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Introduction

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Template for comments and secretariat observations

Date: xx/xx/20xx	Document: ISO/DIS xxxx
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1	2	(3)	4	5	(6)	7
MB	Clause No./ Subclause No./Annex (e.g. 3.1)	Paragraph/ Figure/Table/Note	Type of comment	Comment (justification for change) by the MB	Proposed change by the MB	Secretariat observations on each comment submitted
	3.1	Definition 1	ed	Definition is ambiguous and needs clarifying.	Amend to read '...so that the mains connector to which no connection...'	
	6.4	Paragraph 2	te	The use of the UV photometer as an alternative cannot be supported as serious problems have been encountered in its use in the UK.	Delete reference to UV photometer.	

English Version

Foodstuffs - Determination of saxitoxin-group toxins in
shellfish - HPLC method using pre-column derivatization
with peroxide or periodate oxidation

Produits alimentaires - Détermination de la teneur en
toxines du groupe de la saxitoxine dans les coquillages
- Méthode par CLHP avec dérivation pré-colonne et par
oxydation au peroxyde ou au periodate

Lebensmittel - Bestimmung von Toxinen der
Saxitoxingruppe in Schalentieren - HPLC-Verfahren mit
Vorsäulenderivatisierung und Peroxid- oder
Periodatoxidation

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 275.

If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

This draft European Standard was established by CEN in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

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Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

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COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (prEN 14526:2024) has been prepared by Technical Committee CEN/TC 275 “Food analysis – Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

This document will supersede EN 14526:2017.

prEN 14526:2024 includes the following significant technical changes with respect to EN 14526:2017:

- new Figure 2 detailing a procedure for a large numbers of test samples added;
- mandatory Clause 3 Terms and definitions added;
- new Clause 12 specifying quality controls added;
- new Annex B with example chromatograms added.

Introduction

Paralytic shellfish poisoning (PSP) toxins are derivatives of saxitoxin. These toxins have been detected in filter-feeding bivalve molluscs in various parts of the world. Paralytic shellfish poisoning is characterized by symptoms varying from slight tingling sensation or numbness around the lips to fatal respiratory paralysis. This document describes an analytical method for the quantification of these PSP toxins by extraction from shellfish tissue followed by several clean-up steps and a separation by high performance liquid chromatography (HPLC) with fluorescence detection (FLD).

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1 Scope

This document specifies a method [1] for the quantitative determination of saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), neosaxitoxin (NEO), decarbamoyl neosaxitoxin (dcNEO), gonyautoxin 1 and 4 (GTX1,4; sum of isomers), gonyautoxin 2 and 3 (GTX2,3; sum of isomers), gonyautoxin 5 (GTX5 also called B1), gonyautoxin 6 (GTX6 also called B2), decarbamoyl gonyautoxin 2 and 3 (dcGTX2,3; sum of isomers), N-sulfocarbamoyl gonyautoxin 2 and 3 (C1,2; sum of isomers) and N-sulfocarbamoyl gonyautoxin 1 and 4 (C3,4; sum of isomers) in (raw) mussels, oysters, scallops and clams. Laboratory experience has shown that this document can also be applied to other marine invertebrates [2], [3] and processed products of those species, however, no complete interlaboratory validation study according to ISO 5725-2:1994 has been carried out so far. The method described was validated in an interlaboratory study [4], [5] and was also verified in a European Union Reference Laboratory for Marine Biotoxins (EURLMB)-performance test aiming the total toxicity of the samples [6]. Toxins which were not available in the first interlaboratory study [4], [5] as dcGTX2,3 and dcNEO were validated in two additional interlaboratory studies [7], [8]. The lowest validated levels [4], [5], [8], are given in μg toxin (free base)/kg shellfish tissue and also as μmol /kg shellfish tissue and are listed in Table 1.

Table 1 — Lowest validated levels

Toxin		$\mu\text{g}/\text{kg}$	$\mu\text{mol}/\text{kg}$
saxitoxin (STX) [5]		22 ^c	0,07 ^c
gonyautoxin 2,3 (GTX2,3) [5]		114 ^b	0,29 ^b
gonyautoxin 5 (GTX5) [5]		27 ^c	0,07 ^c
decarbamoyl saxitoxin (dcSTX) [5]		8 ^c	0,03 ^c
neosaxitoxin (NEO) [5]		33 ^c	0,10 ^c
gonyautoxin 1,4 (GTX1,4) [5]		61,4 ^c	0,15 ^c
N-sulfocarbamoyl gonyautoxin 2,3 (C1,2) [5]		93 ^c	0,20 ^c
N-sulfocarbamoyl gonyautoxin 1,4 (C3,4) [5]		725 ^b	1,48 ^b
gonyautoxin 6 (GTX6)	direct [4]	30	0,08
	indirect [9]	834 ^b	2,11 ^b
decarbamoyl gonyautoxin 2,3 (dcGTX2,3) [8]		271 ^a	0,77 ^a
decarbamoyl neosaxitoxin (dcNEO) [8]		594 ^b	2,18 ^b
^a lowest spiked level; mean recovery: 58 % [8] ^b lowest concentration tested ^c lowest concentration tested with a HorRat < 2 [4], [5]			

A quantitative determination of GTX6 was not included in the first interlaboratory study but several laboratories detected this toxin directly after solid phase extraction with ion-exchange (SPE-COOH) clean-up and reported a mass concentration of 30 $\mu\text{g}/\text{kg}$ or higher in certain samples. For that reason, the present method is applicable to quantify GTX6 directly, depending on the availability of the standard substance. Whenever GTX6 standard is not commercially available, it is possible to determine GTX6 after hydrolysis of Fraction 2 of the SPE-COOH clean-up, described in 6.4, as NEO. The indirect quantification of GTX6 was validated in two additional interlaboratory studies [7], [8]. A study to compare direct and indirect GTX6 quantification was conducted at the EURLMB [16].

