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VALIDATION OF AN ANALYTICAL METHOD TO DETERMINE THE CONTENT OF ACRYLAMIDE IN ROASTED COFFEE

REPORT ON THE COLLABORATIVE TRIAL
Determination of Acrylamide in Coffee by Isotope Dilution
High Performance Liquid Chromatography Tandem Mass Spectrometry

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Contents

Summary

An inter-laboratory comparison study was carried out to evaluate the effectiveness of a method that was standardised for the determination of acrylamide in bakery and potato products for the determination of acrylamide in roasted coffee.
The intention was to extend the scope of the standardised method to roasted coffee. Therefore only slight modifications of the standardised method were tolerated.
The method is based on aqueous extraction of the roasted coffee matrix and solid phase extraction (SPE) clean-up followed by isotope dilution high performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).
The test portion of the sample was spiked with isotope labelled acrylamide and extracted on a mechanical shaker with n-hexane and water for one hour. The sample extract was centrifuged, the organic phase was discarded, and a portion of the aqueous extract was further cleaned-up by solid phase extraction on Isolute Multimode followed by Isolute ENV+ columns. The acrylamide-containing fraction eluted from the second SPE column was evaporated to about 500 µL and analysed by reverse-phase high performance liquid chromatography (HPLC) with tandem mass spectrometric detection.

Three coffee samples and one aqueous acrylamide standard solution (quality control sample) were sent to 11 laboratories from 8 EU Member States. All samples were sent as blinded duplicates.

Based on the reported results the relative standard deviations for reproducibility (RSD_R) were 11.5 % at an acrylamide level of 160 µg/kg, 10.1 % at a level of 263 µg/kg, and 9.6 % at a level of 585 µg/kg. The values for RSD_r in those materials ranged from 1 % to 3.5 %.

Introduction

The accurate determination of acrylamide in food requires robust and reliable analytical techniques. A previous collaborative study project revealed that an analytical method based on high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS) performs well for bakery and potato products [1]. This method became recently an international standard [2].

However, at the workshop on acrylamide (Brussels, 16 to 17 March 2006), jointly organised by the European Commission, Directorate General Health and Consumer Protection (EC-DG SANCO) and the European Food and Drink Federation (CIAA), it was felt that standardised methods should be made available also for more complex food matrices [3]. In particular a standardised method for the determination of acrylamide in roasted coffee was requested. Hence it was decided by the European Commission's Joint Research Centre (EC JRC), the Swedish National Food Administration (SNFA), and the Nordic Committee on Food Analysis (NMKL) to organise a second collaborative study, this time focusing on the determination of acrylamide in roasted coffee. For the sake of user-friendliness, it was agreed to extend the scope of the initially collaboratively validated method to roasted coffee instead of proposing a completely different analysis procedure. It was also decided to apply samples with naturally incurred acrylamide in the study, covering the relevant range of acrylamide levels in roasted coffee. Data fed into the EU monitoring database on acrylamide levels in food were used to identify the relevant range of content [4, 5]. About 90 % of the acrylamide contents of roasted coffee samples reported to the database lie between 150 µg/kg and 550 µg/kg. Therefore the collaborative study aimed to evaluate the performance of the analysis method for this range.

Test samples were kindly supplied by Nestlé, or were purchased in local markets.

The method validation by collaborative trial was performed and evaluated according to internationally accepted guidelines [6, 7].

Test materials for the collaborative study

Test samples

For this inter-laboratory comparison exercise a set of four different roasted coffee test materials, covering a wide range of acrylamide content, was kindly prepared by Nestlé (Orbe, Switzerland) in their pilot plants. The roasted coffee beans were shipped from Switzerland to the Institute for Reference Materials and Measurements (IRMM, Belgium), where they were ground, homogenised and bottled.

Initial experiments revealed that depending on the grinding process both the coffee samples and the mill could heat up significantly. Hence a two-stage grinding process applying a Retsch ZM 200 centrifugal mill was conducted in order to avoid heating up of samples which might result in a potential change of the acrylamide content. A 2.0 mm sieve was applied in the first and a 0.5 mm sieve in the second stage. The coffee beans were cooled before and throughout the whole grinding process with liquid nitrogen. This procedure prevented both thermally induced changes of the test material and blockage of the mill.

However, cooling with liquid nitrogen entails condensation of humidity from ambient air on the ground sample, which provokes aggregation of particles and which might lead to the growth of mould. Therefore, the ground coffee samples were freeze-dried between grinding and homogenisation. A Turbula mixer was employed for the latter.

Finally the samples were bottled at below -10°C into 10 mL amber glass vials.

Two commercial ground coffee samples were purchased in local supermarkets. For each of the two samples two packages of 500 g were mixed, homogenised as described before and bottled. Each bottle contained a sample quantity (three to four grams) that was sufficient for only one analysis.

The IUPAC protocol for the design, conduct, and interpretation of method-performance studies specifies the number of test materials to at least five [6]. In contrary, ISO 5725-2:1994 does not fix any particular number of test materials to be included in the collaborative trial [7].

However, since the targeted acrylamide content range was quite narrow, it was decided to limit the number of test materials included in the collaborative study to three, representing the lower, and upper level of the tested range, as well as one level in-between, because significant differences in the performance of the method were not expected within such narrow range of content. The set of test materials shipped to the

participants of the study was composed of one commercial ground coffee samples and two samples prepared on request by Nestlé. The test samples were labelled with numbers between 1 and 1000. Table 1 contains an overview of the test samples. The listed acrylamide contents are mean values calculated from the results of the participants after removal of outliers.

All samples were sent as blinded duplicates. The participants were requested to analyse each sample once.

Table 1: Overview of test samples

Sample number	Kind of sample	Acrylamide content
Sample 1	Commercial ground coffee sample	160 µg/kg
Sample 2	Specially prepared coffee sample	263 µg/kg
Sample 3	Specially prepared coffee sample	585 µg/kg

Trainings sample

A fourth sample was added for familiarisation with the method. The amount of this training sample was sufficient for at least four analyses. The training sample was labelled as such.

Unknown acrylamide standard solution

An aqueous standard solution of acrylamide and D_3-acrylamide was gravimetrically prepared and bottled into 2 mL autosampler vial labelled as "unknown standard solution". The solution was ready to inject and was meant to check the correctness of instrument calibration. For this purpose the D_3-acrylamide concentration (402 ng/ml) was given to the participants. The acrylamide content had to be determined by the participants and had to be reported to the organisers.

Homogeneity of the test materials

According to generally accepted procedures for homogeneity testing 10 randomly selected test samples were analysed for the acrylamide content, applying the analysis procedure that was tested in the collaborative trial.

The obtained data were subjected to one-way analysis of variance (ANOVA). The outcome of the statistical evaluation showed that in all cases the bottled test material was sufficiently homogeneous [8]. Details are given in the Annex 1.

