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A Routine High-Performance Liquid Chromatography Method for Carotenoid Determination In Ultrafrozen Orange Juices

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An isocratic reversed-phase high-performance liquid chromatography method was developed for routine analysis of the main carotenoids related to the color of orange juice, using a more selective wavelength (486 nm) in which the absorption in the red-orange region of the visible spectra is maximum. Separation was carried out using as the mobile phase the mixture methanol:acetonitrile: methylene chloride:water (50:30:15:5, v/v/v/v), to which small amounts of butylated hydroxytoluene and triethylamine were added (0.1%). Identification was made by comparison either with standards obtained by thin-layer chromatography or with spectral data previously reported. The reproducibility of the method was remarkable; coefficients of variation for the most polar xanthophylls were under 1 and 4% for retention times and areas, respectively. Its application to Valencia late ultrafrozen orange juices has shown that major carotenoids are lutein + zeaxanthin (36%), lutein 5,6-epoxide (16%), antheraxanthin (14%), and β-cryptoxanthin (12%).

KEYWORDS: Carotenoids; HPLC; orange juice; Valencia late

INTRODUCTION

Carotenoids are one of the main classes of natural pigments because its distribution in the plant kingdom is extremely wide. Citrus fruits are a complex source of carotenoids, with the largest number of carotenoids found in any fruit. The importance of carotenoids in juice color, along with the growing interest in these pigments due to their health benefits, has stimulated the development of a wide variety of analytical methods for the characterization of these compounds. Thus, carotenoid content in orange juice has been widely discussed (*1*−*6*). Gross et al. (*1*) isolated over 50 carotenoids using a combination of column and thin-layer chromatography (TLC). Stewart (*3*) studied the carotenoid content in juice of several citrus cultures during the fruit maturation period. Recently, some new studies on carotenoid profiles in orange juice have come out (*5*−*7*).

Until the development of liquid chromatography methods, quantification of carotenes and xanthophylls was made employing open columns; however, high-performance liquid chromatography (HPLC) has several advantages in relation to those methods. Isocratic methods applied to carotenoid analysis are well-represented in the bibliography. Reeder and Park (*8*) employed different columns and mobile phases to determine α- and β-carotene and β-cryptoxanthin. Fisher and Rouseff (*9*) used a solid phase extraction technique prior to the analysis in order to select the carotenoids of interest. Recently, another isocratic method, which allows the determination of several carotenoids, has been proposed (*10*).

Although gradient solvent systems improve the resolution of the analysis, isocratic methods show some very important advantages, such as lower total time of analysis, because it is not necessary to equilibrate the column after each injection. So, isocratic methods are more suitable for routine analysis. In addition, the reproducibility of the analysis is higher due to less fluctuation of the retention times.

Most of the isocratic methods mentioned above were used to determine provitamin A carotenoids in orange juice, due to the limitations of this kind of elution. Analysis could be carried out within 15−20 min, but only four or five carotenoids could be determined. The main objective of our study was to develop an isocratic method to achieve the separation of the most important carotenoids related to the color of orange juice, by means of a more selective wavelength for the meaningful carotenoids as far as orange juice color is concerned. Because the color of orange juice is an important parameter in relation to the quality of the product, it would be important for the industry to have routine methods, according to its equipment, for analyzing these compounds. For this purpose, gradient methods are not the most useful, since the analysis time, including reequilibration time, is over 1 h. On the other hand, this study was also aimed at characterizing the profile of these most important carotenoids related to the color in Valencia late ultrafrozen orange juice, including the provitamin A carotenoids β-cryptoxanthin, α-carotene, and β-carotene.

