File No. 1-90/FSSAI/SP (MS&A)/2009

Food Safety and Standards Authority of India

(A statutory Authority established under the Food Safety and Standards Act, 2006)

(Quality Assurance Division)

FDA Bhawan, Kotla Road, New Delhi – 110002

Dated, the 13 July, 2020

ORDER

Subject: **Methods of analysis of Fortificants in Foods (Pyridoxine, Zinc and Folic Acid) and Formulated Supplements for Children - reg.**

The Scientific Panel on Methods of Sampling and Analysis, Scientific Committee and Food Authority has approved the following methods-

(i) Methods of analysis of Fortificants in Foods (Pyridoxine, Folic Acid and Zinc) **[Annexure – I]**

(ii) Methods of analysis of Formulated Supplements for Children **(Annexure – II)**

2. The food testing laboratories are hereby requested to use the aforesaid method, with immediate effect.

Encl: Method

13 JUL 2020

(Sanu Jacob)
Director (QA)

To:

1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories

Annexure – I

Methods of analysis for Fortificants in Foods

A. Pyridoxine

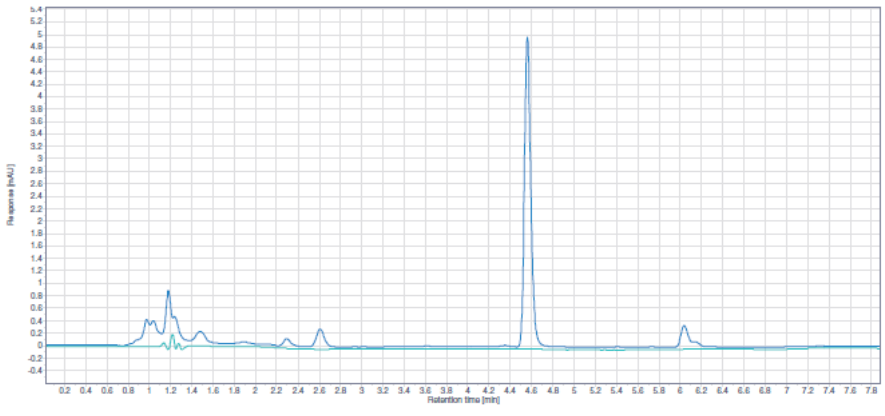
Commodity	Parameter	Test Method	Instrument & Detector/Method
Atta, Maida and Rice	**Pyridoxine (Vitamin B_6)**	EN 14164	HPLC

B. Folic Acid

FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Folic acid (Vitamin B9) in Fortified Rice and Wheat Flour**		
Method No.		**Revision No. & Date**	
Safety and precautions	The method uses an immunoaffinity folic acid kit which needs to be stored at 4°C. Prepare all the standards in dark in low-actinic volumetric glassware and store at 2-8 °C in tightly stoppered volumetric flasks. Acetonitrile is an inflammable liquid, and should be kept away from heat, sparks, open flames, hot surfaces. If spilt on skin (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.		
Scope	The method is applicable for the determination of Folic acid, in fortified rice and wheat flour.		
Principle	Folic acid is extracted from the sample using pancreatin and L-ascorbic acid. After incubation the extract is filtered, diluted with water, and applied to an immuno-affinity column (IAC) containing antibodies specific to folic acid. Folic acid is quantified by reversed-phase liquid chromatography (RP-HPLC) with UV detection.		
Apparatus	1. HPLC system equipped with a quaternary gradient pump, an autosampler (100 µL maximum loop capacity) and a diode array detector with 60 mm path length for the highest sensitivity and 10 mm path flow cell and column oven 2. Solid-phase extraction vacuum manifold apparatus 3. Shaker incubator: 37°C 4. Water-bath- 70°C 5. Vortex mixer 6. Analytical balance -Suitable for weighing samples with accuracy up to 0.1 mg 7. Centrifuge tubes. Autoclavable 50 mL polypropylene amber-colored centrifuge tubes 8. Centrifuge-5000 rpm, holding 50 mL tubes 9. Filter paper Whatman 597.5 filters (185 mm). 10. Screw-capped amber glass bottle-100 mL 11. Measuring cylinder: 10 and 50 mL 12. Amber-colored volumetric flasks- 10 and 100 mL. 13. Micropipettes-Capable of delivering 0.5-10, 2-20, 5-50, 10-100, 20-200, 200-1000 µL, and 1-5 mL of liquids such as vitamin standards, solvents, buffers, and extracts.		
Chemicals	a) Folic acid analytical standard, Sigma Aldrich (CAS No. 59-30-3;		

