Method validation study on determination of melamine and cyanuric acid in food

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1. Introduction

At the request of the European Committee for Standardization (CEN) by RIKILT a standard for the determination of melamine (MEL) and cyanuric acid (CYA) in food was described. In order to validate this new CEN method a ring trial was organized between February 2014 and July 2014. The objective is to assess the comparability of results of melamine and cyanuric acid obtained by the new draft CEN standard in seven samples of food.

2. Participants

Eighteen laboratories registered of which nine reported results. Eight of them are situated in Europe and one in North America.

3. Design of the study

3.1 Sample preparation

Fourteen samples (table 1) have been prepared from regular food products with fortification of MEL and/or CYA. The samples were homogenized by adding methanolic solutions of MEL and CYA to the powdered materials. They were homogenized by adding liquid nitrogen and continuous stirring. Potassium dichromate was added to the whole milk and soy milk samples in order to prevent bacterial growth.

Also, two glass ampoules (materials A and B) containing melamine or cyanuric aid were prepared (table 1).

Table 1: Aimed levels in the materials

		Aimed levels (mg/kg)	
Material	Code	Melamine	Cyanuric acid
Infant formula	C and D	1.2	0.8
Infant formula soy based	E and F	0.7	0.9
Milk powder	G and H	0.9	1.3
Whole milk	I and J	1.4	0.6
Soy milk	K and L	1.0	1.0
Chocolate powder	M and N	0.8	0.9
Milk chocolate	O and P	0.7	1.2

3.2 Sample identification

After homogenization, the materials were divided into sub-portions of 10 g and stored in plastic bottles. The samples for the participants were randomly coded by a web application (Annex 1). Per material ten randomly taken samples were used for homogeneity testing.

3.3 Homogeneity

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [7] and ISO 13528 [8], taking into account the insights discussed by Thompson [9] regarding the Horwitz equation. Ten containers of each material were analysed in duplicate for MEL and CYA. The results are presented in Annex 2. All materials demonstrated to be sufficiently homogenous for use in this study, except MEL in chocolate., but the results for CYA already proved the homogeneity of the material. Chocolate powder was excluded from this study, due to inhomogeneity.

3.4 Sample distribution

Each of the participating laboratories received a randomly assigned laboratory code, generated by the webapplication. The sample sets with the corresponding number, consisting of twelve coded samples and two ampoules (Annex 1) were sent to the participating laboratories on April 22-2014. The sample sets were packed in an polypropylene box and were dispatched to the participants immediately by courier. The samples were accompanied by a letter (Annex 3)

describing the requested analyses and an acknowledgement of receipt form. By e-mail the laboratories received instructions on how to use the web application for reporting results.

The laboratories were asked to store the samples at +4°C until analysis. A single analysis of each sample was requested using the prescribed method "*Food analysis - Determination of melamine and cyanuric acid in foodstuffs LC-MS/MS method*" (Annex 4,). The deadline for submitting the results was May 30 2014, allowing 5 weeks for the analysis.

4. Statistical evaluation of the results

The main objective of this collaborative study is the validation of the new CEN method for the determination of MEL and CYA in foodstuffs (§4.1). In addition, the results of all participating laboratories, using the new CEN method, were evaluated as a proficiency test by assigning z-scores (§4.2).

4.1 Collaborative study

Statistical evaluation of the results was carried out according to the Collaborative Study Guidelines of AOAC International [10] for blind (unpaired) replicates. In these guidelines the HorRat (Horwitz ratio) is described for evaluation of collaborative studies. The HorRat is the ratio of the reproducibility relative standard deviation, expressed as a percent (RSD_R %) to the predicted reproducibility relative standard deviation, expressed as a percent ($PRSD_R$ %,):

$$\text{HorRat} = \frac{RSD_R(\%)}{PRSD_R(\%)}$$

Where: $PRSD\ (\%) = 2C^{-0.1505}$

C = estimated mean concentration expressed as a decimal fraction (10^{-6} for this test)

The acceptable range for the HorRat value is <2.0.

