

## HPLC Method Optimization and Validation for Determination of Lycopene in Tomato (*Lycopersicon esculentum*, Mill.) Fruits

Wondu Garoma Berra

Food Technology and Bioprocess Engineering, College of Engineering and Technology,  
Wollega University, Post Box No: 395, Nekemte, Ethiopia

### Abstract

The aim of this work was to develop efficient and accurate protocol that can measure lycopene in tomato. A total of ten tomato varieties loose and cluster types were selected for the study. All the tomatoes were harvested at commercial ripening stage and prepared after 3 days of storage at 18°C and relative humidity (RH) of 80%. For analysis purpose each tomato was cut, quartered, chopped and frozen in liquid nitrogen and then subjected to grinding. The finely ground tomato samples were immediately filled into air-tight plastic tube and stored in freezer (-80 °C) for about two weeks. Finally, the tomato samples were prepared in different composition of extraction solvent before subjected to HPLC analysis. Mobile phase composition Acetonitrile/Methanol (50:50, v/v) added with Triethylamine 9 µM; extraction solvents hexane/acetone/ethanol (50:25:25 v/v/v); re-dissolving residue in Tetrahydrofuran, followed Acetonitrile/Methanol (15:30:55, v/v/v); flow rate 0.6 mL/min and λ detection near to 472nm were showed most suitable for lycopene determination in tomato, achieving best characteristic spectral profile with precision (RSD<15), accuracy and recovery (≥81.7%), and sensitive detection limit (0.0156 µg/mL) within separation of ~21 minutes. The result showed that HPLC is most accepted and efficient method for determining lycopene in tomato.

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#### \*Corresponding Author:

Wondu Garoma Berra

E-mail:

woundgaroma@gamil.com

### INTRODUCTION

Lycopene is the most abundant carotenoid pigment principally responsible for the characteristic deep-red color of ripe tomato fruits and tomato products. Recently, a lot of attention has been paid to its biological and physicochemical properties, especially related to its effect as a natural antioxidant (Ananthanarayan and Choudhari, 2007; Barba *et al.*, 2006; Qiu *et al.*, 2006; Rao, 2007; Maguer and Shi, 2000), with a singlet oxygen physical quenching rate constant almost twice as high as that of β-carotene and more than ten times higher than that of α-tocopherol (DiMascio *et al.*, 1989; Ali and Rao, 2007; Pol *et al.*, 2004; Shi *et al.*, 2003).

Lycopene, having a high nutraceutical value, has a wide use in pharmaceutical, food, feed, and cosmetic industries (Barba *et al.*, 2006; Maguer and Shi, 2000). Reports from Ananthanarayan

and Choudhari (2007) and Maguer and Shi (2000) indicate the commercial importance of lycopene as natural color ingredient in food formulations from the widespread use of tomato paste as a colorant. As a result, industrial production of lycopene from tomatoes is highly demanded by pharmaceutical companies and for functional food development.

According to Hart and Scott, (1995), tomatoes normally contain about 3 to 5 mg lycopene per 100g of raw material, although concentration varies depending on tomato products (Bicanic *et al.*, 2005), ripening process and storage temperature (Barrett and Garcia, 2006). For example, and temperature higher than 32.2°C during the growing season results in smaller lycopene concentrations in tomatoes (Barrett and Garcia, 2006).

Today, a number of analytical procedures have been developed and applied for the determination of antioxidants in fresh tomato and processed tomato products (Gomez-Romero *et al.*, 2007) in a destructive and non-destructive way including electronic tongue (Rudnitskaya *et al.*, 2001), colorimeter, electronic nose (Berna *et al.*, 2002), the acoustic firmness sensor (AFS), near infrared spectroscopy (NIR) (Peirs *et al.*, 2003; Yong *et al.*, 2005; Baranska *et al.*, 2006), nuclear magnetic resonance (NMR) (Gladden and Alexanderz, 1996), NIR-FT-Raman spectroscopy (Schulz *et al.*, 2005), Fourier transform infrared spectroscopy (FTIR), hyperspectral imaging (Berna, 2006), etc.

However, the analytical methods used for lycopene are rather complicated (Barba *et al.*, 2006), and not all analytical methods available for carotenoid analysis in food products are suitable for lycopene rich foods due to its low solubility and interference with mobile phase in some of the solvents employed. Furthermore, the instability of lycopene during processes of extraction, handling, and elimination of organic solvents makes the sample preparation for lycopene an extremely delicate task, often requiring successive and complex procedures to ensure that all the carotenoids are extracted (Barba *et al.*, 2006; Maguer and Shi, 2000; Xu *et al.*, 2006).

Therefore, optimization of the existing methodologies of extraction/separation and the necessity for a reliable and rapid analysis method for lycopene in tomatoes was importantly considered in this work.

## MATERIALS AND METHODS

### Experimental Design and Plant Materials

A total of ten tomato varieties, 5 loose cultivars (*Growdena*, *Broderna*, *DRW 75-93*, *Admiro* and *Excelsior*), and 5 cluster type (*Tricia*, *Clotilde*, *Bonaparte*, *Plaisance* and *Dirk*) were selected and harvested at commercial ripening for the study. These tomato varieties were obtained from two experimental gardens in Belgium and prepared after 3 days storage at 18°C and 80% RH. For analysis tomatoes were cut, quartered, chopped and frozen in liquid nitrogen, then subjected to grinding. The finely ground tomato samples were immediately filled into air-tight 15 mL plastic tube and stored in freezer (-80°C) for about two weeks before HPLC analysis.

## HPLC Analysis of Lycopene

### Instrumentation

The HPLC apparatus (Hewlett Packard, series 1100) was equipped with a quaternary Isopump Exchange System (G1310A), Degasser (G1379A), ALS autosampler (G1329A), ALS Thermo thermostat (G1330B) column heater and a Thermo Separation Spectra Series UV/Visible diode-array detector (G1315A). Data processing and analysis were carried out using HP Agilent ChemStation software (Agilent Company). The column used for separation was Prevail C18 5 $\mu$  (250 x 3 mm i.d., Alltech), coupled to Prevail C18 5 $\mu$  (7.5x2.1 mm i.d., Alltech) guard column. A Thermo spectronic model Genesys 10 UV (Rochester, NY, USA) spectrophotometer was routinely used for measuring absorbance of the working standard solutions.

