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Bioactive Compounds of Grapefruit (*Citrus paradisi* Cv. Rio Red) Respond Differently to Postharvest Irradiation, Storage, and Freeze Drying

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In the present study, the effect of irradiation, storage, and freeze drying on grapefruit bioactive compounds was investigated. Grapefruits were exposed to one of two irradiation doses: 0 (control) or 300 Gy (^{137}Cs, a proposed treatment against fruit flies) and then stored for up to 6 days. At the last storage time point (6 days after harvest), grapefruit pulp from control and irradiated fruits was freeze-dried. Bioactive compounds were extracted from Rio Red grapefruit pulp and analyzed with reverse phase liquid chromatography while volatile compounds were analyzed using gas chromatography. Freeze-dried pulp from irradiated fruits had a higher ($P \leq 0.05$) flavonoid content (naringin and narirutin) as compared to the freeze-dried pulp from the control fruits. Freeze-drying treatment reduced ($P \leq 0.05$) the lycopene content, but the reduction ($P \leq 0.05$) in β-carotene content occurred only in the control fruit. Reduction in *d*-limonene and myrcene was observed in the irradiated fruits at 6 days after harvest and in the freeze-dried samples. These results warrant testing of the effect of postharvest treatments and processing on bioactive compounds in functional systems as they have varied effects on different bioactive compounds of grapefruit.

KEYWORDS: ***Citrus paradisi*; Rutaceae; grapefruit; irradiation; freeze drying; carotenoids; flavonoids; terpenoids**

INTRODUCTION

Bioactive compounds from fruits and vegetables are widely considered to be valuable for human health. However, the bioactive compounds of fruits and vegetables may be influenced by postharvest treatments and/or processes such as irradiation, storage, and freeze drying. This aspect has received little consideration even in contemporary nutritional and epidemiological studies due to limited scientific data on postharvest effects on bioactive compounds. Citrus fruit is a rich source of bioactive phytochemical constituents such as flavonoids, limonoids and their glucosides, ascorbic acid, folic acid, carotenoids (lycopene and β-carotene), coumarin-related compounds (auraptene), highly fermentable fiber, and potassium. These constituents may serve as chemopreventive agents (*1*) in addition to other beneficial effects on human health. Citrus fruits are good sources of flavonoids (hesperidin and naringenin) and carotenoids (lycopene and β-carotene), which possess antioxidant activity (*2*, *3*). Our recent study demonstrated that naringenin suppresses high multiplicity aberrant crypt foci formation and cell proliferation in rat colon (*4*). Limonin, another grapefruit bioactive compound, has been found to significantly reduce the incidence of colonic adenocarcinomas induced by azoxymethane in male F344 rats (*5*) and also in Sprague−Dawley rats (*6*) by suppressing cell proliferation and enhancing apoptosis.

The content of bioactive compounds in fruits and vegetables may be altered by postharvest treatments such as low dose irradiation (*7*) and freeze drying (*8*). Currently, irradiation is being considered as a versatile and viable alternative to toxic methyl bromide fumigation for treatment against Mexican fruit flies in Texas red grapefruit (*9*).

Freeze drying also has been used extensively to dehydrate bioactive-rich plants in order to test their chemopreventive ability in animal models (*10*, *11*). Several freeze-dried fruit and vegetable products are available as dietary supplements, and the market for these kinds of products seems to be growing (*12*). However, the effect of postharvest processes such as low dose irradiation, storage, and freeze drying on bioactive components of grapefruit is not well-documented. The objective

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of this study was to evaluate the impact of irradiation, storage, and freeze drying on grapefruit bioactive components such as flavonoids, limonoids, volatile components, and carotenoids.

MATERIALS AND METHODS

Fruit Samples. Grapefruits (*Citrus paradisi* Cv. Rio Red) were harvested ($n = 48$) from an orchard at the Texas A&M University-Kingsville Citrus Center's South Farm, and pulp devoid of seeds, albedo, and flavado was collected from six fruits. The remaining 42 grapefruits were washed and waxed in a commercial packing line within 24 h. Twenty-one fruits were exposed to irradiation (300 Gy) 4 days after harvest, and a similar number of fruits served as the control. Both irradiated ($n = 6$) and control ($n = 6$) grapefruit pulp samples were collected on 4 and 6 days. Samples were also collected from six control and six irradiated fruits on day 6 for freeze drying at the NASA, Shuttle and ISS Food Systems, Johnson Space Center (Houston, TX). The freeze-dried material was vacuum packed in airtight plastic bags. The temperature during transport and short storage was 20 °C for the fruits. However, pulp samples were transported with dry ice (−78.5 °C). All samples were stored at −80 °C until analysis.

