Citrus Section

Proc. Fla. State Hort. Soc. 116:197-200. 2003.

DISTRIBUTION OF TOTAL POLYPHENOLICS AND ANTIOXIDANT POTENTIALS IN DIFFERENT TISSUES OF *CITRUS PARADISI*, *CITRUS GRANDIS* AND *CITRUS SINENSIS*

UFUK KOCA, BALA RATHINASABAPATHI[1] AND GLORIA A. MOORE
University of Florida
Horticultural Sciences Department
P.O. Box 110690
Gainesville, FL 32611-0690

Additional index words. ascorbate oxidase, Folin-Ciocalteu, FRAP, total phenolics

Abstract. **Flavonoid glycosides in citrus fruits are known for their antioxidant qualities. Despite the availability of data on flavonoid glycoside levels in citrus fruits and juices, little is known on how various flavonoid glycosides are distributed in citrus plants and how that distribution relates to the tissue's antioxidant potential. The objective of the current study was to test the correlation between the levels of phenolic compounds including flavonoid glycosides in a tissue and the tissue's antioxidant potential in three citrus species. Fruit outer peel, juice vesicles, juice vesicle membranes, flavedo, albedo, young leaves and mature leaves of grapefruit, sweet orange and pummelo tissues were quantitatively extracted using a solvent mixture. The extracts were evaluated for their antioxidant potential and total phenolics. Analysis of the phenolics in pummelo leaf tissue suggested that most UV-positive compounds in the tissue extracts were flavonoid glycosides. Antioxidant potential (AOP), as measured by a ferric reducing/antioxidant power (FRAP) assay, was correlated to the levels of total phenolics in leaf extracts. The significance of species-specific and tissue-specific differences in their antioxidant potentials is discussed.**

Flavonoids are widely-distributed, polyphenolic, secondary compounds in the plant kingdom. Over 4,000 flavonoid compounds have been identified to date (Heim et al., 2002). Epidemiological studies prove that increased consumption of flavonoid abundant diet leads to decrease in mortality from coronary heart disease and certain types of cancer (Hertog et al., 1995; Ross and Kasum, 2002). Although flavonoid glycosides can have a wide range of biological activities, the protective role of flavonoids in living systems is mostly due to their antioxidant potential, which is related to transfer of reactive oxygen species (ROS), chelation of metal catalysts, activation of antioxidant enzymes and inhibition of certain type of oxidases (Heim et al., 2002).

Citrus plants are known to be rich in their flavonoid glycoside content in fruit, fruit juices and leaves (Horowitz and Gentili 1977; Kawaii et al., 1999; Manthey, 2002). A recent study on the antioxidant potential (AOP) of fresh orange juices attributed that potential to their total phenolic content (Rapisarda et al., 1999). Although there are many studies on the antioxidant potential of dietary flavonoids (Ross and Kasum, 2002 for a review) and some on citrus, tissue distribution of antioxidant flavonoids in citrus is not currently well known. Understanding the tissue distribution of antioxidant flavonoids is the first step toward breeding citrus with improved levels of antioxidant flavonoids.

Because many antioxidant flavonoids are thought to have evolved in plants to protect the photosynthetic machinery from oxidative damage (Demmig-Adams and Adams, 2002), we hypothesized that leaf tissue would contain significantly more antioxidant flavonoids than fruit tissues (Manthey et al. 2000). To test this hypothesis we analyzed extracts of various fruit tissues and young and mature leaves for antioxidant potential and total phenolics. Leaf tissue of pummello was analyzed for flavonoid glycosides.

We employed the ferric reducing/antioxidant power (FRAP) assay, a commonly used method for measuring the antioxidant potential of dietary antioxidants (Benzie and Szeto, 1999; Pulido et al., 2000) because it is simple, robust and inexpensive. The method is based on determining the reduction of a ferric-tripyridyltriazine complex to its ferrous, blue colored form by the antioxidant compound(s). Total phenolics were measured in the same extracts and were correlated to the total antioxidant power. HPLC-mass spectral analyses of pummello leaf tissue extracts indicated that flavonoid glycosides indeed constituted a large portion of the total phenolics.

