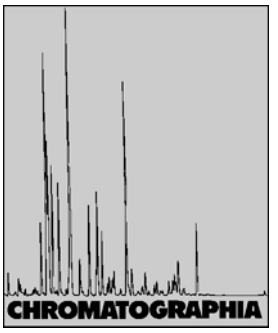
Separation of Lycopene and Its *cis* Isomers by Liquid Chromatography



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Key Words

Column liquid chromatography
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Summary

Lycopene is an important biological compound that is widely distributed in fruits and vegetables. Epidemiological study has shown that the dietary intake of lycopene may reduce the risk of certain types of cancers such as prostate cancer. However, the formation of *cis*-isomers of lycopene during food processing and storage may decrease its biological activity. Thus, it is important to learn about the content of lycopene and its *cis* isomers in foods. In this study we compared two types of columns (C_{18} and C_{30}) and various solvent systems for the separation of lycopene and its *cis* isomers by HPLC. Results showed that all-*trans*-lycopene and its nine *cis* isomers could be resolved by employing a C_{30} column with a mobile phase of n-butanol-acetonitrile-methylene chloride (30:70:10, *v/v/v*) and detection at 476 nm within 35 min. A C_{30} column was found to provide more powerful resolution of lycopene and its *cis* isomers, but the retention time was drastically increased compared to that of a C_{18} column. The *cis*-isomers of lycopene were tentatively identified as 5-*cis*, 9-*cis*, 13-*cis*, 15-*cis*-lycopene, and possibly as four lycopene di-*cis* isomers, based on spectral characteristics and Q ratios as reported in the literature. The method developed in this study could be applied to determine the lycopene content in tomatoes.

Introduction

Lycopene is an important biological compound that is widely distributed in fruits and vegetables. Epidemiological study has shown that the dietary intake of lycopene may reduce the risk of certain type of cancer such as prostate cancer [1]. Because of presence of 11 conjugated carbon-carbon double bonds, lycopene is susceptible to chemical change when exposed to light, heat and oxygen [2]. During food processing, lycopene may undergo degradation and isomerization simultaneously, and the formation of *cis* isomers of lycopene may reduce its color intensity and biological activity [3]. Some recent studies suggested that several *cis* isomers of lycopene such as 9-*cis*-, 13-*cis*- and 15-*cis*-lycopene are present in human serum [4]. In tomato products, all-*trans*-, 13-*cis*- and 15-*cis*-lycopene are reported to occur [5]. As the resonance effect of *cis*-lycopene is lower than all-*trans*-lycopene, the antioxidative ability of the former should be inferior to the latter. Thus, to develop a method for the simultaneous determination of all-*trans*-lycopene and its *cis* isomers in food products is extremely important.

Traditionally, the separation of carotenoids in food samples is often carried out by HPLC with a C_{18} column. However, most HPLC methods employing a C_{18} column failed to resolve all-*trans*-lycopene and its *cis* isomers [6, 7]. To remedy this problem the application of a C_{30} column for separation of all-*trans*-lycopene and its *cis* isomers has been developed [4, 8, 9]. Emenhiser *et al.* [8] developed a mobile phase of methyl tert-butyl ether (MTBE) and methanol in different proportions for the separation of all-*trans* and *cis* forms of α-carotene, β-carotene and lycopene [8]. However, lycopene was not eluted. Yeum *et al.* [4] used a gradient solvent system of methanol-MTBE-water to separate lycopene and its *cis* isomers, and all-*trans*-, 9-*cis*-, 13-*cis*-, and 15-*cis*-lycopene were resolved within 35 min [4]. However, the drawback of this method is that baseline drift occurred, which may affect the quantitation accuracy. In view of this problem the development of an HPLC method for separation of lycopene and its *cis* isomers is necessary. The objective of this study was to compare both C_{18} and C_{30} columns and various mobile phases for the separation and identification of all-*trans* and *cis* forms of lycopene.

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Experimental

Instrumentation

The HPLC instrument consisted of a Phenomenex DG-440 degasser (Torrance, CA, USA), a Rheodyne model 7161 injector (CA, USA), a Jasco PU-980 pump (Jasco Co., Tokyo, Japan) and a Jasco MD-915 photodiode-array detector. Borwin computer software was used to process data. Two C_{18} columns (Vydac 201TP54 and Waters 5C18-AR; 250 mm × 4.6 mm I. D., particle size 5 μm) and a C_{30} column (YMC Carotenoid; 250 mm × 4.6 mm I. D., particle size 5 μm) were used for separation of lycopene and its *cis* isomers.

