COMPARISON BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY OF THE EFFICIENCY OF TWO EXTRACTION PROCEDURES IN THE RECOVERY OF OKADAIC ACID AND DINOPHYSISTOXIN-2 FROM SHELLFISH HOMOGENATES

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1. SUMMARY

1. Mussels collected in September and November 2002 from two sea lochs on the west coast of Scotland were used for a study on the comparison of two extraction procedures for the diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA) and dinophysistoxin-2 (DTX2).

2. The concentrations of OA and DTX2 (DTX1 was not detected) in these mussels were determined prior to the study by the routine Fisheries Research Services (FRS) DSP monitoring method, M1940 (Appendix I). Concentrations ranged from 44 – 157 µg OA/kg for OA and 88 - 350 µg OA equivalents/kg for DTX2 in pooled samples of 10 mussels. This analysis was carried out to enable evaluation of the extraction efficiency of the two procedures over a range of DSP toxin concentrations.

3. The FRS extraction procedure (SOP 2245) was compared with a United Kingdom National Reference Laboratory (UK-NRL) procedure (SOP 2247) to determine the extraction efficiencies, with a view to standardisation of the mouse bioassay (MBA) method.

4. OA and DTX2 concentrations were determined by liquid chromatography-mass spectrometry (LC-MS) at each of the extraction stages for both procedures. In addition, the total extracted OA and DTX2 was calculated.

5. The concentrations of OA recovered from SOP 2247 acetone extraction were higher, but the subsequent losses were greater after liquid/liquid partitioning into diethyl ether (DEE). However, there were generally no statistically significant differences between the total OA concentrations extracted by either procedure.

6. The DTX2 recoveries were similar for SOP 2245 and SOP 2247 acetone extractions and there were also no significant differences in the total DTX2 concentrations extracted by either procedure.
2. INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a well-known and widespread human toxic syndrome causing gastrointestinal problems after consumption of contaminated shellfish.\(^1\)\(^2\) The syndrome was originally associated with the lipophilic compound, okadaic acid and its structurally related derivatives, dinophysistoxins.\(^3\)\(^4\) Over the years several additional lipophilic compounds toxic to mice have been detected and added to the DSP toxin group despite differences in their structure and function. This situation was resolved to an extent when a recent European Commission Decision\(^5\) re-classified the toxins into three groups, a) the DSP toxins, okadaic acid (OA) and dinophysistoxins (DTXs) and pectenotoxins (PTXs), b) yessotoxins (YTXs) and c) azaspiracids (AZAs). The Decision states that the maximum level of OA, DTXs and PTXs together in bivalve molluscs, echinoderms, tunicates and marine gastropods intended for human consumption shall be 160 µg of OA equivalents/kg. For the purpose of this study OA and DTXs are defined as the DSP toxins. EU Decision 2002/225/EC states that “A series of mouse bioassay procedures, differing in the test portion and in the solvents used for the extraction and purification steps can be used for detection of the toxins… Sensitivity and selectivity depend on the choice of the solvents used for the extraction and purification steps….\)” The use of acetone alone can result in false positives due to other compounds being extracted, while diethyl ether (DEE) in the final extraction step facilitates the extraction of diarrhetic toxins, although this solvent has been shown to extract YTXs erratically\(^6\). Chloroform extracts a wider spectrum of toxins including PTXs, YTXs and other, unknown, toxins with neurotoxic effects\(^6\). Although it is widely accepted that the mouse bioassay (MBA) is based on the use of acetone as the primary extraction solvent followed by liquid/liquid partition with diethyl ether, little standardisation of the procedure exists throughout Europe. To address this issue in the UK, the UK National Reference Laboratory (UK-NRL) for Marine Biotoxins Network at their meeting in February 2003 made a series of recommendations to standardise the procedures. As the need for further research into the method was identified the resultant standard operating procedure (SOP) was named SOP 2247. FRS has held UKAS accreditation on the MBA extraction procedure using acetone and diethyl ether since 2002 (SOP 2245). This bioassay meets the requirements of Directive 91/492/EEC\(^7\). In order to change the current procedure over to SOP 2247 data was required to demonstrate that equivalent quantities of OA and DTX2 were recoverable using the two procedures. The main differences between the two procedures are the ratio of acetone to homogenate in the primary extraction, the volume of water remaining after rotary evaporation of the acetone and the number of subsequent liquid/liquid partition steps into DEE. In SOP 2247, 100 g (± 1 g) of homogenised shellfish is extracted with two 100 ml (± 5 ml) volumes of acetone. Three liquid/liquid partition steps with DEE follow this. In contrast, SOP 2245 extracts 75 g (± 1 g) of homogenised shellfish with 225 ml (± 5 ml) of acetone, followed by two liquid/liquid partition steps with DEE. The purpose of this study was therefore to determine, by LC-MS, if SOP 2247 (Appendix II) gave equivalent recoveries of OA and DTX2 when compared to SOP 2245 (Appendix III).
3. METHODOLOGY

3.1 Sample Collection

Mussels (*Mytilus edulis*) were collected from Loch Laxford, Sutherland and Loch Striven, Argyll and Bute in September and November 2002 respectively (Fig. 1), transported to FRS in cool boxes packed with ice and stored, in shell, at -20°C until required for analysis.

