JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Major Anthocyanins and the Color of Red-Fleshed Budd Blood Orange (*Citrus sinensis*)

HYOUNG S. LEE*

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

High-performance liquid chromatography (HPLC) with photodiode array detection was applied for the characterization of anthocyanins in red-fleshed Budd Blood (*Citrus sinensis*) orange. More than seven anthocyanin pigments were separated within 30 min by using a binary gradient (0.1% H_3PO_4 in water and 0.1% H_3PO_4 in acetonitrile) elution on a Prodigy ODS column. Separations by reversed-phase HPLC and semipreparative HPLC on a Prodigy 10-μm ODS Prep column, and acid and alkali hydrolyses were used for identification of anthocyanins. The primary anthocyanins in Budd Blood orange grown in Florida were cyanidin-3-(6″-malonylglucoside) (44.8%) followed by cyanidin-3-glucoside (33.6%). Two other minor pigments were also acylated with malonic acid. Malonated anthocyanins represented the major proportion (>51%) of anthocyanins in Budd Blood orange. Total anthocyanin contents and juice color parameters (CIE *L**,*a**,*b**) were compared with six other Florida-grown blood oranges.

KEYWORDS: Budd Blood orange; HPLC; color; pigment; anthocyanin; malonated anthocyanins

INTRODUCTION

Budd Blood orange is one of the sweet orange cultivars selected from the first 10-year field trials with good juice color along with very good flavor (*1*). Budd Blood is noteworthy for its excellent orange flesh color and the consistent appearance of red coloration. The fruit did not develop the deep red coloration of blood oranges grown in cool climates, and the peel was usually the typical yellow to orange color of Florida oranges (*1*). Budd Blood matures in midseason as do most blood oranges. The box yield and pound solids were similar to Hamlin, which is the principal early-season orange grown in Florida, and began to show red flesh by December with good coloration in January. Also, the juice flavor was excellent and often had some of the distinctive flavor associated with blood oranges (*1*).

Red-colored blood orange is not common in the U.S. market, but because of its unique red pigment, it has been blended with lightly colored blond sweet orange juice to improve the color (*2*). Furthermore, fresh-squeezed unpasteurized blood orange juice is currently marketed throughout the west coast of the United States. Red coloration in sweet orange (*Citrus sinensis*) is mostly caused by the presence of water-soluble anthocyanin pigments not usually found in citrus. Previously, anthocyanins in Italian blood oranges were characterized as cyanidin-3-glucoside, the major anthocyanin, with four minor acylated (cinnamic acids) cyanidin derivatives (*3, 4*). Later, cyanidin-3-rhamnoside was reported as one of the major anthocyanins in commercial Italian blood orange (*5*). However, the anthocyanins in blood oranges grown in Florida have not been studied for a pigment profile.

The red water-soluble anthocyanin pigments in orange fruit are of special interest because recent nutritional and epidemiological studies have described many beneficial health effects (*6*−*8*) of anthocyanins for protection against certain cancers, cardiovascular disease, and aging. The importance of pigments on juice color and the growing interest in possible health benefits of anthocyanins have stimulated efforts to study the pigmentation in Budd Blood, a promising selection for small-scale commercial production. The juice is also valuable for blending with other cultivars having pale color.

The study is aimed at characterization of anthocyanin pigments and the color of the red-fleshed Budd Blood, and determining any significant differences in anthocyanin contents compared with common blood oranges grown in Florida.

MATERIALS AND METHODS

Orange Fruits. Budd Blood fruit were collected from the Florida Citrus Arboretum, Lake Alfred, FL, in early February 2000. Other blood orange cultivar selections (Moro, Moro × Tarocco, Sanguinelli, and Tarocco) were also collected from the Arboretum for comparison. Random samples of 15 fruit each were collected to represent all canopy positions. Moro blood oranges from California were obtained from local stores. Juice was extracted with a household-type electric reamer (Waring, New Hartford, CT) from all fruit in each sample, filtered through cheesecloth, pasteurized (90 °C for 30 s), cooled, and frozen (−20 °C) until used.

Anthocyanin Extracts. Budd Blood orange juice (25 mL) was homogenized (30 s at speed 4) in an Omni mixer homogenizer (Warrenton, VA) with 50 mL of extracting solvent (acetone/ethanol/

* To whom correspondence should be addressed. E-mail: hsl@lal.ufl.edu.

10.1021/jf011205+ CCC: $22.00
Published on Web 01/25/2002

hexane, 25:25:50, v/v/v) and centrifuged. The top hexane layer with carotenoids was removed. Bottom layer with anthocyanin pigments was recovered, concentrated to remove the ethanol and acetone, then passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), eluted with acidified methanol (0.1% HCl/MeOH), and filtered.

