

# Analysis of Vitamin B2 in Saffron Stigmas (*Crocus sativus* L) by Capillary Electrophoresis Coupled with Laser-Induced Fluorescence Detector

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**Abstract** Saffron is the most expensive spice derived from the flower of *Crocus sativus*. The purpose of this study is to determine the vitamin B2 level in saffron using a trustable, easy, and sensitive analytical method and therefore to provide the missing information on the nutritional value of this spice. In our current study, by separating with the fast separation technique capillary electrophoresis (CE) and using a laser-induced fluorescence (LIF) detector which is both sensitive and discriminating from other UV active ingredients, the precise determination of vitamin B2 in saffron was achieved. Saffron samples were extracted by boiling water and directly injected to the separation buffer, borate at pH 9.5. The repeatability of the peak areas (%RSD) of riboflavin for intraday and interday was in the satisfactory range of equal to or less than 2.32 and 4.70 %, respectively. The limit of detection (LOD) and limit of quantification (LOQ) values for the method were 4.15 and 13 nM, respectively. Determination of riboflavin was performed by the standard addition method. Riboflavin contents of five saffron samples from two of the biggest producers in the global market (Iran and Spain) were in the range of 5.02–13.86 µg/g.

**Keywords** Saffron · Riboflavin · Vitamin B2 · Capillary electrophoresis · Laser-induced fluorescence · Determination

## Introduction

Dried stigmas of *Crocus sativus* L. (saffron) are considered to be one of the most expensive spices in the world. In addition to its use as a spice due to its odor, taste, and color, saffron has been used as a folk medicine for a long time. Recently, many reports on the health benefit effects of this valuable spice have been produced. The biological activity of saffron on health problems such as gastric disorders, cardiovascular disease, insulin resistance, depression, premenstrual syndrome, insomnia, anxiety, and anticancer properties has been reviewed by Melnyk et al. (2010). Many studies have shown the pharmacological and biological effects of saffron extracts due to its high antioxidant activity. The studies on the antioxidant activities of saffron were reviewed by Rahaiee et al. (2015). Recently published review articles by Zhang et al. (2013) and Bolhassani et al. (2014) give studies on in vitro and in vivo antitumor effects of saffron.

Consumers have the right and expectation to have the nutritional values of foods determined and revealed. Saffron is a very valuable spice. With the exception of two spectrophotometric studies (Bhat and Broker 1953; Sastry et al. 1955) from the 1950s that claim saffron contains vitamin B2 and that have mutually completely contradictory results, no information exists on saffron. For food samples that contain many ingredients, a trustable result can in fact not be expected for a single analyte from a spectrophotometric study. The results from these two old works have been quoted in two review papers (Sampathu et al. 1984; Rios et al. 1996). One of these results gives a surrealistically high value, in comparison with B2 amounts in food samples that are known to be important B2 sources.

During the last decade, capillary electrophoresis as a separation technique has gained a great popularity in food analysis (Garcia-Canas et al. 2014). Low costs, low sample and reagent consumption, ease of automation, high resolution, and less

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analyzing time are some of the superiorities of this technique. Hyphenation with a laser-induced fluorescence detector has led capillary electrophoresis (CE) to reach higher sensitivities (Başkan et al. 2010; Tezcan et al. 2013; Uzaşçı et al. 2012; Uzaşçı and Erim 2014).

Our work is the first chromatographic study that gives the saffron content of vitamin B2 in a trustable setting. B2 is an essential vitamin that can only be obtained from foods. However, the low vitamin B2 levels in food contents make difficult the quantitative determination of this vitamin. On the other hand, vitamin B2 (riboflavin) has a high fluorescence quantum yield. Because of this characteristic, especially in laser-induced fluorescence (LIF) detectors, low concentration limits can be reached in vitamin B2 detection (Hustad et al. 1999; Pérez-Ruiz et al. 2001; Cataldi et al. 2002, 2003; Hu et al. 2007; Chen et al. 2013).

Our current study has achieved the determination of vitamin B2 in saffron, by separating with the fast separating technique CE and using a LIF detector which is both sensitive and discriminating among other UV active contents.

## Materials and Methods

### Materials

#### Chemicals

Riboflavin and disodium tetraborate decahydrate salt were purchased from Sigma-Aldrich (Steinheim, Germany). Borate buffer was prepared by adjusting to the desired pH with sodium hydroxide or hydrochloric acid. Twenty millimolar borate buffer at pH 9.5 was used as the electrophoretic buffer. All solutions were prepared with water purified by an Elga Purelab Option-7-15 model system (Elga, UK).

#### Plant Samples

Three of the saffron samples in 1-g packages were obtained from local markets in Iran as known commercial brands (I–III). Two Spanish saffron species (IV and V) belonging to the La Mancha region of Spain were purchased in 2-g packages from Amazon.com. According to the claims of providers, all the five samples were first-grade saffron samples.