3.2 Sample Preparation

In May 2003 approximately 1 kg of Loch Laxford mussels, of marketable size (ca. > 15 mm), were thawed at room temperature, mixed and divided to give two sub-samples. 2-3 kg of Loch Striven mussels, of marketable size, were also thawed, mixed and divided to give three sub-samples. A 2-3 kg composite (50% w/w Loch Laxford:Loch Striven mussels) was also created to give a third sample from which three sub-samples were taken. Prior to carrying out the comparison study, the Loch Laxford and Loch Striven mussels (pools of 10 individuals) were analysed in accordance with FRS method M1940 (Appendix I). The concentrations of OA in the Loch Leven and Loch Striven mussels were 44 µg OA/kg and 157 µg OA/kg respectively (DTX2 concentrations were 88 µg OA equivalents/kg and 350 µg OA equivalents/kg respectively). Preparation of mussel homogenates from these two sites provided a range of OA and DTX2 concentrations for the comparison of the extraction efficiency of the two SOPs.

3.3 Extraction

Each of the mussel sub-samples prepared above was shucked and homogenised for 1 minute using a domestic food processor to give enough material for a 100 g (SOP 2247) and 75 g (SOP 2245) sample. Each sub-sample was aliquotted into a container and extracted using either SOP 2247 or SOP 2245. At each stage of the extraction procedure, 1 ml of sample was removed and analysed following the schematic diagram in Figure 2 and detailed below. For the purpose of this study replicates of three separate mussel homogenates were analysed to observe the extraction efficiencies over a range of OA and DTX2 concentrations.

3.4 Analysis of Samples by LC-MS

At each stage of the extraction procedure, 1 ml of sample was removed (Fig. 2) and analysed in duplicate by high performance liquid chromatography (Agilent Technologies, West Lothian, UK) linked to a single quadropole mass spectrometer (API 150EX, Applied Biosystems, Warrington, UK) with a TurboIonspray interface. Acetone extracts of the shellfish homogenate were passed through a 0.2 µm cellulose acetate filter into a 2 ml borosilicate glass vial prior to analysis by LC-MS. DEE extracts (1 ml in a 2 ml borosilicate glass vial) were dried down with nitrogen at room temperature, reconstituted in methanol:water (1 ml, 80:20 v/v) and sonicated for 5 minutes prior to analysis by LC-MS. A base de-activated silica (BDS) reverse phase C8 column (50 x 2.1 mm, 3 µm - Thermo Hypersil-Keystone, Runcorn, UK) was used for all analyses. The mobile phase consisted of 2 mM ammonium formate (Fisher Scientific, Loughborough, UK) and 50 mM formic acid (Sigma-Aldrich, Dorset, UK) in 50% v/v
acetonitrile (Rathburns, Walkerburn, UK) at a flow rate of 0.25 ml min\(^{-1}\). OA and DTX2 have the same relative molecular mass of 804.5 \(g\ \text{mol}^{-1}\) and the mass-to-charge \((m/z)\) ratio of 803.5 (from the \([\text{M-H}]^+\)) was used for identification and quantification of both analytes when analysed by LC-MS. DTX2 was also quantified using OA as the calibrant with a response factor correction that has been previously determined at FRS (data not in report). The full MS parameters for DSP toxin analysis are given in Table 1. Calibration solutions for OA were prepared in methanol from OACS-1 standard (25.3 \(\mu\)g OA/ml, National Research Council, (NRC), Canada). Sets of calibration standards (ranging from 10 to 275 ng OA/ml) were analysed throughout the analysis run and calibration curves were produced using the area of the analyte peak. Calibration curves were linear with a correlation coefficient of greater than 0.99. The concentrations of OA in the extracts were calculated by Analyst version 1.1 software (Applied Biosystems, Cheshire, UK) from the external calibration curve.

### 3.5 Calculations

Shellfish contain a considerable amount of water (mussels up to 85%, pers. comm. L. Stobo) and therefore this was taken into account in the study. The calculations from the primary extractions assumed that the amount of water in the initial acetone extraction was 85\% of the weight of mussel homogenate used. A multiplication \((m)\) factor was therefore applied to results obtained from these extractions as follows:

\[
m = \frac{V_a + (W_h \times 0.85)}{W_h}
\]

Where \(V_a\) = volume of acetone (ml) and \(W_h\) = weight of homogenate extracted (g).

The subsequent acetone extraction in SOP 2247 and fractions obtained from the liquid/liquid partitions into DEE were regarded as containing negligible amounts of water and the following multiplier \((m)\) was therefore applied to results obtained from these extracts:

\[
m = V_s \times W_h
\]

Where \(V_s\) = volume of solvent (ml) and \(W_h\) = weight of homogenate extracted (g).

The value of \(V_s\) in the liquid/liquid partitioning steps corresponded to the volume of water remaining after the acetone fraction had been rotary evaporated. For SOP 2245 samples this was taken as 40 ml, whereas 50 ml of solvent was used for SOP 2247 samples.

Data for each homogenate were analysed in turn using a linear mixed model, where the fixed term was defined by the following groups,

- SOP 2245 single acetone extraction,
- SOP 2247 double acetone extraction,
- SOP 2245 double DEE liquid/liquid partition and
- SOP 2247 triple DEE liquid/liquid partition.
The random terms were samples and replicates. Before carrying out the analyses of variance, distribution of the data was considered. With a maximum of 24 observations in any single analysis it was not possible to demonstrate normality absolutely. However, there was no suggestion of serious non-normality and therefore the data were analysed on the original scale.

