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Quantification of main bioactive metabolites from saffron (*Crocus sativus*) stigmas by a micellar electrokinetic chromatographic (MEKC) method

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Abstract

Saffron is an expensive spice, cultivated in many regions of the world. Its chief metabolites include crocins, which are responsible for the coloring ability, safranal, which is the main essential oil constituent, and picrocrocin which is the main bitter constituent of the spice. A simple micellar capillary electrochromatographic (MEKC) method capable of quantifying all three types of main constituents was established. The pH, sodium dodecyl sulphate (SDS) content and electrolyte concentration of the background electrolyte was optimized. A simple extraction protocol was developed which can extract all metabolites of different polarity from the saffron stigmas. Optimal background electrolyte composed of 20 mM disodium phosphate, 5 mM sodium tetraborate, 100 mM SDS, pH was set 9.5. Optimal extracting solvent was the background electrolyte, incubated with the sample for 60 min. The proposed method allows quantification of picrocrocin, safranal, crocetin- Di-(β-d-gentiobiosyl) ester and crocetin (β-d-glycosyl)-(β-d-gentiobiosyl) ester within 17.5 min, with limit of detection values ranging from 0.006 to 0.04 mg/ml, from a single stigma.

Keywords

Abbreviations

AUC, area under curve; BGE, background electrolyte; CE, capillary electrophoresis; CG, crocetin glycosides; DW, dry weight; LOD, limit of detection; LOQ, limit of quantification; MALDI-TOF, matrix assisted laser desorption and ionization – time-of-flight; MEKC, micellar electrokinetic chromatography; NACE, non-aqueos capillary electrophoresis; RSD, relative standard deviation; tCdG, trans-crocetin di-gentiobiosyl ester; tCGG, trans-crocetin gentiobiosyl glucosil ester

1. Introduction

Saffron is an expensive spice cultivated in many regions of the world. The spice is the dried stigma of the plant *Crocus sativus* L. (Iridaceae). Its major cultivation regions giving high percent of the total world production are Iran, India, Spain, Greece and Morocco [1]. Besides its main application as a spice, it has recently gained interest as a potential source of pharmacologically active metabolites, which are also responsible for the spice characteristics of saffron. Being sterile, it could be only distributed throughout the region of cultivation by means of asexual reproduction, which led to a relative genetic homogeneity [2]. However, different techniques of drying and post-harvest treatment lead to different metabolite patterns [3], that is why the dried spice samples from different regions of cultivation do not have the same concentrations of main constituents. Saffron contains three different main metabolite groups, which are often analyzed by different methods and instruments. The presence of all three metabolite groups is important to achieve the desired organoleptic property of the spice, and all are linked to some potential therapeutic application, as discussed briefly below.

First, the dried stigma is well-known for its coloring ability, which was recognized already in ancient times. The coloring ability is due to the presence of the water-soluble carotenoids, crocins. The crocins are different glycosides (mono-glycosyl or di-glycosyl esters) of a C20 carotenoid aglycon crocetin (Fig. 1). Among the crocetin glycosides (CGs), crocetin digentiobiosyl ester is the most abundant in saffron. Crocetin is actually the precursor of crocin synthesis, its water-solubility is negligible compared to the crocin esters. The fruits of Gardenia jasminoides Ellis also contain some of these water-soluble carotenoids [4], but no other chief constituents of saffron are present. Being polyunsaturated, they act as natural antioxidants by efficiently scavenging reactive oxygen species [5]. This antioxidant activity can be one of the causes of the measured anti-inflammatory activity of saffron extracts, and CGs in particular. Several new potential uses have been established for saffron extracts, and crocins. Crocin and CGs were shown to have inhibitory effects on several cancer cell lines in vitro [6], and also in animal studies. The 80% ethanolic extract of saffron was shown to be effective in the treatment mild to moderate depression in randomized, placebo-controlled clinical trials on outpatients [7], but the active ingredients were not identified. Other results on effects of crocetin and its esters include anti-atherosclerotic effects, and anti-insulin resistance activity, measured mostly in rodent models [6]. The findings above make the spice an excellent candidate for being a functional food. Crocetin and its esters are routinely analyzed by HPLC [8], and a non-aqueous capillary electrophoresis (NACE) method is also published for crocin separation [9]. Typical concentrations of total crocin are found to be as high as 6–16% of dry weight (DW) [6]. Other, more special methods, like Raman spectroscopy were described for estimation of these important compounds [10].

The main component responsible for the bitter taste in saffron is picrocrocin, a terpenoid glycoside (Fig. 1), which is present in 1–13% of DW in Croci stigma. Being bitter, picrocrocin is likely to be responsible for the digestion enhancing activity showed by the aqueous saffron extract on rats. It is also shown to contribute to the cytotoxic activity on some tumor cell lines [6]. Picrocrocin is quite polar, and can be analyzed by HPLC [11].

The characteristic odor of saffron is attributed to safranal (Fig. 1) content, the main compound of the saffron volatile oil (present in 30–70% in the essential oil) [6]. The essential oil also contains other compounds (probably also carotenoid breakdown-products) with similar structure [12] and [13]. Safranal is the product of natural de-glycosylation of picrocrocin, dehydration is important in the release of safranal from picrocrocin via enzymatic activity. Safranal was shown to have anxiolytic activity in mice, and was also shown to inhibit some cancer cell lines [6]. Volatile constituents were typically determined by GC or GC–MS [12] and [13], but in some cases LC–MS is also employed. General (and official) methods of saffron quality management are often time consuming, expensive and many lack sufficient specificity [14].

