



Improved quantification method of crocins in saffron extract using HPLC-DAD after qualification by HPLC-DAD-MS

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ABSTRACT

Crocins in commercial liquid saffron extracts (Saffr'activ®) were identified using high-performance liquid chromatography (HPLC) with a diode array detector (DAD) and mass spectrometry (MS). The impact of storage on the qualities of the saffron extract were studied with HPLC-DAD-MS by exposing *trans*-4-GG crocin to environmental factors. Light and temperature induced degradation after only one week. *Trans*-4-GG crocin was totally hydrolyzed when stored at 60 °C and exposed to light. A quick and reliable method using HPLC-DAD was then developed to improve quantification of crocins in commercial liquid saffron extracts. An internal standard quantification method that uses a response factor, corrected with the molecular weight of each crocin, improved results for old saffron extracts.

1. Introduction

Saffron is produced from the red stigmata of *Crocus sativus*, a plant belonging to the Iridaceae family, and is widely used for cooking. The main compounds in saffron are crocins, crocetin, picrocrocins, and safranal (Mohamadpour et al., 2013). Safranal is an aromatic terpene responsible for the aroma of saffron. Several biological properties of saffron have been unambiguously attributed to safranal, such as antidepressant (Amin et al., 2015; Hosseinzadeh et al., 2004), anxiolytic (Pitsikas et al., 2008), hypnotic (Hossein Hosseinzadeh & Noraei, 2009), and analgesic properties (Erfanparast et al., 2015). The bitterness of saffron can be attributed mainly to picrocrocin, which is a glycosylated compound precursor of safranal (Lozano et al., 2000). Little is known about its biological activities, as picrocrocin is not easily available to conduct studies. Crocetin is a carotenoid-derived diacid responsible for the color of saffron and has demonstrated vasodilator (Llorens et al., 2015), antioxidant (Chen et al., 2016), antidepressant (Amin et al., 2015), and neuroprotective effects (Ahmad et al., 2005). Molecules of the crocin family are constituted of a crocetin moiety linked to sugars via an ester bond. Currently, eight crocins are known, in both *cis*- and *trans*- forms. These 16 isomers are mono- or di-glycosylated crocetins, whereas the main crocin in saffron is the *trans*-4-GG crocin (Table 1, Table S1) (Carmona et al., 2006). Crocins have been proven to

be safe for humans (Mohamadpour et al., 2013).

As one of the most expensive spices in the world, saffron is regularly a subject of fraud and adulteration (Rocchi et al., 2018). ISO 3632.1 and ISO 3632.2 standards have been established to ensure the quality of this plant, the absence of additives or adulterants (pesticides, artificial color, etc.), and to assess the relative amounts of active compounds.

ISO 3632–2 2010 measures, by UV–visible spectrophotometry, the absorbance at 250, 330, and 440 nm, which supposedly are representative of the picrocrocin, safranal, and crocins amount, respectively. However, this method for quantifying the saffron constituents is not a specific technique and has some limitations when applied to assessing authenticity (Rocchi et al., 2018).

Due to their biological properties, the crocin quantity should be determined with accuracy in order to determine the dose for medication. A near-infrared spectroscopy method combined with chemometric techniques has been used to determine the content of crocins (Li et al., 2018), but this method has not been widely adopted in the nutraceutical industry. Indeed, the quantification method requested by the nutraceutical industry has to be simple, rapid, and reliable. The analysis of crocin content by HPLC has already been studied by several authors (Bergonzi et al., 2012; Carmona et al., 2006; Liu et al., 2018; Rocchi et al., 2018), but Sánchez et al., 2008 showed that crocins are sensitive to increasing temperatures. Sanchez also demonstrated that purified

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Table 1
Main characteristics of crocins identified in the saffron extract using HPLC-DAD-MS.

Compound number	Retention time (min)	Observed ions (<i>m/z</i>)	M + Na ⁺	Number of glucose units	Molecular weight (amu)	Raw formula	Maximum of absorption (nm)	Identified crocins
1	18.1	329 ; 675 ; 837	1161	5	1138	C ₅₀ H ₇₄ O ₂₉	326, 440	<i>cis</i> -5-tG
2	19.8	329 ; 491 ; 653 ; 675 ; 994	999	4	976	C ₄₄ H ₆₄ O ₂₄	440	<i>trans</i> -4-GG
3	20.8	329 ; 653 ; 837	999	4	976	C ₄₄ H ₆₄ O ₂₄	440	<i>trans</i> -4-ng
4	21.4	329 ; 491	837	3	814	C ₃₈ H ₅₄ O ₁₉	440	<i>trans</i> -3-Gg
5	25.7	329 ; 675 ; 994	-	4	976	C ₄₄ H ₆₄ O ₂₄	326, 440	<i>cis</i> -4-GG
6	26.5	311 ; 473 ; 491 ; 635 ; 797 ; 959 ; 994	999	4	976	C ₄₄ H ₆₄ O ₂₄	326, 440	<i>cis</i> -4-ng
7	26.8	311 ; 329 ; 473 ; 513	675	2	652	C ₃₂ H ₄₄ O ₁₄	440	<i>trans</i> -2-G
8	27.5	311 ; 329 ; 473 ; 513 ; 635	-	2	652	C ₃₂ H ₄₄ O ₁₄	326, 440	<i>cis</i> -2-G
9	27.7	311 ; 329 ; 473	837	3	814	C ₃₈ H ₅₄ O ₁₉	326, 440	<i>cis</i> -3-Gg
10	29.3	329 ; 473	675	2	652	C ₃₂ H ₄₄ O ₁₄	326, 440	<i>cis</i> -2-gg

crocins are less stable than crude saffron extracts. Thus, standard crocins used for calibration curves are probably sensitive to temperature. In this context, the aim of the study was to find a reliable method that is easy to implement for quantifying crocins in saffron extracts.