4. RESULTS AND DISCUSSION

This study presents LC-MS data on the concentrations of OA and DTX2 recovered from three mussel homogenates at different stages of the DSP toxin extraction procedure as described in SOPs 2245 and 2247. Recoveries of OA using the double acetone extraction procedure of SOP 2247 were higher than for the single acetone extraction of SOP 2245 (Tables 2 and 3, Fig. 3) for all three homogenates. Although the levels of OA extracted after the first acetone step of SOP 2247 were comparable to the levels obtained with SOP 2245, the additional acetone extraction of the former made a significant contribution to the difference in the total OA recovered between the two procedures. SOP 2247 was therefore more efficient at recovering OA from the homogenates analysed. These recoveries were significantly (p < 0.05) higher in homogenates 1 and 2 but not in homogenate 3. This was due to the high degree of variability (%CV), for both procedures, in the concentrations of OA in the sub-samples for this homogenate. The variability of OA concentrations between sub-samples of the same homogenate for the acetone extractions was not consistent throughout the study, ranging from 2.7% to 32.9%, highlighting the difficulty in obtaining a truly homogeneous mix of mussel material. After rotary evaporation of the acetone from the primary extraction a residual volume of water remained which was then subjected to sequential liquid/liquid partitioning with equal volumes of DEE. Partitioning of OA into the first volume of DEE was less efficient in SOP 2247 due to the presence of a higher volume of water (50 ml, as opposed to 40 ml present in SOP 2245). A higher volume of water was present in SOP 2247 sample as 100 g of homogenate was extracted compared to 75 g in SOP 2245. Losses of OA after partitioning into DEE were significant (p < 0.05) for all homogenates extracted using SOP 2247, but only for homogenate 2 using SOP 2245. It should be noted that the proportion of OA in the second DEE partitioning step for this homogenate was much lower than the other two homogenates, resulting in a lower total concentration. However, there were losses of OA in all homogenates for both procedures after the liquid/liquid partitioning steps into DEE. These losses were greater for SOP 2247 for all three samples, although the total OA concentration was only significantly (p < 0.05) different between the two procedures in homogenate 2. This difference can be attributed to the lower level of OA initially extracted in SOP 2245 and the lower proportion of OA recovered after the second DEE step, as mentioned above. In conclusion, the recovery of OA into DEE was generally more efficient in the first partition step of SOP 2245 compared to the first partition step of SOP 2247. This was due to the higher water content in the latter and the formation of an emulsion, which retained some OA. The incorporation of a third liquid/liquid DEE partition step in SOP 2247 compensated for this initial lower recovery, resulting in similar final concentrations of OA for the two procedures.

Recoveries of DTX2 were not significantly different (p > 0.05) between the two procedures, for either the primary, acetone, extraction or the liquid/liquid DEE partitioning step (Tables 4 and 5, Fig. 4). However, there were losses of DTX2 for both procedures during the liquid/liquid partition into DEE (up to 29.6% and 34.7% for SOP
Comparison By Liquid Chromatography –Mass Spectrometry

2245 and SOP 2247 respectively). These losses were significant in both procedures for homogenates 1 and 2. As with OA, the recovery of DTX2 into DEE was higher in the first liquid/liquid partitioning step of SOP 2245 compared with that of SOP 2247 due to the higher water content in the latter. However, the subsequent DEE partitioning steps of SOP 2247 were able to recover comparable levels of DTX2 to SOP 2245, to the extent that there were no significant differences between the final DTX2 concentrations for the two procedures.

After extracting with acetone and rotary evaporation, the solution left was an aqueous emulsion. This emulsion affected the recovery efficiency of the procedure as both OA and DTX2 was retained in solution. In summary, the lower solvent:homogenate ratio and greater weight of homogenate used in SOP 2247 resulted in a higher residual aqueous emulsion volume and therefore less efficient recovery of OA and DTX2 in the first DEE step. This was, however, offset by the incorporation of a third DEE liquid/liquid partition step resulting in similar final OA and DTX2 recovery rates between the two procedures.

The data presented in this study only applies for mussels and the lipophilic toxins, OA and DTX2 and cannot be extrapolated to other toxins and shellfish species.

5. CONCLUSIONS

1. Although the single acetone extraction in SOP 2245 uses a higher ratio of acetone to homogenate, the double acetone extraction in SOP 2247 gave higher recoveries of OA. However, there were no significant differences between the two procedures for the recovery of DTX2.

2. During the liquid/liquid partitioning into DEE a small amount of OA and DTX2 remained in the aqueous fraction due to the formation of an emulsion. This contributed to the losses of OA and DTX2 between the acetone and DEE extractions (up to 31% and 48% for SOP 2245 and SOP 2247 respectively) along with random and systematic error. Both procedures showed losses for OA and DTX2 with SOP 2247 losses being significant for all three homogenates.

3. Throughout the study the variations between replicates were relatively high (>10%). These findings are consistent with other studies carried out on DSP toxins in mussels by FRS and highlights that the variability between sub-samples of the same homogenate exceeds the variability between replicate determinations within a sample.

4. The overall comparison of the two procedures showed that there was generally no significant difference in the total concentration of OA and DTX2 extracted.

6. ACKNOWLEDGEMENTS

The authors would like to thank staff at the FRS Marine Laboratory for their contribution to the analytical work required in this study and also the Food Standards Agency Scotland (FSAS) for funding the study under the Biotoxin Monitoring Programme (SO2007).
7. REFERENCES


8. TABLES

TABLE 1

MS parameters for the analysis of DSP toxins

<table>
<thead>
<tr>
<th>MS parameter</th>
<th>Setting</th>
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<td>Nebuliser gas velocity (l min⁻¹)</td>
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<td>Curtain gas velocity (l min⁻¹)</td>
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<td>Ion – OA &amp; DTX2</td>
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<td>Dwell time (ms)</td>
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TABLE 2

Summary of the mean concentration of okadaic acid (µg OA/kg) in three mussel homogenates using SOP 2245.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>OA</th>
<th>StDev</th>
<th>%CV</th>
<th>OA</th>
<th>StDev</th>
<th>%CV</th>
<th>OA</th>
<th>StDev</th>
<th>%CV</th>
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<td>1° (Acetone) Extraction</td>
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<td>9.70</td>
<td>28.4</td>
<td>70.07</td>
<td>9.61</td>
<td>13.7</td>
<td>141.55</td>
<td>46.62</td>
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<td>1.43</td>
<td>13.5</td>
<td>13.21</td>
<td>2.68</td>
<td>20.3</td>
<td>31.87</td>
<td>8.35</td>
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<td></td>
<td>2</td>
<td>10.60</td>
<td>1.43</td>
<td>13.5</td>
<td>13.21</td>
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# n=3 due to the mis-injection on LC-MS of one replicate.

