STABILITY DEPENDENCE OF BIOACTIVE APOCAROTENOIDS OF KROKOS KOZANIS IN THE PRESENCE OF NATURAL PHENOLIC ANTIOXIDANTS. ITS SIGNIFICANCE IN BIOAVAILABILITY STUDIES

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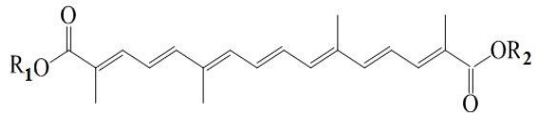
Abstract – The present study aimed at examining the limitations of the protective effect that strong phenolic antioxidants have on the oxidation of saffron apocarotenoids (sugar esters of crocetin or crocins, CRTSEs) using an *in vitro* gastrointestinal digestion model that mimics physiological conditions in the upper digestive tract. The bioaccessibility of CRTSEs from infusions of two commercial blends of saffron with dried fruits, herbs and spices was investigated. Results are discussed with regard to the total phenol content, DPPH radical scavenging activity and also changes in the contents of individual phenolic constituents of the infusions during the digestion procedure. The findings are of importance to establish the minimum phenol concentration required to protect crocins upon encapsulation processes that take place at elevated temperatures (e.g. spray drying).

Keywords: saffron, bioaccessibility, herbal infusions, phenolic compounds

1. INTRODUCTION

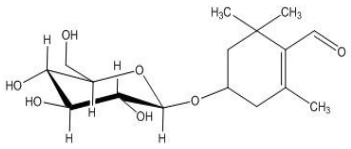
Saffron, the most expensive spice in the world, derives from the dehydrated red stigmas of the plant *Crocus sativus* L. which is cultivated in specific regions in Asia (mainly Northern Iran) and Europe (mainly Greece). The major European producing area is located in a small geographic region around the Greek town of Kozani (North Western Macedonia, Greece) from where the PDO product Krokos Kozanis originates. Except for domestic trade, the particular product is exported to other European countries, the Emirates and Saudi Arabia representing an important figure in the worldwide saffron market.

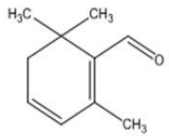
Saffron spice in general is highly valued in the food and beverage industry for the (i) bright orange yellow hues that are attributed to a group of water-soluble apocarotenoids which are sugar esters of crocetin (8,8′-diapocarotene-8,8′-dioic acid) and are known as crocins, (ii) the unique bitter taste that is mainly assigned to the colorless monoterpene glucoside picrocrocin (4-(*β*-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) and (iii) the delicate aroma mainly due to the presence of safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) (Fig. 1) [1]. In cases of high quality saffron, these secondary metabolites account for half of its dry weight [2].



$R_1 = R_2 = H$ (crocetin)

R_1, R_2 = glucose and/or gentiobiose (major crocetin sugar esters)





Picrocrocin Safranal

Fig. 1. Chemical structures of the major saffron bioactive compounds.

There is a growing body of evidence from epidemiological studies of the last decades that saffron extracts and individual constituents may exert multiple biological actions including anticancer, anti-inflammatory, antimicrobial and antioxidant so that it can be considered as a functional spice and a high added-value agricultural product [3]. Though the medicinal properties of saffron are thoroughly examined [4], the bioaccessibility/bioavalability studies of the responsible apocarotenoids are rather limited [5,6,7]. Bioaccessibility of a particular compound depends on interactions with other dietary constituents. In our previous study [8], it was evidenced that strong phenolic antioxidants

(rosmarinic acid, caffeic acid) in excess, positively affect the stability of crocins during *in vitro* gastrointestinal digestion. Thus, crocins from saffron-containing herbal tea infusions rich in hydroxycinnamic acids were 1.1-1.5 times more bioaccessible than those from reference infusion (containing only saffron). This effect was even stronger when more complex phenolic compounds such as flavan-3-ols and derivatives were abundant in the test infusions.

