SLOVENSKI STANDARD
SIST EN 16618:2015

01-julij-2015

Analize živil - Določevanje akrilamida v živilih s tekočinsko kromatografijo s tandemsko masno spektrometrijo (LC-ESI-MS-MS)

Food analysis - Determination of acrylamide in food by liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS)

Lebensmittelanalytik - Bestimmung von Acrylamid in Lebensmitteln mit Flüssigkeitschromatographie und Tandem-Massenspektrometrie (LC-ESI-MS/MS)

Analyse des produits alimentaires - Dosage de l'acrylamide dans les produits alimentaires par chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (CL-ESI-SM-SM)

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

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Food analysis - Determination of acrylamide in food by liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS)

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Lebensmittelanalytik - Bestimmung von Acrylamid in Lebensmitteln mit Flüssigchromatographie und Tandem-Massenspektrometrie (LC-ESI-MS/MS)

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Foreword

This document (EN 16618:2015) 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of an identical text or by endorsement, at the latest by October 2015 and conflicting national standards shall be withdrawn at the latest by October 2015.

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1 Scope

This European Standard specifies a method for the determination of acrylamide in bakery ware such as bread, toasted bread, crisp bread, butter cookies, and biscuits, as well as potato products such as potato chips, potato crisps, and potato pan cake and roasted coffee, by liquid chromatography in combination with electrospray ionization and tandem mass spectrometry (LC-ESI-MS/MS). This method has been validated in an interlaboratory study via the analysis of both naturally contaminated and spiked samples, ranging from 14,3 µg/kg to 9 083 µg/kg. It was developed at the Swedish National Food Administration and validated in a study organized by the Directorate General Joint Research Centre (DG JRC), Swedish National Food Administration and the Nordic Committee on Food Analysis (NMKL), see [1] and [2].

The limit of quantification (LOQ) depends on the type of instrument used and on the actual performance of the instrument. The majority of the laboratories participating in the validation study were able to determine acrylamide in a butter cookie sample at a level of 14,3 µg/kg. Thus, the validation by interlaboratory study showed that LOQ can be expected to be in the range between below 15 µg/kg and 30 µg/kg.

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 1042:1999, *Laboratory glassware - One-mark volumetric flasks (ISO 1042:1998)*

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

3 Principle

Acrylamide is extracted with water and isotopic labelled acrylamide is added. The extract is centrifuged and the supernatant is cleaned up with two solid phase extraction (SPE) columns. The first SPE column contains silica based C18 groups as well as anion and cation exchangers, and since acrylamide is not retained by the column, the extract is just passed and collected. The reason for using this column is to retain as many matrix components as possible (non-polar compounds as well as anions and cations) without retaining acrylamide, i.e. this first SPE column is used as a chemical filter.

The second SPE column contains a polymer based phase with a relatively high capacity to bind acrylamide. The extract is loaded onto the column, the column is washed with water and finally eluted with a mixture of 60 parts per volume of methanol and 40 parts per volume of water. The purpose of this step, apart from further cleaning of the extract, is to concentrate the extract and to obtain low limits of quantification.

After evaporation of the methanol, the extract is analysed by LC-MS/MS. For this purpose an HPLC column with graphitized carbon as stationary phase is used, since the retention factor (k) is relatively high (k = 4 when no organic solvent is added in the mobile phase) compared to other commercially available columns.

4 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696:1995, unless otherwise specified. Solvents shall be of quality for HPLC analysis.

4.1 Acrylamide (CAS 79-06-1), purity not less than 99,9 % mass fraction.

The chemical structure is:

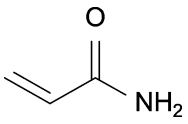

Figure 1 — Acrylamide

WARNING — Acrylamide has been classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans. Protective equipment as laboratory coat, disposable gloves and safety glasses shall be used. All handlings of acrylamide and organic solvents shall be performed in a fume cupboard with adequate air flow.

