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Journal of Chromatography A, 913 (2001) 371–377

JOURNAL OF
CHROMATOGRAPHY A

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High-performance liquid chromatography for the characterization of carotenoids in the new sweet orange (Earlygold) grown in Florida, USA

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Abstract

High-performance liquid chromatography with photodiode array detection was developed for the separation and identification of carotenoids from a new sweet orange, Earlygold. Carotenoid pigments were extracted using hexane–acetone–ethanol and saponified using 10% methanolic potassium hydroxide. More than 25 carotenoid pigments were separated within 40 min using a ternary gradient (acetonitrile–methanol, methyl *tert.*-butyl ether and water) elution on a C_{30} reversed-phase column. The carotenoid pattern of Earlygold was generally similar to the early season Hamlin but with some quantitative differences, especially with violaxanthin. Major carotenoids including violaxanthin, lutein, β-cryptoxanthin, antheraxanthin, luteoxanthin, zeaxanthin, β-carotene, and α-carotene were identified based on on-line spectral data obtained by a photodiode array detector, and comparison to the spectra of the standards and reported values. A numerical notation, the ratio of the peak heights between absorption bands, was also calculated to compare to the literature values. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Fruit juices; Food analysis; Carotenoids

1. Introduction

Color is one of the most important attributes of orange juice products and is largely due to the presence of diverse carotenoid pigments. Considerable attention has been directed towards analysis of carotenoid pigments by HPLC including normal-phase and reversed-phase [1–6]. The carotenoid distribution in citrus is extremely complex and subject to considerable variation. A list of more than 115 different carotenoids has been compiled from different citrus cultivars including Valencia, Washington Navel and Shamouti oranges [7].

Recently documented health benefits of carotenoids have stimulated efforts to develop new a stationary phase for the separation of carotenoids comprising a very diverse class of molecules including non-polar hydrocarbons and the polar xanthophylls. Recently, Sander et al. [8] reported a novel C_{30} bonded phase with significantly enhanced selectivity for carotenoids compared to the monomeric or polymeric C_{18} columns. A good separation of most of the carotenoid standards using a non-aqueous mobile phase gradient (methanol and methyl *tert.*-butyl ether) was achieved [9]. Later, a carotenoid column with the unique C_{30} bonded phase was applied for carotenoids in sweet oranges by Rouseff

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PII: S0021-9673(00)01029-3

et al. [10], and more recently by Mouly et al. [11] using a gradient of water, methanol and methyl *tert*.-butyl ether.

In the Florida citrus industry, Hamlin (*Citrus sinensis*) is the principal early-season sweet orange. This cultivar has the advantage of a superior yield capacity, but its juice color is relatively poor. Therefore, new sweet orange selections with better juice color and flavor have been under evaluation for about 10 years in a cooperative field trial. A report of this trial described a selection named Earlygold as one of the most promising [12]. Earlygold is a Seleta-type orange obtained from a variety collection in Brazil. Earlygold developed a higher juice color score and palatable flavor compared to standard Hamlin orange throughout the growing season in the field trial [12]. The objective of our study was to characterize the carotenoid pigments of the new sweet orange selection, Earlygold, using a HPLC method.

2. Materials and methods

2.1. Orange fruits

Earlygold fruit were sampled from a field trial in St. Cloud, FL, USA during the mid-1997–1998 growing season. Fruits were extracted with a household-type electric hand reamer, and the juice was filtered through cheesecloth, pasteurized (90°C for 30 s) and cooled before analysis.

2.2. Carotenoid extraction

A 25-ml aliquot of juice was homogenized (30 s at speed 4) in an Omni mixer homogenizer (Warrenton, VA, USA) with 50 ml of extracting solvent (hexane–acetone–ethanol, 50:25:25, v/v), and centrifuged for 5 min at 6500 rpm at 5°C (IEC, Needham, MA, USA). 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.

2.3. Saponification

Saponification was carried out according to Noga and Lenz [3] with modification. The hexane extract was concentrated to dryness utilizing a rotary evaporator, redissolved with 2 ml of methyl *tert*.-butyl ether (MTBE), and placed in a 15-ml culture tube to which was added 2 ml of 10% methanolic KOH. The sample was wrapped with aluminum foil to protect it from light. Prior to capping, the tube was gently blanketed with nitrogen, closed, and placed in the dark overnight at room temperature. The sample was removed from the dark and transferred to a separatory funnel to which 5 ml of water was added and 2 ml of 0.1% BHT in MTBE, and the aqueous layer removed. Three additional water rinses were carried out, draining the aqueous layer after each rinse. The MTBE layer was then filtered through an Isolute sodium sulfate drying cartridge (Intl. Sorbent Technol., UK), transferred into a 15 ml centrifuge tube, concentrated by evaporation with nitrogen, and the volume adjusted with 0.1% BHT in MTBE to 1.0 ml. The sample was filtered through a Millipore FHLC 0.5 μm filter (Bedford, MA, USA) before injection to the HPLC. The experiment was conducted under dimmed light and all samples were wrapped in foil.