2. AIM OF THE STUDY

In the present study we examined possible limitations concerning the stability effect of phenolic antioxidants on crocins. The bioaccessibility of crocins from two commercial products representing different herbal tea blends with Krokos Kozanis was investigated. The specific brands were selected on the basis of their rather moderate phenolic content and radical scavenging properties as reported in our previous study [8]. The results are discussed taking into account the complexity in chemical composition of the corresponding infusions, as indicated in Table 1.

Table 1. Ingredients and chemical composition of the examined herbal tea blends with saffron.

Herbal infusion	Major ingredients	Expected major compounds in infusion based on literature	Ref.
 #1	Orange peel, hibiscus, cinnamon, apple, cloves, natural flavours, saffron	Catechin, epicatechin and derivatives, rutin, hydroxycinnamic acid glycosides, cinnamaldehyde, *trans*-4-GG crocetin ester, picrocrocin	[9,10]
 #2	Apple, rosehips, orange leaves, orange peels, natural flavors, lemon peel, sweet blackberry, honey granules, saffron	Proanthocyanidins, catechin, hesperetin, gallic acid, chlorogenic acid, ferulic acid, ascorbic acid, *trans*-4-GG crocetin ester, picrocrocin	[11-13]

3. MATERIALS AND METHODS

3.1. Samples

Commercial herbal tea blends with saffron (1.1% w/w) were purchased from the market (Thessaloniki, Greece). Tea bags that contained only saffron at the same concentration as that claimed on the label of products were also prepared for comparison reasons. The saffron sample used as a reference was of the same batch as that used in the particular lots of the commercial samples and was donated upon request by the manufacturer.

3.2. Standards, reagents and solvents

Trans-crocetin di-(*β*-D-gentiobiosyl) ester (*trans*-4-GG crocetin ester) was laboratory isolated by semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) according to Kyriakoudi and Tsimidou [14]. In brief, the system consisted of two Marathon IV series HPLC pumps (Rigas Labs, Thessaloniki, Greece), a Rheodyne injection valve (model 7125) with a 250 μL fixed loop (Rheodyne, Cotati, CA) and a diode array linear UVIS-206 multiple wavelength detector (Linear Instruments, Fermont, CA). Separation was carried out on a Nucleosil 100 C18 (250 x 10 mm i.d.; 7 μm) chromatographic column (Macherey-Nagel, Düren, Germany). The solvents used were water (A) and methanol (B). The gradient was 0 min, 30% (B), 0-10 min, 45% (B), 10-20 min, 70% (B), 20-30 min, 100% (B), 30-40 min, 100% (B), 40-50 min, 30% (B) and the flow rate 3.0 mL/min. Monitoring was at 440 nm. Purity of isolated *trans*-4-GG crocetin ester (97%) was checked (a) chromatographically by RP-HPLC-DAD in the range of 200 – 550 nm and calculated as the percentage of the total peak area at 440 nm and (b) by Nuclear Magnetic Resonance (NMR) spectroscopy, recording the 1H 1D spectra at 300 MHz on a Brucker 300AM spectrometer (Rheinstetten, Germany). All standards, reagents and solvents were of the highest purity required.

3.3. Preparation of infusions

The preparation of infusions was carried out by immersing one tea bag in 400 mL of boiling water (90 ^{o}C) for 5 min according to the instructions of the manufacturer. For each type of infusions 9 bags from 9 different packages belonging to different lots were used. Aliquots of 100 mL per infusion were combined to form a representative sample (900 mL) that was used in all further analyses.

3.4. Total phenol content estimation using the Folin-Ciocalteu assay

The total phenol content of the infusions was determined spectrophotometrically by the Folin-Ciocalteu (F-C) assay according to [5]. Caffeic acid

(CA) was used as a reference standard, and results were expressed as mg CA equivalents/100 mg dry infusion on the basis of a calibration curve (y = 0.0099x − 0.0996, (10–100 μg/10 mL), R^2 = 0.99 (n = 6)). Briefly, in a 10-mL volumetric flask, 5 mL of water, 0.4 mL of each representative sample and 0.5 mL F-C reagent were mixed. After exactly 3 min, 1.0 mL of saturated sodium carbonate solution (37%, w/v) was added, and the mixture was agitated. The volume was adjusted with water and the flask left in the dark for 1 h at room temperature. The absorbance was measured at 750 nm (U-2000 Hitachi UV-Vis spectrophotometer, Tokyo, Japan) against a blank prepared in the same way with deionized water in the place of the infusion aliquot. All of the measurements were performed in triplicate, and results were expressed as the mean value.

