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Development of two reference materials for all trans-retinol, retinyl palmitate, α- and γ-tocopherol in milk powder and infant formula

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ABSTRACT

Vitamins are important food constituents that can be present in almost every foodstuff. Food quality and safety depends on food surveillance by reliable quantitative analysis enabled by appropriate quality control. Certified matrix reference materials are versatile tools to support quality assurance and control. However, in the case of vitamins, which are important in various foods, there is a lack of matrix reference materials. Two certified reference materials for the determination of all–*trans*-retinol, retinyl palmitate, and α- and γ-tocopherol in milk powder and infant formula have been developed by the National Institute of Standards, Egypt. This article presents the preparation, characterization, homogeneity, and stability testing as well as statistical treatment of data and certified value assignment. The assignment of the certified values and associated uncertainties in the prepared natural-matrix reference materials were based on the widely used approach of combining data from independent and reliable analytical methods.

1. Introduction

Infant formula as a breast-milk substitute must be manufactured to satisfy, by itself, the nutritional requirement of infants during the first months of life up to the introduction of appropriate complementary food. The relationship between nutrient levels and human health requires accurate determination of nutrient levels by laboratories making such measurements. Food control laboratories are required to use validated methods wherever possible. For this reason, analytical methods must be subjected to internal and/or external validation studies. Food and feed reference materials and especially certified reference materials play a key role in internal validation studies. They are a useful tool in the verification of the accuracy of analytical measurements and are employed in analytical quality assurance, quality control

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schemes, laboratory accreditation, as well as in the establishment of traceability in the framework of internationally agreed standards [1–3]. They can also be used for the process of measurement uncertainty estimation or the calibration of analytical instruments. At present, the quality of feed and foodstuffs is estimated not only using ecological indices (concentration of toxic elements, mycotoxins, etc.), but also by the concentration of valuable biologically active substances (amino acids, vitamins, provitamins, etc.). The concentration of biologically active substances, vitamins in particular, in foodstuffs is strictly regulated, because both the deficiency and excess of vitamins can cause harmful effects [4]. Vitamins have been divided into two groups based on their solubilities in fat solvents or in water. Thus, fat-soluble vitamins include A, D, E, and K, while vitamins of the B-complex and C are classified as water-soluble. In general, vitamin A refers to all–*trans*-retinol, which is the most active form of this vitamin, while vitamin E is a collective term for tocopherols (α-, β-, γ-, and δ) and tocotrienols [5,6]. In the present work, the process of preparation and characterization of matrix reference materials from milk powder and infant formula is described. These reference materials were certified for mass fraction of vitamins A and E using independent analytical methods [7] for sample preparation and chromatographic separation. Establishing the traceability of measurement results to SI units in addition to the homogeneity and stability testing of the prepared reference materials were also highlighted.

2. Materials and methods

2.1. Chemicals and reagents

High-performance liquid chromatography (HPLC) grade solvents, methanol, acetonitrile, n-hexane, acetone, petroleum ether, dichloromethane, cyclohexane, isopropanol, diethyl ether, and ethyl acetate were purchased from Fluka and Riedel (Munich, Germany) and were used in all procedures without further purification. Tert-butylhydroquinone was used as an antioxidant and was obtained from Sigma–Aldrich (Munich, Germany). K_2HPO_4 (extra pure), and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Karl Fischer titration reagents were from Scharlau (Sentmenat, Spain) High purity water was obtained through a Milli-Q water purification system (Millipore, Bedford, MA, USA) and was used in all procedures. Vitamins A and E (all–*trans*-retinol, retinyl palmitate, α-tocopherol and γ-tocopherol) were of analytical reagent grade. All the vitamins were supplied by Sigma–Aldrich.

2.2. Chromatographic system used for determination of vitamins A and E

The chromatographic analysis was carried out using HPLC Agilent 1100 integrated system equipped with a G1313A automated injector, a G1311A pump and G1315B multiwavelength diode-array detector (Agilent, Santa Clara, CA, USA). The chromatographic separation of the compounds was achieved with a reversed phase columns, ZORBAX SB-C18 (3.0 mm × 250 mm, 5 μm) from Agilent and (ODS-hypersil 2.1 mm × 100 mm 3 μm) from Thermo Fisher (Waltham, MA, USA) as well as normal phase ZORBAX-RX Sil 4.6 mm × 250 mm 5 μm and Polaris 5 Si 4.6 mm × 100 mm columns from Agilent, operating at constant room temperature (20°C). The chromatographic data were analyzed using Agilent Chemstation Rev. B.02.01-SR1 (260). The compounds under study were identified by their retention times and their UV spectral characteristics.

2.3. UV-visible spectrophotometer

The purity of vitamins was determined by UV-visible spectrophotometry using a Specord 250 Plus (Analytik-Jena, Jena, Germany) equipped with a 15-sample tray.

2.4. Karl Fischer titrator

Moisture content was determined using a volumetric Karl Fischer titrator (852 Titrando; Metrohm, Tampa, FL, USA) in which about 0.2 g samples were accurately weighed and transferred to a Karl Fisher beaker containing methanol as extraction solvent. The method was applied for four bottles and each bottle was measured three times.

2.5. Materials processing and packaging

Milk powder and infant formula samples were obtained from local markets (Cairo, Egypt). Two kilograms of each material were homogenized and packed in 40-g brown glass bottles to prevent light exposure and humidity uptake, the bottles were stored at 4°C in the dark.

