INFANT FORMULA AND ADULT NUTRITIONALS

Improved AOAC First Action 2011.08 for the Analysis of Vitamin B_{12} in Infant Formula and Adult/Pediatric Formulas: First Action 2014.02

ESTHER CAMPOS GIMÉNEZ
Nestlé Research Center, Vers-chez-les-Blanc, CH-1000 Lausanne 24, Switzerland

This report documents improvement and single-laboratory validation performed on AOAC First Action Method 2011.08 for vitamin B_{12} in infant formula and adult/pediatric nutritional formula. The original validation study included a range of fortified products, from infant formulas to breakfast cereals or beverages. Extended validation data, including additional infant formulas and adult/pediatric nutritionals, has now been produced. In addition, the method has been modified to use ultra-HPLC and the calibration range extended in a multilevel calibration curve. Detection and quantification limits were also improved by increasing the sample weight used for analysis and the reconstitution rate adapted to the requirements. The Stakeholder Panel on Infant Formula and Adult Nutritionals Test Material Kit, designed to represent a large range of products within the category (infant formula and adult nutritionals made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein), was used to determine performance characteristics of the method. The modifications included allow now full compliance with standard method performance requirements established for vitamin B_{12} (SMPR 2011.005). LOQ was ≤0.01 µg/100 g, working range between 0.01 and 5.0 µg/100 g, repeatability ≤7%, and recovery in the range 90–110%. The method was granted AOAC First Action status 2014.02.

Based on the data presented in the single-laboratory validation study (SLV) reported by Campos Giménez et al. (1), the method "Determination of Vitamin B_{12} in Infant Formulas and Adult Nutritionals by LC-UV Detection with Immunoaffinity Extraction" was granted First Action status and designated AOAC **2011.08** (2, 3).

The original validation study included a large range of fortified products, not only infant formulas but also breakfast cereals and beverages. The data provided for infant formulas and adult/pediatric nutritionals was limited and needed to be extended to the full set of Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) matrixes. The method has been further improved by introduction of rapid ultra-HPLC (UHPLC), a multilevel external calibration curve, and change of reconstitution rate and sample weight used to comply with SPIFAN requirements. Additional sample preparation for the analysis of amino acid-based products has now been included. These modifications allow full compliance with standard method performance requirements (SMPR) established for vitamin B_{12} (4) in terms of LOQ (≤0.01 µg/100 g), working range (0.01–5.0 µg/100 g), repeatability (≤7%) and recovery (90–110%). The improved method was granted AOAC First Action status **2014.02**.

Received March 29, 2014.
The method was approved by the Expert Review Panel for Infant Formula as First Action.

The Expert Review Panel for Infant Formula invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

Corresponding author's e-mail: esther.campos-gimenez@rdls.nestle.com

An appendix is available on the *J. AOAC Int.* website at http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac

DOI:10.5740/jaoacint.14-119

AOAC Official Method 2014.02
Vitamin B_{12}
in Infant Formula and Adult/Pediatric Formulas
Ultra-High-Performance Liquid Chromatography
First Action 2014

[Applicable for the determination of vitamin B_{12} in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates), made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.]

Caution: The method uses commonly used solvents and reagents. Refer to appropriate manuals or safety data sheets to ensure that the safety guidelines are applied before using chemicals.

Cyanide.—Fatal if swallowed, inhaled, or comes in contact with skin. Wear protective gloves, clothing, and eyewear. Wash hands immediately after handling the product. Cyanide reacts with acids to form highly toxic and rapid acting HCN gas. Use only in effective fume removal device to remove vapors generated. Destroy residues with alkaline NaOCl solution.

Trifluoroacetic acid (TFA).—Causes severe burns and eye damage. Wear protective gloves, clothing, eyewear, and face protection. Use only in effective fume removal device to remove vapors generated.

