Antioxidant Properties of Crocin from *Gardenia jasminoides* Ellis and Study of the Reactions of Crocin with Linoleic Acid and Crocin with Oxygen

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Crocin—a water soluble carotenoid—is found in the fruits of gardenia (*Gardenia jasminoides* Ellis) and in the stigmas of saffron (*Crocus sativus* Linne). For crocin purification, gardenia fruits are extracted with 50% acetone, followed by ether washing, ion exchange, and separation by preparative HPLC. Purified crocin with purity of >99.6% has an antioxidative activity at concentrations up to 40 ppm. At 20 ppm the antioxidative activity of crocin is comparable to that of BHA. The antioxidant property of crocin as evaluated by the thiocyanate method was better than with the thiobarbituric acid method. The adduct between the linoleic acid radical and crocin was detected by LC-MS. When crocin reacted with oxygen in the presence of $FeSO_4$, intermediates such as monohydroperoxides and dihydroperoxides of crocin were formed and detected by LC-MS.

Keywords: *Carotenoids; crocetin derivatives; antioxidant; thiocyanate method; TBA method; HPLC; MS*

INTRODUCTION

Carotenoids have been studied for many years with regard to their important roles in photobiology, photochemistry, and photomedicine. There has been a growing interest in the antioxidant properties of carotenoids in the past few years (Krinsky, 1994; Palozza and Krinsky, 1994; Edge et al., 1997). It has been found that β-carotene reacts with the peroxyl radical to form an adduct (Burton and Ingold, 1984) that can be detected by mass spectrometry (Liebler and McClure, 1996). Carotenoids may act as antioxidants by quenching singlet oxygen (Palozza and Krinsky, 1992) or free radicals (Mortensen and Skibsted, 1997) that are formed during lipid oxidation, such as the lipid radical formed by hydrogen abstraction from an allylic CH_2 group, the peroxyl radical, and the hydroxyl radical (Kappus, 1991; Berset and Cuvelier, 1996). However, information about the mechanism of oxidation of crocin—a water soluble carotenoid—has not yet been well established.

Crocin, the digentiobiosyl ester of crocetin, is the main pigment of the fruit of gardenia (*Gardenia jasminoides* E.) and dried stigmas of saffron (*Crocus sativus* L.) (Figure 1). Crocin is one of the few water soluble carotenoids found in nature; therefore, it might find numerous applications as a food colorant or antioxidant (Francis, 1987, 1992; Ríos et al., 1996). The mechanism of the reaction of crocin with oxygen is not elucidated satisfactorily. Previous works on crocin have dealt with the stability (Tsimidou and Tsatsaroni, 1993; Tsimidou and Biliaderis, 1997; Ichi et al., 1995a,b), the antioxidative activity (Ichi et al., 1995a,b), the superoxide scavenger capacity in sperm cryoconservation (Paramonova et al., 1989), the competitive inhibition of the radical-induced bleaching by various phenolic compounds (Bors et al., 1982, 1983, 1984; Tubaro et al., 1996), and the application of crocetin as a singlet oxygen monitor (Matheson and Rodgers, 1982). No information has been provided on the capacity and the position at which the oxygen or radicals attack crocin and on the oxidized products of crocin.

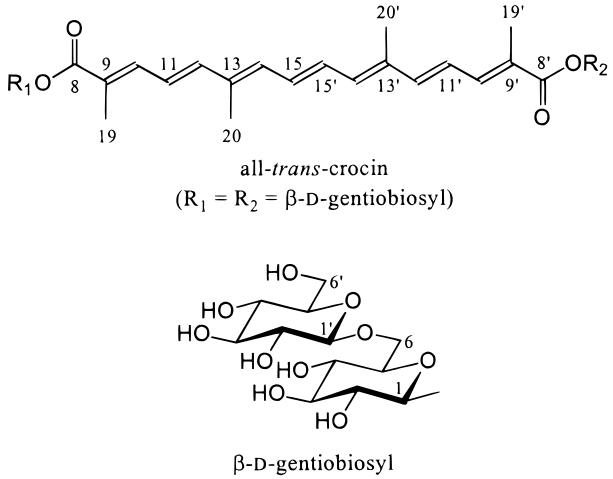

Figure 1. Structure of crocin.

Our objective was to isolate and purify crocin from extracts of gardenia fruits, to study the antioxidant property of crocin and compare it with that of other antioxidants, and to provide information on the reaction of crocin with linoleic acid and also on the reaction of

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crocin with oxygen. In vitro reactions were carried out to characterize the main oxidation products.

EXPERIMENTAL PROCEDURES

Materials. Dried fruits of gardenia were obtained from Vietnam in the provinces of Ninh Thuan, Bac Ninh. Acetone, ether, methanol, ethanol, and 1-butanol were of HPLC grade (Fisher Scientific, Fair Lawn, NJ). 2-Thiobarbituric acid, 1,1,3,3-tetraethoxypropane, Dowex X2-200 strongly acidic cation-exchange resin, Dowex 1X2-200 strongly basic anion-exchange resin, ferrous sulfate, ferrous chloride, ammonium thiocyanate, potassium chloride, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), Trolox, α-tocopherol, and linoleic acid were purchased from Sigma (St. Louis, MO).

Preparation of Crude Extract of Gardenia Fruits and Purification of Crocin. One hundred grams of gardenia fruits was extracted in the dark by triturating in 300 mL of 50% acetone for 24 h at 25 °C, centrifuging, and filtering under vacuum. The residues were re-extracted (100 mL × 3) in the same manner. The total filtrates were pooled and evaporated under vacuum at 20−25 °C using a rotary evaporator to 100 mL. The crude extract of gardenia was dried under a nitrogen stream and lyophilized at −40 °C under vacuum. This dried crude extract was used in the assays.

