Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 106 (2008) 410–414
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Separation and identification of the yellow carotenoids in *Potamogeton crispus* L.

Dandan Ren [a,*], Shenghua Zhang [b]

[a] *Food Engineering Department, Dalian Fisherish University, Dalian 116023, PR China*
[b] *College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China*

Received 12 October 2006; received in revised form 21 May 2007; accepted 29 May 2007

Abstract

It was found that ducks fed with *Potamogeton crispus* L. could lay natural red-yolk eggs with good quality in vast lake areas. *P. crispus* L. was found to be a new resource of carotenoids based on this fact. Carotenoids from *P. crispus* L. were extracted using petroleum ether-acetone and saponified using 40% methanolic potassium hydroxide. Four major yellow carotenoid pigments were obtained by a MgO column and thin-layer chromatography. These carotenoids were identified as neoxanthin, violaxanthin, lutein and β-carotene based on visible spectra in different solvents compared with values reported in the literature, functional group tests and mass spectrum by LC-MS. The content of total carotenoids from *P. crispus* L. was measured to be 231 μg/g (dry weight) by visible absorption spectroscopy.

Keywords: *Potamogeton crispus* L.; Carotenoids; Separation; Identification

1. Introduction

Carotenoids are important biological compounds that are present in all green tissues of higher plants. Carotenoids have many functions such as antioxidation, immunity-regulation, inhibiting proliferation of tumor cells etc. The applications of carotenoids in medicine and cosmetics have been well documented as is their utilization as food additives (colorants, antioxidants).

Potamogeton crispus L. (*P. crispus*) is a submersed herbaceous perennial plant, which could be found in freshwater lakes, ponds, paddy fields and rivers all over the China. It is an important primary producer in freshwater ecosystems, providing good fodder source for herbivorous fishes and poultry (Jian, Li, Wang, & Chen, 2003). We found that ducks fed with *P. crispus* could lay natural red-yolk eggs with good quality. It is known that the color of egg yolk is closely related with the type and concentration of carotenoids in feed. Chemical analytical study and feeding trials proved that carotenoids in the red-yolk eggs mainly derived from *P. crispus* (Wang et al., 2003). *P. crispus* is a new resource of carotenoids, while very few reports were about the identification and classification of carotenoids in *P. crispus*. The red carotenoids in *P. crispus* were separated and identified by thin layer chromatography (TLC), HPLC-MS/MS, Raman micro-spectroscopy, FT-IR sepectroscopy and high resolution EI-MS in our lab. The results showed that the red carotenoids were rhodoxanthin and its *cis*/*trans* isomers (Liu, Zhang, Peng, Wang, & Zhang, 2004; Wang et al., 2004).

Nowadays methods for carotenoids isolation and analysis include thin-layer chromatography (TLC), open column chromatography (OCC) and high-performance liquid chromatography (HPLC). For identification and structure elucidation, visible spectrophotometry, NMR and mass spectrometry are the most recommended (Azevedo-Meleiro & Rodriguez-Amaya, 2004; de Sá & Rodriguez-Amaya, 2003; Hodisan, Socaciu, Ropan, & Neamtu, 1997; Lacker, Strohschein, & Albert, 1999; Oliver & Palou, 2000).

* Corresponding author. Tel.: +86 411 84763559.
E-mail address: rendandanliu@yahoo.com.cn (D. Ren).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.05.074

2. Material and methods

2.1. Chemicals

β-carotene standard and diatomaceous earth are purchased from Sigma Company, USA. Most of solvents and chemicals were of analytical grade (Magnesium oxide, sodium sulfate anhydrous, anhydrous sodium carbonate, methanol, petroleum ether, acetone, anhydrous ethanol, acetone, chloroform, *n*-hexane, carbon bisulfide, BHT). Methanol was of HPLC grade purchased from Fisher Chemicals Company, USA. Silica gel G60 for thin-layer chromatography was obtained from Qingdao Haiyang chemical company, China.

