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Proper Solvent Selection for Lycopene Extraction in Tomatoes and Application to a Rapid Determination

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I Introduction

Trans-lycopene (lycopene), the major carotenoid found in raw red tomatoes (Shi et al., 1999; Zakaria et al., 1979), imparts the characteristic red color. Lycopene is an indicator of maturity (Watada et al., 1976), which is a critical factor for palatability (Iino et al., 1982) and it is also important for its recognized health benefits (e.g., Stahl et al., 1992).

Some extraction solvents have been used for lycopene determination in tomatoes. Acetone has been the most commonly used extraction solvent for lycopene in tomatoes (Beerh et al., 1959; D'Souza et al., 1992; Mencarelli et al., 1988; Schierle et al., 1997; Watada et al., 1976; Zakaria et al., 1979). AOAC method (Williams, 1984) employs acetone/n-hexane (4 : 6, v/v) as the extraction solvent. Sadler et al. (1990) employed n-hexane/acetone/ethanol (2 : 1 : 1, v/v/v), while Khachik et al. (1992) used tetrahydrofran (THF). However, we do not know which solvent is best for lycopene extraction in tomatoes because their extraction efficiencies have not been compared, though Adsule et al. (1979) compared the extraction efficiency among acetone, n-hexane and petroleum ether in tomato. Alternatively, Matsumoto et al. (2007) employed diethylether/methanol (7 : 3, v/v) as the extraction solvent for *Citrus* varieties. This extraction solvent has not been applied to tomatoes, and may be better for lycopene extraction in tomatoes. Use of a solvent with low extraction efficiency would not be appropriate for the quantification of any component. Thus, the extraction efficiency of lycopene from tomatoes should be compared using various extraction solvents to quantify the lycopene content precisely.

The analysis of carotenoids in agricultural products is time-consuming, especially, using high performance liquid chromatography (HPLC)(e.g., Matsumoto et al., 2007). Spectrophotometric measurement of tissue extract obtained with an extraction solvent provides a rapid and simple method. It is also convenient because this method can quantify lycopene content without standard analysis, alternatively it can be done with an absorption coefficient. But the determined values have not been compared with those using other methods. Beerh et al. (1959) developed a rapid spectrophotometric method for estimating lycopene content in tomato ketchup using acetone, while Mencarelli et al. (1988) analyzed lycopene and chlorophyll in tomato using an acetone extraction and visible spectrophotometry. Nagata et al. (1992) developed a simple method for the simultaneous determination of carotenoids and chlorophyll in tomato using acetone/n-hexane (4 : 6, v/v) extracts. Identification of a more efficient extraction solvent may allow for the application of a rapid determination method for lycopene in tomato. The resulting

values should then be compared with those obtained using a proper liquid chromatography (LC), e.g., HPLC method, which is currently the standard for such analysis.

The objectives of this study were to select a highly efficient extraction solvent for lycopene from tomatoes using LC, and then compare the determined values using a rapid determination method with the highly efficient extraction solvent.

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II Materials and Methods

1 Samples

Samples of red or pink tomatoes were purchased from retail shops or cultivated at the headquarters and Taketoyo vegetable research station of the National Institute of Vegetable and Tea Science (NIVTS), NARO. In addition, two samples of tomato juice and one vegetable juice were purchased from a retail store.

