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Coffee and coffee products — Determination of acrylamide — Methods using HPLC-MS/MS and GC-MS after derivatization

Café et de ses dérivés — Dosage de l'acrylamide — Méthodes utilisant CLHP-MS/MS et CG-MS après dérivation

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 15, *Coffee*.

Introduction

When applying this document, all existing safety regulations have to be followed.

Coffee and coffee products — Determination of acrylamide — Methods using HPLC-MS/MS and GC-MS after derivatization

WARNING — The use of this document can involve hazardous materials, operations and equipment. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to take appropriate measures for ensuring the safety and health of the personnel prior to application of this document and to fulfil statutory requirements for this purpose.

1 Scope

This document specifies methods for the determination of acrylamide in coffee and coffee products by extraction with water, clean-up by solid-phase extraction and determination by HPLC-MS/MS and GC-MS. It was validated in a method validation study on roasted coffee, soluble coffee, coffee substitutes and coffee products with ranges from 53 µg/kg to 612,1 µg/kg.

2 Normative references

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

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

No terms and definitions are listed in this document.

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

- IEC Electropedia: available at http://www.electropedia.org/
- ISO Online browsing platform: available at http://www.iso.org/obp

4 Principle

The coffee sample is extracted with water or, in the case of soluble products, dissolved in water. A clean-up by solid phase extraction is employed to remove interfering matrix compounds. Two alternative methods can be used for the determination: high-performance liquid chromatography with mass spectrometric detection (HPLC-MS/MS) or, after a bromination of the acrylamide, gas chromatography with mass spectrometric detection (GC-MS). In both cases, isotopic labelled internal standard solutions are used.

5 Reagents

WARNING — In view of health risks when working with acrylamide, appropriate preventive and protection measures shall be taken, such as using a fume cupboard, aspirating acrylamide-containing solutions only with a pipette, and avoiding skin and eye contact or inhalation of acrylamide-containing vapour.

If available, reagents of “residue analysis grade” or “analytical reagent grade” shall be used. The level of impurities in the reagents that contribute to the blank should be negligibly small. The blank shall be checked regularly.

5.1 Water, of grade 1 according to ISO 3696, MS-grade is recommended.

5.2 Operating gases of high purity, suitable for GC and mass spectrometry according to the instructions of the manufacturer of the apparatus.

5.3 Solvents, such as methanol, ethyl acetate, acetonitrile, n-hexane, MS-grade is recommended.

5.4 Acrylamide, C_3H_5NO, purity >98 %, reference substance.

5.4.1 Acrylamide stock solution, mass concentration ρ = 1 000 µg/ml.

Weigh (0,10 ± 0,001) g of acrylamide into a 100 ml one-mark volumetric flask and swirl it in 30 ml of water in order to dissolve the acrylamide. Fill up to the mark with water and mix well. The stock solution is stable for at least 3 months when stored protected from light at a maximum of 6 °C.

Alternatively, a commercially available solution with a mass concentration of ρ = 1 000 µg/ml may be used. The information of the manufacturer regarding the stability of the solution shall be observed.

5.4.2 Acrylamide calibration solution, ρ = 10 µg/ml.

Using a pipette, transfer (1,0 ± 0,001) ml of the acrylamide stock solution (5.4.1) into a 100 ml one-mark volumetric flask and fill up to the mark with water. This solution shall be stored protected from light at a maximum of 6 °C and shall be freshly prepared every working day. Depending on the working range, more dilution steps might be necessary.

5.5 D3-acrylamide (acrylamide-2,3,3-d3) internal standard solution, $C_3H_2D_3NO$, purity >98 %, reference substance.

5.5.1 D3-acrylamide stock solution (internal standard solution).

Weigh (0,10 ± 0,001) g of D3-acrylamide into a 100 ml one-mark volumetric flask and swirl it in 30 ml of water in order to dissolve the D3-acrylamide. Fill up to the mark with water and mix well. The stock solution is stable for at least 3 months when stored protected from light at a maximum of 6 °C.

Alternatively, a commercially available solution with a mass concentration of ρ = 1 000 µg/ml may be used. The information of the manufacturer regarding the stability of the solution shall be observed.

5.5.2 D3-acrylamide internal standard solution.

Using a pipette, transfer (1,0 ± 0,001) ml of the D3-acrylamide stock solution (5.5.1) into a 100 ml one-mark volumetric flask and fill up to the mark with water. This solution shall be stored protected from light at a maximum of 6 °C and shall be freshly prepared every working day. Depending on the working range, more dilution steps might be necessary.

NOTE 1 For HPLC-MS/MS, the solutions according to 5.4.1 to 5.5.2 can be prepared using the HPLC eluent as a solvent. The stability of these solutions depends on the mobile phase used and has to be validated.

When using GC-MS, all standard solutions according to 5.4.2 and 5.5.2 shall be subjected to the derivatization step according to 8.5.1.

NOTE 2 Instead of D3-acrylamide, it is also possible to use $^{13}C_3$ acrylamide for the preparation of the internal standard solution. However, in the following clauses, the procedure and calculation are described for D3-acrylamide only.

5.6 Saturated bromine water.

Saturate distilled water with bromine in a 100 ml one-mark volumetric flask (with a glass stopper) until a phase of bromine is formed at the bottom of the flask (around 3,5 % of bromine at 4 °C). Acidify the bromine water to a pH of about 1 using concentrated hydrobromic acid, (HBr, with a specific gravity of 1,48 g/cm^3).

