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Analysis of Native Carotenoid Composition of Sweet Bell Peppers by Serially Coupled C_{30} Columns

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Serial coupled columns reversed-phase separations in high-performance liquid chromatography can be a useful tool for the analysis of complex real samples. The great difficulties found when analyzing complex carotenoid samples, due to the high natural variability of these compounds, as well as to the presence of carotenoid esters, are well documented. In the present contribution, the applicability of connecting two C_{30} columns to increase significantly the separation power, resolution and peak capacity for the analysis of carotenoids in a complex carotenoid sample, like sweet bell peppers, has been shown for the first time. By using LC coupled to PDA/APCI-MS detectors, 56 different carotenoids have been detected in red sweet bell peppers. By using two serial coupled C_{30} columns a peak capacity of 95.4 was obtained, compared with 73 achieved using a single column. Moreover, resolution greatly improved between different critical peaks when using two serial coupled C_{30} columns, compared with a single column. Interestingly, free carotenoids, mono-esters and di-esters were quantitatively equally represented (around 33% for each different class) in red sweet bell pepper, showing, therefore, a value for the ratio of mono-esters/di-esters of around 1, which could be considered a parameter of typicality. Free β-carotene (12.6%), capsanthin-C14:0 (8.4%), and capsanthin-C12:0-C14:0 (8.9%) were the most abundant carotenoids in the three different classes of red sweet bell pepper. No carotenoid esters were detected in either yellow or green sweet bell peppers. The application of such methodology in the analysis of other complex carotenoid matrices could be a future objective of research.

Keywords: carotenoids, sweet bell peppers, liquid chromatography-DAD-APCI-MS.

Carotenoids are based on a C_{40} tetraterpenoid skeleton which can undergo a high diversity of modifications, such as cyclization in one or both ends, hydrogenation, dehydrogenation, and addition of lateral groups, among others, resulting in an extremely wide group of compounds. Usually, these compounds are divided into two groups: hydrocarbons (commonly known as carotenes) and oxygenated compounds (generally named xanthophylls). To further increase the natural variability of these compounds, it has to be considered that the carotenoids can be present in nature as either free carotenoids or in a more stable form esterified with fatty acids, in the case of the oxygenated compounds. To simplify to some extent their analysis, a saponification procedure has been traditionally employed to release all the carotenoid esters and to analyze all these compounds in their free form. Although this saponification step acts also as a clean-up procedure, some drawbacks are found, mainly related to the formation of artefacts, as well as to the production of carotenoid degradation. Moreover, as a result of the saponification step, information on the native carotenoid composition of the studied samples is lost. Thus a better approach to carotenoid content is through classifying plant materials depending on either a free or esterified xanthophylls profile. In fact, the carotenoid esters could be used as a marker of authenticity of food products and could be useful as a ripeness degree index; moreover, esters may enhance food product oxidative stability and may improve carotenoid bioavailability. Although esterification does not change the chromophore properties of the carotenoid molecules, it does modify the immediate molecular environment; thus, chemical activities may be altered depending on the kind of fatty acid bound to the xanthophylls. 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 a great deal of attention since they have been described to possess several important functional properties, mainly antioxidant activity [1], as well as prevention of cardio vascular diseases [2,3], cancer [4] and macular degeneration [5]. These properties make these compounds ideal for the always increasing functional food industry, as well as promoting the

required and recommended amounts of carotenoids in the human diet. *Capsicum annuum* is a carotenogenic fruit: during ripening, the transformation of the chloroplast into chromoplast occurs. Chlorophylls disappear and more and new carotenoids are formed. *Capsicum* species uniquely have capsanthin-capsorubin synthase that synthesizes two red pigments, capsanthin and capsorubin. Moreover, esterification greatly increases during the fruit ripening process. *Capsicum* is one of the oldest and most popular vegetables and spices in the world. Considering the carotenoid profile among various *C. annuum* cultivars [6-16], the spicy ones have received greater attention than the non spicy cultivars; the native carotenoid profile in sweet bell peppers had not been previously investigated. Therefore, the aim of this study was to investigate the native carotenoid composition in sweet bell peppers by developing an analytical LC method which allowed the direct identification of the carotenoids in the samples, based on the use of serially connected C_{30} columns coupled with DAD and APCI-MS detectors. High performance liquid chromatography has been selected as the analytical tool for a large number of applications, including carotenoid analysis. However, due to the great complexity of some natural samples containing this kind of compound, conventional LC could not have enough separation power. In this work, serial connection of two columns is proposed as an alternative to conventional LC. The applicability of connecting two C_{30} columns to increase significantly the separation power, resolution and peak capacity for the analysis of carotenoids in sweet bell peppers has been demonstrated. Moreover, here we report the first investigation of the native carotenoid profile in sweet bell peppers at three different ripening stages: green, yellow and red (Figures 1, 2 and 3). As shown in Figure 3B, 56 different carotenoids have been detected in red sweet bell peppers, including many esters (for peak identification see Table 1). No carotenoid esters were detected in either yellow or green sweet bell peppers. The identification of these compounds was carried out combining the information provided by the two detectors employed (i.e., DAD and APCI-MS detectors) and the commercial standards available. As can be observed in Figure 3, the order of elution of the different compounds is highly dependent on the polarity and hydrophobicity of the molecules. Therefore, free xanthophylls elute before mono-esterified ones, and the di-esterified xanthophylls have longer retention times. By considering the different fragmentations in the APCI-MS producing different regioisomers, various xanthophyll di-esters were detected in red bell peppers.