High-Performance Liquid Chromatography Analysis of Anthocyanin and Anthocyanidin. Anthocyanin and anthocyanidin pigments were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with binary gradient elution (*9*). A Prodigy 5-μm ODS 3 column (4.6 × 150 mm, 5 μm) from Phenomenex (Torrance, CA) was used with 0.1% H_3P0_4 in water (eluant A) and 0.1% H_3P0_4 in acetonitrile (eluant B). Detection was at 520 nm, flow rate at 1 mL/min, an injection of 10 μL, and temperature at 25 °C. A gradient program was performed: initial condition was 90% A/10% B; 0−2 min, 90% A/10% B; 2−32 min, 50% A/50% B; 32−37 min, 50% A/50% B; 37−57 min, 30% A/70% B; and back to the initial condition. Chromatography was performed with a Waters Alliance 2690 Separations Module with autoinjector, 996 PDA detector, and a Millennium data system.

Standards of anthocyanin (cyanidin-3-glucoside) and anthocyanidins (cyanidin, pelagonidin, peonidin, and malvidin chlorides) were obtained from Extrasynthese (Genay, France). Cyanidin-3-(6″-malonylglucoside) was obtained from Polyphenols Lab. AS, Norway. Red onion was used as a source of malonated anthocyanins.

Semipreparative Fractionation of Anthocyanin. For the collection of anthocyanin peaks for identification, semipreparative fractionation was performed by using HPLC (Waters 600E model pump) with a semipreparative Prodigy 10-μm ODS Prep column (10 × 250 mm, i.d., 10 μm) from Phenomenex, 0.1% H_3P0_4 in water (eluant A) and 0.1% H_3P0_4 in acetonitrile (eluant B). Initial condition was 80% A/20% B; 0−2 min, 80% A/20% B; 2−32 min, 50% A/50% B; and back to the initial condition. Detection was at 520 nm (Spectra-Physics model 200), flow rate was 2.5 mL/min, and injection volume was 50 μL.

Each peak was collected by using a Gilson fraction collector (Middleton, WI), combined and evaporated under vacuum to remove the acetonitrile. The concentrated extract was passed through a C18 Sep-Pak, washed with ethyl acetate to remove polyphenolics other than anthocyanins as described by Skrede et al. (*10*), and anthocyanins were eluted with 0.1% HCl in methanol. After evaporation of the solvent, acid or alkali hydrolyses were performed.

Acid and Alkali Hydrolyses of Anthocyanins. The acid and alkali hydrolysis procedures previously described by Lee and Wicker (*9*) were used for the preparation of anthocyanidins, sugars, and acids. Acid hydrolysis was performed with 2 N HCl (10 mL) for 60 min in a boiling-water bath. For alkali hydrolysis, concentrated, purified pigment (5 mL) was hydrolyzed in a screw-cap test tube with 10 mL of 10% KOH for 8 min at room temperature in the dark. The resultant hydrolysate was neutralized with 5N HCl, purified through a C18 Sep-Pak, freeze-dried, dissolved in a small volume (ca. 1.5 mL) of water, and filtered.

HPLC Analysis for Sugars and Organic Acids. Analysis of sugar components of the anthocyanins was performed by using an HPLC procedure with a YMC−Pak PA column (4.6 × 250 mm, i.d., 5 μm) from Waters (Milford, MA), using 75% acetonitrile in water, with a flow rate of 1 mL/min (t_R = rhamnose, 6.3 min; fructose, 8.51 min; glucose, 9.49 min; rutinose, 11.87 min), and a refractive index detector (Hewlett-Packard, Wilmington, DE). The hydrolysate was freeze-dried and dissolved in a small volume of water. HPLC analysis of the acylated acid components of the anthocyanin was made after both acid and alkaline hydrolysis. The hydrolysate was injected onto a YMC ODS-AQ column (4.6 × 250 mm) from Waters (Milford, MA), with 2% KH_2P0_4 (pH 2.4), UV at 210 nm, a flow rate of 0.7 mL/min, and injection volume at 20 μL.

Color Analysis. Color (CIE L^*,a^*,b^*) analysis was conducted by using a Macbeth Color-Eye 3100 spectrophotometer (Kollmorgen Instruments Corp., Newburgh, NY) in the reflectance mode as described previously by Lee (*11*). Hue ($\tan^{-1} b^*/a^*$) and chroma [$(a^{*2} + b^{*2})^{1/2}$] were calculated from CIE a^* and b^* values.

