Characterization of Carotenoids in Juice of Red Navel Orange (Cara Cara)

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High-performance liquid chromatography with photodiode array detection was applied for the separation and characterization of carotenoids from a red-fleshed navel orange (Cara Cara). Carotenoid pigments were extracted using hexane/acetone/ethanol and saponified using 10% methanolic potassium hydroxide. More than 29 carotenoid pigments were separated within 60 min using a ternary gradient (75% acetonitrile/25% methanol, methyl *tert*-butyl ether, and water) elution on a C_{30} reversed-phase column. The presence of lycopene (3.9 ± 1.1 ppm) and a relatively large percentage of β-carotene were distinct differences in the pigment profile of this red navel orange juice as compared to the profile of standard navel orange juice. The juice color in the red navel was much deeper orange than that from navel orange; hue angle ranged from 84.1 to 89.4 for red navel compared to 98.2 to 100.5 for standard navel.

Keywords: *Carotenoids; red navel orange; HPLC; color; pigment; lycopene*

INTRODUCTION

The red-fleshed navel orange cultivar (Cara Cara), which originated as a mutation of probably Washington Navel from Venezuela (*1*), was released for propagation in Florida in 1990 (*2*). The fruit is currently marketed in the fresh fruit market and is attractive in salads because of its nearly crimson flesh, which is similar to that of the pink and red grapefruit. This appealing fruit is also valuable for its juice, which can be blended with that of other cultivars that have pale color. This is an extremely attractive addition to the range of navel cultivars, and has created great interest with producers and consumers, as it combines novel internal appearance with fine eating quality (*1*).

Red coloration in sweet orange (*Citrus sinensis*) is mostly due to the presence of anthocyanins such as cyanidin-3-glucoside (*3*). However, previous studies have reported that the red coloration in certain sweet oranges is due to carotenoid pigments rather than anthocyanins which are responsible for the characteristics in blood orange cultivars. Monselise and Halevy (*4*) reported the presence of lycopene from Sarah, a pink bud sport of Shamouti orange from Israel, which has many of the characteristics of pink grapefruit. Later, Stewart et al. (*5*) reported that red coloration in red Valencia orange, which is much deeper than that from standard Valencia, is due to cryptoxanthin. In a recent book on citrus varieties of the world (*1*), it was indicated that the deeply colored flesh of Cara Cara orange is associated with lycopene, which is usually found in Star Ruby grapefruit. The presence of lycopene in sweet orange is not common, but is of special interest because recent nutritional and epidemiological studies have probed the importance of lycopene in preventing certain cancers (*6*).

The importance of carotenoids on juice color and the growing interest in health benefits of carotenoids have stimulated efforts to study the carotenoids and nutrient values in red navels, which have been recently introduced in the market. This study is aimed at characterization of carotenoid pigments and the color of the red-fleshed navel orange (Cara Cara) which has not previously been studied systematically, and ascertaining whether there are significant differences in carotenoid contents compared to those of standard navel oranges grown in Florida.

MATERIALS AND METHODS

Orange Fruits. Oranges (navel and red navel) were obtained from local growers in Florida during the mid-December, 1999−2000 season. The navel orange is an early-maturing fruit and is usually harvested from October to January. Fruits were extracted with a household-type electric hand reamer, and the juice was filtered through cheesecloth (pulp content, 8 vol %), pasteurized (90 °C for 30 s), and cooled before analysis.

Carotenoid Extraction. Carotenoid extraction was carried out according to Lee and Castle (*7*). A 25-mL aliquot of orange juice was homogenized (30 s at speed 4) in an Omni mixer homogenizer (Warrenton, VA) with 50 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v/v), and centrifuged for 5 min at 6500 rpm at 5 °C (IEC, Needham, MA). The top layer of hexane containing the color was recovered and transferred to a 25-mL volumetric flask. The volume of recovered hexane was then adjusted to 25 mL with hexane. Saponification was carried out overnight using aqueous 10% methanolic KOH solution in the dark at room temperature (*7*). Reproducibility of the extraction was less than 3% CV.

