SLOVENSKI STANDARD
SIST ENV 14194:2002

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Živila - Določevanje saksitoksina in dc-saksitoksina v školjkah - Metoda HPLC z uporabo postkolonske derivatizacije

Foodstuffs - Determination of saxitoxin and dc-saxitoxin in mussels - HPLC method using post column derivatisation

Lebensmittel - Bestimmung von Saxitoxin und DC-Saxitoxin in Muscheln - HPLC-Verfahren mit Nachsäulenderivatisierung

Produits alimentaires - Détermination de la teneur en saxitoxine et en dc-saxitoxine dans les moules - Méthode par chromatographie liquide haute performance apres dérivation post-colonne

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English version

Foodstuffs - Determination of saxitoxin and dc-saxitoxin in mussels - HPLC method using post column derivatisation

Produits alimentaires - Détermination de la teneur en saxitoxine et en dc-saxitoxine dans les moules - Méthode par chromatographie liquide haute performance après dérivation post-colonne

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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Foreword

This document (ENV 14194:2002) has been prepared by Technical Committee CEN /TC 275, "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this European Prestandard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Prestandard specifies a method for the quantitative determination of saxitoxin (STX), dc-saxitoxin (dc-STX) and the qualitative determination of neo-saxitoxin, and the gonyautoxins GTX-2 and GTX-3 in mussels. The method can also be used to identify the N-sulfocarbamoyl toxins C-1, C-2, GTX-5 and GTX-6 after hydrolysis and, if these toxins are present, to exclude false positive results for GTX-2, GTX-3, neo-saxitoxin and saxitoxin. For mussel the limit of quantification is for saxitoxin 0,04 mg/kg mussel meat and for dc-saxitoxin 0,03 mg/kg mussel meat (signal/noise = 10).

The limits of detection for C-1, C-2, GTX-2, GTX-3, GTX-5, GTX-6 and neo-saxitoxin have not been determined.

2 Normative references

This European Prestandard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Prestandard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

EN ISO 3696:1995, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*

3 Principle

PSP (Paralytic Shellfish Poisoning) toxins are extracted from mussels homogenate with an acidic aqueous solution. After centrifugation the supernatant is purified by solid phase extraction (SPE) over a C18 clean-up cartridge. Part of the extract is directly injected on the HPLC for toxin determination. After the separation on the analytical column post column derivatisation is used, with oxidation with periodic acid and after acidification the derivatized toxins are detected using fluorimetric detection. HPLC analysis is separated in two parts: a system to separate the saxitoxin group toxins (saxitoxin, dc-saxitoxin and neo-saxitoxin) and a system to separate the GTX group toxins (GTX-2 and GTX-3). Identification after hydrolysis is based on the transformation of the N-sulfo-carbamoyl toxins to their corresponding carbamoyl toxins.

WARNING - PSP toxins are strong neurotoxins. Gloves and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard.

4 Reagents

4.1 General

During the analysis, unless otherwise stated, use only water according to grade 1 of EN ISO 3696:1995.

All chemicals shall be of pro analysis (p.a) quality, unless otherwise indicated.

Reference materials (calibrants of the toxins) originating from other sources than indicated may also be used if well-characterised and with a well-defined mass concentration.

4.2 Methanol

4.3 Acetonitrile, HPLC quality

4.4 Hydrochloric acid, volume concentration $\varphi(HCl) \approx 25$ %, (acidimetric)

4.4.1 Hydrochloric acid solution, substance concentration $c = 1{,}0$ mol/l

Dilute 130 ml of hydrochloric acid solution (4.4) with 870 ml of water.

4.4.2 Hydrochloric acid solution, $c = 0{,}1$ mol/l

Dilute 100 ml of hydrochloric acid solution (4.4.1) with 900 ml of water.

4.5 Octane sulfonic acid solution, $c = 0{,}5$ mol/l

Dissolve 2,9 g of octane sulfonic acid sodium salt monohydrate in 25,0 ml of water.

4.6 Phosphoric acid, the mass fraction is 85 %

4.6.1 Phosphoric acid solution, $c = 0{,}5$ mol/l

Take 6,7 ml of phosphoric acid (4.6) and dilute to 200,0 ml with water.

4.7 Ammonia solution, $\varphi = 25$ % and $\varphi = 1$ %

For the 1 % solution, dilute 40 ml ammonia solution $\varphi = 25$ % with 960 ml of water.

4.8 Periodic acid solution, $c = 0{,}01$ mol/l

Dissolve 1,14 g of periodic acid in water and dilute up to 500 ml with water.

4.9 Acetic acid, 100 %

4.9.1 Acetic acid solution 1, $c = 1$ mol/l

Take 57,2 ml of acetic acid solution (4.9) and dilute to 1,0 l with water.

