Chemical Investigation of Gamma-Irradiated Saffron (*Crocus sativus* L.)

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Changes in aroma and coloring properties of saffron (*Crocus sativus*) after γ-irradiation at doses of 2.5 and 5 kGy (necessary for microbial decontamination) were investigated. The volatile essential oil constituents responsible for aroma of the spice were isolated by steam distillation and then subsequently analyzed by gas chromatography/mass spectrometry (GC/MS). No significant qualitative changes were observed in these constituents upon irradiation, although a trained sensory panel could detect slight quality deterioration at a dose of 5 kGy. Carotene glucosides that impart color to the spice were isolated by solvent extraction and then subjected to thin-layer chromatography and high-performance liquid chromatography (HPLC). Fractionation of the above pigments into aglycon and glucosides was achieved by using ethyl acetate and *n*-butanol, respectively. Analysis of these fractions by HPLC revealed a decrease in glucosides and an increase in aglycon content in irradiated samples. The possibility of degradation of pigments during gamma irradiation is discussed.

Keywords: *Carotene glycosides; gamma irradiation; gas chromatography/mass spectrometry; high-performance liquid chromatography; saffron; volatile oil*

INTRODUCTION

Saffron is the most expensive spice widely used for its aroma and coloring properties. It has been used as a sedative and analgesic in traditional medicinal preparations (*1* and *2*) and has recently been shown to have distinct anticancer activities (*1* and *3*).

The aroma of saffron is attributable to its steam volatile essential oil. The volatile oil constituents of the spice have been exhaustively studied (*4*−*6*), and monoterpene aldehyde safranal was found to be responsible for the distinct odor of the spice (*6*).

The coloring property of the spice is attributed mainly to its water-soluble carotenoids, the crocins, which are glycosyl esters of 8,8′-diapocarotene-8,8′-dioic acid (crocetin) (*2*). Various analytical separation techniques such as thin-layer chromatography, high-performance liquid chromatography (HPLC), and gas chromatography, and spectrometric methods such as nuclear magnetic resonance (NMR), mass spectrometry, and infrared spectroscopy, have been used to isolate and characterize these carotenoids. So far, six carotene glycosides have been reported to be present in saffron (*7*).

Like other spices, saffron is prone to microbial contamination due to improper handling, storage, and transportation. Although fumigation using chemicals such as ethylene oxide is the currently practiced method for decontamination of spices, the process is banned in several countries of the world because of possible toxic residues and potential health hazards for workers in fumigation plants. Exposure to ionizing radiation, such as γ-rays, offers an effective alternative to fumigation, as it is a safe physical process that leaves no detectable toxic residues (*8*). A dose of 5−10 kGy is recommended for decontamination of spices without adversely effecting their flavor quality (*9*).

No report exists so far on the effect of γ-irradiation on the aroma and coloring properties of saffron. The present work, therefore, aims at determining changes, if any, in these properties when the spice is subjected to γ-irradiation at doses necessary for microbial decontamination.

EXPERIMENTAL PROCEDURES

Commercial samples of saffron were obtained from the Nuclear Research Laboratory, Shrinagar, Kashmir, India. Samples were divided into to two equal lots. One lot was kept as the nonirradiated control sample, and the other lot was subjected to γ-irradiation at 25 °C to an overall average dose of 2.5 and 5 kGy at a rate of 18 Gy/min using a ^{60}Co package irradiator (AECL, Ottawa, ON). Samples were analyzed for changes in volatile oil constituents and pigments within one to two days of storage after irradiation. Solvents (analytical reagent grade) were obtained from E. Merck (India), Mumbai, India and were redistilled before use. HPLC-grade methanol, water, and acetic acid were passed through 0.45-μm filters (Millipore, Bedford, MA). All analyses were carried out in triplicate.

Isolation and Analysis of Volatile Oil. Aliquots (10 g) of both control and γ-irradiated samples (whole stigmata) were separately subjected to simultaneous distillation−extraction (*10*) technique for 2 h using peroxide-free diethyl ether as extracting solvent. Solvent was then removed, by passing a slow stream of nitrogen, to obtain the volatile oil for each sample.

