EU Reference Laboratory for Monitoring of Marine Biotoxins

2nd Edition: Revised in April 2026

EU-Harmonised Standard Operating Procedure for determination of domoic acid in shellfish by HPLC-UV

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1. Introduction

The primary toxin responsible for the Amnesic Shellfish Poisoning (ASP) is domoic acid (DA), which is mainly produced by the diatom *Pseudo-nitzschia* spp., but also by species from the genera *Nitzschia* and *Amphora*, as well as certain rhodophyte algae. The DA belongs to a group of amino acids, called kainoids, which are classified as neuroexcitants or excitoxins that interfiere with the neurotransmission mechanisms in the brain.

This Standard Operating Procedure (SOP) is based on a procedure described by Quilliam, Xie and Hardstaff (1995) for the quantitative determination of DA in unsalted seafood [2]. In this procedure, a single-step extraction with 50% aqueous methanol, 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 (HPLC-UV). Considering the results obtained in the validation process, the method described in this SOP allows the elimination of the clean-up step.

2. Significant changes in new edition to previous edition

Compared to the 1st Edition (June 2008), this edition includes format-related updates as well as additional comments regarding quality control measures and the assurance of the validity of the results. The analytical content has not been substantially modified. An obsolete reference to Annex B in Section 2 has been removed. Normative references have been reviewed.

3. Scope

This SOP specifies a method for the quantitative determination of DA in bivalve molluscs. The limit of detection (LOD) depends on UV detector sensitivity, but it should be within the range of 0.05 – 4.0 mg/kg. The limit of quantification (LOQ) shall be at least 2.7 mg/kg.

The method has been tested for DA determination in different matrices such as mussels, clams or scallops, spiked and/or naturally contaminated at levels ranging from1.8 – 150 mg/kg.

4. Normative references

- **Regulation (EC) No 853/2004** of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin
- **Commission Implementing Regulation (EU) 2019/627** of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls

5. Reagents and reference materials

- Deionized water: Grade 1 according to ISO 3696:1987.
- Methanol: HPLC quality.
- Acetonitrile: HPLC quality.
- Extraction solvent: Methanol / Water 50:50 v/v.
- Acetonitrile / Water 1:9 v/v.

- Trifluoroacetic acid (TFA): Spectrophotometric grade (≥99%).
- Mobile phase: Aqueous 10 % acetonitrile with 0.1 % TFA. For single pump systems, mix 100 ml acetonitrile with 400 ml water, add 1.0 ml TFA, and dilute to 1 L with water. Other mobile phases could be used.
- Certified Reference Material (CRM): Certified calibration solution for DA. CRMs shall be stored according to manufacturer's conditions. Prior to opening, each ampoule should be allowed to warm at room temperature. The remaining content of opened ampoules shall be transferred to amber vials and sealed. These vials shall be stored at refrigeration temperature (approx. 4ºC) and darkness for a maximum of three months.
- Calibration solutions: Prepare a series of calibration solutions with increasing concentration of DA + epi-DA, within the mass range of e.g. 0.2 to 25 µg/ml, by diluting the certified calibration solution with acetonitrile:water, 1:9 v/v. Keep solutions in darkness and refrigerated for a maximum of three months. Warm up solutions at room temperature before use.
- Reference material: Mussel tissue reference material for DA. It shall be stored according to manufacturer's conditions.

6. Equipment and material

Standard laboratory apparatus shall be used and shall include the following:

- Analytical balance, capable of weighing to the nearest of 0.1 g.
- Grinder or mixer.
- Centrifuge, capable of reaching 3000 x g (refrigerated at 4°C, if possible).
- Adjustable automatic pipettes, cover in the range from 20µl to 1000 µl.
- Membrana filter, methanol compatible with 0.2 µm or 0.45 µm pore size.

HPLC instrumentation

- Injection system.
- Pump, capable of isocratic elution.
- Column oven, able to reach 40°C ± 2°C.
- 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.
- 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 7.3 "Laboratory procedure, HPLC".
- Glass amber vials, 2 ml or less, with crimp caps (to store domoic acid working calibrations solutions).

