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HPLC Method for the Determination of Lycopene in Crude Oleoresin Extracts

THADIKAMALA SATHISH*†, DORADLA UDAYAKIRAN‡, KUSHALA HIMABINDU§, PATSAMATLA LAKSHMI DURGA SRIDEVI¶, DEVARAPALLI KEZIA§ and PULLASI BHOJARAJU#

Center for Biotechnology, Department of Chemical Engineering
Andhra University, Visakhapatnam-530 003, India
E-mail: satish.tadikamalla@gmail.com

A simple and sensitive isocratic RP-HPLC method with spectrophotometric detection (475 nm) was developed and validated for the determination of lycopene in crude plant extracts. The method utilizes Supelcosil LC-18 column (150 × 4.6 mm), 3 µm particle size and a mobile phase consisting of acetonitrile, methanol and tetrahydrofuran (70:25:5, % v/v) at a flow rate of 1.0 mL/min. The retention times of lycopene and astaxanthin (internal standard) were 14.2 and 3.0 min, respectively and the total run time was 15 min. Experiments were conducted at room temperature in the absence of direct sunlight. Calibration graph of standard lycopene was linear in the concentration range of 200-1000 ng/mL with the detection limit of 50 ng/mL. The method was validated for the concentration range 0.2-1.0 mg/mL. Intra-and inter-day relative standard deviations were less than 3.5.

Key Words: Lycopene, Astaxanthin, HPLC determination.

INTRODUCTION

Lycopene (ψ,ψ-carotene) (Fig. 1), an acyclic carotenoid containing 11 conjugated double bonds, is naturally present in *trans*-form in raw tomatoes[1] and imparts red colour to the tomatoes. It is also one of the most abundant non-vitamin analogues present in human blood from food consumption[2]. Among the common dietary carotenoids, lycopene has the highest single

†*Present address:* Bioengineering and Environmental Engineering Center, Indian Institute of Chemical Technology, Hyderabad-500 007, India.

‡Department of Biotechnology, Sri Venkateswara University, Tirupati-517 502, India.

§Department of Biotechnology, St. Martine Engineering College, Dulapally, Secunderad-500 014, India.

¶Department of Biotechnology, CMR College of Engineering and Technology, H-7, Medchal Road, Hyderabad-501 401, India.

#Shri Vishnu College of Pharmacy, Bhimavaram-534 202, India.

oxygen quenching capacity *in vitro*[3] and its antioxidant properties are probably related to risk reduction of certain types of cancers. Remarkable inverse relationship between lycopene intake or serum lycopene concentrations and cancer risk have been observed[4], especially for prostate, pancreas, intestine, pharynx and to certain extent stomach cancer. Some of the studies[5,6] have shown that lycopene is the only carotenoid associated with cancer risk reduction and its plasma concentration is also considered as a useful clinical parameter associated with myocardial infarction[7]. In addition lycopene may exhibit other physiological activities such as suppression of human cancer cells[8].

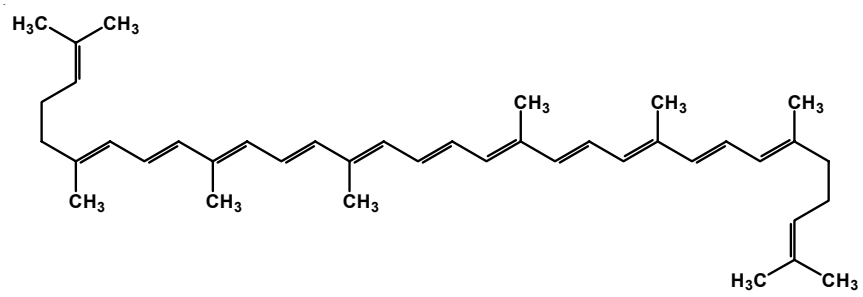

Fig. 1

All *trans*-lycopene may be converted to its *cis*-configuration during food processing[9]. Processing promotes different side reactions that could affect the antioxidant activity of tomato products. Firstly, processing of tomato affects the amount of antioxidants. About 30 % of ascorbic acid, the most reactive antioxidant is lost during tomato juice production[10] and it is almost completely degraded[11] during air drying of tomato at 80 and 110 ºC. Carotenoids have shown to undergo both isomerization and oxidation under model conditions[12]. Secondly, processing of tomato enhances the accessibility of hydroxyl phenol groups. It has been found that, after air-drying at 80 ºC, the number of hydroxyl phenol groups increases owing to the hydrolysis of flavanoid glycosides and/or the release of cell wall phenolics[13]. Several papers have demonstrated that the *cis*-isomers of lycopene could be adsorbed in to body more easily and played a more important role in biological function than all *trans*-lycopene[14,15].

