FOODINTEGRITY HANDBOOK

A GUIDE TO FOOD AUTHENTICITY ISSUES AND ANALYTICAL SOLUTIONS

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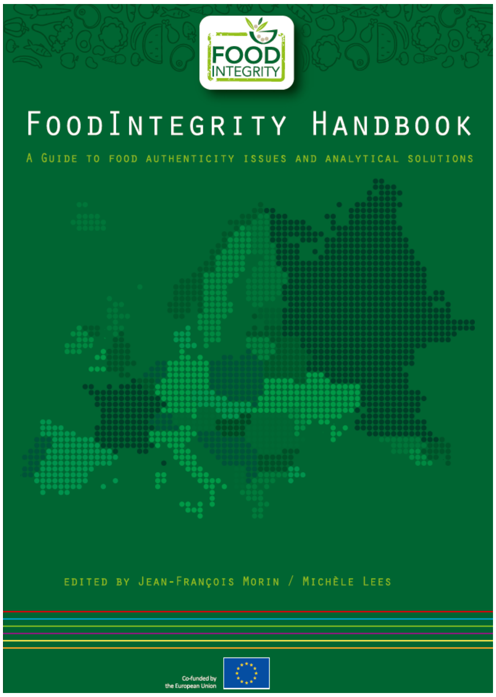

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Saffron

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General overview of the product

Saffron, the most valued spice in the world, is the dried stigmas of the flowers of *Crocus sativus* L., and the only one able to impart colour, flavour and aroma to foods. Very often saffron is confused with other plants, sometimes due to lack of knowledge, but also, which is worse, adulterated for economic gain. For this reason, saffron deserves to be dealt with in a separate chapter in this book.

The cultivation of saffron has been known for more than 3700 years as illustrated in the frescoes of the Minoan goddess Thera where a crocus field appears with women picking and offering flowers of *Crocus sativus* or *Crocus cartwrightianus* [1]. Since those early days, the way the spice is obtained has not evolved as it is still manually processed, without the mechanisation seen for other agricultural products. Once the flower is collected and the stigmas removed, it will be handled differently according to country, production area or even culture; such differences contributing to the real value of the spice [2]. The most prized saffron origin on the international market is from La Mancha (Spain), since it traditionally imparts most colour, flavour and aroma to food [3]. Until the 1990s, Spain was also the largest producer, but following a massive abandonment of agriculture in this country, production fell drastically, and Spanish companies had to turn to Iran as a new supplier of the raw material. However, most of the world distribution continues to be from Spain, due to the know-how that the trading companies have maintained through family tradition.

World production of saffron remains unclear, but it is known that Iran is the largest producer in the world, exceeding 90 %. According to the Iranian Ministry of Agriculture in 2013, 280 tons were produced in this country [4]. Although internal consumption of saffron in Iran is high, it is the largest exporter of this spice with Spain as the largest importer. In Spain, the spice is stored under the suitable conditions and its quality determined. With little information available and its high market price, this spice has been an easy victim of adulteration since ancient times, offering the fraudster considerable economic returns.

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1. Product Identity

1.1. Definition of the product and manufacturing process

The flower of *Crocus sativus* grows from a corm in late October and early November in the northern hemisphere, and in late April and early May in the southern hemisphere. It is picked manually in the field, and depending on the tradition of the production area, either at dawn with the flower closed, or at noon when the flower is open. The stigma and part of the style are separated from the rest of the flower, to a greater or lesser extent according to tradition. Subsequently, the stigmas are dried either using a direct flameless heat source or by leaving them for several days in the sun or in the shade, depending on each producing area. In some places there is a flower market, where the farmer who collects the flowers is not the one who produces the spice. All these factors mean that there is great diversity among the products obtained in different areas and, therefore, different quality products [3].