Irradiation Treatment. Irradiation was carried out with ^{137}Cs self-contained dry storage irradiators (Husman model 521A, Isomedix, Inc., Whippany, NJ) at the U.S. Department of Agriculture facility (Mission, TX). Grapefruits were exposed to 300 Gy with a centerline-absorbed dose of 40 Gy min^{-1}. The fruit temperatures (Noncontact thermometers, Raytek Raynger ST Series, Santa Cruz, CA) before and after irradiation were 20.7 ($n = 16$; SEM = 0.25) and 22 ($n = 16$; SEM = 0.23) °C, respectively. The nonirradiated controls were also transported to the irradiation site along with the samples to be irradiated with the aim of exposing them to similar conditions.

Standards. Naringin (naringenine-7-rhamnosidoglucoside, NAR), narirutin (naringenin-7-rutinoside, NAT), didymin (isosakuranetin-7-rutinoside, DID), neohesperidin (hesperitin 7-neohesperidoside, NEH), and poncerin (isosakuranetin-7-neohesperidoside, PON) were obtained from Indofine Chemical Company, Inc. (Hillsborough, NJ). Ascorbic acid, d-limonene, myrcene, lycopene, and β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). Limonin, nomilin, and obacunone were purified by procedures established in our laboratory (*13*). Dimethyl formamide (DMF) and acetonitrile (ACN) were obtained from VWR Scientific Products (Houston, TX).

Flavanone Analysis. Grapefruit pulp samples were analyzed for flavanone content by reverse phase liquid chromatography with modification of the procedure described by Mouly et al. (*14*). Fresh pulp (5 g) or freeze-dried pulp (2 g) was homogenized with 20 mL of DMF, and subsequently, a 1.5 mL aliquot was centrifuged at 7500*g* for 20 min. This clear solution (20 μL) was injected into the high-performance liquid chromatography (HPLC) system using an autosampler. Separation of flavanone compounds was performed using Altima C-18 column (Alltech Associates, Deerfield, IL; 250 mm × 4.6 mm) and a guard column. A binary solvent system of ACN and water with 4% acetic acid was programmed to start at 0% and end at 70% ACN concentration for a 65 min period. The flavanone peaks were detected at 280 nm. Flavanones were identified by matching their respective spectra and retention times with those of commercially obtained standards from Indofine Chemical Company, Inc. Quantification of the flavanones was done by using known concentrations of external standards from the commercial source.

Limonoid Aglycone Analysis. Freeze-dried grapefruit pulp powder (3 g) of both control and irradiated fruits was homogenized with 50 mL of water, and the pH was adjusted to 2.05. To this solution, 70 mL of ethyl acetate (0.7 M) was added and stirred thoroughly for 30 min. Samples were centrifuged for 10 min at 7500*g*. The supernatant was evaporated under vacuum to dryness and reconstituted with 2 mL of methanol (100%). The filtered samples were injected (10 μL) into the HPLC using an autosampler. The analytical HPLC column for limonoid analysis was a Novapack C-18 column (Alltech Associates; 250 mm × 4.6 mm), and the mobile phase was 10−50% ACN with 3 mM H_3PO_4 for a 70 min period. Limonoids were detected by UV absorption at 210 nm (*15*). Limonoids were identified and quantified by matching their respective spectra and retention times with those of known concentrations of external standards purified by procedures established in our laboratory (*13*).

Terpenoid Analysis. Fresh grapefruit pulp (15 g) or freeze-dried pulp (2 g) samples and 50 mL of distilled water (24 °C) were placed in a 540 mL plastic blender jar. The samples were blended with 200 μL of 5% acetone for 2.5 min at medium speed using an Osterizer brand food blender. Headspace gas samples (1 mL) were injected into a GC (Perkin-Elmer 8700 model) equipped with a flame ionization detector (*16*).

