AOAC Official Method 2014.02
Vitamin B_{12} (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula

Liquid Chromatography-Ultraviolet Detection
First Action 2014
Final Action 2017

[Applicable to determination of vitamin B_{12} (cyanocobalamin) 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: Method uses commonly used solvents and reagents. Refer to the appropriate manuals or safety data sheets to ensure that the safety guidelines are applied before using chemicals.

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

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

Vitamin B_{12}.—Vitamin B_{12} is sensitive to light; conduct operations under subdued light or use amber glassware. Keep all sample and standard solutions away from direct light.

A. Principle

Vitamin B_{12} is extracted from the sample in a sodium acetate buffer (pH 4) containing cyanide at 100°C for 30 min. Extracts are purified and concentrated with an immunoaffinity column. Vitamin B_{12} is determined as cyanocobalamin by ultra-high-performance liquid chromatography (UHPLC) or high-performance liquid chromatography (HPLC) with UV detection at 361 nm. Separation takes place on a C_{18} column using an acetonitrile gradient in water.

B. Apparatus and Materials

(**a**) *Balances*.—With readability of 0.1 mg and 0.01 g.

(**b**) *Sonicator*.

(**c**) *In-line water bath*.—With magnetic stirrers or autoclave.

(**d**) *pH meter.*

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

(**f**) *Heating block*.—With nitrogen evaporation.

(**g**) *Vortex.*

(**h**) *Homogenizer*.—Polytron® PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematica, Lucerne, Switzerland), or equivalent.

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

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

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

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

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

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

(**o**) *Micro LC vials.*—Amber.

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

(**q**) *Electronic digital pipet*.—Variable volume, 200–1000 µL.

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

(**s**) *Immunoaffinity columns*.—EASI-EXTRACT® VITAMIN B_{12} LGE (R-Biopharm AG; Product Code P88).

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

(**u**) *Chromatographic system*.—HPLC or UHPLC system equipped with a quaternary or binary pump, sample injector, UV-VIS detector (or optionally a PDA detector), degassing system, and data software.

(**v**) *Analytical column.*—Depending on the chromatographic system available, use HPLC or UHPLC columns.

(*1*) *UHPLC column*.—Waters Acquity UPLC® BEH C18, 1.7 µm, 2.1 × 100 mm (Waters, Milford, MA, USA), or equivalent.

(*2*) *HPLC column*.—Nucleosil 100-3 C18 HD, 125 × 3.0 mm (Macherey-Nagel, Inc., Oesingen, Switzerland), C18 ACE 3AQ, 150 × 3.0 mm (ACE, Aberdeen, Scotland, UK), or equivalent.

C. Chemicals and Standards

(**a**) *Methanol.*—HPLC grade.

(**b**) *Acetonitrile.*—HPLC grade.

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

(**d**) *Milli-Q water*.—Millipore (Bedford, MA, USA).

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

(**f**) *Sodium acetate trihydrate p.a*.—Merck (Darmstadt, Germany), or equivalent.

(**g**) *Sodium hypochlorite.*—Technical grade.

(**h**) *TFA*.—Merck, or equivalent.

(**i**) *Vitamin B_{12} (cyanocobalamin)*.—Purity >99%; Sigma-Aldrich (St. Louis, MO, USA), or equivalent.

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 it react for 2 days in a fume hood.

(**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 cyanocobalamin 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. This solution is stable for ≥6 months at –20°C.

(**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 separated 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, **D(e)**.

E. Sample Preparation and Extraction

(**a**) *Sample reconstitution for powder samples*.—Weigh 25.0 g (W_1) of sample into a 250 mL beaker. Add 200 g (W_2) water at 40 ± 5°C. Mix with a glass rod until suspension is homogeneous or homogenize with a Polytron®. Proceed as described in **E(d)**.

(**b**) *Sample reconstitution for amino acid based products*.—Weigh 25.0 g (W_1) of powder sample into a 250 mL beaker. Add 190 g (W_2) of water at 40 ± 5°C and 10 g (W_3) skimmed milk powder. Mix with a glass rod until suspension is homogeneous or homogenize with a Polytron. In parallel, run a blank by replacing the sample by water (215 g water + 10 g skimmed milk powder). Dilute both, the reconstituted sample and the blank, twice in water (e.g., 50 g reconstituted sample or blank + 50 g water). Proceed as described in **E(d)**.

