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Determination of lycopene and β-carotene by high-performance liquid chromatography using sudan I as internal standard

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Abstract

A new method, using sudan I as internal standard to determine the content of lycopene or β-carotene in samples, was developed. According to UV–vis absorption spectra, sudan I, lycopene and β-carotene all had absorption peaks at 450 nm. They could be separated absolutely by high-performance liquid chromatography (HPLC) with retention time of 2.7, 6.6 and 10.1 min, respectively. The related equations between sudan I and lycopene or β-carotene content were obtained and verified by determining the content of lycopene or β-carotene in *Blakeslea trispora* cells. The relative error was below 1.4% for determining lycopene content and below 1.9% for β-carotene. Intra-day variability for lycopene determination was less than 3.4% and for β-carotene was less than 1.4%. The mean recovery of lycopene or β-carotene was 96.1 and 97.9%, respectively.

Keywords: Lycopene; β-Carotene; Sudan I; HPLC; Internal standard

1. Introduction

Carotenoids are essential nutrients in human diet because they prevent cardiovascular diseases, regulate the immuno system and are considered as anti-carcinogenic agents, antioxidants and precursors of Vitamin A [1–4]. Also carotenoids are one of the most important group of natural pigments, which are mainly used in the dyeing of different kinds of foodstuffs [5]. Their importance is increasing due to a more extensive use of natural compounds in the food, following the directives of European Community in favor of natural rather than synthetic compounds [6]. The classical, natural sources of carotenoids are fruits, vegetables and microorganisms [7–10].

Lycopene and β-carotene are two kinds of important fat soluble carotenoids, and lycopene is a precursor of β-carotene during the biosynthesis process [11]. Structurally, the carotenoids are polysoprenoid compounds, which are synthesized by tail-to-tail linkage of geranylgeranyl molecules [12]. Lycopene contains eleven conjugated and two non-conjugated double bonds [13]. β-Carotene contains eleven conjugated double bonds [14]. The characteristic conjugated double bond system of carotenoids produces the main problems associated with work and manipulation on carotenoids, that is their particular instability, especially towards light, oxygen, heat, acid and alkaline conditions [12,15]. Each factor may cause the degradation, oxidation and/or the *trans–cis* isomerisation of lycopene or β-carotene. So several precautions are necessary when handling lycopene or β-carotene, for example, the use of antioxidants, dim lighting laboratory environment and dark, low temperature storage [16–18]. However, these measures could only slow down but not inhibit the transformation of lycopene or β-carotene.

Up to now, lycopene and β-carotene have been determined in biological samples mostly by high-performance liquid chromatography (HPLC) [19–21]. It needs standard sample of lycopene or β-carotene to confirm the retention time and content in HPLC. Because of the instability and high price of the lycopene or β-carotene standard, it is necessary to find their substitute. Sudan I, a cheap and stable industrial dye, may be the appropriate one. In this paper, the stability of lycopene, β-carotene and sudan I, their spectral and chromatographic characteristics have been studied and a new method using sudan I as internal standard to analyze the lycopene or β-carotene content in samples has been established.

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2. Experimental

2.1. Reagents and standards

The solvents acetonitrile, dichloromethane, petroleum ether were all HPLC grade. Lycopene standard (>95% purity), β-carotene standard (>96% purity), sudan I (analytical grade) and the antioxidant BHT (2,6-di-*tert*-butyl-4-methylphenol) were purchased from Sigma (USA).

Standard solutions were prepared by dissolving pure individual compound including lycopene, β-carotene or sudan I in petroleum ether containing BHT (1%, w/v), which is as the antioxidant. The concentration of standard solution of lycopene, β-carotene or sudan I was 0.1, 0.3 and 0.3 mg/ml, respectively. The mixture of standard solution was prepared with 0.1 mg/ml individual standard solutions. All solutions were stored in brown flasks at −20 °C.

2.2. Chromatographic conditions

HPLC was performed on a HITACHI Series HPLC system equipped with L-7100 pump and a L-7100 UV–vis detector. Peaks were separated on a Diamonsil C_{18} column using the mobile phase [acetonitrile–dichloromethane (75:25, v/v)], or on a Spherisorb ODS2 column using the mobile phase [acetonitrile–methanol–dichloromethane (71:22:7, v/v/v)] or on a Shim-pack CLC-C8 column using the mobile phase [acetonitrile–methanol–chloroform (47:47:6, v/v/v)]. The flow-rate of the mobile phase was 1.5 ml/min. The absorption of analytes was detected at 450 nm. Samples were injected manually, and the injection volume was 5 μl or as otherwise stated. T2000 software was used for peak integration and calculation.