For this purpose, HPLC-DAD-MS, which is widely available in laboratories, was used to identify individually each crocin of the extract. In this study, the impact of storage conditions on the quality of standard crocins was evaluated. Standard *trans*-4-GG crocin and saffron extract were monitored for one week while exposed to air, light, and heat to determine if the use of the response factor (*k*) of this major crocin to quantify all the crocins is relevant. The second part of the study investigated the influence of a corrected response factor.

2. Materials and methods

2.1. Chemicals and reagents

Saffron stigmata were purchased from a producer of Khorasan region (Iran). Dried stigmata were compliant with European food regulation. Saffron stigmata were category I according to ISO 3632-2 norm with 8.3% of moisture and volatile matter content, picrocrocin E^{1%} of 82, crocin E^{1%} of 212.02 and safranal E^{1%} of 35.4.

Commercial Saffr'Activ® liquid batch SAF 3C 190302L standardized with 3.4% crocins and 2.6% safranal (ISO 3632-2 2010), as determined by spectrometry (ISO 3632-2 2010), and commercial Saffr'Activ® liquid batch SAF 3C 210101L standardized with 7.9% crocins, as determined by HPLC, were prepared by Green Plant Extract (Comercial Quimica Masso, Barcelona, Spain) from saffron stigmata described above.

Standard crocin (*trans* Gen/Gen crocin 99%, reference 80391) were purchased from PhytoLab (Vestenbergsgreuth, Germany). Acetonitrile (HPLC grade, purity > 99.9%, reference 34851), water (HPLC grade, reference 270733), 4-Nitroaniline analytical standard (purity > 98%, reference 31569) and safranal (purity > 90%, reference W338907) were purchased from Sigma-Aldrich (Germany) and formic acid Optima (HPLC grade, purity 99.5%, 10596814) was purchased from Fisher chemical.

2.2. HPLC-DAD-MS

Prior to HPLC analysis, each sample was filtered through a 0.2 µm cellulose acetate membrane (VWR international, USA). 10 µL of sample were injected in a Shimadzu single quadrupole LC-MS-2020 equipped with a SPD-M20A Diode Array. The chromatographic separations were performed on a Sunfire C18 column (250 mm × 4.6 mm i.d., 5 µm particle size) at 30 °C. The mobile phase consisted of water with 0.1% formic acid and acetonitrile with 0.1% formic acid, using the following gradient: 0 to 5 min: 10% acetonitrile; 5 to 17 min: 30% acetonitrile; 17 to 24 min: 40% acetonitrile; 24 to 26 min: 60% acetonitrile; 26 to 27 min: 60% acetonitrile; 27 to 32 min: 80% acetonitrile; 32 to 36 min: 10% acetonitrile; 36 to 42 min: column equilibration. The flow rate was set at

0.4 mL/min.

The UV detection was carried out from 190 nm to 550 nm.

Mass spectra were obtained with electrospray ionization in positive mode. The optimized conditions for ESI source were capillary voltage at 4 kV, capillary temperature at 350 °C and heat block at 300 °C. The desolvation line (DL) voltage was set to 0 V and 250 °C. The nebulizing gas flow and the drying gas flow were respectively set to 1.5 L/min and 15 L/min. The mass spectra were recorded on the quadrupolar analyzer between *m/z* 150 and 1500 at 1364 *uma/sec*.

2.3. Quantification

2.3.1. External standard quantification

The *trans*-4-GG crocin (crocin PhytoLab) was used as standard for external calibration. 10 mg of standard *trans*-4-GG crocin was diluted in 1 mL DMSO (dimethyl sulfoxide) to realize a stock solution and solutions between 1 and 200 mg/L (1:1000 and 1:5) were realized in water/ acetonitrile, 90/10, v/v. Each solution was filtered through a 0.2 µm cellulose acetate membrane (VWR international, USA). All the samples and standards were analyzed in triplicate.

The equation for calibration was [standard in µg] = ([standard area]-b)/a where a is the slope and b is the intercept of the calibration curve. Linear regression was $y = 93339 \times - 19,480$ with $R^2 = 0.99$.

The limit of detection (LOD) and limit of quantification (LOQ) were determined for standard crocin from the calibration curve according to the formulas:

$$\text{LOD} = (3\sigma_a)/a$$

$$\text{LOQ} = (10\sigma_a)/a$$

with σ_a = standard deviation of the slope (Collard et al., 2015; Shrivastava & Gupta, 2011).

LOD of the standard *trans*-gen/gen crocin is 4.49 mg/L and LOQ is 15.4 mg/L, with $\sigma_a = 1456.8 \text{ UA} \cdot \text{ml}/\mu\text{g}$.

