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► **To cite this version:**

Susana Margarida Rodrigues, Paulo Vale, Maria João Botelho, Susana Gomes. Extraction recoveries and stability of DSP toxins in naturally contaminated samples. *Food Additives and Contaminants*, 2009, 26 (02), pp.229-235. 10.1080/02652030802290530 . hal-00577408

**HAL Id: hal-00577408**

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Submitted on 17 Mar 2011

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Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-112.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	17-Jun-2008
Complete List of Authors:	Rodrigues, Susana; Instituto Nacional dos Recursos Biológicos - IPIMAR, U-Amb Vale, Paulo; Instituto Nacional dos Recursos Biológicos - IPIMAR, U-Amb Botelho, Maria João; Instituto Nacional dos Recursos Biológicos - IPIMAR, U-Amb Gomes, Susana; Instituto Nacional dos Recursos Biológicos - IPIMAR, U-Amb
Methods/Techniques:	Chromatography - LC/MS, Extraction, Method validation, Quality assurance
Additives/Contaminants:	Phycotoxins – DSP
Food Types:	Seafood

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## Extraction recoveries and stability of DSP toxins in naturally contaminated samples

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### Abstract

During the last few years the occurrence of a high percentage of esters of DSP toxins has been observed in shellfish from the Portuguese coast. Most of the commercial bivalves contain DSP toxins in ester forms, either acyl derivatives of okadaic acid (OA) or of dinophysistoxin-2 (DTX-2). The stability of these toxins in shellfish tissues and in raw methanol extracts was investigated in two different species naturally contaminated, mussel and carpet shell, over a four week period. The results for both species revealed that DSP toxins were more stable in tissue than in raw methanol extracts. Losses of DSP toxins were seen in the first two weeks and were more than 30%, but after that a period of stabilisation was observed. The decrease was due probably from losses of esters of OA and DTX-2, the free toxins were stable over the period studied. The extraction most commonly used for chemical and biochemical assays relied on methanolic extraction with aqueous 80% methanol. In this work we have tested the extraction solvent on the extractability of DSP toxins from several naturally contaminated species. A single dispersive extraction with methanol, with solvent ratios of 70%, 80%, 90% and 100% were tested. After alkaline hydrolysis of esterified toxins and cleanup with hexane and dichloromethane, the samples were analysed by LC-MS. The recovery of DSP toxins increased with increasing percentage of methanol up to 90%. A decrease in recovery with 100% methanol was observed probably due to problems during the liquid-liquid partitioning.

**Keywords:** Okadaic acid, dinophysistoxin-2, acyl esters, solvent extraction, shellfish

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## Introduction

Diarrheic shellfish poisoning (DSP) is the marine syndrome that affects most recurrently shellfish from the Portuguese coast. Most of the commercial bivalves contain DSP toxins in ester forms, either acyl derivatives of okadaic acid or acyl derivatives of dinophysistoxin-2 (Quilliam *et al.*, 2003, Vale, 2006a). In highly contaminated Portuguese shellfish, it was demonstrated that 98-99% of okadaic acid (OA) and dinophysistoxin-2 (DTX-2) were found in the ester form (DTX-3), being detected only after hydrolysis of shellfish extracts. For mussels (*Mytilus galloprovincialis*) and donax clams (*Donax* spp.) OA and DTX-2 were found both esterified and in free form, with variable percentage of acyl esters (Vale and Sampayo, 2002; Vale, 2004; Vale, 2006b). Dinophysistoxin-1 (DTX-1) was not found until now in Portuguese shellfish (Vale and Sampayo, 2002).

Due to the absence of commercial standards and the large diversity of acyl esters that can be present in shellfish samples, they are indirectly analysed by an alkaline hydrolysis reaction that releases fatty acids from the parent toxins and gives an indirect estimation of their abundance. The hydrolysis procedure is a good option to quantify the amount of toxin esters belonging to OA-group and the effectiveness of hydrolysis depends on the binominous temperature/time of reaction and the base concentration used (Rodrigues, 2007, Villar-González *et al.*, 2008).

Few studies have been conducted about the behaviour of DSP toxins with storage at freezing temperatures immediately after harvesting the samples. The stability of DSP toxins in shellfish tissues and in raw methanol extracts was studied in two different naturally contaminated species, mussel and carpet shell, after harvesting and stored at  $-30^{\circ}\text{C}$  over a four week period.

The extraction most commonly used until recently for chemical and biochemical assays was based on the method of Lee *et al.* (1987) and relied on extraction with aqueous 80% methanol. New information was reported recently about the low extractability of DSP esters with the use of 80% methanol (Holland *et al.*, 2003, McNabb *et al.*, 2005). Due to the high percentage of acyl esters commonly found in shellfish it is important to use efficient procedures that can extract DSP toxins, particularly ester forms, from shellfish tissues. In this work we have also tested the extraction solvent on the extractability of DSP toxins from several naturally contaminated shellfish species.

