INVESTIGATIONS TO ASSESS WHETHER DIETHYL ETHER OR ACETONE CARRY - OVER DURING THE DSP STANDARD OPERATING PROCEDURE IS RESPONSIBLE FOR THE ATYPICAL RESPONSE IN MICE

FSA REPORT

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Contents

Executive summary

1. Diethyl ether (DEE) and acetone are used as extraction solvents in the test method for lipophilic toxins responsible for Diarrhetic Shellfish Poisoning (DSP). It has been suggested that the atypical response seen in the DSP mouse bioassay (MBA) could be caused by DEE or acetone remaining in the final extract at levels which, due to incomplete evaporation, would induce symptoms in the mouse.

2. A number of experiments have been undertaken in 2002 and 2003 by each of the statutory biotoxin monitoring laboratories: Centre for Fisheries, Environment and Aquacultural Science (CEFAS), Department of Agriculture and Rural Development (DARD), Fisheries Research Services (FRS) and by independent contractors Central Science Laboratory (CSL) and Macaulay Institute. The work was carried out to determine whether the methods used at CEFAS, DARD and FRS result in solvents remaining in the final extract after the rotary evaporation steps at levels which may induce symptoms similar to atypical responses sometimes observed in mice during the DSP MBA. This paper summarises the findings of the entire programme of work commissioned by the Agency and describes experiments carried out in 2002 and 2003 by DARD, CEFAS, FRS, CSL and Macaulay Institute to:

    - Compare the symptoms induced in mice administered with increasing doses of DEE with the atypical response observed in the MBA.
    - Semi-quantitatively measure the levels of DEE in the airspace above extracts prior to the MBA
    - Compare levels of DEE carry-over between FRS original and interim SOPs
    - Quantify the amounts of DEE and acetone remaining in extracts produced at each laboratory
    - Investigate whether operation of the interim extraction method results in different levels of solvent carry-over at each laboratory.
    - Investigate whether the levels of solvents remaining in extracts correlate with the incidence of atypical responses in the DSP MBA.

3. The data from these investigations do not show a correlation between the atypical response in the DSP MBA and DEE and acetone levels and therefore, from the data presented in this investigation, it is concluded that these solvents are not responsible for the clinical symptoms sometimes observed in mice during the assay.

4. However, DEE and acetone have been found to be carried over into the final extract in variable quantities. This variation could be attributed to chemical differences in the composition of shellfish samples and/or possibly errors inherent to the evaporation stage of the DSP method.

5. Sample extracts prepared by DARD were consistently low in DEE and acetone content, with an equivalent value of 0.16µl DEE per ml of Tween extract[1]. This is

[1] ìl/ml = [ìg/ml / 1000] / 0.7146
Where specific gravity of DEE=0.7146 g/ml

substantially below the LD_{50} of DEE in mice (68µl), and the level at which DARD report that DEE produces symptoms in mice (10 ìl). Extracts prepared by CEFAS and FRS were found to contain variable and occasionally high levels of DEE and acetone. This raises issues about the way in which the extraction process is being carried out because samples of shellfish extracts prepared for the MBA should not contain significant amounts of DEE or acetone if the evaporation stage has been carried out effectively. The National Reference Laboratory (NRL) will be asked to identify courses of action to address this issue.

6. The amount of solvent carry-over was found to be dependent on shellfish species, mussels containing significantly higher levels than cockles. It should be also be noted that the atypical response, although observed with a few mussel samples, is seen mainly with cockle samples. The differences between species are likely to be real, however there may be other factors (not measured here) that affect the results. The differing chemical composition of shellfish species may influence the efficiency of solvent removal from the extracts. Thus, it cannot be assumed that the solvent evaporation conditions used for one type of shellfish species will necessarily be effective for removal of solvents from extracts of other shellfish species. It is therefore concluded that the final evaporation step should be carefully monitored to ensure effective removal of solvents from extracts.

7. Experimental details from each of the separate phases of work are included as Annexes to this paper.

Background

8. Commission Decision 2002/225/EEC specifies the use of the MBA for DSP testing. The MBA is specified as the reference method but alternative or complementary methods are permitted **provided** that they:

- detect at least the following toxic groups of compounds: okadaic acid (OA), dinophysistoxins (DTXs) and also yessotoxins (YTXs), pectenotoxins (PTXs) and azaspiracids (AZAs) at the levels stated in the Decision,
- provide an equivalent level of health protection,
- have been validated according to international protocols, and
- have been approved by the Commission as required by Article 12 of Council Directive 91/492/EEC.

9. The UK method for the detection of DSP toxins in shellfish is based on the Yasumoto (1984)[2] MBA method. This test complies with EU requirements and the European Commission has recently confirmed that the MBA is the best method currently available because it detects all known toxins and the results obtained using it take precedence over other permitted alternative methods under the same EU legislation[3]. However, a standardised international procedure for the application of the test method does not exist.

[2] Yasumoto et al. Diarrhetic shellfish poisoning. Am. Chem. Soc. 19,207-214.
[3] D Byrne communication.

10. The DSP MBA used in the monitoring programmes involves intraperitoneal (IP) injection of shellfish extract, using clinical symptoms or death as the assay endpoint. During the 2001, 2002 and 2003 algal biotoxin monitoring programmes, atypical positive responses (unusually rapid onset of muscle spasms, convulsions and in some cases mouse death) have been observed in respect of samples extracted from some cockles harvested around the coast of England and Wales by CEFAS and Northern Ireland by DARD, and a small number of mussel samples from England and Wales by CEFAS. No similar atypical responses have been reported in shellfish extracts from Scottish waters by FRS.

11. An extensive programme of work has been put in place by the Agency to investigate the cause of the atypical response in the DSP test and to assess its implications for human health. The first phase of work eliminated a number of suggested causes (outlined below), which were reported in the December 2002 Board Paper[4] (and can be downloaded from the Agency's website www.food.gov.uk). These included a series of experiments to investigate whether the atypical responses could be explained by external or environmental factors:

    - **Heat Trials –** Heat trials at 90°C for 90 seconds of cockle samples from Burry Inlet, to establish the effects of commercial heat processing on the toxin.
    - **Toxin Extraction –** to produce a stable toxin extract
    - **Toxin Identification –** Formally test for all known toxins
    - **Choice of Solvent –** Assess effect of different solvent extraction techniques when used in the DSP mouse bioassay
    - **Algal Studies –** Carry out detailed monitoring of phytoplankton.
    - **Anthropogenic Pollutants –** Examine heavy metals as a cause of the response.

12. During 2001 at a UK-NRL network meeting, it was noted that there were variations in the DSP extraction procedures being used by the monitoring laboratories. At that time, CEFAS and FRS were using variations of the Yasumoto 1984 method and DARD was using a variation of the Yasumoto 1978 method.

13. Towards the end of 2002 the Agency became fully aware of discrepancies in the numbers and volumes of solvent extractions employed by each UK biotoxin monitoring laboratory. At a meeting of the UK NRL network on DSP in February 2003 each of the existing protocols for the DSP test used by the laboratories was discussed. Agreement was reached on how to standardise the different stages of the test method, but some precise details of how stages of the test method were to be carried out remained unresolved. Each laboratory subsequently adapted its existing SOPs to accommodate the decisions made at the meeting which aimed to help improve consistency of the DSP testing procedures across the UK. Once the adjustments were made, the interim procedure was implemented in June 2003.

14. Three stages of the extraction method were discussed in detail at the same meeting of the UK-NRL: the number and volume of the primary acetone extractions, the volume and number of the secondary DEE extractions and the

[4] Updating Report on the Atypical DSP Result in Cockles, December 2002, FSA.

removal of solvents and particulates from the final extract. It was suggested that the differences in execution of these stages may influence the incidence of the atypical responses. It was therefore agreed that work would be commissioned to validate the extraction and particulate removal stages of the DSP test. This work was subsequently commissioned at CSL and has concentrated on the stages of the test that give rise to the greatest concerns and is also seeking to optimise all stages of the procedure for the detection of lipophilic toxins, including all those covered by current EU decisions. At this time, the optimised standard operating procedure for use by the UK statutory monitoring laboratories is still under development by CSL. Once validation work is complete, the method will be ring tested with the statutory monitoring laboratories and if successful, implemented across the UK.

15. Prior to the introduction of the interim SOP on the 2 June 2003, atypical responses in the DSP test had not been observed by FRS. On application of the interim procedure by FRS, 18 samples were tested and 4 resulted in abnormal responses in the MBA. FRS and Rowett Institute vets attributed these abnormal reactions to DEE remaining in the final extract. It was therefore jointly decided (FRS, FSA and the Home Office) that until issues of solvent carry-over associated with the application of the new extraction method used at FRS had been resolved, use of the interim procedure should be suspended at that laboratory.

16. The FSA immediately instigated work to investigate the cause of the abnormal responses seen at FRS and this work is reported in this paper (Parts 5, 6 and 7). Testing procedures at the three laboratories involved in the UK statutory biotoxin monitoring programme were also independently audited on behalf of the Agency. The findings of this work are presented in a report by Makin (2003)[5], which can be downloaded from the Agency's website. Issues associated with the use of DEE were also reported as part of this work.

17. The interim procedure is still in place at the other two statutory testing laboratories, where they are giving comparable results to those seen previously prior to its introduction.

[5] Makin, H.L.J., An audit of methods and procedures for lipophilic toxin analysis used by laboratories at CEFAS, FRS AND DARD, which undertake the statutory monitoring of shellfish toxins in the UK.

PART 1- DETERMINATION OF CLINICAL SIGNS IN MICE WHICH CAN BE ATTRIBUTED TO DEE (DARD AND CEFAS)

18. The LD_{50} represents the dose necessary to cause death in 50% (one half) of animal in an experimental study and is a measure of the lethal effect of a chemical after administration of a single dose.

19. The LD_{50} value for IP administration of DEE in mice has been reported as 2420mg/kg bodyweight[6]. For a 20g mouse this equates to an LD_{50} of approximately 68µl assuming a specific gravity for DEE of 0.7146g/ml[7]. However, it should be noted that the LD_{50} is a mean value and lower doses may be lethal in some animals.

20. Data provided by the NRL, referring to information supplied by Sigma-Aldrich Co, suggests that 55µl of DEE will kill a mouse after IP injection, and that DEE is known to act on the central nervous system. It has been suggested that lower doses of DEE could induce clinical symptoms in mice. It has been speculated that the presence of Tween (polyethylene glycol sorbitan monostearate), used to help solubilise lipophilic toxins in the aqueous final extract, could potentiate the effects of DEE because it may disrupt cell membranes to increase the bioavailability of DEE in the mouse. However, specific information supporting this mechanism are lacking.

21. A limited study was undertaken by DARD in 2002[8] to examine the clinical effects in mice when administering increasing doses of DEE (0-167µl) to mice by IP injection in 1% Tween (one mouse per dose). Although it did not look at the effect of shellfish matrix and DEE combined, this work found that DEE volumes up to 50µl per ml of Tween, resulted in hyperactivity and unsteady gait followed by rapid recovery of the affected animals. Responses after administration of DEE volumes $\geq$ 50µl per ml of Tween included unsteady gait quickly followed by collapse, slow, alternate rear leg movement and shallow rapid breathing. During recovery further slow rear leg twitching/scratching was observed. A DEE volume of >150µl /ml of Tween caused rapid death (see Annex A).

22. In DARD's opinion the responses from administration of DEE in Tween could be distinguished from the atypical responses observed when cockle extracts were administered to mice by the intensity of the clinical signs observed with the latter. It was reported that the atypical responses observed with the cockle extract prior to collapse were much more violent than those from DEE intoxication i.e. rapidity of rear leg and body movements. The cockle extract causing the atypical response also resulted in agonal breathing after collapse (slower and more gasping, with the mouth opening much wider). Recovery from low doses of DEE was also more rapid than from (non-fatal doses of) cockle extract the causing atypical response.

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[6] D304 diethyl ether. The Dictionary of Substances and their Effects. 2nd Edition. Royal Society of Chemistry (1998) pages 433-435.

[7] 1ppm = 1ìg/ml]

[8] Updating Report on the Atypical DSP Result in Cockles, December 2002, FSA.

23. This finding was replicated by CEFAS in July 2003 when it investigated the effects of DEE in mice using two spiked Tween samples, one at 8ìl/ml and a second at 20ìl/ml (one mouse per dose). The 8ìl spike caused no observable effect in the mouse and the 20ìl spike caused some mild hyper-activity (climbing on the cage lid) followed by a period when the mice became slightly subdued. None of the signs associated with atypical responses to the DSP MBA were observed in either of the spiked samples.

24. Although these studies are limited and used spiked Tween rather than shellfish extracts, the findings suggest that on its own, DEE is not the cause of the atypical positives produced by cockle extracts.

PART 2- COMPARISON OF DEE AND DICHLOROMETHANE (DCM) AND ATYPICAL RESPONSES (CEFAS)

25. During 2002 CEFAS extracted 23 cockle samples using either DEE or DCM as the secondary solvent, of which 8 gave atypical responses to the DSP MBA, to establish if the type of secondary solvent used had an affect on the incidence of the atypical responses. This work has previously been reported in the December 2002 Board Paper[9] but is reproduced in Annex B for ease of reference.

26. The results of this work demonstrate the occurrence of atypical responses in the MBA from using DCM as a secondary solvent. However, It is not possible to know if the variability observed between DEE and DCM methods was due to mouse bioassay variability or the efficiency of these different chemicals to extract the component(s) responsible for the atypical clinical symptoms.

PART 3 – SUITABILITY OF GASTEC KITS FOR DETERMINATION OF DEE IN AIRSPACE ABOVE TWEEN SUSPENSIONS OF SAMPLE EXTRACTS (DARD AND CEFAS)

27. To address concerns relating to solvent carry over the Agency recommended that each monitoring laboratory should use GC-MS headspace analysis for the quantification of DEE in shellfish extracts. However, due to lack of technical facilities, the laboratories were not able to conduct the analysis on their premises. Therefore, as a quick, semi-quantitative measurement of DEE in airspace above the extracts, the Agency asked all laboratories to use a rapid detection kit (Gastec, Anachem Ltd), already being used by DARD, to check whether solvents were being carried through to the final extract.

28. The Gastec kits were assessed by DARD and CEFAS to determine suitability for measuring gross levels of DEE in the airspace above sample extracts prior to injection into the mouse. Details of the kit can be found at Annex C.

29. DARD carried out a preliminary investigation to assess whether Gastec kits with a limit of detection (LOD) of 400ppm could be used to detect DEE from shellfish extracts, by assessing the ability of the tubes to measure DEE in the air directly above spiked Tween solutions and above a cockle extract (which had shown an atypical response in mice). Experimental results are reported at Annex C, Table 5.

[9] Updating Report on the Atypical DSP Result in Cockles, December 2002, FSA.

30. The results indicated that the kits were able to detect DEE in the airspace above solutions of Tween and extracts of cockles prior to administration to mice.

31. DARD conducted further calibration of the Gastec kits using DEE and de-ionised water. The calibration curves produced are shown in Annex C, Table 6 and Figure 1. CEFAS used the Gastec kits to construct calibration curves for both detection tube 161 and 161L using a range of Tween extracts spiked with DEE from 0 ìl/ml up to 40ìl/ml. The data are reproduced at Annex C, Table 7. The calibration charts plotted are reproduced at Annex C, Figures 2 and 3, and are based on one set of data per chart. The data show that within the limits of detection of the Gastec kits the relationship between the Gastec readings and the levels of DEE in the spiked samples is linear. In addition, the results show that the more sensitive tubes (161L) correlated better with the levels of DEE in the spiked samples at low concentrations than the less sensitive 161 tubes. Both types of detector were found to provide a reproducible results on repeat analyses.

32. On the basis of these data all laboratories were asked by the Agency on the 26 June 2003 to introduce the Gastec kit 161L into their monitoring procedures as a QC measure.

PART 4 – MEASUREMENT OF DEE USING GASTEC IN THE AIRSPACE ABOVE TWEEN SUSPENSIONS OF EXTRACTS FROM MONITORING SAMPLES (DARD AND CEFAS)

33. The Gastec kits have been used by DARD (kits 161 and 161L) to test a proportion of routine monitoring samples for the presence of DEE prior to administration to mice since June 2003. However, the large number of monitoring samples precluded Gastec analysis of every sample. At the time of writing 20 monitoring samples had been measured. The data are reported at Annex D, Table 8.

34. The majority of samples were screened using the Gastec kit 161 with a LOD of 400ppm, as these samples had been analysed prior to the Agency request to use kits with an LOD of 10ppm. It can be seen that in the majority of cases, if the extracts contained DEE, the levels measured in the airspace above the extracts were below 400ppm. It was shown that in the case of samples containing over 400ppm of DEE, this could be lowered in some cases, by returning the extract to the rotary evaporator for a further 2-5 minutes.

35. CEFAS have also used the more sensitive Gastec kit (161L) with an LOD of 10ppm to measure the concentration of DEE in the airspace above extracts reconstituted in Tween from samples prepared for the routine monitoring programme. Twenty-nine routine samples have been analysed for the presence of DEE in the airspace using the Gastec detection tubes. Fifteen samples tested produced an atypical response in the DSP MBA, and 14 produced negative responses in the DSP MBA. The results are reported at Annex D, Table 9.

36. The Tween extracts produced at CEFAS when analysed using the calibrated 161 and 161L Gastec detector tubes showed the presence of DEE between 1.8 and 10 ìl/ml in 52% of samples analysed. However, the occurrence and concentration

of DEE in these extracts does not correspond with atypical responses in the MBA, with 66% of atypical test results occurring in samples containing no DEE. In addition, over 70% of samples which tested negative in the bioassay were found to contain DEE and the highest levels of DEE found in 4 samples that gave negative response in the MBA. While Gastec only provides an indication of DEE levels, it can be seen that atypical responses do not relate to samples with high DEE levels.

