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Determination of Saffron Flower Metabolites by Near-Infrared Spectroscopy for Quality Control

Jorge F. Escobar-Talavera, María Esther Martínez-Navarro, Gonzalo L. Alonso * and Rosario Sánchez-Gómez *

Cátedra de Química Agrícola, Escuela Técnica Superior de Ingeniería Agronómica y de Montes y Biotecnología (ETSIAMB), Universidad de Castilla-La Mancha, Avda. de España s/n, 02071 Albacete, Spain; jorge.escobar@uclm.es (J.F.E.-T.); mesther.martinez@uclm.es (M.E.M.-N.)

* Correspondence: gonzalo.alonso@uclm.es (G.L.A.); rosario.sgomez@uclm.es (R.S.-G.);

Tel.: +34-926053160 (G.L.A.); +34-926053618 (R.S.-G.)

Abstract: Saffron, obtained by dehydrating the stigmas of the *Crocus sativus* flower, is a spice of great importance. In saffron, the flower emerges before leaf formation, thanks to the nutritional reserves of the corm. Early knowledge of metabolite levels such as crocins, picrocrocin, safranal, anthocyanins, or kaempferols in flowers serves as a guide to evaluate the quality of the corm (coloring power, flavor, aroma, or antioxidant capacity, among others). In this study, near-infrared spectroscopy (NIR) was calibrated and validated to determine the main saffron metabolites, both in stigmas and in floral residue. To achieve this, saffron flowers from different locations of the Denomination of Origin (D.O.) “Azafrán de La Mancha” (Castilla-La Mancha, Spain) were analyzed using NIR spectroscopy. Prior to this, samples were analyzed by RP-HPLC-DAD, where the concentration of all cited metabolites was determined. The development of a predictive model through NIR calibration and validation was successful, achieving high R^2 values, especially in the case of the sum of crocins and kaempferol-3-O- β -sophoroside. Using these predictive models, it is possible to determine the quality of saffron corm by analyzing the flower.

Keywords: corm quality; crocetin esters; kaempferol-3-O- β -sophoroside; near-infrared spectroscopy; partial least squares regression model; picrocrocin; saffron flowers



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1. Introduction

The plant *Crocus sativus* L. has been cultivated since ancient times to obtain the dehydrated stigmas of its flowers, which constitute the spice saffron, known for its culinary use and various bioactive properties [1]. As with almost all spices, it cannot be determined whether it was first used as a medicinal plant or as a culinary condiment. Saffron is valued in cooking for its high content of crocins, substances responsible for its color; picrocrocin, the compound responsible for its bitter taste; and safranal, the main substance responsible for its exquisite aroma [2,3]. When ingested, crocetin, the hydrolysis product of crocins, is detected in the blood [4]. It has been shown that this molecule, in its *trans* form, acts as a blood oxygenator and is capable of crossing the blood–brain barrier [5]. Together with safranal, crocetin is attributed with the bioactive properties of saffron [6].

The floral remnants are a by-product consisting of tepals and stamens [7] which mainly contain anthocyanins and flavonols. These compounds are applied in the cosmetics and parapharmacy industries due to their antioxidant and bioactive properties [8].

In autumn, the flowers emerge from the corm (thickened stem) (Figure 1) before the leaves, a condition known as hysternanth. The corm is planted at the beginning of summer, and during the autumn, winter, and spring months, daughter corms develop and grow, which then bloom the following autumn, while the mother corm dies. In other words, the plant remains alive for two years, with the first year dedicated to growth and development and the second year to offspring production. Normally, in plots where *Crocus sativus* L. is cultivated, the corms are kept buried until the plantation density is high, and soil nutrient

competition prevents the formed corms from growing. This timeframe depends on the fertility characteristics of the production areas. Thus, in Spain, the same corms remain buried for 4 to 5 years. *Crocus sativus* L. is a sterile plant and does not reproduce by seeds but rather through budding [9]. Corms obtained from previous-year plantations act as seeds for new plantations, limiting the rapid spread of cultivation due to their availability. Commercially, corm suppliers certify their good sanitary conditions, but it is not possible to guarantee their quality.

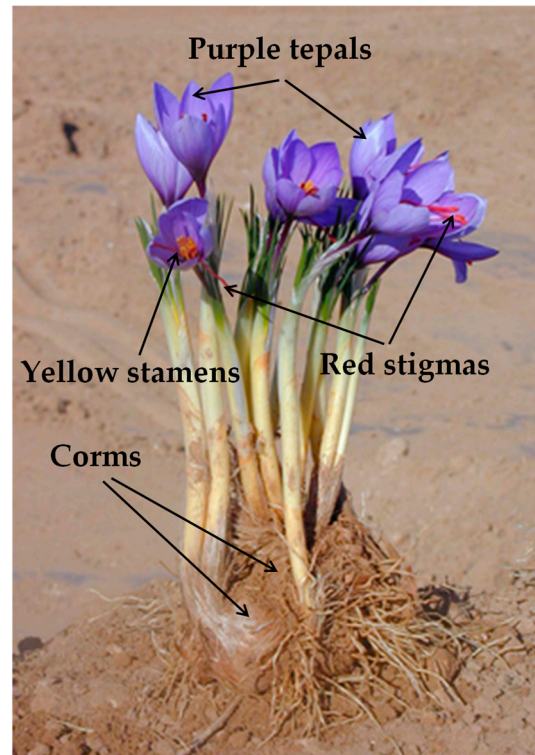


Figure 1. Flowers emerging from a cluster of corms derived from a single corm after three years in the same cultivation soil.

Currently, there is no analytical methodology to assure corm quality, since it can only be measured through the content of metabolites in the product, in both stigmas and floral remnants. Therefore, to indirectly control the quality of a corm, it is necessary to determine the concentration of the main metabolites such as crocins, picrocrocin [10], flavonols, and anthocyanins [11] in the saffron flowers directly emerging from the corm. This is not the case for safranal, since it is a compound that forms during the dehydration process and therefore does not directly derive from the corm.