2.3.2. Internal standard quantification

Internal standard solution was prepared with 250 mg of 4-nitroaniline dissolved in 5 mL methanol. The resulting stock solution was diluted in water/acetonitrile, 90/10, v/v to reach 3.6 mg/mL, 2 mg/mL and 0.1 mg/mL for the standard range. The commercial Saffr'Activ® was diluted at 1/125 in water/acetonitrile, 90/10 v/v and 100 µL of 3.6 mg/mL solution of 4-nitroaniline was added in this commercial solution.

The precision of the experimental procedure was evaluated by studying the repeatability ($s / |\bar{x}|$ where: *s* = the sample standard deviation and \bar{x} = sample mean).

2.4. Degradation study

A standard of *trans*-4-GG crocin stored at -22 °C was used to prepare 5 solutions at 200 mg/L (1:5) in water/acetonitrile 90/10 v/v. The impact of storage conditions on crocins stability were compared by

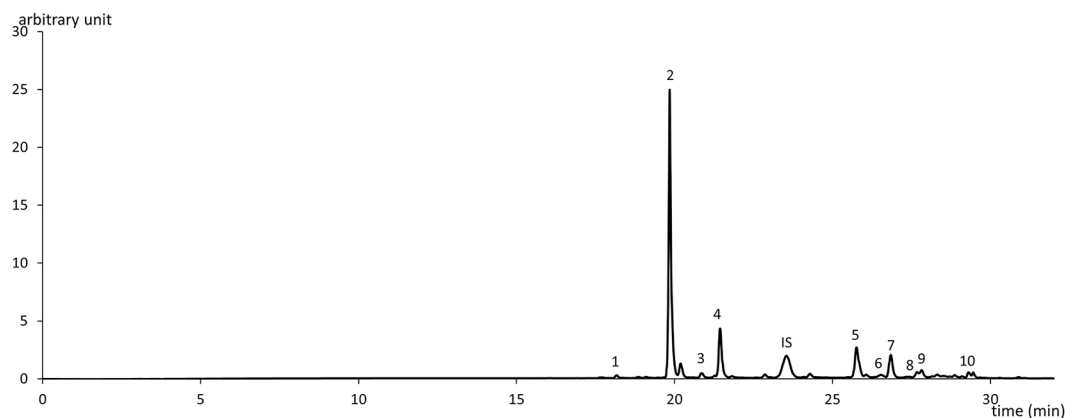


Fig. 1. LC-UV chromatograms (440 nm) of crocetin ester in *Crocus sativus* extract. IS : internal standard (4-nitroanilin).

modulating temperature and light exposure. The results obtained immediately after the crocin sample preparation (Fig. 2a) were compared with those obtained with samples stored for 1 week in different conditions. Sample crocin d,r was stored at room temperature, in a flask protected from the light with alumina foil (Fig. 2b). Sample crocin l,r was stored in a flask exposed to light at room temperature (Fig. 2c). Sample crocin d,60 was stored at 60 °C in a flask protected from the light with alumina foil (Fig. 2d). Sample crocin l,60 was exposed to light and stored at 60 °C (Fig. 2e). The impact of storage conditions on crocins stability in the commercial Saffr'Activ® batch 210101L were also compared by modulating temperature and light exposure. The results obtained immediately after the saffron sample preparation (Fig. 3a) were compared with those obtained with samples stored for 1 week in different conditions. Sample saffron d,r was stored at room temperature, in a flask protected from the light with alumina foil. Sample saffron l,r was stored in a flask exposed to light at room temperature. Sample saffron d,60 was stored at 60 °C in a flask protected from the light with alumina foil. Sample saffron l,60 was exposed to light and stored at 60 °C. After one week, the samples were diluted at 1/125 in water/acetonitrile, 90/10, v/v and analyzed (Fig. 3b, Fig. 3c, Fig. 3d, Fig. 3e).

3. Results and discussion

3.1. Identification of crocins using HPLC- DAD-MS

Crocins were identified in Saffr'Activ® liquid batch SAF 3C 190302L prior to quantification (Fig. 1). The different identified crocins are named with the abbreviation c/t-x-R₁R₂ based on their three main characteristics: *cis* or *trans* isomerization (c/t), total number of glucose from 1 to 5 (x), and the structure of R₁ and R₂ that corresponds to the acid form (H), glucose (g), gentiobiose (G), neapolitanose (n), or triglucose (t) (Cai et al., 2015; Carmona et al., 2006; Liu et al., 2018; Sánchez et al., 2008; Pfister et al., 1996).

Ten crocins were eluted between 16.5 and 29.4 min. 4-Nitroaniline, used as an internal standard, was eluted at 23.4 min (no crocin coeluted with 4-nitroaniline). Crocins were identified by MS, as the number of hexoses can be deduced from the molecular weight provided by the mass spectra. In addition, the retention time and UV-visible spectra provide additional helpful information for confirming the structures. The UV-visible spectra allow for the differentiation of *cis* from *trans* crocetin since the *cis* isomer absorbs at 326 and 440 nm whereas the *trans* isomer only absorbs at 440 nm (Tarantilis et al., 1995). Generally, the *trans* isomers have a higher polarity than the *cis* isomers. The identified crocins and their characteristics are listed in Table 1.