For purification of crocin the crude extract of gardenia fruits was washed with ether (100 mL × 3) and passed through a Dowex X2-200 cation-exchange column (10 mm i.d. × 250 mm length) by elution with methanol (100 mL × 2) and then immediately through a Dowex 1X2-200 anion-exchange column (10 mm i.d. × 250 mm length) by elution with methanol. The extract was further purified by preparative HPLC to separate the crocetin derivatives. The conditions of the preparative HPLC were as follows: column, μBondapak C_{18} 10 μm (25 mm i.d. × 100 mm length) (Waters Chromatography, Milford, MA); injected volume, 5 mL; detector, 440 nm. The mobile phase delivered at a flow rate of 8 mL/min was water for the first 15 min, which was changed linearly to 62% (v/v) methanol over 20 min, maintained at 62% methanol for 15 min, then increased linearly to 100% methanol over 10 min, and finally maintained at 100% methanol for 15 min. The crocin fraction having a retention time of 31.4−32.2 min was collected and evaporated under vacuum and under a nitrogen stream to dryness and then recrystallized with methanol, evaporated under a nitrogen stream, and lyophilized at −40 °C under vacuum. Crocin having a purity of >99.6% as analyzed by analytical HPLC was used in the assays.

HPLC Analysis. The conditions of analytical HPLC (Beckman Gold System, San Ramon, CA) were as follows: column, ODS-2 (C_{18}) 10 μm (4.6 mm i.d. × 250 mm length) (Chromatographic Sciences, Montréal, PQ, Canada); injected volume, 20 μL; detector, photodiode array (440 nm) (from 200 to 500 nm). The mobile phase delivered at a flow rate of 1 mL/min was water for the first 15 min, which was changed linearly to 50% (v/v) methanol over 10 min, changed linearly from 50% methanol to 100% methanol over 30 min, and maintained at 100% methanol for 10 min (Van Calsteren et al., 1997; Dufresne et al., 1997).

All assays were performed in triplicate. Results presented are the means and standard deviations of the replicates.

Antioxidation Assays. In the antioxidation assays the following antioxidants were tested: crocin, crude extract of gardenia fruits, BHA, BHT, Trolox, and α-tocopherol.

Thiocyanate Assay. Thiocyanate assays were performed as described by Haraguchi et al. (1992) and Yagi et al. (1994). Different amounts of samples (crocin or antioxidants) dissolved in 120 μL of ethanol were added to a reaction mixture in screw-cap vials. The reaction mixture consisted of 2.88 mL of 2.51% linoleic acid in ethanol and 9 mL of 40 mM phosphate buffer (pH 7.0). The vials were incubated at 37 °C in the dark. At intervals during incubation, a 0.1-mL aliquot of the reaction mixture was mixed with 9.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid. Precisely 3 min after the addition of ferrous chloride to the reaction mixture, the absorbance at 500 nm was measured.

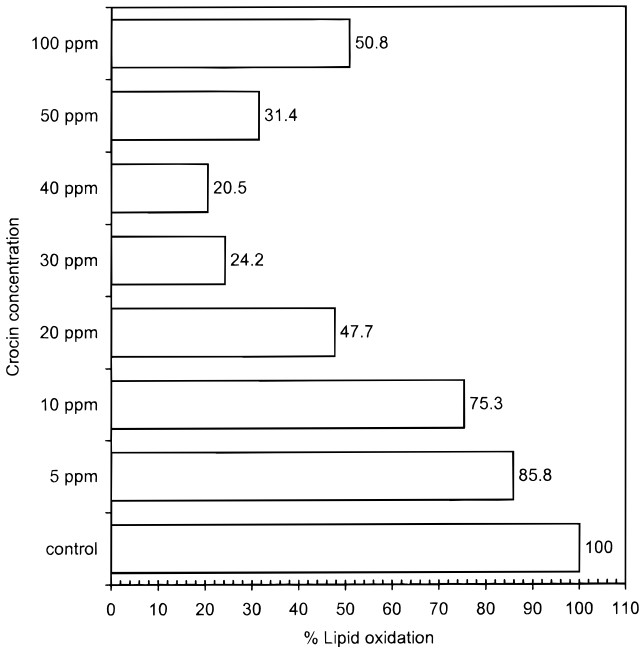

Figure 2. Antioxidative activity of crocin at different concentrations after 6 days of oxidation at 37 °C using the thiocyanate assay.

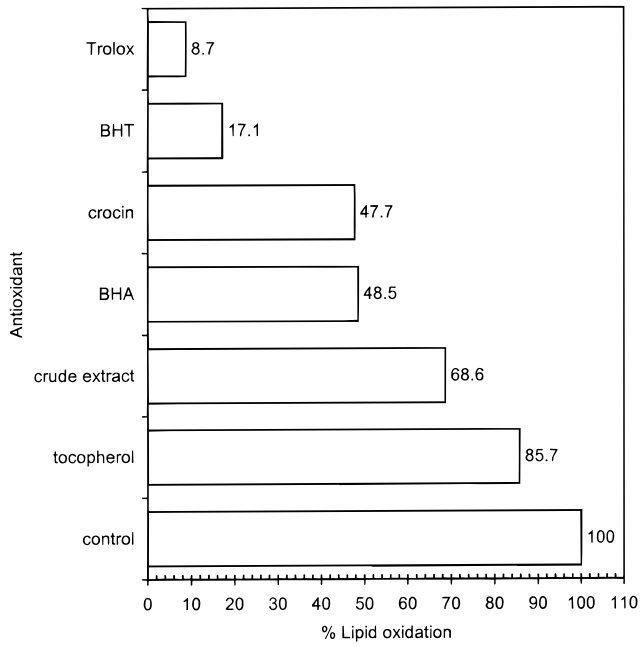

Figure 3. Antioxidative activity of 20 ppm of crocin, crude extract from *G. jasminoides*, and different antioxidants after 6 days of oxidation at 37 °C using the thiocyanate assay.

