Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of the Mass Fractions of Vitamins in Whole Milk Powder

Certified Reference Material ERM®-BD600

EUR 24924 EN – 2011

JRC EUROPEAN COMMISSION

The mission of the JRC-IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Reference materials sales
Retieseweg 111
B-2440 Geel, Belgium
E-mail: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC 66346

EUR 24924 EN
ISBN 978-92-79-21045-7 (pdf)
ISBN 978-92-79-21044-0 (print)
ISSN 1831-9424 (online)
ISSN 1018-5593 (print)
doi:10.2787/50186

Luxembourg: Publications Office of the European Union

CERTIFICATION REPORT

Certification of the Mass Fractions of Vitamins in Whole Milk Powder

Certified Reference Material ERM®-BD600

M. Dabrio Ramos, H. Schimmel, H. Emons

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM), Geel (BE)

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this report to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of the reference material ERM-BD600, a whole milk powder certified for the mass fractions of water- and fat-soluble vitamins. All the required steps during the production were conducted according to ISO Guides 34 and 35 [1,2] to ensure the high quality of the final product, including matrix material processing, homogeneity and stability studies and value assignment. The matrix material, a spray dried whole milk powder, was packed into sachets containing approximately 100 g of powder, and stored at -30 ºC. Dedicated studies confirmed the homogeneity and stability of the vitamins in the milk powder under the conditions evaluated. The characterisation of the material, organised and coordinated by IRMM, was done by an inter-laboratory comparison. The certified and indicative values were obtained as the unweighted mean of the laboratory means of the accepted sets of results for each analyte, and the expanded uncertainty associated (k = 2) includes contributions from the homogeneity, the long term stability and the characterisation of the material. The certified values in ERM-BD600 are the following:

Component	**Certified Value**[1] [mg/kg] dry mass	**U**[2] [mg/kg] dry mass	p
Vitamin A (all-*trans* retinol)	**3.8**	**0.6**	8
A (*all-trans*-retinol and 13-*cis*-retinol)	**4.1**	**0.8**	6
Vitamin B_1 (thiamin)[3]	**4.5**	**0.6**	14
Vitamin B_2 (riboflavin)	**16.7**	**1.4**	14
Vitamin B_{12} (cyanocobalamin)	**0.32**	**0.07**	6
Vitamin C (total ascorbate)	**74**	**11**	15
Vitamin E (α-tocopherol)	**86**	**15**	18

[1]Unweighted mean value of the accepted sets of data (p) obtained in a different laboratory and/or with a different method of determination expressed in mg/kg in dry mass.
[2]Expanded uncertainty with a coverage factor of k = 2, alternatively for vitamins B_{12} and A (*all-trans*-retinol and 13-*cis*-retinol), $t_{(0.05,5)}$=2.57, according to ISO Guide 98-3 [3], to the Expression of Uncertainty in Measurement, corresponding to a level of confidence of approximately 95 %.
[3] Expressed as thiamin hydrochloride

Table of Contents

1. Glossary

α	Confidence level
ANOVA	Analysis of variance
b	Slope of the linear regression
BCR	Community Bureau of Reference
CAP	Certification advisory panel
CEN	European Committee for Standardisation
CI	Confidence interval
CRM	Certified reference material
DAD	Diode array detection
df	Degrees of freedom
DHAA	Dehydroascorbic acid
Em	Emission wavelength
ERM	European Reference Material
Ex	Excitation wavelength
F	*f* value
F_{crit}	Critical *f* value
k	Coverage factor
FL	Fluorescence
FLD	Fluorescence detection
HPLC	High performance liquid chromatography
IDF	International Dairy Federation
IDMS	Isotopic Dilution Mass Spectrometry
IRMM	Institute for Reference Materials and Measurements
IS	Internal standard
KFT	Karl Fischer titration
L	Laboratory code
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOQ	Limit of quantification
LTS	Long term stability
MBA	Microbiological assay
MS_{within}	Mean of squares within groups (ANOVA)
$MS_{between}$	Mean of squares between groups (ANOVA)
MS	Mass spectrometry
n	Number of replicates
n.c.	Not calculable
NP-HPLC	Normal phase high performance liquid chromatography
p	Number of accepted data sets
PL	Pyridoxal
PM	Pyridoxamine
PN	Pyridoxine
PE	Petroleum ether
PETM	Polyethylene terephtalate metallised
PSA	Particle size analysis
RDA	Recommended daily allowance
RP-HPLC	Reverse Phase high performance liquid chromatography
RSD	Relative standard deviation
RSE	Relative standard error
s	Standard deviation
s_b	Standard error of the slope of the linear regression

s_{bb}	Between-ampoule (in)homogeneity standard deviation
s_e	Standard error
s_{wb}	Within-ampoule standard deviation
SI	International System of Units
SS	Sum of squares (ANOVA)
STS	Short term stability
t	*t* value of the Student's *t* distribution
TCEP	Tris [2-carboxyethyl] phosphine
TFA	Trifuoroacetic acid
t_{crit}	Critical *t* value
u_{bb}	Relative standard uncertainty due to between-sachet (in)homogeneity
u^*_{bb}	Relative standard uncertainty due to the inhomogeneity that can be hidden by the method repeatability
u_{char}	Relative standard uncertainty from characterisation
U_{CRM}	Expanded uncertainty of the certified value
u_{lts}	Relative standard uncertainty associated to long-term (in)stability
u_{meas}	Relative standard uncertainty of the measurement result
u_{sts}	Relative standard uncertainty associated to short-term (in)stability
UV	Ultraviolet detection

2. Introduction

General

The Council Directive 90/496/EEC [4] on nutrition labelling for foodstuffs, as amended by Commission Directive 2003/120/EC [5], defines “nutrition labelling” as any information appearing on the label of food products including the energy value and the following nutrients: protein, carbohydrate, fat, fibre, sodium, vitamins and minerals listed in the Annex of the Council Directive. In 2008 a new amendment was introduced [6] as regards recommended daily allowances, energy conversion factors and definitions.

The Annex to Directive 90/496/EEC lists the vitamins and minerals which may be declared as part of the nutrition labelling, specifies their recommended daily allowances (RDAs) and defines a rule of what constitutes a significant amount.

The rule on significant amount becomes a reference in other Community legislation, in particular in Article 8(3) of EU Directive 2002/46/EC [7] on the approximation of the laws of the Member States relating to food supplements. It is also included in the regulations on nutrition and heath claims made on food [8] and on the addition of vitamins and minerals to foods [9]. More recently, the Commission Regulation (EC) No 1170/2009 [10] amended the Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements.

The compliance with the European and other existing international legislations on the vitamin contents implies the need of sufficiently accurate methods for their determination. The implementation of quality systems in analytical laboratories, in particular the accreditation according to ISO 17025 [11], and the developments made on standardisation of methods by organisations as the European Committee for Standardisation (CEN) or AOAC International have contributed to an overall improvement in the quality of results from the control and routine laboratories analysing vitamins. In terms of comparability, existing CRMs have also played an important role as a tool for accuracy determination during the development and validation of methods as well as a quality control tool.

In this frame, this report describes the production of a whole milk powder material and its characterisation of the mass fraction of a total of ten water and fat-soluble vitamins, carried out by the Institute for Reference Materials and Measurements (IRMM).

3. Participants

Processing

• NIZO Food Research, Ede, NL

(NEN-EN-ISO 9001:2000 accreditation; DNV 11694-2006-AQ-ROT-RvA)

• European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

(ISO/IEC 17025, ISO Guide 34 accreditation; BELAC 268-TEST)

Project management & data evaluation

• European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

(ISO/IEC 17025, ISO Guide 34 accreditation; BELAC 268-TEST)

Homogeneity and stability measurements

- Eurofins Steins Laboratorium, Vejen, DK
  (ISO/IEC 17025 accreditation; DANAK 153 and 168)
- Silliker Ibérica, Barcelona, ES
  (ISO/IEC 17025 accreditation; CGA-ENAC-LEC 257/LE413)

Characterisation measurements

The participating laboratories were, in alphabetical order:

- Arotop Food & Environment, Mainz, DE
  (ISO/IEC 17025 accreditation; DAP-PL-3283.00)
- Central Agricultural Office, Budapest, HU
  (ISO/IEC 17025 accreditation; DAP-PL-3101.00)
- DTU, National Food Institute, Søborg, DK
  (ISO/IEC 17025 accreditation; DANAK n. 350)
- Eurofins Steins Laboratorium, Vejen, DK
  (ISO/IEC 17025 accreditation; DANAK n. 153 and 168)
- GBA Gesellschaft für Bioanalytik Hamburg mbH, Hamburg, DE
  (ISO/IEC 17025 accreditation; DAC-PL-0040-97)
- ifp Institut für Produktqualität, Berlin, DE
  (ISO/IEC 17025 accreditation; DAP-PL-3942.00)
- Institut Suisse des Vitamines, Epalinges, CH
  (ISO/IEC 17025 accreditation; STS 462)
- Institut Kuhlmann, Ludwigshafen, DE
  (ISO/IEC 17025 accreditation; DAP-PL-3184.00)
- INZO, Chierry, FR
  (ISO/IEC 17025 accreditation; COFRAC 1-2218)
- Laboratoire AQUANAL, Pessac, FR
  (ISO/IEC 17025 accreditation; COFRAC n. 1-0703)
- Laboratori Agroalimentari, Generalitat de Catalunya, Cabrils, ES
  (ISO/IEC 17025 accreditation; CGA-ENAC-LEC 157/LE309)
- LAREBRON, Illkirch, FR
  (ISO/IEC 17025 accreditation; COFRAC n. 1-1946)
- LGC Limited, Food Chemistry, Teddington, UK

(ISO/IEC 17025 accreditation; UKAS n. 036)
- Livsmedelsverket, Uppsala, SE
  (ISO/IEC 17025 accreditation; SWEDAC 06-3832-51.1457)
- Nestlé Iberia AQAL, Barcelona, ES
  (ISO/IEC 17025 accreditation; CGA-ENAC-LEC 730/LE1466)
- Nestlé Nederland, Nunspeet, NL
  (ISO/IEC 17025 accreditation; RvA L383)
- Silliker Ibérica, Barcelona, ES
  (ISO/IEC 17025 accreditation; CGA-ENAC-LEC 257/LE413)
- Zentralinstitut für Ernährungs- und Lebensmittelforschung (ZIEL), TUM, Freising, DE
  (ISO/IEC 17025 accreditation; DAP-PL-3034.00)

4. Processing of the material

Four thousand litres of bovine whole milk were conditioned, enriched with a vitamin mixture (according to the *a priori* set nominative vitamin levels illustrated in Table 1), pasteurised, homogenised, evaporated and spray-dried at the food grade pilot plant NIZO Food Research (Ede, The Netherlands), resulting in 389 kg of milk powder. The material was packed in plastic laminated paper bags to prevent for light exposure and humidity uptake, and immediately transported to IRMM, where it was stored at 4 ºC in the dark until further processing. The bulk powder was analysed and the results (illustrated in Table 1) were provided by NIZO as part of the processing report.

Table 1. Target vitamin mass fractions in the whole milk powder and initial screening data reported by NIZO Food Research.

Component	Units	Target Value	Screening results reported
Vitamin A (total retinol)	mg/kg	7.0	3.4
Vitamin B_1 (thiamin)[1]	mg/kg	5.5	3.1
Vitamin B_2 (riboflavin)	mg/kg	16	14
Vitamin B_6 (total pyridoxine)	mg/kg	7.5	7.1)
Vitamin B_{12} (cyanocobalamin)	µg/kg	320	360
Niacin[2]	mg/kg	65	< 9.5
Total folate (folic acid)[3]	mg/kg	0.75	0.59
Vitamin D_3 (cholecalciferol)	mg/kg	0.105	0.118
Vitamin E (α-tocopherol)	mg/kg	100	100
Insolubility index (IDF 129 [12])	mL	-	1.0
Fat (%, m/m)	-	-	3.62
Protein (%, m/m)	-	-	3.51
Lactose (%, m/m)	-	-	4.53

[1] Expressed as thiamin hydrochloride
[2] The target level of niacin was not reached due to improper composition of the vitamin mixture used for vitamin fortification.
[3] The target level expressed as total folate. Screening result provided as folic acid.

The bulk material was further processed at IRMM. To prevent segregation of the milk powder, a homogenisation step was introduced using a 700 L stainless steel drum fitted in a

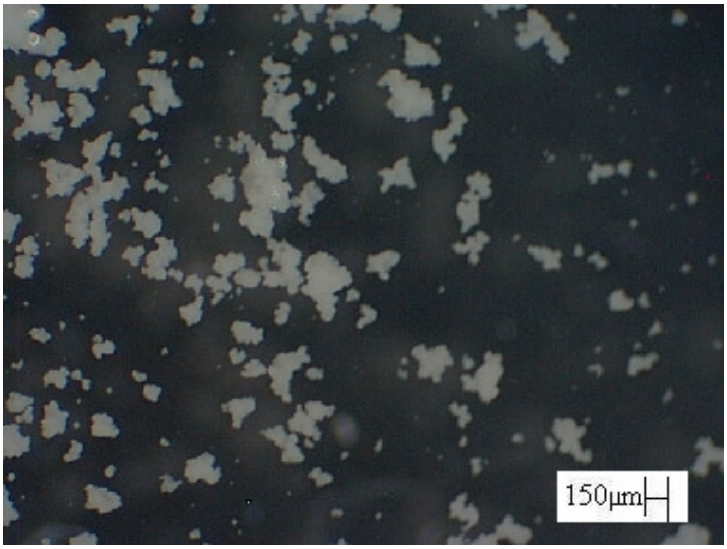
DynaMIX-CM500 three dimensional mixer (WAB, Basel, CH) and operated during one hour. The homogenised material was then manually filled into pouches containing approximately 100 g of milk powder material. The thermo sealed pouches were subsequently introduced in aluminium coated plastic foils (PETM/PE 110), vacuum sealed and stored at -30 ºC.

Material characterisation measurements

Four units of the final material covering initial, intermediate and final stages of the filling sequence were subjected to particle size analysis (PSA) using laser diffraction, to the analysis of water content by Karl Fischer titration (KFT), the measurement of water activity and the shape and size of the particles by micrographs. The results for the particle size distribution showed that for the size classes X_{10}, X_{50} and X_{90} the corresponding particle sizes were 87, 228 and 428 µm, respectively.

The evaluation of PSA results indicated that the batch is homogeneous with respect to the particle size over the whole production. The micrographs taken support the findings of the PSA, where many particles are observed on the range of 250 µm.

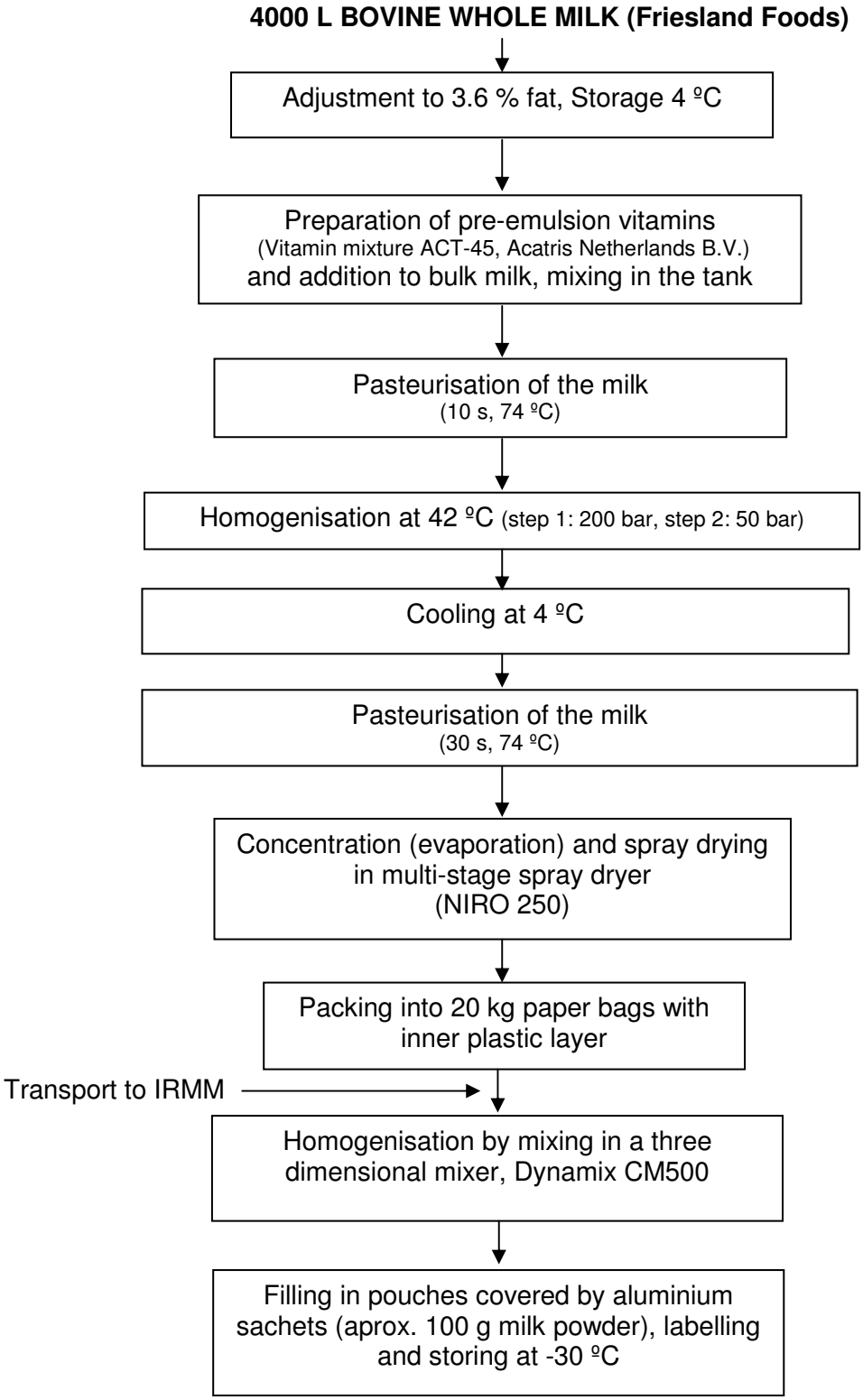
Figure 1. Micrograph of the milk powder final product, ERM-BD600.



The average water content of the whole milk powder material, as determined by KFT [13], is 2.15 ± 0.14 g/100 g. The water activity results are also found normal and acceptable, ranging between 0.113 and 0.169. A too dry matrix would show a lower stability due to oxidation reactions whereas the excess of water would provoke degradation of the material due to microbiological processes.

The main processing steps are summarised in the following flow diagram:

FLOW DIAGRAM FOR PROCESSING OF ERM-BD600



5. Homogeneity study

The aim of a homogeneity study is to assess the regular distribution of the components to be characterised in the whole batch of reference material. The set-up of the study included the analysis of 14 sachets of ERM-BD600 randomly selected. For practicability reasons the analyses were performed under intermediate precision conditions, split in two different days. All the vitamins were determined by Eurofins AS, DK, using validated methodology under the scope of ISO 17025 accreditation. Liquid chromatography combined with fluorimetric detection was applied for vitamins A, E, B_1, B_2 and B_6 (for vitamin D_3, alternatively UV and MS detection were used) whereas niacin, folate, and vitamin B_{12} were determined using microbiological assays. For vitamin C, a spectrofluorometric method was employed. The samples were analysed in triplicate in a randomised order and the results, as well as a brief description of the methodology employed, are illustrated in Annex A.

Table 2. Evaluation of the statistical significance of trends from results of samples analysed for homogeneity

	Filling sequence order	
Component	**Trend**	**Outliers**
Vitamin A (retinol)	no	one[a] (α=0.05)
Vitamin B_1 (thiamin)	yes[b]	none
Vitamin B_2 (riboflavin)	no	none
Vitamin B_6 (total pyridoxine)	no	none
Vitamin B_{12} (cyanocobalamin)	no	none
Niacin (nicotinic acid + nicotinamide)	no	one
Total folate	no	one
Vitamin C	yes[b]	none
Vitamin D_3 (cholecalciferol)	no	none
Vitamin E (α-tocopherol)	no	one[a] (α=0.05)

[a] One single replicate outlier in the analytical sequence provokes one value of the filling sequence (mean of 3 replicates) to become outlier. No abnormality is reported by the laboratory.

[b] Statistical significant trend caused by day-to-day method variation effects. Additional evaluation of an independent set of data confirmed the absence of filling effects.

The data was scrutinised for outliers, as well as for normality and unimodality of the distributions (results not shown). The normally distributed results for all vitamins follow a unimodal distribution, with the exception of vitamin C. Bimodality detected in that case was caused by a day-to-day variation effect of the analytical method, as illustrated in the corresponding graph of Annex A, where samples below and above unit n. 1000 were analysed in two different days. Furthermore the daily variation caused a statistical significant trend of the results for vitamins C and B_1, as indicated in Table 2. To discard any filling effect as a source of heterogeneity, an additional check was performed using independent sets of

results from the short-term stability study at the same temperature. The outcome of the evaluation confirmed the lack of trend with the filling sequence for vitamins C and B_1.

According to the Grubbs tests (Table 2), a number of outlying values were identified in the analytical sequence although no particular irregularity has been reported by the laboratory. For vitamins A and E, having a common sample treatment, the single outlier identified corresponded to analytical results from the same sample, which suggests an anomaly occurred to that particular replicate. Furthermore, when these values were considered, the mean mass fractions for the corresponding sachet in the filling sequence became as well outliers. Analogously, the single outlier detected for niacin in the analytical sequence (single replicate analysis) had an effect on the outlier detected when the filling sequence was assessed (mean of three replicates). Nevertheless, for none of the vitamins any value was discarded for statistical reasons only and consequently all results were submitted to a one-way analysis of variance (ANOVA) for the estimation of the uncertainty contribution from possible heterogeneity [14].

