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 autosampler and 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)

Internal standard: Trans-B-APO-8'-Carotenal (art.nr: 10810-1G – Merck)

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/mL.

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). This only must be done if the system is changed in some way. In all other cases it is enough to check the systems response factor by analyzing a heat-isomerized control solution at least three times during the sequence. Instructions on how to make the control solution, see next section.

Preparation of solution of heat-isomerized astaxanthin (control solution):
Weigh approximately 1,5 mg of all-E-astaxanthin and 0,5 g of BHT to the nearest 0,1 mg and dissolve in a 500 ml volumetric flask in 10 ml of tetrahydrofuran Dilute this solution with 200 ml of a mixture of 86 parts per volume of n-heptane and 14 parts per volume of acetone. Reflux for 1 h in a water bath at a temperature of 80 °C. Cool to room temperature and dilute the solution to the mark with the mixture of n-heptane and acetone. Pour the mixture into a dispenser bottle, mix well, leave at room temperature overnight and dispense into HPLC vials. Immediately seal the vials carefully with septa made from PTFE and silicone and store them at approximately 23 °C in the dark.

Preparation of intern standard solution
Weight in approximately 7,5 mg of trans-beta-apo-8 carotenal and transfer into a 200 mL volumetric flask. Dissolve in acetone, dilute in acetone to volume, and mix.

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 with autosampler
- 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 / 100 / 200 mL volumetric flask
- 10 mL glass tube w/screw cap
- Centrifuge
- 2 mL glass vials w/lid (preferable with a premade cross to avoid vacuum in the vial)

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) Add 8 mL of acetone with BHT, mix, and wait 3 minutes
4) Open the valve and suck the extract through the frit into a 35 mL test tube using vacuum.
5) Repeat extraction + filtration with two additional portions of 8 mL acetone w/BHT
6) Evaporate the combined filtrates under a flow of N_2 in a 50 ºC water bath
7) 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

8) Rinse the tube with 2 x 3 mL heptane and combine the solutions with the extract in the volumetric flask
9) Dilute to the mark with mobile phase (86:14, heptane:acetone), shake and fill an aliquot of the solution to a centrifugation tube
10) Centrifuge at 2500 *g* in 5 min
11) Transfer 900 µL supernatant to a 2 mL glass vial, add 100 µL internal standard solution, put on a lid an mix carefully
12) Analyze sample on HPLC with normal phase column and UV/VIS detection
13) Remember to prepare a control sample by mixing 900 µL heat isomerized control solution with 100 µL internal standard solution. Analyze this sample as the first sample in the sequence, and at least two more times during the sequence to check the response.

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: 1.2 mL/min
- Total analysis time: 16 min per sample
- Injection volume 50 µL (can be increased/decreased if necessary)
- 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 ultimate AS"
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.

8. Calculations

- 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).
- Check the internal standard peak (elutes at approximately 2,8 min)
- Copy/Paste the areas into the excel sheet "beregningsark" which can be found under:
  - labmal- various analyzes – astaxantin
- Divide all AX areas on the area of the internal standard to correct for variations in the injection volume (a problem in normal phase chromatography due to vacuum)
- Check that the astaxanthin standard is calculated to be approximately 2 mg/L
- The sheet now automatically calculates the AX in mg/kg in the samples
- Remember to save the excel sheet on the form; YYMMDD_rekvXX_lastname

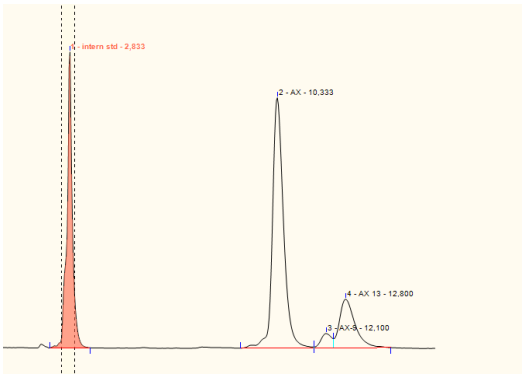

Figure 1: Chromatogram showing the separation of the internal standard and the isomers of astaxanthin

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