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BUREAU OF INDIAN STANDARDS

DRAFT FOR COMMENTS ONLY

खाद्य पदार्थों में विटामिनबी 12 का निर्धारण एलसी-एमएस/एमएस के उपयोग द्वारा - परीक्षण की पद्धति

Draft Indian Standard

DETERMINATION OF VITAMIN B12 IN FOODS USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) – METHOD OF TEST

ICS No 67.050

Test Methods for Food Products, Sectional Committee, FAD 28	Last date of comments 22/02/2025

FOREWORD

(Foreword shall be updated later)

Vitamin B12, or cobalamin, is a water-soluble Vitamin crucial for red blood cell formation, DNA synthesis, and neurological function. VitaminB12 exists in different chemical forms, i.e. adenosylcobalamin, cyanocobalamin, hydroxocobalamin and methylcobalamin. Vitamin B12 is present in foods of animal origin, including fish, meat, poultry, eggs, and dairy products. Plant foods do not naturally contain Vitamin B12. However, fortified breakfast cereals and fortified nutritional yeasts are readily available rich sources of Vitamin B12.

A collaborative Research & Development (R&D) project with an objective to validate test methods of Vitamins in identified food matrices was assigned to CSIR - Central Food Technological Research Institute (CFTRI), Mysuru along with five participating laboratories. This method is an outcome of the R&D project and the single lab validation of the method has been performed by CSIR-CFTRI, for determination of Vitamin B12 in fortified atta (flour) and almonds. The performance characteristics obtained for these matrices have been given in Annex A. The HORRATr values are within recommended value of less than 2.

In reporting the result of a test or analysis made in accordance with this standard, is to be rounded off, it shall be done in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (second revision)’.

1 SCOPE

This document specifies the method for the quantitative determination of Vitamin B12 forms, specifically cyanocobalamin and methylcobalamin, in food matrices such as fortified atta (flour), almonds and similar matrices, using the UHPLC-MS/MS technique. Its application to other food matrices requires appropriate laboratory verification or validation, as applicable.

2 REFERENCES

The standards given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards:

IS No.	*Title*
IS 1070 : 2023	Reagent Grade Water Specification (*fourth revision*)

3 PRINCIPLE

Vitamin B12 is extracted from the sample using sodium acetate buffer (*p*H 4.5) at 105°C. Extracts are purified and concentrated with C18 solid-phase extraction (SPE) cartridges and analyzed with mass spectrometry. Determination of B12 is made by liquid chromatography with multiple reaction monitoring of mass spectrometer.

4 REAGENTS AND MATERIALS

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

4.1 Taka- diastase from *Aspergillus oryzae*, *(~100U/mg), powder, slightly beige,*, EC Number: 232-588-1.

4.2 Pepsin A, Pepsin from hog stomach, powder, ≥250 units/mg solid.

4.3Sodium Acetate Anhydrous or Sodium Acetate Trihydrate, ACS Grade.

4.4 Reagent Grade water, Grade 1 as per IS 1070.

4.5 Methanol, LC-MS Grade.

4.6 Ammonium Formate, LC-MS Grade.

4.7Ascorbic Acid, CAS Registry Number® (CAS RN®):50-81-7, 99 % Purity.

4.8 Vitamin B12 (Cyanocobalamin). CAS Registry Number® (CAS RN®):68-19-9, 99.8 % or HPLC grade.

4.9 Vitamin B12 (Hydroxycobalamin), CAS Registry Number® (CAS RN®):22465-48-1, 99.8 % or HPLC grade.

4.10 Vitamin B12 (Methylcobalamin),CAS Registry Number® (CAS RN®):13422-55-4, purity 99.8% or HPLC grade.

4.11 Filter paper, Whatman 2V or equivalent.

NOTE — CAS Registry Number® is a trademark of CAS Corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by BIS of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5 APPARATUS

5.1 Ultra-HPLC (UHPLC) system, consisting of a binary pump system, a sample injector unit, a degasser unit, and a column oven.

5.2 Triple Quadrupole mass spectrometer or equivalent tandem MS (MS/MS instrument)

5.3 Column, C18 1.7 µm, 2.1*100 mm.

5.4 Oven, Capable of maintaining temperatures of 95 ± 5°C and 105 ± 5°C.

5.5 *p*H meter, with calibration buffer.

