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Quantification of saffron (*Crocus sativus* L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions

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ABSTRACT

The primary goal of this study was to propose saffron as a sustainable substitute crop with high added value in some Moroccan agricultural areas with low and erratic rainfalls, for their socio-economical development. The quality of the saffron spice has to be evaluated prior to recommendation for commercial production. For this purpose, saffron was grown in experimental plots for the first time in eleven different experimental zones with a disparity of altitudes, soils and climates. High-performance liquid chromatography (HPLC) was used to quantify the most important saffron components crocins, picrocrocin, and safranal which are respectively responsible for its colour, taste and odour. The respective average values, in % dry matter, across all sites altogether are 29.01 ± 5.6; 14.04 ± 7.1 and 0.22 ± 0.11. The statistical analysis shows that crocins are stable under each specific environment tested ($p > 5\%$) for 3 years of study. Meanwhile, there was a large variability in safranal content for the same period ($p < 0.05$). This suggests that post-harvest processing of saffron produced under different environments may need to be improved. Analysis of environmental impact on saffron quality showed that just the altitude affects crocins ($R^2 = 0.84$, $p < 0.05$).

1. Introduction

The flowers of saffron (*Crocus sativus* L.), a plant from the family Iridaceae, possess red-orange tripartite stigmas. This triploid sterile monocot species is not known to grow in the wild, but has been cultivated for its stigma for a long time. It is highly valued as a culinary spice for its flavouring and colouring properties (Rios et al., 1996), and is the subject of ongoing scientific research for its potential medicinal properties. Interest in the impact of saffron carotenoids on human health is growing due to their high antioxidant capacity (Abdullaev, 2002; Pham et al., 2000; Verma and Bordia, 1998).

Saffron is a perennial crop well adapted to arid and semi-arid lands which produce stigmas annually. It is also adaptable to temperate and sub-tropical climates, and can be grown on soils varying from sandy to well-drained clay loams. It blooms in autumn and spends a long period of dormancy (aestivation) in the summer. It is said to be native to the Mediterranean environment that is characterized by cool to cold winters, with autumn–winter–spring rainfall, and warm dry summers with very little rainfall. The Mediterranean environment is recognized worldwide as the best region to produce saffron, with regards to its quality, which is attributed to many factors. However, in Europe, especially in the Mediterranean basin, saffron production faces a crisis. The production of saffron has decreased due to the rise in labour costs which has made production unprofitable in spite of its high market price. The result is a significant decrease in areas for growing saffron during the last few decades in some traditional saffron producing regions in Europe (Negbi, 1999). This situation is advantageous to Morocco where this crop requires mostly family labour and, while it is the only country in Africa and in the Middle East that grows saffron.

The use of Moroccan saffron for medicinal purposes has a long history and has been practiced for centuries (Migration et Développement, 2006); however, its area of production is limited to a small region in the south, especially in the Anti-Atlas Mountains, on about 600 ha. The main region of its implantation lies in the Taliouine zone (Altitude 1200–1630 m, latitude 30°26′N and a longitude of 8°25′W), a remote area in the Southwest of Morocco (Ait Oubahou and El Otmani, 1999) with cold winters and hot summers. The temperature varies between −2 °C during winter and 45 °C during summer. Rainfall ranges annually from 100 to 300 mm. The average yield varies from 2 to 6 kg/ha during its 5–6 years of soil occupation, with a maximum reached between

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the third to the fifth year. Higher yields are achieved on some well-maintained plots (10–12 kg/ha). The annual production is about 3 tons. Saffron production in Morocco is carried out traditionally without the use of mineral fertilization or pesticides. The major saffron production is marketed at a national level.

Saffron quality depends on the concentration of its three major metabolites providing the unique colour and flavour to the stigmas. Picrocrocin ($C_{16}H_{26}O_7$) is considered to be the main bitter principle of saffron. It is a monoterpene glycoside precursor of safranal ($C_{10}H_{14}O$), the major volatile oil responsible for the aroma. β-Glucosidase action on picrocrocin liberates the aglycone, 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC, $C_{10}H_{16}O_2$)) which is transformed to safranal by dehydration during the drying process of the plant material (Lozano et al., 2000; Winterhalter and Straubinger, 2000). The UV absorption maximum for picrocrocin is 254 nm (Alonso et al., 1999). The aroma of saffron comes from an essential oil, which is primarily composed of the terpene aldehyde, safranal, being the most abundant volatile component in the stigmas of saffron (>60% of essential oil) (Roedel and Petrzika, 1991; Tarantilis and Polissiou, 1997). The absorbance maximum for safranal is 330 nm. The dye substances collectively referred to as the crocins, come from the water-soluble glycosidic *cis*- and *trans*-carotenoid crocin, glucosyl esters of crocetin. Crocins dissolve easily in water to provide an orange-red solution. This is the reason for its application as a food colorant. The absorbance maxima of crocins are at about 440 nm in distilled water (ISO/TS 3632, 2003).