2 Lycopene analysis

a Lycopene extraction and standard

Individual tomatoes were either cut using a kitchen knife (designated as 'cut' samples) or homogenized using a mixer for home use (MJ-C36, National, Japan) (designated as 'paste' samples). For each sample, 3 g was placed in a 50 ml brown glass centrifuge tube and homogenized in 35 ml of extraction solvent using a homogenizer (ULTRA-TURRAX T25, JANKE & KUNKEL IKA-Labortechnik, Germany). Extraction solvents included diethylether/methanol (7:3, v/v), acetone/n-hexane (4:6, v/v), n-hexane/acetone/ethanol (2:1:1, v/v/v), acetone, ethanol, and THF. All reagents were special grade (Wako Pure Chemical Industries, Japan). After residue settling, the extract was removed to a 100 ml brown glass flask. After adding 15 ml of extraction solvent, the residue was again homogenized. After settling, the extract was removed to the brown flask. The extraction step, consisting of 15 ml solvent additions, was repeated. For lycopene analysis, extracts were combined and the final volume was adjusted to 100 ml using the same solvent. When an extract was given three fractions, fraction #1 consisted of the initial 35 ml extract plus the first 15 ml extract, fraction #2 consisted of the second and third 15 ml extracts, and fraction #3 consisted of the fourth and fifth 15ml extracts. When 6 g samples were analyzed, each of the steps outlined above was scaled up by twofold. The standard solution contained 1 mg of lycopene (L9879, Sigma, USA) dissolved in acetone/n-hexane (4:6, v/v) in a final volume of 100ml. The unknowns and standards were filtered using a 0.2 μ m GL chromatodisk 13N disposable filter (GL Science, Japan), then stored in vials for HPLC or ultra performance liquid chromatography (UPLC) at -20°C before analysis. The septa of the HPLC vials were made of Teflon (surface) and aluminum (back). The septa of the UPLC vials were made of silicon (surface) and aluminum (back).

b HPLC analysis

HPLC-H1 consisted of a 880-50 degasser, 880-PU pump, 850-AS autoinjector, 865-CO column oven, and 870-UV UV-visible detector (Japan Spectroscopic Co., Japan). HPLC-H2 consisted of a DG-980-50 degasser, PU-980 pump, AS-2055plus autoinjector, 860-CO column oven, and UV-970

UV-visible detector (Japan Spectroscopic Co., Japan). Isocratic separation was achieved using one of the following C18 columns: C18-5B (250 × 4.6 mm i.d., 5 μm particle size, Shodex, Japan), TSKgel ODS-120T (250 × 4.6 mm i.d., 5 μm particle size, Tosoh, Japan), or Mightysil RP-18 GP 150-4.6 (150 × 4.6 mm i.d., 5 μm particle size, Kanto Kagaku, Japan). The mobile phase was 100% methanol for HPLC (Wako Pure Chemical Industries, Japan) at a flow rate of 1.0 ml/min. Sample injection volume was 10 μl, column temperature was 25°C (room temperature), and peaks were detected at 450 nm (Nagata et al., 2007).

c UPLC analysis

The UPLC consisted of a pump, autoinjector (partial loop-needle overflow mode with a 10 μl sample loop), column holder, and a 2996 photodiode array detector (Waters, USA). Isocratic separation was achieved using the C18 column, Acquity UPLC™ BEH C18 (100 × 2.1 mm i.d., 1.7 μm particle size, Waters, USA). The mobile phase used was 100% methanol at a flow rate 0.35 ml/min. Sample injection volume was 3 μl, column temperature was 25°C, and peaks were detected at 450 nm.

d Repeatability of LC analysis

The intraday and interday observed precision using HPLC or UPLC was calculated by replicate analysis (n = 3) of the same extract, and expressed as the mean ± standard deviation (SD).

e Purity check of lycopene standard solution

Purity of the lycopene standard was determined by diluting the solution by tenfold in a covered 1 cm path quartz cuvette, then measuring the absorbance at 472 nm (U-2810 spectrophotometer, Hitachi, Japan) using a solvent blank, acetone/n-hexane (4:6, v/v). The concentration was calculated using an absorption coefficient of 3450 %⁻¹ cm⁻¹ (Britton et al., 2004).

f Rapid determination method

To develop a rapid determination method, diethylether/methanol (7:3, v/v) extracts were put through the disposable filter and diluted twofold, and then the absorbance was measured in the covered quartz cuvette at 505 nm using diethylether/methanol (7:3, v/v) as the blank. The lycopene concentration was calculated using an absorption coefficient of 3150 %⁻¹ cm⁻¹ (Nagata et al., 1992). HPLC analysis was performed using HPLC-H1 equipped with a Mightysil RP-18 GP 150-4.6 column (150 × 4.6 mm i.d., 5 μm particle size, Kanto Kagaku, Japan).