Organisation of the collaborative study

About twenty laboratories were invited end of September 2007 for participation in the study. Eleven laboratories followed the invitation. Their participation was confirmed in writing (see Annex). Each participant received begin of November the before mentioned set of test materials and standard solutions and in addition 10 units of both types of solid phase extraction (SPE) cartridges that are specified in the analysis method.

The coffee test samples were dispatched on dry ice whereas the SPE cartridges and the standard solutions were shipped at ambient temperature. The participants were requested to store the coffee test samples at -20 °C.

Each parcel contained a detailed description of the content. A sample receipt form as well as a description of the study was attached to the parcels too. The participants were informed about sample dispatch by email. They received with the email notification pdf-files of the standard operating procedure and the description of the study, and electronic versions of the sample receipt form and the template for reporting of results. Copies of the documents are presented in Annex 2.

The design of the study included beside the measurement of the test samples also training, and quality control aspects. Each participant received a training sample for familiarisation with the analysis method, an unknown standard solution to allow evaluation of the correctness of instrument calibration, and the set of three roasted coffee test materials, which were sent as blinded duplicates. Each roasted coffee test material had to be analysed once, whereas the choice of the number of replicate analyses of the "unknown standard solution" was left to the participants. They were just requested to report the mean value of the replicate determinations.

The deadline for reporting of results was set to 31.12.2007. However, some participants requested an extension due to heavy workload. Receipt of results was finally closed mid of February. Table 3 lists the nine laboratories that reported analysis data until then.

Table 3: List of participants in the inter-laboratory comparison exercise that reported results

Institution
VWA Region South, Eindhoven, The Netherlands
National Food Institute, Dep. Food Chemistry, Søborg, Denmark
Livsmedelsverket, Uppsala, Sweden
Eurofins Analytik GmbH, Hamburg, Germany
Unilever Italia - Laboratorio Centrale, Cisterna di Latina, Italy
Landesuntersuchungsanstalt Sachsen, Dresden, Germany
Finnish Food Safety Authority EVIRA, Helsinki, Finland
ICT Prague, Prague, Czech Republic
Laboratoire Départemental de la Sarthe, Le Mans, France

Method of analysis

The method of analysis that was used in this study can be found in Annex 3.

Deviations from analysis procedure for bakery and potato products

The main differences between the analysis procedure for bakery and potato products, previously validated by collaborative trial, and the analysis procedure employed in this study consisted first of partitioning of fat and other matrix components between n-hexane and the aqueous extract, which is performed simultaneously with the aqueous extraction of the roasted coffee sample, second the clean-up of a smaller volume of extract by solid phase extraction and third the adjustment of the concentrations of calibration standards to the acrylamide content range of coffee samples.

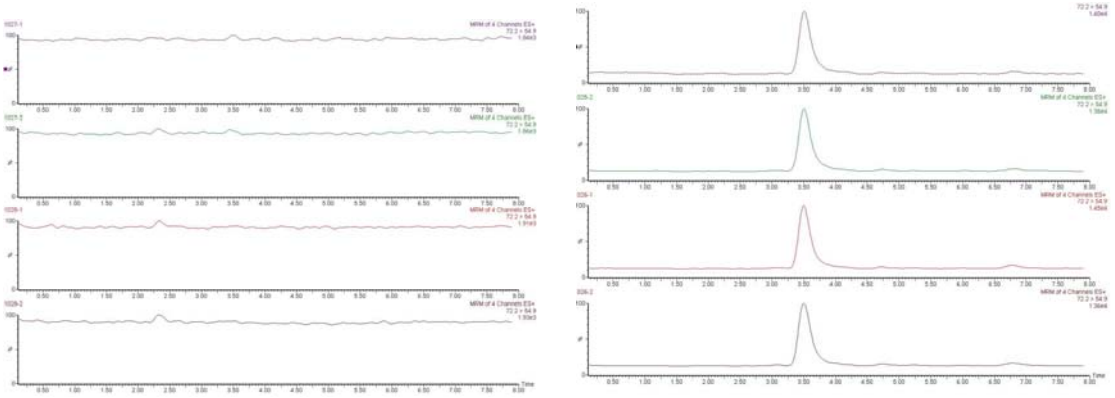
The clean up of the aqueous extract by partitioning of co-extractives into n-hexane proved beneficial from the point of accounted matrix effects in the consecutive LC-MS/MS analysis.

Figure 1 shows the chromatograms for the transition 72>55 of a coffee sample extracted and cleaned up either with or without partitioning into n-hexane. Each preparation was performed twice, and each preparation was injected into the LC-MS/MS instrument twice. Figure 1a shows chromatograms of samples that were not subjected to the partitioning step. Severe matrix effects suppressed the acrylamide signal. This was not the case when liquid/liquid partitioning into n-hexane was applied (Figure 1b).

Figure 1: a) Sample preparation without partitioning into n-hexane

b) Sample preparation with partitioning into n-hexane

a) b)



The second modification concerned the volume of extract that was loaded on the Multimode cartridge. To avoid overloading of the SPE cartridge, it was reduced from 10 mL to 2 mL. This affects of course sample enrichment. However, most commercial roasted coffee samples contain acrylamide at a level at which high enrichment factors are not anymore crucial for analyte detection. Hence lower sample enrichment should not affect the performance of the method dramatically.

The third modification consisted of the adjustment of the concentrations of calibration standards to the tested acrylamide content range. The concentration of acrylamide standards was limited to maximum 1000 ng/mL due to, compared to the analysis procedure for bakery and potato products, lower sample enrichment, and the quite narrow acrylamide content range of roasted coffee samples. However, additional acrylamide concentration levels were introduced to provide sufficient data points for the estimation of the calibration function.

Statistical analysis of results

Appropriateness of instrument calibration was checked before starting calculation on the data for the coffee test samples by scrutinising the results reported for the unknown standard solution for unacceptable large deviations from the gravimetrically established concentration. The tolerated deviation was set to 10 % of the assigned concentration. Exceeding this limit would result in exclusion of the data for the coffee samples from further evaluations. This procedure aimed to minimise the influence of biased standard preparation on the precision estimates.

The data reported by one participant was excluded from the statistical analysis because of non-compliance. The reason was that the respective laboratory reported for all test samples acrylamide contents that were about twice the contents reported by the other participants. The laboratory considered a mistake in the preparation of the isotope labelled standard as the source for the deviations, which led to the data exclusion. Results were also excluded from the data evaluation when only one of the blinded duplicate samples was analysed.

Precision estimates were obtained using a one-way ANOVA approach according to the IUPAC Harmonised Protocol [6]. Details of the average analyte content, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, and the HORRAT ratio is presented in Table 5. The collaborative trial results were also examined using Cochran's and Grubbs' tests ($p<0.025$) for evidence of outliers [6].

Results and Discussion

Table 4 lists the reported values for the acrylamide content of the coffee test materials by laboratory, each row representing one laboratory identified by a code, and the columns representing the different materials. Cells show results as reported, where no value was reported the cell is empty. Non-compliant respectively outlying results are identified by superscripts. The table contains also the results reported for the unknown standard solution. The gravimetrically established acrylamide concentration of the unknown standard solution was 211 µg/kg. All results for the unknown standard solution complied with the 10% deviation criterion. However, the data reported by participant 5 and 9 were close to the lower respectively upper limit.