5.6 Incubator, Capable of maintaining temperatures 37 °C ± 1°C.

5.7 Analytical balance, Capable of weighing 0.00001 g.

5.8 SPE Cartridges, Solid phase extraction cartridges -C18 chemistry.

5.9 Beakers, Glass, assorted sizes.

5.10 Funnels, Plastic, suitable to use with filter paper.

5.11 Disposable syringes, 3 mL.

5.12 Syringe Filters, PTFE, 0.2 µm, 13 mm.

5.13 Centrifuge tubes, Polypropylene, 15 mL.

5.14 Glass Reagent bottles, 1l and 500 mL.

5.15 Pipettes, Variable Volume, 100-1000µl.

5.16 Cryogenic vials, 2mL

5.17 Vacuum manifold, 24 ports with stopcocks or equivalent.

5.18 Volumetric flasks, Assorted sizes.

5.19 HPLC vials, septa and caps

6 PROCEDURE

6.1 STANDARDS AND SOLUTIONS PREPARATION

6.1.1 Mobile Phases and Prepared Solutions

6.1.1.1 *Mobile phase A, (formic acid; 0.1%, v/v)*
To 500 mL water, add 0.5 mL formic acid.

6.1.1.2 *Mobile phase B, (methanol; 100%, v/v);* 500 mL methanol; Expiry is 1 month.

6.1.1.3 *0.1 M sodium acetate buffer*

Dissolve 16.4 g sodium acetate anhydrous or 27.2 g sodium acetate trihydrate in approximately 1800 mL laboratory water. Adjust pH to 4.50 with concentrated acetic acid. Dilute to 2000 mL with laboratory water. Expiration 3 months.

6.1.1.4 *6% Taka-diastase*

Dissolve 0.6 g taka-diastase in 10 mL water. Prepare fresh immediately before use.

6.1.1.5 *Pepsin(1mg/mL)*

Dissolve 10 mg in 10 mL water. Prepare fresh immediately before use.

6.1.2 Standard Stock Solutions

6.1.2.1 *Vitamin B12 stock standard (1 mg/1mL)*

Accurately weigh 10 mg of each B12 standard and transfer it into a 10 mL amber colored volumetric flask. Add 300µl of 0.1 N NH4OH to dissolve it & make up the rest of the volume with reagent grade water and vortex for 2 minutes. Store the solution at 4°C in a light protected area.

6.1.2.2 *Intermediate Stock Solution 1-ISS 1 (0.1 mg/1mL)*

Pipette out 1.0 mL of stock solution to a 10 mL amber colored volumetric flask containing 2 mL of Reagent grade water. Make up the rest of the volume with diluent (25% Methanol) and vortex the solution for 2 minutes.

6.1.2.3 *Intermediate Stock Solution 2-ISS 2 (0.01 mg/ mL)*

Pipette out 1.0 mL of ISS 1 to a 10 mL amber colored volumetric flask containing 2 mL of reagent grade water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

6.1.2.4 *Intermediate Stock Solution 3-ISS 3 (1 ug /mL)*

Pipette out 1.0 mL of ISS 2 to a 10 mL amber colored volumetric flask containing 2mL of reagent grade water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

6.1.2.5 *Intermediate Stock Solution 4-ISS 4 (0.1 ug /mL)*

Pipette out 1.0 mL of ISS 3 to a 10 mL amber colored volumetric flask containing 2 mL of reagent grade water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes. Using this intermediate stock solution prepare a calibration standard ranging from 0.5 ng/mL to 20 ng/ mL.

6.1.2.6 *Bracketing standard solution/ Standard stock solution 4*

Pipette out 0.5 mL of ISS 4 to a 10 mL amber colored volumetric flask containing 2 mL of reagent grade water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes to obtain concentration of 5 ng/mL.

6.2 Sampling

Mix all products thoroughly before sampling. Weigh 3 g of sample. Add 25 mL reagent grade water and mix until all of the powder dissolves. Add 1 mL of 6% taka-diastase if samples contain significant levels of starch. Allow taka-diastase to react with samples for at least 30 minutes before continuing with the extraction.

6.3 Extraction

Add 30 mL 0.1 M sodium acetate buffer with 100mg ascorbic acid (*p*H 4.5), adjust PH with acetic acid to each sample and swirl to mix and add additionally 1 mL pepsin in case of almond (1mg/mL) (pH 1.8-2.0). Heat samples in a 105 °C oven for at least 60 min, but for no more than 120 min. (Oven temperature will drop when the door is opened. Start timing when oven temperature returns to 105 °C.). After at least 60 min, remove samples from oven and immediately cool in ice bath.Make up the volume to 100 mL with Reagent grade water. Mix well. Filter samples through Whatman 2V filter paper and transfer liquid layer to funnels lined with filter paper.