The volatile oils were analyzed using a Shimadzu QP-5050A gas chromatography/mass spectrometry instrument equipped with a GC-17A gas chromatograph and provided with a DB-1 capillary column (length 30 m, i.d. 0.25 mm, film 0.25 μm).

Following are the operating conditions: column temperature, programmed from 60 to 200 °C at the rate of 4 °C/min, held at initial temperature and at 200 °C for 5min, and further to 280 °C at the rate of 10 °C, held at final temperature for 25 min. Injector and interface temperatures were maintained at

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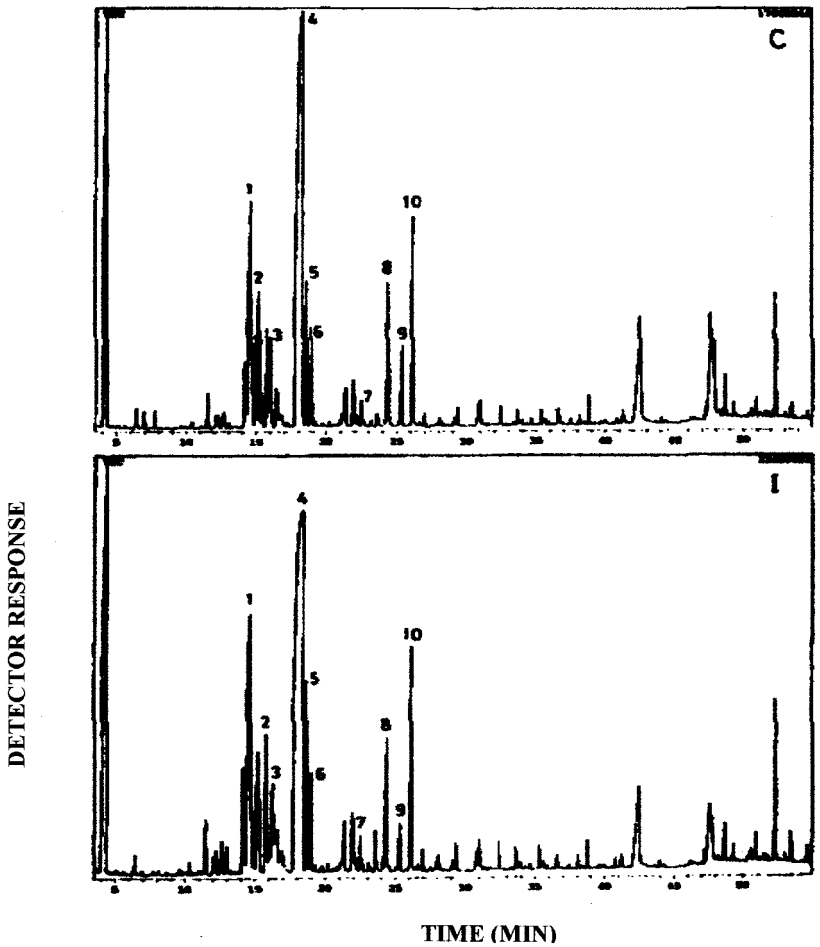

Figure 1. Gas liquid chromatogram of essential oils isolated from control and gamma-irradiated (5 kGy) saffron: C, control; I, irradiated.

Table 1. Relative Distribution of Major Flavor Compounds of Saffron Identified by GC−MS

peak no.	retention time (min)	compound name	relative distribution (%)[a] control	relative distribution (%)[a] irradiated (5 kGy)
1	14.637	α-isophorene	5.25 ± 1.46	6.17 ± 1.54
2	15.234	ketoisophorene	3.17 ± 0.83	3.48 ± 0.62
3	16.049	2,6,6-trimethyl-1,4-cyclohexadione	1.81 ± 0.33	1.78 ± 0.23
4	18.3	safranal	32.93 ± 1.98	19.56 ± 1.76
5	18.627	2,4-cycloheptadiene-1-one-2,6,6-trimethyl (eucarvone)	1.85 ± 0.61	1.92 ± 0.39
6	18.959	3,5,5-trimethyl-2-hydroxy-1,4-cyclohexadione-2-ene	1.67 ± 0.19	1.22 ± 0.15
7	22.544	2,5-dimethyl-2-isopropenyl-l-cyclohexanone	0.35 ± 0.17	0.34 ± 0.12
8	24.437	2,4,4-trimethyl-3-carboxaldehyde-5-hydroxy-1-cyclohexanone 2,5-diene	3.4 ± 0.67	2.8 ± 0.48
9	25.409	2,6,6-trimethyl-4-hydroxy-1-cyclohexene-1-carboxaldehyde	1.57 ± 0.35	0.63 ± 0.22
10	26.198	dihydro-beta-ionene	3.71 ± 0.43	3.43 ± 0.57

