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BSI Standards Publication

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

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National foreword

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

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

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of an identical text or by endorsement, at the latest by October 2015 and conflicting national standards shall be withdrawn at the latest by October 2015.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:

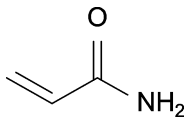

Figure 1 — Acrylamide

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:

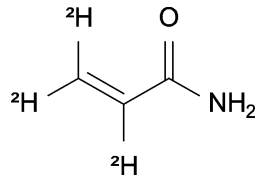

Figure 2 — Deuterium-labelled acrylamide

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

solvent and the residual solvent in the column (by applying a slight vacuum or a slight pressure) in a glass vial of at least 4 ml, which is compatible with the evaporation equipment (5.11).

Evaporate the methanol from the extract, never exceeding 40 °C. This can be achieved by means of e.g. a vortex evaporator under vacuum for approximately 30 min at 40 °C, or by a gentle stream of inert gas, heating the glass vial to a maximum of 40 °C. Do not reduce the volume to less than approximately 500 µl. Transfer the sample to a suitable autosampler vial and perform LC-MS/MS analysis.

6.3.2 Cleanup for coffee samples

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 2 ml of the aqueous phase (6.2.2) through the column, followed by 3 ml of water, and collect the combined eluate, consequently approximately 5 ml in total.

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 5 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 solvent and the residual solvent in the column (by applying a slight vacuum or a slight pressure) in a glass vial of at least 4 ml, which is compatible with the evaporation equipment (5.11).

Evaporate the methanol from the extract, never exceeding 40 °C. This can be achieved by means of e.g. a vortex evaporator under vacuum for approximately 30 min at 40 °C, or by a gentle stream of inert gas, heating the glass vial to a maximum of 40 °C. Do not reduce the volume to less than approximately 500 µl. Transfer the sample to a suitable autosampler vial and perform LC-MS/MS analysis.

7 Measurement

7.1 LC-MS/MS determination

7.1.1 LC-MS/MS conditions

For a successful analysis it is of paramount importance that the instrument is in good condition and that all instrumental parameters are optimized.

Use the HPLC column (5.1.2) and the mobile phase (4.7) at a flow rate of 400 µl/min. The column is held at room temperature (22 °C ± 2 °C). The injection volume is 10 µl. Other chromatography columns may be used, provided system suitability provisions (see 7.1.2) are fulfilled.

Use electrospray ionization, Selected Reaction Monitoring (SRM) and unit resolution. Optimize all parameters as different temperatures, gas flows, voltages and probe position for the detection of acrylamide at the flow rate of the mobile phase. Optimize collision energy individually for each of the following transitions: m/z 72 > 55, 72 > 54, 72 > 44 and 75 > 58. Use optimal settings of dwell time and inter-channel delay to obtain the best sensitivity, avoiding any crosstalk and to obtain chromatograms with at least 15 data points per channel over the peak. Detect acrylamide and the internal standard with the transitions m/z 72 > 55 and 75 > 58 for quantitative purposes, and m/z 72 > 55, 72 > 54 and 72 > 44 for confirmation of the identity of acrylamide. Depending on the type of LC-MS/MS instrument used, other transitions can be available for peak qualification too.

NOTE Definition of selected reaction monitoring: data acquired from one or more specific product ions corresponding to m/z selected precursor ions recorded via two or more stages of mass spectrometry.

The following parameter setting were successfully applied[3)]:

Table 3 — LC-MS/MS conditions

HPLC parameters	
HPLC column	Hypercarb™ column (50 mm x 2,1 mm) equipped with a Hypercarb™ pre-column (10 mm x 2,1 mm)
Column temperature	Room temperature (22 °C ± 2 °C)
Injection volume	10 µl
Mobile phase	0,1 % acetic acid in water
Mobile phase flow	400 µl/min
Total run time	8 min
MS parameters	
Desolvation gas	N_2, 600 l/h
Desolvation temperature	400 °C
Nebulising gas	N_2, fully open
Cone gas	N_2, 200 l/h
Collision gas	Argon, $2,3 \times 10^{-3}$ mbar
Ion source temperature	125 °C
Capillary voltage	2 kV
Cone voltage	20 V
Hexapole voltage	10 V
Collision energies:	
72 > 55 and 75 > 58	9 eV
72 > 44	20 eV
72 > 54	16 eV
Dwell time	0,15 s
Inter channel delay	0,03 s

Inject the extract of each sample preferably twice. Use a run time of 8 min for samples to allow matrix components to elute from the column. If necessary, wash the column with 80 % of acetonitrile in water, as described in 7.1.3, in between the set of samples, or after the end of the batch.

