Application of Diode Array Detection with a C-30 Reversed Phase Column for the Separation and Identification of Saponified Orange Juice Carotenoids

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Thirty-nine carotenoid pigments in saponified orange (*Citrus sinensis*) juice were separated using a water, methanol, methyl-*tert*-butyl ether gradient on a non-endcapped C-30 reversed phase column. Pigments were extracted using the International Fruit Juice Union method for orange juice carotenoids, which employs precipitation with Carrez reagent and resolubilizing with acetone. Chromatographic resolution, R_s, between lutein and zeaxanthin was 2.9. Pigments were identified on the basis of diode array spectral characteristics, retention times, and relative elution order compared to authentic standards and literature values. An examination of the diode array data from the chromatographically resolved peaks indicated that the most useful information could be obtained from monitoring the chromatographic effluent at 350, 430, and 486 nm. More carotenoid peaks were detected at 430 nm, but greater selectivity for several carotenoids was obtained at 350 and 486 nm. At 430 nm the six largest carotenoid peaks observed in orange juice consist of auroxanthin A, mutatoxanthin A, mutatoxanthin B, lutein, zeaxanthin, and isolutein. Whereas open column and thin-layer chromatography required days, the separation and identification of saponified orange juice carotenoids can now be accomplished within 40 min.

Keywords: *Lutein; isolutein; zeaxanthin; β-cryptoxanthin; citrus*

INTRODUCTION

Citrus is a complex source of carotenoids with the largest number of carotenoids found in any fruit (Gross, 1987). Approximately 115 different carotenoids have been reported in citrus including a large number of isomers (Stewart, 1973). Citrus carotenoids were extensively studied in the 1960s and early 1970s by numerous investigators who used open column chromatography to separate and identify these pigments (Curl and Bailey, 1961; Yokoyama and White, 1966; Bernath and Swisher, 1969; Gross et al., 1971). Whereas large amounts of material could be recovered for identification purposes, separation times required several days and artifact formation was a problem since carotenoids are sensitive to heat, light, and oxygen. Stewart and Wheaton (1971) demonstrated that high-pressure liquid chromatography could be used to separate saponified citrus carotenoids in only a few hours. They employed hand-packed MgO columns to separate carotenes. Hand -packed $ZnCO_3$ columns were used to separate xanthophylls. They subsequently used this technique to show that two previously reported citrus carotenoids were artifacts that were unintentionally produced during the saponification step (Stewart and Wheaton, 1973). High-pressure liquid chromatography (HPLC) has since become the method of choice to study carotenoids regardless of their source.

Numerous reversed phase chromatographic systems have been developed for carotenoids in a variety of matrices. However the large numbers and diverse structures of carotenoids in citrus juices have necessitated extraordinary chromatographic systems be developed. If only a few carotenoids are of interest and the range in polarities is not great, isocratic systems can be employed. Neils and De Leenheer (1983) developed an isocratic, nonaqueous reversed phase system employing acetonitrile:dichloromethane:methanol (70:20:10) that could separate nine different carotenoids with polarities ranging from lutein to β-carotene within 32 min. Fisher and Rouseff (1986) optimized a ternary, nonaqueous solvent system to make it suitable for routine analysis of β-carotene and β-cryptoxanthin in orange juice and also developed a solid phase extraction procedure to separate and quantify these and other carotenoids. Quackenbush and Smallidge (1986) developed an isocratic reversed phase separation employing methanol:chloroform (9:1) as mobile phase that could separate and quantify β-carotene in the presence of zeaxanthin, β-cryptoxanthin and some cis isomers in saponified orange juice samples.

