UK-NRL

Title: Standard Operating Procedure for the Quantitation of Amnesic Shellfish Poison in Shellfish by HPLC with UV Detection.

Production Summary

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Reviewed by	UK-NRL Network
Date	December 2020
Issue authorisation	UK NRL for Marine Biotoxins

Distribution of copies

Authorised Recipient	Location

History of Procedure

Issue	Date Issued	Changes
Version 1	20th May 2011	
Version 2	15th December 2020	Section 1: Regulations up-dated.
		Section 9: Sample prep. reworded
		Section 14.5: Application of recovery clarified

1. Aim and Scope

The method described below is applied in support of EC Regulation 853/2004 which lays down the specific hygiene rules for food of animal origin and Regulation 2019/627, which specifies the official methods of analysis. The domoic acid content is reported as the sum of domoic acid and epidomoic acid (measured against a certified domoic acid +epi-domoic acid calibration solution).

This SOP describes the sample storage, preparation and extraction conditions which must be followed for the analysis of domoic acid in shellfish tissue. It describes the method performance criteria which must be met to comply with Commission decision 2002/657/EC.

2. Introduction

Domoic acid is the toxin responsible for incidents of amnesic shellfish poisoning. Domoic acid, a rare naturally occurring amino acid, is a member of a group of potent neurotoxic amino acids that act as agonists to glutamate, a neurotransmitter in the central nervous system. Domoic acid occurs in certain phytoplankton (Pseudo-nitzschia spp), which are ingested by shellfish during normal filter feeding. When the domoic acid content of shellfish is elevated they may pose a significant risk to human health. The above Directives establish a limit of 20µg/g of domoic acid in whole tissue or any part edible separately.

3. Principle of the Method

The method consists of a methanol/water extraction followed by an optional strong anion exchange clean-up. The clean-up protocol is not necessary if the performance criteria specified can be met using the crude methanol extract and the domoic acid and epi-domoic acid elute away from interfering peaks. The resulting eluate is analysed by HPLC with UV detection (based on Quilliam et al 1995).

4. Safety

Reference should be made to individual laboratory risk assessments and COSHH documentation.

5. Environmental Conditions

The bowl and lid of the processor/blender, sieve, knife and spatula are washed, as a minimum, under hot running tap water between samples. Benches are wiped if there is a spillage and cleaned with detergent at the end of the day.

The following storage conditions for samples prior to official testing for monitoring purposes apply:

Stage	Storage conditions
Whole shellfish	Whole shellfish may be stored refrigerated (2-8°C) so that no longer than 72 hours elapses between sample harvest and sample homogenisation
Homogenate	Must not be stored prior to analysis
50 % (v/v) aqueous methanol extract for ASP analysis	May be stored at 2-8°C for up to 5 days
Citrate buffer extract for ASP analysis	May be stored for a maximum of 5 days at 2-8°C.

6. Calibration solutions

6.1 Prepare a calibration series, with at least four points, from NRC Canada (CRM-DA) or Cifga Spain (CRM DA) stock standard solution. Keep calibration solutions in the dark and refrigerated (at approximately 4ºC) when not in use. Do not store calibration series for more than 6 months. Do not freeze the solutions. Allow solutions to equilibrate to room temperature before use. The plot of peak areas against the concentration of the injected DA+ epi-DA calibration solutions must show a linear regression of $r^2 \geq 0.99$.

7. Control material

7.1 Certified matrix reference material for domoic acid, e.g. available from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia, Canada. The material should be stored frozen (-12ºC or lower) and should be used to test the accuracy of the analytical procedure on a routine basis.

7.2 Laboratory matrix reference material, or fortified material containing known amounts of analyte at or near the maximum permitted limit, as well as a procedural blank must be run with each sample batch. Method performance must be monitored for each

batch of samples using spiked material, lab reference material or certified reference material.

8 Apparatus

8.1 Analytical balance, capable of weighing to the nearest of 0.1g.

8.2 Grinder or mixer (Ultra Turrax or vortex).

8.3 Centrifuge, capable of reaching 2500g.

8.4 Centrifuge tubes, nominal volume 30-50ml, with screw tops.

8.5 Membrane filter, methanol compatible with a pore size 0.2µm or 0.45µm.

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

8.7 HPLC instrumentation, comprising the following;

8.8 Injection system.

8.9 Pump, capable of isocratic elution or gradient.

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

NOTE Other LC columns and dimensions may be suitable if the separation and method performance can be achieved. The use of a guard column is recommended.

8.11 UV-spectrophotometric detector set to a wavelength of 242nm.

8.12 HPLC vials.

9. Sample Preparation.

Note: **Refer to the current NRL guidance document on recommended minimum number of animals to be opened for testing.**

9.1 Thoroughly clean the outside of the shellfish with fresh water. Open by cutting the adductor muscle. Rinse inside with fresh water to remove sand and foreign material only if necessary to avoid possible loss of toxin. Remove meat from shell by separating adductor muscles and tissue connecting at hinge.

