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# Geographical characterization by MAE-HPLC and NIR methodologies and carbonic anhydrase inhibition of Saffron components

Riccardo Nescatelli<sup>a\*</sup>, Simone Carradori<sup>b</sup>, Federico Marini<sup>a</sup>, Remo Bucci<sup>a</sup>, Celeste De Monte<sup>c</sup>, Adriano Mollica<sup>b</sup>, Luisa Mannina<sup>c,d</sup>, Mariangela Ceruso<sup>e</sup>, Claudiu T. Supuran<sup>e,f</sup>, Daniela Secci<sup>c</sup>

<sup>a</sup>*Dipartimento di Chimica, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy.*

<sup>b</sup>*"G. D'Annunzio" University of Chieti-Pescara, Department of Pharmacy, Via dei Vestini 31, 66100 Chieti Scalo (CH), Italy.*

<sup>c</sup>*Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy.*

<sup>d</sup>*Istituto di Metodologie Chimiche, Laboratorio di Risonanza Magnetica "Annalaura Segre", CNR, via Salaria km 29.300, 00015 Monterotondo, Rome, Italy.*

<sup>e</sup>*Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, 50019, Florence, Italy.*

<sup>f</sup>*Università degli Studi di Firenze, Neurofarba Department, Section of Pharmaceutical and Nutraceutical Sciences, 50019, Sesto Fiorentino (Florence), Italy.*

## Abstract

A microwave-assisted extraction method was optimized for the recovery of bioactive compounds from *Crocus sativus* L. stigmas using water/ethanol mixture with respect to ISO 3632-2 procedure. HPLC-DAD was employed to evaluate pros and cons of the extraction parameters, namely, solvent type and volume and the duration of the procedure. Microwave-assisted extraction speeded up recovery of these active principles limiting extraction time and solvent waste. Additional NIR experiments were performed to compare spectra in pseudo-absorbance of Saffron samples from different geographical origins by applying chemometric techniques. Biological activity of crocin, safranal and its semisynthetic derivatives as inhibitors of five isoforms of human carbonic anhydrase was also explored.

*Keywords:* *Crocus sativus* L.; hCA inhibitors, microwave-assisted extraction, experimental design, NIR spectroscopy, geographical origin, HPLC-DAD, principal component analysis.

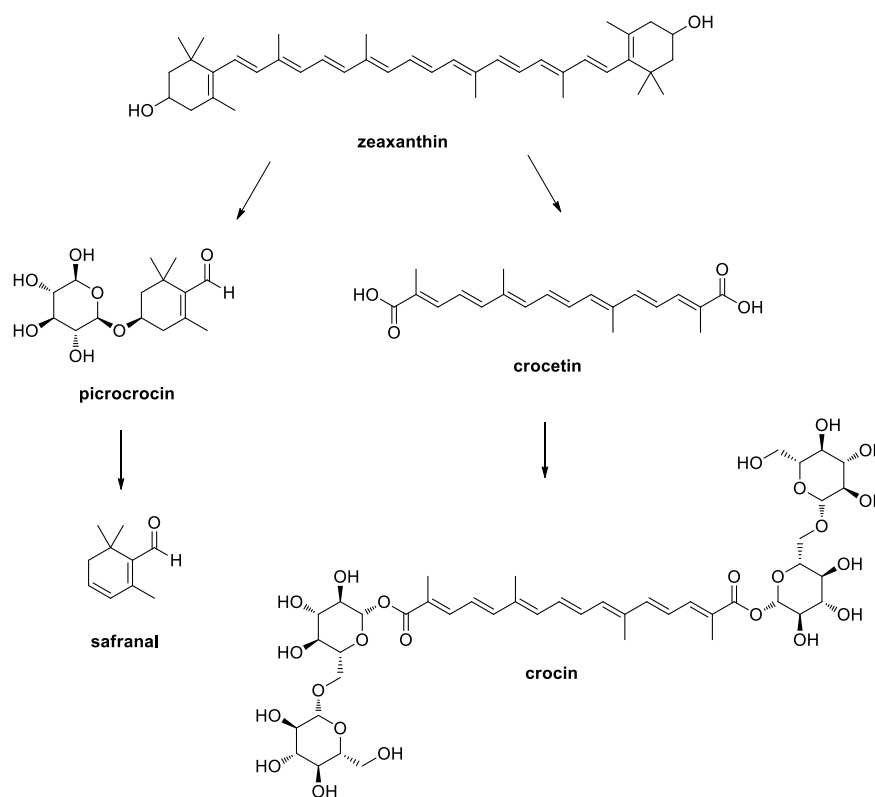
\*Corresponding author: Tel/fax: +39 06 49693242; e-mail: riccardo.nescatelli@uniroma1.it.

### **Highlights**

- Optimization of the simultaneous recovery of crocin, picrocrocin and safranal by HPLC-DAD after microwave extraction
- NIR spectroscopy was applied to Saffron of different geographical origins
- A chemometric method of classification faster than HPLC-DAD analysis was developed
- Biological activity against five isoforms of human carbonic anhydrase was evaluated

## Introduction

Saffron spice, represented by the dried red stigmas of *Crocus sativus* L., is the most expensive spice in the world being already known by ancient people for its characteristic aroma, flavor and taste. The general chemical composition of this spice is known (Pfander et al., 1982). Among the various substances which are present in this natural product, those which mostly attracted the interest are secondary metabolites endowed with promising biological activities: crocin, crocetin, picrocrocin and, the most important component of the volatile fraction, safranal. They are derived from a single precursor, zeaxanthin, after enzymatic cleavage. The compounds that infer colour hues are apocarotenoids (especially crocin 1, *trans*-crocetin (di- $\beta$ -D-gentiobiosyl) ester), whereas the bitter taste and aroma are mainly given by the monoterpene glycoside picrocrocin (4-( $\beta$ -D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexane-1-carboxaldehyde) and the volatile fraction components, *i.e.*, safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) (Figure 1).



**Figure 1.** Biosynthesis of the major components extracted from *Crocus sativus* L. stigmas.

Saffron is produced in many countries of the Mediterranean basin (Spain, Italy, Morocco, Greece, France) and southern Asia (Iran, India). The determination of the geographical origin plays an important role for this product and its quality. Other minor producers are Arzerbaijan, Iraq, Syria, Jordan, Egypt (the latter three are still following the traditional crop), Kashmir, Hungary, China, Lebanon (one of the oldest manufacturers) and Switzerland (Hosseinzadeh et al., 2013).

Dry Saffron and most often its aqueous and alcoholic extracts are well known in the food industry to enrich a variety of products as flavouring and colouring agents. In addition, several experimental studies shed light on therapeutic applications and toxicity of Saffron and its major components (Melnyk et al., 2010).

Due to its high price and limited cultivation yield, Saffron production and commerce give rise to several problems of sophistication and adulterations which must be markedly contrasted. The chemical composition of Saffron is the most important indicator of its quality and of its commercial value in agreement with ISO 3632-1 and -2 (ISO Technical Specifications 3632-1/2. Saffron (*Crocus sativus* L.) Part 1: Specifications, and Part 2: Test methods. 2003). The International Organization for Standardization (ISO) has defined the quality of Saffron establishing three different categories (I, II and III) and the procedures for their assessment.

The quality of Saffron depends on many factors, such as climate and soil of the area of production, harvesting and drying. The drying process represents the most critical phase of its production due to the registered differences among the producing countries. India, Iran and Morocco usually expose Saffron stigmas to direct sunlight, whereas Greece, Italy and Spain prefer drying processes indoor at higher temperature than ambient one. That's what has the strong impact on the amount of active principles and then on the quality of Saffron, due to the volatility and photodegradation processes.