Operating conditions for the GC included injector and detector temperature, 250 °C; air and H_2 pressure, 138 and 105 kPa, respectively; and 30 mL/min of helium as carrier gas. A glass column (2 mm ID and 270 cm long) packed with 80% Carbowax 1500 on Chromosorb WAW-HMDS 80/100 mesh was used for the separation. Oven temperatures were maintained at 50 °C for 0.5 min and raised to 130 °C at the rate of 10 °C/min (total run time of 8.5 min). Terpenoid compounds were purchased from Sigma-Aldrich Corp., and standard retention times were used to confirm the identity of volatile components. External standards (2−10 μg/g) were injected, and standard curves were constructed for quantification of the compounds in the samples.

Carotenoid Analysis. Samples of grapefruit pulp (5 g for fresh tissue and 1 g for freeze-dried) were extracted with acetone (50 mL). The acetone extract was filtered using Miracloth filter (Behring Diagnostics, La Jolla, CA) and mixed with 50 mL of hexane. Double-distilled water (50 mL) was added to this mixture to separate the hexane layer from the acetone mixture, and samples were stored in the dark for 24 h to allow the transfer of carotenoids into the hexane layer. Subsequently, aliquots (750 μL) of the hexane layer were transferred to an HPLC vial. Hexane was evaporated by nitrogen stream, and the carotenoids were resuspended in 750 μL of acetone. An HPLC system with a Perkin-Elmer LC-250B pump, a LC-200 autosampler, and a UV−vis detector was used. A 20 μL sample was injected. The mobile phases were ACN:water (9:1; solvent A) and ethyl acetate (35%; solvent B). For both solvents A and B, 0.1% triethylamine was added to improve interaction of the mobile phase and stationary phases. The column was a Spherisorb ODS-2 C-18, 5 μm (Waters Corporation, Milford, MA; 250 mm × 4.6 mm) with a guard column. The column was kept at room temperature (about 22 °C) with a flow rate of 1 mL min^{-1}, and detection was performed at 450 nm. The standard compounds were purchased from Sigma-Aldrich Corp., and external standards were used for quantification of the samples. *trans*- and *cis*-Lycopene were combined to obtain the total lycopene content.

Ascorbic Acid Analysis. Fresh (5 g) or freeze-dried pulp (2 g) was homogenized with 25 mL of metaphosphoric acid (3%). A 1.5 mL aliquot was centrifuged at 7600 rpm for 20 min. The supernatant (20 μL) was injected onto a Waters Bondpak C-18 column (Waters Corporation; 30 cm × 0.4 cm) with a guard column. The mobile phase was ACN:water (70:30 v/v) with ammonium phosphoric acid (1.15 g/L) at the flow rate of 1.0 mL min^{-1}. Ascorbic acid was detected at 255 nm with a run time of 20 min. Ascorbic acid was identified and quantified by comparison of peak areas with external standards purchased from Sigma Chemical Co.

Statistical Analysis. Statistical analysis was performed using SAS (*17*). This experiment utilized a 2 × 4 factorial design. Significant differences between control and irradiated (two factors) grapefruit bioactive compounds were evaluated over the storage time (four factors) and their interaction using the general linear model at the 5% probability level. At the last storage time point (6 days after harvest), grapefruit pulp was freeze-dried and the bioactive content was presented on a fresh weight basis. Sample means were compared by the least significant difference test at the 5% probability level whenever there was no interaction.

RESULTS AND DISCUSSION

Influence of Irradiation and Freeze Drying on Flavanone Content of Grapefruit Pulp. Figure 1A shows the HPLC chromatogram of flavanones in grapefruit pulp. It shows the NAT, NAR, NEH, DID, and PON along with other peaks, which were not identified in our study. Interaction between irradiation and storage was observed for NAR content (**Figure 2**). A rapid

