Method name: Astaxanthin
BIOVIT-nr.: Arb1017

1. Introduction

Pigments are important ingredients in feed to achieve a desired coloring of salmonids, where it represents an important cost of the feeds. Astaxanthin (AX) is the natural main carotenoid in the meat of wild salmonids. By using an HPLC method instead of more traditional methods like color cards and spectrometry, it is possible to quantify the levels of AX (and canthaxanthin), but also to distinguish between some of the isomers of AX [1,2]

Main instrument: Ultimate 3000 UHPLC with UV detector (Thermo Scientific).

2. Reagents:

Magnesium sulfate, anhydrous, purity > 98 % (art.nr: 63136-250G- VWR)

Acetone, purity > 99 % (art.nr: 1.00020.2500 - VWR)

n-heptane, purity > 99% (art.nr: 1.04379.2500 – VWR)

Reference substance of all-E-astaxanthin, purity > 95 % (art.nr: 41659 – merck)

BHT (2,6-Di-tert-butyl-p-kresol) ≥99.0% (art.nr: TCIAD0228-25G – VWR)

Tetrahydrofuran ≥99.5% stabilized (art.nr: TCIAT0104-500ML – VWR)

HPLC mobile phase (isocratic): 86:14 , acetone:heptane

Preparation of astaxanthin standard solution (1,5 mg/mL):
Weigh approximately 1.5 mg to the nearest 0.1 mg of the reference substance and 1 g of BHT into a 100 mL volumetric flask. Dissolve in 5 mL of tetrahydrofuran and dilute to the mark with tetrahydrofuran. Support dissolution by ultrasonic treatment. Transfer an aliquot of 10 mL of this solution into a 100 mL volumetric flask and add approximately 85 mL of heptane. The mixture cools and contracts. Warm the solution to room temperature and dilute to the mark with heptane. This results in an astaxanthin concentration of approximately 1.5 mg in a mixture of 9 parts per volume of heptane and 1 part per volume of tetrahydrofuran.

Measure by spectrometry immediately after preparation and inject the standard solution into the HPLC. Determine the response factor from the total peak areas of the chromatogram and the concentration measured by spectrometry (more info under calculations).

3. Risk assements:

Wear safety goggles, gloves, and work in fume hood when handling organic solvents. Take extra precautions when preparing the standard solution due to the use of BHT (toxic/causes damage to organs/may damage fertility or the unborn child)) and tetrahydrofuran (Highly flammable/suspected of causing cancer). Read datasheet carefully and work in fume hood with safety googles and gloves.

4. Equipment:

- Ultimate 3000 UHPLC
- Spectrophotometer
- Ultrasonic bath
- Solid phase extraction manifold
- SPE columns, 30 mL reservoirs (art.nr: MANA730034 – VWR)
- Nitrogen flow evaporator with water bath and holder for pipettes
- 10 mL volumetric flask
- 100 mL volumetric flask
- 10 mL glass tube w/screw cap
- Centrifuge
- 2 mL glass vials w/lid

5. Special remarks

To avoid tailing of the astaxanthin peaks, modify the stationary phase by pumping a solution of 1 g of phosphoric acid dissolved in 100 mL of methanol through the column for at least 1 h at a flow rate of 1 mL/min. Wash the column with mobile phase at a flow rate of 1.2 mL/min for at least 5 h. The acid-modification is maintained for more than one year if the stationary phase is not exposed to polar solvents like water.

Astaxanthin molecules are unstable and can quickly be degraded by light and oxygen. Store standard solutions under nitrogen at -20 °C.

6. Sample material

Feed and feces analysis: 0.2 g sample is required. Degree of grinding 1 mm.
Fish muscle analysis: 1-2 g homogenized sample is required.
A control sample containing 3.2 mg/kg should be run with each batch.

Feed samples must be stored from 2 to 8 °C.
Muscle sample must be stored at -20 °C.

