JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Application of Comprehensive Two-Dimensional Liquid Chromatography To Elucidate the Native Carotenoid Composition in Red Orange Essential Oil

PAOLA DUGO,*,† MIGUEL HERRERO,§ DANIELE GIUFFRIDA,† TIINA KUMM,§ GIOVANNI DUGO,† AND LUIGI MONDELLO†,#

Dipartimento di Scienze degli Alimenti e dell'Ambiente, Facoltà di Scienze, Università di Messina, Salita Sperone 31, 98166 Messina, Italy; Dipartimento Farmaco-chimico, Facoltà di Farmacia, Università di Messina, Viale Annunziata, 98168 Messina, Italy; and Campus-Biomedico, Via E. Longoni 47, 00155 Roma, Italy

In the present work, the ability of a LC × LC-DAD/APCI-MS method developed at this laboratory to identify the native composition of carotenoid in an extremely complex matrix such as red orange essential oil was demonstrated. To carry out this task, two independent and orthogonal separation mechanisms were coupled through a 10-port switching valve that simultaneously collected the eluent from a microbore cyano column used as the first dimension in normal phase mode and injected it to a conventional reversed phase monolithic C_{18} column in the second dimension separation. By using this novel analytical technique together with the use of DAD and APCI-MS detectors it was possible to identify in the sample, without the need of any pretreatment, 40 different carotenoids. Among them, 16 carotenoid monoesters were identified, mainly β-cryptoxanthin palmitate ($C_{16:0}$), myristate ($C_{14:0}$), and laureate ($C_{12:0}$) as well as several lutein, violaxanthin, antheraxanthin, and luteoxanthin monoesters. Moreover, 21 carotenoid diesters composed by several antheraxanthin, luteoxanthin, violaxanthin, and auroxanthin diesters were found in the native carotenoid composition of the orange oil. The main carotenoid diesters were the laureate palmitate ($C_{12:0}$, $C_{16:0}$), myristate palmitate ($C_{14:0}$, $C_{16:0}$), and dipalmitate ($C_{16:0}$, $C_{16:0}$) diesters, although other diesters were also identified. Besides, two different free carotenes, ζ-carotene and phytofluene, and a xanthophyll, lutein, were also determined. To the authors' knowledge, this is the first time that carotenoid diesters are described and identified in orange essential oil. Likewise, it has been demonstrated that the LC × LC approach proposed in this study is capable of coping with the direct analysis and identification of a complex natural source of carotenoids such as the orange.

KEYWORDS: Comprehensive LC; carotenoids; esters; orange; native composition

INTRODUCTION

Carotenoids are an important kind of natural pigment that can be widely found in plant-derived food and products. Although these compounds have been traditionally used in the food industry as colorants, nowadays they attract great attention because they have been described to possess several important functional properties, mainly antioxidant activity (*1–3*), as well as prevention of cardiovascular diseases (*4, 5*), cancer (*6, 7*), and macular degeneration (*8*). These properties make these compounds ideal for the always increasing functional food industry as well as promoting the consumption of the natural products in which they are contained. Citrus species are well-known to possess a rich carotenoid pattern and are regarded as the most complex natural source of these types of compounds (*9*). Nevertheless, these compounds are widely distributed in nature and can be found in higher plants, algae, fungi, and bacteria (*10, 11*).

Among the citrus species, orange has been the most studied given its importance in terms of production and its industrial application in juices. In fact, orange juice is probably the most consumed fruit juice worldwide (*12*). The chemical structure of carotenoids is usually based on a C_{40} tetraterpenoid structure with a centrally located, extended conjugated double-bond system, which acts as the light-absorbing chromophore, and is related to the color shown (*13*). Taking into account their chemical structures, these compounds can be divided into two different groups: first, hydrocarbon carotenoids, generally named carotenes; and oxygenated carotenoids, commonly known as

* Corresponding author (e-mail pdugo@pharma.unime.it; telephone +39 090 6766536; fax +39 090 6766532).

† Dipartimento di Scienze degli Alimenti e dell'Ambiente, Università di Messina.

§ Dipartimento Farmaco-chimico, Università di Messina.

Campus-Biomedico.

10.1021/jf800144v CCC: $40.75 © 2008 American Chemical Society
Published on Web 04/30/2008

xanthophylls. This second group is the most complex one in terms of number of compounds and variations in their structure and can be found in either its free form (as are found the carotenes) or in a more stable fatty acid esterified. Thus, in view of the fact that a single carotenoid could be found forming different esters, the already complex natural variability of carotenoids is often increased by the formation of these carotenoid esters.

For this reason, to simplify the analysis of these compounds, the most used approach has consisted of carotenoid analysis after a saponification process. This saponification step allows the release of the carotenoids bound to the fatty acids and their analysis in their free form. Besides, this procedure acts as a cleanup step, eliminating compounds that could interfere in the analysis such as chlorophylls or lipids. In this way the carotenoid composition of citrus (*9, 14, 15*), particularly orange (*12, 16–20*), among other fruits (*2, 10, 21*), has been extensively studied. With regard to the carotenoid composition of orange, violaxanthin, luteoxanthin, lutein, β-cryptoxanthin, mutatoxanthin, and zeaxanthin have been usually identified as the major carotenoids (*9, 12, 14, 16*). However, this approach is not free of drawbacks. In fact, the saponification procedure is well-known to produce carotenoid degradation as well as artifact formation, preventing the discernment of the precise native carotenoid composition. In this sense, a different, more complex, approach could be used, based on the study of the carotenoid ester distribution of the sample as a way to know the real native carotenoid composition. Due to the complexity of this task, this approach has been much less used (*22*) and has been generally aimed to the study of the esters formed by a particular carotenoid. β-Cryptoxanthin esters from different vegetables and fruits (*23*) as well as astaxanthin esters from different matrices (*24, 25*) and zeaxanthin esters in plants (*26*), among others (*22, 27*), have been studied.

The native carotenoid composition of citrus in general and, particularly, orange has not been extensively studied, partially due to the complexity of these samples in their original form. The natural variability of the carotenoids together with the possibility of a particular carotenoid forming different esters as well as the relative instability of these compounds and the limited availability of enough standards makes the analysis and identification of these compounds in their native form a really difficult task. Thus, the data available regarding the orange native carotenoid composition up to now are rather limited (*18, 23*). This natural variability has implied the necessity of powerful analytical methods able to separate and identify these often quite similar compounds. Conventional LC has been the technique generally chosen to carry out carotenoid analysis (*28*). In particular, since their introduction by Sander et al. (*29*), C_{30} stationary phases have been widely employed to this aim (*28*). However, even when these C_{30} stationary phases are used, the separation power of conventional HPLC could be insufficient to analyze complex natural matrices. In this regard, our group has already demonstrated the applicability of comprehensive LC to identify the carotenoids present in saponified (*19*) and intact samples (*30*). Traditionally, the study of the carotenoid esters has been performed by a laborious and time-consuming procedure including the isolation of the ester fraction by column chromatography, saponification, and methylation of the fatty acids prior to their analysis by GC (*31, 32*). Compared to this approach, our present development allows the direct analysis of the orange oil without the need of any sample pretreatment, which implies quite faster analysis and simpler procedure.