[a] Results are SD ± $n = 3$.

210 °C and 230 °C, respectively. Helium was used as carrier gas. Ionization voltage was 70 eV. Electron multiplier voltage was 1kV. Matching their mass-spectral fragmentation pattern with those in the spectral library (Flavor and Fragrance and Wiley/NIST libraries) provided with the instrument identified the compounds of interest.

A panel of 5 judges who were familiar with saffron aroma carried out sensory evaluation of the total volatile oil extracts of each spice sample. The essential oils were appropriately diluted with diethyl ether and then sniffed on filter paper strips.

Isolation and Analysis of Coloring Pigments. *Isolation.* Aliquots of 5 g each of control and irradiated saffron (whole stigmata) were extracted three times with 80% aqueous methanol (3 × 15) at room temperature in an omnimixer (setting at 5 for 3 min). The resulting slurry was filtered under suction and residue re-extracted with 80% aqueous methanol in the same manner as above until the filtrate was colorless. The filtrates of each sample were pooled and then evaporated to dryness under vacuum to obtain a residue that was then brought to a 5% solution in 80% aqueous methanol (total extract). A part of the total extract was appropriately diluted with distilled water and then successively extracted with ethyl acetate and *n*-butanol.The respective organic layers were evaporated to dryness as above and then brought to 1% in methanol and 80% aqueous methanol, respectively.

Analysis. Absorption spectra of ethyl acetate and *n*-butanol fractions were carried out in 80% aqueous methanol on a Shimadzu UV−vis spectrophotometer in the wavelength range of 200−700 nm.

Analytical thin-layer chromatography was carried out on ammonium sulfate (5%) impregnated silicagel G plates (0.25-mm thickness). Plates were developed using *n*-butanol/acetic aci/water (4:1:1), and the separated yellow spots were either visualized directly or by heating the plate for 15 min at 180 °C. Comparing the R_f values with that of standard compounds

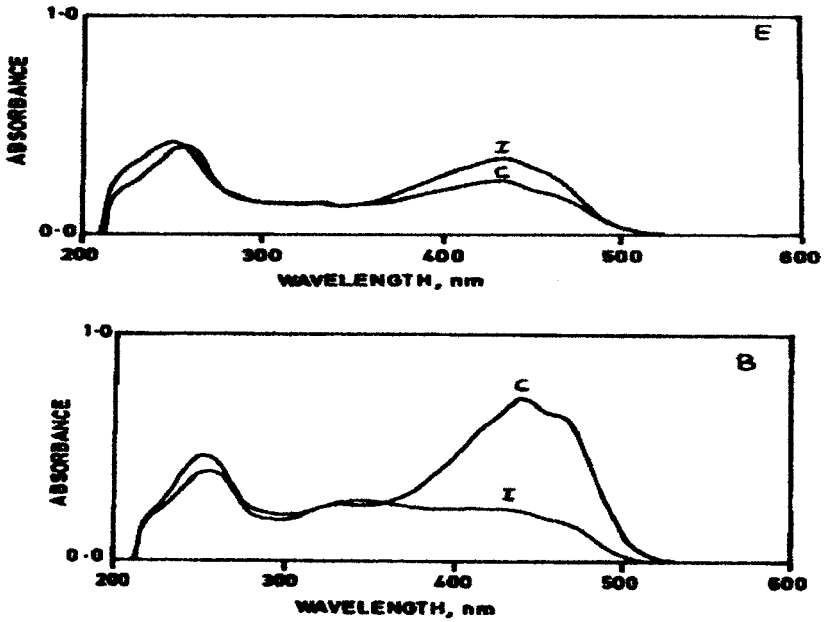

Figure 2. Absorption spectrum of *n*-butanol and ethyl acetate fractions of saffron: top, E, ethyl acetate fraction; bottom, B, butanol fraction; C, control; I, irradiated.