The protective effect has been attributed to antioxidant in tomato, which are believed to inhibit the reactive oxygen species (ROS)-mediated reactions occurring *in vivo* during number of pathological process[16]. It is currently understood that tomato-derived products are almost the exclusive source of the carotenoid lycopene and provide a significant contribution to dietary intake of ascorbic acid[17].

Lycopene has been determined in food or biological samples by many analytical methods, such as UV-Visible spectrophotometry[18], liquid chromatography connected to electrospry-ionization (LC/ESI-MS)[19], reverse phase liquid chromatography[20] (RP-LC), supercritical fluid chromatography[21] (SFC), matrix assisted desorption ionization[22] (MALDI) and especially LC with spectrophotometric detection[23-30].

Majority of existing chromatographic methods describes the determination of number of plasma carotenoids including lycopene and, since they use gradient elution, are time consuming and complicated. Due to the antioxidant properties of lycopene, several attempts are being made to isolate lycopene from tomatoes in large quantities and formulate it in suitable dosage forms for human use in risk reduction of certain types of cancer. For these research activities a fast, reliable, simple HPLC method is required for the determination of lycopene in isolated and purified crude material and in the plasma of experimental animals and humans.

In this paper a fast isocratic reverse-phase high-performance liquid chromatographic method with UV detection is described for the determination of lycopene in crude oleoresin of tomato material. In this method internal standard astaxanthin (Fig. 2) have been used. Literature survey reveals that there is no internal standard method was developed for the determination of lycopene.

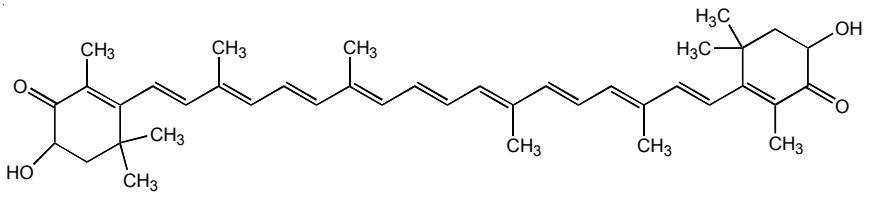

Fig. 2

EXPERIMENTAL

Tomatoes were purchased from a local farm in Hyderabad (India). Lycopene standard was kindly provided by Sami Laboratories (Bangalore, India) and internal standard from Devis Laboratories Ltd. (Hyderabad, India). The HPLC grade solvents, methanol and acetonitrile were purchased from Qualigens Fine Chemicals (Mumbai, India); tetrahydrofuran and astaxanthin were purchased from E. Merck (India) Limited (Mumbai, India).

The chromatographic system consisted of a Shimadzu LC-10 AS system (controller, pumps, injector equipped with a 20 µL sample loop) and an SPD-10A UV-Visible detector (Shimadzu, Japan). The data and chromatograms were collected using C-7RA chromatopac software system (Shimadzu, Japan). The pH of the solution was adjusted by using Digital pH Meter,

Model DI 707 (Digisun Electronics, Hyderabad, India). Dissolution of compounds was enhanced by sonication on Bandelin sonerex (Bandelin, Berlin). UV spectra of lycopene for selecting the working wavelength of detection were scanned using Cintra 5 UV-Visible spectrophotometer (GBC Scientific equipments, Australia).

Preparation of standards: Stock solutions of lycopene and astaxanthin (astaxanthin) for generating standard curves were prepared by dissolving 10 mg of each compound in methanol and THF (50:50, %, v/v) for lycopene and methanol for astaxanthin to yield concentrations of 100 and 40 µg/mL, respectively. Working standard solution of astaxanthin was obtained by diluting 2.5 times.

Chromatographic conditions: A Supelcosil LC-18 analytical column 3 µm, 150 × 4.6 mm i.d (Supelco, USA), the mobile phase consisted of acetonitrile, methanol and tetrahydrofuran (70:25:5, % v/v) and was delivered at flow rate of 1.0 mL/min. Prior to use, the mobile phase was degassed by sonication. Between the samples the injection needle washed with blank solution. The retention times for lycopene and astaxanthin were 14.2 and 3.0 min, respectively. The wavelength was set at 475 nm.