Many methods of saffron component analysis have been described (Tarantilis et al., 1995). The chemical composition of saffron samples from many countries indicates that the values reported are strongly dependent on the methods employed for drying, extraction and analysis (Kanakis et al., 2004; Lozano et al., 2000; Zareena et al., 2001). The method of saffron quality characterization currently recommended by the International Standardization Organization is UV–vis spectrophotometry (ISO/TS 3632, 2003). Unfortunately, the method is non-specific and unable to adequately separate between genuine and adulterated saffron, and thus unable to provide a quality category on the international market (Lozano et al., 1999; Zougagh et al., 2005a). Various analytical methods have been developed including thin-layer chromatography (TLC) (Sampathu et al., 1984); reverse-phase high-performance liquid chromatography (RP-HPLC), coupled with a UV–vis detector or, more often, a photodiode array detector (PDA) (Caballero-Ortega et al., 2007), with mass spectrometry (Tarantilis et al., 1995) and gas chromatography (GC), with a mass spectrometer (MS) detector for the volatiles (Narasimhan et al., 1992; Tarantilis and Polissiou, 1997). Others methods such as near infrared spectroscopy (NIR) (Zalacain et al., 2005), nonaqueous capillary electrophoresis (CE) (Zougagh et al., 2005b) and proton nuclear magnetic resonance (^{1}H NMR) (Assimiadis et al., 1998; Tarantilis and Polissiou, 2004) have been developed with some success.

The method used in this work for the quality determination of saffron is high-performance liquid chromatography (HPLC) with PDA detector. This method is the most efficient analytical technique for the analysis of sensitive compounds in complex extracts of natural products (Alonso et al., 2001).

This research is the first to be conducted on Moroccan saffron for quality analysis under different environments for a possible extension of the regions where this valuable crop can be grown. The main goal is to support a recommendation of saffron as a substitute crops for the socio-economic development of some deprived rural areas. Saffron can be used as an alternative crop for the diversification of agricultural production as a way to improve the quality of farm life by its relatively high profit, especially for women farmers who are most often utilized in the picking of flowers and subsequent stigma sorting. It could also ensure the sustainable use and conservation of arid area since it is a perennial culture which is adapted to erratic environments. The objective of the work is to determine the range of variation in the main saffron compounds as influenced by environment and to determine the region that yields the highest saffron quality based on crocins level.

2. Materials and methods

2.1. Corms collection

Prior survey work on saffron cultural techniques, yields and problems identification in the main saffron growing zone in the south region of Morocco was done in September of 2005. Corms were collected in fields and growers' reserves. In addition, further information on cultural techniques used in the region was recorded from local specialists and through many interviews with farmers so as to conduct the experiments in the same manner as done by farmers.

2.2. Stigma collection

The experiments were conducted on *Crocus* stigmas grown under diverse environments during 2005, 2006 and 2007. The flowers on each experimental plot were picked by hand at approximately the same time of day (from 6 to 8 am). Methods for removal of the stigma from flowers and drying conditions were kept identical to the methods used by farmers in the main saffron Moroccan regions. Stigmas were brought indoors where they were separated by hand shortly after collecting in the field, and were dried, in shade, for 8–10 days. Afterward, stigmas were weighed for yield determinations and analyzed for quality determination.

Commercial Moroccan Saffron from "Taliouin" (Anti-Atlas), the main saffron growing zone in Morocco and "Le Safranier d'Ourika"

Table 1
Characteristics of the trials sites in comparison with control site (Taliouin).

Sites number	Site name	Altitude (m)	Clay (%)	Sand (%)	OM (%)	P (ppm)	K (ppm)	Soil pH (solvent:water)
S1	Marrakech	1300	13	53	4.1	211.1	522.2	7.8
S2	Rabat	75	19	72	3.5	106.5	466.7	7.9
S3	Merchouch	398	35	12	2.2	70.2	524.2	7.6
S4	Koudia	200	16	55	1.9	78.6	575.7	7.5
S5	Larache	47	11	86	2.8	28.1	89.2	6.8
S6	Ouezzen	614	25	50	3.7	24.1	276.0	7.8
S7	Chaouen	600	30	53	2.6	159.8	1087.3	7.0
S8	Oulmes	1135	25	44	4.5	1.2	178.4	6.2
S9	Meknes	714	35	26	5.7	438.2	1157.0	7.7
S10	Settat	397	15	32	3.7	24.12	276.0	7.8
S11	Taounate	509	40	14	1.7	34.8	331.8	7.9
C1 (control 1)	Taliouin	1630	23	22	1.3	17.0	412.5	8.3

OM: organic matter; P: phosphorus; K: potassium.

in Marrakech (High Atlas) were analyzed in order to compare their composition with that of saffron obtained from experimental trials. The samples were bought from known farmers and are not adulterated.

2.3. Experimental conditions

11 sites with a diversity of climates, soils, and altitudes were chosen for saffron cultivation (Table 1, Fig. 1). The study was conducted at five of Morocco's National Institute of Agronomic Research (INRA) experimental field stations and six individual farmers. The sowing trials (100 m^2 in each site) consisted of 2 m × 3 m raised beds with rows 20 cm apart. Three replicates were used. Sets of three corms are planted at a distance of 10 cm within rows. The planting depth is about 15 cm. Sowing density is about 3.5 tons of corms/ha. About 20–30 tons/ha of farm manure were applied during the ploughing. Afterward, manure is applied at a rate of 30 tons/ha after the crop establishment. Irrigation was initiated during the first week of September and applied every 15 days until the flowering period. Two to 3 more irrigations were added till the end of the saffron cycle depending on rainfall. Weeds are controlled by hand. No mineral fertilization was applied for correcting soils. Soil properties analyses were done at some of the experimental sites. Meteorological data were collected from some classical meteorological stations located in the region were experimental sites were located. Meteorological station representative of the experimental site S4 and S6 were not available. Soil properties and climatic data indicate the range of conditions of our trials. The selection of fields' locations at the farm level was done with the active participation of farmers.