The non-targeted near-infrared spectroscopy (NIR) technique can swiftly provide the metabolic fingerprint of agricultural products. Combining this technique with chemometrics can facilitate the data analysis, discrimination, and classification of agricultural products, as well as the prediction of concentrations and parameters related to their quality [12–14]. Supervised chemometric methods are widely known, including Linear Discriminant Analysis (LDA), Partial Least Squares Discriminant Analysis (PLS-DA), partial least squares regression (PLS-R), Soft Independent Modeling of Class Analogy (SIMCA), Support Vector Machine (SVM), Random Forests (RFs), Artificial Neural Networks (ANNs), K-Nearest Neighbors (K-NN), Linear Learning Machine (LLM), and Bayes Linear Discriminant Analysis [15,16], among others. When assessing the quality of an agricultural product, calibration is necessary, which can be performed through univariate, multiple regression, principal component, least squares, cross-validation, and independent test set calibrations, as well as through minimum angle regression and elastic net. The goodness of the models can be evaluated using certain parameters such as the coefficient of regression

(R^2), the Prediction Residual Sum of Squares (PRESS), the residual predictive deviation (RPD), the Standard Error of Prediction (SEP), or the standard error of cross-validation (SECV) [13], principally.

Nowadays, the analysis of the main metabolites of the *Crocus sativus* L. flowers, which encompasses stigmas or floral residue, involves analysis by chromatographic techniques such as Reversed-Phase High-Performance Liquid Chromatography with Diode Array Detection (RP-HPLC-DAD) [11,17]. As is known, these types of techniques are (a) time-consuming; (b) labor-intensive methods; and (c) require sample treatment. Therefore, scaling them up to a large number of samples is not an easy task. In this line, replacing their use by employing the NIR spectroscopy technique for the estimation of any of the main metabolites presents a better option for routine analysis in time due to the following benefits: (a) a fast technique; (b) no sample preparation required, since solids and liquids can be used in pure forms; (c) a low cost per sample, as no chemicals or solvents are needed; (d) an environmentally friendly technique, due to no waste being generated. Taking all of the above into account, the aim of this study is to calibrate NIR equipment to evaluate the quality of *Crocus sativus* L. corms by calibrating the content of the main metabolites in their flowers.

2. Materials and Methods

2.1. Chemicals and Reagents

The solvents and reagents employed were all HPLC purity or analytical grade. Acetonitrile (ACN) (CAS: 75-05-8), trifluoroacetic acid (TFA) (CAS: 7605-1), and hydrochloric acid (HCl) (CAS: 7647-01-0) were supplied from Panreac (Barcelona, Spain). Ultrahigh-purity water was produced using a Milli-Q system (Millipore, Bedford, MA, USA).

For standards, crocetin esters, *trans*-crocetin di-(β -D-gentiobiosyl) ester (*trans*-4-GG) (CAS: 55750-85-1), and *trans*-crocetin (β -D-glucosyl)-(β -D-gentiobiosyl) ester (*trans*-3-Gg) (CAS: 55750-84-0) with purity levels of $\geq 99\%$ were obtained from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany); picrocrocin (synthesized in the laboratory according to Sánchez et al. [3]) and kaempferol-3-O- β -sophoroside (CAS: 19895-95-5) were obtained from BLD Pharmatech (Shanghai, China).

For standards' preparation, a stock solution was prepared, from which, dilutions were made to obtain the different calibration points. For the dilutions, water/hydrochloric acid (100:1, *v/v*) was used in the same way as for the extractions. Storage took place at $-20\text{ }^{\circ}\text{C}$.

2.2. Plant Material

A total of 173 samples of *Crocus sativus* flowers from different locations within the D.O. "Azafrán de La Mancha" (Castilla-La Mancha, Spain) were collected during the 2022 and 2023 harvest seasons. The fresh *Crocus sativus* L. flowers were subjected to a freeze-drying process in the LyoAlfa 6-50 freeze-dryer (Telstar, Terrasa, Spain) for 5 days to ensure complete lyophilization (constant weight). The freeze-dryer conditions were $-50 \pm 2\text{ }^{\circ}\text{C}$ and 10^{-3} mbar. Subsequently, dried *Crocus sativus* L. flowers were ground into a homogeneous powder using a mortar and stored at room temperature ($18 \pm 3\text{ }^{\circ}\text{C}$) in a chamber with silica gel until analysis.

Moisture content analysis was conducted on the samples using a moisture balance equipped with a halogen lamp, specifically the XM-120 T model (Cobos, Barcelona, Spain), operating at a temperature of $105\text{ }^{\circ}\text{C}$. When moisture loss was less than 0.1% in 180 s, it was considered that the samples had reached constant mass.

2.3. Preparation of Extracts of *Crocus sativus* L. Flowers

Extracts of *Crocus sativus* L. flowers were prepared according to Moratalla-Lopez et al.'s method [11]. Briefly, 200 mg of lyophilized and grounded powder from each sample was mixed with 25 mL of water/HCl (100:1, *v/v*) and stirred in the dark at 500 rpm for 1 h. Afterward, extracts underwent centrifugation at 3500 rpm for 5 min (Selecta, Barcelona, Spain). The supernatant was then filtered through a hydrophilic polytetrafluoroethylene

(PTFE) membrane with pores of 0.45 μm (Millipore, Bedford, MA, United States). The resulting filtered extract was carefully transferred to a vial for Reversed-Phase High-Performance Liquid Chromatography with Diode Array Detection (RP-HPLC-DAD) analysis.

2.4. Chromatographic Conditions

The content of *Crocus sativus* L. flowers' metabolites was determined according to a method developed by Moratalla-López et al. [11]. The RP-HPLC-DAD analyses were performed using an Agilent 1200 HPLC chromatograph (Palo Alto, CA, United States). The chromatographic column employed was a Develosil ODS-HG-5 with dimensions of 250 $\times$ 4.6 mm, 5 μm , (Teknokroma, Sant Cugat Del Vallès, Barcelona, Spain), maintained at a temperature of 40 $^{\circ}\text{C}$. The mobile phase consisted of ultrahigh-purity water/trifluoroacetic acid (TFA) (99.5:0.5, *v/v*) (A) and acetonitrile (ACN) (B). The elution gradient was as indicated in Table 1. The flow rate was set at 1.0 mL/min, and the injection volume was 30 μL .

