CERTIFICATION REPORT

The certification of the mass fraction of aflatoxin M_1 in whole milk powders

Certified Reference Material
ERM®-BD282 (zero level)
ERM®-BD283 (low level)
ERM®-BD284 (high level)

Report EUR 21202 EN

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Institute for Reference Materials and Measurements

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EUR Report 21202
Luxembourg: Office for Official Publications of the European Communities

ISBN 92-894-7684-2

Printed in Belgium

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SUMMARY

The mycotoxin aflatoxin M_1 (AfM_1) is a serious food safety hazard for which the European Commission has already established a maximum permissible level of 0.05 µg/kg AfM_1 in milk and milk products.

This report describes the preparation of three whole milk powder reference materials ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) and the certification of their individual aflatoxin M_1 mass fractions. ERM-BD282, ERM-BD283, ERM-BD284 were originally certified as BCR-282R, BCR-283R, BCR-284R.

The certified values were calculated as the unweighted arithmetic mean values of results delivered by a number of experienced labaoratories participating in a collaborative characterisation exercise. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties due to possible inhomogeneity and instability. The certified values are listed below:

ERM No.	Certified value	Uncertainty	No. of accepted sets of results (p)
ERM-BD282	< 0.02 µg/kg	-	9
ERM-BD283	0.111 µg/kg [1)]	0.018 µg/kg [2)]	7
ERM-BD284	0.44 µg/kg [1)]	0.06 µg/kg [2)]	8

1) This value is the unweighted mean of p accepted mean values, independently obtained by p laboratories.
2) The uncertainty is the expanded uncertainty (k = 2) of the value defined in 1).

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GLOSSARY

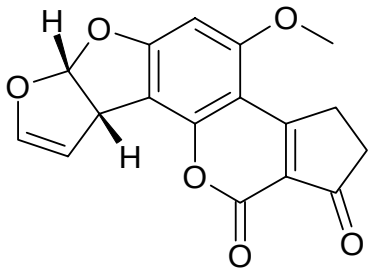
ACN	Acetonitrile
AfB_1	Aflatoxin B_1
AfM_1	Aflatoxin M_1
AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of Variances
APCI	Atmospheric Pressure Chemical Ionization
b.w.	Body Weight
CI	Confidence interval
CRM	Certified Reference Material
CV	Coefficient of Variation
ECD	Electron Capture Detector
ELISA	Enzype Linked Immuno Sorbent Assay
ERM®	Trademark European Reference Material
FLD	Fluorescence Detection
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity Column
KF	Karl Fischer water determination
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
ODS	Octadecyl Silane
PBS	Phosphate Buffered Saline
RM	Reference Material
RP	Reserved phase
RSD	Relative Standard Deviation
RSD_R	Relative between laboratory Standard Deviation
SD	Standard Deviation
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography
U	Uncertainty
u_{bb}	Between-bottle uncertainty
u_{char}	Characterisation uncertainty
u_{lts}	Uncertainty of long-term stability
u_{sts}	Uncertainty of short-term stability
UV	Ultraviolet
u_{wb}	Within-bottle uncertainty

1 INTRODUCTION

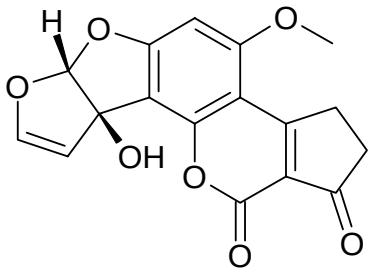
1.1 General

Aflatoxins are a group of mycotoxins, secondary fungal metabolites, which are of greatest significance for the safety of food- and feedstuffs. Aflatoxins are mainly produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus* both pre- and postharvest at relatively high moisture contents and temperatures. They may occur on various agricultural commodities, which enter the food chain either directly or are used for the production of animal feedingstuffs (e.g. peanut, maize, rice and products thereof).

The aflatoxins B_1, B_2, G_1 and G_2 are the main naturally occurring aflatoxins. Aflatoxin B_1 (AfB_1) [(6aR-cis)-2,3,6a,9a-tetrahydro-4-methoxycyclopenta[c]furo[3',2':4,5] furo[2,3,-h][1]benzopyran-1,11-dione, CAS: 1162-65-8] is widely regarded as the most potent liver carcinogen known for a wide variety of mammalian species, including humans [1].



Aflatoxin B_1



Aflatoxin M_1

Figure 1.1: Graphical illustrations of the molecular structure of aflatoxin B_1 and its metabolite aflatoxin M_1

Aflatoxin M_1 (AfM_1) [2,3,6a,9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta[c]furo [3',2':4,5]furo[2,3,-h][1]benzopyran-1,11-dione, CAS: 6795-23-9] is a hydroxylated derivative of AfB_1 which is formed and excreted in the milk of lactating animals after the ingestion of AfB_1 contaminated feed [2]. The molecular structures of AfB_1 and AfM_1 are depicted in Figure 1.1.

The frequent detection of AfM_1 in commercial milk and dairy products, the high consumption of these products, especially in infants and the probable carcinogenicity of AfM_1, led to an increased public awareness and therefore to the establishment of measures to control AfM_1 contamination of food- and feedstuffs. The importance of aflatoxins as a food safety hazard is reflected in the existence of regulations controlling the maximum limits for aflatoxins in foods in 17 countries [3].

The Scientific Committee for Food of the European Community (SCF) points out that aflatoxins are genotoxic carcinogens. Therefore a threshold below which tumour formation would not occur can not be described. In other words, only a zero level of exposure will result in no risk [4]. Also the WHO judged AfM_1 as being possibly carcinogenic to humans. The European Commission has established a maximum permissible level of 0.05 µg/kg AfM_1 in milk and milk for the manufacture of milk-based products and heat-treated milk [5]. The establishment of this low limit was based on applying the ALARA (as low as reasonably achievable) principle.

1.2 Analytical aspects

A wide variety of analytical methods for the determination of AfM_1 in milk and dairy products are currently available.

These methods mainly employ thin-layer chromatography (TLC), high-performance liquid chromatography with fluorescence detection (HPLC-FLD), gas chromatography with mass spectrometry (GC-MS) or enzyme-linked immunosorbent assays (ELISA) subsequent to a liquid/liquid (l/l) extraction, solid-phase extraction (SPE), or immunoaffinity column (IAC) clean-up.

The most widely used procedures for the purification of mycotoxins are the antibody-based immunoaffinity clean-up columns prior to separation and quantification by reversed phase (RP)-HPLC-FLD using excitation and emission wavelengths of e.g. 365 and 435 nm, respectively. Analysis of AfM_1 and its metabolites using IACs is simple, robust and quantitative. Typically, the limit of detection (LOD) of an IAC method is 0.005-0.02 µg AfM_1/kg milk powder corresponding to 0.5-2 ng AfM_1/kg milk. The method gives a high mean recovery rate of 70 - 100 %. From the environmental point of view a major advantage of the IAC clean-up is that in contrast to conventional methods using l/l-extraction the use of a chlorinated solvent (e.g. chloroform) is no longer necessary.

Two RP-HPLC-FLD methods after IAC clean-up were already adopted as standard methods, i.e. EN ISO 14501 (1998) [6] and IDF 171 (1995) [7] by CEN (European Committee for Standardisation), ISO (International Organisation for Standardisation) and IDF (International Dairy Federation). Recently, the AOAC International (Association of Analytical Communities) has adopted an RP-HPLC-FLD method after IAC clean-up as new first action method for AfM_1 in milk at > 0.02 ng/mL which was collaboratively studied by twelve European laboratories [8,9]. The concentration levels studied ranged from 0.023 to 0.103 ng/mL. Values for the relative standard deviation under repeatability conditions (RSD_r) ranged from 8 to 18 % and those obtained under reproducibilty conditions (RSD_R) ranged from 21 to 31 %. The mean recovery for AfM_1 was found to be 74 % at a level of 0.05 ng/mL. The method fulfils a need to those countries that have tolerance levels for AfM_1 set in milk at 0.05 µg/kg, e.g. in the EU [5].

Despite the development of a wide variety of sensitive methods, accurate determination of AfM_1 at the extremely low levels found in milk remains difficult. For this reason CEN published a report that specifies criteria for performance characteristics for guidance to select methods for analysis for mycotoxins, including AfM_1 in milk [10]. The performance characteristics for AfM_1 methods are $RSD_r \leq 30$ %, $RSD_R \leq 50$ % and recovery rates of 60-120 % at concentration levels of 10-50 ng/kg as well as $RSD_r \leq 20$ %, $RSD_R \leq 30$ % and recovery rates of 70-110 % at concentration levels of more than 50 ng/kg. These performance criteria (incl. recovery) for the methods of analysis for AfM_1 in milk were already recommended in an European Commission Directive (1998) [11,12] laying down the sampling methods and the methods of analysis for the official control of the levels for certain contaminants in foodstuffs.

The analytical difficulty and the economic importance of controlling AfM_1 levels in milk and dairy products led the BCR to prepare a series of milk powder CRMs in 1985 [13] and an AfM_1 calibrant RM in 1999 [14]. Because of the good acceptance of these CRMs and to ensure comparability and traceability further on it was required to produce new batches.

2 PARTICIPANTS

Activity	Company / Institute, Department / Division /Unit , Town, Country
P/C	Institute for Reference Materials and Measurements (IRMM), Geel, BE
HS/C	Centrum voor Landbouwkundige Onderzoek (CLO), Melle, BE
HS/C	Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven, NL
C	Central Science Laboratory (CSL), York, UK
C	Direct Laboratories, Wolverhampton, UK
C	Eläinlääkintä - ja elintarviketutkimuslaitos (EELA), Helsinki, FI
C	Eusko Jaurlaritza, Dept. de Sanidad, Bilbao, ES
C	Instituto Nacional de Engenharia e Tecnologia Industrial (INETI), Lisbon, PT
C	Istituto Superiore di Sanità (ISS), Rome, IT
C	Laboratory of the Government Chemist (LGC), Teddington, UK
C	Nederlandse Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek (TNO), Zeist, NL
C	Rijks-Kwaliteitsinstituut voor Land- en Tuinbouwproducten (RIKILT), Wageningen, NL

(P = preparation, HS = homogeneity/stability, C = certification)

3 PREPARATION OF THE MATERIALS

3.1 Preparation of whole milk powder materials

Three new candidate materials were produced to substitute the partly sold-out milk powder materials ERM-BD282, -BD283 and -BD284. ERM-BD282 (zero level) is supposed to be a blank material, a whole milk powder with a very low content of AfM_1. ERM-BD283 (low level) and ERM-BD284 (high level) are whole milk powders containing a low (0.1 µg/kg) and a high (0.4 µg/kg) level of AfM_1.

3.1.1 Base materials

ERM-BD282 (zero level) was prepared from a non-contaminated whole milk powder material. For the preparation of ERM-BD283 (low level) and ERM-BD284 (high level), the same base material was used which was blended with a highly contaminated material. The base materials were analysed at RIVM, Bilthoven, NL for their AfM_1 contents. The concentration of the highly contaminated material was approx. 87 µg/kg AfM_1 whereas the non-contaminated material was found to contain less than 0.02 µg/kg AfM_1.

3.1.2 Processing

For the preparation of ERM-BD282 (zero level), 47 kg of non-contaminated milk powder was added in small portions to 94 L of demineralised water in a 200 L container under constant stirring.

For the preparation of ERM-BD283 (low level) and ERM-BD284 (high level), 45 kg non-contaminated milk powder and 90 L demineralised water were used. When the milk powder was reconstituted completely, an emulsion of 65 g contaminated milk powder dispersed in 500 mL demineralised water (ERM-BD283) and 260 g contaminated milk powder dispersed in 2000 mL demineralised water (ERM-BD284) were added. The mixtures were stirred for another 30 min, transferred to 40 trays (size of one tray: 450 mm x 300 mm x 30 mm), and then frozen. Half of the batches were stored at -18 ºC until the other half of the batches had been freeze-dried in the meantime.

3.1.3 Drying, sieving and bottling

The batches were freeze-dried in an Epsilon freeze-dryer (Christ, DE) for 100-120 hours with a pressure gradient between 1000 mbar and 4×10^{-3} mbar. The sole temperature ranged between -40 ºC and +20 ºC. During the freeze-drying process, water content and water activity were checked.

After freeze-drying, the two sub-batches were sieved (<1 mm) and subsequently 30 min homogenised in a Turbula mixer.

The milk powders were bottled manually (30 g units) into amber glass bottles of 100 mL in a glove box under a dry nitrogen atmosphere. The total amount produced was 1477 bottles for ERM-BD282 (zero level), 1414 bottles for ERM-BD283 (low level) and 1421 bottles for ERM-BD284 (high level). After bottling, the batches were stored at -20 ºC and sufficient aliquots were stored at a reference temperature of -70 ºC.