Thiobarbituric Acid (TBA) Assay. The antioxidative activity of crocin was determined by using the TBA method as described (Tamura and Yamagami, 1994; Ohkawa et al., 1979). The oxidation of linoleic acid was carried out by using a modification of a method reported previously (Tamura and Yamagami, 1994; Tamura and Shibamoto, 1991). Linoleic acid (17.8 μmol) was added into a 30-mL test tube and then diluted with 4.85 mL of Tris-HCl buffer solution (0.25 mM, pH 7.40) containing 0.2% (w/v) sodium dodecyl sulfate (SDS) and 0.75 mM potassium chloride. Lipid peroxidation was initiated by adding 20 mM ferrous sulfate (50 μL) with or without antioxidants. The total volume of the reaction solution was adjusted to 5 mL with the buffer, and the tube was stoppered

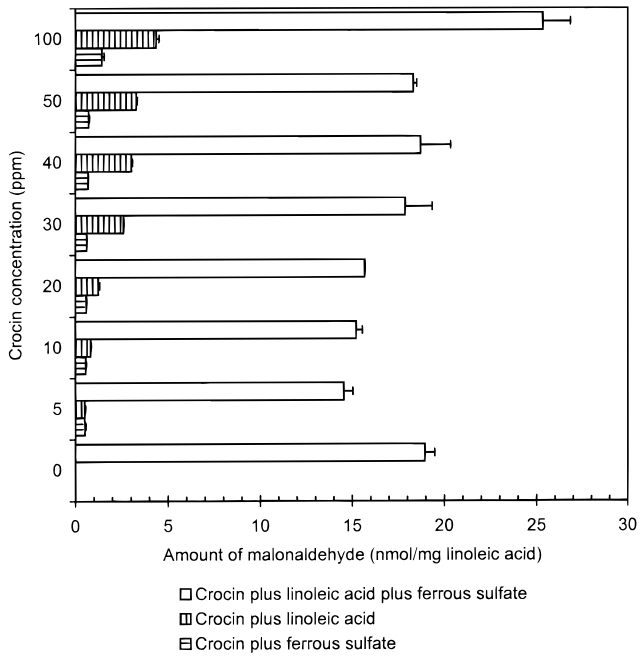

Figure 4. Effect of crocin on the production of malonaldehyde from linoleic acid oxidized by $FeSO_4$ for 16 h in 0.25 mM Tris-HCl buffer (pH 7.4, containing 0.2% SDS and 0.75 mM KCl).

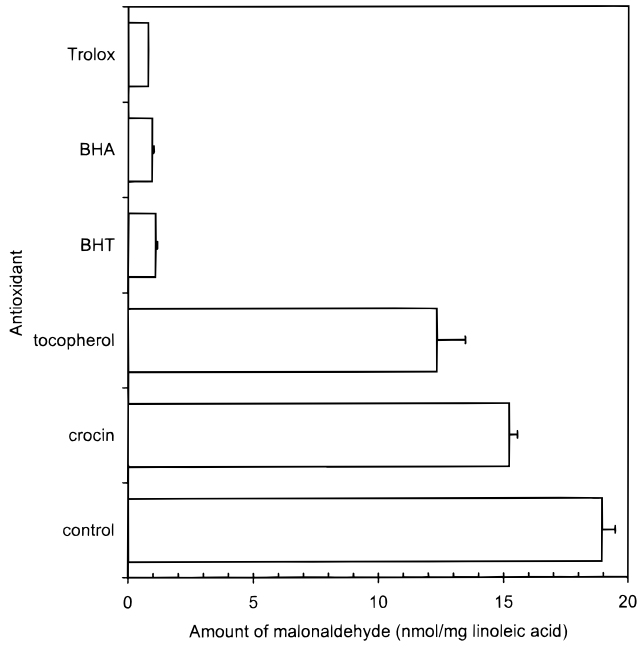

Figure 5. Effect of 10 ppm of crocin and other antioxidants on the production of malonaldehyde from linoleic acid oxidized by $FeSO_4$ for 16 h in 0.25 mM Tris-HCl buffer (pH 7.4, containing 0.2% SDS and 0.75 mM KCl).

and incubated for 16 h at 37 °C in darkness for up to 6 days. The reaction was stopped by adding 100 μL of a 90.8 mM alcoholic solution of BHT to the tube. Each tube contained 2 mg of BHT. A 200-μL aliquot of the reacted solution was incubated with 1 mL of a 0.67% (w/v) TBA solution and 3 mL of 0.05 N HCl for 30 min in a 95 °C water bath. The solution was then cooled on ice for 5 min. The colored substances were extracted by 4 mL of 1-butanol. The absorbance of the 1-butanol layer was measured at 535 nm. The results were expressed in terms of malonaldehyde production; a calibration curve was established by using malonaldehyde bis(diethyl acetal) (1,1,3,3-tetraethoxypropane) as a standard. Malonaldehyde quantitatively prepared by heat-assisted acid hydrolysis (mentioned above) from an aliquot of 1,1,3,3-tetraethoxypropane (10 mmol/mL) was immediately derivatized to a TBA reactive substance to make the standard calibration curve.

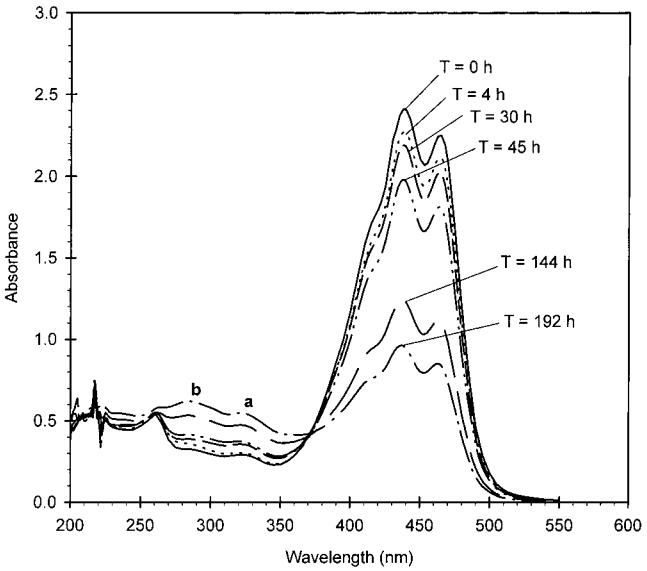

Figure 6. Changes in UV−vis absorbance spectrum during the oxidation of crocin at 37 °C in 40 mM phosphate buffer (pH 6.0 containing $FeSO_4$).