As can be seen in Table 2, by using two serially coupled C_{30} columns a peak capacity of 95.4 was obtained, compared with 73 using a single column. Moreover, the resolution values of some critical pairs (12-13, 29-30 and 32-33) were significantly improved by the coupling of the two C_{30}columns.

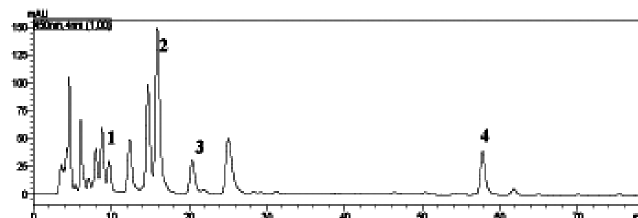

Figure 1: Chromatograms (455 nm) of sweet bell green peppers using one C30 column. Peak identification: 1. Luteoxanthin; 2. Lutein; 3. Zeaxanthin ; 4. β-Carotene.

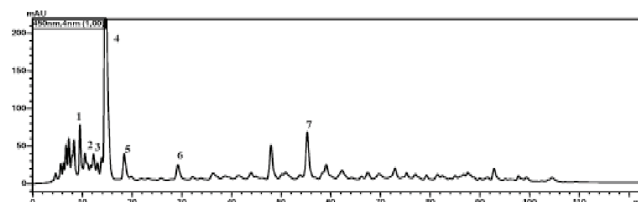

Figure 2: Chromatograms (455 nm) of sweet bell yellow peppers using one C30 column. Peak identification: 1. Luteoxanthin; 2. Auroxanthin; 3. Antheraxanthin ; 4. Lutein; 5. Zeaxanthin; 6. β-cryptoxanthin; 7. β-carotene

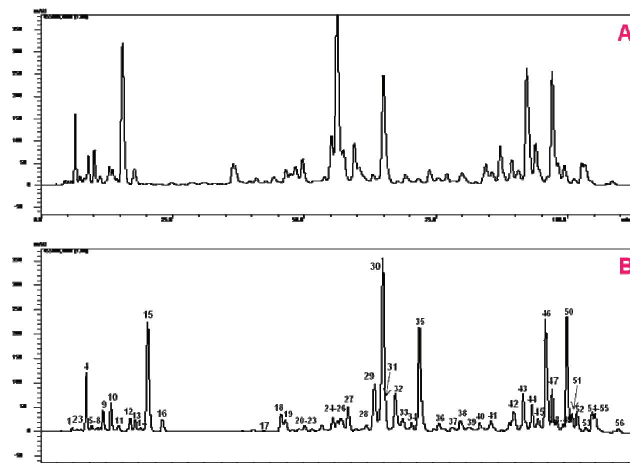

Figure 3: Chromatograms (455 nm) of sweet bell red peppers using one C_{30} column (A), and two C_{30} columns (B). For peak identification see Table 1.

Interestingly, free carotenoids, mono-esters and di-esters were quantitatively equally represented (around 33% for each different class) in red sweet bell pepper, and the ratio of di-esters/mono-esters was around 1, which could be considered a typicality parameter of red peppers. β-Carotene (6%), lutein (20-30%) and zeaxanthin (2-5%) were the most abundant carotenoids present in yellow and green sweet bell peppers, whereas violaxanthin (2.2%) capsanthin (8.1%) and β-carotene (12.6%) characterized the free carotenoid fraction of sweet red bell peppers. Myristic acid (C14:0) was the most abundant fatty acid present as mono-ester with capsanthin (8.4%), capsorubin (3.1%) and antheraxanthin (2.7%) in red bell peppers. Lauric and myristic acids were mainly present in the di-esters of capsanthin and capsorubin in red bell peppers. In particular, the most abundant di-esters in red bell peppers were capsanthin-C12:0, C14:0 (8.9%), capsanthin-C14:0, C14:0 (7.1%) and capsorubin-C14:0, C14:0 (3.3%).

