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**BUREAU OF INDIAN STANDARDS**

**DRAFT FOR COMMENTS ONLY**



*भारतीय मानक मसौदा*

# एचपीएलसी-यूवी विधि का उपयोग करके खाद्य पदार्थों में विटामिन बी$_{12}$ का निर्धारण - परीक्षण की पद्धति

*Draft Indian Standard*

# Determination of Vitamin B12 in Foodstuffs by HPLC- UV – Method of Test

(*First Revision of IS 7529*)

**ICS No 67.050**

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

## FOREWORD

*(Formal clause shall be updated later)*

Vitamin B12, or cobalamin, is a water-soluble Vitamin crucial for red blood cell formation, DNA synthesis, and neurological function. Vitamin B12 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.

This standard was first published in 1975 with a title "*Method for estimation of vitamin B 12 in foodstuffs*" in which microbiological method was described for estimation of VitaminB12. The microbiological method is old and there is a lot of variability in this method so a need was felt to revise the standard. In this revision, the microbiological method has been replaced by the method based on AOAC Official Method 2014.02 "*Vitamin B12 (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula Liquid Chromatography-Ultraviolet Detection*". Accordingly, the title of the standard has also been updated.

For the revision of the standard, 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. A single lab validation of the method described in AOAC 2014.02 has been performed by Nestle India Limited Laboratory Services, Moga, for determination of Vitamin B12 in infant formulas, adult/pediatric formulas, infant and breakfast cereals, pulses, fruits and vegetables, fortified rice kernels, spirulina, beverages, Vitamin premixes, nuts and nut products. 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 a method for the quantitative determination of Vitamin B12 in the form of cyanocobalamin in food matrices, namely, infant formulas; adult/pediatric formulas; infant and breakfast cereals; pulses; fruits and vegetables; fortified rice kernels; spirulina; beverages; Vitamin premixes; nuts and nut products.

## 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 (f*ourth revision*) |

## 3 PRINCIPLE

The Vitamin B12 is extracted from the sample in a sodium acetate buffer (pH 4) containing cyanide at 100°C for 30 min. The extracts are purified and concentrated with an immunoaffinity column. Vitamin B12 is determined as cyanocobalamin by high-performance liquid chromatography (HPLC) with Ultra-violet (UV) detection at 361 nm. The separation takes place on a C18 column using an acetonitrile gradient in water.

## 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 Vitamin B12 (cyanocobalamin),** CAS Registry Number® (CAS RN®): 68-19-9; Purity >99%; Certified Reference Material should be used.

**4.2 Trifluoroacetic Acid (TFA),** CAS Registry Number® (CAS RN®): 76-05-1, suitable for HPLC, Purity ≥99.0%.

**4.3 Sodium Acetate Trihydrate,** CAS Registry Number® (CAS RN®): 6131-90-4 , ACS reagent, Purity ≥99.0%.

**4.4 Cyanide Standard Solution,** 1000 mg/l CN.

NOTE — For single lab validation, Cyanide solution (100mg/L) from Supelco with Product No 1.19533 was used. Equivalent product can be chosen.

**4.5 Methanol,** HPLC grade.

**4.6 Acetonitrile,** HPLC grade.

**4.7 Glacial Acetic Acid,** CAS Registry Number® (CAS RN®): 64-19-7; ≥99.5% glacial extrapure.

**4.8 Reagent Grade Water,** Grade 1 as per IS 1070.

**4.9 α-Amylase from *Bacillus subtilis*,** CAS Registry Number® (CAS RN®): 9000-90-2, ~50 U/mg.

**4.10 Taka diastase from *Aspergillus Oryzae***, 100 U/mg; Check the absence of Vitamin B12 in the amylase or Taka diastase by analyzing 1 g of enzyme. This should be done for each new batch of enzyme.

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 HPLC column,** C18, 150 × 3.0 mm , Particle Size 3µm

**5.2 Immunoaffinity columns,** Column having 10 ml size and capacity of 1 µg.
Ensure the column has not dried out and contains buffer over gel. It is important to note that the antibody included in immunoaffinity column can be denatured by extreme temperature and change in *p*H.

