ADDENDUM
TO

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

January 2004

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. The work carried out on behalf of the Agency to investigate this issue was reported in '*Investigations to assess whether diethyl ether or acetone carry-over during the DSP standard operating procedure is responsible for the atypical response in mice'* which was published on the 2 October 2003 and can be downloaded from the Agency's website[1].

3. Since then, the Agency has been made aware of some additional information which is relevant to its investigations, and the associated issues are covered in this report. The conclusions at paragraph 25 update and supersede those in Parts 6, 7 and 8 of the original report. This section also includes an interpretation of data available from the UK statutory monitoring programme on the presence of known non-DSP shellfish biotoxins and the occurrence of the atypical response in the DSP MBA.

4. On the basis of the data reported in the original report and in this addendum, the Agency considers that no evidence has been generated by the investigations it has commissioned to support the hypothesis that there is a direct causal relationship between DEE and/or acetone levels in the extract injected in the mouse bioassay and the atypical responses recorded.

5. This report has been produced by FSA UK with input from UK-NRL, CSL, CEFAS, DARD and FRS.

[1] http://www.food.gov.uk/science/research/microbioSafety/b16programme/shellfish_toxins

Issue

6. This report includes additional solvent data and addresses some specific stakeholder comments on the original report relating to the possible role of solvents and hydrophilic toxins in the atypical response observed in the DSP MBA. It also includes a section on the analysis of solvent carry-over data.

Solvents

7. The original report recorded that Gastec kits, which are semi-quantitative, are a convenient means of detecting the presence or absence of DEE in shellfish extracts, and that headspace GC-MS is a reliable tool for providing a good assessment of the relative amounts of the solvents in shellfish extracts. Since then, further data has been obtained which confirms that Gastec kits can detect acetone as well as DEE.

8. In view of the need to ensure that any DEE and/or acetone carried over from the extraction procedure is below levels capable of causing biological activity in the mouse, information on toxicity of these solvents was sought. Data for DEE was included in the original report (the LD_{50} value for IP administration of DEE in mice is reported as 2420mg/kg bodyweight[2], which is equivalent to 68µl of DEE for a 20g mouse). Very few data are available on the effects of acetone in mice after intra-peritoneal (ip) administration and the available information does not give a consistent picture. Two LD_{50} values have been reported for acetone administered by the ip route: 1,297mg/kg bodyweight[3], and 3100 mg/kg bodyweight[4], which are equivalent to administration of 33ìl or 79ìl acetone respectively for a 20g mouse. In another study, 2000 mg/kg bodyweight doses of acetone were administered by the ip route on two occasions within 24 hours without apparent lethality[5]. Data on the clinical effects of acetone administered to mice by ip injection over a dose range are not available. No data could be found on the co-administration of acetone and DEE.

9. Data generated from analysis of statutory monitoring samples has shown that Gastec kit 161L can reliably detect DEE and/or acetone at levels below the level necessary to cause biological activity in the mouse. This will be reported separately as part of a forthcoming report on the trial of the UK-NRL DSP SOP.

10. On the basis of the limited data available, and in view of the desire to ensure that solvent levels in the shellfish extracts are as low as practicable, and below levels capable of causing biological effects in the mouse, the Agency takes the view that no solvent should be detectable in the sample extracts using the Gastec kit 161L, at the time of injection into mice. This view has been accepted by the UK-NRL, CEFAS, DARD, FRS, HPA and the Home Office. The appropriate control step,

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

[3] Krasavage, W. J., J. L. O'Donoghue and G. D. Divincenzo. 1982. Acetone. In: Patty's Industrial Hygiene and Toxicology, 3rd rev. ed., vol. 2c., G. D. Clayton and F. E. Clayton, eds. Wiley Interscience, New York. pp. 4720-4727.

[4] Value quoted from Patty's Toxicology (Vol.6), Chapter 1: Acetone, pp 16.

[5] Kurata, N. et al. (1991) Studies on the inhibition and induction of metabolism of ethyl carbamate by acetone and related compounds. Drug Metabolism and Disposition. 19. (2). Pp. 388-939.

which involves checking solvents are not detectable by the Gastec kit prior to injection, has been included in the UK-NRL DSP SOP to achieve this.

11. Data obtained since the UK-NRL DSP SOP came into operation on 17 November 2003 at CEFAS, DARD, FRS, as well as the HPA, confirms that the Gastec kits are being used effectively at these laboratories to detect DEE and acetone, and that levels in the extracts injected have been consistently below those necessary to cause biological activity in the mouse (these data will be detailed in a forthcoming report on the trial of the UK-NRL DSP SOP).

Data on known Shellfish Biotoxins

12. The hypothesis that the atypical response may be linked to the presence of non DSP toxins, such as those responsible for Paralytic Shellfish Poisoning (PSP), which is a hydrophilic toxin, or Amnesic Shellfish Poisoning (ASP) has also been considered.

13. As reported in the December 2002 Board Paper[6] (which can be downloaded from the Agency's website www.food.gov.uk), work was undertaken to formally test for all known shellfish biotoxins in extracts generating the atypical response. No DSP, PSP or ASP toxins were detected in any of the atypical samples tested.

14. Shellfish samples collected for routine DSP monitoring (i.e. from areas which are not subject to temporary prohibition orders) are tested for a range of other toxins which collectively fall within the groups ASP and PSP. Since June 2001, on no occasion was PSP or ASP (above the action level) and the atypical response detected in the same sample (see Table 1 for PSP and Table 2 for ASP). On one occasion, PSP below the action level was detected in a sample which generated the atypical mouse response in the DSP test. On one occasion, ASP below the action level was detected in a sample which generated the atypical mouse response in the DSP test.

Table 1. Occurrence of PSP in atypical samples collected through the monitoring programme.

Year	Number of atypical positives	Number of atypical positives tested for PSP	% of atypical positives tested for PSP	Of those tested for PSP, number which were positive for PSP
2001	79	17	21.5%	0
2002	247	39	15.8%	0
2003	189	36	19.0%	0

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

Table 2. Occurrence of ASP in atypical samples collected through the monitoring programme.

Year	Number of atypical positives	Number of atypical positives tested for ASP	% of atypical positives tested for ASP	Of those tested for ASP, number which were positive for ASP
2001	79	16	20.3%	0
2002	247	45	18.2%	0
2003	189	40	21.2%	0

15. From these data, it is suggested that PSP or ASP are not linked to the atypical response. If they were, the current testing arrangements would have picked them up. On the basis of the data available it would therefore seem highly unlikely that the atypical response is caused by PSP or ASP.