See Table **2014.02A** for samples used during validation of the method. The set is composed of six nonfortified (placebo) products and 12 fortified products. It also includes a Standard Reference Material, SRM 1849a Infant/Adult Nutritional

Table 2014.02A. SPIFAN SLV test materials kit information

	Product description
Fortified products	Infant formula powder, partially hydrolyzed, milk-based
	Infant formula powder, partially hydrolyzed, soy-based
	Infant elemental powder
	Infant formula powder, milk-based
	Infant formula powder, soy-based
	Infant formula ready-to-feed, milk-based
	Child formula powder
	Adult nutritional powder, milk protein-based
	Adult nutritional powder, low-fat
	Adult nutritional ready-to-feed, high-protein
	Adult nutritional ready-to-feed, high-fat
	SRM 1849a Infant/Adult Nutritional Formula
Nonfortified (placebo)	Infant elemental powder
	Infant formula ready-to-feed, milk-based
	Child formula powder
	Adult nutritional ready-to-feed, high-protein
	Adult nutritional ready-to-feed, high-fat

Formula, from the National Institute of Standards and Technology (Gaithersburg, MD) with a reference value for vitamin B_{12}.

A. Principle

Vitamin B_{12} is extracted from the sample using sodium acetate buffer in the presence of sodium cyanide at 100°C for 30 min. Extracts are purified and concentrated with an immunoaffinity column. Vitamin B_{12} is determined by ultra-high-performance liquid chromatography with UV detection at 361 nm.

B. Apparatus and Materials

(**a**) *Balances*.—With readability of 0.1 mg (AT200; Mettler-Toledo Inc., Greifensee, Switzerland) and 0.01 g (PE400; Mettler-Toledo Inc.).

(**b**) *Sonicator*.—Bioblock (Fisher Scientific, Wohlen, Switzerland).

(**c**) *Laboratory oven*.—Heraeus (Hanau, Germany), or water bath.

(**d**) *In-line water bath (with magnetic stirrers) or autoclave*.

(**e**) *pH meter*.—Metrohm 691 (Herisau, Switzerland).

(**f**) *Rotary shaker for biochemistry*.—Labnet International (Edison, NJ) or Stuart LB3 (Barloworld, Bibby Sterilin Ltd, Staffordshire, UK).

(**g**) *Heating block*.—With nitrogen evaporation (Pierce Biotechnology, Inc., Rockford, IL).

(**h**) *Vortex*.—Scientific Industries, Inc. (Bohemia, NY).

(**i**) *Homogenizer*.—Polytron PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematica, Lucerne, Switzerland).

(**j**) *Volumetric flasks*.—Amber glass, 10, 50, 100, 200, 250 mL; clear glass, 2000 mL.

(**k**) *Graduated cylinders*.—50, 100, and 1000 mL.

(**l**) *Beakers*.—Amber glass, 250 mL.

(**m**) *Flat-bottom round flasks or Erlenmeyers*.—Amber glass, 250 mL.

(**n**) *Folded paper filters*.—602 1/2 or 597 1/2 (Whatman Inc., Maidstone, UK).

(**o**) *Amber vials*.—Screw top, 7 or 4 mL (Supelco Inc., Bellefonte, PA).

(**p**) *Micro LC vials*.—Amber (Supelco Inc.).

(**q**) *Pipets*.—Graduated glass, 10 mL, or volumetric glass, 9 mL.

(**r**) *Electronic digital pipet*.—Variable volume, 10–100 µL.

(**s**) *Syringes*.—Disposable, 20 mL, equipped with a perforated rubber stopper attached to the tip.

(**t**) *Immunoaffinity columns*.—EASY-EXTRACT VITAMIN B_{12} LGE (R-Biopharm AG, Darmstadt, Germany, www.r-biopharm.com; Product Code P88).

(**u**) *Immunoaffinity column rack*.—Product Code CR1 (R-Biopharm AG).