2.2. Sample extraction and saponification

Carotenoids in *P. crispus* were extracted with a mixture of acetone and petroleum ether (1:1, v/v) at the room temperature until colorless. After washed for several times with water, the upper phase was collected and combined as crude extract. The crude extract was filtered, evaporated to dryness in a rotavapor and resuspended in petroleum ether. Saponification was carried out in ethereal solution by adding 40% w/v KOH in methanol to a final concentration of 4% w/v KOH. The mixture was kept in the dark for 12 h at room temperature. The extract was washed several times with water until all the KOH is removed. The total carotenoids from *P. crispus* (TCPC) was evaporated on a rotavapor and dissolved in a little petroleum ether.

2.3. Quantitative evaluation of TCPC

The total content of TCPC was determined by spectrophotometry ($\lambda = 450$ nm) and calculated by following formula: TCPC amount (mg) = optical density value × volume (mL) × dilute times × 10÷2500 (mean value $A^{1\%}_{1\ cm} = 2500$ of colored carotenoids).

2.4. Preparation of fractionations of TCPC

An alternative way to characterize the carotenoid composition of *P. crispus* involved a preliminary separation of TCPC on an open column of Magnesium oxide-Diatomaceous Earth (1:2, w/w, activated for 2 h at 110 °C). Small volumes of TCPC (2–3 mL) were put onto the top of column and eluted successively with three solvent systems: 96% petroleum ether and 4% acetone (Band 1); petroleum ether:acetone:methanol (85:15:1, v/v/v) (Band 2) and petroleum ether:acetone:methanol (85:15:2, v/v/v) (Band 3). Each Band was collected separately, evaporated, dissolved in petroleum ether or acetone.

Each band eluted from the MgO column was further purified with TLC plate, using silica gel as adsorbent and developing with mixed solvent, i.e. hexane–ethyl acetate–acetone–methanol (27:4:2:2, v/v/v/v). Then the pigment bands obtained by TLC were marked and scraped into test tubes and dissolved with acetone, and filtered at reduced pressure, respectively. The acetone extract was evaporated to dry under a stream of nitrogen, residues dissolved with a bit of solvents and prepared for analysis. Through the isolation and purification as above methods, the four yellow fractions y_1, y_2, y_3, and y_4 were obtained according to their R_f values at TLC plates.

2.5. Purity of the fractions

Purity of the fractions was checked by reverse phase HPLC. The total carotenoids from *P. crispus* were also checked by HPLC in order to show the proportion of each carotenoid. The HPLC analysis was carried out with a Varian separation module (model 210) equipped with an autosampler (model 410) and a UV–VIS photodiode array detector (varian model 350). A Shim-pack VP-ODS 150 mm × 4.6 i.d. column (Shimadzu co., Kyoto, Japan) was used.

The mobile phase used in gradient separations included solvent A and solvent B. solvent A was methanol–water (90:10, v/v) containing 0.1% triethylamine solvent. Solvent B was ethyl acetate. The mixture of solvents A and B was programmed as follows: 0–20 min (B, from 0 to 80%, 0.8 mL/min), 20–40 min (B, from 80 to 90%, 1 mL/min), 40–45 min (B, from 90 to 0%, 1 mL/min) and 45–50 min (100% A, 1 mL/min). The flow rate was 0.8 mL/min in the first 20 min, while 1.0 mL/min in the last 30 min. The absorbance of the effluent was monitored at a wavelength of 450 nm.

2.6. Identification of yellow carotenoids from P. crispus

The red pigments had been identified as rhodoxanthin and its *cis*/*trans* isomers by our lab (Wang et al., 2004), so we would identify other major yellow carotenoid pigments hereinafter.

The total extract and purified fractions were analysised by TLC and their R_f values were measured. The developing solvent of TLC was a mixture of hexane–ethyl acetate–acetone–methanol (27:4:2:2, v/v/v/v). Visible spectroscopic data for carotenoids from *P. crispus* in different solvents were measured by UV–VIS Spectrophotometer.

Some tests for particular functional groups in carotenoids such as epoxide groups and aldehyde or keto groups were carried out in a UV–VIS spectrophotometer cuvette and monitored for diagnostic changes in the spectrum (Britton, 1995; Vetter, Englert, Rigassi, & Schwieter, 1971). Those tests including acid-catalysed epoxide-furanoid rearrangement and reduction of aldehydes and ketones with $NaBH_4$ were carried out to test if there was any functional group or not.