Table 1. Chromatographic conditions of RP-HPLC-DAD.

Minutes	Acetonitrile (%)	Water/Trifluoroacetic Acid (%)
0	0	100
30	30	70
40–45	80	20
50–55	20	80
55–59	0	100

For all the compounds, identification was performed using the DAD by comparison with the corresponding UV–visible spectra and the retention time of their pure standards. Thus, the DAD detector was set at 440 nm to identify crocetin esters (*trans*-crocetin (β -D-triglucosyl)-(β -D-gentiobiosyl) ester, *trans*-5-tG; *trans*-crocetin (β -D-neapolitanosyl)-(β -D-glucosyl), *trans*-5-ng; *trans*-crocetin di-(β -D-gentiobiosyl) ester, *trans*-4-GG; *trans*-crocetin (β -D-neapolitanosyl)-(β -D-glucosyl) ester, *trans*-4-ng; *trans*-crocetin (β -D-gentiobiosyl), *trans*-2-G; *trans*-crocetin (β -D-glucosyl)-(β -D-gentiobiosyl) ester, *trans*-3-Gg; *cis*-crocetin di-(β -D-gentiobiosyl) ester, *cis*-4-GG; *cis*-crocetin (β -D-glucosyl)-(β -D-gentiobiosyl) ester, *cis*-3-Gg; *cis*-crocetin di-(β -D-gentiobiosyl) ester, *cis*-4-GG; *cis*-crocetin di-(β -D-glucosyl) ester, *cis*-2-gg; and, *cis*-crocetin (β -D-gentiobiosyl) ester, *cis*-2-G), at 250 nm for the detection of picrocrocin, at 266 nm for the detection of flavonols (kaempferol-3-O- β -sophoroside-7-O- β -glucoside, K-3-O-s-7-O-g; kaempferol-3-O- β -sophoroside, K-3-O-s; kaempferol aglycone, K), and at 520 nm for the identification of anthocyanins (delphinidin-3,5-di-O- β -glucoside, D-3,5-di-O-g; petunidin-3,5-di-O- β -glucoside, P-3,5-di-O-g; delphinidin-3-O- β -glucoside, D-3-O-g; malvidin-3,5-di-O- β -glucoside, M-3,5-di-O-g; and petunidin-3-O- β -glucoside, P-3-O-g). Quantification was based on the calibration curves of the respective commercial standards at five different concentrations achieved by a UV–visible signal ($R^2 = 0.9900$ – 0.9999). All analyses were performed in duplicate, with two measurements taken for each replicate [2,18].

2.5. Near-Infrared Spectroscopy Measurements

The powdered saffron flower samples were deposited to completely cover the surface of a 1 cm^2 quartz Petri dish (Perkin-Elmer, Norwalk, CT, USA) and analyzed by Perkin Elmer Spectrum One FT-NIR equipment (Norwalk, CT, USA) coupled with a near-infrared reflectance accessory (NIRA). Data acquisition was carried out over a wavelength range of 10,000–4000 cm^{-1} , with a fixed resolution of 16 cm^{-1} . All samples were scanned in duplicate.

2.6. Data Analysis

The model calibration analysis was performed with the average of two replicated spectra for each *Crocus sativus* L. flower sample. In terms of data processing, a multivariate analysis approach was employed to conduct both quantitative and qualitative assessments. The tracking procedure in the partial least squares regression (PLS-R) modeling generated a systematic profile that incorporated various spectral pretreatment methods to mitigate diverse adverse effects stemming from the physical properties of the sample, technical errors during measurements, or simply instrumental noise. Spectrum-One software version 1.00 was utilized to perform data pretreatment and establish PLS-R models for the studied content. The pretreatment combinations of the spectrum varied depending on the calibrated metabolites, including standard normal variate (SNV), which was applied to all spectra used in the calibration models, which first normalizes a spectrum by calculating the average intensity value and then subtracting this value from the spectrum; smoothing (5, 13, and 19 points); and Savitzky–Golay first and second derivative (5, 9, and 13 points) pre-processing for all measurements to obtain the most robust model.

The model's performance was examined using the coefficient of determination for calibration, R^2_c (1), which indicates the proportion of variance in the dependent variable that the independent one can explain, and the residual predictive deviation, RPD (2); by providing a metric of model validity, higher values correspond to better predictive capacity in the model, as previously described by Luo et al. [19]

$$R^2_c = \left[\left(\sum_i (y_i - \bar{f}_i)^2 / \sum_i (y_i - \bar{y})^2 \right) \right] \quad (1)$$

$$RPD = [\text{Standard deviation of measured extracts} / \text{RMSE}] \quad (2)$$

where RMSE is the root mean square error.

Data pre-processing methods and the selection of wavenumber ranges resulted in high predictability and the precise estimation of *Crocus sativus* L. flower metabolites.

For the calibration process, cross-validation was employed to determine the most suitable number of latent variables and prevent excessive fitting of the equation. Alongside r^2 , the ratio of standard deviation to standard error of cross-validation, RPD_c (3), was used to evaluate the overall accuracy of each equation's fit according to Suchat et al. [20]

$$RPD_c = SD_{cal} / SEC_V \quad (3)$$

The RPD_p (4) index was also calculated. This index represents the relationship between the standard deviation of the reference data and the standard error of cross-validation or test set validation, but specifically using the predicted reference values instead of the actual values. This index provides a measure of the predictive capability of the model relative to the variability in the reference data, which can help determine the usefulness of the model for specific applications. A higher RPD_p value indicates the better predictive capability of the model according to Suchat et al. [20].

$$RPD_p = SD_{cal} / SEP \quad (4)$$

where SD_{cal} refers to the standard deviation obtained from the mean of each parameter of the samples and SEP refers to the Standard Error of Prediction in calibration.

