Determination of Stabilised Canthaxanthin in Premixes and Feedstuffs

1. Introduction

This method is part of a collection of methods suitable for the determination of vitamins and carotenoids in premix and compound feed. The method is appropriate for the use in the feed industry.

2. Keywords

Canthaxanthin, E/Z-isomers, *cis-trans* isomers, premix, feed, HPLC.

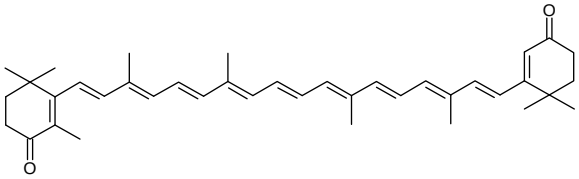
2.1. Subject of Analysis

Determination of stabilised canthaxanthin in premix and feed.

2.2. Matrix

Premix and feed.

2.3. Structure



all-E-Canthaxanthin

3. Table of Contents

4. Scope

This method specifies the determination of the total canthaxanthin in premixes and complete feedstuffs by High Performance Liquid Chromatography (HPLC).

5. Terms and Definitions

- In present method the geometrical isomers of canthaxanthin are identified by the characters Z and E which correspond to the terms *cis* and *trans*, respectively.
- The term *total canthaxanthin* means the total amount of canthaxanthin and corresponds to the sum of all geometrical canthaxanthin isomers detected.

6. Principle

The assay comprises an enzymatic digestion of the formulation followed by extraction with ethanol and dichloromethane. The extract is injected into an isocratic normal-phase HPLC system that is able to resolve the all-E isomer and the main Z isomers of canthaxanthin. Canthaxanthin from carotenes and other xanthophylls possibly present in feed such as apoester (Ethyl-8'-apocarotenoic ester), citranaxanthin, lutein and zeaxanthin. The Z isomers of canthaxanthin are quantified on basis of the response of all-E canthaxanthin. The lower specific absorbance of the main Z-isomers (9Z and 13Z canthaxanthin) is taken into account by correction with an experimentally determined relative response factor.

7. Range

Above 1 mg/kg for feed
Above 0.5 g/kg for premix

8. Safety Notes

- Ethanol is highly flammable.
- n-Hexane, n-heptane, cyclohexane and acetone are highly flammable, irritating to skin and eyes, and harmful by inhalation and if swallowed.
- Diethyl ether is extremely inflammable, may form explosive peroxides, is irritating to skin and eyes and harmful by inhalation and if swallowed.
- Dichloromethane and chloroform are harmful by inhalation and if swallowed. The solvents are irritating to skin and eyes and there is limited evidence of a carcinogenetic effect.
- Butylated hydroxytoluene (BHT) is harmful by inhalation and if swallowed, and irritating to skin and eyes.

Most of these reagents are harmful to aquatic animals. Adequate measures have to be taken to avoid damage to health and environment.

9. Reagents

- Chloroform, puriss. p.a. (e.g. Fluka no. 25690)
- Dichloromethane, p.a. (e.g. Merck no. 6050)
- n-Hexane, p.a. (e.g. Merck no. 4367)
- n-Heptane, p.a. (e.g. Merck no. 4379)
- Cyclohexane, p.a. (e.g. Fluka no. 28932)
- Diethyl ether, stabilized with 0.005% BHT (e.g. Fluka no. 31690)
- Ethanol absolute, p.a. (e.g. Merck no. 983)
- Acetone, p.a. (e.g. Merck no. 14)
- Silica gel 60, particle size 0.2-0.5 mm, 35-70 mesh, for column chromatography (e.g. Merck no. 7733)
- Water, distilled or demineralised
- Maxatase, P440000 encapsulated (Genencor International)
- Protex 6L (Genencor International)
- Butylated hydroxytoluene (BHT, e.g. ICN Biochemicals no. 203824)
- Mobile phase: In a 1000 mL volumetric flask, 70 mL acetone are combined with ca. 900 mL of n-hexane. Mixing these solvents results in a decrease in temperature and volume. The mixture is warmed up to room temperature and then adjusted to volume with n-hexane. The solution is stable at 20-25°C for at least 1 month.
- Reference substance of all-E canthaxanthin, purity (HPLC) > 95% (e.g. Dr Ehrenstorfer GmbH, Augsburg, Germany). The reference substance has to be stored under argon or nitrogen at approx. -20°C.