## Material and methods

Shellfish samples used in this study were collected from the northwest coast of Portugal in the summer of 2005 and were obtained as part of the biotoxins monitoring programme. Samples were received in the laboratory within 24 h of being removed from the field and were analysed as soon as they arrived at the laboratory.

### ***Stability of DSP toxins***

The study on stability of DSP toxins was performed in two different species naturally contaminated with DSP toxins, mussel (*Mytilus galloprovincialis*) and carpet shell (*Venerupis pullastra*). Stability studies were carried out on shellfish tissues and on raw methanol extracts for different times of storage and were performed in triplicate (three aliquots were selected on each day).

Fresh edible tissues were removed from the shell, homogenised and 5g aliquots were weighed into 18 capped polypropylene tubes. All the aliquots were stored at  $-30^{\circ}\text{C}$  with the exception of the aliquots that were processed at the same day (day 0).

Day 0: For each shellfish species 3 tubes with 5g homogenate were extracted with 20 ml of methanol:water (80:20, v/v) using dispersive extraction procedure and centrifuged. From each tube a 2 ml supernatant aliquot was taken for total DSP determination and a 2 ml supernatant aliquot for free DSP toxins determination. The remaining raw methanol extracts were stored at  $-30^{\circ}\text{C}$ . Hydrolysis was performed with 400  $\mu\text{l}$  of 1M NaOH at  $40^{\circ}\text{C}$  for 45 min. and neutralised with 425  $\mu\text{l}$  of 1M HCl.

The cleanup of the hydrolysed and unhydrolysed extracts was performed through double washing with 2 ml hexane, addition of 0.5 ml of water and extracted twice with 2 ml of dichloromethane. The combined dichloromethane extracts were dried at  $38^{\circ}\text{C}$  under reduced pressure on a Rapidvap and resuspended in 500  $\mu\text{l}$  of methanol:water (80:20, v/v). 2.5  $\mu\text{l}$  aliquots were injected in triplicate on the LC-MS system according with the method described by Vale and Sampayo (2002).

Day 4: *Stability on raw methanol extract:* From the raw methanol extracts stored at day 0 a 2 ml supernatant aliquot was taken for total DSP determination and a 2 ml supernatant aliquot for free DSP toxins determination. The hydrolysis and cleanup procedure was the same described above.

*Stability on shellfish tissues:* From the shellfish tissues stored at day 0 a 5 g homogenate were taken in triplicate and extracted with 20 ml of methanol:water (80:20, v/v). After centrifugation a 2 ml supernatant aliquot was taken for total DSP determination and a 2 ml supernatant aliquot for free DSP toxins determination. The hydrolysis and cleanup procedure was the same described above.

Day 7, 14, 21 and 28: The same procedure described for day 4.

### ***Solvent Extraction***

Seven shellfish samples were analysed and included mussel (*Mytilus galloprovincialis*), cockle (*Cerastoderma edule*), grooved carpet shell (*Ruditapes decussates*), carpet shell (*Venerupis pullastra*), surf clam (*Spisula solida*), razor clam (*Ensis* spp.) and donax clam (*Donax* spp.). For each species the

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3 whole shellfish tissues were homogenised and 5 g aliquots were taken into 12 polypropylene tubes.  
4 The extraction procedure was performed in triplicate with methanol:water ratios of 70%, 80%, 90%  
5 and 100% using a single dispersive extraction with 20 ml of the methanol solutions to be tested. A 2  
6 ml aliquot of methanol extracts were hydrolysed with 400 µl of 1M NaOH at 50°C for 1h and  
7 neutralised with 425 µl of 1M HCl. After alkaline hydrolysis of esterified toxins, the extracts were  
8 cleaned twice with 2 ml of hexane and dichloromethane extracted. For 70% and 80% methanolic  
9 extracts 0.5 ml of water was added before dichloromethane cleanup, while for 90% and 100% extracts  
10 1 and 1.5 ml of water was added respectively. The combined dichloromethane layers were dried under  
11 reduced pressure and re-suspended in 500 µl of 80% aqueous methanol. 2.5 µl aliquots were injected  
12 in triplicate into the LC-MS.  
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### 20 21 *Liquid chromatography-mass spectrometry*