If stored at 4 °C and protected from light, the solution can be used for about 4 weeks.

5.7 Potassium bromide, KBr.

5.8 Sodium thiosulfate (pentahydrate), $Na_2S_2O_3 \cdot 5\ H_2O$.

5.9 Triethylamine, $(C_2H_5)_3N$.

5.10 Sodium sulfate (anhydrous, granular), Na_2SO_4.

5.11 Carrez solution I.

Dissolve 10,6 g of potassium hexacyanoferrate trihydrate (II) $K_4[Fe(CN)_6] \cdot 3\ H_2O$ in 100 ml of water. If stored at 4 °C and protected from light, the solution is stable for 6 months.

5.12 Carrez solution II.

Dissolve 21,9 g of zinc acetate dihydrate $Zn(CH_3COO)_2 \cdot 2\ H_2O$ in 100 ml of water. If stored at 4 °C and protected from light, the solution is stable for 6 months.

5.13 Borate buffer, pH 8,6.

Mix 68 ml of a 0,1 molar sodium borate solution (20,12 g $Na_2B_4O_7$ per litre of water) and 32 ml of 0,1 molar hydrochloric acid, $c(HCl) = 0{,}1$ mol/l, in a 100 ml one-mark volumetric flask.

6 Apparatus

Usual laboratory apparatus and, in particular, apparatus according to 6.1 to 6.14 are required.

Apparatus and parts of the apparatus which come into contact with the sample and extract shall be free of residues which can cause blank values. Preferably glassware or equipment made of stainless steel or PTFE (polytetrafluoroethylene) shall be used.

6.1 Analytical balance, capable of weighing to an accuracy of 0,1 mg.

6.2 Coffee mill, suitable for grinding roasted coffee beans.

6.3 Glassware, for collecting and storing the extracts, preferably made of amber glass, as sample vials for manual or automatic use, equipped with an inert seal (e.g. vials with PTFE coated septum).

6.4 Ultrasonic bath, capable of being maintained at 40 °C.

6.5 Laboratory centrifuge, suitable for 15 ml and 50 ml centrifugal tubes and with a minimum *g*-force of 2 000 *g*.

6.6 Centrifuge tubes, of 15 ml and 50 ml.

6.7 One-mark volumetric flask, of 20 ml and 100 ml.

6.8 Pipettes, glass or automatic, suitable for measuring volume ranges of standard solutions and sample extract dilutions.

6.9 Glass or polypropylene cartridges, with sorbents for the solid phase extraction (SPE), and for the clean-up of extracts in 8.3.2 and 8.5.1 (examples are given in Table B.1).

6.10 High performance liquid chromatograph (for the test procedure according to 8.4), equipped with ESI and mass spectrometric detector (HPLC-MS/MS); gas supply as specified by the manufacturer.

6.11 HPLC column (for the test procedure according to 8.4), suitable for acrylamide chromatography (examples are given in Table C.1).

6.12 Gas chromatograph (for the test procedure according to 8.5) with mass spectrometric detector (GC-MS) and operating gas supply (5.2) as specified by the manufacturer.

6.13 GC column, (for the test procedure according to 8.5) capillary column, suitable for acrylamide chromatography (examples are given in Table C.2).

6.14 Membrane filter units, syringe filter (e.g. cellulose acetate filters 0,45 µm pore size) suitable for filtration of sample eluate obtained by solid phase extraction before injection into the chromatographic system.

7 Sampling

Sampling is not part of the method specified in this document. The sampling procedure shall be subject to agreement by the interested parties. A representative, thoroughly mixed sample shall be used, which has not been damaged or adulterated during transport or storage.

In order to exclude changes in the acrylamide levels, the analysis shall be performed shortly after reception of the sample. The samples shall be stored under cool conditions below 6 °C at a maximum of 6 months, under the exclusion of light and they shall be exposed to room temperature only for analysis.

The date of receipt of the sample, as well as the date of roasting or the best-before date, shall be documented along with the date of analysis.

8 Procedure

8.1 General

To avoid losses of the analyte, it is necessary that the samples are protected from light during extraction and further preparation. For this reason, amber glassware shall always be used. Otherwise, the content of the vessels and flasks shall be protected from incident light using aluminium foil.

8.2 Preparation of the sample extract

If necessary, grind the sample in a coffee mill (6.2) and homogenize thoroughly.

Weigh 2 g of the homogenized sample of roasted coffee, soluble coffee or coffee substitute or 5 g of liquid coffee beverage to the nearest 1 mg using an analytical balance (6.1) and transfer it into a 50 ml centrifuge tube (6.6).

Add 2 ml of n-hexane to the test sample and shake briefly. Then spike the test sample with D3-acrylamide as the internal standard solution in a concentration corresponding to the expected acrylamide level of the sample.

EXAMPLE Weigh 2 g of coffee and add 100 µl internal standard solution (ρ = 10 µg/ml), which is equivalent to an acrylamide mass fraction of 500 µg/kg in the coffee sample.

Add 20 ml of distilled water, shake briefly but vigorously, and sonicate (6.4) for 15 min at approximately 40 °C.