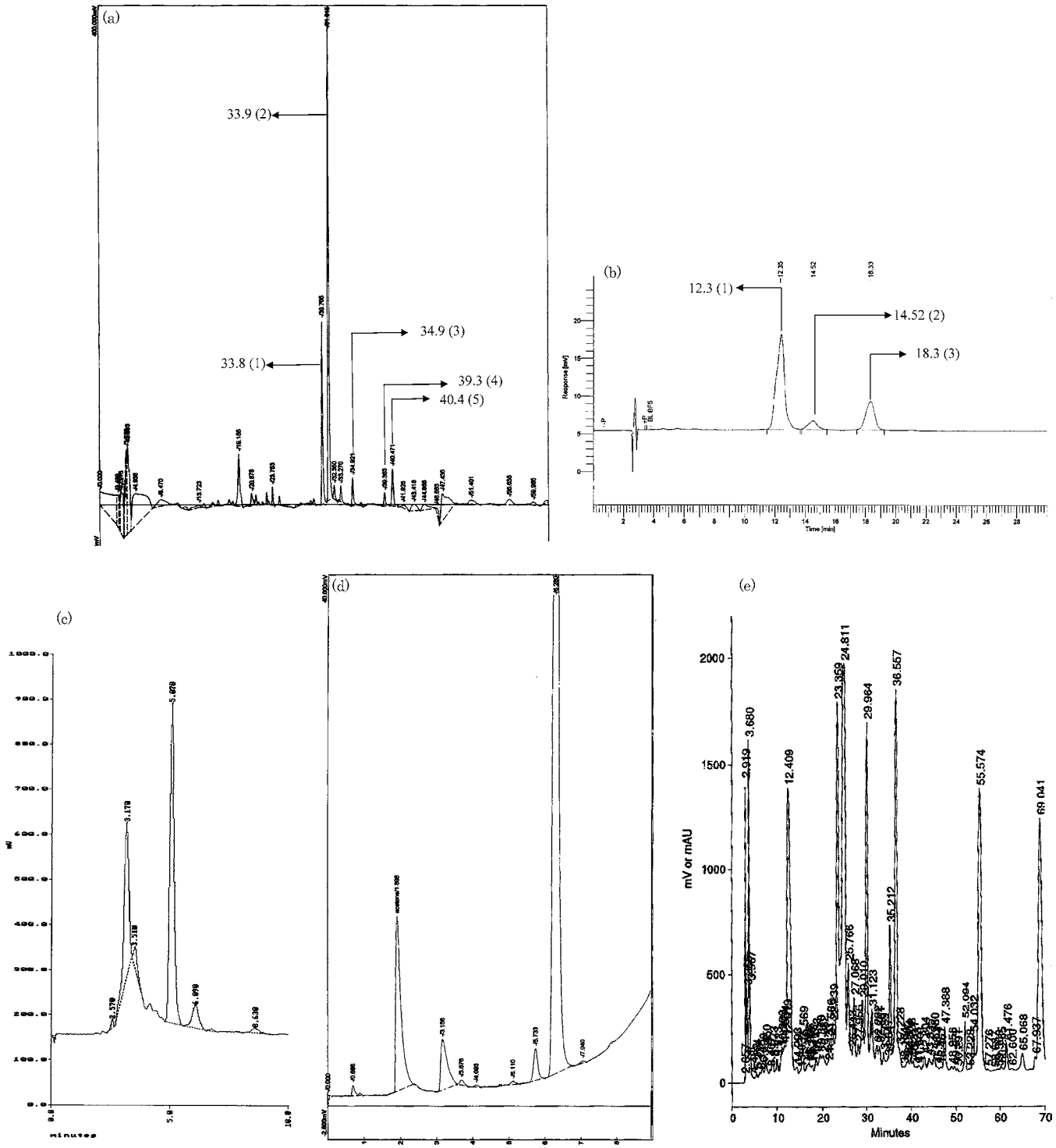

Figure 1. (**A**) HPLC chromatogram of flavonoids from grapefruit pulp using C-18 column and UV detection at 280 nm. Peaks are labeled 1–5 (corresponding retention times clarified in call out boxes): 1, NAT; 2, NAR; 3, NEH; 4, DID; and 5, PON. (**B**) HPLC chromatogram of *trans*- and *cis*-lycopene and β-carotene from grapefruit pulp using C-18 column and visible detection at 450 nm. Peaks are labeled 1–3 (corresponding retention times clarified in call out boxes): 1, *trans*-lycopene; 2, *cis*-lycopene; and 3, β-carotene. (**C**) HPLC chromatogram of ascorbic acid from grapefruit pulp using C-18 column and UV detection at 255 nm (at RT = 5.078). (**D**) GC chromatogram of myrcene (at RT = 5.73) and D-limonene (at RT = 6.28) from grapefruit pulp using a glass column packed with 80% Carbowax 1500 on Chromosorb WAW-HMDS 80/100 mesh. (**E**) HPLC chromatogram of limonin (at RT = 55.57) obacunone (at RT = 69.04), and nomilin (at RT = 62.60) from grapefruit pulp using C-18 column and UV detection at 210 nm.

increase in NAR content of control fruit was observed with storage that declines to initial levels in freeze-dried material. However, in irradiated fruit, the increase is delayed until 6 days but again declines to initial levels in the freeze-dried material.