2.4. Plant materials and chemicals

Saffron stigma samples collected from various locations (zones) in Morocco and two commercial saffron samples were analyzed for quality. Two standards, safranal 88% and crocins were purchased from Sigma–Aldrich (St. Louis, MO). Picrocrocin was purified as described below. HPLC Methanol (MeOH: UV c.o.205) and HPLC acetonitrile (ACN: UV c.o.190) were purchased from Fisher Chemicals (Pittsburgh, PA). Deionized water 18.98 MΩ resistance, used as HPLC mobile phase, was provided by the Solution 2000 water purification system.

2.5. Sample preparation

Saffron stigma were placed in a Pyrex 100 mm × 50 mm glass dish and cut using a razor blade to "grind" the saffron as evenly as possible. Ground plant material was removed by using a spatula and paintbrush into pre-weighed 20 mL scintillation vials. Approximately 50–60 mg of each saffron sample was weighed in a 25 mL volumetric flask for extraction.

For the determination of crocins, safranal and picrocrocin in saffron, samples were extracted with 8 mL of degassed methanol and sonicated for 1 h and then stored overnight. The whole process is carried out in darkness and at room temperature. Samples were removed from darkness, sonicated one more hour, and brought to volume with previously degassed methanol. Each extracted sample was filtered using a 1 mL Tuberculin syringe and a 0.20 μm filter tip into an HPLC vial for HPLC analysis. A 25 μL sample was then injected into an HPLC coupled to a diode array detector.

2.6. HPLC analysis

The HPLC system used for sample harvested during 2006 was an Agilent 1100 series consisting of a degasser, quaternary pump, ALS auto-sampler and PDA detector. An Agilent Zorbax SB-C-18, 4.6 mm × 250 mm, 5 μm column was used for this analysis with a column flow of 1 mL/min. Sample injections were made at 25 μL for all samples and standards. Using a solvent system of H_2O and ACN, a gradient elution was used for analysis. During 2007 we tried to use the same protocol used on 2006 on a Thermo-Electron Surveyor HPLC (San Jose, CA) consisting of a degasser, quaternary pump, PDA detector coupled with a mass spectrophotometer LCQ advantage max. A BDS hypensil C-18 (150 × 4.6) mm × 5 μm column was used with a column flow of 1 mL/min. The analyses were triplicated for each sample. Safranal was detected at 310 nm, all crocins were detected at 440 nm and picrocrocin at 250 nm. The saffron standards are given in Fig. 2A–C, respectively for safranal, crocins and picrocrocin.

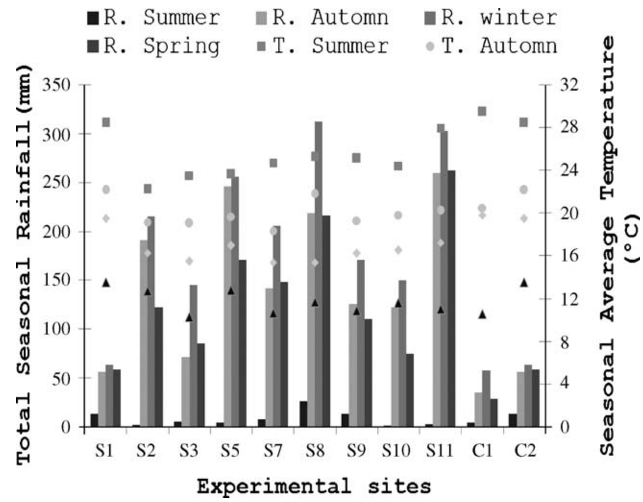

Fig. 1. Seasonal average temperature and total seasonal rainfall of experimental sites compared to data from Taliouin (C1) and "Le safranier de l'Ourika" (C2) (R = Rainfall, T = Temperature) (Average 2000–2006).

2.7. Picrocrocin purification

Picrocrocin purification was performed using preparative HPLC (Waters Delta Prep 4000). 500 μL of saffron methanol extract (20 mg/mL 80% ETOH) was directly injected into a 20 mL/min stream. An HPLC linear gradient was utilized and consisted of 90:10 (H_2O:acetonitrile) to 50:50 over 25 min. Picrocrocin peak elution was monitored at 250 nm. The fraction was concentrated by rotary evaporation and the water removed using solvent exchange through a C-18 Sep-pack. High-resolution mass spectrometry was used to verify both molecular weight and molecular formula. ^{1}H NMR (100 MHz, CD_3OD): δ 10.0 (1H, s), 4.39 (1H, d, J = 6.8), 4.05 (1H, m), 3.81 (1H, d, J = 12.0), 3.63 (1H, dd, J = 4.8, 12.0), 3.30 (1H, m), 3.25 (1H, m), 3.12 (1H, t, J = 7.6), 2.64 (1H, m), 2.27 (1H, m), 2.10 (3H, s), 1.81 (1H, m), 1.49 (1H, t, J = 12.0), 1.19 (3H, s), 1.17 (3H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 193.7, 155.9, 141.3, 102.8, 78.3, 78.1, 75.3, 72.2, 71.8, 62.9, 48.4, 42.5, 36.8, 29.5, 28.1, 19.5. The picrocrocin collected was 92% pure.

2.8. Quantitative analysis

With five concentration points, an external standard least squares regression for quantification was used. The three analytes: safranal, crocins and picrocrocin were used to formulate separate calibration curves. The response factors were calculated using the equation RF = DR/C where DR is the detector response in peak area (PA) and C is the analyte concentration.

The chromatograms of each Saffron sample were compared to the standard injections. The target peaks were confirmed by retention time and mass spectra and/or UV spectral data.

