflor yellow B	42.1	225, 410	1044
VI	Prechartamin	58.7	240, 405	955
VII	Chartamin	68.6	370, 520	909
Curcuma longa (turmeric)				
α	Demethoxycurcumin	63.6	250, 425	337
β	Bisdemethoxycurcumin	63.9	250, 420	307
γ	Curcumin	64.3	260, 430	367

Source: From Sabatino, L., Scordino, M., Gargano, M., Belligno, A., Traulo, P., Gagliano, G., 2011. HPLC/ PDA/ESI-MS evaluation of saffron (*Crocus sativus* L.) adulteration. Nat. Prod. Commun. 6, 1873–1876.

The normative ISO 3632 (ISO 3632-1; ISO 3636-2) employed in the international trade market to determine saffron's quality based on spectrophotometric and chromatographic measurements is clearly insufficient to assess saffron's authenticity when saffron is adulterated with plant foreign matter with similar color and morphology. In fact, the ISO method is not able to detect adulterations with other plants such as safflower, marigold, or turmeric when their content is lower than 20% (Sabatino et al., 2011).

High-performance liquid chromatography (HPLC) coupled with photodiode array (PDA) and electrospray ionization mass spectrometry (ESI-MS) detection has also revealed the addition of plant adulterants at a minimum of 2%–5% (w/w) (Sabatino et al., 2011). The HPLC/PDA/MS technique allowed the unequivocal identification of adulterant characteristic marker molecules that could be recognized by the values of absorbance and mass (Table 20.2). The selection of characteristic ions of each marker molecule revealed concentrations of up to 5%, w/w, for safflower and marigold and up to 2% for turmeric. In addition, the high dyeing power of turmeric allowed the determination of 2%, w/w, addition using exclusively the HPLC/PDA technique.