Method repeatability (s_{wb}) expressed as a relative standard deviation is given as follows:

$$s_{wb} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

MS_{within}: mean square within a sachet from an ANOVA
$\bar{y}$: average of all results of the homogeneity study

Between-unit variability (s_{bb}) expressed as a relative standard deviation is given by the following equation:

$$s_{bb} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$

$MS_{between}$: mean square among sachets from an ANOVA
n: average number of replicates per sachet, equal to 3

The heterogeneity that can be hidden by method repeatability is defined as follows:

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

$\nu_{MSwithin}$: degrees of freedom of MS_{within}

The larger value of s_{bb} or u^*_{bb} was used as uncertainty contribution for homogeneity, u_{bb}

The results obtained are illustrated in Table 3, including the u_{bb} estimate for the different vitamins. According to the present evaluation the milk powder processed is considered as sufficiently homogeneous material at the level of sample intake investigated.

Table 3. Data resulting from evaluation of ERM-BD600 homogeneity set-up

Component	s_{bb} [%]	u^*_{bb} [%]	u_{bb} [%]
Vitamin A (retinol)	2.8	2.8	2.8
Vitamin B_1 (thiamin)	3.4	1.0	3.4
Vitamin B_2 (riboflavin)	1.9	1.2	1.9
Vitamin B_6 (pyridoxine)	n.c.	1.0	1.0
Vitamin B_{12} (cyanocobalamin)	n.c.	1.0	1.0
Niacin	n.c.	1.9	1.9
Total folate	4.4	4.1	4.4
Vitamin C[1]	2.3	0.7	2.3
Vitamin D_3 (cholecalciferol)	n.c.	1.2	1.2
Vitamin E (α-tocopherol)	n.c.	2.1	2.1

[1]Resuls from evaluation of ERM-BD600 material homogeneity reprocessing data sets from the short-term stability study.
n.c. not calculable ($MS_{between}<MS_{within}$)

6. Minimum sample intake

During an earlier production of a milk powder certified reference material (BCR-421), 10 g was established as minimum sample intake for α-tocopherol and cholecalciferol, and 2.5 g for the remaining certified vitamins. More recently, the results of the stability studies performed to the ERM-BD600 material demonstrated that the use of lower quantities of material is, for some of the vitamins, also appropriate. Furthermore, during the certification campaign the participating laboratories applied their own methodologies including the use of specific mass sample intakes. Table 4 indicates the maximum and minimum masses of whole milk powder employed by participant laboratories during the material characterisation. The results from the low end sample intakes used during the characterisation of the material show within laboratory variability comparable with those using higher sample intakes. Whenever the lowest sample intake was uniquely employed by a single laboratory, a conservative approach included establishing higher masses as the minimum sample intakes. This is the case of vitamin C, where quantities of milk powder lower than 1.5 g were tested providing comparable variability with higher sample amounts, although the recommended sample intake was finally set to 2.5 g.

The same criterion was applied for the rest of the vitamins in ERM-BD600, with the following portions of minimum sample recommended for the analysis: 1 g for folate, niacin and vitamin B_{12}; 2.5 g for vitamins B_1, B_2, B_6 and C; 5 g for vitamins A, D and E.

Table 4. Ranges of whole milk powder sample intake covered by the methods used during the certification campaign of ERM-BD600

Component	Minimum [g]	Maximum [g]
Vitamin A	2.5	50
Vitamin B_1	2.5	50
Vitamin B_2	2.5	50
Vitamin B_6	2.0	15
Vitamin B_{12}	1.0	50
Niacin	1.0	5
Folate	1.0	5
Vitamin C	0.5	10
Vitamin D_3	2.0	50
Vitamin E	4.0	50

7. Stability studies

All ten vitamins candidate for certification, both water and fat-soluble, were submitted to systematic stability studies. Given that factors such as moisture, oxidation, exposure to UV light or strong acid/base can affect the stability of the vitamins, care was taken to minimise as much as possible these factors during the material processing and later on for the selection of the packaging material and storage conditions.

Previous experience with the BCR-421 material indicated that -30 ºC was a more appropriate temperature rather than -20 ºC for the normal storage of the milk powder, as it assures a better preservation of the vitamins in the material.

The stability of ERM-BD600 on short and long-term periods was investigated applying isochronous test schemes [15] as described below.

Short-term stability

Once the material was processed, a number of ERM-BD600 sachets were picked by random stratified sampling for the different studies to be conducted. For the short-term stability study, performed for the selection of the material transport conditions, the isochronous testing scheme [15] was applied as follows: three sachets were kept at a certain temperature (4 ºC, 18 ºC or -30 ºC) for 1, 2 or 4 weeks respectively, while another three sachets were kept at the reference temperature, -70 ºC. The complete short-term stability study required a total of 30 units. Three independent sub-samples per sachets were analysed by Eurofins AS, DK using the methodology described in Annex A. Samples were grouped by temperatures and the groups were measured under repeatability conditions in a randomised manner.

The results at each temperature and time point were evaluated as follows:

The results for samples stored at a certain temperature (4 ºC, 18 ºC or -30 ºC) were grouped by time points. The slope of the regression line generated by the variation of vitamin mass fraction present *vs*. the time was calculated and the significance of the slope was evaluated statistically with the aim of detecting any possible trend that would indicate degradation of the material.

The measurement results of the isochronous testing scheme at the three temperatures are detailed in Annex B. Below, Table 5 summarises the outcome of the statistical evaluation of the stability study data in terms of trends as well as the presence or absence of outlying mean values according to the Grubbs test.

Table 5. Evaluation of the statistical significance of positive (+) or negative (-) trends from results of ERM-BD600 samples analysed for short-term stability over a period of one month.

	Temperature					
	-30 ºC		4 ºC		18 ºC	
Component	Trend	Outliers	Trend	Outliers	Trend	Outliers
Vitamin A	no	none	no	one	yes (-)	none
Vitamin B_1	no	none	no	none	no	none
Vitamin B_2	no	one	no	none	yes (+)	none
Vitamin B_6	no	one (α=0.05)	no	none	yes (-)	none
Vitamin B_{12}	no	none	no	one (α=0.05)	no	none
Vitamin C	no	one (α=0.05)	yes[1] (-)	none	yes (-)	none
Vitamin D_3	no	one	no	one (α=0.05)	no	one
Vitamin E	no	one (α=0.05)	no	one	no	none
Niacin	yes (-)	one (α=0.05)	no	none	no	one (α=0.05)
Total folate	no	one	yes[1](+)	none	yes (+)	one (α=0.05)

[1]No significant trend at 99 % confidence interval

At storage temperatures of 4 and 18 ºC statistically significant trends were observed for several vitamins. At the storage temperature of -30 ºC, only niacin appears to degrade with time although no trend is detected at either of the higher temperatures. Given the expected relatively high stability of the vitamin, results at -30 ºC for niacin can be attributable to an analytical artefact, as further confirmed during the long-term stability study. Furthermore, positive temperatures induce degradation for vitamin C (at 4 ºC) confirmed by the data obtained for samples stored at 18 ºC. Similarly, folate has a significant trend at both 4 and 18 ºC, in this case of a positive sign, attributable to a possible incomplete deconjugation during extraction. At the highest temperature the data for vitamins A, B_2 and B_6 show also a significant trend. In this case, taking into account that the method is precise enough to allow meaningful evaluation, it can be concluded that the reference material ERM-BD600 is stable when stored to a temperature of -30 ºC for a period of one month, but not at higher temperatures. Consequently ERM-BD600 has to be kept frozen during transport and will be dispatched on dry ice.

Long-term stability

The assessment of the long-term stability was performed through the analysis of samples submitted to an isochronous scheme [15], in an analogous manner as described in the short-term stability section.

Time-points: 0, 4, 8 and 12 months (at -30 ºC)

Time-points: 0, 2, 4 and 6 months (at +4 ºC)

Reference temperature: -70 ºC

Twenty-one sachets were required for the complete study including 3 samples at each time point at each temperature. Two replicates per sample were analysed by Silliker Ibérica, ES, and the average results as well as details on the analytical methods applied are shown in Annex C. The fact that the methodology employed is not always equivalent to that used for the short-term stability is of no consequence due to the independence of the two studies. The resulting data was evaluated in compliance with the ISO Guide 35 [2]. One outlier was detected for vitamin B_6, both in the 6 months and 12 months schemes, corresponding to one result of a reference sample used in both cases. As no technical reason was found to reject the data point, the value was included in the evaluation. A regression analysis was applied and the results are summarised in Table 6, both at 95 % and 99 % confidence interval.

Table 6. Evaluation of the statistical significance of positive (+) or negative (-) trends from results of ERM-BD600 samples analysed for long-term stability over periods of six and twelve months at 4 ºC and -30 ºC, respectively.

	12 months		6 months	
	-30 ºC		4 ºC	
Component	Trend	Outliers	Trend	Outliers
Vitamin A	no	none	no	none
Vitamin B_1	no	none	no	one (α=0.05)
Vitamin B_2	yes[1] (+)	none	yes (+)	none
Vitamin B_6	no	one	no	one
Vitamin B_{12}	no	one (α=0.05)	no	none
Niacin	no	none	yes[1] (-)	none
Total folate	no	none	no	none
Vitamin C	no	none	no	none
Vitamin D_3	yes[1] (+)	none	yes (+)	one
Vitamin E	no	none	no	none

[1]No trend at 99% confidence interval

The significance of the slope given by the variation of the vitamin mass fraction *vs.* the time was statistically evaluated with the aim of detecting possible degradation. Although niacin shows a significant trend at 4 ºC, the situation at -30 ºC is different, no degradation is detected. In this case there is no additional stability data available for storage at higher temperatures for the same time period or for longer periods at the same temperature, thus it

is difficult to attribute the significant trend to either a degradation or to an analytical artefact. For vitamins D_3 and B_2, a statistically significant trend is detected at the two temperatures. A closer look to the data shows a particularly good repeatability of the results at -30 ºC (e.g. for vitamin D_3 RSD = 0.82 %), causing a statistical significance of the trend when results are minimally deviating. This effect is not technically relevant; it is rather due to an analytical artefact, as supported by the fact that the minimal slope has a positive trend. For the rest of the vitamins no technically relevant trend is observed, thus it can be deduced that there is no degradation of the samples at the normal storage temperature of -30 ºC in the time period under evaluation.

Table 7 illustrates the values of the u_{lts} calculated as described in [16] estimated for the stability scheme of 12 months at the normal storage temperature for a given shelf-life of 24 months.

Table 7. u_{lts} values for ERM-BD600 estimated for a shelf-life of 2 years.

	-30 ºC, 12 months scheme	
Component	u_{lts} [mg/kg]	u_{lts} [%]
Vitamin A	0.431	5.8
Vitamin B_1	0.193	4.1
Vitamin B_2	0.409	2.4
Vitamin B_6	0.121	2.5
Vitamin B_{12}	0.023	7.4
Niacin	1.585	15.6
Total folate	0.035	9.7
Vitamin C	2.746	4.2
Vitamin D_3	0.002	1.2
Vitamin E	6.264	7.4

8. Characterisation

The characterisation of the material was done by a collaborative study. All the laboratories participating in the characterisation study were selected according to quality requirements criteria including demonstration of their competence by providing evidence on sufficient performance for the analysis of vitamins during e.g. participation on proficiency testings, the use of validated methodology and accreditation of the laboratory to ISO 17025 for the relevant field.

The participant laboratories were requested to apply validated methodologies of their own choice, which included chromatographic separation techniques or microbiological assays and the use of their own calibrants. Procedures based on reference standards [17-29] were

extensively selected among the participants. The main details of the analytical methods corresponding to each vitamin are described in Annex D (Tables D.1-D.10).

Three ERM-BD600 units randomly selected from the processed batch and an additional milk powder control sample of undisclosed vitamin composition were sent to each collaborating laboratory. A total of nineteen participants were requested to analyse ERM-BD600 under intermediate precision conditions, i.e. each bottle on a different day in two replicates plus the control sample, using their own validated methodology. Individual results were reported in mg/kg in dry matter for each replicate analysis performed.

Table D.11 in Annex D comprises the individual results corresponding to six independent replicate analyses of ERM-BD600 material reported by the laboratories participating in the certification campaign. Not all laboratories participated in the characterisation of the ten target vitamins, as indicated in the table. According to ISO Guide 35, all the data results were scrutinised for outlying means and variances as well as for the homogeneity of variances and normality of the distributions. The evaluation included the identification of outlying mean values, investigation of potential technical reasons explaining the deviating result and the eventual comparison of the outliers with the parallel results provided by the laboratory for the blind milk powder control sample. Outliers were removed from the evaluation only on the basis of technical reason which invalidated the data concerned; No values were eliminated on statistical grounds alone. The outcome of the evaluation is summarised in Table 8 with some particular remarks made in the text below for each vitamin.

Table 8. Characterisation of vitamins in ERM-BD600. Statistical data evaluation of accepted data sets.

	Vit A (all-*trans* +13-*cis*-retinol)	Vit A (all-*trans* retinol)	Vit B_1	Vit B_2	Vit B_6	Vit B_{12}	Niacin	Total folate	Vit C	Vit. D_3	Vit. E
Number of accepted data sets	6	8	14	14	11	6	10	9	15	15	18
Mean of means [mg/kg]	4.113	3.830	4.464	16.675	7.076	0.324	8.021	0.552	74.278	0.136	86.389
RSE mean of means [%]	3.220	3.386	3.678	2.601	4.314	2.509	6.892	9.425	5.233	8.767	3.929
Outlying means, Dixon test	No	No	No	No	L5	No	No	No	No	L1, L13	No
Outlying means, Grubbs test	No	No	No	L9 (α=0.05)	L5	No	No	No	No	L1	No
Outlying lab variances, Cochran test	No	L7, L9	L17	L8, L9	No	L7 (α=0.05)	L11	L11	L12	L1, L4, L5, L9, L11, L17	L13
Lab variances homogeneous, Barlett test	No	No	No	No	No	Yes (α=0.01)	No	No	No	out of range	No
Distribution of means normal, Skewness & kurtosis, normal probability plot	Yes	Yes	Yes	Yes	Yes (α=0.01)	Yes	Yes	Yes	Yes	No	Yes

Vitamin A is reported in different ways. A number of laboratories provided the total mass fraction as the **sum of all-*trans* retinol and 13-*cis* retinol** whereas other laboratories uniquely quantified the major component, all-*trans* retinol. In both cases, the evaluation of the 12 data sets provided, all based on chromatographic methods, indicates a clusters of the results in two different groups. Individual mass fractions within each data set are detailed in Table D.11, Annex 4. Laboratories applying reverse phase HPLC methods with UV detection reported significantly higher results for vitamin A than those using normal phase combined with either UV or FL detection (FLD). In most methods the UV wavelength was set to a fixed value of 325 nm. When applying RP-HPLC-DAD to analyse the ERM-BD600 sample, one of the laboratories (L6) detected chromatographic interferences with the vitamin A signal at the mentioned wavelength, although no interference was detected on the blind control milk powder sample. As an alternative, the same laboratory used and reported data obtained by RP-HPLC in combination with FLD for the vitamin A quantification, where no interference was detected in either of the samples. Due to this detected interference, doubts are triggered about the validity of datasets obtained by RP-HPLC-UV for the ERM-BD600 samples. The technical issue might as well affect to applied methods using RP-HPLC-FLD. Although the reported results using this approach are more in agreement with the rest of the data sets, the possibility of a quenching effect due to the presence of interferences might not be neglected. Consequently the datasets obtained by RP-HPLC are removed from further evaluation. The statistical evaluation of a total of 6 consistent datasets normally distributed (Fig. 2) reveals no outlying means or variances and thus provides the assigned certified value for the vitamin as the unweighed mean of the laboratory means.

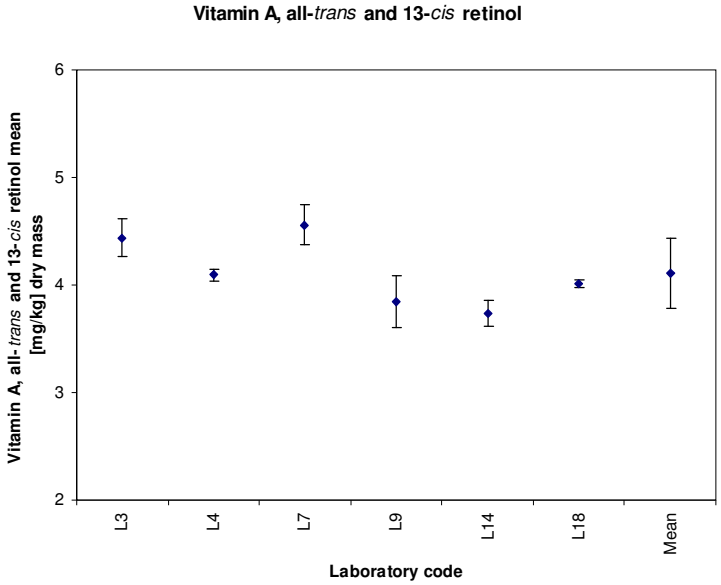


Fig. 2. Accepted data sets for the characterisation of vitamin A (as sum of all-*trans* and 13-*cis*-retinol) in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6

independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

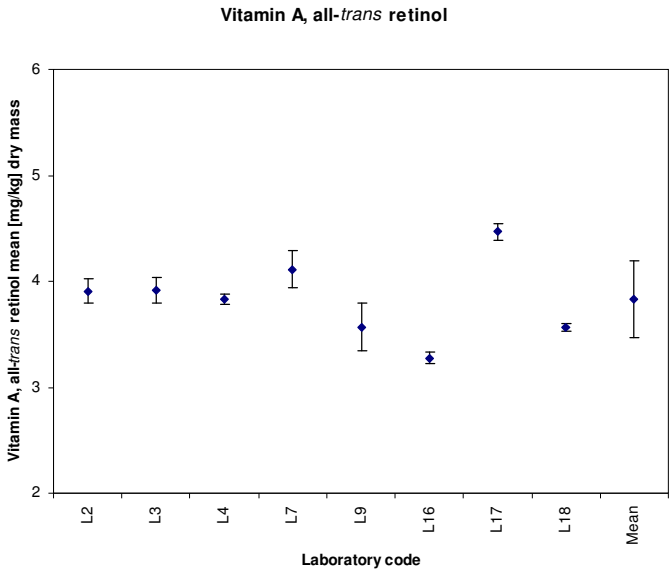


Fig. 3. Accepted data sets for the characterisation of vitamin A (as all-*trans* retinol) in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

For **Vitamin A as all-*trans* retinol**, two among ten participant laboratories reported results obtained by RP-HPLC-UV. One of these laboratories (L19) detected and reported chromatographic interferences during the analysis of the milk powder material, confirming above mentioned observations from a different laboratory. Consequently datasets obtained by RP-HPLC-UV are not considered for evaluation. The rest of the eight data sets, normally distributed with no outliers detected (Fig. 3), are used for certified value assignment.

Vitamin B_1 was analysed by 16 laboratories requested to provide the results as thiamin chloride hydrochloride. A first iteration including all results identified L12 as an outlier. In this case, the laboratory indicated that the values detected were at the level of the LOQ of their method. In fact, the laboratory indicated that out of three milk powder sachets, two had vitamin concentrations below the LOQ of their method and reported a third mass fraction value, used for the present evaluation. Consequently the results of L12 were rejected. The variance for L9 is an outlier according to the Cochran test, although the mean value passes the outlier Dixon's test. In this case the laboratory itself questioned about the validity of the data and therefore the data set was removed.

The methodology employed for the determination of vitamin B_1 included acid hydrolysis of the sample followed by enzymatic dephosphorylation (except in the case of L17) and subsequent application of HPLC with FLD. Laboratory 19 used a different instrumental set up

combining HPLC with MS and no enzymatic dephosphorylation step during sample preparation. The mean value of this dataset, although not identified as outlier, slightly deviates from the apparent mean. The available data is not sufficient to discern whether the LC-MS methodology or the lacking enzymatic digestion have a decisive influence on the result. In fact, the main phosphorylated form of the thiamin, the thiamin pyrophosphate, occurs in food matrices as vegetables, cereals, legumes, meats, yeast and *E. coli* [29] and no phosphorylated forms of thiamin are found in milk powder material [30]. Consequently the lack of phosphatase digestion, regularly included in generic protocols for the determination of vitamin B1 in foodstuffs as the CEN method, is expected not to be critical in the determination of thiamin in the milk powder. Consequently L17 and L19 values are retained with the pool of data used for certified value assignment (Fig. 4).

