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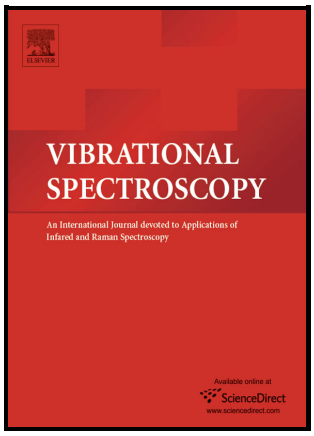

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ATR-FTIR spectroscopy combined with DNA barcoding and GC-MS to assess the quality and purity of saffron (*Crocus Sativus L.)*

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Abstract

Fourier transform infrared spectra of saffron (*Crocus sativus L.*) samples were acquired using attenuated total reflectance (ATR-FTIR). The main objective of the study was to determine the chemical composition of 11 samples of saffron collected from different areas in Morocco using the chemometric analysis of ATR-FTIR fingerprints and identifying the adulterated saffron among samples bought from local markets in different countries (Spain, Iran, and Morocco). The the authenticity and the purity of saffron samples was validated through a molecular analysis (DNA barcoding coupled to sequencing) and chromatographic analysis GC-MS. The results of ATR-FTIR showed vibration intensities of six distinct fingerprint regions displaying statistically significant differences. The spectrum of the sample from Timjicht (Taznakht) showed typical bands due to the vibration in 3000-2800 cm^{-1} (the richest in carbohydrates, lipids, and amino acids) and 1800 to 1725 cm^{-1} region (the richest in carbonyl and ester groups) and was classified a single subset in samples scatter plot. Then samples from Boulmane (S2), Ain Leuh (S3), Taliouine (S6), and Taznakht (S7-S8) were classified close to each other, which indicates the similarity in their vibration intensities mainly in the region of carbohydrates, lipids, amino acids, and esters. Similarities in terms of proteins and hydroxyl groups were revealed between the samples from El Mers (S11) and Taliouine (S1). Finally, the last sub-group contained samples from Ourika, Azilal and Ain Atia, which showed low composition in all components. Furthermore, to detect adulterated saffron from samples of unknown origin, a comparison of the ATR-FTIR spectra were carried out with spectra of pure saffron and results

showed that the peaks at 1706, 1732, and 1225 cm^{-1} (linked to crocin which are present primarily in saffron) were absent in one sample (SI). Interestingly, the use of another plant species named *Arrhenatherum elatius* as materiel for saffron adulteration was confirmed by the molecular study (DNA barcoding) and chromatographic analysis GC-MS

Keywords: Saffron, ATR-FTIR, Phylogeny, Purity and authenticity, Sequencing analysis, GC-MS.

1. Introduction

Saffron, and more specifically the dried red stigma of the *Crocus sativus* L. flowers, is one of the most expensive spices in the world [1,2]. It is an important coloring, and aromatic food agent is also used for its medicinal and pharmacological properties (anti-inflammatory, anticonvulsant, antidepressant, and antitumor agents [3–5]. The rich and versatile composition in carbohydrates (12-15%), proteins (10-14%), total oils (5-9%), fiber (4-5%), ash (4%), and volatiles (0.3-0.8%) is what gives the saffron its unique characteristics [6].

It contains three main chemical components: the glycosyl esters of crocetin (crocins) belonging to the carotenoids family and responsible for the bright yellow-red dye of saffron stigmas, picrocrocin (glycoside of safranal) that is responsible for the bitter taste, however, the characteristic aroma of saffron is due to safranal (monoterpene aldehyde) [7–9]. Crocins ($C_{44}H_{64}O_{24}$) are water-soluble carotenoids that give saffron its distinct and unique color and are one of the few families of carotenoids found in nature that is freely soluble in water [10–12]. Picrocrocin (4 - (β – D - glucopyranosyloxy) -2,6,6 – trimethy l - 1- cyclohexene -1-carboxaldehyde, $C_{16}H_{26}O_7$) is thought to be the foremost contributor to the bitter taste of saffron. According to Sánchez et al. [13], picrocrocin was only identified in the genus *Crocus*, the only edible species of which is *Crocus sativus*. Therefore, this compound can constitute a molecular marker of this spice, the taste of which can not be imitated by other spices and could be used to identify true saffron. With regard to the volatile fraction of saffron responsible for its

aroma, more than 90 compounds were identified, however, safranal $C_{10}H_{14}O$ was the major compound among all of them [8,14]. This compound is the result of picrocrocin conversion by either a two-step enzymatic / dehydration process involving the intermediate HTCC (4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde) or a direct thermal degradation [13,15,16]. According to Himeno and Sano [17] and Iborra [18], safranal was detected in very few plants and it can be generated when certain carotenoids are subjected to a thermal process. Its concentration in saffron is generally between 0.1% and 0.6% [19].

Considering its limited production and cultivation that occurs in cold climatic conditions and high altitudes areas, in addition to the labor-intensive extraction process, saffron is a potential candidate for commercially motivated fraud [20], and it is among the most widely interrogated for purity and authenticity [13].

Various methods are used in the adulteration of saffron in order to obtain excessive profit with low-priced products, including mixing with less valuable species (turmeric), adding various plant materials (corn beard, *Calendila officinalis*, and safflower), and natural or artificial colorants. In addition, the weight of saffron is increased by immersing the fibers in oil honey, glycerin or wax [21,22]. Investigations of authentic and commercial saffron samples of different origins are very important because the biological activity and the pharmaceutical properties of plants are strongly dependent on their structure. Many techniques are used to verify the quality and authenticity of saffron. In addition to conventional methods that involve time-consuming, laborious, costly procedures and chemical reagents, there are new, accurate and fast analytical techniques for evaluating food quality attributes such as FTIR (Fourier transform infrared spectroscopy), NIR (Near-infrared spectroscopy) [23,24]. Furthermore, to detect fraudulent samples and confirm their biological origin, DNA barcoding is useful for the authentication of saffron and remains widely used for the identification of plant species [25,26].