Capillary electrophoresis (CE) separations, and micellar electrokinetic chromatography (MEKC)-related methods in particular, have major advantages. Frequently cited advantages include lower organic solvent consumption (compared to HPLC), the ability to separate compounds of very different polarity, the simplicity of sample preparation, and others which make CE and MEKC an excellent complementary method to HPLC [15].

Our aim was to develop a simple and environmentally friendly extraction protocol, and a single-run capillary MEKC method for routine screening and quality management of saffron samples, that requires only minimal amount of Croci stigma.

2. Materials and methods
2.1. Chemicals
Crocetin and safranal were purchased from Sigma–Aldrich. All used standard reagents (inorganic salts, solvents) etc. were of analytical quality (HPLC grade in the case of solvents) and were purchased from Spektrum-3D kft (Hungary). For all applications, bidistilled water was used.

2.2. Plant material
Dried plant material was obtained from local and internet markets; samples from the four main sites of production–Iran, Spain, Greece and Italy–were purchased. Residual moisture was removed from plant material with adsorbent silica.

2.3. Purification of pircocrocin and crocetin glycosides from saffron
CGs and picrocrocin were purified using a protocol based on [16]. To purify the CGs, the plant material was extracted with MeOH exhaustively, filtered, evaporated to dryness, reuptaken in 10% MeCN, and separated to fractions by RP-HPLC - Shimadzu SPD-M10A HPLC, semipreparative column: Supelco Supelcosil LC-18 column (25 cm × 10 mm, 5 μm), flow rate 3 ml/min, using a water-MeCN linear gradient (from 25 to 100%, 60 min, +0.1% TFA). Elution was monitored at 440 nm. Of the several CGs present, the fractions with the two most prominent ones were collected, and evaporated to dryness. The isolated compounds were identified by UV-Vis spectra, and MALDI-TOF analysis as described in [17]. The most prominent purified CG (31.0 min retention time) showed spectroscopic data identical to that of trans-crocetin di-(β-d-gentiobiosyl) ester (tCdG) (Fig. 1). Another purified fraction (28.5 min retention time) contained trans-crocetin (β-d-glycosyl)-(β-d-gentiobiosyl) ester (tCGG) (Fig. 1). Fragmentation patterns of glycosidic side chains helped to distinguish between possible isomers as described in [17]. Cis- and trans-crocetin glycosides were identified based on UV-Vis characteristics [18] and [19]. Among the CGs of saffron, these two were previously shown to be most prominent by LC/MS [19]. Other crocin fractions were also purified to determine their retention time in CE, but the amounts obtained were not enough for further identification.

Picrocrocin was purified from the same runs as described for crocin isolation. Retention time was 20.0 min, elution was monitored at 250 nm. Identification was done by UV-Vis spectrum and MALDI-TOF analysis.

2.4. Instrumentation, general settings
A PrinCE-C 700 capillary electrophoresis instrument with a diode array detector was used. A fused silica capillary (50 μm i.d., 375 μm o.d., total (effective) length: 60 cm (52 cm)) was used for the experiments. Before measurement with a new buffer, preconditioning was accomplished by washing the capillary with 0.1 M NaOH (3 min), water (3 min), and equilibration with the new BGE (10 min.). Between samples, capillary was washed with 0.1 M NaOH (3 min), water (3 min), and equilibrated

with the BGE (4 min). Applied voltage was +25 kV. Samples were injected hydrodynamically, +50 mbar pressure was applied at the inlet for 0.1 min.

2.5. Optimization of analyte extraction from saffron stigmas

In order to obtain a method capable of extracting all analytes of interest, the plant material was extracted with different types of solvents, including water, methanol, and background electrolyte (optimized BGE: 20 mM disodium phosphate, 5 mM sodium tetraborate, 100 mM SDS, pH 9.5). A single dry stigma was accurately weighed (typical weight of few milligrams), reduced to powder and homogenized with 150 μl of extracting solvent. Thereafter, samples were allowed to stand at room temperature for 30 min, in darkness, followed by subsequent centrifugation at 13,000 rpm for 20 min. The supernatants were used for MEKC analysis directly. After choosing the solvent type (see Section 3.1), extraction time was optimized, i.e. incubation times of 15, 30, 60, 120 min were tested.

Linearity of extraction was also tested by extracting three different amounts of Croci stigma samples. Approximately 10, 20 and 30 mg DW/ml extracts were obtained (n = 3, each). Linearity was estimated by calculating the R2 of the linear regression between the AUCs of the analytes of interest and DW of the samples.

Finally, a recovery study was carried out. Six samples of Croci stigma were extracted with MeOH, three samples with BGE. Three of the MeOH supernatants were evaporated to dryness, and reuptaken in the same amount of BGE. The three other MeOH samples were diluted with 2-fold concentrated BGE (40 mM disodium phosphate, 10 mM sodium tetraborate, 200 mM SDS in water) to obtain a BGE-like solvent. The three types of extracts were analysed with MEKC. The recovery study was done in triplicate.

