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Changes in Total and Individual Crocetin Esters upon in Vitro Gastrointestinal Digestion of Saffron Aqueous Extracts

Anastasia Kyriakoudi,[†] Maria Z. Tsimidou,*[,†] Yvonne C. O'Callaghan,[‡] Karen Galvin,[‡] and Nora M. O'Brien[‡]

[†]Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki (AUTh), 54124 Thessaloniki, Greece

[‡]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Supporting Information

ABSTRACT: Changes that may be expected in crocetin esters (crocins) upon digestion were examined in saffron aqueous extracts for the first time. Chemical characterization of total and individual crocins and other bioactive compounds was achieved by UV−vis spectrophotometry, RP-HPLC-DAD, and LC-ESI-MS. Antioxidant activity was evaluated using in vitro assays and the comet assay. The observed loss for both total and *trans*-crocins was higher in saffron (∼50%) than in gardenia extracts (∼30%), which were also examined for comparison. Loss was lower than that reported for hydrophobic carotenoids. *cis*-Isomers were less affected, leading to the hypothesis that *trans*/*cis* isomerization may occur in parallel to degradation reactions. Monitoring changes in the extracts at oral, gastric, or intestinal phases, separately, verified this view pointing out the critical effect of pH, temperature, and duration of process but not of digestive enzymes. No isomerization and less degradation (<20% loss) was evidenced when pure *trans*-crocetin (di-β-D-gentiobiosyl) ester was subjected to gastric or intestinal conditions.

KEYWORDS: *crocetin esters, saffron, gardenia, in vitro gastrointestinal digestion, stability, bioaccessibility*

INTRODUCTION

Crocetin esters (crocins) are a series of glycosides of a C_{20}-dicarboxylic acid, called crocetin (8,8′-diapocarotene-8,8′-dioic acid). Glucose, gentiobiose, and neapolitanose are the sugar moieties that esterify the ends of crocetin. These esters originate from C_{40}-carotenoids by chain cleavage and due to their reduced size are known as apocarotenoids. They are responsible for the coloring properties of *Crocus sativus* L. dry stigmas (saffron) that comprise the most expensive spice in the world.[1] Crocins are freely water-soluble in contrast to most carotenoids and are highly valued in the food industry and gastronomy for their ability to bestow bright orange to yellow hues. *trans*-Crocetin (di-β-D-gentiobiosyl) ester (*trans*-crocin 1) is the most abundant ester with a high water solubility which is attributed to the sugar moieties. Crocins constitute approximately 30% of the total dry matter of the spice.[2] Levels depend on growing conditions of *Crocus sativus* L., processing technology, and storage conditions.[1] To date, use of saffron at levels typical of the average diet of different populations (Iranian, Indian, Greek, Spanish) is not questioned as unsafe, though available scientific data are not conclusive for its safety status.[3] In addition to their known applications in the food industry, an increasing number of studies indicates a variety of pharmacological actions for crocetin and crocins, for instance, their protective effect against atherosclerosis, tumor cell proliferation, hepatotoxicity, bladder toxicity, ethanol-induced hippocampal disorders, and their role as antioxidant quenchers for free radicals have been reported.[2,3]

To increase our understanding of the potential benefits of crocetin esters, it is important to assess their bioaccessibility.[4] According to O'Sullivan et al.,[5] carotenoid bioaccessibility is defined as "the amount of the ingested carotenoid(s) that are available for absorption in the gut after digestion". The bioaccessibility of β-carotene, lutein, other natural mixtures of lipophilic carotenoids, and bixin has been extensively investigated;[6−8] however, to our knowledge, no such data exist for crocetin esters.

The present study aimed to examine the changes that occur in crocetin esters upon digestion of saffron aqueous extracts. The in vitro digestion assay provides data that may be related to bioavailability of these apocarotenoids (type, isomer form). For further substantiation of observations, changes in crocetin esters upon digestion of aqueous extracts of *Gardenia jasminoides* Ellis dry fruits were also evaluated. The latter is a well-established source of crocetin esters.[9] Our approach is novel as to date (a) changes in total or individual crocetin esters were only examined under conditions mimicking spice storage and cooking[10−13] and (b) absorption studies of glycosidic forms of secondary metabolites have only been reported for phenolic compounds (such as flavonoids).[14−16] To the best of our knowledge, this is the first study investigating the impact of gastrointestinal conditions on edible carotenoid glycosides.

MATERIALS AND METHODS

Samples and Standards. Commercially available authentic Greek saffron was donated by the Saffron Cooperative of Kozani (Greece).

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"Fruits de gardenia" powder was purchased from a pharmacy (Zurich, Switzerland). *trans*-Crocin 1 and geniposide [1,4,5,7-tetrahydro-7-(acetomethyl)-cyclopentapyran-4-carboxylic acid methyl ester] were laboratory-prepared by semipreparative RP-HPLC. Isolation was carried out on a Nucleosil 100 C_{18} (250 × 10 mm i.d.; 7 μm) chromatographic column (Macherey-Nagel, Düren, Germany). A gradient elution was used with water (A) and methanol (B) as eluents. The gradient was 30 to 100% B in 30 min, and the flow rate was 3.0 mL/min. Monitoring was at 350 nm. Picrocrocin [4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexane-1-carboxaldehyde] was isolated according to Sánchez et al.[17] Crocetin (CRT) was prepared from an aqueous extract of saffron by acidic hydrolysis as described in Ordoudi et al.[18] Purity of isolated *trans*-crocin 1 (97%) and picrocrocin (91%) was checked (a) chromatographically by reversed-phase high-performance liquid chromatography diode array detector (RP-HPLC-DAD) in the range of 200−550 nm and calculated as the percentage of the total peak area at 440 and 250 nm, respectively, and (b) by nuclear magnetic resonance (NMR) spectroscopy recording the ^{1}H 1D spectra at 300 MHz on a Bruker 300AM spectrometer (Rheinstetten, Germany). Purity of isolated geniposide (91%) and CRT (90%) was checked chromatographically by RP-HPLC-DAD in the range of 200−550 nm and calculated as the percentage of the total peak area at 240 and 440 nm, respectively. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97%) was from Aldrich Chemical Co. (Steinheim, Germany), and caffeic acid (98%) was from Sigma Chemical Co. (St. Louis, MO, USA).

