



Relative Measurement of Zeaxanthin Stereoisomers by Chiral HPLC

Principle

To measure the relative percentages of the (3R,3'R), (3R,3'S) and (3S,3'S) – stereoisomers of zeaxanthin in dietary ingredient and finished product forms of dietary supplements. Extracted samples are injected and separated on a chiral carbamate-modified amylose HPLC column and detected by absorbance at 450 nm.

Reagents and Supplies

20 mL glass vials with screw top caps

50 and 100 mL volumetric flasks

5 and 10 mL pipets

0.10-1.00 mL micropipet

3 mL disposable syringes and 0.45 micron PTFE syringe filters

HPLC sample vials (if using an autosampler)

Solvents

HPLC: hexane, isopropanol

Extraction: HPLC grade water and HEAT (hexane/ethanol/acetone/toluene) or dichloromethane

Standards

(3R,3'R) – and *meso*-(3R,3'S) – all-E-zeaxanthin (>90% pure)

Saponification materials (optional)

30% methanolic KOH

35% (saturated) aqueous sodium chloride solution

15 mL culture tubes with screw top caps

vortex mixer

Alginate digestion solution (see footnote 3)

Instruments and settings

Analytical balance (± 0.1 mg)

Ultrasonic bath with heating to 50°C

High performance liquid chromatography system with UV/vis diode array detector

Column: Chiralpak AD-H (Chiral Technologies, Inc., Westchester, PA, USA), 250 x 4.6 mm (5 μ m particle size)

Mobile phase table

Time (min)	Flow rate (ml/min)	% n-Hexane	% Isopropanol	Gradient
0	0.8	95	5	
50	0.8	95	5	
55	0.8	50	50	linear
63	0.8	50	50	
65	0.8	95	5	linear
75	0.8	95	5	

Column temperature: Room temperature (approx. 23°C)

Injection volume: 20 µl

Detection: absorbance at 450 nm

Preparation of Sample

Note: All preparations are scaled to provide nominal concentrations of less than 10 ppm and preferably between 1-6 ppm of zeaxanthin in the injection solution. Higher concentrations could lead to errors due to solubility limitations of zeaxanthin in the injection solvent.

(1) Powder concentrates (typically 20-50 wt% zeaxanthin)

Use ultrasonic agitation to dissolve 30-50 mg of sample in 50 mL HEAT¹ or dichloromethane. Add 0.10 mL of this solution to an empty glass vial with at least 15 mL capacity and evaporate residual solvent under nitrogen flow.² Add 10 mL of 5:95 isopropanol:hexane to the vial, cap and mix thoroughly to dissolve. Filter this final solution through 0.45 micron PTFE syringe filter and inject into the HPLC.

(2) Beadlets (about 5-20 wt% zea) and tablets/capsules (about 2-8 mg zea per tablet)

In a 100 mL volumetric flask, combine 50-100 mg of beadlets or one-two pulverized tablets (to provide 4-8 mg of zeaxanthin) with 5 mL water³ and agitate with ultrasonication for 30 min at 50°C. Alternatively, one can grind the sample and water together in a mortar and pestle and transfer the mixture to a 100 mL volumetric flask using HEAT or dichloromethane, if necessary, to rinse the mortar and complete the transfer of solids to the flask. Fill the flask to the mark with one of the following solvent blends: (a) HEAT¹ or (b) 40 mL ethanol and dichloromethane as needed. Sonicate the resulting mixture for 10 min with occasional hand mixing. If using HEAT, allow any undissolved aqueous phase to separate to the bottom of the flask. Dilute 0.50 mL of

¹ HEAT is hexane, ethanol, acetone, and toluene combined in 10:6:7:7 volumetric ratio.

² The manufacturer of the Chiralpak AD-H column advises against injection of methylene chloride or acetone, even in trace quantities. To ensure best column longevity, it is advisable to evaporate the residual solvent from the first dilution prior to addition of the injection solvent.

³ Samples that include alginate-encapsulated zeaxanthin might require more aggressive treatment. For more complete release of zeaxanthin from samples such as these, replace water with the following extraction buffer. To prepare, dissolve 6.8 g potassium dihydrogenphosphate and 1.0 g Na₂EDTA dihydrate in approximately 90 mL water in an Erlenmeyer flask. Add approximately 1.8 g of sodium hydroxide and sonicate for 20 min until the sodium hydroxide is dissolved. Adjust to pH 8.7 ± 0.3 with 4 M NaOH and fill to a volume of 100 mL with water. Add 0.025 g sodium dodecylsulphate and sonicate for 20 min at room temperature (may not be completely dissolved). Prepare daily.

the organic phase into 10 mL of 5:95 isopropanol:hexane and mix thoroughly. Filter this final solution through 0.45 micron PTFE syringe filter and inject into the HPLC.

