Agri-Food & Biosciences Institute

VETERINARY SCIENCES DIVISION

Chemical Surveillance Branch

A Preliminary Investigation into the Applicability of Current Methods of Analysis for the detection of Marine Biotoxins in the Common Periwinkle (*Littorina littorea*).

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Executive Summary

1. Removal of Periwinkle tissue from its shell.

Whilst a lengthy process, "Smash and Grab" was selected as a suitable shucking method as this resulted in tissue which was closest to its natural state. "Boiled for 1 minute" was selected for further investigation as it offered a more efficient method of shucking whilst reducing heat treatment to a minimum. "Freezing overnight" was rejected as it offered no advantage over "smash and grab" and the yield of tissue was much less. The effect of boiling on the toxin concentration and toxin profile in periwinkle tissue requires investigation before this method of shucking could be recommended.

2. Extraction of Periwinkle Tissue for Lipophilic Toxin Determination.

The lipophilic toxin extraction protocols for mouse bioassay are acceptable for use on periwinkle tissue, in that no adverse effects on mice were produced by the extract. However it should be noted that the extraction efficiency of the method has not been evaluated. It should also be noted that the effect of boiling on the toxin concentration of periwinkles requires investigation before this method of shucking could be recommended.

3. LC-MS for lipophilic toxins

The application of the recommended extraction procedure for LC-MS/MS appears to be useable; however recovery evaluation should be undertaken as part of any extension of scope to cover gastropod tissue.

4. Domoic acid determination by biosensor

The ASP Biosensor extraction method (CSD 405) is a feasible method for periwinkle matrix. Evaluation of recovery and matrix effects were outside the scope of the current study but would have to form part of any proposed extension of scope.

5. Domoic Acid determination by HPLC

The extraction procedure for Domoic Acid HPLC (CSD 406) can be applied to periwinkle matrix and there was no indication of potential coeluting peaks in the samples analysed. Preliminary studies on recovery suggest that recovery is similar to

that in bivalve molluscs. Spiked homogenate results suggest that boiling for 1 minute to remove tissue from the shell may have no effect on the levels of domoic acid, however caution should be applied as spiked tissue was used and the technique used required boiling of the tissue in test tubes rather than in-shell.

6. Paralytic Shellfish Poison analysis by HPLC

The extraction procedure for PSP HPLC (CSD 408) may be a feasible method for periwinkle matrix. There is the potential for interference from matrix components in periodate oxidation with a peak at around 6.5 minutes in some of the samples. This may interfere with the detection of C1,2 by periodate oxidation. For quantification of C1,2, peroxide oxidation is used and in the majority of the samples tested, no interfering peaks were observed in the peroxide oxidation. In one of the 3 replicates of sample 2 (Belfast Lough), a peak was observed. This peak did not occur in the corresponding non-oxidised sample.

Of more concern is the apparent low recovery of the one toxin tested. Before this method could be extended for use as a screen or quantitative method for periwinkles, more extensive validation work would be required to determine the recovery for the full range of toxins.

7. Determination of PSP by Mouse Bioassay.

The reaction seen in the mice makes the method unsuitable for use in periwinkles. There would appear to be a component of periwinkle matrix which mice cannot tolerate. This may affect the accuracy of the test. Given the frequency of the adverse reactions, the test should not be used for periwinkles, on ethical grounds.

Introduction

The common periwinkle is a small marine gastropod that is abundant in the intertidal zone. They are found throughout northern Europe. An intertidal species, they are also commonly found in estuaries and can tolerate relatively low salinities. They are herbivores, using their rough tongue-like radula to scrape thin-film algae from rocks and kelp fronds. They also feed on sea lettuce, Ulva lactuca.

Whilst current EC legislation (EC 853/20004 and 853/2004) applies not only to bivalve molluscs but also to echinoderms, tunicates and gastropods, only bivalve molluscs are subject to routine monitoring for the presence of marine biotoxins. As a result, there is little information on the suitability of the current test protocols for the detection of marine toxins in species other than bivalve molluscs. With the potential for the classification of production areas to be extended to non-bivalve species, it is important to know if the current methods of processing and analysis can be applied for routine monitoring. This study investigates the applicability of the current marine biotoxin test protocols to periwinkles and was undertaken to the specifications agreed with Food Standards Agency (Appendix 1).

Methodologies:

Samples were extracted and analysed using the methods specified for the analysis of marine biotoxins under the United Kingdom official monitoring programme. The methods investigated in the study were:

1. Removal of periwinkle tissue from the shell (Section 1)
2. UKNRL standard operating procedures for the processing and extraction of shellfish for the subsequent detection of lipophilic toxins by mouse bioassay (Section 2).
3. Community Reference Laboratory standard operating procedure for the extraction and analysis of lipophilic toxins by LC-MS/MS (Section 3).
4. Chemical Surveillance Branch standard operating procedure for the extraction and analysis of shellfish for the presence of Domoic Acid by biosensor (Section 4).
5. Chemical Surveillance Branch standard operating procedure for the extraction and analysis of shellfish for the presence of Domoic Acid by HPLC with UV detection (Quilliam et al, 1995) (Section 5).

6. UKNRL standard operating procedure for screening for the presence of Paralytic Shellfish Poison by pre-column oxidation and HPLC with fluorescence detection (AOAC 2005.06) (Section 6).

7. UKNRL standard operating procedure for the extraction of shellfish using Hydrochloric acid, for subsequent analysis by mouse bioassay (Section 7).

Samples:

Periwinkles *(Littorina Littorea*) were sampled from three locations: two sites in Strangford Lough (Comber and Portavogie) and one site in Belfast Lough.

1. Periwinkle Shucking

Initially, four methods of removing the periwinkle from its shell were investigated:

- "Smash and Grab": smashing the shell of the animal and extracting the tissue from it.
- Boiling the periwinkles in water for up to one minute.
- Boiling the periwinkles in water for two minutes (to emulate cooking) and running under cold water.
- Freezing the periwinkles overnight at -20ºC and thawing.

For all methods, the sample in-shell was weighed before and after removal to determine what ratio of tissue to sample weight was obtained. The time taken to obtain the tissue was also recorded. A 1 kg sample was divided into 4 sub-samples of approximately 250g and the weights recorded.

Sample A: "Smash and grab".

A nutcracker was used to crack open the shell of the periwinkle and remove the animal. The process was time consuming with animals still having to be teased out using a pin hook and some small fragments of shell removed from the tissue prior to homogenisation.

Weight in shell:	253g
Tissue weight:	39g
Time taken:	32 minutes

Homogenisation of the tissue was straight forward but removal of sufficient sample to undertake all analyses (200g) will be time consuming and significantly longer than that taken for example, for mussels. Care is required to ensure shell fragments are removed as there is the potential for these to cause damage to the Ultra-Turrax. It is anticipated that up to 1 hour would be required to produce sufficient tissue for analyses. Although a more robust method of separation may be quicker, it would result in increased shell fragments.

Sample B: Boiling for 1 minute.

A 1 litre beaker was filled to 500ml with distilled water and brought to the boil. Once boiling, the periwinkles were added and the water brought back to a gentle boil. The

periwinkles were boiled for 1 minute, removed from the water and drained into a sieve. The periwinkles were then easily removed from their shell with a pin hook.

Shell weight:	252g
Tissue weight:	46g
Time taken:	7 minutes (not including initially bringing the water to the boil).

The appearance of the tissue was different from that observed in the fresh, uncooked sample. After boiling, the tissue appeared swollen and of a much drier consistency, making homogenisation more difficult.

Sample C: Boiling for 2 minutes and rinsing in cold water.

A 1 litre beaker was filled to 500ml with distilled water and brought to the boil. Once boiling, the periwinkles were added and the water brought back to a gentle boil. The periwinkles were boiled for 2 minutes, removed from the water, drained into a sieve and rinsed with cold running water. The animals were easily removed from the shell with a pin hook.

Shell weight:	244g
Tissue weight:	44g
Time taken:	8 minutes (not including initially bringing the water to the boil).

The tissue appeared swollen and much drier making homogenisation more difficult although not noticeably different from that produced by boiling for 1 minute.

Sample D: Freezing at -20ºC overnight and thawing.

The periwinkle sample was stored at -20ºC overnight then thawed at room temperature. Removal from the shell was difficult with in most cases only half the animal being removed, resulting in a poor yield of tissue for further processing.

Shell weight:	230g
Tissue weight:	19g
Time taken:	20 minutes (not including the overnight freezing and the time taken to defrost.)

The tissue appeared similar to that produced by the smash and grab method of shucking. No technical problems were identified during homogenisation.

Conclusion

Whilst a lengthy process, “Smash and Grab” was selected as a suitable shucking method as this resulted in tissue which was closest to its natural state. “Boiled for 1 minute” was selected for further investigation as it offered a more efficient method of shucking whilst reducing heat treatment to a minimum. “Freezing overnight” was rejected as it offered no advantage over “smash and grab” and the yield of tissue was much less.

2. Extraction of Periwinkle Tissue for Lipophilic Toxin Determination.

Periwinkle samples of approximately 1.5kg were extracted and analysed as detailed in the relevent UKNRL SOPs. Tissue was extracted from the shells by smash and grab and following boiling for 1 minute, as detailed below.

2.1 Sample Preparation

Each 1.5kg sample was split into 2 x 750g sub-samples and each 750g sub-sample was shucked by "Smash and Grab" and Boiled 1 minute. Details of the tissue obtained and the time taken were,

Smash + Grab

Shell weight: 750g

Tissue weight: 139g

Time taken: 50 – 60 minutes

Boiling for 1 minute

Shell weight: 750g

Tissue weight: 136g

Time taken: 31 minutes

Those periwinkles extracted by boiling were drier than the Smash + Grab animals and were more difficult to homogenise even with the use of an Ultra-turrex homogeniser.

2.1 Extraction for lipophilic toxins

Samples homogenised well with acetone and were sufficiently separated when centrifuged at the speed and time as stated in the UK NRL SOP. Acetone evaporation and volume of aqueous phase remaining were all as expected, as was the ether wash. Ether evaporation was carried out to the point where an oily residue remained. This took no longer than would be expected for bivalve extracts. However readings on the Gastec Diethyl Ether detection tubes showed levels of around 20 – 50 ppm. Flasks were replaced on the rotary evaporator for a further 20-30 minutes to ensure Gastec readings were below 10ppm. The residue was re-suspended and made up to 4ml with Tween. The extended evaporation time could prove to be an issue if a large number of samples were to be tested on one day.

