HPLC Analysis of Carotenoids from Tomatoes Using Cross-Linked C18 Column and MS Detection

Hussein G. Daood1,*, Gyula Bencze2, Gábor Palotás3, Zoltán Pék1, Akmal Sidikov4 and Lajos Helyes1

1Regional University Science Centre, Szent István University, Páter K. u. 1, Gödöllő H-2103, Hungary, 2Vichem Ltd., Herman Ottó u. 15, Budapest H-1022, Hungary, 3Univer Product Co., Szolnoki u. 35, Kecskeméti H-6000, Hungary, and 4Uzbe Research Institute of Cotton Breeding and Seed Production, University Street N1, Ribray District, Tashkent 218112, Uzbekistan

*Author to whom correspondence should be addressed. Email: hdaood682@gmail.com

Received 25 February 2013; revised 21 July 2013

This study was conducted to analyze carotenoids from tomatoes by high-performance liquid chromatography using reversed-phase C18 silica having cross-linked end-capping with diode array and mass spectrometric detection. An efficient gradient elution system was developed to achieve good and reliable separation of both major and minor carotenoids as well as their isomers. Resolution of lycopene, β-carotene and their isomers was 0.91–3.97 and 1.02–2.86 with cross-linked and conventional C18 column, respectively. The % recovery for zeaxanthin, lycopene and β-carotene was found to be in the range of 89–97%. Limits of detection and quantification of 19.44 and 64.79 ng/mL for zeaxanthin, 15.6 and 52.4 ng/mL for lycopene and 8.28 and 27.61 ng/mL for β-carotene were determined. More carotenoid compounds could be separated and detected with the new method as compared with conventional C18 column. Hyphenation of HPLC with photodiode array and mass spectrometry detectors assisted in detection of tetra-dehydrocarotenoid and fatty acid diesters of xanthophylls in tomato products. Content of all-trans-lycopeno, β-carotene and total carotenoid in different industrial tomatoes tested was found to range between 41.87 and 84.65, 0.89 and 1.50 and 53.22 and 112.60 μg/g fresh weight, respectively.

Introduction

The nutritional benefit of tomato-based products has been attributed to their being rich in bioactive compounds such as carotenoids and antioxidant vitamins (vitamins E and C) (1, 2). Most of carotenoids from tomato-based products have been found among those determined in human plasma revealing the biological and nutritional importance of such products as good sources of food constituents with functional properties (3–5). Over the last decades, epidemiological studies have shown a strong association between a high dietary intake of biologically active carotenoids and a reduced risk of serious various cancers and cardiovascular disease (6–11).

Factors most likely to affect content and composition of carotenoids in tomatoes and tomato-based products may include genetic diversion (12, 13), technology (14, 15), fruit ripeness and conditions of cultivation (16, 17). During maturation, post-harvest and processing, carotenoids undergo substantial chemical alteration on their structure forming a wide variety of isomers or derivatives that vary significantly in biological and chemical properties.

To study the chemical composition and alteration of carotenoid pigments, several analytical methods including high-performance liquid chromatography (HPLC) have been elaborated and applied (4). Most of the applied methods based on separation of carotenoids on reversed-phase adsorbents with either isocratic (14) or gradient elution (18, 19). The most widely and efficiently used stationary phase for reversed-phase column is the monomeric C8 and C18 with poor resolution of the geometric cis–trans isomers (20). For better separation and high resolution of geometric isomers of carotenoids, mainly lycopene and β-carotene polymeric C30 adsorbent had been developed (21, 22). Polymeric C30 column has been successfully applied to separate cis–trans isomers of lycopene from tomato extract (23). Lycopene isomers from different citrus fruits have been efficiently separated on C30 column (24). However, application of C30 column has some limitation concerning ease of equilibration, cost and its efficiency to separate only geometrical isomers of carotenoids with limited range of polarity, most likely, less polar carotenones such as lycopene and β-carotene. When applied to the general analysis of carotenoid composition including polar xanthophylls and oxygen non-containing carotenones from any food sample, polymeric C30 gives similar profile to that processed with conventional C18 column, but with longer run time (25).

