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Foreword

This document (TC 275 WI 00275xxx) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This document is a working document.

According to the CEN/CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Introduction

The amnesic shellfish poisoning (ASP) toxin, domoic acid, belongs to a group of amino acids, called the kainoids, which are classed as neuroexcitants or excitoxins that interfere with the neurotransmission mechanisms in the brain. The toxin can be accumulated in shellfish feeding on a number of toxic *Pseudonitzschia* species. Ingestion of seafood contaminated with domoic acid can lead to an intoxication which symptoms include (among others) abdominal cramps, vomiting, disorientation and memory loss (amnesia) and can become severe in certain cases.

High performance liquid chromatography with ultraviolet detection (HPLC-UV) was the first chemical analytical method for domoic acid and is still the most commonly used for monitoring shellfish. Domoic acid detection is facilitated by its strong absorbance at 242 nm [1].

This European Standard is based on a procedure described by Quilliam, Xie and Hardstaff (1995) for the quantitative determination of domoic acid (DA) in unsalted seafood [2]. In this procedure, a single-step extraction with 50% aqueous methanol and a selective clean-up and preconcentration with strong anion exchange solid phase extraction are used. Determination is performed by high performance liquid chromatography with isocratic condition and ultraviolet absorbance detection. The method described in this European Standard allows elimination, in view of results obtained in the validation procedure, of the clean-up step.

Furthermore this document specifies in Annex B a modified method based on a procedure described by Quilliam, M. A., Sim, P. G.; McCulloch, A.W. and McInness, A. G. [3] for the quantitative determination of domoic acid and its isomers e.g. epi-domoic acid (epi-DA) in seafood. In this procedure [3] a single-step extraction with 50% aqueous methanol, a boiling step and a solid phase extraction (SPE) are used. In the described method the SPE clean up step is not done. The boiling step is optional and allows a better decanting of the supernatant. Determination is performed by high performance liquid chromatography with binary gradient and ultraviolet absorbance detection.

Both methods can be applied for the quantitative determination of domoic acid (DA).

1 Scope

This European Standard specifies a method for the quantitative determination of domoic acid in bivalve molluscs and finfish. The limit of detection is about 10-80 ng/ml (0,05-4,0 mg/kg), depending on the UV detector sensitivity. The quantitation limit for domoic acid by this method is, at least, 2,7 mg/kg (or µg/kg). The method has been tested for domoic acid determination in different matrices such as mussels, clams, scallops and anchovies, spiked and/or naturally contaminated at levels ranging from 2,7 to 85,1 mg/kg (or µg/kg).

This European Standard furthermore specifies in Annex B a method for the quantitative determination of domoic acid in mussels and mussel products. This method works without halogenated solvent. After successful testing it may also be applicable to other shellfish, for example scallops. The limit of quantification of domoic acid by this method is 1,0 mg/kg. The method has been tested for domoic acid determination at levels ranging from 5 mg/kg to 12,9 mg/kg

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

3 Principle

Domoic acid is extracted from bivalve tissue with a mixture of methanol and water. The extract is filtered through a membrane filter and measured using HPLC equipment with isocratic elution and UV detection. The amount of domoic acid is calculated by the external standard method.

4 Reagents

During the analysis, unless otherwise stated, use only water according to grade 1 of EN ISO 3696.

All chemicals shall be of pro analysis (p. a.) quality, unless otherwise indicated.

Reference material originating from other sources than indicated may also be used if well-characterised and with a well-defined mass concentration.

4.1 Methanol, HPLC quality

4.2 Acetonitrile, HPLC quality

4.3 Extraction solvent, Methanol/water 50:50, v/v

4.4 Acetonitrile/water, 1:9 v/v

4.5 Trifluoroacetic acid (TFA), spectrophotometric grade (≥99%)

4.6 Eluent

Aqueous 10 % acetonitrile (4.2) with 0,1 % TFA (4.5). For single pump systems, mix 100 ml acetonitrile with ca 400 ml water, add 1,0 ml TFA, and dilute to 1 l with water. Other mobile phases could be used.

4.7 Standard substance

Domoic acid, e.g. available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada [1]. Sealed ampoules should be stored in the dark in a refrigerator (at approximately +4ºC). Do not freeze the solution. Prior to opening, each ampoule should be allowed to warm to room temperature. Once the ampoule has been opened, accurate aliquots should be removed using calibrated volumetric equipment and transferred to other amber containers for dilution and/or analysis as soon as possible. Closed vials should be stored in the dark in a refrigerator (at approximately +4ºC) for no more than 3 months.