22 Liquid chromatography-mass spectrometry (LC-MS) was performed on a Hewlett-Packard (HP)  
23 Model 1100 coupled with an HP model 1946A Series single quadrupole mass spectrometer through an  
24 atmospheric pressure electrospray ionization (ESI) interface operated in the negative ion mode  
25 according with the method described by Vale and Sampayo (2002). Briefly a 2.5 µl aliquot was  
26 separated on a Merck Lichrospher 100 RP-18 column (5 µm, 125×2 mm) protected by a guard column  
27 (5 µm, 4×4 mm) with a mobile phase consisting of acetonitrile: 0.5% acetic acid (65:35, v/v) pumped  
28 at 200 µl/min. The column temperature was kept at 30°C. After 2 min. of separation the effluent was  
29 directly coupled with the mass spectrometer and selected ion monitoring (SIM) was used to record the  
30 signals from the [M-H]<sup>-</sup> ions at *m/z* 803.5 for OA and DTX-2. Identities of OA homologues peaks  
31 were confirmed by matching of retention time with standards compounds. The linear calibration curve  
32 consisted of six standard solution in the range corresponding to 0-0.96 µg/ml of OA in methanol:water  
33 (80:20, v/v), prepared from the OA certified reference material (NRC CRM-OA-b) from Marine  
34 Biosciences Institute (NRC, Canada). For DTX-2 the response was assumed identical to OA on a peak  
35 area basis.  
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## 48 **Results**

### 49 *Stability of DSP toxins*

50 The stability of DSP toxins in shellfish tissues and in raw methanol extracts was evaluated during 28  
51 days of storage. For both species, the concentration of total DSP toxins was determined by LC-MS in  
52 the hydrolysed extracts and represents the concentration of total OA plus total DTX-2 (free OA and  
53 free DTX-2 plus the OA and DTX-2 that resulted from the hydrolysis of acyl esters of OA and acyl  
54 esters of DTX-2). Free DSP toxins (free OA plus free DTX-2) were also quantified by LC-MS in the  
55 unhydrolysed extracts. DTX-1 was not found in these samples. The results for both species are  
56 presented in Figure 1. For carpet shell total DSP concentration decreased 20% in tissues homogenates  
57 and 35% in raw methanolic extract in the first two weeks, and no further decrease was observed in the  
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3 two remaining weeks of storage at  $-30^{\circ}\text{C}$  (Figure 1a and Table 1). For carpet shell the concentrations  
4 of free DSP toxins were very low, near the quantification limit. Like in other reports for this species  
5 OA and DTX-2 were found 97% esterified (Vale and Sampayo, 2002; Vale, 2004; Vale, 2006b). For  
6 free DSP toxins the concentration in raw methanol extracts seems to have increased slightly, while in  
7 shellfish tissue a slight decrease was observed (Figure 1b). A decrease of concentration of DSP esters  
8 (acyl OA and acyl DTX-2) was observed in shellfish tissues and in methanolic extracts, with losses  
9 higher than 20% in shellfish tissue and higher than 35% in methanolic extracts after two weeks (Figure  
10 1c). The stability of total OA was similar to the stability of total DTX-2 both in shellfish tissues and in  
11 raw extracts, as well as the stability of acyl esters of OA and DTX-2.  
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19 For mussels the same behaviour was observed for total DSP toxins in tissues homogenates and raw  
20 methanolic extracts with a decrease of 25% in total DSP concentration in the first two weeks of  
21 storage at  $-30^{\circ}\text{C}$  (Figure 1d and Table 2). The stability of OA and DTX-2 was similar in tissues and in  
22 raw extracts. For free DSP toxins no significant variations occurred in tissues homogenates and the  
23 slight increase observed in the methanolic extracts was not statistically significant (Figure 1e). The  
24 concentration of DSP esters decreased in methanolic extracts from the first week of storage and after  
25 two weeks losses of 40% were observed either in shellfish tissues or in methanolic extracts. In both  
26 cases comparing the stability OA acyl and DTX-2 acyl esters, the decrease of DTX-2 esters  
27 concentration was higher than the observed for OA esters. For acyl OA esters losses of 36% and 40%  
28 were found in tissues and in raw extracts, while for acyl DTX-2 esters the losses were higher than  
29 65%.  
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### 39 *Solvent Extraction*

40 The extraction efficiency was tested for different shellfish matrices with different profiles of DSP  
41 contamination. For this study two shellfish species contaminated with only acyl esters of OA (carpet  
42 shell and donax clam) were chosen and five species contaminated with acyl esters of OA and DTX-2  
43 (cockle, grooved carpet shell, mussel, razor clam and surf clam). The results obtained for the different  
44 methanol:water ratios tested with single dispersive extraction are presented in Table 3 and Figure 2.  
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50 The highest DSP concentrations were obtained with 90% methanol for all the matrices analysed with  
51 the exception of carpet shell and grooved carpet shell. 90% methanol was able to extract 10 to 28%  
52 more DSP toxins from shellfish tissues than 80% methanol (Table 3 and 4). Low recoveries were  
53 obtained for OA and DTX-2 with 70% methanol for all the species analysed, with this extraction  
54 solvent more than 40% of DSP toxins remained in shellfish tissues (Table 4). The DSP concentrations  
55 obtained with 100% methanol were lower than 90% methanol and high variability was observed  
56 between triplicates (Table 3).  
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## Discussion