Material and Methods

Material. Leaf and fruit samples were harvested on 28 Mar. 2003 from 10-year old *Citrus sinensis* Osb, 'Washington', *Citrus* × *paradisi* 'Duncan', and *Citrus grandis* L. trees, grown in the citrus collection shade-house at the University of Florida orchard, Gainesville, Fla. All fine chemicals, reagents and solvents were of the highest purity available and were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Sample preparation. Two to three fruits from each species were washed in deionized water and divided into five tissue sections including outer peel, flavedo, albedo, juice vesicle membranes and juice vesicles. The outer peel tissue was collected as shavings of the orange or yellow-colored tissue of the peel without flavedo and albedo. Young and mature leaves were collected and rinsed in water. Samples, 1 g fresh weight each, were packed in aluminum foil and stored at -80 °C until extraction. Young leaves were identified near the shoot apical meristem from the current season's growth. Mature leaves were fully expanded leaves from previous season's growth. For each tissue, three samples were collected. Additional 1 set of samples from each tissue type for each species were weighed as 1 g fresh weight and dried in an oven to constant

We thank Dr. Jodie Johnson for mass spectral analyses, Dr. Daniel Cantliffe for funding and Ben Grandon for technical assistance. This research was supported by the Florida Agricultural Experiment Station, and approved for publication as Journal Series No. R-09541.

[1]Corresponding author.

weight. Dry weight: fresh weight for outer peel, flavedo, albedo, juice membrane, juice vesicles, young leaves and old leaves respectively were a) for pummelo 0.25, 0.17, 0.18, 0.23, 0.48, 0.24 and 0.41; b) for sweet orange 0.25, 0.30, 0.24, 0.21, 0.14, 0.24 and 0.42; c) for grapefruit 0.24, 0.19, 0.21, 0.21, 0.53, 0.21 and 0.38. Extractions and analyses of these samples were done in four or three independent experiments and the data were pooled for statistical analyses.

Tissue extraction. Frozen tissue samples (1 g fresh weight) were ground in liquid nitrogen in a mortar and pestle. The samples were then transferred to glass tubes and extracted three times with 2 mL methanol:dimethylsulfoxide (1:1 v:v) mixture. Tissue extracts, totaling 6 mL from each sample, were stored at 4 °C in the dark.

FRAP assay. The antioxidant potential (AOP) of citrus tissue samples was estimated with the FRAP assay, using ascorbic acid as standard, with some modifications to the method described by Benzie and Strain (1999). The FRAP reagent was prepared fresh prior to each experiment. The assay period was 30 min and the absorbance at 593 nm was measured using a UV-visible spectrophotometer (Beckman DU-520, Beckman Instruments, Fullerton, Calif.). All FRAP values were converted to nmoles ascorbic acid equivalents. Preliminary trials indicated that the solvent mixture employed did not interfere with the assay, and that a 30 min incubation period was within the linear increase in antioxidant activity in time course experiments (data not shown).

Potential role of ascorbate on AOP. To test whether ascorbic acid in the tissue extracts was responsible for the antioxidant activity, the extracts were assayed for antioxidant activity with and without treating the samples with 2 units of ascorbate oxidase, an enzyme that will oxidize the ascorbate (Benzie and Strain, 1999).

Estimation of total phenolics. Tissue extract (10 µL) was incubated with 500 µL Folin-Ciocalteu phenol reagent for 2 min, then 1.5 mL of 1.9 M Na_2CO_3 was added (Ohno et al., 2000, Vinson et al., 2001). Reaction mixtures were incubated at room temperature for 1h by mixing in a vortex every 20 min. At the end of 1 h, the reaction mixtures were centrifuged briefly and the absorbance at 760 nm was recorded in a UV-Visible spectrophotometer. Tannic acid was used as a standard and all values were converted to tannic acid equivalents.

Statistical treatment of data. Values for total phenolics and the antioxidant potential for tissue extracts were analyzed following analysis of variance using a completely randomized design (SAS, 2001). Means within each species were compared using Duncan's multiple range test (SAS, 2001).

