U.S. Department of Health & Human Services

FDA U.S. Food and Drug Administration

Elemental Analysis Manual

4.15 High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometric Determination of Vitamin B_{12} in Nutritional Products

Version 1.0 (April 2024)

Current Validation Status:
Single Lab Validation: yes
Multi-lab validation: no

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GLOSSARY

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SCOPE AND APPLICATION

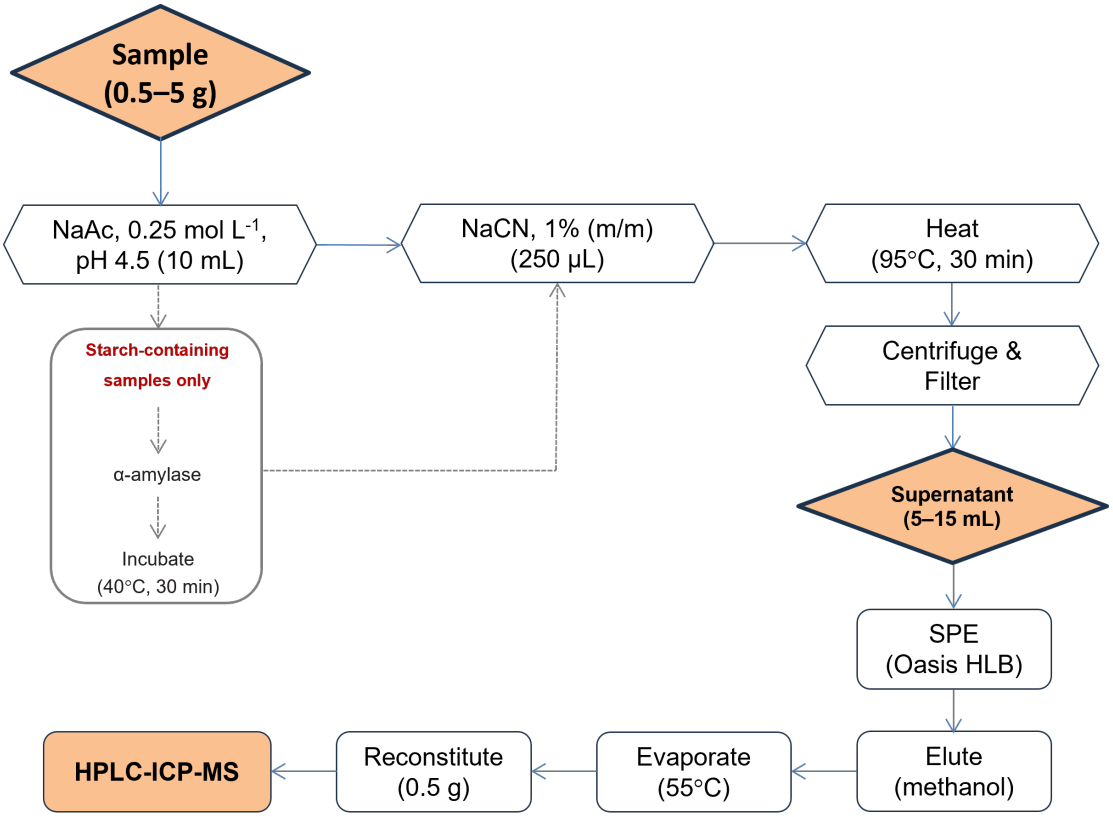
This method describes a procedure to quantitate vitamin B_{12} (cobalamin) in nutritional products (infant formulas, toddler and adult nutritive drinks, milk, protein powders/drinks and breakfast cereals). Various forms of vitamin B_{12} are collectively determined as cyanocobalamin (CNCbl) using high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS). The mass fraction of vitamin B_{12} is reported as µg of CNCbl per 100 g product. Other matrices may be analyzed by this procedure if performance is verified in the matrix at the mass fraction of interest. This method cannot be used to speciate the different forms of vitamin B_{12}.The method is intended solely for use by thoroughly trained analysts and experienced in the analysis of trace elements in food products, as well as in the use of both HPLC and ICP-MS.

4.15.1 SUMMARY OF METHOD

The method is illustrated as a flowchart in 4.15 Figure 1. An analytical portion weighing between 0.5 g and 5.0 g, depending on its form and composition, is mixed with solutions of sodium acetate (NaAc) and sodium cyanide (NaCN) and then subjected to heating in a hot block. Starch-containing samples are treated with α-amylase and incubated in a water bath prior to the addition NaCN and subsequent heating in a hot block. The supernatant obtained following hot block extraction is passed through a C-18 solid phase extraction (SPE) sorbent for cleanup and analyte pre-concentration. CNCbl is eluted from the sorbent with methanol and the solvent is evaporated from the eluate using a centrifugal evaporator. The remaining solid is reconstituted to small volume for subsequent analysis by HPLC-ICP-MS.

CNCbl is separated on a Zorbax Eclipse XDB-C8 column isocratically; see 4.15 Table 2. ICP-MS is used as a Co-specific detector for cobalamin-containing chromatographic eluents. The instrument is operated in O_2/Ar option gas mode to prevent carbon buildup from the organic mobile phase and the collision cell was pressurized with He to eliminate interference from polyatomic ions such as $^{24}Mg^{35}Cl$, $^{43}Ca^{16}O$ and $^{23}Na^{36}Ar$ on Co at *m/z* 59. CNCbl is identified by matching the peak retention time with a reference standard, and its mass fractions are calculated based on peak areas for analytical solutions compared to responses for standard solutions.

4.15 Figure 1. Method flow chart



Typical analytical limits were calculated per EAM §3.2[1] during single laboratory validation of the method and are listed in 4.15 Table 1. The limits will vary depending on the specific instrumentation, analyte pre-concentration factor and actual operating conditions used.

4.15 Table 1. Analytical limits

Sample	Analytical limits	Abbreviation	ASDL	ASQL	LOD	LOQ
Solid	Cyanocobalamin	CNCbl	0.03	0.2	0.004	0.03
Liquid	Cyanocobalamin	CNCbl	0.03	0.2	0.01	0.06

Analytical limits are calculated according to EAM §3.2[1].
Analytical limits are calculated based on 10 replicate injections (n = 10) of a fortified blank.
Analytical methods are calculated based on 2 g (solid) or 5 g (liquid) analytical portion for buffer (NaAc) extraction, and 10 g buffer extract for SPE pre-concentration.
Analytical limits are expressed in µg CNCbl/100 g sample.

