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LIQUID CHROMATOGRAPHIC PROFILES OF MAJOR CAROTENOID ESTERS IN COMMERCIALLY PROCESSED CALIFORNIA NAVEL AND VALENCIA ORANGE JUICE CONCENTRATES

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SUMMARY

A procedure for establishing profiles of major carotenoid esters in commercially processed Valencia and Navel orange juice concentrates by reversed-phase liquid chromatography (LC) using Sudan 1 as internal standard is described. The procedure involved conversion of 5,6-epoxides in heat concentrated citrus juices to more stable 5,8-epoxides by treatment of extracted carotenoids with hydrochloric acid followed by dual wavelength analyses at 400 and 465 nm using LC. The esters of 5,8-furanoids (auroxanthin and mutatoxanthin) were approximately quantitated at 400 nm without interference from other carotenoids. Cryptoxanthin esters and free cryptoxanthin, lutein esters, citraurin esters and carotenes were approximately quantitated at 465 nm without interference from auroxanthin esters. The furanoid esters varied from 60 to 75% of the total carotenoids in the concentrates. The cryptoxanthin esters varied from 5 to 10% of the total carotenoids in Valencia orange juice concentrates and from 10 to 15% of the total carotenoids in Navel orange juice concentrates. Citraurin esters were present only in Navel orange juice concentrates and β-carotene content was less than 5% of the total carotenoids in both concentrates. The total carotenoids and individual carotenoids increased with the advance in season in Navel orange juice concentrates which had less than half the amount of total carotenoids of Valencia orange juice concentrates.

INTRODUCTION

Color is an important quality aspect in commercially processed orange juice concentrates. The color of citrus fruits is due to carotenoids. Valencia orange is the major source of concentrates produced in the U.S.A. Navel oranges and other citrus fruits are also processed for juice for export. The Navel and Valencia oranges belong to sweet orange (*Citrus sinensis*).

Citrus carotenoids have been studied extensively by classical methods using

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open column and thin-layer chromatography (TLC)[1,2], and recently by high-performance liquid chromatography (HPLC)[3,4]. These procedures used saponification of extracted carotenoids prior to chromatographic analyses. Saponification hydrolyzes carotenoids esters and is a severe chemical treatment for labile carotenoids.

In a recent study of citrus fruit carotenoids by LC without saponification and pre-treatment, the authors[5] have found only a limited number of simple carotenoids in oranges and mandarins. All the hydroxylated carotenoids in citrus fruits were acylated with C_{12}–C_{18} saturated fatty acids, and violaxanthin and antheraxanthin (5,6-epoxides) accounted for two-thirds of the total carotenoid content. Eleven esters (seven diesters and four monoesters) each of violaxanthin and antheraxanthin were found in fresh fruits. The 5,8-furanoids of violaxanthin and antheraxanthin were not found in fresh oranges and mandarins. The visible absorption spectra of fresh Navel and Valencia orange juices was essentially that of violaxanthin with a maximum at 436 nm in methanol which shifted to 400 nm on treatment with methanolic hydrochloric acid. The carbonyl carotenoid β-citraurin was not found in Valencia oranges, but was present in Navel oranges and mandarins.

The current analytical procedures, based on open column chromatography on activated magnesium oxide and deactivated alumina and adapted by the Association of Official Analytical Chemists[6] and International Federation of Fruit Juice Producers[7], are tedious and do not distinguish individual carotenoids. This study was undertaken to develop an HPLC procedure for the approximate quantitation of major carotenoid esters in commercially processed California Navel and Valencia orange juice concentrates and to determine the variation in major carotenoid esters in orange juice concentrates from different areas so that a carotenoid profile can be developed for routine quality control and detection of adulteration. Previous attempts to separate citrus concentrate carotenoids by HPLC failed because of the numerous isomers of violaxanthin present in heat processed orange juice concentrates[8]. Preliminary studies on LC separation of orange juice concentrate carotenoid esters by the procedure developed for fresh fruits[5] did not succeed because esters of luteoxanthin, auroxanthin and mutatoxanthin were present in the concentrates, in addition to esters of violaxanthin and antheraxanthin. The 5,8-furanoid esters resolved poorly from the 5,6-epoxide esters in the reversed-phase chromatogram.

EXPERIMENTAL

Samples

The citrus juice concentrates used in this study were processed by Sunkist Growers (Ontario, CA, U.S.A.) at two locations during the 1986–87 season. Four concentrates each of early-season, mid-season and late-season Navel and Valencia were analyzed in duplicate. The °Brix and Brix/Acid ratios of concentrates varied from 65–68 and 10–20, respectively.

Materials and reagents

The chemicals and solvents used were ACS grades, and the solvents used for LC were HPLC grade. Chemicals for fatty acid analyses and β-carotene with defined purity were purchased from Sigma (St. Louis, MO, U.S.A.). Sudan 1 [1-(phenylazo)-2-naphthol] purchased from Aldrich was purified as follows: Commercial grade

Sudan 1 was dissolved in hot ethanol which was filtered hot and cooled slowly. The crystals formed were collected by filtering, washed with cold ethanol and dried at 50–60°C under vacuum to constant weight. The crystals (0.1241 g) were dissolved in acetone–isopropanol (50:50) and diluted to 500 ml in a volumetric flask to make a 1-m*M* stock solution which was stable in the refrigerator over 3 months. The stock solution was diluted 1 to 10 to make 100 ml of 0.1 m*M* working standard which was prepared daily for use as internal standard.

Apparatus

LC analyses were done on Perkin-Elmer Series 4 HPLC with microprocessor controlled solvent delivery, variable-wavelength detector with a 1.4-μl cell and integrator. Gas–liquid chromatographic (GLC) separations of methyl fatty acid esters were done on a Varian 1200 gas chromatograph with flame ionization detection using 15% diethylene glycol succinate on Chromosorb W (6 ft. × 1/8 in. I.D.) as liquid phase. The electronic spectra were obtained by a Gilford Response spectrophotometer using 1-cm cells or a 0.5 ml flow through cell.