Table 5 gives information on the mean value of each sample, the number of laboratories reporting results and the number of laboratories whose results were excluded respectively considered in the data evaluation. It also contains the calculated method performance parameters for repeatability and reproducibility, and the Horrat value for reproducibility [9].

The reproducibility relative standard deviation (RSD_R) was, depending on the acrylamide content level, between 9.6 % and 11.5 %. This translates into Horrat values for reproducibility of 0.5 to 0.6, which is much below the broadly accepted maximum value of 2.0 [9]. However, for all coffee samples large differences between the repeatability relative standard deviations (RSD_r) and reproducibility relative standard deviations were found, which could be caused by two facts. On the one hand the participants had already much experience with the determination of acrylamide in food, which is reflected in small repeatability standard deviations. On the other hand deviations between results reported for coffee test samples and the assigned acrylamide contents of the coffee test samples correlated well with the deviations of results for the unknown standard solution from its gravimetrically established concentration. This becomes clear for the participants 5 and 9. Hence the precision parameter RSD_R that accounts for between laboratory effects is strongly influenced by biased instrument calibration.

The performance of the method for the matrix roasted coffee agrees well with its performance for bakery and potato products [1]. The method performance was investigated for the latter matrices for acrylamide contents between about 40 µg/kg and 9100 µg/kg. Details are given for better illustration in Table 6.

Table 4: Individual results of the acrylamide content of the coffee test samples and the unknown standard solution

Laboratory		Sample 1		Sample 2		Sample 3		Unknown standard
		µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
1		159	176	280	278	572	584	202
2		185	181	292	294	645	648	210
3		281[a]	322[a]	519[a]	499[a]	923[a]	1250[a]	219
4		246[b]	271[b]	415[b]	484[b]	545	548	200
5		130	130	230	220	520[a]		190
6		164	159	261	266	582	569	210
7		159	151	265	269	587	594	213
8		148	144	227	232	493	500	210
9		177	173	277	290	662	658	232

[a] non-compliant result
[b] outlying result

Table 5: Performance parameters for determination of the acrylamide content of roasted coffee at three different content levels

	Mean	N	nc	outl.	n	s_r	RSD_r	s_R	RSD_R	HoR
	µg/kg					µg/kg	%	µg/kg	%	
Sample 1	160	9	1	1	7	5.5	3.5	18.4	11.5	0.5
Sample 2	263	9	1	1	7	4.9	1.9	26.6	10.1	0.5
Sample 3	585	8	1	0	7	5.6	1.0	56.3	9.6	0.6

Legend: Mean –mean mass fraction [µg/kg]; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics; s_r – repeatability standard deviation [µg/kg], RSD_r – relative standard deviation under repeatability conditions [%]; s_R, RSD_R– the respective values for reproducibility, HoR – the Horrat value for reproducibility

Table 6: Performance parameters for the determination of the acrylamide content of bakery and potato products

	Mean	RSD_r	RSD_R	HoR
	µg/kg	%	%	
Butter biscuits (A)	16	-	-	-
Toasted bread	38	5.5	8.5	0.3
Butter biscuits (B)	96	7.8	11.8	0.5
Spiced biscuits	249	3.7	10.4	0.5
Potato crisps (A)	324	6.0	12.7	0.7
Spiked mashed potato powder	500	5.4	8.8	0.5
Commercial potato crisps (A)	628	8.9	13.2	0.8
Crisp bread (ERM®-BD272)	980	3.1	5.4	0.3
Potato crisps (B)	2512	5.9	11.7	0.8
Commercial potato crisps (B)	4051	4.3	8.9	0.7
Potato crisps (C)	9082	5.0	9.1	0.8

Legend: Mean –mean mass fraction [µg/kg]; RSD_r: repeatability relative standard deviation, RSD_R: reproducibility relative standard deviation, HoR: Horrat ratio for reproducibility

Conclusions

The study aimed to extend the scope of an analysis method for the determination of acrylamide in bakery and potato products to roasted coffee. Hence only slight modifications of the original standard operating procedure, which were justified by the analytical performance of the method, were tolerated.

Eleven laboratories participated in the trial. The acrylamide content of the test samples that were provided as blinded duplicates ranged from about 160 µg/kg to about 580 µg/kg. Results were received from nine participants. The results of one of them were excluded from the data evaluation since there was evidence of a systematic error in the standard preparation.

The calculated method performance parameters are satisfying with regard to internationally accepted criteria as Horrat values for reproducibility were between 0.5 and 0.6, which is much below the broadly accepted maximum value of 2.0.

The organisers of the study will submit it to the Nordic Committee on Food Analysis (NMKL) for discussion and possible adoption as NMKL standard.

Acknowledgement

The organisers are very grateful for the support given by Nestlé. We would like to thank in particular Dr. Richard Stadler for the preparation and supply of suitable test materials.

The authors would also like to thank all participants for taking the burden of analysing the test samples and providing comprehensive documentation thereof.

The authors would like to thank NMKL for technical support in the organisation of the study.

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Annex 1: Homogeneity of roasted coffee test materials

Homogeneity test for test material 1

sample id	rep 1	rep 2
1	156	168
2	157	168
3	162	147
4	199	185
5	180	155
6	190	189
7	183	185
8	210	179
9	185	187
10	196	158

				mean	sd	cv
				µg/kg	µg/kg	%
				177,1	17,1	9,7

	ANOVA										Sufficient Homgeneity	
		Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*		*target σ*	*F<Fcrit?*	*s_s/σ*
		Between Groups	3695,7	9	410,6	2,19	0,12	3,02		36,8		0,29
		Within Groups	1870,8	10	187,1							*critical $s_s/\sigma = 0.3$*
		Total	5566,5	19							**ACCEPT**	

Homogeneity test for test material 2

sample id	rep 1	rep 2										
1	212	240										
2	273	280										
3	293	261										
4	261	242										
5	236	246										
6	221	224										
7	249	286										
8	188	262										
9	212	302		mean	sd	cv						
10	240	231		µg/kg	µg/kg	%						
				247,9	29,7	12,0						
	ANOVA									Sufficient Homgeneity		
		Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*		*target* σ	*F<Fcrit?*	s_s/σ
		Between Groups	7964,4	9	884,9	1,01	0,49	3,02		48,8		0,04
		Within Groups	8761,9	10	876,2							*critical* $s_s/\sigma = 0.3$
		Total	16726,3	19							**ACCEPT**	