(**v**) *Chromatographic system*.—Waters Acquity UPLC® including Binary Solvent Manager, Sample Manager, and UV detector (Waters, Milford, MA) or ultra-high-performance chromatography system of equivalent characteristics.

(**w**) *Chromatographic column*.—Waters Acquity UPLC® BEH C18, 1.7 µm, 2.1 × 100 mm (Waters).

C. Chemicals and Solvents

(**a**) *Methanol*.—HPLC grade (Merck, Darmstadt, Germany).

(**b**) *Acetonitrile*.—HPLC grade (Merck).

(**c**) *Acetic acid, glacial*.—Merck.

(**d**) *Milli-Q water*.—Millipore (Bedford, MA). Use throughout where water is specified.

(**e**) *Sodium cyanide puriss*.—Fluka (Buchs, Switzerland).

(**f**) *TFA for spectroscopy*.—Merck.

(**g**) *Vitamin B_{12} (cyanocobalamin), approximately 99%*.—Sigma-Aldrich (St. Louis, MO).

(**h**) *Sodium acetate trihydrate p.a*.—Merck.

(**i**) *Sodium hypochloride*.—Technical grade.

(**j**) *α-Amylase from Bacillus subtilis*.—Approximately 50 units/mg (Sigma-Aldrich); optional.

D. Preparation of Reagents and Standard Solutions

(**a**) *Sodium acetate solution 0.4 M, pH 4.0*.—Into a 2000 mL volumetric flask, weigh 108.8 g sodium acetate trihydrate. Add about 1800 mL water. Dissolve. Add 50 mL acetic acid, and adjust pH to 4.0 with acetic acid. Dilute to volume with water.

(**b**) *Sodium cyanide solution, 1% (w/v)*.—Weigh 0.5 g sodium cyanide into a 50 mL amber glass volumetric flask. Dilute to volume with water. Any excess of 1% sodium cyanide solution must be destroyed by adding 1.5 mL of a 15% solution of sodium hypochlorite per 1 mL sodium cyanide solution. Let react for 2 days in a fume hood. (*Caution:* Sodium cyanide is highly toxic. Avoid contact with skin, and work in a fume hood. Disposal of any unused solutions should comply with local regulations.)

(**c**) *Mobile phase A*.—To 1000 mL water, add 250 µL TFA. Mix well.

(**d**) *Mobile phase B*.—To 1000 mL acetonitrile, add 250 µL TFA. Mix well.

(**e**) *Sample dilution solvent*.—Mix 90 mL mobile phase A with 10 mL mobile phase B.

(**f**) *Vitamin B_{12} stock standard solution (100 µg/mL)*.—Accurately weigh 20.0 mg vitamin B_{12} into a 200 mL amber glass volumetric flask. Add about 150 mL water. Dissolve by sonication and stirring for a few minutes. Dilute to volume with water. Solution is stable for ≥6 months at –20°C. (*Note*: Vitamin B_{12} is sensitive to light. Conduct operations under subdued light, or use amber glassware. Keep all solutions away from direct light.)

(**g**) *Vitamin B_{12} intermediate standard solution (400 ng/mL)*.—Pipet 1 mL vitamin B_{12} stock standard solution into a 250 mL amber glass volumetric flask. Make up to volume with water.

(**h**) *Vitamin B_{12} working standard solutions for calibration (2, 10, 20, 40, 60, 100 ng/mL)*.—Pipet into six separate 10 mL amber glass volumetric flasks, 50, 250, 500, 1000, 1500, and 2500 µL vitamin B_{12} intermediate standard solution. Dilute to volume with sample dilution solvent, (**e**).

E. Sample Preparation and Extraction

(**a**) *Sample reconstitution for powder samples*.—Weigh 25.0 g sample into a 250 mL beaker. Add 200 g water at 40 ± 5°C. Mix with a glass rod until the suspension is homogeneous, or homogenize with a Polytron. Proceed as described in **E**(**d**) *Extraction*.