Some studies on the stability of DSP toxins in shellfish tissues have been conducted with the scope of preparing contaminated shellfish tissues to use as reference materials (Hess *et al.*, 2007; McCarron *et al.*, 2007). McCarron *et al.* (2007) reported in a mussel tissue contaminated with OA and DTX-2 that these compounds were stable during the storage at  $-20^{\circ}\text{C}$ , but the mussel material used was frozen for several years prior to the study.

In the present work, the study was conducted in fresh shellfish tissues after harvesting and the stability of DSP toxins was evaluated during the period of storage. For both species losses of DSP toxins in shellfish tissues up to 20-25% occurred in the first two weeks, after that period a stabilisation was observed. The decrease was due mainly from losses of esters of OA and DTX-2. The free toxins were stable over the period studied, however the concentration in raw methanol extracts seems to have increased slightly probably due to some enzymatic activity in the extract that originate some hydrolysis of esters forms with the release of the parent compounds. A possible route for DSP acyl esters degradation is the oxidation of the carbon chain of acyl groups bounded to OA and DTX-2. The fatty acids attached to OA and DTX-2 found in Portuguese shellfish ranged from a carbon chain of C14 to C22 with saturated and unsaturated carbon bonds (Quilliam *et al.*, 2003, Vale, 2006a, Vale, 2006b). Oxidative mechanisms of fatty acids can occur in the double bond of monoenoic acids, polyenoic acids and in conjugated double-bond systems. The last one oxidizes with considerable ease by oxidation of the terminal carbons in the conjugated system (Streitweiser and Heathcock, 1985).

The results for both species revealed that DSP toxins were more stable in tissue than in raw methanol extracts, losses of OA and DTX-2 were higher than the observed for tissues. These results are important for monitoring proposes when some times it is necessary to repeat the analyses. The study of the stability on raw methanol extract was performed in a way to evaluate the possibility of using methanol extracts. These extracts are routinely kept as safeguard measure in case of laboratory accidents during sample preparation, additionally tissue homogenates are also kept for monitoring and research purposes. To obtain reproducibility and reliable results it is recommended that DSP determination should be performed during the first week of storage and starting from the shellfish tissue rather than from methanolic extract

The study on the extraction efficiency with different methanol ratios revealed a low extractability of DSP toxins with 70% and 80% methanol. Aqueous 90% methanol was more efficient in recovering DSP toxins than 80% methanol as observed by Holland *et al.* (2003) and McNabb *et al.* (2005). According to these authors it's due to an improved extraction of esters forms of OA and DTX-2 from shellfish tissues. The DSP concentrations obtained with pure methanol were lower than with 90% methanol. Poor recoveries were obtained with this solvent probably due to problems of phase



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3 separation during liquid-liquid partitioning. The phase separation of 100% methanol and hexane is not  
4 complete, part of the hexane will stay in the methanol layer and the residual hexane can interfere when  
5 after addition of water a dichloromethane clean-up is applied resulting in problems of phase  
6 separation. The creation of an emulsion during dichloromethane extraction may have occurred. To use  
7 hexane to wash an extract to remove fat a maximum percentage of 80% methanol is required. If water  
8 was added before hexane washing in order to obtain an extract containing 80% methanol there will be  
9 probably less problems in phase separation.  
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16 Some authors have reported that pure methanol could improve efficiency of extraction but the co-  
17 eluting matrix compounds can affect the ionization efficiency and cause poor reproducibility and  
18 accuracy (Goto *et al.*, 2001, Ito and Tsukada, 2001, McNabb *et al.*, 2005). McNabb *et al.* (2005)  
19 recommended the use of 90% methanol because it provides high recoveries and keep the MS signal  
20 enhancement/suppression effects at acceptable levels.  
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### 28 **Acknowledgements**

29 Programme “Safety, Surveillance and Quality of Bivalve Molluscs” (QCAIII/med.4/MARE  
30 Programme) supported this work.  
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## Captions

Figure 1. Stability of DSP toxins in shellfish tissues and raw methanol extracts of carpet shell a), b), c) and mussel d), e), f) during 28 days of storage at  $-30^{\circ}\text{C}$  (each value is the mean  $\pm$  SD). and d) total OA+DTX-2, b) and e) free OA+DTX-2, c) and f) total acyl OA+acyl DTX-2.

