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REVIEW ARTICLE

DETECTION SHELLFISH POISONING, MARINE TOXINS, OF FOOD BY HPLC:PARALYTIC SHELLFISH POISONING.

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Abstract

Shell fish are marine animals which exhibit filter feeding mechanism on some biological foods, such as micro algae, from the sea. This mechanism results in accumulating different toxins which can cause several types of diseases to human beings once consumed without assuring and quantifying the toxic level present inside their body. Shellfish include several types of marine animals, such as oysters, clams, lobsters and muscles. These organisms can produce different toxins that can produce a number of poisonings like PSP, commonly produced by pelagic marine dinoflagellates,up on specific exposure levels. There are about 20 PSP toxin analogs with closely related structures among which,saxitoxin is the most potent toxin.

In order to utilize effectively and efficiently these shell fish and other sea foods by people, there is a critical need to implement effective and efficient marine toxin detection and monitoring programs from the farm to fork. There are several detection methods which have been approved and accepted by regulatory agencies, such HPLC, ELISAand MBA. Detection methods using HPLC are very significant to solve the problemsthat arise from shellfish poisoning toxins including PSP toxins, as this methods is very reliable to detect and quantify target toxin and profile many other toxins at the same time. Moreover, it is used in environmental safety regulations. HPLC method has got verification by the Association of Official Analytical Chemistry (AOAC) for PSP toxins determination in shellfish and is now included in the European Union Directives. Based on the European Union Directive 91/492/EEC, the content of PSP toxins must not exceed 80μg/100g of shellfish flesh in accordance with the biological testing method. Aim of this review is to give an awareness on the paralytic shellfish poisoning and their detection methods via HPLC for the food industries and the consumers at large.

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Introduction:-

Seafoodare very nutritious and rich in micronutrients, minerals, essential fatty acids and proteins. Fish demand in recent years has been growing, not only because of the increasing needs of thegrowing population especially from all over the world, but also due to the growing demand for seafood. They are very susceptible to spoilage by microorganisms, enzymes and physical agents once caught from the sea if no preservation methods are applied. All the fish and shell fish are the categories of seafood, have different feeding mechanisms, such as predation and filter their feed

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Bivalve shellfish, such as oysters, clams and mussels feed exclusively on phytoplankton that they filter from seawater. Shellfish are usually unaffected by toxic algae themselves but can accumulate toxins in their tissue to levels that can be lethal to humans. Paralytic, Amnesic, Neurotoxin and Diarrhetic are the four types of shellfish poisoning. Shell fish can produce paralytic poisoning when they accumulate saxitoxins from marine dinoflagellates and ingestion of contaminated shellfish with PSP toxins can cause serious life-threatening intoxications to human being (Harju*et al*., 2015; Yu *et al.*, 2013). PSP toxins hinder the formation of an action potential by the blocking of the influx of sodium ions (Na^+) through excitable membranes. PSP toxins can cause severe illness, paralysis and death in humans, when present in seafood in sufficient concentrations (Mihali*et al*., 2011). Therefore, PSP toxins are risk to public health that must be detected before serving. There are different analytical detection methods of PSP, such as High Performance Liquid Chromatography(HPLC), Enzyme-linked immunosorbent assays (ELISA), Mouse bioassay (MBA) and some other recently developed methods like, hydrophilic interaction ultra-performance liquid chromatography tandem mass spectrometry (HILC UPLC-MS/MS) (Rey *et al*., 2016; Rossignoli*et al*., 2015). HPLC is one of the most common methods to detect and quantify PSP in seafood. Therefore, detection and quantification of maximum limit of shell fish via HPLC is most required in order to provide safe and sound quality seafood as well as protect the health of consumers.

Given the heightened emphasis on seafood ensuring that these products are compliant, it is pertinent that up to date information is accessible to consumers and industries, regarding suitable test methods of HPLC that can be used by industries for paralytic shell fish toxins. This review has therefore focused to compile supporting information both to industries and consumers, so as to have an awareness on the use of appropriate detection methods while dealing with shell fish, particularly PSP toxins, in marine and other food products. The target of this review is to give awareness and familiarization to industries and consumers with shell fish poisoning in food including their symptoms and control methods as well as to provide informationon the use of HPLC as a detection method of these toxins and its significance.