2.3. UV–vis absorption detection

The visible spectral absorption (360–540 nm) of lycopene, β-carotene and sudan I was measured by a Perkin-Elmer UV-3000 spectrophotometer.

2.4. Sample preparation

Blakeslea trispora cells were cultured in our laboratory. Two kinds of *B. trispora* powder samples were used to extract lycopene or β-carotene. Lycopene was extracted from the cells with addition of an inhibitor during the culture and β-carotene from the cells without addition of the inhibitor [11]. Dried cells (0.6 g) were homogenized in petroleum ether and shaken until they became colourless. After filtration the petroleum ether phase was supplemented to 25 ml and added 1% (w/v) BHT. Then the petroleum ether phase containing lycopene or β-carotene was analyzed by HPLC immediately.

3. Results and discussion

3.1. Stability of lycopene, β-carotene and sudan I

The stability of the standard samples of lycopene, β-carotene and sudan I were determined by HPLC. The peak areas of

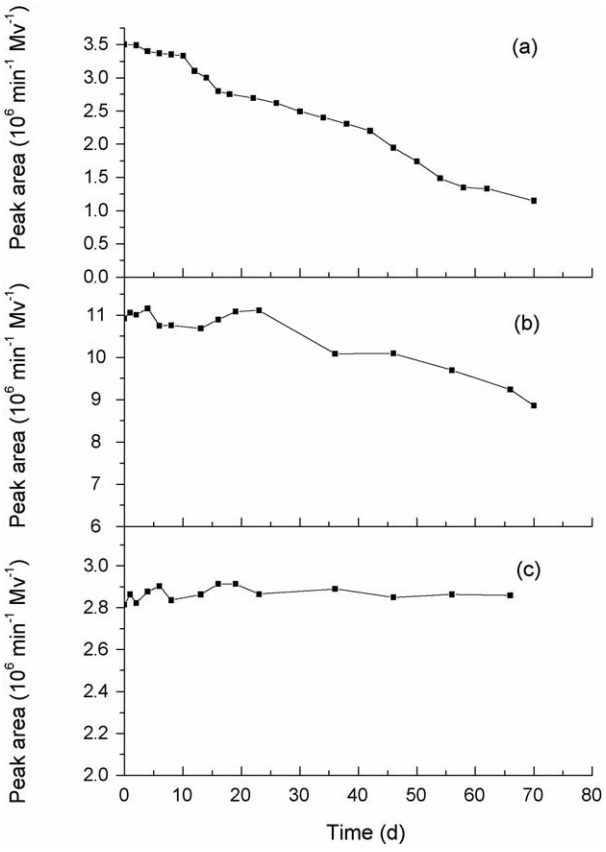

Fig. 1. Degradation of lycopene (a), β-carotene (b) and sudan I (c) with time. The standard solutions were stored in the brown flasks at −20 °C and determined by HPLC. Chromatographic conditions: Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm); mobile phase acetonitrile–dichloromethane (75:25, v/v); injection volume 5 μl.

lycopene, β-carotene and sudan I at different time are shown in Fig. 1. As shown in Fig. 1a, lycopene kept stable in the first 10 days, and declined sharply after 10 days. Comparing with lycopene, the molecular structure of β-carotene was short of two bonds, so β-carotene kept stable during the first 20 days, and after 20 days, the content of β-carotene also decreased (Fig. 1b). After 70 days, the content of lycopene and β-carotene decreased to 33 and 80%, respectively. As standard samples often used in the quantity determination, the instability of lycopene and β-carotene became the limited factor and would give a higher determination result. For ensuring the precision of determination results, standard samples had to be renewed frequently. Moreover, the standard samples of lycopene and β-carotene were very expensive, which made the high analytical cost. Therefore, it was important to find a cheap substitute. As a kind of industrial dye, sudan I was very cheap, quite stable and the concentration kept unchanged during 70 days (Fig. 1c).

