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ScienceDirect
Food Chemistry 109 (2008) 439–446
FOOD CHEMISTRY
www.elsevier.com/locate/foodchem

Analytical Methods

Determination of carotenoids in *Dunaliella salina* cultivated in Taiwan and antioxidant capacity of the algal carotenoid extract

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Received 24 July 2007; received in revised form 17 December 2007; accepted 18 December 2007

Abstract

A simple HPLC method with good separation efficiency was developed to determine all-*trans* and *cis* forms of carotenoids in *Dunaliella salina* cultivated in Taiwan. The analysis used a C30 column (250 × 4.6 mm, 5 μm) and an isocratic solvent system (flow rate = 1 mL/min) mixing methanol–acetonitrile–water (84/14/2, v/v/v) and methylene chloride, (75/25, v/v). Carotenoids were detected at 450 nm. Moreover, the antioxidant capacities of the algal carotenoid extract were also evaluated with Trolox equivalent antioxidant capacity (TEAC) assay, reducing power and 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay. Results showed that 7 carotenoids in the algal extract could be separated simultaneously within 30 min and the total amount of them was 290.77 mg/g algae. The contents of all-*trans*-β-carotene and 9- or 9′-*cis*-β-carotene, the major carotenoids in the algae, were 138.25 and 124.65 mg/g algae, respectively. The contents of all-*trans*-lutein, all-*trans*-zeaxanthin, 13- or 13′-*cis*-β-carotene, all-*trans*-α-carotene and 9- or 9′-*cis*-α-carotene were 6.55, 11.27, 4.95, 2.69, and 2.41 mg/g algae, respectively. The algal carotenoid extract had significantly higher antioxidant activity than all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin in all antioxidant assays. The *cis* forms of carotenoids, especially 9- or 9′-*cis*-β-carotene, might play crucial roles for the antioxidant capacities of the algal extract.

Keywords: Algae; Antioxidant capacity; *Dunaliella salina*; HPLC

1. Introduction

Dunaliella salina is a type of unicellular and halophilic green biflagellate microalga without a rigid cell wall structure (Ben-Amotz & Avron, 1992; Denery, Dragull, Tang, & Li, 2004; García-González, Moreno, Manzano, Florencio, & Guerrero, 2005; Raja, Hemaiswarya, Balasubramanyam, & Rengasamy, 2007). Because *D. salina* contains abundant β-carotene, the algae has been used as a food coloring agent, a pro-vitamin A supplement for food and animal feed, an additive to food and cosmetics, a health food product (antioxidant claim) and so on (Edge, McGarvey, & Truscott, 1997; Johnson, & Schroeder, 1995).

Carotenoids are recognized as efficient antioxidants against oxidative damage (Jimenéz, & Pick, 1993). They could quench singlet oxygen ($^1O_2$), resulting in the suppression of lipid peroxidation (Burton, & Ingold, 1984; Foote, & Denny, 1968). Ben-Amotz (1999) indicated that humans could lower incidence of certain cancers, coronary heart disease and other degenerative diseases through eating carotenoid-rich vegetables and fruits (Ben-Amotz, 1999; Gester, 1993; Ziegler, 1989). For the determination of carotenoids, the reversed-phase high performance liquid chromatography (RP-HPLC) has been used routinely because of its excellent separation efficiency (Chen, Tai, & Chen, 2004; Inbaraj, Chien, & Chen, 2006; Liu, Kao, & Chen, 2004; Tai, & Chen, 2000). There are, however, no thorough reports on the composition and content of carotenoids in *D. salina* cultivated in Taiwan.

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0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.12.043

In the present study, we developed an isocratic RP-HPLC method to determine carotenoids including their isomers in *D. salina*; the amounts of these compounds in the algae were also quantified. The antioxidant activities of the algal carotenoid extract for Trolox equivalent antioxidant capacity (TEAC) assay, reducing power and scavenging ability on 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radicals were also investigated in this work.

2. Materials and methods

2.1. Materials

Spray dried powder of *D. salina* (Dunal) Teod. cultivated in Taiwan was obtained from Gong Bih Enterprise Co., Ltd. (Wunlin, Taiwan).

2.2. Chemicals and standards

All-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin standards were purchased from Sigma Co. (St. Louis, MO, USA). Solvents used for the extraction and determination of carotenoids, such as acetonitrile (ACN), methanol (MeOH), methylene chloride (CH_2Cl_2), ethanol (EtOH), acetone and *n*-hexane were purchased from Merck Co. (Darmstadt, Germany). Deionized water (dd H_2O) was prepared by Ultrapure™ water purification system (Lotun Co., Ltd. Taipei, Taiwan). Potassium hydroxide (KOH) was purchased from Merck Co. (Darmstadt, Germany).

