



BSI Standards Publication

**Animal feeding stuffs: Methods of
sampling and analysis — Determination of
carotenoids in animal compound feed and
premixtures by high performance liquid
chromatography — UV detection (HPLC-UV)**

National foreword

This British Standard is the UK implementation of EN 17550:2021.

The UK participation in its preparation was entrusted to Technical Committee AW/10, Animal feeding stuffs.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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English Version

**Animal feeding stuffs: Methods of sampling and analysis -
Determination of carotenoids in animal compound feed
and premixtures by high performance liquid
chromatography - UV detection (HPLC-UV)**

Aliments des animaux - Méthodes d'échantillonnage et
d'analyse - Détermination de la teneur en caroténoïdes
des aliments composés et des prémélanges pour
animaux par chromatographie liquide à haute
performance couplée à une détection UV (CLHP-UV)

Futtermittel - Probenahme- und
Untersuchungsverfahren - Bestimmung von
Carotinoiden in Mischfuttermitteln und
Vormischungen für Tiere mittels Umkehrphasen-
Hochleistungs-Flüssigchromatographie mit UV-
Detektion (RP HPLC UV)

This European Standard was approved by CEN on 20 September 2021.

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European foreword

This document (EN 17550:2021) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs - Methods of sampling and analysis”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by June 2022, and conflicting national standards shall be withdrawn at the latest by June 2022.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

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Introduction

The method described in this document aims at constituting a tool for the effective control of carotenoids in feed by the competent authorities in the frame of Regulation (EC) No 1831/2003. Making use of the properties of the isosbestic wavelength for quantification, the method allows, in laboratory routine conditions, determining the sum of all isomers for each carotenoid authorized in poultry or fish feed. This approach can result in higher analytical variation of quantitative results in some matrices, and analysis should be repeated by an alternative method if the obtained variation significantly deviates from data presented in A.3 of this document. These alternative methods may be those optimized for the measurement of single carotenoids authorized under Regulation (EC) No 1831/2003 and available from the European Union Reference Laboratory for Feed Additives.

1 Scope

This analytical procedure specifies a reverse phase high performance liquid chromatographic with UV detection (RP-HPLC-UV) method for the simultaneous determination of four authorized carotenoids in fish compound feed and fish premix, namely astaxanthin (AXN), canthaxanthin (CXN), adonirubin (ADR) and astaxanthin dimethyldisuccinate (AXN DMDS), and of six authorized carotenoids in poultry feed and poultry premix, namely canthaxanthin (CXN); capsanthin (CSN), ethyl ester of beta-apo-8'-carotenoic acid (BACARE), citranaxanthin (CIXN), lutein (LUT) and zeaxanthin (ZEA) at levels ranging from approximately 2 mg/kg to approximately 4 500 mg/kg (depending on the carotenoid). Beta-carotene (BCAR), authorized in compound feed and premixes for all animal species, was also added to the scope. The analytical procedure is fit for the purpose of quantitation of declared carotenoids and labelling confirmation. This document is applicable to feed produced using natural and synthetic feed additives.

Xanthophyll esters like those of lutein, zeaxanthin and capsanthin that might be present in feed materials are not authorized feed additives and therefore not part of the scope of this document.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 6498, *Animal feeding stuffs - Guidelines for sample preparation (ISO 6498)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <https://www.electropedia.org/>
- ISO Online browsing platform: available at <https://www.iso.org/obp>

4 Principle

The carotenoids are first disclosed through an enzymatic reaction at 50 °C. The samples are extracted with acetone by means of a pressurized liquid extraction instrument or by liquid solid extraction. The extracts are centrifuged and analysed by reverse phase HPLC with UV or Diode Array Detection. A common isosbestic wavelength of 410 nm is selected for the determination of the target analytes, thus ensuring that the various isomers of each of the carotenoids have similar absorbance coefficients. The quantitation is performed through external calibration.

NOTE The maximum contents of the carotenoids, as established by the European regulations for the authorization of feed additives ([3], [4]), are expressed in terms of the sum of the all-*trans* and *cis* isomers. Therefore, from a legal point of view, it is important to sum up the areas of the corresponding isomers in the HPLC chromatogram prior to quantification of the individual carotenoids.