Figure 2. Concentration of a) total OA, b) total DTX-2 and c) total DSP determined by LC-MS for different shellfish species using different methanol ratios in the extraction procedure (each bar represents mean  $\pm$  SD). For donax clam and grooved carpet shell the extraction was not performed with 70% methanol. DTX-2 was not present in carpet shell and donax clam samples.

Table 1. DSP concentrations obtained in carpet shell tissues and raw methanol extracts during the 28 days of storage at  $-30^{\circ}\text{C}$  (mean  $\pm$  SD).

Table 2. DSP concentrations obtained in mussel tissues and raw methanol extracts during the 28 days of storage at  $-30^{\circ}\text{C}$  (mean  $\pm$  SD).

Table 3. Total OA+DTX-2 concentration obtained in different matrices using different methanol ratios in the extraction procedure (mean  $\pm$  SD).

Table 4. Percentage recoveries of total DSP (OA+DTX-2) obtained in different matrices using different methanol ratios relatively to 90% methanol.

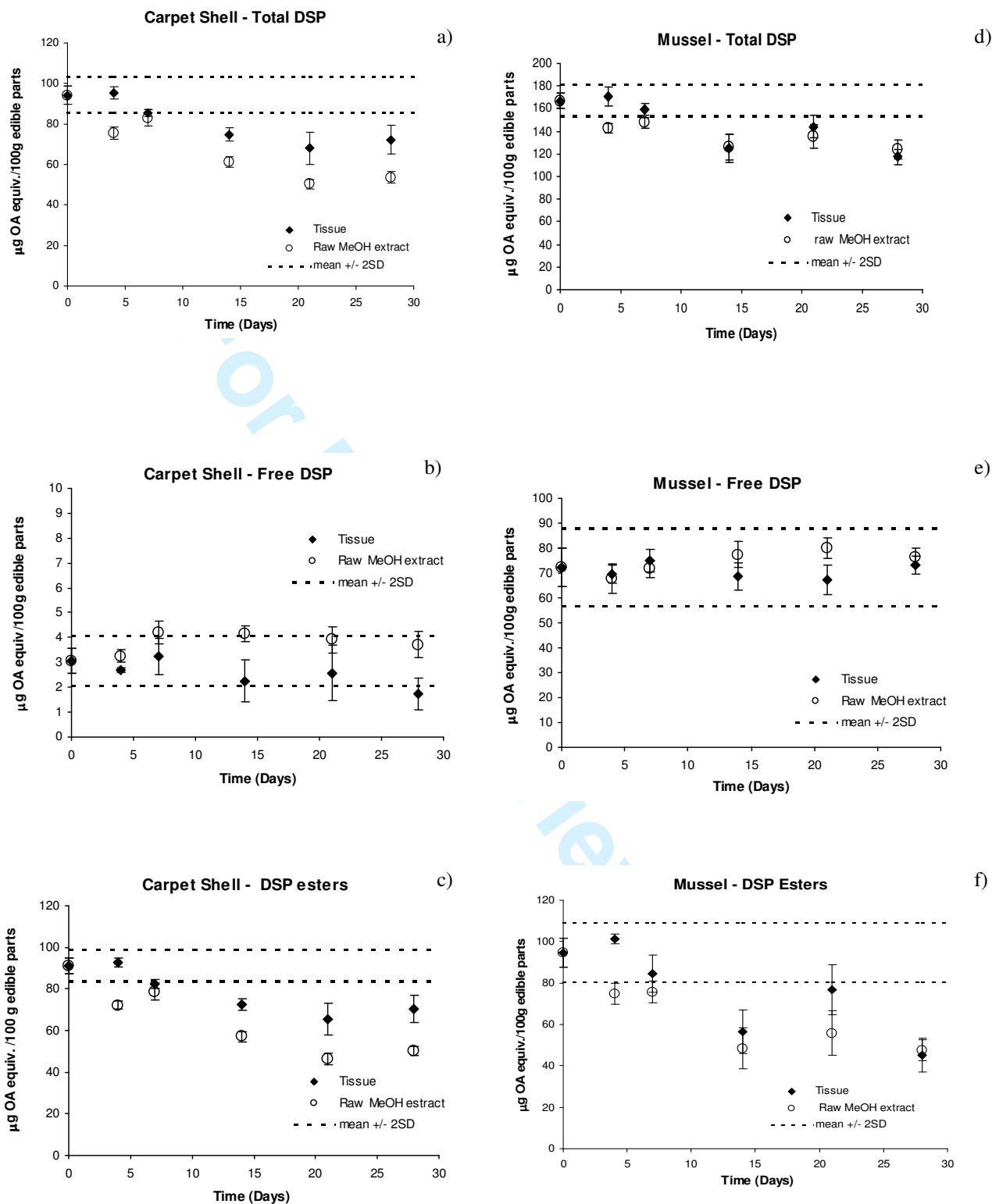


Figure 1. Stability of DSP toxins in shellfish tissues and raw methanol extracts of carpet shell a), b), c) and mussel d), e), f) during 28 days of storage at  $-30^{\circ}\text{C}$  (each value is the mean  $\pm$  SD). a) and d) total OA+DTX-2, b) and e) free OA+DTX-2, c) and f) total acyl OA+acyl DTX-2.