Homogeneity test for test material 3

sample id	rep 1	rep 2
	µg/kg	µg/kg
1	586	629
2	568	546
3	624	581
4	595	560
5	580	563
6	560	587
7	529	571
8	553	597
9	632	623
10	584	610

	mean	sd	cv
	µg/kg	µg/kg	%
	583,9	28,9	5,0

Sufficient Homgeneity

	SS	*df*	*MS*	*F*	*P-value*	*F crit*		*target* σ	*F<Fcrit?*	s_s/σ
Source of Variation										
Between Groups	10390,9	9	1154,5	2,11	0,13	3,02		101,0		0,17
Within Groups	5484,3	10	548,4							*critical* $s_s/\sigma = 0.3$
Total	15875,1	19							**ACCEPT**	

Annex 2: Documents sent to participants

Registration form

Registration for participation in the validation of a LC/MS/MS method for the determination of acrylamide in coffee by collaborative trial

Company/Laboratory name:

Contact person: Mr. ☐ Mrs. ☐

First name: Family name:

E-mail:

Address for sample shipment (no P.O. Box!)**:**

Name:

Street:

Zip-code: City:

Country: Tel.:

Please return registration form to: **e-mail:** thomas.wenzl@ec.europa.eu

or fax: +32-14 571 873

Deadline: 15 September 2007

Confirmation of participation in the study

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Food Safety and Quality Unit

Geel, 30.10.2007

Dear Madame/Sir

I would like to acknowledge your participation in the method validation study by collaborative trial on the determination of acrylamide in coffee samples, which is jointly organised by DG JRC – IRMM, the Swedish National Food Administration, and NMKL.

The samples will be shipped within the next two weeks. However, you will be informed by email when they leave IRMM. The SOP and a detailed description of the study will follow as well.

With best regards

Thomas Wenzl

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Telephone: (32-14) 571 211 – Direct line: 320; Fax: (32-14) 571 783; Email: Thomas.Wenzl@ec.europa.eu
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Description of content of parcel 1

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Food Safety and Quality Unit

Geel, 06.11.2007

Acrylamide in coffee – Method validation study by collaborative trial

Sample description

Dear Madame/Sir!

Please find in this box 6 samples each numbered with numbers between 1 and 1000 and 2 samples labelled as "Training Sample".

Each of the test samples has to be analysed just once!

The "Training Sample" shall be used for familiarisation with the analysis procedure prior to the analysis of the test samples. For that purpose, laboratories are supplied with an excess of SPE cartridges, allowing performing 4 training runs. The organisers of the method validation study would kindly ask all participants to use this training opportunity.

The standard operating procedure (SOP), a detailed description of the study, a results report form as well as a questionnaire on the analysis of the test samples will be sent to you by email.

SPE cartridges, calibration standards, and an unknown acrylamide standard solution are dispatched in a separate package.

All samples were ground, and homogenised at low temperature. They were stored frozen (-20°C) and were dispatched cooled with dry ice. The acrylamide content of coffee samples might change during storage. Therefore we recommend:

STORE ALL SAMPLES AT -20°C

Please confirm receipt of the samples with the sample receipt form that was sent to you by email.

For the organising committee

With best regards

Thomas Wenzl

Retieseweg 111, B-2440 Geel, Belgium
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Description of content of parcel 2

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Food Safety and Quality Unit

Geel, 06.11.2007

Acrylamide in coffee – Method validation study by collaborative trial

Sample description

Dear Madame/Sir!

Please find in this box:

SPE cartridges Isolute ENV+, 500 mg, 6 mL	10 pieces
SPE cartridges Isolute Multimode, 1000 mg, 6 mL	10 pieces
Calibration standards	10 autosampler vials, each containing 1.5 ml of standard solution, all ready for injection Please take the concentration of the standards from the study description that was sent to you by email.
Unknown standard solution	1 vial with acrylamide standard solution in water. Please find details on the internal standard concentration in the study description that was sent to you by email

If analysis is not performed immediately, store the calibration standards and the unknown standard solution at 4°C.

For the organising committee

With best regards

Thomas Wenzl

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Study description

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Food Safety and Quality Unit

Geel, 06.11.2007

Acrylamide in coffee – Method validation study by collaborative trial

Study description

Dear Madame/Sir!

The method validation study by collaborative trial on the determination of acrylamide in coffee, jointly organised by DG JRC, SNFA and NMKL, starts with the receipt of the samples.

A 6 weeks period of time is given for reporting of the results. Results have to be reported applying the template that was sent to you by email.

Deadline for reporting of results is 31 December 2007.

Following measurements have to be performed, according to the supplied standard operating procedure (SOP)

The standard operating procedure (SOP) was sent to you by email. Please confirm receipt of the samples with the sample receipt form.

Training samples:

The training samples (two identical items) are meant for familiarisation with the analysis method. Each participant shall use these samples to get experience with the analysis protocol and to evaluate repeatability of the method.

Test samples:

Each participant received a number of 6 test samples each coded with numbers between 1 and 1000. **Each of the test samples has to be analysed just once!**

Unknown standard solution

Each participant received together with the standard solutions one 2 mL autosampler vial labelled as "unknown standard solution". This solution is ready for injection and shall be analysed with the samples. The internal standard concentration of this solution is: **402 ng/mL**

continued on the back side

Retieseweg 111, B-2440 Geel, Belgium
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http://www.irmm.jrc.be

Calibration standards:

A set of 10 calibration standards were prepared according to the SOP. Pipetted volumns were evaluated gravimetrically. The standards were filled in 2 mL amber glass autosampler vials with crimp caps and are supplied ready for injection. Each participant shall use these standards for checking her/his own calibration. Please take notice that a reliable estimation of reproducibility also includes calibration. **Therefore, please prepare your own calibration standards with your own chemicals according to the SOP, starting with the preparation of stock standards and use them for calculation of the results.**

The analyte concentrations of the standards that were sent to you are given in Table 1:

Table 1:

Name on label	Acrylamide	Internal standard
	ng/mL	ng/ml
Calibration standard 0	0	401
Calibration standard 50	50.7	401
Calibration standard 75	76.3	402
Calibration standard 100	102	402
Calibration standard 150	153	402
Calibration standard 250	253	398
Calibration standard 400	406	402
Calibration standard 500	507	402
Calibration standard 750	760	401
Calibration standard 1000	1014	401

For the organising committee

With best regards

Thomas Wenzl

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http://www.irmm.jrc.be

Sample receipt form

Method validation by collaborative for the determination of acrylamide in coffee, jointly organised by DG JRC, CSL, SNFA and NMKL

TEST MATERIALS RECEIPT FORM

Name of Participant	
Affiliation	

Please ensure that the items listed below have been received undamaged, and then make the relevant statement:

Date of the receipt of the test materials (dd/mm/yy)	
All items have been received undamaged	Yes ☐ / No ☐
Items are missing / damaged.	Yes ☐ / No ☐
Test sample parcel was received cooled	Yes ☐ / No ☐

Remarks:

Content of test samples parcel

a) 6 coffee test materials in 10 mL amber glass vials with crimp cap, coded, sample content 3 to 4 g per vial

b) 2 coffee samples in 10 mL amber glass vials with crimp caps, labelled "Training Sample", sample content 3 to 4 g per vial

c) 1 leaflet containing sample description and sample storage instructions

Content of consumables parcel required for LC-MS-MS analysis

d) 10 SPE cartridges, Isolute ENV+, 500 mg, 6 mL

e) 10 SPE cartridges, Isolute Multimode, 1000 mg, 6 mL

f) 1 set of 10 calibration standards in 2 mL amber glass crimp top autosampler vials, labelled as "Calibration standard 0" to "Calibration standard. 1000", standard volume 1.5 mL, ready for injection

g) One 2 mL amber glass crimp top autosampler vial, labelled as "Unknown standard solution", standard volume 1.5 mL, ready for injection

h) 1 leaflet containing sample description and sample storage instructions

Please return the completed form by email to: Lubomir.Karasek@ec.europa.eu
or fax it to: +32 14 571 783

Template for reporting of results

Method validation study by collaborative trial on the determination of acrylamide in coffee

Results report form

Name and address			
Name:		Street + #	
Company		City	
Department		Country	

Results for test samples								
	Sample code	Date of analysis	Number of injections		Area of internal standard peak	Area of acrylamide peak		Mean acrylamide content
		dd.mm.yy			m/z 75>58	m/z 72>55	m/z 72>44	µg/kg
1								
2								
3								
4								
5								
6								

Results for "Unknown standard solution"					
	Date of analysis	Number of injections		Mean acrylamide content	Standard deviation
	dd.mm.yy			µg/kg	µg/kg
Unknown standard solution					

Results for "Training sample" (optional)					
	Number of analysis			Mean acrylamide content	Standard deviation
				µg/kg	µg/kg
Training sample					

Reporting of the results for the training sample is voluntarily. However, we would appreciate, if you could supply us with those data.

Please fill the template and send it to: irc-irmm-acrylamide@ec.europa.eu

Please print the recorded chromatograms of all test samples showing the transitions m/z 72>55, 72>44 and 75>58 and send them by normal mail to:

Thomas Wenzl
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel
Belgium

Deadline for reporting of results: 31 December 2007

Acrylamide results report form LC-MS-MS 1/3

Annex 3: Standard operating procedure for the determination of acrylamide in roasted coffee by LC-MS/MS

Determination of acrylamide in coffee by liquid chromatography tandem mass spectrometry (LC-MS/MS)

1. SCOPE AND APPLICATION

The method is suitable for determination of acrylamide in coffee.

2. SAFETY

Acrylamide has been classified by IARC as "probably carcinogenic to humans"!

Protective equipment as laboratory coat, disposable gloves, and safety glasses have to be used.
Acrylamide and organic solvents should be handled in a fume hood with adequate air flow**.**

3. PRINCIPLE

A test portion with addition of isotopically labelled acrylamide is defatted and extracted with water. The extract is centrifuged and the supernatant undergoes clean-up with two solid phase extraction (SPE) columns.

The first SPE, (Isolute Multimode), contains silica based C-18 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.* the Multimode column is used as a chemical filter.

The second SPE, (ENV+), contains a polymer based phase with a relatively high capacity to bind acrylamide. The extract is loaded, the column washed with water and finally eluted with 60% methanol in water. The purpose of this step, apart from further cleaning of the extract, is to concentrate the extract and to obtain low levels of LOQ.

After evaporation of the methanol the extract is analysed by liquid chromatography tandem mass spectrometry LC-MS/MS. For this purpose an HPLC column with graphitised 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. APPARATUS

4.1 LC-MS/MS system

4.1.1 Liquid chromatograph consisting of:
Thermostated column compartment
Injector capable of injecting 10 µL of sample
HPLC pump capable of maintaining a mobile phase flow of 0.2 – 0.4 mL/min

4.1.2 HPLC column
Hypercarb, 5 µm, 50 mm x 2.1 mm i.d., with a guard column 5 µm, 10 mm x 2 mm i.d. (Thermo Hypersil-Keystone). Alternatively a Hypercarb, 5 µm, 100 mm x 2.1 mm i.d., with a guard column 5 µm, 10 mm x 2 mm i.d. (Thermo Hypersil-Keystone) may be applied. The stationary phase of these columns is graphitised carbon. Respective chromatograms are depicted in the Annex.
Other columns (e.g. Atlantis C_{18}, 3µm, 150 mm x 2.1 mm i.d., Waters) showing comparable performance to the column specified may be applied as alternative, but equivalence of column performance has to be demonstrated.

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

4.1.4 Data acquisition and data analysis system
Suitable software for data collection and evaluation.

4.2 Solid phase extraction system

4.2.1 Vacuum manifold for solid phase extraction

4.2.2 SPE column "Multimode":
Isolute Multimode, 1000 mg / 6 mL, from IST, Hengoed, Mid Glamorgan, UK.
Alternatives might be used, provided that equivalence is demonstrated.

4.2.3 SPE column "ENV+":
Isolute ENV+, 500 mg / 6 mL, from IST, Hengoed, Mid Glamorgan, UK.
Alternatives might be used, provided that equivalence is demonstrated.

4.3 Analytical balance
Capable of weighing to 0.01 mg.

4.4 Laboratory balance
Capable of weighing to 0.01 g.

4.5 Calibrated precision microliter pipettes
Capacity 200 – 1000 µL, and 1000 – 5000 µL.

4.6 Centrifuge tubes
Falcon tubes, 50 mL, polypropylene, disposable

4.7 Mechanical shaker (e.g. multi-wrist arm shaker) that allows well mixing of different phases.

Adjustable (for maximum sample-extractant agitation), capable of holding 50 mL Falcon tubes.

4.8 Cooled centrifuge

Capable of obtaining 3600xg for 50 mL Falcon tubes, temperature 10°C.

4.9 Equipment for solvent evaporation

Note: The evaporation temperature must not exceed 40°C.

Based on vacuum or a stream of nitrogen.

4.10 Glassware

Volumetric flasks, volume 50 mL, 100 mL etc., according to ISO 1042.

Glass vials with a volume of at least 4 mL, suitable for the evaporation equipment.

Amber glass autosampler vials suitable for the HPLC autosampler.

5. REAGENTS AND STANDARDS

Chemicals should be of the stated quality, although equivalent or higher quality reagents are permitted. Water should be HPLC grade, comply with grade 1 of ISO 3696 (electrical conductivity below 0.1 μS/cm at 25°C).

5.1 Acrylamide:

Purity ≥ 99.9 % (CAS 79-06-1)

5.2 Deuterium-labelled D_3-acrylamide, or C_{13}-labelled acrylamide

Deuterium content >98% (e.g. Polymer Source Inc. Dorval, Quebec, Canada)

5.3 Methanol - HPLC grade

5.4 *n*-Hexane - HPLC grade

5.5 Acetic acid - glacial (CAS 64-19-7)

5.6 ENV+ elution solvent:

Methanol + water solution (60 + 40, $V_1 + V_2$).