reported in the literature identified spots of interest. Preparative thin-layer chromatography (0.5-mm thickness) was performed on silica gel G plates using the same solvent system as above and the separated yellow bands were then scrapped and eluted with 80% aqueous methanol. The individual compounds thus obtained were used as standard in the present study. Part of the individual bands at R_f values of 0.28 and 0.11 were each separately hydrolyzed (1N HCl, 1 h, 100 °C). The hydrolyzate was neutralized with 1N KOH, and the aglycon was then extracted with diethyl ether. The organic and aqueous layers were evaporated to dryness in vacuo and dissolved in 80% aqueous methanol. The former was subjected to analytical thin-layer chromatography as above in order to identify the aglycon, and the latter was analyzed using *n*-butanol/acetic acid/diethyl ether/water (9:6:3:1) in order to ascertain the sugar residue. Crocetin and glucose were the only constituents detected.

High-performance liquid chromatography (Pharmacia LKB Biotechnology, Sweden) was carried out on a 10-μm ODS-2 (C_{18})(0.46 cm i.d., 25 cm length, Shandon Scientific Ltd., UK) provided with a guard column (0.46 i.d., 1 cm length). Pigments were eluted over 40 min with a linear gradient from 1% (v/v) acetic acid in water to 100% methanol at a flow rate of 1 mL/min (*11*). Peaks were monitored on a UV−vis spectrophotometer at 440 nm. Comparing the retention time of individual peaks with those of standard pigments reported in the literature (*11*) identified peaks of interest.

Quantification of individual peaks was carried out by the external standard method using thin-layer chromatography isolated crocetin gentiobiosyl-glucosyl ester (R_f, 0.28) as standard. Aliquots of the standard solution (0.01%) ranging in concentration from 1 to 5 μg were injected under the same experimental conditions as above. A plot of amount of standard vs peak area was used to obtain a standard curve. Individual peaks of interest were quantified by injecting 100 μL of 0.01% solutions of each of the samples (both control and irradiated) under identical conditions as above. The amounts of individual peaks were calculated from the standard curve and expressed in g/kg. All samples were analyzed in triplicate.

RESULTS AND DISCUSSION

Volatile Oil. Steam distillation of saffron yielded a pale yellow oil with a characteristic aroma of the spice. The yield (w/w) of oil obtained (0.6% ± 0.31) from both control and gamma-irradiated samples was comparable with the values reported in the literature (*1*).

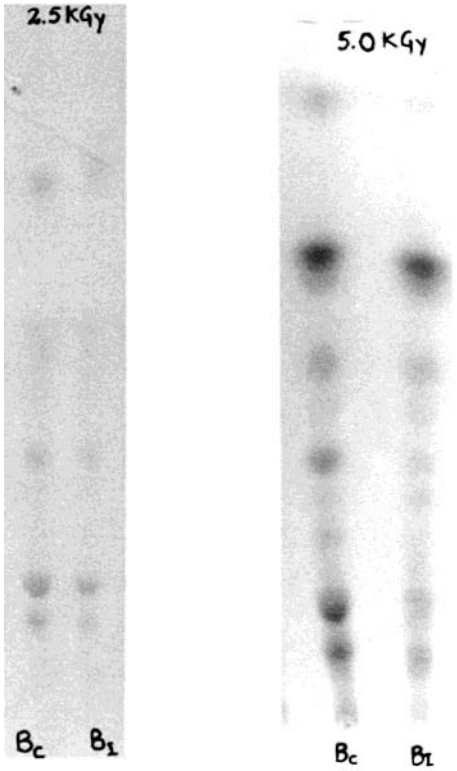

Figure 3. Thin-layer chromatogram of *n*-butanol extracts of saffron: C, control; I, irradiated; B, butanol fraction.

