Antioxidative Activity of Browning Reaction Products Isolated from Storage-Aged Orange Juice†

Hyoung S. Lee

Citrus Research and Education Center, Florida Department of Citrus, 700 Experiment Station Road, Lake Alfred, Florida 33850

The antioxidative activity of methanol extracts of browning reaction products isolated from stored orange juice on the oxidation of linoleic acid was investigated at pH 8.0. The antioxidative activity was assessed by conjugated diene formation from peroxidation of linoleic acid at 40 °C. Quantitative changes in linoleic acid were also measured by reversed-phase HPLC. Browning reaction products were found to possess potent antioxidant activity as measured by peroxidation of linoleic acid. The antioxidative activity of browning reaction products was stronger than that of butylated hydroxyanisole but weaker than that of α-tocopherol at the 0.01% level. The antioxidant effect of browning reaction products appears to increase as juice browning increases. Further fractionation of browning reaction products on a Sep-Pak C_{18} cartridge followed by antioxidative assay revealed that the most effective antioxidant fractions were extracted by 10% and 20% ethyl acetate in hexane and 100% methanol.

INTRODUCTION

Browning reaction products from Maillard-type reactions (between sugars and amino acids), caramelization-type reactions, and heat-processed foods have been shown to have antioxidative activity that retards lipid oxidation in model systems (Kirigaya et al., 1969; Lingnert and Eriksson, 1980; Yamaguchi et al., 1981) as well as in some foods containing fats (Lingnert, 1980; Lingnert and Lundgren, 1980). However, it is also known that the antioxidative effect of the Maillard reaction products was found to be strongly dependent on reactants and reaction conditions (Lingnert and Eriksson, 1980). Citrus browning is unique to the typical Maillard-type browning since citrus fruits have significant amounts of ascorbic acid; the oxidation of ascorbic acid has been considered the major factor in the browning of citrus products (Varsel, 1990). Besides ascorbic acid, large amounts of other organic acids (mainly citric) and their salts create favorable conditions for degradation of sugars, amino acids, and phenolics during processing and upon subsequent storage (Lee and Nagy, 1988). It was therefore of interest to investigate whether citrus browning products also have antioxidative properties.

The purpose of this research was to investigate by model systems the potential antioxidative activity of browning reaction products from storage-aged orange juice using the model system of linoleic acid in aqueous media. Also, it was of interest to see if potential antioxidant activity was related to the extent of juice browning.

MATERIALS AND METHODS

Materials. Glass-packed orange juices were obtained from a local processor. Tris(hydroxymethyl)aminomethane, linoleic acid, butylated hydroxyanisole (BHA), and α-tocopherol (type V) were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions (0.35%) of BHA and α-tocopherol were prepared with methanol. Bondapak C_{18}/Porasil B (37–75 μm) column packing materials and Sep-Pak C_{18} cartridges were obtained from Millipore Co. (Milford, MA).

Browning Reaction Products (BRP) from Orange Juice. Browning reaction products were prepared by aging commercially bottled orange juices in a temperature-controlled storage locker (50 °C) for 15 weeks. For comparison of each different storage time, juice samples were collected at 3-week intervals.

Isolation of Browning Reaction Products. A 100-mL sample of aged (15 week) orange juice was freeze-dried and the dried residue extracted three times with 150 mL of methanol using a wrist-action shaker (Burrell Co., Pittsburgh, PA) for 10 min. The extracts were combined followed by filtration and concentration to dryness in vacuo. The dried residue was dissolved with 5 mL of water and further purified by passing through a glass column (2 × 15 cm) prepacked with 20 g of Bondapak C_{18}/Porasil B. After the column was washed with 50 mL of water, the brown products were eluted with 750 mL of methanol, concentrated to dryness, dissolved with 5 mL of water, and freeze-dried. A stock (0.35%) solution in water was prepared before testing for antioxidative activity.

