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Solid-phase extraction and high-performance liquid chromatography procedures for the analysis of paralytic shellfish toxins

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Abstract

Paralytic shellfish poisoning (PSP) toxins are produced by certain dinoflagellate species such as *Gymnodinium catenatum* and *Alexandrium tamarensis,* during certain periods of the year influenced by several environmental factors, affecting the aquaculture industry and mainly bivalve molluscs. HPLC with fluorescence detection is a powerful analytical technique for the analysis of such toxins; several HPLC alternatives have been developed in order to improve the liquid chromatographic analysis, but due to the complexity of the sample matrix, important work has been focused recently on the clean-up of samples prior to HPLC analysis. Solid-phase extraction procedures offer advantages for this clean-up. In this work we focus on the study of three different clean-up methods prior to HPLC with fluorescence detection analysis of PSP toxins present in contaminated mussel samples; by spiking uncontaminated mussel samples with two different PSP toxin standards and by calculating the recovery values for these experiments. These recoveries must be taken into account in order to quantify the exact amount of PSP toxins present in the contaminated samples. © 1998 Published by Elsevier Science B.V.

Keywords: Sample preparation; Paraltyic shellfish poisoning; Food analysis; Toxins; Saxitoxins

1. Introduction

The mussel industry is one of the most important in the Galician Region (NW of Spain); this industry is seriously affected by the presence of toxic dinoflagellates in marine phytoplankton, which produce toxic compounds such as paralytic shellfish poisoning (PSP) toxins [1]. These are a group of potent marine phycotoxins, including compounds such as saxitoxin (STX), neosaxitoxin (NEO) and several sulphate and N-sulphonate analogues (Fig. 1). A large number of these analogues have been recently found, which justifies the search of an accurate, sensitive and selective analytical method applicable to all these toxins.

A high-performance liquid chromatography (HPLC) method for the analysis of these PSP toxins has been developed and improved by Oshima [2]. This method has the great advantage of higher sensitivity and fairly good specificity for the analysis of PSP toxins.

The complexity of the sample matrix makes the development of clean-up procedures necessary in order to remove interferences, prevent false peaks and give more accurate quantitative results. In addition cleaning-up is also effective in prolonging the lifetime of the column. With this aim, three different clean-up methods have been applied prior to HPLC analysis. These clean-up methods were described in the literature [2–4] and slightly modified. A comparative study of the efficiency of these clean-up methods will be established and the results will also be compared with those obtained by using the

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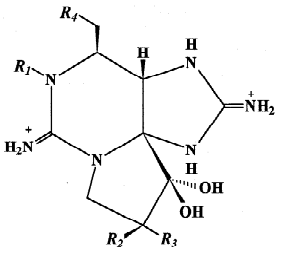

			Carbamate toxins	N-Sulphocarbamoyl toxins	Decarbamoyl toxins
R1	*R2*	*R3*	*R4*= **-OONH$_2$**	**-OONHSO$_3^-$**	**-OH**
H	H	H	STX	GTX5	dcSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	H	NEO	GTX6	dcNEO
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 1. Structure of PSP toxins.

conventional method [5] thus avoiding the clean-up step after extraction of PSP toxins.

In the present study we have established the efficiency of these clean-up methods by means of recovery experiments following the protocol indicated in Section 2 by spiking uncontaminated real mussel samples used as blank material, with two PSP components, STX and decarbamoyl (dc) STX, these standards were the only PSP toxin standards available as pure individual standards in our laboratory at the time of study.

2. Experimental

2.1. Toxin standards and samples

0.03 *M* Acetic acid solutions of STX and dcSTX (20 μg/ml) supplied by RIVM (Bilthoven, Netherlands) for BCR ''standards measurements and testing program certification study'', were used in this study (see Fig. 1 for PSP toxin structures).

Uncontaminated mussel samples from the Ría de La Coruña were kindly provided by ''Dirección Provincial de Saúde'', La Coruña, Consellería de Sanidade, Xunta de Galicia, Spain.