Comprehensive two-dimensional LC (LC × LC) is a novel technique coupling two independent LC separation processes with orthogonal selectivities (*33*). In LC × LC the whole sample is analyzed in the two dimensions independently by using a switching valve as a transfer system between them. In fact, this technique has been already demonstrated as an effective technique to the analysis of complex matrices (*33*). Previously, we have developed a comprehensive LC method coupled to MS and PDA detectors to analyze the carotenoid ester distribution from real samples (*30*). The performance of this new method was tested with a relatively simple citrus sample in terms of carotenoid content, mandarin essential oil (*30*). Besides, other applications in food analysis can be found (*33*). In this work, a further step is achieved, and the native carotenoid esters and free carotenoid composition of a much more complex sample, red orange essential oil, is studied for the first time. Thus, the objective of the present work was to identify the native carotenoid composition, free carotenoids, and carotenoid esters from orange essential oil (extracted from orange peels) by using a comprehensive NPLC × RPLC-PDA/MS method. To the best of our knowledge, this is the first time that the orange carotenoid ester composition is studied as a whole.

MATERIALS AND METHODS

Samples and Chemicals. Ethyl alcohol was purchased from Carlo Erba Reagenti (Milan, Italy). All other solvents were from VWR International Srl (Milan, Italy). All solvents were of HPLC grade.

The sample of genuine red orange essential oil (*Citrus sinensis*, red or blood orange variety) was donated by a local producer and analyzed as received after filtration. Carotenoid standards, namely, β-carotene (hydrocarbon), lutein (diol), β-cryptoxanthin (monool), zeaxanthin (diol), and lutein dipalmitate ($C_{16:0}$, $C_{16:0}$), were purchased from Extrasynthese (Genay, France).

Instruments. All of the LC × LC analyses were carried out using the instrument described elsewhere (*19*): briefly, the system consisted of a Shimadzu Prominence LC-20A^{square} Comprehensive LC system (Shimadzu, Milan, Italy), including a CBM-20A controller, LC-20 AD and LC-20 ADSP dual-plunger parallel-flow pumps (LC1), an LC-20 AB solvent delivery module equipped with two dual-plunger tandem flow pumps (LC2), a DGU-20A5 degasser, an SPD-M20A photodiode array detector, a CTO-20AC column oven, and an SIL-20AC autosampler. To connect the two dimensions, an electronically controlled two-position 10-port Supelpro switching valve (Supelco, Milan, Italy) was employed, placed inside the column oven and equipped with two 20-μL sample loops. Both dimensions and the switching valve were controlled by the LCsolution ver. 1.21 SP1 software (Shimadzu). Besides, an LCMS 2010A MS detector (Shimadzu) was connected online. MS data acquisition was performed by the LCMSsolution ver. 3.30 software (Shimadzu).

The LC data were converted to ASCII data using the function of the LCsolution software. Later, these data were elaborated and visualized in two and three dimensions using Chrom^{square} ver. 1.0 software (Chromaleont, Messina, Italy).

LC × LC Analyses. For the analysis of the carotenoid esters, a Discovery Cyano (250 × 1.0 mm i.d., 5 μm dp) customized column (Supelco) was employed in the first dimension (consisting of cyanopropylsilane particles). The mobile phases selected were *n*-hexane/butyl acetate/acetone 80:15:5 (v/v/v, A) and *n*-hexane (B). The separation was performed under the following linear gradient: 0 min, 100% B; 90 min, 0% B; 120 min, 0% B; 120.1 min, 100% B; 130 min, 100% B. The flow rate was 10 μL/min, maintained by using a Protecol flow splitter (SGE, Milan, Italy) placed between the mixer and the autosampler. Six microliters of red orange essential oil was directly injected without any dilution or sample pretreatment. In the second dimension, a Chromolith Performance RP-18 (100 × 4.6 mm i.d.) monolithic column (Merck, Darmstadt, Germany) was used. The mobile phases employed were 2-propanol (A) and 20% water in acetonitrile (v/v, B). The flow rate was set at 5 mL/min following a linear gradient consisting

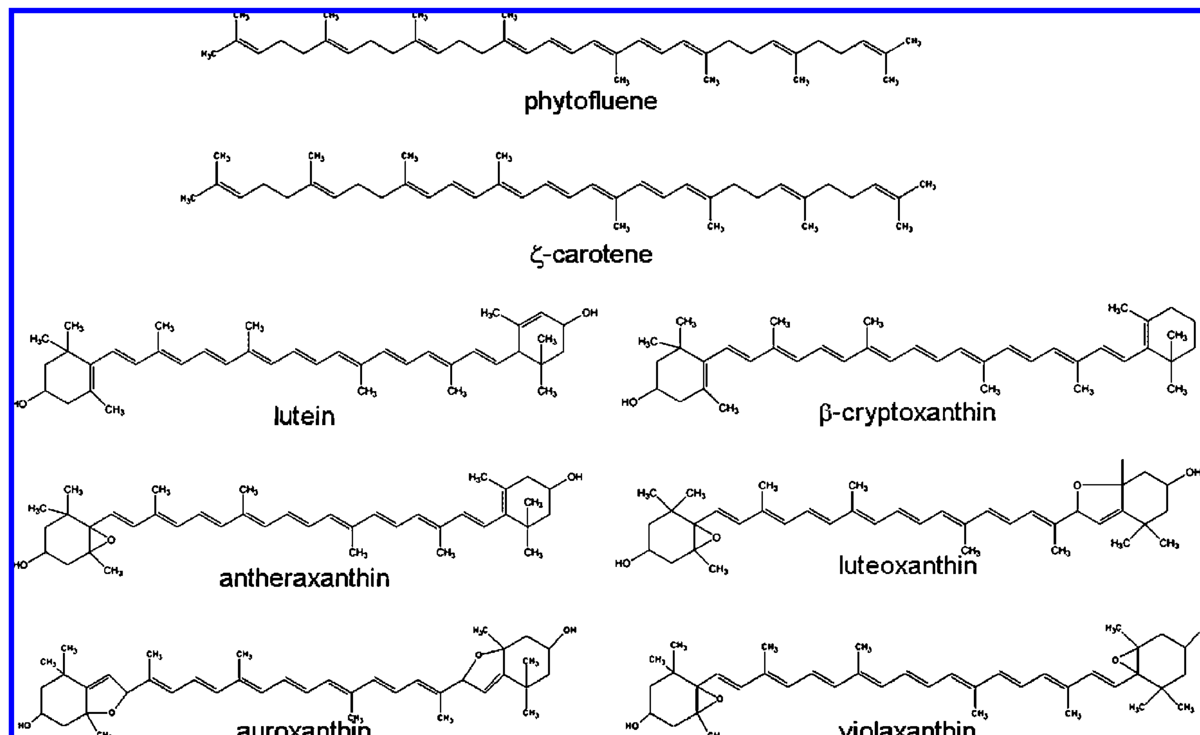

Figure 1. Chemical structures of the main carotenoids identified in the present work.

of 0.1 min, 70% B; 0.4 min, 30% B; 1.5 min, 20% B; 1.71 min 70% B. Two minute gradients were repeated during the whole analysis time, 2 min being the modulation time for the 10-port valve. The injection of the sample in the second dimension was made also through this valve (injection volume = 20 μL).

