Determination of Stabilised β-Carotene in Premixes and Feedstuffs

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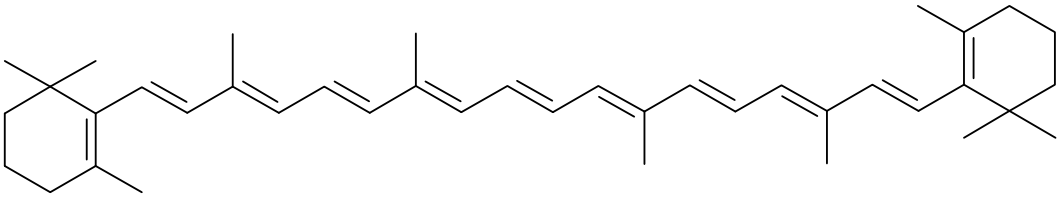
Introduction

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.

Structure:



all-E β-Carotene

Determination of Stabilised β-Carotene in Premixes and Feedstuffs

1 Scope

This method specifies the determination of the total β-carotene in premixes and complete feedstuffs by High performance liquid chromatography (HPLC).

2 Terms and Definitions / Keywords

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

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

3 Principle

The assay comprises an enzymatic digestion of the formulation followed by extraction with ethanol and dichloromethane. The extract is injected into an isocratic reversed-phase HPLC system that is able to resolve the all-E isomer and the main Z isomers of β-carotene. β-Carotene is separated from α-carotene and xanthophylls such as β-cryptoxanthin, lutein and zeaxanthin. The Z isomers of β-carotene are quantified on basis of the response of all-E β-carotene. The lower specific absorbance of Z isomers is taken into account by correction with experimentally determined relative response factors.

4 Safety Notes

- Ethanol is highly flammable.
- Tetrahydrofuran, cyclohexane, methanol, 2-propanol, acetonitrile, and N-ethyldiisopropylamine are higly fammable solvents, 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.

5 Reagents

- Acetonitrile, p.a. (e.g. Rathburn Chemicals no. 1016)
- Ammonium acetate, p.a. (e.g. Fluka no. 09690)
- Ascorbic acid (Vitamin C), p.a. (e.g. Merck no 127)
- Butylated hydroxytoluene (BHT, e.g. ICN Biochemicals no. 203824)
- Chloroform, puriss. p.a. (e.g. Fluka no. 25690)
- Cyclohexane, p.a. (e.g. Fluka no. 28932)
- Dichloromethane, p.a (e.g. Merck no. 6050)
- Ethanol, absolute, p.a. (e.g. Merck no. 983)
- N-Ethyldiisopropylamine, p.a. (e.g. Fluka no. 03440)
- Methanol, p.a. (e.g. Merck no. 6009)
- Maxatase, P440000 encapsulated (Genencor International)
- 2-Propanol, p.a. (e.g. Fluka no. 59300)
- Water, distilled or demineralised
- Protex 6L (Genencor International)
- Tetrahydrofuran, purum (stabilised with 0.025% BHT, e.g. Fluka no. 87368)
- Ammonium acetate (0.2%): 0.5 g of ammonium acetate is dissolved in 250 mL demineralised water
- Mobile phase: In a 1000 mL volumetric flask 50 mg of BHT are dissolved in 20 mL of 2-propanol. 0.2 mL of N-ethyldiisopropylamine, 20 mL of aqueous ammonium acetate (0.2%), 455 mL acetonitrile and approx. 450 mL of methanol are combined with the BHT solution. Mixing these reagents results in a decrease in temperature and volume. The solution is warmed up to room temperature and then adjusted to volume with methanol. It can be used for approx. two days.
- Reference substance of all-E β-carotene, purity (HPLC) > 95% (e.g. Dr Ehrens-torfer GmbH, Augsburg, Germany). The reference substance has to be stored under argon or nitrogen at approx. -20°C.

