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Aflatoxin M_1 in milk powders: Processing, homogeneity and stability testing of certified reference materials

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Abstract
As part of the certification campaign of three candidate reference materials for the determination of aflatoxin M_1 (AfM_1) in whole milk powders, homogeneity, short- and long-term stability tests of naturally contaminated milk powders have been performed. The homogeneity of two AfM_1-contaminated milk powders was studied by taking samples at regular intervals of the filling sequences and analysing in triplicate for their AfM_1 contents by liquid chromatography with fluorescence detection (LC-FLD) using random stratified sampling schemes. The homogeneity testing of an AfM_1 'blank' milk powder material was performed by determining the nitrogen content because AfM_1 levels were below the limit of detection of the most sensitive determination method. The short-term stability of AfM_1-contaminated milk powders was evaluated at three different storage temperatures (4, 18 and 40°C). After storage times of 0, 1, 2 and 4 weeks, samples were investigated using LC-FLD. The long-term stability study comprised of measurements after 0, 6, 12 and 18 months after storage at −20 and 4°C. Analyses were done by LC-FLD. Based on the homogeneity tests, the materials were sufficiently homogenous to serve as certified reference materials. Corresponding uncertainty contributions of 0.23–0.89% were calculated for the homogeneity. The stability measurements showed no significant trends for both short- and long-term stability studies. The long-term stability uncertainties of the AfM_1-contaminated milk powders were 7.4 and 6.3%, respectively, for a shelf-life of 6 years and storage at −20°C. Supplementary stability monitoring schemes over a long period of several years are currently ongoing.

Keywords: *Aflatoxin M_1, mycotoxin, homogeneity, stability, milk powder, certified reference material (CRM).*

Introduction

Aflatoxins are a group of mycotoxins that are of greatest significance for the safety of food and feedstuffs. They are mainly produced by the moulds of the genus *Aspergillus* both pre- and post-harvest at relatively high moisture contents and temperatures. They can occur in various agricultural commodities that enter the food chain either directly or are used for the production of animal feedstuffs.

The main naturally occurring aflatoxins are B_1, B_2, G_1 and G_2. Aflatoxin B_1 is widely regarded as the most potent liver carcinogen known for a wide variety of mammalian species, including humans (Council for Agricultural Science and Technology 2003).

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] is a hydroxylated derivative of AfB_1 that is formed and excreted in the milk of lactating animals after the ingestion of AfB_1 contaminated feed (Van Egmond 1989). 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 (International Agency for Research on Cancer 1993) 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 AfM_1 as a food safety hazard is reflected in the existence of regulations controlling

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the maximum limits for AfM_1 in milk and milk products in about 60 countries (Food and Agriculture Organization of the United Nations 2004). The European Commission (EC) has established a maximum permissible level of $0.05\,\mu g\,kg^{-1}$ AfM_1 in milk and in milk for the manufacture of milk-based products and heat-treated milk (European Commission 2001). Quite recently, this regulation was amended including maximum permissible levels of $0.025\,\mu g\,kg^{-1}$ AfM_1 for infant formulae, follow-on formulae and dietary foods (European Commission 2004). The regulation has foreseen to reduce the maximum level to $0.01\,\mu g\,kg^{-1}$ AfM_1 if a reliable analytical determination in this concentration range is possible.

Analytical difficulties and the economic importance of controlling AfM_1 maximum levels in milk and dairy products already led to the production of a series of CRMs for AfM_1 in whole milk powder in 1992 (Van Egmond and Wagstaffe 1992) and an AfM_1 calibrant RM in 1999 (Van Egmond et al. 1999). These activities were funded by the Measurements & Testing Programme of the EC. Because of the high acceptance of these materials by the analytical community and the need to ensure the comparability and traceability of measurements at lower analyte levels, it was required to produce three new milk powder materials (ERM-BD282, -BD283 and -BD284) containing different AfM_1 mass fraction levels in accordance with the new legal requirements.

The aim of this paper is to provide a detailed description of the material processing, homogeneity, and short- and long-term stability testing of the candidate reference materials. The stability studies were carried out as 'isochronous measurements' (Lamberty et al. 1998). No isochronous stability studies of AfM_1 in milk powder have previously been published. Only a rough idea of the stability could be gained from the studies undertaken by Van Egmond and Wagstaffe (1992). However, accurate knowledge about the uncertainty contributions of the homogeneity (u_{bb}), short-term (u_{sts}) and long-term (u_{lts}) stability to the combined uncertainty of the candidate CRMs for AfM_1 in milk powder is of crucial importance (Van der Veen et al. 2001). Moreover, u_{lts} is associated with the attribution of a certain shelf-life as required by ISO Guides 31 and 34 (International Organization for Standardization 1998, 2000) for storage under specific conditions. The calculation of the uncertainty contributions of the homogeneity, short- and long-term stability, and the determination of the particular shelf-lives are described and discussed below.

Materials and methods

Processing of the materials

Three new candidate milk powder materials for AfM_1 were produced. ERM-BD282 (zero level) is supposed to be a blank material, a whole milk powder with a very low (non-detectable) content of AfM_1. ERM-BD283 (low level) and ERM-BD284 (high level) are whole milk powders containing about 0.1 and $0.4\,\mu g\,kg^{-1}$ AfM_1, respectively, corresponding to about 0.01 and $0.04\,\mu g\,kg^{-1}$ if reconstituted to liquid milk.