The total effluent flow from the second-dimension column was split in two (ca. 1 mL/min to MS and ca. 4 mL/min to PDA) so that the two detectors, MS and PDA, could be connected in parallel. The PDA detector covered a wavelength range from 250 to 550 nm (cell temperature = 40 °C, sampling rate = 12.5 Hz, time constant = 0.025 s), whereas the MS detector was working in APCI-positive mode. The parameters involving the MS detection and APCI ionization were as follows: acquisition mode, scan; nebulizing gas (N_2) flow, 2.5 mL/min; event time, 0.5 s; detector voltage, 2 kV; m/z range, 250–1300 amu; interface voltage, 4.5 kV; interface temperature, 400 °C; CDL voltage, –15 kV; CDL temperature, 250 °C; heat block, 200 °C; Q-array, 20.0, 20.0, 80.0; RF, 150 V; sampling frequency, 2 Hz.

RESULTS AND DISCUSSION

Method Development. In this work, a further development of the methodology of the separation and identification of intact samples by LC × LC is carried out, and the analysis of probably the most complex citrus sample with regard to the carotenoid fraction, that is, orange, has been performed. In **Figure 1**, the chemical structures of the main carotenoids identified in their free and esterified forms in this work are shown.

The method previously developed to analyze the mandarin essential oil (*30*) was modified with the aim of achieving a better resolution of the peaks separated in the first dimension, given the greater complexity and number of carotenoids found in the orange sample. The comprehensive two-dimensional LC approach employed was based on the use of a cyano microbore column in the first dimension to carry out a normal phase separation together with a conventional reversed phase monolithic C_{18} column in the second dimension. The use of a microbore column in the first dimension allowed us to minimize the possible problems that could be found due to solvent incompatibility when using a NPLC−RPLC coupling. This kind of setup (NP-LC) allowed the separation of the carotenoids first into different groups in the first dimension according to their polarity and, then, according to their hydrophobicity in the second dimension.

In this sense, the gradient employed in the first dimension (*30*) was reoptimized. Independently, the second dimension conditions formerly employed were tested to assess their suitability concerning this new sample and were found to be adequate. The typical 2D plot of the orange sample obtained by LC × LC (wavelength = 450 nm) is shown in **Figure 2**. In this figure the different peaks that were possible to identify are numbered, and their assignments can be found in **Tables 1** and **2**.

As can be deduced from what has been above explained, the identification of the carotenoids is quite complicated. In this study the carotenoid identification has been carried out combining the information provided by the two detectors employed (DAD and MS), the relative position of the different peaks in the 2D plane, and the data found in the literature, as well as the use of some standards. However, the limited availability of carotenoid standards, particularly carotenoid ester standards, must be noted. In fact, one of the main strengths of the methodology developed in this work was the possibility of successfully identifying a good number of carotenoids and carotenoid esters by injecting the intact sample without any kind of sample treatment. During a typical saponification procedure, not only are the carotenoids released into their free forms but also a high amount of interfering compounds are eliminated. However, during the analysis of the sample directly injected to identify its native composition, other compounds present in the sample could interfere either in the separation or in the detection, mainly in the MS. Moreover, in this application the use of an MS detector was compulsory because the carotenoid esters cannot be distinguished by their UV–vis spectra among them and from their free carotenoid.

The elution order observed in the second-dimension separation corresponded to a typical behavior in which the more hydrophobic compounds are more retained and elute later. On

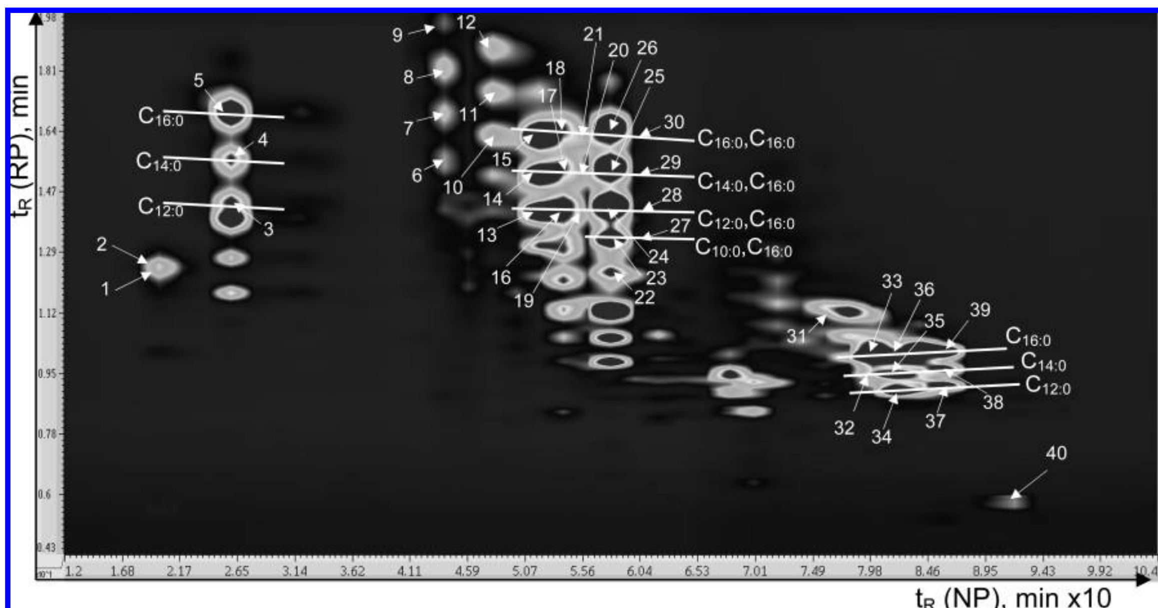

Figure 2. Two-dimensional plot of the intact red orange essential oil comprehensive 2D LC carotenoid analysis. Wavelength = 450 nm. For peak identification, see **Tables 1** and **2**. For experimental conditions, see text.