Extraction

A 10.0-gm sample of citrus juice concentrate was homogenized with 50 ml methanol in a Polytron homogenizer at high speed for 1 min and filtered through medium porosity Buchner funnel (100 ml capacity) under vacuum. The residue in the Buchner funnel was further extracted with 50 ml acetone by mixing with a glass rod under low vacuum, and the cake was washed with additonal 50 ml acetone. A volume of 15 ml 10% methanolic hydrochloric acid was added to the combined filtrate (150 ml) in the Buchner flask. After shaking thoroughly for 30 s, the extract was transferred immediately to a separatory funnel (500 ml capacity) containing 50 ml light petroleum (b.p. 40–60°C). Any insolubles left in the Buchner flask were dissolved in 300 ml distilled water and added to the separatory funnel. The aqueous solvent phase drained off after 5 min. Depending on the amount of total carotenoids, a volume of 5 or 10 ml 0.1 m*M* Sudan 1 working standard and 100 mg of magnesium carbonate were added. The light petroleum layer was washed twice with 100 ml distilled water and the aqueous phases drained off. The light petroleum layer was dried with 10 g anhydrous sodium sulfate, filtered through filter paper and concentrated at low temperature in a rotary evaporator to dryness. The residue was dissolved completely in 0.5 ml acetone and filtered through a 0.45-μm filter. The filtrate (5–10 μl) was then used for HPLC injections. HPLC analyses were done immediately after extraction.

Chromatographic procedure

Reversed-phase HPLC. Octadecyl silane (Waters Resolve C_{18}, 150 × 3.9 mm I.D., stainless steel) was used for HPLC separations. A guard column (2.5 cm) with the same reversed-phase packing was used. The mobile system consisted of methanol with a convex gradient of 0.2 over 15 min to a 50:50 mixture of methanol–ethyl acetate followed by a linear gradient (1.0) to 100% ethyl acetate over 5 min. The gradient was nearly linear between 5 and 15 min. Other conditions were as follows: equilibration, 10 min; flow-rate, 1.0 ml/min; chart speed, 10 mm/min; detection, 400 and 465 nm with an a.u.f.s. of 0.16.

TLC. Thin-layer separations were done on silica gel G. The R_F values were determined on 0.250 mm thin layers, and preparative separations were done on 0.500 mm layers. The solvent consisted of 5.0% acetone in light petroleum (solvent 1) for the separation of carotenes, diesters and cryptoxanthin esters, and 20% acetone in light petroleum (solvent 2) for the separation of monoesters of dihydroxy carotenoids and citraurin esters.

Test for citraurin esters

To a solution of citraurin ester in methanol (5 ml) was added 100 mg sodium borohydride. The spectrum was recorded after 5 min. A hypsochromic shift of 20–30 nm in the absorption maximum indicated the presence of one conjugated in-chain carbonyl group which had been reduced to an alcohol group by sodium borohydride[9].

Qualitative analyses

The carotenoids quantitated in this study were known to be present in citrus fruits[8]. Individual peaks collected from HPLC of purified TLC fractions were identified by their electronic spectra in different solvents, chemical tests, R_F values on silica gel thin layers and retention times on reversed-phase HPLC. Spectral comparisons were made with reported values[9–11]. The *cis*-carotenoids were identified by the presence of absorptions in the 300–350 nm region of the electronic spectrum[12].

Identification of fatty acids

The major carotenoid esters were collected individually from 5–10 HPLC runs and solvent removed under vacuum. They were saponified with 1 ml 10% methanolic potassium hydroxide by heating in a boiling water bath for 2 min in a screw cap test tube. One ml 15% boron trifluoride–methanol (Sigma) was added and heating continued for 3 min. The solution was cooled, 1 ml hexane added and a saturated solution of sodium chloride was added to bring the hexane layer to the top of the test tube. The hexane layer containing the methyl esters of fatty acids were carefully drawn with a pipette and analyzed by GLC. The fatty acids were identified by comparison of retention data with those of authentic methyl esters[13]. The fatty acid analyses were also done on TLC purified fractions containing mixtures of esters of the same xanthophyll. The amount of esters required for fatty acid analyses by this procedure was large and only the major esters were identified. Tentative identification of a number of acyl groups were made from the linear relationship between the number of acyl carbons and HPLC retention times of identified esters[11]. The acyl groups of citraurin, cryptoxanthin and lutein had been identified previously[5].

Quantitative analyses

Quantitative analyses by reversed-phase HPLC using Sudan 1 as internal standard were done in duplicates. Peak area measurements at 400 and 465 nm were used for quantitation. HPLC peaks at 400 nm were calculated as auroxanthin equivalent. Auroxanthin myristate-palmitate was isolated in sufficient quantities by HPLC after a preliminary purification by TLC and the concentration of chromatographically pure auroxanthin ester was determined using an $E_{1\%}$ value of 2550 at 400 nm in ethanol. The reported absorbance value of violaxanthin[9] was used for the calculation

of auroxanthin esters. HPLC peaks at 465 nm were calculated as β-carotene. Response factors for auroxanthin myristate-palmitate at 400 nm and β-carotene at 465 nm were determined by injecting mixtures of known concentrations of these carotenoids and internal standard, and measuring the area responses. The response factor, f, was determined in duplicate for each carotenoids by the equation:

$$\frac{\text{concentration internal standard}}{\text{concentration carotenoid}} = f\frac{\text{area internal standard}}{\text{area of carotenoid}}$$

the response factors for β-carotene and auroxanthin myristate-palmitate were 2.4057 and 4.2751, respectively. Mutatoxanthin ester peaks appeared in the chromatograms measured at both 400 and 465 nm, and this carotenoid was quantified as the sum of concentrations in both the chromatograms, since the sum of absorbance at 400 and 465 nm of mutatoxanthin in methanol was found to be nearly the same as the absorbance at the maximum absorption (421 nm). The total carotenoid content reported included all observed peaks in the chromatogram. The limit of detection at 0.16 a.u.f.s. used in this study was 0.1 μg/g.

The procedure used for quantitation of carotenoid esters was only approximate. All peaks in the chromatogram at 400 nm were calculated as auroxanthin (molecular weight, MW 600) equivalent and the peaks in the chromatogram at 465 nm were calculated as β-carotene (MW 536) equivalent. Though myristate-palmitate ester of auroxanthin was used for determining the response factor by HPLC, the concentrations reported were based on the absorbance of auroxanthin. Approximate concentrations of individual esters can be determined by multiplying by a factor calculated for each ester by the equation: MW of the ester/600 at 400 nm and MW of the ester/536 at 465 nm. Thus the factors calculated for auroxanthin dipalmitate and cryptoxanthin palmitate respectively are 1.79 and 1.47.

RESULTS

Qualitative analysis

Partition. The carotenoids from Navel and Valencia orange juice concentrates were partitioned separately between 96% methanol and light petroleum. The light petroleum and methanol phases were subjected to TLC using solvents 1 and 2 respectively.