OA = okadaic acid; StDev = standard deviation; CV = coefficient of variation; DEE = diethyl ether.

* Loss (%) = ((Acetone Extraction Total – DEE Partition Total)/Acetone Extraction Total) x 100
Summary of the mean concentration of okadaic acid (µg OA/kg) in three mussel homogenates using SOP 2247.

<table>
<thead>
<tr>
<th>Stage (Acetone) Extraction</th>
<th>Step</th>
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<th>Homogenate 2 (n=5#)</th>
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<td>[OA]</td>
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<tr>
<td>1 °</td>
<td>1</td>
<td>39.36</td>
<td>1.06</td>
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<th>Stage Diethyl ether Partition</th>
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<th>Homogenate 2 (n=5#)</th>
<th>Homogenate 3 (n=6)</th>
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<td></td>
<td></td>
<td>[OA]</td>
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# n=5 due to the mis-injection on LC-MS of one replicate.

OA = okadaic acid; StDev = standard deviation; CV = coefficient of variation; DEE = diethyl ether.

* Loss (%) = ((Acetone Extraction Total – DEE Partition Total)/Acetone Extraction Total) x 100
TABLE 4

Summary of the mean concentration of DTX2 (µg OA equivalents/kg) in three mussel homogenates using SOP 2245.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>[DTX2]</th>
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# n=3 due to the mis-injection on LC-MS of one replicate.

DTX2 = Dinophysistoxin-2; StDev = standard deviation; CV = coefficient of variation; DEE = diethyl ether

* Loss (%) = ((Acetone Extraction Total – DEE Partition Total)/Acetone Extraction Total) x 100
TABLE 5

Summary of the mean concentration of DTX2 (µg OA equivalents/kg) in three mussel homogenates using SOP 2247.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Homogenate 1 (n=4)</th>
<th>Homogenate 2 (n=5#)</th>
<th>Homogenate 3 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[DTX2]</td>
<td>StDev</td>
<td>%CV</td>
</tr>
<tr>
<td>1° (Acetone) Extraction</td>
<td>1</td>
<td>74.42</td>
<td>7.43</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>27.30</td>
<td>0.78</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>101.72</td>
<td>7.00</td>
<td>6.9</td>
</tr>
<tr>
<td>Diethyl ether Partition</td>
<td>1</td>
<td>31.31</td>
<td>8.73</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20.14</td>
<td>1.90</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.95</td>
<td>1.47</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>66.40</td>
<td>8.29</td>
<td>12.5</td>
</tr>
<tr>
<td>Loss*</td>
<td></td>
<td>34.7%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# n=5 due to the mis-injection on LC-MS of one replicate.

DTX2 = Dinophysistoxin-2; StDev = standard deviation; CV = coefficient of variation; DEE = diethyl ether.

* Loss (%) = ((Acetone Extraction Total – DEE Partition Total)/Acetone Extraction Total) x 1
9. FIGURES

Figure 1  Location of mussel sampling sites on the West Coast of Scotland.
Figure 2  Flowchart illustrating the extraction procedures and analysis of LC-MS samples for the determination of DSP toxins.

Mussel Homogenate

SOP 2247 (100 g)

Acetone extraction 1 (100 ml) → LC-MS analysis (Fixed volume 1 ml)

Acetone extraction 2 (100 ml) → LC-MS analysis (Fixed volume 1 ml)

DEE extraction 1 Residual volume* → LC-MS analysis (Fixed volume 1 ml)

DEE extraction 2 Residual volume* → LC-MS analysis (Fixed volume 1 ml)

DEE extraction 3 Residual volume* → LC-MS analysis (Fixed volume 1 ml)

SOP 2245 (75 g)

Acetone extraction (225 ml) → LC-MS analysis (Fixed volume 1 ml)

DEE extraction 1 Residual volume* → LC-MS analysis (Fixed volume 1 ml)

DEE extraction 2 Residual volume* → LC-MS analysis (Fixed volume 1 ml)

* The residual DEE volume was 50 ml in SOP 2247 and 40 ml in SOP 2245.
**Figure 3** Total mean okadaic acid concentration (µg OA/kg) in acetone and diethyl ether extracts from three mussel homogenates as determined by LC-MS. Error bars represent the standard deviation.

**Figure 4** Total mean DTX2 concentration (µg OA equivalents/kg) in acetone and diethyl ether extracts from three mussel homogenates as determined by LC-MS. Error bars represent the standard deviation.
10. APPENDIX I

M1940 - Determination of DSP in shellfish by LC-MS

1. Introduction and Scope

Method M1940 describes the determination of diarrhetic shellfish poisons (DSP) in shellfish tissue samples. The method has the ability to quantify okadaic acid, a major diarrhetic shellfish poison, and identify related diarrhetic shellfish poisons. The range of the method is from the limit of detection to 1375 ng g\(^{-1}\).

