BS EN 14526:2017

BSI Standards Publication

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

bsi.

National foreword

This British Standard is the UK implementation of EN 14526:2017. It supersedes BS EN 14526:2004 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Published by BSI Standards Limited 2017

ISBN 978 0 580 89117 5

ICS 67.120.30

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 January 2017.

Amendments/corrigenda issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14526

January 2017

ICS 67.120.30

Supersedes EN 14526:2004

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 European Standard was approved by CEN on 7 November 2016.

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. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists 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.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Ref. No. EN 14526:2017 E

Contents

Page

European foreword ... **3**
Introduction ... **4**
1 Scope ... **5**
2 Normative references ... **6**
3 Principle ... **6**
4 Reagents ... **9**
5 Apparatus ... **12**
6 Procedure ... **14**
7 HPLC determination ... **18**
8 Calibration curve ... **20**
9 Identification ... **20**
10 Calculation ... **20**
11 Precision ... **35**
12 Test report ... **35**
Annex A (informative) Precision data ... **36**
Bibliography ... **64**

European foreword

This document (EN 14526:2017) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2017, and conflicting national standards shall be withdrawn at the latest by July 2017.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 14526:2004.

EN 14526:2017 includes the following significant technical changes with respect to EN 14526:2004:

— the applicability is greater as more samples were tested in interlaboratory studies;

— the extraction procedure in 6.2 has been revised;

— the chromatographic conditions in Clause 7 have been revised;

— guidelines for calculation in presence of several toxins were introduced;

— the method has been additionally validated in several interlaboratory studies, and the precision data in Annex A have been revised.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

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).

WARNING — The use of this standard can involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to take appropriate measures to ensure the safety and health of personnel prior to application of the standard, and fulfil statutory and regulatory requirements for this purpose.

1 Scope

This European standard 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 1 and 2 (C1,2; sum of isomers) and (depending on the availability of certified reference materials (CRMs)) N-sulfocarbamoyl-gonyautoxin 3 and 4 (C3,4; sum of isomers) in (raw) mussels, oysters, scallops and clams. Laboratory experience has shown that it is also be applicable in other shellfish [2], [3] and cooked shellfish products. The method described was validated in an interlaboratory study [4], [5] and was also verified in a EURL-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		**µg/kg**	**µmol/kg**
saxitoxin (STX) [5]		22[c]	0,07[c]
gonyautoxin 2,3 (GTX2,3) [5]		114[b]	0,29[b]
gonyautoxin 5 (GTX5, B1) [5]		27[c]	0,07[c]
dc-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 1,2 (C1,2) [5]		93[c]	0,20[c]
N-sulfocarbamoyl-gonyautoxin 3,4 (C3,4) [5]		725[b]	1,48[b]
gonyautoxin 6 (GTX6, B2)	Direct [4] Indirect [9]	30 834[b]	0,08 2,11[b]
dc-gonyautoxin 2,3 (dcGTX2,3) [8]		271[a]	0,77[a]
dc-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 (B2) 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 µg/kg or higher in certain samples. For that reason, the present method is applicable to quantify GTX6 (B2) directly, depending on the availability of the standard substance. Currently it is possible to determine GTX6 after a 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 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, see [10].

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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)*

3 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 (B1), dcSTX, GTX2,3 and dcGTX2,3) can be quantified after SPE-C18 clean-up[1)].

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 sum of both toxins.

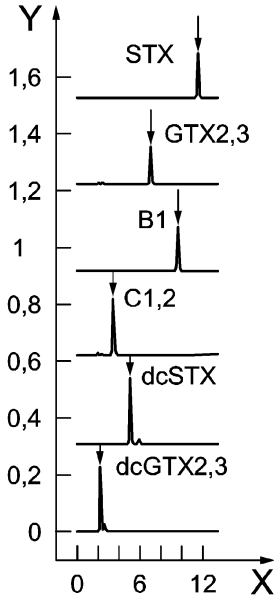
STX reacts to a single specific oxidation product regardless of the kind of oxidation reaction (whether peroxide or periodate). The same is valid for GTX2,3 as well as GTX5 (B1) and C1,2. In contrast, dcSTX and dcGTX2,3 produce each two different oxidation products in both oxidation reactions, see also Table 2. The toxin dcNEO is oxidized into two oxidation products only with the periodate oxidation. Each of the toxins NEO, GTX6 (B2), GTX1,4 and C3,4 produce three peaks after periodate oxidation but only 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). So the chromatograms shall be carefully interpreted after a SPE C18 clean-up.

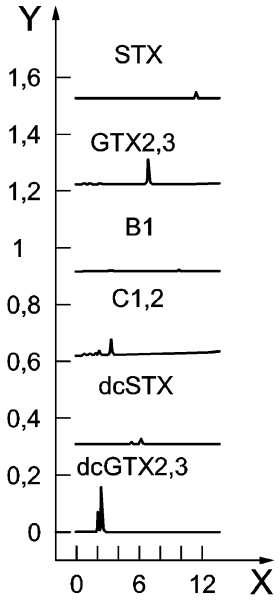
1) This document is based on a procedure described by Lawrence et al. [4] and was also published as AOAC Official Method 2005.06 [1].

Table 2 — Reaction products after oxidation with periodate and peroxide

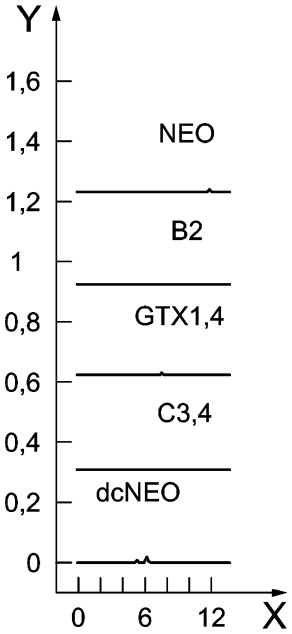
Toxin	Oxidation products and HPLC-eluting order		Intensity		Oxidation product at the same retention time as	
	peroxide	periodate	peroxide	periodate	peroxide	periodate
STX	one	one	++	+	NEO[a] {3} [b]	NEO {3}; GTX6 (B2) {3}
dc-STX	first {1}	first {1}	++	-		dcNEO {1}
	second {2}	second {2}	+	+	NEO[a] {2}	NEO {2}; GTX6 (B2) {2}; dcNEO {2}
NEO	no	first {1}	—	+		GTX6 (B2) {1}
	second {2}	second {2}	-	++	dcSTX {2}	GTX6 (B2) {2}; dcSTX {2}; dcNEO {2}
	third {3}	third {3}	-	+	STX	STX; GTX6 (B2) {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 (B1)	one	one	++	-		
GTX6 (B2)	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 (B2) {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.						



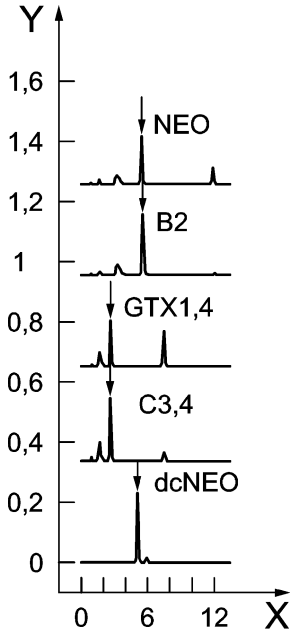
a) Non-hydroxylated toxins: peroxide



b) Non-hydroxylated toxins: periodate



c) N-hydroxylated toxins: peroxide



d) N-hydroxylated toxins: periodate

Key

Y detection response (V)

X time (min)

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

For the quantitative determination of N-hydroxylated toxins, a fractionation by SPE-COOH clean-up is necessary (shown in Table 3) because the oxidation products of some PSP toxins (NEO and GTX6 (B2), 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. The C toxins elute unretained with water, the GTX toxins (GTX1 to GTX6 as well as dcGTX2 and dcGTX3) elute with 0,05 mol/l NaCl while the saxitoxin group (STX, NEO, dcNEO and dc-STX) requires 0,3 mol/l NaCl for elution. These fractions can be analysed by HPLC after oxidation with periodate or peroxide.

Table 3 — Toxin elution order after SPE-COOH clean-up

Fraction	Eluent	Eluting toxin
1	water	C1,2; C3,4
2	0,05 mol/l NaCl	dcGTX2,3; GTX2,3; GTX1,4; GTX5; GTX6 (B2)
3	0,3 mol/l NaCl	dcSTX; STX; NEO; dcNEO

4 Reagents

If not otherwise specified, reagents of pro analysis (p.a.) 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.

4.1 Methanol, HPLC quality.

4.2 Acetonitrile, HPLC quality.

4.3 Ammonium formate solution, substance concentration c = 0,3 mol/l.

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

4.4 Glacial acetic acid:

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

Dilute 1 ml of glacial acetic acid (4.4) to 100 ml with water.

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

Dilute 572 µl of glacial acetic acid (4.4) to 100 ml with water.

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

Dilute 100 µl of acetic acid solution 2 (4.4.2) to 100 ml with water.

4.5 Ammonium acetate:

4.5.1 Ammonium acetate solution 1, c = 0,1 mol/l.

Dissolve 0,77 g of ammonium acetate (4.5) to 100 ml with water.

4.5.2 Ammonium acetate solution 2, c = 0,01 mol/l.

Dilute 10 ml of ammonium acetate solution 1 (4.5.1) to 100 ml with water.

4.6 Sodium chloride:

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

Dissolve 0,29 g of sodium chloride (4.6) to 100 ml with water.

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

Dissolve 1,75 g of sodium chloride (4.6) to 100 ml with water.

4.7 Hydrochloric acid, c = 1 mol/l.

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

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

4.9 Sodium hydroxide:

4.9.1 Sodium hydroxide solution 1, c = 1 mol/l.

Dissolve 4 g of sodium hydroxide (4.9) to 100 ml with water.

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

Dilute 10 ml of sodium hydroxide solution 1 (4.9.1) to 50 ml with water.

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

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

4.11 Periodic acid:

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

Dissolve 0,2279 g of periodic acid (4.11) in 10 ml of water.

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

Dilute 3,4 ml of periodic acid solution 1 (4.11.1) with 6,6 ml of water. Store in a refrigerator in the dark at approximately + 4 °C. Prepare fresh every day.

4.12 Periodate oxidation reagent.

Mix one volume part of periodic acid solution 2 (4.11.2) with one volume part of disodium hydrogenphosphate solution (4.8) and one volume part of ammonium formate solution (4.3). Bring the mixture to pH 8,2 by drop wise adding sodium hydroxide solution 2 (4.9.2) and check the pH by using a pH meter. Prepare fresh every day of analysis.

4.13 PSP toxin standard substances:

4.13.1 PSP toxin stock solutions.

For convenience, standard substances can be combined into three mixtures by appropriate dilution of standard solutions in water. Adjust those solutions to about pH 4 with 0,1 mol/l of acetic acid solution 2 (4.4.2). For the analysis of C1,2, adjust solutions to pH 5 as otherwise degradation has been observed. Table 4 shows suitable concentration for each PSP toxin in three 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 4 — Examples of suitable concentrations for each PSP toxin in three stock solution mixtures

Stock solution mixtures		**Toxin concentration**	
		µg/ml [a]	nmol/ml
Mix 1	GTX1,4	0,192	0,467
	NEO	0,189	0,600
Mix 2	GTX2,3	0,265	0,670
	GTX5 (B1)	0,202	0,532
	STX	0,201	0,672
	dc-STX	0,054	0,211
	dcGTX2,3	0,080	0,227
Mix 3	C1,2	0,203	0,427
	C3,4	0,188	0,383
	dcNEO	0,137	0,503
[a] related to the free base of the toxins			

NOTE Ampoules containing separately STX, NEO, GTX1,4, GTX2,3, C1,2, GTX5, dcGTX2,3, dcNEO, dcSTX standard substances in hydrochloric acid or aqueous acetic acid with concentrations ranging from 100 µg/ml to 2000 µg/ml are commercially available[2].

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 http://aesan.msssi.gob.es/en/CRLMB/web/home.shtml and http://aesan.msssi.gob.es/en/CRLMB/web/estandares_materiales_referencia/materiales_referencia.shtml. 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.

4.13.2 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 (4.13.1) with 0,1 mmol/l of acetic acid solution 3 (4.4.3). 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, and dried.

For the interlaboratory study in 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 (6.5.2 and 6.5.3). 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 (4.4.3).

4.13.3 PSP-solution for recovery check.

Prepare solutions of toxins of the appropriate mass concentration (e.g. in 0,6 % acetic acid) for checking the recovery of the toxins on the SPE-cartridges.

4.14 Matrix modifier 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 6.3.1 and adjust the extract to pH 6,5 with sodium hydroxide solution 1 (4.9.1). The solution can be stored in a refrigerator for 2 days to 3 days to precipitate co-extracted material. Decant the supernatant or filter it using a 0,45 µm filter (5.20) 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).

4.15 HPLC eluents:

4.15.1 Eluent A: Ammonium formate, c = 0,1 mol/l.

Dissolve 6,31 g of ammonium formate in 1 l water and adjust to pH 6,0 by adding approximately 6 ml of acetic acid solution 2 (4.4.2). Filter through a membrane filter (5.18) using vacuum.

4.15.2 Eluent B: Ammonium formate, c = 0,1 mol/l in 5 % acetonitrile.

Dissolve 6,31 g of ammonium formate in 950 ml water and add 50 ml of acetonitrile (4.2). Adjust to pH 6,0 by adding approximately 6 ml of acetic acid solution 2 (4.4.2). Filter through a membrane filter (5.18) using vacuum.

5 Apparatus

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

5.1 Grinder.

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

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

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

5.5 Centrifuge, capable to reach 3 600 g[3)] at the outer end of the centrifuge tubes.

5.6 Pipettes, autopipettes with disposable plastic tips.

5.7 Vortex mixer.

5.8 Water bath or hot plate.

5.9 Graduated conical test tube, (2 ml, 5 ml, 10 ml, 15 ml).

5.10 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 (4.13.3) 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. This check is not possible for GTX6 and C3,4 as standard substances are not yet commercially available.

If the laboratory has shown over time that there is no inter-batch variation in the performance of the SPE cartridges, the following approach may be used: Each new batch of SPE-C18 cartridges shall be checked with sample solutions of well-known concentrations to ensure that minimum recovery of the whole process is the minimum level of the validation data.

5.11 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 (4.13.3) to ensure that minimum recovery obtained with the COOH-cartridge is 80 % and the correct elution patterns are obtained according to 6.3.2. This check is necessary due to experiences gathered during method development as it was observed that variations can occur. This check is not possible for GTX6 and C3,4 as standard substances are not yet commercially available.

If the laboratory has shown over time that there is no inter-batch variation in the performance of the COOH cartridges, the following approach may be used: Each new batch of COOH cartridges shall be checked with sample solutions of well-known concentrations to ensure that minimum recovery of the whole process is the minimum level of the validation data.

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

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

5.14 Reaction tubes, e.g. glass tube with screw cap or vials with 1,5 ml.

5.15 pH indicator paper, able to precisely identify a pH of 6,5 ± 0,3.

5.16 pH meter.

5.17 Rotary evaporator, optionally for samples with low concentrations.

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

---

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

5.19 HPLC vials, e.g. amber glass.

5.20 HPLC system, comprising the following:

5.20.1 Injector, preferably a refrigerated injector, capable of injection up to 100 µl.

5.20.2 Pump, capable of gradient elution**.**

5.20.3 Column oven able to heat to (40 ± 2) °C.

5.20.4 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.

