SLOVENSKI STANDARD
SIST EN 16204:2012

01-september-2012

Živila - Določevanje lipofilnih toksinov alg (skupina toksinov okadaične kisline, jesotoksini, azaspiracidi, pektenotoksini) v školjkah in njihovih proizvodih z uporabo LC-MS/MS (tekočinska kromatografija s tandemsko masno spektrometrijo)

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

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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

Ta slovenski standard je istoveten z: **EN 16204:2012**

ICS:

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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EUROPEAN STANDARD

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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

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

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: Avenue Marnix 17, B-1000 Brussels

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of an identical text or by endorsement, at the latest by November 2012, and conflicting national standards shall be withdrawn at the latest by November 2012.

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

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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.

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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.

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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.

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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

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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.

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4.10 HPLC mobile phase 2 (chromatography under basic conditions)

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4.10.1 Eluent A2

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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.

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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

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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).

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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.

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5.13 Syringe for filter system, e.g. 1 ml.

5.14 High-speed table centrifuge (up to 10 000 × *g*).

6 Procedure

6.1 Preparation of samples

6.1.1 General

Storage of shellfish samples in frozen state (at –18 °C) for up to a year has no negative influence (see [7]) on the results obtained with this method.

6.1.2 Raw samples

After receipt, the raw samples should be shelled and drained, and then frozen until used for analysis or shall be extracted immediately.

6.1.3 Cooked samples

The sample is cooked/steamed after receipt then drained and shelled. If it is not immediately treated, it can be cooled down until 24 h.

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To cook the shellfish, heat a sufficient amount of water (approximately 2 l to 3 l water per 1 kg of shellfish) to the boiling point. When the water is boiling, place shellfish into the water and cook under further addition of heat for approximately 3 min. After cooking, remove the shellfish meat from the open shells and homogenize.

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6.2 Homogenization and extraction

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For the determination of lipophilic algal toxins, homogenize 100 g to 200 g shellfish meat or the meat of 50 to 100 whole shellfish in order to ensure representative data. If only small amounts are available, the whole sample amount is used.

Homogenize shellfish meat by means of a mechanical blender (5.1) and prepare immediately or store at – 18 °C.

Weigh (2,00 ± 0,02) g of homogenate into a centrifuge tube (5.2). Add 9 ml of aqueous methanol (4.1) and mix for 1 min using a blender (5.1) at 13 500 min^{-1}. Subsequently, centrifuge (5.2) the mixture at approximately 2 000 x *g* at room temperature for 5 min. Decant the supernatants into a 20 ml volumetric flask. Repeat the extraction. Decant the second supernatants into the 20 ml volumetric flask and dilute to the mark with aqueous methanol (4.1). Subsequently, filter the extract using a 0,45 µm membrane filter (5.12). As an alternative to filtration, a high-speed table centrifuge with 10 000 × *g* may be used for the same purpose (5.14).

If only a small sample amount is available, (1,00 ± 0,01) g homogenate may be extracted twice with 4,5 ml of methanol (4.1) and made up to 10 ml.

6.3 Hydrolysis

The hydrolysis step is necessary for determination of toxins of the okadaic acid group, bound as esters. From this step, the sum of free and bound toxins of the okadaic acid group is determined. The bound toxins are determined as the difference of the sum (after hydrolysis) and the free toxins (without hydrolysis).

Pipette 1,0 ml of methanolic extract (6.2) into a vial (5.9). Add 125 µl of sodium hydroxide (4.3) and heat in a heat block (5.4) at (76 ± 2) °C for 40 min. After cooling the solution to room temperature, add 125 µl of

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hydrochloric acid (4.4) for neutralization and shake for 30 s (5.3). Subsequently, filter the samples through a 0,45 µm membrane (5.12).

Other amounts can also be used for hydrolysis. Care should be taken to ensure following ratios:

— Crude/raw extract / NaOH (2,5 mol/l) / HCl (2,5 mol/l) corresponding to 1 / 0,125 / 0,125.

During hydrolysis vials shall be closed firmly (boiling point of methanol is 65 °C). The loss of methanol during hydrolysis can be checked by weighing the vials before and after heating.

7 HPLC-MS/MS analysis

7.1 General

The measuring conditions stated are merely indicative; they may be adjusted to the respective local conditions. Triple Quadrupole Tandem Mass Spectrometry should be used. Analytes that cannot be distinguished by mass spectrometry (e.g. OA and DTX-2) shall be baseline separated by means of chromatography.

Before starting measurements, the HPLC system should be thoroughly rinsed (use starting conditions). Moreover, it is recommended to perform a test run for at least one sample before starting the sequence.

