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Impact of two different dehydration methods on saffron quality, concerning the prevalence of *Saffron latent virus* (SaLV) in Iran

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ABSTRACT

The dehydration process is a prerequisite to preserve saffron for a long time. According to this process, saffron shows differences in the main compounds responsible for its quality (colour, taste, aroma, and flavonol content). At present, the freeze-drying method obtains dried products with the highest quality. Viruses can modify the physiology and metabolism of plants, being able to affect the activities of several enzymes. For this reason, the main compounds of saffron have been analyzed under two different dehydrating processes, freeze-drying and dark-drying, considering their infection status with the *Saffron latent virus* (SaLV). Results showed that the picrocrocin and safranal content enables to differ dark-dried samples from freeze-dried ones. Besides, the kaempferol-3-*O*-sophoroside-7-*O*-glucoside content allows differentiating between SaLV-infected ($SaLV^+$) and uninfected ($SaLV^-$) saffron samples. Moreover, our data suggest that the freeze-drying would decrease crocins content, and dark-drying can nullify the adverse effect of SaLV on crocins content.

1. Introduction

Dehydration, a post-harvesting process, is necessary to obtain and preserve saffron from *Crocus sativus* L. stigmas. The stigmas lose around 80% of their weight during the dehydration process, and the International Standard ISO 3632 (2011) recommends a value below 12% of moisture content. In Iran, India, and Morocco, this process is carried out at room temperature directly, under sunlight or in air-ventilated conditions and in Spain, Greece, and Italy, drying at higher temperatures by using a heating source (Carmona et al., 2005).

Post-harvest processing determines the stability, quality, and economic value of the final product. According to ISO 3632 (2011), which exclusively deals with saffron quality, four grades of colour intensity have been established, including IV (poorest saffron quality), III, II, and I (finest saffron quality). The dehydration process is one of the critical determinants of saffron quality as measured by the levels of its secondary metabolites: crocetin esters, picrocrocin, and safranal (Pardo et al., 2002) and also some other compounds such as flavonoids, kaempferol-3-*O*-sophoroside-7-*O*-glucoside, being the major. During the dehydration process, the water-soluble picrocrocin (a biodegradable product of the carotenoid zeaxanthin) is converted to the safranal either by two-step enzymatic/dehydration process involving the intermediate 4-Hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) or directly by dehydration at high temperatures or extreme pH (Carmona et al., 2005).

There are several studies related to the effects of the dehydration process on secondary metabolites of saffron (Acar, Sadikoglu, & Ozkaymak, 2011; Chaouqi et al., 2018; del Campo et al., 2010; Tong et al., 2015). At present, freeze-drying (known as lyophilization) is one of the effective dehydration methods, resulting in the highest spice

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quality. Freeze-drying is a separation process in which the solvent content (particularly water) is sublimated from the material being dried. The heat-sensitive volatile compounds of saffron can be more preserved using freeze-drying in comparison with other conventional drying methods. Moreover, it can be used for dehydration of saffron with minimum safranal (main aroma component) and crocin (colouring agent) loss (Acar, Sadikoglu, & Doymaz, 2015). Accordingly, the high cost of saffron freeze-drying can be compensated by the minimum loss of safranal and crocin contents in the final products.

For most crops, plant diseases can impose considerable production circumscriptions and affect both yield and overall quality of the product, especially modifications of secondary metabolites (Bruni et al., 2005). In addition to pharmaceutical applications of plant secondary metabolites, these compounds possess multiple functions in plants, including disease resistance (Anterola & Lewis, 2002; Dixon, 2001). Plant resistance to pathogen infection is mostly associated with transcriptional activation of defense genes including those related to secondary metabolites, *e.g.*, induction of plant glycosyltransferases (UGTs), leading to localized cell death at the infection site and restriction of pathogen spread (known as hypersensitive response, HR) (Langlois-Meurinne, 2005). However, the effect of viruses on secondary metabolism is not limited to only resistant plants. The presence of different variants of viruses can also modulate the susceptible host's gene expression related to secondary metabolism as reported for different variants of *Tobacco etch virus* infecting tobacco plants (Cervera, Ambros, Bernet, Rodrigo, & Elena, 2018). So far, the influence of viral infection on plant compounds have been assessed for *Tomato mosaic virus* (ToMV, *Tobamovirus*) on *Solanum lycopersicum* M. (López-Gresa et al., 2012), *Alfalfa mosaic virus* (AMV, *Alfamovirus*) on *Ocimum basilicum* L. (Bruni, Bellardi, & Parrella, 2016) and *Cucumber mosaic virus* (CMV, *Cucumovirus*) on *Cucumis melo* L. (Shalitin & Wolf, 2000).

Recently a highly prevalent potyvirus, *Saffron latent virus* (SaLV), has been reported from the *C. sativus* fields of Iran (Parizad et al., 2017a, 2017b, 2018). It has been indicated that edaphoclimatic and cultivation conditions significantly determine the quality of the saffron, and also, the presence of SaLV modifies the content of its metabolites (Parizad et al., 2019). Here, the main purpose of this study was to further our understanding of the effect of two different dehydration processes on the contents of important saffron secondary metabolites in the presence of SaLV infection, using both high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) and UV–vis spectrometry.

