Elucidation of Carotenoid Patterns in Citrus Products by Means of Comprehensive Normal-Phase \times Reversed-Phase Liquid Chromatography

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A novel approach for carotenoid analysis has been developed. Orange essential oil and juice carotenoids were separated by means of comprehensive dual-gradient elution HPLC, using normal phase with a microbore silica column in the first dimension (first D), reversed phase with a monolithic C18 column in the second dimension (second D), and a 10-port switching valve as an interface. An on-line photodiode array detector was used in order to obtain absorption spectra. Peak identification was obtained by combining retention data with the UV–visible spectra.

Food carotenoids, mainly distributed in plant-derived foods, are an important class of natural pigments, notable for their wide distribution, structural diversity, and various functions. In terms of structure, they are usually C40 tetraterpenoids derived from eight C5 isoprenoid units. A characteristic of all carotenoids is that the arrangement of the isoprene units becomes reversed in the center of the carotenoid molecule. Their basic linear and symmetrical skeleton, which can be cyclized at one or both ends, has lateral methyl groups separated by six C atoms at the center and five C atoms elsewhere. A distinctive characteristic is a centrally located, extended conjugated double-bond (DB) system, which serves as the light-absorbing chromophore, being responsible for the yellow, orange, or red color that characterizes these compounds. The extended DB system is also the main factor behind the particular instability of carotenoids toward light, heat, oxygen, and acids. For this reason, several precautions have to be applied when handling carotenoids. For example, the most widespread method is to add antioxidants such as butylated hydroxytoluene (BHT) to the extraction solvent and mobile phase and, when handling, to keep the ambient temperature low and constant.2

Carotenoids are usually classified in two main classes: hydrocarbon carotenoids, known as carotenes (e.g., β-carotene, lycopene), and oxygenated carotenoids, known as xanthophylls (e.g., β-cryptoxanthin, lutein, violaxanthin). Mono- or dihydroxylated carotenoids often occur in an esterified form that is more stable than the free carotenoids.

Figure 1 shows a partial scheme of cyclic carotenoid transformations in nature.1 The composition of carotenoids in nature is greatly variable: cyclization and other modifications (hydrogenation, dehydrogenation, DB migration, chain shortening or extension, rearrangement, isomerization, and introduction of oxygen functions) result in a multitude of structures. Moreover, it depends also on various external factors, such as plant cultivar, ripeness stage, geographic site of production, postharvest handling, and conservation. In fact, ~700 kinds of carotenoids have been isolated in nature.3 The largest number of carotenoids found in any fruit are those of citrus fruits: more than 115 different compounds and their isomers have been reported.4,5 The largest number of studies have regarded carotenoids present in orange juice,6–12 while less

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research work has been made on citrus essential oil composition.14–16 Lutein, violaxanthin, \( \beta \)-cryptoxanthin, antheraxanthin, and zeaxanthin are reported as the major carotenoids in orange juice and oil.6,8–10,14

Due to the complex composition of carotenoids in natural matrices, to their great structural diversity (including a great

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variety of isomers and derivatives with minor differences among the isomers), and to the fact that they are extremely unstable compounds, the elucidation of carotenoid patterns in real world samples is a particularly challenging task. Moreover, the determination is complicated also by the lack of commercially available standards.

High-performance liquid chromatography (HPLC) appears to be the method of choice for carotenoid analysis. In general, both normal-phase (NP) and reversed-phase (RP) HPLC have shown the capability to separate these pigments, but while quite few normal-phase separations have been reported, reversed-phase HPLC, in particular with C18 and C30 stationary phases, has been used widely.\(^{17,18}\) NP HPLC, especially when using the silica-based, nitrile-bonded stationary phase, is more efficient in separating more polar carotenoids such as lutein, zeaxanthin, their geometrical isomers and metabolic byproducts,\(^{20,21}\) but fails in separating monohydroxycarotenoids and carotenones, such as α- and β-carotene,\(^{21,22}\) which can be separated in RP, where retention increases with increasing hydrophobicity and decreasing polarity. C30 and C18 are the most widespread stationary phases in RP for carotenoid analyses, permitting also the separation of geometrical isomers.\(^{23}\) Therefore, in order to obtain the complete determination of carotenoids in real samples, multiple separation mechanisms or systems can greatly help. In fact, multidimensional chromatography offers a higher peak capacity and an enhanced separation power for resolving complex samples.\(^{24-26}\)

