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Djurfoder – Analys av lipofila marina biotoxiner i tvåskaliga blötdjur med LC-MS/MS

Foodstuffs – Determination of lipophilic algal toxins (okadaic acid group toxins, yessotoxins, azaspiracids, pectenotoxins) in shellfish and shellfish products by LC-MS/MS

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The European Standard EN 16204:2012 has the status of a Swedish Standard. This document contains the official version of EN 16204:2012.

Upplysningar om sakinnehållet i standarden lämnas av SIS, Swedish Standards Institute, telefon 08-555 520 00. Standarder kan beställas hos SIS Förlag AB som även lämnar allmänna upplysningar om svensk och utländsk standard.

Information about the content of the standard is available from the Swedish Standards Institute (SIS), telephone +46 8 555 520 00. Standards may be ordered from SIS Förlag AB, who can also provide general information about Swedish and foreign standards.

Denna standard är framtagen av kommittén för Livsmedel och foder, SIS/TK 435.

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EUROPEAN STANDARD
NORME EUROPÉENNE
EUROPÄISCHE NORM

EN 16204

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English Version

Foodstuffs - Determination of lipophilic algal toxins (okadaic acid group toxins, yessotoxins, azaspiracids, pectenotoxins) in shellfish and shellfish products by LC-MS/MS

Produits alimentaires - Dosage des toxines algales lipophiles (toxines du groupe acide okadaïque, yessotoxines, azaspiracides, pecténotoxines) dans les coquillages et les produits à base de coquillages par CL-SM/SM

Lebensmittel - Bestimmung der lipophilen Algentoxine (Okadasäuregruppen-Toxine, Yessotoxine, Azaspirosäuren, Pectenotoxine) in Schalentieren und Schalentiererzeugnissen mit LC-MS/MS

This European Standard was approved by CEN on 20 April 2012.

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Foreword

This document (EN 16204:2012) has been prepared by Technical Committee CEN/TC 275 “Food Analysis - Horizontal methods”, the secretariat of which is held by DIN.

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of an identical text or by endorsement, at the latest by November 2012, and conflicting national standards shall be withdrawn at the latest by November 2012.

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Introduction

Lipophilic marine biotoxins are the most frequently occurring algal toxins in Europe and are produced by certain marine dinoflagellates. They can accumulate in filter-feeding bivalves reaching highly toxic levels and, after consumption, may cause harm in humans such as nausea, vomiting and diarrhoea.

Commission Regulation 15/2011 stipulates LC-MS/MS as the reference methodology and refers to a method validated by the EU-RL Network. The method presented in EN 16204 is proposed as an alternative method to the one validated by EU-RL.

1 Scope

This European Standard specifies a multi-reference method for the determination of lipophilic algal toxins (fat-soluble algal toxins produced by some dinoflagellates) in raw shellfish and shellfish products including cooked shellfish, by liquid chromatography coupled to tandem mass spectrometry LC-MS/MS [1], [2], [3]. This method has been validated in an inter-laboratory study consisting of three parts via the analysis of both naturally contaminated homogenates of blue mussel and spiked extracts of blue mussel, oyster and clam. For further information on the validation, see Annex A. Additional studies have investigated further matrices (see [4], [5]).

The detection limit for toxins of the okadaic acid group, azaspiracids and pectenotoxins was determined to be 6 µg/kg shellfish meat and for yessotoxins 10 µg/kg shellfish meat.

Quantitative determination of okadaic acid (OA), pectenotoxin-2 (PTX-2), azaspiracid-1 (AZA-1) and yessotoxin (YTX) can be carried out directly by means of standard substances available commercially. Assuming an equal response factor, okadaic acid is used for the indirect quantitative determination of the two dinophysistoxins dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2); likewise azaspiracid-1 (AZA-1) is used for the indirect quantitative determination of azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3), while YTX is used for homo-yessotoxin, 45-OH-yessotoxin and 45-OH-homo-yessotoxin, and PTX-2 for pectenotoxin-1 (PTX-1).

The limit of quantification (LOQ) for toxins of the okadaic acid group, azaspiracids and pectenotoxins was determined to be 20 µg/kg shellfish meat and for yessotoxins 35 µg/kg shellfish meat.

By means of hydrolysis [6], the esters of okadaic acid, DTX-1 and DTX-2 can also be determined quantitatively as the corresponding free acids.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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

Remove the shellfish meat from the shell and homogenize the total shellfish meat. Extraction is carried out with aqueous methanol (φ = 80 %). Separation is performed on a HPLC reverse-phase column provided with a binary gradient and detection is carried out by means of tandem mass spectrometry using triple quadrupole technology. The concentration of lipophilic toxins is determined by means of external calibration.

4 Reagents

If not otherwise specified, reagents of analytical grade and solvents suitable for LC-MS/MS shall be used. Water shall be distilled in glass vessels or demineralised before use, or shall be of equivalent purity according to EN ISO 3696:1995. Since the use of this method involves reagents harmful to health, appropriate precautionary and protective measures such as avoiding skin contact and using an extractor hood shall be taken.