2. Materials and methods

2.1. Plant materials

C. sativus plant samples were randomly collected during the growing season 2014 from Tehran province fields.

2.2. Saffron stigmas preparation

Saffron stigma tissues were prepared through two different processes: freeze-drying and dark-drying. In the freeze-drying, samples were snap-frozen in liquid nitrogen and dried using Zirbus VaCo 5 Laboratory Freeze Dryer (ZIRBUS technology GmbH, Bad Grund/Harz, Germany) under the pressure of 0.02 mbar, condenser temperature of −60 °C, in 48 h. Whereas in dark-drying, saffron stigma tissues were placed on a cotton cloth, in a ventilated dark room, at room temperature (23–25 °C), for seven days.

2.3. Saffron extract preparation

Saffron aqueous extracts were prepared essentially following ISO 3632 (2011) with a slight amendment. Briefly, 50 mg instead of 500 mg tissues were taken for HPLC-DAD analysis, as described in previous work (Parizad et al., 2019). The obtained homogenate was filtered through a syringe filter made of polytetrafluoroethylene (PTFE) with a pore size of 0.45 μm (Millipore, Bedford, MA, USA). Subsequently, it was transferred into a vial for HPLC-DAD analysis.

2.4. HPLC-DAD analysis

As outlined in Parizad et al. (2019), though RP-HPLC-DAD, crocetin esters, picrocrocin, safranal, kaempferol glycosides, and HTCC were analyzed by using an Agilent 1200 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm × 4.6 mm inner diameter, 5 μm Phenomenex Luna C_{18} column (Le Pecq Cedex, France) equilibrated at 30 °C. HPLC-grade acetonitrile was obtained from Panreac® (Barcelona, Spain), and ultrahigh-purity water was produced using a Milli-Q system (Millipore, MA, USA). The wavelength of 440 nm for crocetin esters detection, 330 nm for safranal and kaempferols glycosides detection, and 250 nm for picrocrocin and HTCC detection were set in the DAD detector (Hewlett Packard, Waldbronn, Germany). All analyses were performed in duplicate with two measurements taken for each replicate.

2.5. Identification and quantification of crocetin esters, picrocrocin, safranal, kaempferol glycosides, and HTCC

Identification of crocetin esters, picrocrocin and safranal was carried out as previously reported (Carmona, Zalacain, & Alonso, 2006). Quantification was based on the following calibration curves (García-Rodríguez et al., 2014): C_i = (0.00746 ± 0.00004)A_i – (0.00571 ± 0.12863), correlation coefficient (R^2) = 0.9999 for *trans*-5-tG, *trans*-5-nG, and *trans*-4-GG; C_i = (0.00713 ± 0.00003)A_i – (0.00472 ± 0.05608), R^2 = 0.9999 for *trans*-3-Gg, and *trans*-2-gg; C_i = (0.00531 ± 0.0004)A_i – (0.00571 ± 0.12863), R^2 = 0.9999 for *cis*-4-GG; C_i = (0.00500 ± 0.00003)A_i – (0.00331 ± 0.05608), R^2 = 0.9999 for *cis*-3-Gg, and *cis*-2-gg; C_i = (0.02900 ± 0.00002)A_i + (0.51940 ± 0.02631), R^2 = 0.9999 for picrocrocin and HTCC, and C_i = (0.03227 ± 0.00063)A_i + (0.05101 ± 0.03103), R^2 = 0.9989 for safranal. Limits of detection (LOD) and quantification (LOQ) by García-Rodríguez et al. (2014) were taken into consideration. Identification of kaempferol tetrahexoside and kaempferol-3-*O*-sophoroside-7-*O*-glucoside was done according to Carmona et al. (2007). A six-point calibration curve of kaempferol aglycone was used to quantify these flavonols, C_i = (0.01891 ± 0.00019)Ai + (1.14525 ± 0.52291), R^2 = 0.9997. C*i* and A*i* were the concentration (mg/L) and the HPLC peak area of the corresponding compound *i*, respectively.

Safranal (purity ≥88%) and crocetin esters (*trans*-4-GG and *trans*-3-Gg, purity ≥ 99%) were obtained from Sigma-Aldrich (Madrid, Spain) and Phytolab GmbH & Co. KG (Vestenbergs-greuth, Bravaria, Germany), respectively, and picrocrocin was isolated, as described by Sánchez, Carmona, Ordoudi, Tsimidou, and Alonso (2008).

3. Nomenclature for crocetin esters

Abbreviations for crocetin esters (also known as crocins) were adopted from Carmona, Zalacain, Sánchez, Novella, and Alonso (2006): *trans*-5-tG, *trans*-crocetin (β-D-triglucosyl)-(β-D-gentiobiosyl) ester; *trans*-5-nG, *trans*-crocetin (β-D-neapolitanosyl)-(β-D-gentiobiosyl) ester; *trans*-4-GG, *trans*-crocetin di-(β-D-gentiobiosyl) ester; *trans*-3-Gg, *trans*-crocetin (β-D-glucosyl) (β-D-gentiobiosyl) ester; *trans*-2-gg, *trans*-crocetin di-(β-D-glucosyl) ester; *cis*-4-GG, *cis*-crocetin di-(β-D-gentiobiosyl) ester; *cis*-3-Gg, *cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester, and *cis*-2-gg, *cis*-crocetin di-(β-D-glucosyl) ester.

3.1. UV–vis spectrometry analysis

Saffron extracts, after proper dilution (1:10, V/V), were monitored by UV–vis scanning from 190 nm to 700 nm using a Perkin-Elmer

Lambda 25 spectrophotometer (Norwalk, CT, USA) with UV WinLab 2.85.04 software (Perkin-Elmer). All the analyses were performed in duplicate, and two measurements were taken for each replicate. Absorbance values were recorded at 440 nm (as colouring strength), 330 nm, and 257 nm following ISO 3632 (2011) guidelines.