Reagents and Solvents. All of the chemicals from various suppliers were of the highest purity needed for each assay. In particular, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^\bullet$) were from Sigma Chemical Co. Folin-Ciocalteu (F-C) phenol reagent was obtained from Panreac Quimica (Barcelona, Spain). 2,2′-Azobis(2-aminopropane)dihydrochloride (AAPH) was from Fluka Chemie (Buchs, Switzerland). The enzymes used (pancreatin, pepsin, mucin) were from Sigma Chemical Co. Human monocytic blood U937 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Fetal bovine serum was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were from Cruinn Diagnostics (Dublin, Ireland). All other cell culture chemicals and reagents were from Sigma Chemical Co. (Dublin, Ireland). All solvents used were of HPLC grade. Ultrahigh purity water was produced in the laboratory using a Millipore-Milli-Q-system (Barstead International, Dubuque, IA, USA).

Preparation and Characterization of Saffron and Gardenia Dry Fruit Aqueous Extracts. *Apocarotenoid Characterization.* For the apocarotenoid characterization of the plant materials, aqueous extracts were prepared as follows: saffron and gardenia dry fruit were carefully ground with a pestle and mortar just prior to analysis. The finely ground plant material (0.05 g) was extracted with ultrahigh purity water (100 mL) by rigorous agitation (~500 rpm) for 1 h at ambient temperature. All manipulations were performed under subdued (yellow) light to minimize photodecomposition of crocetin esters. The characterization was achieved using UV−vis spectrophotometry, RP-HPLC-DAD, and where necessary liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The UV−vis spectra of all of the extracts after appropriate dilution (1:10, v/v) were recorded in the region 200−600 nm with a spectrophotometer (Shimadzu UV 1601, Kyoto, Japan) equipped with quartz cells (1 × 1 × 4 cm). Absorption measurements in triplicate were obtained for each solution and ranged from 0.6 to 1.2 (photometric range of the instrument = −0.5−3.99). The results were expressed as $E^{1\%}_{\lambda_{max}}$ according to the equation[19] $E^{1\%}_{\lambda_{max}} = D \times 10000/m\ (100 - H)$, where D is the absorbance value; m is mass (g); H is the moisture and volatile content of the sample (%, w/w); and λ_{max} is for crocins at 440 nm. Derivative spectra were calculated using the UVPC 1601 (Personal Spectroscopy Software, v.3.9, Shimadzu) software facilities (smoothing parameter of 17 experimental points, delta lamda of 10). 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). Separation was carried out on a LiChroCART Superspher 100 C_{18} (125 × 4 mm i.d.; 4 μm) column (Merck, Darmstadt, Germany). A gradient elution was used with 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 0.5 mL/min, and the injection volume was 20 μL. Chromatographic data were processed using ChromQuest version 3.0 software (Thermo Separation Products). Monitoring was in the range of 200−550 nm, and quantification of crocetin esters, picrocrocin, and geniposode was carried out by integration of the peak areas at 440, 250, and 240 nm, respectively. Peak identification was based on comparison of retention times, UV−vis spectra matching with those of available standards and literature data.[20,21] LC-ESI-MS (model 2010 EV, Shimadzu, Kyoto, Japan) on the positive and negative ion modes was then employed for the characterization of crocin 1 (*trans* and *cis*), crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester or crocin 2 (*trans* and *cis*),crocin 2 (*trans*), picrocrocin, and geniposide and verification of their presence in saffron and gardenia extracts. Separation was achieved on the same chromatographic column. Quantification of total and individual crocetin esters was accomplished with the aid of a calibration curve of *trans*-crocin 1 within the range of 95−480 ng/10 μL [$y = 34891x - 774339$; $R^2 = 0.99$ ($n = 6$)]. The picrocrocin content of saffron was also determined by constructing a calibration curve within the range of 90−913.5 ng/10 μL [($y = 10325x - 12380$; $R^2 = 0.99$) ($n = 5$)].

Characterization of Other Bioactive Compounds in Saffron and Gardenia Dry Fruit Aqueous Extracts. Aqueous extracts (500 and 5000 mg/L) of finely ground plant material were prepared as aforementioned. The geniposide content of gardenia dry fruit was determined under the same RP-HPLC-DAD conditions using a calibration curve within the range of 100−1000 ng/10 μL [$y = 4949x - 154922$, $R^2 = 0.99$ ($n = 6$)]. The total polar phenol content of saffron and gardenia extracts as well as their reducing power were determined spectrophotometrically by the Folin-Ciocalteu assay as described elsewhere.[22] Caffeic acid and Trolox were used as reference standards. The total phenol content was expressed as microgram caffeic acid/gram dry material, whereas the reducing power of the above-mentioned extracts was expressed as Trolox equivalents (mM) on the basis of calibration curves constructed for the two standards: (i) caffeic acid ($y = 0.0094x + 0.0174$, 1−25 μg/10 mL, $R^2 = 0.99$ ($n = 5$), (ii) Trolox ($y = 0.1777x - 0.0233$, 0.2−4.0 mM, $R^2 = 0.99$ ($n = 6$). The repeatability of measurements calculated for a standard solution and an extract were found to be satisfactory (CV% = 2.0 and 3.0, respectively, $n = 5$). All of the subsequent measurements of test samples were then performed in triplicate.

In Vitro Antioxidant Studies. *DPPH$^\bullet$ Radical Scavenging Activity.* Estimation of the DPPH radical scavenging activity was determined by a modification of the procedure outlined by Nenadis and Tsimidou.[23] Saffron and gardenia aqueous extracts were added to 2.9 mL of a 0.1 mM methanolic solution of DPPH$^\bullet$. The absorbance at 515 nm was recorded following a 30 min incubation. The results were expressed as % radical scavenging activity values (%RSA) by using the formula $\%RSA = [Abs_{515(t=0)} - Abs_{515(t)}] \times 100/Abs_{515(t=0)}$ after correction with an appropriate blank. The repeatability of measurements calculated for the extracts was found to be satisfactory (CV% = 2.3, $n = 5$). Subsequent measurements were then carried out in triplicate for each one of the test samples.