2.2 Mouse Bioassay for lipophilic toxins

The extracts, (Smash + Grab and Boiled 1 minute), were more viscous than that produced by other species of shellfish and were more difficult to draw up through a 25 gauge hypodermic needle. The samples tested were able to be administered through a 25 gauge needle.

Two mice were injected by interperitoneal route with each extract and monitored closely for the first hour post-injection and then monitored hourly for 8 hours, then checked again at 11 and finally 24 hours. The observations were scored as detailed in the UK NRL SOP. There were no adverse reactions in any of the mice and all mice were marked “normal” immediately post-injection and throughout the 24 hour observation period (Appendix 1 details examples from one set of samples).

2.3 Conclusion

The lipophilic toxin extraction protocols for mouse bioassay are acceptable for use on periwinkle tissue, in that no adverse effects on mice were produced by the extract. However it should be noted that the extraction efficiency of the method has not been evaluated. It should also be noted that the effect of boiling on the toxin concentration of periwinkles requires investigation before this method of shucking could be recommended.

3. LC-MS for lipophilic toxins

3.1 Extraction

Samples of both Smash + Grab and Boiled 1 minute periwinkle tissue (Belfast Lough samples), were taken through the extraction procedure for the detection of DSP by LC-MS (Community Reference Laboratory draft SOP). There were no issues identified in the application of this extraction procedure to periwinkle tissue.

3.2 Conclusion

The application of the recommended extraction procedure for LC-MS/MS appears to be useable; however recovery evaluation should be undertaken as part of any extension of scope to cover gastropod tissue.

4. Domoic acid determination by biosensor

Three replicates of both Smash + Grab and Boiled 1 minute periwinkles from each of the three sites were extracted for the detection of ASP by Biosensor SOP CSD 405.

4.1 Extraction

On each occasion both Smash and Grab and Boiled < 1 minute samples mixed well with methanol and were sufficiently separated when centrifuged at the speed and time stated in the standard operating procedure. Evaporation in the dri-block and re-suspension in HBS Buffer was normal and filtering and subsequent mixing with antibody solution required no changes or modifications from the protocol used for bivalve molluscs.

4.2 Biosensor analysis

Subsequent analysis of extracts from shucking procedures, (Smash + Grab and Boiled 1 minute) showed normal characteristics on the biosensor sensogram. The sensogram show the response from the instrument during sample injection and analysis and the trace produced was normal. (Appendix 3)

4.3 Conclusion

The ASP biosensor extraction protocol (CSD 405) is a feasible method for periwinkle matrix. Evaluation of recovery and matrix effects were outside the scope of the current study but would have to form part of any proposed extension of scope.

5. Domoic Acid determination by HPLC

Three replicates from each of the three sites were prepared by each shucking process and extracted following SOP CSD 406 v.4: In addition 2 samples of Smash + Grab and 2 samples of Boiled < 1 minute were spiked with 10µg/g of Domoic Acid.

5.1 Extraction.

During the extraction procedure there were no difficulties with homogenisation, centrifugation or clean-up using SAX SPE cartridges. There appeared to be no difference between periwinkle sample extracts and extracts from bivalve mollusc species. Sample extracts were analysed by HPLC to check for matrix interferences such as co-eluting peaks.

5.2 HPLC Analysis.

Smash + Grab	Peaks at around 7 and 7.5 minutes. (Fig. 1, 5 & 8)
Boiled 1 minute	Peak at around 7 and 7.5 minutes (Fig. 2, 6 & 9)

The peaks observed correspond to similar peaks found in the negative mussel sample (Fig. 3 & 7), recoveries, the LRM, standards and the procedural blank (Fig. 10).

None of the peaks noted in the periwinkle extracts interfered with the Domoic Acid peak at 8.7 minutes. (Fig. 4)

Fig. 1 Smash + Grab

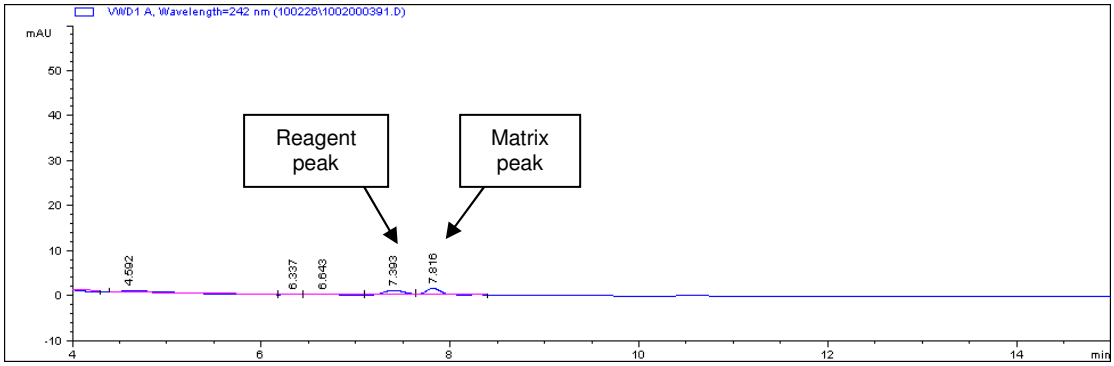


Fig. 2 Boiled 1 minute

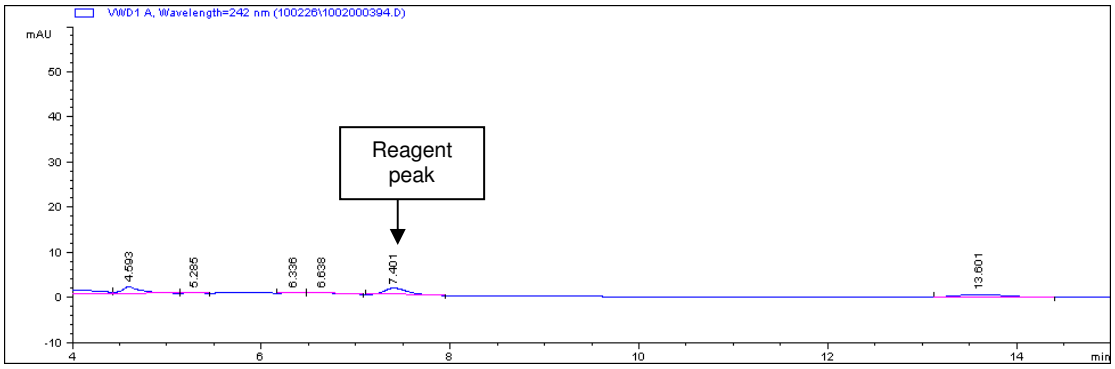


Fig. 3 Negative (Mussel)

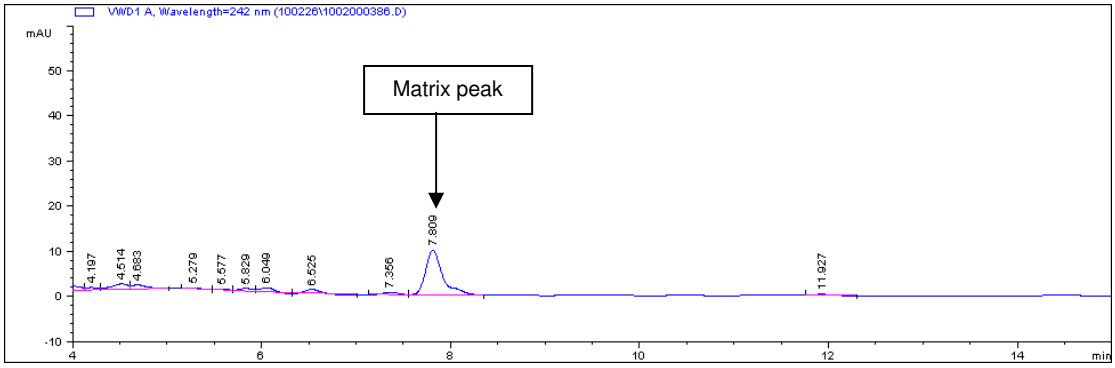


Fig. 4 Domoic Acid Standard

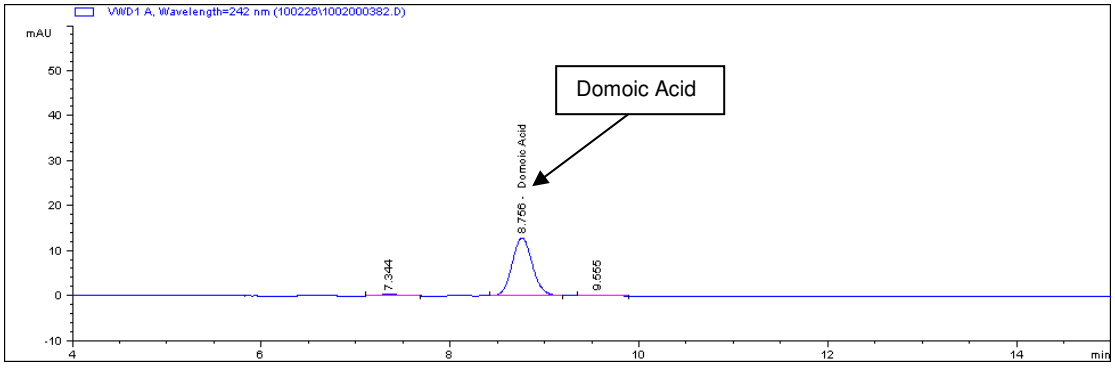


Fig. 5 Smash + Grab (2nd sample)