4.8 Standard solutions

4.8.1 Calibration solutions

Prepare a series of calibration working solutions with increasing concentration of DA + epi-DA, within the mass range of e.g. 0,2 to 24,85 µg/ml, by accurately diluting the certified calibration solution with acetonitrile:water, 1:9 v/v (4.4). Keep solutions in the dark and refrigerated (at approximately 4ºC) when not in use. Do not store them for more than 3 months. Do not freeze the solutions. Warm up solutions to room temperature before use.

4.9 Reference material

Mussel tissue reference material for domoic acid, e.g. available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada[1]. Mussel homogenate should be stored in the freezer (at -12 ºC or lower). When a bottle is opened the entire contents should be used immediately. If sub sampling is necessary, a thorough blending of the bottle contents is required before a sub sample is removed, as some sedimentation occurs over long-term storage. The reference material may be used to test the accuracy of an existing analytical procedure.

5 Apparatus

5.1 General

Use usual laboratory apparatus and, in particular, the following:

5.2 Analytical balance, capable of weighing to the nearest of 0,1 g

5.3 Grinder or mixer

5.4 Centrifuge, capable to reach 3 000 g (refrigerated at 4°C, if possible)

5.5 Centrifuge tubes, nominal volume 30-50 ml, with crew tops

5.6 Membrane filter, methanol compatible with a pore size 0,2 µm or 0,45 µm

5.7 Adjustable automatic pipettes, cover in the range from 20 µl to 1000 µl

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1) This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of the product name. Equivalent products may be used if they can be shown to lead to the same results.

5.8 HPLC instrumentation, comprising the following

5.8.1 Injection system

5.8.2 Pump, capable of isocratic elution

5.8.3 Column oven, able to reach 40ºC ± 2ºC

5.8.4 Analytical column, for example C18 reverse phase, 250 mm x 4.6 mm i.d. packed with 5 µm

NOTE Other LC column and dimensions may be suitable if mobile phase flow and/or injection volumes are adjusted. The use of a guard column is recommended. If the HPLC is millibore compatible, a 250 mmx 2,1 mm i.d. column packed with the same stationary phase can be used with a 5 µl injection volume and a mobile phase flow rate of 0,2-0,3 ml/min.

5.8.5 UV-spectrophotometric detector, set to a wavelength of 242 nm, providing a S/N of 10:1 on injection of a 0,2 µg/ml domoic acid solution using the conditions given in 6.3

5.8.6 Data system

5.8.7 HPLC vials

5.8.8 Glass amber vials, 2 ml or less, with crimp caps (to store domoic acid working calibration solutions)

6 Procedure

6.1 Sample Preparation

Bivalves with shell: Thoroughly clean outside of the shellfish with fresh water. Open by cutting adductor muscle. Rinse inside with fresh water to remove sand and foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge. Do not use heat or anaesthetics to open the shell. After removal from shellfish, drain tissues 5 min in a sieve to remove salt water. For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a grinder or blender [4]. Sub samples from this homogenate can be taken immediately after blending, while still well mixed, or after mixing again. In the case of scallops, at least 10 specimens should be taken (Commission Decision 2002/226/CE) [5].

Bivalves without shell (whole body or any edible part separately): If needed, clean outside with fresh water and allow it to drain. For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a grinder or blender. Sub samples from this homogenate can be taken immediately after blending, while still well-mixed, or after mixing again. In the case of scallops, at lest 10 specimens or individual edible parts (muscle, muscle + gonad) should be taken (Commission Decision 2002/226/CE) [5].

Fish: clean, scale and eviscerate fish. In case of small fish ≤ 15 cm, use 5-10 fish. For intermediate-size fish, remove and discard heads, scales, tails, fins, guts, and inedible bones: fillet fish to obtain all flesh and skin from head to tail and from top of back to belly on both sides [4]. For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a grinder or blender. Sub samples from this homogenate can be taken immediately after blending, while still well-mixed, or after mixing again.

6.2 Extraction procedure

Accurately weigh 4 g ± 0.1 g of tissue homogenate into a graduate centrifuge tube (5.4) or a stainless steel micro-blender cup. Add 16 ml of extraction solvent (methanol:water 50:50, 4.3) and homogenize the sample extensively (3 min. at 10000 rpm). Do not try to recover all the tissue remaining in the homogenizer probe or blender cup, but wash them thoroughly afterwards to prevent contamination of the next sample.

If a blender has been used for homogenization, pour the resulting slurry into a centrifuge tube.