Chemicals used for antioxidant capacity assays including 2-2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), ferrous chloride ($FeCl_3$), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), α-tocopherol, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), horseradish peroxidase, sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Sigma Co. (St. Louis, MO, USA).

2.3. Preparation of carotenoid extract from D. salina

The method was referred to that reported by Subagio, Morita, and Sawada (1996). Under nitrogen gas, 15 g of *D. salina* powder was extracted with 400 mL of hexane/acetone/EtOH (2/1/1, v/v/v) at ambient temperature for 24 h followed by adding 20 mL of 40% methanolic KOH for saponification at ambient temperature for 8 h. After filtering (with 0.45 μm Teflon membrane), the extract was transferred to a separatory funnel, washed with 400 mL of distilled water for 3 times and the solvent was removed with a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) at 30 °C. The yield of carotenoid extract was calculated. The extractive procedure was executed in dim lighting. Ten milligrams of the extract was dissolved in 5 mL of MeOH/CH_2Cl_2 (1/1, v/v) for carotenoids analysis, the rest was kept in the dark under nitrogen at −80 °C until antioxidant capacity was assayed.

2.4. Determination of carotenoids in D. salina

2.4.1. HPLC analysis of carotenoids

A *PrimeLine*™ Gradient Model 500G HPLC pump system (Analytical Scientific Instruments, Inc., El Sobrante, CA, USA) and a S-3210 photodiode-array detector (PDA) (Schambeck SFD GmbH, Bad Honnef, Germany) were used to determine carotenoids in *D. salina*. The analytical conditions were referred to that reported by Inbaraj et al. (2006) and modified further: An YMC C30 column (250 × 4.6 mm, 5 μm) (Waters Co., Milford, MA, USA), isocratic solvent system, methanol–acetonitrile–water (84/14/2, v/v/v)/methylene chloride (75/25, v/v), flow rate = 1 mL/min, detection, 450 nm. Samples were injected using a manual injection valve (Rheodyne™ 7725i, Rheodyne Co., Rohnert Park, CA, USA) (20 μL injection volume). HPLC separation efficiency was evaluated with the separation factor (α) and resolution (Rs). The limits of detection (LODs) and quantification (LOQs) for all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin standards were concluded by the signal-to-noise ratio (S/N) of 3 and 10, respectively. The reproducibility for each carotenoid was determined; the standard deviation was computed from six measurements by run-to-run and day-to-day.

2.4.2. Identification of carotenoids

The identification of various carotenoids in *D. salina* was carried out by (1) comparison of retention time and absorption spectra of unknown peaks with reference standards, (2) addition of carotenoid standards to sample for co-chromatography. In addition, the *cis*-forms of carotenoids identification was according to Inbaraj et al. (2006), who identified these compounds based on the spectral characteristics and *Q*-ratios published in the literatures (Böhm, Puspitasari-Nienaber, Ferruzzi, & Schwartz, 2002; Chen et al., 2004; Inbaraj et al., 2006; Lin, & Chen, 2003; Liu et al., 2004). The peak purity was automatically determined through the S-3210 PDA. For this calculation, the peak maximum (apex) and the half function values on the left (upslope) and the right (downslope) of the peak were compared.

2.4.3. Preparation of standard curve

Six amounts (the range is 0.02–10 μg) of all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin were injected into HPLC (Each standard was dissolved in 1 mL of MeOH/CH_2Cl_2 and the injection volume was 20 μL), the linear regression equation for each standard curve was obtained by plotting the amount of standard compound injected against the peak area. The regression equation and the correlation coefficient (r^2) were calculated with Chrom-Manager Multisystem (Analab Co., Taipei, Taiwan). Due to the absence of commercial *cis*-forms of

carotenoids, the *cis*-isomers were quantified based on the calibration curves of their corresponding all-*trans* form of carotenoid standard, their extinction coefficients were similar (Boileau, Merchen, Wasson, Atkinson, & Erdman, 1999; Lee, & Chen, 2002).

