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Influence of Different Extraction Methods and the Storage Time on Secondary Metabolites of Saffron

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***Abstract*—The spice's quality of saffron depends on the concentration of secondary metabolites, such as crocins, picrocrocin and safranal. This work aims to evaluate the influence of the extraction solvents and the extraction duration on metabolites absorbance of Moroccan saffron. It aims also to determine the storage time effect. The results show that long extraction duration (for example 24 h) causes loss of coloring strength. With alcoholic extracts, a better coloring strength is obtained compared to an aqueous extraction. As regards storage time, the analysis clearly shows that the saffron contents undergo a significant variation of chemical characteristics during the storage.**

***Keywords*— Saffron; crocin; safranal; picrocrocin; extraction; storage time.**

I. INTRODUCTION

Saffron is a spice constituted by the dried stigmas of *Crocus sativus L*. that belongs to the family of Iridaceae. It is the most expensive spice in the word principally cultivated in the Mediterranean region (Morocco, Spain, Greece,..) and in the south western Asia (Iran, India …) [1][2]. The stigmas of saffron have been used for very ancient times as food additive, as a dye in cosmetic preparation, textile and woodwork and also for medicinal purposes. Recent works have confirmed that saffron spice has a variety of pharmacological effects such as antihypertensive, antidepressant…[3] [4] [5]. Additionally, the antioxidant activity constitute one of the important mechanisms for the various health effects of saffron [4] [5][6].

Crocin (for color), picrocrocin (for taste) and safranal (for aroma) are the main chemical constituents of saffron. High levels of those chemical compounds in saffron provide it high quality and determine it commercial price. With an international production extremely limited and a high value in the world market, the saffron has always been the subject of frequent adulteration and fraud [7][8] and subsequently, it must be certified over world market by ISO 3632 standards. In this way, the saffron is commercially categorized on three quality categories. The saffron is classified as having a high quality (category I) when the direct reading absorbance at 440 nm for crocin is greater than 190 [7].

In Africa, Morocco is the main country that grows saffron. Its production area is limited to 1500 ha and is located mainly in the Anti-Atlas Mountains. The three main regions of saffron implantation are Taliouine (Altitude 1200–1630 m, latitude 30° 29.937'N and a longitude of 007° 51.703'W), Askaouen (Altitude 1700–2200 m, latitude 30° 40' 33,5''N and a longitude 007°44'10,7 ''W) and Taznakht zones (Altitude 1500–2000 m, latitude 30° 38.147'N and a longitude of 007° 29.530'W). These areas are climatically characterized by a cold winter and hot summer. Temperature varies between -2°C during winter and reaches 45°C during summer [9].

The present work constitutes the first part of a saffron project which aims to search a chemical identity of Moroccan saffron. This part focalized on physical characterization, aimed to evaluate the influence of the extraction solvents and the extraction duration on saffron metabolites absorbance. It also aimed to determine the storage time effect. For that purpose, authentic samples of saffron provided from Taliouine were used and the standard ISO extraction procedure was also used. The extraction and storage conditions constitute two limiting steeps in saffron quantification process.

II. MATERIALS AND METHODS

A. *Materials and methods*

The saffron samples of *Crocus sativus L*. were provided by the 1.2.3 saffron company as recently harvested dried stigmas. Well conditioned, they were immediately transported under dark conditions to the chemistry laboratory where they were analyzed at room temperature. All organic chemical compounds and solvent used in this study were HPLC grade. The UV-spectrophotometer is UV2300 model.

Two commercial samples, originated from Taliouine region and produced at 2013 were used.

Table 1 describes the different protocols adopted and the specific conditions under which they were applied to determine the levels of crocin, safranal and picrocrocin metabolites in the saffron samples. In these protocols, the previously ground saffron was suspended in various extraction solvents. The suspension was stirred magnetically for different extraction times at different temperatures.