2.6. Sample preparation

2.6.1. Saponification

Five grams of powdered sample were reconstituted with 10 mL of distilled water, and then immersed in warm water (40°C), and mixed for 5 minutes until complete homogenization was achieved. Vortex was used to complete the homogenization of the sample. The sample was transferred to a 250 mL Erlenmeyer flask, and 0.5 mL of a 20% (m/v) hydroquinone solution and 50 mL of KOH ethanol solution were added. The preparation was subjected to continuous shaking for 2 hours at room temperature [8,9].

2.6.2. Extraction

The sample was transferred to a separating funnel and 25 mL of hexane were added and the mixture was shaken for 10 minutes. The upper/organic layer was then transferred to another separating funnel, and the aqueous layer was re-extracted with a further 25 mL of hexane. The two organic extracts were combined and washed twice by shaking for 1 minutes with a mixture of 10 mL ethanol and 25 mL of water. The organic layer was collected and evaporated to dryness in a turbo vap evaporator at 40°C. For the second method, the same steps were applied using ethyl acetate/diethyl ether/petroleum ether mixture with ratio (20:30:50) respectively.

2.6.3. *Chromatographic separations*

After evaporation, residue was redissolved in 1 mL of methanol. The methanol was directly filtered through a 0.45-μm nylon filter and collected in a 1 mL amber glass vial and 10 μL were then injected into the HPLC system. The separation was carried out using ZORBAX SB-C18 (3.0 mm × 250 mm, 5 μm) or ODS-hypersil (2.1 mm × 100 mm, 3 μm) at flow rate 0.5 mL/min using linear and step gradient from methanol and water mixture as shown in Table 1.

For normal phase chromatography, the extract was reconstituted with 1 mL of hexane. The hexane phase was directly filtered through a 0.45-μm nylon filter and collected in a 1 mL amber glass vial, and then 10 μL were injected into the HPLC system. The separation was carried out using ZORBAX-RX Sil 4.6 mm × 250 mm, 5 μm and polaris 5 Si 4.6 mm × 100 mm connected in series at flow rate 1.0 mL/min using isocratic elution of 98.5% hexane and 1.5% isopropanol. Retinols and vitamin E were detected at 325 nm and 296 nm, respectively.

3. Results and discussion

Four methods were used for characterization of vitamins in milk powder and infant formula (Fig. 1). Two different methods of extraction based on solvents of different polarities and two methods of separation based on columns with different selectivity were used. In the first and third methods, mixtures of ethyl acetate/diethyl ether/petroleum ether (20:30:50, respectively) were used for extraction of fat-soluble vitamins from milk and infant formula. Meanwhile separation of the extracted vitamins was carried out by reversed and normal phase liquid chromatography. However, these methods were applied after saponification using alcoholic potassium hydroxide solution. The resulting chromatograms M1 and M3 are shown in Figs. 2 and 3. From these figures, it can be seen that all–*trans*-retinol, α-tocopherol, γ-tocopherol, and retinyl palmitate were separated clearly in both milk powder and infant formula. In the second and fourth methods, normal hexane was used for extraction of vitamins A and E from milk and infant formula. The separation of the extracted vitamins was carried out by normal reversed phase liquid chromatography. However, these methods were applied after saponification using alcoholic potassium hydroxide solution. The resulting chromatograms M2 and M4 are shown in Figs. 2 and 3. From the figures, it can be seen that all–*trans*-retinol, α-tocopherol, γ-tocopherol, and retinyl palmitate were separated clearly in infant formula. Retinyl palmitate was not detected in milk powder by the second method.

Table 1 – Chromatographic separation.

Time (min)	Methanol (%)	Water (%)
0	80	20
5	90	10
10	98	2
20	99	1
25	80	20

3.1. Determination of water content

The water content was measured by volumetric Karl Fischer titration. The average water content of the milk powder and infant formula material, as determined by Karl Fischer, is 2.91 ± 0.16 g/100 g and 2.58 ± 0.13 g/100 g, respectively.

3.2. Homogeneity study

The homogeneity study was designed so that the between-bottle variability and the within-bottle variability can be studied [10]. The experimental set-up of the homogeneity study is explained in Fig. 4, which shows the different bottles and the subsamples of each bottle. For quantitative determination of inhomogeneity of the materials, five bottles were randomly selected from the whole set of 50 bottles for each matrix, and five independent subsamples of each bottle were analyzed. All selected samples were analyzed under repeatability. This scheme was repeated once, leading to a total of five sample preparations per unit. All samples were analyzed by reversed phase liquid chromatography under repeatability conditions ensuring that all samples were quantified against the same calibration. The results of the homogeneity studies in shown in Tables 2 and 3 were analyzed by analysis of variance (ANOVA) in order to evaluate the uncertainty of the material variability [11–13]. From the ANOVA analysis results, the values of $F_{calculated}$ are smaller than those of $F_{critical}$ for all vitamers, indicating good homogeneity between bottles [10].