(**b**) *Sample reconstitution for amino acid-based products.*—Weigh 25.0 g powder sample into a 250 mL beaker. Add 190 g water at 40 ± 5°C and 10 g skimmed milk powder. Mix with a glass rod until the suspension is homogeneous, or homogenize with a Polytron. In parallel, run a blank by replacing the sample by water. Dilute both, reconstituted sample and blank, twice in water (e.g., 50 g reconstituted sample or blank + 50 g water). Proceed as described in **E**(**d**) *Extraction.*

(**c**) *Sample preparation for liquid samples*.—Mix well to ensure homogeneity of the sample portion. Proceed as described in **E**(**d**) *Extraction*.

(**d**) *Extraction*.—Weigh 60.0 g sample suspension, **E**(**a**) and (**b**), blank, **E**(**b**), or liquid sample, **E**(**c**), into a 250 mL flat-bottom amber glass flask or Erlenmeyer with ground glass neck. Add 1 mL of 1% sodium cyanide solution, **D**(**b**). If the sample contains starch, add about 0.05 g α-amylase, mix thoroughly, stopper the flask, and incubate 15 min at 40 ± 5°C. Add 25 mL sodium acetate solution, **D**(**a**). Mix well. Place the flask in a boiling water bath for 30 min (or autoclave 30 min at 100°C). Cool the flask in an ice bath. Quantitatively transfer the content of flask to a 100 mL amber glass volumetric flask. Dilute to volume with water. Filter the solution through a folded paper filter. In the case of high-fat products, and if recovery is low, dilute the filtrate 1:3 in water before cleanup to improve recovery or repeat the extraction by using a smaller sample portion.

(**e**) *Immunoaffinity cleanup*.—Let the immunoaffinity columns warm to room temperature by removing them from refrigeration at least 30 min before use. Place each immunoaffinity column on the rack. Open the caps and let the storage buffer drain by gravity. Close the lower cap. Load the column with 9 mL clear filtrate and close the upper cap. Place the column in a rotary shaker, and mix slowly for 10–15 min. Return the column to the support and let stand for a few minutes. Open the caps to let the liquid drain by gravity. Wash the column with 10 mL water. With a syringe, insert about 40 mL air to dry the column. Elute with 3 mL methanol, and collect eluate in a 4 or 7 mL amber glass reaction vial. Rinse the column with 0.5 mL methanol, and with a syringe, insert about 20 mL air to collect all the methanol in the same vial. Evaporate the eluate at 50°C under a stream of nitrogen. Reconstitute the sample in 0.3 mL sample dilution solvent, **D**(**e**). Mix on a Vortex mixer. Transfer to a micro amber vial.

F. Analysis

(**a**) *Chromatographic conditions*.—Flow rate, 0.4 mL/min; injection volume, 50 µL; detection, UV at 361 nm; gradient elution, *see* Table **2014.02B**.

(**b**) *System suitability test*.—Equilibrate the chromatographic system for at least 15 min. Inject a working standard solution three to six times, and check peak retention times and responses. Inject working standard solutions on a regular basis within a series of analyses. The coefficient of variation should not be higher than 2%.

(**c**) *Analysis*.—Make single injections of standard and test solutions. Measure chromatographic peak response (height).

(**d**) *Identification*.—Identify vitamin B_{12} peak in the chromatograms of the test solution by comparison with the retention time and UV spectrum of the corresponding peak obtained for the standard solution.

(**e**) *Calibration*.—Plot peak responses against concentrations (in ng/mL). Perform regression analysis. Calculate slope and intercept. Check the linearity of the calibration ($R^2 > 0.99$; standard error of calibration < 10%).