5 Reagents and materials

WARNING 1 — Carotenoids are subject to light degradation. Protect analytical work adequately from day light, and keep standard solutions protected from light by using amber glassware, amber vials or aluminium foil.

WARNING 2 — Avoid inhalation of and exposure to the toxic standard materials and solutions thereof. Work under fume hood when handling the solvents and solutions. Wear safety glasses and protective clothing.

WARNING 3 — Always wear a safety mask when handling Hydromatrix™.

Unless otherwise specified, use only reagents of recognized analytical grade.

5.1 Protease with the ability to release carotenoids from their encapsulated form.

NOTE Suitable proteases are available¹.

5.2 Purified water, e.g. Milli-Q or equivalent².

5.3 Butylated hydroxytoluene BHT.

5.4 High purity diatomaceous earth suitable for Pressurized Liquid Extraction (PLE), e.g. Hydromatrix™, bulk support material².

5.5 Acetone, HPLC grade.

5.6 Acetone, spectroscopic grade³.

5.7 Acetonitrile, HPLC grade.

5.8 Methyl *tert*-butyl ether tBME, HPLC grade.

5.9 Tetrahydrofuran (THF) stabilized with 250 mg/l to 350 mg/l butylated hydroxytoluene (BHT), HPLC grade.

5.10 *n*-Hexane, spectroscopic grade³.

5.11 Ethanol, spectroscopic grade³.

5.12 Cyclohexane, spectroscopic grade³.

5.13 Mobile phase for HPLC

5.13.1 Phase A: acetonitrile + methyl *tert*-butyl ether + water mixture 70 + 20 + 10; $V_1 + V_2 + V_3$, stabilized with 1 000 mg/l BHT.

Using a graduated cylinder (6.17), transfer 700 ml of acetonitrile (5.7) into a 1 000 ml bottle. Measure (6.17) and add 200 ml of methyl *tert*-butyl ether (5.8) and 100 ml water (5.2). Add 1,0 g of BHT (5.3). Perform mixing and degassing for 10 min in an ultrasonic bath (6.11). This mobile phase is stable for 28 days.

NOTE The retention time of the carotenoids is strongly influenced by slight differences in the composition of mobile phase A. The use of an HPLC quality control sample (9.1) is crucial for the correct signal allocation.

¹ Alcalase® and Multifect PR 6L have been successfully used for the validation.

² Milli-Q, Hydromatrix™, Alcalase® and Multifect PR 6L are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

³ The exact spectroscopic grade depends on the carotenoid for which the UV standardisation of the standard solution is performed (5.15.2).

Furthermore, the presence of non-targeted carotenoids might interfere with the signals of the targeted analytes when the composition of mobile phase A deviates from 5.13.1.

5.13.2 Phase B: acetonitrile + methyl *tert*-butyl ether mixture (70 + 30; $V_1 + V_2$), stabilized with 1 000 mg/l BHT.

Using a graduated cylinder (6.17), transfer 700 ml of acetonitrile (5.7) into a 1 000 ml bottle. Measure (6.17) and add 300 ml methyl *tert*-butyl ether (5.8). Add 1,0 g of BHT (5.3). Perform mixing and degassing for 10 min in an ultrasonic bath (6.11). This mobile phase is stable for 28 days.

5.14 Reference standards

Guaranteed purity is required for each lot of reference standard:

5.14.1 Astaxanthin (AXN), CAS N° 472-61-7, purity ≥ 970 g/kg.

5.14.2 Canthaxanthin (CXN), CAS N° 514-78-3, purity ≥ 970 g/kg.

5.14.3 Adonirubin (ADR), CAS N° 4418-72-8, purity ≥ 970 g/kg.

5.14.4 Astaxanthin dimethyldisuccinate (AXN DMDS), CAS N° 578006-46-9, purity ≥ 950 g/kg.

5.14.5 Capsanthin (CSN), CAS N° 465-42-9, purity ≥ 950 g/kg.

5.14.6 Ethyl ester of beta-apo-8'-carotenoic acid (BACARE), CAS N° 1109-11-1, purity ≥ 950 g/kg.

5.14.7 Lutein (LUT), CAS N° 127-40-2, purity ≥ 950 g/kg.

5.14.8 Citranaxanthin (CIXN), CAS N° 3604-90-8, purity ≥ 950 g/kg.

5.14.9 Zeaxanthin (ZEA), CAS N° 144-68-3, purity ≥ 950 g/kg.

5.14.10 Beta-carotene (BCAR), CAS N° 7235-40-7, purity ≥ 950 g/kg.

5.15 Standard solutions

Protect all standard solutions from daily light.