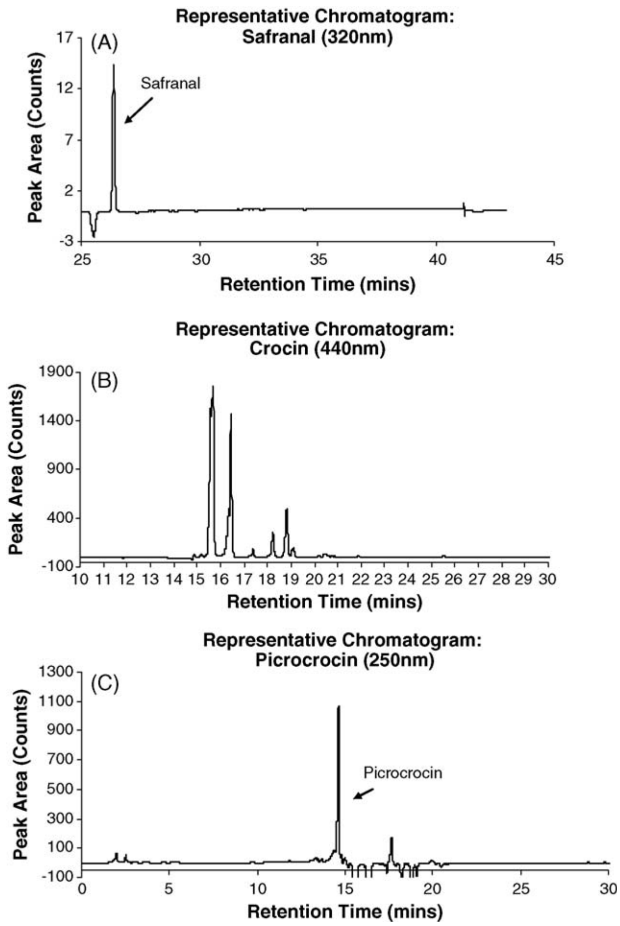

Fig. 2. Representative chromatogram (A) safranal, (B) crocins, (C) Picrocrocin.

Confirmed integrated peaks were then used to determine the analyte percentage. The RF of the target analyte was used to determine the "percent in dry weight" for each sample using the equation: (PA/RF/C) $\times$ 100 = %(Peak Area/Response Factor/Concentration).

The R^2 for the three standard curves is 0.999. The range of injected standard concentrations was between 0.1 and 0.001 mg/mL for safranal; 1 and 0.001 mg/mL for crocins and 1.15 and 0.001 mg/mL for picrocrocin. The limit of quantification for the three components was 0.0001 mg/mL.

2.9. Determination of the main saffron characteristics using UV–vis spectrometric method

Saffron samples were analyzed according to the ISO 3632 trade standard (ISO/TS 3632, 2003). This method allows the determination of the main characteristics of saffron related with picrocrocin, safranal and crocins content. Higher amount of these components means higher quality of saffron. According to ISO, picrocrocin, safranal and crocins are expressed as direct reading of the absorbance of 1% aqueous solution of dried saffron at 257, 330 and 440 respectively.

The saffron samples analyzed are the same samples used for HPLC analysis. Measurements of $E^{1\%}$ of an aqueous saffron extract at 440, 330, and 250 nm, respectively, were done using a 1 cm pathway quartz cell. Results are obtained by direct reading of the absorbance, D, at three wavelengths, as follows:

$E^{1\%}_{1\,\text{cm}}$ 257 nm: absorbance at about 257 nm (maximum absorbance of picrocrocin);
$E^{1\%}_{1\,\text{cm}}$ 330 nm: absorbance at about 330 nm (maximum absorbance of safranal);
$E^{1\%}_{1\,\text{cm}}$ 440 nm: absorbance at about 440 nm (maximum absorbance of crocins):
Where $E^{1\%}_{1\,\text{cm}} = (D \times 10000)/(m \times (100 - H))$.

Where D is the specific absorbance; m is the mass of the saffron sample, in grams; H is the moisture and volatile content of the sample, expressed as a mass fraction.

Moisture and volatile contents were identified by using powdered saffron stigmas. The sample were ground with a pestle and mortar and passed through a 0.5 mm mesh. After weighing, the powdered samples, they were introduced uncovered in an oven set at 103 °C for16 h. The moisture and volatile matter content are expressed as a percentage of the initial sample using the following relation: ((initial mass − constant mass)/initial mass) $\times$ 100.

The reported values are the average values of three replicates. The material used for analysis is a Shimadzu (Tokyo, Japan) UV 310 PC, UV–visible–NIR Scanning spectrophotometer.

2.10. Statistical analysis

Data for all experiments were analyzed using a SAS (r) 9.1 (2007) (SAS Institute INC, Cary, NC, USA) statistical software package and Genstat Release 10.2, copyright 2007 (PC/Windows) (Law Agricultural Trust, Rothamsted Experimental Station). Statistical analysis was performed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) for multiple comparisons. The p-values less than 0.05 were considered statistically significant. Data collected from different sites have been discriminated on quality-basis in relation to environment using Principal Component Analysis (PCA). Only the first 2 PC's are retained. Cluster analysis was conducted to group sites into subsets, which share the same common trait.

3. Results and discussion

There was a great variability of saffron yields obtained under different regions which vary from 0.9 $\pm$ 0.3 (S10) to 9.2 $\pm$ 4.9 kg/ha (S5) as a mean of 2 years of study (Table 2). However, the statistical analysis does not show any difference between sites (Table 2), but differences between years were observed (Table 3). This latter could be explained by the fact that saffron production increases from the first to the third year of cultivation (McGimpsey et al., 1997). Corms remain in the soil during several years, so their number increases over the years of cultivation. In the first year, the yield did not go beyond 1 kg/ha in all the experimental sites because of the corm production

Table 2
Duncan's multiple range test (DMRT) for comparing saffron mean yield through different Moroccan environments (average mean of 2006 and 2007 years) (Alpha = 0.05, N = 11, MSE = 11.6).