4.9.2 Acetic acid solution 2, $c = 0{,}2$ mol/l

Dilute 200 ml of acetic acid solution 1 (4.9.1) with 800 ml of water.

4.9.3 Acetic acid solution 3, $c = 0{,}03$ mol/l

Dilute 150 ml of acetic acid solution 2 (4.9.2) with 850 ml of water.

4.10 Helium

4.11 Saxitoxin standard solutions

4.11.1 Saxitoxin stock solution, mass concentration ρ = 1,0 µg/ml

Prepare a calibration solution containing 1,0 µg/ml of saxitoxin in acetic acid solution 3 (4.9.3). Store the calibration solution cool (at approximately + 4 °C) and in the dark. This solution is stable for at least 6 months.

NOTE 1 An ampoule containing approximately 0,2 ml of saxitoxin calibration solution, mass concentration of saxitoxin of 0,14 mg/ml is for example available from the National Research Council Canada, Halifax, Canada (set of calibrants PSP-1). In order to obtain a calibration solution of 1,0 µg/ml, the content of the ampoule can be quantitatively transferred to 27,8 ml acetic acid 3 (4.9.3) (total volume 28,0 ml). The total volume can be checked and adjusted by weighing (5.9), using density of water for calculations. For this purpose weigh the ampoule before opening, take care that all glass is preserved after opening and weigh again after the calibrant solution is transferred.

NOTE 2 A calibration solution containing GTX-1 and GTX-4 is also available and may be used for qualitatively determination of these toxins. This has not been tested in the certification study of the Standard Measurements & Testing Programme, designed to certify the mass fractions of saxitoxin and dc-saxitoxin in mussel reference materials, see [6].

4.11.2 Saxitoxin calibration solutions, ρ = 0,05 µg/ml to 0,30 µg/ml

Prepare for the determination of saxitoxin a calibration series of increasing concentration in the mass concentration range of 0,05 µg/ml to 0,30 µg/ml from saxitoxin stock solution (4.11.1) in acetic acid solution 3 (4.9.3). Prepare fresh every day of analysis.

4.12 Dc-saxitoxin standard substance

Store cool and in the dark.

4.13 Dc-saxitoxin stock solution, ρ = 1,0 µg/ml

Prepare from dc-saxitoxin standard substance (4.12) a dc-saxitoxin calibration solution 1,0 µg/ml in acetic acid solution 3 (4.9.3) by weighing (5.9), using density of water for calculations. This solution is stable for at least 5 months (see Bibliography, [3]).

4.14 Dc-saxitoxin calibration solution, ρ = 0,05 µg/ml to 0,30 µg/ml

Prepare for the determination of dc-saxitoxin a calibration series of increasing concentration in the mass concentration range of 0,05 µg/ml to 0,30 µg/ml from Dc-saxitoxin stock solution in acetic acid solution 3 (4.9.3). Prepare fresh every day of analysis.

NOTE For the quantitative determination of saxitoxin and dc-saxitoxin a combined calibration solution for these 2 toxins can be made if the valley between the 2 toxin peaks in the HPLC chromatogram is ≤ 10% of the sum of the peak heights. If this condition is fulfilled in the HPLC separation, one combined calibration solution containing saxitoxin and dc-saxitoxin can be prepared in the mass concentration range of 0,05 µg/ml to 0,30 µg/ml (for both toxins) in acetic acid solution 3 (4.9.3).

4.15 Neo-Saxitoxin standard solutions

4.15.1 Neo-saxitoxin calibration solution, ρ = 2,0 µg/ml

Prepare a calibration solution containing 2,0 µg/ml of neo-saxitoxin in acetic acid solution 3 (4.9.3). Store the calibration solution cool (at approximately + 4 °C) and in the dark. This solution is stable for at least 6 months.

NOTE 1 An ampoule containing 0,2 ml neo-saxitoxin calibration solution, mass concentration 0,14 mg/ml is for example available from the National Research Council Canada, Halifax, Canada (set of calibrants PSP-1). In order to obtain a calibration solution of 2,0 µg/ml, the content of the ampoule can be quantitatively transferred to 13,8 ml acetic acid solution 3 (4.9.3) (total volume 14,0 ml). The total volume can be checked and adjusted by weighing (5.9), using density of water for calculations.

NOTE 2 A calibration solution containing GTX-1 and GTX-4 is also available and may be used for qualitatively determination of these toxins. This has not been tested in the certification study of the Standard Measurements & Testing Programme, designed to certify the mass fractions of saxitoxin and dc-saxitoxin in mussel reference materials, see [6].

4.15.2 Neo-saxitoxin calibration solution for qualitative analyses, ρ = 1,0 µg/ml

Add 50 µl of Neo-saxitoxin calibration solution (4.15.1) to 50 µl of acetic acid solution 3 (4.9.3). Prepare fresh every day of analysis.