FT-IR spectroscopy has a strong potential in the analysis and quality control of foods because of its sensitivity, versatility, and speed [27,28]. It uses mid-infrared in ranges from 4000 cm^{-1} to 400cm^{-1}. This tool is non-destructive with an easy sample preparation protocol and does not require any hazardous chemicals, and makes qualitative analysis faster and more reliable [29]. As with all spectroscopic techniques, FTIR can be used to identify compounds or to determine the composition of samples and, therefore, the assessment of the quality of foods and their authenticity [30,31]. Moreover, the FTIR spectra are complex and consist of many related variables (wavenumber) by sample, making visual analysis very difficult and complicated. For this, chemometric approaches are generally applied to simplify or reduce the number of

parameters with minimal loss of the total variance, this facilitating their interpretation [32]. Regarding saffron, FT-IR spectroscopy was first used for the characterization of crocins and related apocarotenoids [10]. This technique was also performed for authentication of saffron in terms of geographical origin [33] and for detecting saffron deterioration due to aging [34]. The FT-IR spectra of saffron were obtained and the vibrational fundamentals from the IR spectrum were analyzed and assigned according to the available literature. Furthermore, several methods developed for the investigation of adulteration of saffron with bulking agents of plant origin are based on molecular tools [35,36]. The use of DNA barcoding coupled with sequencing has demonstrated its potential for *Crocus* spp. differentiation [37] and authentication among saffron adulterants [38]. In addition, four primary genetic regions such as rbcL, matK, trnH-psbA and ITS were widely recognized as standard DNA barcode regions for plant species [39]. Moreover, , gas chromatography-mass spectrometry (GC-MS) is among the well-established and widely used methods to identify food aroma compounds. This method is used to quantify safranal in a range of saffron samples [8,9,40–42], for the detection of adulterants [43], and especially for geographical discrimination of saffron samples [41,42]. Therefore, the present study aimed at studying the chemical composition of 11 saffron samples collected from different areas using the chemometric analysis of ATR-FTIR fingerprints and identifying the adulterated saffron among three samples bought from local markets from different countries (Spain, Iran, and Morocco). In addition, molecular analysis (DNA barcoding coupled to sequencing) and chromatographic analysis (GC-MS) were carried out to validate the authenticity of the saffron.

2. Materials and methods

2.1. Plant materials

Eleven dried saffron samples were collected from different areas of the anti-Atlas (Taliouine, Timjicht, Siroua, Khouzama), the high Atlas (Ourika), and the Middle Atlas (Azilal, Ain leuh, Serghina, Ain Atia, and El Mers) (**Figure 1**). Moreover, three commercial samples of *Crocus sativus* were purchased from the local market from Spain (SE), Iran (SI), and from Morocco (SD).

2.2. FTIR-ATR Analysis

Ground and homogenized saffron samples were scanned in the wavelength range of 4000-400 cm^{-1} with a spectral resolution of 4 cm^{-1} using FTIR spectrometer (Perkin-Elmer, USA) and the characteristic peaks and their functional groups were detected. FTIR peak values were

recorded. Each analysis was repeated three times for spectrum confirmation. Furthermore, with the objective of verifying the authenticity of three samples acquired from the market, a comparison with pure saffron was carried out using ATR-FTIR spectroscopy.

On each spectrum obtained, a correction by attenuated total reflectance (ATR correction) was carried out automatically by the PERKIN ELMER software with a basic correction (baseline) in order to correct the multiplicative interferences. To show the difference in absorbance between different saffron samples, the corrected spectrum and the absorbance of the major peaks were plotted using Origin Pro software version 2019.

2.3. DNA extraction, PCR and sequencing

DNA extraction was carried out on dry saffron stigmas using a modified CTAB procedure protocol [44]. The rbcL genes of the genomic DNA of the plant were amplified using two sets of universal primers rbcL a-f (5'ATG TCACCA CAA ACA GAG ACTAAAGC3') and rbcL a-R (5'GTA AAA TCA AGT CCA CCG CG 3') [45]. PCR reactions were performed in a total volume of 25 µl using EnzimaGoTaq DNA polymerase (Bioline, London, UK) according to the manufacturer's instructions. Each PCR test consists of 5 µL (Buffer 5x), 0.25 µL Taq, 1 µL of forward Primer (10µM), 1 µL of reverse Primer (10 µM). The rest of the volume is completed with sterile distilled water. Amplifications were performed following conditions for 35 cycles (95°C for 4 min, 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 min). PCR products were then observed using 1.5% electrophoresis gel. Sequencing analysis was carried out based on the Sanger method. The sequences were then edited and aligned using BioEdit software (version 7.0.5.3), and similarity was checked in Genbank before being deposited, using the Blast program.

2.4. Volatile profile analysis

The volatile components analysis of saffron was performed by gas chromatography-mass spectrometry (GC-MS) using Agilent 7890A system (Wilmington, DE, USA) with mass selective detector 5975 Network MSD and coupled to an MPS automatic sampling system. Chromatographic separation was performed on HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.17 mm), and the following temperature program was used: 60 °C held for 3min, then increased to 210 °C at a rate of 4 °C/min, then held at 210 °C for 15min, then increased to 300°C at a rate of 10 °C/min, and finally held at 300 °C for 15 min. Helium was used as the carrier gas at a constant flow of 1 ml/min. For quantification, the results are presented as a percentage of the peak area considering a response factor of the fiber.

2.5. Statistical analysis

Statistical analysis was performed by SPSS V25 software (version 25, IBM SPSS Statistics 20, New York, NY, USA) on the basis of the integrated peak areas and the results obtained were expressed as the mean±standard deviation. A variance analysis was performed to check if there was a significant difference between samples from the studied areas. Duncan'multiple analysis was used to compare the mean of sample absorbance at a significance level of $p<0.05$. The principal component analysis (PCA) was performed p according to the correlation coefficients and the Varimax rotation method with Kaizer normalization was used in order to identify the most discriminating variables and the total inertia expressed by the model.

3. Results and Discussion

3.1. Analysis FTIR combined with chemometrics of saffron samples of known origin

Infrared spectroscopy is an effective tool to detect different chemical components in food products [46,47]. So different varieties and different processed products would be distinguished by this tool. The FTIR spectrum was used to identify the functional group of active components based on the peak value in the infrared radiation region. It provides valuable information associated with the main secondary metabolites of saffron, namely crocins and picrocrocins, which can represent more than 50% of dried stigmas.

FTIR-ATR spectra of saffron samples are illustrated in **Figure 2** and their assignments are detailed in **Table 1**. The spectra were obtained in a range of 4000 to 400cm^{-1} and six absorbance zones were identified: 3700-3000cm^{-1}, 3000-2800 cm^{-1}, 1800-1725 cm^{-1}, 1700-1500 cm^{-1}, 1500-1185 cm^{-1} and 1185-900 cm^{-1}. According to the results obtained, all the spectra have a normal appearance compared to the spectrum of pure saffron [23,48]

The absorption band at 3350 cm^{-1} corresponds to the stretching vibration of the O-H hydroxyl group. The 3000 - 2800 cm^{-1} region shows two peaks due to asymmetrical and symmetrical stretch vibrations of methylene. The bands in the region of 1775 - 1725 cm^{-1} are due to stretching of the carbonyl and ester groups (**Table 1**). The vibration of the carbonyl group (C=O) related to the crocin content in the saffron samples was observed at 1732 cm^{-1} [49]. The bands at 1650 cm^{-1}were assigned to the –C=C group and conjugated C=O group (e.g. aldehydic C=O of picrocrocin). Another characteristic vibration of saffron, attributed to the C-O stretch of C(=O)-O in the crocin ester groups, is responsible for the band located at 1225 cm^{-1} [48].