Most of the crocins were identified on the basis of their [M + Na]⁺ ion (Table 1), and for all the crocins, *m/z* 329 and *m/z* 311 corresponded to the crocetin aglycone and the dehydrated crocetin aglycon,

respectively. Characteristic fragments with a difference of *m/z* 162 corresponding to the loss of 1 to 5 glucose units were observed. Fragmentations mainly occurred on the ester function. A loss of water can also occur from these ions, leading to the ions *m/z* 473, 635, 797, and 959 corresponding to [M - nC₆H₁₀O₅ - H₂O + H]⁺ with n = 1 to 5. An ion at *m/z* 994 corresponding to the ammonium adduct [M + NH₄]⁺ was observed in the case of compounds 2, 7, 7', and 8.

Based on these observations, compound 1 was identified as *cis*-5-tG with *m/z* 1161 [M + Na]⁺, *m/z* 837 [M - gentiobiose + Na]⁺, *m/z* 675 [M - triglucose + Na]⁺, and *m/z* 329 [M - triglucose - gentiobiose + H]⁺. Compound 2 was unambiguously identified as *trans*-4-GG by comparison to the chromatographic retention time and mass spectra of the commercial compounds. The fragmentation pattern *m/z* 999 [M + Na]⁺, *m/z* 994 [M + NH₄]⁺, *m/z* 675 [M - gentiobiose + Na]⁺, *m/z* 653 [M - gentiobiose + H]⁺, *m/z* 491 [M - gentiobiose - glucose + H]⁺, and *m/z* 329 [M - gentiobiose - gentiobiose + H]⁺ confirmed the structure of the major *trans*-4-GG crocetin. Compound 3 was identified as *trans*-4-ng crocetin based on the ion *m/z* 837 [M - glucose + Na]⁺ and its UV spectra. The structure of compound 4 was confirmed to be *trans*-3-Gg based on the fragments *m/z* 837 [M + Na]⁺, *m/z* 491 [M - gentiobiose + H]⁺ or *m/z* 473 [M - gentiobiose - H₂O + H]⁺, and *m/z* 329 [M - gentiobiose - glucose + H]⁺. Compound 5 was identified as *cis*-4-GG. Indeed, its fragmentation pattern is similar to compound 2 (*m/z* 994 [M + NH₄]⁺, *m/z* 675 [M - gentiobiose + Na]⁺, *m/z* 491 [M - gentiobiose - glucose + H]⁺, and *m/z* 329 [M - gentiobiose - gentiobiose + H]⁺) and the supplementary absorption band at 326 nm is characteristic of a *cis* isomer. For the same reasons, compound 6 was identified as *cis* 4-ng crocetin, an isomer of compound 3. Compounds 7 and 8 showed comparable mass spectra, with fragments at *m/z* 675 [M + Na]⁺ and *m/z* 635 [M - H₂O + H]⁺, *m/z* 513 [M - glucose + Na]⁺, and *m/z* 473 [M - glucose - H₂O + H]⁺ but different UV-Vis spectra. They were identified as *trans*- and *cis*-2-GH, respectively, which were later confirmed by the degradation experiment (see section 3.2). Compounds 9 and 4 also have the same fragmentation pattern but differ in their UV absorption spectra, indicating that compound 9 is *cis*-3-Gg crocetin. Finally, compound 10 showed a similar fragmentation pattern to compounds 7 and 8, demonstrating the presence of 2 glucose moieties. Based on the UV-Vis absorption at 326 nm, it was identified as *cis*-2-gg. All these crocetin structures correspond to the structures that have also been identified in literature (Carmona et al., 2006).

3.2. Impact of storage on total crocetin concentration

In a first approach, standard *trans*-4-GG was used to evaluate the impact of storage condition on total crocetin concentration. The degradation products of crocins that occurred during storage were identified prior to quantification. The identification of the degradation products was realized by comparison with the retention times, absorbances, and