Allow a few minutes for precipitation and in the case of non-sedimenting samples centrifuge (6.5) for 15 min at 2 000 *g* to separate suspended solids. Before liquid chromatography (8.4) or derivatization and gas chromatographic separation (8.5), take 10 ml from the lower aqueous phase and use it for a further clean-up according to 8.3. Take the lower aqueous phase through the upper hexane phase using a pipette without removing the hexane phase. If necessary, the hexane phase may also be removed cautiously using a Pasteur pipette.

8.3 Clean-up of the extracts

8.3.1 Carrez precipitation

Clean-up the sample extract prepared according to 8.2 by Carrez precipitation. Add 1 000 µl of Carrez solution I (5.11) and shake. Add 1 000 µl of Carrez solution II (5.12) and shake again. After a short exposure time, centrifuge for 4 min at 2 000 *g*. Decant the supernatant, wash the residue with 2 ml to 3 ml of water, centrifuge and decant again. Combine both aqueous solutions.

8.3.2 Solid phase extraction

Clean-up the sample extract after Carrez precipitation (8.3.1) by solid phase extraction (SPE) using two sequential cartridges with adsorber material (examples are given in Table B.1). The first cartridge contains 500 mg of C18 material, the second cartridge 500 mg of ion exchanger. The cartridges can be used in a serial alignment. If appropriate, a combined cartridge can be used.

Condition both SPE columns according to the manufacturer's instructions successively with methanol and distilled water. Place the complete sample extract (8.3.1) on top of the upper (first) SPE column, allow to soak and add 2 ml to 3 ml of water. Collect the eluate until the cartridge is dry. Place the eluate on top of the second or lower conditioned ion exchange column, add 2 ml to 3 ml of water and collect the eluate. A complete elution can be achieved by using a light vacuum or pressure. Collect the eluate including washing water in a 20 ml one-mark volumetric flask and fill up to 20 ml with water.

8.4 HPLC-MS/MS measurement

8.4.1 High-performance liquid chromatography (HPLC)

Prior to the HPLC-MS/MS analysis, add organic solvent to the cleaned-up extract (8.3.2) in order to make up the desired eluent composition and filter through a membrane filter (6.14) before injecting a suitable volume (e.g. 10 µl to 100 µl depending on the column used) onto the HPLC column.

Optimize the device parameters of the HPLC system in accordance with the manufacturer's instructions. The chromatographic conditions shall be adjusted to suit the selected column (examples are given in Table C.1).

The clean-up stages according to 8.3.1 and 8.3.2 are essential for the chromatographic separation of the analyte peaks from the interfering peaks. An example of chromatogram is given in Figures C.1 and C.2.

8.4.2 Identification and quantification by mass spectrometry (HPLC-MS/MS)

Detect acrylamide using MS/MS in the positive ionization mode (electrospray ionization, ESI).

For identification, use the mass transition $m/z = 72 \rightarrow 55$. Acrylamide is identified as present if a signal at the mass track of the daughter ion (m/z 55) appears in the MS/MS chromatogram and the deviation of the retention time from that of an authentic reference substance, analysed under the same HPLC conditions, is less than 5 %.

Possible transitions for acrylamide and D3-acrylamide are given in Table 1.

Quantify the analyte by comparing the abundance of the parent-daughter ions of acrylamide with the isotope-labelled internal standard solution using the mass transitions 72 → 55 (acrylamide) and 75 → 58 (D3-acrylamide).

A third mass transition 72 → 54 may be used for further confirmation of the results. The evaluation of this transition was not part of the interlaboratory study (Annex A).

Table 1 — Mass spectrometric transitions used for the identification and quantification of acrylamide

Reference substance	Selected transitions for the identification and quantification of acrylamide using MS/MS modus m/z	
Acrylamide	72 → 55	Identification and quantification
	72 → 44	Identification (informative, qualifier)
D3-acrylamide	75 → 58	Identification and quantification
	75 → 44	Identification (informative, qualifier)

8.5 Measurement with GC-MS

8.5.1 Derivatization and sample preparation for gas chromatography

Add 3,5 g of potassium bromide (5.7) to the aqueous sample solution (8.3.2). Add 2,5 ml of saturated bromine water (5.6) and allow to react for 2 h at room temperature in the dark. Add a few drops of sodium thiosulfate solution (5.8) to eliminate excessive bromine. When using a one molar solution, a few drops are enough to eliminate the brown colour caused by bromine. A significant overdose of sodium thiosulfate should be avoided.

Apply the extract quantitatively to a polymer resin cartridge (content 500 mg) with polar and strong cationic exchange properties (for examples, see Table B.2), which has previously been conditioned according to the manufacturer's instructions. During this process and in the subsequent rinsing steps, it is necessary to ensure that the column is not running dry. Wash the column subsequently with 3 ml of water, 1 ml of borate buffer of pH 8,6 (5.13) and again with 0,5 ml of water. The column is then to be sucked dry, followed by elution of the analyte with 2 ml of ethyl acetate with a contact time of 1 min to 2 min on the column.

NOTE 1 It can be advantageous to elute with more ethyl acetate to improve the recovery. However, this can amplify the impact of interferences.

Dry the extract by adding the lowest possible amount of sodium sulfate (5.10) to ensure a water free extract and then add 50 µl of triethylamine (5.9). This solution can be used directly for GC analysis.