3.3. Stability study

Vitamins A and E in milk powder and infant formula candidates for certification were submitted to stability studies. Given that factors such as moisture, oxidation, or exposure to UV light can affect the stability of the vitamins, care was taken to minimize these factors as much as possible during the material processing, packaging, and storage. Assessment of the stability was performed through the analysis of samples at four time points (0 months, 2 months, 4 months, and 6 months) at 4°C and 20°C. Five samples were analyzed at each time point at each temperature. Two replicates per sample were analyzed and the average results are shown in Table 4. The results were evaluated for each time point and temperature. Results were screened for single and double outliers at confidence level of 95%. Data were plotted as a function of time and the regression lines were calculated to check for significant trends, possibly indicating degradation of the material. The uncertainties due to stability of the target vitamins were calculated as uncertainty of the slope of the regression line [12–16]. The significance of the slope was evaluated statistically with the aim of detecting any possible trend that would indicate degradation of the material. At storage temperatures of 4°C and 20°C, statistically significant trends were not observed for all vitamers.

3.4. Traceability of measurements

The traceability of the measurement results to the definition of the SI units was achieved by the use of primary calibrator solution. Its concentration values were based on the extinction coefficients, masses, purity assessments, and appropriate

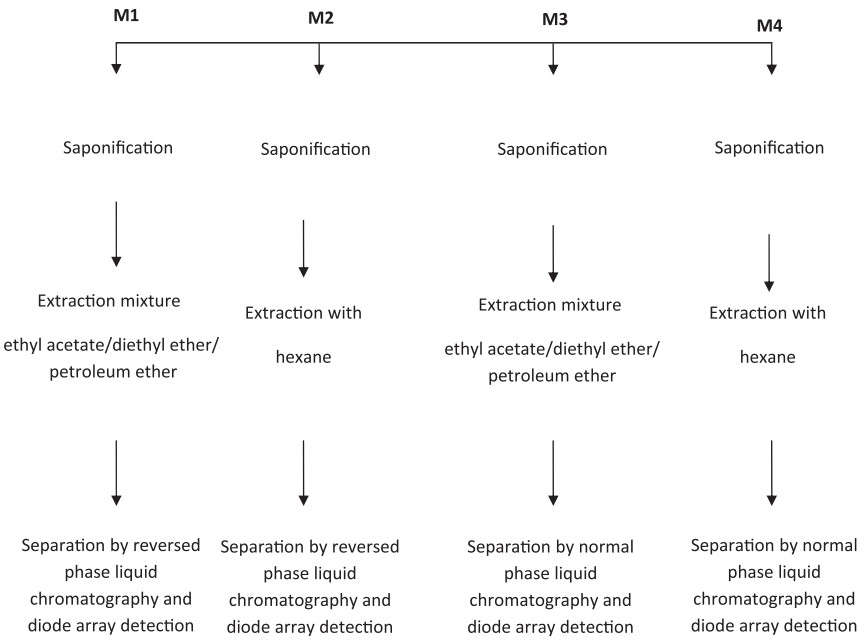

Fig. 1 – Methods of extraction, separation and quantification of fat-soluble vitamins in milk powder and infant formula.

uncertainties [17]. The standard uncertainties for the purity measurements represent the standard deviation of the mean values are provided in Table 5. The concentrations of vitamins A and E in the stock solutions were determined by two methods. In the first method we used dynamic flow HPLC coupled with diode array detector [18–20]. The second method is the static UV/VIS spectrophotometer using Beer's Law [21–27] with purity corrections for impurity absorbed at the same wavelength by liquid chromatography.

3.5. *Statistical treatment of the measurement results*

The results were tested for normality, outliers, equality of means, and homogeneity of variances at 95% confidence level