### Chemicals/Reagents and Standards

Standards of lycopene from tomato (purity 99%), and  $\beta$ -carotene (type II: Synthetic  $\geq$ 95%) were obtained from Sigma Company (St. Louis, MO, USA), while  $\beta$ -apo-8'-carotenal (8'-apo- $\beta$ ,  $\phi$ -caroten-8'-al) was purchased from Fluka Company. HPLC grade solvents were supplied by ACROS organics in the case of methanol (MeOH), n-hexane and Tetrahydrofuran (THF, stabilized with 0.025% butylated hydroxytoluene, BHT). Ethanol absolute and Chloroform were obtained from VWR Company. Acetonitrile (ACN, HPLC gradient grade) was from Fisher Scientific, Acetone from Chemlab and Triethylamine (TEA) from Fluka. High-purity oxygen-free nitrogen gas (purity > 99%) was obtained from Air products (MAP gasses, Belgium). De-mineralized water was prepared regularly supplied to the laboratory through pipeline.

### Preparation of Standard Solutions

#### Lycopene

The *trans*-lycopene (all-E'-lycopene) standard solution was prepared by diluting 1 mg commercial lycopene standard from tomato with hexane to desired concentration and the solutions were transferred to a volumetric flask. To avoid degradation, lycopene solution was divided into 1.5mL aliquots in an air-tight screw-topped brown glass vials, dried under nitrogen and stored at -20°C. Individual stock standard solutions were freshly prepared every day adding a suitable volume of hexane to the vials containing lycopene standard and mixing until complete dissolution. Then, the concentration was determined using Beer's law ( $A = \epsilon bc$ ) from its extinction coefficient ( $E_{1cm}^{1\%}$ , 3450 in hexane at 472 nm, Arroyave *et al.*, 1982). Then, the

concentrations of the standard solutions were calculated as below.

$$C_{all-E'-Lycopene} [mg/L] = \frac{\text{Absorption} \times 10^4}{3450}$$

Individual working solutions were prepared from stock solutions each day by evaporating an aliquot with nitrogen and making to desired volume and concentration with re-dissolution solvents. Efficient solubility of the residue was achieved by using re-dissolving solvent composition THF followed by ACN/MeOH (15:30:55, v/v/v).

### **β-Carotene**

A working solution of β-carotene was prepared by diluting a stock solution of β-carotene with hexane to desired concentration in a similar way as standard lycopene preparation, while considering its extinction coefficient 2590 in hexane at 450nm.

### **β-Apo-8'-Carotenal (Trans)**

According to Hart and Scott (1995), the internal standard β-apo-8'-carotenal was used to assess losses during extraction procedure. A working solution of β-apo-8'-carotenal was prepared directly in solvent composition of THF followed by ACN/MeOH (15:30:55 v/v/v) and used as stock solution for further dilution to obtain the desired concentration for detection at 450 nm.

### **Sample Preparation**

Lycopene extraction from tomato was based on modifications and optimization of Barba *et al.* (2006) method, with solvents mixtures of hexane/acetone/ethanol (50:25:25, v/v/v) for extraction. Sample containing between 1.5–2.5 g of tomato juice was precisely weighed into a 250 mL Erlenmeyer flask, tightly wrapped in aluminum foil to protect it from light and then mixed with 100mL of extraction solvent. The mixture was stirred on a magnetic stirring plate during 30 min. To the extract, 15mL of de-mineralized water was added and mechanically agitated. The solution was subsequently separated into distinct phases of polar and non-polar layers. From lycopene-bearing upper hexane layer, 10mL was placed in a round-bottomed flask and dried in rotary evaporator (~34°C). Different compositions of solvents (THF/ACN/MeOH) were assayed for re-dissolution of the dry extract: (1) THF/ACN/MeOH (15:30:55, v/v/v), (2) THF/ACN/MeOH (15:55:30, v/v/v) and, (3) dissolving in THF and then ACN/MeOH followed (15:30:55, v/v/v). In all the solvents containing Methanol and Acetonitrile, ultrasonic agitation for few minutes was used to

make sure that the solvents were mixed homogeneously.

The lycopene residue was dissolved to final volume of 8mL with solvent system 3 for HPLC analysis. Due to variations in lycopene content among tomato samples, also other volumes i.e., 4mL and 16mL have been considered and tested to keep quantification within optimal calibration range. The final solution was filtered through 0.45 μm membrane filters (13 mm i.d., Alltech) and then 20μL was injected for HPLC analysis.

### **Chromatographic Conditions**

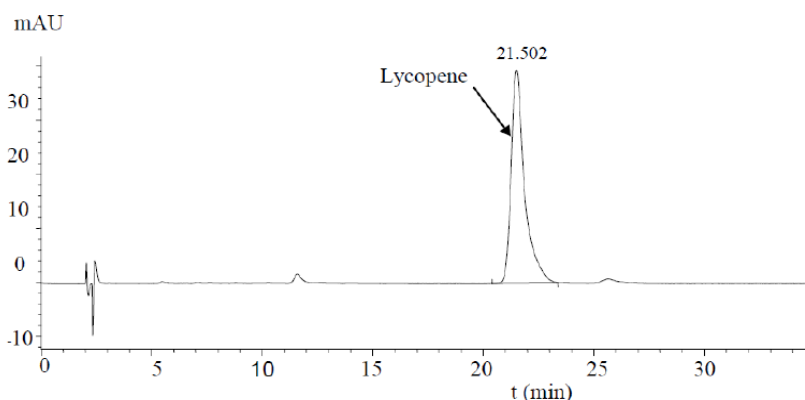
Tomato extracts were analysed for lycopene content by separation followed by quantitative determination using reversed-phase HPLC system consisting of UV/visible diode-array detector separation module, auto-injector, and column temperature regulator. The C18 column was then conditioned with the elution solvent at flow rate of 0.6 mL/min with detection at 472 nm. Other flow rates including 0.9 and 1.5mL/min have also been tested. Based on Barba *et al.* (2006) and [www.alltech.be](http://www.alltech.be), separation of carotenoids was attempted isocratically using various compositions of MeOH and ACN as mobile phase: (1) MeOH /ACN (90:10, v/v) added with 9 μM TEA, (2) ACN/ MeOH (75:25, v/v) added with 9 μM TEA and (3) ACN/MeOH (50:50, v/v) added with 9 μM TEA. Optimal separation was achieved with mobile phase 3. Injection volumes 20 μL and 100 μL were also tested but smaller injection was preferred to avoid overloading on column during analysis.