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TABLE 1: DIFFERENT METHODS OF EXTRACTION OF CROCINS, PICROCROCIN AND SAFRANAL

Method code	Extraction solvent	Extraction duration
Method $A_{0.5h}$	Cold distilled water	30 min
Method A_{1h}		1h
Method A_{2h}		2h
Method A_{24h}		24h
Method A'	Cold distilled water	1h
Method B	warm distilled water (45 °C)	
Method C20% methanol	methanol:water 20%:80% v/v	
Method C50% methanol	Methanol:water 50%:50% v/v	
Method C80% methanol	Methanol:water 80%:20% v/v	
Method C100% methanol	methanol 100%	
Method D20% ethanol	Ethanol:water 20%:80% v/v	
Method D50% ethanol	Ethanol:water 50:50% v/v	
Method D80% ethanol	Ethanol:water 80%:20% v/v	
Method D100% ethanol	ethanol 100%	

B. *ISO extraction procedure*

Five hundred milligrams mass (500 mg) of saffron previously sieved through a 0.5 mm sieve were suspended in a 1 L of distilled water in a closed flask. The suspension was magnetically stirred during 1 h at room temperature in the dark to preserve crocin which is sensitive to UV-light. 20 mL of the obtained solution were completed to 200 mL of distilled water. Once homogenized, the solution was filtered through a polyamide filter of 0.45 µm and analyzed according to the ISO 3632 standard. For the saffron extract, the $E^{1\%}$ maximum value was measured with an UV-Vis. spectrometer (UV2300 model) at 440 nm, 330 and 257 nm respectively for crocin, safranal and picrocrocin compounds.

The absorbance maxima and the results were expressed according to ISO 3632-2-2010 using the equation number 1.

$$E = \frac{DO \times 10000}{m(100 - WMV)} \qquad (1)$$

Where:
DO is the specific absorbance;
m is the mass of the saffron sample, in grams;
W_{MV} is the moisture and volatile content of the sample, expressed as a mass fraction. (ISO-3632-2-2010, Part 2):
To verify the stability of the samples under studied conditions and to observe the reliability of the analyzes, each sample was also three times separately analyzed in the same operating conditions and all experiences were repeated also three times.

C. *Determination of the moisture and volatile contents*

Moisture and volatile contents were determined according to ISO 3632-2-2010. 2,5g of stigmas were thought at 0,0001g then introduced uncovered in an oven set at 103 °C for 16 h. The results were expressed according to the following equation number 2:

$$W_{MV} = (m_0 - m_1) \times 100 \qquad (2)$$

Where:
- W_{MV} is the moisture and volatile matter content of the sample, expressed in %;
- m_0 is the initial sample mass, in grams;
- m_1 is the mass of the sample after drying, in grams.

All experiments were repeated three times [10].

D. *Storage time:*

The study of the storage effect had done by analyzing samples from the same cultivar after their storage for a period of 12 months by the interval of three months.
The quantity of samples was divided on two parts in order to study the effect of temperature and light.
- Effect of the storage temperature: 4°C or room temperature;
- Effect of light: storage in light or darkness.

III. RESULTATS

Tables 2 and 3 present the corresponding amounts of the three compounds of saffron expressed by the formula 1.

A. *Extraction duration*

TABLE 2: EFFECT OF EXTRACTION DURATION OF CROCINS, PICROCROCIN AND SAFRANAL WITH COLD WATER IN AMBIENT TEMPERATURE. THE SAMPLE USED HAD A MOISTURE OF 9.06 %.

Method code	Crocin ($E_{440}^{1\%}$)	Safranal ($E_{330}^{1\%}$)	Picrocrocin ($E_{257}^{1\%}$)
Method $A_{0.5h}$	175.8	39.5	76.8
Method A_{1h}	249.2	52.0	87.9
Method A_{2h}	300.5	54.2	92.3
Method A_{24h}	188.3	48.3	83.5

The average values ranged between 175.8 and 300.5, 76.8 and 92.3 and between 39.5 and 54.2 respectively for crocins, picrocrocin and safranal. The highest mean significant values for all saffron compounds is obtained with extraction duration of 2 hours.