Statistical Analysis. Statistical analysis [descriptive statistics and Fisher's least-significant difference (LSD) test] was conducted with SigmaStat PC software from SPSS, Inc (Chicago, IL). Trends were considered significant when means of compared sets differed at $P < 0.05$.

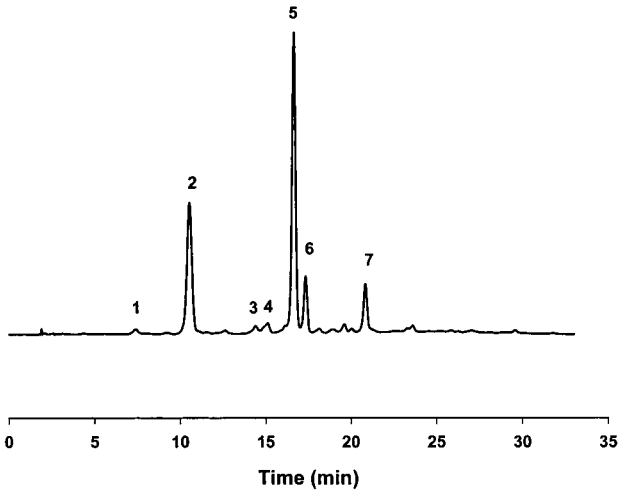

Figure 1. RP-HPLC chromatogram for anthocyanins in Budd Blood orange juice on Prodigy ODS 3 column (4.6 × 150 mm, 5 μm). Mobile phases are 0.1% H_3PO_4. in water and 0.1% H_3PO_4 in acetonitrile. Detection at 520 nm. See **Table 1** for identification.

Table 1. Spectral Data of Some Anthocyanins from Budd Blood Orange Juice

peak	t_R (min)	area (%)	spectral data λ_{max} (nm)	E_{440}/E_{vis} (as %)	E_{320}/E_{vis} (as %)	aglycone	sugar	acylating agent
1	7.3	<1.0	278, 320^{sh}, 524	30.8	21.4			
2	10.5	28.0	278, 515	34.4	12.7	cyanidin	glucose	
3	14.4	<1.0	278, 331, 524	30.0	82.5			
4	15.1	1.4	284, 327, 515	33.3	52.5			
5	16.6	47.6	281, 515	34.5	13.7	cyanidin	glucose	malonic
6	17.3	8.7	281, 518	30.4	17.6	cyanidin	glucose	malonic
7	20.8	7.2	281, 518	31.2	22.7	cyanidin	glucose	malonic

RESULTS AND DISCUSSION

Anthocyanin Profile. Figure 1 shows the HPLC chromatogram of anthocyanins from Budd Blood orange juice purified by C18 Sep-Pak cartridge. Seven peaks were separated, but identity of minor peaks (1 and 3) with an area percentage less than 1%, and a peak (peak 4) with a poor resolution were not considered. Identification of four major peaks (2, 5, 6, and 7) was made from comparison of the retention and spectral data with that of authentic standard, from known sources and from literature values. This was further confirmed by preparative HPLC with acid and alkali hydrolyses, and presented in **Table 1**.

Acid hydrolysis of peak 2 produced cyanidin as aglycone, and sugar analysis showed glucose. Peak 2 had a visible absorption maximum at 515 nm, and $E_{440}/E_{vis-max}$ ratio was 34.4%, which agreed well with those reported for cya-3-glucoside under a similar HPLC condition (*12*). The absorption ratio between 440 nm and λ_{max} is often used to assume the position of glycosides (*13*). $E_{320}/E_{vis-max}$ ratio was less than 20, which agreed well with the spectral property for cyanidin-3-glucoside (*14*). With the use of spectral data and the results of acid and alkaline hydrolyses, peak 2 was assigned as cyanidin-3-glucoside, and further confirmed by cochromatography with authentic cyanidin-3-glucoside.