A quantitative determination of C3,4 was included in the first interlaboratory study. The present method is applicable to quantify C3,4 directly, depending on the availability of the standard substance. If no standard substances are available, C3,4 can only be quantified as GTX1,4 if the same hydrolysis protocol used for GTX6 (6.4) is applied to Fraction 1 of the SPE-COOH clean-up [10]. A study to compare direct and indirect C3,4 quantification was conducted at the EURLMB [16].

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

EN ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025)*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

4 Principle

WARNING — PSP toxins are neurotoxins which can be taken up by inhalation or orally. Therefore, adequate protection measures are to be applied.

Paralytic Shellfish Poisoning (PSP) toxins are extracted from shellfish tissue homogenate by heating with acetic acid. After centrifugation the supernatant is purified by solid phase extraction (SPE) using a C18 clean-up cartridge. It is analysed by HPLC after oxidation with periodate or peroxide with fluorescence detection. Most toxins (STX, C1,2, GTX5, dcSTX, GTX2,3 and dcGTX2,3) can be quantified after SPE-C18 clean-up¹.

Oxidation of PSP toxins leads to several reaction products that are separated by reversed phase HPLC and detected by fluorescence detection. The obtained reaction products for PSP toxins after oxidation with peroxide and periodate are listed in Table 2. Additionally, the corresponding chromatograms are shown in Figure 1.

The gonyautoxins GTX2 and GTX3 as well as GTX1 and GTX4 and decarbamoyl gonyautoxins dcGTX2 and dcGTX3 and the N-sulfocarbamoyl gonyautoxins C1 and C2 as well as C3 and C4 are structural isomers and lead with both oxidation modes to the same reaction products. The amount of structural isomers is determined as the sum of both toxins.

STX reacts to a single specific oxidation product regardless of the kind of oxidation reaction (whether peroxide or periodate). The same is valid for GTX2,3 as well as GTX5 and C1,2. In contrast, dcSTX and dcGTX2,3 produce each two different oxidation products in both oxidation reactions, see also Table 2. The toxin dcNEO is oxidized into two oxidation products only with the periodate oxidation. Each of the

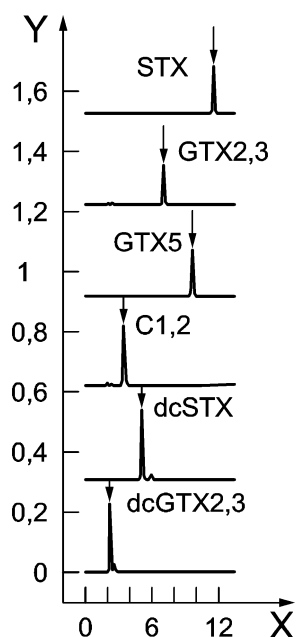
¹ This document is based on a procedure described by Lawrence et al. [4] and was also published as AOAC Official Method 2005.06 [1].

toxins NEO, GTX6, GTX1,4 and C3,4 produce three peaks after periodate oxidation but normally the second eluting peak is used for quantification (peroxide oxidation cannot be used for quantification).

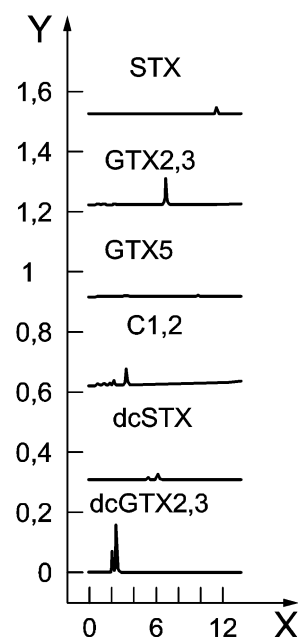
Co-occurrence of different PSP toxins in shellfish can influence the analytical results, because some of the PSP toxins can (partially) lead to the same reaction products (see Table 2 and Annex B). So, the chromatograms shall be carefully interpreted after a SPE C18 clean-up.

Table 2 — Reaction products after oxidation with periodate and peroxide

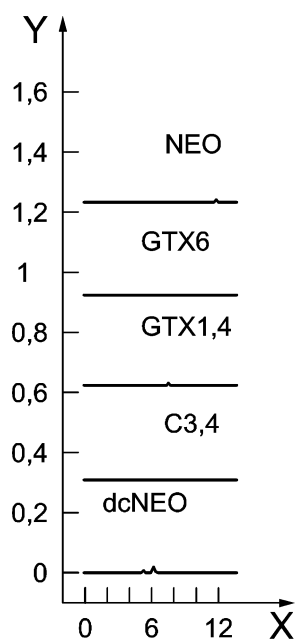
Toxin	Oxidation products and HPLC-eluting order		Intensity		Oxidation product at the same retention time as	
	peroxide	periodate	peroxide	periodate	peroxide	periodate
STX	one	one	++	+	NEO ^a (3) ^b	NEO (3); GTX6 (3)
dcSTX	first (1)	first (1)	++	-		dcNEO (1)
	second (2)	second (2)	+	+	NEO ^a (2)	NEO (2); GTX6 (2); dcNEO (2)
NEO	no	first (1)	—	+		GTX6 (1)
	second (2)	second (2)	-	++	dcSTX (2)	GTX6 (2); dcSTX (2); dcNEO (2)
	third (3)	third (3)	-	+	STX	STX; GTX6 (3)
C1,2	one	one	++	+		
C3,4	no	first (1)	—	+		GTX1,4 (1)
	no	second (2)	—	++		GTX1,4 (2); dcGTX2,3 (2)
	no	third (3)	—	+		GTX1,4 (3); GTX2,3
GTX1,4	no	first (1)	—	+		C3,4 (1)
	no	second (2)	—	++		C3,4 (2); dcGTX2,3 (2)
	third (3)	third (3)	-	++	GTX2,3	C3,4 (3); GTX2,3
GTX2,3	one	one	++	++	GTX1,4 ^a (3)	C3,4 (3); GTX1,4 (3)
GTX5	one	one	++	-		
GTX6	no	first (1)	—	+		NEO (1)
	no	second (2)	—	++		NEO (2); dcSTX (2); dcNEO (2)
	no	third (3)	—	-		NEO (3); STX
dcGTX2,3	first (1)	first (1)	++	+		
	second (2)	second (2)	+	++		C3,4 (2); GTX1,4 (2)
dcNEO	first (1)	first (1)	-	++		dcSTX (1)
	second (2)	second (2)	-	+	dcSTX (2)	dcSTX (2); NEO (2); GTX6 (2)
Intensity: — not visible - very low + low ++ high						
^a High concentration of the indicated toxin may influence the quantification by simulating an increased content. ^b Numbers in curly brackets are the elution order.						