Paralytic Shell Fish Poisoning and its Consequences:-

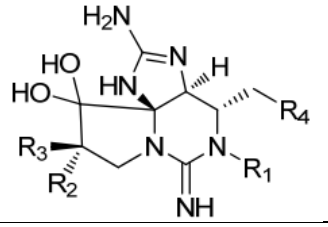
Paralytic shell fish poisoning is naturally occurring marine toxin produced by marine plants, phytoplankton. People could get poisoned after eating the filter feeding animals which have accumulated the toxin. Basically, such toxins in food have no detectable taste or odor and cannot be removed by cleaning nor destroyed by cooking (Yu *et al.*, 2013; Batmomolin Benyamin *et al*., 2016).

Several forms of shellfish (Table 1) poisoning may occur after ingesting filter-feeding bivalve mollusks (such as mussels, oysters, clams, scallops, and cockles) that contain potent toxins. The toxins originate in small marine organisms (dinoflagellates or diatoms) that are ingested and are concentrated by shellfish. There are different types of shell fish that can produce different types of toxins due to improper preservation; and some of them if they ingest certain types of algae (Wang, 2008). The toxins can be produced by certain bacteria and some bio-accumulate in filter-feeding shellfish, resulting in several types of human illness (Zhang *et al*., 2015). Different types of shell fish poisoning could cause human illness, such as paralytic shellfish poisoning (PSP), neurotoxin shellfish poisoning, amnesic shellfish poisoning (ASP), diarrheic shellfish poisoning (DSP), ciguatera shellfish poisoning (CFP) and azaspiracid shellfish poisoning (AZP) (Polletti*et al*., 2003). In addition to the human illnesses caused by ingestion of contaminated seafood. Several shell fish toxins possess the potential for use in bioterrorism, including the conotoxins and tetrodotoxin (Anderson, 2012; Batmomolin Benyamin *et al*., 2016).

Paralytic shellfish poisoning (PSP) is the most common and most severe form of shellfish poisoning. PSP is caused by eating shellfish contaminated with saxitoxins produced by dinoflagellates from *Aelxandrium*, *Pyrodinium*, and *Gymnodinium* genera (Anchorage, 2012). Microalgae grow in an environment which is suitable to them, then are able to produce blooms in high concentrations which can discolor the seawater, a bloom known as "red tide". The filter feeders are dependent on these microalgae for food. Through time, more toxins can be accumulated and

pass to the predator via the food chain . Long years ago in 1957 red tides were identified and confirmed that they can cause symptoms and death in laboratory-tested animals after feeding with shell fish, such as mice.

This toxin was isolated by Sommer and Meyer using ion exchange chromatography. After several years its structure was identified (Fig. 1) by Jon Border and Colleagues and Rossignol*et al.* (2015) and was reported that,PSP hastetrahydropurine structure which help to block excitation of nerve and muscle cells causing paralysis. Gracee*et al.* (2001) reported that, HPLC method is the only one which can detect the lowest level of this toxin, even though mouse bioassay was developed first for its detection. Some of these toxins are 1,000 times more potent than cyanide, and toxin levels contained in a single shellfish can be serious to humans. After eating toxic shellfish symptoms like numbness and tingling of the face, lips, tongue, arms, and legs can be shown. There may be headache, nausea, vomiting, and diarrhea. Severe cases could be associated with ingestion of large doses of toxin and clinical features such as ataxia, dysphagia, mental status changes, flaccid paralysis, and respiratory failure (Stacey, 2010; Rossignol*et al*., 2015; Batmomolin Benyamin *et al*., 2016).



Compound	R_1	R_2	R_3	R_4	Relative toxicity
Saxitoxin (STX)	H	H	H	$OCONH_2$	1.0000
Decarbamoylsaxitoxin (dc STX)	H	H	H	OH	0.5131
Neosaxitoxin (NEO)	OH	H	H	$OCONH_2$	0.9243
Gonyautoxin 1 (GTX1)	OH	H	OSO3H	$OCONH_2$	0.9940
Gonyautoxin 2 (GTX2)	H	H	OSO3H	$OCONH_2$	0.3592
Gonyautoxin 3 (GTX3)	H	OSO_3H	H	$OCONH_2$	0.6379
Gonyautoxin 4 (GTX4)	OH	OSO_3H	H	$OCONH_2$	0.7261
Gonyautoxin 5 (GTX5)	H	H	H	$OCONHSO_3H$	0.0644

Figure 1:- Statures and toxicity of Paralytic Shell Fish Poisoning (Harju*et al.,* 2015 ;Gracee*et al*., 2001)

Table 1:- list of some common Shell fish toxins and the seafood they are commonly associated with (Canadian food inspection agency, 2014).