The bands in the 1500-1250cm^{-1}region, due to C=C and C-C stretch, are linked to the polyene chain present in the structure of crocetin, known as the main chromophore of saffron [50]. The 1200-700cm^{-1}region corresponds to sugars and polysaccharides, the absorption bands are mainly attributed to the stretching vibrations of the C-O group in saccharides. According to Ordoudi et al. [23], the region between 1175 and 1157 cm^{-1} provides information on the extent of hydrolysis of the main oligosaccharides (gentiobiosis andnapolitanose) and/or polysaccahrides due to storage. Also, the bands at 1028 cm^{-1} associated with the presence of glucose group, as well as intensities in the 1175-1157 cm^{-1} region related to the breakdown of glycosidic bonds, were most useful for monitoring the effects of storage and detection of saffron deterioration.

The main vibration regions are shown separately in **Figure 3** and the average areas obtained by integration are shown in **Table 2**. In the first vibration region (3700-3000 cm^{-1}), the samples showed significant differences ($p<0.01$) with a mean of 14.89 ± 5.31. In the second vibration region (3000-2800 cm^{-1}) which exhibits two peaks due to asymmetric and symmetrical stretching vibrations of methylene, the sample S4 showed the highest absorbance with an average peak area of 7.6 ± 0.10; indicates that the saffron from Timjicht (Taznakht) is rich in carbohydrates, lipids and amino acids (**Figure 3**), followed by S3 and S1 from Ain Leuh (Ifrane) and Askouan (Taliouine) with an average area of 6.50 ± 0.50 and 6.30 ± 2.85, respectively. This finding is in accordance with the results of assays on secondary metabolites, particularly picrocrocins. Furthermore, the analysis of variance of integrated intensities corresponding to the absorption in this region showed significant differences among samples ($p<0.05$). In the region of 1800 to 1725 cm^{-1} corresponding to the carbonyl and ester groups, the highest absorbance and peak area were recorded in sample S4 (Timjicht/Taznakht) followed by S1 and S2 from Taliouine and Serghina (Boulmane), respectively. The vibration of the carbonyl group (C=O) is related to the crocin content as confirmed by [49], using UV spectroscopy. For the bands corresponding to proteins (1700-1500 cm^{-1} and 1500-1185 cm^{-1}),to sugars and polysaccahrides (1185-900 cm^{-1}), the samples with the highest absorbances and areas remain S4 and S1 from Timjicht (Taznakht) and Askouan (Taliouine) followed by S3 and S2 from Ain leuh and Serghina (Boulmane) respectively. Interestingly, the analysis of variance of integrated areas corresponding to the absorption in these regions showed significant differences.

Based on the areas calculated from the regions of vibrations, ATR-FTIR spectroscopy combined with principal component analysis (PCA) allows reducing the size of the data

(multitude of wavelengths) with minimal risk of loss of total variance. Furthermore, PCA is used for preliminary exploratory analysis to visualize the sample distribution and investigate possible patterns owing to saffron purity. In our study, only a principal component load greater than 0.7 was considered to be significant for each factor. The PCA revealed two components that express 95.86% of the variance (**Table 3**). The first component PC1 represented 53.13% of the total variance and was mainly explained by vibration regions attributed to carbohydrates, lipids, amino acids, carbonyl and ester groups, sugars and polysaccahrides. As for the second component PC2, it represented 42.73% of the total variance and corresponded to the absorbance region of proteins and the hydroxyl group.

The scatter plot was built on the basis of two principal components, PC1 and PC2 whose respective expressed variances are 53.13 and 42.73%. Four groups were revealed. The sample from Timjicht (S4) was classified as a single subset in the samples scatter plot, and then samples from Boulmane (S2), Ain Leuh (S3), Taliouine (S6), and Taznakht (S7-S8) were classified close to each other, which indicates the similarity in their vibration intensities mainly in the region of carbohydrates, lipids, amino acids and esters. Similarities in terms of proteins and hydroxyl groups were revealed between the samples from El Mers (S11) and Taliouine (S1). Finally, the last sub-group contained samples from Ourika, Azilal, and Ain Atia, which showed low composition in all components.

3.2. Determination of saffron fraud

3.2.1. ATR-FTIR spectroscopy

To detect adulterated saffron from samples of unknown origin, a comparison of the ATR-FTIR spectra was carried out with spectra of pure saffron. Three samples were acquired from local markets: SD, SI, and SE, and their FTIR spectra were compared with the spectra of the pure saffron (S1 acquired from a producer in Askaoun/Taliouine). The results are presented in **Figure 5**.

As shown in **Figure 6**, FTIR spectra provide important information to differentiate between saffron and other plant material, although common characteristics can be found. The absorption bands at 1706, 1732 and 1225 cm^{-1} are linked to crocin. These characteristic peaks are absent in SI which is assumed to be from Iran. The characteristic absorption band of –C=C group and conjugated C=O group (e.g. aldehydic C=O of picrocrocin) located at 1650 cm^{-1} in saffron is shifted to 1654 cm^{-1}. This means that it is another plant material other than the *Crocus sativus*. Regarding the sample originating from Spain (SE), the FTIR spectra show the same appearance

as that of pure saffron (S1). The peak characterizing the saffron in the 2000-500 cm $^{-1}$ region and providing important information on secondary metabolites in particular crocins and picrocrocins are also present which demonstrates its authenticity.

For the last one supposed to originate from Morocco (SD), the FTIR spectra show some difference, especially at 1225 cm^{-1}. The 1200–700 cm^{-1} range is mostly correlated with the presence of sugars and polysaccharides and the spectra in this region are different from that of pure saffron. This can lead to the conclusion that SD could be saffron mixed with another plant material.

3.3. DNA barcoding

To verify the aforementioned results, tests for species authentication were performed using the rbcl marker DNA barcoding method as described below. The phylogenetic relationships of the different samples are shown in **Figure 7**. The results showed that the 13 samples were identified as saffron plants, namely *Crocus sativus*; and only one was identified as *Arrhenatherum elatius* which is the sample 'SI' bought from the local market and supposedly from Iran. Phylogeny tree analysis (**Figure 7**) has shown that all the samples identified as saffron were clustered with the reference *Crocus sativus* (MN606017), while the sample that was not saffron was clustered with its reference *Arrhenatherum elatius* (MF596585). This confirms our results obtained by FTIR-ATR spectroscopy, which revealed that the spectrum of SI is different from that of *Crocus sativus*. **Table 4** shows the identity of the samples and their GenBank accession number and the percentage of similarity.

Currently, consumer awareness is growing on food safety, and they are increasingly interested in knowing the source, the origin, and the quality of the foods they eat. For this and in order to keep them informed regarding their diet and the type of food they buy from retailers, food traceability and validation have become key issues [51]. Saffron is one of the most expensive spices used in the food industry and it is highly regarded for its properties of taste, flavor, color, and health benefits. These valuable properties, combined with a limited production, increased its market value and induced various adulterations [20].

To detect fraudulent samples and confirm their biological origin, DNA barcoding is a useful tool for the authentication of saffron and remains widely used for the identification of plant species) [25]. In our study, the rbcL gene was used as a barcode gene for the identification of saffron. This approach has proven to be effective in determining the samples' origin and their

quality, detecting adulteration of substandard materials present in the food, and protecting the consumer from these unfair practices [52*]*.