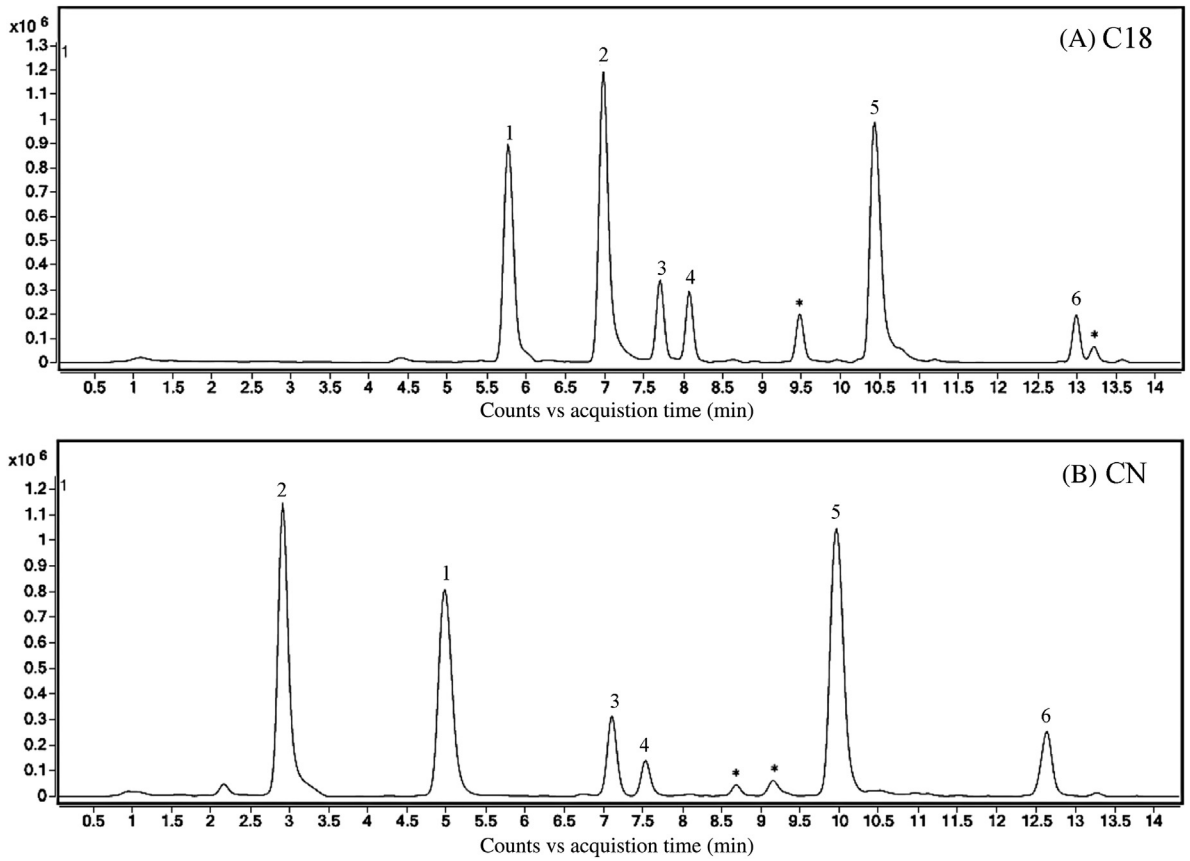

FIGURE 20.3 LC-MS extracted ion chromatogram of glycosylated kaempferol derivatives and geniposide from an authentic saffron sample adulterated with 5% of gardenia extract in C18 (A) and cyano (B) columns. LC conditions: flow rate, 0.4 mL min^{-1}; mobile phases, water containing 0.1% formic acid (solvent A), and acetonitrile containing 0.1% formic acid (solvent B); elution gradient: 5%−17.5% B in 10 min; 17.5%−50% B in 2 min; 50% B for 4 min, 50%−5% B for 1 min, and 5% B for 10 min in order to reequilibrate the column at the initial conditions; injected volume, 5 μL; temperature 40°C. MS conditions in ESI − : capillary voltage, 3000 V; nozzle voltage, 0 V; drying gas conditions, 10 L min^{-1} and 300°C; nebulizer pressure, 1.7 bar; sheath gas conditions, 6.5 L min^{-1} and 300°C; fragmentator, 175 V; skimmer, 60 V; octapole voltage, 750 V. Peak identification: (1) Kaempferol 3,7,4′-O-triglucoside, (2) Geniposide, (3) Kaempferol 3-*O*-sophoroside-7-*O*-glucoside, (4) Kaempferol 3,7-*O*-diglucoside, (5) Kaempferol 3-*O*-sophoroside, and (6) Kaempferol 3-*O*-glucoside. (*) Different kaempferol derivatives not identified. *From Guijarro-Díez, M., Castro-Puyana, M., Crego, A.L., Marina, M.L., 2017. A novel method for the quality control of saffron through the simultaneous analysis of authenticity and adulteration markers by liquid chromatography-(quadrupole-time of flight)-mass spectrometry. Food Chem. 228, 403−410.*

A liquid chromatography-(quadrupole-time of flight)-mass spectrometry (LC-MS) methodology was developed by Guijarro-Díez et al. (2017) to assess the authenticity of saffron through the analysis of a group of glycosylated kaempferol derivatives proposed as novel authenticity markers as a result of a metabolomic study of saffron (Fig. 20.3). A strategy was proposed to evaluate the minimum quantifiable adulteration percentage, which was established at 0.2% regardless of the adulterant employed. The determination of characteristic and endogenous compounds such as glycosylated kaempferols as authenticity markers could be a highly effective tool when detecting adulterations regardless of the adulterant employed. For example, the existence of a new adulteration method of saffron with gardenia is of interest to the developed methodology since it allows the detection of geniposide as an adulteration marker in saffron. The developed LC-MS methodology was successfully applied to the analysis of 19 commercial saffron samples through the analysis of glycosylated kaempferols and geniposide shown to be specific and suitable for the routine analysis because of its sensitivity, accuracy, and reproducibility.

It is assumed that saffron authentication through established methodology is a challenging task, as saffron of higher quality may be intentionally blended with plant-derived substitutes to disguise fraud. Therefore, the development of analytical methodologies for reliable saffron quality control is of high interest for consumer protection and fraud prevention.

20.2.2 Infrared spectroscopy

Infrared (IR) spectroscopy is often used as a simple, fast, and green method for the adulteration screening of botanical materials for foods and herbs. IR spectroscopy provides another versatile and cost-effective option for the high-

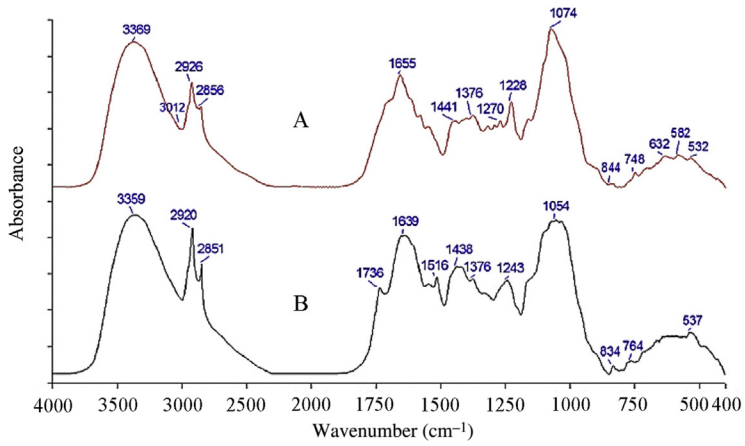

FIGURE 20.4 Original MIR spectra of (A) saffron stigmas and (B) safflower petals. *From Chen, J.-B., Zhou, Q., Sun, S.-Q., 2016. Adulteration screening of botanical materials by a sensitive and model-free approach using infrared spectroscopic imaging and two dimensional correlation infrared spectroscopy. J. Mol. Struct. 1124, 262–267.*

