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Rapid direct resolution of the stereoisomers of all-*trans* astaxanthin on a Pirkle covalent L-leucine column

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ABSTRACT

Astaxanthin, 3,3'-dihydroxy-β,β-carotene-4,4'-dione, gives salmon flesh its distinctive color. Synthetic all-*trans* astaxanthin consists of a racemic mixture of two enantiomers (3*R*,3'*R* and 3*S*,3'*S*) and a meso form (3*R*,3'*S*). The stereoisomeric composition of endogenous astaxanthin in wild salmon differs from that of synthetic astaxanthin added to the fish feed of pond-bred salmon. In order to determine the origin of astaxanthin in salmon flesh, a method is needed that can distinguish the various isomeric forms. HPLC conditions are described for the rapid direct resolution of the three stereoisomers of all-*trans* astaxanthin on a Pirkle covalent L-leucine column. This method also partially resolves the stereoisomeric forms of the major *cis* isomer.

INTRODUCTION

The endogenous color of the flesh of Salmonid fish may be duplicated by the addition of oxycarotenoid color additives to the fish feed. However, to meet FDA requirements, color additives must be specifically listed for this use in the Code of Federal Regulations (CFR).

Two oxycarotenoids are believed to be widely used as color additives in fish feed to enhance the color of pond-bred salmonids —canthaxanthin and astaxanthin (Fig. 1). Canthaxanthin can be easily distinguished from astaxanthin by TLC [1] and HPLC [2]. Astaxanthin, but not canthaxanthin, is normally found in wild salmonids (*Salmo salar* and *Oncorhynchus*). The distribution of astaxanthin stereoisomers found in wild salmonids (endogenous astaxanthin) differs from that in synthetic astaxanthin added to fish feed. Endogenous astaxanthin is reported to consist of 78–85% of the (3*S*,3'*S*) enantiomer, 12–17% of the (3*R*,3'*R*) enantiomer and 2–6% of the meso form [3]. In contrast, synthetic astaxanthin consists of 25% of each enantiomer and 50% of the meso form. This difference in stereoisomer distribution can be used to differentiate synthetic and endogenous astaxanthin in commercial fish.

Maoka *et al.* [4] resolved all-*trans* astaxanthin on a Pirkle covalent D-phenylglycine type column manufactured in Japan; however, the analysis time was 70 min.

Mobile phases of similar polarity have been found that allow the stereoisomers of all-*trans* astaxanthin to be directly resolved on a Pirkle covalent L-leucine column in 10–15 min. With this modified method, it should be possible to distinguish between synthetic astaxanthin extracted from the flesh of pond-bred salmon and endogenous astaxanthin, by comparison of their chromatographic profiles.

The 13-*cis* isomer of astaxanthin is more strongly retained and better resolved than the all-*trans* isomer under the same chromatographic conditions. To resolve a mixture of the two isomers, a 20-min analysis time is required.

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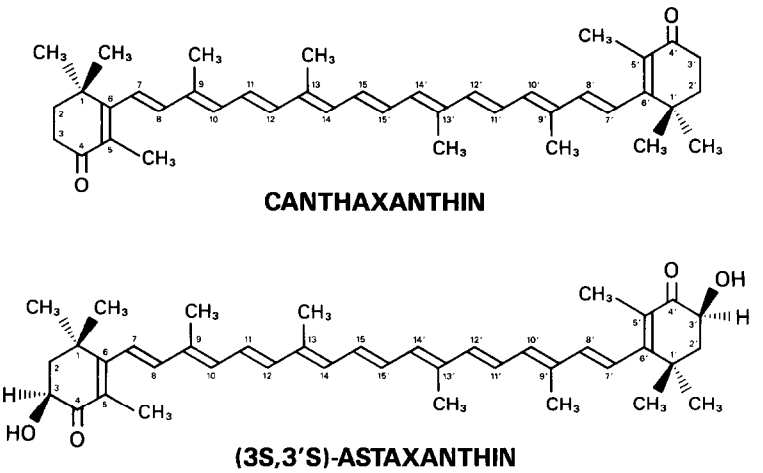

Fig. 1. Structures of astaxanthin and canthaxanthin.

EXPERIMENTAL

Materials

Synthetic astaxanthin was obtained from Hoffmann-La Roche, Nutley, NJ, USA. Hexane (Baxter), tetrahydrofuran (THF) (Baxter), acetonitrile (Baxter), methanol (Baker), ethanol (EM Science), 2-propanol (Baxter), 1-butanol (Fisher), *tert.*-butanol (EM Science), triethylamine (Fluka) and diethylamine (Fluka) were HPLC grade. Trioctylamine (Aldrich) was ACS reagent grade.

Apparatus

The analysis was performed on a computer-controlled Hewlett-Packard 1090 Series II/M liquid chromatograph, equipped with an auto-injector and autosampler, a diode array detector, and ternary solvent delivery system, with low-volume metering for each of the three solvent channels, and low-pressure mixing of the metered solvent.

HPLC conditions

A Pirkle covalent L-leucine column (5 μm), 250 × 4.6 mm I.D. (Regis Chemical Company, Morton Grove, IL, USA), was used under isocratic conditions. The flow-rate was 1.5 ml/min and the monitoring wavelength was 470 nm. Solvents were filtered, and sparged with He as recommended by the instrument manufacturer. All mobile phases contained 77% hexane and 15–22% THF, with the balance supplied by other modifiers as listed. The modifiers were chosen so that the polarity of the mobile phase remained unchanged. Other modifiers that were used, either separately or together, included acetonitrile (ACN) and various alcohols and amines, each ranging in concentration from 0.1 to 6%. Alcohols that were used are methanol, ethanol, isopropanol (IPA), *tert.*-butanol and 1-butanol. Amines that were used are diethyl-, triethyl- (Et_3N) and tri-*n*-octyl amine.