According to the results obtained, 13 samples, including 2 saffron bought respectively at the local market (SD) and at the supermarket (SE of Spain) are real saffron identical to the species *Crocus sativus* while the sample bought in the local market and supposed to be an Iranian origin and which turns out to be of another plant species named *Arrhenatherum elatius* used as adulterants with saffron. This DNA barcode approach is widely applicable because it is based on the genetic traceability of plant material using sequences from the DNA segment of the plastid genome found in each cell of a plant. Previous reports indicate that plant materials commonly used in the adulteration of saffron are corn beard, *Calendila officinalis, Carthamus tinctorius (*safflower), *Curcuma longa L.* (turmeric) [13,21,53], other illegitimate materials have been reported such us : *Buddleja officinalis, Gardenia jasminoides, Arnica montana*, petals of *Hemerocallis* spp, seeds of *Bixa Orellana* [36], and *Morhusalba, Pistaciavera, Triticum aestivum* and *Erichloa spp.* [52]. Finally, in this research, new plant material (*Arrhenatherum elatius*) was identified as an adulterant of saffron. To our knowledge, it was never reported by other authors.

3.4. Gaz chromatography –Mass spectrometry

In order to validate the results obtained by both the FTIR-ATR analysis and the molecular analysis, the volatile profile of the four samples was analyzed by GC-MS in order to detect the adulterated samples. The results are presented in Table 5. The results showed that S1 and SE samples contain several volatile compounds, which are naturally present in saffron. The terpenes were the most abundant in both samples, with monoterpenic aldehydes (such as Safranal) and monoterpenic ketones (such as isophorone, and 4-ketoisophorone) being the most represented groups, which are in line with reported data [8,54]. This finding confirmed our results obtained by FTIR and barcoding techniques.

Safranal is the most volatile component of saffron and constitutes about 60% of the volatile fraction [55]. Our study revealed that the content of safranal was 52.21% and 83.53% in S1 and SE, respectively. As for isophorone, their values were 15.41% and 2.14% in S1 and SE, respectively. The SD sample, whose FTIR spectrum was different from the normal spectrum of saffron, had a low level of safranal (10.17%). The results revealed the presence of other compounds, which are not naturally occurring in saffron, in particular Alpha-muurolene, Beta-bisabolene and Delta-Cadinene. These were inconsistent with molecular analysis results, which

showed that SD was a *Crocus sativus*. This demonstrates that barcoding DNA can be trusted alone as not a discriminating technique when saffron samples are blended with other plant species because PCR amplification is done from a molecule. Furthermore, the SI sample, which is not pure saffron as shown by both spectral and molecular analyses, exhibited a different volatile profile with the presence of strange compounds that not occurring naturally in saffron, in particular menthol (46.77%) and p-Menth-3-ene (2.21%). The SI sample had also low level of safranal (39.93%) and isophorone (1.48%). Thus, validating results obtained by FTIR analysis and barcoding DNA. The presence of safranal and isophorone in low content confirms that it is adulterated saffron.

4. Conclusion

Fourier transform infrared (FTIR) spectroscopy is a non-destructive, rapid, and promising method for the assessment of the quality of saffron as well as detecting any potential adulteration. The ATR-FTIR spectra analysis revealed six fingerprint regions in which, all saffron samples showed statistically different vibrations intensity expected in 1800-1725 cm^{-1}. The sample of Timjicht (Taznakht) showed a typical difference, which exhibited the highest carbohydrates, lipids, amino acids, carbonyl and ester groups. ATR-FTIR analysis revealed its strong potential in the determination of saffron fraud compared with traditional methods, which are costly and time-consuming. Furthermore, the rbcL marker DNA barcoding method can remain indiscriminate in the case of a mixture of plant species and made it possible to identify *Arrhenatherum elatius*is as an adulterant of saffron. Therefore, it was concluded that the FTIR analysis validated by the robust GC-MS analysis, gave more reliable results than the barcoding DNA in the detection of adulteration in saffron samples.