In one of the first nonaqueous, reversed phase, gradient systems applied to citrus carotenoids, Noga and Lenz (1983) used two 25 cm, 10 μm, C-18 columns to separate several saponified mandarin (*C. reticulata*) peel carotenoids within 40 min. Their ternary solvent system consisted of a methanol, water, and acetone gradient. Lutein and zeaxanthin were not resolved. Moreover, the quantitative values reported by these authors are in sharp disagreement with other literature values (Farin et al., 1983; Gross, 1981; Stewart, 1973). Philip and Chen (1988b) developed a binary methanol:ethyl acetate reversed phase gradient to separate and characterize numerous orange juice carotenoid esters. Perfetti et al. (1988) developed a reversed phase gradient separation using an acetonitrile:methanol:methylene chloride mobile phase for characterizing unsaponified orange juice carotenoids, with the ultimate aim of detecting adulteration of orange juice. Few peaks were

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identified as their purpose was solely to generate peak patterns for computer pattern recognition analyses. Khachik and co-workers (1989) developed a reversed phase gradient system for saponified and unsaponified carotenoids in foods using a solvent system consisting of methanol: acetonitrile:methylene chloride:hexane (4:34:1:1 to 4:18:9:9). Although many vegetables and fruit were analyzed, pink grapefruit was the only citrus examined.

The few complete reports of orange juice carotenoid composition are thus based on either open column technology (Curl and Bailey, 1956), a combination of column and thin-layer chromatography (Gross et al., 1972), or normal phase HPLC with self-constructed columns (Stewart, 1977). The carotenoid compositional values in these studies are in notable disagreement with each other. The few reports which have utilized modern, high-efficiency reversed phase columns have reported values for only a selected few carotenoids (Bushway, 1986; Fisher and Rouseff, 1986).

Photodiode array detection (PAD) has been used extensively for the study of carotenoids because full spectrum data can be obtained for each chromatographic peak. Tentative identification can usually be achieved because carotenoid spectra are moderately characteristic. Fisher and Rouseff (1986) used PAD to identify and determine β-cryptoxanthin and α- and β-carotene in orange and mandarin juices. Tan (1988) used PAD to identify specific cis-*trans* carotenoid isomers in tomato paste monitoring column effluent at 286, 350, 450, and 470 nm. Khachik and co-workers (1989) used PAD to identify and quantify the major carotenoids in extracts of apricots, peaches, cantaloupe, and pink grapefruit. Chen and co-workers (1993) used PAD for their study of carotenoid and chlorophyll stability during microwave cooking.

Therefore the purpose of this study was to develop a stable, high-efficiency separation that would allow the rapid, effective separation and identification of orange juice carotenoids. A second goal was to employ photodiode array detection to determine chromatographic peak purity and to identify the carotenoids from their spectral characteristics.

METHOD AND MATERIALS

Reagents and Standards. All reagents used were HPLC grade from Fisher Scientific (USA) or from Merck (Germany). One mixture of standards purchased from Sigma Chemical Co. (St. Louis, MO) consisted of canthaxanthin, ethyl-β-apo-8′-carotenoate, β-apo-8′-carotenal, lycopene, and β-carotene. Another set of standards consisting of β-cryptoxanthin, α-carotene, β-carotene, lutein, and zeaxanthin was obtained from Dr. Gerry Spinwall of Hoffmann-La Roche.

Sample Preparation. Twenty-five milliliters of single strength (ss) orange juice or 5.0 g of orange juice concentrate was precipitated with Carrez solution (zinc cyanoferrate(II)). Two milliliters of $ZnSO_4 \cdot H_2O$ solution was added to 25 mL of juice and mixed. Two milliliters of $K_4[Fe(CN)_6 \cdot H_2O$ was added with agitation. After mixing, the solution was allowed to stand 10 min before being centrifuged. The supernatant was decanted and discarded. A small amount of acetone was added to solubilize the carotenoids in the precipitate. The acetone layer was removed and washed by shaking with petroleum ether and water in a separatory funnel. The petroleum ether layer was separated and evaporated to dryness. The residue was dissolved in 6 mL of diethyl ether and 6 mL of 10% methanolic KOH. After standing overnight, protected from light at room temperature, it was extracted with 20 mL of diethyl ether. One hundred milliliters of a 10% NaCl solution was added to the separatory funnel. After shaking the ether layer was removed and washed with distilled water until free of alkali. The ether layer was dried with sodium sulfate and evaporated to dryness under vacuum. Carotenoids were dissolved with 0.5 mL of acetone or methyl *tert*-butyl ether (MTBE) and diluted in 1.0 mL of methanol and placed in sealed amber vials until analysis.

