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Mini Review

Chiral high performance liquid chromatography of astaxanthin's stereoisomers

Tomas Rezanka[1,*] and Valery M. Dembitsky[2]

[1]Institute of Microbiology, Videnska 1083, 142 20 Prague, Czech Republic. [2]Graduate School of Applied Sciences and Technology, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

ABSTRACT

The separation of astaxanthins from carotenoids from freshwater amphipod, yeast, green alga and flower plant using by HPLC is described. The stereoisomers of astaxanthins were separated by means of chiral column and their structures were confirmed by circular dichroism spectra. The resolution of single stereoisomers of astaxanthin has α=1.048 and α=1.056 respectively. The future scope of chiral HPLC, including the separation of the former consistent astaxanthins from green alga and yeast is also discussed.

INTRODUCTION

Astaxanthin is a highly oxidized carotenoid which is found in marine, freshwater animal tissues [1,2], algae, higher plants, fruits [3,4] and isolated from marine bacterium [5-7]. Recently we identified novel carotenoid glycoside ester from freshwater amphipod [8], and the present work is a continuation of our earlier studies detailing the carotenoid composition some organisms.

The major carotenoids in aquatic animals are 3-hydroxy-4-oxocarotenoids, *e.g.*, astaxanthins. These carotenoids have one or two asymmetric centers and so they exist in stereoisomeric forms. The methods for their isolation, most frequently using HPLC, were described [9-15], but some difficulties during isolation and identification often occurred.

SEPARATION OF ISOMERS

The worst one is stereoselective separation of the diastereoisomers of di-(-)-camphanates of 3-hydroxy-4-oxocarotenoids. Some studies about chromatographic resolution of these diastereoisomers were published [9,10,16,17].

The later separation of all three stereoisomers of astaxanthin (*i.e.* 3S,3'S-astaxanthin, 3R,3'R-astaxanthin and 3RS,3'RS-astaxanthin) in free forms without any converting to their corresponding diastereoisomers di-(-)-camphanates by HPLC on optical resolution column has been achieved [18]. The aim of this paper is to discuss the separation of stereoisomers of astaxanthins and their occurrence in plant, alga, yeast and mainly in freshwater amphipod.

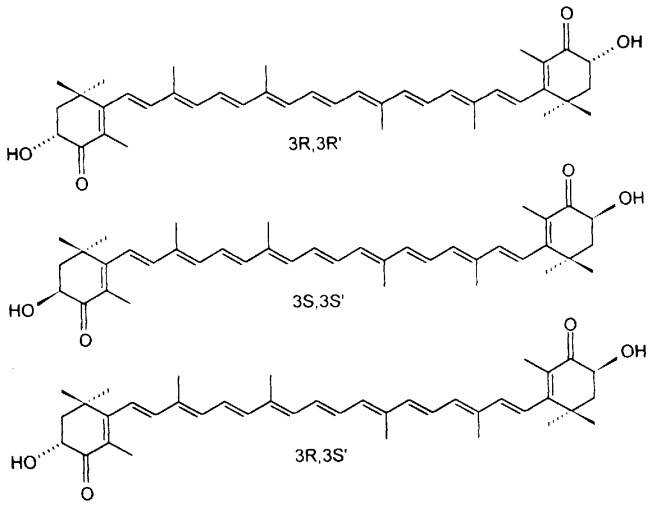
Rapid procedure for direct isolation of astaxanthins and their resolution into stereoisomers is described. This procedure is based on HPLC separation of a total extract. On the first column (silica) the mixture was separated by polarity of single compounds and on the second one (chiral) only appropriate stereoisomers were analysed.

Amphipod *Acanthogammarus grewingkii* was collected in the lake Baikal (Russia) [8], green alga *Scenedesmus sp.* was obtained from our department in Tebo (Czech Republic), yeast *Phaffia rhodozyma* CCY-77-1-1 was sent from Culture of Yeast (Bratislava, Slovakia) and pheasants-eye was bought in the local market-hall.

The total lipids were extracted with chloroform-methanol (1:1, v/v). The extract was washed with 50 mM Tris buffer [19], evaporated to 1/10 volume and crude mixture was analysed by HPLC.

Two liquid chromatographs, *i.e.* the semi-preparative Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml/ min), an SCL-6A system controller, an SPD ultraviolet detector (220 nm, respective 481 nm), an SIL-1A sample injector and a C-R3A data processor, with preparative column SGX (spherical silica gel) 250 mm x 8 mm ID; 7 µm particles (Tessek, Prague, Czech Republic) and analytical apparatus Sigma 3B (Perkin Elmer, Norwalk,

* Corresponding author
E-mail: rezanka@biomed.cas.cz



CT, USA) with an analytical column 250 mm x 4.6 mm ID, packed with Supelcosil LC-(R)-DNBPG ((R)-dinitrobenzoyl phenyl-glycine) (represented by Sigma-Aldrich s.r.o., Czech Republic). After injection of 100 µl of solution of total crude extract (5 mg/ml) a gradient of aceto-nitrile-methanol (convex gradient from 100 : 0 to 90:10 for 20 min) was applied. The eluate from the semipreparative column was injected into the second apparatus (Sigma) and then was eluted with an isocratic mixture hexane-dichloromethane-ethanol (74:25:1).

Identification of astaxanthins peaks was achieved by means of visible spectra that were measured in ethanol with Shimadzu UV-160A and by ^{1}H-NMR. NMR spectra were recorded with a Bruker AM 500 pulsed Fourier transform spectrometer (Karlsrue, Germany) operating at 500 MHz for ^{1}H at ambient temperature in $CDCl_3$. The CD spectra were measured in CH_2Cl_2 by means of Jasco instrument (Jasco, Japan).