5.20.5 Fluorescence detector, excitation wavelength of λ = 340 nm and emission wavelength of λ = 395 nm and capable to detect a peroxide-processed (6.5.3) 400 pg STX standard substance as a free base on the column with a signal-to-noise ratio of at least 10:1.

6 Procedure

6.1 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 (5.1). At least 100 g to 150 g of pooled homogenized shellfish tissue should be taken. If not directly proceeding with the analysis the homogenized shellfish tissue can be frozen.

6.2 Extraction procedure

Defrost the homogenized sample in the refrigerator or at room temperature or use it directly after grinding (6.1).

Do not heat the sample.

Keep all extracts and solutions refrigerated when not in use.

Weigh a test portion of 5 g ± 0,1 g of homogenized shellfish in a 50 ml centrifuge tube (5.4) and mix with 3 ml of 1 % acetic acid solution 1 (4.4.1) on a vortex mixer. Cap it loosely to avoid pressure build up during heating and place in a boiling water bath (100 °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.

Remove samples from the water bath, remix 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 (5.9).

Add 3 ml of 1 % acetic acid solution 1 (4.5.1) to the centrifuge tube containing the once extracted sample (solid residue), mix well 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 (5.9) 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 extract has to be stored in a refrigerator.

6.3 Sample purification

6.3.1 SPE-C18 clean-up

Condition the cartridge according to the manufacturers' instructions, e.g. condition the 3 ml SPE-C18 cartridge (5.10) with 6 ml of methanol followed by 6 ml of water. Discard the solutions which have passed the cartridge. Place a 5 ml graduated conical test tube (5.9) under the cartridge. Add 1 ml (0,5 g shellfish tissue equivalent) of the crude extract (6.2) 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 with 1 mol/l of NaOH (4.9.1) using pH-indicator paper (5.15) or pH meter (5.16) 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 usually stable for more than one year in a freezer, however, this has to be verified.

Aliquots of this extract may be used for oxidation with periodate and peroxide as described in 6.5.2 and 6.5.3.

Additionally, an aliquot of this extract 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. Furthermore an aliquot of the sample extract from SPE-C18 clean-up has to be mixed with matrix modifier and water (instead of periodate oxidant) as control-sample for the periodate oxidation and will be analysed. 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.

If N-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 recovery of GTX1,4 and NEO [3].

6.3.2 SPE-COOH clean-up (fractionation)

Fractionate only extracts from SPE-C18 clean-up that contain N-hydroxylated PSP toxins (e.g. NEO, dcNEO, C3,4; GTX6 (B2) 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 (5.11) by passing 10 ml of 0,01 mol/l ammonium acetate solution 2 (4.6.2) 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 (6.3.1) through the cartridge and collect the eluate in a 10 ml graduated conical test tube 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 6.5.2 and/or 6.5.3 for the oxidation steps. If C3,4 is present, proceed to 6.4.

Fraction 2: Pass 4 ml of 0,05 mol/l NaCl solution 1 (4.6.1) through the same cartridge and collect the eluate (labelled as Fraction 2) in a 5 ml graduated conical test tube. Adjust final volume to 4 ml. This fraction contains the toxins GTX1,4, GTX2,3, GTX5 (B1), GTX6 (B2) and dcGTX2,3. Proceed to 6.5.2 and/or 6.5.3 for the oxidation steps. If GTX6 (B2) is present, proceed to 6.4.

Fraction 3: Pass 5 ml of 0,3 mol/l NaCl solution 2 (4.6.2) through the cartridge and collect in 5 ml graduated conical test tube marked as Fraction 3. Adjust final volume to 5 ml. This fraction contains STX, NEO, dcNEO and dcSTX. Proceed to 6.5.2 and/or 6.5.3 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 50 ml round bottom flasks instead of graduated conical test tubes 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 5 ml graduated conical test tube using a Pasteur pipette. Rinse the 50 ml round bottom flask 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 6.5.2 and/or 6.5.3.

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 6.3.3).

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.

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

To improve the sensitivity and to reduce the carry over in the adjoining fractions of low PSP-toxin contents, 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 6.3.2. In contrast to [1], only the concentration of the sodium chloride solutions and the volume for the elution is different (see Table 5).

Table 5 — Comparison of fractionation conditions [2], [11]

		COOH-SPE (e.g. Bakerbond Carboxylic Acidsilane)		**Weak cation exchanger** (e.g. Strata-X-CW, Phenomenex)	
Fraction	**Toxins**	**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	3,0 ml
F3	STX; NEO; dcNEO; dcSTX	0,3 mol/l NaCl	5,0 ml	2,0 mol/l NaCl	3,0 ml

6.4 Conversion of GTX6 (B2) into NEO and/or C3,4 into GTX1,4

6.4.1 General

Currently a GTX6 (B2) standard substance is not available. Therefore, in order to analyse GTX6 (B2), the toxin shall be hydrolysed into NEO which can be quantified [7], [8]. GTX6 (B2) content can be indirectly determined by hydrolysis of Fraction 2 of the SPE-COOH clean-up where NEO does not occur.

Currently a C3,4 standard substance is not commercially available. The toxin, present in Fraction 1 of the SPE-COOH clean-up, can be indirectly determined by hydrolysis to GTX1,4 [10].

6.4.2 Hydrolysis of SPE-COOH Fraction 1 or 2

Add 75 µl of 1 mol/l HCl to 300 µl of SPE-COOH Fraction 2 (6.3.2) 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 (4.9.1) 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. However if the oxidized hydrolized fraction does not reach pH 8,2 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 after periodate oxidation as described in 6.5.2.

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 and after reaction 225 µl of 1 mol/l NaOH solution 1).

6.5 Oxidation procedure

6.5.1 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 paragraph 6 of 6.3.1.

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 3 of 6.5.2 can be used.

6.5.2 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 prior to the periodate oxidation [12].

Add 100 µl of standard solution or sample extract after SPE C18 clean-up (6.3.1) or fraction of SPE-COOH clean-up (6.3.2) to 100 µl of matrix modifier solution in a 1,5 ml vial. Then add 500 µl of periodate oxidant (4.13) 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 (4.4) and mix. Allow the mixture to stand for at least 10 min at room temperature before injecting 50 µl to 100 µl into the HPLC system.

The solution is stable for at least one day for the non-hydroxylated toxins. However, for NEO, GTX6 (B2), 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 3 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 (4.14) 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 (4.11.2), 0,3 mol/l ammonium formate (4.3) and 0,3 mol/l sodium hydrogen phosphate (4.8) with proportions of

5:1:1 respectively, adjusting the pH to 8,2 prior to use. Also the sensitivity was raised with the use of high injection volumes (100 µl) [3].

6.5.3 Peroxide oxidation

Add 25 µl of 10 % (w/v) aqueous H_2O_2 (4.10) to 250 µl of 1 mol/l NaOH (4.9.1) in a 1,5 ml vial and mix. Then add 100 µl of standard solution or sample extract after SPE C18 (6.3.1) or SPE-COOH clean-up (6.3.2). Mix and allow reacting for 2 min at room temperature. Add 20 µl of glacial acetic acid (4.4) and mix the solution. Inject 25 µl to 50 µl of this solution into the HPLC system. The solution is stable for at least 8 h at room temperature, see [5].

NOTE Injecting more than 50 µl can cause peak broadening.

7 HPLC determination

Check your system stability (% of peak area variation and % of retention time variation) against your own HPLC specifications. Inject PSP oxidation products and chromatographically separate to allow individual identification and quantification of the toxins.

Note that dcGTX2,3 and dcSTX produce each two peaks as the peroxide oxidation leads to two different oxidation products, which shall be chromatographically separated with a resolution of greater than 1,5. The FLD should be capable to detect a peroxide-processed (6.5.3) 400 pg of STX standard substance as free base on the column with a signal-to-noise ratio of at least 10:1.

The following HPLC conditions led to satisfying results:

Column:	C18 reversed phase, 5 µm, 150 mm x 4,6 mm
Temperature:	40 °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 6.

Table 6 — Recommended mobile phase gradient at a flow of 2 ml/min

Time min	**Eluent A** %	**Eluent B** %
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 pH of the eluents A and B are unstable over the time. Therefore, prepare eluents A and B fresh before each analytical series [12].

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 again.

NOTE 2 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 (e.g. +6 °C) [12].

Analyse extracts after SPE C18 clean-up (6.3.1) or fractions of SPE-COOH clean-up 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 (6.3.1). Extracts with PSP toxin contents exceeding the highest calibration standard have to be diluted with water. Note the dilution factors.

To efficiently apply this method to routine analysis conditions where large numbers of test samples need to be analysed, the procedure in Figure 2 is recommended.

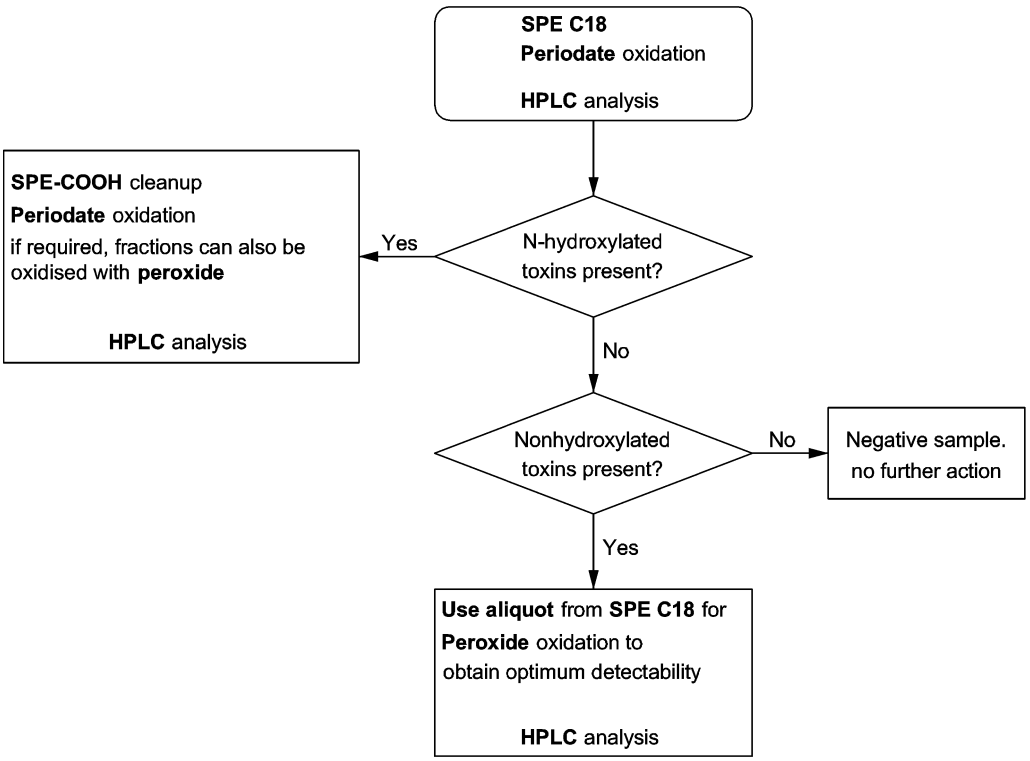


Figure 2 — Schematic diagram of procedure [4]

Analyse extracts after SPE C18 clean-up and periodate oxidation. If no peaks correspond to any of the PSP standard substances, the test sample is negative and no further analyses are required.

If any of the non-hydroxylated toxins (STX, dcSTX, GTX2,3, dcGTX2,3, GTX5 (B1) or C1,2) are present and in the absence of the N-hydroxylated toxins (NEO, dcNEO, GTX1,4, GTX6 (B2) and C3,4), then the non-hydroxylated toxins can be quantified by direct comparison to known standard solutions derivatised by the periodate oxidation. However, the peroxide oxidation reaction is much more sensitive for GTX5 (B1), C1,2 and dcSTX and is preferred if optimum sensitivity is required.

If both N-hydroxylated and non-hydroxylated toxins are present, the non-hydroxylated toxins are quantified after another aliquot of the extract after SPE C18 clean-up is oxidized using peroxide, and the peaks are compared to equally oxidized standard solutions. The reasons for this are that with periodate oxidation some of the N-hydroxylated toxins react to the same products as their non-hydroxylated analogues. Further, for some toxins (especially GTX5 (B1), C1,2 and dcSTX) peroxide oxidation yields much better sensitivity.

For quantification of any N-hydroxylated toxins, a further aliquot of the extract after SPE C18 clean-up has to be fractionated (6.3.2) and oxidized with periodate. This step separates C3,4 from GTX1,4 and GTX6 (B2) from NEO, enabling all toxins to be quantified by direct comparison to standard substances carried through the same periodate oxidation procedure, see [5].

8 Calibration curve

Prepare a calibration curve at the beginning of the analysis and whenever the chromatographic conditions change. Plot the peak response against the mass concentrations of the injected PSP toxin calibration solutions. The coefficient of determination should be at least 0,98 for all toxins. Determine the concentration of PSP toxins directly with the calibration graph (see 10.2.2) or with direct comparison to analytical standards at similar concentrations as anticipated in the sample [5] (see 10.2.3).

9 Identification

Identify the PSP toxins by comparing the retention times and the number of oxidation products of the sample with the standard substances.

10 Calculation

10.1 General

Each toxin is quantitatively determined in shellfish tissue by using the calibration curve (10.2.2). As alternative, peak areas of sample test solutions are compared with peak areas of standard solutions with similar mass concentrations as anticipated in the sample (10.2.3). For convenience, three standard mixtures can be used for quantification of the toxins as described in Table 4. Calculate the result according to 10.2 except for the cases listed in Table 7.

Table 7 — Calculation to be applied for the different toxin compositions

Toxin	Neo		C3,4	GTX1,4	GTX6	dcNeo
In presence of	dcSTX	STX, dcSTX, dcNeo		GTX2,3, dcGTX2,3		dcSTX
Calculation	10.3	10.8	10.5	10.7	10.4	10.6

After calculating the toxin concentrations, these should be expressed in STX 2HCl-eq by applying the equation mentioned in 10.9.

STX reacts to a single specific oxidation product regardless of the kind of oxidation reaction. The same is valid for GTX2,3 as well as GTX5 (B1) and C1,2. In contrast, dcSTX and dcGTX2,3 produce each two different oxidation products in both oxidation reactions, see also Clause 3 and Table 2.

The toxin dcNEO is oxidized into two oxidation products only with the periodate oxidation. Each of the toxins NEO, GTX6 (B2), GTX1,4 and C3,4 produce three peaks after periodate oxidation but only the second eluting peak is used for quantification (peroxide oxidation cannot be used for quantification)

(Table 2). Because some PSP toxins (NEO and GTX6 (B2), GTX1,4 and C3,4) give the same oxidation products, their quantitative determination can only be made after separation by SPE-COOH clean-up as described (6.3.2 or 6.3.3). If present in high concentration, dcSTX will interfere with NEO quantification after periodate oxidation. NEO can be quantified by peak ratios after periodate and peroxide oxidation (10.3). Due to the lack of standard substances, GTX6 can be quantified after hydrolysis to NEO (6.4.2), (10.4). C3,4 can be quantified after hydrolysis to GTX1,4 (6.4.2), (10.5). Since dcNEO and dcSTX have two common peaks when subjected to periodate oxidation, the quantification is described in 10.6.

