German Federal Institute for Risk Assessment

Report on the method validation study to standardize a method for the

Determination of melamine and cyanuric acid in animal feed by LC-MS/MS

In the frame of CEN/TC 327 third mandate M/522 Part II

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Determination of melamine and cyanuric acid in animal feed by
LC-MS/MS

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1 Introduction

The project was performed in the framework of the CEN/TC 327 third mandate M/522 Part II to standardize a method for the

Determination of melamine and cyanuric acid in animal feed by LC-MS/MS.

Melamine (2,4,6-triamino-1,3,5-triazine) a heterocyclic triazine is present in the environment and may be detected in food and feed at low concentrations. This background level is caused by the widespread use of melamine in the synthesis of melamine-formaldehyde resins for the fabrication of e.g. plastics, glues, laminates. Migration from melamine containing products e.g. kitchen- and tableware or packaging into food is also possible. Additionally melamine occurs as metabolite or degradation product of the pesticide and veterinary drug cyromazine and as fire-retardant.

The oxytriazine analogues cyanuric acid, ammelin and ammelide may be produced as by-products in melamine synthesis and depending on the purification process melamine may contain different levels of these structurally related substances. Cyanuric acid is also a dissociation product of the water disinfectant dichloroisocyanurates and together with melamine an impurity of urea-based feed additives for ruminants (e.g. biuret).

Melamine contaminated wheat gluten and later also rice protein concentrate used as ingredient for pet food were identified as cause for the sickness and death of dogs and cats 2007 in the USA, Canada and South Africa. Contaminated infant formula was the reason for illness of almost 300000 children in China 2008. These adulterations were performed since especially melamine but also the oxytriazine analogues are rich in nitrogen and therefore enhancing the apparent protein content of the feed. The methods to analyse the protein content in feed based on the determination of the nitrogen content.

To distinguish between the unavoidable background presence of melamine and unacceptable adulteration a concentration of 2.5 mg kg^{-1} in food and feed was established as an appropriate maximum level. For infant formula a level of 1 mg kg^{-1} was established.

In the meantime various methods are available for the analysis of melamine and also cyanuric acid in different matrices.

The main question is the accuracy of the methods. One area of concern is the extraction procedure because of the limited solubility of melamine, cyanuric acid and the potentially present melamine: cyanuric acid complex. Solubility of melamine and cyanuric acid in water is 2-3 g l^{-1} and for the melamine: cyanuric acid complex 0.01 g l^{-1}.

Although the solubility problem should not arise at the 2.5 mg kg^{-1} action level in food, care must be taken during clean-up and analysis.

A high-performance liquid chromatographic (HPLC) mass spectrometric (MS) method for the analysis of melamine and cyanuric acid in the concentration range between 1 mg kg^{-1} and 100 mg kg^{-1} in feed was developed. In a first step screening has to be performedto check for the presence of melamine and cyanuric acid in samples. In this method samples are extracted by a mixture of trichloroacetic acid, acetonitrile and water. The extract is further diluted with acetonitrile, centrifuged and injected into the LC-MS/MS system. For samples tested positive in the screening method a confirmatory method has to be performed. For the confirmatory method an internal standard is added, extraction is performed with a mixture of trichloroacetic acid, acetonitrile and water and twice by a mixture of acetonitrile and water.

After dilution and centrifugation the supernatant is injected into the LC-MS/MS system. The quantification of melamine and / or cyanuric acid is carried out by means of standard addition.

The limit of quantification of the method has been demonstrated to be better than 0.3 mg kg^{-1} for melamine and 0.6 mg kg^{-1} for cyanuric acid. The quantification of concentrations above 100 mg kg^{-1} is possible but the method has to be validated by the operator.

2 Scope

To standardize a method in the frame of the CEN/TC 327 third mandate, a method validation study is mandatory. For the present method it was decided to perform a training study before realisation of the main study. The training study was performed to assess the transferability of the method to other laboratories and to define possible problems during implementation of the method in other laboratories.

Therefore the participants were encouraged to send their observations and comments on the method together with their results to the project leader.

Finally 13 laboratories, including the laboratory of the Federal Institute for Risk Assessment (BfR), expressed their readiness to participate in the method validation training study. Four test samples, differing in terms of concentrations of melamine and cyanuric acid and the type of feed material, were sent to the participants. 8 laboratories sent back results.

The results were evaluated according to DIN ISO 5725-2: 2002-12

14 laboratories expressed their willingness to participate in the main study. Eight of these 14 laboratories participated in the training study and six laboratories were additional acquired. Eight test samples differing in terms of the type of animal feed and concentrations of melamine and cyanuric acid were prepared. The materials (16 unknown samples and one blank sample as quality control sample) were sent to the participants as blind duplicates altogether. Additionally the revised method and the evaluation templates were sent. From 14 laboratories 12 data sets were sent back.

The results were evaluated according to DIN ISO 5725-2: 2002-12

3 Design of the method validation study

3.1 Design and results of training study

For the training study of the method validation study 4 feed materials were prepared. One material was a blank material, two materials contained melamine and cyanuric acid and one material only melamine. The samples were dispatched to the participants at 27-07-2015 and should be analysed in duplicate. With the samples also standards were sent to the participants. To assess the homogeneity of the prepared material after portioning 10 samples were randomly selected and the homogeneity was determined. All tested materials were homogenous. In the complete feed for rabbits a mean concentration of melamine of 101.8 mg kg-1 and of cyanuric acid of 5.46 mg kg^{-1} was measured.

In soy meal a mean concentration of melamine of 10.44 mg kg^{-1} was measured no cyanuric acid was added. The measured concentrations were 2.72 mg kg^{-1} for melamine in cat feed and 10.36 mg kg^{-1} for cyanuric acid. In complete feed for laying hens no melamine and no cyanuric acid were measured (blank material).

The material was sent to 13 laboratories (including the laboratory of the project leader). 8 laboratories (annex 1) sent back results. One laboratory (L004) was excluded from the evaluation since they used their own method. The results for melamine in feed are given in 14.1.1, for cyanuric acid in feed in 14.1.2 and the results of the statistical evaluation in 14.1.3 in the annex 1.

Conclusions of the training study were:

1) The numbers of participating laboratories sending valid results are too low to validate the method in a method validation study, especially for cyanuric acid.
2) For analysing cyanuric acid the used instruments must be in a good condition.
3) The participants used different LC-MS systems. The results as far as can be assessed are not dependent on the instrument. Therefore the type of LC-MS system has not to be mandatory codified as part of the method.
4) The handling of the method has to be further simplified.
5) The results of the estimated statistical parameters are not sufficient to meet the requirements for a method validation. Comparing the results of the participants the correct implementation of the method in the respective laboratory is an important factor.
6) For cyanuric acid even fewer results were delivered. The statistical parameters obtained from these results were slightly better in comparison to melamine.

Further steps were identified and performed:

1) Finding more participants
2) Delivery of the fifth draft of the method, including the suggestions of the participants
3) Preparation of the material for the main study
4) Realisation of the main study

3.2 Design of the main study

The in the training study identified necessary steps, as recruiting further participants, revision of the method, preparation and delivery of the samples were performed.

For the main study 8 feed materials were prepared. One material was a blank material, five materials contained melamine and cyanuric acid, one material only melamine and one material only cyanuric acid. For the preparation of the samples the feed material was spiked with

the analyte(s), grounded, divided into portions and the homogeneity was checked. After ensuring the homogeneity the material was labelled and stored at +4°C respectively at -30 °C (pet food for cats) until dispatching. Each participant received 16 samples, the samples should be analysed once. For quality control purposes (spiking) an additional blank material (rape meal) was sent. A receipt of acknowledgment, a letter with instructions, the method (electronically) and the reporting sheet (electronically) were sent to the participants.

4 Time frame

For recruiting more participants laboratories known to be involved in the analysis of melamine were contacted directly by the project leader via e-mail. Six laboratories agreed to participate in the main study. Two laboratories of the training study withdrew their agreement to participate because of the workload in their laboratories. Overall the number of laboratories declaring their willingness to participate in the study was 14.

The fifth draft of the method including the comments of the participants of the training study and discussions in the Working Group 1 of CEN/TC 327. The final draft of the method for the main trial dated 11-05-2016.

The samples were dispatched to the participants at 11-05-2016. The laboratories not participating in the training study received additionally three samples from the training study for quality control purposes.

The samples arrived at the laboratories between 12-05-2016 and 13-05-2016. 12 laboratories sent back the acknowledgment of receipt. Some participants reported that vials of standard solutions cracked during transport or storage. These participants received new standard solutions and if necessary additional new samples.

The deadline for submitting the results was set to 04-07-2016.

5 Test items

5.1 Samples

Eight samples were prepared.

A complete feed for laying hens was chosen as blank material. All materials were grinded to a diameter of 1.0 mm and mixed.

Before fortification of the material all feed samples were analysed to exclude contamination with melamine or cyanuric acid. In some feed samples analysed in the laboratory of the project leader concentrations around the LOD (0.1 to 0.2 mg kg^{-1}) of melamine and cyanuric acid were detected. This is possibly due to migration of melamine from melamine containing packaging material.

To prepare contaminated material seven different feeds were selected.

- Wheat bran as feed material
- Soy meal as feed material
- Complete feed for laying hens
- Pet food for dogs (dry)
- Pet food for cats (wet)
- Milk replacer
- Complementary feed for milk cows

The material was prepared between end of September 2015 and end of November 2015.
For spiking of the materials two different procedures depending on the water content of the feed material were chosen.

5.1.1 Feed material with a moisture content below 20 %

Feed material with a moisture content < 20 % was grinded, spiked, lyophilised, divided into portions and samples taken for homogeneity testing.

First, the material was grinded to a diameter of 1 mm and mixed. About 1 to 1.5 kg of the material was weighed into a cooking food mixer. The required amount of melamine and cyanuric acid was weighed and dissolved in warm water. The solution was completely transferred to the feed and the slurry was mixed two hours in the cooking machine. The material was transferred into aluminium dishes, refrigerated first to -30°C and then to -75°C and subsequently lyophilised. After lyophilisation the material was mixed in a Grindomix GM 200 first 15 s at 2500 rpm, then at 3000 rpm and finally at 3500 rpm.

The mixed feed was portioned into 20 g portions in 100 ml PP-plastic containers and randomly 10 samples were taken for determination of the homogeneity. The spiked concentrations are given in table 1.

Table 1: Prepared feeds and the spiked concentrations

Feedstuff		melamine	cyanuric acid
	Internal sample number	mg/kg	mg/kg
Complete feed for laying hens	Blank		
Wheat bran	FC-144	1.5	8.0
Soy meal	FC-145	30	5.0
Complete feed for laying hens	FC-041	1.0	1.0
Pet food for dogs (dry)	FC-151	-	80
Complementary feed for milk cows	FC-153	6.0	4.0
Milk replacer	FC-166	2.5	2.5
Pet food for cats (wet)	FC-180	1.0	-
Rape meal for quality control	Blank FC-055	-	-

5.2 Material with a moisture content above 75 %

Feed material with water content > 75 % was mixed, lyophilised, spiked, divided into portions and samples for the homogeneity testing were taken.

Pet food for cats was mixed in a cooking food mixer and transferred into aluminium dishes. Each dish was weighed without and with the material. The material was refrigerated first at -30°C then at -75°C and subsequently lyophilised. After lyophilisation the dishes were weighed and the dry matter calculated. The dried material was mixed and the spiking solution added. For preparation of the spiking solution melamine and cyanuric acid were weighed and dissolved in warm water. The amount of water corresponds to the calculated water content of the material. The material was mixed again for 2 hours in a cooking machine.

The spiked concentrations are given in table 1.

After preparation of the material aliquots were portioned. Therefore 50 g portions of the pet food were weighed into 100 ml PP-plastic containers.

All samples were labelled with a unique number which consisted of a randomly selected four digit number.

Sample 1
Sample No. BfR-XXXX
CEN TC 327
Method validation study
Melamine & cyanuric acid in animal feed
Main study

5.3 Standard material

With the samples also standards were sent to the participants.

1.2 ml standard solutions of melamine and cyanuric acid, and 20 ml $^{13}C_3$-melamine and $^{13}C_3$-cyanuric acid at a concentration of 0.1 mg ml^{-1} were dispatched.

6 Homogeneity and stability

6.1 Determination of the homogeneity

To assess the homogeneity of the prepared material after portioning 10 samples were randomly selected and the homogeneity was tested.
From each of the 10 samples 2 subsamples were weighed and analysed according to the protocol for the confirmatory method.
The homogeneity of the samples was determined between October 2015 and November 2015.

The results of the analysis are given in detail in annex 2 (14.2). The results were evaluated by one-way Anova. If the calculated F-value obtained from the data was lower than the critical F-value and if the calculated p-value is above the significance level of α=0.05 the sample material is considered homogeneous in respect to the analytes.
All tested materials were homogenous.

The average concentrations for the feedstuffs determined during testing the homogeneity are shown in table 2.

Table 2: Mean concentrations (n=20) of the different feedstuffs as determined in the homogeneity study

Feedstuff		melamine	cyanuric acid
	Internal sample number	mg/kg	mg/kg
Complete feed for laying hens	Blank	-	-
Wheat bran	FC-144	1.73	9.17
Soy meal	FC-145	32.46	5.56
Complete feed for laying hens	FC-041	1.06	1.08
Pet food for dogs (dry)	FC-151	-	87.17
Complementary feed for milk cows	FC-153	6.62	4.59
Milk replacer	FC-166	2.62	2.42
Pet food for cats (wet)	FC-180	1.1	-

6.2 Determination of the stability

The samples for the study were prepared between September and November 2015 and the homogeneity of the samples was determined between October and November 2015. At this time the concentrations of the materials were determined. The dried feed samples were stored at +4°C and the wet feed at -30°C all the time. The stability of the prepared samples was determined between April and July 2016.

For determination of the stability from the stored samples 6 samples were selected randomly and analysed. The results were compared with the results from the homogeneity study. The variances of the series were compared by F-test (one–tailed). No significant differences in the variances were observed for the samples. The mean of the two data sets was compared by t-test (one-tailed). The results showed no significant differences during storage. The results are shown in annex 3 (14.3).

7 Distribution of the samples and standards and instructions to the participants

Portions of the samples were packed together with the standards into parcels and were kept cool by dry ice.

The identification numbers of samples sent to the participants are given in annex 4 (14.4). With the samples also a covering letter including detailed instructions regarding to the participants was sent.

The instructions to the participants included (annex 5, 14.5)

- Information on sample handling and storage
- Information on the performance of the analyses
- Acknowledgment of reception

The samples were dispatched to the participants at 11-05-2016. The laboratories not participating in the training study received additionally three samples from the training study for quality control purposes.

12 laboratories sent back the acknowledgment of receipt.

The samples arrived at the laboratories between 12-05-2016 and 13-05-2016. The vials with the internal standard were damaged in two packages. In one package one sample vial and the internal standard vials were broken and additionally the samples were delivered at room temperature. The two laboratories received new vials with internal standards and the other laboratory received a new parcel with a new set of samples and standards.

The deadline for submitting the results was set to 04-07-2016.

The method protocol was sent to the participants via e-mail on 12-05-2016 (annex 6). The certificates of the standard substances were also included. The reporting sheet for the results (ProLab templates) and an Excel sheet for details about method parameter were sent to the participants on 25-05-2016 by e-mail (annex 7, 14.7).

Due to problems with their equipment one laboratory asked for an extension of the deadline. Last results arrived at the 02-08-2016. Due to the low number of participants the late arrival of the results was accepted.

8 Evaluation of the results

8.1 General observations

The material was sent to 14 laboratories (including the laboratory of the project leader). 12 laboratories (annex 8, 14.8) sent back results. One laboratory (L004) was excluded from the evaluation since they used their own method. The single test results for melamine in feed are given in table 3 and figure 1 to 6 and for cyanuric acid in feed in table 4 and figure 7 to 12.

The LC-MS/MS conditions described in the method protocol are given only as a guideline and therefore the participants could choose their own method of analysis. The different conditions for LC-MS/MS are given in annex 9 (14.9). As can be seen the used conditions are quite different e.g. most participants used a HILIC column for separation also a porous graphite carbon column was used. The MS equipment was different distinguishing both in sensitivity but also in technique.

For analysis of melamine but especially cyanuric acid it is important to ensure that the LC-MS/MS equipment is suitable and in excellent working order.

The reported deviations from the method protocol are in one case another dilution due to the sensitivity of the equipment and in another case an additional filtration step. The reported deviations from the method protocol are given in annex 10 (14.10).

Overall these modifications were considered as minor and the results were taken into account for the evaluation.

Table 3: Results of the analysis of melamine in feed

	Wheat bran		Soy meal		Complete feed for laying hens		Pet food for dogs (dry)		Complementary feed for milk cows		Milk replacer		Pet food for cats (wet)	
	FC 144		FC 145		FC 041		FC 151		FC 153		FC 166		FC 180	
Sample No	1	12	7	13	9	14	4	8	15	3	10	16	5	6
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
LC001	1,20	1,23	27,06	27,12	0,91	0,91	nq (<0,5)	nq (<0,5)	5,78	5,39	2,39	2,34	1,03	0,87
LC004	1,90	3,10	39,20	39,8	1,10	1,20	nd (<0,1)	nd (<0,1)	7,70	7,40	2,50	3,30	1,20	1,20
LC005	1,45	1,44	30,86	30,81	0,66	0,71	nd (<0,1)	nd (<0,1)	5,27	6,98	2,56	2,55	0,93	0,92
LC006	1,62	1,51	34,15	26,35	1,05	0,89			6,07	6,47	2,49	2,56	1,06	1,07
LC007	1,68	1,75	24,26	40,20	1,06	1,17			9*)	nd(0,5)	1,74	2,74	1,17	1,04
LC009	1,59	1,76	31,10	29,90	0,98	0,97	nq (<0,5)	nq (<0,5)	6,28	6,47	2,46	2,3	1,21	1,09
LC013	1,82	1,57	32,27	32,19	0,99	1,01	nd (<0,1)	nd (<0,1)	7,07	6,77	2,65	2,7	1,21	1,15
LC014	1,75	1,56	27,60	27,02	0,94	0,91	nd (<0,1)	nd (<0,1)	5,27	6,11	2,68	2,92	1,12	1,20
LC016	1,70	1,77	29,74	31,44	0,97	1,02	nq <0,06	nq <0,06	5,96	6,38	2,53	2,57	1,09	1,07
LC017			28,54	25,11					5,33	4,9	3,43	3,37	1,21	0,94
LC018	1,67	1,53	33,4	34,2	0,87	0,82	nd <0,15	nd <0,15	6,32	6,45	2,34	2,61	1,10	1,09
LC019	1,78	1,69	32,39	31,49	1,08	1,02	nd (<0,1)	nd (<0,1)	6,51	6,65	2,62	2,67	1,10	1,08

Laboratory was excluded from further evaluation since they used their own method

Cochran outlier, confirmed with Mandel's k-statistik (α=0,01)

Grubbs outlier, confirmed with Mandel's h-statistik (α=0,01)

*) confirmed with Mandel's h-statistik (α=0,05), because of the large difference between the analysis (9,0 mg/kg and <0,5mg/kg),the value has been eliminated

nd = not detected (below LOD) nq = below LOQ

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
0,95	16,1	13,0	5,29

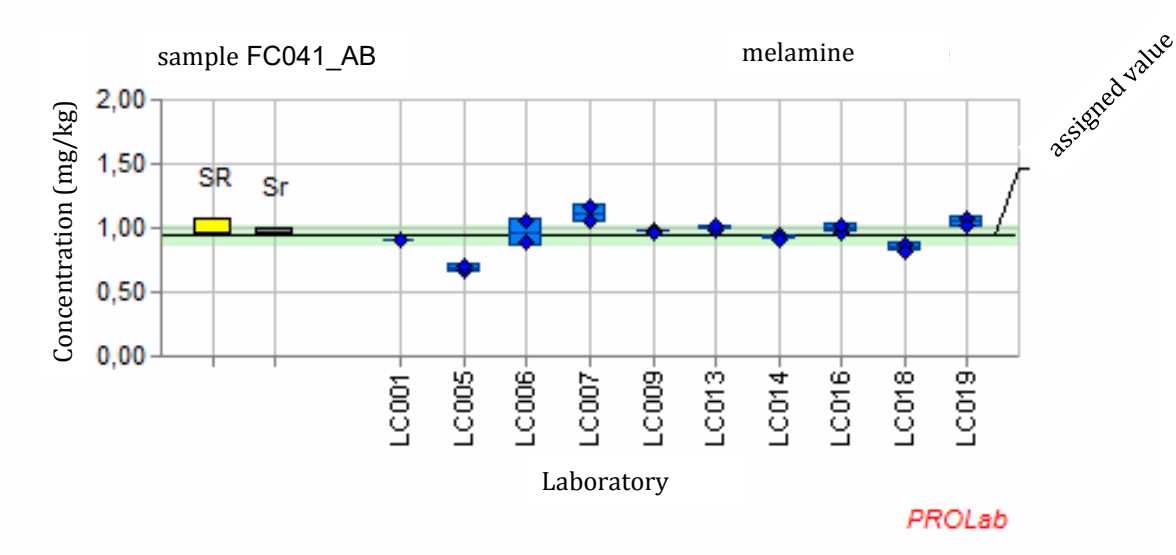


Figure 1: Results of the analysis of melamine in feed for laying hens (Sample FC-041)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
1,60	14,90	11,02	5,89