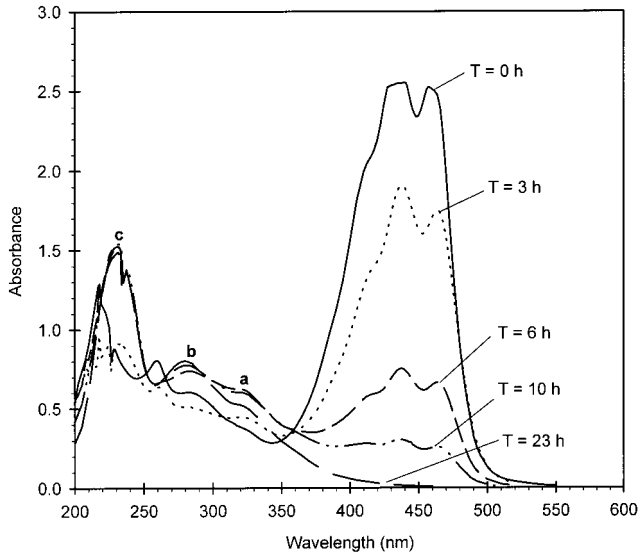

Figure 7. Changes in UV−vis absorbance spectrum during the co-oxidation of crocin with linoleic acid (1:2 molar ratio) at 37 °C in 40 mM phosphate buffer (pH 6.0 containing $FeSO_4$).

Crocin Oxidation Assay. The crocin oxidation reaction mixture consisted of 4 mg of crocin in 0.5 mL of ethanol, 1.5 mL of 40 mM phosphate buffer (pH 6.0), and 20 μL of 20 mM $FeSO_4$, with or without 1.2−12 μL of linoleic acid. The reaction mixture was placed in a 25-mL cap vial and incubated at 37 °C in the dark. After a given period of time a 100-μL aliquot of the reaction solution was withdrawn to measure the UV−visible spectra. The aliquot was diluted with 0.9 mL of distilled water and placed in a 1-cm light path quartz cuvette, and the UV−visible spectra were obtained on a Beckman DU-7 spectrophotometer in the 200−600 nm range. The rest of the reaction mixture was diluted with 8 mL of water and washed with ether (10 mL × 3) to eliminate unreacted linoleic acid. The reaction mixture was applied onto a PrepSep-C_{18} (Fisher Scientific). Products adsorbed on C_{18}-PrepSep were washed with water (20 mL × 10) and eluted with methanol (10 mL × 3). The eluate was evaporated under a nitrogen stream, lyophilized at −40 °C, and used for HPLC and LC-MS analyses.