Equipment. The chromatographic equipment consisted primarily of a Hewlett-Packard 1090M microprocessor controlled integrated chromatographic system consisting of three low-pressure syringe metering devices for ternary gradient formation, a single high-pressure diaphram pump with pulse dampener, a variable-volume autosampler, and a photodiode array detector. The photodiode array detector was set to scan from 250 to 550 nm. Four separate data channels were set to record the absorbances at 290, 350, 430, and 486 nm with spectral bandwidths of 8 nm. Data were collected, stored, and integrated using the Hewlett-Packard HP-79994 chromatographic work station and related software.

Chromatographic Conditions. The 4.6 mm i.d. × 25 cm YMC column (Hampsted, NC) consists of polymeric C-30 material chemically bonded to 5m silica and is not endcapped. The initial solvent composition consisted of 90% MeOH, 5% water, and 5% MTBE. The solvent composition changed in a linear fashion to 95% MeOH and 5% MTBE at 12 min. During the next 8 min, (20 min running time) the solvent composition was changed to 86% MeOH and 14% MTBE. After reaching this concentration the solvent was gradually changed to 75% MeOH and 25% MTBE at 30 min. Final composition was reached at 50 min and consisted of 50% MeOH and 50% MTBE. All solvent changes were made in a linear fashion. Initial conditions were reestablished in 2 min and reequlibrated for 15 min before the next injection. Flow rate was 1 mL/min Injection volume was 20 mL.

RESULTS AND DISCUSSION

Although citrus carotenoids have been studied extensively using open column technology, little has been done to utilize the tremendous improvements in separation technology since the first separations with handmade columns (Stewart and Wheaton, 1971). The majority of citrus carotenoid studies have involved peel carotenoids for two reasons. First, these pigments are responsible for the desirable color of the fruit, and second, peel is the most concentrated source of these pigments in the fruit. Compared to peel, juice is a much less concentrated source of carotenoid pigments. Isolation and concentration techniques are more critical. HPLC has been employed to separate specific juice carotenoid vitamin A precursors for nutritional studies (Stewart, 1977; Philip and Chen, 1988a). The color of citrus juice has long been a measure of its perceived quality. The U.S. Department of Agriculture (USDA) assesses both color and flavor equally at 40% of total in their grading system. Citrus carotenoids are the major pigments in orange juices, but only a few studies have attempted to examine the individual carotenoids. This is due in large part because juice carotenoids are complex and there have been no simple, rapid measuring procedures available.

Choice of Stationary Phase (Column) Material. The vast majority of chromatographic methods developed for carotenoids in the last decade have utilized C-18 material (Craft, 1992). Carotenoids are lipid soluble and of very limited solubility in aqueous solutions due to their predominate hydrocarbon composition. Neils and De Leenheer (1983) reported bonded phase material shorter than C-18 (hexyl or octyl) had an unfavorable effect on carotenoid retention and separation. Sandler and Wise (1990) compared the behavior of carotenoids with the various forms of C-18 material and observed significant differences. Factors such as

particle size, shape, pore dimensions, degree of carbon loading, endcapping and monomeric versus polymeric bonding had striking effects on the resulting carotenoid separation. Carotenoid polarity can vary considerably due to the considerable structural variation observed with naturally occurring carotenoids. Thus, while one column type might separate carotenes effectively, it might not separate the polar xanthophylls such as lutein and zeaxanthin adequately. While other researchers used a multitude of solvent selectivity combinations, Sander and Wise (1987) proposed modifying the stationary phase by increasing the carbon chain length to between C-20 and C-30 to achieve the desired resolution. The column material they synthesized demonstrated excellent resolving properties for polyaromatic hydrocarbons and serum carotenoids, and the column material has recently become a commercial product.