The application of a divert valve (optional) is recommended in order to increase instrument stability and times between maintenance interventions. By applying a divert valve, the effluent from the chromatographic column

3) A Quattro Ultima PT from Micromass ® was used for the measurements. This is an example of a suitable product 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.

is directed to the mass spectrometer only between expected retention time of acrylamide minus 0,5 min and expected retention time of acrylamide plus 1,5 min, and signals are recorded only between these two times.

7.1.2 System suitability

The response of the LC-MS can vary from day to day or over longer periods. Also the HPLC column can deteriorate after having been used several times, or just once, depending on the number of injections and type of samples analysed. Therefore, the system should be checked prior to each series of analysis in the following way.

Equilibrate the column with mobile phase and the mass spectrometer for, e.g. 30 min. Inject at least three times one of the standard solutions to check the response of the LC-MS equipment as well as retention time, peak shape and peak width of the acrylamide and internal standard peaks. The response should be similar as after the optimization. If not, the interface needs to be cleaned and/or the mass spectrometer needs to be re-optimized. The retention time of acrylamide should be on the Hypercarb™ column (5.1.2) above 1,7 min (at a flow of 0,4 ml/min), and the peak width at half height should be below 0,2 min. The retention factor (k) should be above 3,5. Tailing occurs even for fresh columns, but the distance from peak maximum to tailing edge of peak (measured at 10 % height) should not be more than twice the distance from peak maximum to the leading edge of the peak. For better illustration, a typical chromatogram for potato products is shown in Annex A.

Columns not fulfilling these requirements could be regenerated by washing (see 7.1.3). If regeneration was not successful, the column shall be exchanged.

Inject pure water to check for possible contamination of the system. No traces of acrylamide should be detected.

7.1.3 Regeneration of HPLC column

If the performance of the HPLC column (5.1.2) is significantly worse than expected or required (see 7.1.2) it may be restored by regeneration of the column. Regeneration of columns shall be performed according to the recommendations given by the column supplier.

For graphitized carbon columns the following procedures are recommended.

The column can be flushed with 80 % acetonitrile in water at 0,4 ml/min for 30 min in line with the LC-MS. This may be done routinely after each day or even between each set of injections. Make sure to give time for equilibration with the mobile phase.

For more severe cases, the following washing procedure, preferably performed off-line the LC-MS, can be used. Dispose of the guard column. Change the flow direction and flush the column at room temperature at 0,2 ml/min in a consecutive order for:

a) two h with a mixture of 50 % tetrahydrofuran (THF), 10 % ammonia and 40 % water. Consider that some polymers used for HPLC tubings are not resistant against THF;

b) 30 min with pure methanol;

c) 30 min with the mobile phase for equilibration.

7.2 Batch (sequence) composition

— Analyse at least six calibration standards starting from the lowest concentration at the beginning of each batch and the resulting average response factor is used to quantify that set of samples. Additional calibration standards may be included in the sequence whenever deemed necessary.

— Analyse extracts of the test samples in random order.

— Analyse at least one procedural blank sample with each batch of samples.

— Analyse a sample for the determination of recovery with each batch of samples (see 7.4).

— At the end of each batch, a mid-point standard should be analysed to monitor instrument drift. Acceptable values are ± 5 % of original concentration.

Pure methanol may be injected for monitoring of carry over whenever deemed necessary.

NOTE A procedural blank sample is a blank sample made up of all reagents foreseen for the preparation of the test portion and processed in all respects as the sample. This kind of blank, tests the purity of the reagents, but also identifies other possible sources of contamination, like the glassware and the analytical instrument (for this European Standard, the procedural blank sample consists only of 40 ml of water, to which 400 µl of the internal standard solution is added). It is subjected to the whole extraction and cleanup procedure and serves to determine background levels of acrylamide.