2. Principle of the Method

Diarrhetic shellfish poisons are extracted into solution and then filtered to remove particulate matter. The samples are transferred to glass vials for analysis by liquid chromatography mass spectrometry (LC-MS). The instrument then detects okadaic acid and quantifies this compound by comparison to a standard curve. Okadaic acid has a relative molecular mass of 804.5 g\(\text{mol}^{-1}\) and the mass-to-charge ratio 803.5 (from the \([\text{M-H}\]^{-}\)) is used to identify it when analysed by LC-MS. The LC-MS is able to distinguish ions of differing mass-to-charge ratios and quantify such ions.

3. Reference Material

MUS-2, NRC CRM (Blue mussel tissue homogenate).

4. Reagents

Deionised water (Millipore, ultrapure 18.2 M\(\Omega\))
Methanol (HPLC grade)
Ice
80\% (v/v) methanol/water solution
40\% (v/v) methanol/water solution

5. Major Equipment

Centrifuge
Ultraturrax\textsuperscript{®} (speed setting 2)
Two-place balance or better
Vortex mixer
Vacuum manifold with cartridge holder
Waters Oasis\textsuperscript{®} HLB SPE cartridges (60 mg, 3 ml)
MS Applied Biosystems API 150EX
HPLC (Agilent) comprising:
Solvent reservoir and degasser
Agilent 1100 quaternary pump
Agilent 1100 autosampler for 2 ml vials
Reverse Phase C8 column
Waste chemicals reservoir
Micro-centrifuge
6. Environmental Control

The bench is cleaned at the end of each batch with a disinfectant such as virkon®. Solvent reservoir and solvent waste bottles have stoppers and only small leakages due to evaporation are possible. Samples can be reused for later injections and are stored in the freezer (-20°C). Once the samples are ready to be disposed of, they can be poured into the non-chlorinated solvent waste and the glass vials are disposed of into the glass waste bin.

7. Interferences

With some samples the filter may become blocked with the particulate matter and it may not be possible to collect more than half a vial of filtrate. In this case, a new filter may be attached to the syringe or alternatively at least 100 µl of filtrate is pipetted into a glass insert vial.

8. Sampling and Sample Preparation

Samples are the homogenised tissue of shellfish flesh. The homogenate can be prepared freshly or from frozen shellfish.

9. Analytical Procedure

The sample is logged in and if it is not to be analysed immediately then it is placed in a freezer until analysis, for a maximum of one month. When analysis is to be carried out the sample is allowed to defrost at room temperature for at least one hour or alternatively it is stored refrigerated overnight.

9.1 Extraction

The homogenised shellfish tissue (4 g ± 0.5 g) is weighed into a centrifuge tube (50 ml). 16 ml (± 0.16 ml) of 80% (v/v) methanol/water is added using a calibrated dispenser, and the total amount is blended, on ice, with an Ultraturrax® for 1 min (± 15 seconds). The centrifuge tube is then centrifuged for at least 10 minutes at a setting of 3000 rpm. The supernatant is decanted into a 30 ml universal container. The container is closed, and shaken quickly by hand and then passed three times on a vortex mixer. If the sample is not immediately analysed it is stored in a freezer (-20°C) until required. The Ultraturrax® is then rinsed with clean tap water in a plastic beaker and dried with a paper towel. An aliquot of the extract prepared is then filtered into a glass vial and sealed.

9.2 Filtering

1-2 ml of the shellfish extract is taken up by syringe and the filter is attached. The extract is passed through the filter and at least half a 2 ml borosilicate glass vial is filled. The vial is sealed using a PTFE septum and screw cap. The sample is now ready for analysis. Alternatively, 0.5 ml of shellfish extract is placed in a centrifugal filter and closed. It is then placed in a micro-centrifuge and spun for 1 minute (± 0.5 minutes) at 14000 rpm. The sample is then placed in a glass vial with insert and closed using a screw top with PTFE septum. The sample is now ready for analysis by LC-MS with Analyst® software to quantify the amount of okadaic acid present in each sample.
9.3 **Calibration and Quality Control**

At the start of each run a system suitability check (20 ng ml\(^{-1}\) okadaic acid standard) is injected. The value of okadaic acid signal to noise ratio must be greater than 20. Thereafter a series of calibration standards are analysed every 18 samples along with a method blank and LRM/CRM extract or clean up extract. The calibration series is interspersed evenly throughout the run and a set of calibration standards should cover a maximum of 18 samples.

The calibration model is linear and the \(r^2\) (correlation coefficient) must be in the range of 0.990 - 1.000. The LRM/CRM value must lie within the limits of the Shewhart chart. If any of the quality criteria are not met then the batch is referred to Technical Management and a quality query raised. Samples may be reanalysed, if necessary.

