Monitoring Paralytic Toxins in Shellfish of Morocain Mediterranean Coast by Assaying Their Toxicity Using The HPLC Method

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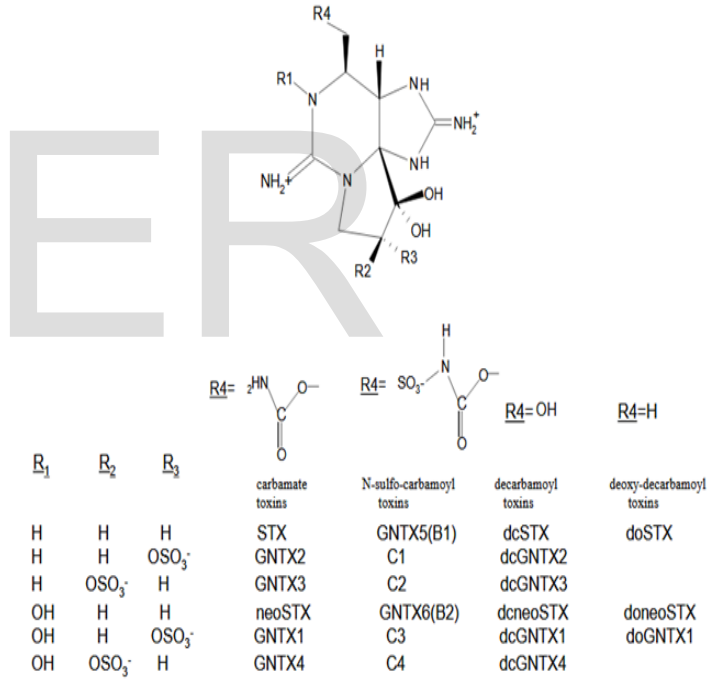
Abstract— Increasing the risk of consumer poisoning by digestion of bivalve molluscs contaminated with Saxitoxins and its analogues, requires both monitoring on the marine coasts and within the laboratory for the development of new methods to cope with this problem. Admittedly, the development of a method is always linked to its validation, as it requires the validation of the precolumn HPLC assay method for the identification and quantification of these toxins.

Index Terms — Paralytic toxins, Saxitonies, Validation, Morocain Mediterranean Coast, Bivalve molluscs, HPLC.

1 INTRODUCTION

IN the presence of several factors, the ocean surface forms blooms caused by the multiplication of micro-algae. The proliferation of genera of these microorganisms produces paralytic toxins (Paralytic Shellfish Poisons (PSPs)) that are likely to accumulate in filtering organisms, such as bivalve molluscs. The ingestion of these organisms by humans leads to serious poisoning. PSPs act by blocking the voltage-gated sodium channels, thereby avoiding the spread of nerve impulses, causing paralysis [1]. The molecule responsible for this toxicity is saxitoxins (STX) and its derivatives (dcSTX, GTX2 & 3, dcGTX2 & 3, C1 & 2, NEO, dcNEO, GTX1 & 4).Fig1. [2], [3].

The first case of intoxication in Morocco due to the ingestion of shellfish accumulating these toxins was recorded in 1961 [4]. In order to cope with the health risks related to these biotoxins, the National Institute for Halieutic Research (INRH) has set up, since 1992, a network for monitoring the safety of the coast. The role of this network is to control the level of contamination by these biotoxins, regularely taking samples of water and shellfish samples, and forbid fishing in contaminated areas until significatif reduction of toxins concentrations.
Therefore the regional laboratory of analysis and research in Tangier aims to develop and validate the analysis chemical method HPLC / FD for the detection, quantification and identification of PSP.



R1	R2	R3	carbamate toxins	N-sulfo-carbamoyl toxins	decarbamoyl toxins	deoxy-decarbamoyl toxins
H	H	H	STX	GNTX5(B1)	dcSTX	doSTX
H	H	OSO_3^-	GNTX2	C1	dcGNTX2	
H	OSO_3^-	H	GNTX3	C2	dcGNTX3	
OH	H	H	neoSTX	GNTX6(B2)	dcneoSTX	doneoSTX
OH	H	OSO_3^-	GNTX1	C3	dcGNTX1	doGNTX1
OH	OSO_3^-	H	GNTX4	C4	dcGNTX4	

Fig. 1. Structur of Saxitonie and its derivatives [2], [3].

2 PREFERENCE METHOD FOR PARALYTIC SHELLFESH POISONS (PSP) DETECTION

2.1 Biological Test

The official method used for the detection of PSP is a biological test performed on mice (bioassay, MBA). The lethal dose is of the order of 800 µg equivalent STX per kg of flesh [5].

This test gives an approximated quantification in saxitoxin equivalent (STX the most toxic molecule of the PSP family) evaluated by the survival time of the mice after intraperitoneal

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injection of an extract of a total ground meat from the sample to analyze. Nevertheless, this method remains qualitative and non-specific since it does not give an idea about the nature of the toxin present. In addition, the current policy of the European Commission aims to eliminate, as far as possible, toxicological tests on animals, on the one hand for ethical reasons (taking into account the suffering of the animal) and on the other hand, because of the scientific community's argument that animal testing would not be appropriate for all classes of toxins [6]. Thus, the use of other more sensitive and accurate confirmation analysis methods becomes necessary.

3 ALTERNATIVE METHOD

3.1 Chemical Test

Several chemical methods for the detection of PSP have been developed such as ELISA, Receptor binding assays, Cytotoxicity / cell, culture assays. However high performance liquid chromatography was the first to be developed [7].