10. Equipment

- Grinder (e.g. coffee grinder MX 32/MXK; Braun AG., Frankfurt/M., Germany)
- Ultrasonic water bath, 150 W at 35 kHz (e.g. TUC-150, Telsonic, Bronschhofen, Switzerland)
- Rotary evaporator (e.g. Rotavapor, Büchi, Flawil, Switzerland)
- Spectrophotometer (e.g. UVICON 930, Kontron, Zürich, Switzerland)
- Centrifuge (e.g. Megafuge 1.0, Heraeus, Zürich, Switzerland)
- Balances (e.g. PM 2000 and AT 261 Delta Range, Mettler-Toledo)
- 10 mL funnel-shaped SPE columns (e.g. SPE columns, No. 120-1005-HX empty columns fitted with single frits, ICT, Basel, Switzerland)
- Solid-phase extraction manifold (e.g. SPE manifold, Visiprep® No. 5-7030; 0.90x55-mm steel needles are attached to the outlets, Supelco, Buchs, Switzerland)
- Multi-pipette (e.g. Eppendorf Multipette® plus No. 4980000.015 with 10 or 50 mL Combitips No. 30069.269 or 30069.277, Vaudaux, Schönenbuch, Switzerland)
- Electric dispenser (e.g. Microlab®500 - 3.5 mL extract is aspirated and 1 mL dispensed, acetone as flushing solvent, Hamilton, Bonaduz, Switzerland)
- Vortex (e.g. MS1 IKA Minishaker , Instrumenten-Gesellschaft AG, Zürich, Switzerland)
- SpeedVac (e.g. SpeedVac® Plus, SC210A with RH48-18-125 rotor for 48 test tubes, (Savant Instruments, NY, USA)
- HPLC modules:
  - Autosampler (e.g. Mod. 717, Waters)
  - Pump (e.g. PU-1580, Jasco)
  - UV/VIS detector (e.g. UV2070plus, Jasco)
  - Integrator (e.g. Atlas Chromatography Data System, Thermo Lab Systems)

11. Sampling

Samples of approx. 100 g are taken from premix. From feed pellets or mash feed samples of approx. 250 g are taken.

12. Standard Solutions and Calibration

12.1. Preparation of standard solution

Weigh approx. 3 mg of crystalline all-E canthaxanthin and 1 g BHT into a 100 mL volumetric flask. Add approx. 20 mL of chloroform and put the flask in ultrasonic water bath at ambient temperature for approx. 30 sec. Then make up to volume with cyclohexane and mix well. Pipette 10.0 mL of this solution into a second 100 mL volumetric flask and fill up to volume, yielding a concentration of approx. 3 mg of canthaxanthin per litre cyclohexane/chloroform (98:2; v/v).

12.2. Spectrophotometry of standard solution:

Immediately after preparation, measure the absorption of the standard solution against cyclohexane at the maximum (approx. 466 nm) by a spectrophotometer (measure the maximum). Calculate the canthaxanthin concentration according to formula 1 (see section 15.1.)

12.3. HPLC of standard solution

Immediately after preparation, repeatedly inject 20 µl aliquots of the standard solution into the HPLC system. Determine the total peak areas of the chromatograms (excluding the solvent peak) and calculate the average of all chromatograms. Calculate the response factor for all-E canthaxanthin from the averaged total peak areas and the spectrophotometrically measured canthaxanthin concentration according to formula 2 (see 15.2.).

12.4. Constancy of the HPLC system

Calibrations can be routinely performed e.g. every three months. During the interval between calibrations, the constancy of the HPLC system is controlled via control solutions analysed along with each set of samples. These controls are solutions of heat-isomerised canthaxanthin, concentrations of which have been found to be stable at approx. 4°C in darkness over at least 3 months.