10. **Calculation of Results**

The concentration in the sample solution is calculated by the Analyst\(^\text{®}\) software from the calibration curve and the counts per second value obtained for each sample. A calculation is then performed on the concentration value to convert it into the required units of ng/g. The formula for the calculation is:

\[
C_s = C_i \times \frac{(V_e + m_s)}{m_s}
\]

Where:

- \(C_s\) = concentration of analyte in sample (ng OA g\(^{-1}\))
- \(C_i\) = concentration of analyte in sample solution analysed (ng OA ml\(^{-1}\))
- \(m_s\) = mass of sample extracted (g)
- \(V_e\) = volume of 80% (v/v) methanol/water solution used for extraction (ml)

11. **Precision, Bias and Limit of Determination**

Lower limit of determination (LOD):

\[
LOD = C_L \frac{(V_e + m_s)}{m_s}
\]

Higher limit of determination (HOD):

\[
HOD = C_H \frac{(V_e + m_s)}{m_s}
\]

- \(C_L\) = concentration of lowest standard (ng OA ml\(^{-1}\))
- \(C_H\) = concentration of highest standard ng OA ml\(^{-1}\))
- \(m_s\) = mass of sample extracted (g)
- \(V_e\) = volume of 80% methanol/water solution used for extraction (ml)
12. Method Validation

a) Recovery (CRM MUS-2)

<table>
<thead>
<tr>
<th>SID No.</th>
<th>Batch No.</th>
<th>Okadaic Acid Concentration (ng OA g⁻¹)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>12065/00</td>
<td>1191</td>
<td>10401.0</td>
<td>94.6</td>
</tr>
<tr>
<td>12066/00</td>
<td>1191</td>
<td>11268.6</td>
<td>102.4</td>
</tr>
<tr>
<td>12067/00</td>
<td>1191</td>
<td>9276.1</td>
<td>84.3</td>
</tr>
<tr>
<td>12068/00</td>
<td>1191</td>
<td>10259.8</td>
<td>93.3</td>
</tr>
<tr>
<td>3198/01</td>
<td>1191</td>
<td>12072.1</td>
<td>109.7</td>
</tr>
<tr>
<td>3255/01</td>
<td>1201</td>
<td>10412.9</td>
<td>94.7</td>
</tr>
<tr>
<td>3293/01</td>
<td>1219</td>
<td>10949.9</td>
<td>99.5</td>
</tr>
<tr>
<td>3318/01</td>
<td>1219</td>
<td>11850.7</td>
<td>107.7</td>
</tr>
<tr>
<td>3319/01</td>
<td>1219</td>
<td>11642.1</td>
<td>105.8</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>10903.7</td>
<td>99.1</td>
</tr>
<tr>
<td>StDev</td>
<td></td>
<td>880.5</td>
<td>8.0</td>
</tr>
<tr>
<td>%RSD</td>
<td></td>
<td>8.1</td>
<td></td>
</tr>
</tbody>
</table>

b) Limit of Detection

<table>
<thead>
<tr>
<th>Blank Replicate No.</th>
<th>[OA] (ng OA ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.683</td>
</tr>
<tr>
<td>2</td>
<td>6.882</td>
</tr>
<tr>
<td>3</td>
<td>7.551</td>
</tr>
<tr>
<td>4</td>
<td>7.731</td>
</tr>
<tr>
<td>5</td>
<td>7.592</td>
</tr>
<tr>
<td>6</td>
<td>6.633</td>
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<tr>
<td>7</td>
<td>7.123</td>
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<tr>
<td>8</td>
<td>7.608</td>
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<tr>
<td>9</td>
<td>7.834</td>
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<tr>
<td>10</td>
<td>7.991</td>
</tr>
<tr>
<td>Mean</td>
<td>7.463</td>
</tr>
<tr>
<td>StDev</td>
<td>0.438</td>
</tr>
<tr>
<td>%RSD</td>
<td>5.87</td>
</tr>
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</table>

LOD = 10.2ng/g (where a 4g sample is used and LOD is 4.65 x StDev.).
c) **Precision (10% and 90% of Calibration Range Standards)**

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>Batch</th>
<th>10% of Calibration Range Standard (ng OA ml(^{-1}))</th>
<th>90% of Calibration Range Standard (ng OA ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1191</td>
<td>26.11</td>
<td>237.70</td>
</tr>
<tr>
<td>2</td>
<td>1191</td>
<td>29.03</td>
<td>237.00</td>
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<td>3</td>
<td>1191</td>
<td>27.27</td>
<td>253.80</td>
</tr>
<tr>
<td>4</td>
<td>1191</td>
<td>26.03</td>
<td>241.20</td>
</tr>
<tr>
<td>5</td>
<td>1191</td>
<td>25.86</td>
<td>237.70</td>
</tr>
<tr>
<td>6</td>
<td>1191</td>
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<td>232.20</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>26.43</td>
<td>239.93</td>
</tr>
<tr>
<td>StDev</td>
<td></td>
<td>1.59</td>
<td>7.38</td>
</tr>
<tr>
<td>%RSD</td>
<td></td>
<td>6.00</td>
<td>3.08</td>
</tr>
</tbody>
</table>

13. **Uncertainty of Measurement**

**Sources on Uncertainty:**
- **Sampling**
  Shellfish samples are received whole and a selection are shucked and homogenised to obtain the necessary weight. There will be differences between the individual shellfish such as size, state of gonads etc., but sampling errors are discounted as the reported result is based on the samples received.
- **Sub-sampling**
  A sub-sample (approximately 4g) of the homogenate is taken for OA analysis. It is sometimes extremely difficult to obtain a completely homogeneous sample (notably scallop gonads and mussels) and therefore there will be differences in okadaic acid concentrations between sub-samples. This will also contribute to levels of uncertainty and is accounted for in the precision data for the Certified Reference Materials.
- **Storage conditions**
  Samples are transported in cool boxes or lunch boxes to the FRS and the transportation time and temperature may have an effect on OA content.
- **Reagent purity**
  All solvents used are HPLC Grade and prepared solutions are labelled with preparation and expiry dates. Stock certified standards are obtained from the NRC, Canada. Final concentrations of calibration solutions have not been corrected for purity as >99%, therefore negligible. Any uncertainty is accounted for in the validation data.
- **Instrument effects**
  The calibration curve is monitored (typically > 0.999 for LC-MS) and any uncertainty is accounted for in the QC and validation data. The instrument is maintained by trained staff and approved engineer. System suitability checks are always analysed before batch analysis.
- **Weight**
  Check weight is used on balance (tolerance generally < 1%) and is sufficient for the accuracy required. Uncertainty is accounted for in the QC and validation data.
- **Volume**
  Calibrated pipettes and dispensers are used for the preparation of reagents and have a tolerance of 1% and 10% respectively. They are checked annually and one-volume checked every month. Uncertainty is accounted for in the QC and validation data.
• **Time**
  Only calibrated timers are used. There is a negligible contribution to uncertainty.