Table 1. UV−Vis and MS Information and Identification of the Free Carotenoids and Carotenoid Monoesters Found in the Red Orange Essential Oil

ID	retention time (min)	identification	UV−vis maxima	$[M + H]^+$	$[M + H - FA]^+$
1	21.2	ζ-carotene	381, 401, 426	541	
2	21.2	phytofluene	334, 350, 369	543	
3	27.4	β-cryptoxanthin laureate ($C_{12:0}$)	426s,[a] 452, 480	735	535
4	27.6	β-cryptoxanthin myristate ($C_{14:0}$)	426s, 452, 480	763	535
5	27.7	β-cryptoxanthin palmitate ($C_{16:0}$)	426s, 452, 480	791	535
6	45.6	lutein laureate ($C_{12:0}$)	420, 448, 474	733	533
7	45.7	lutein myristate ($C_{14:0}$)	420, 448, 474	761	533
8	45.8	lutein palmitate ($C_{16:0}$)	420, 448, 474	789	533
9	45.9	lutein stearate ($C_{18:0}$)	420, 448, 474	835	533
31	77.1	antheraxanthin palmitate ($C_{16:0}$)	422, 443, 472	823	567
32	80.9	luteoxanthin (b) myristate ($C_{14:0}$)	401, 420, 446	811	583
33	81.0	luteoxanthin (b) palmitate ($C_{16:0}$)	401, 420, 446	839	583
34	82.9	luteoxanthin (a) laureate ($C_{12:0}$)	401, 423, 448	783	583
35	82.9	luteoxanthin (a) myristate ($C_{14:0}$)	401, 423, 448	811	583
36	83.0	luteoxanthin (a) palmitate ($C_{16:0}$)	401, 423, 448	839	583
37	86.9	violaxanthin laureate ($C_{12:0}$)	419, 440, 468	783	583
38	87.0	violaxanthin myristate ($C_{14:0}$)	419, 440, 468	812	583
39	87.0	violaxanthin palmitate ($C_{16:0}$)	419, 440, 468	839	583
40	92.6	lutein	422, 446, 474	551[b]	

[a] s, spectral shoulder. [b] $[M + H - H_2O]^+$.

the other hand, from the identification carried out and the 2D plot shown in **Figure 2**, it is possible to deduce the elution order in the normal phase separation carried out in the first dimension. It can be appreciated that the first eluted compounds were the hydrocarbons. Regarding the rest of the compounds, it could be thought that according to their polarity, diesters would elute more quickly than monoesters. However, this was the case only when considering a particular carotenoid. Thus, the carotenoid structure was found to be of great importance because the presence of free polar groups had a stronger influence than the presence of two fatty acids. Therefore, the elution order of the several mono- and diesters found corresponded to a combination between their esterification degree and the polarity of the carotenoid bound to their structure.

Carotenoids in Red Orange Essential Oil. In **Table 1** the identification of the free carotenoids and carotenoid monoesters found is shown. In the first place, it was possible to identify two hydrocarbons, ζ-carotene (7,8,7′8′-tetrahydro-ψ,ψ-carotene) and phytofluene (7,7′,8,8′,11,12-hexahydro-15-*cis*-ψ,ψ-carotene). These carotenoids are quite similar (see **Figure 1**) and coeluted in the two dimensions. However, thanks to the differences between their UV−vis spectra (maxima at 381, 401, and 426 nm for ζ-carotene and at 334, 350, and 369 nm for phytofluene) and molecular weights, it was possible to correctly identify these two compounds in the first peak eluted in the 2D plot (**Figure 2**). The retention time of the β-carotene standard was similar to those of these two compounds. However, β-carotene was not detected in the sample. Next to these compounds, several β-cryptoxanthin monoesters eluted from the first-dimension separation. Although these compounds coeluted in the first dimension, they were adequately separated in the second dimension. That is, it was possible to identify β-cryptoxanthin laureate ($C_{12:0}$), myristate ($C_{14:0}$), and palmitate ($C_{16:0}$) (peaks 3, 4, and 5, respectively). The identification of these esters agreed with the data found in the literature (*34*), these three esters being considered the most common β-cryptoxanthin esters in plants. Besides, as can be appreciated in **Figure 2**, the β-cryptoxanthin palmitate seemed to be the most abundant β-cryptoxanthin ester, followed by its myristate ester and finally the β-cryptoxanthin laureate. Again, these data ratified the previous studies published regarding the esters found in orange (*23*).

Table 2. UV−Vis and MS Information and Identification of the Carotenoid Diesters Found in the Red Orange Essential Oil

ID	retention time (min)	identification	UV−vis maxima	$[M + H]^+$	$[M + H - FA_1 - FA_2]^+$ and other main ions
10	49.6	antheraxanthin laureate palmitate ($C_{12:0}$, $C_{16:0}$)	422, 444, 469	1005	531,[b] 805 ($[M + H - C_{12:0}]^+$)
11	49.8	antheraxanthin myristate palmitate ($C_{14:0}$, $C_{16:0}$)	422, 444, 469	1033	531,[b] 777 ($[M + H - C_{16:0}]^+$)
12	49.9	antheraxanthin dipalmitate ($C_{16:0}$, $C_{16:0}$)	422, 444, 469	1061	531,[b] 805 ($[M + H - C_{16:0}]^+$)
13	53.4	luteoxanthin (a) laureate palmitate ($C_{12:0}$, $C_{16:0}$)	397, 420, 446	1021	565, 821 ($[M + H - C_{12:0}]^+$)
14	53.5	luteoxanthin (a)-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	397, 420, 446	1049	565, 803 ($[M + H - C_{14:0}]^+$)
15	53.6	luteoxanthin (a) dipalmitate ($C_{16:0}$, $C_{16:0}$)	397, 419, 446	1059[a]	565, 803 ($[M + H - C_{16:0} - H_2O]^+$)
16	55.4	violaxanthin laureate palmitate ($C_{12:0}$, $C_{16:0}$)	415, 437, 466	1003[a]	565, 765 ($[M + H - C_{16:0}]^+$), 821($[M + H - C_{12:0}]^+$)
17	55.5	violaxanthin myristate palmitate ($C_{14:0}$, $C_{16:0}$)	415, 437, 466	1049	565, 775 ($[M + H - C_{16:0} - H_2O]^+$), 803 ($[M + H - C_{14:0}$-$H_2O]^+$)
18	55.7	violaxanthin dipalmitate ($C_{16:0}$, $C_{16:0}$)	415, 437, 466	1059[a]	565, 803 ($[M + H - C_{16:0} - H_2O]^+$)
19	57.4	auroxanthin laureate palmitate ($C_{12:0}$, $C_{16:0}$)	381, 401, 425	1021	565, 1003 ($[M + H - H_2O]^+$), 803 ($[M + H - C_{12:0} - H_2O]^+$), 747 ($[M + H - C_{16:0} - H_2O]^+$)
20	57.5	auroxanthin myristate palmitate ($C_{14:0}$, $C_{16:0}$)	381, 401, 425	1049	565, 1031 ($[M + H - H_2O]^+$), 803 ($[M + H - C_{14:0} - H_2O]^+$), 775 ($[M + H - C_{16:0} - H_2O]^+$)
21	57.6	auroxanthin dipalmitate ($C_{16:0}$, $C_{16:0}$)	381, 402, 426	1078	565, 821 ($[M + H - C_{16:0}]^+$)
22	59.2	luteoxanthin (b) dilaureate ($C_{12:0}$, $C_{12:0}$)	401, 420, 447	965	565, 765 ($[M + H - C_{12:0}]^+$)
23	59.3	luteoxanthin (b) caproate palmitate ($C_{10:0}$, $C_{16:0}$)	400, 420, 446	993	565, 975 ($[M + H - H_2O]^+$)
24	59.4	luteoxanthin (b) laureate palmitate ($C_{12:0}$, $C_{16:0}$)	398, 420, 446	1003[a]	565, 747 ($[M + H - C_{16:0} - H_2O]^+$), 803 ($[M + H - C_{12:0} - H_2O]^+$)
25	59.5	luteoxanthin (b) myristate palmitate ($C_{14:0}$, $C_{16:0}$)	398, 420, 446	1049	565, 1031 ($[M + H - H_2O]^+$), 803 ($[M + H - C_{14:0} - H_2O]^+$), 775 ($[M + H - C_{16:0} - H_2O]^+$)
26	59.6	luteoxanthin (b) dipalmitate ($C_{16:0}$, $C_{16:0}$)	400, 420, 446	1077	565, 803 ($[M + H - C_{16:0} - H_2O]^+$)
27	61.3	auroxanthin isomer caproate palmitate ($C_{10:0}$, $C_{16:0}$)	382, 403, 429	993	565, 975 ($[M + H - H_2O]^+$)
28	61.4	auroxanthin isomer laureate palmitate ($C_{12:0}$, $C_{16:0}$)	382, 403, 429	1021	565, 1003 ($[M + H - H_2O]^+$), 747 ($[M + H - C_{16:0} - H_2O]^+$), 803 ($[M + H - C_{12:0} - H_2O]^+$)
29	61.5	auroxanthin isomer myristate palmitate ($C_{14:0}$, $C_{16:0}$)	382, 403, 429	1049	565, 1033 ($[M + H - H_2O]^+$), 775 ($[M + H - C_{16:0} - H_2O]^+$)
30	61.6	auroxanthin isomer dipalmitate ($C_{16:0}$, $C_{16:0}$)	382, 403, 429	1077	565, 1059 ($[M + H - H_2O]^+$), 821 ($[M + H - C_{16:0}]^+$)