Chemicals

The all-*trans*-lycopene standard was purchased from Extrasynthese Co. (Genay, France). The HPLC-grade solvents, including methanol and methylene chloride, were purchased from Merck Co. (Darmstadt, Germany). Both 2-propanol and 1-butanol were from Labscan Co. (Bangkok, Thailand). Acetonitrile was from Mallinckrodt Co. (Paris, KY, USA). All of the solvents were degassed by sonication prior to use.

Method

Separation and Identification

Various binary solvent systems containing water-acetonitrile or methanol-acetonitrile in different proportions (15:85, 20:80, 10:90 or 5:95, *v/v*) as well as 2-propanol-acetonitrile (30:70, *v/v*) were used for comparing the separation efficiency of lycopene and its *cis* isomers. Also, various ternary solvent systems containing 2-propanol-acetonitrile-methylene chloride in different ratios (30:70:5, 30:70:10 or 30:70:20, *v/v/v*) and 1-butanol-acetonitrile-methylene chloride (30:70:10, *v/v/v*) were employed. Two C_{18} and one C_{30} columns were compared for all of these solvent systems, with flow rate of 1.0 mL min^{-1} and detection at 476 nm. The concentration of the working solution of lycopene standard was 200 μg mL^{-1}. The injection volume was 20 μL and the sensitivity was 0.005 AUFS. Each peak was scanned between 200–650 nm with a Jasco MD-915 photodiode-array detector. The *k* value (capacity factor) was used to assess the solvent strength of each mobile phase. The detection limit was determined based on a method described by the International Conference on Harmonization [10]. Three lycopene standard concentrations of 10, 25 and 50 ppm were prepared and each was injected three times. Three calibration curves were obtained by plotting concentration against area. The means of the slopes (S) and standard deviation of the intercepts (σ) were calculated. The lowest detection limit (DL) and quantitation limit (QL) were calculated as follows:

$$DL = 3.3 \times \sigma / S$$
$$QL = 10 \times \sigma / S$$

In addition, both the DL and QL of several cis isomers of lycopene were also determined.

All-trans lycopene was identified by comparing the retention time and absorption spectrum with a reference standard. In addition, *cis*-lycopene was tentatively identified based on the following rules:

1. The mono-*cis* isomers of lycopene results in a hypsochromic shift of about 4 nm when compared to the parent all-trans forms [11, 12].
2. The central *cis* isomers of lycopene such as 13-*cis*, 13′-*cis*, 15-*cis* and 15′-*cis* have a strong peak in the UV region at about 340 nm [2].
3. The *cis* isomers of lycopene have smaller extinction coefficients and reduced fine structure [12].
4. The mono-*cis* or di-*cis* isomers of lycopene could be tentatively identified by the Q ratio. The Q ratio could be defined as the height ratio of the *cis* peak to the main absorption peak.
5. The di-*cis* isomers of lycopene may be shifted to shorter wavelength than its mono-*cis* form [2].

Extraction and Purification of Lycopene from Tomatoes

To illustrate the separation capability of the developed solvent system, tomatoes were used as a reference sample for the extraction and separation of lycopene, using a method similar to that described by Shi *et al.* (1999) [13]. Three tomatoes weighing about 150 gm were cut into pieces and blended in a blender for 5 min, after which the puree was homogenized with a polytron. Ten gm of puree was collected and transferred to a flask, which was wrapped with aluminum foil to exclude light. A solution (100 mL) containing hexane-acetone-ethanol (2:1:1, *v/v/v*) was added to the flask and the mixture was agitated for 10 min in a shaker, after which 15 mL water was added for another 5-min shaking. After shaking, the mixture was allowed to stand in the dark for 1 hr until two phases separated. The upper phase containing the lycopene solution was collected, evaporated to dryness and dissolved in 2 mL of hexane-acetone-ethanol (2:1:1, *v/v/v*) for open-column chromatography. A mixture containing magnesium oxide (15 gm) and diatomaceous earth (15 gm) was suspended in 100 mL of hexane, and the suspension was poured into a glass column (30 cm × 25 mm I. D.) with suction at the bottom to allow the adsorbent to be packed firmly. Anhydrous sodium sulfate (5 gm) was placed above the adsorbent to form a 2-cm layer. Two mL of carotenoid extract was poured onto the column and the various fractions of pigments were eluted with the solvent systems in order of increasing polarity. The elution process started with 100 mL of 100% hexane, followed by 100 mL each of hexane-acetone (95:5, 90:10 or 80:20, *v/v*), and hexane-acetone-ethanol (84:15:1 or 83:15:2, *v/v/v*). The sixth fraction with the deepest red color was collected, evaporated to dryness and dissolved in 2 mL of n-butanol-acetonitrile-methylene chloride (30:70:10, *v/v/v*). The solution was filtered through a 0.2-μm membrane filter for HPLC analysis. The concentration of mono-*cis* isomer of lycopene was quantified based on the standard curve of all-*trans*-lycopene, which was prepared by plotting ten concentrations (0,25, 50, 75, 100, 125, 150, 175, 200, 225, 250 $\mu g \cdot g^{-1}$) against area.

Results and Discussion

The separation of carotenoids and their *cis* isomers are routinely conducted by HPLC using a C_{18} column [6, 7, 14]. However, the HPLC conditions used by these authors failed to resolve all-trans and *cis* forms of lycopene. In this study we used a C_{18} column (Vydac 201TP54) initially and a mobile phase of methanol-acetonitrile (15:85, *v/v*) with flow rate of 1.0 mL min^{-1} and detection at 476 nm. All-trans-lycopene was eluted within 15 min, but all the *cis* isomers of lycopene were eluted together as one peak. By changing the proportions of methanol-acetonitrile as 20–80, 10–90 or 5–95 (*v/v*), no improvement

was observed. A solvent system of acetonitrile-water of different ratios also did not show good separation. Similar results were obtained with a Waters C_{18} column and the same mobile phases, with the exception that the retention time of the all-trans-lycopene was drastically increased. These results indicated that even with the same types of column and the same particle size, the retention behavior and separation efficiency of lycopene and its *cis* isomers could be changed, probably because of differences in the porosity, carbon load and polymerization of the C_{18} stationary phase [9, 15].

Because of the difficulty in resolving lycopene and its *cis* isomers by using a C_{18} column, it was necessary to employ a C_{30} column instead. Initially, when a solvent system of methanol-acetonitrile (15:85, *v/v*) was used at a flow rate of 1.0 mL min^{-1} and detection at 476 nm, lycopene was eluted in 250 min. Apparently, the greater hydrophobicity of the C_{30} stationary phase resulted in a longer retention time for lycopene and its *cis* isomers. However, by replacing methanol with 2-propanol, three peaks were resolved within 250 min and were tentatively identified as the *cis* isomers of lycopene. By changing the mobile phase to 2-propanol-acetonitrile (30:70, *v/v*), the retention time could be reduced to under 100 min. It has been reported that by adding a modifier such as methylene chloride to the mobile phase for the reversed-phase separation, it is possible to shorten the retention time. Thus, by adding different proportions (5, 10 and 20%) of methylene chloride to the mobile phase, the solvent strength was greatly enhanced. However, the major drawback was that the resolution was reduced. For the purpose of increasing resolution and reducing retention time simultaneously,

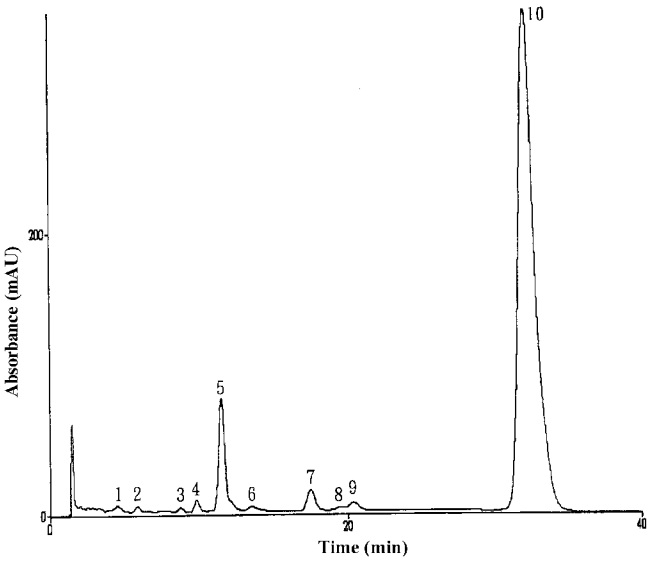

Figure 1. HPLC chromatogram of lycopene standards. Chromatographic conditions described in text. Peaks: **1** = unknown; **2** = di-*cis*-lycopene; **3** = di-*cis*-lycopene; **4** = 15-*cis*-lycopene; **5** = 13-*cis*-lycopene; **6** = di-*cis*-lycopene; **7** = 9-*cis*-lycopene; **8** = di-*cis*-lycopene; **9** = 5-*cis*-lycopene, **10** = all-*trans*-lycopene.