The RMSEC (Root Mean Square Error of Calibration) (5) was also calculated, which is a measure used to evaluate the quality of a calibration model. The lower the RMSEC value, the better the predictive capability of the model. This value represents the standard deviation of the residuals between the predicted values and the actual values in the calibration dataset.

$$RMSEC = \left[\sum_{i=0}^n (f_i - y_i)^2 / n \right]^{1/2} \quad (5)$$

3. Results and Discussion

3.1. *Crocus sativus* Flower Metabolite Content (RP-HPLC-DAD) for Set of Samples

The entire set of *Crocus sativus* L. flower samples (173) used in the present study, from all the localities included under the D.O. “Azafrán de La Mancha”, were of two ages (2022–2023) and submitted to different agronomic practices to obtain a more representative trial. The descriptive statistic data for the main metabolite content are shown in Table 2.

Table 2. Descriptive statistics for *Crocus sativus* L. flower metabolites (RP-HPLC-DAD data).

Compound	Range (g kg ^{−1} Flower Dry Weight)	Mean	SD	CV (%)
<i>cis</i> -4-GG	0.56–2.30	1.31	0.58	44
<i>cis</i> -2-G	0.03–0.52	0.26	0.09	15
<i>cis</i> -3-Gg	0.58–2.28	2.43	0.79	32
<i>cis</i> -2-gg	0.03–0.07	0.03	0.01	33
<i>trans</i> -5-tG	0.15–0.51	0.28	0.13	16
<i>trans</i> -5-ng	0.22–0.35	0.24	0.12	50
<i>trans</i> -4-GG	8.32–33.89	16.84	8.54	50
<i>trans</i> -4-ng	0.17–2.30	0.20	0.08	40
<i>trans</i> -3-Gg	3.94–25.06	9.56	4.47	46
<i>trans</i> -2-G	0.03–0.38	0.52	0.31	59
Picrocrocin	2.03–49.5	30.14	6.44	21
K-3-O-β-s	0.42–50.2	35.28	7.65	21
K-3-O-s-7-O-g	3.42–7.40	5.58	1.15	20
K	0.01–0.19	0.09	0.06	66
D-3,5-di-O-g	1.09–9.1	6.21	1.04	16
P-3,5-di-O-g	0.27–0.45	0.3	0.11	36
D-3-O-g	0.76–2.23	1.08	0.27	25
M-3,5-di-O-g	0.07–4.64	2.67	1.24	46
P-3-O-g	0.24–0.26	0.21	0.09	42

cis-4-GG: *cis*-crocetin di-(β-D-gentiobiosyl) ester; *cis*-2-G: *cis*-crocetin (β-D-gentiobiosyl) ester; *cis*-3-Gg: *cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester; *cis*-2-gg: *cis*-crocetin di-(β-D-glucosyl) ester; *trans*-5-tG: *trans*-crocetin (β-D-triglucosyl)-(β-D-gentiobiosyl) ester; *trans*-5-ng: *trans*-crocetin (β-D-neapolitanosyl)-(β-D-; *trans*-4-GG: *trans*-crocetin di-(β-D-gentiobiosyl) ester; *trans*-4-ng: *trans*-crocetin (β-D-neapolitanosyl)-(β-D-glucosyl) ester; *trans*-3-Gg: *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester; *trans*-2-G: *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester; K-3-O-β-s: kaempferol-3-O-β-sophoroside; K-3-O-s-7-O-g: kaempferol-3-O-β-sophoroside-7-O-β-glucoside; K: kaempferol aglycone; D-3,5-di-O-g: delphinidin-3,5-di-O-β-glucoside; P-3,5-di-O-g: petunidin-3,5-di-O-β-glucoside; D-3-O-g: delphinidin-3-O-β-glucoside; M-3,5-di-O-g: malvidin-3,5-di-O-β-glucoside; P-3-O-g: petunidin-3-O-β-glucoside.

In Table 2, the concentration ranges, mean, standard deviation, and coefficient of variation (in %, calculated by dividing the standard deviation by the mean of each compound, per 100) of each compound for the groups analyzed can be observed: crocins, picrocrocin, flavonols, and anthocyanins. The results obtained in the RP-HPLC-DAD analysis show that the upper concentration ranges of the main compounds increased compared to the values found in the literature using the same method. For example, for the major crocin, *trans*-4-GG, the highest concentration value found in the analyses was 33.89 g/kg, which was higher than the levels found in the study of Moratalla et al. [11], where the maximum concentration value found for this crocin was 18.98 g/kg. The same was observed in the case of one of the other major crocins, *trans*-3-Gg, where the maximum concentration value found was 25.06 g/kg, while the value found previously by Moratalla et al. [11] was 13.51 g/kg.

Since the sample analyzed in this work was fresh flowers and not saffron spice, to compare the metabolite content, it was necessary to perform a conversion, as certain metabolites such as crocins are more concentrated in the spice. To carry out the conversion, the value obtained in the flowers was multiplied by 9.2 [8]. Thus, for example, the value of 33.89 g/kg in *trans*-4-GG crocin would be equivalent to 315.17 g/kg of the same crocin in the spice. The most relevant kaempferol was k-3-O- β -s, since its concentration was much higher than the rest of the kaempferols. Due to its high concentration, it is the only kaempferol among those analyzed for which proper calibration and validation were achieved. The picrocrocin average was higher than that determined by Kabiri et al. [21], as multiplying the values found in the present study by 9.2 to determine the corresponding content in spice revealed a value of 277 g/kg, higher than the one of 120 g/kg determined by Kabiri et al. [21]. As for the content of k-3-O- β -s, a higher concentration (35.8 g/kg, reaching values of 50.2 g/kg) was determined compared to that determined by Carmona et al. [22] (3.13 g/kg), mainly because the sample in that study was saffron spice and not the whole flower. Since it is in the floral residue where the highest concentration of kaempferol is found, the result is a concentration of k-3-O- β -s more than ten times higher than in the spice. If the maximum value obtained for k-3-O- β -s (50.2 g/kg) is divided by 0.78, the equivalent concentration that the tepals of flowers would have can be determined [23], rendering us able to compare such values with those obtained by Vago et al. [24]. In this way, the higher concentration of k-3-O- β -s would be 64.3 mg/g in tepals, which would be a value consistent with those determined by Vago et al. [24]. In relation to the coefficients of variation (CV) obtained (Table 2), in most cases, values greater than 30% were observed, which could demonstrate the great variability found in the different flowers.