The advent of new chromatographic materials with specificity, high steric selectivity and high efficiency opened up the possibilities to achieve simple, reliable and efficient separation and sensitive determination of different isomers and artifacts of fat-soluble pigments.

The objective of the present work was to determine carotenoids composition and content from tomatoes by HPLC using reversed-phase column having cross-linked end-capping and combined photodiode array (PAD) mass spectroscopic (MS) detection.

Materials and Methods

Materials

Standard all-trans β-carotene, lycopene and zeaxanthin were purchased from Sigma-Aldrich (Budapest, Hungary). Authentic standard materials for β-cryoprotein and lycopene were prepared by the Institute of Bio- and Medical Chemistry of the medical School of the University of Pécs (Hungary) by applying a protocol including isolation and crystallization (26). HPLC and analytical grade organic solvents were from Merck (Darmstadt, Germany). A cross-linked Nucleodur Isis C-18 HPLC column was from Macherey Nagel (Düren, Germany).
Tomato fruits of different varieties cultivated with water and potassium supply treatments were obtained from experimental field of the Szent István University (Gödöllő, Hungary). Tomato plants were grown in brown forest soil under conventional cultivation conditions and applying two different irrigation treatments (regular irrigation, cutoff irrigation, in which water supply was stopped 30 days before harvest and unirrigated control). Basic nutrients were supplied when plants were transplanted with Agroblen 18-8-16 (nitrogen–phosphorus–potassium) that provided 266 kg ha\(^{-1}\) K fertilizer. Additionally, more potassium fertilizer was applied with KNO₃ at fruit set, resulting in two different potassium supplies of 454 and 555 (+K) kg ha\(^{-1}\), respectively.

The freshly harvested ripe fruits (1 kg from each replication block) were homogenized in a warring blender and stored after flushing with nitrogen in a plastic jars at \(-20\) °C when not immediately analyzed.

**HPLC analysis**

**Extraction of carotenoids**

Five grams of tomato fruit samples were crushed in a crucible mortar in presence of 1 g quartz sand and 0.5 g ascorbic acid. The extraction procedure started with binding of water with methanol according to a previously described procedure (27) followed by extraction of carotenoids by 1,2-dichloroethane in a liquid–liquid partitioning. The polar and non-polar phases were separated by addition of 1 mL of doubly distilled water and mechanical shaking for 15 min. The lower non-polar phase was separated in a separating funnel, dried over anhydrous Na₂SO₄ and evaporated at 30 °C under vacuum. The residue were redissolved in HPLC grade acetone or 30% dichloromethane in methanol and then filtered through a 0.45-µm Teflon (PTFE) syringe filter before injection onto HPLC system.

Peak identification was based on comparison of retention time, spectral characteristics and mass spectrum data with those of available standards or with data from literature as shown in Section Results and Discussion.

**Instrument and HPLC conditions.** A Waters Alliance liquid chromatographic instrument consisting of a Model 2696 Separation Module (Gradient pump, autosampler and column heater) and a Model 2695 photodiode array detector was used. Operation and data processing were performed by Empower software.

Separation of carotenoids was performed using two procedures (i) on Nucleodur ISIL, 1.8 µm, 5 cm \(\times\) 4.6 mm, column with either gradient elution of (A) water and (B) acetone, in which elution started with 20% A in B, which changed to 12% A in B in 12 min then to 5% A in B in 5 min and stayed isocratic for 5 min and turned to 20% A in B in 3 min or with gradient elution of (A) methanol–water (93:7), (B) methanol and (C) 30% 1,2-dichloromethane in methanol, in which elution started with 100% A and changed to 100% B in 5 min then changed to 100% C in 20 min and finally turned to 100% A in 5 min; (ii) on conventional Nucleosil C18, 3 µm, 250 \(\times\) 4.6 mm column eluted with aforementioned gradient elution systems. The flow rate was 0.7 mL min\(^{-1}\) in each method used. PAD spectrum of carotenoids was displayed between 200 and 700 nm. For quantification peak, area of each compound was taken at the maximum wavelength of its absorption spectrum.