Analysis of solvent data

16. Variable solvent levels originally reported for cockle and mussel extracts prepared from certain batches of samples from CEFAS were noted, and an investigation into the possible causes of these variations was undertaken. On reviewing the CEFAS GC-MS data, CSL found that the peak area for the internal standard for samples extracted on 11 and 12 September 2003 was 10 times smaller than 'normal'. This was found to be due to operator error, which resulted in a new working internal standard (IS) being made up from a previously diluted standard.

17. In the circumstances, CSL recommended (having demonstrated acceptable calibration) that the data for the affected period (i.e. data for week 3 samples from Part 6, and mussel samples for Part 7, of the original report) be re-analysed using an external standard approach.

18. This approach is recommended (over applying the external standard approach to all the data) because it is generally accepted by analytical chemists to be an appropriate way of dealing with data where the IS data for a proportion of the whole dataset has been compromised. Details of both approaches can be found at Annex 1.

19. In addition, results of week 3 FRS monitoring samples (which were previously unavailable) have been analysed for solvent levels by GC-MS.

20. Statistical analysis of the re-evaluated CEFAS data, along with the extra data from FRS samples prepared for week 3, was undertaken and is reported in Annexes 2 and 3.

21. However, to address industry concern that use of the recommended approach may have materially altered the findings of the report, the Agency agreed to re-analyse all the data using the external standard approach. The results of the statistical analysis on the external standard approach are shown in brackets in Annexes 2 and 3, alongside data from the recommended approach. The dataset generated using the external standard approach is also shown at Annex 4.

22. Of the 5 issues considered in Part 6 of the original report (amended version at Annex 2), re-analysis using the external approach did not affect any of the conclusions.

23. Of the 2 issues considered in Part 7 of the original report (amended version at Annex 3), the re-analysis using the external standard approach affected one of the conclusions. It found that cockle extracts contained significantly lower amounts of diethyl ether (DEE) and acetone than mussel extracts, whereas the recommended approach found there to be no significant difference in the concentrations of DEE and acetone between extracts of cockles and mussels.

24. Irrespective of the approach taken, the statistical analysis of the data confirms that based on the studies carried out, there were no statistically significant differences in the levels of DEE and acetone present in extracts generating atypical responses, and those generating negative responses, in mice.

Conclusions

25. The conclusions which can be drawn from the complete dataset are set out below. They update and supersede those in Parts 6, 7 and 8 of the original report. Most of the conclusions in that original report are unaffected by the additional analysis reported in this Addendum.

    i. Analysis of the dataset found no statistically significant differences in the levels of DEE and acetone present in extracts generating atypical responses, and those generating negative responses, in mice.

    ii. DEE and acetone were found in the final extracts of cockles and mussels irrespective of the SOP used (interim method as used by all labs or the original FRS method). There is no statistical correlation between DEE and acetone carry over levels (measured by GC-MS) with the interim SOP used by all laboratories, and the original method used by FRS.

    iii. Significant differences in DEE and acetone carry-over levels were found between the original and interim methods used at FRS, but this may be explained by the fact that extracts prepared using the original method were left open to the atmosphere overnight to allow further evaporation of solvents. This practice is not recommended as overnight storage prior to re-suspension in Tween could lead to potential adsorption of toxins onto surfaces of containers and deterioration of toxins. Since introduction of the UK-NRL DSP SOP, all laboratories re-suspend extracts in Tween prior to storage.

    iv. There were no significant differences in the levels of DEE and acetone carried over in the extracts of cockles and mussels.

    v. None of the samples prepared by the laboratories contained DEE or acetone above the respective intraperitoneal LD_{50} (DEE=68ìl, acetone=33ìl).

    vi. FRS samples prepared using the interim method had significantly higher levels of DEE as measured by GC-MS than samples from CEFAS ($P<0.05$) and

DARD ($P<0.01$). There was no statistical difference between DEE levels in stoppered extracts prepared by FRS using the original and those prepared using the interim SOP.

vii. DARD extracts contained the lowest levels of DEE and acetone, substantially lower than the IP LD_{50} of DEE and acetone (equivalent to 68µl and 33ìl respectively when injected into a 20g mouse). DARD samples had significantly lower levels of acetone than samples prepared by either FRS or CEFAS ($P<0.01$).

viii. One CEFAS sample was found to contain DEE exceeding 10ìl/ml (16.8 ìl/ml), while another CEFAS sample contained acetone exceeding 10 ìl/ml (13.8 ìl/ml). Both of these samples, when tested in the mouse, gave a negative result. All other samples (n=129) prepared by the laboratories were below 10µl/ml for both DEE and acetone.

ix. The re-analysed data for week 3 CEFAS samples falls within the range of DEE and acetone levels found in samples prepared during weeks 1 and 2.

x. Atypical responses were observed when dichloromethane (DCM) was used instead of DEE in the extraction procedure.

xi. Administration of DEE in Tween to mice at concentrations up to 150µl (107,190ìg/ml) were considered not to produce the atypical symptoms reported during shellfish toxin monitoring.

xii. DEE presence as determined by Gastec, and levels as determined by GC-MS, showed a positive correlation, however the data was poorly distributed across the possible range of values.

xiii. Since the introduction of the UK-NRL DSP SOP, Gastec kits have been successfully used to ensure that solvent levels in extracts are minimal and consistently below those levels necessary to cause biological activity in the mouse.

xiv. PSP or ASP have not been detected in those samples tested which show the atypical response.

26. 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.

Fish and Shellfish Branch
Microbiological Safety Division
30 January 2004

Annex 1

Application of internal and external standard approach in GC-MS data analysis

For the internal standard (IS) procedure, a fixed amount (50ìl) of IS (d10-DEE at ca. 4,000 mg/l) was added to a fixed volume of sample extract in Tween (150ìl) and sealed in a headspace vial for HS-GC-MS analysis. The HS-GC-MS was calibrated by preparing a series of mixed acetone and DEE solutions in Tween (ca. 7 - 4,000 mg/l) and adding 150ìl to a HS vial containing 50ul of IS solution. The ratios of both the DEE and acetone peak areas to the IS peak area were plotted against DEE/acetone concentration to provide separate DEE and acetone calibration lines (R2 always > 0.99). The peak areas of acetone and DEE in the samples were divided by the peak area of the IS in the samples to obtain the sample analyte/IS peak area ratio. The corresponding concentration of DEE/acetone was then determined from the calibration equation directly.

For the external standard approach, the absolute peak areas of DEE and acetone in the calibration standards prepared above were plotted against analyte concentration to give a linear calibration line. The concentration of DEE and acetone in the samples was calculated from the DEE or acetone absolute peak areas obtained from the calibration line.

