

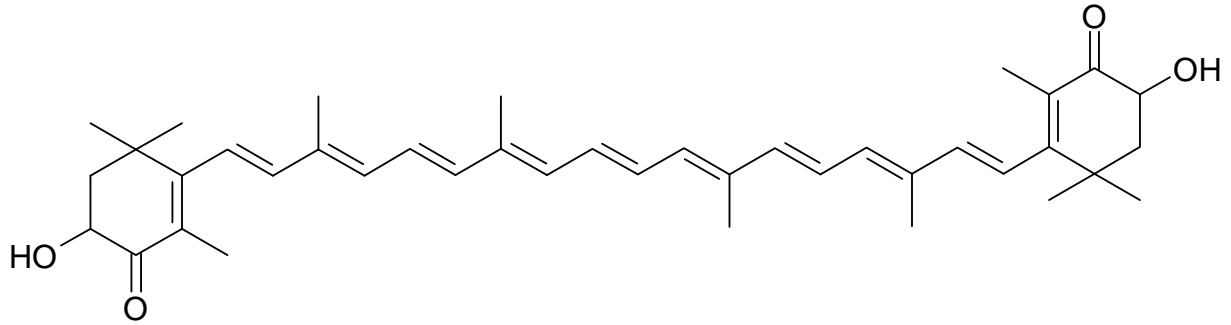
Determination of Stabilised Astaxanthin in Premixes and Feedstuffs

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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 Astaxanthin

Determination of Stabilised Astaxanthin in Premixes and Feedstuffs

1 Scope

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

2 Terms and Definitions / Keywords

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

- In present method the geometrical isomers of astaxanthin are identified by the characters Z and E which correspond to the terms *cis* and *trans*, respectively.
- The term *total astaxanthin* means the total amount of astaxanthin and corresponds to the sum of all geometrical astaxanthin 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 normal-phase HPLC system that is able to resolve the all-E isomer and the main Z isomers of astaxanthin. Astaxanthin is separated from its oxidation products astacene and semiastacene as well as from carotenes and other xanthophylls possibly present in feed such as canthaxanthin, lutein and zeaxanthin. The Z isomers of astaxanthin are quantified on basis of the response of all-E astaxanthin. The lower specific absorbance of the 9Z and 13Z isomer is taken into account by correction with experimentally determined relative response factors.

4 Safety Notes

Ethanol is highly flammable.

n-Hexane, n-heptane, acetone, and methanol are highly flammable, irritating to skin and eyes, and harmful by inhalation and if swallowed.

Diethyl ether is extremely flammable, 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 carcinogenic effect.

Butylated hydroxytoluene (BHT) is harmful by inhalation and if swallowed, and irritating to skin and eyes.

Ortho phosphoric acid can cause burns.

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

5 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)
- 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)
- Methanol, p.a. (e.g. Merck no. 6009)
- Ortho phosphoric acid, 85% (e.g. Merck no. 573)
- 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, 140 mL acetone are combined with ca. 820 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 substances of all-E astaxanthin, purity (HPLC) > 95% (e.g. Dr Ehrenstorfer GmbH, Augsburg, Germany). The reference substances have 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)
- 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 Multipipette® 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)
- HPLC modules: 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)

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 require pre-grinding before use.

7.2 Extraction

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

7.2.1 Premixes and feed with a declared astaxanthin content of 1000 mg/kg and more

Weigh accurately approx. 2-3 g of sample with a declared astaxanthin content of \geq 1000 mg/kg into a tared 100 mL volumetric flask. Add approx. 100 mg of BHT, approx. 500 mg of Maxatase or 500 μ L 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-heptane/acetone (86:14; 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 μ L into the HPLC.

7.2.2 Feed with declared astaxanthin content between 20 and 1000 mg/kg

7.2.2.1 Pelleted or extruded feed

Weigh accurately approx. 2-3 g of ground sample with a declared astaxanthin 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 μ L 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. Purify the extract as described below.

7.2.2.2 Mash feed

Weigh accurately approx. 10 g of mash feed with a declared astaxanthin 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 μ L 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.

7.2.3 Feed with declared astaxanthin content lower than 20 mg/kg

Weigh accurately approx. 50 g of mash feed with a declared astaxanthin 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.

7.3 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.

7.3.1 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 (86:14; v/v). Transfer aliquots of the solution into HPLC vials, centrifuge at 4000 rpm for 5 min (if necessary) and analyse by HPLC.

7.3.2 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 (86:14; 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.