The column used in this study was intentionally not endcapped. Currently, most C-18 columns are endcapped to cover residual silanol sites of the silica surface to avoid "mixed mode" separations. However, we have observed that many polar xanthophylls are poorly retained unless silanol sites are available. Craft (1992) compared the separation of Neils and De Leenheer (1983) which had been developed with polymeric nonendcapped C-18 material with the identical column from the same manufacturer which is now endcapped. Zeaxanthin and lutein were no longer resolved and relative retention had drastically decreased. However, even with non-endcapped material we found it necessary to add a small amount of water to the mobile phase at the beginning of the chromatogram to increase the retention of the most polar xanthophylls in orange juice.

Chromatographic Solvents. We evaluated a variety of published solvent systems and found them lacking adequate resolution and/or chromatographic stability for the carotenoids found in citrus. In a recent survey of reversed phase chromatographic methods for carotenoids (Craft, 1992), it was shown that the strong solvent used to elute carotenoids was either a chlorinated hydrocarbon (chloroform or methylene chloride), acetone, ethyl acetate or tetrahydrofuran (THF) or combinations thereof. We tested various combinations of water, acetonitrile and methanol as the weak solvent in combinations with methylene chloride, ethyl acetate, THF, acetone, and hexane as the strong solvent. Methanol is inexpensive and relatively nontoxic, but suffers from viscosity problems in the presence of water. Acetonitrile has lower viscosity and a lower UV cutoff but is more toxic and expensive. In addition it has been reported that lower carotenoid recoveries are observed with acetonitrile-based solvents than with methanol (Epler et al., 1992).

Combined with hexane, MTBE has been used to separate carotenoids on silica columns (Kamber and Pfander, 1984). It seems to be an excellent solubilizing agent for carotenoids and its UV cutoff is lower than both ethyl acetate and acetone. No miscibility problems have been observed with methanol or acetonitrile alone or containing low amounts (5%) of water. Our best previous separations had been achieved with combinations of methanol and acetonitrile with either THF or acetone as the strong solvent. The use of MTBE appears to offer selectivity advantages as the strong solvent over acetone or THF. This is not too surprising since it is in a different solvent group (Snyder, 1979). Initial gradients consisted of binary mixtures of methanol and MTBE from 5 to 95% MTBE in 30 min. The

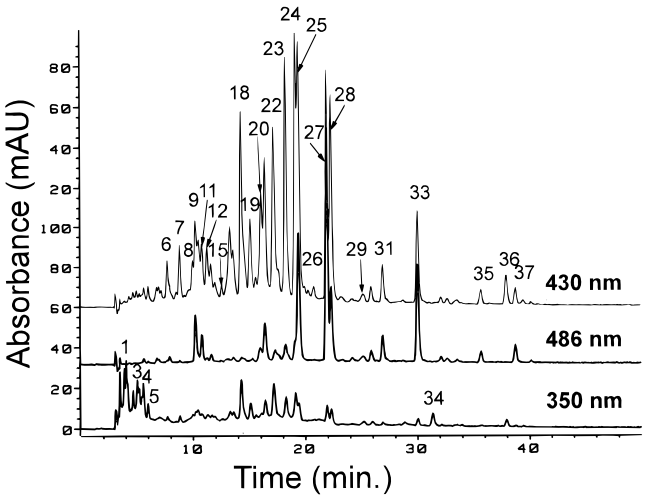

Figure 1. Saponified carotenoids in orange juice. Peaks are labeled and identified in Table 1. Chromatographic conditions are given in text. Chromatograms from absorbance monitoring at 430, 486, and 350 nm, respectively, are shown, all at identical attenuations.

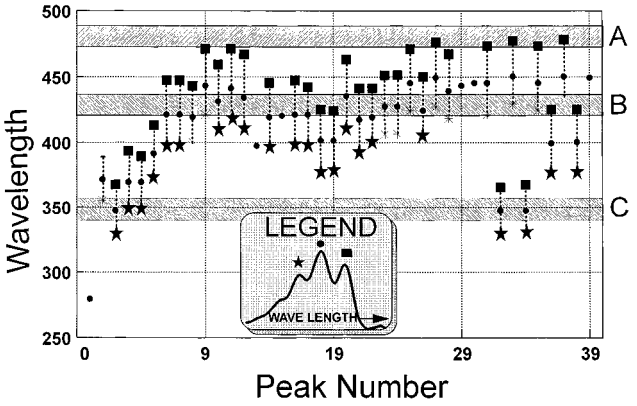

Figure 2. Plot of carotenoid absorbance maxima for each peak observed in Figure 1. Spectral bandpasses of ±8 nm for each monitoring wavelength (labeled A, B, and C corresponding to 486, 430, and 350 nm) are shown as shaded bands. Peak symbols are shown in the legend. The asterisk is used to indicate where a shoulder was present.