ABTS$^{\bullet+}$ Assay. The radical scavenging activity of saffron and gardenia extracts against ABTS$^{\bullet+}$ was evaluated according to the protocol of Re et al.[24] after suitable modifications. The results were expressed as Trolox equivalents (mM Trolox). The repeatability of the measurements calculated for the extracts was found to be satisfactory (CV% = 2.8, $n = 5$). Subsequent measurements were then performed in triplicate.

Cupric Ion Reducing Antioxidant Capacity Assay (CUPRAC). The Cu(II) reducing capacity of saffron and gardenia extracts was measured according to the protocol of Apak et al.[25] Briefly, 1 mL of a 0.02 M solution of copper(II) chloride, 1 mL of a 0.0075 M neocuproine solution, and 1 mL of a 1 M ammonium acetate buffer (pH = 7.0) were mixed with 100 μL of the aqueous extracts. After the

addition of 1.0 mL of deionized water (to a final volume of 4.1 mL), the mixture was shaken for 15 s. The absorbance at 450 nm was measured after the solution had been allowed to stand in the dark for 30 min. The results were expressed as Trolox equivalents (mM) after correction with an appropriate blank. The repeatability of measurements calculated for the extracts was found to be satisfactory (CV% = 4.8, n = 5). All of the measurements were then performed in triplicate.

Ex Vivo Antioxidant Studies. *Cell Culture.* Human monocytic blood, U937, cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS). Cells were incubated in an atmosphere of CO_2−air (5:95, v/v) at 37 °C and were maintained in the absence of antibiotics. U937 cells were plated at a density of 1×10^5 cells/mL in RPMI containing 2.5% FBS for each experiment.

Preparation of Extracts for Cellular Antioxidant Studies. An appropriate portion of finely ground saffron (0.05 g) and gardenia dry fruit (0.2 g) was extracted with 10 mL of an ethanol−water mixture (50:50, v/v) in a sonication water bath.

Cell Viability. U937 cells were supplemented with increasing concentrations of saffron and gardenia fruits extracts (0−50 and 0−250 μg/mL, respectively) taking into account the different crocetin esters content in 6-well plates with a final volume of 2 mL and incubated for 24 h. Cells (25 μL) were placed in an Eppendorf flask and mixed with 25 μL of fluorescein diacetate/ethidium bromide (FDA/EtBr) solution. The mixture was incubated for 5 min at 37 °C, and 25 μL of the mixture was placed on a microscope slide. Immediately, 100 cells were scored using the blue filter on a Nikon optiphot-2 fluorescent microscope (Micron Optical Co. Ltd., County Wexford, Ireland). Live cells appeared green, and dead cells were red. Measurements were performed in duplicate, and the results were expressed as the average percentage live cells.

Determination of DNA Damage (Comet Assay). U937 cells were supplemented with 50 μg/mL saffron extract and 200 μg/mL gardenia extract for 24 h in 6-well plates with a final volume of 2 mL. Following incubation, the cells were treated with or without 40 μM H_2O_2 for 30 min at 37 °C. Cells were harvested, suspended in a low melting point (LMP) agarose, placed on microscope slides, and allowed to solidify. Slides were placed in cold lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH = 10, 1% (w/v) sodium sarcosinate], with 1% (v/v) Triton X-100 and 10% (v/v) DMSO added freshly before each use, for 1.5 h. Slides were then placed in a horizontal gel electrophoresis tank containing fresh electrophoresis solution (1 mM EDTA, 300 mM NaOH) for 30 min. Subsequently, electrophoresis was carried out for 25 min at 4 °C with a current of 25 V (300 mA) using a compact power supply. After electrophoresis, the slides were washed three times with neutralizing buffer (0.4 M Tris, pH = 7.5) at 4 °C for 5 min each. Slides were stained with ethidium bromide (20 μg/mL) and covered with coverslips. Komet 5.5 image analysis software (Kinetic Imaging Ltd., Bromborough, UK) was utilized to measure the level of DNA damage which was expressed as percentage tail DNA.

In Vitro Gastrointestinal Digestion Procedure. Saffron and gardenia aqueous extracts were prepared to the same total crocetin esters content (15 and 7.5 mg total crocetin esters/L extract). The extracts were used directly for the in vitro digestion procedure which was carried out according to O'Connell et al.[26] with some modifications in order to adjust the protocol to the phytochemical characteristics of the tested plant materials. Briefly, aliquots of aqueous extracts of both plant materials were transferred into amber bottles, and Hank's balanced salt solution (HBSS) was added to a final volume of 20 mL. To each bottle was added 1 mL of freshly prepared pepsin (0.04 g pepsin/0.1 mol/L HCl), 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 4.100g using a SL 16R centrifuge (Thermo Scientific, MA, USA) for 20 min at 4 °C,[27] and the supernatants were collected and filtered through a 0.45 μm membrane filter and stored at −18 °C until further analysis. In subsequent experiments, the gastric and small intestinal phases of digestion have been performed as described above unless stated otherwise.

For the simulation of the oral phase of digestion for the second series of experiments, 18 mL of artificial saliva [$NaHCO_3$ (1.302 g), $K_2HPO_4 \cdot 3H_2O$ (0.343 g), NaCl (0.220 g), KCl (0.112 g), $CaCl_2 \cdot H_2O$ (0.441 g), mucin (0.540 g), and α-amylase (0.145 g) in 250 mL of distilled water (pH = 7)][28] was added to 2 mL of an aqueous saffron extract or 100 μL of pure crocin 1, and the mixture was incubated at 37 °C for 5 min in a shaking water bath at 55 rpm.[29] Before the experiment, the saliva was centrifuged at 4.480g for 10 min to remove any undissolved material.