TLC. The major bands from the thin layer were scraped off the plate and extracted with acetone; the solvent was removed and the components were tentatively identified by their spectra. The methanol-phase carotenoids isolated were free cryptoxanthin (R_F value 0.65), *trans*- and *cis*-citraurin esters (R_F values 0.85 and 0.90), mutatoxanthin monoesters (R_F value 0.70) and auroxanthin monoesters (R_F value 0.60). The light petroleum phase carotenoids were carotenes (R_F value 0.95), cryptoxanthin esters (R_F value 0.85), *trans*- and *cis*-lutein diesters (R_F values 0.70 and 0.75), mutatoxanthin diesters (R_F value 0.25) and auroxanthin diesters (R_F value 0.15). Since the TLC separation was based on functional groups, the individual esters of the same carotenoid did not separate.

HPLC. The TLC-separated carotenoids were further separated into individual esters by reversed-phase HPLC. Figs. 1 and 2 show HPLC chromatograms of seven

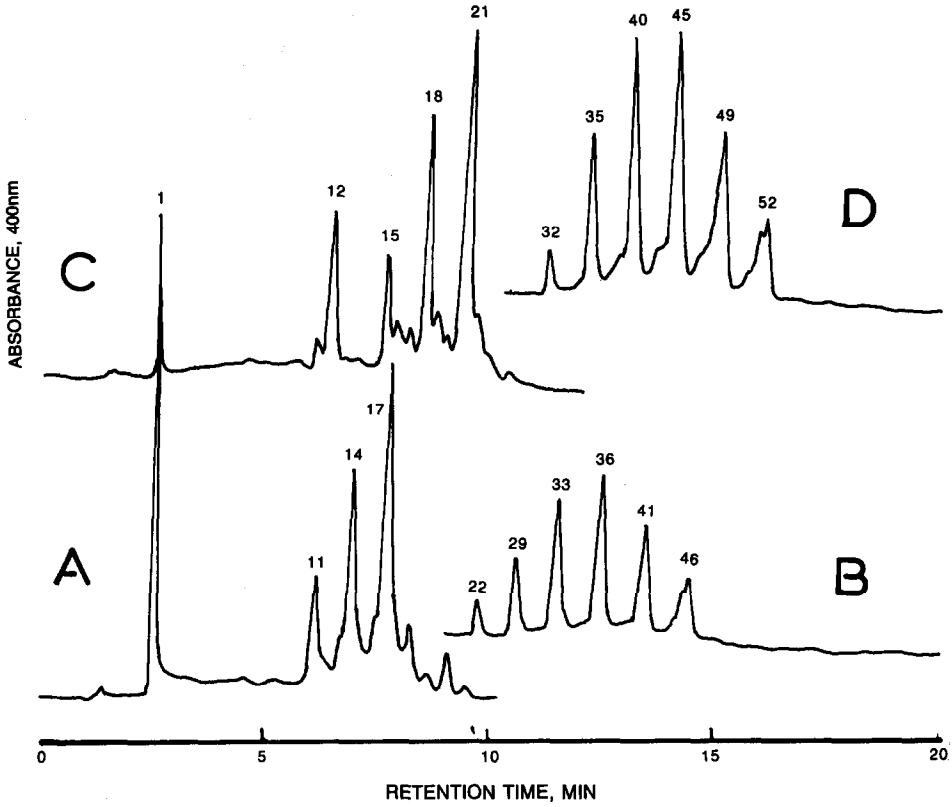

Fig. 1. HPLC separation of: (A) auroxanthin monoesters, (B) auroxanthin diesters, (C) mutatoxanthin monoesters and (D) mutatoxanthin diesters isolated by TLC and measured at 400 nm.

different ester bands isolated from TLC and β-carotene. These compounds accounted for nearly all the major carotenoid ester peaks found in Navel and Valencia orange juice concentrate extracts. The peak identification numbers, retention times and peak identities are given in Table I.

The reversed phase and the mobile phase used in this study were developed for the separation of carotenoid esters[11]. The free xanthophylls eluted between 2 and 6 min, monoesters of dihydroxy carotenoids and citraurin esters between 6 and 10 min, hydrocarbons between 9 and 10 min, and diesters of dihydroxy carotenoids and cryptoxanthin esters between 10 and 20 min. The conditions used did not resolve satisfactorily α and β forms of carotenes and cryptoxanthins, and *trans* and *cis* forms of carotenoids with the exception of lutein diesters. However the esters of the same carotenoid differing by only two acyl carbons separated with a resolution of 3, and there was a linear relationship between the number of acyl carbons and retention times[11].

Spectrophotometry. The HPLC separated carotenoid esters were collected individually in sufficient quantities for recording their electronic spectra. Figs. 3 and 4 show the electronic spectra of carotenoids isolated from Navel and Valencia concentrates and Sudan 1. Acylation does not alter the shape or location of peak maxima