5.7 LC mobile phase solvent:

Acetic acid + water solution (1 + 1000, $V_1 + V_2$).

5.7 Standards

It is recommended to prepare standards gravimetrically.

Record the weight of the empty flask and then all weights of consequent additions of D_3-acrylamide, acrylamide and water. Calculate the final concentrations of D_3-acrylamide and acrylamide in the solution using tabulated density values of water for the given temperature.

Examples of standards preparation:

5.7.1 Stock standard solutions of acrylamide and D_3-acrylamide (1000 µg/mL):
Weigh to the nearest of 0.05 mg about 100 mg acrylamide and D_3-acrylamide respectively into volumetric flasks, dissolve in water and dilute to 100 mL. Solutions scan be stored at 4°C for at least 3 months.

5.7.2 Intermediate D_3-acrylamide internal standard solution (10 µg/mL)
Transfer 1000 µL of stock solution of D_3-acrylamide 5.7.1 (1000 µg/mL) to a 100 mL volumetric flask and dilute to marked volume with water.

5.7.3 D_3-acrylamide internal standard solution (1000 ng/mL)
Transfer 5000 µL of intermediate internal standard solution 5.7.2 (10 µg/mL) to a 50 mL volumetric flask and dilute to marked volume with water.

5.7.4 Acrylamide intermediate standard solution (100 µg/mL).
Transfer 5000 µL of stock solution of acrylamide 5.7.1 (1000 µg/mL) to a 50 mL volumetric flask and dilute to marked volume with water.

5.7.5 Acrylamide intermediate standard solution (10 µg/mL).
Transfer 5000 µL of intermediate standard solution 5.7.4 (100 µg/mL) to a 50 mL volumetric flask and dilute to marked volume with water

5.7.6 LC-MS calibration standards.
Dilute aliquots from standard solutions 5.7.2, 5.7.4 and 5.7.5 with water to give for example 0, 50, 75, 100, 150, 250, 400, 550, 750 and 1000 ng/mL respectively of acrylamide, all containing 400 ng/mL of D_3-acrylamide.
The analysis of an even higher number of calibration standards, distributed properly over the whole range of concentration, is advisable if a broad range of concentration has to be covered.

Calibration standards

Standard 0 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL) and dilute to marked volume with water.

Standard 50 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 500 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 75 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 750 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 100 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 1000 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 150 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 1500 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 250 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 2500 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 400 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 4000 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 500 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 5000 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 750 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 7500 µL of acrylamide standard solution 5.7.5 (10 µg/mL) and dilute to marked volume with water.

Standard 1000 ng/mL: Add to a 100 mL volumetric flask 4000 µL of internal standard solution 5.7.2 (10 µg/mL), 1000 µL of acrylamide standard solution 5.7.4 (100 µg/mL) and dilute to marked volume with water.

6. PROCEDURE

6.1 General notes

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

6.1.2 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 this is not a problem for HPLC analysis, make sure never to exceed 40°C during extraction or the work-up process.

6.1.3 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 < 1mm before extraction and use, if necessary, a mechanical device for preparation of homogeneous slurry, e.g. Ultra Turrax or Waring blender.

6.1.4 Sample extracts might 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 peaks of acrylamide and internal standard are large enough for a safe quantification in the end.

6.2 Extraction

Weigh, to nearest of 0.01 g, 2.0 g test portion into a 50 mL Falcon tube. Add 5 mL of *n*-hexane (alternatively cyclohexane). Add 40 mL of water. Add 400 µL of internal standard solution (1000 ng/mL) (5.7.3). Shake intensively for 15-30 s by hand and 10 – 15 s with a Vortex shaker, and then for 60 min on a mechanical shaker that allows well mixing of different solvents (e.g. a multi-wrist arm shaker or similar device), adjusted to maximum sample-extractant agitation. Centrifuge in a cooled centrifuge (10 °C, 3600 rpm, 20 min). Check proper phase separation of *n*-hexane (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.

6.3 Clean-up

For all steps adjust the flow of the SPE columns to let the liquid elute drop wise.
Check the completeness of elution of acrylamide from the ENV+ SPE cartridge by recording the elution profile, at least for each new batch of cartridges.

Fit the **Multimode column** (4.2.2) to the vacuum manifold. Condition the column with 3 mL of methanol and 2 x 6 mL of water. Pass 2 mL of the supernatant through the column, followed by 3 mL of water, and collect the combined eluate, consequently approximately 5 mL in total.

Fit the **ENV+ column** (4.2.3) to the vacuum manifold. Pre-treat the column with 5 mL methanol and 5 mL water. Load the extract (approximately 5 mL) from the previous column and discard eluate. Rinse the column once with 4 mL of water and discard the rinsing solvent. Assure that there is no eluate left in the valves/flow channels of the vacuum manifold by e.g. placing the cartridge on another (dry) position of the vacuum manifold. Rinsing solvent that is left in the valves might contain co-extracts that could interfere with the internal standard peak. After rinsing, elute the acrylamide with 2 mL of

60% methanol in water. Collect the elution solvent and the residual solvent in the cartridge (by applying a slight vacuum or a slight pressure) in a glass vial of at least 4mL, which is suitable for the evaporation equipment (4.9).

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 nitrogen, 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.

6.4 LC/MS-MS determination

6.4.1 LC-MS/MS conditions

For a successful analysis it is of high importance that the instrument is in good conditions and that all instrumental parameters are optimised.

Use the Hypercarb or other suitable column (4.1.2) and the mobile phase (5.6) at a flow rate of 200 - 400 µL/min. The column is held at room temperature. The injection volume is 10 - 20 µL.
Use electrospray ionisation in positive mode, selected reaction monitoring (SRM) and unit resolution. Optimise all parameters as probe temperatures, gas flows, voltages, and probe-position for the detection of acrylamide at the flow rate of the mobile phase. Optimise 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. Using the Quattro Ultima instrument (Waters, Micromass) the following parameter settings were successfully applied:

HPLC parameters	
HPLC Column	Hypercarb column 50x2,1 mm equipped with a Hypercarb pre-column (10x2,1 mm)
Column temperature	Room temperature
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 dm^3/h
Desolvation temperature	400°C
Nebulising gas	N_2, fully open
Cone gas	N_2, 200 dm^3/h
Collision gas	Argon, 2,3 mbar
Ion source temp	125°C
Capillary voltage	2 kV
Cone voltage	20 V
Collision energies:	
72>55 and 75>58	9 eV
72>44	20 eV
72>54	16 eV
Dwell time	0,15 sec
Inter channel delay	0,03 sec

Analyse the sample extract in duplicate, preferably triplicate. Due to possible changes of response factor during a sequence, it is recommended to run samples together with standards in short sequences of one replicate per vial. Repeat the "short sequence" for the second measurement of the sample extract. Inject the standard solutions in total at least three, preferably four times. Mix the standards and sample extract injections in the sequence. Use sufficient run time for samples to allow matrix components to elute from the column. If necessary, wash the column with 80% acetonitrile in water, as described under 6.4.3 Washing of HPLC column, in between the set of samples, or after the end of the batch.