3.2. NIR Interpretation for *Crocus sativus* L. Flower Metabolites

The analysis of the main metabolites of the *Crocus sativus* L. flowers, which reveals the quality of the corms, involves the analysis of either the stigma or the floral residue, having to resort in both cases to chromatographic techniques such as RP-HPLC-DAD [11,17]. This technique, among others, is a time-consuming, labor-intensive method and requires sample treatment and is not easily scaled up to hundreds of samples. Replacing its use by employing the near-infrared (NIR) spectroscopy technique for the estimation of any of the main metabolites presents a better option for routine analysis in time. Samples were fully scanned in the range of 10,000–4000 cm^{-1} (750–2500 nm) to fine-tune the scan to generate the best PLS-R model.

Figure 2 shows the total spectra obtained by NIR spectroscopy, represented by different colors, and, as can be seen, all the samples show a similar spectrum, with higher or lower absorbance, depending on the regions. In NIR spectroscopy, there are different measurement ranges associated with different types of chemical bonds. Among the distinctive features observed, there are three prominent bands at 8331, 6853, and 5164 cm^{-1} , reflecting the fundamental and overtone vibrations of the O-H group present in water molecules. Another significant band at 5798 cm^{-1} corresponds to the fundamental vibration of the C-H group, typically indicative of the presence of lipids, oils, and aromatic compounds [25]. It is worth noting the signal centered at 4670 cm^{-1} , frequently associated with C-H groups found in aromatic compounds, especially those with phenolic characteristics [26].

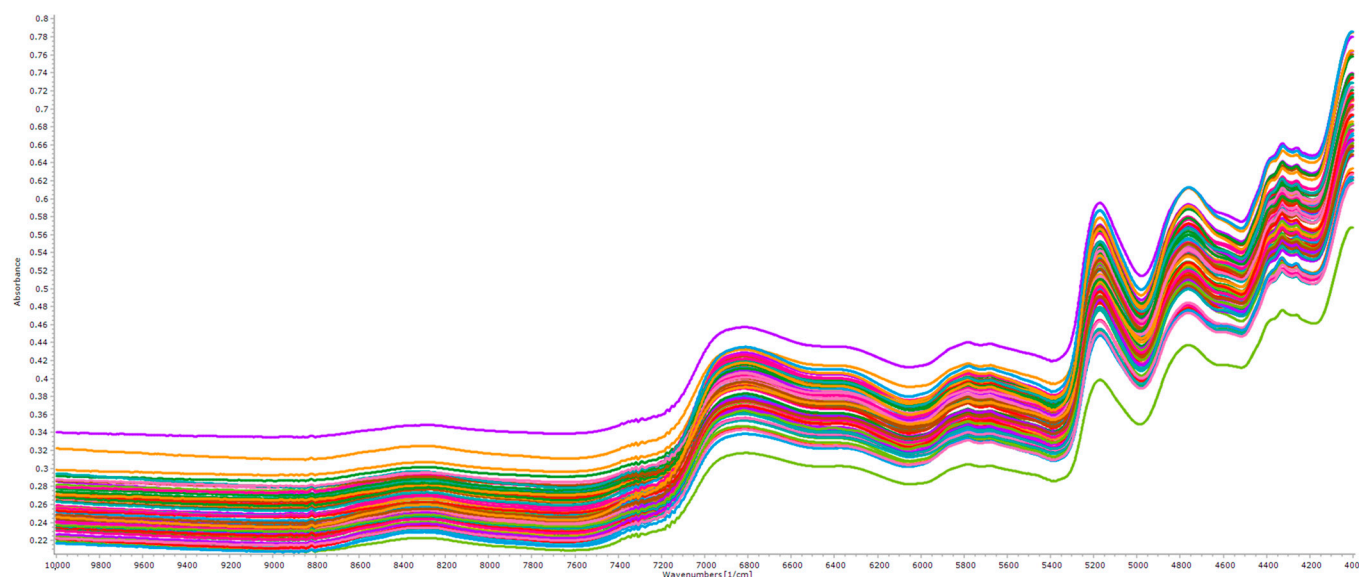


Figure 2. Near-infrared spectrum of *Crocus sativus* flower samples.

3.3. NIR Calibration and Validation

As seen in Table 2, many compounds were quantified in the flower samples. However, it was not possible to obtain positive calibration–validation results. Thus, the best results were obtained for the following combinations/compounds: (a) Σ cis-crocins, which includes *cis*-4-GG, *cis*-3-Gg, *cis*-2-gg, and *cis*-2-G; (b) Σ trans-crocins, which encompasses *trans*-5-tG, *trans*-5-ng, *trans*-4-GG, *trans*-4-ng, *trans*-3-Gg, and *trans*-2-g; (c) Σ crocins, as the sum of the previous *cis* and *trans*; (d) picrocrocin; and (e) kaempferol-3-O- β -sophoroside, which, due to its high concentration among the flavonols analyzed, proper calibration and validation had been achieved. Related to the anthocyanin compound, it was not possible to achieve good calibration for any of those analyzed, nor for all of them.

Table 3 shows the statistics of the calibration set of the metabolites above in all samples analyzed. For the calibration model's development, the entire infrared spectral region (10,000–4000 cm^{-1}) was considered for spectral acquisition after eliminating the redundant spectra. This was mainly because the target molecules in this study contain several types of bonds, which makes it difficult to select a narrower range than the one chosen for calibration–validation. As shown in Table 2, a wide-ranging concentration value was found in the *Crocus sativus* L. flowers for each metabolite, indicating good scattering for such model development. In the initial attempt, it was sought to calibrate the different compounds by focusing on the ranges corresponding to the mentioned peaks. However, the calibrations yielded highly deficient results, with excessively low R^2 values. This outcome can likely be attributed, together with the above, to the intrinsic nature of the samples, since saffron flower is a heterogeneous sample, containing a myriad of compounds. These compounds may vary in composition and concentration, leading to potential interference in the detection of the target compounds. The presence of such diverse compounds within the saffron flower could introduce complexities and challenges in accurately quantifying the different metabolites. Factors such as the varying chemical properties, solubilities, and reactivities of these compounds may contribute to the observed difficulties in achieving robust calibration. Therefore, further refinement and optimization of the calibration methodology may be necessary to account for the complexities inherent in the saffron flower sample and to improve the accuracy and reliability of calibration.