3.2. UV–vis absorption spectra and HPLC chromatogram of lycopene, β-carotene and sudan I

The spectrophotometric features of the carotenoids are produced by the conjugated double bond. As shown in Fig. 2, lycopene and β-carotene contained three characteristic absorption peaks respectively (λ = 450, 470, 505 nm for

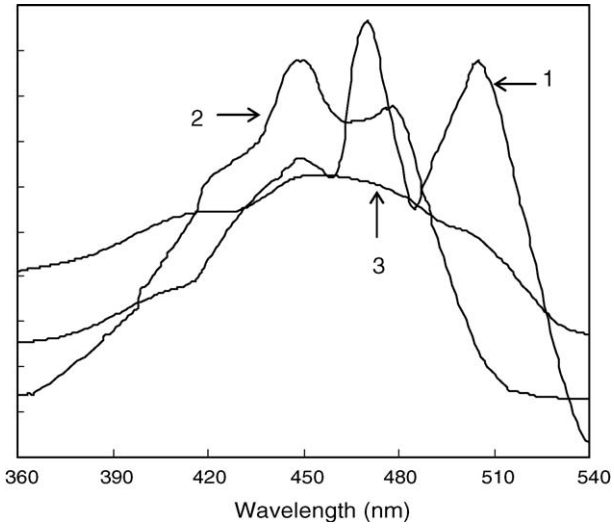

Fig. 2. UV–vis absorption spectra (360–540 nm) of lycopene (1), β-carotene (2) and sudan I (3).

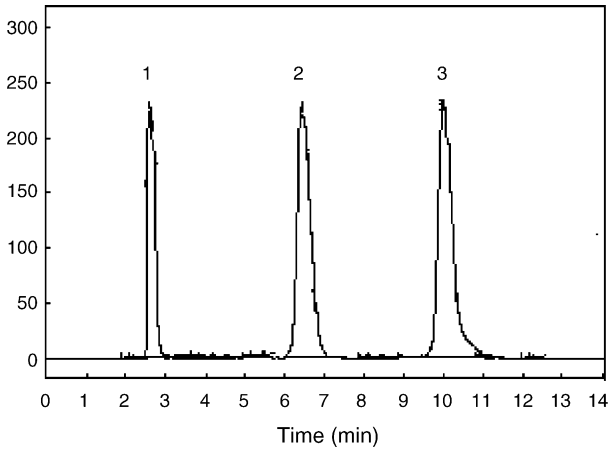

Fig. 3. HPLC chromatogram of the mixed standard solutions including 0.1 mg/ml sudan I (1), lycopene (2) and β-carotene (3). Chromatographic conditions were the same as Fig. 1.

lycopene, and 417, 450, 478 nm for β-carotene). Also sudan I showed three absorption peaks and the obvious absorption peak was at 450 nm. The visible absorption spectra indicated lycopene, β-carotene and sudan I had the similar absorption characteristics and could be determined simultaneously at 450 nm.

Typical HPLC chromatogram of the mixed standard solution including 0.1 mg/ml lycopene, β-carotene and sudan I is shown in Fig. 3. Sudan I, lycopene and β-carotene could be separated absolutely, and the retention time of sudan I was shorter than that of lycopene and β-carotene. The retention time of sudan I, lycopene and β-carotene was 2.7, 6.6 and 10.1 min, respectively. Repeated experiments showed that although the state of the chromatographic column was different, the retention time of the peak was almost the same. UV–vis absorption spectra and HPLC chromatogram of sudan I, lycopene and β-carotene indicated it was feasible using sudan I as internal standard during the determination of lycopene or β-carotene by HPLC.

3.3. Determination of corrected coefficient

The peak areas of lycopene and β-carotene were different from that of the sudan I, and appeared the proportion relationship. The corrected coefficients were obtained according to the injection volumes and peak areas of standard samples, and the results are summarized in Tables 1 and 2.

The corrected coefficient using sudan I substituting for lycopene as internal standard was 3.4491. The equation calculating the lycopene content in the samples can be obtained:

$$C_i = \frac{A_i \times C_S \times V_S}{3.4491 \times A_S \times V_i} \quad (1)$$

Table 1
Peak areas and corrected coefficients of sudan I and lycopene with different injection volumes

	Injection volume (μl)					
	1	2	3	4	5	6
Peak area of sudan I	194237	395807	597557	804584	1020052	1203790
Peak area of lycopene	672719	1354887	2073342	2767045	3504898	4168846
Corrected coefficient	3.4634	3.4231	3.4697	3.4391	3.4360	3.4631
Mean of corrected coefficient			3.4491 ± 0.026			

Chromatographic conditions: Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm); mobile phase acetonitrile–dichloromethane (75:25, v/v).

Table 2
Peak areas and corrected coefficients of sudan I and β-carotene with different injection volumes

	Injection volume (μl)					
	1	2	3	4	5	6
Peak area of sudan I	195108	394538	594954	792366	1036492	1208066
Peak area of β-carotene	736257	1483226	2288285	3107318	3956078	4719009
Corrected coefficient	3.7736	3.7594	3.8462	3.9216	3.8168	3.9063
Mean of corrected coefficient			3.8373 ± 0.0843			

Chromatographic conditions were the same as Table 1.