NOTE 1 It is important to note that after the SPE-COOH clean-up, some toxins cannot completely be separated into one of the three fractions and sometimes small amounts can occur in the adjoining fraction.

NOTE 2 It is suggested to express calibrants in µmol/ml, as this may reduce the risk of calculation errors after analysis and makes calculations simpler.

10.2 Toxin calculation

10.2.1 General

There are two calculation methods to quantify the toxin concentration:

10.2.2 Calculation method with standard calibration curve

Calculate the substance concentration c in µmol/kg or mass concentration w in µg (free base)/kg of the toxin using the calibration curve and Formula (1).

$$c \text{ or } w = \frac{A_x - b}{a} \times \frac{V_x \times F_d \times 1000}{m} \quad (1)$$

where

A_X is the peak area of the toxin in the extract or fraction analysed;

a is the slope of calibration curve of the toxin;

b is the intercept of the calibration curve;

V_X is the final volume of extract analysed (in millilitre);

F_d is the dilution factor (for those extracts or fractions where PSP toxins are above the most concentrated standard mixture, dilute with water);

m is the mass of test portion carried through clean-up procedure, in gramm, usually 0,5 g for SPE-C18 clean-up and 0,25 g for SPE-COOH clean-up;

1 000 is the factor to convert result from µmol/g to µmol/kg or µg/g to µg/kg.

Due to the multiple charged functional groups the toxins can occur in different forms. Therefore it is important to specify the exact salt form (molecular weight) if the results are expressed in µg/kg.

10.2.3 Calculation alternative method using one point calibration [4], [5]

Calculate the concentration of each toxin in the sample by comparison the respective peak area with that of the standard substance provided in measurements. The injection of the standard solution should be timely close to injection of the unknown sample (before or after), and furthermore the concentration of both should be similar.

Calculate the substance concentration c in µmol/kg or the mass fraction w in microgram (free base) per kilogram of the toxin according to Formula (2), see also [4], [5].

$$c \text{ or } w = \frac{A_x \times C_s \times V_x \times F_d \times 1000}{A_s \times m} \quad (2)$$

where

A_x is the peak area of the toxin in the extract or fraction analysed;

A_S is the peak area of the nearest standard solution (time and concentration);

Cs is the substance concentration or mass concentration of standard solution, in µmol/ml or µg/ml;

Vx is the final volume of extract or fraction analysed, in millilitre;

F_d is the dilution factor (for those extracts where PSP toxins are above the most concentrated standard mixture, dilute with water);

m is the mass of test portion carried through clean-up procedure, in grams (0,5 g for SPE C18 clean-up, 0,25 g for SPE-COOH clean-up);

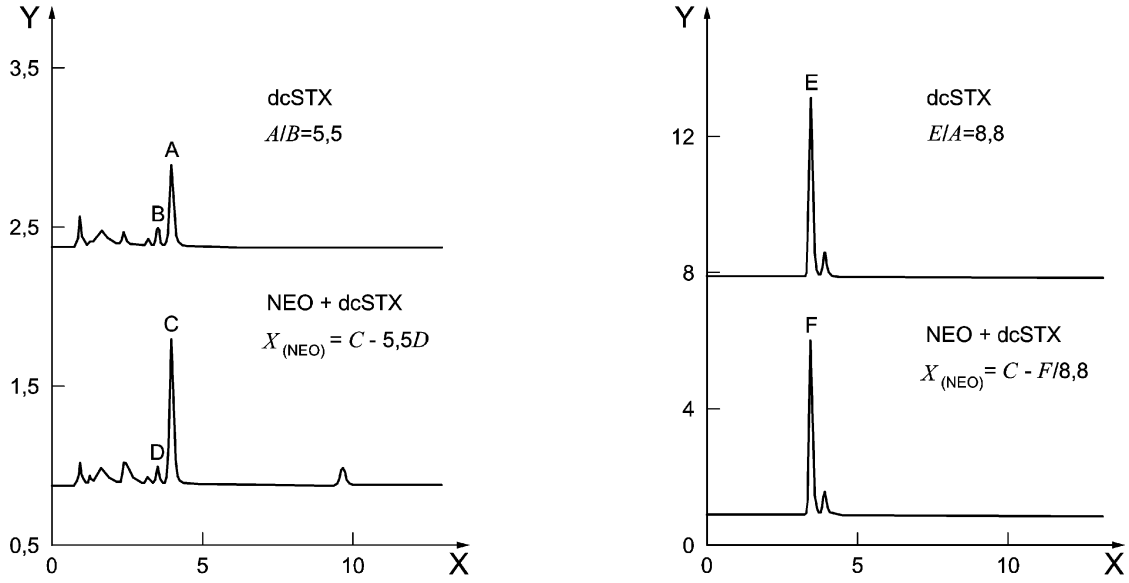
1 000 is the factor to convert result from µmol/g to µmol/kg or µg/g to µg/kg.

10.3 Calculation of NEO in the presence of dcSTX [4], [5]

10.3.1 General

Due to the fact that one of the reaction products of NEO and dcSTX is identical, a strategy for quantifying NEO in the presence of dcSTX is necessary. The second oxidation product of NEO has the same retention time as the second oxidation product of dcSTX. The peak area of NEO in a sample containing both NEO and dcSTX may be calculated in two ways. Figure 3 shows chromatograms after periodate and peroxide oxidation of a dcSTX standard solution and an unknown sample containing a mixture of NEO and dcSTX.

Method 1 and Method 2 are not applicable for the calculation of NEO in the presence of dcSTX and dcNEO. The only way to quantify is to use the third periodate peak of NEO when STX is absent. If also STX is present, the content of STX is calculated after peroxide oxidation. The portion of NEO in the peroxide peak needs to be neglected. The reached content of STX has converted into the corresponding area of the periodate oxidation and subtracted from the third periodate oxidation peak area of NEO. The residual area correlates to the content of NEO, see 10.8.



a) periodate oxidation **b) peroxide oxidation**

Key

Upper Chromatogram	dcSTX standard solution
Lower Chromatogram	unknown sample
dcSTX standard solution	A = second (larger) peak, periodate oxidation
	B = first (smaller) peak, periodate oxidation
	E = first (larger) peak, peroxide oxidation
Unknown sample	C = second (larger) peak, periodate oxidation
	D = first (smaller) peak, periodate oxidation
	F = first (larger) peak, peroxide oxidation
Y	detector response (Luminescence, Lu)
X	time (min)

Figure 3 — Chromatograms explaining the calculation of NEO in the presence of dcSTX (shown peak ratios are just examples)

10.3.2 Method 1

If the concentration of dcSTX in the sample containing a mixture of NEO and dcSTX is significant enough to produce a well-integrated first peak (peak D, Figure 3) with periodate oxidation, then the peak area corresponding to NEO ($X_{(NEO)}$) can be calculated from the ratio of the two dcSTX peak areas produced with periodate oxidation of the standard.

For the dcSTX standard solution analysed after periodate oxidation, calculate the ratio of the two peak areas using Formula (3).

$$\frac{A}{B}=\frac{X_{(dcSTX)}}{D}\Rightarrow X_{(dcSTX)}=\frac{D\times A}{B} \quad (3)$$

where

A is the peak area of the second (larger) peak of dcSTX from periodate oxidation;

B is the peak area of the first (smaller) peak of dcSTX from periodate oxidation;

D is the peak area of the first (smaller) peak of dcSTX in the unknown sample from periodate oxidation;

$X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

Calculate the peak area of NEO by using Formula (4).

$$X_{(NEO)}=C-X_{(dcSTX)}=C-\frac{D\times A}{B} \quad (4)$$

where

C is the peak area of the second (larger) peak of dcSTX+ NEO in the unknown sample from periodate oxidation.

For the calculation of the NEO mass and/or substance concentration use Formula (1) and/or (2).

10.3.3 Method 2

If the concentration of dcSTX in the sample containing NEO and dcSTX is not significant enough to produce a well-integrated first peak (peak D, Figure 3) with periodate oxidation, then calculate the peak area corresponding to NEO ($X_{(NEO)}$) from the ratio of the dcSTX peak produced with periodate oxidation (peak A) and the dcSTX peak area produced with peroxide oxidation (peak E) in the standard solution.

For a dcSTX standard solution analysed after periodate and peroxide oxidation, calculate the ratio of the two peak areas with Formula 5.

$$\frac{E}{A}=\frac{F}{X_{(dcSTX)}}\Rightarrow X_{(dcSTX)}=\frac{F\times A}{E} \quad (5)$$

where

E is the peak area of the first (larger) peak of dcSTX from peroxide oxidation;

F is the peak area of the first (larger) peak of dcSTX in the unknown sample from peroxide oxidation;

$X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

Calculate the peak area of NEO with Formula (6):

$$X_{(NEO)}=C-X_{(dcSTX)}=C-\frac{F\times A}{E} \quad (6)$$

For the calculation of the NEO mass and/or substance concentration use Formula (1) and/or (2).

NOTE The results of the two methods differ. Method 2 might be more accurate since the peak areas (peaks E and F) used in the calculations is much larger than peaks B and D used in method 1.

10.4 Calculation of GTX6 (B2)

If GTX6 (B2) is detected, use the periodate oxidation of the hydrolysed Fraction 2 for quantification. The hydrolysis converts GTX6 (B2) into NEO. For quantification, use the calibration in µmol/kg of the second eluting peak of NEO. The molarity of the GTX6 (B2) toxin is equal to the molarity of the NEO toxin present on Fraction 2 after hydrolysis.

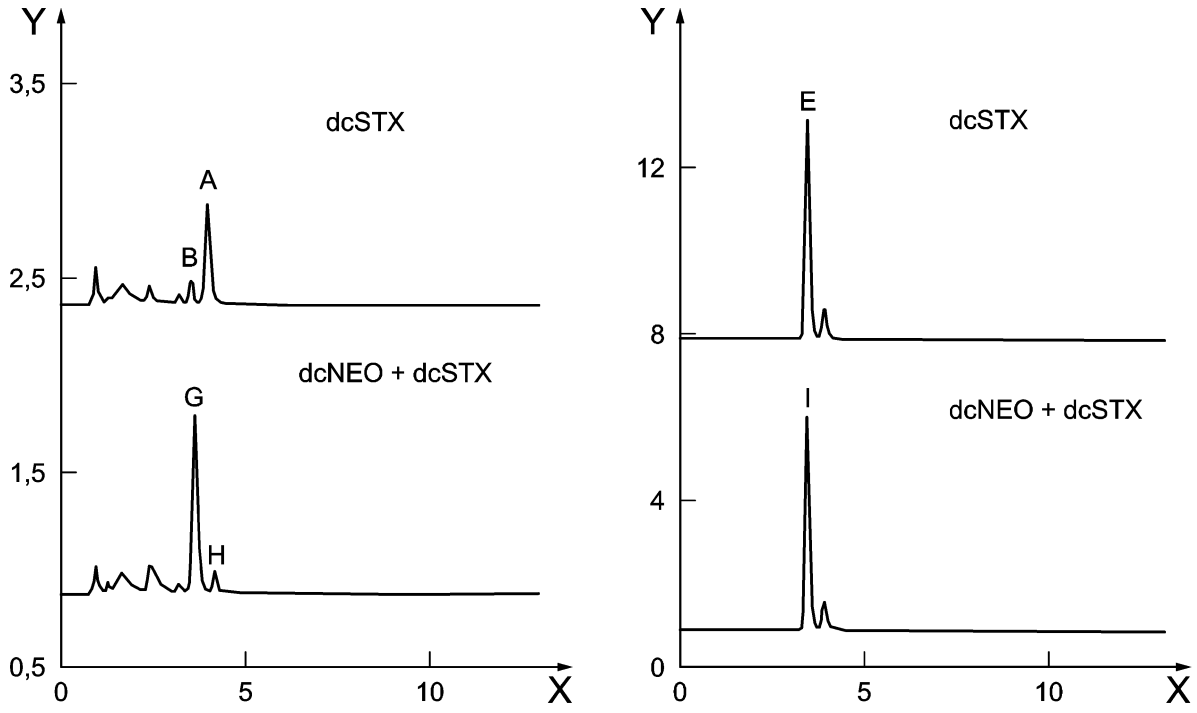
10.5 Calculation of C3,4

If C3,4 is detected, use the periodate oxidation of the hydrolysed Fraction 1 for quantification. The hydrolysis converts C3,4 into GTX1,4. For quantification, use the calibration in µmol/kg of the second eluting peak of GTX1,4. The molarity of the C3,4 toxin is equal to the molarity of the GTX1,4 toxin present on Fraction 1 after hydrolysis.

10.6 Calculation of dcNEO in the presence of dcSTX

10.6.1 General

Due to the fact that both of the reaction products of the periodate oxidation of dcNEO and dcSTX (Peak G and H in Figure 4) are identical, a strategy for quantifying dcNEO in the presence of dcSTX is necessary. There are two methods (method 3 and method 4) to quantify the toxin concentration.



a) periodate oxidation **b) peroxide oxidation**

Key

upper Chromatogram	dcSTX standard solution
Lower Chromatogram	unknown sample
dcSTX standard solution	B = first (smaller) peak, periodate oxidation
	A = second (larger) peak, periodate oxidation
	E = first (larger) peak, peroxide oxidation
Unknown sample	G = first (larger) peak, periodate oxidation
	H = second (smaller) peak, periodate oxidation
	I = first (larger) peak, peroxide oxidation
Y	detector response (Luminescence, Lu)
X	time (min)

Figure 4 — Chromatograms explaining the calculation of dcNEO in the presence of dcSTX

10.6.2 Method 3

If the concentration of dcSTX in the sample containing a mixture of dcNEO and dcSTX is significant enough to produce a well-integrated first peak (peak G, Figure 4) with periodate oxidation and the contribution of dcNEO in the peroxide oxidation is negligible, then the peak area corresponding to dcNEO ($X_{(dcNEO)}$) can be calculated from the ratio of the two dcSTX peak areas produced with peroxide and periodate oxidation of the standard solution (peak B and E) and the unknown sample (peak I).

$$\frac{E}{B}=\frac{I}{X_{(dcSTX\_Period)}}\Rightarrow X_{(dcSTX\_Period)}=\frac{I\times B}{E} \quad (7)$$

where

B is the peak area of the first (smaller) peak of dcSTX from periodate oxidation;

E is the peak area of the first (larger) peak of dcSTX from peroxide oxidation;

I is the peak area of the first (larger) peak of dcSTX in the unknown sample from peroxide oxidation;

$X_{(dcSTX\text{-}Period)}$ is the peak area of dcSTX in peak G.

Calculate the peak area of dcNEO in peak G with Formula (8).

$$X_{(dcNEO)}=G-X_{(dcSTX\_Period)}=G-\frac{I\times B}{E} \quad (8)$$

where

G is the peak area of the first (larger) peak of dcSTX+ dcNEO of the unknown sample from periodate oxidation.

For the calculation of the dcNEO mass and/or substance concentration use Formula (1) and/or (2).