Annex 2

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

48. The data was re-analysed to determine:

i. Whether DEE measurements by Gastec and GC-MS correlate for the interim and FRS original method.
ii. Whether there is a statistically significant difference in the levels of DEE and acetone between MBA negative extracts and extracts giving an atypical response produced using the interim method by CEFAS and DARD.
iii. Whether there are any statistically significant differences in DEE and acetone concentrations between laboratories when extracts are produced using the interim method.
iv. Whether there are any statistically significant differences in DEE and acetone concentrations between the original and interim methods used at FRS.
v. Whether there is a correlation between the DEE and acetone concentrations for the interim method applied at CEFAS, DARD and FRS and original method applied at FRS.

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

i. Pearson correlation ($\hat{\rho}$) 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). Results were confirmed using non-parametric tests. The advantage of the non-parametric approach is that it does not assume any particular distribution from the data. In all tests carried out in the report, the non-parametric tests agreed with the parametric ones.
iii. One-way analysis of variance (ANOVA) to compare the three different laboratories. Due to the high variability between laboratories, comparisons were carried out assuming unequal variances across the laboratories. All tests were corrected for multiple comparisons.
iv. Independent sample t-test to compare the two methods (interim and FRS original). 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. Thus, to re-balance the design, averages were used as the unit for analysis. Using averages rather than the median also allows the worst-case scenario for samples with high variability within the replicates.
- 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.
- Every time test assumptions were violated, a corresponding non-parametric test was used.
- For Part 6, power analysis was carried out *a posteriori*, since little information was available prior to the study on solvent carry-over levels. For a power of 90% and a 5% significance level the minimum differences in solvent levels that would have been identified between atypical and negative responses in the MBA for DEE are approximately 1.2ìl/ml (0.6ìl/ml using external standards) and for acetone are approximately 1.6ìl/ml ((0.6ìl/ml using external standards). For a power of 90% and a 1% significance level the minimum differences in solvent levels that would have been identified between laboratories for DEE are approximately 7.1ìl/ml (4.7ìl/ml using external standards) and for acetone are approximately 1.9ìl/ml (0.9ìl/ml using external standards).

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 correlation between Gastec and GC-MS measurements of DEE (ñ = 0.56) when the interim method is used for all laboratories and data is considered together *(r = 0.47 when external standards data used)*. Looking closer at the data there is a poor distribution of observations across the range of values (i.e. the majority have very low Gastec and GC-MS DEE values and a few with very large Gastec and GC-MS DEE values).**There is a weak correlation for acetone measured by GC-MS and Gastec measurements (ñ = 0.002)** *(r = 0.067 when external standards data used).*

The FRS original and interim method data display weaker correlation between Gastec and GC-MS measurements. This may be attributed to FRS using different methods over the course of the experiment to measure levels of DEE. Gastec kits with a higher LOD (400µg/ml) were used in the first week, and lower LOD (10µg/ml) in the remaining weeks.

The apparently lower concentrations of DEE measured by Gastec when compared with GC-MS analysis of the same samples may be explained by the differences in the way the headspace gas is obtained. Gastec kits measure the DEE in the headspace arising from passive diffusion from the sample matrix in a vessel open to the atmosphere, whereas headspace GC-MS samples are heated to 60°C for 5 minutes in a sealed system. One would therefore expect that headspace GC-MS conditions would drive the solvents out of the sample matrix resulting in higher concentrations of solvent in the headspace gas compared with passive diffusion under ambient conditions.

Gastec tubes 161L are also able to detect acetone[7]. Therefore Gastec measurements allow an estimation of the total solvent presence (i.e. a contribution from both DEE and acetone) in a sample extract. The data suggest that Gastec kits have a role as an indicative test to describe the presence of DEE and/or acetone in an environment above a particular level, however they are not suitable to accurately measure DEE and acetone levels. GC-MS provides a robust, quantitative measure of DEE and acetone.

ii. Whether there is a statistically significant difference in the levels of DEE and acetone between MBA negative extracts and extracts giving an atypical response 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). FRS data from samples extracted by the interim SOP could not be included in this analysis as these extracts were not tested in the MBA. All data from CEFAS and DARD laboratories were combined before analysis. During the investigation CEFAS were the only laboratory to report atypical responses in the MBA (8 atypical responses in 45 samples).

Statistical analysis using non-parametric tests of CEFAS and DARD data shows that there were no significant differences in DEE (P=0.13) *(P=0.30 when external standards data used)* **and acetone (P=0.86)** *(P=0.88 when external standards data used)* **levels for atypical and negative MBA results (i.e. atypical MBA responses do not occur with high levels of solvent)**. The average concentration of DEE measured by GC-MS for atypical MBA responses was 0.08ìl/ml, however for negative MBA responses the average was 0.91 ìl/ml. The average concentration of acetone measured by GC-MS for atypical MBA responses was 0.69ìl/ml, however for negative MBA responses the average was 2.57ìl/ml.

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

iii. Whether there are any statistically significant differences in DEE and acetone concentrations between laboratories when extracts are produced using the interim method.

Concentrations of DEE and acetone as measured by headspace GC-MS remained in many of the sample extracts prepared by all laboratories at levels above the level of quantification (10µg/ml (0.01ìl/ml)[8]). The levels of DEE and acetone remaining in extracts varied between each laboratory. Summary statistics are reported in Table 1 *(summary statistics calculated using the external standard approach can be found at Table 3, Annex 4).* 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.

[7] Information provided by Anachem Ltd.

[8] ìl/ml = [ìg/ml / 1000] / 0.7146 where specific gravity of DEE=0.7146 g/ml
ìl/ml = [ìg/ml / 1000] / 0.780 where specific gravity of acetone=0.780 g/ml

Using the interim method there were significant differences in the concentrations of DEE and acetone measured by GC-MS between the laboratories (P<0.01) *(P<0.02 when external standards data used)***. Comparing the different laboratories (correcting for multiple comparisons) it can be shown that FRS has significantly higher levels of GC-MS DEE than CEFAS (P<0.05)** *(P=0.028 when external standards data used)* **and DARD (P<0.01)** *(P<0.01 when external standards data used)***. Further, DARD has significantly lower levels of acetone than FRS and CEFAS (p<0.01)** *(P<0.001 when external standards data used).*

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 of the original report. 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 who both have a higher throughput of samples than DARD. This may provide an explanation for 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 FRS sample prepared using the interim method, at 28,169ìg/ml of DEE in the extract using headspace GC-MS. Using a conversion factor[9], this is equivalent to 39ìl of DEE per ml of Tween extract. The highest average DEE level recorded by the laboratories was from FRS (4,588µg/ml of DEE in the extract), which is equivalent to 6.4ìl 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 in the original report, while limited, shows that a level of DEE >150ìl (107,190ìg) per ml of Tween 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.

