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Determination of carotenoids and their esters in fruits of *Lycium barbarum* Linnaeus by HPLC–DAD–APCI–MS

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ABSTRACT

The fruit of *Lycium barbarum* Linnaeus, a traditional Chinese herb containing functional components such as carotenoids, flavonoids and polysaccharides, has been widely used in the health food industry because of its possible role in the prevention of chronic disease like age-related macular degeneration. The objectives of this study were to develop a high performance liquid chromatography–photo diode array detection–mass spectrometry (HPLC–DAD–MS) method with atmospheric pressure chemical ionization (APCI) mode for qualitative and quantitative analyses of carotenoids in fruits of *L. barbarum*. Dried samples of *L. barbarum* were subjected to extraction without saponification or extraction followed by saponification. A C30 column with a gradient mobile phase of methylene chloride (100%) and methanol–acetonitrile–water (81:14:5, v/v/v) was used to separate carotenoids, with a total of 11 free carotenoids and 7 carotenoid esters being resolved from unsaponified and saponified *L. barbarum* extracts within 51 and 41 min, respectively. The fatty acid composition of carotenoid esters was confirmed by gas chromatography. Zeaxanthin dipalmitate (1143.7 μg/g) was present in the largest amount, followed by β-cryptoxanthin monopalmitate and its two isomers (32.9–68.5 μg/g), zeaxanthin monopalmitate and its two isomers (11.3–62.8 μg/g), all-*trans*-β-carotene (23.7 μg/g) and all-*trans*-zeaxanthin (1.4 μg/g).

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1. Introduction

Fruit of *Lycium barbarum* Linnaeus (Family: Solanaceae), a popular traditional Chinese herbal medicine, has been used as a functional food for centuries because of its possible beneficial effect in the prevention of chronic diseases such as age-related macular degeneration, which can be due to the presence of lutein and zeaxanthin [1]. In addition, it may possess antioxidant and antitumor activities [2,3], neuroprotective effect [4], and enhance immunity [3] as well. The presence of various functional components like polysaccharides, flavonoids and carotenoids in *L. barbarum* fruits is believed to be responsible for these effects [5–8]. The composition of polysaccharides and flavonoids has been studied extensively, however, the amount and variety of carotenoids still remain uncertain.

Carotenoids, a group of lipid-soluble compounds with color ranging from yellow to red, have been shown to be present in large quantity in fruits of *L. barbarum* [9–11]. Of the various carotenoids, zeaxanthin and its esters were reported to dominate, but the exact profile of carotenoids is questionable because of limitation and difference in analytical techniques employed. Peng et al. [9] used a thin-layer chromatographic (TLC) method to determine various carotenoids in *L. barbarum* fruits, and identified only zeaxanthin, β-cryptoxanthin and β-carotene. The major drawback of this method is that the resolution of carotenoids is poor and no quantitation is carried out. In a similar study Lee et al. [10] used a high performance liquid chromatographic (HPLC) method to determine the carotenoids in *L. barbarum* fruits, and a total of ten carotenoids, including zeaxanthin, β-cryptoxanthin, violaxanthin and their esters were identified. But the identification could only be tentative due to lack of any mass spectral data for these carotenoids. In a later study, Weller and Breithaupt [11] developed a liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) technique to analyze zeaxanthin esters in several plants, and a total of four zeaxanthin monoesters plus four zeaxanthin diesters were resolved in 50 min and identified. However, for wolfberry (*L. barbarum*) sample, only one zeaxanthin diester (zeaxanthin dipalmitate) was identified and quantified, which should be inadequate as the presence of some other free carotenoids and carotenoid esters remain unknown.

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In view of the possible impact of carotenoids on human health, our objective was to develop a better HPLC–DAD–APCI–MS method to determine both free carotenoids and carotenoid esters in fruits of *L. barbarum*. Also, the fatty acid composition of carotenoid esters was analyzed using a gas chromatographic (GC) method.

2. Experimental

2.1. Materials

A total of 1.8 kg of *L. barbarum* fruits was purchased from a local drug store in Taipei, Taiwan. Carotenoids standards, including all-*trans*-zeaxanthin and all-*trans*-β-cryptoxanthin were from Extrasynthese (Genay, France), and all-*trans*-β-carotene was from Sigma (St. Louis, MO, USA). Internal standard β-*apo*-8′-carotenal was from Fluka (Buchs, Switzerland). The purity of all these standards were >95% as indicated by the manufacturer. Fatty acid methyl ester standards, including stearic acid methyl ester (18:0), oleic acid methyl ester (18:1) and linoleic acid methyl ester (18:2) were from Sigma, and palmitic acid methyl ester (16:0) was from Nu-Chek-Prep (Elysian, MN, USA). Reagent 2,2-dimethoxypropane was also from Sigma. The HPLC grade solvents such as methanol, toluene, acetone, *n*-hexane, acetonitrile, dichloromethane and tetrahydrofuran were from Lab-Scan (Dublin, Ireland). The analytical grade solvent *n*-hexane was from Grand Chemical (Taipei, Taiwan). Ethanol (95%) was from Taiwan Tobacco and Wine Board (Tainan, Taiwan). Deionized water was made using a Milli-Q purification system (Millipore Co., Bedford, MA, USA). Sodium hydroxide and anhydrous sodium sulfate were from Riedel-de Haën (Barcelona, Spain). A polymeric C30 column (250 mm × 4.6 mm i.d., 5 μm particle) used to separate carotenoids was from Waters (Milford, MA, USA). An HP-88 capillary column (100 m × 0.25 mm i.d., 0.2 μm film thickness) containing (88% cyanopropyl)-methylarylpolysiloxane was from Agilent Technologies (Palo Alto, CA, USA).