(3) Oil suspensions or oleoresins (typically 5-20% zeaxanthin)

(Note: See preparation 5 for products that may contain zeaxanthin esters.)

Use ultrasonic agitation to dissolve 100-150 mg of liquid sample in 50 mL HEAT¹ or dichloromethane. Add 0.10 mL of this solution to an empty glass vial with at least 15 mL capacity and evaporate residual solvent under nitrogen flow.² Add 10 mL of 5:95 isopropanol:hexane to the vial, cap and mix thoroughly to dissolve. Filter this final solution through 0.45 micron PTFE syringe filter and inject into the HPLC.

(4) Soft gels (typically 2-4 mg zeaxanthin per capsule)

(Note: See preparation 5 for products that may contain zeaxanthin esters.)

Carefully slice two soft gel capsules lengthwise and place both together into in an open glass vessel (these will provide about 500-700 mg of liquid fill). Add 50 mL of HEAT¹ or dichloromethane and apply ultrasonic agitation for 5 min to dissolve the xanthophylls. Add 0.10 mL of this solution to an empty glass vial with at least 15 mL capacity and evaporate residual solvent under nitrogen flow.² Add 10 mL of 5:95 isopropanol:hexane to the vial, cap and mix thoroughly to dissolve. Filter this final solution through 0.45 micron PTFE syringe filter and inject into the HPLC.

(5) Saponification procedure.

Some liquid extracts may contain naturally-occurring zeaxanthin esters. Because meso-forms are generally not prepared in ester-form, analysis of these samples without saponification could underestimate the relative amount of natural (3R,3R') isomer or overestimate the relative amount of meso (3R,3S') isomer. If accurate determination of minimum meso isomer level is necessary in these extracts, the following method should be used to hydrolyze the natural esters prior to HPLC analysis.

- a) In a 10 mL culture tube with sealable cap, combine 600 µL of ethanol and 250 µL of 30% methanolic KOH.
- b) Add 100 µL of the first dilution made in preparation 4 or 5 above.
- c) Vortex this mixture for 15 s and let rest at 22-28°C for 30 min or 2-10°C for 18 h.
- d) Allow the sample to return to room temperature. Uncap sample and add 2.0 mL of 35% saturated sodium chloride solution to the culture tube.
- e) Add 4.5 mL of 5:95 isopropanol/hexane and vortex sample for 30 s.
- f) Let sit for 3-5 minutes for two layers to appear.
- g) Transfer the top layer from the culture tube into a 10.0 mL volumetric flask. Make sure that no aqueous (bottom) layer is transferred.
- h) Repeat steps e – g and combine both top layer fractions.
- i) Fill the 10.0 mL volumetric flask containing supernatant to the mark with 5:95 isopropanol:hexane and mix well.
- j) Filter the solution through a 0.45 µm Teflon™ (PTFE) into an amber 2 mL vial and analyze by HPLC.

Peak Identification

The peaks of the (3R,3'R) –, *meso*-(3R,3'S) – and (3S,3'S) – all-E-zeaxanthin isomers and lutein are identified by injection of reference substance of at least one of these stereoisomers and use of the relative retention times given in the table below. The retention time for (3R,3'R)-E-zeaxanthin is typically between 30-40 min. In complex mixtures it is recommended to confirm the identity of the peaks by comparison of their UV/VIS-spectra with that of the reference substance using a diode array detector (DAD). Reference standards for (3R,3'R) – E-zeaxanthin and lutein are available from several research chemical suppliers. The ZTA is presently working to provide a commercially-available *meso*-(3R,3'S) – all -E-zeaxanthin standard.