PART 5 – INVESTIGATION BY FRS AND MACAULAY INSTITUTE, ABERDEEN

37. In July 2003 FRS and the Macaulay Institute in Aberdeen were commissioned by FSA Scotland to carry out a small ad-hoc study to investigate whether DEE was being carried over into final extracts prepared by FRS using their original extraction method and the interim procedure. Using the original extraction method samples were left overnight to aid DEE removal from the sample. This step was not included in the interim method and therefore it is not possible to directly compare the DEE levels in the final extracts from the two methods. A full report of this work can be found at Annex E.

38. Samples of shellfish extracts prepared by FRS from mussels and scallops using the interim SOP and the original FRS SOP were sent to Macaulay Institute for analysis of their DEE content using a range of different methods: by Fourier Transform Infra Red Spectroscopy (FTIR), Gastec tubes and qualitative Gas Chromatography - Flame Ionisation Detection (GC-FID). A comparison of the DEE concentrations between samples stoppered immediately after rotary evaporation of the extract and those left open to the atmosphere for a period of time was also undertaken.

39. Although FTIR was not found to be suitable for DEE detection, it showed that all the extracts contained significant amounts of water.

40. DEE was detected by GC-FID in all extracts prepared by FRS using the interim and original procedures. Samples which were stoppered immediately after preparation were found to contain higher levels of DEE than those which were left un-stoppered, irrespective of the procedure used.

41. Additional rotary evaporation at the Macaulay Institute for even a few minutes was found to decrease the GC-FID response for DEE. When the same extract was filtered through sodium sulphate to remove traces of water, and the DEE removed by rotary evaporation as previously, water was removed and the level of DEE was further reduced. One suggestion for these results may be that the rotary evaporation step is not be being applied effectively at FRS. Other possibilities include incomplete separation of organic and aqueous phases during extraction, and the presence of water acting as a sink for DEE.

PART 6 – MEASUREMENT BY GC-MS HEADSPACE ANALYSIS OF DEE AND ACETONE IN SAMPLE EXTRACTS PREPARED BY CEFAS, DARD AND FRS

42. Based on the work completed in Parts 1-5, which did not preclude the possibility of DEE being the cause of the atypical response in mice, further work was commissioned by the Agency to give a definitive view on this matter and this is reported in Parts 6 and 7.

43. In August 2003 the Agency commissioned CSL to accurately quantify levels of DEE and acetone in extracts prepared by CEFAS, DARD and FRS using Gas Chromatography – Mass Spectrometry (GC-MS) analysis of the headspace gas. The samples analysed for DEE were prepared following the protocol that can be found at Annex F.1. The analytical method used was developed by CSL and is outlined at Annex F.2.

44. CEFAS, DARD and FRS were each asked to send 45 extracts prepared using the extraction method used by their laboratory (interim at CEFAS and DARD, original at FRS) to CSL over a 3 week period (i.e.15 extracts/week) for DEE and acetone analysis by GC-MS. Extracts of both cockles and mussels were requested from each laboratory. DARD was unable to collect the required number of samples due to the small number of samples they receive from their monitoring programme. FRS was unable to collect the required number of extracts and also could not collect a significant number of cockle extracts as cockles are not frequently harvested in Scotland. The rotary evaporations conditions used at each laboratory during preparation of the samples are presented in Annex F.3.

45. Each shellfish sample was taken through the routine extraction procedure at each monitoring laboratory, and the residue was re-suspended in 4ml of 1% Tween. Three 150µl aliquots (replicates) of Tween suspension were then removed and placed in separate headspace vials provided by CSL. An appropriate quantity of internal standard (isotope of DEE), supplied by CSL, was added to each sample vial, to check whether DEE losses were occurring during storage and transport of the vials to CSL. Designated vials were used for spiked samples. All vials were sealed immediately and sent under controlled conditions to CSL on a weekly basis for GC-MS headspace analysis of DEE and acetone. Each laboratory was also asked to use the Gastec system to measure DEE in the airspace above the sample extracts to allow comparison of the Gastec and GC-MS measurements and provide an estimate of the accuracy of Gastec to monitor DEE levels routinely.

46. FRS was asked to prepare duplicate samples using both the interim and their original method. The interim procedure requires extracts to be immediately re-suspended in Tween following evaporation of DEE using a rotary evaporator. The original FRS SOP says extracts may be left over-night following rotary evaporation to allow further evaporation of DEE prior to re-suspension in Tween – a step that had been introduced prior to 1996 because DEE had occasionally been found in samples in the past, when testing was carried out by Torry Research Services (TRS). Hence DEE measurements on samples prepared with the original FRS SOP, using Gastec and GC-MS, were conducted on Tween samples prepared after extracts had been left open to the atmosphere over-night.

DEE measurements on samples prepared by FRS with the interim SOP, using Gastec and GC-MS, were also conducted on extracts which had been immediately re-suspended in Tween following evaporation, using a similar procedure in operation at DARD and CEFAS, as Gastec was used by other laboratories after resuspension in Tween.

47. All sample extracts prepared (except those prepared by FRS using the interim procedure) were subsequently injected into the mouse for routine monitoring purposes, and the responses recorded. Data from FRS obtained using the interim procedure are only presented for the first two weeks of samples collected. Analysis of week 3 interim samples was not available at the time of writing.

48. The data generated by the GC-MS analysis are reported in Annex F.3. Statistical analysis of the data was undertaken to determine:

    i. Whether DEE measurements by Gastec and GC-MS correlate for the interim and FRS original method.
    ii. Whether there is a difference between MBA negative and atypical responses for concentration levels of DEE and acetone in extracts produced using the interim method by CEFAS and DARD.
    iii. Whether there are any differences between laboratories for extracts prepared using the interim method for DEE and acetone concentrations.
    iv. Whether there are any differences between FRS original and interim methods for DEE and acetone concentrations.
    v. Whether there is a relationship between the DEE and acetone concentrations for interim and original method.

49. The statistical methods used to address the questions above were:

    i. Pearson correlation (ñ) to evaluate the correlation between GC-MS DEE and Gastec. The Pearson correlation varies between -1 and 1, where a correlation of -1 is a perfect negative correlation and a correlation of 1 is a perfect positive correlation.
    ii. Independent sample t-test to compare the two types of MBA responses (negatives and atypical). Due to normality issues results were confirmed using non-parametric tests.
    iii. One-way analysis of variance (ANOVA) to compare the three different laboratories. Due to the high variability of the data, results were investigated further using different assumptions, such as unequal variances across the laboratories.
    iv. Independent sample t-test to compare the two methods (interim and FRS original). Due to normality issues results were confirmed using non-parametric tests.
    v. Pearson correlation to evaluate the correlation between GC-MS DEE and acetone.

50. Study limitations and assumptions:

- Due to time constraints and labelling problems, not all three replicates per sample were available. Using averages allows a re-balance of the design, as the unit of

analysis is the average of the available replicates rather than the replicates themselves.

- P-values lower than 0.05 were considered to be significant throughout the analysis.
- All values below the limit of quantification (LOQ) were replaced by the value of the LOQ.
- All results extrapolated from calibration curves, and results where peaks were found but ion ratio confirmation criteria was not satisfied, were used as accurate values. These data cannot be reported with the same level of confidence as results that satisfy all of the QA parameters, however they are still robust.
- Every time test assumptions were violated, a corresponding non-parametric test was used.

51. The findings of this work to address the various questions are reported below.

i. Whether DEE measurements by Gastec and GC-MS correlate for the interim and FRS original method.

There is a weak correlation between Gastec and GC-MS measurements of DEE (ñ = 0.11) when the interim method is used for all laboratories (when data is considered together). Nevertheless, Gastec is a convenient means of detecting the presence or absence of DEE and has some potential use as a QC measure.

CEFAS data considered on its own shows a weak correlation. Week 3 data from CEFAS differs considerably from weeks 1 and 2, and this difference reduces the strength of the correlation substantially. The FRS original and interim method produces similar results, i.e. a weak correlation. This may be attributed to the use of differing Gastec kits with a higher LOD (400µg/ml) in the first week, and lower LOD (10µg/ml) in the remaining weeks.

There is some evidence that the Gastec measurements of DEE were affected by the acetone in the samples. However, due to the use of different Gastec kits between labs it is difficult to assess the true affect of this.

The lower level of DEE measured by Gastec as opposed to GC-MS may be explained by the lack of equilibration time for DEE to partition equally between the liquid (Tween) and gas (headspace) phases for Gastec measurement. Gastec kits measure the DEE in the headspace arising from passive diffusion from the sample matrix, whereas headspace GC-MS samples were heated to 60°C for 5 minutes in a sealed system. One would therefore expect that headspace GC-MS to conditions drive the solvents out of the sample matrix over a shorter period of time, which would allow higher DEE levels to be detected.

The data suggest that Gastec kits, which are only semi-quantitative, may have a role as an indicative test to describe the presence or absence of DEE in an environment, but they are not be suitable to accurately measure DEE levels. GC-MS provides a reliable, quantitative measure of DEE and acetone.

ii. Whether there is a difference between MBA negative and atypical responses for concentration levels of DEE and acetone in extracts produced using the interim method by CEFAS and DARD.

Data analysis was restricted to those laboratories that used the interim method and carried out the MBA (i.e. CEFAS and DARD). All data from both laboratories was combined before analysis as differences between laboratories was not investigated at this stage. During the investigation CEFAS were the only laboratory to report atypical responses in the MBA, these being in 8 of 45 samples.

Statistical analysis of CEFAS and DARD data shows that there were no significant differences in DEE (P=0.39 for the non-parametric test) and acetone (P=0.88 for the non-parametric test) levels for atypical and negative MBA results (i.e. atypical MBA responses do not occur with high levels of solvent). FRS data from samples produced by the interim SOP were not included in this analysis as these extracts were not tested in the MBA.

Analysis of this dataset does not show a relationship between DEE or acetone and the atypical response.

iii. Whether there are any differences between laboratories for extracts prepared using the interim method for DEE and acetone concentrations.

Concentrations of DEE and acetone remained in many of the sample extracts prepared by all laboratories at levels above the level of quantification (10μg/ml). DEE and acetone remaining in extracts varied between each laboratory. Summary statistics are reported in Table 1. All samples from FRS prepared using the original SOP were excluded from the analysis because sample extracts were left overnight to allow evaporation of solvents.

Using the interim method there were no significant differences in the concentrations of DEE measured by GC-MS between the laboratories. However, looking closer at the data it can be seen that the spread of the data is NOT equal across different laboratories (e.g., DARD varies between 0 to 1,000 whilst CEFAS varies between 0 and 100,000). Therefore, when testing for multiple comparisons we can assume that variances are unequal across the different laboratories. **The approach shows that DARD has significantly lower GC-MS DEE than CEFAS (P<0.05) and FRS (P<0.01).**

Differences in solvent levels remaining in extracts prepared by each laboratory possibly originate from differences in evaporation procedure. The conditions used at each laboratory are summarised in Annex F.3. It can be noted that the rotary speed used at DARD is much lower and the length of evaporation time longer, than either CEFAS or FRS. This may provide an explanation to the differences in solvent levels experienced by all laboratories, however, it is likely that differences in the equipment used, possibly evaporator pressure may be a contributing factor.

DEE measured in the sample extract by headspace GC-MS can be converted to an equivalent amount per ml of Tween and allow comparison with data relating to the LD_{50} in Part 1. However, the LD_{50} is based on data from the administration of neat DEE rather than an aqueous mixture (i.e. DEE and Tween).

The highest level of DEE was recorded from a CEFAS sample, at 87,310ìg/ml of DEE in the extract. Using a conversion factor[10], this is equivalent to 122ìl of DEE per ml of Tween extract. The highest average DEE level recorded by all three laboratories was a CEFAS sample at 7,256µg/ml of DEE in the extract. This is equivalent to a level of 10ìl of DEE per ml of Tween extract. DARD recorded the lowest average DEE level in extracts at 111µg/ml, equivalent to 0.16µl DEE per ml of Tween extract.

The work conducted by DARD in Annex A, while limited, shows that a level of DEE >150ìl (107,190ìg) per ml of Tween DARD was required to kill a mouse following IP injection. However, a level of 10ìl (7,146ìg) DEE per ml of Tween had no obvious symptoms. Levels of DEE have been recorded in some samples (6.7%) from CEFAS, which are higher than the LD_{50}, yet no symptoms in mice were recorded. A possible explanation for this could be the delay in time between preparing the sample extracts for GC-MS analysis and injection of the sample in mice, and this may have allowed significant levels of DEE to evaporate.

Samples of shellfish extracts prepared for the MBA should not contain significant amounts of DEE or acetone, if the evaporation stage has been carried out effectively. It is recommended that CEFAS and FRS should adopt the DARD approach to the evaporation procedure, thereby standardising measures to reduce potential solvent carry over. Overnight storage prior to re-suspension in Tween is not recommended as this could lead to potential degradation of lipophilic substances[11,12]. However, with all changes to procedure, care should be taken to ensure that they do not affect the ability of the procedure to detect the atypical response.

iv. Whether there are any differences between FRS original and interim methods for DEE and acetone concentrations.

There are significant differences in extracts for DEE and acetone between the original and interim methods used at FRS (P < 0.01). The interim method produces significantly higher DEE and acetone measurements than the original method. However, samples prepared using the original SOP are left over-night to allow DEE to evaporate before re-suspending in Tween and subsequent DEE measurement. Leaving extracts overnight to allow DEE and acetone to

[10] ìl/ml = [ìg/ml / 1000] / 0.7146
Where specific gravity of DEE=0.7146 g/ml

[11] Hyenstrand, P., J. S. Metcalf, K. A. Beattie and G. A. Codd (2001). "Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin microcystin-LR by high performance liquid chromatography." Water Research 35(14): 3508-3511.

[12] Hyenstrand, P., J. S. Metcalf, K. A. Beattie and G. A. Codd (2001). "Losses of the cyanobacterial toxin microcystin-LR from aqueous solution by adsorption during laboratory manipulations." Toxicon 39(4): 589-594.

evaporate, may lead to potential degradation of lipophilic substances and should not be practised.

Table 1. Summary statistics of solvent concentrations in cockle and mussel extracts prepared by each laboratory.

Solvent		DARD Interim SOP	CEFAS Interim SOP	FRS	
				Interim SOP	Original SOP
Number of samples		17	45	33	37
DEE (µg/ml) by GC-MS	**Mean**	111	7,256	4,788	259
	Median	48	179	1,900	10
	Range	10-917	10-87,310	57-28,169	10-6,989
Acetone (µg/ml) by GC-MS	**Mean**	44	7,317	878	97
	Median	37	1,526	555	78
	Range	10-94	76-108,345	27-4,195	10-598
DEE (µg/ml) by Gastec	**Mean**	12	35	2,835	200
	Median	10	10	400	25
	Range	10-50	10-350	10-10,000	10-400

v. Whether there is a relationship between the DEE and acetone concentrations for interim and original method.

For the interim method there is a strong positive correlation (ñ = 0.86) between concentrations of DEE and acetone within each sample, i.e. amount of DEE increases with increasing amounts of acetone, but this does not apply for data from the original FRS method (ñ = 0.23).

52. The results of the experiments reported in Part 6, show that varying amounts of DEE can remain in extracts of shellfish to varying degrees and that these levels do not appear to relate with atypical responses observed in mice. DARD produced samples with consistently low levels of DEE and acetone. Extracts prepared by CEFAS and FRS however, were found to contain variable and occasionally high levels of DEE and acetone.

PART 7 – MEASUREMENT OF DEE AND ACETONE IN REPLICATE SAMPLES OF COCKLES AND MUSSELS TO DETERMINE WHETHER SOLVENT LEVELS IN EXTRACTS ARE SPECIES DEPENDENT

53. The results of the experiments reported in Part 6, show that varying amounts of DEE can remain in extracts of shellfish and that the varying levels do not appear to relate with atypical responses observed in mice. It was suggested that the

variability in the quantity of DEE could be due to differences in shellfish matrix and so work was undertaken to address this point.

54. To determine whether the amount of DEE and acetone carry-over is species dependent or whether the extraction procedure, or operator implementation, introduces variability in the quantity of DEE and acetone remaining in Tween extracts, 16 replicate samples each of cockles and mussels were extracted and GC-MS headspace analysis undertaken.

55. CEFAS prepared a batch of cockle samples collected from one harvesting area so that differences within species that may be caused by environmental effects could be minimised. Enough homogenate was prepared to produce 16 replicate samples. The replicate samples of cockles were extracted by CEFAS using the interim SOP. Aliquots of Tween extracts (3x150µl) from each sample were separated into headspace vials and, following addition of internal standard, vials were sealed immediately and sent to CSL for analysis. Spiked samples, also prepared at CEFAS, were used as QC samples under controlled conditions. CEFAS repeated this experiment for mussel samples collected from the same harvesting area. The experimental protocol followed by CEFAS is at in Annex G.1.

56. The data collected from this study of DEE and acetone concentrations in replicate samples are reported in Annex G.2. Statistical analysis of the data was undertaken to determine:

    i. Whether DEE and acetone carry-over were dependent upon species.
    ii. Whether variations in DEE and acetone were substantial within replicate samples of the same species.

57. The statistical methods employed were:

    i. Analysis of variance (ANOVA) controlling for the time for rotary evaporation and the pressure at the end of rotary evaporation.
    ii. The coefficient of variation (CV), which measures, in percentage terms, the relative variability of the data. Instead of reporting the overall variability (standard deviation), which is dependent on unit and the range of the data, the CV provides a relative value of the variability by dividing the standard deviation by the mean. A CV of around 200% means that the standard deviation is twice the size of the mean, i.e. highly variable data.

58. Study limitations and assumptions:

    - On occasion, due to compromised seals on vials, not all three replicates per sample were available. Using averages allows a re-balance of the design, as the unit of analysis is the average of the available replicates rather than the replicates themselves.
    - P-values lower than 0.05 were considered as significant throughout the analyses.
    - All values below the limit of quantification (LOQ) were replaced by the value of the LOQ.