LC-MS (MSD, Agilent, waldbronn, Germany) was also used for positive identification and with the following parameters: elcetrospray ionization (ESI) with a drying gas flow of 6.0 L/min, nebulizer pressure of 12.0 psi and

drying gas temperature of 250 °C. The ionizing voltage was 70 eV. The detection was done in positive mode in the mass range of 100–700 AMU. For LC, Zorbax SB-C_{18}column (150 mm × 2.1 i.d.) was used and the mobile phase was acetonitrile–methanol (70:30, v/v) containing 0.02 mol/L ammonium acetate in methanol part. The flow rate was 0.2 mL/min and the absorbance was measured at a wavelength of 450 nm.

3. Results and discussion

3.1. Purity of the isolated fractions

The HPLC chromatograms of the isolated carotenoids fractions showed that each fraction has a single peak. Photodiode array measurements of spectral properties for the individual peaks were determined at the upslope, apex and downslope. The matching of the three spectra indicated the degree of peak purity. The results showed the purity of fractions was highly satisfactory.

3.2. TLC of carotenoids from P. crispus

The thin layer chromatogram of TCPC and B_1, B_2 and B_3 obtained from TCPC by column chromatography showed that five fractions were separated from B_2 by silica-TLC. It was found that the main composition of B_1 was y_4, B_2 included y_1, y_2 and y_3 etc. fractions and B_3 included r_1, r_2 and r_3 which were identified as rhodoxanthin and its *cis*/*trans* isomers by our lab (Wang et al., 2004).

It was found that there was a single spot in the TLC of each fraction by the thin layer chromatogram of TCPC and fractions y_1, y_2, y_3 and y_4. Their R_f values are 0.087, 0.160 0.247 and 0.960 respectively. The R_f value of y_4 matched the value of β-carotene standard very well. So y_4 was likely β-carotene.

3.3. Visible spectra of carotenoids from P. crispus

The visible spectra of carotenoids y_1, y_2, y_3 and y_4 from *P. crispus* showed three more-or-less distinct peaks, which was the typical spectrum of carotenoids. Table 1 showed that the visible spectra data for the carotenoids of *P. crispus* separated by TLC. the wavelengths of maximum absorption and spectral fine structural values (%III/II) obtained from this study agreed well with those of the literature (Britton, 1995).

3.4. Chemical reactions of carotenoids from P. crispus

The aldehyde or keto group test showed that there was no aldehyde or keto group in y_1, y_2, y_3 and y_4. As shown in Table 1, the results showed that there was no epoxide group in y_3 and y_4, one epoxy group in y_1. A hypsochromic shift of 40 nm on addition of HCl confirmed the presence of two epoxides in y_2. According to those studies above, we presumed that y_1, y_2, y_3 and y_4 were neoxanthin, violaxanthin, lutein and β-carotene, respectively.

3.5. LC-MS of carotenoids from P. crispus

The HPLC-ESI-MS of carotenoids from *P. crispus* y_1, y_2, y_3 and y_4 were shown in Fig. 1a–d, respectively.

The molecular ions and the mass fragments are in Table 2. The identification of each carotenoid was described in detail as follows:

y_1-*Neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol)*: In Fig. 1a, in positive ion mode, the *m*/*z* 601 ion corresponding to the $[M + H]^+$ was the most prominent ion in the mass spectrum of y_1 in full scan mode. So we could presume that the relative molecular weight of y_1 was 600. Other ions in positive ionization mode were the sodium adduct of the ion $[M + Na]^+$ (*m*/*z* 623) and also the dehydrated protonated ions

Table 1
Visible absorption spectra and epoxide test of carotenoids from *P. crispus*

