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BSI Standards Publication

Foodstuffs — Determination of domoic acid in raw shellfish, raw finfish and cooked mussels by RP-HPLC using UV detection

bsi.

National foreword

This British Standard is the UK implementation of EN 14176:2017. It supersedes BS EN 14176:2003 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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Foodstuffs - Determination of domoic acid in raw shellfish, raw finfish and cooked mussels by RP-HPLC using UV detection

Produits alimentaires - Dosage de l'acide domoïque dans les coquillages crus, les poissons crus et les moules cuites par CLHP en phase inverse couplée à la détection UV

Lebensmittel - Bestimmung von Domoinsäure in rohen Schalentieren, rohen Fischen und gekochten Miesmuscheln mit RP-HPLC und UV-Detektion

This European Standard was approved by CEN on 7 November 2016.

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European foreword

This document (EN 14176:2017) 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of an identical text or by endorsement, at the latest by July 2017, and conflicting national standards shall be withdrawn at the latest by July 2017.

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

This document supersedes EN 14176:2003.

EN 14176:2017 includes the following significant technical changes with respect to EN 14176:2003:

— the extraction procedure in 6.2 has been revised;

— the chromatographic conditions in 6.3 have been revised;

— the method has been re-validated, and the validation data in Annex A have been revised.

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

Introduction

The amnesic shellfish poisoning (ASP) toxin, domoic acid (DA), 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 DA 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 DA and is still the most commonly used for monitoring shellfish. DA detection is possible by its strong absorbance at 242 nm [1].

This European Standard is based on two, comparable procedures. One procedure for the quantitative determination of DA and its isomers e.g. epi-domoic acid (epi-DA) in unsalted raw seafood (Method A) is described in [2]. The other procedure for the quantitative determination of DA and its isomers e.g. epi-DA in cooked mussel (Method B) is described in [3].

Method A uses a single-step extraction with 50 % aqueous methanol and an optional selective clean-up and concentration step with strong anion exchange solid phase extraction (SPE). Taking into account results of the validation procedure, the optional clean-up step of Method A as published under [2] is not described in this standard. Analytes are determined by high performance liquid chromatography (HPLC) under isocratic conditions with ultraviolet absorbance detection.

Method B uses a single-step extraction with 50 % aqueous methanol and an optional heating step which allows a better decanting of the supernatant. However, it has been observed that heating can degrade DA and epi-DA. DA and epi-DA are determined by HPLC with binary gradient and ultraviolet absorbance detection.

Both methods can be applied for the quantitative determination of DA.

WARNING — The use of this standard can involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to take appropriate measures to ensure the safety and health of personnel prior to application of the standard, and fulfil statutory and regulatory requirements for this purpose.

1 Scope

This European Standard specifies methods for the quantitative determination of domoic acid in raw bivalve molluscs and finfish as well as in cooked mussels. The limit of detection is about 10 ng/ml to 80 ng/ml (0,05 mg/kg to 0,4 mg/kg), depending on the UV detector sensitivity. Method A has been validated for the determination of DA in different raw matrices such as mussels, clams, scallops and anchovies, spiked and/or naturally contaminated at levels ranging from 2,7 mg/kg to 85,1 mg/kg. Method B has been validated for the determination of DA at levels ranging from 5 mg/kg to 12,9 mg/kg in cooked blue mussels.

For further information on validation data, see Clause 8 and Annex A.

Laboratory experience has shown that this standard can also be applied to other shellfish species, however, no complete validation study according to ISO 5725 has been carried out so far.

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, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

3 Principle

DA and epi-DA are extracted from sample tissue with a mixture of methanol and water. The extract is filtered through a membrane filter and measured using HPLC equipment with isocratic (Method A) or gradient (Method B) elution and detection by UV absorption. The amount of DA is calculated by the method of external standard calibration.

WARNING — ASP toxins are neurotoxins which can be taken up by inhalation or orally. Therefore, adequate protection measures are to be applied.