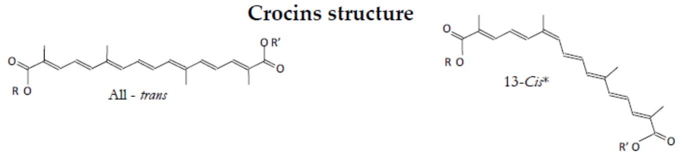
In La Mancha saffron, the three filaments are joined together with a small part of the yellowish-white style that contributes nothing. Italian saffron is very similar and although in Greece stigmas are also held together, Greek saffron is usually accompanied by flower pollen which contributes other flavour and aroma characteristics. In Iran, traditionally, the stigma is accompanied by a long part of the style and is dried in the sun or in the shade, as in Morocco. In India, the stigmas, after being separated, are rubbed together to obtain a homogenous darker colour and therefore quality.

Saffron is marketed for its colour, flavour and aroma with major metabolites that determine the quality of saffron. These are currently controlled by ISO 3632 [5] and used in all commercial transactions with saffron. The substances responsible for the colouring properties of saffron are the glycosidic esters of the carotenoid dicarboxylic crocetin (2E, 4E, 6E, 8E, 10E, 12E, 14E)-2,6,11,15-tetramethylhexadeca-2,4,6,8,10,12,14-heptaenedioic acid, $C_{20}H_{24}O_4$). The glycosides bound to crocetin are gentiobiose, glucose, neopolitanose and triglycose [6–9], which in saffron are found in their *trans* (majority) and *cis* (minority) forms. All these compounds are referred to in the literature as crocins, although in fact crocin is only *trans*-crocetin di (β-*D*-gentiobiosyl) ester. Figure 1 describes the names that have been accepted in recent years by the scientific community. The colouring strength of the spice depends on the concentration of these compounds, which ranges between 16-28 % in the dried stigma of *Crocus sativus* L., reaching concentrations up to 30 % in some years.

The substance responsible for the characteristic bitter taste of saffron is picrocrocin (4- (*β-D*-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, $C_{16}H_{26}O_7$). Up to now, this compound has not been detected in any other raw material, whether from plant or animal origin, and it is therefore considered to be a molecular marker of true saffron [11]. Its concentration is usually between 7-16 % [12], although in some samples it can reach 20 %.

With respect to saffron aroma, safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde, $C_{10}H_{14}O$) is the main compound [13] and the aglycone of picrocrocin. It has been detected in very few plant products and can be also generated when certain carotenoids undergo a thermal process. Safranal concentration is much lower than crocins and picrocrocin, usually between 0.1-0.6 % [14].

Crocins structure



Nº Glucose Moieties (R + R′)	Moiety Distribution (R/R′)	Isomer	Named as
5	triglucoside/gentiobioside	*trans*	*trans*-5-tG
		cis	*cis*-5-tG
	neapolitanoside/gentiobioside	*trans*	*trans*-5-nG
		cis	*cis*-5-nG
4	neapolitanoside/glucoside	*trans*	*trans*-4-ng
		cis	*cis*-4-ng
	gentiobioside/gentiobioside	*trans*	*trans*-4-GG
		cis	*cis*-4-GG
3	gentiobioside/glucoside	*trans*	*trans*-3-Gg
		cis	*cis*-3-Gg
2	gentiobiose/hydrogen	*trans*	*trans*-2-G
		cis	*cis*-2-G
	glucose/glucose	*trans*	*trans*-2-gg
		cis	*cis*-2-gg
1	glucose	*trans*	*trans*-1-g
		cis	*cis*-1-g

Figure 1: Simplified names of the glycosidic esters, crocins, of the carotenoid crocetin introduced by Carmona et al. [3]. Meaning of the abbreviations: t is Triglucose; G is Gentiobiose; n is Neapolitanose; g is Glucose [10]