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Declarations

Conflict of interest: There are no conflicts to declare

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Figure captions

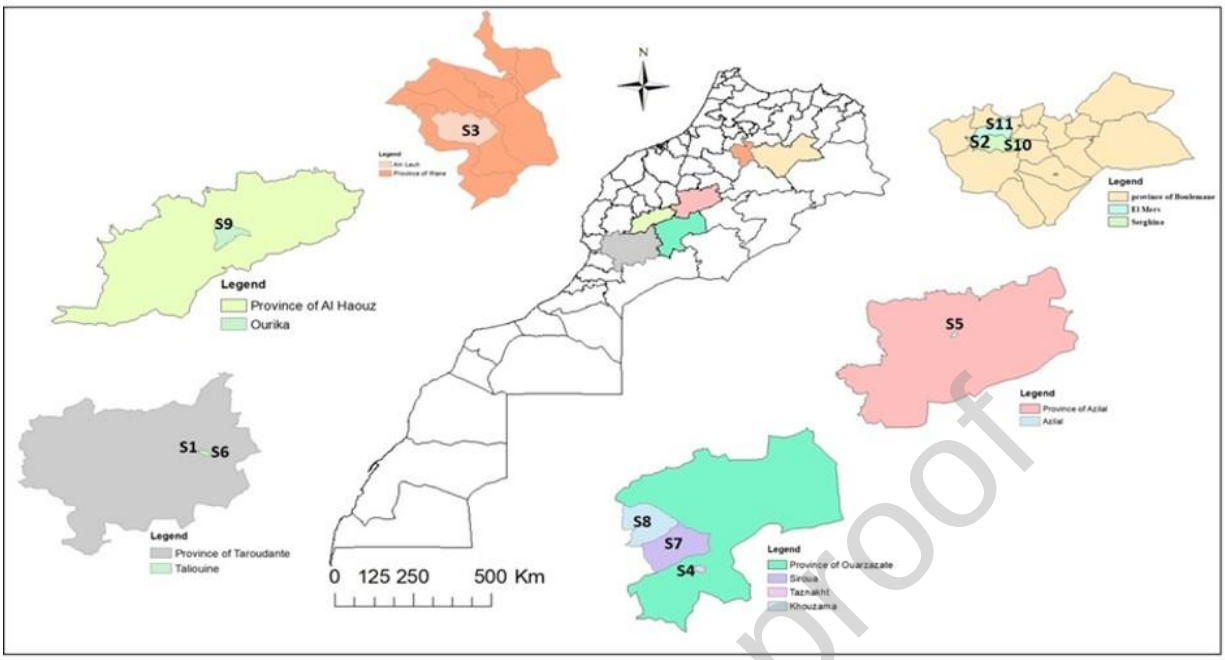

Figure 1. Map showing the location of sample location zones

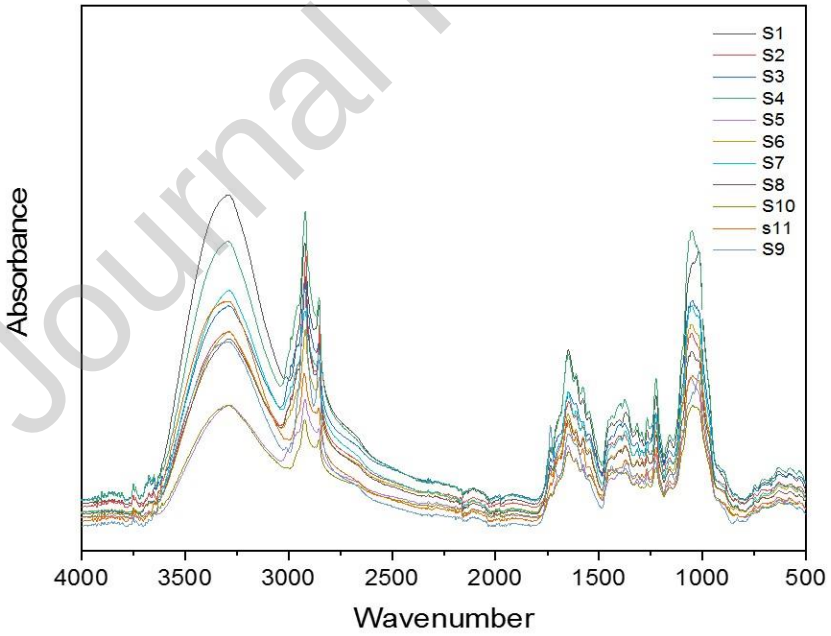

Figure 2. FTIR spectra of saffron samples with absorbance value of 4000-500 cm^{-1}

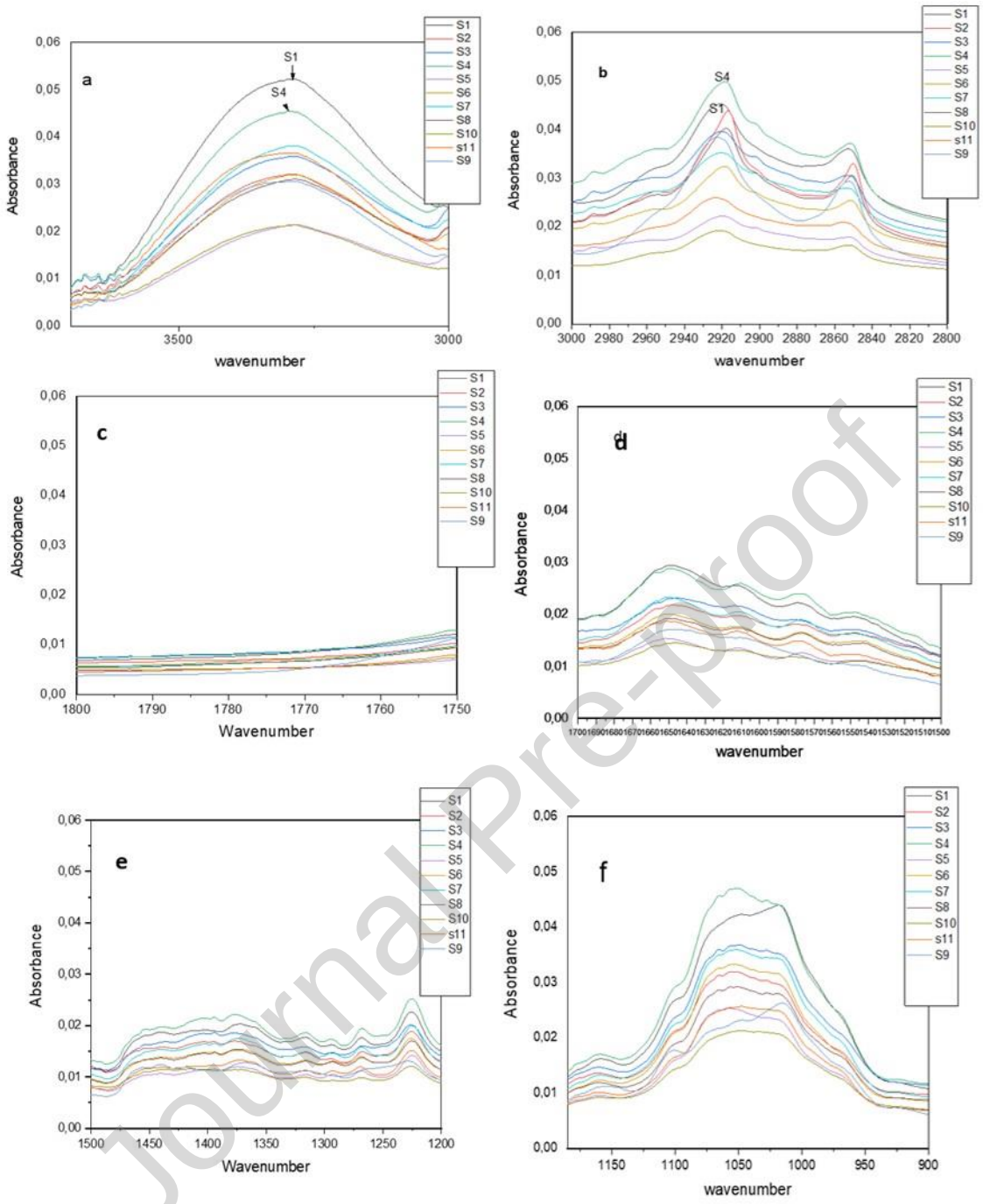

Figure 3. Profils of the FTIR spectrum of different saddron samples: a-f refer to the following major vibration regions: 3700 -3000, 3000-2800, 1800-1725, 1700-1500, 1500-1185 and 1185-900 cm^{-1}

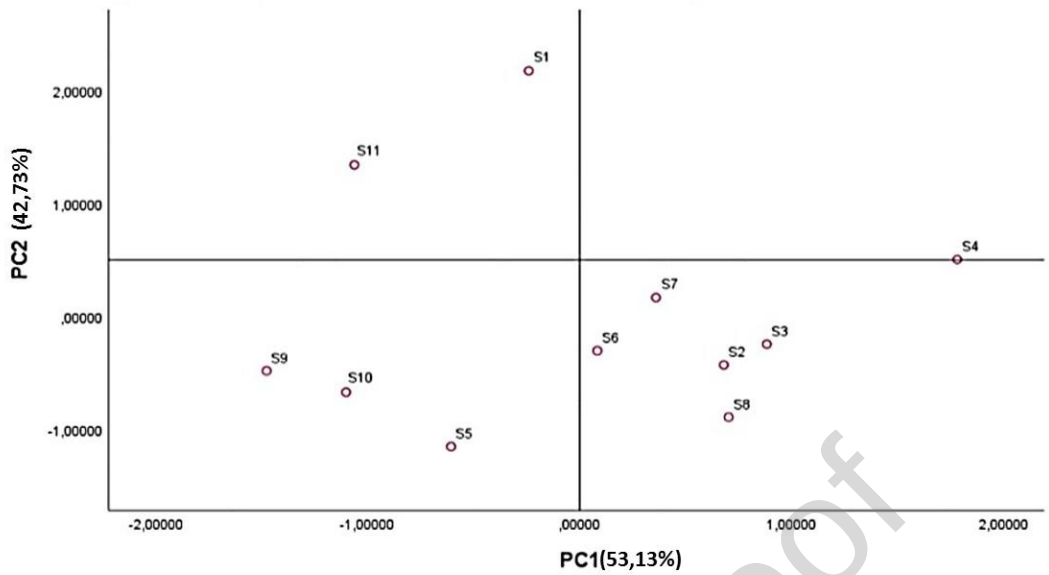

Figure 4. Scatter plots of saffron samples performed on average integrated areas of each fingerprint regions (3700–3000, 3000–2800, 1800–1725, 1700–1500, 1500–1185 and 1185–900 cm^{-1}) collected using ATR–FTIR spectra