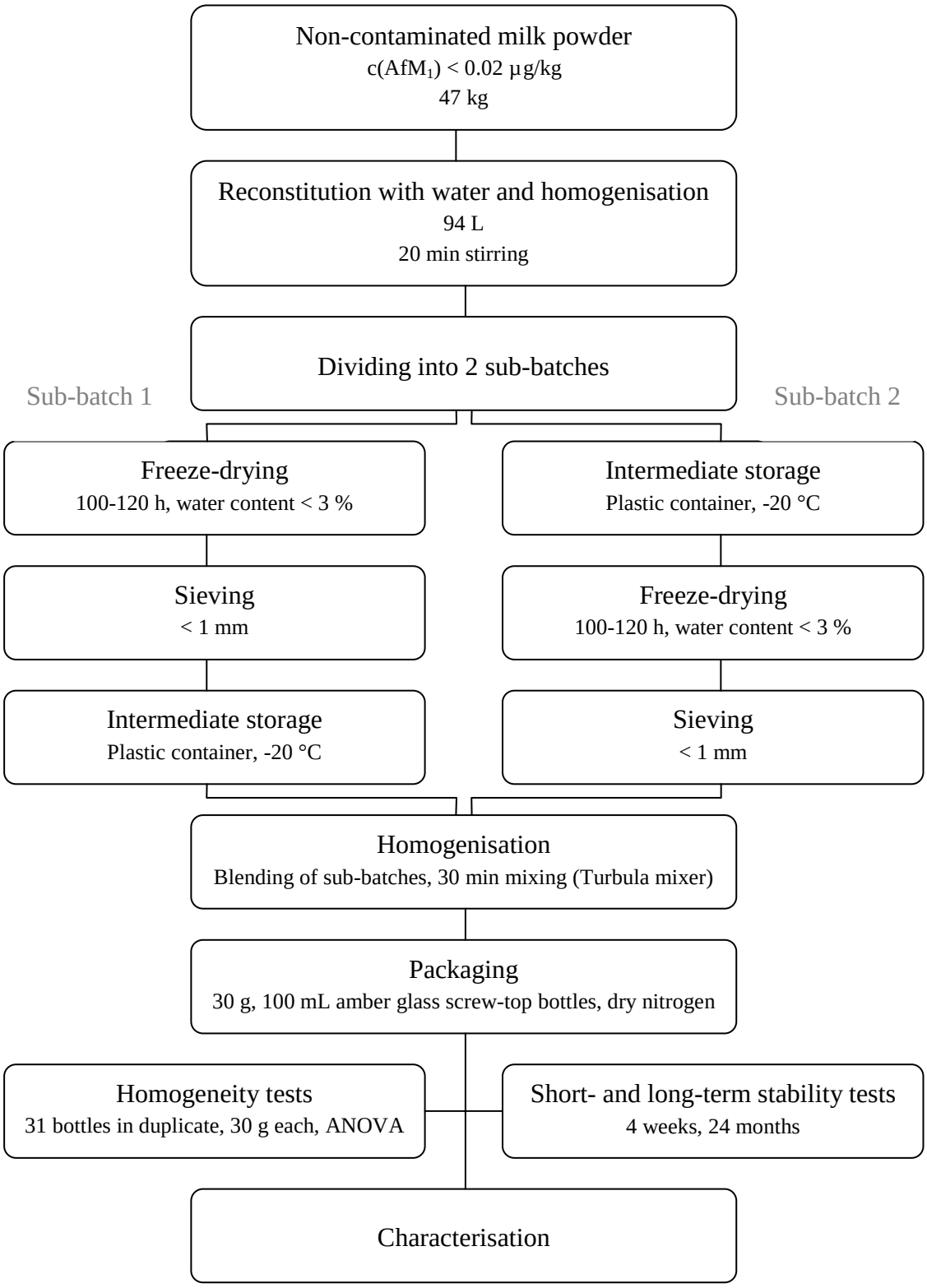


Figure 3.1: Processing and testing of AfM_1 in milk powder ERM-BD282 (zero level)

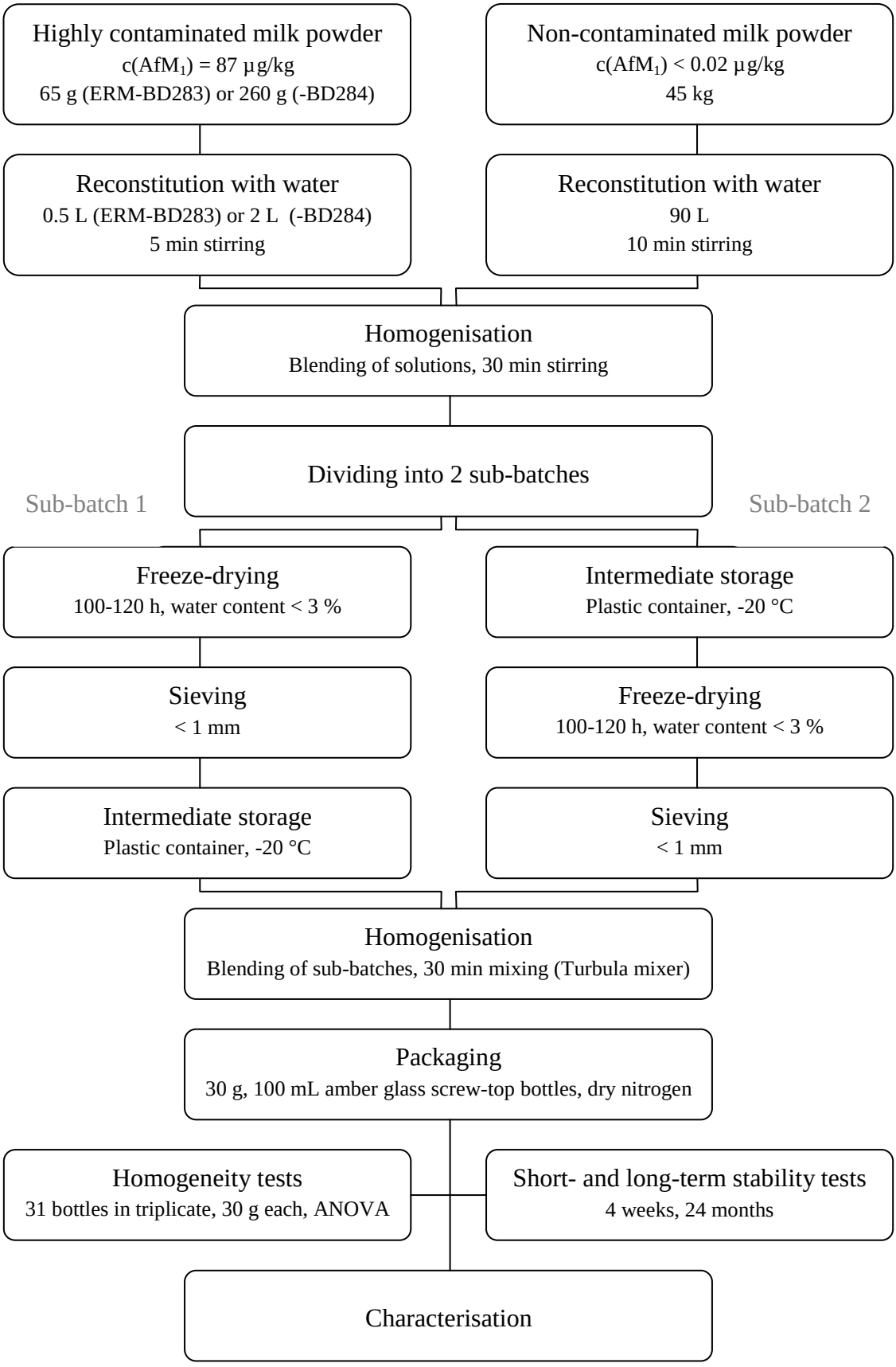


Figure 3.2: Processing and testing of AfM_1 in milk powder ERM-BD283 (low level) and ERM-BD284 (high level)

3.1.4 Additional specification measurements

3.1.4.1 Moisture content

The water contents of the milk powders ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) were measured by Karl Fischer titration. For ERM-BD282 (zero level) nine selected bottles were analysed in duplicate showing an average water content of 2.3 ± 0.5 % and a water activity of less than 0.07. For both ERM-BD283 (low level) and ERM-BD284 (high level) ten selected bottles were analysed in duplicate showing an average water content of 1.8 ± 0.4 % and a water activity of less than 0.07, respectively. The individual results are given in Table 3.1 to Table 3.3.

Table 3.1: Water activity and water analysis of ERM-BD282 (zero level).

ERM-BD282	Water (%)			Water activity (a_w)
Bottle ID	Replicate 1	Replicate 2	Mean	
14	1.9	1.9	1.9	< 0.07
181	2.5	2.6	2.6	< 0.07
297	2.0	1.9	2.0	< 0.07
659	2.7	2.6	2.6	< 0.07
771	2.1	2.1	2.1	< 0.07
887	2.4	2.3	2.4	< 0.07
1007	2.3	2.3	2.3	< 0.07
1126	2.1	2.1	2.1	< 0.07
1363	2.5	2.6	2.5	< 0.07
Mean ± CI (95 %)			2.3 ± 0.5	< 0.07

Table 3.2: Water activity and water analysis of ERM-BD283 (low level).

ERM-BD283	Water (%)			Water activity (a_w)
Bottle ID	Replicate 1	Replicate 2	Mean	
7	2.0	2.0	2.0	< 0.07
164	2.0	1.6	1.8	< 0.07
325	1.9	2.1	2.0	< 0.07
446	1.7	2.1	1.9	< 0.07
561	2.4	1.6	2.0	< 0.07
738	1.7	1.4	1.6	< 0.07
917	2.0	1.8	1.9	< 0.07
1068	1.7	1.6	1.7	< 0.07
1260	1.8	2.0	1.9	< 0.07
1369	2.0	2.2	2.1	< 0.07
Mean ± CI (95 %)			1.8 ± 0.4	< 0.07

Table 3.3: Water activity and water analysis of ERM-BD284 (high level).

ERM-BD284 Bottle ID	Water (%) Replicate 1	Replicate 2	Mean	Water activity (a_w)
31	1.5	1.4	1.4	< 0.07
184	1.5	1.4	1.5	< 0.07
337	1.5	1.6	1.5	< 0.07
490	2.1	2.1	2.1	< 0.07
643	1.8	1.7	1.8	< 0.07
795	1.8	1.8	1.8	< 0.07
949	1.8	1.6	1.7	< 0.07
1102	2.1	2.2	2.2	< 0.07
1255	1.8	1.8	1.8	< 0.07
1408	1.9	1.9	1.9	< 0.07
Mean ± CI (95 %)			1.8 ± 0.4	< 0.07

3.1.4.2 Particle size measurements

Ten particle size analyses have been carried out for each of the milk powder materials employing a laser diffraction measuring device from Sympatec, DE. The powders were dispersed in methanol and the particle size distribution measured over a range of 0.5 to 875 µm. A sonication duration of 20 s applying a stirring rate of 1200 rpm with a measuring duration of 10 s was used.

Representative graphs of the particle size distributions for the milk powder materials are depicted in Figure 3.3 showing e.g. for ERM-BD282 (zero level) that 99 % and 50 % of the milk powder particles were smaller than 214 µm (top particle size) and 60 µm (median), respectively.

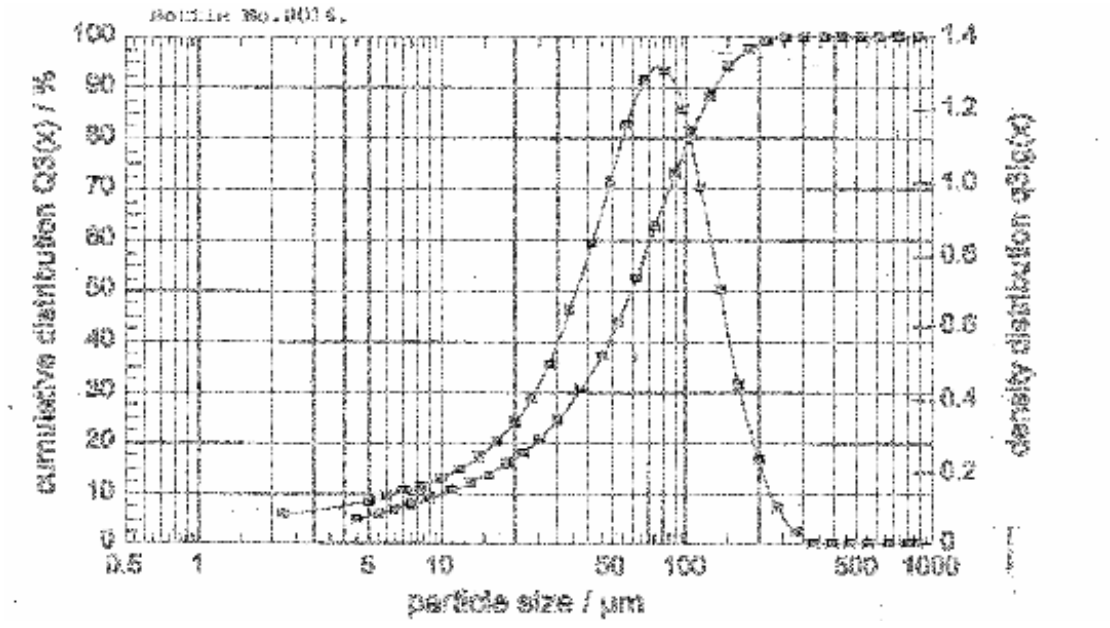


Figure 3.3: Representative particle size distribution of milk powder ERM-BD282 (zero level)

3.1.4.3 Microscopic examination

In addition, three micrographs were taken for ERM-BD282, ERM-BD283 and ERM-BD284 employing a Stemi 2000-C microscope from Zeiss, DE equipped with a MicroCam from Polaroid, USA.

The milk powder samples were applied on microscope slides for visual inspection. A representative micrograph for the milk powder materials, e.g. ERM-BD282 (zero level), is presented in Figure 3.4. The micrographs confirmed the findings of the particle size distribution.

