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A new validated SPE-HPLC method for monitoring crocetin in human plasma—Application after saffron tea consumption

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ABSTRACT

Saffron (stigmas of *Crocus sativus* L.) is a well-known spice with many attributed therapeutic uses throughout centuries. Although studies have demonstrated that crocetin and crocins from saffron have various biological functions, issues concerning the route and way of saffron administration, the absorption and metabolism of saffron carotenoids in humans have not been answered yet. In the present study, an isocratic reversed-phase liquid chromatographic method was developed and validated for the determination of crocetin in plasma. Samples were pre-treated by solid phase extraction (recoveries >72%) and were chromatographed on a Luna C-18 column (4.6 mm × 250 mm, 5 μm) with a mobile phase consisting of methanol–water–trifluoroacetic acid (75.0:24.5:0.5, v/v/v) at a flow rate of 1.0 mL min^{-1}. The HPLC method developed resulted in sharp peaks at 10.7 (*trans*-crocetin) and 18.6 min (*cis*-crocetin), whereas the calibration curve of total crocetin in plasma displayed a good linearity for concentrations of 0.020–20 μM (R^2 = 0.999). Specificity, precision, accuracy and stability were studied with spiked plasma samples and were acceptable. The developed method was applied to the determination of crocetin levels in plasma of four healthy human volunteers before and after consumption of one cup of saffron tea (200 mg of saffron in 80 °C water for 5 min). Results showed that the concentration of crocetin was high after 2 h (1.24–3.67 μM) and still determined after 24 h (0.10–0.24). Interestingly, the percentage of the *cis*-isomer ranges from 25 to 50%, suggesting *in vivo* isomerization.

1. Introduction

Saffron, the dried stigmas (the term styles is also used by certain botanologists [1]) of *Crocus sativus* flowers, is a spice important in the Mediterranean, Indian and Chinese diet and has been continuously used in folk remedies for more than 90 diseases for over 3000 years [2]. Recent research on its biomedical properties supports its use for many of these indications, although the great majority of the studies are conducted *in vitro* and in experimental animals [3]. Its composition is also quite unique: the main constituents are hydrophilic carotenoids, the crocins, which are mono- and diglycosyl-esters of crocetin and are responsible for the characteristic golden yellow color they confer.

Besides culinary uses, saffron is consumed as a herbal tea *per se* or is added in other herbal infusions for its unique flavor characteristics and its attributed medicinal uses. According to the WHO monograph issued in 2007, daily doses of up to 1.5 g of saffron are thought to be safe; at doses of 5.0 g or more, Stigma Croci may cause serious adverse reactions and overdose (12.0–20.0 g/day) may be fatal [3]. Intraperitoneal administration of saffron ethanolic extract to Wistar rats at the high doses of 0.35, 0.70 and 1.05 g kg^{-1} body weight for two weeks caused mild to severe hepatic and renal tissue injuries, weight loss, significant reductions in the haemoglobin and haematocrite levels and total red blood cell counts; intraperitoneal median lethal dose (LD50) value of ethanolic extracts was found to be 3.5 g kg^{-1} body weight [4]. Modaghegh et al. showed that consumption of 200 and 400 mg saffron tablets by healthy humans for 7 days is safe [5]. Administration of 200 mg saffron capsules to men with erectile dysfunction for 10 days was safe and had a positive effect on sexual function [6], whereas administration of 30 mg capsules to patients with mild-to-moderate Alzheimer's disease [7] and to women with symptoms of premenstrual syndrome [8] had a favorable outcome. A reduction in lipoprotein oxidation in 20 human subjects was also reported after administration of 50 mg saffron in milk twice a day for 3–6 weeks [9].

However, issues concerning the route and way of saffron administration, the absorption and metabolism of saffron carotenoids in humans have not been answered. Experiments conducted in rodents, showed that orally administered crocins are hydrolysed to crocetin before being incorporated into blood circulation and

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that crocetin is likely to be metabolized to glucuronide conjugates both in the intestinal mucosa and in the liver of mice [10,11]. The aim of this study was the development and validation of a chromatographic analytical method for the determination of crocetin in human plasma and application of that method to the determination of plasma crocetin levels after saffron tea consumption by healthy volunteers. In this context, we screened saffron tea composition to investigate if tea preparation induces changes in saffron components, i.e. carotenoid isomerization.