2.2. Extraction, clean-up and analysis of PSP toxins

Six hundred μl of STX and 600 μl of dcSTX standard calibration solutions (20 μg/ml), were added to 15 g of uncontaminated mussel sample to give a final concentration of 0.80 mg/kg mussel. After extraction with 15 ml of 0.2 *M* acetic acid and subsequent homogenization and centrifugation, the spiked extract was passed through a reversed-phase cartridge (Sep-Pak Plus C_{18} cartridges, Part. No. WAT 020515), according to the following procedures:

2.2.1. Procedure 1

The conditions of this procedure were described by Oshima [2] with slight modifications: through a C_{18} cartridge previously conditioned with methanol and equilibrated with water, 3 ml of extract were loaded and 1.5–2.0 ml of eluate were collected for the analysis.

2.2.2. Procedure 2

The conditions of this procedure were described by Lawrence et al. [3] and subsequently slightly modified. A 1-ml aliquot of spiked acetic extract was passed through a reversed-phase C_{18} cartridge (Waters) previously conditioned with 6 ml of methanol followed by 6 ml of 0.2 *M* acetic acid. The effluent and an additional 2 ml water wash were collected for the analysis.

2.2.3. Procedure 3

The conditions of this procedure were described by Locke and Thibault [4] and after some slight modifications, were applied to our samples. 0.5 ml of spiked acetic extract was passed through a reversed-phase C_{18} SPE cartridge (Waters) and eluted with 1 ml of 0.2 *M* acetic acid. The cartridge had been preconditioned with 2 ml each of methanol, water and 0.2 *M* acetic acid.

After these clean-up procedures, all the extracts were ultrafiltered through a polysulphone membrane (Ultrafree-MC, 10 000 NMWL, Millipore filters). Twenty μl of purified extracts were analyzed by post-column HPLC fluorescence detection analysis under the conditions described in Table 1, which were also described in Ref. [6].

Table 1
HPLC-fluorescence detection conditions for analysis of PSP toxins

HPLC instrument	Perkin-Elmer series 10-LC Column: Reversed-phase, Prodigy 5 μm C_8 (Phenomenex) 150×4.6 mm
Mobile phases: flow-rate 0.8 ml/min for STX group	2 m*M* sodium 1-heptanesulphonate in 30 m*M* ammonium phosphate pH 7.1–acetonitrile (100:5)
Oxidizing reagent: flow-rate 0.4 ml/min	7 m*M* potassium periodate in 50 m*M* potassium phosphate buffer, pH 9.0
Reaction:	in 10 m PTFE tubing (0.5 mm I.D.) at 65°C in water bath
Acid solution flow-rate at 0.4 ml/min	0.5 *M* acetic acid
Detection	Hitachi F1000 fluorescence detector, double monochromator Excitation wavelength 330 nm Emission wavelength 390 nm

3. Results and discussion

Liquid chromatographic analysis with fluorescence detection was carried out under the conditions described above. Fig. 2 shows an example of the chromatograms obtained for the uncontaminated mussel samples with or without clean-up under the abovementioned conditions; spiked extracts with standard solutions of STX and dcSTX were also analyzed under the same conditions. This analysis was carried out in triplicate in order to check the reproducibility of the whole procedure. The spiked extracts were passed through C_{18} cartridges to be cleaned-up following the conditions described in Sections 2.2.1–2.2.3. The differences that we have introduced to the conventional methods used by Oshima [2], Lawrence et al. [3] and Locke and Thibault [4], are mainly in the acid used for extraction and elution of the toxins. As we have mentioned previously the standards used for this study were used in an intercalibration exercise in which the use of acetic acid for extraction was recommended. In our experience it is important to make the extraction and clean-up under the same acid conditions wherein, the concentration of the acid used is also critical; for this reason we have tried to optimize the sample preparation step according to our previous data on the analysis of PSP toxins.