No carotenoid esters were detected in either green or yellow sweet bell peppers. Interestingly, Minguez-Mosquera [6]

Table 1: UV-Vis, MS data and identification of the different carotenoids separated in sweet bell red pepper using two serially connected C_{30} columns.

No.	Peak identification	λ_{max} (nm)	$[M + H]^+$
1	Neoxanthin	416,440,469	601,583
2	n.i.	419,446,467	601,583
3	β-Apo-10-carotenal	436	376,361
4	Violaxanthin	418,441,470	601,583
5	Luteoxanthin	400,422,448	601,583
6	n.i.	418,440,467	613,595
7	n.i.	400,424,448	601,583
8	n.i.	400,423,448	615,597
9	Capsanthin-5,6-epoxide	469,486	601,583,565
10	Anteraxanthin	424s,445,474	585,567
11	n.i.	357,448,469	585,567
12	Mutatoxanthin A	406,429,451	585,567,549
13	n.i.	457s,479,507	601,583,567,491
14	Mutatoxanthin B	406,429,452	585,567,549
15	Capsanthin	474	585,567,479
16	Zeaxanthin	427,451,477	569,551,476
17	Phytofluene	332,348,367	543
18	β-Cryptoxanthin	426,451,477	553,535
19	Anteraxanthin-laureate	422,447,474	767,749,567
20	n.i.	400,424,448	875,597
21	n.i.	424,443,469	875,597,565
22	n.i.	451,467	823,583
23	n.i.	425s,447,467	877,823,597
24	n.i.	466	879,599,583
25	n.i.	400,424,448	879,793,597
26	Anteraxanthin-myristate	424,446,474	795,777,567
27	n.i.	425,446,474	876,777
28	Mutatoxanthin-laureate	408,425,452	767,749,567
29	Capsorubin-myristate	479	811,583,565
30	β-carotene	452,478	537
31	Capsanthin-laureate	474	767,567,549
32	Anteraxanthin-myristate	425,447,474	795,777,567
33	n.i.	424s,448,471	853,795,567
34	Mutatoxanthin-myristate	406,429,452	795,777,567
35	Capsanthin-myristate	474	795,777,567
36	n.i.	460	795,567,549
37	n.i.	426,451,474	879,684,533
38	n.i.	424,443,469	961,821,547
39	n.i.	466,472	823,567,549
40	β-Cryptoxanthin-laureate	426,451,477	735,535
41	n.i.	469	765,549
42	β-Cryptoxanthin-miristate	425,452,479	765,535
43	Capsanthin-di-laureate (C12:0,C12:0)	473	949,749,549
44	Capsorubin-laureate-myristate (C12:0,C14:0)	479	993,793,765,565
45	n.i.	428,451,470	1021,793,565
46	Capsanthin-laureate-myristate (C12:0,C14:0)	474	977,777,749,549
47	Capsorubin-di-myristate (C14:0,C14:0)	481	1021,793,565
48	Mutatoxanthin-laureate-myristate (C12:0,C14:0)	406,429,454	977,793,765,565
49	Zeaxanthin-laureate-myristate (C12:0,C14:0)	425,451,478	961,761,733,533
50	Capsanthin-di-myristate (C14:0,C14:0)	474	1005,777,549
51	n.i.	469	1005,777,749,549
52	Capsorubin-myristate-palmitate (C14:0-C16:0)	479	1049,821,793,565
53	Zeaxanthin-di-myristate (C14:0,C14:0)	427,452,477	989,761,533
54	Capsanthin-myristate-palmitate (C14:0,C16:0)	474	1033,805,777,549
55	n.i.	469	805,777,549
56	n.i.	469	805,713,549,551

Table 2: Values of Peak capacity (Pc) and resolution (Rs) between selected peaks for the analysis of sweet bell red peppers using two different set ups.