4.15.2 EQUIPMENT AND SUPPLIES

Disclaimer: The use of trade names in this method constitutes neither endorsement nor recommendation by the U.S. Food and Drug Administration. Equivalent performance may be achievable using apparatus and materials other than those cited here.

This method was developed using an Agilent 7900 ICP-MS equipped with MassHunter software, and an Agilent 1260 HPLC. As such, several instrumentation-specific and software-specific instructions are provided. Other instrument brands and/or models may be used but their performance characteristics must meet or exceed those provided by these instruments. If different equipment is used, operational details such as how to proceed through the software will be different and is permitted. Any modification of the analysis procedures given herein would require validation according to FDA guidelines and the quality control elements detailed below in §4.15.8 must pass.

Refer to the instrument manuals for safety precautions regarding use.

(1) Inductively coupled plasma-mass spectrometer (ICP-MS)—Equipped with collision/reaction cell that can be pressurized with helium and kinetic energy discrimination for polyatomic interference attenuation. Must have option gas introduction (Agilent specific nomenclature; other manufacturers will have different names) to burn carbon in organic solvent phase by introducing O_2/Ar mixture.

(2) Torch (recommended for organic solvent introduction)—Quartz torch with 1.5 mm i.d. injector (Agilent part no. G3280-80080). ICP-MS organic solvent sample introduction kit (Agilent part no. G3280-60580) includes torch and recommended peristaltic pump tubing and connectors.

(3) Nebulizer and cones (recommended for organic solvent introduction)—Concentric nebulizer (Agilent part no. G1820-65138), platinum sampler cone (Agilent part no. G3280-67036), platinum skimmer cone (Agilent part no. G3280-67063), and brass skimmer base for x-lens (Agilent part no. G3280-60621).

(4) Liquid chromatograph (HPLC)—Equipped with pump, autosampler, and degasser. The HPLC must be interfaced with the ICP-MS such that it will trigger the ICP-MS to start recording data after each injection.

(5) Peristaltic pump—Integrated into the ICP-MS or a freestanding model; used to recirculate post-column marker (internal standard, ISTD) solution through switching valve.

(6) 6-port switching valve—Integrated in the HPLC column compartment or external but

controlled via HPLC external contact board; used to inject ISTD solution.

(7) Water bath—Capable of agitating and heating 50 mL centrifuge tubes to 40°C; used to incubate amylase-treated samples.

(8) Hot block—Capable of heating 50 mL centrifuge tubes to 95°C, requires uniform temperature control; used to heat samples mixed with extraction solutions.

(9) Laboratory centrifuge—Capable of spinning 50 mL centrifuge tubes at minimum 1960xg.

(10) Vacuum concentrator—Used to remove organic solvent from SPE eluates; requires cold trap to recover solvents in accordance with safety and environmental rules.

(11) Analytical balance—Capable of measuring to 0.0001 g.

(12) Top loading balance—Capable of measuring from 0.01 g to 1000 g.

(13) pH meter—With calibration buffers (pH 4, 7 and 10).

(14) Vortex mixer—Used to agitate extracts and solutions.

(15) HPLC column—Zorbax Eclipse XDB-C8 column 150 mm x 3 mm, 3.5 µm particle size (Agilent part no. 963954-306).

(16) Column in-line filter—Frit, 0.2 µm (e.g., Waters part no. 700005698) and filter assembly kit (e.g., Waters part no. 205000343).

(17) SPE cartridges—Oasis HLB, 6 cc, 500 mg sorbent per cartridge, 60 µm (Waters part no. 186000115).

(18) SPE rack—Used to hold SPE cartridges during extraction (e.g., rBiopharm part no. RBRCR).

(19) Syringe filters (PVDF)—Disposable; 0.2 µm, 13 mm diameter (e.g., MilliporeSigma product no. SLGNX13NL) and 0.45 µm, 30–33 mm diameter (e.g., MilliporeSigma product no. SLHN033NB) with Luer-Loc inlet; used to filter extracts.

(20) Plastic syringes—Disposable; general use, non-sterile, 5 or 10 mL, Luer-Loc tip; used to filter extracts and push air through the SPE cartridge.

(21) Pipettes—Adjustable; volume ranges from 10 µL to 10 mL, accuracy ±10%.

(22) Pipette tips—Volume ranges from 10 µL to 10 mL, accuracy ±10%.

(23) Disposable transfer pipets—Plastic; used to transfer analytical portions and supernatants.

(24) Centrifuge tubes—Amber; high density polypropylene (HDPE) conical tubes with caps, 15 mL (e.g. Eppendorf Catalog No. 0030122194) and 50 mL (e.g. Eppendorf Catalog No. 0030122224), used for extraction and to store standard solutions and extracts.

(25) Evaporation tubes—Compatible with the vacuum concentrator; must have sample visibility to check evaporation has reached dryness.

(26) Autosampler vials—Amber, 2 mL; conical vials are recommended to make sure the HPLC injection needle reaches the extract in the vial (e.g., Thermo part no. 6PSV9-03FIVAP). Use vial inserts (e.g., Agilent part no. 5181-3377) for flat-bottom vials.

(27) Clean air hood/canopy—Polypropylene metal free hoods/canopies are recommended for sample handling.

4.15.3 REAGENTS AND STANDARDS

Safety: Reagents should be regarded as potential health hazards and exposure to these materials should be minimized. Use appropriate personal protective equipment including safety glasses, gloves and lab coat while handling reagents. Analysts should consult and must be familiar with their lab's chemical hygiene and safety plan and Material Safety Data Sheets for all reagents and standards listed. All waste generated must be handled appropriately.

Cyanide is fatal if swallowed, inhaled, or comes in contact with skin. It reacts with acids to form highly toxic and rapid-acting hydrogen cyanide gas. An exhausting hood must be used when working with NaCN. ***Discard cyanide-containing waste into acid-free containers.*** *Destroy excess 1% (m/m) NaCN solution with alkaline sodium hypochlorite (NaOCl) solution (in a fume hood, add 1.5 mL of 10–15% NaOCl solution per 1 mL NaCN solution and let the mixture react for two days before discarding).*

Reagents

(1) Reagent water—Water that meets specifications for ASTM Type I water[2], such as 18.2 MΩ•cm de-ionized water (DIW).

(2) Argon supply—High purity (99.99%).

(3) Helium for collision cell—Ultra high purity (99.999%).

(4) Oxygen-argon mixture (20:80)—Ultra high purity (99.999%).

(5) Sodium acetate, anhydrous $C_2H_3NaO_2$—CAS 127-09-3, F.W. 82.03 g mol^{-1}, purity ≥ 99%.