Comprehensive two-dimensional LC (LC\(_x\)\(\times\)LC) is a modern technique based on the combination of two independent separation steps with orthogonal selectivities. In LC\(_x\)\(\times\)LC, a primary column is connected to one or more secondary columns by means of a switching valve as interface. The function of the latter is to isolate continuous fractions of the first column effluent and then release them onto the second-dimension column. The effectiveness of comprehensive LC separations has been demonstrated in several works, where size exclusion or ion-exchange chromatography hyphenated to RP have been used\(^{27-29}\) or even NP combined with RP, where problems related to mobile-phase immiscibility were overcome.\(^{30-34}\)

In the present research, a novel approach based on the use of comprehensive LC for carotenoid separation has been developed. The carotenoids in orange essential oil and juice were separated by comprehensive dual-gradient elution HPLC, using NP with a microbore silica column in the first dimension and RP with monolithic C18 column in the second dimension. Tentative identification of carotenoids, for which authentic standards were not available, was carried out by using the combination of chromatographic retention data and visible absorption spectra obtained with an on-line photodiode array (PDA) detector.

**EXPERIMENTAL SECTION**

**Materials.** (1) Chemicals. Ethyl alcohol was purchased from Carlo Erba Reagenti (Milan, Italy). All other solvents were from VWR International S.r.l. (Milan, Italy). All solvents were HPLC grade.

(2) Samples. Pure standard β-carotene was purchased from Sigma-Aldrich S.r.l. (Milan, Italy). The analyses were carried out on genuine sweet orange essential oil and red orange juice samples, donated by a local producer.

**Methods.** (1) Sample Preparation. Saponification. Prior to HPLC analyses, alkaline hydrolysis was performed in order to hydrolyze the carotenol esters: 5 g of essential oil, stabilized with 0.1% w/w of BHT, was treated with 5 mL of methanolic potassium hydroxide (2 N). The mixture was left under stirring for 16 h at room temperature (22 °C), in darkness. The free carotenoids were then extracted with three aliquots of 5 mL of diethyl ether. The organic extracts were combined and washed several times with water until neutral pH. The solvent was evaporated under vacuum at 30 °C to constant volume of the residue (~4–4.5 mL). The saponified essential oil sample was injected without further dilution. Orange juice was subjected to extraction before saponification: 100 mL of juice was extracted three times with a 60-mL mixture of petroleum ether and THF (8:10 v/v). The organic extract was reduced to a small volume using a vacuum evaporator and hydrolyzed as in the case of the essential oil. The solvent was evaporated under vacuum at 30 °C, and the residue was dissolved in THF to a final volume of 5 mL for the chromatographic analysis.