4 Reagents

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

If not otherwise indicated, all chemicals shall be of pro analysis (p. a.) quality.

Reference materials (certified, if available) and standard substances originating from other sources as indicated may also be used if well-characterized and with a well-defined mass concentration.

If not already specified, stability of solutions should be determined by the laboratory.

4.1 Methanol, HPLC quality

4.2 Acetonitrile, HPLC quality

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

4.4 Acetonitrile/water, 10:90 v/v (Method A)

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

4.6 Formic acid, mass concentration ≥ 98 % (Method B)

4.7 Eluents

4.7.1 Eluent 1 (isocratic conditions)

Aqueous 10 % v/v acetonitrile (4.4) with 0,1 % v/v TFA (4.5). For single pump systems, mix 100 ml acetonitrile with approximately 400 ml water, add 1,0 ml TFA, and dilute to 1 l with water.

4.7.2 Eluent 2 (gradient conditions)

Mix 100 ml acetonitrile (4.2) with 900 ml water and adjust pH to 2,5 using formic acid (4.6).

4.7.3 Eluent 3 (gradient conditions)

Mix 300 ml acetonitrile (4.2) with 700 ml water and adjust pH to 2,5 using formic acid (4.6).

4.8 Standard substances

4.8.1 Domoic acid as certified calibration solution[1)]

Sealed ampoules should be stored in the dark in a refrigerator (at approximately +4 °C). Do not freeze the solution. Prior to opening, each ampoule should be at room temperature. Once the ampoule has been opened, accurate aliquots should be removed using calibrated volumetric equipment and transferred to other amber glass vial for dilution and/or analysis as soon as possible. Closed vials should be stored in the dark in a refrigerator (at approximately +4 °C) for no more than 3 months.

NOTE Epi-DA is contained as a minor component in the certified calibration solution from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada[1)]

4.8.2 **Domoic acid, as crystalline powder,** purity of > 95 %

4.9 Standard solutions

4.9.1 General

Either use commercially available certified calibration solutions (4.8.1) or prepare calibration solutions by dissolving crystalline DA powder (4.8.2) and subsequently dilute. Both procedures have been proven to lead to successful validation data.

4.9.2 Stock solution

Weigh (5.1) DA crystalline powder (4.8.2) into a volumetric flask and 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 °C).

4.9.3 Standard solution

Dilute the stock solution (4.9.2) with methanol to a final concentration of 50 µg/ml. Check the mass concentration of this solution by comparing with certified calibration solutions (4.8.1).

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1) Information on suitable calibration solutions are e. g. available on http://aesan.msssi.gob.es/en/CRLMB/web/home.shtml and http://aesan.msssi.gob.es/en/CRLMB/web/estandares_materiales_referencia/materiales_referencia.shtml. This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of the products referred to in the websites or available from Canada. Equivalent products may be used if they can be shown to lead to the same results.

4.9.4 Calibration solutions

Prepare calibration solutions of appropriate mass concentrations of DA and epi-DA, either by diluting the standard solution (4.9.3) or by diluting the certified calibration solution (4.8.1).

For the validation of Method A with isocratic elution, calibration solutions were prepared in the range of 0,2 µg/ml to 25 µg/ml by diluting the certified calibration solution (4.8.1) with acetonitrile:water, 10:90 v/v (4.4).

For the validation of Method B with gradient elution, calibration solutions were prepared in the range of 0,5 µg/ml to 10 µg/ml by diluting the standard solution (4.9.3) with a methanol/water solution (4.3).

Keep solutions in the dark and refrigerated (at approximately + 4 °C) 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.

4.10 Reference material[2)]

Mussel tissue reference material should be stored according to the manufacturers specifications. Each bottle should be allowed to warm to room temperature prior to opening and the contents thoroughly mixed by vortexing for a minimum of 2 min. When a bottle is opened the entire contents should be used immediately. The reference material can be used to test the accuracy of an existing analytical procedure. Extraction of the reference material should be performed according to the procedure described in 6.2.1 or 6.2.2.