An extract without clean-up was also analyzed in order to establish a comparison between the results obtained. An example of these results is shown in Fig. 3. From the qualitative point of view, clean-up methods help to obtain cleaner chromatograms as we can observe by comparing Fig. 3a and Fig. 3b, c and d, although in these cases the peaks which appear due to interferences do not elute at retention times close to those associated with the toxins analyzed (STX and dcSTX); The example shown in Fig. 3 is probably not the most appropriate to show the influence of the interferences in these two PSP toxin standards at least from the qualitative point of view, this situation could be better represented if we were trying to analyze some other PSP toxins such as NEO, which elutes at a retention time close to those due to interferences. The reason for focusing this study only on these two standards, is due to their availability as individual and pure standards at the time of the study.

At this point it is interesting to remark that sample preparation for HPLC analysis of PSP toxins includes extraction and clean-up. Both are common steps for the next three isocratic HPLC analyses of the three different groups of PSP toxins, in order to know the efficiency of this procedure independent of the toxin to be analyzed.

The study of the efficiency of the clean-up methods was carried out by means of recoveries, as already mentioned. The whole procedure was applied to three identical samples and this procedure was

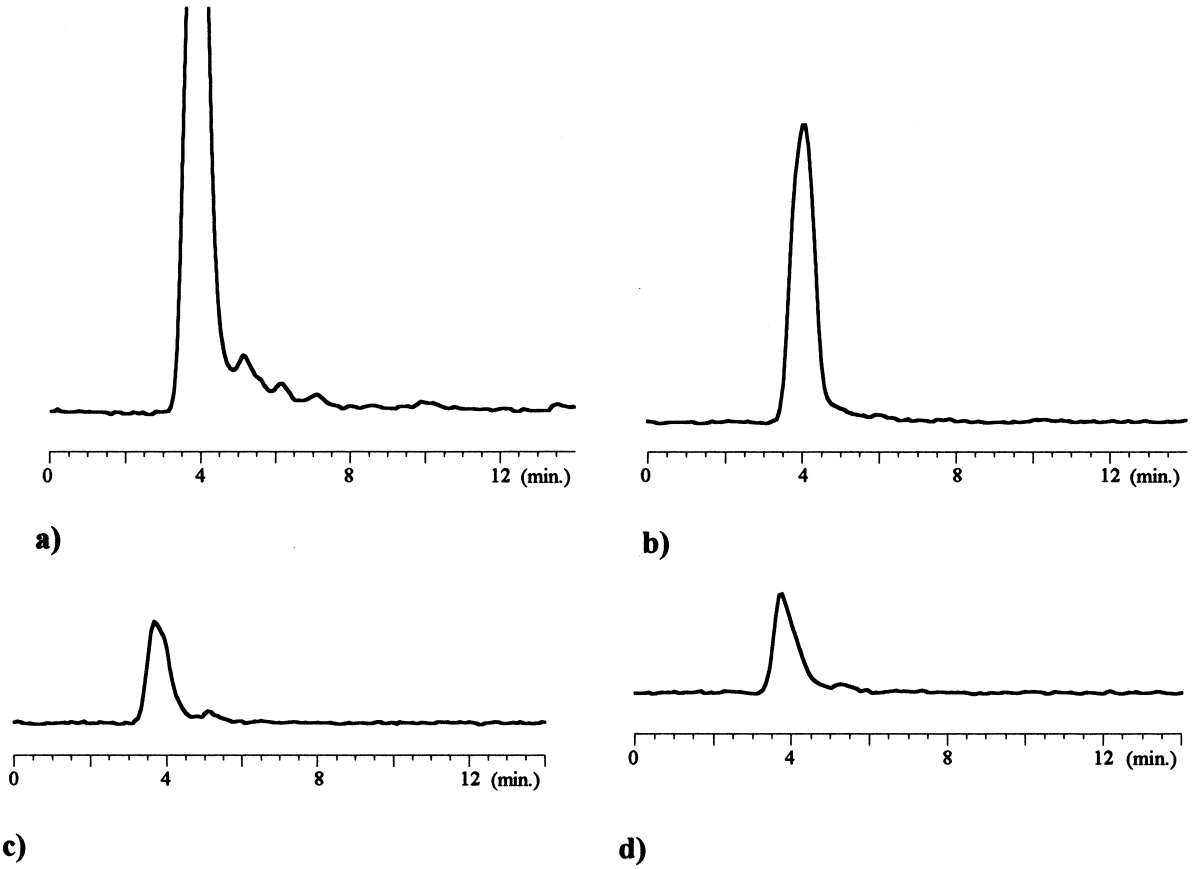

Fig. 2. Chromatograms obtained for uncontaminated mussels (a) without clean-up; (b) clean-up 1; (c) clean-up 2; (d) clean-up 3.