2.4.4. Recoveries of carotenoids

The recoveries were measured by adding a mixture of all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin standards (each weighing 0.25, 0.5 and 1 mg) to 0.5 g of *D. salina* powder. And then it was extracted with 13.33 mL of hexane/acetone/EtOH (2/1/1, v/v/v) at ambient temperature for 24 h followed by adding 0.67 mL of 40% methanolic KOH for saponification at ambient temperature for 8 h. The procedure was carried out in dim lighting under nitrogen gas. After filtering (with 0.45 μm Teflon membrane), the extract was transferred to a separatory funnel, washed with 10 mL of distilled water for 3 times and the solvent was removed with a rotary evaporator at 30 °C. After HPLC analysis, the recovery for each all-*trans* form carotenoid standard was calculated from the measured result and the original quantity of carotenoid used. The recoveries of *cis* isomers were also assumed to be equivalent to their corresponding all-*trans* forms of carotenoids.

2.4.5. Statistical analysis

The standard calibration equations of carotenoids, recoveries and quantitative analyses were performed in triplicate and the mean values were calculated. The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests to resolve significance between means, at a level of $p < 0.05$.

2.5. Antioxidant capacity assay of the carotenoid extract from D. salina

All-*trans* forms of lutein, zeaxanthin, α-carotene and β-carotene and α-tocopherol standards were used as controls in all assays.

2.5.1. DPPH radical scavenging activity

The method was referred to that reported by Shimada, Fujikawa, Yahara, and Nakamura (1992) and Epsin, Soler-Rivas, and Wichers (2000). An aliquot of each sample (30 μL, 0.5–40 mg/mL) in acetone/MeOH (1/1, v/v) was mixed with 200 μL of 100 μM DPPH (prepared with methanol). The mixture was shaken vigorously and then left to stand at room temperature for 60 min in the dark. The absorbance was measured spectrophotometrically at 520 nm against an acetone/MeOH (1/1, v/v) blank. The lower absorbance indicated the stronger scavenging activity. EC_{50} value (mg sample/mL), the effective concentration at which 50% of the DPPH radicals were scavenged, was obtained from the plot of scavenging activity against the concentration of sample. The scavenging activity was calculated based on the percentage of DPPH radical scavenged.

2.5.2. TEAC assay

TEAC assay was based on that reported by Arnao, Casas, Del Río, Acosta, and García-Cánovas (1990) and Scalzo, Politi, Pellegrini, Mezzetti, and Battino (2005). ABTS is a chromogen (colorless) that would be converted to blue–green colored $ABTS^{\cdot+}$ radical cation by an oxidative reagent. $ABTS^{\cdot+}$ could also be reduced to its colorless form by antioxidant. The absorbance was measured spectrophotometrically at 734 nm as a function of concentration and the scavenging percentage of $ABTS^{\cdot+}$ was calculated relative to Trolox, a water-soluble analog of vitamin E used as an antioxidant standard. Antioxidant activity was presented as micromole of Trolox equivalent per gram of sample weight. The $ABTS^{\cdot+}$ solution ($OD_{734} = 0.7 \pm 0.02$) was prepared by mixing ABTS, peroxidase and H_2O_2 with the final concentrations of 100 μM, 4.4 unit/mL and 50 μM, respectively, and then reacted at 30 °C for 6 min. The dose–response curve was derived for Trolox (0, 0.125, 0.25, 0.5, 1, 2 and 4 mmol/L) that was diluted in 1 mL of $ABTS^{\cdot+}$ solution.

2.5.3. Reducing power

The reducing power was measured according to that reported by Oyaizu (1986) and Yen and Chung (1999). An aliquot of each sample (0.5 mL, 0.5–10 mg/mL) in acetone/MeOH (1/1, v/v) was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% $K_3Fe(CN)_6$ followed by incubating at 50 °C for 20 min. After adding 0.5 mL of 10% trichloroacetic acid, the mixture was centrifuged at 3750*g* for 10 min (Hermle Z300K centrifuge, Hermle Labortechnik GmbH, Wehingen Württ, Germany). The supernatant (0.1 mL) was then taken out and mixed with 0.1 mL of methanol and 20 μL of 0.1% ferric chloride immediately. After 10 min reaction, the absorbance was determined at 700 nm against a blank. The higher absorbance indicated the stronger reducing power.

2.5.4. Statistical analysis

All antioxidant capacity assays were carried out in triplicate and the mean values were calculated. The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests were used to assess differences between means. A significant difference was considered at a level of $p < 0.05$.