8 Standard Solutions and Calibration

8.1 Preparation of standard solution

Weigh approx. 1.5 mg of crystalline all-E astaxanthin and 1 g BHT into a 100 mL volumetric flask. Add approx. 5 mL of chloroform and put the flask in ultrasonic water bath at ambient temperature for approx. 30 sec. Then make up to volume with chloroform and mix well. Pipette 10.0 mL of this solution into a second 100 mL volumetric flask and combine with approx. 85 mL of n-hexane. Mixture cools and contracts. Bring the solution to room temperature and fill up to volume with n-hexane yielding a concentration of approx. 1.5 mg of astaxanthin per litre n-hexane/chloroform (9:1; v/v).

8.2 Spectrophotometry of standard solution:

Immediately after preparation, measure the absorption of the standard solution against n-hexane at the maximum (approx. 470 nm) by a spectrophotometer. Calculate the astaxanthin concentration according to formula 1 (see section 10.1.)

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 astaxanthin from the averaged total peak areas and the spectrophotometrically measured astaxanthin concentration according to formula 2 (see 10.2.).

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 astaxanthin, 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 crystalline astaxanthin (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 (86:14; 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 (86:14; 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.

9 HPLC

9.1 Conditions

- Column: LiChrosorb Si60, 5 µm, 125 x 4 mm (Merck)
- Mobile phase: n-Heptane/acetone (86:14; v/v), isocratic
- Flow rate: 1.2 mL/min
- Pressure: 50-80 bar
- Temperature: ambient (e.g. 23 °C)
- Injection volume: 10-200 µL
- Detection: VIS at 470 nm
- Run time: 15 min

In order to avoid tailing of the astaxanthin peaks, the stationary phase is modified by pumping a solution of ortho-phosphoric acid in methanol (1%; w/v) through the packed column for approx. 1 h at a flow rate of 1 mL/min. The column is then washed with mobile phase at a flow rate of 1.2 mL/min for at least 5 hours. Afterwards the column is inserted into the HPLC system. This acid-modification of the stationary phase is maintained for more than a year if not exposed to polar solvents such as water.

9.2 Retention times

Retention of all-E-astaxanthin: 7-12 min

Approximative relative retention times (in relation to all-E-astaxanthin):

Unidentified Z-Astaxanthin isomers: 0.89–0.94

9Z-Astaxanthin: 1.16

13Z-Astaxanthin: 1.22

Astaxanthin esters*: 0.10–0.40

all-E-β-Carotene: 0.14

all-E-Echinenone: 0.20

all-E-Canthaxanthin: 0.35

all-E-β-Cryptoxanthin: 0.35

all-E-Astacene: 0.46

all-E-Adonirubin: 0.56

all-E-Semiastacene: 0.66

all-E-Lutein: 1.8

all-E-Zeaxanthin: 1.9

- fatty acid esters of astaxanthin and other xanthophylls

10 Calculations

10.1 Spectrophotometric astaxanthin concentration of the standard solution:

$$\text{Astaxanthin [mg/L]} = \frac{\text{Absorption} \cdot 10000}{2100} \quad \text{formula 1}$$

10.2 Response factor of all-E astaxanthin:

$$RF_{\text{all-E astaxanthin}} [\text{mVsL/mg}] = \frac{A_{\text{tot}}}{c} \quad \text{formula 2}$$

10.3 Astaxanthin content in feed or premix samples:

$$\text{Astaxanthin} [\text{mg/kg}] = \frac{(A_{\text{all-E}} + A_{\text{xZ}} + A_{\text{9Z}} \cdot 1.2^* + A_{\text{13Z}} \cdot 1.6^*) \cdot V}{m \cdot RF_{\text{all-E}}} \quad \text{formula 3}$$

2100: E (1%/1cm) = Theoretical Absorption of an 1% astaxanthin solution (w/v) in an 1 cm cell at the maximum of absorption (approx. 470 nm) in n-hexane [ref. 1]

10 000: Scaling factor.

A_{tot} : Mean total peak area of the chromatograms of standard solution [mVs]

$A_{\text{all-E}}$: Area of all-E astaxanthin [mVs]

A_{9Z} : Area of 9Z astaxanthin [mVs]

A_{13Z} : Area of 13Z astaxanthin [mVs]

A_{xZ} : Area of other non-identified Z isomer(s) of astaxanthin [mVs]

c : Spectrophotometrically determined astaxanthin concentration in standard solution [mg/L] (see above)

m : Sample weight [g]

$RF_{\text{all-E}}$: Response factor of all-E astaxanthin [mVsL/mg]

