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HPLC/PDA/ESI-MS Evaluation of Saffron (*Crocus sativus L.*) Adulteration

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The present study evaluated the reliability of the ISO/TS 3632-2 UV-Vis spectrometric method for saffron classification, making experiments on saffron samples to which were added increasing concentrations of common saffron spice adulterants (safflower, marigold and turmeric). The results showed that the ISO/TS 3632-2 method is not able to detect addition of up to 10-20%, w/w, of saffron adulterants. For additions from 20 to 50%, w/w, of the three adulterants, saffron was classified in a wrong category; addition of higher than 50%, w/w, determined variations in the investigated parameters that did not allow identification of the product as "saffron". In all cases, the method did not permit the recognition of the nature of the adulterant. On the contrary, the specificity of the HPLC/PDA/MS technique allowed the unequivocal identification of adulterant characteristic marker molecules that could be recognized by the values of absorbance and mass. The selection of characteristic ions of each marker molecule has revealed concentrations of up to 5%, w/w, for safflower and marigold and up to 2% for turmeric. In addition, the high dyeing power of turmeric allowed the determination of 2%, w/w, addition using exclusively the HPLC/PDA technique.

Keywords: spice, saffron, safflower, marigold, turmeric, fraud.

Saffron (*Crocus sativus* L., family Iridaceae) is the most valuable spice in the world. The species is a perennial meadow plant that reaches 10 to 25 cm and grows from bulbs. The cultivation of saffron requires a Mediterranean continental climate with cold winters, warm and dry summers, and a dry Mediterranean humidity regime. The plant is resistant to extreme temperatures in the summer and winter [1a]. Saffron is a spice that adds color, taste and aroma to various foods. The odor is related to its essential oil, a component of which is cineole. The bitter taste of saffron is due to picrocrocin and picrocrozioide, and its color from crocin esters that produce glucose and crocetin after hydrolysis [1a].

The world production of saffron is about 180 tons per year of which 90% is produced in Iran and the remainder in India, Greece, Morocco, Italy, Spain and other countries. The biggest worldwide exporters are Iran, followed by Spain. In Italy the annual production is around 400 kg [1a]. Saffron is cultivated mainly in Sardinia and Abruzzo, with about 35 ha and 7 ha, respectively, and to a lesser extent in Umbria, Tuscany, Liguria and Sicily. Some productions (i.e. Abruzzese di Navelli and San Gimignano) have been awarded the Protected Designation of Origin (PDO). The commercialization costs, including the different process stages, could exceed 1,000 €/kg.

Nowadays saffron production faces a crisis, but all nations involved are traditionally committed to saffron cultivation and preserve it actively. In the Mediterranean basin production of saffron has been decreased due to rising standards of living and, inevitably, due to the rise in labor costs. However, Mediterranean saffron bears the best quality features worldwide, which is attributed to deep cultivation knowledge and careful treatment by all European producers.

Due to its high value, saffron spice has been subjected to many adulterants throughout history, such as mixing of extraneous materials, immersing with vegetable oil or glycerin, and addition of various mineral substances, artificial colorants and less valuable colored spices [1a]. Saffron quality is determined after a series of characteristic parameters for the spice itself (moisture content, flower residue, foreign material, ash content, soluble condensate, coloring power, etc) combined with necessary external conditions (absence of parts from other plants, micro-biological flora and pesticide residues). Methods applied for quality assurance are widely known and enterprises are able to use the necessary technology in order to guarantee the product quality to consumers. Since 1980 a standard procedure (ISO/TS 3632) allows the quality for saffron classification. ISO/TS 3632 was updated in 2003 with the

Table 1: Specifications of ISO/TS 3632 "Saffron" [1b].

