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Review

Phycotoxins in seafood —toxicological and chromatographic aspects

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ABSTRACT

Two typical clinical types of algae-related seafood poisoning have attracted medical and scientific attention: paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP). Therefore, it became necessary to establish methods for the evaluation of possible hazards caused by contamination of seafood with these phycotoxins. Bioassays with mice or rats are the common methods for the determination of the toxin content of seafood. However, biological tests are not completely satisfactory because of a lack of sensitivity and pronounced variations. Additionally, there is growing opposition against animal testing. Therefore, many efforts have been undertaken to determine phycotoxins by chromatographic methods. PSP determination is mainly based on high-performance liquid chromatographic (HPLC) separation by ion-pair chromatography followed by postcolumn oxidation of the underivatized toxins in alkaline solution and fluorescence detection. HPLC methods for the determination of the DSP toxins okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are characterized by precolumn derivatization with 9-anthryldiazomethane (ADAM) and/or 4-bromomethyl-7-methoxycoumarin (Br-Mmc), followed by chromatographic separation of the DSP esters formed and fluorescence detection. The chromatographic methods discussed in this review allow the rapid, sensitive and non-ambiguous determination of individual species of the two most important phycotoxins in seafood, PSP and DSP.

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1. INTRODUCTION

It has been known since ancient times that certain fish and shellfish may be poisonous and can cause death after consumpion [1]. The chemical nature and biological basis for these food-borne intoxications have been elucidated during the last 50 years. It is now evident that certain microscopic algae produce very potent toxins (phycotoxins or algal toxins). The concentrations of toxins in the sea are highest during algal blooms. The phycotoxins are taken up by predators feeding on plankton, either directly as with molluscs or through several trophic levels as in fish. These food items are then consumed by man (Fig. 1).

As fish and shellfish constitute an important part of the world's food supplies, the apparently increasing contamination of food by algal toxins constitutes a specific chemical hazard requiring appropriate attention [3,4].

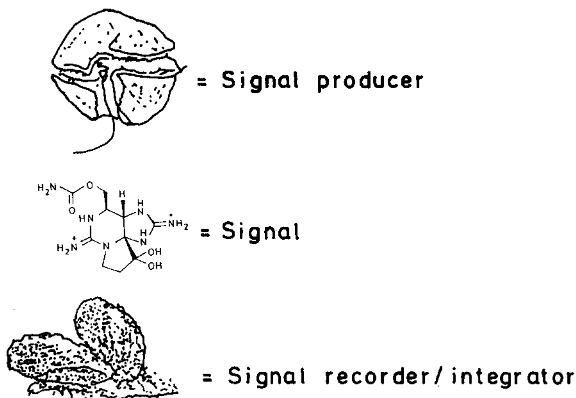


Fig. 1. The common mussel, found world-wide, is used as the signal recorder/integrator [2].

1.1. *Paralytic shellfish poisoning (PSP)*

1.1.1. *Occurrence in seafood*

The PSP toxins are produced by certain unicellular marine algae known as dinoflagellates. Most of the PSP-producing dinoflagellates are found in the genus *Alexandrium* [5]. Contamination of shellfish with PSP toxins has traditionally been associated with the appearance of an algal bloom, the so-called "red tide". It is also important to note, however, that not all red tides are associated with toxic phycoplankton and contamination of shellfish. On the other hand, shellfish can still contain PSP when *Alexandrium* spp. concentrations in the sea are already far below those found in algal blooms.

The PSP compounds are transferred to shellfish (*e.g.*, mussels, clams, scallops) during filter-feeding. Digestion takes place in the intestinal tract and is associated with the hepatopancreas complex. The highest concentrations of PSP have been found in these digestive glands [4].

1.1.2. *Chemical structure and toxicity*

PSP symptoms include perioral tingling sensations, prickly sensations in the finger tips and toes, progressing to arms and legs, then general weakness and slight respiratory insufficiency, followed by muscular paralysis, severe respiratory difficulty and finally death unless ventilatory support is given [6].

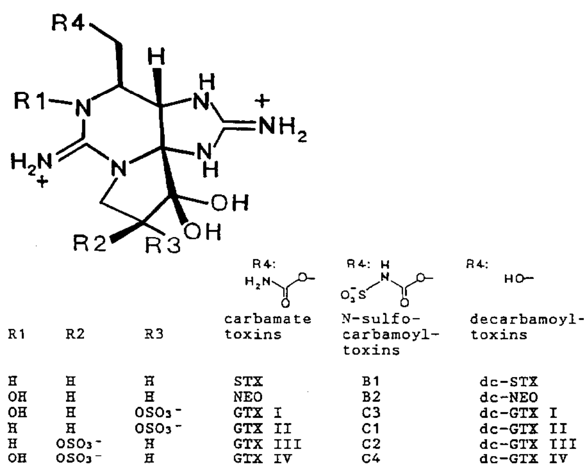
The first PSP component to be chemically characterized was saxitoxin (STX), which, although it was initially discovered in shellfish in California, has since been found in the greatest concentrations in the Alaska butterclam, *Saxidomus giganteus*, from which the name was derived [7]. Saxitoxin is a hygroscopic solid, soluble in water and methanol, but almost insoluble in most non-polar organic solvents [8].

Subsequently, several other toxins of the PSP group have been characterized chemically, including 1-hydroxysaxitoxin (NEO) and the epimers of 11-hydroxysaxitoxin sulphate and 11-hydroxyneosaxitoxin sulphate [9–12]. The last compounds, named gonyautoxin II, III, I and IV (GTX), are less basic, but otherwise their properties are similar to those of STX.

In addition, PSP compounds with a sulphocarbamoyl group have been isolated from both dinoflagellates and shellfish, and decarbamoyl toxins, *e.g.*, decarbamoylsaxitoxin (dc-STX), which previously had been made only in the laboratory, has been found in nature [13–17].

Thus, referring to their chemical structure, three groups of PSP toxins (N-sulphocarbamoyl, carbamate and decarbamoyl toxins) are known (Fig. 2).

The nature and number of PSP compounds depend on the toxin patterns produced by the dinoflagellates, their storage and their metabolism in shellfish [18,19]. In addition to the differences in chemical structure, the individual PSP toxins show various toxicity. Originally, toxicities were expressed in mouse units (MU, or the amount of toxin that kills a 20-g mouse in 15 min), but with the introduction of pure saxitoxin as a standard substance for toxicity control this was refined to 1 microgram saxitoxin equivalents, usually being referred to 100 g of shellfish meat [14]. Th absolute toxicity of STX was assigned to be 5500 MU/mg,



R1	R2	R3	R4: carbamate toxins	R4: N-sulfo-carbamoyl-toxins	R4: decarbamoyl-toxins
H	H	H	STX	B1	dc-STX
OH	H	H	NEO	B2	dc-NEO
OH	H	OSO_3^-	GTX I	C3	dc-GTX I
H	H	OSO_3^-	GTX II	C1	dc-GTX II
H	OSO_3^-	H	GTX III	C2	dc-GTX III
OH	OSO_3^-	H	GTX IV	C4	dc-GTX IV

Fig. 2. Structure of naturally occurring PSP components [16]. STX = saxitoxin; NEO = neosaxitoxin; GTX = gonyautoxins.

where 1 MU is equivalent to 0.18 μg of STX as the dihydrochloride [11]. The value of 1.0 was established for the relative toxicity of STX [14].