The peak responses were measured at 472 nm for lycopene and 450 nm for β-carotene with the help of a variable wavelength UV/vis monitor coupled diode-array detector, with an output to chromatographic data handling system (Agilent ChemStation software) which permitted manual manipulation of peak integration.

The identification of the peaks was carried out by comparing the retention times with those obtained with a standard solution of all-trans lycopene (Figure 1). The quantification was performed using calibration curves made with different injected amounts of external standard, all-trans-lycopene; in a similar proportion as in the samples.

### **Peak Identification and Quantification**

Identification of carotenoids was carried out by comparison of HPLC retention times with corresponding standards and chromatographic properties with standards of lycopene and β-carotene. Additional identification was carried out



**Figure 1:** Chromatogram of lycopene standard solution under chromatographic condition: Prevail C<sub>18</sub>5 $\mu$  (250x3mm, i.d.), coupled to Prevail C<sub>18</sub>5 $\mu$  (7.5x2.1mm, i.d.) guard column; mobile phase MeOH/ACN (50:50, v/v) added with TEA 9  $\mu$ M; flow rate 0.6 ml/min; column temperature 30 °C;  $\lambda_{\text{detection}} = 472$  nm.

comparing spectral data obtained with DAD array detector G1315A with reported values (Schierle *et al.*, 2003). In general, quantitative analysis of carotenoids is carried out by external calibration curve method, generated from measurements made with many concentrations of pure standards (0.039-2.8  $\mu$ g/mL). Then lycopene content in tomato was calculated according to formula 2 here below. Finally, the data were expressed as milligram of each carotenoid per 100g fresh weight of the tomato analyzed.

$$\text{Lycopene [mg/g]} = \frac{A_{\text{Sa}} V_{\text{T}} V_2}{m \cdot \text{RF} \cdot V_1}$$

Where, **Asa**: Peak areas of lycopene from sample solution [mAU], **m**: Amount of sample [g], **RF**: Response factor [mAU/mg], **VT**: Total volume of the upper lycopene bearing hexane layer [mL], **V1**: Volume of aliquot of extract, which is evaporated [mL] and **V2**: Volume of final sample solution [mL]

### In-house Validation of the Established Methods

The established method was validated for major performance parameters (precision, accuracy, linearity, limit of detection and quantification).

Seven pointed calibration curves were constructed to test linearity within working range: 0.039-2.80  $\mu$ g/mL. This was performed six times (n=6). The limit of detection (LOD) defined as amount of the carotenoid, resulting in a peak height three times the baseline noise (Hulshof *et al.* 1997) was calculated.

However, this concept is not practical because often time, noise level on a detector during the method development phase may be different when samples are assayed on different detectors, etc (CDER, 1994). So, for reliable

quantification (LOQ) the lowest standard concentration level in the calibration range was considered as stated by www.iupac.org. Limits of quantification (LOQ) were set at 2.5 times the LOD (Hulshof *et al.*, 1997). Based on this relation, LOD was calculated.

The accuracy of the HPLC analysis was monitored by means of a recovery assay. Fresh tomato samples were spiked with internal standard solutions of 0.055 mg/mL  $\beta$ -apocarotenal. The spiked samples (n=8) were subjected to the entire process of extraction and HPLC analyses. The % recovery of carotenoids, calculated below.

In order to check the precision of the method the injection repeatability, the calibration repeatability and the extraction repeatability were calculated in terms of relative standard deviations (RSD).

$$\text{Recovery (\%)} = \frac{\text{Analyzed Amount}}{\text{Added Amount}} \times 100$$

### Statistical Analysis

All tomato data from HPLC-analysis were subjected to analysis of variance (ANOVA) to determine statistically significant differences in lycopene content between loose and cluster, and between different cultivars at  $p < 0.05$  using SAS/STAT® version 9.1 (SAS institute, Inc. Cary, NC, United States).

$$\text{SEP} = \sqrt{\frac{1}{1p-1} \sum_{i=1}^{1p} (Y_i - y_i - \text{bias})^2}$$

$$\text{where bias} = \frac{1}{1p} \sum_{i=1}^{1p} (Y_i - y_i)$$

Where, **Y<sub>i</sub>** is the predicted value of the **ith** observation, **y<sub>i</sub>** is the measured value of the **ith** observation and **1p** is the number of observations in the validation.

## RESULTS AND DISCUSSION

### HPLC Method Development and Validation

#### Optimization of Method

A wide range of series of analyses has been carried out to develop efficient and accurate methodology using HPLC for lycopene analysis in tomato starting with Barb *et al.* (2006) protocol. According to this protocol the best composition were: mobile phase was MeOH/ACN (90:10, v/v) added with 9 $\mu$ M TEA, extraction solvent hexane/acetone/ethanol (50:25:25, v/v/v), re-dissolution solvent was THF/CAN/MeOH (15:30:55, v/v/v) and flow rate was 0.9 mL/minute. However, in this solvent combination the obtained chromatogram results (Figure 2) showed completely poor resolution, peaks were not baseline separated and also impure spectra were seen. Such different outcome might have arisen due to the difference in chromatographic apparatus such as column type and size used during our analysis and that of Barba *et al.* (2006).

Probably using this mobile phase composition, a balance between methanol and non-polar solvent may not be achieved due to a high Methanol (MeOH) proportion which of course may result in great polarity difference. It has also clearly been stated that the presence of TEA has no effect on selectivity; instead it improves the response of carotenoids and reduces or eliminates on-column degradation (Hart and Scott, 1995; Rodriguez, 2001). The butylated hydroxytoluene (BHT) found in small amount (0.025 %) in THF employed in our solvents used for extraction and separation of lycopene only serves as antioxidant by controlling oxidation and isomerization reactions (Ananthanarayan and Choudhari, 2007). To our knowledge, the presence of BHT is not related with the problem of selectivity using this mobile phase. The result reveals that mobile phase composition, MeOH/ACN (90:10 v/v) + 9 $\mu$ M TEA has no good selectivity with respect to our column system and chromatographic setting.