Increased NAR content at the 4th (42%) and 6th (31%) day after harvest as compared to the levels at harvest (0 days) in control fruits was thought to be due to enhanced phenylalanine ammonia-lyase (PAL) activity (*18*, *19*). PAL catalyzes the deamination of L-phenylalanine to form *trans*-cinnamic acid, a precursor for flavonoids and tannins (*20*). Irradiation has been shown to induce PAL activity in a variety of fruits, including citrus (*7*, *21*). These results suggest that the stress induced by storage in combination with irradiation synergistically increases PAL activity.

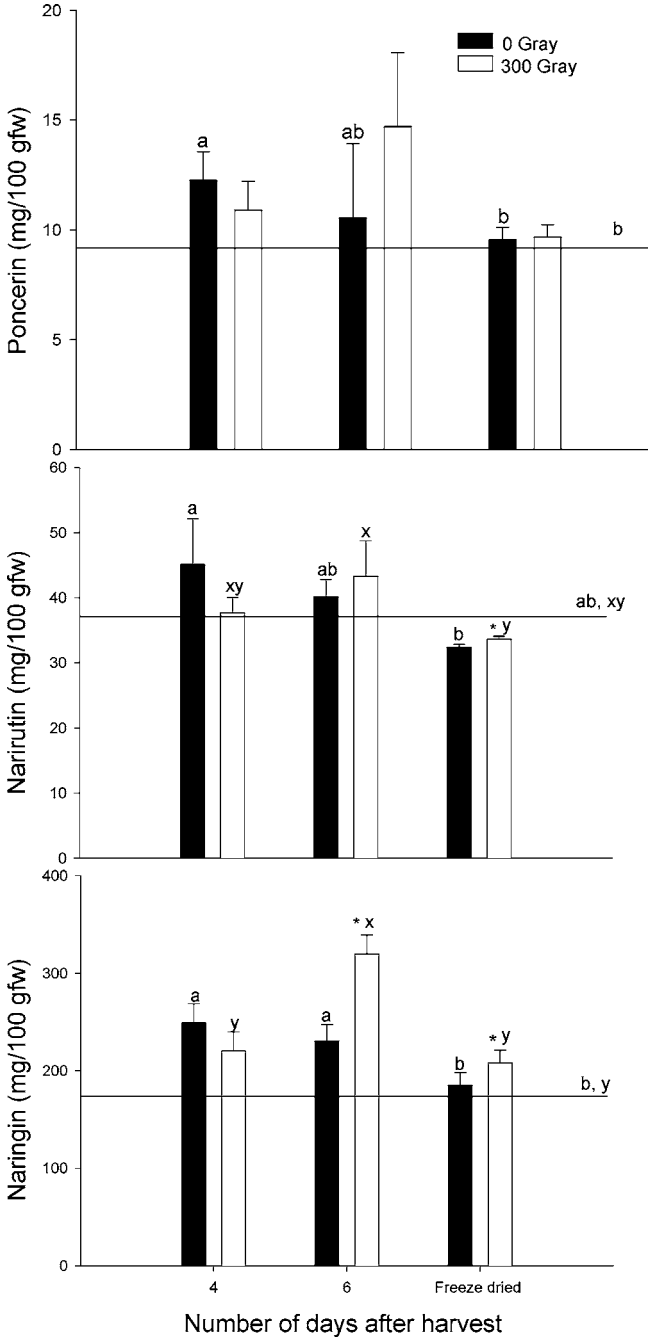

Figure 2. Irradiation, storage, and freeze-drying effects on NAR, NAT, and PON contents of Rio Red grapefruit ($n = 6$). *Indicates differences between the mean values of control and irradiated fruits at the same time interval ($P \leq 0.05$). The same letter on the bar for control and irradiated fruits indicates no differences over time ($P \leq 0.05$). The horizontal line indicates the mean value on the day of harvest (SEM: NAR = 8.24; NAT = 1.92; and PON = 0.97). Interaction between irradiation and storage was observed for NAR ($P \leq 0.05$).

