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Identification and Quantification of Carotenoids Including Geometrical Isomers in Fruit and Vegetable Juices by Liquid Chromatography with Ultraviolet−Diode Array Detection

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A method was established for the identification and quantification of carotenoids including geometrical isomers in fruit and vegetable juices by liquid chromatography with an ultraviolet−diode array detector, using a C_{18} Vydac 201TP54 column. The mobile phase used was the ternary methanol mixture (0.1 M ammonium acetate), *tert*-butyl methyl ether and water, in a concentration gradient, and a temperature gradient was applied. Retinol palmitate was added as an internal standard. An extraction process (ethanol/hexane, 4:3, v/v) was performed, followed by saponification with diethyl ether/methanolic KOH (0.1%, w/v, BHT) (1:1, v/v) for 0.5 h at room temperature. Seventeen different (*cis* and *trans*) carotenoids were identified by UV−vis spectra and retention times in HPLC in the juices analyzed. The analytic parameters show that the method proposed is sensitive, reliable, accurate, and reproducible.

KEYWORDS: Carrot juice; *cis*−*trans* carotenoids; citric juice; *Citrus sinensis*; food analysis; liquid chromatography

INTRODUCTION

Adapting to new trends and consumer demands has been one of the primary objectives of orange juice producers, so that for some years these manufacturers have been producing juices with mild pasteurization, marketed in refrigerated conditions and with limited shelf life. New products are being produced in this line with juice mixtures that provide increased quality (nutritive value, color, etc.), this being the factor that contributes most to consumer acceptance and an increase in the value added to the product.

Citrus is a complex source of carotenoids, with the largest number of carotenoids found in any fruit (*1*). About 700 kinds of carotenoids have been isolated in nature (*2*). The carotenoids present in citrus are a complex mixture of >115 natural substances (*3*). Various carotenoids, including α-carotene, β-carotene, and β-cryptoxanthin (**Figure 1**), have provitamin A activity, being transformed into retinal by mammals. The xanthophylls (oxocarotenoids) lutein and zeaxanthin (**Figure 1**) are also known to provide protection against macular degeneration connected with age, through their ability to capture free oxygen and blue light in the retina (*4*). Carotenoids have a range of important and well-documented biological activities. They are potent antioxidants and free radical scavengers (*5*), and they modulate the pathogenesis of cancers (*6*, *7*) and coronary heart disease (*8*). The application of various industrial treatments can lead to the formation of cis isomers, which do not have the same vitamin activity as the all-trans isomers. This is vital for the accurate determination of the dietary intake of these micronutrients and in the development of comprehensive food tables (*9*, *10*).

Health professionals consider the consumption of fruit and vegetables to be of great importance as a means of protection against illnesses such as cancer and coronary heart disease, partially because of their high content of antioxidant vitamins, such as vitamins C and E, phenolic compounds, and carotenoids (*10*). Vitamin A is an essential nutrient for man and all mammalian species because it cannot be synthesized within the body. Citric fruits such as oranges contain a certain carotenoid number, but not all of them are precursors of vitamin A. However, carotenoids are important in the prevention of certain degenerative diseases including coronary heart disease and cancers.

Various procedures have been described for the determination of carotenoids. High-performance liquid chromatography (HPLC) is considered to be the method of choice for the separation, identification, and quantification of carotenoids found in biological tissues (*11*−*14*). On the other hand, determination of the complete carotenoid composition is complicated, costly, and time-consuming (*15*). The best separation of carotenoids is achieved with C_{18} columns (*11*, *14*, *16*−*20*). The reasons for choosing the C_{18} package include compatibility with many solvents, usability for the complete polarity range of carotenoids, and wide commercial availability. The good separation of geometrical isomers of carotenoids by the polymeric phases of C_{18} is evident (*21*, *22*). Many authors have used a 5-μm

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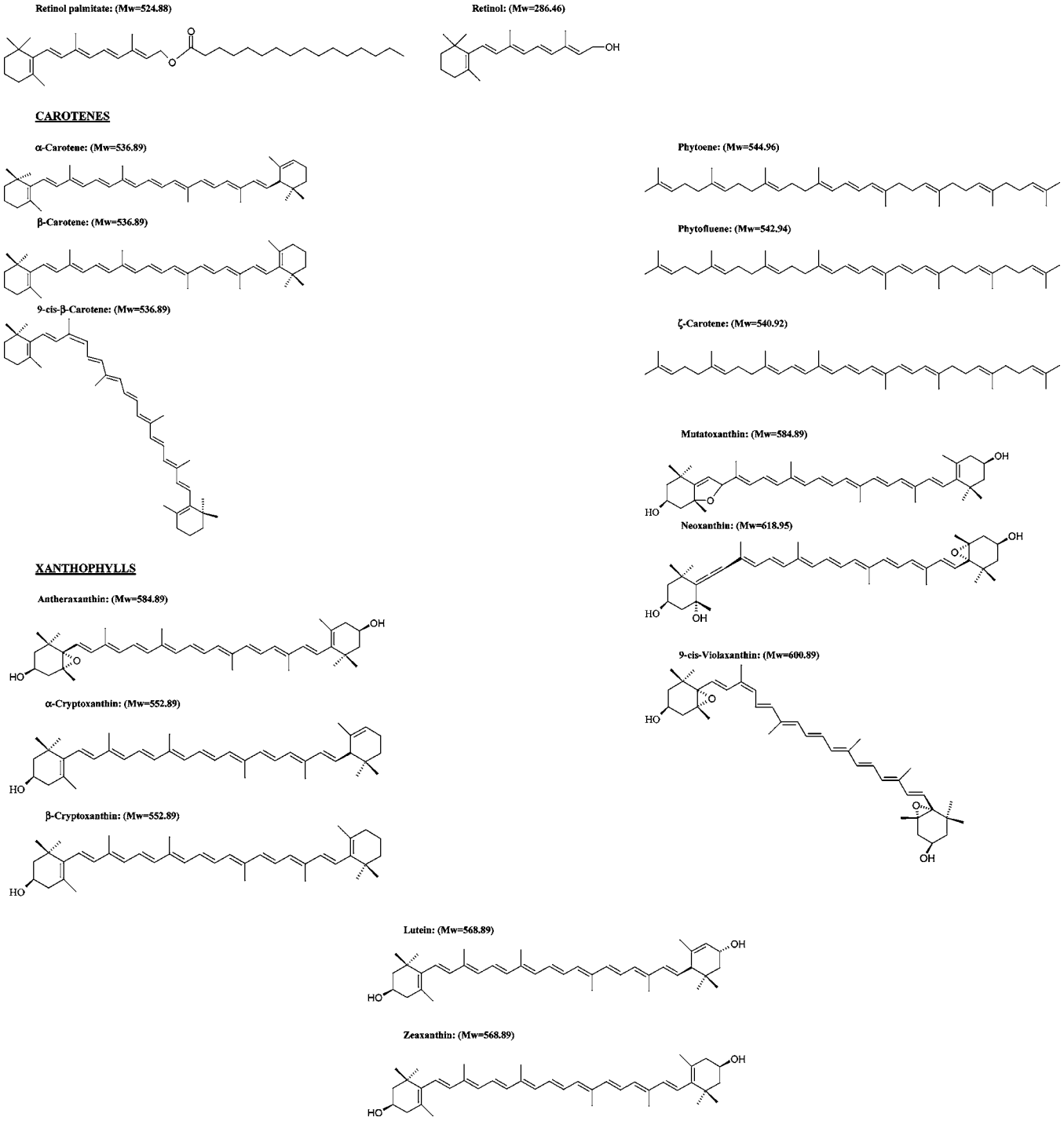