2.2. Instrumentation

The Agilent HPLC system (Palo Alto, CA, USA) was equipped with a quaternary pump (Agilent G1311A), an online degasser (Agilent G1379A) and a column oven controller (Agilent G1316A), which was connected in series with a photo diode array detector (DAD) (Agilent G1315B) and a single quadrupole mass spectrometer (Agilent 6130) with multi-ionization source (APCI/ESI). The gas chromatograph equipped with flame ionization detecter (HP 6890) was also from Agilent Technologies. The N-1 rotary evaporator was from Eyela (Tokyo, Japan). The high-speed centrifuge (model 5810) was from Eppendorf (Hamburg, Germany). The sonicator (model DC400H) was from Chuan-Hua Co. (Taipei, Taiwan).

2.3. Analysis of carotenoids

2.3.1. Simultaneous extraction and saponification

A method based on Chen et al. [12] was used to extract carotenoids from *L. barbarum* fruits. Briefly, 1 g powder sample of *L. barbarum* was mixed with 20 mL of hexane–ethanol–acetone–toluene (10:6:7:7, v/v/v/v) in a 40-mL brown vial and shaken for 1 h. One milliliter of 40% methanolic potassium hydroxide was added to the mixture and kept for saponification in the dark for 12 h. A stream of nitrogen was flushed into the vial to avoid isomerization or degradation of carotenoids during saponification. Next, 15 mL of hexane was added and shaken for 1 min, followed by adding 15 mL of 10% anhydrous sodium sulfate solution. After shaking for 1 min, the mixture was allowed to settle until two layers were formed. The supernatant (carotenoid layer) was collected and the residue was mixed again with 15 mL of hexane for further partition. This procedure was repeated five times and all the supernatants were combined for evaporation to dryness. The residue was dissolved in 5-mL dichloromethane, filtered through a 0.22-μm membrane filter, and 20-mL was injected for HPLC analysis.

2.3.2. Extraction without saponification

The extraction procedure was the same as described above with the exception that no methanolic potassium hydroxide was added for saponification.

2.3.3. Extraction followed by saponification

One g powder sample of *L. barbarum* was mixed with 20 mL of hexane–ethanol–acetone–toluene (10:6:7:7, v/v/v/v) in a brown vial, after which the mixture was shaken for 1 h, followed by shaking with 15-mL of 10% anhydrous sodium sulfate solution for 1 min and the supernatant was collected. The residue was partitioned with 15 mL of hexane repeatedly for five times and all the supernatants were combined for evaporation to dryness. Subsequently, the residue was dissolved in 20 mL of hexane–ethanol–acetone–toluene (10:6:7:7, v/v/v/v) and 1 mL of 40% methanolic potassium hydroxide was added for saponification for 2, 4, 6 and 8 h in the dark under nitrogen. After saponification, the upper layer was collected and the lower aqueous layer was repeatedly partitioned three times with 20-mL hexane. The supernatants were pooled, vacuum dried, dissolved in 5-mL dichloromethane, filtered through a 0.22-μm membrane filter and 20 mL was injected for HPLC analysis.

2.3.4. HPLC–DAD–APCI–MS analysis

A method based on Inbaraj et al. [13] was modified to separate the various carotenoids in *L. barbarum* fruits. A total of 7 carotenoid esters and 11 free carotenoids were resolved within 51 and 41 min, respectively, in unsaponified and saponified extracts by using a C30 column and a gradient mobile phase of dichloromethane (A) and methanol–acetonitrile–water (84:14:5, v/v/v) (B): 16% A and 84% B initially, increased to 17% A in 22 min, 55% A in 40 min, 75% A in 55 min, and returned to 16% A in 60 min. The column temperature was maintained at 25 °C, flow rate at 1 mL/min and detection wavelength at 450 nm. The identification of carotenoids was performed by comparing retention time, UV spectra and mass spectra of unknown peaks with reference standards and values reported in the literature. For LC–MS, the positive ion mode (APCI) was used to detect carotenoids and their esters, with total ion current (TIC) scanning range 400–1200 *m*/*z*, corona current 4 μA, capillary voltage 2000 V, charging voltage 2000 V and nitrogen as nebulizer gas (purity 99.9% and flow rate 7 L/min) and vaporizer temperature at 330 °C. The identification of *cis*-isomers of carotenoids was based on spectral characteristics and *Q*-ratios as described in several previous studies [12–14]. In addition, the mass spectra of *cis*-isomers of carotenoids were determined and compared with those of *trans*-carotenoids for confirmation. The purity of each peak was automatically determined using the DAD.

2.4. Photoisomerization of carotenoid standards

For further identification of *cis*-isomers of carotenoids, 1 mg of all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene standards were each dissolved in 10 mL of dichloromethane separately in a 10-mL glass vial for a concentration of 100 μg/mL. All the vials were placed in an incubator at 25 °C and illuminated for 12 h with four fluorescent light tubes (55 cm long and 20 W each) at a light intensity of 2000–3000 lx

Table 1
Calibration data, detection limit and quantitation limit for carotenoid standards

Carotenoid	Calibration data[a]		DL[b] (μg/mL)	QL[c] (μg/mL)
	Regression equation	R^2		
All-*trans*-zeaxanthin	$y = 0.6689x - 0.0484$	0.9982	0.05	0.15
All-*trans*-β-cryptoxanthin	$y = 0.7869x - 0.0175$	0.9985	0.025	0.075
All-*trans*-β-carotene	$y = 0.9425x - 0.0293$	0.9949	0.025	0.075

[a] Based on standard curves prepared by plotting ratio of carotenoid standard to internal standard (β-*apo*-8′-carotenal) against its area ratio.
[b] DL: detection limit.
[c] QL: quantitation limit.

and illumination distance of 30 cm. After illumination, each standard solution was collected, filtered through a 0.22-μm membrane filter and 20 μL injected for HPLC analysis. The UV spectrum and mass spectrum of each peak of isomerized standards were compared with those of unknown peaks on the HPLC chromatogram of *L. barbarum* fruit extract.