#### Standard Preparation

Stock standard solution of riboflavin was prepared in the concentration of 50  $\mu\text{M}$  in distilled water. Working standards were prepared daily by gradual dilution of stock standard solution by distilled water in the linear calibration concentration range (0.01–1  $\mu\text{M}$ ). Stock solutions and daily standard solutions are stored in amber glasses. Riboflavin standard

solutions were prepared daily. When any difference in signal intensities of riboflavin standards from the intensity of the working standard of the first day was observed, the stock solution was refreshed.

#### Sample Preparation

The whole dried stigmas of saffron flower in commercial boxes (1 or 2 g) were well grinded and 100-mg portions were extracted with 10 ml of boiling water and then infused for 5 min in water bath. The extracted supernatant was passed through a 45- $\mu\text{m}$  pore-sized microfilters and after appropriate dilution with water injected to the CE-LIF system.

#### Apparatus and Operating Conditions

Agilent 1600 capillary electrophoresis system (Waldbronn, Germany) equipped with a ZETALIF 2000 laser-induced fluorescence detector (Picometrics, Montlaur, France) was used for the determination of riboflavin in the saffron samples. The excitation was performed by a 488 Ar-ion laser and emission at 520 nm. Agilent ChemStation software was utilized for data processing. Injections were made at 50 mbar for 6 s and the separation voltage was set at 25 kV. The temperature was adjusted to 25  $^{\circ}\text{C}$ .

The fused silica capillary obtained from Polymicro Technologies (Phoenix, AZ, USA) was 75  $\mu\text{m}$  I.D. with the total length of 65 cm (50 cm to the detector). Prior to daily use, the capillary column was flushed successively by 1 M NaOH for 15 min, water for 2 min, 0.1 M NaOH for 2 min, water for 2 min, and buffer for 5 min. Between runs, 2 min of flushing with 0.1 M NaOH, 2 min of water, and 5 min of buffer were performed.

## Results and Discussion

### Separation Condition

Riboflavin is a fluorophore, water-soluble compound with a pKa value of 9.69 which can easily turn to a negatively charged molecule in basic solution. Considering the aforementioned characteristic of riboflavin, the desirable peak height, symmetry, and shape for riboflavin were obtained with 20 mM borate buffer at its natural pH value of 9.5. Voltage was set at 25 kV, and it was observed that at this separation condition, there was no significant interference in real samples.

### Method Validation

The calibration curve exhibited a linear relationship between riboflavin concentration and fluorescence intensity in 0.01,

0.05, 0.1, 0.25, 0.5, and 1  $\mu\text{M}$  riboflavin with 0.9931 correlation coefficient. The repeatability of the method was determined by consecutive injections in the same day ( $n=6$ ) and in three different non-consecutive days ( $n=18$ ). The intraday RSD of riboflavin according to corrected peak areas was 2.32 % and the interday RSD was 4.70 %. The limit of detection (LOD) was calculated as three times the average noise taken for three different baseline areas. LOD value of the method for riboflavin was 4.15 nM. Consequently, 10 times the average signal to noise ratio was taken as the limit of quantification (LOQ), and it was 13 nM. The term of precision, given by relative standard deviations for real sample analysis, was between 2.37 and 4.37 %. Results are summarized in Table 1.

### Riboflavin Content of Real Samples

The riboflavin content of saffron extract was found from the established traditional calibration curve by using riboflavin standards. However, the recovery tests did not give satisfactory results. Owing to the fact that there is no blank sample for saffron, the standard addition method was used for assaying the true riboflavin content of samples. Standard addition experiments were performed for the extracts of five saffron samples defined in the “Plant Samples” section. This method involves adding the standards directly to the aliquots of saffron extracts in the three different levels of approximately half, same, and double of the sample concentrations. One portion of each extract was left untreated and then each portion was injected to the capillary. The plots between the added standards and peak areas obtained were extrapolated to  $y=0$  to obtain native analyte concentration in the extracts. Standard additions were repeated two times for each extract. Best straight lines were obtained for every sample by the least square method. The correlation coefficients of all standard addition curves were above ( $R^2$  0.992). Comparison of the outcomes of two experiments, external standard curve and

**Table 1** Analytical performance of the proposed method

Parameter	RF
Intraday precision ( $n=6$ )	
Corrected peak area (RSD %)	2.32
Interday precision ( $n=3 \times 6$ )	
Corrected peak area (RSD %)	4.7
Linearity	
Linear range ( $\mu\text{M}$ )	0.01–1
Coefficient of regression	0.993
LOD (nM)	4.15
LOQ (nM)	13

LOD limit of detection, LOQ limit of quantification, RSD relative standard deviation, RF riboflavin