Total Carotenoids. Estimation of total carotenoid content was carried out on an aliquot of the hexane extract, as described under the procedure for carotenoid extraction, by measuring the absorbance at 450 nm using a Genesis-5 Spectronic spectrophotometer (Rochester, NY). Total carotenoid content was calculated according to De Ritter and Purcell (*8*) using an extinction coefficient of β-carotene, $E^{1\%} = 2505$.

HPLC Analysis of Carotenoids. Carotenoid pigments were analyzed by RP-HPLC using a ternary gradient elution

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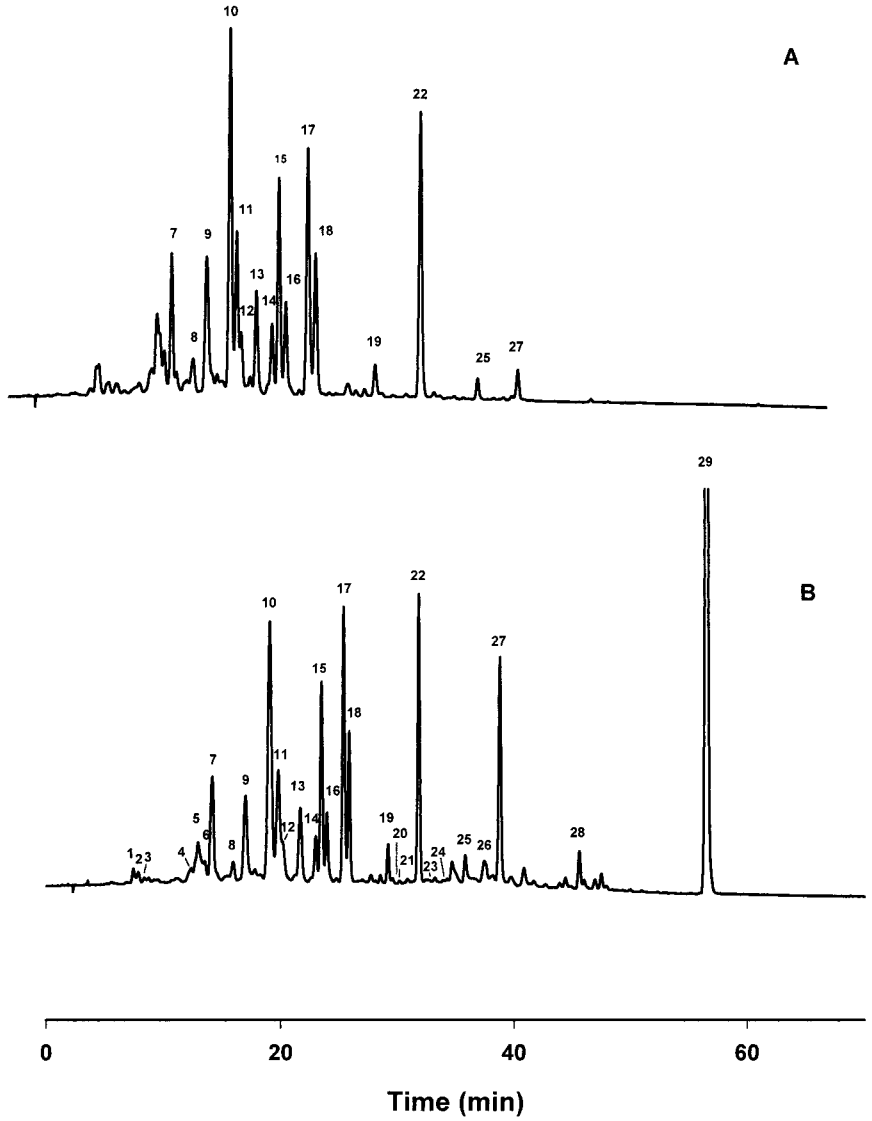

Figure 1. RP−HPLC chromatogram for carotenoid in navel orange juice on YMC C_{30} Carotenoid column (4.6 × 150 cm, 3 μm). Mobile phases are 75% MeCN/25% MeOH, MTBE, and water. The mobile phase contained 0.01% BHT and 0.05% TEA as modifiers. Detection at 450 nm. See Table 1 for identification. Standard navel (A) and red navel (B) orange juices.