(**f**) *Quantitation*.—Calculate the concentration of vitamin B_{12}, in µg/100 g of product as reconstituted, as follows:

$$\frac{(A-I)\times V_0\times V_2\times 100}{S\times m\times V_1\times 1000}$$

where A = response (height) of the peak obtained for the sample solution, I = intercept of the calibration curve, S = slope of the calibration curve, V_0 = volume of the test solution (volume used to dissolve the test portion) in mL (100 mL), V_2 = volume in which the aliquot of sample solution is reconstituted after immunoaffinity cleanup (0.3 mL), m = weight of the test portion, as reconstituted, in g (60 g), and V_1 = volume of the aliquot of sample solution loaded onto the affinity column (9 mL). For amino acid-based products calculate the vitamin B_{12} content on the sample and on the blank, **E**(**e**); take into account the additional dilution factor 1/5 in the calculations. Deduct the amount of vitamin B_{12} in the blank to the amount in the sample.

Table 2014.02B. Gradient elution

Time, min	Mobile phase A, %	Mobile phase B, %
0.0	90	10
1.7	90	10
2.5	75	25
2.9	10	90
3.9	10	90
4.0	90	10
8.0	90	10

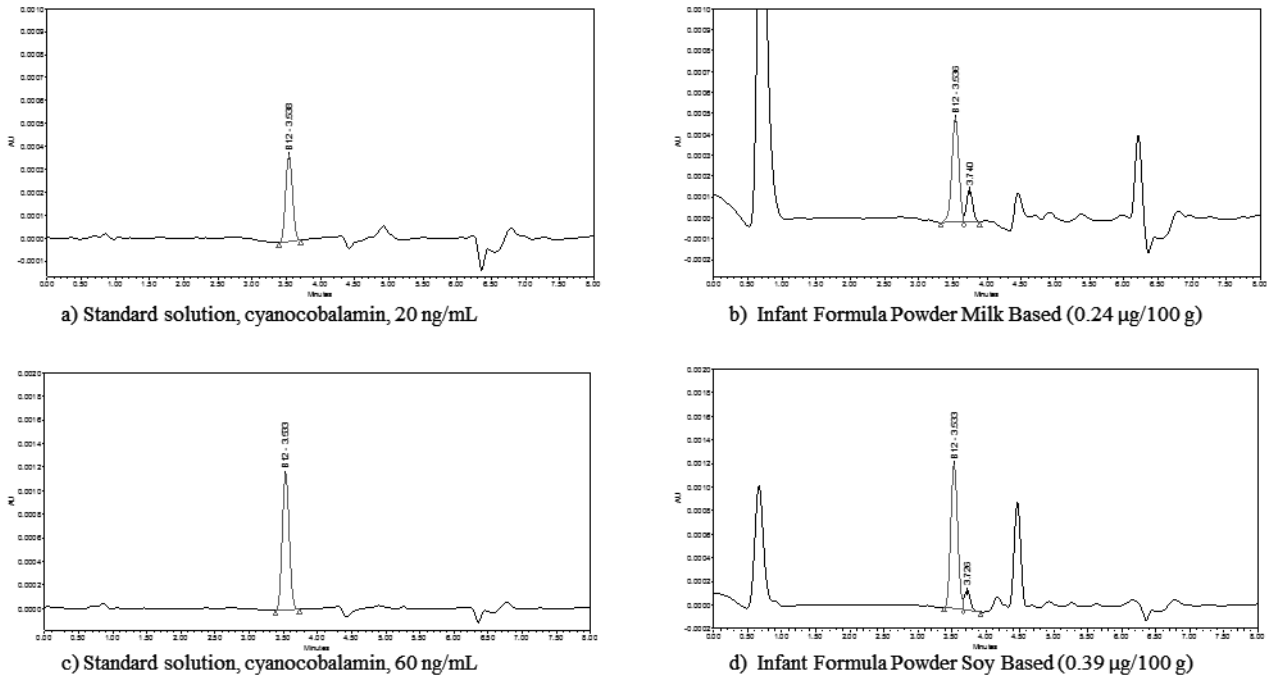


Figure 1. Example chromatograms of standard solutions at 20 (a) and 60 ng/mL (c) and infant formula powders (b, d).

(**g**) *Reporting*.—Report results with two decimal points as cyanocobalamin, in µg/100 g of reconstituted product. Reconstitution rates are 25 g/225 g for powder products, 50 g/100 g for concentrates, and 1 g/1 g for ready-to-feed formulas.

Validation Protocol

(**a**) *Linearity*.—Three independent stock solutions of cyanocobalamin were prepared at a concentration of 100 µg/mL. Working solutions at different levels prepared from dilution of stock solutions were injected in triplicate.

(**b**) *LOD/LOQ*.—Ten independent analysis of a nonfortified liquid sample, overdiluted to obtain a final concentration of about 0.005 µg/100 g, were used for determination of LOD and LOQ as LOD = blank mean + 3 SD and LOQ = blank mean + 10 SD.

(**c**) *Trueness*.—Reference material (SRM 1849a) was analyzed in duplicate over 6 days by two different analysts. Overall mean was calculated and compared to the reference value.

(**d**) *Recovery*.—Spiking experiments were performed at 50 and 100% of typical target levels in infant formula, on three selected nonfortified products. Spiked and nonspiked samples were analyzed in duplicate on 3 different days by two different analysts. The rest of the nonfortified products were spiked and analyzed in duplicate on a single day. The overall mean of unspiked samples was used to compute recoveries.

(**e**) *Precision studies*.—Six fortified samples, including SRM 1849a, were selected for precision studies and analyzed in duplicate on 6 different days by two analysts. Fresh reagents and working standards were prepared each day. Repeatability was verified on the rest of the samples by analyzing them in duplicate on a single day; this was due to insufficient amount of sample available to run on multiple days.

(**f**) *Statistics*.—SD of repeatability (S_r) and SD of intermediate reproducibility (S_{iR}) were used as measures of within-day and between-day variability, respectively. They were calculated from the data obtained in the precision studies as:

$$S_r=\sqrt{\frac{\sum_{i=1}^{n}(x_{i1}-x_{i2})^2}{2n}} \text{ and } S_{iR}=\sqrt{SD^2(b)+\frac{1}{2}Sr^2}$$

where n is the number of duplicate determinations; x_{i1} and x_{i2} are the two single results with i going from 1 to n and $SD^2(b)$ is the SD between the means of duplicates. Recovery rates (%) were calculated from spiking experiments as:

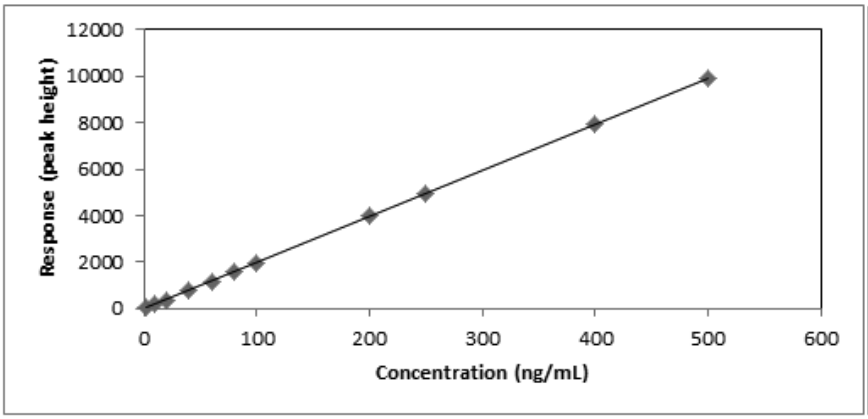
$$\text{Recovery (\%)} = \frac{C_{spiked}-C_{native}}{C_{added}} \times 100$$

where C_{spiked} is the concentration measured in the spiked sample; C_{native} is the concentration measured in the nonfortified sample (overall mean of unspiked samples); and C_{added} is the concentration of analyte added.

Validation Results

Chromatography.—Example chromatograms using the newly validated conditions (UHPLC) are shown in Figure 1. Chromatographic time has been reduced from the previously reported 16 min to about 8 min.