Site number	Site name	Mean yield (a) (kg/ha)	DMRT (b) (5%)
S1	Marrakech	3.8 $\pm$ 2.8	a
S2	Rabat	1.6 $\pm$ 1.1	a
S3	Merchouch	3.4 $\pm$ 2.2	a
S4	Koudia	1.8 $\pm$ 0.9	a
S5	Larache	9.2 $\pm$ 3.5	a
S6	Ouezzen	6.0 $\pm$ 5.6	a
S7	Chaouen	5.5 $\pm$ 4.5	a
S8	Oulmes	7.8 $\pm$ 2.8	a
S9	Meknes	4.8 $\pm$ 0.8	a
S10	Settat	0.9 $\pm$ 0.3	a
S11	Taounate	1.6 $\pm$ 1.0	a

Data are expressed as the mean $\pm$ standard error based on three replicates at each site. (a): Average of 2 years of experimentation. (b): any two means having the same letter are not significantly different at the 5% level of significance.

Table 3
Analysis of variance of saffron yield between sites and years (3 years) ($N = 11$, $R^2 = 0.63$).

Yield		
Source	DF	Pr > F
SITE	10	0.67
Year	2	0.004

was very small (lower than 2 g weight/corm). The second year, the yield increased up to 5.7 kg/ha, while in the third year, the maximum yield obtained reached 12.7 kg/ha. This maximal yield value is achieved in the S5 site during 2007 (12.7 kg/ha) (Fig. 3) with the highest mean value of 9.2 kg /ha obtained in the same site (Table 2). This highest yield obtained could be explained by the dimension and weight of corms (Fig. 4) measured during the summer of 2007, which have higher weights (21.6 ± 4.3 g) and greater size (3.7 ± 0.3 cm) than the corms collected from different trials during the same period. The S5 site has a sandy soil (86%) (Table 1), which facilitates the growth of corms. Many researchers reported that saffron production is influenced by dimension of corms (De Mastro and Ruta, 1993; Lombardo et al., 2005). According to the results obtained by (Mashayekhi et al., 2007), an increase in corm weight resulted in more flower production with a threshold corm weight of around 20 g, and that small corms are not guaranteed to flower. Yield is rather a difficult parameter to predict in saffron because it is a function of many agronomic, biological and environmental factors able to exercise a great influence on production (Gresta et al., 2008). Yield reported varies greatly depending on the factors cited above. In irrigated Moroccan main saffron growing zones, yields of about 2.5–6 kg/ha are obtained (Ait Oubahou and El Otmani, 1999). In Iran, the average yield of saffron is around 5.4 kg/ha (Behzad et al., 1992). However, higher yields have been reached in other parts of the world, such as 24 kg/ha in New Zealand (McGimpsey et al., 1997) and 29 kg/ha in Navelli (Italy) (Tammaro, 1999), but these yields were obtained with an annual cropping system using only the largest corms planted every year.

Saffron quality, which depends on the concentration of its major metabolites, is analyzed in different environments and results are presented in Table 4.

There was a significant difference ($p < 5\%$) in all saffron components among experimental sites and between sites and commercials controls. The average values for crocins ranged between 18% and 37% on a dry weight basis, with an average of

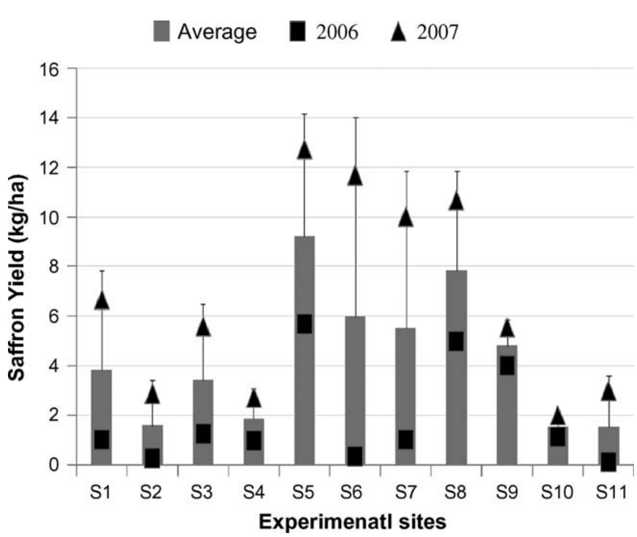

Fig. 3. Evolution of saffron yields in sites during 2 years of experimentation (2006 and 2007).

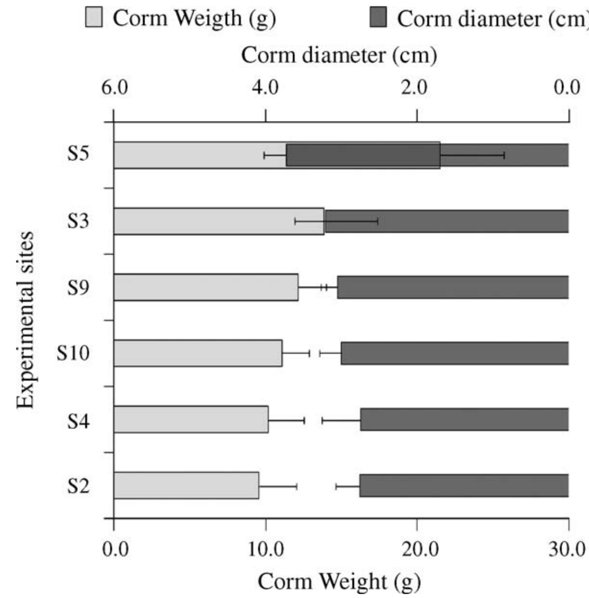

Fig. 4. Saffron corms dimensions measured during summer 2007 for some experimental sites. (Mean values ± SD of 300 corms.)