9.2. After removal from shellfish, drain the tissue in a sieve.

9.3. Transfer to a blender and blend until homogeneous.

10. Extraction procedure

10.1 Accurately weigh 4g ± 0.1g of tissue homogenate into a graduated centrifuge tube or a stainless steel micro-blender cup.

10.2 Add 16ml of extraction solvent (methanol: water 50:50 (v/v)) and homogenize the sample. An alternative extraction protocol may be used provided it has been internally validated and shown to be fit for purpose, in terms of achieving suitable and repeatable recoveries. 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.

10.3 Centrifuge at 2500g or higher for ≥10min.

10.4 If not using SPE clean-up, filter a portion of the supernatant through a dry methanol compatible 0.2µm filter. Sample extracts should be analysed as soon as possible.

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

10.6 For screening samples with a known or suspected high level of contamination it may be necessary to bring sample extract within the standard curve calibration range. A measured aliquot of the extract can be diluted to a fixed volume with the appropriate diluent using a volumetric flask, calibrated pipette or micropipettes. Mix thoroughly before analysis.

11. Sample Clean-Up (Optional):

The conditioning, washing and elution volumes are given as examples and may vary depending on the cartridges used. Refer to the manufacturer's instructions. The sample clean-up procedure may be automated.

11.1 Condition a SAX cartridge by passing sequentially 6ml methanol followed by 3ml water and finally 3ml of extraction solvent.

11.2 Load 5mL of supernatant on to the cartridge. For manual operation, the flow rate should be ca. 1 drop/second. Stop the flow as soon as the meniscus reaches the top of the cartridge packing and discard the effluent.

11.3 Wash the cartridge with 5ml of cartridge wash solution (Acetonitrile: water 1:9 (v/v)) and discard the effluent.

11.4 Add 0.3mL of citrate buffer and allow to flow slowly onto the cartridge until the meniscus reaches the top of the cartridge packing and discard the effluent.

11.5 Add 5mL of citrate buffer and elute (ca. 1 drop/second) and collect the effluent in a suitable glass container until the meniscus reaches the top of the cartridge packing.

11.6 Mix the solution well and transfer a suitable aliquot into a vial for HPLC analysis.

Note: The cleaned extracts should be analysed as soon as possible. If analysis is not performed immediately, the extract may be stored in a tightly sealed container in the dark at 2 – 8°C for a maximum of 5 days.

12. HPLC measurement

12.1 Determination of the domoic acid content in a sample is performed after chromatographic separation on a reversed phase column using isocratic or gradient conditions. The following example of HPLC conditions will produce acceptable results:

Isocratic conditions using mobile phase, 15% Acetonitrile, 0.1% TFA in UPLC grade water.

Column: C18 reversed phase, 5µm, 250mm x 4.6mm.

Temperature: 40°C

Flow: 1ml/min

Injection volume: 20µl

UV detector: 242nm

12.2 Operating conditions must be such that,

- the ratio of the chromatographic retention time of the analyte must correspond to that of the calibration solution at a tolerance of ± 2.5%
- The minimum acceptable retention time for the analyte is twice the retention time corresponding to the void volume of the column.

13. Evaluation of results

13.1 Identification: Identify domoic acid and epi-domoic acid by comparing the retention times of the sample with that of the standards. Epi-domoic acid may not be identifiable in the standard solutions as they may not resolve under certain chromatographic conditions.

13.2 Quantification: The concentration of domoic acid present in the sample is reported as the sum of domoic acid and epi-domoic acid.

14. Essential Performance Criteria

14.1 In the case of repeated analyses of a certified reference material, the guideline range for the deviation of the experimentally determined recovery corrected value is a minimum of -20% to +10% of the certified value.

14.2 The method must achieve within-laboratory reproducibility not greater than the corresponding reproducibility CV (inter-laboratory) at a concentration at or around 0.5 x the permitted level (i.e.10µg/g). For example, from data reported for Quasimeme Round 51, the mean between - laboratory CV of 16% is reported (the mean of the figures from the three statistical models quoted) at a concentration of ~20ug/g. Therefore the within-laboratory reproducibility measured should not be greater than 16%.

14.3 Procedural blank. The chromatogram from the procedural blank should not show a peak at the same retention time as DA (or Epi-DA).

14.4 Positive control. When using a laboratory reference material (LRM), the combined concentration of DA and Epi-DA should be within the upper and lower quality control limits assigned during LRM characterisation.

14.5 Recovery correction. The recovery value must be within the range 80-110%. If recovery is not 100% the reported value should be corrected accordingly and recovery details included on reports.

14.6 Acceptability. In assessing the acceptability of results, both internal QC and HPLC system suitability criteria must also be considered and a judgement made by the competent person.

14.7 The decision level for the test is that level above which there is a ≥ 95% statistical certainty that the permitted limit has been truly exceeded and this must be quoted on all reports. It may be quoted as the uncertainty of measurement associated with the method.