SLOVENSKI STANDARD
SIST-TS CEN/TS 16233-1:2011

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Živila - Metoda HPCL za določevanje ksantofilov v ribjem mesu - 1. del: Določevanje astaksantina in kantaksantina

Foodstuffs - HPLC method for the determination of xanthophylls in fish flesh - Part 1: Determination of astaxanthin and canthaxanthin

Lebensmittel - HPLC-Verfahren für die Bestimmung von Xanthophyllen in Fischfleisch - Teil 1: Bestimmung von Astaxanthin und Canthaxanthin

Produits alimentaires - Méthode de dosage des xanthophylles dans la chair de poisson par CLHP - Partie 1: Dosage de l'astaxanthine et de la canthaxantine

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English Version

Foodstuffs - HPLC method for the determination of xanthophylls in fish flesh - Part 1: Determination of astaxanthin and canthaxanthin

Produits alimentaires - Méthode de dosage des xanthophylles dans la chair de poisson par CLHP - Partie 1: Dosage de l'astaxanthine et de la canthaxantine

Lebensmittel - HPLC-Verfahren für die Bestimmung von Xanthophyllen in Fischfleisch - Teil 1: Bestimmung von Astaxanthin und Canthaxanthin

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Foreword

This document (CEN/TS 16233-1:2011) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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1 Scope

This Technical Specification specifies a method for the determination of canthaxanthin and astaxanthin in fish flesh by high performance liquid chromatography (HPLC). The method can be applied at a range above 0,02 mg/kg. The method should not be applied to the determination of canthaxanthin in poultry tissues, egg yolks and shrimp tissues due to a possible interference of canthaxanthin with cryptoxanthin and xanthophyll esters sometimes present in these matrices.

2 Normative references

The following referenced documents are indispensable for the application 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 3696:1995, *Water for analytical laboratory use – Specification and test methods (ISO 3696:1987).*

3 Principle

Extract fish flesh by homogenising the tissue in acetone. Filter the extract and evaporate at reduced pressure using a rotary evaporator or a flow of nitrogen at 50 °C. Dissolve the residue in a mixture of *n*-heptane and acetone and analyse by an isocratic normal-phase HPLC.

The HPLC system described effectively separates astaxanthin and canthaxanthin and these both xanthophylls from other carotenoids found in fish flesh as e.g. β-carotene, lutein and zeaxanthin. The main geometrical isomers of astaxanthin are separated from each other and from oxidation products of astaxanthin, astacene and semi-astacene. In contrast, the isomers of canthaxanthin are not separated.

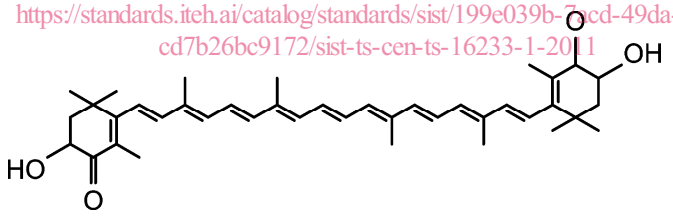

Figure 1 — all-E-Astaxanthin

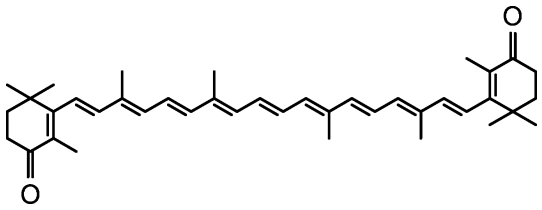

Figure 2 — all-E-Canthaxanthin

4 Reagents

During the analysis, unless otherwise stated, use only water complying with grade 1 of EN ISO 3696:1995 and reagents of recognized analytical grade, e.g. pro analysis (p.a.).

4.1 Magnesium sulfate, anhydrous, purity (complexometric) > 98 %.

4.2 Phosphoric acid, volume fraction φ is 85 %, purity (acidimetric) ≥ 85 %.

4.3 Butylated hydroxytoluene (BHT), purity (GC) > 99 %.

4.4 Tetrahydrofuran, purity (GC) > 99 %, stabilized with 0,025 % 2,6-di-tert-butyl-p-cresol (BHT).

4.5 Cyclohexane, purity (GC): > 99 %.

4.6 *n*-heptane, purity (GC): > 99 %.

4.7 Acetone, purity (GC): > 99 %.

4.8 Ethanol, absolute, purity (GC): > 99 %.

4.9 Methanol, purity (GC): > 99 %.

4.10 Reference substances of all-E-astaxanthin and all-E-canthaxanthin, purity (HPLC): > 95 %.

Store reference substances under nitrogen or argon at approximately -20 °C. Traces of oxygen destroy the substances.

4.11 HPLC mobile phase solvent, isocratic.

Mix 86 parts per volume of *n*-heptane (4.6) with 14 parts per volume of acetone (4.7).

4.12 Preparation of astaxanthin standard solution, ρ = 1,5 mg/ml.

Weigh approximately 1,5 mg to the nearest 0,1 mg of the reference substance of all-E-astaxanthin (4.10) and 1 g of BHT (4.3) into a 100 ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran (4.4) 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 *n*-heptane (4.6). The mixture cools and contracts. Warm the solution to room temperature and dilute to the mark with *n*-heptane. This results in an astaxanthin concentration of approximately 1,5 mg/l in a mixture of 9 parts per volume of *n*-heptane and 1 part per volume of tetrahydrofuran.