1.2: Relative response factor of 9Z astaxanthin

1.6: Relative response factor of 13Z astaxanthin

V : Dilution [mL] (= theoretical volume in which the sample is dissolved)

$$\text{For extraction variant 7.2.1.} \quad V = \frac{100 \cdot 100}{5} = 2000 \quad \text{formula 4}$$

$$\text{For extraction variant 7.2.2.1.} \quad V = \frac{100 \cdot V_2 \cdot 20}{V_1 \cdot V_{\text{Inj}}} \quad \text{formula 5}$$

$$\text{For extraction variant 7.2.2.2} \quad V = \frac{W_1 \cdot 100 \cdot V_2 \cdot 20}{W_2 \cdot V_1 \cdot V_{\text{Inj}}} \quad \text{formula 6}$$

$$\text{For extraction variant 7.2.3} \quad V = \frac{W_1 \cdot 250 \cdot V_2 \cdot 20}{W_2 \cdot V_1 \cdot V_{\text{Inj}}} \quad \text{formula 7}$$

11 Repetition of Analyses

All analyses are conducted in duplicates. The results of the two determinations are compared by calculating the mean and the 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 outlying 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%.

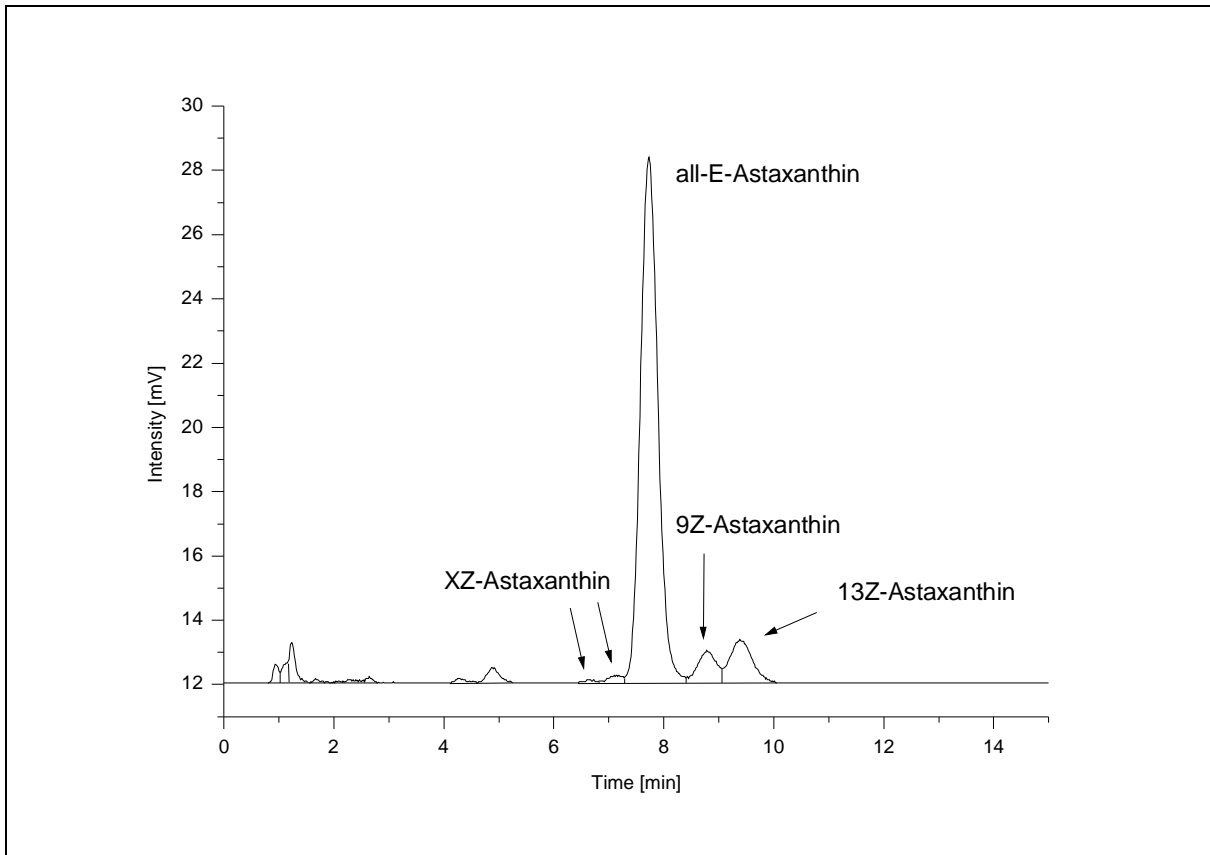
$$\text{Expanded Measurement Uncertainty} = 2 RSD_R(\%) = 4C^{(-0.15)}:$$

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 μ g/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 astaxanthin.



14 Notes

The relative response factors (section 10) are experimentally determined correction factors for the lower specific absorbance of 9Z and 13Z astaxanthin compared to all-E astaxanthin [ref. 1]).

Bibliography

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