2.4. Total carotenoids

Total carotenoid content was determined on an aliquot of the hexane extract, as described under procedure for carotenoid extraction, by measuring the absorbance at 450 nm in a Genesis-5 Spectronic spectrophotometer (Rochester, NY, USA). Total carotenoid content was calculated according to De Ritter and Purcell [13] using an extinction coefficient of β-carotene, $E^{1\%}=2505$.

2.5. HPLC analysis of carotenoids

Carotenoid pigments were analyzed by RP-HPLC using the ternary gradient elution procedure with modification from a previous work [10]. Chromatography was carried out with a Waters liquid chromatography system equipped with a model 600E solvent delivery system, a model 996 photo diode array detection (DAD) system, a model 717 plus autosampler, and a Millennium Chromatography Manager. A C_{30} Carotenoid column (150×4.6 mm I.D., 3

μm) from YMC (Wilmington, NC, USA) was used with MeCN–MeOH (75:25, v/v, eluent A), 100% MTBE (eluent B) and water (eluent C) as mobile phases. The eluent contained 0.01% BHT and 0.05% TEA (triethylamine) as modifiers [5] in order to prevent the degradation of carotenoids on the column. Flow rate was 1 ml/min, with the column temperature set at 25°C, and an injection volume of 10 μl. The linear gradient program was performed as follows: Initial condition was A–C (95:5); 0–10 min, A–B (95:5); 10–19 min, A–B (86:14); 19–29 min, A–B (75:25); 29–54 min, A–B (50:50); 54–66 min, A–B (26:74) and back to the initial condition for re-equilibration. Analysis was conducted under subdued light to avoid carotenoid degradation during analysis. DAD measurements of spectral properties for the individual peaks (from 300 to 600 nm) were determined up slope, apex, and down slope. The match of these spectra indicated the degree of peak purity.

The β-carotene and α-carotene standards were obtained from Sigma (St. Louis, MO, USA). The β-cryptoxanthin, lutein, and zeaxanthin were obtained from Extrasynthese (Genay, France). One mixture of standards obtained from Henkel (Kankakee, IL, USA) 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. [14].

3. Results and discussion

3.1. Total carotenoids

For the isolation of carotenoids from orange juice for HPLC, carotenoids are often extracted directly with methanol and acetone [4], ethyl acetate [5], diethyl ether [3,15], or precipitated through adsorption by means of zinc cyanoferrate(II), then extracted from the precipitate with acetone and dissolved in light petroleum [10,11]. In this study, carotenoid pigment was directly extracted from juice using the mixture of ethanol–acetone–hexane [16] with the aid of homogenization to facilitate the pigment extraction. Previously, a mixture of ethanol and hexane was suggested as the most suitable for extraction of carotenoids from the various matrixes with low biological hazard [17]. The total carotenoids were expressed as the sum of carotenoid concentrations measured at 450 nm (as β-carotene). Reproducibility (RSD) of the extraction was less than 3%, and extraction at medium speed (speed 4) for 30 s in an Omni mixer homogenizer appeared to be satisfactory.

The total carotenoid pigment content of Earlygold juice was in the range of 8.3–8.8 μg/ml in January during the 1997–1998 season, and was more than two times higher than the values found in Hamlin, which is the principal early-season orange in Florida.

3.2. Pigments in juice

More than 25 carotenoid pigments were identified in Earlygold juice (Fig. 1). The addition of modifiers such as triethylamine to the mobile phase was shown to improve the recovery of carotenoids from the column [18]. Reproducibility of peak areas was satisfactory, less than 7% RSD except for peaks 5 and 7. The relatively high RSDs for peaks 5 and 7 are mainly due to poor resolution of these peaks and the low concentrations of these peaks in the sample. Retention times of the individual carotenoids detected in Earlygold varied slightly. The mean value