NOTE — Immunoaffinity columns used in Single lab validation were EASI-EXTRACT® VITAMIN B12 LGE (R-Biopharm AG; Product Code P88) & Immunoaffinity column rack was from R-Biopharm AG, Product Code CR1. Equivalent products may be chosen.

**5.3 Immunoaffinity column rack**

**5.4 Chromatographic system,** HPLC system equipped with a quaternary or binary pump, sample injector, UV VIS detector (or optionally a PDA/DAD detector), degassing system, and data software.

**5.5 Balances,** with readability of 0.1 mg and 0.01 g.

**5.6 Sonicator**

**5.7 Water bath,** having magnetic stirrers.

**5.8 pH meter**

**5.9 Rotary shaker**

**5.10 Heating block,** with nitrogen evaporation.

**5.11 Vortex**

**5.12 Homogenizer**

**5.13 Volumetric flasks,** Amber glass, 10, 50, 100, 200, 250 mL; clear glass, 2000 mL.

**5.14 Graduated cylinders,** 50, 100, and 1000 mL.

**5.15 Beakers,** Amber glass, 250 mL.

**5.16 Flat-bottom round flasks or Erlenmeyers,** Amber glass, 250 mL.

**5.17 Folded paper filters,** 602H 1/2 or 597 1/2 or equivalent.

**5.18 Amber vials,** Screw top, 7 or 4 mL.

**5.19 Micro LC vials,** Amber.

**5.20 Pipets,** Graduated glass - 10 mL or volumetric glass - 9 mL.

**5.21 Electronic digital pipet**, Variable volume, 200–1000 µL.

**5.22 Syringes**, Disposable, 20 mL, equipped with a perforated rubber stopper attached to the tip.

## 6 PROCEDURE

### 6.1 Standard and Solution Preparation

**6.1.1** *Mobile phases and prepared solutions*

**6.1.1.1** *Mobile phase A*
To 1000 mL water, add 250 µL TFA. Mix well.

**6.1.1.2** *Mobile phase B*
To 1000 mL acetonitrile, add 250 µL TFA. Mix well.

**6.1.1.3** *Sample dilution solvent,* Mix 90 mL mobile phase A with 10 mL mobile phase B.

**6.1.1.4** *Sodium acetate solution, 0.4 M and pH 4.0*
To prepare this solution weigh 108.8 g sodium acetate trihydrate (**4.3**) into a 2000 mL volumetric flask. Add about 1800 mL water and dissolve. Now, add 50 mL acetic acid (**4.7**) and adjust pH to 4.0 with acetic acid. Dilute to volume with water.

**6.1.2** *Preparation of standard solutions*

**6.1.2.1** *Vitamin B12 stock standard solution (100 µg/mL)*

Accurately weigh 20.0 mg cyanocobalamin (**4.1**) into a 200 mL amber glass volumetric flask. Add about 150 mL water. Dissolve by sonication (**5.6**) and stirring for a few minutes. Dilute to volume with water. This solution is stable for ≥6 months at –20°C.

NOTE — Please consider purity of Standard for calculating standard concentration. Vitamin B12 is sensitive to light. Conduct operations under subdued light or use amber glassware. Keep all solutions away from direct light.

**6.1.2.2** *Vitamin B12 intermediate standard solution (400 ng/mL***)**

Pipette 1 mL Vitamin B12 stock standard solution (**6.1.2.1)** into a 250 mL amber glass volumetric flask. Make up to volume with water.

**6.1.2.3** *Vitamin B12 working standard solutions for calibration (2,10, 20, 40, 60, 80 ng/mL)*

Pipet into six separated 10 mL amber glass volumetric flasks 50, 250, 500, 1000, 1500, and 2000 µL Vitamin B12 intermediate standard solution (**6.1.2.2**.) Dilute to volume with sample dilution solvent (**6.1.1.3**).

