SLOVENSKI STANDARD
SIST EN 14526:2017

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Nadomešča:
SIST EN 14526:2005

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 - Dosage 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

Ta slovenski standard je istoveten z: **EN 14526:2017**

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.120.30	Ribe in ribji proizvodi	Fish and fishery products

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EUROPEAN STANDARD

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

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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.

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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of an identical text or by endorsement, at the latest by July 2017, and conflicting national standards shall be withdrawn at the latest by July 2017.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].

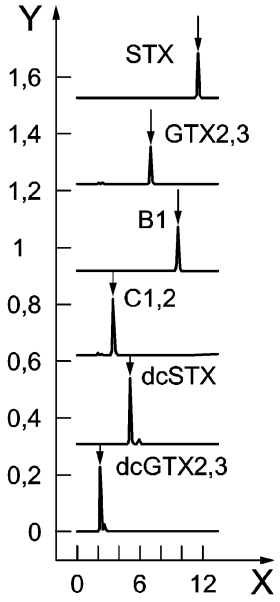
Table 2 — Reaction products after oxidation with periodate and peroxide

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}

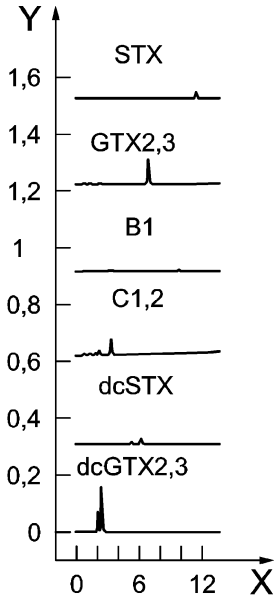
Intensity: — not visible
- very low
+ low
++ high

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

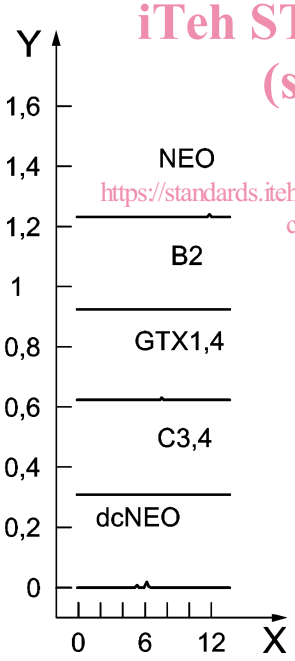
[b] Numbers in curly brackets are the elution order.



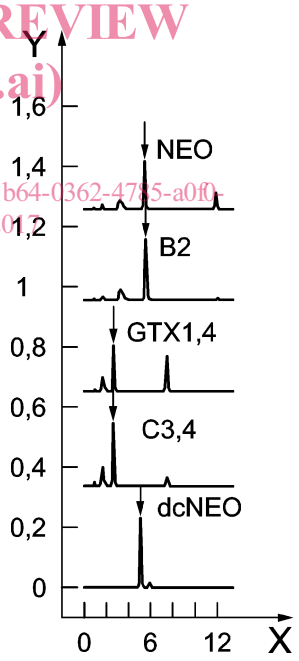
a) Non-hydroxylated toxins: peroxide



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.

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