[a] $[M + H - H_2O]^+$. [b] $[M + H - FA_1 - FA_2 - H_2O]^+$.

β-Cryptoxanthin has been identified as a major carotenoid in orange saponified samples (*14*) and has great importance from a health point of view. Several health benefits have been described related to β-cryptoxanthin such as prevention of bone loss (*35*) as well as provitamin A effect (*36*) and antioxidant properties (*2*). Coeluting in the first-dimension separation with these esters (peaks 3−5) were separated three other compounds in the second dimension (see peaks below peak 3 in **Figure 2**). The UV−vis signal recorded for these three compounds was not extremely good but allowed us to distinguish the typical carotenoid profile with maxima at 402, 425, and 450 nm. The three MS spectra of these compounds presented a main peak of *m*/*z* 401. Together with this ion it was possible to detect others of *m*/*z* 657, 629, and 601 for the peak immediately below peak 3 and the peaks underneath. Thus, the UV−vis spectra and the ions found could correspond to a fragmentation pattern of monoesters formed by a C_{30}-based carotenoid, β-citraurinene esterified with palmitic ($C_{16:0}$), myristic ($C_{14:0}$), and lauric ($C_{12:0}$) acids, respectively. On the other hand, the lack of free polar groups in its structure would have motivated their elution together with the β-cryptoxanthin esters in the first dimension; its shorter chain would have aided the faster elution in the second-dimension reversed phase separation compared to those other monoesters. This carotenoid has been described in orange (*37*), but there are no more recent reports confirming its presence in this fruit. However, although these kinds of C_{30} carotenoids, usually called apo-carotenoids, have been described in citrus peels (*38*), they have never been proposed to form carotenoid esters in any matrix. Therefore, we did not conclude an unequivocal identification of these three peaks. The next group of eluting compounds in the first-dimension analysis were several lutein monoesters (peaks 6–9). The higher retention of these compounds in the normal phase separation compared to the β-cryptoxanthin esters is explained by the presence of a free hydroxyl group in their structure, therefore, conferring higher polarity. In this case, combining the information provided by the MS and DAD detectors, it was possible to identify lutein laureate ($C_{12:0,}$ peak 6), myristate ($C_{14:0,}$ peak 7), palmitate ($C_{16:0,}$ peak 8), and stearate ($C_{18:0,}$ peak 9). Again, the palmitate monoester was the most abundant. On the contrary, lutein stearate was the lutein monoester found in less quantity. The main property related to this xanthophyll is age-related macular degradation prevention (*39, 40*), and it has been also identified as one of the main carotenoids in orange (*14*).

Close to these compounds eluted a wide group (in an 8 min gap) of compounds formed by diesters. Several carotenoid

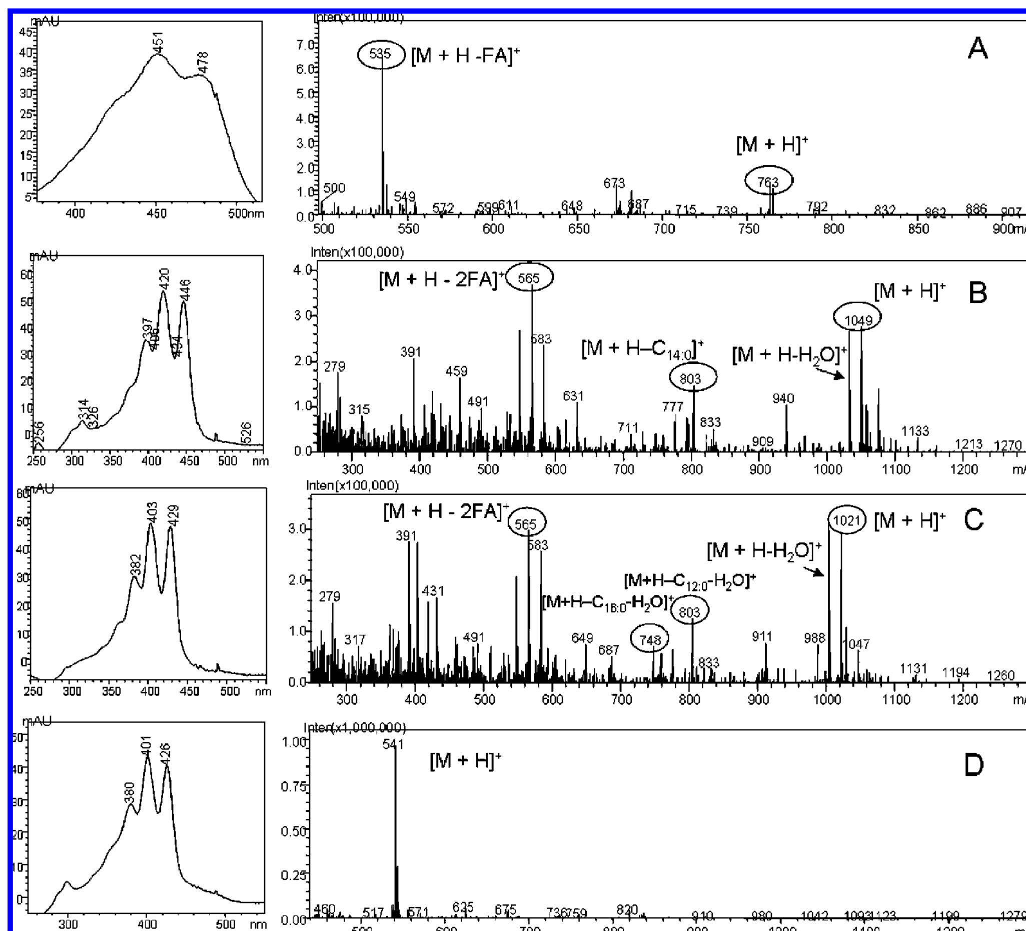

Figure 3. Examples of the determination of different carotenoid esters combining the UV−vis and MS information: (**A**) β-cryptoxanthin myristate ($C_{14:0}$); (**B**) luteoxanthin (a) myristate palmitate ($C_{14:0}$, $C_{16:0}$); (**C**) auroxanthin laureate palmitate ($C_{12:0}$, $C_{16:0}$); (**D**) ζ-carotene.