Among the factors related to the quality of Saffron, the determination of crocin, picrocrocin and safranal are the most important parameters. In the literature, different extraction techniques have been reported, on the basis of the component to be extracted. Recovery of the bioactive compounds from Saffron is affected by more than one factor, such as extraction technique and solvent, temperature and length of the process, ground stigmas particle size and location of the compounds in the cell. Depending on aim of the study, large arrays of polar and non polar solvents as well as their mixtures have been used so far for the extraction of Saffron metabolites (Jiang et al., 2014).

The classical extraction method, described by ISO 2003, provides 0.5 g of Saffron using H<sub>2</sub>O (1 L) under magnetic stirring for 1 hour at room temperature for the extraction of crocin, picrocrocin, safranal. In the literature, there are also articles reporting ultrasonic extraction (Jalali-Heravi et al., 2009).

Conversely, microwave-assisted extraction, which has been successfully applied to the extraction of a wide range of hydrophilic and hydrophobic plant bioactive components in shorter time and at a low cost, has found limited use in the recovery of Saffron bioactive constituents till now. Furthermore, microwave-assisted extraction could provide better isolation of the secondary metabolites after the complete swelling of the subcellular structures, reducing solvent waste and allowing an accurate control of different parameters (Zhang et al., 2011).

Analytical techniques, being *a posteriori* techniques, are essential for food safety, food quality control and for the traceability and authentication of food products. In addition they are effective internationally and constitute a valid tool in order to prevent frauds and to confirm the authenticity of products. The most commonly used analytical techniques for food authentication and traceability are spectroscopic and chemometric ones, especially if used in combination (Moore et al., 2012).

For quality control of Saffron, different parameters and evaluation methods based on different principles are chosen. In particular, as far as the authentication of the quality of Saffron, rapid and accurate controls methods are usually preferred, through the use of NIRS (near-infrared spectroscopy, range 12500 and 4000  $\text{cm}^{-1}$ ) and HPLC (high performance liquid chromatography) but their choice depends on many factors (Scot et al., 1994; Sikorski, 1996; Springett, 2000).

The electromagnetic radiation used is of different frequency, the response of the instrument consists of absorption bands due to chemical compounds, that can be observed, in the spectral regions of the NIR, as a result of molecular vibrations of these compounds thus giving rise to spectral signatures which are characteristic of the food composition and which may be considered as “fingerprints” of the food (Pizarro et al., 2013; Bevilacqua et al., 2012).

Chromatographic methods are also widely used for the measurement of the ‘fingerprints’ of foodstuffs (Gutiérrez et al., 2012; Gao et al., 2012). High performance liquid chromatography

provides high-resolution compound separations and can be used in conjunction with different detectors such as a diode array detector (DAD).

However, the term “fingerprinting techniques” describes a variety of analytical methods that can measure the composition of foodstuffs in a non-selective way such as by collecting a spectrum or a chromatogram. Mathematical processing and classification models of the large volume of information contained in such fingerprints may permit the characterization of foodstuffs (Brereton, 2010).

As a matter of this, the first part of this work is focused on the development of an analytical method which could allow a fast, cheap and reliable analysis of the Saffron permitting to control and to guarantee the quality of the product. Therefore, a procedure for the determination of crocin, picrocrocin and safranal, after the microwave-assisted extraction (MAE) of the samples followed by HPLC-DAD analysis has been developed and optimized. For comparison, these results will be compared to those obtained by NIR spectroscopy.

The second part of this work analyzed the biological activity of crocin, safranal and some semisynthetic derivatives of safranal as inhibitors of five isoforms of human carbonic anhydrase (hCA) due to the importance of such natural products as biologically active metabolites and as lead compounds for the design of new chemical entities (Moshiri et al., 2014).

## **Materials and methods**

### *Chemicals and standards*

Commercial standards of all *trans*-crocin and safranal (>88%) were purchased from Sigma-Aldrich (Italy), and picrocrocin (>98%) from Biotain PHARMA CO.LTD (China). Safranal was purified by column chromatography on silica gel (230-400 mesh, G60 Merck) using ethyl acetate:*n*-hexane (1:3) as the eluent. <sup>1</sup>H, <sup>13</sup>C NMR and IR spectra of the purified product were in agreement with those reported in the literature. 4-Nitroaniline, used as internal standard (IS), was purchased from

Sigma-Aldrich (Italy). HPLC grade (<99.9%) methanol and ethanol were purchased from Carlo Erba (Italy). Ultrapure water generated by the MilliQ system (Millipore, Bedford, MA) was used.

One hundred six samples were collected from the best representative leading producers with particular interest towards Italian Saffron. In particular, 20 samples from Greece, 25 samples from Turkey, 19 samples from Latium (Italy), 19 samples from Sardinia (Italy), 23 samples from Spain were collected. They were obtained under the guarantee of their quality in according to ISO 3632 guidelines.

#### *Sample preparation*

Dried Saffron stigmas (10 mg) were ground manually with an agate pestle and mortar and passed through a 0.4 mm sieve just before further analysis.

#### *Experimental design for the selection of microwave-assisted extraction conditions*

Microwave-assisted extraction was performed by using an automatic Biotage Initiator™ 2.0 (Uppsala, Sweden). The internal vial temperature is strictly controlled by an IR sensor probe. Saffron ground stigmas (about 10 mg) were placed in a 10 mL sealed vessel suitable for an automatic single-mode microwave reactor (2.45 GHz high-frequency microwaves, power range 0-300 W) and the proper amount of solvent was added to the sample to form an orange suspension. The mixture was pre-stirred magnetically for 10 s and then heated by microwave irradiation for 1, 10 and 19 min at 40 °C (irradiation power reaches its maximum at the beginning of reaction, then it decreases to lower and quite constant values). After cooling with pressurized air, each extract was filtered through a PTFE filter (0.45 mm; Whatman-Merck, Darmstadt, Germany) and subsequently analyzed by HPLC-DAD.

#### *HPLC-DAD analysis*

The extracts were analyzed by HPLC-DAD with a Thermo Quest Spectrasystem LC (Thermo Fisher Scientific, Waltham, MA) equipped with a P4000 pump, a UV6000 UV-Vis Diode Array Detector,

and a SN4000 interface to be operated via a personal computer. Extracted compounds were separated using an Eclipse XDB-C18 analytical column (4.6x250 mm, 5  $\mu\text{m}$  particle size; Agilent Technologies, Santa Clara, CA) protected by a guard cartridge of the same packing, operating at 25  $^{\circ}\text{C}$ . Separation was carried out using gradient elution with a mixture of water (A):acetonitrile (B) (30–70% B in 20 min) at a flow rate of 0.9  $\text{mL min}^{-1}$ . Injection volumes were 10  $\mu\text{L}$  for all samples and standards. Multiwavelength detection was in the range of 200–550 nm and quantification was carried out by integration of the peak areas at 250 nm (picrocrocin), 310 nm (safranal) and 440 nm (crocin).