RESULTS AND DISCUSSION

Resolution of the three stereoisomers of all-*trans* astaxanthin was obtained with the following mobile phases:

(a) hexane–THF–Et_3N (77:19:2), $\alpha_{RR/SS}$ = 1.29;
(b) hexane–THF–Et_3N–ACN (77:17:2:2), $\alpha_{RR/SS}$ = 1.34 (see Fig. 2);
(c) hexane–THF–IPA–ACN (77:17:2:2), $\alpha_{RR/SS}$ = 1.2; and
(d) hexane–THF–Et_3N–ACN–methanol (77:20.5:1:1:0.5), $\alpha_{RR/SS}$ = 1.23.

Like other carotenoids, all-*trans* astaxanthin undergoes *trans/cis* isomerization when exposed to light or heat [5]. To avoid isomerization, the astaxanthin solution should be placed in an amber vial and analysed soon after dissolution.

When a solution of all-*trans* astaxanthin in a sealed vial was exposed to available light (daylight

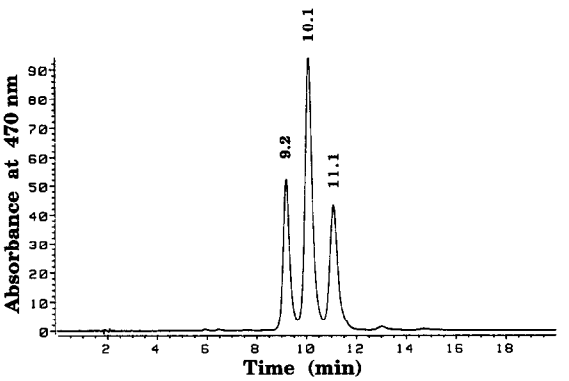

Fig. 2. Elution profile of all-*trans* astaxanthin on a Pirkle covalent L-leucine column (5 μm), 250 × 4.6 mm I.D. Mobile phase: hexane–THF–Et_3N–ACN (77:19:2:2); $\alpha_{RR/SS}$ = 1.34.

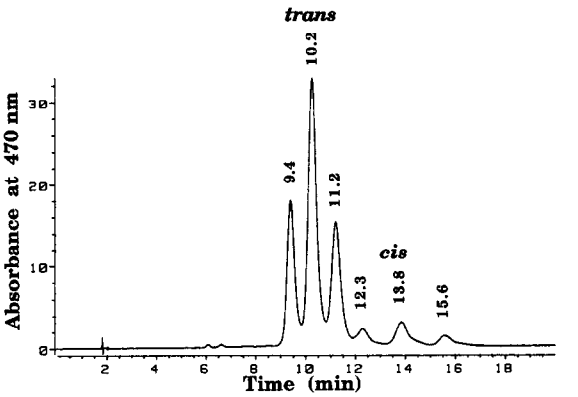

Fig. 3. Elution profile of all-*trans* astaxanthin with a minor amount of the 13-*cis* isomer, on a Pirkle covalent L-leucine column (5 μm), 250 × 4.6 mm I.D. Mobile phase: hexane–THF–IPA–Et_3N (77:20:1.5:1.5); $\alpha_{(trans)RR/SS}$ = 1.26, $\alpha_{(cis)RR/SS}$ = 1.32.

and fluorescent) in the laboratory, 13-*cis* and 15-*cis* astaxanthin peaks appeared in the chromatograms, overlapping the peaks of the all-*trans* components in mobile phases (a) to (d) above. The mobile phases were therefore further modified in order to resolve the nine stereoisomers of all-*trans*, 13-*cis*, and 15-*cis* astaxanthin. The three all-*trans* isomers were resolved from the *cis* isomers, and the *cis* stereoisomers were resolved from each other. However, the 15-*cis* stereoisomer peaks appeared as shoulders on the 13-*cis* isomer peaks [6] (Fig. 3).

Representative mobile phases are as follows:

(e) hexane–THF–IPA–Et_3N (77:20:1.5:1.5), $\alpha_{(trans)RR/SS}$ = 1.26, $\alpha_{(cis)RR/SS}$ = 1.32 (see Fig. 3);

(f) hexane–THF–IPA–Et_3N–methanol–1-butanol (77:19:0.8:1.6:0.8:0.8), $\alpha_{(trans)RR/SS}$ = 1.2, $\alpha_{(cis)RR/SS}$ = 1.25.

This method can therefore be used to quickly detect *trans*/*cis* isomerization of all-*trans* astaxanthin.

For the determination of the stereoisomer ratio of all-*trans* astaxanthin, mobile phase (b) may be used. However, if *trans*/*cis* isomerization occurs, then mobile phase (e) is recommended.

Preliminary results indicate that by using this method, synthetic astaxanthin extracted from the flesh of pond-bred salmon can be quickly distinguished from endogenous astaxanthin by comparison of their chromatographic profiles [7]. Being less polar than astaxanthin, other associated carotenoids found in small amounts in the skin of salmon, such as the mono- and diesters of astaxanthin, elute in the first 5 min and therefore do not interfere with the resolution of astaxanthin. A statistical study is under way to validate the distribution of the stereoisomers of endogenous all-*trans* astaxanthin extracted from the flesh of six subspecies of authenticated wild salmon.

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