For comparison of antioxidative activity between each different storage period, a simplified procedure using the Sep-Pak (vac/1 cm^3) C_{18} cartridge was used. A 2-mL portion of the centrifuged juice was pipetted into a syringe and passed through a Sep-Pak C_{18} cartridge which had been prewetted with 3 mL of methanol followed by 5 mL of water. After the cartridge was washed with 3 mL of water, the brown compounds were eluted with 3 mL of methanol and filtered through a 1.2-μm filter cartridge (Gelman Science, Inc., Ann Arbor, MI). The browning intensity of these methanol eluants was compared by measurement of absorption at 420 nm with a Shimadzu Model UV-160 UV–visible spectrophotometer.

Fractionation. A 5-mL portion of stock BRP solution (0.35%) was loaded onto the Sep-Pak (vac/3 cm^3) C_{18} cartridge. The cartridge was eluted stepwise with 5 mL each of 100% hexane; 10%, 20%, 30%, 50%, 70%, and 100% ethyl acetate in hexane; and 100% methanol. The separated fractions were added to 1 mL of water, the solvent removed by flushing with N_2 gas, and the volume adjusted to 5 mL with methanol.

Incubation Procedure for the Test of Oxidation. In the preparation of lipid substrate, the mixture of linoleic acid (0.316 g) in 50 mL of 0.1 M Tris-HCl buffer, pH 8.0, was thoroughly emulsified by aid of an ultrasonic bath and then diluted to 200 mL with the same buffer. Fresh lipid substrate was prepared for every model. In the incubation, the mixtures containing 31 mL of linoleic acid, BRP (0–2 mL), and water in 35 mL total volume were equally divided (1 mL) into small vials and incubated at 40 °C in the dark.

Measurement of Antioxidative Effects. The antioxidative effect of the BRP was evaluated both by the spectrophotometric method and by a HPLC method. In the spectrophotometric method, peroxidation of linoleic acid was measured as an increase in 234-nm absorbance due to conjugated diene

† Florida Agricultural Experiment Station, Journal Series No. R-01791.

0021-8561/92/1440-0550$03.00/0

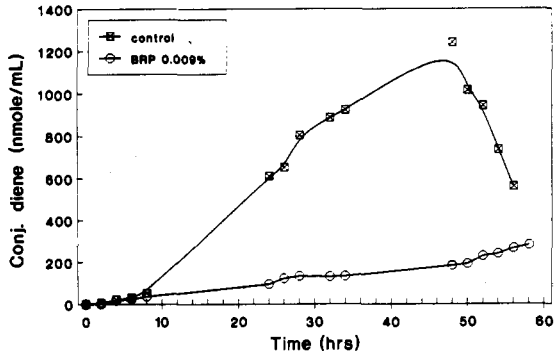

Figure 1. Changes in conjugated diene level during oxidation of linoleic acid with or without browning reaction product (BRP).

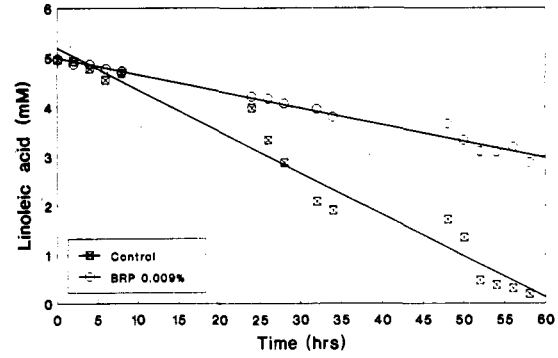

Figure 2. Measurement of linoleic acid by HPLC during its oxidation with or without browning reaction product (BRP).

formation. Aliquots of the test solution (0.1 mL) were diluted at intervals with 3.4 mL of methanol and vortexed, and the absorbance was measured at 234 nm by using the Shimadzu Model UV-160 spectrophotometer (Columbia, MD). The difference in absorbance between a fresh and an incubated sample was calculated, and the conjugated diene was estimated by using a molar extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} (Uchida et al., 1990). HPLC analysis for linoleic acid was according to a procedure slightly modified from that reported by Uchida et al. (1990). Conditions were as follows: Zorbax RX-C18 column (4.6 mm i.d. × 250 mm) and guard column from Mac-Mod Analytical Inc. (Chadds Ford, PA); isocratic elution with 0.1% trifluoroacetic acid in water/acetonitrile (10:90 v/v); flow rate of 1.0 mL/min; detection at 210 nm; injection volume of 15 μL. HPLC systems were Waters 600 E system controller/pump, Shimadzu Model SIL-6A autoinjector, Spectra Physics Model 200 UV–vis detector, and Shimadzu C-R3A integrator. Linoleic acid eluted at 9.23 ± 0.13 min. All test results are the average of duplicate samples.