As a result mobile phase composition, MeOH/ACN (90:10 v/v) added with 9 $\mu$ M TEA was replaced by ACN/MeOH (75:25, v/v) added with 9 $\mu$ M TEA, as suggested in Alltech ([www.alltech.be](http://www.alltech.be)) for fat soluble vitamins keeping other procedures more or less as Barba *et al.* (2006). Here, chromatogram reveals that the peak of lycopene was well separated from other. However, the changed composition of mobile phase resulted in slightly extended retention time compared to the earlier composition, though not a major problem at this level of study.

At this stage the signals were well separated and broad, but with this mobile phase composition, ACN/MeOH (75:25, v/v) added with 9 $\mu$ M TEA spectrum showed impurity (Figure 4). Different flow rates have also been tested aiming to solve spectral impurity. Finally, flow rate of 0.6 mL/minute improved the spectral profile of lycopene when compared to other flow rates (Figure 1).

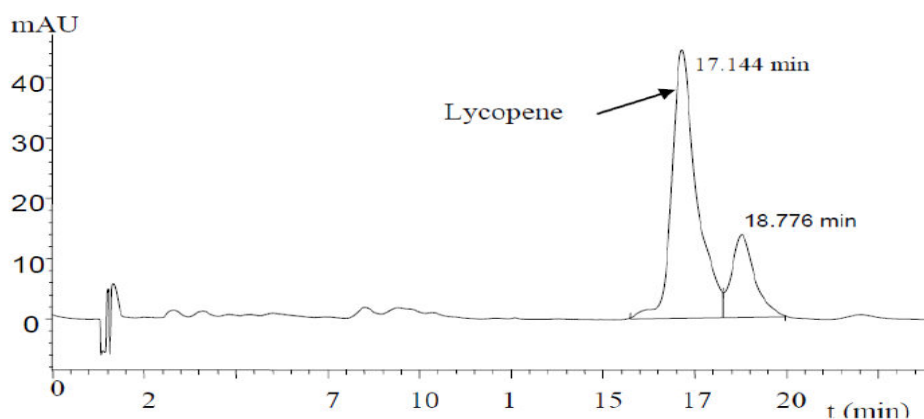
However, spectral characteristic of  $\beta$ -carotene was better at 0.9 mL/minute. As lycopene is the compound of interest in this study, the flow rate of 0.6 mL/minute was further used.

The chromatographic signal presentation in Figure 3 doesn't indicate any impurity of the peak; the spectrum evaluation (Figure 4), however, identified the peak of lycopene as impure even at a flow rate 0.6 mL/minute. According to the HPLC manual (Agilent company) spectral impurities can be caused by changes in solvent composition (gradients) or in peaks that are not baseline separated. Hence, the mobile phase, ACN/ MeOH (75:25, v/v) added with TEA 9  $\mu$ M was changed into a new composition, ACN/ MeOH (50:50, v/v) added with TEA 9  $\mu$ M. This new composition aimed to balance between methanol and non-polar solvents, as a high methanol proportion can cause lycopene precipitation in the extracts (Barba *et al.*, 2006), and a high non-polar solvent content caused deformation of the chromatographic characteristic (Figure 4) due to the great polarity difference.

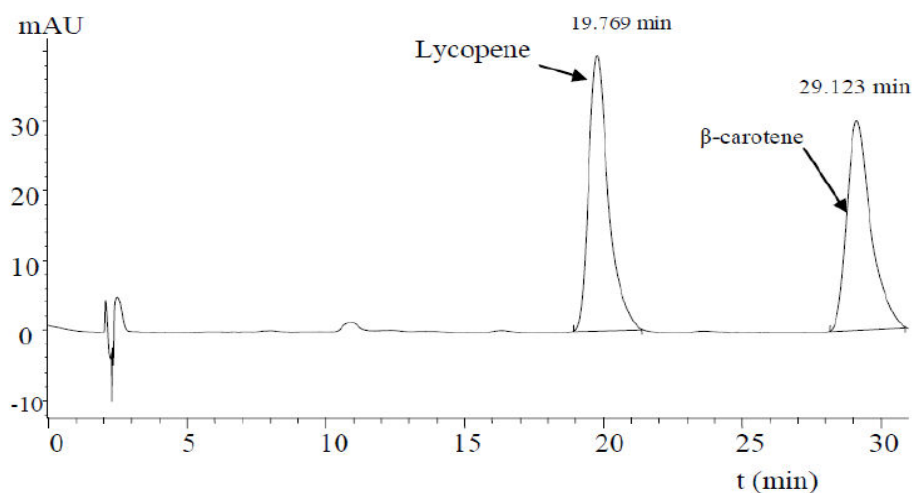
As clearly observed from the chromatogram and spectral characteristic (Figure 5, 6 and 7), the new mobile phase which consists of equal composition of ACN and MeOH has removed most of the defects including spectral impurity that was observed during the two previous mobile phase systems.

During optimization it was also seen that the re-dissolution solvent mixture THF/ACN/ MeOH(15:30:55, v/v/v) resulted in lycopene precipitation. The precipitation was formed at the bottom of vial even after prolonged agitation, which indicates that the desired compound cannot dissolve completely with this re-dissolution solvent combination affecting the recovery of lycopene when observed from the peak area obtained.