#### *NIR analysis*

For the acquisition of spectra in the near infrared range, a Nicolet 6700 FT-NIR instrument (Thermo Scientific Inc., Madison, WI), equipped with a tungsten–halogen source and an InGaAs detector, was used. The spectra were acquired at room temperature and without any further sample treatment, in reflectance mode, through the use of an integrating sphere (Thermo Scientific Inc., Madison, WI). Operationally, for the acquisition of each spectrum, the proper amount of Saffron (approximately 10 mg) was placed inside a cylindrical glass sample holder (19 mm internal diameter, 2.7 cm in height), which was then positioned on the hole of the integrating sphere. The spectra were acquired between 10,000 and 4000  $\text{cm}^{-1}$ , collecting 82 scans at a nominal resolution of 4  $\text{cm}^{-1}$ . For each sample four spectra were acquired for a total of 424 spectra (106\*4). The data were then exported from Omnic Suite software (Thermo Fisher Scientific Inc., Waltham, MA) as an ASCII file, which was then imported into MATLAB (release R2009b, The MathWorks Inc., Natick, MA), for the successive chemometric analysis.

#### *Statistical analysis*

The chromatographic and spectroscopic data were processed with different chemometric methods. The MAE-HPLC-DAD results collected on the available samples were also used to build a model to

discriminate the 5 classes of samples (Greece, Latium, Sardinia, Spain, Turkey). The method used for discriminant classification was linear discriminant analysis (LDA) (Fisher, 1936). Aim of Linear Discriminant Analysis is to find one or more linear combinations of parameters which allow to discriminate optimally the various groups of samples (Greece, Latium, Sardinia, Spain, Turkey). In this way it is possible to assign an observation (sample) in a given class on the basis of measurements of crocin, picrocrocin and safranal.

### *Chemistry*

The semisynthetic compounds (**1-7**) have been prepared by us as reported in a previous paper (De Monte et al., 2014).

### *CA inhibition assay*

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various human CA isozymes (I, II, V(A), IX and XII) as previously reported by us [De Monte et al., 2014]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nM, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na<sub>2</sub>SO<sub>4</sub> or NaClO<sub>4</sub> (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration), following the CA-catalyzed CO<sub>2</sub> hydration reaction for a period of 5-10 s. Saturated CO<sub>2</sub> solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO-water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. At least 7 different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least squares methods using PRISM 3, from Lineweaver-Burk plots as

reported earlier (D'Ascenzio et al., 2014) and represent means from at least three different determinations.

## **Results**

### *Optimization of microwave-assisted extraction of Saffron*

Microwave-assisted extraction parameters were selected by applying a simple experimental design, supported by HPLC-DAD, for designing experiments and building predictive models through the evaluation of the effects of several factors. To the best of our knowledge, there is no published report on the use of MAE for the concomitant recovery of crocin, safranal and picrocrocin from Saffron. To reach our goal, at the beginning we tested different extraction solvents (methanol, acetone, diethyl ether, dichloromethane, ethanol, ethyl acetate, methanol:water 50:50, ethanol:water 50:50) applying variable microwave irradiation (40 °C) for ten minutes to different samples (10 mg in 10 mL of solvent). From this screening and comparing the results to classic extraction, we selected the four best solvents to be applied to all samples of Saffron (methanol, ethanol, methanol/water 50:50, ethanol/water 50:50). HPLC-DAD was employed to follow pros and cons of the process.

Two of the parameters to be set to perform the microwave-assisted extraction are the temperature of the extraction solvent and the irradiation power. In order to avoid thermal degradation of the analytes, after preliminary tests carried out at different temperatures, it has been decided to set a constant temperature of 40 °C for the entire duration of the extraction. In addition to setting the temperature and the irradiation power, the extraction time must also be regulated. In particular, 3 levels were considered for the extraction time: 1 minute, 10 minutes and 19 minutes. Also, the following solvents were tested: MeOH, EtOH and mixtures H<sub>2</sub>O:MeOH=50:50 and H<sub>2</sub>O:EtOH=50:50. Lastly, for each solvent, the influence of the extraction volume was also tested by considering two different levels: 2 mL and 10 mL of solvent.

In order to optimize the extraction time, the type and volume of solvent, a full factorial design with 24 experiments ( $3 \times 4 \times 2 = 24$ ) was carried out. In Table 1 we schematically showed all the experimental variables and the levels considered for each of them.

**Table 1.** Experimental variables and levels considered.

| <b>Solvent</b>                  | <b>Extraction time</b> | <b>Solvent volume</b> |
|---------------------------------|------------------------|-----------------------|
| Ethanol (e)                     | 1 minute (-1)          | 2 mL (-1)             |
| Methanol (m)                    | 10 minutes (0)         | 10 mL (1)             |
| Methanol:H <sub>2</sub> O (m/w) | 19 minutes (1)         |                       |
| Ethanol:H <sub>2</sub> O (e/w)  |                        |                       |

The operating conditions for the microwave-assisted extraction of the analytes from Saffron samples were optimized using an experimental design. In particular, for each of the 24 designed experiments (for all extraction tests the same Saffron sample was used), the extracts were subjected to HPLC analysis and the area of safranal, crocin and picrocrocin were integrated. Obviously, each area was normalized according to the area of the internal standard added (10 µg I.S./10 mg of Saffron) before each extraction. The conditions were optimized to maximize both the quantity of each analyte extracted in general and to maximize the concentration of each analyte extracted in the extracting solution. Indeed, on one hand it is important to assess the efficiency of the process in order to develop a procedure able to extract as much crocin, picrocrocin and safranal as possible. On the other hand, it is important to maximize the concentration of the analytes in the extract, to be able to analyze very dilute solutions and analytes in trace. In fact, even if the developed method allows to extract almost all of the analytes using big volume of solvents, often it is necessary to add a step of concentration. In this specific case, however, it is not possible to make a concentration of the sample extract. In fact the use of a rotavapor or nitrogen flow to facilitate the elimination of the solvent leads to a significant loss of the more volatile compounds, such as safranal.

Tables 2 and 3 showed the normalized area (max=1) relative to safranal, crocin and picrocrocin. To determine which of the experiments was the best to extract the maximum quantity of safranal, crocin and picrocrocin, the area of each analyte was normalized in function of sample weight and area of the internal standard (10 µg I.S./10 mg of Saffron). Then these areas (for each analyte) were normalized to 1.

**Table 2.** Normalized quantity of extracted safranal, picrocrocin and crocin; 1.00 means maximum quantity in the extract.

| Extraction solvent             | Solvent volume (mL) | Extraction time (min)<br>Area norm. safranal |      |      | Extraction time (min)<br>Area norm. picrocrocin |      |      | Extraction time (min)<br>Area norm. crocin |      |      |
|--------------------------------|---------------------|--|------|------|---|------|------|--|------|------|
|                                |                     | 1  | 10   | 19   | 1   | 10   | 19   | 1  | 10   | 19   |
| EtOH                           | 10                  | 0.68   | 0.66 | 0.66 | 0.08  | 0.09 | 0.09 | 0.13                                       | 0.13 | 0.14 |
|                                | 2                   | 0.61   | 0.63 | 0.61 | 0.04  | 0.07 | 0.10 | 0.07                                       | 0.11 | 0.15 |
| MeOH                           | 10                  | 0.73   | 0.68 | 0.68 | 0.75  | 0.70 | 0.69 | 0.71                                       | 0.81 | 0.78 |
|                                | 2                   | 0.68   | 0.63 | 0.63 | 0.65  | 0.61 | 0.58 | 0.61                                       | 0.71 | 0.68 |
| MeOH:H <sub>2</sub> O<br>50:50 | 10                  | 0.88   | 0.83 | 0.80 | 1.00  | 0.99 | 0.90 | 0.93                                       | 1.00 | 0.86 |
|                                | 2                   | 0.80   | 0.78 | 0.76 | 0.94  | 0.91 | 0.82 | 0.86                                       | 0.92 | 0.80 |
| EtOH:H <sub>2</sub> O<br>50:50 | 10                  | 1.00   | 0.93 | 0.90 | 0.93  | 0.91 | 0.85 | 0.76                                       | 0.82 | 0.78 |
|                                | 2                   | 0.93   | 0.85 | 0.85 | 0.85  | 0.82 | 0.78 | 0.73                                       | 0.82 | 0.78 |

Table 2 showed that the best MAE to reach the greatest amount of safranal from sample is obtained extracting for 1 minute and using 10 mL EtOH:H<sub>2</sub>O=50:50, for crocin extracting for 10 minutes and using 10 mL MeOH:H<sub>2</sub>O=50:50 and for picrocrocin extracting for 1 minute and using 10 mL MeOH:H<sub>2</sub>O=50:50.