Calibration of standards: Calibration curves were prepared by spiking 200-1000 ng/mL of working standard solutions in 2 mL of blank solution (final volume) to yield 200, 400, 600, 800, 1000 ng/mL for each analysis. To each above solution, 1 mL of working I.S was added. Another stock solutions separately prepared for quality control. All the standard curves were checked using quality control samples. The stock solutions were stored at -20 ºC in refrigerator. All the work carried in the absence of direct sunlight.

A 20 µL aliquot was injected in to the analytical column. Quantitative analysis based on peak area measurements as ratios towards the peak area of internal standard.

Search for internal standard: Several carotenoids whose structure and solubility is resembles to standard lycopene, were added to standard solution of lycopene and next to the crude extracts and the resulting mixture was subjected to analysis according to the procedure.

Method of validation

Specificity and selectivity: The specificity of the method was evaluated with regard to interference compounds. Five different samples were injected and studied. To establish the range of linearity between compound concentrations and detector response the compound concentrations of 0.2, 0.4, 0.6 and 1.0 µg/mL were used.

Linearity: To establish the range of linearity between drug concentration and detector response, the lycopene concentrations of 200, 400, 600, 800 and 1000 ng/mL were used. Five replicates of analyte were measured.

Limit of detection (LOD): The LOD can be defined as the smallest level of analyte that gives a measurable response. The LOD is based on S/N ratio typically 3.0 for HPLC methods. Five replicate of analyte were measured.

Limit of quantification (LOQ): Limit of quantification was defined as the lowest concentration at which the precision expressed by relative standard deviation (RSD) is less than 20 % and accuracy expressed by relative difference in the measured and true value was also less than 20 %. In other words, the analyte response is 10 times greater than to the noise response. Five replicate of analyte were measured and quantified.

Recovery: Percentage recoveries of lycopene from spiked blank were determined and represented as mean ± standard deviation by injecting quality control samples of five replicates.

Precision: To assess the precision of the method, intra-day and inter-day (days 7) measurements of lycopene were completed with computation of the coefficient of variation (CV %) for replicate samples (n = 5) using concentrations of 200, 400, 600 and 1000 ng/mL. Both intra-day and inter-day samples were calibrated with standard curve concurrently prepared on the day of analysis.

Accuracy: Intra-day and inter-day accuracy was evaluated by assaying quality control with different concentrations of lycopene. Intra-day and inter-day accuracy assessed by analyzing four quality control samples at each concentration on the same day and mean values of four samples for 7 days, respectively. Accuracy was presented as per cent error (relative error), [(measured concentration-added concentration)/added concentration] × 100 (%).

System suitability: It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. For this, parameters like plate number (N), tailing factor (k), resolution (R) and relative retention time (α), HETP, capacity factor (k^I), plates per meter and peak symmetry of samples were measured.

Robustness: The optimum HPLC conditions set for this method have been slightly modified for samples of lycopene (mg/mL) dissolved in the crude extracts as a means to evaluate the method robustness. The small changes include the mobile phase ratio, the flow rate, the detection wavelength, the sonication time, the filtration system and the column.

Analysis of crude extracts: 10 mg of lycopene was quantitatively transferred to a 10 mL volumetric flask and dissolved in methanol and THF (50:50, % v/v) the final volume was made up to the mark with methanol. Then, this solution was subjected to sonication for 1 h. After getting clear solution, the solution was filtered through 0.2 µm membrane filter. Further dilutions were provided.

RESULTS AND DISCUSSION

Choice of stationary phase and mobile phase: For the selection of stationary phase several columns such as C-8 and C-18 were used, among which non-polar C-18 analytical chromatographic column was chosen as the stationary phase for the separation and determination of the non-polar hydrophobic lycopene molecules. The 3 µm particle size provides well separated narrow peaks.

For the mobile phase a number of eluting systems were examined. The use of binary mixtures of methanol and acetonitrile, at any ratio, solvent systems most frequently used for the separation/determination of carotenoids, resulted in prolonged retention time of the analyte. Therefore, the addition of a third solvent, in which lycopene is more soluble, was found to be essential. Taking into consideration the solubility characteristics of lycopene, the most suitable solvents to be used were chloroform and THF. The use of chloroform was avoided, due to its higher toxicity. For the optimization of the composition of the mobile phase, many different ternary mixtures consisting of methanol, acetonitrile and THF (60:25:15, 70:20:10, 70:25:5) were examined. The choice of optimum composition was based on the so-called chromatographic response factor. A composition of (70:25:5, % v/v) for acetonitrile, methanol and THF, respectively, gave the highest value of the chromatographic response factor and provided an efficient separation of lycopene with a sufficient retention time. A flow rate of 1.0 mL min^{-1} was found to be optimum from a studied range of 0.5-1.5 mL min^{-1} as a compromise between an optimum retention time, baseline stability and noise.