(6) Sodium cyanide NaCN—CAS 143-33-9, F.W. 49.01 g mol^{-1}, purity ≥ 98%.

(7) Methanol CH_3OH—CAS 67-56-1, F.W. 32.04 g mol^{-1}, purity ≥ 99.9%, Optima LC-MS grade.

(8) Formic acid CH_2O_2—CAS 64-18-6, F.W. 46.03 g mol^{-1}, purity ≥ 99%, Optima LC-MS grade.

(9) Acetic acid, glacial $C_2H_4O_2$—CAS 64-19-7, F.W. 60.05 g mol^{-1}, Optima grade.

(10) α-amylase—(e.g. MilliporeSigma part no. 10065 (powder) or MilliporeSigma part no. A8220 (solution)).

(11) Sodium hypochlorite NaOCl—CAS 7681-52-9, F.W. 74.44 g mol^{-1}, 10–15% active chlorine.

Standards

(1) Cyanocobalamin (CNCbl), powder—CAS 68-19-9, F.W. 1355.37 g mol^{-1}, meets USP testing specifications.

(2) (Optional) Cyanocobalamin (CNCbl), solution—CAS 68-19-9, F.W. 1355.37 g mol^{-1} (e.g., MilliporeSigma product no. V-019).

(3) Hydroxocobalamin (OHCbl), powder—CAS 59461-30-2, F.W. 1382.82 g mol^{-1}, meets USP testing specifications.

4.15 Table 2. HPLC Settings

HPLC	Condition
Filter	0.2 µm frit
Column	Agilent Zorbax Eclipse XDB-C8 (3 x 150 mm, 3.5 µm)
Mobile phase	0.1% (v/v) formic acid in 30% (v/v) methanol in DIW
Injection volume	20 µL
Column temperature	Ambient
Flow rate	0.4 mL min^{-1}
Acquisition time	360 s (6 min)

4.15.4 EXTRACTION SOLUTION, MOBILE PHASE, STANDARD SOLUTION AND DILUTION SOLUTION PREPARATION

Note: Unless specified otherwise, calculations are gravimetric. For masses up to 100 g, record the mass to 0.0001 g. If over 100 g, record to 0.1 g. Liquids are typically transferred via pipetting. For all aqueous solutions, assume unit density to determine nominal volumes (e.g., if an instruction states to transfer 20 mg, then 20 µL should be pipetted). ***Mass, however, is always recorded for use in calculations.*** *When commercially made solutions are given in mass/volume units (e.g., µg/mL), use density and convert to mass/mass units (e.g., µg/g) for calculations.*

Note: The following operations should be performed in a clean environment to reduce contamination. Conduct experiments under subdued light or use amber tubes

and vials for sample handling. ***Vitamin B_{12} is sensitive to light****; prepare standard solutions and extracts under subdued light or use amber tubes. Keep solutions away from direct light. Bring solutions to room temperature and mix well prior to use.*

Extraction Solutions

Extraction Solution 1: 0.25 mol L^{-1} NaAc, pH 4.5±0.05—Tare a 1L HPLC reservoir bottle, add 20.51 g NaAc (anhydrous) followed by DIW to bring mass to 980 g. Adjust pH to 4.5±0.05 with glacial acetic acid and add DIW until a total solution mass of 1000±5 g is reached.

Extraction Solution 2: 1% (m/m) NaCN—Tare a 100 mL bottle, add 1.0 g NaCN, and add DIW until a total solution mass of 100±1 g is reached.

Mobile Phase Solution

0.1% (v/v) formic acid in 30% (v/v) methanol in DIW—Tare a 1L HPLC reservoir bottle, add 1.22 g (1.0 mL, d = 1.22 g mL^{-1}) formic acid followed by 237.3 g (300 mL, d = 0.791 g mL^{-1}) methanol, and add DIW until a total solution mass of 937.5±1 g (1000.0 mL) is reached.

Rinsing Solution

95% (v/v) methanol in DIW—Tare a 1L HPLC reservoir bottle, transfer 751.5 g (950 mL, d = 0.791 g mL^{-1}) methanol, and add DIW until a total solution mass of 801.5±1 g (1000.0 mL) is reached.

Dilution Solution

0.1% (v/v) formic acid in DIW—Tare a 100 mL bottle, add 0.12 g (0.1 mL, d = 1.22 g mL^{-1}) formic acid, and fill bottle with DIW until a total solution mass of 100.02±1 g (100 mL) is reached.

Standard Solutions

The solutions used for quantitative purposes include *stock standard solution*, *intermediate standard solution*, *calibration standard solutions*, and an *ISTD solution*.

Stock standard solution is made in the laboratory from pure solid CNCbl powder.

Intermediate standard solution is prepared from the *stock standard* on the day of use to make *calibration standards* and fortify analytical portions.

Calculations for the preparation of standards are based on elemental Co (as opposed to compound) and expressed on a mass fraction basis (i.e., mass/mass). Record masses for all standard preparation procedures.

(1) Stock Standard Solution (Stk Std):

Prepare the Stk Std (100 µg g^{-1} Co) from solid CNCbl powder. Tare a 50 mL amber centrifuge tube, transfer 60 mg CNCbl powder and dilute to 25 g with DIW. Tightly seal the tube, vortex for 30 s.

a. Prepare a dilute solution (10–25 ng g^{-1} Co) from the Stk Std and analyze by ICP-MS to verify the total Co mass fraction. Use a standard solution with verified Co concentration for calibration.

b. (Optional) Prepare a dilute solution (10–25 ng g^{-1} Co) from the Stk Std and analyze against a commercially available CNCbl standard solution (see §4.15.3) by HPLC-ICP-MS (see 4.15 Table 2) to confirm the CNCbl concentration. Commercial CNCbl standard solutions prepared in methanol require careful handling because of volatility; do not use these solutions as Stk Std for routine calibration.

CNCbl is light-sensitive and lacks stability over time; reevaluate its stability biweekly by monitoring the formation of OHCbl (4.15 Figure 2). Discard solution if impurity (OHCbl) is > 2% relative to the CNCbl peak (quantify OHCbl based on CNCbl calibration curve).

(2) Intermediate Standard Solution (Intm Std):

Prepare the Intm Std (100 ng g^{-1} Co) on the day of use. Tare a 50 mL amber tube, transfer 50 mg of the Stk Std, and dilute to 50 g with DIW.