6 Apparatus

- 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)
- HPLC modules:
  - Autosampler (e.g. Waters WISP 717, Millipore Corp.)
  - Pump (e.g. L-6200, Merck)
  - Degasser (e.g. Degassex DG-440, Phenomenex)
  - Column thermostat (e.g. Jetstream 2 plus, Thermotechnic products, Konton)
  - VIS detector (e.g. Severn Analytical, Mod SA 6504, Stagroma AG)
  - Integrator (e.g. Atlas Chromatography Data System, Thermo Lab Systems)

7 Sample Preparation / Procedure

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

7.1 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.

7.2 Extraction

The extraction procedure depends on the β-carotene concentration in the sample.

7.2.1 Premixes and feed with a declared β-carotene content of 100 mg/kg and more

Weigh accurately approx. 1 g premix or feed with a declared β-carotene content of ≥ 1000 mg/kg or 3 g feed with a declared β-carotene content between 100 and 1000 mg/kg into a 250 mL volumetric flask. Add approx. 100 mg vitamin C, approx. 100 mg of Maxatase or 100 µl of Protex 6L, and 10 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, add 130 mL of dichloromethane and shake again. Mixing the solvents results in a decrease of temperature and a reduction in volume of the mixture. 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 and mix well. Fill an aliquot of the turbid 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.

7.2.2 Feed with declared β-carotene content of between 20 and 100 mg/kg

Accurately weigh approx. 10 g of feed into a tared 100 mL volumetric flask through a wide funnel. Add approx. 100 mg of vitamin C, approx. 100 mg of Maxatase or approx. 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 ethanol to the warm suspension, cool to ambient temperature and adjust to the mark with demineralised water. Weigh again, shake vigorously and immediately pour 8 - 12 g of the mixture into a tared 100 mL flask through a funnel. Weigh the transferred aliquot of the suspension. Add 35 mL of ethanol, shake, add 50 mL of dichloromethane and shake again. Mixing the solvents results in a decrease of temperature and a reduction in volume of the mixture. Leave the flask in the dark 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. Fill an aliquot of the supernatant into a LC vial and centrifuge at approx. 4000 rpm for 5 min with a standard laboratory centrifuge. Inject 20 µL into the HPLC.

7.2.3 Feed with declared β-carotene content less than 20 mg/kg

Accurately weigh approx. 50 g of feed into a tared 250 mL volumetric flask through a wide funnel. Add approx. 100 mg of vitamin C, 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, cool to ambient temperature, and adjust to the mark with demineralised water. Weigh again, shake vigorously and immediately pour 8 - 12 g of the mixture into a tared 100 mL flask through a funnel. Weigh the transferred aliquot of the suspension. Add 35 mL of ethanol, shake, add 50 mL of dichloromethane and shake again. Mixing the solvents results in a decrease of temperature and a reduction in volume of the mixture. Leave the flask in the dark until ambient temperature is reached and the volume has increased again (approx. 1-2 h).

Make up the flask to volume with dichloromethane, mix well, and let solids settle. Fill an aliquot of the supernatant into a LC vial and centrifuge at approx. 4000 rpm for 5 min in a standard laboratory centrifuge. Inject 20 µL into the HPLC.

8 Standard Solutions and Calibration

8.1 Preparation of standard solution

Weigh approx. 4 mg of crystalline all-E β-carotene into a 100 mL volumetric flask. Add approx. 20 mL of tetrahydrofuran and put the flask in ultrasonic water bath at ambient temperature for approx. 30 sec. Then make up to volume with tetrahydrofuran and mix well. Pipette 5.0 mL of this solution into a second 100 mL volumetric flask and dilute to volume with cyclohexane. Thus, a concentration of approx. 1.5 mg of β-carotene per litre cyclohexane/tetrahydrofuran (95:5; v/v) is achieved.

8.2 Spectrophotometry of standard solution:

Immediately after preparation, measure the absorption of the standard solution against cyclohexane at the maximum (approx. 456 nm) by a spectrophotometer. Calculate the β-carotene concentration according to formula 1 (see 10.1, section Calculation).

8.3 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 average. Calculate the response factor for all-E β-carotene from the averaged total peak areas and the spectrophotometrically measured β-carotene concentration according to formula 2 (see 10.2., section Calculation).