3.2. Serological and molecular assays

Serological tests were used to detect the presence of viruses previously reported from *Crocus* spp. in leaf tissue samples of *C. sativus* plants, as described by Parizad et al. (2018). Since there is no species-specific antibody provided against recently described SaLV, reverse transcription-polymerase chain reaction (RT-PCR) was performed to check the SaLV infection status of selected saffron samples. Overall, total RNA extraction, cDNA synthesis, RT-PCR, and sequencing were performed according to Parizad et al. (2019).

3.3. Statistical analysis

One-way analysis of variance (ANOVA) was performed with mean values compared by Duncan's test at $p < 0.05$ and discriminant function analysis using the statistical software package SPSS 24.0 (SPSS INC., Chicago, IL, USA).

4. Results and discussion

Saffron carotenoids (*e.g.*, crocetin esters, picrocrocin, safranal) accumulate in the stigma tissues, along with the differentiation of plastids (Bouvier, Suire, Mutterer, & Camara, 2003). As plastids are one of the potential replication sites of plant viruses, virus infections can modulate the differentiation of plastids such as chloroplast/ chromoplast transition (Almasi, Harsanyi, & Gaborjanyi, 2001), leading to probable effect on carotenoid metabolism.

To quantify the probable effects of a recently described potyvirus (SaLV) infection (Parizad et al., 2018) on carotenoid metabolism in saffron stigmas which were dried in the different dehydration process, stigma tissues of *C. sativus* L. were randomly selected either for freeze-drying or dark-drying and were analyzed through HPLC-DAD and UV–vis spectrophotometry.

Results of HPLC-DAD and UV–vis spectrophotometric analyses of saffron samples with different dehydration processes are shown in Tables 1A and 1B. Significant differences in several metabolite concentrations were found. Freeze-dried saffron samples obtained a greater concentration of picrocrocin and safranal than the dark-dried ones (Table 1A). On the contrary, *trans*-5-tG, *trans*-5-nG, *trans*-4-GG, and *cis*-4-GG recorded less concentration in the freeze-dried saffron samples. The sum of crocetin esters did not show significant differences, but its content in dark-dried saffron samples was about 17% more than freeze-dried ones. There were no significant differences in the content of the main kaempferol between the two drying processes, while the kaempferol tetrahexoside reported greater content in the freeze-dried samples. Regarding the UV–vis results (Table 1B), there were significant differences in $A^{1\%}_{1cm}$ 330 nm and $A^{1\%}_{1cm}$ 440 nm; absorbance in relative to freeze-dried samples was greater in the dark-dried samples. Freeze-dried samples did not belong to Category III, while the dark-dried ones belonged to Category III. Due to the interferences occurring in an aqueous extract of saffron, according to ISO 3632 (2011) parameters $A^{1\%}_{1cm}$ 257 nm and $A^{1\%}_{1cm}$ 330 nm (García-Rodríguez et al., 2017, 2014), there was no concordance which kind of dehydration process obtained a greater content of picrocrocin and safranal, depending on the techniques used, either HPLC-DAD or UV–vis (Tables 1A and 1B). It was noticed that there were significant differences in the value of $A^{1\%}_{1cm}$ 440 nm parameter, while there were no significant differences regarding the sum of crocetin esters between these two different dehydration processes. However, as mentioned above a greater content of the sum of crocetin esters in dark-dried saffron samples was recorded (Table 1A). In a similar study by Acar et al. (2011), the safranal content of the commercial saffron samples dried in a freeze dryer was five times higher than that of naturally sun-dried samples, while crocetin esters' content of freeze-dried saffron samples was about 40% higher.

Table 1A
The content of the main compounds of saffron samples with different dehydration processes by HPLC-DAD analysis.

Compound (mmol·(100 g)$^{-1}$ saffron ± SD)*	Dehydration process	
	Freeze-Dried	Dark-Dried
Picrocrocin	33.88 ± 1.61b	15.14 ± 1.54a
Safranal	0.84 ± 0.34b	0.41 ± 0.07a
HTCC	20.20 ± 11.99a	16.82 ± 4.30a
trans-5-tG	0.07 ± 0.02a	0.09 ± 0.02b
trans-5-nG	$0.06{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$a	$0.08{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$b
trans-4-GG	3.53 ± 1.26a	4.59 ± 1.43b
trans-3-Gg	3.81 ± 1.86a	3.71 ± 1.35a
trans-2-gg	1.17 ± 1.58a	0.74 ± 0.41a
cis-4-GG	0.24 ± 0.13a	0.35 ± 0.11b
cis-3-Gg	0.17 ± 0.12a	0.20 ± 0.07a
cis-2-gg	0.05 ± 0.10a	0.04 ± 0.02a
Σ Crocins	9.91 ± 3.50a	11.95 ± 4.42a
K1	0.31 ± 0.07b	0.26 ± 0.02a
K2	0.93 ± 0.27a	0.80 ± 0.17a

*Values are the mean of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. K1, kaempferol tetrahexoside, and K2, kaempferol 3-*O*-sophoroside-7-*O*-glucoside. One-way analysis of variance (ANOVA) for each column is included. Different letters (a, b) within each row represent statistically significant differences ($p < 0.05$) across different dehydration processes for each metabolite's concentration.