**MS conditions.** The samples were injected into a PerkinElmer Series 200 HPLC System, connected to an AB Sciex 4000 QTRAP mass spectrometer with electrospray ionization interface. The curtain gas was set to 20, temperature was 450 °C. The ion spray voltage was 4500 V with declustering potential 50 in positive ion mode, while in negative ion mode they were \(-4500\) and \(-100\) V, respectively. In selected reaction monitoring mode, the collision gas was set to medium, and the collision cell exit potential was 12. The data was collected and analyzed with Analyst® 1.6 Software.

**Chemical tests of carotenoids**

Acetone extract of tomato carotenoids was applied to a thin-layer chromatography as described previously (28) using MN silica gel plates and development with n-hexane–acetone–acetic acid. The bands of different carotenoids were subjected to acetylation, epoxidation and cis-isomer tests as described by Bauernfeind (29).

**Validation**

A well-homogenized, chopped tomato samples were spiked with 10 and 100 µg zeaxanthin and β-carotene per gram food using stock solution containing 100 µg mL\(^{-1}\). Addition of standard material was at the first step of extraction procedure. After analysis, the recovery was obtained by dividing the calculated concentration over the added concentration performing three replications for each concentration.

Precision test was done by repeating five times the analysis of the same sample (well homogenized) on the same day (intraday). Precision was expressed as relative standard deviation (RSD). Limits of detection (LOD) and quantification (LOQ) were calculated by measuring the concentration of standard materials when the peak signal is three times and five times higher than that of the noise, respectively.

For calibration, four working solutions between 0 and 100 µg for β-carotene and lycopene were prepared and injected under the same conditions used for the analysis of the samples. The peak areas were recorded at the maximum wavelength absorbance of each standard material and plotted against concentration to get calibration curves.

**Statistical analysis**

One way analysis of variance was used to determine the degree of significance between cultivars and treatments in the concentration of different carotenoids.

**Results**

Figure 1 shows the HPLC profile of tomato carotenoids as separated on conventional Nucleosil 100-5 C18, 3 µm column with gradient elution system including decreasing polarity of eluent from highly polar water in methanol to less polar 1,2-dichloromethane in methanol in 25 min. With such a gradient elution only two peaks representing lycopene derivatives
appeared with marked overlapping with other minor carotenes, while three derivatives of \( \beta \)-carotene could be detected.

With cross-linked C18 column retention and partitioning of lycopene (open chain structure) (Figure 2) was higher that of \( \beta \)-carotene (\( \beta \)-ionone ring containing) giving the possibility for the later to elute with shorter retention time than that of the former. The elution order of oxygen-containing xanthophylls remained unchanged.

To achieve better separation of derivatives of both lycopene and \( \beta \)-carotene, Nucleodur ISIS, the highly spherical and pure type of cross-linked chromatographic material was used with gradient elution of water in acetone. The newly elaborated HPLC method provided excellent separation and detection of different derivatives of the dominant carotenoids such as lycopene and \( \beta \)-carotene as well as oxygen-containing polar xanthophylls (Figure 3). Under these conditions, the elution pattern of caroteneoids was similar to that found with usual reversed-phase C18 end-capped column.

A comparison, in terms of chromatographic parameters, between conventional and cross-linked columns is shown in Table I. All chromatographic parameters, particularly resolution (Rs) indicated that cross-linked end-capped column is more efficient than conventional end-capped C18 one for the separation of carotenoids of tomato extract. Resolution value of 3.97 recorded for the separation of lycopene and \( \beta \)-carotene gave the possibility for the isomers of both compounds to partition and elute with Rs value higher than 1. In case of conventional C18 column of the same dimensions, although capacity factor values were higher, lycopene and \( \beta \)-carotene were separated from each other with much lower Rs value, therefore, \( \beta \)-zaxanthin overlapped with \( \beta \)-dehydrocarotenoid, which could be separated completely from all-trans lycopene and its cis isomers using cross-linked column.