## 6.2 Sample Preparation

**6.2.1** *Sample reconstitution for powder samples (Infant formula, Adult Nutrition Formula, Cereals, Fortified Rice Kernels, spirulina, Nuts & Nut products)*

Weigh 25.0 g ($W_1$) of sample into a 250 mL beaker. Add 200 g ($W_2$) water at 40 ± 5°C. Mix with a glass rod until suspension is homogeneous or homogenize with a homogenizer (**5.12**). Proceed as described in **6.3**.
Wherever content of Vitamin B12 is very high (e.g. Spirulina), lower the sample weight.

**6.2.2** *Sample preparation for liquid samples (e.g. liquid milk, fruit juices, Ready to drink beverages etc)*

Mix well to ensure homogeneity of the sample portion and take sample weight as 60 g and proceed as described in **6.3**.

**6.2.3** *Sample preparation for Vitamin Premixes*

Into a 100 mL amber glass volumetric flask, weigh to the nearest 0.1 mg, 1 g of Vitamin premix. To it add 50 mL of water at 40 °C ± 5 °C and mix well. Sonicate for 5 minutes. Then, make up to volume with water. Filter through a folded paper filter. Pipette an aliquot of the filtrate into a 100-mL amber volumetric flask. Add 50 mL sodium acetate buffer *p*H (**6.1.1.4**). Make up to volume with water. Final concentration of Vitamin B12 should be between 2-80 ng/mL, otherwise dilute appropriately with water. Proceed directly as described under section **6.4**.

## 6.3 Extraction

Weigh 60.0 g (m) sample suspension, blank or liquid sample into a 250 mL flat-bottom amber glass flask or Erlenmeyer with ground glass neck. Add 10 mL of 1000 mg/L Cyanide solution. If the sample contains starch, add about 0.05 g α-amylase or Taka diastase and mix thoroughly. For matrices containing high starch, content of Taka diastase can be increased upto 1 gm. Stopper the flask and incubate 15 min at 40 ± 5°C. Add 25 mL sodium acetate solution (**6.1.1.4**), Mix well. Place flask in a boiling water bath for 30 min. Cool flask in ice bath or let stand at room temperature. Quantitatively transfer content of flask to a 100 mL ($V_1$) amber glass volumetric flask. Dilute to volume with water. Filter solution through folded paper filter.

## 6.4 Immunoaffinity cleanup

Let the immunoaffinity columns warm to room temperature by removing them from refrigeration at least 30 min before use. Place each immunoaffinity column on the rack. Open caps and let storage buffer drain by gravity. Close the lower cap. Load column with 9 mL ($V_2$) of clear filtrate and close the upper cap. Place column in a rotary shaker and mix slowly for 10 to15 min. Return column to the

support and let it stand for a few minutes. Open the caps to let liquid drain by gravity. Wash column with 10 mL water. With a syringe, insert about 40 mL air to dry the column. Elute with 3 mL methanol and collect the eluate in a 4 or a 7 mL amber glass reaction vial. Rinse the column with 0.5 mL methanol, and with a syringe, insert about 20 mL air to collect all the methanol in the same vial. Evaporate at 50°C under a stream of nitrogen. Reconstitute sample in 0.3 mL ($V_3$) sample dilution solvent. If content is high, dilution can be prepared to bring the sample concentration within linearity range. Mix on a vortex mixer. Transfer to a micro amber vial.

**6.5 HPLC Chromatography Conditions**

**6.5.1** *Flow rate,* 0.250 mL/min.

**6.5.2** *Injection volume***,** 100 µL.

**6.5.3** *Detection,* UV at 361 nm (alternatively 550 nm can be monitored); gradient elution (*See* **Table 1**).

**6.5.4** *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.

**6.5.5** *Analysis*

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

**6.5.6** *Identification*

Identify Vitamin B12 peak in the chromatograms of the test solution by comparison with the retention time and UV spectrum of the corresponding peak obtained for the standard solution.

