



# Validation by Collaborative Trial of an Isotope Dilution Liquid Chromatographic Tandem Mass Spectrometric Method to Determine the Content of Acrylamide in Roasted Coffee

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**Validation by Collaborative Trial of an Isotope Dilution Liquid Chromatographic Tandem Mass Spectrometric Method to Determine the Content of Acrylamide in Roasted Coffee**

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## Abstract

A method for the determination of acrylamide in roasted coffee was subjected to method validation by collaborative trial. The aim was to extend the scope of a method already standardised for the determination of acrylamide in bakery and potato products to include roasted coffee. Modifications of the already standardised method were therefore kept to a minimum. The method was 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 stable isotope labelled acrylamide and extracted on a mechanical shaker with n-hexane and water for 1 hour. The sample extract was centrifuged, the organic phase was discarded, and a portion of the aqueous extract was further cleaned-up by SPE on an Isolute Multimode cartridge followed by a second clean-up step on an Isolute ENV+ cartridge. The volume of the acrylamide-containing fraction eluted from the second SPE column was reduced by evaporation and analysed by LC-MS/MS. Three coffee samples and one aqueous acrylamide standard solution were sent to 11 laboratories from 8 EU Member States. All samples were sent as blind duplicates. Based on the reported results the relative standard deviations for reproducibility ( $RSD_R$ ) were 11.5 % at a level of 160  $\mu\text{g/kg}$ , 10.1 % at a level of 263  $\mu\text{g/kg}$ , and 9.6 % at a level of 585  $\mu\text{g/kg}$ . The values for repeatability ( $RSD_r$ ) in those materials ranged from 1 % to 3.5 %. The method performance parameters are satisfying with regard to internationally accepted criteria. Hence the method would be suitable for the enforcement of regulatory limits.

**Introduction**

The accurate determination of acrylamide in food requires robust and reliable analytical methods. Still today workshops are conducted to discuss amongst others the state-of-the-art of analytical procedures for this purpose (e.g. EFSA's Colloquium 11, Tabiano, Italy, 22 to 23 May 2008). The number of papers published so far on analytical aspects of the determination of acrylamide in food is large and led to four review papers (Wenzl et al. 2003; Castle and Eriksson 2005; Zhang et al. 2005; Wenzl et al. 2007). The most frequently applied measurement techniques are gas chromatography – mass spectrometry (GC-MS) after derivatisation of acrylamide, and liquid chromatography – tandem mass spectrometry (LC-MS/MS).

A previous collaborative trial revealed that an analytical method based on LC-MS/MS performs well for bakery and potato products (Wenzl et al. 2006). This method was developed at the Swedish National Food Administration and became recently an international standard (Rosén et al. 2007; Nordic Committee on Food Analysis (NMKL) 2007).

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 challenging food matrices (Guenther et al. 2007). In particular a standardised method for the determination of acrylamide in roasted coffee was requested, as problems with the analysis of coffee samples were reported in the literature (Roach et al. 2003). Roach et al. experienced deterioration of the performance of the chromatographic method after injection of coffee extracts at room temperature within a few hours (Roach et al. 2003). Riediker and Stadler (2003) reported difficulties in the analysis of coffee samples caused by matrix interferences. The sample clean up procedure, which they applied for many types of food, was based on dual stage solid phase extraction (SPE) applying Isolute Multimode® (500mg, 3 mL, Biotage AB, Uppsala, Sweden) and AccuBond II SCX® (100 mg, 1 mL, Agilent, Palo Alto, CA, USA). However, it turned out to be not suitable for difficult food matrices such as roasted coffee or cocoa powder. An additional liquid/liquid extraction step employing ethyl acetate had to be included in the sample clean up procedure for the determination of acrylamide in these food matrices. Severe ion suppression was also noted by Granby and Fagt (2004) in the determination of acrylamide in coffee. Similarly to Riediker and Stadler (2003), they applied also Isolute Multimode® SPE cartridges for sample clean up, but in a different format (300 mg, 3 mL). The capacity of these cartridges was limited to a volume of 500 µL of coffee extract, corresponding to 20 mg of roasted

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2  
3 coffee. Loading of higher volumes of coffee extract caused severe matrix interferences.  
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5 However, the proposed clean-up procedure could not completely remove the colour from the  
6  
7 extract (Granby and Fagt 2004).