	Sigma PN, 47866 Supelco). b) Acetonitrile-HPLC grade c) Water: HPLC grade ≥18 megohm in resistivity d) Absolute ethanol (99.8%) e) Sodium hydroxide pellets f) L-ascorbic acid g) Sodium phosphate monobasic (NaH_2PO_4) h) Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) i) Trifluoroacetic acid (HPLC grade) j) Pancreatin (CAS No. 8049-47-6) (4 × USP specifications) k) Folic acid Immunoaffinity kit (R-Biopharm (Darmstadt, Germany or equivalent).
Preparation of reagents.	*0.1 M phosphate buffer*: Dissolve 9.36 g of monobasic sodium phosphate (anhydrous) and 32.74 g sodium phosphate dibasic heptahydrate in 2 L distilled water. When fully dissolved, adjust the pH of the buffer to 7.0 with a few drops of orthophosphoric acid. *10% L-Ascorbic acid solution*: Dissolve 10 g L-ascorbic acid in 100 mL distilled water in a volumetric flask. Mix well, and store in an amber glassware. Prepare fresh every week. *Elution solution*: 30% acetonitrile containing 0.2% TFA. Add 70 mL water containing 200 µL TFA to 30 mL acetonitrile. *Folic acid diluent*: Prepare 15% acetonitrile in water with 0.1% TFA in a 100 mL volumetric flask. Add 85 mL water containing 100 µL TFA to 15 mL acetonitrile. *1 M NaOH solution*: Dissolve 400 mg sodium hydroxide pellets in 10 mL water in a volumetric flask. *1 M HCl solution*: Dilute 880 µL HCl (approximately 37%) to 10 mL with water in a volumetric flask.
Preparation of standards	a) Prepare all the standards in dark in low-actinic volumetric glassware and store at 2-8 °C in tightly stoppered volumetric flasks. b) Prepare the stock solution (200 mg/L) by dissolving 20 mg (accurately weighed) folic acid with 200 µL 1 M sodium hydroxide in a 15 mL tube. c) To this add 10 mL distilled water, and adjust the solution pH to 6.0 with 1 M HCl (by adding approximately 150 µL 1 M HCl). d) Mix thoroughly on a vortex mixer for 30 s and transfer to a 100 mL amber volumetric flask. Add 10 mL distilled water and 20 mL

	ethanol. e) The final volume is made up to 100 mL mark with distilled water (200 mg/L) in amber colored volumetric flask. f) Prepare stock solution fresh every week. Store in the dark at 2-8°C. g) The calibration standards of 800, 400, 200, 100, and 50 µg/L concentrations are prepared daily by serial dilutions from the stock solutions using 15% acetonitrile in water containing 0.1% TFA).
Preparation of test samples	a) All glassware used for analysis must be made of low-actinic glass. b) Grind the samples to a fine powder. c) Weigh (5 g) of the sample directly into a 100 mL screw-capped amber colored glass bottle, add 50 mL 0.1 M phosphate buffer (pH 7.0), mix thoroughly (~15 min). d) Add 1 g pancreatin (4 × USP specifications**)** and allow to dissolve (~5 min). Add 6 mL, 10% L-ascorbic acid solution. Mix well on a vortex mixer for 5 min. Incubate in a shaking incubator at 37°C for 2 h. Then incubate at 70°C in a water bath for 20 min for inactivate enzyme. e) Cool to room temperature (25 ±2 °C). f) Transfer the contents to a 100 mL amber-colored volumetric flask, and make up to the mark with 0.1 M phosphate buffer. g) Transfer the sample to 2 × 50 mL centrifuge tubes h) Centrifuge at 5000 rpm (~5500 × g) for 10 min. i) Filter the supernatant through a Whatman S&S 597.5 filter. j) Use 15 mL aliquots of filtrate for immunoaffinity cleanup
Immunoaffinity Chromatography	a) Bring the immunoaffinity cartridges to room temperature (25 ±2 °C) before use. b) Place the cartridges vertically in a vacuum manifold. Pass 15 mL of the filtrate through the cartridge at a flow rate of 2-3 mL/min. c) A steady pressure is maintained for the optimal interaction of folic acid with the antibody in the immunoaffinity cartridges. d) With the help of a glass syringe barrel, pass 10 mL of distilled water pass through the immunoaffinity cartridge. e) Any remaining traces of water are removed from the cartridge under vacuum. f) Place an amber-colored vial (2 mL) directly beneath the column. Elute Folic acid with 1 mL elution solution (30% acetonitrile: 70% water containing 0.2% TFA) at a flow rate of one drop per second or