diesters have been identified thanks to the data provided by the MS detector (molecular ions and several losses and complementary ions that helped the identification) as well as by their typical UV−vis spectra (peaks 10–30). In **Figure 3**, some examples of identification of these diesters (**Figure 3B**,**C**) together with a monoester (**Figure 3A**) and a free carotenoid (**Figure 3D**) are shown. For these compounds typical ions formed by the pseudomolecular ion ($[M + H]^+$) and losses of one ($[M + H - FA_1]^+$, $[M + H - FA_2]^+$) or two fatty acids ($[M + H - FA_1 - FA_2]^+$) as well as several losses of water ($[M + H - H_2O]^+$, $[M + H - FA - H_2O]^+$) were produced, enabling the identification of the fatty acids implied in the molecule. Each group of carotenoid diesters coeluted in the first dimension, as occurred with the monoesters, and was separated under RP conditions in the second dimension. Besides, as can be observed in **Figure 2**, the elution from the first-dimension separation of these compounds was very close. This was due to the fact that almost all of the carotenoids found forming diesters were very similar in their chemical structure, having a fatty acid esterified and an epoxide group in each of their two rings (see **Figure 1**). This chemical structure was also responsible for the slower elution of these diesters compared to the above-described β-cryptoxanthin and lutein monoesters. The fatty acid by far most abundant bound to almost all diesters was palmitic acid. The first group of diesters was formed by antheraxanthin diesters, namely, antheraxanthin laureate palmitate ($C_{12:0}$, $C_{16:0}$), myristate palmitate ($C_{14:0}$, $C_{16:0}$), and antheraxanthin dipalmitate ($C_{16:0}$, $C_{16:0}$) (see compounds **10–12** in **Table 2**). These diesters were more retained in the second-dimension separation due to the higher hydrophobicity of antheraxanthin compared to the other carotenoids forming diesters. The next group of diesters were formed by luteoxanthin (a) (see compounds **13–15** in **Table 2**). Namely, the luteoxanthin (a) laureate palmitate ($C_{12:0}$, $C_{16:0}$), myristate palmitate ($C_{14:0}$, $C_{16:0}$), and dipalmitate ($C_{16:0}$, $C_{16:0}$) were identified. Exactly the same diesters were found eluting closely for violaxanthin (peaks 16–18) and auroxanthin (peaks 19–21). Whereas luteoxanthin and violaxanthin have been described as main carotenoids in orange (*12*), this was not the case for auroxanthin, although it has been also identified in orange samples (*14, 41*). The same kind of violaxanthin diesters have been similarly identified in potato (*42*), whereas some of the auroxanthin diesters were described using LC combined with GC (*32*). The other two groups of carotenoid diesters identified were formed by luteoxanthin (b) and auroxanthin isomer (peaks 22–26 and 27–30, respectively). In this case, in addition to the types of diesters previously found, a new caproate palmitate ($C_{10:0}$, $C_{16:0}$) diester could be identified [peaks 23 and 27 for luteoxanthin (b) and auroxanthin, respectively]. Besides, luteoxanthin (b) dilaureate ($C_{12:0}$, $C_{12:0}$) could be also identified with a pseudomolecular ion $[M + H]^+$ 956 and fragments of *m*/*z* 765 and 565 corresponding to $[M + H - C_{12:0}]^+$ and $[M + H - 2FA]^+$, respectively. This type of diester did not appear among the rest of the carotenoids and was the only diester not including, at least, an esterified palmitic acid. Moreover, some other peaks coeluting with the luteoxanthin (b) diesters (see peaks below peak 22 in **Figure 2** were separated in the second-dimension analysis. Although these compounds presented a UV−vis spectrum typical of apocarotenoids, with a single absorption maximum, their identification could not be unequivocally

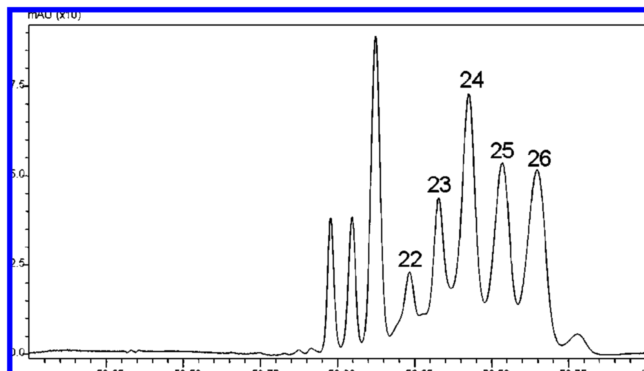

Figure 4. Second-dimension chromatogram (450 nm) corresponding to a 2 min analysis. For peak identification, see **Table 2**.

achieved. However, the separation power of the present methodology can be deduced from **Figure 4**. In this figure, the chromatogram corresponding to one second-dimension separation shows, concretely, the separation of the luteoxanthin (b) diesters together with the above-mentioned unidentified compounds. In total, in this particular separation up to nine compounds are adequately separated in a short analysis time.

The last group of compounds eluting from the first-dimension separation was formed by carotenoid monoesters. In this case, as can be observed in **Table 1**, all of the compounds could be identified according to their diverse UV−vis spectra as well as the detection of the pseudomolecular ion ($[M + H]^+$) and the loss of the fatty acid bound to the carotenoid ($[H + H - FA]^+$). In this sense, antheraxanthin palmitate ($C_{16:0}$) (peak 31) was identified in first place. Eluting close to this compound, luteoxanthin (b) myristate ($C_{14:0}$) (peak 32) and palmitate ($C_{16:0}$) (peak 33) were also determined. Later, two groups of monoesters were found, which coeluted in the first-dimension separation. However, these monoesters were separated in the second-dimension separation, allowing their identification as luteoxanthin (a) laureate ($C_{12:0}$), myristate ($C_{14:0}$), and palmitate ($C_{16:0}$) (peaks 34, 35, and 36, respectively) and violaxanthin laureate ($C_{12:0}$), myristate ($C_{14:0}$), and palmitate ($C_{16:0}$) (peaks 37, 38, and 39, respectively). To our knowledge, up to now, these carotenoid monoesters have not been described in orange, although their free carotenoids, antheraxanthin, luteoxanthin, and violaxanthin, have been extensively determined in this matrix. Finally, although the UV−vis spectra obtained were not of high quality, thanks to the use of its commercial standard and its MS spectra, peak 40 was identified as lutein. This free xanthophyll possesses two free hydroxyl groups, which allowed its higher retention in the first dimension and, at the same time, a quite fast elution in the second dimension. Concretely, this was the only free xanthophyll detected in sufficient amount to be identified in the red orange essential oil.