2.6. MEKC method development

2.6.1. Effect of BGE composition on separation of *Crocus* metabolites

First, BGE pH was optimized. To test the effect of pH on the separation of the *Crocus* metabolites, a starting background electrolyte (25 mM disodium phosphate and 100 mM SDS) was set to different pHs, 6.0, 7.0, 8.0, 9.0, 9.5, 10.0. After optimization of pH, different concentrations of SDS were tested to achieve better separation of the analytes of interest. Concentrations 12.5, 25, 50, 75, 100 and 200 mM SDS were tested in a BGE containing 25 mM phosphate at pH 9.5 (1 M NaOH). To test the effect of phosphate concentration on the separation of the *Crocus* metabolites, a series of background electrolytes with pH 9.5, and 100 mM SDS was made, with phosphate concentrations 12.5, 25, 50 mM. Phosphate alone has very limited buffering capacity at pH 9.5, which may lead to pH shift in the inlet and outlet, and alteration of separation parameters [20]. After optimization of phosphate concentration, different phosphate/borate ratios were tested to achieve the same separation efficacy while increasing buffering capacity of the BGE. Different ratios of disodium phosphate/sodium tetraborate (in particular, 25/0, 20/5, 15/10, 10/15, 5/20, and 0/25 mM) concentrations were tested.

2.6.2. Testing performance of the proposed method

To test the method for the ability to separate common minor peaks in *Crocus sativus*, samples from different regions were tested with the described separation procedure, and their metabolite contents quantified. The method's ability to separate minor CG peaks from chief ones was tested by evaluation of HPLC and MEKC chromatograms in a high concentration range. A 25.0 mg of plant material was extracted exhaustively with MeOH (3 × 1000 μl), pooled, evaporated to dryness. A part of the dry extract was reuptaken in BGE so that the resulting solution was about 125 mg DW equivalent/ml concentrated. The supernatant was analyzed by the proposed MEKC method and checked for interfering CG peaks. Another part of the dry extract was analyzed by HPLC. The same

instrumentation and main parameters were used, as described for metabolite purification, but a much smaller amount (equivalent DW: 100 μg, in 10 μl 20% MeCN) of the extract was injected.

2.6.3. Validation
For quantification, five-point standard calibration curves were used for all analytes, standards dissolved in BGE. Picrocrocin was detected at 250 nm, CGs and crocetin at 440 nm, safranal at 310 nm.

Precision was calculated as RSD values with respect to retention time and AUC. The values were determined in the range that contains the common concentrations of the real samples. Intraday repeatability was measured from 6 replicates. Interday repeatability was measured by calculating the RSD between the means of within-day results for both retention time and AUC, for all analytes', data of three days were used.

Sensitivity was expressed as LOD and LOQ values. Upper limit of quantification was assumed to be the most concentrated standard solution tested. Linearity was expressed as R^2 of the linear regression function between area under curve values and concentrations of standard solutions.

Accuracy was calculated by spiking the extracts with known amount of standards (50, 100 and 150% of the nominal concentrations, within linear range), and the recoveries calculated for all analytes (n = 3).

3. Results and discussion
3.1. Optimal extraction parameters
The saffron stigmas contain metabolites of a wide polarity spectrum, i.e. safranal is an apolar constituent, an essential oil compound immiscible with water, but picrocrocin and CGs are glycosidic (Fig. 1) and therefore dissolve excellently in water. Thus, different solvents were tested for extraction.

The background electrolyte (20 mM disodium phosphate, 5 mM sodium tetraborate, 100 mM SDS in water, pH 9.5) was found to extract most metabolites from the stigmas, whereas methanol and water extracted less metabolites, especially in the case of safranal. Direct injection of methanol extracts resulted in very poor general resolution. In water extracts, the peak of crocetin (present only in traces in BGE or MeOH extracts) was also not detected. Therefore, BGE was chosen as extracting solvent, and extraction time for BGE was optimized.

It was found, that the metabolite concentration–time functions follow a saturation curve. The 120 min extract contained equal amount of safranal, picrocrocin and only insignificantly more crocin esters than the 60 min extract (p > 0.05 for all analytes, ANOVA, n = 3). The plant material visibly lost its color at the end of the 60 min incubation. Shorter incubation times resulted in much less metabolite concentration in the supernatant. The 30 min extract contained 30–60% less metabolites (area under curve), than the 60 min extract. Therefore, 60 min extraction time was chosen for further experiments as a compromise.

Linearity of the extraction was tested by showing linear relationship between area under curve values of analytes and plant material DW mass in extracts of different concentrations (10, 20 and 30 mg DW/ml). Linearity was confirmed by the high R2 values of the linear regression, R2 values were 0.9932, 0.9934, 0.9908 and 0.9807 for picrocrocin, safranal, tCdG and tCGG, respectively (p < 0.001). Extraction was found to be linear at least up to 30 mg DW/ml.

Extraction efficiency using the proposed BGE was compared with that achieved using MeOH. MeOH extracts were evaporated to dryness, and the dried extract was reuptaken in BGE, and analysed with the

proposed MEKC method. Safranal could not be recovered this way, because this volatile substance evaporated during vacuum drying of the MeOH extract, causing significant reduction of the recovered amount. Therefore, three other replicates of MeOH extracts were diluted with double-concentration BGE, and analysed. Presence of MeOH in the sample did not significantly alter the resolution of safranal. Recoveries were 107, 108, 119 and 116% for picrocrocin, safranal, tCdG and tCGG, respectively (n = 3). The BGE extraction solvent was found to be as efficient as MeOH–the difference was not found to be significant (p > 0.05).

Samples in BGE were found to be stable for at least 24 h after extraction, at room temperature, in darkness.