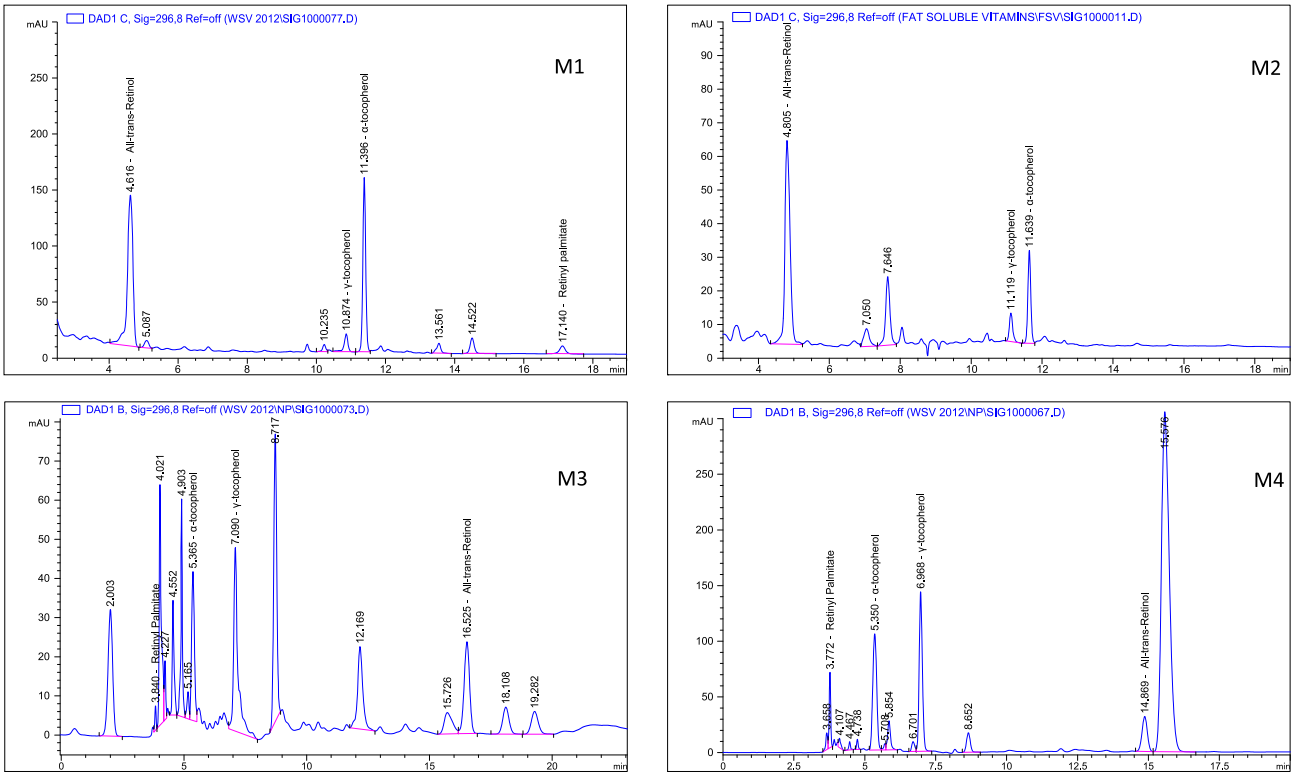

Fig. 2 – Chromatograms of vitamins A and E in milk powder by the four independent analytical methods.

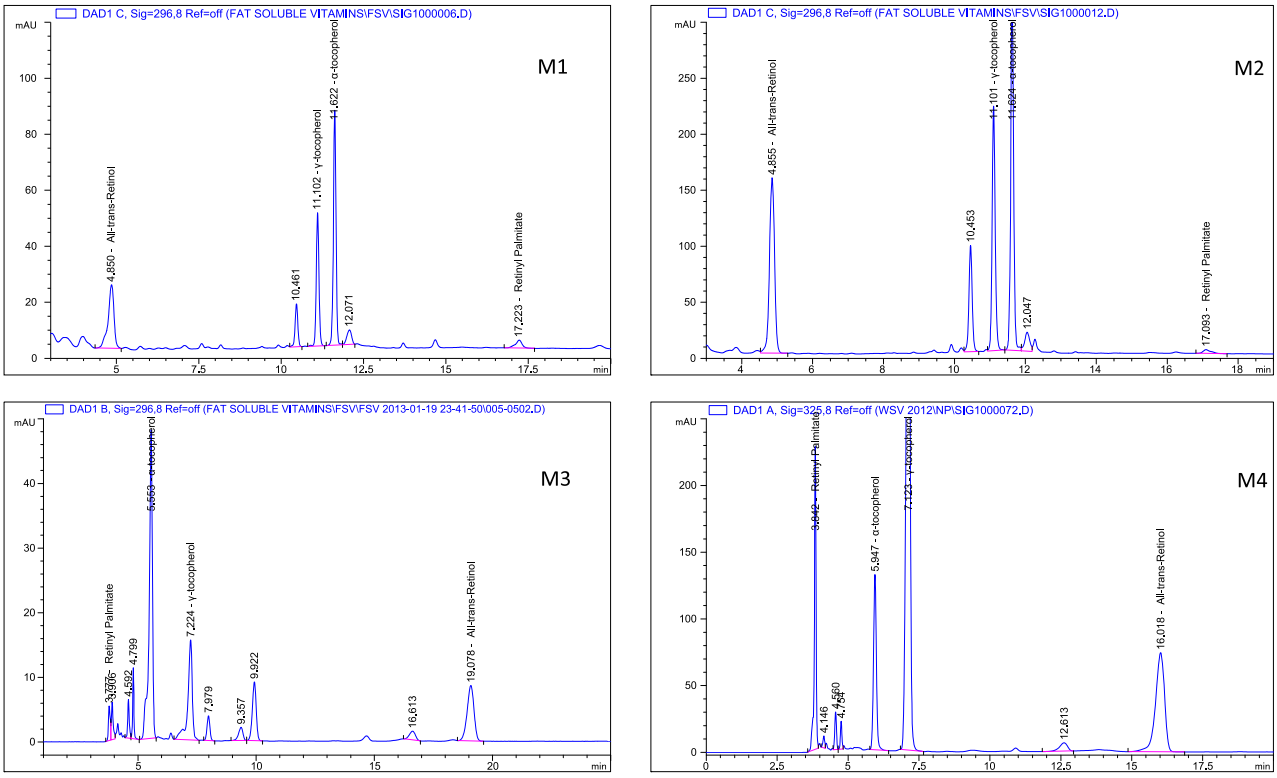

Fig. 3 – Chromatograms of vitamins A and E in infant formula by the four independent analytical methods.

using the statistical packages: Minitab 16 (Minitab Inc. Brandon Court, Progress Way, Coventry, UK), Costat statistical software (CoHort Software, Pacific Grove, CA, U.S.A), Microsoft Excel 2010 (Microsoft, Redmond, WA, USA), and XLSTAT 7.5.2 (Addinsoft, New York, USA), respectively. The result indicates that the data were normal except for retinyl palmitate in milk powder, free from outliers, and methods variances were homogenous except for data of retinyl palmitate in milk powder and all–*trans*-retinol in infant formula.