7. Procedure

7.1 Sample Preparation

- Bivalves with shells: Thoroughly clean outside of the shellfish with fresh water. Open the shells by cutting the adductor muscle. Rinse inside with fresh water to remove sand and foreign material. Remove meat from the shell by separating adductor muscles and tissue connecting at the hinge. Do not use heat or anesthetics to open the shells. After removal, drain tissues for 5 min in a sieve to remove salt water. For representative sampling, at least 100-150 g of pooled tissue should be homogenized using a grinder or blender [4]. Subsamples 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 collected [5].
- Bivalves without shells (whole body or any edible part separately): If necessary, clean the exterior with fresh water and allow it to drain. For representative sampling, at least 100-150 g of pooled tissue should be homogenized using a grinder or blender. Subsamples 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 collected [5].
- Fish: Clean, scale and eviscerate fish. In case of small fish ≤ 15 cm, use 5-10 individuals. For intermediate-sized fish, remove and discard heads, scales, tails, fins, viscera, and inedible bones. Fillet the fish to obtain all flesh and skin from head to tail and from the dorsal to the ventral side on both sides [4]. For representative sampling, at least 100-150 g of pooled tissue should be homogenized using a grinder or blender. Subsamples from this homogenate can be taken immediately after blending, while still well-mixed, or after mixing again.

7.2 Extraction procedure

Accurately weigh 4 ± 0.1 g of tissue homogenate into a graduate centrifuge tube or a stainless steel micro-blender cup. Add 16 ml of extraction solvent (methanol:water 50:50) and homogenize the sample thoroughly (3 min. at 10,000 rpm). Do not try to recover all the tissue remaining on the homogenizer probe or in the blender cup; instead, wash them thoroughly afterwards to prevent contamination of subsequent samples. If a blender has been used for homogenization, transfer 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 for the 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 diluted 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.

7.3 HPLC measurement

Determination of the DA content in a sample is performed after chromatographic separation on a reversed-phase column under isocratic conditions. The following HPLC conditions have been found to provide satisfactory 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 / 220 nm*

* For quality control, it is recommended to compare the 242/220 nm area ratio obtained in calibration standards with that of the samples, to ensure that the observed peaks correspond to DA.

NOTE: High injection volumes may require diluting the extract with water to avoid broadening of the DA peak associated, which can occur when injecting a solvent with stronger elution strength than the mobile phase (the “solvent wash out” effect).

7.4 Calibration curve

Prepare a calibration curve, with at least four points, each day of analysis and/or whenever chromatographic conditions change. Plot peak area versus the concentration of the injected DA+ epi-DA calibration solutions. Ensure that the calibration curve shows a linear regression with a coefficient of determination (r^2) ≥ 0.99. Also, the residual criterion shall be verified: the difference between the experimental and theoretical values should fall within 90–110%.

7.5 Sample injection

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

8. Evaluation of results

8.1 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 isomerize, especially to the C5´-diasteomer, epi-domoic acid (epi-DA); therefore, a mixture will inevitably form during long-term storage of any standard solution. 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 any time. Under some LC conditions, DA and epi-DA do not resolve; this

does not present a problem and, in fact, simplifies the analysis. Analysts should base instrument calibration and quantification on the sum of both DA and epi-DA peak areas [3].

8.2 Quantification

After determining each sample concentration (DA+epi-DA) using the calibration curve, calculate the level of DA+epi-DA in the sample using the following formula:

$$\mathbf{concentration}\ (\mu\mathbf{g\ DA} + \mathbf{\ epi} - \mathbf{DA/g}) = \frac{\boldsymbol{\mu gDA + epiDA/ml\ injected\ extract}}{\boldsymbol{w}} \times \boldsymbol{D} \times \boldsymbol{V_t}$$

- Vt: total volume of homogenate and extracting solvent in ml
- W: sample weight
- D: dilution factor (if extract has been diluted)

8.3 Precision

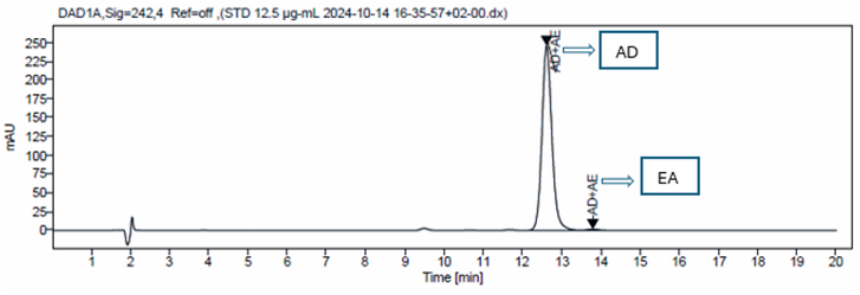
The method has been validated in a formal collaborative study involving 13 participating laboratories. Details of the interlaboratory study on the precision of the method are provided in Annex A. The values derived from this interlaboratory study may not be applicable to concentration ranges and matrices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

9. 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 Standard Operating Procedure 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.