<table>
<tr><td></td><td>by gravity.
g) Add 1 mL distilled water and repeat the same elution procedure.
h) The elute is then analyzed by HPLC after appropriate dilution.</td></tr>
<tr><td>Chromatography Analysis</td><td>a) The HPLC system (e.g. Agilent 1260 Infinity II Prime) should consist of a quaternary gradient pump, an autosampler (100 µL maximum loop capacity) and a diode array detector with 60 mm path length for the highest sensitivity and 10 mm path flow cell.
b) C18 column (e.g. Poroshell SB-C18 column, 3.0 × 100 mm, 2.7 µm or equivalent). The column oven temperature is maintained at 30°C.
c) Flow rate 0.6 mL/min and detection at 280 nm
d) Injection volume: 10 µL
e) Mobile phase A (0.1% TFA in water) and B (acetonitrile)
f) Gradient program:
<table>
<tr><td>Time (mins)</td><td>% B</td></tr>
<tr><td>0</td><td>7</td></tr>
<tr><td>5</td><td>20</td></tr>
<tr><td>6</td><td>20</td></tr>
<tr><td>7</td><td>65</td></tr>
<tr><td>9</td><td>65</td></tr>
<tr><td>10</td><td>7</td></tr>
<tr><td>12</td><td>7</td></tr>
</table></td></tr>
<tr><td>Results</td><td> 
Figure 1. Representative chromatogram of folic acid in rice sample after immunoaffinity cartridge extraction and cleanup.</td></tr>
<tr><td>Calculation</td><td>a) Carry out a regression analysis and calculate Regression coefficient (R^2) by analyzing the calibration standards (800, 400, 200, 100, and 50 µg/L) by fitting the data into a linear regression curve,</td></tr>
</table>

	including zero as the response for the reagent blank. b) Calculate the folic acid content by using the following equation: $$\textit{Folic acid (mg/kg)} = \frac{\textit{Folic acid IC} \times \textit{Makeup volume} \times \textit{Dilutions} \times \textit{Standard purity}}{\textit{Sample weight (g)} \times 100}$$ Where, makeup volume = 100 mL; dilutions = 0.133; sample weight = approximately 5 g; and folic acid IC = folic acid concentration in sample measured against a calibration curve. c) The LOD and LOQ are determined by considering the S/N of 3 and 10, respectively, for the folic acid signal in the matrix. d) Determine the recovery of folic acid by the external spiking method at three different spike levels (75, 100, and 150 μg/kg) in six replicates. Calculate the recovery value using the following equation: $$\textit{Recovery (\%)} = \frac{(A - B)}{C} \times 100$$ where A = the concentration of folic acid in the spiked sample (micrograms per kilogram); B = the natural content of folic acid in the control sample (micrograms per kilogram); C = the spiked concentration of folic acid (micrograms per kilogram;
LOD LOQ	a) LOD 20 μg/Kg in Matrix. b) LOQ 50 μg/Kg in Matrix.
Reference	Mahato A., Vyas S., Chatterjee N. (2020). HPLC-UV Estimation of Folic Acid in Fortified Rice and Wheat Flour using Enzymatic Extraction and Immunoaffinity Chromatography Enrichment: An Interlaboratory Validation Study. *Journal of AOAC International* 103 (1): 73-77. DOI: https://doi.org/10.5740/jaoacint.19-0207
Approved by	Scientific Panel on Methods of Sampling and Analysis