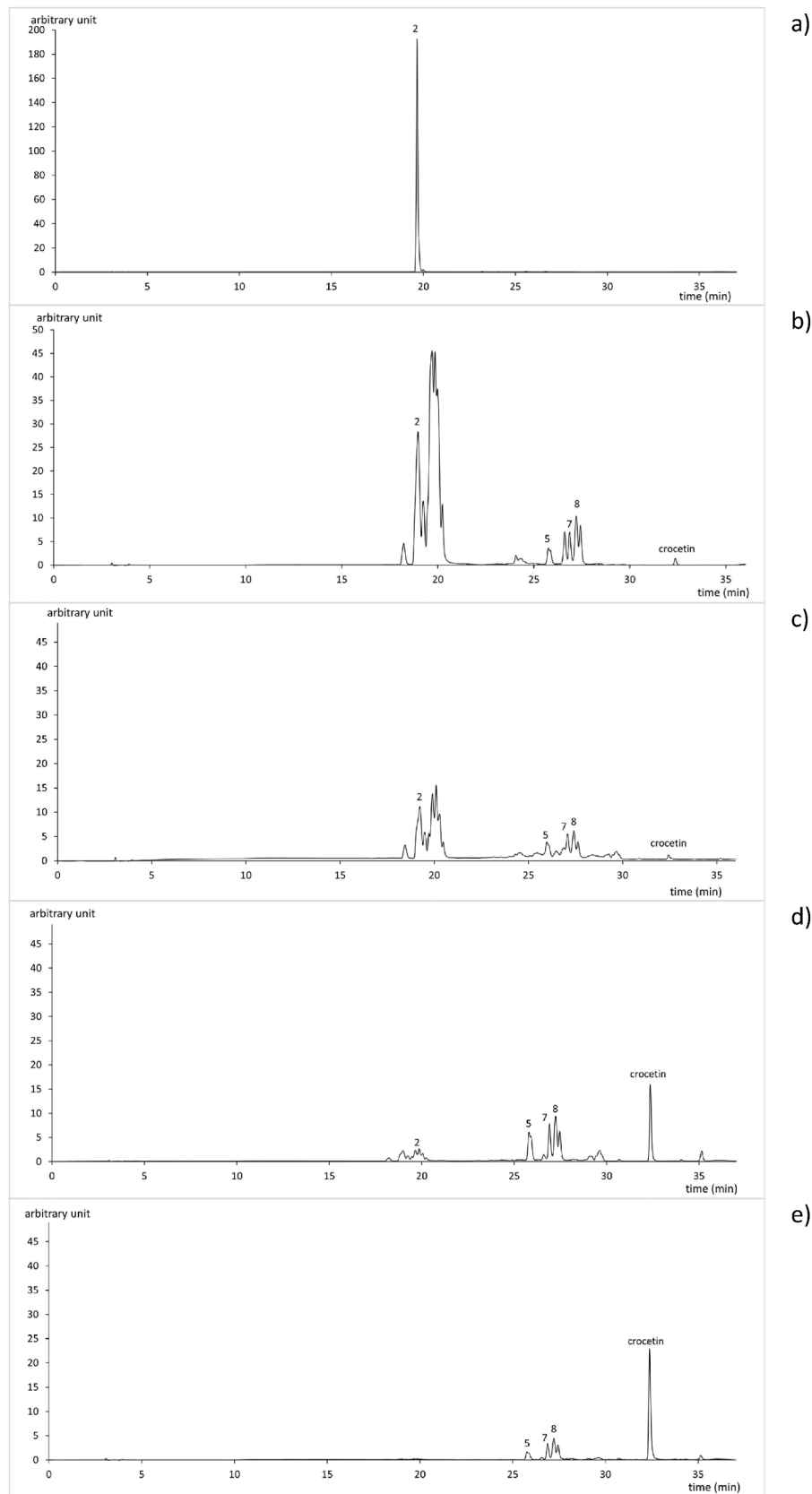
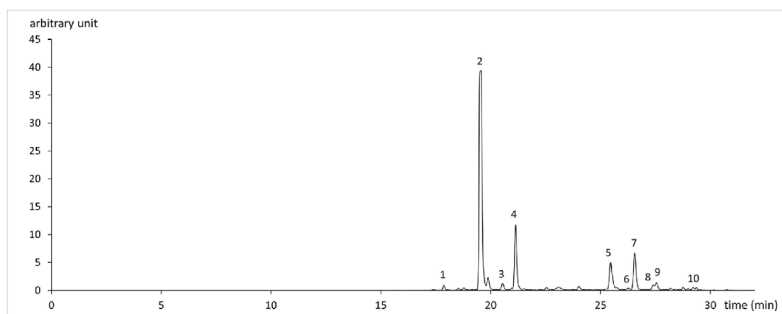
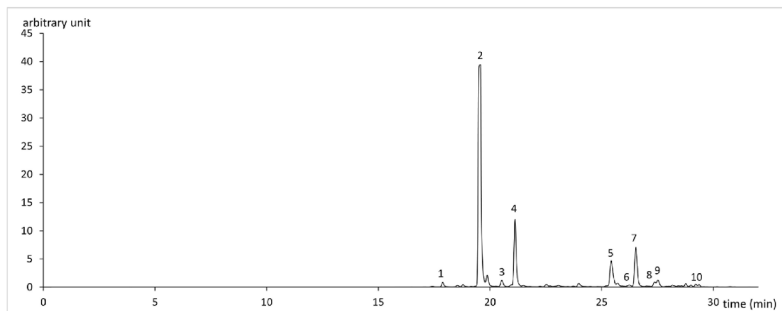


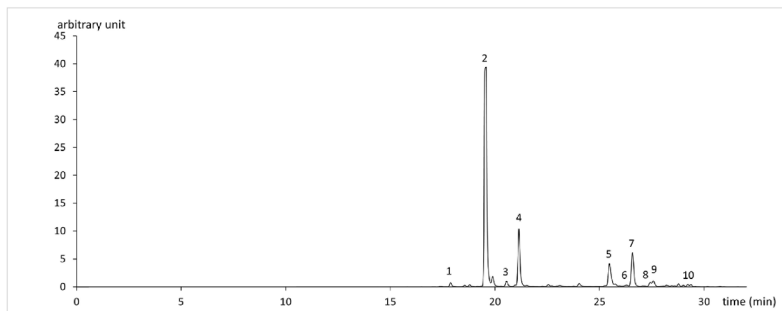
Fig. 2. LC-UV chromatograms (440 nm) of the samples of *trans*-4-GG crocin stored in different conditions for 1 week. (a) fresh sample, (b) protected from light at room temperature, (c) exposed to light at room temperature, (d) protected from light at 60 °C, (e) exposed to light at 60 °C.