The results show that changes in the extraction duration affected the spectrometric values as we see in figure 1, the long extraction periods cause significant loss of the coloring strength. The degradation of saffron pigments seemed to be fast, so this step of analysis should be short. In the recent IS0 3632-2-2010, the extraction period was severely reduced to one hour of magnetic stirring that may influenced the concentration of the secondary metabolites of saffron.

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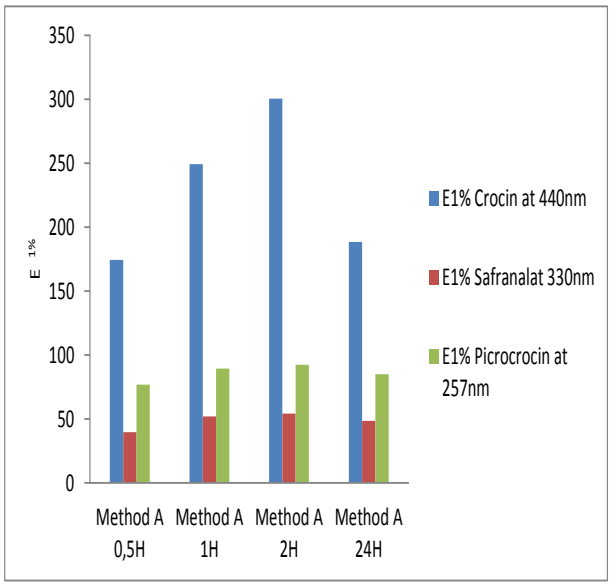


Fig 1: Effect of extraction duration of crocins, picrocrocin and safranal with cold water in ambient temperature. The sample used has moisture of 9.06 %.

B. Extraction solvents

Different extraction protocols with different conditions were applied to determine the amounts of the main chemical compounds of saffron. We had investigated the effects of the extraction solvents on the crocin, picrocrocin and safranal levels. The table N°3 presents the results.

TABLE3: EFFECT OF SOLVENTS IN THE EXTRACTION PROTOCOL OF CROCINS, PICROCROCIN AND SAFRANAL WITH 1 HOUR STIRRING. THE SAMPLE USED WAS S2 AND IT HAS A MOISTURE OF 9.4 %.

Method code	**Extraction solvent**	**$E_{440}^{1\%}$**	**$E_{330}^{1\%}$**	**$E_{257}^{1\%}$**
Method A'	Cold water	59.4	15.4	30.8
Method B	warm water at (45 °C)	94.6	17.6	37.4
Method $C_{20\%}$ methanol	methanol:water 20% :80% v/v	90.2	19.8	37.4
Method $C_{50\%}$ methanol	methanol:water 50%:50% v/v	112.2	22.0	39.6
Method $C_{80\%}$ methanol	methanol:water 80%:20% v/v	125.4	22.0	39.6
Method $C_{100\%}$ methanol	methanol 100%	112.2	17.6	253
Method $D_{20\%}$ ethanol	ethanol/water 20%:80% v/v	74.8	17.6	35.2
Method $D_{50\%}$ ethanol	ethanol/water 50:50% v/v	140.8	28.6	48.4
Method $D_{80\%}$ ethanol	ethanol/water 80%:20% v/v	140.8	28.6	48.4
Method $D_{100\%}$ ethanol	ethanol 100%	37.4	6.6	19.8

Those results indicate that the higher coloring strength values were obtained both with the 50% and 80% ethanol extraction protocol. The water 50%-ethanol 50% showed the most efficient crocin, safranal and picrocrocin extraction.

The graph reported in the figure 2 represents the comparison of the crocin, safranal and picrocrocin levels obtained after different methods of extraction using the UV–Vis spectrometer.