In the test for acylated anthocyanins by mild alkaline hydrolysis with KOH, peaks 5−7 nearly disappeared, indicating

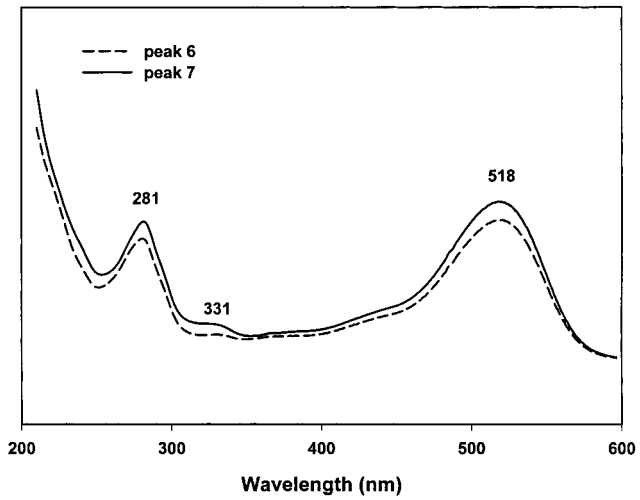

Figure 2. UV–Visible spectra of peaks 5 and 7 obtained by on-line HPLC–PDA.

either acylated anthocyanin or an alkaline-labile anthocyanin. The on-line spectrum of peaks 5−7 did not show any additional distinct peak or shoulder in UV between 310 and 340 nm (**Figure 2**), which suggested peaks 5−7 were not acylated with hydroxylated, aromatic organic acids. It was suspected that peaks 5−7 were acylated with aliphatic carboxylic acids, such as acetic or malonic acid, because of their late retention time. Acylation increases the retention time of anthocyanins under reversed-phase (RP) HPLC (*15*).

Peak 5 had a visible absorption maximum at 515 nm, and the $E_{440}/E_{vis-max}$ ratio was 34.5%. Acid hydrolysis yielded cyanidin and glucose. Alkaline hydrolysis yielded malonic acid. The malonate of cyanidin-3-glucoside retains longer than cyanidin-3-glucoside under RP-HPLC with acidified methanol elution (*16*). With the spectral data and results of acid and alkaline hydrolyses, peak 5 was assigned as cyanidin-3-glucoside malonyl ester, which has malonic acid attached to the C-6 of glucose, because this acyl substituent is generally bound to C-6 sugar (*17*). Peak 5 was further confirmed by cochromatography with authentic cyanidin-3-(6″-malonylglucoside).

The occurrence of malonyl ester of cyanidin-3-glucoside is seldom reported in fruits, but it has been frequently found in *Allium* species (*18*) including red onions and plants. The very diverse malonate anthocyanins were also reported from petals of *Hibiscus syriacus* (*19*), in plants of compositae (*15*), and in flowers of maize (*20*). Cyanidin-3-(6″-malonylglucoside) has been reported in the Italian Moro blood orange juice (*21*). It was initially designated as cyanidin-3-glucoside acylated with acetic acid (*4*), or cyanidin-3-rutinoside (*5*), but later, this inconsistency was further reinvestigated and its structure elucidated as cyanidin-3-(6″-malonyl)-β-glucoside (*21*).

Pigments 6 and 7 gave λ_{max} at 518 nm (**Figure 2**) and $E_{440}/E_{vis-max}$ of 30.4 and 31.2%, respectively. Complete acid hydrolyses of peaks 6 and 7 yielded only glucose as the sugar component, and aglycone analysis showed cyanidin. In addition, alkaline treatment of peaks 6 and 7 yielded malonic acid (t_R = 7.29 ± 0.2 min) as the acidic component. On the basis of this chemical evidence, peaks 6 and 7 were assumed to be the malonyl esters of peak 2 (cyanidin-3-glucoside). Previously, four different malonated cyanidins were known to be present in major varieties of red onions (*18*). Thus, pigment extract from red onion was compared under the same HPLC conditions. On the basis of the elution pattern, and spectral data compared with the anthocyanins from the red onion, peaks 6 and 7 were thought of as malonated cyanidins with dimalonic substitution or probably as positional isomers. The spectral properties of these two anthocyanins were reported as λ_{max} at 520 nm and $E_{440}/E_{vis-max}$ of 29.1% (*18*). These two malonated anthocyanins were not previously reported in red-fleshed blood oranges.

Table 2. Color and Pigments in Blood Orange Juices

blood orange juices	Budd blood	Moro[a]	Moro[b]	Moro[c]	Moro × Tarocco	Sanguinelli[d]	Sanguinelli[e]
monomeric anthocyanin (mg/L)	50.3	21.9	9.7	103.8	13.7	28.4	64.9
Cya-3-glucoside (%)	33.6	29.4	28.9	38.8	28.2	30.2	30.9
Cya-3-(6″-malonyl-glucoside) (%)	44.8	48.2	37.8	37.2	39.8	46.0	45.0
color density	2.8	2.4	2.2	3.6	3.0	2.6	4.0
polymeric color	2.0	2.0	1.2	2.7	2.9	0.8	3.0
CIE *L**	33.2	38.8	38.7	29.2	39.2	35.5	32.7
CIE *a**	18.1	14.7	5.8	15.9	6.6	11.9	17.0
CIE *b**	8.2	11.0	13.3	5.3	12.2	12.2	9.3
hue	24.6	38.6	65.9	20.6	61.2	45.1	28.4
chroma	20.0	18.8	14.9	15.8	13.9	17.7	19.7

[a] Moro, Moro s/o. [b]Moro, Moro cleo. [c]Moro, from California. [d]Sanguinelli, Sanguinelli s/o; [e]Sanguinelli, Sanguinelli s/m.