Table 1B
UV–vis spectrophotometric parameter values of saffron samples with different dehydration processes.

($A^{1\%}_{1cm}$ ± SD)*	Dehydration process	
	Freeze-Dried	Dark-Dried
$A^{1\%}_{1cm}$257 nm	71 ± 25a	73 ± 7a
$A^{1\%}_{1cm}$330 nm	24 ± 9a	34 ± 3b
$A^{1\%}_{1cm}$440 nm	105 ± 41a	153 ± 37b

* Values are the mean of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. One-way analysis of variance (ANOVA) for each column is included. Different letters (a, b) within each row represent statistically significant differences ($p < 0.05$) across different dehydration processes for each $\boldsymbol{A^{1\%}_{1cm}}$ parameter.

Next, the plants from where stigmas were collected for HPLC-DAD and UV–vis spectrophotometry analyses, were checked for SaLV infection. Following ELISA and RT-PCR, SaLV infection of 19, and SaLV-free status of six *C. sativus* plants were verified. Due to the random selection of stigmas for the dehydration process, we found that out of 19 SaLV infected samples (SaLV$^+$), 16 were in the freeze-drying, and three in the dark-drying. All six samples detected with no SaLV infection (SaLV$^-$) were found in the freeze-drying process.

To study the effects of SaLV infection on saffron quality, an ANOVA of freeze-dried SaLV$^+$ and SaLV$^-$ saffron samples was performed (Tables 2A and 2B). SaLV$^+$ samples obtained a greater content of safranal, but a lesser content of picrocrocin, *trans*-5-tG, *trans*-5-nG, *trans*-4-GG, and *cis*-4-GG. Moreover, freeze-dried saffron samples in the presence of SaLV showed lesser kaempferol-3-*O*-sophoroside-7-*O*-glucoside contents. In spite of the greater content of several crocetin esters in saffron samples in the absence of SaLV, the sum of crocetin esters did not show significant differences. However, greater content (around 15.2%) in SaLV$^-$ samples were recorded. There was no concordance again between the safranal content obtained from HPLC-DAD and

Table 2A
The content of the main compounds of freeze-dried SaLV$^+$ and SaLV$^-$ saffron samples by HPLC-DAD analysis.

Compound (mmol·(100 g)$^{-1}$ saffron ± SD)*	Freeze-dried saffron samples	
	SaLV$^+$	SaLV$^-$
Picrocrocin	29.85 ± 16.43a	44.64 ± 9.11b
Safranal	0.93 ± 0.35b	0.62 ± 0.17a
HTCC	19.36 ± 13.84a	22.44 ± 3.59a
trans-5-tG	0.06 ± 0.02a	0.08 ± 0.02b
trans-5-nG	$0.50{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$a	$0.07{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$b
trans-4-GG	3.11 ± 1.12a	4.62 ± 0.90b
trans-3-Gg	3.65 ± 1.95a	4.24 ± 1.56a
trans-2-gg	1.23 ± 1.79a	0.99 ± 0.84a
cis-4-GG	0.21 ± 0.11a	0.34 ± 0.13b
cis-3-Gg	0.15 ± 0.13a	0.23 ± 0.08a
cis-2-gg	0.06 ± 0.11a	0.04 ± 0.03a
Σ Crocins	9.45 ± 3.53a	11.15 ± 3.19a
K1	0.30 ± 0.07a	0.32 ± 0.07a
K2	0.88 ± 0.22a	1.08 ± 0.34b

*Values are the mean of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. K1, kaempferol tetrahexoside, and K2, kaempferol 3-*O*-sophoroside-7-*O*-glucoside. One-way analysis of variance (ANOVA) for each column is included. Different letters (a, b) within each row represent statistically significant differences ($p < 0.05$) across SaLV-infected (SaLV$^+$) and uninfected (SaLV$^-$) saffron samples for each metabolite's concentration.

Table 2B
UV–vis spectrophotometric parameters value of freeze-dried SaLV$^+$ and SaLV$^-$.

($A_{1cm}^{1\%}$ ± SD)*	Freeze-dried saffron samples	
	SaLV$^+$	SaLV$^-$
$A_{1cm}^{1\%}$257 nm	65 ± 26a	87 ± 16b
$A_{1cm}^{1\%}$330 nm	21 ± 7a	31 ± 9b
$A_{1cm}^{1\%}$440 nm	94 ± 40a	137 ± 26b

* Values are the mean of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. One-way analysis of variance (ANOVA) for each column is included. Different letters (a, b) within each row represent statistically significant differences ($p < 0.05$) across SaLV-infected (SaLV$^+$) and uninfected (SaLV$^-$) saffron samples for each $\boldsymbol{A_{1cm}^{1\%}}$ parameter.

UV–vis spectrophotometric value of $A_{1cm}^{1\%}$ 330, since in HPLC analysis SaLV$^+$ samples obtained higher content of safranal than SaLV$^-$, while the value of $A_{1cm}^{1\%}$ 330 nm for SaLV$^+$ was lesser than for SaLV$^-$. Moreover, the coluring strength ($A_{1cm}^{1\%}$ 440) showed significant differences, in contrast to the sum of crocins (Tables 2A and 2B). The SaLV$^-$ samples belonged to Category III, according to ISO 3632 (2011) and due to the less value of $A_{1cm}^{1\%}$ 440 nm, the SaLV$^+$ samples did not belong to Category III. It would seem that the presence of SaLV in saffron samples produced an effect in the chemical composition since, except safranal, the rest of the metabolites, which showed significant differences, obtained lower content as measured through both HPLC-DAD and spectrophotometric (UV–vis).