NOTE 2 To check the completeness of the conversion reaction, methacrylamide (e.g. 100 µl of an aqueous 0,5 µg/ml solution) can be added to the sample solution prior to the bromination step. If the recovery for the methacrylamide derivative is satisfactory, it is assumed that acrylamide was completely brominated.

NOTE 3 By adding triethylamine, the unstable dibromopropionamide is quantitatively converted to the stable monobromopropenamide, which is detected by GC-MS. Without this conversion, uncontrolled HBr-elimination can occur in the further course of analysis (e.g. at active sites in the injector), giving results that are not reproducible.

8.5.2 Gas chromatography

Dilute the purified extract (8.5.1) with ethyl acetate (5.3) according to the expected analyte concentration and, if necessary, filter using membrane filter (6.14) prior to GC/MS analysis.

The gas chromatograph (6.12) shall be set up in accordance with the manufacturer's instructions and the device parameters shall be optimized. The chromatographic settings shall be adjusted to suit the selected column. Examples are given in Tables C.2 and C.5 and example chromatograms are given in Figures C.3, C.4 and C.5.

8.5.3 Identification and quantification by mass spectrometry

The analytes are transferred to positive ions (molecular ions) and characteristic fragment ions by electron impact ionization (EI). Use these ions for the quantification and identification.

Quantify by comparing the peak area of ions deriving from bromopropenamide with those from the isotope-labelled internal standard solution (see Table 2).

Table 2 — Observed ions

Original compound	Observed ions *m*/*z*	
Bromopropenamide	149: $[C_3H_4{}^{79}BrON]^+$	Identification and quantification
	106: $[C_2H_3{}^{79}Br]^+$	Identification
Bromopropenamide-D_2	153: $[C_3{}^2H_2{}^1H_2{}^{81}BrON]^+$	Identification and quantification
	110: $[C_2{}^2H_2{}^1H_1{}^{81}Br]^+$	Identification
NOTE Due to the natural isotope distribution of bromine, occurrences of ions with *m*/*z* 151 and *m*/*z* 108 are not capable of being evaluated.		

9 Calibration

9.1 General advice

Before application of the GC method, all calibration solutions shall be subject to the derivatization procedure according to 8.5.1.

To obtain an accurate calibration of the test procedure according to 8.4, it is necessary to determine the retention time of acrylamide (LC-MS/MS) or, for procedures according to 8.5, to determine the retention time of bromopropenamide (GC-MS). Identify the retention times using an aqueous solution of the reference substance under the specified chromatographic conditions.

9.2 Determination of linearity and definition of the working range

Determine linearity and working range by analysis of solutions of acrylamide (5.4.2) at different concentration levels spiked with D3-acrylamide at the same level as given in 8.2, e.g. 0,5 µg/ml. These data are applied to set up a calibration function to calculate linearity range for acrylamide (working range).

9.3 Calibration with internal standard solution

Add defined amounts of internal standard solution (5.5.2) to several test portions of a selected coffee sample at different concentration levels covering the working range. Then carry out the analytical process (8.2). Use the data so determined to calculate a matrix-dependent calibration function with the internal standard solution. This determination shall be carried out separately for each type of sample matrix (coffee extract, surrogates).

NOTE The determination of the analyte content in unknown samples is generally based on matrix- and sample-specific properties using the internal standard solution that is added to each sample prior to the analysis (see 8.2 and 10.2).

9.4 Determination of the laboratory specific recovery

Add defined amounts of acrylamide calibration solution (5.4.2) to several test portions of a coffee sample at different concentration levels. Then carry out the analytical process (8.2). Determine the average recovery rate of the laboratory using the values obtained. If available, recovery can be determined by analysing a reference sample with certified or otherwise known acrylamide content. It is recommended to use such material as a recovery sample in routine analysis.

10 Evaluation

10.1 Criteria for identification

See Tables 1 and 2.

10.2 Calculation and final results

Use the calibrated set prepared according to 9.2 in order to plot the peak ratio of acrylamide to internal standard solution against the concentration ratio of acrylamide to internal standard solution and perform a linear regression.

Calculate the peak ratio of acrylamide to internal standard solution for the sample with unknown acrylamide content. The concentration ratio of acrylamide to internal standard solution for the sample can be calculated using the general linear equation. Calculate the mass fraction of acrylamide, w, in the sample, in µg/kg using Formula (1). The result is automatically corrected by the recovery rate.

$$w = K \cdot \frac{\left(yi - b\right)}{a} \cdot \frac{V}{m \cdot 1\,000} \tag{1}$$

where

w is the acrylamide mass fraction of the sample in microgram per kilogram (µg/kg);

K is the target mass concentration of the internal standard solution in the unknown sample, in microgram per millilitre (µg/ml);

yi is the peak area of acrylamide divided by the peak area of the internal standard solution in the unknown sample;

a is the slope of the regression line of peak area ratio versus concentration ratio;

b is the intercept of the regression line of peak area ratio versus concentration ratio;

V is the final volume of sample preparation, in millilitre (ml);

m is the sample mass in in gram (g).

Alternatively, a simplified calculation method can be used. With this method, it is assumed that the response of the D3-acrylamide peak of a given sample corresponds to the internal standard solution concentration of 500 mg/kg (see example in 8.2). The corresponding concentration of acrylamide can be calculated from the response of the peak for the native acrylamide using the rule of three, and the result can be multiplied by a factor of 0,974 (molecular mass of acrylamide/molecular mass of D3-acrylamide). When this method is applied, it is strongly recommended that at least one sample of known acrylamide content in each series is analysed in order to ensure the basic functionality of the measuring device.