**6.5.7** *Calibration*

Plot peak responses against concentrations (in ng/mL). Perform regression analysis. Calculate slope and intercept. Check the linearity of the calibration ($R2 > 0.99$; standard error of calibration < 10 %)**.**

**6.5.8** *Sequence set up*

Inject a working standard after every 6-8 samples to monitor system stability. The coefficient of variation of retention times and peak response should not be higher than 2 %.

**Table 1 Summary of gradient program**
*(Clause 6.5.3)*

| Time, *Min*<br>(1) | % A<br>(2) | %B<br>(3) |
|---|---|---|
| 0.0 | 90 | 10 |
| 0.5 | 90 | 10 |
| 4.0 | 75 | 25 |
| 5.0 | 10 | 90 |
| 9 | 10 | 90 |
| 11 | 90 | 10 |
| 16 | 90 | 10 |

NOTE — Single Lab validation was conducted using Waters Alliance HPLC system. The chromatographic conditions given here may be slightly modified depending on the system used.

### 6.6 Critical points and Precautions

The following precautions should be taken while performing the test:

a) Samples should be properly homogenized.

b) For cereal based samples, add α-amylase or Taka diastase otherwise sample will not be properly filtered. Taka diastase may be preferred over α-amylase. Check the absence of Vitamin B12 in each lot of amylase/Taka diastase by performing a blank.

c) Ensure that water bath should be used at boiling temperature as lower temperatures can lead to under extraction of Vitamin B12 and thus lead to lower results.

d) For Vitamin premixes, sonication and proper mixing is very critical.

e) The TFA causes severe burns and eye damage. Wear protective gloves, clothing, eye wear, and face protection. The TFA should be used only in effective fume removal device to remove vapors generated.

f) Vitamin B12 is sensitive to light; conduct operations under subdued light or use amber glassware. Keep all sample and standard solutions away from direct light.

g) After a series of analyses wash the column for at least 1 hour with 50 % mobile phase A and 50 % mobile phase B. For long-term storage of the column follow supplier's recommendations.

h) The recovery of the immunoaffinity clean-up should be checked for each batch of columns. Run a standard solution containing known amount and proceed to the immunoaffinity clean-up.

## 7 CALCULATIONS

### 7.1 Quantitation (liquid and powder samples)

Calculate the concentration of Vitamin B12 in µg/100 g of product as follows:

$$\frac{(\mathrm{A}-\mathrm{I}) \times (W_1 + W_2) \times V_1 \times V_3 \times 100}{\mathrm{S} \times W_1 \times \mathrm{m} \times V_2 \times 1000}$$

Where

A = response (height or area) of the peak obtained for the sample solution,

I = intercept of the calibration curve,

S = slope of the calibration curve,

$W_1$ = weight of powder sample used for reconstitution (25 g),

$W_2$ = weight of water used for reconstitution (200 g),

m = weight of sample suspension (60 g),

$V_1$ = volume of the test solution (volume used to dissolve the test portion) in mL (100 mL),

$V_2$ = volume of the aliquot of the sample solution loaded onto the affinity column (9 mL)

$V_3$ = volume in which the aliquot of the sample solution is reconstituted after immunoaffinity cleanup (0.3 mL).

| | | |
|---|---|---|
| 100 | = | factor to convert in 100 g basis |
| 1000 | = | factor to convert nanogram to microgram |

**7.2 Vitamin premixes for human nutrition:** Calculate the mass fraction, w, of the Vitamin B12 (cyanocobalamin), in microgram per 100 grams of sample, using the equation as follows:

$$\frac{(A_p - \mathrm{I}) \times V_1 \times V_3 \times D \times 100}{\mathrm{S} \times \mathrm{m} \times V_2 \times 1000}$$

$A_p$ = Peak height of the cyanocobalamin in the sample solution (**6.2**)

I = intercept of the calibration curve

S = slope of the calibration curve

$V_1$ = volume, in mL, in which the test portion has been dissolved (in general 100 mL)

$V_3$ = volume, in mL, in which the final extract has been reconstituted (i.e. 0.3 mL)

D = Dilution factor (if applicable)

m = mass of the test portion, in grams (1 g)

$V_2$ = volume, in mL, of the aliquot loaded onto the immunoaffinity column (9 mL)

100 = factor to convert in 100 g basis

1000 = factor to convert nanogram to microgram

### 7.3 Reporting of results

Report results with two decimal points as cyanocobalamin in µg/100 g of product.