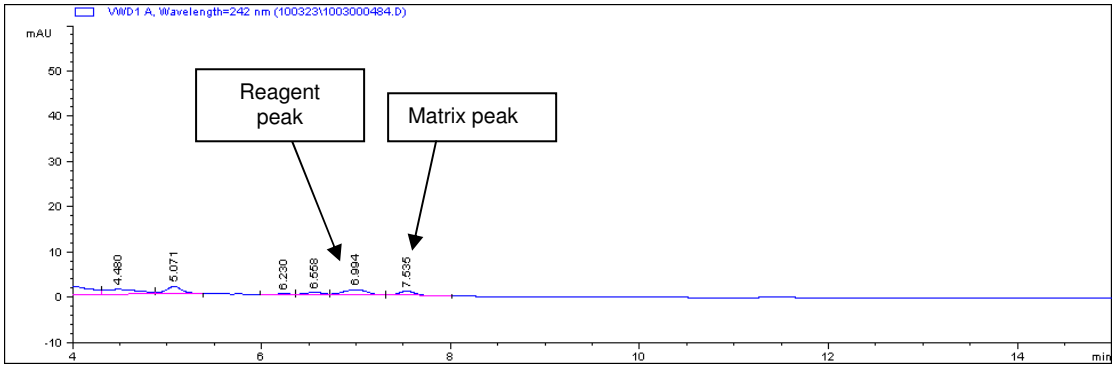


Fig. 6 Boiled 1 minute (2nd sample)

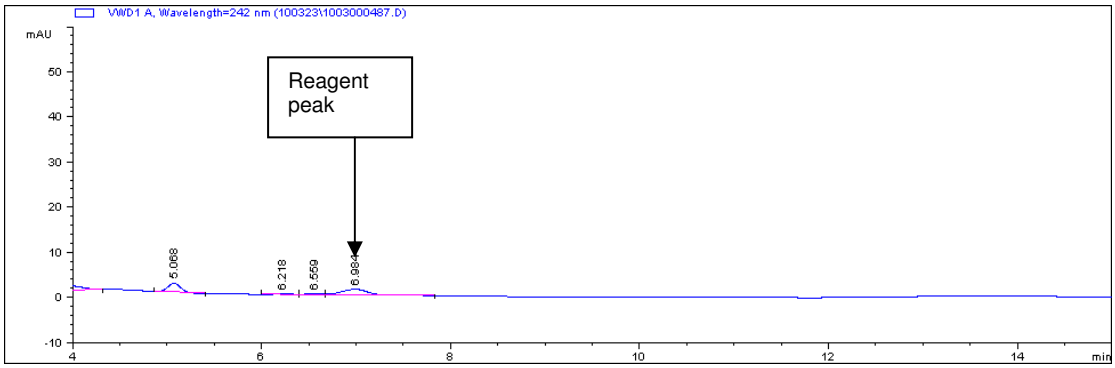


Fig. 7 Negative (Mussel 2nd sample)

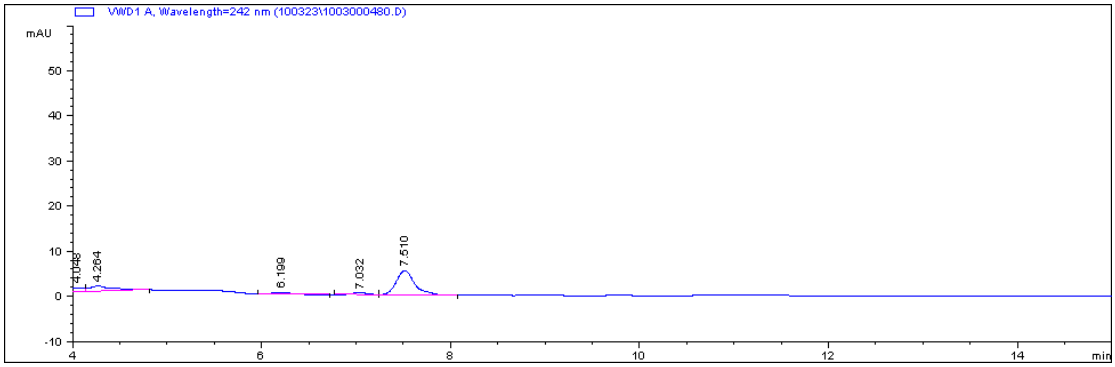


Fig. 8 Smash + Grab (3rd sample)

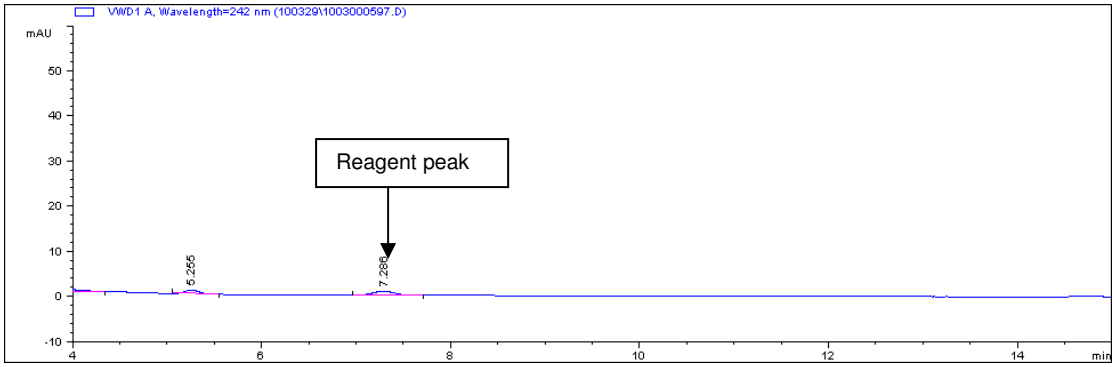


Fig. 9 Boiled 1 minute (3rd sample)

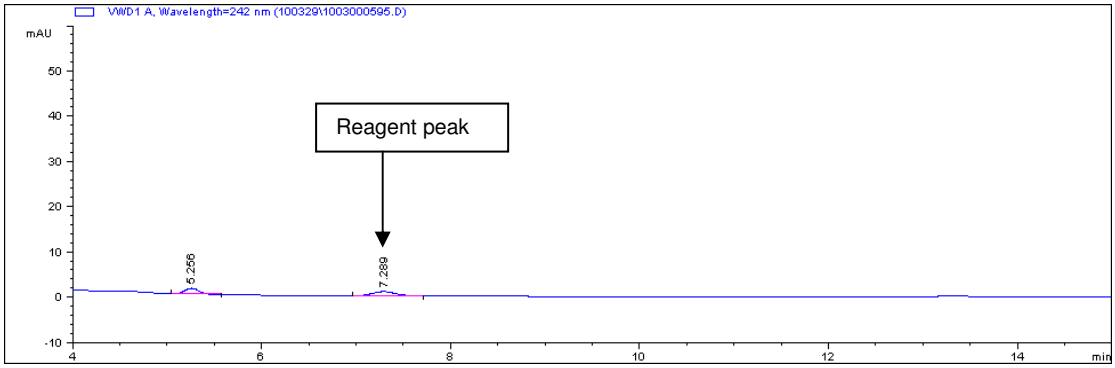
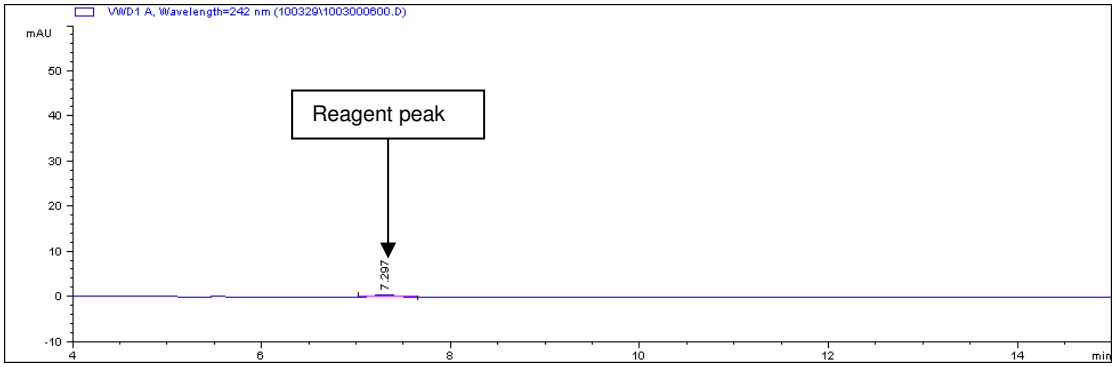


Fig. 10 Procedural Blank (3rd sample)



5.3 Spiked Periwinkle Extract

Extract obtained by both shucking methods was spiked at 10µg/g and analysed as detailed in the standard operating procedure. The calculated concentrations suggest no significant matrix enhancement or suppression; however more detailed information would be required before extending the scope of the method.

Smash + Grab	1:	9.33 µg/g	at 8.76 minutes (Fig. 12)
	2:	9.77 µg/g	at 8.83 minutes
Boiled <1min	1:	9.90 µg/g	at 8.78 minutes (Fig. 13)
	2:	10.80 µg/g	at 8.82 minutes

Fig. 11 Smash + Grab Spike

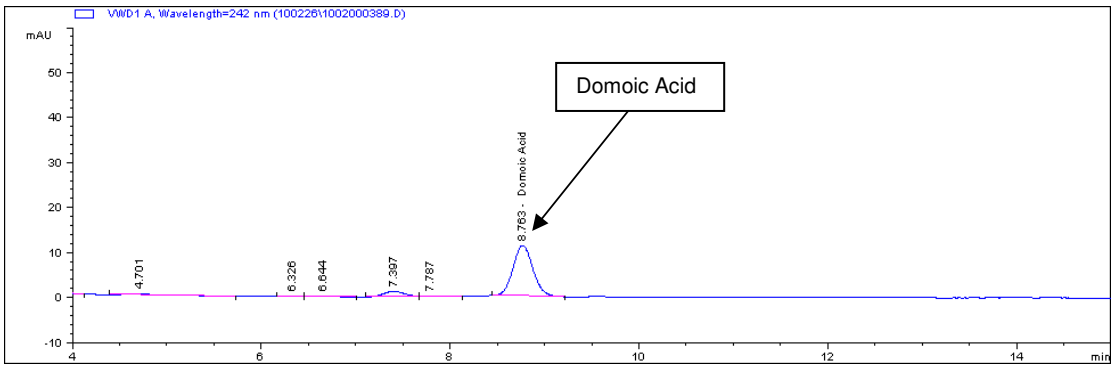
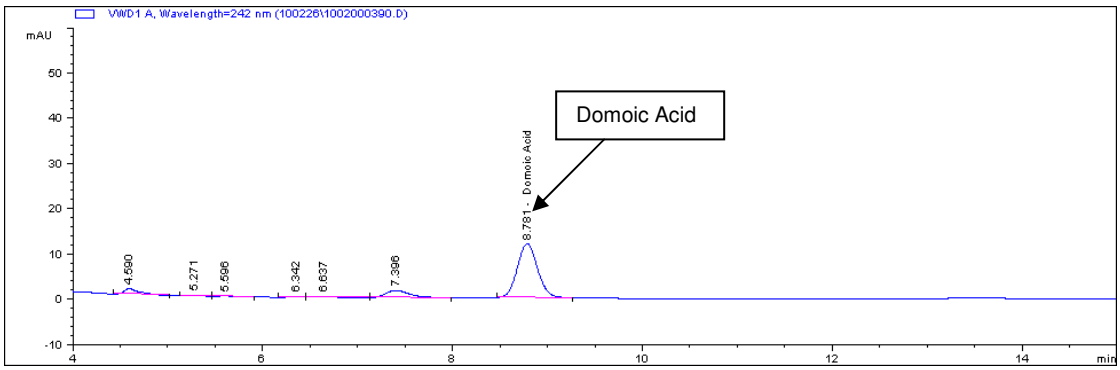