A representative gas liquid chromatogram of essential oil obtained from the above samples is shown in Figure 1. Identification of the major essential oil constituents was achieved using gas chromatography/mass spectrometry (Table 1). For the controls, safranal was the major constituent identified, accounting for 30% of the oil, which is far lower than the reported literature values (60-70%). α-Isophorene (5%), ketoisophorene (3%), dihydro-beta-ionene(3.71%), and 2,4,4-trimethyl-3-carboxaldehyde 5-hydroxy-1-cyclohexanone 2,5-diene (3.4%) were the other major compounds identified in the oil. Tarantilis and Polissiou (*6*) have earlier reported the

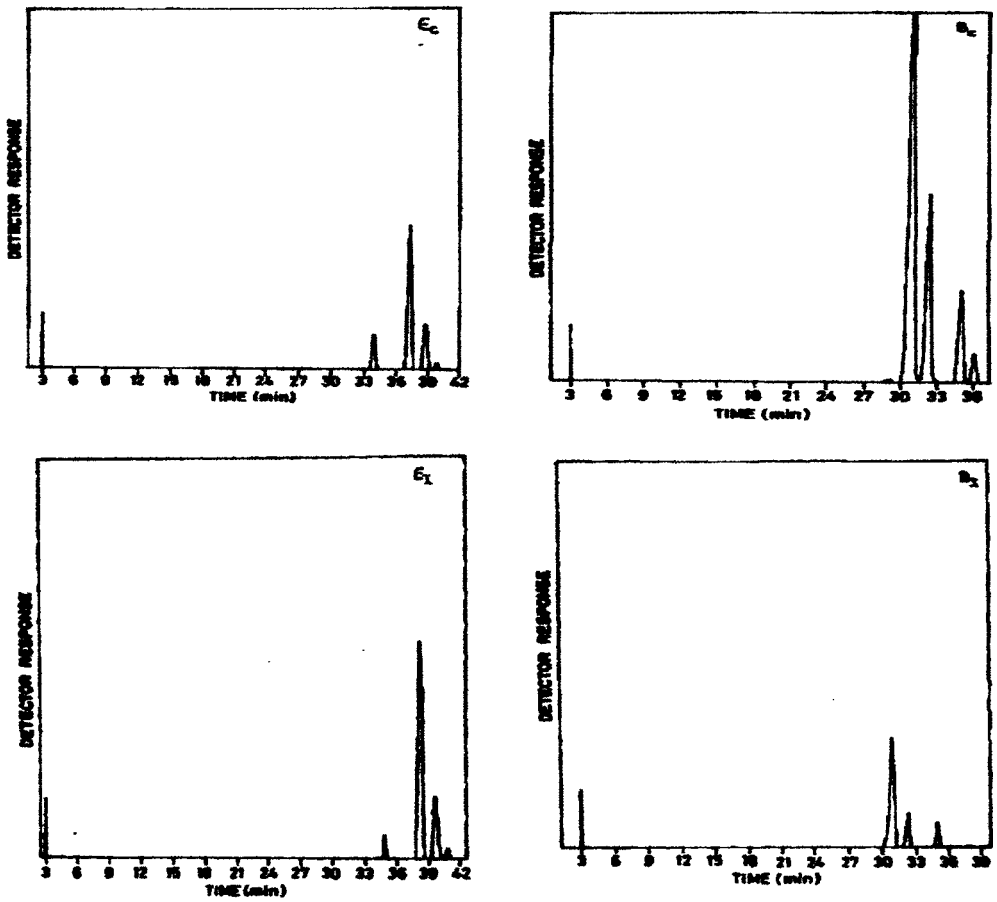

Figure 4. High-performance liquid chromatogram of *n*-butanol and ethyl acetate extracts of saffron: left panels, E, ethyl acetate fraction; right panels, B, butanol fraction; C, control; I, irradiated.

effect of distillation techniques on the composition of the essential oil of saffron. Drastic conditions employed in steam distillation and micro-simultaneous distillation–extraction methods resulted in volatile oil over-enriched with high-boiling-point components because of degradation of low-boiling sensitive constituents. Such effects, besides the origin or source of sample, could account for the low content of safranal in the oil presently studied. No detectable qualitative changes in the volatile oil constituents could be observed between the control and irradiated samples (Figure 1).

