Chromatographic Purification of Natural Lycopene

Sari H. Hakala and I. Marina Heinonen*

Department of Applied Chemistry and Microbiology, P.O. Box 27, FIN-00014 University of Helsinki, Finland

The natural lycopene isolation method was developed in three steps. First, carotenoids were extracted from tomato purée with petroleum ether; next, the carotenoid extract was prepurified from all carotenoids except lycopene with solid-phase extraction (SPE) using silica cartridges. Lycopene was then further purified three times with semipreparative high-performance liquid chromatography (HPLC) using a μBondapak C_{18} column. The purity and recovery of the lycopene were checked after each step with analytical HPLC using a Zorbax ODS column. A solvent mixture of acetonitrile–dichloromethane–methanol (45:10:45) was used in all HPLC evaluations. The shoulder of the lycopene peak was identified with diode array detection. Also, spectral scans for peak purity were made. After extraction, 30 mg of lycopene was recovered from 10 g of tomato purée and the lycopene content amounted to 87% of the total carotenoids. After the SPE, 21 mg of the extracted lycopene remained, the lycopene content being 93% of total carotenoids and that of *all-trans*-lycopene 80%. The amount of lycopene after the first and third HPLC fractionations declined from 9 to 6 mg, respectively. With the method developed, 6 mg of lycopene was isolated from 10 g of tomato purée. However, the natural lycopene isolated contained about 20% lycopene cis isomers and 3% xanthophylls.

INTRODUCTION

Lycopene is a tetraterpenic C_{40} carotenoid that plays an important role in photosynthesis. Carotenoids protect the organism from photo-oxidative damage and act as accessory light-harvesting pigments in the antenna systems (Codgell, 1985).

Interest in natural coloring substances has increased as we have gained more knowledge of the harmful effects of artificial food colorants. Lycopene absorbs light in the red region and imparts the red color to fruits such as tomato and watermelon, in which it is the predominant carotenoid (Heinonen et al., 1989). For industrial uses, natural lycopene could readily be purified from tomato-processing wastes (Al-Wandawi et al., 1985).

Carotenoids have long aroused interest owing to their wide-ranging roles in photochemistry, photobiology, and photomedicine and their possible use as a chemopreventative treatment for cancer (Truscott, 1990). Although it has no provitamin A activity, lycopene does exhibit a physical quenching rate constant with singlet oxygen almost twice as high as that of β-carotene (Di Mascio et al., 1989; Conn et al., 1991; Devasagayam et al., 1992). In its molecular structure, lycopene has 11 conjugated double bonds and thus is able to function as an antioxidant. Evaluation of carotenoids should, therefore, be extended to include other compounds such as lycopene (Di Mascio et al., 1989; Truscott, 1990).

The aim of the study was to develop a method for purifying natural lycopene. The purity of the lycopene available is only 90–95%, and information is lacking about the impurities. Moreover, the standard is quite expensive. In this study the term lycopene refers to all stereoisomers of lycopene. The terms all-trans and cis isomers of lycopene are used when specific lycopene configurations are discussed.

EXPERIMENTAL PROCEDURES

Apparatus. The solid-phase extractions (SPE) were made using a Baker SPE-21 apparatus (J. T. Baker Inc.) with silica and amino cartridges (3 cm^3, Varian Analytichem Bond Elut). A Waters Delta Prep 3000 liquid chromatograph (Waters Millipore) equipped with a Waters 484 UV–vis detector and a Varian 4270 integrator was used in semipreparative and analytical high-performance liquid chromatography (HPLC). The data were stored and processed using a Hewlett-Packard computing system (Series 9000). Semipreparative purifications were made on a μBondapak C_{18} column [10 μm, 150 × 19 mm (i.d.)] (Waters), and a Zorbax ODS [5–6 μm, 250 × 4.6 mm (i.d.)] column (DuPont) was used in the analytical HPLC. Both columns were protected with a C_{18} guard column [10 μm, 50 × 4.9 mm (i.d.)] (Waters).

Reagents and Materials. The reference samples of lycopene (90–95%), lutein (Sigma, St. Louis, MO), and β-carotene (Hoffman-La Roche, Basel, Switzerland) were used without further purification. The HPLC grade solvents—methanol, acetonitrile, methylene chloride, hexane, and chloroform (Rathburn, Scotland)—were also used without further purification. Tomato purée (Marti, Hungary) was purchased from a local supermarket.

Extraction. Carotenoids were extracted from aliquots of 10 g of tomato purée with petroleum ether (PE) at least three times (3 × 100 mL) using a Bamix for mechanical disruption and using Na_2SO_4 as desiccant, which was then followed by vacuum filtration. An extraction procedure using acetone (3 × 100 mL) in homogenization (Heinonen et al., 1989) was also tested.