Fig. 12 Boiled 1minute Spike



5.4 Spiked Periwinkle Homogenate by Domoic Acid HPLC

An investigation was undertaken to determine if the method of shucking had an effect on any Domoic acid toxins contained in the periwinkles. Naturally contaminated periwinkles were unavailable therefore two, 2g aliquots of periwinkle tissue which been shucked using the Smash + Grab method were spiked with Domoic Acid at 10µg/g. One, 2g aliquot was placed in a 50ml centrifuge tube, loosely capped and placed in a water bath and boiled for 1 minute to emulate a naturally contaminated sample under-going the shucking method of "Boiled 1 minute." Both 2g aliquots were then extracted for Domoic acid by HPLC as per SOP CSB 406 v.4. Results indicated that both aliquots contained domoic acid at around the spiked value of 10µg/g.

Smash + Grab spike 9.77µg/g D.A. (Fig. 14)

Boiled 1 minute spike 9.97µg/g D.A. (Fig. 15)

Fig. 14 Smash + Grab spike

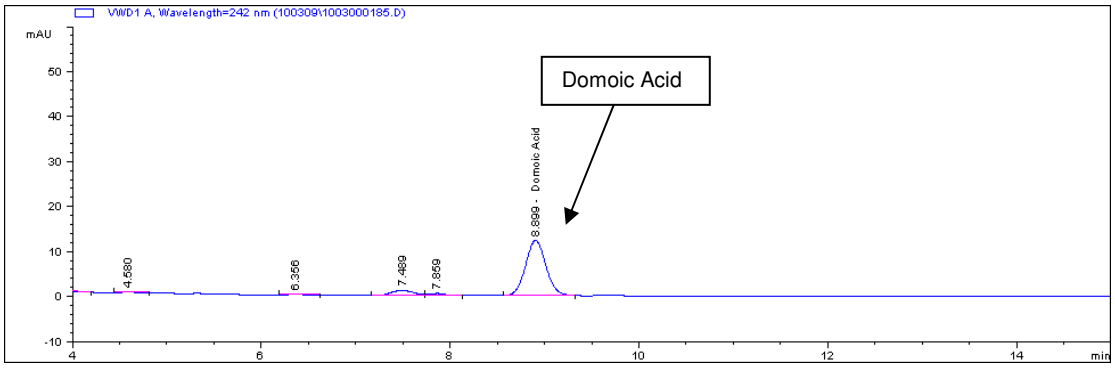
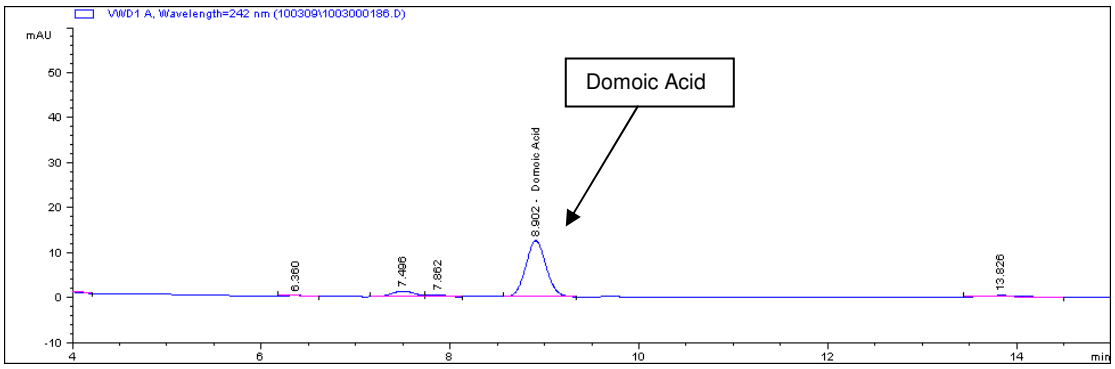


Fig. 15 Boiled < 1 minute spike



5.5 Conclusion

The extraction procedure for Domoic Acid HPLC (CSD 406) can be applied to periwinkle matrix and there was no indication of potential coeluting peaks in the samples analysed. Preliminary studies on recovery suggest that recovery is similar to that in bivalve molluscs. Spiked homogenate results suggest that boiling for 1 minute to remove tissue from the shell may have no effect on the levels of domoic acid, however caution should be applied as spiked tissue was used and the technique used required boiling of the tissue in test tubes rather than in-shell.

6. Paralytic Shellfish Poison analysis by HPLC

Three samples were each tested by the UKNRL SOP for the screening of PSP in Bivalve molluscs, following extraction from the shell by “Smash and Grab” and “Boiled 1 minute.” In addition 2 replicates of Smash + Grab and 2 replicates of Boiled 1 minute were spiked at 1/5 Action Level (160 µg STXdiHydrochloride equivalents/kg) with GTX 2, 3.

6.1 Extraction.

During the extraction procedure there were no difficulties with vortex mixing or boiling the periwinkle acetic acid extracts. After centrifugation there was a difference between the periwinkle extracts which had been removed by “Boiled 1 min” and those removed by “Smash + Grab.”

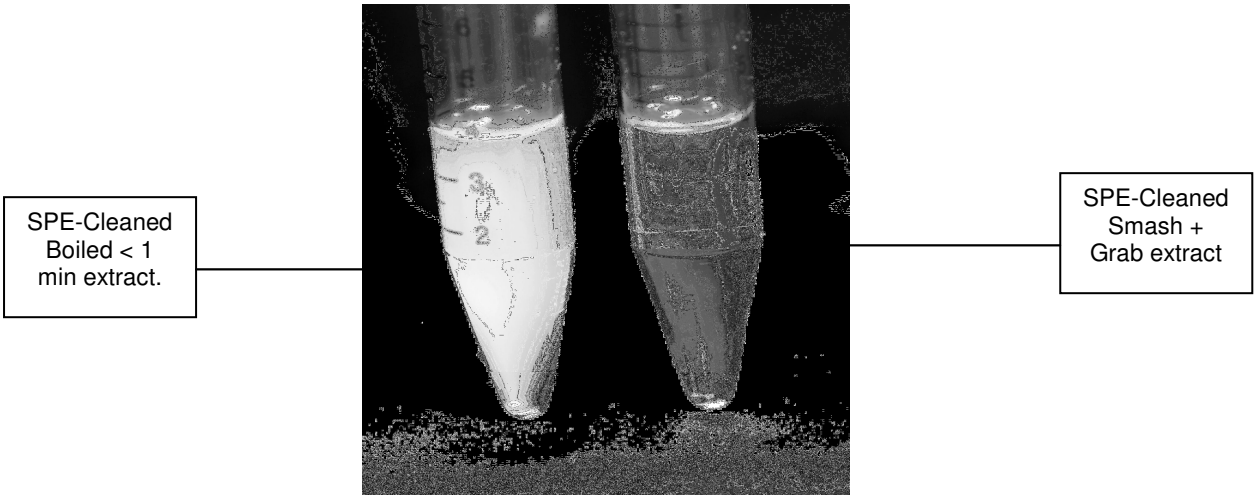
The centrifuged acetic acid extracts of the periwinkle tissue which had been shucked by “boiled 1 minute” appeared cloudy and had small particulate matter suspended. The extract appeared more viscous than the extract from the tissue which had been shucked by “smash and grab” which were clear. Although there was some suspended particulate matter, this settled quickly and did not pose a problem in obtaining a clear subsample.

Extracts were then made up to 5ml with water. There was a difference at this stage between periwinkles and other species, with the supernatant from periwinkles only measuring between 2-3ml whereas other species measuring between 3-4ml.

Solid phase (SPE) clean-up of the periwinkle extracts through C18 SPE columns was undertaken by gravity rather than by the automated system (ASPEC) to ensure the process could be observed closely. The SPE-clean up was a slower process than the clean-up of the negative and positive control samples (both mussel tissue). The packing in the SPE columns became very dirty especially for those extracts that had been boiled for shucking and the final water stage on all the periwinkle extracts had to be pushed through by positive pressure unlike the control samples which dripped though by gravity.

The cleaned extracts of Smash + Grab periwinkles were all quite clear and transparent whereas those for Boiled < 1min were very cloudy, opaque suspensions (Fig. 16).

Fig 16.



The pH of samples before adjustment differed between the periwinkle extracts and other species and also depending on how the periwinkles had been shucked:

Smash + Grab pH before adjustment 5.6

Boiled 1 minute pH before adjustment 5.6

Other species pH before adjustment 4.5 – 5.0

Samples were oxidised as per the SOP however matrix modifier was replaced with UPLC grade water.

6.2 Analysis

Oxidised sample extracts were then analysed by HPLC to check for any matrix interferences such as co-eluting peaks. (Table 1)

Table 1

	Periodate Peaks	**Peroxide Peaks**
Smash + Grab	2.4 mins and 6.4 mins	2.4 mins
Boiled 1 minute	No peaks observed	No peaks observed

Periodate Oxidation

In Periodate oxidation samples "Boiled 1 minute" show no unusual peaks. Matrix peaks, similar to those observed in bivalve molluscs and in the non-oxidised samples were observed (Fig. 17 & 18)

The peak observed at around 2.3 – 2.4 minutes in the Smash + Grab sample chromatogram (Fig. 19) is comparable to the naturally fluorescent peak observed in the chromatogram of the non-oxidized sample (Fig. 20).