MATERIALS AND METHODS

Standards. The isolation and purification of standards was carried out by TLC on plates of silica gel 60 F_{254} (20 cm × 20 cm, thickness

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0.7 mm) (Merck, Darmstadt, Germany), according to previous works (*11*, *12*). Lutein, neoxanthin, and violaxanthin were obtained from a saponified extract of mint (*Mentha* sp.). β-Cryptoxanthin and β-carotene were isolated from saponified extracts of papaya (*Carica papaya* L.) and carrot (*Daucus carota* L.), respectively. The solvent systems used were as follows: petroleum ether (bp 65−95 °C)−acetone−diethylamine (10:4:1) for mint and papaya extracts and petroleum ether (bp 65−95 °C) for carrot extract.

After calculating the R_f, each band was scraped from the plate and placed into a spin-drier tube with an appropriate solvent (acetone, ethanol). After the mixtures were spin-dried for 5 min at 3000 rpm, the supernatants were filtered through Millipore PVDF Millex filters (13 mm × 0.45 μm) (Bedford, MA). Standards were placed in amber vials and kept at −20 °C until purity analyses were carried out.

HPLC. The HPLC apparatus employed for routine analysis consisted of a Hewlett-Packard 1050 system, equipped with an isocratic pump and a UV−vis detector. A 50 μL loop was used for injection. The column was a C_{18} Kromasil 5 μm (250 mm × 4.6 mm) with a guard column of the same material (10 mm × 4 mm) (Hichrom Ltd., Reading, U.K.).

The mobile phase was a quaternary mixture of methanol:acetonitrile:methylene chloride:water (50:30:15:5, v/v/v/v) to which 0.1% butylated hydroxytoluene (BHT) and 0.1% triethylamine were added. All solvents were HPLC grade.

The column was kept at room temperature (23 °C), and the flow rate was 2.5 mL min^{-1}. The wavelength was adjusted to 486 nm. The chromatograms were registered using ChemStation software (Hewlett-Packard, Palo Alto, CA). After three injections, the column was washed with methylene chloride and then equilibrated with the mobile phase.

To identify and characterize the carotenoids determined in this study, a Hewlett-Packard 1100 chromatographic system was used, equipped with a photodiode array detector. The spectrum from 350 to 800 nm was recorded and stored. The chromatographic conditions were the same as mentioned above.

Peak purity was established by recalling the spectra that were obtained at the upslope (at peak half-height), apex, and downslope (at peak half-height) against a baseline reference. The spectra were normalized and overlaid for comparison. The spectra of the standards obtained by TLC were used for identification purposes whenever possible. Other chromatographic peaks were tentatively identified by means of comparison of their spectral characteristics with those reported in the literature.

Quantitative Analysis. The concentrations of carotenoid pigments were expressed as relative percentage of total peak area. To calculate the absolute concentrations of the provitamin A carotenoids, β-carotene was used as external standard. For this purpose, four solutions of β-carotene in acetone were prepared, taking successively greater aliquots of the stock solution. The concentrations of these solutions were determined spectrophotometrically using a value of $\epsilon_0 = 134.4$ cm^{-1} mM^{-1} (*14*). The absorbance measurements were carried out at 454 nm. Quantification of β-cryptoxanthin and α-carotene was also carried out based on a β-carotene standard curve, since they all have an identical chromophore.

Reproducibility, Linearity of Response, and Limit of Detection (LOD). To study the stability of the retention times and the peak areas, three consecutive injections of a single sample were made. Linearity of response was evaluated taking into account the β-carotene standard curve. The LOD was calculated from the equation of the calibration curve according to the formula $LOD = a + 2s_{y/x}$ (*15*).

Samples. After the selection of the mobile phase, 17 ultrafrozen Valencia late orange juices, obtained from the industry as representative samples of the 2000 season, were analyzed for carotenoid determination. Oranges were harvested in Huelva (Atlantic Coast of southern Spain). In the industry, the juices are cooled and immediately frozen using liquid nitrogen in an industrial freezing tunnel. The commercial samples are kept in freezing chambers between −18 and −21 °C until its distribution. The samples analyzed in this study were kept at −21 °C until its analysis in the laboratory. Thawing was carried out at room temperature (23 °C) for 24 h.