3.5. DPPH radical scavenging activity

The DPPH radical scavenging activity of representative samples was assessed according to the procedure presented by Nenadis and Tsimidou [15]. In brief, samples (0.05 mL) were added to 2.9 mL of a 0.1 mM methanolic solution of DPPH·. The absorbance at 515 nm was recorded at the start and after 30 min of incubation. A calibration curve of rosmarinic acid (RA) was prepared, and the results were expressed as mg RA equivalents/100 mg dry infusion. Measurements were carried out in triplicate.

3.6. Liquid chromatographic analysis

The crocetin sugar esters (CRTSEs) present in the examined infusions were identified and quantified at 440 nm by high performance liquid chromatography (HPLC) as previously described by Kyriakoudi, Chrysanthou, Mantzouridou and Tsimidou [2]. In brief, the HPLC system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA, USA), a Midas autosampler (Spark, Emmen, The Netherlands) and a UV 6000 LP diode array detector (DAD) (Thermo Separation Products, San Jose, CA, USA). Separation was carried out on a Discovery HS C18 (250 x 4.6 mm i.d.; 5 μm) column (Supelco, Bellefonte, USA). The solvents used were a mixture of water:acetic acid (1%, v/v) (A) and acetonitrile (B). The linear gradient was 20 to 100% B in 20 min. The flow rate was 0.8 mL/min. The injection volume was 20 μL. The analytical samples were prepared after proper dilution with deionized water (1:2, v/v) and filtration through a 0.45 μm membrane filter. Chromatographic data were processed using the ChromQuest Version 3.0 software (Thermo Separation Products, San Jose, CA, USA). Monitoring was in the range of 200-550 nm. Quantification of the total CRTSEs content was accomplished with the aid of a proper calibration curve of *trans*-4-GG crocetin ester. Analysis of phenolic compounds and peak identification was carried out under the same chromatographic conditions with those for CRTSEs. Quantification in the UV-Vis region of individual groups of phenolic compounds, i.e. hydroxycinnamic acids, flavanones, flavan-3-ols, was carried out using proper calibration curves of rosmarinic acid (λ_{max} = 330 nm), naringenin (λ_{max} = 285 nm), and (-)-epicatechin (λ_{max} = 270 nm), respectively. The results were expressed as mg/100 mg dry infusion.

3.7. In vitro gastrointestinal digestion procedure

Infusions prepared from the two different blends of herbs with saffron were used directly for the *in vitro* digestion procedure, which mimics the physiological conditions in the upper digestive tract (stomach and small intestine), according to [5]. Briefly, aliquots of the infusions were transferred into amber bottles and Hank's balanced salt solution (HBSS) was added to a final volume of 20 mL. To each bottle, 1 mL of freshly prepared pepsin (0.04 g pepsin/0.1 mol/L HCl) was added and the pH was acidified to 2.0 using 1 mol/L HCl. The samples were overlaid with nitrogen gas and incubated at 37 °C for 1 h in a shaking water bath at 95 rpm to mimic the gastric phase of human digestion. The intestinal phase involved increasing the pH to 5.3 with 0.9 mol/L sodium bicarbonate followed by the addition of 200 μL of bile salts glycodeoxycholate (0.8 mmol/L), taurodeoxycholate (0.45 mmol/L) and taurocholate (0.75 mmol/L) and 100 μL of porcine pancreatin (0.08 g/mL HBSS). The final pH was adjusted to 7.4 using 1 mol/L NaOH. Samples were overlaid with a layer of nitrogen gas and incubated for 2.5 h at 37 °C to mimic the duodenal phase of human digestion. After the intestinal phase, the digestate was centrifuged at 4.100 g using a SL 16R centrifuge (Thermo Scientific, Massachusetts, USA) for 15 min at 4 °C, the supernatants were collected and filtered through a 0.45 μm membrane filter

(Sartorius Stedim Biotech GmbH, Goettingen, Germany) and stored at -18 °C until further analysis. Bioaccessibility of CRTSEs was calculated using the (1).

$$Bioaccessibility(\%) = \left(\frac{C_{digested}}{C_{undigested}} \right) x100 \qquad (1)$$

where $C_{digested}$: total CRTSEs concentration determined by HPLC after digestion, $C_{undigested}$: total CRTSEs concentration before digestion.