Centrifuge at 3 000 g or higher for 10 min. Filter a portion of the supernatant through a dry methanol-compatible 0,45 µm or 0,2 µm filter. Sample extracts should be analyzed as soon as possible. If analysis is not performed immediately, the extract may be stored in a tightly sealed screw-capped storage container in a freezer at c.a. –12ºC.

Extraction blank: perform the extraction procedure (see above) except substitute water in place of sample tissue (chromatograms should be free of peaks eluting near domoic acid or causing excessive baseline slope).

For screening samples with a known or suspected high level of contamination, a measured aliquot of the extract can be dilute to a fixed volume with water, using a calibrated volumetric flask, graduated pipette or micropipettes, mix and analyze. For salted samples, deliver 1.0 ml of extract into a volumetric flask or graduated cylinder, dilute to 5 ml with water, mix and analyze.

6.3 HPLC measurement

Determination of the domoic acid content in a sample is performed after chromatographic separation on a reversed phase column using isocratic conditions (4.6).

The following HPLC conditions led to satisfying results:

Isocratic conditions

Column:	C18 reversed phase, 5 µm, 250 mm x 4,6 mm
Temperature:	40°C
Flow:	1 ml/min
Injection volume:	20 µl
UV detector:	242 nm

NOTE High injection volumes may require diluting the extract with water to avoid broadening of the domoic acid peak associated with injecting a solvent of stronger elution power than the mobile phase (“solvent wash-out” effect).

6.4 Calibration graph

Prepare a calibration graph, with at least four points, each day of the analysis and/or whenever the chromatographic conditions change. Plot the peak area against the concentration of the injected DA+ epi-DA calibration solutions. Ensure that the coefficient of correlation of calibration curve shows a linear regression ($r \geq 0,99$; % Yi/Xi 100 % ± 10 %).

6.5 Sample injection

Inject samples in duplicate. Replicate single injections should have a coefficient of variation (CV) < 5 %. Avoid carry-over between injections of different samples by washing the injector loop.

7 Evaluation of results

7.1 Identification

Identify domoic acid and epi-domoic acid by comparing the retention times of the sample with that of the standards. DA identity should be confirmed (e.g. co-chromatography, spectral analysis, monitoring the column eluent at two different wavelengths).

NOTE Pure domoic acid in solution has been found to gradually isomerise, especially to the C5´-diasteromer, epi-domoic acid (epi-DA); therefore, a mixture will inevitably result on long-term storage of any standard. Since epi-DA has a UV spectrum identical with that of DA, the relative molar response factors in LC with UV detection are identical and relative proportions can be recalculated at a time. Under some LC conditions, DA and epi-DA do not resolve; this does not present a problem and in fact makes analysis simpler. Analysts should base their instrument calibration and quantification on the sum of both DA and epi-DA areas [3].

7.2 Quantification

After determining each sample concentration (DA+epi-DA), (external standard method) with the calibration graph, calculate the level of domoic acid + epidomoic acid in the sample using the following formula:

$$\text{concentration (µg DA+ epi-DA/g)} = \frac{\mu gDA + epiDA / ml\ injected\ extract}{W} \times D \times Vt$$

Vt - total volume of homogenate and extracting solvent in ml

W - sample tissue weigh (usually 4 g)

D - dilution factor (if extract has been diluted)

7.3 Precision

The method has been validated in a formal collaborative study with 13 participating laboratories. Details of the interlaboratory test on the precision of the method are given in annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given in annex A.

8 Test report

The test report shall contain the following data:

— all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

— all information necessary for the identification of the calibrant;

— a reference to this European Standard or to the test method used;

— the date and type of sampling procedure (if known);

— the date of receipt of the sample;

— the date of test;

— the test results and the units in which they have been expressed;

— any particular points observed in the course of the test;

— any operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

Precision data

The following data were obtained from the “EU validation study of domoic acid determination by HPLC-UV”, organised by the EU Community Reference Laboratory for Marine Biotoxins according to the Guidelines of Collaborative Study Procedures to Validate Characteristics of the Method of Analysis (AOAC, 2002) and using different matrices and contamination levels with blind duplicates.