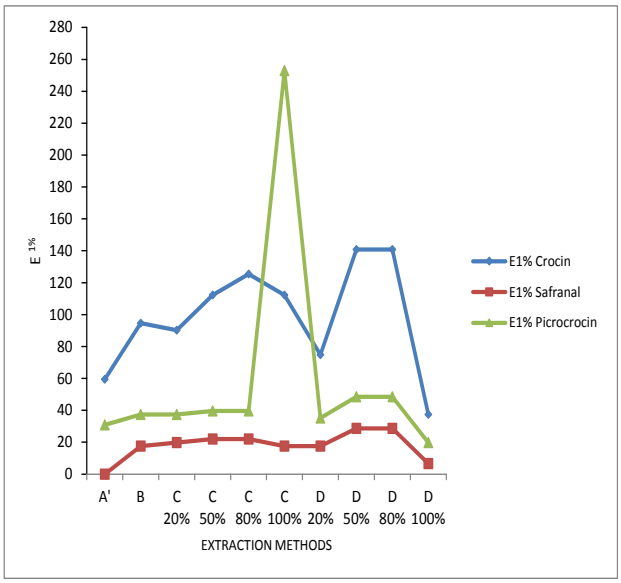
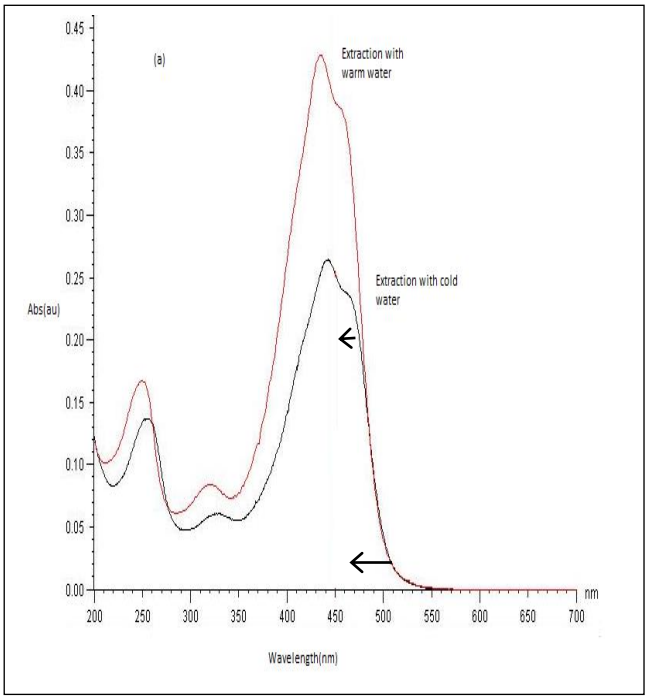


Fig 2: Effect of solvents in the extraction protocol of crocins, picrocrocin and safranal with 1 hour stirring.

We can see that for crocin and safranal, the best solvent is ethanol 50% -water 50% but for the picrocrocin, the best is methanol 100%. We can see also that the extraction with warm water was better than the extraction with cold water.

The graphs reported in the figure 3 represent the comparison of the crocin, safranal and picrocrocin levels obtained after extraction by warm water and cold water and by 50% of water/ ethanol and 50% of water/ methanol.



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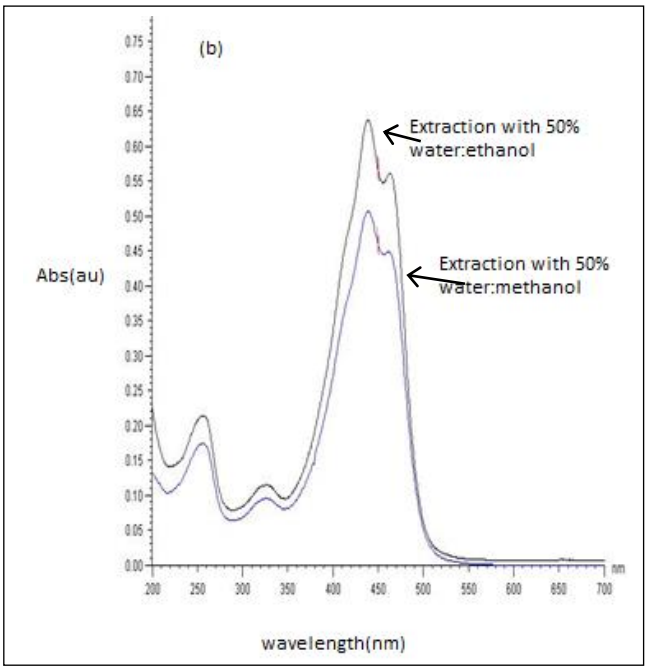