(3) Calibration Standard Solutions (Cal Std):

Cal Stds are diluted with the dilution solution. Prepare the standards on the day of use.

a. Cal Std 5 (20 ng g^{-1} Co)—Tare 2 mL autosampler vial, transfer 200 mg of the Intm Std, and dilute to 1 g.

b. Cal Std 4 (10 ng g^{-1} Co)—Tare 2 mL autosampler vial, transfer 100 mg of the Intm Std, and dilute to 1 g.

c. Cal Std 3 (5 ng g^{-1} Co)—Tare 2 mL autosampler vial, transfer 50 mg of the Intm Std, and dilute to 1 g.

d. Cal Std 2 (1 ng g^{-1} Co)—Tare 2 mL autosampler vial, transfer 100 mg of Cal Std 4, and dilute to 1 g.

e. Cal Std 1 (0.5 ng g^{-1} Co)—Tare 2 mL autosampler vial, transfer 50 mg of Cal Std 4, and dilute to 1 g (the mass fraction of Cal Std 1 needs to be at or slightly above the laboratory's ASQL).

(4) Standard Blank—Transfer 1 g of the dilution solution to a 2 mL autosampler vial. Include the Standard Blank as a point on the calibration curve (0 ng g^{-1} Co).

(5) Continuing Calibration Verification (CCV)—Use Cal Std 3 as CCV.

(6) Continuing Calibration Blank (CCB)—Use the Standard Blank as CCB.

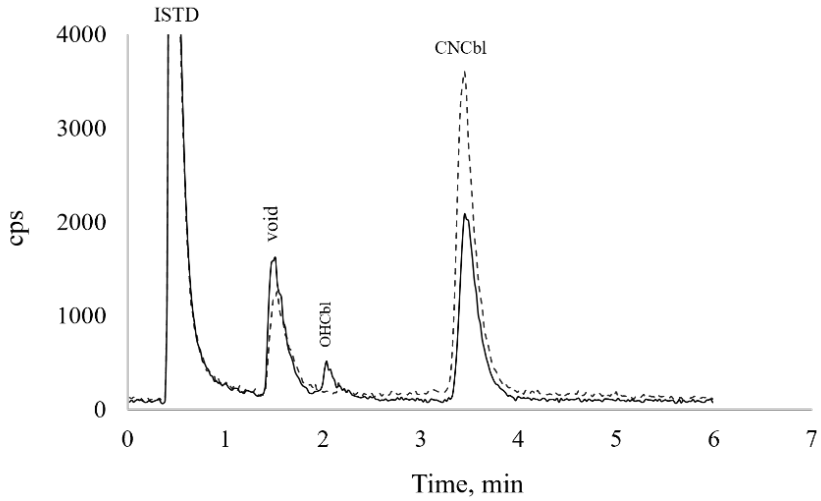
(7) Post-Column Marker Solution, ISTD (5 ng g^{-1} Co)—Tare a 250 mL bottle, transfer 12.5 g of the Intm Std, and dilute to 250 g with the dilution solution. Prepare fresh solution if the signal obtained is decreased significantly.

(8) OHCbl Standard (1 ng g^{-1} Co)—Follow procedures described in (1) above to prepare a standard solution of OHCbl (10 ng g^{-1} Co). The standard will only be used to establish the retention time of OHCbl to monitor the stability of the CNCbl Stk Std.

Fortification Standard

Prepare the fortification standard (5–10 ng g^{-1} Co recommended) by diluting the required amount of the Intm Std in DIW.

4.15 Figure 2. Example HPLC-ICP-MS chromatogram.



Chromatograms showing separation of CNCbl in a standard solution (solid line) and an extract generated from an infant formula sample (dashed line). ISTD – post column marker solution.

4.15.5 SAMPLE HOMOGENIZATION AND EXTRACTION PROCEDURES

Definition of Terms: start here

An 'analytical portion' is defined as portion of a test sample that is being processed for a particular laboratory test.

An 'analytical solution' is defined as an extract generated from an analytical portion and is ready for analysis.

An 'extraction batch' is defined as a group of analytical portions (including method blank) extracted using the same extraction protocol at the same time.

An 'analytical batch' consists of blanks, standards, and analytical solutions analyzed in a single sequence with one calibration. An analytical batch may contain analytical solutions from more than one extraction batch.

Sample Homogenization

Food preparation and homogenization procedures are found in EAM §2.1[3] through EAM §2.2.2. For solid sample, homogenize 25 g and preserve homogenate in amber centrifuge tube at temperature recommended by the manufacturer. Typically, solid samples are analyzed without moisture correction. Shake liquid and ready-to-drink/feed samples before taking analytical portions. Store samples as recommended by the manufacturer. Bring refrigerated samples to room temperature before use. Reference materials should be analyzed according to the manufacturer's recommendations.

Analytical Portion

Take 0.5–5.0 g analytical portion (depending on expected analyte level). Take 0.5–2.0 g for solid samples and reference materials. 5.0 g is recommended for liquid and ready-to-drink/feed samples.

Extraction Procedure

(1) Liquid Extraction

a. Weigh a 50 mL centrifuge tube with cap and record mass.

b. Remove cap, tare tube, transfer required amount of sample, and record mass.

c. Pipette 10 mL of 0.25 mol L^{-1} NaAc solution (pH 4.5) into the tube. If sample ***does not*** contain starch (based on label ingredient list), proceed as described in (f).

d. Add 0.05 g (powder) or 300 µL (solution) α-amylase, cap tube, and vortex for 30 seconds.

e. Incubate in a water bath at 40°C for 30 min.

f. Add 250 µL of 1% (m/m) NaCN solution, cap tube, vortex for 30 seconds, and record mass.

g. Place the tubes on a pre-heated (95±5°C) hot block for 30 min.

h. Remove tubes from the hot block, allow to cool to room temperature, and centrifuge for 15 min at 1960xg.

i. Weigh 15 mL centrifuge tube with cap and record mass.

j. Filter the extract through a 0.45 µm syringe filter into the pre-weighed 15 mL tube, cap tube, and record mass.