4. RESULTS AND DISCUSSION

The manufacturer's instructions were adopted in all experiments in order the results for the bioaccessibility of crocins from the corresponding infusions to have a common basis for comparison. A reference infusion containing only saffron was also prepared and included in the study. These results are presented in Table 2.

Table 2. % Bioaccessibility of total crocetin sugar esters (CRTSEs) in infusions of herbal tea blends containing saffron.

Infusion	% Bioaccessibilty of total CRTSEs (as *trans*-4-GG)[a,b]
#1	83.9 ± 9.2[a]
#2	85.3 ± 5.7[a]
Reference infusion	*60.9 ± 2.2*[b]

[a]Mean value of three independent measurements ± sd. [b]Different lowercase letters within the same column indicate significant differences among infusions according to Duncan's test ($p < 0.05$).

As shown in Table 2, crocins from the infusions of the two herbal teas were quite stable under the conditions of *in vitro* digestion and no significant differences were evidenced regarding their % bioaccessibility values. Moreover, the stability of crocins was found be enhanced by almost 1.4 times in the infusions of herbal tea blends containing saffron compared with the reference infusion (containing only saffron). It should be mentioned that the obtained values for the infusions of the two examined products were of the same size with those reported for a series of commercial herbal blends with saffron that contained peppermint, black or green tea, as the major ingredient [8]. Infusions from *Mentha × piperita* L. (peppermint) are rich in flavanone and flavone glycosides, as well as hydroxycinnamic acids, e.g., rosmarinic acid [16], but do not seem to contain any flavan-3-ols (catechin and epicatechin derivatives). The latter are found in abundance in *Camelia sinensis* leaves (tea) [17]. On the other hand, the infusions #1 and #2 derived from blends that are labeled to contain orange peel and rose hips (> 50% w/w) and apple and hibiscus (> 50% w/w), respectively, as the major ingredients. In those cases more complex phenols such as proanthocyanidins (oligomers and polymers of flavan 3-ol monomer units), anthocyanins, flavonoids (e.g. hesperetin, rutin) and ascorbic acid are expected to prevail. Fig. 2. shows the structures of some of the major constituents identified in infusions #1 and #2.

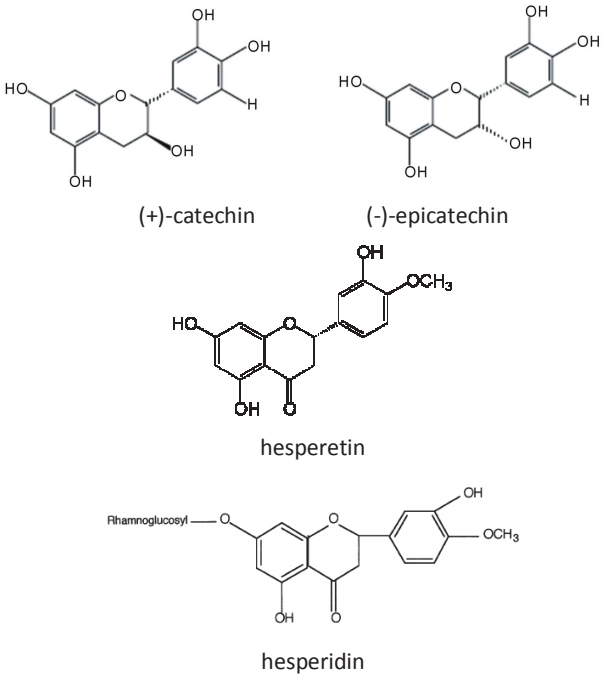


Fig. 2. Chemical structures of the major phenolic compounds present in infusions #1 and #2.