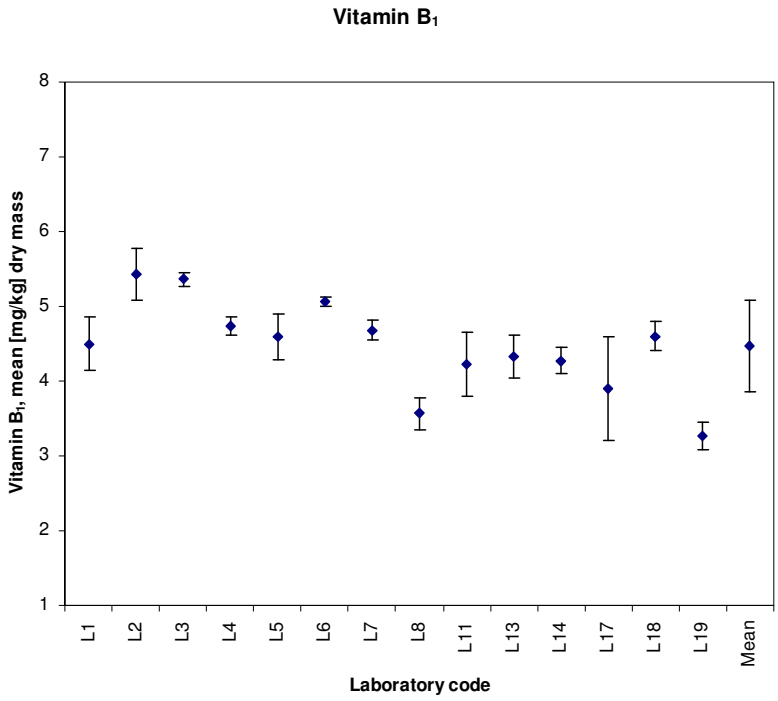


Fig. 4. Accepted data sets for the characterisation of vitamin B_1 in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Sixteen laboratories provided results for the mass fraction of **vitamin B_2** expressed as riboflavin. The LC protocols used for the analysis involve the acidic hydrolysis of the sample under different conditions. Except in two cases, this is continued with an enzymatic treatment at different incubation conditions where enzymes such as e.g. clara-diastase, takadiastase or a cocktail of enzymes are used having activities as protease, phosphatase and amylase, as part of a general procedure for the determination of vitamin B_2 applicable to foodstuffs. The

laboratory mean values are normally distributed and the complete group of data sets successfully pass the outliers test indicating no evidence of a grouping of datasets influenced by the methods of determination used. However, in the milk powder matrix, riboflavin is expected to be present essentially in the free form as well as phosphorylated in a minor percentage [30]. Consequently the absence of enzymatic treatment could trigger the underestimation of the vitamin B_2 present in the sample, therefore L17 and L19 datasets are not considered for further evaluation. The evaluation of the 14 remaining normally distributed datasets (Fig. 5) reveals the presence of a straggler value (L9) although there is no technical reason to eliminate this result, which is retained for the value assignment.

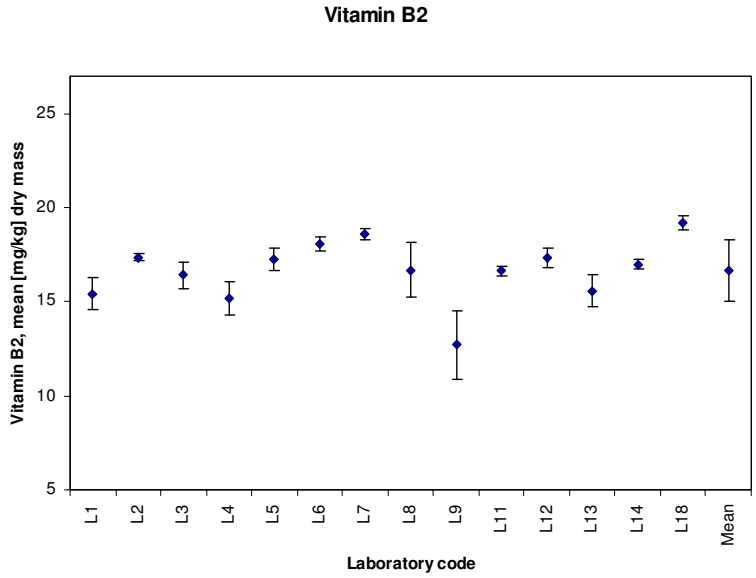


Fig. 5. Accepted data sets for the characterisation of vitamin B_2 in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Vitamin B_6 is reported by 13 collaborators as total pyridoxine, including pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and the phosphorylated derivatives. Some of the methods used by the laboratories cover as well the determination of β-glycosylated forms, although these forms are mostly present in plants but not in animal-derived foods [31]. As separation method HPLC is a common denominator to all the procedures, in most cases followed by FLD with the exceptional application of MS by two of the participants. Specific details on sample treatments and chromatographic and detection conditions employed are listed in Table D.4, Annex D. By far the most common sample treatment applied was acidic hydrolysis at different conditions followed by an enzymatic digestion, using enzymes with

phosphatase activity, and continued with pre or post-column derivatisation in combination with LC separation. A reduced number of participants did not apply enzymatic treatment. Among them, one laboratory used a very simple protocol consisting on a sample dilution, followed by centrifugation and filtration prior to LC-MS/MS analysis. As for vitamin B_2, the enzymatic treatment plays a role on the determination of vitamin B_6 when it is present as phosphorylated form. In milk powder approximately 25 % of the vitamin B_6 is expected to be phosphorylated [30], therefore the absence of enzymatic digestion is regarded as a technical reason to withdraw L17 and L19 from further evaluation. The statistical assessment of the remaining data revealed one outlier among the results, although no technical issues have been identified in relation to the dataset determination. Consequently it is not possible to assign a certified value for the material. Instead the value is provided as additional material information.

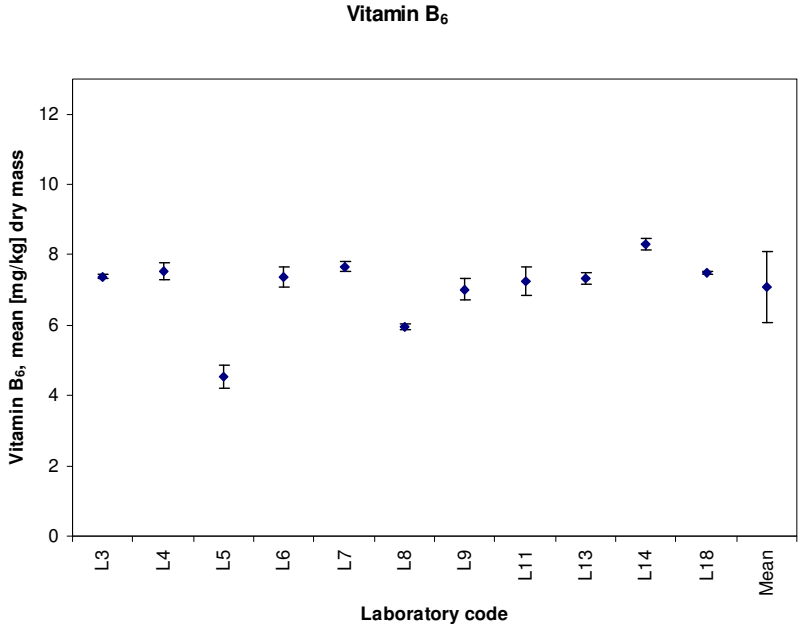


Fig. 6. Accepted data sets for the characterisation of vitamin B_6 in ERM-BD600 Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Eleven participants submitted results for **Vitamin B12** as cyanocobalamin. Methodologies applied in this case include the use of techniques as diverse as LC with UV detection, microbiological assays with turbidimetric determination and affinity based inmunosensor monitored by surface plasmon resonance. A preliminary screening of the results identifies L6 and L1 as outlying laboratory means. In neither case technical anomalies were detected or reported by the laboratories in relation with these microbiological analyses. The data scatter

indicates lacking robustness of the indirect microbiological methods. Therefore the certified value is based on the datasets obtained by direct chemical methods (p=6, Fig. 7).

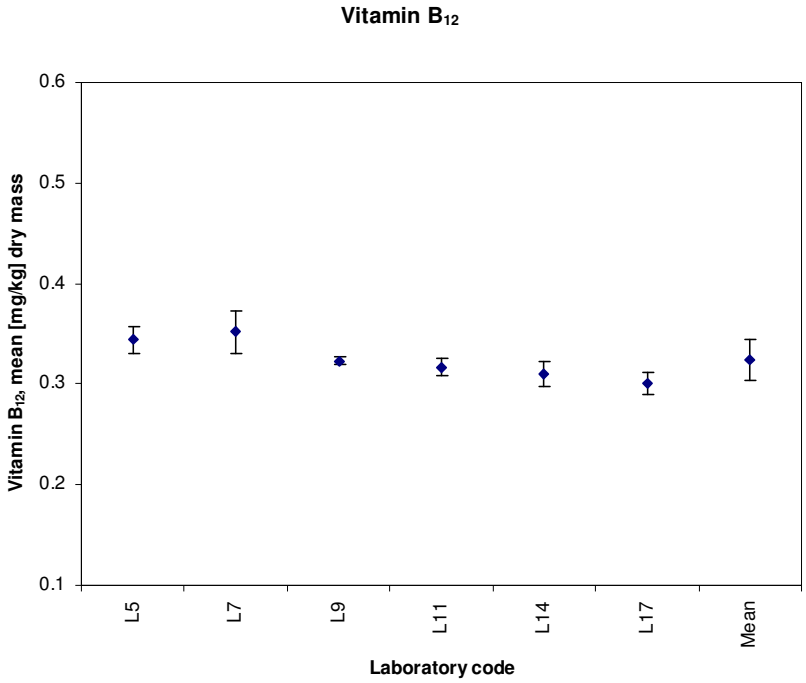


Fig. 7. Accepted data sets for the characterisation of vitamin B_{12} in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Niacin results were reported by 11 laboratories (Fig. 8) employing methodologies including either LC techniques with FL/ MS detection or microbiological assays, as detailed in Table D.6, Annex D. Acid hydrolysis was used by all laboratories except one, using enzymatic hydrolysis (L4), as it converts nicotinamide to nicotinic acid allowing for the measurement of total available niacin. A visual inspection of all data sets identifies two suspicious laboratory means, L5 and L17, although no outlying laboratory means were statistically detected. L5 applied a microbiological method whereas L17 dissolved the sample in water, centrifuged and filtered it prior to LC-MS/MS analysis. In neither of the two cases particular technical issues were reported by the laboratories although the lack of acidic treatment of the sample possibly induced an incomplete sample extraction that could justify the low niacin value obtained by L17, thus excluded for further assessment. No other dependence of the measurement results in relation to the methodology applied was found. Consequently all remaining ten data sets were evaluated. Due to the large scatter of the accepted data on top of the substantial uncertainty contribution of the long term stability of the vitamin, the niacin

mass fraction can not be certified with an acceptable level of uncertainty, therefore niacin in the milk powder material is provided as indicative value.

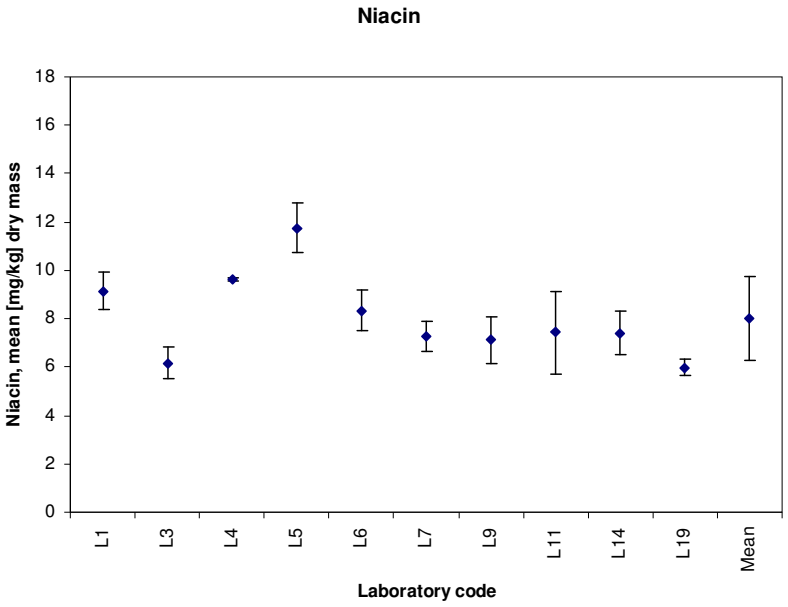


Fig. 8. Accepted data sets for the characterisation of Niacin in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Eleven sets of values were reported as **total folate** by the participant laboratories. A diversity of strategies applied for the quantification included isotopic dilution mass spectrometry (IDMS) among the chromatographic methods and the use of microbiological assay (MBA) protocols which allow the measurement of total folate in the milk samples. The activity of the lactic bacteria used within the MBA, *Lactobacillus casei* or *Lacobacillus rhamnosus*, is known to have an analogous response to most folate derivatives, including mono- and polyglutamates, although the response is slower for the long-chain derivatives. On the other hand, HPLC methods offer a high selectivity towards folate derivatives in the folate extract, even though it is not suitable for either di- and polyglutamates, therefore may result on lower folate values (as probably the case of L17) unless they are converted into mono- or diglutamates by a conjugase or folate hydrolase as γ-glutamylcarboxyipeptidase. Both the sample extraction conditions and the deconjugase enzymes employed for deconjugation of folate are factors reported to influence the final analysis result. Despite the variety of methodologies and the individual sets of conditions applied, no outliers are identified among the normally distributed laboratories means reported. Nevertheless it is decided to focus the evaluation on MB based methods only (Fig. 9) as well as to provide the one chromatographic IDMS dataset result for total folate (L13) as additional material information. Similarly to the

previous case, the level of uncertainty estimated for folate exceeds the acceptable levels for a certified value, and consequently the total folate mass fraction is provided as an indicative value.

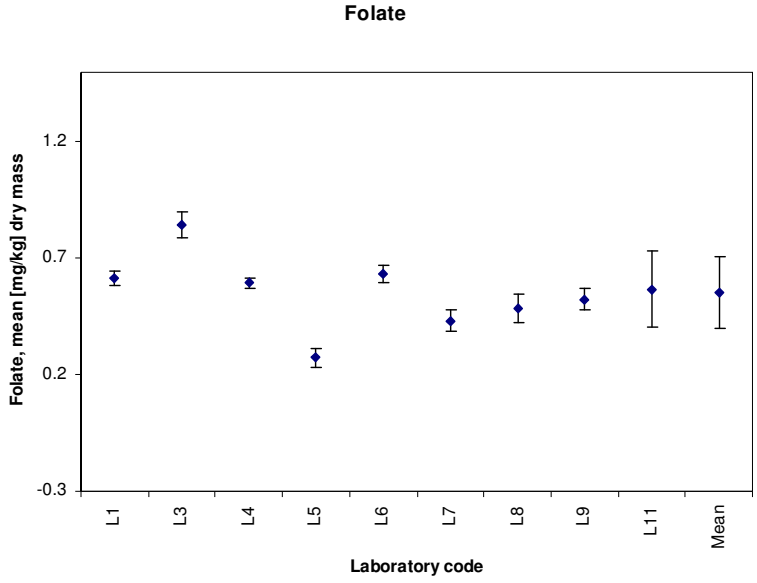


Fig. 9. Accepted data sets for the characterisation of total folate in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Vitamin C results of 15 laboratories were expressed as total ascorbate mass fraction including ascorbic acid and dehydroascorbic acid (DHAA). After acidic extraction, a reduction step for DHAA is introduced prior to determination by HPLC-UV or electrochemical detection. Alternatively, the ascorbic acid was oxidised to DHAA followed by derivatisation and HPLC-FLD determination. The visual inspection of the data shown in Fig. 10 reveals two suspicious datasets (L8, L17), although the statistical scrutiny of the data by Dixon's and Grubbs's tests does not identify them as outliers. L12 shows a large variance, confirmed as outlier by the Cochran test, which is associated to a day to day variation of the results. None of the laboratories (L8, L17, L12) indicated technical issues related to the specific measurements. Therefore the values are retained for certified value assignment.

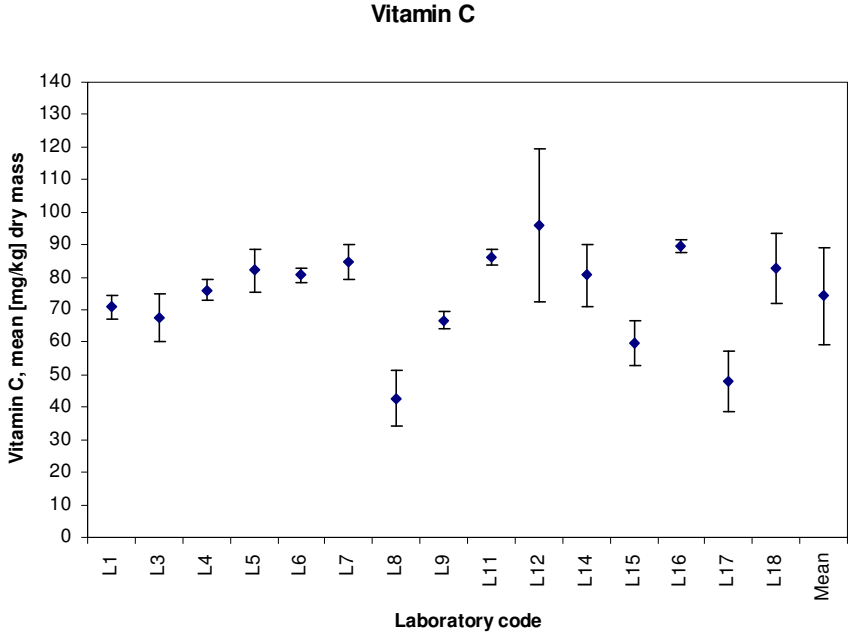


Fig. 10. Accepted data sets for the characterisation of vitamin C in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Cholecalciferol, **vitamin D_3**, is analysed in the milk powder material by 16 laboratories. The methodologies used basically consist on the saponification of the samples in alcoholic media prior to liquid-liquid extraction and/or clean-up procedures using e.g. SPE, followed by RP-HPLC separation and detection by UV or MS using an internal standard.

The result of laboratory L8 for vitamin D_3 largely exceeded those provided by the rest of the laboratories. Laboratory 8 confirmed the reported value. However the bias identified for the result for ERM-BD600 material was consistent with that observed for the control sample, exceeding about three orders of magnitude the vitamin D_3 content in the control sample, as well as the values reported by other laboratories for ERM-BD600. Consequently the dataset of L8 is excluded from the value assignment. Furthermore, among the 15 datasets left for evaluation, shown in Fig. 11, two laboratory means were identified as outliers, L1 and L13, although no specific technical reason was reported nor identified to explain the deviating results. Due to the presence of outlier laboratory means it is only possible to provide results for vitamin D_3 as additional material information.

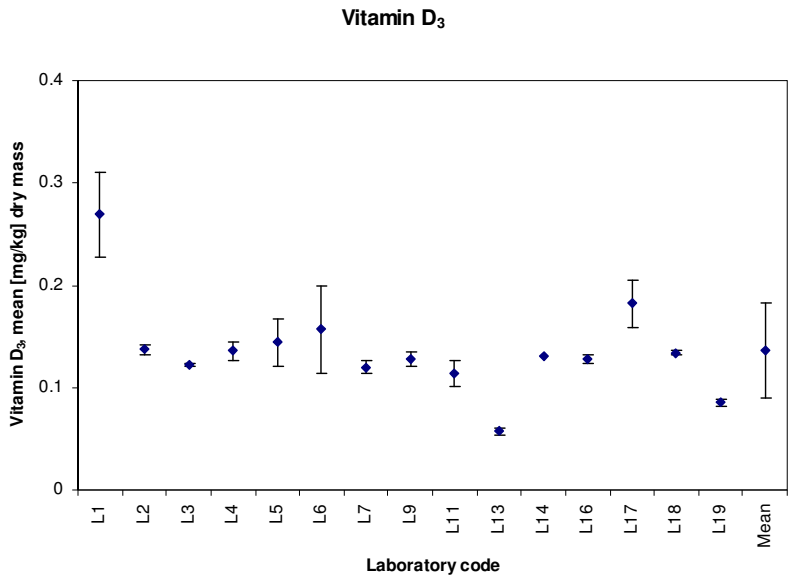


Fig. 11. Accepted data sets for the characterisation of vitamin D_3 in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

Vitamin E was expressed as α-tocopherol mass fraction by a total of 18 collaborating laboratories. The methodology selected by the laboratories for the analysis of this vitamin, as for most of the target vitamins in this characterisation, is also very much in line with the international standard EN 12822. The milk powder sample is submitted to alkaline saponification followed by appropriate extraction. For the determination of the vitamin, laboratories combine either reverse phase or normal phase HPLC with UV or FL detection and external standard calibration is applied by most participants. The submission of the data to the different statistical tests reveals no outliers, either for the laboratory means or for the related variances. Consequently the 18 normally distributed datasets (Fig. 12) are used for value assignment.

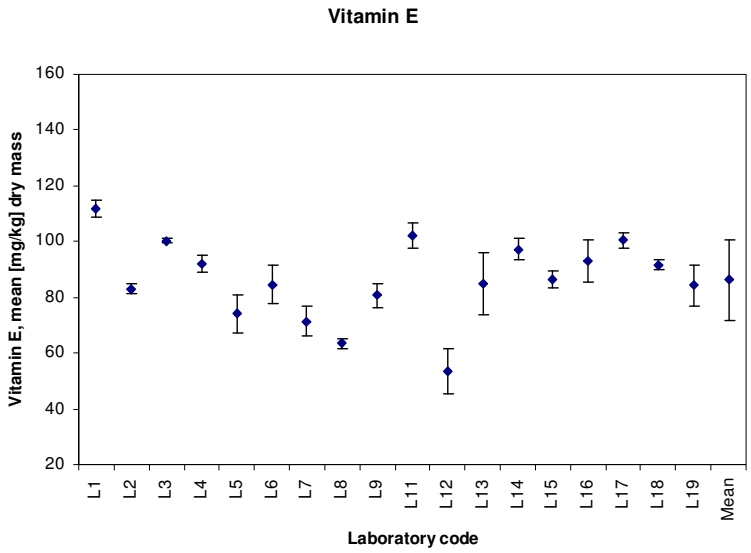


Fig. 12. Accepted data sets for the characterisation of vitamin E in ERM-BD600. Individual results are plotted as the laboratories mean values obtained for 6 independent replicates with error bars representing the standard deviation of the results. The mean of the laboratory means is also indicated with error bars corresponding to the standard deviation.