(2) LC\(_x\)\(\times\)LC Analyses. Instrumentation and Software. The LC\(_x\)\(\times\)LC analyses were performed on a Shimadzu Prominance LC-20A\(^{29}\)Comprehensive LC system (Shimadzu, Milan, Italy), which consists of a CBM-20A controller, LC-20 AD and LC-20 ADSP dual-plunger parallel-flow pumps (LC 1), an LC-20 AB solvent delivery module, equipped with two dual-plunger tandem-flow pumps (LC 2), a DGU-20A5 degasser, an SPD-M20A photodiode array detector, an SPD-20A UV detector, a CTO-20AC column oven, and an SIL-20AC autosampler. The two dimensions were connected by using an electronically controlled 2-position, 10-port Supelpro switching valve (Supelco, Milan, Italy), placed inside the column oven and equipped with two 20-μL sample loops. Both dimensions and the switching valve were controlled by the LCsolution Version 1.21 SP1 software (Shimadzu). With regard
Experimental Conditions. First Dimension. The chromatographic column used in the first dimension was a Supelcosil LC-
Si, 300 × 1.0 mm i.d., 5 μm dp (Supelco) installed into the column oven. The mobile phase consisted of n-hexane (A) and ethyl alcohol (B) in gradient mode. The gradient (linear between two steps) was as following: 0 min: 1.1% B; 1 min: 2% B; 30 min: 4% B; 45 min: 5% B; 75 – 160 min: 9.9% B. The flow rate was 10 μL/min, maintained by using a Protocel flow-splitter (SGE, Milan, Italy) between the mixer and the column. The 3-μL injections were made by the autosampler.

Second Dimension. The analytical column was a Chromolith Performance RP-18 (100 × 4.6 mm i.d.) monolithic column (Merck, Darmstadt, Germany). As mobile phase, 2-propanol (A) and 20% of water in acetonitrile v/v (B) in gradient mode were used. Considering the retention times for the first-eluting peaks in the first dimension (obtained with preliminary analyses), a gradient in the second dimension was applied from 16 to 160 min. Two different repetitive 2-min gradients were used as shown in Table 1 (the concentration of the mobile phase between two steps changes linearly). The flow rate was 4.7 mL/min, the injection volume was 20 μL (made by the 10-port valve), and the modulation time was 2 min.

The temperature of the column oven was 30 °C. As detection system, the combination of the PDA and UV–visible detector was used, connected in series at the exit of the monolithic column. The PDA detector was connected at the exit of the first D column only for preliminary analyses.

UV–visible conditions: polarity, +; response, 1.0 s; cell temperature, 30 °C; wavelength, 450 nm; sampling rate, 25 Hz.

PDA conditions: wavelength range, 250–550 nm; cell temperature, 30 °C; sampling rate, 12.5 Hz; time constant, 0.320 s.

RESULTS AND DISCUSSION

Before comprehensive NP–RP HPLC analysis of carotenoids, the optimization of the single dimensions were carried out.

First-Dimension Separation. A silica micro-HPLC column was used in the first dimension. Under normal-phase conditions, the components were separated according to their increasing polarity. The aim of this step was the separation of carotenoids into groups of different polarity (hydrocarbons, monools, diols, etc.). During the optimization step, various solvent concentrations, under isocratic and gradient conditions, have been tested. At first the isocratic elution was tested. This appeared to be not suitable: using the mobile phase with lower ethyl alcohol content, the polyols were not eluted, while with a higher ethyl alcohol content, the separation of hydrocarbons and monools was not achieved. Thus, the best results were obtained using n-hexane and ethyl alcohol in gradient mode, increasing the ethyl alcohol concentration during the analysis. This enables the separation of all groups of carotenoids. The flow rate in the first dimension was set at 10 μL/min in order to obtain 3–4 second D sampleings across a first-dimension peak, according to Murphy et al.,36 considering a 2-min modulation period (optimized analysis time for the second dimension), and to ensure the transfer of small amounts of the first-dimension effluent onto the second D column to avoid problems of solvent immiscibility.30,36

At the beginning, no flow splitter was used, but under gradient mode, one of the two pumps was operated at a very low flow rate (from 0.1 to 1 μL/min). A flow splitter was located between the first D mixer and the first D column, to avoid problems related with repeatability. The total flow rate at the pumps was set at 93 μL/min, thus ensuring better repeatability under gradient conditions. The CV values for retention times, calculated on the basis of three consecutive analyses, were lower than 2% for all peaks, while they ranged from 2 to 6% without the use of the flow splitter.