Instead, to maximize the concentration we need to take into account the volume of solvent. Table 3 refers to the maximization of concentration of the analytes in each extract. The area of each analyte is divided by the area of the internal standard (10 µg I.S./10 mg of Saffron) and by the volume of extracting solvent used for extraction (areas are normalized to 1; max. area = 1).

**Table 3.** Normalized concentration of extracted safranal, picrocrocin, and crocin; 1.00 means maximum concentration in the extract.

| Extraction solvent             | Solvent volume (mL) | Extraction time (min)<br>Area norm. safranal |      |      | Extraction time (min)<br>Area norm. picrocrocin |      |      | Extraction time (min)<br>Area norm. crocin |      |      |
|--------------------------------|---------------------|--|------|------|---|------|------|--|------|------|
|                                |                     | 1  | 10   | 19   | 1   | 10   | 19   | 1  | 10   | 19   |
|                                |                     | EtOH   | 10   | 0.15 | 0.14  | 0.14 | 0.02 | 0.02                                       | 0.02 | 0.03 |
| 2                              | 0.66                |  | 0.68 | 0.66 | 0.05  | 0.08 | 0.11 | 0.08                                       | 0.12 | 0.16 |
| MeOH                           | 10                  | 0.16   | 0.15 | 0.15 | 0.16  | 0.15 | 0.15 | 0.15                                       | 0.18 | 0.17 |
|                                | 2                   | 0.74   | 0.68 | 0.68 | 0.69  | 0.65 | 0.62 | 0.67                                       | 0.77 | 0.74 |
| MeOH:H <sub>2</sub> O<br>50:50 | 10                  | 0.19   | 0.18 | 0.17 | 0.21  | 0.21 | 0.19 | 0.20                                       | 0.22 | 0.19 |
|                                | 2                   | 0.87   | 0.84 | 0.82 | 1.00  | 0.97 | 0.88 | 0.93                                       | 1.00 | 0.88 |
| EtOH:H <sub>2</sub> O<br>50:50 | 10                  | 0.22   | 0.20 | 0.19 | 0.20  | 0.19 | 0.18 | 0.17                                       | 0.18 | 0.17 |
|                                | 2                   | 1.00   | 0.92 | 0.92 | 0.91  | 0.88 | 0.83 | 0.80                                       | 0.89 | 0.84 |

The MAE procedure which allows obtaining the highest concentration of safranal in the extract corresponds to extraction for 1 minute and using 2 mL of EtOH:H<sub>2</sub>O. The MAE procedure which allows to obtain the highest concentration of crocin in the extract corresponds to extraction for 10 minutes and using 2 mL MeOH:H<sub>2</sub>O=50:50. Finally, the MAE procedure which allows obtaining the highest concentration of picrocrocin in the extract corresponds to extraction for 1 minute and using 2 mL MeOH:H<sub>2</sub>O=50:50. As can be noted we must use different mixtures of solvents (MeOH:H<sub>2</sub>O for crocin and picrocrocin, EtOH:H<sub>2</sub>O for safranal) and different extraction time (1 minute for safranal and picrocrocin, 10 minutes for crocin) depending on which compound we want to optimize.

Regarding the volume of extracting solvent, the use of 2 mL of solvent allows to obtain much higher concentrations of the analytes than using 10 mL. Indeed the use of 10 mL, even if the volume is 5 times greater than 2 mL, does not lead to a comparable increase in the amount of analytes extracted. On the basis of these results, for the analysis of safranal, crocin and picrocrocin in available Saffron samples, it was decided to use the best conditions for the extraction of safranal (2 mL EtOH:H<sub>2</sub>O and 1 minute of extraction) because they provided a limited solvent waste balanced by a suitable

extract concentration, reduced extraction times limiting the degradation of the active principles and the best recovery of safranal which is normally present in very low concentrations.

*Validation of HPLC-DAD analysis of the Saffron extracts*

The developed method, based on the microwave-assisted extraction, was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), reproducibility and recovery (Table 4).

**Table 4.** Validation parameters – 2 mL EtOH:H<sub>2</sub>O 50:50 and 1 min extraction time.

| compound    | linearity (µg/mL)                | LOD (µg/mL) | LOQ (µg/mL) | reproducibility (RSD%) <sup>a</sup> | recovery (%) <sup>b</sup> |
|-------------|----------------------------------|-------------|-------------|-------------------------------------|---------------------------|
| safranal    | 0.5–55<br>R <sup>2</sup> =0.9999 | 0.15        | 0.50        | <3                                  | 87 ± 2                    |
| picrocrocin | 0.5–50<br>R <sup>2</sup> =0.9999 | 0.15        | 0.50        | <3                                  | 80 ± 2                    |
| crocin      | 2.0–85<br>R <sup>2</sup> =0.9999 | 0.15        | 0.50        | <3                                  | 68 ± 2                    |

<sup>a</sup>Intermediate precision determined by different analysts on six separate weeks; <sup>b</sup>N=6.

As it is possible to see, crocin recovery due to the short extraction time is not so high; however, this analyte is still extracted in large amount thanks to its abundant presence in this spice. The calibration curves for safranal, crocin and picrocrocin are reported in the Supporting Information. Since analyte-free Saffron does not exist, LOD and LOQ were calculated as the average signal plus, respectively, 3 times and 10 times the standard deviation of a solution of EtOH: H<sub>2</sub>O = 50:50.

LOD=0.15 µg/mL; LOD=30 µg/g

LOQ=0.50 µg/mL; LOQ=100 µg/g

An RSD%<2 indicated that repeatability of procedure was satisfactory (N=6). Intermediate precision determined by different analysts on six separate weeks was also found satisfactory (RSD%<3).

Test for recoveries were performed on three different Saffron samples (1 from Greece, 1 from Sardinia and 1 from Latium) comparing different extraction methods, such as ISO 2003 method.

Specifically the following extractions were compared for the recovery of safranal:

- a) One cycle of extraction with 2 mL (1x2 mL) of EtOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- b) One cycle of extraction with 10 mL (1x10 mL) of EtOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- c) Two cycles of extraction with 10 mL (2x10 mL) of EtOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- d) Three cycles of extraction with 10 mL (3 x 10 mL) of EtOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- e) ISO 2003 Method: 0.5 g Saffron in 1 L of H<sub>2</sub>O, room temperature, with stirring for 1 hour.

Tests were carried out with more extraction cycles to assess the efficiency and relative recoveries for safranal. Furthermore the different methods were compared with ISO 2003 method. Comparative results are reported in Supporting Information (Table S4).

