# भारतीय मानक

***Indian Standard***

***Determination Of Vitamin B12 In Foods***

***Using LC-MS/MS***

 BIS 2024

भारतीय मानक ब्यरू ो

BUREAU OF INDIAN STANDARDS

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Test Methods for Food Products Sectional Committee, FAD 28

**FOREWORD**

This Indian Standard (First Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Test Methods for Food Products Sectional Committee had been approved by the Agricultural and Food Products Division Council.

Vitamin B12, or cobalamin, is a water-soluble vitamin crucial for red blood cell formation, DNA synthesis, and neurological function. It exists in three primary forms: methylcobalamin, adenosylcobalamin, and hydroxocobalamin. Methylcobalamin is involved in homocysteine metabolism and neurotransmitter function, adenosylcobalamin is key for fatty acid metabolism and energy production in mitochondria, and hydroxocobalamin is used in detoxification processes.

This standard was originally published in 1976. While bringing out first revision of this standard, new auxiliary food products have been added which may be used for palate clearing. The standard has been brought out in the latest style and format of Indian Standard, and references to Indian Standards, wherever applicable, have been updated.

The composition of the Committee responsible for the formulation of this standard is given in Annex A.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated expressing the result of a test or analysis, shall be rounded off in accordance with IS 2: 2022 ‘Rules for rounding off numerical values (*second revision*)’. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

**DETERMINATION OF VITAMIN 12**

**IN FOODS USING LC-MS/MS**

## **SCOPE**

This document specifies a method for the quantitative determination of Vitamin B12 forms that is cyanocobalmine and methylcyanocobalmine in foods including fortified atta and almonds

1. **REFERENCES**

There are no normative references in this document.

1. **TERMINOLOGY**

For the purpose of this standard, the definitions given shall apply.

1. **PRINCIPLE**

Vitamin B12 is extracted from the sample using sodium acetate buffer (pH 4.5) at 105°C. Extracts are purified and concentrated with C8 or 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.

## **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.

* 1. **Taka- diastase,** Sigma 86247
  2. **Sodium Acetate Anhydrous or Sodium Acetate Trihydrate,** ACS
  3. **Milli Q water**
  4. **Methanol,** LC/MS Grade
  5. **Ascorbic Acid,** Sigma A92902
  6. **Pepsin**, Sigma P7000
  7. **Ammonium Formate,** LC/MS Grade
  8. **Vitamin B12 (Cyanocobalamine),** CRM PHR1234
  9. **Vitamin B12 (Hydroxycobalamine),** CRM PHR3186
  10. **Vitamin B12 (Methylcobalamine**), CRM PHR3410

1. **STANDARD AND SOLUTION PREPARATION**

### Mobile Phases and Prepared Solutions

* + 1. **Mobile phase A**, **(formic acid; 0.1%, v/v),** To 500 mL water, add 0.5 mL formic acid.
    2. **Mobile phase B**, Methanol 100%
    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.
    4. **6% Taka-diastase,** Dissolve 0.6 g taka-diastase in 10 mL water. Prepare fresh immediately before use.
    5. **Pepsin,** 1mg/ml

### Stable Isotope Labelled Compounds, Individual, Internal Standard Stock Solutions

* + 1. **Vitamin B12 stock standard (10000 ppm),**

Accurately weigh 10 mg of the 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 Milli Q water and vortex for 2 minutes. Store the solution at 4°C in a light protected area.

* + 1. **Intermediate Stock Solution 1-ISS 1 (100 ppm)**

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

* + 1. **Intermediate Stock Solution 2-ISS 2 (10ppm)**

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

* + 1. **Intermediate Stock Solution 3-ISS 3 (1 ppm)**

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

* + 1. **Intermediate Stock Solution 4-ISS 3 (100 ppb)**

Pipette out 1.0 ml of ISS 3 to a 10 ml amber colored volumetric flask containing 2 ml of Milli Q water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

* + 1. **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

Milli Q water. Make up the rest of the volume with diluent and vortex the solution for 2 minutes.