3. Results and discussion

3.1. HPLC analysis of carotenoids in D. salina

Fig. 1 is the HPLC chromatogram of carotenoid extract from *D. salina*. Seven carotenoids (the spectral characteristics and *Q*-ratios of the seven resolved peaks corresponded to carotenoids) in the extract could be separated simultaneously within 30 min. Table 1 shows the assignment data for all-*trans* and *cis* forms of carotenoids in *D. salina*. Peaks 1, 2, 4 and 6 were certainly assigned as all-*trans* forms of lutein, zeaxanthin, α-carotene and β-carotene, respectively

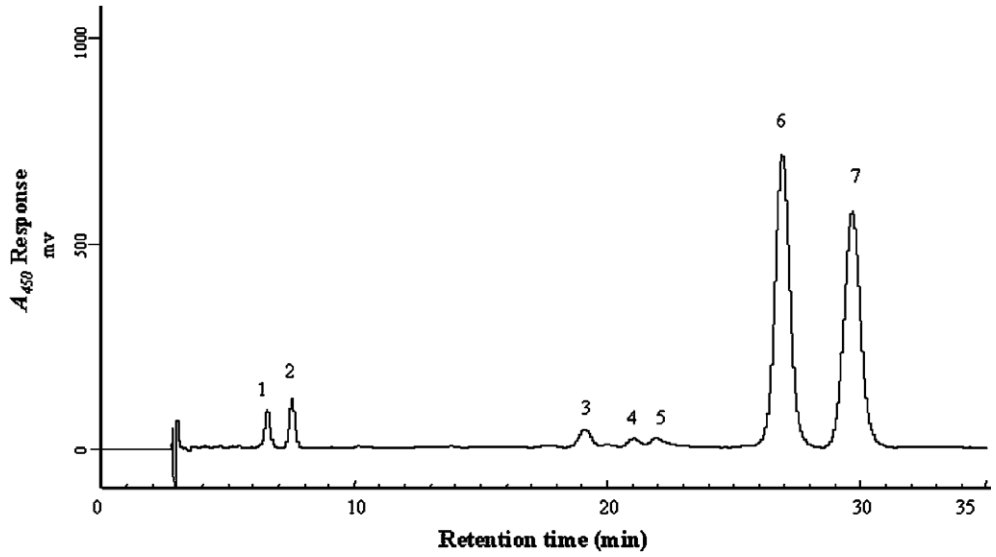

Fig. 1. HPLC chromatogram of carotenoid extract from *D. salina*. HPLC conditions: Column, YMC C30 (250 × 4.6 mm, 5 μm), mobile phase, methanol–acetonitrile–water (84/14/2, v/v/v)/methylene chloride (75/25, v/v), flow rate, 1 mL/min, detection, 450 nm. See Table 1 for the assignment of peaks.

Table 1
Assignment data for all-*trans* and *cis* forms of carotenoids in *D. salina*

Peak no.	Compound	Retention time (min)	λ (nm)				*Q*-ratio[a]
1	All-*trans*-lutein	6.6	332	424	446	470	0.11
2	All-*trans*-zeaxanthin	7.6	344	427	451	475	0.12
3	13- or 13′-*cis*-β-carotene	19.1	344	422	444	475	0.44
4	All-*trans*-α-carotene	21.1	344	425	449	476	0.10
5	9- or 9′-*cis*-α-carotene	21.9	344	422	446	470	0.22
6	All-*trans*-β-carotene	26.9	349	429	458	481	0.12
7	9- or 9′-*cis*-β-carotene	29.7	344	428	452	476	0.19

[a] *Q*-ratio: the height ratio of the *cis* peak to the main absorption peak.

based on the criteria expounded in Section 2.4.2. Peak 3 was tentatively assigned as 13- or 13′-*cis*-β-carotene according to those reported by Lin and Chen (2003) and Inbaraj et al. (2006). Peak 5 was tentatively assigned as 9- or 9′-*cis*-α-carotene by the references of Böhm et al. (2002) and Inbaraj et al. (2006). Peak 7 was tentatively assigned as 9- or 9′-*cis*-β-carotene through comparing with the reports of Liu et al. (2004) and Inbaraj et al. (2006).

Table 2 shows that the separation factors (α) and resolutions (Rs) of carotenoids in the algae for all peaks were higher than 1. The purities of all peaks were higher than 97%. Furthermore, reproducibility was good for these carotenoids as presented in the analytical results, R.S.D. < 2.12% for retention times and R.S.D. < 3.12% for integrated areas (Table 3). Therefore, the isocratic solvent system was practical to determine the carotenoids in *D. salina*.