12.4.1. Preparation of the control solution

In a 500 mL volumetric flask, dissolve approx. 1.5 mg of crystalline canthaxanthin (e.g. reference substance) and 0.5 g of BHT in 10 mL of chloroform. The solution is diluted with approx. 200 mL of n-heptane/acetone (93:7; v/v) and refluxed for 2 h at a water bath temperature of 80°C. After cooling, the solution is made up to volume with n-hexane/acetone (93:7; v/v). The mixture is poured into a dispenser bottle, mixed well, left over night at ambient temperature and then apportioned in a number of LC vials. Immediately after filling, the vials are carefully sealed with Teflon/silicone septa and stored at approx. 4°C in the dark.

13. Procedure

13.1. Preparation of samples:

Grind approx. 30 - 40 g of pellets in a coffee grinder. Mash feed and premix do not required pre-grinding before use.

13.2. Extraction:

The extraction procedure depends on the canthaxanthin concentration in the sample.

13.2.1. Premixes and feed with a declared canthaxanthin content of 1000 mg/kg and more

Weigh accurately approx. 2-3 g of sample with a declared canthaxanthin content of ≥ 1000 mg/kg into a tared 100 mL volumetric flask. Add approx. 100 mg of BHT, approx. 500 mg of Maxatase or 500 µL of Protex 6L, and 6 mL of demineralised water. Shake in a way that all solids are covered by water and place the flask for 30 min in an ultrasonic water bath at 50°C. Add 40 mL of ethanol to the warm suspension, shake, add 50 mL of dichloromethane and shake again. Mixture cools and contracts. Leave the flask in darkness until ambient temperature is reached and the volume has increased again (approx. 1-2 h). Make up to volume with dichloromethane, mix well, and let solids settle. Pipette 5 mL of the extract into a 100-mL volumetric flask and adjust to volume with n-hexane/acetone (93:7; v/v). Fill an aliquot of the solution into a LC vial and centrifuge at approx. 4000 rpm for 5 min in a standard laboratory centrifuge. Inject 20 µL into the HPLC.

13.2.2. Feed with declared canthaxanthin content between 20 and 1000 mg/kg

13.2.2.1. Pelleted or extruded feed

Weigh accurately approx. 2-3 g of ground sample with a declared canthaxanthin content between 20 and 1000 mg/kg into a tared 100 mL volumetric flask. Add approx. 100 mg of BHT, approx. 100 mg of Maxatase or 100 µL of Protex 6L, and 6 mL of de-mineralised water. Shake in a way that all solids are covered by water and place the flask for 30 min in an ultrasonic water bath at 50°C. Add 40 mL of ethanol to the warm suspension, shake, add 50 mL of dichloromethane and shake again. Mixture cools and contracts. Leave the flask in darkness until ambient temperature is reached and the volume has increased again (approx. 1-2 h). Make up to volume with dichloromethane, mix well, and let solids settle. Purify the extract as described below.

13.2.2.2. Mash feed

Weigh accurately approx. 10 g of mash feed with a declared canthaxanthin content between 20 and 1000 mg/kg into a weighed 100 mL volumetric flask using a funnel. Add approx. 500 mg of BHT, approx. 100 mg of Maxatase or 100 µL of Protex 6L, and 40 mL of demineralised water. Shake in a way that all solids are covered by water and place the flask for 30 min in an ultrasonic water bath at 50°C. Add 50 mL of ethanol to the warm suspension, shake, cool to ambient temperature, and adjust to volume with demineralised water. Weigh the flask again in order to calculate the weight of the aqueous-alcoholic suspension (W_1), shake vigorously, and pour immediately 8-12 g of the mixture into a tared 100 mL volumetric flask. Weigh the transferred aliquot of the suspension (W_2). Add 35 mL of ethanol, shake, add 50 mL of dichloromethane and

shake again. Mixture cools and contracts. Leave the flask in darkness until ambient temperature is reached and the volume has increased again (approx. 1-2 h). Make up to volume with dichloromethane, mix well, and let solids settle. Purify the extract as described below.