Table 1 gives the specific toxicities of individual PSP toxins (absolute toxicity, expressed in MU/μM). The toxicity of the N-sulphocarbamoyl toxins is relatively low, that of the carbamate toxins is significantly higher and the decarbamoyl toxins exhibit intermediate toxicity [20].

In general, PSP toxins are heat stable at slightly acidic pH, but unstable and easily oxidized under alkaline conditions [4]. It is possible that PSP toxins are converted by enzymatic processes: the hydrolysis of N-sulphocarbamoyl toxins in clams leads to the more toxic decarbamoyl toxins. At the end of the metabolic process often only dc-STX is present [21]. One may assume that in man gastric hydrochloric acid also converts N-sulphocarbamoyl toxins into carbamate and decarbamoyl toxins, thus increasing the toxicity of PSP-contaminated seafood [16].

1.2. Diarrhetic shellfish poisoning (DSP)

1.2.1. Occurrence in seafood

After the consumption of shellfish which have been feeding on toxic dinoflagellates of the *Dinophysis* or *Prorocentrum* genera, intestinal disturbances such as diarrhoea, nausea, vomiting, abdominal pain and chills may occur. As in most cases diarrhoea is the predominant symptom, the syndrome is called diarrhetic shellfish poisoning (DSP) [22,23]. Species of DSP-producing algae are widely distributed but seldom form red tides. *Dinophysis fortii* has been identified as a producer of DSP in Japan [24], whereas *Dinophysis acuminata* is suspected to be the toxin producer in recent outbreaks in Netherlands and Germany [25–27]. It has been reported that in the presence of *Dinophysis fortii* at a low cell density of 200 cells/l, mussels and scallops became toxic enough to affect man [27].

1.2.2. Chemical structure and toxicity

The heat-stable, lipophilic substances of the DSP complex are classified into three groups: okadaic acid (OA) and its derivatives named dinophysistoxins (DTX), the pectenotoxins (PTX) and yessotoxin (YTX) [23] (Fig. 3).

OA and DTX were obtained from the digestive

TABLE 1

SPECIFIC TOXICITY OF PSP TOXINS [20]

N-Sulphocarbamoyl toxins		Carbamate toxins		Decarbamoyl toxins	
Toxin	Specific toxicity (MU/μM)	Toxin	Specific toxicity (MU/μM)	Toxin	Specific toxicity (MU/μM)
B1	250	STX	2100	dc-STX	900
B2	250	NEO	2300	dc-NEO	900
C1	17	GTX2	1000	dc-GTX2	380
C2	258	GTX3	1600	dc-GTX3	380
		GTX1	1900	dc-GTX1	950
		GTX4	1900	dc-GTX4	950

glands (hepatopancreas) of the blue mussel *Mytilus edulis* and some of the toxic compounds PTX and YTX have been identified in the digestive glands of the scallops *Patinopecten yessoensis* [28–31]. Among these toxins, only OA, DTX-1 and DTX-3 have been proved to induce diarrhoea in man [32]. Further measurements revealed that OA is the principal toxin in European mussels, whereas DTX-1 predominates in Japanese scallops [33,34]. Therefore, the monitoring of seafood for DSP toxins with high-performance liquid chromatographic (HPLC) methods is restricted to OA and DTX-1 determination [35].

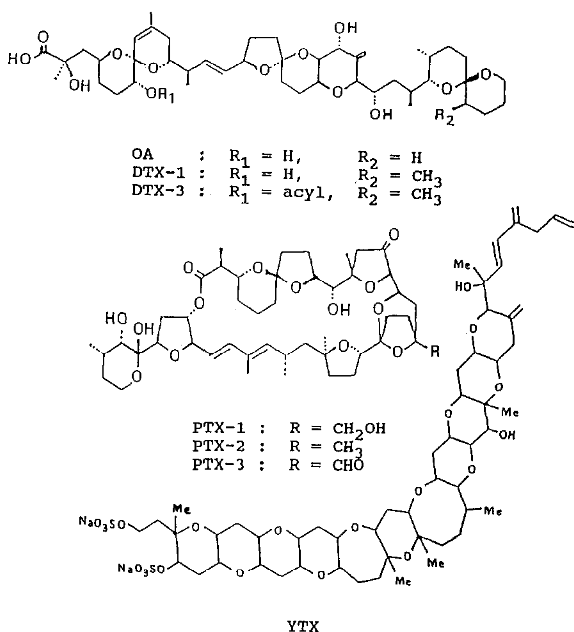


Fig. 3. Structure of DSP components [28]. OA = okadaic acid; DTX = dinophysistoxin; PTX = pectenotoxin; YTX = yessotoxin.

1.3. Regulations for phycotoxins in seafood

Marine phycotoxin regulations may involve environmental surveillance for toxic algal species in areas where shellfish are grown and legal actions to ensure that toxin-contaminated shellfish do not reach the consumer. Therefore, several countries have monitoring programmes to check for the occurrence of toxic phytoplankton [28].

Weighing the various factors that play a role in the decision-making process of establishing phytotoxin tolerances may not be easy. Despite this dilemma, a number of countries have established limits and regulations for marine phycotoxins [36]. Regulations exist for PSP and specifically for saxitoxin, and for DSP and specifically for okadaic acid [37].

Actual or proposed tolerance levels for PSP are typically 400 MU per 100 g; 40–80 μg PSP per 100 g and 40–80 μg saxitoxin per 100 g. For DSP the tolerance levels also vary: 5 MU per 100 g, not detectable in rat bioassay, and 20–60 μg DSP per 100 g (Table 2).

Different concentration limits are used to express the tolerance level for PSP: MU per 100 g and μg per 100 g. The latter unit currently seems to be less appropriate in the countries that use the mouse bioassay, because they actually test for toxicity only in the mouse [37]. Expression of a tolerance level for PSP in μg per 100 g would be valuable if the various PSP exhibit the same toxicity, but they do not [38].

TABLE 2

SUMMARY OF REGULATIONS FOR PSP AND DSP [37]

	PSP	DSP
Products	Molluscs, shellfish, bivalves, mussels	Molluscs, shellfish, bivalves, mussels
Limits	400 MU per 100 g 40–80 μg PSP per 100 g 40–80 μg saxitoxin per 100 g	5 MU per 100 g 20–60 μg per 100 g Not detectable in a rat bioassay
Methods	Mouse bioassay Spectrometry HPLC	Mouse bioassay Rat bioassay HPLC

To overcome the differences between the findings of the mouse bioassay and the results of PSP determinations by HPLC, accurate HPLC methods permitting the exact determination of all individual PSP components in seafood samples must be available [39–41]. The tolerance levels for DSP were generally set at the limit of detection of the analytical method used, most often a mouse bioassay; a few countries relied on a rat bioassay and incidentally HPLC in addition.

The most modern approach for the determination of both PSP and DSP is HPLC [37]. The application of HPLC for regulatory purposes, however, is hampered by the lack of validated analytical methodology, pure analytical standards of the various toxins and reference samples for analytical purpose. Neither the bioassays nor the HPLC procedures have been validated in collaborative studies. Therefore, developments in this area deserve strong support, because the enforcement of phycotoxin regulations ultimately depends on reliable analytical measurements [42].