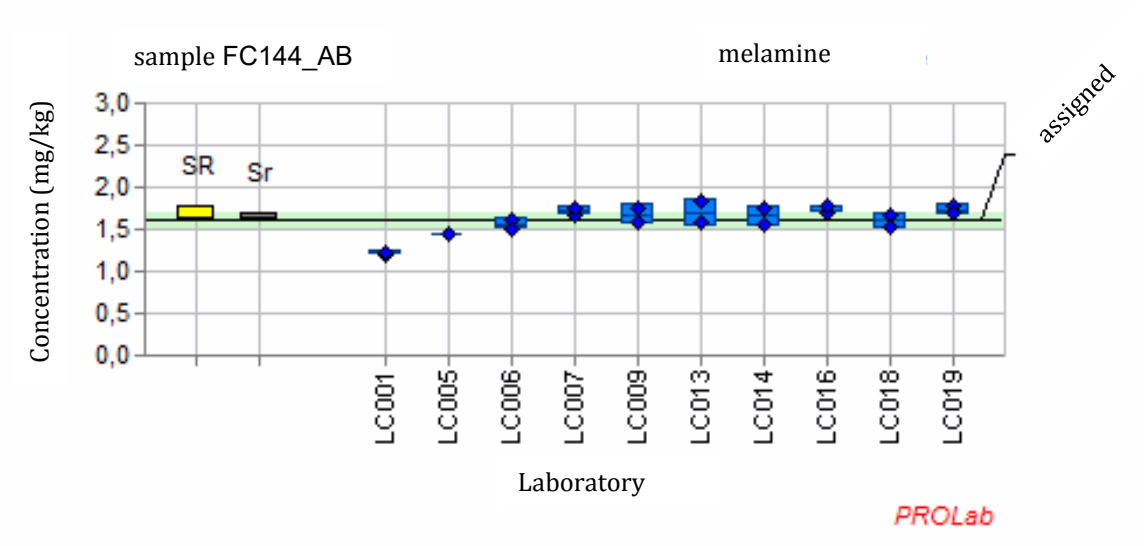


Figure 2: Results of the analysis of melamine in wheat bran (FC-144_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
30,14	9,58	9,11	6,58

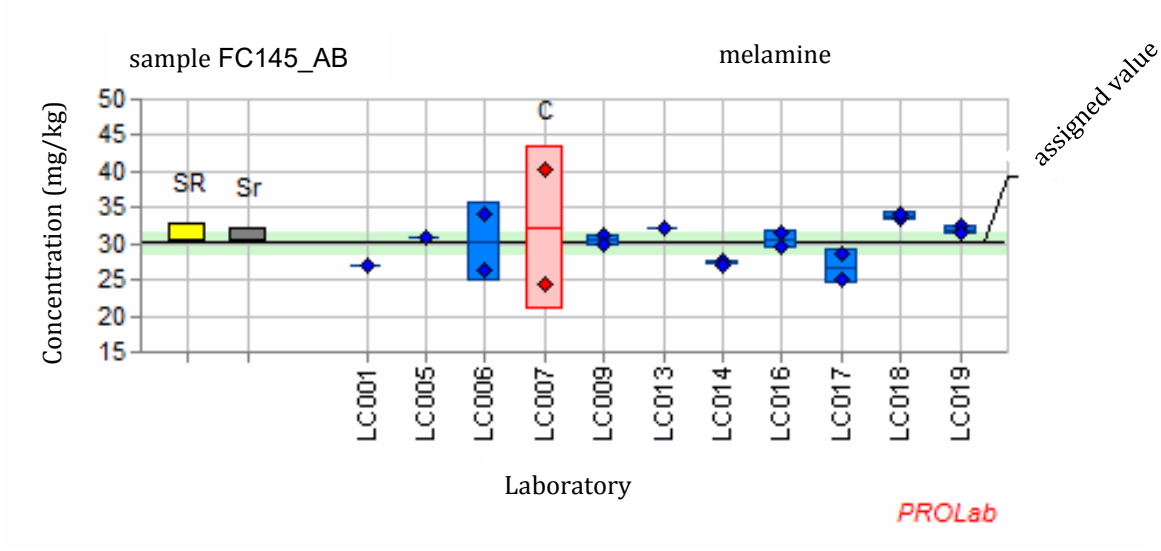


Figure 3: Results of the analysis of melamine in soy meal (FC-145_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
6,12	12,18	10,18	7,72

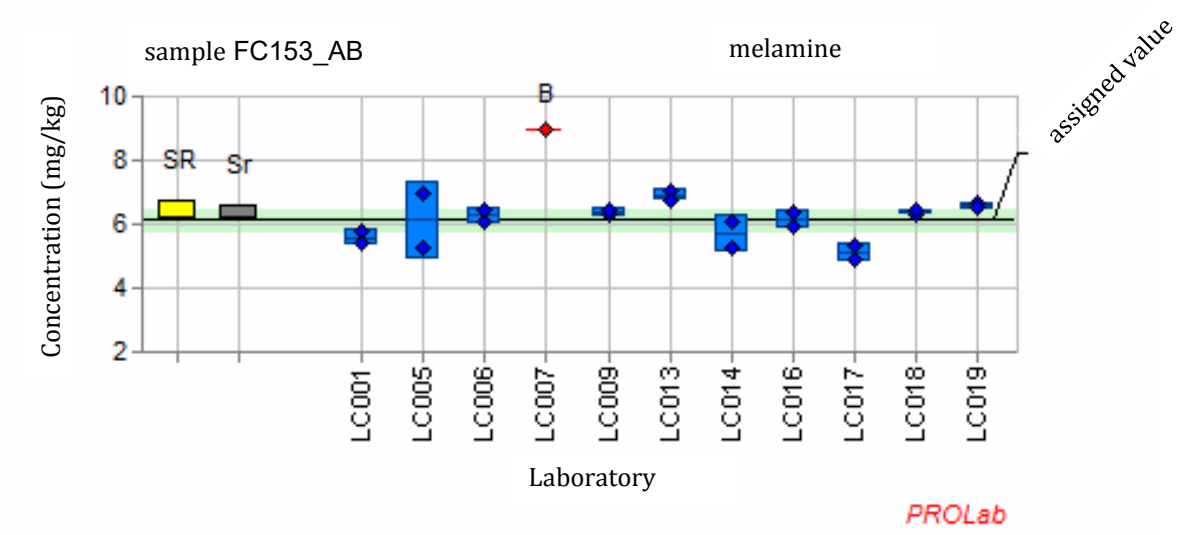


Figure 4: Results of the analysis of melamine in complementary feed for milk cows (FC-153_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
2,55	13,89	6,11	3,81

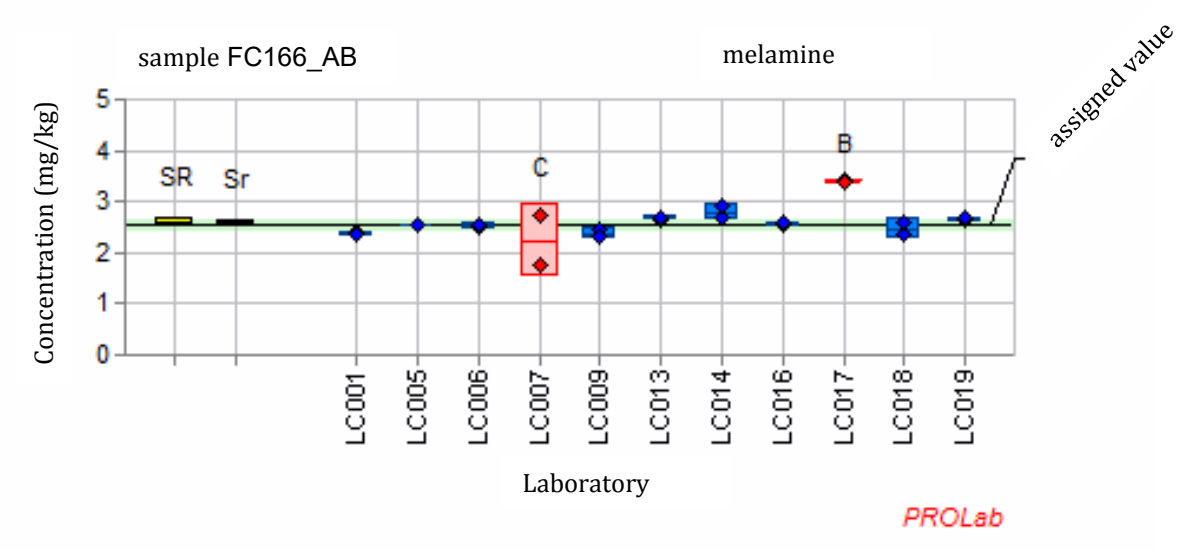


Figure 5: Results of the analysis of melamine in milk replacer (FC-166_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
1,08	15,81	9,05	7,41

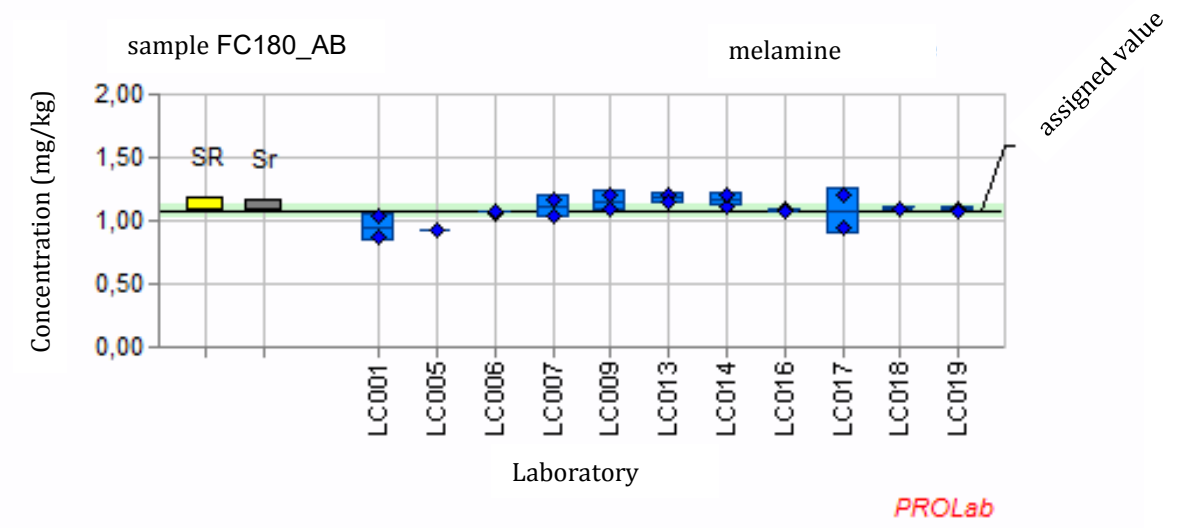


Figure 6: Results of the analysis of melamine in cat feed (FC-180_AB)

Table 4: Results of the analysis of cyanuric acid in feed

	Wheat bran		Soy meal		Complete feed for laying hens		Pet food for dogs (dry)		Complementary feed for milk cows		Milk replacer		Pet food for cats (wet)	
	FC 144		FC 145		FC 041		FC 151		FC 153		FC 166		FC 180	
Sample No	1	12	7	13	9	14	4	8	15	3	10	16	5	6
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
LC001	8,72	8,33	5,35	5,6	1,01	1,2	97,3	78,1	4,54	4,6	2,4	2,58	nq	nq
LC004	11,5	9,5	7	6,5	0,9	1,1	105,4	93,4	5	4,4	2,4	2,5	<0.1	<0.1
LC005	8,97	9,01	5,16	5,34	nd <0,5	nd <0,5	86,29	85,52	4,86	4,76	nd <0,5	nd <0,5	nd <0,5	nd <0,5
LC006			5,46	6,28										
LC007[1)]	4,4	2,12	nd <0,5	1,03		nd <0,5	14,88	2,28	nd <0,5	nd <0,5	nd <0,5	nd <0,5		
LC009	8,71	8,84	5,85	7,79	0,87	0,62	82,8	89,4	5,39	2,85	3,42	2,86	nq <0,5	nq <0,5
LC013	9,45	9,11	5,37	5,38	1,16	1,23	84,94	89,37	4,08	4,2	2,7	2,9	nd <0,2	nd <0,2
LC014	6,79	4,59	2,45	3,8	0,97	0,76	22,96	22,91	2,75	1,84	1,79	1,9	nd <0,3	nd <0,3
LC016	9	8,65	5,77	5,32	1,08	1,08	73,89	80,93	3,96	4,45	2,62	2,37	nd <0,15	nd <0,15
LC017														
LC018	9,06	9,21	5,56	5,71	1,02	1,06	87,7	87,7	4,46	4,42	2,49	2,55	nd <0,15	nd <0,15
LC019	8,53	8,70	5,40	5,74	0,89	1,31	86,04	81,70	4,46	4,45	3,18	2,95	nd <0,2	nd <0,2

Laboratory was excluded from further evaluation since they used their own method

Cochran outlier, confirmed with Mandel's k-statistik (α=0,01)

Grubbs outlier, confirmed with Mandel's h-statistik (α=0,01)

[1)] The laboratory showed abnormalities in Mandel's h- statistics. The h-values of the results exceeded the critical h-values on an error probability of level α=0,05 respectively 0,01. The laboratory was excluded.

nd = not detected (below LOD) nq = below LOQ

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
1,02	15,95	18,70	14,97

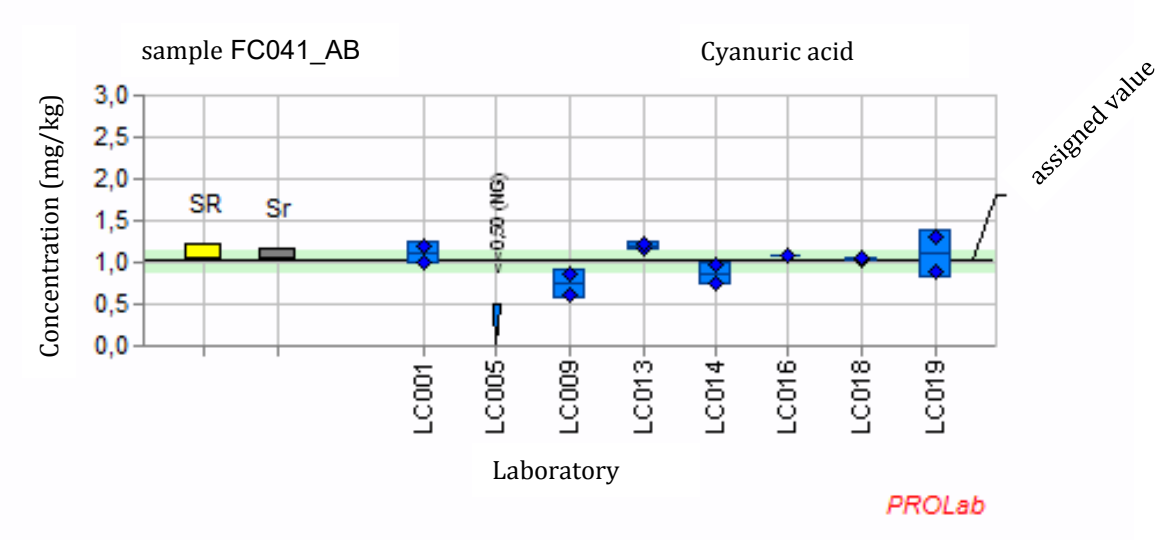


Figure 7: Results of the analysis of cyanuric acid in feed for laying hens (FC0-041_AB)

Mean (mg kg-1)	sH (%)	sR (%)	sr (%)
8,88	11,52	3,40	2,04

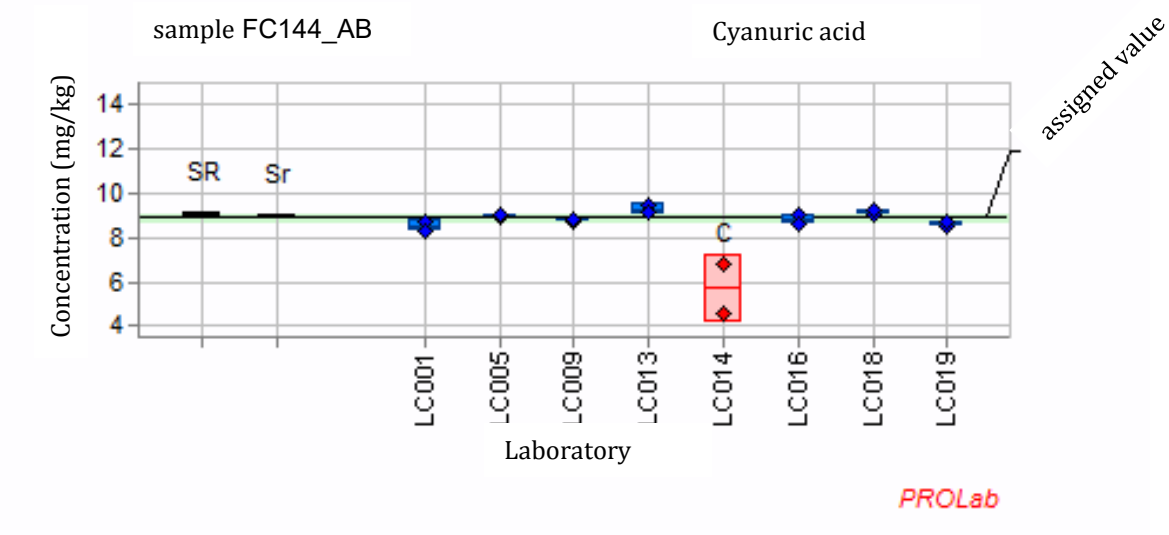


Figure 8: Results of the analysis of cyanuric acid in wheat bran (FC-144_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
5,41	12,41	19,65	11,28

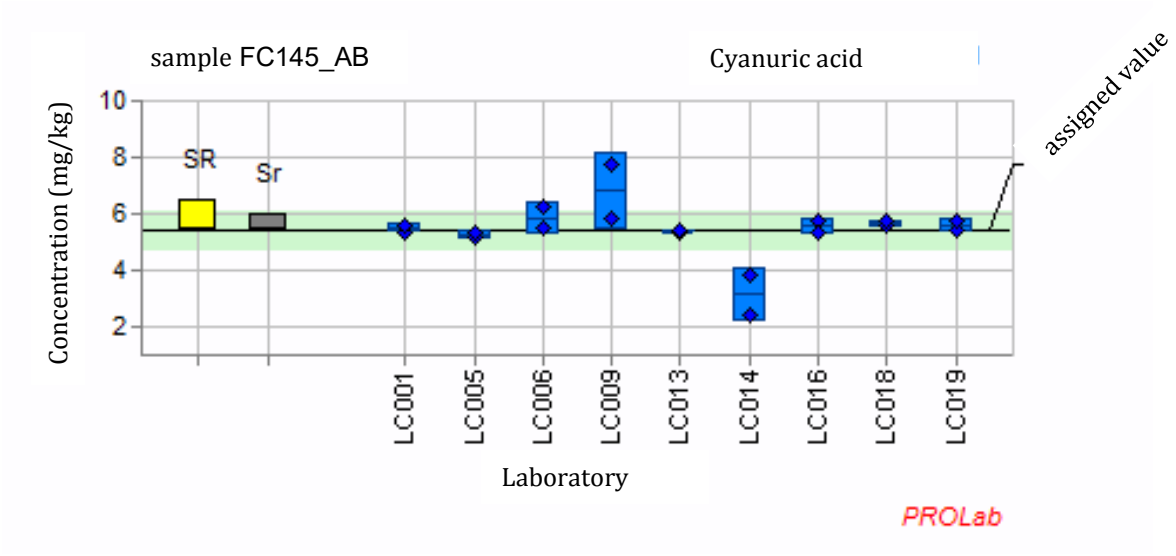


Figure 9: Results of the analysis of cyanuric acid in soy meal (FC-145_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
85,12	8,20	7,03	7,03