Fig. 17 Boiled < 1 minute Periodate

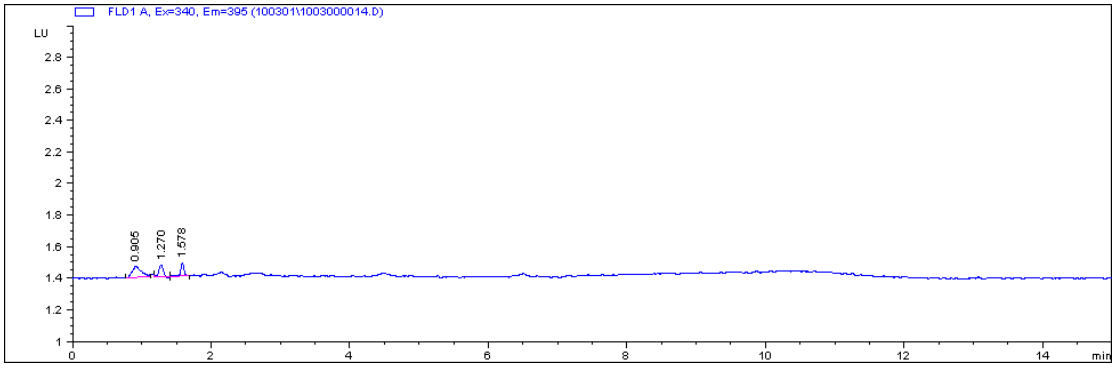


Fig. 18 Boiled < 1 minute Non-oxidised

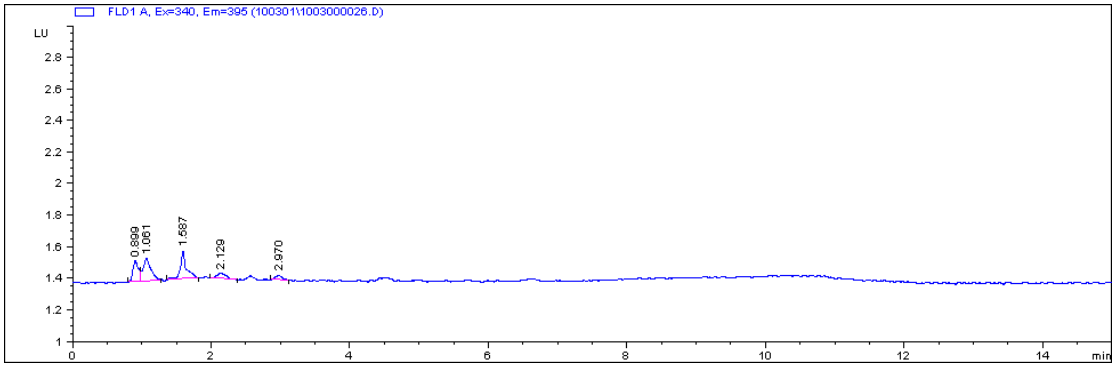


Fig. 19 Smash + Grab Periodate

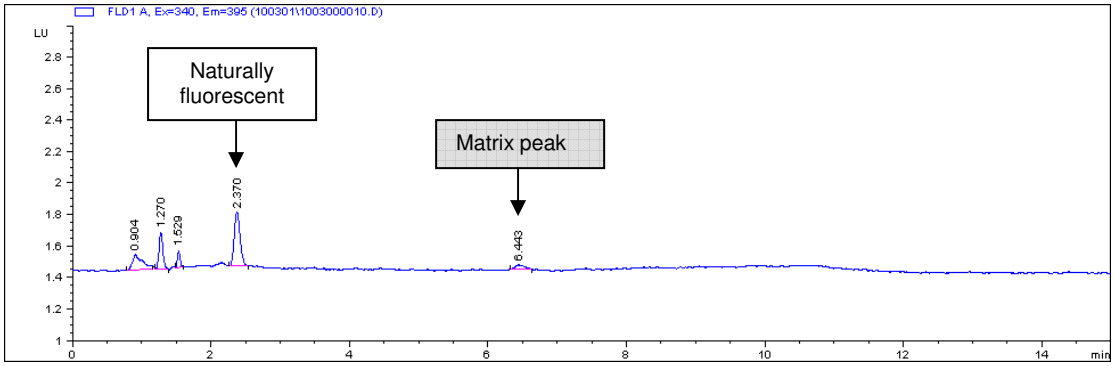
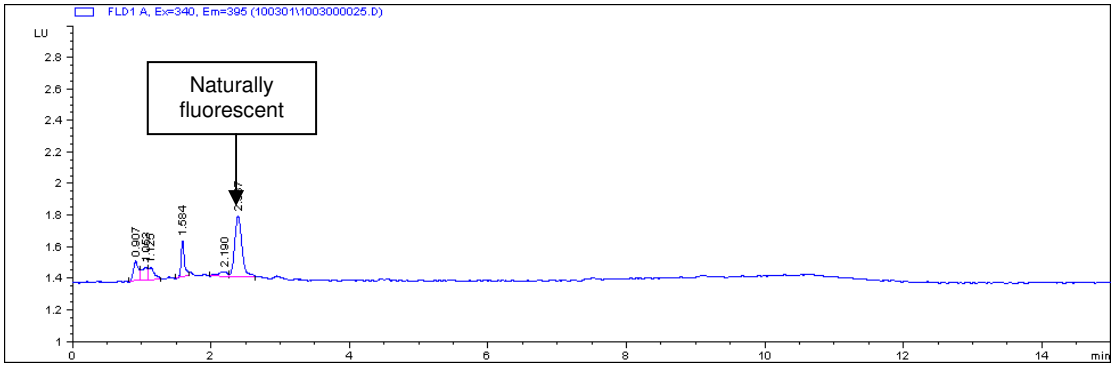


Fig. 20 Smash + Grab Non-oxidised



The matrix peak seen at around 6.4 – 6.5 minutes (Fig. 19) does not interfere with any of the standard peaks in Mix 1 & 2 (Fig. 21, 22), but may interfere with the peak for the toxin C1,2 in Mix 3 (Fig.23).

Fig. 21 Standard Mix 1 Periodate

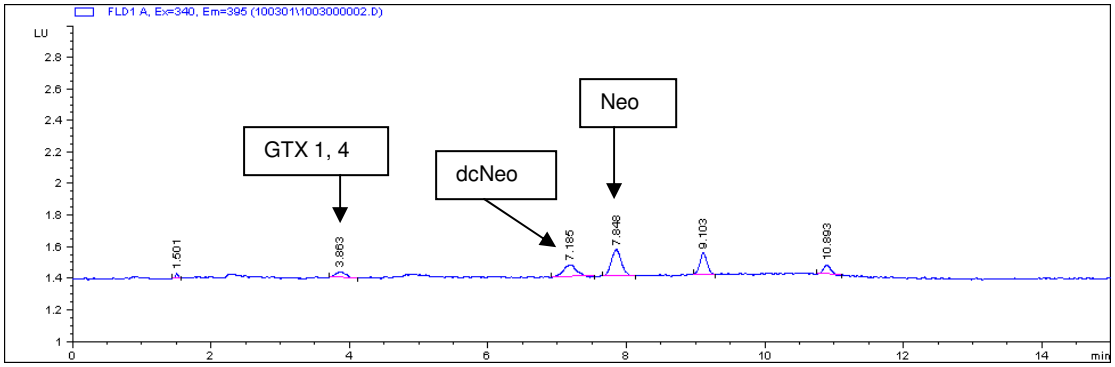


Fig. 22 Standard Mix 2 Periodate

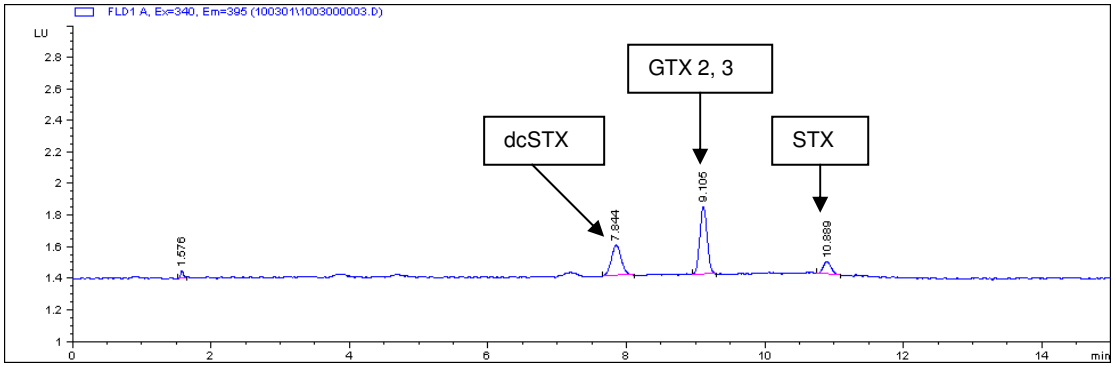
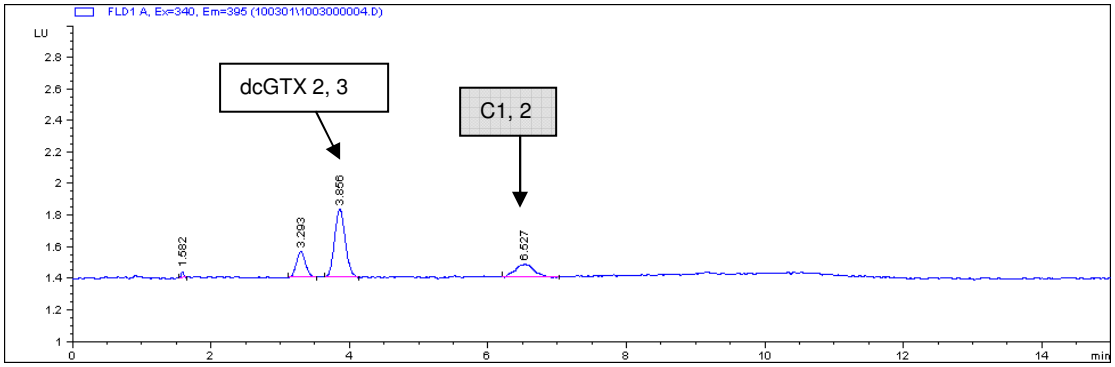


Fig. 23 Standard Mix 3 Periodate



Peroxide oxidation

In the peroxide oxidation of the sample extracts, a fluorescence peak at 2.3 to 2.4 minutes was observed. This matches the retention time of a peak observed in non-oxidised and periodate –oxidised sample extracts. This may be a naturally occurring fluorescent component of the sample matrix (Fig. 24). Periwinkle sample 2 (Belfast Lough) showed a peak in the peroxide oxidation at 6.6mins in the extract obtained from Smash + Grab protocol (Fig. 28). This peak would interfere with the peak for the toxin C1, 2. However this was the only occurrence of an interfering peak in the peroxide oxidation in any of the three periwinkle extractions. No other potentially interfering peaks were observed (Fig. 26 & 27).