Fig 3: Comparison of the saffron's UV-Visible specters: (a) extraction by warm and cold water and (b) extraction by the 50% of water/ethanol and 50% of water/methanol

C. Effect of the storage time

To study the effect of storage time, light and temperature on the characteristics of saffron, the sample was divided into four groups. The chemical compounds were measured using the extraction procedure of ISO-3632-2.

The coloring strength changed significantly with time of the storage, the temperature and light had strong effect on the crocin and picrocrocin degradation. The coloring strength decreased from 235 to 112 and the bitterness decreased from 92 to 70 during 12 months of storage. While safranal content showed an increasing trend during storage from 55 to 70.

As can be seen in the figures below, crocin and picrocrocin show a decreasing while safranal and moisture content showed an increasing during storage.

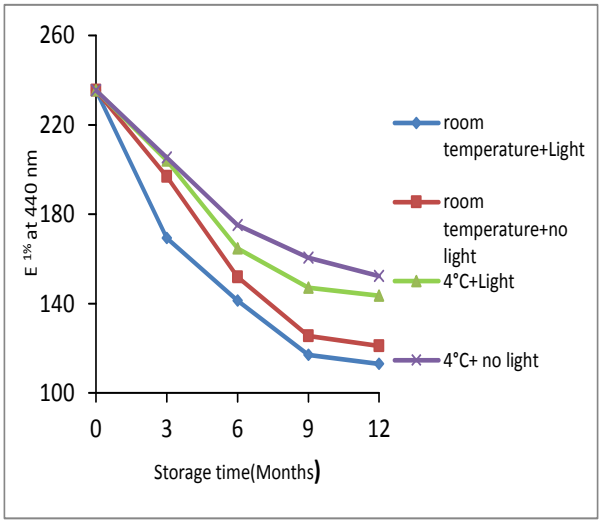

Fig 4: Effect of the storage time on crocins content

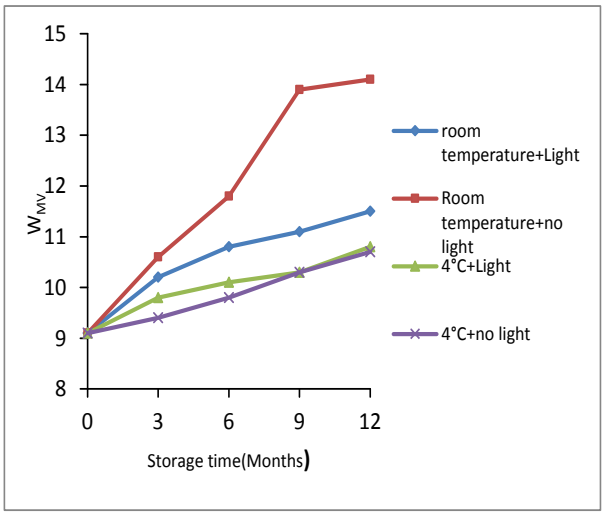

Fig 5: Effect of the storage time on moisture and volatile content (WMV)