7.3 Identification and calculation of results

The peak identity is confirmed by comparison of the peak area ratios for the transitions m/z 72 > 54 / 72 > 55, and 72 > 44 / 72 > 55 from sample extracts and standard solutions. The ratios should not differ more than ± 20 % from those obtained for standard solutions.

The transition 72 > 27 may be used for peak identification too. However, the presence, visibility and abundance of this transition depends on the type of mass spectrometer used.

7.4 Recovery

Determine the recovery in each batch of samples using certified reference materials or other methods approved by international guidelines, e.g. spiking, see [4] and [5]. Recovery is for information only. Recovery factors are not applied for correction of results.

NOTE The following certified reference materials are available (not exhaustive): ERM® BD273, Acrylamide in toasted bread, available from the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium, (http://irmm.jrc.ec.europa.eu) and ERM® BD272, Acrylamide in crispbread, available from Federal Institute for Materials Research and Testing (BAM), Berlin, Germany, (www.bam.de).

8 Determination of concentrations

Calibration by internal standardization is applied for the determination of acrylamide. This calibration requires the determination of response factors *f* defined using Formula (1):

$$f_i = \frac{A_{SA} \times c_{[d3]A}}{A_{[d3]A} \times c_{SA}} \quad (1)$$

where

f_i is the response factor of acrylamide and acrylamide-2,3,3-D_3 determined by the analysis of calibration standard *i*;

A_{SA} is the area of the unlabelled acrylamide peak as SRM mass trace m/z 72 > 55 in the calibration standard;

$A_{[d3]A}$ is the area of labelled acrylamide-2,3,3-D_3 peak as SRM mass trace m/z 75 > 58;

$c_{[d3]A}$ is the acrylamide-2,3,3-D_3 concentration of the calibration solution;

c_{SA} is the acrylamide concentration of the calibration solution.

$$\overline{f} = \frac{\sum_{i=1}^{i=n} f_i}{n} \quad (2)$$

where

$\overline{f}$ is the average of n response factors of acrylamide and acrylamide-2,3,3-D_3 determined from n calibration solutions.

Attention shall be paid to the linearity of the instrument response, which could be different for different instruments. If the linear range of the instrument is exceeded, appropriate measures shall be taken, such as restriction of calibration to a smaller concentration range and consequently dilution of samples with high acrylamide levels.

Calculate for each sample the average of the amount of acrylamide that was extracted from the sample (X_A) for the N replicate injections that were performed for the respective sample using Formula (3):

$$X_A = \frac{1}{N} \times \sum_{1}^{N} \frac{A_A \times X_{[d3]A}}{A_{[d3]A} \times \overline{f}} \quad (3)$$

where

X_A is the amount of acrylamide (in nanogram) that was extracted from the sample;

A_A is the area of unlabelled acrylamide peak for SRM mass trace 72 > 55 of the sample;

$A_{[d3]A}$ is the area of the acrylamide-2,2,3-D_3 peak for SRM mass trace 75 > 58 of the sample;

$X_{[d3]A}$ is the absolute amount (in nanogram) of internal standard (acrylamide-2,3,3-D_3) added to the sample;

$\overline{f}$ is the average of n response factors of acrylamide and acrylamide-2,3,3-D_3 determined from n calibration standards.

Calculate the mass fraction of acrylamide, c_S, in micrograms per kilogram, in the sample using Formula (4):

$$c_S = \frac{X_A}{W_S} \quad (4)$$

where

X_A is the absolute amount, in nanograms, of acrylamide extracted from the sample;

W_S is the sample mass, in grams.

Results shall be reported to three significant figures and reporting units are µg/kg.

9 Precision

9.1 General

Details of the interlaboratory test of the precision of the method are summarized in Annex B. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than given in Annex B.

9.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in not more than 5 % of the cases.