C. Zinc

fssai FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Method for determination of Zinc in fortified wheat & rice flour using ICP-MS**		
Method No.		**Revision No. & Date**	
Note	Applicable for the determination of zinc at trace levels in fortified wheat and rice flour. The method is capable of determining 2.5-50 mg of Zinc/kg flour, depending on instrument design. This method is not applicable to milk powder.		
Caution	Chemicals used are common use solvents, acids and reagents that are harmful if inhaled, swallowed or absorbed through the skin. Digestion vessels must cool for an appropriate time before opening in order to avoid burns from hot and corrosive vapours. Always gently add acid to water. Concentrated Nitric acid and Hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear including eye protection, gloves with appropriate resistance and a laboratory coat. Use an adequate fume hood while working with acids. Microwave operation involves hot pressurized acid solution. Use appropriate personal protective equipment, such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Hydrogen peroxide is a strong oxidizer and can react aggressively with organic material to give off oxygen gas and heat. Adequate protective gear should be worn. Refer to Material Safety data Sheets (MSDSs) to ensure that the safety guidelines are applied before using chemicals. The Inductively coupled plasma-mass spectrometer (ICP-MS) emits UV light when the plasma is on. Wear UV resistant goggles should be worn if working near the plasma. The instrument generates high levels of radio frequency (RF) energy and is hot when the plasma is on.		
Principle	Nitric acid, and hydrogen peroxide are added to homogenized wheat/rice flour sample in microwave vessels, and digested using a preprogrammed temperature control. Analysis is performed by ICP-MS. Polyatomic interferences with the low mass elements are reduced or eliminated by analysis in He collision mode using kinetic energy discrimination (KED). Quantitation of Zinc is achieved essentially simultaneously by comparing the analyte–ISTD response ratios in the unknown samples with a standard curve constructed from response ratios of calibration standards.		
Equipment	a) ICP mass spectrometer: equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. A quadrupole mass spectrometer must have mass		

	range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system should achieve 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). b) Microwave Digestion System: Commercial microwave designed for laboratory use at 0–300°C, with closed-vessel system and controlled temperature ramping capability. The microwave should be appropriately vented and corrosion resistant. The microwave field is directed to the sample region and adapts itself to the number of filled or empty positions and the filling state of the vessel. The cavity should provide highly efficient heating in a compact system. Venting system should enable precise pressure control in each vessel. c) Fume hood d) Analytical balance capable of measuring to 0.1 mg e) Volumetric flasks (Class A): 5 - 1000 mL f) Micropipettes - fixed or variable, covering ranges 10 - 5000 µL g) Polypropylene bottles - 50 mL h) Polytetrafluoroethylene (PTFE)-coated magnetic stir bars i) Argon gas, high purity grade (99.999%) j) Helium gas, ultra-high purity ≥ 99.999% k) Clean-room gloves: Tested and certified to be low in the metals of interest l) Spatulas: To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO3 prior to use
Chemicals	Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use. a) Ultra-pure water - Deionized water polished to ASTM Type 1 specification or better (resistance ≥ 18 megaohms), demonstrated to be free from the metals of interest and potentially interfering substances b) Nitric acid (HNO_3) 65% concentrated: Trace metal grade c) Hydrogen peroxide (H_2O_2): 30%ACS reagent grade d) Auto tuning solution (1 µg/L) – Ba, Bi, Ce, Co, In, Li and U in 2.5% (v/v) HNO3 and 0.5 % (v/v) HCl e) Zinc Stock standard solutions: Obtained from a reputable and professional commercial source with traceability f) Internal standard solution-Germanium (Ge) g) Daily optimization, tuning and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer's suggestions
Preparation of reagents/ standards	a) Calibration standards- Prepare fresh everyday. Dilute the single element zinc stock standard solutions into 50 mL pre-cleaned autosampler vials with 5% (v/v) HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of

quantitation (LOQ) when recalculated in units specific to the reported sample results. See below for recommended concentrations for the calibration curve.

b) The linearity standards (0, 0.005, 0.02, 0.04, 0.06, 0.08 and 0.10 mg/L) containing 10 µg/L of Ge internal standard, prepared from stock of 1000 mg/L Zn standard.

c) Internal standard stock solution (ISTD)- Ge at 5 mg/L in 2% (v/v) HNO_3

d) Nitric acid rinse solution (2%, v/v) for autosampler rinse port with Tergitol added. Mix 30.77 mL of concentrated nitric acid (65%) with 20 mL Tergitol solution, and ultra-pure water to prepare a total volume of 1000 mL. Expiration: 3 months when stored at room temperature.

e) All autosampler vials should be soaked in 2% HNO_3 overnight and then rinsed three times with ultra-pure water. Then dry vials in a clean hood before use.

f) Volumetric flasks should be soaked in about 5% HNO_3 (v/v) overnight prior to use.

g) Nitric acid rinse solution (5%, v/v) for volumetric flask. Mix 76.92 mL of concentrated nitric acid (65%) with 20 mL Tergitol solution, and ultra-pure water to prepare a total volume of 1000 mL. Expiration: 3 months when stored at room temperature.