from a previous work (*7*). Chromatography was carried out with a Waters liquid chromatography system equipped with a 600E pump with a model 996 photodiode array detector, and Millennium Chromatography Manager (ver. 3.2). A C_{30} carotenoid column (150 × 4.6 mm i.d., 3 μm) from YMC Inc. (Wilmington, NC) was used with MeCN/MeOH (75:25, v/v, eluent A), methyl *tert*-butyl ether (eluent B), and water (eluent C) as mobile phases. The eluent contained 0.01% BHT and 0.05% TEA (triethylamine) as modifiers in order to prevent the degradation of carotenoids on the column (*9*). The flow rate was 1 mL/min, column temperature set at 25 °C, and injection volume was 10 μL. Detection was at 450 nm. Following is the gradient program that was used : initial condition was 95% A/5% C; 0−10 min, 95% A/5% B; 10−19 min, 86% A/14% B; 19−29 min, 75% A/25% B; 29−54 min, 50% A/50% B; 54−66 min, 26% A/74% B; and back to the initial condition for reequilibration. Analysis was conducted under subdued light to avoid carotenoid degradation during analysis. PDA measurements of spectral properties for the individual peaks (from 230 to 600 nm) were determined up-slope, apex, and down-slope. The match of these spectra indicated the degree of peak purity. Quantitative estimation of β-carotene and lycopene contents in juice was conducted by HPLC using the external standard method without saponification.

The β-carotene (β,β-carotene), α-carotene (β,ϵ-carotene), and lycopene (ψ,ψ-carotene) standards were obtained from Sigma (St. Louis, MO). The β-cryptoxanthin (β,β-caroten-3-ol), lutein (β,ϵ-carotene-3, 3′-diol), and zeaxanthin (β,β-carotene-3, 3′-diol) were obtained from Extrasynthese (Genay, France). One mixture of standards obtained from Henkel Corp. (Kankakee, IL) consisted of lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-carotene. Mango was used as a source of violaxanthin as described by Mercadante et al. (*10*).

Color Analysis. Color (CIE L*, a*, b*) analysis was conducted using a Macbeth Color-Eye 3100 spectrophotometer (Kollmorgen Instruments Corp., Newburgh, NY) in the reflectance mode as previously described by Lee and Castle (*7*). Hue ($\tan^{-1}$ b*/a*) and chroma [$(a^{*2}+b^{*2})^{1/2}$] were calculated from CIE a* and b* values. The orange color score (CN) was also measured using a Hunter model D-45 citrus colorimeter (Hunter Associates Laboratories, Inc., Reston, VA).

Statistical Analysis. Statistical analyses (descriptive, correlation, and *t*-test) were conducted using SigmaStat software from SPSS, Inc. (Chicago, IL). Trends were considered significant when means of compared sets differed at $P < 0.05$.

RESULTS AND DISCUSSION

Juice Pigment Profile. The RP-HPLC chromatograms in Figure 1A and 1B show the carotenoid pattern in standard navel (1A) and red navel (1B) orange juices

Table 1. Chromatographic and Spectroscopic Characteristics of Carotenoid Pigments in Red Navel Orange Juice

