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Short communication

Preparative isolation and characterization of some minor impurities of astaxanthin by high-performance liquid chromatography

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Abstract

A simple and rapid preparative high-performance liquid chromatography (HPLC) method has been developed to isolate and characterize some minor impurities of astaxanthin using a normal-phase Lichrosorb silica column with *n*-hexane–acetone–tetrahydrofuran (90:2:8, v/v/v) as mobile-phase and detection at 475 nm. The isolated impurities were characterized as astacene, dehydro astacene and apoastaxanthinal by UV–vis, ESI–MS, ^{1}H and ^{13}C NMR spectroscopy and the molecular structures were assigned. The impurities collected using the developed conditions were over 98% pure.
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1. Introduction

Carotenoids are naturally occurring tetraterpenes found in various fruits, vegetables, plants, algae and bacteria. These are not only essential for human health but also effective as cancer preventing agents, life extenders, and inhibitors of ulcers, heart attacks and coronary artery diseases [1–4]. Carotenoids act as antioxidants through a free radical mechanism by quenching singlet oxygen and oxidizing species in the prevention of cellular damages [5]. The antioxidant activity of astaxanthin was reported to be ten times stronger than that of other carotenoids, viz., zeaxanthin, lutein, canthaxanthin and β-carotene [6]. This particular carotenoid is of considerable academic and practical interest not only because of its abundance in nature but also high economic value as a pigment in fish feeds [7]. It is widely used as an additive in fish feed to enhance the colour of pond-bred salmonids. It is extracted from certain algae which are known to be a continuous and reliable source of astaxanthin, because of their possible cultivation in bioreactors on a large scale [8,9]. While isolating astaxanthin from the source materials as well as the synthesis, several of its analogues, viz., astacene, etc. are generally formed as artifacts or degradation products reducing its quality significantly [10]. Therefore, the development of a chromatographic system for separation and determination of astaxanthin and its minor impurities/degradation products is of great importance for quality assurance and control purposes.

Over the years, non-aqueous reversed-phase liquid chromatography (NARP) on Zorbax ODS with typical eluents containing acetonitrile, dichloromethane and methanol was used for separation of carotenoids in a variety of biological materials, viz., orange juice, serum, bacteria, algae and artemisia which is an active antimalarial constituent of a traditional Chinese medicinal herb [11–13]. Under these conditions, the acidic astaxanthin failed to yield a symmetrical peak while astacene could not be eluted from the column. Vecchi et al. have used a silica column coated with phosphoric

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acid to overcome the problem of peak tailing while resolving astacene, semi-astacene and astaxanthin [14]. Willy et al. have extended this method for determination of astaxanthin in fish feed successfully [15]. Turujman has used a Pirkle covalent L-leucine column for rapid and direct resolution of the three stereioisomers of all-*trans* astaxanthin to distinguish between pond–bred aqua cultured and marine caught authenticated wild salmon [16]. However, the reproducibility of this method was found to be very poor as it exhibited different selectivity towards the stereoisomers of astaxanthin when repeated with an identical L-leucine column. Columns packed with Pirkle covalent L-leucine and D-phenylglycine stationary phases were tested to examine the reproducibility of separation and significant differences were found for columns packed by different manufacturers [17]. Under these chromatographic conditions, the stereo isomeric forms of *cis* isomer of astaxanthin were also resolved partially. Subsequently, the same group of workers have developed a chiral HPLC method not only for separation of all-*trans* (3*S*, 3′*S*), (3*R*, 3′*R*), (3*S*, 3′*R*) and (3*R*, 3′*S*) stereoisomers from each other but also from 9-*cis*-astaxanthin [18]. Sander et al. have developed a polymeric C_{30} phase for separation of *cis*/*trans* isomers of different carotenoids [19]. This column was subsequently used for on-line coupling of atmospheric pressure chemical ionisation–mass spectrometry (APCI–MS)[20]. Among all the LC–API–MS methods, LC–TurboIonspray–MS is the most sensitive technique and it proved to be adequate for qualitative and quantitative assay of astaxanthin in complex matrixes such as natural products [21]. Recently, the use of high-speed counter-current chromatography for preparative isolation and purification of astaxanthin from microalgae was reported [22]. However, to the best of our knowledge there are no reports on isolation and characterization of related impurities of astaxanthin in the literature. In the present paper, we describe a simple and rapid preparative HPLC method for isolation and identification of impurities of astaxanthin using normal-phase silica with *n*-hexane–acetone–tetrahydrofuran (90:2:8, v/v/v) as mobile-phase and detection at UV 475 nm at ambient temperature.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise. HPLC-grade hexane and THF (Ranbaxy, SASNagar, India) were used. Samples of astaxanthin provided by M/s. Divis Laboratories Limited, Hyderabad were used.