8.4 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 β-carotene, concentrations of which have been found to be stable at approx. 4°C in darkness over at least 3 months.

8.4.1 Preparation of the control solution

In a 500 mL volumetric flask, dissolve approx. 1.5 mg of β-carotene reference substance and 0.5 g of BHT in 10 mL of chloroform. The solution is diluted with approx. 200 mL of ethanol/tetrahydrofuran (9:1; 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 ethanol/tetrahydrofuran (9:1; v/v). The mixture is poured into a dispenser bottle, mixed well, left over night at ambient temperature and then apportioned in a large 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.

9 HPLC

9.1 Conditions

- Column: Suplex pKb-100, Supelco, 5 µm, 250x4.6 mm
- Mobile phase: 2-Propanol/ammonium acetate/acetonitrile/methanol including BHT and N-ethyldiisopropylamin (see 5., section Reagents)
- Column temperature: 30 °C
- Autosampler temperature: 15 °C
- Flow rate: 0.8 mL/min
- Pressure: approx. 33 bar
- Injection volume: 20 µL
- Detection: 448 nm
- Run time: 35 min

9.2 Retention times

Absolute retention time of all-E β-carotene: approx. 20-25 min
Relative retention times (in relation to all-E β-carotene):

- all-E Lutein: approx. 0.30
- all-E Zeaxanthin: approx. 0.32
- all-E Cryptoxanthin: approx. 0.60
- all-E Lycopene: approx. 0.67
- Z Lycopene isomers approx. 0.69 - 0.79
- all-E α-carotene: approx. 0.93
- all-E β-carotene: approx. 1.00
- 9Z β-carotene: approx. 1.07
- other Z β-carotene isomers: approx. 1.10
- 13Z β-carotene: approx. 1.17
- 15Z β-carotene: approx. 1.21

10 Calculation

10.1 Spectrophotometric β-carotene concentration of the standard solution:

$$\beta\text{-Carotene [mg/l]} = \frac{\text{Absorption} \cdot 10000}{2500} \qquad \textit{formula 1}$$

2500: E (1%/1cm) = Absorption of a 1% β-carotene solution (w/v) in a 1 cm cell at the maximum of absorption (approx. 456 nm) in cyclohexane [ref. 2, 3, 4]
10 000: Scaling factor.

10.2 Response factor of all-E β-carotene:

$$RF_{\text{all-E β-Carotene}}\ [\text{mVsl/mg}] = \frac{A_{tot}}{c} \qquad \textit{formula 2}$$

10.3 β-Carotene content in feed or premix samples:

$$\beta\text{-Carotene [mg/kg]} = \frac{(A_{\text{all-E}} + A_{9Z} + A_{XZ} + A_{13Z} \cdot 1.2^{*} + A_{15Z} \cdot 1.4^{*}) \cdot V}{m \cdot RF_{\text{all-E}}} \qquad \textit{formula 3}$$

A_{tot}: Mean total peak area of standard solution [mVs]
$A_{all\text{-}E}$: Area of all-E β-carotene [mVs]
A_{9Z}: Area of 9Z β-carotene [mVs]
A_{XZ}: Area of other non-identified Z isomer(s) of β-carotene [mVs]
A_{13Z}: Area of 13Z β-carotene [mVs]
A_{15Z}: Area of 15Z β-carotene [mVs]
c: Spectrophotometrically determined β-carotene concentration in standard solution [mg/l] (see above)
m: Sample weight [g]
$RF_{all\text{-}E}$: Response factor of all-E β-carotene [mVsl/mg]
1.2: Relative response factor of 13Z β-carotene
1.4: Relative response factor of 9Z β-carotene
(the relative response factors are experimentally determined correction factors for the lower specific absorbance of 13Z and 15Z β-carotene compared to all-E β-carotene [ref. 1])
V: Dilution [mL] (= theoretical volume in which the sample is dissolved)