Second-Dimension Separation. For the second D, a monolithic C18 column was used, in order to obtain fast separations without loss of resolution. Under RP conditions, carotenoids are eluted according to their increasing hydrophobicity and decreasing polarity (polyols, diols, monools, and hydrocarbons). Preliminary analyses, carried out under gradient elution, gave an acceptable separation in less than 2 min using a flow rate of 4.7 mL/min. According to this, the modulation time (sampling frequency) in the 2D system was set at 2 min.

Considering the retention times of carotenoids in the first D, the 2D analyses were performed in the 16–160-min time window. Due to the wide differences in polarity and hydrophobicity between hydrocarbons (carotenes) and their oxygenated derivates (xanthophylls), two different gradient programs were developed to ensure a better separation of components of the same group in the second dimension. For the separation of carotenes, a faster initial gradient with a longer isocratic step at a high concentration of 2-propanol was used, due to the high hydrophobicity of these compounds. This gradient was applied in the first part of the comprehensive LC×LC analysis (between 16 and 26 min) where carotenes were eluted from the NP column. For the separation of the more polar and less hydrophobic xanthophylls, a slower gradient was used from 26 min onward.

Comprehensive Two-Dimensional Analyses. Figures 2 and 3 show the comprehensive LC×LC chromatogram of the carotenoid fraction of an orange essential oil (Figure 2) and a juice (Figure 3) after saponification, obtained at 450 nm, with all determined peaks numbered, and the location of the carotenoid groups in the 2D space. Most of the components present in orange samples present a maximum of absorption in the 450-nm zone.

Table 1. Second-Dimension Gradient

<table>
<thead>
<tr>
<th>total analysis time, min</th>
<th>second dimension time (0–2 min), s</th>
<th>concn of B, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>16–26</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>95</td>
</tr>
<tr>
<td>26–160</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>95</td>
</tr>
</tbody>
</table>

* Mobile phase 2-propanol (A) and 20% water in acetonitrile (B).
thus, this wavelength was chosen for 2D visualization. Fifty-seven peaks have been detected in the two-dimensional space for the orange oil and 59 peaks in the orange juice. In total, 75 carotenoids have been detected in the two samples, 37 of which were identified. Table 2 reports all the components detected in the two samples with their average retention times ($t_R$) and spectral data. In the case of identified components, comparison with spectral characteristics found in literature is also reported. Average retention times reported in Table 2 were obtained by performing 6 LC×LC runs (3 on sweet orange essential oil and 3 on red orange juice). Retention times in comprehensive LC are obtained by the combination of the first D retention time and the second D retention time. In this work, a modulation frequency of 2 min was used. Even slight variations of first-dimension retention times can give values different in 2 min, due to the fact that the most abundant cut of the peak can be moved of one modulation. However, LC×LC retention times showed very good repeatability: most of the CV values in the first D were lower than 2% (59 of 75), only one peak had CV higher than 5% and others less than 3%; all CV values in the second D were smaller than 1%, and most of them (48 of 75) were less than 0.5%.

Identification was performed on the basis of spectroscopic data, position of the peaks in the 2D plot, and comparison with literature data.