Characteristics	Specifications Categories I	II	III	Test method
Moisture and volatile matter, % (w/w), max.	12[a] 10[b]	12[a] 10[b]	12[a] 10[b]	ISO/TS 3632-2:2003, Clause 7
Total ash, % (w/w), on dry basis, max.	8	8	8	ISO 928:1997. Clause 8, and ISO/TS 3632-2:2003, Clause 12
Acid-insoluble ash, % (w/w), on dry basis, max.	1	1	1.5	ISO 930:1997. Clause 7, and ISO/TS 3632-2:2003, Clause 13
Bitterness, expressed as direct reading of the absorbance of picrocrocine at about 257 nm, on dry basis, min.	70	55	40	ISO/TS 3632-2:2003, Clause 14
Safranal, expressed as direct reading of the absorbance at about 330 nm, on dry wt basis min. max.	20 50	20 50	20 50	ISO/TS 3632-2:2003, Clause 14
Coloring strength, expressed as direct reading of the absorbance of crocine at about 440 nm, on dry wt basis, min.	190	150	100	ISO/TS 3632-2:2003, Clause 14
Artificial water soluble acid colorants	Absent	Absent	Absent	ISO/TS 3632-2:2003, Clause 16 and/or Clasue17

[a]filaments; [b] powder.

text that currently governs the product quality [1b]. This regulation is applicable to saffron strands, ground saffron and dust. The rule divides saffron into different categories based primarily on physical-chemical criteria (Table 1).

European saffron is considered the best in the world due to its chemical, physical and organoleptic features, as measured by certain parameters. New high quality verification standards and new evaluation methods should be introduced in order to determine accurately color and to prevent fraud, as reported by many scientific works [1c,2a,2b,3].

The present study evaluated the reliability of the ISO/TS 3632 UV-Vis spectrometric method for saffron classification, making analyses on samples of saffron blended with different concentrations of safflower, marigold and turmeric, widely used as saffron spice adulterants [1a,2a]. The results of the spectrometric method were compared and integrated with the HPLC/PDA/ESI-MS technique for the unequivocal identification of adulterants through the identification of specific marker compounds.

In Table 2 are reported the data related to the different parameters measured according to the ISO/TS 3632 (2003) spectrometric method for saffron category assignment performed on unadulterated and on spice-spiked San Gavino ISO Category II saffron. Five independent additions at different concentrations (10-67%) of adulterants (safflower, marigold and turmeric) were made.

Results showed that the ISO/TS 3632-2 spectrometric method is not able to detect the addition of up to 10-20%, w/w, of saffron adulterants, resulting in a correct

Table 2: Results of ISO/TS 3632 UV-vis spectrophotometry on the analyzed mixes.

Mix composition w/w	$E^{1\%}$ 257 nm	$E^{1\%}$ 330 nm	$E^{1\%}$ 440 nm	ISO Category
Saffron 100%	66	32	170	II
90% Saffron - 10% Turmeric	59	27	150	II
80% Saffron - 20% Turmeric	53	25	132	III
67% Saffron - 33% Turmeric	45	21	111	III
50% Saffron - 50% Turmeric	34	15	81	-
33% Saffron - 67% Turmeric	22	9	49	-
90% Saffron - 10% Safflower	63	31	152	II
80% Saffron - 20% Safflower	59	30	136	III
67% Saffron - 33% Safflower	53	29	113	III
50% Saffron - 50% Safflower	48	27	86	-
33% Saffron - 67% Safflower	42	26	60	-
90% Saffron - 10% Marigold	61	30	151	II
80% Saffron - 20% Marigold	55	29	135	III
67% Saffron - 33% Marigold	49	25	112	III
50% Saffron - 50% Marigold	39	22	83	-
33% Saffron - 67% Marigold	31	18	54	-

$E^{1\%}$ absorbency at λ_{max} for a 1% solution of the test sample for a 1cm cell

classification of the mixes as saffron ISO category II. For additions from 20 to 50%, w/w, of the three adulterants studied, the mixes were classified as worse than ISO category III saffron. Spikes higher than 50%, w/w, determined variations of the investigated parameters that did not allow the identification of the mixes as "saffron". In all cases the method did not permit the recognition of the kind of adulterant.

Therefore, the use of only the spectrometric technique may underestimate the saffron fraud occurrence due to the addition of less valuable spices. The hyphenated techniques like high performance liquid chromatography coupled with UV-Vis spectrophotometers and mass spectrometry may allow a better assessment of the quality of the saffron products.