5 Apparatus

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

5.1 Analytical balance, capable of weighing to the nearest 0,1 mg

5.2 Balance, capable of weighing to the nearest 0,01 g

5.3 Homogenizer (e.g. grinding or blending machine)

5.4 Mechanical mixer, high speed at 8 000 min^{-1} to 45 000 min^{-1} (e.g. Ultra turrax)

5.5 Centrifuge, capable of effectively separating the liquid and solid phase, e.g. 3 000 g [3)] (refrigerated at + 4 °C, if possible)

5.6 Centrifuge tubes, nominal volume 30 ml to 50 ml, with screw caps

5.7 Membrane filter, methanol compatible with a pore size of 0,2 µm or 0,45 µm, e.g. surfactant-free cellulose acetate with glass fibre pre-filter

5.8 Adjustable automatic pipettes, covering the range from 20 µl to 1000 µl

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2) Suitable reference material is e. g. available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia-Canada. Epi-DA is contained as a minor component in this certified reference material. 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 named. Equivalent products may be used if they can be shown to lead to the same results.

3) $g = 9{,}81\ m \cdot s^{-2}$.

5.9 Electrical heater, (Method B)

5.10 HPLC vials

5.11 Glass amber vials, 2 ml or less, e.g. with crimp caps (to store DA calibration solutions)

5.12 HPLC system, comprising the following

5.12.1 Injector

NOTE A refrigerated injector can prevent DA degradation over time, especially if room temperatures are high.

5.12.2 Pump, capable of isocratic elution (Method A) or gradient elution (Method B).

5.12.3 Column oven able to heat to (40 ± 2) °C (Method A) or (35 ± 2) °C (Method B).

5.12.4 Analytical column, C18 reversed phase column, e.g. 250 mm x 4,6 mm i.d. (Method A) or 4,0 mm i.d. (Method B) packed with 5 µm particles.

NOTE Other LC columns and dimensions can be suitable if mobile phase flow and/or injection volumes are adjusted. The use of a guard column is recommended. Suitable results can also be obtained with the following conditions: C18-column of 250 mm x 2,1 mm i.d., packed with a comparable stationary phase, with a 5 µl injection volume and a mobile phase flow rate of 0,2 ml/min to 0,3 ml/min.

5.12.5 UV-detector, if possible a diode array detector, set to 242 nm, providing a signal to noise ratio (S/N) of 10:1 on injection of a 0,2 µg/ml DA solution using the conditions given in 6.3.

6 Procedure

6.1 Sample preparation

6.1.1 Raw bivalves with shell

Thoroughly clean the outside of the shellfish with tap water. Open by cutting the adductor muscle. Rinse inside with tap water to remove sand and foreign material. Remove tissue 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. Homogenize (5.3) at least 100 g to 150 g of pooled tissue [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 according to [5].

6.1.2 Raw bivalves without shell (whole body or any edible part separately)

If needed, clean outside with tap water and allow it to drain. Homogenize (5.3) at least 100 g to 150 g of pooled tissue. 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 or individual edible parts (muscle, muscle + gonad) should be taken according to [5].

6.1.3 Raw fish

Clean, scale and eviscerate fish. In case of small fish ≤ 15 cm, use 5 to 10 fishes. 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]. Homogenize (5.3) at least 100 g to 150 g of pooled tissue. Sub-samples from this homogenate can be taken immediately after blending, while still well-mixed, or after mixing again.

6.1.4 Cooked mussels[4)]

Homogenize (5.3) at least 100 g to 150 g of pooled cooked mussel tissue. Sub-samples from this homogenate can be taken immediately after blending, while still well-mixed, or after mixing again. If the laboratory receives raw mussels but wishes to apply Method B, the mussels shall be cooked first until the shells are open. Remove the shells.

6.2 Extraction procedure

NOTE It is essential to verify in-house changing extraction conditions prior to use.