Set Up	Pc	Rs (12-13)	Rs (29-30)	Rs (32-33)
1 x C30	73.0	0.23	0.66	0.33
2 x C30	95.4	0.85	1.28	0.87

reported that in the yellow spicy *C. annuum* cultivar Bola, 50% of the carotenoids were esterified, and that at the fully ripened red stage the percentages of the free carotenoid pigments and the partially and totally esterified forms of these were 21.3%, 35.6%, and 43.1%, respectively, and, therefore, different from the percentages reported in this work for red sweet bell peppers, where the three fractions were equally represented. The increase in xanthophyll esterification during ripening reported in this work is in agreement with the report by Hornero-Mendez [8] for various cultivars of spicy *C. annuum* cultivars. This process has been related to a phenomenon intimately linked with and inherent to the degeneration of chloroplast and formation of chromoplast . Such a phenomenon might be the result of hydrophobicity requirements on the part of the carotenoid, so that, with all its hydroxyl groups esterified, it will be included more readily in the lipid matrix of chromoplast membranes and organelles (plastoglobules) [8]. Schweiggert *et al*. [9] reported that capsanthin and β-carotene were the main free carotenoids in spicy red peppers, in agreement with the results reported in this work for sweet red bell peppers, and they reported also a similar esters profile for spicy red pepper pods, although the sweet bell red peppers analyzed in this work showed a higher degree of esterification with capsanthin rather than with capsorubin. Apart from a very early report by Gregory *et al*. [11] on the carotenoid esters in sweet red bell peppers carried out by gas chromatographic analysis of the transesterified fatty acids obtained after saponification and which reported some generic tentative identifications, the work here reported is the first direct study of the native carotenoid composition of sweet bell peppers using a liquid chromatographic methodology. In fact, previous works on the carotenoid composition of sweet bell peppers [12-16] were carried out after a saponification step.

Although the application of the methodology reported in this work is instrumentally quite simple, it has been demonstrated to be a valid way to further improve resolution and efficiency in LC.

Experimental

Samples and chemicals: Fresh samples of green, yellow and red sweet bell peppers were supplied by a local producer. Carotenoid standards, namely, β-carotene, lutein, β-cryptoxanthin, zeaxanthin, capsanthin and lutein-di-palmitate ($C_{16:0}$, $C_{16:0}$) were purchased from Extrasynthese (Genay, France). All the carotenoid standards were stored protected from light at -18°C. All the solvents used, namely, methanol, methyl tert-butyl ether (MTBE) and water, were HPLC grade and purchased from Sigma-Aldrich (Milan, Italy). BHT (butylatedhydroxy

toluene) and potassium hydroxide were obtained from Sigma-Aldrich (Milan, Italy).

Carotenoid extraction: The sweet bell pepper samples (200 g) were homogenized, and the carotenoids extracted 4 times with methanol/ethyl acetate/light petroleum (1:1:1). The upper phase was kept and ca. 2 mg of BHT was added prior to evaporation under vacuum until dryness. The dry residue was then resuspended in a given volume of MTBE/methanol (1:1) and stored protected from light at -18°C until use.

LC-DAD/APCI-MS analyses: To carry out the analyses a Shimadzu HPLC instrument (Shimadzu, Milan, Italy) was employed including a SCL-10A-*VP* system controller, two LC-10AD-*VP* pumps and a SPD-10A*vp* diode array detector. Besides a Shimadzu mass spectrometer LCMS-2010 equipped with an APCI interface in the positive ion mode was installed in parallel. The APCI parameters were set as follows: probe voltage (kV), 4.5; probe temperature, 400°C; block temperature, 200°C; CDL temperature, 250°C; Q array voltage, 20 and 80 V; gas flow, 2.5 L/min. Data acquisition and system control was performed by the LCMSsolutionver 3.30 software (Shimadzu). The injections were made manually through a Rheodyne injection valve (injection volume 20 μL). The C_{30} columns employed consisted of two YMC 30 analytical columns (YMC Europe, Schermbeck, Germany) with 5 μm C_{30} reversed-phase material (250 × 4.6 mm I.D.), including a pre-column YMC 30 (S-5 μm, 10 × 4.0 mm I.D.). The HPLC solvent systems were (A) methanol/MTBE/water (83:15:2, v/v/v) and (B) methanol/MTBE/water (8:90:2, v/v/v) used following a linear gradient depending on the use of one or two serial coupled columns. Gradient for the separation with 1 x C_{30} column: 0-20 min, 0% B; 20-160 min 100% B; then reconditioning. Gradient for the separation with 2 x C_{30} columns: gradient times were doubled keeping unchanged the B%. The flow rate employed was 1 mL/min and the chromatograms were recorded at 450 nm and the UV-Vis spectra were recorded in the range from 250 to 600 nm (sampling rate: 12.5 Hz; time constant: 0.64 s). The column oven temperature was 40°C.

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