3.6. *Assignment of reference value and uncertainty*

Data from the different analytical methods were used for computation of the weighted mean and its associated

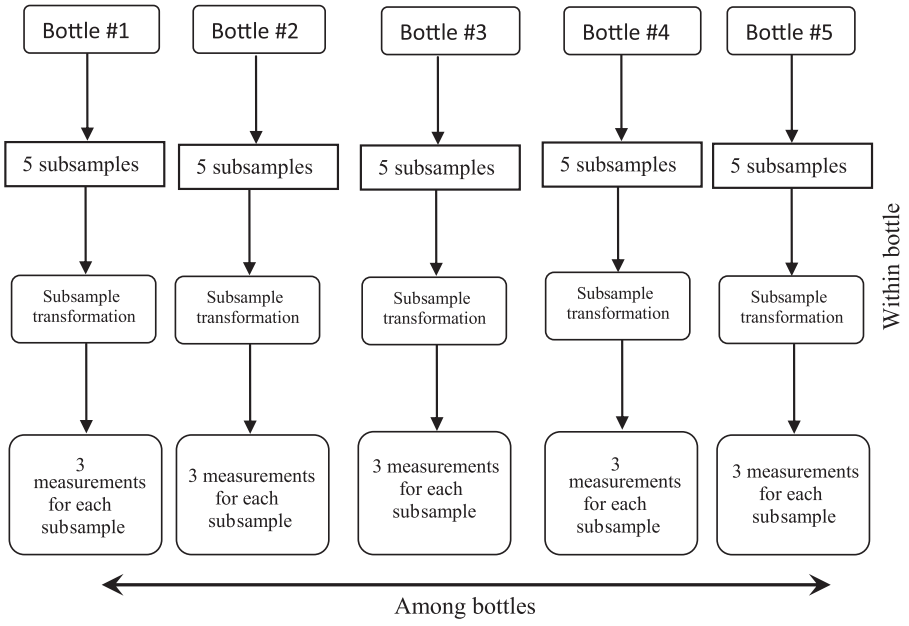

Fig. 4 – Layout for the homogeneity study of the within and among bottles.

Table 2 – Results of the between-bottle homogeneity study for vitamins A and E in milk powder reference material.

Bottle	All–*trans*-retinol (mg/kg)					α-tocopherol (mg/kg)				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
	5.17	4.94	5.15	5.34	5.02	78.11	81.96	83.41	79.76	77.63
	4.81	5.31	5.24	5.11	4.96	82.84	75.83	72.82	77.89	79.48
	4.88	5.24	5.52	5.23	5.09	79.41	84.31	79.29	73.41	84.88
	5.34	4.97	5.04	5.07	5.23	83.06	76.61	77.88	80.62	77.24
	5.09	4.83	5.16	5.19	5.13	76.17	82.83	82.62	79.71	80.72
Bottle	γ-tocopherol (mg/kg)					Retinyl palmitate (mg/kg)				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
	51.01	49.07	50.61	51.94	52.81	0.24	0.19	0.19	0.27	0.22
	50.92	53.99	51.49	51.25	50.53	0.25	0.29	0.24	0.29	0.27
	50.84	48.43	49.01	50.32	49.55	0.23	0.21	0.25	0.28	0.31
	50.26	51.39	48.73	49.47	48.17	0.21	0.18	0.27	0.26	0.21
	53.07	53.63	51.21	50.48	51.97	0.27	0.19	0.18	0.22	0.20

uncertainty. The uncertainty takes into consideration the random error within each method and the error due to the disagreement between methods. The strategy of combining data to derive the certified values and its uncertainties is: (1) estimation of the uncertainty of the method mean for each method; (2) estimation of the between-method variance and the weights of each method; (3) calculation of the weighted mean and its variance; (4) estimation of the uncertainty due to the material variability; (5) calculation of the effective degrees of freedom; and (6) estimation of the bias allowance.

3.6.1. *Calibration uncertainty*

The uncertainty of the calibration process is a combined contribution from the calibration curve, random variations in measurements of unknown and standards, gravimetric dilution of the stock solution, and uncertainty of the assigned reference values. The uncertainty budget of the calibration process was evaluated in accordance with ISO GUM and EURACHEM guides [28,29]. The estimation of each of the three contributions is explained below.

3.6.1.1. Random variations in measurement of y. The uncertainty $u(x_{pred}, y)$ in a predicted value x_{pred} due to variability in y can be estimated from the calibration data in Equations (1) and (2):

$$\mathrm{var}_{(x_{pred})} = \mathrm{var}(y_{obs}) \Big/ b_1^2 + \frac{S^2}{b_1^2} \cdot \left(\frac{1}{\sum w_i} + \frac{(x_{pred} - \bar{x})^2}{(\sum (w_i x_i^2) - (\sum (w_i x_i)^2 / \sum w_i} \right) \quad (1)$$

$$u_{(x_{pred})} = \sqrt{\mathrm{var}(x_{pred})} \quad (2)$$

Where S^2 is the residual error, b_1 is the calculated best fit gradient, w_i is the weight assigned to y_i and is the difference between x_{pred} and the mean x of the n values x_1, x_2....x_n.