10.6.3 Method 4

The sensitivity of the peroxide mode for dcNEO is very poor, therefore the content of dcNEO is negligible for the calculation of dcSTX after peroxide oxidation. The portion of dcSTX in the area of Peak G is calculated with the calibration curve Formula (10) or (11), respectively.

$$\rho_{(dcSTX-H2O2)}=\frac{I-b_{dcSTX-H2O2}}{a_{dcSTX-H2O2}}=\rho_{(dcSTX-Period)} \quad (9)$$

$$X_{(dcSTX-Period)}=\rho_{(dcSTX-H2O2)}\times a_{dcSTX-Period}+b_{dcSTX-Period} \quad (10)$$

$$X_{(dcSTX-Period)}=\left(\frac{(I-b_{dcSTX-H2O2})\times a_{dcSTX-Period}}{a_{dcSTX-H2O2}}\right)+b_{dcSTX-Period} \quad (11)$$

where

$\rho_{(dcSTX\text{-}H2O2)}$ is the mass concentration of dcSTX in Peak I;

I is the peak area of I

$\rho_{(dcSTX\text{-}Period)}$ is the mass concentration of dcSTX in Peak G;

$X_{(dcSTX\text{-}Period)}$ is the peak area of dcSTX in peak G;

$a_{dcSTX\text{-}H2O2}$ is the slope of calibration curve of dcSTX oxidized with peroxide;

$a_{dcSTX\text{-}Period}$ is the slope of calibration curve of dcSTX oxidized with periodate;

$b_{dcSTX\text{-}H2O2}$ is the intersection of the calibration curve of dcSTX oxidized with peroxide;

$b_{dcSTX\text{-}Period}$ is the intersection of the calibration curve of dcSTX oxidized with periodate.

Calculate the peak area of dcNEO in peak G with Formula (12).

$$X_{(dcNEO)} = G - X_{(dcSTX-Period)} = G - \left(\frac{\left(I - b_{dcSTX-H2O2}\right) \times a_{dcSTX-Period}}{a_{dcSTX-H2O2}} \right) + b_{dcSTX-Period} \quad (12)$$

For the calculation of the dcNEO mass and/or substance concentration use the Formula (1) and/or (2).

10.7 Calculation of GTX1,4 in the presence of GTX2,3 and dcGTX2,3

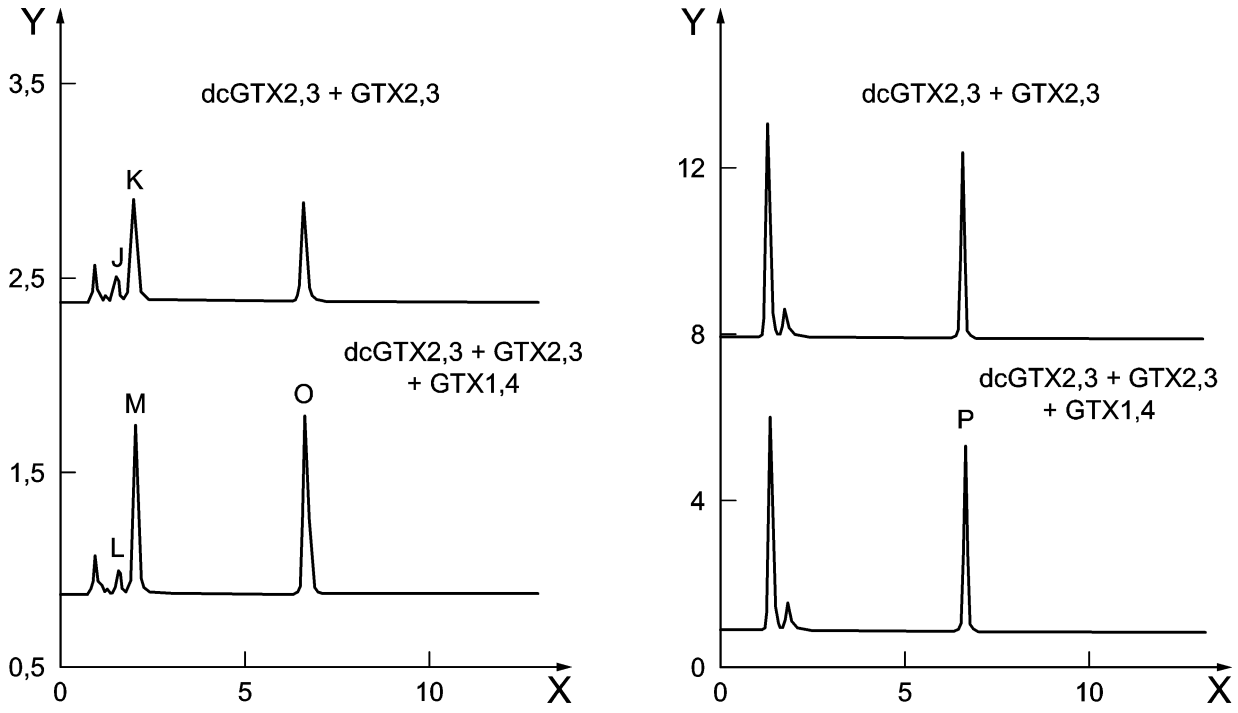
10.7.1 General

Due to the fact that some of the reaction products of GTX1,4 and GTX2,3 as well as GTX1,4 and dcGTX2,3 are identical, a strategy for quantifying GTX1,4 in the presence of GTX2,3 and dcGTX2,3 is necessary, **although this situation is unlikely to occur**. The second oxidation product of GTX1,4 (peak M, Figure 5) has the same retention time as the second oxidation product of dcGTX2,3. The third oxidation product of GTX1,4 (peak O) has the same retention time as the one oxidation product of GTX2,3.

If only GTX2,3 is present, the content of GTX1,4 can be calculated using the area of the second oxidation peak of GTX1,4.

If only dcGTX2,3 is present, the content of GTX1,4 can be calculated using the area of the third oxidation peak of GTX1,4.

The amount of GTX1,4 in a sample containing GTX1,4, dcGTX2,3 and GTX2,3 can be calculated with Method 5 and Method 6.



a) periodate oxidation b) peroxide oxidation

Key

Upper Chromatogram	standard solution containing GTX2,3 and dcGTX2,3
Lower Chromatogram	unknown sample
dcGTX2,3 standard solution	J = first (smaller) peak, periodate oxidation
	K = second (larger) peak, periodate oxidation
Unknown sample	L = first (smaller) peak, periodate oxidation
	M = second (larger) peak, periodate oxidation
	O = third (larger) peak, periodate oxidation
	P = third (larger) peak; peroxide oxidation
Y	detector response (Lu)
X	time (min)

Figure 5 — Chromatograms explaining the calculation of GTX1,4 in the presence of GTX2,3 and dcGTX2,3

10.7.2 Method 5

If the concentration of dcGTX2,3 in the sample containing a mixture of GTX1,4 and dcGTX2,3 is significant enough to produce a well-integrated first peak (peak L, Figure 5) with periodate oxidation, then the peak area corresponding to GTX1,4 ($X_{(GTX1,4)}$) can be calculated from the ratio of the two dcGTX2,3 peak areas produced with periodate oxidation of the standard (use Formula (13)).

$$\frac{K}{J}=\frac{X_{(\mathrm{dcGTX2,3})}}{L}\Rightarrow X_{(\mathrm{dcGTX2,3})}\frac{K\times L}{J} \tag{13}$$

where

J is the peak area of the first (smaller) peak of dcGTX2,3 from periodate oxidation;

K is the peak area of the second (larger) peak of dcGTX2,3 from periodate oxidation;

L is the peak area of the first (smaller) peak of dcGTX2,3 in the unknown sample from periodate oxidation;

$X_{(dcGTX2,3)}$ is the peak area of dcGTX2, 3 in peak M.

Calculate the peak area of GTX1,4 with Formula (14).

$$X_{(GTX1,4)}=M-X_{(dcGTX2,3)}=M-\frac{L\times K}{J} \tag{14}$$

where

M is the peak area of the second (larger) peak of dcGTX2,3 and GTX1,4 in the unknown sample from periodate oxidation.

For the calculation of the GTX1,4 mass and/or substance concentration use Formula (1) and/or (2).

10.7.3 Method 6

The sensitivity of the peroxide mode for GTX1,4 is very poor, therefore the content of GTX1,4 is negligible for the calculation of GTX2,3 after peroxide oxidation. The portion of GTX2,3 in the area of Peak O is calculated with the calibration curve Formula (16), or (17), respectively.

$$\rho_{(GTX2,3-H2O2)}=\frac{P-b_{GTX2,3-H2O2}}{a_{GTX2,3-H2O2}}=\rho_{(GTX2,3-Period)} \tag{15}$$

$$X_{(GTX2,3-Period)}=\rho_{(GTX2,3-H2O2)}\times a_{GTX2,3-Period}+b_{GTX2,3-Period} \tag{16}$$

$$X_{(GTX2,3-Period)}=\left(\frac{\left(P-b_{GTX2,3-H2O2}\right)\times a_{GTX2,3-Period}}{a_{GTX2,3-H2O2}}\right)+b_{GTX2,3-Period} \tag{17}$$

where

$\rho_{(GTX2,3\text{-}H2O2)}$ is the mass concentration of GTX2,3 in Peak P;

$\rho_{(GTX2,3\text{-}Period)}$ is the mass concentration of GTX2,3 in Peak O;

P is the peak area of P;

$X_{(GTX2,3\text{-}Period)}$ is the peak area of GTX2,3 in peak O;

$a_{GTX2,3\text{-}H2O2}$ is the slope of calibration curve of GTX2,3 oxidized with peroxide;

$a_{GTX2,3\text{-}Period}$ is the slope of calibration curve of GTX2,3 oxidized with periodate;

$b_{GTX2,3\text{-}H2O2}$ is the intersection of the calibration curve of GTX2,3 oxidized with peroxide;

$b_{GTX2,3\text{-}Period}$ is the intersection of the calibration curve of GTX2,3 oxidized with periodate.

Calculate the peak area of GTX1,4 in peak O by using Formula (18).

$$X_{(GTX1,4)} = O - X_{(GTX2,3-Period)} = O - \left(\frac{\left(P - b_{GTX2,3-H2O2}\right) \times a_{GTX2,3-Period}}{a_{GTX2,3-H2O2}} \right) + b_{GTX2,3-Period} \quad (18)$$

where

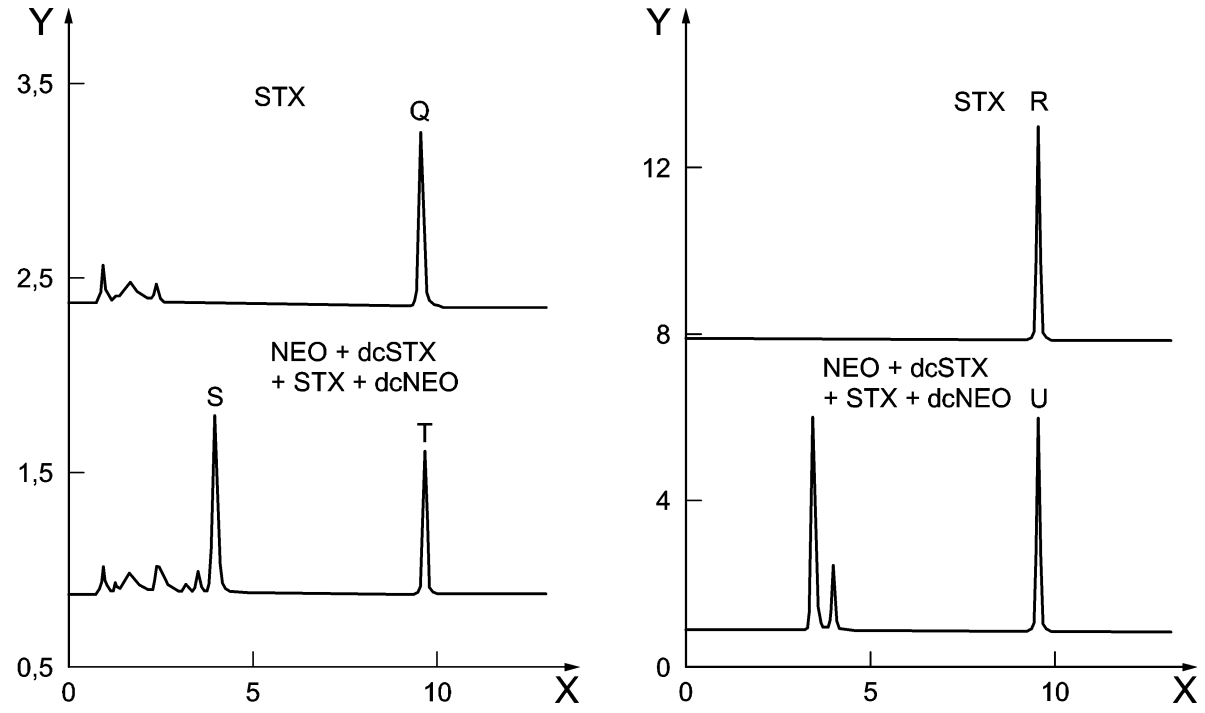
O is the peak area of the third (larger) peak of GTX2,3 and GTX1,4 in the unknown sample from periodate oxidation.

For the calculation of the GTX1,4 mass and/or substance concentration use Formula (1) and/or (2).

10.8 Calculation of NEO in the presence of dcSTX, dcNEO and STX (optional)

10.8.1 General

Due to the fact that some of the reaction products of NEO, dcNEO, STX and dcSTX are identical, a strategy for quantifying NEO in the presence of dcNEO, dcSTX and STX can become necessary, although this situation is unlikely to occur. The second oxidation product of NEO has the same retention time as the second oxidation product of dcSTX and dcNEO (Peak S, Figure 6). The third oxidation product of NEO has the same retention time as the oxidation product of STX (Peak T). The peak area of NEO in a sample containing NEO, dcNEO, dcSTX and STX may be calculated by the following two methods (method 7 and method 8). Figure 6 shows chromatograms after periodate and peroxide oxidation of a STX standard solution and an unknown sample containing a mixture of NEO and dcNEO, dcSTX and STX.



a) periodate oxidation **b) peroxide oxidation**

Key

Upper Chromatogram	STX standard solution
Lower Chromatogram	unknown sample
STX standard solution	Q = peak, periodate oxidation
	R = peak, peroxide oxidation
Unknown sample	S = second (larger) peak, periodate oxidation
	T = third (larger) peak, periodate oxidation
	U = third (larger) peak, peroxide oxidation
Y	detector response (Lu)
X	time (min)

Figure 6 — Chromatograms explaining the calculation of NEO in the presence of dcSTX, dcNEO and STX

10.8.2 Method 7

If the sample containing NEO and dcSTX, dcNEO and STX (peak T: NEO and STX are together, peak S: NEO, dcNEO and dcSTX are together, Figure 6) and the contribution of NEO in peak U in the peroxide oxidation is negligible, then calculate the peak area corresponding to NEO ($X_{(NEO)}$) from the ratio of the STX peaks produced with periodate oxidation (peak Q) and peroxide oxidation (peak R) in the standard solution.

For a STX standard solution analysed after periodate and peroxide oxidations, calculate the ratio of the two peak areas with Formula (19).

$$\frac{R}{Q}=\frac{U}{X_{(STX)}}\Rightarrow X_{(STX)}=\frac{U\times Q}{R} \tag{19}$$

where

Q is the peak area of the peak of STX from periodate oxidation;

R is the peak area of the peak of STX from peroxide oxidation;

U is the peak area of the peak of STX in the unknown sample from peroxide oxidation;

$X_{(STX)}$ is the peak area of STX in peak T.