1.2. Current standards of identity or related legislation

1.2.1. ISO Standard

Major metabolites determine the quality of saffron, which is actually controlled by ISO 3632 [5], and used in all commercial transactions with saffron. The methodology still used to control saffron quality is UV-vis spectrophometry, although there are several scientific studies [14] that have shown that safranal and picrocrocin are overestimated by this methods. Despite this, no changes have been undertaken by ISO. Even in the draft amendment of Codex Alimentarius [5], the spectrophotometric methodology used in the ISO 3632 standard is proposed for the determination of saffron quality, but this time high performance liquid chromatography has been included in order to detect the adulteration of saffron spice with water-soluble dyes. The question that arises now is: Why not determine the quality of saffron, in terms of quantification of their metabolites, by means of liquid chromatography? There are enough scientific papers which show that such a technique can contribute to a correct quality determination [6,15,12,16]. A specific approach was carried out by García-Rodríguez et al. [17] who developed an analytical protocol, in which extraction of the compounds is based on ISO 3632 [5], but where identification and quantification of the saffron metabolites is done using liquid chromatography equipment with aligned diode detector (HPLC-DAD) and quantification performed using the commercial patterns (esters of crocetin *trans*-4-GG and *trans*-3-Gg, safranal) and the picrocrocin isolated by the same group using the methodology described by Sánchez et al. [11].

1.2.2. EU Protected Designations of Origin

In recent years, there has been increased interest in guaranteeing and defending the quality of saffron produced in certain historical regions. As a result, there are six Protected Designations of Origin (PDOs) in Europe: "Krokos Kozanis" in Greece [18], "Azafrán de La Mancha" in Spain [19], "Zafferano dell'Aquila" in Italy [20], "Zafferano di San Gimignano" in Italy [21], "Zafferano di Sardegna" in Italy [22] and "Munder Safran" in Switzerland. The drawback is that, to demonstrate their quality, most of these PDOs use the spectrophotometric method of ISO 3632, which is based on an erroneous analytical technique as already mentioned.

2. Authenticity issues

The main problem for the saffron consumer is the lack of knowledge about the shape of the product, which is the reason why some plant products such as *Carthamus tinctorius* are on offer on the market that are not true saffron. Also by not knowing the product in certain regions, it is easy to confuse consumers with fibres coloured with artificial dyes.

2.1. Identification of current authenticity issues

Throughout history, the adulteration of saffron has been prosecuted, and for many centuries and in diverse cultures, adulteration of the spice carried the death penalty [23]. Nowadays, the most frequent adulteration is to dye fibres, plants or animals, with food colorants, simulating the colour of saffron stigma [24]. True saffron is also frequently confused with the petals of the flowers of *Carthamus tinctorius,* sometimes referred to as "bastard saffron", which is also considered a spice but which does not impart to food the colour, flavour and aroma of true saffron [25]. However, the most difficult way of adulterating this spice is when the saffron is sold as whole, where the filaments that form the stigma are seen, and which are joined or cut according to the production area.

Adulterants and substitutes mainly consist of parts of the plant of *Crocus sativus* or from other plants such as marigold (*Calendula officinalis*) or arnica (*Arnica Montana*), which might have been dyed. Many diverse plant materials use the name “saffron” all over the world, for example safflower (*Carthamus tinctorius*) is called bastard saffron or saffron thistle; marigold is also known as Indian saffron, American saffron or Mexican saffron. These names contribute to the misidentification of saffron in filaments. Turmeric *(Curcuma longa* L.) may be misidentified as powered saffron. The mixture with extracted, recoloured exhausted saffron or old saffron also constitutes a fraudulent practice. In addition, increasing moisture and adding substances like honey, starch, meat fibres, coloured artificial fibres or even inorganic compounds to increase weight constitute known adulterations [25]. Nowadays, the addition of artificial colorants is the most common type of fraud. The aim of this practice is to mislead the consumer by improving or changing the appearance of old and low quality saffron, or of other extraneous materials added, to increase weight or use as substitutes. Gardenia has been found among the latest adulterants and substitutes of saffron due to its content in crocetin esters [25,26].

The content of crocins and picrocrocin in saffron is very high; there is no other spice that has such a high content of these metabolites, which is the reason why this spice is so appreciated by the consumer. As the crocin content is higher than 16 %, it would not be economically profitable to adulterate saffron with another product by adding crocins to reach these concentrations; the fraud would be more expensive than saffron itself. In addition, given that the picrocrocin content has to be higher than 7 % to be considered saffron, and that this compound is a molecular marker since it is only found in saffron, the fraud would be even more expensive. In other words, simply by changing the method of determining the quality of the spice, all types of fraud could be avoided.