(**c**) *Sample preparation for liquid samples*.—Mix well to ensure homogeneity of the sample portion. Proceed as described in **E(d)**. In the case of high-fat nutritional products, if recovery is low, samples can be diluted in water (e.g., 50 g sample + 50 g water) before extraction to improve recovery.

(**d**) *Extraction*.—Weigh 60.0 g (*m*) sample suspension **E(a)**, **E(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 and mix thoroughly. Stopper the flask and incubate 15 min at 40 ± 5°C. Add 25 mL sodium acetate solution **D(a)**. Mix well. Place flask in a boiling water bath for 30 min (or autoclave 30 min at 100°C). Cool flask in ice bath or let stand at room temperature. Quantitatively transfer content of flask to a 100 mL (V_1) amber glass volumetric flask. Dilute to volume with water. Filter solution through folded paper filter.

(**e**) *Immunoaffinity cleanup*.—Let 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 caps and let storage buffer drain by gravity. Close the lower cap. Load column with 9 mL (V_2) of clear filtrate and close the upper cap. Place column in a rotary shaker and mix slowly for 10–15 min. Return column to the support and let stand for a few minutes. Open the caps to let liquid drain by gravity. Wash column with 10 mL water. With a syringe, insert about 40 mL air to dry the column. Elute with 3 mL methanol, and collect the eluate in a 4 or a 7 mL amber glass reaction vial. Rinse 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 at 50°C under a stream of nitrogen. Reconstitute sample in 0.3 mL (V_3) sample dilution solvent **D(e)**. Mix on a vortex mixer. Transfer to a micro amber vial.

F. Analysis

(**a**) *UHPLC conditions*.—(*1*) *Flow rate*.—0.4 mL/min.

(*2*) *Injection volume*.—50 µL.

Table 2014.02A. UHPLC gradient elution table

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

Table 2014.02B. HPLC gradient elution table

Time, min	Mobile phase A, %	Mobile phase B, %
0.0	90	10
0.5	90	10
4.0	75	25
5.0	10	90
9.0	10	90
11.0	90	10
16.0	90	10

(*3*) *Detection*.—UV at 361 nm (alternatively 550 nm can be monitored); gradient elution (Table **2014.02A**).

(**b**) *HPLC conditions*.—(*1*) *Flow rate*.—0.25 mL/min.

(*2*) *Injection volume*.—100 µL.

(*3*) *Detection*.—UV at 361 nm (alternatively 550 nm can be monitored); gradient elution (Table **2014.02B**).

(**c**) *System suitability test*.—Equilibrate the chromatographic system for at least 15 min at the initial conditions. 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.

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

(**e**) *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.

(**f**) *Calibration*.—Plot peak responses against concentrations (in ng/mL). Perform regression analysis. Calculate slope and intercept.

(**g**) *Quantitation (liquid and powder samples)*.—Calculate the concentration of vitamin B_{12} in µg/100 g of product as follows:

$$\frac{(A-I)\times(W_1+W_2)\times V_1\times V_3\times 100}{S\times W_1\times m\times V_2\times 100}$$

where A = response (height or area) of the peak obtained for the sample solution, I = intercept of the calibration curve, S = slope of the calibration curve, W_1 = weight of powder sample used for reconstitution (25 g), W_2 = weight of water used for reconstitution (200 g), m = weight of sample suspension (60 g), V_1 = volume of the test solution (volume used to dissolve the test portion) in mL (100 mL), V_2 = volume of the aliquot of the sample solution loaded onto the affinity column (9 mL), and V_3 = volume in which the aliquot of the sample solution is reconstituted after immunoaffinity cleanup (0.3 mL).