- All results extrapolated from calibration curves, and results where peaks were found but ion ratio confirmation criteria was not satisfied, were used as accurate values. These data cannot be reported with the same level of confidence as results that satisfy all of the QA parameters, however they are still robust.

59. The findings of this work are reported below:

i. Whether DEE and acetone carry-over were dependent upon species.

A summary of DEE and acetone concentrations between species (cockles and mussels) is in Table 2. **The concentration of DEE and acetone was found to be significantly less in the cockle samples than in the mussel samples (P < 0.05 for DEE and P < 0.01 for acetone)**. This suggests that the physico-chemical nature of mussel matrix may preferentially absorb larger quantities of these solvents in comparison to cockle matrices. Shellfish matrices would therefore seem to be a contributory factor to the amount of solvent carried over to the final extract.

Variation between species may be a result of the differences in fat content of the two species. Mussels have a higher fat content than cockles and this may affect the amount of solvents which are carried through to the final extract. However, since rotary evaporation is the final stage of the method prior to re-suspension in Tween, minimal amounts of solvents should remain in the final extract before being injected into the mouse.

Table 2. Summary statistics of concentrations of solvents from 16 replicate samples of each shellfish species prepared by CEFAS.

	Cockles extracted using interim SOP		Mussels extracted using interim SOP	
	DEE (µg/ml)	Acetone (µg/ml)	DEE (µg/ml)	Acetone (µg/ml)
Mean	786	3,695	15,610	40,828
Median	39	1,991	1,419	7,101
Min	10	92	117	757
Max	3,556	9,804	74,929	163,166

ii. Whether variations in DEE and acetone were substantial within replicate samples of the same species.

DEE and acetone concentrations vary considerably between replicate samples of each species (CV = 222% for DEE and CV = 193% for acetone).

Possible causes for the variability of solvents between replicate samples are postulated below:

- Multi operator bias can not be ruled out and may contribute to the variability of data between samples, particularly in relation to the application of the rotary

evaporator stage. Since determination of the end point is subjective this could result in residual acetone and water in the extract which may have the effect of trapping or partitioning DEE into the dissolved phase of the extract itself, and thereby mean that not all the DEE is removed.

- Lack of homogeneity of the bulk sample used to prepare the replicate samples. If the bulk sample is not entirely homogeneous, solvents may be associated with certain samples which have a higher fat content.

PART 8 – CONCLUSIONS

60. From the data presented in this report the following conclusions can be drawn:

- There is no relationship between the occurrence of atypical responses seen in mice on injection with samples extracted from cockles and the level of DEE or acetone which may remain within the extract. Therefore, there does not appear to be a direct causal relationship between DEE and acetone levels and the atypical responses recorded during these investigations. In addition, atypical responses were still observed when DCM was used instead of DEE in the extraction procedure.

- DEE and acetone remained in many of the extracts prepared by each laboratory. DARD extracts contained significantly lower concentrations of DEE and acetone, substantially lower than the LD_{50} of DEE at 68µl, and below that of CEFAS or FRS. Evaporation conditions differ between laboratories, DARD having a notably lower rotary evaporation speed (rpm) and longer evaporation times than CEFAS or FRS. This may provide an explanation to the differences in solvent levels experienced by all laboratories, however, it is likely that differences in the equipment used, possibly evaporator pressure may be a contributing factor.

- Administration of DEE in Tween to mice at concentrations up to 150µl did not produce the atypical symptoms which are reported during shellfish toxin monitoring.

- The higher levels of DEE measured in a number of extracts did not appear to induce obvious symptoms in mice and therefore this finding supports the view that solvent is not responsible for atypical responses. It is possible that there is a cause and effect issue to address with respect to the dissipation of the sample extract in 1% Tween, with some samples lending themselves to acceptable dissipation (and hence acceptable injection into the mouse).

- There is a weak correlation between Gastec and GC-MS measurements of DEE when the interim method is used for all laboratories. Nevertheless, Gastec is a convenient means of detecting the presence or absence of DEE and has some potential use as a QC measure.

- DEE and acetone are carried through to the final extract irrespective of the SOP used (interim or original FRS method). Based on the findings at Annex E which measured DEE in stoppered extracts prepared using both the original

and interim SOP, the interim SOP does not result in higher levels of DEE in the final extract than using the FRS original SOP. However, there are significant differences in DEE and acetone levels between the original and interim methods used at FRS, when measurements taken from the original SOP extracts are done so after overnight evaporation.

- Inter-species differences in matrix are a possible factor in the variable amounts of DEE and acetone remaining in the final extract following evaporation and re-suspension in Tween. The concentration of DEE and acetone is significantly lower in cockle extracts compared with mussel extracts, which may be due to the differing fat content of the species. This also supports the view that solvents are not responsible for the atypical responses as they are predominantly found when testing cockles.

- There is a strong positive correlation between DEE and acetone for the interim SOP.

61. The findings of this report have been shared and discussed with CEFAS, DARD, FRS and CSL and will be used to inform future development of the DSP SOP. In addition, measures will be introduced to ensure effective removal of solvents before the extract is tested in the mouse. This work will be over-seen and monitored by the UK-NRL.

62. This report was prepared by the Agency to present and interpret the results of the studies it funded. The Agency considers that it has carried out sufficient work to rule out solvent carry-over as the cause of the atypical response in the DSP MBA.

63. The Agency accepts that there may be additional information that could be relevant to the above investigations of which it is not aware. Should any relevant new evidence based work come to light once this report has been published, the Agency will review it and consider an appropriate response.

ANNEX A

DETERMINATION OF CLINICAL SIGNS IN MICE WHICH CAN BE ATTRIBUTED TO ETHER (DARD 2002)

64. DARD has examined the clinical effects in mice when administering increasing doses of DEE (0-167µl) in 1% Tween by IP injection to single mice.

65. DEE (5-400µL) was added to 1% aqueous Tween (2ml) and mixed by hand and capped immediately to prevent evaporation. Mice (16-23g) were injected IP with 1ml of DEE/1% Tween mixture. The mice were observed until death or full recovery.

Table 3. Responses in mice after IP injection of DEE/1% Tween mix

DEE (µl per ml of Tween)	DEE (µg/ml)	Mouse bodyweight (g)	Effect in mice on injection of 1ml DEE/1% Tween mixture
0 (Tween only)	0	17	Normal.
2.5	1,787	16.6	Rapid breathing at 3-4 minutes, no other symptoms.
5.0	3,573	16	Hyper activity at 3-4 minutes, rapid grooming and random movement around cage.
9.9	7,075	18.3	No obvious symptoms.
24.4	17,436	23.7	Hyperactive, rapid movement around cage, unsteady swaying at minutes. Did not collapse. Recovered.
47.6	34,015	17.4	Collapse at <1 minute, rapid, shallow breathing, alternate slow, rear leg twitching at ~6 minutes. Signs of movement by ~ 11 minutes. Full recovery by 15 minutes.
90.9	64,957	18	Hyperactive, grooming, rapid movement around cage, 1-2 minutes unsteady, rolling gait, collapse 2-3 minutes, shallow breathing, recovery evident by ~20 minutes.
166.7	119,124	17.2	Collapse <1 minute, death by 2 minutes. No observed general symptoms.

ìl/ml = [ìg/ml / 1000] / 0.7146
Where specific gravity of DEE=0.7146 g/ml

66. While the data in Table 3 is for a limited data set (n=1/dose), and Tween spiked with DEE may not be comparable to cockle extract contaminated with DEE, DARD were of the view that there were significant differences between an atypical response observed during shellfish toxin monitoring and the DEE induced response.

ANNEX B

COMPARISON OF DEE AND DICHLOROMETHANE (DCM) AND ATYPICAL RESPONSES (CEFAS)

67. CEFAS extracted 23 cockle samples using both DEE and DCM as the secondary solvent establish if the type of secondary solvent used had an affect on the incidence of the atypical responses. The results of the samples are shown in Table 4.

Table 4. DSP MBA results using DCM and DEE to extract cockle samples

	Mouse bioassay result for atypical response				
Sample Number	**DCM**	**DEE**	**Sample Number**	**DCM**	**DEE**
BTX 2002/215	Negative	Negative [2]	BTX 2002/513	Negative[1]	Negative[1]
BTX 2002/218	Negative	Negative	BTX 2002/533	Negative	Negative
BTX 2002/219	Negative	Negative	BTX 2002/539	Negative[1]	Negative
BTX 2002/248	Negative	Negative	BTX 2002/216	**Positive**	Negative
BTX 2002/249	Negative	Negative	BTX 2002/217	**Positive**	Negative
BTX 2002/250	Negative	Negative	BTX 2002/220	**Positive**	Negative
BTX 2002/267	Negative	Negative	BTX 2002/448	**Positive**	**Positive**
BTX 2002/447	Negative[2]	Negative	BTX 2002/502	Negative[3]	**Positive**
BTX 2002/455	Negative	Negative	BTX 2002/512	**Positive**	**Positive**
BTX 2002/489	Negative	Negative	BTX 2002/530	Negative[1]	**Positive**
BTX 2002/490	Negative	Negative	BTX 2002/563	**Positive**	Negative
BTX 2002/511	Negative[3]	Negative			

[1]negative with moderate mouse clinical signs
[2]negative with severe mouse clinical signs
[3]negative with mild clinical signs

68. The results of this work demonstrate that atypical responses in the MBA occur when either DEE or DCM is used as a secondary solvent. However, it is not possible to establish if the different incidence of atypical responses observed between DEE and DCM used was due to variability in the MBA or the ability of the different solvents to extract shellfish components.

ANNEX C

SUITABILITY OF GASTEC KITS FOR DETERMINATION OF DEE IN AIRSPACE OF SAMPLE EXTRACTS (CEFAS AND DARD)

69. Gastec vapour detection tubes (Anachem Ltd) measure the concentration of DEE vapour using a colorimetric indicator. This system has been developed to measure gaseous DEE concentrations and applications range from occupational hygiene process control, environmental measurement and COSHH assessments.

70. The Gastec kit, which is available in two formats[13], provides a semi-quantitative measure of DEE and is a useful indicator of its presence. The DEE vapour is detected by a colour change in the indicator - potassium dichromate (orange) being converted to reduced chromic sulfate (green). However, other solvents may also react with the indicator as such the results are indicative of the total reducing activity of the sample. The length of the indicator filled tube that changes colour is proportional to the concentration of DEE present. Using a calibration scale printed on the tube, the concentration may be read immediately without the need for further laboratory analysis.

71. A preliminary investigation to assess whether Gastec kits with an LOD of 400ppm could be applied to the detection of DEE from shellfish extracts was carried out by DARD, by assessing the ability of the tubes to measure DEE in the air directly above spiked Tween solutions and a cockle extract (which had shown an atypical response in mice).

    - 1% Tween (4ml) was spiked with 0-20µl aliquots of DEE. The Tween/DEE solutions were tested with Gastec tubes to determine DEE presence after each addition of DEE.

    - Cockle tissue (100g) was extracted using the interim procedure. The airspace above the extract was tested for the presence of DEE using the Gastec to assess whether DEE was carried over from the extraction stage. Following addition of 1% Tween (4ml) to the extract, the extract was spiked with 0-20µl aliquots of DEE.

72. The kits were found to be able to detect DEE in the airspace above solutions of Tween and extracts of cockles prior to administration to mice (Table 5).

73. DARD calibrated the Gastec tubes by adding aliquots of DEE to 5ml of de-ionised water, capping and shaking the vials and measuring the DEE concentration in the airspace above the samples (as described in more detail in paragraph 78). However, since water and DEE do not mix it is considered that the vapour content may depend on the degree of mixing applied to the test sample, therefore this data can only be used to estimate levels of DEE. The results are shown in Table 6 and Figure 1.

[13] Gastec kit catalogue number 161 operates over range 0.04-1.0%, while Gastec kit catalogue number 161L operates over range 10-1200ppm.
Conversion factors: ppm=%x10000.

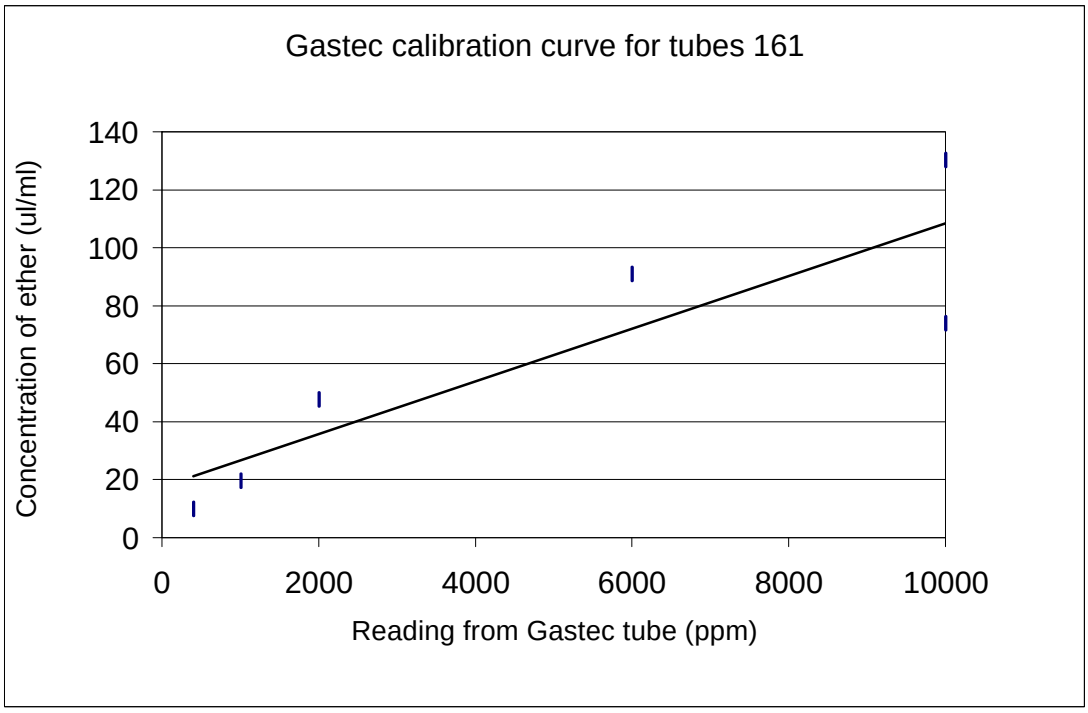
Table 5: Gastec kit (161) analysis of Tween and an atypical cockle extract spiked with DEE by DARD.

Sample	Tween spiked with DEE	Cockle extract spiked with DEE
DEE (㎖/ml)	Gastec estimate of DEE level (ppm)	Gastec estimate of DEE level (ppm)
0	<400	<400
1.25	<400	<400
2.5	400	400
3.75	400-1,000	400-1,000
5	1,000	1,000

Table 6. Calibration of Gastec tubes 161 by DARD using DEE/de-ionised water mixtures.

Volume (㎖) of DEE added to 5ml de-ionised water	Final volume (ml)	DEE concentration in spiked sample (㎖/ml)	DEE concentration as estimated by Gastec (ppm)
50	5.05	9.90	400
100	5.10	19.61	1,000
250	5.25	47.62	2,000
400	5.40	74.07	10,000
500	5.50	90.91	6,000
750	5.75	130.43	>10,000

Figure 1. Calibration curve of Gastec detection tube 161 by DARD



74. CEFAS also calibrated the Gastec kits and constructed calibration curves for both detection tube types (161 and 161L) using a range of Tween extracts spiked with DEE from 0 ìl/ml up to 40 ìl/ml (Table 7).

75. The spiked solutions were prepared by adding 2 ml of Tween to a graduated vial, adding the DEE and making up the volume to 4ml using Tween. The detector tubes were then used to sample the air space at the top of each tube, and the results plotted to give an indication of the response (Figures 2 and 3).

Table 7. Calibration of Gastec tubes 161 and 161L by CEFAS using DEE/Tween mixtures.