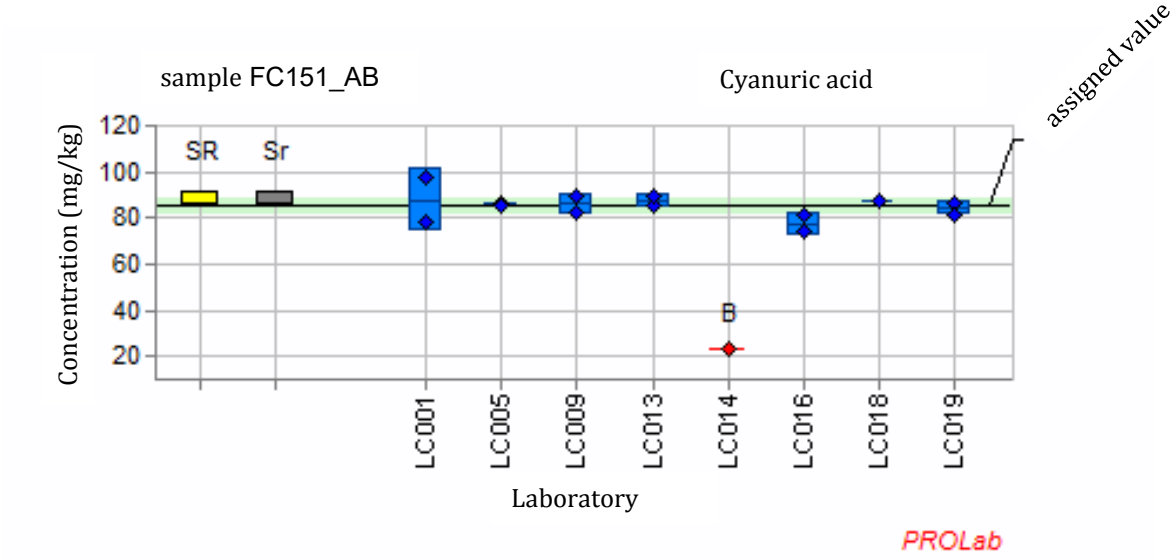


Figure 10: Results of the analysis of cyanuric acid in dog feed (FC-151_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
4,44	12,78	6,01	3,38

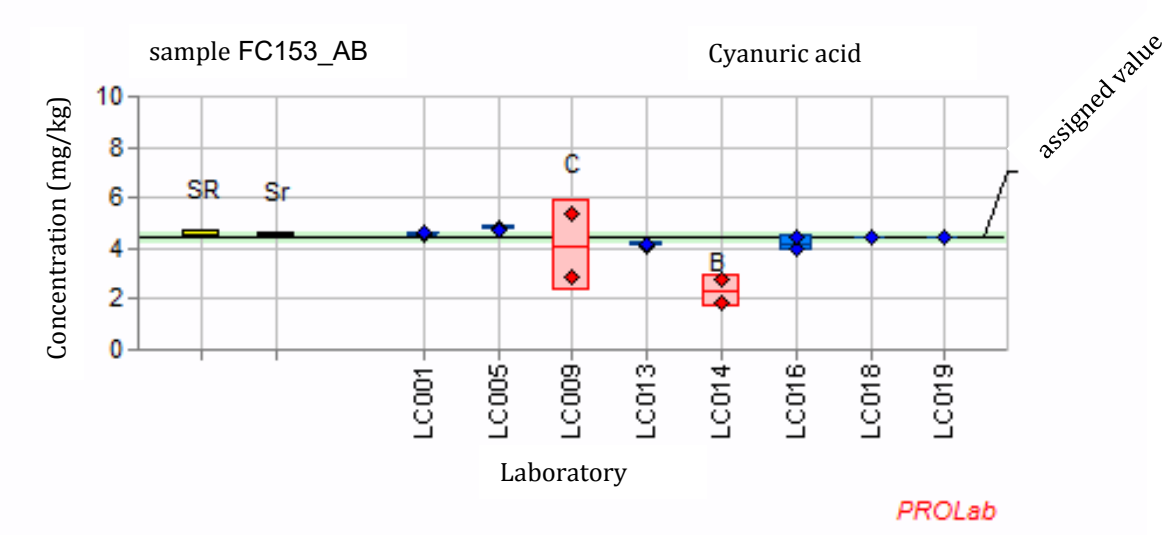


Figure 11: Results of the analysis of cyanuric acid in complementary feed for milk cows (FC-153_AB)

Mean (mg kg^{-1})	sH (%)	s_R (%)	s_r (%)
2,62	13,84	17,44	7,33

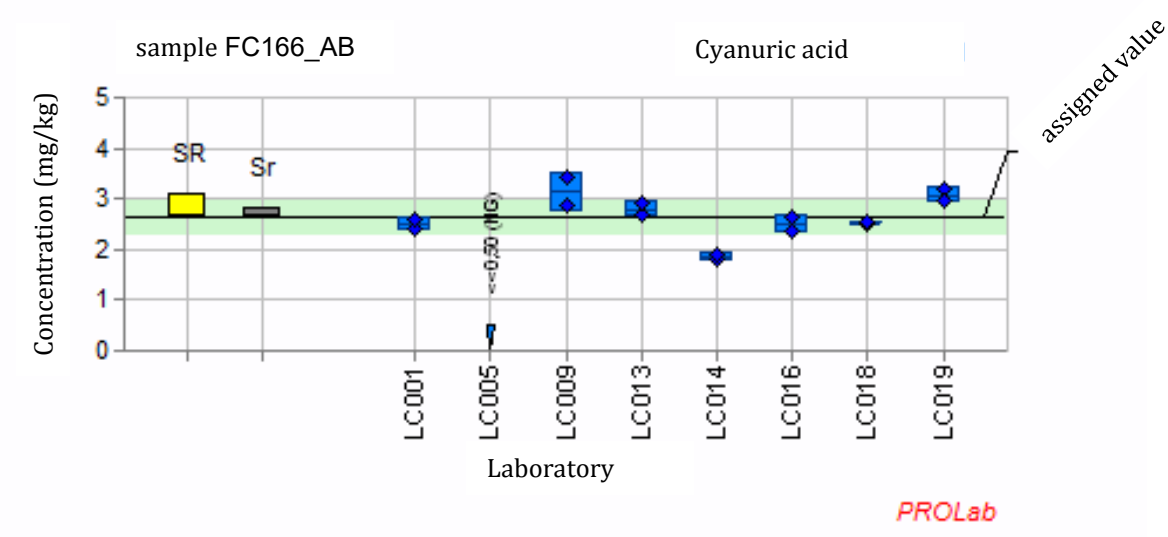


Figure 12: Results of the analysis of cyanuric acid in milk replacer (FC-166_AB)

8.2 Statistical evaluation of the results

The evaluation of the samples was performed according DIN ISO 5725-2: 2002-12.

Before calculation of the statistical parameters the results were tested for the presence of outliers by means of graphical and numerical test methods and checked for their normal distribution. The Mandel's h and Mandel's k statistics were used as graphical consistency techniques. If results or repeatability of a laboratory revealed as strongly different from results or repeatability of other laboratories the data of these laboratory were removed. The numerical outlier tests of Cochran and of Grubbs were performed. Data which were recognized as outliers were rejected. The outlier corrected values were tested for normal distribution by the Shapiro-Wilk test.

After outlier elimination, test for normal distribution the statistical parameters such as the assigned value (Mean), the predicted relative Horwitz standard deviation, the reproducibility standard deviation and the repeatability standard deviation were calculated.

8.2.1 Mandel's between laboratory consistency test statistic (Mandel's h statistic and Mandel's k-statistic)

Before calculation of the statistical parameters the results of the laboratories were plotted and the consistency of the data was examined. This test may indicate that specific laboratories exhibit patterns of results strikingly different from the other of the experiment.

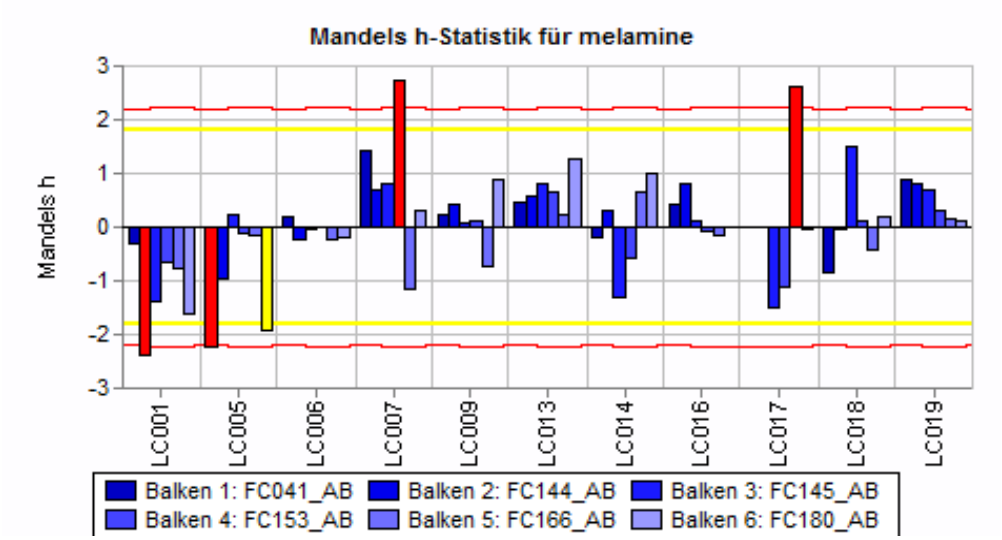
Mandel's h statistic assesses deviation of the laboratory mean value in comparison to the mean value of all laboratories. If the h-value of a laboratory exceeds the critical value across many materials a systematic error of the mean of this laboratory will be indicated in comparison to the general mean of all laboratories. In consequence of the data of this laboratory is removed.

The within laboratory consistency statistic is assessed by Mandel's k statistic. If the k values of a laboratory exceed the critical value across many materials this will be an indication of a systematic higher deviation of this laboratory in comparison to the other laboratories. The data of this laboratory is removed too.

After that the Mandel's h statistic and the Mandel's k statistic will be repeated.

The results of first Mandel's h and k statistics are given in figure 13a and 13b.

The data of laboratory L007 were eliminated from statistical evaluation of cyanuric acid because of the h values of all samples had been exceeded the critical h values for the respective material.



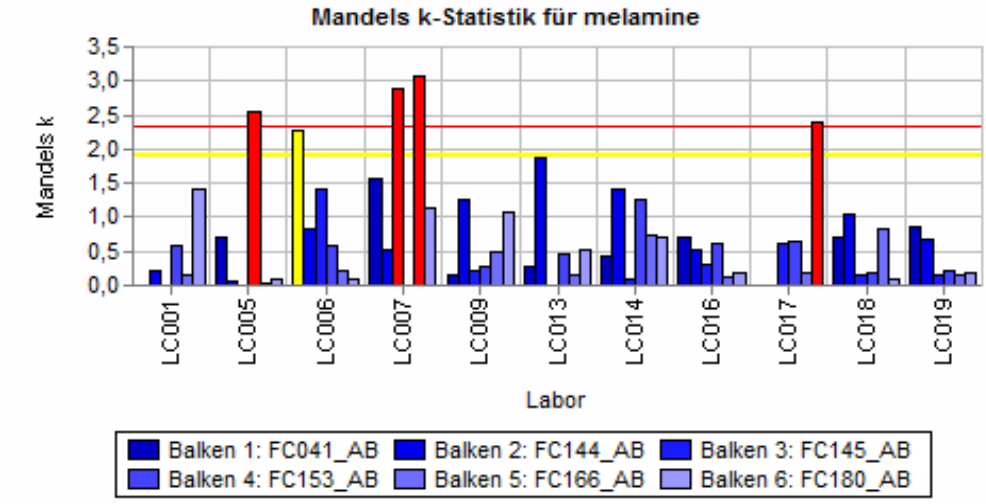
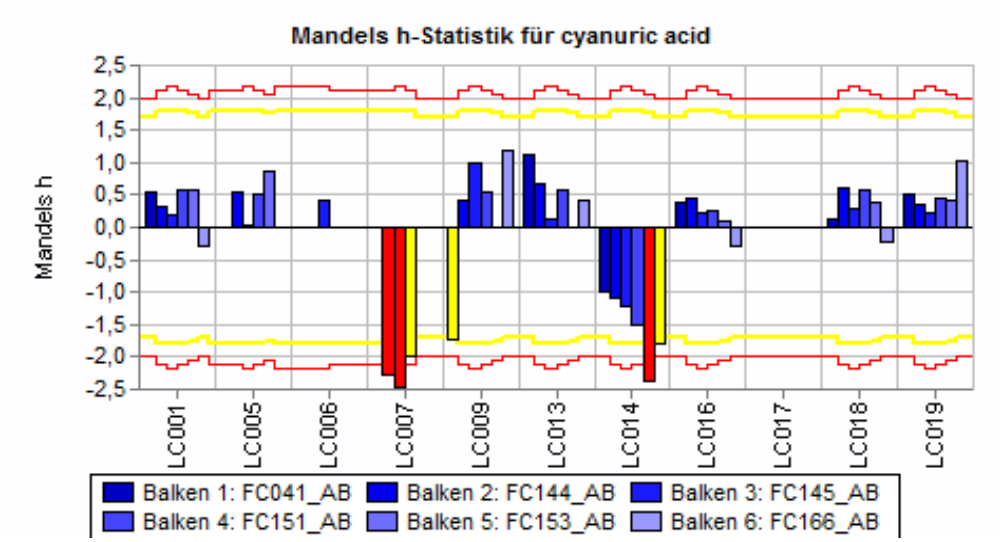


Figure 13a: Mandel's h- and Mandel's k-statistic to assess deviations of laboratories for the analysis of melamine in different feed materials

(FC-041_AB: feed for laying hens, FC-144_AB: wheat bran, FC-145_AB: soy meal, FC-151_AB: dog feed, FC-153_AB: Complementary feed for milk cows, FC-166_AB: Milk replacer, FC-180_AB: cat feed)



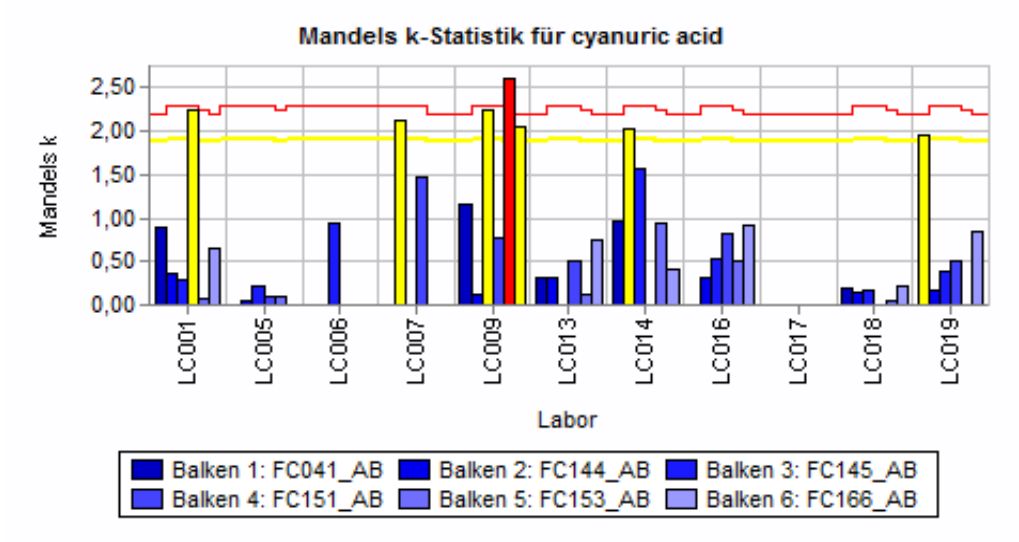


Figure 13b: Mandel's h- and Mandel's k-statistic to assess deviations of laboratories for cyanuric acid in different feed materials

(FC0-41_AB: feed for laying hens, FC-144_AB: wheat bran, FC-145_AB: soy meal, FC-151_AB: dog feed, FC-153_AB: Complementary feed for milk cows, FC-166_AB: Milk replacer, FC-180_AB: cat feed)

8.2.2 Grubbs and Cochran tests for outliers

After the Mandel statistics the numerical outlier tests of Grubbs and Cochran for each material and each analyte were performed. To identify outliers the following approach was chosen. The laboratory will be identified and eliminated as an outlier if the variance of the laboratory within the measurement results between the two samples will exceed the critical Cochran test value (α = 0.01) and this deviation will be confirmed by the critical value in Mandel's k statistic.

Results will be rejected as a Grubbs outlier if a significant deviation of the mean of the laboratory (α = 0.01) is determined by the Grubbs test. Additional the Mandel's h-statistics has to confirm this deviation.

The outliers are given in table 5

After elimination of the identified outliers the outlier corrected values were tested for normal distribution by Shapiro-Wilk test.

Table 5: Analytical results that had been identified as an outlier

laboratory	sample	substance	type of outlier
LC007	FC145	melamine	Cochran / Mandel-k
LC007	FC153	melamine	Grubbs / Mandel-h
LC007	FC166	melamine	Cochran / Mandel-k
LC017	FC166	melamine	Grubbs / Mandel-h
LC014	FC144	cyanuric acid	Cochran / Mandel-k
LC014	FC151	cyanuric acid	Grubbs / Mandel-h
LC014	FC153	cyanuric acid	Grubbs / Mandel-h
LC009	FC153	cyanuric acid	Cochran / Mandel-k

8.2.3 Calculation of statistical parameters

After outlier elimination the arithmetic mean of each material was calculated from the laboratory mean of duplicate analysis. This mean was defined as assigned value because no certified materials in respect of melamine or cyanuric acid were available and could be used for the validation study.

The Horwitz standard deviation according to the Horwitz function modified by Thompson served as assigned value for standard deviation (sHa) of the method. In the present concentration range of 120 µg kg-1 ≤ c < 138 g kg-1 the standard deviation is calculated according to the modified Horwitz function:

$$sHa = 0.02 * c^{0.8495}$$ [kg/kg]

c: dimensionless mass fraction of the analyte to the sample [kg/kg]

In a second step repeatability standard deviation (sr) and reproducibility standard deviation (sR) were calculated for each material. The different number of the submitted results was taken into consideration for the calculation of the statistical parameters. Repeatability stand-

ard deviation (sr) and reproducibility standard deviation (sR) as well as repeatability limit (r) and reproducibility limit (R) characterize the precision of a standard measurement method.

The statistical parameters calculated for melamine and cyanuric acid are given in table 6 and table 7.

8.3 Summary of statistical evaluation

From the calculated statistical parameters given in table 6 and 7 it could be seen that the number of laboratory results for cyanuric acid is too low for five out of six sample materials to make a conclusion on the statistical parameters. Additionally results of sample FC-145 and FC-151 are not normally distributed at a significance level of 5 %. They only correspond to a statistical normal distribution at a significance level of 1 %.

The statistical parameters for melamine allow the conclusion that the data submitted by the laboratories show a good precision and a good reproducibility. For all materials the values calculated for reproducibility are lower in comparison to the predicted Horwitz standard deviation. The ratio reproducibility standard deviation to the repeatability standard deviation is in five of the six materials lower than 2. Normally this ratio lies between 2 and 3.

Analytical data of sample FC-144 for melamine is not normally distributed at the significance level of 5 %. The outlier tests performed gave mixed results for the data obtained from laboratory LC001. By Mandel's h statistic at level of 1 % the results were recognized as significant deviation, but not confirmed as an outlier in Grubbs test at the same significance level. Only at a significant level of 5 % in the Grubbs test the values were identified as outlier. Therefore the data were not rejected from evaluation and the results for sample FC-144 corresponded to normal distribution only at a significance level of 1 %. The results of all laboratories differ only slightly from one another. Therefore the slightly higher deviation from the laboratory in relation to the others is recognized as significant by the statistical tests. Data of LC001 weren't eliminated and the differing level of significance of normal distribution accepted.

Table 6: Results for the calculation of the values for repeatability and reproducibility for melamine

		Melamine					
		FC-041	**FC-144*)**	**FC-145**	**FC-153**	**FC-166**	**FC-180**
		Complete feed for laying hens	**Wheat bran**	**Soy meal**	**Complementary feed for milk cows**	**Milk replacer**	**Pet food for cats (wet)**
Number of laboratories providing results		10	10	11	11	11	11
Number of participants		11	11	11	11	11	11
Assigned value (Mean)	mg/kg	0.95	1.60	30.59	6.12	2.55	1.08
Predicted relative Horwitz standard deviation.	mg/kg	0.15	0.24	2.89	0.75	0.35	0.17
Reproducibility standard deviation	mg/kg	0.12	0.18	2.75	0.62	0.16	0.10
Repeatability standard deviation	mg/kg	0.05	0.09	1.98	0.47	0.10	0.08
Relative predicted Horwitz standard deviation	**%**	**16.13**	**14.90**	**9.58**	**12.18**	**13.89**	**15.81**
Relative reproducibility standard deviation	**%**	**13.05**	**11.02**	**9.11**	**10.18**	**6.11**	**9.05**
Relative repeatability standard deviation	**%**	**5.29**	**5.89**	**6.58**	**7.72**	**3.81**	**7.41**
Relative limit of repeatability(2,80*s_r)	%	14.81	16.48	18.43	21.61	10.67	20.76
Relative limit of reproducibility(2,80*s_R)	%	36.54	30.84	25.51	28.52	17.10	25.34
Number of outliers		0	0	1	1	2	0
Number of laboratories after elimination of outliers		10	10	10	10	9	11
Total number of data		20	20	20	20	18	22
HorRat		**0.81**	**0.74**	**0.95**	**0.84**	**0.44**	**0.57**
s_R/s_r		2,47	1,87	1,38	1,32	1,60	1,22

*) the results of sample FC144 didn't correspond to a normal distribution at significance level α=0.05. They correspond to normal distribution at a significance level α=0.01.

Table 7: Results for the calculation of the values for repeatability and reproducibility cyanuric acid

		Cyanuric acid					
		FC-041	FC-144	FC-145*)	FC-151*)	FC-153	FC-166
		Complete feed for laying hens	Wheat bran	Soy meal	Pet food for dogs (dry)	Complementary feed for milk cows	Milk replacer
Number of laboratories providing results		8	8	9	8	8	8
Number of participants		11	11	11	11	11	11
Assigned value (Mean)	mg/kg	1.02	8.88	5.41	85.12	4.44	2.62
Predicted relative Horwitz standard deviation.	mg/kg	0.16	1.02	0.67	6.98	0.57	0.36
Reproducibility standard deviation	mg/kg	0.19	0.30	1.06	5.98	0.27	0.46
Repeatability standard deviation	mg/kg	0.15	0.18	0.61	5.98	0.15	0.19
Relative predicted Horwitz standard deviation	**%**	**15.95**	**11.52**	**12.41**	**8.20**	**12.78**	**13.84**
Relative reproducibility standard deviation	**%**	**18.70**	**3.40**	**19.65**	**7.03**	**6.01**	**17.44**
Relative repeatability standard deviation	**%**	**14.97**	**2.04**	**11.28**	**7.03**	**3.38**	**7.33**
Relative limit of repeatability($2{,}80*s_r$)	%	41.92	5.72	31.58	19.67	9.46	20.52
Relative limit of reproducibility($2{,}80*s_R$)	%	52.36	9.51	55.03	19.67	16.82	48.83
Number of outliers		0	1	0	1	2	0
Number of laboratories after elimination of outliers		7	7	9	7	6	7
Total number of data		14	14	18	14	12	14
HorRat		**1.17**	**0.30**	**1.58**	**0.86**	**0.47**	**1.26**
$\mathbf{s_R/s_r}$		1,25	1,66	1,74	1,00	1,78	2,38

*) the results of sample FC145 and FC151 didn't correspond to a normal distribution at significance level α=0.05. They correspond to normal distribution at a significance level α=0.01.

8.4 Comments from the participants

All comments received from the participants are grouped and given in annex 10 (14.10).

The following topics were comprised by the comments

- Suggestions for a simplification of the method and of handling
- Deviations from the method protocol
- Suggestions for an optimisation of the description of the method protocol
- Problems with the equipment

In general no participant reported that it was impossible to implement the method in the laboratory.

9 Summary and conclusions

14 laboratories declared their willingness to participate in the method validation study. From these laboratories 12 laboratories send back results. One laboratory used its own method, so the results had to be omitted from the evaluation. Overall 11 data sets were available to perform a statistical evaluation.

From these data 4 laboratories didn't participate in the training study. Therefore they received additional samples from the training study with known concentrations.

The number of participating laboratories and additionally the number of submitted results for the analysis of melamine in feed are sufficient for statistical evaluation. The test on normal distribution on a significance level of $\alpha = 0.05$ show that the results for melamine are with the exception of sample FC-144 normal distributed. All calculated values for the relative reproducibility standard deviation are below the predicted relative Horwitz standard deviation.

The ratio of the reproducibility standard deviation to the repeatability standard deviation is with the exception of one sample below 2, typically the value is between 2 and 3.

Overall the implementation of the method, also in the newly participating laboratories was successful and the calculated statistical parameters fulfilled the requirements.

Some laboratories had problems to detect cyanuric acid in the samples. They were correlated to the sensitivity of the instrument respectively for cyanuric acid or may be to an early elution from the column, using especially UPLC conditions. The number of laboratories sending results for cyanuric acid is just sufficient for some samples. After performing the outlier tests only for one sample the number of valid results exceed 8.

Overall from the results of the method validation study the following conclusions are warranted:

The method is suitable to analyse melamine in animal feed.

The number of data for cyanuric acid is regarded insufficient to allow a valid statistical evaluation, although the results indicate that the method is also suitable to analyse cyanuric acid in animal feed.

And to all participants of the study:

thank you very much for your cooperation and your valuable comments on the method!

10 References

(1) Commission Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed, Official Journal of the European Communities L140 (2002) 10-21

(2) Commission Regulation (EU) No 574/2011 of 16 June 2011 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for nitrite, melamine, Ambrosia spp. and carry-over of certain coccidiostats and histomonostats and consolidating Annexes I and II thereto, Official Journal of the European Union L159 (2011) 7-24

(3) Commission Regulation (EU) No 107/2013 of 5 February 2013 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for melamine in canned pet food, Official Journal of the European Union L35 (2013) 1-2

(4) ISO 5725:1994, “Accuracy (trueness and precision) of measurement methods and results, part 1 to 6”. In “Statistical methods for quality control.” Geneva: ISO Standards Handbook, International Standardization Organisation.

(5) Association of Analytical Communities, Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis”, AOAC INTERNATIONAL, (2002)

(6) Horwitz, W., Albert, R., Deutsch, M.J., Thompson JN, Precision parameters of methods of analysis required for nutrition labeling. Part I. Major nutrients, J. AOAC Int. (1990), 73, 661–680

(7) Horwitz W, Albert R., The Horwitz ratio (HorRat): A useful index of method performance with respect to precision, J AOAC Int., (2006), 89(4), 1095-109

(8) M. Thompson, Limitations of the Application of the Horwitz equation, A rebuttal”, Trends in Analytical Chemistry, (2007), 26 (7), 659 – 661

11 List of abbreviations and definitions

CEN	European Committee for Standardization
TC	Technical Committee
HPLC	High-performance liquid chromatographic method
MS	Mass spectrometric detection
JRC	Joint Research Center
IRMM	Institute for Reference Materials and Measurements
PP	Polypropylene
s_r	Repeatability standard deviation
s_R	Reproducibility standard deviation

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14 Annexes

14.1 Annex 1: Results of the training study

14.1.1 Analysis of melamine in animal feed

Sample No	70		67		62		71	
	Complete feed for laying hens		Complete feed for rabbits		Cat feed		Soy meal	
	value 1	value 2	value 1	value 2	value 1	value 2	value 1	value 2
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
LC001			104.71	100.34	2.60	2.57	9.67	9.57
LC002	< 0,5		66.00	73.16	2.44	2.66	8.48	8.42
LC004	<0.1		110.10		2.85		10.35	
LC005	0.06	0.07	35.56	40.32	0.74	1.09	3.44	3.24
LC006	NN	NN	110.4	108.2	3.52	3.39	12.69	12.76
LC007			91.07	92.72	2.25	2.38	7.65	9.46
LC009	1.12		101.64		2.70		9	
LC011	0.18	0.34			6.05	6.25		
LC012			105.79	81.38	2.66	2.62	8.92	9.29
LC013	0.00	0.00	102.46	102.98	2.80	2.90	10.19	9.92

Laboratory was excluded from further evaluation since they used their own method

Cochran outlier

14.1.2 Analysis of cyanuric acid in animal feed

Sample No	70		67		62		71	
	Complete feed for laying hens		Complete feed for rabbits		Cat feed		Soy meal	
	value 1	value 2	value 1	value 2	value 1	value 2	value 1	value 2
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
LC001			5.98	7.24	9.71	8.09	0.50	0.68
LC002								
LC004	0.65		6.10		10.10		0.60	
LC005								
LC006	NN	NN	5.31	4.85	10.81	9.93		
LC007			6.03	5.64	12.09	10.41		
LC009	<0,1		4.32		10.02		0.10	
LC011								
LC012			4.20	4.74	11.35	8.67	NN	NN
LC013	0.00	0.00	5.48	5.32	11.16	10.75	0.00	0.00

Laboratory was excluded from further evaluation since they used their own method

Cochran outlier

14.1.3 Results for the calculation of the values for repeatability and reproducibility (without Lab 005) for the training phase

		Melamine				Cyanuric acid			
		Blank	Feed for rabbits	Cat feed	Soy meal	Blank	Feed for rabbits	Cat feed	Soy meal
		70	67	62	71	70	67	62	71
Number of laboratories providing results		6	8	9	8	3	6	6	4
Number of participants		9	9	9	9	9	9	9	9
Assigned value (Mean)	mg/kg		97.32	2.71	9.45		5.29	10.25	0.35
Predicted relative Horwitz standard deviation.	mg/kg		7.82	0.37	1.08		0.66	1,16	
Reproducibility standard deviation	mg/kg		11.83	0.43	1.26		1,18	1,7	
Repeatability standard deviation	mg/kg		3,4	0,13	0,19		0,48	1,17	
Relative predicted relative Horwitz standard deviation	**%**	**17,72**	**8.03**	**13.77**	**11.41**		**12,45**	**11,27**	**18,78**
Relative Reproducibility standard deviation	**%**		**12.16**	**15.69**	**13.39**		**22.25**	**16.57**	
Relative repeatability standard deviation	**%**		**3.54**	**4.83**	**2.05**		**9.12**	**16.57**	
Number of Cochran outliers			1		1				
Number of laboratories after elimination of outliers			6	8	6		6	6	2
Total number of data		6	13	15	13	1	11	11	3
HorRat			**1.51**	**1.14**	**1.17**		**1.79**	**1.47**	

14.1.4 Discussion and conclusions from the training study

13 laboratories declared their willingness to participate in the method validation study. From these laboratories 9 laboratories send back results. One laboratory used its own method, so the results had to be omitted from the evaluation.

The number of participating laboratories and additionally the number of submitted results is not sufficient for statistical evaluation. Despite the results were evaluated to give an indication on the performance of the method.

Some laboratories had problems to detect cyanuric acid in the sample. Some problems were of technical nature (problems with negative ESI), others were correlated to the sensitivity of the instrument respectively for cyanuric acid. Therefore the number of laboratories sending valid results for cyanuric acid is even lower in comparison to melamine.

The test on normal distribution on a significance level of $\alpha = 0.05$ show that the results for melamine in sample 67 and sample 62 are not normal distributed. Therefore it is not valid to evaluate the results according DIN ISO 5725-2. Since melamine is homogenously distributed in the material the results indicate that the method is not equally implemented in all laboratories. Along the same line indicate the calculated value for the reproducibility exceeding the predicted relative Horwitz standard deviation about threefold.

For cyanuric acid the valid results were even lower than for melamine. The calculated values of reproducibility for the samples 67 and 62 were sufficient, but the repeatability normally in the range between one third and a half of the reproducibility is equal to the value of the reproducibility in sample 62.

In the comments from the participants some proposals regarding simplification measures were given (number of points for standard addition, handling of dilution of the samples). These proposals were tested by the project leader. As far as practicable these measures will be adopted in a new draft of the method.

Conclusions

1. The numbers of participating laboratories sending valid results are far too low to validate the method in a method validation study, especially for cyanuric acid.
2. For analysing cyanuric acid the used instruments must be in a good condition.
3. The participants used different LC-MS systems. The results as far as can be assessed are not dependent on the instrument. Therefore the type of LC-MS system has not to be mandatory codified as part of the method.
4. Due to comments from the participants the handling of the method is now further simplified.
5. The results of the estimated statistical parameters are not sufficient to meet the requirements for a method validation. But comparing the results of the participants the implementation of the method in the respective laboratory is an important factor.
6. For cyanuric acid even fewer results were delivered. The statistical parameters obtained from these results were slightly better in comparison to melamine.

Further steps

1. Discussion on the continuation of the project, since the number of participants must considerably increase.
2. Finding more participants

3. Delivery of the fifth draft of the method
4. Preparation of the material for the main study
5. Realisation of the main study

14.2 Annex 2: Results of testing the homogeneity

14.2.1 Homogeneity Testing – Wheat bran, results for melamine

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
144-15-1	1.79	1.76
144-15-2	1.71	1.83
144-15-3	1.70	1.72
144-15-4	1.67	1.77
144-15-5	1.75	1.72
144-15-6	1.65	1.76
144-15-7	1.69	1.70
144-15-8	1.73	1.70
144-15-9	1.84	1.74
144-15-10	1.71	1.75

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	3.55	1.78	0.000
2	2	3.54	1.77	0.007
3	2	3.42	1.71	0.000
4	2	3.44	1.72	0.005
5	2	3.47	1.74	0.000
6	2	3.41	1.71	0.006
7	2	3.39	1.70	0.000
8	2	3.43	1.72	0.000
9	2	3.58	1.79	0.005
10	2	3.46	1.73	0.001

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.019	9	0.002	0.83	0.60	3.02
Between groups	0.026	10	0.003			
Total	0.045	19				

14.2.2 Homogeneity Testing - Wheat bran, results for cyanuric acid

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
144-15-1	9.21	9.05
144-15-2	8.96	9.70
144-15-3	9.17	8.96
144-15-4	9.30	9.09
144-15-5	9.05	9.02
144-15-6	8.65	9.43
144-15-7	8.74	9.11
144-15-8	9.32	9.45
144-15-9	9.41	9.22
144-15-10	9.27	9.29

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	18.26	9.13	0.013
2	2	18.66	9.33	0.274
3	2	18.13	9.07	0.022
4	2	18.39	9.20	0.022
5	2	18.07	9.04	0.000
6	2	18.08	9.04	0.304
7	2	17.85	8.93	0.068
8	2	18.77	9.39	0.008
9	2	18.63	9.32	0.018
10	2	18.56	9.28	0.000

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.427	9	0.047	**0.65**	**0.74**	**3.02**
Between groups	0.730	10	0.073			
Total	1.157	19				

14.2.3 Homogeneity Testing – Soy meal, results for melamine

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
145-15-1	31.91	31.22
145-15-2	31.39	32.80
145-15-3	32.71	32.95
145-15-4	32.90	32.86
145-15-5	31.78	32.50
145-15-6	32.23	32.81
145-15-7	34.23	30.41
145-15-8	33.75	32.77
145-15-9	33.54	31.52
145-15-10	32.15	32.72

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	63.13	31.565	0.2381
2	2	64.19	32.095	0.9940
3	2	65.66	32.83	0.0288
4	2	65.76	32.88	0.0008
5	2	64.28	32.14	0.2592
6	2	65.04	32.52	0.1682
7	2	64.64	32.32	7.2962
8	2	66.52	33.26	0.4802
9	2	65.06	32.53	2.0402
10	2	64.87	32.435	0.1625

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	4.037	9	0.449	**0.38**	**0.92**	**3.02**
Between groups	11.668	10	1.167			
Total	15.705	19				

14.2.4 Homogeneity Testing – Soy meal, results for cyanuric acid

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
145-15-1	5.23	5.74
145-15-2	5.43	5.51
145-15-3	5.79	5.41
145-15-4	5.67	5.81
145-15-5	5.43	5.51
145-15-6	5.45	5.43
145-15-7	5.48	5.48
145-15-8	5.67	5.57
145-15-9	5.68	5.69
145-15-10	5.58	5.67

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	10.97	5.49	0.1301
2	2	10.94	5.47	0.0032
3	2	11.2	5.60	0.0722
4	2	11.48	5.74	0.0098
5	2	10.94	5.47	0.0032
6	2	10.88	5.44	0.0002
7	2	10.96	5.48	0.0000
8	2	11.24	5.62	0.0050
9	2	11.37	5.69	0.0001
10	2	11.25	5.63	0.0040

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.200	9	0.02	**0.98**	**0.51**	**3.02**
Between groups	0.228	10	0.02			
Total	0.428	19				

14.2.5 Homogeneity Testing –pet food for dogs (dry), results for cyanuric acid

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
151-15-1	88.19	88.34
151-15-2	88.87	86.56
151-15-3	87.02	90.63
151-15-4	88.16	89.30
151-15-5	89.33	86.81
151-15-6	84.52	86.29
151-15-7	85.09	85.85
151-15-8	86.23	85.85
151-15-9	89.49	86.55
151-15-10	84.75	87.69

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	176.53	88.265	0.011
2	2	175.43	87.715	2.668
3	2	177.65	88.825	6.516
4	2	177.46	88.73	0.650
5	2	176.14	88.07	3.175
6	2	170.81	85.405	1.566
7	2	170.94	85.47	0.289
8	2	172.08	86.04	0.072
9	2	176.04	88.02	4.322
10	2	172.44	86.22	4.322

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	32.547	9	3.62	**1.53**	0.26	**3.02**
Between groups	23.591	10	2.36			
Total	56.138	19				

14.2.6 Homogeneity Testing – complementary feed for milk cows, results for melamine

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
153-15-1	6.80	6.42
153-15-2	6.37	6.43
153-15-3	6.50	6.62
153-15-4	6.78	6.44
153-15-5	6.63	6.52
153-15-6	6.48	6.68
153-15-7	6.66	6.77
153-15-8	6.68	6.82
153-15-9	6.62	6.76
153-15-10	6.83	6.56

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	13.22	6.61	0.072
2	2	12.80	6.40	0.002
3	2	13.12	6.56	0.007
4	2	13.22	6.61	0.058
5	2	13.15	6.58	0.006
6	2	13.16	6.58	0.020
7	2	13.43	6.72	0.006
8	2	13.50	6.75	0.010
9	2	13.38	6.69	0.010
10	2	13.39	6.70	0.036

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.185	9	0.02	**0.90**	0.56	**3.02**
Between groups	0.227	10	0.02			
Total	0.412	19				

14.2.7 Homogeneity Testing – complementary feed for milk cows, results for cyanuric acid

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
153-15-1	4.62	4.68
153-15-2	4.73	4.74
153-15-3	4.76	4.52
153-15-4	4.47	4.50
153-15-5	4.46	4.46
153-15-6	4.65	4.52
153-15-7	4.64	4.63
153-15-8	4.53	4.63
153-15-9	4.55	4.49
153-15-10	4.60	4.60

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	9.30	4.65	0.002
2	2	9.47	4.74	0.000
3	2	9.28	4.64	0.029
4	2	8.97	4.49	0.000
5	2	8.92	4.46	0.000
6	2	9.17	4.59	0.008
7	2	9.27	4.64	0.000
8	2	9.16	4.58	0.005
9	2	9.04	4.52	0.002
10	2	9.20	4.60	0.000

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.124	9	0.014	**2.98**	0.05	**3.02**
Between groups	0.046	10	0.005			
Total	0.171	19				

14.2.8 Homogeneity Testing – milk replacer, results for melamine

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
166-15-1	2.68	2.65
166-15-2	2.69	2.55
166-15-3	2.74	2.63
166-15-4	2.71	2.53
166-15-5	2.59	2.65
166-15-6	2.66	2.49
166-15-7	2.63	2.62
166-15-8	2.58	2.62
166-15-9	2.63	2.58
166-15-10	2.52	2.63

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	5.33	2.67	0.000
2	2	5.24	2.62	0.010
3	2	5.37	2.69	0.006
4	2	5.24	2.62	0.016
5	2	5.24	2.62	0.002
6	2	5.15	2.58	0.014
7	2	5.25	2.63	0.000
8	2	5.20	2.60	0.001
9	2	5.21	2.61	0.001
10	2	5.15	2.58	0.006

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.022	9	0.002	**0.43**	0.89	**3.02**
Between groups	0.057	10	0.006			
Total	0.079	19				

14.2.9 Homogeneity Testing – milk replacer, results for cyanuric acid

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
166-15-1	2.20	2.49
166-15-2	2.33	2.57
166-15-3	2.43	2.43
166-15-4	2.40	2.34
166-15-5	2.27	2.41
166-15-6	2.43	2.48
166-15-7	2.50	2.48
166-15-8	2.41	2.42
166-15-9	2.39	2.41
166-15-10	2.41	2.66

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	4.69	2.35	0.0421
2	2	4.9	2.45	0.0288
3	2	4.86	2.43	0.0000
4	2	4.74	2.37	0.0018
5	2	4.68	2.34	0.0098
6	2	4.91	2.46	0.0012
7	2	4.98	2.49	0.0002
8	2	4.83	2.42	0.0000
9	2	4.8	2.40	0.0002
10	2	5.07	2.54	0.0313

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.070	9	0.008	**0.68**	**0.71**	**3.02**
Between groups	0.115	10	0.012			
Total	0.186	19				

14.2.10 Homogeneity Testing – Pet food for cats (wet), results for melamine

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
180-15-1	1.05	1.10
180-15-2	1.03	1.20
180-15-3	1.09	1.10
180-15-4	1.08	1.18
180-15-5	0.92	0.99
180-15-6	0.77	1.18
180-15-7	1.09	1.05
180-15-8	1.06	1.25
180-15-9	1.38	1.23
180-15-10	1.14	1.12

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	2.15	1.07	0.001
2	2	2.23	1.11	0.014
3	2	2.18	1.09	0.000
4	2	2.26	1.13	0.006
5	2	1.90	0.95	0.002
6	2	1.95	0.98	0.085
7	2	2.14	1.07	0.001
8	2	2.31	1.15	0.018
9	2	2.61	1.31	0.012
10	2	2.26	1.13	0.000

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.171	9	0.019	**1.37**	0.32	**3.02**
Between groups	0.139	10	0.014			
Total	0.310	19				

14.2.11 Homogeneity Testing – Complete feed for laying hens, results for melamine

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
041-14-1	1.12	1.13
041-14-2	1.12	1.14
041-14-3	1.14	1.04
041-14-4	1.00	0.99
041-14-5	1.05	1.01
041-14-6	1.06	1.13
041-14-7	1.01	1.06
041-14-8	0.99	1.06
041-14-9	1.03	1.05
041-14-10	1.06	1.08

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	2.25	1.13	3.715E-05
2	2	2.26	1.13	3.033E-04
3	2	2.19	1.09	5.372E-03
4	2	1.99	1.00	1.538E-05
5	2	2.05	1.03	7.122E-04
6	2	2.20	1.10	2.503E-03
7	2	2.07	1.03	1.024E-03
8	2	2.05	1.03	2.135E-03
9	2	2.08	1.04	1.729E-04
10	2	2.14	1.07	2.891E-04

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.039	9	0.004	**3.42**	0.03	**3.02**
Between groups	0.013	10	0.001			
Total	0.051	19				

14.2.12 Homogeneity Testing – Complete feed for laying hens, results for cyanuric acid

Samples	Determination 1 mg kg^{-1}	Determination 2 mg kg^{-1}
041-14-1	1.03	1.29
041-14-2	1.12	1.10
041-14-3	0.95	1.07
041-14-4	1.10	1.06
041-14-5	1.14	1.20
041-14-6	1.07	1.08
041-14-7	1.07	1.08
041-14-8	1.09	1.09
041-14-9	1.11	1.04
041-14-10	1.10	1.10

Statistical evaluation: ANOVA – One way

Summary				
Row	Count	Sum	Mean	Variance
1	2	2.32	1.16	0.035
2	2	2.22	1.11	0.000
3	2	2.02	1.01	0.007
4	2	2.16	1.08	0.001
5	2	2.34	1.17	0.002
6	2	2.15	1.07	0.000
7	2	2.15	1.07	0.000
8	2	2.18	1.09	0.000
9	2	2.16	1.08	0.003
10	2	2.20	1.10	0.000

Source of Variation	Square sum (SS)	Degrees of freedom (df)	Mean Square (MS)	Calculated F-value	p-value	critical F-value
Differences within groups	0.037	9	0.004	**0.88**	0.57	**3.02**
Between groups	0.047	10	0.005			
Total	0.085	19				

14.3 Annex 3: Stability of the test samples

14.3.1 Melamine

Determination of the stability 7 – 8 month after preparation of the samples

Melamine	Wheat bran		Soy meal		Pet food for dogs (dry)		Complementary feed for milk cows		milk replacer		Pet food for cats (wet)		Complete feed for laying hens	
	FC144-15		FC145-15		FC151-15		FC153-15		FC166-15		FC180-15		FC041-14	
Time of analysis	Nov. 2015	Apr. 2016	Oct. 2015	Jul. 2016	Dec. 2015	Jul. 2016	Nov. 2015	Jul. 2016	Nov. 2015	Apr. 2016	Dez. 2015	Apr. 2016	Oct. 2015	Apr. 2016
Mean (mg kg^{-1})	**1.72**	**1.69**	**32.26**	**31.61**			**6.64**	**6.57**	**2.64**	**2.63**	**1.15**	**1.10**	**1.03**	**1.04**
number of samples	10	6	10	6			10	6	10	6	10	6	10	6
standard deviation (mg kg^{-1})	0.057	0.035	0.883	0.626			0.150	0.119	0.067	0.055	0.063	0.030	0.031	0.014
t-Test	**1.44**		**1.56**				**0.86**		**0.30**		**1.90**		**0.50**	
critical value	2.51		2.51				2.51		2.51		2.51		2.51	
	stable		stable				stable		stable		stable		stable	
F-test	**2.63**		**1.99**				**1.57**		**1.49**		**4.46**		**4.96**	
critical value	6.68		6.68				6.68		6.68		6.68		6.68	
	variances are homogen		variances are homogen				variances are homogen		variances are homogen		variances are homogen		variances are homogen	

14.3.2 Cyanuric acid

Determination of the stability 7 – 8 month after preparation of the samples

Cyanuric acid	Wheat bran		Soy meal		Pet food for dogs (dry)		Complementary feed for milk cows		milk replacer		Pet food for cats (wet)		Complete feed for laying hens	
	FC144-15		FC145-15		FC151-15		FC153-15		FC166-15		FC180-15		FC041-14	
Time of analysis	Nov. 2015	Apr. 2016	Oct. 2015	Jul. 2016	Dec. 2015	Jul. 2016	Nov. 2015	Jul. 2016	Nov. 2015	Apr. 2016	Dez. 2015	Apr. 2016	Oct. 2015	Apr. 2016
Mean (mg kg^{-1})	**9.11**	**8.91**	**5.54**	**5.44**	**87.39**	**86.38**	**4.60**	**4.67**	**2.47**	**2.55**			**1.08**	**1.12**
number of samples	10	6	10	6	10	6	10	6	10	6	10	6	10	6
standard deviation (mg kg^{-1})	0.255	0.205	0.166	0.272	1.592	1.095	0.100	0.119	0.091	0.074			0.054	0.061
t-Test (one-tailed)	**1.62**		**0.90**		**1.36**		**1.21**		**1.88**				**1.32**	
critical value	2.51		2.51		2.51		2.51		2.51				2.51	
	stable		stable		stable		stable		stable				stable	
F-test (one-tailed)	**1.55**		**2.67**		**2.11**		**1.40**		**1.54**				**1.28**	**1.55**
critical value	6.68		6.68		6.68		6.68		6.68				6.68	
	variances are homogen		variances are homogen		variances are homogen		variances are homogen		variances are homogen				variances are homogen	

14.4 Annex 4: Numbers of distributed samples

Sam-ple	Type of feeding stuff		LC001	LC002	LC004	LC005	LC006	LC007	LC009	LC013	LC014	LC015	LC016	LC017	LC018	LC019
Sam-ple 2	rapeseed meal	BLANK_A	BfR-0034	BfR-0040	BfR-0171	BfR-0099	BfR-0120	BfR-0125	BfR-0004	BfR-0015	BfR-0019	BfR-0255	BfR-0254	BfR-0114	BfR-0211	BfR-0298
Sam-ple 11	rapeseed meal	BLANK_B	BfR-0071	BfR-0072	BfR-0080	BfR-0194	BfR-0122	BfR-0036	BfR-0152	BfR-0048	BfR-0164	BfR-0154	BfR-0128	BfR-0276	BfR-0170	BfR-0308
Sam-ple 1	Wheat bran	144-15	BfR-0126	BfR-0280	BfR-0223	BfR-0282	BfR-0010	BfR-0248	BfR-0284	BfR-0231	BfR-0063	BfR-0188	BfR-0286	BfR-0285	BfR-0086	BfR-0302
Sam-ple 12	Wheat bran	144-15	BfR-0016	BfR-0189	BfR-0230	BfR-0104	BfR-0202	BfR-0193	BfR-0119	BfR-0111	BfR-0185	BfR-0055	BfR-0203	BfR-0017	BfR-0044	BfR-0310
Sam-ple 7	soy meal	145-15	BfR-0153	BfR-0172	BfR-0003	BfR-0177	BfR-0213	BfR-0176	BfR-0031	BfR-0166	BfR-0281	BfR-0105	BfR-0239	BfR-0240	BfR-0259	BfR-0309
Sam-ple 13	soy meal	145-15	BfR-0197	BfR-0134	BfR-0129	BfR-0247	BfR-0095	BfR-0236	BfR-0183	BfR-0157	BfR-0252	BfR-0278	BfR-0271	BfR-0140	BfR-0204	BfR-0301
Sam-ple 9	complete feed for laying hens	041-14	BfR-0115	BfR-0168	BfR-0242	BfR-0130	BfR-0263	BfR-0192	BfR-0147	BfR-0013	BfR-0144	BfR-0009	BfR-0057	BfR-0068	BfR-0241	BfR-0300
Sam-ple 14	complete feed for laying hens	041-14	BfR-0060	BfR-0182	BfR-0148	BfR-0124	BfR-0138	BfR-0228	BfR-0007	BfR-0151	BfR-0156	BfR-0288	BfR-0090	BfR-0167	BfR-0067	BfR-0311
Sam-ple 4	Pet food for dogs (dry)	151-15	BfR-0098	BfR-0187	BfR-0133	BfR-0035	BfR-0262	BfR-0221	BfR-0279	BfR-0137	BfR-0018	BfR-0209	BfR-0100	BfR-0028	BfR-0127	BfR-0312
Sam-ple 8	Pet food for dogs (dry)	151-15	BfR-0274	BfR-0169	BfR-0073	BfR-0121	BfR-0186	BfR-0227	BfR-0277	BfR-0222	BfR-0014	BfR-0260	BfR-0094	BfR-0224	BfR-0216	BfR-0304
Sam-ple 15	complementary feed for milk cows	153-15	BfR-0113	BfR-0181	BfR-0021	BfR-0022	BfR-0212	BfR-0237	BfR-0089	BfR-0001	BfR-0155	BfR-0243	BfR-0258	BfR-0269	BfR-0220	BfR-0313
Sam-ple 3	complementary feed for milk cows	153-15	BfR-0226	BfR-0041	BfR-0162	BfR-0143	BfR-0201	BfR-0251	BfR-0264	BfR-0200	BfR-0112	BfR-0178	BfR-0079	BfR-0074	BfR-0033	BfR-0305
Sam-ple 10	milk replacer	166-15	BfR-0051	BfR-0175	BfR-0179	BfR-0199	BfR-0238	BfR-0215	BfR-0165	BfR-0097	BfR-0069	BfR-0064	BfR-0270	BfR-0106	BfR-0078	BfR-0307

Sam-ple	Type of feeding stuff		LC001	LC002	LC004	LC005	LC006	LC007	LC009	LC013	LC014	LC015	LC016	LC017	LC018	LC019
Sam-ple 16	milk replacer	166-15	BfR-0196	BfR-0268	BfR-0233	BfR-0110	BfR-0149	BfR-0047	BfR-0256	BfR-0283	BfR-0225	BfR-0163	BfR-0116	BfR-0229	BfR-0101	BfR-0303
Sam-ple 5	Pet food for cats (wet)	180-15	BfR-0184	BfR-0039	BfR-0030	BfR-0092	BfR-0275	BfR-0190	BfR-0056	BfR-0234	BfR-0214	BfR-0249	BfR-0245	BfR-0207	BfR-0265	BfR-0306
Sam-ple 6	Pet food for cats (wet)	180-15	BfR-0219	BfR-0131	BfR-0118	BfR-0029	BfR-0081	BfR-0077	BfR-0102	BfR-0210	BfR-0142	BfR-0103	BfR-0206	BfR-0037	BfR-0084	BfR-0299
Blank		055-15	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

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	82-3820-00-8876593	-2359	10.05.2016	82 Hildburg Fry

Collaborative study for the validation of the method "Determination of melamine and cyanuric acid in animal feed by LC-MS/MS"

Dear Participant,

thank you very much for your willingness to participate in the main study of our collaborative trial.

This collaborative trial is organized by the Federal Institute for Risk Assessment, Unit Contaminants within the 3rd Mandate of the European Commission to the European Standardisation body CEN/TC327.

Aim of the study

Aim of the study is the standardisation of a method for the determination of melamine and cyanuric acid in feed. Therefore the validation of this method in a collaborative trial is necessary.

Design of the study

The first part was the training period which was intended to give the laboratories the opportunity to become familiar with the method. In this period four samples were distributed to the laboratories, the report of this part was sent to the participants at 9th of March 2016.

The second part is the validation study started on 10th of May 2016.

Contents of the package

The package sent to you for the validation study contains:

- 17 samples of animal feed
- One sample of the 17 samples is a blank material, labelled as Blank sample. This sample could be used as blank material and for spiking.

- The isotopically labelled standard solutions and melamine and cyanuric acid standard solutions (including MSDS and certificate per e-mail)
- Acknowledgment of reception for samples and standards

- **A description of the method (referred to as draft for the validation study) will be sent in the next days by e-mail.**

An excel sheet for reporting of your results will be sent to you later.

Since the aim of the study is the validation of the method in a collaborative trial you are requested to perform the analysis strictly according to the method description.

If you have any questions to the method please do not hesitate to contact us by e-mail Hildburg.Fry@bfr.bund.de

The deadline for reporting the results of the collaborative trial is **04. July 2016**.

Your LAB Code is: LC014

Instruction sheet

1) Samples and Sample Handling

The sample material is sufficient to allow at least one analysis per sample for the screening method and one analysis per sample for the confirmation method.
You receive 17 animal feed samples, the isotopically labelled standard substances and the standard substances.

Acknowledgment of reception

a) Please check the contents of the parcel carefully and acknowledge receipt and conformity of the samples and standards.

b) Please send the acknowledgment of reception to:
Hildburg.Fry@bfr.bund.de

Storage of samples and standards

c) Store the samples at +4 °C except sample 5 and sample 6 which have to be stored at - 20 °C. Store the standards at -20 °C on arrival. Before starting analysis, the samples and the standards have to be left at room temperature until ambient temperature is reached.

Handling of samples and standards

d) All samples are ground and homogenised. Before analysis mix the samples again: Dry samples with e.g. an overhead shaker for 15 minutes and the samples with a wet content > 75 % by shaking thoroughly by hand and with a spatula.

e) After homogenisation of the samples weigh the appropriate amount immediately and perform the analyses as described in the method. Please record the weights with an accuracy of one digit after the decimal point.

f) The concentration of all standard solutions is 0.1 mg ml^{-1}.

Analysis of samples

g) In a first step you have to analyse the samples **with the screening method** and then to verify positive findings by performing the **confirmatory method**. Positive samples have to be analysed **once**.

h) In the case you do not analyse the samples on the same day, please analyse first sample 1 to 10 and then sample 11 to 16.

i) For quantification please use the standards sent to you. Results shall be stated in mg kg^{-1} with an accuracy of two digits after the decimal point.

j) In case of values below the limit of quantification or the limit of detection, please specify these particular limits.

Reporting of results

k) We wish to point out that your reporting on the particulars for each method is most profitable and valuable for the outcome of the study. In the same context you are also requested to meticulously report any irregularities or disturbance that may occur during the measurement. Equally must be mentioned any change of operator, as the case may be, reasons for missing results.

l) Please send a chromatogram of a sample of your choice that comprises all relevant ion traces and a total ion chromatogram (TIC).
Please clearly indicate your Lab Code on the chromatogram.

2) Quality Control Samples

For additional quality control a blank material (Blank) is sent to you. Further details are given in the method protocol.

3) Reporting of results

For reporting your data, we will send you data form templates by e-mail.
(**Reporting-sheet-XXX**). Please insert the details and save the file adding your lab code (replacing XXX) to the data file name.

Please return the files with your inserted data (**Reporting-sheet-XXX.xlsx**) not later than

04. July 2016

to the following e-mail address:
Hildburg.Fry@bfr.bund.de

We wish you success analysing the samples!

Your LAB Code is: LC014

On behalf

Hildburg Fry

Acknowledgment of reception

Please find below the list of content of samples and standard substances for the collaborative trial **"Determination of melamine and cyanuric acid in animal feed by LC-MS/MS"**.

Please check the content of the package carefully and return the completed form to

Hildburg.Fry@bfr.bund.de

Name:
Institute:
LAB-Code: LC014

Date of arrival of samples: _____

Date of checking samples: _____

Test Material	**Sample Code**	**Condition at Arrival**	
Animal feed Sample 1	BfR_0063	□ o.k.	□ damaged
Animal feed Sample 2	BfR_0019	□ o.k.	□ damaged
Animal feed Sample 3	BfR_0112	□ o.k.	□ damaged
Animal feed Sample 4	BfR_0018	□ o.k.	□ damaged
Animal feed Sample 5	BfR_0214	□ o.k.	□ damaged
Animal feed Sample 6	BfR_0142	□ o.k.	□ damaged
Animal feed Sample 7	BfR_0281	□ o.k.	□ damaged
Animal feed Sample 8	BfR_0014	□ o.k.	□ damaged
Animal feed Sample 9	BfR_0144	□ o.k.	□ damaged
Animal feed Sample 10	BfR_0069	□ o.k.	□ damaged
Animal feed Sample 11	BfR_0164	□ o.k.	□ damaged
Animal feed Sample 12	BfR_0185	□ o.k.	□ damaged
Animal feed Sample 13	BfR_0252	□ o.k.	□ damaged
Animal feed Sample 14	BfR_0156	□ o.k.	□ damaged
Animal feed Sample 15	BfR_0155	□ o.k.	□ damaged
Animal feed Sample 16	BfR_0225	□ o.k.	□ damaged
Animal feed (blank)	Blank	□ o.k.	□ damaged
^{13}-C_3-melamine standard solution		□ o.k.	□ damaged
^{13}-C_3-cyanuric acid standard solution		□ o.k.	□ damaged
Melamine standard standard solution		□ o.k.	□ damaged
Cyanuric acid standard standard solution		□ o.k.	□ damaged

Comments:

Date: _____ Signature: _____

14.6 Annex 6: draft of the method for the main trial (2016-05-11): Determination of melamine and cyanuric acid in animal feed by LC-MS/MS (used for the main trial)

Animal Feeding stuffs –
Determination of melamine and cyanuric acid content by liquid chromatographic method with mass spectrometric detection (LC-MS/MS)

Futtermittel –
Bestimmung des Melamin- und Cyanursäuregehaltes mit Flüssigkeitschromatographie und massenspektrometrischer Detektion (LC-MS/MS)

Aliments pour animaux –
Dosage de la melamine et l'acide cyanurique par de chromatographie liquide couplée à un spectromètre de masse (CL-SM/SM)

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Warning:The use of this standard involves hazardous materials, operations and equipment. This standard does not purpose to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This European norm defines a high-performance liquid chromatographic (HPLC) mass spectrometric (MS) method for the analysis of melamine and cyanuric acid in the concentration range between < 1 mg kg^{-1} and 100 mg kg^{-1} in feed. The method is suitable both for screening and for confirmation (quantification) purposes. The limit of quantification of the method has been demonstrated to be better than 0.3 mg kg^{-1} for melamine and 0.6 mg kg^{-1} for cyanuric acid. The quantification of concentrations above 100 mg kg^{-1} is possible but the method has to be validated by the operator.

2 Normative References

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references the latest edition of the references document (including any amendments) applies.

3 Principle

To check for the presence of melamine and cyanuric acid in a screening method, samples are extracted by a mixture of trichloroacetic acid, acetonitrile and water. The extract is further diluted with acetonitrile, centrifuged and injected into the LC-MS/MS system. For samples tested positive in the screening method a confirmatory method has to be performed. For the confirmatory method an internal standard is added, extraction is performed with a mixture of trichloroacetic acid, acetonitrile and water and twice by a mixture of acetonitrile and water. After dilution and centrifugation the supernatant is injected into the LC-MS/MS system. The quantification of melamine and / or cyanuric acid is carried out by means of standard addition.

4 Reagents

WARNING: Avoid inhalation of and exposure to the toxic materials and solutions thereof. Work in a fume-hood when handling the solvents and solutions. Wear safety glasses, protective clothing and avoid skin contact.

Use only reagents of recognized analytical grade or higher unless stated otherwise.

4.1 Diethylamine (DEA)

4.2 Trichloroacetic acid (TCA)

4.3 Water, purity grade for HPLC or comparable quality (e.g. with Milli-Q purified water)

4.4 Acetonitrile, hypergrade for LCMS

4.5 Methanol, hypergrade for LCMS

4.6 Extraction solvent A: Weigh 80 g TCA (4.2) and add 600 ml acetonitrile (4.4) and water (4.3) at the rate of 10 + 30 (v/v) (solvent B 4.7).

4.7 Extraction solvent B: Mix acetonitrile (4.4) and water (4.3) at a rate of 10 + 30 (v/v)

4.8 LC-MS/MS

4.8.1 Ammonium acetate

4.8.1.1 Ammonium acetate solution, c = 10 mmol/l

Weigh 0.77 g ammonium acetate (4.8.1) to the nearest 0.01 g into a 1 000 ml volumetric flask (5.10) and dissolve in water (4.3). Add up to 1000 ml with water (4.3). Prepare new solutions weekly.

4.8.2 Mobile Phase for HPLC

4.8.2.1 Mobile Phase A

Mix acetonitrile (4.4) and ammonium acetate solution (4.8.1.1) at the rate of 95 + 5 (v/v).

4.8.2.2 Mobile Phase B

Mix ammonium acetate solution (4.8.1.1) and acetonitrile (4.4) at the rate of 95 + 5 (v/v).

4.8.3 Reference substances

Specification of the purity of each batch of the reference standards is necessary.
Note: The standard substances or standard solutions can be purchased from Sigma-Aldrich, Restek or Witega. This is an example for suitable products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of these products. Comparable products could be used, if the results are comparable.

4.8.4 Melamine (2,4,6-Triamino-1,3,5-triazine, sym-Triaminotriazine)

4.8.4.1 Melamine standard substance purity at least 99 %

4.8.4.2 Alternatively Melamine standard solution c = 1 mg ml^{-1} or c = 0.1 mg ml^{-1}

4.8.5 Cyanuric acid (1,3,5-Triazine-2,4,6-triol, 2,4,6-Trihydroxy-1,3,5-triazine)

4.8.5.1 Cyanuric acid standard substance purity at least 98 %

4.8.5.2 Alternatively Cyanuric standard solution c = 1 mg ml^{-1} or c = 0.1 mg ml^{-1}

4.8.6 $^{13}C_3$-Melamine

4.8.6.1 $^{13}C_3$-Melamine standard substance purity at least 95 %

4.8.6.2 Alternatively $^{13}C_3$-Melamine standard solution c = 0.1 mg ml^{-1}

4.8.7 $^{13}C_3$-Cyanuric acid

4.8.7.1 $^{13}C_3$-Cyanuric acid standard substance purity at least 97 %

4.8.7.2 Alternatively $^{13}C_3$-Cyanuric acid standard solution c = 0.1 mg ml^{-1}

4.9 Standard solutions

Note 1: The preparation of mixture solutions of melamine and cyanuric acid is not advisable.

4.9.1 Melamine stock solution 1, c = 1 mg ml^{-1}

Weigh 10 mg to the nearest 0.1 mg melamine reference standard (4.8.4.1) into a 10 ml volumetric flask (5.10). The true weight has to be recorded. Dissolve the standard in 2.5 ml DEA (4.1) and about 5 ml water (4.3), place in an ultrasonic bath (5.7) for 15 – 30 min and dilute with water (4.3) up to 10 ml. The stock solution is stored at -20 °C and has to be prepared fresh after six months.
Alternatively: Use commercially available standard solutions (4.8.4.2)

4.9.2 Melamine stock solution 2, c = 0.1 mg ml^{-1}

Alternatively to 4.9.1 weigh 10 mg to the nearest 0.1 mg melamine reference standard (4.8.4.1) into a 100 ml volumetric flask (5.10). The true weight has to be recorded. Dissolve the standard in water (4.3), place in an ultrasonic bath (5.7) for 15 – 30 min and dilute with water (4.3) up to 100 ml. The stock solution is stored at +4 °C and has to be prepared fresh after six months.
Alternatively: Use commercially available standard solutions (4.8.4.2)

4.9.3 Melamine working solution 1, c = 0.01 mg ml^{-1}

Pipette 1 ml of the melamine stock solution 2 (4.9.2) or alternatively 100 µl of the melamine stock solution 1 (4.9.1) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) to 10 ml. The solution is stored at +4 °C and has to be prepared fresh after three months.

4.9.4 Melamine working solution 2, c = 0.001 mg ml^{-1}

Pipette 1 ml of the melamine working solution 1 (4.9.3) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °C and has to be prepared fresh after three months.

4.9.5 Melamine working solution 3, c = 0.1 µg ml^{-1}

Pipette 1 ml of the melamine working solution 2 (4.9.4) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °C and has to be prepared fresh after three months.

4.9.6 Cyanuric acid stock solution 1, c = 1 mg ml^{-1}

Weigh 10 mg to the nearest 0.1 mg cyanuric acid reference standard (4.8.5.1) into a 10 ml volumetric flask (5.10). The true weight has to be recorded. Dissolve the standard in 1 ml DEA (4.1) and about 5 ml water (4.3), place in an ultrasonic bath (5.7) for 15 – 30 min and dilute with water (4.3) up to 10 ml. The solution is stored at -20 °C and has to be prepared fresh after six month.
Alternatively: Use commercially available standard solutions (4.8.5.2)

4.9.7 Cyanuric acid stock solution 2, c = 0.1 mg ml^{-1}

Alternatively to 4.9.6 weigh 10 mg to the nearest 0.1 mg cyanuric acid reference standard (4.8.5.1) into a 100 ml volumetric flask (5.10). The true weight has to be recorded. Dissolve the standard in water (4.3), place in an ultrasonic bath (5.7) for 15 – 30 min and dilute with water (4.3) up to 100 ml. The stock solution is stored at +4 °C and has to be prepared fresh after six months.
Alternatively: Use commercially available standard solutions (4.8.5.2)

4.9.8 Cyanuric acid working solution 1, c = 0.01 mg ml^{-1}

Pipette 1 ml of the cyanuric acid stock solution 2 (4.9.7) or alternatively 100 µl of the cyanuric acid stock solution 1 (4.9.6) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10)

and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °C and has to be prepared fresh after three months.

4.9.9 Cyanuric acid working solution 2, c = 0.001 mg ml^{-1}

Pipette 1 ml of the cyanuric acid working solution 1 (4.9.8) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °C and has to be prepared fresh after three months.

4.9.10 Cyanuric acid working solution 3, c = 0. 1 µg ml^{-1}

Pipette 1 ml of the cyanuric acid working solution 2 (4.9.9) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °C and has to be prepared fresh after three months.

4.9.11 $^{13}C_3$-Melamine stock solution c = 0.1 mg ml^{-1}

Weigh 10 mg to the nearest 0.1 mg $^{13}C_3$-melamine reference standard (4.8.6.1) into a 100 ml volumetric flask (5.10). The true weight has to be recorded. Dissolve the standard in water (4.3), place in an ultrasonic bath (5.7) for 15 – 30 min and dilute with water (4.3) up to 100 ml. The stock solution is stored at +4 °C.
Alternatively: Use commercially available standard solutions (4.8.6.2)

4.9.12 $^{13}C_3$-Melamine working solution c = 0.01 mg ml^{-1}

Pipette 1 ml of the $^{13}C_3$-melamine stock solution (4.9.11) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °.

4.9.13 $^{13}C_3$-Cyanuric acid stock solution c = 0.1 mg ml^{-1}

Weigh 10 mg to the nearest 0.1 mg $^{13}C_3$-cyanuric acid reference standard (4.8.7.1) into a 100 ml volumetric flask (5.10). The true weight has to be recorded. Dissolve the standard in water (4.3), place in an ultrasonic bath (5.7) for 15 – 30 min and dilute with water (4.3) up to 100 ml. The stock solution is stored at +4 °C.
Alternatively: Use commercially available standard solutions (4.8.7.2)

4.9.14 $^{13}C_3$-Cyanuric acid working solution c = 0.01 mg ml^{-1}

Pipette 1 ml of the $^{13}C_3$-cyanuric acid stock solution (4.9.13) with a suitable pipette (5.4) into a 10 ml volumetric flask (5.10) and dilute with water (4.3) up to 10 ml. The solution is stored at +4 °C.

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

5.1 HPLC-system capable of gradient elution consisting of

5.1.1 Pump

5.1.2 Vacuum degasser

5.1.3 LC- Injection system

5.1.4 Thermostatic column compartment

5.1.5 Triple-Quadrupole mass spectrometer with electrospray ionization and MRM mode

5.1.6 Computer system for data collection and evaluation

5.1.7 Column, ZIC®-HILIC 150 mm (length) x 2.1 mm (diameter), 5 µm particle size, 200 Å (other HILIC columns are possible but the selectivity may differ)

5.1.8 Pre-column, SeQuant® ZIC®-HILIC Guard PEEK 20 x 2.1 mm, metal-free guard column (recommended)

5.2 2 ml clear HPLC vials

5.3 11 mm Crimp cap with PTFE/silicone seals

5.4 Automatic pipettes or glass pipettes (class A)

5.5 Cooling Bench top centrifuge

5.6 Vortex mixer

5.7 Ultrasonic bath

5.8 Centrifuge conical polypropylene tubes with screw caps, capacity 50 ml and 15 ml

5.9 Shaker for sample homogenisation and extraction, speed continuously adjustable

5.10 Volumetric flask, 10 ml, 50 ml, 100 ml, 1000 ml

5.11 Funnel

5.12 Dispenser

5.13 Graduated cylinder, different volumes

5.14 Microcentrifuge for at least 20000 × g

5.15 20 ml Storage screw top glass vial with solid-top screw cap and septum

Note 2: To avoid migration, the material of the caps should contain no or only small amounts of melamine.

5.16 2 ml Polypropylene microcentrifuge tubes with lock

5.17 Analytical balance, accuracy to the nearest 0.1 mg

5.18 Mill with stainless-steel rotor for grinding to at least 1 mm

5.19 Chopper for feed with a high moisture content (e.g. pet food)

6 Preparation of sample

The size of the sample shall be sufficient and the packing, the transport and the storage shall maintain the integrity of the sample.
Sampling is not part of this document. A recommended sampling procedure is given in EN ISO 6497.

6.1 General

Prepare the sample according to EN ISO 6498.

6.2 Laboratory sample

Grind the laboratory sample (typically 50 g) to a particle size of at least 1 mm in the mill (5.18) in order to ensure representative data. Mix the sample thoroughly.

For samples with a moisture content > 75 % crush the sample in a chopper (5.19)

6.3 Test material

Homogenize the laboratory sample (6.2) for at least 10 minutes in a shaker (5.9) at slow speed before further processing. Laboratory samples with a moisture content > 75 % were mixed with a spatula.

6.4 Sample material

Sample material with a moisture content < 20 % weigh 2.0 g, to the nearest 0.1 g of the test material (6.3) into a 50 ml centrifuge tube (5.8). Record the weight of the sample in g.
Sample material with a moisture content > 75 % weigh 10.0 g, to the nearest 0.1 g of the test material (6.3) into a 50 ml centrifuge tube (5.8). Record the weight of the sample in g.
The sample is further purified by the described procedure.

7 Procedure

In a screening method the presence of melamine and cyanuric acid is examined. Testing positive for the analytes in the screening method the confirmatory method has to be performed. Due to the diversity of animal feed samples different matrix effects (enhancement / suppression) were observed. These effects are not always compensated by isotopically labelled standards. Therefore in samples tested positive, the quantification is realised by means of a three point standard addition.

Note 3: It is possible to identify ammeline and ammelide with this method. Quantification has to be considered problematic since standard substances of these analytes are often contaminated with melamine and / or cyanuric acid.

7.1 Screening method

For the screening method no internal standard is added.

7.1.1 Sample extraction

Add 15 ml of extraction solvent A (4.6) with a dispenser (5.12) to the sample (6.4) and mix for 15 s on a vortex mixer (5.6). The sample is placed in an ultrasonic bath (5.7) for 15 minutes. Subsequently centrifuge the mixture at approximately 4000 x g at 15 °C for 10 min in the centrifuge (5.5). Decant the supernatant into a 50 ml volumetric flask (5.10) and dilute to the mark with extraction solvent B (4.7).

7.1.2 Sample dilution

Pipette first 900 µl acetonitrile (4.4) into the 2 ml polypropylene microcentrifuge tubes (5.16), add 100 µl sample extract (7.1.1), agitate on a vortex mixer (5.6) for 15 s and centrifuge at 20000 x g for 10 min at + 4 °C in the microcentrifuge (5.14).

7.1.3 Injection solution for LC-MS/MS

Decant the supernatant carefully without possible occurring precipitate (7.1.2) into the HPLC vial (5.2) and inject this solution into the LC-MS/MS system (5.1).

7.2 Confirmatory method

7.2.1 Addition of internal standard

For the confirmatory method for samples with an expected concentration below 10 mg kg^{-1} add 0.15 ml melamine internal standard solution (4.9.11) and 0.15 ml cyanuric acid internal standard solution (4.9.13) to the samples (6.4).
For samples with an expected concentration between 10 mg kg^{-1} and 100 mg kg^{-1} add 1.5 ml melamine internal standard solution (4.9.11) and 1.5 ml cyanuric acid internal standard solution (4.9.13) to the samples (6.4).

Note 4: If one analyte is > 10 mg kg^{-1} and the other < 10 mg kg^{-1} the volume of the added internal standards differs from each other.

7.2.2 Sample extraction

Add 15 ml of extraction solvent A (4.6) with a dispenser (5.12) to the sample (7.2.1) and mix for 15 s on a vortex mixer (5.6). The sample is placed in an ultrasonic bath (5.7) for 15 minutes. Subsequently centrifuge the mixture at approximately 4000 x g at 15 °C for 10 min in the centrifuge (5.5). Decant the supernatant into a 50 ml volumetric flask (5.10).

Additionally for a second extraction add 15 ml extraction solvent B (4.7) with a dispenser (5.12) to the remaining residue, agitate strongly on a vortex mixer (5.6) until the residue is shaken up and place the sample in an overhead shaker (5.9) for 15 min. Subsequently centrifuge the mixture at approximately 4000 x g at 15 °C for 10 min in the centrifuge (5.5). Combine the second supernatant with the first extract in the 50 ml volumetric flask (5.10) and repeat the second extraction with 10 ml extraction solvent B (4.7).

Finally dilute the combined supernatants in the 50 ml volumetric flask (5.10) to the mark with extraction solvent B (4.7).

Note 5: The extraction of some animal feeding stuffs leads to small particles and fat on the surface of the liquid. These small particles shall be above the mark of the volumetric flask after fill up.

7.2.3 Sample dilution and standard addition

For the confirmatory method in this step the standard addition is performed. For samples with expected concentrations below 10 mg kg^{-1} pipette 1.0 ml of extract (7.2.2) into four 15 ml polypropylene tubes (5.8) each. Pipette to the extracts 9 ml acetonitrile (4.4).
For samples with expected concentrations between 10 mg kg^{-1} and 100 mg kg^{-1} pipette 0.1 ml of the extract (7.2.2) into four 15 ml polypropylene tubes (5.8) each. Pipette to the extracts 9.9 ml acetonitrile (4.4).

In three tubes add standard solutions in different concentrations. The recommended added concentration levels should be in the range of 20 %, 60 % and 100 % of the expected concentration in equidistant steps. In one tube no standard is added. An example is given in table 1. The added volume of the standard solution should not exceed 100 µl for each analyte (altogether 200 µl).

Note 6: Samples in the concentration range between 10 mg kg^{-1} and 100 mg kg^{-1} are tenfold more diluted (0.1 ml sample extract in 10 ml) in comparison to samples with concentrations < 10 mg kg^{-1} (1.0 ml sample extract in 10 ml) in this step. Therefore the added standard solutions are the same as between 1 mg kg^{-1} and 10 mg kg^{-1}.

Note 7: If one analyte is > 10 mg kg^{-1} and the other < 10 mg kg^{-1} perform the standard addition for each analyte separately in two separate sequences.

Note 8: A white precipitation can appear (possibly TCA). Before performing 7.2.4 wait until the precipitation has settled.

7.2.4 Injection solution for LC-MS/MS

Pipette 0.25 ml sample extract (7.2.3) into the 2 ml polypropylene microcentrifuge tubes (5.16), add 0.75 ml acetonitrile (4.4), agitate on a vortex mixer (5.6) for 15 s and centrifuge at 20000 x g for 10 min in the microcentrifuge (5.14).

Decant the supernatant into the HPLC vial (5.2) and inject this solution into the LC-MS/MS system (5.1).

Table 1a – Example of a standard addition for a sample with a moisture content < 20 % (sample weight 2 g) and with an estimated concentration of 1 mg kg^{-1} and 10 mg kg^{-1} . Pipetting scheme for melamine and cyanuric acid (add into a 10 ml volumetric flask)

standard curve	Expected concentration + added concentration mg kg^{-1}	Expected concentration + added concentration mg kg^{-1}	Added concentration in injection solution (7.2.4) pg $µl^{-1}$	volume analyte solution MEL CYA µl	working solution analyte
MC - 0	1 + 0.0	10 + 0	0.0	+ 0	
MC – 1	1 + 0.2	10 + 2	0.2	+ 80 + 80	3 (4.9.5) 3 (4.9.10)
MC – 2	1 + 0.6	10 + 6	0.6	+ 24 + 24	2 (4.9.4) 2 (4.9.9)
MC – 3	1 + 1.0	10 + 10	1.0	+ 40 + 40	2 (4.9.4) 2 (4.9.9)

Table 1b – Example of a standard addition for a sample with a moisture content > 75 % (sample weight 10 g) and with an estimated concentration of 1 mg kg^{-1} and 10 mg kg^{-1} . Pipetting scheme for melamine and cyanuric acid (add into a 10 ml volumetric flask)

standard curve	Expected concentration + added concentration mg kg^{-1}	Expected concentration + added concentration mg kg^{-1}	Added concentration in injection solution (7.2.4) pg μl^{-1}	volume analyte solution MEL CYA µl	working solution analyte
MC - 0	1 + 0.0	10 + 0	0.0	+ 0	
MC – 1	1 + 0.2	10 + 2	1.0	+ 40 + 40	2 (4.9.4) 2 (4.9.9)
MC – 2	1 + 0.6	10 + 6	3.0	+ 12 + 12	1 (4.9.3) 1 (4.9.8)
MC – 3	1 + 1.0	10 + 10	5.0	+ 20 + 20	1 (4.9.3) 1 (4.9.8)

7.3 Quality control measures for screening method

7.3.1 Blank extract

It has to be ensured that the material is free of melamine and cyanuric acid.
Therefore prepare the 50 ml blank material extract as described under 7.1.

7.3.2 Control standards

In the screening method the quality control measures are used to estimate the concentration level of potential positive findings. Therefore standards at a concentration level of 20 pg μl^{-1} and of 2.0 pg μl^{-1} are prepared. This corresponds to concentrations of 0.5 mg kg^{-1} and 5 mg kg^{-1} respectively in the samples. Due to matrix suppression this lower concentration of the standard was chosen to avoid an underestimation of the concentration in the sample.

Preparation of a control standard (c = 20 pg μl^{-1})
The concentration in the standard solution corresponds to a concentration of 5 mg kg^{-1} in the samples.
Pipette 20 µl melamine working solution 1 (4.9.3) and 20 µl cyanuric acid working solution 1 (4.9.8) into a 10 ml volumetric flask (5.10). Additionally add 10 µl internal standard solutions (4.9.12 and 4.9.14) and fill up to volume with acetonitrile (4.4).
If the peak area of the sample is higher than the peak area of the standard perform the method for samples above 10 mg kg^{-1}.

Preparation of a control standard (c = 2.0 pg μl^{-1})
The concentration in the standard solution corresponds to a concentration of 0.5 mg kg^{-1} in the samples.
Pipette 20 µl melamine working solution 2 (4.9.4) and 20 µl cyanuric working solution 2 (4.9.9) into a 10 ml volumetric flask. Additionally add 10 µl internal standard solutions (4.9.12 and 4.9.14) and fill up to volume with acetonitrile (4.4).
If the peak area of the sample is higher than the peak area of the standard perform the confirmatory method.

Note 9: For samples with a moisture content > 75 % the weight is fivefold higher. Therefore the peak area of the samples is approximately also fivefold higher.

7.4 Quality control measures for confirmatory method

7.4.1 Blank extract

Before it has to be ensured that the material is free of melamine and cyanuric acid.

Add 0.15 ml melamine internal standard solution (4.9.11) and 0.15 ml cyanuric acid internal standard solution (4.9.13) as described under 7.2.1 to the weighed blank material (6.4). Prepare the 50 ml blank material extract as described under 7.2.2.

7.4.2 Spiked matrix sample

To check the within-run performance, it has proven necessary to run at least one QM sample per sequence in the confirmatory method. This sample should contain a known concentration of melamine and cyanuric acid such as a sample with an assigned value used in an inter-laboratory trial, a laboratory reference material or a blank material spiked with standards. The concentration of the quality sample should be in the expected range of the samples. Also the injection of a standard solution without matrix is recommended. For all quality control measures the setting up and maintaining of control charts is recommended.

Example for the preparation of a matrix sample (1 mg kg^{-1}).
To the blank material add as described under 7.2.1 0.15 ml melamine internal standard solution (4.9.11), 0.15 ml cyanuric acid internal standard solution (4.9.13), additionally 200 µl melamine working solution 1 (4.9.3) and 200 µl cyanuric acid working solution 1 (4.9.8). Extraction and dilution of the sample is performed as described under 7.2.2 and 7.2.3.

7.4.3 Control standards

Preparation of a control standard (c = 2.0 pg $µl^{-1}$)
Use the standard solution prepared under 7.3.2 for the screening method, but the concentration in the confirmatory method corresponds to 2 mg kg^{-1} in the samples.

8 HPLC-MS/MS analysis

8.1 General

The measuring conditions stated are merely indicative; they may be adjusted to the respective local conditions. Triple Quadrupole Tandem Mass spectrometry should be used. Since cyanuric acid is measured in ESI (-) mode and melamine in ESI (+) mode the chromatographic separation shall be sufficient to allow switching of mode.
Using polarity switching a separation of the analytes is not necessary, but it has to be ensured that the sensitivity and the given parameters (e.g. LOQ and LOD) are fulfilled.
Before starting measurements, the HPLC system shall be thoroughly rinsed (using starting conditions). It is recommended to perform at least one standard injection before starting the sequence.

8.2 HPLC operating conditions

Chromatographic conditions may be chosen freely. The acceptable minimum retention time is twice the retention time of the void volume of the column.

When using the HILIC-column specified under (5.1.7) and the mobile phases A (4.8.2.1) and B (4.8.2.2) conditions in Table 2 were found to be appropriate.

Table 2 – HPLC operating conditions

Time min	**Mobile phase A** %	**Mobile phase B** %
0.0	100.0	0.0
5.0	100.0	0.0
7.0	80.0	20.0
8.0	80.0	20.0
9.0	50.0	50.0
12.0	50.0	50.0
12.5	100.0	0.0
20.0	100.0	0.0

Flow rate mobile phase: 0.3 ml/min
Injection volume: 5 µl
Column oven temperature: 30 °C
To avoid carry-over during the sequence it is recommended to use an automatic injector with internal and / or external needle wash.

8.3 Determination of melamine and cyanuric acid in sample test solutions

Inject aliquots of the sample test solutions and quality control measures into the HPLC system in an appropriate sequence.

8.3.1 Example of an analysis sequence for screening method

In the following an example of an analysis sequence is given:

- Control standard (c = 20 pg μl^{-1}) (7.3.2) inject twice
- Control standard (c = 2.0 pg μl^{-1}) (7.3.2)
- Blank Extract (7.3.1)
- Sample solutions (7.1.3)
- Blank Extract (7.3.1)
- Control standard (c = 20 pg μl^{-1}) (7.3.2)
- Control standard (c = 2.0 pg μl^{-1}) (7.3.2)

8.3.2 Samples tested positive in the screening method

Integrate the peak area of the control standards (c = 2.0 pg μl^{-1}) injected at the beginning and at the end of the sequence. If the peak area of the sample tested positive for melamine and / or cyanuric acid in the screening method is greater than the lowest peak area of the control standard then perform the confirmatory method.
If the peak area of the sample tested is greater than the peak area of the control standard (c = 20 pg μl^{-1}) than perform the procedure for samples > 10 mg kg^{-1}

8.3.3 Example of an analysis sequence for confirmatory method

In the following an example of an analysis sequence is given:

- Control standard (c = 2.0 pg μl^{-1}) (7.4.3) inject twice
- Blank Extract (7.4.1)
- Spiked matrix sample (e.g. 1 mg kg^{-1})(7.4.2)
- Sample solutions (7.2.4)

- Blank Extract (7.4.1)
- Spiked matrix sample (e.g. 1 mg kg^{-1}) (7.4.2)
- Control standard (c = 2.0 pg μl^{-1}) (7.4.3)

9 Calculation

9.1 Peak identification

For qualitative detection in the screening method the deviation of the retention time of the analytes should not exceed 5 % in comparison to the standards.

In the confirmatory method the detection of the analytes is carried out by comparison of the ratio of the retention time of the analytes to that of the internal standards in the sample with the ratio in the standard solutions. The deviation should be less than 2.5 %.

At least one confirmatory transition is acquired for definitive identification of the analyte. The relative ion intensities of the quantification transition to the confirmatory transition shall correspond to those of the calibration standard and should not exceed 25 %.

9.2 Quantification (Confirmatory method)

The quantitative determination is performed according to the method of standard addition. Integrate the peak areas of the most intense ions of the analytes and of the corresponding internal standard and calculate the ratio.

$$Ratio\,(R) = \frac{A_{MEL}}{A_{IS-MEL}}$$

A_{MEL} Peak area of melamine (cyanuric acid)
$A_{IS\text{-}MEL}$ Peak area of the internal standard $^{13}C_3$-melamine ($^{13}C_3$-cyanuric acid)
R is ratio of the peak area of the analyte to the peak area of the internal standard

Evaluation is based on the linear equation of the regression line of the analytes.

For the standard addition line use y = R and x = the added concentrations of the standard addition in pg per µl injected sample solution. The concentration of the analyte in pg per µl injected sample solution resulted from the extrapolation of the standard addition line up to the intercept with the x-axis (y = 0) from the y-intercept and the slope a, the mass concentration of the analyte in the sample in mg kg^{-1} sample is calculated by the following formula.

$$c = \frac{b \times E}{a \times w \times F \times 1000}$$

c is the mass concentration of the analyte in mg analyte per kilogram of sample
b is the Y-intercept of the regression line
a is the slope of the regression line
E is the volume of sample extract (ml) (7.2.2)
F is the dilution factor

$$F = \frac{Aliquot\ (7.2.3)\ from\ extract\ \ (ml)(7.2.2)}{Volume\ of\ sample\ dilution\ (ml)(7.2.3)} \times \frac{Aliquot\ (7.2.4) from\ extract\ (ml)(7.2.3)}{Volume\ of\ injection\ solution\ (ml)(7.2.4)}$$

w is the weight of the sample (g)
1000 is the conversion factor from µg kg^{-1} in mg kg^{-1} sample

Annex A

Examples for suitable MS detection conditions

A1 Example suitable for SCIEX API 4000 Q-Trap or API 4000

Source parameters and general mass spectrometric specifications (see table A.1)

- Ionisation		ESI
- Temperature		600 °C
- Curtain gas	positive mode	20 psi
- Curtain gas	negative mode	30 psi
- Collision gas		Medium
- Entrance potential (EP)	positive mode	10 V
- Entrance potential (EP)	negative mode	- 10 V
- Ion spray voltage:	positive mode	5500 V
- Ion spray voltage:	negative mode	- 4500 V
- Ion source gas 1 and 2	positive mode	60 psi
- Ion source gas 1 and 2	negative mode	60 psi
- Q1 Resolution		Unit
- Q3 Resolution		Unit

Table A.1 - Substance-specific mass spectrometric conditions

Substance	Polarity	Q1 Mass	Q3 Mass	Dwell [ms]	DP [a] [V]	CE [b] [eV]	CXP [c] [V]
Melamine	positive	127	85	150	71	27	14
		127	68	150	71	43	10
		127	60	150	71	27	10
$^{13}C_3$-Melamine	positive	130	87	150	51	27	14
		130	70	150	51	41	12
Cyanuric acid	negative	128	42	150	-40	-32	-5
		128	85	150	-40	-14	-5
$^{13}C_3$-Cyanuric acid	negative	131	43	150	-30	-30	-5
		131	87	150	-30	-14	-5
Ammelide	negative	127	84	150	-80	-16	-5
		127	42	150	-80	-37	-5
	positive	129	87	100	120	53	10
Ammeline	positive	128	86	100	61	25	14
		128	69	100	61	43	12
		128	43	100	61	51	6
	negative	126	83	150	-50	-16	-5

[a] Declustering potential
[b] Collision energy
[c] Cell exit potential

Annex B

Results of the in-house validation of the method

Table B.1 - Limit of detection and limit of quantification of the method

	Melamine mg kg^{-1}		Cyanuric acid mg kg^{-1}		Determined by
Feed	LOD	LOQ	LOD	LOQ	
Complete feed for cats (wet)	0.125	0.385	0.358	0.912	ISO 11843-2
Complete feed for laying hens	0.063	0.208	0.247	0.694	ISO 11843-2
Milk replacer	0.089	0.284	0.125	0.387	ISO 11843-2
Wheat bran	0.138	0.422	0.279	0.768	ISO 11843-2
Complete feed for dogs (dry)	0.073	0.238	0.168	0.499	ISO 11843-2
Soya meal	0.032	0.111	0.114	0.355	ISO 11843-2
Complete feed for pigs	0.085	0.272	0.086	0.277	ISO 11843-2
Complete feed for cats (dry)	0.105	0.33	0.137	0.419	ISO 11843-2

Table B.2 – Recovery and precision of the method

Feed	Melamine		Cyanuric acid		Single blank samples spiked with
	Recovery %	Coefficient of variation %	Recovery %	Coefficient of variation %	
Complete feed for cats (wet) (n=6)	100	1.1	94	2.2	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for cats (wet) (n=6)	93	4.0	94	2.2	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1} (second operator)
Milk replacer (n=6)	91	3.1	102	3.7	MEL 1.0 mg kg^{-1} CYA 10 mg kg^{-1}
Milk replacer (n=6)	93	2.3	100	1.5	MEL 1.0 mg kg^{-1} CYA 10 mg kg^{-1} (second operator)
Complete feed for laying hens (n=6)	89	3.7	91	3.0	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}
Soya meal (n=6)	100	2.0	100	2.0	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for pigs (n=6)	105	2.1	99	2.8	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}

Table B.3 – Recovery and precision of the method by analysing spiked bulk material

Feed	Melamine		Cyanuric acid		1 kg blank material spiked with
	Recovery [%]	Coefficient of variation [%]	Recovery [%]	Coefficient of variation [%]	
Milk replacer (n=6)	94	1.7	92	2.5	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for laying hens (n=10)	98	1.5	101	3.3	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for laying hens (n=6)	105	1.3	106	3.0	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1} (second operator)
Complete feed for laying hens (n=10)	98	2.9	105	4.4	MEL 2.5 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for laying hens (n=10)	110	2.9	97	5.6	MEL 1.0 mg kg^{-1} CYA 1.0 mg kg^{-1}
Complete feed for laying hens (n=6)	107	2.8	107	3.1	MEL 1.0 mg kg^{-1} CYA 1.0 mg kg^{-1} (second operator)
Complete feed for laying hens (n=10)	100	4.7	100	5.2	MEL 100 mg kg^{-1} CYA 100 mg kg^{-1}
Complete feed for laying hens (n=6)	110	2.3	110	1.1	MEL 100 mg kg^{-1} CYA 100 mg kg^{-1} (second operator)
Complete feed for laying hens (n=10)	103	3.4	96	2.6	MEL 10 mg kg^{-1} CYA 100 mg kg^{-1}
Complete feed for laying hens (n=10)	104	2.9	107	1.7	MEL 10 mg kg^{-1} CYA 10 mg kg^{-1}
Complete feed for laying hens (n=10)	97	4.8			MEL 2.5 mg kg^{-1}

Feed	Melamine		Cyanuric acid		1 kg blank material spiked with
	Recovery [%]	Coefficient of variation [%]	Recovery [%]	Coefficient of variation [%]	
Complete feed for laying hens (n=10)			93	8.3	CYA 2.5 mg kg^{-1}
Complete feed for cats (wet) (n=3)	101	1.7			MEL 2.5 mg kg^{-1}
Complete feed for cats (wet) (n=3)	95	0.6	106	3.1	MEL 1.0 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for cats (wet) (n=3)	100	0.8	110	0.9	MEL 2.5 mg kg^{-1} CYA 100 mg kg^{-1}
Complete feed for cats (wet) (n=6)	101	1.0	103	0.8	MEL 1.0 mg kg^{-1} CYA 2.5 mg kg^{-1}
Complete feed for cats (wet) (n=6)	101	0.9			MEL 2.5 mg kg^{-1}

Annex C
(informative)

Typical chromatogram

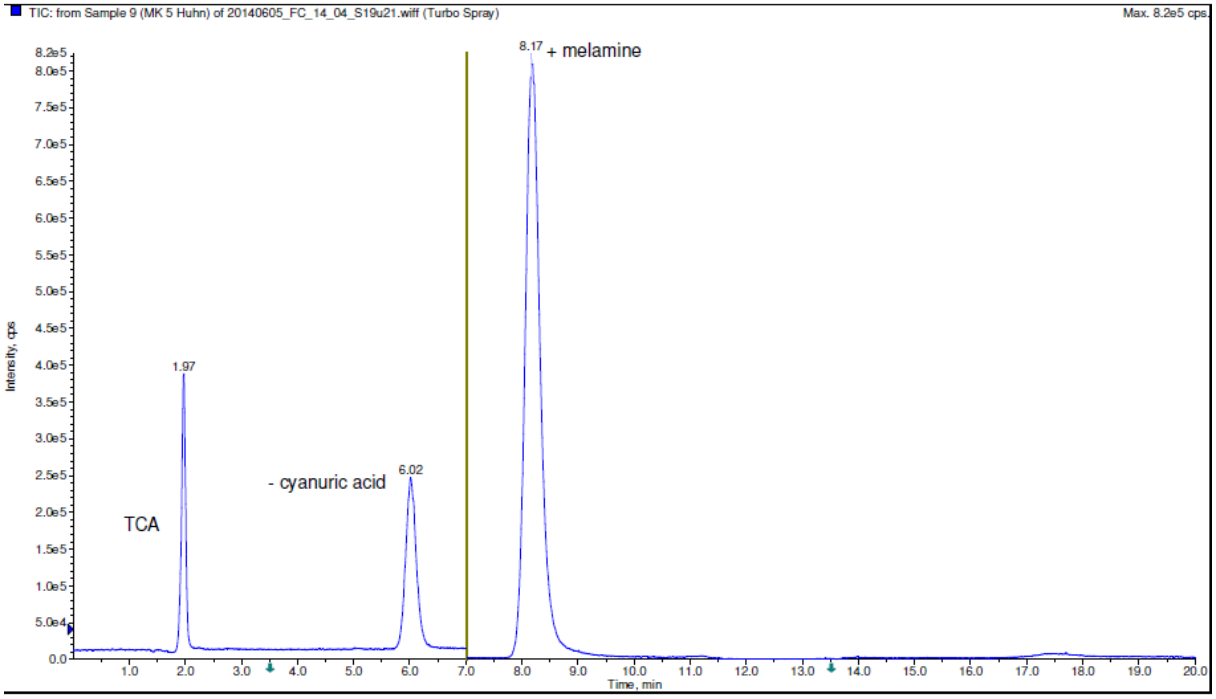

Figure C.1: - Typical chromatogram of a spiked blank feed material (melamine concentration 2.5 mg kg^{-1} and cyanuric acid concentration 2.5 mg kg^{-1})

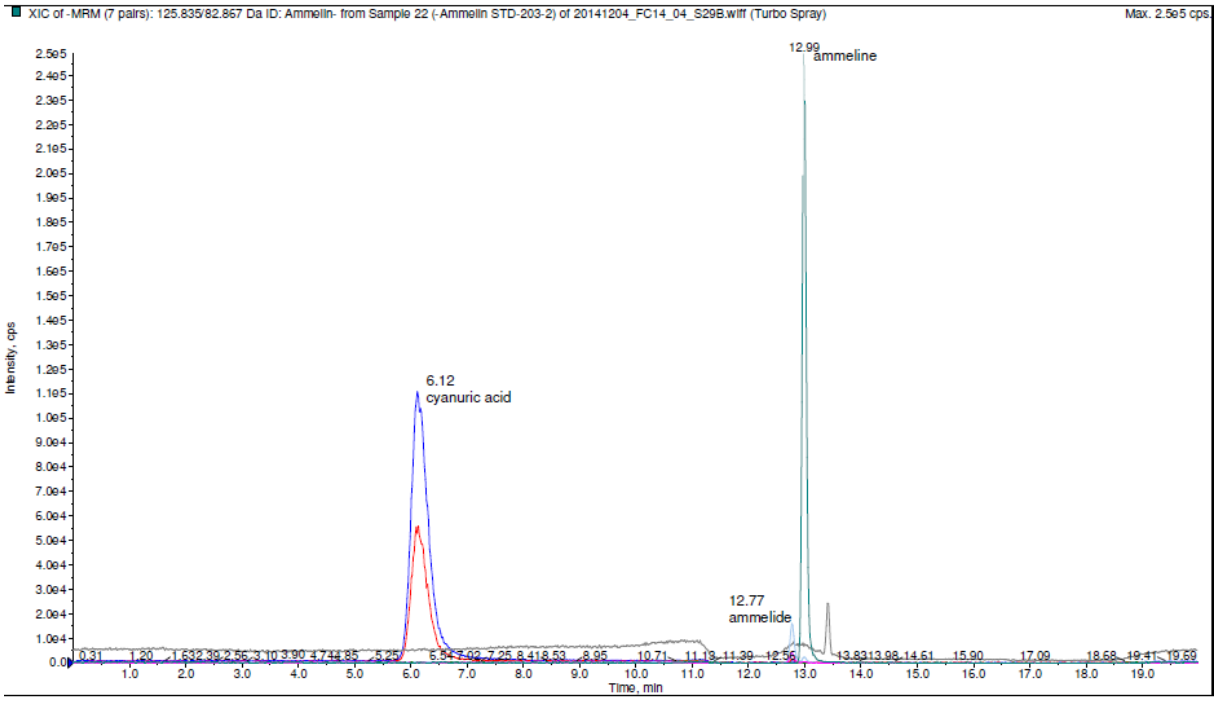

Figure C.2: - Typical chromatogram of an ammeline standard: Impurities of cyanuric acid and ammelide in the standard substance

Bibliography

[1] EN ISO 6497, Animal feeding stuffs – Sampling (ISO 6497)

[2] EN ISO 6498 Animal feeding stuffs – Guidelines for sample preparation (ISO 6498)

[3] ISO 11843-2, Capability of detection – Part 2: Methodology in the linear calibration case

14.7.1 Instructions on installation of the template files for electronic recording of the results

User Guide for Data Reporting – Electronic Recording

You will receive the following two template files. Please use these templates to report your results:

LCXXX.LAB (Results table, **LCXXX** is your lab code)
LCXXX.LA2 (configuration file, includes sample information and legend)

To enter your data you need the configurable data input module called “RingDat”.
Please download the file “RingDat.exe” from the following URL address:
http://quodata.de/fileadmin/RingDat/ringdat4_en.zip. The download is free of charge.

To report your data, please proceed as follows:

1. Copy the provided data files on a selected directory on your hard disk.

2. If desired, you may voluntarily report results obtained from more than one analytical method. If so, please rename the file from**LCXXX.LAB** to **LCXXX_1.LAB, LCXXX_2.LAB** and so on. Skip this point, if you want to submit a single data set.

3. Open the file RINGDAT4.exe with a double-click: The window “Entry of the test result“ will open.

4. Click on “Open“ in the menu bar. A menu window will appear where you can find the file **LCXXX.LAB** in the corresponding directory. Open that file now, the table “Method validation 2016“ will appear.

5. The following identifiers are used:
   BfR_XXX: Results of the samples

   Please enter the respective sample mass (net weight stated in g) by filling in the column “net weight [g]“ with an accuracy of three significant digits.

6. Enter your analytical results (stated in mg/kg) into column “MWx“ (x = 1;2;3) with an accuracy of two digits after the decimal point (e.g. 45.3). Please enter “ <(LOD) “ (**LOD** = limit of detection, e.g. **“<< 0,10”**) into column “MWx” if an analyte is not detectable. Enter your limit of detection (LOD) into column “LOD“ (e.g. “0,10”)
   If you get levels below the limit of quantification (LOQ), please enter “< (LOQ)“ (**LOQ** = value of LOQ, e.g. **“< 100”**) into column “MWx” and in column “LOQ” the limit of quantification (e.g. “100”)

7. If you have not analysed selected sample or analyte do not fill in anything in the row.

8. Save your file by using the command “Save data”.
   If you have **finished** data entry, finalize the file using “Finish input”.

9. Reduce every column to a minimum size, still allowing for legibility though, by positioning the cursor on top of the column and drawing the width to a smaller scale. It is necessary to change the display format to make a print-out of the result table (protocol) by clicking on “Protocol”. Otherwise an error message may be displayed.

Please return the templates after inserting your data

LCXXX.LAB,
LCXXX.LA2,

by email to

hildburg.fry@bfr.bund.de

Please send the result table (protocol) with your signature also to the mail-address given above.

Please submit your results before **04.July 2016**.

Please send the sheet with your signature to the address given in the documents.

Federal Institute for Risk Assessment (BfR)
FG 82 - Contaminants

Method validation study 2016: melamine and cyanuric acid in animal feed

Analyseergebnisse

Laborcode: LC013

Probencode	analyt	name	unit	net weight [g]	MW1 LOQ	LOD
BfR_0001	MEL	melamine	mg/kg			
BfR_0001	CYS	cyanuric acid	mg/kg			
BfR_0013	MEL	melamine	mg/kg			
BfR_0013	CYS	cyanuric acid	mg/kg			
BfR_0015	MEL	melamine	mg/kg			
BfR_0015	CYS	cyanuric acid	mg/kg			
BfR_0048	MEL	melamine	mg/kg			
BfR_0048	CYS	cyanuric acid	mg/kg			
BfR_0097	MEL	melamine	mg/kg			
BfR_0097	CYS	cyanuric acid	mg/kg			
BfR_0111	MEL	melamine	mg/kg			
BfR_0111	CYS	cyanuric acid	mg/kg			
BfR_0137	MEL	melamine	mg/kg			
BfR_0137	CYS	cyanuric acid	mg/kg			
BfR_0151	MEL	melamine	mg/kg			
BfR_0151	CYS	cyanuric acid	mg/kg			
BfR_0157	MEL	melamine	mg/kg			
BfR_0157	CYS	cyanuric acid	mg/kg			
BfR_0166	MEL	melamine	mg/kg			
BfR_0166	CYS	cyanuric acid	mg/kg			
BfR_0200	MEL	melamine	mg/kg			
BfR_0200	CYS	cyanuric acid	mg/kg			
BfR_0210	MEL	melamine	mg/kg			
BfR_0210	CYS	cyanuric acid	mg/kg			
BfR_0222	MEL	melamine	mg/kg			
BfR_0222	CYS	cyanuric acid	mg/kg			
BfR_0231	MEL	melamine	mg/kg			
BfR_0231	CYS	cyanuric acid	mg/kg			
BfR_0234	MEL	melamine	mg/kg			
BfR_0234	CYS	cyanuric acid	mg/kg			
BfR_0283	MEL	melamine	mg/kg			
BfR_0283	CYS	cyanuric acid	mg/kg			

Die Eingabe der Werte in RingDat wurde noch nicht abgeschlossen.
Bitte betätigen Sie die Taste "Datei abschließen", bevor das Protokoll gedruckt wird.

Please save the Excel sheet under Method-Sheet-LC-LAB-CODE

Participant

Study	Determination of melamine and cyanuric acid in animal feed by LC-MS/MS - CEN/TC 327
LAB-CODE	
Institute/Organisation	
Street / Post Box	
Postal Code	
City	
Country	
Contact	
Telephone	
e-mail	

Sample Extraction and Clean-up

LAB-CODE 0

Sample extraktion and Clean-up according to method description

☐ yes ☐ no

If no, please describe the deviations

Comments

Quantification

LAB-CODE 0

Quantification according to method description

☐ yes ☐ no

If no, please describe the deviations:

Limit of detection and Limit of Quantification

Determination of LOQ/LOD by

☐ Signal to Noise

☐ other

Please specify

LOD melamine

LOD cyanuric acid

LOQ melamine

LOQ cyanuric acid

HPLC Method

LAB-CODE 0

☐ HPLC ☐ UHPLC

Instrument

Supplier

Column

☐ Normal Phase ☐ Reversed Phase

☐ HILIC

☐ Other Please specify

Type
Particle size (µm)
Diameter (mm)
Length (mm)
Supplier

Mobile Phase

☐ isocratic ☐ gradient

Solvent

Solvent A

Solvent B

Solvent C

Gradient Elution

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)

MS/MS Parameters

LAB-CODE		0	
Type of MS		If other please specify	
Supplier Type			
Ionisation		If other please specify	
Scan		If other please specify	
Polarity	melamine		cyanuric acid

MS or MS/MS Parameter (general)

(please specify)	Parameter	Value	Unit

Measured transitions and substance specific MS conditions

			Parameter	Parameter	Parameter
			(unit)	(unit)	(unit)
		precursor-product			
Melamine	Transition 1 Transition 2 Transition 3				
Cyanuric acid	Transition 1 Transition 2 Transition 3				
$^{13}C_3$-melamine	Transition 1				
$^{13}C_3$-cyanuric acid	Transition 1				

Results of screening method / Standard addition

LAB-CODE 0

		Screening method		Range of added standard	
	Sample No	Melamine mg kg^{-1}	Cyanuric acid mg kg^{-1}	Melamine mg kg^{-1}	Cyanuric acid mg kg^{-1}
Sample 1					
Sample 2					
Sample 3					
Sample 4					
Sample 5					
Sample 6					
Sample 7					
Sample 8					
Sample 9					
Sample 10					
Sample 11					
Sample 12					
Sample 13					
Sample 14					
Sample 15					
Sample 16					
Sample 17					

Comments

14.8 Annex 8: Participants (in alphabetical order)

Institute	City	Country
Covance Food Solutions	Harrogate	United Kingdom
CVUA Rhein Ruhr Wupper	Krefeld	Germany
Federal Institute for Risk Assessment	Berlin	Germany
INVIVO LABS site de Saint Nolff	Saint Nolff	France
Istituto Zooprofilattico Sperimentale della Sardegna	Sassari	Italy
JRC/ IRMM/ Standards for Food Bioscience	Geel	Belgium
LAVES Futtermittelinstitut Stade	Stade	Germany
MARS Petcare Central Laboratory	Aimargues	France
Merieux NutriSciences Italia	Resana	Italy
Nutreco Nederland BV - MasterLab	Boxmeer	Netherlands
RIKILT	Wageningen	Netherlands
The State Laboratory	Celbridge, Co Kildare	Ireland
ÚKZUZ	Brno	Czech Republic

14.9 Annex 9: LC-MS/MS conditions used by the participants

	LC0004	LC0005	LC0006	LC0007	LC0009	LC0013	LC0014	LC0015	LC0016	LC0017	LC0018	LC0019
HPLC	HPLC	HPLC	HPLC	HPLC	HPLC	HPLC	UPLC	UPLC	HPLC	HPLC	UPLC	HPLC
	Thermo	Agilent	Agilent 1260 infinity	Shimadzu 2x LC-20ADXR, SIL-30AC, CTO-30, HP gradi-ent	Shimadzu	Agilent1260	Agilent 1290 Infinity	Waters UPLC I-class	Agilent 1200 series model	Shimadzu Pro-minence	Agilent 1290	Thermo Ultramode
MS	Triple Quad	Triple Quad	Triple Quad	Triple Quad	Triple Quad	Triple Quad	Triple Quad	Triple Quad	Triple Quad	Ion-Trap	Triple Quad	Triple Quad
	Thermo	Agilent Technolo-gies	AB Sciex	Thermo Scientific	Waters	AB Sciex	Agilent Technolo-gies	Waters	AB Sciex	AB Sciex	Agilent Technolo-gies	Thermo Scientific
		6420	Q Trap 5500	TSQ Quantum	Ultima	Q Trap 4000	6490 LC-MS	Quattro Premier XE	API 4000	Q Trap 5500	6490	Quantiva
Column	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC	HILIC
Type	alltima hp hilic	ZIC®-HILIC	ZIC-HILIC	ZIC-HILIC	TKSgel Amide-80	ZIC-Hilic	Acquity BEH HI-LIC	BEH	Nucleodur	Kinetex HILIC	Column, ZIC®-HILIC	ZIC-Hilic
Particle size [mm}	5	5	5	3,5	3	5	1,8	1,7	3	2,6	5	5
Diameter [mm]	2,1	2,1	2,1	2,1	3	2,1	2,1	2,1	4,6	2,1	2,1	2,1
Length[mm]	150	150	150	100	100	150	150	100	15	50	150	150
Supplier	grace	SeQuant	SeQuant	Merck	TOSOH	SeQuant	Waters	Waters	Macherey-Nagel	Pheno-menex	200 Å	SeQuant
Mobile Phase	isocratic	gradient	gradient	gradient	gradient	gradient	gradient	gradient	isocratic	gradient	gradient	gradient
Composition Mobile phase A	10 mmol acatat	10mM NH4OAc	10mM NH4OAc-buffer pH 7,2	ACN:H2O (95:5), 10mM NH4OAc	4.8.2.1 from me-thod	ACN / NH4OAc (95+5)	10 mM NH4OAc-buffer at pH 4.0	5mM NH4OAc in ACN:H20 (99:1)	10mM NH4OAc	95:5 ACN:10m M NH4OAc	10 mM NH4OAc: ACN 5:95	ACN / NH4OAc (95+5)
Composition Mobile phase B	ACN	ACN	ACN	ACN/H2O (5/95),	4.8.2.2 from me-	ACN: NH4OAc	10mM NH4OAc	10mM NH4OAc	ACN	10mM NH4OAc:	10mM NH4OAc:	ACN: NH4OAc

	LC0004	LC0005	LC0006	LC0007	LC0009	LC0013	LC0014	LC0015	LC0016	LC0017	LC0018	LC0019
				10mM NH4OAc	thod	(5:95)	in 97:3 (v/v) ACN - H20	in H2O		ACN 95:5	ACN 95:5	(5:95)
Gradient elution	0 // 2 / 98	0 // 5 / 95	0 // 5 / 95	0 // 92 / 8	0 // 94 / 6	0 // 100 / 0	0 // 0 / 100	0 // 100 / 0	0 // 10 / 90	0,01 // 90 / 10	0 // 100 / 0	0 // 100 / 0
(time [min] //	17 // 2 / 98	3 // 5 / 95	10 // 20 / 80	11 // 92 / 8	8 // 94 / 6	5 // 100 / 0	1 // 0 / 100	0,8 // 100 / 0	14,5 // 10 / 90	2 // 90 / 10	5 // 100 / 0	5 // 100 / 0
Mobile phase A [%] /		3.5 // 9.27 / 90.73	15 // 40 / 60	11,01 // 50 /50	13 // 33 / 67	7 // 80 / 20	3,2 // 6 / 94	2,3 // 78 / 22		2,5 // 50 / 50	7 // 80 / 20	7 // 80 / 20
Mobile phase B [%]		4.5 // 90 / 10	25 // 40 / 60	14 // 50 / 50	14 // 6 / 94	8 // 80 / 20	3,5 // 50 / 50	4 // 78 / 22		3 //50 / 50	8 // 80 / 20	8 // 80 / 20
		5.5 // 90 / 10	30 // 5 / 95	14,01 // 92 / 8	15 // 6 / 94	9 // 50 / 50	4 // 50 / 50	5 // 100 / 0		3,5 // 90 / 10	9 // 50 / 50	9 // 50 / 50
		5.6 // 5 / 95	40 // 5 / 95	20 // 92 / 8	15,5 // 94 / 6	12 // 50 / 50	4,1 // 0 / 100	6 // 100 / 0		5 // 90 / 10	12 // 50 / 50	12 // 50 / 50
		8 // 5 / 95				12,5 // 100 / 0	8 // 0 / 100				12,5 // 100 / 0	12,5 // 100 / 0
						20 // 100 / 0					20 // 100 / 0	20 // 100 / 0
Flow rate [ml/min]	0,4	0,7	0,3	0,1	0,25	0,3	0,7	0,3	0,8	0,3	0,3	0,3
Injection volume [µl]	3	5	5	3	10	5	10	5	5	5	5	5
Injector temperature [°C]	7	ambient	8	30	10	ambient	15		20	12	room tempera-ture	ambient
Column temperature [°C]		30	40	30	40	30	50	30	35	30	30	30
needle wash external		yes	yes	yes	yes	yes	yes	yes	no	yes	yes	
internal	yes	yes	yes	yes	yes		yes	yes	yes	yes	yes	yes
Retention time [min]												
Melamin standard	7	2,9		8,29	11,08	6,1	3,05	3,45	7,1	0,89	7,44	
Sample	7	2,9	6	8,26	11,08	6,1	3,05	3,46	8,1	0,88	7,43	

	LC0004	LC0005	LC0006	LC0007	LC0009	LC0013	LC0014	LC0015	LC0016	LC0017	LC0018	LC0019
Cyanuric acid standard	2	2,2		7	6,34	4,3	1,25	1,21	3,2	0,58	5,55	6,1
sample	2	2,1	5,5	7,14	6,34	4,4	1,25		3,8	0,58	5,54	6,1

ACN: acetonitrile
NH4OAc: ammonium acetate
H2O: water

LC01 didn’t provide a method sheet

14.10 Annex 10: Comments from the Participants

14.10.1 Problems with equipment or observations

	Comments from the participants	Comments from project leader	Lab No
1.	for cyanuric acid our triple quad Quattro Premier XE has not sufficient sensitivity(> 20 ng/ml) to perform analysis according to sample extraction and sample dilution described in the method		015

14.10.2 Modification of the method by the participants

	Comments from the participants	Comments from project leader	Lab No
	Extraction with acid and water dilution in acetonitril.		004
2.	For the conformation method the additional dilution before LC injection is left out (7.2.4 in the method). Instead 1 ml extract is centrifuged in a micro centrifuge. This due to sensitivity of the LCMSMS system used.		009
3.	We centrifuged with 16000 rpm, some samples were cloudy, we filtered this samples with PTFE 0,2 µm	During method development we tested also the PTFE filters. They are suitable, so may be a remark in the method description should be included	006

14.10.3 Proposals for the improvement of the method

	Comments from the participants	Comments from project leader	Lab No
4.	The TCA in the injection solution has a detrimental effect on the ESI probe and spray stability across long sequences. A note in the method protocol to divert the elution volume containing the TCA would be helpful. Reequilibration of the column to starting conditions was done at 0.3 mL/min from 11 to 20 min run time.	That could be dependent on the type of ESI probe we didn't observe unusual abrasion.	007

14.10.4 Comments to description of the method

	Comments from the participants	Comments from project leader	Lab No
5.	The description of the sample preparation is rather confusing and should be revised. Is it really necessary to have two different concentration ranges of just one order of magnitude each? Triple quadrupole MS have a sufficiently large dynamic range to deal with concentrations from 1 to 100 mg/kg without having to additionally dilute the upper range.		007

14.10.5 Comments to description of the method

	Comments from the participants	Comments from project leader	Lab No
6.	Micro centrifugation was done at 12,000rpm in parts 7.1.2 and 7.2.4. On arrival the standards we stored at -20C as per instruction sheet. When removed from the freezer the internal standard glass containers cracked. The internal standards were allowed to thaw in beakers and were then transferred to new containers.	Other participants also observed problems especially during thawing with the glass vials.	017
7.	In the screening run the following samples had peak areas greater than the 2pg/ul Melamine control standard; 3, 5, 6, 10, 15, 16. Samples 7 and 13 had peak areas greater than the 20pg/ul Melamine control standard. Samples 4 and 8 had peak areas greater than the 2pg/ul Cyanuric acid standard. We analysed pre-trial QCs as our QC for the confirmation run,sample 62 for Melamine and got a result of 2.58mg/kg and sample 67 for Cyanuric acid which gave a result of 5.43mg/kg. We are concerned that the solvent standard is not representative of the concentration in matrix as the 2pg/ul control solvent standard ran with the confirmation run had a greater peak area than both of the QCs and a 1mg/kg spike had an area approximately 20 times less than the control standard.	That is a little bit strange, since the overall recovery of the analytes, including the matrix effects is around 50 %. Therefore the signal of a 2 g μl^{-1} standard should be lower than the decision limit to perform the confirmation method.	017
8.	For the screening method no internal standard is added and no correction for internal standard or recovery was made. Standard addition: indicated standard additions were used for the confirmatory method.		018
9.	recovery: melamine 95% / cyanuric acid 105% added 13C standard is 0.1mg/l in final solution before injection.		004
10.	cyanuric acid is unsure		006

Some general remarks in regard to the analysis of cyanuric acid:

For the LC-MS/MS instruments we tested the sensitivity for cyanuric acid is lower in comparison to melamine. The limit of quantification in the range of 1 mg/kg especially in some feed materials is therefore more challenging.

The sensitivity of the method depends also on the used HILIC column. For some columns we tested the signal was only half of the signal of the HILIC which is given in the method.

May be additional problems occur if the retention time is very short, as with UHPLC.
Last but not least, the analysis of cyanuric acid requires also an instrument which is in a really good condition. In our experience cyanuric acid reacts earlier than melamine to deviations from the optimum conditions.