Table A.1 — Precision data

Sample	1	2	3	4	5	6
Matrix	Clam spiked	Clam naturally contaminated	Mussel spiked	Gonad scallop naturally contaminated	Whole body scallop naturally contaminated	Anchovy naturally contaminated
Year	2003	2003	2003	2003	2003	2003
Number of laboratories	12	12	12	13	13	12
Number of laboratories retained after eliminating outliers	12	12	11	13	13	11
Number of outliers (laboratories)	0	0	1	0	0	1
Number of accepted results	12	12	11	13	13	11
Mean value, µg/g	3,38	17,7	11,6	2,72	9,63	85,1
Repeatability standard deviation s_r, µg/g	0,28	0,40	0,37	0,12	0,18	1,1
Repeatability limit r [r = 2,8 x s_r), µg/g	8,3	2,3	3,2	4,3	1,9	1,3
Reproducibility standard deviation s_R, µg/g	0,51	1,6	2,0	0,62	1,2	6,6
Reproducibility limit R [r = 2,8 x s_R), µg/g	15	9,0	17	23	12	7,7
Horrat	1,1	0,87	1,6	1,7	1,0	0,94
Recovery, %	79		84			

Annex B
(normative)

Method using non-halogenated eluent

B.1 Principle

Domoic acid is extracted from mussel tissue with a mixture of methanol and water. The extract is filtered through a membrane filter and measured using HPLC equipment with binary gradient and detection via UV absorption. The amount of domoic acid is calculated by the method of external standard.

B.2 Reagents

During the analysis, unless otherwise stated, use only water according to grade 1 of EN ISO 3696.

All chemicals shall be of pro analysis (p. a.) quality, unless otherwise indicated.

Reference material originating from other sources than indicated may also be used if well-characterised and with a well-defined mass concentration.

B.2.1 **Methanol,** HPLC quality

B.2.2 **Acetonitrile,** HPLC quality

B.2.3 Extraction solvent, Methanol/water 50:50, v/v

B.2.4 Formic acid, mass concentration ≥ 98 %

B.2.5 Eluents

B.2.5.1 Eluent A

Mix 100 ml acetonitrile (B.2.2) with 900 ml water and adjust pH to 2,5 using formic acid (B.2.4).

B.2.5.2 Eluent B

Mix 300 ml acetonitrile (B.2.2) with 700 ml water and adjust pH to 2,5 using formic acid (B.2.4).

B.2.6 Standard substance

Domoic acid, e.g. available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada [2]. Sealed ampoules should be stored in the dark in a refrigerator (at approximately +4ºC). Do not freeze the solution. Prior to opening, each ampoule should be allowed to warm to room temperature.

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2) This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of the product name. Equivalent products may be used if they can be shown to lead to the same results.

B.2.7 Standard solutions

B.2.7.1 Stock solution

Weigh domoic acid (B.2.6) into a volumetric flask and/or dissolve in methanol to a final concentration of 500 µg/ml. Closed vials should be stored in the dark in a refrigerator (at approximately +4 ºC).

B.2.7.2 Calibration solutions

Dilute stock solution (B.2.7.1) with methanol to a final concentration of 50 µg/ml. Further dilute with methanol to following calibration solutions: 0,5 µg/ml; 1,0 µg/ml; 2,0 µg/ml; 4,0 µg/ml; 6,0 µg/ml; 8,0 µg/ml and 10 µg/ml. These solutions correspond to following domoic acid concentrations in mussel homogenate: 2,0 mg/kg; 4,0 mg/kg; 8,0 mg/kg; 16,0 mg/kg; 24 mg/kg and 32 mg/kg. Closed vials should be stored in the dark in a refrigerator (at approximately +4 ºC) for no more than 3 months.

B.2.8 Reference material

Mussel tissue reference material for domoic acid, e.g. available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada [3]. Mussel homogenate should be stored in the freezer (at -12 ºC or lower). The reference material may be used to test the accuracy of an existing analytical procedure.

B.3 Apparatus

B.3.1 General

B.3.2 Use usual laboratory apparatus and, in particular, the following:Grinder or mixer

B.3.4 Centrifuge, capable to reach 3 000 g**Graduated centrifuge tubes,** nominal volume ≥ 30 ml, with crew tops

B.3.6 Mechanical mixer, high speed at 8000 to 45000 rpm (e.g. Ultra turrax)**Membrane filter,** 0,2 µm and/or 0,45 µm**Electrical heater**

B.3.9 Analytical balance, capable of weighing to the nearest of 0,1 g**HPLC instrumentation,** capable of gradient elution, column oven able to reach 35°C**UV-spectrophotometric detector,** set to a wavelength of 242 nm**Analytical column,** for example C18 reverse phase, 250 mm x 4 mm i.d. packed with 5 µm

B.3.13 HPLC vials

B.3.14 Glass amber vials, 2 ml or less, with crimp caps (to store domoic acid working calibration solutions)

B.4 Procedure

B.4.1 Sample Preparation

For representative sampling, at least 100-150 g of pooled mussel tissue should be homogenized in a grinder or blender (B.3.3). In the case of scallops, at least 10 specimens should be taken (Commission Decision 2002/226/CE) [5]. Weigh a test portion of 5,0g ± 0,1 g of the homogeneous mussel material into a graduated centrifuge tube (B.3.5). Add methanol/water mixture (B.2.3) and make up to a volume of 20 ml; mix for approx. 2 min using a mechanical mixer (B.3.6).