4.2 Deuterium-labelled acrylamide – acrylamide-2,3,3-D_3 (CAS 122775-19-3).

The chemical structure is:

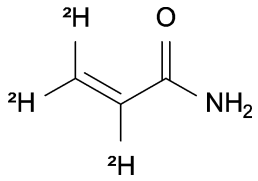

Figure 2 — Deuterium-labelled acrylamide

Alternatively, ^{13}C-labelled acrylamide (acrylamide-$^{13}C_3$, CAS 287399-26-2) may be used.

4.3 Methanol (CAS 67-56-1).

4.4 Glacial acetic acid (CAS 64-19-7).

4.5 *n*-Hexane (CAS 110-54-3).

Alternatively, cyclohexane (CAS 110-82-7) may be used.

4.6 Eluent for SPE column 2 (5.2.3)

Mix 60 parts per volume of methanol (4.3) with 40 parts per volume of water.

4.7 HPLC mobile phase

Mix 1 part per volume of glacial acetic acid (4.4) with 1 000 parts per volume of water.

4.8 Stock solutions of acrylamide and acrylamide-2,3,3-D3, mass concentration ρ = 1 000 µg/ml.

Weigh, to the nearest 0,05 mg, approximately 100 mg of acrylamide and acrylamide-2,3,3-D_3 respectively into separate 100 ml volumetric flasks, dissolve in water and dilute to 100 ml. Solutions can be stored at 4 °C for at least 3 months.

4.9 Internal standard solution 1, ρ = 10 µg/ml.

Transfer 1 000 µl of the stock solution of acrylamide-2,3,3-D_3 (4.8) to a 100 ml volumetric flask and dilute to the mark with water.

4.10 Internal standard solution 2, ρ = 1 000 ng/ml.

Transfer 5 000 µl of the internal standard solution 1 (4.9) to a 50 ml volumetric flask and dilute to the mark with water.

4.11 Acrylamide standard solution 1, ρ = 100 µg/ml.

Transfer 5 000 µl of the stock solution of acrylamide (4.8) to a 50 ml volumetric flask and dilute to the mark with water.

4.12 Acrylamide standard solution 2, ρ = 10 µg/ml.

Transfer 5 000 µl of the acrylamide standard solution 1 (4.11) to a 50 ml volumetric flask and dilute to the mark with water.

4.13 Acrylamide standard solution 3, ρ = 100 ng/ml.

Transfer 1 000 µl of the acrylamide standard solution 2 (4.12) to a 100 ml volumetric flask and dilute to the mark with water.

4.14 LC-MS calibration solutions

Dilute aliquots from standard solutions (4.9), (4.11), (4.12) and (4.13) with water to give calibration solutions of e.g. 0 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml, 500 ng/ml, 1 000 ng/ml, 2 000 ng/ml, 5 000 ng/ml and 10 000 ng/ml respectively of acrylamide, all containing 400 ng/ml of acrylamide-2,3,3-D_3. Examples for the preparation of calibration solutions are given in Table 1. Table 2 indicates the relation between calibration solution concentrations and acrylamide contents of food samples. Calibration shall be performed on at least six concentration levels distributed properly over the working range. The analysis of an even higher number of calibration solutions should be analysed if such a broad range of concentrations (0 µg/kg to 10 000 µg/kg) shall be covered.

Table 1 — Preparation of LC-MS calibration solutions

Calibration solution ng/ml	**Volumetric flask** ml	**Internal standard solution (4.9)** µl	**Acrylamide standard solution** µl
0	100	4 000	0
5	100	4 000	5 000 of (4.13)
10	100	4 000	10 000 of (4.13)
20	100	4 000	200 of (4.12)
50	100	4 000	500 of (4.12)
100	100	4 000	1 000 of (4.12)
250	100	4 000	2 500 of (4.12)
500	100	4 000	5 000 of (4.12)
1 000	100	4 000	1 000 of (4.11)
2 000	100	4 000	2 000 of (4.11)
5 000	100	4 000	5 000 of (4.11)
10 000	50	4 000	5 000 of (4.11)