RESULTS AND DISCUSSION

Antioxidative Activity of the Browning Reaction Products. In the model lipid system using linoleic acid, formation of conjugated dienes as a function of incubation time at 40 °C is shown in Figure 1. The oxidation of linoleic acid was accompanied in the early stage by the formation of hydroperoxides with a conjugated diene system which exhibited an absorption at 234 nm (Frankel, 1962). In the control, which did not contain the BRP, the rate of peroxidation of linoleic acid was rapid, reaching the maximum level within about 50 h of incubation (Figure 1). The addition of BRP at a concentration of 0.009% significantly slowed the rate of conjugated diene formation. Thus, at 48 h of incubation, the level of conjugated diene in the control was about 1240 nmol, while with BRP the level of conjugated diene was very low, forming less than 190 nmol under the same condition. In the quantitative analysis of unoxidized linoleic acid by HPLC, the antioxidative effect of BRP was even more apparent. More than 65% of the linoleic acid in the control was consumed after 48 h, and over 90% was degraded after 60 h of incubation. The degradation rate of linoleic acid was greatly inhibited in the presence of BRP at the concentration of 0.009%, losing less than 27% of the linoleic acid through oxidation during the entire 60-h period (Figure 2). These results clearly suggest that the BRP obtained from storage-aged orange juice contain potent antioxidative activity which can retard the peroxidation of linoleic acid in aqueous media.

Comparative Antioxidative Activity. The antioxidant activity of the BRP obtained from orange juice stored for 15 weeks at 50 °C was compared with that of commercial antioxidants BHA and α-tocopherol. The decrease in concentration of linoleic acid for each antioxidant as a function of incubation time is illustrated in Figure 3. The

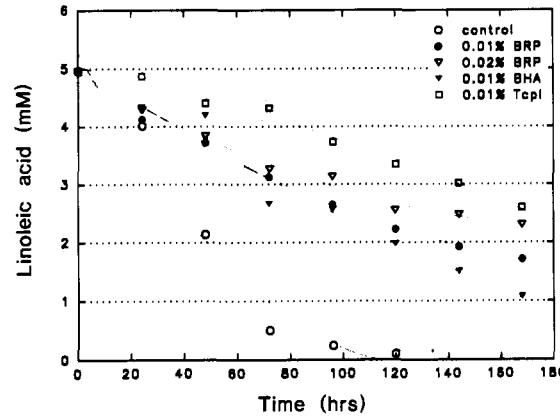

Figure 3. Comparison of the antioxidative activity of browning reaction product (BRP) with that of BHA and α-tocopherol (Tcpl).

0.01% level of BRP was found to be more effective against the peroxidation of linoleic acid than BHA but less effective than α-tocopherol at the same level (Figure 3). From polynomial regression equations for best curve fittings for each antioxidant, the time required for 50% oxidation of linoleic acid was calculated to make comparison of each antioxidant easy (Table I). The control, which did not have any antioxidants, lost 50% of its original level within 39.6 h, while in the samples containing 0.01% of each antioxidant the times were about 105 h for BRP, 97.5 h for BHA, and up to 172.3 h of protection against 50% loss of linoleic acid for α-tocopherol. From these data (Table I), antioxidative activity of the BRP at 0.01% was nearly 1.1 times as effective as BHA and 0.61 times as effective that of α-tocopherol at 0.01% level. However, it should be considered that BRP was still a crude mixture, the active components needing further purification for better comparison.