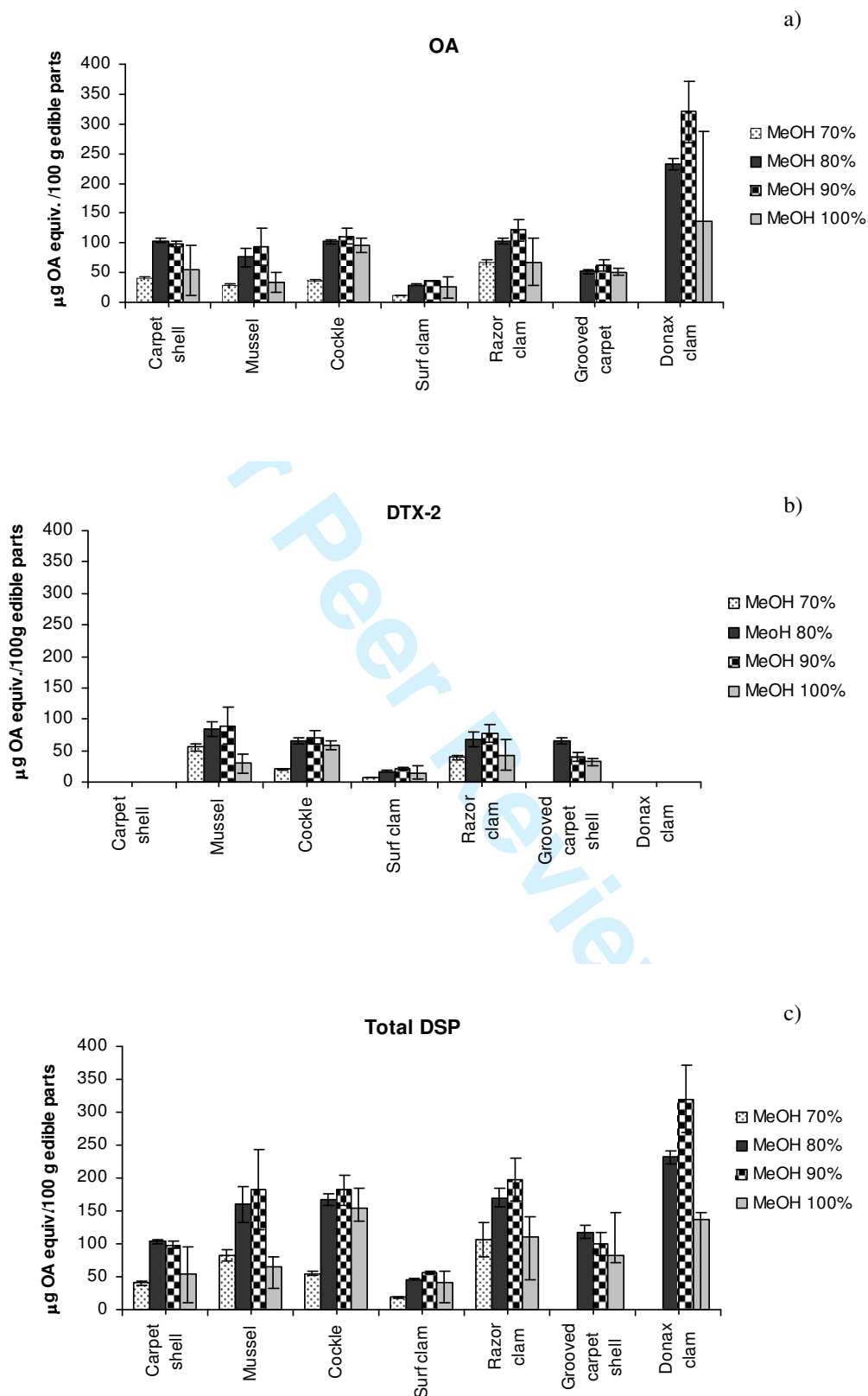


Figure 2. Concentration of a) total OA, b) total DTX-2 and c) total OA+DTX-2 determined by LC-MS for different shellfish species using different methanol ratios in the extraction procedure (each bar represents mean  $\pm$  SD). For donax clam and grooved carpet shell the extraction was not performed with 70% methanol. DTX-2 was not present in carpet shell and donax clam samples.