Calculate the peak area of NEO according to Formula (20).

$$X_{(NEO)}=T-X_{(STX)}=T-\frac{U\times Q}{R} \tag{20}$$

where

T is the peak area of the third (larger) peak of STX and NEO in the unknown sample from periodate oxidation.

For the calculation of the NEO mass and/or substance concentration use Formula (1) and/or (2).

10.8.3 Method 8

The sensitivity of the peroxide mode for NEO is very poor; therefore the content of NEO is negligible for the calculation of STX after peroxide oxidation. The portion of STX in the area of Peak T is calculated with the calibration curve Formula (22) or (23), respectively

$$\rho_{(STX)-H2O2}=\frac{U-b_{(STX-H2O2)}}{a_{(STX-H2O2)}}=\rho_{(STX-Period)} \tag{21}$$

$$X_{(STX-Period)}=\rho_{(STX-H2O2)}\times a_{(STX-Period)}+b_{(STX-Period)} \tag{22}$$

$$X_{(STX-Period)}=\left(\frac{\left(U-b_{(STX-H2O2)}\right)\times a_{STX-Period}}{a_{STX-H2O2}}\right)+b_{STX-Period} \tag{23}$$

where

$\rho_{(STX\text{-}H2O2)}$ is the mass concentration of STX in Peak U;

$\rho_{(STX\text{-}Period)}$ is the mass concentration of STX in Peak T;

U is the area of Peak U;

$X_{(STX\text{-}Period)}$ is the peak area of STX in peak T;

$a_{STX\text{-}H2O2}$ is the slope of calibration curve of STX oxidized with peroxide;

$a_{STX\text{-}Period}$ is the slope of calibration curve of STX oxidized with periodate;

$b_{STX\text{-}H2O2}$ is the intersection of the calibration curve of STX oxidized with peroxide;

$b_{STX\text{-}Period}$ is the intersection of the calibration curve of STX oxidized with periodate.

Calculate the peak area of NEO in peak T with Formula (24).

$$X_{(NEO)} = T - X_{(STX-Period)} = T - \left(\frac{\left(U - b_{(STX-H2O2)}\right) \times a_{(STX-Period)}}{a_{(STX-H2O2)}} \right) + b_{(STX-Period)} \quad (24)$$

For the calculation of the NEO mass and/or substance concentration use Formula (1) and/or (2).

10.9 Conversion to STX 2HCl equivalents

To control the legal limit for PSP toxins in shellfish tissue it is necessary to calculate the total toxicity as STX 2HCl equivalents. Therefore each PSP toxin mass fraction has to be converted into the substance concentration in µmol/kg with Formula (25) if not already done in 10.2.

$$\mathrm{Ci}\left(\mu\mathrm{mol}/\mathrm{kg}\right) = \frac{w}{M} \quad (25)$$

where

w is the mass fraction of toxin (µg/kg) (calculated in 10.2);

M is the molecular weight of the toxin (µg/µmol).

The total toxicity in the tissue is given by Formula (26).

$$\mu\mathrm{g\ STX\ 2HCl\ equivalents\ /kg} = \left(\sum_{i=1}^{n} C_i \times T_i \right) \times 372{,}2 \quad (26)$$

where

C_i is the toxin substance concentration (µmol/kg) of each detected toxin;

T_i is the toxicity equivalent factor of the PSP toxins according to Table 8, see [13]; [14];

372,2 is the molecular weight from STX 2HCl (g/mol).

For those toxins that are determined together, since they co-elute, (dcGTX2 and dcGTX3; GTX1 and GTX4; GTX2 and GTX3; C1 and C2; C3 and C4) it shall be chosen the highest toxicity factor of the two isomers to calculate the toxicity contribution of each toxin.

NOTE The European working group on the determination of PSP toxins by the AOAC Method 2005.06 conducted by the European Reference Laboratory for Marine Biotoxins (EURLMB) agreed on the 3rd meeting 2009 to use the toxicity equivalent factors (TEFs) indicated in Table 8 as given by EFSA.

Other TEFs than those listed in Table 8 may be used provided that they have been internationally accepted and their use has been also accepted by the EU commission, EURLMB and their NRLs Network.

Table 8 — Toxicity equivalent factors (TEFs) of the PSP Toxins

Name	**TEFs according to EFSA opinion** [14]	**TEFs according to Oshima** [13]
GTX5 (B1)	0,1	0,0644
GTX6 (B2)	0,1	0,0644
C1	0,0	0,0060
C2	0,1	0,0963
C3	0,0	0,0133
C4	0,1	0,0576
dcGTX2	0,2	0,1538
dcGTX3	0,4	0,3766
dcNeo	0,4	not available
dcSTX	1	0,5131
GTX1	1	0,9940
GTX2	0,4	0,3592
GTX3	0,6	0,6379
GTX4	0,7	0,7261
11-hydroxy-STX	0,3	0,3186
NEO	1	0,9243
STX	1	1,0000

11 Precision

Details on the interlaboratory studies and method performance of the method are given in Annex A.

The values derived from these interlaboratory studies may not be applicable to concentration ranges and matrices other than those given in Annex A.

12 Test report

The test report should contain the necessary data according to EN ISO/IEC 17025 and the following:

a) all information necessary for the identification of the sample (kind of sample [e.g. cooked, processed or raw], origin of sample, designation);

b) a reference to the European Standard;

c) the date and type of sampling procedure (if known);

d) the date of receipt of the sample;

e) the date of test;

f) the test results and the units in which they have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

Precision data

A.1 Interlaboratory study organized by the Health Canada (2001)

The following data were obtained in an interlaboratory study organized by the Health Canada, Food Research Division, Ottawa, Canada in accordance with the AOAC® Official Methods Validation Program [15] in 2001. For this interlaboratory study, three calibration points were used (4.13.2). Samples of mussels, both blank and naturally contaminated, were mixed and homogenized to provide a variety of PSP toxin mixtures and concentration levels. The same procedure was followed with samples of clams, oysters, and scallops. 21 samples in total were sent to 21 collaborators but 3 laboratories withdrew after receiving the test materials. Results were obtained from 18 laboratories representing 14 different countries. Data from two laboratories were not included in the report, because insufficient information was provided to verify results. Data (not corrected for recovery) of the remaining 16 laboratories were statistically analysed and summarized in Table A.5 to Table A.19. Table A.20 and Table A.21 show results obtained from blind duplicates for all toxins studied. All results were acceptable with HorRat values below 2, see [4] and [5].

The interlaboratory study applied the Dixon test to each of the individual test samples having a sufficient number of data points to obtain the interlaboratory statistical data for the test samples. HorRat values (acceptable values are < 2) indicate that variability of the results for most of the toxins at various concentrations is in the acceptable range.

This interlaboratory study material included shellfish tissue from Spain, New Zealand, and east and west coasts from Canada, and contained a variety of PSP toxin patterns to test the method with geographically different matrices and mixtures of PSP toxins, see Table A.1.

GTX6 (B2) was not included in the interlaboratory study for quantitative determination because the amount of standard substance available was insufficient. However, after SPE-COOH clean-up, most laboratories successfully detected and reported GTX6 (B2) in certain samples at a concentration of about 30 µg/kg or greater. This indicates that once analytical standard is available, this method should be applicable to the quantitative detection of this toxin.

The following abbreviations are used for all tables in this Annex A:

nd	not determined;
Year	Year of interlaboratory study
Labs without outliers	Number of laboratories retained after eliminating outliers
Accepted results	Number of accepted results
$\overline{x}$	Mean value
s_r	Repeatability standard deviation
RSD_r	Repeatability relative standard deviation
r	Repeatability limit
s_R	Reproducibility standard deviation
RSD_R	Reproducibility relative standard deviation
R	Reproducibility limit
σ	Target standard deviation for proficiency assessment

The following sample data are valid for all precision data tables.

Table A.1 — Characteristics of samples

Sample No	Matrix	characteristics
Practice	mussel, naturally contaminated	
1	scallop, naturally contaminated	
2	clams, naturally contaminated	
3	oyster, blank	
4	mussel, blank	
5	clams, naturally contaminated	blind duplicate of 5D
5D	clams, naturally contaminated	blind duplicate of 5
6	clams, blank	
7	mussel, naturally contaminated	
8	mussel, naturally contaminated	blind duplicate of 8D
8D	mussel, naturally contaminated	blind duplicate of 8
9	mussel, naturally contaminated	
10	oyster, naturally contaminated	blind duplicate of 10D
10D	oyster, naturally contaminated	blind duplicate of 10
11	oyster, naturally contaminated	
12	mussel, naturally contaminated	
Spike 1	mussel, artificially contaminated (spiked)	spiked with NEO; STX; GTX1,4; GTX2,3
Spike 2	mussel, artificially contaminated (spiked)	spiked with NEO; STX; GTX1,4; GTX2,3; C1,2; C3,4
Spike 3	mussel, artificially contaminated (spiked)	spiked with dcSTX; STX; GTX5
Spike 4	mussel, artificially contaminated (spiked)	spiked with dcSTX; STX; GTX5
Spike 5	mussel, artificially contaminated (spiked)	spiked with dcSTX; GTX2,3; GTX5; C1,2; C3,4

A.2 Performance test for the total toxicity organized by the Community Reference Laboratory of Marine Biotoxins (CRLMB) 2006[4)]

The following data were obtained in an performance test [6] organized by the Community Reference Laboratory of Marine Biotoxins (CRLMB), in accordance with the AOAC Official Methods Validation Program [15] in 2006 on (raw and frozen) samples. It was a study to evaluate the “fitness for purpose”

4) The current name is European Union Reference Laboratory for Marine Biotoxins (EURLMB), Agencia Española de Consumo, Seguridad Alimentaria y Nutrición. Vigo, Spain.

of Lawrence HPLC-FLD method for the Official Control of PSP toxins in the European laboratories. The performance of the EU participant laboratories in the application of this method was compared with that obtained in the interlaboratory Study organized by Lawrence [5]. Lawrence method [1] indicates the way to calculate the concentration of each PSP toxin (in µg free base of the toxin/kg shellfish tissue), but it does not indicate how to proceed to report results in total toxicity. The performance test expresses the results in µg equivalents STX 2HCl/100 g shellfish tissue. In this European Standard, the values were converted in µg equivalents STX 2HCl/kg shellfish tissue. The specific toxicities of the PSP toxins (Oshima, 1995, [13] as also appear in "Supplemental Information for PSP toxin CRMs") had to be used for calculations.

Samples of mussels, scallop, clam and *Acanthocardia tuberculata* (giant cockle) naturally contaminated (see Table A.2), were homogenized. Samples containing C1,2 (standard substance not available at that moment), dc-STX, GTX2,3, GTX5, STX and dcGTX2,3 can be quantified after SPE-C18. Extracts contained also GTX1,4, C3,4 (standard substance not available), NEO, and GTX6 (standard substance not available).

Six samples in total were sent to 21 collaborators but three laboratories did not return their results in time. Results were obtained from 18 laboratories. For those laboratories that confounded one PSP toxin with a different one (i.e. GTX6 for NEO), their result was considered as non-valid data and were removed prior to the statistical evaluation. Outliers' identification was carried out by the Cochran and Grubbs tests, for samples CRL/06/P/01 and CRL/06/P/05 (blind duplicates). For the rest of the samples, Dixon test ($\alpha = 0,05$) was used instead.

The precision data are shown in Table A.22 and Table A.23.

Table A.2 —Characteristics of samples from performance test

sample No	matrix	characteristics
CRL/06/P/01	mussel, naturally contaminated	blind duplicate of CRL/06/P/05
CRL/06/P/02	*Acanthocardia tuberculata*, giant cockle naturally contaminated	
CRL/06/P/03	clams, naturally contaminated	
CRL/06/P/04	scallop, naturally contaminated	
CRL/06/P/05	mussel, naturally contaminated	blind duplicate of CRL/06/P/01
CRL/06/P/06	mussel, naturally contaminated	

A.3 Determination of PSP toxins in shellfish including GTX6 after hydrolysis organized by the Community Reference Laboratory of Marine Biotoxins (CRLMB)[4)] 2007

The interlaboratory study [7] was organized by the Community Reference Laboratory for Marine Biotoxins (CRLMB), Agencia Espanola de Seguridad Alimentaria y Nutricion (AESAN) on raw samples. Essential innovations compared with the previous study was the availability of C1,2 toxins standard substance and the determination of GTX6 (B2) through acidic hydrolysis. It should be established in a small scale whether the hydrolysis protocol is practicable for indirect determination of GTX6 (B2). Therefore, two samples (frozen and homogenized mussels; see Table A.3) were analysed by seven EU laboratories. The Lawrence HPLC method was used and additionally an acidic hydrolysis of the purified COOH-SPE-Fraction 2 had to be proceeded to determine indirectly the GTX6 (B2) toxin (see 6.4).

Table A.3 —Characteristics of samples

Sample No	Matrix	Species	origin	notes
CRL/07/HYDR/01	mussels	*Mytilus galloprovincialis*	Galicia (E)	naturally contaminated
CRL/07/HYDR/02	mussels	*Mytilus galloprovincialis*	Galicia (E)	naturally contaminated

The determination of GTX6 (B2), GTX5 (B1), dcGTX2,3, C1,2, dcSTX and the total toxicity were evaluated. The specific toxicities of the PSP toxins (Oshima, 1995, [13] as also appear in "Supplemental Information for PSP toxin CRMs") had to be used for calculations of the total toxicity. All laboratory data were included for the statistical evaluation. Outlier were identified by Dixon Test (α = 0,05) and were excluded from the calculated mean-value of the participants. The obtained data are shown in Table A.24 to Table A.27, Table A.29 and Table A.30.

A.4 Extension of the validation including dcGTX2,3 and dcNEO provided by the Community Reference Laboratory of Marine Biotoxins (CRLMB)[4)] 2008

The Community Reference Laboratory for Marine Biotoxins (CRLMB), Agencia Espanola de Seguridad Alimentaria y Nutricion (AESAN), conducted an interlaboratory study for the determination of PSP toxins in raw and frozen shellfish [8]. Thereby, the existing AOAC *Official Method* 2005.06 should be extended by dcGTX2,3 and dcNEO. Furthermore the acidic hydrolysis protocol to convert GTX6 (B2) to NEO was included, which has been proven to be useful for the quantification of GTX6 [7].

Different naturally contaminated or spiked clams and mussels were homogenized and used as test material for the interlaboratory study (see Table A.4). Eleven samples in total were sent to 11 collaborators. The obtained data from 10 different laboratories were statistical evaluated to identify outliers which were excluded from further calculations. Outliers' identification was carried out by the Cochran and Grubbs tests, for samples (blind duplicates). For the rest of the samples, Dixon test (α = 0,05) was used instead.

Finally, validation data for dcGTX2,3 were identified during this interlaboratory study. The determination of GTX6 (B2), GTX5 (B1), dcGTX2,3, C1,2, dcSTX, dcNEO and the total toxicity were evaluated. The specific toxicities of the PSP toxins [13] had to be used for calculations of the total toxicity. The obtained data are shown in Table A.24 to Table A.31.

The complicate calculation to quantify dcNEO and GTX6 (B2) could be the reason for the poor performance of some of the laboratories in this interlaboratory study.