This storage and/or irradiation induced de novo synthesis of NAR may be responsible for a higher (12.5%) NAR content in freeze-dried grapefruit pulp from irradiated fruits as compared to the control (*22*). While NAR (35%) and NAT (22%) contents of irradiated fruits decreased due to freeze drying as compared to 6 days after storage, their contents were comparable to the day of harvest. NAT (29%) and PON (20%) declined in the freeze-dried control samples as compared to the levels 4 days after harvest (**Figure 2**). Previous reports suggest that flavanones such as NAR and NAT are sensitive to freeze drying (*22*).

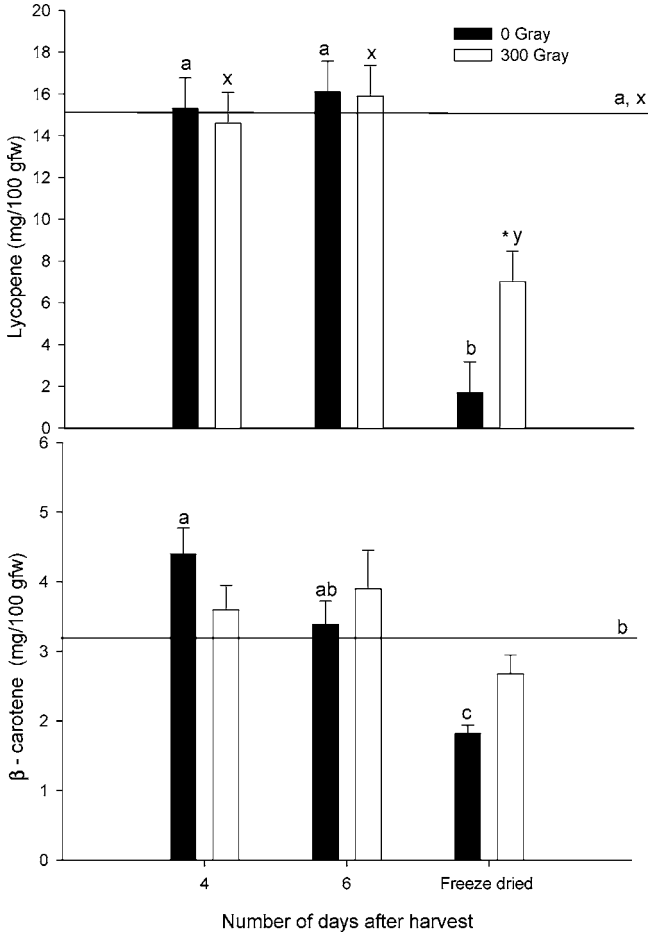

Figure 3. Irradiation, storage, and freeze-drying effects on β-carotene and lycopene contents of Rio Red grapefruit ($n = 6$). *Indicates differences between the mean values of control and irradiated fruits at the same time interval ($P \leq 0.05$). The same letter on the bar for control and irradiated fruits indicates no differences over time ($P \leq 0.05$). The horizontal line indicates the mean value on the day of harvest (SEM: β-carotene = 0.16; and lycopene = 1.6). Interaction between irradiation and storage was for lycopene ($P \leq 0.05$).

However, the NAR and NAT contents in freeze-dried controls were not different from the day of harvest.

DID and NEH contents in grapefruit pulp ranged from 1.76 to 3.69 and 1.43−3.15 mg/100 gfw, respectively (data not shown). These values are almost double as compared to the levels found in grapefruit juices (*14*). DID and NEH were not affected by irradiation, freeze drying, or the number of days after harvest. The differential response of these flavonoids to irradiation may be due to substituents linked to the flavanone skeleton (*7*).

Influence of Irradiation and Freeze Drying on Lycopene, β-Carotene and Ascorbic Acid Content of Grapefruit Pulp. **Figure 1B**,**C** shows the HPLC chromatogram of carotenoids (lycopene and β-carotene) and ascorbic acid in the extracted samples of grapefruit pulp, respectively. Interaction between irradiation and storage was significant for lycopene content. The lycopene contents in freeze-dried pulp from irradiated and control fruits were less ($P \leq 0.05$) than the fruits at the 0, 4, and 6 days after harvest (**Figure 3**). However, freeze-dried pulp from irradiated fruit had a higher (312%) lycopene content as compared to control fruit. β-Carotene was increased (34%) by the 4th day after harvest in control fruits. However, freeze drying reduced (44%) the β-carotene content below the initial levels. Interestingly, no significant differences were observed