11 Precision data

11.1 General

Details of the interlaboratory test of the precision of the method are summarized in Tables 3 and A.1. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than those given in Tables 3 and A.1.

11.2 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.

11.3 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.

Table 3 — Precision data for the determination of acrylamide in coffee

Sample	$\bar{x}$ µg/kg	r µg/kg	R µg/kg
Sample A (coffee extract)	612,1	51,86	328,44
Sample B (coffee surrogate extract)	204,1	34,80	141,26
Sample C (roasted coffee)	53,74	12,13	38,576
Sample D (roasted coffee)	217,1	26,49	109,20

11.4 Recovery

For the HPLC method, recovery should be in the range of 80 % and 120 % with regard to the matrix-based calibration curve. For the GC method, recovery should be in the range of 70 % and 120 %.

12 Measurement uncertainty

The laboratory-specific measurement uncertainty should be determined separately for each matrix.

13 Test report

The test report should contain the data according to ISO/IEC 17025 and at least the following:

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

b) a reference to this document, i.e. ISO 18862;

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)

Performance characteristics

An interlaboratory study was carried out by the Working Committee NA 057-05-09 *Kaffee* of the food and agricultural products standards committee of DIN with the participation of other laboratories. The study was performed following the instructions described in this document over an analysis period of not more than 10 days. The precision of the method was determined observing the statistical requirements of ISO 5725-1 and ISO 5725-2. The results are given in Table A.1.

Table A.1 — Interlaboratory study results (for both HPLC-and GC variants)[a]

Parameter	Sample A	Sample B	Sample C	Sample D
Year of interlaboratory study	2009	2009	2009	2009
Number of participating laboratories	13	13	13	13
Number of laboratories after removing outliers	10	11	11	11
Number of accepted results	30	33	32	33
Number of outliers	7	4	5	4
Mean value $\overline{x}$ in µg/kg	612,1	204,1	53,74	217,1
Repeatability standard deviation, s_r (µg/kg)	18,52	12,43	4,333	9,46
Relative repeatability standard deviation RSD_r (%)	3,0	6,1	8,1	4,4
Repeatability, r $(2{,}8 \cdot s_r)$ (µg/kg)	51,86	34,80	12,132	26,49
Reproducibility standard deviation, s_R (µg/kg)	117,30	50,45	13,777	39,00
Relative reproducibility standard deviation RSD_R (%)	19,2	24,7	25,6	18,0
Reproducibility, R $(2{,}8 \cdot s_R)$ (µg/kg)	328,44	141,26	38,576	109,20
Reproducibility according to Horwitz value R_{Horr}	294,79	116,01	37,317	122,18
Horrat value R/R_{Horr}	1,12	1,22	1,03	0,90

[a] A separate statistical evaluation of HPLC-MS/MS and GC-MS results revealed no significant difference between the ranges of measured values.

Annex B
(informative)

Examples of absorber materials

Table B.1 — Examples of suitable sorbents for the SPE clean-up (HPLC and GC; see 8.3)

Product (or manufacturer)[a]	Sorbents (description)	Function
Chromabond ABC18 500 mg	Octadecyl silica phase with ion exchange functions	Combined cartridge
Isolute® Multimode 500 mg	Mixed-mode containing non-polar (C_{18}), strong cationic exchange ($-SO_3^-$) and strong anion exchange ($-NR_3^+$) functional groups	Combined cartridge
Bond Elut 500 mg	C18 hydrophobic silica-based sorbent	C18
Strata C18E 500 mg	C18 hydrophobic silica-based sorbent, end capped	C18
Strata™-X-C 33u 500 mg	Benzenesulfonic acid-modified polymeric cationic exchanger for the SPE	Strong ion exchange
Cromabond HR-XC 500 mg	Benzenesulfonic acid-modified polymeric cationic exchanger for the SPE	Strong ion exchange

[a] The named materials are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Table B.2 — Examples of suitable sorbents for the SPE clean-up of the GC extract (see 8.5.1)

Product (or manufacturer)[a]	Sorbents (description)	Function
Strata™-X-C 33u 500 mg	Benzenesulfonic acid-modified polymeric cationic exchanger for SPE	Strong ion exchange
Cromabond HR-XC 500 mg	Benzenesulfonic acid-modified polymeric cationic exchanger for SPE	Strong ion exchange

[a] The named materials are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Annex C
(informative)

Examples of columns and analysis conditions

C.1 Generally suitable chromatographic columns

Chromatographic columns with their respective analysis conditions are listed in Tables C.1 and C.2. Different column dimensions may be used after adjustment of the flow rate.

