SLOVENSKI STANDARD
SIST-TS CEN/TS 16233-2:2011

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Živila - Metoda HPCL za določevanje ksantofilov v ribjem mesu - 2. del: Določanje razmerja enantiomer astaksantina

Foodstuffs - HPLC method for the determination of xanthophylls in fish flesh - Part 2: Identification of the enantiomer ratio of astaxanthin

Lebensmittel - HPLC-Verfahren zur Bestimmung von Xanthophyllen in Fischfleisch - Teil 2: Bestimmung des Enantiomerenverhältnisses von Astaxanthin

Produits alimentaires - Méthode de dosage des xanthophylles dans la chair de poisson par CLHP - Partie 2: Identification de la distribution énantiomérique de l'astaxanthine

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.120.30	Ribe in ribji proizvodi	Fish and fishery products

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TECHNICAL SPECIFICATION

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English Version

Foodstuffs - HPLC method for the determination of xanthophylls in fish flesh - Part 2: Identification of the enantiomer ratio of astaxanthin

Produits alimentaires - Méthode de dosage des xanthophylles dans la chair de poisson par CLHP - Partie 2: Identification de la distribution énantiomérique de l'astaxanthine

Lebensmittel - HPLC-Verfahren zur Bestimmung von Xanthophyllen in Fischfleisch - Teil 2: Bestimmung des Enantiomerenverhältnisses von Astaxanthin

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Contents

Page

Foreword

This document (CEN/TS 16233-2:2011) 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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Introduction

Three stereoisomers of all-E-astaxanthin (two enantiomers and a meso-form) exist, named (3R,3'R)-, (3S,3'S)- and (3R,3'S, meso)-all-E-astaxanthin, see Figure 1. These isomers can be determined by chromatography on a chiral HPLC column. Salmonids receive their astaxanthin through the diet and the flesh isomer composition reflects the dietary source of astaxanthin. Synthetically produced (racemic) astaxanthin and alternative organic forms of astaxanthin (produced by micro-organisms like *Xanthophyllomyces dendrorhous - formerly Phaffia rhodozyma* and *Haematococcus pluvialis*) have different stereoisomer profiles, see Figure 2. Even though there are minor differences in bioavailability of theses forms in fish [1], [2], the resulting flesh isomer profile can be used to distinguish wild from farmed salmon, and to determine which form of astaxanthin that has been supplemented the feed to farmed species [3], [4], [5] . Precautions should be taken since the method can sometimes not discriminate groups of farmed salmon that have been fed diets with mixed astaxanthin sources or fed different sources during periods of the production cycle [6]. In addition, racemic astaxanthin mixtures have been observed in the shrimp species Penaeus and also in some other orders of higher crustaceans. Therefore, fish exclusively fed with shrimp meal could also contain racemic astaxanthin [7], [8], and could erroneously be regarded as fish fed with synthetic astaxanthin.

1 Scope

This Technical Specification describes a method for the determination of the astaxanthin enantiomer ratio in fish flesh by high performance liquid chromatography (HPLC).

2 Normative references

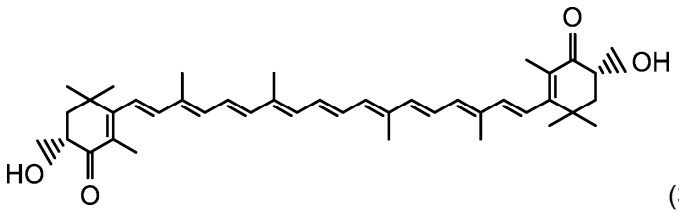
The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, *Water for analytical laboratory use – Specification and test methods (ISO 3696:1987).*

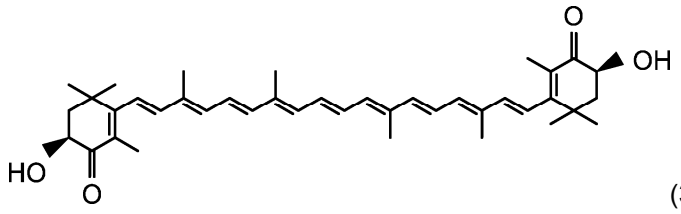
3 Principle

Extract fish flesh by homogenizing the tissue in acetone. Filter the extract and evaporate at reduced pressure using a rotary evaporator or a flow of nitrogen at 50 °C. Finally, dissolve the residue in the mobile phase, a mixture of *n*-heptane, dichloromethane and ethanol.