Table 2 presents the percentages of each anthocyanin in the Budd Blood orange juice. Cyanidin-3-(6″-malonylglucoside) was the most predominant anthocyanin, comprising more than 44% of the total anthocyanin content, followed by cyanidin-3-glucoside (34%). Quantitatively, malonated anthocyanins (peaks 5, 6, and 7) account for more than half (51.3%) of the total anthocyanin contents in Budd Blood.

Budd Blood showed a relative quantitative anthocyanin pattern similar to the other blood oranges grown in Florida (**Table 2**). However, a difference exists in major anthocyanin profiles, such as cyanidin-3-glucoside (peak 2) and its malonyl ester (peak 5), between Florida-grown blood oranges and blood oranges from Italy reported in the literature. Unlike the Florida-grown blood orange samples presented in **Table 2**, cyanidin-3-glucoside was reported as the major anthocyanin, comprising between 40% and 60% of the total anthocyanin in blood oranges grown in Italy (*4*). The blood orange from Italy is pigmented mainly by cyanidin-3-glucoside (50%) and its acetylated derivatives (18%) with a spread of eight minor components (*4*). Also, cyanidin-3-glucoside was the major anthocyanin in the California-grown blood orange sample in this study (**Table 2**). In 1958, Chandler (*22*) reported cyanidin-3-glucoside as the major (>90%) anthocyanin from California blood oranges (Moro variety) by thin-layer chromatography. Thus, it is speculated that different geographic growing regions, as well as fruit maturity, probably had influence on the anthocyanin profiles of red-pigmented blood oranges.

Table 2 also presents the mean values of total anthocyanin contents obtained by the pH differential method. The values from other Florida-grown blood samples were also included for comparison. Budd Blood orange is highly pigmented; the mean value of monomeric anthocyanin content was 50.3 ± 12.8 mg/L, which is higher (LSD =20.8, $P < 0.05$) than the values found in other Florida-grown blood oranges such as Moro, or Moro × Tarocco varieties (**Table 2**). However, these Budd Blood anthocyanins were far less developed than the major Italian blood orange varieties, Sanguinelli (87.2−118.5 mg/L) or Moro (105−164 mg/L) reported by Rapisarda and Giuffrida (*23*), or than the California Moro blood orange samples, 96−166 mg/L (*24*). Anthocyanin pigmentation may well be highly dependent on climate and temperature, and it is well-known that most anthocyanin-containing fruits develop higher coloration in cooler

regions (*25*). Furthermore, variability in the analytical methodologies for the pigment contents should be considered.

Table 2 presents the mean values of juice color parameters (CIE L^*,a^*,b^*) of six different red-pigmented blood oranges grown in Florida. All CIE color parameters summarized in **Table 2** supported the deep color of Budd Blood juice. Hue angle (h^*), which is the attribute of color that is perceived, was highly correlated to anthocyanin content ($r = -0.927$). Hue angle was significantly different from the values from other blood orange juices grown in Florida (LSD = 10.1, $P < 0.05$). Most of the blood orange juices from Moro s/o, Moro Cleo, Moro × Tarocco, and Sanguinelli s/o showed higher hue angles than Budd Blood except Moro blood oranges from California (**Table 2**). Budd Blood orange juice color was similar to the Sanguinelli Sweet Milan. Chroma, which attributes the dimension of saturation, was not significantly correlated with the anthocyanin contents.

In conclusion, the red color of the Budd Blood is characterized by the presence of anthocyanins and is highly pigmented compared with most other Florida-grown blood orange samples. In addition, a relatively higher percentage of cyanidin-3-(6″-malonylglucoside) with considerable amounts of two more malonated anthocyanins was found in Budd Blood orange. Malonated anthocyanins represented the major proportion of anthocyanins in the Budd Blood orange.

ACKNOWLEDGMENT

The technical assistance provided by Mr. Gary Coates in the experiment is gratefully acknowledged.

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Received for review September 11, 2001. Revised manuscript received November 27, 2001. Accepted November 29, 2001.

JF011205+