It was also found that in some examined samples, the HPLC profile of tomato carotenoids separated on cross-linked column contained two more peaks with retention times higher than those of the less polar carotenes like phytoene. These peaks disappeared when the extract was subjected to alkaline hydrolysis revealing their being fatty acid esters of xanthophylls.

**MS and PAD identification**

Table II shows important information that assisted in achieving satisfactory identification for carotenoids separated and detected by both MS and PAD detectors particularly those detected for the first time in tomato extract. For acute identification, both PAD and MS data were combined and used. The first polar
Table II
MS and PAB Detection Data for Identification of Carotenoids Separated from Tomato on Cross-Linked ISIS Column with Water in Acetone Gradient Elution, See Text

<table>
<thead>
<tr>
<th>m/z</th>
<th>Carotenoids</th>
<th>Ionization</th>
<th>Maximum absorption (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>601</td>
<td>Neoxanthin</td>
<td>+</td>
<td>413 437 466</td>
</tr>
<tr>
<td>570</td>
<td>Zeaxanthin</td>
<td>+</td>
<td>421 452 477</td>
</tr>
<tr>
<td>568</td>
<td>Lycoxanthin epoxide</td>
<td>+</td>
<td>433 459 492</td>
</tr>
<tr>
<td>568</td>
<td>cis-Dihydroxy-lycopene</td>
<td>+ 360</td>
<td>432 460 493</td>
</tr>
<tr>
<td>552</td>
<td>Lycopoxanthin</td>
<td>+</td>
<td>448 475 506</td>
</tr>
<tr>
<td>536</td>
<td>All-trans-Lycopene</td>
<td>+</td>
<td>449 475 506</td>
</tr>
<tr>
<td>536</td>
<td>9-cis-Lycopene</td>
<td>+ 360</td>
<td>432 461 490</td>
</tr>
<tr>
<td>536</td>
<td>13-cis-Lycopene</td>
<td>+ 370</td>
<td>430 460 488</td>
</tr>
<tr>
<td>531</td>
<td>Tetra-dehydrocarotenoid</td>
<td>-</td>
<td>449 475 504</td>
</tr>
<tr>
<td>395</td>
<td>Unidentified</td>
<td>-</td>
<td>433 459 492</td>
</tr>
<tr>
<td>536</td>
<td>β-Carotene</td>
<td>+</td>
<td>428 455 482</td>
</tr>
<tr>
<td>536</td>
<td>cis-β-Carotene</td>
<td>+ 340</td>
<td>417 434 471</td>
</tr>
<tr>
<td>878</td>
<td>Lycopophyll diester</td>
<td>+</td>
<td>433 461 492</td>
</tr>
<tr>
<td>890</td>
<td>Anthexanthin diester</td>
<td>+</td>
<td>421 444 473</td>
</tr>
</tbody>
</table>

Method validation parameter
After spiking tomato sample with known quantities of zeaxanthin, lycopene and β-carotene, the % recovery was found to be in the range of 89–97%. LOD and LOQ of 19.44 and 64.79 ng/mL for zeaxanthin, 15.6 and 52.4 ng/mL for lycopene and 8.28 and 27.61 ng/mL for β-carotene were low, indicating the high sensitivity of the method developed in this work.

Precision expressed as RSD from five replications of the same sample was 2.36 for zeaxanthin, 4.33 for lycopene and 6.27 for β-carotene.