(2) Solid Phase Extraction (SPE)

Note: Pass the supernatant and solvents through the SPE cartridge by gravity, do not use external means to avoid fast drain.

a. Condition the SPE sorbent (Oasis HLB) with 3 mL of methanol and rinse it with 3 mL of DIW after the methanol has drained.

b. Load the filtered and weighed extract from item (1 j) on the cartridge quantitatively using a transfer pipette.

c. After all the filtrate has passed, wash the sorbent with 3 mL DIW.

d. Weigh an empty evaporation tube and record its mass.

e. After the DIW is drained in item (2 c), place the pre-weighed evaporation tube under the SPE cartridge and load the cartridge with 3 mL methanol.

f. After the methanol has drained, rinse sorbent with 0.5 mL methanol and collect eluate in the evaporation tube. Push air through the sorbent using a syringe to transfer residual solvent into the evaporation tube.

g. Evaporate the methanol from the SPE eluate to dryness in a vacuum concentrator at 55±5°C.

h. Pipette 0.5 mL of the dilution solution into the evaporation tube and record mass. Mix thoroughly until the leftover dissolves completely. If there are undissolved particles, filter extract through a 0.2 µm syringe filter.

i. Keep the reconstituted extract (also called analytical solution) refrigerated away from light until analysis. Analytical solutions must be analyzed within three days after reconstitution.

(3) Fortified Analytical Portion (FAP)

Note: Fortifications shall be performed by addition of standards to the analytical portion prior to adding the extraction solutions. It is recommended that unfortified and fortified analytical portions of a product be prepared from the same homogenized laboratory test sample.

a. Weigh sample into a pre-weighed and tared 50 mL centrifuge tube.

b. Fortify the analytical portion with the required amount of the fortification standard at 50–300% of the native vitamin B_{12} mass fraction. For example, a sample containing 2.53 µg CNCbl/100 g (equivalent to 1.1 ng g^{-1} Co) would be fortified at 100% of the native analyte mass fraction by taking a 2.0 g analytical portion and spiking with 0.22 g of 23.0 µg CNCbl/100 g fortification standard (equivalent to 10.0 ng g^{-1} Co).

c. Take the fortified sample through the extraction procedures described in items (1) and (2).

(4) Method Blank (MBK)

a. Take 2.0 g DIW through the extraction procedures described in items (1) and (2).

b. Include a method blank (MBK) in each extraction batch.

4.15.6 DETERMINATION PROCEDURE

Instrument Setup - Hardware

(1) HPLC

a. Details of the chromatographic method are provided in 4.15 Table 2.

b. If the HPLC system has been used for other separation(s) than the current method, flush it thoroughly with the rinsing solution before use.

a. Flush the column with the rinsing solution at 0.4 mL min^{-1} for 20–30 min just before the start of every analytical batch. New Zorbax XDB-C8 columns (and columns used with other eluents) must be conditioned with the rinsing solution at 0.4 mL min^{-1} for 3 hours followed by the mobile phase for 1 hour.

Note: Typical back pressure for the Zorbax Eclipse XDB column with the current chromatographic condition is in the range 2500–3000 psi. Significantly different column pressures should be investigated; check for leaks and clogs or replace the column.

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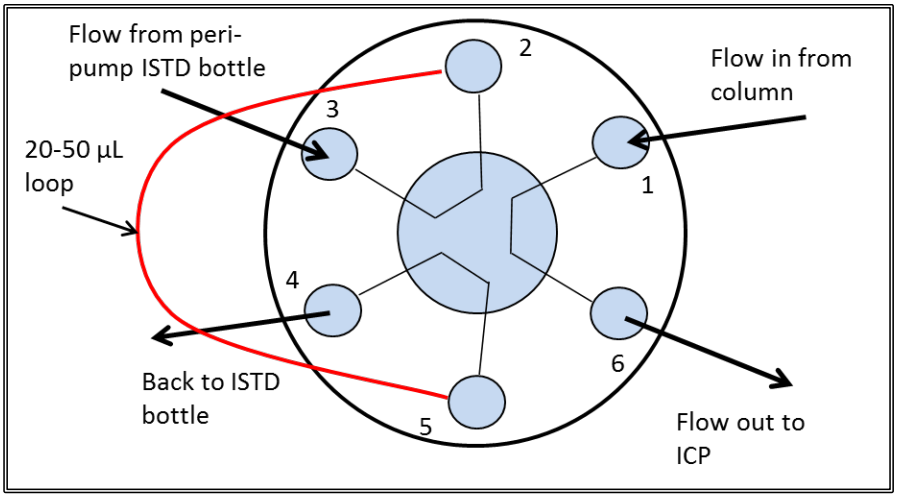
b. To introduce ISTD, connect a small (20 to 50 µL) loop across 2 ports of the 6-way 2 position column switching valve, with HPLC flow and peristaltic pump post-column reservoir flow tubes connected similar to 4.15 Figure 3. Connect tubing to peristaltic pump to begin solution circulation and verify that no bubbles are present. In the HPLC column compartment control window, set the column switching valve to change position at 0.25 min and switch back to original position at 0.3 min. Check that the peristaltic pump flow rate is sufficient to refill the injection loop between injections (0.3 mL min^{-1} is sufficient).

4.15 Figure 3. Set-up for post-column marker (ISTD) introduction.



(2) ICP-MS

Note: The method was developed using Agilent 7900 ICP-MS. Other instruments with collision cell and option gas introduction can be used.

a. See EAM §3.6.4[1] for additional details on ICP-MS.

b. Perform manufacturer recommended or laboratory start-up and initialization procedures.

c. Tune instrument using a 10 ng g^{-1} tune solution prepared in 30% (v/v) methanol in DIW. Use helium mode and kinetic energy discrimination and add 20% of O_2/Ar (20:80) option gas upstream to the plasma. Put the internal standard tubing in 30% (v/v) methanol in DIW during tuning. Typical ICP-MS operating parameters are provided in 4.15 Table 3.

d. Ensure that the signal for *m/z* 59 is within normal range for the instrument compared to its historical performance with similar instrument settings.

e. Connect the HPLC column outflow (port 6 in 4.15 Figure 3) to the ICP-MS nebulizer via PEEK tubing and start HPLC flow (0.4 mL min^{-1}).

f. Connect the ISTD line to the peristaltic pump. If the ISTD peristaltic pump is integrated into the ICP-MS, take off the tuning solution tubing and replace it with the ISTD line.

g. Connect trigger and LAN cables.

h. Ensure drainage is sufficient to prevent spray chamber flooding.

i. Ensure 'Sample Introduction' is switched to HPLC in the software.

4.15 Table 3. Typical ICP-MS Operating Parameters

ICP-MS	Condition
RF power	1550 W
RF matching	1.4 V
Sampling depth	8.0 mm
Plasma gas flow	15 L min^{-1}
Nebulizer gas flow	0.7 L min^{-1}
Make up gas flow	0.15 L min^{-1}
Helium flow	2.0 mL min^{-1}
Option gas (O_2/Ar (20:80))	20%
Spray chamber temperature	2°C
Peristaltic pump speed	0.2 rps
Plasma ignition mode	Organic solvent
Data acquisition mode	Time resolved
Integration time	1.0 sec (m/z 59), 0.1 sec (m/z 61)
Replicate per ion	1

Instrument Setup - Software

(1) Set ICP-MS acquisition method for time-resolved collection of *m/z* 59 and *m/z* 61 with integration (dwell) times of 1.0 sec and 0.1 sec, respectively. Enter appropriate HPLC conditions described in 4.15 Table 2.