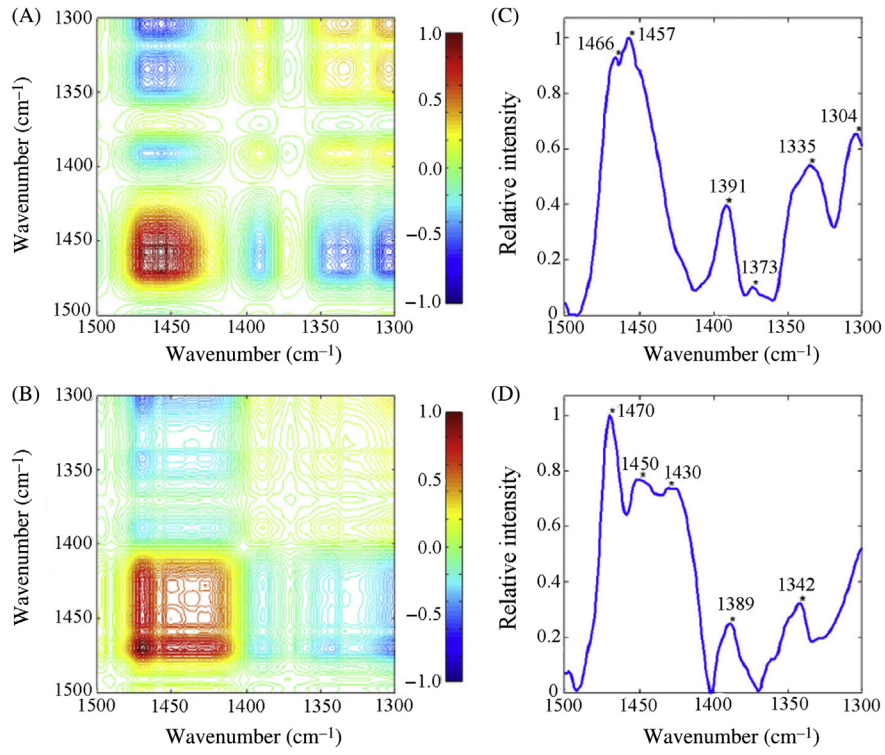

FIGURE 20.5 Normalized synchronous 2D correlation MIR spectra of (A) saffron stigmas and (B) safflower petals in the range of 1500−1300 cm^{-1}. The autopeak spectra of (C) saffron stigmas and (D) safflower petals clearly show the autopeaks on the diagonals of the synchronous 2D correlation spectra. *From Chen, J.-B., Zhou, Q., Sun, S.-Q., 2016. Adulteration screening of botanical materials by a sensitive and model-free approach using infrared spectroscopic imaging and two dimensional correlation infrared spectroscopy. J. Mol. Struct. 1124, 262–267.*

throughput analysis of a diverse range of foods and herbs (Karoui et al., 2010). The increasingly recognized potential of portable/handheld IR spectroscopy renders this technique an effective fingerprinting tool (Ellis et al., 2012) for either laboratory or on-site analysis along complex supply networks. However, the overlapping of absorption signals of various substances significantly decreases the sensitivity and specificity of IR spectroscopy in the detection of adulterated samples. However, measuring the entities separately makes it possible to cluster the authentic and adulterant entities in a single sample. In comparison, near-infrared (NIR) spectroscopic imaging is more suitable for the measurement of plant samples, because most entities can be measured directly in the transmission or reflection mode. Therefore, NIR spectroscopic imaging can be used for the exploratory clustering analysis of the entities in plant samples.

The applicability of IR spectroscopy for screening saffron adulteration with plant adulterants was also investigated by Chen et al. (2016) using IR spectroscopic imaging, 2D correlation IR spectroscopy, and principal component analysis (PCA) (Fig. 20.4). According to chemical compositions revealed by the original, second derivative, and 2D correlation mid-infrared (MIR) spectra, the cluster containing crocetin and crocins should be saffron, while the other cluster without crocetin or crocins should be the adulterant (Fig. 20.5). The feasibility of this approach was proven by the simulated