Figure 1. Structures of internal standard and carotenoids.

polymeric C_{18} column, a Vydac 201TP54 (*23*−*29*). Historically, the efficiency of the separation has been improved by using smaller particle sizes, changing the shape of the particles from irregular to spherical, ensuring uniform particle size, and making the columns more reproducible. The advantages of a small particle size include high efficiency and low consumption of solvents because shorter columns are used. The disadvantage is the decrease in column life as a result of obstruction and the use of high pressure. This disadvantage can be reduced by filtering the samples and by using precolumns (*21*, *25*, *29*).

Diode array detectors (DAD) were used commonly for the identification of carotenoids in citrus and vegetables due to the specific spectral data of many of these pigments (*1*, *15*, *18*, *30*).

In the selection of the mobile phase gradient, it is necessary to take into account the fact that the main solvent should be slightly organic and have low viscosity; it should also allow suitable solubility of carotenoids and permit the work to be performed in low-pressure conditions. These criteria limit the choice to acetonitrile and methanol. There have been reports that in most columns solvents based on methanol provide greater recovery of carotenoids than those based on acetonitrile, and they also have a lower cost and toxicity (*31*, *32*).

Carotenoids are fat soluble but because of the high moisture content of plant tissues, a preliminary extraction solvent miscible with water is generally necessary to allow for penetration of the solvent. Methanol is often used as an initial extractant (*2*).

Table 1. Extinction Coefficients of Various Standards

compound	solvent	λ (nm)	$E^{1\%}$
lutein	ethanol	445	2550
zeaxanthin	ethanol	452	2480
β-carotene	ethanol	453	2620
retinol palmitate	ethanol	328	975

Water-immiscible solvents can be used after the sample is dehydrated; however, it has been found that more efficient extractants are composed of a slightly polar solvent in addition to the nonpolar solvent (*33*).

Saponification is used to evaluate the presence of carotenoid esters, because the saponification of esterified carotenoids with fatty acids gives rise to their hydroxycarotenoid derivatives, considerably simplifying the chromatogram profile (*20*). Saponification causes a clear transformation of the carotenoid profile, with considerable loss of carotenoids, especially xanthophylls (*19*). Saponification of carotenoid extracts may be necessary to remove neutral fats, chlorophylls, and chlorophyll derivatives that interfere in the spectrophotometric assay of carotenoids and in the release of esterified xanthophylls (*2*, *34*).

Various compounds are normally used as internal standard: β-apo-8′-carotenal (trans) (*35*, *36*), Sudan I (*20*, *29*), cantha-xanthin (*37*, *38*), echinenone (*39*−*41*), nonapreno-β-carotene (*42*), or *all-trans*-retinol palmitate (*43*−*45*).

In this work we established and validated a method for the determination of the various (trans and cis) carotenoids in a fresh orange−carrot juice mixture and in fresh orange juice in order to evaluate their nutritive qualities.

MATERIALS AND METHODS

Reagents. β-Carotene, *all-trans*-retinol palmitate, and *tert*-butyl hydroxytoluene (BHT) (special grade) were purchased from Sigma (Steinheim, Germany). Lutein and zeaxanthin were provided free as standard substances by Roche (Basel, Switzerland). Ammonium acetate (HPLC grade), petroleum ether, hexane (HPLC grade), potassium hydroxide (85%), and *tert*-butyl methyl ether (TBME) (HPLC grade) were purchased from Scharlau (Barcelona, Spain); acetonitrile (special grade) and magnesium hydroxide carbonate (40−45%) from Panreac (Barcelona, Spain); and ethanol, diethyl ether, methanol, and sodium chloride (special grade) from Baker (Deventer, The Netherlands). Chloroform was obtained from Merck (Darmstadt, Germany).

Samples. Natural orange juice (produced from *Citrus sinensis*) and an orange−carrot juice mixture (80:20, v/v) were provided by the manufacturer involved in the project. The juices were packaged aseptically and frozen at −40 °C until the time for their analysis. We verified that their vitamin profiles did not change during the storage period.

Preparation of Stock Standard Solutions. To prepare the stock standard solution, 12.5 mg of β-carotene, 4 mg of lutein, 5.2 mg of zeaxanthin, and 35 mg of retinol palmitate were weighed and dissolved in 25 mL of chloroform with 0.1% BHT (w/v). They were stored in sealed amber vials under N_2, at the lowest temperature possible (< −20 °C), for use over an extended period.

The precise concentration of each of the standards was checked periodically by its extinction coefficient. To do this, 100 μL of the stock standard solution of β-carotene, lutein, zeaxanthin, and retinol palmitate was taken and evaporated with N_2, and in each case the result was redissolved with 10 mL of the appropriate solvent (see **Table 1**) for subsequent spectrophotometric measurement after filtration of the solution (*40*). The concentration of each standard was calculated by applying eq 1

$$C\ (\mu\text{g/mL}) = A \times 10^6/E^{1\%} \quad (1)$$

where C is concentration, A is absorbance, and $E^{1\%}$ is the extinction coefficient.