2.2. Potential threat to public health

Some adulterants may be dangerous for human health, as for example the so-called autumn saffron (*Colchicum autumnale*) which is extremely toxic. In principle, almost all adulterations of saffron can cause health problems. If saffron is adulterated with coloured fibres from other plants, allergy problems can occur since the consumer is not able to correctly identify the product being consumed which may be an allergen. If saffron is adulterated using artificial food colours to dye fibres that confuse the consumer, problems can be generated since their innocuous use is not admitted for this purpose. If it is with artificial colours, the problems can be greater, because these are not food grade products and can be toxic in varying degrees. The dust of metallic red oxides that are confused with ground saffron and that are very toxic and carcinogenic have also been detected. However saffron was and is still also valued as a medicinal plant with important biomedical applications. In the last 15 years, its bioactivity has been demonstrated and published in high impact scientific journals [10].

3. Analytical methods used to test for authenticity

3.1. Officially recognised methods

Non-saffron samples are not currently detected when evaluated by spectrophotometric measurements according to ISO 3632:2011 [5]. An approach to detect artificial colorants in saffron using derivative UV-Vis spectrometry was carried out in 2005, but the quantification limits reported were far from the actual market situation [27]. All this confusion would be greatly minimized, if the quality of the saffron were to be determined according to the content of crocins, picrocrocin and safranal by liquid chromatography.

As previously described, the method used in commercial transactions is the one described in ISO 3632-Part 2 [5], which is based on spectrophotometric measurements of aqueous saffron extracts. This methodology leads to erroneous results on two of the three main saffron metabolites due to the low selectivity in the determination of safranal and picrocrocin. This method is not selective because the absorbance at 330 nm and 257 nm, which are used to determine safranal and picrocrocin respectively, are wavelengths where other compounds also absorb. The misunderstanding due to the use of such methodology is producing significant errors. For example, in saffron-extract tablets used as dietary supplements, safranal is being quantified with a content higher than 5 % which means that the tablet would be toxic and taking several of them would lead to irreversible health problems for the consumer [14].

The problem about using the wrong standard (ISO 3632 Part 2 [5]) is that it has been extended to other official country standards, PDOs, Codex Alimentarius, etc. with the consequent global confusion of the chemical characteristics of the spice.

3.2. Other commonly used methods

The methods used that are proposed are not actually included in any of the official standards, as mentioned before, but could be used on a routine basis. Proper saffron quality determination should be carried out by the detailed quantification of the crocetin esters, picrocrocin and safranal metabolites by means of chromatography devices. LC-based methods with DAD detector may be used to determine water-soluble compounds such as crocins and picrocrocin. To determine the aromatic composition, GC-MS methods with different extraction and injection systems are used [15]. If only the most abundant volatile need to be quantified (safranal), HPLC-DAD methods can also be used [17]. An example of a saffron fingerprint, shown in Figure 2, which may help to define the authentication of the product [28] includes the chromatograms obtained at wavelengths of 440, 330 and 250 nm. At 440 nm, the fingerprint of the four mayor crocins, *trans*-4-GG, *trans*-3-Gg, *cis*-4-GG and cis-3-Gg, can be observed; the other peaks that appear at 440 nm are the rest of crocins named in the Figure 1, which may or may not be present. At 330 nm, the small peak of the safranal can be seen at the end of the chromatogram. At 250 nm, the peak of picrocrocin is observed.