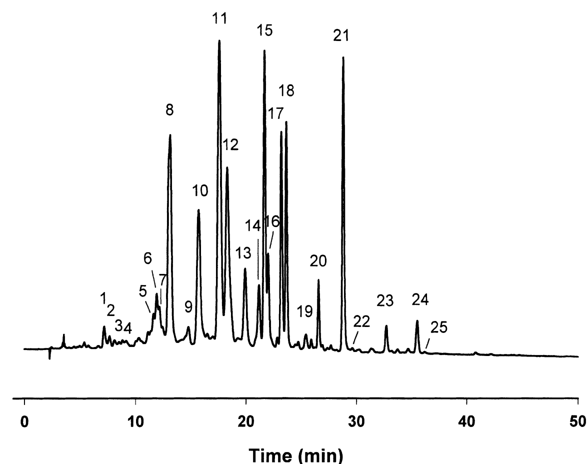

Fig. 1. RP-HPLC chromatogram for carotenoid in Earlygold orange juice (mid-January, 1997–1998 season). YMC C_{30} Carotenoid column (4.6×150 mm, 3 μm). Mobile phases are MeCN–MeOH (75:25), MTBE and water. The mobile phase contained 0.01% BHT and 0.05% TEA as modifiers. Detection at 450 nm. See Table 1 for identification.

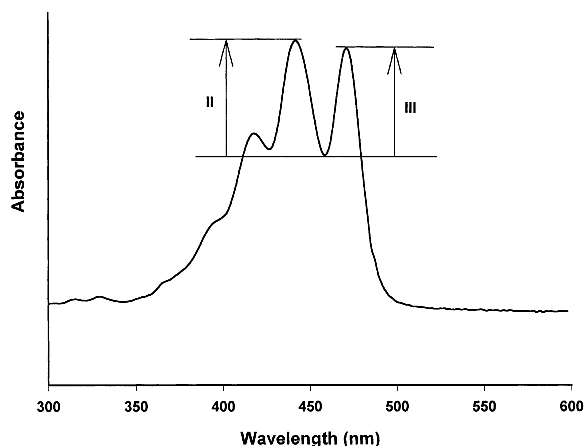

Fig. 2. Spectral fine structure (as %III/II) of violaxanthin.

of the RSDs for the retention times was less than 3% within the same day of analysis.

Due to the complex composition of carotenoid extracts and minor shape differences among carotenoid isomers, identification of carotenoids is difficult. Tentative identification of carotenoids, for which authentic standards are not available, was carried out by HPLC through the combined used of chromatographic retention as discussed in previous works [2,3,10,15,19,20], 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 λ_{max}, band II) as a percentage, was also used for identification (Fig. 2). The ratio of peak heights has proven useful to compare the spectra fine structure as described by Britton [19]. Table 1 reports the chromatographic retention of carotenoids and spectral characteristics obtained by on-line DAD from Earlygold. Table 1 also lists the spectral fine structural values, which are reported in the literature [19] as well as the values calculated from authentic standards analyzed from this study. Values (%III/II) obtained from this study

Table 1
Chromatographic and spectroscopic characteristics of Earlygold orange juice carotenoids[a]

Peak No.	t_R (min)	%*	λ_{max} (nm)	%III/II	%III/II**	Tentative identification
1	7.1	0.8	418,441,470	81.6	80–85[1]	Neoxanthin *a*
2	7.6	0.4	419,441,470	63.3		Neoxanthin *b*
3	8.1	0.5	401,423,449	70.7	75[1]	Neochrome
4	8.8	0.5	401,424,450	72.9		Trollichrome
5	11.6	0.7	324,414,438,466	81.3		
6	11.9	1.4	329,419[sh],446,472	67.0		
7	12.1	0.6	419[sh],440,467	65.5		
8	13.1	10.1	414,442,472	90.0	95–98[1]	Violaxanthin
9	14.8	5.0	335,439,465	20.4		
10	15.7	7.3	400,424,450	97.9		Luteoxanthin *a*
11	17.6	16.1	328,414,438,466	95.1	97[s]	*cis*-Violaxanthin
12	18.3	10.9	422[sh],447,475	52.4	55–60[1]	Antheraxanthin
13	19.9	3.4	397,419,445	100.0		Luteoxanthin *b*
14	21.1	2.6	404,423,455	35.0	45–50[1]	Mutatoxanthin
15	21.6	10.4	426[sh],447,474	50.0	60[1], 67[s]	Lutein
16	22.0	3.3	409[sh],430,451	55.8		
17	23.2	6.8	333,420[sh],444,471	55.9		Lutein isomer
18	23.7	6.5	428[sh],452,481	28.0	25–26[1], 36[s]	Zeaxanthin
19	25.4	0.6	342,427[sh],445,469	17.5		
20	26.6	1.8	425[sh],447,477	59.1	60[1]	α-Cryptoxanthin
21	28.8	9.1	429[sh],452,479	28.8	27[1], 29[s]	β-Cryptoxanthin
22	30.3	0.1	379,400,424	85.9	95[1]	ξ-Carotene
23	32.7	0.9	424[sh],443,476	55.6	55[1], 65[s]	α-Carotene
24	35.5	1.2	429[sh],454,479	26.1	25[1], 31[s]	β-Carotene
25	36.1	0.1	342,424[sh],446,472	33.3		9-*cis*-β-Carotene