The *working solution* was prepared each day from the stock solution of each of the standards. To do so, we took 25, 150, 90, and 100 μL of β-carotene, lutein, zeaxanthin, and retinol palmitate, respectively, evaporated them with N_2, and diluted them with 1 mL of methanol/TBME (70:30, v/v). The concentration of each solution was checked by its extinction coefficient.

Instrumentation. The LC system consisted of a series 1050 chromatograph with a quaternary pump system, a diode array detector (Hewlett-Packard, 1100 series), a column thermostat (Agilent 1100 series), an on-line degassing system, and a ChemStation (series A.06.03) data system (Hewlett-Packard, Waldbronn, Germany).

A Vydac 201TP54 column with a particle size of 5 μm, 250 × 4.6 mm, reverse phase C_{18} and a precolumn (guard column) Vydac 201TP (4.6 mm i.d. cartridge with 5-μm particles) (Hesperia, CA) were used.

RESULTS AND DISCUSSION

Method Development and Evaluation. A number of factors that affect the chromatographic responses of carotenoids and contribute to analytical variations and inaccuracies in their quantitative determination are discussed and solutions provided. The following gives the result of our method development and assesses the "robustness" of the method.

Selection of the Internal Standard. The incorporation of an internal standard is highly recommended because for the quantitative measurement of carotenoids it is essential to take into account losses due to incomplete recovery, evaporation, and variability in the injection. A suitable internal standard should not be present in the sample to be determined, but it should have a similar structure and, therefore, behave similarly to the compound that it is desired to extract. Also, it should not appear at a retention time that could interfere with other components present in the sample.

Retinol palmitate was selected as the internal standard (**Figure 1**), and it also served to indicate whether saponification was complete, because it is transformed into retinol (**Figure 1**), which appears at much lower retention times (*43*). **Figure 2** shows the chromatogram before and after saponification of the internal standard, retinol palmitate (λ = 350.8 nm).

Extraction and Saponification of Carotenoids. When we applied the extraction and subsequent saponification proposed by Rouseff et al. (*1*), we observed the separation of distinct chromatographic peaks, with a clear identification of β-carotene, although there was baseline drift. To avoid precipitation with Carrez reagents and the use of solvents (petroleum ether and acetone) with oxidant power, we applied and modified the extraction method proposed by Taungbodhitham et al. (*46*). We increased the sample size to 25 g of juice and added BHT (0.1%, w/v), 100 μL of internal standard, 0.05 g of magnesium hydroxycarbonate, and 35 mL of ethanol/hexane (4:3, v/v). We studied several agitation times (5, 15 and 30 min), obtaining the best results with a time of 15 min (under N_2 and protected from the light), and filtered in a low-pressure. The residue was washed with 35 mL of ethanol/hexane (4:3, v/v) and filtered. The residue was washed twice with 12.5 mL of ethanol and finally with 12.5 mL of hexane (until it was colorless). All of the liquid filtrates were combined and washed twice with 50 mL of 10% NaCl in an amber decanting funnel and then with 50 mL of water (three times). The organic phase was evaporated at 40 °C in a rotary evaporator (Eyela NE-1). We saponified the residue obtained and varied the volumes of diethyl ether (5, 10, and 15 mL) and methanolic KOH (5, 10, and 15 mL) added for the saponification, because completion of saponification varied with the kind of juice (orange−carrot, plain orange, etc.), and finally we added 10 mL of diethyl ether and 10 mL of (0.5 M) methanolic KOH with 0.1% BHT (w/v) and left it

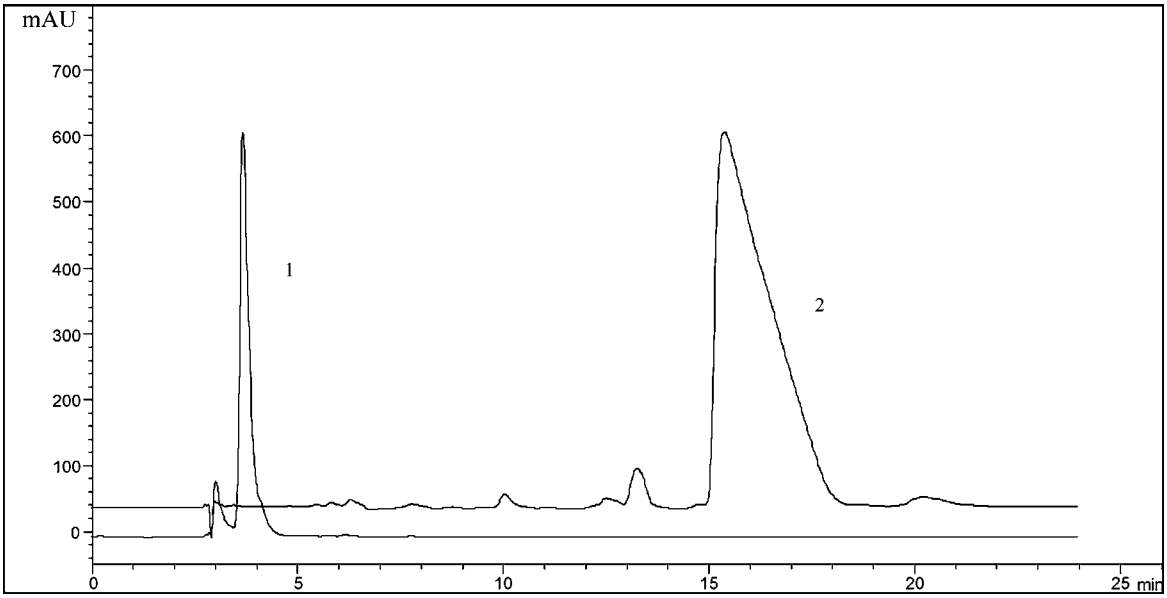

Figure 2. Chromatogram before (**2**) and after (**1**) saponification of the internal standard, retinol palmitate (λ = 350.8 nm).

overnight at room temperature protected from the light. To avoid possible alterations (oxidations) and reduce the time for saponification, we studied various saponification times: 0.5, 1, 3, and 24 h. We verified that saponification was complete after 30 min, and so this was the time selected. We performed the saponification in an inert atmosphere (N_2).