A substantial decrease in the content of safranal and 2,6,6-trimethyl-4-hydroxy-1-cyclohexene-1-carboxaldehyde was, however, noted in the irradiated sample (Table 1). Zarghami and Heinz (*4*) have earlier reported oxidative breakdown of safranal under ultraviolet light resulting in formation of isophorene-related compounds. These workers have also predicted a mechanism to explain such oxidative transformation. The possibility of such reactions occurring during exposure to gamma-radiation (5 kGy) could account for the decrease in content of safranal in the irradiated sample. Sensory evaluation of the distilled oils also indicated a perceptible deterioration in the organoleptic property of the volatile oil obtained from irradiated spice treated at doses above 5 kGy. Hence, the spice was not treated beyond this dose.

Coloring Pigments. Absorption spectra of *n*-butanol and ethyl acetate fractions obtained from both control and irradiated (5 kGy) samples are shown in Figure 2. All the samples showed a λ_{max} at 440.5 nm. However, a substantial decrease in the absorbance of the butanol fraction and a distinct increase in the absorbance of the ethyl acetate fraction at the above wavelength were noted in the irradiated sample when it was compared to that of the control. A radiation-induced breakdown of carotene glucoside could thus be predicted, giving rise to carotene and sugar residue, with the former being extracted into ethyl acetate.

Upon analytical thin-layer chromatography, total extract of all the above samples resolved into five major spots that tentatively corresponded to crocetin (R_f, 0.86 and 0.72), picrocrocin (R_f, 0.4), crocetin gentiobiosyl-glucosyl ester (R_f, 0.28), and crocetin di-gentiobiosyl ester (R_f, 0.11). Identity of crocetin (R_f, 0.86) was further confirmed by gas chromatography/mass spectrometry as its acetyl derivative. Besides these major compounds, a few minor colored spots also appeared at R_f values above 0.86. Coulson (*12*) had earlier reported the presence of β-carotene and zeazanthin in saffron. However, as crocin and its glycosides are the main coloring constituents of saffron, changes in only these components during irradiation were of interest in the present study.

No qualitative changes in the carotene glucosides were noted between the control and irradiated samples. Fractionation of the total extract into ethyl acetate and *n*-butanol permitted separation of aglycons and glucosides as revealed on thin-layer chromatography, with the former being extracted into ethyl acetate. The butanol extract containing mainly crocin showed significant quantitative differences in the irradiated sample. The spots at R_f values 0.28 and 0.11 almost disappeared in the 5 kGy irradiated sample (Figure 3). These results

Table 2. Effect of Gamma Irradiation on the Pigment Content of Saffron Stigmata as Estimated by HPLC

compound name	retention time (min)	concentration, g/Kg[a] control	concentration, g/Kg[a] irradiated (5 kGy)
crocetin di-gentiobiosyl ester	27.6	40.033 ± 2.77	4.396 ± 0.214
crocetin gentiobiosyl-glucosyl ester	29.0	9.360 ± 1.790	1.027 ± 0.183
crocetin neapolitanosyl ester	32.0	4.540 ± 0.240	0.740 ± 0.192
crocetin gentiobiosyl ester	32.6	0.980 ± 0.104	not detectable
crocetin	33.8	0.891 ± 0.124	1.963 ± 0.223

[a] Results are SD ± $n = 3$.

are similar to those observed with spectrometry. The results were further confirmed by HPLC.

A representative HPLC profile of ethyl acetate and *n*-butanol fraction obtained from the 5 kGy irradiated sample is shown in Figure 4. The chromatogram of the butanol fraction is similar to that of saffron stigmata extract reported earlier (*11*). This fraction showed four distinct peaks at R_t 27.6, 29.0, 32.0, and 32.6 min that were tentatively assigned as crocetin di-gentiobiosyl ester, crocetin gentiobiosyl-glucosyl ester, crocetin neapolitanosyl ester, and crocetin gentiobiosyl ester, respectively, by comparison with literature data. Chromatograms of irradiated samples were characterized by an appreciable decrease in content of the above compounds (by ~90%), although the relative distribution of these pigments remained unchanged (Figure 4). The ethyl acetate fraction on the other hand showed a major peak at R_t 33.8 min that corresponded to crocetin. The area of this peak was found to increase in the irradiated sample. Glycosidic linkages that connect monosaccharides in a polysaccharide chain are known to undergo cleavage by a free radical mechanism upon exposure to ionizing radiation (*8*). Such effects could possibly explain the breakdown of crocetin glucosides presently observed.