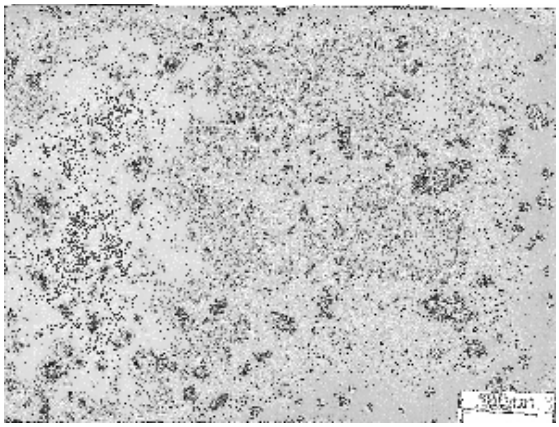


Figure 3.4: Representative micrograph of milk powder ERM-BD282 (zero level)

3.1.5 AfM_1 content

Furthermore, the three candidate CRMs were analysed in triplicate for their AfM_1 contents employing HPLC-FLD after IAC clean-up. These preliminary in-house measurements indicated that the target values were reached. The AfM_1 content in the milk powder ERM-BD282 (zero level) was found to be less than 0.03 µg/kg (LOQ). Both contaminated milk powder materials ERM-BD283 (low level) and ERM-BD284 (high level) contained 0.10 µg/kg and 0.39 µg/kg AfM_1, respectively.

3.2 Homogeneity studies

3.2.1 AfM_1 in milk powder ERM-BD282

3.2.1.1 Design of the homogeneity study

The homogeneity testing of the AfM_1 in milk powder ERM-BD282 (zero level) was performed by selecting about every 50^{th} bottle (N = 31, 30 g each) of the 1477 bottles during the filling sequence (November 2001).

3.2.1.2 Method of analysis

The homogeneity measurements of AfM_1 in milk powder ERM-BD282 (zero level) were made by determination of the Kjeldahl-nitrogen content because there was no indication for AfM_1 contamination based on preliminary analyses. The measurements were performed in duplicate (n = 2) employing a digestion method (macro method) according to the IDF 20B/2 standard [15]. Prior to analysis the bottles were allowed to reach room temperature before opening. The analytical sequence for the bottles was selected using a random stratified scheme to allow the evaluation of any trends due to the filling sequence. The analytical sequence was analysed over a period of seven days and was, therefore, divided in seven subsequences.

3.2.1.3 Results of the homogeneity study

The individual data of the homogeneity study were normalised with respect to the averages of the analytical subsequences to ensure repeatability conditions and are presented in Annex B-1.

The normalised data of the homogeneity testing were evaluated as described elsewhere [16] by ANOVA, which allows the separation of the method variation (s_{wb}) from the experimental averages over one unit ($u_{c,bb}$) to obtain an estimation for the real variation between units (s_{bb}) as shown in the following equation:

$$u^2_{c,bb} = s^2_{bb} + \frac{s^2_{wb}}{n}$$

The standard deviation between the units was used as the estimator for the between-units variance. The measurement variation sets a lower limit u^*_{bb} to this estimator, which is given by the equation, mentioned below:

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{\nu_{MSwithin}}}$$

with MS_{within}, n and $\nu_{MSwithin}$ being the mean squares within units, the number of measurements per unit (n = 2 or n = 3 for duplicate or triplicate analysis), and the degrees of freedom of MS_{within}, respectively. The uncertainty of the homogeneity (u_{bb}) is consequently estimated as s_{bb} or u^*_{bb}, depending on which of these is larger.

The ANOVA results of the homogeneity testing for ERM-BD282 (zero level) are summarised in Table 3.4 together with the outcome of the homogeneity testing of ERM-BD283 (low level) and ERM-BD284 (high level). For the ERM-BD282 (zero level) a difference in the within- and between-sample variances was detected by the F-test at the 95 % confidence level due to the high precision of RSD = 0.26 % of the nitrogen determination method. However, the material was regarded to be homogeneous since the RSD of 0.26 % and the u_{bb} of 0.23 % were very small compared to typical method repeatabilities of more than 10 % for the determination AfM_1 in milk powders. Nevertheless, the determination of the homogeneity by

use of the nitrogen content can only serve as an estimate because it is not possible to determine any AfM_1 in the milk powder ERM-BD282 (zero level).

Additionally, linear regression analyses were carried out for the normalised results in order to detect effects due to filling and analysis order. The slopes of the lines were tested for significance on a 95 % confidence level to check for significant trends. No significant trends due to filling or analysis order were observed. The normalised results due to the filling sequence are presented in Figure 3.5.

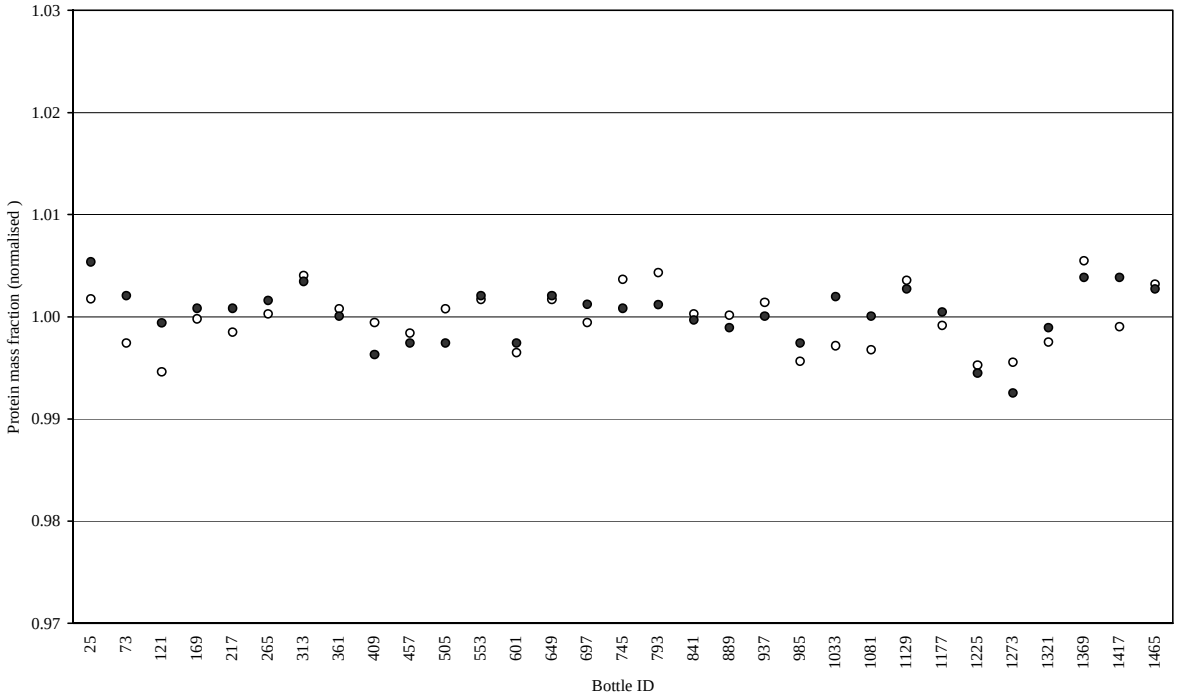


Figure 3.5: AfM_1 in milk powder ERM-BD282 (zero level): Results of the homogeneity study normalised with respect to the averages of the analytical subsequences - Filling sequence.

3.2.2 AfM_1 in milk powder ERM-BD283 and ERM-BD284

3.2.2.1 Design of the homogeneity studies

For the homogeneity studies (October 2000), which was conducted prior to the certification study, 31 bottles (N = 31) of ERM-BD283 (low level) and 31 bottles (N = 31) of ERM-BD284 (high level) were taken at regular intervals of the filling sequence and analysed in triplicate (n = 3).

3.2.2.2 Method of analysis

Prior to analysis the bottles were allowed to reach room temperature before opening. The analytical sequence for the bottles was selected using a random stratified scheme to allow the evaluation of any trends due to the filling sequence. The analytical sequences were divided in eight sub-sequences each because not more than fourteen AfM_1 determinations could be carried out per day.

For the determination of AfM_1 milk powder (10.0 g) was mixed with celite (10.0 g) and glass pearls (4 g). The mixture was extracted with 100 mL chloroform and 10 mL water by shaking on a horizontal table shaker for 30 min. The extract was filtered through a Macherey-Nagel 617 ¼ folded filter. An aliquot of the eluate (50 mL) was evaporated to dryness using a rotary

evaporator at 50 °C. The residue was successively dissolved and transferred to a separation funnel using 10 mL of methanol and 60 mL of n-pentane. Liquid-liquid extraction was performed in three separation steps of 30 s employing additional 40 mL of water and 50 mL of n-pentane. The combined methanol-water phases were passed through a Whatman microfibre filter GF/C. An aliquot of the filtrate (40 mL) was diluted with water (40 mL).

The AflaprepM IAC (Rhone Diagnostic Technologies LTD) with the pre-connected solvent reservoir was placed on an SPE station (VacMaster). The diluted well-mixed filtrate was filled into the solvent reservoir and passed through the IAC with a flow rate of about 1 drop/s. Afterwards the column was washed with water (10 mL). After the washing steps, the IAC was dried with nitrogen and the washings were discarded.

AfM_1 was eluted by transferring two times 800 µL methanol onto the IAC. The eluates were collected in a 10 mL graduated flask. After the methanol had passed the IAC, the flask was filled up to the mark with water and mixed well.

An aliquot of the well-mixed eluate (2.5 mL) was injected automatically in the HPLC system. The HPLC separation was done with a Prodigy ODS 100 Å column (150×4.6 mm, 5 µm) from Phenomenex at 25 °C and with acetonitrile/water (25+75, v+v) as mobile phase at a flow rate of 1.0 mL/min (isocratic). Separated compounds were detected with a fluorescence detector (Jasco 821-FP) at a wavelength of 365 nm for excitation and 435 nm for emission.

The method performance characteristics obtained from the method validation study were an LOQ of 0.036 µg/kg AfM_1, expanded measurement uncertainty (U) of 10 % (at about 0.1 µg/kg AfM_1) and an average recovery of 99 %.

3.2.2.3 Results of the homogeneity studies

a) AfM_1 in milk powder ERM-BD283 (low level)

Subsequent to packaging the homogeneity measurements for the AfM_1 in milk powder ERM-BD283 (low level) were evaluated by ANOVA as described in section 3.2.1 according to Linsinger et al. [16]. The individual data of the homogeneity study were normalised with respect to the averages of the analytical sub-sequences to ensure repeatability conditions. The normalised individual data are given in the Annex B-2 and the results of the ANOVA are summarised in Table 3.4. No difference in the within- and between-sample variances could be detected by the F-test at the 95 % confidence level. The material could be regarded as homogeneous. The s_{bb} was not calculable due to the fact that $MS_{between}$ was smaller than MS_{within}. Therefore, the u^*_{bb} of 0.89 % was adopted as an estimate for the uncertainty contribution due to potential inhomogeneity.

Additionally, linear regression functions were calculated for the results due to filling and analysis order. The slopes of the lines were tested for significance on a 95 % confidence level to check for significant trends. No significant trends due to analysis order have been observed. However, a slight trend has been observed for the filling order, which is already fully covered by the uncertainty due to possible inhomogeneity (u_{bb}) of 0.89 %. The normalised results due to the filling sequence are presented in Figure 3.6.

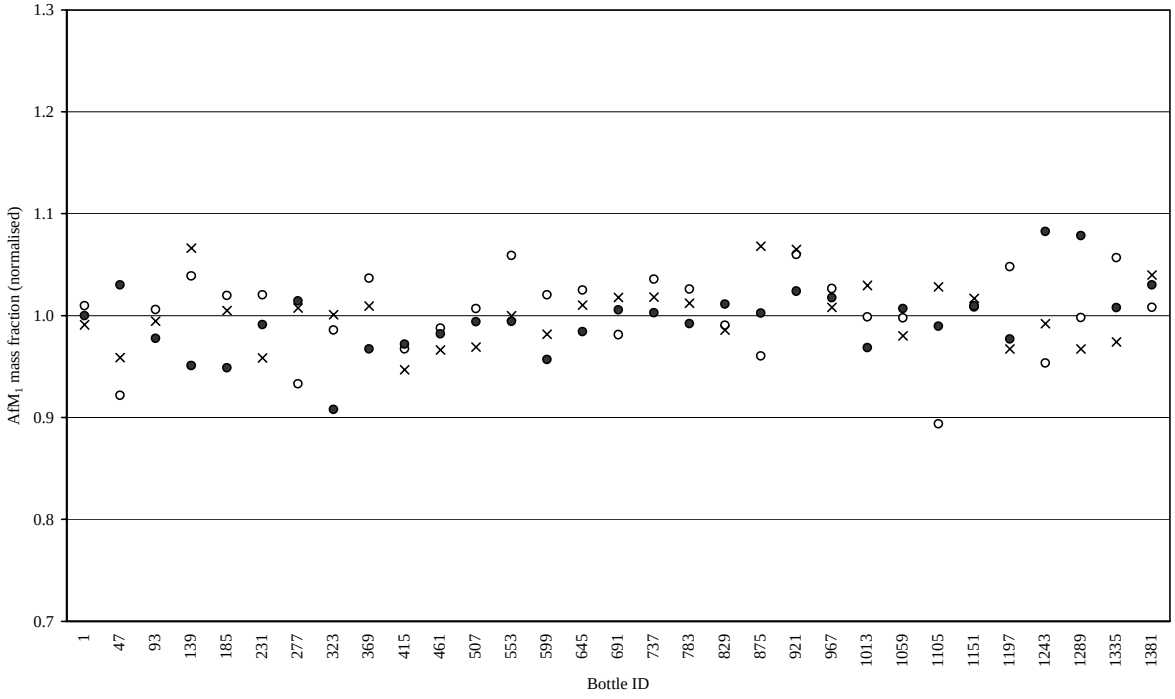


Figure 3.6: AfM_1 in milk powder ERM-BD283 (low level): Results of the homogeneity study normalised with respect to the averages of the analytical subsequences - Filling sequence.

b) AfM_1 in milk powder ERM-BD284 (high level)

The final homogeneity measurements for AfM_1 in ERM-BD284 (high level) were also subjected to ANOVA. Individual data of the homogeneity study given in the Annex B-3 were normalised with respect to the averages of the analytical sub-sequences. The s_{wb} and u_{bb} were calculated as described in section 3.2.1. The results of the ANOVA are summarised in Table 3.4. No difference in the within- and between-sample variances could be detected by the F-test at a 95 % confidence level. The material could be regarded as homogeneous. The s_{bb} was not calculable due to the fact that $MS_{between}$ was smaller than MS_{within}. Therefore, the u^*_{bb} of 0.77 % was adopted as estimator for the uncertainty due to potential inhomogeneity.