3.2. Separation of *Crocus* analytes
For method development, sample aliquots obtained from Croci stigma by extraction with BGE for 60 min were used. Metabolite identification was accomplished by standard addition to the plant samples.

3.2.1. Effect of pH on the separation of *Crocus* metabolites
First, experiments were run to determine the optimal pH for the separation of the metabolites of interest. A pH series including 6, 7, 8, 9, 9.5 and 10 was tested, the BGE contained 100 mM SDS and 25 mM phosphate. Changes in pH generally have great impact on EOF, dissociation of analytes and BGE components, complexes with BGE components, and thereby resolution [21].

As picrocrocin does not contain any dissociable functional groups, its retention time is only affected by interactions with micelles. The retention time of picrocrocin was not seriously affected by pH, it was around 5 min, but baseline separation from an unidentified impurity with higher retention time was not achieved at pHs 8 and 9.

Retention time of safranal was also not influenced by pH in the higher pH range. Safranal does not have any dissociable functional groups, its separation is only influenced by interactions with SDS micelles and EOF, but is relatively more apolar, than picrocrocin, resulting in more intensive micelle interaction and higher retention time.

Separation of CGs from each other was influenced by pH in spite of not being able to dissociate at the pH-s tested (Fig. 2). Resolution became a little better at higher pH-s between tCdG and tCGG (peaks 3 and 4 on Fig. 2), best separation of the crocin esters was achieved at pH 9.5 (Fig. 2d) and pH 10 (not shown). The retention time of an unidentified trans-crocin (peak 1 on Fig. 2) was more effected, at pH 10 this compound could not be separated from the main crocin esters (tCdG and tCGG), lower pH values resulted in lower retention times. Resolution between tCdG and an unidentified compound possessing cis-crocin UV-Vis spectrum (peak 2 in Fig. 2) was also best at high pH (9.5 or above, Fig. 2d). Finally, pH 9.5 BGE was selected for further experimentation because of increased resolution between the different crocins, and between picrocrocin and the impurities.

3.2.2. Effect of SDS concentration on the separation of *Crocus* metabolites
After optimization of pH, different SDS concentrations were tested. SDS concentration had great impact on retention times. In the case of picrocrocin, SDS concentration affected separation from impurities and retention time. At higher SDS concentration, higher retention times and better separation was obtained. Picrocrocin was separated from impurities at 50 mM SDS or higher concentration. As picrocrocin is uncharged and semipolar, its partition between the micellar and water phase greatly influences the retention time and separation.

The retention times of safranal was also influenced by SDS concentration. Higher SDS concentrations led to higher retention times. At the highest tested concentration (200 mM), safranal peak was not observed, retention time must have increased above total run time (30 min).

At lower concentrations (50 mM or below), crocin separation was not satisfactory (Fig. 3a–c). Higher concentrations of SDS increased the retention time of CGs. Resolution between tCdG and tCGG (peaks 3 and 4 on Fig. 3) was best at 100 mM SDS, as some other minor impurities (an unidentified cis-crocin, and other unidentified trans-crocins) merged into one or more crocin peak complexes at lower SDS concentrations (Fig. 3a–c). Separation of tCdG from an unidentified cis-crocin (peak 2 on Fig. 3) was only achieved at 100 mM SDS (Fig. 3d). Though unidentified, a method's capability of separating cis-crocins is important because abundance of these molecules (i.e. ratio of cis/trans crocins) can interfere with routine spectrophotometric quality management of the spice [22], as they have an absorption maximum around 330 nm. The paper [9] was also dealing with crocin separation, but no clear statement of cis-crocin separation was mentioned. At 200 mM, crocin esters were not detected, probably because retention time has increased above total run time. As a sum, 100 mM SDS was selected for further optimization, mainly because it separated CGs sufficiently.

3.2.3. Effect of phosphate concentration on the separation of *Crocus* metabolites
Changes in phosphate concentration did not alter retention time of picrocrocin, as it was expected. In the case of safranal, changes were also minimal. Higher phosphate concentration (50 mM) increased current, and resulted in a general loss of resolution. Lower phosphate concentrations (12.5 mM) resulted in loss of resolution between CGs. Thus, 25 mM phosphate was selected as optimal electrolyte concentration.

3.2.4. Effect of disodium phosphate/sodium tetraborate ratio on the separation of *Crocus* analytes
The phosphate-based BGE at pH 9.5 has very limited buffering capacity; therefore it may theoretically be susceptible to pH changes in the inlet and the outlet, thereby affecting reproducibility [20]. Shifts in pH need to be minimized to increase the amount of samples that can be run without changing the BGE. Though no significant shift in retention times after 10 sample determinations was observed, the BGE was further optimized to increase its buffering capacity. Borate also may offer increased selectivity due to complexation of sugar side-chains of glycosides [23].

As BGEs with higher total electrolyte concentrations could only be used at lower voltage, which leads to unwanted significant increase in retention times, this strategy was not tested. Instead, the BGE was further optimized by partially replacing disodium phosphate with sodium tetraborate, that has significant buffering capacity at this pH. All borate-containing BGEs were found to have significantly more (at least 20-fold) buffering capacity at pH 9.5 than the BGE based solely on disodium phosphate (determined by pH meter); detailed description of the issue is available in [24].

Replacing 25 mM phosphate with 20 mM phosphate and 5 mM borate did not influence the resolution of CGs. Higher borate concentrations (10 mM borate/15 mM phosphate), however, led to decreased resolution between the major cis-CG and the main trans-CGs, and also increased retention times of all analytes. Peak broadening also occurred at high sodium tetraborate concentrations. Major effects on other analytes' resolution were not observed.