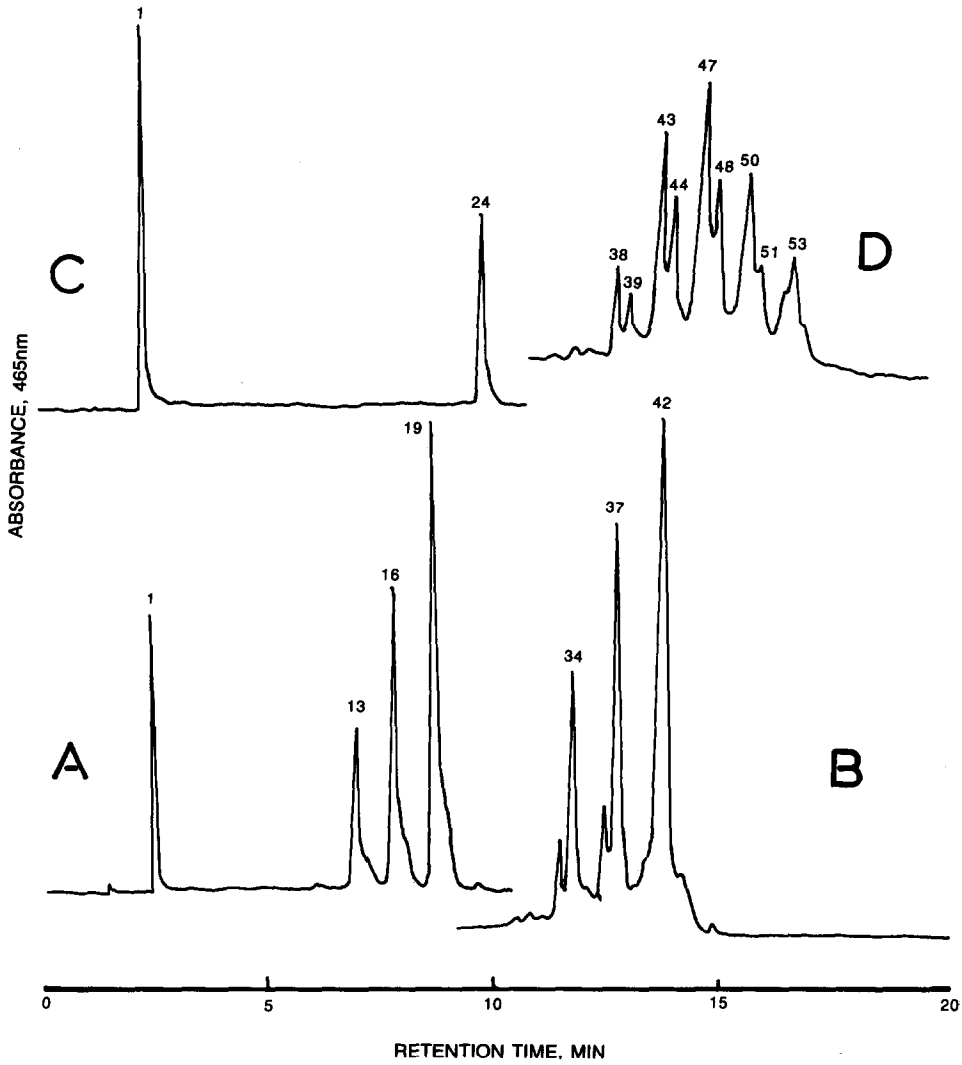

Fig. 2. HPLC chromatograms of: (A) citraurin esters, (B) cryptoxanthin esters, (C) β-carotene and (D) lutein diesters isolated by TLC and measured at 465 nm.

of carotenoid spectra[14]. The visible light absorption maxima of carotenoids in methanol were as follows: auroxanthin esters, 378, 400 and 425 nm; mutatoxanthin esters, 397, 421 and 447 nm; *cis*-citraurin esters, 336 (*cis* peak) and 452 nm; *trans*-citraurin esters, 459 nm; β-cryptoxanthin esters, 450 and 478 nm; β-carotene, 453 and 480 nm; *cis*-lutein esters, 330 (*cis*- peak), 415, 442 and 470 nm; and *trans*-lutein ester, 420, 444 and 473 nm. These maxima obtained were comparable to those reported[9].

Identification of carotenoids. The structures of carotenoids reported to be present in Navel and Valencia orange juice concentrates[8] are given in Fig. 5. Only the esters of auroxanthin, mutatoxanthin, cryptoxanthin, lutein and citraurin, and β-carotene were characterized in this report.

TABLE I

HPLC PEAK IDENTIFICATION NUMBERS AND RETENTION DATA OF CAROTENOID ESTERS OF VALENCIA AND NAVEL ORANGE JUICE CONCENTRATES AFTER TREATMENT WITH HYDROCHLORIC ACID

Not all observed peaks were numbered.

Peak No.	*Wavelength of measurement (nm)*	*Peak identity*	*Retention time (min)*
1	400 and 465	Sudan 1	2.47
2 to 9	400 and 465	Xanthophylls unidentified	2.50–6.00
10	465	Free cryptoxanthin	6.23
11	400	Auroxanthin monolaurate	6.26
12	400	Unidentified	6.40
13	465	Citraurin laurate	6.92
14	400	Auroxanthin monomyristate	7.12
15	400 and 465	Mutatoxanthin monolaurate	7.63
16	465	Citraurin myristate	7.76
17	400	Auroxanthin monopalmitate	7.96
18	400 and 465	Mutatoxanthin monomyristate	8.48
19	465	Citraurin palmitate	8.67
20	400 and 465	Unidentified	9.04
21	400	Mutatoxanthin monopalmitate	9.52
21	465	Mutatoxanthin monopalmitate	9.52
22	400	Auroxanthin dilaurate	9.78
23	465	Carotenes (isomers)*	9.54
24	465	β-Carotene	9.82
28	465	Mutatoxanthin dilaurate*	10.56
29	400	Auroxanthin laurate-myristate	10.75
30	465	Cryptoxanthin caprate*	10.91
31	465	Unidentified	11.09
32	465	Mutatoxanthin laurate-myristate	11.23
33	400	Auroxanthin dimyristate	11.70
34	465	Cryptoxanthin laurate	11.91
35	400 and 465	Mutatoxanthin dimyristate	12.29
36	400	Auroxanthin myristate-palmitate	12.68
37	465	Cryptoxanthin myristate	12.95
38	465	Lutein dilaurate (*trans*)	12.96
39	465	Lutein dilaurate (*cis*)	13.25
40	400 and 465	Mutatoxanthin myristate-palmitate	13.60
41	400	Auroxanthin dipalmitate	13.68
42	465	Cryptoxanthin palmitate	13.98
43	465	Lutein laurate-myristate (*trans*)	13.90
44	465	Lutein laurate-myristate (*cis*)	14.28
45	400 and 465	Mutatoxanthin dipalmitate	14.60
46	400	Auroxanthin palmitate-stearate	14.66
47	465	Lutein dimyristate (*trans*)	14.96
48	465	Lutein dimyristate (*cis*)	15.28
49	400 and 465	Mutatoxanthin palmitate-stearate*	15.60
50A	400	Auroxanthin disterarate*	
50	465	Lutein myristate-palmitate (*trans*)	15.99
51	465	Lutein myristate-palmitate (*cis*)	16.32
52	400 and 465	Mutatoxanthin distereate*	16.61
53	465	Lutein dipalmitate (*trans*)	17.10
54	465	Lutein dipalmitate (*cis*)	17.36

* Identification tentative.