The following extractions were compared for the recovery of picrocrocin:

- a) One cycle of extraction with 2 mL (1x2 mL) of MeOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- b) One cycle of extraction with 10 mL (1x10 mL) of MeOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- c) Two cycles of extraction with 10 mL (2x10 mL) of MeOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- d) Three cycles of extraction with 10 mL (3x10 mL) of MeOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.
- e) ISO 2003 Method: 0.5 g Saffron in one liter of H<sub>2</sub>O, room temperature, with stirring for 1 hour.

- f) One cycle of extraction with 2 mL (1x2 mL) of EtOH:H<sub>2</sub>O=50:50; 1 minute per cycle; 10 mg Saffron.

Tests were carried out with more extraction cycles to assess the efficiency and relative recoveries for picrocrocin. Furthermore the different methods were compared with ISO 2003 method. Comparative results are reported in Supporting Information (Table S5).

The following extractions were compared for the recovery of crocin:

- a) One cycle of extraction with 2 mL (1x2 mL) of MeOH:H<sub>2</sub>O=50:50; 10 minutes per cycle; 10 mg Saffron.
- b) One cycle of extraction with 10 mL (1x10 mL) of MeOH:H<sub>2</sub>O=50:50; 10 minutes per cycle; 10 mg Saffron.
- c) Two cycles of extraction with 10 mL (2x10 mL) of MeOH:H<sub>2</sub>O=50:50; 10 minutes per cycle; 10 mg Saffron.
- d) Three cycles of extraction with 10 mL (3x10 mL) of MeOH:H<sub>2</sub>O=50:50; 10 minutes per cycle; 10 mg Saffron.
- e) ISO 2003 Method: 0.5 g Saffron in one liter of H<sub>2</sub>O, room temperature, with stirring for 1 hour.
- f) One cycle of extraction with 2 mL (1x2 mL) of EtOH:H<sub>2</sub>O=50:50; 10 minutes per cycle; 10 mg Saffron.

Tests were carried out with more extraction cycles to assess the efficiency and relative recoveries for crocin. Furthermore the different methods were compared with ISO 2003 method. Comparative results are reported in Supporting Information (Table S6).

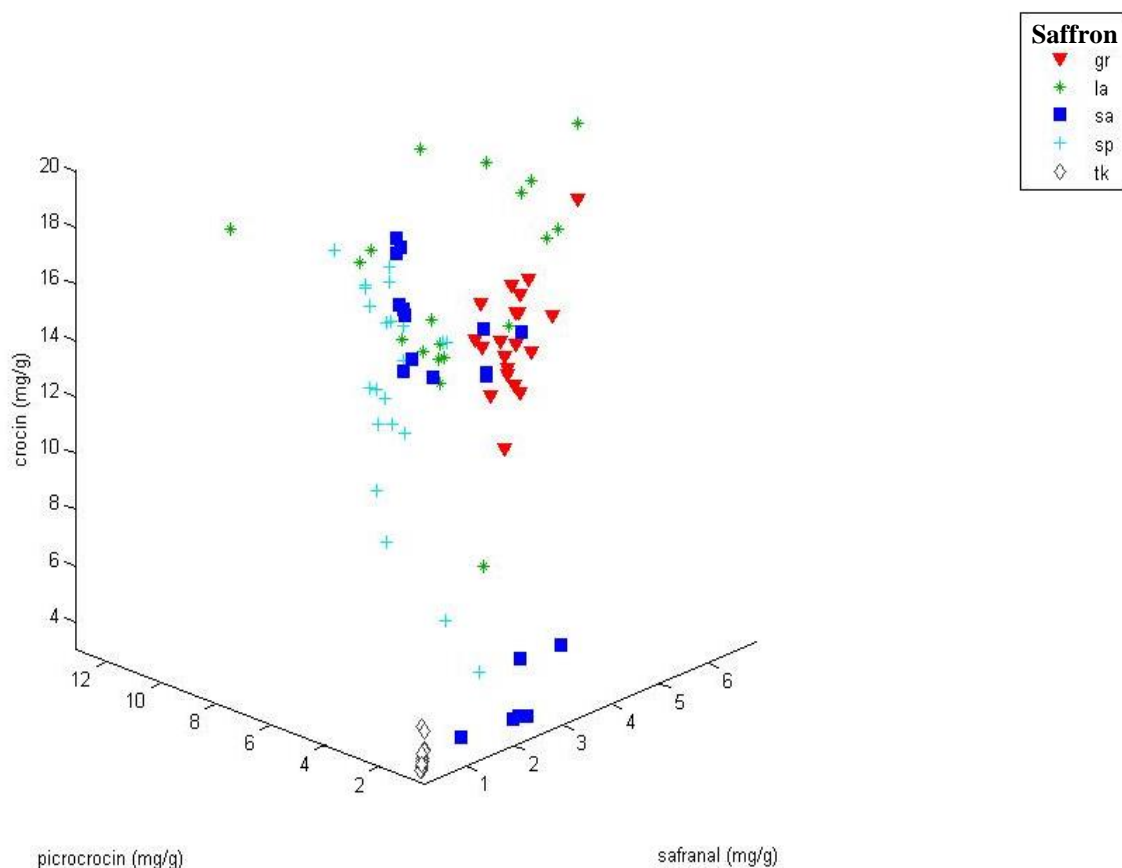
## **Discussion**

A microwave-assisted extraction system for biologically active compounds has many advantages over other conventional extraction methods. Microwave-assisted extraction methods required

shorter time, less solvents, providing higher extraction rates and better products with lower costs. The method (previously described and validated), which allows to obtain the maximum concentration of safranal in the extract, was then applied for the determination of constituents related to the quality of Saffron (safranal, picrocrocin and crocin) in all the available samples.

10 mg of ground Saffron were placed in a 10 mL sealed vessel suitable for an automatic single-mode microwave reactor and 2 mL of EtOH:H<sub>2</sub>O were added to the sample. The extraction temperature was set at 40 °C and the extraction time to 1 minute. The extract was then filtered through a PTFE filter and 10 µL were injected into the HPLC system. Under these experimental conditions, we obtained high recovery for safranal which is normally present in very low concentrations.

The 106 Saffron samples were analyzed by MAE-HPLC-DAD and plotted in the space of experimental variables (safranal, crocin and picrocrocin). Figure 2 shows the distribution of the analyzed Saffron samples. In Supporting Information (Table S7) results for each sample are reported.



**Figure 2.** Representation of the 106 samples in the space of the variables (safranal, crocin and picrocrocin).

Figure 2 shows how the Turkish (tk) samples are grouped in a clearly limited area and that they are relatively far from all the other samples produced in other geographical areas such as Greece (gr), Spain (sp) and the Italian districts Latium (la) and Sardinia (sa). The graphical representation of the results also shows how the Greeks samples and the Spanish samples are well separated in the space of the three experimental variables.

In Table 5 we report the error in cross-validation evaluated by linear discriminant analysis for each distinct Saffron.