3.6.1.2. Uncertainty of the assigned reference values x_i. The reference values x_i have uncertainties that propagate through to the final result (Equation (3)). An approximate estimate of the uncertainty $u(x_{pred}, x_i)$ in a predicted value x_{pred} due to uncertainty in a particular reference value x_i is:

$$u(x_{pred}, x_i) \approx u(x_i)/n \quad (3)$$

where n is the number of x_i values used in the calibration.

3.6.1.3. Gravimetric dilution of the stock solution. The values of x and y are subject to a constant unknown offset arising from serial dilution of the stock solution. If the standard

Table 3 – Results of the between-bottle homogeneity study for vitamins A and E in infant formula reference material.

Bottle	All–*trans*-retinol (mg/kg)					α-tocopherol (mg/kg)				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
	6.63	6.53	7.13	6.55	7.01	75.78	69.93	72.14	69.15	72.18
	6.84	7.11	7.03	6.92	6.86	72.51	77.89	73.49	76.55	69.95
	7.02	6.63	6.79	7.29	7.11	71.34	69.57	66.19	71.69	70.81
	6.54	6.62	6.94	6.81	6.73	72.17	74.09	68.08	72.32	73.14
	6.41	6.84	7.16	7.57	7.19	75.06	76.01	69.85	78.47	72.9
Bottle	γ-tocopherol (mg/kg)					Retinyl palmitate (mg/kg)				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
	50.70	52.37	49.06	51.21	48.19	0.80	0.82	0.81	1.02	0.93
	48.22	48.39	51.52	50.18	51.30	0.74	1.07	1.01	0.83	1.03
	51.07	49.65	49.90	51.23	51.06	0.79	1.05	0.61	0.71	1.09
	52.57	50.21	53.44	52.58	53.19	0.94	0.72	0.69	0.96	0.92
	50.65	50.70	49.65	54.16	50.68	0.82	0.77	0.75	1.01	0.86

Table 4 – Summary results of stability study at 4°C and 20°C expressed as dry mass fraction (mg/kg).

Matrix	Analyte	Temp.	Months				Regression	
			0	2	4	6	Slope	S
Milk powder	All–*trans*-retinol	20°C	5.25	5.23	5.02	4.89	−0.06	0.01
	α-tocopherol		77.91	78.51	76.74	79.50	0.15	0.30
	γ-tocopherol		51.06	50.11	51.81	49.89	−0.09	0.23
	Retinyl palmitate		0.28	0.23	0.25	0.19	−0.01	0.01
	All–*trans*-retinol	4°C	5.02	5.05	4.92	5.08	0.00	0.02
	α-tocopherol		79.91	79.84	80.88	79.50	−0.01	0.16
	γ-tocopherol		51.26	49.88	51.05	50.83	−0.01	0.17
	Retinyl palmitate		0.28	0.19	0.25	0.23	0.00	0.01
Infant formula	All–*trans*-retinol	20°C	7.31	7.28	7.14	7.06	−0.04	0.01
	α-tocopherol		78.49	79.27	79.19	80.99	0.37	0.13
	γ-tocopherol		50.20	51.11	51.43	50.92	0.12	0.11
	Retinyl palmitate		0.98	0.81	0.78	1.03	0.01	0.03
	All–*trans*-retinol	4°C	7.11	6.94	7.06	7.13	0.01	0.02
	α-tocopherol		78.49	76.02	77.10	72.92	−0.78	0.34
	γ-tocopherol		50.20	49.40	48.86	50.32	−0.01	0.19
	Retinyl palmitate		0.98	0.73	0.98	0.94	0.01	0.03

uncertainties (Equation (4)) on y and x from these effects are *u(y, const)* and *u(x, const)*, then the uncertainty on the interpolated value x_{pred} is given by:

$$u(x_{pred})^2 = u(x, const)^2 + (u(y, const)/b_1)^2 + \mathrm{var}(x) \quad (4)$$

3.6.2. *Uncertainty of measurements of the mean of each method*

Since more than one analytical method is compared to determine the certified value, it is important that the variability of the mean for each method is estimated correctly. In order to estimate the standard uncertainty of the mean, a two-way fully nested ANOVA was used to determine which design factors have significant effect on the measurements [30]. The randomized complete block design model explained in Equations (5)–(7) was used to calculate the type An uncertainty of each of the method means.

$$Var(\bar{y}) = \frac{MS.sample + MS.run - MS.error}{Total.number.of.measurements} \quad (5)$$

The type A standard uncertainty is

$$\sqrt{Var(\bar{y})} \quad (6)$$

Type A combined standard uncertainty is composed from type A standard uncertainty and calibration uncertainty. The results obtained are shown in Tables 6 and 7 for each matrix.

$$u_c = \sqrt{(Type\ A)^2 + (Calibration)^2} \quad (7)$$

3.6.3. *Between method variance and method weights*

The measured values produced by each method are modeled as the sum of the true property value, method bias, and random error (Equation (8)):

$$m + b_i + e_{ij} \quad (8)$$

Method weights are derived by assuming that the random errors (e_i) are independent, have mean = 0, and have different variance for each method. The variance σ_b^2 may be estimated from the between-method differences. Under this model, the variance of the average of n_i measurements from the ith method is:

$$\frac{\sigma_i^2}{n_i} + \sigma_b^2 \quad (9)$$

Averaging the method means using weights proportional to the inverse of this variance leads to an estimate of the true value with minimum variance. The between method variance are illustrated in Tables 6 and 7 for each matrix. The weight for each method is inversely proportional to the sum of the variance of its mean (Equations (10) and (11)) and the between-method variance. The Paule–Mandel weighting scheme involves use of an algorithm for estimating the between method variance, σ_b^2 and the square of its combined standard

Table 5 – Results of purity determination by UV/VIS spectrophotometer and LC and associated standard uncertainty for vitamins A and E.