At the end of this time we added 20 mL of diethyl ether and extracted twice with 50 mL of 10% NaCl (w/v). The ether phase was washed three times with 50 mL of H_2O until a neutral pH was obtained. It was filtered in the presence of anhydrous Na_2SO_4, but we observed that in some of the samples there were remains of water in the ether phase. Therefore, to ensure that the water was totally eliminated, we added 10 mL of absolute ethanol (after testing various volumes: 2, 5, and 10 mL), and in some samples it was necessary to add a further 5 mL to achieve dryness, evaporating at 45 °C. The residue was dissolved with 4 mL of diethyl ether and placed in an amber glass flask, the solvent was evaporated under N_2, and the residue was stored at −20 °C until time for chromatographic determination (we verified that the results remained invariable up to 4 days after extraction). **Figure 3** shows a diagram of the final extraction process.

The carotenoid extract was reconstituted with 1 mL of MeOH/TBME (70:30, v/v) at the moment of injection.

Some authors employ 2, 3, 5, and 6 h (refs *27*, *42*, *47*, and *30* respectively) to carry out the saponification process in the dark at room temperature. Other authors employ >12 h (*1*, *22*, *24*, *37*, *38*, *49*−*51*). We have checked that 30 min was adequate to carry out the saponification, the same time used by Wingerath et al. (*52*).

Mobile Phase. Carotenoids may undergo losses or degradation on the column. Studies have indicated that compounds which act as modifiers improve the recovery of carotenoids from the column and reduce or eliminate on-column degradation. Ammonium acetate has also been reported to improve column recovery. Addition of ammonium acetate to MeOH used in the mobile phase increased the recovery from all columns (*14*, *32*). First, methanol was chosen, and ammonium acetate was added to it in various concentrations of 0.05, 0.1, and 0.2 M. The best results were obtained with a concentration of 0.1 M, as there was an increasing improvement in the separation of the different chromatographic peaks and at the same time a better recovery of them.

TBME encourages elution of the more apolar compounds, and therefore it was introduced in a small proportion in the first minutes and gradually increased to 25% in the final minutes of the chromatogram. We observed that in the first minutes the chromatographic peaks did not resolve correctly, and therefore we decided to add a small percentage of H_2O during the first 10 min, taking care not to have water and TBME at the same time (to avoid formation of small bubbles). In the end we selected the introduction of 5% water with 95% methanol during the first 3 min, after which the water was eliminated and we eluted with methanol. TBME was then introduced and gradually increased to 25% at 15 min, after which it was decreased so as to have 100% methanol at the end of the chromatogram. **Table 2** shows the mobile phase gradient selected.

The influence of ambient temperature on the elution of carotenoids was standardized by selecting a temperature gradient (0 min, 20 °C; 6 min, 30 °C; 22 min, 20 °C). Some authors use column temperatures from 20 to 40 °C (*10*, *29*, *38*, *39*, *41*, *50*, *51*), but above 40 °C, the separation of α-carotene and β-carotene is poor, where a curvature around 40°C is caused by a phase change of polymeric ODS from solid-like state to liquid-like state (*22*).

Figures 4 and **5** show the chromatograms obtained by applying the method established for the separation of carotenoids in orange−carrot juice mixtures and orange juices. The most common retention times and wavelengths are shown in **Table 3**.

Identification and Quantification of Carotenoids. Basically because of the lack of commercial standards of carotenoids, especially of cis isomers, we used various identification techniques employed by many authors, including the following:

The UV−vis spectra and the maximum λ values of the carotenoids were compared, which indirectly indicates the greater or lesser conjugation between their bonds, because the greater the conjugation, the higher the maximum λ values are.

The polarity of the carotenoids was examined because in reverse phase liquid chromatography the retention times follow the order from greater to lesser polarity.

Various spectral fine structural values were determined, including the following:

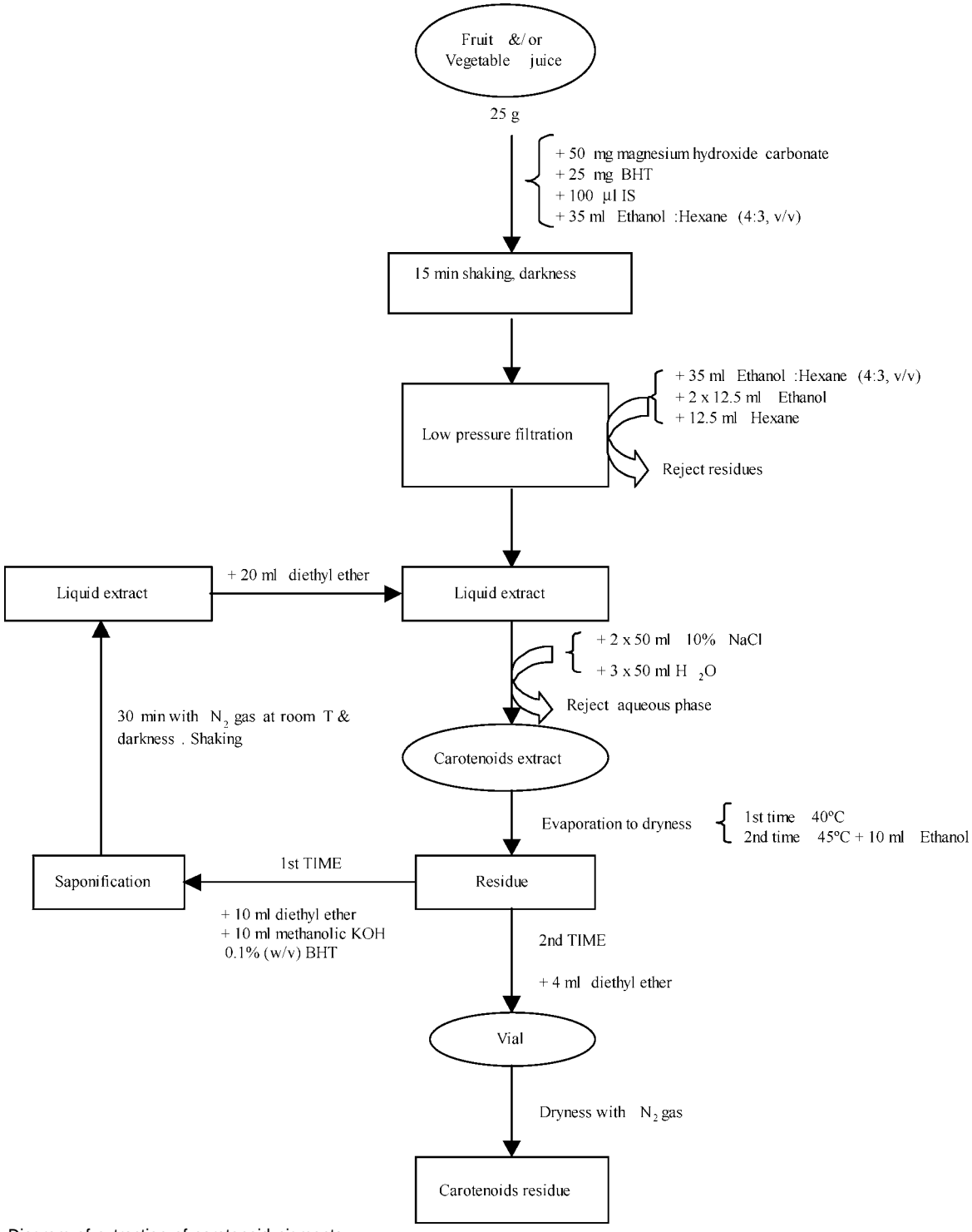

Figure 3. Diagram of extraction of carotenoid pigments.

%III/II is the percentage of the quotient between band III and band II (normally λ_{max}), taking the trough between the two bands as the baseline. It is used to identify carotenoids (*49*−*51*, *53*, *54*).

$\%D_B/D_{II}$ is the percentage of the quotient between the cis peak band and band II (normally λ_{max}). It is used to identify the different cis isomers of carotenoids (*1*, *31*, *32*, *40*, *43*, *46*).

The Q ratio = D_{IImax}/D_B is the quotient between band II (normally λ_{max}) and the cis peak band. This is also used to identify the different cis isomers of carotenoids (*23*−*25*, *47*, *55*).

With all of these techniques, it was possible to identify the most characteristic carotenoids in orange and orange−carrot juices.

Table 2. Mobile Phase Gradient for Determination of Carotenoids by HPLC

time (min)	MeOH + AA[a] (%)	H_2O (%)	TBME (%)
0	95	5	0
3	100	0	0
5	95	0	5
10	86	0	14
15	75	0	25
22	95	0	5
23	100	0	0

[a] AA, 0.1 M ammonium acetate.

Lutein and zeaxanthin (peaks 6 and 7, respectively) are

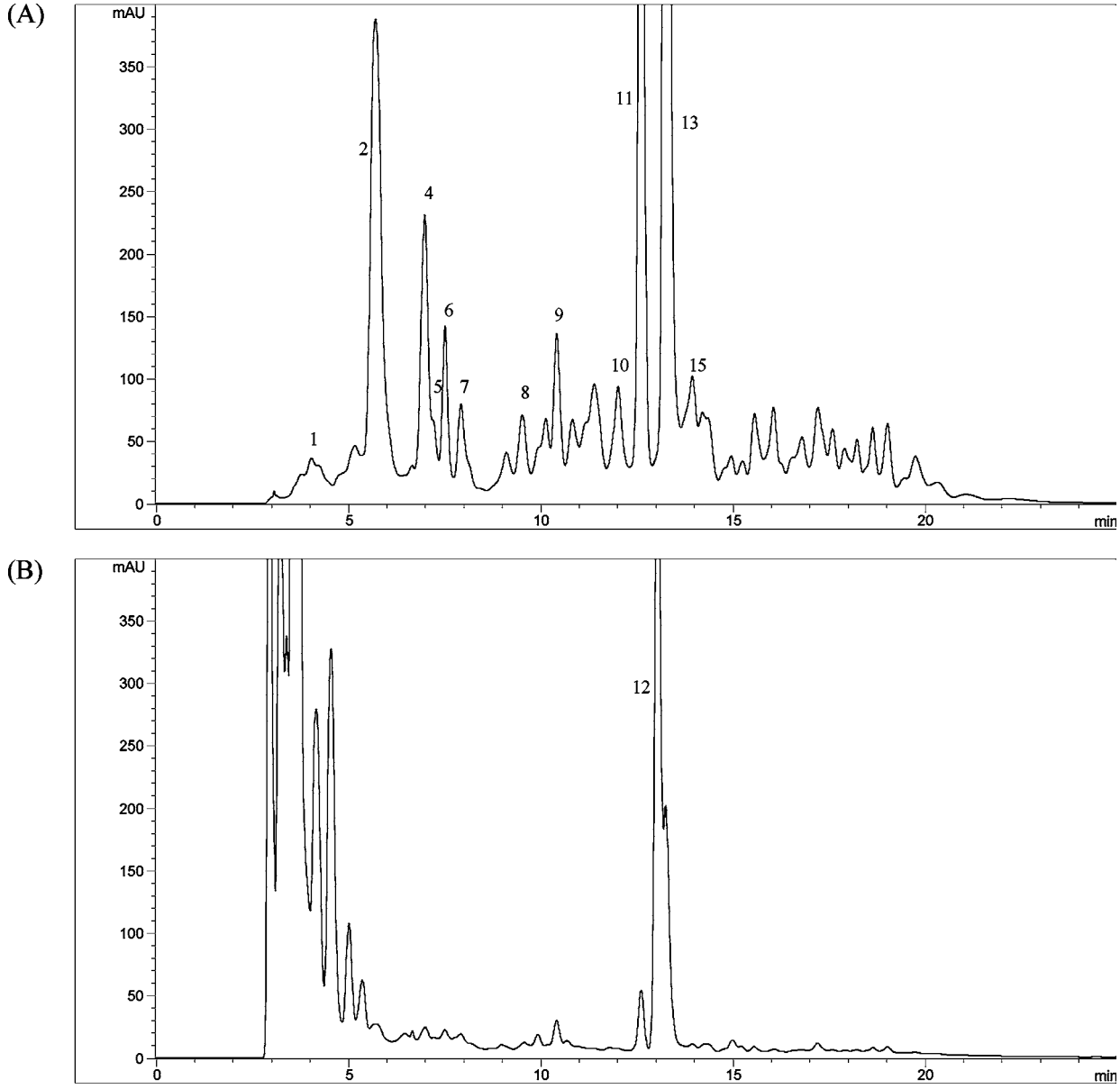

Figure 4. Chromatogram of orange–carrot juice mixture at $\lambda = 450$ nm (**A**) and $\lambda = 290$ nm (**B**).

usually very difficult to resolve as they differ only in the position of a single bond in one of the terminal rings (**Figure 1**), and some authors cannot resolve them (*35*, *38*), but they are completely resolved with this chromatographic system. However, 9-*cis*-violaxanthin is not completely resolved from neoxanthin (peak 2). The coelution of these peaks is due to the similar structures (**Figure 1**) and therefore similar spectral characteristics (**Table 3**). The chromatographic purity of this incompletely resolved carotenoid pair is shown in **Figure 6**.