Table 2
Separation factor (α), resolution (Rs) and purity of carotenoids in *D. salina*

Peak no.	Compound	Retention time (min)	α[a]	*Rs*[b]	Peak purity (%)
1	All-*trans*-lutein	6.6	–	–	98.2
2	All-*trans*-zeaxanthin	7.5	1.3 (1/2)	1.5 (1/2)	99.6
3	13- or 13′-*cis*-β-carotene	19.1	3.5 (2/3)	15.4 (2/3)	99.3
4	All-*trans*-α-carotene	21.1	1.1 (3/4)	2.3 (3/4)	97.7
5	9- or 9′-*cis*-α-carotene	21.9	1.1 (4/5)	1.0 (4/5)	97.4
6	All-*trans*-β-carotene	26.9	1.3 (5/6)	3.6 (5/6)	99.9
7	9- or 9′-*cis*-β-carotene	29.7	1.1 (6/7)	1.4 (6/7)	99.9

Values in parentheses represent two neighboring peaks.
[a] $\alpha = t_{R2}-t_0/t_{R1}-t_0$, where t_{Rn} is the retention time of an analyte, t_0 is the retention time of an unretained peak.
[b] $Rs = 2\,(t_{R2}-t_{R1})/(w_1 + w_2)$, where w_n is the band width of an analyte at the baseline.

Herrero, Jaime, Martín-Álvarez, Cifuentes, and Ibáñez (2006) utilized a C30 column and a gradient solvent system composing of methanol–water–triethylamine (90/10/0.1, v/v/v)/methyl *tert*-butyl ether–chloride–methanol–water–triethylamine (90/6/4/0.1, v/v/v/v) (from 93.5: 6.5 to 0: 100, v/v) to determine carotenoids in *D. salina* from NBT Ltd. (Jerusalem, Israel) within 34 min. Although the condition could resolve 5 carotenoids (α-carotene, 13-*cis*-β-carotene, all-*trans*-β-carotene, 15-*cis*-β-carotene and 9-*cis*-β-carotene), the separation efficiency for these compounds was not very good. Abd El-Baky, El-Baz, and El-Baroty

Table 3
Reproducibility of the carotenoids in *D. salina*

Peak no.	Compound	R.S.D.[a] (%)			
		Retention time		Integrated area	
		Run-to-run	Day-to-day	Run-to-run	Day-to-day
1	All-*trans*-lutein	0.56	1.51	1.68	2.39
2	All-*trans*-zeaxanthin	0.52	1.36	1.75	2.42
3	13- or 13′-*cis*-β-carotene	0.41	1.73	1.84	3.07
4	All-*trans*-α-carotene	0.62	2.12	1.95	2.69
5	9- or 9′-*cis*-α-carotene	0.64	2.06	2.24	2.92
6	All-*trans*-β-carotene	0.48	1.51	1.76	3.12
7	9- or 9′-*cis*-β-carotene	0.50	1.67	1.84	2.46

The analytical conditions are shown in Section 2.4.1.
[a] The result was obtained from 5 mg carotenoid extract/mL with six measurements.

(2004) used a C18 column and an isocratic solvent system, acetonitrile–methanol (80/10, v/v) to determine β-carotene, lutein, zeaxanthin, astaxanthin and cryptoxanthin in *D. salina* obtained from the Culture Collection of Botany Department, Texas University (Austin, Texas, USA). Although the five carotenoids could be separated within 15 min, their *cis* isomers were not measured in the investigation. A simple HPLC method developed in our experiment had appropriate separation time and desirable separation efficiency for carotenoids (including all-*trans* and *cis* isomers) of *D. salina*.

3.2. Carotenoid content in D. salina

The LODs and LOQs were 3.5 and 11.6 ng for all-*trans* lutein, 3.1 and 10.5 ng for all-*trans* zeaxanthin, 0.3 and 1.2 ng for all-*trans* α-carotene, and 0.5 and 2.3 ng for all-*trans* β-carotene, respectively. Solutions that contained 0.02–10 μg of these carotenoid standards were used to establish the standard calibration curves, which were linear and reproducible. Carotenoids were detected at 450 nm. Table 4 shows the linear regression equations of these compounds, all of their correlation coefficients (r^2) were above 0.999.

The yield of *D. salina* extract was 29.12% after extraction. The recoveries of added all-*trans* forms of lutein, zeaxanthin, α-carotene and β-carotene (each weighing 0.25–1 mg) in *D. salina* were all above 98.6% (Table 5). Regardless of amounts added, the recoveries were not significantly different between these compounds.