Table 1. DSP concentrations obtained in carpet shell tissues and raw methanol extracts during the 28 days of storage at  $-30^{\circ}\text{C}$  (mean  $\pm$  SD).

Day	Tissue ( $\mu\text{g OA equiv./100g}$ )					
	Free OA	Free DTX-2	Acyl OA <sup>1</sup>	Acyl DTX-2 <sup>1</sup>	Total OA	Total DTX-2
0	1.9 $\pm$ 0.3	1.2 $\pm$ 0.3	57.8 $\pm$ 2.5	33.4 $\pm$ 1.3	59.7 $\pm$ 2.8	34.6 $\pm$ 1.9
4	1.9 $\pm$ 0.2	0.8 $\pm$ 0.1	59.1 $\pm$ 1.1	33.5 $\pm$ 1.0	61.0 $\pm$ 2.1	34.3 $\pm$ 1.1
7	2.1 $\pm$ 0.4	1.1 $\pm$ 0.3	51.2 $\pm$ 1.8	31.0 $\pm$ 0.5	53.3 $\pm$ 2.3	32.0 $\pm$ 1.0
14	1.5 $\pm$ 0.3	0.8 $\pm$ 0.6	45.6 $\pm$ 3.3	27.0 $\pm$ 0.8	47.1 $\pm$ 3.8	27.8 $\pm$ 1.0
21	1.4 $\pm$ 0.5	1.2 $\pm$ 0.7	40.4 $\pm$ 5.3	25.0 $\pm$ 3.2	41.8 $\pm$ 5.4	26.2 $\pm$ 3.2
28	1.1 $\pm$ 0.6	0.6 $\pm$ 0.1	45.7 $\pm$ 4.1	24.8 $\pm$ 2.5	46.8 $\pm$ 4.2	25.4 $\pm$ 2.9

Day	Methanol extract ( $\mu\text{g OA equiv./100g}$ )					
	Free OA	Free DTX-2	Acyl OA <sup>1</sup>	Acyl DTX-2 <sup>1</sup>	Total OA	Total DTX-2
0	1.9 $\pm$ 0.3	1.2 $\pm$ 0.3	57.8 $\pm$ 2.5	33.4 $\pm$ 1.3	59.7 $\pm$ 2.8	34.6 $\pm$ 1.9
4	2.3 $\pm$ 0.3	1.0 $\pm$ 0.2	46.5 $\pm$ 1.2	25.7 $\pm$ 0.6	48.8 $\pm$ 1.7	26.7 $\pm$ 1.6
7	3.1 $\pm$ 0.4	1.1 $\pm$ 0.4	50.0 $\pm$ 2.6	28.8 $\pm$ 1.6	53.1 $\pm$ 3.1	29.9 $\pm$ 1.5
14	3.2 $\pm$ 0.1	1.0 $\pm$ 0.3	36.2 $\pm$ 1.0	20.9 $\pm$ 1.7	39.4 $\pm$ 1.9	21.9 $\pm$ 1.6
21	2.6 $\pm$ 0.4	1.3 $\pm$ 0.3	29.6 $\pm$ 3.1	16.8 $\pm$ 1.0	32.2 $\pm$ 2.6	18.1 $\pm$ 1.4
28	2.7 $\pm$ 0.3	1.0 $\pm$ 0.2	33.2 $\pm$ 1.2	16.8 $\pm$ 1.0	36.0 $\pm$ 1.7	17.8 $\pm$ 1.1

<sup>1</sup>The acyl OA and acyl DTX-2 concentrations were obtained by the difference between total OA equivalents (with hydrolysis) and free OA and free DTX-2 (without hydrolysis), respectively.

Table 2. DSP concentrations obtained in mussel tissues and raw methanol extracts during the 28 days of storage at  $-30^{\circ}\text{C}$  (mean  $\pm$  SD).

Day	Tissue ( $\mu\text{g OA equiv./100g}$ )					
	Free OA	Free DTX-2	Acyl OA <sup>1</sup>	Acyl DTX-2 <sup>1</sup>	Total OA	Total DTX-2
0	16.4 $\pm$ 2.1	55.8 $\pm$ 5.6	63.6 $\pm$ 2.6	31.1 $\pm$ 4.6	80.0 $\pm$ 2.5	86.9 $\pm$ 4.6
4	15.6 $\pm$ 0.8	53.9 $\pm$ 2.9	69.0 $\pm$ 1.1	32.4 $\pm$ 2.3	84.6 $\pm$ 3.9	86.3 $\pm$ 4.5
7	17.3 $\pm$ 1.1	57.7 $\pm$ 3.5	61.2 $\pm$ 4.1	23.3 $\pm$ 5.0	78.4 $\pm$ 3.5	81.0 $\pm$ 2.4
14	14.4 $\pm$ 1.4	54.2 $\pm$ 4.2	42.9 $\pm$ 3.9	17.3 $\pm$ 1.8	57.3 $\pm$ 4.1	71.5 $\pm$ 8.9
21	14.5 $\pm$ 1.3	52.7 $\pm$ 4.7	54.4 $\pm$ 4.9	22.4 $\pm$ 7.1	68.9 $\pm$ 4.4	75.1 $\pm$ 5.8
28	13.6 $\pm$ 0.8	59.6 $\pm$ 3.0	40.9 $\pm$ 3.3	6.4 $\pm$ 4.3	54.5 $\pm$ 2.8	66.0 $\pm$ 4.4