2. ANALYTICAL METHODOLOGY

2.1. Methods for PSP determination

2.1.1. Bioassay

The highly potent and unpredictable nature of PSP necessitates constant monitoring of the toxin content of shellfish from beaches in affected areas. These monitoring programmes use the standard mouse bioassay for PSP determination as prescribed by the AOAC for determining the level of toxicity [43]. It consists of the intraperitoneal (i.p.) injection into mice of an acidified and heated extract of shellfish tissues and the determination of the time until death occurs [44]. By standardizing the conditions for the bioassay (mouse mass, pH of extract and salt concentration) a fairly reliable routine procedure was established [4]. Because different strains of mice differ in their susceptibility to the PSP toxins, the sensitivity of the mouse colony used must first be determined by calculating a correction factor (CF value) obtained after i.p. injection of the STX standard. The acidified extracts of shellfish are screen-tested in a few mice in order to determine the dilution of the extract that will kill mice of 19–21 g body mass within 5–7 min, the conditions under which the assay is most sensitive. PSP levels of samples as low as about 400 μg STX equivalent per kilogram of seafood can be detected [45].

The requirement for a large number and constant supply of mice is often cited as the main drawback of this bioassay [46]. An additional problem is the protective effect of NaCl; Schantz *et al.* [44] studied the effect of salt on the death times of mice and found that, at the 0.5% NaCl level, a 30% lower value was obtained for PSP toxin concentration. Further, the bioassay detects lethal toxicity in a sample, regardless of the chemical structure of the toxins. This may have advantages from a regulary standpoint but is a disadvantage when individual PSP toxins are to be determined. In view of these facts, chromatographic techniques have been developed to separate individual PSP toxins [47,48].

2.1.2. HPLC for PSP determination

HPLC techniques allow the separation and sensitive detection of individual PSP toxins irrespective of their number and group. Therefore, HPLC methods have opened up a new dimension in phycotoxin analysis [14]. However, the results obtained have to be comparable to those of the mouse bioassay [40]. This requirement is partly fulfilled by the application of identical procedures for sample preparation (Fig. 4).

Additionally, accurate HPLC determination of the various PSP components in the samples is a necessity.

The concentrations of individual PSP toxins were calculated on the basis of the PSP peaks in the HPLC traces, converted into their STX equivalent

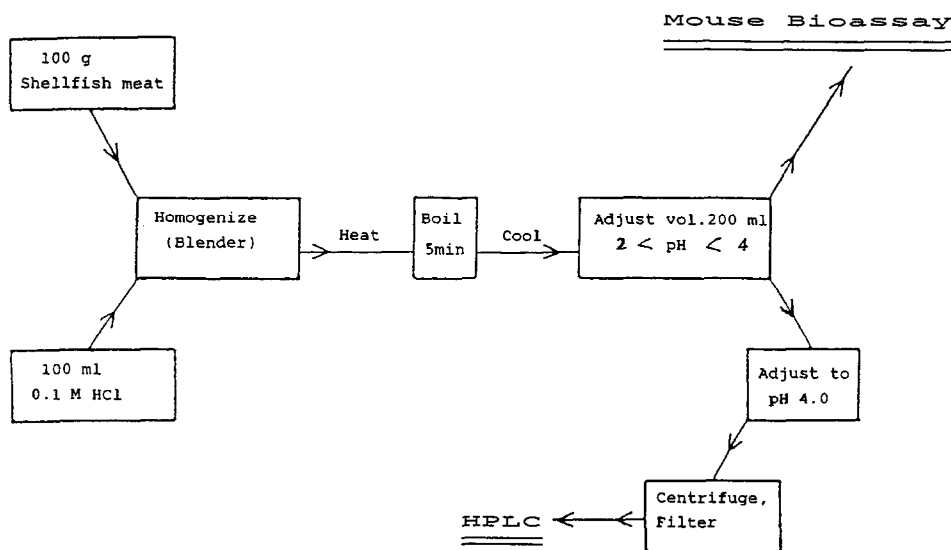


Fig. 4. Sample preparation procedure for comparative studies involving HPLC and mouse bioassay [39].

and summed for comparison with bioassay values [39]. Individual contributions to sample toxicity (G) were calculated for each toxin using the following equation [40]:

$$G = CTD/100$$

where C = toxin concentration (μm per 100 g), T = toxicity factor (μg STX/μM toxin) and D = dilution of sample (ml per 100 g of shellfish meat).

2.1.2.1. Toxin detection

The detection of the PSP toxins is based on the fluorimetric assay described by Bates and co-workers [50,51]. As PSP toxins show neither UV absorption nor fluorescence, STX was oxidized in alkaline solution to obtain derivatives detectable by common HPLC detectors. The derivatization reaction is based on the oxidation of STX to 8-amino-6-hydroxymethyl-2-aminopurine-3-propionic acid, which reacts in acidic solution to give a fluorescent pyrimidopurine (Fig. 5).

For PSP detection some workers have used this reaction and measured directly the fluorescence of the oxidation products [52,53]; others first separated the oxidation products by chromatography before subsequent fluorescence detection [54,55]. It should be noted that the application of the oxidation reaction for precolumn derivatization has several drawbacks. For example, our experiments with STX revealed that following oxidation with NaOH–H_2O_2 more than one peak appeared in the HPLC traces. In addition, the analysis of canned mussels contaminated with PSP revealed the presence of large amounts of decarbamoyl toxins in addition to carbamate toxins. However, up to now no chromatograms have been published showing the chromatographic separation of defined oxidation products of decarbamoyl toxins after precolumn derivatization [56,57]. Therefore, the oxidation of PSP toxins is usually carried out as a postcolumn reaction [58]. When this technique is applied the number of oxidation products of individual PSP

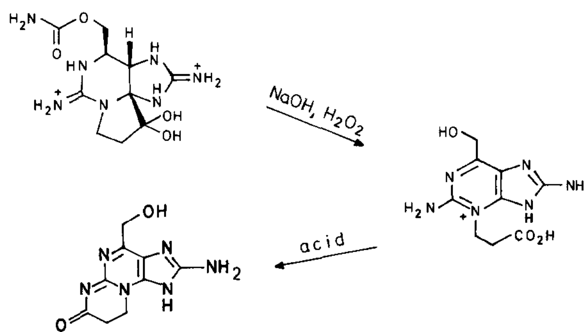


Fig. 5. Oxidation of saxitoxin to fluorescent pyrimidopurine [50].

components is not important, because the chromatographic separation is finished and the fluorescence detector records the oxidation products as a sum [59].

Various PSP toxins exhibit different fluorescence intensities after their oxidation, which must be taken into account in the evaluation of the chromatograms for the determination of PSP toxicity (Table 3).

2.1.2.2. Toxin separation

Many chromatographic techniques have been developed for separating PSP toxins in their underivatized form. At first these separations were carried out using ion-exchange and/or gel permeation techniques [60–62] and later silica-based HPLC-columns (amino or cyano columns) were applied [21,39,59]. However, the breakthrough in HPLC for PSP separation was the introduction of ion-pair chromatography [63].

Sullivan and Wekell [64] utilized an HPLC system equipped with a postcolumn derivatization unit and a fluorescence detector. HPLC separation of the PSP toxins is carried out on a polystyrene–divinylbenzene resin column (PRP-1; Hamilton, Darmstadt, Germany) and gradient elution with phosphate buffer solution, containing heptane and hexanesulphonic acids as ion-pair reagents. The carbamate toxins are well separated. However, the separation of the N-sulphocarbamoyl toxins C1–C4 is poor (Fig. 6).