The total amount of carotenoids in *D. salina* was 290.77 mg/g algae (Table 6). The contents of all-*trans*-β-carotene and 9- or 9′-*cis*-β-carotene were 138.25 and 124.65 mg/g algae, respectively, they were 90.42% of the total carotenoid in *D. salina* and were the major carotenoids in the algae. The contents of all-*trans*-lutein, all-*trans*-zeaxanthin, 13- or 13′-*cis*-β-carotene, all-*trans*-α-carotene and 9- or 9′-*cis*-α-carotene were 6.55, 11.27, 4.95, 2.69 and 2.41 mg/g algae, respectively. García-González et al. (2005) pointed out that *D. salina* contained 9-*cis*-β-carotene and lutein. Ben-Amotz, Katz, and Avron (1982) observed that *D. salina* contained abundant all-*trans*-β-carotene and 9-*cis*-β-carotene. Yokthongwattana, Savechenko, Polle, and Melis (2005) found that long-term acclimatization to stress due to irradiation caused substantial accumulation of zeaxanthin in *D. salina* along with a

Table 4
The linear regression equations of the all-*trans* forms of carotenoid standards

Compound	Detection, 450 nm		
	Linear range (ng)	Linear regression equations[a]	Correlation coefficient (r^2)
All-*trans*-lutein	20–10,000	$Y = 50.298X + 717.71$[b]	0.9997
All-*trans*-zeaxanthin	20–10,000	$Y = 41.411X - 252.98$	0.9993
All-*trans*-α-carotene	20–10,000	$Y = 50.488X + 804.04$	0.9992
All-*trans*-β-carotene	20–10,000	$Y = 51.647X + 645.42$	0.9995

[a] All data are the means of triplicate analyses.
[b] Y is the value of the peak area, X is the value of sample quantity (ng).

Table 5
The recoveries of added carotenoids in *D. salina* after extraction

Added amount (mg)	Recovery% (CV%)[A,B] Compound			
	All-*trans*-lutein	All-*trans*-zeaxanthin	All-*trans*-α-carotene	All-*trans*-β-carotene
0.25	98.62 (3.24) a	98.65 (2.95) a	98.97 (2.82) a	98.82 (3.01) a
0.50	99.20 (2.72) a	99.24 (3.13) a	99.24 (2.94) a	99.16 (2.62) a
1.00	99.35 (2.35) a	99.37 (2.07) a	99.41 (3.04) a	99.47 (3.10) a

Values bearing different letters in the same column are significantly different ($p < 0.05$).
[A] All values are the means of triplicate analyses.
[B] Values in parentheses are the coefficient of variation (%).

Table 6
Carotenoid contents in *D. salina*

Peak no.	Compound	Content (mg/g algae)[a]
1	All-*trans*-lutein	6.55 ± 0.92
2	All-*trans*-zeaxanthin	11.27 ± 1.58
3	13- or 13′-*cis*-β-carotene	4.95 ± 0.83
4	All-*trans*-α-carotene	2.69 ± 0.45
5	9- or 9′-*cis*-α-carotene	2.41 ± 0.32
6	All-*trans*-β-carotene	138.25 ± 10.03
7	9- or 9′-*cis*-β-carotene	124.65 ± 9.91
Total amount		290.77

[a] All values are mean ± SD obtained by triplicate analyses.

lowering in the relative amount of other pigments, including chlorophylls and several carotenoids. Abd El-Baky et al. (2004) found β-carotene, astaxanthin, zeaxanthin, lutein and cryptoxanthin in *D. salina* cultivated under nitrogen deficiency combined with NaCl stress and ultraviolet B radiation (290–320 nm). They indicated that the cultivation condition could potentially increase carotenoids in the algae. In our investigation, 7 of all-*trans* and *cis* forms of carotenoids were determined in *D. salina* cultivated in Taiwan. The amounts of all-*trans*-β-carotene and 9- or 9′-*cis*-β-carotene were much higher than other carotenoids. The algae also contained all-*trans* forms of lutein and zeaxanthin but no astaxanthin and cryptoxanthin. Abalde, Fabregas, and Herrero (1991) demonstrated that different cultivation conditions and environments would affect the content and composition of compounds in algae.