Results from HPLC-DAD and UV–vis spectrophotometry were further subjected to discriminant function analysis (Fig. 1) with two functions. 70.9% of the variance was attributed to the dehydration process separating freeze-dried saffron samples from those dried in dark conditions. Function two, further separated SaLV$^+$ saffron samples from SaLV$^-$ ones, with 100% of the cumulative variance, except for one SaLV$^-$ sample, which was grouped as SaLV$^+$. This may be due to the low concentration of SaLV in the sample and the inability of the serological assay to detect the virus. Function one depends on picrocrocin mainly, which could be due to the dehydration process, as shown in several other studies, the drying methods for obtaining saffron has influence in the content of picrocrocin (Carmona et al., 2005; Chaouqi et al., 2018; del Campo et al., 2010; Pardo et al., 2002). Function two depends on *cis*-3-Gg, *cis*-2-gg, *trans*-3-Gg, and *trans*-2-gg, principally. In this case, the mechanism of glycosyltransferase enzymes could have been modified due to the effects of SaLV infection.

To study the effects of SaLV infection on saffron quality along with the dehydration process, an ANOVA with freeze-dried (SaLV$^+$/ SaLV$^-$), and dark-dried (SaLV$^+$) saffron samples were performed (Tables 3 and 4). As expected, there were significant differences in the same secondary metabolites, as observed previously: picrocrocin, safranal, *trans*-5-tG, *trans*-5-nG, *trans*-4-GG, *cis*-4-GG, and kaempferol-3-*O*-sophoroside-7-*O*-glucoside (Table 3). Except for safranal, the presence of SaLV reduced the content of the compounds mentioned above in freeze-dried SaLV$^+$ saffron samples. Therefore, it would seem that the concentration of saffrońs metabolites was affected by the presence of SaLV. Considering only the significant differences obtained for SaLV-infected samples, freeze-dried SaLV$^+$ samples showed greater content of safranal and picrocrocin than dark-dried samples. However, *trans*-5-tG, *trans*-5-nG, *trans*-4-GG, *cis*-4-GG obtained lower content in freeze-dried samples. It seems that the dehydration process is involved in the picrocrocin content as other studies have reported (Carmona et al., 2005; Chaouqi et al., 2018; del Campo et al., 2010; Pardo et al., 2002). In dark conditions, the enzymes responsible for the glycosylation would be in contact with crocetin esters for a longer time, which may lead to high glycosylated crocetin esters.

Regarding the safranal content, it is known that high temperature during the process of saffron dehydration causes a high content of safranal (Carmona et al., 2005). Though Acar et al. (2011) reported that saffron samples dehydrated using the freeze-drying method obtained a five-time higher content of safranal than the samples dried naturally under the sun. So far, it is not known what could be the reason for that. One possible explanation could be as follows: according to the accepted hypothesis for the generation of saffron volatiles where all compounds responsible for the attributes of this spice would be formed from a unique precursor (zeaxanthin), the volatile compounds would be obtained in the last step of the metabolic pathway (Carmona, Zalacain, & Alonso, 2006). In this hypothesis, zeaxanthin is broken down by an enzyme at both ends to generate crocetin dialdehyde and picrocrocin. Thus, each molecule of zeaxanthin would produce one molecule of crocetin dialdehyde and two of picrocrocin. Subsequently, crocetin dialdehyde would be oxidized and esterified to generate the crocetin esters. Later, picrocrocin would be converted to safranal either by a two-step enzymatic/dehydration process involving the intermediate 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) or directly by thermal degradation. Thus, to understand in greater depth the metabolic pathway for safranal production, the content of the sum of picrocrocin, safranal, and HTCC was obtained (Table 3). In spite of using two different dehydration process, there were no significant differences in HTCC content. Thus, it would seem that these drying processes do not have a differentiating effect on the formation of this compound. In the case of freeze-dried SaLV$^+$ samples, as its picrocrocin content was lower than SaLV$^-$ ones, and its safranal content was higher, this could be speculated that a part of picrocrocin would be getting converted into safranal. Till now, the only known fact related to safranal production is that it could be due to the enzymatic/dehydration or thermal degradation process, and by freeze-drying, these particular conditions are not provided. Thus, our data suggest that the significant differences in the sum of picrocrocin, safranal, and HTCC were due to the different content of safranal and picrocrocin, where the presence of SaLV could be responsible.

In addition, the values of the sum of picrocrocin, safranal, and HTCC, along with the sum of all crocetin esters were also studied, and the molar ratio was calculated to compare the obtained data with that of the accepted hypothesis. Thus, the molar ratio (picrocrocin + safranal + HTCC/sum of all crocetin esters) of freeze-dried SaLV$^+$ and SaLV$^-$ along with dark-dried SaLV$^+$ saffron samples were obtained (Table 5). In accordance with the accepted hypothesis