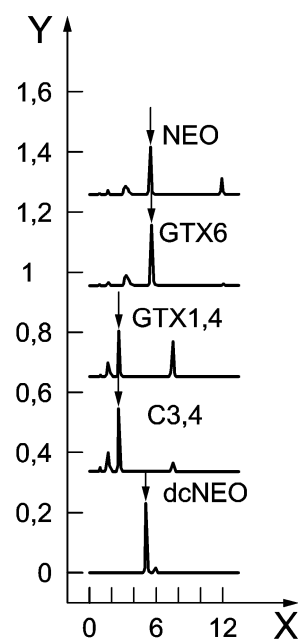
a) Non N1-hydroxylated toxins: peroxide



b) Non N1-hydroxylated toxins: periodate



c) N1-hydroxylated toxins: peroxide



d) N1-hydroxylated toxins: periodate

Key

X detection response (V)

Y time (min)

Figure 1 — Reaction products after derivatization with peroxide and periodate (peaks for quantification are marked with arrows)

For the quantitative determination of most N1-hydroxylated toxins, a fractionation by SPE-COOH clean-up is necessary (shown in Table 4) because the oxidation products of some PSP toxins (NEO and GTX6, GTX1,4 and C3,4) are identical. This step separates the PSP toxins into three distinct groups, namely the C toxins, the GTX toxins and the saxitoxin group by elution with mobile phases of different ionic strength. For example, with Bakerbond, the C toxins elute unretained with water in Fraction 1, the GTX toxins (GTX1 to GTX6 as well as dcGTX2 and dcGTX3) elute with 0,05 mol/l NaCl in Fraction 2 while the saxitoxin group (STX, NEO, dcNEO and dcSTX) requires 0,3 mol/l NaCl for elution in Fraction 3. These fractions can be analysed by HPLC-FLD after oxidation with periodate or peroxide.

5 Reagents

If not otherwise specified, reagents of pro analysis and solvents suitable for HPLC-FLD shall be used.

Water shall be distilled in glass vessels or demineralized before use or shall be of equivalent purity according to EN ISO 3696.

If not already specified, stability of solutions should be determined by the laboratory.

5.1 Methanol, HPLC quality.

5.2 Acetonitrile, HPLC quality.

5.3 Ammonium formate:

5.3.1 Ammonium formate solution, substance concentration $c = 0,3 \text{ mol/l}$.

Dissolve 1,892 g of ammonium formate (crystalline powder) (5.3) in 100 ml of water.

5.4 Glacial acetic acid:

5.4.1 Acetic acid solution 1, mass fraction $p \approx 1 \%$.

Dilute 1 ml of glacial acetic acid (5.4) to 100 ml with water.

5.4.2 Acetic acid solution 2, $c \approx 0,1 \text{ mol/l}$.

Dilute 572 μl of glacial acetic acid (5.4) to 100 ml with water.

5.4.3 Acetic acid solution 3, $c \approx 0,1 \text{ mmol/l}$.

Dilute 100 μl of acetic acid solution 2 (5.4.2) to 100 ml with water.

5.4.4 Acetic acid solution 4, mass fraction $p \approx 0,6 \%$.

Dilute 0,6 ml of glacial acetic acid (5.4) to 100 ml with water.

5.5 Ammonium acetate:

5.5.1 Ammonium acetate solution 1, $c = 0,1 \text{ mol/l}$.

Dissolve 0,77 g of ammonium acetate (5.5) to 100 ml with water.

5.5.2 Ammonium acetate solution 2, $c = 0,01 \text{ mol/l}$.

Dilute 10 ml of ammonium acetate solution 1 (5.5.1) to 100 ml with water.

5.6 Sodium chloride:

5.6.1 Sodium chloride solution 1, $c = 0,05$ mol/l.

Dissolve 0,29 g of sodium chloride (5.6) to 100 ml with water.

5.6.2 Sodium chloride solution 2, $c = 0,3$ mol/l.

Dissolve 1,75 g of sodium chloride (5.6) to 100 ml with water.

5.7 Hydrochloric acid, $c = 1$ mol/l.

5.8 Disodium hydrogenphosphate or disodium hydrogenphosphate 7-hydrate:

5.8.1 Disodium hydrogenphosphate solution, $c = 0,3$ mol/l.

Dissolve 4,26 g of disodium hydrogenphosphate (5.8) in 100 ml water or dissolve 8,04 g of disodium hydrogenphosphate 7-hydrate (5.8) in 100 ml water.

5.9 Sodium hydroxide:

5.9.1 Sodium hydroxide solution 1, $c = 1$ mol/l.

Dissolve 4 g of sodium hydroxide (5.9) to 100 ml with water.

5.9.2 Sodium hydroxide solution 2, $c = 0,2$ mol/l.

Dilute 10 ml of sodium hydroxide solution 1 (5.9.1) to 50 ml with water.