also carried out in triplicate. Tables 2 and 3 show the results found for the mean recoveries for the three identical samples spiked with dcSTX and STX, respectively. The analysis of the results led us to conclude that both clean-up 1 and the conventional method without clean-up allowed one to obtain highest recoveries, when compared with those obtained for methods with clean-up 2 and 3 for dcSTX as well as for STX.

In Table 4 we summarized the mean recoveries of dcSTX and STX in all cases (without clean-up and with the three different clean-up procedures). These results clearly show that the clean-up procedures carried out involve a partial elution of the toxins from the column, being considerably lower in clean-up procedures 2 and 3. This hypothesis was later confirmed by analyzing the subsequent elution fractions, although this experiment was not absolutely successful, due to the additional dilution factors introduced in the clean-up procedures, which makes the detection and quantitation of such diluted toxins difficult. Several real contaminated mussel samples were also used to repeat the experiment. These samples had a higher amount of toxins, making their detection in the diluted fractions easier. The results obtained with this experiment agree with those obtained for the spiked extracts in terms of recoveries, but also by analyzing the subsequent elution fractions, we confirmed the partial retention of the abovementioned toxins (data not shown).

The conventional method without clean-up gives the highest recovery. If we were trying to analyze STX and dcSTX, in this concrete example, we could avoid the clean-up step, just as a conclusion of the obtained results, but we previously mentioned the need of a common sample preparation step, which