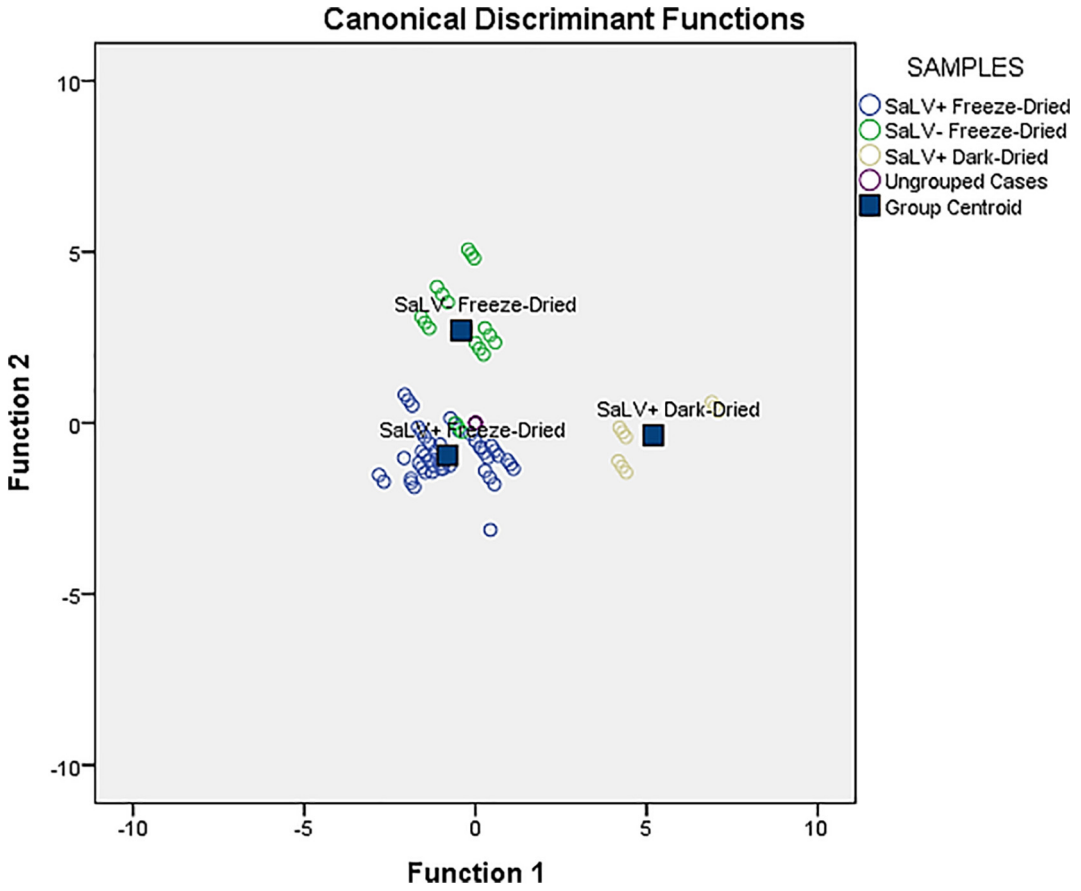

Fig. 1. A plot of the results from the stepwise canonical analysis of $SaLV^+$ and $SaLV^-$ samples obtained through different dehydration conditions.

Table 3
Content of the main compounds of $SaLV^+$ and $SaLV^-$ saffron samples with different dehydration processes by HPLC-DAD analysis.

Compound (mmol·(100 g)$^{-1}$ saffron ± SD)*	Saffron Samples		
	$SaLV^+$ Freeze-Dried	$SaLV^-$ Freeze-Dried	$SaLV^+$ Dark-Dried
Picrocrocin (P)	29.85 ± 16.43b	44.64 ± 9.11c	15.14 ± 1.54a
Safranal (S)	0.93 ± 0.35b	0.62 ± 0.17a	0.41 ± 0.07a
HTCC	19.36 ± 13.84a	22.44 ± 3.59a	16.82 ± 4.30a
P + S + HTCC	50.13 ± 29.49b	67.70 ± 11.12c	32.37 ± 5.87a
trans-5-tG	0.06 ± 0.02a	0.08 ± 0.02b	0.09 ± 0.02b
trans-5-nG	$0.05{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$a	$0.07{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$b	$0.08{\cdot}10^{-3}$ ± $0.02{\cdot}10^{-3}$b
trans-4-GG	3.11 ± 1.12a	4.62 ± 0.90b	4.59 ± 1.43b
trans-3-Gg	3.65 ± 1.95a	4.24 ± 1.56a	3.71 ± 1.35a
trans-2-gg	1.23 ± 1.79a	0.99 ± 0.84a	0.74 ± 0.41a
cis-4-GG	0.21 ± 0.11a	0.34 ± 0.13b	0.35 ± 0.11b
cis-3-Gg	0.15 ± 0.13a	0.23 ± 0.08a	0.80 ± 0.07a
cis-2-gg	0.06 ± 0.11a	0.04 ± 0.03a	0.04 ± 0.02a
Σ Crocins	9.45 ± 3.53a	11.15 ± 3.19a	11.95 ± 4.42a
K1	0.30 ± 0.07a,b	0.32 ± 0.07b	0.26 ± 0.02a
K2	0.88 ± 0.22a	1.08 ± 0.34b	0.80 ± 0.17a

*Values are the mean of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. K1, kaempferol tetrahexoside, and K2, kaempferol 3-*O*-sophoroside-7-*O*-glucoside. One-way analysis of variance (ANOVA) for each column is included. Different letters (a, b, c) within each row represent statistically significant differences ($p < 0.05$) across different dehydration processes with SaLV-infected ($SaLV^+$) and uninfected ($SaLV^-$) for each metabolite's concentration.

for the generation of saffron volatiles, the expected molar ratio would be 2:1 (picrocrocin + safranal + HTCC/sum of all crocetin esters). Dark-dried saffron samples showed a closer molar ratio value to the accepted hypothesis than freeze-dried ones. In this case, the high content of crocetin esters, which were more glycosylated and thus found more stability, might be the reason. On the other hand, the high molar ratio for freeze-dried saffron samples could be due to a technical limitation as it is difficult to solubilize crocetin esters once they are frozen, causing insoluble molecular conglomerates. Indeed, it was observed in saffron samples through the optical microscope that the stigmas contained some square molecules, which were crocetins and had not been solubilized (Naess et al., 2006). Moreover, the content of picrocrocin and safranal was recorded high (molar ratio around 6: < 1) in freeze-dried saffron samples, possibly due to either the effects of the presence of SaLV or the dehydration process or both.