2.2. Apparatus

The HPLC system composed of two LC-10AT VP pumps, one LC-8A pump, an SPD-10AVP diode array detector an SIL-10AD VP auto injector, a DGU-12A degasser and SCL-10A VP system controller (all from Shimadzu, Kyoto, Japan). A normal-phase Lichrosorb silica (Thermo Quest Hypersil, Runcorn, UK) column (15 cm × 4.6 mm i.d.; particle size 5 μm) and silica (YMC, Kyoto, Japan) column (250 mm × 20 mm i.d.; particle size 5 μm) were used for separation and isolation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system.

2.3. Chromatographic conditions

2.3.1. Analytical HPLC

Analytical HPLC was performed with Lichrosorb silica (Thermo Quest Hypersil, Runcorn, UK) column (15 cm × 4.6 mm i.d.; particle size 5 μm) using two LC-10AT VP pumps. The mobile-phase was *n*-hexane–acetone–tetrahydrofuran (90:4:6, v/v/v). Before delivering in to the system it was filtered through 0.45 μm, PTFE filter and degassed using vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0 ml/min at room temperature (28 °C). Chromatograms were recorded at 475 nm using a SPD-10A VP diode array detector.

2.3.2. Preparative HPLC

Preparative HPLC was performed with LC-8A pump and YMC-silica (250 mm × 20 mm, 5 μm particle size) column using *n*-hexane–acetone–tetrahydrofuran (90:2:8, v/v/v) as mobile-phase at a flow rate of 20 ml/min. The pooled fractions were concentrated by rotary evaporator and each fraction was analyzed by analytical HPLC to check the purity prior to characterization.

2.4. ESI–MS

Mass spectra were recorded using a quadrupole mass spectrometer (QuatroLC, Micromass, UK) equipped with an electro spray ion source. The mass spectra were acquired in positive ion mode over the mass range 50–1000 Da.

3. Results and discussion

Analytical HPLC was used initially to develop the conditions for separation of astaxanthin and its impurities. Two solvents, viz., acetone and THF were tried with *n*-hexane in different compositions. When the content of acetone was 14%, the separation was found to be good but the solubility of astaxanthin was poor in the mobile solvent. Small quantities of THF were added to different compositions of hexane–acetone mixtures to study its effect on the retention of astaxanthin and its related impurities. The mobile-phase containing *n*-hexane–acetone–tetrahydrofuran (90:4:6, v/v/v) has resulted in optimum separation of all the impurities. The three minor impurities and astaxanthin eluted at retention times of 3.46, 4.92, 5.86, and 9.43 min, respectively. The developed conditions have been transported on

to a preparative chromatograph and optimized for injecting large amounts of sample for isolation of the impurities in sufficient quantities for further characterization. A normal-phase column of YMC Silica 250 mm × 20 mm i.d., 5 μm particle size with mobile-phase comprising of 90% *n*-hexane, 2% acetone and 8% tetrahydrofuran has been used. The flow rate, the sample loading capacity and its concentration were optimized to collect maximum amount of impurities of highest purity in a single run. The maximum flow rate used in this system was 20 ml/min. The THF content was slightly increased relative to the analytical conditions. It has increased the solubility of astaxanthin as well as reduced the total run time to less than 1 h. Under the preparative conditions astaxanthin has eluted at 26.0 min and good separations were still achieved.

The present work is directed towards preparative isolation of the above impurities and their characterization by spectroscopic techniques like UV, NMR and mass spectrometry. After the collection of each fraction, the solvent is evaporated under vacuum and the purity checked by analytical HPLC. All the collected impurities were found to be over 98% pure. Later the fractions were analyzed by ^{1}H and ^{13}C NMR spectrometry. The spectral data has been used for characterization of the impurities collected by preparative HPLC. The ESI–MS data of the fractions has provided evidence of molecular masses. The ^{1}H NMR data of these impurities has been compared with the values reported in the literature and found to be in good agreement with the minor impurities of astaxanthin [23,24].