Table 4 shows the chromatographic and spectral characteristics of carotenoids obtained in orange juices. Comparative chromatographic and spectral characteristics of carotenoids in orange juice obtained from the literature are shown in **Table 5**.

For the quantification of the various carotenoids identified in the samples studied, we quantified the different carotenoids in accordance with their similarity in terms of chemical−structural behavior and polarity with respect to the validated standards for which we had a calibration curve: lutein, zeaxanthin, and β-carotene. Accordingly, for the quantification we proceeded as follows:

The chromatogram was separated into three parts; all of the carotenoids up to and including lutein were quantified as such, and the remaining xanthophylls were quantified as zeaxanthin. The carotenes were quantified as β-carotene.

Each carotenoid was quantified in accordance with whether its maximum λ was close to 290, 350, 430, or 450 nm. The content of each carotenoid in micrograms per 100 g of sample was determined by applying eq 2:

$$(A_{\text{sample}}/A_{\text{IS}}) \times \mu\text{g of standard} \times 100/(A_{\text{standard}}/A_{\text{IS}}) \times \text{g of sample} \quad (2)$$

(IS is internal standard).

Analytical Parameters. To check the reliability and usefulness of the proposed method, the analytical parameters were determined for the carotenoids for which commercial standards were available: lutein, zeaxanthin, and β-carotene.

The responses were linear in the following ranges and equations: 2.02−56.55 μg/mL ($n = 7$) with an equation $y = 0.022x + 0.0425$ ($r = 0.991$) for lutein, 2.56−51.29 μg/mL ($n = 7$) with an equation $y = 0.026x - 3 \times 10^{-5}$ ($r = 0.992$) for

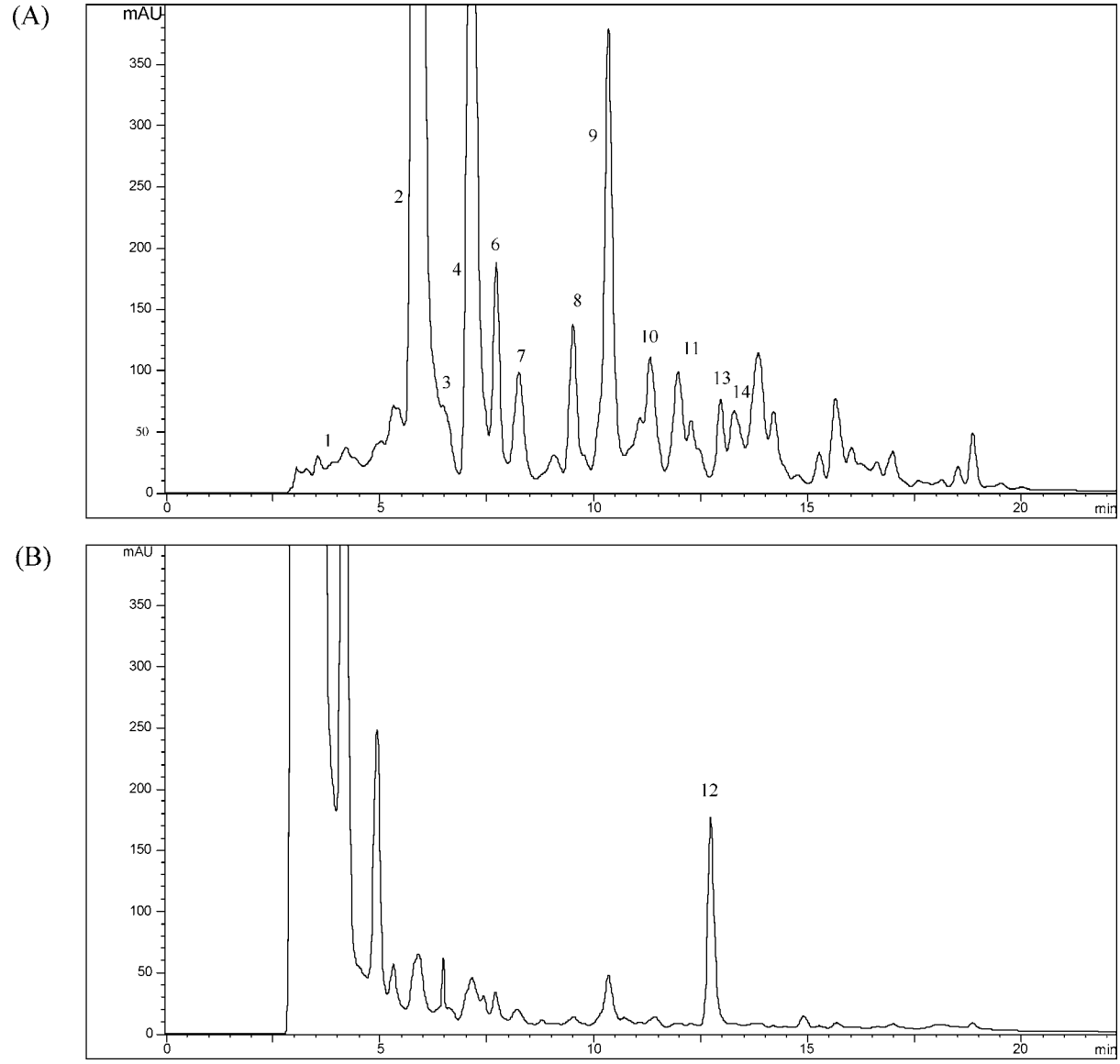

Figure 5. Chromatogram of orange juice at $\lambda = 450$ nm (**A**) and $\lambda = 290$ nm (**B**).