Volume of DEE added to Tween (ìl)	Final volume (ml) of Tween/DEE mix	DEE concentration in spiked Tween sample
0	4	0
5	4	1.25
10	4	2.5
30	4	7.5
40	4	10
60	4	15
80	4	20
160	4	40

Figure 2. Calibration curve of Gastec detection tube 161L by CEFAS

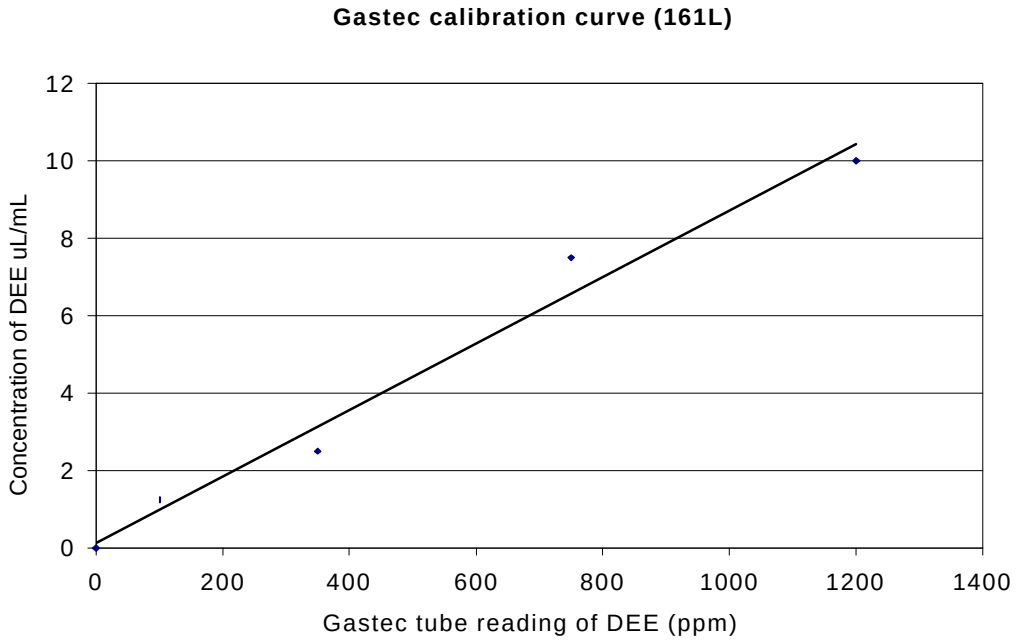
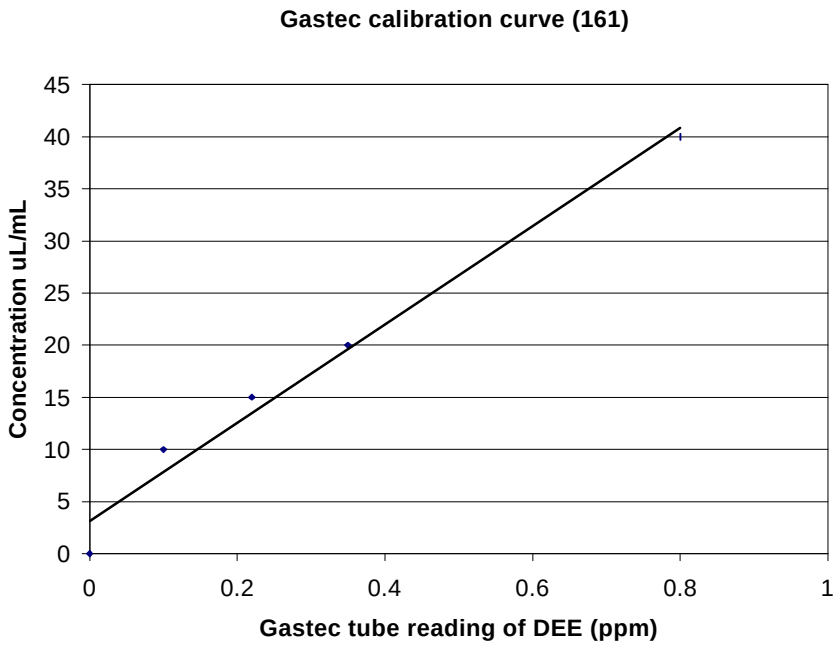


Figure 3. Calibration curve of Gastec detection tube 161 by CEFAS



ANNEX D

MEASUREMENT OF DEE IN THE AIRSPACE ABOVE MONITORING SAMPLES USING GASTEC (DARD AND CEFAS)

76. The Gastec kits have been applied by DARD (kits 161 and 161L) to test routine monitoring samples for the presence of DEE prior to administration to mice.

77. When DEE was judged to have evaporated (by sight and smell), the Gastec tube was attached to the sampling syringe and placed close to the dried extract at the bottom of the flask. The air was sampled and the reading taken when staining of the indicator reagent in the tube stopped (Flask 1). Where a positive reading was obtained, the flask was returned to the rotary evaporator and evaporation continued for 2-5 minutes. Testing was repeated (Flask 2). The shellfish residue was suspended in Tween as detailed in the interim procedure and transferred to glass vials. DEE detection was carried out by placing the sample tube close to the surface of the Tween. Results are shown in Table 8.

Table 8. Results of use of Gastec tubes at DARD to detect DEE in airspace above extracts prepared using the interim arrangements prior to IP injection.

Sample	Date	Species	Site	DEE concentration as estimated by Gastec (ppm)			DSP MBA Result
				Flask 1	Flask 2	Tween	
0305602	18/06/03	Oyster	Carlingford	<400	N/A	<400	Negative
0305603	18/06/03	Oyster	Carlingford	<400	N/A	<400	Negative
0305604	18/06/03	Oyster	Carlingford	<400	N/A	<400	Negative
0305605	18/06/03	Mussel	Foyle	<400	N/A	<400	Negative
0305606	18/06/03	Mussel	Foyle	<400	N/A	<400	Negative
0305949	01/07/03	Oyster	Strangford	<400	N/A	<400	Negative
0306269	08/07/03	Mussel	Larne	<400	N/A	<400	Negative
0306270	08/07/03	Mussel	Larne	<400	N/A	<400	Negative
0306271	08/07/03	Mussel	Larne	<400	N/A	<400	Negative
0306272	08/07/03	Oyster	Larne	<400	N/A	<400	Negative
0306322	09/07/03	Mussel	Belfast	<400	N/A	<400	Negative
0306323	09/07/03	Mussel	Belfast	<400	N/A	<400	Negative
0306494	21/07/03	Cockle	Strangford	400	Negative	400	Negative
0306502	21/07/03	Scallop	Strangford	1000	Negative	<400	Negative
0306503	21/07/03	Mussel	Strangford	400	Negative	Negative	Positive (typical)
0306508	24/07/03	Oyster	Strangford	400	Negative	<400	Negative
0306537	23/07/03	Oyster	Larne	<400	N/A	<400	Negative
0306551	24/07/03	Mussel	Belfast	<400	N/A	<400	Negative
0306553	24/07/03	Cockle	Dundrum	<400	N/A	<400	Negative
Tween*	29/07/03	N/A	N/A	N/A	N/A	<10	N/A
0306747*	29/07/03	Mussel	Strangford	20	N/A	<10	Negative

*Refers to samples analysed with tubes 161L with an LOD=10ppm. All other samples were analysed with tubes161, with an LOD=400ppm.

78. CEFAS also used the Gastec kit (161L) to measure the concentration of DEE in the airspace above extracts suspended in Tween from samples prepared for the routine monitoring programme. Twenty-nine routine samples have been analysed for the presence of DEE in the airspace using the Gastec detection tubes (Table 9). The samples tested consisted of 15 that produced an atypical DSP responses in the MBA and 14 negative extracts. Fifteen of the extracts tested were found to contain DEE levels between 1.8 and 10ìl/mL and 14 extracts were found to contain no DEE. Of the extracts that gave atypical responses, 33% produced a positive reaction in the detector tubes but no DEE could be detected in the remaining 66% of the extracts that gave atypical responses. Of the negative atypical Tween extracts, 71% were found to contain DEE, the remaining 29% 7were found not to contain DEE.

Table 9. Results of DEE screening using Gastec kits 161L (LOD=10ppm) by CEFAS

Sample Number	Sample Site	DSP MBA Result	DEE concentration as estimated by Gastec in headspace (ppm)	Concentration equivalent of DEE (㎖/ml) in extract from calibration curve	Shellfish species
BTX/2003/0513	Henn Point	Atypical	300	2.8	Mussels
BTX/2003/0518	Minnis Bay	Atypical	400	3.5	Cockles
BTX/2003/0522	Zone 1 The wash	Atypical	<10	0	Cockles
BTX/2003/0523	Zone 2 The Wash	Atypical	300	2.8	Cockles
BTX/2003/0524	Zone 3 The Wash	Atypical	<10	0	Cockles
BTX/2003/0544	W Shoebury	Atypical	<10	0	Cockles
BTX/2003/0545	Foulness	Atypical	<10	0	Cockles
BTX/2003/0547	NE Maplin	Atypical	<10	0	Cockles
BTX/2003/0548	Minnis Bay	Atypical	<10	0	Cockles
BTX/2003/0549	Pegwell Bay	Atypical	<10	0	Cockles
BTX/2003/0553	Zone 2 The Wash	Atypical	180	1.8	Cockles
BTX/2003/0554	Zone 3 The Wash	Atypical	1,200	10	Cockles
BTX/2003/0566	Burry NW	Atypical	0	0	Cockles
BTX/2003/0567	Burry SW	Atypical	0	0	Cockles
BTX/2003/0574	Ray Sands	Atypical	0	0	Cockles
BTX/2003/0520	Burry SE	Negative	1,200	10	Cockles
BTX/2003/0525	Caldy Blacks	Negative	300	2.8	Mussels
AT (CSL Tween)	Burry NW	Negative	200	2	Cockles
CT (CSL Tween)	Burry NW	Negative	100	1	Cockles
BTX/2003/0546	Phoenix	Negative	1,200	10	Cockles
BTX/2003/0550	W Kirby	Negative	<10	0	Cockles
BTX/2003/0551	Mostyn Bank	Negative	<10	0	Cockles
BTX/2003/0552	Zone 1 The wash	Negative	<10	0	Cockles

Sample Number	Sample Site	DSP MBA Result	DEE concentration as estimated by Gastec in headspace (ppm)	Concentration equivalent of DEE (ml/ml) in extract from calibration curve	Shellfish species
BTX/2003/0560	Strood Channel	Negative	1,200	10	Native oyster
BTX/2003/0559	Saltmead Ledge	Negative	600	5	Native oyster
BTX/2003/0563	Calamansac	Negative	600	5	Native oyster
BTX/2003/0568	Burry SE	Negative	1,200	10	Mussels
BTX/2003/0570	Waddeton	Negative	0	0	Mussels
BTX/2003/0572	Pyefleet Spit	Negative	400	3.5	Pacific oyster

79. In summary, these results suggest that the presence of DEE is not responsible for the occurrence of atypical responses in the MBA.

ANNEX E

INVESTIGATION BY MACAULAY INSTITUTE, ABERDEEN

FINAL REPORT OF A STUDY FOR

THE FOOD STANDARDS AGENCY SCOTLAND

Investigation of unusual mouse responses to lipophilic extracts of shellfish on application of the UK Interim standard operating procedure at FRS Marine Laboratory

Susan Gallacher*, Joyce Petrie*, Tony Fraser, Alistair Smith****

***FRS Marine Laboratory, Victoria Road, Torry, Aberdeen**
****** The Macaulay Institute, Craigiebuckler, Aberdeen**

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Contents

Executive Summary

The Interim standard operating procedure (SOP) for lipophilic toxins in shellfish was introduced at FRS in June 2003 at the request of the Food Standards Agency. Unusual mouse symptoms were observed after intraperitoneal injection of shellfish extracts prepared following this SOP. Experienced monitoring and veterinary staff noted that the extracts smelled of the solvent diethyl ether (DEE). The mouse reactions were similar to those observed in the early 1990's from DEE carry over in shellfish extracts (monitoring staff) and exposure to sub-optimal anaesthetic levels (veterinary staff). The need for investigative work was identified and a collaborative *ad-hoc* experiment was established between chemists from the Macaulay Institute in Aberdeen and FRS with funding from the Food Standards Agency Scotland.

Gas chromatography with flame ionisation detection comparative analysis demonstrated that DEE was present in the headspace of extracts prepared following both the original FRS SOP and the new Interim SOP in all samples sealed immediately after the final DEE extraction. Samples prepared using the original FRS SOP and left overnight in unsealed flasks at room temperature contained trace levels of DEE in the extract headspace. The data was supported by results obtained from the application of GASTEC commercial kits for the detection of DEE in the air above the extract (headspace).

It was concluded that further research was required to determine why DEE remained in the headspace of shellfish extracts at FRS and whether this occurs at the other UK biotoxin monitoring laboratories. Any SOP used in the extraction procedure for the detection of lipophilic compounds in shellfish monitoring should ensure removal of DEE from extracts.

Introduction

Recommendations for an amended DSP extraction method were agreed at the UK-National Reference Laboratory (NRL) Network meeting on 18 February 2003. At the request of the Food Standards Agency Scotland these recommendations were incorporated by Fisheries Research Services (FRS) into a revised standard operating procedure (SOP). Upon approval by the United Kingdom Accreditation Service (UKAS) this procedure, termed the "Interim SOP", was implemented at FRS in the week commencing 2 June 2003.

Shellfish (mussels and surf clams) prepared by the method described in the Interim SOP (Appendix 1) were tested on 5 June 2003 at FRS and gave mouse reactions which differed from the typical reaction usually observed for okadaic acid and dinophysistoxins. Staff noted that the response in mice was variable for different extracts and ranged from no symptoms, to minor symptoms with the mice recovering, to severe symptoms where the animals were in such obvious distress that they were euthanased. Of particular interest was the onset of these symptoms within 1-2 minutes post injection. Experienced monitoring and veterinary staff noted that many of the extracts smelled of diethyl ether (DEE). The clinical signs presented by the mice were similar to those occasionally observed in the early 1990's from DEE carry-over (monitoring staff) and sub-optimal exposure to anaesthetics (veterinary staff).

Major differences between the original FRS SOP and the Interim SOP can be summarised as follows:

- The Interim method uses 2 x acetone extractions (100 ml each) and 3 DEE extractions (ca. 150 ml in total) compared to the original FRS method which uses 1 x acetone extraction (225 ml) and 2 x DEE extractions (ca. 90 ml in total)

- The ratio of shellfish flesh to solvent differs. The original SOP requires 75 g of shellfish in 225 ml of acetone (i.e. 1:3 ratio) whereas the Interim SOP requires 100 g shellfish to an initial acetone volume of 100 ml (i.e. initial extraction of 1:1 shellfish:acetone). This ratio difference results in a disparity between the percentages of acetone in the initial extract (73% and 54% v/v acetone for the original SOP and Interim SOPs respectively) which will influence the compounds extracted. More lipophilic compounds will be extracted in the higher percentage of acetone.

- Samples were left overnight, at room temperature, after the final DEE extraction prior to the addition of Tween, in the original FRS method. With samples extracted using the Interim method Tween was added immediately after rotary evaporation of the final DEE extract (i.e. in the Interim SOP samples are not left overnight to allow DEE evaporation)

Following discussion of the unusual mouse responses observed at FRS possible reasons were summarised as:

- DEE carry-over
- Uncharacterised substance(s) toxic to mice by intraperitoneal (i.p.) injection
- A combination of both of the above

The need for investigative work was identified and a collaborative experiment was established in July 2003 between chemists from the Macaulay Institute (MI) in Aberdeen and FRS with funding from the Food Standards Agency Scotland.

Objective

The objective of this *ad hoc* experiment was to determine if DEE remained in shellfish samples extracted using the methods described in the original FRS SOP and the Interim SOP. Methods applied for DEE detection included gas chromatography with flame ionisation detection (GC-FID), Fourier Transform infra-red spectroscopy (FTIR) and the commercial GASTEC tubes.

Material and methods

Samples used

Two separate batches of shellfish farm mussels were obtained from Loch Etive and Dornoch and scallops from an off-shore location on the west coast of Scotland (Box H8). The mussels were transported to FRS by first class post and scallops by courier (as in FRS' standard procedure). Samples collected less than two weeks previously from the mussel sites were negative by mouse bioassay using the original FRS extraction procedure. Upon arrival at FRS the three batches of shellfish were immediately shucked and extracted as detailed below.

Extraction procedure

Two 100 g aliquots of each mussel and scallop sample were processed using the Interim SOP 2247 (Appendix 1). A further two 75 g aliquots of each mussel batch and the scallop batch were processed using the original FRS extraction method as detailed in SOP 2245 (Appendix 2). Variations to the treatment of the samples after the final DEE extraction are described below:

- One flask from each shellfish sample extracted by the original FRS SOP was left open to the air, in a fume hood overnight, at room temperature after the final DEE extraction. The flasks were subsequently transported to MI unsealed with approximately 36 hours elapsing between extraction and analysis.

- One flask, from each shellfish sample extracted by the original FRS SOP, was sealed immediately after the final DEE extraction using a ground glass stopper.

- One flask, from each shellfish sample extracted by the Interim SOP, was sealed immediately after the final DEE extraction using a ground glass stopper.

- After the final DEE extraction one flask from each shellfish sample extracted by the Interim SOP was flushed with nitrogen overnight. The flasks were subsequently transported to MI unsealed with approximately 36 hours elapsing between extraction and analysis.

Extraction of shellfish samples was undertaken on 8 July 2003 by FRS staff with the samples transported by car at ambient temperature to the MI on 9 July. These were

subsequently stored at room temperature before analysis on 10 July 2003. The samples were coded by FRS and therefore analysis at MI was undertaken “blind.”

GC-FID

GC-FID conditions were established (30 m x 0.25 mm id fused silca column coated with SE30 (0.25 µm thickness); Temperature 28 °C; splitless injection) such that DEE was eluted from the column with a retention time of about 3 minutes. The headspace gas from each of the flasks was sampled using a 5 µl syringe and that volume was injected on to the GC column. GC was used solely for comparative purposes and no attempt was made to construct calibration curves or obtain quantitative data. Comparative data were based on the detector response (µV)/unit volume of headspace injected on to the GC column.

GASTEC

GASTEC tubes with a measurement range of 400-10,000 ppm (Anachem Tube no. 161) and 10-1200 ppm (Tube no. 161L) were used in accordance with the manufacturer’s instructions with the pump set for sampling 50 ml of headspace.

FTIR

Approximately 1-2 ml aliquots from all of the shellfish extracts were transferred to the trough plate of a horizontal attenuated total reflectance (HATR) accessory in the form of a thin film and infrared (IR) spectra were recorded using a Nicolet Magna-IR 550 Fourier Transform infrared spectrometer in accordance with the UKAS accredited: test method I002

Further processing of samples at the MI

At MI, one of the samples (obtained using the Interim SOP and sealed immediately after the final DEE extraction) was rotary evaporated at a temperature of 48 °C for 3 minutes, sealed and heated to 48 °C for a further 2 minutes (to drive any remaining DEE in the extract into the headspace) at which point DEE was measured in the sample headspace.

Subsequently, the sample was further processed by dissolving it in DEE and filtering through sodium sulphate (~5 g) to remove water. The filtrate was evaporated to dryness and after stoppering the flask and heating (as described above) the headspace gases were analysed for the presence of DEE.

A further sample(obtained using the original SOP and sealed immediately after the final DEE extraction) was subjected to water removal using sodium sulphate as described above and analysed for DEE in the headspace.

Results

Visual appearance and odour

Visual examination showed that each shellfish extract contained water (immiscibility with the extracted oil). A strong smell of DEE was noted in those flasks sealed

immediately after rotary evaporation (Table 1). Unsealed flasks did not smell of DEE (Tables 2 and 3).