The more accurate calibration model, for each metabolite or sum of them, obtained with NIR spectral data against their RP-HPLC-DAD determination is summarized in Table 4. Thus, the final model selected and presented was based on the analyses of some error parameters: higher RPD and lower standard errors given by RMSECV (root mean square error of cross-validation) and RMSEP (root mean square error of prediction) (Table 4).

Table 3. NIR statistical parameters for the calibration data of the measured *Crocus sativus* flower metabolites.

	N	Spectral Range (cm ⁻¹)	Pre-Process *	R ² _c
Σ <i>cis</i> -Crocins	173	9000.9–4000	2.13.13	1.00
Σ <i>trans</i> -Crocins	173	9000.9–4000	2.13.19	0.97
Σ Crocins	173	9000.9–4000	1.13.13	0.99
Picrocrocin	77	9000.9–4000	1.9.5	0.95
K-3-O-β-s	77	10,000–4000	1.5.13	0.99

* The three-digit code (a.b.c.), where ‘a’ refers to the number of the derivative (1 and 2), ‘b’ is the interval over which the derivative is calculated (5, 9, and 13), and ‘c’ corresponds to the number of data points in a running mean or smoothing (5, 13, and 19). R²_c: coefficient of determination in calibration; RMSEC: standard error of calibration. Σ *cis*-Crocins: *cis*-4-GG + *cis*-3-Gg + *cis*-2-gg + *cis*-2-G; Σ *trans*-Crocins: *trans*-5-tG + *trans*-5-ng + *trans*-4-GG + *trans*-4-ng + *trans*-3-Gg + *trans*-2-g. K-3-β-O-s: kaempferol-3-O-β-sophoroside.

Table 4. NIR statistical parameters for the cross-validation data of the measured *Crocus sativus* L. flower metabolites.

Compound	R ² _{cv}	RMSECV	RMSEC	RPD	RPD _c	RPD _p
Σ <i>cis</i> -Crocins	0.90	0.48	0.11	2.47	2.31	1.25
Σ <i>trans</i> -Crocins	0.84	2.21	4.01	6.51	2.45	1.16
Σ Crocins	0.98	1.24	0.78	8.83	8.83	5.45
Picrocrocin	0.82	3.1	5.2	7.2	9.23	7.27
K-3-O-β-s	0.80	3.46	0.001	2.21	2.18	1.22

R²_{cv}: coefficient of determination in cross validation RMSECV: root mean squared error of cross validation; RMSEC: root mean squared error of calibration; RPD: calculated according to Luo et al. [19]; RPD_c: residual predictive deviation (SD/SECV); RPD_p: SDcal/SEP. Σ *cis*-Crocins: *cis*-4-GG + *cis*-3-Gg + *cis*-2-gg + *cis*-2-G; Σ *trans*-Crocins: *trans*-5-tG + *trans*-5-ng + *trans*-4-GG + *trans*-4-ng + *trans*-3-Gg + *trans*-2-g. K-3-β-O-s: kaempferol-3-O-β-sophoroside.

PLS-R was used to perform the calibration model with the more appropriate pretreatments as above to increase the performance of the predictive models in the selected spectral range, which are shown in Table 3, where different spectral ranges were identified for each model depending on the specific metabolite analyzed. Notably, for the major kaempferol in *Crocus sativus* L. flowers (kaempferol-3-O-β-sophoroside), the entire range of measurements (10,000–4000 cm⁻¹) was considered. However, for crocins and picrocrocin, a narrower spectral range of 9000.9–4000 cm⁻¹ was deemed suitable for calibration and validation.

In the calibration process for picrocrocin, a first derivative with 9 points was applied, followed by smoothing with 5 points. This method resulted in an R²_{cv} value of 82.9%. Similarly, for the sum of crocins, which includes both *cis* and *trans*-crocins, the same spectral range was used. Employing a first derivative with 13 points and a smoothing of 13 points, an R²_{cv} of 98.83% was achieved. However, it is worth noting that individual calibrations were conducted for *cis* and *trans*-crocins, since it is known that their bioactivity is different [27]; so, for this reason, it was considered necessary to perform a separate calibration of them. In this case, the use of higher derivatives and additional smoothing points to mitigate noise during derivation was required. Thus, *trans*-crocins were calibrated within the same spectral range as crocins but with a higher derivative (second with 13 points) and more smoothing (19 points), yielding an R²_{cv} of 84.05%. On the other hand, calibration for *cis*-crocins was also within the same spectral range but with different smoothing (13 points), resulting in an R²_{cv} of 90.81%. If the value obtained for the calibration of the sum of crocins is compared with other calibration values used in the food industry, it can be observed that the R² value obtained in the present work is equal to or even higher than some of the R² values of those calibrations. For example, Kawasaki et al. (2008) [28] obtained a calibration for the fat content of raw milk with a validation R² of 0.95. Another example

is the calibration of the sugar content in pears conducted by Xu, Qi, Sun, Fu, and Ying (2012) [29], which obtained a validation R^2 value of 0.87. Finally, for the calibration of kaempferol-3-O- β -sophoroside, a first derivative with 5 points was applied, followed by smoothing with 13 points, resulting in an R^2_{cv} of 80.51%.

In previous studies related to NIR calibration in saffron spice, good results to determine the content of the main crocins and picrocrocin was achieved [30]. Since saffron flower is a sample containing many more interferences than the spice and has different analyte concentrations due to being a more complex matrix, the calibrations for the spice were not suitable for flower calibration. In the present research, it was started with the range of 10,000–4000 cm^{-1} for sample measurement using NIR, achieving R^2_{cv} values between 0.82 and 0.98.