Samples of shellfish extracts prepared for the MBA should not contain solvent above levels capable of causing biological effects in the mouse, if the evaporation stage has been carried out effectively. It was recommended that CEFAS and FRS would adopt the DARD approach to the evaporation procedure, thereby standardising measures to minimise potential solvent carry over. Overnight storage prior to re-suspension in Tween is not recommended as this could lead to potential adsorption of toxins onto surfaces and degradation of lipophilic toxins[10,11].

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

[10] 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.

iv. Whether there are any statistically significant differences in DEE and acetone concentrations between the original and interim methods used at FRS.

There are significant differences between the original and interim methods used at FRS with respect to the concentrations of DEE and acetone (P < 0.01) *(P<0.01 when external standards data used).* 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 evaporate, may lead to potential adsorption of toxins onto surfaces and/or possible 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 and method			DARD Interim SOP	CEFAS Interim SOP	FRS	
					Interim SOP	Original SOP
Number of samples			16	45	33	37
DEE by GC-MS	Mean	ìl/ml	**0.16**	**1.03**	**6.42**	**0.39**
	Median	ìl/ml	**0.07**	**0.14**	**2.59**	**0.01**
	Range	ìl/ml	**0.01-1.28**	**0.01-16.80**	**0.04-39.42**	**0.01-9.78**
Acetone by GC-MS	Mean	ìl/ml	**0.06**	**2.24**	**1.11**	**0.13**
	Median	ìl/ml	**0.05**	**1.13**	**0.73**	**0.11**
	Range	ìl/ml	**0.01-0.12**	**0.08-13.76**	**0.03-5.10**	**0.01-0.77**
DEE (mg/ml) by Gastec	Mean		13	35	2,813	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 weak correlation (ñ=-0.02) *(r = 0.046 when external standards data used*) **between concentrations of DEE and acetone within each sample. This is a reflection of the differences between the results observed at CEFAS and FRS. FRS data contains samples with high concentrations of DEE but lower concentrations of acetone; in contrast, CEFAS data contains samples with lower concentrations of DEE but**

[11] 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.

higher concentrations of acetone. The correlation between DEE and acetone when using the original FRS method is weak (ñ = 0.24) *(r=0.175 when external standards data used).*

52. The results of the experiments reported in Part 6, show that varying amounts of DEE, as measured by headspace GC-MS, 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.

CEFAS data for week 3 samples recalculated using the external standard approach.

LOD (µg/ml) DEE=5, Acetone=5
LOQ (µg/ml) DEE=10, Acetone=10.
[ìl/ml = (ìg/ml / 1000)/specific gravity of solvent]. DEE=0.7146 g/ml, acetone=0.780g/ml.
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.

CEFAS data week 3

Sample code	Replicate	Species	Gastec DEE (ìg/ml)	GC-MS DEE (ìg/ml)	GC-MS acetone (ìg/ml)	GC-MS DEE (ìl/ml)	GC-MS acetone (ìl/ml)	MBA
BTX/2003/0749	1	Cockles	250	1562	780	2.19	1.00	Negative
	2			1586	803	2.22	1.03	
	3			1570	774	2.20	0.99	
BTX/2003/0750	1	Cockles	<10	68	76	0.10	0.10	Positive (Atypical)
	2			65	63	0.09	0.08	
	3			75	76	0.10	0.10	
BTX/2003/0751	1	Cockles	<10	86	110	0.12	0.14	Positive (Atypical)
	2			93	82	0.13	0.11	
	3			91	94	0.13	0.12	
BTX/2003/0752	1	Cockles	<10	68	87	0.10	0.11	Negative
	2			76	73	0.11	0.09	
	3			74	98	0.10	0.13	
BTX/2003/0753	1	Cockles	<10	88	135	0.12	0.17	Positive (Atypical)
	2			96	108	0.13	0.14	
	3			94	131	0.13	0.17	
BTX/2003/0754	1	Cockles	20	924	532	1.29	0.68	Negative
	2			863	516	1.21	0.66	
	3			851	487	1.19	0.62	
BTX/2003/0755	1	Mussels	<10	102	56	0.14	0.07	Negative
	2			99	54	0.14	0.07	
	3			112	75	0.16	0.10	
BTX/2003/0756	1	Mussels	80	1714	816	2.40	1.05	Negative
	2			2036	843	2.85	1.08	
	3			2090	813	2.92	1.04	
BTX/2003/0757	1	Mussels	15	1352	1481	1.89	1.90	Negative
	2			1820	1795	2.55	2.30	
	3			1682	1662	2.35	2.13	
BTX/2003/0758	1	Cockles	<10	72	103	0.10	0.13	Positive (Atypical)
	2			89	132	0.12	0.17	
	3			87	145	0.12	0.19	
BTX/2003/0767	1	Cockles	<10	363	332	0.51	0.43	Negative
	2			416	349	0.58	0.45	
	3			370	305	0.52	0.39	
BTX/2003/0768	1	Mussels	<10	126	82	0.18	0.11	Negative
	2			140	90	0.20	0.12	
	3			130	86	0.18	0.11	
BTX/2003/0769	1	Mussels	10	437	282	0.61	0.36	Negative
	2			497	274	0.70	0.35	
	3			229	363	0.32	0.47	
BTX/2003/0770	1	Mussels	40	2421	3083	3.39	3.95	Negative
	2			2674	3144	3.74	4.03	
	3			2647	3186	3.70	4.08	
BTX/2003/0771	1	Mussels	<10	183	347	0.26	0.44	Negative
	2			446	260	0.62	0.33	
	3			212	360	0.30	0.46	

Additional FRS data for week 3 samples which was not available at the time of writing the original report (calculated using internal standard).

FRS Interim method, week 3 – additional data.								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
10486	A	Mussels	>400	7322*	472	10.25	0.61	N/A
	B			8213*	469	11.49	0.60	
	C			8245*	530	11.54	0.68	
5812	A	Mussels	>400	7664*	737	10.72	0.94	N/A
	B			7941*	895	11.11	1.15	
	C			7732*	1057	10.82	1.36	
5820	A	Mussels	300	471	1026	0.66	1.32	N/A
	B			510	1171	0.71	1.50	
	C			512	1120	0.72	1.44	
5821	A	Mussels	100	230	1905	0.32	2.44	N/A
	B			250	1998	0.35	2.56	
	C			256	2173	0.36	2.79	
5824	A	Mussels	>400	1345	1481	1.88	1.90	N/A
	B			1573	1577	2.20	2.02	
	C			1579	1537	2.21	1.97	
5828	A	Mussels	>400	4881*	409	6.83	0.52	N/A
	B			5706*	1138	7.98	1.46	
	C				No sample			
10509	A	Mussels	>400	2302	418	3.22	0.54	N/A
	B			2902	471	4.06	0.60	
	C			2694	470	3.77	0.60	
10522	A	Cockles	35	115#	381	0.16	0.49	N/A
	B			140#	469	0.20	0.60	
	C			117#	421	0.16	0.54	
10528	A	Mussels	10	<LOQ	717		0.92	N/A
	B			77#	759	0.11	0.97	
	C			<LOQ	764		0.98	

Annex 3

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

56. The data was re-analysed 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) to test whether DEE and acetone carry-over were dependent upon species. The ANOVA takes into account two factors that may confound the differences between species: length of 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 larger than 100% means that the size of the standard deviation is larger than the mean, and therefore, we would consider the data to be variable.