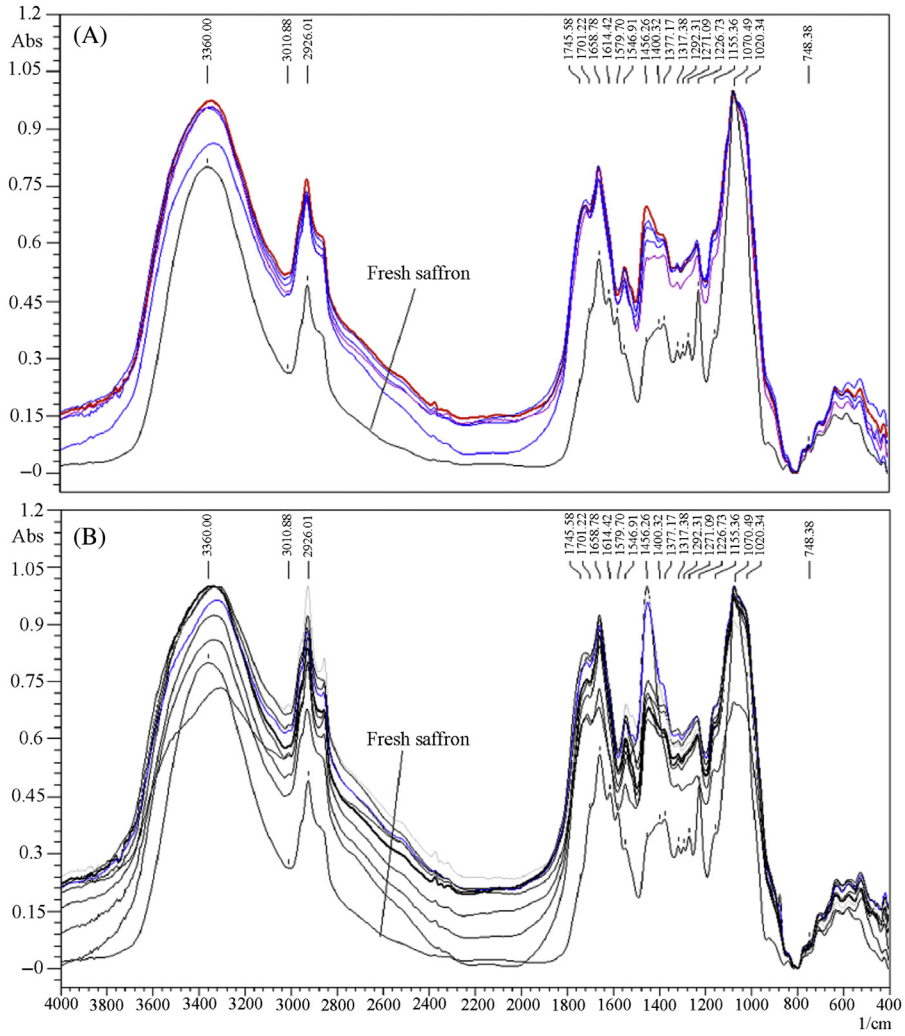

FIGURE 20.6 FTIR spectra of test set; (A) five samples from individual producers and (B) ten samples from blends of stigmas obtained from different producers. *From Ordoudi, S.A., de los Mozos Pascual, M., Tsimidou, M.Z., 2014. On the quality, control of traded saffron by means of transmission Fourier-transform mid-infrared (FT-MIR) spectroscopy and chemometrics. Food Chem. 150, 414–421.*

adulterated sample of saffron. Accordingly, saffron adulterated by adding other plant materials can be detected by a simple, fast, sensitive, and green screening approach using IR spectroscopic imaging, 2D correlation spectroscopy, and necessary chemometrics techniques. However, information from both NIR microspectroscopic imaging and transmission Fourier transform infrared (FTIR) was required and only one adulterant (i.e., safflower) was evaluated.

Fourier transform spectrometers have replaced dispersive instruments for most applications due to their superior speed and sensitivity. They have greatly extended the capabilities of infrared spectroscopy and have been applied to many areas that are very difficult or nearly impossible to analyze by dispersive instruments. Instead of viewing each component frequency sequentially, as in a dispersive IR spectrometer, all frequencies are examined simultaneously in Fourier transform infrared (FTIR) spectroscopy.

FTIR spectroscopy is a simple analytical technique largely applied for its rapidity and reproducibility in food fraud detection. FTIR spectroscopy was first employed for the characterization of crocins and related apocarotenoids in saffron (Tarantilis et al., 1998). The quality and authenticity issues of saffron using IR spectroscopic techniques have also been studied by Zalacain et al. (2005), Anastasaki et al. (2010), and Ordoudi et al. (2014). Transmission FTIR spectroscopy has also been used for detecting saffron adulteration or contamination with several colorants (Ordoudi et al., 2014) (Fig. 20.6).

Karimi et al. (2016) reported the capability of FTIR spectroscopy combined with appropriate chemometric techniques to detect and quantify six different artificial colorants including tartrazine, Sunset yellow, Azorubine, Quinolone yellow, Allura red, and Sudan II in Iranian saffron. Analysis of the selected clusters of variables indicates that three regions band are responsible for differentiation of standard samples from their fraud ones. Their study showed that the combination of FTIR and clustering concept resulted in the best performance for calibration and external test set with 100% sensitivity and specificity.

Within the techniques used for measurements over the mid-IR region, where structural information related to the fundamental vibrations occurs (Lohumi et al., 2015), diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) has proved its potential for the determination of quality and authenticity parameters of foods and herbs (Saltas et al., 2013). Petrakis and Polissiou (2017) presented an application of DRIFTS and chemometric techniques for evaluating adulteration of saffron with six characteristic adulterants of plant origin: *Crocus sativus* stamens, calendula, safflower, turmeric, buddleja, and gardenia. The proposed method involved a three-step process for the detection of adulteration as well as for the identification and quantification of adulterants. The results obtained illustrate that this strategy based on DRIFTS has the potential to complement existing methodologies for the rapid and cost-effective assessment of typical saffron frauds. The possibility of portable or handheld instrumentation may further enhance the potential of this approach for on-site analysis and fraud detection within long and complicated supply chains.

Another advanced method, namely laser-induced breakdown spectroscopy, provides a rapid elemental analysis of the sample and can have application in food analysis (Bilge et al., 2015; Tiwari et al., 2013). FTIR and Raman spectroscopy are complementary methods that give molecular information about the sample, whereas LIBS identifies the elemental composition. Varliklioz et al. (2017) compared three spectroscopic techniques, namely, attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy, Raman spectroscopy, and laser-induced breakdown spectroscopy (LIBS), and the superiority of the techniques was investigated by using PCA. Among these methods, LIBS was considered to be a promising tool for quantifying the level of adulteration in saffron samples with a simple and accurate process.

20.2.3 ^{1}H nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique largely applied for its rapidity and reproducibility, having the potential for high-throughput analyses with minimal sample pretreatment (Longobardi et al., 2013). The development of NMR spectrometers with high-field magnets as well as advances in probe technology have enabled the analysis of numerous compounds in low concentrations, with high precision and accuracy (Bharti and Roy, 2012; Ohtsuki et al., 2013). NMR-based metabolite fingerprinting may identify the subtle differences that often exist between authentic and fraudulent products. This metabolomic approach has been explored to discriminate authentic saffron from commercial samples; the results indicated relative amounts of picrocrocin and the sum of different crocetin glycosides as the characteristic metabolites of authentic saffron (Yilmaz et al., 2010).

The evaluation of adulteration with typical plant-bulking agents can be rapidly performed using ^{1}H nuclear magnetic resonance (^{1}H NMR) metabolite fingerprinting (Petrakis et al., 2015). This approach led to the development of reliable classification models for the detection and identification of adulterants utilizing the ^{1}H NMR data obtained from the DMSO-d6 extracts of samples. Taking into account the deficiency of established methodologies to detect saffron adulteration with plant adulterants, the method reliably assessed the type of adulteration and could be viable for detecting saffron fraud at a minimum level of 20% (w/w).