5.15.1 Carotenoid stock standard solution, ca. 200 $\mu\text{g/ml}$.

Prepare fresh and measure immediately.

NOTE Possible carotenoids are astaxanthin (AXN), canthaxanthin (CXN), adonirubin (ADR), astaxanthin dimethyldisuccinate (AXN DMDS), capsanthin (CSN), ethyl ester of beta-apo-8'-carotenoic acid (BACARE), citranaxanthin (CIXN), lutein (LUT), zeaxanthin (ZEA) and beta-carotene (BCAR).

Accurately weigh 1,0 mg of the carotenoid standard (5.14) (note down the mass of standard) (or transfer quantitatively the whole content of the container containing the standard substance (5.14)) in a 5,0 ml volumetric flask.

Dissolve and make up to the mark with THF/BHT (5.9). Mix well using a vortex mixer (6.27) and an ultrasonic bath (6.11). The accurate mass fraction needs to be standardized using the spectrophotometer (6.2) as described in detail in 5.15.2. Reserve a 1 ml portion for the isomerisation procedure (5.15.4), if needed. Reserve another aliquot for the identification of the analyte as described in 9.5.3.2 and store it in the freezer.

5.15.2 UV standardization of the standard solutions

Pipette 100 µl of the selected carotenoid stock standard solution (5.15.1) into a 10 ml volumetric flask (6.8) and make up to the mark with the appropriate solvent (Annex A). The nominal value of the obtained solution is ca. 2 µg/ml. Scan the spectrum of this solution from 300 nm to 550 nm and measure the absorption of this solution against the pure solvent, at the maximum, using the spectrophotometer (6.2). The maximum is solvent specific and is given as an approximate value in Annex A.

The exact content of the selected carotenoid is given by Formula (1).

$$\text{Carotenoid } \mu\text{g/ml} = E_{\lambda_{\text{max}}} \times 10\,000 / E^{1\%}_{1\text{ cm}} \quad (1)$$

EXAMPLE For all-*E* AXN, the solvent suggested in the table in Annex A is *n*-hexane. For this solvent, the wavelength of measurement is approximately 470 nm and the $E^{1\%}_{1\text{ cm}}$ (or $A^{1\%}_{1\text{ cm}}$) is 2 100.

Pipette with a suited pipette 100 µl of the astaxanthin stock standard solution (5.15.1) into a 10 ml volumetric flask and make up to the mark with *n*-hexane. The nominal value of the obtained standard measuring solution is ca. 2 µg/ml. Measure the UV spectrum of this solution against pure *n*-hexane, using the spectrophotometer (6.2) set at the wavelength of maximum absorption (approx. 470 nm).

NOTE The solvent of the measured solution is not pure as there is a small presence of THF/BHT (5.9). However, the effect is considered as negligible and the same tabulated extinction coefficient is applied, given that the contribution of THF/BHT (5.9) is very small (1 %).

The content of AXN is given by Formula (2).

$$\text{AXN } \mu\text{g/ml} = E_{\text{max}} \times 10\,000 / 2\,100 \quad (2)$$

A solution of the same concentration, 100 µl of the astaxanthin stock standard solution (5.15.1) pipetted into a 10 ml volumetric flask and made up to the mark with acetone (5.5), shall be injected simultaneously in the HPLC (see 5.15.3).

5.15.3 HPLC standard calibration curve

Pipette with a suited pipette 100 µl of carotenoid stock standard solution (5.15.1) into a 10 ml volumetric flask and make up to the mark with acetone (5.5).

5.15.4 Isomerisation

5.15.4.1 General

It is recommended to perform this step when ambiguity occurs regarding the presence of possible isomers.

5.15.4.2 Isomerisation of the standard solutions

The standards prepared according to 5.15.1 could be isomerised in order to have a profile of the all-trans and cis isomers of each carotenoid, which are present in the equilibrium phase.