2.5. Quantitation of carotenoids

Internal standard β-*apo*-8′-carotenal used for quantitation was dissolved in dichloromethane for a concentration of 1000 μg/mL. For preparation of standard curves, ten concentrations of all-*trans*-zeaxanthin (0.5, 1, 5, 10, 20, 30, 50, 100, 150 and 170 μg/mL), seven concentrations (0.1, 0.5, 1, 5, 10, 20 and 30 μg/mL) of all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene were prepared separately and mixed with β-*apo*-8′-carotenal for a final concentration of 15 μg/mL. Three standard curves were each prepared by plotting concentration ratio of carotenoid standard to internal standard against its area ratio. The regression equations and correlation coefficients (R^2) of the standard curves obtained for all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene are summarized in Table 1. Because of unavailability of commercial standards for *cis*-carotenoids, the quantitation of *cis*-isomers of zeaxanthin, β-cryptoxanthin and β-carotene were based on the standard curves of their corresponding all-*trans* forms, while neoxanthin was quantified by multiplying concentration of β-*apo*-8′-carotenal and peak area ratio of neoxanthin to β-*apo*-8′-carotenal. Similar to *cis*-carotenoids, an approximate quantification of semi-quantification was carried out for carotenoid esters.

2.6. Detection and quantitation limits

Three concentrations of 0.025, 0.05 and 0.1 μg/mL each for all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene were prepared and injected into HPLC. The detection limit (DL) calculated based on S/N ≧ 3 and quantitation limit (QL) based on S/N ≧ 10 are presented in Table 1.

2.7. Precision study

For reproducibility study, the intra-day variability was carried out by injecting 1 μg/mL of all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene separately three times for a total of nine times on the same day, while the inter-day variability was performed by injecting standards at the same concentration three times per week for a total of 3 weeks. The coefficient of variation (CV, %) of the intra-day variability for all-*trans* forms of zeaxanthin, β-cryptoxanthin and β-carotene were calculated to be 1.31, 1.58 and 2.27%, respectively, and the inter-day variability were 1.83, 2.18 and 3.46%.

2.8. Accuracy study

For recovery study, two concentrations of all-*trans*-zeaxanthin (20 and 80 μg/mL), all-*trans*-β-cryptoxanthin (10 and 40 μg/mL) and all-*trans*-β-carotene (5 and 20 μg/mL) were each added to 1 g powder sample for extraction and HPLC analysis. After quantitation, the recovery of each carotenoid was calculated based on the ratio of the concentration of carotenoid standard after and before HPLC analysis. Because of absence of commercial standards

Table 2
Tentative identification data, purities and contents of all-*trans* plus *cis* forms of saponified carotenoids in *Lycium barbarum* fruits

Peak no.	Carotenoid	Retention time (min)	λ (nm) (in-line)[a]				λ (nm) (reported)				Q-Ratio found [f]	Q-Ratio reported	Peak purity (%)	Content (μg/g)[h]
1	Neoxanthin	8.09		416	440	470		416	442	468[b]	0.33	–	98.1	11.9 ± 0.0
2	9- or 9′-*cis*-zeaxanthin	11.90	338	422	446	474	340	424	450	474[c]	0.18	–	99.1	30.4 ± 2.6
3	13- or 13′-*cis*-zeaxanthin	15.60	338	424	446	472	338	419	446	472[c]	0.37	–	91.8	4.4 ± 0.7
4	15- or 15′-*cis*-zeaxanthin	16.92	338	422	446	470	338	426	450	478[c]	0.45	–	97.9	29.6 ± 2.4
5	All-*trans*-zeaxanthin	19.52		426	450	478		425	454	478[d]	0.08	0.06[d]	99.8	1196.8 ± 13.1
IS [g]	β-*Apo*-8′-carotenal	27.03										–		
6	9- or 9′-*cis*-zeaxanthin	29.20	338	422	446	474	340	424	450	474[c]	0.12	–	90.8	5.1 ± 2.4
7	All-*trans*-β-cryptoxanthin	33.67		428	454	480		428	454	480[c]	0.16	–	99.6	48.1 ± 0.7
8	9- or 9′-*cis*-β-cryptoxanthin	35.26		430	452	–		424	450	476[c]	0.11	–	87.9	5.1 ± 0.0
9	13- or 13′-*cis*-β-carotene	37.65	342	422	448	474	344	422	446	476[e]	0.43	0.46[e]	93.1	8.1 ± 0.0
10	All-*trans*-β-carotene	40.18		426	456	480		430	458	482[e]	0.09	0.08[e]	99.3	15.0 ± 0.3
11	9- or 9′-*cis*-β-carotene	40.87	342	426	452	478	344	428	452	476[e]	0.13	0.13[e]	98.2	9.3 ± 0.2

[a] A gradient mobile phase of methanol–acetonitrile–water (81:14:5, v/v/v) and methylene chloride (100%) was used in this study.
[b] A mobile phase of methanol and 0.1% triethylamine/methyl-*tert*-butyl-ether was used by De Rosso and Mercadante [18].
[c] A mobile phase of acetonitrile–methanol–methylene chloride–hexane was used by Khachik et al. [17].
[d] A mobile phase of methanol–methylene chloride–2-propanol (89:1:10, v/v/v) was used by Tai and Chen [14].
[e] A gradient mobile phase of methanol–acetonitrile–water (84:14:2, v/v/v) and methylene chloride (100%) was used by Inbaraj et al. [13].
[f] *Q*-Ratio is defined as the height ratio of the *cis* peak to the main absorption peak.
[g] IS: internal standard.
[h] Average of duplicate analyses ± standard deviation.