NMR metabolite fingerprinting has shown to be efficient for determining and identifying fraudulent additions of bulking agents to saffron, especially when plant adulterants are involved and the spice is commercialized in powder form. The obtained results confirmed the combined use of ^{1}H NMR spectroscopy and multivariate data analysis as a valid and powerful tool to investigate quality and authenticity of food products. The use of advanced spectroscopic techniques such as ^{1}H NMR may enable the rapid, nondestructive screening of quality and authenticity of a product with minimal or no sample preparation.

Petrakis et al. (2017) used an NMR-based approach to identify and determine the adulteration of saffron with Sudan dyes (Fig. 20.7). They revealed that the high linearity, accuracy, and rapidity of investigation enable high-resolution ^{1}H NMR spectroscopy to be used for evaluation of saffron adulteration with Sudan dyes. Accordingly, the main advantage of using NMR is the minimal sample preparation and null chemical treatment. In this respect, water-soluble artificial colorants, usually analyzed by other spectroscopic techniques, such as UV-vis and liquid chromatography, could be investigated as well, avoiding the drawbacks of sample pretreatment and the additional problems derived from the matrix effect that might influence the chromatographic resolution.

20.2.4 Molecular techniques

The cheaper availability of biomolecular assays has made these DNA-related techniques affordable in a wide array of food-related applications, allowing the achievement of operating costs similar to those of UV detection (Dhanya and

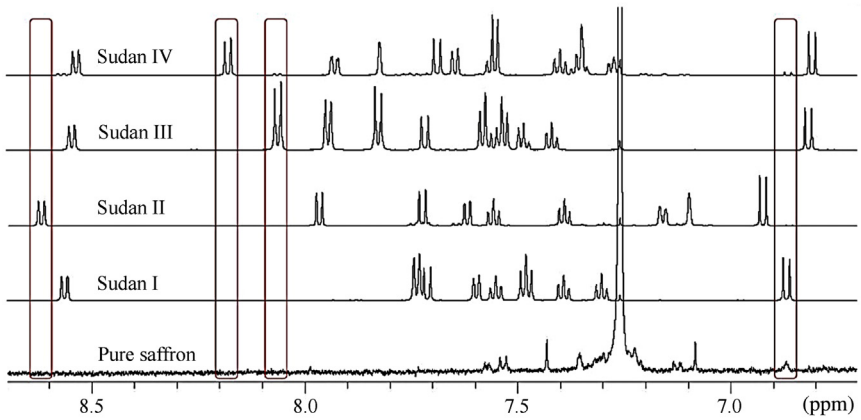

FIGURE 20.7 Aromatic region of ^{1}H NMR spectra of Sudan I–IV dyes and the pure Greek saffron analyzed. From the bottom to the top pure saffron, Sudan I, Sudan II, Sudan III, and Sudan IV are represented. Specific signals for the identification of each Sudan dye in adulterated saffron are highlighted. *From Petrakis, E.A., Cagliani, L.R., Tarantilis, P.A., Polissiou, M.G., Consonni, R., 2017. Sudan dyes in adulterated saffron (*Crocus sativus *L.): Identification and quantification by ^{1}H NMR. Food Chem. 217, 418–424.*

Sasikumar, 2010). As a consequence, DNA markers have become a popular means for the identification and authentication of a steadily increasing range of food products, spices, and medicinal or aromatic plants (Halima et al., 2007).

Molecular markers can detect differences at the DNA level and offer numerous advantages over conventional phenotype-based alternatives because they are stable and detectable in all tissue types, regardless of the growth environment, development state, or differentiation status (Agarwal et al., 2008). The advantages of this approach include high sample throughput, low detection limit, and good interlaboratory reproducibility. As techniques develop, DNA-based methods could be used in the analysis of processed foodstuff, patent drugs, fixed paraffin, adequate extraction, or vegetable oil (Costa et al., 2012). Thus, molecular markers offer several advantages for the determination of food authenticity in routine quality control (Marmiroli et al., 2013).

Currently, the majority of the methods developed for investigating saffron adulteration with bulking agents of plant origin rely on molecular techniques. Although very sensitive, such methods usually require extensive sample preparation and cannot easily address adulteration with plant extracts, probably due to the absence of recoverable DNA (Soffritti et al., 2016). Several PCR-based methods have been used to detect saffron adulteration, including allele-specific PCR (AS-PCR) (Mao et al., 2007), DNA sequence analysis (Ma et al., 2001), and RAPD-derived SCAR markers (Javanmardi et al., 2012; Marieschi et al., 2012). According to Marieschi et al. (2012) sequence-characterized amplified regions (SCAR) markers may represent a fast, sensitive, reliable, and low-cost screening method for the authentication of dried commercial saffron material. The method enabled the unequivocal detection of low amounts (up to 1%) of each adulterant, allowing the preemptive rejection of suspect samples (Fig. 20.8). Its enforcement limits the number of samples to be subjected to further evaluation with pharmacognostic or phytochemical analyses, especially when multiple batches have to be evaluated in a short time.