13.2.3. Feed with declared canthaxanthin content lower than 20 mg/kg

Weigh accurately approx. 50 g of feed with a declared canthaxanthin content lower than 20 mg/kg into a wide-neck 250 mL volumetric flask using a funnel. Add approx. 500 mg of BHT, approx. 500 mg of Maxatase or 500 µL of Protex 6L, and 110 mL of demineralised water. Shake in a way that all solids are covered by water and place the flask for 30 min in an ultrasonic water bath at 50°C. Add 100 mL of ethanol to the warm suspension, shake, cool to ambient temperature, and adjust to volume with demineralised water. Weigh the flask again in order to calculate the weight of the aqueous-alcoholic suspension (W_1), shake vigorously, and pour immediately 8-12 g of the mixture into a tared 100 mL volumetric flask. Weigh the transferred aliquot of the suspension (W_2). Add 35 mL of ethanol, shake, add 50 mL of dichloromethane and shake again. Mixture cools and contracts. Leave the flask in darkness until ambient temperature is reached and the volume has increased again (approx. 1-2 h). Make up to volume with dichloromethane, mix well, and let solids settle. Purify the extract as described below.

13.3. Purification of the extract

The extract is purified by open-column chromatography on silica gel. The silica gel must be fresh (stored at a dry place) and fulfil exactly the given specifications. The material can be used only once. Two alternative procedures can be used.

13.3.1. Large scale procedure

Fill a chromatography tube with approx. 10 mL of n-hexane/diethyl ether (1:1; v/v) and add 5 g of silica gel. Disperse the silica gel (e.g. with a jet of n-hexane/diethyl ether (1:1; v/v) from a dispenser) so that all air bubbles are removed. Drain out and waste the solvent until the surface of the silica gel is just covered with solvent. Pipette 25.0 mL (V_1) of the water-ethanol-dichloromethane extract onto the silica gel and elute with 120 mL of the mixture n-hexane/diethyl ether (1:1; v/v). Collect the eluate in a 250 mL round-bottom flask and remove the solvent by means of a rotary evaporator under reduced pressure at 50 °C for approx. 10 min. Dissolve the residue in 5 or 10 mL (V_2) of n-heptane/acetone (93:7; v/v). Transfer aliquots of the solution into HPLC vials, centrifuge at 4000 rpm for 5 min (if necessary) and analyse by HPLC.

13.3.2. Small scale procedure

Insert 10 mL funnel-shaped SPE columns into the flow control valves of a solid-phase extraction manifold and fill each of the columns with 300 mg of silica gel. Disperse the silica gel (e.g. with a jet of approx. 3 mL of n-hexane/diethyl ether (1:1; v/v) from a dispenser) to remove any air bubbles. Drain out and waste the solvent until the solvent just covers the silica gel. Transfer 1.00 mL (V_1) of the water-ethanol-dichloromethane extract with a sufficiently precise pipetting device (e.g. electric dispenser) onto the column. Let the extract penetrate the silica gel completely and elute the carotenoids with 5 mL n-hexane/diethyl ether (1:1; v/v) without using vacuum (flow rate is adjusted by the diameter of the outlet needles). Collect the eluate in a 10 mL test tube, mix well

on a Vortex (necessary in order to avoid bumping) and evaporate the solvent in a SpeedVac at approx. 40°C under reduced pressure for approx. 45 min. Add 1.00 mL (V_2) of n-heptane/acetone (93:7; v/v) (e.g. with Eppendorf Multipette), close the tube and agitate on a test-tube shaker to dissolve the residue. Transfer aliquots of the solution into HPLC vials, centrifuge at 4000 rpm for 5 min (if necessary) and analyse by HPLC.