Five point calibrations of lycopene and β-carotene concentration versus peak area indicated the straight relationship between 0 and 20 μg/mL for both compounds. The linear regression equations y = 1.3x − 0.08 and y = 0.7925x − 0.28 with correlation coefficient (R²) values of 0.9999 and 0.9978 for lycopene and β-carotene were obtained, respectively. These values denote that the method developed is adequate for sensitive determination of carotenoids even at low concentration of carotenoids in the working solutions prepared for the samples before injection onto the HPLC column.

Carotenoids in fresh tomatoes
As application of the new HPLC method freshly harvested tomato fruits were obtained and analyzed. Table III summarizes the results for the major carotenoids and those detected with considerable level in tomato extract. All the obtained samples were of tomato for processing cultivars produced under conventional conditions with different K and water treatment. It was found that there are significant differences (P = 0.01) in carotenoid content, but not composition, between different cultivars with Brigade having the highest level of carotenoids. Treating this cultivar with different K doses and cutoff (no irrigation 45 days before harvest) resulted in a light increase in carotenoid content of the fruits, while usual water supply in addition to rainfall caused carotenoid level to substantially (P = 0.05) decrease in Brigade cultivar.
Discussion

With conventional reversed-phase, end-capped chromatographic adsorbent, elution of carotenoids is affected, to a high extent, by polarity of both eluents and mobile phase. Therefore, elution, in terms of retention time, of tomato carotenoids was found to be in the order of polar xanthophylls $> \beta$-carotene. The separation of different isomers of lycopene and $\beta$-carotene, the major carotenoid compounds in tomato products, required conventional gradient elution.

In case of base-deactivated C18 column with cross-linked end-capping, the carbon skeleton results in high carbon content and a non-polar phase that exhibits a high steric selectivity. The change observed in elution order of lycopene and $\beta$-carotene has also been observed with C30 RP column (25). It is of interest that four derivatives of lycopene could be separated from the dominant all-trans form, while cis isomers of $\beta$-carotene and some other minor carotenoids eluted together and, in some extract, overlapped with all-trans lycopene. The unchanged retention pattern of polar xanthophylls on cross-linked column indicates that the polarity of compounds is still a limiting factor in their dissociation and association on such reversed-phase adsorbent irrespectively of the type of end-capping. Ikoma and
coworkers (31) achieved close separation pattern using C30 reversed-phase column with gradient elution that needed relatively longer time for the complete separation of oxygen non-containing carotenoids.

The application of MS detection assisted in finding of new carotenoid that has not ever been reported in carotenoid extract of tomato products. The m/z less by four protons than that of lycopene denotes that one carotenoid compound underwent dehydrogenation at two positions in the polyene chain. Although Steiger et al. (32) and Mjits et al. (33) reported occurrence of di- and tetra-dehydrolycopene in microorganisms, lycopene is excluded from this because if it undergoes dehydrogenation, the number of conjugated double bond will be increased to 13, and thereby, the maximum absorption wavelength should be higher by, at least, 14 nm. Most probably, the unknown compound dues to a tetrahydro-γ-carotene with two more double bonds, one locates inside another other outside the conjugated system. The additional chromophor can cause the light absorption the γ-carotene to be close to that of all-trans lycopene. However, correct and final identification needs further investigation applying suitable structural elucidation means such as NMR.

The MS and PAD identification of tomato carotenoids agrees, to a high extent, with that described by some authors who used tandem MS for the identification based on fragmentation of carotenoids also (34, 35, 36).

The range achieved for the recovery of carotenoids points out to the fact that carotenoids are sensitive to molecular oxygen and light, the factors most likely to induce degradation during preinjection preparation of carotenoid extract, which should be optimized to be performed with minimal loss. This hold true for the low accuracy with regard to β-carotene as compared with that found with zeaxanthin and lycopene, which had higher stability under the conditions used.

The lowest level of carotenoids in food extracts is well above than the LOQ estimated in the new method developed in this work. With analytical column of 3–5 μm particles, the LOQ has been found to be ~10 times higher than the values achieved with UPLC column having cross-linked end-capping (13, 37) revealing the increased sensitivity of determination by such a method.