8  
9 To reduce interferences, the application of LC-MS with atmospheric pressure chemical  
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11 ionisation (APCI) was proposed for the determination of acrylamide in coffee by Senyuva and  
12  
13 Gökmen (2005). In a more recent paper, Aguas et al. (2006) published an analysis procedure  
14  
15 for the determination of acrylamide in coffee that is based on liquid/liquid partitioning and  
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17 SPE on aminopropyl cartridges (500 mg, 4 mL, Alltech Associates, Deerfield, IL, USA). The  
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19 authors achieved high levels of precision. However, the analysis protocol included azeotropic  
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21 drying of the aqueous extract, which might cause losses of the analyte, and measurement of  
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23 the samples by LC-APCI-MS/MS. Since APCI is primarily used for the determination of non-  
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25 polar compounds, it is compared to electrospray ionisation less frequently applied in food  
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27 control laboratories, which limits the broad applicability of such methods.

28  
29 However, as many problems were reported in the analysis of acrylamide in coffee and as  
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31 none of the analytical procedures proposed for that purpose was tested in a collaborative trial,  
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33 information on the precision of any analysis procedure under reproducibility conditions was  
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35 not available. Hence it was decided by the European Commission's Joint Research Centre (EC  
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37 JRC), the Swedish National Food Administration (SNFA), and the Nordic Committee on  
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39 Food Analysis (NMKL) to organise a second collaborative trial, this time focusing on the  
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41 determination of acrylamide in roasted coffee. For the sake of user-friendliness, it was agreed  
42  
43 to extend the scope of the initially collaboratively validated method to roasted coffee instead  
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45 of proposing a completely different analysis procedure. It was also decided to apply samples  
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47 with naturally incurred acrylamide in the study, covering the relevant range of acrylamide  
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49 levels in roasted coffee. Data available from the EU monitoring database on acrylamide levels  
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51 in food were used to identify the relevant contamination range (Wenzl and Anklaam 2007;  
52  
53 European Commission 2009). About 90 % of the acrylamide content of roasted coffee  
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55 samples reported to the database lie between  $150 \mu\text{g kg}^{-1}$  and  $550 \mu\text{g kg}^{-1}$ . Therefore, the  
56  
57 collaborative trial aimed to evaluate the performance of the analytical method for this content  
58  
59 range.  
60

This paper reports the set up and the outcome of the method validation study by  
collaborative trial of a LC-MS/MS method for the determination of acrylamide in roasted  
coffee, and compares the method performance data for the analysis of coffee samples to data  
gained for bakery and potato products.

**Materials and Methods**

***Design of the study***

Twenty laboratories from 12 EU Member States with experience in acrylamide analysis by LC-MS/MS were contacted to participate in the study. Of these, 11 expressed their interest in participation in the study and were therefore supplied with test materials. Finally, nine laboratories submitted results. The study was designed as a blind duplicate study according to international guidelines (International Union for Pure and Applied Chemistry (IUPAC) 1995; International Organization for Standardization (ISO) 1994). The participants were provided with the method protocol, instruction guidelines, and the test samples. They were requested to follow the method protocol as much as possible. Any deviation from the protocol had to be reported to the organisers of the collaborative trial.

The participants applied for the analyses of the test samples different models of LC-MS/MS instruments from three different suppliers. Hence any contribution of the type of instrument to the precision of the analysis method is included in the precision estimates.

***Test samples***

Samples were either purchased in local markets or specially prepared and supplied by Nestlé (Orbe, Switzerland). Commercial samples were purchased as ground coffee, whereas grinding was required in the case of the specially prepared materials. A Retsch ZM 200 centrifugal mill (Haan, DE) was applied for this purpose. Since no information on the influence of the grinding process on the acrylamide content of roasted coffee was available, a dual-stage cryo-grinding procedure was applied as a precautionary measure. 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.