• **Centrifuge**
  Serviced annually. Suitable for the method and any uncertainty is accounted for in the QC and validation data.

• **Computational effects**
  Concentrations are calculated by instrument software that has been manually checked and acceptable. There is a negligible contribution to uncertainty.

• **Blank correction**
  A method (solvent) blank is run with each batch of samples. Uncertainty is accounted for in the QC and validation data.

• **Environment conditions**
  Cross-contamination is minimised by rinsing the Ultraturrax after each sample homogenisation as well as analysing a method blank with each batch of analysis. Uncertainty is accounted for in the QC and validation data. Freezer temperatures are monitored and connected to an alarm system.

• **Operator effect**
  Only trained personnel can carry out the method unsupervised and all standard operating procedures used in the method are fully documented and the latest version available to all personnel. Variations between operators are accounted for in the QC samples run with each batch of samples. Uncertainty is accounted for in the QC and validation data.

• **Matrix effects**
  CRM materials are representative of samples analysed. Uncertainty accounted for in the QC and validation data.

• **Random effects**
  These are accounted for by the validation and QC data.

**Summary of validation data:**

<table>
<thead>
<tr>
<th></th>
<th>LC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of Detection:</td>
<td>0.010 µg OA/g</td>
</tr>
<tr>
<td><strong>Coefficient of Variance:</strong></td>
<td></td>
</tr>
<tr>
<td>Blank:</td>
<td>5.9%</td>
</tr>
<tr>
<td>Low Standard (0.5µg/ml):</td>
<td>6.0%</td>
</tr>
<tr>
<td>High Standard (4.5µg/ml):</td>
<td>3.1%</td>
</tr>
<tr>
<td>Precision of CRM:</td>
<td>8.0%</td>
</tr>
<tr>
<td>Variance of Shewhart Chart:</td>
<td>8.6%</td>
</tr>
<tr>
<td>CRM I.D.:</td>
<td>MUS-2</td>
</tr>
<tr>
<td>Recovery of CRM:</td>
<td>99.1%</td>
</tr>
</tbody>
</table>
Combined uncertainty:

<table>
<thead>
<tr>
<th></th>
<th>LC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systematic component</td>
<td>0.9 ÷ 2</td>
</tr>
<tr>
<td>(Recovery from CRM):</td>
<td></td>
</tr>
<tr>
<td></td>
<td>= 0.5%</td>
</tr>
<tr>
<td>Random component</td>
<td>8.6%</td>
</tr>
<tr>
<td>(Shewhart Chart variance):</td>
<td></td>
</tr>
</tbody>
</table>

Assume linear summation and a value of k = 2.

Combined standard uncertainty:

LC-MS (MUS-2) = 2 × [(0.5^2 + 8.6^2)]^{0.5} = 12.2%

The reported expanded uncertainty is based on an uncertainty multiplied by a coverage factor of k = 2, providing a level of confidence of approximately 95.
11. APPENDIX II

SOP 2247 - Extraction of DSP toxins from shellfish tissues for bioassay – UKNRL method (Interim SOP)

1. Introduction and Scope

This procedure covers the extraction of toxin from processed shellfish tissue homogenate. The extraction is then analysed to assess the level of DSP toxin.

2. Principle of the Method

This process involves homogenised shellfish tissues being blended together with acetone using an Ultra-Turrax®, filtration of the resulting mixture, evaporation, ether separation, further evaporation and suspension in Tween to yield the extract for analysis.

3. Reference Material

Not applicable.

4. Reagents

Acetone (AnalaR)
Diethyl ether (AnalaR)
1% Tween 60
Distilled water

5. Major Equipment

Calibrated balance
Water bath
Rotary evaporator
Ultra-Turrax®
Sonicator

6. Environmental Control

Not applicable.

7. Interferences

Not applicable.

8. Sampling and Sample Preparation

Shellfish samples are prepared and an aliquot is received in a wide necked screw capped bottle labelled with sample number.

9. Analytical Procedure

Before starting the extraction, the sample number is recorded on record sheet. Each further stage is also recorded and initialled on this record sheet.
9.1 To 100g ± 1 g tissue in screw capped bottle add 100 ml ± 5 ml acetone using dispenser pump on Winchester bottle

9.2 Homogenise using Ultra-Turrax ® at speed setting 3 (13500 rpm) for at least 30 seconds.

9.3 In a fume hood, using a large plastic funnel and 113V fluted filter paper, filter homogenate into a narrow necked screw capped bottle labelled with sample number

9.4 Remove tissue from filter paper and re-suspend in a further 100ml ± 5 ml acetone. Repeat 9.2 and 9.3

9.5 To clean Ultra-Turrax ® between samples, wipe with tissue to remove excess shellfish tissue from openings, remove stuck tissue with forceps and rinse by operating with acetone only in a spare screw capped bottle at setting 3 for 10-15 seconds

9.6 Pour filtrate into labelled, 500 ml, glass, round bottomed flask and evaporate off acetone. Acetone is completely evaporated when there are no obvious signs of liquid running back into the flask.