Fill a LC vial with 1 ml standard. Close tightly and check that the vial is well sealed and the cap does not turn. Heat up the vial at about 80 °C for about 2 h to achieve the equilibrium between the isomers. A vials block heater (6.30) can be used for this purpose.

Inject a dilution of the isomerised standard (e.g. 1 → 200).

The isomerised standard is stable for several months at room temperature.

5.16 HPLC positive quality control sample (QCS)

Weigh approximately 0,8 mg AXN (5.14.1), 0,4 mg CXN (5.14.2) and 0,5 g BHT (5.3) in a round bottom flask (6.9). Add 200 ml of an acetone (5.5) + water (5.2) 75 + 25 V1 + V2 solution. Fit a condenser (6.10) to the flask and immerse the flask in a heating mantle or heated bath (6.28) with a magnetic stirrer (6.29). Heat to boiling and allow to refluxing for about 1,5 h to 2 h.

NOTE Alternatively, the mixture can be heated at 80 °C in a pressure-resistant tube.

This reaction will cause the formation of a stable isomers ratio. Transfer to a 500 ml volumetric flask (6.8) and make up to volume with acetone (5.5). Mix well and transfer immediately in HPLC vials. The filling should be fast and each vial should be immediately closed to avoid evaporation. Store the vials at room temperature and away from light. This solution, when protected from oxygen, is stable for several months.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 HPLC system, consisting of the following.

6.1.1 Pump, pulse free, capable of maintaining a volume flow rate from 0,1 ml/min to 2,0 ml/min.

6.1.2 Injection system, manual or autosampler.

If an autosampler is used, a cooled one is recommended although it was not used during the validation of the method.

6.1.3 UV/VIS detector, variable wavelength, suitable for reliable measurements at 410 nm, or UV/VIS photodiode array detector (DAD).

6.1.4 Computer data system.

6.1.5 Analytical column, Supelco® Suplex pKb-100 5 µm, 250 mm × 4,6 mm or equivalent⁴.

6.1.6 Guard column, 5 µm, 2 cm × 4 mm SUPELCOSIL™ Suplex™ pKb-100 Supelguard™ Cartridge or equivalent⁴.

6.2 Spectrophotometer, with 1 mm apertures.

6.3 Grinding instrument.

6.4 Sieve, with 1 mm apertures.

6.5 Balances, one analytical, of 10 g capacity or greater with 0,1 mg readability, and one, of 100 g capacity or greater with 0,01 g readability.

6.6 Polypropylene containers, 100 ml with lids.

⁴ Supelco® Suplex pKb-100, SUPELCOSIL™ Suplex™ pKb-100 Supelguard™ Cartridge, ASE 300 Dionex®, Büchi SpeedExtractor E-914, Eppendorf® tubes, Techne Dri-Block Heater and Ultra-Turrax® mixer are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

- 6.7 Variable-volume positive displacement piston pipettes**, suitable for pipetting volumes ranging from 50 µl to 100 µl.
- 6.8 Glass volumetric flasks** of, 5 ml, 10 ml, 150 ml, 500 ml and 1 000 ml.
- 6.9 Round bottom flasks** of 500 ml.
- 6.10 Allihn condenser.**
- 6.11 Ultrasonic bath**, temperature controlled.
- 6.12 Flat spatulas.**
- 6.13 PLE**, pressurized solvent extraction system, ASE 300 Dionex®, Büchi SpeedExtractor E-914 or equivalent⁴.
- 6.14 PLE cells**, suitable for the extraction unit used, 66 ml or larger.
- 6.15 Cellulose filters** for PLE cells.
- 6.16 PLE vials**, for the extraction unit used, at least 240 ml.
- 6.17 Graduated cylinder** of 250 ml and 1 000 ml.
- 6.18 Microcentrifuge safe-lock tubes**, 1,8 ml, Eppendorf® tubes or equivalent⁴.
- 6.19 Microcentrifuge.**
- 6.20 1,5 ml HPLC amber glass vials.**
- 6.21 HPLC glass vials crimper.**
- 6.22 Centrifuge.**
- 6.23 Centrifuge tubes**, 50 ml or 100 ml.
- 6.24 Ultra-Turrax® mixer**⁴.
- 6.25 PLE Funnels** for PLE cells.
- 6.26 Powder funnels.**
- 6.27 Vortex mixer.**
- 6.28 Heating mantle or heated bath.**
- 6.29 Magnetic stirrer.**
- 6.30 Vials block heater**, Techne Dri-Block Heater or equivalent⁴.