6.2.1 Method A

Accurately weigh 4,0 g ± 0,1 g of tissue homogenate into a centrifuge tube (5.6) or a stainless steel micro-blender cup. Add 16 ml of the methanol/water mixture (4.3) and homogenize (5.4) the sample extensively (3 min at 10 000 min^{-1}). It is not necessary to recover all the tissue remaining in the dispersing element 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.

6.2.2 Method B

Weigh a test portion of 5,0 g ± 0,1 g of the homogeneous mussel material into a centrifuge tube (5.6). Add 15 ml of the methanol/water mixture (4.3); mix for approximately 2 min using a mixer (5.4).

Heating extracts improves decanting of the supernatant. In this case, close the tube and heat the mixture at 60 °C to 70 °C for 15 min [3]. Let the sample cool down to room temperature. Be aware that heating the extract can produce some DA degradation.

6.2.3 Centrifugation, dilution and storage

Centrifuge at 3000 *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 analysed 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 refrigerator at approximately +4 °C.

Extraction blank: perform the extraction procedure (see 6.2.1 or 6.2.2) by substituting sample tissue with water (chromatograms should be free of peaks eluting near DA or causing excessive baseline slope).

For screening samples with a known or suspected high level of DA contamination, the extract can be diluted with methanol/water (50:50) (4.3).

6.3 HPLC determination

6.3.1 General

For the validation studies, samples extracted according to 6.2.1 were determined with isocratic HPLC conditions described in 6.3.2, and samples extracted according to 6.2.2 were determined with *gradient* HPLC conditions described in 6.3.2. However, laboratory experience has shown that the HPLC conditions specified for Method A and Method B can be applied to both extraction protocols. A typical chromatogram is shown in Annex B.

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[4] Laboratory experiences have shown that cooked bivalves, such as scallops can also be prepared according to 6.1.4, however, this has not been validated in the interlaboratory study. In the case of scallops, at least 10 specimens should be taken [5].

6.3.2 HPLC-conditions

Determination of the DA content in a sample is performed after chromatographic separation on a reversed phase column either under isocratic conditions with eluent 1 (4.7.1) or under *gradient conditions* with eluent 2 (4.7.2) and eluent 3 (4.7.3).

The following HPLC conditions led to satisfying results:

Column: C18 reversed phase, 5 µm, 250 mm x 4,6 mm *or 4,0 mm*

Temperature: 40 °C *or 35 °C*

Flow: 1 ml/min *or 0,7 ml/min*

Injection volume: 5 µl to 20 µl

UV detector: 242 nm

Isocratic run time: approximately 15 min to 20 min.

NOTE These conditions were applied in the interlaboratory study with Method A under isocratic conditions, however, the values in italics were applied in the interlaboratory study with Method B under gradient conditions.

If an alternative chromatographic column is used, it can be necessary to adjust chromatographic conditions.

Appropriate gradient conditions are described in Table 1.

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

Time min	Eluent 2 %	Eluent 3 %
0,00	90	10
30,00	90	10
30,10	60	40
40,00	60	40
40,10	90	10
50,00	stop	

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 and epi-DA calibration solutions. Ensure that the coefficient of correlation of calibration curve shows a linear regression (Method A: $r \geq 0,99$ and outlier test of the calibration points (calculation of the percentage ratio between each peak area calibration point (Yi) to the corresponding mass calibration point (Xi) correlated to the mean of all ratios) Yi/Xi [%] between 100 % ± 10 %.).

6.5 Sample injection

Inject samples in duplicate unless the injection repeatability is sufficient. Replicate single injections should have a coefficient of variation (CV) < 5 %. In order to check the stability of the system, inject the calibration solutions and/or solutions with fixed concentration of reference material (4.10) at regular intervals.

7 Evaluation of results

7.1 Identification

Identify DA and epi-DA by comparing the retention times of the sample with those of the standard substances.