Preparation of Test Samples

1) All digestions must be prepared in triplicate.

2) Weigh 0.5 g of homogenized wheat/rice flour accurately to the nearest 0.001 g into a clean microwave digestion vessel.

3) Add 0.5mL Ge ISTD using a calibrated digital pipet.

4) Add 5 mL concentrated HNO_3 and 2 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

5) Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave digestion unit following the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

6) Digest samples at a minimum temperature of 180-200°C for a minimum time of 20 minutes each stage as per the below table. (Appropriate ramp times and cool down times should be included in the microwave program, depending on the model of microwave digestion system. Follow the programme shown in Table 1.

Table 1 Microwave digestion temperature program for wheat/rice flour samples.

Stage	Step	Temp.,^{0}C	Ramp. min.	Hold, min
I	1	180	20	20
	2	Cool down	NA	20
II	3	200	20	20
	4	Cool down	NA	20

7) After digestion, place vessels in a fume hood, unscrew the cap/venting

	nut slowly to gradually release pressure, and then completely remove the cap. 8) Remove the cap, rinse with ultra-pure water. Slowly add approximately 20 mL ultra-pure water to the contents of the vessel, swirl to mix, make up to 50 mL in a volumetric flask with ultra-pure water. Shake briefly. 9) Method Blank: Follow the above steps of 3, 4 and then step 7. Specifically, do not microwave digest the method blank, which can subject the blank to contamination. *Note: During preparation of samples the transfer or final volume does not need to be quantitative because ISTDs are added prior to digestion due to which the analyte ISTD ratios will be constant*.
Determination	1) Perform instrument startup routine and initial checks following the manufacturer recommendations. 2) Ignite the plasma and start the peristaltic pump. 3) Allow plasma and system to stabilize for at least 30 min. 4) Perform an optimization of the sample introduction system (e.g., X-Y and Z optimizations) to ensure maximum sensitivity. 5) Perform the instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements to ensure the instrument's quadrupole mass filtering performance is adequate. 6) Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications. 7) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios. 8) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. 9) Analyze test solutions using an ICP-MS instrument standardized with the indicated standard solutions. 10) Internal standards must be present in all samples, standards, and blanks at identical concentrations. 11) Start the analysis of samples. Immediately following the calibration, a method blank should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination. *Note: The order of analysis should be calibration standards, followed by rinse, blank check, check standard, control sample, sample, sample triplicate, and finally a repeated check standard.*

Calculation	Sample concentrations in ng/g (ppb) are automatically calculated by the software using a non weighted least –squares linear regression calibration analysis to produce a best fit curve. Y = ax + blank $$\mathbf{x\ (ng/g)} = \frac{Y - \text{blank}}{a} \times DF$$ where x = analyte concentration (ng/g); Y = analyte-to-ISTD intensity ratio, which is the measured count of each analyte's standard solution data point in the calibration curve divided by the counts of the ISTD at the same level; blank = analyte to- ISTD intensity ratio, which is the measured count of the blank standard solution data point in the calibration curve divided by the counts of the ISTD at the same level as the blank standard solution; a = slope of the calibration curve (mL/ng); DF = dilution factor, or the volume of the sample solution (mL) divided by sample weight (g).
Reference	AOAC Official Method 2015.06. Minerals and Trace Elements in Milk, Milk Products, Infant Formula, and Adult/Pediatric Nutritional Formula ICP-MS Method. Final Action 2017
Approved by	Scientific Panel on Methods of Sampling and Analysis

Annexure – II

Method of analysis for Formulated Supplements for Children mentioned in Regulation No. 2.4.11.4 of FSS (Food Product Standards and Food Additives) Regulation, 2011