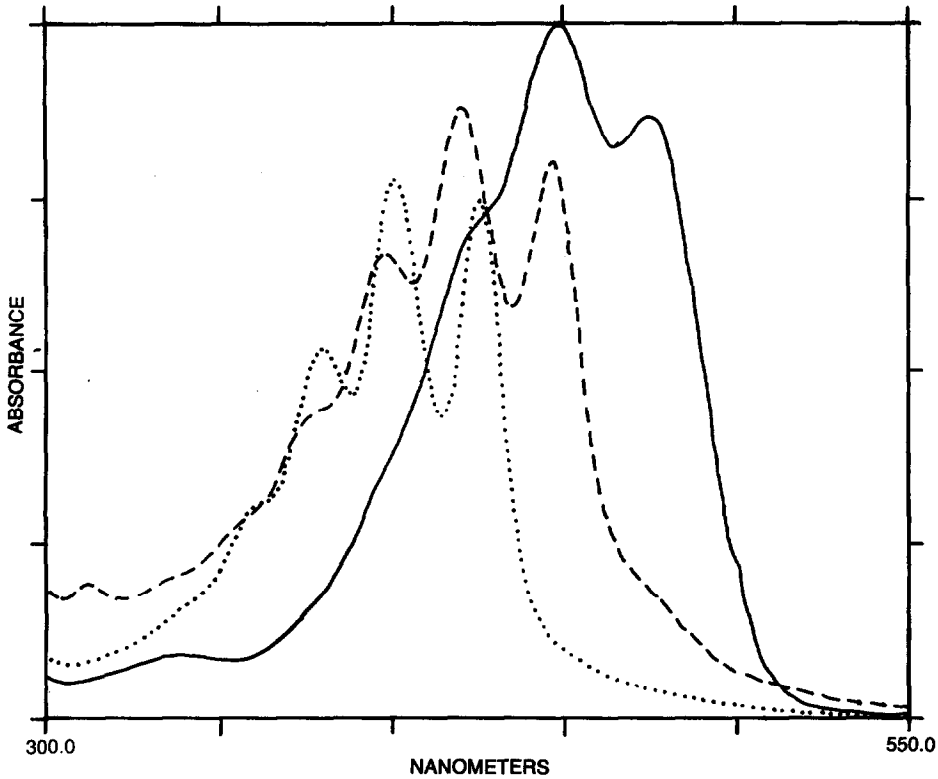

Fig. 3. The electronic spectra (qualitative) of auroxanthin myristate-palmitate (·····), cryptoxanthin myristate (———) and mutatoxanthin dimyristate (– – –) in methanol.

The central *cis* forms were readily identified by their absorptions in the 300–350 nm region (Fig. 4A and 4B). The carbonyl carotenoids were characterized by broad absorption maximum (Fig. 4C). Reduction of carbonyl carotenoids with sodium borohydride resulted in a hypsochromic shift of 30 nm on the absorption maximum with the appearance of detail (Fig. 4C). The 5,8-furanoid carotenoids were characterized by the typical three-banded spectrum with strong longest wavelength absorptions (Fig. 3).

The relative movement of carotenoids on thin-layer silica gel were indicative of their functional groups. The monoesters of dihydroxy carotenoids had R_F values less than 0.1 in 5% acetone in light petroleum. Cryptoxanthin esters and lutein diesters were well separated from diesters of auroxanthin and mutatoxanthin, although their separations were difficult to achieve by HPLC. Citraurin esters were distinctively red (*trans*) and pink (*cis*) on the thin-layer plates. Auroxanthin esters were blue-green on dried thin-layer plates. The *cis* and *trans* forms of lutein separated by TLC and HPLC.

Identification of fatty acids. The fatty acids identified by GLC after alkaline hydrolysis of the esters were lauric, myristic, palmitic and stearic acids. Caprate was tentatively identified as a minor ester of cryptoxanthin. The linear relationship be-

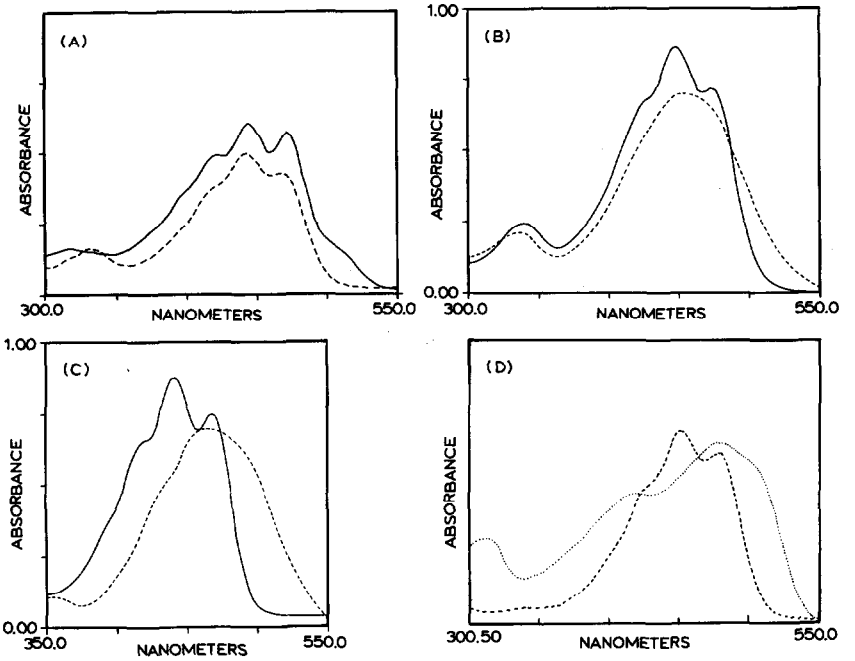

Fig. 4. The electronic spectra (qualitative) of: (A) *trans-* (———) and *cis-* (– – –) lutein diesters; (B) *cis*-cryptoxanthin (———) and citraurin (– – –); (C) citraurin (– – –) and reduced citraurin (citraurol) (———); and (D) synthetic β-carotene (– – –) and Sudan 1 (.....) in methanol.

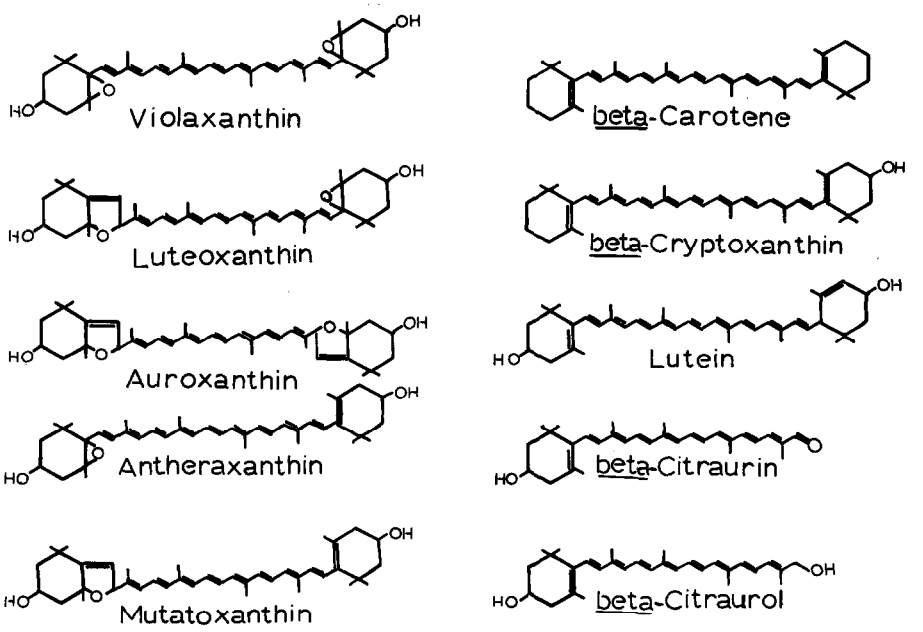

Fig. 5. The structures of major reported citrus carotenoids.