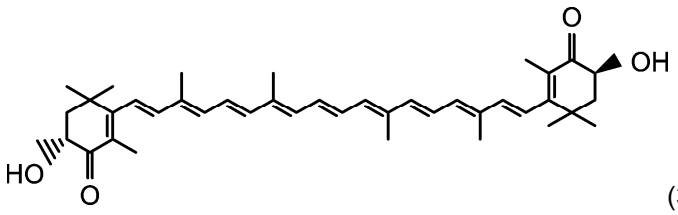
Determine the ratio of (3R,3'R)-, (3R,3'S, meso)- and (3S,3'S)-all-E-astaxanthin by chromatography on a chiral HPLC column.



(3R,3'R) all-E-astaxanthin



(3S,3'S) all-E-astaxanthin



(3R,3'S) all-E-astaxanthin

Figure 1 — Enantiomers and meso-form of all-E-astaxanthin

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

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

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

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

4.6 Dichloromethane, p.a., purity (GC) : > 99 %.

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

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

4.9 Chiral HPLC mobile phase solvent, isocratic.

Mix 24 parts per volume of *n*-heptane (4.5) with 58 parts per volume of dichloromethane (4.6) and 0,3 parts per volume of ethanol (4.8).

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 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 (4.2) into a 100 ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran (4.3) 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 (4.5). 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.

4.12 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 (4.2) into a 100 ml volumetric flask. Dissolve in 5 ml of tetrahydrofuran (4.3) 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 (4.4). 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.

4.13 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 (4.2) to the nearest 0,1 mg and dissolve in a 500 ml volumetric flask in 10 ml of tetrahydrofuran (4.3). Dilute this solution with 200 ml of a mixture of 86 parts per volume of n-heptane (4.5) 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 SPE columns, 25 ml reservoirs, plastic, equipped with a 10 micron bottom frits.

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

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

6 Sample preparation and extraction

6.1 Large scale extraction

Cut approximately 100 g of tissue in pieces of about 1 cm in length using a knife or a pair of scissors. Weigh 10 g to 20 g of the minced tissue to the nearest 0,01 g into a 200 ml beaker. Add 5 g of magnesium sulfate (4.1), 50 mg of BHT (4.2) and 40 ml of acetone (4.7). Homogenize the mixture with a dispersing instrument (5.3.1) and filter through a sintered glass frit (5.2) using vacuum. Scrape off the residue and homogenize again with a fresh portion of approximately 40 ml of acetone. Repeat this procedure until the filtrate is colourless (usually 2 extractions). Combine the filtrates, add 20 ml ethanol (4.8) and evaporate the mixture at 50 °C using a rotary evaporator (5.4). Dry the residue by addition and evaporation of approximately 5 ml of ethanol. Dissolve the residue in 10 ml to 50 ml of mobile phase (4.9) and fill an aliquot of the turbid solution into a HPLC vial. Centrifuge the vial at approximately 2 500 *g* and use the clear supernatant for HPLC-analysis.

6.2 Small scale extraction

Combine approximately 100 g of fish tissue with 1 g of BHT (4.2) and homogenize the mixture with a knife mill (5.1). Weigh, to the nearest 1 mg, approximately 1 g of the homogenate and approximately 1 g of magnesium sulfate (4.1) into an empty 25 ml plastic SPE-column equipped with a 10 µm frit at the bottom (5.9). Insert the column into a closed valve of a solid-phase extraction manifold (5.10). Add 8 ml of acetone (4.7) and homogenize the mixture with a dispersing instrument (5.3.2). Open the valve and suck the extract through the frit into a 35 ml test tube using vacuum. Repeat extraction and filtration with two additional portions of 8 ml acetone. Evaporate the combined filtrates under a flow of nitrogen at 50 °C (5.5). Dissolve the residue in 5 ml to 10 ml of the mobile phase (4.9) and fill an aliquot of the turbid solution into a HPLC vial. Centrifuge the vial at approximately 2 500 *g* and use the clear supernatant for HPLC-analysis.

7 HPLC

7.1 Identification

Identify the racemats of the xanthophylls by injecting 20 µl of the clear supernatant taken from the centrifuged HPLC vial according to 6.1 or 6.2 into the HPLC system under the conditions according to 7.2.

For identification of the astaxanthin enantiomers and the meso-form, inject the standard solution of synthetic all-E-astaxanthin (4.11) in to the HPLC-system. In synthetic all-E-astaxanthin the (3R,3’R)-, (3R,3’S, meso)-, and (3S,3’S)- isomers are present in a ratio of approximately 1:2:1 (racemat). They elute in this sequence, see Figure 2.

7.2 Conditions

The following conditions have been shown to lead to acceptable results.