The values for butter cookies are:

$\bar{x}$ = 14,3 µg/kg r = 3,4 µg/kg

$\bar{x}$ = 95,7 µg/kg r = 20,9 µg/kg

The values for toasted bread are:

$\bar{x}$ = 37,9 µg/kg r = 5,9 µg/kg

The values for crisp bread are:

$\bar{x}$ = 980 µg/kg r = 86 µg/kg

The values for spiced biscuits are:

$\bar{x}$ = 249 µg/kg r = 25,5 µg/kg

The values for potato crisps are:

$\bar{x}$ = 324 µg/kg r = 54,6 µg/kg

$\bar{x}$ = 628 µg/kg r = 157 µg/kg

$\bar{x}$ = 2 512 µg/kg r = 418 µg/kg

$\bar{x}$ = 4 051 µg/kg r = 485 µg/kg

$\bar{x}$ = 9 083 µg/kg r = 1 260 µg/kg

The values for mashed potato (fortified)are:

$\bar{x}$ = 500 µg/kg r = 76,0 µg/kg

The values for roasted coffee are:

$\bar{x}$ = 160 µg/kg r = 15,4 µg/kg

$\bar{x}$ = 263 µg/kg r = 13,7 µg/kg

$\bar{x}$ = 585 µg/kg r = 15,7 µg/kg

9.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit *R* in not more than 5 % of the cases.

The values for butter cookies are:

$\bar{x}$ = 14,3 µg/kg R = 5,6 µg/kg

$\bar{x}$ = 95,7 µg/kg R = 31,6 µg/kg

The values for toasted bread are:

$\bar{x}$ = 37,9 µg/kg R = 9,0 µg/kg

The values for crisp bread are:

$\bar{x}$ = 980 µg/kg R = 148 µg/kg

The values for spiced biscuits are:

$\bar{x}$ = 249 µg/kg R = 72,6 µg/kg

The values for potato crisps are:

$\bar{x}$ = 324 µg/kg R = 115 µg/kg

$\bar{x}$ = 628 µg/kg R = 232 µg/kg

$\bar{x}$ = 2 512 µg/kg R = 826 µg/kg

$\bar{x}$ = 4 051 µg/kg R = 1 010 µg/kg

$\bar{x}$ = 9 083 µg/kg R = 2 312 µg/kg

The values for mashed potato (fortified) are:

$\bar{x}$ = 500 µg/kg R = 123 µg/kg

The values for roasted coffee are:

$\bar{x}$ = 160 µg/kg R = 51,5 µg/kg

$\bar{x}$ = 263 µg/kg R = 74,5 µg/kg

$\bar{x}$ = 585 µg/kg R = 157,6 µg/kg

The relationship between R and $\bar{x}$ can be sufficiently approximated by the following formula:

R(µg/kg) = 0,258 × $\bar{x}$ (µg/kg)

with coefficient of determination (R^2 = 0,99), meaning that the reproducibility relative standard deviation is constant over the working range.

10 Test report

The test report shall contain the following data:

a) all information necessary for the identification of the sample (type of sample, origin and designation of the sample);

b) a reference to this European Standard;

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

d) the date of receipt;