3 Statistical analysis

The significance of the differences was tested using a paired t-test (Microsoft Excel 2000).

III Results and Discussion

Lycopene was dissolved in diethylether/methanol (7:3, v/v), acetone/n-hexane (4:6, v/v), petroleum ether, acetone, ethanol, and benzene. The lycopene dissolved rapidly in acetone/n-hexane (4:6, v/v), but dissolving lycopene in ethanol was difficult. Assessing the separation of the standard solution using the UPLC method, which evolved from the former HPLC method

(Villiers et al., 2006), showed that the strongest lycopene signal (i.e., peak area) was observed when acetone/n-hexane (4:6, v/v) was used as the solvent. Use of the petroleum ether solvent caused a splitting of the peak. Diethylether/methanol (7:3 v/v) took a long time to dissolve the lycopene, and there was also the risk of decomposition. Therefore, lycopene was dissolved in acetone/n-hexane (4:6 v/v) and the standard solution was injected into the LC.

The influences of sample preparation ('cut' or 'paste') and extraction solvent [acetone/n-hexane (4:6) or diethylether/methanol (7:3)] on the determined lycopene values were investigated using UPLC. Results showed that the extraction efficiency obtained using diethylether/methanol was superior to that of acetone/n-hexane, irrespective of the sample preparation. No difference in lycopene values was observed between the 3 or 6 g paste samples in the diethylether/methanol solvent (Table 1). Therefore, 3 g pastes were adopted as the standard sample preparation to obtain good sample uniformity, while also saving solvent volume.

Among the solvents, diethylether/methanol (7:3, v/v), acetone/n-hexane (4:6, v/v), acetone, and ethanol, the diethylether/methanol provided the best extraction efficiency from the tomato using HPLC (Table 2). Diethylether/methanol was superior to acetone/n-hexane like results using UPLC. Comparisons were also made among diethylether/methanol (7:3, v/v), n-hexane/acetone/ethanol (2:1:1, v/v/v), and THF as the extraction solvent for lycopene in a tomato. Again, we observed that diethylether/methanol had the best extraction efficiency using HPLC, though the column used was different from table 2 (Table 3). The efficiency observed using acetone/n-hexane

Table 1. Influence of sample preparation and solvents on quantified lycopene values of tomatoes ('Nitakikoma').

sample preparation ^a	solvent ^b	lycopene(%) ^c (Mean±SD)
cut 6g	4:6	64±6*
cut 6g	7:3	100±19
cut 6g	7:3	98±12
paste 6g	4:6	42±4*
paste 6g	7:3	100±20
paste 6g	7:3	100±20
paste 3g	7:3	97±14

^a 'Cut' means that part of a tomato was cut and a solvent was added.

'Paste' means that a tomato was homogenized using a mixer before a solvent was added.

^b '4:6' means acetone/n-hexane(4:6 v/v).

'7:3' means diethylether/methanol(7:3 v/v).

^c Mean±standard deviation(SD) when extract was injected into UPLC 3 times. The maximum value in each experiment was expressed as 100%.

* Significantly different vs. each maximum value at $p<0.05$.

Table 2. Influence of solvents on quantified lycopene values of a tomato ('Nitakikoma').

solvent ^a	lycopene (mg/100g) ^b Mean±SD
7:3	12.76±0.33
4:6	7.33±0.05**
Acetone	11.93±0.22*
Ethanol	2.68±0.09**

^a '4:6' means acetone/n-hexane(4:6 v/v).

'7:3' means diethylether/methanol(7:3 v/v).

^b HPLC-H1 equipped with a Shodex C18-5B column.