Preliminarily, this study has characterized separately the fingerprint HPLC/PDA/ESI-MS of acidified water-methanol extracts of saffron, marigold, safflower and turmeric. Figure 1 showed the UV-Vis chromatograms of the studied spices. In Table 3 are reported the assignment of the characteristic molecules of each botanical species as a function of retention times, UV-Vis and mass properties.

Crocetin glycosides are responsible for the saffron yellow color; their UV-Vis spectra are characterized by an absorption maximum at about 440-460 nm depending on the molecule. *Trans*- and *cis*-crocetin glycoside showed a different spectroscopic behavior because *cis*-crocetins presented an additional absorption band around 325 nm in their UV-Vis spectrum in comparison with their *trans*-isomers. Six crocetin glycosides (**2-7**), together with colorless picrocrocin [4-(*α*-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde] (**1**), were identified in the analyzed San Gavino saffron. As confirmed by ESI-MS analysis, in agreement with literature data [2b,4], *trans*-crocetin di-(*β*-D-gentibiosyl) ester (**3**), *cis*-crocetin (*β*-D-glucosyl)-(*β*-D-gentibiosyl)

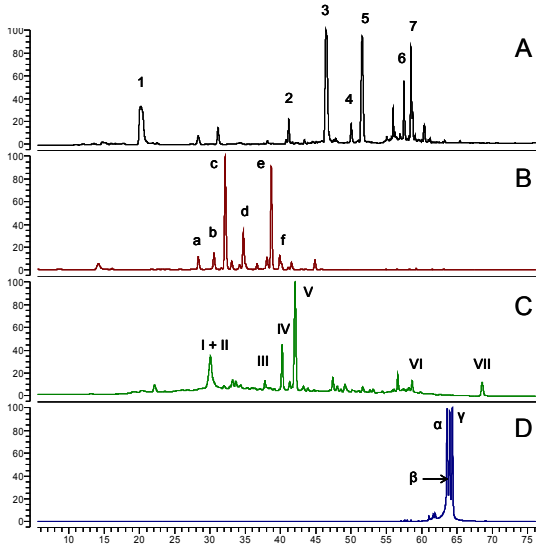

Figure 1: UV-Vis chromatograms of analyzed extracts. **A:** Saffron (λ 250 nm + λ 440 nm); **B:** Marigold (λ 350 nm); **C:** Safflower (λ 410 nm + λ 520 nm); **D:** Turmeric (λ 425 nm). For peak identification see Table 3.

Table 3: HPLC/PDA/MS chemical characterization of studied extracts.

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

ester (**5**) and *cis*-crocetin di-(*β*-D-glucosyl) ester (**7**) were the most abundant crocetin derivatives, followed by *cis*-crocetin di-(*β*-D-gentibiosyl) ester (**6**), *trans*-crocetin (*β*-D-neapolitanosyl)-(*β*-D-gentibiosyl) ester (**2**) and *trans*-crocetin (*β*-D-glucosyl)-(*β*-D-neapolitanosyl) ester (**4**).

Marigold extract was characterized by six main peaks (**a-f**), which displayed identical UV absorptions with maxima at about 255 and 350 nm, typical of flavonols. The ESI-MS $[M-H]^-$ molecular ions, together with comparison with scientific references [5], permitted their unequivocal assignation to quercetin 3-*O*-rutinosylrhamnoside (**a**), quercetin 3-*O*-rutinoside (**b**), isorhamnetin-3-*O*-rutinosylrhamnoside (**c**), narcissin (**d**), isorhamnetin 3-*O*-neohesperidoside (**e**), and isorhamnetin-3-*O*-glucoside (**f**). Among them, the isoramnetin derivatives predominated in the analyzed sample.