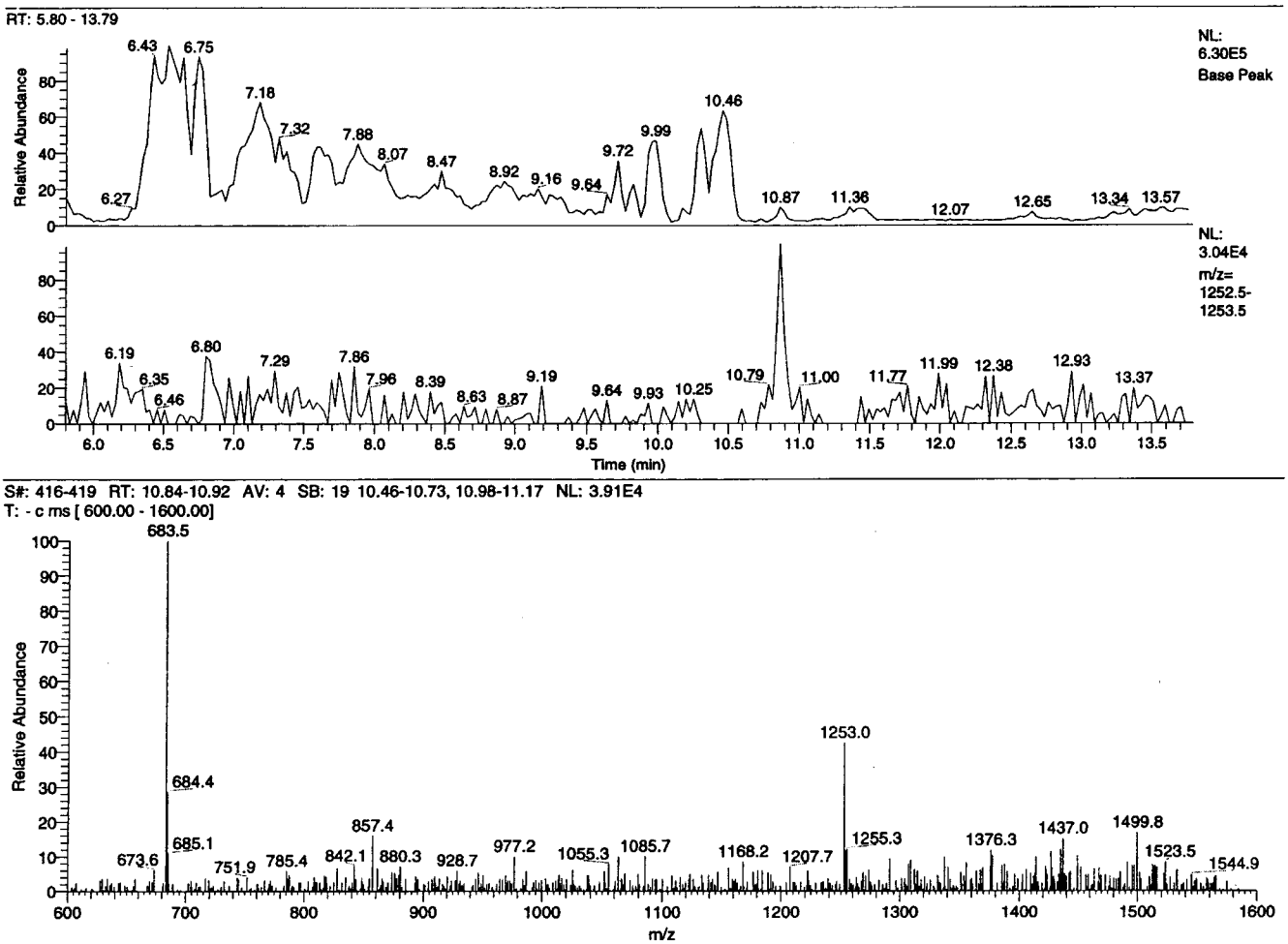

Figure 8. LC-MS analysis of crocin oxidation products at 37 °C at pH 6.0 after 16 h of incubation with linoleic acid (crocin/linoleic acid molar ratio = 1:1) in the presence of $FeSO_4$: (top) total ion current (upper) and *m/z* 1253 selected ion (lower) chromatograms; (bottom) mass spectrum at retention time of 10.84−10.92 min.

LC-MS Analysis. Analysis of the reaction mixture samples was performed using a Finnigan LCQ liquid chromatograph mass spectrometer (LC-MS). The HPLC conditions were as for the HPLC analysis above, and detection was in the negative mode with the electron spray ionization method.

LC-MS-MS Analysis. A Micromass Quattro LC LC-MS-MS mass spectrometer was used to obtain full-scan spectra. Negative ion full scans were done on LC injections of the samples. The spectrometer was scanned from *m/z* 100 to 1500 every 2 s. The liquid chromatography was done on a C18 1.0 × 150 mm column with a gradient. The gradient started at 5% (90:10 acetonitrile/water, 0.07% formic acid) and 95% (0.07% formic acid aqueous). It was increased linearly to 100% (90:10 acetonitrile/water, 0.07% formic acid) over a 20-min period. The LC runs had a cycle time of 55 min.

RESULTS AND DISCUSSION

The results of the thiocyanate assays are shown in Figures 2 and 3. The antioxidative activity of crocin increased with concentration up to 40 ppm (w/v) and decreased at higher concentrations of crocin, that is, 50−100 ppm (Figure 2). This might be explained by the fact that at higher concentrations crocin acts as an oxygen-carrying agent (Holloway and Gainer, 1988) and serves as a pro-oxidant in the co-oxidation of linoleic acid. The thiocyanate test showed that at a concentration of 40 ppm the inhibition of oxidation of linoleic acid was 80% as compared to the blank. This result is similar to that reported by Ichi et al. (1995a,b).

Of all the antioxidants at a concentration of 20 ppm, Trolox—a water soluble α-tocopherol—was the most efficient in preventing the oxidation of linoleic acid over a 6-day period. Trolox had a 91.3% antioxidant efficiency, whereas the antioxidant efficiency of crocin was only 52.3%. The crude extract of gardenia had the weakest antioxidative activity because of the small contents of crocin (0.120 ± 0.016 mg/mL) and other crocetin glycosyl esters, but its antioxidative activity was still more efficient than that of α-tocopherol.

The antioxidative activity of crocin as determined by the TBA method (Figures 4 and 5) was smaller than that measured by the thiocyanate method. The higher TBA value (inversely proportional to the antioxidative activity) may be explained by the formation of not only malonaldehyde but also other aldehydes that can react with TBA to form colored complexes having their maximum wavelength in the same region of 530 nm (Hoyland and Taylor, 1991; Guillén and Guzmán-Chozas, 1998). Moreover, the presence of sugars such as gentiobiose, which was formed by hydrolysis of crocin, interfered in the TBA assay, with an absorption band located between 450 and 460 nm (Yu and Sinnhuber, 1962). The oxidation of crocin with $FeSO_4$ showed that there may also be formation of a colored complex between the oxidation products of crocin and TBA in the TBA test (Figure 4). The effect of this colored complex was significant and expressed equivalently in