2. Materials and methods

2.1. Reagents and chemicals

Methanol (MeOH) and acetonitrile (AcCN) of HPLC grade were supplied from Merck KGaA (Darmstadt, Germany). Dimethylsulphoxide (DMSO), ammonia solution, hydrochloric acid and solid reagents (ammonium acetate, sodium chloride and sodium phosphate), of analytical grade were also purchased from Merck. Trifluoroacetic acid (TFA) and butylated hydroxytoluene were obtained from Sigma–Aldrich (St. Louis, USA) whereas *trans*-crocetin [(2E,4E,6E,8E,10E,12E,14E)-2,6,11,15-tetramethyl-2,4,6,8,10,12,14-hexadecaheptaenedioic acid] (purity >98% for the sum of both isomers) was purchased from Extrasynthese (Genay Cedex, France). Saffron in the form of dried styles was supplied from Cooperative de Saffron (Krokos Kozanis, Greece). The dried plant material was identified by Professor Gregoris Iatrou, Department of Biology, University of Patras, Greece.

2.2. Preparation of crocetin standards

Stock solutions of crocetin were prepared in DMSO at an initial concentration of 10 mM and diluted to working concentrations (0.005–100 μM) in methanol. Methanolic solutions of the analyte were kept at −20 °C in a dark place, until further use. The solutions were stable for at least one month. Pooled plasma, obtained from young healthy volunteers (25–35 years old), was used for the preparation of spiked plasma standards. For spiked solutions of crocetin in plasma, 25 μL of crocetin was diluted in plasma to a final volume of 500 μL.

2.3. Plasma sample pretreatment

During the optimization of plasma sample pretreatment, samples were submitted to solid phase extraction (SPE) procedure for the removal of matrix interferences during the analysis. Reversed phase Strata-X cartridges (200 mg/3 mL) consisting of a surface modified with styrene–divinylbenzene polymer were obtained from Phenomenex (Torrance, CA, USA). The cartridges were conditioned with methanol and equilibrated in loading buffer. In the proposed method, 500 μL of plasma sample was diluted in 500 μL of MeOH:CH_3COOH (25 mM) containing 10% NaCl (w/v) (10:90, v/v), centrifuged and the supernatant was loaded onto the cartridges. Washing was performed with 1 mL of MeOH–CH_3COOH (25 mM) (5:95, v/v). The retained crocetin was eluted with 6 mL NH_3–MeOH–water (5:90:5, v/v/v). Finally, the eluate was evaporated to dryness in a speed Vac system (Labconco Corp., Kansas city, MO, USA). The residues were stored at −20 °C until the next day. Dry residues were redissolved in 100 μL of the HPLC mobile phase.

2.4. HPLC determination of crocetin in plasma

The chromatographic system consisted of an Ultimate 3000 Pump (Pump LPG-3400 A, Dionex Corporation, Sunnyvale, CA, USA) with a 20 μL Rheodyne 8125 injector (Rheodyne, Ronhert Park, CA, USA). The Column Compartment (TCC-3100) was stabilized at 40 °C and UV-detection was performed with a diode array detector (DAD), Ultimate DAD-3000. Data were collected, stored and integrated on a Chromeleon v 6.80 Systems software. Separation of analytes was performed on a Luna C-18 reversed-phase column (250 mm × 4.6 mm I.D., 5 μm particle size) from Phenomenex (Torrance, CA, USA). Elution was performed with methanol–water–TFA (75.0:24.5:0.5, v/v/v) for 30 min. The flow rate of the mobile phase was 1 mL min^{-1}. Spectra were monitored by the diode array detector.