Table A.4 —Characteristics of samples

Sample No	Matrix	Species	origin	notes
CRL/08/P01 and CRL/08/P/07	clam	*Venuerupis pullarstra*	Obidos Lagoon (P), Galicia (E)	Blind duplicate blank clam (Galicia) was mixed with naturally contaminated clam (P)
CRL/08/P/02	mussel	*Mytilus galloprovincialis*	Galicia (E)	Blank sample, aquired at retail market
CRL/08/P03 and CRL/08/P/08	mussel	*Mytilus galloprovincialis*	Galicia (E)	Blind duplicates, blank material were mixed with naturally contaminated ones
CRL/08/P/04	clam	*Spisula solida*	Sines (P)	Naturally contaminated
CRL/08/P/05	mussel	*Mytilus galloprovincialis*	Galicia (E)	Sample CRL/08/P/02 spiked with NRC-dcGTX2,3-b at 0,807 µmol/kg sample
CRL/08/P/06	clam	*Meretryx lyrata*	Vietnam	Sample CRL/08/P/10 spiked with NRC-dcGTX2,3-b at 0,773 µmol/kg sample
CRL/08/P/09	mussel	*Mytilus galloprovincialis*	Galicia (E)	Sample CRL/08/P/02 spiked with NRC-dcGTX2,3-b at 1,84 µmol/kg sample
CRL/08/P/10	clam	*Meretryx lyrata*	Vietnam	Blank sample
CRL/08/P/11	clam	*Meretryx lyrata*	Vietnam	Sample CRL/08/P/10 spiked with NRC-dcGTX2,3-b at 1,80 µmol/kg sample

Table A.5 —Precision data for STX after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5[a]
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	12	15	15	15	15	15	15	15	14	15	15	15	15	15	15	15	15	15	15	15	11
Laboratories without outliers	11	15	15	15	15	15	15	15	14	15	15	15	15	15	15	15	15	15	15	15	11
Number of outliers (laboratories)	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Accepted results	11	15	15	15	15	15	15	15	14	15	15	15	15	15	15	15	15	15	15	15	11
$\overline{x}$, µg/kg	471	1048	338	nd	nd	522	518	nd	23,4	310	316	132	136	144	258	784	223	447	51,3	169	13,2
s_R, µg/kg	41,2	229	90,9			69,4	73,9		8,48	78,1	70,3	33,4	46,1	41,6	59,5	215	52,5	112	17,0	43,0	8,65
RSD_R, %	8,74	21,9	26,9			13,3	14,3		36,3	25,2	22,3	25,2	33,9	28,8	23,1	27,5	23,5	25,2	33,1	25,4	65,6
R [$R = 2{,}8 \times s_R$], µg/kg	115	642	254			194	207		23,7	219	197	93,5	129	117	167	603	147	315	47,5	120	24,2
HorRat	0,49	1,38	1,43			0,75	0,81		1,29	1,32	1,17	1,16	1,57	1,35	1,18	1,65	1,17	1,39	1,32	1,22	2,14
Recovery, %																	74,4	74,5	93,2	76,8	

[a] It is pointed out that for this sample, HorRat values exceed 2.

Table A.6 — Precision data for STX after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	15	15	14	14	14	15	15	14	12	15	13	14	14	15	15	14	13	15	11	15	9
Laboratories without outliers	15	15	14	14	14	15	15	14	12	15	13	14	14	15	15	14	13	15	11	15	9
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Accepted results	15	15	14	14	14	15	15	14	12	15	13	14	14	15	15	14	13	15	11	15	9
$\overline{x}$, µg/kg	452	956	295	nd	nd	510	474	nd	21,3	270	269	120	140	124	233	644	191	409	43,6	148	10,4
s_R, µg/kg	122	188	82,8			85,8	82,6		6,80	68,8	49,8	38,2	45,2	26,9	37,9	78,9	39,2	123	11,4	31,4	4,48
RSD_R, %	26,9	19,6	28,1			16,8	17,4		31,9	25,5	18,5	31,9	32,3	21,8	16,3	12,3	20,5	30,0	26,1	21,2	42,9
R [$R = 2{,}8 \times s_R$], µg/kg	341	526	232			240	231		19,0	193	139	107	127	75,4	106	221	110	343	31,8	88,0	12,5
HorRat	1,49	1,22	1,46			0,95	0,97		1,12	1,31	0,95	1,45	1,50	0,99	0,82	0,72	1,00	1,64	1,02	1,00	1,35
Recovery, %																	63,7	68,2	79,2	67,2	

Table A.7 — Precision data for NEO after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice	3	4	5	5D	6	8	8D	9	12	Spike 1	Spike 2	Spike 3 [a]	Spike 4 [b]	Spike 5 [b c]
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	12	15	15	8	10	15	14	14	13	15	14	15	14	12	12
Laboratories without outliers	10	15	15	7	9	15	14	14	13	15	14	15	14	12	12
Number of outliers (laboratories)	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Accepted results	10	15	15	7	9	15	14	14	13	15	14	15	14	12	12
$\overline{x}$, µg/kg	566	nd	nd	33,1	40,0	nd	284	278	140	871	223	455	158	213	344
s_R µg/kg	113			12,8	17,4		91,8	70,6	45,9	228	88,0	117	39,4	85,8	164
RSD_R, %	20,0			38,5	43,5		32,3	25,4	32,8	26,1	39,5	25,7	24,9	40,2	47,6
R [$R = 2{,}8 \times s_R$] µg/kg	317			35,7	48,8		257	198	128	637	246	327	110	240	458
HorRat	1,15			1,44	1,68		1,67	1,31	1,52	1,60	1,97	1,43	1,18	1,99	2,53
Recovery %											55,7	56,9			

[a] NEO together with dcSTX but calculated as NEO.

[b] No NEO present in the samples, Peak due to dcSTX but calculated as NEO.

c It is pointed out that for this sample, HorRat values exceed 2.

Table A.8 — Precision data for NEO after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice	3	4	5	5D	6	8	8D	9[a]	12	Spike 1	Spike 2
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	15	14	14	10	10	14	14	15	14	15	14	15
Laboratories without outliers	15	14	14	10	10	14	13	15	14	14	14	15
Number of outliers (laboratories)	0	0	0	0	0	0	1	0	0	1	0	0
Accepted results	15	14	14	10	10	14	13	15	14	14	14	15
$\overline{x}$, µg/kg	643	nd	nd	38,8	42,2	nd	255	266	97,6	843	212	492
s_R µg/kg	148			13,0	18,3		89,9	92,9	46,4	199	68,1	159,6
RSD_R, %	23,0			33,6	43,3		35,3	34,9	47,5	23,7	32,1	32,4
R [$R = 2{,}8 \times s_R$] µg/kg	414			36,5	51,2		252	260	130	558	191	447
HorRat	1,35			1,29	1,68		1,80	1,79	2,09	1,44	1,59	1,82
Recovery %											53,1	61,6

[a] It is pointed out that for this sample, HorRat values exceed 2.

Table A.9 — Precision data for dcSTX after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice > HR	1	3	4	5	5D	6	7	11	12a	Spike 3[b]	Spike 4	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	9	13	15	15	14	13	15	12	11	7	15	15	15
Laboratories without outliers	9	13	15	15	14	12	15	11	11	-	15	15	14
Number of outliers (laboratories)	0	0	0	0	0	1	0	1	0		0	0	1
Accepted results													
$\overline{x}$, µg/kg	18,8	12,3	nd	nd	8,14	7,42	nd	10,6	3,45		83,7	294	386
s_R µg/kg	21,6	4,33			2,71	1,83		2,02	1,63		41,2	63,4	40,6
RSD_R, %	115	35,2			33,3	24,7		19,2	47,3		49,2	21,6	10,5
R [R = 2,8 × s_R] µg/kg	60,5	12,1			7,59	5,12		5,66	4,56		115	177	114
HorRat	3,95	1,13			1,01	0,74		0,60	1,26		2,12	1,12	0,57
Recovery %											83,7	73,5	64,3

[a] Not calculated, Only 7 laboratories reported numerical data, the remainder reported as not detected.

[b] It is pointed out that for this sample, HorRat values exceed 2.

Table A.10 — Precision data for dcSTX after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice[b]	1	3	4	5	5D	6	7	11[a]	12	Spike 3	Spike 4	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	10	13	14	14	11	10	14	13	6	10	13	15	15
Laboratories without outliers	10	12	14	14	11	10	14	13	-	10	13	15	15
Number of outliers (laboratories)	0	1	0	0	0	0	0	0		0	0	0	0
Accepted results	10	12	14	14	11	10	14	13		10	13	15	15
$\overline{x}$, µg/kg	12,6	9,88	nd	nd	8,36	6,80	nd	11,6		7,80	69,1	276	396
s_R µg/kg	12,4	3,89			4,86	3,33		3,04		3,22	17,1	52,8	93,7
RSD_R, %	98,3	39,4			58,1	49,0		26,2		41,3	24,7	19,1	23,7
R [$R = 2{,}8 \times s_R$] µg/kg	34,7	10,9			13,6	9,32		8,51		9,02	47,9	148	262
HorRat	3,18	1,23			1,77	1,44					1,03	0,99	1,29
Recovery %											69,1	69,0	66,0

[a] Not calculated, Only 6 laboratories reported numerical data; the remainder reported as not detected.

[b] It is pointed out that for this sample, HorRat values exceed 2.

Table A.11 — Precision data for GTX1,4 (together) after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7a	8	8D	9	11	12a	Spike 1	Spike 2[a]	Spike 5[a]
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	11	10	12	16	16	11	11	16	15	12	13	13	12	10	10	11	13
Laboratories without outliers	11	10	11	16	16	11	11	16	15	12	13	13	12	10	10	11	12
Number of outliers (laboratories)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Accepted results	11	10	11	16	16	11	11	16	15	12	13	13	12	10	10	11	12
$\overline{x}$, µg/kg	1 438	199	285	nd	nd	61,4	62,1	nd	1 489	573	672	254	81,0	1 816	240	1 440	2 236
s_R µg/kg	650	170	60,1			28,5	17,7		417	157	164	69,6	54,9	428	116	275	572
RSD_R, %	45,2	85,4	21,1			46,4	28,6		28,0	27,4	24,4	27,3	67,7	23,6	48,3	19,1	25,6
R [$R = 2{,}8 \times s_R$] µg/kg	1 819	476	168			79,7	49,6		1 168	439	459	195	154	1198	324	770	1 602
HorRat	2,98	4,19	1,09			1,91	1,17		1,86	1,57	1,44	1,39	2,90	1,61	2,43	1,26	1,80
Recovery %															60,0		

[a] GTX1,4 together with C3,4 but calculated as GTX1,4.

Table A.12 — Precision data for GTX1,4 (together) after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice	1[a]	2	3	4	5	5D	6	7	8	8D	9	11	12	Spike 1	Spike 2	Spike 5[b]
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	16		14	15	15	13	13	16	15	16	16	14	9	16	14	15	14
Laboratories without outliers	16		13	15	15	13	13	16	14	15	15	14	8	16	14	15	14
Number of outliers (laboratories)	0	-	1	0	0	0	0	0	1	1	1	0	1	0	0	0	0
Accepted results	16		13	15	15	13	13	16	14	15	15	14	8	16	14	15	14
$\overline{x}$, µg/kg	1 279		364	nd	nd	73,5	71,5	nd	240	645	675	236	48,9	2 079	268	628	265
s_R µg/kg	317		141			17,2	22,6		64,5	148	121	44,9	13,0	395	86,8	167	68,8
RSD_R, %	24,8		38,8			23,4	31,6		26,9	22,9	17,9	19,1	26,7	19,0	32,5	26,6	25,9
R [$R = 2{,}8 \times s_R$] µg/kg	887		395			48,1	63,3		181	414	339	126	36,5	1 106	243	468	193
HorRat	1,61		2,08			0,99	1,33		1,35	1,34	1,06	0,96	1,06	1,33	1,66	1,55	1,33
Recovery %															66,9	78,5	

[a] Not calculated, Half of the laboratories reported not detected.

[b] No GTX1,4 present in this sample, Peaks due to dcGTX2,3 (confirmed by peroxide oxidation) but calculated as GTX1,4.

Table A.13 — Precision data for GTX2,3 (together) after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 1	Spike 2	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	13	16	16	16	16	16	16	16	14	16	16	16	16	16	16	16	15	16	16
Laboratories without outliers	12	16	15	16	16	16	16	16	14	16	16	16	16	15	15	15	14	16	16
Number of outliers (laboratories)	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
Accepted results	12	16	15	16	16	16	16	16	14	16	16	16	16	15	15	15	14	16	16
$\overline{X}$, µg/kg	3 291	2 526	2 538	nd	nd	120	114	nd	349	813	757	316	367	336	673	1 856	227	529	954
s_R µg/kg	524	652	479			21,8	24,4		93,0	253	181	66,0	89,0	78,6	182	505	42,7	167	293
RSD_R, %	15,9	25,8	18,9			18,2	21,5		26,7	31,1	23,8	20,8	24,2	23,4	27,1	27,2	18,8	31,6	30,8
R [$R = 2{,}8 \times s_R$] µg/kg	1 467	1 826	1 342			61,0	68,4		260	708	506	185	249	220	510	1415	120	469	822
HorRat	1,19	1,86	1,36			0,83	0,97		1,42	1,89	1,43	1,10	1,30	1,24	1,59	1,87	0,94	1,80	1,91
Recovery %																	75,8	88,2	79,5

Table A.14 — Precision data for GTX2,3 (together) after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 1	Spike 2	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	16	16	16	15	14	16	16	15	15	16	16	16	16	15	16	16	15	16	16
Laboratories without outliers	15	16	16	15	14	15	16	15	15	16	16	16	15	15	16	16	15	16	16
Number of outliers (laboratories)	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Accepted results	15	16	16	15	14	15	16	15	15	16	16	16	15	15	16	16	15	16	16
$\overline{x}$, µg/kg	3 314	2 348	2 698	nd	nd	132	116	nd	343	805	746	321	369	342	701	1 874	261	564	1 025
s_R µg/kg	594	522	506			25,3	28,4		80,2	164	165	58,2	71,4	86,1	204	345	42,7	117	175
RSD_R, %	17,9	22,2	18,8			19,2	24,5		23,4	20,4	22,1	18,1	19,3	25,2	29,1	18,4	16,4	20,7	17,1
R [$R = 2{,}8 \times s_R$] µg/kg	1 665	1 462	1 417			70,9	79,6		225	460	461	163	200	241	571	966	120	327	489
HorRat	1,34	1,58	1,36			0,88	1,11		1,25	1,24	1,32	0,96	1,04	1,34	1,72	1,27	0,84	1,19	1,07
Recovery, %																	87,1	93,9	85,4