58. Study limitations and assumptions:

- On occasion, due to compromised seals on vials, not all three replicates per sample were available. Thus, to re-balance the design, averages were used as the unit for analysis. Using averages also allows the worst-case scenario for samples with high variability within the replicates.
- 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.
- Power analysis was carried out for Part 7 as *a priori* information on the prevalence of atypical results in cockles (approximately 40%) and mussels (approximately 1%) was available. For a power of 80% and a 5% significance level, a sample size greater than 15 samples would be enough to shown significantly greater concentrations in cockles when comparing against mussels.

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 detailed in Table 2. **There were no significant differences in the concentration of DEE (P = 0.88)** *(P=0.02 when external standards data used)* **and acetone between cockles and mussels (P = 0.20)** *(P=0.93 when external standards data used)*. On the basis of the dataset, differences in shellfish matrices do not appear to influence the volume of solvent carried over into the final extract as measured by headspace GC-MS.

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 (ìl/ml)	Acetone (ìl/ml)	DEE (ìl/ml)	Acetone (ìl/ml)
Mean	1.10	4.74	1.19	2.76
Median	0.05	2.55	0.19	0.56
Min	0.01	0.18	0.09	0.07
Max	4.98	12.57	4.15	11.70

ii. Whether variations in DEE and acetone varied substantially within replicate samples of the same species.

DEE and acetone concentrations vary considerably between replicate samples of each species. DEE CV=154% for cockles and 129% for mussels. Acetone CV=101% for cockles and 132 % for mussels. *When using external standard data, DEE CV=140% for cockles and 129% for mussels. Acetone CV=113% for cockles and 132% for mussels***.**

Possible causes for the variability in concentration 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.

Data collected on DEE and acetone concentrations in replicate samples of mussels recalculated using the external standard approach

LOD (µg/ml) DEE=5, Acetone=5
LOQ (µg/ml) DEE=10, Acetone=10
[ìl/ml = (ìg/ml / 1000)/specific gravity of solvent]. DEE=0.7146 g/ml, acetone=0.780g/ml.
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.

MUSSELS

Sample number	Replicate	GASTEC DEE (ug/ml)	GC-MS DEE (ug/ml)	GC-MS acetone (ug/ml)	DEE (ìl/ml)	Acetone (ìl/ml)
1	1	30	1403	3149	1.96	4.04
	2		1409	3175	1.97	4.07
	3		1453	3206	2.03	4.11
2	1	<10	91	280	0.13	0.36
	2		88	276	0.12	0.35
	3		86	272	0.12	0.35
3	1	10	1063	2717	1.49	3.48
	2		1048	2721	1.47	3.49
	3		1056	2739	1.48	3.51
4	1	50	2942	6558	4.12	8.41
	2		2926	6506	4.09	8.34
	3		3030	6736	4.24	8.64
5	1	<10	69	107	0.10	0.14
	2		67	105	0.09	0.13
	3		67	107	0.09	0.14
6	1	20	2727	9137	3.82	11.71
	2		2849	8937	3.99	11.46
	3		2812	9297	3.94	11.92
7	1	35	2221	5062	3.11	6.49
	2		2131	5021	2.98	6.44
	3		2212	5077	3.10	6.51
8	1	20	86	287	0.12	0.37
	2		80	284	0.11	0.36
	3		89	303	0.12	0.39
9	1	<10	67	52	0.09	0.07
	2		66	55	0.09	0.07
	3		64	51	0.09	0.07
10	1	<10	77	203	0.11	0.26
	2		76	196	0.11	0.25
	3		81	202	0.11	0.26
11	1	<10	85	887	0.12	1.14
	2		145	1130	0.20	1.45
	3		133	1099	0.19	1.41
12	1	<10	137	159	0.19	0.20
	2		152	172	0.21	0.22
	3		158	175	0.22	0.22
13	1	<10	64	126	0.09	0.16
	2		73	128	0.10	0.16
	3		68	124	0.10	0.16
14	1	<10	150	584	0.21	0.75
	2		145	569	0.20	0.73
	3		151	576	0.21	0.74
15	1	15	2346	4810	3.28	6.17
	2		2149	4690	3.01	6.01
	3		2211	4765	3.09	6.11
16	1	<10		No sample		
	2		70	145	0.10	0.19
	3		66	145	0.09	0.19

Annex 4

Ether and acetone data recalculated using the external standard approach for comparative purposes only

LOD (µg/ml) DEE=5, Acetone=5
LOQ (µg/ml) DEE=10, Acetone=10
[ìl/ml = (ìg/ml / 1000)/specific gravity of solvent]. DEE=0.7146 g/ml, acetone=0.780g/ml.
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.

DARD data

WEEK 1								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
0307435	A	Cockles	<10	65	89	0.09	0.11	Negative
	B			48	86	0.07	0.11	
	C			55	89	0.08	0.11	
0307436	A	Cockles	<10	45	54	0.06	0.07	Negative
	B			44	55	0.06	0.07	
	C			27	49	0.04	0.06	
0307437	A	Cockles	<10	37	36	0.05	0.05	Negative
	B			34	37	0.05	0.05	
	C			35	36	0.05	0.05	

WEEK 2								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
307485	A	Cockles	<10	< LOQ	15	0.01	0.02	Negative
	B			< LOQ	13	0.01	0.02	
	C			< LOQ	15	0.01	0.02	
307486	A	Cockles	<10	< LOQ	< LOQ	0.01	0.01	Negative
	B			< LOQ	< LOQ	0.01	0.01	
	C			< LOQ	< LOQ	0.01	0.01	
307638	A	Cockles	<10	72#	62	0.10	0.08	Negative
	B			57#	67	0.08	0.09	
	C			60#	58	0.08	0.07	
307639	A	Cockles	<10	< LOQ	55	0.01	0.07	Negative
	B			< LOQ	60	0.01	0.08	
	C			< LOQ	56	0.01	0.07	
307640	A	Cockles	<10	60#	53	0.08	0.07	Negative
	B			65#	57	0.09	0.07	
	C			63#	53	0.09	0.07	
307667	A	Mussel	<10	55#	14	0.08	0.02	Negative
	B			59#	15	0.08	0.02	
	C			66#	16	0.09	0.02	
307668	A	Mussel	50	775	10	1.08	0.01	Negative
	B			673	11	0.94	0.01	
	C			601	11	0.84	0.01	
307677	A	Cockles	<10	58#	96	0.08	0.12	Negative
	B			57#	73	0.08	0.09	
	C			59#	92	0.08	0.12	