*** Significantly different vs. maximum value(7:3) at $p<0.05$ and $p<0.01$, respectively.

(1:1, v/v) was only 36% of that observed from the acetone extract, and the efficiency of n-hexane was also low (41%) (Adsule et al., 1979). Thus, the low extraction efficiencies observed with acetone/n-hexane (4:6) and n-hexane/acetone/ethanol (2:1:1) were likely due to the presence of n-hexane. In addition, diethylether/methanol (7:3) extracted the lycopene from a tomato more

Table 3. Influence of solvents on quantified lycopene values of a tomato ('Nitakikoma').

solvent ^a	lycopene (mg/100g) ^b
	Mean ± SD
7:3	6.78 ± 0.28
2:1:1	5.48 ± 0.06 *
THF	6.45 ± 0.08

^a '7:3' means diethylether/methanol(7:3 v/v).

'2:1:1' means n-hexane/acetone/ethanol (2:1:1 v/v/v)

'THF' means tetrahydrofran.

^b HPLC-H1 equipped with a Kanto Kagaku Mightysil RP-18 GP 150-4.6 column.

* Significantly different vs. maximum value (7:3) at $p < 0.05$.

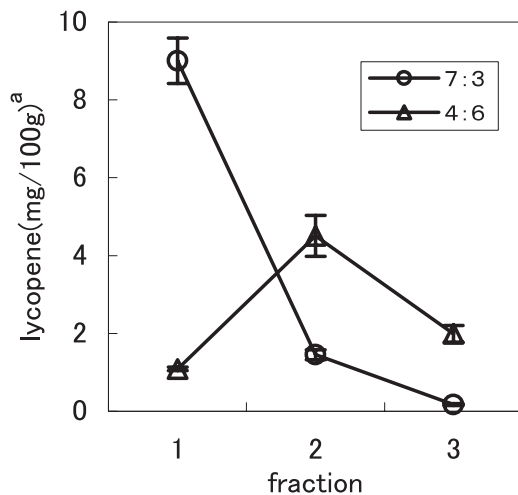


Fig.1 Lycopene content of fractionated tomato ('Nitakikoma') extracts with diethylether/methanol (7:3 v/v)(7:3) or acetone/n-hexane (4:6 v/v)(4:6).

When an extract was given three fractions, fraction #1 consisted of the initial 35ml extract plus the first 15ml extract, fraction #2 consisted of the second and third 15ml extracts, and fraction #3 consisted of the fourth and fifth 15ml extracts.

^aHPLC-H1 equipped with a Shodex C18-5B column.

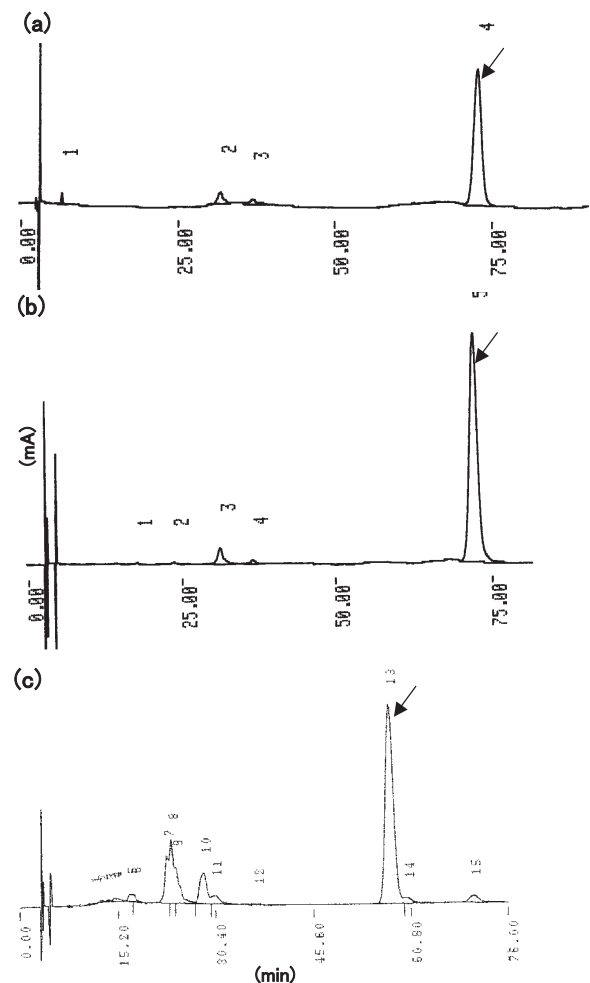


Fig.2 HPLC chromatogram.