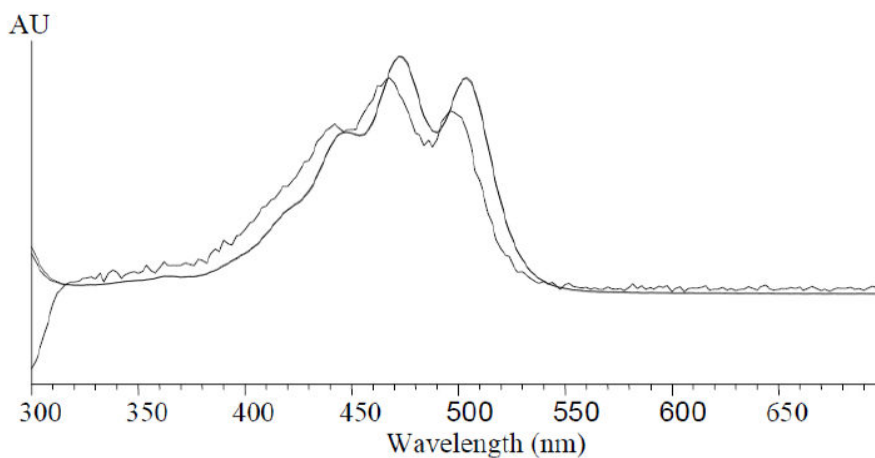
Besides, the re-dissolution solvent combination THF/ACN/MeOH (15:30:55, v/v/v) showed lack solubility for lycopene. Later when



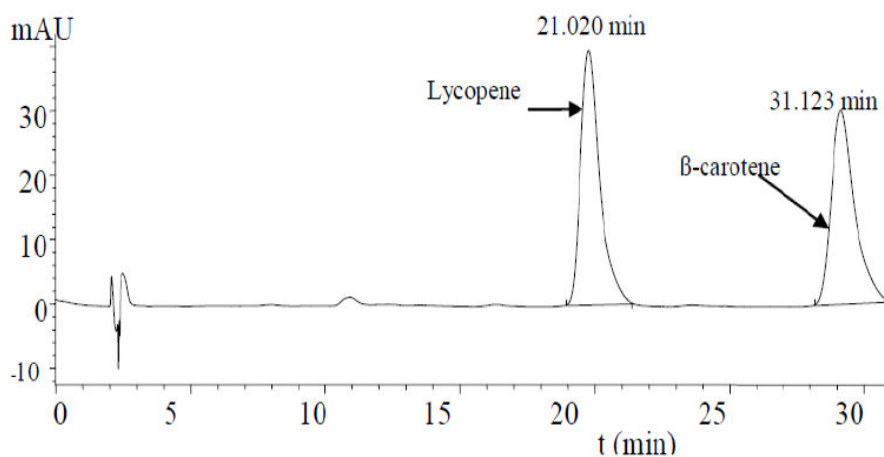
**Figure 2:** Chromatogram of tomato extract under chromatographic condition: Prevail  $C_{18}5\mu$  (250x3mm, i.d.), coupled to Prevail  $C_{18}5\mu$  (7.5x2.1mm, i.d.) guard column; mobile phase MeOH/ACN (50:50, v/v) added with TEA  $9\ \mu\text{M}$ ; flow rate 0.9 ml/min; column temperature  $30\ ^\circ\text{C}$ ;  $\lambda_{\text{detection}} = 472\ \text{nm}$  (where y-axis shows response in mAU).



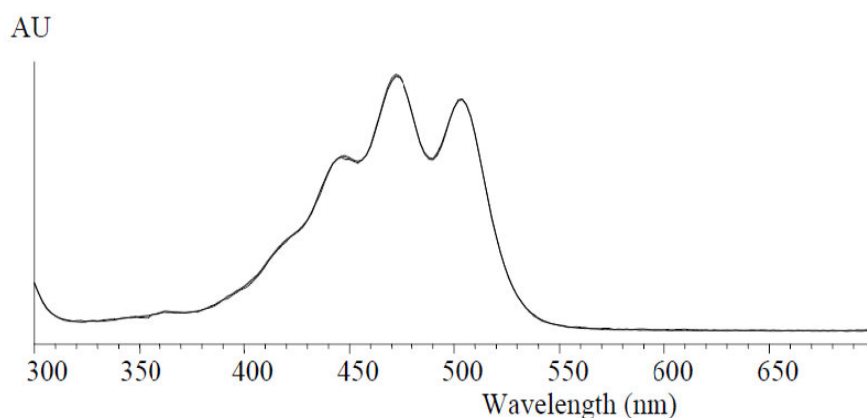
**Figure 3:** Chromatogram of tomato extract mixed with standards (lycopene and  $\beta$ -carotene) under chromatographic condition: Prevail  $C_{18}5\mu$  (250x3mm, i.d.), coupled to Prevail  $C_{18}5\mu$  (7.5x2.1mm, i.d.) guard column; mobile phase ACN/MeOH (75:25, v/v) added with TEA  $9\ \mu\text{M}$ ; flow rate 0.6 ml/min; column temperature  $30\ ^\circ\text{C}$ ;  $\lambda_{\text{detection}} = 472\ \text{nm}$  and  $450\ \text{nm}$  respectively.



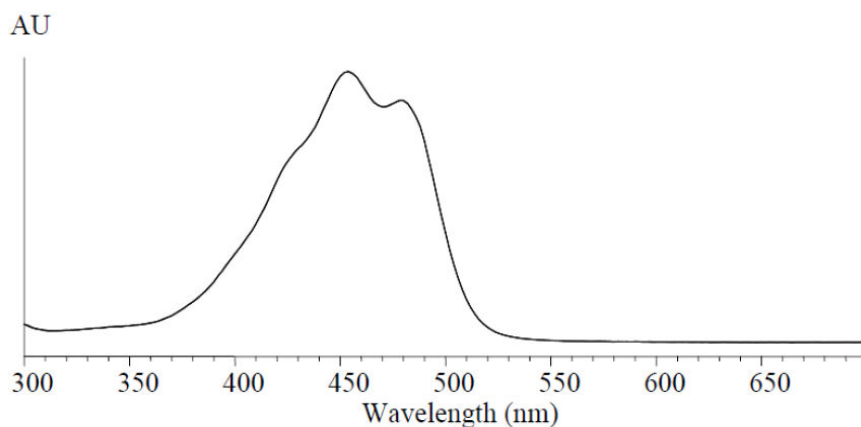
**Figure 4:** The UV/Vis-diode array impure spectrum (at  $472\ \text{nm}$ ,  $0.6\ \text{mL/min}$ ) of lycopene of the first peak of figure 1.3 (y-axis shows absorbance)



**Figure 5:** Chromatogram of tomato with lycopene and  $\beta$ -carotene under chromatographic condition: Prevail  $C_{18}5\mu$  (250x3mm, i.d.), coupled to Prevail  $C_{18}5\mu$  (7.5x2.1mm, i.d.) guard column; mobile phase ACN/MeOH (50:50, v/v) added with TEA 9  $\mu$ M; flow rate 0.6 ml/min; column temperature 30  $^{\circ}$ C;  $\lambda_{\text{detection}} = 472$  nm and 450 nm respectively.