Group	Solvent	λ_{max} (nm)	%III/II	Hypsochromic shift (nm)	Results of epoxide test	Tentative identification
y_1	Chloroform	423 447 476	–	17	Monoepoxide	Neoxanthin
	Ethanol	415 437 466	82			
	CS_2	463 494	–			
	Acetone	416 439 468	85			
y_2	Chloroform	426 450 479	–	40	Diepoxide	Violaxanthin
	Ethanol	417 440 470	96			
	CS_2	446 470 499	–			
y_3	Chloroform	432 455 485	–	None	Nonepoxide	Lutein
	Ethanol	423 445 474	61			
	CS_2	474 505	–			
y_4	Chloroform	462 487	–	None	Nonepoxide	β-Carotene
	Ethanol	448 473	25			
	Pertoleum ether	449 476	24			
	Acetone	453 479	15			

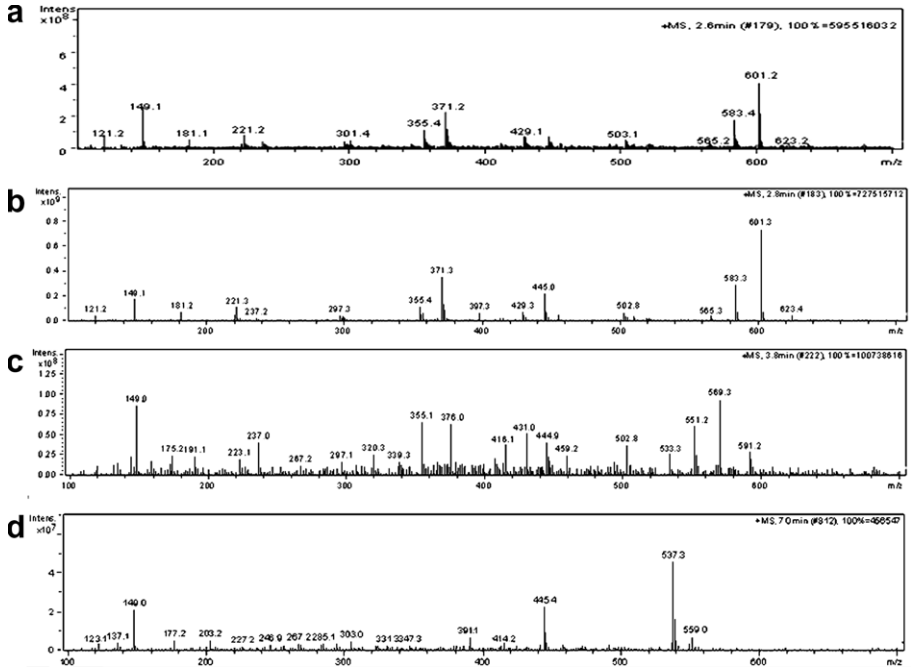

Fig. 1. HPLC-ESI-MS of carotenoids from *P. crispus*. Elcetrospray ionization (ESI) with a drying gas flow of 6.0 L/min, nebulizer pressure of 12.0 psi and drying gas temperature of 250 °C. The ionizing voltage was 70 eV. The detection was done in positive mode in the mass range of 100–700 AMU. The mass spectrums of y_1, y_2, y_3 and y_4 were shown in a–d, respectively.

Table 2
Molecular ions and characteristic fragments of the carotenoids from *P. crispus*

Carotenoid	*m/z*	
	Molecular ion $[M + H]^+$	Fragments
Neoxanthin	601	583, 565, 221,181
Violaxanthin	601	583, 565, 221, 181
Lutein	569	551, 533, 416, 376
β-Carotene	537	445, 203, 177, 149, 137, 123

$[M + H–H_2O]^+$ (*m/z* 583) and $[M + H–2H_2O]^+$ (*m/z* 565). The fragments at *m/z* 221 and 181 were due to the elimination of a β-ring with epoxide and hydroxyl groups.