Table A.15 — Precision data for GTX5 (B1) after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 2	Spike 3	Spike 4	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	11	13	11	16	16	14	14	16	15	15	15	15	12	14	14	15	14	15	15	15
Laboratories without outliers	11	13	11	16	16	13	13	16	15	15	15	15	12	14	14	15	14	15	15	14
Number of outliers (laboratories)	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Accepted results	11	13	11	16	16	13	13	16	15	15	15	15	12	14	14	15	14	15	15	14
$\overline{x}$, µg/kg	435	91,1	31,0	nd	nd	42,8	40,5	nd	145	328	334	120	37,7	39,6	73,1	947	53,4	311	1 213	691
s_R µg/kg	47,8	25,1	7,36			8,36	7,22		27,1	47,1	51,5	25,3	9,26	13,0	16,5	264	16,0	70,0	240	223
RSD_R, %	11,0	27,6	23,7			19,6	17,8		18,7	14,4	15,4	21,2	24,6	32,8	22,5	27,9	30,0	22,5	19,8	32,3
R [$R = 2{,}8 \times s_R$] µg/kg	134	70,3	20,6			23,4	20,2		75,9	132	144	71,0	25,9	36,3	46,1	739	44,8	196	671	626
HorRat	0,61	1,20	0,88			0,76	0,69		0,87	0,76	0,82	0,96	0,94	1,26	0,95	1,73	1,21	1,18	1,27	1,91
Recovery, %																		77,8	75,8	86,4

Table A.16 — Precision data for GTX5 (B1) after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice	1	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 2	Spike 3	Spike 4	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	14	13	9	14	14	12	12	14	13	14	14	14	10	12	12	14	13	13	14	15
Laboratories without outliers	14	12	9	14	14	12	12	14	13	14	14	14	10	12	12	14	13	13	14	15
Number of outliers (laboratories)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Accepted results	14	12	9	14	14	12	12	14	13	14	14	14	10	12	12	14	13	13	14	15
$\overline{x}$, µg/kg	350	71,8	27,1	nd	nd	42,0	40,8	nd	121	286	274	124	30,6	37,6	63,0	785	46,0	293	1 065	620
s_R µg/kg	58,8	13,0	4,43			11,1	9,73		17,7	43,7	52,5	29,1	5,99	10,9	11,1	73,4	8,78	43,0	147	85,4
RSD_R, %	16,8	18,2	16,3			26,4	23,9		14,7	15,3	19,1	23,5	19,6	29,1	17,6	9,35	19,1	14,7	13,8	13,8
R [$R = 2{,}8 \times s_R$] µg/kg	165	36,5	12,4			31,0	27,2		49,6	122	147	81,5	16,8	30,6	31,1	206	24,6	120	413	239
HorRat	0,90	0,76	0,59			1,02	0,92		0,67	0,79	0,98	1,07	0,72	1,11	0,73	0,56	0,75	0,76	0,87	0,80
Recovery, %																		73,1	66,5	77,5

Table A.17 — Precision data for C1,2 (together) after SPE C18 clean-up obtained by the interlaboratory study A.1

Sample	Practice	2	3	4	5	5D	6	7	8	8D	9	10	10D	11	12	Spike 2	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	12	14	16	15	16	16	16	15	12	11	9	11	15	16	16	14	15
Laboratories without outliers	12	13	16	15	16	15	16	15	12	10	9	11	15	16	16	13	14
Number of outliers (laboratories)	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1
Accepted results	12	13	16	15	16	15	16	15	12	10	9	11	15	16	16	13	14
$\overline{X}$, µg/kg	299	254	nd	nd	257	239	nd	912	118	96,3	76,6	162	190	358	257	735	1 567
s_R µg/kg	50,8	56,8			74,7	50,8		302	63,8	24,1	80,3	43,7	58,5	58,2	67,4	109	228
RSD_R, %	17,0	22,4			29,1	21,3		33,1	54,0	25,0	105	27,0	30,8	16,3	26,3	14,8	14,5
R [$R = 2{,}8 \times s_R$] µg/kg	142	159			209	142		845	179	67,5	225	122	164	163	189	305	638
HorRat	0,89	1,14			1,48	1,07		2,04	2,45	1,10	4,46	1,28	1,50	0,87	1,34	0,88	0,97
Recovery, %																73,5	78,3

Table A.18 — Precision data for C1,2 (together) after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	Practice	2	3	4	5	5D	6	7	8	8D	9 [a]	10	10D	11	12	Spike 2	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	14	15	14	14	15	15	15	15	9	10		14	14	15	13	14	15
Laboratories without outliers	14	14	14	14	14	15	15	15	9	10		14	14	15	12	14	15
Number of outliers (laboratories)	0	1	0	0	1	0	0	0	0	0	-	0	0	0	1	0	0
Accepted results	14	14	14	14	14	15	15	15	9	10		14	14	15	12	14	15
$\overline{x}$, µg/kg	301	261	nd	nd	237	238	nd	983	109	93,4		204	201	349	258	860	1 687
s_R µg/kg	77,8	66,2			64,3	80,3		255	40,3	35,8		57,4	48,5	81,3	39,2	205	342
RSD_R, %	25,8	25,4			27,1	33,8		25,9	37,1	38,3		28,1	24,2	23,3	15,2	23,8	20,3
R [$R = 2{,}8 \times s_R$] µg/kg	218	185			180	225		714	113	100		161	136	228	110	573	959
HorRat	1,35	1,30			1,37	1,70		1,62	1,66	1,67		1,38	1,19	1,24	0,78	1,45	1,37
Recovery, %																86,0	84,4

[a] Not calculated, Only 3 laboratories reported numerical data, the remainder reported as not detected.

Table A.19 — Precision data for C3,4 (together) after SPE-COOH clean-up obtained by the interlaboratory study A.1

Sample	3	4	6	7	12	Spike 2	Spike 5
Year	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	15	15	15	13	11	9	15
Laboratories without outliers	15	15	15	13	10	9	15
Number of outliers (laboratories)	0	0	0	0	1	0	0
Accepted results	15	15	15	13	10	9	15
$\overline{x}$, µg/kg	nd	nd	nd	837	238	725	1 425
s_R µg/kg				227	106	181	302
RSD_R, %				27,1	44,8	25,0	21,2
R [$R = 2{,}8 \times s_R$] µg/kg				636	298	506	846
HorRat				1,65	2,25	1,49	1,40
Recovery, %						80,5	79,2

Table A.20 — Precision data for the blind duplicates after SPE C18 clean-up obtained by the interlaboratory study A.1

PSP toxin	STX			NEO	dcSTX	GTX1,4		GTX2,3			GTX5 (B1)			C1,2		
Sample [a]	5/5D	8/8D	10/10D	8/8D	5/5D	5/5D	8/8D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	15	15	15	13	12	9	11	16	16	15	13	15	12	15	8	11
Laboratories without outliers	15	15	15	13	12	9	11	16	16	15	13	15	12	15	8	11
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Accepted results	15	15	15	13	12	9	11	16	16	15	13	15	12	15	8	11
$\overline{x}$, µg/kg	520	313	140	280	7,46	64,6	601	117	785	347	41,7	331	38,6	241	101	169
s_r µg/kg	31,4	67,8	25,0	43,1	0,68	12,9	120	15,8	136	66,6	5,26	27,9	8,35	37,5	28,6	53,5
RSD_r, %	6,03	21,7	17,8	15,4	9,08	19,9	20,0	13,5	17,3	19,2	12,6	8,42	21,6	15,5	28,3	31,7
r [$r = 2{,}8 \times s_r$] µg/kg	87,9	190	69,9	121	1,90	36,0	336	44,2	381	187	14,7	78,0	23,4	105	80,1	150
s_R µg/kg	71,5	73,2	43,9	83,8	2,02	24,1	156	23,2	220	81,1	7,82	49,2	11,7	53,4	38,4	53,5
RSD_R, %	13,7	23,4	31,3	29,9	27,0	37,4	25,9	19,8	28,1	23,4	18, 8	14,9	30,3	22,1	38,0	31,7
R [$R = 2{,}8 \times s_R$] µg/kg	200	205	123	235	5,65	67,6	435	64,8	617	227	21,9	138	32,7	149	107	150
HorRat	0,78	1,23	1,46	1,55	0,81	1,55	1,50	0,90	1,69	1,25	0,73	0,79	1,16	1,12	1,68	1,52
Recovery, %																

[a] Youden pairs

Table A.21 — Precision data for the blind duplicates after SPE-COOH clean-up obtained by the interlaboratory study A.1

PSP toxin	STX			NEO		dcSTX	GTX1,4		GTX2,3			GTX5 (B1)			C1,2		
Sample [a]	5/5D	8/8D	10/10D	8/8D	5/5D	5/5D	5/5D	8/8D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D
Year	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002	2002
Number of laboratories	15	13	14	13	10	9	12	15	15	16	14	11	14	10	14	8	14
Laboratories without outliers	15	13	14	13	10	9	12	15	15	16	14	11	14	10	14	8	14
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Accepted results	15	13	14	13	10	9	12	15	15	16	14	11	14	10	14	8	14
$\overline{x}$, µg/kg	492	273	132	263	40,5	6,89	73,9	660	124	775	357	40,7	280	35,3	230	101	203
s_r µg/kg	44,0	36,2	32,9	67,7	7,82	0,88	11,1	85,8	19,9	91,1	84,3	5,04	40,6	8,49	46,1	20,0	37,1
RSD_r, %	8,94	13,2	24,9	25,8	19,3	12,8	15,0	13,0	16,0	11,8	23,6	12,4	14,5	24,1	20,0	19,8	18,3
r [$r = 2{,}8 \times s_r$] µg/kg	123	101	92,1	190	21,9	2,47	31,1	240	55,7	381	236	14,1	114	23,8	129	56,0	104
s_R µg/kg	85,7	60,1	37,9	90,0	15,9	3,15	19,4	135	28,1	167	84,3	10,4	48,0	9,56	62,1	40,5	52,7
RSD_R, %	17,4	22,0	28,7	34,2	39,2	45,8	26,2	20,5	22,6	21,5	23,6	25,5	17,2	27,1	27,0	40,1	26,0
R [$R = 2{,}8 \times s_R$] µg/kg	240	168	106	252	44,4	8,83	54,2	378	78,8	617	236	29,0	134	26,8	174	113	147
HorRat	0,98	1,13	1,32	1,75	1,51	1,35	1,11	1,20	1,03	1,69	1,26	0,98	0,89	1,02	1,35	1,78	1,28
Recovery, %																	

a Youden pairs

Table A.22 — Precision data for total toxicity obtained by performance test A.2

Sample	CRL/06/P/01 [a]	CRL/06/P/02	CRL/06/P/03	CRL/06/P/04	CRL/06/P/05 [a]	CRL/06/P/06
Year	2006	2006	2006	2006	2006	2006
Number of laboratories	18	18	16	17	18	13
Laboratories without outliers	16	17	15	15	16	11
Number of outliers (laboratories)	2	1	1	2	2	2
Accepted results	16	17	15	15	16	11
$\bar{x}$, µg STX 2HCl eq,/kg	1708,3	1033,9	2829,4	319,8	1524,9	105,4 [b]
σ µg STX 2HCl eq,/kg	469,1	277,8	610,0	82,6	418,7	34,1
s_R [c], µg STX 2HCl eq,/kg	476,4	249,2	551,2	57,3	340,8	26,1
RSD_R, %	27,89	24,10	19,48	17,92	22,35	24,80
R [c] [$R = 2{,}8 \times s_R$], µg STX 2HCl eq,/kg	1334,0	697,7	1543,3	160,5	954,3	73,2
HorRat	1,88	1,51	1,42	0,95	1,49	1,10
Recovery, %						

[a] blind duplicates

[b] The total toxicity of this sample could not be evaluated for the lack of standard substances e,g, GTX6 (B2), C3,4 and dcNEO.

[c] The calculation is based on the reproducibility relative standard deviation RSD_R in %.

Table A.23 — Precision data for total toxicity for the blind duplicates obtained by performance test A.2

Sample	CRL/06/P/01 and CRL/06/P/05
Year	2006
Number of laboratories	18
Laboratories without outliers	16
Number of outliers (laboratories)	2
Accepted results	16
$\bar{x}$, µg STX 2HCl eq,/kg	1616
s_r, µg STX 2HCl eq,/kg	210,6
RSD_r, %	13,03
r [$r = 2{,}8 \times s_r$], µg STX 2HCl eq,/kg	589,6
s_R, µg STX 2HCl eq,/kg	423,3
RSD_R, %	26,19
R [$R = 2{,}8 \times s_R$], µg STX 2HCl eq,/kg	1185
HorRat	1,31
Recovery, %	

Table A.24 — Precision data for dcGTX2,3 obtained by interlaboratory study A.3 and A.4

Sample	CRL/07/ HYDR/02	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/04	CRL/08/ P/05	CRL/08/ P/06	CRL/08/ P/07	CRL/08/ P/08	CRL/08/ P/09	CRL/08/ P/11
Year	2007	2008	2008	2008	2008	2008	2008	2008	2008	2008
Number of laboratories	6	10	10	10	9	10	10	10	10	10
Laboratories without outliers	6	10	9	10	9	10	10	9	10	10
Number of outliers (laboratories)	0	0	1	0	0	0	0	1	0	0
Accepted results	6	10	9	10	9	10	10	9	10	10
$\bar{x}$, µmol/kg	1,19	2,07	2,48	3,84	0,43	0,45	2,45	2,62	0,99	0,96
σ, µmol/kg	0,42	0,62	0,75	1,15			0,74	0,79		
s_R, [a] µmol/kg	0,55	0,73	0,86	1,08	0,13	0,13	0,99	1,20	0,29	0,38
RSD_R, %	46,1	35,5	34,7	28,2	30,3	29,9	40,4	45,7	29,1	39,6
R [a] [$R = 2{,}8 \times s_R$], µmol/kg	1,54	2,06	2,41	3,03	0,36	0,38	2,77	3,35	0,81	1,06
HorRat	2,53	2,12	2,13	1,85	1,57	1,54	2,48	2,82	1,71	2,31
Recovery %					53,6	58,6			53,9	53,1

[a] The calculation is based on the Reproducibility relative standard deviation RSD_R in %.

Table A.25 — Precision data for C1,2 obtained by the interlaboratory study A.3 and A.4

Sample	CRL/07/ HYDR/01	CRL/07/ HYDR/02	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/07	CRL/08/ P/08
Year	2007	2007	2008	2008	2008	2008
Number of laboratories	6	6	10	10	10	10
Laboratories without outliers	6	6	9	10	9	10
Number of outliers (laboratories)	0	0	1	0	1	0
Accepted results	6	6	9	10	9	10
$\bar{x}$, µmol/kg	1,96	7,93	15,3	19,1	15,9	20,8
σ, µmol/kg	0,65	1,15	2,23	2,78	2,32	2,72
s_R, [a] µmol/kg	0,87	3,36	2,97	3,57	3,13	5,55
RSD_R, %	44,4	42,4	19,4	18,7	19,7	26,7
R [a] [$R = 2{,}8 \times s_R$], µmol/kg	2,44	9,41	8,31	10,0	8,77	15,5
HorRat	2,74	3,24	1,63	1,63	1,67	2,36
Recovery %						

[a] The calculation is based on the Reproducibility relative standard deviation RSD_R, in %.

Table A.26 — Precision data for dcSTX obtained by the interlaboratory study A.3 and A.4

Sample	CRL/07/ HYDR/01	CRL/07/ HYDR/02	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/04	CRL/08/ P/07	CRL/08/ P/08
Year	2007	2007	2008	2008	2008	2008	2008
Number of laboratories	8	7	10	10	10	10	10
Laboratories without outliers	7	7	10	9	10	10	9
Number of outliers (laboratories)	1	0	0	1	0	0	1
Accepted results	7	7	10	9	10	10	9
$\bar{x}$, µmol/kg	0,327	2,18	4,23	3,97	3,44	4,31	3,88
σ, µmol/kg	0,11	0,47	0,45	0,42	0,36	0,45	0,41
s_R, [a] µmol/kg	0,085	0,78	0,65	0,67	0,56	1,38	0,66
RSD_R, %	26,1	35,6	15,6	16,8	16,4	32,1	16,9
R [a] [$R = 2{,}8 \times s_R$], µmol/kg	0,24	2,17	1,85	1,87	1,58	3,87	1,84
HorRat	1,12	2,04	0,99	1,05	1,01	2,03	1,06
Recovery %							

[a] The calculation is based on the Reproducibility relative standard deviation RSD_R, in %.