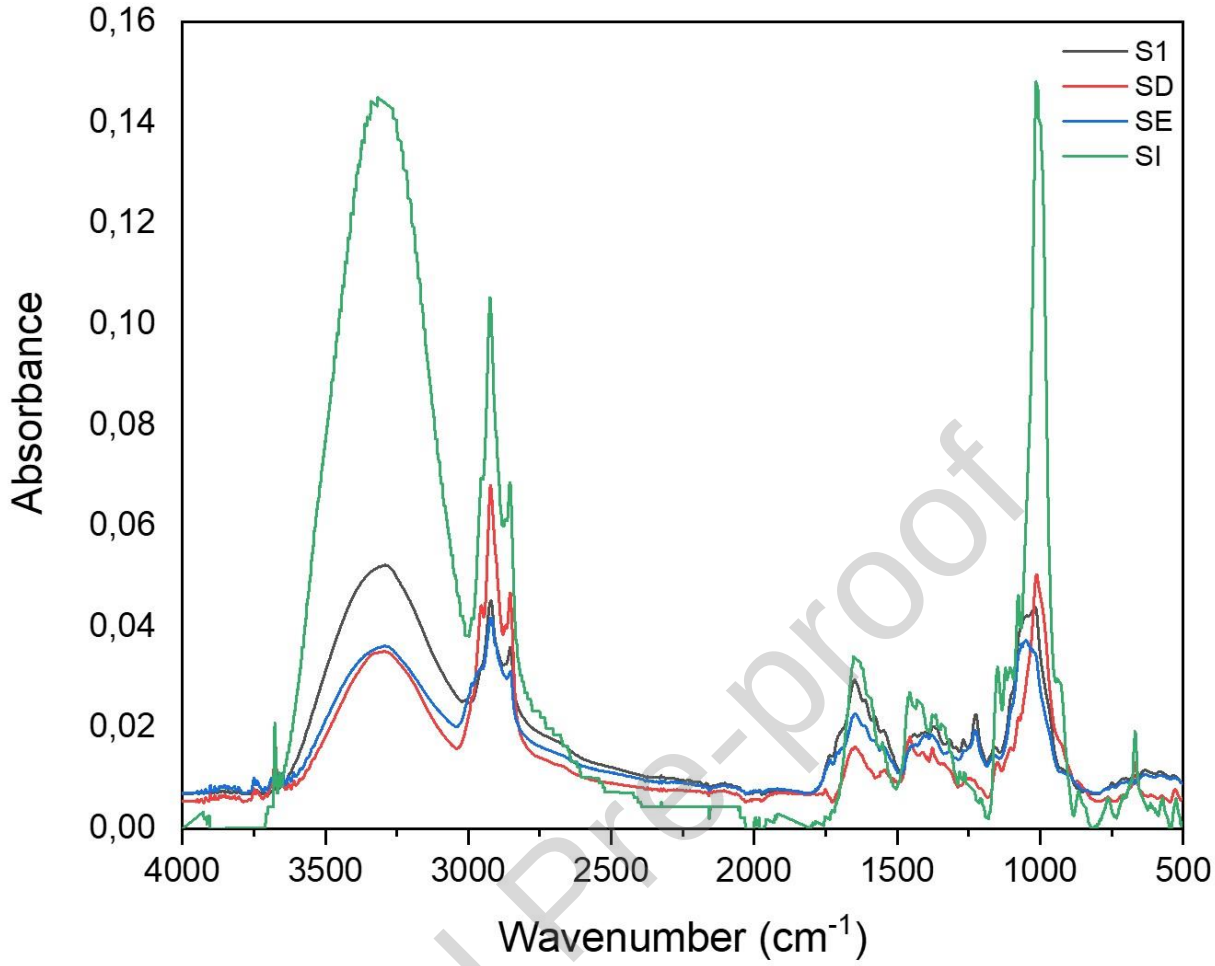

Figure 5. FTIR spectra of pure saffron (black line) and other samples acquired from the local market (SD, SE, and SI) over the spectral region 4000–400 cm^{-1}

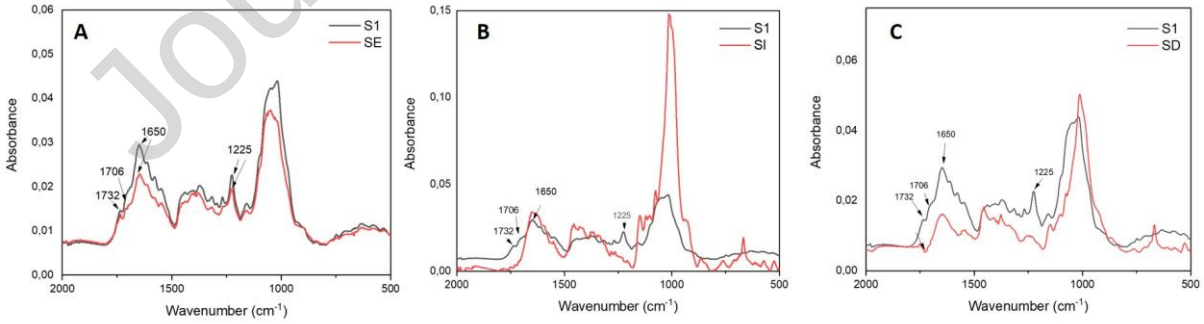

Figure 6. FTIR spectra of pure saffron (black line) and other samples (Redline) acquired from the local market (SD, SE, and SI) over the spectral region 2000–500 cm^{-1}