Statistical Analysis. Statistical comparisons of the mean values were performed by one-way analysis of variance (ANOVA), followed by the multiple comparison Duncan test ($p < 0.05$ confidence level) using the SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Characterization of Saffron and Gardenia Aqueous Extracts. *Chemical Characterization.* Zero- and second-order derivative spectra of the extracts in the region of 400−500 nm showed that the typical carotenoid triplet band presented the same λ_{max} (413, 439, 469 nm) while differences were found only below 300 nm (Figure 1A). Moreover, the RP-HPLC

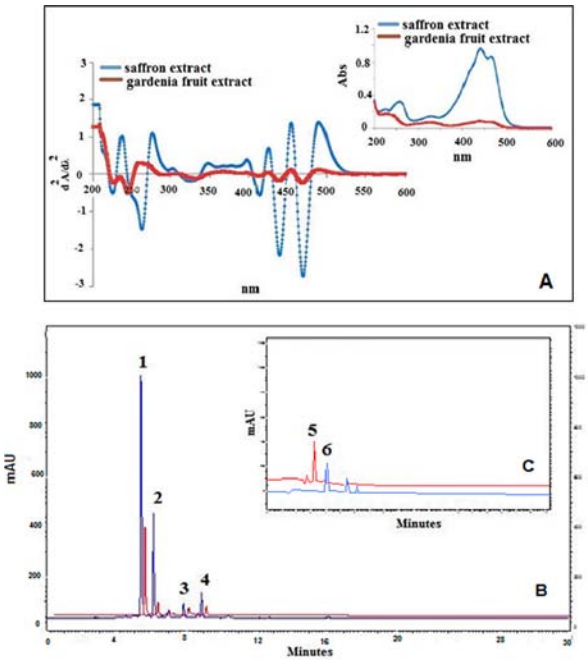

Figure 1. UV−vis second derivative spectra (A) and RP-HPLC profiles (B, C) of saffron and gardenia aqueous extracts at various wavelengths. Peak assignment: peak 1, *trans*-crocin 1; peak 2, *trans*-crocin 2; peak 3, *cis*-crocin 1; peak 4, *cis*-crocin 2; peak 5, geniposide; peak 6, picrocrocin. Chromatographic conditions and peak assignment as described in Materials and Methods.

profile at 440 nm indicated the presence of the same crocetin esters, which differed only quantitatively (Figure 1B). Picrocrocin and geniposide were detected and quantified at 250 and 240 nm, respectively (Figure 1C). On the basis of the results in Table 1, the levels of total and major individual crocetin esters found in the saffron extract were at least 3-fold higher than those in gardenia extract, similar to previously

Table 1. Total and Major Individual Crocetin Esters Content, Picrocrocin and Geniposide Content of Saffron and Gardenia Aqueous Extracts

	saffron extract[a,b,c] (g/100 g dry powder)	gardenia extract[a,b,c] (g/100 g dry powder)
total crocetin esters	30.1 ± 2.0 a	10.1 ± 0.1 b
trans-crocin 1[d]	16.0 ± 0.3 c	4.0 ± 0.8 d
trans-crocin 2[d]	8.9 ± 0.2 e	3.3 ± 1.2 f
picrocrocin	11.9 ± 1.0	
geniposide		19.1 ± 0.4

[a]Different lower case letters within the same row differ significantly according to Duncan's test at $p < 0.05$. [b]Each value is the mean of triplicate determinations ± SD. [c]Expressed as *trans*-crocin 1. [d]*trans*-Crocin 1 + *trans*-crocin 2 content >80% of total crocetin esters content.

reported data.[9,13,20,21,30−33] Based in Figure 1B and Table 1 data, it can be argued that the two extracts are closely related matrices with regard to apocarotenoid composition. The picrocrocin content of saffron (12 g/100 g dry powder) was similar to published data.[17] The geniposide content of gardenia dry fruit (19 g/100 g dry powder) was higher than that observed in the literature (3.7−11.0 g/100 g dry powder), probably due to the different extraction procedure.[31,33] The content of phenolic compounds in both extracts was low (saffron = 2.4 ± 0.3 and gardenia = 0.6 ± 0.1 g caffeic acid/100 g dry material).

Reducing and Radical Scavenging Activity. Reducing and radical scavenging activity data for the two extracts at two different concentrations (500 and 5000 mg/L) are shown in Table 2. Saffron exhibited almost no reducing activity (expressed as mM Trolox) toward the F-C reagent (0.25 ± 0.12) and Cu^{II} (0.16 ± 0.12). The observed reactivity was concentration-dependent. When a 10-fold higher concentration of saffron extract (5000 mg/L) was tested, the F-C and the CUPRAC values were increased by almost 10-fold (2.40 ± 0.11) and 14-fold (2.36 ± 0.71), respectively. A similar trend was observed for gardenia extracts. Regarding radical scavenging activity, saffron extracts at both concentrations demonstrated no $DPPH^{\bullet}$ scavenging activity in line with previous reports.[18] Gardenia extracts exhibited some radical scavenging activity that was concentration-dependent (5% at 500 mg/mL and 33.3% at 5000 mg/mL). Higher antioxidant activity of gardenia extracts in comparison to that of saffron extracts may be attributed to the presence of constituents other than crocetin esters.[28] Moreover, saffron extracts exhibited low though dose-dependent scavenging activity toward the $ABTS^{\bullet+}$ radical (500 mg/L, 0.15 ± 0.11; 5000 mg/L, 1.21 ± 0.14). The same trend was also observed for gardenia extracts (Table 2) in accordance to those reported by He et al.[34] using an online HPLC-$ABTS^{\bullet+}$ system, who did not verify any contribution of crocin 1 or geniposide to the overall activity. The authors agreed with the hypothesis of Ordoudi et al.[18] that this may be partially related to adverse stereochemical effects due to the presence of glycosidic moieties.