**Table 5.** LDA of Saffron samples; the quantity (mg/g) of safranal, crocin and picrocrocin were used; cross validation (CV)= venetian blinds w/5 splits.

|                                  | <b>Greece</b> | <b>Latium</b> | <b>Sardinia</b> | <b>Spain</b> | <b>Turkey</b> |
|----------------------------------|---------------|---------------|-----------------|--------------|---------------|
| <b>Error in Cross-Validation</b> | 1             | 13            | 13              | 4            | 0             |
| <b>number of samples</b>         | 20            | 19            | 19              | 23           | 25            |

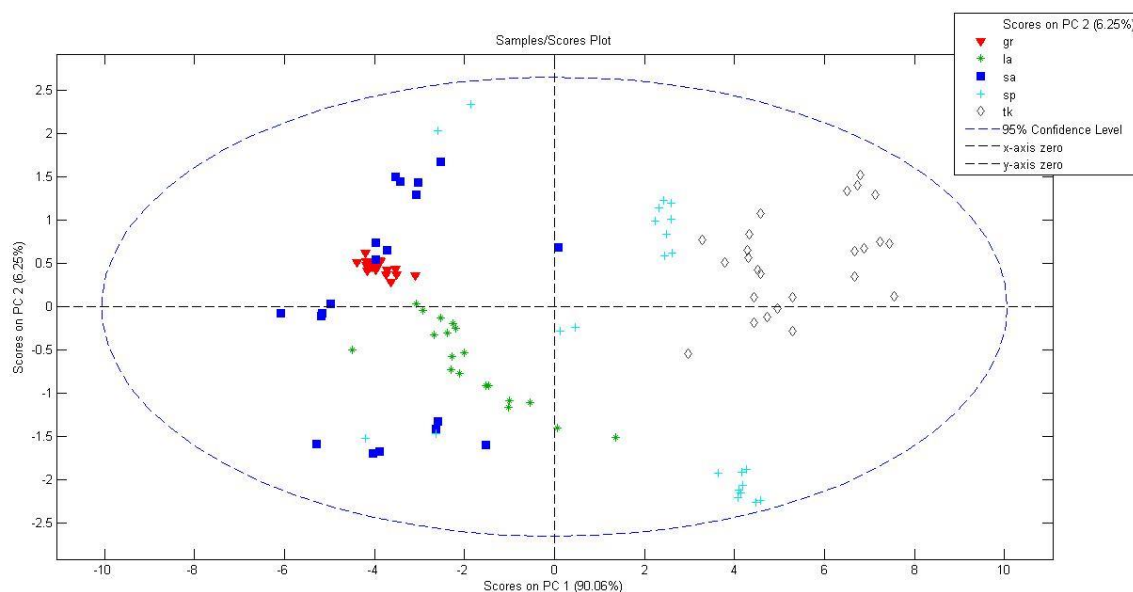
LDA provided a classification on the basis of the corresponding content of crocin, picrocrocin and safranal in different Saffron samples. Saffron from Latium and Greece presented the bigger concentrations of crocin, picrocrocin and safranal. Saffron from Sardinia was split into two clusters with completely different amounts of their active principles. Spanish Saffron is the most scattered and in general is characterized by a low concentration of safranal. Turkish Saffron displayed the lowest concentrations of the active principles and this could be due to the specific drying procedure. The method developed, based on microwave-assisted extraction, allows a limited use of solvents and efficient use of Saffron, allowing a rapid quality control. In fact, the amount of picrocrocin, crocin and safranal indicate the quality of Saffron being related on taste, smell and color. The amount of crocin, picrocrocin and safranal are also related to the geographical origins of production. In fact, type of soil, climatic conditions, modes of production and storage, change in function of different geographical areas of production. Saffron is dried differently (shade, heating system, electric ovens, sunlight, etc.) in various regions of the world, and drying practices are known to affect the final composition of Saffron. Crocins and picrocrocin compounds degrade naturally in the cells of stigmas during drying and storage (Straubinger et al., 1998).

Even if the MAE-HPLC-DAD method compared to other methods presents in the literature allows a rapid analysis and a limited use of solvents, a method based on NIR spectroscopy for discriminant classification of Saffron was also developed. NIR spectroscopy is a non-destructive, non-invasive, rapid, and it does not require any pre-treatment of the sample. There are many advantages from its

use, in addition to those already mentioned. From an environmental point of view, it results zero impact: Saffron can be analyzed without any pretreatment and therefore without the use of solvents. NIR spectroscopic analysis is also economic because it does not need reagents.

The "raw" spectral signals obtained are affected by various undesirable phenomena, such as the presence of shift in the base line, or effects due to the scattering. Since these contributions to the signal are unwanted sources of variability (*i.e.*, they are not related to the phenomenon of interest), before chemometric analysis a pre-treatment of the signals was necessary. Therefore, after being exported, the spectral data were converted in pseudo-absorbance and pretreated with the SNV (Standard Normal Variate) algorithm (Barnes et al., 1989). After pretreatment, spectral data matrices were built by averaging the pretreated signals of the four replicated measurements for each sample. The set of NIR spectra measured after SNV pretreatment are reported in Supporting Information (Figure S8).

The 106 samples were then plotted in the space of the principal components (PC). Figure 3 showed the samples produced in different ways and in different geographical areas, in the space of the first two PCs.



**Figure 3.** Representation of the samples in the space of the first two principal components.

The analysis of the principal components shows how the samples from the same geographical area of production are well grouped.

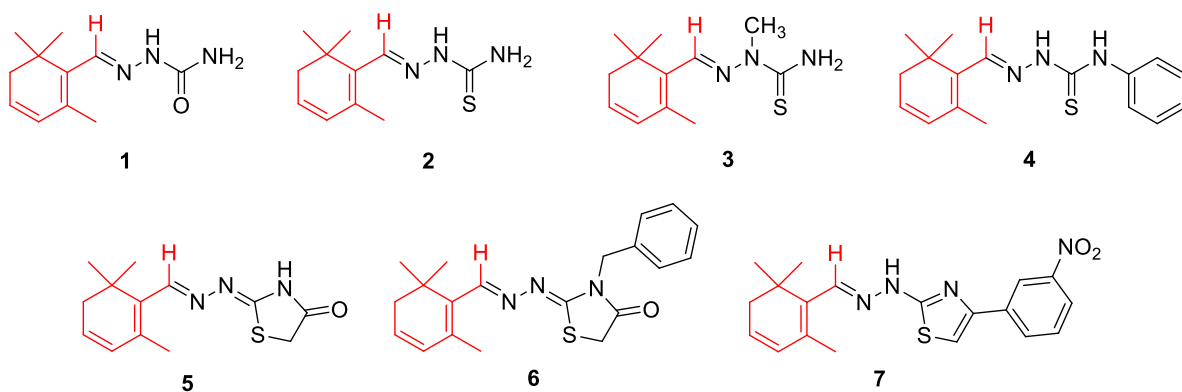
As can be seen from the representation of the 106 samples in the space of the safranal, crocin and picrocrocin variables (Figure 2), also in this case (Figure 3), with the NIRS data is possible to note for the Greek samples a cluster in a narrow space of the PC. Even the Turkish samples in the space of PC can be grouped together and separated from the other samples. The same analysis can be made for Latium samples, which form a distinct cluster from the other samples. With the NIR data, in addition to exploratory analysis, in order to discriminate the different geographical origins and production of Saffron we developed a classification model (PLS-DA).

**Table 6.** PLS-DA model, latent variable=9, cross validation (CV)= venetian blinds w/10 splits.

| NIR data         | Greece | Latium | Sardinia | Spain | Turkey |
|------------------|--------|--------|----------|-------|--------|
| sensitivity cal. | 1.000  | 1.000  | 1.000    | 0.913 | 1.000  |
| specificity cal. | 1.000  | 1.000  | 0.966    | 1.000 | 1.000  |
| sensitivity CV   | 1.000  | 1.000  | 0.947    | 0.913 | 1.000  |
| specificity CV   | 0.988  | 1.000  | 0.954    | 0.976 | 0.988  |

The low number of samples did not allow an external validation of the model. However, the developed model permits an almost perfect classification in cross validation of all the five available classes of samples (Table 6).