5.10 Hydrogen peroxide, $w = 30$ %:

5.10.1 Hydrogen peroxide solution, $w \approx 10$ %.

Dilute 3 ml of hydrogen peroxide solution (5.10), of mass fraction $w = 30$ % with 6 ml of water. Prepare fresh every day. Store both solutions in the dark at approximately +4 °C.

5.11 Periodic acid:

5.11.1 Periodic acid solution 1, $c = 0,1$ mol/l.

Dissolve 0,2279 g of periodic acid (5.11) in 10 ml of water.

5.11.2 Periodic acid solution 2, $c = 0,034$ mol/l.

Dilute 3,4 ml of periodic acid solution 1 (5.11.1) with 6,6 ml of water. Store in a refrigerator in the dark at approximately +4 °C. Prepare fresh every day.

5.12 Periodate oxidation reagent.

Mix one volume part of periodic acid solution 2 (5.11.2) with one volume part of disodium hydrogenphosphate solution (5.8.1) and one volume part of ammonium formate solution (5.3.1). Bring the mixture to pH $8,2 \pm 0,3$ by drop wise adding sodium hydroxide solution 2 (5.9.2) and check the pH by using a pH meter. Prepare fresh every day of analysis.

5.13 PSP toxin standard substances:

5.13.1 PSP toxin stock solutions.

For convenience, standard substances can be combined into four or more mixtures by appropriate dilution of standard solutions in water. Table 3 shows suitable concentration for each PSP toxin in four stock solution mixtures. Store the solutions in the dark at approximately +4 °C and check the mass concentrations regularly after 2 weeks or store in the dark at approximately –18 °C or below and check the mass concentrations regularly after 6 months.

Table 3 — Example of suitable compositions and concentrations for each PSP toxin in four stock solution mixtures

Stock solution mixtures		Toxin concentration	
		µg/ml ^a	nmol/ml
Mix 1	GTX1,4	0,41	1,0
	NEO	0,32	1,0
Mix 2	GTX2,3	0,30	1,0
	GTX5	0,40	1,0
	STX	0,38	1,0
	dcSTX	0,26	1,0
	dcGTX2,3	0,35	1,0
	C1,2	0,48	1,0
Mix 3	dcNEO	0,27	1,0
Mix 4	GTX6	0,40	1,0
	C3,4	0,49	1,0
^a related to the free base of the toxins			

NOTE 1 Ampoules containing separately GTX1,4, NEO, GTX2,3, GTX5, STX, dcSTX, dcGTX2,3, C1,2, dcNEO, GTX6, C3,4 standard substances in aqueous hydrochloric acid or aqueous acetic acid with concentrations ranging from 10 µmol/l to 200 µmol/l are currently commercially available².

NOTE 2 See chromatograms for the mixtures in Table 3 in Annex B.

Some of the standard substances can be contaminated with other PSP toxins; therefore, the impurities shall be taken into account for calibration purposes (by quantifying impurities, running different calibration curves or including it in uncertainty measurements).

² Suitable calibration solutions can be obtained from the National Research Council Canada, Halifax, Canada. Further information on suitable calibration solutions is e. g. available on the homepage of the European Reference Laboratory on Marine Biotoxins https://www.aesan.gob.es/en/CRLMB/web/public_documents/seccion/materiales_referencia.htm. This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of this source of supply. Equivalent products may be used if they can be shown to lead to the same results.

5.13.2 PSP toxin calibration solutions.

Prepare a calibration with at least five points for the determination of PSP toxins for example undiluted, 2,5 fold, 5 fold, 7,5 fold and 10 fold dilution of the PSP stock solution (5.13.1) with 0,1 mmol/l of acetic acid solution 3 (5.4.3). PSP toxin calibration solutions may be also prepared by diluting stock solution mixtures with water (as long as the pH is acidic). Store in the dark at -18°C and check the mass concentration regularly after 6 months.

NOTE 1 It is important to store diluted standard solutions in plastic vials or in deactivated glass containers which can e.g. be achieved by soaking the vials overnight in sodium hydroxide, rinsed with water followed by methanol (5.1), and dried.

For the interlaboratory study described in Clause A.1 [4], [5], three calibration points were used. However, in order to increase the robustness of the method, it is advised to use at least five calibration points.

NOTE 2 Another method to prepare the calibration solution is to implement this in the oxidation step (7.5.2 and 7.5.3). Different aliquots from the PSP toxin stock solution are used and made up to 100 μl final volume with 0,1 mmol/l acetic acid solution 3 (5.4.3).

5.13.3 PSP-solution for checking the efficiency of the cartridges

Prepare solutions of toxins of the appropriate mass concentration for checking the recovery of the toxins on the SPE-cartridges.

One option is to prepare the mix in 0,6 % acetic acid solution 4 (5.4.4) to check recovery for SPE-C18 and prepare the mix in water adjusted to $\text{pH } 6,5 \pm 0,3$ to check SPE-COOH.

A second option is to prepare the mix in 0,6 % acetic acid solution 4 (5.4.4); Toxins quantified after peroxide oxidation: dcGTx2,3; C1,2; dcSTx; GTx2,3; GTx5 and STx will be only checked after SPE-C18.

For all other toxins checks will be done after both SPE-C18 and SPE-COOH clean-ups. For this, standard solutions will be passed through both clean-ups sequentially after the SPE-C18 the pH is adjusted to $6,5 \pm 0,3$ and recovery will be evaluated after each clean-up. For example, the standards used for these checks can have a 0,8 μM concentration.