Cellular Antioxidant Activity. The absence or low antioxidant activity of plant extracts as determined by chemical reaction studies does not always correlate with the antioxidant effects observed by in vivo or ex vivo tests. For this reason, an additional assay was performed. First, the cytotoxic effects of the saffron and gardenia extracts were determined by the fluorescein diacetate/ethidium bromide (FDA/EtBr) assay. FDA is converted to the fluorescent compound, fluorescein, by a cytosolic esterase present only in living cells.[35] EtBr cannot cross the cell membrane when the cells are alive, but it can penetrate the damaged membranes of dead cells and binds itself to DNA or RNA and fluoresces red.[36] Cells remained viable following exposure to saffron extract up to a concentration of 50 μg/mL (Figure 2A) and to gardenia extract up to a concentration of 200 μg/mL (Figure 2B). These were the concentrations of the extracts used in the comet assay. The latter is a sensitive and rapid tool for DNA strand break detection in individual cells. Comets are formed when the broken ends of the negatively charged DNA molecule become free to migrate in the electric field toward the anode.[37] To date, evidence about the protective effect against DNA damage induced by hydrogen peroxide has been reported only for crocin 1.[38] In the present study, the addition of 40 μM H_2O_2 to U937 cells caused an increase in DNA damage to approximately 52.1% tail DNA compared to the unchallenged control with approximately 5% tail DNA (Figure 2C). Cell supplementation with saffron or gardenia extracts exhibited a significant decrease ($p < 0.05$) in DNA damage to a similar extent (13.2 and 15.8% tail DNA, respectively). Indeed, there were no significant differences ($p < 0.05$) between the protective effects of the two plant materials. Previous studies employing different cell model systems such as monocytes (saffron[18]), serum/glucose-deprived PC-12 cells (crocin 1[39]), 6- hydroxydopamine (6-OHDA) model of rat Parkinsonism (crocetin[40]), AGS, and SUN638 gastric cancer cells (gardenia[41]) indicated high bioactivity which was not observed using in vitro assays. Our data are novel and interesting as the comet assay has been used for the first time and has revealed a similar potential bioactivity for both plant extracts which are rich in the same apocarotenoids.

Characterization of the two plant extracts indicated that these were good candidates for further examination of total and

Table 2. Reducing and Radical Scavenging Activity of Saffron and Gardenia Aqueous Extracts

	reducing activity		radical scavenging activity	
plant extract (mg/L)	F-C (mM Trolox)[a,b]	CUPRAC (mM Trolox)[a,b]	$DPPH^{\bullet}$ (% RSA)[a,b]	$ABTS^{\bullet+}$ (mM Trolox)[a,b]
saffron				
500	0.25 ± 0.12 a	0.16 ± 0.12 b	0 (−) a	0.15 ± 0.11 a
5000	2.40 ± 0.11 c	2.36 ± 0.71 d	0 (−) a	1.21 ± 0.14 c
gardenia				
500	0.17 ± 0.03 a	0.01 ± 0.00 a	5.0 ± 0.09 b	0.20 ± 0.11 a
5000	1.39 ± 0.54 b	0.76 ± 0.61 c	33.3 ± 1.73 c	1.06 ± 0.20 b

[a]Different lower case letters within the same column for each sample differ significantly according to Duncan's test at $p < 0.05$. [b]Each value is the mean of triplicate determinations ± SD.

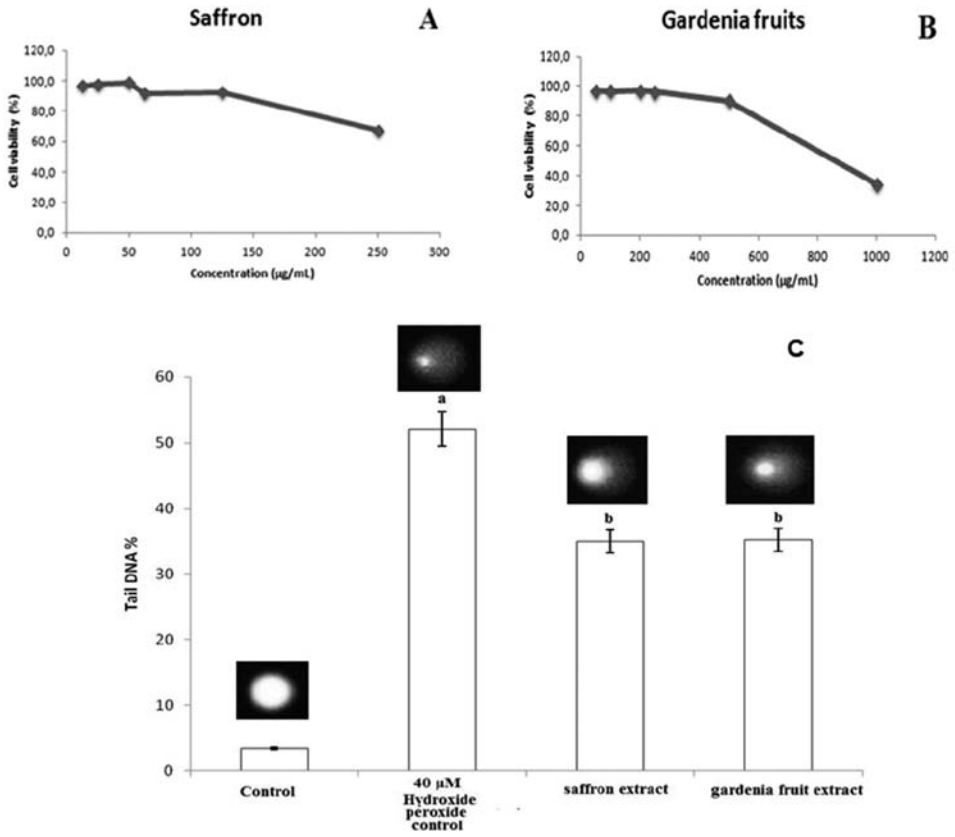

Figure 2. Viability of U937 cells following addition of increasing levels of saffron extract (0−250 μg/mL) (A) and gardenia extract (0−1000 μg/mL) (B) and DNA damage in U937 cells following pretreatment with or without saffron (50 μg/mL) and gardenia extracts (200 μg/mL) (C) for 24 h and then exposure to 40 μM H_2O_2 for 30 min at 37 °C. Data are the means of three independent experiments, with standard errors represented by vertical bars. Statistical comparisons of the mean values were performed by one-way ANOVA, followed by Duncan's test. [a,b] Mean value was significantly different from that for H_2O_2-challenged cells ($p < 0.05$).

individual crocetin esters under conditions simulating the human gastrointestine.

Changes in Crocetin Esters under in Vitro Gastrointestinal Conditions. Both plant extracts were prepared to the same total crocetin esters content, and their changes, upon digestion, were investigated at two different concentrations (15 and 7.5 mg total crocetin esters/L extract). UV−vis spectrophotometry and RP-HPLC-DAD were employed to follow the alteration that crocetin esters undergo during the complete simulated gastrointestinal digestion.