$29.01 \pm 5.6\%$. The highest mean significant value is achieved in both Moroccan commercial saffron and in Marrakech experimental site (S1) (Table 4). The variation coefficient in crocins average content between all the sites and the commercial sample analyzed over the 3 years of study is high ($cv = 22$) which means that there is not homogeneity of crocins content in different samples studied.

For most of the saffron samples (commercial and under experimentation), picrocrocin ranged between 4.2% and 28.8% on a dry weight basis with the highest content found in commercial saffron (C2). Meanwhile, safranal is slightly lower in commercial saffron compared to some experimental sites, as S11 and S1, where average safranal obtained is the highest. The average safranal values range between 0.04% and 0.48% (Table 4).

A broad range of values is reported for these saffron components and the amount varies greatly from country to country. Reported values for crocins vary from 0.85% to 32.4% dry weight (Alonso et al., 2001). Other values reported vary between 2.9 mg% (29 mg/g) (Li et al., 1999) and 4.6 mg% (45.99 mg/g) (Caballero-Ortega et al., 2004) for Iranian saffron and 6.7 mg% (67.3 mg/g) (Sujata et al., 1992) for Indian saffron. Safranal levels reported by some researcher are around 0.80 mg% (8 mg/g) (Sujata et al., 1992). Other values reported for safranal vary between a minimum of 0.06 mg/g and a maximum of 0.29 mg/g (Hadizadeh et al., 2007). The picrocrocin content, ranges between 0.79% and 12.94% in Spanish saffron, 1.07–2.16% in Indian, and 2.18–6.15% in Iranian saffron. The decomposition of picrocrocin gives rise to the safranal which takes place during processing (drying, storage) of saffron (Rios et al., 1996; Straubinger et al., 1998). Saffron is dried differently (shade, heating system, electric ovens, sunlight, etc.) in various regions of the world, and drying practices are known to affect the final composition of saffron. Crocins and picrocrocin compounds degrade naturally in the cells of stigmas during drying, storage and extraction (Straubinger et al., 1998). The degree of degradation depends on temperature, humidity, light irradiation and other compounds in the milieu.

As we have just discussed above, there is an inconsistency in the value of saffron components reported. The different protocols for the determination of saffron components in different countries indicate that the calculated amounts depend on the drying, extraction, separation and quantification methods (Caballero-Ortega et al., 2007). For this reason, the compositions of saffron

Table 4
Determination of saffron constituents (% dry weight) under different environment in comparison to two commercials control (C1 and C2) using HPLC method: Crocins and safranal values are average of 3 years of experimentation (2005, 2006 and 2007), picrocrocin are values obtained during harvest 2006. (Sites are classified from the lowest to the highest altitude).

Site number	Site name	Sites altitude	Crocins (%)		Safranal (%)		Picrocrocin (%)	
S5	Larache	46.7	17.90	d	0.21	bc	11.92	f
S2	Rabat	75.3	25.84	abcd	0.11	bc	14.25	ef
S4	Koudia	200	25.14	bcd	0.18	bc	6.75	h
S10	Settat	397	26.70	abcd	0.04	c	15.75	d
S3	Merchouch	398	24.46	bcd	0.19	bc	10.67	fg
S11	Taounate	509	24.00	cd	0.48	a	–	
S7	Chaouen	*600*	31.17	abc	0.19	bc	17.84	cd
S6	Ouezzen	614	29.63	abc	0.10	bc	4.23	i
S9	Meknes	714	25.13	bcd	0.28	ab	10.01	gh
C2	"Safranier d'Ourika"	1100	37.23	a	0.24	bc	28.78	a
S8	Oulmes	1135	31.88	ab	0.27	abc	9.11	gh
S1	Marrakech	1300	34.25	a	0.35	ab	14.69	ef
C1	Taliouin	1630	36.27	a	0.17	bc	24.52	b

Means with different letters in a column show differences at a significance level of 5% according to DMRT.

from other studies were not compared to those obtained in this study. Nevertheless, the comparison of the Moroccan saffron in this study is valued because it was conducted in the same way for all the experimental Moroccan sites, with the same processing and storage method used by Moroccan saffron growers and submitted to the same extraction, separation and quantification method. Also, the genetic material from the main saffron zone in the south of Morocco (Taliouin region) was used in all of our studies. The difference among sites and between sites and commercials is normally due to the environment. However, in the concern to have an idea on the saffron quality according to ISO standard, the evaluation of saffron collected from different experimental sites was done according to the limit set by the ISO 3632/TS normative (ISO/TS 3632-1/2, 2003). The major focus of this measure is to establish whether the product meets the ISO standard parameters, to get an indication of the range of quality being produced under different environment compared to the two commercials Moroccan control and to have a comparison basis with saffron from others countries. Results showed that 73% of the samples collected from different sites, had high colour and belong to category I, and all the experimental samples had higher aroma strength (Table 5).