FTIR

DEE was not detected in any of the shellfish extracts upon analysis by FTIR probably due to the inherent lack of sensitivity of the technique for solid/liquid phase samples. (Typical IR limits for solid/liquid organic samples are approximately 1-5% weight basis). The FTIR technique is more sensitive for samples in the gas phase (T. Fraser pers. comm) but transfer of headspace gas to an appropriate gas cell was anticipated to be problematic. As there was no guarantee that the limited IR sensitivity would allow detection of DEE analysis of headspace this method was not pursued.

However, significant amounts of water were detected in all of the shellfish extracts by FTIR. Examples of the occurrence of water in the IR spectra for two samples extracted by the original and Interim SOPs are shown on Figure 1. Full quantification of the water content was not feasible as the heterogeneous nature of the extract (a mixture of an oil-type liquid and water) made it impossible to remove an aliquot (1-2 ml) which was truly representative of the whole extract.

GC-FID and GASTEC results

Results from GC-FID analysis are summarised in Table 1-4 and a typical chromatogram shown on Figure 2. DEE was detected in the headspace of all three shellfish extracts from flasks sealed immediately after the final DEE extraction with both the original FRS and Interim SOP. GC-FID responses for the DEE peak ranged between 680-980 µV (Table 1). Using the GASTEC tubes the concentration of DEE in the headspace of these samples was estimated as >10,000 ppm. There was no evidence to indicate that the extracts produced using the Interim SOP contained more DEE in the headspace than those extracts from the original FRS SOP (Table 1). It should be noted however that no attempt was made to carry out GC analysis on a quantitative basis (i.e. construction of calibration curves etc).
Conversely, shellfish extracts left unsealed in the fume-hood overnight (Table 2) and those flushed with nitrogen overnight (Table 3) did not smell of DEE and the GASTEC tubes gave colour changes corresponding to <10 ppm DEE. GC-FID analysis of the headspace gave µV readings between 0.3-0.8 (Tables 2 and 3). However, as the flasks were open to the atmosphere for approximately 36 hours (i.e time between sample extraction, being left overnight and analysis at MI) responses from the GASTEC tubes may be due to compound(s) other than DEE.

Additional processing of the extracts at MI

After further rotary evaporation at MI of one of the samples obtained using the Interim SOP (sealed immediately after the final DEE extraction) the GC response reduced to 2.3 µV from the initial value of 820 µV. This response was further decreased to 0.8 µV (Table 4) when the extract was taken up in DEE, filtered through sodium sulphate and the DEE removed by rotary evaporation.

The GC response for a sample obtained using the original SOP (sealed immediately after the final DEE extraction) reduced from the initial value of 820 µV to 1µV (Table 4) after the extract was taken up in DEE and filtered through sodium sulphate as above.

GASTEC analyses of both samples after the additional treatments described above gave only a trace of colour.

It was noted that the 0.8 and 1 µV responses by GC-FID were no more than a “blip” on the baseline of the chromatogram and any colour change on the GASTEC tubes may have been due to some other reducing compound in the headspace gas.

Tables and Figures

Table 1: GC-FID, GASTEC and sensory results for headspace analysis of shellfish extracts sealed immediately after the final DEE extraction

Shellfish	SOP used	GC-FID peak height*** (uV)	GASTEC result (ppm)	Smell of DEE
LE mussel	Original*	980	>10,000	+
	Interim**	680	>10,000	+
D mussel	Original*	820	>10,000	+
	Interim**	820	>10,000	+
Scallop	Original*	730	>10,000	+
	Interim**	680	>10,000	+

*Original refers to FRS SOP number 2245
**Interim refers to FRS SOP number 2247
***Indicative values as calibration curves were not prepared and estimation of uncertainty of measurement was not carried out
Shellfish source: LE is Loch Etive; D is Dornoch
+ DEE detected; -DEE not detected

Table 2: GC-FID, GASTEC and sensory results for headspace analysis of shellfish extracts prepared using the original FRS SOP and left in the fume hood overnight after the final DEE extraction

Shellfish	SOP used	GC-FID peak height** (uV)	GASTEC result (ppm)	Smell of DEE
LE mussel	Original*	0.3	<10	-
D mussel	Original*	0.4	<10	-
Scallop	Original*	0.6	<10	-

*Original refers to FRS SOP number 2245
**Indicative values as calibration curves were not prepared and estimation of uncertainty of measurement was not carried out
Shellfish source: LE is Loch Etive; D is Dornoch
+ DEE detected; -DEE not detected

Table 3: GC-FID, GASTEC and sensory results for headspace analysis of shellfish extracts prepared using the Interim SOP and flushed with nitrogen overnight after the final DEE extraction

Shellfish	SOP used*	GC-FID peak height**(uV)	GASTEC result (ppm)	Smell of DEE
LE mussel	Interim	0.8	<10	-
D mussel	Interim	0.6	<10	-
scallop	Interim	0.5	<10	-

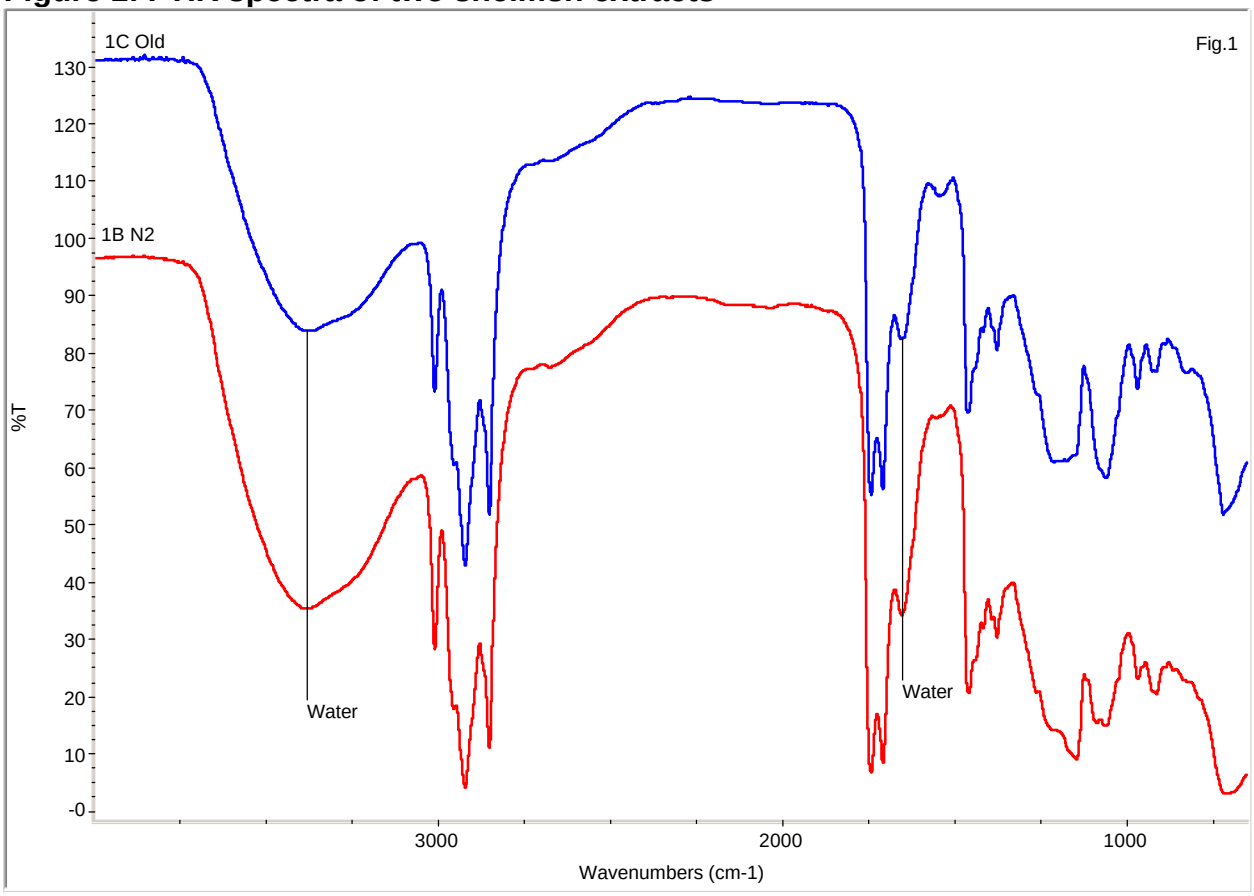
*Interim refers to FRS SOP number 2247
**Indicative values as calibration curves were not prepared and estimation of uncertainty of measurement was not carried out
Shellfish source: LE is Loch Etive; D is Dornoch
+ DEE detected; -DEE not detected

Table 4: GC-FID, GASTEC and sensory results for headspace analysis of mussel extracts after further processing at MI compared to the original FRS preparation

SOP used	Process	GC-FID peak height*** (uV)	GASTEC result (ppm)	Smell of DEE
Original*	Sealed immediately at FRS	820	>10,000	+
	Sodium sulphate treated at MI	1	40	-
Interim**	Sealed immediately at FRS	820	>10,000	+
	Further evaporation at MI	2.3	40	Faint
	Sodium sulphate treated at MI	0.8	<10	-

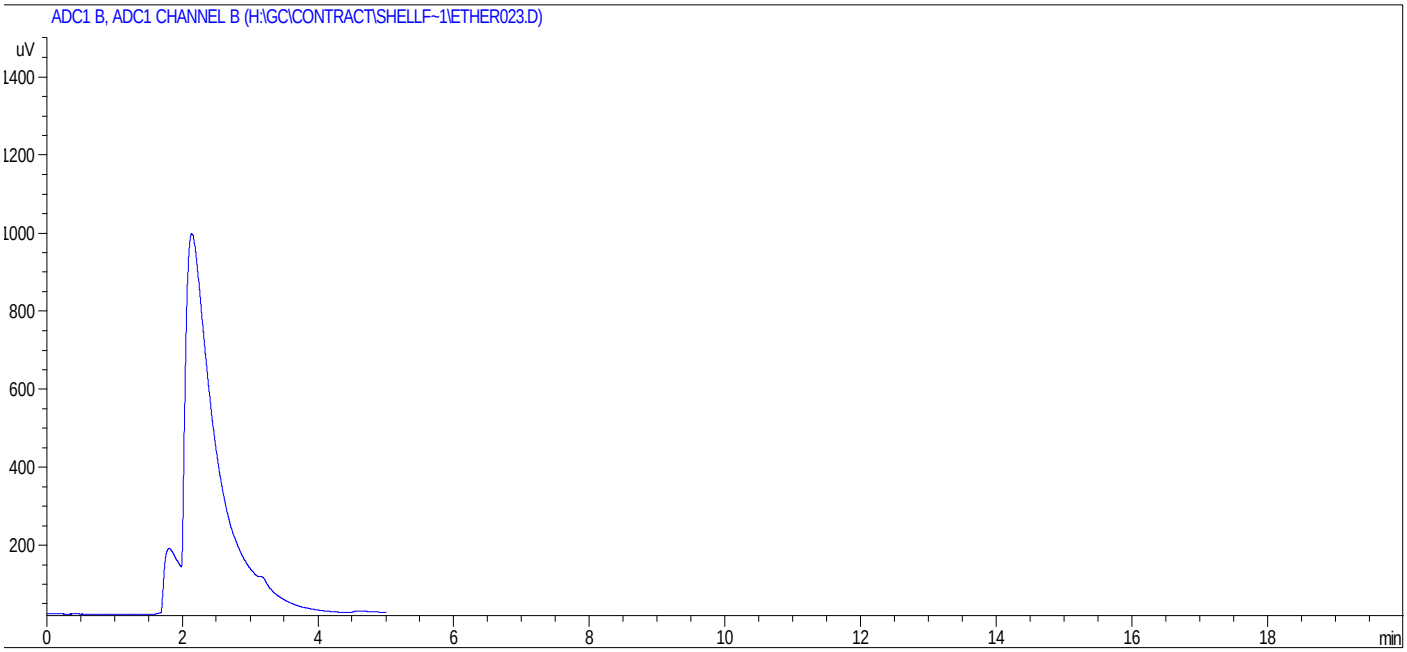
*Original refers to FRS SOP number 2245;
**Interim refers to FRS SOP number 2247;
***Indicative values as calibration curves were not prepared and estimation of uncertainty of measurement was not carried out
+ DEE detected; -DEE not detected

Figure 1: FTIR spectra of two shellfish extracts



Original SOP-blue line; Interim SOP-red line

Figure 2: Gas chromatogram of DEE



Discussion

Using GC-FID comparative analysis this *ad-hoc* study[14] demonstrated that DEE was present in the headspace of shellfish extracts, sealed immediately after the final DEE extraction, using both the original FRS and Interim extraction SOPs. The data was supported by results obtained using the commercial GASTEC tubes for the detection of DEE.

It was hypothesised that the presence of DEE in the extract headspace may be due to:

- Incomplete separation of the organic and aqueous phases during the extraction procedure
- Incomplete removal of solvent by rotary evaporation
- The presence of water in the extracts which may act as a "sink" for the DEE.

The extraction method should be examined in detail to ensure removal of DEE from the extracts. Preliminary data from this study would suggest that the inclusion of a step involving filtration through sodium sulphate removed water from the extract and vastly reduced the quantity of residual headspace DEE.[15]

It would be beneficial to determine if the results from this study can be reproduced by other researchers using quantitative GC-MS analysis and also if DEE remains in shellfish extracts from other UK biotoxin monitoring laboratories[16]. Since it is not currently feasible to measure DEE directly in the extract further studies would again require headspace measurements, which by definition, cannot give a direct quantitative measure of the amount of DEE in the actual extract. Careful consideration would therefore be required to define the conditions to drive DEE from the extract into the headspace in order to achieve an equilibrium between the liquid and gas phases.

The Sigma chemical data sheet on DEE (July 2003) lists the central nervous system (CNS) as a target organ for adverse effects of DEE. However, little toxicology data is available in the literature on the effect of DEE in mice after i.p. exposure. DEE in sample the headspace, as found in this study, infers that the solvent was present in the shellfish extracts which were sealed immediately after the final rotary evaporation stage of both the original and Interim SOP. This may provide some circumstantial evidence that DEE contamination of shellfish extracts could have been a possible cause of the unusual mouse symptoms observed at FRS when the Interim SOP was introduced in June 2003. An experiment to assist in proving/disproving this hypothesis may involve modifying the extraction procedure to ensure total removal of DEE from shellfish extracts followed by observation of mouse reactions upon exposure to these extracts. This type of approach is ethically preferable to experiments involving direct exposure of mice to DEE.
It is also important to note that in samples prepared using the Interim SOP, at FRS in June 2003, the intensity of the unusual clinical signs in mice varied. If DEE was

[14] This study was undertaken during July 2003
[15] Subsequent work carried out on behalf of FSA UK at the Central Science Laboratory (CSL) on the extraction method supports this finding.
[16] CSL has subsequently been commissioned by FSA UK to investigate the presence of acetone and DEE using GC-MS in shellfish extracts in samples prepared at the UK monitoring laboratories.

responsible for these responses the results would suggest varying levels of DEE in the extracts. This could be examined by conducting a quantitative study as mentioned above. Factors which in theory could influence the quantity of DEE remaining in the extract include:

- Inter/intra shellfish species differences as the consistency of shellfish matrices including their water/lipid content may vary depending on their spawning condition
- Differences in the reproducibility of DEE carry-over on replicate extractions of the same sample

Therefore it is feasible that DEE was trapped to varying extent in the shellfish depending on the matrix and/or slight variations in the application of individual extractions. These factors should be considered when designing future work.

Conclusions

From observations made by experienced monitoring and veterinary staff and from the outcome of the experiment undertaken at MI the following conclusions can be made:

- DEE was present in the headspace of shellfish extracts sealed immediately after the final DEE extraction using both the original FRS and Interim extraction SOPs.

- Further research is required to determine why DEE remained in the headspace of shellfish extracts at FRS and whether this occurs at the other UK monitoring laboratories.

- The extraction procedure for the detection of lipophilic compounds by mouse bioassay should ensure removal of DEE from shellfish extracts.

- Future experiments should examine the reproducibility in the quantity of trapped DEE upon replicate extraction of the same sample and different shellfish samples.[17]

Acknowledgements

This work was funded by FSAS. The authors would like to thank FRS monitoring staff for the preparation of samples and Dr. E.A. Smith, Dr. C. Moffat, Ms. L.Stobo, FSAS and FSA UK for editorial comment.

References

SOP 2247: Extraction of DSP toxin from shellfish tissue for bioassay (UK NRL method)

SOP 2245: Extraction of DSP toxin from shellfish tissue for bioassay

Sigma-Aldrich: Chemical information sheet Diethyl ether (product no. 31700; valid 05/2003-07/2003)

Appendix 1: SOP for the interim DSP extraction method

[17] This work has subsequently been commissioned by FSA UK and is reported separately.

Fisheries Research Services

SOP 2247 (Version 9)

Extraction of DSP toxin from shellfish tissue for bioassay (UK NRL method)

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 (SOP 2220)
distilled water

5. Equipment

disposable gloves
calibrated balance
wide necked screw capped bottles
narrow necked screw capped bottles
113V 100cm fluted filter paper
large plastic funnel
graduated tubes
100ml measuring cylinder
disposable pastette
water bath
rotary evaporator
glass flask 100ml and 500ml
conical flask
separating funnel + stopper
cork ring
clamp stand
Ultra-Turrax ®
calibrated dispenser
calibrated timer
sonicator

6. Environmental Control

Not applicable

7. Interferences

Not applicable

8. Sampling and Sample Preparation

Shellfish samples are prepared as per SOP 2215 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 (Rowett 4). Each further stage is also recorded and initialled on this record sheet

9.1 To 100g ± 1g tissue in screw capped bottle add 100ml ± 5ml acetone using dispenser pump on Winchester bottle

9.2 Homogenise using Ultra-Turrax ® at speed setting 3 (13500rpm) 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 resuspend in a further 100ml ± 5ml 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, 500ml, glass, round bottomed flask and evaporate off acetone (SOP 2230). 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 100ml 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 100ml glass round bottomed flask and evaporate (SOP 2230) until dry + 5-10 mins.