Table 3
LC–MS–APCI data of carotenoids and their esters in *L. barbarum* fruits

Peak no.	Carotenoid	*m/z* found	*m/z* reported
1	Neoxanthin	601.4 $[M+H]^+$, 583.4 $[M+H-H_2O]^+$	601 $[M+H]^+$, 583 $[M+H-H_2O]^+$, 565 $[M+H-2H_2O]^+$, 547 $[M+H-3H]^+$, 509 $[M+H-92]^+$, 491 $[M+H-H_2O-92]^+$, 393, 221[a]
2	9- or 9′-*cis*-zeaxanthin	569.4 $[M+H]^+$, 551.4 $[M+H-H_2O]^+$	–
3	13- or 13′-*cis*-zeaxanthin	569.4 $[M+H]^+$	–
4	15- or 15′-*cis*-zeaxanthin	569.4 $[M+H]^+$, 551.4 $[M+H-H_2O]^+$	568 [M] [b]
5	All-*trans*-zeaxanthin	569.4 $[M+H]^+$, 551.4 $[M+H-H_2O]^+$	569 $[M+H]^+$, 551 $[M+H-H_2O]^+$, 533 $[M+H-2H_2O]^+$, 463 $[M+H-106]^+$[a]
6	9- or 9′-*cis*-zeaxanthin	569.4 $[M+H]^+$, 551.4 $[M+H-H_2O]^+$	–
7	All-*trans*-β-cryptoxanthin	553.4 $[M+H]^+$, 535.4 $[M+H-H_2O]^+$	553 $[M+H]^+$, 535 $[M+H-H_2O]^+$, 461 $[M+H-92]^+$[a]
8	9- or 9′-*cis*-β-cryptoxanthin	553.4 $[M+H]^+$	–
9	13- or 13′-*cis*-β-carotene	537.3 $[M+H]^+$	537 $[M+H]^+$, 444 $[M+H-92]^+$[a]
10	All-*trans*-β-carotene	537.4 $[M+H]^+$	537 $[M+H]^+$, 444 $[M+H-92]^+$[a]
11	9- or 9′-*cis*-β-carotene	537.4 $[M+H]^+$	537 $[M+H]^+$, 444 $[M+H-92]^+$[a]
12	Zeaxanthin monopalmitate	807.7 $[M+H]^+$, 551.4 $[M+H-FA]^+$[e]	807.7 $[M+H]^+$, 551.4 $[M+H-FA]^+$[c]
13	Zeaxanthin monopalmitate	807.7 $[M+H]^+$, 551.4 $[M+H-FA]^+$	807.7 $[M+H]^+$, 551.4 $[M+H-FA]^+$[c]
14	β-Cryptoxanthin monopalmitate	791.6 $[M+H]^+$	790 $[M]^+$[d]
15	Zeaxanthin monopalmitate	807.7 $[M+H]^+$, 551.4 $[M+H-FA]^+$	807.7 $[M+H]^+$, 551.4 $[M+H-FA]^+$[c]
16	β-Cryptoxanthin monopalmitate	791.6 $[M+H]^+$	790 $[M]^+$[d]
17	β-Cryptoxanthin monopalmitate	791.5 $[M+H]^+$	790 $[M]^+$[d]
18	Zeaxanthin dipalmitate	1045.9 $[M+H]^+$, 789.7 $[M+H-FA]^+$, 533.4 $[M+H-2FA]^+$	1045.9 $[M+H]^+$, 789.7 $[M+H-FA]^+$, 533.4 $[M+H-2FA]^+$[c]

[a] Based on a report by De Rosso and Mercadante [18].
[b] Updike and Schwartz [20].
[c] Weller and Breithaupt [11].
[d] Wingerath et al. [19].
[e] FA, fatty acid.

for *cis*-carotenoids, the quantitation of *cis*-carotenoids was based on the recovery of all-*trans*-carotenoids. Without saponification, the recoveries of all-*trans* forms of zeaxanthin, β-cryptoxanthin and β-carotene were 92, 92 and 87%, respectively, and were used for quantitation of carotenoid esters, but with saponification, the recoveries dropped to 83, 74 and 74%, which should be caused by prolonged saponification time. However, when *cis*-isomers of carotenoids were taken into account, the recoveries of all-*trans* forms of zeaxanthin, β-cryptoxanthin and β-carotene rose to 88, 80 and 85% respectively, indicating a portion of all-*trans*-carotenoids were converted into their corresponding *cis*-isomers during saponification. Thus, the quantitation of each all-*trans*-carotenoid was based on the recovery of all-*trans* plus *cis*-forms of each carotenoid.