Table 2 — Relation between acrylamide contents of calibration solutions and contents in food

Calibration solution ng/ml	Bakery and potato products µg/kg	Roasted coffee µg/kg
10	10	50

5 Apparatus

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

5.1 LC-MS/MS system

5.1.1 HPLC apparatus, comprising the following:

5.1.1.1 Thermostated column compartment.

5.1.1.2 Injection system, capable of injecting 10 µl of sample.

5.1.1.3 Mobile phase pump, capable of maintaining a mobile phase flow of 0,4 ml/min.

5.1.2 HPLC column

The stationary phase of the column is graphitized carbon[1a)], particle size 5 µm, 50 mm x 2,1 mm with a guard column[1a)], particle size 5 µm, 10 mm x 2 mm.

Alternative columns/stationary phases may be applied provided that similar performance to the graphitized carbon column can be demonstrated.

5.1.3 Mass spectrometer

Triple quadrupole mass spectrometer operating in positive electrospray and, selected reaction monitoring mode (SRM), set to obtain unit resolution.

5.1.4 Data acquisition and analysis system

Suitable data collection and evaluation software.

5.1.5 Divert valve (optional)

HPLC valve installed between HPLC column and mass spectrometer in order to direct the HPLC effluent either to waste or to the mass spectrometer, see 7.1.1.

5.2 Solid phase extraction system

5.2.1 Vacuum manifold for solid phase extraction

1) a) Hypercarb™ column, Thermo Hypersil-Keystone® column, b) ISOLUTE ® Multimode SPE column and c) ISOLUTE ® ENV+ SPE column from Biotage® are examples of suitable products available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

5.2.2 SPE column 1

Multimode SPE column comprising non-polar, strong anion exchange, and strong cation exchange properties[1b)], 1 000 mg/6 ml.

5.2.3 SPE column 2

Crosslinked polystyrene-divinylbenzene copolymer for the extraction of polar analytes from aqueous samples[1c)], 500 mg/6 ml.

5.3 Analytical balance, accuracy to the nearest 0,01 mg.

5.4 Laboratory balance, accuracy to the nearest 0,01 g.

5.5 Calibrated precision microlitre pipettes, of 200 µl to 1 000 µl, and of 1 000 µl to 5 000 µl capacity.

5.6 Centrifuge tubes, volume of 50 ml, polypropylene, disposable.

5.7 Mechanical shaker, e.g. wrist arm shaker, allowing well mixing of different phases, capable of holding 50 ml centrifuge tubes.

5.8 Vortex mixer.

5.9 Cooled centrifuge, capable of a centrifugal force of 3 600 *g* for 50 ml centrifuge tubes.

5.10 Volumetric flasks, volume of 50 ml, 100 ml, etc. according to EN ISO 1042:1999.

5.11 Glass vials, volume of at least 4 ml, suitable for the evaporation equipment.

5.12 Amber glass autosampler vials, suitable for the HPLC autosampler.

5.13 Evaporation equipment, based on vacuum or a stream of inert gas.

The evaporation temperature shall not exceed 40 °C.

6 Sample preparation

6.1 General

Residues of acrylamide have sometimes been found in laboratory ware as e.g. filters. Make sure the laboratory ware does not contain any measureable amounts of acrylamide, and include procedural blank samples as controls in each series of samples.

Acrylamide has been found to be formed as an artefact in some analytical procedures for acrylamide, e.g. during extraction or in the injection port of GC instruments. Even if this is not a problem for HPLC analysis, make sure to never exceed 40 °C during extraction or the work-up process.

It has been proven that acrylamide is efficiently extracted from various types of food by shaking with water if the particles of the samples are small enough. Make sure that the particles are < 1 mm before extraction and use, if necessary, a mechanical device for preparation of homogenous slurry[2)].