**Annex A**
(*Foreword*)

## PERFORMANCE CHARACTERISTICS OBTAINED DURING SINGLE LAB VALIDATION

**Table 2 Performance Characteristics of Vitamin B12 for New Matrices**

| | **Spirulina** | **Beverages** | **Fortified Rice Kernel** | **Breakfast Cereal** | **Vitamin Premix** |
|---|---|---|---|---|---|
| **Mean** | 132.381 | 5.030 | 13.937 | 0.642 | 319.6 |
| **SDr** | 4.520 | 0.020 | 0.164 | 0.014 | 9.605 |
| **Cvr %** | 3.4% | 0.4% | 1.2% | 2.2% | 3.0% |
| **SDiR** | 5.076 | 0.031 | 0.150 | 0.019 | 8.017 |
| **CViR %** | 4% | 1% | 1% | 3% | 3% |
| **HORRATR** | 0.5 | 0.05 | 0.10 | 0.17 | 0.37 |

**Table 3 Performance Characteristics of Vitamin B12 for spiked samples**

| | **Fortified Rice Kernel Spiked 6 mcg/100 g** | **Nuts Spiked 0.12 mcg/ 100 g** | **Juice Spiked 0.12 mcg/ 100 g** | **Nuts Spiked 0.6 mcg/100 g** | **Juice Spiked 0.6 mcg/100 g** |
|---|---|---|---|---|---|
| Mean | 20.041 | 0.119 | 0.116 | 0.544 | 0.581 |
| SDr | 0.250 | 0.010 | 0.012 | 0.020 | 0.029 |
| Cvr % | 1.2% | 8.3% | 10.0% | 3.7% | 5.1% |
| SDiR | 0.222 | 0.009 | 0.011 | 0.015 | 0.029 |
| CViR % | 1% | 3% | 9% | 3% | 5% |
| **HORRATR** | 0.11 | 0.34 | 0.43 | 0.16 | 0.29 |

## Annex B
*(Informative)*

## EXAMPLE CHROMATOGRAMS FOR 20 ng/ml STANDARD

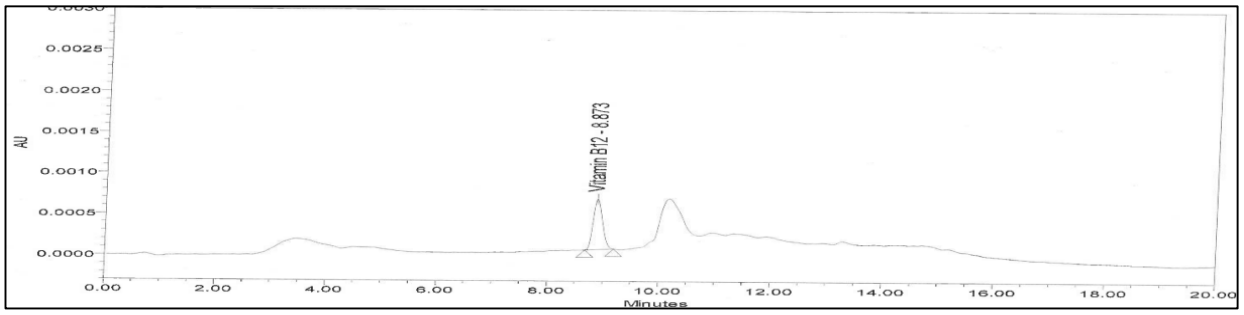


FIG.1 EXAMPLE CHROMATOGRAMS FOR 20 NG/ML STANDARD