9.7 Remove flask from evaporator and continue process in fume cupboard.

9.8 Pour contents of flask into 100 ml measuring cylinder and measure volume. Return liquid to flask, add equal volume of ether, mix well, transfer to separating funnel and allow to separate.

9.9 Run bottom layer into original round bottomed flask and transfer top layer to labelled conical flask. Place same volume of ether as above into original flask, mix well, transfer to separating funnel and allow to separate.

9.10 Repeat 9.9, discard bottom layer into round-bottomed flask and add portion in conical flask to separating funnel.

9.11 Using a wash bottle, add at least 5ml distilled water to separating funnel, stopper and mix well. (release pressure in separating funnel by opening tap when funnel is inverted). Leave to separate then discard bottom layer into round-bottomed flask. Repeat 9.11. Pour contents of round-bottomed flask into Winchester bottle labelled ‘waste chemicals’.

9.12 Run remaining contents of separating funnel into labelled 100 ml glass round bottomed flask and evaporate until dry + 5-10 mins.

9.13 Using a disposable pipette add 3 ml Tween 60 to flask and mix with contents of flask. Squirt contents up and down using a pastette until contents are suspended in Tween 60 and sides of flask are clean. Sonicator may be used to help dislodge contents – pour warm water into sonicator and switch on. Immerse flask in water two or three times for at least 20 seconds each time.

9.14 Transfer Tween suspension to labelled graduated tube and make volume up to 4ml mark with 1% Tween. Tween samples may be stored in refrigeratored for up to 5 days – record storage time on record sheet.
10. **Calculation of Results**

Not applicable

11. **Precision, Bias and Limit of Determination**

Not applicable

12. **Reports**

Not applicable

13. **Safety**

Wear laboratory coat and disposable gloves and safety specs if required.

14. **Literature References**

Yasumoto et al., Diarrhetic Shellfish Poisoning, American Chemical Society, 1984, pp207-214.

12. APPENDIX III

SOP 2245 - Extraction of DSP toxins from shellfish tissues for bioassay

1. Introduction and Scope

This procedure covers the extraction of toxin from processed shellfish tissue homogenate. The extraction is then analysed to assess the level of DSP toxin.

2. Principle of the Method

This process involves homogenised shellfish tissues being blended together with acetone using an Ultra-Turrax®, filtration of the resulting mixture, evaporation, ether separation, further evaporation and suspension in Tween to yield the extract for analysis.

3. Reference Material

Not applicable

4. Reagents

- Acetone (AnalaR)
- Diethyl ether (AnalaR)
- 1% Tween 60
- Distilled water

5. Major Equipment

- Calibrated balance
- Water bath
- Rotary evaporator
- Ultra-Turrax®
- Sonicator

6. Environmental Control

Not applicable

7. Interferences

Not applicable

8. Sampling and Sample Preparation

Shellfish samples are prepared and an aliquot is received in a wide necked screw capped bottle labelled with sample number.

9. Analytical Procedure

Before starting the extraction, the sample number is recorded on record sheet. Each further stage is also recorded and initialled on this record sheet.

9.1 To 75 g ± 1 g tissue in screw capped bottle add 225 ml ± 5 ml acetone using dispenser pump on Winchester bottle.
9.2 Homogenise using Ultra-Turrax® at speed setting 3, for at least 30 seconds.

9.3 To clean Ultra-Turrax® between samples, wipe with tissue to remove excess shellfish tissue from openings, remove stuck tissue with forceps and rinse by operating with acetone only in a spare screw capped bottle at setting 3 for 10-15 seconds.

9.4 In a fume hood, using a large plastic funnel and 113V fluted filter paper, filter homogenate into a narrow necked screw capped bottle labelled with sample number.

9.5 Pour the filtrate into a labelled, 500ml, glass, round bottomed flask and evaporate off acetone. Acetone is completely evaporated when there are no obvious signs of liquid running back into the flask.

9.6 Remove flask from evaporator and continue process in fume cupboard.

9.7 Pour contents of flask into 50 ml measuring cylinder and measure volume. Return liquid to flask, add equal volume of ether, mix well, transfer to separating funnel and allow to separate.

9.8 Place same volume of ether as above into original round bottomed flask and add bottom layer from separating funnel. Allow to separate and transfer top layer from separating funnel to labelled conical flask.

9.9 Mix contents of round bottomed flask, transfer to separating funnel and allow to separate again. Discard bottom layer into round-bottomed flask and add portion in conical flask to separating funnel.

9.10 Using a wash bottle add at least 5 ml distilled water to separating funnel, stopper and mix well. (release pressure in separating funnel by opening tap when funnel is inverted). Leave to separate then discard bottom layer into round-bottomed flask. Repeat 9.9 and pour contents of round-bottomed flask into Winchester bottle labelled ‘waste chemicals’.

9.11 Run remaining contents of separating funnel into labelled 100ml glass round bottomed flask and evaporate until dry + 5-10 mins. Flask may be left overnight in fume cupboard to ensure thoroughly dry.

9.12 Using a disposable pipette add 3 ml ± 10% Tween 60 to flask and mix with contents of flask. Squirt contents up and down using a pastette until contents are suspended in Tween 60 and sides of flask are clean. Sonicator may be used to help dislodge contents – pour warm water into sonicator and switch on. Immerse flask in water two or three times for at least 20 seconds each time.

10. Calculation of Results

Not applicable

11. Precision, Bias and Limit of Determination

Not applicable
12. **Reports**

Not applicable

13. **Safety**

Wear laboratory coat and disposable gloves and safety specs if required.

14. **Literature References**