5.14 Matrix modifier (MM) for periodate oxidation.

Use a blank extract (PSP free) from oysters as described in 6.1 and 6.2. If stored frozen at -20°C , this initial PSP-free crude oyster extract is stable and can be used within at least two months. For use as matrix modifier, clean-up according to 7.3.1 and adjust the extract to $\text{pH } 6,5 \pm 0,3$ with sodium hydroxide solution 1 (5.9.1). Filter the supernatant using a 0,45 μm filter (6.18) and store the obtained matrix modifier in a refrigerator. Analyse the matrix modifier for PSP toxins by periodate and peroxide oxidation to ensure absence of toxins before use. It shall be prepared every two weeks (i.e. again cleaned up from the crude extract).

NOTE Different batches of matrix modifier could affect enormously the recovery of N-1 hydroxylated toxins, in particular NEO, due to matrix effects. Therefore, when selecting matrix modifier for periodate oxidation it is important to check if the matrix modifier is affecting the recovery of these toxins.

5.15 HPLC eluents:

Prepare eluents A and B fresh before each analytical series [12].

5.15.1 Eluent A: Ammonium formate, $c = 0,1 \text{ mol/l}$.

Dissolve 6,31 g of ammonium formate (5.3) in 1 l water and adjust to $\text{pH } 6,0 \pm 0,3$ by adding approximately 6 ml of acetic acid solution 2 (5.4.2).

5.15.2 Eluent B: Ammonium formate, $c = 0,1 \text{ mol/l}$ in 5 % acetonitrile.

Dissolve 6,31 g of ammonium formate (5.3) in 950 ml water and add 50 ml of acetonitrile (5.2). Adjust to pH $6,0 \pm 0,3$ by adding approximately 6 ml of acetic acid solution 2 (5.4.2). The composition can be adjusted.

6 Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

6.1 Grinder.

6.2 Balance, capable of weighing to the nearest 0,01 g.

6.3 Analytical balance, capable of weighing to the nearest 0,1 mg.

6.4 Plastic centrifuge tubes, polypropylene, 50 ml, with caps.

6.5 Centrifuge, capable to reach $3\,600\text{ g}^3$ at the outer end of the centrifuge tubes.

6.6 Pipettes, autopipettes with disposable plastic tips.

6.7 Vortex mixer.

6.8 Water bath or hot plate.

6.9 Graduated conical test tube, 15 ml.

6.10 SPE-C18 cartridges, e.g. 500 mg per 3 ml volume.

Check each new batch of SPE-C18 cartridges (e.g. Supelcoclean LC18) with standard solutions (5.13.3) to ensure that minimum recovery obtained with the C18-cartridge is 80 %. This check is necessary due to experiences gathered during method development as it was observed that variations can occur.

6.11 SPE-COOH ion exchange cartridges, e.g. 500 mg per 3 ml volume.

Check each new batch of SPE-COOH cartridges (e.g. Bakerbond Carboxylic Acidsilane or, optional, a weak cation exchanger, e.g. Strata-X[®] from Phenomenex) with standard solutions (5.13.3) to ensure that minimum recovery obtained with the COOH-cartridge is 80 % and the correct elution patterns are obtained according to 7.3.2. This check is necessary due to experiences gathered during method development as it was observed that variations can occur.

6.12 Manifold or automatic SPE station (for the SPE clean-ups).

6.13 Block heater (or similar) for the hydrolysis step.

6.14 Reaction tubes, e.g. polypropylene vials with 1,5 ml.

6.15 pH indicator paper, able to precisely identify a pH of $6,5 \pm 0,3$.

6.16 pH meter, with micro-electrode.

³ $g = 9,81 \text{ m} \cdot \text{s}^{-2}$.

6.17 Rotary evaporator or similar equipment with suitable glass containers, optionally for samples with low concentrations.

6.18 Membrane filter, for aqueous solutions, with a pore size of 0,45 µm, e.g. regenerated cellulose.

6.19 HPLC vials, e.g. amber glass or polypropylene vials.

6.20 HPLC system, comprising the following:

6.20.1 Injector, preferably a refrigerated injector, capable of refrigerating at $(6 \pm 2) ^\circ\text{C}$ and injection up to 100 µl.

NOTE Some toxin oxidation products are unstable when using an auto sampler at room temperature, therefore the responses of these toxins (e.g. dcNEO and NEO) can decrease during a sequence. This could be improved by using a cooled auto sampler [12].

6.20.2 Pump, capable of gradient elution.

6.20.3 Column oven able to heat between $(30 \pm 2) ^\circ\text{C}$ and $(45 \pm 2) ^\circ\text{C}$.

6.20.4 Analytical column, e.g. RP C18, particle size 5 µm, 150 mm (length) × 4,6 mm (diameter).

The measurement may be carried out with different separation columns (dimension, manufacturer). However, the PSP oxidation products shall be chromatographically baseline separated.

6.20.5 Fluorescence detector, excitation wavelength of $\lambda = 340 \text{ nm}$ and emission wavelength of $\lambda = 395 \text{ nm}$.

7 Procedure

7.1 Sample preparation

Thoroughly clean outside of the shellfish with tap water. Open by cutting adductor muscle. Rinse shells with tap water once to remove sand and foreign material. Remove the shellfish tissue from shells by separating adductor muscles and tissue connecting at the hinge. After removal from shellfish, drain tissues 5 min in a sieve. Homogenize the shellfish tissue in a grinder (6.1). At least 100 g of pooled homogenized shellfish tissue should be taken. If not directly proceeding with the analysis it is advised to store the homogenized shellfish in the freezer.

7.2 Extraction procedure

Defrost the homogenized sample in the refrigerator or at room temperature or use it directly after grinding (7.1).

Do not heat the sample.

Keep all extracts and solutions refrigerated when not in use.

Weigh a test portion of $5 \text{ g} \pm 0,1 \text{ g}$ of homogenized shellfish in a 50 ml centrifuge tube (6.4) and mix for at least 30 s with 3 ml of 1 % acetic acid solution 1 (5.4.1) on a vortex mixer. Cap it loosely to avoid pressure build up during heating and place in a boiling water bath ($100 ^\circ\text{C}$) so that the contents of the tube are below the water line. Heat the samples for 5 min.