2.9. Fatty acid composition in carotenoid esters

A method described by Coral-Hinostroza and Bjerkeng [15] was modified to determine fatty acid composition in carotenoid esters. One milliliter of unsaponified carotenoid extract from the powdered fruit sample of *L. barbarum* was mixed with 2 mL of benzene, 2 mL of 0.5 M methanolic hydrochloric acid solution and 0.2 mL of 2,2-dimethoxypropane in a vial, after which the mixture was kept at room temperature for reaction to proceed until 12 h and then 4 mL of 6% sodium bicarbonate aqueous solution was added to terminate the reaction. Hexane (15 mL) was then added for extraction of fatty acid methyl esters and the upper layer was collected. The procedure was repeated three times and all the supernatants were combined for evaporation to dryness. The residue was dissolved in 1-mL of hexane, filtered through a 0.22-μm membrane filter, and 1 μL was injected into GC for fatty acid analysis. An Agilent high-polar HP-88 column (100 m × 0.25 mm i.d., 0.2 μm film thickness) was used to separate the various fatty acids with flame ionization detection and the following temperature programming was used: 170 °C initially, maintained for 24 min, raised to 220 °C at 7.5 °C/min, to 230 °C at 10 °C/min, and maintained for 5 min. The injector temperature was 240 °C and the detector temperature 250 °C, with flow rate of nitrogen gas at 3 mL/min and split-ratio at 10:1. The various fatty acids in carotenoid esters were identified by comparing retention times of unknown peaks with reference standards and cochromatography with added standards.

2.10. Statistical analysis

All the data were subjected to analysis of variance using one-way ANOVA and Duncan's multiple range test for significance ($\alpha = 0.05$) evaluation based on a software system by SAS [16].

3. Results and discussion

Initially, a HPLC gradient mobile phase system developed by Inbaraj et al. [13] was used for separation of carotenoids in *L. barbarum*. However, this method failed to resolve carotenoids in *L. barbarum* fruits, which should be due to the presence of a different variety of carotenoids. After numerous studies, a new HPLC gradient solvent system was developed: 16% dichloromethane (A) and 84% methanol–acetonitrile–water (84:14:5, v/v/v) (B) initially, increased to 17% A in 22 min, 55% A in 40 min, 75% A in 55 min and returned to 16% A in 60 min. A total of 11 free carotenoids and 7 carotenoid esters were separated within 51 and 41 min, respectively, in unsaponified and saponified extracts with column temperature at 25 °C, flow rate at 1 mL/min and detection wavelength at 450 nm. Fig. 1A shows the HPLC chromatogram of carotenoid extracts from dried *L. barbarum* fruits with saponification for 6 h. After comparing UV spectra, retention times and mass spectra of unknown peaks with those in the literature [11–14,17–20], a total of 11 carotenoids, including neoxanthin (peak 1), 9- or 9′-*cis*-zeaxanthin (peak 2), 13- or 13′-*cis*-zeaxanthin (peak 3), 15- or 15′-*cis*-zeaxanthin (peak 4), all-*trans*-zeaxanthin (peak 5), 9- or 9′-*cis*-zeaxanthin (peak 6), all-*trans*-β-cryptoxanthin (peak 7), 9 or 9′-*cis*-β-cryptoxanthin (peak 8), 13- or 13′-*cis*-β-carotene (peak 9), all-*trans*-β-carotene

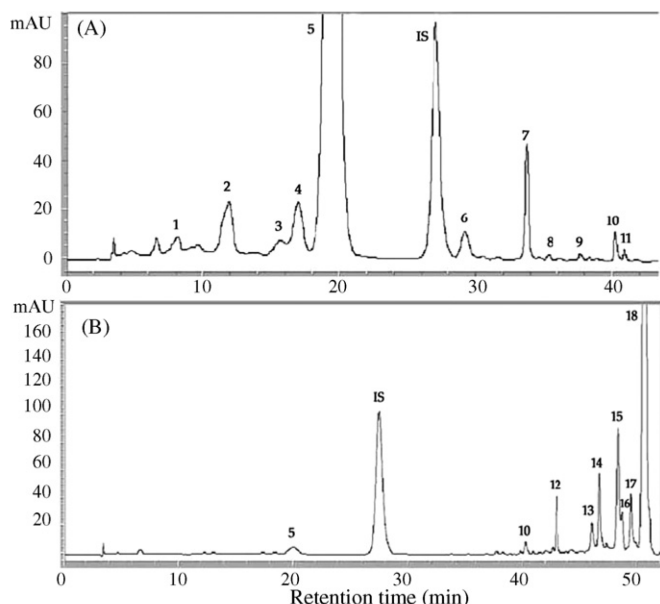

Fig. 1. HPLC chromatogram of saponified (A) and unsaponified (B) carotenoid extracts from dried *Lycium barbarum* fruits. Peaks: 1, neoxanthin; 2, 9- or 9′-*cis*-zeaxanthin; 3, 13- or 13′-*cis*-zeaxanthin; 4, 15- or 15′-*cis*-zeaxanthin; 5, all-*trans*-zeaxanthin; 6, 9- or 9′-*cis*-zeaxanthin; 7, all-*trans*-β-cryptoxanthin; 8, 9- or 9′-*cis*-β-cryptoxanthin; 9, 13- or 13′-*cis*-β-carotene; 10, all-*trans*-β-carotene; 11, 9- or 9′-*cis*-β-carotene; 12, zeaxanthin monopalmitate; 13, zeaxanthin monopalmitate; 14, β-cryptoxanthin monopalmitate; 15, zeaxanthin monopalmitate; 16, β-cryptoxanthin monopalmitate; 17, β-cryptoxanthin monopalmitate; 18, zeaxanthin dipalmitate; IS: β-*apo*-8′-carotenal.