R^2_{cv} values between 0.6 and 0.8 provide an indication of an approximate predictive model, while values between 0.8 and 0.9 suggest that the model is suitable. However, when the R^2_c surpasses the threshold of 0.9, it can be said that the model is excellent in terms of its predictive capability [31]. The R^2_{cv} value obtained for total crocins' validation was 0.98 (Table 4), suggesting that the method is excellent in terms of predictive capacity. The validations of the sum of *cis*-crocins and the sum of *trans*-crocins resulted in R^2_{cv} values of 0.90 and 0.84, respectively, which suggest a good predictive model, being excellent for *cis*-crocins. Related to picrocrocin, the R^2_{cv} value obtained was 0.82, indicating that the predictive capacity of this method is good, and, for kaempferol-3-O- β -sophoroside, an R^2_{cv} of 0.80 was obtained, also considered indicative of a correct predictive model.

The Ratio of Performance to Deviation (RPD) index, which reports the ratio between the standard deviation of the reference data of the validation set and the standard error of cross-validation prediction or the test set validation, has been employed as an essential measure to assess the accuracy of the model. This parameter is crucial for determining the applicability of the model. Different threshold values for the accuracy of the model given by RPD have been found in the bibliograph. According to Prieto et al. [32], RPD values exceeding 3 are deemed suitable for screening purposes, while those surpassing 5 are considered appropriate for quality control. Values surpassing 8 are deemed exceptional for all analytical tasks. In the validation of the sum of crocins, RPD values exceeding 8 were attained. Similarly, in the validations of picrocrocin and *trans*-crocins, both RPD values exceeded 5, with picrocrocin registering a higher RPD value of 7.27. These results imply that both validations are suitable for a range of applications, including analytical purposes and quality control [32]. However, RPD values obtained for the *cis*-crocins and kaempferol-3-O- β -sophoroside fell below 3, indicating that their predictive capabilities are not as robust as the previous ones. Nonetheless, given their high R^2_{cv} values, such models may still serve a useful purpose in preliminary screening processes.

As observed in Figure 3, differences are observed in the peaks at 7000 cm^{-1} and 5300 cm^{-1} when overlaying the spectra of three selected samples with different concentrations of total crocins. As it can be observed in the two peaks indicated, there was a difference in the area under the curve which could have been due to the differences in the total crocin amounts present in the three samples.

Figure 4 shows scatter plots of the values of different compounds from saffron flower obtained by RP-HPLC-DAD versus predicted by NIR. Regression between laboratory values and those predicted by NIR resulted in good correlations: 0.98 for Σ crocins, 0.96 for Σ *trans*-crocins, 0.85 for Σ *cis*-crocins, 0.95 for picrocrocin, and 0.81 for kaempferol-3-O- β -sophoroside. The differences between results from the different metabolites could not have been due to their concentration in the flower samples, since, although for the crocins the predictive values obtained were good and this can be related to their higher content in the flowers, in the case of the kaempferol-3-O- β -sophoroside, such favorable predictive values were not observed.

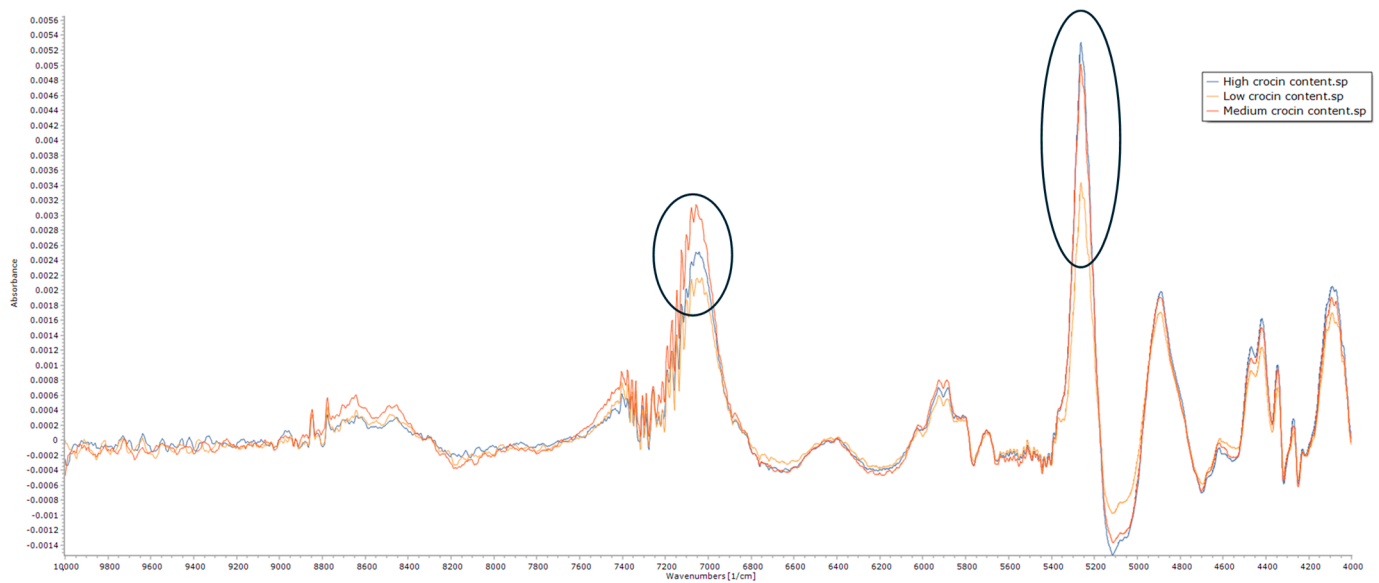


Figure 3. First derivative of 3 spectra with different crocin contents (high, medium, and low). The rounded areas correspond to the peaks with the highest signal.