The first group eluting from the NP column consists in hydrocarbons. The separation of these components under NP conditions is very difficult and isomers such as α- and β-carotene coelute. On the contrary, these components can be easily separated under RP conditions. Under NP conditions, acyclic components elute slightly earlier than cyclic components. As a consequence, the acyclic ζ-carotene, phytoene, and phytofluene (coeluted under peak 1) elute before cyclic α- and β-carotene (peaks 7 and 8, respectively). The elution order on the C18 monolithic column depends on the type of cyclization (position
Table 2. Peaks Determined in Orange Essential Oil and Juice Samples with Their First- and Second-Dimension Retention Times\(^{a,b}\)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>1(^{st}) D</th>
<th>2(^{nd}) D</th>
<th>Literature data</th>
<th>Orange oil</th>
<th>Orange juice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>lit. (R_t)</td>
<td>lit. (R_t)</td>
<td>(\lambda_{max})</td>
<td>(\lambda_{max})</td>
<td>(\lambda_{max})</td>
</tr>
<tr>
<td>1</td>
<td>1,2-Cymene</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>425.0</td>
<td>399.0</td>
</tr>
<tr>
<td>2</td>
<td>Phenol</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>0.0</td>
<td>425.0</td>
</tr>
<tr>
<td>3</td>
<td>Thymol</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
<tr>
<td>4</td>
<td>Camphene</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>0.0</td>
<td>425.0</td>
</tr>
<tr>
<td>5</td>
<td>Camphor</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
<tr>
<td>6</td>
<td>Caryophyllene</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
<tr>
<td>7</td>
<td>Linalool</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
<tr>
<td>8</td>
<td>Citral</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
<tr>
<td>9</td>
<td>α-pinene</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
<tr>
<td>10</td>
<td>β-pinene</td>
<td>1.4</td>
<td>83.5</td>
<td>0.5</td>
<td>337.0</td>
<td>337.0</td>
</tr>
</tbody>
</table>

- Average of three consecutive analyses of orange oil and three of orange juice sample with corresponding CV Values, experimental spectral data obtained by PDA detector, and spectral characteristics found in the literature. \(^{2,6-8,10,13-16,37,38}\)
- H, hydrocarbon; M, monool; MME, monool monoepoxide; D, diol; DME, diol monoepoxide; DDE, diol diepoxide; PME, polyol monoepoxides; s, spectral shoulder; +, present in the sample; −, not present in the sample.
of DB in the cycle and number of cycles). α-Carotene (β,γ-cycles) elutes before β-carotene (β,β-cycles), while acyclic γ-carotene (ψ,ψ)-ends elutes later, together with phytoene and phytofluene. These three acyclic carotenes differ only in the number of DBs (9 in phytoene, 10 in phytofluene, and 11 in γ-carotene). In this case, the difference in the DB number is not sufficient to allow the separation of these three compounds on the short monolithic column. However, these compounds have very different absorption spectra with different \( \lambda_{\text{max}} \). At the wavelength of 450 nm, only the γ-carotene peak (\( \lambda_{\text{max}} = 378, 400, 425 \) nm) is visible, as the other two compounds do not absorb in this range (phytoene: \( \lambda_{\text{max}} = 276, 286, 297 \) nm, phytofluene: \( \lambda_{\text{max}} = 331, 348, 367 \) nm); the different UV–visible spectra allow their detection.

The second group eluting from the NP column consisted of monools and their epoxides. Of these, α-cryptoxanthin (peak 13), β-cryptoxanthin (peak 14), and cryptoxanthin-5,6-epoxide (peak 12) were identified in both samples. The presence of the additional oxygen in the epoxide ring increases the sorption affinity of monool epoxides on the silica column, and due to this, they elute slightly later in NP and earlier in RP mode. In fact, cryptoxanthin-5,6-epoxide is located to the right and underneath α-cryptoxanthin on the 2D plane. On the C18 column, α- and β-cryptoxanthin, which coeluted on the silica column, are well separated. As in the case of hydrocarbons, in RP mode α-cryptoxanthin, with a \( \beta,\gamma \)-cyclization, elutes before β-cryptoxanthin with a \( \beta,\beta \)-cyclization. With regard to epoxides, the presence of another oxygen in the molecule affects not only the retention times but also the absorption spectra. The presence of an epoxide ring in the 5,6 position shifts \( \lambda_{\text{max}} \) to a \( \sim 5 \) nm shorter wavelength (β-cryptoxanthin, \( \lambda_{\text{max}} = 428, 450, 478 \) nm; cryptoxanthin-5,6-epoxide, \( \lambda_{\text{max}} = 420, 444, 473 \) nm). This shift is a result of the elimination of one double bond from the cycle, thus reducing conjugation.