peak no.	t_R (min)	%[a]	%[b]	λ_{max} (nm)	% III/II	%/III/Il	carotenoid[g]
1	7.47	0.6	0.2	418,441,470	81.6	85[c]	* neoxanthin a
2	7.91	0.4	0.2	419,441,470	63.3	85[c]	* neoxanthin b
3	8.40	0.2	<0.1	401,423,449	70.7	75[c]	* neochrome
4	12.39	0.5	0.4	324,414,438,466	81.3		
5	13.00	2.2	1.0	329,419[s],446,472	67.0		
6	13.56	0.5	0.6	419[s],440,467	65.5		
7	14.20	3.6	2.6	414,442,472	90.0	98[c]	* violaxanthin
8	15.99	0.6	0.3	332,438,467	20.4		
9	17.08	3.1	1.7	400,424,450	94.4		* luteoxanthin-like
10	19.11	9.7	9.0	328,414,438,466	95.1	98[c]	* cis-violaxanthin
11	19.84	5.2	2.2	422,447,475	52.4	60[c]	* antheraxanthin
12	20.16	1.3	0.7	397,419,445	92.4		* luteoxanthin a
13	21.73	1.9	1.5	397,419,445	92.9		* luteoxanthin b
14	23.06	1.4	0.8	404,423,455	40.9	45[c]	* mutatoxanthin
15	23.54	5.1	2.6	426[s],443,474	50.0	60[c], 67[e]	lutein
16	24.02	2.1	1.0	409[s],430,451	55.8		* mutatoxanthin-like
17	25.44	6.7	4.0	333,420[s],444,471	62.5	85[c], 59.7[f]	* isolutein
18	25.92	3.7	1.8	428[s],452,481	31.2	25[c], 36[e], 34.8[f]	zeaxanthin
19	29.27	0.9	0.3	425[s],447,477	59.1	60[c], 67[e]	* α-cryptoxanthin
20	30.48	<0.1	20.7	276,287,298	10.0	10[c]	* phytoene
21	30.73	<0.1	12.0	334,350,368	68.0	87[d]	* phytofluene a
22	31.88	6.9	3.4	429[s],452,479	28.8	25[c], 29[e]	*β*-cryptoxanthin
23	32.85	<0.1	0.3	333,350,369	98.0	87[d]	* phytofluene b
24	33.30	<0.1	1.0	379,400,424	85.9	95[c]	* *ξ*-carotene
25	35.87	0.7	0.3	419[s],449,476	55.6	55[c]	α-carotene
26	37.53	1.0	0.4	415,437,465	46.2		
27	38.85	6.6	3.2	429[s],454,479	26.0	25[c]	*β*-carotene
28	45.68	1.0	0.5	358,438,465,493	45.0		* lycopene cis-isomer
29	56.57	30.0	23.8	448,474,506	73.7	65[c]	lycopene

[a] Based on peak area at 450 nm. [b] Based on a Maxplot chromatogram. [c] From Britton (*14*). [d] Mercadante et al. (*15*) in petroleum ether or hexane. [e] Obtained from authentic standards under this HPLC condition. [f] Calculated values from the spectra presented by Rouseff et al. (*11*). [g] *, tentative identification.

obtained in mid-December 1999. More than 29 carotenoids were separated from red navel, and the chromatographic profile of carotenoids showed marked differences from those of standard navel orange juice. Reproducibility of analysis was satisfactory; mean value of the coefficients of variation for the retention times was less than 3% within the same day of analysis.

In the present study with reversed-phase HPLC, the nonpolar carotenoids were eluted according to decreasing polarity, such as polyols > diols > monols, and then hydrocarbons. Thus, less polar hydrocarbon carotenoids, such as α-carotene, *β*-carotene, and more unsaturated lycopene, were retained longer than xanthophylls (Figure 1b). The *ξ*-carotene (λ_{max} = 379, 400, 424 nm), which eluted between α-carotene and *β*-carotene in a previous work with MeOH/MTBE/water (*11*,*12*), eluted earlier than α-carotene for this condition. Differences in mobile phase strength in this work probably affected the selectivity.

Tentative identification of carotenoids for which authentic standards are not available was carried out by HPLC through the combined use of chromatographic retention and by coelution of standards as discussed in previous works (*13*), and visible absorption spectrum obtained with an on-line photodiode array detector. A numerical notation (% III/II), which describes the ratio of the peak height of the longest-wavelength absorption band (band III) to that of the middle absorption band (usually λmax, band II) as a percentage, was also used for identification. The ratio of peak heights has proven useful to compare the spectra fine structure as described by Britton (*14*) and Mercadante et al. (*15*). Table 1 reports the chromatographic retention of carotenoids and spectral characteristics obtained by on-line PDA from red navel. Table 1 also lists the spectral fine structural values, which are reported in the literature (*14*), as well as the values calculated from authentic standards analyzed from this study. Values (% III/II) obtained from this study were comparable to the values found in the literature (*14*).