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 (WAB Maschinenfabrik, Muttenz, CH) was employed for the latter. Finally the samples were bottled at below -10°C into 10 mL amber glass vials.

The IUPAC protocol for the design, conduct, and interpretation of method-performance studies specifies the number of test materials to at least five (IUPAC 1995). In contrary, ISO 5725-2:1994 does not fix any particular number of test materials to be included in the collaborative trial (ISO 1994).

However, since the targeted acrylamide content range was quite narrow, it was decided to limit the number of test materials included in the collaborative trial 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 a 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.

Homogeneity of the test samples was tested according to generally accepted procedures for homogeneity testing (Thompson et al. 2006). Ten glass vials containing the test samples were randomly selected and analysed in duplicate for the acrylamide content, applying the analysis procedure that was tested in the collaborative trial.

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 (Fearn and Thompson 2001)

All samples were sent as blinded duplicates. The participants were requested to analyse each sample once, and report results within eight weeks after reception of the test samples. However the deadline for reporting of results had to be extended due to the high workload of some participants.

Besides test materials, participants received a training sample and an unknown standard solution. The training sample, was a coffee sample prepared in the same way as the test materials and was supplied for familiarisation with the analytical method. Its amount was sufficient to perform at least four independent analyses. Reporting of results for the test sample was not mandatory. The unknown standard aimed for evaluating correctness of instrument calibrations. Participants were requested to report the acrylamide concentration of this solution to the organisers. For that purpose the internal standard concentration (402 ng mL<sup>-1</sup> of d<sub>3</sub>-acrylamide) was given to them. The unknown standard solution was bottled into 2 mL auto-sampler vials and was ready to inject.

### Analytical method

The analytical method was to a large extent identical with the analysis procedure developed at the Swedish National Food Administration that previously was validated by collaborative trial for bakery and potato products (Wenzl et al. 2007; NMKL 2007). In particular 2.0 g of roasted coffee test sample are extracted in a 50 mL falcon tube with 40 mL of water. A quantity of 400 ng isotope labelled acrylamide (deuterium content > 98 %) is



added as internal standard to the extractant. Additionally 5 mL of n-hexane are added to the falcon tube for simultaneous clean up of the aqueous extract. The extraction is performed by first vigorously shaking by hand (for 15 sec) followed by shaking on a mechanical shaker (for 60 min at maximum agitation speed). The application of a wrist-arm shaker was proposed, but not mandatory. However, orbital shakers were applied by some participants instead of the proposed type. The three phases (solid, aqueous, and organic) are separated by centrifugation for 20 min at 10 °C and 3250 xg. The organic phase is discarded and 2 ml of the aqueous phase is passed through a preconditioned Isolute Multimode<sup>®</sup> solid phase extraction cartridge (6 mL, 500 mg, Biotage AB, Uppsala, Sweden). The eluate is collected and loaded on a Isolute ENV+<sup>®</sup> solid phase extraction cartridge (6 mL, 500 mg, Biotage AB). The cartridge is rinsed with 4 mL of water followed by the elution of acrylamide with 2 mL of 60 % methanol in water. The elution solvent volume is reduced by e.g. a stream of nitrogen to a final volume of about 500 µL. A quantity of 10 µL is injected on a Hypercarb column (e.g. 50 mm x 2.1 mm, Thermo Electron Corporation, Waltham, MA, USA), run isocratically with 0.1 % acetic acid in water at a flow rate of 400 µL min<sup>-1</sup>. The participants were only free to choose the length of the Hypercarb column, which is available in 50 mm or 100 mm formats, because other phases do not show the same retention characteristics of both acrylamide and potential interferences. The detection of acrylamide had to be performed by all participants by tandem quadrupole LC-MS/MS and electrospray ionisation in positive ion mode. However, the parameter settings of the mass spectrometer and the interface were not specified, since they are a matter of tuning and change from one instrument to the other significantly. Therefore the participants were only requested to optimise sensitivity of their instrument prior to the analysis of the test samples. For acrylamide the transitions  $m/z$  72>55, 72>54 and 72>44 were recorded, whereas the transition 75>58 was used for the isotope labelled internal standard.