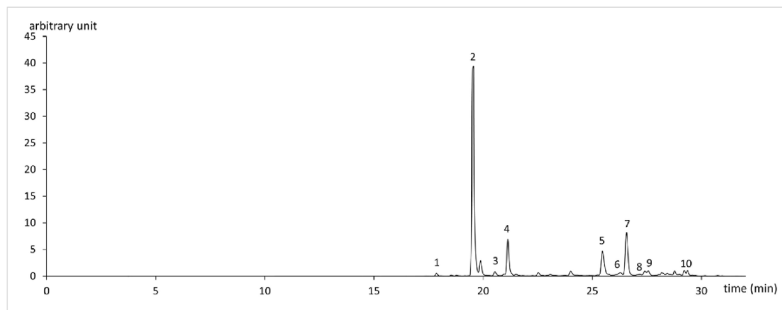
a)



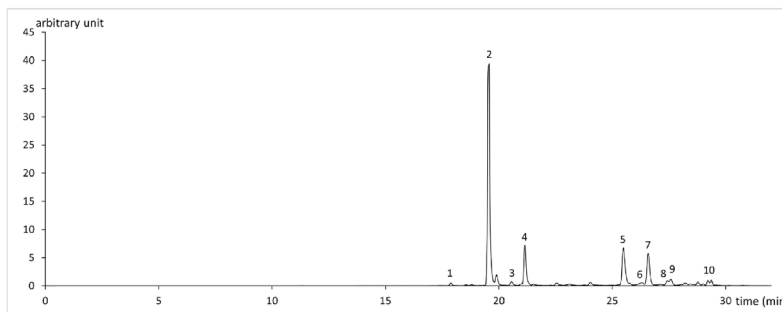
b)



c)



d)



e)

Fig. 3. LC-UV chromatograms (440 nm) of the samples of saffron stored in different conditions for 1 week. (a) fresh sample, (b) protected from light at room temperature, (c) exposed to light at room temperature, (d) protected from light at 60 °C, (e) exposed to light at 60 °C.

Table 2
Main characteristics and relative percentages of the degradation products of *trans*-4-GG crocin.

retention time	<i>m/z</i> (ESI +)	Compound	Crocin	Crocin d,r	Crocin l,r	Crocin d,60	Crocin l,60
19.8	311, 455, 491, 617, 635, 797, 959, 999	<i>trans</i> -4-GG crocin (standard)	100%	68%	45%	7%	0%
25.7	311, 473, 491, 675, 797, 994	<i>cis</i> -4-GG crocin	0%	5%	21%	15%	8%
26.8	311, 473, 491, 675	<i>trans</i> -2-G crocin	0%	11%	19%	22%	11%
27.5	311, 491, 635	<i>cis</i> -2-G crocin	0%	14%	13%	18%	11%
32.5	329	crocetin	0%	2%	3%	37%	70%

fragmentations obtained with the Saffr'Activ®. The peaks at 19.8 min and 25.7 min were attributed to *trans* and *cis*-4-GG crocins, and the peaks at 26.8 min and 27.5 min corresponded to the partially hydrolyzed *trans* and *cis*-2-G crocins. Finally, the peak at 32.5 min, which exhibits a molecular ion *m/z* 329, was identified as the free crocetin resulting from the total hydrolysis of the crocins (Table 2).

Analysis of the freshly prepared standard *trans*-4-GG crocin (sample crocin) showed a single peak (Table 2, Fig. 2a). After one week stored at room temperature and protected from the light (sample crocin d,r) the standard crocin represented 68% of the mixture (Fig. 2b), and the degradation products were mainly crocins (Table 2). The main compound is eluted at 19.8 min., while badly resolved peaks appeared at 25.7, 26.8, and 27.5 min. After 1 week stored at room temperature and exposed to light (Table 2, Fig. 2c) (sample crocin l,r), the standard crocin represented 45% of the mixture. The standard *trans*-4-GG crocin is totally degraded when stored at 60 °C (Fig. 2e) and exposed (sample crocin l,60) to light.

The decrease in the initial *trans*-4-GG crocin and the increase in degradation products, especially crocetin, clearly show the impact of light and temperature exposure on *trans*-4-GG crocin stability (Table 2). From these observations, one can conclude that storage conditions greatly influence the fate of the crocins.

Trans-4-GG crocin quantified using external calibration represented 198 mg/l of the fresh sample (sample crocin). For degradation compounds at 440 nm, all the compounds obtained were considered to be crocins, and the response factor determined for *trans*-4-GG crocin was used to quantify all the crocins (Lozano 2000). In the 1 to 200 mg/L concentration range, a good linearity was obtained ($R^2 = 0.99$), and the linear regression was $y = 93339 \times - 19480$. A 42% loss of *trans*-4-GG crocin (115 mg/L) was observed when stored for 1 week in the dark at room temperature (sample crocin d,r). The exposure to light at room temperature (sample crocin l,r) induced 75% loss of *trans*-4-GG crocin (50 mg/L). A storage at 60 °C in the dark (sample crocin d,60) resulted in 96.5% loss (7 mg/L), whereas *trans*-4-GG crocin was totally degraded in the sample exposed to light for 1 week at 60 °C (sample crocin l,60).