(a) Tomato 'Nitakikoma' extracted with diethylether/methanol (7:3 v/v) (HPLC-H1 equipped with a Shodex C18-5B column.)

(b)(c) Lycopene standard solution in acetone / n-hexane(4:6 v/v)
(b) HPLC-H1 equipped with a Shodex C18-5B column.)

((c) HPLC-H2 equipped with a Toso TSKgel ODS-120T column.)

Each arrow shows the peak of lycopene.

readily than acetone/n-hexane (4:6; Fig. 1).

A HPLC chromatogram of a tomato extract using diethylether/methanol (7:3) is shown in Fig. 2a. The recovery of lycopene from *Citrus* fruit in diethylether/methanol (7:3) was 92% using a HPLC system (Matsumoto et al., 2007). The lycopene extracted with diethylether/methanol (7:3) from a tomato was stable over a period of 2 weeks (Fig. 3).

When the standard solution of lycopene was injected into LC, the rate of the peak area varied among the LC systems using a C18 column (Fig. 2b and 2c): the bigger the rate of the peak area, the lower the calculated lycopene value of a tomato. The lycopene value was high using UPLC or HPLC-1 because the standard had a low rate at the peak area (Table 4). The lycopene appeared to be partially degraded after the standard solution was injected. When the rate of the peak area was low, cis-lycopene and oxidative degradation products may increase (Shi et al., 1999) because some peaks were relatively increased (Fig.2c). These peaks were not identified because they were low and unseparated peaks. The methanol mobile phase used typically provided higher recoveries compared to the acetonitrile mobile phase (Epler et al., 1992). Therefore, LC systems with a low rate of peak areas were not adopted for the quantitative determination. Thus, we recommend the use of a C18 column with a high rate of lycopene peak areas. The Shodex C18-5B column provided the highest rate of the peak area (Table 4, Fig. 2a and 2b), but it is no longer available. Using HPLC-H1 equipped with a Kanto Kagaku Mightysil RP-18 GP 150-4.6 column, the values determined were almost the same as those from HPLC-2(HPLC-H1 equipped with the Shodex C18-5B column) in Table 4.

A rapid determination method using visible spectrophotometry was applied with diethylether/methanol (7:3) as the solvent using an absorption coefficient of $3150\%^{-1}\text{cm}^{-1}$ at 505 nm. The correlation coefficient between the values determined using the proper HPLC and the rapid method was 0.987 ($n = 32$; Fig. 4). Beerh et al. (1959) extracted lycopene with acetone and then transferred it to petroleum ether before the absorbance was measured at 503 nm. However,

Table 4. Influence of lycopene peak area(%) of standard solution on quantified lycopene values of a tomato ('Nitakikoma') using LC.

LC system	peak area (%)	lycopene (mg/100g)
	of lycopene	Mean \pm SD
UPLC	46.64	21.51 \pm 0.54**
HPLC-1 ^a	63.50	20.41 \pm 2.37*
HPLC-3 ^b	87.09	12.97 \pm 0.19*
HPLC-2 ^c	90.90	11.40 \pm 0.30

^a HPLC-1: HPLC-H2 equipped with a TOSO TSKgel ODS-120T column.

^b HPLC-3: HPLC-H2 equipped with a Kanto Kagaku Mightysil RP-18 GP 150-4.6 column.

^c HPLC-2: HPLC-H1 equipped with a Shodex C-18 5B column.

*,** Significantly different vs. minimum value (HPLC-2) at $p < 0.05$ and $p < 0.01$, respectively.