1. Impurity isolated at retention time 3.46 min (rrt 0.36). UV–vis λ_{max} 476 nm (*n*-hexane), MS (ESI) *m*/*z* 593.6 (M + H), ^{1}H NMR (200 MHz) (solvent: $CDCl_3$) δ ppm: 1.25–1.29 (s, 12H, H-16, H-17, H-16′, H-17′), 2.00–2.29 (m, 18H, H-18, H-19, H-20, H-18′, H-19′, H-20′), 6.04 (s, 2H, H-2, H-2′), 6.30–6.67 (m, 16H, H-7, H-10, H-14, H-8, H-12, 3-OH, H-11, H-15, H-7′, H-10′, H-14′, H-8′, H-12′, 3-OH′, H-11′, H-15′). ^{13}C NMR (50.28 MHz) (Solvent: $CDCl_3$) δ ppm: 12.56 (C-19, C-19′), 12.78 (C-20, C-20′), 13.59 (C-18, C-18′), 28.14 (C-16, C-17, C-16′, C-17′), 39.26 (C-1, C1′), 123.09 (C-7, C7′), 124.63 (C-11, C-11′), 125.56 (C-2, C-2′), 128.37 (C-5, C-5′), 133.83 (C-14, C14′), 134.63 (C-9, C-9′), 135.31 (C-10, C-10′), 136.71 (C-13, C13′), 139.73 (C-12, C-12′), 142.37 (C-8, C-8′), 144.57 (C-3, C3′), 161.32 (C-6, C-6′), 182.48 (C-4, C-4′).
2. Impurity isolated at retention time 4.92 min (rrt 0.52) UV–vis λ_{max} 429 nm (*n*-hexane), MS (ESI) *m*/*z* 380.5 (M + H), ^{1}H NMR (200 MHz) (solvent: $CDCl_3$) δ ppm: 1.22–1.39 (2xs, 6H, H-16, H-17), 1.85–2.20 (m, 14H, H-18, H-19, H-20, H-20′, H-2), 3.70 (s, 1H, 3-OH), 4.25–4.35 (dd, 1H, H-3, J = 1.81 Hz), 6.1–7.3 (m, 9H, H-7, H-8, H-10, H-11, H-12, H-14, H-15, H-15′, H-14′), 9.43 (s, 1H, H-12′). ^{13}C NMR (50.28 MHz) (solvent: $CDCl_3$) δ ppm: 9.59 (C-20′), 12.63 (C-19), 13.00 (C-20), 13.90 (C-18), 26.10 (C-16), 30.68 (C-17), 36.78 (C-1), 45.46 (C-2), 69.20 (C-3), 124.20 (C-7), 126.79 (C-5), 126.79 (C-11), 128.15 (C-13), 132.16 (C-15), 134.51 (C-9), 136.07 (C-10), 137.33 (2xC-12, 13), 138.81 (C-14), 141.13 (C-14′), 141.88 (C-8), 148.47 (C-13′), 161.92 (C-6), 194.30 (C-12′), 200.37 (C-4).
3. Impurity isolated at retention time 5.86 min (rrt 0.62), UV–vis λ_{max} 470 nm (*n*-hexane), MS (ESI) *m*/*z* 596 (M + H), ^{1}H NMR (200 MHz) (solvent: $CDCl_3$) δ ppm: 1.21–1.30 (s, 12H, H-16, H-17, H-16′, H-17′), 1.89–2.09 (m, 20H, H-18, H-19, H-18′, H-19′, H-2, H-20, H-20′), 3.70 (s, 1H, 3-OH), 4.33 (d, 1H, H-3), 6.05 (s, 1H, H-2′), 6.33–6.66 (m, 15H, H-7, H-8, H-10, H-11, H-12, H-14, H-15, H-3′, –OH, H-7′, H-8′, H-10′, H-11′, H-12′, H-14′, H-15′). ^{13}C NMR (50.28 MHz $CDCl_3$) δ ppm: 9.58–13.90 (C-19, C-19′, C-20, C-20′, C18, C-18′), 26.14 (C-16), 30.7 (C-17), 28.14 (C-16′, C-17′), 36.79 (C-1), 39.24 (C-1′), 45.51 (C-2), 69.21 (C-3), 123.30 (C-7, C-7′), 124.62 (C-11, C-11′), 125.53 (C-2′), 126.88 (C5), 128 (C5′), 130.69 (C-15), 132.18 (C-15′), 133.81 (C-14, C-14′), 134.54 (C-9, C-9′), 135.14 (C-10, C-16′), 136.68–137.28 (C-13, C-13′), 139.73 (C-12, C-12′), 142.26 (C-8, C-8′), 144.65 (C-3′), 161.28 (C-6′), 162.12 (C-6), 182.87 (C-4′), 200 (C-4).