9.13 Using a disposable pipette add 3ml Tween 60 (SOP 2220) 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 fridge 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. See risk assessment RL2

14. Literature References

Yasumoto *et al*., Diarrhetic Shellfish Poisoning, American Chemical Society, 1984, pp207-214.
Yasumoto *et al.,* Occurrence of a New Type of Shellfish Poisoning in the Tohoku District, *Bull. Japan. Soc .Sci. Fish.,* **44**(11), 1978, pp1249-1255

Appendix 2: SOP for the original FRS DSP extraction Method

Fisheries Research Services

SOP 2245 (Version 10)

Extraction of DSP toxin from shellfish tissue 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 (SOP 2220)
distilled water

5. Equipment

disposable gloves
calibrated balance
wide necked screw capped bottles
narrow necked screw capped bottles
113V 100cm fluted filter paper
large plastic funnel
universal tubes
50ml measuring cylinder
disposable pastette
water bath
rotary evaporator
glass flask 100ml and 500ml
conical flask
separating funnel + stopper
cork ring
clamp stand
Ultra-Turrax ®
calibrated dispenser
calibrated timer
sonicator

7. Environmental Control

Not applicable

7. Interferences

Not applicable

10. Sampling and Sample Preparation

Shellfish samples are prepared as per SOP 2215 and an aliquot is received in a wide necked screw capped bottle labelled with sample number.

11. Analytical Procedure

Before starting the extraction, the sample number is recorded on record sheet (Rowett 4). Each further stage is also recorded and initialled on this record sheet

9.1 To 75g ± 1g tissue in screw capped bottle add 225ml ± 5ml 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 filtrate into labelled, 500ml, glass, round bottomed flask and evaporate off acetone (SOP 2230). 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 50ml 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 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 9.10. 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 (SOP 2230) until dry + 5-10 mins. Flask may be left overnight in fume cupboard to ensure thoroughly dry.

9.12 Using a disposable pipette add 3ml ± 10% Tween 60 (SOP 2220) 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. See risk assessment RL2

14. Literature References

Yasumoto *et al*., Diarrhetic Shellfish Poisoning, American Chemical Society, 1984, pp207-214.
Yasumoto *et al.,* Occurrence of a New Type of Shellfish Poisoning in the Tohoku District, *Bull. Japan. Soc .Sci. Fish.,* **44**(11), 1978, pp1249-1255

ANNEX F

F.1 PROTOCOL FOR THE MEASUREMENT OF DEE AND ACETONE IN SAMPLE EXTRACTS FROM EACH LABORATORY BY GC-MS HEADSPACE ANALYSIS

1. Objective

1.1 To determine if diethyl ether (DEE) and acetone are present in Tween extracts of UK shellfish monitoring samples.

2. Outline

2.1 FRS, CEFAS and DARD have noted the presence of DEE in shellfish extracts to varying extents.
2.2 In order to examine the frequency of occurrence and quantity of DEE in the headspace of routine shellfish extracts, at the different Institutes, each monitoring laboratory will send a total of 45 extracts prepared using the SOP currently in use at their laboratory to the CSL over a 3 week period (15/week).
2.3 The samples will be analysed for DEE using headspace-gas chromatography-mass spectrometry (HS-GC-MS)
2.4 Mouse bioassay (MBA) results for the samples will also be recorded
2.5 At this point of time FRS do not use the Interim arrangements. In order to provide data for comparison with the other laboratories FRS will prepare parallel samples using the Interim arrangements for submission to the CSL
2.6 Semi-quantitative data on DEE concentrations in the headspace of the these shellfish extracts, as determined with the Gastec 161L kit, will also be assessed by each monitoring laboratory
2.7 In order to provide further information on the potential of solvents to contaminate the shellfish extracts the levels of acetone in the shellfish extract's headspace will also be measured by HS-GS-MS
2.8 An overview of the experiment is given in Figure 4.

The data will provide the following information

2.9 Determine if DEE and/or acetone is present in the headspace of shellfish extracts, resuspended in Tween, in monitoring samples prepared at the 3 monitoring laboratories.
2.10 Determine if significant variations in DEE concentrations in the headspace of shellfish extracts, resuspended in Tween, exist at the 3 monitoring laboratories when the Interim arrangements are used.
2.11 Provide a comparison of the quantity of DEE present in shellfish extract headspace in samples prepared using FRS' current method and the interim arrangements.
2.12 Data on the concentration of DEE and acetone in shellfish extract headspace in relation to MBA responses.
2.13 A comparison of DEE concentrations determined by Gastec 161L (performed by the monitoring laboratories) and HS-GC-MS performed by CSL

3. Participants

Monitoring laboratories:	CEFAS, DARD and FRS
Chemical analysis:	CSL
Advice/co-ordination:	UK-NRL/FSA
Statistics:	FSA
Report writing:	CSL/FSA

4. Methodology

4.1 Target shellfish species

Mussels (*Mytilus edulis*) and cockles (*Cerastoderma edule*).

4.2 Supply and storage of shellfish

For the purpose of this experiment samples should be prepared from shellfish obtained from the routine monitoring programme at each laboratory. The shellfish should be stored, if required, as per the normal practise at each laboratory.

4.3 Preparation of GC vials, standards and stock solutions at the monitoring laboratories

4.3.1 Vial preparation and capping procedure:

Vials, caps and septa must be handled using laboratory gloves (see Note 1). The septa are PTFE-lined butyl rubber and must be placed grey side (PTFE) down so that the blue face is visible through the hole in the cap. Caps must be crimped by applying the crimping tool using a steady even pressure so that the cap does not turn freely after crimping.

4.3.2 Internal standard solution preparation

Carefully break the ampoule containing the d^{10}-DEE along the score line and immediately transfer the contents to a small (1-2 ml) glass vial fitted with a screw cap using a Pasteur pipette. Seal the vial cap with parafilm and store in an upright position at –20°C until required. Before use, allow the vial to attain ambient temperature.

4.3.3 d^{10}-DEE in Tween stock internal standard solution

Using a calibrated pipette, add 9.5 ml 1% Tween solution to a preweighed glass screw cap vial (with a maximum volume of 20 ml) and record the weight to 0.0001g in Form 1. Using a calibrated pipette (or microlitre syringe), add 500 µl of d^{10} DEE solution to the vial, immediately replace cap and record the weight to 0.0001g in Form 1. Seal vial cap with parafilm and store in an upright position at –20°C until required. Shake thoroughly before use.

4.3.4 d^{10}-DEE in Tween working internal standard solution

Using a calibrated pipette, add 9.0 ml 1% Tween solution to a glass screw cap vial (with a maximum volume of 20 ml) and add 1.0 ml of d^{10} DEE in Tween stock solution (5.3.3) to the vial, immediately replace the cap and mix thoroughly. Seal vial cap with parafilm and store in an upright position at –20°C until required. Shake thoroughly before use.

4.3.5 DEE/acetone in Tween stock spiking solution

Prepare a solution of DEE and acetone in 1% Tween using the procedure given in 5.3.3, by adding 500 µl each of DEE and acetone to 9.0 ml of 1% Tween. Prepare fresh on day of use. *Note: Volumes used in the preparation of this solution may be adjusted as the experiment progresses depending on the data generated*. *CSL will inform participants of any amendments.*

4.3.6 DEE/acetone in Tween working spiking solution

Using a calibrated pipette, add 9.0 ml 1% Tween solution to a glass screw cap vial (with a maximum volume of 20 ml) and add 1.0 ml of DEE/acetone in Tween stock spiking solution (5.3.5) to the vial, immediately replace the cap and mix thoroughly. Seal vial cap with parafilm and store in an upright position at –20°C until required. Shake thoroughly before use.

Note 1: CSL experience with HS-GC-MS analysis of volatiles at ppb levels has shown that vial rinsing is not necessary. Analysis of empty vials and vials containing only Tween solution has shown no significant interference arising from the vials, caps or septa if handled in the prescribed way.

4.4 Homogenate preparation at the monitoring laboratories

4.4.1 Location

If possible, steps should be taken to maximise the distance between the areas in which shellfish extraction and cleanup are carried out, and where sample extracts are prepared for HS-GC-MS analysis in order to prevent adventitious contamination from airborne solvent(s).

4.4.2 Preparation of shellfish extracts

Forty five shellfish samples from each laboratory (15/week) should be extracted as detailed in the SOPs listed in Table 2. Add 1 % Tween to the shellfish extract after the DEE stage as detailed in the individual SOPs (Note: FRS will extract the samples using both their current SOP and the interim arrangements)

Three variations to the normal procedure involve recording additional data as detailed below:

a) record the volume of solution remaining after the removal of acetone by rotary evaporation on Form 2
b) Weigh the empty flask prior to the rotary evaporation DEE stage and after rotary evaporation and hence calculate the weight of the residue. Record weights on Form 2

c) Record the weight of Tween added to the residue after the DEE rotary evaporation stage on Form 2.

SOPS to be applied at each laboratory

Laboratory	SOP Title	SOP no	Version
CEFAS	DSP Extraction from Shellfish	12	9
DARD	Mouse bioassay for the detection of DSP	212	5
FRS (I)	Extraction of DSP toxin from shellfish tissues for bioassay	2245	10
FRS (2)	Extraction of DSP toxin from shellfish tissues for bioassay (UK NRL method)	2247	9

4.4.3 Preparation of extracts for shipment to CSL

4.4.3.1 Place the GC vial and cap on the balance and pipette 150 µl of well-mixed shellfish extract (in 1% Tween) into the vial and record the weight of the extract on Form 2.

4.4.3.2 While the GC vial and cap remains on the balance, using a calibrated pipette, add 50 µl of d^{10}-DEE working internal standard solution (5.3.4) to the vial, crimp the cap securely (unless a spike is to be prepared) and reweigh. Record weight on Form 2. Swirl the vial gently to mix contents.

4.4.3.3 Repeat steps 5.4.3.1 and 5.4.3.2 a further 2 times to produce 3 aliquots of the shellfish extract spiked with the internal standard in separate GC vials.

4.4.3.4 Store the samples in an upright position at –20°C immediately prior to shipment to CSL.

4.4.3.5.Ideally samples should be sent by Wednesday evening of each week at the latest.

4.5 Gastec measurements

Measure the DEE concentration in the headspace of the remaining Tween extract by holding the GASTEC 161L tube as close as possible to the extract surface whilst avoiding the possibility of drawing liquid into the tube. Follow the manufacturers

instructions for operation of the kit and use two pump strokes set at a sample volume of 100 ml. Record the result on Form 3.

4.5 Mouse bioassay

Conduct the MBA assay on the remaining Tween extract in accordance with each laboratory's SOPs (FRS will do the MBA on samples from their current SOP only). Record the results on Form 3.

4.6 Preparation of spikes, blanks and controls at monitoring laboratories

An analytical batch is defined as the number of samples each laboratory can process on any given day. A reagent blank and duplicate spiked samples should be included with every analytical batch by the monitoring laboratories. All extracts, internal standards and spike solutions must be added to the vial and the vial crimped **before** proceeding to the next vial, otherwise analyte losses will be incurred.

4.6.1 The reagent blank

For each analytical batch the reagent blank should consist of 75 ml of water substituted for the shellfish sample and taken through the extraction procedure with resuspension in Tween as detailed in each laboratories SOP's (Table 2)

4.6.2 The DEE / acetone spike

For each analytical batch a single shellfish sample is extracted and resuspended in Tween as detailed in section 4.4.2 and 4.4.3, in accordance with each laboratories own SOPs. Aliquots of 150 µl of the Tween extract is added to six vials. Three of these vials are then spiked with DEE and acetone at levels chosen by the participant but unknown to the CSL. The spike volume can be between 50-200 µl of the spiking solution (see 5.3.6). All volumes and weights should be recorded on Form 4. The DEE/acetone spike solution should be added immediately following addition and weighing of the internal standard solution. Add the required volume of spike solution (5.3.4) to the vial using a calibrated pipette, crimp the cap securely and reweigh. Record weight on Form 4. Swirl the vial gently to mix contents. *Note: Volumes used in the preparation of the spike may be adjusted as the experiment progresses depending on the data generated*. *CSL will inform participants of any amendments*

4.6.3 Hygiene check

An empty crimped vial and a vial containing 1% Tween solution only should be included with the first batch of samples.

4.6.4 Solvent integrity check

A small aliquot (3-4ml) of both the acetone and the DEE used in the extraction procedure should be placed in separate vials, sealed using the crimper and sent with the first batch of samples.

Note 2: Preliminary experiments at CSL have shown that DEE readily disperses in Tween solution and is stable enough for use as a spiking solution (or as an internal standard for d^{10}-DEE).

4.7 HS-GC-MS analysis by CSL

Analysis of samples by HS-GC-MS will be conducted at CSL as detailed in the SOP: *Headspace GC-MS determination of diethyl ether and acetone residues in shellfish extracts intended for mouse bioassay*

5. Summary of Information to be recorded by the monitoring laboratories

Four forms should be completed by the monitoring laboratories and submitted to the FSA. These should provide the following detail:

Form 1: Internal standard solution
- Weight of Tween
- Weight of d^{10}-DEE

Form 2: Weight of material at various stages in the sample preparation
- Volume of solution remaining after the removal of acetone by rotary evaporation
- Weight of residue after DEE rotary evaporation
- Weight of Tween added to the residue
- Weight of extract added to GC vial
- Weight of internal standard solution added to GC vial

Form 3: Mouse bioassay and GASTEC data
- Sample no
- Species
- Date extraction
- Extraction operator
- Date MBA conducted
- MBA operator
- No mice used
- MBA symptoms
- MBA end-point
- MBA result issued
- GASTEC result

Form 4: Preparation of acetone/DEE spike
- Volume of DEE and acetone spike solution added
- Weight of DEE and acetone spike solution added

6. Materials required by the laboratories, which are additional to routine monitoring requirements

GC vials (to be supplied by CSL)
GC vial caps (to be supplied by CSL)

GC vial septa (to be supplied by CSL)
crimper device (to be supplied by monitoring laboratories)
Internal standard d^{10}-DEE (to be supplied by CSL)
GASTEC 161 L tubes (to be purchased by monitoring laboratories)

7. Transport of samples from the monitoring laboratories to The CSL

All vials containing material from experiment 1 should be sealed in an upright position in a tamper proof insulated container. Samples should be kept as cold as possible during transit using dry ice or freezer blocks/bags. Temperature loggers (or min/max thermometers) should be inserted into the package next to the vials. Containers should be marked clearly that they should be maintained in an upright position and stored on receipt at –20 °C. If feasible same day delivery of samples by courier should be used but if not possible then overnight delivery. A list of unique sample codes should be provided with the package. Despatch notes should be retained by the monitoring laboratories. The package should be addressed to:

Room 10GA08
DEFRA Central Science Laboratory
Sand Hutton
York
YO41 1LZ

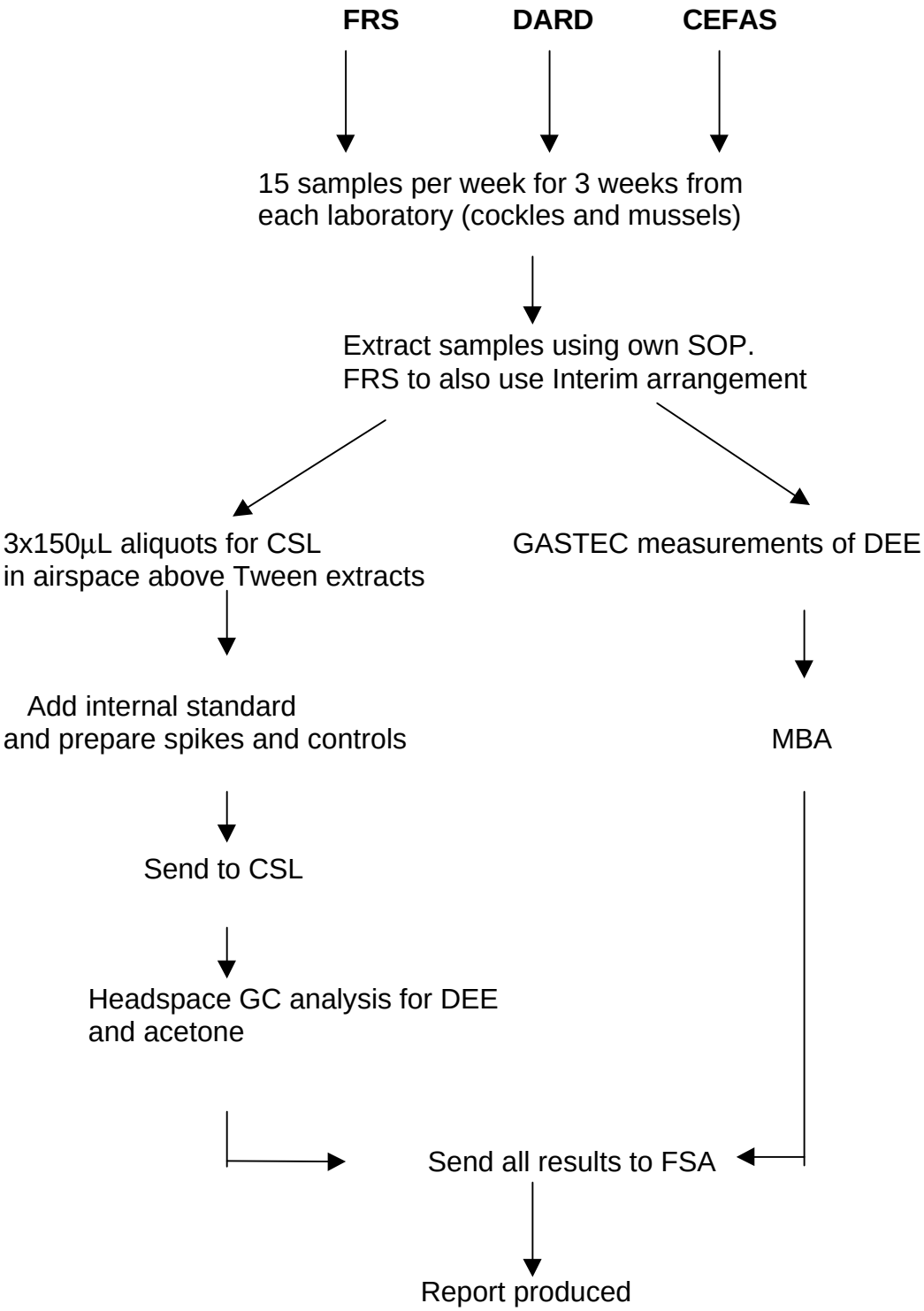
Please notify CSL by phone or e-mail prior to dispatch:

Samples can be received at CSL outwith 9am-5pm. If this is likely to occur please advise CSL as soon as possible.