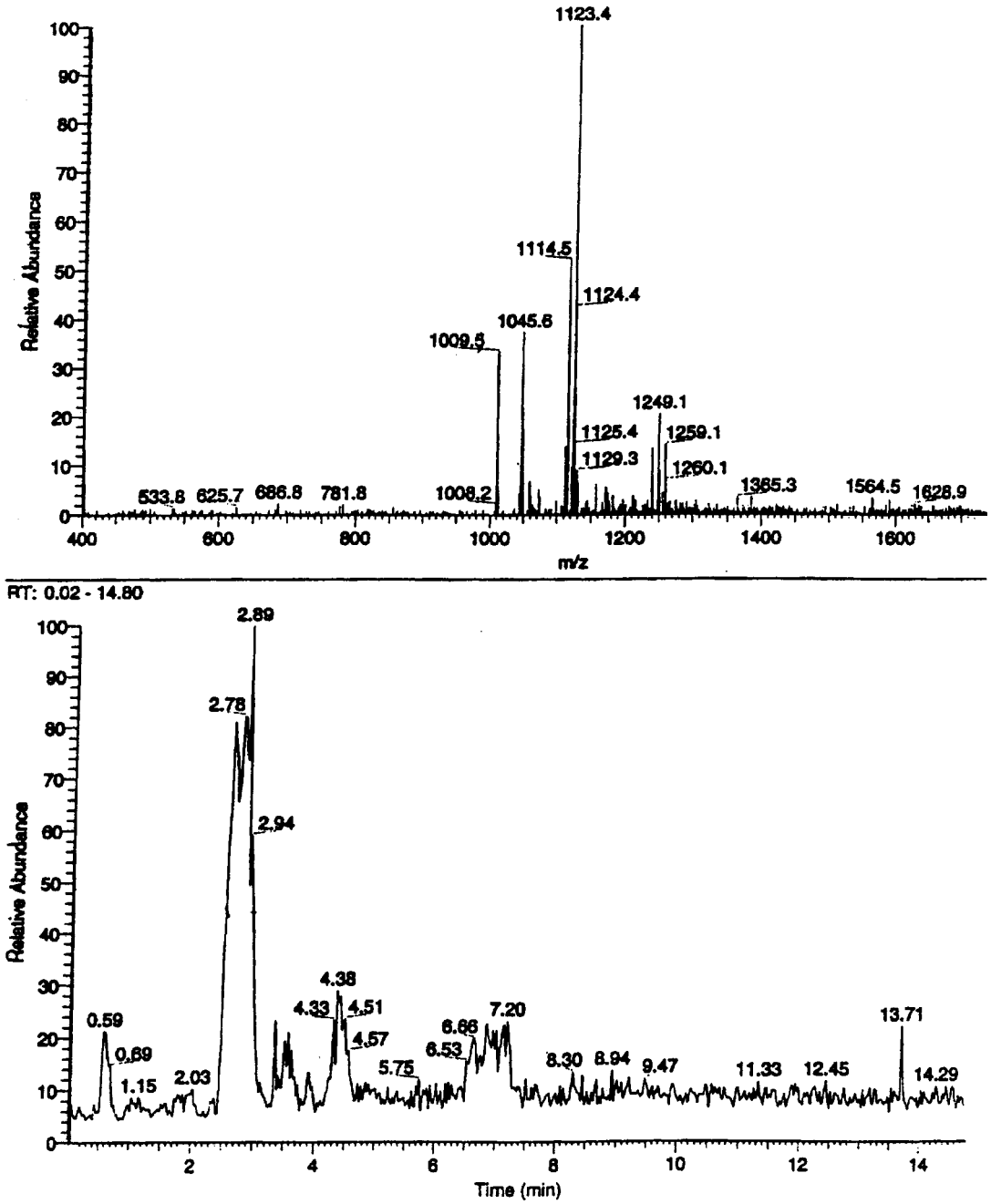

Figure 9. LC-MS analysis of crocin oxidation products at 37 °C at pH 6.0 after 6 days of incubation in the presence of $FeSO_4$: (bottom) total ion current chromatogram; (top) mass spectrum at retention time of 2.52−2.97 min.

the unit of amount of malonaldehyde (nanomoles) per milligram of linoleic acid (crocin plus $FeSO_4$). The presence of $FeSO_4$ also accelerated the co-oxidation of crocin and linoleic acid. Of all the antioxidants at a concentration of 10 ppm in the TBA assay (Figure 5), Trolox was again the most efficient; crocin was the least efficient due to the oxidation products of crocin, which might also react with TBA to cause the higher TBA value.

When crocin was oxidized by oxygen, it lost its conjugated carbon−carbon double-bond system (Britton et al., 1995) to cause a loss of optical intensity and changes in wavelengths. Depending on the number of conjugated carbon−carbon double bonds remaining after oxidation, the maximum wavelength of the intermediates is shifted to shorter wavelengths (hypsochromic shift) (Kohler, 1995). The UV−visible spectra during the reaction of crocin and oxygen showed that the absorbance at 440 nm of crocin decreased and that in the region of 200−350 nm there were two small peaks due to intermediates at 280−285 and 320−325 nm (peaks b and a, respectively, in Figure 6). However, the latter peak might not be totally due to oxidized products because the cis peak of crocin is also in the region of 315−330 nm (Van Calsteren et al., 1997).

During the assays crocin was co-oxidized and lost its absorbance more rapidly in the presence of linoleic acid than in the absence of linoleic acid (Figure 7). Possibly radicals formed when linoleic acid was oxidized reacted with crocin at the carbon−carbon conjugated double-bond system (Burton and Ingold, 1984; Liebler and McClure, 1996; Kohler, 1995). The UV−visible spectra during the co-oxidation of crocin and linoleic acid showed that in the region of 200−350 nm there were