14. HPLC

14.1 Conditions

- Column: LiChrosorb Si60, 5 µm, 250 x 4 mm (VWR)
- Mobile phase: n-Heptane/acetone (93:7; v/v), isocratic
- Flow rate: 1.5 mL/min
- Pressure: approx. 80 bar
- Temperature: ambient (e.g. 23 °C)
- Injection volume: 20-200 µL
- Detection: VIS at 466 nm
- Run time: 18 min

14.2. Retention times

Retention times:

Retention of all-E-canthaxanthin:	approx. 11 min
Approximative relative retention times (in relation to all-E-canthaxanthin):	
α-Carotene:	0.15 (all-E- and Z-isomers)
β-Carotene:	0.15 (all-E- and Z-isomers)
Ethyl 8'-apo-β-carotenoate (apoester):	0.23 (all-E- and Z-isomers)
Z-Citranaxanthin:	0.32 - 0.41
all-E-Citranaxanthin:	0.44
Z-Canthaxanthin:	0.84-0.91
all-E-Canthaxanthin:	1.00
9Z- and 13Z-Canthaxanthin:	1.10
Z-β-Cryptoxanthin:	0.82-0.99
all-E-β-Cryptoxanthin:	1.08
all-E-Lutein:	13
all-E-Zeaxanthin:	15

15. Calculations

15.1. Spectrophotometric canthaxanthin concentration of the standard solution:

$$\text{Canthaxanthin [mg/L]} = \frac{\text{Absorption} \cdot 10000}{2200} \qquad \textit{formula 1}$$

15.2. Response factor of all-E canthaxanthin:

$$RF_{\text{all-E canthaxanthin}}\ [\text{mVsL/mg}] = \frac{A_{total}}{c} \qquad \textit{formula 2}$$

15.3. Canthaxanthin content in feed or premix samples:

$$\text{Canthaxanthin [mg/kg]} = \frac{(A_{\text{all-E}} + A_Z \cdot 1.3) \cdot V}{m \cdot RF_{\text{all-E}}} \qquad \textit{formula 3}$$

2200: E (1%/1cm) = Theoretical Absorption of an 1% canthaxanthin solution (w/v) in an 1 cm cell at the maximum of absorption (approx. 466 nm) in cyclohexane [ref. 1]
10 000: Scaling factor.
A_{total}: Averaged total peak area of chromatograms of standard solution)excluding the solvent pekas) [mVs]
$A_{all\text{-}E}$: Peak area of all-E canthaxanthin [mVs]
A_Z: Peak area of Z isomers of canthaxanthin [mVs]
c: Spectrophotometrically determined canthaxanthin concentration in standard solution [mg/L] (see above)
m: Sample weight [g]
$RF_{all\text{-}E}$: Response factor of all-E canthaxanthin [mVsL/mg]
1.3: Relative response factor of Z isomer fraction of canthaxanthin
V: Dilution [mL] (= theoretical volume in which the sample is dissolved)

For extraction variant 13.2.1. $V = \frac{100 \cdot 100}{5} = 2000$ *formula 4*

For extraction variant 13.2.2.1. $V = \frac{100 \cdot V_2 \cdot 20}{V_1 \cdot V_{Inj}}$ *formula 5*