Furthermore, linear regression functions were calculated for the results due to filling and analysis order. The slopes of the lines were tested for significance at a 95 % confidence level to check for significant trends. No significant trends due to filling or analysis order have been observed. The normalised results due to the filling and analysis sequence are presented in Figure 3.6.

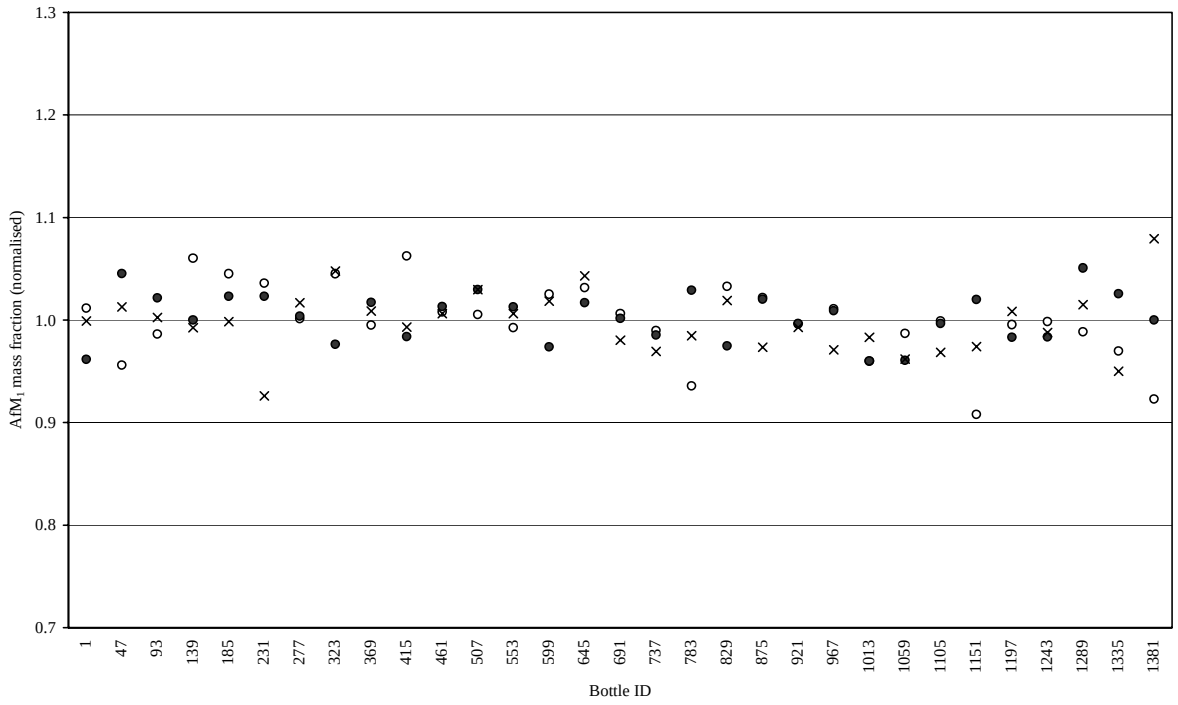


Figure 3.7: AfM_1 in milk powder ERM-BD284 (high level): Results of the homogeneity study normalised with respect to the averages of the analytical subsequences - Filling sequence.

3.2.3 Conclusion

The results of the homogeneity studies are summarised in Table 3.4. Uncertainty contributions due to possible inhomogeneity (u_{bb}) of 0.23 %, 0.89 % and 0.77 % were used for the evaluation of the combined uncertainty of ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level), respectively (cf. section 4.4).

Since a sample amount of 10 g milk powder was employed in the described homogeneity studies a minimum amount of 10 g milk powder has to be analysed by future users of the produced milk powder reference materials ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level).

Table 3.4: Summarised results of the homogeneity testings of ERM-BD282, ERM-BD283 and ERM-BD284

	Protein content	AfM_1 content	
	ERM-BD282 (zero level)	ERM-BD283 (low level)	ERM-BD284 (high level)
Mean of means	26.50 g/100 g	0.107 µg/kg	0.425 µg/kg
SD	0.07 g/100 g	0.003 µg/kg	0.010 µg/kg
RSD	0.26 %	3.1 %	2.3 %
N	31	31	31
s_{wb}	0.18 %	3.6 %	3.2 %
s_{bb}	0.23 %	not calculable ($MS_{between} < MS_{within}$)	not calculable ($MS_{between} < MS_{within}$)
u^*_{bb}	0.063 %	0.89 %	0.77 %
$\mathbf{u_{bb}}$ [(1)]	**0.23 %**	**0.89 %**	**0.77 %**
F	4.29	0.84	0.80
F_{crit}	1.83	1.64	1.64

(1) Higher value (u^*_{bb} or s_{bb}) taken as uncertainty estimate for potential inhomogeneity.

3.3 Stability studies

3.3.1 Design of the stability studies

The short-term stability of AfM_1 in both milk powder materials ERM-BD283 (low level) and ERM-BD284 (high level) was evaluated under three different conditions (+4 °C, +18 °C, and +40 °C) in May 2002. Four storage periods of 0, 1, 2, and 4 weeks were investigated. AfM_1 was quantified using an HPLC-FLD method.

The long-term stability studies (October 2001-May 2003) were conducted at storage periods of 0, 6, 12, and 18 months at -20 °C and +4 °C using an HPLC-FLD method for quantification.

The stability studies were carried out as "isochronous measurements" [17]. Therefore, the bottles were removed after their allocated storage times at the temperature conditions mentioned above and set to -70 °C (2 bottles at each time and temperature). The pooled samples were then analysed together under repeatability conditions after 4 weeks for the short-term stability studies. For the long-term stability studies the measurements were carried out after 18 months.

3.3.2 Method used for the determination of AfM_1 in ERM-BD283 and ERM-BD284

The determination of AfM_1 in milk powder by HPLC-FLD was performed as described already for the homogeneity measurements. Prior to analysis the bottles were allowed to reach room temperature before opening. The analytical sequences for the bottles were selected using a random stratified scheme. The analytical sequences were divided in two sub-sequences because not more than fourteen AfM_1 determinations could be carried out per day.

3.3.3 Results of the stability studies

The objective for the short-term stability studies of AfM_1 in ERM-BD283 (low level) and ERM-BD284 (high level) was to evaluate whether special care must be taken during transport. The stabilities at certain storage temperatures were assessed by the long-term stability studies. Statistical analyses of the results were carried out to investigate if there were significant trends in the AfM_1 mass fraction due to storage [18]. Therefore, the slopes of the fitted regression functions were tested for significance at a 95 % level. In case that the slope of the regression lines did not differ significantly from zero the standard error of the slopes were multiplied by the chosen time for which the certificate is valid, in this case six years, to derive an estimate for the uncertainty due to possible instability of the materials. The validity of the certificate can be prolonged, if additional data are obtained.

Additionally, statistical analyses have been done to investigate if there were any significant trends due to the filling sequence or analysis sequence of the bottles. However, no trends were observed for the filling sequence or analysis sequence of the bottles.

a) AfM_1 in milk powder ERM-BD283 (low level)

The results of the short-term stability studies conducted at +40 °C for AfM_1 in ERM-BD283 (low level) are presented graphically in Figure 3.8. All AfM_1 mass fractions were normalised with respect to the average mass fraction of the reference samples (stored at -70 °C, week 0). The analyses of the isochronous measurement scheme at +4 °C was omitted because AfM_1 in milk powder ERM-BD283 (low level) was already found to be stable for four weeks at higher temperatures of +18 °C and +40 °C. No trends were observed for each of the storage conditions, filling sequences or analysis sequences of the material. The slopes were found to be not significant at a 95 % confidence level. Normalised individual results of these studies are listed in Annex C-1.

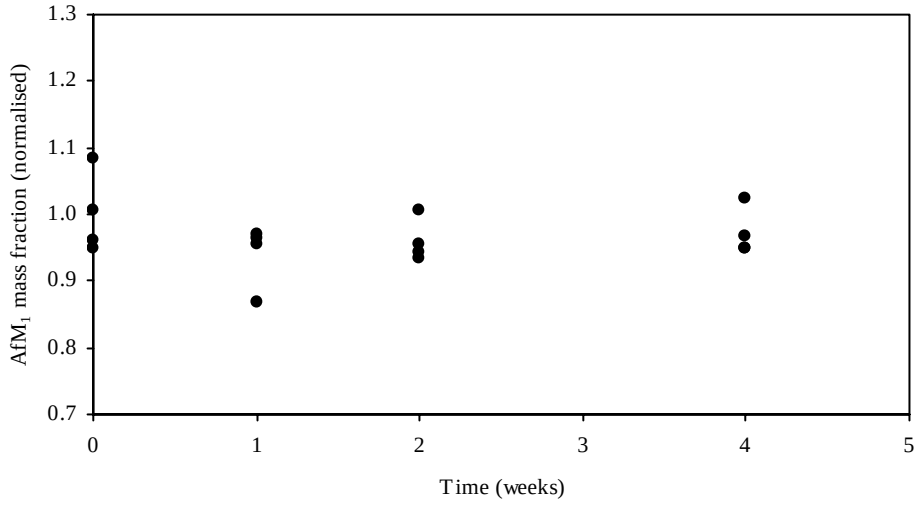


Figure 3.8: AfM_1 in milk powder ERM-BD283 (low level): Results of the short-term stability study at +40 °C normalised with respect to the average result of measurements at reference temperature (0 weeks).

The stability of AfM_1 in milk powder ERM-BD283 (low level) at a certain storage temperature is assessed by the long-term stability studies. The normalised results for the long-term stability study conducted at -20 °C are presented graphically in Figure 3.9. All AfM_1 mass fractions were normalised with respect to the average mass fraction of the reference samples (stored at -70 °C, week 0). Normalisation served only as a means to make the studies comparable. The slopes of the lines were calculated and tested for significance at a 95 % confidence level. They were found to be statistically not significant. Therefore, the standard error of the normalised values was used to estimate the uncertainty. Dotted lines in the long-term stability show the uncertainty of the certified value of the reference material due to possible instability. For a shelf life of 6 years, the u_{lts} for AfM_1 in milk powder ERM-BD283 (low level) amounts to 11.4 % at +4 °C and 7.4 % at -20 °C. Normalised individual results of these studies are listed in Annex C-3.

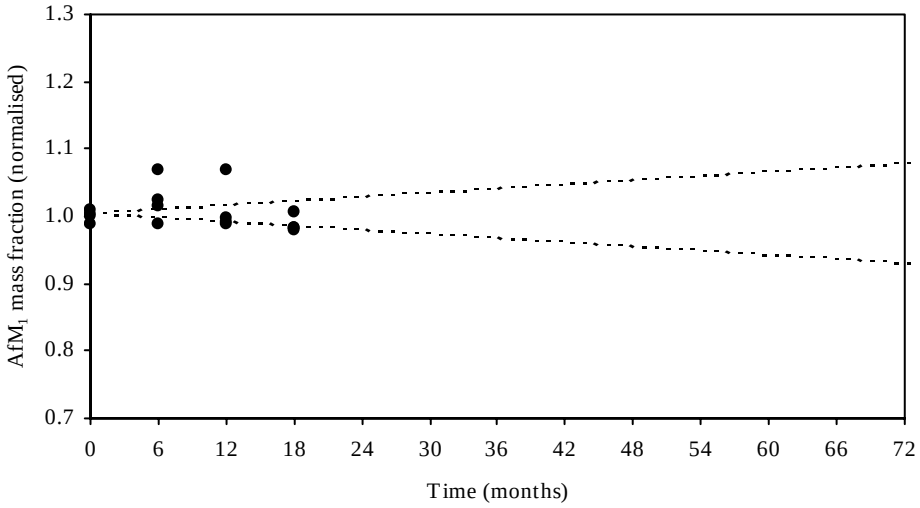


Figure 3.9: AfM_1 in milk powder ERM-BD283 (low level): Results of the long-term stability study at -20 °C normalised with respect to the average result of measurements at reference temperature (0 months).

b) AfM_1 in milk powder ERM-BD284 (high level)

The results of the short-term stability studies conducted at +40 °C for AfM_1 in ERM-BD284 (high level) are presented graphically in Figure 3.10. All AfM_1 mass fractions were normalised with respect to the average mass fraction of the reference samples (stored at -70 °C, week 0). The analyses of the isochronous measurement scheme at +4 °C was omitted because AfM_1 in milk powder ERM-BD283 (low level) was as well found to be stable for four weeks at higher temperatures of +18 °C and +40 °C. No trends were observed for each of the storage conditions, filling sequences or analysis sequences of the material. The slopes were found to be not significant at all on a 95 % level. Normalised individual results of these studies are listed in Annex C-2.

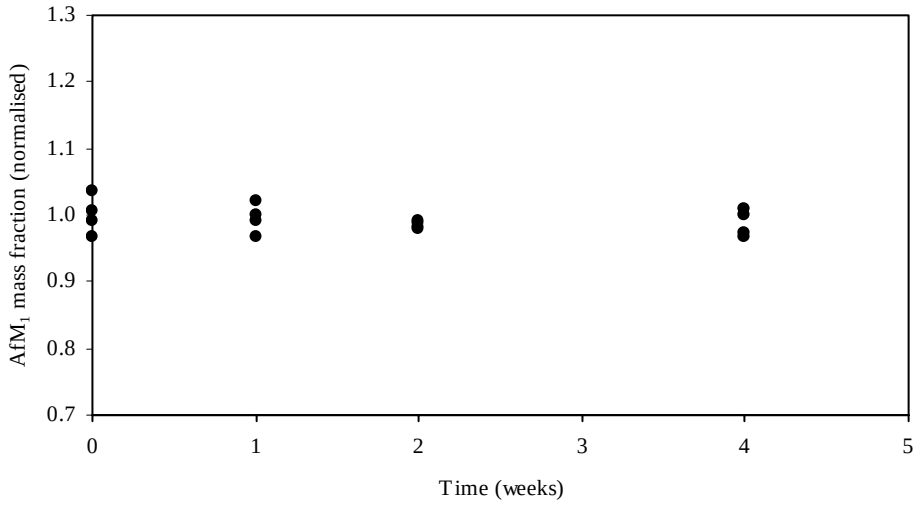


Figure 3.10: AfM_1 in in milk powder ERM-BD284 (high level): Results of the short-term stability study at +40 °C normalised with respect to the average result of measurements at reference temperature (0 weeks).

The normalised results for the long-term stability study of AfM_1 in ERM-BD284 (high level) conducted at -20 °C are presented graphically in Figure 3.11. AfM_1 mass fractions were also normalised with respect to the average mass fraction of the reference samples (stored at -70 °C, week 0). Normalisation served only as a means to make the studies comparable. The slopes of the lines were calculated and tested for significance at a 95 % confidence level. They were found to be statistically not significant. Therefore, the standard error of the normalised values was used to estimate the uncertainty. Dotted lines in the long-term stability show the uncertainty of the certified value of the reference material due to possible instability. For a shelf life of 6 years, the u_{lts} for AfM_1 in milk powder ERM-BD284 (high level) amounts to 8.5 % at +4 °C and 6.3 % at -20 °C. Normalised individual results of these studies are listed in Annex C-4.

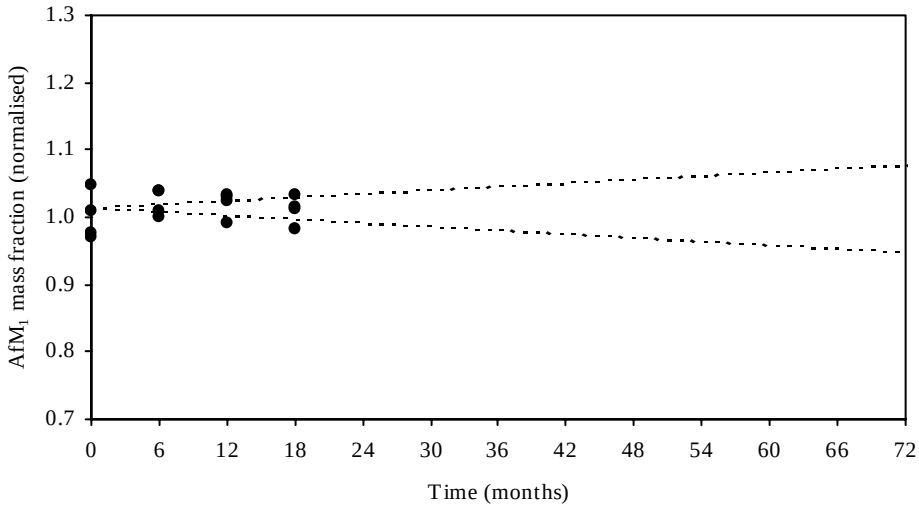


Figure 3.11: AfM_1 in in milk powder ERM-BD284 (high level): Results of the long-term stability study at -20 °C normalised with respect to the average result of measurements at reference temperature (0 months).

3.3.4 Conclusions of the stability studies

The determination of AfM_1 in ERM-BD283 (low level) and ERM-BD284 (high level) employing HPLC-FLD showed no significant trends for both short-term and long-term stability studies.

For AfM_1 in ERM-BD282 (zero level) no stability studies were performed with respect to the AfM_1 content, because AfM_1 was not detectable ($c(AfM_1) < 0.02$ µg/kg). In additon, no increase of AfM_1 can be expected due to the chemical properties and the way of formation. Therefore, ERM-BD282 (zero level) will be stored and shipped under the same conditions as ERM-BD283 (low level) and ERM-BD284 (high level).

Based on the results of the short-term stability studies ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) can be transported at ambient temperature. The uncertainty of the short-term stability (u_{sts}) is assumed to be negligible since no degradation is expected to happen during this short time.

On the basis of the long-term stability measurements, it can be recommended to store ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) at a temperature of -20 °C. The long-term stability uncertainty (u_{lts}) was calculated as described. For a shelf life of 6 years, the u_{lts} for AfM_1 in both milk powders ERM-BD283 (low level) and ERM-BD284 (high level) amounts to 7.4 % and 6.3 %, respectively. The validity of the certificate can be prolonged, if additional data are obtained.

4 THE CERTIFICATION EXERCISE

4.1 Design of the study

The certification exercise (July - October 2002) was performed on the basis of the experiences gained during the project for the certification of AfM_1 in four milk powder samples [13] and additional interlaboratory studies [8]. Particular emphasis was placed on calibration (for which purpose a primary AfM_1 calibrant was provided), adequate and repeatable recoveries, a demonstration of the absence of matrix effects and acceptable 'blank' values.

For the final certification exercise the mass fraction of AfM_1 in the candidate reference materials ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) was measured by a number of external collaborators. The analytical work was carried out by twelve carefully selected participants. One of them employed two different methods. The laboratories had to prove their measurement capabilities and had to demonstrate experience in the concerned analytical field by providing documentary evidence of having successfully participated in certification exercises organised by an EC body and/or documentation of successful participation in other national and/or international interlaboratory studies or laboratory accreditation. Furthermore, laboratories had to provide a full description of the analytical method and documentary evidence of the performance characteristics of their methods.

The following samples were sent to each of the twelve participants:

- 9 bottles of ERM-BD282, "Aflatoxin M_1 in whole milk powder (Zero level)" (>30 g each);
- 3 bottles of ERM-BD283, "Aflatoxin M_1 in whole milk powder (Low level)" (>30 g each);
- 3 bottles of ERM-BD284, "Aflatoxin M_1 in whole milk powder (High level)" (>30 g each);
- 3 ampoules of the common calibrant RM-423, "AfM_1 in chloroform" (2.5 mL each);
- temperature indicators.

a) AfM_1 in milk powder ERM-BD282 (zero level)

First, the 'blank' "AfM_1 in whole milk powder (Zero level)" – ERM-BD282 had to be analysed. Six replicate measurements using independent sample preparations had to be performed on three different days (two measurements on one bottle per day).

b) Recovery check

In order to determine the recovery rates of the participants analytical procedures in the concentration range of the contaminated candidate reference materials, ERM-BD282 (zero level) had to be spiked with the AfM_1 working solution, which had to be prepared using RM-423, according to a provided protocol. Thus, mass fractions of about 0.1 and 0.4 µg/kg of AfM_1 in the spiked 'blank' milk powders were obtained. Nine replicate measurements had to be carried out on three different days (three measurements per day for both concentration levels). Prior to the quantification of the spiked milk powder materials the obtained peak area/height of the 'blank' milk powder material - if present - was subtracted. Since the certification results were corrected by the daily recovery factor, analyses of the 'blank', spiked and naturally contaminated material had to be performed together on one day.

The individual results of the recoveries obtained for the low and high concentration spiking levels are depicted in Annex D-3 and in Annex D-5.

c) AfM_1 in milk powders ERM-BD283 (low level) and ERM-BD284 (high level)

The three bottels of ERM-BD283 and ERM-BD284 supplied to the participants had to be analysed on three different days using independent sample preparations (two measurements on one bottle per day), giving in total six replicate measurement results per candidate CRM.

The results obtained for the naturally contaminated milk powders had to be corrected for the recovery rate of the method at the target concentration level for each working day. The mean recovery obtained of the low spiking level and of the high spiking level had to be used for recovery correction of the ERM-BD283 and ERM-BD284 analyses.

In general, analyses of the 'blank', spiked and contaminated milk powders had to be performed as one analytical sequence carried out on one single day of analysis (day 1, day 2 and day 3 with at least 24 hours in-between). In case that reconstitution of the milk powders and sample clean-up could not be performed on the same day as the chromatographic analyses the purified sample extracts had to be stored in the freezer (below -18 °C) and had to be analysed as soon as possible.

The provided common AfM_1 calibrant had to be used for external calibration. Therefore, a five point external calibration curve (four injections each) had to be established on three different days. The calibration curve had to cover the AfM_1 concentration range of about 0.05 - 0.6 µg/kg in milk powder matrix. The calculation by use of peak area or height was up to the participants, but had to be kept during the characterisation exercise. The dilution and preparation of a working solution of the common AfM_1 calibrants were performed according to a protocol and to the provided 'Instructions for use' given in the BCR information on the "Aflatoxin M_1 in chloroform" [14]. Further, the linearity of the calibration curves had to be checked on the basis of linearity plots.

4.2 Results of the certification exercise and technical evaluation

In total twelve labs participated in the certification for ERM-BD282, ERM-BD283 and ERM-BD284. One of them employed two different methods. Thus, in total 13 sets of results were obtained.

Subsequent to the practical work the various sets of results of the participants were subject to a technical evaluation. All results, even those agreeing well with the overall set of results were scrutinised for potential systematic errors. In this way the possibility of incorrect certification is minimised. For the value assignment the sets of results provided by seven and eight laboratories were accepted for ERM-BD283 and ERM-BD284, respectively.

The great majority of the participants selected for the certification employed slightly modified versions of the EN ISO 14501:1999 [6] or IDF 171:1995 [7] standard methods for the determination of AfM_1 in milk and milk powder. Most participants used water for the extraction of AfM_1 from milk powder (eight methods). Two participants utilised chloroform/water mixtures as extraction solvent. In all cases clean-up was carried out with immunoaffinity columns (eight participants used products from R-Biopharm Rhône, two from Vicam). One laboratory used additional liquid/liquid-extraction for purification of the samples. All methods employed HPLC-FLD for the separation and detection. Various types of C18 RP-HPLC columns were employed. The participants used only isocratic solvent systems with mainly water/acetonitrile and water/acetonitrile/methanol solvent mixtures. Fluorescence detection was carried out with excitation wavelengths in the range between

360 nm and 365 nm and emission wavelengths in the range between 420 nm and 435 nm. Details of the methods of analysis chosen for the certification exercise are given in the Annex A.

The individual results submitted for the certification of AfM_1 in ERM-BD283 (low level) and ERM-BD284 (high level) after recovery correction are given in Annex D-2 and Annex D-4. The individual results of the recoveries obtained for the low and high concentration spiking levels are depicted in Annex D-3 and Annex D-5. The individual results submitted for the 'blank' milk powder samples ERM-BD282 are given in Annex D-1.

The overall agreement between the results of the laboratories can be considered as being highly satisfactory, compared with the results from previous intercomparison studies. There is no indication of any systematic effect associated with the analytical methods employed. At an evaluation meeting, where all except two participating laboratories were represented, the following method performance criteria were used as guidelines in evaluating the results of the milk powder materials containing significant amounts of AfM_1. They were based on the criteria established by CEN [10] for AfM_1 analysis at concentration levels of 0.01-0.05 ng/L milk (corresponding to about 0.1-0.5 µg/kg milk powder) and on the discussions with the project participants:

- An allowed recovery range of 60-120 %;
- Relative within-laboratory standard deviation of $RSD_r \leq 10$ %;
- Relative between-laboratory standard deviation of $RSD_R \leq 15$ %;
- Linear calibrations (linearity plots).

The technical evaluation led to the remarks and conclusions listed in the next sections.

4.2.1 AfM_1 in milk powder ERM-BD282 (zero level)

ERM-BD282 is intended to approximate a 'blank' material. The results were examined in detail by the participants and the following observations were made:

On the basis of the results obtained, participants concluded that the AfM_1 mass fraction is less than 0.02 µg/kg. The level of contamination found was generally lower than 0.02 µg/kg or even below the LOD, which had to be at least 0.02 µg/kg at a signal to noise ratio of 3:1.

Remarks concerning the performance of some participants:

Lab 3: The entire set of results was rejected for certification because the within day variation and the variation of the recoveries (spiking) were higher than acceptable.

Lab 5: The results were rejected since the method showed an LOD of more than 0.02 µg/kg.

Lab 9: The whole set of results was rejected since the participant did not attend the certification meeting.

Lab 13: The whole set of results was rejected since the participant did not attend the certification meeting.

On two days lab 2 had detected AfM_1 at a concentrations higher than the LOD of 0.005 µg/kg but lower than the LOQ of 0.008 µg/kg which still guarantees the AfM_1 level to be less than 0.02 µg/kg. All remaining eight labs could not even detect AfM_1 in the milk powder, although their methods employed showed LODs less than 0.02 µg/kg. The individual results are given in Annex D-1.

4.2.2 AfM_1 in milk powder ERM-BD283 (low level)

Remarks concerning the performance of some participants:

Lab 3: The entire set of results was rejected for certification because the within day variation and the variation of the recoveries (spiking) were larger than the acceptance criteria.

Lab 5: The certification measurements showed a high variation. The set of results was not accepted because some of the results were lower than the LOQ of the method.

Lab 7: The recoveries were lower than the acceptance criterion and showed a high variation due to method problems. Therefore, the participant withdrew the results.

Lab 9: The whole set of results was rejected since the participant did not attend the certification meeting.

Lab 11: No data were provided for day 1. Very low recoveries with high variation and certification results with inconsistencies were observed because of serious temperature problems during analysis. The participant withdrew the whole set of results.

Lab 13: The whole set of results was rejected since the participant did not attend the certification meeting.

All remaining seven sets were accepted on technical grounds. The individual results are given in Annex D-2.

4.2.3 AfM_1 in milk powder ERM-BD284 (high level)

Remarks concerning the performance of some participants:

Lab 3: The entire set of results was rejected for certification because the within day variation and the variation of the recoveries (spiking) were larger than the acceptance criteria.

Lab 7: The recoveries were lower than the acceptance criterion and showed a high variation due to method problems. Therefore, the participant withdrew the results.

Lab 9: The whole set of results was rejected since the participant did not attend the certification meeting.

Lab 11: No data were provided for day 1. Very low recoveries with high variation and certification results with inconsistencies were observed because of serious ambient temperature problems during analysis. The participant withdrew the whole set of results.

Lab 13: The whole set of results was rejected since the participant did not attend the certification meeting.

All remaining eight sets were accepted on technical grounds. The individual results are given in Annex D-4.

4.3 Data evaluation

The results for the determination of AfM_1 in ERM-BD283 (low level) and ERM-BD284 (high level) accepted on technical grounds were subjected to the following statistical treatment.

For each accepted set of results (i), the mean value $\bar{x}_i$ was calculated as the arithmetic mean of the individual measurements.

The sets of results found acceptable on technical grounds were submitted to the following statistical tests:

- Kolmogorov-Smirnov-Liliefors test to assess the conformity of the distributions of

individual results and set means to the normal distribution;

- Nalimov test to detect 'outlying' values in the population of the individual results and the population of set means;
- Cochran test to detect 'outlying' values in the laboratory variances (s_i);
- One way analysis of variance (ANOVA) to compare and estimate the between- and within-set components of the overall variance of all individual results.

Application of the F-test to the within and between laboratory variances estimated by ANOVA showed that the variation for AfM_1 in ERM-BD284 (high level) between labs was significantly larger than the within lab variation. The variation between labs were not found to be statistically significant for AfM_1 in ERM-BD283 (low level). Moreover, the Bartlett test showed that the variances were not homogeneous. Therefore, the laboratory mean values (and not the individual results) were used for the calculation of the overall means of the results for AfM_1 in both ERM-BD283 (low level) and ERM-BD284 (high level).

The results of the normality and Nalimov test applied to the laboratory means are reported in Table 4.1 together with the results of the Cochran test on the set variance.

For Cochran and Nalimov tests, a value is an 'outlier' when the hypothesis that it belongs to the population of results considered, can be rejected with a 1 % risk of error. A value is termed a 'straggler' when the risk of erroneous rejection lies between 1 and 5 %.

The certified value was calculated as the arithmetic mean of means ($\bar{x}$) using the number of sets accepted for the certification (p) after both statistical and technical scrutiny. The mean of means $\bar{x}$ is presented as a solid line in Figure 4.1 and Figure 4.2.

The half-with of the 95 % confidence interval of the mean of means $\bar{x}$ with the following limits was calculated as:

$$\bar{x} - t^{\tau}_{1-\alpha/2} \cdot \frac{s}{\sqrt{p}} \quad \text{and} \quad \bar{x} + t^{\tau}_{1-\alpha/2} \cdot \frac{s}{\sqrt{p}}$$

$t^{\tau}_{1-\alpha/2}$ is the value of the Student's t distribution for $\tau = p\text{-}1$ degrees of freedom and a significance level $\alpha = 0.05$; s is the standard deviation of the distribution of the laboratory mean values.

It is estimated as:

$$s = \sqrt{\frac{\sum_{i=1}^{p} \left(\bar{x}_i - \bar{x}\right)^2}{p-1}}$$

The variance of laboratory 2 was found to be an outlier for AfM_1 in milk powder ERM-BD283 (low level). Since the rejection of this result could not be justified from the technical evaluation, the result was retained.

Table 4.1: Summary of the statistical data for AfM_1 in ERM-BD283 (low level) and ERM-BD284 (high level)

	AfM_1 in milk powder	
	ERM-BD283 (low level)	ERM-BD284 (high level)
Number of data sets	7	8
Number of replicate measurements	42	48
Mean of means (µg/kg)	0.111	0.442
SD of means (µg/kg)	0.007	0.021
Standard error of overall mean (=s/$\sqrt{n}$) (µg/kg)	0.0025	0.007
Relative standard error (%)	2.2	1.7
Half-width of the 95 % CI (µg/kg)	0.006	0.018
Outlying or straggling mean values? (Nalimov + Dixon test)	no	no
Outlying or straggling variances? (Cochran test)	Lab 2	no
Variances homogeneous? (Bartlett test)	no (1)	yes
Distribution of means normal? (Kolmogorov-Smirnov-Liliefors test)	no	yes

(1) at 5 % significance.

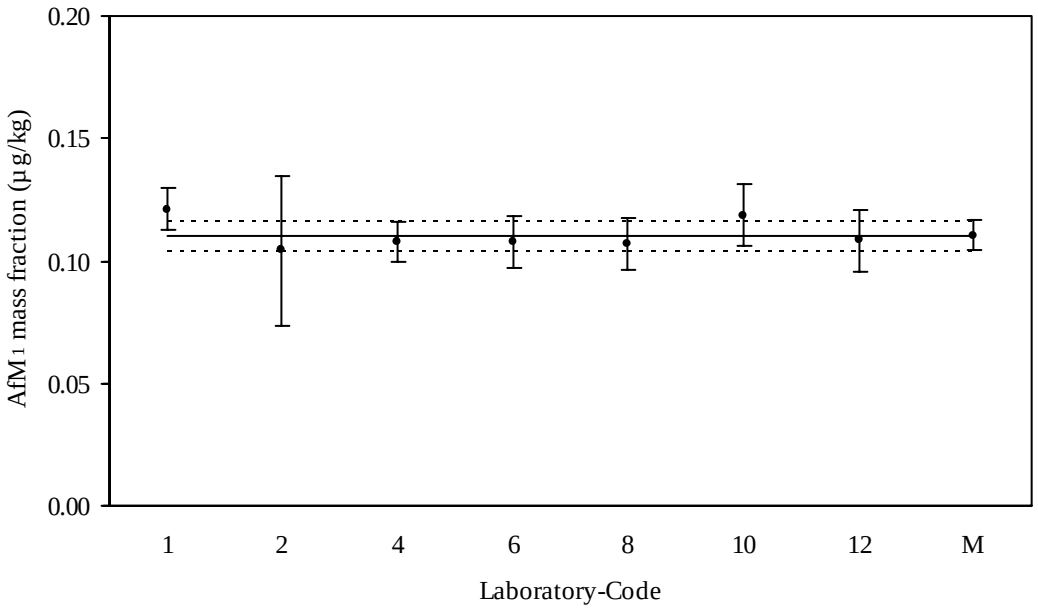


Figure 4.1: Lab means accepted for the certification of AfM_1 in ERM-BD283 (error bars are 95 % CI's of the lab means, dotted lines are 95 % CI of the mean of means (M) of 0.111 µg/kg)

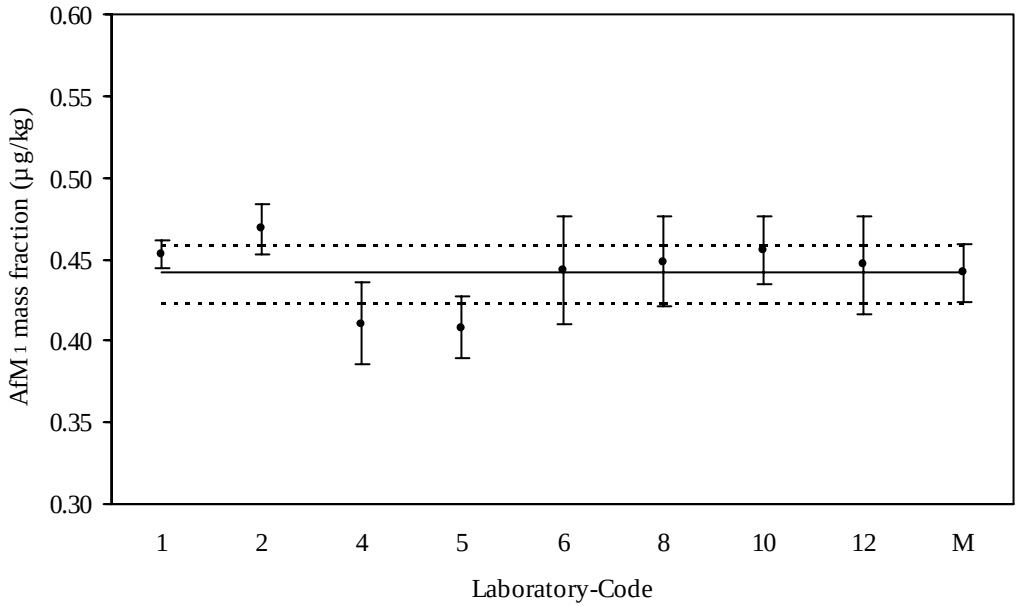


Figure 4.2: Lab means accepted for the certification of AfM_1 in ERM-BD284 (error bars are 95 % CI's of the lab means, dotted lines are 95 % CI of the mean of means (M) of 0.442 µg/kg)

4.4 The certified values and uncertainties

The uncertainty of a CRM can be written as [18]:

$$U_{CRM} = k \cdot \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2 + u_{sts}^2}$$

where U_{CRM} is the expanded uncertainty of the CRM, k is the coverage factor (k = 2), u_{char} is the uncertainty of the certification study, u_{bb} is the between-bottle inhomogeneity, u_{lts} is the uncertainty of long-term stability (storage), and u_{sts} is the uncertainty of the short-term stability (transport).

The standard errors between laboratory means were used as estimation for the uncertainties of the characterisation of the AfM_1 mass fraction of the milk powder materials ERM-BD283 (low level) and ERM-BD284 (high level). Since a common AfM_1 calibrant was used during the characterisation study of ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) the relative standard uncertainty ($u_c(c)$) of 0.13 % as calculated in Josephs et al. [19] should be added as fifth contribution to the combined uncertainties of ERM-BD283 (low level) and ERM-BD284 (high level). However, the uncertainty contribution of the common AfM_1 calibrant has no significant influence on the results of the combined uncertainties of ERM-BD283 (low level) and ERM-BD284 (high level) and was therefore not considered according to paragraph 3.4.4 of the 'GUM' [20].

The standard deviations between bottles were used as estimations of the uncertainties due to possible inhomogeneity (u_{bb}). Uncertainty contributions due to short-term stability (u_{sts}) were assumed to be negligible, as the materials will be shipped at ambient conditions and no degradation is expected to happen during this short time. The estimations of u_{lts} were derived

from isochronous long-term stability studies after 18 months, as explained in section 3.3. Six years was chosen as a proper time of validity of the certificates. A coverage factor of k = 2 was applied to obtain expanded uncertainties. The individual uncertainty components, the combined standard uncertainties and the expanded uncertainties for AfM_1 in ERM-BD283 and ERM-BD284 are presented in Table 4.2. The certified values are valid until October 2007. The validities can be extended, if further stability tests do not indicate degradation. The certified values and the expanded uncertainties for ERM-BD283 (low level) and ERM-BD284 (high level) are summarised in Table 4.3.

Table 4.2: Evaluation of the expanded uncertainties for the AfM_1 content of ERM-BD283 (low level) and ERM-BD284 (high level)

	AfM_1 in milk powder	
	ERM-BD283 (low level)	ERM-BD284 (high level)
u_{char} (%)	2.2	1.7
u_{bb} (%)	0.89	0.77
u_{lts} (%)	7.4	6.3
Combined uncertainty (%)	7.8	6.6
Certified value (µg/kg)	0.111	0.442
Combined uncertainty (µg/kg)	0.0086	0.0291
Expanded uncertainty [1)] (µg/kg)	0.018	0.059
Expanded uncertainty [1)] (%)	15.6	13.2

1) A coverage factor (k = 2) was applied to obtain expanded uncertainties.

It was not possible to certify the AfM_1 content for ERM-BD282 (zero level) because of the very low level of contamination, which was less than 0.02 µg/kg or even below the LODs of less than 0.02 µg/kg of the participating laboratories. It was however possible, to certify the AfM_1 level as being below 0.02 µg/kg (cf. Table 4.3) on the basis of the technical evaluation of the results presented in Annex D-1.

Table 4.3: Certified AfM_1 contents of ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) presented as mass fractions (µg/kg)

BCR No.	Certified value	Uncertainty	No. of accepted sets of results (p)
ERM-BD282	< 0.02 µg/kg	-	9
ERM-BD283	0.111 µg/kg [1)]	0.018 µg/kg [2)]	7
ERM-BD284	0.44 µg/kg [1)]	0.06 µg/kg [2)]	8

1) This value is the unweighted mean of p accepted mean values, independently obtained by p laboratories.

2) The uncertainty is the expanded uncertainty (k = 2) of the value defined in 1).