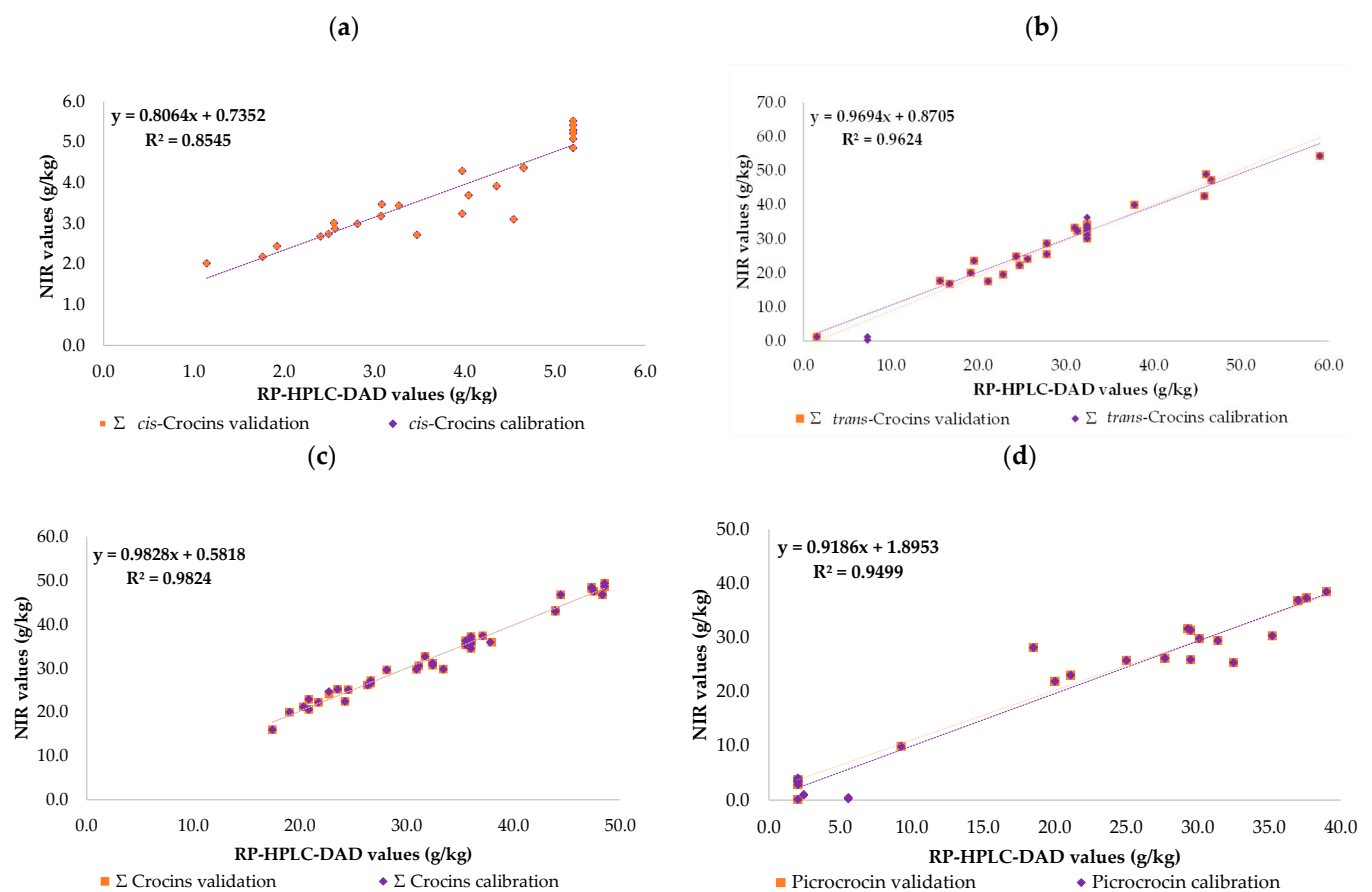


Figure 4. Cont.

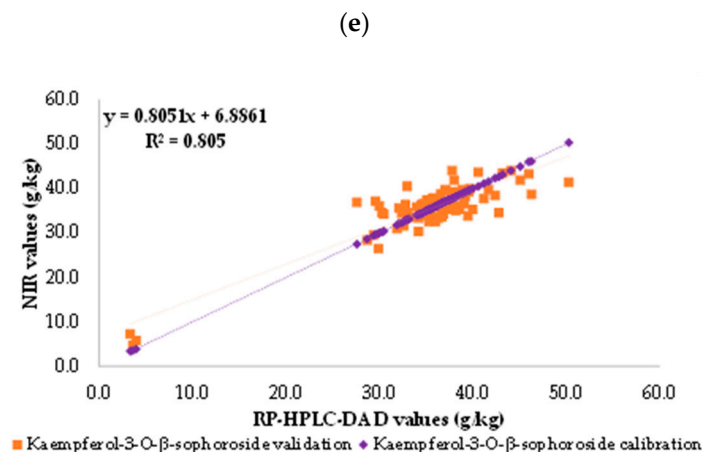


Figure 4. Scatter plot obtained with RP-HPLC-DAD versus values predicted by the NIR in saffron flower samples using PLS-R: (a) Σ *cis*-crocin; (b) Σ *trans*-crocin; (c) Σ crocin; (d) picrocrocin; (e) kaempferol-3-O-β-sophoroside.

4. Conclusions

Good predictive models have been developed in the NIR calibrations of the principal saffron flower metabolites. Excellent predictive models have been obtained for the calibrations of Σ crocin and picrocrocin, with an RPD close to 8, which qualifies the model for quality control purposes. Although the calibrations of Σ *cis*-crocin (*cis*-4-GG + *cis*-3-Gg + *cis*-2-gg + *cis*-2-G), Σ *trans*-crocin (*trans*-5-tG + *trans*-5-ng + *trans*-4-GG + *trans*-4-ng + *trans*-3-Gg + *trans*-2-g), and kaempferol-3-O-β-sophoroside did not achieve as high R^2_{cv} or RPD values, the models are still suitable for screening purposes.

Measuring parameters such as the sum of total crocin or picrocrocin in saffron flower allows for an indirect measurement of corm quality since the metabolites present in the flower originate from the nutrients stored in the corm the previous year.

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