[a] Sh=indicates the shoulder instead of peak. *Percentage of peak area. **1=values obtained from Ref. [19], and s=from standards.

agree well with the values found in the literature [19] except for the antheraxanthin and mutatoxanthin. Values obtained for those two carotenoids are slightly lower than published values. This discrepancy is probably due to the purity of peak as well as calculation of fine structure of carotenoid spectrum. Slight interference to the mutatoxanthin peak (peak 14) by the early elution of a trace compound λ_{max} 404 nm (383, 404, 428 nm) might affect the value. The antheraxanthin (peak 12) peak tailed slightly which is probably due to the interference by a late eluting trace carotenoid (λ_{max}, 419 nm).

Carotenoids appear to be more sensitive to changes in HPLC conditions than many other classes of compounds. Initially, analysis was conducted using a ternary gradient with MeOH–MTBE–water which was the mobile phase for separation of carotenoids as presented in previous works on C_{30} column [9–11]. However, the peak purity test indicated that peak 15 [15,16] is not a spectrally homogeneous peak, indicating the presence of a co-elution. Also, the two major peaks [11,12] were poorly resolved (R_s<0.6). Separation of such poorly resolved peaks and co-eluting peaks needed improvement. Thus, later, this mobile phase composition was changed from 100% MeOH to MeCN–MeOH (75:25) in an attempt to improve the resolution and band spacing for these carotenoid peaks. In Fig. 1 with MeCN–MeOH (75:25), separation is improved, especially for poorly resolved peaks 11 and 12, which are separated as two peaks. This change in mobile phase also showed simultaneous improvement in the resolution of peaks 15 and 16 to help to characterize these peaks for identification in this sample. Even though baseline resolution could not be obtained for peak 15 from a co-eluting peak, changes of mobile phase indicated a co-elution of two spectrally distinct compounds as peak 15 and peak 16 (Fig. 1).

Due to slight differences in carotenoid structure, optimization of the mobile-phase composition was not achieved for complete baseline resolution of all interested carotenoid peaks, but changes of mobile phase strength from 100% MeOH to MeCN–MeOH (75:25) with the addition of TEA in this study resulted in dramatic improvement in resolution. A change from methanol to acetonitrile in reversed-phase HPLC often results in large changes in mobile phase selectivity (α) by substituting the strong solvent for methanol. Lutein (β,ε-carotene-3,3′-diol) and zeaxanthin (β,β-carotene-3,3′-diol) are usually very difficult to resolve [3], having the same chemical formula, but are completely resolved with this condition. Also, a lutein isomer (peak 17), probably the *cis*-isomer of isolutein, was clearly resolved from the zeaxanthin peak under this system. In a previous RP-HPLC on a C_{30} column with mobile phase of MeOH–MTBE–water gradient, elution could not resolve isolutein from zeaxanthin [11]. The addition of a small amount (0.05%, v/v) of TEA for this mobile phase appeared to improve the peak shape. Furthermore, use of a column with a smaller particle size of 3 μm in this study (Fig. 1) instead of a 5 μm column [10,11] probably enhanced the resolution slightly for this study as described by Emenheiser et al. [21].

In previous separations of various xanthophylls on a C_{18} reversed-phase column, early elution of neoxanthin among xanthophylls, and also a longer retention of antheraxanthin than violaxanthin were reported [2,3,20,22]. However, a different selectivity of neoxanthin and antheraxanthin from RP-HPLC on the C_{30} phase was reported by Rouseff et al. [10]. Recently, Mouly et al. [11], who used the same conditions as the HPLC method by Rouseff et al. [10], also reported the same early elution pattern of antheraxanthin (diol monoepoxide) than other xanthophylls such as neoxanthin (polyol) and violanthin (diol diepoxide). Furthermore, isolutein (lutein-5,6-epoxide), which has two hydroxy groups and one epoxy group, was reported to retain longer than lutein (two epoxy groups) in the previous works [10,11]. It was interesting to observe the different selectivity of carotenoids on C_{30} phases compared to the C_{18} phase. As usual in RP-HPLC, the elution order is according to their polarity, however, the relative retention behavior of carotenoid is reported to be difficult to explain structurally [10]. In a present study with reversed-phase HPLC, the non-polar carotenoids are expected to elute according to decreasing polarity such as polyols>diols>monols> and then hydrocarbons. Thus, antheraxanthin, which has ten conjugated double bonds (nine in the chain and one in the ring), was considered to be retained longer than violaxanthin, which has nine conjugated double bonds in the chain, and also to be retained

longer than neoxanthin in this system. Peak 4 is assigned as *trans*-violaxanthin using a carotenoid extract from matured mango, which is known to have *trans*-violaxanthin and its *cis*-isomers as major carotenoids [14].