Internal standard approach: In order to minimize the contribution of sample preparation, injection variation and column deterioration to the final results, the internal standard mode of quantification applied. For this several compounds were tested. Amongst astaxanthin was considered as internal standard, because it fulfills the requirements for good internal standard; it possess similar physicochemical properties, go through all the steps of procedures and elute close to analyte.

Method of validation

Chromatography: A representative chromatogram of blank spiked with 100 ng/mL of lycopene and 200 ng/mL astaxanthin (as an internal standard) was shown in Fig. 3. Both compounds were well separated from each other and from the solvent peaks. The chromatogram of sample does not show any interference peaks.

Linearity: The linearity of calibration curves (peak area *vs.* concentration) for lycopene in pure solutions was checked over the concentration ranges of about 200-1000 ng/mL and found to be linear with correlation

coefficients of better than 0.999 in most cases. Furthermore, the linearity of the calibration curves for the compounds related to lycopene were also studied over the ranges between 200 and 1000 ng/mL and found to be linear with correlation coefficients of better than 0.9999 for all the cases in either pure solutions or in solutions comprising the crude extracts.

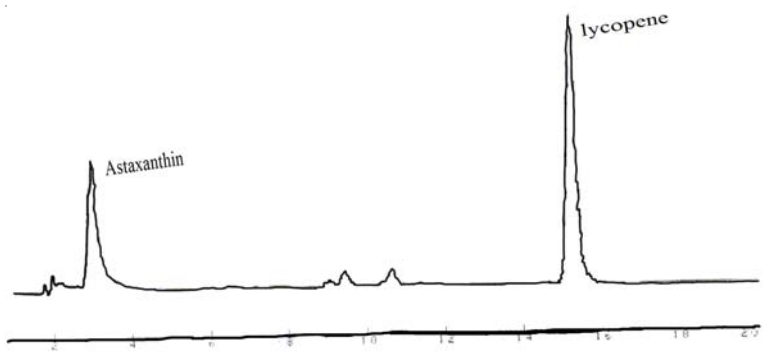

Fig. 3. HPLC chromatogram of blank spiked with 100 ng/mL of lycopene and 200 ng/mL of astaxanthin (IS)

Specificity and selectivity: Interference studies were conducted and investigated by determining the retention times in this system. The retention times of β-carotene and other carotenoids were 18.5 *etc.*, respectively. No peaks were observed until 18 min.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD and LOQ of the developed method were calculated on the basis of 3.3 and 10 times, respectively, the standard deviations of most dilute standard or more accurately, standard error of regression ($S_{Y/x}$). For crude material, samples are dissolved in methanol, THF (50:50, % v/v) and injected. LOD and LOQ of crude samples were found to be 50 ng/mL and 100 ng/mL.

Recovery: Recovery from crude extracts was calculated by comparing the peak heights of pure standards prepared in methanol and THF (50:50, % v/v) and injected directly into the analytical column with those of samples containing the same amount of test sample (n = 5). Recoveries were determined at four different concentrations ranging from 200-1000 ng/mL.

Precision and accuracy: Intra-and inter-day precision and accuracy were evaluated by assaying quality control with different concentrations of lycopene. Intra-and inter-day precision were assessed by analyzing four quality control samples at each concentration on the same day and mean values of quality controls for 7 d, respectively (Tables 1 and 2). Intra-and inter-day relative standard deviations were less than 3.0 and 3.1, respectively in the concentration range of 200-1000 ng/mL. Accuracy represented

TABLE-1
INTRA-DAY PRECISION (CV) AND ACCURACY
(RELATIVE ERROR) MEASUREMENTS OF LYCOPENE

Concentration (ng/mL)	Measured concentration (ng/mL) ± SD	CV (%)	Relative error (%)
200	190.01 ± 5.6	2.91	-5.00
400	392.31 ± 7.0	1.84	-1.93
600	600.90 ± 8.0	1.32	+0.10
1000	968.90 ± 10.5	1.19	-3.10

TABLE-2
INTER-DAY PRECISION (CV) AND ACCURACY
(RELATIVE ERROR) MEASUREMENTS OF LYCOPENE

Concentration (ng/mL)	Measured concentration (ng/mL) ± SD	CV (%)	Relative error (%)
200	186.48 ± 5.81	3.11	-6.76
400	392.12 ± 8.02	2.03	-1.97
600	606.60 ± 8.30	1.36	+1.10
1000	965.90 ± 11.00	1.25	-3.21

as per cent error (relative error) [(measured concentration-added concentration)/added concentration] × 100 (%), while precision was measured by calculating intra- and inter-day C.V values.