A quantitative distribution of the pigments, namely crocetin gentiobiosyl-glucosyl ester, crocetin di-gentiobiosyl ester, crocetin neapolitanosyl ester, crocetin gentiobiosyl ester, and crocetin, is summarized in Table 2. Total contents of these pigments in the control samples were similar to those reported earlier (*13*) in which it was shown that there is a considerable decrease (80−83%) in the content of crocin pigments during processing and storage. In the present case, the concentration of crocetin increased by 2.2 times, whereas that of crocins decreased approximately 83% to 89% in the irradiated samples compared to the control samples. Thus, radiation-induced degradation of crocetin glucosides is suggested, resulting in partial release of crocetins that are subsequently extracted into ethyl acetate. These results are in agreement with those obtained from spectrometry and thin-layer chromatography.

Thus, although a dose of 10 kGy is recommended for achieving commercial sterility of spices, exposure to doses greater than 5 kGy results in deterioration in the organoleptic property of saffron. This restricts radiation processing of the spice beyond this dose making the process feasible only when microbial loads are low.

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LITERATURE CITED

(1) Rios, J. L.; Recio, M. C.; Giner, R. M.; Manez, S. An update review of saffron and its active constituents. *Phytotherapy Res.* **1996**, *10*, 189−193.
(2) Sampathu, S. R., Shivashankar, S., Lewis, Y. S. Saffron (*Crocus sativus* L.) − Cultivation, processing, chemistry, and standardization. CRC Crit. Rev. Food Sci. Nutr. **1984**, *20*, 123−157.
(3) Nair, S. C.; Pannikar, B.; Pannikar, K. R. Antitumor activity of saffron (*Crocus sativus*). *Canc. Lett.* **1991**, *57*, 109−114.
(4) Zarghami, N. S.; Heinz, D. E. Monoterpene aldehydes and isophorene-related compounds of saffron. *Phytochemistry* **1971**, *10*, 2755−2761.
(5) Rodel, W.; Petrzika, M. Analysis of volatile components of saffron. *J. High Resolut.ion Chromatogr.* **1991**, *14*, 771−774.
(6) Tarantilis, P. A.; Polissiou, M. G. Isolation and identification of the aroma components from saffron. *J. Agric. Food Chem.* **1997**, *45*, 459−462.
(7) Pfander, H.; Rychener, M. Separation of crocetin glycosyl esters by high-performance liquid chromatography. *J. Chromatogr.* **1982**, *234*, 443−447.
(8) Diehl, J. F. *Safety of Irradiated Foods*; Marcel Dekker: London, 1995; pp 173−223.
(9) Farkas, T. *Irradiation of Dry Food Ingredients*. CRC Press: Boca Raton, FL, 1988; pp 1−9 and 25−36.
(10) Nickerson, G. B.; Likens, S. T. Gas chromatography evidence for the occurrence of hop oil components in beer. *J. Chromatogr.* **1966**, *21*, 1−5.
(11) Dufresne, C.; Cormier, F.; Dorion, S.; Niggli, U. A.; Pfister, S.; Pfander, H. Glycosylation of enacapsulated crocetin by a *Crocus sativus L.* cell culture. *Enzyme Microb. Technol.* **1999**, *24*, 453−462.
(12) Coulson. Miscellaneous Naturally Occurring Colouring Materials for Foodstuffs. In *Developments in Food Colours, Vol. 1*; Walford, J., Ed.; Applied Science: London, 1980; pp 189−21.
(13) Raina, B. L.; Agarwal, G.; Bhatia, A. K.; Govind, S. G. Changes in pigments and volatiles of saffron (*Crocus sativus*) during processing and storage. *J. Sci. Food Agric.* **1995**, *71*, 27−32.

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