Peak I has a spectra similar to that of a violaxanthin at slightly shorter wavelength and with slightly reduced fine structure, probably neoxanthin. Peak 7 is assigned as violaxanthin using a carotenoid extract from mature mango, which is known to have *trans*-violaxanthin and its *cis*-isomers as major carotenoids (*10*). The *β*-carotene and its hydroxy derivatives, such as zeaxanthin or *β*-cryptoxanthin, have the same chromophore and therefore showed almost identical spectra as presented in Table 1. The slight differences in % III/II values in Table 1 are probably due to the calculation of fine structure of the carotenoid spectrum.

The spectrum of peak 12 was almost identical to that of peak 13, which is assigned as an isomer of luteoxanthin. The similarity of the absorption spectrum of peak 16 to that of mutatoxanthin (peak 14) strongly suggests that peak 16, like mutatoxanthin, possesses eight conjugated double bonds in its chromophoric system. Peak 28 is assigned as a lycopene *cis*-isomer, based on the *cis*-peak (358 nm), and spectral data (λ_{max} = 438, 465, 493 nm) which agrees well with the previous work on lycopene *cis*-isomers by Schierle et al. (*16*).

The percentages of each of the carotenoids in pigment extracts from red navel orange juice are also presented in Table 1. The primary pigments in red navel were lycopene (30%), followed by *cis*-violaxanthin (9.7%), *β*-cryptoxanthin (6.9%), isolutein (6.7%), and *β*-carotene (6.6%). It is most interesting to note that in the red navel there was a large amount of red-colored lycopene as the major carotenoid which is not present in standard navel orange or other major sweet orange cultivars grown in Florida.

Table 2. Color and Pigment Contents in Navel Orange Juices

orange juices[a]	CIE L*	CIE a*	CIE b*	hue	chroma	CN[b]	β-carotene (ppm)	lycopene (ppm)	total carotenoids[c] (ppm)
red navel									
min	40.61	0.22	16.88	79.97	16.88	37.20	0.7	2.7	6.0
max	42.99	3.54	20.52	89.38	20.52	40.70	1.3	5.4	9.9
mean	41.76	1.51	19.13	85.58	19.23	39.01	1.0	3.9	7.7
SD	0.70	1.33	1.23	3.82	1.26	1.19	0.3	1.1	1.2
navel									
min	43.81	−4.63	22.44	98.20	22.83	36.20	0.1	0.0	3.8
max	47.22	−4.09	28.39	100.59	28.68	37.40	0.1	0.0	5.7
mean	46.06	−4.29	26.06	99.40	26.42	36.86	0.1	0.0	4.7
SD	1.33	0.22	2.19	0.94	2.16	0.43	0.0	0.0	0.8

[a] Based on one-half bushel of fruit. [b] CN is orange juice color number based on Hunter Citrus Colorimeter. [c] Total carotenoids are estimated using the extinction coefficient of β-carotene, 2505 (*8*).

Table 2 presents the quantitative data on two major hydrocarbon carotenoids (β-carotene and lycopene) from both red and standard navel orange juices. Because there were significant losses (>25%) in lycopene during saponification as previously reported by Muller (*17*), the approximate concentrations of β-carotene and lycopene are measured by HPLC without saponification. However, the losses of β-carotene were not significant as previously observed by Khachik et al. (*18*). The mean value for lycopene in red navel is 3.9 ppm, and is higher than values found from Ruby Red grapefruit juice, which is the most widely grown colored grapefruit in Florida (*19*). Lycopene is the major colored pigment in red cultivars of grapefruit and is responsible for the pleasant red hue in pink and red grapefruit juice (*19*). Furthermore, lycopene is known to be more potent than other carotenoids in antioxidant ability and is a very efficient quencher of singlet oxygen, neutralizing harmful free radicals which damage body cells (*20*). Besides the presence of lycopene, it is also interesting to note the relatively high content of β-carotene in red navel compared to that in the standard navel: about 10-fold in the mean value. Red navel orange is a better source for β-carotene, and the dual role of β-carotene to provide both color and provitamin A activity could be an important functionality in red navel juice.