Crocins and safranal content based on HPLC methods over the 3 years of study are in Table 6. Picrocrocin was not included because we have only data for 1 year of study (2006). The statistical analysis shows that crocins are stable under each specific environment tested ($p > 5\%$). Meanwhile, there was a large variability in safranal content for the same period ($p < 0.05$). This suggests that post-harvest processing of saffron produced under different environments may need to be improved.

The Pearson's correlation coefficient matrix of all the measured variables is reported in Table 7. Only in very few cases a significant positive correlations were found (reported in bold for $p < 5\%$) between altitude and crocins; soil texture (clay) and safranal. The best soils for saffron production have been reported to be the well-drained clay-calcareous and deep soil (Skrubis, 1990), well-ploughed sandy-loamy soil (Sampathu et al., 1984) or a well-drained clay soil (Fernandez, 2004; Sampathu et al., 1984). Saffron is also cultivated on sandy soil in Azerbaijan (Azizbekova and Milyaeva, 1999). Saffron quality does not appear to be affected by the soil chemical composition and temperatures (Table 7). However, as reported in the literature about pH and temperatures, optimum soil pH for saffron apparently ranges from neutral to slightly alkaline (Gresta et al., 2008) and saffron can tolerate cold temperatures (−18 °C) (Mollafilabi, 2004). Some other reports include a large range of temperatures, including 2–10 °C during winter and 20–25 °C during summer (Gresta et al., 2008). Furthermore, Indian saffron is cultivated in sub-tropical areas (Sampathu et al., 1984).

Regarding the relationship between saffron quantity (yield) and quality under different altitudes, quality, yield and altitude were analyzed by employing Principal Components Analyses (PCA). Combined, PC1 (47%) and PC2 (26%) accounted for 69% of the total variance of the data (Fig. 5). The first PC (PC1) is characterized by altitude and crocins (Table 8). This is appropriate regarding that Pearson correlation showed significant correlation between altitude and crocins (Table 9). The second PC (PC2) is characterized by a positive relationship of yield with safranal (Table 8). The Pearson correlation showed no significant correlation between those two variables (Table 9). The value of correlation coefficient (r) did not go beyond 0.19. The 11 sites had distributed scores all around the centre indicating a big variability in variable studied. Three different environments could be identified: one included locations with high crocins content, but also a high yield (S7 and S8). The other environment covered locations which were

Table 5
Comparison of $E^{1\%}_{\lambda_{max}}$ values of Picrocrocin, safranal and crocins obtained in different experimental sites with ISO procedure. Crocins and safranal values are average of 2 years of experimentation (2006 and 2007), picrocrocin are values obtained during harvest 2006. (Sites are classified from the lowest to the highest altitude).

Sites number	UV–visible[a]	
	$E^{1\%}_{330}$	$E^{1\%}_{440}$
S5	50 ± 1	128 + 1.1
S2	48 ± 0.2	254 + 0.3
S4	38 ± 0.3	243 + 0.4
S10	48 ± 0.3	231 + 0.7
S3	46 ± 0.3	139 + 0.4
S11	47 ± 0.3	117 + 0.3
S7	50 ± 0.7	268 + 0.1
S6	43 ± 0.01	275 + 0.2
S9	47 ± 0.7	202 + 0.4
C2	40 ± 2	350 + 1.6
S8	50 ± 0.6	256 + 1.9
S1	36 + 0.7	287 + 0.5
C1	38 + 0.01	276 + 0.7

[a] Extraction according to ISO 3632 method with distilled water as the reference liquid.

Table 6
Analysis of variance of saffron quality between sites and years (3 years, $N = 13$).

Crocins			Safranal		
Source	DF	Pr > F	Source	DF	Pr > F
Site	12	0.045	Site	12	0.049
Year	2	0.55	Year	2	0.0001

Table 7
Correlation matrix of Pearson coefficients between quality and environmental factors ($p < 5\%$, $N = 13$).

	Crocins	p	Safranal	p	Picrocrocin	p
Altitude	**0.84**	**0.0012**	0.14	0.6813	0.44	0.17545
Clay	0.07	0.8484	**0.71**	**0.0136**	0.20	0.54739
Sand	−0.21	0.5257	−0.43	0.1851	−0.39	0.24019
OM	−0.07	0.8413	−0.13	0.7107	−0.43	0.18907
K	0.14	0.6767	0.09	0.8026	0.11	0.74763
P	−0.14	0.6847	0.15	0.6505	−0.09	0.78370
pH	0.11	0.7403	−0.15	0.6598	0.31	0.35398
TM[a]A[b]	0.35	0.2852	0.32	0.3312	0.08	0.80910
TMW	−0.01	0.9829	0.32	0.3404	0.22	0.52471
TMSp	0.34	0.3069	0.24	0.4764	0.44	0.17383
TMSu	0.50	0.1189	0.20	0.5502	0.24	0.47344
Tm[c]A	−0.25	0.4508	−0.14	0.6745	−0.43	0.18271
TmW	−0.48	0.1331	−0.27	0.4209	−0.50	0.12057
TmSp	−0.19	0.5812	−0.14	0.6833	−0.30	0.36373
TmSu	0.33	0.3236	0.06	0.8649	0.22	0.52005
Crocins	0.00	1.0000	−0.19	0.5760	0.41	0.20759
Safranal	−0.19	0.5760	0.00	1.0000	−0.02	0.94565
Picrocrocin	0.41	0.2075	−0.02	0.9456	0.00	1.00000

Bolded values are significant at $p < 0.05$.
[a] TM: Average maximal seasonal temperature.
[b] Su: summer; A: autumn; W: Winter; Sp: spring.
[c] T mi: Average minimal seasonal temperature.