Ilness	Toxin	Seafood
Paralytic shellfish poisoning (PSP)	Saxitoxin	Oysters, clams, scallops, mussels, cockles, whelks
Diarrhetic shellfish poisoning (DSP)	Okadaic acid	Various shellfish, cockles, mussels, oysters
Tetrodotoxin poisoning	Tetrodotoxin	Pufferfish, California newt, parrotfish, octopus, starfish, angelfish, and xanthid crabs
Neurotoxic Shellfish Poisoning	Brevetoxin	oysters, clams, and mussels
Amnesic shellfish poisoning (ASP)	Domoic acid	Bivalve molluscan shellfish, clams, mussels, oysters, scallops

Disease Caused and its Symptoms':-

Paralytic shell fish poisoning toxins are more prevailing of sodium blocking agents, mainly the carbamates type (Zhang *et al*., 2015; Schirone*et al*., 2011). They hinder the formation of an action potential by the blocking of the influx of sodium ions (Na^+) through excitable membranes (Harju*et al*., 2015). PSP symptoms begin within a few minutes to a few hours after eating toxic shellfish. PSP toxins can cause tingling of lips and tongue as early symptoms and progress to tingling of fingers and toes and then loss of control of arms and legs, followed by difficulty in breathing, slurred speech and problems in swallowing, paralysis, weakness, ataxia, floating/dissociative feeling, nausea, dizziness, vomiting, headache, dysphagia, and dysarthria and death in humans, when present in seafood in sufficient concentrations (Gessner*et al*., 1997; Yu *et al.*, 2013). Gessner*et al*.(1997) also reported diastolic and systolic hyper- tension in almost all patients. Generally shell fish toxins can cause different types of diseases to the consumers and have symptoms which is highly dependent on the toxin produced. the consequence of

toxin is form simple to highly server acute and has relation on the immune system of the people, pregnant women, children and immune suppressed, who are victim of these toxins. Hence, PSP toxins are very dangerous to public health, and thus consumersand fishery industries should be careful and vigilant all the time.

Overview of Detection Methods:-

In order to utilize effectively and efficiently these shell fish, sea food, by the people there is a critical need to implement effective and efficient marine toxin detection and monitoring programs. These methods are very crucial in assuring safety and quality of the shell fish, such as, shrimp, lobster, mollusks, and oyster, and preventing health of consumers at large. Different chemical or biochemical methods have been developed for quantification of paralytic shell fish toxins (PSTs). Customarily paralytic shell fish toxins were determined using mouse bioassay.however, because of ethical considerations, the use of test animals (mammals) in assay, high-performance liquid chromatography (HPLC) with fluorimetric detection has been chosen to identify and quantify PSP toxins present in seafood. Recently, a liquid chromatography (LC), high liquid chromatography (HPLC), method are developed and validated by the Association of Official Analytical Chemists (AOAC) for PST determination in shellfish (Rey*et al*., 2016; Lawrence *et al.,* 2005) and is at present included in the European Union Directives (Commission Regulation (EC) No 1664/2006) to detect the food borne hazards from marine toxins (FAO, 2004).

Shellfish toxins can be detected and quantified via several methods of detection.These detection methods have different quantification levels, sensitivities and other properties. In order to use the analytical methods depending on the targeted toxin and maximum level determination and quantification, certain criteria have been stated in earlier studies on shellfish poisoning, such as accuracy, applicability (matrix and concentration range), limit of detection, limit of determination, precision, repeatability, reproducibility, recovery, selectivity, sensitivity, linearity, and measurement of uncertainty, ruggedness.