In agreement with the chromatographic behavior above explained, in all of those monoester compounds (peaks 32–39), the presence of a hydroxyl group together with two epoxy groups increased their relative polarity, making these compounds more retained than their respective diesters and also than the other less polar monoesters (i.e., β-cryptoxanthin and lutein monoesters). Interestingly, as can be observed in **Figure 2**, the respective group of monoesters eluted in the second dimension at similar retention times when the carotenoid included in the molecule was similar. That is, in a hypothetical single reversed phase separation, all of the palmitate monoesters of luteoxanthin (b), luteoxanthin (a), and violaxanthin would be coeluted, as well as their myristate monoesters and laureate monoesters. The same would be valid for the same kind of diesters. However, in this case, the presence of two fatty acids made the retention times in the second dimension higher, as would be expected considering their higher hydrophobicity. On the other hand, less polar carotenoid monoesters, such as those formed by β-cryptoxanthin, had retention times comparable to those of the diesters found.

Using this modified LC × LC method a theoretical peak capacity of 551 was calculated (*43*), corresponding to individual peak capacities of 29 and 19 for the first NP and the second RP separations, respectively. This value effectively represents the great capabilities of the developed LC × LC-DAD/APCI-MS method to separate and identify the native carotenoid composition of orange essential oil without any kind of sample pretreatment. Although the first-dimension peak capacity could seem relatively too low considering the long analysis times, the low flow rates employed (10 μlL/min) together with the fact that wide peaks were desired (to be sampled more than once in the second dimension) prevented the achievement of higher peak capacities in this first dimension.

In summary, the ability of a modified LC × LC-DAD/APCI-MS method previously developed at our laboratory to analyze and identify the native carotenoid composition of an extremely complex matrix such as red orange essential oil was demonstrated. By using the present methodology, it was possible to identify 40 different carotenoids in the orange oil including 3 free carotenoids, 16 carotenoid monoesters, and 21 carotenoid diesters, analyzed without any kind of sample pretreatment. The most abundant carotenoids were β-cryptoxanthin, forming palmitate, myristate, and laureate monoesters, and luteoxanthin, forming different mono- and diesters. To the best of our knowledge, this is the first time that carotenoid diesters are described and identified in orange as well as antheraxanthin, luteoxanthin, and violaxanthin monoesters. Moreover, this is the first application of LC × LC devoted to the analysis of such a complex source of carotenoids as the native carotenoid fraction of orange. The present optimized methodology could be effectively applied to the identification of the intact carotenoid pattern of other interesting natural matrices.

ACKNOWLEDGMENT

We gratefully thank Shimadzu and Sigma-Aldrich/Supelco Corporations for their continuous support.

LITERATURE CITED

(1) Leach, G.; Olivera, R.; Morais, R. Production of a carotenoid-rich product by alginate entrapment and fluid-bed drying of *Dunaliella salina*. *J. Sci. Food Agric* **1998**, *76*, 298–302.
(2) de Quiros, A. R. B.; Costa, H. S. Analysis of carotenoids in vegetable and plasma samples: a review. *J. Food Compos. Anal.* **2006**, *19*, 97–111.
(3) Beutner, S.; Bloedorn, B.; Frixel, S.; Hernandez Blanco, I.; Hoffman, T.; Martin, H. D.; Mayer, B.; Noack, P.; Ruck, C.; Schmidt, M.; Schulke, I.; Sell, S.; Ernst, H.; Haremza, S.; Seybold, G.; Sies, H.; Stahl, W.; Walsh, R. Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of β-carotene in antioxidant functions. *J. Sci. Food Agric.* **2001**, *81*, 559–568.
(4) Arab, L.; Steck, S. Lycopene and cardiovascular disease. *Am. J. Clin. Nutr.* **2000**, *71*, 1691S–1695S
(5) Rao, A. V.; Rao, R. G. Carotenoids and human health. *Pharm. Res.* **2007**, *55*, 207–216.
(6) Omoni, A. O.; Aluko, R. E. The anti-carcinogenic and anti-atherogenic effects of lycopene: a review macular degeneration. *Trends Food Sci. Technol.* **2005**, *16*, 344–350.