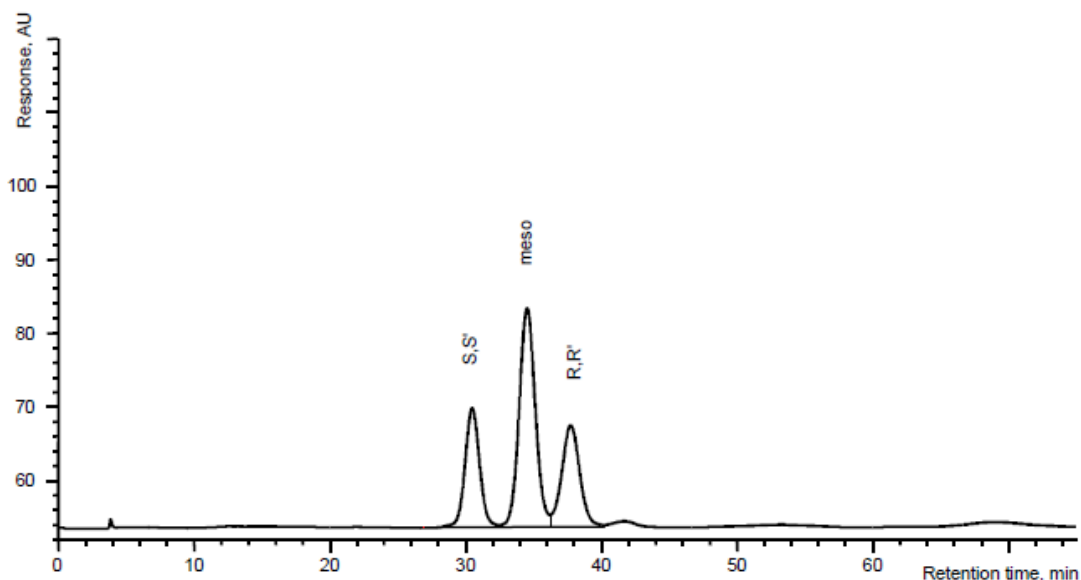
Component	Retention time / retention time of 3R,3'R
(3S,3'S)-E-Zeaxanthin	0.82
<i>meso</i> -(3R, 3'S)-E-Zeaxanthin	0.92
(3R,3'R)-E-Zeaxanthin	1.00
(3R,3'R,6'R)-E-Lutein	1.12

Quantitation

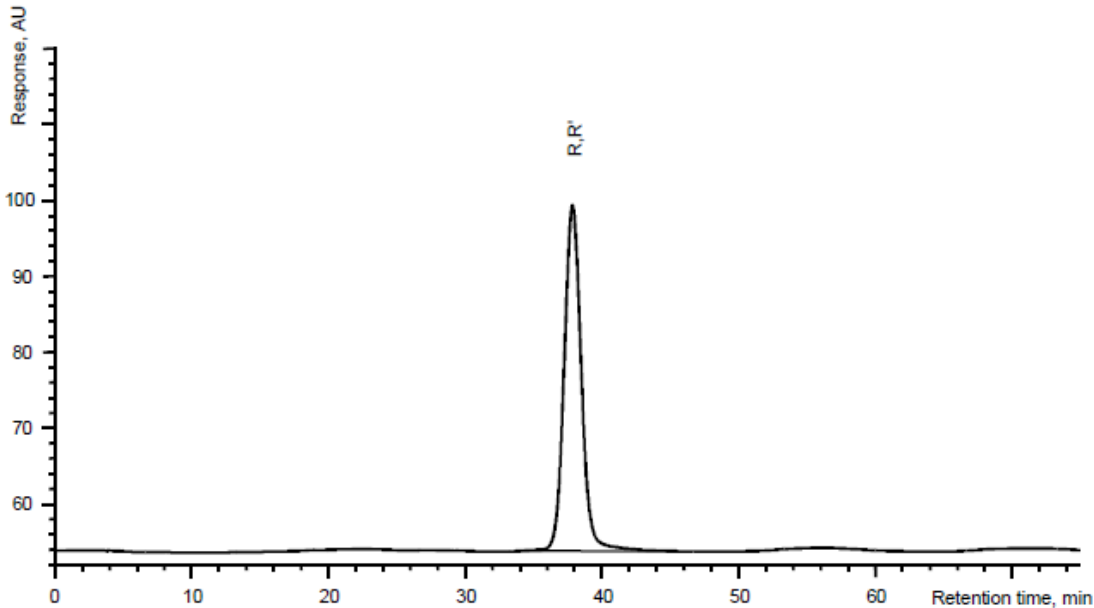
No calibration is necessary. Results are reported as the relative area percent of the three stereoisomers of E-zeaxanthin (normalized to a sum total of 100%).

Representative Results

Typical Chromatogram of Racemic all-E-Zeaxanthin:



Typical Chromatogram of (3R,3'R) all-E-Zeaxanthin:



Chiral chromatography of 20 ppm RS-Zea, RR-Zea and Lutein before isomerization:

