Detailed report on the second European inter-laboratory comparison study on the determination of acrylamide in food - Acrylamide in crispbread samples

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Introduction

Acrylamide (AA) is a substance that has found widespread application in industry, e.g. for the purification of drinking water and in food packaging. Due to its toxicological properties, legal limits have been set for both drinking water and for migration into food [1-5].

Since the finding of elevated levels of acrylamide in heat-treated potato products and other goods was reported by the Swedish National Food Authority in April 2002, concerted efforts have been made to try to improve the image of the nutritional uptake of this substance by monitoring its content in different kinds of food [6, 7].

Following a request of the participants of the European workshop on "Analytical methods for the acrylamide determination in food" (April 2003, Oud-Turnhout, Belgium), the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Directorate General Joint Research Centre (DG JRC) organised a first inter-laboratory comparison test on the determination of AA in butter cookies and crispbread samples, including raw and spiked crumb extracts (July 2003) [8-10]. From this first test, it became clear that additional training efforts would be necessary for a significant number of laboratories. Therefore, a second trial was organised by the JRC to evaluate the progress of the laboratories. The second trial was scheduled for March 2004 and focused this time on the determination of acrylamide from different crispbread samples and crispbread extracts only. The set of samples was completed by AA standard solutions, which were prepared by dissolution of solid AA in appropriate solvents by the coordinator.

The study was a dedicated collaborative trial and was again free of charge for the participants. The organisation of the study as well as the evaluation of the results was done according to "The International Harmonised Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories", further-on denoted as "Harmonised Protocol" [11]. It was announced via the Directorate General Health and Consumer Protection (DG SANCO) to the national food authorities of EU Member States and EU Candidate Countries. Additionally all participants of the first round were informed by email (see Annex 1). Information concerning the application procedure for the study was also available on the homepage of the Food Safety and Quality Unit (FSQ) of JRC-IRMM.

In order to facilitate the application procedure, a special application form was sent to the interested laboratories (see Annex 2).

Forty-three laboratories subscribed for participation in the ring trial. Most of them belonged to 14 European countries. Receipt of the test samples was confirmed by the participants via the sample receipt form (see Annex 3).

The participants were asked to determine the AA content in the test samples by application of their usual in-house analysis methods.

In total, 42 data sets with the results of at least one sample were reported to the organisers of the study. A special report form, which was made available to the participants (see Annex 4), had to be used for reporting. One participant could not meet the deadline for analysis due to the breakdown of the instrument. In order to keep confidentiality, the identity of the laboratories were coded by a unique number between 1 and 100, which will be used further on. Details regarding the analytical methods used were requested from the participants. A summery of the applied methods is given in Annex 6.

Test Materials

Commercial brands of crispbread were purchased in German and Belgian local markets. The crispbread was coarsely ground with a Romer Analytical Sampling Mill (Romer Labs Inc., Union MO, USA) before subsequent grinding with a Baumeister UDL VA mill (1 mm hole screen) (Baumeister Verfahrenstechnik GmbH, Norderstedt, Germany). The resulting powder was homogenised in a cement mixer for 1 h. Both materials were split into portions of approximately 50 g in amber glass vials, which were stored at +4 °C. Each vial was individually numbered. The homogeneity of the samples was tested as it is described below.

Extracts from crispbread

Crispbread sample 1 was extracted with water in the following way: 1000 g of crispbread was weighed into a 15 L bucket. 10 L of water was poured over the sample, which was extracted by means of an Ultra Turrax for 30 minutes at room temperature. The extract was centrifuged for 10 minutes at 10 °C at 2000 x g in portions of 250 mL. The aqueous extract was collected in another bucket and finally divided into two equal portions. One portion remained untreated and was filled into 50 mL brown glass vials, while the other portion was spiked with an aqueous AA standard solution to give a spiking level of 50.8 ng/mL. The spiked extract was

homogenised by intensive stirring and was also portioned into 50 mL brown glass vials. To avoid additional alteration of the matrix, neither the raw extract nor the spiked extract were stabilised. All vials were filled close to the rim, tightly sealed with PTFE coated butyl septa in aluminium crimp caps and labelled with self-adhesive paper labels that contained the sample name and a short sample description.

Acrylamide standards solutions

The standard solutions were prepared by weighing of about 40 mg acrylamide of minimum 99 % purity (Sigma, Sigma-Aldrich CO, St. Louis, MO, USA) into 200 mL volumetric flasks and dissolving in high purity water (MilliQ, Millipore, Brussels, Belgium) for HPLC or ethyl acetate (EtAc), quality SupraSolv^{TM}, (Merck, Darmstadt, Germany) for GC/MS measurement without derivatisation of AA.

The standards were diluted to give final AA concentrations of 60.9 ng/mL (Standard A) and 40.7 ng/mL (Standard B) for the aqueous solutions, and 607.6 ng/mL (Standard C) and 444.4 ng/mL (Standard D) for the solutions in EtAc. Amber glass vials (25 mL) were filled with the standard solutions and tightly sealed with PTFE coated butyl septa and aluminium crimp caps. The vials were labelled as "Acrylamide Standard Solution"; the solvent used was also mentioned. The acrylamide contents of standard A and C were given on the label.

All vials were put after filling immediately into a refrigerator and were stored at 4 °C.

Dispatch of samples

All samples were sent via express mail in polystyrene boxes, equipped with a cooling cell, which was pre-cooled to -20 °C. Most of the participants reported sample receipt within 24 hours after sending.

Homogeneity of samples

Homogeneity was tested according to the Harmonised Protocol [11].

Crispbread and crispbread extracts

Ten randomly selected packages of each test sample were analysed in duplicate applying the following method: Two g of the homogenised sample was defatted with *n*-hexane. Internal standard, d3-acrylamide, (200 ng) was added and after an equilibration time of 30min, 20 mL of water was admixed. The sample/water mixture was homogenised by means of an Ultra Turrax homogeniser. Acrylamide was extracted in a sonicator at 60 °C for 30 min. The sample was purified by adding 500 µL of Carrez I (potassium hexacyanoferrat (II), c = 150 g/L) and Carrez II (zinc acetate, c = 300 g/L) solutions. The sample was centrifuged at 4500 x g for 20 min and the supernatant was decanted. Two mL of the extract were pipetted onto preconditioned Isolute Multimode SPE cartridges (size: 3 mL, 300 mg) (International Sorbent Technology, Hengoed, Mid Glamorgan, UK). The first mL of the eluate was discarded, the second was collected and analysed.

The quantification of acrylamide was performed by liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) with electrospray ionisation in positive mode. Acrylamide was identified by multiple reaction monitoring (MRM) set to record m/z 72>72, 72>55 and 72>44. Monitored transitions for the internal standard were m/z 75>75, 75>58 and 75>44. Quantification was performed by comparison of the peak area ratio of acrylamide with the internal standard d_3-acrylamide, monitored by using the MRM transition m/z 72>55 (acrylamide) and 75>58 (d_3-acrylamide).

Ten randomly selected vials of each extract were analysed in duplicate by LC/MS/MS applying a modification of the mentioned protocol. The internal standard solution was directly added to the aqueous sample (100 ng to 10 mL of extract).

Standard solutions

The standard solutions were homogenised by vigorously shaking, therefore sufficient homogeneity could be assumed. The AA content of the standard solutions was checked by six-fold LC/MS/MS measurement of the aqueous standard and six-fold GC/MS measurement of the standard in EtAc. Since the standard solutions did not contain any internal standard (ISTD), d_3-AA, 98% deuterium (Cambridge Isotope Laboratories, Andover, MA, USA) was

added prior to the measurements. The measurement results confirmed the calculated AA contents.

The homogeneity of the test samples were proved by subjecting the results of the duplicate measurements to one-way "analysis of variance" (ANOVA). The results are given in Table 1-5 of Annex 5. Sufficient homogeneity was found for the crispbread samples, as well as for the crispbread extracts.

Statistical evaluation of the results

Assigned value

The assigned concentration of AA in the test materials was calculated for the respective test sample from the reported mean values of the duplicate determinations of the participants by application of robust statistics. The striking advantages of robust statistics compared to the traditional approach has recently been demonstrated by the Analytical Methods Committee of Royal Society of Chemistry (AMC) [12]. It has the advantage that the detection and rejection of outliers is not necessary, thus the impact of extreme values on the average and the standard deviation is down weighted. Furthermore, the methods work well with data distributions that deviate significantly from the Gaussian distribution, as it was the case in this study. The robust mean values and robust standard deviations were computed by application of a MS Excel® macro that was written by the AMC. The respective figures are tabulated for each test sample in the following sections of the report. The reliability of the calculated robust mean value was counterchecked by visualising the data distribution by kernel density estimation [13]

Performance indicator and target standard deviation

The performance of laboratory *i* is expressed by the z_i-score, which is calculated according to equation 1.

$$z_i = \frac{x_i - \overline{X}}{\sigma} \qquad \text{Equation 1}$$

z_i: z-score of laboratory *i* for the respective sample; x_i reported AA content of laboratory *i* for that sample, expressed as the mean of duplicate determinations; $\overline{X}$: assigned value for the respective sample, σ: target standard deviation

The target standard deviation was calculated according to a proposal of Thompson, which applies a concentration dependent modification of the Horwitz equation [12]. Below an assigned value of 120 µg/kg, the target standard deviation was set to 22 % of the assigned value. Above that border value, it was calculated according to equation 2, which includes the assigned value, expressed as dimensionless mass ratio (1 µg/kg ~ 1 ppb = 1.10^{-9}).

$$\sigma = 0{,}02\frac{\left(\overline{X}*1.10^{-9}\right)^{0{,}8495}}{1.10^{-9}} \qquad \text{Equation 2}$$

σ: target standard deviation; $\overline{X}$: assigned value (µg/kg)

Since the target standard deviation depends only on the assigned value, it is not influenced by the width of the distribution of the reported analysis results. Consequently, the comparison of different proficiency tests (PTs) on the same analyte/matrix combination is facilitated.

z-Scores were calculated for the crispbread samples, the raw and the spiked crispbread extract samples. They were not computed for the AA standard solutions, because this would not reflect the proficiency of the laboratories in the determination of AA in food. The acceptability of a laboratory's performance was evaluated according to the following generally accepted limits [9]:

$\lvert z\rvert \leq 2.0$	satisfactory
$2.0 < \lvert z\rvert < 3.0$	questionable
$\lvert z\rvert \geq 3.0$	unsatisfactory

A z-score was not assigned, if the reported AA content was below the limit of quantification (LOQ).

Evaluation of the analysis data for the crispbread samples

Overview

Laboratories that reported numeric values for the AA content of the samples were considered in the statistical evaluation of the results, except those that reported numeric values below the LOQ of the applied method. These were discarded from the evaluation of the respective sample. Also the results that were given as “below LOQ” were excluded from the evaluation of the respective sample. The latter two are marked by a “x” in the following tables.

According to the Harmonised Protocol, robust statistics was applied for the evaluation of the results of analysis, Therefore, it was not necessary to exclude outliers from the statistical evaluation, although some results were identified being outliers.

The distribution of the results was checked by kernel density estimation. This analysis is also capable of determining multimodality. In general the results of analysis were not normally distributed and the respective kernel density plots showed at least 3 different modes.

Assigned value and target standard deviation

The assigned value was determined by different procedures, all of them based on robust statistics. The simplest robust estimate of the mean value is the median. A more elaborated estimation is represented by an iterative approach that is known as Huber H15. These two estimates were compared with the major mode of the kernel density plots.

However, the evaluations confirmed that the median of the data sets could be selected as the assigned value.

Consequently, the target standard deviation was calculated from this value.

z-Scores of the participants

The mean values of the duplicate determinations of AA in the crispbread sample are tabulated with the corresponding z-score in tables 2, 4 and 6; summary statistics are presented in tables 1, 3 and 5. Figures 1-3 show the plot of z-scores in ascending order. z-Scores were not attributed to results that were reported as ”below LOQ”, indicated in the tables by a “x”.

Crispbread sample 1

Table 1: Summary statistics for crispbread 1

Number of results		40		
Range of results	µg/kg	11.0	to	390.0
Median	µg/kg	45.8		
Huber H15	µg/kg	46.1		
Major mode	µg/kg	44.3		
Assigned value	µg/kg	45.8		
Target standard deviation	µg/kg	10.1		
Number of not satisfactory performing laboratories		6		

Figure 1: Plot of z-scores for the crispbread sample 1

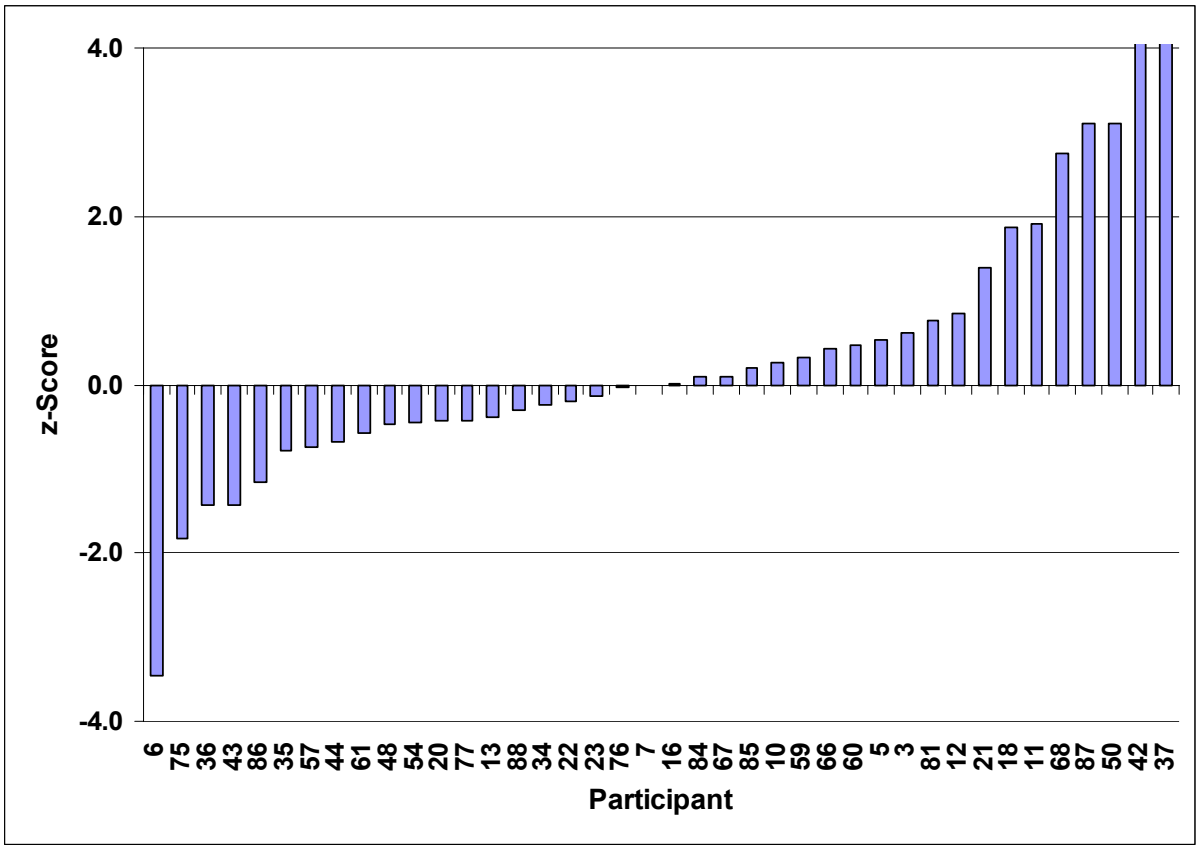

Table 2: Results of analysis and z-scores for the crispbread sample 1; bold printed z-scores mark unsatisfactory results, "x" indicates "below LOQ"

Participant	Reported result	z-Score		Participant	Reported result	z-Score
	µg/kg				µg/kg	
3	52.05	0.62		**44**	39.00	-0.68
5	51.20	0.53		**48**	41.10	-0.47
6	11.00	**-3.45**		**49**	x	x
7	45.65	-0.02		**50**	77.00	**3.09**
10	48.50	0.27		**54**	41.32	-0.45
11	65.00	1.90		**57**	38.35	-0.74
12	54.38	0.85		**59**	49.00	0.31
13	42.00	-0.38		**60**	50.50	0.46
16	46.00	0.02		**61**	40.00	-0.58
18	64.63	1.87		**66**	50.14	0.43
20	41.50	-0.43		**67**	46.85	0.10
21	59.75	1.38		**68**	73.50	**2.75**
22	43.90	-0.19		**75**	27.50	-1.82
23	44.55	-0.13		**76**	45.50	-0.03
34	43.50	-0.23		**77**	41.50	-0.43
35	38.00	-0.78		**81**	53.42	0.75
36	31.40	-1.43		**84**	46.75	0.09
37	390.00	**34.14**		**85**	47.90	0.21
38	x	x		**86**	34.22	-1.15
42	355.50	**30.72**		**87**	77.00	**3.09**
43	31.50	-1.42		**88**	42.85	-0.30

Crispbread sample 2

Table 3: Summary statistics for crispbread 2

Number of results		42		
Range of results	µg/kg	229.2	to	1100.0
Median	µg/kg	497.5		
Huber H15	µg/kg	497.7		
Major mode	µg/kg	496.9		
Assigned value	µg/kg	497.5		
Target standard deviation	µg/kg	88.4		
Number of not satisfactory performing laboratories		4		

Figure 2: Plot of z-scores for the crispbread sample 2

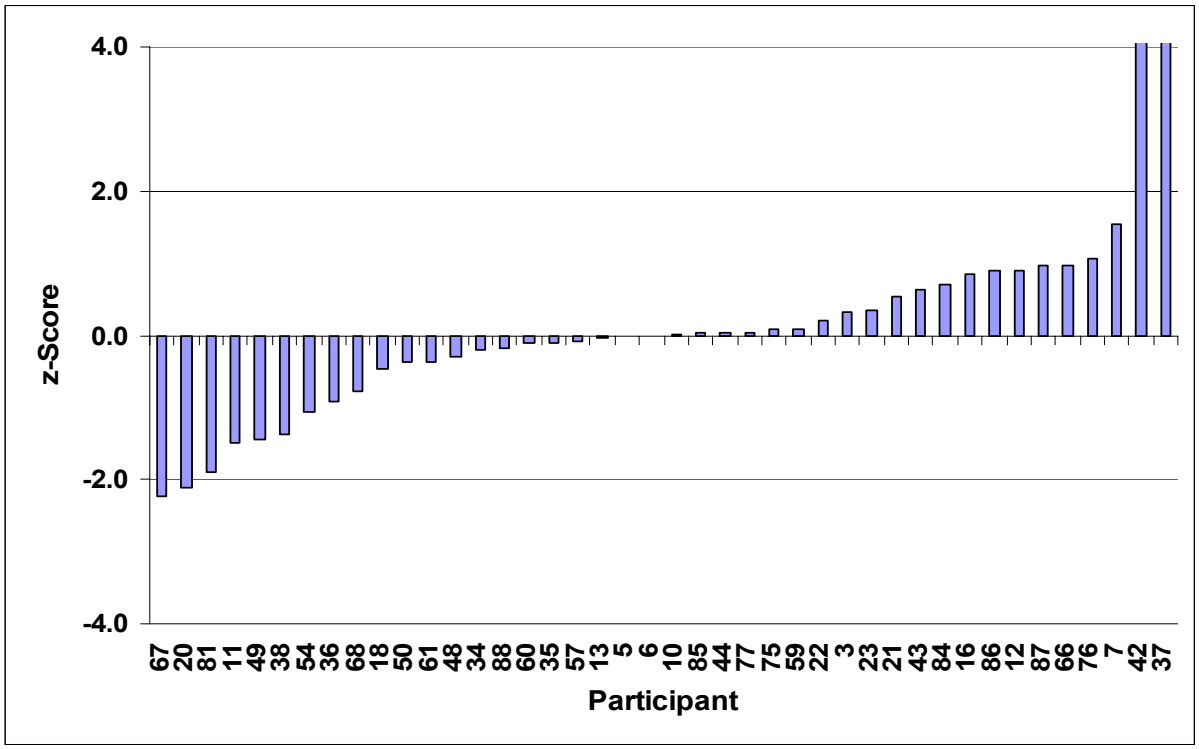

Table 4: Results of analysis and z-scores for the crispbread sample 2; bold printed z-scores mark results outside the acceptable range

Participant	Reported result	z-Score		Participant	Reported result	z-Score
	µg/kg				µg/kg	
3	526.50	0.33		**44**	500.00	0.03
5	496.48	-0.01		**48**	470.50	-0.31
6	497.50	0.00		**49**	369.00	-1.45
7	633.00	1.53		**50**	464.00	-0.38
10	499.00	0.02		**54**	403.68	-1.06
11	365.00	-1.50		**57**	489.50	-0.09
12	577.15	0.90		**59**	505.00	0.08
13	494.00	-0.04		**60**	487.00	-0.12
16	571.50	0.84		**61**	465.00	-0.37
18	456.05	-0.47		**66**	583.20	0.97
20	310.00	**-2.12**		**67**	299.25	**-2.24**
21	545.70	0.55		**68**	428.00	-0.79
22	515.00	0.20		**75**	505.00	0.08
23	528.25	0.35		**76**	591.50	1.06
34	479.50	-0.20		**77**	500.00	0.03
35	488.00	-0.11		**81**	330.29	-1.89
36	415.50	-0.93		**84**	559.50	0.70
37	1100.00	**6.82**		**85**	499.70	0.02
38	375.75	-1.38		**86**	576.38	0.89
42	1041.70	**6.16**		**87**	583.00	0.97
43	553.50	0.63		**88**	481.95	-0.18

Crispbread sample 3

Table 5: Summary statistics for crispbread 3

Number of results		42		
Range of results	µg/kg	248.8	to	905.4
Median	µg/kg	413.5		
Huber H15	µg/kg	422.0		
Major mode	µg/kg	401.6		
Assigned value	µg/kg	413.5		
Target standard deviation	µg/kg	75.5		
Number of not satisfactory performing laboratories		8		

The broader range between the different estimates of the mean, compared to crispbread sample 2, is certainly the consequence of a broader distribution of the results of analysis than it was found for crispbread sample 2. This is indicated by the robust relative standard deviation, which was calculated by using the median and the robust equivalent of the standard deviation, the adjusted median absolute deviation (MADe). For the crispbread sample 2 the robust relative standard deviation was 12.4 % and for crispbread sample 3 14.7 %.

Figure 3: Plot of z-scores for the crispbread sample 3

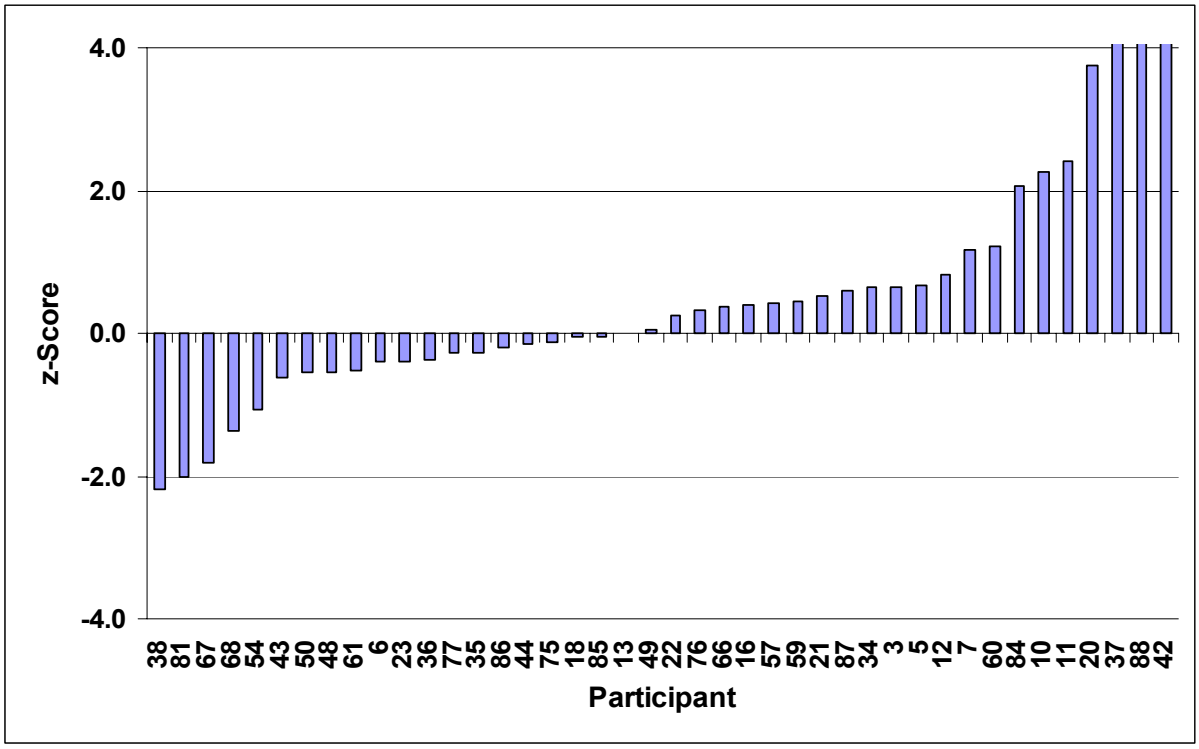

Table 6: Results of analysis and z-scores for the crispbread sample 3; bold printed z-scores mark results outside the acceptable range

Participant	Reported result	z-Score		Participant	Reported result	z-Score
	µg/kg				µg/kg	
3	462.50	0.65		**44**	401.50	-0.16
5	464.66	0.68		**48**	373.00	-0.54
6	383.50	-0.40		**49**	417.50	0.05
7	502.00	1.17		**50**	372.50	-0.54
10	585.00	**2.27**		**54**	333.36	-1.06
11	595.00	**2.40**		**57**	445.50	0.42
12	475.05	0.81		**59**	448.00	0.46
13	413.50	0.00		**60**	506.00	1.22
16	443.00	0.39		**61**	375.00	-0.51
18	409.49	-0.05		**66**	441.56	0.37
20	696.00	**3.74**		**67**	276.35	-1.82
21	452.34	0.51		**68**	311.00	-1.36
22	433.00	0.26		**75**	403.50	-0.13
23	384.05	-0.39		**76**	438.00	0.32
34	462.50	0.65		**77**	392.50	-0.28
35	393.50	-0.26		**81**	261.50	-2.01
36	385.00	-0.38		**84**	569.00	**2.06**
37	790.00	**4.98**		**85**	409.85	-0.05
38	248.85	**-2.18**		**86**	398.74	-0.20
42	905.40	**6.51**		**87**	458.00	0.59
43	367.00	-0.62		**88**	858.80	**5.89**

Evaluation of the analysis data for the crispbread extracts

The determination of the assigned value, the target standard deviation as well as the z-scores was done as described for the crispbread samples.

Laboratories that reported very high values for these samples were contacted by the organiser to check for calculation and/or reporting mistakes, e.g. reporting in the wrong units (µg/kg instead of ng/mL). The latter could happen if the results were produced by an automatic routine that considers a tenfold lower AA content in the extract compared to the solid sample. This is the case in many analytical protocols for the determination of the AA content of food by LC/MS/MS (see Appendix 6). As far as this request for clarification was answered by the respective participants, the correctness of the units was confirmed.

Raw crispbread extract

Overview

The raw extract was prepared by extraction of 1000 g of crispbread with 10 L of water. Therefore, the reported results of analysis have to be multiplied by a factor of 10 to get the acrylamide content of the "dry" sample (natural humidity was not removed prior to the extraction).

Eleven participants stated an AA content of the sample below the LOQ of their method. The residual 31 laboratories reported figures for the raw extract. Results from 6 laboratories were discarded because the given figures were below the LOQ that was reported by the participant for the respective analysis method.

The residual 25 results of analysis ranged over more than one order of magnitude.

Table 7: Summary statistics for the raw crispbread extract

Number of results		25		
Range of results	ng/mL	3.0	to	64.5
Median	ng/mL	4.9		
Huber H15	ng/mL	5.1		
Major mode	ng/mL	4.6		
Assigned value	ng/mL	4.9		
Target standard deviation	ng/mL	1.08		
Number of not satisfactory performing laboratories		6		

Figure 4: Plot of z-scores for the raw crispbread extract

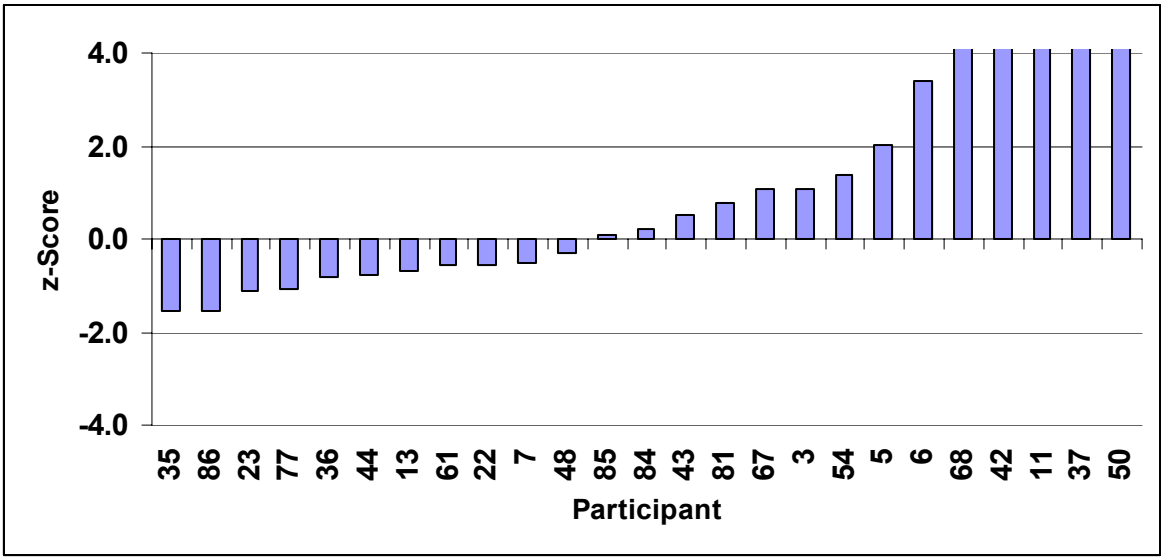

Table 8: Results of analysis and z-scores for the raw crispbread extract; bold printed z-scores mark results outside the acceptable range, results below the LOQ of the applied method are marked by a "x"

Participant	Reported result	z-Score		Participant	Reported result	z-Score
	ng/mL				ng/mL	
3	5.65	1.06		**44**	3.80	-0.77
5	6.60	2.00		**48**	4.26	-0.32
6	8.00	**3.39**		**49**	x	x
7	4.05	-0.53		**50**	64.65	**59.62**
10	x	x		**54**	5.98	1.39
11	40.00	**35.15**		**57**	x	x
12	x	x		**59**	x	x
13	3.89	-0.68		**60**	x	x
16	x	x		**61**	4.00	-0.58
18	x	x		**66**	x	x
20	x	x		**67**	5.65	1.06
21	x	x		**68**	33.00	**28.21**
22	4.02	-0.56		**75**	x	x
23	3.45	-1.12		**76**	x	x
34	x	x		**77**	3.50	-1.07
35	3.00	-1.57		**81**	5.35	0.76
36	3.75	-0.83		**84**	4.81	0.23
37	45.00	**40.12**		**85**	4.65	0.07
38	x	x		**86**	3.03	-1.54
42	35.25	**30.44**		**87**	x	x
43	5.12	0.53		**88**	x	x

Spiked crispbread extract

Overview

The spiked extract was prepared by addition of an AA standard solution to an aliquot of the raw crispbread extract. The spiking level was 50.8 ng/mL aqueous extract.

The values of the raw extract sample were subtracted from the results of analysis of the spiked extract. As it was mentioned in the previous section, this could be done with the data of 25 participants. The figures given below were calculated from corrected results!

Table 9: Summary statistics for the raw crispbread extract

Number of results		42		
Range of results	ng/mL	16.5	to	595.0
Median	ng/mL	51.2		
Huber H15	ng/mL	51.8		
Major mode	ng/mL	49.9		
Assigned value	ng/mL	51.2		
Target standard deviation	ng/mL	12.8		
Number of not satisfactory performing laboratories		8		

Figure 5: Plot of z-scores for the spiked crispbread extract, determination of the z-scores is based on corrected mean values.

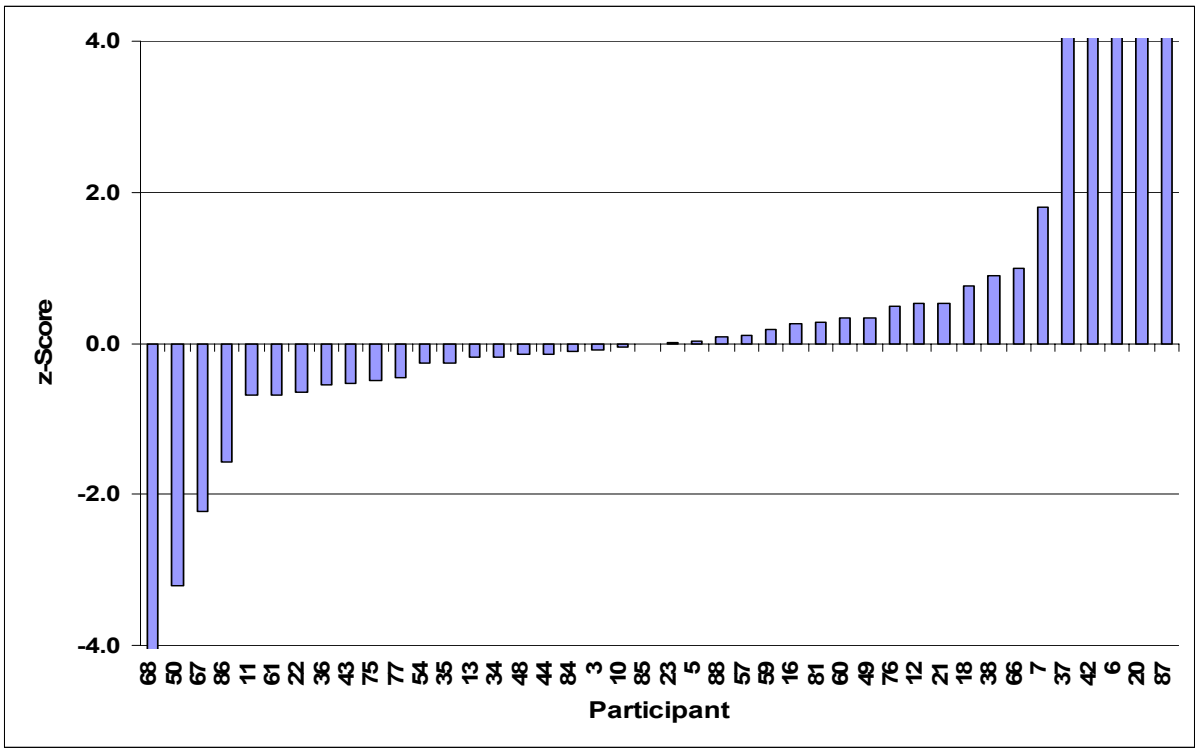

Table 10: Corrected results of analysis and z-scores for the spiked crispbread extract; bold printed z-scores mark results outside the acceptable range

Participant	Corrected result	z-Score		Participant	Corrected result	z-Score
	ng/mL				ng/mL	
3	50.10	-0.09		**44**	49.45	-0.14
5	51.73	0.04		**48**	49.30	-0.15
6	164.50	**8.83**		**49**	55.65	0.34
7	74.40	1.81		**50**	10.20	**-3.20**
10	50.67	-0.05		**54**	47.84	-0.27
11	42.50	-0.68		**57**	52.60	0.11
12	57.96	0.52		**59**	53.50	0.18
13	48.86	-0.19		**60**	55.50	0.33
16	54.50	0.25		**61**	42.50	-0.68
18	61.12	0.77		**66**	64.03	1.00
20	420.00	**28.76**		**67**	22.63	**-2.23**
21	58.12	0.54		**68**	-16.50	**-5.28**
22	42.93	-0.65		**75**	45.00	-0.49
23	51.45	0.02		**76**	57.50	0.49
34	49.00	-0.18		**77**	45.50	-0.45
35	48.00	-0.25		**81**	54.78	0.27
36	44.31	-0.54		**84**	49.94	-0.10
37	115.00	**4.97**		**85**	51.05	-0.02
38	62.80	0.90		**86**	31.07	-1.57
42	121.65	**5.49**		**87**	595.00	**42.41**
43	44.39	-0.54		**88**	52.39	0.09

Evaluation of the analysis data for the AA standard solutions

Overview

Two aqueous AA solutions and two AA standards in EtAc were prepared by the metrological division of IRMM. The concentrations of the standard solutions were adjusted according to the enrichment factors of the methods they were prepared for. The aqueous standards (40.7 ng/mL and 60.9 ng/mL) were sent to laboratories that applied LC/MS/MS, LC/MS, LC/LC/DAD and GC/MS including derivatisation of AA. The standards in the organic solvent (444.4 ng/mL and 607.6 ng/mL) were meant for laboratories that determine the AA content of food samples by GC/MS without prior derivatisation of AA. Due to the different chemical nature of the internal standards that are normally used by the participants, an internal standard was not added to the standard solutions.

Twenty-nine results were considered in the data evaluation of the aqueous AA solutions and 12 in the evaluation of the organic standard. One laboratory did not report results.

Results

Instead of calculating z-scores, the percentage of the deviation of the reported values from the calculated AA content of the standard solutions was determined. The respective values for the aqueous solution are listed in table 11, whereas those for the standard in EtAc are shown in table 12

Table 11: Aqueous standards: Results of analysis and deviation from calculated AA content

Participant	Standard A (60.9 ng/mL)		Standard B (40.7 ng/mL)	
	Reported result	Deviation	Reported result	Deviation
	ng/mL	%	ng/mL	%
5	59.85	-1.72	41.46	1.88
7	59.70	-1.97	48.75	19.81
10	66.57	9.31	50.96	25.23
11	48.40	-20.53	35.80	-12.02
12	60.01	-1.47	42.81	5.20
13	61.95	1.72	40.50	-0.47
16	58.00	-4.76	35.50	-12.75
22	64.70	6.24	39.50	-2.92
23	60.25	-1.07	40.60	-0.22
34	59.50	-2.30	39.50	-2.92
36	51.85	-14.86	38.35	-5.75
38	61.40	0.82	24.65	-39.42
42	123.80	103.28	50.74	24.70
43	60.00	-1.48	39.00	-4.15
44	60.70	-0.33	41.25	1.38
48	58.70	-3.61	39.15	-3.78
49	60.40	-0.82	38.30	-5.87
50	79.50	30.54	56.40	38.61
57	60.75	-0.25	42.05	3.34
60	60.50	-0.66	44.00	8.13
61	61.00	0.16	41.50	1.99
66	72.20	18.56	50.96	25.23
75	52.00	-14.61	38.00	-6.61
81	59.70	-1.97	34.23	-15.88
84	61.50	0.99	40.90	0.52
85	64.30	5.58	41.60	2.24
86	68.55	12.56	50.47	24.04
87	59.00	-3.12	39.00	-4.15
88	60.80	-0.16	43.60	7.15

Table 12: Organic standards: Results of analysis and deviation from calculated AA content

Participant	Standard C (607.6 ng/mL)		Standard B (444.4 ng/mL)	
	Reported result	Deviation	Reported result	Deviation
	ng/mL	%	ng/mL	%
6	517.50	-14.83	285.50	-35.65
18	603.06	-0.75	427.92	-3.56
20	700.00	15.21	520.00	17.20
21	603.01	-0.76	427.92	-3.56
35	479.00	-21.17	x	x
37	490.00	-19.35	350.00	-21.12
54	609.40	0.30	439.80	-0.88
59	567.50	-6.60	414.00	-6.69
67	404.90	-33.36	334.80	-24.54
68	535.00	-11.95	348.50	-21.46
75	479.00	-21.17	367.00	-17.29
77	445.00	-26.76	302.50	-31.82

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Annex

Annex 1: Announcement of Study

EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
IRMM

Geel, 17.12.2003

The Directorate General Joint Research Centre IRMM is going to organise a second study on the determination of acrylamide in crispbread. The start of the study is planned for end of January 2004. If you are interested in participating in the forthcoming study, please fill in the attached application form and send it to Thomas.Wenzl@cec.eu.int on **12 January 2004** at the latest. The participation is free of charge. As the number of participants is limited to about 70, we can not assure participation for every interested laboratory. For this reason respond in time.

Short description of the sample composition of the forthcoming inter-laboratory comparison study:

1) Due to calibration problems of some laboratories in the first study, we will add two acrylamide standard solutions to the samples. The acrylamide content of one standard solution will be given, in order to enable the participants to check their instrument calibration, while the concentration of the second standard solution will remain unknown.
2) A spiked sample extract will be included into the study in order to enable the evaluation of the influence of the clean-up procedure onto the results of analysis.
3) Three crispbread samples have to be analysed from the participants. We expect to get a better picture about the influence of the analyte concentration level onto the distribution of the analysis results. In addition, the influence of the extraction procedure onto the results of analysis will be investigated.

All samples have to be analysed in duplicate.

A method description form will be sent to the participants by email, in parallel to the samples (end of January). Only results of participants that transmitted the requested information will be evaluated. Deadline for the report of the analysis results will be one month after sample shipping (end of February).

We confirm confidentiality of all information concerning the identity of the participants.

The evaluation of the study will be performed according to the International Harmonised Protocol for Proficiency Testing of Analytical Laboratories.

Retieseweg, B-2440 Geel, Belgium
Tel.: +32-(0)14-571 211 - Direct line: ..... •Fax: +32-(0)14-584 273
http://www.irmm.jrc.be

Annex 2: Application form

Questionnaire for the participation in an European interlaboratory comparison study on the analysis of acrylamide from crispbread

Laboratory name:	
Contact person: Mr. ☐ Mrs. ☐	
First name:	Family name:
E-mail:	
Address: Street:	
Zip-code:	City:
Country:	Tel.:

Analysis method:	**LC/MS/MS** ☐ **LC/MS** ☐ **GC/MS (derivatisation)** ☐ **GC/MS (without derivatisation)** ☐
Extraction:	**Solvent:** Water ☐ Water/organic ☐ Organic ☐ Specify composition: **Volume:** mL **Temperature:** °C, **Time:** min
	Weighed sample quantity / analysis: g
Clean-up (mark all applied techniques)	SPE ☐ which cartridge(s): , Carrez ☐ Liquid/liquid extraction ☐ Ultracentrifugation ☐ Other ☐ (please specify):
Quantification	Internal standardisation ☐ / external standardisation ☐
Internal standard (ISTD)	d_3-acrylamide ☐ $^{13}C_3$-acrylamide ☐ $^{13}C_1$-acrylamide ☐ Methacrylamide ☐ Propionamide ☐ N,N-dimethylacrylamide ☐ **Equilibration of ISTD/sample** (before extraction)**: Yes** ☐ **No** ☐ **Equilibration time:** min
Concentration of ISTD in injection solution	**ng/mL**
Solvent composition of injection solution	(e.g. 100% H_2O)
	LOD (µg/kg): **LOQ (µg/kg):** (typical for crispbread samples)
Acrylamide analysis per month	<10 ☐ 10-25 ☐ 26-50 ☐ 51-100 ☐ 101-200 ☐ >201 ☐
In-house validation of method	Yes ☐ No ☐
Accredidation status	Laboratory is accredided: **Yes** ☐ **No** ☐ Laboratory accredided for acrylamide analysis: **Yes** ☐ **No** ☐

Please return questionnaire to: thomas.wenzl@cec.eu.int

Deadline: 12th January 2004

Annex 3: Sample receipt form

Interlaboratory comparison test on the analysis of acrylamide from crispbread samples

TEST MATERIALS RECEIPT FORM

Name of Participant	
Affiliation	

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

Date of the receipt of the test materials	
All items have been received undamaged	Yes ☐ / No ☐
Items are missing/damaged.	Yes ☐ / No ☐

Remarks:

Content of parcel

a) 3 test materials coded as "Crispbread Test Material"(at least 50 g per package)

b) At least one amber vial identified as "Raw Extract of Crispbread" (50 mL)

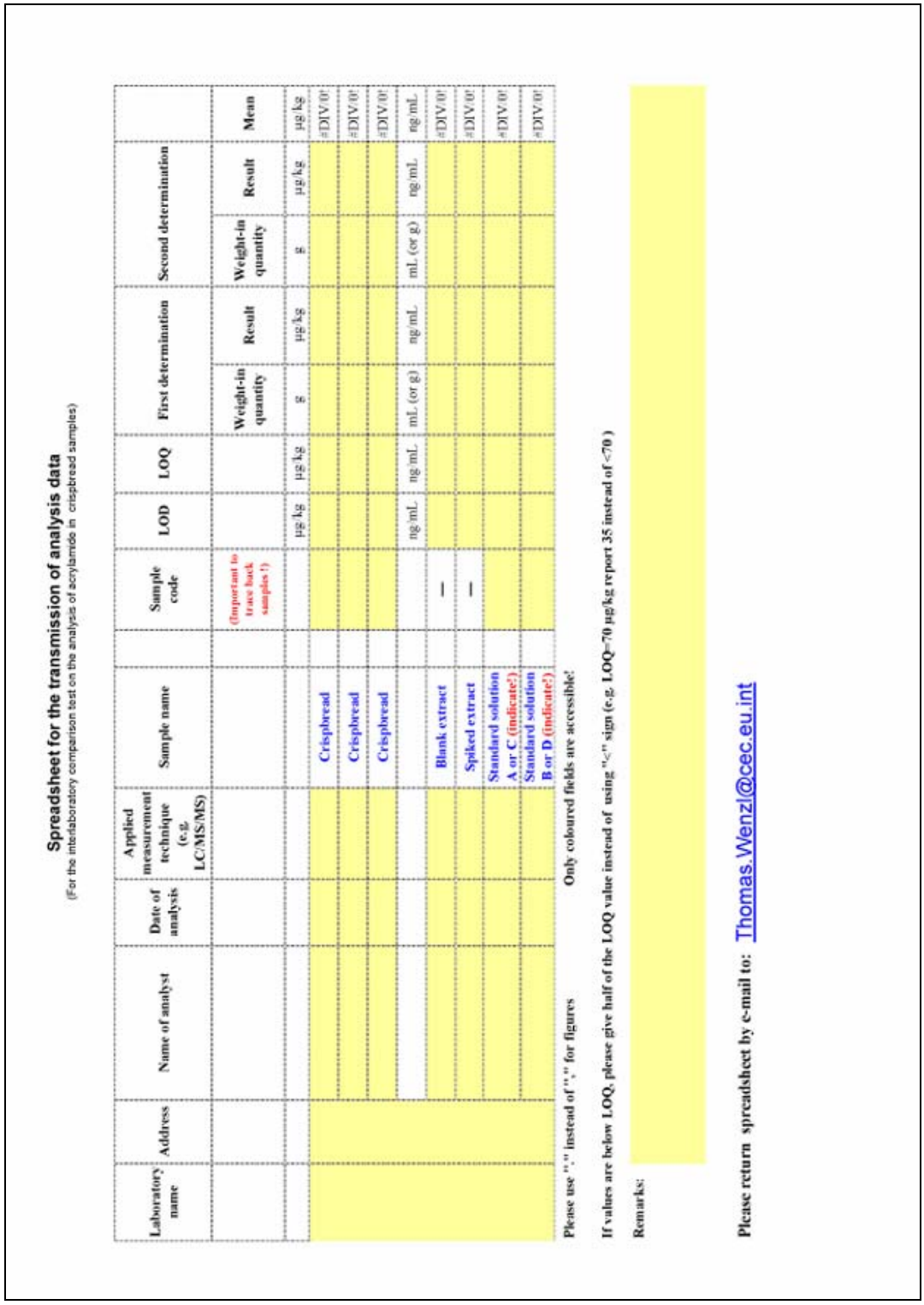
c) At least one amber vial identified as "Spiked Crispbread Extract" (50 mL)

d) At least two amber vials identified as "Acrylamide standard solution" and a letter from A to D (at least 15 mL)

e) One accompanying letter

Please return the completed form to: Thomas.Wenzl@cec.eu.int

Annex 4: Analysis results report form



Spreadsheet for the transmission of analysis data

(For the interlaboratory comparison test on the analysis of acrylamide in crispbread samples)

Laboratory name	Address	Name of analyst	Date of analysis	Applied measurement technique (e.g. LC/MS/MS)	Sample name		Sample code	LOD	LOQ	First determination		Second determination		
										Weight-in quantity	Result	Weight-in quantity	Result	Mean
							(Important to trace back samples !)	µg/kg	µg/kg	g	µg/kg	g	µg/kg	µg/kg
					Crispbread									#DIV/0!
					Crispbread									#DIV/0!
					Crispbread									#DIV/0!
								ng/mL	ng/mL	mL (or g)	ng/mL	mL (or g)	ng/mL	ng/mL
					Blank extract		—							#DIV/0!
					Spiked extract		—							#DIV/0!
					Standard solution	A or C (indicate!)								#DIV/0!
					Standard solution	B or D (indicate!)								#DIV/0!

Please use "." instead of "," for figures Only coloured fields are accessible!

If values are below LOQ, please give half of the LOQ value instead of using "<" sign (e.g. LOQ=70 µg/kg report 35 instead of <70)

Remarks:

Please return spreadsheet by e-mail to: Thomas.Wenzl@cec.eu.int

Annex 5: Homogeneity data

Table 1: Homogeneity data for the crispbread sample 1

sample id	**acrylamide (µg/kg)**	
	replicate 1	replicate 2
1	42.0	45.2
2	48.1	44.1
3	44.8	41.5
4	44.9	51.3
5	42.1	47.8
6	40.3	46.5
7	48.7	47.3
8	45.3	49.1
9	40.4	46.9
10	47.6	49.1
mean	45.7	

ref. for σ	Horwitz
target σ	11.6
s_a	3.2
F	0.88
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Table 2: Homogeneity data for the crispbread sample 2

sample id	**acrylamide (µg/kg)**	
	replicate 1	replicate 2
1	499.2	479.3
2	504.5	485.0
3	504.1	488.9
4	509.4	493.1
5	487.5	502.3
6	511.0	510.0
7	471.6	496.2
8	495.3	463.9
9	488.0	483.2
10	497.4	504.6
mean	493.7	

ref. for σ	Horwitz
target σ	87.9
s_a	12.6
F	1.10
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Table 3: Homogeneity data for the crispbread sample 3

sample id	acrylamide (µg/kg)	
	replicate 1	replicate 2
1	407	413
2	403	425
3	442	414
4	427	423
5	426	433
6	424	447
7	431	430
8	470	444
9	429	428
10	426	431
mean	428.7	

ref. for σ	Horwitz
target σ	78.02
s_a	11.4
F	2.41
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Table 4: Homogeneity data for the raw crispbread extract sample

sample id	acrylamide (µg/kg)	
	replicate 1	replicate 2
1	5.1	4.6
2	5.0	4.7
3	4.7	4.9
4	5.2	4.6
5	4.4	5.1
6	5.1	4.9
7	4.5	5.1
8	5.0	5.3
9	4.3	4.9
10	5.2	5.1
mean	4.88	

ref. for σ	Horwitz
target σ	1.74
s_a	0.3
F	0.69
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Table 5: Homogeneity data for the spiked crispbread extract sample, values are corrected with mean value of raw extract

sample id	**acrylamide (ng/mL)**	
	replicate 1	replicate 2
1	47.5	51.6
2	51.0	48.9
3	48.4	50.1
4	52.2	52.6
5	49.9	53.2
6	52.0	50.4
7	49.4	51.6
8	51.0	52.3
9	47.6	49.5
10	51.5	51.9
mean	50.6	

ref. for σ	Horwitz
target σ	12.7
s_a	1.6
F	1.31
F critical	3.02
F<Fcrit?	PASS
s_s	
s_s/σ	
critical s_s/σ	0.3
s_s/σ<critical s_s/σ?	

Annex 6: Analytical methods applied by the participants

The method details are tabulated as they were reported by the participants. Not tabulated information was not submitted. It should be noted that the authors do not claim completeness of the given method details.

The following abbreviations are used:

AA	Acrylamide
AcN	Acetonitrile
CI	Chemical ionisation
EI	Electron impact ionisation
ESI+	Electrospray ionisation positive mode
EtAc	Ethyl acetate
I.D.	Internal diameter
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass/charge ratio
MeOH	Methanol
MP	Mobile phase
PCI	Positive chemical ionisation
RT	Room temperature
t-BME	tert-Buthyl methyl ether

Table 6.1: LC/MS/MS - Standardisation and Extraction

Participant		3	7	10	11	13	22	23	34	36	44	48	49	57	60	61	66	75	84	85	87
Internal Standisation		Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
External Standisation			Yes																	Yes	
Internal Standard		D3-AA		D3-AA	D3-AA	13C3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA	D3-AA, Methacryl-amide	D3-AA	D3-AA	D3-AA
Equilibration of internal standard with sample		No	No	Yes	No	No	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	No	No	No	No
Equilibration time	min												10	10	5	30	15				
Weight-in quantity	g	5.0	1.0	1.0	4.0	5.0	1.0	5.0	2.0	4.0	5.0	4.0	5.0	1.0	2.5	2.0	1.0	20.0	1.0	2.0	5.0
Extraction solvent		Water	Water	Water	Water	Water	Water	Water	Water	Water	5% MeOH	Water	Water	Water	Water	Water	Water	1-Propanol	5% MeOH	Water	5% MeOH
Solvent volumn	mL	100	10	9.5	30	100	10	45	20	40	100	40	100	20	21	20	10	50	10	10	100
Extraction temp	°C	40	80	RT	RT	RT	RT	80	60	RT	RT	RT	75	40	RT	60	RT	25	20	60	RT
Extract. time	min	10	60	20	60	2	5	120	30	1	60	2	20	10	30	15	20	60	5	1	60
Maceration time	min																				60
Sample / solvent ratio	g/mL	0.05	0.10	0.11	0.13	0.05	0.10	0.11	0.10	0.10	0.05	0.10	0.05	0.05	0.12	0.10	0.10	0.40	0.10	0.20	0.05
Addition of amylase										Yes											

Table 6.2: LC/MS/MS - Sample clean-up

Participant		3	7	10	11	13	22	23	34	36	44
Freezing after extraction			Yes		Yes						
Defatting		Yes							Yes		
Defatting solvent		iso-Hexane / t-BME = 95/5							iso-Hexane		
Centrifugation of extract			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ultrafiltration					Yes		Yes			Yes	
Carrez precipitation		Yes							Yes		
Volumns of Carrez solutions I + II	mL + mL	1+1							0,5+0,5		
SPE		Yes		Yes	Yes	Yes	Yes			Yes	
Cartridges		M6N ABC 18		Bond Elut Accucat 200mg / 3mL	IS MM 300mg / 3mL	IS MM 300mg / 3mL	IS MM 300mg / 3ccm			IS MM 300mg / 3mL	
				OASIS HLB 200mg / 6mL		OASIS HLB 200mg / 6mL	OASIS HLB 200mg / 6mL				
Liquid/liquid extraction									Yes		
no special clean-up			Yes								Yes
Filtration			Yes	Yes		Yes		Yes			

Table 6.2: Continued

Participant		**48**	**49**	**57**	**60**	**61**	**66**	**75**	**84**	**85**	**87**
Freezing after extraction				Yes			Yes				
Defatting			Yes	Yes	Yes		Yes	Yes		Yes	
Defatting solvent			Hexane	Cyclohexane/Butylmethylether=95/5	Hexane		Hexane	Hexane		CH2Cl2	
Centrifugation of extract		Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes
Ultrafiltration											
Carrez precipitation				Yes	Yes	Yes				Yes	
Volumns of Carrez solutions I + II	mL + mL			0.5+0.5	1.25+1.25	0.5+0.5				1+1	
SPE		Yes					Yes	Yes		Yes	
Cartridges		IS MM 300mg / 3mL					Bond Elut Accucat 200mg / 3mL	Charcoal/Aluminiumoxide/Celite		IS MM 300mg / 3mL	
		ENV+					OASIS HLB 200mg / 6mL				
Liquid/liquid extraction											
no special clean-up											Yes
Filtration			Yes		Yes	Yes	Yes		Yes	Yes	

Table 6.3: LC/MS/MS - Chromatographic parameters

Participant		3	7	10	11	13	22	23	34	36	44
Inj. Vol	µL	20	10	100	10	20	20	10	40	50	5
Sample amount / injection	g/mL	0.05	0.10	0.10		0.05	0.10	0.10	0.05	0.10	0.05
Column supplier		Phenomenex	Waters	Waters	Thermo Hypersil	Thermo	Thermo Hypersil	Thermo Hypersil	Merck	Thermo Hypersil	Alltech
Type		Hypercarb	Atlantis C18	µBondapak C18	Hypercarb	Hypercarb	Hypercarb	Hypercarb	Lichrospher 100 CN	Hypercarb	Alltima C18
Lenght	mm	100	150	300	100	50	50	50	250	100	150
I.D.	mm	2.00	2.10	3.90	2.00	2.10	2.10	2.10	4.00	2.10	3.20
Particle size	µm	5.00	3.00	10.00	5.00		5.00	5.00	5.00	5.00	5.00
Mobile phase		0,05% FA, 1% AcN	0,1% Acetic acid, 2,1% AcN	0,1% Acetic acid	5% MeOH	Water	Gradient water/MeOH 80/20 - 60/40	0,01M FA in water /MeOH gradient	AcN / 1% acetic acid gradient zu 100% AcN	2% AcN	5% AcN in 5 mM FA
MP flow	mL/min	0.20	0.25	0.6, split 2/1	0.20	0.20	0.20	0.20	0.70	0.20	0.30
Column temp	°C	30	RT	RT	26			30	25	30	30
Net-retention time	min	4.00	3.10	8.10	5.00	4.00	1.10	1.70	3.60	4.00	4.00
Ionisation		ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+
Recorded Ions		72>55 72>54 72>44	72>55	72>72 72>55	71>54 71>25 74>57 74>29	72>55 72>54 75>58	72>72 72>55 75>58	72>72 72>55 72>54 75>58	72>72 72>55 72>44 75>58 75>75 75>44	72>55 72>44 75>58	72>55 72>44 75>58
LOD	µg/kg	10.00	2.50	30.00	20.00	3.00	3.00	3.00	10.00		10.00
LOQ	µg/kg	30	25	30	40	10	10	30	30		20

Table 6.3: Continued

Participant		**48**	**49**	**57**	**60**	**61**	**66**	**75**	**84**	**85**	**87**
Inj. Vol	µL	10	20	50	15	20	20	20	20	60	10
Sample amount / injection	g/mL	1.00	0.05	0.01	0.05	0.05	0.100	3.50	0.07	1.33	0.05
Column supplier		Thermo Hypersil	Phenomenex	Thermo Hypersil	Merck	Merck	Waters	Thermo Hypersil / Phenomenex	Alltech	Showa Denko	Alltech
Type		Hypercarb	Luna	Hypercarb	Lichrosphere CN 100	Lichrosphere CN 100	Atlantis dC18	Hypercarb / Luna C18	Prevail C18	Shodex Rspack DE 413 L	Altima C18
Lenght	mm	50	150	100	250	250	150	150	100	250	50
I.D.	mm	2.10	3.00	3.00	4.00	4.00	2.10	2.00	2.10	4.60	3.20
Particle size	µm	5.00	3.00	5.00		5.00	3.00	3.00	3.00		5.00
Mobile phase		0,1% acetic acid	5% MeOH in 0,1% FA	0.05%FA/MeOH=90/10	0.5% ACN, 0.1% FA	ACN/FA/H2O	5% MeOH, 0,1% Acetic acid	1% AcN + 0,5% FA	0.1%FA/ACN =95/5	gradient water/MeOH= 9:1 to 6:4 in 12 min, 0.01% FA	0.05 mM FA, 0.1% FA in ACN
MP flow	mL/min	0.40	0.25	0.25	0.25	0.25	0.15	0.20	0.10	0.60	0.30
Column temp	°C	RT	60	60	RT		RT	30	RT	40	30
Net-retention time	min	2.00	4.45	3.80	11.80	11.00	4.80	3.20	4.10	4.50	6.03
Ionisation		ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+	ESI+
Recorded Ions		72>55 72>54 75>58	72>55 72>54 75>58	72>55 72>54 72>44 75>58	72>55 75>58	72>72 72>55 75>58 75>75	72>72 72>55 75>58	72>55 75>58 86>58	72>55 75>58	72>55 72>54 72>27 75>58 75>30	72>55 72>44 75>58
LOD	µg/kg		50.00	10.00	20.00	10.00	15.00	5.00	17.00	7.00	
LOQ	µg/kg	2	100	30	40	30	35	10	34	21	

Table 6.4: GC/MS with derivatisation - Standardisation and Extraction

Participant		**5**	**16**	**42**	**43**	**50**	**81**	**86**
Internal Standisation		Yes	Yes	Yes	Yes	Yes	Yes	Yes
External Standisation								
Internal Standard		D3-AA	D3-AA	Methacrylamide	D3-AA	D3-AA	D3-AA	Methacrylamide
Equilibration of internal standard with sample		Yes	Yes	No	No	No	Yes	No
Equilibration time	min	10	15				15	
Weight-in quantity	g	2.0	10 to 20	10.0	7.0	5.0	2.0	5.0
Extraction solvent		Water	Water	Water	Water	Water	Water/MeOH=2/1	Water
Volumn	mL	40	200	100	60	100	30+15	50+25
Extraction temp	°C	RT	80	80	RT	40	RT	RT
Extract. time	min	10	60	120	15	10	2*60	
Addition of amylase			Yes					

Table 6.5: GC/MS with derivatisation - Sample clean-up

Participant		5	16	42	43	50	81	86	88
Defatting		Yes				Yes			
Defatting solvent		Hexane				Cyclohexan/Butylmethylether (95/5)			
Centrifugation		Yes	Yes	Yes		Yes	Yes	Yes	Yes
Carrez precipitation			Yes			Yes			
Volumns of Carrez solutions	mL + mL		1+1			1+1			
Derivatisation reagent		KBr/HBr/Br2	KBr/HBr/Br2			KBr/HBr/Br2	KBr/HBr/Br2	KBr/HBr/Br2	KBr/HBr/Br2
Reaction time							1 min	over night	
Reaction temp	°C						4	0	
Extraction solvent			EtAc				EtAc	EtAc	EtAc
Extraction solvent volumn	mL + mL		40				20 + 20	20 + 10	10
SPE							Silicagel activity I	Silicagel activity I	
Liquid/liquid			Yes			Yes	Yes		
Extrelut NT20					Yes				
Final volumn	mL		1				1	0.1	1

Table 6.6: GC/MS with derivatisation - Chromatographic parameters

Participant		**5**	**16**	**42**	**43**	**50**	**81**	**86**	**88**
Inj. Vol		1	2	2	1	5	2	3	2
Sample amount / injection	g/mL	0.50	5.00	3.75	20.00	0.10	1.00		2.00
Injection technique		Splitless	Splitless	Splitless	Split	PTV/splitless	Splitless	PTV/splitless	Splitless
Column supplier		J&W	SGE	Supelco	J&W	J&W	J&W	Varian	J&W
Type		DB-5MS	BPX50	SPB50	DB17	DB-1701	DB5MS	CP Sil 24 CB	DB-17 MS
Lenght	m	30	30	30	30	30	60	30	30
I.D.	mm	0.32	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Film thickness	µm	0.25	0.25	0.30	5.00	0.25	0.25	0.25	0.25
Mobile phase		He	He		He	He	He	He	
MP flow	mL/min	1.00	1.00			1.00			
Temp. Program		65/1-15-250/10	55/2-25-175/6-50-280/6	85/1-25-175/6-40/250/10	65-5-?	80/1-5-180/11	65/2-8-120/0-9 280/5	60/4-20-120/2-5-150/1-20-270/8	65/2-10-250/10
Net-retention time	min	7.50	9.00	8.24	20.00	20.50	13.28	18.90	8.10
Ionisation		EI	EI	EI	EI	EI	EI	EI	EI

Recorded ions	m/z	106, 108, 150, 152	150, 155; 106; 133	106, 108, 150, 152	109, 111, 153, 156	106, 108, 111, 152	106, 109, 153	106, 108, 150, 152	133, 138, 149, 151, 154, 135
LOD	µg/kg	10	20	0.8		10			10
LOQ	µg/kg	30	40	2	< 30	30			30

Table 6.7: GC/MS without derivatisation - Standardisation and Extraction

Participant		**18**	**20**	**21**	**37**	**54**	**59**	**67**	**68**	**76**	**77**
Internal Standisation		Yes	Yes	Yes	Yes		Yes	Yes	Yes	Yes	Yes
External Standisation						Yes		Yes	Yes		
Standards		D3-AA	D3-AA	D3-AA	Methacrylamide		D3-AA	D3-AA	D3-AA	D3-AA	D3-AA
								Methacrylamide	Methacrylamide	Methacrylamide	Methacrylamide
Equilibration of internal standard with sample		Yes	No	Yes	No		Yes	No	No	No	Yes
Equilibration time	min	15		15			5				30
Weight-in quantity	g	5.0	20.0	10.0	0.5	10.0	5.0	5.0	5.0	20.0	20.0
Extraction solvent		80% n-Propanol	Water	85% AcN	MeOH/H2O = 9/1	Water	Water	ACN/water = 85/15	n-Propanol	n-Propanol	Water/n-propanol
Volumn	mL	80	240	150	1	100	20	50	20	50	40
Extraction temp	°C	25	60	25	60	60	RT	RT	RT	25	70
Extract. time	min	30	30	30	30	60	30	1	1	60	30
Maceration									30 min / 70°C		

Table 6.8: GC/MS without derivatisation - Sample clean-up

Participant	20	21	18	37	54	59	67	68	76	77
Defatting		Yes	Yes			Yes		Yes	Yes	Yes
Defatting solvent		Hexane	Hexane			Hexane		Hexane	Hexane	Hexane
Centrifugation	Yes		Yes	Yes	Yes	Yes	Yes	Yes	Yes	
SPE									Charcoal / Aluminiumoxide / Celite	
Carrez	Yes	Yes				Yes				
Volumns		5.5				1,25+1,25				
Liquid/liquid					Yes	Yes		Yes		Yes
ChemElut							Yes			
Extrelut NT20	Yes	Yes	Yes							

Table 6.9: GC/MS without derivatisation - Chromatographic parameters

Participant		18	20	21	37	54	59	67	68	76	77
Inj. Vol	µL	2	2	2	1.5	2	3	2.5	1	2	3
Sample amount/injection	g/mL	5.00	12.50	10.00	0.50		20.00	1.00	0.17	3.50	0.17
Injection technique		On-column	Splitless	On-column	Splitless	Splitless	PTV/splitless	Splitless	Splitless	Splitless	On-column
Column supplier		J&W	Phenomenex	J&W	J&W	Supelco	Agilent	Varian	J&W	J&W	Homemade
Type		FFAP	ZB Wax	FFAP	DB Wax	Suwax 10	Innowax	VF-5MS	FFAP	Carbowax	Carbowax
Lenght	m	30	30	30	30	60	25	30	30	60	10
I.D.	mm	0.32	0.25	0.32	0.25	0.25	0.20	0.25	0.25	0.25	0.25
Film thickness	µm	0.25	0.25	0.25	0.25	0.25	0.40	0.25	0.25	0.25	0.40
Mobile phase		He	He	He	He	He	He	He	He	He	He
MP flow	mL/min	1.00		1.00	1.00		1.00	0.50	1.00	2.00	2.70
Temp. Program	°C	60/2-10-240/10	50/1-35-255/15	60/2-10-240/10	80/2-8-250/0	70/2-10-250/5	80/5-10-200/5-20-240/10	50/0.8-20-70/0-6-110/0	50/3-50-240/9	70/2-20-220/0-6-280/1	70/2-15-220/2
Net-retention time	min	15.00	6.00	15.00	12.00	21.10	20.00	6.00	12.10	10.80	8.60
Ionisation		CI	CI	CI	EI	PCI	EI	PCI	EI	PCI	PCI
Reactant gas		MeOH		MeOH		NH4		CH4		CH4	CH4
Recorded ions	m/z	72, 75	72, 55	72, 75	55, 71, 69, 85	89, 72	71, 55, 74, 58	72	71, 44, 55; 74, 58, 47; 85, 41, 44, 69	70, 73, 84	72, 75, 86, 88
LOD	µg/kg	7	10	7	100		20	5	4	5	10
LOQ	µg/kg	21	20	21			40	15	12	10	40

Table 6.10: Other techniques - Standardisation and Extraction

Participant		6	12	38
Technique		GC/MS/MS	LC-MS derivatisation	LC/MS
Internal Standisation		Yes	Yes	Yes
Internal Standard		13C3-AA	13C3-AA	13C3-AA
Equilibration		No	Yes	Yes
Equilibration time	min			
Weight-in quantity	g	5.0	3.0	0.5
Extraction solvent		Water	Water	Water
Volumn	mL	80	40	5
Extraction temp	°C	60	25	25
Extract. time	min	30	30	30

Table 6.11: Other techniques - Sample clean-up

Participant		6	12	38
Technique		GC/MS/MS	LC-MS derivatisation	LC/MS
Defatting		Yes	Yes	
Defatting solvent		Hexane	Hexane	
Centrifugation		Yes	Yes	Yes
Derivatisation reagent			2-Mercaptobenzoic acid	
SPE				Bond Elut Accucat 200mg / 3mL
Extrelut NT20		Yes		
Filtration		Yes		Yes

Table 6.12: Other techniques - Chromatographic parameters

Participant		6	12	38
Technique		GC/MS/MS	LC-MS derivatisation	LC/MS
Inj. Vol	µL	1	10	20
Sample amount / injection		1.25	12.00	0.10
Injection technique	g/mL	On-column		
Column supplier		BGB	Phenomenex	Phenomenex
Type		BGB Wax	Synergi Polar-RP	Synergy polar RP
Lenght	m	30	150	150
I.D.	mm	0.25	2.00	4.60
Film thickness resp. particle size	µm	0.25	4.00	4.00
mobile Phase		He	ACN/Water gradient 30:70 - 60:40 +0.1%Hac	H2O/ACN/HAc (79/11/10) / H2O = 2/98
MP flow	mL/min		0.20	0.50
Column temp	°C			25
Temp. Program	°C	60/0-8-210/0		
Net-retention time	min	13.00	3.50	2.80
Ionisation		CI	ESI+	ESI+
Reactant gas				
Recorded ions	m/z	72>55	226>191	72, 75
	m/z	75>58	229>194	
LOD	mg/kg	5	4	8.8
LOQ	µg/kg	10	12	31.9