From a pharmacological point of view, few data are available in literature about Saffron spice and/or its components regarding the **human** carbonic anhydrase (hCA, EC 4.2.1.1) inhibition. Given our interest in this field (D'Ascenzio et al., 2014; Carradori et al., 2013), we explored the potential anti-hCA activity (five isoforms) of crocin and safranal, which are two important bioactive products found in *C. sativus*, and we also tested the semisynthetic derivatives of safranal (**1-7**, **Scheme 1**) in order to establish which modifications in the chemical structures could improve the biological activity to provide new leads for the development of promising biologically active agents (Table 7).



**Scheme 1.** Structures of the semisynthetic safranal derivatives **1-7**.

**Table 7.** Inhibitory activity of crocin, safranal, its semisynthetic derivatives **1-7** and reference compound (acetazolamide) against five selected hCA isoforms by stopped-flow CO<sub>2</sub> hydrase assay.

| Compound                   | <i>K<sub>i</sub></i> (μM)* |        |        |        |         |
|----------------------------|----------------------------|--------|--------|--------|---------|
|                            | hCA I                      | hCA II | hCA VA | hCA IX | hCA XII |
| <b>Crocin</b>              | >10                        | >10    | 0.26   | >10    | 0.029   |
| <b>Safranal</b>            | >10                        | >10    | 0.17   | >10    | 0.033   |
| <b>1</b>                   | >10                        | >10    | 0.043  | 4.16   | 0.032   |
| <b>2</b>                   | >10                        | >10    | 0.046  | 2.86   | 0.039   |
| <b>3</b>                   | >10                        | >10    | 0.21   | 3.82   | 0.034   |
| <b>4</b>                   | >10                        | >10    | 0.046  | 0.044  | 0.028   |
| <b>5</b>                   | >10                        | >10    | 0.047  | 2.85   | 0.031   |
| <b>6</b>                   | >10                        | >10    | 0.043  | 0.044  | 0.032   |
| <b>7</b>                   | >10                        | >10    | 0.19   | 3.91   | 0.030   |
| <b>Acetazolamide (AAZ)</b> | 0.10                       | 0.008  | 0.38   | 0.041  | 0.038   |

\*Mean from 3 different assay, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).

Crocin, safranal and all the semisynthetic derivatives of safranal (**1-7**) were completely inactive against the cytosolic, widespread hCA I and II off-target isoforms. Moreover, crocin and safranal were also not active against hCA IX displaying a good selectivity towards hCA XII. Both these isoforms are validated targets for the treatment of hypoxic cancers. As regards the other derivatives, **4** and **6** possessed a strong inhibitory activity against the two tumor-associated CA isoforms, in the nanomolar range, whereas the other compounds were more active against hCA XII (hCA IX inhibition values in the micromolar range). A special consideration might be attributed to hCA VA inhibition by both natural and semisynthetic compounds. This isozyme is involved in lipo- and gluconeogenesis and could have a role in the glucose level control (Hasani-Ranjbar et al., 2013; Gouta et al., 2010; Kang et al., 2012). Indeed, hCA VA (and hCA VB) isoforms are expressed only in the mitochondria, assisting the catalytic activity of the mitochondrial pyruvate carboxylase enzyme (PC) (Carta et al EOTP 2013; Supuran EOED 2012, etc). PC uses bicarbonate ions, which are incorporated in the pyruvate, to form oxaloacetate which in turn is then converted into citrate via the reaction with acetyl coenzyme A (Ac-CoA) (Carta et al EOTP 2013; Supuran EOED 2012,

Arechederra RL, Waheed A, Sly WS, et al. Effect of Sulfonamides as Selective Carbonic Anhydrase Va and Vb Inhibitors on Mitochondrial Metabolic Energy Conversion. *Bioorg Med Chem* 2013; 21:). Citrate, unlike oxaloacetate and Ac-CoA, is able to translocate from the mitochondria into the cytosol by means of the tricarboxylate transporter, and once *in loco* gets degraded back into oxaloacetate and CoA by the ATP-cytrate lisase. The oxaloacetate is then decarboxylated to afford pyruvate which is re-taken into the mitochondria by the pyruvate carboxylase transporter. The cytosolic activated CoA is then converted into malonyl-CoA by means of the cytosolic acetyl-coenzyme A carboxylase (ACC). The malonyl-CoA units are then elongated in the same manner to afford fatty acids (7, 8). It has been demonstrated that sulfonamide/sulfamate CA inhibitors also inhibit this process *in vitro* and *in vivo* showing significant antiobesity action (Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nature Rev Drug Discov* 2008; 7: 168-18; Supuran CT. Carbonic anhydrase inhibitors in the treatment and prophylaxis of obesity. *Expert Opin Ther Pat* 2003; 13: 1545-50). Crocin and safranal investigated here, inhibited this isoform with  $K_i$  values in the nanomolar range, whereas compounds **1**, **2**, **4-6** demonstrated to be more potent than their parent compound displaying also a good selectivity over the first two isoforms. These are among the very few non-sulfonamide derivatives showing this interesting biological activity, which might be useful to design antiobesity agents with a novel mechanism of action.

## Conclusion

The developed method allows high recovery, high reproducibility and samples analysis with very low concentration of safranal, crocin and picrocrocin. This method, thanks to the high concentration factor (only 2 mL of solvent), can be used for the analysis of compounds and derivatives with similar characteristics of safranal, crocin and picrocrocin even if they are present in very low

concentrations. By using microwave-assisted extraction, we obtained concentrations of extracts approximately 10 times higher compared to the ISO 2003 method and to methods which use ultrasounds for the extraction. Moreover, with the developed method, it is possible to simultaneously analyze both safranal, and crocin/picrocrocin, while the works reported in the literature are focused on the determination of either safranal or crocin and picrocrocin (Kyriakoudi et al., 2012; Maggi et al., 2011). Although a perfect geographical classification could not be performed only with the quantitative analysis of crocin, picrocrocin and safranal, we can assert, by graphical analysis of the samples in the space of these three parameters (Figure 2) and by the results of the linear discriminant analysis in cross validation (Table 5), that the content of these bioactive compounds is strongly linked to the production area.

A discriminant method of classification faster than HPLC-DAD analysis was developed. The analysis of the NIR spectra of the Saffron samples allowed to discriminate in a rapid, non-destructive and zero impact way, samples from the different production areas such as Turkey, Greece, Spain and Italy (Latium and Sardinia districts). Our proposal for solvent selection should be considered in a forthcoming revision of the ISO 3632-2 technical standard as it accelerates extraction of bioactive compounds from stigma tissues under microwave-assisted conditions

Lastly, a selective inhibitory activity against five selected hCA isoforms (especially towards hCA V(A) and XII) was registered with crocin, safranal and some safranal semisynthetic derivatives, opening a new scenario in the medicinal value of this spice.

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**Supplementary data.** Supplementary data associated with this article can be found, in the online version.