AS-PCR and SCAR markers are derived from specific fragments. These markers require screening new fragments or alleles to design specific primers for any new or unfamiliar adulterants, which limits their application in the detection of new adulterants. Therefore, these techniques require time-consuming sequencing or have limited application. New molecular markers, particularly DNA barcode-based universal primers methods, have been proposed and are being rapidly developed (Jiang et al., 2014).

To develop a straightforward, nonsequencing method for rapid, sensitive, and discriminating detection of these adulterants in traded saffron, Jiang et al. (2014) proposed a barcoding melting curve analysis method (Bar-MCA) that uses the universal chloroplast plant DNA barcoding region trnH-psbA to identify adulterants. Melting curve analysis is a fast and sensitive method for differentiating PCR production by fluorescence monitoring of the melting curve of the double-stranded DNA that is intercalated by the dye SYBR Green I in a real-time PCR system (Ririe et al., 1997). Results can be obtained without additional post-PCR processing in less than 2 hours. By amplification with the barcoding primer pair psbAF/trnHR and performing melting curve analysis, saffron was distinguished from the adulterants or detected in mixtures based on its melting temperature. This technique could detect the presence of an expected plant material and adulterant materials in close-tube reactions. Compared with other sequence-based species discrimination methods, melting curve analysis is a very promising technique, particularly in terms of costs and time.

Villa et al. (2017) used an EvaGreen real-time PCR approach as a simple, fast, highly sensitive, and reliable method for the detection and quantification of safflower in saffron. Based on their research the normalized real-time PCR system could be used for the detection and quantification of even smaller amounts of safflower, since the technique enables positive amplification of the target down to 2 pg of DNA ($\sim$1.4 DNA copies) (Fig. 20.9). In this work, qualitative PCR

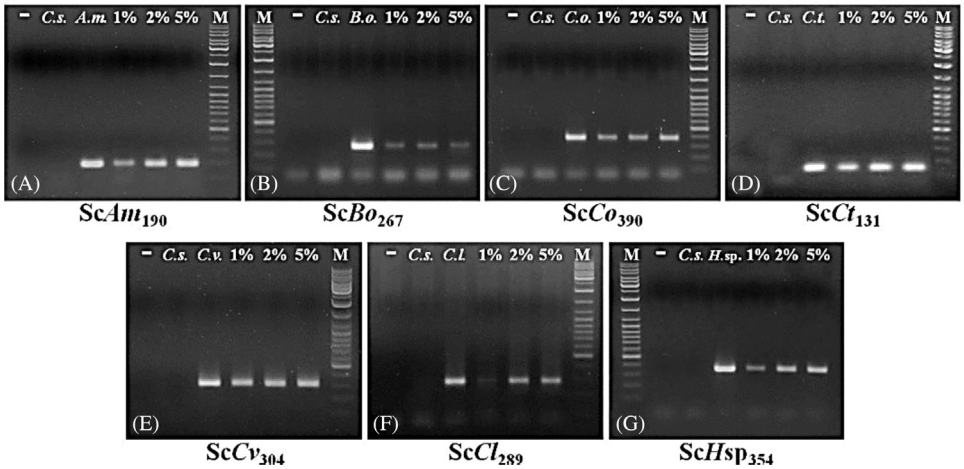

FIGURE 20.8 SCAR marker sensitivity assay: (A) PCR performed with $ScAm_{190}$ primer pair specific for *Arnica montana* (1%, 2%, and 5% DNA from mixtures of *C. sativus* and *A. montana*). (B) PCR performed with $ScBo_{267}$ primer pair specific for *Bixa orellana* (1%, 2%, and 5% DNA from mixtures of *C. sativus* and *B. orellana*). (C) PCR performed with $ScCo_{390}$ primer pair specific for *Calendula officinalis* (1%, 2%, and 5% DNA from mixtures of *C. sativus* and *C. officinalis*). (D) PCR performed with $ScCt_{131}$ primer pair specific for *Carthamus tinctorius* (1%, 2%, and 5% DNA from mixtures of *C. sativus* and *C. tinctorius*). (E) PCR performed with $ScCv_{304}$ primer pair specific for *Crocus vernus* (1%, 2%, and 5% DNA from mixtures of *C. sativus* and *C. vernus*). (F) PCR performed with $ScCl_{289}$ primer pair specific for *Curcuma longa*. (G) PCR performed with $ScHsp_{354}$ primer pair specific for *Hemerocallis* sp. (1%, 2%, and 5% DNA from mixtures of *C. sativus* and *Hemerocallis* sp.). − , negative control, amplification with no template DNA; *C.s.*, DNA from dried stigmas of *C. sativus*, as further negative control; *A.m.*, DNA from dried flowers of *A. montana*, as positive control; *B.o.*, DNA from dried seeds of *B. orellana*; *C.o.*, DNA from dried flowers of *C. officinalis*; *C.t.*, DNA from dried flowers of *C. tinctorius*; *C.v.*, DNA from dried stigmas of *C. vernus*; *C.l.*, DNA from dried rhizomes of *C. longa* (1%, 2%, and 5%), DNA from mixtures of *C. sativus* and *C. longa*; *H.* sp., DNA from dried tepals of *Hemerocallis* sp.; *M*, 100 bp DNA ladder. *From Marieschi, M., Torelli, A., Bruni, R., 2012. Quality control of saffron (*Crocus sativus *L.): Development of SCAR markers for the detection of plant adulterants used as bulking agents. J. Agric. Food Chem. 60, 10998 − 11004.*