CONCLUSIONS

Extracts from all four organisms were separated by HPLC and the peaks correspon-ding to free carotenoids and their glycosides were obtained [8]. Separation by means of chiral phase is eminent. Resolution of all three peaks has values α_1=1.048 and α_2=1.056 respectively. As it was described in our previous paper [8], just from this amphipod a broad spectrum of different carotenoids and their glycosides was isolated. Based on the literature data [4,9-11,18] the presence of astaxanthin stereo-isomers in amphipod was assumed.

For additional validation of structures, other natural sources of astaxanthins were used. Yeast is known as a producer of mainly R,R stereoisomer [21,22], on the contrary green plants, *i.e.* higher plant and also green algae contained S,S stereoisomer [23,24]. Renstrom et al. [11] studied carotenoid composition of shrimp *Pandalus borealis* and found some optical astaxanthin's isomers (3S,3'S), (3R,3'S; meso) and (3R,3'R). Astaxanthin isolated from lobster eggs (*Homarus gammarus*) [10] was unexpectedly found to be a mixture of all three optical isomers also as determined by HPLC.

From these above-mentioned sources the mixtures of astaxanthins were analysed, as is illustrated by Fig. 1. The above chromatogram (crab) shoves three peaks, *i.e.* all three possible stereoisomers. In the middle and also bottom chromatograms it was obtained by one peak (stereoisomer), while in the green alga the traces of R,R and meso were proved. There were identified not only both R,R and S,S stereoisomers, but also the meso-form. All isomers were obtained in negligible amounts.

The structures of single stereoisomers were confirmed by circular dichroism spectra (structures of stereoisomers see below). The competent curves are presented in Figure 2. The results suggested that in the possible food of invertebrate are included as algae as fungi (yeast). We cannot say if the change of configuration occurs earlier in the food chain or in the amphipod body. The individuality of the animals from the lake, see also our previous papers, does not enable us to make an unambiguous conclusions about sources of all three stereoisomers.

This chromatographic separation makes possible in very

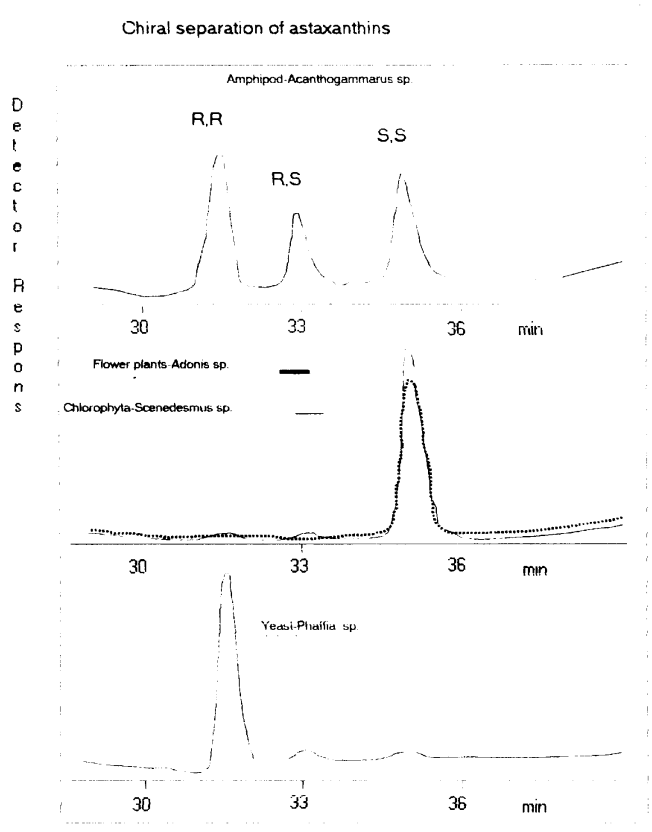


Fig. 1.Chiral separation of different stereoisomers of astaxanthins from various sources. HPLC conditions, see SEPARATION OF ISOMERS.

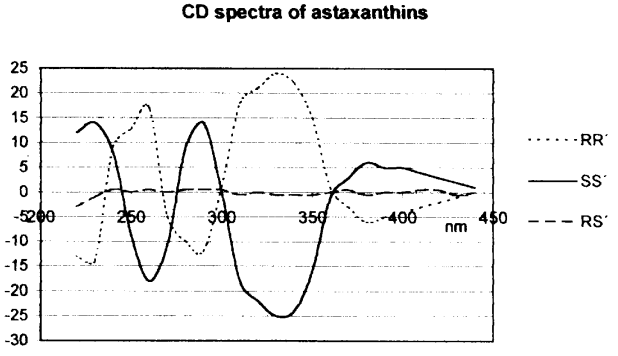


Fig. 2. CD spectra of 3R, 3′R-astaxanthin (dotted), 3S, 3′S-astaxanthin (solid) and 3R, 3′S-astaxanthin(dashed)

short time (about 1 hour) and without degradation of sensitive carotenoids to analyse complex mixture of carotenoids including resolution of their stereoisomers. The major contribution of chiral HPLC is the discovery new ways in separation and identi-fication of former consistent compounds. As we see from Figure 2, it is possible to identify all stereoisomers by means of CD spectra. In all cases it was possible to identify three stereoisomers, *i.e.* R,R, S,S and meso-form (R,S or S,R) using chiral HPLC.

Future utilisation of chiral HPLC might bring interesting results. Traces of one of meso and R,R stereoisomers or S,S diastereoisomer were obtained from both alga and yeast, see Fig. 1, what has not been described.

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