There are three types of HPLC most used for the detection of STX and its analogs which are HPLC pre-column and post column, the first two are coupled to a fluorimeter detector and liquid chromatography coupled to mass spectrophotometry LC-MS , their principle is generally the same consisted in the quantification of the toxins by the assay either of their fluorescence after periodate or peroxide oxidation (according to the grouping of the toxins to be analyzed) after a stage of extraction in solid phase on C18 or by mass spectrophotometry after conversion of molecules into ions are treated based on their charge and mass [8].

4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This method allows the separation between the molecules contained in a mixture to be analyzed, in our study, corresponds to the saxitoxins and its derivatives present in the chair of bivalve molluscs.

The extraction is done with 1% acetic acid followed by heating at 100 degrees Celsius for 5 minutes then cooling with cold water for 5 minutes. The extract is then centrifuged at 4550 g for 10 min and the supernatant is collected in another tube, this step is repeated twice for good recovery of toxins existing in the pulpit. 1ml of extract is then purified in C18 by solid phase extraction followed by washing with 2ml of ultra pure water the pH of the mixture is adjusted to 6.5 and the volume is also adjusted according to the weighing of the flesh ex : 2g \ 4ml [9].

Before going on to read, the sample to be analyzed is derivatized at the expense of the type of toxins to look for - STX, dcSTX, GTX2 & 3, dcGTX2 & 3 and C1 & 2 peroxide and NEO, dcNEO and GTX1 & 4 to periodate.

The derivatisation is done at 100ul of the sample after alkalinization of the medium with 1M NaOH and then adding the peroxide, the reaction is then stopped by glacial acetic acid after 2min.

At the level of the apparatus the column is reverse phase type C18, 5um (4.6 * 250 or 150) mm, the flow is equal to 1 or 2ml \ min depending on the column used. The first mobile phase is a mixture of 0.1M ammonium formate diluted in ultrapure water and the second is ammonium formate diluted in 5% Acetonitrile. The pH of both phases is adjusted to 6 and are used as a gradient. In case of the presence of the N-1-hydroxylated toxins the sample is purified on a COOH column and the elution is made according to the nature of toxins:

1. By water for the recovery of N-sulfocarbamoyl-gonyautoxine 1 & 2 (C1 & 2) ;
2. By 0.05M NaCl for the recovery of Gonyautoxines;
3. And finally by 0.3M NaCl for the recovery of STX.

The chromatogram which exposes different peaks of dcGTX2 & 3, C1 & 2, dcSTX, GTX2 & 3 and SXT respectively is represented by fig2. above.

The identification of the peaks is done by a first injection of standard NRC type toxins, individually to set the retention time of each and then a mixture is prepared for the entire group.

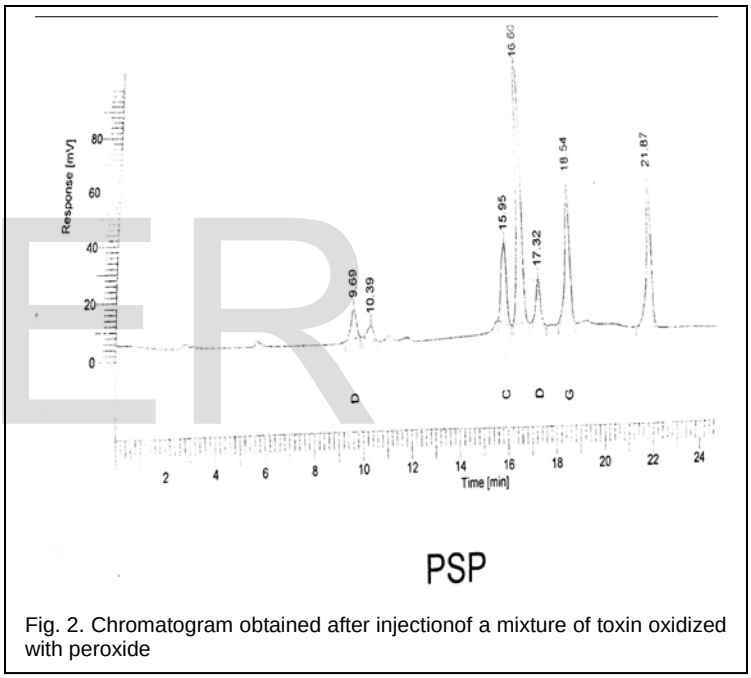

Fig. 2. Chromatogram obtained after injectionof a mixture of toxin oxidized with peroxide

5 SYNTHESIS AND RECOMMENDATION

The risk of mortality through the digestion of shellfish contaminated with biotoxins is no longer negligible in the case of poisoning related to its toxins. Four cases of death in Morocco are noted related to these paralytic toxins [4], [10]. hence the need of protective measures for consumers by the surveillance of all marine environments causing the increase of this risk. The techniques currently used for the detection of these toxins are not all adopted by the laboratories, among which we note those who require more time, maintenance (MBA) for their use ... and other that costs very expensive. Admittedly, speed, efficiency and specificity are very important criteria for a good separation and quantification of saxitoxins and its analogues, and high performance liquid chromatography covers these criteria provided that the pH is well controlled in each step of

the process, the gradient of the mobile phase, the time of the derivatization and the conditioning of the column.

6 Conclusion

The reliability of the results depends on the credibility of the method used, for the application and implementation of the latter requires validation for each type of mollusc present in the Mediterranean coast especially for an accredited laboratory.

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