Make sure that the water bath has reached the boiling point before inserting samples into it and starting to count the heating time. Do not place too many tubes in the bath at once in order to avoid that the water bath stops boiling for more than 30 s. Start measuring the 5 min as soon as water resumed boiling.

Remove samples from the water bath, remix for at least 30 s on a Vortex mixer and cool it by placing in a refrigerator or a beaker of cold water for 5 min and centrifuge for 10 min at 3 600 *g*. Decant the supernatant into a 15 ml graduated conical test tube (6.9).

Add 3 ml of 1 % acetic acid solution 1 (5.4.1) to the centrifuge tube containing the once extracted sample (solid residue), mix for at least 30 s on a Vortex mixer and centrifuge again for 10 min at 3 600 *g*. Decant and collect the supernatant into the same graduated conical test tube (6.9) that contains the first portion of the crude extract and adjust accurately to 10 ml with water.

The procedure can be stopped at this point and the crude extract can be stored for at least of 5 days in a refrigerator 2 °C to 8 °C or frozen a longer period.

NOTE Crude extracts can be kept frozen (–20 °C) for at least 3 years except for bivalve mollusc species with high PSP biotransformation ability like *Spisula solida* [17], [18] or razor clams (*Ensis siliqua*, *Ensis ensis* and *Solen marginatus*).

7.3 Sample purification

7.3.1 SPE-C18 clean-up

Condition the cartridge according to the manufacturers' instructions, e.g. condition the 3 ml SPE-C18 cartridge (6.10) with 6 ml of methanol (5.1) followed by 6 ml of water. Discard the solutions which have passed the cartridge. Place a graduated conical test tube (6.9) under the cartridge. Add 1 ml (0,5 g shellfish tissue equivalent) of the crude extract (7.2) to the cartridge. Keep the flow rate between 2 ml/min to 3 ml/min for all elutions. Collect the eluate in the graduated conical test tube. Wash the cartridge with 2 ml of water and combine the washings with the eluate to get the purified extract.

Avoid running dry the cartridges during the complete process.

Adjust this purified extract to pH $6,5 \pm 0,3$ with 1 mol/l of NaOH (5.9.1) using pH meter (6.16) and then adjust the volume exactly to 4 ml with water.

For screening purposes the sample purification can be stopped at this point. The extract from SPE-C18 clean-up is stable for at least one year at ≤ -18 °C.

Aliquots of this extract may be used for oxidation with periodate and peroxide as described in 7.5.2 and 7.5.3.

If N1-hydroxylated PSP toxins are detected in this extract, continue with the SPE-COOH ion exchange clean-up as described below.

NOTE For investigation of whole king scallops (*Pecten maximus*) and whole queen scallops (*Aequipecten opercularis*) contaminated with NEO and GTX1,4, the use of 1,5 ml of the crude extract for the SPE-C18 clean-up increases the sensitivity of GTX1,4 and NEO [3].

7.3.2 SPE-COOH clean-up (fractionation)

Fractionate only extracts from SPE-C18 clean-up that contain N1-hydroxylated PSP toxins (e.g. NEO, dcNEO, C3,4; GTX6 and GTX1,4) after SPE-C18 clean-up.

Condition the cartridge according to the manufacturers' instructions, e.g. condition the 3 ml SPE-COOH cartridge (6.11) by passing 10 ml of 0,01 mol/l ammonium acetate solution 2 (5.5.2) through it. Keep the flow rate between 2 ml/min to 3 ml/min for all elutions. Discard the eluate.

Fraction 1: Pass a 2 ml aliquot (0,25 g shellfish tissue equivalent) of shellfish extract from SPE-C18 clean-up (7.3.1) through the cartridge and collect the eluate in a graduated conical test tube (6.9) labelled as Fraction 1. Then pass 4 ml of water through the cartridge and collect into the same tube. Adjust final volume to 6 ml in total. This fraction contains the C toxins. Proceed to 7.5.2 and/or 7.5.3 for the oxidation steps. If C3,4 is present and C3,4 standards are not commercially available, proceed to 7.4.

Fraction 2: Pass 4 ml of 0,05 mol/l NaCl solution 1 (5.6.1) through the same cartridge and collect the eluate (labelled as Fraction 2) in a graduated conical test tube (6.9). Adjust final volume to 4 ml. This fraction contains the toxins GTX1,4, GTX2,3, GTX5, GTX6 and dcGTX2,3. Proceed to 7.5.2 and/or 7.5.3 for the oxidation steps. If GTX6 is present and GTX6 standards are not commercially available, proceed to 7.4.

Fraction 3: Pass 5 ml of 0,3 mol/l NaCl solution 2 (5.6.2) through the cartridge and collect in a graduated conical test tube (6.9) marked as Fraction 3. Adjust final volume to 5 ml. This fraction contains STX, NEO, dcNEO and dcSTX. Proceed to 7.5.2 and/or 7.5.3 for the oxidation steps.

Avoid running dry the cartridges during the complete process.

If problems with detector sensitivity are encountered each fraction can be concentrated. A suggested concentration step is to collect each fraction from SPE-COOH-clean-up into a glass container suitable for the evaporation equipment (6.17) and evaporate to approximately 1 ml on e.g. a rotary evaporator or other adequate evaporators with a water bath set at 45 °C. Transfer the solution into a graduated conical test tube (6.9) using a Pasteur pipette. Rinse the glass container 3 times with about 0,2 ml to 0,3 ml of water each time, transferring the rinse into the graduated tube just so the final volume of SPE-COOH cleaned-up fraction is 2 ml. Analyse Fractions 1, 2 and 3 by HPLC after periodate and peroxide oxidations as described in 7.5.2 and/or 7.5.3.