The impact of the harsh digestive environment was found to be critical for the stability of crocetin esters at both concentrations tested. Half of the original amount of total crocetin esters of the saffron aqueous extract—expressed either as $E^{1\%}_{440nm}$ or mg *trans*-crocin 1 by HPLC—was lost after digestion. Such a loss is attributed to a combination of factors which include (a) the physiological temperature of the human body (37 °C), (b) the acidic or basic pH, characteristic for each phase of digestion (gastric and small intestinal, respectively), and (c) the activity of digestive enzymes. The importance of the combined effect of pH and temperature to the hydrolytic and oxidative degradation of apocarotenoids has been reported in the past,[11] but no information is available for the effect of digestive enzymes. Decrease in $E^{1\%}_{440nm}$ values was followed by a concomitant increase in $E^{1\%}_{330nm}$ values for both of the plant extracts tested (Table I in the Supporting Information). Such a change could be related to partial transformation of *trans*- to *cis*-isomers that absorb at 320−330 nm (mainly due to 13-*cis* bonds).[20] Such a possibility was enhanced by the increased amounts of peak no. 3 (Figure 1B) that was assigned to *cis*-crocin 1 by LC-ESI-MS [signal at m/z 508 in the positive ion mode corresponding to $[M + K]^+/2$ (molecular mass 976)] and of peak no. 4 (Figure 1B) that was tentatively assigned to *cis*-crocin 2 on the basis of RP-HPLC-DAD and literature data[20,21] (Figure 3A,B).

Changes in the content of individual crocetin esters accounted for at least 50% of that of *trans*-crocin 1. Loss of *trans*-crocin 2 was somewhat less (∼45%) (Table 3), indicating a possible relationship between the degree of glycosylation and crocetin ester stability. Regarding *cis*-isomers, observations of chromatographic profile (Figure 3A,B) indicated that their presence was less affected by gastrointestinal conditions, which led us to the hypothesis that *trans*/*cis* isomerization occurs in parallel to degradation reactions. Kinetics of the two types of reactions is obviously different. Similar results were obtained when the amount digested was decreased from 15 to 7.5 mg (Table II in the Supporting Information).

Isomerization along with degradation during the digestion process has been reported for a series of carotenoids as well as for norbixin, the apocarotenoid of annatto. More specifically, both *cis*- and *trans*-isomers of norbixin were detected after simulated digestion, suggesting isomerization to a considerable extent (∼90%).[8] The same phenomenon has also been reported for lycopene. Even though lycopene occurs in foods mainly in the *trans* form, the *cis*-isomer has been detected in blood samples reaching 60−80% of total lycopene.[42] No sign

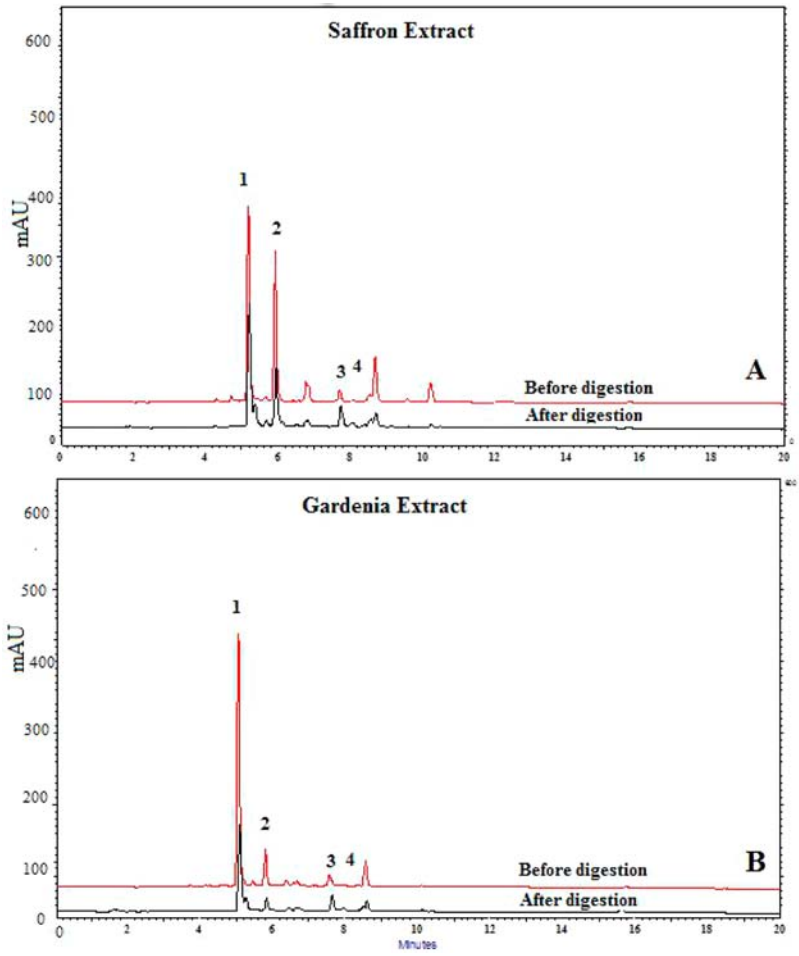

Figure 3. RP-HPLC-DAD profile of aqueous saffron (A) and gardenia (B) extracts before and after complete simulated digestion. Peak assignment: peak 1, *trans*-crocin 1; peak 2, *trans*-crocin 2; peak 3, *cis*-crocin 1; peak 4, *cis*-crocin 2. Chromatographic conditions and peak assignment as described in the Materials and Methods.