For extraction variant 7.2.1: $V = 250$ *formula 4*

For variants 7.2.2 and 7.2.3: $V = \frac{W_1 \cdot 100}{W_2}$ *formula 5*

250: Volume of the volumetric flask used in extraction variant 7.2.1 [mL]
W_1: Weight of the aqueous/ethanolic suspension in the first flask used in extraction variants 7.2.2 and 7.2.3 [g]
W_2: Weight of the aliquot of aqueous/ethanolic suspension (8-12 g) transferred into the second 100 mL flask used in extraction variants 7.2.2 and 7.2.3 [g]
100: Volume of the flask used for dichloromethane extraction in variants 7.2.2 and 7.2.3 [mL]

11 Repetition of Analyses

All analyses are conducted in duplicates. The results of the two determinations are compared by calculating the relative residual standard deviation (RSD%). The mean of the double determination is reported as result if the RSD% is below the limit for repetition (see below). If the RSD% exceeds this limit a further double determination is conducted. The mean of all four determinations is then reported, except one of the four values is clearly identifiable as outlier. In this case, the outlaying value is not taken into account and the mean calculated from the three values left.

Limits for repetition (RSD%, n = 2):
5% for samples with contents ≥ 1000 ppm
10% for samples with contents between 20 and 1000 ppm
15% for samples with contents ≤ 20 ppm

12 Measurement Uncertainty

The measurement uncertainty of results generated with the present method may be estimated from the Horwitz predicted relative residual standard deviation:

$RSD_R(\%) = 2C^{(-0.15)}$.

Doubling this concentration-dependent value gives the expanded measurement uncertainty for a confidence level of approximately 95%**.**

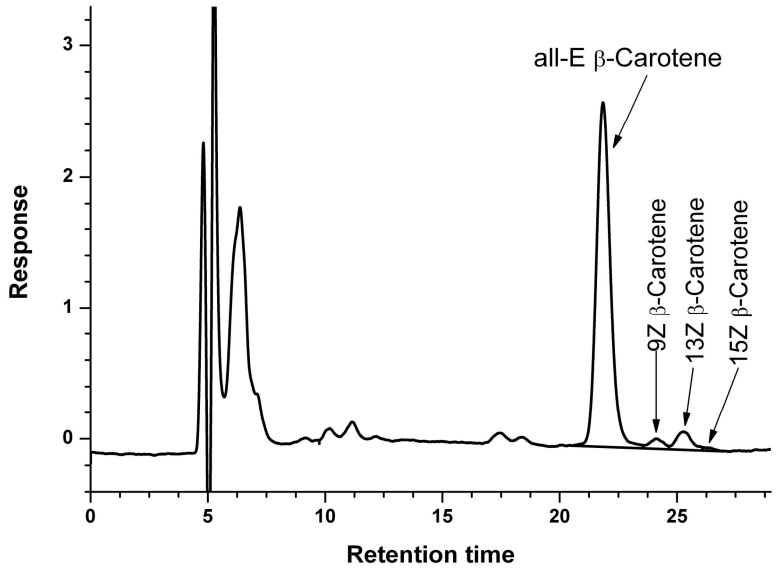
Expanded Measurement Uncertainty = 2 $RSD_R(\%) = 4C^{(-0.15)}$:

Concentration C	C (in decimals)	Expanded Uncertainty (%)
10 g/kg	0.01	± 8
1 g/kg	0.001	± 11
100 mg/kg	0.0001	± 16
10 mg/kg	0.00001	± 22*
1 mg/kg	0.000001	± 32
< 100 µg/kg	0.0000001	± 44

* e.g. range of acceptable concentrations = 7.8 – 12.2 mg/kg

13 Typical Chromatogram

13.1 Chromatogram of an extract of feed containing stabilized β-carotene.



14 Notes

- The extraction procedure of the present method does not quantitatively extract endogenous β-carotene from plant cells with thick cellulose walls. For such matrices, extraction by direct saponification would be preferable.
- In the present HPLC system, esters of xanthophylls with fatty acids, rarely present in premixes and feeds in critical amounts, can co-elute with the β-carotene isomers. This interference could be avoided by including a saponification step in extraction or work-up.
- Injection volumes above 20 µl are not compatible with the mobile phase and can lead to peak deformation (double peaks)

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