SLOVENSKI STANDARD
oSIST prEN 14526:2025

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Živila - Določevanje toksinov iz skupine saksitoksina v školjkah - Metoda HPLC z uporabo predkolonske derivatizacije s peroksidno ali perjodatno oksidacijo

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

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

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

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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

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European foreword

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

This document is currently submitted to the CEN Enquiry.

This document will supersede EN 14526:2017.

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

— new Figure 2 detailing a procedure for a large numbers of test samples added;

— mandatory Clause 3 Terms and definitions added;

— new Clause 12 specifying quality controls added;

— new Annex B with example chromatograms added.

Introduction

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

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1 Scope

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

Table 1 — Lowest validated levels

Toxin		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) [5]		27[c]	0,07[c]
decarbamoyl saxitoxin (dcSTX) [5]		8[c]	0,03[c]
neosaxitoxin (NEO) [5]		33[c]	0,10[c]
gonyautoxin 1,4 (GTX1,4) [5]		61,4[c]	0,15[c]
N-sulfocarbamoyl gonyautoxin 2,3 (C1,2) [5]		93[c]	0,20[c]
N-sulfocarbamoyl gonyautoxin 1,4 (C3,4) [5]		725[b]	1,48[b]
gonyautoxin 6 (GTX6)	direct [4]	30	0,08
	indirect [9]	834[b]	2,11[b]
decarbamoyl gonyautoxin 2,3 (dcGTX2,3) [8]		271[a]	0,77[a]
decarbamoyl neosaxitoxin (dcNEO) [8]		594[b]	2,18[b]

a lowest spiked level; mean recovery: 58 % [8]

b lowest concentration tested

c lowest concentration tested with a HorRat < 2 [4], [5]

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

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

2 Normative references

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

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

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

3 Terms and definitions

No terms and definitions are listed in this document.

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

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

4 Principle

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

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

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

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

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

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

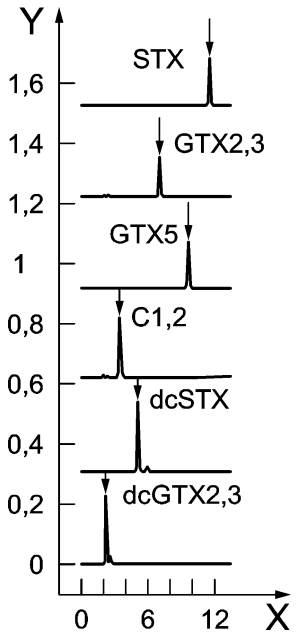
Table 2 — Reaction products after oxidation with periodate and peroxide

Toxin	Oxidation products and HPLC-eluting order		Intensity		Oxidation product at the same retention time as	
	peroxide	periodate	peroxide	periodate	peroxide	periodate
STX	one	one	++	+	NEO[a] (3)[b]	NEO (3); GTX6 (3)
dcSTX	first (1)	first (1)	++	-		dcNEO (1)
	second (2)	second (2)	+	+	NEO[a] (2)	NEO (2); GTX6 (2); dcNEO (2)
NEO	no	first (1)	—	+		GTX6 (1)
	second (2)	second (2)	-	++	dcSTX (2)	GTX6 (2); dcSTX (2); dcNEO (2)
	third (3)	third (3)	-	+	STX	STX; GTX6 (3)
C1,2	one	one	++	+		
C3,4	no	first (1)	—	+		GTX1,4 (1)
	no	second (2)	—	++		GTX1,4 (2); dcGTX2,3 (2)
	no	third (3)	—	+		GTX1,4 (3); GTX2,3
GTX1,4	no	first (1)	—	+		C3,4 (1)
	no	second (2)	—	++		C3,4 (2); dcGTX2,3 (2)
	third (3)	third (3)	-	++	GTX2,3	C3,4 (3); GTX2,3
GTX2,3	one	one	++	++	GTX1,4[a] (3)	C3,4 (3); GTX1,4 (3)
GTX5	one	one	++	-		
GTX6	no	first (1)	—	+		NEO (1)
	no	second (2)	—	++		NEO (2); dcSTX (2); dcNEO (2)
	no	third (3)	—	-		NEO (3); STX
dcGTX2,3	first (1)	first (1)	++	+		
	second (2)	second (2)	+	++		C3,4 (2); GTX1,4 (2)
dcNEO	first (1)	first (1)	-	++		dcSTX (1)
	second (2)	second (2)	-	+	dcSTX (2)	dcSTX (2); NEO (2); GTX6 (2)

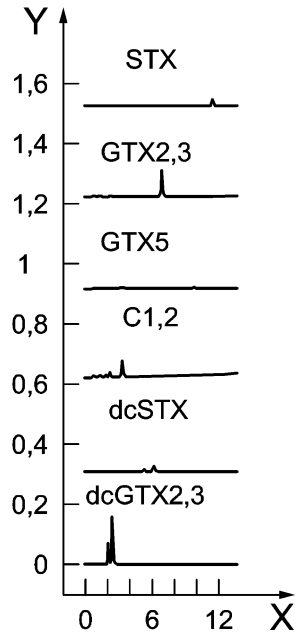
Intensity: — not visible
- very low
+ low
++ high

[a] High concentration of the indicated toxin may influence the quantification by simulating an increased content.

[b] Numbers in curly brackets are the elution order.

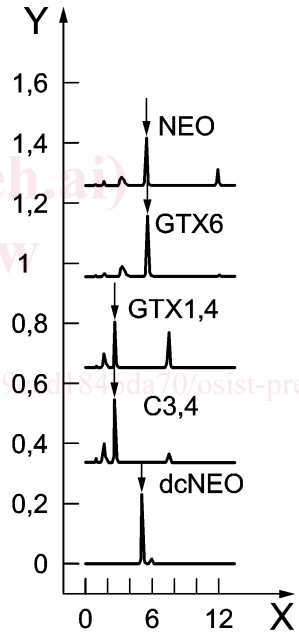


a) Non N1-hydroxylated toxins: peroxide



b) Non N1-hydroxylated toxins: periodate

c) N1-hydroxylated toxins: peroxide



d) N1-hydroxylated toxins: periodate

Key

X detection response (V)

Y time (min)

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

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

5 Reagents

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

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

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

5.1 Methanol, HPLC quality.

5.2 Acetonitrile, HPLC quality.

5.3 Ammonium formate:

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

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

5.4 Glacial acetic acid:

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

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

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

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

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

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

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

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

5.5 Ammonium acetate:

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

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

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

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

5.6 Sodium chloride:

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

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

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

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

5.7 Hydrochloric acid, c = 1 mol/l.

5.8 Disodium hydrogenphosphate or disodium hydrogenphosphate 7-hydrate:

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

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

5.9 Sodium hydroxide:

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

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

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

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

5.10 Hydrogen peroxide, w = 30 %:

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

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

5.11 Periodic acid:

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

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

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

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

5.12 Periodate oxidation reagent.

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

5.13 PSP toxin standard substances:

5.13.1 PSP toxin stock solutions.

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

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

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

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

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

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

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