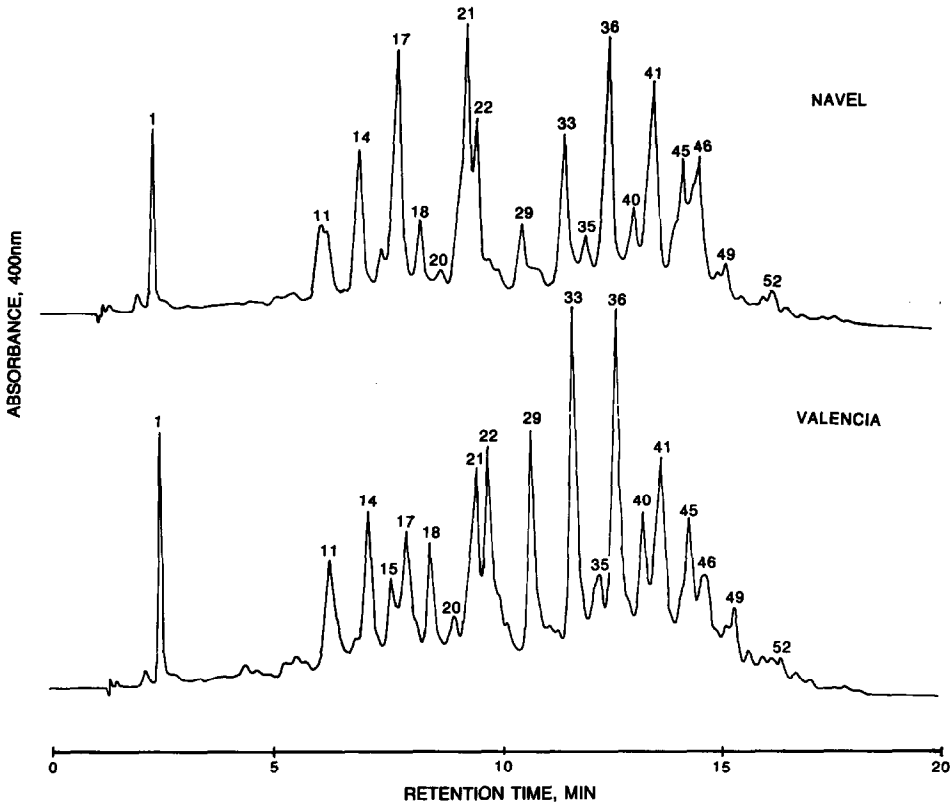

Fig. 6. HPLC chromatograms of Valencia and Navel concentrate carotenoids at 400 nm.

tween number of acyl groups and retention times on reversed phase HPLC was used for the tentative identification of some unknown small peaks.

Quantitative analysis

Procedure. The presence of numerous isomers of 5,6-epoxides in heat concentrated orange juice concentrates with close retention times on reversed-phase HPLC made it impossible to resolve the carotenoid esters by the HPLC procedure developed for fresh fruits which contained only 5,6-epoxides[5]. Therefore the procedure was modified to convert all the 5,6-mono and diepoxides (violaxanthin, luteoxanthin and antheraxanthin) to stable 5,8-furanoids (auroxanthin and mutatoxanthin) by treatment of extracted carotenoids with hydrochloric acid prior to HPLC. This procedure not only reduced the number of peaks attributed to isomers of violaxanthin and antheraxanthin esters but also avoided the task of resolving these isomers with very close retention times. The transformations of violaxanthin and antheraxanthin occurred immediately and quantitatively on treatment with hydrochloric acid using a procedure adapted from a test developed for 5,6-epoxides[15] and is reported for the first time.

TABLE II

QUANTITATIVE DATA ON CAROTENOIDS OF VALENCIA AND NAVEL ORANGE JUICE CONCENTRATES AT 400 nm AFTER TREATMENT WITH HYDROCHLORIC ACID (FIG. 6)

The concentrations are reported as auroxanthin equivalents.

Peak No.	*Peak identity*	*Concentration (μg/g)*	
		Valencia	*Navel*
11*	Auroxanthin monolaurate	3.54	1.94
14	Auroxanthin monomyristate	3.56	2.08
15	Mutatoxanthin monolaurate	1.81	0.63
17	Auroxanthin monopalmitate	3.70	3.39
18	Mutatoxanthin monomyristate	2.90	1.23
20	Unidentified	1.37	0.69
21*	Mutatoxanthin monopalmitate	4.50	3.28
22*	Auroxanthin dilaurate	6.12	2.31
29	Auroxanthin laurate myristate	4.71	1.40
32	Mutatoxanthin laurate-myristate	1.51	0.00
33	Auroxanthin dimyristate	6.77	2.43
35	Mutatoxanthin dimyristate	2.51	1.10
36	Auroxanthin myristate-palmitate	7.81	3.66
40	Mutatoxanthin myristate-palmitate	3.12	1.57
41	Auroxanthin dipalmitate	5.49	3.24
45	Mutatoxanthin dipalmitate	3.75	2.23
46	Auroxanthin palmitate-stearate	3.31	2.57
49	Mutatoxanthin palmitate stearate**	2.18	1.04
50A	Auroxanthin distearate**	0.78	0.33
52	Mutatoxanthin distearate	0.61	1.07

* Contained more than one carotenoid.
** Identification tentative.

Auroxanthin and mutatoxanthin had absorption maxima at 400 and 421 nm respectively in methanol. The minor orange juice concentrate carotenoids *beta*-cryptoxanthin, *beta*-citraurin, *beta*-carotene and lutein had absorption maxima in methanol at 450, 459, 453 and 444 nm, respectively. The absorption maxima of the minor carotenoids of citrus concentrates were spectrally separated from auroxanthin by over 40 nm. Thus HPLC peak measurements at 400 nm would quantitate auroxanthin and a second peak measurement at a wavelength above 450 nm would quantitate the minor carotenoids without interference from auroxanthin. Mutatoxanthin with an absorption maximum at 421 nm in methanol would appear in both the chromatograms depending on its relative absorbance at 400 and 450 nm. Fig. 3 shows the spectra of auroxanthin, mutatoxanthin and cryptoxanthin isolated from Navel and Valencia orange juice concentrates in methanol. Auroxanthin had no absorbance above 450 nm. Though lutein, cryptoxanthin, β-carotene and citraurin had some absorbance at 400 nm, their concentration in orange juice concentrates relative to auroxanthin was too small to be detectable at 400 nm at the a.u.f.s. (0.16) of auroxanthin myristate-palmitate used for quantitation in this study.