7 Sampling

It is important that the laboratory receives a sample that is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this document. A recommended sampling method is given in EN ISO 6497 [1].

8 Preparation of test sample

8.1 General

Prepare the test sample in accordance with EN ISO 6498.

8.2 Laboratory sample

Grind the laboratory sample (usually 50 g) so that it passes completely through a sieve with 1 mm apertures (6.4) or until a fine paste is obtained. Mix thoroughly.

8.3 Test sample

The test sample consists of a representative and homogenized aliquot of the ground laboratory sample (8.2) of at least 10 g.

8.4 Test portion

Accurately weigh 5,0 g to the nearest 0,1 g of the thoroughly mixed test sample (8.3) into a 100 ml polypropylene container (6.6) for extraction procedure 9.3.1 or into a 50 ml or 100 ml centrifuge tube (6.23) for extraction procedure 9.3.2. Note down the mass expressed in g. Submit it to the analysis procedure (Clause 9).

9 Procedure

9.1 General

The complete procedure (9.2 to 9.4) should be applied to two test portions (8.4) of the same test sample in order to perform two independent and parallel determinations.

The use of quality control samples is recommended.

The QCS (5.16) shall be injected before and after the complete sequence samples each day of analysis, in order to assess the suitability of the HPLC/DAD system.

The QCS (5.16) contains two carotenoids:

- 1) CXN, a stable and more soluble carotenoid, and
- 2) AXN, a less stable and less soluble carotenoid.

Depending on the analytes to be determined, a relevant carotenoid may be added to this mix.

The mixture is isomerized until a constant ratio of the isomers is reached. This solution, when protected from oxygen, is stable for several months.

9.2 Enzymatic disclosure

Add 0,2 g of BHT (5.3), 100 µl of enzyme (5.1) and 15 ml of purified water (5.2) to the test portion (8.4). Close tightly and shake vigorously to ensure that all the feed is permeated with water. Place in an ultrasonic bath (6.11) with the temperature set at 50 °C for 15 min to 20 min. Shake every 5 min.

9.3 Extraction

9.3.1 Extraction using PLE

Remove the lid of the test portion after step 9.2, taking care of not losing sample drops. Add approximately 13 g of high purity diatomaceous earth suitable for PLE (5.4), close tightly and hand-shake vigorously until the wet feed sample looks all adsorbed on the diatomaceous earth beadlets and detaches well from the polypropylene container's wall.

NOTE Stomping the container on the table can help.

Open the containers carefully, remove the sample particles from the lid into the container using a flat spatula (6.12). Mix well with the spatula (6.12), making sure that there are no sample clusters left. Place two cellulose filters (6.15) on the bottom of the PLE cell (6.14). Transfer all the material in the PLE cell (6.14) using a funnel (6.25). Top up with diatomaceous earth (5.4) if needed. Close the cell and extract according to the parameters in Table 1.

Table 1 — Extraction parameters

Extraction solvent	Acetone (5.5)
Pressure (bar)	103,4
Pressure (psi)	1 500
Temperature (°C)	58
Preheat time (min)	0
Heat time (min)	0
Static time (min)	7
Flush volume (%)	120
Purge time (s)	60
Static cycles	3

Each feed extraction lasts approximately 30 min. The instrument can run up to 12 samples in an automated way, e.g. overnight.

Take the tube containing the raw extract. Mix well and record the total volume (V_{ext}) (10) of extract using a graduated cylinder (6.17).

9.3.2 Conventional liquid solid extraction (LSE)

Add 50 ml of acetone (5.5) to the test portion and shake for 1 min. Centrifuge (6.22) at approximately 1 800 g to 1 900 g (approximately 3 100 rpm) for 5 min. Collect the supernatant (first extract) and transfer into a 150 ml volumetric flask (6.8).