(peak 10) and 9- or 9′-*cis*-β-carotene (peak 11) were identified (Tables 1 and 2). The retention factor (k) value ranged from 1.38 to 10.99, indicating that a proper solvent strength was controlled, while separation factor (α) value from 1.02 to 1.80 implied that an appropriate selectivity of solvent system to sample components was attained. Table 2 also presents the purities and contents of carotenoids in saponified *L. barbarum* fruit extract. With the exception of 9- or 9′-*cis*-β-cryptoxanthin (87.9%), the peak purities of all the carotenoids (90.8–99.8%) were >90%. All-*trans*-zeaxanthin was found to be present in largest amount (1196.8 μg/g), followed by *cis*-zeaxanthin (69.5 μg/g), all-*trans*-β-cryptoxanthin (48.1 μg/g), *cis*-β-carotene (17.4 μg/g), all-*trans*-β-carotene (15.0 μg/g), neoxanthin (11.9 μg/g) and *cis*-β-cryptoxanthin (5.1 μg/g).

Fig. 1B shows the HPLC chromatogram of carotenoid extracts from dried *L. barbarum* fruits without saponification. Carotenoid esters were identified based on the fragment ions at m/z 551.4 [M+H – 256.2], 535.4 [M+H – 256.2] and 533.5 [M+H – 512.4], due to the loss of one or two palmitic acids (MW 256.4 for palmitic acid) from the molecular ions at m/z 807.6, 791.6 and 1045.9 for zeaxanthin monopalmitate, β-cryptoxanthin monopalmitate and zeaxanthin dipalmitate, respectively. In addition, the mass spectra were compared with those reported in the literature [11,18–20] and a total of two free carotenoids and seven carotenoid esters, namely, all-*trans*-zeaxanthin (peak 5), all-*trans*-β-carotene (peak 10), three zeaxanthin monopalmitates (peaks 12, 13 and 15), three β-cryptoxanthin monopalmitates (peaks 14, 16 and 17) and one zeaxanthin dipalmitate (peak 18) were resolved and identified (Tables 2 and 3; Fig. 2). On the basis of GC analysis, four fatty acids including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) were present in oil-soluble portion of *L. barbarum* fruit extract (chromatogram not shown). Table 4 also presents the purities and contents of unsaponified carotenoids in *L. barbarum* fruits. Compared to saponified carotenoids, carotenoid esters showed a much larger k value

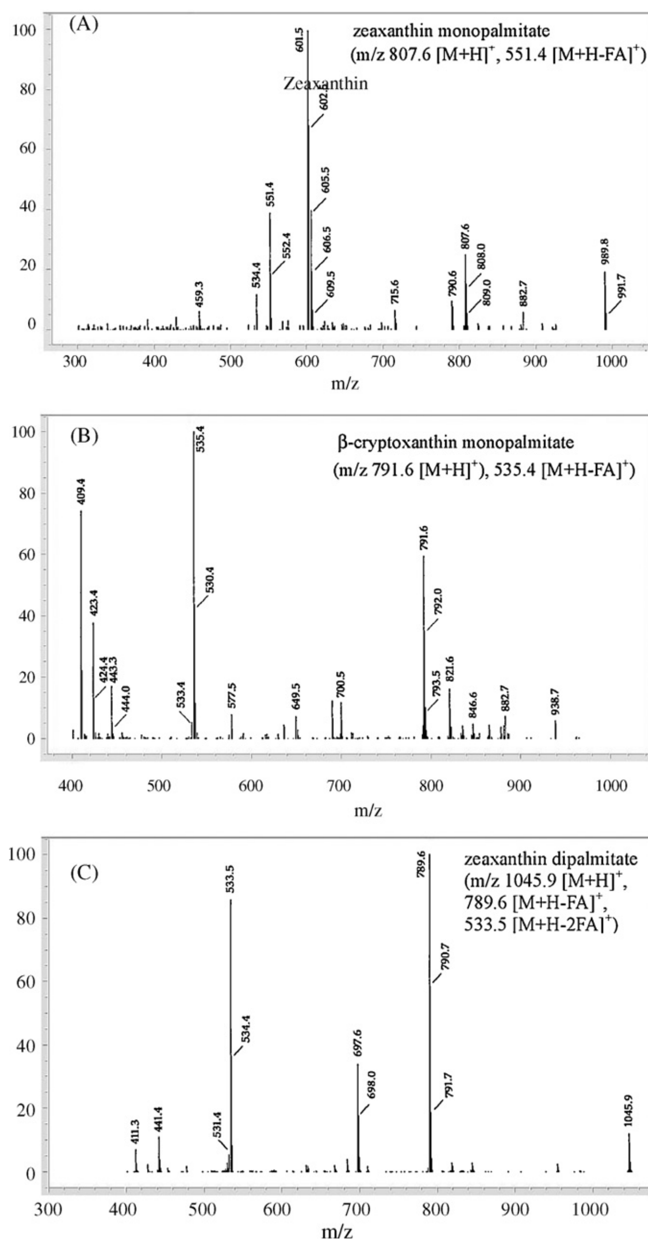

Fig. 2. Mass spectra of carotenoid esters in unsaponified fruit extract of *L. barbarum*. FA, fatty acid.

(4.86–13.83), which should be caused by their low polarity resulting in a greater interaction with the C30 stationary phase. The α values and peak purities of all the carotenoid esters ranged from 1.01 to 1.08 and 99.3 to 99.9%, respectively. Zeaxanthin dipalmitate was found to be present in largest amount (1143.7 μg/g), followed by β-cryptoxanthin monopalmitate (165.8 μg/g), zeaxanthin monopalmitate (86.0 μg/g), all-*trans*-β-carotene (23.7 μg/g) and all-*trans*-zeaxanthin (1.4 μg/g).