y_2-*Violaxanthin (5,6,5′,6′-diepoxy-5,6,5′, 6′-tetrahydro-β,β-carotene-3,3′-diol)*: Fig. 1b showed the ESI+ mass spectra of y_2. The mass spectrum revealed the relative molecular weight of y_2 was 600 according to *m/z* 601 $[M + H]^+$ and *m/z* 623 $[M + Na]^+$. Fragments at *m/z* 583 $[M\text{-}17]^+$ and *m/z* 565 $[M\text{-}35]^+$ represented the elimination of one H_2O and two H_2O molecules, respectively. There were also fragments at *m/z* 221 and 181, indicating that the epoxide was in a ring with a hydroxyl substituent, the cleavage being between carbons 10 and 11, 13′ and 12′, respectively, of the polyene chain.

y_3-*Lutein (β,ε-carotene-3,3′-diol)*: In Fig. 1c, the base peak at *m/z* 569 belonged to the protonated molecule, $[M + H]^+$. *M/z* 591, *m/z* 551 and *m/z* 533 were the sodium adduct of the ion $[M + Na]^+$, the dehydrated protonated ions $[M + H–H_2O]^+$ and $[M + H–2H_2O]^+$, respectively. The relative molecular weight of y_3 was 568. Ions such as *m/z* 416 $[M + H - 153]^+$ and *m/z* 376 $[M + H - 193]^+$ were due to loss of a b-ring with a hydroxyl function (cleavage between carbons 7 and 8, and 9 and 10, respectively, of the polyene chain).

y_4-β-*Carotene (β,β-carotene)*: Fig. 1d was the mass spectrum of y_4 in positive ion mode. The relative molecular weight of y_4 was 536 because of the *m/z* 537 $[M + H]^+$ and *m/z* 559 $[M + Na]^+$. Fragments at *m/z* 445 was corresponding to $[M + H - 92]^+$ (elimination of toluene). Also fragments at *m/z* 203, *m/z* 177, *m/z* 149, *m/z* 137 and *m/z* 123 were due to cleavage between carbon 11 and 12, 9 and 10, 8 and 9, 7 and 8, and 6 and 7, respectively, of the polyene chain.

From the mass spectrums of y_1, y_2, y_3 and y_4, the information of functional groups could be found. The fragments of $[M + H - 18]^+$ and $[M + H - 36]^+$, which were commonly in the spectra of hydroxylated carotenoids, appeared in the mass spectrums of y_1, y_2 and y_3, not in the spectra of y_4. It indicated that there were at least two hydroxyl groups in the structures of y_1, y_2 and y_3 and no hydroxyl group in y_4. The results were exactly accordant with the structures of neoxanthin, violaxanthin, lutein and β-carotene.

According to the results of TLC, visible spectra, chemical reactions and LC-MS, we concluded that y_1, y_2, y_3 and y_4 were neoxanthin, violaxanthin, lutein and β-carotene, respectively.

3.6. Carotenoids of P. crispus

The content of total carotenoids from *P. crispus* L. was 231 μg/g (dry weight) measured by spectroscopy.

The HPLC chromatogram of TCPC was shown in Fig. 2. According to visible spectra of TCPC from DAD

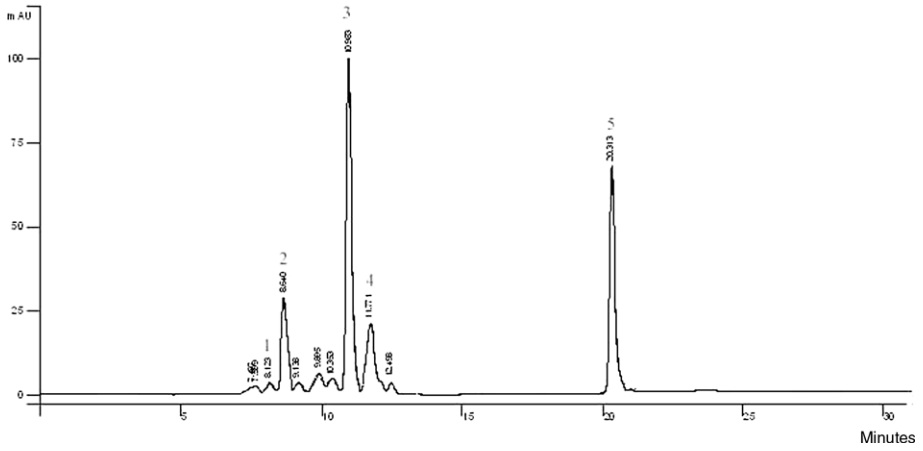

Fig. 2. RP-HPLC chromatogram of total carotenoids from *P. crispus*. Peak identification: (1) neoxanthin, (2) violaxanthin, (3) lutein, (4) rhodoxanthin, (5) β-carotene. HPLC conditions are described in the text.