HPLC-Mass spectrometry. Tissue extracts (20 µL) of young and mature leaf samples were analyzed by reverse phase C-18 HPLC (RP-18, 2.1 × 150 mm) in tandem with positive ion ESI electrospray ionization (ESI)-mass spectrometry (Berhow, 2002; Manthey, 2002) using a mass spectrometer at 3.5 kV spray voltage (Thermo Finnigan, San Jose, CA). The samples were loaded in solvent A (5 mM ammonium acetate in 0.5% v/v glacial acetic acid in water) and eluted using a gradient of solvent B (0.5%, v/v, glacial acetic acid in methanol) at a flow rate of 0.1 mL·min^{-1}.

Results and Discussion

Leaves have the most antioxidant potential. Various tissues from three citrus species were examined for their antioxidant potential by using FRAP assay (Table 1). Extracts of young and old leaf tissue displayed the highest antioxidant potential among the other tissue types in all three citrus species examined (Table 1). In grapefruit, young leaves had significantly higher levels of antioxidant potential than the mature leaves. In the other two species young leaves did not differ significantly from the mature leaves (Table 1). Our results support the hypothesis that photosynthetic tissues have more antioxidant compounds compared to fruit. These results also suggest that young leaves of citrus may be used for extraction of antioxidant compounds for potential use in medicine or other applications.

Table 1. Total antioxidant potential of fruit and leaf tissues-extracts of pummelo, sweet orange and grapefruit as determined by the FRAP assay. The values are means and standard errors for four determinations. For each column, means followed by the same letter are not significantly different (P = 0.05) within a species, by Duncan's multiple range test.

	Total antioxidant potential (nmoles ascorbic acid equivalents per g fresh wt.)		
Tissue	Pummelo	Sweet orange	Grapefruit
Outer peel	1406 ± 122 a	1845 ± 47 a	1511 ± 21 a
Flavedo	673 ± 186 a	1622 ± 57 a	831 ± 63 a
Albedo	849 ± 9 a	2079 ± 23 a	512 ± 57 a
Juice membranes	807 ± 1 a	1471 ± 22 a	692 ± 27 a
Juice vesicles	1110 ± 36 a	815 ± 122 a	1535 ± 207 a
Young leaves	3779 ± 344 b	5608 ± 157 b	5441 ± 353 b
Mature leaves	2883 ± 287 b	4497 ± 230 b	3439 ± 189 c

Antioxidant potentials in citrus fruits. The antioxidant potentials were of varying levels in different fruit tissues. The highest antioxidant potential was found in outer peel in pummelo, albedo in sweet orange and outer peel and juice vesicles in grapefruit. These results may have implications in processing citrus for juice, an important industry in Florida with an annual value of $1.8 billion dollars (Hodges et al., 2001). It is possible to use different fruit tissue portions to blend into fruit juices during processing to increase the antioxidant potential of the juice.

Ascorbic acid is not contributing to the antioxidant potential of citrus tissue extracts. Citrus juice is known for its high content of ascorbic acid, a compound known for its antioxidant potential. To examine whether ascorbic acid contributes to the antioxidant potential observed, the tissue extract samples were subjected to ascorbate oxidase activity prior to FRAP assays. Ascorbate oxidase specifically reduces the L-ascorbic acid in a reaction mixture. Results indicated that ascorbate oxidase addition did not reduce the antioxidant potential of

Table 2. Total phenolics in fruit and leaf tissues extracts of pummelo, sweet orange and grapefruit as determined by the Folin-Ciocalteu assay. The values are means and standard errors for three determinations. For each column, means followed by the same letter are not significantly different within a species (P = 0.05) by Duncan's multiple range test.