Violaxanthin was the next predominant carotenoid. Violaxanthin is a yellow pigment with the main cis-form in citrus (*21*) having visible absorption maxima of 414, 438, 466, and 328 nm. Violaxanthin showed high values for fine structure value (% III/II) > 90% and agreed well with the literature value (Table 1).

Violaxanthin is known to form from epoxidation of zeaxanthin via antheraxanthin (*21*), and is a precursor of neoxanthin and luteoxanthin. Violaxanthin is a common carotenoid in sweet oranges, and three different violaxanthin *cis*-isomers were previously reported from the sweet orange (*22*). In fact, violaxanthin (trans plus cis) was the dominant carotenoid (>45% of the total) in the pulp of navel orange (*23*), and in the juice (>33% of the total) of Hamlin orange (*24*), which is the principal early season orange grown in Florida. In this study, *cis*-violaxanthin was the major carotenoid (>14%), followed by β-cryptoxanthin and isolutein in standard navel orange juice. The general pattern of the xanthophylls in two navel cultivars was similar as presented in Figure 1a, 1b, but the relatively large percentage of *cis*-violaxanthin in standard navel orange juice is notable (Figure 1a).

The β-cryptoxanthin is the third most predominant pigment in red navel, comparable to the percentages of lutein isomer and β-carotene. The β-cryptoxanthin is an orange carotenoid having visible absorption maxima of 429, 452, and 479 nm, which is almost identical to the spectrum of β-carotene (Table 1). Previously, Stewart et al. (*5*) described that β-cryptoxanthin is responsible for the much deeper orange juice color in red Valencia orange than that from known types of this cultivar. Large amounts of this pigment in juice could impart a desirable bright deep orange color. In sweet orange juice, the relatively large amount of β-cryptoxanthin was considered as the main source of provitamin A activity in juice (*24*). Provitamin A activity comes from a limited number of carotenoids which possess one unsubstituted β-end group (*24*). Because of the relatively large percentages of β-carotene and β-cryptoxanthin in red navel juice, these two carotenoids are considered as the main source of the provitamin A activity occurring in red navel juice.

Lutein and zeaxanthin are usually very difficult to resolve (*25*), as they have the same chemical formula (*10*) ($C_{40}H_{56}0_2$), but they are completely resolved with this condition. Lutein and isolutein, an epoxy derivative of lutein (*21*), are the two major pigments of β,ϵ-series carotenoids found in navel orange juice. Lutein is a typical basic chloroplast pigment (*21*) and is predominant in dark green vegetables (*26*). Recent reports on the strong protective effect of lutein against age-related macular degeneration (ARMD), and reducing the risks of certain cancers, has attracted attention to its content in foods (*26*, *27*). Lutein content was slightly more than 5% in total carotenoid content in the red navel orange juice (Table 1). Lutein was known to gradually disappear and was replaced by typical chromoplast pigments such as β-cryptoxanthin during ripening (*21*).

The red navel also contains the colorless precursors phytofluene (λ_{max} = 350 nm) and phytoene (λ_{max} = 287 nm). HPLC under multiwavelength detection (extracted at 310 nm) by PDA was able to detect a large proportion of noncolored carotenoids, such as phytoene and phytofluene, as presented in Figure 2. Phytoene is an intermediate in the biosynthesis of carotenoids, and is known to be present predominantly as the cis-form in nature (*28*). Phytoene (λ_{max} = 276, 287, 298 nm) gives a spectrum with no distinctive peak height at band III, and produces a less defined fine structure (Figure 2), thus, the spectral fine structure (% III/II) value was estimated as 10 (Table 1).