Table 3. Total and Major Individual Crocetin Ester Content (mg/L Extract), Picrocrocin Content (mg/L Extract), and Geniposide Content (mg/L Extract) at a Concentration of 15 mg Crocetin Esters/L Extract of Saffron and Gardenia Aqueous Extracts before and after Complete Simulated Digestion

	saffron[a,b,c]			gardenia[a,b,c]		
	before digestion	after digestion	% loss	before digestion	after digestion	% loss
total crocetin esters	13.1 ± 0.8 a	6.0 ± 1.9 b	54.4 ± 1.5	10.5 ± 0.1 a	7.1 ± 1.4 b	32.3 ± 1.2
trans-crocin 1	8.1 ± 0.8 c	3.6 ± 1.4 d	55.1 ± 2.6	8.3 ± 0.8 c	5.8 ± 1.1 d	30.1 ± 1.6
trans-crocin 2	4.5 ± 0.3 e	2.5 ± 1.1 f	45.2 ± 2.2	3.8 ± 0.5 e	3.4 ± 1.0 e	10.5 ± 2.0
picrocrocin	9.4 ± 0.3 g	6.8 ± 3.2 h	27.6 ± 1.1			
geniposide				55.3 ± 3.8 g	23.2 ± 1.4 h	58.0 ± 1.0

[a]Different lower case letters within the same row for each material tested before and after digestion differ significantly according to Duncan's test at $p < 0.05$. [b]Each value is the mean of triplicate determinations ± SD. [c]Expressed as *trans*-crocin 1.

for complete hydrolysis of crocetin esters to form water-insoluble crocetin was evidenced under the in vitro digestion conditions. The presence of crocetin after the oral administration of crocetin esters to rodents could be justified by activity of intestinal microflora.[43−45]

Picrocrocin, was found to be more stable than the major crocetin esters (27.6% loss, Table 3). On the other hand, the secoiridoid glycoside, geniposide, was unstable, and half of its initial content was lost after digestion (58.0% loss, Table 3). This finding is important considering the fact that its hydrolysis product, the aglycone genipin, has been reported to possess genotoxicity.[46]

Results so far indicate that an important amount of crocetin esters (~50% of the original material) is available for absorption (i.e., bioaccessible). This may be a characteristic of hydrophilic carotenoids such as the norbixin for which the authors postulated that its high bioaccessibility must be attributed to its hydrophilic character.[8] Crocetin esters are more bioaccessible than lipophilic carotenoids (e.g., β-carotene, lutein, lycopene, zeaxanthin, β-cryptoxanthin) from different types of foods (e.g., fruits, vegetables, herbs)[6,26] with lutein being the most bioaccessible (52.6 to 95.5% in tomatoes) due to its lower lipophilicity.[47] Though further research would be required to determine the bioavailability of crocetin esters, the

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present study may be used as an indication of the potential bioavailability because higher bioaccessibility is generally associated with a higher bioavailability.[48,49]

To clarify further the contribution of each phase of digestion to the actual changes in crocetin esters, additional experiments were carried out for a saffron aqueous extract (15 mg total crocetin esters/L extract) and for pure *trans*-crocin 1 aqueous solution at a concentration similar to that found in the extract (~8 mg/L extract). Besides mimicking separately the gastric and the small intestinal phases, at this stage, it was considered useful to examine also any possible changes in the content of individual crocetin esters and pure *trans*-crocin 1 as a result of ingestion through the mouth cavity. Data for all of the above experiments are summarized in Table 4 as % loss of total and major individual crocetin esters.

Table 4. Changes in Total and Major Individual Crocetin Ester Content (mg/L Extract) after the Simulated Oral Phase of Digestion (Artificial Saliva, 5 min, 37 °C, 55 rpm), the Gastric Phase of Digestion (HCl, 1 h, 37 °C, 95 rpm, pH = 2.0), in the Presence or Absence of Pepsin and the Simulated Small Intestinal Phase of Digestion (2 h, 37 °C, 95 rpm, pH = 7.4) in the Presence or Absence of α-Amylase and Bile Salts[a,b]

	Oral Phase		
	before simulation of oral phase of digestion		after simulation of oral phase of digestion
total crocetin esters	14.9 ± 0.2 a		14.0 ± 0.4 a
trans-crocin 1	9.0 ± 0.1 b		8.0 ± 0.1 b
trans- crocin 2	5.0 ± 0.2 c		4.0 ± 0.1 c
	Gastric Phase		
	before gastric phase of digestion	after simulation of gastric phase of digestion in the absence of pepsin	after simulation of gastric phase of digestion in the presence of pepsin
total crocetin esters	13.0 ± 0.8 a	11.0 ± 1.3 b	10.7 ± 0.4 b
trans-crocin 1	8.0 ± 0.8 c	7.3 ± 0.7 d	6.9 ± 0.2 d
trans-crocin 2	4.3 ± 0.3 e	3.8 ± 0.3 f	3.7 ± 0.1 f
	Small Intestinal Phase		
	before simulation of small intestinal phase of digestion	after simulation of small intestinal phase of digestion in the presence of α-amylase and bile salts	after simulation of small intestinal phase of digestion in the absence of α-amylase
total crocetin esters	19.2 ± 1.1 a	12.4 ± 0.2 b	13.4 ± 0.6 c
trans-crocin 1	11.2 ± 0.3 d	8.0 ± 0.1 e	8.6 ± 0.3 e
trans-crocin 2	6.1 ± 0.6 f	4.0 ± 0.1 g	4.2 ± 0.2 g

[a]Different lower case letters within the same row for each phase of digestion differ significantly according to Duncan's test at $p < 0.05$. [b]Each value is the mean of triplicate determinations ± SD.

Changes under the Oral Phase of Digestion. The main function of the mouth is to convert foods into suitable forms for swallowing. As has been pointed out,[50] foods or liquids undergo several processes such as mixing with saliva, change in their pH and temperature, the action of digestive enzymes, and the interactions with the tongue and other surfaces of the mouth. The most important interaction is that with saliva. Under artificial saliva conditions (pH = 6.8 ± 0.3, 37 °C, 55 rpm, 5 min), no statistically significant differences were observed for the total and individual crocetin esters of the saffron extract. Liquids rapidly pass through the mouth cavity since they do not need to be broken down to smaller pieces.[50] Moreover, saliva consisting of water (~99%), minerals (<1%), and a complex fraction of proteins (0.1−0.2%) including enzymes (such as α-amylase), antibacterial, and glycosylated proteins (such as mucin) did not affect these apocarotenoids. α-Amylase, responsible for the breakage of α-glycosidic bonds of polysaccharides,[51] did not affect those of crocetin esters, and mucin had no substrate as crocetin esters are not bound to proteins in the extracts. Moreover, artificial saliva was not found to affect pure *trans*-crocin 1 (7.2 ± 0.1 mg/L before oral phase and 7.1 ± 0.1 mg/L after). Neither degradation nor isomerization was evident at this phase of digestion.