In tables 2 and 3 the results of the collaborative study are presented by means of the grand mean and the HorRat values.

Table 2. Results for melamine

Material	Code	# labs	Grand mean (mg/kg)	RSD_r (%)	RSD_R (%)	HorRat
Infant formula	C and D	9	1.26	10.1	19.4	1.26
Infant formula soy based	E and F	9	0.73	23.1	30.7	1.83
Milk powder	G and H	9	1.04	6.51	21.2	1.33
Whole milk	I and J	9	1.43	3.00	11.7	0.77
Soy milk	K and L	9	1.06	13.9	21.0	1.32
Milk chocolate	O and P	9	0.71	16.9	35.1	**2.08**

Table 3. Results for cyanuric acid

Material	Code	# labs	Grand mean (mg/kg)	RSD_r (%)	RSD_R (%)	HorRat
Infant formula	C and D	5	0.83	7.51	17.3	1.05
Infant formula soy based	E and F	6	0.87	12.0	18.5	1.13
Milk powder	G and H	5	1.45	9.23	9.23	0.61
Whole milk	I and J	5	0.57	6.47	13.4	0.77
Soy milk	K and L	6	0.91	15.0	25.1	1.53
Milk chocolate	O and P	5	0.96	13.5	13.5	0.84

The grand means of the materials are very much comparable to the aimed levels: from 80% (0.96/1.2) for cyanuric acid in chocolate to 116% (1.04/0.9) for melamine in milk powder.

All results were included for calculations, except for cyanuric acid: the results of PT371 were not taken into account, due to much higher concentrations and/or poor replicates. For an overview of the reported concentrations, see Annex 7. Modifications of the participants are presented in Annex 6. No major modifications were applied, mainly small chromatographic or mass spectrometric settings.

4.2 Proficiency test

The statistical evaluation of the proficiency test was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [7], elaborated by ISO, IUPAC and AOAC and ISO 13528 [8] in combination with the insights published by the Analytical Methods Committee [11,12] regarding robust statistics. For the evaluation the consensus value

(X), the uncertainty of the consensus value (u), a standard deviation for proficiency assessment (σ_P) and z-scores were calculated (see Annex 5 for statistical procedure). Annex 8 shows the z-scores of the participants and in Annex 9 the results are presented graphically.

For melamine four questionable z-scores ($2<|z|<3$) were reported by labs PT365 (in infant formula, infant formula soy based and soy milk) and PT379 (infant formula soy based). Statistical evaluation of melamine in milk chocolate was not appropriate, since the results show a high variation.

For cyanuric acid seven unsatisfactory z-scores ($|z|>3$) were reported by labs PT371 (all matrices) and lab PT367 (soy milk).

5. Conclusions

Within this collaborative study for melamine and cyanuric acid in food products a prescribed method was tested by nine laboratories. From their results it can be concluded that:

- The method is suitable for the analysis of melamine in infant formula (milk or soy based), milk powder, milk and soy milk (HorRat values varied from 0.77-1.83)
- The method is just outside the required HorRat of 2.0 for the analysis of melamine in chocolate and therefore not suitable.
- The method is suitable for the analysis of cyanuric acid in all matrices.

Annexes:

1. Codification of the samples	Annex 1 Codification of the samples.pdf
2. Homogeneity	Annex 2 Homogeneity.pdf
3. Instruction letter	Annex 3 Instructions.pdf
4. Method description	Annex 4 Final CEN standard MEL and CY
5. Statistical procedure for proficiency test	Annex 5 [illegible] statistics.pdf
6. Modifications to method by participants	Annex 6 Modifications to meth
7. Reported concentrations	Annex 7 Reported concentrations.pdf
8. z_a-scores	Annex [illegible] z-scores.pdf
9. Graphical overview z_a-scores	Annex 9 Graphs z.pdf

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