Day	Methanol extract ( $\mu\text{g OA equiv./100g}$ )					
	Free OA	Free DTX-2	Acyl OA <sup>1</sup>	Acyl DTX-2 <sup>1</sup>	Total OA	Total DTX-2
0	16.4 $\pm$ 2.1	55.8 $\pm$ 5.6	63.6 $\pm$ 2.6	31.1 $\pm$ 4.6	80.0 $\pm$ 2.5	86.9 $\pm$ 4.6
4	16.6 $\pm$ 1.7	51.1 $\pm$ 4.2	52.9 $\pm$ 1.1	21.9 $\pm$ 4.4	69.5 $\pm$ 1.7	73.1 $\pm$ 3.2
7	18.5 $\pm$ 1.7	53.2 $\pm$ 2.7	53.8 $\pm$ 4.3	21.9 $\pm$ 2.7	72.3 $\pm$ 2.9	75.2 $\pm$ 2.1
14	22.4 $\pm$ 1.4	55.1 $\pm$ 3.6	39.0 $\pm$ 6.7	9.4 $\pm$ 3.8	61.4 $\pm$ 7.0	64.6 $\pm$ 4.4
21	19.7 $\pm$ 1.3	60.2 $\pm$ 2.8	43.5 $\pm$ 4.3	16.1 $\pm$ 2.6	63.2 $\pm$ 4.2	72.6 $\pm$ 6.5
28	21.4 $\pm$ 0.8	55.0 $\pm$ 2.8	37.8 $\pm$ 1.7	9.8 $\pm$ 3.5	59.3 $\pm$ 2.7	64.8 $\pm$ 5.8

<sup>1</sup>The acyl OA and acyl DTX-2 concentrations were obtained by the difference between total OA equivalents (with hydrolysis) and free OA and free DTX-2 (without hydrolysis), respectively.

Table 3. Total OA+DTX-2 concentration obtained in different matrices using different methanol ratios in the extraction procedure (mean  $\pm$  SD).

Species	Total OA + DTX-2 ( $\mu\text{g OA equiv./100g}$ )			
	70% MeOH	80% MeOH	90% MeOH	100% MeOH
Mussel	83.2 $\pm$ 8.7	160.0 $\pm$ 28	183.1 $\pm$ 60.8	64.2 $\pm$ 32.3
Cockle	55.2 $\pm$ 2.7	167.6 $\pm$ 8.0	181.9 $\pm$ 23.3	153.4 $\pm$ 17.7
Grooved carpet shell	--- <sup>2</sup>	118.0 $\pm$ 9.3	100.6 $\pm$ 17.2	82.9 $\pm$ 11.0
Razor clam	106.5 $\pm$ 25.3	170.4 $\pm$ 14.1	197.8 $\pm$ 31.7	111.3 $\pm$ 65.6
Surf clam	18.6 $\pm$ 10.7	46.3 $\pm$ 0.9	56.9 $\pm$ 1.9	40.9 $\pm$ 40.0
Carpet shell	40.3 $\pm$ 2.5	103.7 $\pm$ 2.9	98.7 $\pm$ 5.5	54.1 $\pm$ 42.2
Donax clam	---- <sup>2</sup>	232.0 $\pm$ 9.3	320.4 $\pm$ 51.1	135.9 $\pm$ 152.1

<sup>2</sup> The extraction with 70% methanol was not performed for grooved carpet shell and donax clam.

Table 4. Percentage recoveries of total DSP (OA+DTX-2) obtained in different matrices using different methanol ratios relatively to 90% methanol.

Species	Recovery (%)		
	70% MeOH	80% MeOH	100% MeOH
Mussel	45.4	87.3	35.0
Cockle	30.3	92.2	84.3
Grooved carpet shell	--- <sup>2</sup>	117.2	82.4
Razor clam	53.9	86.2	56.3
Surf clam	32.7	81.3	71.8
Carpet shell	40.8	105.1	54.8
Donax clam	---- <sup>2</sup>	72.4	42.4

<sup>2</sup> The extraction with 70% methanol was not performed for grooved carpet shell and donax clam.