It should be noted that laboratories were specifically inquired to specify the calibration employed and to report details on the particular substances used for calibration. Generally, calibrants were systematically checked in-house for their purity or the concentration of the calibrants was determined by spectrophotometry at appropriate wavelengths.

9. Certified values and uncertainties

The certified values for ERM-BD600 are calculated as the mean of means of the accepted data sets. The standard error of the mean of means was used as an estimation of the uncertainty contribution of the characterisation exercise. The standard error is calculated as the standard deviation divided by the square root of the number of accepted data sets. Table 9 shows the certified vitamin mass fractions as well as their associated uncertainties, estimated by combining the different contributions as

$$U_{CRM} = k\sqrt{u^2_{bb} + u^2_{lts} + u^2_{char}}$$

Where U_{CRM} is the expanded uncertainty of the certified quantity value

k is the coverage factor (a factor of 2 is applied to give approximately 95% confidence)

u_{char} is the uncertainty associated to the characterisation of the material u_{bb} is the uncertainty associated to the between bottle variation

u_{lts} is the uncertainty contribution from the estimation of the long-term stability study

Table 9. Certified values and uncertainty contributions for vitamin content in milk powder, ERM-BD600.

Component	u_{char} (%)	u_{bb} (%)	u_{lts}(%), (X_{shelf}= 24 months)	U* (%)	**Certified Value** [mg/kg] dry mass	**U*** [mg/kg] dry mass	p
Vitamin A (all-*trans* retinol)	3.4	2.8	5.8	14.6	**3.8**	**0.6**	8
Vitamin A (all-*trans*+13-*cis*-retinol)	3.2	2.8	5.8	18.5	**4.1**	**0.8**	6
Vitamin B_1 (thiamin)	3.7	3.4	4.1	12.96	**4.5**	**0.6**	14
Vitamin B_2 (riboflavin)	2.6	1.9	2.4	8.0	**16.7**	**1.4**	14
Vitamin B_{12} (cyanocobalamin)	2.5	1.0	7.4	20.2	**0.32**	**0.07**	6
Vitamin C (total ascorbate)	5.2	2.3	4.2	14.2	**74**	**11**	15
Vitamin E (α-tocopherol)	3.9	2.1	7.4	17.3	**86**	**15**	18

*k=2 for all vitamins except for vitamins A (all-*trans* + 13-*cis*-retinol) and B_{12} where $t_{(0.05,5)}$=2.57

10. Indicative values

For the reasons expressed in section 8, two vitamin mass fraction values assigned to the material are provided as indicative.

Table 10. Indicative values and uncertainty budget for vitamins in ERM-BD600

Component	**Indicative Value** [mg/kg] dry mass	**U** [mg/kg] dry mass	p
Niacin	**8.0**	**2.8**	10
Total folate	**0.55**	**0.16**	9

11. Additional material data

This information can be used to complement the information of the certificate of this material. The values for vitamins B_6 and D_3 could not be certified due to the presence of outliers provoking a lack of agreement between the results, whereas total folate as obtained by LC-IDMS is provided as complementary information. The data given in Table 11 is to be taken as informative values only.

Table 11. Additional material information for vitamin D_3 in ERM-BD600. These results are not certified values and are informative only.

Component	**Mass fraction range** [mg/kg] dry mass	p
Vitamin B_6 (total pyridoxine)[1]	4.53-8.31	11
Vitamin D_3 (cholecalciferol)	0.057-0.269	15
Total folate	0.74[2]	1

[1] Expressed as pyridoxine base
[2] Mean value from a single dataset obtained by LC-IDMS/MS

12. Metrological Traceability

The range of vitamin determinations in ERM-BD600 was carried out by independent laboratories with demonstrated qualification and accreditation in the field. They employed well characterised, understood and validated methodology among which existing standard methods were extensively applied. In addition the laboratories employed calibrants of their own choice which are commercially available from various sources. In many cases the laboratories performed additional in-house purity checks to verify supplier certificates prior to the measurements.

For **vitamin A** the milk sample underwent alkaline saponification at different conditions, in some cases including antioxidants and nitrogen flushing. Then it continued by liquid-liquid or solid phase extraction with a variety of organic solvents mixtures and subsequently followed LC separation, employing several normal phase chromatographic columns, with different types of detection. The final determination was achieved by external standard calibration. The measurand identity is operationally defined, by the use of normal phase LC and the quantity value is traceable to the SI as appropriately characterised standards were used.

For the water soluble **vitamin B_1**, the method involved acidic extraction at different conditions followed by enzymatic hydrolysis with a range of enzymes and incubation conditions. HPLC separation involved the use of various chromatographic columns and detectors; a pre- or post-column derivatisation step was added prior to FLD. External standard calibration was applied using pure substances from diverse sources. Therefore the measurand is operationally defined by the use of HPLC methods, and the quantity value is traceable to the SI.

Vitamin B_2 was extracted by acid hydrolysis followed by enzymatic digestion applied with different enzymes under diverse incubation conditions. HPLC separation involved the use of different chromatographic columns; in some cases a post-oxidation step was added prior to detection by FLD. External standard calibration was applied using pure substances from

diverse sources. Therefore the measurand is operationally defined by the use of HPLC methods including enzymatic sample treatment with the quantity value traceable to the SI.

The determination of **vitamin B_6**, as total pyridoxine, included the sample extraction in acidic media at different conditions as well as diverse enzymatic treatments for dephosphorylation. HPLC separation involved the use of different chromatographic columns at different experimental conditions prior to detection by FLD. External standard calibration was applied using pure substances from diverse sources. Therefore the measurand is operationally defined by the use of HPLC methods including enzymatic sample treatment, and the quantity value is traceable to the SI.

Vitamin B_{12} value is established by results predominantly obtained by a number of liquid chromatographic methods although very well correlated with those from a biosensor protein-binding assay using surface plasmon resonance. Different procedures employed for sample preparation included buffer extraction in the presence of cyanide followed by enzymatic digestion using different enzymes. In some cases immunoaffinity colums are employed for purification prior to HPLC. The measurand is structurally defined because of the use of different methods and traceable to the SI.

Niacin is determined by methodologies including either liquid chromatography with FL or MS detection or microbiological assays. Post-column derivatisation was performed prior to FLD and *L. plantarum* was the microorganism used for the turbidimetric detection. Sample treatment comprised the vitamin extraction in different conditions, including acidic or enzymatic hydrolysis. In this case the measurand is structurally defined because of the use of independent methods. Characterised calibrants were used, and the quantity value is traceable to the SI.

The determination of **total folate** is based on the use of microbiological assays. These were performed by extraction of the sample using different buffers, different enzymes and followed by turbidimetric bacterial growth detection using external standard calibration, in this way defining the measurand. Characterised calibrants were used, and the quantity value is traceable to the SI.

After acidic extraction of the milk sample, the determination of **vitamin C** involved a reduction step for DHAA prior to determination by HPLC-UV or electrochemical detection. Alternatively, the ascorbic acid was oxidised to DHAA followed by derivatisation and HPLC-FLD determination using different conditions. The measurand identity is operationally defined, by the use of liquid chromatography with the quantity value traceable to the SI as appropriately characterised standards were used.

The determination of **vitamin D_3** involved different sample preparation procedures. Subsequent to saponification of the milk sample, at times in the presence of antioxidants, liquid-liquid extraction with different solvents and/or SPE clean up was frequently applied, followed by preparative chromatography at different conditions prior to UV or MS detection. An internal standard was used for quantification. The measurand identity is operationally defined, by the use of HPLC with the quantity value traceable to the SI as appropriately characterised standards were employed.

For **vitamin E** determination the milk sample underwent alkaline saponification at different conditions, in several cases including antioxidants and nitrogen flushing. Then it continued by liquid-liquid or solid phase extraction with a variety of organic solvents mixtures and subsequently followed by LC separation employing a range of chromatographic columns and different detectors. The final determination was achieved by external standard calibration. The measurand identity is operationally defined, by the use of LC with the quantity value traceable to the SI as appropriately characterised standards were used.

13. Instructions for use

Safety precautions

The usual laboratory safety precautions apply.

Minimum sample intake

Before opening, samples should be allowed to equilibrate to room temperature.
The minimum amount of sample to be used for each vitamin is: 1 g for folate and vitamin B_{12}; 2.5 g for vitamins B_1, B_2, B_6 and C; 5 g for vitamins A, D and E.

Correction for dry mass content

The certified and indicative values are assigned to the material applying dry mass correction. The dry matter correction must be made on a separate subsample taken from the same sachet used for the analysis of vitamins. The loss on drying is determined in parallel to the vitamin analysis by drying an aliquot of 1-3 g of milk powder sample in a well ventilated oven at atmospheric pressure for 2 h at 103 ± 2 ºC [32].

Intended use

This material is intended to be used for method performance control and validation purposes (trueness determination). For assessing the method performance, the measured values of the CRMs are compared with the certified values following a procedure described in ERM application note 1 [33]. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_m).
- Combine measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty (u_{Δ}) using a coverage factor of two ($k = 2$), corresponding to a confidence interval of approximately 95 %
- If $\Delta_m \leq U_{\Delta}$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

It should be borne in mind that the methods used in the characterisation are methods routinely applied for measuring vitamins in milk. The agreement of results from different methods demonstrates that the processing did not affect any properties relevant for these methods and that ERM-BD600 behaves like a real sample.

Dispatch and storage

ERM-BD600 samples are dispatched on dry ice. Upon receipt, the material shall be stored at a temperature of -30 ± 5 ºC to ensure stability until analysis.

14. Acknowledgements

The authors would like to thank Alexander Bernreuther and Reinhard Zeleny, from IRMM, and P. Finglas (Institute of Food Research, UK), S.P.J. van Leeuwen (Institute for Environmental Studies (IVM), VU University, Amsterdam, NL) and E. Nordkvist (National Veterinary Institute, SE), as Certification Advisory Panel members, for actively participating in the reviewing of this report.
Likewise the authors would like to thank Katherine Sharpless (NIST, US) for fruitful discussions.

15. References

[1] ISO Guide 34:2000, General requirements for the competence of reference materials producers

[2] ISO Guide 35:2006, Reference materials - General and statistical principles for certification

[3] ISO/IEC Guide 98-3:2008 Uncertainty of measurement -- Part 3: Guide to the expression of uncertainty in measurement (GUM:1995)

[4] Council Directive 90/496/EEC on nutrition labelling for foodstuffs, OJ L 276, 6.10.1990, p. 40–44

[5] Commission Directive 2003/120/EC amending Directive 90/496/EEC on nutrition labelling for foodstuffs, OJ L 333, 20.12.2003, p. 51

[6] Commission Directive 2008/100/EC, OJ L 285, 29.10.2008, p. 9–12

[7] Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements OJ L 183, 12.7.2002, p. 51

[8] Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods, OJ L 404, 30.12.2006, p. 9–25

[9] Regulation (EC) No 1925/2006 of the European Parliament and of the Council on the addition of vitamins and minerals and of certain other substances to foods, OJ L 404, 20.12.2006 p. 26-38

[10] EC no 1170/2009

[11] ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories.

[12] ISO 8156:2005, IDF 129:2005, Dried milk an dried milk products-determination of insolubility index

[13] Kestens, V., Conneely, P., Bernreuther, A. Vaporisation coulometric Karl Fischer titration: A perfect tool for water content determination of difficult matrix reference materials. Food Chem. 106 (2008) 1454

[14] A.M.H. van der Veen, T. Linsinger, J. Pauwels, Uncertainty calculations in the certification of reference materials, 2. Homogeneity study. Accred. Qual. Assur., 6 (2001) 26-30

[15] A. Lamberty, H. Schimmel, J. Pauwels, The study of the stability of reference materials by isochronous measurements, Fres. J. Anal. Chem., 360 (1998) 359-361

[16] T. Linsinger, J. Pauwels, A. Lamberty, H. Schimmel, AMH van der Veen, L. Siekmann. Fres. J. Anal. Chem., 370 (2001) 183-188

[17] European Committee for Standardisation, (2000) Foodstuffs-determination of vitamin A by high performance liquid chromatography – Part 1: measurement of all-*trans*-retinol and 13-*cis*-retinol, EN 12823-1.

[18] European Committee for Standardisation, (2000) Foodstuffs-determination of vitamin D by high performance liquid chromatography: measurement of cholecalciferol (D_3) and ergocalciferol (D_2), EN 12821.

[19] European Committee for Standardisation, (2000) Foodstuffs-determination of vitamin E by high performance liquid chromatography: measurement of α, β,γ and δ-Tocopherols, EN 12822.

[20] European Committee for Standardisation, (2003) Foodstuffs-determination of vitamin C by HPLC, EN 14130.

[21] European Committee for Standardisation, (2003) Foodstuffs-determination of vitamin B_1 by HPLC, EN 14122.

[22] European Committee for Standardisation, (2003) Foodstuffs-determination of vitamin B_2 by HPLC, EN 14152.

[23] Official Methods of Analysis (2000) 17th Ed., AOAC International, Gaithersburg, MD, Method 944.13

[24] Official Methods of Analysis (2000) 17th Ed., AOAC International, Gaithersburg, MD, Methods 985.34

[25] Official Methods of Analysis (2000) 17th Ed., AOAC International, Gaithersburg, MD, Method 961.14

[26] Official Methods of Analysis (2000) 17th Ed., AOAC International, Gaithersburg, MD, Method 975.41

[27] Official Methods of Analysis (2000) 17th Ed., AOAC International, Gaithersburg, MD, Method 981.16

[28] Official Methods of Analysis (2000) 17th Ed., AOAC International, Gaithersburg, MD, Method 968.32

[29] The EFSA Journal (2008) 864, 1-31. http://www.efsa.europa.eu/en/scdocs/doc/ans_ej864_Benfotiamine_op_en.pdf?ssbinary=true

[30] S. Ndaw, M. Bergaentzlé, D. Aoudé-Werner, C. Hasselmann, Extraction procedures for the liquid chromatographic determination of thiamin, riboflavin and vitamin B6 in foodstuffs, Food Chemistry 71 (2000) 129-138

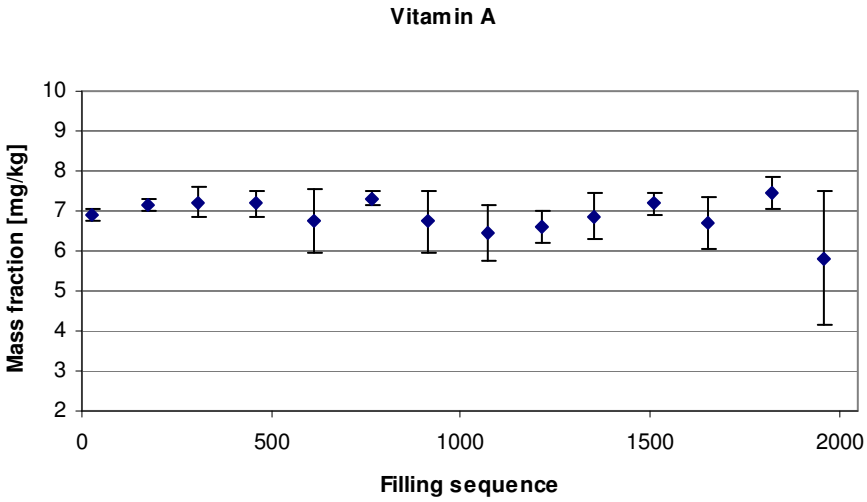
[31] Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts and Minor Constituents. P. L. H. McSweeney, Patrick F. Fox Eds., 2009, p.609. ISBN: 978-0-378-84864-8

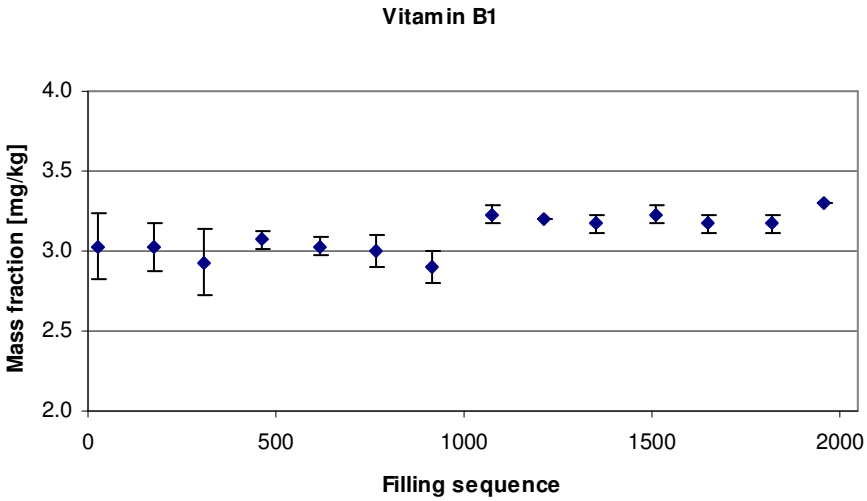
[32] IDF Standard 26A:1993 (ISO/CD 5537), Dried milk and dried cream determination of water content.

[33] Linsinger, T.P.J. Comparison of measurement result with the certified value, ERM Application Note 1, July 2005, http://www.erm-crm.org

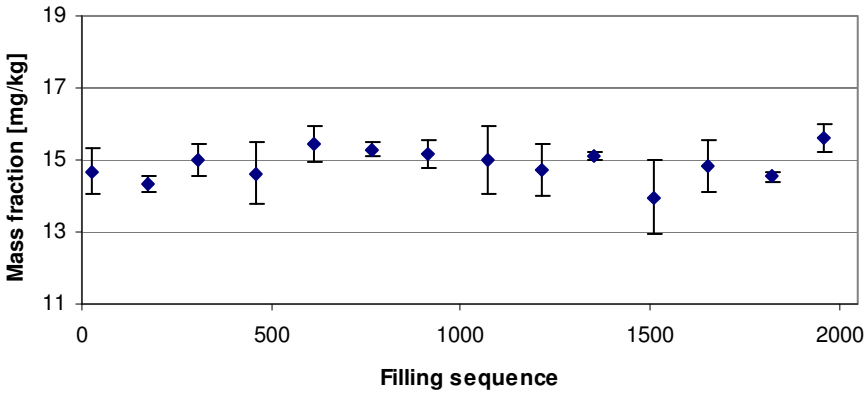
Annex A. Homogeneity results

Results from the homogeneity study of vitamins in milk powder ERM-BD600, as mean mass fraction values of 3 independent replicates per sachet.

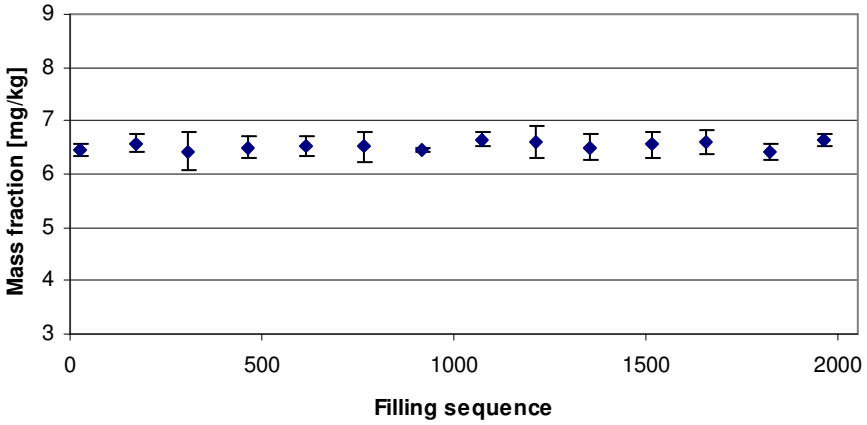




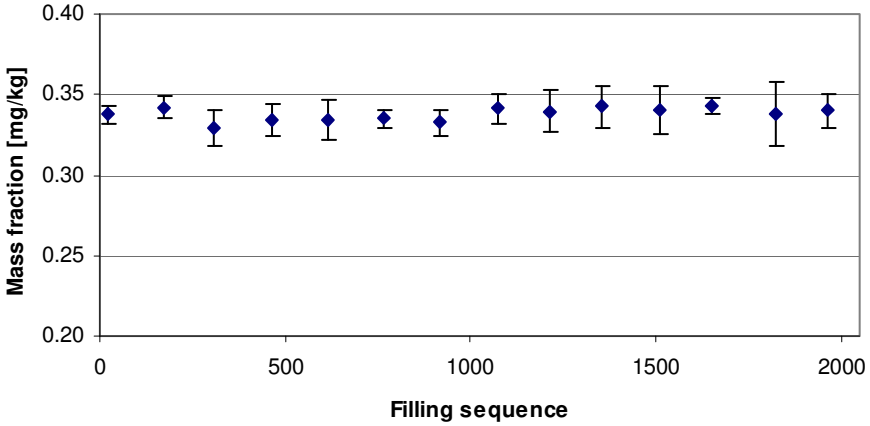
Vitamin B2



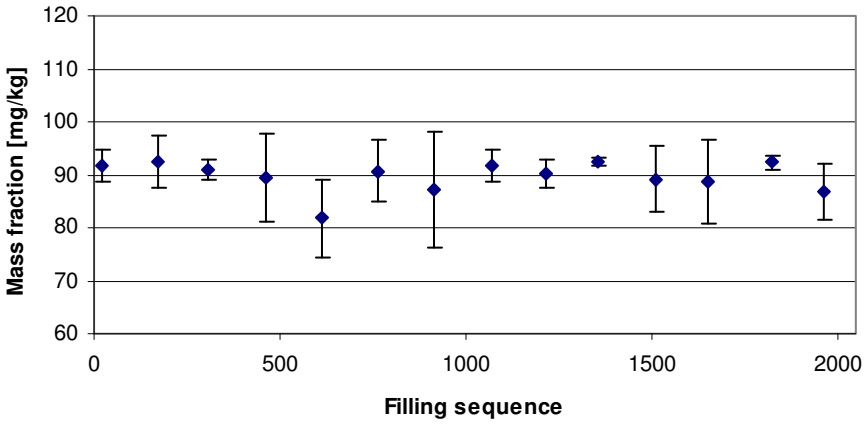
Vitamin B6



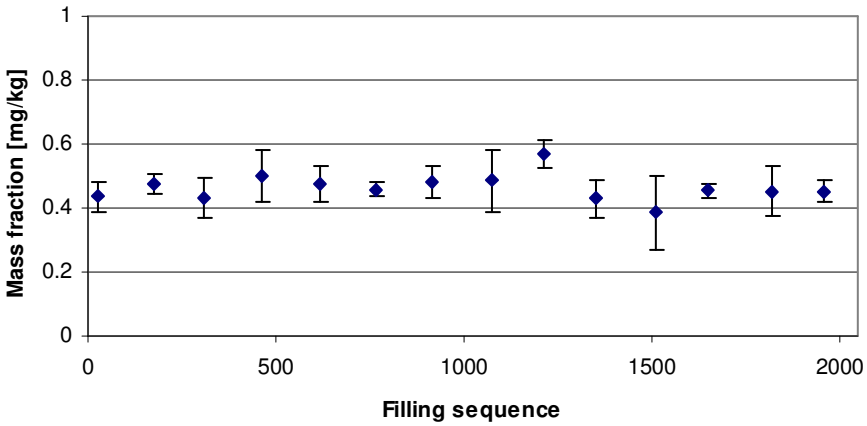
Vitamin B12



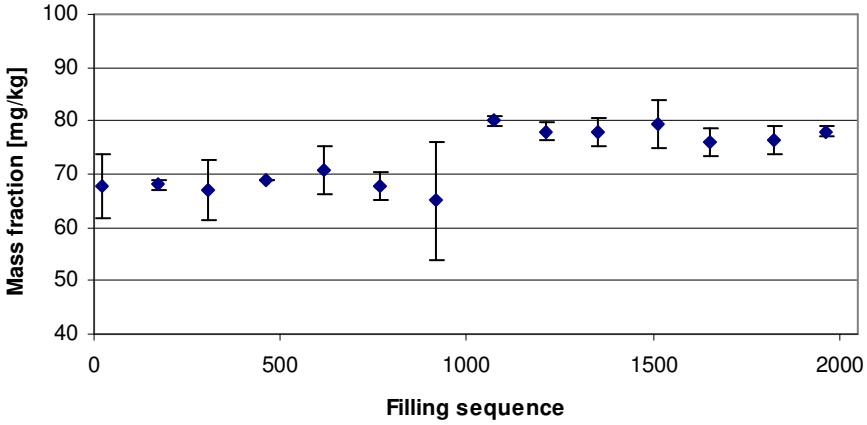
NIACIN

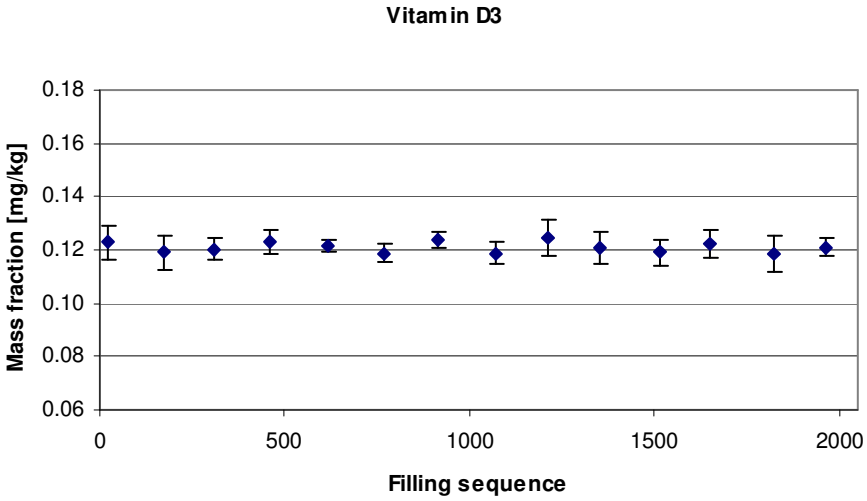


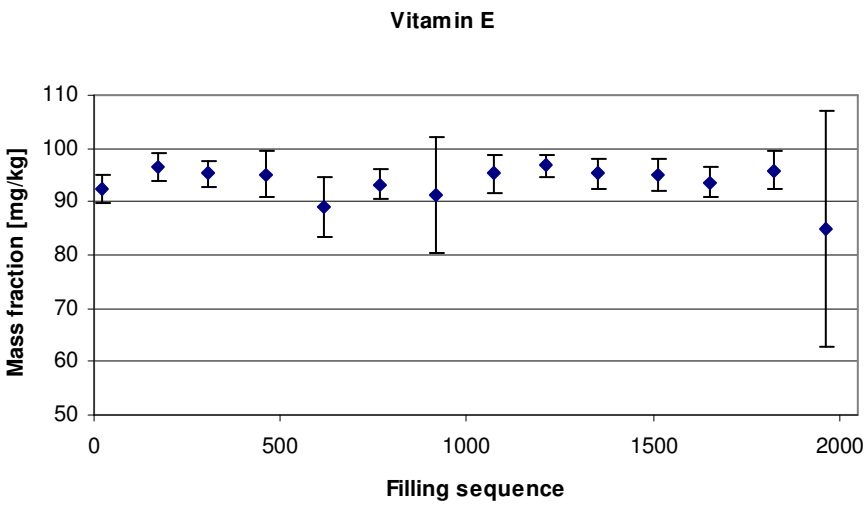
FOLATE



Vitamin C




Vitamin D3
Mass fraction [mg/kg]
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0
500
1000
1500
2000
Filling sequence


Vitamin E
Mass fraction [mg/kg]
110
100
90
80
70
60
50
0
500
1000
1500
2000
Filling sequence

Brief description of methods applied during homogeneity and short term stability studies

Vitamins A and E (reference methods EN 12823-1 and EN 12822)
Vitamins A and E are saponified by using ethanolic potassium hydroxide solution and extracted two times with hexane:ethylacetate (85:15 v/v). The determination is carried out by RP-HPLC with fluorometric detection vitamin A (Ex: 325 nm, Em:475 nm) and vitamin E (Ex: 290 nm, Em:327 nm). Sample intake, 10 g

Vitamin B_1 (reference method EN 14122)
Vitamin B1 is extracted from the sample in an autoclave using acid hydrolysis followed by enzymatic dephosphorylation and quantified by RP-HPLC with flourimetric detection (Ex:368 nm, Em 440 nm) after post column oxidation to thiochrome. Sample intake, 2.5 g

Vitamin B_2 (reference method EN 14152)
Vitamin B2 is extracted from the sample in an autoclave using acid hydrolysis followed by enzymatic dephosphorylation and quantified by RP-HPLC with fluorimetric detection (Ex: 468 nm, Em 520 nm). Sample intake, 2.5 g

Vitamin B_6 (reference method EN 14164)
Vitamin B_6 is extracted from the sample in an autoclave using acid hydrolysis followed by enzymatic dephosphorylation. By reaction with glyoxylic acid in the presence of Fe^{2+} as catalyst, pyridoxamine is transformed into pyridoxal, which is then reduced to pyridoxine by the action of sodium borohydride in alkaline medium. Pyridoxine is finally quantified by HPLC with fluorimetric detection (Ex: 290 nm, Em: 395 nm). Sample intake, 2.5 g

Vitamin B_{12} (reference method AOAC 952.20 / 45.2.02)
Vitamin B_{12} is extracted from the sample in an autoclave using a buffered solution. After dilution with basal medium (containing all required growth nutrients except cobalamin) the growth response of Latobacillus leichmanii (ATCC 7830) to extracted cobalamin is measured turbidimetrically. This is compared to calibration solutions with known concentrations. Sample intake, 2.5 g

Vitamin C (reference method AOAC 967.22)
The ascorbic acid is extracted in an acidic solution and oxidized to dehydroascorbic acid in the presence of Norit. Oxidized form reacts with o-phenylenediamine to produce fluorophor having activation maximum at ca 350 nm and fluorescence maximum at ca 430 nm. Fluorescence intensity is proportional to concentration. Sample intake, 2.5 g

Niacin (Reference method: AOAC 944.13)

Niacin is extracted from the sample by autoclaving using acid hydrolysis. The sample is diluted with basal media, containing all required growth nutrients except nicotinic amid and nicotinic acid. The growth response of *Lactobacilius plantarum* (ATCC 8014) is measured turbidmetrically and compared to calibration solutions with known concentrations. The niacin is measured as a sum of nicotinic acid and nicotinic amide. Sample intake, 2.5 g

Folate (reference method AOAC 944)

Folate (including folic acid) is extracted from the sample in an autoclave using a buffer solution, followed by an enzymatic digestion with human plasma and pancrease V and finally by a second autoclave treatment. After dilution with basal medium containing all required growth nutrients except folic acid and growth response of *Lactobacillus rhamnosus* (ATCC 8043) to extracted folate is measures turbidimetrically and compared to calibration solutions with known concentrations. Sample intake, 2.5 g

Vitamin D (reference method EN 12821)

Vitamin D is saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted with dieethylether. The extract is concentrated and cleaned up by SPE , followed by normal phase semi-preparative. The amount of vitamin D_2 or D_3 is determined by reverese phase HPLC with DAD (265 nm) and MS detection. Sample intake, 10 g

Dry mass – Karl Fisher method:

The method determines the actual water contents of fats, oils and many foods by titration with Karl Fisher reagent, which reacts quantitatively with water.

Annex B. Short-term stability results

Summary results table of ERM-BD600 at -30, 4 and 18 ºC expressed as dry mass fraction (mg/kg) of vitamins, mean of triplicate analysis

Vitamin		-70 ºC	Temperature -30 ºC			Temperature +4 ºC			Temperature +18 ºC		
		Ref	Time [weeks]			Time [weeks]			Time [weeks]		
[mg/kg] dry mass	samples	Time 0	1	2	4	1	2	4	1	2	4
A	1	7.73	8.47	8.14	8.23	8.31	7.63	7.76	7.08	7.24	6.84
	2	7.46	8.33	8.15	8.21	7.88	7.69	6.98	7.12	7.28	6.78
	3	7.52	8.41	8.06	7.88	7.75	7.57	7.49	6.95	7.00	6.77
B1	1	3.33	3.28	3.27	3.34	3.35	3.35	3.38	3.43	3.42	3.38
	2	3.40	3.25	3.35	3.28	3.32	3.35	3.38	3.39	3.41	3.44
	3	3.33	3.28	3.26	3.28	3.39	3.35	3.43	3.36	3.38	3.39
B2	1	15.08	16.06	16.08	15.83	15.98	15.74	14.96	16.17	16.66	16.17
	2	15.75	15.89	15.83	15.75	15.65	15.71	15.48	16.12	16.29	16.56
	3	15.27	15.90	15.62	15.87	15.79	14.58	15.63	16.01	15.43	16.35
B6	1	7.39	7.17	7.03	6.88	7.00	7.04	7.09	6.08	6.50	5.91
	2	7.10	7.19	7.11	6.97	7.32	7.05	6.88	6.29	6.76	6.31
	3	6.86	7.18	7.00	7.08	6.81	6.86	7.00	6.50	6.16	5.89
B12	1	0.36	0.36	0.36	0.36	0.36	0.37	0.38	0.36	0.36	0.36
	2	0.37	0.36	0.36	0.36	0.36	0.37	0.36	0.35	0.36	0.36
	3	0.37	0.36	0.36	0.37	0.36	0.37	0.36	0.36	0.36	0.36
Niacin	1	10.89	10.56	9.36	8.37	10.96	9.46	9.82	12.13	10.92	11.61
	2	11.08	9.88	9.59	8.39	9.51	9.97	9.67	11.82	13.51	11.91
	3	10.58	9.01	9.20	7.16	9.72	9.54	9.94	11.38	12.33	14.74
Folate	1	0.41	0.42	0.39	0.38	0.41	0.41	0.45	0.55	0.67	0.66
	2	0.45	0.46	0.39	0.35	0.44	0.42	0.48	0.66	0.54	0.68
	3	0.40	0.45	0.38	0.51	0.43	0.43	0.46	0.63	0.74	0.59
C	1	82.98	83.37	80.4	84.94	81.85	82.26	80.66	79.02	79.93	76.07
	2	82.93	83.34	81.37	81.3	81.68	82.18	81.23	80.12	78.37	76.66
	3	82.58	85.21	80.7	82.03	82.54	82.89	81.41	79.51	76.9	77.65
D3	1	0.12	0.12	0.12	0.11	0.11	0.12	0.11	0.12	0.12	0.12
	2	0.11	0.12	0.11	0.12	0.11	0.11	0.12	0.14	0.12	0.11
	3	0.11	0.12	0.12	0.11	0.11	0.12	0.12	0.12	0.12	0.12
E	1	104.55	108.06	105.15	106.51	111.52	101.92	105.99	98.65	102.35	99.89
	2	98.57	108.01	105.42	105.35	106.60	103.25	95.45	101.57	101.73	100.37
	3	100.96	106.58	105.90	100.64	105.55	102.75	104.06	100.35	101.42	101.27

Annex C Long-term stability results

Summary results of ERM-BD600 at -30 and 4 ºC expressed as dry mass fraction (mg/kg) of vitamins, mean of duplicate analysis.

Vitamin		-70 ºC Ref	Temperature -30 ºC Time [months]			Temperature +4 ºC Time [months]		
[mg/kg] dry mass	samples	Time 0	4	8	12	2	4	6
A	1	7.3	7.2	6.5	7.3	6.6	7.1	6.8
	2	6.8	7.0	6.8	7.4	6.2	6.9	6.4
	3	7.2	6.6	7.3	7.5	7.5	6.6	7.1
B1	1	4.8	4.7	4.6	4.8	5.0	4.9	5.1
	2	4.9	4.6	4.7	4.8	5.2	5.0	4.9
	3	4.7	4.8	4.9	4.6	4.7	4.9	5.1
B2	1	17.1	17.1	17.1	17.2	18.0	18.1	18.0
	2	17.2	16.6	17.2	17.1	17.4	18.3	18.2
	3	16.9	17.0	17.8	18.0	18.1	18.2	18.6
B6	1	5.0	4.9	4.8	4.8	4.8	4.7	4.7
	2	4.8	4.8	4.7	4.8	4.7	4.6	4.7
	3	4.7	4.8	4.8	4.8	4.7	4.7	4.7
B12	1	0.296	0.303	0.335	0.326	0.309	0.304	0.316
	2	0.288	0.334	0.327	0.326	0.279	0.305	0.308
	3	0.326	0.327	0.328	0.296	0.296	0.302	0.312
Niacin	1	10.6	9.8	9.9	8.9	10.3	12.0	9.4
	2	10.3	10.3	10.1	9.7	10.3	9.4	8.6
	3	12.1	10.8	9.1	10.7	11.5	11.2	9.4
Folate	1	0.349	0.356	0.337	0.355	0.378	0.329	0.312
	2	0.352	0.360	0.346	0.354	0.401	0.358	0.365
	3	0.383	0.370	0.338	0.374	0.345	0.369	0.348
C	1	62.4	66.1	67.1	65.9	74.0	73.5	72.0
	2	66.8	65.7	66.3	68.5	76.0	71.6	68.8
	3	64.9	64.4	66.5	65.0	73.4	71.7	70.3
D3	1	0.124	0.123	0.125	0.126	0.131	0.131	0.140
	2	0.124	0.125	0.126	0.126	0.143	0.134	0.134
	3	0.126	0.125	0.126	0.126	0.130	0.137	0.149
E	1	90.6	85.6	76.3	87.4	80.9	82.2	92.2
	2	81.8	83.0	79.6	87.7	75.9	89.2	85.8
	3	85.9	76.6	86.1	91.7	85.2	91.3	93.4

Brief description of methods applied during long term stability studies

Vitamin A

Sample mass: 2.5 g. Saponification with KOH aqueous solution (50:50); acid ascorbic solution and ethanol. Reflux 30 minutes. Extraction with PE (80 ml + 40 ml + 40 ml). Wash KOH aqueous-alcoholic solution (30gr KOH+100ml EtOH+900 ml water) and then with water to neutral pH.

Purified fraction is evaporated and dissolved in methanol.

RP-HPLC, Atlantis® dC18 3µm 4.6x20 mm. Water:Acetonitrile (5:95) with 0.1% TFA acid. Temperature: 25 ºC. UV at 325 nm

External calibration, vitamin A Alcohol (all-trans retinol) Fluka, based on peak area.

Vitamin B1

Sample mass: 2.5 g. Acid digestion of the sample with HCl 0.1 M (30 minutes, 121ºC). Treatment with claradiastase for 16 h at 37 ºC. Derivatisation with potassium ferrocyanide solution (1%)

RP-HPLC, Atlantis dC18 5 µm 4.6x150 mm, Water-Methanol (65:35). FLD: E_{ex} = 365 nm E_{em} = 435 nm

External calibration, thiamin chloride hydrochloride (Sigma), based on peak area.

Vitamin B2

Sample mass: 2.5 g. Acid digestion of the sample with HCl 0.1 M (30 min, 121 ºC). Treatment with claradiastase for 16 h at 37 ºC.

RP-HPLC , Atlantis dC18 5 µm 4.6x150 mm, Water-Methanol (65:35). FLD: E_{ex} = 450 nm E_{em} = 510 nm

External calibration, riboflavin (Sigma), based on peak area.

Vitamin B6

Sample mass: 2.5 g. Acid digestion of the sample with HCl 1 M (30 min, 121 ºC). Treatment with claradiastase for 16 h at 37 ºC, at pH = 4,5 +/- 0,1. Derivatization with glyoxilic acid, ferrous sulfate, and sodium borhydride.

RP-HPLC, Atlantis ® C18 5 µm 4.6x150 mm, gradient: 0.1% Trifluoroacetic acid in water, 0.1% Trifluoroacetic acid in acetonitrile. Temperature: 40 ºC. FLD: E_{ex} = 292 nm E_{em} = 398 nm

External calibration, pyridoxine hydrochloride (Fluka), based on peak area.

Vitamin B12

Sample mass: 2.5 g. Digestion of the sample in presence of sodium acetate solution, potassium cyanide solution (1%), α-amylase and pepsin (37 ºC during 30 min, and then 84 ºC during 30 minutes). Filter and purification with inmunoaffinity column (R-Biopharm Rhône). Elution with methanol, evaporation and reconstitution in 0.025 % TFA in water.

RP-HPLC, ACE 3 AQ 3.0x150 mm, gradient: 0.025 % TFA acid in water, acetonitrile. Temperature: 30 ºC. DAD: at 361 nm

External calibration, cyanocobalamin (Sigma), based on peak area.

Niacin

Sample mass: 2.5 g. Extraction of the sample with chlorhydric acid 0,5 N, 60 min at 121 ºC. Cool and adjust pH at 6.8 with sodium hydroxide solution 15 %. Filter.

Microbiological method. Organism growth using *Lactobacillus plantarum* (CECT 748, ATCC14917), pH = 6,8 +/-0,1. Incubation for 15 h at 37 ºC

Spectrophotometric detection at 546 nm

External calibration, nicotinamide (Sigma)

Total Folate

Sample mass: 2.5 g. Extraction of the sample with phosphate buffer 0.05 N (pH=7,2), 15 minutes at 121 ºC. Cool and treat with common pancreatine (Merck), 24 h at 37 ºC. Inactive 5 minutes at 121 ºC, cool and filter.

Microbiological method. Organism growth using *Lactobacillus casei* (CECT 278), pH = 6,8 +/-0,1. Incubation for 15 h at 37 ºC

Spectrophotometric detection at 546 nm

External calibration, folic acid (Sigma)

Vitamin C

Sample mass: 2.5 g.Treatment with extraction solution (metaphosphoric acid 4% in water: Metanol), filtration and injection

RP-HPLC, Waters Atlantis C18 3 µm 4.6x150 mm, TFA 0.1 % in water

UV-VIS at 254nm

External calibration, ascorbic acid (Fluka), based on peak area.

Vitamin D_3

Sample mass t: 2.5 g. Addition of internal standard (vitamin D_2 solution). Saponification with 50 ml of potassium hydroxide (KOH) aqueous solution (50:50); 10 ml of acid ascorbic solution (3,5 grams of ascorbic acid in 20 ml of NaOH 1N solution) and 50 ml of ethanol. Reflux 30

minutes. Extraction with PE (80 ml + 40ml + 40 ml). Wash with 3 portions of 40 ml of KOH aqueous-alcoholic solution (30 g KOH+100 ml EtOH+900 ml water) and then with water to neutral pH. Clean-up with NP-HPLC.

RP-HPLC Nova-Pak® C18 4□m 4.6x250 mm with pre-column Nova-Pak® C18 4 µm

Detector UV: 265 nm.