(2) Fill in the software's 'Sample List' with the list of standards and analytical solutions to be analyzed. An example of a typical analytical batch is shown in 4.15 Table 4.

a. Verify the purity of standard and the analyte retention time by analyzing the highest mass fraction calibration standard. If impurity (OHCbl) is > 2% relative to the CNCbl peak, prepare calibration standards from fresh Intm Std, or prepare new Stk Std if necessary. If retention time is not reproducible with previous analyses, flush the column with the rinsing solution for a few more minutes followed by the mobile phases and reanalyze the check standard.

b. Calibrate using the standard blank and at least four calibration standards.

c. Analyze the MBK followed by the reference material(s).

d. Analyze ten analytical solutions (includes sample replicates and FAPs).

e. Analyze CCV and CCB after every ten analytical solutions (including replicates and FAPs) and at the end of the analytical batch.

4.15 Table 4. Typical Analytical Batch Sequence

Solution	Purpose	QC criteria
DIW blank	Verify clean auto-sampler vials	≤ ASDL
Check	Verify purity and retention time	OHCbl peak < 2% of CNCbl peak; reproducible retention times (within ±0.3 min)
Calibration standards	Standardize instrument	$r^2 \geq 0.996$
MBK	Verify absence of contamination	≤ ASDL
Reference material	Demonstrate accuracy	Recovery 100±20%
Ten (10) analytical solutions (includes replicates and FAPs)	Determine CNCbl conc (as Co)	within calib. range, RPD ≤ 20% FAP recovery 100±20%
Continuing calibration verification (CCV)[a]	Verify standardization	100±15% of expected
Continuing calibration blank (CCB)	Verify absence of contamination	≤ ASDL
Ten (10) analytical solutions (includes replicates and FAPs)	Determine CNCbl conc (as Co)	within calib. range, RPD ≤ 20% FAP recovery 100±20%
Continuing calibration verification (CCV)	Verify standardization	100±15% of expected
Continuing calibration blank (CCB)	Verify absence of contamination	≤ ASDL
[a]This and any subsequent standard solution injections must agree with previous standard solution injections with in ±15%.		

Chromatograms

(1) Check retention times, peak shape and response of both the ISTD and CNCbl in the *m/z* 59 chromatograms. Compare retention times and peak shapes to the example chromatogram in 4.15 Figure 2. To some extent, the retention time and peak shape are dependent on the age and performance of the HPLC column. However, significant differences between analyte retention time in standards and test materials (including FAPs) **within the same batch** are not anticipated and should be investigated and corrected if noted.

(2) Check *m/z* 61 chromatogram for indications of possible interference from $^{24}Mg^{35}Cl^{+}$, $^{43}Ca^{16}O^{+}$ and $^{23}Na^{36}Ar^{+}$ on Co at *m/z* 59. Peaks detected in the *m/z* 61 chromatogram arising from $^{24}Mg^{37}Cl^{+}$, $^{43}Ca^{18}O^{+}$ and $^{23}Na^{38}Ar^{+}$ will also have peaks with matching

retention time in the *m/z* 59 chromatogram. Peaks may also be observed in the *m/z* 61 chromatograms without corresponding peaks at *m/z* 59; for example, due to $^{61}Ni^{+}$, but do not represent interference.

(3) Integrate Chromatograms - *m/z* 59

a. Automatic integration is preferred to ensure consistency and minimize bias; however, because auto-integrators are not always reliable, visually inspect all chromatograms with manual integration applied when necessary to maintain accuracy. It is important to verify that peaks are properly identified by the integrator and imperative that manual integrations be as consistent as possible, especially within the same analytical batch. For the auto-integrator, ensure that the integration window is wide enough to capture the entire peak, but not so wide as to catch any part of neighboring peak(s). Try adjusting the integration window if a peak goes unintegrated when the batch is processed. Manually integrate if the auto-integrator cannot be adjusted to reliably find the peak.

b. For very small peaks, follow software instructions to determine signal-to-noise ratio (S/N). Questionable peaks must have a S/N > 3 to be considered detected. Peaks with S/N < 3 shall be treated as non-detected.

c. Examine chromatograms to ensure that all analyte peaks are correctly identified and that no unrelated peaks have been misidentified as the analyte.

4.15.7 CALCULATIONS

Calibration

(1) Calibrate using the standard blank and at least 4 calibration standards.

(2) Use a weighted calibration curve (e.g., $1/x^2$) to calculate analyte mass fraction from the integrated peak areas of the analytical solutions (weighted regressions often work well for samples with mass fractions towards the bottom of the calibration range). Use the 'IGNORE' option for y-intercept. Select the post-column marker as ISTD.

Calculations for Analyte Mass Fraction

The mass fraction of CNCbl in the analytical portion ($MF_{CNCbl(s)}$) is calculated from the mass fraction of Co in the analytical solution ($MF_{Co(as)}$), which is the HPLC-ICP-MS result, with an accounting for analyte pre-concentration after the liquid and solid phase extraction steps. $MF_{CNCbl(s)}$ is reported as µg/100 g.

Step 1. Calculate mass fraction of Co in the analytical portion ($MF_{Co(s)}$) in µg g^{-1}

$$MF_{Co(s)} = (MF_{Co(as)})(PF)\left(\frac{1\ \mu g}{10^3\ ng}\right)$$

where: $MF_{Co(as)}$ = mass fraction of Co in the analytical solution (ng g^{-1})

PF = pre-concentration factor (unitless)

$$PF = \left(\frac{M_{(s)} + M_{(es)}}{M_{(s)}}\right)\left(\frac{M_{(as)}}{M_{(e)}}\right)$$

where: $M_{(s)}$ = mass of analytical portion (g)

$M_{(es)}$ = mass of extraction solutions (NaAc, NaCN and α-amylase) (g)

$M_{(e)}$ = mass of extract loaded on the SPE cartridge (g)

$M_{(as)}$ = mass of analytical solution (reconstituted extract) (g)

Step 2. Convert $MF_{Co(s)}$ to $MF_{CNCbl(s)}$ (µg/100 g)

$$MF_{CNCbl(s)} = (MF_{Co(s)})\left(\frac{M_{CNCbl}}{M_{Co}}\right)(100)$$

where: M_{CNCbl} = molecular mass of CNCbl (1355.37 g mol^{-1})

M_{Co} = molar mass of Co (58.93 g mol^{-1})

Note: For the sake of simplicity, $MF_{CNCbl(s)}$ is also referred to as MF_s in this document.