Sample Preparation. Sample preparation was done following a modification of the Fisher and Rouseff (*9*) method. A 10 mL sample of ultrafrozen orange juice was centrifuged for 5 min at 5000 rpm. The clarified juice was discarded, and the pellet containing the carotenoids was successively centrifuged, as described above, with methanol until no more color remained. The extractant contained a small proportion of BHT (0.004%), to protect the carotenoids from oxidation during the sample manipulation. BHT has been widely used for this purpose before (*5*, *10*, *16*, *17*). All of the methanolic extracts were combined and used in the saponification reaction. The washing of the coloring material just before the first centrifugation, described in the original method, was not done because losses of pigments were observed. For the saponification of the extract, 10 mL of methanolic potassium hydroxide (10%, w/v) was added and the mixture was allowed to stand in the dark at room temperature for 1 h.

The mixture was transferred to a separatory funnel, and carotenoids were extracted with 30 mL of methylene chloride containing BHT (0.004%). The potassium hydroxide was removed by washing four times with water. The yellow extract was evaporated to dryness at room temperature and finally redissolved in 1.5 mL of methanol−acetone (2:1, v/v). Before the chromatographic analysis, the aliquot was filtered using Millipore PVDF Millex filters (13 mm × 0.45 μm). Operations were carried out under dim light, and all samples were analyzed in triplicate.

RESULTS AND DISCUSSION

Standards Isolation. The identification of the compounds was made first on the basis of their R_f, which agreed well with those reported by other authors (*12*), except in the case of β-carotene, probably due to the usage of petroleum ether (bp 65−95 °C) instead of light petroleum ether (bp 40−60 °C). This first identification was checked by spectroscopy, by means of comparison of the absorption maxima in different solvents with those reported in the literature (*18*). The purity of the standards isolated was checked by HPLC following the method described previously.

HPLC. *Selection of Monitoring Wavelength.* Carotenoids were usually monitored at 450 nm, although diode array detectors allowed the selection of several wavelengths of interest. Several authors (*13*, *19*−*21*) have recorded the chromatograms at 486 nm, because this is a more selective wavelength for the meaningful orange juice carotenoids as far as color is concerned (*19*), since the absorption in the red-orange region of the visible spectrum is maximum at this wavelength. In relation to this fact, a great simplification in the chromatograms of Valencia orange juice carotenoids can be observed clearly when a wavelength of 486 nm is selected (*13*). It is also possible to achieve a more accurate determination of β-carotene monitoring at 486 nm, because at this wavelength ζ-carotene, which cannot be separated easily from the former one at other wavelengths, is not detected.

Selection of Mobile Phase. Three different sets of solvent systems were evaluated in order to achieve the separation of the carotenoids of interest. Separation between α- and β-carotene and between α- and β-cryptoxanthin was good in any of the mobile phases used, so we concentrated on separating the most polar xanthophylls.

The first set included solvent systems similar to those reported by several authors and consisted in ternary mixtures of acetonitrile (in the highest proportion), methylene chloride, and methanol (in the lowest proportion) (*9*, *22*, *23*). When these mobile phases were employed, only carotenes and the least polar xanthophylls could be separated. To decrease the strength of the mobile phase and achieve a greater retention, the contents in acetonitrile and methanol were increased while the proportion of methylene chloride was kept around 5%. A better separation was achieved, but the low proportion of methylene chloride involved analyses over 1 h, which was not suitable for a routine