2.5. Method validation

The proposed method was validated according to the official guidelines of FDA for bioanalytical methods [12]. Selectivity was investigated by analysis of blank samples (plasma) from at least 6 healthy individuals. The calibration curve was obtained by plotting the sum of peak areas (y) of *trans*- and *cis*-crocetin versus their concentration (x). Nine different concentrations (0.002, 0.02, 0.2, 0.5, 1, 2, 5, 10, 20 μM) were used for total crocetin. Good linearity was determined through the correlation coefficient (R^2), which should be better than 0.99. The LLOD was determined as the analyte concentration yielding signal with a signal-to-noise (S/N) ratio of 3:1, whereas the LLOQ was defined as the analyte concentration yielding signal with S/N ratio 10:1. The noise was evaluated as the largest deviation of detector signal of baseline. Intra-day accuracy and precision were estimated with five analyses on the same day of plasma samples spiked with crocetin at three different concentration levels: 0.15, 3, 15 μM. The accuracy was calculated by comparison of each concentration measured to the nominal concentration of the standard solution and was expressed as % of the relative error values whereas precision was numerically expressed by relative standard deviation (RSD) of values. Inter-day accuracy and precision of the method were estimated with three analyses of the plasma samples spiked with crocetin (0.15, 3, and 15 μM) ten days later. Precision and accuracy should be less than 15% except for the LLOQ where the values should not exceed 20%. The stability of crocetin is determined by analysis of the low (0.15 μM) and the high (15 μM) spiked plasma standards after three freeze (at −20 °C and −70 °C) and thaw cycles, and after 24-h storage at room temperature. SPE recovery of total crocetin was calculated by comparison of the determined concentrations obtained from analysis of spiked plasma samples after SPE to those calculated from the analysis of the respective concentrations of crocetin standards in methanol (5 times the concentration of spiked plasma samples).

2.6. HPLC analysis of various saffron infusions

Saffron infusion was prepared by adding 150 mL of hot water (80 °C) to 200 mg of saffron and leaving the stigmas steep for 5 min. The infusion was centrifuged and the supernatant was used directly for HPLC analysis. In order to compare the HPLC profile to that of the standard aqueous methanol extract, saffron was extracted with methanol:water (1:1, v/v) (3 mL/50 mg) for 4 h at 25 °C with continuous stirring. The extract was centrifuged, filtered through a 0.2-μm filter and evaporated to dryness. The residue was stored at −20 °C until further use and redissolved in methanol:water (1:1, v/v). All procedures were performed in the absence of light.

Chromatographic analysis was performed on a Supelcosil C18 (5 μm, 25 cm × 4.6 mm, Sigma–Aldrich) column on a Mod.10 AKTA instrument (Amersham Biosciences, Piscataway USA) as described previously by minor modifications [13,14]. Elution was performed with methanol:water (20:80, v/v) for 2 min, a gradient of methanol (20–70%) for 50 min, with a gradient of methanol (70–100%) for 5 min and 100% methanol for 1 min with a flow rate 0.7 mL/min. Both solvents (water and methanol) contained 1% (v/v) acetic

acid. Detection wavelength was 440 nm. Semi-quantification was carried out by multiplying the peak area of each crocin with the respective molecular coefficient absorbance value (89,000 for *trans*- and 63,350 for *cis*-crocins) and expressed as the percentage of each crocin in relation to the total crocin content [15].

2.7. Method application: study design and biologic material

For the preparation of saffron infusions, hot water (80 °C, about 150 mL) was added to one cup containing 200 mg saffron stigmas which were allowed to steep for 5 min. Four young (25–35 years old, 3 female and 1 male, normal BMI) healthy volunteers participated in the study and their written informed consent was obtained. The collection of the biologic material and the procedure followed conformed to the ethical standards of the Helsinki Declaration of 1975 and its latest revisions. The study was approved by the local Bioethics Committee of the University of Patras and all procedures took place in the University Hospital of Patras under medical supervision. All volunteers drank the saffron tea at about 09:00–10:00 a.m. after overnight fasting. Blood was collected in EDTA coated tubes immediately before (0 h), 2 and 24 h after tea consumption. Plasma was prepared by centrifugation of blood at 1000 × *g* for 15 min at 4 °C and then kept at −20 °C till analysis (next day).

3. Results and discussion

3.1. Method development

Initial experiments were performed with pure crocetin standards in methanol. For the determination of the mobile phase, a number of elution systems were examined. The use of a gradient of AcCN:water (15–75% of the organic phase in 25 min) showed a very broad peak at 5 min. Addition of ammonium acetate at the final percentage of 0.1% (w/v) in both solvents resulted in two broad and tailing peaks (a main one at 13 and a minor at 20 min). Better HPLC profiles with good resolutions but still broad and tailing peaks (main peak at 9 and minor at 20 min) were observed with isocratic elution with MeOH–water–acetic acid (75:23:2, v/v/v), as earlier suggested [11]. Decrease in the percentage of acetic acid led to longer elution times. Replacing acetic acid with TFA to the optimum mobile phase composition of MeOH–water–TFA (75.0:24.5:0.5, v/v/v) resulted in sharp peaks at 10.7 and 18.6 min (Fig. 1). A higher amount (>0.5%) of TFA causes broadening of the peaks and increase in the peak area of the late-eluting substance.