rate of change in MTBE was adjusted by adding several steps in the gradient program to provide optimal resolution. Water was added to the initial solvent composition to increase the retention time and improve resolution of the early eluting, polar xanthophylls. The separation achieved with the C-30 column and the solvent gradient system is shown in Figure 1.

Chromatographic Reproducibility. We have observed with other solvent systems using conventional C-18 columns that retention times were not stable. We made triplicate injections of a single sample over a 5 h period to determine retention time stability. Using three rather late eluting peaks, i.e., zeaxanthin, isolutein, and β-cryptoxanthin, the percent standard deviation in retention times was 2.29, 1.57, and 0.09% respectively. Peak area reproducibility was also very good, even for the peaks that were not well resolved. For example, the relative standard deviation in peak area for zeaxanthin was 5.5% and that for isolutein was 7.7.

Chromatographic Resolution. Lutein and zeaxanthin (peaks 25 and 27) are two xanthophylls of very similar structure that are difficult to resolve chromatographically. The resolution between these two carotenoids has been used as a measure of the effectiveness

Table 1. Spectral Characteristics of Orange Juice Carotenoids

			observed (nm)			literature (nm)			
peak no.	carotenoid	RT[a] (min)	peak I	peak II	peak III	peak I	peak II	peak III	ref[b]
1		4.87	s355.5	371.5					
2		5.07	332.5	347.5	367.5				
3		5.3	349.5	369.5	393.5				
4	valenciaxanthin	5.53	351.5	369.5	389.5	351	369	390	A
5		5.99	374.5	391.5	413.5				
6	neochrome	7.68	399.5	421.5	447.5	397	420	444	B
7	trollichrome	8.83	397.5	421.5	447.5	396	422	448	C
8		9.98	s400.5	419.5	443.5				
9	antherxanthin	10.25	s421.5	443.5	471.5	416	441	468	D
10		10.49		431.5	459.5				
11	*cis*-antherxanthin	10.79	418.5	441.5	471.5	418	442	470	B
12	neoxanthin	11.25	414.5	434.5	481.5	413.5	435.5	465.5	C
13		11.68		397.5					
14		11.95	396.5	419.5	445.5				
15		12.56		420.5					
16		13.25	397.5	421.5	447.5				
17		13.56	400.5	421.5	442.5				
18	auroxanthin A	14.25	379.5	401.5	425.5	380	400	425	E
19	auroxanthin B	15.1	379.5	401.5	424.5	380	400	425	E
20	*cis*-violaxanthin	16.06	411.5	435.5	463.5	414	437	464	D
21		16.41	395.5	417.5	441.5				
22	leutoxanthin	17.17	399.5	419.5	441.5	398	420	444	B
23	mutatoxanthin A	18.21	s406.5	427.5	451.5	404	427	452	B
24	mutatoxanthin B	19.1	s406.5	427.5	451.5	404	427	452	B
25	lutein	19.39	s424.5	445.5	471.5	s423.5	445.5	473.5	C
26		20.77	405.5	424.5	450.5				
27	zeaxanthin	21.93	s427.5	449.5	476.5	s425.5	451.5	477.5	C
28	isolutein	22.31	s418.5	439.5	467.5	416	440	470	E
29		25.15		443.5					
30		25.88		445.5					
31	α-cryptoxanthin	26.87	s421.5	445.5	473.5	420	444	472	B
32		28.84	332.5	347.5	365.5				
33	*β*-cryptoxanthin	30.01	s429.5	450.5	477.5	426	452	478	D
34	phytofluene	31.36	333.5	347.5	367.5	330	348	367	B
35	α-carotene	35.66	s424.5	445.5	473.5	s423.5	445.5	473.5	C
36	*ζ*-carotene	37.93	379.5	399.5	425.5	378	400	422	B
37	*β*-carotene	38.77		450.5	478.5	s429.5	451.5	477.5	C
38		39.54	379.5	400.5	425.5				
39		40.12		449.5					