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2) Ultra Turrax® and Waring blender® are examples of suitable products available commercially. This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

Sample extracts can sometimes cause problems by e.g. clogging the SPE columns. The amount of extract loaded on the SPE columns can be reduced provided that the abundances of the peaks of both acrylamide and internal standard are large enough to comply with quantification criteria.

NOTE Complementary information regarding sample preparation and chromatographic separation of acrylamide is given by Petersson et al. [3] and Rosén et al. [1].

6.2 Extraction

6.2.1 Extraction procedure for bakery ware and potato product samples

Weigh, to the nearest 0,01 g, a 2,0 g test portion into a 50 ml centrifuge tube (5.6). Add 40 ml of water. Add 400 µl of internal standard solution 2, *c* = 1 000 ng/ml (4.10). Shake intensively for 15 s to 30 s by hand and 10 s to 15 s with a vortex mixer (5.8), and then for 60 min on a mechanical shaker (5.7) adjusted to maximum sample-extractant agitation. Centrifuge in a cooled centrifuge (5.9) at 10 °C, 3 600 x *g* for 20 min and take off 10 ml of the aqueous phase to a clean test tube. Avoid to transfer parts of the fat layer that will be formed and found on the top, depending of the fat content of the sample.

Take care that a homogenous slurry is formed and that the whole sample is in contact with the extractant. If the described procedure is for any reason not sufficient to produce a homogenous slurry, additional mechanical forces shall be applied by e.g. application of a device for preparation of homogenous slurry.

6.2.2 Extraction procedure for coffee samples

Weigh, to the nearest 0,01 g, a 2,0 g test portion into a 50 ml centrifuge tube (5.6). Add 5 ml of *n*-hexane (alternatively cyclohexane). Add 40 ml of water. Add 400 µl of internal standard solution 2, *c* = 1 000 ng/ml (4.10). Shake intensively for 15 s to 30 s by hand and 10 s to 15 s with a vortex mixer (5.8), and then for 60 min on a mechanical shaker (5.7), adjusted to maximum sample-extractant agitation. Centrifuge in a cooled centrifuge (5.9) at 10 °C, 3 600 x *g* for 20 min. Check for proper phase separation of *n*-hexane (or cyclohexane), aqueous and solid phase. Remove and discard the organic solvent phase (*n*-hexane or cyclohexane), and transfer 10 ml of the aqueous phase to a clean test tube.

Take care that a homogenous slurry is formed and that the whole sample is in contact with the extractant. If the described procedure is for any reason not sufficient to produce a homogenous slurry, additional mechanical forces shall be applied by, e.g. application of a device for preparation of homogenous slurry.

NOTE Cyclohexane is a suitable alternative for *n*-hexane.

6.3 Cleanup

6.3.1 Cleanup for bakery and potato product sample

For all steps adjust the flow of the SPE columns to let the liquid elute drop wise (about 30 drops per min). Check the completeness of elution of acrylamide from the SPE column 2 (5.2.3) by recording the elution profile, at least for each new batch of columns.

Fit SPE column 1 (5.2.2) to the vacuum manifold (5.2.1). Condition the column with 3 ml of methanol and 2 times with 6 ml of water. Pass 10 ml of the aqueous extract (6.2.1) through the column and collect the eluate.

Fit SPE column 2 (5.2.3) to the vacuum manifold (5.2.1). Condition the column with 5 ml of methanol and 5 ml of water. Load the extract (approximately 10 ml) from the previous column and discard the eluate. Rinse the column once with 4 ml of water and discard the rinsing solvent. Assure that no eluate is left in the valves or flow channels of the vacuum manifold by e.g. placing the column on another (dry) position of the vacuum manifold. Rinsing solvent that is left in the valves could contain co-extracts that could interfere with the internal standard peak. After rinsing, elute acrylamide with 2 ml of 60 % methanol in water (4.6). Collect the elution