Monitoring of spectra of the two peaks by the diode array detector showed that both of them share maximum double peaks at 420–460 nm, characteristic of the carotenoids; the earlier eluting peak has another maximum at 250 nm whereas the late-eluting peak has two maxima at 250 nm and 310–330 nm (Fig. 1). Thus, the peak at 10.7 min corresponds to *trans*-crocetin and the second one at 18.6 min to *cis*-crocetin.

This is the first report stressing that during HPLC analysis of *trans*-crocetin (high purity standard both commercially available and that prepared by alkaline hydrolysis of saffron extract as earlier described [10,16,17]), a small amount (about 10%) of the *cis*-isomer always appears irrespective of the matrix (i.e. in pure crocetin standards or in plasma spiked with crocetin), and is well resolved from the *trans*-isomer, i.e. it elutes much later; in our optimum conditions it elutes 8 min later. The existence of the *cis*-isomer is probably a result of the susceptibility of carotenoids to various factors, e.g. light and acids. Earlier HPLC studies on determination of plasma crocetin by Xi et al. and Asai et al. do not report the existence of the *cis*-isomer [10,11].

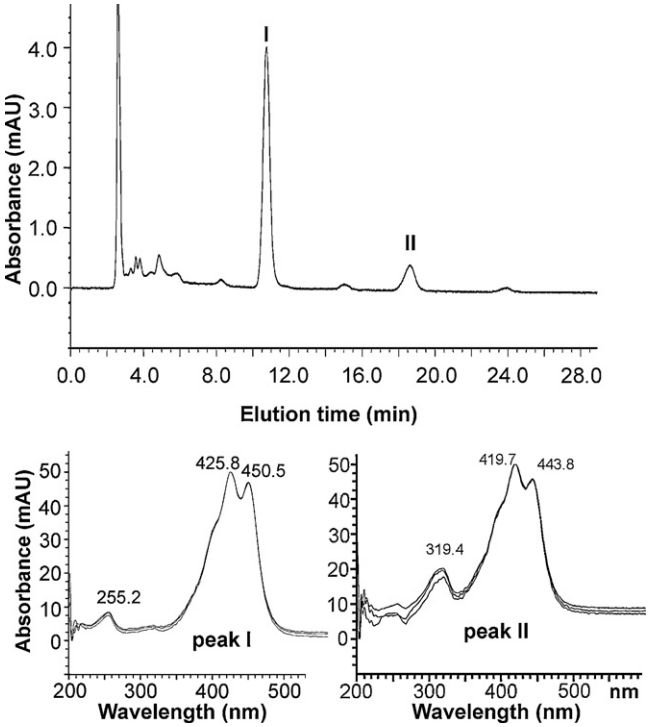

Fig. 1. Crocetin determination. HPLC chromatogram of plasma spiked with crocetin (upper panel, I: *trans*-crocetin, II: *cis*-crocetin) and UV–visible absorption spectra (lower panel). Absorbance maximum of peak I appear at approximately 423 nm whereas peak II displays additional maxima at 320 nm.

Purification and condensation of plasma samples were accomplished via SPE. All experiments were conducted with plasma samples spiked with crocetin. Having in mind the acid–base balance of the carboxylic groups of crocetin at different pH values, reversed phase SPE columns were loaded with 500 μL plasma diluted in water (1:1, v/v), washed with MeOH–phosphate buffer (50 mM, pH 3.0) (20:80, v/v) (2 mL), and eluted with 6 mL NH_3–MeOH–water (5:90:5, v/v/v). About 50% of crocetin was recovered but nearly 30% of crocetin was lost in the washing procedure, probably because of strong interactions with plasma proteins eluted at that step. Indeed, repetition of the procedure without MeOH in the washing step had no difference on recovery values. Non-specific binding of crocetin to human serum albumin via H-bonding, the stability of those complexes and the changes on protein secondary structure have been earlier shown by Kanakis et al. [18]. In order to disrupt those complexes, a high concentration of salt, a small percentage of methanol and low pH values were used. In the proposed method, 500 μL of plasma sample was diluted in 500 mL of MeOH–CH_3COOH (25 mM) containing 10% NaCl (w/v) (10:90, v/v). Samples were loaded on cartridges after centrifugation, and washing was performed with 1 mL of MeOH–CH_3COOH (25 mM) (5:95, v/v). The retained crocetin was eluted with 6 mL NH_3–MeOH–water (5:90:5, v/v/v), as in previous tests. Those changes led to high recovery values; 94.8% at the concentration of 0.2 μM, 83.3% at 0.5 μM and 72.3% at 20 μM.