**Figure 6:** UV/Vis-diode array spectrum of the lycopene peak at 472 nm confirming spectral profile of lycopene (Qiu *et al.*, 2006) (y-axis shows absorbance).



**Figure 7:** UV/Vis-diode array spectrum of  $\beta$ -carotene peak at 450 nm.

the solubility of the tomato lycopene standard was tested, THF was very efficient in dissolving lycopene while the solubility of lycopene in MeOH was very poor. The presence of MeOH hampered the extraction of lycopene due to the poor solubility of this compound in Methanol. Re-dissolving the residue first in THF followed by the remaining (ACN and MeOH) maintaining 15:30:55 composition was found as best combination. Although the solubility of lycopene must be ensured, filtering of the solution to establish concentrations for absorbance reading were not necessary as all the solvents used for our study were HPLC gradient grade. In all cases, extraction solvent hexane/acetone/ethanol (50:25:25) was used without any modification.

In conclusion, the mobile phase composition ACN/MeOH (50:50, v/v) added with TEA 9  $\mu$ M, extraction solvents hexane/acetone/ethanol (50:25:25 v/v/v) and re-dissolving the residue in THF, followed by ACN/MeOH (15:30:55, v/v/v) at flow rate 0.6 mL/min showed best results achieving the characteristic spectral profile of lycopene with a repeatable extraction of carotenoids in tomato in a good recovery.

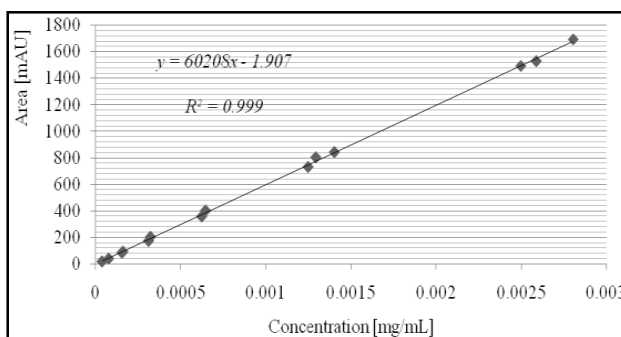
### Validation of the Established Method

#### Linearity and Calibration Curve

For HPLC, a series of tomato lycopene standards were dissolved in the same conditions as mentioned in the method and was injected in the chromatographic system. An external calibration curve for the tomato lycopene standard is represented graphically in the Figure 8 showing the area vs. concentration. The response of lycopene was linear between 0.039–2.8  $\mu$ g/mL with a coefficient of determination ( $R^2$ ) of 0.999. In validation guideline from Khachik *et al.*, (1992) recommends, for validity of standards and instrumentation correlation coefficient ( $r$ ) of  $> 0.9$  and an intercept very close to zero. In another guideline by Mantoura *et al.* (1997) a correlation coefficient ( $r$ )  $> 0.95$  is also mentioned for validation of linearity. So based on this guidelines the linearity between 0.039 and 2.8  $\mu$ g/mL fulfills the requirements. The relative standard deviation ( $n=6$ ) of the regression (calculated as standard error of the estimate divided by average concentration of the standards multiplied by 100) was about 3.2%. Once again this is within the recommended range which is suggested  $< 5\%$  (Khachik *et al.*, 1992).

The limit of quantification (LOQ) was considered at the lowest standard concentration level in the calibration, was 0.039  $\mu$ g/mL. The

limit of detection (LOD), calculated as LOQ divided by 2.5 (Hulshof *et al.*, 1997) was 0.0156  $\mu$ g/mL.



**Figure 8:** Seven pointed ( $n=2$ ) calibration curve for lycopene standard showing area vs. Concentration.

#### Precision

The precision of the instrumental technique ( $n=10$ ) was evaluated by analyzing standard samples prepared by serial dilution within the working range each day for about two weeks. In this way the repeatability calculated as standard deviation (RSD) of the slope of the calibration was 1.1%. This indicates the variability observed within a laboratory, over a short time period given that all analyses were done with same items of equipments and by same operator. The repeatability of the instrument during the entire analysis was also determined as intermediate precision (RSD=2.1%) (Table 1). A method's intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these factors (www.labcompliance.com).

**Table 1:** Precision of the HPLC method for analysis of lycopene in tomato.

Precision	RSD (%)
Injection repeatability ( $n = 64$ )	$< 0.65$
Repeatability of slope of calibration curve	
Response factor RSD	
Short – term ( $n=10$ )	1.1
Intermediate ( $n=16$ )	2.1
Retention time RSD ( $n = 8$ )	2.0
Extraction repeatability ( $n = 32$ )	0.53

In order to check injection repeatability of method within and between run, eight tomato sample determinations within a day were assessed for about two weeks. The overall



injection repeatability (RSD) within and between run ( $n=64$ ) was  $<0.65\%$ , which is acceptable. As part of methods validation, a minimum of 5 injections with RSD of  $\leq 1\%$  is recommended (CDER, 1994). Injection precision expressed as RSD indicates the performance of HPLC, including plumbing, column and environmental conditions during sample analysis. It is obvious that sample preparation and manufacturing variations are not considered here.

For the whole sample extraction and instrumental analysis, the extraction repeatability were calculated as relative standard deviation (RSD =  $0.53\%$ ). This was obtained by performing two extractions from samples of same tomato fruits and analyzing several samples of tomato each day for about two weeks by using the established methods. Since keeping same sample to determine over the following days for tomato were considered not useful as the lycopene contents easily degrades after a short time, fresh tomato samples were examined every day.

Most of RSD values obtained in this study (Table 1) were below the limits of  $15\%$  recommended for analysis of biological samples for substances around  $1 \mu\text{g/mL}$  recommended by AOAC (1993).

### Accuracy and Recovery

The accuracy of the method and possible matrix effects of HPLC analysis were monitored by means of a recovery assay. Tomato samples were spiked with internal standard solutions of  $0.05445 \text{ mg/mL}$   $\beta$ -apo-8'-carotenal. The spiked samples ( $n=8$ ) were subjected to the entire process of extraction and HPLC analysis. The amount of  $\beta$ -apo-8'-carotenal obtained after analysis was calculated relative to the amount added at the beginning using the formula indicated in the method.