4.16 GTX-2/GTX-3 standard solutions

4.16.1 GTX-2/GTX-3 calibration solution, ρ = 2,0 µg/ml and 0,5 µg/ml respectively

Prepare a calibration solution containing 2,0 µg/ml of GTX-2 and 0,5 µg/ml of GTX-3 in acetic acid solution 3 (4.9.3). Store the calibration solution cool (at approximately + 4 °C) and in the dark. This solution is stable for at least 6 months.

NOTE 1 An ampoule containing approximately 0,2 ml GTX-2 (0,12 mg/ml) and GTX-3 (0,029 mg/ml) standard substance is for example available from the National Research Council Canada, Halifax, Canada (set of calibrants PSP-1). In order to obtain a calibration solution of 2,0 µg/ml of GTX-2 and of 0,5 µg/ml of GTX-3, the content of the ampoule can be quantitatively transferred to 11,8 ml acetic acid solution 3 (4.9.3) (total volume 12,0 ml). The total volume can be checked and adjusted by weighing (5.9), using density of water for calculations. For this purpose weigh the ampoule before opening, take care that all glass is preserved after opening and weigh again after the calibrant solution is transferred.

NOTE 2 A calibration solution containing GTX-1 and GTX-4 is also available and may be used for qualitatively determination of these toxins. This has not been tested in the certification study of the Standard Measurements & Testing Programme, designed to certify the mass fractions of saxitoxin and dc-saxitoxin in mussel reference materials, see [6].

4.16.2 GTX-2/GTX-3 calibration solution for qualitative analysis, GTX-2 (0,1 µg/ml) and GTX-3 (0,024 µg/ml)

Add 50 µl of GTX-2/GTX-3 calibration solution (4.16.1) to 950 µl of acetic acid solution 3 (4.9.3). Prepare fresh every day of analysis.

4.17 HPLC eluent A for the STX group

To 6 ml of octane sulfonic acid solution (4.5), add 20 ml of phosphoric acid solution (4.6.1). Add water to a volume of 950 ml. Adjust to pH 7,2 with 25 % ammonia solution (4.7), dilute to 1,0 l with water and filter (5.8).

To 920 ml of this solution, add 80 ml of acetonitrile (4.3). Degas the eluent before use with helium purging (4.10) for approximately 10 min. This solution is stable for 2 weeks.

4.18 HPLC eluent B for the GTX group

To 3 ml of octane sulfonic acid solution (4.5), add 20 ml of phosphoric acid solution (4.6.1). Add water to a volume of 950 ml. Adjust to pH 7,0 with 25 % ammonia solution (4.7), dilute to 1,0 l with water and filter (5.8). Degas the eluent before use with helium purging (4.10) for approximately 10 min. This solution is stable for 3 days.

4.19 Post column derivatisation reagent

Add 50 ml of periodic acid solution (4.8) to 50 ml of 1 % ammonia solution (4.7). Prepare fresh every day of analysis.

4.20 Sodium acetate solution, c = 1,0 mol/l

Dissolve 13,6 g of sodium acetate trihydrate in 100,0 ml water.

5 Apparatus

Use usual laboratory apparatus and, in particular, the following.

5.1 Grinder

5.2 Vacuum manifold for SPE columns

5.3 C18 SPE columns, 1 ml, 100 mg sorbent

5.4 Adjustable automatic pipettes, 20 µl, 100 µl and 1000 µl

5.5 HPLC vials, 1 ml, with caps and septum

5.6 HPLC apparatus, see also figure 1 for system configuration

5.6.1 Injector

5.6.2 Detector, e.g. fluorescence detector with dual monochromator

5.6.3 Integrator

5.6.4 HPLC pump

5.6.5 For saxitoxin group: analytical column, for example C18 ODS Hypersil 5 µm (100 mm x 3 mm)

5.6.6 For GTX toxin group: analytical column, for example C18 ODS Hypersil 5 µm (200 mm x 3 mm)

5.6.7 Post column derivatization system with the components:

5.6.7.1 Pump for adding postcolumn derivatisation reagents with 2 channels

5.6.7.2 Postcolumn reaction coil (with oven), reaction coil length approximately 1 m, volume 1 ml

5.7 Membrane filter, for example pore size of 0,45 mm, diameter 47 mm

5.8 Filter unit to filter HPLC eluents, glass filter holder, for example diameter 47 mm, Borosilicate glass funnel with provision for filter inlay ; anodised aluminium spring clamp in combination with a filtering flask of 1 l.

5.9 Analytical balance

6 Procedure

6.1 Sample preparation

Let mussels thaw if necessary. Rinse shell with tap-water once to remove sand and let the mussels drain to remove excess of water. Remove the meat from the shells. Homogenize approximately 100 g of the mussel meat in a grinder (5.1). Freeze the homogenised mussels if not directly proceeding with the analysis.