TABLE 3

RELATIVE FLUORESCENCE INTENSITY OF PSP TOXINS AFTER OXIDATION UNDER ALKALINE CONDITIONS [53]

Toxin	Relative fluorescence intensity	Relative toxicity
Saxitoxin	1.00	1.00
Neosaxitoxin	0.04	1.00
Gonyautoxin I	0.05	0.73
Gonyautoxin II	1.80	0.42
Gonyautoxin III	1.80	0.67
Gonyautoxin IV	0.05	0.27
B1	0.41	<0.05
B2	0.05	0.09
C1	0.48	0.06
C2	0.48	0.02

When decarbamoyl toxins are present in the samples the chromatograms are ambiguous. Especially the separation of dc-STX and STX is not possible by application of HPLC according to Sullivan and Wekell [64] (Fig. 7). Therefore, it became an urgent issue to improve HPLC methods that would permit the complete separation of carbamate and decarbamoyl toxins [57].

Oshima *et al.* [66] proposed the application of three chromatographic runs for PSP determination. Three groups of toxins categorized by their basicity (group I, C1–C4; group II, GTX I–IV, B1 and B2, dc-GTX I–IV; group III, NEO, dc-STX, STX) are separated in three HPLC systems under isocratic conditions. A reversed-phase column (Develosil C_8-5; Nomura Chemicals) is used, and the eluents for carbamate–decarbamoyl separation contain heptanesulphonic acid as ion-pair reagent and tetrabutylammonium phosphate for the separation of the N-sulphocarbamoyl-11-hydroxysulphate toxins (because of their acidic nature). The method avoids gradient elution, and reliable results are obtained [67]. However, the expensive HPLC equipment and time-consuming prechromatographic steps are serious drawbacks of this method. Therefore, at pres-

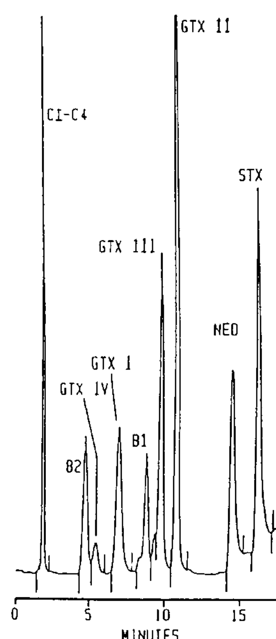


Fig. 6. HPLC illustrating the separation of PSP toxins by the method of Sullivan and Wekell [64].

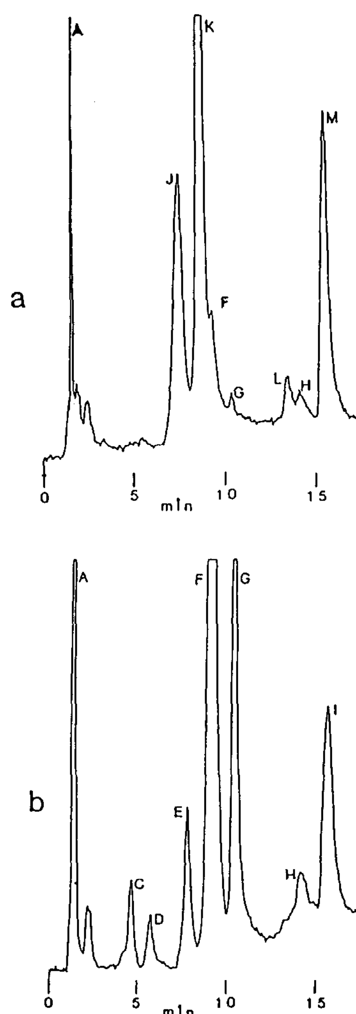


Fig. 7. HPLC of PSP toxins in shellfish extracts according to the method of Sullivan *et al.* [20]. (a) Littleneck clams (*Protothaca staminea*); (b) mussels (*Mytilus edulis*). A = C1 and C2; C = GTX IV; D = GTX I; E = B1; J = dc-GTX III; K = dc-GTX II; F = GTX III; G = GTX II; L = dc-NEO; M = dc-STX; I = STX.

ent, HPLC with gradient elution according to Sullivan and Wekell [64] is the most widely applied method for PSP determination [37,68].

Luckas *et al.* [57] proposed ion-pair chromatography on an RP-C_{18} column (Nucleosil 7-C_{18}; Macherey–Nagel, Düren, Germany) with octanesulphonic acid in the phosphate buffer eluent and isocratic elution to overcome the problem of dc-STX–STX separation. As the application of this method led to interferences in the chromatograms at the retention times of gonyautoxins, the chromatographic conditions were modified: the application of an RP-C_{18} column and two phosphate buffers containing octanesulphonic acid and acetonitrile–tetrahydrofuran as eluent was proposed [69]. A two-step elution allows the separation of carbamate and decarbamoyl toxins, and good resolution of the more strongly retained toxins (NEO, dc-STX, STX) is achieved (Fig. 8).

The separation dc-STX and STX is important because in shellfish N-sulphocarbamoyl toxins are metabolized into carbamate toxins in a first step [70], then the carbamate toxins are partially converted into decarbamoyl toxins, especially into dc-

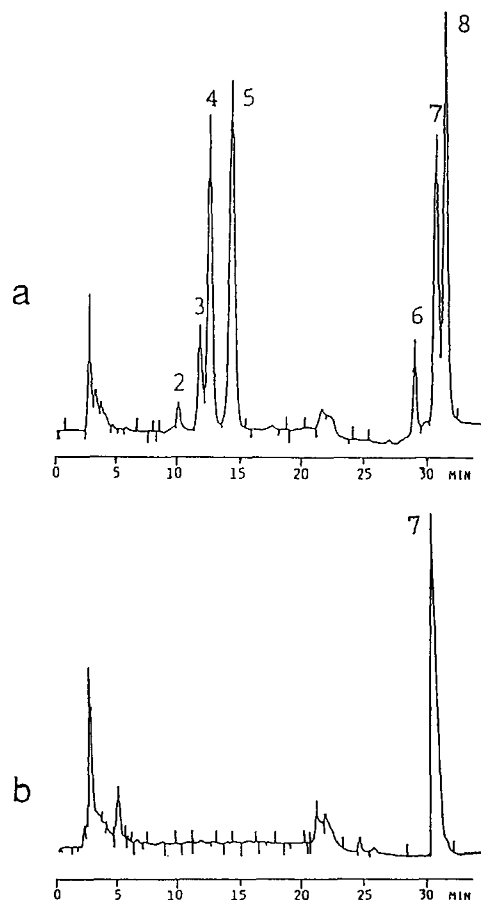


Fig. 8. HPLC of PSP toxins in shellfish extracts according to the method of Thielert *et al.* [69]. (a) Fresh mussels (*Mytilus edulis*, Spain); (b) canned mussels (*Mytilus edulis*, Spain). 2 = dc-GTX II; 3 = dc-GTX III; 4 = GTX II; 5 = GTX III; 6 = NEO; 7 = dc-STX; 8 = STX.

STX [21,71]. Therefore, in seafood carbamate toxins in additon to decarbamoyl toxins, especially dc-STX, have to be determined [16].