Changes under the Gastric Phase of Digestion. Next, changes were monitored under gastric phase conditions (i.e., HCl, 37 °C, 95 rpm, 1 h, pH = 2) in the presence or absence of pepsin. Pepsin, present in the stomach, is a protease, responsible for breaking down proteins into smaller peptides.[51] Like mucin, pepsin is not expected to act on crocetin esters. Lipophilic carotenoids such as carotenes and lycopene, located in the chromoplasts and lutein located in the chloroplasts,[4] are found in food matrices as complexes with proteins. Thus, their bioaccessibility is strongly related to the effective release of these compounds from the protein moiety. There is no such need for the polar crocetin glycosides, which being located in the vacuoles[52] are readily extracted with water.[53] Indeed, under conditions simulating exclusively the gastric phase of digestion, no statistically significant differences were observed for the total or individual crocetin ester content in the presence or absence of pepsin (Table 4). Therefore, the loss observed could be attributed more to the acidic conditions and the elevated temperature than to the action of pepsin. The sensitivity of crocetin esters to acidity is well-established. As reported,[11] the crocetin esters, expressed as $E^{1\%}_{440nm}$, of a cold water extract stored at 40 °C at pH = 3 were mostly degraded within 40 h. This phenomenon is probably related to the high glycosylation degree of crocetin because less polar carotenoids are more stable at low acidic conditions.[54] As the pH value in the course of the gastric phase of simulated digestion is ~2, hydrolysis of apocarotenoid glycosides should be accelerated in comparison to changes during the small intestinal phase ones (pH ~ 7). For example, an 8-fold increase in the total crocin degradation rate constant between pH = 7 (5.8×10^{-3} h^{-1}) and pH = 3 (43.0×10^{-3} h^{-1}) has been found.[11] Moreover, crocetin ester degradation is strongly dependent on temperature. Increasing temperature accelerates the rate of crocetin esters degradation.[12] As found then, the degradation rate constant of the total crocetin esters (expressed as $E^{1\%}_{443nm}$) at 40 °C was 0.4×10^3 $days^{-1}$, whereas at 25 °C it could not be calculated due to very slow reaction kinetics. When pure *trans*-crocin 1 was treated under the conditions simulating those of the gastric phase, a statistically significant decrease in its content was evidenced (7.5 ± 0.1 mg/L before gastric phase and 6.4 ± 0.2 mg/L after). The loss observed (~15%) was similar to that found for *trans*-crocin 1 in the examined saffron extract (~14%). Despite the fact that degradation of *trans*-crocin 1 was observed at this phase of digestion, no isomerization took place.

Changes under the Small Intestinal Phase of Digestion. Pancreatin, present in the small intestine of humans, is a mixture of several digestive enzymes including lipase, protease, and α-amylase.[55] The latter, as has been previously stated, is not expected to break down the β-glycosidic bonds of crocetin ester molecules. Indeed, under conditions simulating exclusively the small intestinal phase (37 °C, 95 rpm, 2 h, pH = 7.4) in the

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presence or absence of bile salts, replacement of pancreatin by purchased pure α-amylase showed no enzymatic activity beyond that related to temperature and pH effects. This observation is in agreement with literature according to which human enzymes cannot digest bound phenols in the form of β-glycosides. Therefore, the latter can survive gastric and small intestinal digestion.[55] Tsimidou and Tsatsaroni[11] pointed out that total crocetin esters (expressed as $E^{1\%}_{440nm}$) were more stable at pH = 7 than at pH = 3. The loss during the small intestinal phase was higher than that during the gastric phase as a result of longer thermal treatment (2 h vs 1 h). In contrast to lipophilic carotenoids, the water-soluble crocetin esters do not need to be incorporated into bile salt micelles (i.e., micellarization) to be absorbed into the enterocyte and exert their physiological function(s).[5] In the case of lipophilic carotenoids, such as α- and β-carotenes and lutein, the presence of bile salts is so crucial that omission of them during the small intestinal phase of in vitro digestion can result in no detectable levels of the carotenoids in the aqueous fraction (i.e., low bioaccessibility).[48] When pure *trans*-crocin 1 was treated under small intestinal conditions, a statistically significant decrease in its content was evidenced (7.7 ± 0.2 mg/L before gastric phase and 6.5 ± 0.1 mg/L after). The loss observed (~15%) was lower from the respective one in the examined saffron extract (~28%), suggesting the potential influence of other coexisting compounds in the latter. Similar to the gastric phase, no isomerization was evidenced at this phase.

When pure *trans*-crocin 1 was treated under the conditions of complete digestion process, the loss observed (~20%) was approximately half of that found for crocin 1 (~50%) when a saffron extract was treated under the same conditions. Isomerization of pure *trans*-crocin 1 was only observed (*trans*-crocin 1/*cis*-crocin 1 = 1.2 ± 0.3) during the complete digestion process probably due to the prolonged procedure.

In conclusion, under the conditions mimicking those of human gastrointestinal tract, the exposure of saffron aqueous extracts to acidic pH in combination with physiological temperature (37 °C) resulted in 50% loss of crocetin esters. Their bioaccessibility can be considered as high compared to that reported for hydrophobic carotenoids. It cannot be precluded that, after reaching the small intestine, crocetin esters are absorbed into blood circulation in the glycosidic form as has been reported for other glycosides (e.g., flavonoid glycosides).[56]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables I and II. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +302310997796. Fax: +302310997847. E-mail: tsimidou@chem.auth.gr.

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■ ABBREVIATIONS USED

RP-HPLC-DAD, reversed-phase high-performance liquid chromatography-diode array detector; NMR, nuclear magnetic resonance; CRT, crocetin; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; F-C, Folin-Ciocalteu; CUPRAC, cupric ion reducing antioxidant capacity assay; FDA/EtBr, fluorescein diacetate/ethidium bromide; LMP, low melting point; ANOVA, analysis of variance

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