307678	A	Cockles	<10	71#	88	0.10	0.11	Negative
	B			71#	92	0.10	0.12	
	C			73#	98	0.10	0.13	

WEEK 3								
Sample code	**Replicate**	**Species**	**GASTEC DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
307803	A	Cockles	<10	98#	36	0.14	0.05	Negative
	B			96#	36	0.13	0.05	
	C			98#	28	0.14	0.04	
307804	A	Cockles	<10	134#	31	0.19	0.04	Negative
	B			128#	40	0.18	0.05	
	C			131#	40	0.18	0.05	
307805	A	Cockles	<10	< LOQ	36	0.01	0.05	Negative
	B			< LOQ	32	0.01	0.04	
	C			< LOQ	41	0.01	0.05	
307845	A	Cockles	<10	101#	26	0.14	0.03	Negative
	B			122#	19	0.17	0.02	
	C			113#	19	0.16	0.02	
307916	A	Cockles	<10					Negative
	B							
	C							

CEFAS data

WEEK 1								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
BTX/2003/0700	1	Cockles	10	< LOQ	696	0.01	0.89	Positive (Atypical)
	2			71	376	0.10	0.48	
	3			81	438	0.11	0.56	
BTX/2003/0701	1	Cockles	<10	< LOQ	43	0.01	0.06	Negative
	2			< LOQ	37	0.01	0.05	
	3			< LOQ	69	0.01	0.09	
BTX/2003/0702	1	Cockles	20	122	1429	0.17	1.83	Negative
	2			73	694	0.10	0.89	
	3			47#	438	0.07	0.56	
BTX/2003/0703	1	Cockles	<10	< LOQ	81	0.01	0.10	Positive (Atypical)
	2			< LOQ	78	0.01	0.10	
	3			< LOQ	115	0.01	0.15	
BTX/2003/0704	1	Cockles	<10	< LOQ	58	0.01	0.07	Negative
	2			< LOQ	45	0.01	0.06	
	3			39#	66	0.05	0.09	
BTX/2003/0705	1	Cockles	<10	< LOQ	69	0.01	0.09	Negative
	2			< LOQ	82	0.01	0.10	
	3			< LOQ	68	0.01	0.09	
BTX/2003/0706	1	Mussels	50	556	438	0.78	0.56	Negative
	2			107	122	0.15	0.16	
	3			415	242	0.58	0.31	

BTX/2003/0707	1	Mussels	125	2049	1417	2.87	1.82	Negative
	2			Seal broken				
	3			2165	859	3.03	1.10	
BTX/2003/0708	1	Mussels	20	509	261	0.71	0.33	Negative
	2			275	128	0.38	0.16	
	3			118	87	0.17	0.11	
BTX/2003/0710	1	Mussels	<10	67	120	0.09	0.15	Negative
	2			43 #	74	0.06	0.09	
	3			53#	78	0.07	0.10	
BTX/2003/0711	1	Cockles	10	97	255	0.14	0.33	Negative
	2			< LOQ	112	0.01	0.14	
	3			59	163	0.08	0.21	
BTX/2003/0714	1	Cockles	40	343	364	0.48	0.47	Negative
	2			403	307	0.56	0.39	
	3			239	222	0.33	0.28	
BTX/2003/0715	1	Cockles	<10	< LOQ	52	0.01	0.07	Positive (Atypical)
	2			< LOQ	43	0.01	0.06	
	3			< LOQ	66	0.01	0.08	
BTX/2003/0716	1	Cockles	<10	< LOQ	129	0.01	0.16	Negative
	2			< LOQ	94	0.01	0.12	
	3			< LOQ	107	0.01	0.14	
BTX/2003/0719	1	Mussels	350	5552	346	7.77	0.44	Negative
	2			5227	212	7.32	0.27	
	3			7156	280	10.01	0.36	

WEEK 2								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
BTX/2003/0724	1	Cockles	55	< LOQ	1891	0.01	2.42	Negative
	2			41#	2873	0.06	3.68	
	3			42#	1840	0.06	2.36	
BTX/2003/0725	1	Cockles	100	328	3898*	0.46	5.00	Negative
	2			309	3882*	0.43	4.98	
	3			321	3752*	0.45	4.81	
BTX/2003/0726	1	Cockles	20	75	1918	0.10	2.46	Negative
	2			64	1981	0.09	2.54	
	3			76	2955	0.11	3.79	
BTX/2003/0727	1	Cockles	10	< LOQ	450	0.01	0.58	Negative
	2			< LOQ	685	0.01	0.88	
	3			< LOQ	490	0.01	0.63	
BTX/2003/0728	1	Cockles	15	< LOQ	399	0.01	0.51	Negative
	2			< LOQ	383	0.01	0.49	
	3			< LOQ	381	0.01	0.49	
BTX/2003/0729	1	Cockles	30	40#	1088	0.06	1.40	Negative
	2			68	1611	0.09	2.07	
	3			63	1821	0.09	2.33	
BTX/2003/0730	1	Cockles	20	69	1258	0.10	1.61	Negative
	2			69	1208	0.10	1.55	
	3			88	1429	0.12	1.83	
BTX/2003/0731	1	Cockles	20	76	495	0.11	0.64	Negative
	2			49#	458	0.07	0.59	
	3			< LOQ	548	0.01	0.70	

BTX/2003/0732	1	Cockles	<10	< LOQ	243	0.01	0.31	Negative
	2			< LOQ	222	0.01	0.28	
	3			< LOQ	266	0.01	0.34	
BTX/2003/0733	1	Cockles	<10	< LOQ	359	0.01	0.46	Positive (Atypical)
	2			< LOQ	374	0.01	0.48	
	3			< LOQ	381	0.01	0.49	
BTX/2003/0734	1	Mussels	10	< LOQ	223	0.01	0.29	Negative
	2			< LOQ	190	0.01	0.24	
	3			< LOQ	206	0.01	0.26	
BTX/2003/0735	1	Mussels	<10	< LOQ	638	0.01	0.82	Negative
	2			< LOQ	624	0.01	0.80	
	3			< LOQ	644	0.01	0.83	
BTX/2003/0736	1	Mussels	50	209	2957	0.29	3.79	Negative
	2			186	2861	0.26	3.67	
	3			257	4004*	0.36	5.13	
BTX/2003/0737	1	Mussels	<10	< LOQ	47	0.01	0.06	Negative
	2			< LOQ	45	0.01	0.06	
	3			< LOQ	61	0.01	0.08	
BTX/2003/0738	1	Mussels	<10	< LOQ	29	0.01	0.04	Negative
	2			< LOQ	32	0.01	0.04	
	3			< LOQ	32	0.01	0.04	

CEFAS week 3 data can be found on page 15.