Thielert *et al.* [69] proposed extracting the samples with 0.03 *M* acetic acid to avoid destruction of the toxins. After ultrafiltration the extracts are injected directly into the chromatograph. To support the data, aliquots of the solution are treated with hydrochloric acid. The N-sulphocarbamoyl toxins are thus converted into their corresponding carbamate toxins, and higher concentrations of carbamate toxins, measured after HCl treatment, are good indicators of the N-sulphocarbamoyl content of the samples. Additionally, the calculation of the sum of STX compounds (*i.e.*, STX, GTX II and GTX III) and OH-STX compounds (*i.e.*, NEO, GTX I and GTX IV) makes it possible to typify PSP producers [72–75].

Irrespective of the PSP determination method applied, the sample preparation plays an important role in obtaining reasonable results. Obviously, differing data obtained with the mouse bioassay and HPLC may be explained by partial destruction of some toxins by the extraction procedure with 0.1 *M* HCl, which is recommended for both the mouse bioassay [43] and HPLC [64]. In addition, the inaccurate determination of decarbamoyl toxins by HPLC [16,39,40,53] may lead to disagreements between the methods. The influence of pH, temperature and storage time of the extracts on the variability of the mouse bioassay is well known [49], whereas the effect of acid treatment and heating time during the extraction process has been studied only recently [76]. Based on these data, it has been proposed to mix a homogenate with an equal volume of 1.0 *M* HCl, to heat in boiling water for 5 min and to apply the supernatant for PSP determination.

Among the advantages of the HPLC methods over the bioassay are greater sample throughput and significantly better sensitivity, in addition to the ability to determine individual PSP toxins (Table 4). Therefore, HPLC methods appear to be a viable alternative to bioassays for PSP determination in seafood.

TABLE 4

DETECTION LIMITS FOR PSP TOXINS BY HPLC AND MOUSE BIOASSAY [20]

Toxin	Detection limit (μM)	
	HPLC[a]	Mouse bioassay[b]
B1	0.040	6.7
B2	0.150	6.7
C1	0.006	59.0
C2	0.006	3.9
GTX I	0.100	0.5
GTX II	0.006	1.0
GTX III	0.006	0.6
GTX IV	0.100	0.5
NEO	0.065	0.4
STX	0.014	0.5

[a] Based on a 20-μl injection; twice baseline noise.
[b] Based on absolute toxicities for each toxin.

2.1.3. Mass spectrometry and HPLC–MS

Mass spectrometry (MS) is a powerful technique that also has an important future for the analysis of marine toxins. In additon to high sensitivity and selectivity, MS can provide structural information useful for the confirmation of toxin identity and the identification of new toxins [77]. Fast atom bombardment (FAB) MS has been investigated for PSP toxins and has proved useful as a means of structural confirmation at moderate sensitivity [78–80]. Recently, Quilliam *et al.* [81] have shown that HPLC–ion-spray MS is an excellent technique for the analysis of marine toxins. The ion formation mechanism is based on the ion evaporation phenomenon [82]. The technique can be used with a wide range of flow-rates and thus can be used on-line with HPLC and for direct sample introduction. PSP toxins are also well suited to ion-spray MS, whereby an abundant $[M+H]^+$ ion is observed.

However, combined HPLC–MS of PSP toxins has proved more challenging. Unfortunately, alkanesulphonates used as ion-pair reagents in the HPLC separation methods interfered with the ion-spray method. Therefore, various stationary phases and eluents are currently being investigated to improve the chromatographic separation [83]. HPLC separation on a PRP-1 column with an ammonium formate buffer and mass chromatograms of PSP toxins are illustrated in Fig. 9.

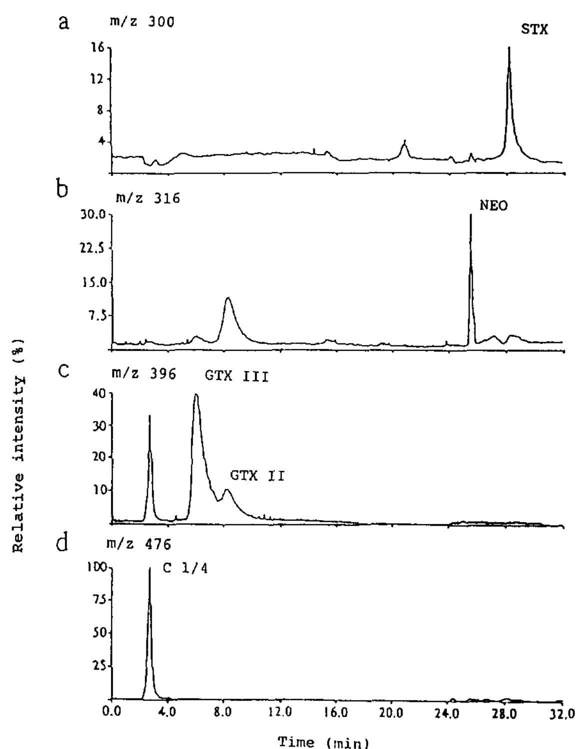


Fig. 9. Mass chromatograms from the HPLC–ion spray MS analysis of a mixture of PSP toxins [83].

2.2. Methods for DSP determination

Control measures to protect humans against DSP contaminated seafood are carried out by application of biological and chemical procedures [84]. The biological methods include the microscopy of certain algae, bioassays with mice and rats and immunological assays [*e.g.*, enzyme-linked immunosorbent assay, (ELISA)] [85]. Two bioassays have been extensively employed for control purposes, the mouse bioassay and the rat bioassay (Table 5). The mouse bioassay includes the i.p. injection of a purified extract. All DSP components are measurable. In the rat bioassay shellfish tissue is mixed with a normal diet and offered to the animals. The diarrhetic effects of OA, DTX-1, DTX-3 and in principle all compounds with similar effects are detected by this procedure. The chemical methods involve HPLC separation followed by fluorescence measurement. HPLC methods allow the sensitive determination of the individual DSP toxins OA and DTX-1. However, no collaborative study of any of the methods has yet been conducted, hence there are no internationally recommended procedures for DSP determination [28].

2.2.1. Bioassays

2.2.1.1. Mouse bioassay

The procedure consists in an extraction of shellfish soft tissue followed by i.p. injection into mice. The animals are observed for 24 h, the end-point being death. In cases of death, dilutions of the extract are tested in order to calculate the concentration of DSP, expressed as mouse units (MU) per gram or 100 g of shellfish. The procedure is unspe-

TABLE 5

METHODS FOR DSP DETERMINATION [84]

Country	Statutory limits	Methods of analysis
Denmark	No detectable amount	Mouse bioassay Rat bioassay
Germany	No detectable amount	Rat bioassay
France	0.2–0.4 MU/g digestive glands	Mouse bioassay
Ireland	No detectable amount	Rat bioassay, HPLC
Japan	5 MU per 100 g soft tissue	Mouse bioassay
Netherlands	No detectable amount	Rat bioassay
Norway	5–6 MU per 100 g soft tissue	Mouse bioassay
Portugal	No detectable amount	Mouse bioassay
Spain	No detectable amount	Mouse bioassay
Sweden	60 μg per 100 g soft tissue	HPLC, mouse bioassay

cific, as no signs except death are observed; the precision and sensitivity are unknown [86,87]. OA, DTX-1, DTX-3, PTX and YTX are detected, so a positive result does not necessarily mean the presence of a diarrhetic toxin.