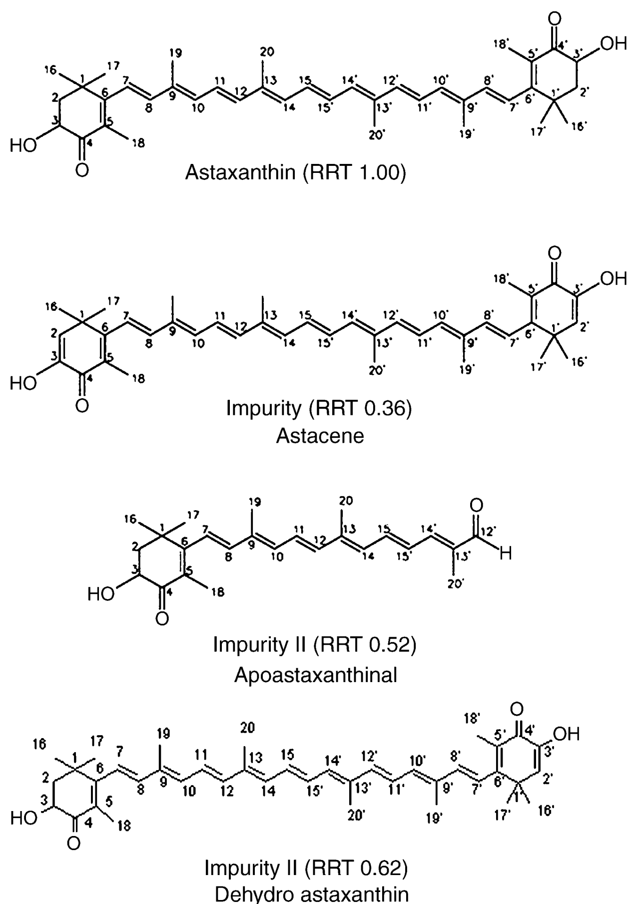

Fig. 1. Chemical structures of astaxanthin and its impurities isolated by preparative HPLC and characterized by NMR and mass spectrometry.

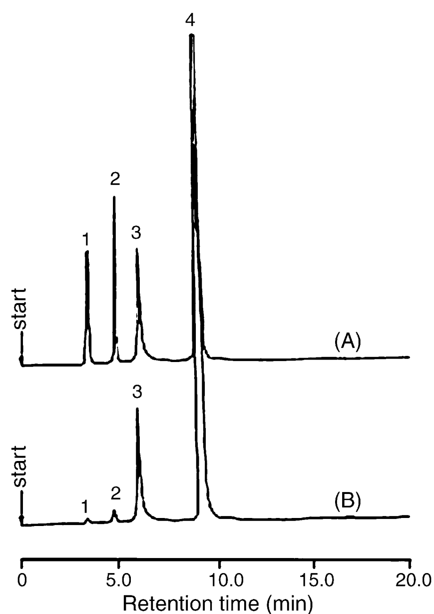

Fig. 2. Analytical HPLC profiles of: (A) a synthetic mixture and (B) a typical sample of astaxanthin. Peak identification: (1) astacene; (2) apoastaxanthinal; (3) dehydro astaxanthin; and (4) astaxanthin.

With the help of the above spectral data, the molecular structures of all the impurities have been assigned and shown in Fig. 1. Fig. 2A shows the typical chromatogram of a synthetic mixture of astaxanthin, astacene, dehydro astacene and apoastaxanthinal. Excellent selectivity and sensitivity were obtained for all the impurities as well as astaxanthin in the combined standard. A commercial sample of astaxanthin chromatographed under the same conditions is shown in Fig. 2B. Peaks were identified by spiking the sample with individual standards and comparing the retention times. These results indicate that the developed conditions are not only suitable for analytical determination but also preparative isolation of some of the minor impurities of astaxanthin.

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