4.15.8 QUALITY CONTROL ELEMENTS

Prior to Analysis of Test Samples

(1) Verify the purity of standards and the retention time of the analyte.

(2) Establish the HPLC-ICP-MS Analytical Solution Detection Limits (ASDL) and Analytical Solution Quantitation Limits (ASQL) according to EAM §3.2[1] based on the standard deviation of replicate (n = 10) analyses of a low-level CNCbl standard solution. Prepare the standard at a mass fraction just above the estimated ASDL.

Calculate ASDL and ASQL for CNCbl as follows:

$$\text{ASDL} = (2)(t_{95})(s)\sqrt{1 + \frac{1}{\text{n}}}$$

$$\text{ASQL} = 30(s)$$

where: t_{95} = one-sided Student's t at 95% confidence level

n = number of measurements

s = standard deviation of replicate measurements (µg/100 g)

(3) Calculate the method Limit of Detection (LOD) and Limit of Quantitation (LOQ) by multiplying ASDL and ASQL, respectively, with the pre-concentration factor (PF).

Note: Due to variability between labs and instrumentation, LOD and LOQ should be determined in each lab. The ASDL and ASQL values in 4.15 Table 1 are presented only as examples.

LOD as found here is different than using a S/N > 3 procedure to examine a chromatogram and decide whether a peak is 'detected'. The latter is a qualitative decision that specifically focuses on the chromatogram whereas LOD is a characteristic of the entire method.

Analysis of Test Samples

(1) Post-Column Marker (ISTD)

The ISTD solution helps to compensate for instrumental drift. The control limit for the ISTD response is 80–120%; reanalyze analytical solutions where ISTD recovery falls out of this range.

(2) Calibration Curve

A minimum of five calibration levels (including standard blank) shall be used. The calibration curve must be linear with $r^2 \geq 0.996$. Inspect the line fit and prepare new standards, if necessary. If the analyte mass fraction in the analytical solution exceeds that of the highest calibration standard, dilute the solution gravimetrically as necessary and reanalyze.

(3) Continuing Calibration Verification (CCV)

A calibration check standard shall be analyzed after every 10th analytical solution and

after the final analytical solution in the batch to monitor retention time and quantitative accuracy. The CCV should be run at a level that is near the mid-point of the analytical calibration curve. The control limit for the calibration check standard is 100±15% of the analyte mass fraction in the standard. A shift in retention time of up to 0.3 min is acceptable when compared to the highest Cal Std. Should these criteria not be met, the check standard may undergo a re-evaluation once. Additional failure requires recalibration and reanalysis of the analytical solutions that were analyzed after the last acceptable CCV result.

(4) Continuing Calibration Blank (CCB)

No peak should be detected (S/N > 3) in the CCB at the retention time of the analyte. If the CCB fails, reanalyze it one more time. If it fails again, rinse the HPLC system and column with the rinsing solution and restart analytical batch.

(5) Method Blanks (MBKs)

No peak should be detected (S/N > 3) in the MBK at the retention time of the analyte. If detected, identify and correct the possible sources of contamination (including reagents, labware, etc.) prior to continuing with the analysis.

(6) Replicate Analytical Portions

Use duplicate analytical portions for each nutritional product. The control limit for relative percentage difference (RPD) is 20% for analyte mass fraction ≥ LOQ. If RPD is above 20%, reanalyze the analytical solutions once. If acceptable RPD is still not achieved, the source of the imprecision should be investigated. Reanalysis of samples analyzed after the last sample with an acceptable RPD may be required. If RPD remains unacceptable, prepare new analytical solutions.

$$\mathrm{RPD}(\%) = \left(\frac{\left|\mathrm{MF}_{(s1)} - \mathrm{MF}_{(s2)}\right|}{\mathrm{MF}_{(av)}}\right)(100\%)$$

where: $\mathrm{MF}_{(s1)}$ = analyte mass fraction in replicate 1 (µg/100 g)

$\mathrm{MF}_{(s2)}$ = analyte mass fraction in replicate 2 (µg/100 g)

$\mathrm{MF}_{(av)}$ = mass fraction average for replicates 1 and 2 (µg/100 g)

(7) Fortified Analytical Portion (FAP)

For each analytical batch and at least once for each separate matrix type, prepare and analyze one FAP to verify peak identification and quantitative recovery. Unfortified and fortified analytical portions of a test sample must be analyzed in the same analytical

batch.

Control limit for FAP recovery is 100±20%. If the recovery is not acceptable, ensure that the fortification level is appropriate and reanalyze the analytical solution once. If FAP fails again, prepare and analyze another FAP (from the same homogenized laboratory test sample).

Compare chromatograms for the unfortified and fortified analytical portions of a product; an appropriate increase in peak area must be observed. In addition, the peak shape in the FAP chromatograms should be similar to that for the unfortified analytical portion, exhibiting no significant band broadening, shoulders or unexpected peaks. Retention time shift of up to 0.3 minutes is tolerated when comparing chromatograms of the unfortified and fortified analytical portions with that of the highest calibration standard.

Note: FAP recovery can fail due to inappropriate fortification levels. Since label claims often underestimate the actual levels of vitamin B_{12} present in nutritional products[4], it is recommended to first analyze the test sample as a 'check sample' to identify the correct level of fortification (do not report the check result in the final analysis report). The vitamin B_{12} mass fraction added by fortification into the analytical portion can be 50–300% of the native analyte mass fraction.

The following equation demonstrates how to calculate FAP recovery.

$$\%\ \text{Recovery} = \left[\frac{\mathrm{MF}_{(\mathrm{FAP})} - \mathrm{MF}_{(\mathrm{s})}}{\left(\frac{(\mathrm{MF}_{(\mathrm{x})})(\mathrm{M}_{(\mathrm{x})})}{\mathrm{M}_{(\mathrm{s})}}\right)}\right](100\%)$$

where: $\mathrm{MF}_{(\mathrm{FAP})}$ = analyte mass fraction in FAP (µg/100 g)

$\mathrm{MF}_{(\mathrm{s})}$ = analyte mass fraction in unfortified analytical portion (µg/100 g)

$\mathrm{MF}_{(\mathrm{x})}$ = analyte mass fraction in fortification solution (µg/100 g)

$\mathrm{M}_{(\mathrm{x})}$ = mass of fortification solution added to analytical portion (g)

$\mathrm{M}_{(\mathrm{s})}$ = mass of analytical portion (g)

(8) Reference Materials

Each extraction batch must include at least one reference material or in-house reference material. Traceable certified or standard reference materials (CRM or SRM) should be

used when available; see 4.15 Table 5 for suggested materials. In the absence of a CRM or SRM, in-house reference materials may be used after suitable characterization[1]. Match the reference material matrix as closely as possible with the sample matrix.