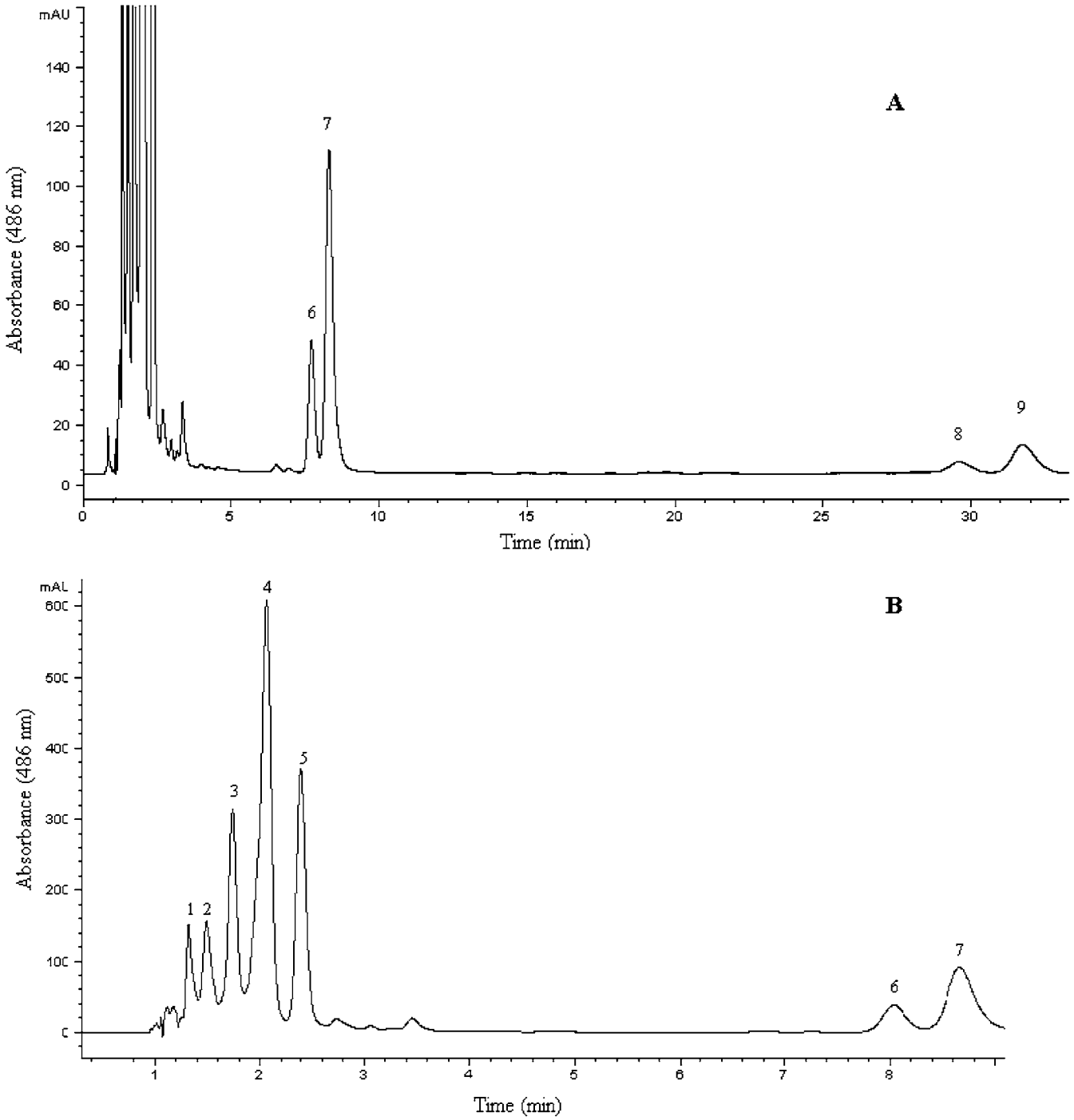

Figure 1. (**A**) Chromatogram of carotenoids in Valencia late ultrafrozen orange juices. (**B**) Detail of the separation of xanthophylls. For peak numbers, see **Table 1**.