NOTE To improve the sensitivity of the method, an alternative ion-exchange SPE-clean-up procedure was developed during a single-laboratory validation [2], [11], (procedure see 7.3.3).

The sample purification can be stopped at this point. Continue immediately with oxidation and HPLC-FLD analysis or store the SPE-COOH cleaned-up fraction in a refrigerator for not more than one week or not more than one year in a freezer.

7.3.3 Alternative weak cation exchange SPE clean-up [2], [11]

To improve the sensitivity and to reduce the carry-over of PSP-toxin contents in the adjoining fractions, the following modified clean-up after the SPE C18 clean-up can be used, however, this procedure has only been in-house-validated.

The fractionation procedure is the same as described in 7.3.2. In contrast to [1], only the concentration of the sodium chloride solutions and the volume for the elution is different (see Table 4).

Table 4 — Options of fractionation conditions [2], [11]

Fraction	Toxins	COOH-SPE (e.g. Bakerbond Carboxylic Acidsilane)		Weak cation exchanger (e.g. Strata-X-CW, Phenomenex)	
		Elution solvent	Volume	Elution solvent	Volume
F1	C1,2; C3,4	Water	6,0 ml	Water	5,0 ml
F2	GTX1,4; GTX2,3; dcGTX2,3; GTX5 GTX6	0,05 mol/l NaCl	4,0 ml	0,3 mol/l NaCl or 0,4 mol/l NaCl	3,0 ml
F3	STX; NEO; dcNEO; dcSTX	0,3 mol/l NaCl	5,0 ml	2,0 mol/l NaCl or 2,5 mol/l NaCl	3,0 ml

7.4 Conversion of GTX6 into NEO and/or C3,4 into GTX1,4

7.4.1 General

If no standard is commercially available GTX6 can alternatively be quantified by hydrolysis to NEO. Therefore, in order to analyse GTX6, the toxin shall be hydrolysed into NEO which can be quantified [7], [8]. GTX6 content can be indirectly determined by hydrolysis of Fraction 2 of the SPE-COOH clean-up where NEO does not occur.

If no standard is commercially available C3,4 can alternatively be quantified by hydrolysis to GTX 1,4. The toxin, present in Fraction 1 of the SPE-COOH clean-up, can be indirectly determined by hydrolysis to GTX1,4 [10].

7.4.2 Hydrolysis of SPE-COOH Fraction 1 or 2

Add 75 µl of 1 mol/l HCl (5.7) to 300 µl of SPE-COOH Fraction 2 (7.3.2) in an HPLC vial or in a glass tube with screw cap. Close the vial/tube well and weigh it. Heat the mixture for 20 min at 90 °C in a water bath or block heater. Cool down the mixture to room temperature and weigh the vial again to check for possible evaporation (if evaporation has occurred add water to the original weight). Add e.g. five times small volumes of 15 µl of 1 mol/l NaOH (5.9.1) and mix each time until the added volume is in total 75 µl. These steps are necessary to neutralize the acid while avoiding an over-alkalinisation of the hydrolysed Fraction 2. Adjust to neutral pH.

During periodate oxidation (combination of hydrolyzed Fraction 2, matrix modifier and periodate derivatising agent) the mixture should change to pH $8,2 \pm 0,3$. However if the oxidized hydrolyzed fraction does not reach pH $8,2 \pm 0,3$ then the hydrolyzed Fraction 2 needs to be adjusted by adding a little more NaOH and the extra amount noted for calculations [12].

Analyse the sample by HPLC-FLD after periodate oxidation as described in 7.5.2.

The procedure can also be applied to Fraction 1 of the SPE-COOH clean-up.

NOTE Depending on the minimum measured volume of the used pH-meter it is possible to scale up all the reagents. (i.e. 900 µl of SPE-COOH Fraction 2 (filtered) +225 µl of 1 mol/l HCl (5.7) and after reaction 225 µl of 1 mol/l NaOH solution 1).

7.5 Oxidation procedure

7.5.1 General

During the oxidation reactions, it is important that the time of the reaction is kept the same for all sample and standard mixtures, since variations can influence the efficiency of the oxidation and the yield of oxidation products thus affecting the repeatability of the results. A control sample to prove the absence of naturally fluorescent compounds is necessary as indicated in 7.5.2 and 7.5.3.

NOTE For investigation of whole king scallops (*Pecten maximus*) and whole queen scallops (*Aequipecten opercularis*) contaminated with NEO and GTX1,4, this approach can decrease the sensitivity. To improve the sensitivity the modification in the periodate oxidation step described in Note of 7.5.2 can be used.

7.5.2 Periodate oxidation

All reagents and solutions used in the oxidation reactions are dispensed using auto pipettes with disposable plastic tips.

It is very important to adjust the pH to $6,5 \pm 0,3$ of the samples and standard mixtures prior to the periodate oxidation [12].

Add 100 µl of standard solution or sample extract after SPE C18 clean-up (7.3.1) or fraction of SPE-COOH clean-up (7.3.2) to 100 µl of matrix modifier solution (5.14) in a 1,5 ml vial. Then add 500 µl of periodate

oxidant (5.12) and mix well on a vortex mixer. Allow the solution to react at room temperature for 1 min and then add 5 µl of glacial acetic acid (5.4) and mix. Allow the mixture to stand for at least 10 min at room temperature before injecting into the HPLC system.

The solution is stable for at least one day for the non-N1-hydroxylated toxins. However, for NEO, GTX6, GTX1,4, a slow degradation of oxidation products occurs. Despite this degradation, the toxins may be quantified if the standard solutions and test solutions are stored and analysed under the same conditions, see [5].

NOTE 1 The solution is stable for at least 72 h when a refrigerated injector at $(6 \pm 2) ^\circ\text{C}$ is used.