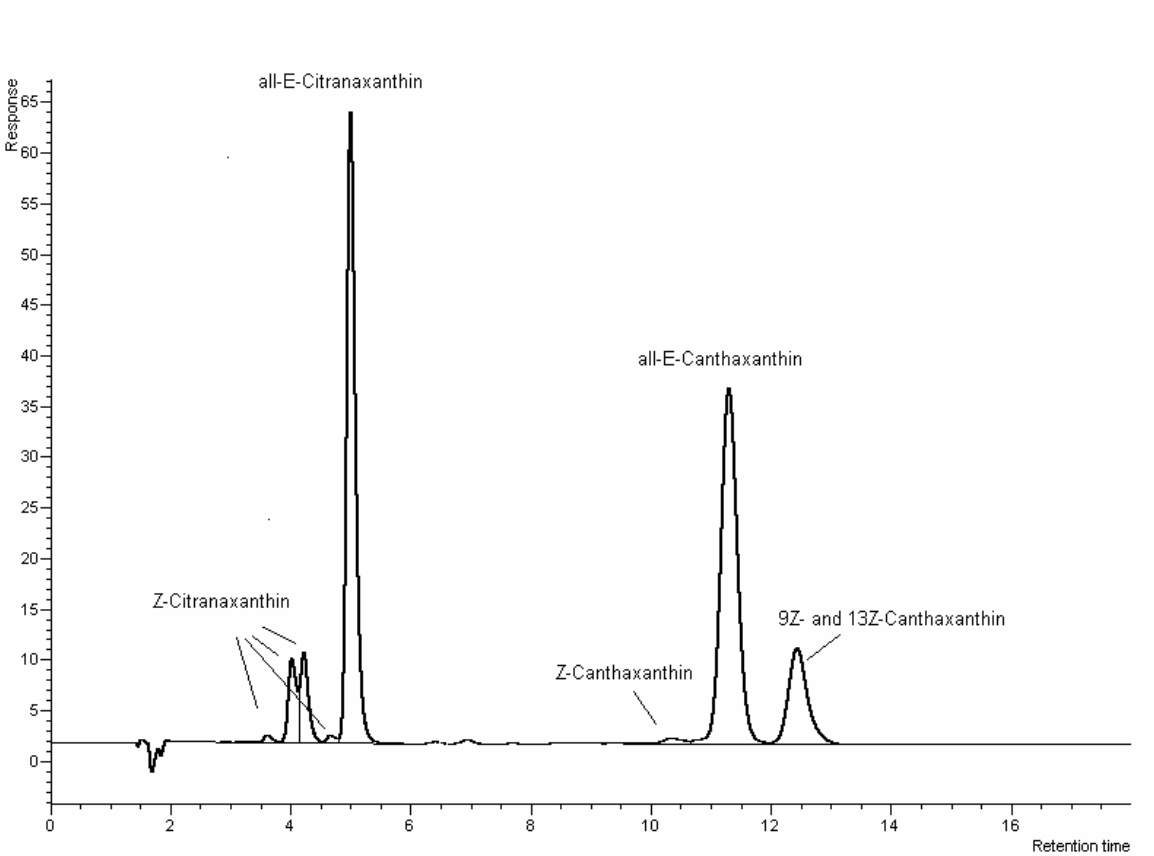
For extraction variant 13.2.2.2. $V = \frac{W_1 \cdot 100 \cdot V_2 \cdot 20}{W_2 \cdot V_1 \cdot V_{Inj}}$ *formula 6*

For extraction variant 13.2.3. $V = \frac{W_1 \cdot 250 \cdot V_2 \cdot 20}{W_2 \cdot V_1 \cdot V_{Inj}}$ *formula 7*

V_1: Volume of the aliquot of extract which is transferred onto the silica-gel column [mL]
V_2: Volume of the final sample (test) solution [mL]
W_1: Weight of the aqueous-alcoholic suspension in the first flask [g]
W_2: Weight of the aliquot of aqueous/alcoholic suspension (8-12 g) transferred to the 100 mL flask [g]
5: Volume of the aliquot of ethanol-dichloromethane extract to be diluted [mL]
100: Volume of volumetric flask [mL]
250: Volume of volumetric flask [mL]
20: Injection volume of the standard solution used for calibration [µL]
V_{Inj}: Injection volume of sample (test) solution [µL]

16. Chromatogram

Chromatogram of an extract of feed containing stabilized canthaxanthin (besides citranaxanthin).



17. Notes

The relative response factor (section 15.) is an experimentally determined correction factor for the lower specific absorbance of 9Z and 13Z canthaxanthin compared to all-E canthaxanthin.

18. Bibliography

[1] Carotenoids, Handbook, G. Britton, S. Liaaen-Jensen, H. Pfander, Birkhäuser Verlag, Basel, Boston, Berlin (2004), ISBN 3-7643-6180-8