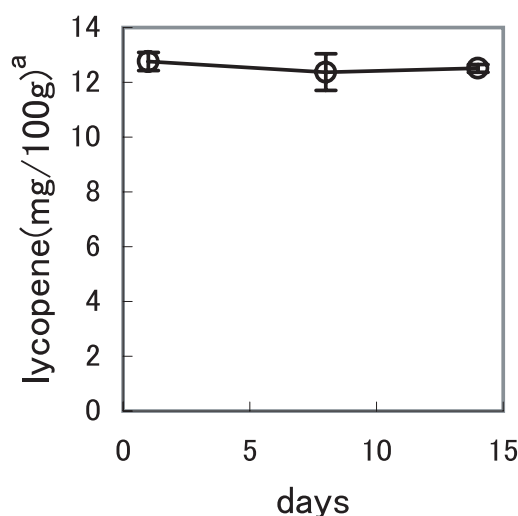


Fig.3 The lycopene values of tomato ('Nitakikoma') extract with diethylether/methanol(7:3 v/v) during storage at -20°C.

^aHPLC-H1 equipped with a Shodex C18-5B column.

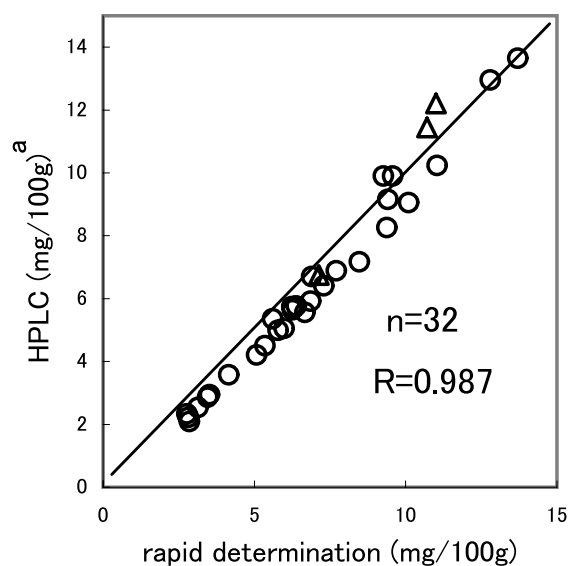


Fig.4 Plots of HPLC vs. rapid-determined lycopene values. Extraction solvent used diethylether/methanol (7:3 v/v). ○:tomato, △:juice.

^aHPLC-H1 equipped with a Kanto Kagaku Mightysil RP-18 GP 150-4.6 column.

petroleum ether also had a low extraction efficiency (Adsule et al., 1979). Use of diethylether/methanol (7:3) provided a more efficient extraction than acetone (Table 2). Measuring the absorbance at 505 nm can improve the risk of contamination by the β -carotene signal at 503 nm, as β -carotene is absorbed in the lower visible range. Even at 503 nm, β -carotene has negligible absorption in red tomato extract (Adsule et al., 1979).

Summary

The best extraction efficiency of lycopene from tomatoes was achieved by employing diethylether/methanol (7:3, v/v) using HPLC. When the standard solution of lycopene was injected into the LC, the rate of the peak area varied among C18 columns. Thus, the use of a C18 column with a high rate of lycopene peak area is recommended. A rapid determination method using a visible spectrophotometer was applied using the best extraction solvent at 505 nm and the absorption coefficient of $3150 \text{ \%}^{-1} \text{ cm}^{-1}$. The correlation coefficient between the values determined using the HPLC versus the rapid determination method was 0.987 ($n = 32$).

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トマトのリコペン抽出に適した溶媒の選択と迅速定量法への応用

伊藤 秀和・堀江 秀樹

摘 要

トマトのリコペン分析法において、抽出効率の高い溶媒を検討したところ、ジエチルエーテル/メタノール(7 : 3、容積比)が最も抽出効率が高かった。リコペン標準液は供試したC18カラムにおいてリコペンのピーク面積値割合が異なったので、本成分のピーク面積値割合の高いカラムを使用する必要がある。また、ジエチルエーテル/メタノール(7 : 3)を抽出溶媒とし、505nmにおける吸収係数 $3150\%^{-1}\text{cm}^{-1}$ を利用する迅速定量法を提案した。HPLCと迅速定量法による定量値の相関係数は0.987(n=32)であった。