method. Quaternary mixtures were also tested in order to improve the separation of early eluting xanthophylls, so an extremely polar solvent such as water was used in the mobile phase. Water has been used in several gradient methods at the beginning of the analysis for this purpose (*7*, *13*, *19*, *21*). No miscibility problems were observed with methylene chloride at low amounts (5%). As a result of this modification, a better separation was achieved. It was also observed that the higher content in methanol, the higher absorption in the mobile phase, so methanol content was adjusted to 50% to achieve a higher absorption of the pigments. Methylene chloride was decreased to 15% to obtain a better resolution between the three earliest eluted peaks. The final composition of the mobile phase was methanol:acetonitrile:methylene chloride:water (50:30:15:5, v/v/v/v). Small amounts of BHT and triethylamine (0.1%) were added in order to avoid degradation of carotenoids during the chromatographic analysis. The analysis time was about half an hour. A chromatogram of carotenoids achieved with this solvent system is shown in **Figure 1**.

Peak Purity and Identification. **Table 1** reports the chromatographic and spectral characteristics obtained by on-line diode array detection for the samples analyzed. It was observed that peaks 3 and 5−9 were pure. The remaining peaks showed spectra, which suggested that more than one single component were present. Chemical structures of the carotenoids determined are shown in **Figure 2**.

Peak 1 was tentatively identified as neoxanthin. Although it was not pure, its retention time fitted in completely with that

Table 1. Chromatographic and Spectroscopic Characteristics of Carotenoid Pigments in the Valencia Late Ultrafrozen Orange Juices ($n = 17$)

peak	RT (min)	%[a]	λ_{max} (nm)[b]	λ_{max} (nm)[c]	carotenoid
1	1.30 ± 0.01	5.73 ± 0.60	416, 438, 468	415, 437.7, 467[d]	neoxanthin
2	1.48 ± 0.02	7.26 ± 0.65	418, 442, 472	414, 442, 472[e]	violaxanthin
3	1.71 ± 0.03	14.33 ± 0.94	426, 450, 476	422, 447, 475[e]	anteraxanthin
4	2.02 ± 0.04	35.65 ± 1.92			lutein + zeaxanthin
5	2.34 ± 0.05	15.69 ± 1.07	420, 444, 472	420, 442, 471[f]	lutein 5,6-epoxide
6	7.70 ± 0.33	4.66 ± 0.77	424, 450, 476	425, 447, 477[e]	α-cryptoxanthin
7	8.29 ± 0.36	11.68 ± 2.45	454, 482[g]	452, 479[e]	β-cryptoxanthin
8	29.37 ± 1.84	1.57 ± 0.69	451, 473	449, 476[e]	α-carotene
9	31.48 ± 1.98	3.40 ± 0.52	456, 482[g]	454, 479[e]	β-carotene

[a] Based on peak area at 486 nm. [b] Maxima observed. [c] Maxima cited in the literature. [d] Ref *13*. [e] Ref *6*. [f] Ref *18*. [g] Spectra identical to those corresponding to standards.