2.2.1.2. Suckling mouse bioassay

Shellfish extracts are administered intra-gastrically to 4–5-day old mice using Teflon tubing, and the mice are kept for 4 h. After killing the mice, the whole intestine is removed and the fluid accumulation ratio (FAR) is expressed as the ratio of intestine mass to that of the remaining body. FAR values above 0.8–0.9 indicate a positive reaction. OA, DTX-1 and DTX-3 produce a positive reaction and PTX does not [88].

2.2.1.3. Rat bioassay

Soft tissue of shellfish (or digestive glands) is mixed with normal rat feed and offered to rats that have been starved for 24 h. After a 16-h period signs of diarrhoea and feed refusal signs are noted, and a semi-quantitative estimate of DSP toxicity is made based on these data. OA, DTX-1 and DTX-3 can be detected by this procedure. PTX and YTX give no reaction [26].

2.2.2. HPLC for DSP determination

In addition to the bioassays, many efforts have been made to determine DSP toxins OA and DTX-1 by HPLC. The HPLC method of Lee *et al.* [89] involves precolumn derivatization of the DSP toxins with 9-anthryldiazomethane (ADAM) and fluorescence detection. However, the application of the reaction with ADAM requires an additional clean-up step after derivatization. To avoid this time-consuming procedure the chromatographic equipment was modified by incorporating a column-switching system [90]. However, problems still arise from the instability of the ADAM reagent. Therefore, the reaction with 4-bromomethyl-7-methoxycoumarin (Br-Mmc) for derivatization of DSP toxins is proposed [91].

2.2.2.1. Sample preparation

The sample preparation procedure suggested by Lee *et al.* [89] can be improved. The first step consists in extraction of the homogenized sample with methanol–water (80:20) followed by cleaning the raw extract with *n*-hexane. By application of Br-Mmc determination the purification with *n*-hexane can be omitted, but additional clean-up by solid-phase extraction (SPE) with silica gel must be carried out. After sample preparation the DSP toxins are dissolved in dichloromethane. This solution is suitable for both derivatizations, the esterification with ADAM and the reaction with Br-Mmc [92].

2.2.2.2. Derivatization procedures

2.2.2.2.1. Derivatization with ADAM. ADAM reacts with carboxylic acids to give the 9-anthrylmethyl esters (Fig. 10). The reaction is carried out without catalyst at room temperature in 60 min [93]. The resulting ADAM derivatives of OA and DTX-1 are detectable with high sensitivity by HPLC with fluorimetric detection (excitation at 365 nm, emission at 412 nm).

2.2.2.2.2. Derivatization with Br-Mmc. Br-Mmc has been extensively used as a label for the derivatization of acidic compounds [94,95]. A convenient one-vial procedure using Br-Mmc for esterification of OA and DTX-1 to give fluorescent coumarin esters (excitation at 325 nm, emission at 390 nm) has been developed [91]. The derivatization of the DSP toxins with Br-Mmc is performed with a crown ether as catalyst in alkaline solution (Fig. 11).

In contrast to the ADAM esterification, the substances used for the Br-Mmc reaction are stable; no interfering peaks due to the derivatization reagent appear in the chromatograms [92].

2.2.2.3. HPLC separation

2.2.2.3.1. HPLC of ADAM derivatives. Usually the fluorescent ADAM derivatives of OA and DTX-1 are separated after clean-up on silica gel by HPLC on a C_{18} reversed-phase column using acetonitrile–methanol–water (8:1:1) as eluent. Both DSP toxins are clearly separable. The retention of OA was about 15 min and that of DTX-1 about 24 min [89]. In Fig. 12 an example of the application of this method is given [96].

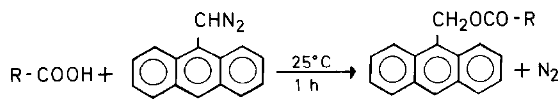


Fig. 10. Derivatization of OA and DTX-1 with ADAM [93].

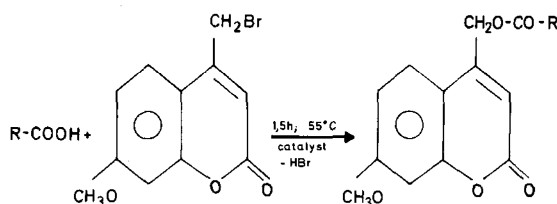


Fig. 11. Derivatization of OA and DTX-1 with Br-Mmc.

The purification of extracts containing ADAM derivatives is necessary to avoid interferences in the chromatograms. This procedure, however, performed by SPE, is time consuming and has negative effects on the reproducibility of data. To avoid the clean-up step, *i.e.*, to inject the derivatives directly into the chromatograph, the HPLC equipment was modified [90]. Two reversed-phase columns (column A, 25-cm RP-C_8; column B, 25-cm RP-C_{18}) and an enrichment column (5-cm RP-C_{18}) are combined with switching valves. After separation on the RP-C_{18} column A (pump A, eluent A) and cutting, either OA or DTX-1 is fixed on the enrichment column. By switching the valves again, the DSP toxins are eluted from the enrichment column and analysed by HPLC on the RP-C_{18} column B (pump B, eluent B) and subjected to fluorescence detection (Fig. 13). This method allows the injection of DSP-containing extracts into the chromatograph immediately after derivatization. In the chromatograms no interferences are visible and the toxin derivatives appear as sharp peaks (Fig. 14).

2.2.2.3.2. HPLC of Br-Mmc derivatives. For the HPLC of the Br-Mmc derivatives the use of a

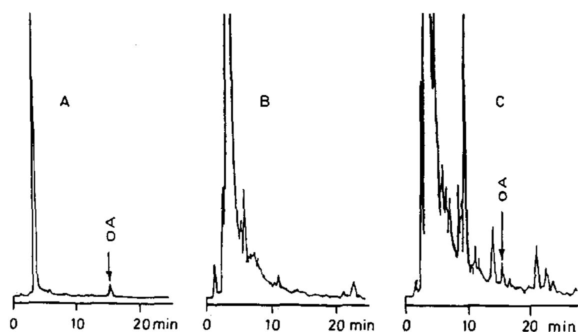


Fig. 12. HPLC of ADAM derivatives [96]: (A) okadaic acid; (B) uncontaminated mussels (Antifer, January 1988); (C) contaminated mussels (Antifer, August 1988).

C_{18} reversed-phase column and isocratic elution with acetonitrile–water (70:30) is suggested [91], and no interfering peaks appear at the retention time of the DSP toxins (Fig. 15). Br-Mmc derivatives of OA and DTX-1 are stable and an additional clean-up step after the derivatization is not required [92].

2.2.3. Mass spectrometry and HPLC–MS

Mass spectrometry using FAB ionization has been used by several workers for the mass spectral characterization of DSP toxins and the ADAM–OA derivative [97–99]. One report also included an attempt at liquid chromatography–mass spectrometry (LC–MS) coupled to a continuous-flow FAB interface [98]. Although a negative-ion chromatogram of the deprotonated molecular species of DTX-1 (m/z 817) was presented, the toxin eluted very early with a peak width of almost 5 min and no details of the sensitivity of the method were given [100]. In a more recent investigation, the presence of OA was confirmed by an impressive combination of electron impact (EI), chemical ionization (CI) and FAB mass spectrometry of the underivatized, and trimethylsilyl (TMS) and pentafluorobenzyl (PFB) derivatized toxin introduced via a direct probe [101].

Quilliam *et al.* [81] recently reported on an investigation of ion-spray mass spectrometry for the analysis of marine toxins, and concluded that this ionization process has great potential for the analysis of trace levels of polar marine toxins by mass spectrometry (and tandem mass spectrometry) combined with either direct flow injection or HPLC techniques.