6.4.2 System suitability

The response of the LC-MS/MS might vary during a sequence, from day to day or over longer periods. Also the HPLC column might 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:

Equilibrate the column with mobile phase and the mass spectrometer for e.g. 30 minutes. 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. The response should be similar as after the optimisation. If not, the interface needs to be cleaned and/or the mass spectrometer needs to be re-optimised. The retention time should not be below 1.5 min (when using Hypercarb 50mm x 2.1 mm at a flow rate of 0.4 mL/min), and the peak width at half height should be below 0.2 min. 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 coffee is shown in Annex. 1.
The respective chromatogram of a coffee sample analysed on a 100 mm x 2.1 mm i.d. Hypercarb column is depicted in Annex 2.
Columns not fulfilling these requirements might be reconditioned by washing (see 6.4.3). If reconditioning was not successful, the column has to be exchanged. The three injections of the standard solution should give nearly identical results.
Inject pure water to check for possible contamination of the system. No traces of acrylamide should be detected.

6.4.3 Washing of HPLC column

If the performance of the HPLC column is significantly worse than expected or required (see "system suitability") it might be restored by washing. The column can be flushed with 80% acetonitrile in water at 0.4 mL/min for 30 min in line with the LCMS. This might 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/MS, might be used:
Dispose off the guard column. Change flow direction and flush the column at room temperature and 0.2 mL/min in a consecutive order for:
(a) two hours with a mixture of 50% tetrahydrofuran (THF), 10% ammonia and 40% water. Please, consider that some polymers used for HPLC tubing are not resistant to THF
(b) 30 min with pure methanol
(c) 30 min with the mobile phase for equilibration

6.5 Analyte 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 [5].

Calibration by the internal standardisation is applied for the determination of acrylamide. This calibration requires the determination of response factors R_f defined by following equation:

$$Rf_i = \frac{A_{SAA} \times c_{[d3]AA}}{A_{[d3]AA} \times c_{SAA}} \qquad \text{Equation 1}$$

where

Rf_i is the response factor of acrylamide and d_3-acrylamide determined by the analysis of calibration standard i**.**

A_{SAA} is the area of the unlabeled acrylamide peak as SRM mass trace m/z 72>55 in the calibration standard i

$A_{[d3]AA}$ is the area of labelled d_3-acrylamide peak as SRM mass trace m/z 75>58 in the calibration standard i

$c_{[d3]AA}$ is the d_3-acrylamide concentration of the standard solution i

c_{SAA} is the acrylamide concentration of the calibration standard i

$$\overline{Rf} = \frac{\sum_{i=1}^{i=n} Rf_i}{n} \qquad \text{Equation 2}$$

where

$\overline{Rf}$ is the average of n response factors of acrylamide and d_3-acrylamide determined from n calibration standards**.**

Calculate for each sample the average of the amount of acrylamide that was extracted from the sample (X_{AA}) from the N replicate injections that were performed for the respective sample using the following equation:

$$X_{AA} = \frac{1}{N} \sum_{1}^{N} \frac{A_{AA} \times X_{[d3]AA}}{A_{[d3]AA} \times \overline{Rf}} \qquad \text{Equation 3}$$

where

X_{AA} is the amount of acrylamide (in ng) that was extracted from the sample.

A_{AA} is the area of unlabeled acrylamide peak for transition 72>55 of the sample

$A_{[d3]AA}$ is the area of the d_3-acrylamide peak for transition 75>58 of the sample

$X_{[d3]AA}$ is the absolute amount (in ng) of internal standard (d_3-acrylamide) added to the sample

$\overline{Rf}$ is the average of n response factors of acrylamide and d_3-acrylamide determined from n calibration standards**.**

The mass fraction of acrylamide in the sample c_S (µg/kg) is obtained from equation 4:

$$c_S(\mu g/kg) = \frac{X_{AA}}{W_S} \qquad \text{Equation 4}$$

where
X_{AA} is the absolute amount (in ng) of acrylamide that was extracted from the sample.
W_S is the sample weight in g

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

7. QUALITY CONTROL

For each batch of samples the following controls are used:

7.1 Calibration standards (5.7.7)

Calibration standards are analysed together with each batch and the resulting response factors are used to quantify that set of samples. At the end of each batch a mid-point verification standard should be analysed to monitor instrument drift. Acceptable values shall not deviate by more than 5% from the original concentration.

7.2 Reference materials

7.2.1 Certified reference materials

There is no certified reference material for the determination of acrylamide in coffee available on the market.
However other certified reference materials exist:
ERM BD 273, Acrylamide in toasted bread, available from the Institute for Reference Materials and Measurements (IRMM), Geel, Belgium, (www.irmm.jrc.be)
ERM BD 272, Acrylamide in crisp bread, available from Federal Institute for Materials Research and Testing (BAM), Berlin, Germany, (www.bam.de).

7.2.2 Laboratory reference materials

Commercial coffee samples could be use as laboratory internal reference materials.

7.3 Replicates

Each sample should be measured at least in duplicate. The absolute difference between replicate values for a given sample, gained by one operator, with one instrument within the shortest time interval, must be in 95 % of all cases within the repeatability standard deviation.

8. METHOD PERFORMANCE

The following data shall be generated under the conditions described in this method:

8.1 Recovery

The recovery shall be determined in each batch of samples using CRMs or other methods provided by international guidelines, e.g. spiking method [6, 7].

8.2 Repeatability and reproducibility

For analysis carried out under repeatability conditions, the intra-laboratory coefficient of variation should be between one half and two thirds of the value derived from the modified Horwitz equation [8].

For analyses carried out under intermediate (within-laboratory) reproducibility conditions, the standard deviation shall not be greater than the value derived from the modified Horwitz equation [8]:

Below an acrylamide content of 120 µg/kg, the standard deviation is set to 22 % of the acrylamide content. Above that border value, it is calculated according to equation 4, which includes the mean acrylamide content of the respective sample, expressed as dimensionless mass ratio (1 µg/kg ~ 1 ppb = 1.10^{-9}).

$$\sigma = 0{,}02\frac{\left(\overline{X}*1.10^{-9}\right)^{0{,}8495}}{1.10^{-9}} \qquad \text{Equation 4}$$

σ: target standard deviation; $\overline{X}$: mean analyte content (µg/kg)

Examples for the modified Horwitz standard deviation are:

Mean acrylamide content	Modified Horwitz standard deviation
µg/kg	µg/kg
100	22.0
200	40.8
500	88.8
1000	160.0
2000	288.2

However it has been shown that significantly lower values of the standard deviation under intermediate reproducibility conditions (compared to the values presented in the table above) can be obtained with a slight modification of the present method [9].