4.5 Traceabilty

The certified values for the AfM_1 mass fractions of the reference materials are traceable to the common AfM_1 calibrant (AfM_1 concentration 9.9248 µg/mL, standard uncertainty 0.0128 µg/mL) employed by all participants. The relative standard uncertainty of the concentration of the common AfM_1 calibrant $u_c(c)$ was derived on the basis of the results obtained by another interlaboratory study [14]. Only UV spectrophotometers calibrated according to the AOAC Official Method for the preparation of standards for mycotoxins were employed in the interlaboratory study. The calibrant is traceable to the molar absorption coefficient of AfM_1 $\varepsilon = 1995\ m^2/mol$ in chloroform at $\lambda_{max} = 365$ nm and, therefore, to the International System of Units (SI).

5 INSTRUCTIONS FOR USE

5.1 Instructions for the physical handling of the sample

5.1.1 Safety warnings

The milk powder materials ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) are supplied in units of at least 30 g of freeze-dried powders in amber glass bottles filled and sealed under nitrogen.

Aflatoxin M_1 is a known carcinogen and should be handled with extreme caution. The bottles should be used only by personnel who are trained in the safe handling and use of the contents.

Normal safety precautions should be followed and in particular the following. The bottles should be opened inside a safety cabinet or fume cupboard. Normal laboratory safety wear including protective clothing (laboratory coat), safety glasses and gloves should be worn.

5.1.2 Storage and transport

The bottles should be stored unopened at -20 °C or less and shipped under ambient conditions.

5.1.3 Physical handling of the CRMs

The bottles should be allowed to warm to room temperature before opening to avoid water condensation. Before sub-samples are taken the content should be thoroughly mixed. The materials may be used as received or after reconstitution with water to simulate liquid milk. If analysis requires reconstitution of the milk powder by addition of water (8-10 times the weight of the milk powder) the following procedure is recommended:

Weigh 10.0 g of the sample to the nearest into a 250 mL beaker. Add 50 mL water prewarmed to 50 °C in small amounts to the milk powder. Mix using a stirring rod until a homogeneous mixture is obtained. If the milk powder is not completely dissolved, place the beaker in a water bath at 50 °C for at least 30 min. Mix carefully. Allow the solution to cool to 20 °C and then quantitatively transfer it to a 100 mL volumetric flask using small amounts of water. Dilute to the 100 mL mark with water. If necessary, filter enough recostituted milk through filter paper or centrifuge.

The certified values relate to the milk powder as received i.e. no corrections are to be made for the moisture content.

5.1.4 Minimum sample intake

A minimum sample amount of 10 g is recommended when analysing ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) for AfM_1 since an amount of 10 g milk powder was employed during the homogeneity studies.

5.2 Guidelines for the use of the CRM in quality control

If the reference materials are to be used for the verification of an analytical procedure or the performance of a method, the user can refer to the results of this certification exercise after having ascertained that the repeatability of his laboratory method is satisfactory.

The user may assess the laboratory bias from the difference between the mean value of replicate measurements (X) and the certified value (μ): $X - \mu$.

The criterion for acceptance is given in ISO Guide 33 [21] as follows:

$$- a_2 - 2\sigma_L < X - \mu < a_1 + 2\sigma_L$$

in which a_1 and a_2 are the adjusted values, chosen by the user according to economic or technical limitations or stipulations and σ_L is the long-term within-laboratory standard deviation.

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ANNEX A

Summary of methods used for the determination of AfM_1 in milk powder BCR-282R, -283R and -284R

Table A-1: Summary of methods used for the determination of AfM_1 in milk powders BCR-282R, -283R and -284R

Lab-Code	1 [A, B, C]	2 [A, B, C]	4 [A, B, C]	5 [C]
Sample size [g]	10	10	10	10
Solvent composition (v/v)	Water	Chloroform/water (90/10)	Water	Water (warm)
Volume [mL]	100	100	100	80
Type	Shake then stir	Shaker	Magnetic stirrer	Shaker
Duration [min]	30	30	15	
Additional steps	Centrifugation	l/l-extraction	Centrifugation (10000 rpm, 25 min, 10 °C)	Centrifugation
Type of clean-up	AflaprepM (Rhone)	AflaprepM (Rhone)	AflaprepM (Rhone)	Aflatest (Vicam)
Column composition	IAC	IAC	IAC	IAC
Extract volume [mL]	100	-	-	80
Dilution of extract	-	-	-	-
Dilution ratio	-	-	-	-
Volume on column [mL]	50	80 (Water/methanol (90/10))	50	80
Eluent composition (v/v)	Acetonitrile	Methanol	Acetonitrile	Acetonitrile/methanol (60/40)
Volume [mL]	3	1.5 (Diluted with water to 10 mL)	4	1.25
Evaporation	Nitrogen	-	Nitrogen	-
Evaporation temperature [°C]	Ambient	-	50	-
Reconstitution solvent (v/v)	Water/acetonitrile/methanol (50/30/20)	-	Water/acetonitrile (75/25)	Water addition
Volume [mL]	2.5	-	0.4	3
Relative sample amount [g]	5	4.07	5	10
Additional clean-up	-	-	Microfilter 0.4 µm	-
HPLC apparatus	Waters 600 controller, pump and 717 autosampler, Jasco FLD, Agilent Chemstation	Pharmacia LKB, gradient pump 2249	Waters 2690 separations module, Alliance	Agilent HP1050 and 1046A
Type of column	Spherisorb ODS2	Prodigy 5 µ ODS3 100A, Phenomenex	ODS-Hypersil, Agilent	Spherisorb ODS2
Dim (l x id, size) [mm x mm; µm]	250 x 4.6; 5	150 x 4.6; 5	250 x 4; 5	250 x 4.6; 5
Flow conditions	Isocratic	Isocratic	Isocratic	Isocratic
Mobile phase (v/v)	Water/acetonitrile/methanol (50/30/20)	Water/acetonitrile (75/25)	Water/acetonitrile (75/25)	Water/acetonitrile/methanol (60/30/10)
Solvent flow [mL/min]	1.0	1.0	0.9	0.8
Temperature [°C]	20	40	Ambient	Ambient
Injection volume [µl]	150	2500	50	250
Relative sample amount [g]	0.3	1.017	0.625	0.8333
Detector	FLD	FLD	FLD	FLD
Ex. /em. wavelength [nm]	360/430	365/435	360/420	360/429
LOD [µg/kg]	0.0086	0.005	0.02	0.06
Uncertainty [%]*	9.7 / 6.8 (9.88)	10.2 / 7.5 (18)	22.5 / 15.8	n/a / 8.8
Recovery [%]	91	99	78	85.9

[A] accepted for the certification of BCR-282R, [B] accepted for the certification of BCR-283R, [C] accepted for the certification of BCR-284R

* Measurement uncertainties calculated according to Josephs et al. [21] for AfM_1 levels of 0.1 µg/kg and 0.4 µg/kg, respectively; measurement uncertainties provided by laboratories given in brackets.

Table A-2: Summary of methods used for the determination of AfM_1 in milk powders BCR-282R, -283R and -284R

Lab-Code	6 [A, B, C]	7 [A]	8 [A, B, C]	10 [A, B, C]
Sample size [g]	5	10	5	10.00
Solvent composition (v/v)	Water	Chloroform/saturated NaCl (150/2)	Water (50 °C)	Water (30 °C)
Volume [mL]	50	150	50	60 (fill up to 100 mL)
Type	Warming in waterbath (40 °C)	Blender (Ultra Turrax)	Shaker	Stirrer
Duration [min]	10	3 - 4	10	30
Additional steps	Centrifugation (4000 rpm)	Vacuum filter (Whatman No. 4)	Elimination of fat after centrifugation (1500 rpm, 15 min)	Centrifugation (4000 rpm, 30 min)
Type of clean-up	AflaprepM (Rhone)	Aflatoxin EASI-extract (Rhone)	AflaprepM (Rhone)	AflaprepM (Rhone)
Column composition	IAC	IAC	IAC	IAC
Extract volume [mL]	50	100	50	50
Dilution of extract	-	10 g (equivalent)	-	-
Dilution ratio	1	in 100 mL PBS/methanol (98/2)	-	-
Volume on column [mL]	50	50	50	50
Eluent composition (v/v)	Acetonitrile	Acetonitrile	Acetonitrile	Acetonitrile/methanol (60/40)
Volume [mL]	3.5	1.5	4	1.0
Evaporation	Nitrogen	Nitrogen	Nitrogen	-
Evaporation temperature [°C]	35	40 - 50	40	-
Reconstitution solvent (v/v)	Water/acetonitrile/methanol (65/25/10)	Water/acetonitrile (85/15)	Water/acetonitrile (90/10)	dilute with 1.0 mL
Volume [mL]	0.7	4	5	2
Relative sample amount [g]	5	5	5	5
Additional clean-up	-	Chloroform evaporated after filtration, reconstituted and defatted (n-hexane)	-	-
HPLC apparatus	Jasco	Gilson 231 and 401 diluter, Jasco FP1520 FLD	Gilson 307 pump and 234 automatic injection system	Waters 2690, Alliance
Type of column	Luna C18	Spherisorb ODS2-excel (Hichrom)	Chromospher C18	Waters Spherisorb S5 ODS2
Dim (l x id, size) [mm x mm; µm]	250 x 4.6; 5	250 x 4.6; 5	2 x (100 x 4.6; 5)	250 mm x 4.6 mm, 5 µm
Flow conditions	Isocratic	Isocratic	Isocratic	Isocratic
Mobile phase (v/v)	Water/acetonitrile/methanol (65/25/10)	Water/acetonitrile/methanol (60/30/10)	Water/acetonitrile (75/25)	Water/acetonitrile/methanol (50/30/20)
Solvent flow [mL/min]	0.8	1.0	0.5	1.0
Temperature [°C]	40	Ambient	Ambient	30 ± 1 (7 ± 1 sample)
Injection volume [µL]	100	400	100	100
Relative sample amount [g]	0.71	0.5	0.1	0.25
Detector	FLD	FLD	FLD	FLD
Ex./em. wavelength [nm]	365/435	364/434	360/435	360/430
LOD [µg/kg]	0.005	0.02	0.011	0.007
Uncertainty [%]	18.4 / 8.6	(15)	7.7 / 13.8	23.7 / 19.7 (3)
Recovery [%]		70 - 110	80 - 110	80.5 - 87.5

[A] accepted for the certification of BCR-282R, [B] accepted for the certification of BCR-283R, [C] accepted for the certification of BCR-284R

* Measurement uncertainties calculated according to Josephs et al. [21] for AfM_1 levels of 0.1 µg/kg and 0.4 µg/kg, respectively; measurement uncertainties provided by laboratories given in brackets.

Table A-3: Summary of methods used for the determination of AfM_1 in milk powders BCR-282R, -283R and -284R

Lab-Code	11 [A]	12 [A, B, C]
Sample size [g]	10.0	10.0
Solvent composition (v/v)	Water (50 °C)	Water (40 °C)
Volume [mL]	100	50 (Addition of 50 mL)
Type	Stirrer	Shaker
Duration [min]	Until dissolved	30
Additional steps	Centrifugation (4000 rpm, 15 min)	Centrifugation (3000 rpm, 10 min, 20 °C), 1 min in ultrasonic bath
Type of clean-up	Aflatest (Vicam)	AflaprepM (Rhone)
Column composition	IAC	IAC
Extract volume [mL]	50	50
Dilution of extract	-	-
Dilution ratio	-	-
Volume on column [mL]	50	50
Eluent composition (v/v)	Acetonitrile	Acetonitrile
Volume [mL]	4	4
Evaporation	Vacuum	Nitrogen
Evaporation temperature [°C]	30	< 50
Reconstitution solvent (v/v)	Water/acetonitrile (80/20)	Water/acetonitrile (75/25)
Volume [mL]	2	0.250
Relative sample amount [g]	5	5
Additional clean-up	Microfilter 0.45 µm	-
HPLC apparatus	Waters 474 and FLD	Dionex GP40 pump and AS3500 sampler, VDS thermostat, Jasco FP92 FLD
Type of column	Waters Spherisorb ODS2	Discovery C18 (guard column)
Dim (l x id, size) [mm x mm; µm]	250 x 4.6; 5	250 x 4.6; 5 (20 x 4.6; 5)
Flow conditions	Isocratic	Isocratic
Mobile phase (v/v)	Water/acetonitrile (75/25)	Water/acetonitrile (75/25)
Solvent flow [mL/min]	1.2	1.0
Temperature [°C]	20	25
Injection volume [µL]	200	100
Relative sample amount [g]	0.5	2
Detector	FLD	FLD
Ex../em. wavelength [nm]	365/435	365/435
LOD [µg/kg]	0.009	0.02
Uncertainty [%]	(6)	19.0 / 11.6
Recovery [%]	96	91

[A] accepted for the certification of BCR-282R, [B] accepted for the certification of BCR-283R, [C] accepted for the certification of BCR-284R

* Measurement uncertainties calculated according to Josephs et al. [21] for AfM_1 levels of 0.1 µg/kg and 0.4 µg/kg, respectively; measurement uncertainties provided by laboratories given in brackets.