Pleasance *et al.* [100] described the application of a combined LC–MS method using ion-spray ionization for the sensitive determination of OA in natural populations of dinoflagellates. Recently, Quilliam and Pleasance [83] reported a study of the confirmation of an incident of DSP in Eastern Canada. Analysis of whole mussel tissue extracts by combined LC–MS confirmed the presence of DTX-1 (Fig. 16).

Although HPLC–MS equipment is expensive, possibilities of automation can begin to justify such an investment if large numbers of samples can be analysed quickly with high-speed methods. For research studies, of course, the amount of informa-

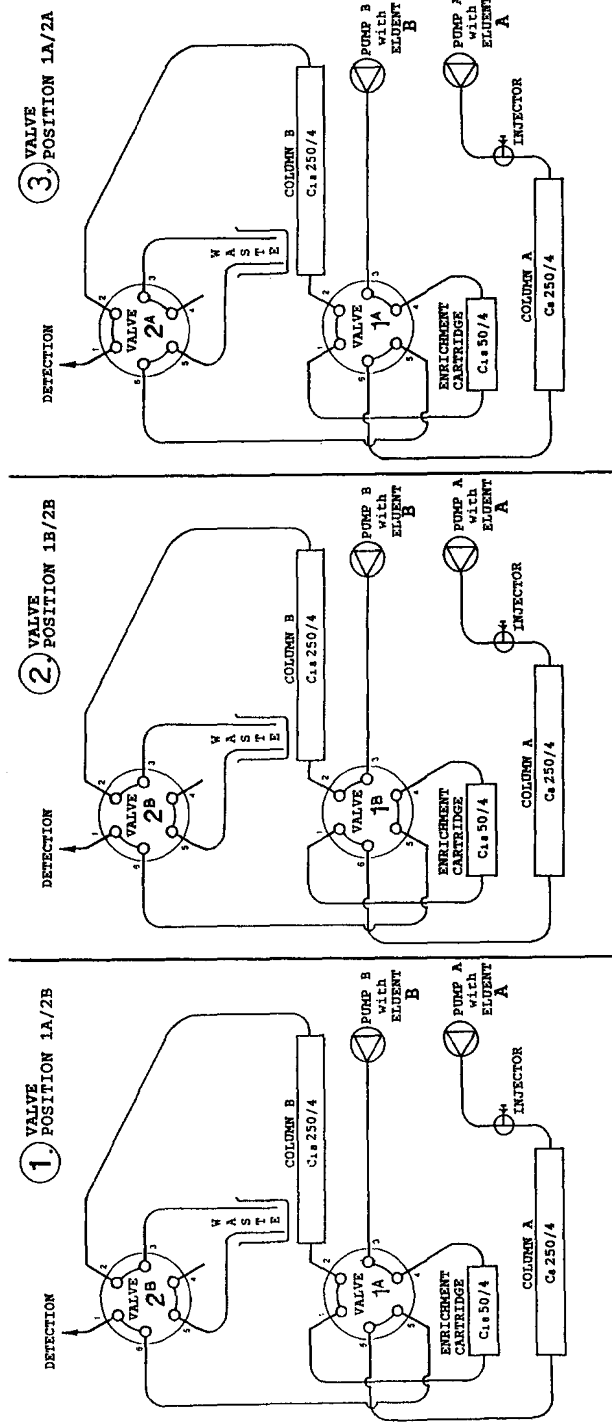


Fig. 13. Column-switching system for HPLC of ADAM derivatives [90].

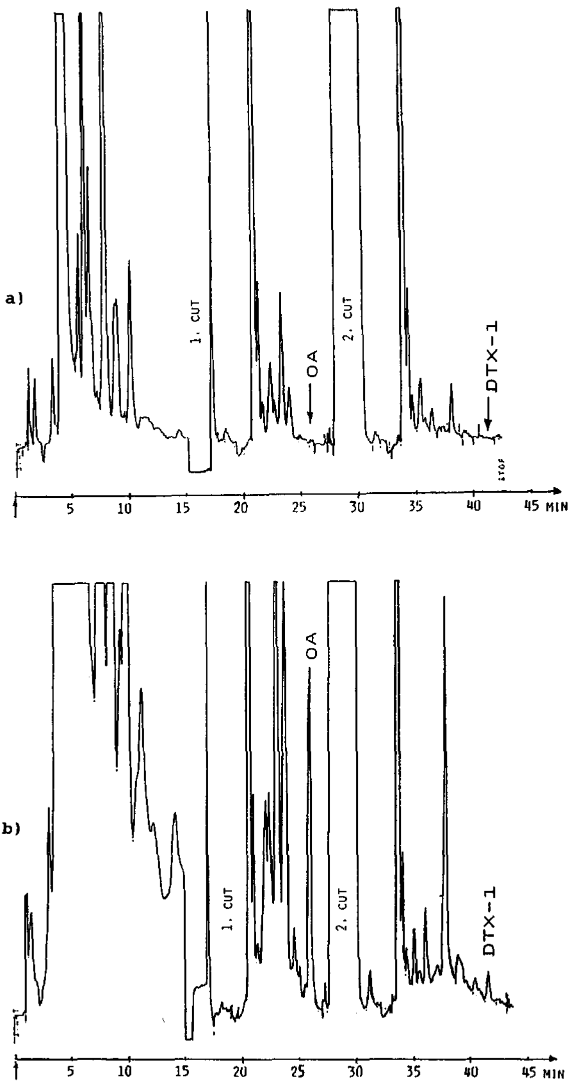


Fig. 14. HPLC of ADAM derivatives [91]. Column-switching system with two cuts. (a) Hepatopancreas (mussels, North Sea, uncontaminated); (b) hepatopancreas (mussels, Limfjord, contaminated).

tion provided by an HPLC–MS analysis is unsurpassed and will facilitate a greater understanding of the chemistry and biochemistry of seafood toxins.

3. CONCLUSIONS

Numerous cases of seafood poisoning occur worldwide each year especially due to the consumption of shellfish contaminated with high levels of toxins produced by marine dinoflagellates. As such incidents present a serious threat to public health and to the economy, there is a need for a better understanding the chemistry and biochemistry of seafood toxins and to develop analytical methods in order to guarantee safe, high-quality seafood products.

The most effective preventive measure is a monitoring programme with control at the source of the harvesting area. Production areas should be closed to harvesting when the toxin level in the shellfish approaches the established guideline or tolerance.