As application of the newly developed method, some samples from an experiments carried out in the university were analyzed for their carotenoid composition and content as well as for the change caused by the cultivation conditions. All tomatoes used were from industrial varieties that are expected to have high lycopene content. The level of carotenoids found was lower than the range estimated in a previous work (13) most likely due to the differences in the varieties and the technology applied in the two works. Also in the previous work, we could not separate and determine tetra-dehydrocarotenoid that has been eluted together with all-trans lycopene. The significant differences found between cultivars are a good basis for selecting the suitable industrial cultivar of tomatoes that rich in bioactive carotenoids and respond positively to the change in cultivation conditions particularly K and water supply, which seem to have an impact on carotenoid biosynthesis in such a crop.

Conclusion

Excellent separation of tomato carotenoids including their isomers can be achieved on the less polar reversed-phase chromatographic material having cross-linked end-capping. Separation and detection of tetra-dehydrocarotenoid from the all-trans and cis isomers of lycopene make it necessary to newly evaluate lycopene and its derivatives in fresh or processed tomato products. It is also necessary to apply more efficient techniques such as tandem MS for the identification of the unidentified carotenoids after accurate separation that ensure their sufficient resolution. Because of being simple and enough sensitive, the newly developed method can be successfully applied in different laboratories with or without hyphenation with MS detectors. With the new procedure, the real concentration of the vital and biologically active all-trans carotenoids of tomato can be correctly estimated and, thereby, data base in this field may need to be substantially corrected.

Funding

The authors thank the National Development Agency (NFU) for financial support under project grant no. TECH-09-A3-2009-0230, USOK2009 and TÁMOP-4.2.1. B-11/2/KMR-2011.

References

2. Lenucci, M.S., Cadinu, D., Taurino, M., Piro, G., Dalessandro, G.J.; Antioxidant composition in cherry and high-pigment tomato cultivars; *Journal of Agricultural and Food Chemistry*; (2006); 54: 206–2063.


4. Rodríguez-Bernaldo de Quirós, A., Costa, H.S.; Analysis of carotenoids in vegetables and plasma samples: a review; *Journal of Food Composition and Analysis*; (2006); 19: 97–111.


8. Johnson, E.J.; The role of lutein in disease prevention; *Nutritional and Clinical Care*; (2000); 3: 289–293.


11. Stacewicz-Spantzikis, M., Bowen, P.E.; Role of lycopene and tomato products in prostate health; *Biochimica et Biophysica Acta*; (2005); 1740: 202–205.

12. Rubio-Diaz, D.E., De Nardo, T., Santos, A., de Jesus, S., Drancis, F.; Profiling of nutritionally important carotenoids from genetically diverse tomatoes by infrared spectroscopy; *Food Chemistry*; (2010); 120: 282–289.


15. Lessin, W., Catigani, L., Schwartz, S.; Quantification of cis-trans isomers of provitamin A carotenoids in fresh and processed fruits and vegetables; *Journal of Agricultural and Food Chemistry*; (1997); 45: 3728–3732.


23. Gómez-Prieto, M.S., Caja, M.M., Herrera, M., Santa-Maria, G.; Supercritical fluid extraction of all-trans-lycopene from tomato; *Journal of Agricultural and Food Chemistry*; (2003); 51: 3–7.


32. Steiger, S., Takaichi, S., Sandmann, G.; Heterologous production of two unusual carotenoids, 1,1′-dihydroxy-3,4-didehydrolycopen-1,1′-hydroxy-3,4,3′,4′-tetradehydrolycopene by combination of the crfC and crfD genes from Rhodobacter and Rubrivivax.; *Journal of Biotechnology*; (2002); 97: 51–58.


36. van Breemen, R., Dong, L., Pajkovic, N.; Atomic pressure chemical ionisation tandem mass spectrometry of carotenoids and their oxidation products in the extract of human plasma; *Analytical Chemistry*; (1992); 64: 2111–2122.