detector compared with those of each fraction, we concluded that peak 1 was neoxanthin, peak 2 was violaxanthin, peak 3 was lutein, peak 4 was rhodoxanthin and peak 5 was β-carotene. Lutein and β-carotene were the major carotenoids, followed by rhodoxanthin and violaxanthin. Neoxanthin was at very low levels. The carotenoid composition of *P. crispus* has not been reported.

4. Conclusions

P. crispus is a new resource of carotenoids. In this study, four major yellow carotenoid pigments in *P. crispus* were separated by a MgO column and thin-layer chromatography. Theose carotenoids were identified as neoxanthin, violaxanthin, lutein and β-carotene based on visible spectra in different solvents compared with values reported in the literature, functional group tests and mass spectrum by LC-MS. The content which measured by visible absorption spectroscopy to be 231 μg/g (dry weight) showed that there are plenty of carotenoids in *P. crispus*.

Acknowledgement

This work was supported by a grant from the Ph.D. Programs Foundation of Ministry of Education of China (No. 20020504008).

References

Azevedo-Meleiro, C. H., & Rodriguez-Amaya, D. B. (2004). Confirmation of the identity of the carotenoids of tropical fruits by HPLC-DAD and HPLC-MS. *Journal of Food Composition and Analysis, 17*, 385–396.

Britton, G. (1995). UV/Visible spectroscopy. In G. Britton, S. Liaaen-Jensen, & H. Pfander (Eds.), *Carotenoids* (pp. 16–17). Basel: Birkhauser Verlag (pp. 46–56).

de Sá, M. C., & Rodriguez-Amaya, D. B. (2003). Carotenoid composition of cooked green vegetables from restaurants. *Food Chemistry, 83*, 595–600.

Hodisan, T., Socaciu, C., Ropan, I., & Neamtu, G. (1997). Carotenoid composition of *Rosa canina* fruits determined by thin-layer chromatography and high-performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis, 16*, 521–528.

Jian, Y. X., Li, B., Wang, J. B., & Chen, J. K. (2003). Control of turion germination in *Potamogeton crispus*. *Aquatic Botany, 75*, 59–69.

Lacker, T., Strohschein, S., & Albert, K. (1999). Separation and identification of various carotenoids by C_{30} reversed-phase high-performance liquid chromatography coupled to UV and atmospheric pressure chemical ionization mass spectrometric detection. *Journal of Chromatography A, 854*, 37–44.

Liu, L. Z., Zhang, M., Peng, G. H., Wang, H. B., & Zhang, S. H. (2004). Separation and identification of Red pigments in natural red yolk of duck's eggs by HPLC-MS-MS. *Chinese Journal of Chromatography, 22*, 197–201.

Oliver, J., & Palou, A. (2000). Chromatographic determination of carotenoids in foods. *Journal of Chromatography A, 881*, 543–555.

Vetter, W., Englert, G., Rigassi, N., & Schwieter, U. (1971). Spectroscopic methods. In O. Isler, H. Gutmann, & U. Solms (Eds.), *Carotenoids* (pp. 190). Basel: Birkhauser Verlag.

Wang, H. B., Peng, G. H., Liu, L. Z., Zhang, M., Ren, D. D., Shi, J. Y., et al. (2003). Studies on the carotenoids in *Potamogeton crispus* – Comparisons on the characteristics of carotenoids between *Potamogeton crispus* and red-yolk duck eggs. *Food Science, 24*, 41–45.

Wang, H. B., Ren, D. D., Liu, L. Z., Peng, G. H., Zhang, M., & Zhang, S. H. (2004). Preliminary identification of red carotenoids from *Potamogeton crispus* L.. *Scientia Agricultura Sinica, 37*, 1363–1368.