Vitamin	UV/VIS spectrophotometric method		Liquid chromatographic method		Selected uncertainty (%)
	Purity (%)	Uncertainty (%)	Purity (%)	Uncertainty (%)	
Retinyl palmitate (*trans*)	98.47	0.68	98.21	0.79	0.79
All–*trans*-retinol	94.95	0.84	94.76	0.71	0.84
α-tocopherol	96.78	0.66	97.68	0.81	0.81
γ-tocopherol	95.30	0.71	94.97	0.63	0.71

Table 6 – Methods athematic mean, type A standard uncertainty, between method variance, method weights (W_i), and weighing factor for each method used in the characterization of vitamins A and E in milk powder reference material.

Matrix	Analyte	Method	Mean	Type A standard uncertainty	Combined standard uncertainty	Between method variance	W_i	Weighing factor	Material Variability
Milk Powder	All trans-Retinol	M1	5.35	0.010	0.190	0.1	7.34	0.24	0.04
		M2	5.16	0.021	0.191	0.1	7.32	0.24	
		M3	5.00	0.014	0.167	0.1	7.82	0.26	
		M4	5.22	0.015	0.167	0.1	7.82	0.26	
	α-tocopherol	M1	80.11	6.010	6.111	1.72	0.03	0.05	0.87
		M2	77.33	2.270	2.525	1.72	0.12	0.22	
		M3	79.47	2.210	2.495	1.72	0.13	0.23	
		M4	80.46	0.740	1.375	1.72	0.28	0.50	
	γ-tocopherol	M1	48.40	0.220	1.005	2.3	0.30	0.36	0.41
		M2	47.70	0.660	1.182	2.3	0.27	0.32	
		M3	51.78	2.330	2.559	2.3	0.11	0.14	
		M4	52.61	1.830	2.114	2.3	0.15	0.18	
	Retinyl Palmitate	M1	0.26	0.030	0.057	0.01	75.73	0.32	0.01
		M2	NA	NA	NA			0.00	
		M3	0.25	0.020	0.046	0.01	82.38	0.35	
		M4	0.25	0.040	0.058	0.01	74.97	0.32	

uncertainty S_i^2 Then the method weights are defined implicitly as follows:

$$W_i = \left[\frac{1}{S_i^2 + \widehat{\sigma}_b^2}\right] \tag{10}$$

The weights are

$$w_i = \frac{W_i}{\sum_1^M W_j} \tag{11}$$

3.6.4. *Material variability*

The uncertainty due to the between-bottle variability u_{bb} can be estimated from the difference between the total variability and the within bottle variability [12,30–34]. Uncertainty due to homogeneity can be calculated using Equation (12) or (13).

$$\sigma_h(u_{bb}) = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \tag{12}$$

$$\sigma_h(u_{bb}) = \sqrt{MS_{within}/n} \cdot \sqrt[4]{2/(\nu MS_{within})} \tag{13}$$

If $MS_{between}$ is larger than MS_{within}, either Equation (12) was used, or both equations were applied and the largest uncertainty value is chosen. However, if $MS_{between}$ is smaller than MS_{within}, which indicates poor repeatability of the measurement method, only Equation (13) can be used.

3.6.5. *Certified value (weighted mean) and its uncertainty*

Results in Tables 8 and 9 were combined to investigate whether they provide certified, reference, or information values. To combine the results, a weighted average of the method means is computed according (Equation (14)) to the

Table 7 – Methods athematic mean, type A standard uncertainty, between method variance, method weights (W_i), and weighing factor for each method used in the characterization of vitamins A and E in infant formula reference material.

Analyte	Method	Mean	Type A standard uncertainty	Combined standard uncertainty	Between method variance	W_i	Weighing factor	Material variability
All–*trans*-retinol	M1	6.76	0.020	0.191	0.07	9.38	0.24	0.11
	M2	6.88	0.030	0.193	0.07	9.34	0.24	
	M3	6.87	0.015	0.167	0.07	10.22	0.26	
	M4	6.92	0.014	0.167	0.07	10.22	0.26	
α-tocopherol	M1	74.80	0.680	1.298	0.82	0.40	0.28	0.90
	M2	73.36	1.320	1.722	0.82	0.26	0.18	
	M3	72.99	0.720	1.364	0.82	0.37	0.26	
	M4	71.58	0.610	1.309	0.82	0.39	0.28	
γ-tocopherol	M1	48.92	0.180	0.997	1.5	0.40	0.29	0.41
	M2	48.51	0.330	1.035	1.5	0.39	0.28	
	M3	51.09	0.860	1.364	1.5	0.30	0.22	
	M4	52.11	0.990	1.450	1.5	0.28	0.20	
Retinyl palmitate	M1	0.86	0.020	0.052	0.02	44.05	0.25	0.05
	M2	0.81	0.010	0.049	0.02	44.63	0.25	
	M3	0.85	0.040	0.058	0.02	42.85	0.24	
	M4	0.81	0.030	0.051	0.02	44.17	0.25	

Table 8 – Certified value and expanded uncertainty for each analyte in milk powder reference material.