	Total Phenolics (µg Tannic acid equivalents per g fresh wt.)		
Tissue	Pummelo	Sweet orange	Grapefruit
Outer Peel	1499 ± 8 a	1295 ± 49 a,b	1533 ± 58 a
Flavedo	1669 ± 244 a,d	1551 ± 109 b	1648 ± 542 a
Albedo	2948 ± 193 b	2805 ± 236 c	2751 ± 172 b
Juice membranes	1735 ± 116 a,d	1879 ± 94 b	1338 ± 29 a
Juice vesicles	908 ± 17 c	806 ± 179 a	986 ± 23 a
Young leaves	4243 ± 218 d	6558 ± 339 c	5758 ± 320 b
Mature leaves	2813 ± 238 a	3728 ± 76 b	2793 ± 136 a

the extracts employed (data not shown). These results suggested that the antioxidant potential of the tissue extracts examined were not due to ascorbate but due to other compounds, similar to that found in another study on small fruits (Kalt et al., 1999). An explanation as to why we did not find ascorbate in the extracts is that ascorbate was not extracted or preserved adequately in the tissue extracts using the current method.

Total phenolics in leaves may be the source of antioxidant potential in citrus. In all analyzed tissue types of citrus species, young leaves showed the highest total phenolics followed by mature leaf tissues and albedo of the fruits (Table 2). Leaf total phenolic contents were correlated to their antioxidant potentials with an r^2 of 0.8634 (n = 6) (Fig. 1) but the total phenolic contents in fruit tissues were poorly correlated to their antioxidant potentials (r^2 = 0.185, n = 6). This suggests that the phenolic compounds in leaves have higher antioxidant potentials than compounds found in fruit tissues.

Correlative evidence for antioxidant flavonoids in citrus leaves. The leaf extracts of the three species of *Citrus* were analyzed by HPLC-mass spectrometry to identify the major phenolics

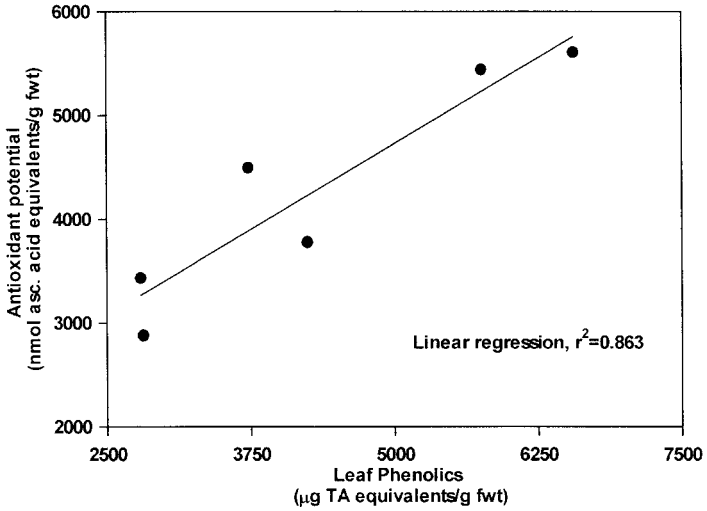

Fig. 1. Correlation of total phenolic levels in leaves of Citrus (Table 2) to their antioxidant potential (Table 1).