Fig. 24 Smash + Grab Peroxide

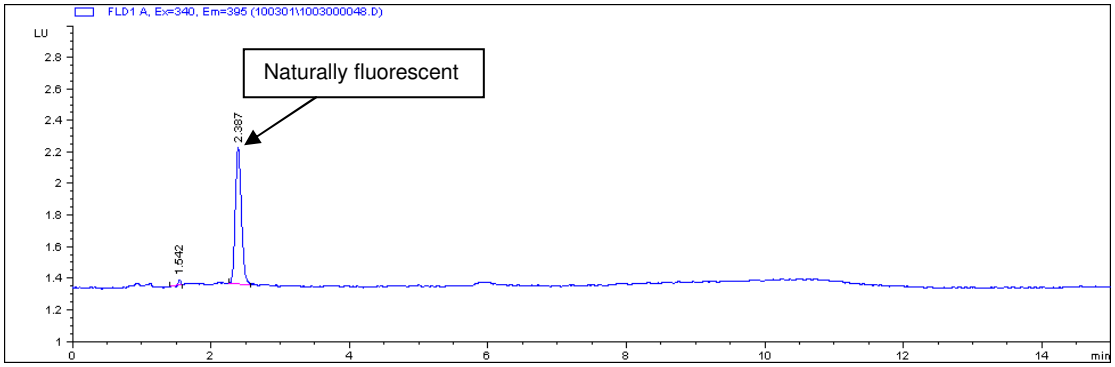


Fig. 25 Boiled < 1 minute Peroxide

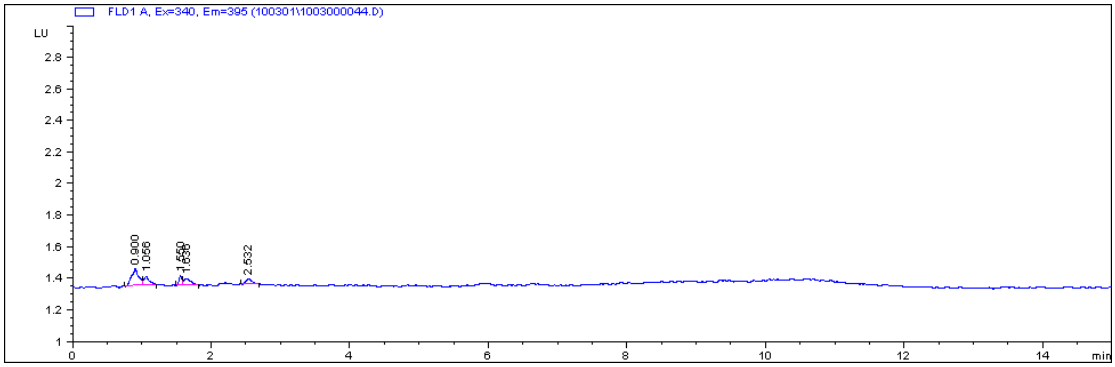


Fig. 26 Standard Mix 2 Peroxide

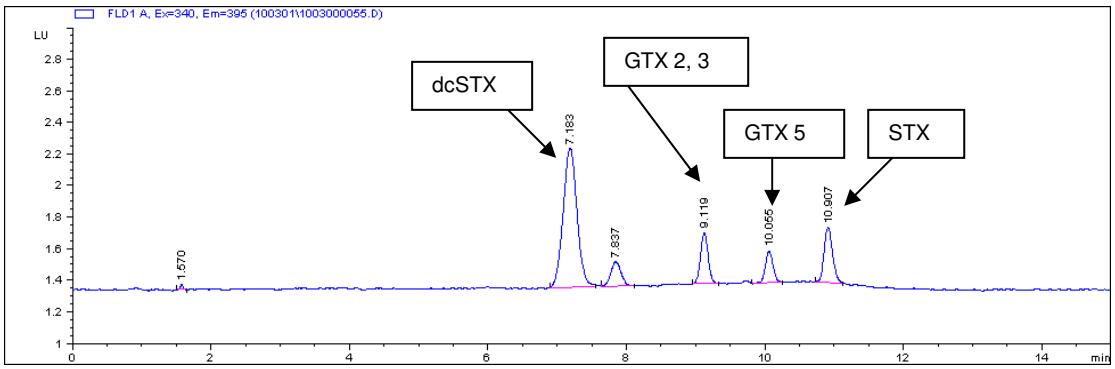


Fig. 27 Standard Mix 3 Peroxide

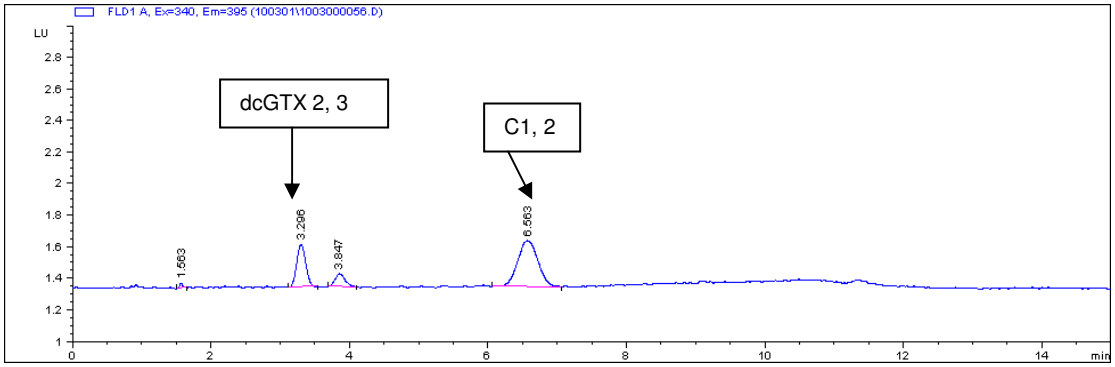
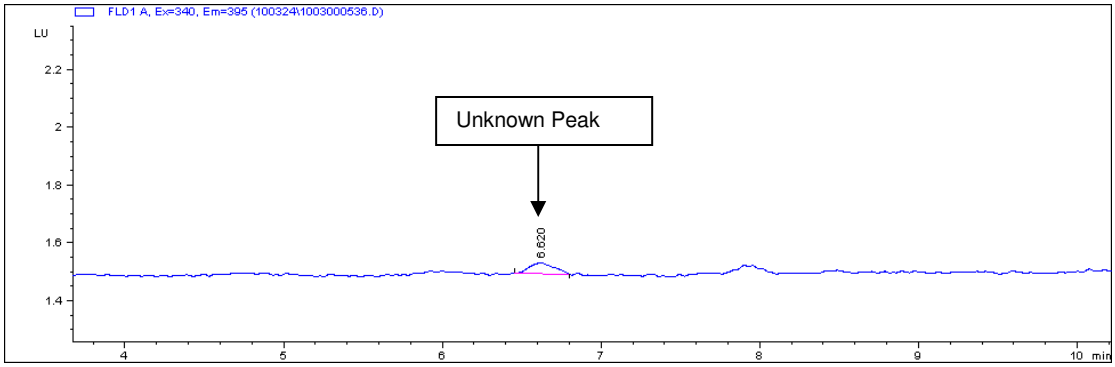


Fig. 28 Sample 2 Smash + Grab Peroxide



6.3 Spiked Periwinkle Homogenate by PSP HPLC

A preliminary investigation was undertaken to determine if the method of shucking might have an effect on the PSP toxins contained in the periwinkles. Naturally contaminated periwinkles were unavailable therefore spiked tissue was used. GTX2,3 was selected as it gave peaks in periodate and peroxide oxidations.

Two 2.5g aliquots of periwinkle tissue that had been shucked using the Smash + Grab method were spiked with GTX 2, 3 at 0.2 x A.L. (160µg STXdiHCl / kg). One 2.5g aliquot was placed in a 50ml centrifuge tube, capped loosely and placed in a boiling water bath for 1minute. This was designed to emulate a naturally contaminated sample under-going the shucking method of "Boiled 1 minute." Both 2.5g aliquots were then extracted as detailed in the UKNRL SOP. Results show that both aliquots contained GTX 2, 3 at approximately 50% of the spiked level measured by periodate and approximately 25% measured by peroxide.

Periodate

Smash + Grab spike	7.04µg/100g (Fig. 33)
Boiled 1 minute spike	8.29µg/100g (Fig. 34)

Fig. 33 Smash + Grab spike Periodate.