## **APPARATUS**

* 1. **Instrument**, UHPLC with Triple Quadrupole mass spectrometer
  2. **Column,** C18 1.7 µm, 2.1\*150 mm or 100 mm
  3. **Oven**, Capable of maintaining temperatures of 95 ± 5°C and 105 ± 5°C.
  4. **pH meter,** With calibration buffer.
  5. **Analytical balance,** Capable of weighing 0.00001 g.
  6. **Beakers,** Glass, assorted sizes.
  7. **Filter paper,** Whatman 2V or equivalent.
  8. **Funnels,** Plastic, suitable to use with fi lter paper.
  9. **Disposable syringes,** 3ml
  10. **Syringe Filters,** PTFE, O.2 µm, 13 mm
  11. **Eppendorf vials,** 2 ml
  12. **Pipets,** Variable Volume, 100-1000µl
  13. **Cryogenic vials,** 2ml
  14. **Vacuum manifold,** 24 ports with stopcocks or equivalent.
  15. **Volumetric flasks,** Assorted sizes.
  16. **LC vials**, septa and caps

This is an example of a suitable product available commercially. 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

## **PROCEDURE**

### Sample Preparation

#### Sampling

Mix all products thoroughly before sampling. Weigh 3 g of sample. Add 25 mL Milli Q 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.

### Extraction

Add 30 mL 0.1 M sodium acetate buffer with 100mg ascorbic acid (pH 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) 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 Milli Q. Mix well.Filter samples through Whatman 2V filter paper and transfer liquid layer to funnels lined with filter paper

### 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 eluant. After all of the sample filtrate has passed through the cartridge, rinse each cartridge with 10 mL laboratory water and discard eluant. 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 vial.

### UHPLC-MS/MS analysis

#### UHPLC Conditions

#### 8.4.1.1 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.

#### 8.4.1.2 Analysis Make single injections of standard and test solutions. Measure chromatographic peak response (height or area).

#### Identification Identify vitamin B12 peak in the chromatograms of the test solution by comparison with the retention time and Multiple reaction monitoring transitions of the corresponding peak obtained for the standard solution.

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

#### Table 1: Summary of gradient program

|  |  |  |  |
| --- | --- | --- | --- |
| TIME | FLOW (ML/Min) | % A | %B |
| 0.00 | 0.2 | 90 | 10 |
| 2.00 | 0.2 | 90 | 10 |
| 4.00 | 0.2 | 10 | 90 |
| 5.00 | 0.2 | 90 | 10 |
| 7.00 | 0.2 | 90 | 10 |

* + 1. **Mass transitions** Mass transitions for each vitamin and its corresponding internal standard are given in Table 2. Retention time windows are also given in the table. Like the tune parameters, these parameters may need adjusted based upon instrument model.

**Table 2: MS/MS transitions for B12**

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

**Limit of Quantification: 0.5 μg/kg**

* + 1. **Mass spectrometer conditions** Mass spectrometry was performed on an Waters XEVO TQ-XS MS in ESI+ 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.

**Table 3: Instrument Conditions**

|  |  |
| --- | --- |
| Instrument | WATERS XEVO TQ-XS |
| Detector | Mass Detector |
| Column | C18 1.7µm, 2.1\*100mm |
| Run time | 7 minutes |
| Column Temperature | 40 °C |
| Flow Rate | 0.2 ml |
| Injection Volume | 5 µL- 20 µl |
| Mobile phase A | 0.1% (v/v) formic acid in water |
| Mobile phase B | Methanol |
| Desolvation Temperature | 200 °C |
| CE | 20 eV |
| CV | 40V |
| Source | ESI +ve |

Note that Collison 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. 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.