Add 50 ml of acetone (5.5) to the solid residue of the first extraction. Use the Ultra-Turrax® mixer (6.24) if needed to achieve dissolution and shake for 1 min. Centrifuge (6.22) at approximately 1 800 g to 1 900 g (approximately 3 100 rpm) for 5 min. Collect the supernatant (second extract) and pool with the first extract in the 150-ml volumetric flask.

Add 40 ml of acetone (5.5) to the residue of the second extraction and shake for 1 min. Centrifuge (6.22) at approximately 1 800 g to 1 900 g (approximately 3 100 rpm) for 5 min. Collect the supernatant (third extract) and pool with the two first extracts in the 150 ml volumetric flask. Make up to volume (V_{ext}) (10) with acetone and shake vigorously.

If it is not possible to find or use 100 ml centrifuge tubes, the more common 50 ml centrifuge tubes may be used. In this case however, the three extraction steps with 50 ml acetone shall be replaced by four extraction steps with 35 ml to 37 ml acetone (5.5) each.

Similarly, if a 150 ml volumetric flask (6.8) is not available, record the final volume V_{ext} (10) using a graduated cylinder (6.17).

Transfer a 1,8 ml aliquot of the raw extract into a microcentrifuge safe-lock tube (6.18).

9.4 Centrifugation

Centrifuge (6.19) for 1 min at high relative centrifugal acceleration (e.g. 13 000 g, approximately 11 800 rpm).

NOTE Alternatively, filter the extract through a 0,45 μm membrane filter (suitable for acetone).

Transfer the supernatant to a HPLC amber vial (6.20) and proceed to the HPLC analysis (9.5).

9.5 HPLC analysis

9.5.1 Analytical conditions

The following conditions are provided for guidance. Other conditions may be used provided they yield to equivalent results.

9.5.1.1 HPLC column as in 6.1.5.

9.5.1.2 Guard column as in 6.1.6.

9.5.1.3 Mobile phase as in 5.13, flow rate: 0,5 ml/min.

9.5.1.4 Injection volume, 5 μl .

9.5.1.5 Column temperature, 20 $^{\circ}\text{C}$.

9.5.1.6 Detection wavelength, isosbestic wavelength 410 nm [5].

9.5.2 HPLC determination

9.5.2.1 Carotenoids elution

Perform the elution of the carotenoids in gradient mode. The gradient to be applied is displayed in Table 2.

9.5.2.2 External calibration curves

Inject each calibration solution described in 5.15.3 immediately after preparation in the HPLC system (6.1) and in parallel with the spectrophotometric standardization (5.15.2). Perform, at least, two parallel determinations (on separate 1 $\rightarrow$ 100 dilutions) or inject at least twice the external calibrant (5.15.3). Note the mean area of the peak, A . The concentration of this solution, c , correlated to the area of the peak is the exact content determined using the spectrophotometer (in $\mu\text{g}/\text{ml}$).

The calibration regression line is plot with the following two points:

- Calibration point 1: concentration 0 $\mu\text{g}/\text{ml}$; area 0 (Arbitrary Unit, AU);
- Calibration point 2: concentration c = exact content determined using the spectrophotometer in $\mu\text{g}/\text{ml}$; determined area A , in AU.

Determine the equation of the calibration regression line according to Formula (3).

$$a = \frac{A}{c} \quad (3)$$

where

a is the slope coefficient of the calibration curve obtained from the standard solutions;

A is the area of the peak;

c is the concentration of the solution, in µg/ml.

Table 2 — Elution gradient

Time min	% of mobile phase A	% of mobile phase B
0	100	0
10	100	0
14	40	60
30	40	60
30,1	100	0
45	100	0

Using these conditions the retention times for various carotenoids should approximately be as in Table 3.

Table 3 — Approximate retention times with the conditions as in 9.5.1

Carotenoid	retention time min
AXN DMDS, astaxanthin dimethyldisuccinate	8,0
AXN, astaxanthin	13,5
ADR, adonirubin	14,5
CXN, canthaxanthin	15,8
CSN, capsanthin	18,6
CIXN, citranaxanthin	20,9
BACARE, ethyl ester of beta-apo-8'-carotenoic acid	21,4
LUT, lutein	24,5
ZEA, zeaxanthin	25,8
BCAR, beta-carotene	32,0