Plant resistance response to pathogens always correlates with the activation of various sets of specified defense mechanisms. The response involves transcriptional activation of defense-related genes and

Table 4
UV–vis spectrophotometric parameters value of SaLV$^+$ and SaLV$^-$ saffron samples with different dehydration processes.

($A^{1\%}_{1cm}$ ± SD)*	Samples		
	SaLV$^+$ Freeze-Dried	SaLV$^-$ Freeze-Dried	SaLV$^+$ Dark-Dried
$A^{1\%}_{1cm}$257 nm	65 ± 26a	87 ± 16a,b	73 ± 7 a,b
$A^{1\%}_{1cm}$330 nm	21 ± 8a	31 ± 9b	34 ± 3b
$A^{1\%}_{1cm}$440 nm	94 ± 40a	137 ± 26b	153 ± 37b

* Values are the mean of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. One-way analysis of variance (ANOVA) for each column is included. Different letters (a, b) within each row represent statistically significant differences ($p < 0.05$) across the dehydration process with SaLV-infected (SaLV$^+$) and uninfected (SaLV$^-$) for each $A^{1\%}_{1cm}$ parameter.

Table 5
The molar ratio value of SaLV$^+$ and SaLV$^-$ saffron samples with different dehydration processes.

	Samples		
	SaLV$^+$ Freeze-Dried	SaLV$^-$ Freeze-Dried	SaLV$^+$ Dark-Dried
Molar ratio	5.50 ± 2.79b	6.46 ± 1.57b	3.24 ± 1.81a

activation of enzymes to specifically modify primary and secondary metabolism. For instance, in *Nicotiana tabacum* L. transgenic plants constitutively expressing a viral protein, a significant induction of several enzymes of anaplerotic (involved in plant primary and secondary metabolism under stress conditions) and antioxidant enzymes were observed (Doubnerová, Janošková, Synková, Subr, Čeřovská, & Ryšlavá, 2007). Thus, a viral protein can influence the enzymes involved in the metabolic pathway. Here, the SaLV effects on metabolites could have happened due to a direct or indirect interaction of SaLV with the enzymes involved in different steps of the metabolic pathway, as reported for some other plants (Cervera et al., 2018). The increase in safranal in freeze-dried SaLV$^+$ than SaLV$^-$ *C. sativus* plants could be SaLV-mediated by inducing more enzymes involved in the metabolic pathway. The speculation of SaLV modulating enzymes is reasonable as there are well-established studies on the impact of viruses on phytohormones, which is also a product of secondary metabolism (Bera, Blundell, Liang, Crowder, & Casteel, 2020; Chisholm, Eigenbrode, Clark, Basu, & Crowder, 2019; Dilworth, Riley, & Stennett, 2017; Lewsey et al., 2010). Numerous studies have shown that viruses are capable of inducing or suppressing enzymes that are critical for the synthesis of salicylic acid, and jasmonic acid (Bera, Blundell, Liang, Crowder, & Casteel, 2020; Chisholm et al., 2019; Lewsey et al., 2010). Interestingly, in our study, all other metabolites decreased in freeze-dried SaLV$^+$ samples which are acting as a precursor for safranal (*e.g.*, picrocrocin), except safranal, this indicates a possibility of another anabolic pathway for safranal regulated by SaLV, or it might be that SaLV increases the rate of safranal synthesis by breaking down its precursor at a faster rate.

Crocetin esters are the principal pigment of *C. sativus* stigma and the most significant quality parameter of spice. In the anabolic pathway of different crocetin esters, crocetin dialdehyde will form different crocetin esters after undergoing successive modifications by an aldehyde oxidoreductase and UDPG-glucosyltransferases (GTASEs) (Dufresne, Cormier, & Dorion, 1997; Moraga, Nohales, Fernández-Pérez, & Gómez-Gómez, 2004). During these steps, different enzymes and pathways are involved in the biosynthesis of these compounds (Dufresne et al., 1997) that may get affected due to the virus activity, and thus, results in lower accumulation of crocetin esters in SaLV$^+$ than SaLV$^-$ plants.