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

7.2 Quantification

Quantify the amount of DA and epi-DA in an unknown sample using the method of external standard by integration of the peak area in relation to the calibration graph of DA standards (4.10). Calculate the DA and epi-DA mass fraction in the sample, *w*, in milligrams per kilogram using Formula (1):

$$w = \frac{peak\ area - b}{a} \frac{D}{m} V_t \qquad (1)$$

where

- a is the slope of calibration graph;
- b is the intercept of the calibration graph;
- D is the dilution factor (if extract has been diluted);
- m is the sample mass, in gram, usually 4 g (Method A) or 5 g (Method B);
- V_t is the total volume of homogenate and extracting solvent, in millilitre.

8 Precision

Method A using isocratic conditions has been validated in an interlaboratory study with 13 participating laboratories. Details of the interlaboratory study on the precision of the method are given in Annex A, Table A.1.

Method B using gradient conditions has been validated in an interlaboratory study with 11 participating laboratories. Details of the interlaboratory study on the precision of the method are given in Annex A, Table A.2.

See also Annex A, Table A.3 listing data obtained from proficiency testing schemes.

The values derived from these interlaboratory studies may not be applicable to concentration ranges and matrices other than those given in Annex A.

9 Test report

The test report should contain the necessary data according to EN ISO/IEC 17025 and the following:

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

— a reference to this European Standard;

— 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

A.1 Precision data for Method A using isocratic elution

The data in Table A.1 were obtained from the "EU validation study of DA determination by HPLC-UV", organized by the EU Reference Laboratory for Marine Biotoxins (EURLMB) according to [6] and using different matrices and contamination levels with blind duplicates.

Table A.1 — Precision data Method A using isocratic elution

Sample	Clam spiked	Clam nc[a]	Mussel spiked	Gonad scallop nc [a]	Whole body scallop nc [a]	Anchovy nc [a]
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 $\bar{x}$, mg/kg	3,38	17,7	11,6	2,72	9,63	85,1
Repeatability standard deviation s_r, mg/kg	0,28	0,40	0,37	0,12	0,18	1,1
Repeatability relative standard deviation RSD_r, %	8,3	2,3	3,2	4,4	1,9	1,3
Repeatability limit r [$r = 2{,}8 \times s_r$], mg/kg	0,78	1,12	1,04	0,34	0,50	3,08
Reproducibility standard deviation s_R, mg/kg	0,51	1,6	2,0	0,62	1,2	6,6
Reproducibility relative standard deviation RSD_R, %	15	9,0	17	23	12	7,8
Reproducibility limit R [$r = 2{,}8 \times s_R$], mg/kg	1,43	4,48	5,60	1,74	3,36	18,5
HorRat	1,1	0,87	1,6	1,7	1,0	0,94
Recovery, %	79		84			

[a] nc = naturally contaminated

A.2 Precision data for Method B using gradient elution

The data in Table A.2 were obtained in interlaboratory studys [7] 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 gradient HPLC conditions were used. Five sub-samples were measured by each participant and if the Grubbs test was failed, three more samples were determined.

Table A.2 — Precision data Method B using gradient elution

Sample	Mussel tissue A	Mussel tissue 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 $\bar{x}$, mg/kg	12,9	5,0
Repeatability standard deviation s_r, mg/kg	0,5	0,2
Repeatability limit r [$r = 2{,}8 \times s_r$], mg/kg	1,4	0,5
Reproducibility standard deviation s_R, mg/kg	1,4	0,6
Reproducibility limit R [$r = 2{,}8 \times s_R$], mg/kg	3,9	1,7
HorRat	1,0	0,96
Recovery, %	85 to 92[a]	81 to 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		

A.3 Precision data from EURLMB Proficiency Testing Schemes

The data in Table A.3 were obtained from the proficiency testing schemes of DA, organized by the EU Reference Laboratory for Marine Biotoxins in 2011 to 2013. Only values from those laboratories which used the HPLC-UV methods were collected in Table A.3.