3.3. Antioxidant activities of the carotenoid extract from D. salina

D. salina contained all-*trans* forms of lutein, zeaxanthin, α-carotene and β-carotene, which were commercial carotenoids. The antioxidant activities of the algal carotenoid extract were determined using 3 methods against those pure carotenoids. For DPPH radical scavenging assay, Fig. 2 shows that the algal extract had significantly higher effect than each carotenoid. At 10 mg/mL, the scavenging abilities on DPPH radicals were 64.14%, 26.23%, 25.75%, 24.38%, 24.01% and 82.41% for the algal extract, all-*trans* forms of zeaxanthin, lutein, β-carotene and α-carotene, and α-tocopherol, respectively. At 40 mg/mL, the scavenging abilities could be increased to 77.37%, 70.12%, 68.83%, 67.32% and 66.14% for the algal extract, and all-*trans* forms of zeaxanthin, lutein, β-carotene and α-carotene, respectively. Table 7 presents that the EC_{50} values of scavenging DPPH radicals of the algal extract, all-*trans* forms of zeaxanthin, lutein, β-carotene and α-carotene, and α-tocopherol were 8.36, 22.82, 23.04, 24.17, 24.54 and 2.88 mg/mL, respectively. With regard to TEAC assay, the TEAC values of the algal extract, all-*trans* forms of zeaxanthin, lutein, β-carotene and α-carotene, and α-tocopherol were 0.76, 0.44, 0.43, 0.45, 0.45 and 1.42 mmol Trolox equivalent/g, respectively (Table 7). The antioxidant capacity of the algal extract was significantly higher than that of each all-*trans* carotenoid. Reducing power was increased with increasing concentration of each experimental sample (Fig. 3). Similarly, the algal extract had higher reducing power than each carotenoid.

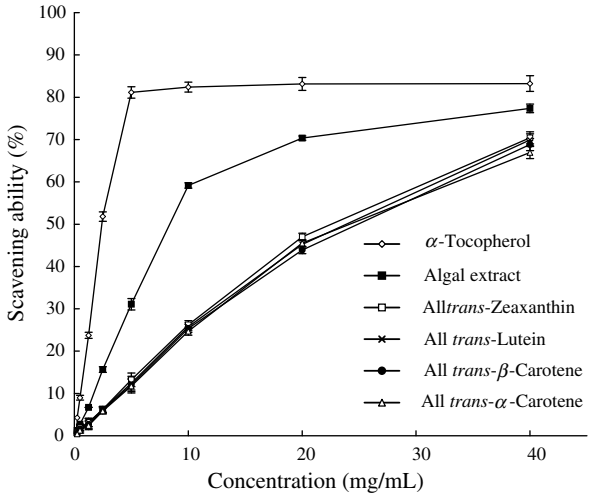

Fig. 2. DPPH radical scavenging effects of *D. salina* carotenoid extract, all-*trans*-forms of α-carotene, β-carotene, lutein and zeaxanthin, and α-tocopherol. Each value is expressed as mean ± SD ($n = 3$).

Table 7
EC_{50} of DPPH radical quenching activity and antioxidant activity (TEAC assay) of the *D. salina* carotenoid extract, all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin, and α-tocopherol

Sample	EC_{50}[A] (mg sample/mL)	TEAC (mmol TE[B]/g sample)
α-Tocopherol	2.88 ± 0.11 d	1.42 ± 0.09 a
Algal extract	8.36 ± 0.75 c	0.76 ± 0.07 b
All-*trans*-zeaxanthin	22.82 ± 2.08 b	0.44 ± 0.06 c
All-*trans*-lutein	23.04 ± 2.41 b	0.43 ± 0.07 c
All-*trans*-β-carotene	24.17 ± 2.64 a	0.45 ± 0.08 c
All-*trans*-α-carotene	24.54 ± 1.83 a	0.45 ± 0.06 c

Values (mean ± SD, $n = 3$) in the same column that are followed by a different letter are significantly different ($p < 0.05$) by Duncan's multiple range tests.
[A] EC_{50} means the effective concentration of sample that can decrease DPPH concentration by 50%.
[B] TE, Trolox equivalent.