(7) Nishino, H.; Tokuda, H.; Satomi, Y.; Masuda, M.; Bu, P.; Onozuka, M.; Yamaguchi, S.; Okuda, Y.; Takayasu, J.; Tsuruta, J.; Okuda, M.; Ichiishi, E.; Murakoshi, M.; Kato, T.; Misawa, N.; Narisawa, T.; Takasuka, N.; Yano, M. Cancer prevention by carotenoids. *Pure Appl. Chem.* **1999**, *12*, 2273–2278.
(8) Snodderly, M. D. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am. J. Clin. Nutr.* **1995**, *62*, S1448–S1461.
(9) Goodner, K. L.; Rouseff, R. L.; Hofsomer, H. J. Orange, mandarin and hybrid classification using multivariate statistics based on carotenoid profiles. *J. Agric. Food Chem.* **2001**, *49*, 1146–1150.
(10) Mendes-Pinto, M. M.; Silva Ferreira, A. C.; Oliveira, M. B. P. P.; Guedes de Pinto, P. Evaluation of some carotenoids in grapes by reversed and normal-phase liquid chromatography: a qualitative analysis. *J. Agric. Food Chem.* **2004**, *52*, 3182–3188.
(11) Jaime, L.; Mendiola, J. A.; Ibañez, E.; Martin-Alvarez, P. J.; Cifuentes, A.; Reglero, G.; Señorans, F. J. β-Carotene isomer composition of sub- and supercritical carbon dioxide extracts. Antioxidant activity measurement. *J. Agric. Food Chem.* **2007**, *55*, 10585–10590.
(12) Melendez-Martinez, A. J.; Vicario, I. M.; Heredia, F. J. A routine high-performance liquid chromatography method for carotenoid determination in ultrafrozen orange juices. *J. Agric. Food Chem.* **2003**, *51*, 4219–4224.
(13) Melendez-Martinez, A. J.; Britton, G.; Vicario, I. M.; Heredia, F. J. Relationship between the colour and the chemical structure of carotenoid pigments. *Food Chem.* **2007**, *101*, 1145–1150.
(14) Agocs, A.; Nagy, V.; Szabo, Z.; Mark, L.; Ohmacht, R.; Deli, J. Comparative study on the carotenoids composition of the peel and the pulp of different citrus species. *Innovative Food Sci. Emerg. Technol.* **2007**, *8*, 390–394.
(15) Matsumoto, H.; Ikoma, Y.; Kato, M.; Kuniga, T.; Nakagima, N.; Yoshida, T. Quantification of carotenoids in citrus fruit by LC-MS and comparison patterns of seasonal changes for carotenoids among citrus varieties. *J. Agric. Food Chem.* **2007**, *55*, 2356–2368.
(16) Rouseff, R.; Raley, L. Application of diode array detection with C-30 reversed phase column for the separation and identification of saponified orange juice carotenoids. *J. Agric. Food Chem.* **1996**, *44*, 2176–2181.
(17) Melendez-Martinez, A. J.; Britton, G.; Vicario, I. M.; Heredia, F. J. Identification of isolutein (lutein epoxide) as *cis*-antheroxanthin in orange juice. *J. Agric. Food Chem.* **2005**, *53*, 9369–9373.
(18) Schlatterer, J.; Breithaupt, D. E. Cryptoxanthin structural isomers in orange, orange juice, and other fruits. *J. Agric. Food Chem.* **2005**, *53*, 635–6361.
(19) Dugo, P.; Skerikova, V.; Kumm, T.; Trozzi, A.; Jandera, P.; Mondelo, L. Elucidation of carotenoid patterns in citrus products by means of comprehensive normal-phase × reversed-phase liquid chromatography. *Anal. Chem.* **2006**, *78*, 7743–7750.
(20) Melendez-Martinez, A. J.; Vicario, I. M.; Heredia, F. J. Geometrical isomers of violaxanthin in orange juice. *Food Chem.* **2007**, *104*, 169–175.
(21) de Rosso, V. V.; Mercadante, A. Z. Identification and quantification of carotenoids by HPLC-PDA-MS/MS from Amazonian fruits. *J. Agric. Food Chem.* **2007**, *55*, 5062–5072.
(22) Giuffrida, D.; La Torre, L.; Stelitano, M.; Pellicanò, T. M.; Dugo, G. Application of HPLC-APCI-MS with a C-30 reversed phase column for the characterization of carotenoid esters in mandarin essential oil. *Flavour Fragrance J.* **2006**, *21*, 319–323.
(23) Breithaupt, D. E.; Bamedi, A. Carotenoid esters in vegetables and fruits: a screening with emphasis on β-cryptoxanthin esters. *J. Agric. Food Chem.* **2001**, *49*, 2064–2070.
(24) Breithaupt, D. E. Identification and quantitation of astaxanthin esters in shrimp (*Pandulus borealis*) and in microalga (*Haematococcus pluvialis*) by liquid chromatography−mass spectrometry using negative ion atmospheric pressure chemical ionization. *J. Agric. Food Chem.* **2004**, *52*, 3870–3875.
(25) Miao, F.; Lu, D.; Li, Y.; Zeng, M. Characterization of astaxanthin esters in *Haematococcus pluvialis* by liquid-chromatography−atmospheric pressure chemical ionization mass spectrometry. *Anal. Biochem.* **2006**, *352*, 176–181.
(26) Weller, P.; Breithaupt, D. E. Identification and quantification of zeaxanthin esters in plants using liquid chromatography−mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 7044–7049.
(27) Pott, I.; Breithaupt, D. E.; Carle, R. Detection of unusual carotenoid esters in fresh mango (*Mangifera indica* L. cv. 'Kent'). *Phytochemistry* **2003**, *64*, 82–829.
(28) Melendez-Martinez, A. J.; Vicario, I. M.; Heredia, F. J. Analysis of carotenoids in orange juice. *J. Food Compos. Anal* **2007**, *20*, 638–649.
(29) Sander, L. C.; Sharpless, K. E.; Craft, N. E.; Wise, S. A. Development of engineered stationary phases for the separation of carotenoid isomers. *Anal. Chem.* **1994**, *66*, 1667–1674.
(30) Dugo, P.; Herrero, M.; Kumm, T.; Giuffrida, D.; Dugo, G.; Mondello, L. Comprehensive normal-phase × reversed-phase liquid chromatography coupled to photodiode array and mass spectrometry detectors for the analysis of free carotenoids and carotenoid esters from mandarin. *J. Chromatogr., A* **2008**, *1189*,196−206.
(31) Zonta, F.; Stancher, B.; Marletta, G. P. Simultaneous high-performance liquid chromatographic analysis of free carotenoids and carotenoid esters. *J. Chromatogr.* **1987**, *403*, 207–215.
(32) Philip, T.; Chen, T. S.; Nelson, D. B. Liquid chromatographic profiles of major carotenoid esters in commercially processed California navel and Valencia orange juice concentrates. *J. Chromatogr.* **1988**, *442*, 249–265.
(33) Dugo, P.; Cacciola, F.; Kumm, T.; Dugo,G.; Mondello, L. Comprehensive multidimensional liquid chromatography: theory and applications. *J. Chromatogr., A* **2008**, *1184*,353−368.
(34) Breithaupt, D. E. *J. Agric. Food Chem.* **2001**, *49*, 5064–5070.
(35) Yamaguchi, M.; Igarashi, A.; Uchiyama, S.; Sugawara, K.; Sumida, T.; Morita, S.; Ogawa, H.; Nishitani, M.; Kajimoto, Y. Effect of β-cryptoxanthin on circulating bone metabolic markers: Intake of juice (*Citrus unshiu*) supplemented with β-cryptoxanthin has an effect in menopausal women. *J. Health Sci.* **2006**, *52*, 758–768.
(36) Uchiyama, S.; Yamaguchi, M. Oral administration of β-cryptoxanthin prevents bone loss on streptozotocin-diabetic rats in vivo. *Biol. Pharm. Bull.* **2005**, *28*, 1766–1769.
(37) Leuenberger, U.; Stewart, I. β-Citraurinene, a new C30-citrus carotenoid. *Phytochemistry* **1976**, *15*, 227–229.
(38) Rodrigo, M. J.; Marcos, J. F.; Alférez, F.; Mallent, M. D.; Zacarías, L. Characterization of pinalate, a novel *Citrus sinensis* mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. *J. Exp. Bot.* **2003**, *54*, 727–738.
(39) Mares-Perlman, J. A.; Millen, A. E.; Ficek, T. L.; Hankinson, S. E. The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. *J. Nutr.* **2002**, *132*, 518S–524S
(40) Beatty, S.; Boulton, M.; Henson, D.; Koh, H. H.; Murray, I. J. Macular pigment and age-related macular degeneration. *Br. J. Ophthalmol.* **1999**, *83*, 867–877.
(41) Gama, J. J. T.; Sylos, C. M. Major carotenoid composition of Brazilian Valencia orange juice: identification and quantification by HPLC. *Food Res. Int.* **2005**, *38*, 899–903.
(42) Breithaupt, D. E.; Bamedi, A. Carotenoids and carotenoid esters in potatoes (*Solanun tuberosum* L.): new insights into an ancient vegetable. *J. Agric. Food Chem.* **2002**, *50*, 7175–7181.
(43) Neue, U. D. Theory of peak capacity in gradient elution. *J. Chromatogr., A* **2005**, *1079*, 153–161.

Received for review January 16, 2008. Revised manuscript received March 6, 2008. Accepted March 8, 2008. The project was funded by the Italian Ministry for the University and Research (MUR) with a PNR 2005-2007 Project RBIP06SXMR "Sviluppo di metodologie innovative per l'analisi di prodotti agroalimentari". M.H. thanks the Spanish Ministry of Education and Science for a postdoctoral fellowship.

JF800144V