Table 5 shows the contents (μg/mL) of carotenoids in *L. barbarum* fruits as affected by saponification time. Saponification is often necessary to remove chlorophylls, lipids, water-soluble impurities and de-esterify carotenoid esters during extraction of free carotenoids. According to AOAC method [21], both hot saponification (56 °C, 20 min) and cold saponification (25 °C, 16 h) can be used to extract free carotenoids, but the former may result in isomerization and degradation of carotenoids and the latter is lengthy.

Table 4
Tentative identification data, purities and contents of all-*trans* plus *cis* form of unsaponified carotenoids in *L. barbarum* fruits

Peak no.	Carotenoid	Retention time (min)	λ (nm) (in-line)[a]			λ (nm) (reported)			Q-Ratio found	Q-Ratio reported	Peak purity (%)	Content (μg/g)[f]
5	All-*trans*-zeaxanthin	19.97	426	450	478	425	454	478[b]	0.08	0.06 [b]	99.0	1.4 ± 0.1
IS[e]	β-*Apo*-8′-carotenal	27.45										
10	All-*trans*-β-carotene	40.20	426	456	480	430	458	482[c]	0.09	0.08 [c]	98.7	23.7 ± 0.3
12	Zeaxanthin monopalmitate	42.95	432	456	482		451[d]		0.16	–	99.9	11.9 ± 0.3
13	Zeaxanthin monopalmitate	46.07	422	446	474		451[d]		0.22	–	99.9	11.3 ± 0.1
14	β-Cryptoxanthin monopalmitate	46.69	432	456	482		450[d]		0.16	–	99.9	64.4 ± 0.0
15	Zeaxanthin monopalmitate	48.31	428	452	476		451[d]		0.17	–	99.6	62.8 ± 0.2
16	β-Cryptoxanthin monopalmitate	48.64	432	456	482		450[d]		0.16	–	99.7	32.9 ± 0.5
17	β-Cryptoxanthin monopalmitate	49.43	432	456	484		450[d]		0.17	–	99.3	68.5 ± 0.5
18	Zeaxanthin dipalmitate	50.57	432	456	482		451[d]		0.14	–	99.8	1143.7 ± 9.7

[a] A gradient mobile phase of methanol–acetonitrile–water (81:14:5, v/v/v)and methylene chloride (100%) was used.
[b] A mobile phase of methanol–methylene chloride–2-propanol (89:1:10, v/v/v) was used by Tai and Chen [14].
[c] A gradient mobile phase of methanol–acetonitrile–water and methylene chloride was used by Inbaraj et al. [13].
[d] A mobile phase of methanol/acetonitrile/dichloromethane/*n*-hexane was used by Wingerath et al. [19].
[e] IS: internal standard.
[f] Average of duplicate analyses ± standard deviation.

In a later study, Chen and Yang [22] reported that by combining extraction and saponification for 16 h, the yield of carotenoids was substantially increased. Chen et al. [23] further demonstrated that for simultaneous extraction and saponification of free carotenoids in Taiwanese mango, a time length of 12 h was adequate for cold saponification. However, in our study, a large amount of carotenoid esters were still present after simultaneous extraction and saponification for 12 h (Table 5). This outcome implied that the simultaneous extraction and saponification method was not applicable to extract free carotenoids in *L. barbarum* fruits. Thus, the extraction and saponification steps should be carried out separately for *L. barbarum* samples. The contents of carotenoid esters were shown to decrease gradually following a rise in saponification time (Table 5). No carotenoid esters were detected after saponification time reached 6 and 8 h. Nevertheless, a longer saponification time (8 h) resulted in a lower yield of carotenoids than that for 6 h, probably because of carotenoid degradation after prolonged saponification. Therefore, the most appropriate saponification time for free carotenoids in *L. barbarum* fruits was selected to be 6 h. We have to point out here that the free carotenoid contents in Table 5 were different from that in Table 2 for the same saponification time (6 h), simply because a different batch of sample was used for the latter.

For further identification of *cis*-carotenoids, all-*trans* forms of zeaxanthin, β-cryptoxanthin and β-carotene were illuminated at 2000–3000 lx for 12 h to promote formation of carotenoid isomers. Illuminated carotenoid standards containing different isomers were separated on a C30 column using the same HPLC gradient mobile phase system (chromatograms not shown). By comparing UV spectra and *Q*-ratios of unknown peaks with reference values in the literature [13,14,17,23], five *cis*-isomers of all-*trans*-zeaxanthin, including two 9- or 9′-*cis*-zeaxanthins, one unidentified mono-*cis*-zeaxanthin, 13- or 13′-*cis*-zeaxanthin and 15- or 15′-*cis*-zeaxanthin were tentatively identified. Similarly, six *cis*-isomers of all-*trans*-β-cryptoxanthin, including one 15- or 15′-*cis*-β-cryptoxanthin, two 13- or 13′-*cis*-β-cryptoxanthins, two *cis*-isomers of β-cryptoxanthin, and one 9- or 9′-*cis*-β-cryptoxanthin were identified. Various *cis*-isomers of all-*trans*-β-carotene identified include four unidentified mono-*cis*-β-carotenes, 15- or 15′-*cis*-β-carotene, 13- or 13′-*cis*-β-carotene and 9- or 9′-*cis*-β-carotene. The mass spectra of all these isomers were also compared with those of unknown peaks on the HPLC chromatogram for both unsaponified and saponified carotenoid extracts of *L. barbarum* fruits, with the former containing no carotenoid isomers and the latter containing two 9- or 9′-*cis*-zeaxanthins (peaks 2 and 6), 13- or 13′-*cis*-zeaxanthin (peak 3), 15- or 15′-*cis*-zeaxanthin (peak 4), 9-