Typical chromatograms of the major transitions of acrylamide and isotope labelled acrylamide are depicted in Figure 1.

The main differences between the previously collaborative trial tested procedure and the current procedure 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 a smaller aliquot of the aqueous extract was cleaned up by solid phase extraction and third the concentrations of calibration standards were adjusted to the lower concentration of the final extract.

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

analysis. Figure 2 shows the chromatograms for the transition 72>55 of a coffee sample extracted and cleaned up at JRC 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 2a 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 2b). However, SNFA did not face severe matrix effects when omitting the partitioning step (Petersson et al. 2006). Hence the lower sensitivity towards matrix effects at SNFA might be reasoned by constructive differences of the LC-MS/MS instrument that was applied for the measurements. Since the partitioning step was necessary in one laboratory it was decided to include it in the study. However, since the application of n-hexane is not permitted in some laboratories, the partitioning step might be omitted after having demonstrated that the method performance is comparable to the outcome of the present study.

The second modification concerned the volume of extract that was loaded on the Isolute Multimode<sup>®</sup> cartridge. To avoid overloading of the SPE cartridge with matrix constituents, the applied extract volume was reduced from 10 mL to 2 mL. This of course affects sample enrichment. However, most commercial roasted coffee samples contain acrylamide at a level at which high enrichment factors are no longer 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 100 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.

## Results and Discussion

The first part of the data evaluation aimed at identifying non-compliant or outlying results from the reported dataset. Appropriateness of instrument calibration was checked before performing any statistics by scrutinising the results reported for the unknown standard solution for unacceptable large deviations from the gravimetric preparation value (211 ng/mL). 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. All reported results were within the acceptable range, although the results of two participants were at the limits.

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.

The collaborative trial results, which are listed for the individual participant in Table 1, were examined using Cochran's and Grubbs' tests ( $p < 0.025$ ) for evidence of outliers. Data identified by these tests as outliers were also excluded from the data evaluation.

Precision estimates were obtained in the second part of the data evaluation by using a one-way ANOVA approach according to the IUPAC Harmonised Protocol (IUPAC 1995). Details of the average analyte content, the standard deviations for repeatability ( $s_r$ ) and for reproducibility ( $s_R$ ), as well as the respective relative standard deviations ( $RSD_r$  and  $RSD_R$ ), the number of outliers, and the HORRAT value (HoR) for reproducibility are presented in Table 2. The HoR value is frequently used as a fitness-for-purpose criterion for the selection of appropriate methods of analysis for the official control of food and feed (Commission of the European Communities 2007).

The reproducibility relative standard deviation ( $RSD_R$ ) was, depending on the acrylamide content level, between 9.6 % and 11.5 %. This translates into HoR values for reproducibility of 0.5 to 0.6, which is significantly below the broadly accepted maximum value of 2.0 (Horwitz and Albert 1991). However, for all coffee samples large differences between the repeatability and reproducibility relative standard deviations were found, which could be caused by two factors. 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 by the participants 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 become clear for participants 5 and 9, whose results deviated from the assigned values by -11.1 % and -18.8 %, and by 7.8 % and 12.8 % for the coffee samples, and by -10.0 % and 10.0 % for the unknown standard solution. Hence, the under-respectively overestimation of the analyte contents of the coffee samples is at least partially caused by biased instrument calibration. This shortcoming affects only the precision

parameter that accounts for between-laboratory effects, which consequently explains the large difference between intra-laboratory and inter-laboratory precision estimates. Therefore special attention has to be given to correct preparation of calibration standards.

The performance criteria of the method for roasted coffee agree well with those for bakery and potato products (Wenzl et al. 2006). The method performance was investigated for the latter matrices for acrylamide contents between about  $40 \mu\text{g kg}^{-1}$  and  $9100 \mu\text{g kg}^{-1}$ . Details are given for better illustration in Table 3. The HoR values for reproducibility were for all samples below 1.0, which means that the precision of the method is better than the precision value predicted by the Horwitz equation. Hence the method would be suitable for the enforcement of regulatory limits.