the possibility of substituting 2-propanol with 1-butanol was investigated. Thus, by developing a mobile phase of 1-butanol-acetonitrile-methylene chloride (30:70:10, *v/v/v*) with flow rate at 2.0 mL min^{-1} and detection at 476 nm, lycopene and its nine *cis* isomers were resolved within 35 min (Figure 1). However, by using the same solvent system and a C_{18} column, all the *trans* and *cis* forms of lycopene were eluted together within 3 min. This result further demonstrated that a C_{30} column could provide more powerful resolution for lycopene and its *cis* isomers, but with drastically increased retention times compared to those with a C_{18} column. In a study dealing with the evaluation of stationary phases for the HPLC separation of cis-trans carotenoid isomers, Emenhiser *et al.* [16] concluded that the polymeric C_{30} phase provides excellent shape selectivity towards both the positional and geometrical isomers of carotenoids. Both the detection and quantitation limits of all-*trans*-lycopene were found to be 2.55 and 7.73 $\mu g \cdot g^{-1}$, respectively. In addition, the DL for 9-*cis*-, 13-*cis*- and 15-*cis* lycopene were 0.02, 0.12 and 0.03 $\mu g \cdot g^{-1}$, respectively, whereas the QL were 0.06, 0.38 and 0.11 $\mu g \cdot g^{-1}$.

Table I shows the retention times, capacity factors (k), spectral characteristics and Q ratios of all-trans-lycopene and its cis isomers. The k values for all the compounds ranged from 2–20. It has been well established that for the optimum separation the k values for all of the peaks should be controlled between 2 and 10 [17]. However, in some instances a large k range (2–20) could also be accepted [17]. Peak **10** was identified as all-*trans*-lycopene. Peaks **2**, **3**, **6** and **8** were tentatively

Table I. Tentative identification data for all-*trans* and *cis* forms of lycopene standards.

Peak No.	Compound	k[a]	λ (nm) (in-line)[b]				λ (nm) reported					Q-ratio found	Q-ratio reported
1	unknown	2.0			452				–[c]			–	–
2	di-*cis*-lycopene	2.9	350		458				–			–	–
3	di-*cis*-lycopene	4.7	350		464	488			–			–	–
4	15-*cis*-lycopene	5.4	362	446	470	506	360	437		466	494[e]	0.75	0.75 (Yeum *et al.*, 1996)
5	13-*cis*-lycopene	6.5	362	446	470	500	360	437		463	494[d]	0.54	0.55 (Schierle *et al.*, 1997)
6	di-*cis*-lycopene	7.9		434	464	494			–			–	–
7	9-*cis*-lycopene	10.5	362	446	470	500	360	438		464	494[d]	0.24	0.12 (Schierle *et al.*, 1997)
8	di-*cis*-lycopene	11.8	368	440	470	500			–			0.47	–
9	5-*cis*-lycopene	12.3	368	452	476	506	362	442		470	502[d]	0.34	0.06 (Schierle *et al.*, 1997)
10	all-*trans*-lycopene	20.0		452	476	506	362	444		470	502[d]	–	0.06 (Schierle *et al.*, 1997)

[a] k: Capacity factor; [b] A mobile phase of 1-butanol-acetonitrile-methylene chloride (30:70:10, *v/v/v*) was used. [c] "–" Data not available; [d] A mobile phase of n-hexane-n-ethyldiisopropylamine (99.85:0.15, *v/v*) was used by Schierle *et al.* (1997). [e] A gradient solvent system of methanol-MTBE-water (ammonium acetate) was used by Yeum *et al.* (1996).