Table C.1 — Analytical conditions for selected HPLC columns

HPLC column[a]	Length mm	Internal diameter mm	Chromatographic settings	
			Eluent	Flow rate ml/min
Synergi™ Hydro-RP A	250	4,6	acetic acid, dil., pH 2,6	1,0
Phenomenex Luna C18, 3 µm	150	3	water : methanol 9:1 (volume fraction), with 0,1 % formic acid	0,4
LiChrospher® 100 CN, 5 µm	250	4	acetonitrile : acetic acid 1 % 1:1 (volume fraction)	0,7
Phenomenex Luna Phenyl-Hexyl	250	4,6	methanol : formic acid 1 %	0,8
Shodex RSpak DE-413, 4 µm	150	4,6	A: Water, formic acid 0,01 %, B: methanol Gradient: 0 min, 90 % A; 5 min, 60 % A, 6 min, 10 % A, 12 min 10 % A, 16 min 90 % A	0,6
Hypercarb®, 3 µm	100	2,1	water: formic acid 1 %	0,15

[a] The named materials are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Table C.2 — GC capillary columns

GC column[a]	Phase material	Length m	Internal diameter mm	Film thickness µm
Optima WAX	Polyethylene glycol 20 000 Da	30	0,25	0,25
RTX-200	Crossbond® trifluoropropylmethyl polysiloxane	60	0,25	0,25

[a] The named materials are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

C.2 Example method and approximate retention time for HPLC-MS/MS

C.2.1 Example A

Inject 20 µl of sample solution onto a Phenomenex Luna Phenyl-Hexyl column[1] (see Table C.1; 5 µm, 250 mm × 4,6 mm) under the chromatographic conditions in Table C.3.

Table C.3 — Chromatographic conditions for example A

Eluent	**Eluent A** **formic acid** 1 %	**Eluent B** **methanol**	**Flow rate** 0,8 ml/min
Gradient	(0 to 3) min, 95 % A; (5 to 8) min, 2 % A		
Sample run	8 min		
Retention time	4,7 min		

C.2.2 Example B

Inject 20 µl sample solution onto a LiChrospher® 100 CN column[1] (see Table C.1) with a LiChrospher® 100 RP-18 pre-column cartridge (4 mm × 4 mm) under the chromatographic conditions in Table C.4.

Table C.4 — Chromatographic conditions for example B

Eluent	**Eluent A** acetonitrile	**Eluent B** acetic acid 1 %	**Flow rate** 0,7 ml/min
Gradient	(0 to 5) min, 50 % A; (6 to 9) min, 100 % A, (10 to 20) min, 50 % A		
Analysis time	20 min		
Temperature of auto sampler	20 °C		
Temperature of column oven	25 °C		
Split	approximately 1:5		
Acrylamide retention time	3,7 min		

C.3 Example of suitable conditions and approximate retention time for GC-MS analysis

Inject 2 µl sample solution in the “pulsed splitless mode” on an Optima WAX column[1] (see Table C.2) under the following chromatographic conditions in Table C.5.

Table C.5 — GC method: Example of suitable conditions and approximate retention time for GC-MS

Injector	250 °C	—
Temperature program	50 °C	1 min hold
	15 °C/min	240 °C
	11 min hold	—
Gas flow	1,0 ml/min	(constant flow)
Bromopropenamid retention time (approximately)	4,4 min	—

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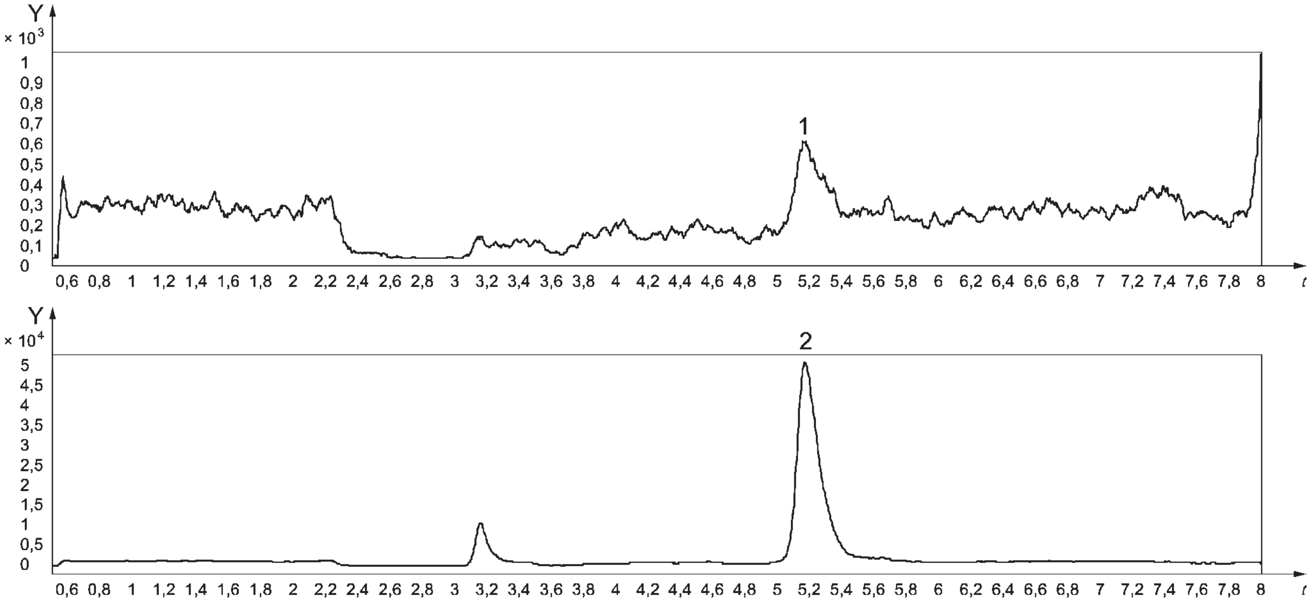
[1] The named materials are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

