P66: CURRENT STATUS AND FUTURE NEEDS IN THE ASSESMENT OF SAFFRON QUALITY PARAMETERS INCLUDED IN THE ISO 3632: "SAFFRON (*CROCUS SATIVUS* L.)"

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Abstract – For more than two decades, commercial transactions of the highly valuable spice saffron rely on the quality parameters specified in the trade standard ISO 3632, that are (a) coloring strength, (b) flavor and (c) aroma strength. These traits, associated with the content of saffron aqueous extracts in sugar esters of *trans*- and *cis*-crocetin (crocins), picrocrocin and safranal are tentatively estimated and expressed as $E^{1\%}$ values at 440 nm, 257 nm and 330 nm, respectively. However, the ISO-suggested methodology has been repeatedly criticized in the relevant scientific literature as non-specific and non-accurate. In this study, we propose an improvement for the quantification of total crocetin esters and picrocrocin content using the ISO-suggested extraction protocol along with UV-Vis spectrophotometry and external standard method with in-house isolated compounds (*trans*-crocetin (di-β-D-gentiobiosyl) ester and picrocrocin). The results, expressed as absolute weight values are compared with those obtained using the external standard method after RP-HPLC-DAD analysis. Our approach is in line with the current food legislation requirements for accurate determination of analytes and avoidance of estimations.

Keywords: quantification, crocetin esters, picrocrocin

1. INTRODUCTION

Saffron, the most expensive spice in the world is produced from the red stigmas of the flower of the plant *Crocus sativus* L. after dehydration. For more than two decades, commercial transactions of the product rely on the ISO 3632 specifications of quality [1] according to which saffron is classified in three trade categories. Classification is mainly based on certain physicochemical parameters of quality that are, (a) the coloring strength, (b) the flavor strength and (c) the aroma strength, associated with the content in sugar esters of *trans*- and *cis*-crocetin (crocins), picrocrocin [4-(β-D glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexane-1-carboxaldehyde] and safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde), respectively. These contents are tentatively estimated in saffron aqueous extracts using UV-Vis spectrophotometry and are expressed as $E^{1\%}$ values at 440 nm, 250 nm and 330 nm, respectively. However, the ISO-suggested methodology for the quantification of saffron major constituents has been repeatedly criticized in the relevant scientific literature [2,3] since it presents several analytical pitfalls. For example, absorption at 257 nm is not specific for picrocrocin while that at 330 nm does not quantitatively express the content of the poorly water-soluble safranal. Furthermore, given that standard crocins and picrocrocin of high purity are still not commonly available in the chemicals market, quantification of the latter saffron constituents involves the use of the mass extinction coefficient values ($E^{1\%}$) instead of the net content ones (% w/w). As it has been evidenced already, specific determination of total and individual crocins as well as picrocrocin can be achieved after RP-HPLC-DAD analysis [4] but no such protocol has been adopted from the relevant ISO technical committee (ISO TE34/SC7) yet. In this study, we propose an improvement for the quantification of total crocetin esters and picrocrocin content using the ISO-suggested extraction protocol and UV-Vis spectrophotometry. The procedure engages the application of the external standard method using in-house isolated compounds (*trans*-crocetin (di-β-D-gentiobiosyl) ester and picrocrocin) of high and consistent purity. The coloring and flavor strengths were expressed as absolute weight values, that is, g

of total crocetin esters or picrocrocin/100 g of dry plant material. These values are also compared with those obtained using the external standard method after RP-HPLC-DAD analysis [4].

2. EXPERIMENTAL

2.1. Samples, reagents and solvents

Saffron samples (*n* = 26) were supplied by the "World Saffron collection" (CROCUS BANK, Quenca, Spain). The samples corresponded to 10 different accessions of *C. sativus* L. stigmas, originating from corms cultivated in the same field and processed under the same conditions. Methanol, acetonitrile (Chem-Lab, Zedelgen, Belgium) and acetic acid (Fluka Chemie, Buchs, Switzerland) used were HPLC grade. Ultrahigh purity water was produced using a SG 2002 v.1.01 system (Barsbuttel, Germany).

2.2. Apocarotenoid isolation

Trans-4-GG crocetin ester [trans-crocetin (di-β-D-gentiobiosyl) ester] was isolated by semi-preparative HPLC and checked for purity (97%), according to [4]. Picrocrocin [4-(β-D glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexane-1- carboxaldehyde] was isolated by Solid Phase Extraction (SPE) according to [5] and checked for purity (91%) as previously described [4].

2.3. Saffron extract preparation

Saffron samples were subjected to ultrasonication (Ultrasonic Homogenizers HD 2070, Berlin, Germany) for 30 min and 0.2 s cycles, at 100% amplitude, according to the optimum procedure for the extraction of crocins, described by [4]. Sample (g) to solvent (mL) ratio was 1:1800, as recommended in the ISO 3632-2 test methods [6].