Table 5
Contents (μg/g) of carotenoids in fruits of *L. barbarum* as affected by extraction and saponification

Peak no.	Carotenoid	Simultaneous extraction and saponification (12 h)	Extraction followed by saponification for varied length of time			
			2 h	4 h	6 h	8 h
1	Neoxanthin	6.1 ± 0.2[b]	11.3 ± 0.8[a]	10.2 ± 0.7[a]	12.1 ± 1.2[a]	11.8 ± 1.1[a]
2	9- or 9′-*cis*-zeaxanthin	20.6 ± 1.1[b]	33.6 ± 6.0[a]	31.9 ± 4.6[a]	32.7 ± 2.9[a]	34.6 ± 3.5[a]
3	13- or 13′-*cis*-zeaxanthin	10.5 ± 0.5[a]	9.3 ± 0.5[bc]	10.4 ± 0.5[ab]	7.2 ± 0.3[d]	8.8 ± 0.4[c]
4	15- or 15′-*cis*-zeaxanthin	12.3 ± 0.6[b]	32.8 ± 3.8[a]	29.5 ± 2.4[a]	30.8 ± 3.3[a]	33.8 ± 3.9[a]
5	All-*trans*-zeaxanthin	419.8 ± 9.4[e]	753.4 ± 10.6[d]	859.7 ± 11.6[c]	1056.4 ± 13.1[a]	938.1 ± 14.2[b]
6	9- or 9′-*cis*-zeaxanthin	9.8 ± 1.1[b]	16.7 ± 1.8[a]	14.9 ± 1.3[a]	8.8 ± 0.5[b]	15.4 ± 1.5[a]
7	All-*trans*-β-cryptoxanthin	18.6 ± 2.0[b]	41.8 ± 2.6[a]	47.6 ± 3.8[a]	45.1 ± 3.0[a]	41.3 ± 4.3[a]
8	9- or 9′-*cis*-β-cryptoxanthin	1.8 ± 0.1[b]	2.0 ± 0.2[b]	2.1 ± 0.2[b]	3.5 ± 0.1[a]	2.0 ± 0.1[b]
9	13- or13′-*cis*-β-carotene	7.2 ± 0.9[a]	7.4 ± 0.8[a]	6.9 ± 0.6[a]	7.6 ± 0.8[a]	7.1 ± 0.6[a]
10	All-*trans*-β-carotene	15.4 ± 1.2[a]	17.3 ± 2.2[a]	17.1 ± 1.8[a]	16.0 ± 1.0[a]	15.7 ± 0.7[a]
11	9- or 9′-*cis*-β-carotene	7.1 ± 0.3[a]	8.3 ± 0.7[a]	8.0 ± 0.7[a]	8.0 ± 0.4[a]	8.0 ± 0.6[a]
12	Zeaxanthin monopalmitate	30.3 ± 2.0[a]	26.7 ± 2.2[b]	5.7 ± 0.2[c]	ND	ND
13	Zeaxanthin monopalmitate	6.0 ± 0.2[a]	5.5 ± 0.2[a]	ND	ND	ND
14	β-Cryptoxanthin monopalmitate	6.1 ± 0.4[a]	ND	ND	ND	ND
15	Zeaxanthin monopalmitate	9.2 ± 0.6[a]	ND	ND	ND	ND
16	β-Cryptoxanthin monopalmitate	4.4 ± 0.6[a]	ND	ND	ND	ND
17	β-Cryptoxanthin monopalmitate	4.3 ± 0.4[a]	ND	ND	ND	ND
18	Zeaxanthin dipalmitate	135.1 ± 6.7[a]	ND	ND	ND	ND

[a–e]Symbols bearing different letters in the same row are significantly different ($p < 0.05$); ND: not detected.

or 9′-*cis*-β-cryptoxanthin (peak 8), 13- or 13′-*cis*-β-carotene (peak 9) and 9- or 9′-*cis*-β-carotene (peak 11).

Comparatively, both zeaxanthin and β-cryptoxanthin esters dominated in unsaponified extracts of *L. barbarum* samples, whereas free zeaxanthin and β-cryptoxanthin were mainly present in saponified extracts. Several reports have also revealed the presence of carotenoid esters in fruits of *L. barbarum* [9–11]. However, only three carotenoid esters, zeaxanthin monopalmitate, zeaxanthin dipalmitate and β-cryptoxanthin monopalmitate were separated and identified by TLC and no quantitation was performed [9]. Moreover, the identification can only be tentative as no mass spectra were determined. Similarly, in another study, a total of ten carotenoids were identified and quantified in fruits of *L. barbarum* by HPLC without mass spectra determination [10]. Recently, Weller and Breithaupt [11] reported the presence of only one zeaxanthin dipalmitate in *L. barbarum* fruits by LC–MS, which remained inadequate. In the present study, by determining the mass spectrum of each carotenoid ester by LC–MS–APCI technique, the fatty acids in carotenoid esters were positively identified (Table 3). The *m*/*z* values of free carotenoids and carotenoid esters reported in several previous studies [11,18–20] were used for confirmation.

4. Conclusion

An HPLC–DAD–APCI–MS method was developed to separate the free carotenoids and their esters in fruits of *L. barbarum* by employing a C30 column and a gradient mobile phase. A total of two free carotenoids and seven carotenoid esters were identified in the unsaponified fraction by HPLC–DAD–APCI–MS and GC–FID techniques, with zeaxanthin dipalmitate present in the largest amount, followed by β-cryptoxanthin monopalmitate, zeaxanthin monopalmitate, β-carotene and zeaxanthin. This method may be applied to analyze free carotenoid and carotenoid esters in both foods and nutraceuticals.

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