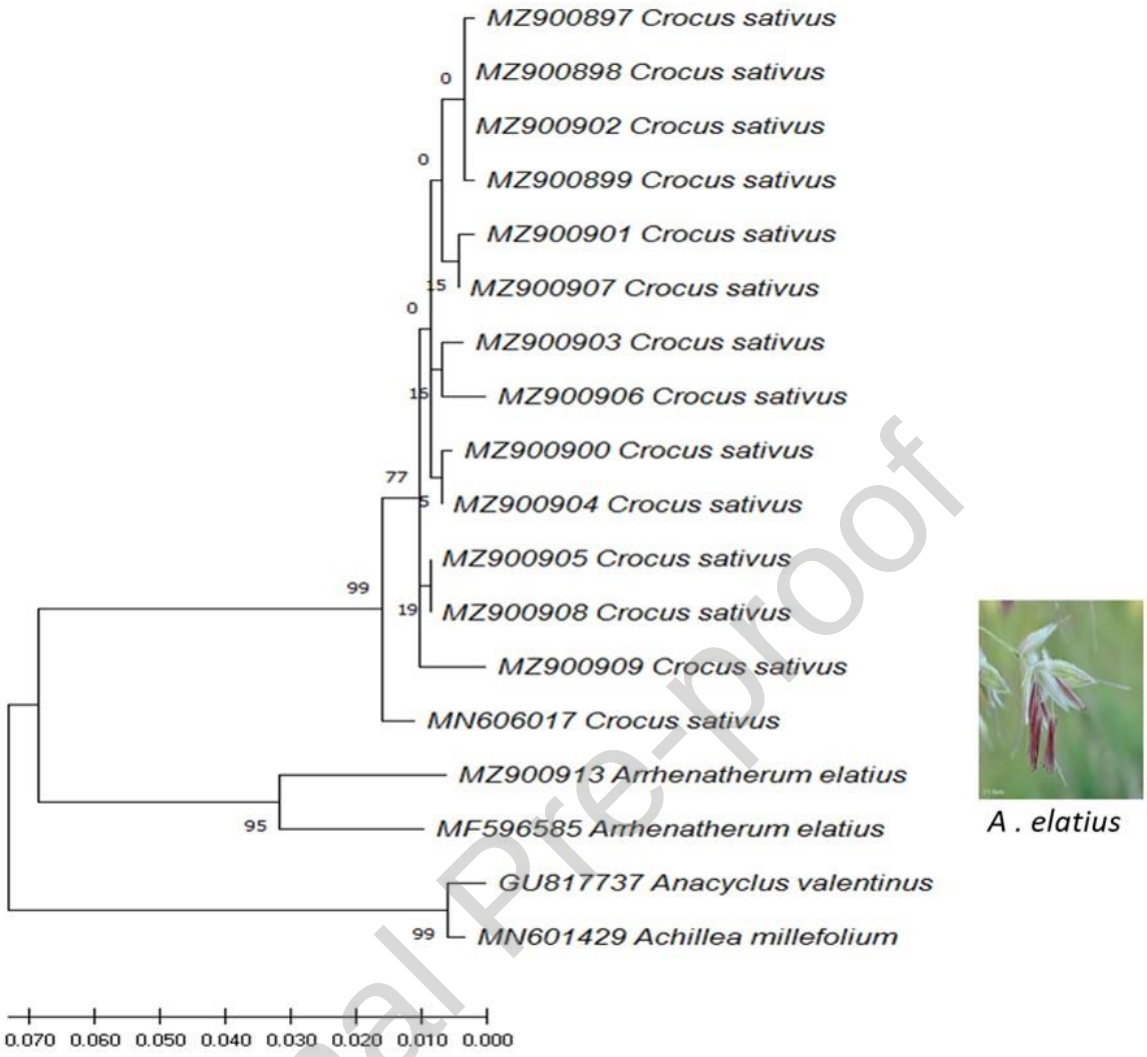

Figure 7. Phylogenetic identification of saffron and its adulterant

Table 1. Wavenumber ranges and their tentatively dominating chemical compounds summarized from Wilson et al. (2000) , Naumann et al. (2010) and Lee et al. (2015) [29,49,56].

Wavenumber range (cm^{-1})	**Assignments to chemical-compound**	**Functional groups**
3700-2996	Various	O–H. N–H
2996-2800	Lipids, carbohydrates	C–H,

1800-1485	Proteins	Amid I + II
1485-1185	Proteins, lipids. phosphate	CH2, CH3 P= O.
1775-1725	Groups of carbonyls and esters	C=O
1185-900	Cellulose, hemicelluloses. sugars	C−O−C, C−O, C−C, C−H

Table 2. Integrated ATR spectra in the major absorption bands of saffron samples

Samples	**Main vibration regions (wavenumbers cm^{-1})**					
	3700-3000	**3000- 2800**	**1800-1725**	**1700-1500**	**1500-1185**	**1185- 900**
S1	23.31±1.02^{d}	6.30±2.85^{de}	0.97±0.42^{bc}	4.41±1.70^{a}	5.37±1.95^{e}	7.17±2.74^{e}
S2	14.41±1.49^{abc}	5.72±1.16^{de}	0.92±0.16^{bc}	3.54±0.32^{abc}	4.54±0.49^{de}	5.45±0.44^{cde}
S3	15.86±0.90^{abc}	6.50±0.50^{de}	0.79±0.01^{ab}	3.76±0.24^{bc}	5.01±0.30^{de}	6.28±0.33^{de}
S4	19.84±0.37^{cd}	7.60±0.10^{e}	1.21±0.07^{c}	4.59±0.18^{c}	5.72±0.09^{e}	7.57±0.10^{e}
S5	9.19±1.85^{a}	3.77±0.49^{abc}	0.56±0.12^{a}	2.51±0.49^{a}	3.14±0.52^{abc}	4.07±0.62^{abc}
S6	13.76±0.99^{abc}	5.02±0.24^{cd}	0.82±0.17^{ab}	3.25±0.20^{ab}	4.07±0.25^{cde}	5.37±0.29^{cde}
S7	16.29±2.36^{dc}	5.83±0.67^{de}	0.92±0.11^{bc}	3.72±0.47^{bc}	4.43±0.53^{cde}	5.82±0.72^{cde}
S8	13.43±1.29^{abc}	5.79±0.39^{de}	0.92±0.09^{bc}	3.16±0.26^{ab}	3.96±0.31^{bcd}	5±0.39^{abc}
S9	9.46±1.26^{ab}	2.57±0.31^{a}	0.66±0.08^{ab}	2.55±0.0^{a}	2.65±0.37^{a}	3.37±0.58^{a}
S10	9.64±1.74^{ab}	2.92±0.56^{ab}	0.66±0.08^{ab}	2.49±0.38^{a}	3.05±0.46^{ab}	3.75±0.60^{ab}
S11	18.58±1.66^{cd}	4.61±0.74^{bcd}	0.89±0.16^{bc}	3.52±0.43^{abc}	4.03±0.55^{cde}	5.22±0.84^{bcd}
Average	14.89±5.31	5.15±1.72	0.85±0.22	3.41±0.85	4.18±1.11	5.37±1.51
Average square ANOVA	61.53**	7.25**	0.09	1.53*	2.83**	5.27**

*,significant difference at the 0.05 level, **, significant difference at the 0.01 level. Data represent mean ± standard deviation (SD). Values followed by different letters (a- b- c- d-e) in the same column are significantly different according to Duncan's test ($p < 0.05$)

Table 3. Principal component (PC) variation load factor in the studied saffron samples

Main vibration regions	Component	
	PC1	PC2
3700-3000 cm^{-1}	0.443	**0.892**
3000-2800 cm^{-1}	**0.902**	0.409
1800-1725 cm^{-1}	**0.727**	0.555
1700-1500 cm^{-1}	0.684	**0.724**
1500-1185 cm^{-1}	**0.788**	0.595
1185-900 cm^{-1}	**0.749**	0.644
Variance (%)	53.13	42.73
Cumulative (%)	**95.86%**	

Eigenvalues higher than 0.7 are mentioned in bold

Table 4. Identified species and their accession number in the Genbank.