Control limits for reference materials are based on certificate values (e.g., see 4.15 Table 5). For materials with non-certified reference values, the limit is set at 100±20% of the certificate value. If acceptable values are not obtained, the analytical solution may be reanalyzed once. If the control limit is still not met, recalibrate and reanalyze the entire analytical batch. Control limits for in-house reference materials should be established as described in EAM §3.5[1].

Note: Several reference materials are available for vitamin B_{12} from the National Institute of Standards Technology (NIST) and the European Commission Joint Research Centre. SRM 1869 has a certified reference value for vitamin B_{12} while RM 8260 and RM 8261 have reference values (non-certified). These materials are recommended for use in analytical batches containing infant formulas and toddler and adult nutritive drinks. SRM 1549a and ERM-BD600 are certified for vitamin B_{12} and can be used with milk samples. SRM 3235 is a non-diary milk product (soy milk) with a certified reference value for vitamin B_{12}.

4.15 Table 5. Control Limits for Vitamin B_{12} Mass Fraction in Reference Materials.

Reference material	Number	Vitamin B_{12}, mg/kg
Infant formula	SRM 1846	0.039±0.003[a]
Infant/adult formula II, milk/whey/soy-based	SRM 1869	0.0447±0.0049[a]
Infant formula, hydrolyzed milk-based	RM 8260	0.0029[b,c]
Adult nutritional formula, high protein	RM 8261	0.0029[b,c]
Whole milk powder	SRM 1549a	0.032±0.002[a]
Whole milk powder	ERM-BD600	0.32±0.07[a]
Soy milk	SRM 3235	0.0147±0.0011[a]

[a]Certified value with Uncertainty expressed as a 95% Confidence Interval or 95% Confidence Interval plus an allowance for systematic error.

[b]Mass fraction in mg/100 g.

[c]Reference value, material not certified for vitamin B_{12}.

4.15.9 REPORT

(1) Report results only when quality control criteria for a batch have been satisfactorily met.

(2) Report average mass fractions of CNCbl for replicate analytical portions in no more than three significant figures. Express mass fractions as µg/100 g.

(3) Report results that are ≥ LOQ as the mass fraction followed by the unit of measurement. Report results that are ≥ LOD and < LOQ as the mass fraction followed by the unit of measurement and the 'trace' qualifier (TR) to indicate this is a level below the limit of quantification which has greater uncertainty and is therefore less reliable. Report results that are < LOD as 0 followed by the unit of measurement.

Example:

LOQ = 0.05 µg/100 g; LOD = 0.02 µg/100 g.

Vitamin B_{12} mass fractions from three different analyses were 0.1 µg/100 g, 0.03 µg/100 g and 0.01 µg/100 g.

0.1 µg/100 g is ≥ LOQ; report 0.1 µg/100 g

0.03 µg/100 g is ≥ LOD but also < LOQ; report (TR)

0.01 µg/100 g is < LOD; report 0 µg/kg

4.15.10 METHOD VALIDATION

In-house (single laboratory) validation

EAM 4.15 has undergone a level 2 single lab validation[4] following the procedures set forth in FDA's *Guidelines for the Validation of Chemical Methods for the FDA Foods Program*[5]. The method was validated by analyzing seven infant formulas, ten toddler and adult nutritive drinks, six milks, eight protein powders/drinks, and seven breakfast cereals purchased from local stores and online. Care was taken in the selection of samples to represent the disparate matrix categories across nutritional products. All samples were analyzed in triplicate (n = 3) and fortified at three levels in duplicate (n = 2). The three-tiered spikes corresponded to 50% (low level), 100% (medium level) and 150% (high level) of the native analyte mass fraction in the product. Reference materials of matrix types matching each group of nutritional products were used (4.15 Table 5). SRM 3252 (protein mix drink), which previously had a reference value for vitamin B_{12}, and SRM 3233 (fortified breakfast cereal) with vitamin B_{12} value published in a

study from NIST[6] were also used.

Method performance characteristics including LOD and LOQ, repeatability, accuracy (reference material and spike recovery), and measurement uncertainty were evaluated. The inter-day precision of the method was evaluated by preparing and analyzing replicate analytical solutions from non-fortified and fortified analytical portions of each product over the course of three days. The reproducibility of the method was < 15% relative standard deviation (RSD) for analyte mass fractions > LOQ. Recovery values mostly ranged between 80% and 120% for fortified samples and reference materials.

4.15.11 METHOD REVISION HISTORY

November 2023 – Single-lab validation data collection completed.

April 2024 – EAM 4.15 draft version 0.1 completed and submitted to EASC.

Multi-lab validation – Under development.

4.15.12 REFERENCES

(1) FDA. Elemental Analysis Manual for Food and Related Products, Section 3: General Analytical Operations and Information 2021 https://www.fda.gov/food/laboratory-methods-food/elemental-analysis-manual-eam-food-and-related-products.

(2) ASTM. ASTM D1193-24, Standard Specification for Reagent Water 2026 http://www.astm.org

(3) FDA. Elemental Analysis Manual for Food and Related Products, Section 2: Sample Preparation 2021 https://www.fda.gov/food/laboratory-methods-food/elemental-analysis-manual-eam-food-and-related-products.

(4) Wolle, M. M.; Escavage, J.; Gray, P. J. Development and Validation of a Method for Vitamin B12 Measurement in Nutritional Products by HPLC-ICP-MS. *Food Analytical Methods* **2025**. DOI: 10.1007/s12161-025-02882-z.

(5) FDA. Guidelines for validation of chemical methods for the FDA Foods and Veterinary Medicine Program 2019 https://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM273418.pdf.

(6) Raju, C. S. K.; Yu, L. L.; Schiel, J. E.; Long, S. E. A simple and sensitive LC-ICP-MS method for the accurate determination of vitamin B12 in fortified breakfast cereals and multivitamin tablets. *Journal of Analytical Atomic Spectrometry* **2013**, *28* (6), 901-907. DOI: 10.1039/C3JA30383G.