FRS data using interim SOP

WEEK 1								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
5732	A	Mussels	>10000	2537	590	3.25	0.76	N/A
	B			3268	647	4.19	0.83	
	C							
5733	A	Mussels	5000	1007	724	1.41	0.93	N/A
	B			1378	780	1.93	1.00	
	C							
5735	A	Mussels	>10000					N/A
	B			3048	896	4.27	1.15	
	C			4467	1477	6.25	1.89	
5737	A	Mussels	8000	1349	415	1.89	0.53	N/A
	B			640	190	0.90	0.24	
	C			1381	424	1.93	0.54	
5738	A	Mussels	1000	1021	485	1.43	0.62	N/A
	B			445	113	0.62	0.14	
	C							
5740	A	Mussels	>10,000	8409	2262	11.77	2.90	N/A
	B			6136	2146	8.59	2.75	
	C					16.59	0.00	
5742	A	Mussels	>10000	1176	92	1.65	0.12	N/A
	B			1476	108	2.06	0.14	
	C			1102	107	1.54	0.14	

5744	A	Mussels	<400	35#	38	0.05	0.05	N/A
	B			34#	41	0.05	0.05	
	C							
5745	A	Mussels	2000	250	1097	0.35	1.41	N/A
	B			160	825	0.22	1.06	
	C			268	1159	0.37	1.49	
5746	A	Mussels	4000	639	1261	0.89	1.62	N/A
	B			714	1206	1.00	1.55	
	C			565	1127	0.79	1.44	
5749	A	Mussels	>10000	11464	84	16.04	0.11	N/A
	B							
	C							
5750	A	Mussels	>10000	11152	85	15.61	0.11	N/A
	B			12942	96	18.11	0.12	
	C			24479	130	34.26	0.17	
5751	A	Mussels						N/A
	B							
	C							
5752	A	Mussels	7000					N/A
	B			1025	131	1.43	0.17	
	C			908	123	1.27	0.16	
5754	A	Mussels	400					N/A
	B			114	39	0.16	0.05	
	C			92	43	0.13	0.06	

WEEK 2								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
5796	A	Mussels	>400	324	20	0.45	0.03	N/A
	B			267	17	0.37	0.02	
	C							
5799	A	Mussels	>400	25932*	375	36.29	0.48	N/A
	B			16289*	431	22.79	0.55	
	C			18012*	433	25.21	0.56	
5800	A	Mussels	>400					N/A
	B			3668*	471	5.13	0.60	
	C			3426	442	4.79	0.57	
5801	A	Mussels	>400	1318	1107	1.84	1.42	N/A
	B			1371	1181	1.92	1.51	
	C			1404	1196	1.96	1.53	
5802	A	Mussels	50					N/A
	B			93	223	0.13	0.29	
	C							
5803	A	Mussels	80	266	241	0.37	0.31	N/A
	B							
	C			287	290	0.40	0.37	
5804	A	Mussels	>400	1269	283	1.78	0.36	N/A
	B			1319	299	1.85	0.38	
	C							
5805	A	Mussels	50	187	261	0.26	0.33	N/A
	B			198	290	0.28	0.37	
	C			197	294	0.28	0.38	

5807	A	Mussels	100	336	411	0.47	0.53	N/A
	B							
	C			345	432	0.48	0.55	
5808	A	Mussels	300	412	726	0.58	0.93	N/A
	B			468	790	0.65	1.01	
	C			500	795	0.70	1.02	

WEEK 3								
Sample code	**Replicate**	**Species**	**GASTEC DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
10486	A	Mussels	>400	5081*	339	7.11	0.43	N/A
	B			6545*	385	9.16	0.49	
	C			5000*	331	7.00	0.42	
5812	A	Mussels	>400	5327*	508	7.45	0.65	N/A
	B			4582*	508	6.41	0.65	
	C			3982*	531	5.57	0.68	
5820	A	Mussels	300	356	745	0.50	0.96	N/A
	B			402	848	0.56	1.09	
	C			357	741	0.50	0.95	
5821	A	Mussels	100	254	2108	0.35	2.70	N/A
	B			250	1961	0.35	2.51	
	C			244	1805	0.34	2.31	
5824	A	Mussels	>400	1211	1271	1.69	1.63	N/A
	B			1184	1125	1.66	1.44	
	C			1199	1108	1.68	1.42	
5828	A	Mussels	>400	2004	175	2.80	0.22	N/A
	B			752	154	1.05	0.20	
	C							
10509	A	Mussels	>400	1127	218	1.58	0.28	N/A
	B			1189	196	1.66	0.25	
	C			1220	224	1.71	0.29	
10522	A	Cockles	35	76#	222	0.11	0.28	N/A
	B			78	207	0.11	0.27	
	C			80#	261	0.11	0.33	
10528	A	Mussels	10	< LOQ	366	0.01	0.47	N/A
	B			54	376	0.11	0.48	
	C			< LOQ	390	0.01	0.50	

FRS data using original SOP

WEEK 1								
Sample code	**Replicate**	**Species**	**GASTEC DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
5732	A	Mussels	<400	< LOQ	24	0.01	0.03	Negative
	B			37	23	0.05	0.03	
	C						0.00	
5733	A	Mussels	<400	1203	767	1.68	0.98	Negative
	B							
	C			< LOQ	< LOQ	0.01	0.01	