2.4. UV-Vis analysis

The UV–vis spectra of all of the solutions and extracts after proper dilution (1:10, v/v) were recorded in the region 200–600 nm with a spectrophotometer (Shimadzu UV 1601, Kyoto, Japan). The absorbance values at 440 nm were used for the determination of total crocetin sugar ester content by means of a six-point regression curve (r2 > 0.993) using trans-4-GG as reference external standard in a concentration range between 6.3 μg/mL to 25.0 μg/mL. LOD (Limit of Detection) was 2.1 μg/mL and Limit of Quantification (LOQ) was 6.3 μg/mL. For the quantification of picrocrocin, a six-point regression curve (r2 > 0.999) was used based on the absorbance values of standard picrocrocin at 257 nm within a concentration range of 3.3 μg/mL to 50.0 μg/mL. LOD (Limit of Detection) was 1.1 μg/mL and Limit of Quantification (LOQ) was 3.3 μg/mL. Results were expressed as g total crocetin esters or picrocrocin / 100 g saffron. The absorbance values at 440 and 257 nm were also used to estimate mass extinction coefficient values ($E^{1\%}$) using the Lambert-Beer law and according to ISO 3632-2 [6]

2.5. HPLC analysis

Reverse phase High Performance Liquid Chromatography (RP-HPLC) was also performed to determine the contents of crocins and picrocrocin. The HPLC system was consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA, USA), a Midas autosampler (Spark, Emmen, The Netherlands) and a UV 6000 LP diode array detector (DAD; Thermo Separation Products). Separation was carried out on a LiChroCART Superspher 100 C18 (125 mm × 4 mm i.d., 4 μm) column (Merck, Darmstadt, Germany) using the gradient elution with a mixture of water:acetic acid 1%, v/v (A):acetonitrile (B) (20–100% B in 20 min) at a flow rate of 0.5 mL min^{-1}. Injection volume was 20 μL. Monitoring was in the range of 200–550 nm and quantification was carried out by integration of the peak areas at 250 nm (picrocrocin) and 440 nm (crocins), as described in [4].

2.6. Statistical Analysis

Statistical comparisons of the mean values were performed by one-way ANOVA, followed by the multiple Duncan test ($p < 0.05$ confidence level) using the SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

Prior to the calculation of the net content values of total crocetin esters and picrocrocin, the mass extinction coefficient values corresponding to the colouring strength and bitterness of the spice (***$E^{1\%}$*** 440 nm and $E^{1\%}$ 257 nm, respectively) were estimated [6]. The $E^{1\%}$ 440 nm values ranged from

207±1.1 to 276±1.1 while those at 257 nm ranged from 75±0.5 to 95±1.1, indicating that all the studied samples belong to the ISO category I. The absorbance values of the samples at 440 and 257 nm were then used to calculate the % (w/w) concentration of total crocetin esters and picrocrocin, respectively, based on the corresponding calibration curves. These values are presented in Table 1, along with the $E^{1\%}$ ones.

Table 1. Spectrophotometric data for the contents of total crocetin esters and picrocrocin, expressed as % (w/w) and $E^{1\%}$ values

Sample	**% (w/w) trans-4-GG**	**$E^{1\%}_{440nm}$ (colouring strength)**	**% (w/w) picrocrocin**	**$E^{1\%}_{257nm}$** (bitterness)
1	39.0±0.2	276±1.1	15.5±0.2	95±1.1
2	35.2±0.4	248±4.2	14.5±0.2	89±2.0
3	34.5±1.0	242±5.9	13.8±0.4	84±3.0
4	31.9±0.4	223±0.0	13.4±0.2	82±1.2
5	32.8±0.2	230±0.2	13.5±0.0	83±0.2
6	30.0±0.1	209±0.6	12.7±0.7	78±4.5
7	30.9±0.4	216±2.9	12.4±0.3	76±1.7
8	36.3±0.5	256±1.1	14.5±0.2	89±1.4
9	35.8±0.7	252±3.6	14.7±0.5	90±3.3
10	34.3±0.2	241±0.0	13.6±0.1	84±0.5
11	29.7±0.1	207±1.1	12.8±0.2	78±1.2
12	30.0±0.2	209±0.1	12.2±0.1	75±0.5
13	33.2±0.3	233±1.0	13.5±0.1	83±0.4
14	36.1±0.9	255±8.1	14.2±0.4	87±2.3
15	34.7±3.9	244±0.8	14.8±0.4	91±2.3
16	33.8±0.2	237±0.8	13.9±0.1	85±0.4
17	31.2±0.3	218±2.8	12.6±0.1	77±0.7
18	33.0±0.2	231±1.6	13.9±0.2	85±1.4
19	32.1±0.4	225±3.6	12.9±0.2	79±1.3
20	33.6±0.2	236±1.4	13.2±0.1	81±0.8
21	34.4±0.4	242±2.4	13.8±0.1	85±0.5
22	34.5±0.6	242±1.6	13.8±0.3	85±1.8
23	31.0±0.7	217±4.0	12.5±0.4	77±2.2
24	36.1±0.4	256±2.2	15.2±0.2	94±1.0
25	36.8±0.4	259±2.2	14.8±0.1	91±0.4
26	36.5±1.1	257±1.2	14.2±0.0	87±0.3