Antioxidative Activity vs Browning Index. The relationship between antioxidative activity and the browning intensity of the juice from each different aging period at 50 °C is illustrated in Figure 4. As was expected, the visual color of the orange juice became more brown as storage time increased. The browning index, measuring the absorption at 420 nm, increased linearly with storage time with a value of 0.59 for 3 weeks and after 15 weeks increased about 3-fold to 1.75 (Figure 4). The level of conjugated diene formed from linoleic acid after 48 h of incubation was compared for each aging period. As can be seen in Figure 4, less conjugated diene formed as the aging period increased. The high correlation coefficient ($r = -0.912$) indicates that the two parameters, browning index and conjugated diene, have an inverse relationship; the antioxidative activity, as measured by the level of conjugated diene formation, increased as the juice became more and more discolored. This result is in agreement

Table I. Comparative Antioxidant Activity on Linoleic Acid Peroxidation

antioxidant (% by wt)	regression equation	r	antioxidant index[a]
none	$Y = 5.2767 - 0.08336X + 0.0003251X^2$	0.9851	39.6
0.01% BHA[b]	$Y = 5.0550 - 0.02989X + 0.0000358X^2$	0.9847	97.5
0.01% BRP[b]	$Y = 4.9183 - 0.02973X + 0.0000626X^2$	0.9986	105.4
0.02% BRP[b]	$Y = 4.9404 - 0.02694X + 0.0000669X^2$	0.9958	139.5
0.01% Tcpl[b]	$Y = 5.0250 - 0.01004X - 0.0000275X^2$	0.9942	172.3

[a] Antioxidant index refers to the time (hours) required for 50% disappearance of linoleic acid. [b] Abbreviations: BHA, butylated hydroxyanisole; BRP, browning reaction products; Tcpl, α-tocopherol.

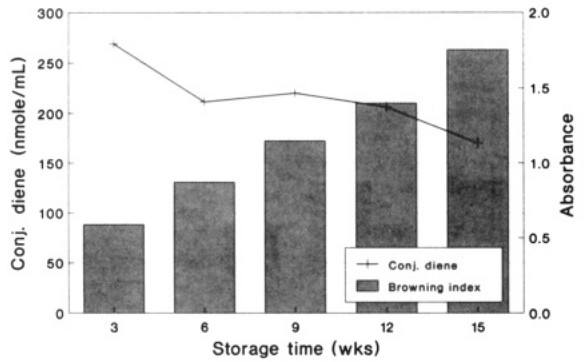

Figure 4. Relationship between conjugated diene levels and browning intensity (A_{420nm}). Conjugated diene was measured after 48 h of incubation at 40 °C.

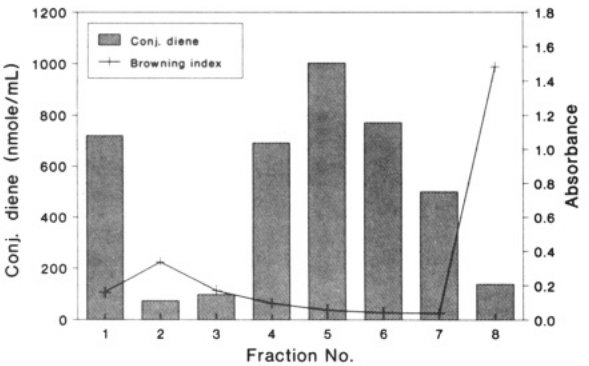

Figure 5. Conjugated diene and browning intensity (A_{420nm}) in each fraction. Conjugated diene was measured after 48 h of incubation at 40 °C.

with earlier observations by Kirigaya et al. (1968) and Yamaguchi et al. (1981), who found a close relationship between the extent of browning and the antioxidative action of Maillard reaction products.