## Conclusions

A method for the determination of acrylamide in roasted coffee samples by LC/MS-MS was validated by collaborative trial. The trial was organised and evaluated according to internationally accepted standards. Nine laboratories from eight EU Member States reported analytical results for roasted coffee test samples covering the acrylamide content range of  $160 \mu\text{g kg}^{-1}$  to  $585 \mu\text{g kg}^{-1}$ . The corresponding reproducibility relative standard deviations were between 11.5 % and 9.6 %. The calculated method performance parameters satisfy internationally accepted criteria with Horrat values for reproducibility between 0.5 and 0.6, which is much below the generally accepted maximum value of 2.0. However, the inter-laboratory precision estimate was significantly higher than the intra-laboratory precision, which was most likely the consequence of biased instrument calibration. Hence special regard shall be given to this part of the analytical procedure.

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**Figure captions**

Figure 1: Typical chromatogram of the LC-MS/MS detection of acrylamide (m/z 72>55) and d3-acrylamide (m/z 75>58) in coffee on a 100 mm x 2.1 mm i.d. Hypercarb column, flow rate 400 µL/min

Deleted: Figure 1: Scheme of sample preparation procedure

Deleted: 2

Deleted: Typical chromatogram for the determination of acrylamide in coffee

Figure 2: SRM chromatograms of the trace 72>55 of a roasted coffee sample both cleaned up and injected twice: a) without partitioning into n-hexane and b) including partitioning into n-hexane