HPLC analyses at 400 nm. Fig. 6 shows the HPLC separation measured at 400 nm of extracts from Navel and Valencia orange juice concentrates treated with hy-

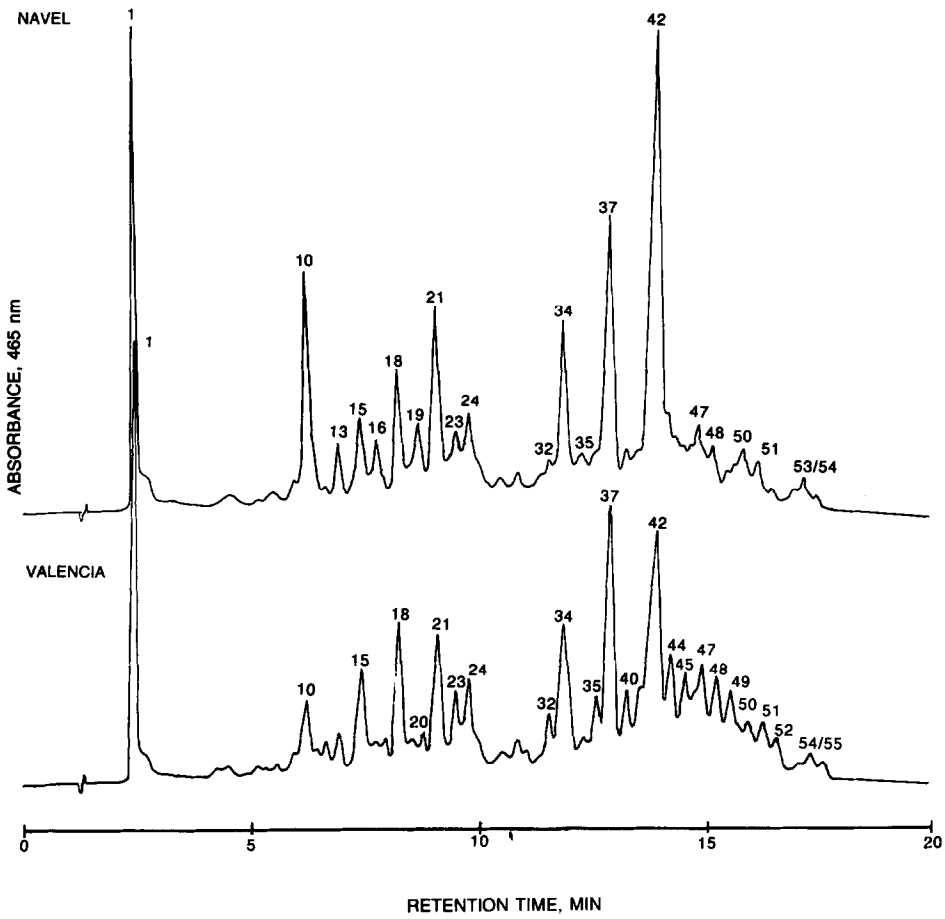

Fig. 7. HPLC chromatograms of Valencia and Navel concentrate carotenoids at 465 nm.

drochloric acid. The identification of peaks and calculated concentrations as auroxanthin equivalents are given in Table II. The esters of auroxanthin (5,8-difuranoid form of violaxanthin) and mutatoxanthin (5,8-furanoid form of antheraxanthin), and colorless hydrocarbons (phytoene, phytofluene and ζ-carotene) are the carotenoids measured at 400 nm. There were some unidentified minor carotenoids.

HPLC analyses at 465 nm. Fig. 7 shows the HPLC chromatograms measured at 465 nm of extracts from Navel and Valencia orange juice concentrates treated with hydrochlorid acid. The identification of peaks and calculated concentrations as β-carotene equivalents are given in Table III. The esters of cryptoxanthin, mutatoxanthin, lutein and citraurin, and carotene are the carotenoids measured at 465 nm.

Laurate, myristate and palmitate esters of cryptoxanthin (peaks 34, 37 and 42) predominated in the chromatogram at 465 nm. The *trans*-lutein dilaurate (peak 38) and lutein laurate-myristate (peak 43) did not resolve from the cryptoxanthin myristate and palmitate peaks. Based on the relative HPLC peak areas of *cis*- and *trans*-lutein esters isolated from thin-layer plates, an approximate correction to this error

TABLE III

QUANTITATIVE DATA ON CAROTENOIDS OF VALENCIA AND NAVEL ORANGE JUICE CONCENTRATES AT 465 nm AFTER TREATMENT WITH HYDROCHLORIC ACID (FIG. 7)

The concentrations are reported as β-carotene equivalents.

Peak No.	*Peak identity*	*Concentration (μg/g)*	
		Valencia	*Navel*
10	Free cryptoxanthin	1.35	1.56
13	Citraurin laurate	0.00	0.46
15	Mutatoxanthin monolaurate	1.90	0.78
16	Citraurin myristate	0.00	0.63
18	Mutatoxanthin monomyristate	2.06	0.98
19	Citraurin palmitate	0.00	0.82
21*	Mutatoxanthin monopalmitate	2.17	1.52
23*	Carotenes (isomers)**	1.29	0.63
24	β-carotene	2.01	1.20
28	Mutatoxanthin dilaurate**	0.72	0.33
30	Cryptoxanthin caprate**	0.66	0.50
32	Mutatoxanthin laurate-myristate	1.17	0.64
34	Cryptoxanthin laurate	2.72	1.47
35	Mutatoxanthin dimyristate	1.17	0.54
37*	Cryptoxanthin myristate	3.64	2.60
40	Mutatoxanthin myristate-palmitate	1.18	0.78
42*	Cryptoxanthin palmitate	5.55	5.25
44	Lutein laurate-myristate (*cis*)	1.66	0.95
45	Mutatoxanthin dipalmitate	0.73	0.45
47	Lutein dimyristate (*trans*)	2.30	0.22
48	Lutein dimyristate (*cis*)	1.51	0.24
49	Mutatoxanthin palmitate-stearate**	1.69	0.53
50	Lutein myristate-palmitate (*trans*)	0.90	0.38
51	Lutein myristate-palmitate (*cis*)	0.94	0.18
53	Lutein dipalmitate (*trans*)	0.67	0.29
54	Lutein dipalmitate (*cis*)	0.75	0.19

* Contained more than one carotenoid.
** Identification tentative.

was applied by reducing twice the peak area of *cis*-lutein myristate-palmitate (peak 44) from the peak areas of cryptoxanthin myristate and palmitate peaks. The reduced area was then added to the *trans*-lutein ester peak areas. The *cis*-lutein esters (peaks 39, 44, 48 and 54) resolved from the *trans*-lutein esters (peaks 38, 43, 47 and 53).