NOTE 2 For investigation of whole king scallops (*Pecten maximus*) and whole queen scallops (*Aequipecten opercularis*) contaminated with NEO and GTX1,4, the use of a king scallop matrix modifier instead of oyster modifier (5.14) for the oxidation of the calibration standards was shown to give good recoveries. A modified periodate reagent containing 5 times the normal relative amounts of periodic acid showed good sensitivity. The periodate used for oxidation of all standard solutions and samples was prepared using 0,03 mol/l periodic acid (5.11.2), 0,3 mol/l ammonium formate (5.3) and 0,3 mol/l sodium hydrogen phosphate (5.8) with proportions of 5:1:1 respectively, adjusting the pH to $8,2 \pm 0,3$ prior to use. Also the sensitivity was raised with the use of high injection volumes (100 µl) [3].

Additionally, an aliquot of the sample extract after C18 clean-up (fractions of SPE-COOH are optional) will be analysed without oxidation as control-sample for the periodate oxidation to verify that peaks in the chromatogram of the oxidized sample are caused by PSP toxins and not by naturally fluorescent compounds. Add 100 µl of sample extract (7.3.1 or 7.3.2) to 100 µl matrix modifier (5.14) in a 1,5 ml vial. Then add 505 µl water and mix well on a vortex mixer. Inject into the HPLC system. The resulting chromatogram will enable the identification of peaks arising from naturally fluorescent sample co-extractives. PSP toxins do not produce peaks under these conditions.

NOTE 3 Instead of replacing all reagents of the oxidation with water the following mix can be used as an alternative oxidation sample reagent. Mix one volume part of disodium hydrogenphosphate solution (5.8.1) with one volume part of ammonium formate solution (5.3.1) and one volume part of water. Adjust the pH of the mixture to $8,2 \pm 0,3$ by adding sodium hydroxide solution 2 (5.9.2) and check the pH using a pH meter. Prepare fresh every day of analysis. Complete this procedure with the addition of 5 µl glacial acetic acid (5.4).

7.5.3 Peroxide oxidation

Add 25 µl of 10 % (w/v) aqueous H_2O_2 (5.10.1) to 250 µl of 1 mol/l NaOH (5.9.1) in a 1,5 ml vial and mix. Then add 100 µl of standard solution or sample extract after SPE C18 (7.3.1) or SPE-COOH clean-up (7.3.2). Mix and allow reacting for 2 min at room temperature. Add 20 µl of glacial acetic acid (5.4) and mix the solution. Inject this solution into the HPLC system. The solution is stable for at least 8 h at room temperature [5].

NOTE 1 The solution is stable for at least 72 h when a refrigerated injector at $(6 \pm 2) ^\circ\text{C}$ is used.

Additionally, an aliquot of the sample extract after C18 clean-up (fractions of SPE-COOH are optional) will be analysed without oxidation as control-sample for the peroxide oxidation to verify that peaks in the chromatogram of the oxidized sample are caused by PSP toxins and not by naturally fluorescent compounds. Add 100 µl of sample extract (7.3.1 or 7.3.2) to 295 µl water in a 1,5 ml vial and mix well on a vortex mixer. Inject into the HPLC system. The resulting chromatogram will enable the identification of peaks arising from naturally fluorescent sample co-extractives. PSP toxins do not produce peaks under these conditions.

NOTE 2 Injecting more than 50 µl can cause peak broadening.

8 HPLC determination

Check the system stability before starting the series of analysis by injecting one standard solution mix or a quality control in triplicate. Quantitative peak area variation shall be no more than 5 % and the retention time variation no more than 2 %.

All peaks shall be chromatographically separated with a resolution greater than 1,5. The fluorescence detector (FLD) shall be capable to detect a peroxide-processed (7.5.3) or periodate-processed (7.5.2) extracts with individual toxins concentrations equivalent to 1/5 of the specified maximum level (ML) [19] with a signal-to-noise ratio of at least 10:1. This is equivalent to a standard solution of 0,054 µM STX after C18 clean-up (dilution factor 8) and 0,021 µM NEO after COOH clean-up (dilution factor 20).

The following HPLC conditions are exemplary and led to satisfactory results:

Column:	C18 reversed phase, 5 µm, 150 mm x 4,6 mm
Oven temperature:	(40 ± 2) °C
Autosampler temperature:	(6 ± 2) °C
Flow:	2 ml/min
Injection volume:	25 µl to 50 µl (for peroxide oxidation) or 50 µl to 100 µl (for periodate oxidation)
Fluorescence detector:	Excitation 340 nm Emission: 395 nm

An appropriate HPLC gradient with a flow rate of 2 ml/min is listed in Table 5.

Table 5 — Recommended mobile phase gradient at a flow of 2 ml/min

Time min	Eluent A (5.15.1) %	Eluent B (5.15.2) %
0	100	0
5	95	5
9	30	70
11	100	0
15	100	0

NOTE 1 The measurement can be carried out with different separation columns (dimension, manufacturer).

The following injection scheme has been proven to be successful: before injecting a series of samples, inject a series of calibration solutions. During analysis of the samples, inject the calibration solutions and/or a solution with defined concentration with regular intervals to check the stability of the system (for example, every fourth injection a solution with fixed concentration). After completion of the sample series, inject the calibration series or at least one point of the calibration series again.

Analyse extracts after SPE C18 clean-up (7.3.1) or fractions of SPE-COOH clean-up (7.3.2) oxidized with periodate and peroxide. Extracts after SPE C18 clean-up without oxidation should be analysed with the periodate and peroxide sequence to verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP and not to naturally fluorescent sample co-extractives (see 7.5.2 or 7.5.3). Extracts with PSP toxin contents exceeding the highest calibration standard have to be diluted with water. Note the dilution factors.