Solid-Phase Extraction (SPE). The PE extract of carotenoids was prepurified from carotenoids other than lycopene with SPE using silica cartridges with a modified version of the method of Tan (1988). The carotenoid extract in PE (50–100 mL) was transferred to the silica cartridge, and PE (100%) solvent was eluted through the cartridge until the lycopene (distinct red) reached the end of it. The lycopene was eluted with chloroform. To minimize impurities in the collected lycopene fraction, collection was stopped as soon as the deepest red fraction had eluted. The capacity of the silica cartridges was determined.

Semipreparative HPLC. Lycopene was further purified with semipreparative HPLC. Different elution mixtures of acetonitrile, dichloromethane, and methanol were tested (70:20:10, 60:30:10, 60:10:30, 65:5:30, 45:10:45) (Nelis and De Leenheer, 1983; Heinonen et al., 1989). Elution mixtures of methanol and chloroform (94:6, 90:10) (Quackenbush, 1987) and acetonitrile, methanol, dichloromethane, and water (7:7:2:0.16) (Stahl et al., 1992) were also tested. The flow rate was 4 mL/min. The injection volume varied between 2 and 3 mL, and the loading was 1.5 mg of lycopene in 8 mL of mobile phase. Only the central part of the lycopene fraction was collected manually.

Analytical HPLC. The purity and recovery of lycopene were checked after every step with analytical HPLC using a mixture

* Author to whom correspondence should be addressed.

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Table 1. Recovery and Purity of Lycopene after the Different Purification Steps

purification step	recovery of lycopene (mg/100 g)	proportion (%) of lycopene relative to total carotenoids	cis isomers of lycopene (%)
extraction with acetone	26	76	*a*
extraction with PE	30	87	*a*
solid-phase extraction	21	93	13
first HPLC fractionation	9	95	15
second HPLC fractionation	7	95	18
third HPLC fractionation	6	97	20

[a] The shoulder of the lycopene peak could be seen, but separation was poor.

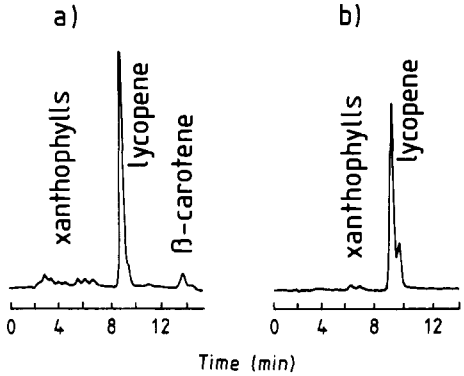

Figure 1. Chromatograms of natural lycopene after (a) extraction with PE and (b) the third fractionation using semipreparative HPLC. The analytical HPLC column was Zorbax ODS and the mobile phase a mixture of acetonitrile, dichloromethane, and methanol (45:10:45) with a flow rate of 2 mL/min. The detection was set at 470 nm.

of acetonitrile, dichloromethane, and methanol (45:10:45) as the solvent; the flow rate was 2 mL/min. Lycopene, β-carotene, and lutein were identified by comparing their retention times with those of authentic standards. Quantitation of lycopene was based on an external standard method. The HPLC chromatograms were monitored at 470 nm.

Checking of Peak Purity with Spectral Scans. A HP-1090 (Hewlett-Packard) liquid chromatograph equipped with a UV–vis diode array detector (DAD) was used to check the peak purity. DAD was, for example, used to identify the shoulder of the lycopene peak by comparing the spectral data measured with data given in the literature. Spectral scans (280–550 nm) of carotenoid extracts collected in the SPE purification and in the semipreparative HPLC were also made using a Perkin-Elmer Lambda 3 UV–vis spectrophotometer (Coleman Instrument Division).

RESULTS AND DISCUSSION

Extraction. Recovery and purity of the lycopene were both better in the sample extracted with PE compared to that extracted with acetone (Table 1). The chromatogram of the PE-extracted purée sample is shown in Figure 1a. It is seen that extraction of lycopene from tomato purée does not require a water-soluble penetrating solvent such as acetone. When carotenoids are extracted with relatively unpolar PE, the amount of polar xanthophylls decreases and the proportion of lycopene relative to carotenoids increases. According to Hobson and Davies (1981), 66% of the lycopene in the tomato juice occurs in the extracellular granule fraction, and according to Lindner et al. (1984), 50% of the lycopene is located in the extracellular fraction of insoluble tomato solids.