ERM-BD282 (zero level) was prepared from a non-contaminated whole milk powder material pre-tested to contain less than $0.02\,\mu g\,kg^{-1}$ AfM_1. For the preparation of ERM-BD283 (low level) and -BD284 (high level), the same base material was used, which was blended with a highly contaminated material pre-checked to contain $87\,\mu g\,kg^{-1}$ AfM_1. Both non-contaminated and highly contaminated base materials were provided by a dairy cooperative.

For the preparation of ERM-BD283 and -BD284, 45 kg non-contaminated milk powder were added in small portions to 90 litres demineralized water in a 200-litre container under constant stirring according to the scheme presented in Figure 1. As soon as the milk powder was reconstituted completely, an emulsion of 65 g contaminated milk powder dispersed in 500 ml demineralized water (ERM-BD283) and 260 g contaminated milk powder dispersed in 2000 ml demineralized water (ERM-BD284) were added. The mixtures were stirred for another 30 min, transferred to 40 trays (size of one tray: $450 \times 300 \times 30$ mm), and then frozen. The freeze-drying was carried out on two sub-batches according to Figure 1. Lyophilization was performed in an Epsilon freeze-dryer (Christ, Germany) for 100–120 h with a pressure gradient between 1000 and 0.004 mbar. The coolant temperature ranged between -40 and $20^{\circ}C$. 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 homogenized for 30 min in a Turbula mixer.

ERM-BD282 (zero level) was prepared in the same way by use of 47 kg non-contaminated milk powder and 94 litres demineralized water.

The milk powders were bottled manually (30 g units) into 100-ml amber glass bottles in a glove box under a dry nitrogen atmosphere. The total amounts produced were 1477 bottles for ERM-BD282, 1414 bottles for ERM-BD283 and 1421 bottles for ERM-BD284. After bottling, the batches were stored at $-20^{\circ}C$ and sufficient aliquots were stored at a reference temperature of $-70^{\circ}C$.

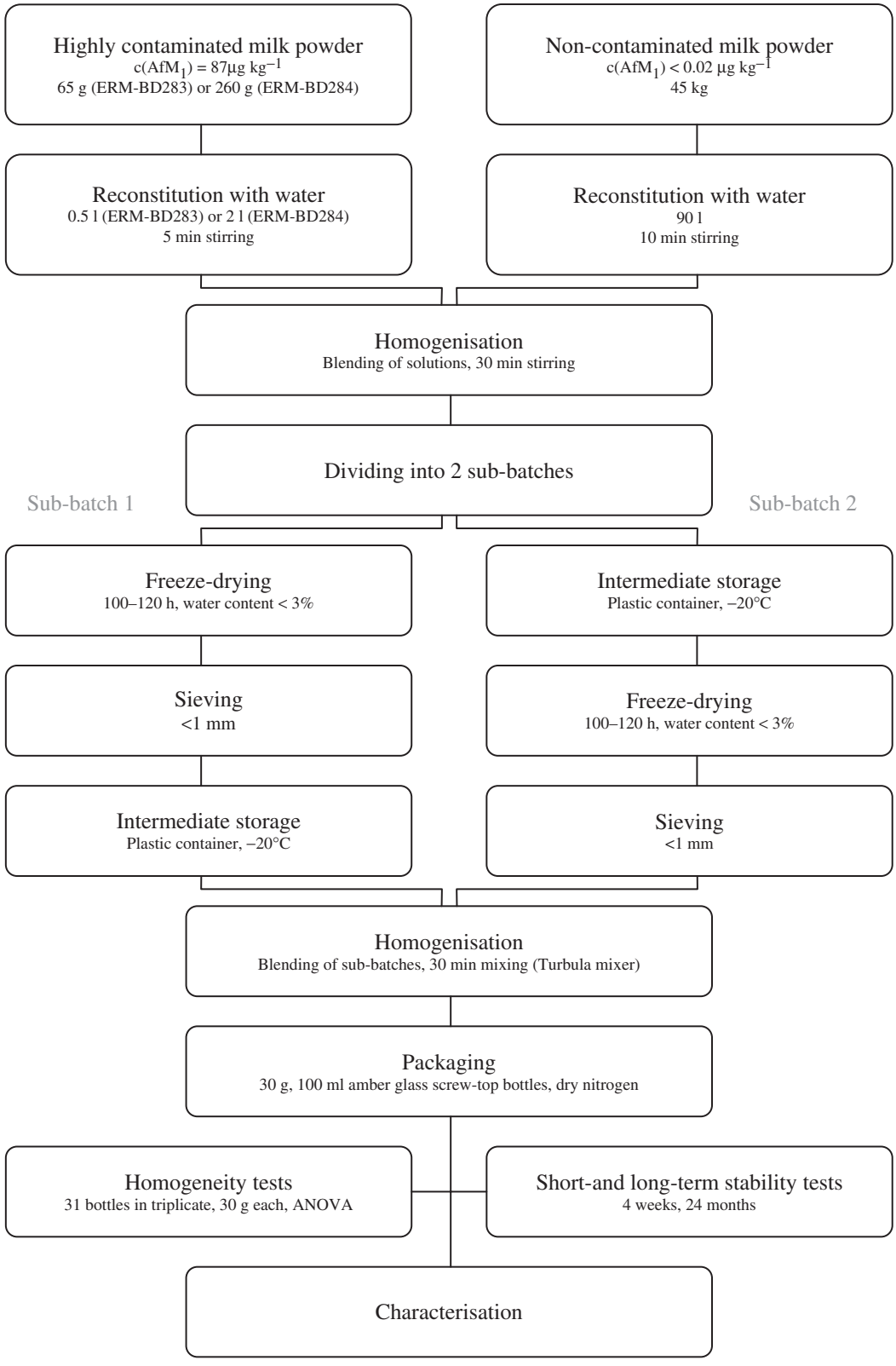

Figure 1. Processing and testing scheme of AfM_1 in milk powder ERM-BD283 and -BD284.