ANNEX B

Individual data of the homogeneity studies

Table B-1: Normalised nitrogen results of the homogeneity study for AfM_1 in milk powder BCR-282R (zero level)

Bottle ID	Nitrogen content (normalised)	
	Replicate 1	Replicate 2
25	1.0054	1.0018
73	1.0020	0.9974
121	0.9994	0.9946
169	1.0008	0.9998
217	1.0008	0.9985
265	1.0016	1.0003
313	1.0035	1.0040
361	1.0001	1.0008
409	0.9963	0.9994
457	0.9974	0.9984
505	0.9974	1.0008
553	1.0020	1.0017
601	0.9974	0.9965
649	1.0020	1.0017
697	1.0012	0.9994
745	1.0008	1.0036
793	1.0012	1.0043
841	0.9997	1.0003
889	0.9989	1.0002
937	1.0001	1.0014
985	0.9974	0.9956
1033	1.0020	0.9971
1081	1.0001	0.9968
1129	1.0027	1.0036
1177	1.0005	0.9991
1225	0.9945	0.9953
1273	0.9925	0.9955
1321	0.9989	0.9975
1369	1.0038	1.0055
1417	1.0038	0.9990
1465	1.0027	1.0032

Table B-2: Normalised results of the homogeneity study for AfM_1 in milk powder BCR-283R (low level)

Bottle ID	AfM_1 mass fraction (normalised)		
	Replicate 1	Replicate 2	Replicate 3
1	1.010	1.000	0.991
47	0.922	1.030	0.959
93	1.006	0.978	0.995
139	1.039	0.951	1.066
185	1.020	0.949	1.005
231	1.020	0.991	0.959
277	0.933	1.014	1.008
323	0.986	0.908	1.001
369	1.037	0.967	1.009
415	0.967	0.972	0.947
461	0.988	0.982	0.966
507	1.007	0.994	0.969
553	1.059	0.994	1.000
599	1.020	0.957	0.982
645	1.025	0.984	1.010
691	0.981	1.006	1.018
737	1.036	1.003	1.018
783	1.026	0.992	1.012
829	0.991	1.011	0.986
875	0.960	1.002	1.068
921	1.060	1.024	1.065
967	1.027	1.018	1.008
1013	0.999	0.969	1.030
1059	0.998	1.007	0.980
1105	0.894	0.990	1.028
1151	1.009	1.009	1.017
1197	1.048	0.977	0.967
1243	0.953	1.083	0.992
1289	0.998	1.079	0.967
1335	1.057	1.008	0.974
1381	1.008	1.030	1.040

Table B-3: Normalised results of the homogeneity study for AfM_1 in milk powder BCR-284R (high level)

Bottle ID	AfM_1 mass fraction (normalised)		
	Replicate 1	Replicate 2	Replicate 3
1	1.012	0.962	0.999
47	0.956	1.045	1.013
93	0.986	1.022	1.002
139	1.060	1.000	0.993
185	1.045	1.023	0.998
231	1.036	1.023	0.926
277	1.001	1.004	1.017
323	1.045	0.976	1.048
369	0.995	1.017	1.009
415	1.063	0.984	0.993
461	1.008	1.013	1.006
507	1.005	1.029	1.029
553	0.992	1.013	1.006
599	1.025	0.974	1.018
645	1.032	1.017	1.043
691	1.006	1.002	0.980
737	0.990	0.985	0.969
783	0.936	1.029	0.985
829	1.033	0.975	1.019
875	1.022	1.020	0.973
921	0.996	0.997	0.993
967	1.011	1.009	0.971
1013	0.960	0.960	0.983
1059	0.987	0.961	0.962
1105	0.999	0.996	0.969
1151	0.908	1.020	0.974
1197	0.996	0.983	1.008
1243	0.999	0.983	0.988
1289	0.988	1.051	1.015
1335	0.970	1.025	0.950
1381	0.923	1.000	1.079

ANNEX C

Individual data of the stability studies

Table C-1: Normalised HPLC-FLD results of the short-term stability studies for AfM_1 in milk powder BCR-283R (low level)

Storage time (weeks)	Temperature (°C)	Bottle ID	AfM_1 mass fraction (normalised)	
			Replicate 1	Replicate 2
0	-70	19	0.96	1.08
0	-70	759	0.95	1.01
1	40	426	0.87	0.96
1	40	1166	0.97	0.95
2	40	204	0.94	1.01
2	40	1314	0.93	0.95
4	40	278	0.97	0.95
4	40	1018	1.02	0.95
1	18	352	1.01	0.96
1	18	1092	1.00	0.95
2	18	94	0.89	0.94
2	18	944	0.98	0.94
4	18	167	0.95	0.96
4	18	1277	0.96	0.95
0	-70	389	n/a	n/a
0	-70	1129	n/a	n/a
1	4	500	n/a	n/a
1	4	1240	n/a	n/a
2	4	241	n/a	n/a
2	4	871	n/a	n/a
4	4	315	n/a	n/a
4	4	1055	n/a	n/a

n/a: not measured

Table C-2: Normalised HPLC-FLD results of the short-term stability studies for AfM_1 in milk powder BCR-284R (high level)

Storage time (weeks)	Temperature (°C)	Bottle ID	AfM_1 mass fraction (normalised)	
			Replicate 1	Replicate 2
0	-70	19	1.04	0.99
0	-70	759	0.97	1.00
1	40	426	1.02	0.99
1	40	1166	1.00	0.97
2	40	204	0.98	0.99
2	40	1315	0.99	0.98
4	40	278	1.01	1.00
4	40	1018	0.97	0.97
1	18	352	1.03	0.99
1	18	1092	1.05	0.96
2	18	94	1.02	1.01
2	18	944	0.99	1.02
4	18	167	1.03	0.94
4	18	1277	1.01	0.97
0	-70	389	n/a	n/a
0	-70	1129	n/a	n/a
1	4	500	n/a	n/a
1	4	1240	n/a	n/a
2	4	241	n/a	n/a
2	4	870	n/a	n/a
4	4	315	n/a	n/a
4	4	1055	n/a	n/a

n/a: not measured

Table C-3: Normalised HPLC-FLD results of the long-term stability studies for AfM_1 in milk powder BCR-283R (low level)

Storage time (months)	Temperature (°C)	Bottle ID	AfM_1 mass fraction (normalised)	
			Replicate 1	Replicate 2
0	-70	131	1.00	1.01
0	-70	685	0.97	1.02
6	4	537	0.97	1.04
6	4	907	0.94	0.91
12	4	463	0.98	0.91
12	4	1203	1.00	1.03
18	4	56	0.96	1.01
18	4	796	0.99	0.94
0	-70	575	1.00	1.00
0	-70	981	1.01	0.99
6	-20	722	1.07	1.02
6	-20	833	0.99	1.02
12	-20	648	1.07	1.00
12	-20	1388	0.99	0.99
18	-20	611	0.98	0.98
18	-20	1351	1.01	0.98

n/a: not measured

Table C-4: Normalised HPLC-FLD results of the long-term stability studies for AfM_1 in milk powder BCR-284R (high level)

Storage time (months)	Temperature (°C)	Bottle ID	AfM_1 mass fraction (normalised)	
			Replicate 1	Replicate 2
0	-70	131	0.99	1.03
0	-70	685	0.98	1.00
6	4	537	1.01	1.02
6	4	907	1.00	1.01
12	4	463	1.01	1.04
12	4	1203	0.98	0.98
18	4	56	0.97	1.10
18	4	796	0.99	0.99
0	-70	575	1.01	1.05
0	-70	981	0.97	0.98
6	-20	722	1.04	1.00
6	-20	833	1.04	1.01
12	-20	648	1.03	1.03
12	-20	1388	1.02	0.99
18	-20	611	1.03	1.02
18	-20	1351	1.01	0.98

n/a: not measured

ANNEX D

Individual data of the certification study

Table D-1: Accepted results for the certification of the AfM_1 content in milk powder BCR-282R (zero level) in µg/kg and not corrected for recovery

Lab-Code	AfM_1 mass fraction (µg/kg)						
	Day 1		Day 2		Day 3		LOD
1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0086
2	<LOD	<LOD	<LOQ*	<LOQ*	<LOQ*	<LOQ*	0.005
4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.02
6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.005
7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.02
8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.011
10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.007
11	n/a	n/a	<LOD	<LOD	<LOD	<LOD	0.009
12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.02

n/a: not measured

*: AfM_1 mass fractions of more than LOD of 0.005 µg/kg, but still less than LOQ of 0.008 µg/kg were observed.

Table D-2: Accepted results for the certification of the AfM_1 content in milk powder BCR-283R (low level) in µg/kg corrected for recoveries

Lab-Code	AfM_1 mass fraction (µg/kg)							
	Day 1		Day 2		Day 3		Overall mean	1 SD
1	0.109	0.115	0.125	0.125	0.130	0.123	0.121	0.008
2	0.0764	0.0594	0.1303	0.1253	0.1165	0.1168	0.104	0.029
4	0.0985	0.0989	0.1154	0.1082	0.1152	0.1107	0.108	0.008
6	0.098	0.094	0.110	0.108	0.122	0.113	0.108	0.010
8	0.098	0.094	0.104	0.117	0.108	0.119	0.107	0.010
10	0.115	0.110	0.141	0.123	0.109	0.114	0.119	0.012
12	0.095	0.092	0.119	0.109	0.119	0.115	0.108	0.012

Table D-3: Accepted recovery rates in % obtained by the participants during the certification exercise after spiking milk powder BCR-282R (zero level) at AfM_1 levels of 0.1 µg/kg and subsequent to the subtraction of possible 'blank' values

Lab-Code	AfM_1 recovery rates (%)										
	Day 1			Day 2			Day 3			Overall mean	1 SD
1	84.6	85.7	87.6	77.0	80.4	84.7	75.9	74.8	75.3	80.7	5.1
2	94.8	99.2	99.2	83.3	88.9	87.3	88.8	89.4	89.7	91.2	5.4
4	88.2	83.7	81.3	71.9	66.1	70.7	67.5	59.7	59.7	72.1	10.3
6	72	69	99	76	70	83	75	69	69	75.8	9.9
8	93.1	102.7	95.6	103.7	96.5	98.8	97.3	101.5	96.8	98.4	3.5
10	101.8	105.2	111.9	67.6	77.7	77.0	88.6	106.8	100.0	93.0	15.7
12	70.0	69.1	71.0	77.6	83.5	74.5	94.9	86.0	85.9	79.2	8.9

Table D-4: Accepted results for the certification of the AfM_1 content in milk powder BCR-284R (high level) in µg/kg corrected for recoveries

Lab-Code	AfM_1 mass fraction (µg/kg)							
	Day 1		Day 2		Day 3		Overall mean	1 SD
1	0.459	0.445	0.451	0.458	0.442	0.462	0.453	0.008
2	0.4777	0.4591	0.4791	0.4843	0.4456	0.4654	0.469	0.015
4	0.414	0.411	0.420	0.397	0.374	0.446	0.410	0.024
5	0.390	0.419	0.429	0.419	0.382	0.411	0.408	0.018
6	0.407	0.432	0.462	0.477	0.408	0.472	0.443	0.032
8	0.459	0.443	0.418	0.421	0.483	0.468	0.449	0.026
10	0.455	0.444	0.427	0.479	0.478	0.449	0.455	0.020
12	0.413	0.458	0.431	0.422	0.475	0.480	0.447	0.028

Table D-5: Accepted recovery rates in % obtained by the participants during the certification exercise after spiking milk powder BCR-282R (zero level) at AfM_1 levels of 0.4 µg/kg and subsequent to the subtraction of possible 'blank' values

Lab-Code	AfM_1 recovery rates (%)										
	Day 1			Day 2			Day 3			Overall mean	1 SD
1	91.4	91.2	94.0	93.9	96.8	95.3	85.9	84.7	85.9	91.0	4.5
2	98.9	105.0	102.5	92.6	91.4	85.2	88.1	86.9	84.6	92.8	7.6
4	77.3	89.1	91.7	70.9	73.1	63.5	68.0	77.9	70.0	75.7	9.4
5	84.0	78.2	76.4	84.4	91.2	90.3	83.1	76.7	78.4	82.5	5.6
6	70	68	74	85	79	73	70	69	77	73.9	5.6
8	79.0	79.0	79.8	98.0	100.5	100.2	89.7	86.4	87.3	88.9	8.9
10	101.9	102.9	101.7	73.7	75.8	76.8	84.8	79.5	85.9	87.0	12.0
12	71.1	70.0	73.1	79.9	82.8	69.8	75.8	77.3	66.6	74.0	5.3

EUR 21202 – DG Joint Research Centre, Institute for Reference Materials and Measurements –
The certification of the mass fraction of aflatoxin M_1 in whole milk powders –
ERM®-BD282 (zero level), ERM®-BD283 (low level), ERM®-BD284 (high level)
Authors: R.D. Josephs, R. Koeber, A. Bernreuther, H. Schimmel, F. Ulberth
Luxembourg: Office for Official Publications of the European Communities
2005 – 36 pp. – 21.0 x 29.7 cm
Scientific and Technical Research series
ISBN 92-894-7684-2

Abstract

The mycotoxin aflatoxin M_1 (AfM_1) is a serious food safety hazard for which the European Commission has already established a maximum permissible level of 0.05 µg/kg AfM_1 in milk and milk products.

This report describes the preparation of three whole milk powder reference materials ERM-BD282 (zero level), ERM-BD283 (low level) and ERM-BD284 (high level) and the certification of their individual aflatoxin M_1 mass fractions. ERM-BD282, ERM-BD283, ERM-BD284 were originally certified as BCR-282R, BCR-283R, BCR-284R.

The certified values were calculated as the unweighted arithmetic mean values of results delivered by a number of experienced labaoratories participating in a collaborative characterisation exercise. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties due to possible inhomogeneity and instability. The certified values are listed below:

ERM No.	Certified value	Uncertainty	No. of accepted sets of results (p)
ERM-BD282	< 0.02 µg/kg	-	9
ERM-BD283	0.111 µg/kg [1)]	0.018 µg/kg [2)]	7
ERM-BD284	0.44 µg/kg [1)]	0.06 µg/kg [2)]	8

1) This value is the unweighted mean of p accepted mean values, independently obtained by p laboratories.
2) The uncertainty is the expanded uncertainty (k = 2) of the value defined in 1).

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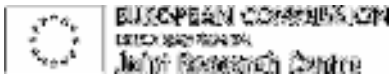

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