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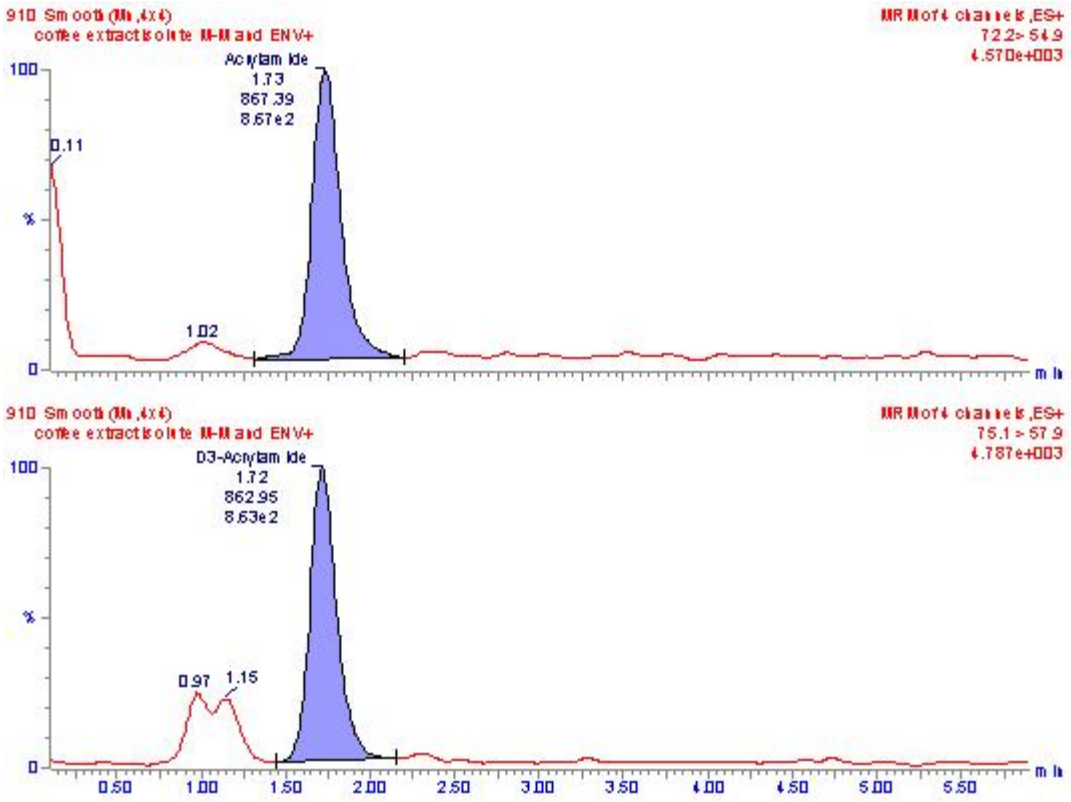
Annex

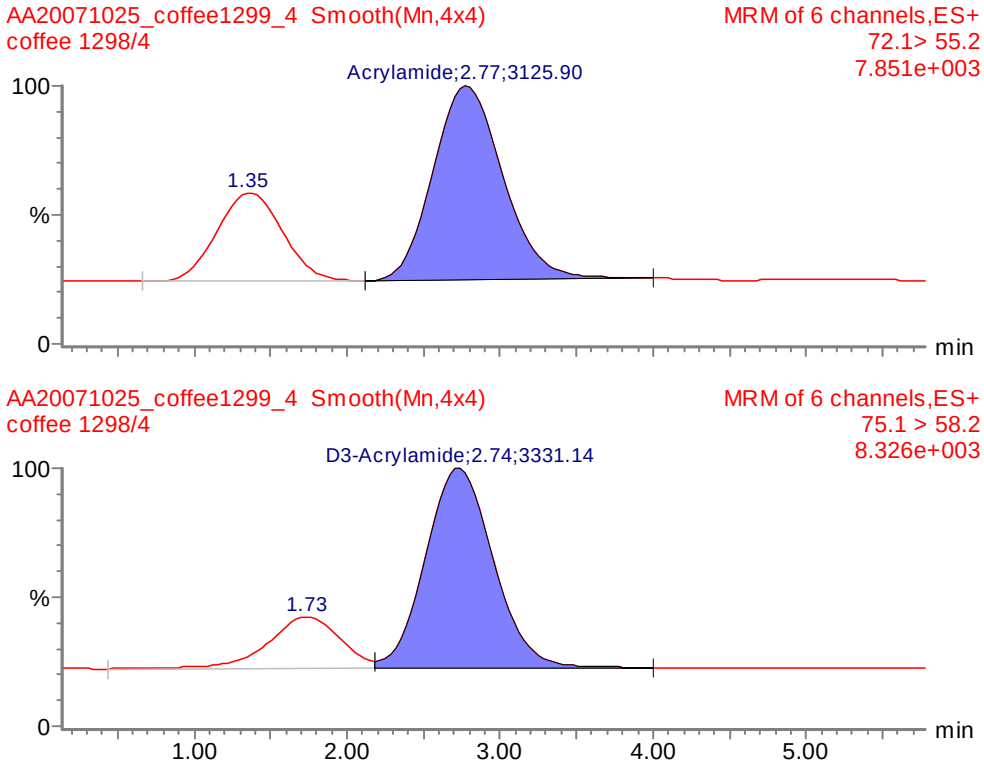
Annex 1:

Typical chromatogram for the determination of acrylamide in coffee on 50 mm x 2.1 mm i.d. Hypercarb column, flow rate 400 µL/min



Annex 2

Typical chromatogram for the determination of acrylamide in coffee on 100 mm x 2.1 mm i.d. Hypercarb column, flow rate 400 µL/min



European Commission

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DOI 10.2787/54267

Abstract
An inter-laboratory comparison study was carried out to evaluate the effectiveness of a method that was standardised for the determination of acrylamide in bakery and potato products for the determination of acrylamide in roasted coffee.
The intention was to extend the scope of the standardised method to roasted coffee. Therefore only slight modifications of the standardised method were tolerated and were introduced in the tested method only when from the analytical point of view required.
The method is based on aqueous extraction of the roasted coffee matrix and solid phase extraction (SPE) clean-up followed by isotope dilution high performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).
The test portion of the sample was spiked with isotope labelled acrylamide and extracted on a mechanical shaker with n-hexane and water for one hour. The sample extract was centrifuged, the organic phase was discarded, and a portion of the aqueous extract was further cleaned-up by solid phase extraction on first Isolute Multimode and second Isolute ENV+ columns. The acrylamide-containing fraction eluted from the second SPE column was evaporated to about 500 µL and analysed by reverse-phase high performance liquid chromatography (HPLC) with tandem mass spectrometric detection.

Three coffee samples and one aqueous acrylamide standard solution were sent to 11 laboratories from 8 EU Member States. All samples were sent as blinded duplicates.

Based on the reported results the relative standard deviations for reproducibility (RSD_R) were 11.5 % at a level of 160 µg/kg, 10.1 % at a level of 263 µg/kg, and 9.6 % at a level of 585 µg/kg. The values for RSD_r in those materials ranged from 1 % to 3.5 %.

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