10. Summary of the procedure

Sample preparation	Bivalves with shell: Clean, open, meat removal and drain Bivalves without shell: Clean and dry (if needed) Sampling: Homogenize 100-150g of pooled tissue For scallops at least 10 specimens or edible parts
Extraction	Weigh: 4 g± 0.1 g ⟶ Add solvent: 16 ml MeOH:H_2O (50:50) Homogenize: Blender 3 minutes at 10,000 rpm and centrifuge: ≥3,000x g for 10 min Filter: Supernatant with 0.45 µm or 0.2 µm methanol-compatible filter Storage: Analyze immediately or store extract in a sealed container at ~-12°C Extraction blank: perform extraction with water instead of tissue
HPLC Chromatographic conditions	Column: C18 reversed phase, 5 µm, 250 mm x 4.6 mm Temperature: 40°C Flow rate: 1ml/min Injection volume: 20 µl
HPLC Calibration curve	4 points daily or after changes in conditions Plot peak area vs DA+epi-DA concentration Ensure $r^2 \geq 0.99$
HPLC Sample injection	Inject samples in duplicate (ensure CV<5%) Prevent carry-over by cleaning injector loop
Evaluation of results: Identification	Identify DA and epi-DA by comparing retention times to standards Notes on DA/epi-DA: DA isomerizes into epi-DA over time. Calibration and quantification are based on the sum of DA + epi-DA
Evaluation of results: Quantification	Calculate the level of DA + epi-DA using the following formula: $$\textbf{concentration}\ (\mu\textbf{g DA} + \textbf{epiDA/g}) = \frac{\boldsymbol{\mu gDA + epiDA/ml\ injected\ extract}}{\boldsymbol{w}} \times \boldsymbol{D} \times \boldsymbol{V_t}$$ Vt: total volume of homogenate + extract solvent in ml W: Sample tissue weight D: Dilution factor
Evaluation of results: Chromatograms	The peak that first appears in the chromatogram corresponds to DA. As mentioned above, DA isomerizes into epi-DA over time; therefore, in some cases, a small peak may be observed after the DA peak. 
Precision	The method has been validated in a formal collaborative study with 13 participating laboratories. Details of the interlaboratory study are given in Annex A. The values derived from this study may not be applicable to concentration ranges and matrices other than those specified in Annex A.

11. Bibliography

[1] Quilliam, M.A. “Chemical methods for domoic acid, the amnesic shellfish poisoning (ASP) toxin”. In Manual on harmful marine microalgae, ch. 9, 247-265 (2004). Hallegraeff, G.M., Anderson, D.M., and Cembella, A.D., Eds.

[2] Quilliam, M.A., Xie, M., and Hardstaff, W.R. “Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood”. Journal of AOAC International, 78(2), 543-554 (1995).

[3] Quilliam, M. A., Sim, P. G.; McCulloch, A.W. and McInness, A. G. “High performance liquid chromatography of domoic acid, a marine neurotoxin, with application to shellfish and plankton” Int. J. Environ. Anal. Chem. 36, 139-154 (1989).

[4] Official Methods of Analysis of the Association of Official Analytical Chemists. 15th ED, sec 959.08 and 937.07, Volume II. AOAC, Arlintong, VA, U.S.A. 1990.

[5] Commission Decision of 15 March 2002 establishing special health checks for the harvesting of certain bivalve molluscs with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive91/492/EEC (2002/226/EC). O.J.E.C. L75 of 16.3.2002, 65-66.

12. Annex A: Precision data

The following data were obtained from the "EU validation study of domoic acid determination by HPLC-UV", organised by the EU 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.

Sample	1	2	3	4	5	6
Matrix	Spiked clam	Naturally contaminated clam	Spiked mussel	Naturally contaminated gonad scallop	Naturally contaminated whole body scallop	Naturally contaminated Anchovy
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, µ/g	3.38	17.7	11.6	2.72	9.63	85.1
Repeatibility standard deviation S_r	0.28	0.40	0.37	0.12	0.18	1.1
Repeatability limit r [r = 2,8 x S_r)	8.3	2.3	3.2	4.3	1.9	1.3
Reproducibility limit R [r = 2,8 x S_R)	0.51	1.6	2.0	0.62	1.2	6.6
Horrat	1.1	0.87	1.6	1.7	1.0	0.94