Isolate	Identity	Accession number	Similarity (%)
S1	*Crocus sativus*	MZ900897	99
S2	*Crocus sativus*	MZ900898	99
S3	*Crocus sativus*	MZ900899	99
S4	*Crocus sativus*	MZ900900	99
S5	*Crocus sativus*	MZ900901	99
S6	*Crocus sativus*	MZ900902	99
S7	*Crocus sativus*	MZ900903	99
S8	*Crocus sativus*	MZ900904	99
S9	*Crocus sativus*	MZ900905	99
S10	*Crocus sativus*	MZ900906	99
S11	*Crocus sativus*	MZ900907	99
SD	*Crocus sativus*	MZ900908	99
SE	*Crocus sativus*	MZ900909	99

SI	*Arrhenatherum elatius*	MZ900913	99

Table 5. List of the identified volatile compounds in saffron samples

Sample	Compound	CAS number	RT	RI calculated	% Peak area
S1	2(5H)-Furanone	497-23-4	6.752	918	1.62
	2-ethyl-1-hexanol	104-76-7	9.575	1032	0.34
	Nonanal	124-19-6	11.534	1106	0.43
	2,6,6-trimethylcyclohexa-1,4-dienecarbaldehyde	162376-82-1	11.634	1110	0.71
	3,5,5-trimethyl-2-cyclohexenone (isophorone)	78-59-1	12.116	1130	15.41
	2,6,6-trimethyl-2-cyclohexene-1,4-dione (ketoisophorone)	1125-21-9	12.722	1154	14.48
	2-hydroxy-3,5,5-trimethylcyclohex-2-enone	4883-60-7	12.781	1156	0.29
	2-hydroxy-4,4,6-trimethylcyclohexa-2,5-dienone	28750-52-9	13.145	1170	0.28
	2,6,6-trimethyl-1,4-cyclohexanedione	20547-99-3	13.393	1179	9.26
	3,4-dimethylbenzaldehyde	5779-95-3	13.757	1192	0.20
	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal)	116-26-7	14.275	1213	52.21
	4-methylene-3,5,5-trimethylcyclohex-2-enone	20548-00-9	14.616	1228	0.57
	2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	35692-98-9	14.904	1240	0.50
	4-hydroxy-3,5,5-trimethylcyclohex-2-enone	14203-59-9	16.733	1313	0.94
	2,4,4-trimethyl-3-carboxaldehyde-5-hydroxy-2,5-cyclohexadien-1-one	35692-95-6	18.716	1398	1.79
	4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde	35692-94-5	19.357	1428	0.30

	2,6-Di-tert-butyl-1,4-benzoquinone	719-22-2	20.398	1474	0.16
	1-(3,5-di-tert-butyl-4-hydroxy-phenyl)	14035-34-8	23.957	1645	0.51
SI	1-carboxaldehyde-5,5-dimethyl-2-methylene-3-cyclohexene	691012-58-5	11.616	1109	1.16
	3,5,5-trimethylcyclohex-2-en-1-one (isophorone)	78-59-1	12.005	1125	1.48
	3,5,5-trimethyl-2-cyclohexene-1,4-dione	1125-21-9	12.587	1149	2.40
	Isomenthone	491-07-6	12.869	1160	0.45
	2,6,6-trimethyl-1,4-cyclohexanedione	20547-99-3	13.205	1172	2.91
	Menthol	15356-70-4	13.416	1180	46.77
	dodecane	112-40-3	13.963	1200	0.97
	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal)	116-26-7	14.116	1206	39.93
	2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	35692-98-9	14.875	1239	0.62
	p-Menth-3-ene	500-00-5	16.351	1297	2.21
	2,4,4-trimethyl-3-carboxaldehyde-5-hydroxy-2,5-cyclohexadien-1-one	35692-95-6	18.645	1395	0.58
	4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde	35692-94-5	19.339	1427	0.52
SD	3,7-dimethyldecane	17312-54-8	11.457	1102	16.96
	1-carboxaldehyde-5,5-dimethyl-2-methylene-3-cyclohexene	691012-58-5	11.593	1108	9.69
	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal)	116-26-7	14.057	1204	10.17
	4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde	35692-94-5	19.422	1431	24.03
	Alpha-muurolene	31983-22-9	21.151	1508	15.70
	Beta-bisabolene	495-61-4	21.274	1514	11.27

	Delta-cadinene	483-76-1	21.645	1532	12.18
SE	3,7-dimethyldecane	17312-54-8	11.458	1102	0.365
	1-carboxaldehyde-5,5-dimethyl-2-methylene-3-cyclohexene	691012-58-5	11.628	1110	2.011
	3,5,5-trimethylcyclohex-2-en-1-one (Isophorone)	78-59-1	12.01	1126	2.14
	3,5,5-trimethyl-2-cyclohexene-1,4-dione	1125-21-9	12.587	1149	3.025
	2,6,6-trimethyl-1,4-cyclohexanedione (Kétoisophorone)	20547-99-3	13.21	1172	0.765
	2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde (safranal)	116-26-7	14.175	1209	83.537
	4-methylene-3,5,5-trimethylcyclohex-2-enone	20548-00-9	14.575	1226	1.269
	2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione	35692-98-9	14.881	1239	1.267
	4-hydroxy-3,5,5-trimethylcyclohex-2-enone	14203-59-9	16.687	1311	1.429
	2,4,4-trimethyl-3-carboxaldehyde-5-hydroxy-2,5-cyclohexadien-1-one	35692-95-6	18.663	1396	1.143
	4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde	35692-94-5	19.345	1427	1.122

Author Statement

Nadia Naim: Conceptualization, Methodology, Software, data curation, Writing original draft preparation. **Nabil Ennahli**: Data curation, Writing- Original draft preparation. **Hanine Hafida**: Superivion. **Rachid Lahlali**: Visualization, Investigation, Supervision, Writing- Reviewing and Editing. **Abdessalem Tahiri***:* Visualization, Investigation. **Marie Laure Fauconnier**: Software, Validation, Data Curation. **Ilham Madani**: Supervision. **Said Ennahli:** Visualization, Investigation, Supervision, Writing- Reviewing and Editing,

Declaration of interests

☒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.

Highlights

- FTIR characterization of 11 samples of Moroccan saffron
- Identification of the adulterated saffron among 3 samples bought from local markets in different countries (Spain, Iran and Morocco)
- Validation of the authenticity and the purity through a molecular analysis (DNA barcoding coupled to sequencing)
- GC-MS confirmed the results obtained by FTIR