## **CALCULATIONS**

Cp= Ci × D1 ÷ SS × D2 ÷ V

where Cp = product concentration in µg/kg; 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)

# AnnexA

(Informative)

Inter Laboratory Data

# Fortified Wheat Atta

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Method Performance Criteria for Cyanocobalamin in Fortified Wheat flour atta** | | | | | | | |
| Element | Cyanocobalamin | |  |  | 0.66\*22 IF Conc. <0.12 ppm,  0.66x2xpower(C,-0.1505) if Conc. >0.12ppm | | |
| Spike Conc. | 0.5 mcg/kg |  |  |  |
|  | All values in mcg/kg | |  |  |
| **Day - Rep.** | **Result** | **Mean** | **SD** | **Repeatability RSD% (Observed)** | **Predicted RSDr% (Horwitz)** | **HORRATr** | **Remarks HORRATr<2** |
| Day 1 - Rep 1 | 0.55 | 0.540 | 0.011 | **2.078** | **11.583** | **0.179** | Within Recommended value |
| Day 1 - Rep 2 | 0.55 |
| Day 1 - Rep 3 | 0.53 |
| Day 1 - Rep 4 | 0.53 |
| Day 1 - Rep 5 | 0.55 |
| Day 1 - Rep 6 | 0.53 |
| Day 2 - Rep 1 | 0.47 | 0.430 | 0.027 | **6.277** | **11.986** | **0.524** | Within Recommended value |
| Day 2 - Rep 2 | 0.44 |
| Day 2 - Rep 3 | 0.44 |
| Day 2 - Rep 4 | 0.40 |
| Day 2 - Rep 5 | 0.40 |
| Day 2 - Rep 6 | 0.43 |
| Mean | **0.485** |  |  |  |  |  |  |
| SD | 0.061 |  |  |  |  |  |  |
| Reproducibility RSDR % (Observed) | **12.50** |  |  |  |  |  |  |
| Predicted RSDR% (Horwitz) | **17.84** | 22% If Avg. Conc. <0.12 ppm,  2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm | | | | | |
| HORRATR | **0.70** |  |  |  |  |  |  |
| **Remarks** | Within Recommended value |  |  |  |  |  |  |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Method Performance Criteria for Cyanocobalamin in Wheat flour atta** | | | | | | | |
| Element | Cyanocobalamin | |  |  | 0.66\*22 IF Conc. <0.12 ppm,  0.66x2xpower(C,-0.1505) if Conc. >0.12ppm | | |
| Spike Conc. | 5 mcg/kg | |  |  |
|  | All values in mcg/kg | | |  |
| **Day - Rep.** | **Result** | **Mean** | **SD** | **Repeatability RSD% (Observed)** | **Predicted RSDr% (Horwitz)** | **HORRATr** | **Remarks HORRATr<2** |
| Day 1 - Rep 1 | 4.740 | 4.577 | 0.092 | **2.019** | **8.398** | **0.240** | Within Recommended value |
| Day 1 - Rep 2 | 4.604 |
| Day 1 - Rep 3 | 4.600 |
| Day 1 - Rep 4 | 4.510 |
| Day 1 - Rep 5 | 4.510 |
| Day 1 - Rep 6 | 4.500 |
| Day 2 - Rep 1 | 4.040 | 3.883 | 0.084 | **2.172** | **8.608** | **0.252** | Within Recommended value |
| Day 2 - Rep 2 | 3.818 |
| Day 2 - Rep 3 | 3.810 |
| Day 2 - Rep 4 | 3.850 |
| Day 2 - Rep 5 | 3.900 |
| Day 2 - Rep 6 | 3.880 |
| Mean | **4.230** |  |  |  |  |  |  |
| SD | 0.372 |  |  |  |  |  |  |
| Reproducibility RSDR % (Observed) | **8.80** |  |  |  |  |  |  |
| Predicted RSDR% (Horwitz) | **12.88** | 22% If Avg. Conc. <0.12 ppm,  2xpower(Avg.Conc./1000000,-0.1505) if Avg. Conc. >0.12ppm | | | | | |
| HORRATR | **0.68** |  |  |  |  |  |  |
| **Remarks** | Within Recommended value |  |  |  |  |  |  |

**Bibliography**

**IS 17669 : 2021**

**ISO 21470 : 2020**

**17**

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