3.2. Method validation

Method's quality parameters were studied under optimum separation parameter conditions using plasma spiked with crocetin at final concentrations ranging from 0.002 to 20 μM. The calibration curve of total crocetin (sum of areas of two peaks) displayed a good linearity for concentrations of 0.020–20 μM ($R^2 = 0.999$, $y = 1.131x - 0.054$). In detail, calibration curves were linear from

Table 1
Accuracy and precision data (intra- and inter-day) for analysis of plasma samples spiked with crocetin expressed as relative error (% RE) and relative standard deviation (% RSD), respectively.

	Concentration (μM)	Accuracy (% RE)	Precision (% RSD)
Intra-day	0.15	5.55	6.34
	3	−1.25	2.11
	15	5.72	1.05
Inter-day	0.15	12.73	16.64
	3	−0.07	3.56
	15	6.52	1.32

Table 2
Percentages (%) of crocins extracted by methanol–water (1:1, v/v) and by hot water (infusion).

Crocins	50% aqueous MeOH for 4 h	Infusion in 80 °C water for 5 min
Trans-crocin-4	50.1 ± 1.4	59.5 ± 0.2
Trans-crocin-3	21.1 ± 0.5	21.0 ± 0.4
Cis-crocin-4	9.1 ± 2.9	5.0 ± 0.1
Trans-crocin-2	3.8 ± 1.3	1.3 ± 0.2
Cis-crocin-3	7.8 ± 2.2	8.2 ± 0.2
Cis-crocin-1	1.8 ± 0.4	1.1 ± 0.3

Crocin-4, crocetin-di-(β-D-gentiobiosyl) ester; crocin-3, crocetin–(β-D-glycosyl)-(β-D-gentiobiosyl) ester; crocin-2, crocetin (β-D-gentiobiosyl) ester; crocin-1, crocetin (β-D-glycosyl) ester.

0.020 to 20 μM for *trans*-crocetin (R^2 = 0.999) and from 0.569 to 10 μM for *cis*-crocetin (R^2 = 0.978). LLOD values are 0.002 and 0.170 μM for *trans*- and *cis*-crocetin, and the respective LLOQ values are 0.020 and 0.569 μM.

The selectivity of the method was determined by analysis of blank plasma samples of six different healthy individuals. HPLC analysis showed no endogenous peaks at the retention times of the crocetin isomers (Fig. 2A).

The intra and inter-day accuracy and precision for the total amount of crocetin were less than 15% and at the LLOQ less than 20% (Table 1). Results are presented as sum of all isomers. These values show that the proposed methodology is reproducible and suitable for the quantitative determination of crocetin in human plasma samples. Peak asymmetry and theoretical plates for *trans*-crocetin are 1.249 ± 0.134 and 3966 ± 408, respectively, whereas for *cis*-crocetin the respective values are 1.126 ± 0.093 and 4327 ± 437.

The short-term (up to 24 h in room temperature) and freeze–thaw (after three cycles) stability was also studied by comparing the mean back-calculated concentrations of treated samples to those of freshly prepared ones. Low-concentration samples (0.15 μM) were not stable at room temperature for 24 h and after three freeze (at −20 °C)–thaw cycles since differences were around 30 and 48%, respectively. High concentration samples (15 μM) were stable in all conditions (differences < ± 15%).

3.3. Analysis of saffron infusions

Saffron infusions were analyzed by HPLC and the fingerprint obtained is in complete agreement with previous reports [14,15,19]. Ten peaks could be identified: two absorbing at 250 nm (picrocrocin and HTCC) and eight absorbing at 440 nm (*trans*-crocin-4, *trans*-crocin-3, *trans*-crocin-2′, *cis*-crocin-5, *cis*-crocin-4, *trans*-crocin-2, *cis*-crocin-3, *cis*-crocin-2). Semi-quantification showed that saffron tea preparation does not induce crocin isomerization and hydrolysis (Table 2). Indeed, in saffron infusions, the only noteworthy changes concern *trans*- and *cis*-crocin-4: content of *trans*-crocin-4 is higher and of *cis*-crocin-4 is lower. The lower percentage of *trans*-crocin-4 in aqueous methanolic extracts might be explained by the higher extractability of less polar crocins in the presence of methanol; the low-degree formation of methyl esters in the presence of methanol has been put forward [20] although Kanakis et al. showed the requirement of non-aqueous conditions for the preparation of dimethylcrocetin [18].