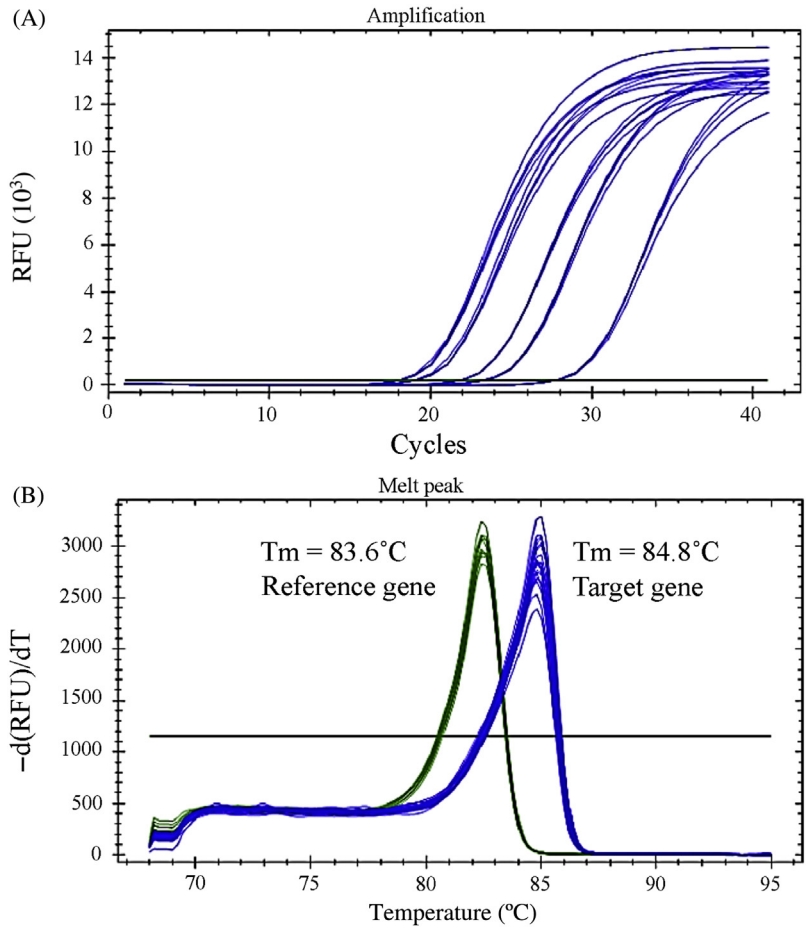

FIGURE 20.9 Real-time PCR amplification with EvaGreen intercalating dye targeting the ITS region of safflower using binary reference mixtures [20%, 10%, 5%, 1%, 0.1% (w/w)] of safflower in saffron. (A) Amplification curves and (B) melting curves (targeting both ITS region of safflower and the eukaryotic gene). *From Villa, C., Costa, J., Oliveira, M.B.P.P., Mafra, I., 2017. Novel quantitative real-time PCR approach to determine safflower (*Carthamus tinctorius*) adulteration in saffron (*Crocus sativus*). Food Chem. 229, 680−687.*

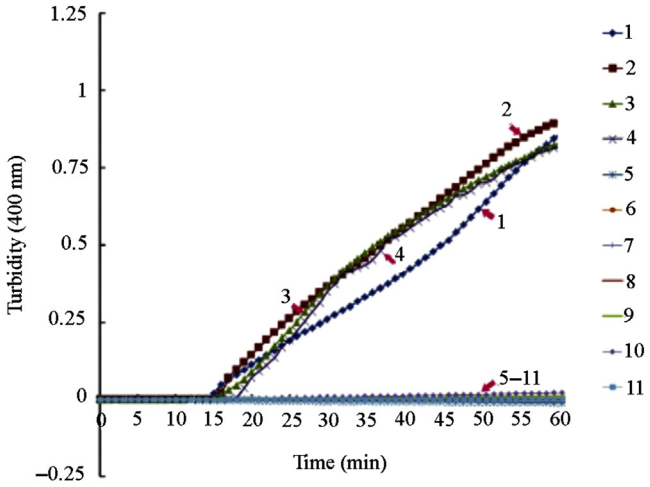

FIGURE 20.10 Specificity of the LAMP assay for the authentication of saffron and its adulterants. Turbidity was monitored with a Loopamp Realtime Turbidimeter at 400 nm every 6 s. 1 μL of calcein (a fluorescent detection reagent) was added to 25 μL of the LAMP reaction mixture before the LAMP reaction. Lines and tubes: *1*, *C. sativus* 01; *2*, *C. sativus* 11; *3*, *C. sativus* 12; *4*, *C. sativus* 13; *5*, *C. officinalis* 01; *6*, *C. tinctorius* 01; *7*, *C. longa* 01; *8*, *D. carota* 01; *9*, *N. nucifera* 01; *10*, *Z. mays* 01; and *11*, Neg (negative control, ddH_2O). *From Zhao, M., Shi, Y., Wu, L., Guo, L., Liu, W., Xiong, C., et al., 2016. Rapid authentication of the precious herb saffron by loop-mediated isothermal amplification (LAMP) based on internal transcribed spacer 2 (ITS2) sequence. Sci. Rep. 6, 25370. doi: 10.1038/srep25370.*

and real-time PCR methods were proposed as specific, sensitive, accurate, and powerful tools for detection and quantification of safflower adulteration in commercial samples of saffron.