Diols and their mono- and diepoxides are the most abundant components in both samples. In NP, diols elute before their corresponding diepoxides. Diol monooxepoxides elute between these groups, with some of them eluting at the same time as the diols. As previously observed for the other carotenoids, diols that differ only in the \( \beta,\gamma \)- and \( \beta,\beta \)-cyclization, such as lutein (peak 23) and zeaxanthin (peak 25), coelute in NP mode but are well separated under RP conditions, with the \( \beta,\beta \)-isomer (zeaxanthin) eluting after the \( \beta,\gamma \) one (lutein). Different cyclization affects also the absorption spectra and \( \lambda_{\text{max}} \). Lutein and zeaxanthin contain 11 DBs in their structure. In zeaxanthin (\( \beta,\beta \)-cyclization), all double bonds are conjugated, while in lutein (\( \beta,\gamma \)-cyclization), only 10 DBs are conjugated. Due to this, the \( \lambda_{\text{max}} \) of zeaxanthin are at a higher wavelength (427.5, 449.5, 476.5 nm) than the \( \lambda_{\text{max}} \) of lutein (422, 445, 474 nm). As it can be seen from Table 2, other components have been detected in both samples and identified as “lutein isomers” (peaks 17, 21, 28, 30, 32). This can be due to the fact that the highly unsaturated carotenoids are prone to isomerization and oxidation. In plants, the all-trans orientation is preferred, but many factors such as heat, light, acids, adsorption on active surfaces, or even the saponification procedure may promote isomerization of trans-carotenoids to the cis form. This results in loss of color (change of \( \lambda_{\text{max}} \) in absorption spectra) and in a different chromatographic behavior, in both NP and RP modes. In this work, due to the lack of standards, in a group of components presenting the same spectral characteristics and a defined position in the 2D plane, the most abundant has been identified as the all-trans isomer, while the others have been indicated as “isomers”. Observing the group of diol monooxepoxides, flavoxanthin (peak 16, \( \beta,\gamma \)-cyclization and epoxy ring in position 5,8), a large number of mutatoxanthin isomers at 95 min and of antheraxanthin isomers at 110 min elute from the 1D column. Mutatoxanthin (peak 38) and antheraxanthin (peak 56) have both \( \beta,\gamma \)-cyclization but, the first one has an epoxy ring in position 5,8 while the second presents a 5,6-epoxy ring. 5,8-Epoxide contains one DB less in the polyene chain in comparison with the corresponding non-epoxide compound. The DB is now located in the 5,8-epoxide cycle. \( \lambda_{\text{max}} \) is shifted \( \sim 20 \) nm to a shorter wavelength (mutatoxanthin, 409, 427, 457 nm; antheraxanthin, 422, 444, 472 nm).

As for the epoxide ring, also the trans and cis isomerization affects the chromatographic behavior and the UV–visible spectra. The presence of a cis double bond causes a hypsochromic shift of \( \lambda_{\text{max}} \) from about 2–6 nm and the appearance of a \( \alpha \) cis peak in or near the ultraviolet region. On the basis of their absorption spectra, antheraxanthin and cis-antheraxanthin (peak 54) were identified in sweet orange essential oil and juice. These two compounds are partially coeluted on the silica column, but were well separated on the C18 column. Elution orders of diol monooxepoxides on both columns were as follow: flavoxanthin with \( \beta,\gamma \)-cyclization and epoxide bond in position 5,8 eluted first, then mutatoxanthin with \( \beta,\gamma \)-cyclization and epoxide bond in position 5,8, followed by antheraxanthin with \( \beta,\beta \)-cyclization and epoxide bond in position 5,6. Once again, on the RP column, the \( \beta,\gamma \)-isomer is less retained than the corresponding \( \beta,\beta \).