In Table 1, the percentage of each carotenoid based on a Maxplot chromatogram, which plots each carotenoid at its maximum absorbance wavelength, is also included. Under Maxplot, phytoene (20.7%) and phytofluene (12%) represent the relatively large percentage of carotenoids, including noncolored carotenoids, in red

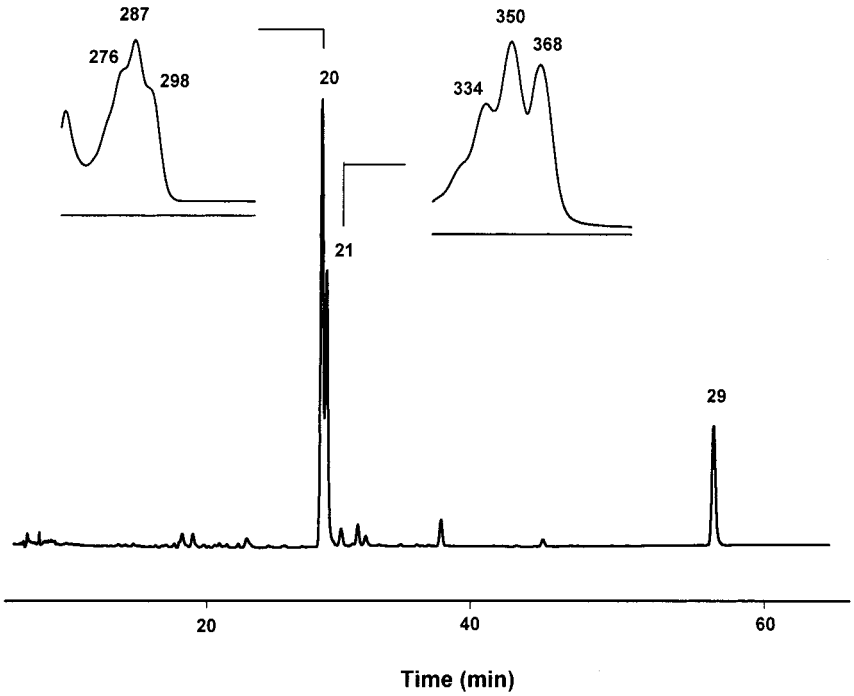

Figure 2. RP−HPLC chromatogram and UV−Visible spectra for carotenoid in navel orange juice at 310 nm.

navel juice. These two colorless pigments make up about $^1/_3$ of the total carotenoids in the Maxplot. In the standard navel orange, substantial amounts of phytoene and phytofluene were also observed as previously reported by Curl and Bailey (*23*). The percentages of the remaining minor carotenoids in red navel are presented in Table 1.

The total juice carotenoids were expressed as the sum of carotenoid concentrations measured at 450 nm (as β-carotene) and presented in Table 2. The total carotenoid pigment content of the red navel juice was in the range of 6.0−9.9 ppm in December during the 1998−99 season. The mean value of total carotenoid pigment contents of red navel juices was found to be more than about 1.6-fold higher than the mean value found in the juices of standard navel orange. Thus, the high content of total carotenoids could contribute to the deep color in red navel orange juice.

Juice Color. All CIE color parameters summarized in Table 2 supported the deeper color of red navel juice compared to the standard navel juice and color values are significantly different ($P < 0.05$) from the color parameters in the standard navel orange juice. The presence of the red pigment, lycopene, in red navel could be responsible ($r = 0.902$, $P < 0.05$) for the higher juice color score of CIE a* values (redness) compared to that of the standard navel juice. In addition, the relatively large percentage of β-carotene in the red navel probably contributed to the more orange-red hue compared to that of the juice from standard navel. Both lycopene ($r = -0.913$) and β-carotene ($r = -0.825$) showed highly negative correlations to the juice hue. Carotenoid pigment does not contribute equally to the color of orange juice (*24*), and the contribution of each is not known. However, the relatively high content of the yellow pigment, *cis*-violaxanthin, in the standard navel orange juice, probably contributed to the higher CIE b* value compared to that of the red navel orange juice.

Orange juice color number (CN) was also included in Table 2. The color number was developed for orange juice products, and is often used for color grading of processed orange juice products in the Florida citrus industry. Orange juice must have a color number of 36−40 points to be classified as Grade A (*24*), and most red navel juices had higher than 36, and provided an average of 39, which is sufficient color for grade A. The CN value showed the highest correlation coefficient with the total juice carotenoid content ($r = 0.946$, $P < 0.05$).

In conclusion, the red color of the red navel is characterized by the presence of lycopene, which is absent in the standard navel. In addition, a relatively high percentage of β-carotene in red navel was found to be one of the major differences in pigment profile compared to that of the standard navel orange.

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