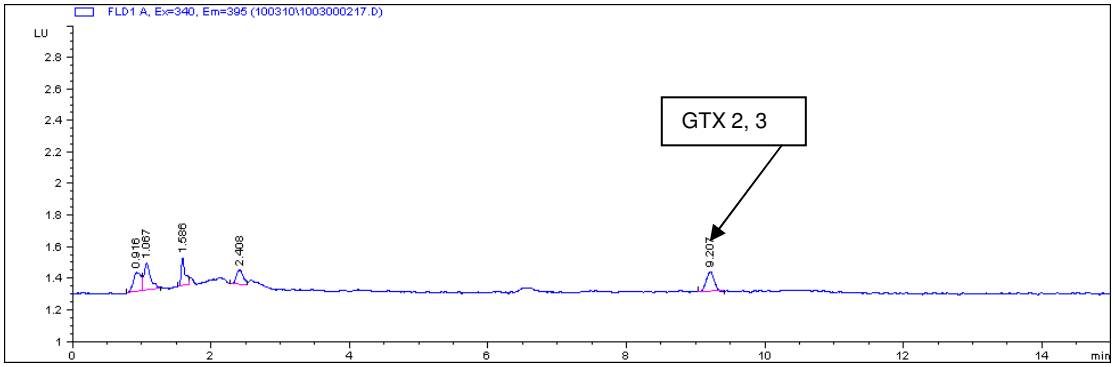
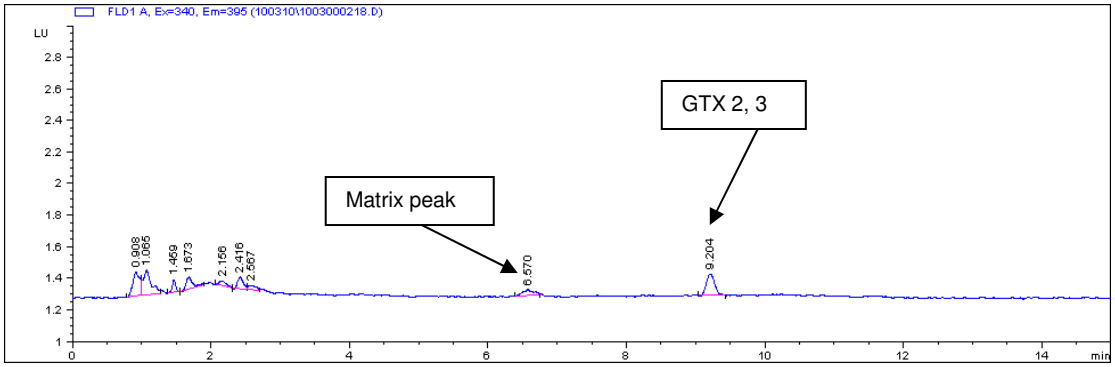


Fig. 34 Boiled < 1 minute spike Periodate.



Peroxide

Smash + Grab spike 4.25µg/100g (Fig. 38)

Boiled < 1 minute spike 5.89µg/100g (Fig. 39)

Fig. 38 Smash + Grab spike Peroxide.

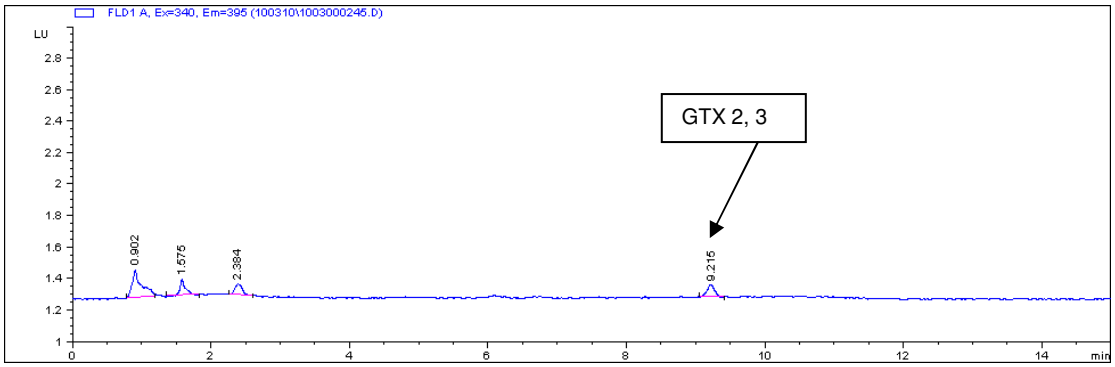
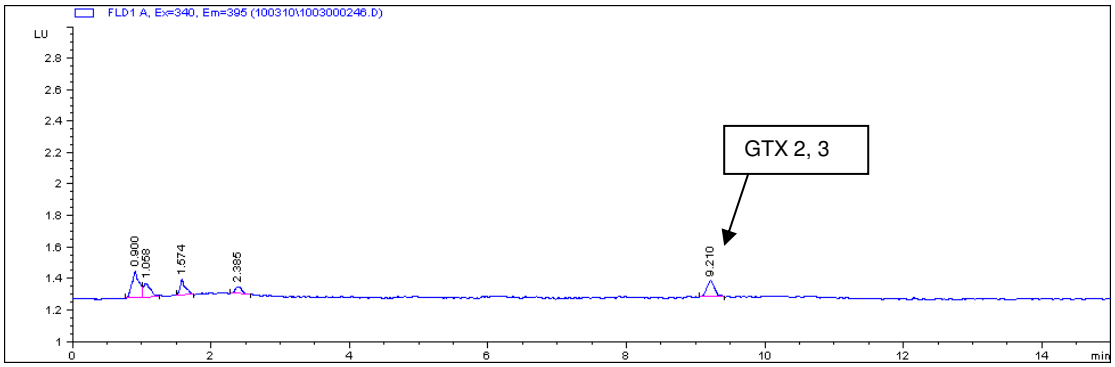


Fig. 39 Boiled < 1 minute spike Peroxide.



6.4 Conclusion

The extraction procedure for PSP HPLC (CSD 408) may be a feasible method for periwinkle matrix. There is the potential for interference from matrix components in periodate oxidation with a peak at around 6.5 minutes in some of the samples. This may interfere with the detection of C1,2 by periodate oxidation. For quantification of C1,2, peroxide oxidation is used and in the majority of the samples tested, no interfering peaks were observed in the peroxide oxidation. In one of the 3 replicates of sample 2 (Belfast Lough), a peak was observed. This peak did not occur in the corresponding non-oxidised sample.

Of more concern is the apparent low recovery of the one toxin tested. Before this method could be extended for use as a screen or quantitative method for periwinkles, more extensive validation work would be required to determine the recovery for the full range of toxins.

7. Determination of PSP by Mouse Bioassay.

Two replicates of a sample of periwinkles from Strangford Lough were prepared by "Smash and Grab" and extracted for determination of PSP by mouse bioassay.

7.1 Extraction

Two mice were injected per sample. There were no issues identified with the extraction protocol and no modifications were required.

7.2 Analysis

Of the four mice injected, all showed significant signs of distress within 4-5 minutes of injection. All were subdued and were breathing rapidly and were walking with an unsteady gait. By 10 minutes, the animals were showing increased difficulty in moving their rear legs. The rear legs became splayed and the animals were prostrate up to 20 minutes, when the test was ended and the mice sacrificed. Given the severity of the reaction, no further samples were tested.

7.3 Conclusion.

The reaction seen in the mice makes the method unsuitable for use in periwinkles. There would appear to be a component of periwinkle matrix which mice cannot tolerate. This may affect the accuracy of the test. Given the frequency of the adverse reactions, the test should not be used for periwinkles, on ethical grounds.

Appendix 1

Agreed Scope

Investigation into Suitability of Current Methods for the Extraction and Determination of Marine Biotoxins in Gastropod Tissue.

It is proposed to undertake the initial investigation on periwinkles and to repeat the protocol on whelks and abalone.
For each species, the investigations will be run as discrete experiments, i.e.
Experiment 1: Tissue from fresh samples with no pre-treatment
Experiment 2: Tissue from boiled samples
Experiment 3: Mechanical removal of the shellfish tissue

A small pilot study will be undertaken to determine if the above are viable options for removing the animal from its shell. Depending on the experiences gained, the above protocols may be changed accordingly.

Number of Replicates:
If possible, samples from three different sites will be obtained. If this is not possible, a sample from a single source will be divided into 3, prior to removal from the shell.

Sample Preparation:
Three methods of extracting the tissue from the shell will be examined: (TBC following pilot study)
Removal from fresh samples,
Removal from boiled samples
Removal by breaking the shells and separation of the tissue from shell fragments.
Removal following freezing.

Information to be gathered:
The number of animals required to provide sufficient tissue for all analysis.
The time taken in sample preparation.
Details on any modifications required to the standard protocol.

Sample Extraction:
The samples will be extracted using,
UKNRL procedure for determination of Lipophilic Toxins.
CRL procedure for the determination of LT by LCMS (as used in the 2008 CRL pre-validation study).
UKNRL extraction protocol for determination of PSP by MBA.
UKNRL extraction procedure for determination of PSP by HPLC (AOAC2005.06)
UKNRL extraction procedure for determination of ASP by HPLC.

Information to be gathered.
All deviations or modifications of the method required to produce the test extracts.
A comparison of the time taken to produce the extracts compared with that for bivalve molluscs.

A qualitative assessment of the extract as to its fitness for subsequent analysis.

Sample Analysis
Subject to DHSS&PS licence approval, sample extracts for lipophilic toxins and PSP will be tested by Mouse Bioassay. The PSP extract will also be tested by HPLC.
Extracts prepared for lipophilic toxins by LCMS will be analysed if deemed suitable.
Extracts for PSP by HPLC will be analysed using the UKNRL procedure.
Extracts for ASP by HPLC will be analysed using the UKNRL procedure.

Information to be gathered.
All mice will be observed as detailed in the relevent UK NRL SOP and all clinical signs recorded.
All chromatographic / LCMS runs will be subject to the routine in-house quality control and all data generated will be compared with data from bivalve molluscs. Evidence will be recorded on potential interfering peaks.

At this stage no information will be gathered on quantitative analysis, i.e. matrix enhancement, linearity etc will not be undertaken.

A final report will be prepared and submitted to Food Standards Agency.

Appendix 2

DSP Mouse Bioassay Observation sheet.

RU73a

RESIDUES
MOUSE BIOASSAY FOR DSP SOP: CSD 402 V8

N = Normal
S = Subdued (quiet, hunched respond when prodded)
Clinical signs: Record which signs are observed and the time of observation – unresponsive, cold, cyanotic (blue colouration to tail, feet, nose)
UC (+ve) = All clinical signs must be present (unresponsive, cold, cyanotic, loss of grip – slips off ungloved hand at 45 degrees)
P = Prostrate/coma (legs splayed, cyanotic)
D = Dead

Site Location: PERIWINKLES..... **Date of extraction:** ..10/3/10.....