AfM_1 determination

Before analysis, the bottles were allowed to reach room temperature before opening. For the determination of AfM_1 milk powder, 10.0 g were mixed with celite (10.0 g) and glass pearls (4 g). The mixture was extracted with 100 ml chloroform and 10 ml distilled water by shaking on a horizontal table

shaker for 30 min. The extract was filtered through a Macherey-Nagel $617\frac{1}{4}$ 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 methanol and 60 ml *n*-pentane. Liquid–liquid extraction was performed in three separation steps of 30 s employing additional 40 ml water and 50 ml *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).

An AflaprepM immuno-affinity column (IAC) (Rhone Diagnostic Technologies Ltd, United Kingdom) with a pre-connected solvent reservoir was placed on an SPE station (VacMaster, IST, VA, USA). The diluted well-mixed filtrate was filled into the solvent reservoir and passed through the IAC with a flow rate of about one drop per 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 twice 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 was well mixed.

An aliquot of the well-mixed eluate (2.5 ml) was injected automatically in the LC system. The LC 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, vv) as the mobile phase at a flow rate of 1.0 ml min^{-1} (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^{-1} AfM_1, expanded measurement uncertainty (U) of 10% (at about 0.1 µg kg^{-1} AfM_1) and an average recovery rate of 99%. This method was thoroughly scrutinized in several interlaboratory comparison studies (preliminary, homogeneity, stability and characterization studies) within the scope of the certification campaign of the first series of CRMs (BCR-282, -283, -284 and -285) for AfM_1 in milk powders (Van Egmond and Wagstaffe 1992).

Nitrogen determination

The Kjeldahl nitrogen measurements were performed in duplicate ($n = 2$) employing a digestion method (macro method) according to the IDF 20B/2 standard (International Dairy Federation 1993). Before analysis, the bottles were allowed to reach room temperature before opening.

Outline of the homogeneity studies

The homogeneity of CRMs was tested by use of highly repeatable methods and its uncertainty contributions were evaluated by an ANOVA approach (Linsinger et al. 2001a), which allowed the separation of the method variation (s_{wb}) from the experimental variation of the averages over one sample unit ($u_{c,bb}$) to obtain an estimation for the real variation between sample units (s_{bb}):

$$u^2_{c,bb} = s^2_{bb} + \frac{s^2_{wb}}{n} \quad (1)$$

The standard deviation between the sample units was used as the estimator for the between-units variance. The measurement variation sets a lower limit u^*_{bb} to this estimator:

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} 4 \sqrt{\frac{2}{\nu_{MSwithin}}} \quad (2)$$

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

In case of the homogeneity measurements of both AfM_1 contaminated milk powder materials ERM-BD283 and -BD284, 31 bottles taken at regular intervals from the filling sequence were analysed in triplicate ($n = 3$) in randomly stratified order for the AfM_1 content by use of the LC-FLD method described above.

The homogeneity testing of ERM-BD282 was performed by determination of the Kjeldahl nitrogen because there was no indication for AfM_1 contamination based on preliminary analyses. Therefore, 31 bottles taken at regular intervals from the filling sequence were analysed in duplicate ($n = 2$) in randomly stratified order for the nitrogen content.

Outline of the stability studies

The objective for the short-term stability studies of AfM_1 in both contaminated milk powder materials ERM-BD283 and -BD284 was to evaluate whether special care must be taken during transport. Short-term stability of AfM_1 in ERM-BD283 and -BD284 was evaluated under three different conditions (4, 18 and 40°C) and four storage periods of 0, 1, 2 and 4 weeks. The stability studies

were carried out as 'isochronous measurements' (Lamberty et al. 1998). Hence, the bottles were removed after their allocated storage times at the temperature conditions mentioned above and set to −70°C (two bottles at each time and temperature). The collected samples were then analysed together after 4 weeks for the short-term stability to enable measurements repeatability conditions using the LC-FLD method described above.

The stabilities at certain storage temperatures were assessed by the long-term stability studies. Therefore, AfM_1 in ERM-BD283 and -BD284 was evaluated under two different conditions (−20 and 4°C) and at longer storage periods of 0, 6, 12 and 18 months. The long-term stability studies were also carried out as isochronous measurements as already explained for the short-term stability studies. The collected samples were then analysed together after 18 months for the long-term stability studies under repeatability conditions using the LC-FLD method described above.

Statistical analyses of the results of all stability studies were carried out to investigate if there were significant trends in the AfM_1 mass fraction due to storage (Linsinger et al. 2001b). 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 to derive an estimate for the uncertainty due to possible instability of the materials.

Additionally, statistical analyses were performed to investigate if there were any significant trends due to the filling sequence or analysis sequence of the bottles.

Results and discussion

Homogeneity studies

The final homogeneity measurements for ERM-BD282 were subjected to ANOVA. 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 with typical method repeatabilities of more than 10% for the determination of AfM_1 in milk powders (Josephs et al. 2004). Nevertheless, the determination of the homogeneity by use of the nitrogen content can only serve as an estimate because it was not possible to determine any AfM_1 in the milk powder ERM-BD282.