Relation of peak height between internal standard (vitamin D_2, Fluka (Sigma-Aldrich)) and vitamin D_3, Fluka (Sigma-Aldrich).

Vitamin E

Sample mass: 2.5 g. Saponification with 50 ml of KOH aqueous solution (50:50); 10 ml of acid ascorbic solution (3,5 grams of ascorbic acid in 20 ml of NaOH 1 N) and 50 ml of ethanol. Reflux 30 minutes. Extraction with PE (80 ml + 40ml + 40 ml). Wash with 3 portions of 40 ml of KOH aqueous-alcoholic solution (30 gr KOH+100 ml EtOH+900 ml water) and then with water to neutral pH. Purified fraction is evaporated and dissolved in methanol.

RP-HPLC, Atlantis® dC18 3 µm 4.6x20 mm, Acetonitrile (5:95) with 0.1% TFA acid

FLD: E_{ex} = 295 nm E_{em} = 340 nm

External calibration, DL-α-tocopherol (Aldrich), based on peak area.

Dry matter

The water content of the sample is determined by the loss of weight of the sample after 2 h in a ventilated oven, at atmospheric pressure, at 103ºC+/- 2ºC.

Annex D. Characterisation methods and results

Table D.1. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin A in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	10	Homogenise with H2O containing ascorbic acid. Saponify with KOH:H2O:EtOH, N2 flushing, 30 min at 70ºC; extract into PE; wash with water and evaporate to dryness; redissolve in MeOH	HPLC. RP18 5µm; MeOH:H2O 95:5; UV at 325 nm	External calibration using all-trans retinol standard treated as sample; (no saponification); peak area
2	50	Slurry preparation, saponification (30min at 85ºC), SPE clean-up, elution, evaporation of eluate to dryness, take up in mobile phase .	NP-HPLC. Tracer Extrasil Si 250x4.6mm 5µm ref. TR-016064. No T program. UV at 326 nm	Standard: Retinol Sigma ref. 95144. Purity corrected by UV spectrophometry. Working standard solutions in hexane and ethanol. Quantitation through linear regression (peak heigh vs retinol content).
3	10	Alkaline saponification, liquid/liquid extraction (pentan:ethylacetate 80:20).	NP-HPLC-UV at 325 nm.	External standard, all-trans retinol, BioChemika. 13-cis retinol assess due to the difference in absortion coefficient of 1,10 compared to all-trans retinol (Stancher & Zonta, J Chrom, 1984, 212, 423-434
4	10	Suspension with water, saponification with 5N ethanolic KOH, Na-sulfide, ascorbate	automatic sampler, 20 µl NP HPLC, isocratic Merck Lichorosorb 60 250/4; 5 µm Fluorescence; Ex: 325, Em: 470	External calibration, photometric determination of standard concentration
5	5-10	Saponification with KOH and extraction with PE	HPLC. RP18 5µm; MeOH:H2O 95:5; UV at 325 nm	External calibration using all-trans retinol standard; peak area
6	10	Saponification followed by extraction into mixed ethers. Extract evaporated to dryness and residue redissolved in methanol.	HPLC, Partisil 10 ODS2 with FLD detection	All-trans retinol calibration standards. 13-cis retinol calculated against all-trans-retinol with appropriate correction for response.
7	50	Saponification with KOH and Na-sulphide in alcohol-water mixture, and Na-ascorbate as antioxidant. Extraction with PE. Evaporation of organic phase. SPE, *Chromabond* cartridges.	Injection volume 50µl. Partial loop. NP-HPLC,Lichrospher Diol 5µm, 15cm 250-4 Guard column: Flow: 1,1 ml/min Pressure: ~50bar UV at 326nm and FLD detection	Calibration curve Standard Retinol (all trans): Sigma (95144) ≥ 99%

8	4	Saponification with KOH methanolic solution, adding pyrogallol 30 min at 75°C, extraction with PE	Inj. Vol. 20 µl at room temperature RP-HPLC Lichrospher 100RP18 (125x 4mm, 5µm) in Methanol/water eluant UV at 325 nm	External calibration-calibrant:retinol acetate (VWR)
9	15	Saponification with KOH methanolic solution, followed by liquid-liquid extraction	V = 100µl HPLC Lichrospher Si 60, 5.0µm, 250 x 4.0 mm, UV at 325 nm	External calibration, retinol BioChemika 95144 (>99%)
10				
11	10	Saponified by using ethanolic potassium hydroxide solution and 2 extractions with hexane:ethylacetate (85:15 v/v).	RP-HPLC UV/DAD at 325 nm.	External calibration curve. Sigma, purity > 98 %. Purity determined spectrofotometrically (Vitamin A 325 nm)
12	10	Saponification with KOH and extraction with THF/ethanol	HPLC-FLD	External calibration
13	20	Saponification, extraction with PE,	RP-HPLC, UV	External calibration with commercial standards
14	20	saponification, liquid-liquid purification	HPLC-FLD	Calibration at the beginning of all sequence
15				
16	5-10	Saponification, liquid-liquid extraction	HPLC-NP; silica phase column with guard column. Substance identification: retention time (fluorescence and UV detection)	External standard calibration. Photometric verification of the standard solution.
17	5	Saponification , extraction with diethylether	HPLC-DAD	External calibration
18	2.5	Ascorbic acid. Saponification with KOH, 30 min at 95°C. Extraction with cyclohexane on a Chem Elut (polar) column.	HPLC: Reprosil Amino column, 150 × 4.6 mm, 3 µm particles. Mobile phase: 2 % isopropanol in n-heptane. UV at 325 nm.	External, trans-retinol (Fluka) and 13-cis-retinol (Sigma)
19	5	Saponification (EN ISO 14565:2000, 9.2.) reflux with KOH + EtOH (ca 80 ^{0}C) for 60 min under nitrogen; Extract with PE /40-70/; Change of solvent to MeOH under nitrogen	HPLC-DAD 325 nm, Waters symmetry C18 3.5µm 4.6*75mm Eluent 5% water 95% MeOH	External calibration Retinol-acetate (Dr. Ehrenstrorfen) Photometric verification of the standard solution

Table D.2. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin B_1 in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	2.5	Extract with HCl. Autoclave at 121 ºC/5 hours. Adjust pH to 4.5 , add takadiastase. Incubate at 37 ºC for 16 h. Pre column oxidation with alkaline hexacyanoferrate solution	HPLC RP-8 5 µm MeOH:acetate buffer 40:60	External calibration using thiamin standard treated as sample
2	50	Slurry preparation and dilution, enzymatic hydrolisis followed by acid hydrolisis with HCl (30min at100ºC), SPE clean-up, oxidation to thiochrome.	Automatic injector., V= 10µL. No temperature control. HPLC column Spherisorb ODS 5µm 250x4.6mm Waters ref. PSS831915. No T program. FLD excitation at 368 nm emission at 440 nm.	Quantitation through linear regression (peak heigh vs thiamin mononitrate content). Standard: Thiamin mononitrate DSM ref. 0418943.
3	2.5	Acid hydrolysis with HCl followed by enzymatic with phosphatase, proteinase and amylase.	Post-column derivatisation followed by fluorescence detection (Ex. 368 nm/Em. 420 nm).	External standard. Thiamin hydrocholoride, USP reference from Rockville, MS, USA
4	3	Acidic extraction with 0,1N HCl, enzymatic digestion with phosphatase	Automatic sampler, 5 µl RP HPLC, Macherey Nagel Nucleodur C18 Gravity 50/4.6; 5 µm FLD Ex: 365, Em: 436	External calibration, photometric determination of standard concentration
5	2.5	Extract with HCl (0.1 mol/l). Autoclave at 121 ºC/ 30 min. Treatment with claradiastase at 37 ºC for 16 h. Derivatisation with potassium ferrocyanide solution (1 %)	HPLC separation using Atlantis dC18 5µm; FLD lex 365 nm, lem 435 nm	External calibration using thiamin chloride hydrochloride, peak area
6	5	Acid hydrolysis, enzyme treatment and oxidation to thiochrome with ferricyanide.	RP HPLC, Supelco LC 18-DB, with FLD detection	Range of thiamin HCl standard solutions oxidised to thiochrome
7	5	Extraction by enzymatic treatment with takadiastase followed by acid hydrolysis. Oxidation to thiochrome by potassium ferricyanide in alkaline medium. Clean-up on strong cation-exchange SPE Column.	Injection volume 20µl Partial loop Column: ODS 3µ Hypersil 100x4,6mm Guard Column Betabasic 18 3µm 10x3mm PN Thermo Flow 1,75 ml/min, Max pressure 300bar Temp. 30ºC FLD Ex=368nm Em=440nm	External calibration, Thiamin: Acros (14899) 98,5-101,5%
8	4	Acid hydrolysis,enzymatic treatment, oxydation of thiamin in thiochrome	injection of 20 µl at room temperature	Lichrospher 60 RPSelectB,250X4mm-5µm in Methanol/water eluant

9	2,0	Extraction by acid hydrolisis followed by dephosphorilation by enzymatic hydrolisis.	Injection volume = 10µl RP-HPLC C8 – 4.6 mm x150 mm-5µm and post-column derivatisation FLD ex: 366 nm em: 435 nm	External calibration with thiamin chlorhydrate (PM = 337,3) Sigma T-4625 (>99%)
10				
11	2,5 g	Extraction using acid hydrolysis followed by enzymatic dephosphorylation.	RP-HPLC with FLD (Ex:368 nm, Em 440 nm) after post column oxidation to thiochrome.	3-point calibration curve. Calibration standards from Sigma, purity > 98 %. Purity determined by spectrofotometry (UV 247 nm).
12	3	Acid and enzymatic hydrolysis and extraction with sodium acetate	HPLC-FLD	External calibration
13	1-10	Liberation with sulphuric acid and diastase treatment, reaction to thiochrome and RP-HPLC	RP-HPLC-FLD	External calibration with commercial standards
14	5	Enzymatic extraction	HPLC, derivatisation post column , FLD	External calibration
15				
16				
17	2.5	Acidic hydrolysis with sulphuric acid, aqueous extraction, Clean-up by ion exchange extraction, Thiochrome formation	HPLC-FLD	External calibration
18	2.5	Acidic hydrolysis, 0.1 M HCl 121°C 30 min and enzymatic treatment, Takadiastase 45°C 18 h	HPLC:Phenomenex Gemini C18 250x4.6mm 5 µm 110 Å. Mobile phase MeOH:H2O 40:60 pH 9.0 FLD after pre-column derivatisation with K3Fe(CN)6	External, Thiaminchloride hydrochloride (Sigma)
19	5	Extract with 1% HCl aq. 50 0C for 1 hour (0,5 hour in ultrasonic bath + 0,5 hour with temperature controlled shaker)	HPLC-MS SIM Column Macherey Nagel NUCLEODUR C18 Gravity 5 µm 150mm*4.6mm Eluent: A 2g/l Ammoniumcarbonate B MEOH gradient: 0-0.5 min 10% B 0.5-5 min. 60% B 5-11 min 60% B post time: 6 min	External calibration Name: Thiamin-HCl Purity: >= 98,5% Provider: ROTH

Table D.3. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin B_2 in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	2.5	Extract with HCl . Autoclave at 121 ºC. Adjust pH to 4.5 using sodium acetate, add takadiastase. Incubate at 37 ºC for 16 h.	HPLC-FLD	External calibration using riboflavin standard treated as sample; peak area
2	50	Slurry preparation and dilution, enzymatic hydrolisis followed by an acid hydrolisis with HCl (30min at 100ºC).	Inj. Vol. 10µL. HPLC-FLD, column Spherisorb ODS 5µm 250 x 4.6mm Waters. No T program. FLD: Ex at 450 nm Em at 530 nm.	standard: Riboflavin Fluka Quantitation through linear regression (peak heigh vs riboflavin content).
3	2.5	Acid hydrolysation with HCl followed by enzymatic treatment with phosphatase, proteinase and amylase.	FLD (Ex. 468 nm/Em 520 nm). Ref.: Jakobsen, J., Food Chem, 2008, 106, 1209-1217.	External standard, Riboflavin
4	3	Acidic extraction with HCl, enzymatic digestion with phosphatase	Automatic sampler, 20 µl RP-HPLC, isocratic. Macherey Nagel Nucleodur C18 125/4; 5 µm FLD: Ex: 465, Em: 525	External calibration, photometric determination of standard concentration
5	2.5	Extract with HCl. Autoclave at 121 ºC. Treatment with claradiastase at 37 ºC for 16 h. Derivatisation with potassium ferrocyanide solution (1 %)	HPLC, Atlantis dC18 5µm; FLD Ex at 450 nm, Em at 510 nm	External calibration using riboflavine, peak area
6	5	Acid hydrolysis and enzyme treatment.	RP-HPLC with FLD	Riboflavin external standard calibrant
7	5	Extraction by enzymatic treatment with takadiastase followed by acid hydrolysis.	Inj. vol. 20µl, Partial loop HPLC. Column: ODS 3µ Hypersil 100x4,6mm Guard Column Betabasic 18 3µm 10x3mm PN FLD Ex at 368nm Em at 440nm	External calibration Standard Ribloflavin ≥ 99%, Sigma Control standard Ribloflavin 98%, Duchefa (R0613)
8	4	Acid hydrolysis, enzymatic treatment	V = 20 µl at room temperature Lichrospher 60 RPSelectB, 250 X 4 mm-5 µm in Methanol/water eluant FLD: Ex at 422 nm, Em at 540 nm	External calibration-calibrant: riboflavin (Sigma)
9	2	Acid hydrolisis followed by enzymatic dephosphorylation	V = 20 µl RP-HPLC Lichrosher 100 RP-18 - 5 µm - 250 mm x 4,6 mm FLD: Ex at 422 nm, Em at 522 nm	External calibration-calibrant: riboflavin >98% (Sigma)
10				

11	2,5	Extraction from the sample in an autoclave using acid hydrolysis followed by enzymatic dephosphorylation.	RP-HPLC with FLD (Ex:468 nm, Em 520 nm) after post column oxidation to thiochrome.	External calibration ,Standards from Sigma, purity > 98 %. The purity determined spectrofotometrically (UV 444 nm).
12	3	Acid and enzymatic hydrolysis and extraction with sodium acetate	HPLC- FLD	External calibration-calibrant: riboflavin (Sigma)
13	1-10	Liberation with sulphuric acid and diastase treatment.	RP-HPLC-FLD	External calibration with commercial standards
14	5	Enzymatic extraction	HPLC- FLD	External calibration
15				
16				
17	2.5	Diluted sulphuric acid; ultrasonic bath; autoclave at 121 °C; addition of sodium acetate solution (pH 4.0 – 4.5); centrifugation	HPLC-FLD	External calibration, riboflavin reference solution (0.5 mg/l in water) Purity check prior to every use photometrically
18	2.5	Acidic hydrolysis with HCl 121 °C 30 min and enzymatic treatment, takadiastase 45°C 18 h	HPLC:Chromasil C18 250x4.6mm 5 µm 110 Å. Mobile phase MeOH:H2O 35:65 pH 3.5 FLD	External, Riboflavin (Fluka)
19	5	Extraction with HCl aq. 50 °C for 1 hour	HPLC-DAD 448 nm Column Macherey Nagel NUCLEODUR C18 Gravity 5 µm 150mm*4.6mm Eluent: A 2g/l Ammoniumcarbonate B MEOH gradient: 0-0.5 min 10% B 0.5-5 min. 60% B 5-11 min 60% B post time: 6 min	External calibration with Riboflavin Purity: >= 98% Provider: ROTH

Table D.4. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin B_6 in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1				
2				
3	2.5	Acid hydrolysation with HCl followed by enzymatic treatment with phosphatase.	Post-column derivatisation, HPLC-FLD (Ex 333 nm/em. 375 nm). Reference: Kall, M, Food Chemistry, 2003, 82, 315-327.	External standard, Pyridoxolhydrochloride, Pyridoxalhydrochloride and Pyridoxaminedihydrochloride from Calbiochem
4	3	Acidic extraction with 0,1M HCl, enzymatic digestion with phosphatase/glucosidase	V = 30 µl RP-HPLC, isocratic. Phenomenex Luna C18 (2) 250/4.6; 5 µm FLD: Ex: 290, Em: 390	External calibration, photometric determination of standard concentration
5	2.5	Extract with HCl (1 mol/l). Autoclave at 121 ºC/ 30 min. Treatment with claradiastase at 37 ºC for 16 h. Derivatisation with glycoxilic acid, ferrous sulfate and sodium borhydride	HPLC Atlantis dC18 5µm; fluorometric detection Ex 292 nm, Em 398 nm	External calibration using pyridoxine hydrochloride, peak area
6	2.5-5	Extraction using enzyme hydrolisis	HPLC, LiChospher 60RP C8 Select, FLD	Pyridoxine from Sigma
7	15	Extraction by trichloroacetic acid. After filtration, adjustment to pH 7,0. Hydrolisation of B6 phosphorus-esters by alkaline phosphatase.	V =20µl, Partial loop RP-HPLC, Lichrospher 100 RP 18 endcapped (5µm) Guard Column RP18 5 micron 4*4mm FLD, Ex=320nm Em=390nm Room temperature	External calibration , Pyridoxamine dichlorid: Sigma (P9380) ≥ 98%. Pyridocal hydrochlorid: Sigma (P9130) ≥ 99% (HPLC), Pyridoxol hydrochlorid: Merck (545072) ≥ 99% (by titration). Internal standard 4-Deoxypyridoxine hydrochlorid: Sigma (D0501)
8	4	Acid hydrolysis, enzymatic treatment, transformation of pyridoxaminin to pyridoxal with glyoxylic acid, reduction of pyridoxal in pyridoxol with sodium borohydrure	V = 20 µl at room temperature RP-HPLC Lichrospher 60 RPSelectB,250X4mm-5µm in Methanol/water with heptanesulfonate eluant FLD: Ex at 290nm, Em at 395nm	External calibration with pyridoxol,HCl (VWR)
9	2,0	Acid hydrolisis, enzymatic treatment by acid phosphatase (dephosphorylation) to obtain pyridoxamine, pyridoxal and free pyridoxine. Derivatisation with glycoxilic acid, ferrous sulfate and sodium borhydride.	V = 20 µl RP-HPLC Lichrosher 100 RP-18 -5 µm- 250 mm x 4,6 mm FLD Ex at 290 nm, Em at 395 nm	External calibration, chlorhydrate de pyridoxine >98%, Sigma
10				

11	2,5	Extracted using acid hydrolysis, enzymatic dephosphorylation. Reaction with glyoxylic acid in the presence of Fe2+, (pyridoxamine into pyridoxal), reduction to pyridoxine by sodium borohydride in alkaline medium.	HPLC-FLD (Ex at 290 nm, Em at 395 nm)	External calibration, pure compounds from Sigma, purity > 98 %. Purity of the standards is determined by spectrofotometric measurement (at UV 288 nm).
12				
13	1-10	Liberation with sulfuric acid treatment,	RP-HPLC-FLD	External calibration, commercial standards of pyridoxal, pyridoxol and pyridoxamine
14	2.5	Enzymatic extraction	RP-HPLC-FLD	External calibration
15				
16				
17	2.5	Addition of water, stirring, centrifugation and membrane filtration.	HPLC-MS-MS	External calibration, pyridoxal hydrochloride and pyridoxin hydrochloride
18	2,5	Acidic hydrolysis, 0.1 M HCl 121°C 30 min and enzymatic treatment, phosphatase 45°C 18 h.	HPLC Phenomenex Hypersil C18, 150x4.6 mm, 3 µm. Mobile phase ACN:phosphate buffer 7:93 pH 2.75. FLD after post-column pH-adjustment with phosphate buffer pH 7.5.	External calibration, Pyridoxamin-hydrochloride, Pyrdoxine-hydrochloride, Pyridoxal-hydrochloride (Sigma)
19	5	Extract with 1% HCl aq. 50 0C for 1 hour (0,5 hour in ultrasonic bath + 0,5 hour with temperature controlled shaker)	HPLC-MS SIM Column Macherey Nagel NUCLEODUR C18 Gravity 5 µm 150mm*4.6mm Eluent: A 2g/l Ammoniumcarbonate B MEOH gradient: 0-0.5 min 10% B 0.5-5 min. 60% B 5-11 min 60% B post time: 6 min	External calibration Pyridoxine-HCl Purity: >= 99% Provider: ROTH

Table D.5. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin B_{12} in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	5	Extraction with 20 ml acetate buffer. Treatment with cyanide. Heat on water bath for 30 min. Adjust to pH 4.5	Microbiological assay using *L. leichmaniis* (ATCC 7830) pH 4.6. Turbidimetric detection with ELISA reader	Calibrarion with SRM B12 standard treated as sample.
2				
3	5	Acetic acid-cyanid extraction	microorganism *Lactobacillus delbrueckii*	
4	1	Water/buffer extraction with cyanide, enzymatic digestion with diastase	Turbidimetric measurement in a Microtiterplate photometer (610-630 nm)	External calibration, commercial standard with purity specification
5	2.5	Digestion in sodium acetate, potassium cyanide solution (1 %), amilase and pepsin at 37 ºC for 30 min and then 84 ºC for 30 min. Purification with inmunoaffinity column.	HPLC ACE 3 AQ 3.0x150 mm; UV at 361 nm	External calibration using cyanocobalamin, peak area
6	1	Buffer extraction,centrifuge, dilution and incubation.	Turbidity	Standards included in the kit for all vitamins. Supplied by RBR. Calibration has five different levels and a blank
7	50	Release of natural vitamin B_{12} forms associated with proteins and conversion into cyanocobalamin by heating in presence of cyanide, at pH 4. Filtration and clean-up on a highly selective immuno-affinity cartridge. Elution with methanol. Evaporation under nitrogen. Dilution in water acetonitrile TFA mixture	Injection volume 100µl Partial loop HPLC, C18, ACE 3AQ 150x3,0mm Guard column: ACE 3µm AQ Flow 0,25 ml/min Pressure ~100 bar Temperature 25ºC UV, 361nm	External Calibration curve Standard Cyanocobalamine: Sigma (V2876) ~99% Control standard Cyanocobalamin: Fluka (43107)
8	1	Extraction with NaCN, takadiastase, incubation 1h at 37ºC, then at 95ºC 30 min	Microbiological test, reading of the Elisa plate at 610 nm	Comparison of growing of *Lactobacillus* with standard curve,
9	2,0	Extraction by enzymatic hydrolysis, Immuno-affinity column. Elution with methanol	Vol injection = 100 µl HPLC, C18 , 150mm x 3.0mm UV at 360 nm	External calibration cyanocobalamine Sigma V-2876 (>98,5%)
10				