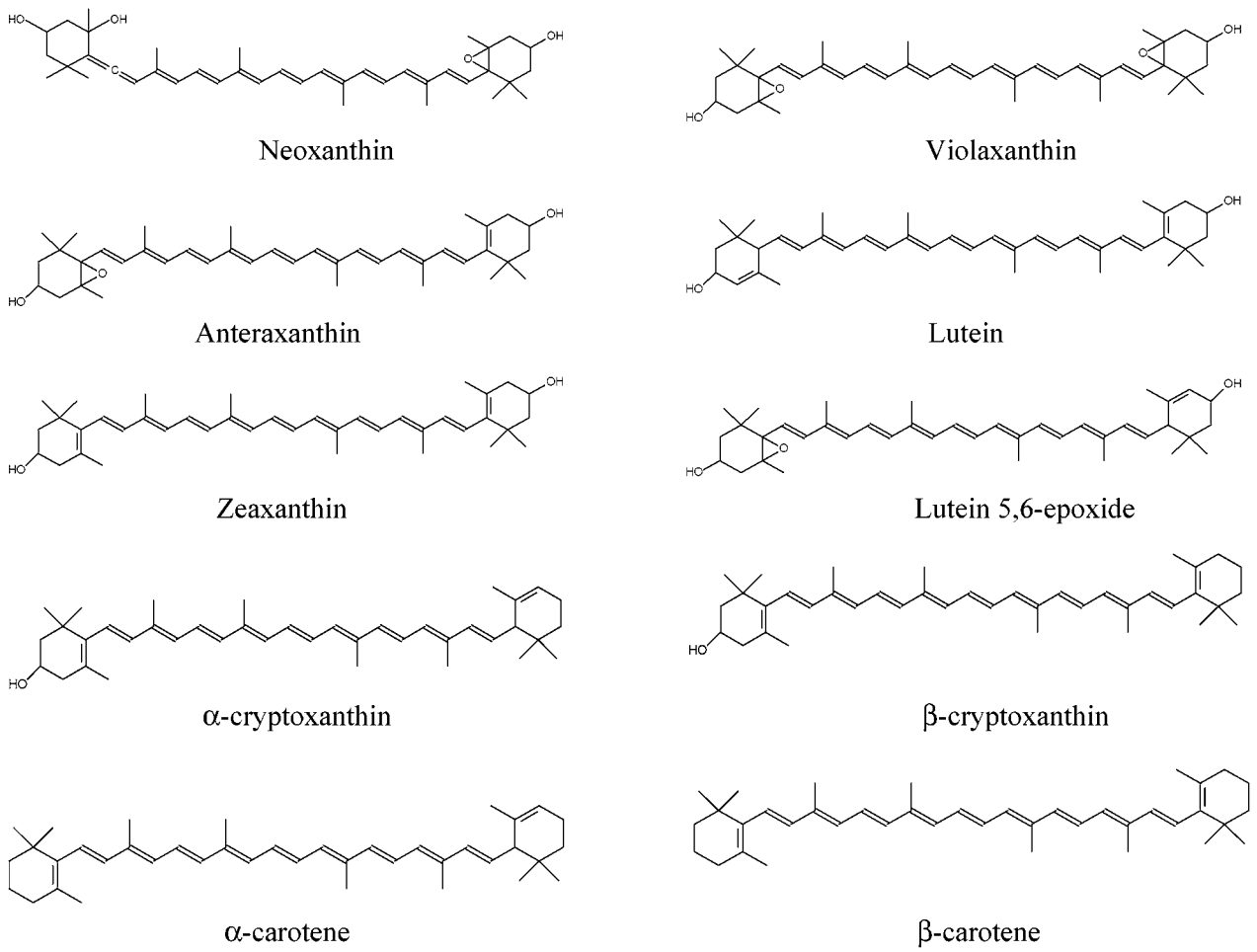

Figure 2. Chemical structures of the carotenoids determined.

of the neoxanthin standard. The chromatographic peak impurity could be due to coelution of the two isomers reported in the literature, neoxanthin a and b (*6*, *7*). Peak 2 was identified as violaxanthin, and its impurity could be explained in the same way, since the presence of three different violaxanthin isomers in Valencia orange juice has been previously reported in the literature (*2*). Peaks 3, 5, 6, and 8 were pure, and their spectral characteristics fitted in with antheraxanthin, lutein 5,6-epoxide, α-cryptoxanthin, and α-carotene, respectively (*6*, *18*).

Peak 4 showed a pronounced shoulder that indicated beforehand its impurity, which was corroborated by the purity analysis. Its spectral characteristics are not shown in **Table 1** because the average spectra lacked a fine structure. Its retention time fitted in with the lutein standard, so it was tentatively identified as a mixture of lutein and zeaxanthin. Both pigments have the same empirical formula, and their structures are almost identical; the only difference between them is the position of a double bond in one of the rings of the molecule. The separation of these xanthophylls by HPLC is extremely difficult so the resolution between them has been used as a measure of the effectiveness of the chromatographic separation (*19*).