Zhao et al. (2016) introduced a loop-mediated isothermal amplification (LAMP) technique for the differentiation of saffron from its adulterants (Fig. 20.10). The LAMP method is a novel nucleic acid amplification technology that is quick, simple, and highly specific. LAMP is based on a complex methodology requiring four to six different primers that are specifically designed to recognize six to eight precise gene sequences. DNA amplification is accomplished by a DNA polymerase with strand-displacing activity, thus obviating the need for a thermal denaturation step to obtain single-stranded DNA (Notomi et al., 2015). The use of isothermal conditions in the LAMP technique allows for reactions to occur in less time because no temperature changes are required. This novel technique was sensitive, efficient, and simple for saffron adulteration detection.

20.2.5 Electronic nose

Another study indicated the ability of an electronic nose system combined with PCA and artificial neural networks to differentiate nonadulterated and adulterated saffron (Heidarbeigi et al., 2015; Kiani et al., 2017). Saffron metabolic processes at the processing and storage duration make gases, which exist in the saffron aroma. It is therefore possible to determine the fake and original saffron by sensing these compounds in the headspace.

An electronic nose is a device that identifies the specific components of an odor and analyzes its chemical makeup to identify it. An electronic nose consists of a mechanism for chemical detection, such as an array of electronic sensors, and a mechanism for pattern recognition, such as a neural network. Electronic noses include three major parts: a sample delivery system, a detection system, and a computing system. The sample delivery system enables the generation of the headspace (volatile compounds) of a sample, which is the fraction analyzed (Fig. 20.11). The system then injects this headspace into the detection system of the electronic nose. The sample delivery system is essential to guarantee constant operating conditions.

The detection system, which consists of a sensor set, is the "reactive" part of the instrument. When in contact with volatile compounds, the sensors react, which means they experience a change of electrical properties.

Heidarbeigi et al. (2015) used an electronic nose based on six metal oxide semiconductor sensors to detect the aroma fingerprints of saffron, saffron with yellow styles, and safflower and dyed corn stigma. They revealed that the system can recognize the saffron adulteration satisfactorily. The electronic nose was able to successfully differentiate nonadulterated and adulterated saffron at higher than 10% adulteration level.

Advances and developments in sensor technology, chemometrics, and artificial intelligence make it possible to develop instruments based on artificial senses such as computer vision (CVS) and electronic nose (e-nose) systems

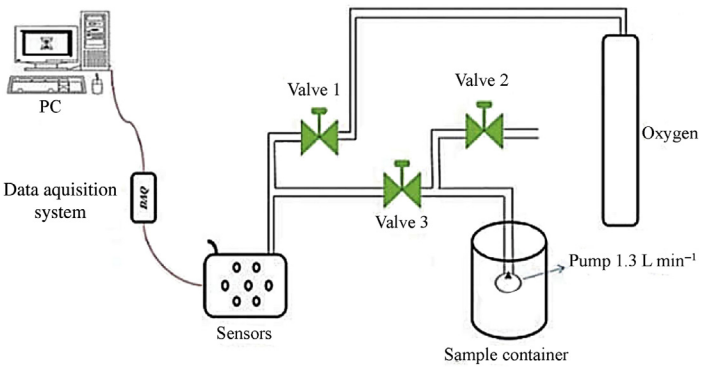

FIGURE 20.11 Schematic view of designed e-nose system. *From Heidarbeigi, K., Mohtasebi, S.S., Foroughirad, A., Ghasemi-Varnamkhasti, M., Rafiee, S., Rezaei, K., 2015. Detection of adulteration in saffron samples using electronic nose. Int. J.* Food *Prop. 18, 1391–1401.*

capable of measuring and characterizing color and aroma. Kiani et al. (2017) developed an integrated system based on CVS and electronic nose (e-nose) for saffron adulteration detection. Both CVS and e-nose techniques require little sample preparation and allow large data sets to be acquired in a short time. The CVS used was comprised of a CCD digital camera, standard lighting system, and software for image processing, which was also equipped with an e-nose system. They concluded that aroma characteristic variables were a little more effective than the color variables in detecting saffron adulteration.

20.3 Conclusion

Saffron authentication is a challenging task since saffron of higher quality may be blended with other materials to hide the fraud. Therefore, using accurate analytical methods to control saffron quality is of high interest for fraud prevention. Methods used to determine the saffron's alteration based on spectrophotometric and chromatographic techniques are insufficient when saffron is adulterated with plants such as safflower, marigold, or turmeric. Saffron adulterated by the addition of other plant materials can be detected using infrared spectroscopy techniques. However, when using this method only one adulterant can be evaluated. Among the methods used for detection of saffron adulteration, NMR spectrometery with high-field magnets is an accurate and precise method for analysis of compounds in low concentrations. This method is reliable for assessing the type of adulteration and could be reliable for dealing with extensive saffron fraud at a minimum level of 20% (w/w). NMR metabolite fingerprinting is efficient for saffron powder especially when plant adulterants are involved. The majority of the methods used for investigation of saffron adulteration with bulking agents of plant origin rely on molecular techniques. These methods are very sensitive but usually require extensive sample preparation and cannot easily address adulteration with plant extracts. The LAMP method is a nucleic acid amplification technology that is quick, simple, and highly specific. Using saffron aroma in the headspace of the electronic nose is another possible method to determine fraudulent saffron. An electronic nose can identify the specific components of saffron odor and analyze its chemical makeup. Although several methods have been used to identify fraud in saffron, there is still no single method used to identify all the fraudulent in the samples.

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