11	2,5	Extraction by autoclaving samples with phosphate buffer (pH 4,5). A heat resistant amylase is added to degrade starch. Cyanide to convert cobalamines-except methylcobalamin- to di-cyanocobalamin.	Total cobalamins is measured with an inhibition assay on a Biacore instrument. Sample extract is mixed with a specific Vitamin Binding Protein. Excess VPB is bound on a chip to an immobilised cobalamin analogue. The amount of bound VPB is measured by surface plasmon resonance.	External calibration. The calibration standard used is USP reference standard.
12				
13				
14	3.5	Enzymatic extraction, purification on immunoaffinity columns	HPLC-UV	External calibration Cyanocobalamine ≥ 99%;
15				
16				
17	2.5	Addition of water and stirring; adjustment to pH 6.5 – 7.5; clean-up using immunoaffinity columns; elution with methanol; evaporation to dryness; reconstitution of the residue in water and diluted potassium cyanide solution	HPLC-MS-MS	External calibration, cyanocobalamine. Purity check of the reference solution: prior to every use photometrically
18				
19				

Table D.6. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Niacin (B_3) in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	5	Extraction with 50 ml HCl (0.1 mol/l). Autoclave at 121 ºC/20 min. Adjust pH to 4.5 with sodium acetate 2.5 mol/l.	Turbidimetric detection, ELISA reader. Microbiological assay using *L. plantarum* (ATCC 8014), pH 6.8	External calibration using piridoxal; standard treated as sample
2				
3	5	Acid hydrolysis	Microorganism *Lactobacillus plantarum*	
4	1	Water/buffer extraction, enzymatic digestion with diastase	Turbidimetric measurement in microtiterplate photometer (610-630 nm)	External calibration, commercial standard with purity specification
5	5	Extraction with HCl 0,5N, 60 min at 121 ºC. Cool and adjust pH at 6.8 with sodium hydroxide solution 15%. Filter.	Microbiological method *Lactobacillus plantarum* (CECT 748, ATCC14917), pH = 6,8 +/-0,1 Incubation for 15h at 37ºC Spectrophotometric detection at 546 nm	External calibration, nicotinamide (Sigma). Standards prepared using extinction coefficient and treated as a sample.
6	1	Buffer extraction, centrifuge, dilution and incubation.	Commercial kit. Turbidity	Standards included in the kit for all vitamins. Supplied by RBR. External calibration
7	5	Extraction of nicotinic acid and nicotinamide by acid-hydrolysis with 0,1M HCl at 100 ºC. Filtration after adjustment of the pH to 4,6.	V = 30µl, partial loop RP-HPLC. ODS-3 µ5 250x4,6 Guard column: ODS-3 Post-column derivatization by UV-irradiation. FLD Ex at 322nm, Em at 380nm Room temperature	External Calibration Nicotinic acid: Acros (12829) 99,5% Nicotinamide: Acros (12827) 99% Control standard Nicotinic acid: Sigma (72309) ≥99,5% (HPLC) Control standard Nicotinamide: Sigma (N3376) ≥98% (TLC)
8	5	Acid hydrolysis.	V = 20 µl, room temperature. HPLC Lichrospher 60 RP SelectB, 250x4mm-5µm in phophate/water eluant. Post- column UV derivation FLD : Ex at 322nm, Em at 380nm	External calibration Nicotinamid and Nicotinic acid (Sigma)
9	2,0	Extraction by acid-hydrolysis	V = 30 µl HPLC-FLD after post-column derivatisation by UV-irradiation in the presence of Cu(II) and H_2O_2. C18 - 5,0 µm -125 mm x 4,6 mm. FLD Ex at 322 nm, Em at 380 nm	External calibration, nicotinic acid Sigma N-4126 (>98%) and nicotinamide Sigma N-3376 (>98%)

10				
11	2,5	Extraction by autoclaving using acid hydrolysis. Dilution with basal media, containing all required growth nutrients except nicotinamide and nicotinic acid.	Growth response of *Lactobacilius plantarum* (ATCC 8014) measured turbidmetrically Niacin is measured as a sum of nicotinic acid and nicotinamide.	External calibration USP standard.
12				
13				
14	2.5	Acid followed by basic extraction	HPLC, derivation post column, FLD	External calibration
15				
16				
17	2.5	Addition of water and stirring; centrifugation and if required membrane filtration; appropriate dilution with water	HPLC-MS-MS	External calibration (single point), nicotinamide
18				
19	5	Extract with 1% HCl aq. 50 ºC for 1 hour (0,5 hour in ultrasonic bath + 0,5 hour with temperature controlled shaker)	HPLC-MS SIM Column Macherey Nagel NUCLEODUR C18 Gravity 5 µm 150mm*4.6mm Eluent: A 2g/l Ammoniumcarbonate B MEOH gradient: 0-0.5 min 10% B 0.5-5 min. 60% B 5-11 min 60% B post time: 6 min	External calibration Nicotinamide Purity: 99% Provider: ChemService

Table D.7. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Total folate in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	5	Add ascorbic acid. Extraction with phosphate buffer pH 6.1 (0.05 mol/l) and pancreatin. 1 hour at 40 ºC. Add NaOH 1 N, 20 min at 100 ºC.Adjust pH to 4.5 using HCl 1N.	Turbidimetric detection Microbiological assay using *Lactobacillus casei* (ATCC7469); pH 6.2: ELISA reader	External calibration using folic acid; standard treated as sample.
2				
3	5	Extraction with ascorbic acid/fosfate buffer, enzyme human plasma/Pancreas V	*Lactobacillus casei*	NIST INFANT FORMULA 1849 - within limits
4	1	Water/buffer extraction, enzymatic digestion with chicken pancreatin	Turbidimetric measurement, microtiterplate photometer (610-630 nm)	External calibration, commercial standard with purity specification
5	5	Extraction with phosphate buffer (pH=7,2), 15 min at 121 ºC. Cool and treat with common pancreatine (Merck), 24 h at 37 ºC. Inactive 5 minutes at 121 ºC, cool and filter.	Microbiological method Organism growth using *Lactobacillus casei* (CECT 278), pH = 6,8 +/-0,1 Incubation for 15 h at 37 ºC Spectrophotometric detection at 546 nm	External calibration, folic acid (Sigma). Standards prepared using extinction coefficient and treated as a sample
6	1	Buffer extraction, centrifuge, dilution and incubation.	Turbidity, commecial kit	External calibration Standards included in the kit for all vitamins.
7	5	Extraction in weakly acidic conditions by hydrolysis at 102 °C. Filtration after adjustment of the pH to 4,6. Addition of the extract to a medium free of the vitamin to be measured. Sterilisation.	Inoculation with *Lactobacillus casei*. Incubation at 37 °C. Measurement of growth by turbidimetry. Photometer, 575nm (absorbance)	External calibration Standard: Sigma (F-7876) ≥ 97% Control standard: Fluka (47620) ≥ 97% (HPLC)
8	1	Extraction with pancreatin, incubation 2 h at 37 °C, then at 95 °C 30 min	Microbiological microplate test, reading of the Elisa plate at 620 nm	Comparison of the growing of *Lactobacillus* with a standard curve.
9	2	Suspension in phosphate buffer pH 6.1. Extraction at 110°C in the presence of α-amylase and protease. Deconjugation by α-glutamyl-carboxypeptidases or conjugases. Dilution. Inoculation of *Lactobacillus casei, subsp. rhamnosus* (ATCC 7469).	Turbidity on microbiological microplate test	Folic acid, Sigma F-7876 (>97%)
10				

11	2,5	Folate (including folic acid) extracted in autoclave using a buffer solution. Enzymatic digestion with human plasma and pancrease V and finally by a second autoclave treatment.	After dilution with basal medium containing all required growth nutrients except folic acid and growth response of *Lactobacillus rhamnosus* (ATCC 8043) to extracted folate is measures turbidimetrically.	External calibration, USP standard.
12				
13	0,1-1	Cleanup over SPE	LC-MS/MS	Stable isotope dilution assay with deuterated folates as internal standards
14				
15				
16				
17	2.5	Extraction with potassium hydroxide at 60 °C; if required centrifugation and membrane filtration; dilution with water; addition of acetic acid	HPLC-MS-MS	External calibration (single point) against folic acid reference solution. Photometric purity check of the reference solution
18				
19				

Table D.8. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin C in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	2.5	Extract with acetic acid 1% at room temperature; filter	HPLC-UV, RP18 5 µm KH_2PO_4 0.005 mol/l pH 2.6;UV at 245 nm	External calibration, ascorbic acid; standard treated as sample; peak height
2				
3	2.5	Extraction with metaphosphoric acid	UV for ascorbic acid (245 nm) and post-column derivatisation ,FLD (Ex at 350 nm, Em at 430 nm). Reference: Kall & Andersen, J Chrom B, 1999, 730, 101-111.	External calibration, L-ascorbic acid from Sigma
4	1.5	Acidic extraction with antioxidants	V = 50 µl RP-HPLC, gradient. Phenomenex Luna C18 (2) 250/4.6; 5 µm, UV at 240 nm	External calibration, photometric determination of standard concentration
5	2.5	Extraction with meta-phosporic acid in methanol	HPLC Atlantis C18 3 µm with UV detection at 254 nm	External calibration with ascorbic acid; peak area
6	2.5	Blend and extract with metaphosphoric/acid EDTA solution. Oxidise to DHAA acid and complex with o-phenylene diamine	RP-HPLC, Genesis C18, with FLD	Ascorbic acid standard solutions treated as with samples; converted to dehydroascorbic acid and complexed with o-phenylene diamine
7	10	Extraction of total ascorbic and isoascorbic acids in acid conditions in the presence of TCEP·HCl.	V = 20 µl, partial loop Total ascorbic acid content by HPLC. Column: RP-18 5µm 4,6x250 Guard column: RP-18 Flow: 1,0 ml/min, pressure: ~130bar Temperature 20ºC UV at 265 nm.	External calibration Standard L(+)-Ascorbic acid: Prolabo (20150,231) 99,0-100,5% Control standard L-Ascorbic acid: Sigma (33034) 99,7-100,5% Control standard D-(-)-Isoascorbic acid: Sigma (58320) ≥ 99%
8	10	Extraction with metaphosphoric acid, reduction of DHAA acid in AA with L cystein	V = 20 µl at room temperature HPLC, Lichrospher 100RP18 (250X4,6mm-5µm) UV at 265 nm	External calibration, ascorbic acid (Sigma)
9	2,5	Extraction with metaphosphoric acid, reduction of DHAA acid in AA with L cystein	V = 30µl HPLC electrochemical detection Lichrosher 100RP18 e, 5µm, 250 mm x 4.0 mm	External calibration, L-ascorbic acid Sigma A-7506 (>98%)
10				

11	2,5	Extraction in an acidic solution and oxidized to DHAA in the presence of Norit.	Oxidized form is reacted with o-phenylenediamine to produce flourophor Ex = 350 nm and Em = 430 nm.	External calibration, pure compounds from Sigma, purity > 95 %.
12	5	Extraction with meta-phosporic acid in methanol	HPLC with UV detection	External calibration
13				
14	5	Acidic extraction, reduction	HPLC with UV detection	External calibration
15	0,5	Dissolve in water, add 3 ml 15 % meta-phosphoric acid	HPLC with UV detection	External calibration
16	2.5	Extraction with metaphosphoric acid, cystein reduction; dilution.	HPLC-RP; C18 encapped UV detection	External calibration.
17	1.5	Extraction with phosphate buffer, treatment with DL- homocysteine, addition of meta-phosphoric acid	HPLC-DAD	External calibration, ascorbic acid
18	2.5	Extraction with 150 ml 2 % m-phosphoric acid:0.5 % oxalic acid. Turmixed in N_2-atmosphere 3 min.	HPLC: Phenomenex Gemini C18, 250x4.6 mm, 5 µm, 110 Å. Mobile phase phosphate-acetate buffer pH 4.5 with Na_2EDTA and dodecyltrimethylammonium-chloride. Electrochemical detection of ascorbic acid, fluorescence detection of dehydroascorbic acid after post-column derivatisation with O-phenyldiamindihydro-chloride.	External, bracketed calibration Ascorbic acid (Merck), dehydroascorbic acid prepared from the ascorbic acid calibrant.
19				

Table D.9. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin D_3 in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1		Homogenise with H2O containing ascorbic acid and saponify using KOH:H2O:EtOH; IS added; 30 min at 70 ºC; extract with PE; wash with water; SPE clean up using Lichrosorb, HEX:IPA	HPLC separation using RP-, 18 5 µm MeOH:H2O 95:5. UV at 265 nm	External calibration using vitamin D standard treated as sample
2	50	Slurry preparation, saponification at 85ºC, SPE clean-up, elution, evaporation to dryness, taking up with mobile phase and preparative chromatography. Fractions collected evaporated and taken up with mobile phase.	Preparative HPLC: 500µL. Analytical HPLC: 100µL. No T control. Preparative HPLC: Tracer Extrasil Si 250x4.6mm 5µm ref. TR-016064. Analytical HPLC: Kromasil 100 C18 250x4.6mm 5µm ref. 3232 No T program. UV at 265nm.	Internal standard addition, ergocalcipherol. Cholecalcipherol Sigma ref. C9756. Purity corrected by UV spectrophometry.
3	2	Alkaline saponification, liquid/liquid extraction (ethylether:petroleum ether 50:50). Silica SPE and preparative HPLC silica/amino column with heptane:isopropanol (gradient program)	HPLC-UV at 265 nm.	Internal standard. Ergocalciferol and Cholecalciferol from Sigma
4	10	Suspension with water, saponification with 5N ethanolic KOH, Na-sulfide, ascorbate, preparative HPLC	Automatic sampler, 100 µl Preparative HPLC: Normal phase, isocratic. Merck Lichorosorb Si 80 250/4; 5 µm; Analytical HPLC: Reversed phase, isocratic. Merck Lichosorb Si 60/250 4; 5 µm UV at 264	
5	10	IS vit. D2. Saponification with 50 ml KOH aqueous solution, ascorbic acid solution and ethanol. Extraction with PE and wash with KOH aqueous-alcoholic solution.	HPLC NP Kromasil 100 Si 5µm 4.6x250 mm. UV detection 265 nm	Internal standard D2
6	10	Saponification followed by extraction into mixed ethers. Clean-up by Si SPE and semi-preparative HPLC.	RP HPLC, Partisil 5 PAC, with UV detection	D3 calibration standards, vitamin D2 as an internal standard.

7	50	Saponification with KOH and sodium sulphide in an alcohol-water mixture (sodium ascorbate as antioxidant), by using a rotating heating block. Extraction with PE, evaporation of organic phase. SPE using Chromabond cartridges. Evaporation, resolved in the mobile phase. Semi preparative NP-HPLC.	V prep step 500µl, partial loop, V analytical step 300µl, partial loop RP-HPLC Column prep step: Lichrospher 100 250-4 SI-60 5µm. Guard column prep step: Analytical column: Lichrospher 100RP 18 250-4. Guard column analytical step UV at 265nm	External calibration Standard Ergocalciferol: Sigma (E5750) Standard Cholecalciferol: Acros (14094) ³ 99%
8	5	Saponification with KOH methanolic solution, 30 min at 85°C, extraction with hexane, semi preparative HPLC on Si60 column	V = 500 µl at room temperature RP-HPLC, Lichrospher 100RP18 (250X4,6mm-5µm) UV at 264 nm	External calibration-calibrant: Cholecalciferol (Acros)
9	15	Saponification with KOH methanolic solution, extraction. Semi preparative NP- HPLC	Semi-prep column: Licrospher Si 60 lichrocart 250-4, RP-HPLC Analytical column: Purospher RP-18 e lichrocart 250-4 UV at 265 nm	IS Calibration Vitamine D_2 (IS) Sigma E-5750 (>97%) Vitamin D_3 (>98%)
10				
11	10	Saponification with KOH methanolic solution and extraction with diethylether. Concentration and clean up by SPE, semi-preparative NP-HPLC.	RP-HPLC-DAD (265 nm) / MS (SIM)	External calibration, USP standards. Purity determined spectrofotometrically (UV 265 nm).
12				
13	20	Saponification, extraction with petrol ether, NP-HPLC	HPLC-UV	IS calibration with D_2 as internal standard, commercial standard D_3
14	20	Saponification, liquid liquid purification	double HPLC-UV detection	External calibration
15				
16	5	Saponification, liquid-liquid extraction, isolation by semi-preparative NP-HPLC	RP-HPLC C18 endcapped; UV detection.	Internal Standard Calibration with D_2. Photometric verification of the standard solution .
17	5	Ascorbic acid as antioxidant. Vitamin D_2 as internal standard. Saponification with KOH at 85°C. Extraction with diethylether; SPE clean-up on a silica gel column; elution with ethylacetate/isooctane; evaporation and reconstitution with methanol	HPLC-MS	Internal standard, vitamin D_2 Photometric purity check of the reference solution
18	10 g	Ascorbic acid added as antioxidant. Vitamin D2 added as internal standard. Saponification with KOH for 30 min at 95°C. Extraction with n-heptane. Sample clean-up with semi-preparative HPLC (silica).	HPLC: Vydac 201TP54 column (C18), 250 × 4.6 mm, 5 µm particles. Mobile phase: 20 % methanol in acetonitrile. UV detection (265 nm).	Internal standard, vitamin D2 (Merck)
19	5	Saponification (according to EN ISO 14565:2000, 9.2.) reflux with 2 ml 50% of KOH + 200 ml EtOH (ca 80 0C) for 60 min under nitrogen; extract with PE /40-70/ , change of solvent to methanol.	HPLC-MS SIM Column: Waters symmetry C18 3.5µm 4.6*75mm Eluent 5% water 95% MEOH	External calibration, vitamine D3 Purity: 99,5% Provider: ChemService

Table D.10. Methods used by participant laboratories during the characterisation campaign of ERM-BD600 whole milk powder

Vitamin E in milk powder

Laboratory code	Sample size [g]	Sample treatment	Method of final determination	Method of calibration
1	10	Homogenise with H2O containing ascorbic acid. Saponify with KOH:H2O:EtOH (10:10ml:50ml), at 70ºC; extract into PE; wash with water and evaporate to dryness; redisolve in MeOH	HPLC. RP18 5µm; MeOH:H2O 95:5; UV at 292 nm	External calibration using DL-α-tocopherol treated as sample; (no saponification); peak area
2	50	Slurry preparation, saponification (30min at 85ºC), SPE clean-up, elution, evaporation of eluate to dryness, taking up with mobile phase	Inj. Vol. 10µL. No temperature control. HPLC, Tracer Extrasil Si 250x4.6mm 5µm ref. TR-016064. FLD: Ex at 294 nm, Em at 326 nm.	External calibration, dl-α-tocopherol FLUKA ref. 95240. Purity corrected by UV spectrophometry.
3	10	Alkaline saponification, liquid/liquid extraction (pentan:ethylacetate 80:20).	HPLC-FLD Ex at 290 nm Em at 327 nm).	External calibration, alfa-tocopherol from Calbiochem
4	10	Suspension with water, saponification with ethanolic KOH, Na-sulfide, ascorbate	V = 20 µl NP-HPLC, isocratic Merck Lichorosorb 60 250/4; 5 µm FLD: Ex at 290 nm, Em at 333 nm	External calibration, photometric determination of standard concentration
5	5-10	Saponification with KOH, ascorbic acid, ethanol. Extraction with petroleum/ether	HPLC. RP18 5µm; H2O:ACN 5:95, 0.1% TFA; Atlantis dC18 3µm 4.6x20mm. FLD Ex at 295 nm, Em at 340 nm	
6	10	Saponification followed by extraction into mixed ethers. Extract evaporated to dryness and residue redissolved in hexane: propan-2-ol	HPLC, Lichrosorb Si60, FLD.	External calibration, α-tocopherol calibration standards.
7	50	Saponification with KOH and sodium sulphide in alcohol-water and sodium ascorbate. Extraction with PE. Evaporation. SPE using Chromabond cartridges. Resolved in the mobile phase.	Injection volume 50µl, Partial loop NP-HPLC-UV and FLD, Lichrospher Diol 5µm, 15cm 250-4 Guard column: Flow: 1,1 ml/min Pressure: ~50bar Room temperature FLD: Ex at 294nm, Em at 325nm	External calibration Standard dl-alpha-Tocopherol: Sigma (T3251) ≥ 96% (HPLC)

8	4	Saponification with KOH methanolic solution, with adding pyrogallol 30 min at 75 °C, extraction with PE	V = 20 µl HPLC, Lichrospher 100RP18 (125X4mm-5µm)in Methanol/water eluant UV at 292 nm	External calibration, alpha tocopherol
9	10 - 15 g	Saponification with KOH methanolic solution, extraction	NP-HPLC, Lichrospher Si 60, 5.0µm, 250mm x 4.0 mm FLD: Ex at 295 nm, Em at 330 nm	External calibration, -dl-a-tocopherol : M (C29H50O2) = 430.7 Calbiochem cat N°613420 (>98%)
10				
11	10	Saponification with KOH methanolic solution, and extracted twice with hexane:ethylacetate (85:15 v/v).	RP-HPLC-FLD (Ex at 290 nm, Em at 327 nm).	External calibration, pure compounds from Sigma, purity > 98 %. The purity of the standards determined by spectrofotometry
12	10	Saponification with KOH and extraction with THF/ethanol	HPLC-FLD	External calibration
13	20	Saponification, extraction with petrol ether, RP-HPLC	HPLC-UV	External calibration with commercial standards
14	20	Saponification	HPLC-FLD	External calibration
15	1-2	Heat treatment, extraction hexane, rotation	HPLC-FLD	External calibration
16	5	Saponification, liquid-liquid extraction	RP-HPLC C18 endcapped; substance identification: retention time (F and UV detection) and UV spectra. Substance quantification by external standard calibration	External standard calibration. Photometric verification of the standard solution
17	5	Ascorbic acid in methanol ,internal standard δ-tocopherol; stirring; saponifiction KOH at 85 °C; extraction with diethylether; evaporation and reconstitution with methanol;	HPLC-DAD	External calibration, α-tocopherol. Photometric verification of the standard solution
18	10	Ascorbic acid as antioxidant. Saponification with KOH for 30 min at 95°C. Extraction with cyclohexane on a Chem Elut (polar) column.	HPLC: Reprosil Amino column, 150 × 4.6 mm, 3 µm particles. Mobile phase: 2 % isopropanol in n-heptane. FLD Ex at 295 nm, Em at 327 nm.	External calibration, alpha-tocopherol (Calbiochem)
19	5	Saponification (according to EN ISO 14565:2000, 9.2.) reflux with 2 ml 50 % of KOH + 200 ml EtOH (ca 80 ºC) for 60 min under nitrogen; extract with PE /40-70/ to final volume 250 ml; change of solvent to MeOH	HPLC-DAD 292 nm Column: Waters symmetry C18 3.5µm 4.6*75mm Eluent 5% water 95% MeOH	ESTD Name: Tocopherol-acetate Purity: 97,7% Provider: ROTH

Table D.11. All data sets reported by the participating laboratories to the certification campaign of ERM-BD600 material. Vitamin mass fractions are expressed as the mean values of the results of 6 independent replicates and their standard deviations, as mg/kg dry matter. Bold figures indicate values accepted for material value assignment.