The late elution of α-cryptoxanthin regarding the other xanthophylls is due to the presence of only a hydroxy group. Its absorption maximums fitted in almost completely with those corresponding to the lutein standard, so its elution order was also considered for identification purposes. Peak 7 was assigned to β-cryptoxanthin. Identification was carried out by comparison with the characteristics of the standard obtained by TLC. The last peak was assigned to β-carotene by comparison to the standard.

The elution order in reversed-phase HPLC is expected to be according to decreasing polarity: epoxy-polyols > dyols >

Table 2. Summary of the Study of Reproducibility of the HPLC Method ($n = 3$)

carotenoid	RT (min)	CV (%)	area	CV (%)
neoxanthin	1.29 ± 0.01	0.77	725.82 ± 14.37	1.98
violaxanthin	1.47 ± 0.01	0.68	814.03 ± 31.95	3.92
anteraxanthin	1.68 ± 0.01	0.59	1572.27 ± 35.85	2.28
lutein + zeaxanthin	1.98 ± 0.02	1.01	3968.01 ± 113.87	2.87
isolutein	2.28 ± 0.02	0.88	1681.19 ± 41.42	2.46
α-cryptoxanthin	7.32 ± 0.08	1.09	729.93 ± 45.20	6.19
β-cryptoxanthin	7.89 ± 0.09	1.14	2102.54 ± 60.99	2.90
α-carotene	27.70 ± 0.4	1.44	221.59 ± 46.94	21.18
β-carotene	29.74 ± 0.5	1.68	589.05 ± 25.57	4.34

monols > hydrocarbons, although the relative retention behavior is difficult to explain structurally, as it has been pointed out before (*19*). Lutein 5,6-epoxide (dyol with an epoxy group), which is retained longer than the mixture lutein + zeaxanthin (dyols), is a clear example; however, a similar elution behavior has been reported previously (*19*, *21*).

Chromatographic Reproducibility. Retention times showed a good reproducibility (**Table 2**), particularly for the most polar xanthophylls (SD ≤ 0.02). The peak areas reproducibility, except for α-carotene, was also good enough. These results indicate that the method proposed could be considered suitable for routine analysis, since each sample can be analyzed in triplicate without significant losses of pigments.

The stability of the retention times in the course of the whole study was remarkable, as it can be observed in **Table 1**. Standard deviations for the most polar xanthophylls were quite low. Fluctuations in the retention times of α- and β-carotene were higher, as is typical in reversed-phase HPLC for the later eluting peaks.

Linearity of Response and LOD. β-Carotene showed a linear response within the range studied as follows: 0.15−0.45 mg β-carotene/L orange juice ($r = 0.9997$). The LOD was 0.12 mg β-carotene/L orange juice.

Quantitative Composition. In **Table 1**, the relative carotenoid content (expressed as relative percentage of total peak area) of the Valencia late ultrafrozen orange juices analyzed is shown. A previous study (*3*) showed that the major carotenoids in sweet orange juice were violaxanthin, antheraxanthin, lutein, α-cryptoxanthin, β-cryptoxanthin, α-carotene, and β-carotene. All of them were determined in the Valencia late orange juice samples analyzed in this study: diol xanthophylls and diol monoepoxide xanthophylls were the predominant carotenoids; the most abundant were lutein + zeaxanthin (36%), lutein 5,6-epoxide (16%), antheraxanthin (14%), and β-cryptoxanthin (12%). Lutein is the main carotenoid found in dark green vegetables, whereas zeaxanthin is responsible for the color of corn. High contents of lutein in foods are quite desirable due to its preventive effects against certain cancers (*24*). The amount of violaxanthin (7%), which is known to form from epoxidation of zeaxanthin, was lower than previously reported values for Valencia orange juices from Spain (22.4%), Belize (18.6%), Florida (11.1%), and Cuba (16.5%) (*13*).