C.4 Example chromatograms for HPLC-MS/MS

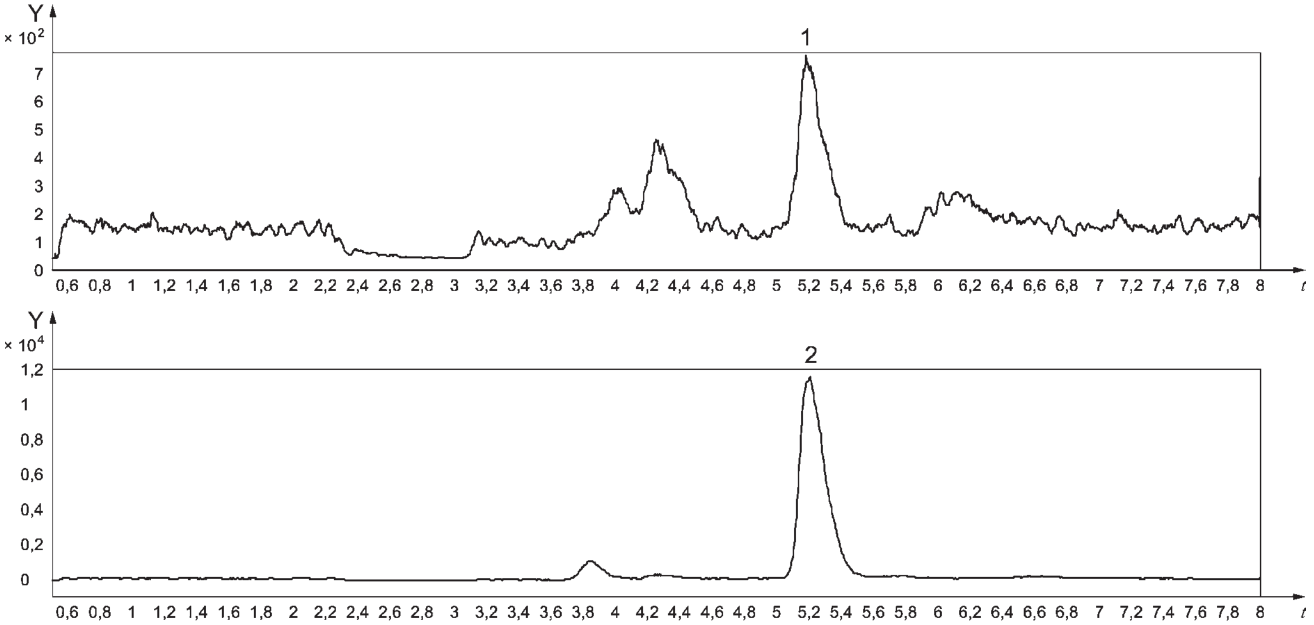
The chromatograms shown in Figures C.1 to C.5 derive from analyses of roasted coffee samples with an approximate acrylamide content of 240 µg/kg. The concentration of the added D3-acrylamide is 500 µg/kg (GC) and 1 000 µg/kg (HPLC).



Key

Y	abundance
t	time in min
1	at *t* = 5,2 min MRM 75,0 → 44,0 (qualifier ion)
2	at *t* = 5,2 min MRM 75,0 → 58,0 (quantifier ion)