Linearity.—An extended calibration range (from 2 to 500 ng/mL) was used for linearity demonstration (Figure 2). Calibration curves were plotted and linearity demonstrated by $R^2 > 0.9999$ and calibration errors well below 5% for all levels



ng/mL	Response	% Error
2	36	8.6%
10	193	0.7%
20	388	0.5%
40	784	0.3%
60	1189	0.4%
80	1576	0.3%
100	1980	0.1%
200	3967	0.1%
250	4956	0.0%
400	7904	0.4%
500	9939	0.2%

Figure 2. Multilevel calibration curve example including calibration error estimates.

except the lowest concentration (2 ng/mL; corresponding to lower LOQ 0.01 µg/100 g), which showed, in some cases, calibration errors 10–20%. It was considered acceptable at this low level.

During routine analysis, a reduced calibration range from 2 to 100 ng/mL, which covers the range 0.01–0.55 µg/100 g, is recommended. This range can be extended as needed.

LOD/LOQ.—Due to the absence of a matrix devoid of vitamin B_{12} in the SPIFAN kit to be used in establishing LOD and LOQ, a nonfortified product over-diluted to contain about 0.005 µg/100 g was used. The results from 10 independent analyses showed an average of 0.006 µg/100 g, with SD of 0.0007 µg/100 g. Thus, LOD was estimated at 0.008 µg/100 g and LOQ at 0.013 µg/100 g.

Trueness.—Results on SRM 1849a (Infant/Adult Nutritional Formula) are shown in Table 1. The overall mean of duplicate analysis was 0.435 µg/100 g, with $SD_{(b)}$ (SD of the mean of duplicates) of 0.010 µg/100 g, which is well within the reference range of 0.482 ± 0.085 µg/100 g.

Recovery.—Results of spiking experiments are shown in Table 2. Most recoveries obtained using the method as previously described complied with requirements (90–100%), except for the Adult Nutritional ready-to-feed (RTF) High Fat and Infant Elemental Powder, with recoveries around 80% (data not shown).

For those two samples, sample preparation was adapted to allow better recovery rates. Briefly, the Adult Nutritional RTF High Fat was diluted three times in water to reduce matrix effect before extraction; while in the case of the amino acid-based (elemental) product, a source of intact protein (skimmed milk powder) was added to mimic regular matrixes. These adaptations allowed obtaining recovery rates within acceptable ranges. After adaptation, recovery rates in all samples ranged from 87.8 to 98.3%. Mean recovery was 91.7 ± 4.0% (mean ± SD).

Precision.—Precision data are shown in Tables 1 and 3. RSD of repeatability, S_r, was below 7%, except for Infant Formula Powder (S_r = 8.2%) and RSD of intermediate reproducibility, S_{iR}, was not higher than 11%. Repeatability was confirmed on the rest of the matrixes (fortified or not) by duplicate analysis on a single day. Only the Child Formula Powder (nonfortified) showed differences between duplicates higher than 7%.

Conclusions

The adaptations provided to the method allow meeting all requirements specified in the SMPR. Response was linear in the range 2–500 ng/mL, which corresponds to 0.01–2.8 µg/100 g (as reconstituted product); this range can easily be extended by dilution of sample extracts. LOD and LOQ were 0.008 and 0.013 µg/100 g, respectively. Accuracy of the method was proven by successful analysis of a Certified Reference Material (SRM 1849a Infant/Adult Nutritional Formula), as well as by recovery rates generally within 90–110% at 50 and 100% target values for infant formulas. Precision estimations (S_r and S_{iR}) determined in the range 0.2–1.2 µg/100 g were below 7 and 11%, respectively, for all matrixes tested (six selected products) except for Infant Formula Powder Milk Based (S_r = 8.2%).