Esterbauer, Geblicki, Puhl, and Jürgens (1992) pointed out that the carotenoids could suppress LDL oxidation induced by Cu^{2+} (the sequence of activity being lutein/zeaxanthin > α, β-carotene), nevertheless, Romanchik, Harrison, and Morel (1997) illustrated that β-carotene could protect LDL from Cu^{2+} induced oxidation in vitro but lutein could not. Lutein had higher antioxidant property than β-carotene for 2,2′-azobis-amidinopropane dihydrochloride (AAPH)-initiated oxidation of food emulsions (Kiokias, & Oreopoulou, 2006). Miller, Sampson, Candeias, Bramley, and Rice-Evans (1996) found that the sequence for $ABTS^{\cdot+}$ radical scavenging ability was β-carotene > lutein ≈ zeaxanthin > α-carotene. Nevertheless, our results were similar to those reported by Böhm et al. (2002). They indicated that Trolox equivalent antioxidant capacities of all-*trans* forms of α-carotene, β-carotene and zeaxanthin did not reveal significant differences. In our investigation, the TEAC values among all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin did not show significant differences (Table 7). These compounds also had similar reducing power (Fig. 3). The DPPH radical quenching activities of all-*trans* forms of lutein and zeaxanthin were significantly higher than those of all-*trans* forms of α-carotene and β-carotene.

Except all-*trans* forms of carotenoids described above, *D. salina* also contained abundant *cis*-forms of carotenoids

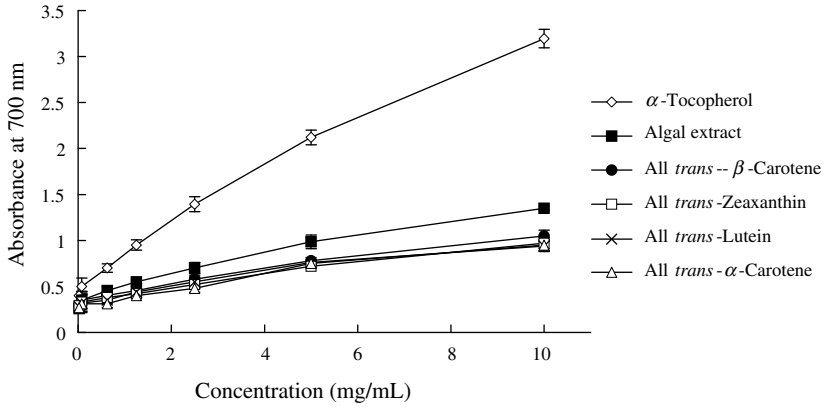

Fig. 3. Reducing power of *D. salina* carotenoid extract, all-*trans*-forms of α-carotene, β-carotene, lutein and zeaxanthin, and α-tocopherol. Each value is expressed as mean ± SD ($n = 3$).

(about 45.40% of total weight of the algal extract). For these *cis*-isomers, 9- or 9′-*cis*-β-carotene was the major compound (42.87% of total weight of the algal extract) and the others were 9- or 9′-*cis*-α-carotene (0.83% of total weight of the algal extract) and 13- or 13′-*cis*-β-carotene (1.70% of total weight of the algal extract). Levin and Mokady (1994) clarified that 9-*cis*-β-carotene displayed higher antioxidant activity in vitro compared to that of all-*trans*-β-carotene. Jimenéz and Pick (1993) described that 9-*cis*-β-carotene was more effective than its all-*trans* isomer for quenching reactive oxygen species. Böhm et al. (2002) also reported that 9-*cis*-α-carotene and 13-*cis*-β-carotene had good Trolox equivalent antioxidant capacity. Therefore, these *cis*-forms of carotenoids, especially 9- or 9′-*cis*-β-carotene should play important roles for the antioxidant activities of *D. salina*. Werman, Ben-Amotz, and Mokady (1999) found that *Dunaliella bardawil* powder (42% all-*trans*-β-carotene and 43% 9-*cis*-β-carotene) inhibited formation of hepatic conjugated dienes after alcohol-induced oxidative stress in rats significantly better than synthetic β-carotene (97% all-*trans* β-carotene, 3% 15-*cis*-β-carotene).

4. Conclusion

In this investigation, seven carotenoids were determined in *D. salina* cultivated in Taiwan using a HPLC method with isocratic solvent system. The algal carotenoid extract had remarkably higher antioxidant activity than all-*trans* forms of α-carotene, β-carotene, lutein and zeaxanthin. The *cis* forms of carotenoids in the extract, especially 9- or 9′-*cis*-β-carotene, might play crucial roles in the antioxidant activity. Therefore, our work provides the necessary information to exploit *D. salina* as a health food.

Acknowledgment

This work was supported by Chun Shan Medical University, Taichung, Taiwan (Project No. CSMU-82-B-013 and CSMU 94-OM-A-163).

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