Table 3
Retention times, peak areas and corrected coefficients of the mixed standard solutions including 0.1 mg/ml sudan I, lycopene and β-carotene with different HPLC columns and mobile phase

	HPLC columns			
	Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) Acetonitrile–dichloromethane (75:25, v/v)[a]	Spherisorb ODS2 column (150 mm × 4.6 mm, 3 μm) Acetonitrile–methanol–dichloromethane (71:22:7, v/v/v)[a]	Diamonsil C_{18} column (150 mm × 4.6 mm, 5 μm) Acetonitrile–dichloromethane (75:25, v/v)[a]	Shim-pack CLC-C8 column (150 mm × 4.6 mm, 5 μm) Acetonitrile–methanol–chloroform (47:47:6, v/v/v)[a]
Retention time (min)				
Sudan I	2.7	3.2	1.6	1.4
Lycopene	6.6	7.9	3.9	1.9
β-Carotene	10.1	12.1	6.4	2.6
Peak area				
Sudan I	1032651	1071807	1464107	1518471
Lycopene	3551391	3748267	5055709	5043145
β-Carotene	3953711	4185191	5695524	5628821
Corrected coefficient				
Lycopene	3.4391	3.4971	3.4531	3.3212
β-Carotene	3.8287	3.9048	3.8901	3.7069

The injection volume was 5 μl.
[a] Mobile phase.

where C_i is the concentration of lycopene in the sample, V_i is injection volume of the sample and A_i is peak area of lycopene; C_S, V_S, A_S is the concentration, injection volume and peak area of sudan I, respectively.

The corrected coefficient using sudan I substituting for β-carotene as internal standard was 3.8373. The equation calculating the β-carotene content in the samples can be obtained:

$$C_j = \frac{A_j \times C_S \times V_S}{3.8373 \times A_S \times V_j} \quad (2)$$

where C_j is the concentration of β-carotene in the sample, V_j is injection volume of the sample and A_j is peak area of β-carotene.

The same substance had different retention behavior with different batch of HPLC columns, thus the corrected coefficients for lycopene and β-carotene were determined with different HPLC columns and mobile phase (see Table 3). The retention times and peak areas of sudan I, lycopene and β-carotene were different from varied HPLC columns and mobile phase. However, the corrected coefficients for lycopene or β-carotene were almost constant. The mixed standard solutions including lycopene, β-carotene and sudan I were determined at different time using the same column. The corrected coefficients changed little, and the standard deviation was less than 0.04 for lycopene and 0.07 for β-carotene (see Table 4).

3.4. Verification of equations

To verify the accuracy of the equations, lycopene or β-carotene contents of *B. trispora* powder samples were determined by HPLC. Carotenoids concentrations of samples were obtained from two paths: (1) the ratios of peak-areas of lycopene or β-carotene of samples to that of the corresponding lycopene or β-carotene standard at certain concentration, i.e. determination concentrations; (2) calculation from Eqs. (1) and (2) using sudan I as internal standard, i.e. calculation concentrations. The HPLC chromatograms of the extract containing lycopene or β-carotene from *B. trispora* cells with sudan I as internal standard are shown in Fig. 4. The relative error was calculated as percentage deviation of calculation concentration from determination concentrations. The relative error was below 1.4% at all concentration levels studied in the determination of lycopene content with sudan I as internal standard (see Table 5). In the range from 5.9 to 43.7 mg/ml, the highest relative error for β-carotene content determination was 1.9% with sudan I as internal standard (see Table 6). So the equations were accurate for sudan I substituting lycopene or β-carotene as standard sample.

Table 4
Retention times, peak areas and corrected coefficients of the mixed standard solutions including 0.1 mg/ml sudan I, lycopene and β-carotene at different determination time

Determination time (days)					
	2	4	6	8	10
Retention time (min)					
Sudan I	2.7	2.8	2.7	2.3	3.1
Lycopene	6.6	6.5	6.8	6.1	7.0
β-Carotene	10.1	9.8	10.2	9.8	11.3
Peak area					
Sudan I	1037291	1006224	1040171	1175442	1032853
Lycopene	3591390	3446016	3631576	4046811	3527812
β-Carotene	3993711	3886642	4067321	4465973	3935792
Corrected coefficient					
Lycopene	3.4623	3.4247	3.4913	3.4428	3.4156
β-Carotene	3.8501	3.8626	3.9102	3.7994	3.8106

Chromatographic conditions: Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm); mobile phase acetonitrile–dichloromethane (75:25, v/v); injection volume 5 μl.