Table I. Results of the homogeneity testing of ERM-BD282, -BD283 and -BD284.

	Protein content ERM-BD282	AfM_1 content	
		ERM-BD283	ERM-BD284
Mean of means	26.50 g 100 g^{-1}	0.107 µg kg^{-1}	0.425 µg kg^{-1}
SD	0.07 g 100 g^{-1}	0.003 µg kg^{-1}	0.010 µg kg^{-1}
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
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.

The ANOVA results of the homogeneity testing for ERM-BD282 are summarized in Table I together with the outcome of the homogeneity testing of ERM-BD283 and -BD284. Additionally, linear regression analyses were carried out for the normalized results in order to detect effects due to sequences of filling and analysis. The slopes of the lines were tested for significance at a 95% confidence level, but no significant trends were observed. The normalized results due to the filling sequence for ERM-BD282 are shown in Figure 2.

After packaging, the homogeneity measurements for the AfM_1 in both contaminated milk powders ERM-BD283 and -BD284 were evaluated. The individual data of the homogeneity study were normalized with respect to the averages of the analytical sub-sequences to ensure repeatability conditions. The results of the ANOVA are also summarized in Table I. No difference in the within- and between-sample variances could be detected by the *F*-test at the 95% confidence level. The materials could be regarded as homogeneous. In both cases, the s_{bb} could not be calculated because $MS_{between}$ was smaller than MS_{within}. Therefore, the u^*_{bb} of 0.89 and 0.77% was adopted for ERM-BD283 and -BD284, respectively, 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 was observed for the filling order of ERM-BD283, which is already fully covered by the uncertainty due to possible inhomogeneity (u_{bb})

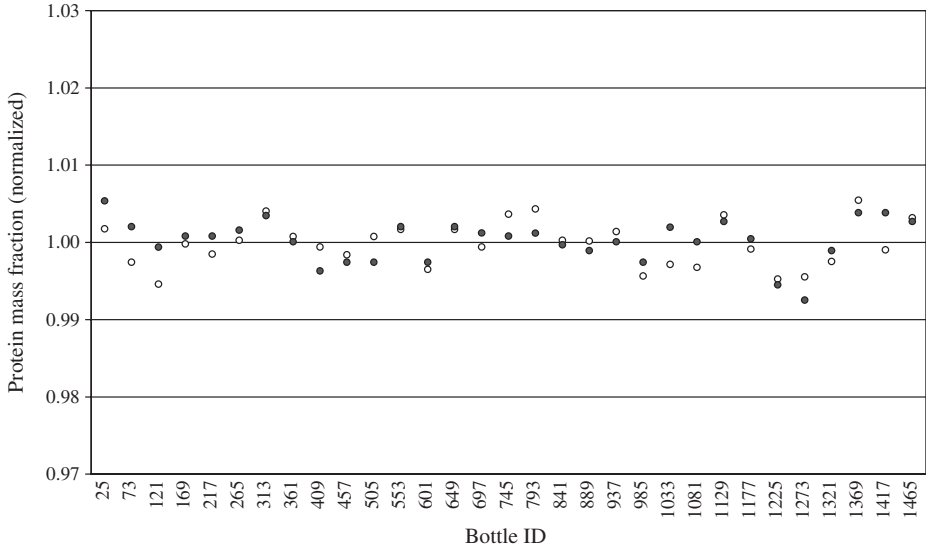

Figure 2. AfM_1 in milk powder ERM-BD282 (zero level): normalized protein mass fractions of the homogeneity study–filling sequence.

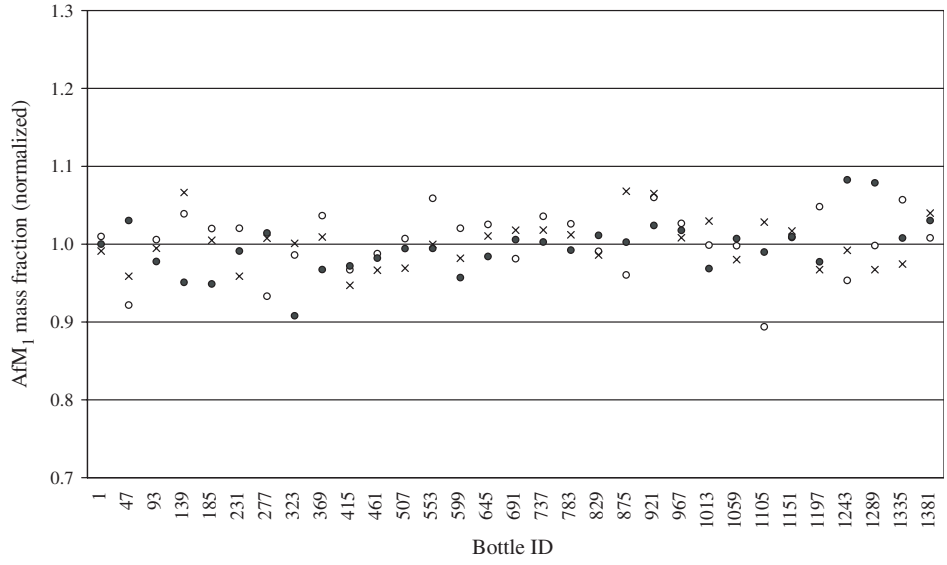

Figure 3. AfM_1 in milk powder ERM-BD283 (low level): normalized results of the homogeneity study–filling sequence.

of 0.89%. The normalized results due to the filling sequences for ERM-BD283 and -BD284 are shown in Figures 3 and 4.