Parameter	Specifications	Test Method	Title
Vitamin A as retinol	120-400 µg/100gm	EN-12823-1	Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 1: Measurement of all-E-retinol and 13-Z-retinol
Vitamin D (expressed as Cholecalciferol or Ergocalciferol)	3-10 µg/100gm	ISO 20636:2018 (equivalent to AOAC 2016.05)	Infant formula and adult nutritionals -- Determination of vitamin D by liquid chromatography-mass spectrometry
Vitamin C	12-40 mg/100gm	ISO 20635:2018 (equivalent to AOAC 2012.22) (for method applicable for foods in general see Fontannaz et al. Food Chemistry 94 (2006) 626–631)	Infant formula and adult nutritionals -- Determination of vitamin C by (ultra) high performance liquid chromatography with ultraviolet detection ((U)HPLC-UV)
Thiamine (Vitamin B_1)	250-500 µg/100gm	EN 14122:2014	Foodstuffs - Determination of vitamin B1 by high performance liquid chromatography
Riboflavin (Vitamin B_2)	180-600 µg/100gm	EN 14152:2014	Foodstuffs - Determination of vitamin B2 by high performance liquid chromatography
Niacin (Vitamin B_3)	2.5-8 mg/100gm	EN 15652:2009	Foodstuffs - Determination of niacin by HPLC
Pyridoxine (Vitamin B_6)	270-900 µg/100gm	EN 14164:2014 (preferred over AOAC 2004.07 due to	Foodstuffs - Determination of vitamin B6 by high performance

		horizontal validation in different matrices)	chromatography
Folic acid (Vitamin B_9)	14.5-48 µg/100gm	AOAC 2011.06	Total Folates in Infant Formula and Adult Nutritionals
Pantothenic acid (Vitamin B_5)	0.6-2 mg/100gm	ISO 20639:2015 (equivalent to AOAC 2012.16)	Infant formula and adult nutritionals -- Determination of pantothenic acid by ultra high performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS)
Vitamin B_{12} (Cyanocobalamin)	0.15-0.5 µg/100gm	AOAC 2014.02 (preferred over AOAC 2011.10 because of simplicity in execution)	Vitamin B12 in Infant Formula and Adult/ Pediatric Formulas
Choline	>32 mg/100gm	AOAC 2015.10	Free and Total Choline and Carnitine in Infant Formula and Adult/ Pediatric Nutritional Formula
Vitamin K	4.5-15 µg/100gm	ISO 21446 (in press)	Infant formula and adult nutritionals — Determination of trans and total (cis + trans) vitamin K1 content — Normal phase HPLC
Biotin	2.5-8 µg/100gm	AOAC 2016.02	Biotin in Infant Formula and Adult/Pediatric Nutritional Formulas
Vitamin E as L-Tocopherols	1.5-5 mg/100gm	EN-12822:2014	Foodstuffs - Determination of vitamin E by high performance liquid chromatography - Measurement of α-, ß-, y- and δ- tocopherol

Sodium	90-300 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Potassium	270-900 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Chloride	240-800 mg/100gm	AOAC 2016.03 (Alternative method is AOAC 986.26 which is validated for Foods)	Chloride in Milk, Milk Powder, Whey Powder, Infant Formula, and Adult Nutritionals
Calcium	180-600 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry

Phosphorous	135-450 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Magnesium	15-50 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Iron	2.5-9 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Iodine	27-90 µg/100gm	ISO 20647:2015 (equivalent to AOAC 2012.15)	Infant formula and adult nutritionals -- Determination of total iodine -- Inductively coupled plasma mass spectrometry (ICP-MS)

Copper	102-340 µg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Zinc	1.5-5.0 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Manganese	0.3-1.2 mg/100gm	AOAC 2011.14 (equivalent ISO method under development-ISO/DIS 15151); (Alternative ICP-MS based method is AOAC 2015.06 for which equivalent ISO method is also under development-ISO/DIS 21424)	Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Phosphorus, Sodium, and Zinc in Fortified Food Products. Microwave Digestion and Inductively Coupled Plasma-Optical Emission Spectrometry
Selenium	5-17 µg/100gm	ISO 20649:2015 (equivalent to AOAC 2011.19)	Infant formula and adult nutritionals -- Determination of chromium, selenium and molybdenum -- Inductively coupled plasma mass spectrometry (ICP-MS)