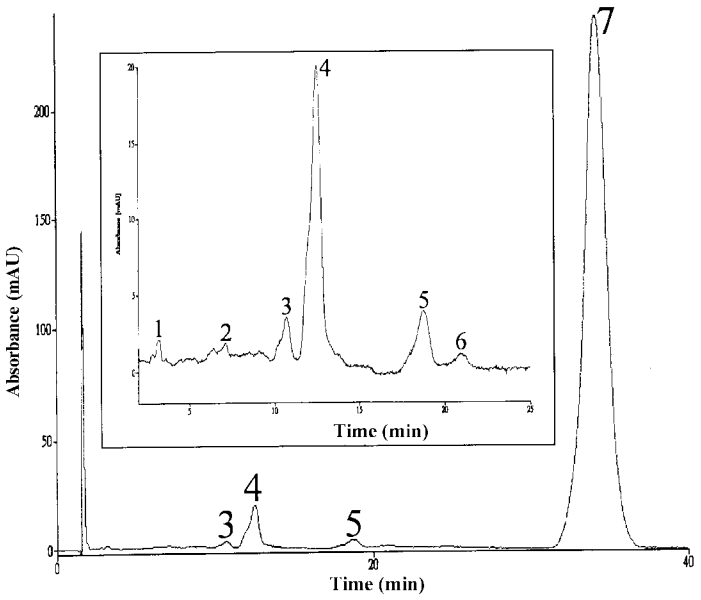

Figure 2. HPLC chromatogram of lycopene and its *cis* isomers extracted from fresh tomato. Chromatographic conditions described in text. Peaks: **1** = *cis*-lutein; **2** = di-*cis*-lycopene; **3** = 15-*cis*-lycopene; **4** = 13-*cis*-lycopene; **5** = 9-*cis*-lycopene; **6** = di-*cis*-lycopene; **7** = all-*trans*-lycopene. The insert represents the enlargement of several peaks that did not show up in the original chromatogram because of low concentrations.

identified as di-cis isomers of lycopene because a stronger hypsochromic shift of 18, 12, 12 and 6 nm had occurred, respectively, when compared to the parent *trans*-compound. It has been suggested that the di-*cis*-carotenoids could be shifted to shorter wavelength of a larger magnitude than mono-*cis*-carotenoids [2]. Peaks **4**, **5** and **7** were tentatively identified as 15-*cis*-, 13-*cis*, and 9-*cis*-lycopene, respectively, which were based on a hypsochromic shift of 6 nm and a cis peak at 362 nm, when compared to all-*trans*-lycopene. The Q ratios were 0.75, 0.54 and 0.24, respectively, which were similar to those reported by the other authors [4, 18]. Peak **9** was tentatively identified as 5-*cis*-lycopene because the spectrum was identical to all-trans-lycopene [18]. Figure 2 shows the HPLC chromatogram of carotenoid pigments in tomato. Table II shows the tentative identification data of carotenoid pigments in tomato. Peak **1** was identified as *cis*-lutein, which was similar to that reported by Tai and Chen (2000) [19]. Peak **7** was identified as all-*trans*-lycopene, based on chromatography with added standards and by comparison of the absorption spectra of the unknown peak with the reference standard. Peaks **3**, **4** and **5** were tentatively identified as 15-*cis*-, 13-*cis*-and 9-*cis*-lycopene. Peaks **2** and **6** were tentatively identified as di-*cis*-lycopene. Fewer peaks were observed in the HPLC chromatogram from the tomato extract when compared to the lycopene standard. This is probably because open-column chromatography was employed for the purification of lycopene, and thus some impurities were removed from the tomato extract. The amounts of 15-*cis*-, 13-*cis*-, 9-*cis*-, and all-*trans*-lycopene in tomato were 0.13, 0.80, 0.18 and 19.65 $\mu g \cdot g^{-1}$, respectively.

Acknowledgement

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Table II. Tentative identification data for pigments in tomato.

Peak No.	Lycopene	k[a]	λ (nm) (in-line)[b]				λ (nm) reported				Q-ratio found	Q-ratio reported
1	*cis*-lutein	0.9	350		448				–[c]		0.38	–
2	di-*cis*-lycopene	3.2	353		448				–		0.58	–
3	15-*cis*-lycopene	5.4	362	445	473	503	360	437	466	494[d]	0.70	0.75 (Yeum *et al.*, 1996)[e]
4	13-*cis*-lycopene	6.4	362	443	469	500	360	437	463	494[d]	0.53	0.55 (Schierle *et al.*, 1997)
5	9-*cis*-lycopene	10.2	362	443	469	503	360	438	464	494[d]	0.28	0.12 (Schierle *et al.*, 1997)
6	di-*cis*-lycopene	11.5	362	445	463	503			–		0.43	–
7	all-*trans*-lycopene	19.4		449	476	508	362	444	470	502[d]	–	0.06 (Schierle *et al.*, 1997)

[a] k: Capacity factor; [b] A mobile phase of 1-butanol-acetonitrile-methylene chloride (30:70:10, *v/v/v*) was used. [c] "–" Data not available; [d] A mobile phase of n-hexane-n-ethyldiisopropylamine (99.85:0.15, *v/v*) was used by Schierle *et al.* (1997). [e] A gradient solvent system of methanol-MTBE-water (ammonium acetate) was used by Yeum *et al.* (1996).

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