Quantitative analyses. Table IV summarizes the results of quantitative analyses of carotenoids in commercially processed California Navel and Valencia orange juice concentrates. The auroxanthin ester concentrations were the sum of violaxanthin, luteoxanthin and auroxanthin ester concentrations and the mutatoxanthin ester concentrations were the sum of antheraxanthin and mutatoxanthin ester concentrations. The Valencia orange juice concentrates had higher total carotenoids than those of Navel orange juice concentrates, and the carotenoid composition was not significantly affected by the season, whereas the total carotenoid content increased in the latter with the advance in season, the early season samples being very low in color

TABLE IV

SUMMARY OF COMPOSITION OF CAROTENOIDS AND CAROTENOID ESTERS (μg/g) IN COMMERCIALLY PROCESSED CALIFORNIA VALENCIA AND NAVEL ORANGE JUICE CONCENTRATES DETERMINED BY LC AFTER TREATMENT WITH HYDROCHLORIC ACID

Auroxanthin esters were calculated as auroxanthin equivalents based on peak measurements at 400 nm. Cryptoxanthin, lutein and citraurin esters, and carotene were calculated as β-carotene equivalents based on peak measurements at 465 nm. Mutatoxanthin esters were calculated based on the sum of concentrations measured at 400 nm (as auroxanthin) and 465 nm (as β-carotene).

	Early season				*Mid season*				*Late season*			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Cryptoxanthin, total												
Valencia	12.0	10.3	7.9	12.2	10.9	11.1	12.4	14.2	6.2	9.5	7.6	12.3
Navel	2.7	1.7	3.2	2.1	9.5	6.3	5.7	4.8	8.1	11.2	8.6	11.3
Carotene, β												
Valencia	1.9	2.0	2.5	2.2	3.2	3.6	3.8	3.8	3.9	3.3	3.0	4.0
Navel	0.5	0.4	0.5	0.3	1.2	0.6	0.6	0.5	1.0	1.6	0.9	1.8
Auroxanthin esters												
Valencia	37.1	45.8	44.0	52.7	46.9	49.3	44.3	40.7	19.4	41.8	20.7	47.0
Navel	7.0	6.7	10.6	8.6	24.8	20.6	16.3	13.6	26.0	38.8	21.9	36.4
Mutatoxanthin esters												
Valencia	47.2	38.8	34.3	40.4	47.5	48.7	43.9	42.4	23.9	42.3	24.9	51.5
Navel	5.0	4.8	6.6	4.4	17.3	11.9	10.4	7.0	17.3	19.5	16.4	24.3
Lutein esters												
Valencia	9.8	11.7	9.2	9.8	10.4	15.4	13.4	17.4	7.0	14.1	7.9	21.0
Navel	2.2	2.2	2.2	1.3	4.3	3.5	2.1	2.0	4.1	5.8	4.0	7.7
Citraurin esters												
Navel	1.0	0.6	0.8	0.5	1.9	1.2	1.2	0.9	1.3	1.7	1.3	3.0
*Total (μg/qm)**												
Valencia	111	126	119	129	136	152	144	140	71	133	82	161
Navel	27	20	28	20	67	59	42	32	69	87	61	101

* Total carotenoids were calculated as the sum of concentrations of all observed peaks measured at 400 nm (as auroxanthin) and at 465 nm (as β-carotene).

and carotenoid content. The β-carotene concentration was low both in Navel and Valencia orange juice concentrates. Cryptoxanthin, the major provitamin A carotenoid in citrus accounted for 5–10% of the total carotenoids in Valencia and 10–15% of the total carotenoids in Navel orange juice concentrates.

DISCUSSION

An HPLC procedure suitable for routine analyses carotenoid esters in commercially processed Valencia and Navel orange juice concentrates is described. The procedure is rapid and is suitable for routine quality control. The hydrochloric acid treatment of extracted carotenoids converts 5,6-epoxides to 5,8-furanoids quantitatively and the procedure does not distinguish between esters of 5,6-epoxide and 5,8-furanoids both of which are known to be present in orange juice concentrates. The dual wavelength analyses of extracts after treatment with hydrochloric acid at

400 and 465 nm simplified the chromatograms, and the cryptoxanthin ester and free cryptoxanthin peaks were readily distinguishable from other peaks. Dual wavelength analyses also permitted quantitation of minor carotenoids which otherwise would be difficult to detect. Accurate measurement of cryptoxanthin is important in evaluating the provitamin A content of citrus juices and in determining adulteration of orange juices with mandarin juices. Sudan 1 which has absorptions in the 400–500 nm region of the spectrum is used as internal standard as well as a marker for correcting small variations in retention times. The quantitative data reported in this study are only approximate. In addition to the response factors for β-carotene and auroxanthin myristate-palmitate, response factors for all major carotenoid esters can be determined. Individual esters can be quantitated by using correction factors for their molecular weight. Calculation of individual peaks as Sudan 1 equivalents would be sufficient for routine quality control.

The visible spectra of Valencia and Navel orange juice concentrate carotenoids without the hydrochloric acid treatment showed maxima at 425 nm in methanol which shifted to 400 nm on treatment with hydrochloric acid. Previous study[5] found only violaxanthin and antheraxanthin in fresh Valencias and Navel oranges and their visible spectra were characterized by maxima at 438 nm in methanol. These evidences and the difficulty encountered in separating the concentrate carotenoid esters without hydrochloric acid treatment on reversed-phase HPLC suggested that the conversion of 5,6-epoxides to 5,8-furanoids was the major change during heat concentration of orange juice. Luteoxanthin and mutatoxanthin esters appeared to be the major carotenoids in the heat concentrated orange juices. The acidic conditions and heat promoted furanoid isomerization, and the pulp afforded some protection against complete conversion of violaxanthin to nearly colorless auroxanthin. Conversion of 5,6-epoxides to 5,8-furanoids results in large decreases in visual color without quantitative losses of carotenoids. Quantitative losses of carotenoids during heat concentration are difficult to determine unless the concentrate and juice from the same batch were analyzed.

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