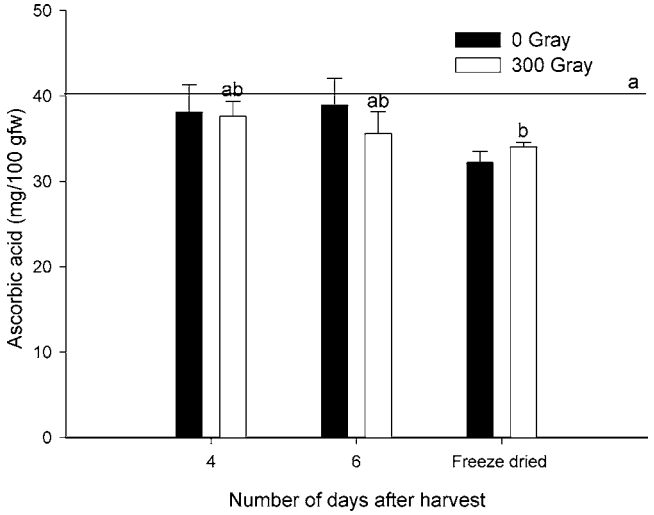

Figure 4. Irradiation, storage, and freeze-drying effects on ascorbic acid content of Rio Red grapefruit ($n = 6$). The same letter on the bar for irradiated fruits indicates no differences over time ($P \leq 0.05$). The horizontal line indicates the mean value on the day of harvest (SEM = 3.0).

in irradiated fruits. Grapefruit carotenoids, especially lycopene, degrade when the fruit pulp or juice is extracted from the fruit (*23*). Previous studies (*24*, *25*) have also shown that plant products lose 12−30% of β-carotene due to freeze drying. However, our previous studies showed numerically higher β-carotene contents in grapefruits exposed to γ-radiation (*26*). This may explain why freeze-dried pulp from irradiated fruits did not show a significant difference in the β-carotene content as compared to the fruits on the 0, 4, and 6 days after harvest.

Freeze drying reduced (15%) the ascorbic acid content in irradiated grapefruit pulp powder as compared to the initial levels. However, the reduction was not observed in control fruit (**Figure 4**). Minimal loss of ascorbic acid has also been recorded in natural strawberries after freeze drying (*8*). Oxidative stress induced by irradiation along with freeze drying may be responsible for reduction in ascorbic acid content in freeze-dried pulp from irradiated fruits as compared to the day of harvest (0 day).

Influence of Irradiation and Freeze Drying on Volatile Compounds. Figure 1D shows the GC chromatogram of volatile compounds (*d*-Limonene and myrcene) in the extracted samples of grapefruit pulp. The content of *d*-limonene was reduced with storage in both irradiated and control fruits (**Figure 5**). However, reduction in *d*-limonene content continued from 4 to 6 days of storage in irradiated fruits but not in control fruits. This resulted in a significantly lower (31%) *d*-limonene content in irradiated fruits as compared to control at 6 days after harvest. Freeze drying resulted in a marked reduction in *d*-limonene content in both control and irradiated fruits. Furthermore, myrcene was reduced to nondetectable levels in the freeze-dried fruits. Bos et al. (*27*) also reported a significant reduction in volatile compounds of cow parsley, *Anthriscus sylvestris* (L.) Hoffm, after freeze drying. Freeze drying of parsley, *Petroselinum crispum* L., also resulted in a marked decrease in the majority of volatile compounds (*28*). The myrcene content was lower in irradiated fruit at the 4th (57%) and 6th (58%) days after harvest as compared to immediately after harvest. Nunez Selles et al. (*29*) also reported a numerical reduction in the volatile compounds including *d*-limonene and myrcene in irradiated grapefruit (1000 Gy).