NOTE You can heat the extract for better decanting the supernatant. Close the tube and heat the mixture at 60 °C to 70 °C for 15 min. Let the sample cool down to room temperature

Centrifuge for 10 min at 2 000 *g*. Decant the supernatant into a tube and filter (B.3.7) an aliquot into a HPLC vial (B.3.13). Sample extracts should be analyzed as soon as possible. If analysis is not performed immediately, the extract may be stored in a freezer at *c.a.* –12 ºC or lower.

B.4.2 HPLC measurement

Determination of the domoic acid content in a sample is performed after chromatographic separation with gradient conditions on a reversed phase column using eluent A (B.2.5.1) and eluent B (B.2.5.2). The following HPLC gradient conditions led to satisfying results:

Gradient conditions

Table B.1 — Time gradient programme for chromatography and column re-equilibration

time (min)	% eluent B
0,00	10
30,00	10
30,10	40
40,00	40
40,10	10
50,00	stop

Column:	C18 reversed phase, 5 µm, 250 mm x 4,0 mm
Temperature:	35 °C
Injection volume:	5 µl
Flow:	0,7 ml/min
UV detector:	242 nm

B.4.3 Calibration graph

Prepare a calibration graph at the beginning of the analysis and/or whenever the chromatographic conditions change. Plot the peak response against the mass concentrations of the injected domoic acid calibration solutions. Ensure that the slope of the calibration curve shows a linear regression. Determine the concentration the domoic acid with the calibration graph.

B.5 Evaluation of results

B.5.1 Identification

Identify domoic acid and epi-domoic acid by comparing the retention times of the sample with that of the standard substances.

NOTE Under some LC conditions, DA and epi-DA do not resolve; this does not present a problem and in fact makes analysis simpler. Analysts should base their instrument calibration and quantification on the sum of both DA and epi-DA areas [3].

B.5.2 Quantification

Quantify the amount of domoic acid and epi-domoic acid in an unknown sample using the method of external standard by integration of the peak area or by determination of the peak height in relation to the calibration curve of domoic acid standards (B.2.7.2). Calculate the domoic acid content in the sample, *w*, in milligrams per kilogram using equation:

$$w = \frac{Peak\ area\ (or\ peak\ height) - b}{a} \times D$$

where:

a is the slope of calibration curve;

b is the intersection of the calibration curve;

D dilution: 4 is the factor of 5 g sample in 20 ml solution.

B.5.3 Precision

The method has been validated in a formal collaborative study with 11 participating laboratories. Details of the interlaboratory test on the precision of the method are given in Annex C. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given in Annex C.

Annex C
(informative)

Precision data of method described in Annex B

The following data were obtained in interlaboratory tests [6] according to the guidelines of the German act of food and feed law (§64-LFBG) and conducted by the German §64-LFBG working group “Muscheltoxine” using two spiked and one blank mussel matrix. The blank sample was identified from all participants. In this study the described method was used. The extract was heated and it was used the gradient HPLC condition. Five sub samples were measured by each participant and if the Grubbs test was failed three samples more were determinate.

Table C.1 — Precision data

Sample	A	B
Year	2001	2001
Number of laboratories	11	11
Number of laboratories retained after eliminating outliers	10	10
Number of outliers (laboratories)	1	1
Number of accepted results	10	10
Mean value, µg/g	12,9	5,0
Repeatability standard deviation s_r, µg/g	0,5	0,2
Repeatability limit r [$r = 2,8 \times s_r$), µg/g	1,4	0,5
Reproducibility standard deviation s_R, µg/g	1,4	0,6
Reproducibility limit R [$r = 2,8 \times s_R$), µg/g	3,9	1,7
Horrat	1,0	0,96
Recovery, %	85-92[a)]	81-90 [a)]
Recovery, % recovery rates found by participants	86	83

a) obtained in homogeneity and stability studies prior to the main part of the interlaboratory study

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