Table A.3 — Precision data from EURLMB Proficiency Testing Schemes, HPLC-UV methods

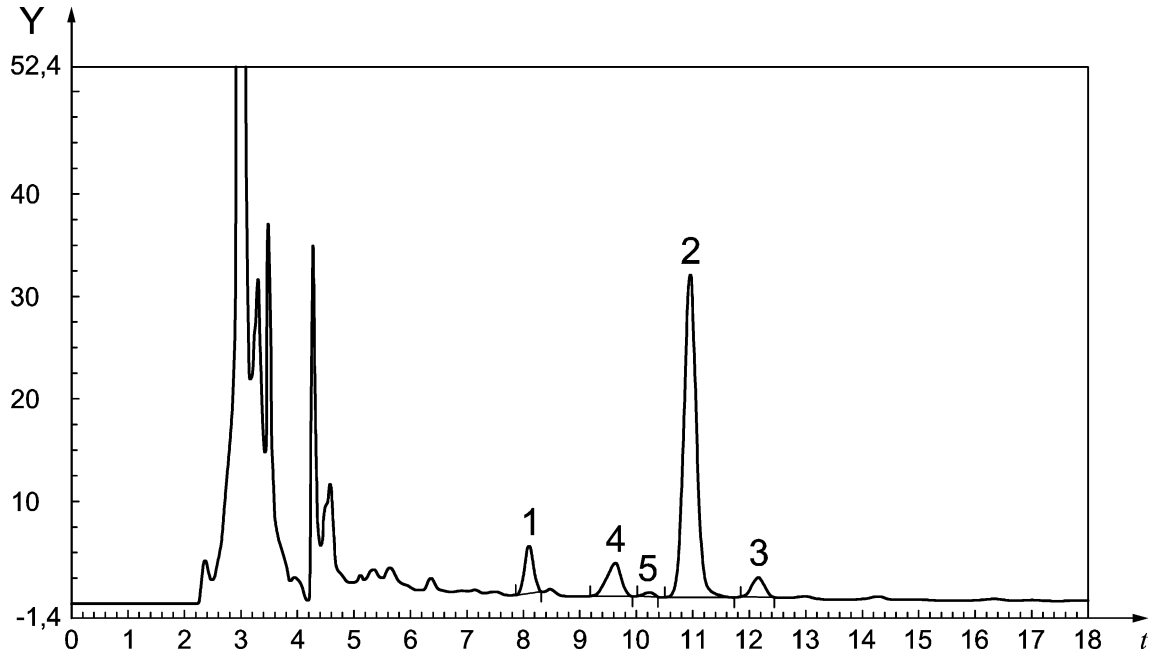
Sample	1	2	3	4	5	6
Matrix	Scallop, nc[a]	Mussel, nc[a]	Scallop, nc[a]	Clam nc[a]	Mussel, nc[a]	Scallop, without hepatopancreas, nc [a]
Year	2011	2011	2012	2012	2013	2013
Number of data submitted	24	24	26	26	25	25
Assigned value[b], mg/kg	15,8	4,7	35,7	22,4	30,5	8,8
Uncertainty of assigned value, mg/kg	0,2	0,1	0,6	0,6	0,5	0,3
Reproducibility standard deviation s_R, mg/kg	1,2	0,6	3,3	2,3	2,6	1,1
Reproducibility limit R [$r = 2,8 \times s_R$], mg/kg	3,4	1,7	9,2	6,4	7,3	3,1
HorRat	0,7	0,9	1,0	1,0	0,9	1,1

[a] nc = naturally contaminated

[b] Calculated using Robust Statistics: algorithm A (ISO 13528:2015*)*

Annex B
(informative)

Typical chromatogram



Key

Chromatography on Dionex-HPLC with PDA100, using Nucleosil C18 (250 mm x 4,6 mm, 5 µm), isocratic condition

Y relative intensity, in %;

t time, in min;

1 L-Tryptophan

2 Domoic acid

3 epi-domoic acid

4 iso-domoic acid D

5 iso-domoic acid E

Figure B.1 — Typical chromatogram of MUS1b reference material

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