4.13 Preparation of canthaxanthin standard solution, ρ = 1,5 mg/ml.

Weigh approximately 1,5 mg to the nearest 0,1 mg of the reference substance of all-E-canthaxanthin (4.10) and 1 g of BHT (4.3) into a 100 ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran (4.4) 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 cyclohexane (4.5). The mixture cools and contracts. Warm the solution to room temperature and dilute to the mark with cyclohexane. This results in a canthaxanthin concentration of approximately 1,5 mg/l in a mixture of 9 parts per volume of cyclohexane and 1 part per volume of tetrahydrofuran.

4.14 Preparation of solution of heat-isomerized carotenoids (control solution)

Weigh approximately 1,5 mg of all-E-astaxanthin (4.10), 1,5 mg of all-E-canthaxanthin (4.10) and 0,5 g of BHT (4.3) to the nearest 0,1 mg and dissolve in a 500 ml volumetric flask in 10 ml of tetrahydrofuran (4.4). Dilute this solution with 200 ml of a mixture of 86 parts per volume of *n*-heptane (4.6) and 14 parts per volume of acetone (4.7). 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 a large number of HPLC vials. Immediately seal the vials carefully with septa made from polytetrafluoroethylene (PTFE) and silicone and store them at approximately 23 °C in the dark.

5 Apparatus

Usual laboratory apparatus, glassware, and the following:

5.1 Knife mill, suitable for food with grinding chamber volume of approximately 1 000 ml.

5.2 Sintered glass frit, porosity 3 (16 µm to 40 µm), diameter: approximately 6 cm.

5.3 Dispersing instrument.

5.3.1 Bench-top dispersing instrument for approximately 1 ml to 2 000 ml e.g. with 20 mm diameter aggregate.

5.3.2 Hand-held dispersing instrument for approximately 1 ml to 250 ml e.g. with 12 mm diameter aggregate.

5.4 Rotary evaporator e.g. 20 °C to 100 °C.

5.5 Nitrogen flow evaporator, with heating block and holder for pipettes.

5.6 Spectrometer, wavelength range 190 nm to 900 nm, wavelength accuracy: ≤ 1 nm.

5.7 Centrifuge, bench laboratory centrifuge for at least 2 500 *g*.

5.8 Balances.

5.8.1 Balance with readability of 0,01 g, precision (std dev.) of ± 0,005 g, capacity of 2 100 g.

5.8.2 Balance with readability of 0,01 mg, precision (std dev.) of ± 0,015 mg, capacity of 205 g.

5.9 Solid phase extraction manifold, steel needles (0,90 mm x 55 mm) attached to the valve outlets.

5.10 SPE columns, 25 ml reservoirs, plastic, equipped with 10 µm bottom fritts.

5.11 HPLC chromatographic system, with column thermostat and UV/visible or diode array detector.

6 Calibration

6.1 General

Prepare standard solutions at single concentrations (4.12 and 4.13), measure by spectrometry immediately after preparation (6.2), and inject the standard solutions into the HPLC (6.3). Determine the response factors of the carotenoids from the total peak areas of the chromatograms and the concentrations measured by spectrometry.

Since the method involves one-level calibrations it is recommended to control the linearity of the HPLC after implementation or any change of the system. For this purpose, the standard solutions can be diluted with a mixture of 86 parts per volume of *n*-heptane (4.6) and 14 parts per volume of acetone (4.7). The correlation coefficient of the regression line (forced through zero) should exceed 0,98.

6.2 Determination concentration of standard solution with spectrometry

Within 1 h after preparation, measure the concentration of all-E-astaxanthin or all-E-canthaxanthin by spectrometry at the respective absorption maximum using *n*-heptane (4.6) as a blank. Calculate the mass concentration (ρ) in milligram per millilitre of the standard solution using Equations (1) and (2):

$$\rho_{\text{all–E–astaxanthin}} = \frac{A_{\max} \times 10\,000}{2\,100} \tag{1}$$

where

$A_{\max}$ is the absorbance value at the absorption maximum;

10 000 is the scaling factor;

2 100 is the $E_{1\text{cm}}^{1\%}$ value of all-E-astaxanthin. The $E_{1\text{cm}}^{1\%}$ value is the theoretical absorption of a 1 % solution of all-E-astaxanthin in a 1 cm cell at approximately 470 nm ($\lambda_{\max}$) in *n*-heptane, see [1].

$$\rho_{\text{all–E–canthaxanthin}} = \frac{A_{\max} \times 10\,000}{2\,200} \tag{2}$$

where

$A_{\max}$ is the absorbance value at the absorption maximum;

10 000 is the scaling factor;

2 200 is the $E_{1\text{cm}}^{1\%}$ value of of all-E-canthaxanthin. The $E_{1\text{cm}}^{1\%}$ value is the theoretical absorption of a 1 % solution of all-E-canthaxanthin in a 1 cm cell at approximately 466 nm ($\lambda_{\max}$) in cyclohexane, see [2].

6.3 Determination response factor of standard solution with HPLC

Within 3 h after preparation, inject at least six aliquots of 20 µl of each standard solution into the HPLC system. Determine the total peak areas of the chromatograms (including the peaks of the all-E-isomer, of possibly present Z-isomers and impurities, but excluding the solvent peak). The peak area of all-E-astaxanthin or all-E-canthaxanthin should exceed 95 % of the respective total peak area of the chromatogram.