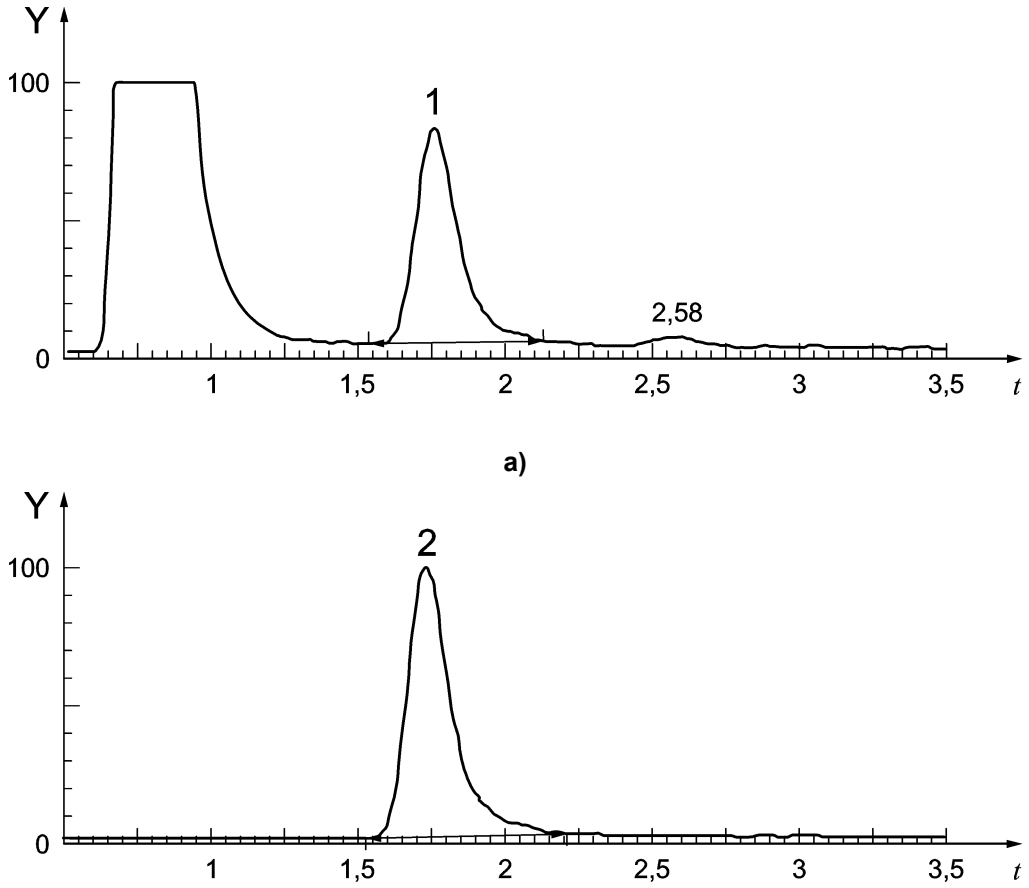
e) the date of test;

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

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

Annex A
(informative)

Typical chromatograms



Key

a) acrylamide (transition 72 > 55; retention time = 1,77 min)

t time, in min

Y abundance units

1 acrylamide

b) acrylamide-2,3,3-D_3 (transition 75 > 58; retention time = 1,75 min)

t time, in min

Y abundance units

2 acrylamide-2,3,3-D_3

Figure A.1 — Typical chromatogram of a potato chips sample containing acrylamide

Operating conditions:

Injection volume:	10 µl
Column:	Hypercarb™, 5 µm, 50 mm x 2,1 mm, with a guard column 5 µm, 10 mm x 2 mm (Thermo Hypersil-Keystone®)
Flow rate:	400 µl/min
Mobile phase:	Mixture of 1 part per volume of acetic acid and 1 000 parts per volume of water

Annex B
(informative)

Precision data

The following data were obtained in an interlaboratory test according to ISO 5725-2 [8] and the AOAC Guidelines [9] for collaborative study procedures to validate characteristics of a method of analysis.

Assigned values were calculated from the results of the participants after removal of outliers, with the exceptions of the spiked mashed potato powder and the candidate reference material for which the spiking level, respectively the preliminary certified value were used.

Table B.1 — Precision data for acrylamide in butter cookies, toasted bread and spiced biscuits

Sample	Butter cookies	Toasted bread	Butter cookies	Spiced biscuits
Source	RS [a]	RS [a]	RS [a]	RS [a]
Year of interlaboratory test	2007	2007	2007	2007
Number of laboratories	16	16	16	16
Number of non-compliant laboratories	4	1	1	1
Number of laboratories retained after eliminating outliers	10	12	13	12
Number of outliers (laboratories)	2	3	2	3
Number of accepted results	10	12	13	12
Mean value, $\bar{x}$, µg/kg	14,3	37,9	95,7	249
Repeatability standard deviation s_r, µg/kg	1,2	2,1	7,5	9,1
Repeatability relative standard deviation, RSD_r, %	8,4	5,5	7,8	3,7
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	3,4	5,9	20,9	25,5
Reproducibility standard deviation s_R, µg/kg	2,0	3,2	11,3	25,9
Reproducibility relative standard deviation, RSD_R, %	14,1	8,5	11,8	10,4
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	5,6	9,0	31,6	72,6
Recovery, %	- [b]	- [b]	- [b]	- [b]
HorRat value, according to [6]	0,5	0,3	0,5	0,5
HorRat value, according to [7]	0,6	0,4	0,6	0,5

[a] RS = from retail store.