Figure C.1 — D3-acrylamide



Key

Y abundance

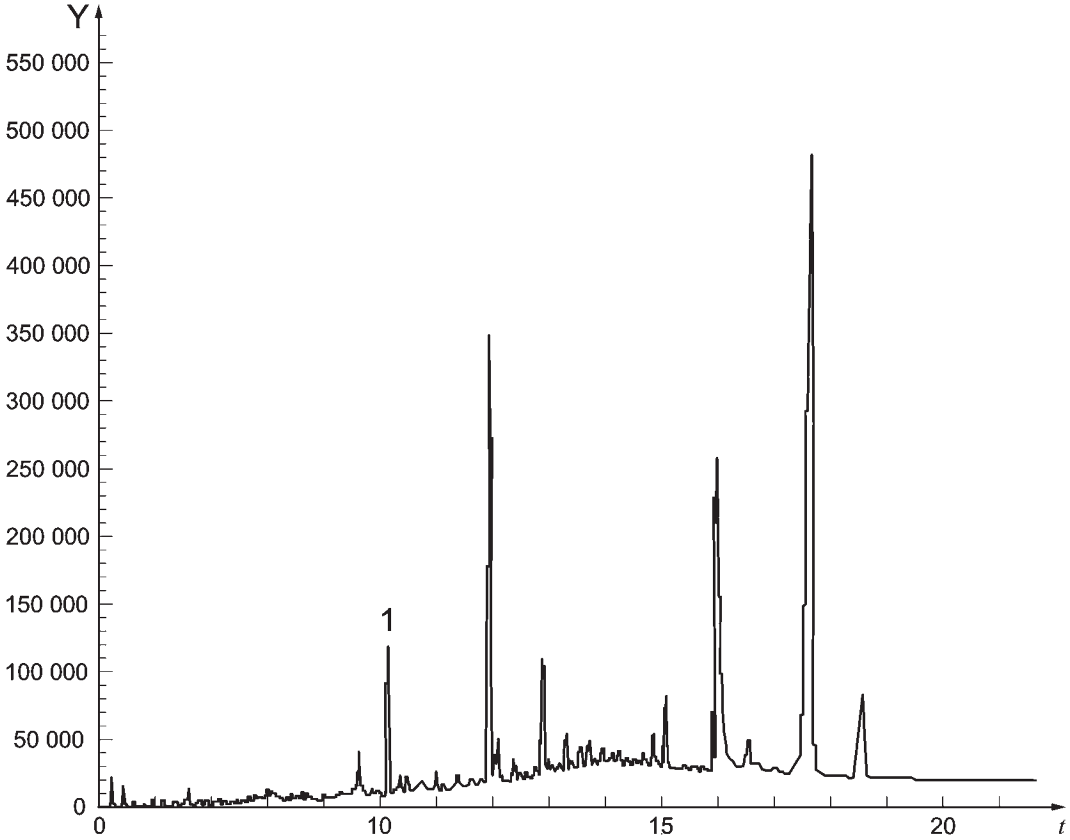
t time in min

1 at *t* = 5,2 min MRM 72,0 → 44,0 (qualifier ion)

2 at *t* = 5,2 min MRM 72,0 → 55,0 (quantifier ion)

Figure C.2 — Acrylamide, native

C.5 Example of chromatogram for GC-MS



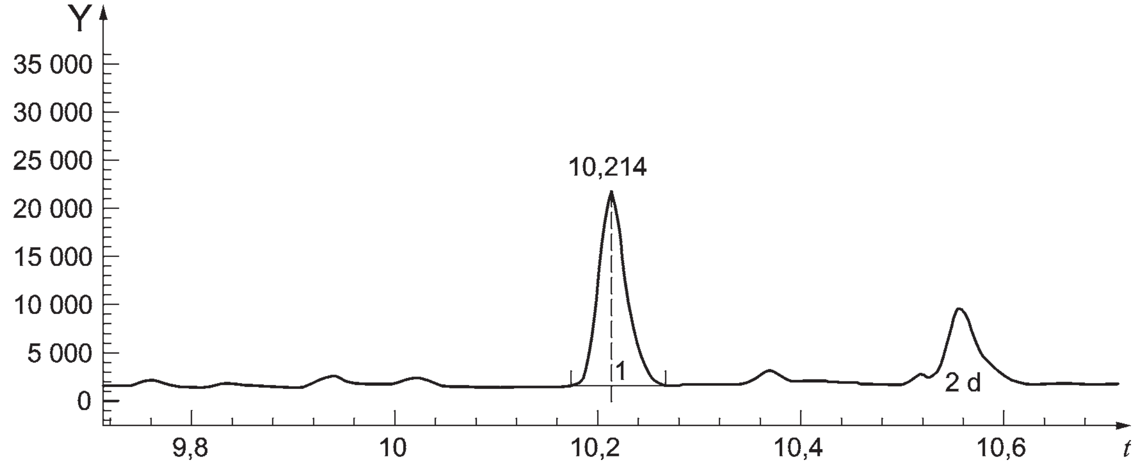
Key

Y abundance

t time in min

1 bromopropenamide

Figure C.3 — Total ion chromatogram (scan mode)



Key

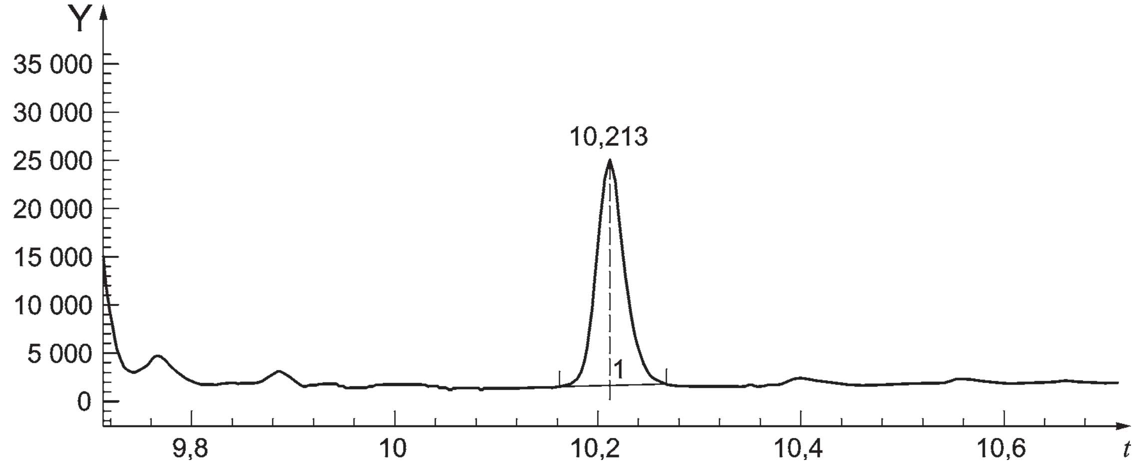
Y abundance

t time in min

1 at *t* = 10,214 min, bromopropenamide mass = 149 $[C_3H_4{}^{79}Br]^+$

2d impurity

Figure C.4 — Extracted ion, mass 149 (quantifier ion)



Key

Y abundance

t time in min

1 at *t* = 10,213 min, bromopropenamide mass = 106 $[C_2H_3{}^{79}Br]^+$

Figure C.5 — Extracted ion, mass 106 (qualifier ion)

Bibliography

[1] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[2] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[3] ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

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