4.1 **Aqueous methanol** (φ= 80 %).

NOTE The validation data of this method have been elaborated with 80 % aqueous methanol. However, it has been shown (see [4], [5]) that equivalent results can be obtained when using 100 % methanol.

4.2 **Acetonitrile**

4.3 Sodium hydoxide, c(NaOH) = 2,5 mol/l.

4.4 Hydrochloric acid, c(HCl) = 2,5 mol/l.

4.5 Formic acid, 98 % to 100 % w/w.

4.6 Ammonium formate

4.7 Nitrogen, gaseous, min purity: 5,0.

4.8 Ammonium hydrogen carbonate

4.9 HPLC mobile phase 1 (chromatography under acidic conditions)

4.9.1 Eluent A1

Dissolve 126 mg (to give a 2 mmol/l solution) of ammonium formate (4.6) and 2 ml (to give a 50 mmol/l solution) of formic acid (4.5) in 50 ml of water and fill up to 1 000 ml with water. If necessary, filter the eluent using a 0,45 µm membrane filter.

4.9.2 Eluent B1

Dissolve 126 mg (to give a 2 mmol/l solution) ammonium formate (4.6) and 2 ml (to give a 50 mmol/l solution) of formic acid (4.5) in 50 ml of water. Add 950 ml of acetonitrile (4.2) and filter the eluent using a 0,45 µm membrane filter, if required.

4.10 HPLC mobile phase 2 (chromatography under basic conditions)

4.10.1 Eluent A2

Dissolve 395 mg (to give a 5 mmol/l solution) of ammonium hydrogen carbonate (4.8) in 1 000 ml of water. If necessary, filter the eluent using a 0,45 µm membrane filter.

4.10.2 Eluent B2

Dissolve 395 mg (to give a 5 mmol/l solution) of ammonium hydrogen carbonate (4.8) in 50 ml water. Add 950 ml of acetonitrile (4.2) in portions of about 100 ml. Shake vigorously after each portion added. If necessary, filter the eluent using a 0,45 µm membrane filter.

4.11 Toxin-free shellfish homogenate

To estimate and determine matrix effects, standard solutions in matrix are prepared. The shellfish homogenate required for this purpose is prepared from shellfishes that have been proved free from toxins.

4.12 Reference substances[1)]

NOTE During the validation study, the following reference substances were available. If other certified reference substances should become available in the future, the use of these substances is recommended.

4.12.1 Okadaic acid (OA)

4.12.2 Pectenotoxin-2 (PTX-2)

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1) Reference substances can be purchased from National Research Council Canada (NRC), Institute for Marine Bioscience, Halifax, for instance (http://www.nrc-cnrc.gc.ca). This is an example for suitable products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of these products.

4.12.3 Azaspiracid-1 (AZA-1)

4.12.4 Yessotoxin (YTX)

4.12.5 Multi-toxin standard solutions

Prepare standard calibration solutions (multi-toxin standard) in aqueous methanol (4.1). The following concentrations are recommended:

— OA, AZA-1, PTX-2: 1,5 ng/ml; 2,5 ng/ml; 5,0 ng/ml; 10,0 ng/ml; 15,0 ng/ml and 25,0 ng/ml;

— YTX: 3,0 ng/ml; 5,0 ng/ml; 10,0 ng/ml; 20,0 ng/ml; 30,0 ng/ml; and 50,0 ng/ml.

4.12.6 Multi-toxin standard solutions in matrix

Prepare a solution with a standard concentration level by dilution in a toxin-free methanolic shellfish extract (6.2) to estimate or determine matrix effects. The recommended concentration levels are:

— OA, AZA-1, PTX-2: 15,0 ng/ml;

— YTX: 30,0 ng/ml.

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

5.1 Mechanical high-speed blender or homogenizer (e.g. Grindomix, Ultra-Turrax®)[2)].

5.2 Centrifuge (working at minimum 2 000 g) **and centrifuge tubes** (volume 20 ml).

5.3 Shaker (e.g. Vortex).

5.4 Heat block.

5.5 Analytical balance, accuracy to the nearest 0,1 mg.

5.6 Volumetric flask, 20 ml or 10 ml.

5.7 Graduated cylinder and suitable pipettes.

5.8 High Performance Liquid Chromatography (HPLC) system, capable of gradient elution.

5.9 HPLC vials.

5.10 Triple-Quadrupol-LC-MS/MS system.

5.11 Analytical Reversed Phase Column, e.g. RP C18, particle size 3 µm, 150 mm (length) × 2 mm (diameter) or C8, 50 mm (length) x 2 mm (diameter), 3 µm particle size, (only for acidic conditions).

Optionally, an appropriate guard column may be used.

5.12 Syringe or membrane filter (e.g. regenerated cellulose membranes with a pore diameter of 0,45 µm).

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2) Ultra Turrax® is an example for a suitable product available commercially from various suppliers. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.