Table 3. Wavelengths and Retention Times of Orange–Carrot Juice Mixture and Orange Juice

			t_R[a] (min) ± SD	
peak	carotenoid	λ (nm)	orange–carrot	orange
1	valenciaxanthin	430	3.78 ± 0.01	3.86 ± 0.03
2	9-*cis*-violaxanthin + neoxanthin	430	5.75 ± 0.04	5.88 ± 0.06
3	*cis*-antheraxanthin	430	ND[b]	6.46 ± 0.04
4	antheraxanthin	450	7.01 ± 0.03	7.13 ± 0.05
5	mutatoxanthin	430	7.20 ± 0.01	ND
6	lutein	450	7.54 ± 0.02	7.71 ± 0.05
7	zeaxanthin	450	7.96 ± 0.04	8.23 ± 0.08
8	α-cryptoxanthin	450	9.53 ± 0.03	9.61 ± 0.06
9	β-cryptoxanthin	450	10.45 ± 0.04	10.49 ± 0.05
10	9-*cis*-α-carotene	430	12.06 ± 0.04	12.12 ± 0.05
11	α-carotene	450	12.63 ± 0.03	12.89 ± 0.03
12	phytoene + phytofluene	290	13.06 ± 0.02	13.44 ± 0.03
13	β-carotene	450	13.28 ± 0.03	13.57 ± 0.03
14	9-*cis*-β-carotene	450	ND	13.77 ± 0.04
15	ζ-carotene	430	14.28 ± 0.04	ND

[a] t_R, retention time. [b] ND, not detected.

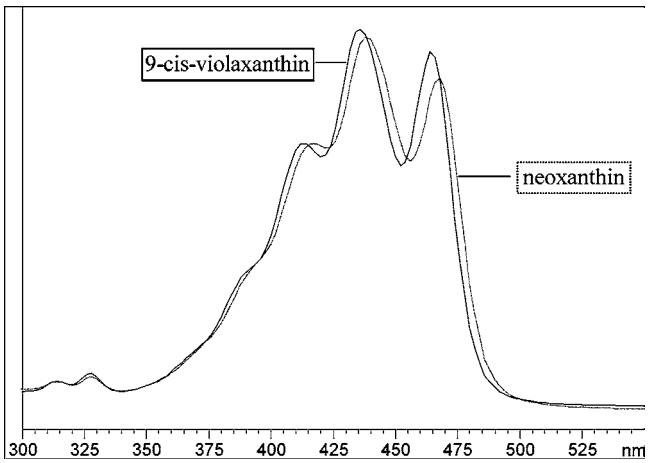

Figure 6. Absorbance spectra for 9-*cis*-violaxanthin and neoxanthin (peak 2) ($\lambda = 430$ nm).

zeaxanthin, and 5.54−1776.15 μg/mL ($n = 7$) with an equation $y = 0.0034x + 0.7654$ ($r = 0.989$) for β-carotene. The concentration of carotenoids in all samples is included in the studied concentration interval.

The limit of detection was calculated by preparing five reagent standard solutions and applying the quotient between 3 times the standard deviation and the slope of the calibration curve

Table 4. Chromatographic and Spectral Characteristics of Carotenoids in Orange Juice

peak	carotenoids	λ_{cis}	λ_1	λ_2	λ_3	Q ratio	%III/II
1	valenciaxanthin		372	392	414	1.49	51.4
2	9-*cis*-violaxanthin	328	414	436	464	11.03	95.1
2	neoxanthin		414	436	464	11.00	79.7
3	*cis*-antheraxanthin	330	(418)	438	464	2.14	22.2
4	antheraxanthin		420	442	468	10.50	52.9
5	mutatoxanthin		(404)	428	452	11.47	55.7
6	lutein		(424)	444	470	5.90	54.6
7	zeaxanthin		(422)	444	472	10.08	31.0
8	α-cryptoxanthin		(416)	438	466	7.06	59.5
9	β-cryptoxanthin		(430)	450	478	26.20	25.2
10	9-*cis*-α-carotene	328	414	436	464	6.40	76.6
11	α-carotene		424	446	474	27.07	55.6
12	phytoene		(276)	286	(298)		9.9
12	phytofluene		332	348	368		89
13	β-carotene		(424)	446	472	12.03	26.3
14	9-*cis*-β-carotene	330	(422)	442	470	8.85	26.5
15	ζ-carotene		380	400	426	14.70	96.2

Table 5. Chromatographic and Spectral Characteristics Obtained from the Literature of Carotenoids in Orange Juice

carotenoid	λ_{cis}	λ_1	λ_2	λ_3	Q ratio	%III/II	refs
valenciaxanthin		351	369	389			*1*
9-*cis*-violaxanthin	328	414	438	466		95.1–98	*42*; *53*; *51*; *56*
neoxanthin		415	437	465		80–85	*42*; *53*; *57*
cis-antheraxanthin		416	438	464			*38*
antheraxanthin		421	443	471		52.4–60	*32*; *42*; *53*
mutatoxanthin		406	427	451		40.9–50	*1*; *42*; *53*
lutein		(424)	444	470	11.5	50–60	*25*; *42*; *53*
zeaxanthin		424	449	477		26–31.2	*38*; *42*; *53*
α-cryptoxanthin		421	443	471		60	*38*; *53*
β-cryptoxanthin		429	450	477		27–28.8	*1*; *42*; *53*
9-*cis*-α-carotene		421	442	468	8.3		*25*
α-carotene		424	445	473		55	*1*; *53*
phytoene		277	287	298		10	*42*; *58*
phytofluene		333	347	367		87–98	*1*; *42*
β-carotene		(423)	444	470	12.7	25–26	*42*; *53*; *59*
9-*cis*-β-carotene	342	(421)	444	471	8.7		*24*
ζ-carotene		379	399	425		85.9–95	*1*; *42*

(LOD = $3 \times S_{n-1}/m$). The detection limits were 0.0196, 0.0166, and 0.1404 μg/mL for lutein, zeaxanthin, and β-carotene, respectively. The limit of quantitation corresponds to the minimum quantity with which it is possible to quantify without uncertainty (LOQ = $10 \times S_{n-1}/m$). The limits of quantitation were 0.0652, 0.0552, and 0.4680 μg/mL for lutein, zeaxanthin, and β-carotene, respectively. To calculate the LOD and LOQ parameters, we used the slope of the external calibration curve and the standard deviation based on the maximum peaks of the reagents standard solutions at 450 nm.