It was hypothesized, that increased heating because of higher current (at higher borate concentration) may be in the background of resolution loss. However, lower concentration BGEs (e.g. 10 mM sodium tetraborate, 5 mM disodium phosphate, 100 mM SDS, pH 9.5) also produced poor resolution between CGs. Thus, it was concluded, that higher disodium phosphate concentrations are required for

acceptable CG separation. A small concentration (5 mM) of sodium tetraborate was found to be necessary to increase the buffering capacity of the BGE to ensure long-term system stability. Final optimal BGE composition was: 20 mM disodium phosphate, 5 mM sodium tetraborate, 100 mM SDS, pH 9.5. A typical chromatogram of a real sample analysed with the final method is plotted in Fig. 4.

3.3. Testing of the method

Samples from dried spice products from Greek, Iranian, Italian and Spanian saffron were subjected to analysis using the extraction and MEKC method proposed above, the results are summarized in Table 1. Picrocrocin and safranal concentrations were comparable to those formerly described for *Crocus* stigmas [16]. Data on concentrations of individual crocetin glycosides (tCdG and tCGG) can be found in [9] and [25], the concentrations of these chief CGs were comparable to those measured in this work. The ratio of different CGs differs in the samples from different geographical sources, also the content of other metabolites was found to be related to the source. The quantitative comparative analysis of *Crocus* samples between production regions, and estimation of variance within regions was beyond the scope of this study. Our main goal was achieved however, by showing that the proposed method separated impurities from analytes of interest – regardless of geographical origin.

Minor crocins could be also detected from a preconcentrated sample (equivalent to 125 mg DW/ml) at 440 nm, separated from the main crocins. Higher concentration was necessary to prove that the minor peaks have CG-like UV-Vis spectra. The electropherograms of samples with the proposed extraction concentration also show most of these minor CG peaks separated from the main analytes of interest (Fig. 4). The minor CG peaks made up only 9.7% of total 440 nm AUC. This concentration was within the linear range of determination.

The HPLC chromatograms of the concentrated MeOH extracts of the stigmas also showed four main crocins, that could be assigned to the four main peaks in the chromatogram (*Crocus* extracts were spiked with the purified, unidentified major CGs as well). The sum of the AUCs of the minor peaks in HPLC contributed very little to the total crocin AUC. The HPLC pattern found was comparable to that published in [19]. We concluded, that the minor CGs do not cause any significant interference during the determination of chief CG analytes.

The described method is also capable of detecting crocetin (extract spiked with standard). None of the stigma samples contained significant concentrations, some contained trace amounts. Crocins were well separated from crocetin, as crocetin dissociates at the BGE pH.

The validation results for the proposed analytical method are summarized in Table 2. The method showed acceptable reproducibility, the extraction protocol as well as the measurement method itself were shown to be efficient. LOD values and RSD values were comparable to those obtained by a NACE method for crocin determination [9]. The proposed method can be an inexpensive means of screening and quantification. It can be a complementary analytical procedure to the published HPLC methods [16] and [19], as it allows quantification of all main constituents (picrocrocin, the two main crocetin glycosides: tCdG and tCGG, and safranal) within reasonable time, from a single stigma of the *Crocus sativus* plant. The proposed method can thus be used for plant-to-plant variability studies.

4. Conclusions

A simple protocol for determination of all main saffron metabolite groups of high consumer interest was established. The proposed extraction method and subsequent MEKC quantification allows determination of two crocetin glycosides (responsible for coloring ability), picrocrocin (main bitter

compound), and safranal (chief constituent of the essential oil, hence, smell). The protocol is carried out in an environmentally friendly fashion (uses no organic solvent), the used reagents are rather inexpensive. The short time required for analysis of a sample allows application as a routine screening and quality-management technique for saffron. The chromatogram pattern also helps estimating cis-crocin to trans-crocin ratios. The low sample demand (single stigma) of the proposed method allows it to be applied for studying plant-to-plant variability.

Acknowledgements

References

[1] L. Maggi, M. Carmona, S.D. Kelly, N. Marigheto, G.L. Alonso, Geographical origin differentiation of saffron spice (*Crocus sativus* L. stigmas) – preliminary investigation using chemical and multi-element (H, C, N) stable isotope analysis, Food Chem. 128 (2011) 543–548.

[2] M.G. Caiola, P. Caputo, R. Zanier, RAPD analysis in *Crocus sativus* L. accessions and related *Crocus* species, Biol. Plant. 48 (2004) 375–380.

[3] M. Gregory, R. Menary, N. Davies, Effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron, J. Agr. Food Chem. 53 (2005) 5969–5975.

[4] A. Ozaki, M. Kitano, N. Furusawa, H. Yamaguchi, K. Kuroda, G. Endo, Genotoxicity of gardenia yellow and its components, Food Chem. Toxicol. 40 (2002) 1603–1610.

[5] A.N. Assimopoulou, Z. Sinakos, V.P. Papageorgiou, Radical scavenging activity of *Crocus sativus* L. extract and its bioactive constituents, Phytother. Res. 19 (2005) 997–1000.

[6] J.P. Melnyk, S. Wang, M.F. Marcone, Chemical and biological properties of the world's most expensive spice: saffron, Food Res. Int. 43 (2010) 1981–1989.

[7] S. Akhondzadeh, H. Fallah-Pour, K. Afkham, A.-H. Jamshidi, F. Khalighi-Cigaroudi, Comparison of *Crocus sativus* L. and imipramine in the treatment of mild to moderate depression: a pilot double-blind randomized trial [ISRCTN45683816], BMC Complem. Altern. Med. 4 (2004) 12.

[8] H. Caballero-Ortega, R. Pereda-Miranda, F. Abdullaev, HPLC quantification of major active components from 11 different saffron (*Crocus sativus* L.) sources, Food Chem. 100 (2007) 1126–1131.

[9] M. Zougagh, B. Simonet, A. Rios, M. Valcarcel, Use of non-aqueous capillary electrophoresis for the quality control of commercial saffron samples, J. Chromatogr. A 1085 (2005) 293–298.

[10] E.G. Anastasaki, C.D. Kanakis, C. Pappas, L. Maggi, A. Zalacain, M. Carmona, G.L. Alonso, M.G. Polissiou, et al., Quantification of crocetin esters in saffron (*Crocus sativus* L.) using Raman spectroscopy and chemometrics, J. Agr. Food Chem. 58 (2010) 6011–6017.

[11] C.P. del Campo, M. Carmona, L. Maggi, C.D. Kanakis, E.G. Anastasaki, P.A. Tarantilis, M.G. Polissiou, G.L. Alonso, et al., Picrocrocin content and quality categories in different (345) worldwide samples of saffron (*Crocus sativus* L.), J. Agr. Food Chem. 58 (2010) 1305–1312.

[12] M. Jalali-Heravi, H. Parastar, H. Ebrahimi-Najafabadi, Characterization of volatile components of Iranian saffron using factorial-based response surface modeling of ultrasonic extraction combined with gas chromatography-mass spectrometry analysis, J Chromatogr. A 1216 (2009) 6088–6097.

[13] C.D. Kanakis, D.J. Daferera, P.A. Tarantilis, M.G. Polissiou, Qualitative determination of volatile compounds and quantitative evaluation of safranal and 4-hydroxy-2, 6, 6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) in Greek saffron, J. Agr. Food Chem. 52 (2004) 4515–4521.

[14] R.S. Verma, D. Middha, Analysis of saffron (*Crocus sativus* L. Stigma) components by LC–MS–MS, Chromatographia 71 (2010) 117–123.

[15] L. Suntornsuk, Recent advances of capillary electrophoresis in pharmaceutical analysis, Anal. Bioanal. Chem. 398 (2010) 29–52.

[16] M. Lage, C.L. Cantrell, Quantification of saffron (*Crocus sativus* L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions, Sci. Hortic. 121 (2009) 366–373.

[17] H. Zhang, Y. Zeng, F. Yan, F. Chen, X. Zhang, M. Liu, W. Liu, et al., Semi-preparative isolation of crocins from saffron (*Crocus sativus* L.), Chromatographia 59 (2004) 691–696.

[18] M.K. Assimiadis, P.A. Tarantilis, M.G. Polissiou, UV-Vis, FT-Raman and ^{1}H NMR spectroscopies of cis – trans carotenoids from saffron (*Crocus sativus* L.), Appl. Spectrosc. 52 (1998) 519–522.

[19] P.A. Tarantilis, G. Tsoupras, M. Polissiou, Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry, J. Chromatogr. A 699 (1995) 107–118.

[20] D. de Jesus, J. Brito-Neto, E. Richter, L. Angnes, I. Gutz, C. do Lago, Extending the lifetime of the running electrolyte in capillary electrophoresis by using additional compartments for external electrolysis RID B-3091-2008 RID G-5711-2011 RID A-1852-2008, Anal. Chem. 77 (2005) 607–614.

[21] S.E. Deeb, M.A. Iriban, R. Gust, MEKC as a powerful growing analytical technique, Electrophoresis 32 (2011) 166–183.

[22] M. Zougagh, A. Rios, M. Valcarcel, Determination of total safranal by in situ acid hydrolysis in supercritical fluid media: application to the quality control of commercial saffron, Anal. Chim. Acta 578 (2006) 117–121.

[23] P. Morin, F. Villard, M. Dreux, P. André, Borate complexation of flavonoid-O-glycosides in capillary electrophoresis. II. Separation of flavonoid-3-O-glycosides differing in their sugar moiety, J. Chromatogr. A 628 (1993) 161–169.

[24] J.C. Reijenga, T.P.E.M. Verheggen, J.H.P.A. Martens, F.M. Everaerts, Buffer capacity, ionic strength and heat dissipation in capillary electrophoresis, J. Chromatogr. A 744 (1996) 147–153.

[25] S.A. Ordoudi, M.Z. Tsimidou, Saffron quality effect of agricultural practices, processing and storage, in: R. Dris, S.M. Jain (Eds.), Production Practices and Quality Assessment of Food Crops: Preharvest Practice, Springer, 2004, pp.209–260.
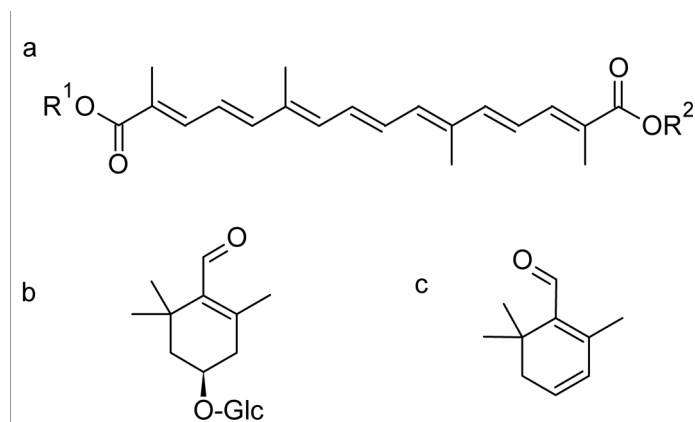
Fig. 1.
Chemical structures of the studied saffron metabolites. (a) Crocetin: $R^1=R^2=H$, crocetin (β-d-glycosyl)-(β-d-gentiobiosyl) ester (tCGG): R^1=glucosyl, R^2=gentiobiosyl; crocetin di-(β-d-gentiobiosyl) ester (tCdG): $R^1=R^2$=gentiobiosyl; (b) picrocrocin; and (c) safranal.
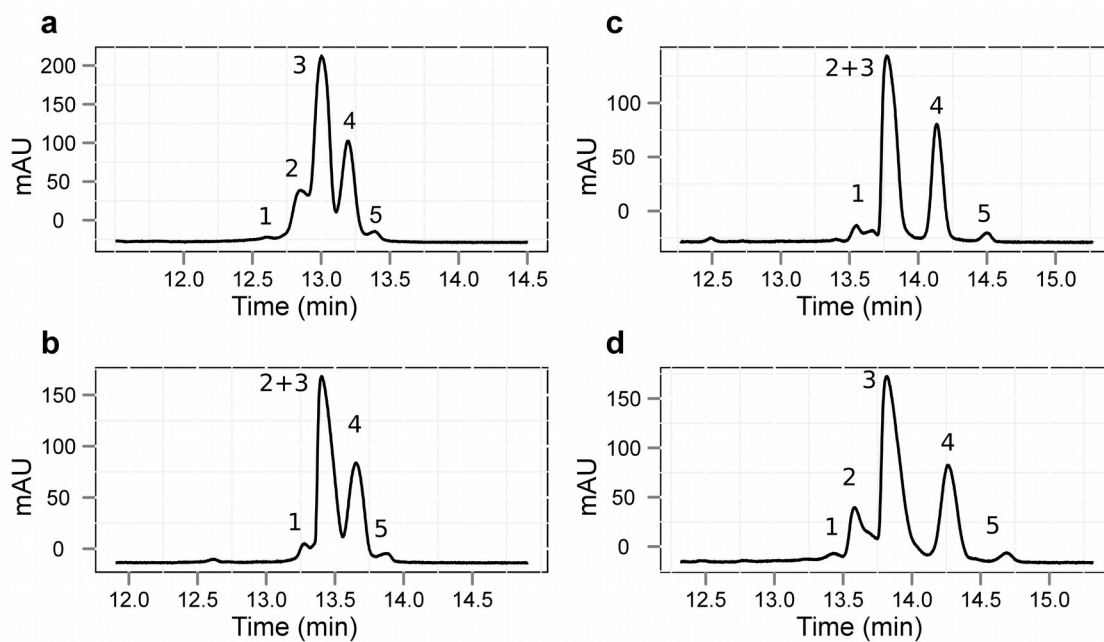

Fig. 2.
Effect of background electrolyte pH on the separation of different crocetin glycosides. BGE contained 100 mM SDS and 25 mM phosphate. Detection was done at 440 nm. Subplots: (a) pH 7.0; (b) pH 8.0; (c) pH 9.0; and (d) pH 9.5. Metabolites: (1) unidentified trans-crocin; (2) unidentified cis-crocin; (3) tCdG; (4) tCGG; and (5) unidentified trans-crocin.
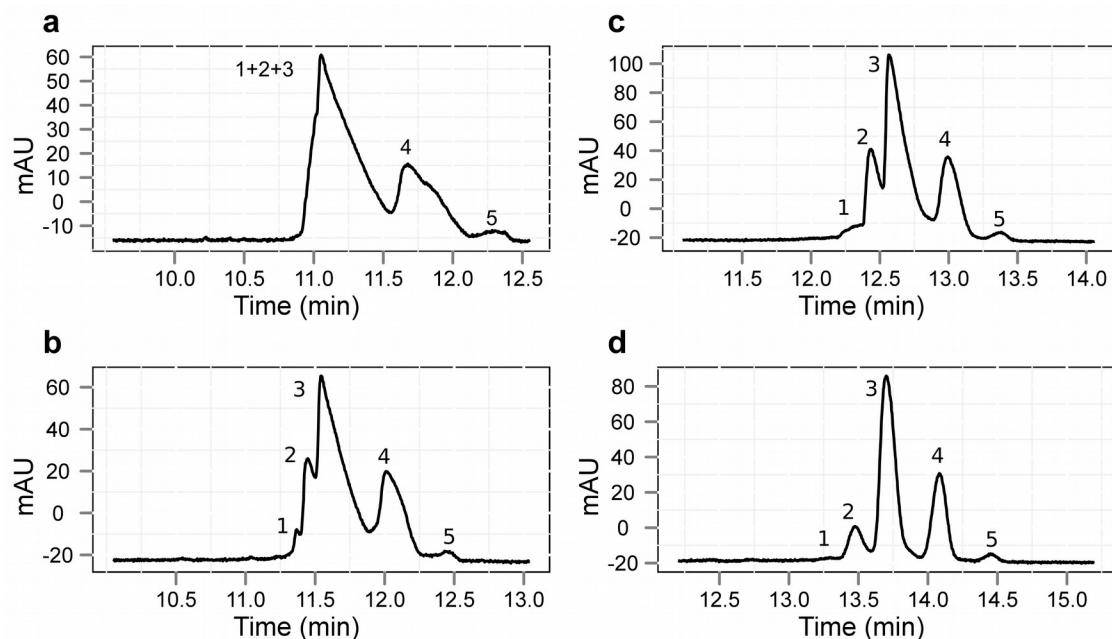
Fig. 3.
Effect of SDS concentration on the separation of different crocetin glycosides. BGE contained 25 mM phosphate, pH was set 9.5. Detection was done at 440 nm. Subplots: (a) 12.5 mM SDS; (b) 25 mM SDS; (c) 50 mM SDS; and (d) 100 mM SDS. Metabolites: (1) unidentified trans-crocin; (2) unidentified cis-crocin; (3) tCdG; (4) tCGG; and (5) unidentified trans-crocin.
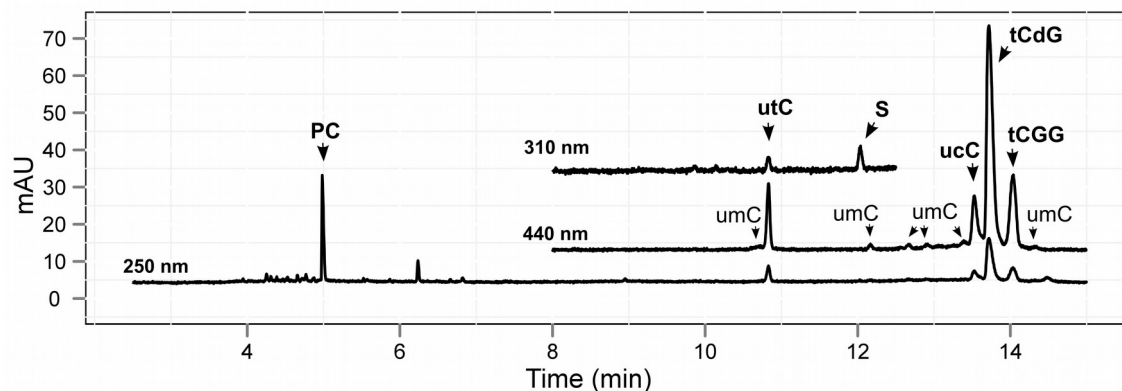

Fig. 4.
MEKC chromatogram obtained from a Greek Crocus sample using the proposed analytical procedure. Detection was done on 250, 310 and 440 nm wavelengths. The 310 nm chromatogram is raised by a factor of 2, the chromatogram at 440 nm is reduced by a factor of 0.5 to allow better emplacement. Chief constituents are marked with arrows. Abbreviations: PC, picrocrocin; S, safranal; umC, unidentified minor crocin; utC, unidentified trans crocin; ucC, unidentified cis crocin, tCGG: crocetin (β-d-glycosyl)-(β-d-gentiobiosyl) ester; tCdG: crocetin di-(β-d-gentiobiosyl) ester.

Table 1.
Results of a single-extraction screening of saffron samples from different regions. Values are given on dry weight basis. Presented data are mean ± SD from three determinations (n = 3). Abbreviations: tCGG: crocetin (β-d-glycosyl)-(β-d-gentiobiosyl) ester; tCdG: crocetin di-(β-d-gentiobiosyl) ester.

Analyte	Greek (%)	Iranian (%)	Italian (%)	Spanish (%)
Picrocrocin	3.993±0.118	2.286±0.153	5.809±0.232	8.026±0.261
tCdG	4.473±0.146	2.208±0.020	3.076±0.073	6.370±0.195
tCGG	0.715±0.052	0.448±0.032	0.919±0.021	1.393±0.033
Safranal	0.051±0.002	0.019±0.003	0.158±0.003	0.081±0.009

Table 2.
Analytical performance of the optimized capillary electrophoretic procedure for quantification of saffron chief metabolites. Abbreviations: AUC, area under curve; DW, dry weight; RSD, relative standard deviation; tCGG, crocetin (β-d-glycosyl)-(β-d-gentiobiosyl) ester; tCdG, crocetin di-(β-d-gentiobiosyl) ester; tR, retention time.

	Picrocrocin	Safranal	tCdG	tCGG
Accuracy (mean of recoveries, n=3×3)	105.3%	101.4%	106.7%	96.9%
Precision (tR, intraday, n=6), RSD	0.67%	0.76%	0.70%	0.73%
Precision (tR, interday, n=3), RSD	0.14%	1.03%	1.35%	1.42%
Precision (AUC, intraday, n=6), RSD	2.65%	2.40%	2.29%	3.54%
Precision (AUC, interday, n=3), RSD	2.27%	1.62%	0.44%	2.50%
Linear regression equation	1.1411x −0.0379	2.0379x −0.0115	5.3137x +1.1446	7.6559x +0.0309
Linearity (R2 of linear regression)	0.9969	0.9999	0.9981	0.9999
LOD (mg/ml)	0.012	0.002	0.003	0.002
LOQ (mg/ml)	0.040	0.006	0.010	0.006
Range (upper limit of quantification) (mg/ml)	1.2	1	1.7	0.8
Typical extract content (1.5mg DW/100μl), (mg/ml)	0.3199	0.0363	0.8070	0.1681