[b] Not determined as recovery is corrected by isotopic labelled acrylamide.

Table B.2 — Precision data for acrylamide in potato crisps, mashed potato powder and crisp bread

Sample	Potato crisps	Fortified mashed potato powder	Potato crisps	Crisp bread
Source	SP [a]	RS [a]	RS [a]	ERM®-BD272
Year of interlaboratory test	2007	2007	2007	2007
Number of laboratories	16	16	16	16
Number of non-compliant laboratories	1	0	0	0
Number of laboratories retained after eliminating outliers	14	16	14	13
Number of outliers (laboratories)	1	0	2	3
Number of accepted results	14	16	14	13
Mean value, $\bar{x}$, µg/kg	324	500	628	980
Repeatability standard deviation s_r, µg/kg	19,5	27,1	55,9	30,7
Repeatability relative standard deviation, RSD_r, %	6,0	5,4	8,9	3,1
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	54,6	76,0	157	86,0
Reproducibility standard deviation s_R, µg/kg	41,0	43,9	82,7	52,9
Reproducibility relative standard deviation, RSD_R, %	12,7	8,8	13,2	5,4
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	115	123	232	148
Recovery, %	- [b]	- [b]	- [b]	- [b]
HorRat value, according to [6]	0,7	0,5	0,8	0,3
HorRat value, according to [7]	0,7	0,5	0,8	0,3

[a] SP = specially prepared for the study, RS = from retail store.

[b] Not determined as recovery is corrected by isotopic labelled acrylamide.

Table B.3 — Precision data for acrylamide in potato crisps

Sample	Potato crisps	Potato crisps	Potato crisps
Source	SP [a]	RS [a]	SP [a]
Year of interlaboratory test	2007	2007	2007
Number of laboratories	16	16	16
Number of non-compliant laboratories	1	1	0
Number of laboratories retained after eliminating outliers	15	15	14
Number of outliers (laboratories)	0	0	2
Number of accepted results	15	15	14
Mean value, $\bar{x}$, µg/kg	2 512	4 051	9 083
Repeatability standard deviation s_r, µg/kg	149	173	450
Repeatability relative standard deviation, RSD_r, %	5,9	4,3	5,0
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	418	485	1 260
Reproducibility standard deviation s_R, µg/kg	295	361	826
Reproducibility relative standard deviation, RSD_R, %	11,7	8,9	9,1
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	826	1 010	2 312
Recovery, %	- [b]	- [b]	- [b]
HorRat value, according to [6]	0,8	0,7	0,8
HorRat value, according to [7]	0,8	0,7	0,8

[a] SP = specially prepared for the study, RS = from retail store.

[b] Not determined as recovery is corrected by isotopic labelled acrylamide.

Table B.4 — Precision data for acrylamide in roasted coffee

Sample	Roasted coffee	Roasted coffee	Roasted coffee
Source	RS [a]	SP [a]	SP [a]
Year of interlaboratory test	2007	2007	2007
Number of laboratories	9	9	8
Number of non-compliant laboratories	1	1	1
Number of laboratories retained after eliminating outliers	7	7	7
Number of outliers (laboratories)	1	1	0
Number of accepted results	7	7	7
Mean value, $\bar{x}$, µg/kg	160	263	585
Repeatability standard deviation s_r, µg/kg	5,5	4,9	5,6
Repeatability relative standard deviation, RSD_r, %	3,5	1,9	1,0
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	15,4	13,7	15,7
Reproducibility standard deviation s_R, µg/kg	18,4	26,6	56,3
Reproducibility relative standard deviation, RSD_R, %	11,5	10,1	9,6
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	51,5	74,5	157,6
Recovery, %	- [b]	- [b]	- [b]
HorRat value, according to [6]	0,5	0,5	0,6
HorRat value, according to [7]	0,5	0,5	0,6

[a] SP = specially prepared for the study, RS = from retail store.

[b] Not determined as recovery is corrected by isotopic labelled acrylamide.

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