Deleted: 3



Figure 1

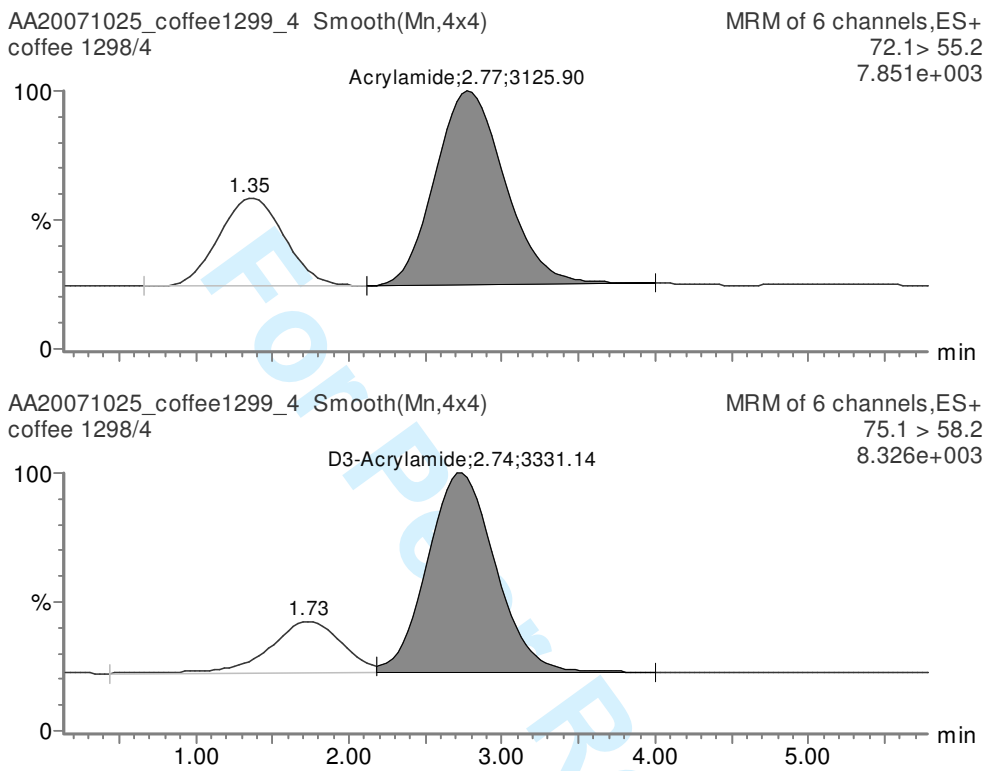


Figure 2

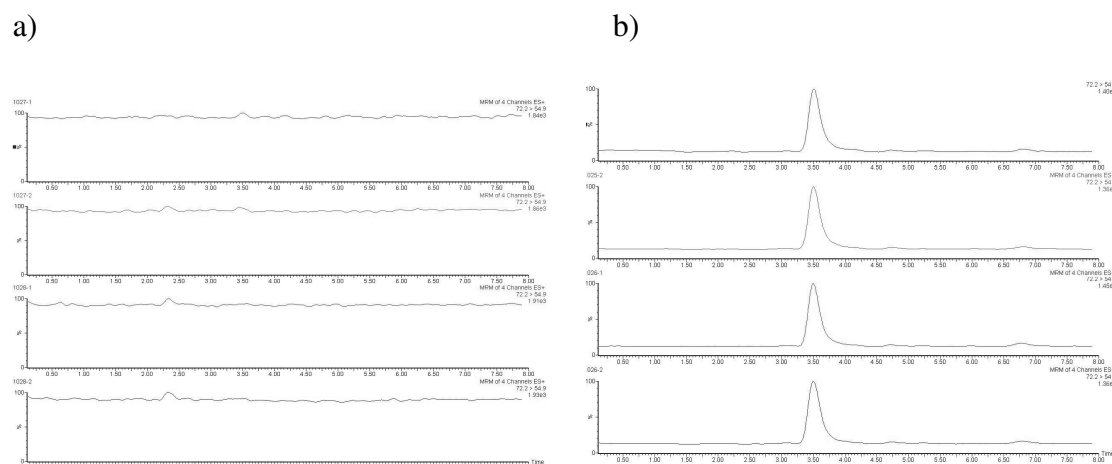


Table 1: Individual results of the acrylamide content of the coffee test samples and the unknown standard solution

Laboratory		Sample 1		Sample 2		Sample 3		Unknown standard
		$\mu\text{g kg}^{-1}$	$\mu\text{g kg}^{-1}$	$\mu\text{g kg}^{-1}$	$\mu\text{g kg}^{-1}$	$\mu\text{g kg}^{-1}$	$\mu\text{g kg}^{-1}$	$\mu\text{g kg}^{-1}$
1		159	176	280	278	572	584	202
2		185	181	292	294	645	648	210
3		281 <sup>a</sup>	322 <sup>a</sup>	519 <sup>a</sup>	499 <sup>a</sup>	923 <sup>a</sup>	1250 <sup>a</sup>	219
4		246 <sup>b</sup>	271 <sup>b</sup>	415 <sup>b</sup>	484 <sup>b</sup>	545	548	200
5		130	130	230	220	520 <sup>a</sup>	-	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

<sup>a</sup> non-compliant result

<sup>b</sup> outlying result

Table 2: Performance parameters for the 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
	$\mu\text{g kg}^{-1}$					$\mu\text{g kg}^{-1}$	%	$\mu\text{g kg}^{-1}$	%	
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 [ $\mu\text{g kg}^{-1}$ ]; N – number of labs; nc – non-compliant laboratories; outl. – outlying laboratories; n – number of laboratories used for statistics;  $s_r$  – repeatability standard deviation [ $\mu\text{g kg}^{-1}$ ],  $RSD_r$  – relative standard deviation under repeatability conditions [%];  $s_R$ ,  $RSD_R$  – relative standard deviation reproducibility conditions, HoR – the Horrat value for reproducibility

Table 3: Performance parameters for the determination of the acrylamide content of bakery and potato products (Wenzl et al. 2006)

	Mean	RSD <sub>r</sub>	RSD <sub>R</sub>	HoR
	µg kg <sup>-1</sup>	%	%	
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 <sup>®</sup> -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<sup>-1</sup>]; RSD<sub>r</sub>: repeatability relative standard deviation, RSD<sub>R</sub>: reproducibility relative standard deviation, HoR: Horrat ratio for reproducibility