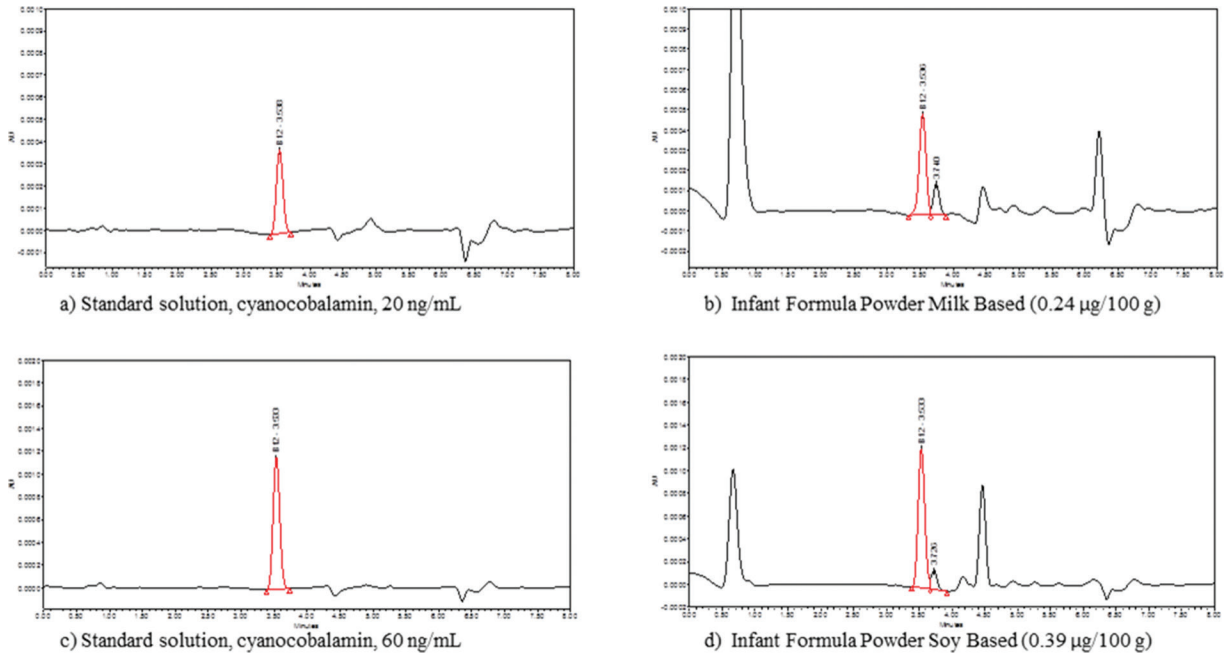

Figure 2014.02. Example chromatograms on selected products and standard solutions. Color figures are available online as supplemental information at: http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac

(**h**) *Quantitation (amino-acid-based products)*.—Calculate the concentration of vitamin B_{12} in the sample in µg/100 g of product as follows:

$$C_{\text{sample}} = \left[\left(A_{\text{blank}} - A_{\text{sample}}\right) - I\right] \times \frac{(W_1 + W_2 + W_3) \times V_1 \times V_3 \times D \times 100}{S \times W_1 \times \boldsymbol{m} \times V_2 \times 1000}$$

where A_{blank} = response (height or area) of the peak in the blank, A_{sample} = response (height or area) of the peak in the sample, I = intercept of the calibration curve, S = slope of the calibration curve, W_1 = weight of sample used for reconstitution (25 g), W_2 = weight of water used for reconstitution (190 g), W_3 = weight of skimmed milk powder used for reconstitution (10 g), m = weight of sample suspension (60 g), V_1 = volume of the test solution (volume used to dissolve the test portion) in milliliters (100 mL), V_2 = volume of the aliquot of sample solution loaded onto the affinity column (9 mL), V_3 = volume in which the aliquot of sample solution is reconstituted after immunoaffinity cleanup (0.3 mL), and D = dilution factor (e.g., 2).

(**i**) *Reporting*.—Report results with two decimal points as cyanocobalamin in µg/100 g of product.

G. Example Chromatograms

See Figure **2014.02**.

References: Campos-Giménez, E., Fontannaz, P., Trisconi, M.J., Kilinc, T., Gimenez, C., & Andrieux, P. (2012) *J. AOAC Int*. **95**, 307–312
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