Table A.27 — Precision data for GTX5 (B1) obtained by the interlaboratory study A.3 and A.4

Sample	CRL/07/ HYDR/01	CRL/07/ HYDR/02	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/07	CRL/08/ P/08
Year	2007	2007	2008	2008	2008	2008
Number of laboratories	8	8	10	10	10	10
Laboratories without outliers	7	7	10	10	10	10
Number of outliers (laboratories)	1	1	0	0	0	0
Accepted results	7	7	10	10	10	10
$\bar{x}$, µmol/kg	1,26	5,42	13,7	9,50	14,3	9,50
σ, µmol/kg	0,20	1,07	2,71	1,88	2,82	1,88
s_R, [a] µmol/kg	0,15	0,39	2,30	2,18	2,50	1,85
RSD_R, %	11,6	7,27	16,8	22,9	17,5	19,5
R [a] [$R = 2{,}8 \times s_R$], µmol/kg	0,41	1,10	6,44	6,09	7,01	5,19
HorRat	0,65	0,51	1,25	1,74	1,41	1,48
Recovery %						
[a] The calculation is based on the Reproducibility relative standard deviation RSD_R, in %.						

Table A.28 — Precision data for dcNEO obtained by the interlaboratory study A.4

Sample	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/04	CRL/08/ P/07	CRL/08/ P/08
Year	2008	2008	2008	2008	2008
Number of laboratories	9	8	8	9	8
Laboratories without outliers	9	8	8	9	8
Number of outliers (laboratories)	0	0	0	0	0
Accepted results	9	8	8	9	8
$\bar{x}$, µmol/kg	4,61	4,67	2,18	4,78	5,22
σ, µmol/kg	1,38	1,40	0,65	1,43	1,57
s_R [a], µmol/kg	3,34	3,29	1,23	2,58	2,54
RSD_R, %	72,4	70,4	56,4	53,9	48,7
R [a] [$R = 2{,}8 \times s_R$], µmol/kg	9,35	9,21	3,44	7,21	7,12
HorRat	4,68	4,57	3,26	3,51	3,21
Recovery %					
[a] The calculation is based on the Reproducibility relative standard deviation RSD_R, in %.					

Table A.29 — Precision data for GTX6 (B2) obtained by the interlaboratory study A.3 and A.4

Sample	CRL/07/ HYDR/01	CRL/07/ HYDR/02	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/07	CRL/08/ P/08
Year	2007	2007	2008	2008	2008	2008
Number of laboratories	6	6	9	9	9	9
Laboratories without outliers	6	6	8	8	8	8
Number of outliers (laboratories)	0	0	1	1	1	1
Accepted results	6	6	8	8	8	8
$\bar{x}$, µmol/kg	2,64	11,7	12,8	18,8	13,3	17,4
σ, µmol/kg	0,92	4,08	3,85	5,65	3,98	5,22
s_R, [a] µmol/kg	0,55	5,34	4,83	7,37	4,11	5,95
RSD_R, %	20,9	45,6	37,7	39,2	30,9	34,2
R [a] [$R = 2{,}8 \times s_R$], µmol/kg	1,54	15,0	13,5	20,6	11,5	16,7
HorRat	1,31	3,59	3,01	3,31	2,48	2,86
Recovery %						

[a] The calculation is based on the Reproducibility relative standard deviation RSD_R, in %.

Table A.30 — Precision data for total toxicity obtained by the interlaboratory study A.3 and A.4

Sample	CRL/07/ HYDR/01	CRL/07/ HYDR/02	CRL/08/ P/01	CRL/08/ P/03	CRL/08/ P/04	CRL/08/ P/07	CRL/08/ P/08
Year	2007	2007	2008	2008	2008	2008	2008
Number of laboratories	8	7	10	10	10	10	10
Laboratories without outliers	7	7	9	8	9	9	8
Number of outliers (laboratories)	1	0	1	2	1	1	2
Accepted results	7	7	9	8	9	9	8
$\bar{x}$, µg STX 2HCl eq,/kg	260	1261	3277	3030	1436	3322	3078
σ, µg STX 2HCl eq,/kg	90,9	441,3	983	908	430	996	923
s_R, [a] µg STX 2HCl eq,/kg	43,4	421,1	1294	642	304	897	871
RSD_R, %	16,7	33,4	39,5	21,2	21,2	27,0	28,3
R a [$R = 2{,}8 \times s_R$], µg STX 2HCl eq,/kg	122	1179	3624	1798	852	2511	2439
HorRat	0,85	2,16	2,95	1,57	1,39	2,02	2,09
Recovery %							

[a] The calculation is based on the Reproducibility relative standard deviation RSD_R, in %.

Table A.31 — Precision data for the blind duplicates obtained by the interlaboratory study A.4

PSP toxin	dcGTX2,3		C1,2		dcSTX		GTX5 (B1)		dcNEO		GTX6		Total toxicity [b]	
Sample [a]	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8	CRL/O8/P/1 and 7	CRL/O8/P/3 and 8
Year	2008	2008	2008	2008	2008	2008	2008	2008	2008	2008	2008	2008	2008	2008
Number of laboratories	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Laboratories without outliers	10	8	9	10	9	9	10	10	8	7	8	8	10	10
Number of outliers (laboratories)	1	3	2	1	2	2	1	1	3	4	3	3	1	1
Accepted results	10	8	9	10	9	9	10	10	8	7	8	8	10	10
$\overline{x}$, µmol/kg	2,3	2,3	15,2	20,2	4,1	3,9	14,0	9,5	4,0	4,1	13,0	18,1	2976,8	3120,6
s_r, µmol/kg	0,70	0,36	3,25	2,98	0,61	0,31	1,46	0,65	0,93	1,36	1,36	2,74	395,4	631,2
RSD_r, %	30,9	15,7	21,4	14,9	15,0	7,99	10,5	6,82	23,5	32,9	10,4	15,1	13,3	20,2
r [$r = 2{,}8 \times s_r$], µmol/kg	1,96	1,02	9,11	8,35	1,71	0,88	4,10	1,81	2,60	3,81	3,81	7,67	1107,1	1767,2
s_R, µmol/kg	0,86	0,75	2,91	4,65	0,70	0,65	2,37	2,01	2,04	1,79	4,46	6,66	1456,6	1736,3
RSD_R, %	38,2	32,4	19,1	23,3	17,1	16,7	16,9	21,1	51,4	43,4	34,2	36,8	48,9	55,6
R [$R = 2{,}8 \times s_R$], µmol/kg	2,42	2,10	8,14	13,02	1,95	1,83	6,63	5,62	5,70	5,02	12,49	18,65	4078,6	4861,6

[a] Souce: [8]

[b] the unit of the total toxicity is given in µg STX 2HCl eq/kg

Bibliography

[1] AOAC Official Method 2005.06. Paralytic Shellfish Poisoning Toxins in Shellfish. Prechromatographic Oxidation and Liquid Chromatography with Fluorescence". First Action 2005 (2005) Official Methods of Analysis of AOAC International. 19

[2] TURNER A.D., HATFIELD R.G., RAPKOVA-DHANJI M., NORTON D.M., ALGOET M. and LEES D.N. (2010) "Single-laboratory validation of a refined AOAC HPLC method 2005.06 for oysters. cockles. and clams in U.K. shellfish". J AOAC Int 93. 1482-1493

[3] TURNER A.D., HATFIELD R.G (2012). "Refinement of AOAC *Official Method*SM 2005.06 Liquid Chromatography-Fluorescence Detection Method to Improve Performance Characteristics for the Determination of Paralytic Shellfish Toxins in King and Queen Scallops J AOAC Int. 95(1):129-142

[4] LAWRENCE J.F., NIEDZWIADEK B., MENARD C. (2004) „Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: interlaboratory study". J AOAC Int. 87. No 1. 83-100

[5] LAWRENCE J.F., NIEDZWIADEK B., MENARD C. (2005) „Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study". J AOAC Int. 88. No 6. 1714-1732

[6] BEN-GIGIREY B., RODRÍGUEZ-VELASCO M.L., VILLAR-GONZÁLES A., BOTANA L.M. (2007) "Influence of the sample toxic profile on the suitability of a high performance liquid chromatography method for official paralytic shellfish toxins control". J Chromatgr. A. 1140. 78–87

[7] COMMUNITY REFERENCE LABORATORY FOR MARINE BIOTOXINS (CRLMB). (2007) "Report on the Study on the Determination of PSP Toxins in Shellfish including GTX6 after Hydrolysis (AOAC Official Method 2005.06)". http://aesan.msssi.gob.es/CRLMB/docs/docs/ayuda_cientifica/PSP-Hydrolysis_study-2007-Report.pdf

[8] BEN-GIGIREY B., RODRÍGUEZ-VELASCO M.L., GAGO-MARTINEZ A. (2012) "Extension of the validation of AOAC Official Method 2005.06 for dcGTX2.3: interlaboratory study". J AOAC Int 95. 111-121

[9] COMMUNITY REFERENCE LABORATORY FOR MARINE BIOTOXINS (CRLMB). (2009) "Report on the CRLMB 2009 proficiency testing on saxitoxin group (PSP) toxin determination, available at http://aesan.msssi.gob.es/CRLMB/docs/docs/laboratorio_nacional_referencia/actividades/intercomparativo/intercomparative_CRLMB-ProficiencyTesting-2009-PSP-REPORT_LNR.pdf

[10] BEN GIGIREY B., TURNER A., GAGO-MARTÍNEZ A. Instrumental Methods for Paralytic Shellfish Toxins. In: Toxinology. Marine and Freshwater Toxins, edited by P. Gopalakrishnakone, Editorial: Springer (ISBN 978-94-007-6419-4)

[11] TURNER A.D., NORTON D.M., HATFIELD R.G., MORRIS S., REESE A.R., ALGOET M. and LEES D.N. (2009) "Refinement and extension of AOAC Method 2005.06 to include additional toxins in mussels: single-laboratory validation". J AOAC Int 92. 190-207

[12] COMMUNITY REFERENCE LABORATORY FOR MARINE BIOTOXINS (CRLMB). (2009) "Practical aspects of the implementation of 2005.06 AOAC Official Method for the Official Control of PSP toxins: technical issues and pitfalls",

http://aesan.msssi.gob.es/CRLMB/docs/docs/ayuda_cientifica/CRLMB-WG_PSP-HPLC_Working_Document_Technical_issues_and_pitfalls_AOAC_2005_06.pdf

[13] OSHIMA Y. (1995). "Post column derivatization liquid chromatographic method for paralytic shellfish toxins". Journal of AOAC International. 78. No 2. 528-532; some errors in the original data values have been corrected (Y. Oshima. personal communication)

[14] EFSA - SCIENTIFIC OPINION OF THE PANEL ON CONTAMINANTS IN THE FOOD CHAIN. Marine biotoxins in shellfish – Saxitoxin group. *The EFSA Journal.* 2009, **1019** pp. 1–76

[15] AOAC Guidelines for collaborative study procedures to validate characteristics of a method analysis (appendix D). 2002. AOAC International

This page deliberately left blank

This page deliberately left blank

British Standards Institution (BSI)

BSI is the national body responsible for preparing British Standards and other standards-related publications, information and services.

BSI is incorporated by Royal Charter. British Standards and other standardization products are published by BSI Standards Limited.

About us

We bring together business, industry, government, consumers, innovators and others to shape their combined experience and expertise into standards -based solutions.

The knowledge embodied in our standards has been carefully assembled in a dependable format and refined through our open consultation process. Organizations of all sizes and across all sectors choose standards to help them achieve their goals.

Information on standards

We can provide you with the knowledge that your organization needs to succeed. Find out more about British Standards by visiting our website at bsigroup.com/standards or contacting our Customer Services team or Knowledge Centre.

Buying standards

You can buy and download PDF versions of BSI publications, including British and adopted European and international standards, through our website at bsigroup.com/shop, where hard copies can also be purchased.

If you need international and foreign standards from other Standards Development Organizations, hard copies can be ordered from our Customer Services team.

Copyright in BSI publications

All the content in BSI publications, including British Standards, is the property of and copyrighted by BSI or some person or entity that owns copyright in the information used (such as the international standardization bodies) and has formally licensed such information to BSI for commercial publication and use.

Save for the provisions below, you may not transfer, share or disseminate any portion of the standard to any other person. You may not adapt, distribute, commercially exploit, or publicly display the standard or any portion thereof in any manner whatsoever without BSI's prior written consent.

Storing and using standards

Standards purchased in soft copy format:

- A British Standard purchased in soft copy format is licensed to a sole named user for personal or internal company use only.
- The standard may be stored on more than 1 device provided that it is accessible by the sole named user only and that only 1 copy is accessed at any one time.
- A single paper copy may be printed for personal or internal company use only.

Standards purchased in hard copy format:

- A British Standard purchased in hard copy format is for personal or internal company use only.
- It may not be further reproduced – in any format – to create an additional copy. This includes scanning of the document.

If you need more than 1 copy of the document, or if you wish to share the document on an internal network, you can save money by choosing a subscription product (see 'Subscriptions').

Reproducing extracts

For permission to reproduce content from BSI publications contact the BSI Copyright & Licensing team.

Subscriptions

Our range of subscription services are designed to make using standards easier for you. For further information on our subscription products go to bsigroup.com/subscriptions.

With **British Standards Online (BSOL)** you'll have instant access to over 55,000 British and adopted European and international standards from your desktop. It's available 24/7 and is refreshed daily so you'll always be up to date.

You can keep in touch with standards developments and receive substantial discounts on the purchase price of standards, both in single copy and subscription format, by becoming a **BSI Subscribing Member**.

PLUS is an updating service exclusive to BSI Subscribing Members. You will automatically receive the latest hard copy of your standards when they're revised or replaced.

To find out more about becoming a BSI Subscribing Member and the benefits of membership, please visit bsigroup.com/shop.

With a **Multi-User Network Licence (MUNL)** you are able to host standards publications on your intranet. Licences can cover as few or as many users as you wish. With updates supplied as soon as they're available, you can be sure your documentation is current. For further information, email subscriptions@bsigroup.com.

Revisions

Our British Standards and other publications are updated by amendment or revision.

We continually improve the quality of our products and services to benefit your business. If you find an inaccuracy or ambiguity within a British Standard or other BSI publication please inform the Knowledge Centre.

Useful Contacts

Customer Services
Tel: +44 345 086 9001
Email (orders): orders@bsigroup.com
Email (enquiries): cservices@bsigroup.com

Subscriptions
Tel: +44 345 086 9001
Email: subscriptions@bsigroup.com

Knowledge Centre
Tel: +44 20 8996 7004
Email: knowledgecentre@bsigroup.com

Copyright & Licensing
Tel: +44 20 8996 7070
Email: copyright@bsigroup.com

BSI Group Headquarters

389 Chiswick High Road London W4 4AL UK

bsi.