Accordingly, mean recovery percentages for HPLC method ranged between  $81.7\%$  and  $84.5\%$ . This value is in the interval accepted by AOAC (1993) which recommends for substances around  $10 \mu\text{g/mL}$  recoveries between  $80\text{--}110\%$ . The mean recovery of the internal standard obtained using the procedures of the established method were  $83.0\%$  (Table 2).

As the summary in Table 2 indicated, recovery percentage of the analytes obtained were found to be unaffected by the composition of the matrices. Water and tomato juice both centrifuged and un-centrifuged were analyzed. In conclusion, results of this study show that, it is

possible to use the established method for the routine analysis of carotenoids in tomato samples with a good recovery and accuracy.

### System Suitability Parameters

Although system suitability specifications and tests are parameters which provide assistance in achieving accuracy and precision of the HPLC data collected, most of these parameters were not well assessed in this study. The result on injection repeatability was already presented in Table 1 which is within the maximum limit recommended by CDER (1994).

The capacity factor (a measure of where the peak of interest is located with respect to the void volume, i.e., elution time of the non-retained components) was estimated to be higher than  $3.2$ . This indicated that our target peak was well-resolved from other peaks and void volume. The value of capacity factor ( $k'$ ) should generally be higher than  $2$  (CDER, 1994).

### Application of the Method for Selected Tomato Cultivars

The developed HPLC method was applied in screening of 10 tomato cultivars for their lycopene content. A representative chromatographic output of 2 tomato cultivars (each from cluster and loose type) are shown in Figure 9 (a and b). A summary of the results obtained from the extraction of the selected tomato cultivars are also presented in table 3.

The identification of lycopene peak in the tomato samples was not that difficult even though the retention times showed slight fluctuation up to  $2\%$  variability calculated as RSD (Table 1) for all the analyses including for the standard. As far as the tomatoes analyzed in our study is concerned, there was no other carotenoid than  $\beta$ -carotene which appears on chromatogram result (Figure 9 (a and b)) along with lycopene. Of course in tomato these are the two dominant carotenoids with lycopene representing  $80\text{--}90\%$  (Nguyen and Schwartz 1999) and  $\beta$ -carotene  $7\text{--}10\%$  (Gould, 1974).

Since the time between extraction and HPLC analysis was considerably minimized, no degradation of carotenoid happened during analysis. In case of degradation we would have seen a number of peaks of cis-lycopene and cis- $\beta$ -carotene on the chromatogram. Therefore, comparing retention time with the corresponding pure lycopene standard and relying on the spectral characteristic was found to be sufficient for identification of lycopene from tomato samples. Actually, additional identification was

**Table 2:** Recovery of  $\beta$ -apo-8' carotenal added to tomato sample (juice).

Analyzed and spiked samples	n	Initial added (mg/mL)	Area of ISt* Recovered	Amount of ISt Recovered (mg/mL)	% Recovery
Tomato sample + ISt	1	0.05445	575.2	0.04485	82.4
Tomato sample + ISt + H <sub>2</sub> O	2	0.05445	577.1	0.04498	82.6
Tomato sample + ISt + Tomato juice	2	0.05445	578.0	0.04507	82.8
Tomato sample + ISt + Centrifuged tomato juice	3	0.05445	587.5	0.04583	82.2
<b>Mean % Recovery</b>					<b>83.0</b>

\* ISt: Internal standard ( $\beta$ -apo-carotenal), amount of ISt used was 1 mL; amount of H<sub>2</sub>O and juice spiked was 2 mL.

**Table 3:** Lycopene content in mg/100 g fresh weigh in selected tomato cultivars before and after correction for recovery.

Tomato Types	Lycopene content (mg/100 g fresh weight)			
	Before correction		After correction	
	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD
<b>Cluster cultivars</b>				
Tricia	2.94 – 5.16	4.32 $\pm$ 0.87	3.55 – 6.22	5.20 $\pm$ 1.05
Clotilde	4.31 – 5.02	4.63 $\pm$ 0.26	5.20 – 6.05	5.58 $\pm$ 0.31
Bonaparte	4.39 – 6.83	5.51 $\pm$ 0.92	5.28 – 8.23	6.64 $\pm$ 0.53
Plaisance	3.73 – 4.99	4.17 $\pm$ 0.44	4.49 – 6.01	5.02 $\pm$ 0.71
Dirk (*)	-	-	-	-
<b>Loose cultivars</b>				
Growdena	2.47 – 3.65	3.13 $\pm$ 0.44	2.97 – 4.40	3.77 $\pm$ 0.53
Brodna	1.59 – 3.12	2.34 $\pm$ 0.60	1.91 – 3.76	2.81 $\pm$ 0.73
DRW 75 – 93	2.15 – 3.85	2.96 $\pm$ 0.59	2.59 – 4.64	3.56 $\pm$ 0.71
Excelsior	1.80 – 3.59	2.67 $\pm$ 0.59	2.17 – 4.32	3.22 $\pm$ 0.71
Admire*	-	-	-	-

\*Lost data after HPLC analysis and couldn't be retrieved at later stage

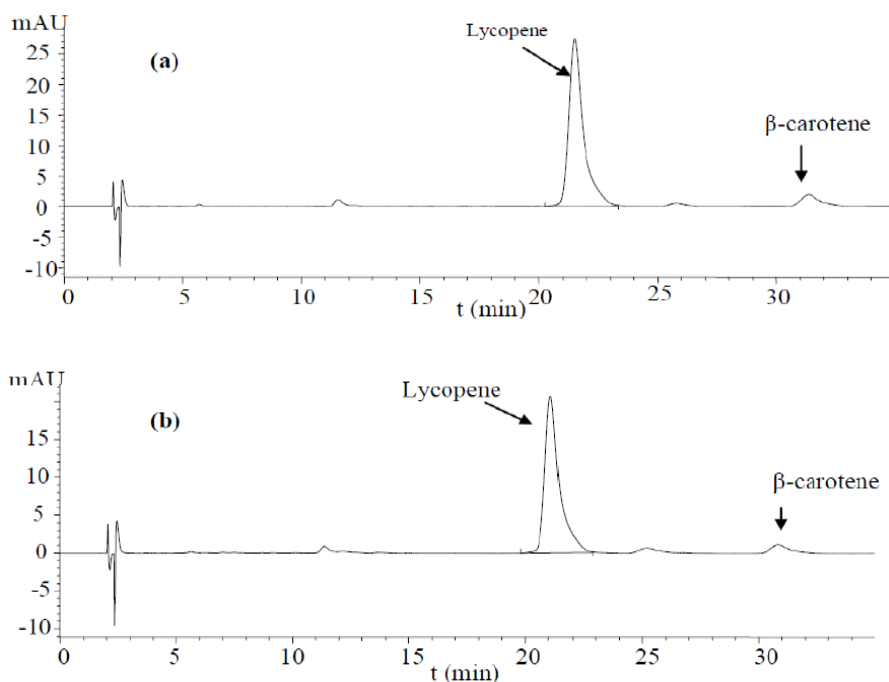
also carried out in our study by comparing the obtained spectral data with reported data of Schierle *et al.* (2003).