7. Job description

Small scale extraction (for large scale – see reference method [3])

1) Weigh in appr. 1 g homogenized sample and 1 g Magnesium sulphate in a 30 mL SPE-column equipped with a 10 µm-frit at the bottom
    - Note the exact weight of the sample with 3 decimals
2) Insert the column into a closed valve of a SPE manifold
3) Mix well with a spatula
4) Add 8 mL of acetone with BHT, mix, and wait 3 minutes
5) Open the valve and suck the extract through the frit into a 35 mL test tube using vacuum.
6) Repeat extraction + filtration with two additional portions of 8 mL acetone w/BHT
7) Evaporate the combined filtrates under a flow of N_2 in a 50 ºC water bath
8) Dissolve the dry and often oily residue in 3 mL of mobile phase (86:14, heptane:acetone) and transfer to a 10 mL volumetric flask
9) Rinse the tube with 2 x 3 mL heptane and combine the solutions with the extract in the volumetric flask
10) Dilute to the mark with mobile phase (86:14, heptane:acetone), shake and fill an aliquot of the solution to a centrifugation tube
11) Centrifuge at 2500 *g* in 5 min
12) Transfer 1 mL supernatant to a 2 mL glass vial with lid
13) Analyze sample on HPLC with normal phase column and UV/VIS detection

HPLC analysis

- Column: LiChrospher Silica-60 (4.6 mm, 25 cm, 5 µm)
- Column temperature: room temperature
- Mobile phase: 86 % heptan: 14 % acetone
- HPLC system: Ultimate 3000 UHPLC system (Thermo Scientific)
- Detector: UV/VIS (470 nm)
- Flow rate: 1.5 mL/min
- Total analysis time: appr. 15 min per sample
- Injection volume 100 µL (Manuel injection)
- Software: Chromeleon

Setting up a sequence in Chromeleon

1. Open Chromeleon – DesktopV5KTA95\ ChromeleonLocal - Instrument data - Ultimate 3000 - sequence - AX
2. Copy a previous sequence (right-click + copy, and paste under the desired folder)
3. Check that the method is called "AX kun UV"
4. Mark all the samples in the sequence (everything should be black).
5. Press Ctrl + c / Ctrl + v - (goes from «finished» to «idle»).
6. Give the sequence the following name; YYMMDD_rekvXX_lastname.

8. Calculations

Within 1h after preparation, measure the concentration of all-E AX by spectrometry at the respective absorption maximum using heptane as a blank. Calculate the mass concentration in mg/mL using equation:

$$\rho_{\text{all-E-astaxanthin}} = \frac{A_{\text{max}} \times 10\,000}{2\,100}$$

Where

- Amax is the absorbance value at the absorption maximum
- 10 000 is the scaling factor
- 2100 is the theoretical absorption of a 1% solution of all-E-astaxanthin in a 1 cm cell at approximately 470 nm (γ_{max}) in heptane.

- Open the chromatograms and check that the baselines are laid correctly
- Open the “interactive results” and check that the peaks for All E-AX, 9Z-AX and 13Z-AX is correctly identified (see figure 1).
- Copy/Paste the areas into the excel sheet "beregningsark" which can be found under:
  - labmal- various analyzes - astaxantin
- Enter the area of the standard (sum of all 3 peaks) to get the correct response factor

  $RF = A_{total} / \rho$

  RF = response factor

  Atotal = the total area of the 3 peaks for All E-AX, 9Z-AX and 13Z-AX

  ρ = the spectrometrically measured AX concentration in the standard solution, in mg/l.
- The sheet now automatically calculates the AX in mg/kg
- Remember to save the excel sheet on the form; YYMMDD_rekvXX_lastname

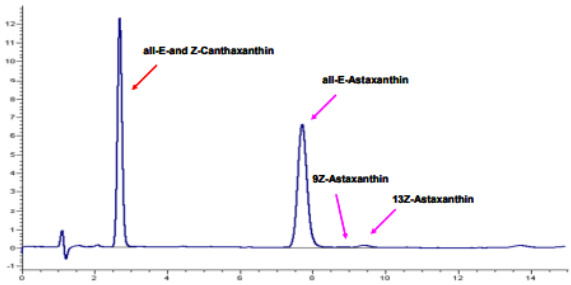

Figure 1: Chromatogram showing the separation of canthaxanthin and the isomers of astaxanthin [1]

9. Literature:

1) Østerlie, Sluttrapport: Utvikling av metode for analyse av pigment i muskel hos laksefisk. Høgskolen i Sør-Trøndelag (2010)

2) Darias Hernández, Tania. "Astaxanthin determination in marine biological samples: an overview." (2013)

3) CEN/TS 16233-1:2011 (E) - HPLC method for the determination of xanthophylls in fish flesh. Part 1: Determination of astaxanthin and canthaxanthin