6.4 Sample Concentration

For each sample that will be cleaned up and concentrated, insert a 500 mg SPE cartridge onto the stopcock of the vacuum Condition each cartridge with at least 20 mL Methanol and rinse each cartridge with at least 10 mL laboratory water. Add 80 mL sample. If necessary, apply enough vacuum so that the samples drip steadily through the cartridges. Discard eluent. After all of the sample filtrate has passed through the cartridge, rinse each cartridge with 10 mL laboratory water and discard eluent. Air-dry each cartridge by pulling a vacuum until no more effluent is observed. Close each stopcock. Collect 10 mL aliquot Filter an aliquot of each

standard and prepared sample through a 0.22 µm syringe filter into an autosampler vials.

6.5 UHPLC-MS/MS ANALYSIS

6.5.1 UHPLC Conditions

The UHPLC conditions are as follows:

a) Source, ESI +ve.
b) Injection Volume, 5 µL- 20 µL.
c) Flow Rate, 0.2 mL.
d) Desolvation Temperature, 200 °C.
e) Column Temperature, 40 °C.
f) Mobile phase A,0.1% (v/v) formic acid in water.
g) Mobile phase B, Methanol.

The gradient program for the column is given in Table 1.

6.5.1.1 *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.

6.5.1.2 Analysis make single injections of standard and test solutions. Measure chromatographic peak response (height or area).

6.5.1.3 Calibration Plot peak responses against concentration (in ng/ mL). Perform regression analysis. Calculate plot and intercept.

NOTE— The chromatographic conditions given here may be slightly modified depending on the system used.

Table 1 Gradient program
(Clause 6.5.1)

Time	Flow (mL/min)	% A	%B
0.00	0.25	50	50
2.00	0.25	20	80
4.00	0.25	10	90
5.00	0.25	20	80
6.00	0.25	50	50
9.00	0.25	50	50

6.5.5 *Mass spectrometer conditions*
Mass spectrometry to be performed in ESI positive mode operating at unit resolution. ESI capillary voltage was set at 2.8 kV; Cone voltage; 40V; Desolvation temperature, 400°C; Desolvation gas flow, 800 L/HR; nebulizer pressure,7.0 psi. Multiple-reaction monitoring mode was applied for quantification and compound identification confirmation.

6.5.6 *Mass transitions*

Mass transitions for each vitamin and its corresponding internal standard are given in Table 2. Like the tune parameters, these parameters may need to be adjusted based upon instrument model.

Table 2 MRM transitions
(*Clause 6.5.6*)

		Precursor ion, (m/z)	**Product ion, (m/z)**	**Cone (V)**	**Collision (eV)**
Methylcobalamin	Analyte quantifier	673.1000	147.13 359.14 665.53	10 10 10	38 18 18
Cynacobalamin	Analyte quantifier	678.8000	147.100 359.200	20 30	40 25
Hydroxycobalamin	Analyte quantifier	664.7000	147.300 359.200	6 2	55 24

NOTE—Collision energy and fragment voltage has to be tuned according to the make and model of mass spectrometer. This is an example of a suitable product available commercially.

6.5.7 *Limit of Quantification (LOQ)*

Limit of Quantification of the method is 0.5 µg/kg. With each batch one spike sample at LOQ level shall be run to verify the performance of the method.

7 CALCULATIONS

Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly. Calculate the ion ratio of the response. The relative or ratios of selective ions, expressed as a ratio relative to the most intense ion that are used for identification, should match with the reference ion ratio. The reference ion ratio is the average obtained from solvent standards measured in the same sequence and under the same conditions as the samples.

$$VitaminB12\left(\frac{ug}{Kg}\right) = (Ci * D1 * V)/(ss * D2)$$

Where

Ci = vitamin B12 concentration of the injected sample preparation extrapolated from standard curve in µg/L;

D1 = volume of the first dilution in mL (100 mL);

ss = sample size in g;

D2 = volume of the second (final) dilution in mL (10 mL);

V = volume of filtrate loaded onto the cartridge in mL (80mL).

Annex A
(Foreword)

PERFORMANCE CHARACTERISTICS OBTAINED DURING SINGLE LAB VALIDATION

Table 3 Performance Characteristics of Single Laboratory Study

	FortifiedAttaSpike 0.5mcg/kg	**FortifiedattaSpike 5mcg/kg**	**Almonds**
Mean	0.485	4.230	0.186
SDr (Repeatibility Standard Deviation)	0.027	0.092	0.006
Repeatability RSDr%(Observed)	6.277	2.172	3.446
Predicted RSDr%(Horwitz)	11.986	8.608	13.597
$HORRAT_r$	**0.52**	**0.252**	**0.253**
SD_R (Reproducibility Standard Deviation)	0.061	0.372	0.008
Reproducibility RSD_R%(Observed)	12.50	8.80	4.42
Predicted RSD_R%(Horwitz)	17.84	12.88	20.60
$HORRAT_R$	**0.70**	**0.68**	**0.21**
Remark	$HORRAT_r$ and $HORRAT_R$ within Recommended value of less than 2		