8. Analysis of results and production of report

CEFAS, FRS and DARD should submit Form 1, 2, 3 and 4 to the FSA at the end of each sampling week (Table 1). CSL should submit their DEE and acetone HS-GC-MS results to FSA at the end of each sampling week (Table 1), including information on blanks/spikes and calibration curves. FSA will circulate a summary of the data to the monitoring laboratories, CSL and NRL weekly (Table 1). CSL will complete a draft report for comment on the same day by the monitoring laboratories, UK-NRL and FSA. CSL/FSA will undertake revisions of the report with FSA issuing the final report.

Figure 4: Outline of Experiment



F.2 HEADSPACE GC-MS DETERMINATION OF DIETHYL ETHER AND ACETONE RESIDUES IN SHELLFISH EXTRACTS INTENDED FOR MOUSE BIOASSAY - ABSTRACT

Scope and field of application

This standard operating procedure (SOP) has been developed from a CSL SOP for the determination of butadiene in foodstuffs, which itself has been developed from a current European Standard (CEN) HS-GC-FID method for butadiene in food contact materials and uses GC-MS as a confirmatory procedure. The procedure has been developed to address the uncertainties relating to the presence of solvent residues of diethyl ether (DEE) and acetone in extracts of shellfish intended for testing using the mouse bioassay (MBA) DSP test, and is applicable to extracts obtained from shelled bivalve molluscs (cockles and mussels).

Principle

Shellfish extract is dispersed in 1% Tween 60 solution and a 150 ìl aliquot placed in a headspace vial to which 50 ìl internal standard (d_{10}-DEE in 1% Tween 60) is added. The sealed vial is heated at 60°C for 5 minutes with agitation, and a 250 ìl aliquot (1:25 injector split ratio) of the headspace is analysed for DEE and acetone by capillary gas chromatography with mass spectrometric detection.

Quality assurance and calibration

The retention times of the analyte peaks are verified by analysis of the high calibration standard with confirmatory analysis by reference to Wiley library ion fragmentation patterns. The relative retention time (RRT) of analytes in sample extracts should agree to +- 5% of that obtained from standards prepared in the absence of sample extract. The ion (m/z) abundance ratios (%) used for analyte confirmation are:

DEE:	74 (41), 59 (68)
d_{10}-DEE:	84 (49), 66 (87)
Acetone	58 (15), 43 (100)

The ion ratios should agree to within +- 25% of that obtained from standards prepared in the absence of sample extract.

A series of DEE/acetone calibration solutions between 0 and *ca*. 4000 ìg/ml were prepared by series dilution in 1% Tween solution. Exactly 150ìl of each standard was placed in a separate headspace vial with 50 ìl of d_{10}-DEE working internal standard solution. While matrix-matched calibration standards are preferred, in the absence of enough suitably ‘blank’ matrix, the use of standards made up in 1% Tween was necessary. The limits of detection (LOD) and of quantification (LOQ) are calculated from an analyte peak with a signal to noise ratio of 5 and 10 respectively. These values vary slightly between batches and are dependent upon the GC-MS instrument used and are generally around 5 and 10 ìg/ml respectively. The LOQ for DEE is usually limited to *ca.* 200 ìg/ml when the ion ratio parameters were not satisfied. Values below this were regarded as semi-quantitative. Separate calibration graphs for DEE and acetone standards are constructed by plotting the peak area ratio of analyte (at the appropriate m/z value) to d_{10}-DEE internal standard against concentration (ìg/ml in 1% Tween). Analyte(s) calibration correlation coefficient should be >0.99. A reagent blank and duplicate spiked and control samples (the latter only if available) should be included with every analytical batch.

F.3 ROTARY EVAPORATION CONDITIONS FOR ACETONE AND DEE REMOVAL AT CEFAS, DARD AND FRS.

Acetone Extraction

Condition	CEFAS	DARD	FRS
Water bath temp	45 ± 2^{o}C	47 ± 2^{o}C	Set to 48^{o}C, must remain 46 – 50^{o}C
How is temp. checked?	Calibrated thermometer	Calibrated thermometer	Calibrated thermometer
Start pressure	420 mbar	No means of knowing	556 mbar
Length of evaporation	15-20 min	25-30mins	~20min
End pressure	100-120 mbar	No means of knowing	~ 100mbar
Rotary speed	Estimated to be 150 – 170rpm)	~27 rpm	~190 rpm
How is complete evaporation determined?	Distillation ceased Pressure 100-120 mbar No obvious smell of acetone can be detected	Distillation ceased No obvious smell of acetone – if acetone detected returned to rotary evaporator.	No obvious signs of liquid running back into flask

DEE Extraction

Condition	CEFAS	DARD	FRS
Water bath temp	45 ± 2^{o}C	47 ± 2^{o}C	Set to 48^{o}C, must remain 46 – 50^{o}C
How is temp. checked?	Calibrated Thermometer	Calibrated thermometer	Calibrated thermometer
Start pressure	420 mbar	No means of knowing	556 mbar
Time of evaporation	~ 10 min or until visible film appears in round bottomed flask and dryness is apparent.	~10 minutes or until dryness / syrup	~ 5 – 10min
End pressure	~ 90 mbar	No means of knowing	less than 100mbar
Rotary speed	~150 – 170rpm	~27 rpm	~190 rpm
How is complete evaporation determined?	Distillation ceased Pressure ~ 90 mbar No residual liquid present (not always possible when oily/lipid extract is present). Sample appears dry No obvious smell of DEE can be detected	Distillation ceased No residual liquid (not always possible) Sample is dry No obvious smell of ether detected - any doubt, returned to rotary evaporator	Pressure less than 100 mbar Appears dry

F.4. Data collected on DEE and acetone concentrations and results of MBA

LOD (µg/ml) DEE=5, Acetone=5
LOQ (µg/ml) DEE=10, Acetone=10
Peak found but ion ratio confirmation criteria not satisfied.
* Results extrapolated from calibration with a range from 7 to 3550ìg/ml DEE and 8 to 4000ìg/ml acetone in 1% Tween solution.

F.4.1 DARD

WEEK 1

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
0307435	A	Cockles	<10	77	96	Negative
	B			59	94	
	C			63	92	
0307436	A	Cockles	<10	54	59	Negative
	B			55	62	
	C			32	52	
0307437	A	Cockles	<10	46	40	Negative
	B			43	42	
	C			44	40	

WEEK 2

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
307485	A	Cockles	<10	<LOQ	11	Negative
	B			<LOQ	<LOQ	
	C			<LOQ	<LOQ	
307486	A	Cockles	<10	<LOQ	<LOQ	Negative
	B			<LOQ	<LOQ	
	C			<LOQ	<LOQ	
307638	A	Cockles	<10	103	82	Negative
	B			69	77	
	C			79	72	
307639	A	Cockles	<10	<LOQ	62	Negative
	B			<LOQ	69	
	C			<LOQ	82	
307640	A	Cockles	<10	86	73	Negative
	B			87	70	
	C			89	72	
307667	A	Mussel	<10	28	<LOQ	Negative
	B			33	<LOQ	
	C			39	<LOQ	
307668	A	Mussel	50	977	<LOQ	Negative
	B			898	<LOQ	
	C			874	<LOQ	
307677	A	Cockles	<10	31	84	Negative
	B			36	70	
	C			32	77	
307678	A	Cockles	<10	51	81	Negative
	B			49	80	
	C			49	83	

WEEK 3

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
307803	A	Cockles	<10	93	23	Negative
	B			93	27	
	C			101	17	
307804	A	Cockles	<10	147	15	Negative
	B			138	29	
	C			145	29	
307805	A	Cockles	<10	<LOQ	45	Negative
	B			<LOQ	22	
	C			<LOQ	32	
307845	A	Cockles	<10	131	31	Negative
	B			137	<LOQ	
	C			134	<LOQ	
307916	A	Cockles	<10			Negative
	B					
	C					

F.4.2 CEFAS

WEEK 1

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml))	GC-MS acetone (mg/ml)	MBA
BTX/2003/0700	1	Cockles	10	<LOQ	1,519	Positive (Atypical)
	2			65	2,018	
	3			193	1,041	
BTX/2003/0701	1	Cockles	<10	< LOQ	220	Negative
	2			<LOQ	994	
	3			<LOQ	242	
BTX/2003/0702	1	Cockles	20	154	3,390	Negative
	2			137	9,758	
	3			688	9,107	
BTX/2003/0703	1	Cockles	<10	< LOQ	294	Positive (Atypical)
	2			<LOQ	2,313	
	3			<LOQ	299	
BTX/2003/0704	1	Cockles	<10	< LOQ	236	Negative
	2			<LOQ	345	
	3			184	530	
BTX/2003/0705	1	Cockles	<10	< LOQ	235	Negative
	2			<LOQ	407	
	3			<LOQ	222	
BTX/2003/0706	1	Mussels	50	906	1,107	Negative
	2			325	3,000	
	3			1,634	783	
BTX/2003/0707	1	Mussels	125	3,673	3,482	Negative
	2			seal broken		
	3			7,950	2,504	
BTX/2003/0708	1	Mussels	20	867	745	Negative
	2			332	1,096	
	3			1,313	959	
BTX/2003/0710	1	Mussels	<10	59	404	Negative
	2			51	675	
	3			183	332	
BTX/2003/0711	1	Cockles	10	119	757	Negative
	2			<LOQ	6,717	
	3			255	832	
BTX/2003/0714	1	Cockles	40	514	903	Negative
	2			265	1294	
	3			925	730	
BTX/2003/0715	1	Cockles	<10	< LOQ	270	Positive (Atypical)
	2			<LOQ	286	
	3			<LOQ	198	
BTX/2003/0716	1	Cockles	<10	< LOQ	427	Negative
	2			<LOQ	583	
	3			<LOQ	327	
BTX/2003/0719	1	Mussels	350	9,332	876	Negative
	2			4,158	1,146	
	3			22,517	687	

WEEK 2

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
BTX/2003/0724	1			<LOQ	5,213	
	2	Cockles	55	#29	7,368	Negative
	3			32	4,990	
BTX/2003/0725	1			917	11,121	
	2	Cockles	100	812	10,474	Negative
	3			888	10,598	
BTX/2003/0726	1			#140	5,568	
	2	Cockles	20	#106	5,696	Negative
	3			#127	7,755	
BTX/2003/0727	1			<LOQ	1,690	
	2	Cockles	10	<LOQ	2,254	Negative
	3			<LOQ	1,791	
BTX/2003/0728	1			<LOQ	1,395	
	2	Cockles	15	<LOQ	1,361	Negative
	3			<LOQ	1,372	
BTX/2003/0729	1			#36	3,788	
	2	Cockles	30	#135	5,378	Negative
	3			#106	5,512	
BTX/2003/0730	1			175	4,612	
	2	Cockles	20	195	4,870	Negative
	3			216	4,622	
BTX/2003/0731	1			221	1,954	
	2	Cockles	20	#132	2,111	Negative
	3			<LOQ	1,745	
BTX/2003/0732	1			<LOQ	878	
	2	Cockles	<10	<LOQ	808	Negative
	3			<LOQ	961	
BTX/2003/0733	1			<LOQ	1,166	
	2	Cockles	<10	<LOQ	1,092	Positive (Atypical)
	3			<LOQ	1,250	
BTX/2003/0734	1			<LOQ	730	
	2	Mussels	10	<LOQ	764	Negative
	3			<LOQ	734	
BTX/2003/0735	1			<LOQ	1,817	
	2	Mussels	<10	<LOQ	1,860	Negative
	3			<LOQ	1,905	
BTX/2003/0736	1			316	4,962	
	2	Mussels	50	277	4,813	Negative
	3			309	5,267	
BTX/2003/0737	1			<LOQ	130	
	2	Mussels	<10	<LOQ	137	Negative
	3			<LOQ	178	
BTX/2003/0738	1			<LOQ	68	
	2	Mussels	<10	<LOQ	70	Negative
	3			<LOQ	89	

WEEK 3

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
BTX/2003/0749	1	Cockles	250	50,620	26,039	Negative
	2			52,522	27,388	
	3			49,134	24,819	
BTX/2003/0750	1	Cockles	<10	#82	710	Positive (Atypical)
	2			<LOQ	457	
	3			#128	598	
BTX/2003/0751	1	Cockles	<10	281	1,083	Positive (Atypical)
	2			327	708	
	3			325	883	
BTX/2003/0752	1	Cockles	<10	#118	1,053	Negative
	2			#263	1,282	
	3			#156	1,119	
BTX/2003/0753	1	Cockles	<10	325	1,460	Positive (Atypical)
	2			378	1,036	
	3			547	2,109	
BTX/2003/0754	1	Cockles	20	38,533	23,399	Negative
	2			24,314	15,329	
	3			24,146	14,546	
BTX/2003/0755	1	Mussels	<10	574	655	Negative
	2			555	623	
	3			569	690	
BTX/2003/0756	1	Mussels	80	69,402	33,935	Negative
	2			71,095	29,911	
	3			75,285	29,723	
BTX/2003/0757	1	Mussels	15	45,353	51,675	Negative
	2			50,817	51,517	
	3			47,518	48,367	
BTX/2003/0758	1	Cockles	<10	#151	1,179	Positive (Atypical)
	2			287	1,260	
	3			287	1,490	
BTX/2003/0767	1	Cockles	<10	5,721	6,096	Negative
	2			6,012	5,714	
	3			5,907	5,622	
BTX/2003/0768	1	Mussels	<10	1,105	1,232	Negative
	2			1,193	1,185	
	3			1,106	1,197	
BTX/2003/0769	1	Mussels	10	10,626	7,753	Negative
	2			11,572	6,999	
	3			2,185	4,572	
BTX/2003/0770	1	Mussels	40	83,478	108,635	Negative
	2			87,088	104,321	
	3			91,366	112,080	
BTX/2003/0771	1	Mussels	<10	1,596	4,362	Negative
	2			12,011	7,789	
	3			2,001	4,622	

F.4.3 FRS Interim SOP

WEEK 1

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
5732	A	Mussels	>10000	7,794*	1,084	N/A
	B			7,652*	904	
	C					
5733	A	Mussels	5000	2,659	1,168	N/A
	B			3,034	1,039	
	C					
5735	A	Mussels	>10000			N/A
	B			5,579*	1,582	
	C			11,207*	2,135	
5737	A	Mussels	8000	3,545	681	N/A
	B			1,849	474	
	C			4,064	774	
5738	A	Mussels	1000	1,542	973	N/A
	B			1,081	223	
	C					
5740	A	Mussels	>10,000	12,164*	3,523	N/A
	B			11,653*	3,544	
	C			11,856*	4,866	
5742	A	Mussels	>10000	3,249	279	N/A
	B			3,462	243	
	C			3,437	317	
5744	A	Mussels	<400	60#	105	N/A
	B			54#	108	
	C					
5745	A	Mussels	2000	319	1,304	N/A
	B			313	1,403	
	C			325	1,170	
5746	A	Mussels	4000	1,443	3,167	N/A
	B			1,445	2,185	
	C			1,571	2,827	
5749	A	Mussels	>10000	19,326*	195	N/A
	B					
	C					
5750	A	Mussels	>10000	20,622*	212	N/A
	B			22,502*	165	
	C			27,646*	193	
5752	A	Mussels	7000			N/A
	B			2,278	259	
	C			2,283	388	
5754	A	Mussels	400			N/A
	B			258	116	
	C			300	185	

WEEK 2

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
5796	A			776	24	
	B	Mussels	>400	769	30	N/A
	C					
5799	A			29,317*	388	
	B	Mussels	>400	27,077*	679	N/A
	C			28,113*	612	
5800	A					
	B	Mussels	>400	4,675*	548	N/A
	C			5,007*	592	
5801	A			1,690	1,383	
	B	Mussels	>400	1,918	1,616	N/A
	C			1,952	1,627	
5802	A					
	B	Mussels	50	107	310	N/A
	C					
5803	A			369	352	
	B	Mussels	80			N/A
	C			363	363	
5804	A			1,864	375	
	B	Mussels	>400	2,030	412	N/A
	C					
5805	A			226	338	
	B	Mussels	50	245	387	N/A
	C			245	394	
5807	A			431	534	
	B	Mussels	100			N/A
	C			460	567	
5808	A			552	971	
	B	Mussels	300	632	1,090	N/A
	C			642	1,006	