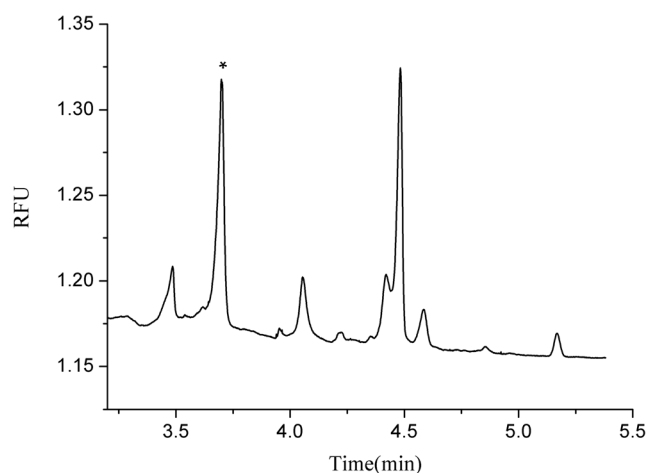
**Table 2** Riboflavin content in real saffron samples

Sample	Mean ( $\mu\text{g/g}$ )	RSD (%)
I	13.86	4.37
II	11.40	2.37
III	10.05	4.25
IV	10.60	2.52
V	5.02	3.36

standard addition method, illustrated that the matrix has an enhancing effect on the fluorescence intensity of riboflavin in real samples. The levels of riboflavin found from the standard addition method in five saffron samples are listed in Table 2. Samples I–III are from Iran and samples IV–V are from Spain.

### Discussion

In the literature, only two very old reports (1953 and 1955) exist on the riboflavin content of saffron samples. The first one was reported by Bhat and Broker (1953). The riboflavin contents of four saffron samples were given as between 56.4 and 138  $\mu\text{g/g}$ . The second report was found in a general review article on saffron by Sampathu et al. (1984), based on the article by Sastry et al. (1955). In that report, the riboflavin contents of two market species from India were given as 3.4 and 5.6  $\mu\text{g/g}$ . The results of the first study were obtained with a spectrofluorometric method applied to saffron extracts. However, as seen from our electropherogram (Fig. 1), saffron extracts contain other fluorophore species together with riboflavin. This might be the main cause of the very high riboflavin content reported. The second data are less than but quite near our results. Since these data were taken from the review



**Fig. 1** Electropherogram of a real sample of saffron. Buffer 20 mM borate at pH 9.5. Injection 50 mbar, 6 s; voltage—25 kV; detection—excitation at 488 nm and emission at 520 nm. RFU relative fluorescence unit. Riboflavin peak is shown with an asterisk

article mentioned above and could not be reached in the original article, the analysis method applied is not known.

Good sources of vitamin B2 are eggs, liver, cereals, and green leafy vegetables. Many analytical reports have been illuminating riboflavin contents of several food stuffs. Riboflavin contents of raw egg white, raw egg yolk, and egg powder were reported by HPLC analysis as 3.27, 2.95, and 12.73  $\mu\text{g/g}$ , respectively (Gliszczynska-Świgło and Koziołowa 2000). HPLC analysis gave 2.45 and 23.4  $\mu\text{g/g}$  riboflavin contents for meat and fresh liver, respectively (Tang et al. 2006). Riboflavin contents of cereal grains were found between 0.17 and 3.63  $\mu\text{g/g}$  by CE-LIF (Chen et al. 2013). Riboflavin contents of several vegetables were reported between 0.34 and 1.67  $\mu\text{g/g}$  by CE-LIF (Cataldi et al. 2003). Riboflavin in mushrooms was found as 5.61–6.69  $\mu\text{g/g}$  by LC (Maria-José Esteve et al. 2001) and in green tea as 2.8–5.4  $\mu\text{g/g}$  by CE-LIF (Hu et al. 2007).

The world's most expensive spice deserves to be known in whole nutritional value. Comparing riboflavin contents of saffron samples of this research with the reported riboflavin contents of other food sources in the literature, we can say that saffron is one of the most riboflavin-rich foods. We believe that in the near future, other reliable techniques for the quantitative assay of riboflavin in saffron samples will support our findings.

## Conclusion

In this study, the vitamin B2 level in saffron was determined using a trustable, easy, and sensitive analytical method, thereby providing the missing information on the nutritional value of this spice. Saffron samples from two of the biggest producers in the global market (Iran and Spain) were analyzed. Our findings show that this valuable spice is a rich source of vitamin B2.

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## Compliance with Ethical Standards

**Funding** This study was funded by the Research Foundation of Istanbul Technical University.

**Conflict of Interest** F. Bedia Erim declares that she has no conflict of interest. Parya Hashemi declares that she has no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Not applicable.

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