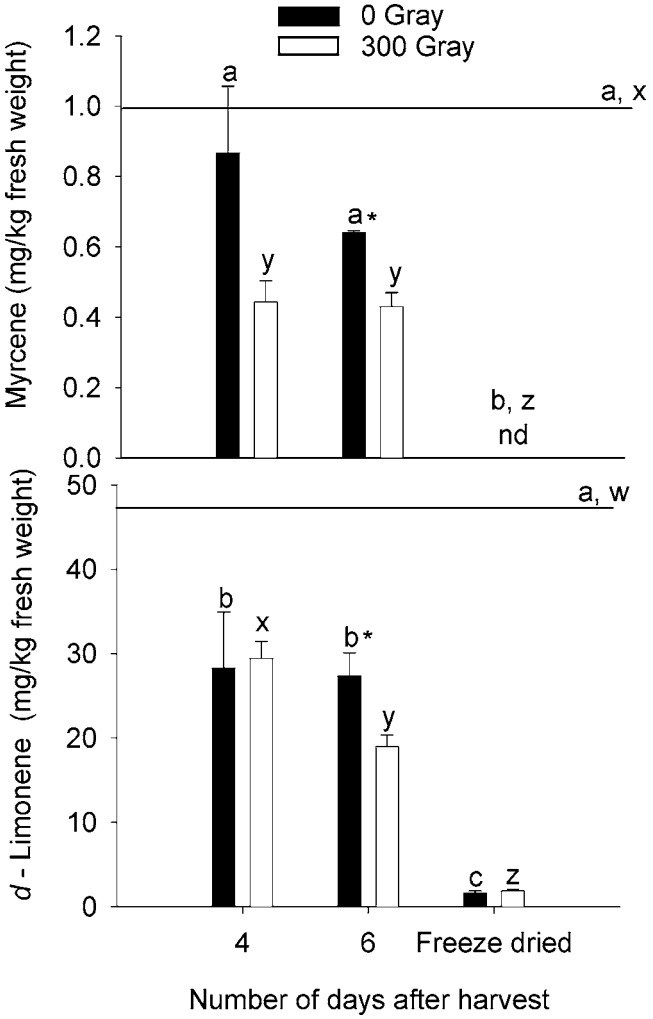

Figure 5. Irradiation, storage, and freeze-drying effects on *d*-limonene and myrcene contents of Rio Red grapefruit ($n = 6$). *Indicates differences between the mean values of control and irradiated fruits at the same time interval ($P \leq 0.05$). The same letter on the bar for control and irradiated fruits indicates no differences over time ($P \leq 0.05$). The horizontal line indicates the mean value on the day of harvest (SEM: *d*-Limonene = 2.74; and myrcene = 0.17). nd means not detected.

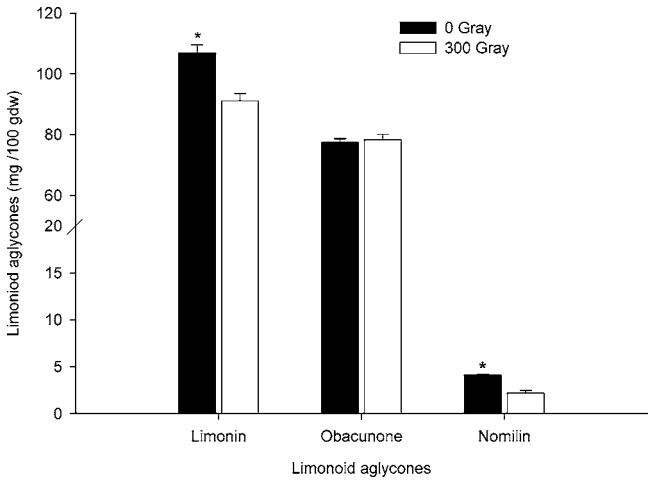

Figure 6. Limonin, obacunone, and nomilin (mg/100 gdw) contents of freeze-dried Rio Red grapefruit pulp ($n = 3$). *Indicates differences between the compound mean values for control and irradiated fruit ($P \leq 0.05$).

Influence of Irradiation and Freeze Drying on Limonoid Aglycones. **Figure 1E** shows the HPLC chromatogram of limonoid aglycones (limonin, nomilin, and obacunone) in the extracted samples of grapefruit pulp. Limonin (15%) and nomilin (47%) contents were reduced in irradiated freeze-dried pulp as compared to freeze-dried controls (**Figure 6**). However, no significant differences were observed in obacunone for irradiated and control fruits. To the best of our knowledge, this is the first study to demonstrate an effect of irradiation on limonin, obacunone, and nomilin.

In summary, our results suggest that postharvest irradiation, storage, and freeze drying have significant effects on the bioactive components of grapefruit. Thus, it is important to take the postharvest processes into consideration when fruit- and vegetable-based dietary supplements are being developed. To accurately assess results for the chemopreventive ability of fruit and vegetable components, it is essential to use suitable methods for the preservation of bioactive compounds.

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