Analyte	Method	Weighted mean	Weighted uncertainty	Certified value (mg/kg)	Bias allowance	u_c (mg/kg)	U_{exp} (mg/kg)
All–*trans*-retinol	M1	1.25	0.09	5.18	0.18	0.28	0.57
	M2	1.29					
	M3	1.35					
	M4	3.71					
α-tocopherol	M1	17.31	1.09	79.52	2.18	3.58	7.37
	M2	18.12					
	M3	40.38					
	M4	17.55					
γ-tocopherol	M1	15.48	0.74	49.38	2.39	3.23	6.66
	M2	7.02					
	M3	9.32					
	M4	0.08					
Retinyl palmitate	M1	0.00	0.03	0.25	0.01	0.04	0.08
	M2	0.09					
	M3	0.08					
	M4	1.25					

weighting algorithm of Paule and Mandel, which is often implemented for combining data from independent chemical analysis methods [30–34]. The weight for each method is inversely proportional to the sum of the variance of its mean and the between-method variance. The weighted average $\tilde{X}$ of the $\overline{X}_i$.

$$\tilde{X} = \sum_{1}^{M} w_i \overline{X}_i \tag{14}$$

Using this weighting scheme, the method weights, the weighted means, and the average weighted mean for each analyte have been calculated and the results are shown in Tables 8 and 9 for milk powder and infant formula, respectively. The uncertainty budget reported on the certification is the expanded uncertainty, which was evaluated in accordance with ISO GUM and EURACHEM guides [28,29]. All possible sources of uncertainty are taken into account.

The combined standard uncertainty, S, for the weighted mean is:

$$S = \sqrt{\sum_{1}^{M} W_i^2 S_i^2} \tag{15}$$

Bias allowance (Equation (16)) is a systematic error, due to the difference in methods. It is taken as the maximum absolute deviation of any method mean from the weighted mean.

$$\textit{Bias allowance} = \max_i |\overline{X}_i - \tilde{X}| \tag{16}$$

Prior to the estimation of interval for the certified value the effective degrees of freedom of the total variance are needed. The degree of freedom is:

$$df\,(\textit{effective}) = \left(\sum_{1}^{M} w_i^2 S_i^2 + \hat{\sigma}_h^2\right)^2 \Big/ \left(\sum_{1}^{M} \frac{(w_i^2 S_i^2)}{n_i - 1} + \frac{\hat{\sigma}_h^4}{df_h}\right) \tag{17}$$

where *df*, is the number of samples measured in duplicate for material variability estimation minus one. The results of the weighted uncertainties calculated according to the scheme explained above are given in Tables 8 and 9 for milk powder

Table 9 – Certified value and expanded uncertainty for each analyte in infant formula reference material.

Matrix	Analyte	Method	Weighted mean	Weighted uncertainty	Certified value (mg/kg)	Bias allowance	u_c (mg/kg)	U_{exp} (mg/kg)
Infant Formula	All trans-Retinol	M1	1.62	0.09	6.86	0.10	0.25	0.51
		M2	1.64					
		M3	1.79					
		M4	1.81					
	α-tocopherol	M1	20.86	0.70	73.17	1.62	2.76	5.68
		M2	13.54					
		M3	19.03					
		M4	19.74					
	γ-tocopherol	M1	14.37	0.59	49.92	2.19	2.91	5.99
		M2	13.82					
		M3	11.13					
		M4	10.60					
	Retinyl Palmitate	M1	0.21	0.03	0.83	0.03	0.08	0.17
		M2	0.21					
		M3	0.21					
		M4	0.20					

and infant formula. The degrees of freedom were calculated and the t-multiplier was identified to be 2.06. The total expanded uncertainty (U_{exp}) associated with the combined concentration value was calculated according to Equation (18) including the estimate of between method bias.

$$U = t_{1-\alpha/2}\sqrt{S^2(\tilde{X}) + \sigma_h^2} + Bias\ allowance \quad (18)$$

where $S^2(\tilde{X})$ is the weighted combined standard uncertainty and σ_h^2 is the uncertainty of the material inhomogeneity.

4. Conclusion

Milk powder and infant formula are matrix certified reference materials that have been certified for the content of all–*trans*-retinol, retinyl palmitate, α-tocopherol, and γ-tocopherol by four independent analytical methods. Two different methods of extraction using solvent of different polarities and two methods of separation using columns with different selectivity. Certification was carried out in full compliance with ISO Guides 30–35 and measurement results were statistically treated and the certified values of all–*trans*-retinol, retinyl palmitate, α-tocopherol, and γ-tocopherol in milk powder and infant formula were assigned and their associated expanded uncertainties were estimated as: 5.18 ± 0.57 mg/kg, 0.25 ± 0.08 mg/kg, 79.52 ± 7.37 mg/kg, and 49.38 ± 6.66 mg/kg; and 6.86 ± 0.51 mg/kg, 0.83 ± 0.17 mg/kg, 73.13 ± 5.68 mg/kg, and 49.92 ± 5.99 mg/kg, respectively. The two reference materials provide a basis for further improvements in quality assurance and quality control of vitamin analysis in milk powder and infant formula. Their importance is not only attributed to the possibility of laboratories internal and external quality control but also to the facilitation of method validation.

Conflicts of interest

All authors declare no conflicts of interest.

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