The total phenol content of the infusions #1 and #2, estimated using the F-C assay was rather low (4.7 ± 0.2 and 5.9 ± 0.1 mg CA equivalents /100 mg dry infusion, respectively). These values represent approximately 20-40 % of those reported for the infusions that are rich in peppermint, black or green tea. In line with this result, the DPPH radical scavenging activity was also very low (3.0 ± 0.6 and 3.7 ± 0.1 RA equivalents/100 mg dry infusion, respectively). Even so, the radical scavenging activity of the examined products is most probably due to the presence of phenolic constituents from herbs, fruits and spices other than saffron; the

aqueous extracts of the latter are not expected to contain appreciable amounts of phenolic compounds or to exhibit *in vitro* radical scavenging activity [5,18] but are known to exhibit *in vivo* antiradical activity as previously shown [18].

These results indicate that the greater stability of crocins from the infusions #1 and #2 during digestion cannot be easily interpreted as an effect of the radical scavenging properties of the co-present phenolic compounds, as suggested previously. Fig. 3 that illustrates the HPLC profiles of infusion #1 at 270 and 285 nm (λ_{max} of flavan-3-ols and flavanones, respectively) before and after digestion provides some evidence that higher molecular weight phenols (e.g. proanthocyanidins) may degrade during the digestion to form smaller molecules that bear catechin moieties. This degradation process might also play a role against crocin oxidation. % Losses in total flavan-3-ols from infusion #1 an #2 were 19.8 ± 5.7 and 20.3 ± 10.4 %, respectively, more than five times those reported for structurally similar compounds in infusions from black and green teas [8]. The concentration of total flavan-3-ols in the examined infusions exceeded that of total crocins by three times in #1 and six times in #2. This finding signifies once again, that phenolic compounds in excess may have a positive effect on crocin stability during *in vitro* digestion.

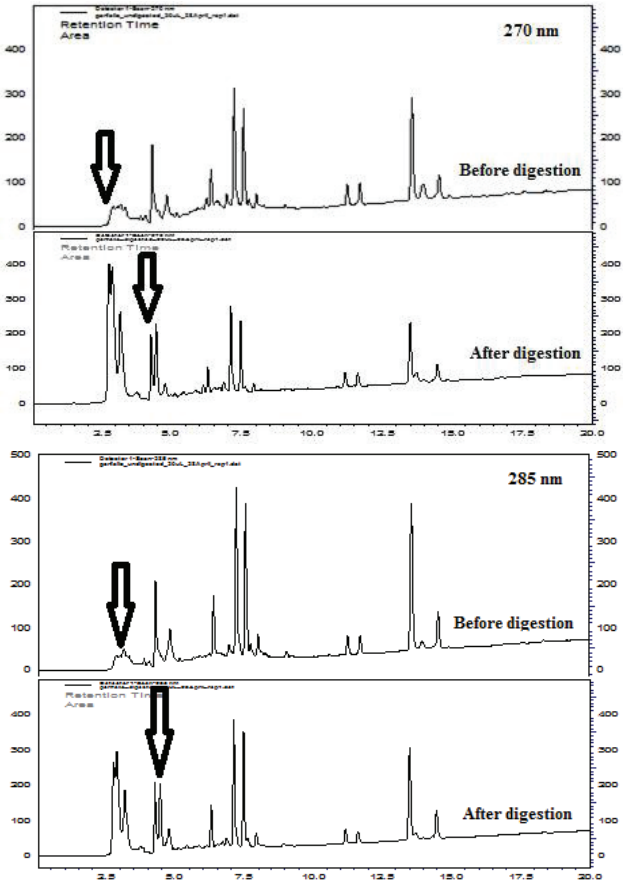


Fig. 3. HPLC-DAD profile of infusion #1 at 270 nm and 285 nm before and after *in vitro* digestion.

5. CONCLUSIONS

The study of the two blends of saffron with different herbs signify that enhanced stability of crocins during the digestion process may be achieved when polyphenols or strong radical scavengers are present in excess. This knowledge is of importance to establish the minimum phenol concentration required to protect crocins upon encapsulation processes that take place at elevated temperatures (e.g. spray drying).

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