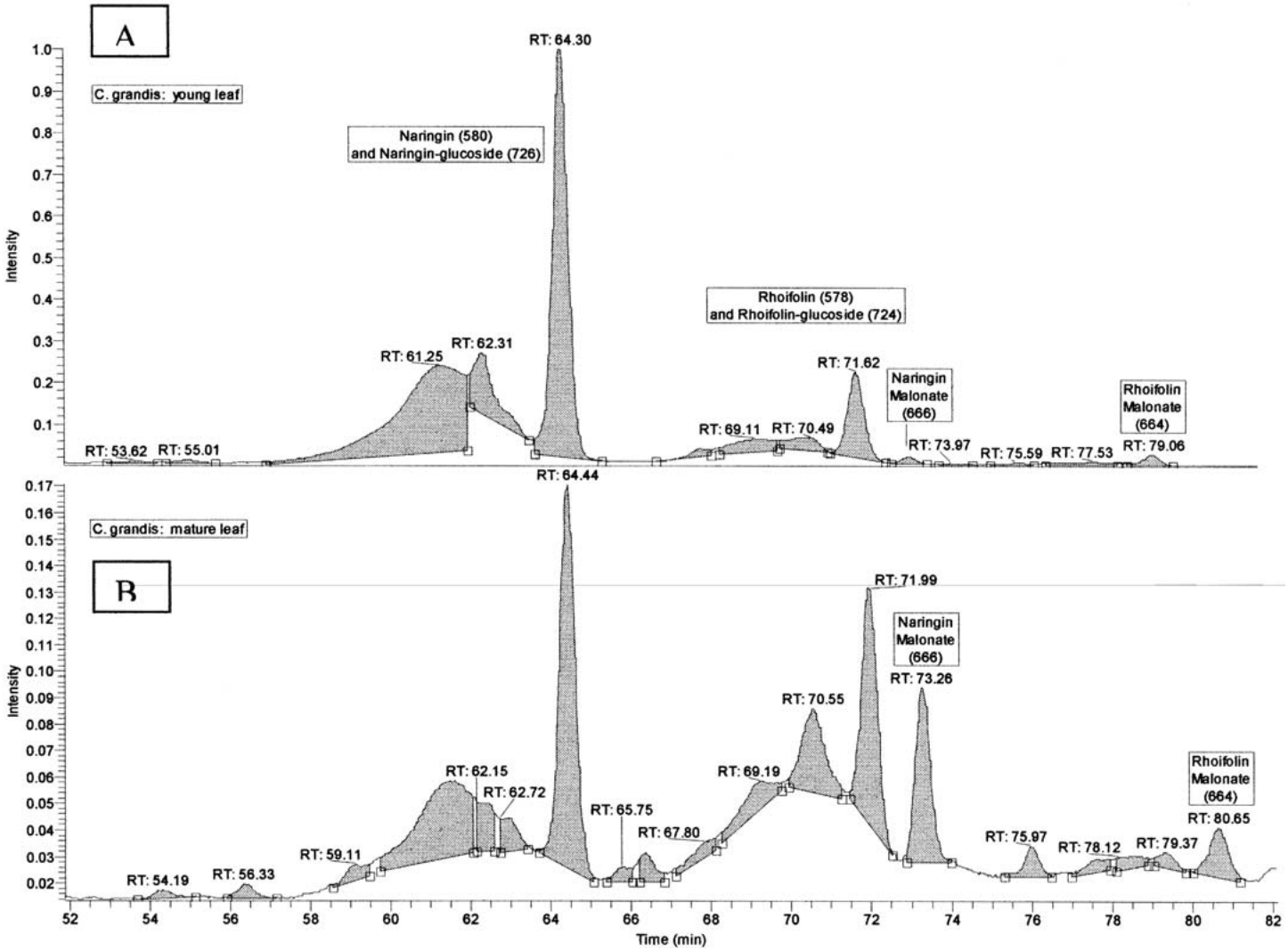

Fig. 2. Comparison of HPLC/UV of flavonoid region of extracts from young leaf (Panel A) and mature leaf (Panel B) of pummelo. Equal volumes of extracts representing equal fresh weights of tissue (20 μL) were analyzed for A and B. Note that y axis scale is different for A and B. The peaks were annotated for the molecular weight and the identity of compounds based on the mass-spectral analysis of individual peaks (data not shown).

(Berhow et al., 1998). Several UV-positive compounds were separated by HPLC. Not all of the UV-positive compounds would be flavonoid glycosides. However, when the major UV-positive peaks were identified by mass spectrometry, the m/z ratios of peaks were identical to known flavonoid glycosides, suggesting that flavonoid glycosides are the major polyphenolics found in these extracts. A sample HPLC chromatogram, annotated with mass spectral data, is shown in Figure 2 for young and mature leaves of pummelo. In this species, young leaves had 3.3-fold higher naringin-4'-glucoside, 2-fold higher naringin-related glycoside (MW 580), and 4-fold higher glycoside of MW 594. Qualitatively young and mature leaves differed in their composition also. A peak with MW 610, corresponding to hesperidin, was found in young leaves and absent in the mature leaf. Several pummelo varieties are known to produce some or trace amounts of hesperidin in their leaves (Berhow et al. 1998). A flavonoid malonate (MW 664; aglycone 270) was present in the mature tissue but not detected in young leaves.