The most successful chromatographic methods developed for the determination of shell fish toxins and other marine toxins include fluorescence detection (FLD) and mass spectrometric (MS) detection which both provide high sensitivity. HPLC fixed with electro-spray ionization tandem spectrometry (MS/MS) has the impending to become the most potent tool for detecting PSP toxins, particularly for confirmatory analysis (Dell Aversano*et al*., 2005). Lawrence *et al*. (2005) has developed the HPLC-fluorescent light detection (FLD) to use as an optional method because of its sensitivity and fully automated.

Detection Method and Principles of HPLC:-

A quick, sensitive and specific method is required to determine the presence of the paralytic shell fish poisoning toxin which is the potential hazard to humans. The techniques of HPLC for the analysis PSPtoxins provide the separation efficiency required for complicated matrices such as the different types of shell fish samples as well as the high resolution required to determine the toxins present in contaminated samples with very low detection limits, which is the main criteria required from the detection methods. In order to obtain accurate and precise quantitative results from PSP, extraction and clean-up steps prior to the chromatographic analysis are the most essential ones (Rey *et al*., 2015).

The chromatographic technique is used to separate different mixtures of compounds of toxins of chemical and biological origin from seafood and other types of foods with the purpose of quantifying, and identifying the individual components of the mixture in many broad fields, such as analytical chemistry and biochemistry is the HPLC. It involves the injection of a small volume of liquid sample into a tube packed with tiny particles which is called the non moving phase (3 to 5 micron (μm) in diameter called the stationary phase). Where individual components of the sample are moved down the packed tube (column) with a liquid of the moving phase, known as a carrier sample, mobile phase, the aliquot is forced through the column by high pressure delivered by a pump that is used to manage the flow rate of mobile phase substance (Rey *et al.,* 2016; Case *et al*., 2013). These components of toxins are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles, more over their affinity and molecular mass. These distinguished components of PSP toxins are detected at the exit of the tube (column) by a flow-through device termed as a detector, which measures their amount, whereby separation of toxins depend on /occur due to difference on their speed of motion (fig. 2). An output from this detector is called a "liquid chromatogram (Case *et al*., 2013). HPLC involves different separation modes, therefore it is necessary to select a mode that is appropriate for the target samples. The spectroscopy, refractive index and fluorescence detection principles are used to detect the compounds eluting from an HPLC column (Torgony*et al*., 2015). Separation modes is highly dependent on parameters of solubility and molecular weight (size exclusion), and chemical properties of the compounds.

A Mass Spectrum (MS) detector senses a compound eluting from the HPLC column first by ionizing it then by measuring it's mass and/or fragmenting the molecule into smaller pieces that are unique to the compound. The MS

detector can sometimes identify the compound directly since its mass spectrum is like a fingerprint and is quite unique to that compound (Buckley *et al*. 1978).

Other type of separation mode of HPLC is the refractive index detection, which has ability of a compound or solvent to deflect light provides a way to detect it. It is a measure of molecule's ability to deflect light in a flowing mobile phase in a flow cell relative to a static mobile phase contained in a reference flow cell and detector, but it is not very sensitive. The quantity of deflection is relative to concentration (Quilliam*et al*., 1995). Fluorescence detectors sense only those substances that fluoresce. Compared to UV-V's detectors fluorescence detectors offer a higher sensitivity and selectivity that allows to quantify and identify compounds and impurities in complex matrices at extremely low concentration levels.

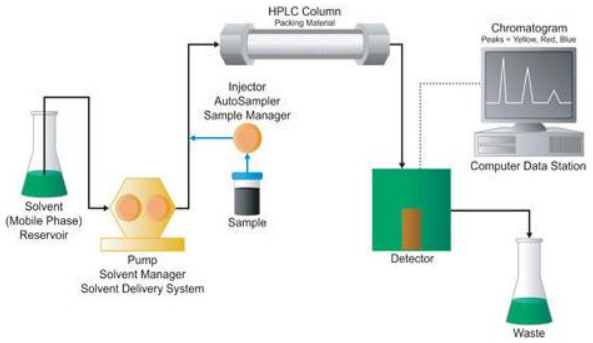


Figure 2:- Basic flow diagram of HPLC system (http://www.waters.com/waters/en_US)