Fractionation of BRP. Stepwise fractionation of BRP on C_{18} cartridge was done to isolate components which might have most of the antioxidative activity by gradually increasing the polarity of the eluant. The antioxidative activity of each of the eight fractions against peroxidation of linoleic acid is illustrated in Figure 5. The levels of conjugated dienes formed after 48 h of incubation were compared. The most active fractions were fraction 2 (10% ethyl acetate in hexane) with the lowest conjugated diene values followed by fraction 3 (20% ethyl acetate in hexane) and fraction 8 (100% methanol). This indicates that possibly several compounds differing in chemical structure formed by browning reaction in orange juice can exhibit antioxidative properties. There were no significant differences between fractions 1, 4, and 6 in conjugated diene level. Fraction 5 (50% ethyl acetate in hexane) was the least effective fraction, showing the highest conjugated diene value, which was close to the value of the control, 1005 nmol/mL. The percent remaining of unoxidized linoleic acid by HPLC ranged from 70.8% for fraction 2 to 24.7% for fraction 5. As can be seen by Figure 5, all of the effective fractions were more or less brown colored. Fraction 8 had the highest browning index; most of the brown compounds seem to be eluted in fraction 8. Fractions 2 and 3 have slight visual brown color. Thus, it appears that most of the compounds responsible for antioxidative activity of BRP from stored orange juice seem to be distributed over the brown fractions. The mechanism of the antioxidative effect by browning reaction products cannot be fully understood, but different opinions seem to suggest that the colorless intermediates, such as reductones (Yamaguchi and Koyama, 1967; Rhee and Kim, 1975; Eichner, 1981), or brown pigments (Kirigaya et al., 1968; Yamaguchi and Fujimaki, 1974; Yamaguchi et al., 1981; Lingnert et al., 1983) play an important role in the antioxidative effect in the system.

LITERATURE CITED

Eichner, K. Antioxidative effect of maillard reaction intermediates. *Prog. Food Nutr. Sci.* **1981**, *5*, 441–451.

Frankel, E. N. Hydroperoxides. In *Symposium on Foods: Lipids and their oxidation*; Schultz, H. W., Day, E. A., Sinnhuber, R. O., Eds.; AVI Publishing: Westport, CT, 1962; Chapter 3.

Kirigaya, N.; Kato, H.; Fujimaki, M. Studies on antioxidant activity of nonenzymic browning reaction products. Part 1. Relations of color intensity and reductones with antioxidant activity of browning reaction products. *Agric. Biol. Chem.* **1968**, *32*, 287–290.

Lee, H. S.; Nagy, S. Relationship of sugar degradation to detrimental changes in citrus juice quality. *Food Technol.* **1988**, 91–94, 97.

Lingnert, H. Antioxidative Maillard reaction products. III. Application in cookies. *J. Food Process. Preserv.* **1980**, *4*, 219–233.

Lingnert, H.; Eriksson, C. E. Antioxidative Maillard reaction products. I. Products from sugars and free amino acids. *J. Food Process. Preserv.* **1980**, *4*, 161–172.

Lingnert, H.; Eriksson, C. E. Antioxidative effect of Maillard reaction products. *Prog. Food Nutr. Sci.* **1981**, *5*, 453–466.

Lingnert, H.; Lundgren, B. Antioxidative Maillard reaction products. IV. Application in sausage. *J. Food Process. Preserv.* **1980**, *4*, 235–246.

Rhee, C.; Kim, D. H. Antioxidant activity of acetone extracts obtained from a caramelization type browning reaction. *J. Food Sci.* **1975**, *40*, 460–462.

Uchida, K.; Haraguchi, K.; Mitsui, M.; Kawakishi, S. Stimulatory effect of histamine on the peroxidation of linoleic acid. *J. Agric. Food Chem.* **1990**, *38*, 1491–1493.

Varsel, C. Citrus juice processing as related to quality and nutrition. In *Citrus Nutrition and Quality*; Nagy, S., Attaway, J. A., Eds.; ACS Symposium Series 143; American Chemical Society: Washington, DC, 1980; pp 225–271.

Yamaguchi, N.; Fujimaki, M. Studies on browning reaction products from reducing sugars and amino acids. Part XV. Comparison of antioxidative activity of melanoidin with that of each tocopherol homologue and synergistic effect of melanoidin on tocopherols. *J. Food Sci. Technol.* **1974**, *21*, 13–18.

Yamaguchi, N.; Koyama, Y.; Fujimaki, M. Fractionation and antioxidative activity of browning reaction products between d-xylose and glycine. *Prog. Food Nutr. Sci.* **1981**, *5*, 429–439.

Received for review July 22, 1991. Revised manuscript received December 18, 1991. Accepted January 21, 1992.