In order to see the real mean differences in lycopene content within and between cultivars, analysis of variance (ANOVA) was performed taking into account cultivars, number of extraction and fruit number subjected to extraction and any combination of them. According to several literature (Maguer and Shi, 2000; Pol *et al.*, 2004; Sahlin *et al.*, 2004), the amount of lycopene in tomato varies considerably between cultivars, stage of maturity and growing conditions.

To assess the variation in lycopene content between loose and cluster tomatoes and within

cultivars itself and between extractions, 4 cluster and 4 loose type cultivars were analyzed. Per cultivar four fruits were taken and two extractions per fruit were carried out. The injection run was requested in triplicate for each extraction. The ranges of lycopene content obtained in tomato samples are from 3.55 to 8.23 mg/100g in cluster tomato and 1.91 to 4.64 mg/100g in loose tomatoes as presented in Table 3.

The largest lycopene concentration obtained was 8.23 mg/100g fresh weight in cultivar Bonaparte which is a cluster tomato type. Conversely, smallest lycopene concentration detected was 1.91 mg/100g fresh weight in Brodna, a loose type tomato cultivar. The mean lycopene content values during this study ranged



**Figure 9:** Carotenoids profile of tomato cultivars (a) Bonaparte (cluster type) and (b) Growdena (loose type).

from 5.02 to 6.64 mg/100g fresh weight in cluster tomatoes and from 2.81 to 3.77 mg/100g fresh weight in loose type. The range of composition observed in tomato cultivars analyzed in this study was in general agreement with reported values (3.1 to 7.74 mg/100g fresh weight) by Nguyen and Schwartz (1999). More specifically, the average range of lycopene concentration in loose tomato exactly matches value obtained by different laboratories (0.88 to 4.20 mg/100g fresh weight) reviewed by Roldan-Gutierrez and Castro (2007). The lycopene content for the cluster types (average value ranged up to 6.64 mg/100 g of fresh weight) was quite close to the literature report average value of 7.1 mg/100 g of fresh weight (Frusciante *et al.*, 2007).

In general, cluster type tomatoes contained higher lycopene than loose tomatoes. The variation in lycopene concentration between cluster and loose tomatoes is up to 2-fold as clearly seen from Table 3. This difference was not surprising due to the fact that lycopene content of tomatoes are a function of genotype, and cluster tomatoes naturally among those supposed to have high carotenoids content (George *et al.*, 2004).

Although the number of tomato cultivars analyzed was small, our study results are also clearly outlined by statistical analysis. The mean

difference in lycopene concentration between tomato cultivars, fruits, extractions and their combinations were well considered. The analysis of variance shows that there is a highly significant difference ( $p=0.0015$ ) in lycopene content between loose and cluster tomato. The composition is significantly higher for all cluster tomatoes analyzed compared to loose types. It is interesting to point out that no statistical significant ( $p = 0.1680$ ) interaction was observed between cultivars. This unexpected result for cultivar (type) interaction might have been attributed to the closeness in lycopene content within cultivar types. Of course, the small number of tomato cultivars (only eight) involved for statistical analyses may also have an influence.

The interaction of fruit (within cultivar and type) was highly significant ( $p<0.0001$ ). The ANOVA calculation also identified the interaction of extraction highly significant ( $p<0.0001$ ) (within cultivar, type and fruits). Consequently, the extraction system used for lycopene analysis combined with other variables can also contribute significantly to the variability obtained within same cultivar (fruit). Data of the tomato samples considered for this study confirmed that concentration of lycopene can be very different (from 1.91 to 8.23 mg/100 g of fresh weight) also for tomatoes having a similar intensity of red color.

## CONCLUSION

This work has provided sufficient information to the present demand of high-purity lycopene, particularly the analytical methods and careful validation procedures available for its extraction, separation, detection and preparative isolation.

Among the several HPLC conditions tested and evaluated; the mobile phase composition ACN/MeOH (50:50, v/v) added with TEA 9  $\mu$ M, extraction solvents hexane/acetone/ethanol (50:25:25v/v/v) and re-dissolving the residue in THF, followed by ACN/MeOH (15:30:55, v/v/v) at flow rate 0.6 mL/min have showed best results in achieving the characteristic spectral profile of lycopene in tomato tissue with precision and acceptable recovery.

The new analytical method provides a means for more rapid separation and quantification of the lycopene extracted from tomato. A sample can be analyzed in ca. 21 min without the necessity of employing a linear gradient or a gradient programme for the mobile phase. This eliminates the need to equilibrate the column between samples, which implies that no additional time is required to carry out such procedures. The two peaks, lycopene and  $\beta$ -carotene were well separated and identified but only lycopene was quantified in this study. The method has been evaluated against different validity parameters and applied in the screening of ten tomato cultivars for their lycopene content. The lycopene content ranged from 3.55 to 8.23 mg/100g in fresh cluster tomatoes and 1.91 to 4.64 mg/100g in fresh loose tomatoes. The largest lycopene concentration, 8.23 mg/100g fresh weight obtained was in cultivar Bonaparte which is a cluster tomato. Conversely, the smallest lycopene concentration, 1.91 mg/100g fresh weight detected was in Brodena, a loose type tomato cultivar. To conclude, it can be said that HPLC technique is a widely accepted, efficient and accurate method of choice for the determination of lycopene in tomatoes, though sample preparation requires prolonged time.

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