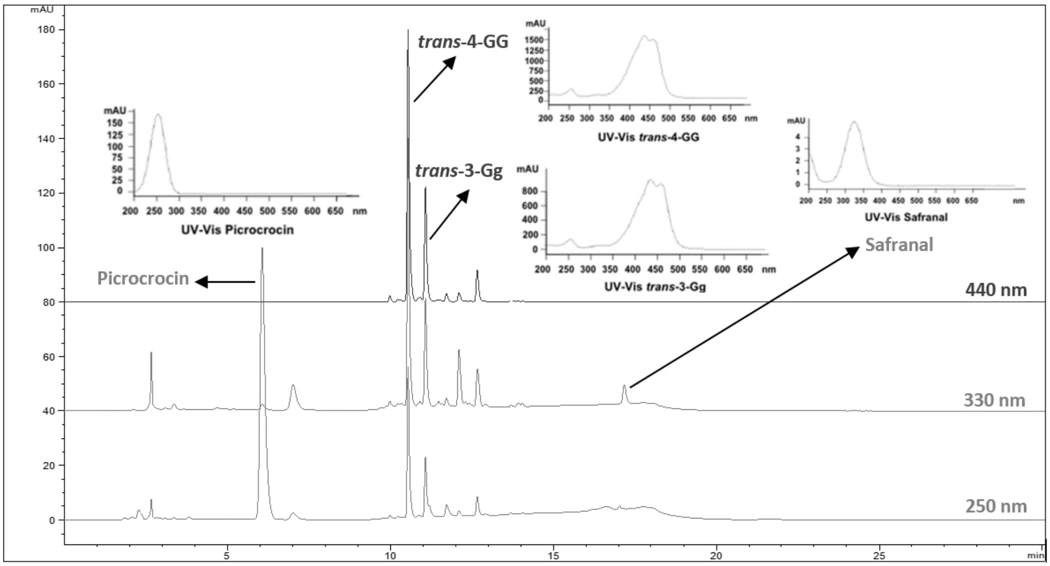


Figure 2: Saffron fingerprint obtained by HPLC, chromatograms at 440, 330 and 250 nm, and their UV-vis spectrum. The peaks corresponding to the major metabolites (crocins, safranal and picrocrocin) are indicated

4. Overview of methods for authenticity testing

The following table provides a summary of the methods and the authenticity issues they address.

Analytical technique	Indicative data or analyte	Authenticity issue / information
Spectrophotometry UV-Vis	Colouring strength	Authentication not possible / detection of artificial colorants in saffron (in g/kg)
Second derivative UV-Vis spectrophotometry	Artificial colorants water soluble	Detection of artificial colorants in saffron (in ppm)
HPLC-DAD	Crocins, picrocrocin and safranal	Saffron authentication (fingerprint) / quality
	Artificial colorants	Detection of artificial colorants in saffron
	Carminic acid	Not be present in Kosher and Halal foods
TLC	Artificial colorants	Detection of artificial colorants in saffron / disused technique
LC/DAD/MS/MS	Crocins, picrocrocin and flavonoids	Identification metabolites of saffron
UHPLC-MS/MS	Crocins	Differentiation of process obtaining saffron
DHS-GC-MS	Safranal and other aroma compounds	Fingerprint of saffron aroma / quality aroma
e-Nose	Volatiles of saffron as a whole	Determination geographical origin
Ultrasound extraction-GC-MS	Volatile compounds	Geographical differentiation of saffron
PTR-TOFMS	Volatile compounds	Identification volatile / quality aroma
Raman spectroscopy	Sum crocins and colouring strength	Quality of saffron
NIR spectroscopy	Saffron quality control	Determination of chemical composition and geographical origin
MIR spectroscopy	FT-IR spectra saffron filaments	Determination geographical origin
Tristimulus colorimetry	Colour	Quality of saffron
SBSE-GC-MS	Multi-residue	Contaminants and pollutants determination
Derivatisation-HPLC-DAD	Free amino acids and ammonium	Determination geographical origin
Stable isotopes H, C and N	Analysis of stable isotopes hydrogen, carbon and nitrogen	Determination geographical origin

5. Conclusion

To control the quality of saffron and avoid adulteration, it is necessary to introduce analytical methodologies in the compulsory standards, as well as in ISO 3632, which determine in detail the metabolites: crocins, picrocrocin and safranal, responsible for colour, taste and aroma, respectively. At this time and in the near future the methodology based on HPLC-DAD is the most appropriate, fastest and cheapest.

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