The uncertainty contributions due to possible inhomogeneity (u_{bb}) to the combined uncertainties of ERM-BD282, -BD283 and -BD284 were 0.23, 0.89 and 0.77% (Table I). 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 ERM-BD282, -BD283 and -BD284.

Stability studies

The measurements of the short- and long-term stability studies of AfM_1 in ERM-BD283 (low level) and -BD284 (high level) were evaluated statistically. The results of the short-term stability studies conducted at 18 and 40°C for AfM_1 in ERM-BD283 and -BD284 are shown in Figures 5 and 6.

All AfM_1 mass fractions were normalized with respect to the average mass fraction of the reference samples (stored at −70°C, week 0). The analyses of the isochronous measurement schemes at 4°C were omitted because AfM_1 in milk powder ERM-BD283 and -BD284 was already found to be stable for 4 weeks at higher temperatures of 18 and 40°C. No trends were observed for each of the storage conditions, filling sequences or analysis sequences of both materials. The slopes were non-significant at a 95% confidence level.

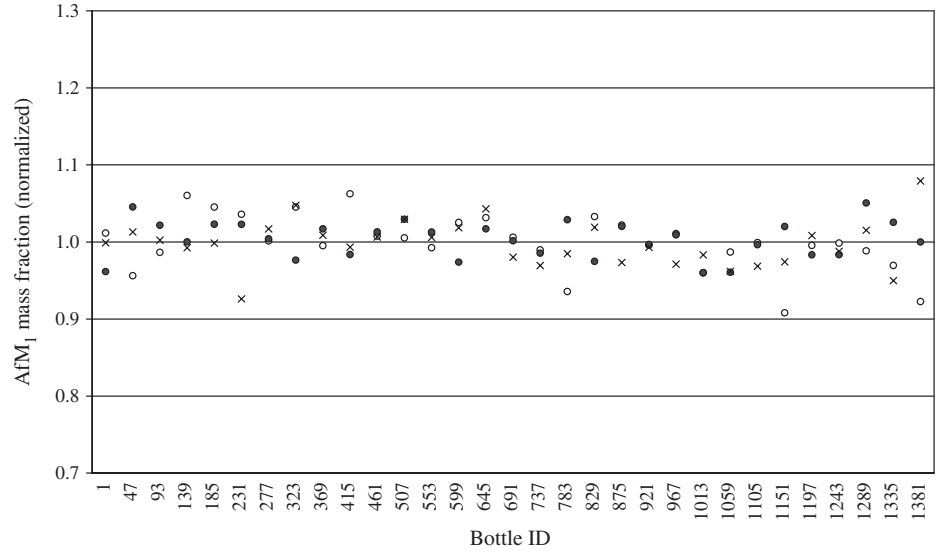

Figure 4. AfM_1 in milk powder ERM-BD284 (high level): normalized results of the homogeneity study–filling sequence.

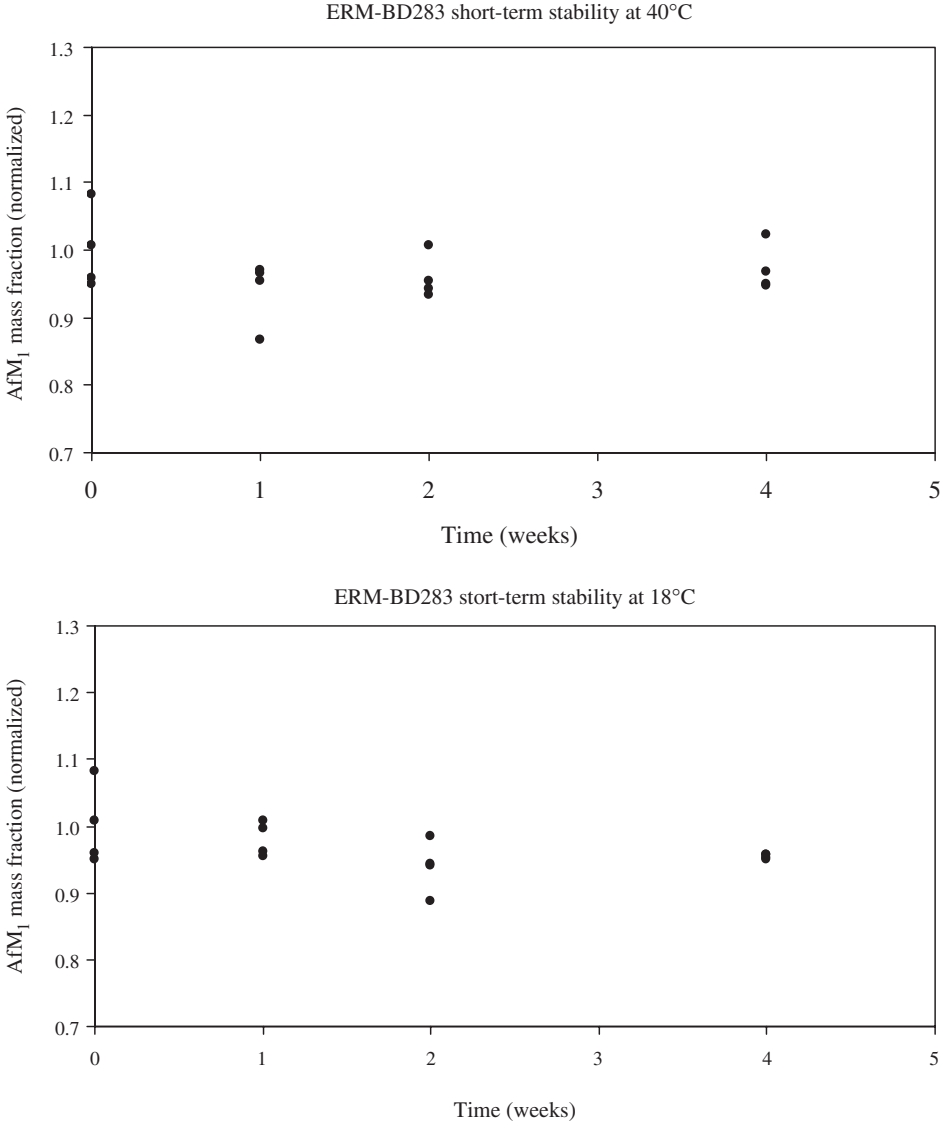

Figure 5. AfM_1 in milk powder ERM-BD283 (low level): normalized results of the short-term stability studies at 40 and 18°C.

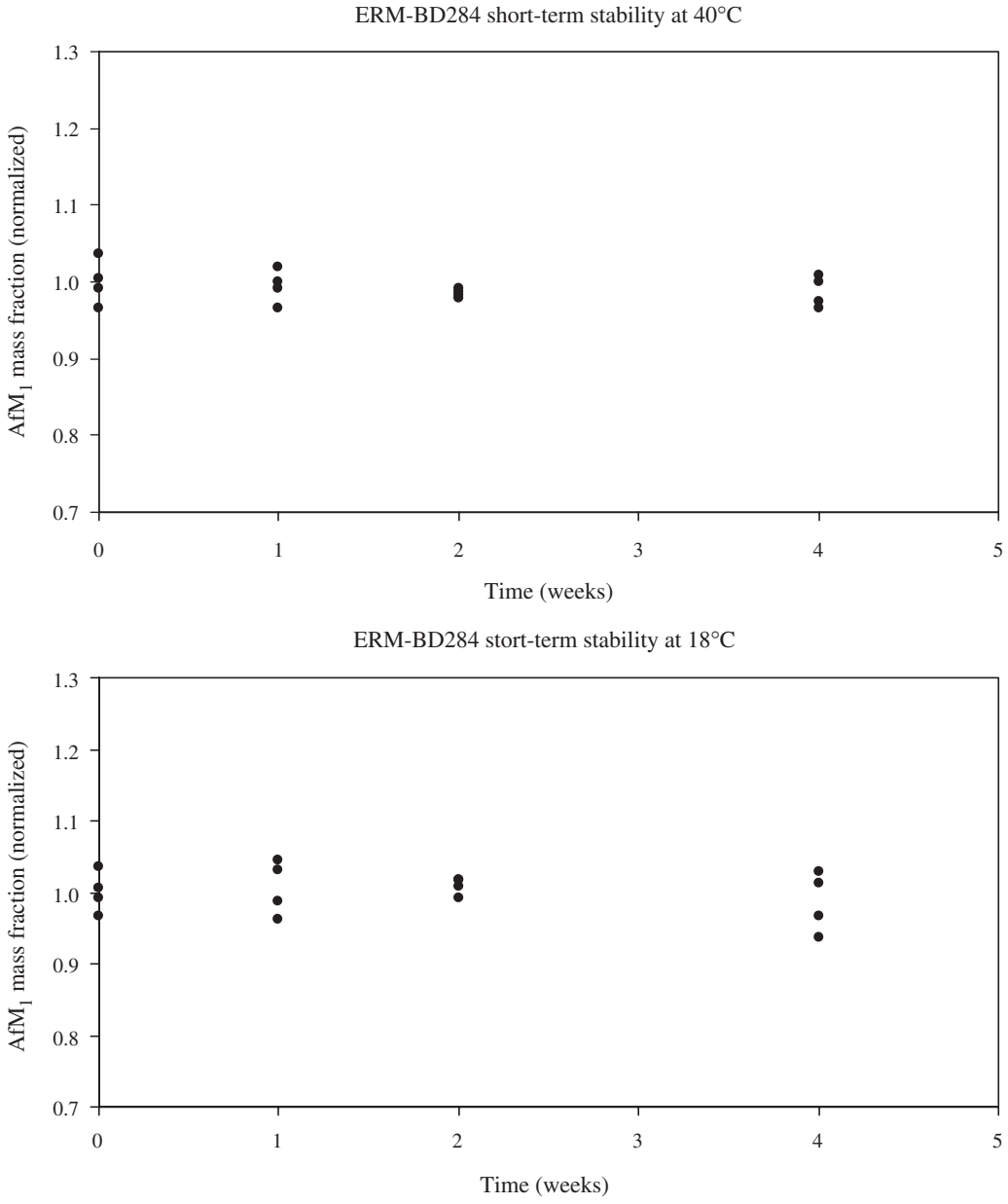

Figure 6. AfM_1 in milk powder ERM-BD284 (high level): normalized results of the short-term stability studies at 40 and 18°C.

The stability of AfM_1 in milk powder ERM-BD283 and -BD284 at certain storage temperatures was assessed by long-term stability studies. The normalized results for the long-term stability studies of both materials conducted at 4 and −20°C are shown in Figures 7 and 8.