Peak I was assigned as neoxanthin, which is known to have a spectrum similar to that of a violaxanthin at slightly shorter wavelengths and with slightly, reduced fine structure [19]. Less polar hydrocarbon carotenoids such as α-carotene and β-carotene and more saturated ξ-carotene were retained longer than xanthophylls (Fig. 1). ξ-Carotene (379, 400, 424 nm), which is eluted between α-carotene and β-carotene in previous work with MeOH–MTBE–water [10,11], eluted earlier than α-carotene for this condition. Changes in mobile phase strength probably affected the selectivity. β-Carotene and its hydroxy derivatives such as zeaxanthin or β-cryptoxanthin have the same chromophore and therefore showed identical spectra as presented in Table 1. The spectrum of peak 12 was almost identical to peak 15. Peak 25 is assigned as a β-carotene isomer, 9-*cis*-β-carotene, based on elution order, and spectral data (342, 424, 446, 472 nm).

The pigment with the highest concentration in Earlygold was *cis*-violaxanthin (16.1%) (Table 1). Violaxanthin is a yellow pigment with the main *cis*-form in citrus [23] having a visible absorption maximum of 466, 438, 414 and 328 nm. Violaxanthins 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 [23], and is a precursor of neoxanthin and luteoxanthin. Three different violaxanthin cis-isomers were previously reported from the Valencia sweet orange which is know to have the most complex pigment pattern [1] among sweet orange varieties. It was also reported that diol diepoxides (mainly violaxanthin) dominate the carotenoid pattern in sweet oranges; more than one third in pulp and more than half in orange peel carotenoids [23].

The percentages of antheraxanthin (10.9%), lutein (10.4%), and β-cryptoxanthin (9.1%) were similar in mature fruits of Earlygold. Lutein is a typical basic chloroplast pigment, which predominates in unripe fruits [23], and is probably converted to epoxy carotenoids as ripening proceeds [24]. Increased biosynthesis of β-cryptoxanthin is known to be one of the principal events occurring during ripening of sweet orange. β-Cryptoxanthin is an orange carotenoid having visible absorption maxima of 479, 452 and 429 nm, which is almost identical to the spectrum of β-carotene (Table 1). Large amounts of this pigment in juice could impart a desirable bright deep orange color. Accumulation of β-cryptoxanthin, which is the main source of provitamin A in sweet orange juice, was comparable to the other sweet orange cultivars [6]. The percentages of the remaining carotenoids in Earlygold were less than 7%, and their relative contribution to juice pigmentation probably varies with fruit maturity.

The general carotenoid composition of the Earlygold juice and the relatively small percentages of hydrocarbon carotenes are characteristic of sweet orange. The percentage of hydrocarbons in Earlygold orange juice was smaller (<5%) compared to xanthophylls, and is probably due to the synthesis of orange and yellow xanthophylls in orange fruit via hydroxylation and epoxidation of hydrocarbon [7]. A non-colored hydrocarbon carotenoid such as phytofluene (λ_{max}=349 nm) was also detected but quantitation was not attempted for this study.

4. Conclusions

Major carotenoids were separated within 40 min with ternary gradient elution. Considerable improvement in resolution was obtained by eluting with a mixture of acetonitrile–methanol (75:25) with an addition of 0.05% TEA instead of 100% MeOH. Calculation of fine structure from on-line DAD spectra was effectively applied to characterize the carotenoids. HPLC indicated that the pigment pattern in the newly released early-season sweet orange, Earlygold, is complicated, but was comparable to other sweet oranges. The major carotenoids typically found in sweet orange are antheraxanthin, violaxanthin, cryptoxanthin, lutein, and carotenes. The diols and their epoxides were predominant, and a noteworthy feature is the relatively high percentage of violaxanthin in Earlygold.

Acknowledgements

The authors are grateful to Dr. Harold Pollack

(Tropicana Products, Inc.) for the grant, and to Mr. Orie M. Lee (St. Cloud, FL, USA) for fruits used in this study. Support was also provided from Florida Production Research Advisory Council Project 928-27. Florida Agricultural Experiment Station Journal Series No. R-07629.

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