Solid-Phase Extraction (SPE). Lycopene eluted as a sharp red zone on the silica cartridge, whereas the amino cartridge was totally unable to retain lycopene when the sample was eluted with PE. The recovery and purity of lycopene are presented in Table 1. The shoulder of the

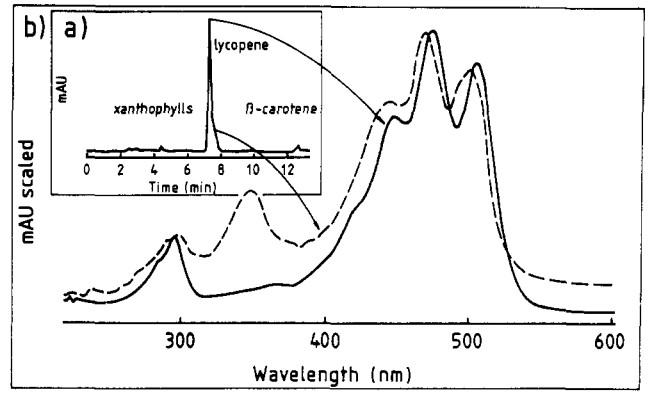

Figure 2. (a) Chromatogram of natural lycopene after extraction with PE. For chromatographic conditions see Figure 1. (b) UV–vis absorption spectra of lycopene peak and its shoulder.

lycopene peak grew slightly during SPE and was tentatively identified as the cis isomer(s) of lycopene (Figure 2). This tentative identification of cis isomer(s) of lycopene was based primarily on the characteristic subsidiary peak in the near-ultraviolet region (Khachik et al., 1989). The increase in cis isomers during SPE might be due to the adsorbent (Braumann and Grimme, 1981; Taylor and Little, 1983; Stalcup et al., 1990), although lycopene was exposed to the silica adsorbent for a relatively short time. The maximum capacity of the silica cartridge was about 31 mg of lycopene in 105 mL of PE.

Semipreparative HPLC. Problems encountered in the semipreparative HPLC involved the separation of the unknown compounds eluting before lycopene and the separation of the cis isomers of lycopene eluting as a shoulder of the lycopene peak (Figure 1b). β-Carotene and xanthophylls such as lutein could be completely removed from the lycopene-containing fraction. The recoveries and purity of lycopene after the three fractionations are presented in Table 1. Separation of lycopene from the compounds eluting prior to it was improved in the μBondapak column by increasing the polarity of the solvent mixture of acetonitrile, dichloromethane, and methanol (70:20:10), by decreasing the amount of dichloromethane (5%), and by increasing the percentage of methanol (25%). The same outcome was also reported by Saleh and Tan (1991), although they used an analytical reversed-phase column. A decrease in the percentage of the unpolar solvent to under 10% can cause the sample to precipitate in the column when the sample contains concentrated unpolar carotenoids (Ng and Tan, 1988; Lesellier et al., 1989). In our study, the proportion of relatively unpolar dichloromethane was kept at 10% to improve the solubility of the sample in the solvent mixture and to extend the lifetime of the column. The proportions of both acetonitrile and methanol were 45%. Although lycopene was collected only from the middle fraction of the highest absorbance of lycopene, the unknown compounds eluting prior to lycopene and the cis isomers of lycopene could not be completely removed. After repetition of the HPLC purification, the recovery of lycopene declined but the purity of lycopene improved only slightly. According to Khachik et al. (1992), carotenoid epoxides may be among the xanthophylls eluting before lycopene.

Good separation of lycopene from its cis isomers and other carotenoids has been achieved in vegetable samples on analytical reversed-phase columns with solvent mixtures of methanol and chloroform (94:6) (Quackenbush, 1987) and in plasma samples with acetonitrile, methanol, dichloromethane, and water (7:7:2:0.16) (Stahl et al., 1991). However, when these solvent mixtures were tested on the

semipreparative μBondapak column, the resolution was poor and the chromatography times were very long. It would be useful to investigate normal-phase semipreparative HPLC techniques to further eliminate cis isomers from *all-trans*-lycopene. In some studies *cis–trans*-carotenoid isomers have been successfully separated and determined by HPLC using an analytical $Ca(OH)_2$ column (Tsukida et al., 1982; Chandler and Schwartz, 1987; Koyama et al., 1988). According to Lesellier et al. (1989), the separation of carotenoid stereoisomers at room temperature by the reversed-phase HPLC technique requires the use of the polymeric ODS phase.

Conclusions. With the method developed, 6 mg of natural lycopene were isolated from 10 g of tomato purée. However, the natural lycopene purified contained 20% cis isomers and 3% other unknown compounds. To further eliminate unknown compounds, in particular cis isomers, from *all-trans*-lycopene, a chromatographic isolation technique using a normal-phase [$Ca(OH)_2$-column] preparative HPLC method should be investigated.

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