If the diol diepoxide zone is considered, the following consideration can be made relative to the elution order on the silica column: violaxanthin (peak 53, \( \beta,\beta \)-cyclization, 5,6,5′,6′-epoxide bond), luteoxanthin (peak 57, \( \beta,\beta \)-cyclization, 5,6,5′,8′-epoxide bond) overlap with auraxanthin (peak 55, \( \beta,\beta \)-cyclization, 5,8,5′,8′-epoxide bond). Other intense peaks are those of auraxanthin and luteoxanthin isomers (peaks 60 and 61). On the C18 column, luteoxanthin coelutes with auraxanthin and is followed by violaxanthin. On the RP column, a structure-related elution behavior can be observed, with 5,8-epoxy eluting before the corresponding 5,6-epoxy structures (both for diol monooxepoxides and diol di-epoxides). As in the case of monooxepoxides, the second epoxide bond affects the absorption spectra. Epoxy bonds in positions 5,6,5′,6′ cause an hypsochromic effect of \( \sim 10 \) nm. If epoxide bonds are in positions 5,6,5′,8′, this shift is \( \sim 25 \) and \( \sim 50 \) nm in the case of epoxide bonds in positions 5,8,5′,8′, as a result of the loss of two DBs from the polyene chain.

The last group of components eluting from the silica column is represented by polyols. From data available, neoxanthin (peak 68, 5′,6′-epoxy \( \beta,\beta \)-triol) and trollichrome (peak 75, 5′,8′-epoxy \( \beta,\beta \)-triol) were identified in both samples. As previously observed, also in this case, under RP conditions the 5′,8′-epoxide elutes before the corresponding 5′,6′-epoxide.

**Comparison of PDA and UV Detectors.** After the second D column, PDA and UV detectors were connected. The UV detector was used for the creation of the 2D plot, extracted at 450 nm, because the sensitivity was higher than that of PDA detector, which was used to obtain absorption spectra of the peaks.
Comparing the results obtained with these two detectors, retention times obtained were practically the same, due to the high second-dimension flow rate and to the minimum volume used to connect the two detectors (~10 µL). Experimental data, obtained by using the PDA detector, were used for creating two-dimensional plots at wavelengths other than 450 nm, for the better visualization of some carotenoids, such as the colorless phytoene and phytofluene (visible at 280 nm), which contain only three and five conjugated double bonds, respectively. The purity of spectra was also controlled with this detector. Due to the fact that absorption spectra of carotenoids have typically three maximums, the presence of a fourth maximum indicated a coelution.

Even though LC×LC analysis provided a much increased resolving power, some pairs, such as luteoxanthin (peak 57) and auraxanthin (peak 55), resulted partially coeluted. In this case, identification was possible by observing PDA chromatograms extracted at wavelengths other than 450 nm, as shown in Figure 4. Although in the UV chromatogram at 450 nm only one peak was visible, two appeared in the PDA chromatogram extracted at 400 nm (a). Figure 4 also shows the spectra of the two identified carotenoids extracted at 100.79 (b) and 100.81 min (c); as can be seen, they presented different \( \lambda_{\text{max}} \) in accordance with the structure of these two diol diepoxides. Luteoxanthin (that is a \( \beta,\beta'-5,6,5',8'-\text{diepoxo-diol} \)) presents \( \lambda_{\text{max}} \sim 20 \) nm higher than auraxanthin (that is a \( \beta,\beta'-5,8,5',8'-\text{diepoxo-diol} \)).

CONCLUSIONS

In the present work, tentative identification of carotenoids, for which authentic standards were not available, was carried out by using the combination of LC×LC retention data and UV–visible absorption spectra obtained with an online photodiode array detector.

The comprehensive LC system used allowed temperature control of both the columns and the valve used as interface. All the operations were carried out in a fully automated way, to ensure maximum repeatability.

Even though the total analysis time was quite long (more than 160 min), the amount of information obtained was very high. A high number of carotenoids were separated in the two samples, and many of them were also identified. Even though the use of a PDA as a detector represented a very powerful tool in carotenoid identification, further work will be carried out using the mass spectrometer as a detector, in order to have additional information useful for components confirmation. The data collected in this research will also be used in the analysis of other matrices rich in carotenoids.

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