7.2 HPLC operating conditions 1 (chromatography under acidic conditions)

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Chromatographic conditions may be chosen freely. The acceptable minimum retention time (for the analyte under examination) is twice the retention time for the void volume of the column.

EXAMPLE 1 When using the C18-column specified in 5.11 and the mobile phases A1 (4.9.1) and B1 (4.9.2), the conditions in Table 1 were found to be appropriate:

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Table 1 — HPLC operating conditions 1

Time min	Flow rate ml/min	Mobile phase A1 %	Mobile phase B1 %
0	0,2	60	40
6,0	0,2	10	90
14,5	0,2	10	90
15,5	0,2	60	40
21,0	0,2	60	40

— Flow rate mobile phase (column): 0,2 ml/min

— Injection volume: 5 µl to 10 µl

— Column oven temperature (including the guard column): 40 °C

EXAMPLE 2 When using the C8 column, specified in 5.11, and the mobile phases A1 (4.9.1) and B1 (4.9.2), the conditions in Table 2 were found to be appropriate:

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Table 2 — HPLC operating conditions 2

Time min	Flow rate ml/min	Mobile phase A1 %	Mobile phase B1 %
0	0,2	70	30
8,0	0,2	10	90
11,0	0,2	10	90
11,5	0,2	70	30
16,0	0,2	70	30

— Flow rate mobile phase (column): 0,2 ml/min

— Injection volume: 5 µl to 10 µl

— Column oven temperature (including the guard column): 40 °C

7.3 HPLC operating conditions 2 (chromatography under basic conditions)

When using the column specified in 5.11 and the mobile phases A2 (4.10.1) and B2 (4.10.2), the conditions in Table 3 were found to be appropriate (see also Figure C.1):

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Table 3 — HPLC operating conditions 3

Time min	Flow rate ml/min	Mobile phase A2 %	Mobile phase B2 %
0	0,2	80	20
15,0	0,2	10	90
20,0	0,2	10	90
21,0	0,2	80	20
26,0	0,2	80	20

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— Flow rate mobile phase (column): 0,2 ml/min

— Injection volume: 5 µl to 10 µl

— Column oven temperature (including the guard column): 40 °C

7.4 Mass spectrometric operating conditions

The measurement may be performed using various instruments and instrument parameters. Some instrument parameters are listed in Annex B. These conditions have been shown to provide satisfactory results.

7.5 Calibration curve

Prepare a calibration curve which includes at least 6 concentration levels (excluding the origin) and which covers a concentration range from e.g. 1,5 ng/ml to 25 ng/ml (or 3,0 ng/ml to 50 ng/ml for YTX). If a larger

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concentration range is required, the calibration line may be sectioned for several ranges or a weighted calibration may be carried out.

The lowest standard should be at or equal to the LOQ.

Samples more highly concentrated than the highest calibration level can also be diluted with blank shellfish extract to obtain the calibration range.

The ratio between intercept and slope is calculated and used as a quality parameter. The absolute value of this ratio corresponds to the concentration plotted on the x-axis and shall not be greater than half the quantification limit. Additionally, the correlation coefficient (r^2) should be equal to or greater than 0,985.

7.6 Determination of algal toxins in sample test solutions

Inject aliquots of the sample test solution (6.2), (6.3) into the HPLC system in an appropriate sequence.

7.7 Quality control measures for sequences

All samples subject to quantitative determination of lipophilic algal toxins shall be analyzed at least by duplicate injection. The repeatability limit of the duplicate injection shall be smaller than $2{,}8 \times r$, where, r stands for the repeatability standard deviation of the extract.

To check the quality of chemical analysis, it has proven necessary to run simultaneously at least one QM sample per sequence. This sample should contain a known concentration of lipophilic algal toxins such as a sample with assigned value used in an inter-laboratory trial, a certified reference material (e.g. NRC Mus-b), a laboratory reference material, or a blank extract/blank homogenate spiked with standards. When tolerance or warning limits are defined, the different repeatability standard deviations of extracts and homogenates shall be taken into account.

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The matrix effects expected within a sequence shall be corrected as described in 8.3.

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For the determination of lipophilic algal toxins the adoption of a defined sequence has been proven suitable.

The following operations layout is recommended:

a) For sequences including more than 20 samples or exceeding a run time of 12 h:

   1) calibration standards;

   2) matrix correction standard;

   3) QM sample;

   4) samples (n > 20, first injection);

   5) calibration standards;

   6) matrix correction standard;

   7) QM sample;

   8) samples (n > 20, second injection);

   9) optionally: one calibration standard.

b) For sequences including less than 20 samples or a run time below 12 h:

   1) samples (n < 20, first injection);