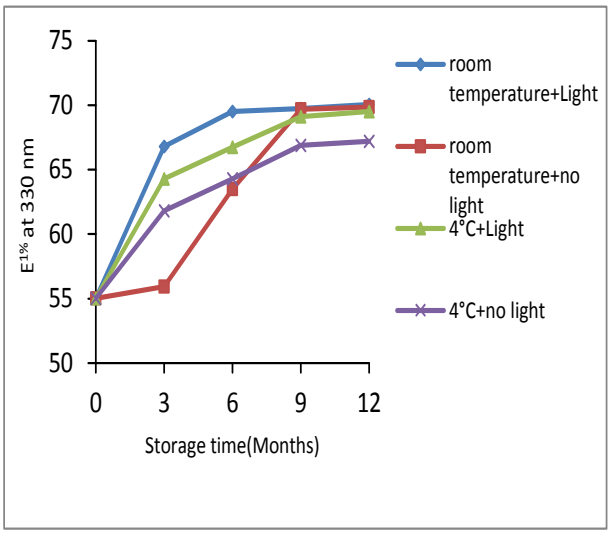

Fig 6: Effect of the storage time on safranal content

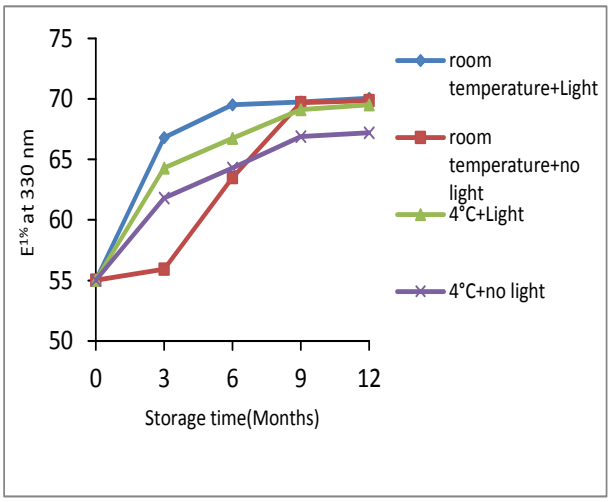

Fig 7: Effect of the storage time on picrocrocin content

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V. DISCUSSION

A. *Extraction protocols*

In order to compare the extraction ability of the solvents, the concentrations of the three main compounds of saffron were investigated. The results indicate that the higher coloring strength values were obtained with the 50% water:ethanol extraction method, the viscosity of the solvent plays the major role in the efficiency of the stigmata extraction because the polarity, molecular coefficient and viscosity of solvent have an important effect on the extraction.

The mixtures of water:ethanol and water:methanol solvents have been used by some authors for the extraction of saffron coloring matter [08][10][11][12][13]. Our results indicate that the solvent water/ethanol is best than water/methanol and this last is better than water because the polar carotenoids of saffron are not freely soluble in cold water but it's soluble in alcoholic solutions.

B. *Effect of storage time*

Saffron is very hygroscopic, it must be preserved after drying in a dry place and away from the air because the crocin content decreases significantly with increasing W_{MV} According to Alonso [09], the degradation of crocin is explained by the protective function of the carotenoids in the cells. Generating oxygen in singlet form, they initiate the auto oxidation process. In addition, the solubility of crocin in water, unlike most carotenoids, promotes its contact with oxygen. Crocin is hydrolyzed in crocetin (colorless) within the stigma. Also light acts as a catalyser for the crocin degradation.

Picrocrocin content decreases slightly while the safranal increases slightly, there is an inverse relationship between their contents. Also picrocrocin is thermolabile compound and it were converted to safranal by enzymatic process or thermic process.

These results are in agreement with the study of Tsimidou [14] and Bolandi [15] which reported that a high humidity degrades crocin and picrocrocin but allows the development of the aroma of saffron, safranal being a hydrolysis product of the picrocrocin.

VI. CONCLUSION

Various methodologies and analytical tools have been applied to analysis of saffron chemicals originating from Taliouine area. The results indicate that the amount of constituents depends highly on processing, method for extraction, drying and qualification.

The extraction of saffron is optimized by the solvent, temperature and stirring time used in the process. We conclude that the different extraction protocols influence absorbance readings which will influence the quality of saffron samples also we conclude that the saffron is very hygroscopic and sensitive to light and temperature, it must be stored in a dark and cold place.

For this, the best solvent of the extraction is ethanol 50%-water 50% and the best storage conditions are the dark at 4°C.

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