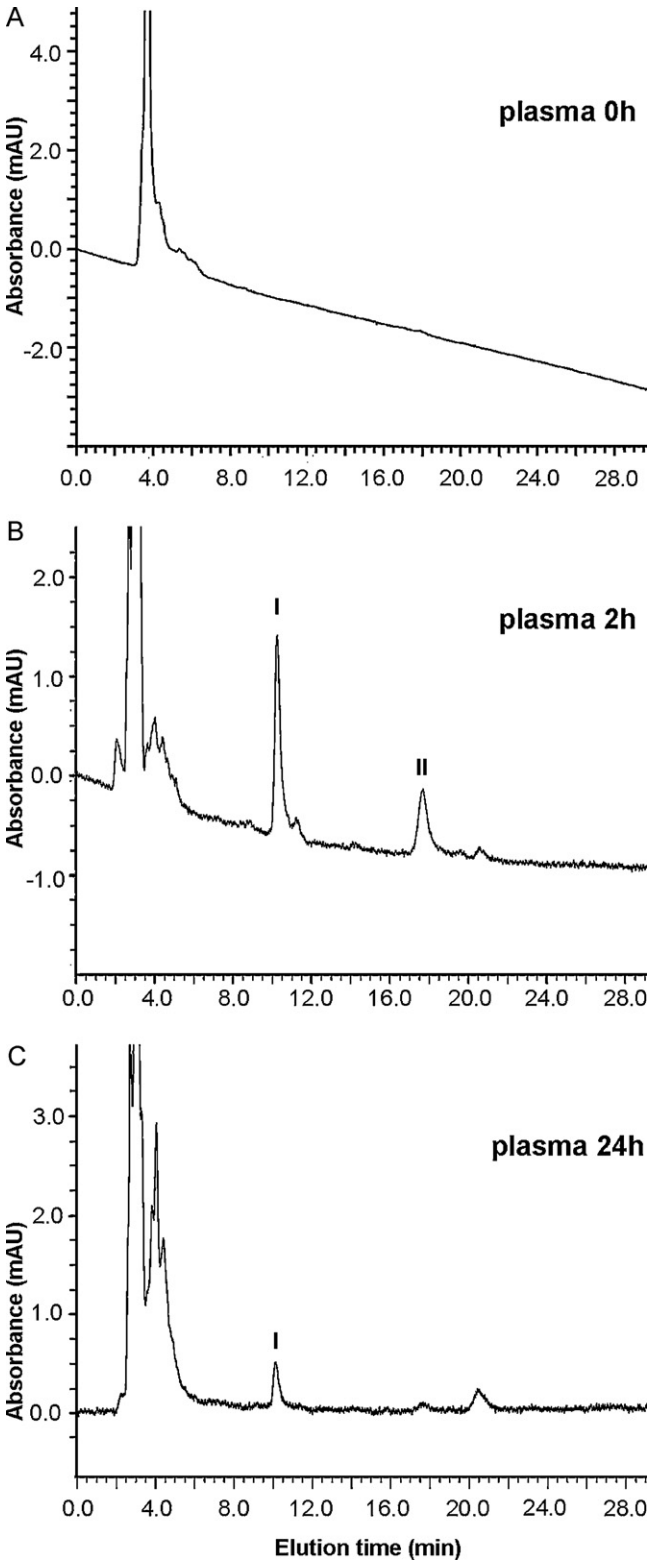

Fig. 2. Plasma analysis before and after saffron tea consumption. HPLC chromatograms of human plasma before saffron administration (A) after 2 h (B) and after 24 h (C). I: *trans*-crocetin, II: *cis*-crocetin.

Table 3
Concentration (μM) of total crocetin determined by SPE-HPLC-DAD analysis in human plasma samples and % percentage of *cis*- isomers.

Sample	Total crocetin (μM)		*Cis*-crocetin/total crocetin (%)	
	2 h	24 h	2 h	24 h
1	3.67	0.12	49.4	ND[a]
2	2.00	0.10	24.1	ND[a]
3	1.24	0.24	27.5	ND[a]
4	1.27	0.13	24.5	ND[a]

[a] ND, *cis*-crocetin was not detected.