[a] RT = retention time. s = spectral shoulder. [b] A = Curl and Bailey, 1961; B = Farin et al., 1983; C = Van Heukelem et al., 1992; D = DeRitter and Prucell, 1981; E = Gross et al., 1972.

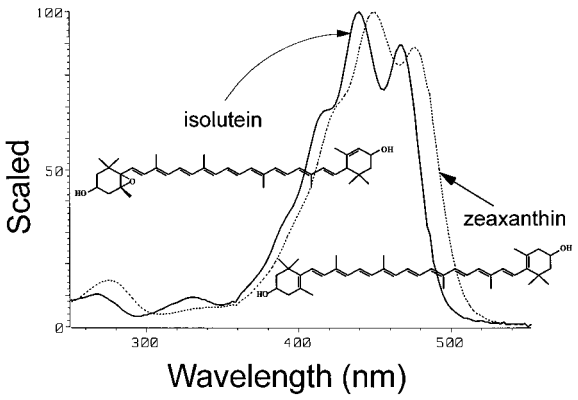

Figure 3. Absorbance spectra for zeaxanthin and isolutein, peaks 27 and 28, respectively. Retention times were 21.93 and 22.31, respectively. Structures for zeaxanthin (a diol) and isolutein (a diol epoxide) are also given.

of a chromatographic separation. In Figure 1 it can be seen that lutein and zeaxanthin are fully resolved (R_s = 2.9). But zeaxanthin is not completely resolved from isolutein (peaks 27 and 28), and lutein is barely resolved from mutaxanthin (peaks 25 and 24, respectively). The chromatographic purity of these incompletely resolved carotenoid pairs at their apex as indicated from their absorbance spectra is shown in Figure 3 for zeaxanthin and isolutein. The relative retention behavior is difficult to explain structurally. Lutein and zeaxanthin are usually very difficult to resolve as they differ only in the position of a single double bond in one of the terminal rings, yet are completely resolved with this chromatographic system. However, both zeaxanthin and isoleutin are barely resolved even though isoleutin contains an extra epoxide group.The photodiode array data indicated that all the peaks shown in Figure 1 were not chromatographically pure. Peaks 3, 8, 10, and 13 produced spectra which suggested that more than a single component was present. Other peaks, such as peaks 32, 38, and 39 were well resolved chromatographically, but provided poor spectra because of a low signal compared to relatively high background noise.

As shown in Figure 4, the spectra of *ζ*-carotene and *β*-carotene (peaks 36 and 37) are distinctly different. The reason can be deduced from the vastly different structural arrangement of these structural isomers (also shown in Figure 4). Whereas *ζ*-carotene is a linear molecule, *β*-carotene has the ends of the molecule looped into six-membered rings. The result is that *β*-carotene has its absorbance bands at longer wavelengths and has lost much of the "fine" structure in the absorbance bands.

β-Carotene and *ζ*-carotene are often difficult to resolve chromatographically. It can be seen in Figure 1 that *ζ*-carotene (peak 36) can only be detected at 430 and not 486 nm. Thus in situations where these two compounds are not well resolved, *β*-carotene would be