As reported by many scientific papers [6,7], the main component of safflower red pigments is carthamin, composed of two chalconoids with conjugated bonds; it is derived from the yellow colored precarthamin by decarboxylation. Both compounds were detected in the analyzed safflower sample as peaks **VI** and **VII**. Safflomin A (**I**), safflor yellow B (**IV**) and anhydrosafflor yellow B (**V**) were identified as the quinochalcone *C*-glycosides responsible for the yellow color of the sample. Moreover, two kaempferol derivatives were identified as 6-hydroxy-kaempferol 3-*O*-*β*-D-glucoside (**II**) and kaempferol 3-*O*-*β*-rutinoside (**III**). Identification was confirmed by spectroscopic and mass spectral data. Three molecules (**α-γ**) were identified as responsible for the yellow distinctive color of turmeric: all of them clearly possess a maximum absorption wavelength near 420 nm. According to literature data [8], these compounds were identified as the dicinnamoylmethane derivatives demethoxycurcumin (**α**), bisdemethoxycurcumin (**β**) and curcumin (**γ**) on the basis of $[M-H]^-$ molecular ions generated by the ESI-MS negative soft ionization. Subsequently, the above-mentioned analytical technique was applied to saffron samples mixed with different concentrations of turmeric, marigold and safflower in the range 2-20%, w/w. For each adulterant, marker molecules have been chosen for their unambiguous identification in the mixture: it was established that their detection was not influenced by the saffron matrix effect.

Extraction of the ion with m/z 623, corresponding to isorhamnetin 3-*O*-neohesperidoside (**e**), is able to detect the presence of marigold. In the case of safflower, the marker molecules for its identification in the mixture were anydrosafflor yellow B (**V**) and chartamin (**VII**) with ions at m/z 1044 and 909, respectively. The marker molecules could be revealed at concentrations of up to 5%, w/w, of both marigold and safflower. The characteristic turmeric curcuminoids triplet due to the presence of demethoxy-curcumin (**α**), bisdemethoxy-curcumin (**β**) and curcumin (**γ**) could easily identify its presence in the mix with saffron, and also at concentrations of 2%, even using only the UV-Vis detector. The above discussed method is currently applied by the Catania Laboratory of Central Inspectorate for Quality Control of Agricultural and Food Productions (ICQRF) on samples of saffron collected in the Italian market in the framework of the Ministry of Agriculture and Forestry institutional quality control activity.

In conclusion, the results of the present study should force the legislative authorities to release new standards for the saffron sector for the maintenance of product purity in order to avoid adulteration and fraud. The methods applied nowadays are outdated, while newer ones are not positively accepted and are rarely used, even though some of them have proven to be efficient in the field. All national and international quality control standards should be reinforced in order to limit the spread of adulterated saffron in the European market deriving from countries that affect considerably the competitiveness of European saffron.

Experimental

Plant material, reagents and standards: Strands of saffron (*Crocus sativus* L.) from San Gavino (Sardinia, Italy), turmeric (*Curcuma longa*) powder, calendula (*Calendula officinalis*) flowers, and safflower (*Carthamus tinctorius*) strands were taken in Italy by ICQRF officials. The plant material was treated in accordance with the specifications of ISO/TS 3632-2:2003 sample preparation for the official analysis [1b]. Different mixes were prepared with saffron with percentages varying from 2 to 70% by weight of each adulterant studied. HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system.

UV-Vis spectrophotometry: The method used was based on the technical specification ISO/TS 3632-2:2003 [1b] and allowed determination of the main characteristics of saffron. The method is based on the spectrophotometric assessment absorption at 3 wavelengths (λmax): 257 nm (maximum absorption of picrocrocin), 330 nm (maximum absorption of safranal) and 440 nm (maximum absorption of crocin).

HPLC/PDA/ESI-MS fingerprint: 20±2 mg of sample was extracted with 2 mL of 50% aqueous methanol containing 0.1% formic acid at room temperature in an ultrasonic bath for 30 min. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant decanted and used for the LC analyses, after filtration through 0.45 µm PTFE filters (LabService Analytica, Bologna, Italy). The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Gemini C18 150 x 2.1 mm i.d. 3µm (Phenomenex); the flow rate was 200 µL/min, the column temperature 30°C and the injection volume 10 µL. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43 % B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 200–700 nm and the mass scan range was 100-1600 m/z. Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 3.5 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 14 (arbitrary units). The main compounds of analyzed plant materials were characterized in terms of retention times, lambda max and MS data.

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