3.4. Application to determination of plasma crocetin after saffron tea consumption

The developed SPE-HPLC analytical methodology was applied for the quantification of crocetin in plasma of healthy humans. Plasma was taken before (0 h) and after (2 and 24 h) saffron tea consumption. The results show that, after consumption of a cup of tea from 200 mg saffron, crocetin is detected in blood stream and trace amounts are found even after 24 h (Fig. 2, Table 3).

This is the first report showing crocetin concentrations in human plasma after saffron consumption. These results confirm previous studies performed in rodents, which showed that orally administered crocetin is quickly absorbed into blood plasma whereas crocins are hydrolysed to crocetin in the gastrointestinal tract before entering blood circulation [10,11]. Asai et al. observed a peak in crocetin concentration at 30 min after administration of a mixed micelle solution (0.2 mL) containing crocetin or crocins (40 nmol each) [10] whereas Xi et al. showed that plasma concentrations of crocetin in rats began to decline after 1 h [11]. In the study of Asai et al., crocetin glucuronides are also identified probably indicating the partial metabolism of crocetin to the glucuronide conjugates in the intestinal mucosa, in the liver, or in both [10]. However, in our experiments, spectra monitoring of early eluting peaks does not reveal the existence of glucuronides in the chromatograms. This does not contradict earlier findings since glucuronides might be lost during SPE. Treatment with glucuronidase before SPE as suggested by Asai et al. will aid future pharmacokinetic studies.

Both isomers were detected in blood plasma 2 h after saffron tea drinking, whereas the *cis*-isomer was not detected after 24 h because it was below LLOD. In three out of four individuals, the *cis*-isomer accounted for about 25% of total crocetin, whereas in the other one it accounts for nearly 50%. The higher determined percentages of the *cis*-isomer in blood samples after saffron tea consumption compared to the usual 10% we observed in pure *trans*-crocetin standards and spiked plasma samples may be the combined result of *in vivo* isomerization and hydrolysis of ingested *cis*-crocins (about 15%) present in saffron infusion in the gastrointestinal tract. Various isomers of lycopene (mainly *cis*- and *trans*-) are also found in blood samples, with the ratio of the *cis*-isomer reaching the 60–80% of total lycopene [21] even though lycopene in foods occurs mainly in the all-*trans* form. The mechanisms underlying the formation, interconversion or biological roles of various crocetin isomers remain enigmatic.

4. Concluding remarks

An analytical SPE-HPLC method was developed for total crocetin determination in human plasma, which displays good selectivity, linearity, sensitivity, accuracy and precision. The method was applied to the determination of crocetin in blood plasma of healthy human individuals before and after saffron tea consumption. Crocetin was not detectable before tea drinking, but its concentration was high after 2 h (1.24–3.67 μM) and still determined after 24 h (0.10–0.24). This first study in humans confirms findings of previous pharmacokinetic studies in rodents showing that orally ingested crocins are hydrolysed to crocetin before entering blood circulation. This is the first report showing the determination of the *cis*-isomer of crocetin in small percentages (10%) in pure crocetin or spiked plasma samples due to interconversion of the *trans*-isomer during analysis to a small degree. The significance of this observation is manifested in the determination of crocetin in healthy human individuals after saffron tea consumption; percentages of the *cis*-isomer range from 25 to 50% suggesting *in vivo* isomerization. At last, we showed that saffron tea preparation does not induce changes in saffron composition concerning the presence of individual crocins. This HPLC method enables the determination of crocetin concentrations in humans and the knowledge of actual concentrations and the percentages of the *trans*- and *cis*-isomers after saffron consumption will aid future nutritional or pharmacokinetic studies.