5734	A	Mussels	<400	< LOQ	27	0.01	0.03	Negative
	B			< LOQ	17	0.01	0.02	
	C			< LOQ	13	0.01	0.02	
5735	A	Mussels	<400	< LOQ	18	0.01	0.02	Positive (typical)
	B							
	C							
5736	A	Mussels	<400	49	121	0.07	0.16	Negative
	B			47	111	0.07	0.14	
	C			30#	120	0.04	0.15	
5737	A	Mussels	<400	< LOQ	< LOQ	0.01	0.01	Negative
	B							
	C			39	< LOQ	0.06	0.01	
5738	A	Mussels	<400	381	115	0.53	0.15	Negative
	B							
	C			417	101	0.58	0.13	
5740	A	Mussels	<400	35#	30	0.05	0.04	Negative
	B			32#	28	0.04	0.04	
	C			26#	24	0.04	0.03	
5741	A	Mussels	<400	39#	28	0.05	0.04	Negative
	B			< LOQ	35	0.01	0.05	
	C			< LOQ	21	0.01	0.03	
5742	A	Mussels	<400	34#	29	0.05	0.04	Negative
	B			38	21	0.05	0.03	
	C							
5744	A	Mussels	<400	< LOQ	13	0.01	0.02	Negative
	B			35#	27	0.05	0.04	
	C			37#	29	0.05	0.04	
5745	A	Mussels	<400	34#	24	0.05	0.03	Negative
	B							
	C			42	37	0.06	0.05	
5746	A	Mussels	<400	36#	38	0.05	0.05	Negative
	B			49	35	0.07	0.04	
	C			37#	42	0.05	0.05	
5749	A	Mussels	<400	< LOQ	18	0.01	0.02	Negative
	B			9881	87	13.83	0.11	
	C			< LOQ	19	0.01	0.02	
5750	A	Mussels	<400	46	24	0.06	0.03	Negative
	B			30#	21	0.04	0.03	
	C			26#	21	0.04	0.03	
5751	A	Mussels	<400	28#	23	0.04	0.03	Negative
	B			34#	28	0.05	0.04	
	C			32#	26	0.04	0.03	
5752	A	Mussels	<400	< LOQ	17	0.01	0.02	Negative
	B			< LOQ	20	0.01	0.03	
	C							
5754	A	Mussels	<400	34	71	0.05	0.09	Negative
	B			33#	78	0.05	0.10	
	C			< LOQ	129	0.01	0.17	

WEEK 2								
Sample code	**Replicate**	**Species**	**GASTEC DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
5796	A	Mussels	<10	< LOQ	< LOQ	0.01	0.01	Negative
	B			< LOQ	< LOQ	0.01	0.01	
	C							
5799	A	Mussels	<10	< LOQ	20	0.01	0.03	Negative
	B			< LOQ	< LOQ	0.01	0.01	
	C			< LOQ	< LOQ	0.01	0.01	
5800	A	Mussels	<10	< LOQ	42	0.01	0.05	Negative
	B			< LOQ	36	0.01	0.05	
	C			33	33	0.05	0.04	
5801	A	Mussels	<10	< LOQ	66	0.01	0.08	Negative
	B							
	C			< LOQ	70	0.01	0.09	
5802	A	Mussels	<10	< LOQ	32	0.01	0.04	Negative
	B			< LOQ	44	0.01	0.06	
	C			< LOQ	44	0.01	0.06	
5803	A	Mussels	<10	< LOQ	28	0.01	0.04	Negative
	B							
	C			< LOQ	17	0.01	0.02	
5804	A	Mussels	<10	< LOQ	14	0.01	0.02	Negative
	B			< LOQ	16	0.01	0.02	
	C							
5805	A	Mussels	<10	< LOQ	16	0.01	0.02	Negative
	B			< LOQ	22	0.01	0.03	
	C			< LOQ	16	0.01	0.02	
5807	A	Mussels	<10	< LOQ	26	0.01	0.03	Negative
	B			< LOQ	22	0.01	0.03	
	C			< LOQ	14	0.01	0.02	
5808	A	Mussels	25	64#	65	0.09	0.08	Negative
	B			95#	54	0.13	0.07	
	C			63#	57	0.09	0.07	

WEEK 3								
Sample code	**Replicate**	**Species**	**Gastec DEE (ìg/ml)**	**GC-MS DEE (ìg/ml)**	**GC-MS acetone (ìg/ml)**	**DEE (ìl/ml)**	**Acetone (ìl/ml)**	**MBA**
10486	A	Mussels	<10	< LOQ	19	0.01	0.02	Negative
	B			< LOQ	20	0.01	0.03	
	C			< LOQ	19	0.01	0.02	
5812	A	Mussels	<10	74	22	0.10	0.03	Negative
	B			< LOQ	22	0.01	0.03	
	C			< LOQ	23	0.01	0.03	
5820	A	Mussels	<10	< LOQ	20	0.01	0.03	Negative
	B			< LOQ	20	0.01	0.01	
	C			< LOQ	21	0.01	0.01	
5821	A	Mussels	<10	< LOQ	20	0.01	0.03	Negative
	B			< LOQ	37	0.01	0.05	
	C			< LOQ	32	0.01	0.04	
5824	A	Mussels	<10			0.01	0.00	Negative
	B			< LOQ	32	0.01	0.04	
	C			< LOQ	31	0.01	0.04	

5828	A	Mussels	<10	< LOQ	23	0.01	0.03	Negative
	B			< LOQ	22	0.01	0.03	
	C			< LOQ	28	0.01	0.04	
10509	A	Mussels	10	87	54	0.12	0.07	Negative
	B			90	79	0.13	0.10	
	C			80	49	0.11	0.06	
10522	A	Cockles	<10	< LOQ	30	0.01	0.04	Negative
	B			< LOQ	28	0.01	0.04	
	C			< LOQ	28	0.01	0.04	
10528	A	Mussels	<10	< LOQ	58	0.01	0.07	Negative
	B			< LOQ	54	0.01	0.07	
	C			< LOQ	50	0.01	0.06	

Table 3. Summary statistics of solvent concentrations in cockle and mussel extracts prepared by each laboratory using the **external standard approach**.

Solvent and method			DARD Interim SOP	CEFAS Interim SOP	FRS	
					Interim SOP	Original SOP
Number of samples			16	45	33	37
DEE by GC-MS	Mean	ìl/ml	0.13	0.64	3.89	0.19
	Median	ìl/ml	0.08	0.10	1.57	0.02
	Range	ìl/ml	0.01-0.96	0.01-8.37	0.05-28.10	0.01-4.62
Acetone by GC-MS	Mean	ìl/ml	0.06	0.86	0.67	0.06
	Median	ìl/ml	0.05	0.38	0.45	0.03
	Range	ìl/ml	0.01-0.12	0.04-4.93	0.02-2.51	0.01-0.50
DEE (mg/ml) by Gastec	Mean		13	35	2,813	200
	Median		10	10	400	25
	Range		10-50	10-350	10-10,000	10-400

Table 4 Summary statistics of concentrations of solvents from 16 replicate samples of each shellfish species prepared by CEFAS using the **external standard approach**.

	Cockles extracted using interim SOP		Mussels extracted using interim SOP	
	DEE (ìl/ml)	Acetone (ìl/ml)	DEE (ìl/ml)	Acetone (ìl/ml)
Mean	0.71	3.04	1.19	2.76
Median	0.07	1.07	0.19	0.56
Min	0.01	0.05	0.09	0.07
Max	2.52	11.52	4.15	11.70