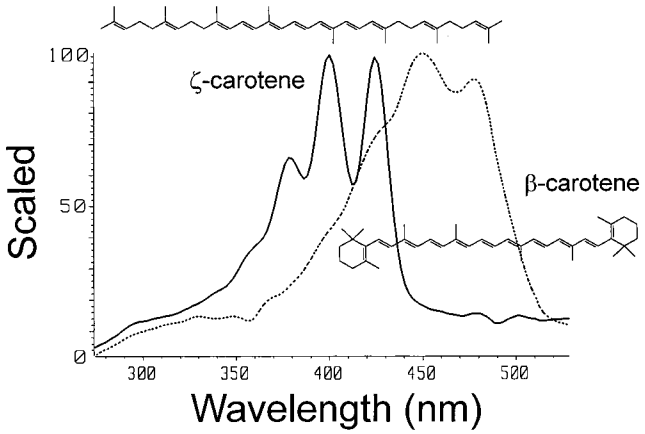

Figure 4. Absorbance spectra for ζ-carotene and β-carotene, peaks 36 and 37, respectively. Retention times were 37.93 and 38.77, respectively. Structures for these carotene carotenoids are also shown.

most accurately determined using the peak area obtained at 486 nm.

Photodiode Array Detection (PAD). The multidimensional nature of diode array data makes it an extremely significant tool for the study of complex natural products such as citrus carotenoids. Whereas earlier workers were usually limited to one detector wavelength, multiple wavelength detection is possible with PAD. Multiple detection wavelengths must be employed to detect carotenoids with diverse spectral characteristics from phytoene (λ_{max} 290 nm) to lycopene (λ_{max} 465 nm). The simultaneous acquisition of complete spectra as peaks elute also provides sufficient information for tentative peak identification by comparing the spectrum of the unknown to that of known compounds. The spectral information can also be used to determine chromatographic peak purity, provided the impurity has a spectrum sufficiently different from that of the parent compound.

Choice of Monitoring Wavelengths. The spectral peak maxima for the 39 separated carotenoids are plotted in Figure 2. Also shown in this figure are the three monitoring wavelengths 486, 430, and 350 nm (each with a 8 nm bandpass) labeled A, B, and C, respectively. Historically carotenoids have been determined at 440 or 450 nm. However, it can be seen that the majority of citrus carotenoids will have one of their absorbance bands in the region of 430 nm. Since this wavelength gave the greatest response for the most orange juice carotenoid peaks, it was also used to measure chromatographic resolution. As shown in Figure 1, phytofluene (peak 34) could not be detected at 430 or 486 nm. Thus the 350 nm wavelength was used to monitor this carotene and valenciaxanthin, a polar, trihydroxy carotenoid (peak 4) as well as peaks 1−3 and peak 32. The 486 nm wavelength was used to determine only those carotenoids, such as β-cryptoxanthin, which would contribute to the desirable deep orange-red color of orange juice. This was a more selective wavelength for the meaningful juice carotenoids as far as color was concerned. As noted in Figure 1, chromatograms obtained at 486 nm were considerably simpler than those obtained at 430 nm.

Spectral Characteristics of Chromatographic Peaks. The spectral characteristics of the 39 separated peaks are shown in Table 1. Twenty of these peaks have been provisionally identified on the basis of spectral matching of observed versus literature spectral data. Spectral shoulders are indicated by an s. Using the central absorbance peak (band) as a primary reference it can be seen that no identified peak differed from its reference value by more than 2 nm. This is within the spectral limits for the spectrophotometer. The wavelengths assigned to shoulders are more difficult to assign accurately. With the exceptions of cryptoxanthin and antherxanthin, most spectral shoulders are within the 2 nm limit. These two peaks were given their respective assignments as the observed values for peaks II and III were within 3−4 nm of the reported values and the differences were consistent.

As a further aid in identification, the retention times of standards were compared with the retention time of the proposed peak. For example, the retention time for β-carotene compared within 0.05 min of the orange juice peaks with identical spectral characteristics. If no other source of comparison was available, identifications were based upon comparison of spectral characteristics of other carotenoids which have previously been reported in orange juices coupled with expected elution order based on polarity or the elution order reported in the literature for similar reversed phase separations.

CONCLUSION

The major carotenoids in orange citrus juice can be separated with increased accuracy due to the improved speed and resolution of this chromatographic system. Thirty-nine chromatographically resolved peaks have been spectrally characterized. All the major carotenoids reported by other investigators have been identified using this more rapid procedure. Twenty of the 39 orange juice carotenoid peaks have been identified using spectral and retention time data from authentic standards or literature values.

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