Conclusion

Our results show that leaf tissue of citrus has the maximum antioxidant potential. This potential is due, at least in part, to the total phenolics. Among these phenolics, certain flavonoid glycosides may play a central role in imparting antioxidant potential. Further studies are in progress to analyze species-specific variations of flavonoid glycosides in citrus and how they differ in their antioxidant potential.

Literature Cited

Benzie, I. F. F. and J. J. Strain. 1999. Ferric Reducing/Antioxidant Power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299:15-27.

Benzie, I. F. F. and T. Szeto. 1999. Total antioxidant potential of teas by the ferric reducing/antioxidant power assay. J. Agric. Food Chem. 47:633-636.

Berhow, M. A., B. Tisserat, K. Kanes, and C. Vandercook. 1998 Survey of phenolic compounds produced in citrus. United States Department of Agriculture, Agricultural Research Service, Technical Bulletin Number 1856.

Berhow, M. A. 2002. Modern analytical techniques for flavonoid determination, pp. 61-76. In B. S. Buslig and J. A. Manthey (eds.). Flavonoids in Cell Function. Kluwer Academic/Plenum Publishers, NY.

Demmig-Adams B. and W. W. Adams. 2002. Antioxidants in photosynthesis and human nutrition. Science 298:2149-2153.

Heim, K. E., A. R. Tagliaferro, and D. J. Bobilya. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13: 572-584.

Hertog, M. G. L., D. Kromhout, C. Aravanis, H. Blackburn, R. Buzina, F. Fidanza, S. Giampaoli, A. Jansen, A. Menotti, S. Nedeljkovic, M. Pekkarin, B. S. Simic, H. Toshima, E. J. M. Feskens, P. C. H. Hollman, and M. B. Kantan. 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch. Intern. Med. 155:381-386.

Hodges, A., E. Philippakos, D. Mulkey, T. Spreen, and R. Muraro. 2001. Economic impact of Florida's Citrus Industry, 1999-2000. EDIS document FE 307. http://edis.ifas.ufl.edu.

Horowitz, R. M. and B. Gentili. 1977. Flavonoid Constituents of Citrus, pp 397-426. In S. Nagy, P. E. Shaw, and M. K. Veldhuis (eds.). Citrus Science and Technology, AVI Publishing Westport, CT.

Kalt, W., C. F. Forney, A. Martin, and R. L. Prior. 1999. Antioxidant capacity, vitamin C, phenolics and anthocyanins after fresh storage of small fruits. J. Agric. Food Chem. 47:4638-4644.

Kawaii, S., Y. Tomono, E. Katase, O. Kazunori, and M. Yano. 1999. Quantitation of flavonoid constituents in citrus fruits. J. Agric. Food Chem. 47:3565-3571.

Manthey, J. A., K. Grohman, M. A. Berhow, and B. Tisserat. 2000. Changes in citrus leaf flavonoid concentrations resulting from blight induced zinc-deficiency. Plant Physiol. Biochem. 38:333-343.

Manthey, J. A. 2002. Analysis of flavonoids and hydroxycinnamates in citrus processing byproducts by high performance liquid chromatography-electrospray ionization-mass spectrometry. Proc. Fla. State Hort. Soc. 115:292-297.

Ohno, T., K. Doolan, L. M. Zibilske, M. Liebman, E. R. Gallandt, and C. Berube. 2000. Phytotoxic effects of red clover amended soils on wild mustard seedling growth. Agriculture, Ecosystems and Environ. 78:187-192

Pulido R., L. Bravo, and F. Saura-Calixto. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agric. Food Chem. 48:3396-3402.

Rapisarda, P., A. Tomaino, R. Lo Cascio, F. Bonina, A. Pasquale, and A. Saija. 1999. Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. J. Agric. Food Chem. 47:4718-4723.

Ross, J. A. and C. M. Kasum. 2002. Dietary flavonoids: Bioavailability, metabolic effects, and safety. Annu. Rev. Nutr. 22:19-34.

SAS. 2001. SAS Software release 8.2, SAS Institute, Inc., Cary, NC, U.S.A.

Vinson, J. A., X. Su, L. Zubik, and P. Bose. 2001. Phenol antioxidant quantity and quality in foods: fruits. J. Agric. Food Chem. 49:5315-5321.