Our data here improves the understanding of processing methods for saffron production. The study of Acar et al. (2015) showed that freeze-drying is a much better method than any conventional one, which gives better spice quality and offsets the high cost. Contrary to this, our current study demonstrates that the quantity of crocins was significantly more in dark-dried samples, which can be considered under the conventional drying method (Table 1A). We re-analyzed the data by considering the molar ratio comparisons that also showed the dark-drying method is better for crocetins esters content (Table 5). As we compared our dataset between SaLV-infected samples (freeze-dried and dark-dried), this is of significance for Irańs saffron industry (producing 90% of the worlds saffron) as till now there is no large scale testing to check for SaLV infection in fields. Therefore, if the end product usage needs more crocins, then freeze-drying would decrease crocins content and should not be done to save the cost of processing. Furthermore, our previous study (Parizad et al., 2018) showed freeze-dried SaLV$^-$ samples have more crocins level relative to freeze-dried SaLV$^+$ samples. In this study, we demonstrate that there was no difference in crocins content between freeze-dried SaLV$^-$ samples and dark-dried SaLV$^+$ samples (*trans*-5-tG, *trans*-5-nG, *trans*-4-GG, *cis*-4-GG in Table 3) though both of them have a significantly higher content than freeze-dried SaLV$^+$ samples. Thus, by using a dark-drying process, we can nullify the adverse effect of SaLV on crocins content (*trans*-5-tG, *trans*-5-nG, *trans*-4-GG, *cis*-4-GG in Table 3).

Apart from carotenoid derivatives, flavonoid derivatives were also quantified through HPLC-DAD and UV–vis spectrophotometry, as they are also crucial for the quality of saffron. Kaempferol aglycone and its glycosides (kaempferol-3-*O*-sophoroside-7-*O*-glucoside) are synthesized through the flavonoid biosynthesis pathway from phenylalanine ammonia-lyase (Gondor et al., 2016; Moratalla-López, Lorenzo, Alonso, & Sánchez, 2016). Three different glucosyltransferases (UGT707B1, UGT75P1, and UGT703B1) have been characterized in *C. sativus* L., isolated from *C. sativus* stigmas (Moratalla-López et al., 2016). In this study, kaempferol-3-*O*-sophoroside-7-*O*-glucoside's concentration was affected by the presence of SaLV, indicating that SaLV could have influenced the related enzymes (*e.g.*, chalcone synthase, chalcone isomerase, flavanone-3-dioxygenase, flavonol synthase, 2″-*O*-glucosyltransferase, UGT707B1, UGT703B1, UGT75P1) and/ or intermediates (*e.g.*, cinnamic acid).

It has been shown that in transgenic tobacco plants, up-regulation of tobacco glucosyltransferase (TOGT1) resulted in the over-accumulation of scopoletin and its glucoside form, scopolin, which caused the early formation of necrotic lesions during the hypersensitive response (HR) to *Tobacco mosaic virus* (TMV) (Gachon, Baltz, & Saindrenan, 2004). HR is considered as an indication of plant resistance towards viruses and can be an important step in the limitation of viral pathogens (Matros & Mock, 2004). In general, viral infection leads to suppression of secondary metabolism and development of the plant as the plant invests more resources on defense against virus infection (Cervera et al., 2018). Here our study agrees with these findings, but some analyses showed that depending on plant-virus interaction, there might be a positive effect on metabolism, as was the case for safranal. It had been proved elsewhere that interactions between viruses and their natural host plants are complex, which affects both viral traits and also the host genes' expression (positively or negatively) (Cervera et al., 2018). It has been shown that the viral infection in the host plant has an especial modulation in the host gene expression profile. If the virulence of the virus changes (e.g., mild or severe variants of the virus), this will cause a change in host gene expression profile. Our recent publications (Parizad et al., 2018, 2019) showed that SaLV is the prevalent *C. sativus* plant virus in Iran, which develops only mild mosaic symptoms on leaves. Considering the ancient *C. sativus* cultivation in Iran and the lack of any known SaLV resistance in *C. sativus* plants, it is advisable to screen *C. sativus* populations/genotypes against SaLV to find any possible resistance sources.

5. Conclusions

Evaluation of the saffron compounds, including picrocrocin, safranal, crocetin esters, and kaempferol-3-*O*-sophoroside-7-*O*-glucoside content, was performed in two different dehydration processes, freeze-drying, and dark-drying, using HPLC-DAD and spectrophotometry. The different dehydration processes could affect the chemical composition of some secondary metabolites of saffron. Moreover, the SaLV infection of *C. sativus* plants can influence these compounds in stigma, and thus the spice quality. We demonstrated here that the presence or absence of SaLV in the studied saffron samples produced a differentiation effect in the analyzed chemical composition of saffrons depending upon different dehydration methods. Thus, the concentration of important saffron secondary metabolites, under two different dehydration processes, was differentially affected by SaLV infection.

The results of HPLC-DAD and UV–vis spectrophotometric analyses sometimes were not in accordance, since HPLC-DAD is more specific analytical technique than spectrophotometry. Moreover, spectrophotometry is not able to determine an exact measurement of picrocrocin and safranal due to some interferences in the determination procedures at 250 and 330 nm (García-Rodríguez et al., 2014). Hence, the use of HPLC-DAD technique is suggested to properly evaluate the saffron quality and the effects of any agents, including virus infection. Nevertheless, a more detailed study is required to estimate the exact impact of the SaLV infection by complementary studies, especially genomics, to identify the accurate target points of the virus and evaluate the expression profiles of the corresponding genes.

CRediT authorship contribution statement

Natalia Moratalla-López: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Shirin Parizad:** Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Mina Koohi Habibi:** Investigation. **Stephan Winter:** Conceptualization, Methodology, Resources. **Siamak Kalantari:** Conceptualization, Methodology, Resources. **Sayanta Bera:** Conceptualization, Methodology, Resources. **Candida Lorenzo:** Formal analysis, Resources. **M. Valle García-Rodríguez:** Conceptualization, Methodology, Validation, Formal analysis, Data curation. **Akbar Dizadji:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Gonzalo L. Alonso:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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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