Separation Techniques for the Analysis of Paralytic Shell FishPoisoning Toxins:-

Some studies on aqueous extracts of shellfish tissue indicated that the toxin and several of its isomers could be separated (and isolated in sufficient amounts for subsequent structural identification) by reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) diode array detection (DAD) which has a range of 210-400nm (Case*et al*., 2013). Aqueous acetonitrile containing 0.1% v/v trifluoro-acetic acid has been used as mobile phase. As the retention time and characteristic UV absorption spectrum of permit unequivocal identification, the HPLC-DAD procedure can be refined with a microbore column to give a rapid (5 min), sensitive (0.3ng detection limit) and reproducible assay method for the determination of shellfish tissue (Yu *et al*., 2013). Extraction can be accomplished by boiling homogenized shellfish tissue for 5 min with distilled water (Ujievic*et al*., 2012). Extracts should be taken through an octadecyl silica solid phase extraction clean-up prior to HPLC. This method has been applied to a variety of shellfish and phytoplankton samples (Michael *et al*., 2016; Rey *et al*., 2016).

The most common chemical method used for the analysis of PSP toxins is the combination of liquid chromatography (LC) with online post-column oxidation and fluorescence detection, using two different isocratic and gradient elution modes. Rey *et al.* (2015) has reported that, this method is important to quantify for the limit of detection and limit of quantification toxins of PSP in different shellfish at different matrix as it is shown in Table 2. The post column method of HPLC is popular as it can chromatographically separate many samples at the same time and use to establish toxins profile based on the geographical area, the phytoplankton species and the shellfish species (Abdul Keyon, 2014). Rey *et al.* (2015) reported that concentration of different types PSP toxins from different shellfish , such as oyster, clam, mussels, and scallop, can be chromatographically separated at the same time and investigate the matrix from different geographical areas using the heptane sulfonate concentration in mobile phase. And HPLC is very important in monitoring and regulating environment, since it gives reliable results than other methods, such as MBA method (Yu *et al.,* 2013).

An alternative LC method employing pre chromatographic oxidation has been reported by Boyer *et al.* (1999) has an improved results for separation and quantification of PSP analogues. All the high performance liquid chromatography (HPLC) methods have resulted in valid approach for the control of these toxic compounds, but although these methods offer good sensitivity and dynamic range, the sensitivity is dependent on parameters such as reagent concentration, reaction times, pH and temperature of the oxidation reaction. To solve this problem mass spectrophotometer with elector spry ionization can be used. This technique has shown excellent sensitivity for PSP as well as for other marine toxins (Rey *et al.,* 2016).

Earlier studies reported that (Elizabeth *et al*., 2006), currently there are at least 22 known PSP toxin derivatives split into three categories: carbamate, N-sulphocarbamoyl, and decarbamoyl. The structures of PSP toxins , which is giving in figure 1, are based on a tetrahydropurine skeleton with two permanent guanidinium functions. Substitutions at four distinct positions around the basic PSP toxin structure categorise the different PSP toxin analogues. Saxitoxin (STX) a carbamate toxin is generally considered the most potent (Harju*et al.,* 2015). Other highly potent carbamate toxins include neosaxitoxin (NEO) and the gonyautoxins 1 to 4 (GTX1, GTX2, GTX3, GTX4). While the N-sulphocarbomoyl (C) toxins are generally considered to be less potent. The low toxicity compounds can be converted to more potent toxins by heat and acidity. It follows, therefore, that food preparation may increase the toxicity of shellfish contaminated with PSP toxins.

Table 2:-Limit of detection and limit of quantification (mg STX eq/kg) **(**Rey *et al*., 2015)

	Mussels		**Clams**		**Scallops**		**Oysters**	
Toxin	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ
GTX4	0.0183	0.0549	0.0201	0.0603	0.0221	0.0663	0.048	0.144
GTX1	0.0642	0.1926	0.0320	0.0960	0.0488	0.1464	0.057	0.171
dcGTX3	0.0071	0.0213	0.0096	0.0288	0.0097	0.0291	0.003	0.009
GTX5	0.0011	0.0053	0.0088	0.0264	0.0298	0.0894	0.025	0.075
dcGTX2	0.0210	0.0630	0.0181	0.0543	0.0244	0.0732	0.030	0.090
GTX3	0.0055	0.0065	0.0072	0.0216	0.0073	0.0219	0.011	0.053
GTX2	0.0099	0.0297	0.0306	0.0918	0.0199	0.0597	0.023	0.069
NEO	0.0239	0.0717	0.0329	0.0987	0.0375	0.1125	0.018	0.054
dcSTX	0.0047	0.0141	0.0172	0.0516	0.0074	0.0222	0.0005	0.001
STX	0.0100	0.0300	0.0394	0.1182	0.0111	0.0333	0.009	0.027
CL2	0.0001	0.0003						
CL2	0.0011	0.0033						