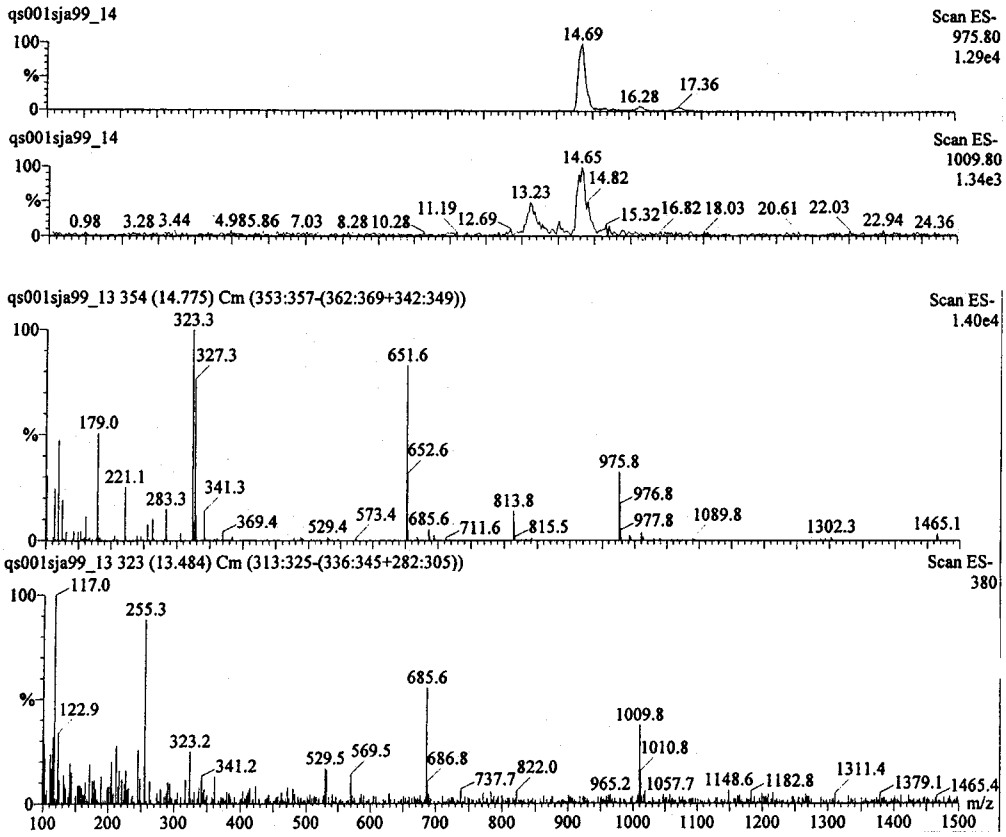

Figure 10. LC-MS-MS analysis of oxidation products at 37 °C at pH 6.0 after 3 days of incubation in the presence of $FeSO_4$: (top) *m/z* 975 (upper) and *m/z* 1009 (lower) selected ion chromatograms; (bottom) mass spectra at retention time of 14.775 min (upper) and 13.484 min (lower).

three small peaks due to intermediates at 230−235 nm (peak c), 275−285 nm (peak b), and 315−320 nm (peak a) (Figure 7). Peak c might be the peak of hydroperoxides of linoleic acid as reported by Koskas et al. (1983).

LC-MS analysis of the reaction mixture of crocin and linoleic acid (1:1 molar ratio) at 37 °C (pH 6.0) after 16 h contained the signal $[M - H]^-$ *m/z* 1253, which corresponded in mass to an adduct (R−Cro$^\bullet$) between crocin (Cro) and linoleic acid radical (R$^\bullet$) (Figure 8). This may indicate that crocin acts as an antioxidant by scavenging not only the peroxyl radical ROO$^\bullet$ (Burton and Ingold, 1984; Liebler and McClure, 1996) but also the linoleic acid radical R$^\bullet$ at the initiation of the autoxidation of linoleic acid. This also confirms the fact that linoleic acid behaves like alkyl radicals that are expected to be scavenged most efficiently as compared to alkoxyl and peroxyl radicals (Liebler and McClure, 1996).

LC-MS analysis of a reaction mixture composed of crocin in 40 mM phosphate buffer (pH 6.0) with $FeSO_4$ at 37 °C after 6 days contained several groups of signals of various retention times (Figure 9). The predominant group at retention time of 2.52−2.97 min contained the main signals of *m/z* 1009, 1045, 1114, and 1123. The first two peaks tentatively correspond in mass to monohydroperoxycrocin (MW 1010.97) and dihydroperoxycrocin (MW 1045.00). This may be explained by the long incubation time, that is, 6 days, that enables oxygen to react with crocin at several carbon−carbon double bonds to form the polyhydroperoxy products.

A reaction mixture similar to the one above but after 3 days of reaction was analyzed by LC-MS-MS. Scanning for *m/z* 975 and 1009 revealed peaks at 14.7 and 13.2 min, respectively (Figure 10). These peaks correspond to crocin and monohydroperoxycrocin. They fragment to reveal peaks at 651 and 685, respectively, which correspond to a loss of 324 mass units characteristic of a gentiobiosyl moiety.

CONCLUSIONS

The antioxidative activity of crocin is concentration dependent. Crocin acts as an antioxidant at concentration levels up to 40 ppm. The thiocyanate test showed that at the concentration of 40 ppm the inhibition of oxidation by crocin was 80% when compared with the blank.

At 20 ppm the antioxidative activity of crocin is comparable to that of BHA. This confers practical relevance to the results and points out the possible utilization of crocin as an antioxidant at the concentrations tested in the study.

The antioxidative activity of crocin measured by the TBA assay is lower than that measured by the thiocyanate assay. This may be explained by the formation of aldehydes other than malonaldehyde in the TBA method that brings about an overestimation.

When crocin is co-oxidized with linoleic acid, it can form an adduct between crocin and the linoleic acid radical.

When crocin reacted with oxygen, the formation of the monohydroperoxide and dihydroperoxide adducts of crocin could be identified by LC-MS.

ACKNOWLEDGMENT

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LITERATURE CITED

Berset, C.; Cuvelier, M.-E. Méthodes d'évaluation du degré d'oxydation des lipides et de mesure du pouvoir antioxydant. *Sci. Aliments* **1996**, *16*, 219−245.

Bors, W.; Saran, M.; Michel, C. Radical intermediates involved in the bleaching of carotenoid crocin. Hydroxyl radicals, superoxide anions and hydrated electrons. *Int. J. Radiat. Biol.* **1982**, *41*, 493−501.

Bors, W.; Michel, C.; Saran, M. Antioxidants: Their function and mechanism as radical scavengers. In *Oxy Radicals and Their Scavenger Systems—Vol. I: Molecular Aspects*; Cohen, G., Greenwalds, R. A., Eds.; Elsevier Science: London, U.K., 1983; pp 38−43.

Bors, W.; Michel, C.; Saran, M. Inhibition of bleaching of the carotenoid crocin—A rapid test for quantifying antioxidant activity. *Biochim. Biophys. Acta* **1984**, *796*, 312−319.

Britton, G.; Liaaen-Jensen, S.; Pfander, H. Carotenoids today and challenges for the future. In *Carotenoids—Vol. 1A: Isolation and Analysis*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhäuser: Basel, Switzerland, 1995; Chapter 2, pp 13−26.

Burton, G. W.; Ingold, K. U. β-Carotene: An unusual type of lipid antioxidant. *Science* **1984**, *224*, 569−573.

Dufresne, C.; Cormier, F.; Dorion, S. *In vitro* formation of crocetin glucosyl esters by *Crocus sativus* callus extract. *Planta Med.* **1997**, *63*, 150−153.

Edge, R.; McGarvey, D. J.; Truscott, T. G. New trends in photobiology (invited review): The carotenoids as antioxidants—A review. *J. Photochem. Photobiol. B Biol.* **1997**, *41*, 189−200.