Sample Ref No./Source	Mouse No.	Mouse Weight (g)	Mouse Sex (M/F)	Start Time	Observations (Symptoms, time to death)											Comments
					Inj.	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	11hr	24hr	
PERI S+G	A	20.0	F	8.55	N	N	N	N	N	N	N	N	N	N	N	
	B	19.6	F		N	N	N	N	N	N	N	N	N	N	N	
PERI B<1	A	20.4	F	8.58	N	N	N	N	N	N	N	N	N	N	N	
	B	18.4	F		N	N	N	N	N	N	N	N	N	D	N	
	A															
	B															
	A															
	B															
	A															
	B															

BE BE EMD. EMD. EMJ. EMJ. LW EMJ. R R R

Check Weight	22.2g
Syringe Batch No.	0901021

Analyst:.....B. Emerson..... Date of injection:...11/3/10.....

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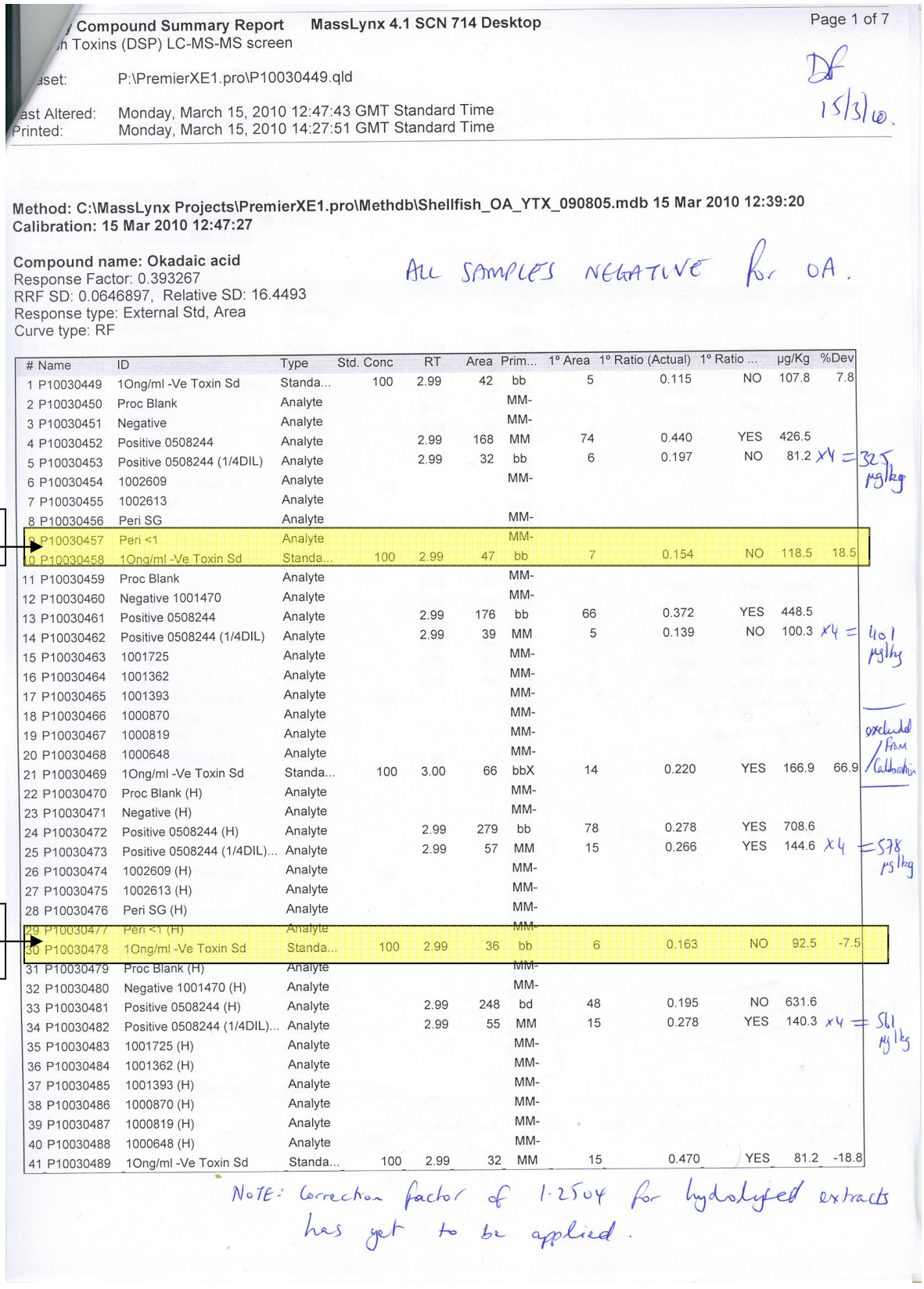
Appendix 3

LCMS/MS results sheet



Compound Summary Report MassLynx 4.1 SCN 714 Desktop Page 1 of 7
Toxins (DSP) LC-MS-MS screen

Dataset: P:\PremierXE1.pro\P10030449.qld
Last Altered: Monday, March 15, 2010 12:47:43 GMT Standard Time
Printed: Monday, March 15, 2010 14:27:51 GMT Standard Time

Df 15/3/10.

Method: C:\MassLynx Projects\PremierXE1.pro\Methdb\Shellfish_OA_YTX_090805.mdb 15 Mar 2010 12:39:20
Calibration: 15 Mar 2010 12:47:27

Compound name: Okadaic acid
Response Factor: 0.393267
RRF SD: 0.0646897, Relative SD: 16.4493
Response type: External Std, Area
Curve type: RF

ALL SAMPLES NEGATIVE for OA.

#	Name	ID	Type	Std. Conc	RT	Area	Prim...	1° Area	1° Ratio (Actual)	1° Ratio ...	µg/Kg	%Dev
1	P10030449	1Ong/ml -Ve Toxin Sd	Standa...	100	2.99	42	bb	5	0.115	NO	107.8	7.8
2	P10030450	Proc Blank	Analyte				MM-					
3	P10030451	Negative	Analyte				MM-					
4	P10030452	Positive 0508244	Analyte		2.99	168	MM	74	0.440	YES	426.5	
5	P10030453	Positive 0508244 (1/4DIL)	Analyte		2.99	32	bb	6	0.197	NO	81.2	x4 = 325 µg/kg
6	P10030454	1002609	Analyte				MM-					
7	P10030455	1002613	Analyte									
8	P10030456	Peri SG	Analyte				MM-					
9	P10030457	Peri <1	Analyte				MM-					
10	P10030458	1Ong/ml -Ve Toxin Sd	Standa...	100	2.99	47	bb	7	0.154	NO	118.5	18.5
11	P10030459	Proc Blank	Analyte				MM-					
12	P10030460	Negative 1001470	Analyte				MM-					
13	P10030461	Positive 0508244	Analyte		2.99	176	bb	66	0.372	YES	448.5	
14	P10030462	Positive 0508244 (1/4DIL)	Analyte		2.99	39	MM	5	0.139	NO	100.3	x4 = 401 µg/kg
15	P10030463	1001725	Analyte				MM-					
16	P10030464	1001362	Analyte				MM-					
17	P10030465	1001393	Analyte				MM-					
18	P10030466	1000870	Analyte				MM-					
19	P10030467	1000819	Analyte				MM-					
20	P10030468	1000648	Analyte				MM-					
21	P10030469	1Ong/ml -Ve Toxin Sd	Standa...	100	3.00	66	bbX	14	0.220	YES	166.9	66.9
22	P10030470	Proc Blank (H)	Analyte				MM-					
23	P10030471	Negative (H)	Analyte				MM-					
24	P10030472	Positive 0508244 (H)	Analyte		2.99	279	bb	78	0.278	YES	708.6	
25	P10030473	Positive 0508244 (1/4DIL)...	Analyte		2.99	57	MM	15	0.266	YES	144.6	x4 = 578 µg/kg
26	P10030474	1002609 (H)	Analyte				MM-					
27	P10030475	1002613 (H)	Analyte				MM-					
28	P10030476	Peri SG (H)	Analyte				MM-					
29	P10030477	Peri <1 (H)	Analyte				MM-					
30	P10030478	1Ong/ml -Ve Toxin Sd	Standa...	100	2.99	36	bb	6	0.163	NO	92.5	-7.5
31	P10030479	Proc Blank (H)	Analyte				MM-					
32	P10030480	Negative 1001470 (H)	Analyte				MM-					
33	P10030481	Positive 0508244 (H)	Analyte		2.99	248	bd	48	0.195	NO	631.6	
34	P10030482	Positive 0508244 (1/4DIL)...	Analyte		2.99	55	MM	15	0.278	YES	140.3	x4 = 561 µg/kg
35	P10030483	1001725 (H)	Analyte				MM-					
36	P10030484	1001362 (H)	Analyte				MM-					
37	P10030485	1001393 (H)	Analyte				MM-					
38	P10030486	1000870 (H)	Analyte				MM-					
39	P10030487	1000819 (H)	Analyte				MM-					
40	P10030488	1000648 (H)	Analyte				MM-					
41	P10030489	1Ong/ml -Ve Toxin Sd	Standa...	100	2.99	32	MM	15	0.470	YES	81.2	-18.8

Periwinkle samples

Periwinkle samples Hydrolysed

excluded from Calibration

NOTE: Correction factor of 1.2504 for hydrolysed extracts has yet to be applied.

Appendix 4

Biosensor results sheet

(Untitled)

Cycle	Unknown	RelResp	Calc da	%cv da
4	cond	229.6	Low	
5	cond	228.1	Low	
AVG.		228.85	#N/A	
14	control1	224.7	Low	
15	control1	227.3	Low	
AVG.		226	#N/A	
18	control2	164.8	8.22	
19	control2	164.8	8.22	
AVG.		164.8	8.22	0.0
20	sB<11	230.5	Low	
21	sB<11	228.5	Low	
AVG.		229.5	#N/A	
22	sB<12	224.8	Low	
23	sB<12	225.2	Low	
AVG.		225	#N/A	
24	sB<13	222.7	0.45	
25	sB<13	225.5	Low	
AVG.		224.1	0.45	#N/A
26	sS&G1	227	Low	
27	sS&G1	228.1	Low	
AVG.		227.55	#N/A	
28	sS&G2	226.9	Low	
29	sS&G2	229.7	Low	
AVG.		228.3	#N/A	
30	sS&G3	227.9	Low	
31	sS&G3	227.1	Low	
AVG.		227.5	#N/A	
16	spiked	131.1	16.69	
17	spiked	131.7	16.46	
AVG.		131.4	16.58	0.99

Periwinkle samples