In order to avoid degradation of crocins in saffron extract, it is thus recommended to store the solution at a low temperature and in the dark. Indeed, *trans*-4-GG crocin was degraded even when stored at ambient temperature in the dark. The crocin was more degraded when exposed to a higher temperature than to light and totally degraded when the two factors were combined. These results are in accordance with those obtained by Sánchez et al., 2008, who showed that degradation increased with increasing temperature.

In a second step, the saffron extract batch 210101L was used to evaluate the impact of storage conditions on *trans*-4-GG crocins in saffron extract. The results were then compared with those obtained with the standard *trans*-4-GG crocin. *Trans*-4-GG crocin quantified using external calibration represented 432 mg/L of the fresh sample (sample saffron). A 2.3% loss of *trans*-4-GG crocin (422 mg/L) was observed when stored for 1 week in the dark at room temperature (sample saffron d,r). The exposure to light at room temperature (sample saffron l,r) induced 8.3% loss of *trans*-4-GG crocin (396 mg/L). A storage at 60 °C in the dark (sample saffron d,60) resulted in 19.2% loss (349 mg/L), while 24.5% loss of *trans*-4-GG crocin (326 mg/L) was observed when the sample was exposed to light for 1 week at 60 °C (sample saffron l,60) (Fig. 3).

Contrary to what was observed for pure *trans*-4-GG crocin, in saffron extract, the *trans*-4-GG crocin is very stable at room temperature even when exposed to light. At 60 °C, light induced degradation is observed, which is lower than what was observed for pure *trans*-4-GG crocin. Those results seem to indicate that crocins are less likely to be degraded in saffron extract, which probably contains components that increase their stability towards light and temperature.

Most of the existing analytical methods use standard *trans*-4-GG crocin to quantify all the crocins; however, the degradation of this standard could induce approximations on the obtained results. Thus, using an internal standard could be a good solution to avoid errors. Moreover, with an internal standard, the absolute quantities obtained for crocins do not depend on the injection volume or on the sample dilution.

3.3. Quantification of crocins in commercial saffron extract

The ISO 3632-2 2010 method is commonly used to quantify crocins in saffron extract, but this method has some limitations when applied to assessing authenticity. Rocchi et al., 2018 demonstrated a poor correlation between the total content of crocins obtained by the ISO 3632-2 method and by UHPLC-MS/MS. The *cis*-isomers do not absorb at 440 nm, and the UV-visible method is not specific (Rocchi et al., 2018).

Crocins can be identified and quantified (Lozano et al., 2000) using MS coupled to HPLC and to DAD (Carmona et al., 2006; Moras et al., 2018). Crocins in saffron extract are generally quantified using the response factor (*k*) of *trans*-4-GG crocin for all the crocins (Lozano et al., 2000; Moras et al., 2018). This normalization on *trans*-4-GG crocin is necessary due to the lack of commercial standards for all the crocins. Thus, the same molar extinction coefficient or response factor (*k* crocin) is applied to all the crocins, which induces approximations in the results, mostly in saffron extract of poor quality. Indeed, *trans*-4-GG crocin is not the major compound in a saffron extract of poor quality. Previous methods (Lozano et al., 2000; Moras et al., 2018) use the response factor of *trans*-4-GG crocin to quantify the total crocins content. Rocchi et al., 2018 only obtain a relative percentage based on the total crocins area. The results can be improved by determining specific response factors for each crocin that has been isolated with semi-preparative HPLC and positively identified by HPLC-DAD-MS and NMR (Koulakiotis et al., 2015). This method is very accurate, but long and fastidious. However, for the nutraceutical industry, a simple and rapid method of absolute quantification is needed. Internal calibration presents the advantage of calibrating sample preparation errors and matrix effects. 4-Nitroaniline has been used as an internal standard (Koulakiotis et al., 2015) for crocin quantification in saffron extract since it absorbs at all the wavelengths used for quantifying saffron compounds and does not co-elute with these compounds. Thus, calculating a corrected response factor for each crocin and using it in an internal method could be a simpler and more rapid method. This last method of quantification was compared with an internal method using normalization on *trans*-4-GG crocin on fresh and old samples.

In the fresh sample, the response factor (*k*) determined for *trans*-4-GG crocins with the calibration curve is used for all the crocins, and the total amount of crocins was 38.5 ± 4.3 mg/g of Saffr'Activ® in Saffr'Activ® liquid batch SAF 3C 190302L.

This previous method is compared to the method using a corrected

Table 3Concentrations of crocins obtained with k crocin equivalent *trans*-4-GG and with the corrected k crocin.