Francis, F. J. Lesser-known food colorants. *Food Technol.* **1987**, *41* (4), 62−68.

Francis, F. J. Miscellaneous colorants. In *Natural Food Colorants*; Hendry, G. A. F., Houghton, J. D., Eds.; Blackie: Glasgow, Scotland, 1992; Chapter 7, pp 242−272.

Guillén-Sans, R.; Guzmán-Chozas, M. The thiobarbituric acid (TBA) reaction in foods: A review. *CRC Crit. Rev. Food Sci. Nutr.* **1998**, *38*, 315−330.

Haraguchi, H.; Hashimoto, K.; Yagi, A. Antioxidative substances in leaves of *Polygonum hydropiper*. *J. Agric. Food Chem.* **1992**, *40*, 1349−1351.

Holloway, G. M.; Gainer, J. L. The carotenoid crocetin enhances pulmonary oxygenation. *J. Appl. Physiol.* **1988**, *65*, 683−686.

Hoyland, D. V.; Taylor, A. J. A review of the methodology of the 2-thiobarbituric acid test. *Food Chem.* **1991**, *40*, 271−291.

Ichi, T.; Higashimura, Y.; Katayama, T.; Koda, T.; Shimizu, T.; Tada, M. Food chemical properties of crocetin derivatives in gardenia (*Gardenia jasminoides* Ellis) yellow color. *J. Jpn. Soc. Food Sci. Technol.* **1995a**, *42*, 784−789.

Ichi, T.; Higashimura, Y.; Koda, T. Food chemical properties of gardenia yellow pigments. *Foods Food Ingredients J. Jpn.* **1995b**, *165*, 96−103.

Kappus, H. Lipid peroxidation: Mechanism and biological relevance. In *Free Radicals and Food Additives*; Aruoma, O. I., Halliwell, B., Eds.; Taylor and Francis: London, U.K., 1991; Chapter 4, pp 59−75.

Kohler, B. E. Electronic structure of carotenoids. In *Carotenoids—Vol. 1 B: Spectroscopy*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhäuser: Basel, Switzerland, 1995; Chapter 1, pp 1−12.

Koskas, J. P.; Cillard, J.; Cillard, P. Direct high-performance liquid chromatographic separation of hydroperoxide isomers of linoleic acid. *J. Chromatogr.* **1983**, *258*, 280−283.

Krinsky, N. I. The biological properties of carotenoids. *Pure Appl. Chem.* **1994**, *66*, 1003−1010.

Liebler, D. C.; McClure, T. D. Antioxidant reactions of β-carotene: Identification of carotenoid-radical adducts. *Chem. Res. Toxicol.* **1996**, *9*, 8−11.

Matheson, I. B. C.; Rodgers, M. A. J. Crocetin, a water soluble carotenoid monitor for singlet molecular oxygen. *Photochem. Photobiol.* **1982**, *36*, 1−4.

Mortensen, A.; Skibsted, L. H. Importance of carotenoid structure in radical-scavenging reactions. *J. Agric. Food Chem.* **1997**, *45*, 2970−2977.

Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351−358.

Palozza, P.; Krinsky, N. I. Antioxidant effects of carotenoids *in vivo* and *in vitro*: An overview. *Methods Enzymol.* **1992**, *213*, 403−420.

Palozza, P.; Krinsky, N. I. Antioxidant properties of carotenoids. In *Retinoids: From Basic Science to Clinical Applications*; Livrea, M. A., Vidali, G., Eds.; Birkhäuser: Basel, Switzerland, 1994.

Paramonova, L. I.; Revina, A. A.; Lizunkov, A. F. Interaction of carotenoids with the superoxide anion radical in relation to their stabilizing effect during cryoconservation of sperm. *Dokl. Vses. Akad. S.'kh. Nauk im. V. I. Lenina* **1989**, *11*, 30−32; *Chem. Abstr.* **1990**, *112*, 52954q.

Ríos, J. L.; Recio, M. C.; Giner, R. M.; Máñez, S. An update review of saffron and its active constituents. *Phytother. Res.* **1996**, *10*, 189−193.

Tamura, H.; Shibamoto, T. Gas chromatographic analysis of malonaldehyde and 4-hydroxy-2-(*E*)-nonenal produced from arachidonic acid and linoleic acid in a lipid peroxidation model system. *Lipids* **1991**, *26*, 170−173.

Tamura, H.; Yamagami, A. Antioxidative activity of monoacylated anthocyanins isolated from Muscat Bailey A grape. *J. Agric. Food Chem.* **1994**, *42*, 1612−1615.

Tsimidou, M.; Biliaderis, C. G. Kinetic studies of saffron (*Crocus sativus* L.) quality deterioration. *J. Agric. Food Chem.* **1997**, *45*, 2890−2898.

Tsimidou, M.; Tsatsaroni, E. Stability of saffron pigments in aqueous extracts. *J. Food Sci.* **1993**, *58*, 1073−1075.

Tubaro, F.; Micossi, E.; Ursini, F. The antioxidant capacity of complex mixtures by kinetic analysis of crocin bleaching inhibition. *J. Am. Oil Chem. Soc.* **1996**, *73*, 173−179.

Van Calsteren, M.-R.; Bissonnette, M. C.; Cormier, F.; Dufresne, C.; Ichi, T.; Leblanc, J. C. Y.; Perreault, D.; Roewer, I. Spectroscopic characterization of crocetin derivatives from *Crocus sativus* and *Gardenia jasminoides*. *J. Agric. Food Chem.* **1997**, *45*, 1055−1061.

Yagi, A.; Uemura, T.; Okamura, N.; Haraguchi, H.; Imoto, T.; Hashimoto, K. Antioxidative sulphated flavonoids in leaves of *Polygonum hydropiper*. *Phytochemistry* **1994**, *35*, 885−887.

Yu, T. C.; Sinnhuber, R. O. Removal of interfering pigments in determining malonaldehyde in the 2-thiobarbituric acid reaction. *Food Technol.* **1962**, *16*, 115−117.

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