The detection and quantification limits described above show that the method is very sensitive for the carotenoids studied.

Accuracy was estimated by means of recovery assays. A sample (16.69, 14.14, and 17.02 μg for lutein, zeaxanthin, and β-carotene, respectively) to which known amounts of standard lutein, zeaxanthin, and β-carotene (38.30, 30.32, and 12.37 μg, respectively) had been added was subjected to the entire extraction and determination process. The recovery percentages obtained were 94.0, 89.2, and 92.8 for lutein, zeaxanthin, and β-carotene, respectively.

Instrumental precision was checked from six consecutive injections of a standard solution, and the relative standard deviations (RSDs) obtained were 0.55, 0.34, and 5.33% for lutein, zeaxanthin, and β-carotene, respectively. When the standard solution was prepared and measured on alternate days, the RSD values were 0.57, 0.51, 5.67% for lutein, zeaxanthin, and β-carotene, respectively. Instrumental precision was also checked from six consecutive injections of a sample extract (**Table 6**). The precision of the method was determined by preparing six aliquots of the same sample of orange juice and six aliquots of the same sample of orange−carrot juice. In both cases, precision was expressed as a coefficient of variation. The results obtained are shown in **Table 6**.

Table 6. Range of Instrumental Relative Standard Deviation (RSD%) and Relative Standard Deviation of the Method for Orange Juice and Orange–Carrot Juice Mixture

		precision (RSD%)[a]	
peak	carotene	instrumental	method
1	valenciaxanthin	3.11–5.86	3.14–8.32
2	9-*cis*-violaxanthin + neoxanthin	1.72–4.06	2.62–4.41
3	*cis*-antheraxanthin	2.13–4.87	2.47–5.01
4	antheraxanthin	2.52–3.01	2.59–3.68
5	mutatoxanthin	3.21–7.14	4.23–7.87
6	lutein	1.77–4.47	3.77–5.84
7	zeaxanthin	1.69–3.31	4.31–6.53
8	α-cryptoxanthin	1.73–3.88	2.28–4.24
9	β-cryptoxanthin	2.17–4.40	2.57–5.26
10	9-*cis*-α-carotene	2.13–5.54	2.84–6.33
11	α-carotene	1.08–4.64	2.78–4.85
12	phytone + phytofluene	4.32–5.65	4.81–5.83
13	β-carotene	1.98–3.04	7.16–10.74
14	9-*cis*-β-carotene	2.47–4.44	2.68–6.30
15	ζ-carotene	1.99–2.83	4.07–8.05

[a] $n = 6$.

It can be seen that the instrumental and method precision levels obtained are acceptable both in standards and in sample (orange−carrot juice) for the carotenoids studied.

Analytical parameters are similar to those found by Chen et al. (*24*), Hart et al. (*40*), and Konings et al. (*27*), highlighting that the linearity interval studied is wider in this work.

Figures 4 and **5** show the chromatograms of the two types of samples that we analyzed: an orange−carrot juice mixture and orange juice, respectively. The differences between the two samples can be seen clearly: the mixed juice has a greater quantity of carotenoids, especially of α- and β-carotene, but the most representative carotenoids (9-*cis*-violaxanthin, neoxanthin, antheraxanthin, lutein, zeaxanthin, α- and β-cryptoxanthin, 9-*cis*-α-carotene, phytoene, and phytofluene) are present in both juices. The most common retention times and wavelengths are shown in **Table 3**. A slight variation can be seen in the retention times obtained for the sample of orange−carrot juice mixture and the sample of orange juice, because they are different matrices. It must also be emphasized that not all of the cis isomers were found in all of the samples analyzed, and the particular isomers obtained depended on each matrix and on their different conservation treatments.

The contents of the various carotenoids identified in accordance with the proposed method in orange juice and in an orange−carrot juice mixture are shown in **Table 7**.

Conclusions. The utilization of photodiode array detection is a valuable tool for characterization of *cis*- and *trans*-carotenoids in vegetable and citric juices (orange juice and an orange−carrot juice mixture). Using the rapid procedure described, the major carotenoids have been characterized from spectral and retention time data obtained with authentic standards or literature values. Major carotenoids were separated within 22 min with ternary gradient elution. The low toxicity and danger of most of the reagents and products used during the extraction and separation of carotenoids do not call for special safety measures in the laboratory. As the analytical parameters

Table 7. Quantification of Carotenoids in Orange–Carrot Juice Mixture and in Orange Juice

peak	carotenoid	μg/100 g ± SD[a] orange–carrot	orange
1	valenciaxanthin	21.57 ± 0.94	14.44 ± 1.27
2	9-*cis*-violaxanthin + neoxanthin	164.52 ± 1.45	738.96 ± 2.65
3	*cis*-antheraxanthin	ND[b]	26.88 ± 3.40
4	antheraxanthin	61.98 ± 0.05	236.89 ± 1.31
5	mutatoxanthin	16.38 ± 1.21	ND
6	lutein	38.56 ± 1.69	53.55 ± 4.69
7	zeaxanthin	32.44 ± 1.98	35.09 ± 3.39
8	α-cryptoxanthin	30.54 ± 2.14	42.29 ± 2.34
9	β-cryptoxanthin	36.80 ± 2.18	118.31 ± 3.61
10	9-*cis*-α-carotene	149.68 ± 8.59	41.45 ± 1.93
11	α-carotene	867.62 ± 8.65	22.41 ± 3.25
12	phytoene + phytofluene	423.15 ± 3.95	36.54 ± 2.61
13	β-carotene	2253.27 ± 13.55	20.63 ± 3.12
14	ζ-carotene	205.50 ± 6.25	ND
15	9-*cis*-β-carotene	ND	22.72 ± 1.74

[a] SD, standard deviation. [b] ND, not detected.

show, the proposed method permits determination of *cis*- and *trans*-carotenoids in vegetable juices, because the method is sensitive, reliable, accurate, and reproducible.

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