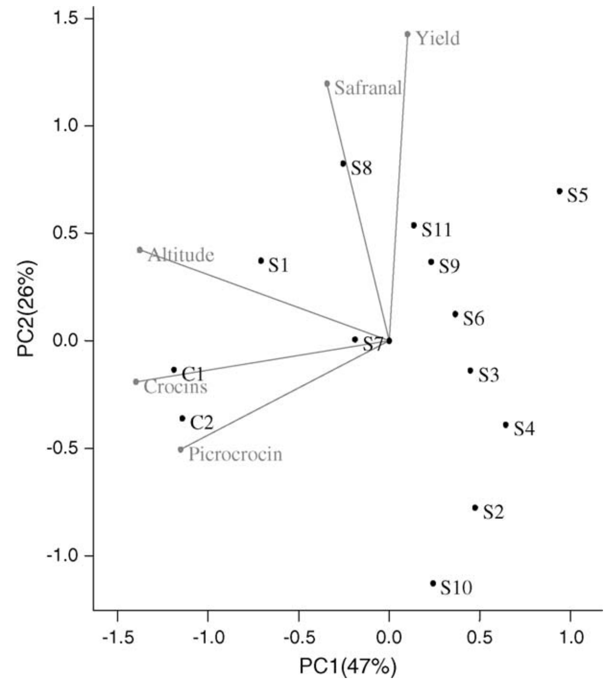

Fig. 5. Principal components analyses for yield and quality and the eleven sites compared to two controls (C1 and C2).

Table 8
Loading of the first two principal components (PC's) for yield, altitude and quality under standard practices.

Variables	PC1	PC2
Altitude	−0.59809	−0.21295
Crocins	−0.60671	0.09621
Picrocrocin	−0.5000	0.25486
Safranal	−0.1492	−0.60319
Yield	0.04391	−0.71875

characterized through a higher level of crocins content but low yield (S1) and a third environment with low crocins content and a variable yield, these were all the other sites (Fig. 5).

Based on results obtained between crocins and altitude that showed a positive correlation coefficient, cluster analysis was performed to group sites into subsets, which share the same common trait based on crocins content and altitude (Fig. 6). Two groups were identified. Group 1, which includes S1, S8, C1 and C2; group 2 that include all the other sites not including S5. We notice that the first group has an altitude over 1000 m. The second group has an altitude lower than 1000 m (Table 1, Fig. 6). The site S5 represents a coastal site with sandy and poor soil based on P and K and its pH is slightly acidic compared to other sites (pH 6.8), but we do not have statistical analysis for proof so as to show that those factors influence saffron quality. This denotes that we would need to have multiple sites in the same region to highlight others factors that influence crocins production with altitude. Furthermore, for each site we have to have a meteorological station to study the real effect of micrometeorological data on saffron quality because the climatic data presented in this study are taken from national meteorological stations that are not in the experimental sites studied. Other climatic data relative to humidity, solar radiation and length of the day have to be considered during further future analysis as a complement for this study.

Altitude has a positive effect on crocins content. However, further research on the impact of the environment on saffron quality and the identification of region enabling the production of high quality saffron is needed. Nevertheless, this multi-year study

Table 9
Correlation matrix of Pearson coefficients between altitude, quality and yield ($p < 5\%$, $N = 13$).

Variables	Altitude	Crocins	Safranal	Picrocrocin	Yield
Altitude	1.00	**0.86**	0.27	0.49	0.14
p		**0.0002**	*0.3648*	*0.0850*	*0.6407*
Crocins	0.86	1.00	0.011	0.58	−0.10
p	*0.0002*		*0.9696*	*0.0338*	*0.7437*
Safranal	0.27	0.011	1.00	0.05	0.18
p	*0.3648*	*0.9696*		*0.8572*	*0.5443*
Picrocrocin	0.49	0.58	0.05	1.00	−0.22
p	*0.0850*	*0.0338*	*0.8572*		*0.4640*
Yield	0.14	−0.10	0.18	−0.22	1.00
p	*0.6407*	*0.7437*	*0.5443*	*0.4640*	

Bolded values are significant at $p < 0.05$.

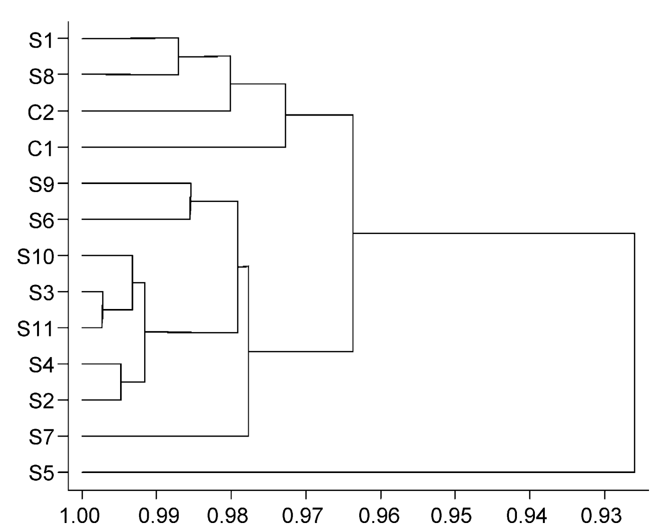

Fig. 6. Dendogram of saffron sites gathered on altitude and crocins basis.

provides important information about the region to grow saffron with high crocins content. Also this study suggests that for the experimental saffron produced under different environments, considerable improvements in the aroma content should be done without losing colour by using other drying methods. It is important to emphasize that this work constitutes the first study conducted on saffron quality determination in Morocco under diverse environments.

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