F.4.4 FRS original SOP

WEEK 1

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
5732	A	Mussels	<400	<LOQ	108	Negative
	B			<LOQ	94	
	C					
5733	A	Mussels	<400	2,932	1,134	Negative
	B					
	C			<LOQ	63	
5734	A	Mussels	<400	<LOQ	110	Negative
	B			<LOQ	73	
	C			<LOQ	76	
5735	A	Mussels	<400	<LOQ	70	Positive (typical)
	B					
	C					
5736	A	Mussels	<400	<LOQ	295	Negative
	B			<LOQ	298	
	C			28#	355	
5737	A	Mussels	<400	<LOQ	57	Negative
	B					
	C			<LOQ	45	
5738	A	Mussels	<400	992	246	Negative
	B					
	C			1,099	221	
5740	A	Mussels	<400	40#	96	Negative
	B			31#	89	
	C			37#	79	
5741	A	Mussels	<400	58#	70	Negative
	B			<LOQ	125	
	C			<LOQ	112	
5742	A	Mussels	<400	32#	74	Negative
	B			<LOQ	101	
	C					
5744	A	Mussels	<400	<LOQ	92	Negative
	B			40#	86	
	C			49#	98	
5745	A	Mussels	<400	41#	88	Negative
	B					
	C			240	468	
5746	A	Mussels	<400	42#	154	Negative
	B			13	156	
	C			64#	111	
5749	A	Mussels	<400	<LOQ	119	Negative
	B			20947*	199	
	C			<LOQ	122	
5750	A	Mussels	<400	<LOQ	105	Negative
	B			32#	118	
	C			26#	130	
5751	A	Mussels	<400	41#	74	Negative

	B			38#	92	
	C			35#	93	
5752	A			<LOQ	124	
	B	Mussels	<400	<LOQ	116	Negative
	C			Compromised seal on vial		
5754	A			46#	256	
	B	Mussels	<400	39#	255	Negative
	C			<LOQ	391	

WEEK 2

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
5796	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C					
5799	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	<LOQ	
5800	A			<LOQ	24	
	B	Mussels	<10	<LOQ	25	Negative
	C			<LOQ	22	
5801	A			<LOQ	87	
	B	Mussels	<10			Negative
	C			<LOQ	82	
5802	A			<LOQ	25	
	B	Mussels	<10	<LOQ	20	Negative
	C			<LOQ	25	
5803	A			<LOQ	<LOQ	
	B	Mussels	<10			Negative
	C			<LOQ	<LOQ	
5804	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C					
5805	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	<LOQ	
5807	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	<LOQ	
5808	A			#66	75	
	B	Mussels	25	#103	71	Negative
	C			#65	63	

WEEK 3

Sample code	Replicate	Species	GASTEC DEE (mg/ml)	GC-MS DEE (mg/ml)	GC-MS acetone (mg/ml)	MBA
10486	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	<LOQ	
5812	A			#57	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	<LOQ	
5820	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	<LOQ	
5821	A			<LOQ	19	
	B	Mussels	<10	<LOQ	31	Negative
	C			<LOQ	18	
5824	A			<LOQ	32	
	B	Mussels	<10	<LOQ	33	Negative
	C			<LOQ	31	
5828	A			<LOQ	<LOQ	
	B	Mussels	<10	<LOQ	<LOQ	Negative
	C			<LOQ	18	
10509	A			#109	111	
	B	Mussels	10	#106	162	Negative
	C			143	237	
10522	A			<LOQ	37	
	B	Cockles	<10	<LOQ	46	Negative
	C			<LOQ	39	
10528	A			<LOQ	144	
	B	Mussels	<10	<LOQ	120	Negative
	C			<LOQ	126	

ANNEX G

G.1 PROTOCOL FOR THE MEASUREMENT OF DEE AND ACETONE IN REPLICATE SAMPLES OF COCKLES AND MUSSELS TO DETERMINE WHETHER SOLVENT AMOUNT IS SPECIES DEPENDENT

1. Objective

To determine if the Interim extraction procedure introduces variability in the quantity of DEE and acetone remaining in the headspace of Tween extracts from cockles and mussels.

2. Outline

- DEE and acetone are not naturally present in shellfish samples. Therefore, the presence of either of these solvents in a shellfish extract must be an artefact of the extraction procedure which may/may not be linked to the sample matrix.
- In recent DARD and CEFAS experiments some samples clearly contained DEE at the end of the extraction procedure (as measured using GASTEC tubes) whereas others did not. The question arises as to why DEE remained in some of the samples and not in others? This is a pertinent question if the issue of DEE in relation to atypical MBA responses is to be resolved.
- Shellfish samples prepared from the monitoring programme have been extracted once per sample. Therefore, routine monitoring samples will not provide an answer to the above question. To address this (in terms of the extraction procedure) multiple extractions of the same sample are required.
- Variability in the quantity of trapped DEE could also be due to differences in the matrix from different shellfish species or even within species (depending in their biological state).
- Although data on DEE levels from the two species will be provided from experiment 1, the usefulness of the data in determining if species influence the quantity of trapped DEE will be dependent on whether or not replicate extractions of the same sample produce consistent data. In a preliminary investigation of this issue the experiment detailed below will be conducted using cockles and mussels
- As shellfish from different geographical areas are likely to show variation in the constituents of their matrix (e.g. dependent on spawning state, food source etc.) it is feasible that this could influence the quantity of trapped DEE. The importance of intra-species differences compared to variations due to the procedure or shellfish species is unknown. Due to resource limitations this factor will not be examined at this stage.
- The variability in DEE concentrations due to the extraction procedure or shellfish matrix will be examined in the first instance by analysing DEE by HS-GC-MS in 16 replicate homogenates (32 in total) in TWEEN of one sample of mussels and one sample of cockles. GASTEC tubes will also be used to gain a semi-quantitative measurement of headspace DEE.
- It is apparent that the rotary evaporator conditions at the various laboratories differ and that it would be impractical for CSL to attempt to reproduce them for this experiment. Therefore the samples will be prepared at CEFAS using SOP 12 version 9 (DSP extraction).
- An overview of the experiment is given in Figure 5.

2.1 The data will provide the following information

- Information on the variation in DEE and acetone concentration in the headspace of Tween extracts in relation to the extraction procedure.

3. Participants

Monitoring laboratory:	CEFAS
Chemical analysis:	CSL
Advice and co-ordination	UK-NRL/FSA
Statistics:	FSA
Report writing	FSA/CSL

4. Methodology

4.1 Target shellfish species

Mussels (*Mytilus edulis*) and cockles (*Cerastoderma edule*).

4.2 Supply and storage of shellfish

CEFAS will:

a) Identify a location where cockles and mussels are available e.g Burry Inlet SW
b) Liase with the local PHA to arrange collection and transit of shellfish to the Weymouth Laboratory
c) A large sample of cockles will be collected and dispatched 3/9/03. These samples will be extracted on the 4 and 5th September (8 per day with a total of 16 replicates)
d) Point c will be repeated the following week for mussels.

4.3 Preparation of GC vials, standards and stock solutions at CEFAS

As detailed in experiment 1

4.4 Homogenate preparation at CEFAS

4.4.1 SOP and equipment

The Interim arrangements in use at CEFAS (SOP number 12, version 9, DSP extraction) will be followed. Equipment and conditions of the rotary evaporation stage are as detailed below:

Equipment

Buchi V800 Vacuum Controller
Buchi R200 Rotovapor Rotary Evaporator
Buchi B490 Heating Bath
Buchi V500 Vacuum Pump

Rotary evaporation procedure

During acetone extraction, samples are set on "Run" this is pre-programmed to evaporate at a set pressure of 420 mbar. Samples are evaporated this way for approximately 15 minutes or until there is a visible film in the round bottom flask. The vacuum controller can then be set to "Continuous", this applies a continuous vacuum to the samples. To determine complete evaporation, the following criteria have to be met: Distillation has ceased, the pressure on the vacuum controller is approximately 120 mbar and upon removal from the evaporation unit, acetone cannot be detected by a cursory "sniff".
For evaporation of DEE, the vacuum controller is set on "Continuous". Full evaporation is met by a combination of the following criteria: Distillation has ceased, No residual liquid present (sometimes this cannot be met), the pressure on the vacuum controller is 90 mbar or less, the sample appears dry and DEE cannot be detected by a cursory "sniff".

4.4.2 Preparation of shellfish extracts

4.4.2.1 Cockles and mussels will be shucked and homogenised in the timescale detailed in Table 3 above. Each homogenate will be split into 16 x 75 g aliquots and extracted by CEFAS SOP 12. This means that a total of 32 aliquots will be prepared; 16 for each species.

4.4.2.2 Upon completion of the DEE evaporation step add 1 % Tween to the shellfish extract as detailed in CEFAS SOP 12

4.4.2.3 Variations to the normal procedure involve recording additional data as detailed below:

d) record the volume of solution remaining after the removal of acetone by rotary evaporation on Form 2
e) Weigh the empty flask prior to the rotary evaporation DEE stage and after rotary evaporation and hence calculate the weight of the residue. Record weights on Form 2
f) Record the weight of Tween added to the residue after the DEE rotary evaporation stage on Form 2.
g) Record the timing of the acetone and DEE rotary evaporation stage, residue appearance, final pressure of DEE rotary evaporation stage on Form 5
h) Record the time from addition of Tween to the sealing of vials for GC analysis on Form 5
i) Record the time GASTEC measurements taken on Form 5

4.4.3 Preparation of extracts for shipment to CSL

As detailed in Experiment 1 with the exception that 3 x 250 µl aliquots should be prepared for each extract (i.e the aliquot volume is 150 µl in experiment 1 due to restrictions on available sample volumes required to conduct mouse bioassays).

4.5 Use of GASTEC tubes

As detailed in Annex F.2.

4.6 Preparation of spikes, blanks and controls at CEFAS

As detailed in Annex F.2.

4.7 HS-GC-MS analysis by CSL

As detailed in Annex F.2.

5. Summary of information to be recorded by CEFAS

As within Annex F.2 with the addition of:

Form 5: Time of stages

- Time acetone evaporation commenced
- Time acetone evaporation finished
- Time DEE evaporation commenced
- Time DEE rotart evaporation finished
- Residue appearance
- Final pressure at end of rotary evaporation
- Time Tween added
- Time internal standard added
- Time GC vials sealed
- Time GASTEC measurement taken
- Date GC vials shipped to CSL

6. Materials required by CEFAS, which are additional to routine monitoring requirements

As detailed in Annex F.2.

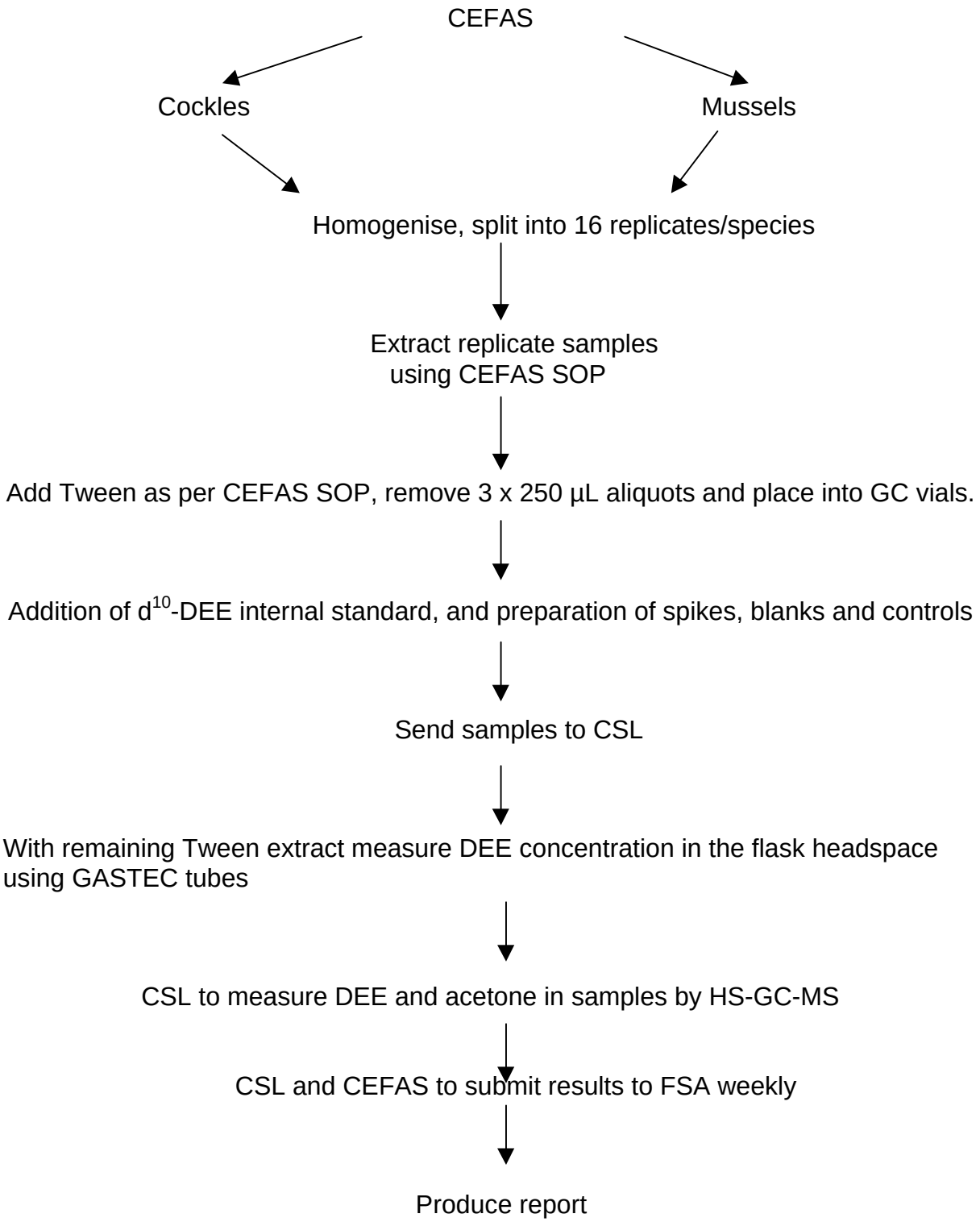
7. Transport of samples from monitoring laboratories to CSL

As detailed in Annex F.2.

8. Analysis of results and production of report

CEFAS, should submit Forms 1-5 to the FSA weekly. CSL to submit their DEE HS-GC-MS results for the samples to FSA weekly including information on blanks/spikes and calibration curves. FSA to issue a summary of the data weekly. CSL/FSA to prepare a draft report with comments from monitoring laboratories. Final report produced by FSA.

Figure 5: Outline of Experiment



G.2 Data collected on DEE and acetone concentrations in replicate samples of cockles and mussels.

LOD (µg/ml) DEE=5, Acetone=5
LOQ (µg/ml) DEE=10, Acetone=10
Peak found but ion ratio confirmation criteria not satisfied.
* Results extrapolated from calibration with a range from 7 to 3550ìg/ml DEE and 8 to 4000ìg/ml acetone in 1% Tween solution.

COCKLES				
Sample number	**Replicate**	**GASTEC DEE (mg/ml)**	**GC-MS DEE (mg/ml)**	**GC-MS acetone (mg/ml)**
1	1	<10	<LOQ	1,273
	2		<LOQ	1,473
	3		<LOQ	1,473
2	1	10	417	4,995*
	2		426	4,948*
	3		429	4,899*
3	1	20	457	5,113*
	2		2,403	10,860*
	3		2,416	10,990*
4	1	<10	#41	2,868
	2		#50	2,928
	3		#45	2,891
5	1	30	3,179	8,821*
	2		-	-
	3		-	-
6	1	50	3,796*	9,989*
	2		3,774*	10,061*
	3		3,097	9,361*
7	1	<10	<LOQ	773
	2		<LOQ	723
	3		<LOQ	762
8	1	<10	#85	1,521
	2		#76	1,539
	3		#59	1,502
9	1	<10	<LOQ	374
	2		<LOQ	401
	3		<LOQ	446
10	1	<10	<LOQ	120
	2		<LOQ	86
	3		<LOQ	121
11	1	20	#36	2,497
	2		#34	2,533
	3		#28	2,355
12	1	20	1,701	8,541*
	2		1,718	8,422*
	3		1,598	8,133*
13	1	<10	<LOQ	542
	2		<LOQ	538
	3		<LOQ	525
14	1	20	1,735	7,536*
	2		1,748	7,675*
	3		1,797	7,695*
15	1	10	<LOQ	393
	2		<LOQ	376
	3		<LOQ	386
16	1	<10	<LOQ	91
	2		<LOQ	89
	3		<LOQ	96

MUSSELS

Sample number	Replicate	GASTEC DEE (ug/ml)	GC-MS DEE (ug/ml)	GC-MS acetone (ug/ml)
1	1	30	21,444*	50,103*
	2		20,307*	47,639*
	3		22,652*	51,944*
2	1	<10	378	3,216
	2		352	3,274
	3		338	3,305
3	1	10	13,366*	36,049*
	2		13,411*	36,791*
	3		13,994*	38,340*
4	1	50	50,891*	115,584*
	2		53,586*	121,420*
	3		55,961*	126,688*
5	1	<10	139#	1,395
	2		120#	1,399
	3		111#	1,377
6	1	20	37,320*	127,656*
	2		41,328*	132,224*
	3		39,781*	134,164*
7	1	35	40,237*	94,034*
	2		40,895*	98,913*
	3		41,126*	96,771*
8	1	20	370	3,798
	2		293#	3,852
	3		408	4,035*
9	1	<10	139#	721
	2		114#	804
	3		98#	747
10	1	<10	267#	2,870
	2		249#	2,809
	3		327#	2,832
11	1	<10	1,150	36,352*
	2		1,310	16,988*
	3		1,255	18,351*
12	1	<10	1,426	2,784
	2		1,601	2,829
	3		1,772	3,008
13	1	<10	96#	1,966
	2		240#	2,067
	3		166#	2,105
14	1	<10	1,607	10,172*
	2		1,545	10,089*
	3		1,735	10,661*
15	1	15	83,205*	174,627*
	2		68,744*	153,916*
	3		72,837*	160,955*
16	1	<10	Seal broken	
	2		220#	2,605
	3		166#	2,822