All AfM_1 mass fractions were normalized with respect to the average mass fractions of the reference samples (stored at −70°C, week 0). Normalization 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 statistically non-significant. Therefore, the standard error of the normalized values was used to estimate the uncertainties. Dotted lines in the long-term stability study show the uncertainty of the certified value of the CRM due to possible instability. Uncertainties of long-term stability studies (u_{lts}) for given shelf-lives of 2, 4, 5 and 6 years are presented in Table II.

In general, the determination of AfM_1 in ERM-BD283 and -BD284 employing LC-FLD showed no significant trends for both short- and long-term stability studies. For AfM_1 in ERM-BD282, no stability studies were performed with respect to the AfM_1 content, because AfM_1 was not detectable ($c(AfM_1) < 0.02\ \mu g\ kg^{-1}$). In addition, an increase of AfM_1 cannot be expected due to

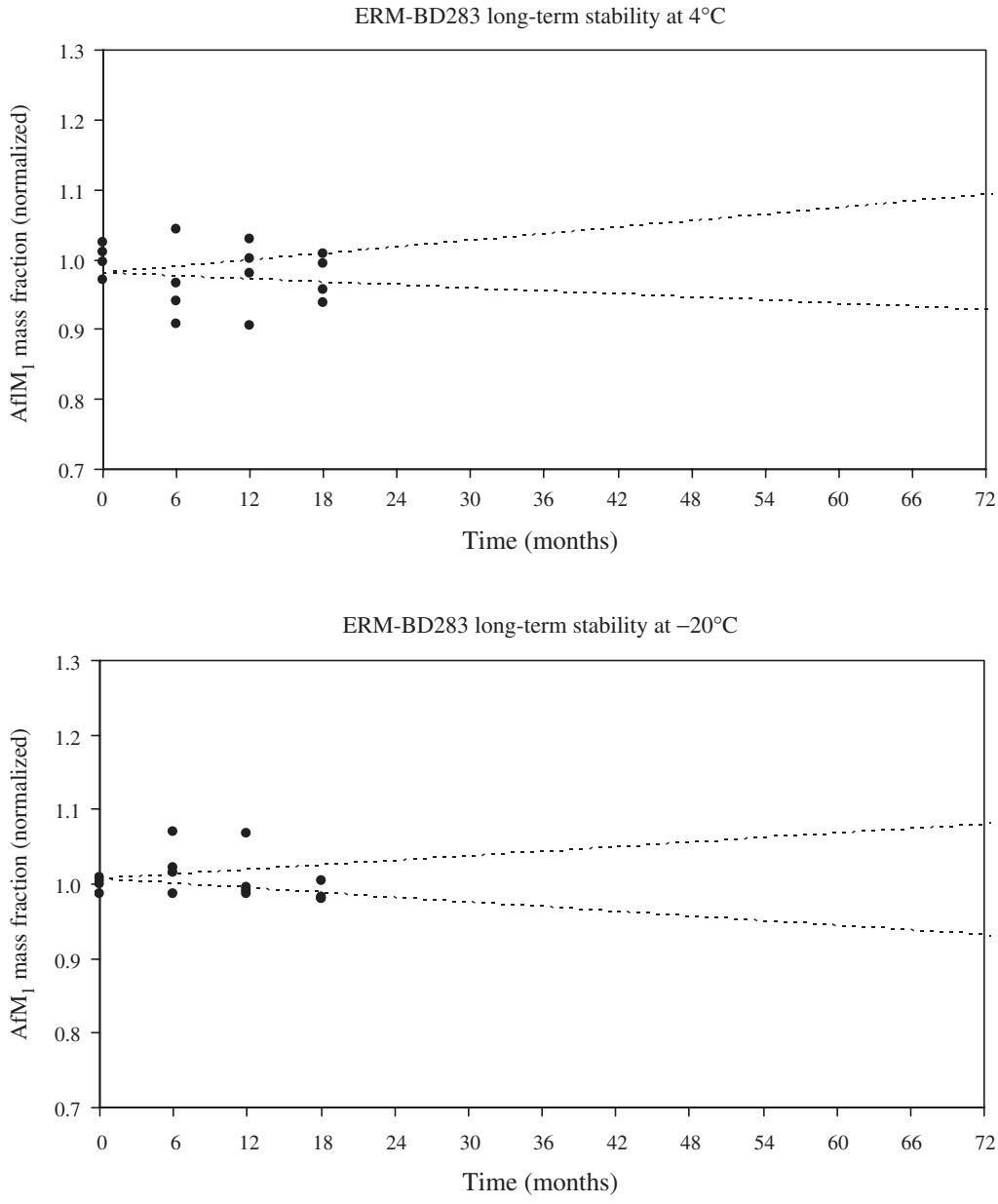

Figure 7. AfM_1 in milk powder ERM-BD283 (low level): normalized results of the long-term stability studies at 4 and −20°C.

the specific mechanism of its formation. Therefore, ERM-BD282 will be stored and shipped under the same conditions as ERM-BD283 and -BD284.

Based on the results of the short-term stability studies ERM-BD282, -BD283 and -BD284 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 was decided to store ERM-BD282, -BD283 and -BD284 at a temperature of −20°C resulting in lower u_{lts} compared with storage at 4°C. This will also result in lower combined uncertainties because u_{lts} is one of the major contributors to the combined uncertainty of certified property values. Consequently, the u_{lts} for AfM_1 in both milk powders ERM-BD283 and -BD284 amounts to 7.4 and 6.3%, respectively, for a shelf-life of 6 years. The validity of the certificates can be prolonged if additional data are obtained from post-certification stability monitoring. Stability monitoring schemes over a long period of several years are currently ongoing. For ERM-BD283 and -BD284, a post-certification monitoring for 9 years has been planned. The materials will be tested for the AfM_1 content employing three isochronous designs at times of 0, 12, 24 and 36 months, 0, 24, 48 and 72 months, as well as 0, 36, 72 and 108 months for −20°C, respectively.