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References

[1] B. Matthew, Crocus: A Revision of the Genus *Crocus*, Timber Press, 1983.
[2] S.C. Ferrence, G. Bendersky, Therapy with saffron and the goddess at Thera, Perspect. Biol. Med. 47 (2004) 199–226.
[3] WHO Monographs on Selected Medicinal Plants, vol. 3, World Health Organization, 2007, pp. 126–135.
[4] D. Mohajeri, G. Mousavi, M. Mesgari, Y. Doustar, M. Khayat Nouri, Subacute toxicity of *Crocus sativus* L. (Saffron) stigma ethanolic extract in rats, Am. J. Pharm. Toxicol. 2 (2007) 189–193.
[5] M.H. Modaghegh, M. Shahabian, H.A. Esmaeili, O. Rajbai, H. Hosseinzadeh, Safety evaluation of saffron (*Crocus sativus*) tablets in healthy volunteers, Phytomedicine 15 (2008) 1032–1037.
[6] A. Shamsa, H. Hosseinzadeh, M. Molaei, M.T. Shakeri, O. Rajabi, Evaluation of *Crocus sativus* L. (saffron) on male erectile dysfunction: a pilot study, Phytomedicine 16 (2009) 690–693.
[7] S. Akhondzadeh, M. Shafiee Sabet, M.H. Harirchian, M. Togha, H. Cheraghmakani, S. Razeghi, S.S. Hejaz, M.H. Yousefi, R. Alimardani, A. Jamshidi, S.A. Rezazadeh, A. Yousefi, F. Zare, A. Moradi, A. Vossoughi, A 22-week multicenter, randomized, double-blind controlled trial of *Crocus sativus* in the treatment of mild-to-moderate Alzheimer's disease, Psychopharmacology (Berl.) 207 (2010) 637–643.
[8] M. Agha-Hosseini, L. Kashani, A. Aleyaseen, A. Ghoreishi, H. Rahmanpour, A.R. Zarrinara, S. Akhondzadeh, *Crocus sativus* L. (saffron) in the treatment of premenstrual syndrome: a double-blind, randomised and placebo-controlled trial, BJOG 115 (2008) 515–519.
[9] S.K. Verma, A. Bordia, Antioxidant property of Saffron in man, Indian J. Med. Sci. 52 (1998) 205–207.
[10] A. Asai, T. Nakano, M. Takahashi, A. Nagao, Orally administered crocetin and crocins are absorbed into blood plasma as crocetin and its glucuronide conjugates in mice, J. Agric. Food Chem. 53 (2005) 7302–7306.
[11] L. Xi, Z. Qian, P. Du, J. Fu, Pharmacokinetic properties of crocin (crocetin digentiobiose ester) following oral administration in rats, Phytomedicine 14 (2007) 633–636.
[12] Guidance for Industry – Bioanalytical Method Validation, U.S. Department of Health and Human Services – Food and Drug Administration (CDER), 2001.
[13] P. Lozano, M.R. Castellar, M.J. Simancas, J.L. Iborra, Quantitative high-performance liquid chromatographic method to analyse commercial saffron (*Crocus sativus L.*) products, J. Chromatogr. A 830 (1999) 477–483.
[14] 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.
[15] M. Carmona, A. Zalacain, J.E. Pardo, E. Lopez, A. Alvarruiz, G.L. Alonso, Influence of different drying and aging conditions on saffron constituents, J. Agric. Food Chem. 53 (2005) 3974–3979.
[16] P.A. Tarantilis, M. Polissiou, M. Manfait, Separation of picrocrocin, *cis*–trans-crocins and safranal of saffron using high-performance liquid chromatography with photodiode-array detection, J. Chromatogr. A 664 (1994) 55–61.
[17] C.D. Kanakis, P.A. Tarantilis, H.A. Tajmir-Riahi, M.G. Polissiou, Interaction of tRNA with safranal, crocetin, and dimethylcrocetin, J. Biomol. Struct. Dyn. 24 (2007) 537–546.

[18] C.D. Kanakis, P.A. Tarantilis, H.A. Tajmir-Riahi, M.G. Polissiou, Crocetin dimethylcrocetin, and safranal bind human serum albumin: stability and antioxidative properties, J. Agric. Food Chem. 55 (2007) 970–977.
[19] M.A. Papandreou, C.D. Kanakis, M.G. Polissiou, S. Efthimiopoulos, P. Cordopatis, M. Margarity, F.N. Lamari, Inhibitory activity on amyloid-beta aggregation and antioxidant properties of *Crocus sativus* stigmas extract and its crocin constituents, J. Agric. Food Chem. 54 (2006) 8762–8768.
[20] H. Pfander, Carotenoid glycosides, Pure Appl. Chem. 47 (1976) 121–128.
[21] K. Wu, S.J. Schwartz, E.A. Platz, S.K. Clinton, J.W. Erdman Jr., M.G. Ferruzzi, W.C. Willett, E.L. Giovannucci, Variations in plasma lycopene and specific isomers over time in a cohort of U.S. men, J. Nutr. 133 (2003) 1930–1936.