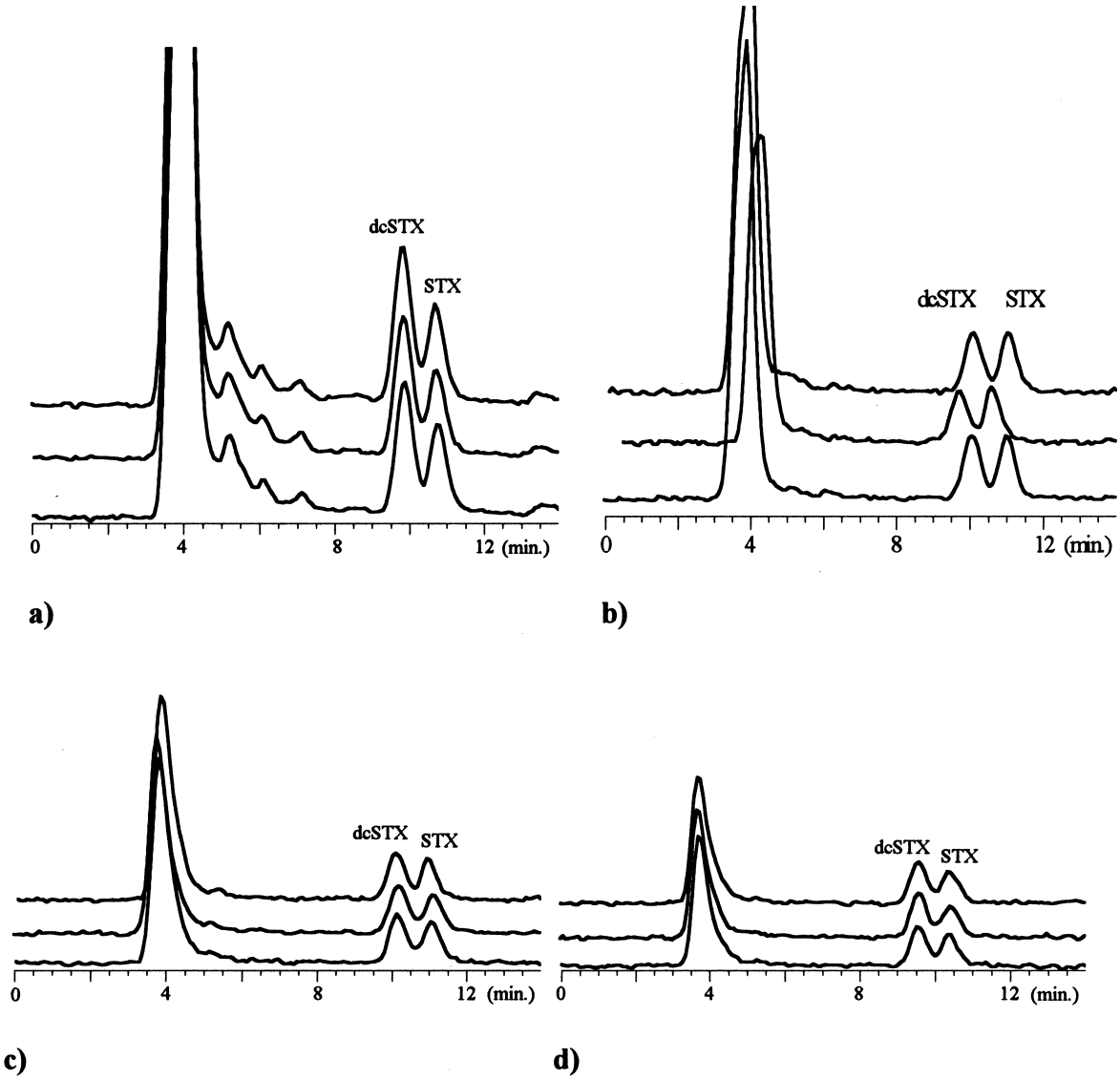

Fig. 3. Chromatograms obtained for uncontaminated mussels spiked with dcSTX and STX (a) without clean-up; (b) clean-up 1; (c) clean-up 2; (d) clean-up 3.

Table 2
Recovery results obtained for dcSTX by spiking uncontaminated mussel tissue with standard of dcSTX

Sample	Recovery (%)							
	Without clean-up		Clean-up 1		Clean-up 2		Clean-up 3	
	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)
1	88.72	4.16	79.35	0.81	46.17	1.52	42.31	3.33
2	81.98	1.82	85.31	2.58	44.03	1.86	34.98	2.23
3	90.55	1.59	78.93	1.63	42.70	1.52	34.81	0.86

Table 3
Recovery results obtained for STX by spiking uncontaminated mussel tissue with standard of STX

Sample	Recovery (%)							
	Without clean-up		Clean-up 1		Clean-up 2		Clean-up 3	
	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)
1	89.96	1.45	66.42	2.95	65.67	0.11	56.97	0.65
2	94.49	0.78	80.91	1.48	62.56	0.56	55.84	1.49
3	94.90	1.61	75.95	1.62	57.13	2.43	53.69	1.81

Table 4
Mean of the recovery results obtained for STX and dcSTX

Sample	Recovery (%)							
	Without clean-up		Clean-up 1		Clean-up 2		Clean-up 3	
	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)	Mean	R.S.D. (n=3)
dcSTX	87.08	5.18	81.20	4.40	44.30	3.95	37.37	11.45
STX	93.12	2.94	74.43	8.89	61.89	7.21	55.50	3.00

includes clean-up; the need of this clean-up to remove interferences, which affect other different PSP components and prevent false results, to prolong the column life and also to give more accurate quantitative results is necessary; for this reason we can conclude that clean-up 1 should be more suitable in terms of achieving the highest recovery and consequently the highest efficiency for the analysis of STX and dcSTX present in real contaminated samples.

4. Conclusions

The need for clean-up methods to remove interferences in biological complicated matrix such as mussels as well as to prolong the life of the chromatographic column is well reported in the literature, as a conclusion of the results obtained in this study, it is strictly necessary to make recovery experiments on these clean-up procedures, in order to determine their efficiency and consequently to know the exact amount of PSP toxins present in the contaminated samples.

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