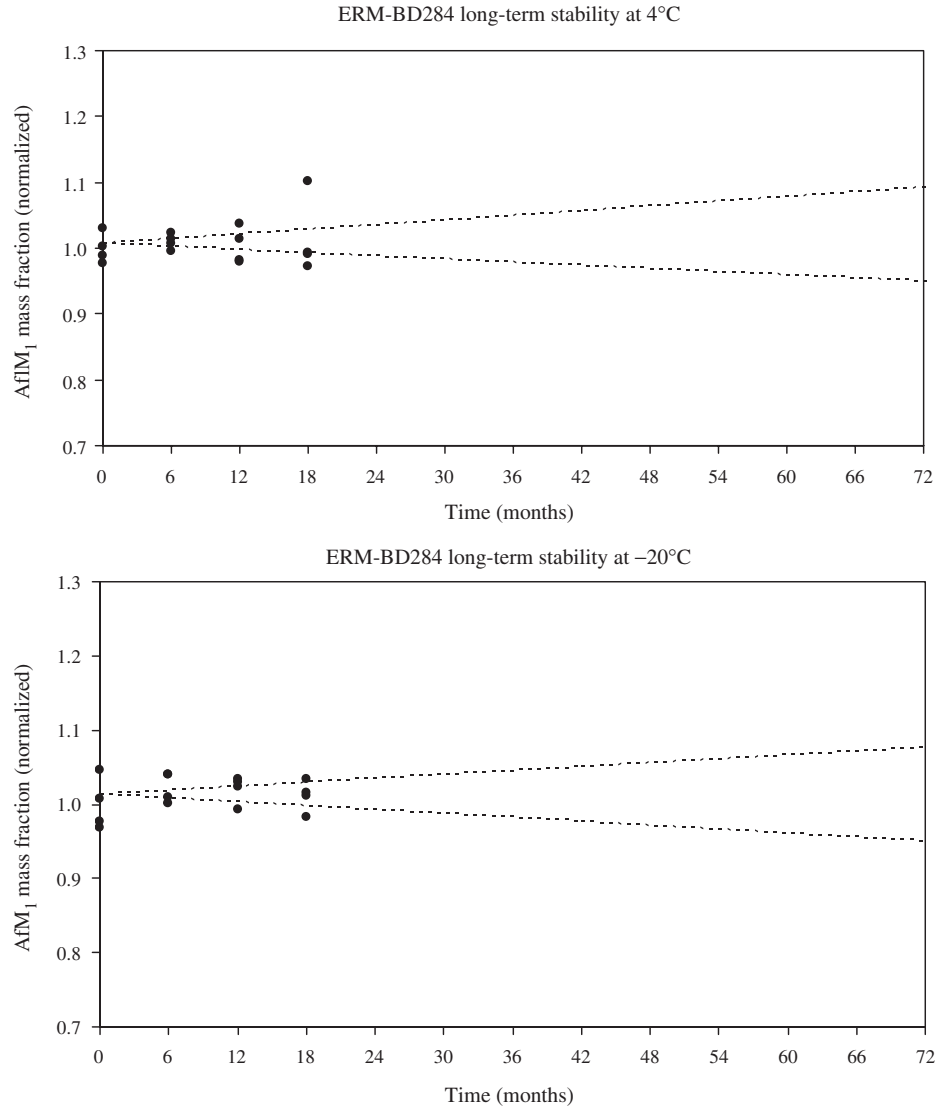

Figure 8. AfM_1 in milk powder ERM-BD284 (high level): normalized results of the long-term stability studies at 4 and −20°C.

Table II. Estimated uncertainties of long-term stability (u_{lts}) of ERM-BD283 and -BD284 for given shelf-lives.

	u_{lts} (%)			
	ERM-BD283		ERM-BD284	
Time (months)	4°C	−20°C	4°C	−20°C
24	3.8	2.5	2.8	2.1
48	7.6	4.9	5.7	4.2
60	9.5	6.2	7.1	5.3
72	11.4	7.4	8.5	6.3

Conclusions

The successful processing and testing of three new CRMs for AfM_1 in milk powder replacing the reference materials BCR-282, -283 and -284 was demonstrated. All operations were planned and performed in full compliance with the ISO Guide 30–35 series. The project contributed to the successful accreditation of the Reference Materials Unit of the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre as reference materials producer according to ISO Guide 34 (International Organization for Standardization 2000) in 2004.

AfM_1 in milk powder was scrutinized for the first time for homogeneity by an ANOVA approach and isochronous stability testing resulting in a reliable estimation of corresponding uncertainties. Based on the results of the homogeneity tests, the materials were sufficiently homogenous to serve as CRMs. Respective uncertainty contributions (u_{bb}) of 0.23–0.89% were calculated for homogeneity. The stability measurements showed no significant trends for both short- and long-term stability studies.

The long-term stability uncertainties (u_{lts}) of the AfM_1 contaminated milk powders ERM-BD283 and -BD284 were 7.4 and 6.3% for a shelf-life of 6 years and storage at a temperature of $-20^{\circ}C$.

Individual data on homogeneity and stability testing are published in the certification report for ERM-BD282, -BD283 and -BD284 (Josephs et al. 2004).

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