where LOQ: Limit of quantification

Regulations and Detection Level for Shellfish Toxins:-

Regulation on determining the maximum level of shell fish toxins are very necessary to protect health of consumers all over the world. Accordingly, consumers can have know-how on acceptable limit of different marine toxins and they can prevent their health themselves too.

Based on to the European Union Directive 91/492/EEC, the content of PSP toxins must not exceed 80μg/100 g of shellfish flesh in accordance with the biological testing method. Using the HPLC method, the European Union permits a maximum level of 20μg/g for the total ASP toxins content in the edible parts of mollusk (Council of the European Communities 1991; Yu *et al.,* 2013; Rossignoli*et al*., 2015).

European Food Safety authority (2008), also reported that, live bivalve molluscs placed in the market by fishermen or food business operators for human consumption should not contain marine bio-toxins that exceed the limit of 800 micrograms per kilogram for paralytic shellfish poison (PSP), 20 milligrams of domoic acid per kilogram for amnesic shellfish poison (ASP), 160 micrograms of okadaic acid equivalents 7 per kilogram for okadaic acid, dinophysistoxins and pectenotoxins in combination, 1milligram of yessotoxin equivalents per kilogram for yessotoxins, 160 micrograms of azaspiracid equivalents per kilogram for azaspiracids.

The risk management linked to Paralytic shellfish toxin contamination of seafood and food products is based on monitoring of toxins in material and preservation methods intended for human consumption (Rossignoli*et al*., 2015; Rossini 2005). Increasing and serious concerns about seafood safety particularly shellfish, filter feeders have much opportunities to accumulate toxins, and human health protection have made evident the necessity of rapid, robust, specific and sensitive analytical methods for the detection of marine toxins and several methods are currently used to assess the contamination of seafood by toxic agents (Campas*et al*., 2007).

Future Perspectives ofHPLC:-

The techniques of HPLC for the analysis of all the shell fish toxins of food provide the separation efficiency required for complicated matrices such as marine samples as well as the high resolution required to determine the

toxins present in contaminated samples of food with very low detection limits. Extraction and cleanup steps prior to the chromatographic analysis are essential in order to obtain accurate quantitative and qualitative results and also to prevent interferences that can cause false positives results. New, fast and on-line automated procedure could give shorter and more accurate analyses of all seafood. The possibility to combine this automation with simple methods is also desirable, especially for routine control purposes. Taking into account the lack of standards for all toxins and also the appearance of new, unknown toxic compounds from seafood, the development of confirmatory techniques for their detection is an important research field of shellfish toxins in preventing health of consumers. The development and optimization of coupling techniques, such as LC-MS, HPLC-FLD or DAD, and using different detectors at one time for same or different sample of obtain more precise, reliable result and the development of adequate interfaces as well as efficient ionization modes is a high priority.

Conclusion:-

To ensure consumer safety, monitoring of toxin levels in all commercially harvested sea food using the analytical techniques such as HPLC, LC-MS, immunoassay, cellular bioassays and molecular probes is highly essential. Monitoring of shellfish supplies for toxins has proved very successful results in limiting human shellfish poisoning all over the world; and this monitoring should continue at a large scale. Furthermore, effective risk assessment programs for the identification of the environmental conditions and organisms responsible for toxin production in shellfish harvesting areas must be developed to prevent shellfish toxin contamination. Efforts to minimize coastal pollution and the associated nutrient load that it causes should also be continued. This will not only reduce the threat posed by marine toxins to consumers and fishery industries, but also promote greater health and sustainability for the valuable marine ecosystems. Therefore, fishermen, consumers and food industries should have know-how on shellfish products and follow current set regulations by the responsible organizations on maximum limit of the toxins, ways of preserving methods and current reliable method of detection of seafood.

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