The best known hazard is paralytic shellfish poisoning (PSP). The accepted public health guideline recognized by most countries is 80 μg of PSP per 100 g of shellfish meat, using the AOAC mouse bioassay. This mouse bioassay has an acceptable

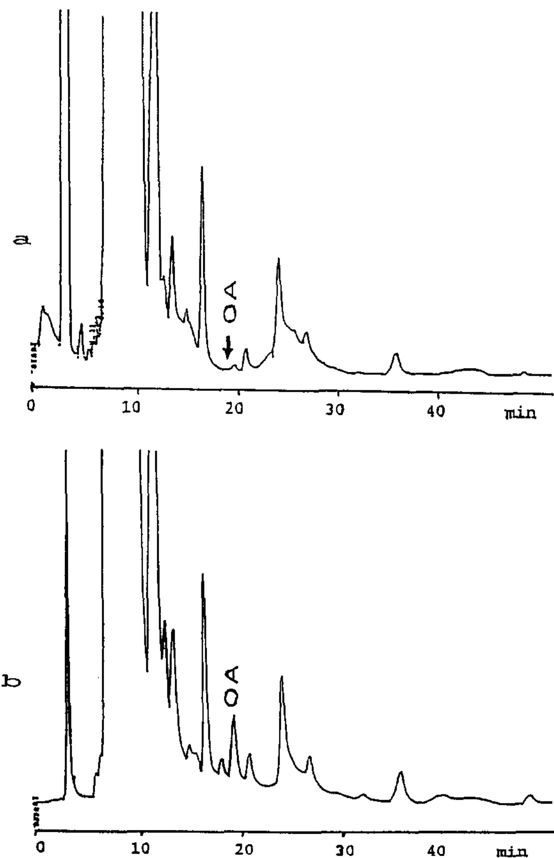


Fig. 15. HPLC of Br-Mmc derivatives [91]. (a) Hepatopancreas (mussels, North Sea, uncontaminated); (b) hepatopancreas (mussels, Limfjord, contaminated).

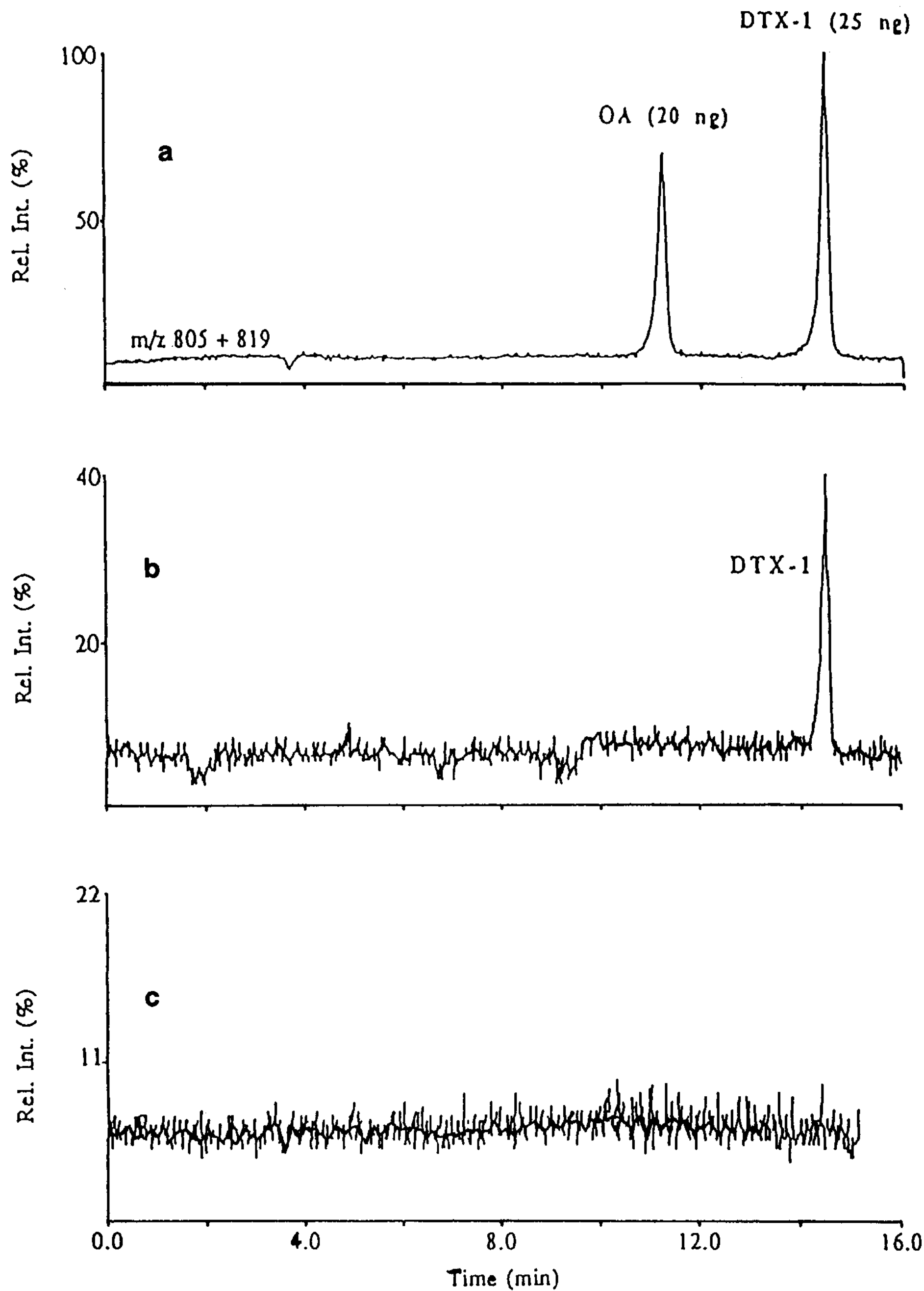


Fig. 16. LC–MS analysis of (a) DSP toxin standards and the extract of (b) suspect and (c) control mussel tissue extracts [83].

precision, but is lacking in sensitivity; 40 μg of PSP per 100 g shellfish meat are detectable. However, HPLC methods have been developed which can detect individual PSP toxins below the 1 μg per 100 g level.

Diarrhetic shellfish poisoning (DSP) is a more recently recognized problem. Okadaic acid (OA) and its derivatives (DTX-1 and DTX-3) are the principal toxins responsible for the diarrhetic symptoms.

The bioassays for DSP determination are not quantitative, the detection limit being 10 μg per rat. Compared with the bioassays, the HPLC method with fluorimetric detection for DSP provide advantages in terms of rapidity, accuracy, specificity and sensitivity.

The application of HPLC to the determination of the acidic components of DSP complex allows the detection of 10 μg of OA and/or DTX-1 per 100 g of shellfish meat. Therefore, HPLC analysis of these DSP components can act as indicators of DSP contamination, as no case of DSP contamination is known without the presence of at least one of these acidic components.

The acceptable levels for PSP and DSP differ significantly between countries. It is desirable for international organizations to evaluate the hazards caused by marine phycotoxins in order to provide a common basis for risk assessment, *i.e.*, to establish international toxin tolerances. For such an evaluation toxicity data are needed based on reliable analytical methodology. The further development of analytical methods for marine phycotoxins is especially needed as the enforcement of phycotoxin legislation is ultimately based on the ability of analysts to identify and determine these toxins accurately in seafood products. The HPLC methods discussed in this review are appropriate for solving these problems.

4. ABBREVIATIONS

Phycotoxins

PSP	Paralytic shellfish poisoning
B1–2, C1–4	N-Sulphocarbamoyl toxins
GTX I–IV	Gonyautoxin I–IV
dc-GTX I–IV	Decarbamoylgonyautoxin I–IV
NEO	Neosaxitoxin
dc-NEO	Decarbamoylneosaxitoxin
STX	Saxitoxin
dc-STX	Decarbamoylsaxitoxin
DSP	Diarrhetic shellfish poisoning
DTX	Dinophysistoxin
OA	Okadaic acid
PTX	Pectenotoxin
YTX	Yessotoxin

Derivatization reagents

ADAM	9-Anthryldiazomethane
Br-Mmc	4-Bromomethyl-7-methoxycoumarin

Units

MU	Mouse units

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