Inositol	<0.40 gm/L	ISO 20637:2015	Infant formula and adult nutritionals -- Determination of myo-inositol by liquid chromatography and pulsed amperometry
Docosahexaenoic acid	<50 mg/100gm	ISO 16958:2015 (alternative to AOAC 2012.13)	Milk, milk products, infant formula and adult nutritionals -- Determination of fatty acids composition -- Capillary gas chromatographic method
Arachidonic acid	<50 mg/100gm	ISO 16958:2015 (alternative to AOAC 2012.13)	Milk, milk products, infant formula and adult nutritionals -- Determination of fatty acids composition -- Capillary gas chromatographic method
Eicosapentaenoic acid	<50 mg/100gm	ISO 16958:2015 (alternative to AOAC 2012.13)	Milk, milk products, infant formula and adult nutritionals -- Determination of fatty acids composition -- Capillary gas chromatographic method
Taurine	<60 mg/100gm	AOAC 997.05	Taurine in Powdered Milk and Powdered Infant Formula
Essential amino acids	>9 mg/L	ISO 13903:2005 (animal feed, no tryptophan), ISO 13904:2016 (animal feed, tryptophan), AOAC 994.12 (feed, no tyrosine, no tryptophan) (AOAC 2017.03 first action - under development)	ISO 13903:2005 - Animal feeding stuffs -- Determination of amino acids content; ISO 13904:2016 - Animal feeding stuffs -- Determination of tryptophan content; AOAC 994.12 - Amino Acids in Feeds

Protein	min 15%	ISO 8968-1:2014 (Alternative to AOAC 991.20 for milk; AOAC 979.09 for grains and AOAC 976.05 for animal feed and pet food)	Milk and milk products -- Determination of nitrogen content -- Part 1: Kjeldahl principle and crude protein calculation
Protein Efficiency ratio	2 when Protein is min 15% 1.75 when protein is 20%	AOAC 960.48 (Alternative to AOAC 982.30 which is a calculation method)	Protein Efficiency Ratio
Moisture	Max 8.0 %	ISO 5537:2004	Dried milk - Determination of moisture content (this is for milk powder, and generally used for solid products by oven. It should be noted that for other matrices for example roasted coffee other methods are available: ISO 11817-1994: Roasted ground coffee – Determination of moisture content – Karl Fischer method
Fat	Min 7.5 %	ISO 8381:2008 (Alternative to ISO 8262)	Milk-based infant foods -- Determination of fat content -- Gravimetric method
Total Ash	Max 7.5 %	AOAC 942.05	Ash of Animal Feed
Antioxidants			
Mixed Tocopherols Concentrate	300 mg/kg fat or oil basis, singly or in combination	EN-12822:2014	Foodstuffs - Determination of vitamin E by high performance liquid chromatography - Measurement of α-, ß-, y- and δ- tocopherol

Alpha Tocopherol	300 mg/kg fat or oil basis, singly or in combination	EN-12822:2014	Foodstuffs - Determination of vitamin E by high performance liquid chromatography - Measurement of α-, ß-, y- and δ- tocopherol
L-Ascorbic acid	50 mg	ISO 20635:2018 (Vitamin C method)	Infant formula and adult nutritionals -- Determination of vitamin C by (ultra) high performance liquid chromatography with ultraviolet detection ((U)HPLC-UV)
Sodium Ascorbate	50 mg	ISO 20635:2018 (Vitamin C method results expressed as sodium ascorbate instead of ascorbic acid)	Infant formula and adult nutritionals -- Determination of vitamin C by (ultra) high performance liquid chromatography with ultraviolet detection ((U)HPLC-UV)
Potassium ascorbate	50 mg	ISO 20635:2018 (Vitamin C method results expressed as potassium ascorbate instead of ascorbic acid)	Infant formula and adult nutritionals -- Determination of vitamin C by (ultra) high performance liquid chromatography with ultraviolet detection ((U)HPLC-UV)
Calcium ascorbate	20 mg	ISO 20635:2018 (Vitamin C method results expressed as calcium ascorbate instead of ascorbic acid)	Infant formula and adult nutritionals -- Determination of vitamin C by (ultra) high performance liquid chromatography with ultraviolet detection ((U)HPLC-UV)