	Vitamin A (all-*trans* + 13-*cis*-retinol)						Vitamin A (all-*trans*-retinol)					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Laboratory code												
1	8.29	7.98	7.57	7.57	7.88	7.98	-	-	-	-	-	-
2	-	-	-	-	-	-	**3.74**	**4.01**	**3.95**	**3.79**	**3.96**	**3.99**
3	**4.23**	**4.33**	**4.39**	**4.74**	**4.50**	**4.43**	**3.79**	**3.84**	**3.87**	**4.13**	**3.94**	**3.90**
4	**4.002**	**4.124**	**4.118**	**4.114**	**4.134**	**4.057**	**3.747**	**3.859**	**3.853**	**3.839**	**3.869**	**3.802**
5	-	-	-	-	-	-	6.7	7.4	6.4	6.6	7.1	6.2
6	4.48	4.09	3.77	4.51	-	-	2.69	2.73	2.36	2.67	-	-
7	**4.62**	**4.76**	**4.42**	**4.40**	**4.77**	**4.37**	**4.19**	**4.3**	**3.98**	**3.97**	**4.31**	**3.93**
8	6.69	6.1	5.95	6.42	6.4	6.04	-	-	-	-	-	-
9	**3.839**	**4.223**	**3.594**	**3.969**	**3.868**	**3.573**	**3.549**	**3.925**	**3.333**	**3.671**	**3.624**	**3.314**
10	-	-	-	-	-	-	-	-	-	-	-	-
11	10.11	8.04	7.32	10.09	7.94	7.55	-	-	-	-	-	-
12	9.2	6.6	9.6	9.4	6.2	9.3	-	-	-	-	-	-
13	8.7	8.7	8.6	7.8	8.3	8.2	-	-	-	-	-	-
14	**3.83**	**3.62**	**3.93**	**3.63**	**3.66**	**3.75**	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	**3.22**	**3.35**	**3.25**	**3.23**	**3.31**	**3.31**
17	-	-	-	-	-	-	**4.6**	**4.4**	**4.4**	**4.5**	**4.5**	**4.4**
18	**4.00**	**4.03**	**4.01**	**4.07**	**3.96**	**4.00**	**3.57**	**3.57**	**3.56**	**3.63**	**3.51**	**3.54**
19	-	-	-	-	-	-	7.29	7.6	7.55	6.16	8.06	6.9

Table D.11 (cont.). All data sets reported by the participating laboratories to the certification campaign of ERM-BD600 material. Vitamin mass fractions are expressed as the mean values of the results of 6 independent replicates and their standard deviations, as mg/kg dry matter. Bold figures indicate values accepted for material value assignment.

	Vitamin B_1						Vitamin B_2					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Laboratory code												
1	**4.4**	**4.1**	**4.7**	**5.1**	**4.5**	**4.2**	**16.33**	**15.63**	**16.14**	**14.23**	**14.98**	**14.95**
2	**5.2**	**5.3**	**6.1**	**5.3**	**5.2**	**5.5**	**17.2**	**17.2**	**17.4**	**17.6**	**17.2**	**17.5**
3	**5.30**	**5.29**	**5.46**	**5.40**	**5.27**	**5.47**	**16.4**	**16.1**	**16.0**	**15.7**	**16.7**	**17.7**
4	**4.532**	**4.633**	**4.781**	**4.868**	**4.776**	**4.791**	**15.183**	**14.124**	**16.014**	**15.397**	**14.155**	**16.218**
5	**4.0**	**4.9**	**4.6**	**4.6**	**4.7**	**4.7**	**16.3**	**17.3**	**17.2**	**17.0**	**17.7**	**18.1**
6	**5.12**	**5.07**	**4.98**	**5.07**	**5.13**	**4.99**	**17.82**	**17.67**	**18.46**	**18.24**	**17.78**	**18.51**
7	**4.90**	**4.51**	**4.64**	**4.77**	**4.64**	**4.64**	**19.0**	**18.6**	**18.2**	**18.8**	**18.6**	**18.3**
8	**3.36**	**3.45**	**3.93**	**3.46**	**3.45**	**3.73**	**17.8**	**14.8**	**17.6**	**17.8**	**14.8**	**17.4**
9	6.13	6.82	5.45	6.13	6.7	5.45	**12.95**	**14.67**	**10.52**	**12.85**	**14.57**	**10.63**
10	-	-	-	-	-	-	-	**-**	**-**	**-**	**-**	**-**
11	**4.34**	**3.9**	**3.9**	**4.34**	**3.9**	**4.97**	**16.88**	**16.5**	**16.26**	**16.98**	**16.6**	**16.67**
12	7.7	7.2	< LOQ	< LOQ	< LOQ	< LOQ	**17**	**17**	**17**	**17**	**18**	**18**
13	**4.218**	**4.661**	**4.129**	**4.128**	**4.707**	**4.119**	**15.7**	**16.3**	**14.4**	**16.2**	**16.2**	**14.6**
14	**4.53**	**4.10**	**4.28**	**4.41**	**4.12**	**4.17**	**17.1**	**17.03**	**16.6**	**17.2**	**17.29**	**16.7**
15	-	-	-	-	-	-	-	**-**	**-**	**-**	**-**	**-**
16	-	-	-	-	-	-	-	**-**	**-**	**-**	**-**	**-**
17	**3.6**	**5.1**	**3.3**	**3.3**	**4.3**	**3.8**	12.10	20.7	14.7	12.0	20.9	13.7
18	**4.78**	**4.33**	**4.51**	**4.87**	**4.52**	**4.56**	**19.0**	**19.5**	**18.8**	**19.1**	**19.8**	**19**
19	**3.28**	**3.43**	**3.51**	**3.26**	**3.03**	**3.1**	12.60	12.6	12.2	14.1	13.8	14.5

Table D.11 (cont.). All data sets reported by the participating laboratories to the certification campaign of ERM-BD600 material. Vitamin mass fractions are expressed as the mean values of the results of 6 independent replicates and their standard deviations, as mg/kg dry matter. Bold figures indicate values accepted for material value assignment.

	Vitamin B_6						Vitamin B_{12}					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Laboratory code												
1	-	**-**	**-**	**-**	**-**	**-**	0.103	0.114	0.109	0.108	0.116	0.106
2	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
3	**7.35**	**7.48**	**7.38**	**7.33**	**7.34**	**7.39**	0.323	0.359	0.387	0.290	0.344	0.380
4	**7.301**	**7.749**	**7.268**	**7.363**	**7.688**	**7.778**	0.387	0.346	0.336	0.336	0.346	0.336
5	**4.7**	**4.7**	**4.0**	**4.7**	**4.8**	**4.3**	**0.334**	**0.355**	**0.353**	**0.324**	**0.357**	**0.341**
6	**7.06**	**7.47**	**7.4**	**7.13**	**7.36**	**7.84**	0.467	0.453	0.494	0.518	0.497	0.504
7	**7.62**	**7.72**	**7.61**	**7.52**	**7.92**	**7.61**	**0.322**	**0.354**	**0.368**	**0.336**	**0.349**	**0.38**
8	**5.83**	**6.00**	**5.99**	**5.93**	**6.00**	**5.99**	0.387	0.388	0.380	0.427	0.404	0.367
9	**7.08**	**6.88**	**6.88**	**7.59**	**6.88**	**6.78**	**0.322**	**0.328**	**0.323**	**0.317**	**0.325**	**0.321**
10	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
11	**7.16**	**7.71**	**7.22**	**7.47**	**7.42**	**6.55**	**0.32**	**0.31**	**0.31**	**0.33**	**0.31**	**0.32**
12	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
13	**7.28**	**7.55**	**7.19**	**7.24**	**7.55**	**7.18**	-	**-**	**-**	**-**	**-**	**-**
14	**8.24**	**8.41**	**8.15**	**8.4**	**8.54**	**8.15**	**0.32**	**0.31**	**0.30**	**0.30**	**0.33**	**0.30**
15	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
16	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
17	**4.8**	**9.8**	**9.0**	**10.0**	**8.6**	**7.4**	**0.28**	**0.31**	**0.30**	**0.31**	**0.30**	**0.30**
18	7.54	7.52	7.5	7.43	7.43	7.46	-	**-**	**-**	**-**	**-**	**-**
19	4.24	4.12	4.06	4.56	3.91	4.43	-	**-**	**-**	**-**	**-**	**-**

Table D.11 (cont.). All data sets reported by the participating laboratories to the certification campaign of ERM-BD600 material. Vitamin mass fractions are expressed as the mean values of the results of 6 independent replicates and their standard deviations, as mg/kg dry matter. Bold figures indicate values accepted for material value assignment.

	Niacin						Total folate					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Laboratory code												
1	**9.5**	**10.3**	**8.6**	**9.6**	**8.7**	**8.2**	**0.623**	**0.571**	**0.617**	**0.660**	**0.606**	**0.611**
2	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
3	**5.345**	**6.120**	**7.191**	**5.892**	**5.917**	**6.582**	**0.798**	**0.818**	**0.948**	**0.826**	**0.877**	**0.799**
4	**9.572**	**9.572**	**9.582**	**9.572**	**9.674**	**9.684**	**0.57**	**0.631**	**0.591**	**0.591**	**0.611**	**0.571**
5	**12.1**	**13.5**	**11.7**	**11.1**	**11.6**	**10.5**	**0.265**	**0.291**	**0.223**	**0.244**	**0.345**	**0.270**
6	**7.573**	**8.960**	**7.454**	**9.215**	**9.165**	**7.696**	**0.674**	**0.663**	**0.585**	**0.620**	**0.653**	**0.601**
7	**6.50**	**7.11**	**7.92**	**6.60**	**7.41**	**7.92**	**0.427**	**0.477**	**0.396**	**0.417**	**0.498**	**0.376**
8	-	**-**	**-**	**-**	**-**	**-**	**0.48**	**0.425**	**0.561**	**0.47**	**0.426**	**0.56**
9	**6.88**	**6.17**	**8.30**	**6.88**	**6.17**	**8.30**	**0.495**	**0.578**	**0.522**	**0.461**	**0.583**	**0.513**
10	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
11	**6.31**	**10.25**	**6.86**	**5.96**	**8.81**	**6.37**	**0.46**	**0.46**	**0.79**	**0.47**	**0.46**	**0.77**
12	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
13	-	**-**	**-**	**-**	**-**	**-**	0.77	0.68	0.78	0.75	0.68	0.77
14	**6.13**	**8.79**	**7.26**	**7.18**	**7.09**	**7.99**	-	**-**	**-**	**-**	**-**	**-**
15	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
16	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
17	4.2	4.2	4.3	4.1	4.1	4.5	0.25	0.26	0.26	0.25	0.26	0.24
18	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
19	**6.13**	**6.01**	**5.26**	**6.22**	**6.17**	**6.14**	-	**-**	**-**	**-**	**-**	**-**

Table D.11 (cont.). All data sets reported by the participating laboratories to the certification campaign of ERM-BD600 material. Vitamin mass fractions are expressed as the mean values of the results of 6 independent replicates and their standard deviations, as mg/kg dry matter. Bold figures indicate values accepted for material value assignment.

	Vitamin C						Vitamin D_3					
Replicate	1	2	3	4	5	6	1	2	3	4	5	6
Laboratory code												
1	**74.9**	**71.2**	**66.6**	**74.5**	**71.8**	**66.6**	**0.236**	**0.266**	**0.236**	**0.266**	**0.348**	**0.26**
2	-	**-**	**-**	**-**	**-**	**-**	**0.128**	**0.141**	**0.140**	**0.139**	**0.135**	**0.140**
3	**60.3**	**66.0**	**77.3**	**61.2**	**65.1**	**76.4**	**0.123**	**0.123**	**0.123**	**0.122**	**0.122**	**0.121**
4	**77.189**	**70.061**	**77.472**	**78.615**	**74.847**	**78.287**	**0.132**	**0.132**	**0.143**	**0.122**	**0.143**	**0.143**
5	**89**	**85**	**77**	**72**	**87**	**82**	**0.175**	**0.124**	**0.136**	**0.170**	**0.123**	**0.135**
6	**82.31**	**80.21**	**81.09**	**83.94**	**77.76**	**78.95**	**0.133**	**0.117**	**0.17**	**0.196**	**0.11**	**0.214**
7	**91.4**	**81.3**	**81.2**	**91.4**	**81.3**	**81.2**	**0.111**	**0.120**	**0.126**	**0.118**	**0.126**	**0.118**
8	**56.3**	**35.5**	**39.3**	**50.4**	**36.5**	**38.3**	76.0	81.8	73.7	82.0	78.9	72.7
9	**63.4**	**66.59**	**66.39**	**66.5**	**71.45**	**65.98**	**0.129**	**0.137**	**0.121**	**0.130**	**0.135**	**0.119**
10	-	**-**	**-**	**-**	**-**	**-**	-	**-**	**-**	**-**	**-**	**-**
11	**82.84**	**86.08**	**87.47**	**85.95**	**85.06**	**89.52**	**0.12**	**0.1**	**-**	**0.12**	**0.1**	**0.13**
12	**126**	**78**	**83**	**126**	**78**	**84**	-	**-**	**-**	**-**	**-**	**-**
13	-	**-**	**-**	**-**	**-**	**-**	**0.062**	**0.053**	**0.057**	**0.062**	**0.053**	**0.057**
14	**83.8**	**72.6**	**76.7**	**81.1**	**72.0**	**97.3**	**0.13**	**0.13**	**0.13**	**0.13**	**0.13**	**0.13**
15	**57.8**	**62.8**	**48.6**	**57.8**	**62.8**	**68.9**	-	**-**	**-**	**-**	**-**	**-**
16	**86.7**	**89.2**	**90.1**	**88.8**	**92.8**	**90.4**	**0.13**	**0.13**	**0.13**	**0.12**	**0.13**	**0.13**
17	**51.7**	**37.4**	**59.3**	**52.3**	**35.9**	**51.3**	**0.22**	**0.16**	**0.17**	**0.2**	**0.17**	**0.17**
18	**79.2**	**83.1**	**86.8**	**66.0**	**98.7**	**82.2**	**0.131**	**0.132**	**0.133**	**0.134**	**0.135**	**0.136**
19	-	**-**	**-**	**-**	**-**	**-**	**0.0898**	**0.0804**	**0.0891**	**0.0796**	**0.0842**	**0.0843**

Table D.11 (cont.). All data sets reported by the participating laboratories to the certification campaign of ERM-BD600 material. Vitamin mass fractions are expressed as the mean values of the results of 6 independent replicates and their standard deviations, as mg/kg dry matter. Bold figures indicate values accepted for material value assignment.

	Vitamin E					
Replicate	1	2	3	4	5	6
Laboratory code						
1	**116.7**	**110.3**	**109.3**	**109.6**	**109.6**	**114**
2	**84**	**82.4**	**79.8**	**84.8**	**83.2**	**83.6**
3	**100.1**	**100.1**	**101.3**	**99.1**	**100.8**	**100.8**
4	**94.705**	**89.817**	**91.233**	**96.232**	**88.9**	**91.233**
5	**67.6**	**81.8**	**78.0**	**66.7**	**80.2**	**70.0**
6	**73.88**	**82.74**	**94.02**	**82.31**	**85.89**	**88.76**
7	**72.2**	**75.9**	**62.8**	**71.6**	**77.3**	**68.4**
8	**66.2**	**62.0**	**61.9**	**64.2**	**65.0**	**61.9**
9	**82.78**	**79.74**	**77.01**	**88.04**	**80.66**	**76.3**
10	-	-	-	-	-	-
11	**106.66**	**99.71**	**95.29**	**107.69**	**100.94**	**102.59**
12	**49**	**65**	**49**	**49**	**63**	**47**
13	**89.6**	**62.6**	**86.5**	**91.7**	**90.1**	**89.2**
14	**100**	**97.3**	**103**	**105**	**96.4**	**97.1**
15	**86.5**	**82.1**	**86.9**	**83.9**	**88.1**	**90.4**
16	**103.9**	**87.0**	**87.6**	**101.8**	**87.6**	**89.9**
17	**99.2**	**100**	**103**	**95.8**	**101**	**104**
18	**91.8**	**89.0**	**91.7**	**94.5**	**91.9**	**91.4**
19	**89.1**	**91**	**91.3**	**75.8**	**81.9**	**75.9**

European Commission

EUR 24924 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of the Mass Fractions of Vitamins in Whole Milk Powder - Certified Reference Materials ERM®-BD600
Author(s): M. Dabrio Ramos, H. Schimmel, H. Emons
Luxembourg: Publications Office of the European Union
2011 – 75 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1831-9424 (online), ISSN 1018-5593 (print)
ISBN 978-92-79-21045-7 (pdf)
ISBN 978-92-79-21044-0 (print)
doi:10.2787/50186

Abstract

This report describes the production of the reference material ERM-BD600, a whole milk powder certified for the mass fractions of water- and fat-soluble vitamins. All the required steps during the production were conducted according to ISO Guides 34 and 35 [1,2] to ensure the high quality of the final product, including matrix material processing, homogeneity and stability studies and value assignment. The matrix material, a spray dried whole milk powder, was packed into sachets containing approximately 100 g of powder, and stored at -30 ºC. Dedicated studies confirmed the homogeneity and stability of the vitamins in the milk powder under the conditions evaluated. The characterisation of the material, organised and coordinated by IRMM, was done by an inter-laboratory comparison. The certified and indicative values were obtained as the unweighted mean of the laboratory means of the accepted sets of results for each analyte, and the expanded uncertainty associated (k = 2) includes contributions from the homogeneity, the long term stability and the characterisation of the material. The certified values in ERM-BD600 are the following:

Component	**Certified Value**[1] [mg/kg] dry mass	**U**[2] [mg/kg] dry mass	p
Vitamin A (all-*trans* retinol)	**3.8**	**0.6**	8
A (*all-trans*-retinol and 13-*cis*-retinol)	**4.1**	**0.8**	6
Vitamin B_1 (thiamin)[3]	**4.5**	**0.6**	14
Vitamin B_2 (riboflavin)	**16.7**	**1.4**	14
Vitamin B_{12} (cyanocobalamin)	**0.32**	**0.07**	6
Vitamin C (total ascorbate)	**74**	**11**	15
Vitamin E (α-tocopherol)	**86**	**15**	18

[1]Unweighted mean value of the accepted sets of data (p) obtained in a different laboratory and/or with a different method of determination expressed in mg/kg in dry mass.
[2]Expanded uncertainty with a coverage factor of $k = 2$, alternatively for vitamins B_{12} and A (*all-trans*-retinol and 13-*cis*-retinol), $t_{(0.05,5)}$=2.57, according to ISO Guide 98-3 [3], to the Expression of Uncertainty in Measurement, corresponding to a level of confidence of approximately 95 %.
[3] Expressed as thiamin hydrochloride

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LA-NA-24924-EN-N

Publications Office