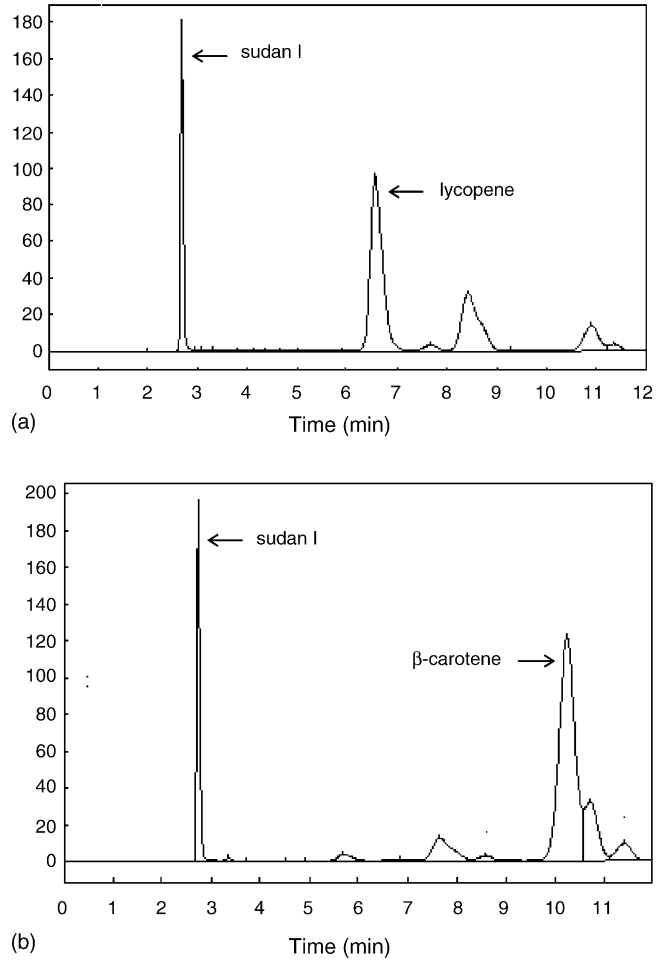

Fig. 4. HPLC chromatograms of the extract containing lycopene (a) or β-carotene (b) from *Blakeslea trispora* cells with sudan I as internal standard. Chromatographic conditions were the same as Fig. 1.

Table 5
Determination of lycopene contents of *Blakeslea trispora* cells with different standard samples

	Lycopene concentration (mg/ml)			
	Sample 1	Sample 2	Sample 3	Sample 4
Determination concentration	6.3	9.8	10.1	13.9
Calculation concentration	6.4	9.8	10.0	13.9
Relative error (%)	1.4	0.3	−1.1	0.4

The relative error is expressed as percentage deviation of calculation concentrations from determination concentrations. Chromatographic conditions were the same as Table 4.

Table 6
Determination of β-carotene contents of *Blakeslea trispora* cells with different standard samples

	β-Carotene concentration (mg/ml)			
	Sample 1	Sample 2	Sample 3	Sample 4
Determination concentration	5.9	15.9	30.9	43.7
Calculation concentration	6.0	15.9	30.9	43.8
Relative error (%)	1.9	0.4	0.2	0.3

The relative error is expressed as percentage deviation of calculation concentrations from determination concentrations. Chromatographic conditions were the same as Table 4.

3.5. Precision and recovery

To examine the precision of the proposed equations, intra-day variability was assessed by determining the same concentration of lycopene or β-carotene in the extract of *B. trispora* cells in pentuplicate. The variability was expressed as coefficient of variation. Results showed that intra-day variability for lycopene determination of *B. trispora* cells was less than 3.4% and for β-carotene was less than 1.4%.

Mean recovery of lycopene or β-carotene from samples were determined by spiking the extract of *B. trispora* cells with different volumes of lycopene or β-carotene standard solutions. Recovery values were calculated by comparing the amount of lycopene or β-carotene found in the samples to the amount added. The mean recovery of lycopene was 96.1%, and that of β-carotene was 97.9%. The data indicated that the assay method showed good repeatability.

4. Conclusion

The results presented in this work showed that sudan I, lycopene and β-carotene had the similar absorbance characteristics and could be separated absolutely in the HPLC chromatogram. So sudan I was feasible to substitute lycopene or β-carotene as internal standard. The equations using sudan I as internal standard to determine lycopene or β-carotene contents in samples by HPLC were established. By verification and examination, the equations were accurate and reproducible. This simple and efficient method overcame the instability of lycopene and β-carotene, improved the accuracy of the results and lowered the analytical cost.

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