6.2 Extraction procedure

Weigh a test portion of 25 g to the nearest 100 mg of the thawed homogeneous mussel mixture in a beaker. Add 25 ml of acetic acid solution 2 (4.9.2) to the beaker. Mix 10 min under constant stirring with a magnetic stirrer. Filter the total mixture of a fluted filter paper. Check the pH with pH paper. If the pH of the mixture is not between pH = 3 and pH = 5 dropwise add hydrochloric acid solution (4.4.1) until it is.

If not proceeding directly with the analysis, the extract may be stored cool (approximately + 4 °C) and in the dark for a maximum of 5 days (risk of fungal growth in extract).

NOTE If stored longer than 5 days, there is a risk of fungal growth in the extract.

6.3 Sample purification

Place an SPE-column (5.3) on a vacuum manifold system (5.2) and apply a mild vacuum.

Condition the column (5.3) successively with: 3 ml of methanol (4.2), 3 ml of water and 1,5 ml of hydrochloric acid solution (4.4.2). Ensure that the column does not run dry during these operations.

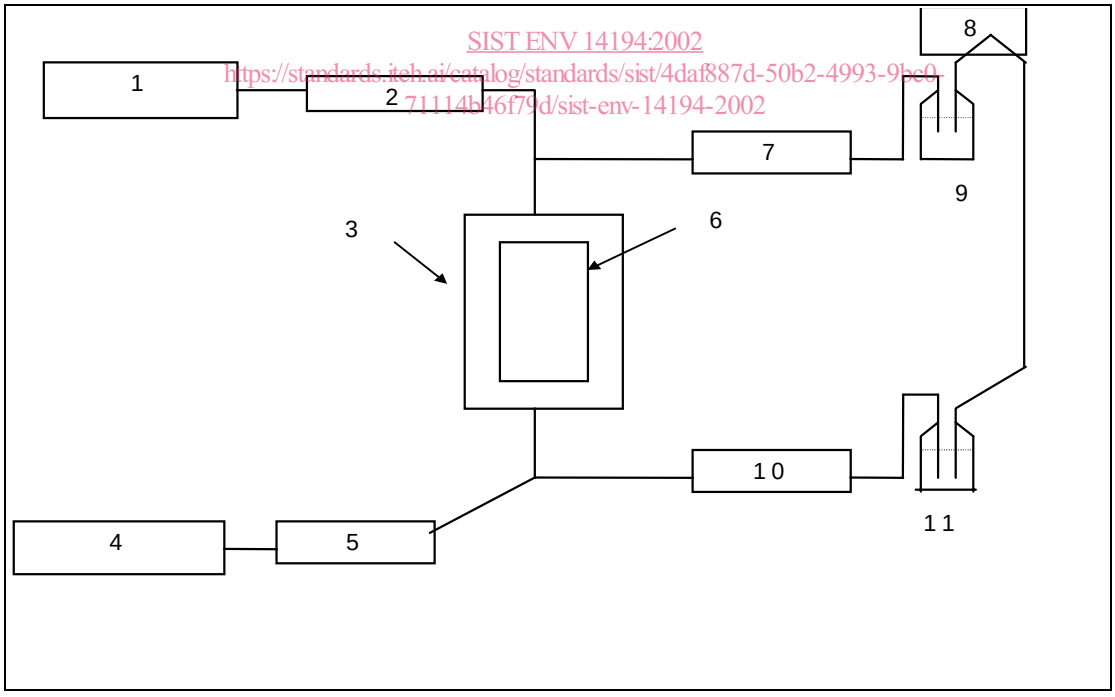
Remove vacuum and place a collection tube under the column and apply vacuum. Apply 0,5 ml (V_c) of the extract on the column. Let the extract be absorbed on the column and collect the effluent in a collection tube and take care that the column does not run dry during these steps.

Rinse the column with 2,0 ml of water and combine the eluate with the effluent in the collection tube. After this step let the column run dry to remove all the liquid from the column. Remove vacuum and remove the collection tube containing the purified extract from the vacuum manifold system. Take the combined fractions and dilute to 3,0 ml with water and mix well (V_e). Transfer approximately 0,5 ml of the extract to an HPLC vial for analysis. This is the sample test solution.

7 HPLC

7.1 HPLC conditions

The HPLC conditions are different for the two groups of toxins. For a schematic overview of the system, see figure 1, [4].



Key

1	HPLC pump	5	Detector	9	Oxidant
2	Column	6	Reaction coil	10	Pump B
3	Block heater	7	Pump A	11	Acid
4	Data system	8	Helium		

Figure 1 — Configuration of HPLC system