Interestingly, all the studied samples were found to contain more than 29.0 % of total crocetin esters on dry basis. This content is much higher than the respective values reported by other researchers for high quality saffron from different production areas of the world [7]. The published data were produced using a mathematical equation that employs mass extinction coefficient values at 440 nm along with chromatographic peak areas of crocetin esters and represents another type of tentative estimation of these metabolites. In the present study, the ascending order of samples based on their total crocetin ester content was generally in line with that based on colouring strength values. Regression analysis of $E^{1\%}_{440}$ versus the % content values for total crocetin esters in saffron gave the equation: $E^{1\%}_{440} = 7.4308x - 13.798$; $R^2 = 0.9994$, signifying a good correlation among the two quantification approaches. In any case, the use of calibration curves using high purity standards is superior as it gives absolute values of the content of the tested materials and not arbitrary numbers that can be only understood by comparison to the max or min ones set in the trade standard.

As far as picrocrocin determination is concerned, the % values shown in Table 1 ranged

from 12.2 to 15.0 g/100 g saffron. These contents are in line with those reported in [7] for saffron of ISO category I, probably because calculations in the latter study had been carried out with the external standard method, too. The ascending order of samples based on $E^{1\%}$257 nm values or the net picrocrocin content (g/100 g saffron) presented more inconsistencies than those evidenced in the case of total crocetin esters although the regression curve between the two parameters was found fairly linear: $E^{1\%}$257 nm = 6.2336x – 1.3371; R^2 =0.9968.

As a next step, liquid chromatographic analysis was carried out to separate the compounds of interest and then accurately determine their contents using chromatographic peak areas and the external standard method. The % values for total crocetin esters or picrocrocin that were calculated after HPLC analysis were then plotted against the respective values that were calculated spectrophotometrically. The regression curves are illustrated in Figures 1 and 2.

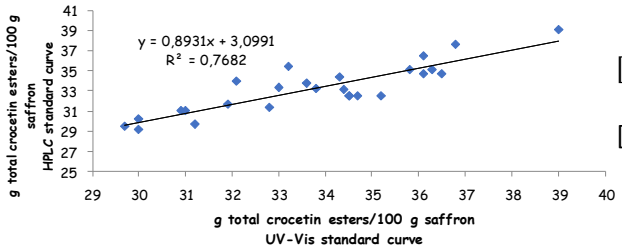

Fig. 1. Regression curve between spectrophotometric and chromatographic data for the % total crocetin ester content (w/w), expressed as *trans*-4-GG crocetin ester

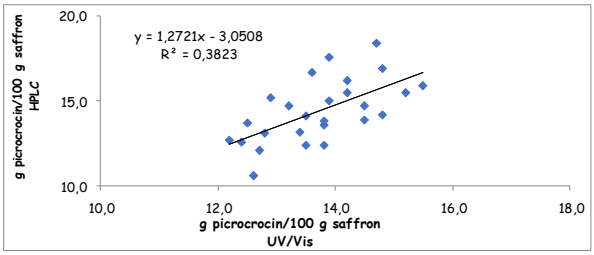

Fig. 2 Regression curve between spectrophotometric and chromatographic data for the % content of picrocrocin (w/w).

Based on the linearity of the regression curve between chromatographic and spectrophotometric data for % content in total crocetin esters, it seems that UV-Vis examination of saffron extracts may be employed instead of RP-HPLC analysis provided that available standard exists. This would allow high-throughput analysis of samples for quality control purposes (~ 1 min vs 1 h, respectively).

On the other hand, the spectrophotometric data for % content in picrocrocin were poorly correlated with those calculated after HPLC analysis, most probably because of the poor specificity of the A257 values. These results signify the importance of chromatographic separation of picrocrocin and determination of its accurate content through a properly constructed calibration curve.

Overall, our approach is in line with the current needs in food legislation to accurately quantify crocetin esters and picrocrocin and avoid estimations.

AKNOWLEDGMENT

The curator of the World Saffron collection (CROCUS BANK, Quenca, Spain) is deeply acknowledged for providing authentic samples upon request.

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