System suitability: For system suitability, five replicates of standard samples were injected and studied the parameters like plate number (N), tailing factor (k), resolution (R) and relative retention time (α), HETP, capacity factor (k^1), plates per meter and peak symmetry of samples.

Robustness: It shows that the per cent recoveries of lycopene was good under most conditions and didn't show a significant change when the critical parameters were modified. The tailing factor for lycopene was always less than 1.0 and the components were well separated under all the changes carried out. Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experiments at room temperature may conclude that the method conditions are robust.

Application of the method to crude plant extract: The developed method is applied for the determination of lycopene in crude material isolated from tomatoes. The percentage purity of crude oleoresin sample is 3.8 % and a RSD of 3.4. It is also useful in the bioavailability experiments using an under development of lycopene formulation with good results. A representative chromatogram of lycopene in crude sample is shown in Fig. 4.

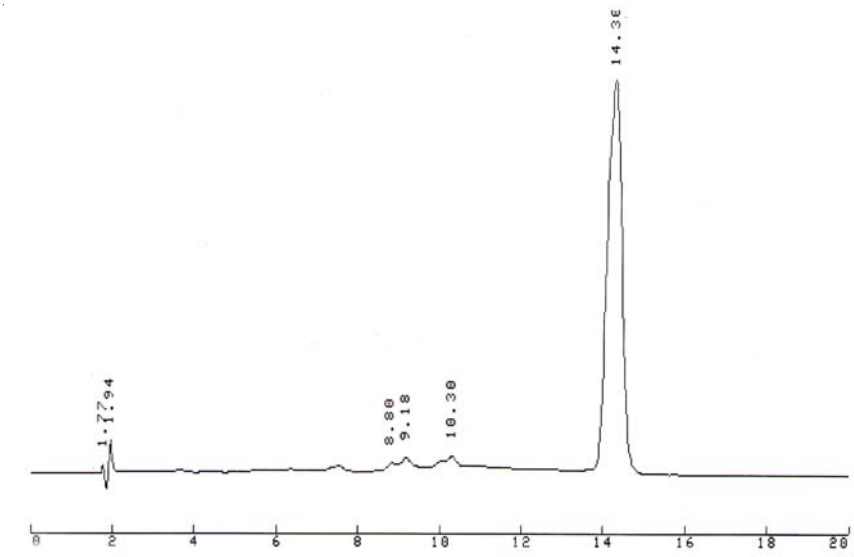

Fig. 4. HPLC chromatogram of lycopene in crude plant extracts

TABLE-3
PURITY STUDIES OF LYCOPENE FROM CRUDE PLANT EXTRACTS

Name of the crude extract	Added amount (mg)	Observed amount (mg) ± SD	Purity (%)
Hexane	10	0.386 ± 0.41	3.86

Changing various parameters, such as composition of mobile phase, flow rate and stationary phases (column) optimized the chromatographic method. THF has more pronounced effect on solubility and elution of the compound. Under the presently prescribed conditions the recoveries of lycopene and astaxanthin were good. A low concentration of THF was used.

No single internal standard method have been reported for determination of lycopene. The differences of % CV less than 3.0 for both the inter- and intra-day data reflect the accuracy of the method. The observations of C.V less than 3.11 for both inter- and intra-day measurements also indicates high degree of precision.

In the present study, we have established a linearity range of 0.2-1.0 µg/mL. This linearity range covers all the strengths of lycopene. Hence this method can be applied for quantifying all the levels of lycopene in extracts and other pharmacokinetic studies.

Conclusion

A reliable, sensitive, accurate, fast isocratic reversed-phase HPLC method for the determination of lycopene in crude sample has been developed, optimized and validated. It has been shown to be precise and sensitive. Attempting to isolate lycopene from tomatoes and prepare a suitable formulation, the method is useful for the content determination and bioavailability studies.

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