## References

- Barnes, R. J., Dhanoa, M. S., & Lister, S. J. (1989). Standard normal variate transformation and detrending of near-infrared diffuse reflectance spectra. *Applied Spectroscopy*, *43*, 772-777.
- Bevilacqua, M., Bucci, R., Magrì, A. D., Magrì, A. L., & Marini, F. (2012). Tracing the origin of extra virgin olive oils by infrared spectroscopy and chemometrics: a case study. *Analytica Chimica Acta*, *717*, 39-51.
- Brereton, P. (2010). Preface to the special issue "Food authenticity and traceability". *Food Chemistry*, *118*, 887.
- Carradori, S., De Monte, C., D'Ascenzio, M., Secci, D., Celik, G., Ceruso, M., Vullo, D., Scozzafava, A., & Supuran, C. T. (2013). Salen and tetrahydrosalen derivatives act as effective inhibitors of the tumor-associated carbonic anhydrase XII - A new scaffold for designing isoform-selective inhibitors. *Bioorganic and Medicinal Chemistry Letters*, *23*, 6759-6763.
- D'Ascenzio, M., Carradori, S., De Monte, C., Secci, D., Ceruso, M., & Supuran, C. T. (2014). Design, synthesis and evaluation of *N*-substituted saccharin derivatives as selective inhibitors of tumor-associated carbonic anhydrase XII. *Bioorganic and Medicinal Chemistry*, *22*, 1821-1831.
- D'Ascenzio, M., Carradori, S., Secci, D., Vullo, D., Ceruso, M., Akdemir, A., & Supuran, C. T. (2014). Selective inhibition of human carbonic anhydrases by novel amide derivatives of probenecid: synthesis, biological evaluation and molecular modelling studies. *Bioorganic and Medicinal Chemistry*, *22*, 3982-3988.
- De Monte, C., Carradori, S., Chimenti, P., Secci, D., Mannina, L., Alcaro, F., Petzer, A., N'Da, C. I., Gidaro, M. C., Costa, G., Alcaro, S., & Petzer, J. P. (2014). New insights into the biological properties of *Crocus sativus* L.: chemical modifications, human monoamine oxidases inhibition and molecular modeling studies. *European Journal of Medicinal Chemistry*, *82*, 164-171.

- De Monte, C., Carradori, S., Secci, D., D'Ascenzio, M., Vullo, D., Ceruso, M., & Supuran, C. T. (2014). Cyclic tertiary sulfamates: selective inhibition of the tumor-associated carbonic anhydrases IX and XII by *N*- and *O*-substituted acesulfame derivatives. *European Journal of Medicinal Chemistry*, *84*, 240-246.
- Fisher, R. A. (1936). The use of multiple measurements in taxonomic problems. *Annals of Eugenics*, *7*, 179-188.
- Gao, W., Yang, H., Qi, L.-W., Liu, E.-H., Ren, M.-T., Yan, Y.-T., Chen, J., & Li, P. (2012). Unbiased metabolite profiling by liquid chromatography-quadrupole time-of-flight mass spectrometry and multivariate data analysis for herbal authentication: classification of seven *Lonicera* species flower buds. *Journal of Chromatography A*, *1245*, 109-116.
- Gouta, B., Bourges, C., & Paineau-Dubreuil, S. (2010). Satiereal, a *Crocus sativus* L extract, reduces snacking and increases satiety in a randomized placebo-controlled study of mildly overweight, healthy women. *Nutrition Research*, *30*, 305-313.
- Gutiérrez, J., Coello, J., & MasPOCH, S. (2012). Enhanced chromatographic fingerprinting of herb materials by multi-wavelength selection and chemometrics. *Analytica Chimica Acta*, *710*, 40-49.
- Hasani-Ranjbar, S., Jouyandeh, Z., & Abdollahi, M. (2013). A systematic review of anti-obesity medicinal plants - an update. *Journal of Diabetes & Metabolic Disorders*, *12*, 28.
- Hosseinzadeh, H., & Nassiri-Asl, M. (2013). Avicenna's (Ibn Sina) the Canon of Medicine and Saffron (*Crocus sativus*): a review. *Phytotherapy Research*, *27*, 475-483.
- ISO 3632-1, Saffron (*Crocus sativus* Linneaus). Part 1: Specifications, International Organisation for Standardization, Geneva, 2011.
- ISO 3632-2, Saffron (*Crocus sativus* Linneaus). Part 2: Test Methods, International Organisation for Standardization, Geneva, 2010.

- Jalali-Heravi, M., Parastar, H., & Ebrahimi-Najafabadi, H. (2009). Characterization of volatile components of Iranian Saffron using factorial-based response surface modeling of ultrasonic extraction combined with gas chromatography–mass spectrometry analysis. *Journal of Chromatography A*, *1216*, 6088–6097.
- Jiang, C., Cao, L., Yuan, Y., Jin, Y., & Huang, L. (2014). Barcoding melting curve analysis for rapid, sensitive, and discriminating authentication of Saffron (*Crocus sativus* L.) from its adulterants. *Biomedical Research International*, 809037.
- Kang, C., Lee, H., Jung, E.-S., Seyedian, R., Jo, M., Kim, J., Kim, J.-S., & Kim, E. (2012). Saffron (*Crocus sativus* L.) increases glucose uptake and insulin sensitivity in muscle cells via multipathway mechanisms. *Food Chemistry*, *135*, 2350-2358.
- Kyriakoudi, A., Chrysanthou, A., Mantzouridou, F., & Tsimidou, M. Z. (2012). Revisiting extraction of bioactive apocarotenoids from *Crocus sativus* L. dry stigmas (Saffron). *Analytica Chimica Acta*, *755*, 77-85.
- Maggi, M., Sánchez, A. M., Carmona, M., Kanakis, C. D., Anastasaki, E., Tarantilis, P. A., Polissiou, M. G., & Alonzo, G. L. (2011). Rapid determination of safranal in the quality control of Saffron spice (*Crocus sativus* L.). *Food Chemistry*, *127*, 369-373.
- Melnyk, J. P., Wang, S., & Marcone, M. F. (2010). Chemical and biological properties of the world's most expensive spice: Saffron. *Food Research International*, *43*, 1981-1989.
- Moore, J. C., Spink, J., & Lipp, M. (2012). Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *Journal of Food Science*, *77*, R118-R126.
- Moshiri, M., Vahabzadeh M., & Hosseinzadeh H. Clinical applications of Saffron (*Crocus sativus*) and its constituents: a review. *Drug Research (Stuttg)*, 2014 May 21 [Epub ahead of print].

- Pfander, H., & Schurtenberger, H. (1982). Biosynthesis of C<sub>20</sub>-carotenoids in *Crocus sativus*. *Phytochemistry*, *21*, 1039-1042.
- Pizarro, C., Rodríguez-Tecedor, S., Pérez-del-Notario, N., Esteban-Díez, D., & González-Sáiz, J. M. (2013). Classification of Spanish extra virgin olive oils by data fusion of visible spectroscopic fingerprints and chemical descriptors. *Food Chemistry*, *138*, 915-922.
- Scot, P. M. Official methods of analysis of AOAC international. 1994, XVI edition. Arlington, VA: AOAC International Press.
- Sikorski, Z. E. Chemical and functional properties of food components. 1996, Lancaster, PA: Technomic Publishing Co.
- Springett, M. B. Raw ingredient quality in processed foods: the influence of agricultural principles and practices. 2000, Gaithersburg, MA: Aspen Publishers.
- Straubinger, M., Bau, B., Eckstein, S., Fink, M., & Winterhalter, P. (1998). Identification of novel glycosidic precursors in Saffron. *Journal of Agricultural and Food Chemistry*, *46*, 3238–3243.
- Zhang, H.-F., Yang, X.-H., & Wang, Y. (2011). Microwave assisted extraction of secondary metabolites from plants: Current status and future directions. *Trends in Food Science Technology*, *22*, 672-688.