Table 1. Precision data for infant formula and adult/pediatric formulas[a]

	Mean, *n* = 12	$SD_{(b)}$	S_r	CV_r, %	S_{iR}	CV_{iR}, %
Infant formula powder, partially hydrolyzed, milk-based	0.35	0.019	0.012	3.4	0.021	3.5
Infant formula powder, partially hydrolyzed, soy-based	0.26	0.074	0.007	2.7	0.009	3.3
Infant formula powder, milk-based	0.24	0.017	0.020	8.2	0.022	9.0
Infant formula powder, soy-based	0.43	0.031	0.013	3.0	0.032	7.4
Adult nutritional RTF, high-protein	1.18	0.046	0.042	3.6	0.055	4.6
SRM1849a Infant/Adult Nutritional Formula	0.435	0.010	0.019	4.4	0.017	3.8

[a] All results reported in µg/100 g of reconstituted product (reconstitution rate 25 g + 200 g water) or ready-to-feed. Mean of duplicate analysis performed by two different analysts on 6 different days. $SD_{(b)}$ = SD of mean of duplicates; S_r = SD of repeatability; CV_r = RSD of repeatability; S_{iR} = SD of intermediate reproducibility; CV_{iR} = RSD of intermediate reproducibility.

Table 2. Recovery results in nonfortified samples[a]

			Level 1		Level 2	
	n	Native content	Recovery, %	CV, %	Recovery, %	CV, %
Child formula powder	6	0.10	96.8	4.3	98.3	3.5
Adult nutritional RTF, high-protein	2	0.03	89.7	4.7	89.8	2.4
Infant formula RTF, milk-based	6	0.05	92.6	4.9	93.3	7.1
Adult nutritional RTF, high-fat	6	0.04	87.8	3.1	87.8	3.0
Infant elemental powder	6	0.00	90.2	3.5	91.1	3.0

[a] *n* = Number of days. Levels 1 and 2 are 0.15 and 0.30 µg/100 g for all products except infant elemental powder, for which level 1 is 2.25 µg/100 g and Level 2 is 4.50 µg/100 g. Native content is reported in µg/100 g of reconstituted or RTF product (reconstitution rate 25 g + 225 g water).

Table 3. Precision verification for infant formula and adult/pediatric formulas[a]

	Mean, *n* = 2	SD, %
Adult nutritional powder, milk protein-based	0.31	3.9
Adult nutritional powder, low-fat	0.67	1.3
Child formula powder	0.94	1.9
Infant elemental powder	0.60	0.5
Adult nutritional RTF, high-fat[b]	1.40	12.2
Infant formula RTF, milk-based	0.32	6.3
Child formula powder (nonfortified)	0.10	11.5
Adult nutritional RTF, high-protein (nonfortified)	0.03	3.9
Adult nutritional RTF, high-fat (nonfortified)[b]	0.04	5.6

[a] Mean of duplicate analysis on a single day. All results reported in µg/100 g of reconstituted product (reconstitution rate 25 g + 200 g water) or RTF.

[b] Results obtained without further dilution of sample previous to extraction.

The method was found suitable for the determination of vitamin B_{12}, in the form of cyanocobalamin, as well as the naturally occurring forms (mainly hydroxyl-, adenosyl-, and methylcobalamin) in infant formula and adult/pediatric formula.

References

(1) Campos Giménez, E., Fontannaz, P., Trisconi, M.J., Kilinc, T., Gimenez, C., & Andrieux, P. (2008) *J. AOAC Int.* **91**, 786–793

(2) Campos Giménez, E., Fontannaz, P., Trisconi, M.J., Kilinc, T., Gimenez, C., & Andrieux, P. (2012) *J. AOAC Int.* **95**, 307–312. http://dx.doi.org/10.5740/jaoacint.CS201108

(3) *Official Methods of Analysis* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.08**

(4) AOAC SMPR **2011.005** (2012) *J. AOAC Int*. **95**, 293. http://dx.doi.org/10.5740/jaoacint.11-0441