Compound	Molecular Weight	k crocin (x 10 ⁵)[eq. <i>trans</i> -4-GG]	Conc. crocins (mg/g)[eq. <i>trans</i> -4-GG]	Standard deviation	k crocins (x 10 ⁵) [Corrected]	Conc. crocins (mg/g) [Corrected]	Standard deviation
1	1138	1.20	0.3	±0.04	1.45	0.4	±0.05
2	976	1.20	23.9	±3.85	1.20	23.9	±3.85
3	976	1.20	0.6	±0.06	1.20	0.6	±0.06
4	814	1.20	4.5	±0.89	1.04	3.9	±0.77
5	976	1.20	3.7	±0.36	1.20	3.7	±0.36
6	976	1.20	0.7	±0.08	1.20	0.7	±0.08
7	652	1.20	2.6	±0.29	0.83	1.8	±0.20
8	652	1.20	0.6	±0.06	0.83	0.4	±0.04
9	814	1.20	0.9	±0.10	1.04	0.8	±0.09
10	652	1.20	0.5	±0.06	0.83	0.4	±0.04
IS	138	–	–	–	–	–	–
Total	–	–	38.5	±4.47	–	36.6	±4.34

response factor of each crocin in the fresh sample. In order to achieve this, a theoretical k was calculated for each crocin. Because the conjugation in each crocin remains nearly unchanged, their response factor is similar for a given molar concentration. Thus, this response factor is valuable for each crocin at one molar concentration according to the equations below:

$$Cm_{\text{crocin 4-GG}} = k_{\text{crocin 4-GG}} \times A_{\text{crocin 4-GG}} \text{ and } Cm_{\text{crocin 4-GG}} = CM_{\text{crocin 4-GG}} \times MW_{\text{crocin 4-GG}}$$

So, $Cm_{\text{crocin}} = k_{\text{crocin}} \times A_{\text{crocin}}$ and $Cm_{\text{crocin}} = CM_{\text{crocin}} \times MW_{\text{crocin}}$
Supposing for one area, all crocins have an identical response:

$$CM_{\text{crocin 4-GG}} = CM_{\text{crocin}} \text{ for } A_{\text{crocin 4-GG}} = A_{\text{crocin}}$$

$$\text{Whence, } k_{\text{crocin}} = Cm_{\text{crocin}} / A_{\text{crocin}}$$

$$k_{\text{crocin}} = Cm_{\text{crocin}} / A_{\text{crocin 4-GG}}$$

$$k_{\text{crocin}} = (CM_{\text{crocin}} \times MW_{\text{crocin}}) / A_{\text{crocin 4-GG}}$$

$$k_{\text{crocin}} = (CM_{\text{crocin 4-GG}} \times MW_{\text{crocin}}) / A_{\text{crocin 4-GG}}$$

$$\text{As a result, } k_{\text{crocin}} = (Cm_{\text{crocin 4-GG}} / MW_{\text{crocin 4-GG}}) \times (MW_{\text{crocin}} / A_{\text{crocin 4-GG}})$$

With A the area, Cm the mass concentration in g/L, CM the molar concentration in mol/L, and MW the molecular weight in g/mol.

Using these corrected k values, each crocin that has been identified previously can be quantified more precisely. The results obtained with normalization on *trans*-4-GG crocin and those obtained with the corrected response factor are summarized in Table 3.

In fresh Saffr'Activ® liquid batch SAF 3C 190302L, a total crocin concentration of 36.64 ± 4.5 mg/g (3.7%) of Saffr'Activ® was obtained with a corrected k instead of 38.46 ± 4.3 mg/g (3.8%) of Saffr'Activ® obtained with the normalization on *trans*-4-GG crocins, which represent a difference of less than 5% between these two methods. The two methods of calculation were applied to a saffron extract, batch SAF 3C 190302L, which has been stored for 22 months at 4 °C. A total crocin concentration of 32.52 mg/g ± 0.4 (3.3%) and 25.64 ± 0.3 mg/g (2.6%) of Saffr'activ® were obtained with normalization on *trans*-4-GG crocin and with the corrected response factor method, respectively. A difference of 21% is obtained between these two methods.

The presence of less glycosylated crocins tends to decrease the mass of the crocins, which explains the difference between the two calculation methods. In a fresh saffron extract of good quality, each of these methods can be used equally because the sample contains a large proportion of crocins with four glucose moieties; as such, the difference between the two calculation methods is particularly subtle. On the other hand, in the old batch, the major crocin is degraded, thus, normalization on *trans*-4-GG crocin overestimates the total crocin concentration. Normalization on *trans*-4-GG crocin is acceptable for a fresh sample, but it is not reliable for quantifying crocins in a degraded or old sample. As a result, the use of a corrected k is necessary for an accurate quantification of crocins in old saffron extracts.

4. Conclusion

In this study, 10 crocins have been identified in saffron extract using HPLC-DAD-MS. The stability to light, air, and temperature of *trans*-4-GG crocin, which is generally used as a standard for crocin quantification, has been evaluated. It has been demonstrated that temperature induces degradation of crocin more severely than light exposure. This instability could lead to false quantification results. Moreover, it has been shown that crocins are more stable in saffron extract. To address this issue, the 10 crocins identified above in saffron extract were quantified using internal standard quantification methods with normalization on *trans*-4-GG crocin. A corrected response factor was applied to the internal standard quantification method, leading to more accurate results for an old saffron extract when compared to results obtained with normalization on *trans*-4-GG crocin.

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Marlène Suchareau: Validation, Investigation, Writing - original draft, Writing - review & editing. **Alexandra Bordes:** Writing - review & editing. **Laurent Lemée:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.130199>.

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