The hydrocarbons α- and β-carotene occurred in very low proportions, probably due to the biosynthesis of xanthophylls by oxidation and/or epoxidation from them (*12*). α- and β-Carotenes, along with the monol β-cryptoxanthin, have provitamin A activity. The latter one is considered the main source of provitamin A in sweet orange juices. The contents of provitamin A carotenoids in the juices analyzed are summarized in **Table 3**. These contents are in agreement with those reported by Reeder and Park (*8*) in Valencia orange juices, except in the case of β-cryptoxanthin, since these authors did not distinguish between the isomers α and β.

Table 3. Levels of Provitamin A Carotenoids in Valencia Late Ultrafrozen Orange Juices ($n = 17$)

carotenoid	average content (mg/L)	range
β-cryptoxanthin	0.69 ± 0.27	0.33–1.21
α-carotene	0.11 ± 0.05	nd–0.23
β-carotene	0.21 ± 0.07	0.11–0.36

However, provitamin A carotenoid contents were significantly higher than those reported by Stewart (*16*), in Florida frozen concentrated orange juice (FCOJ) diluted to single strength, and by Pupin et al. (*10*), in both hand-squeezed Valencia orange juices (0.02, 0.05, and 0.04 mg/L, for β-cryptoxanthin, α-carotene, and β-carotene, respectively) and FCOJ (0.07, 0.02, and 0.04 mg/L, for β-cryptoxanthin, α-carotene, and β-carotene, respectively) from Brazil.

Carotenoid content in oranges depends on biochemical and climatic factors, such as maturity at harvest and temperature during the growth, among others. It has also been suggested that carotenoid patterns in orange juices are more related to the fruit origin than to the variety (*25*). For instance, the levels of provitamin A carotenoids determined in this study as compared to those reported in the bibliography in Valencia juices from different geographic origins (*13*) showed important differences. The contents of β-cryptoxanthin, α-carotene, and β-carotene in the ultrafrozen orange juices analyzed (0.69, 0.11, and 0.21 mg/L, respectively) were quite lower than those reported in Valencia orange juices from Mediterranean areas (2.03, 0.19, and 0.33 mg/L in juices from Spain and 1.75, 0.25, and 0.38 mg/L in juices from Israel, for β-cryptoxanthin, α-carotene, and β-carotene, respectively). However, the contents of α- and β-carotene (0.11 and 0.21 mg/L, respectively) were similar to those reported in Valencia juices from Florida (0.14 and 0.22 mg/L, respectively). On the other hand, the levels of β-cryptoxanthin in the ultrafrozen orange juices analyzed (0.69 mg/L) were similar to those corresponding to Valencia orange juices from Cuba (0.74 mg/L), although the levels of α- and β-carotene in these last ones were lower (0.08 and 0.14 mg/L, respectively) in comparison with the ultrafrozen orange juices analyzed in this paper.

CONCLUSIONS

The isocratic HPLC method proposed in this study has shown to be suitable for routine analysis of the main carotenoids related to the color of orange juice, including the provitamin A carotenoids β-cryptoxanthin, α-carotene, and β-carotene. Detection at 486 nm allows the determination of those carotenoids more related to the color of orange juice, since at this wavelength the absorption in the red-orange region of the visible spectra is maximum. Whereas gradient methods for the separation of carotenoids last over 1 h, the method proposed in this paper allows one to analyze the carotenoids of interest within half an hour, so analyses in triplicate can be carried out without significant losses of pigments. It is a rapid and accurate method suitable for quality control purposes in the industry. Although analysis time in the method proposed in this paper is a bit longer (about half an hour) in comparison to other isocratic methods (about 20 min), eight carotenoids plus a mixture of two more can be determined.

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