BUREAU OF INDIAN STANDARDS

DRAFT FOR COMMENTS ONLY

भारतीय मानक मसौदा

विटामिन बी5 (पैंटोथेनिक एसिड) का निर्धारण अति-उच्च हाई परफ़ॉर्मेंस तरल क्रोमैटोग्राफी और टेंडेम मास स्पेक्ट्रोमेट्री - परीक्षण की पद्धति

Draft Indian Standard

Determination of Vitamin B5 (pantothenic acid) in foodstuffs by ultra-high performance liquid chromatography and tandem mass spectrometry (UHPLCMS/MS) — Method of Test

(*First Revision of IS 9840*)

ICS No 67.050

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

FOREWORD

(Formal clause will be added later)

Pantothenic acid, commonly known as Vitamin B5, is an essential water-soluble vitamin trace nutrient that functions as the obligate precursor of coenzyme A (CoA), through which it plays key roles in myriad biological processes, including many that regulate carbohydrate, lipid, protein, and nucleic acid metabolism. The rich sources of Vitamin B5 are yeast and organ meats (liver, kidney, heart, brain), but eggs, milk, vegetables, nuts and whole-grain cereals are also common sources of pantothenic acid.

This standard was first published in 1981 with a title "*Method for estimation of pantothenic acid in foodstuffs*" in which microbiological method was described for estimation of pantothenic acid in food stuffs. 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 In the first revision of the standard, the microbiological method has been replaced by the method based on ISO 20639:2015 *"Infant formula and adult nutritionals — Determination of pantothenic acid by ultra-high performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS)"*.

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 ISO 20639 has been performed by Nestle India Limited Laboratory Services, Moga on food matrices namely, infant and breakfast cereals, beverages, nuts, fruits and vegetables. The performance characteristics obtained for these matrices have been given in Annex A. The $HORRAT_r$ 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 standard specifies a method for the quantitative determination of pantothenic acid (PA) in food stuffs using ultra high-performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS). The food matrices to which the standard is applicable are infant formula and adult nutritional; infant & breakfast cereals; beverages; fruits and vegetable; nuts and nut products. 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

Pantothenic acid is extracted using a 0.4 mol/litre ammonium acetate buffer solution. After filtration, the final solution is subjected to ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

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 Standards/ Reference Material

4.1.1 *Calcium d-pantothenate*, CAS Registry Number® (CAS RN®): 137-08-6, purity >98%, Certified Reference Material should be used.

4.1.2 *Calcium pantothenate* [$^{13}C_6$, $^{15}N_2$], CAS Registry Number® (CAS RN®): 356786-94-2.

4.2 *α-Amylase from Bacillus subtilis* **(**~50 U/mg, CAS Registry Number® (CAS RN®): 9000-90-2) or *Taka diastase from Aspergillus Oryzae* (100U/mg, CAS Registry Number® (CAS RN®): 9001-19-8)

4.3 Solvent

4.3.1 *Acetonitrile,* LCMSMS Grade or equivalent.

4.3.2 *Ammonium acetate***,** CAS Registry Number® (CAS RN®): 631-61-8; ACS grade ; > 98 % purity.

4.3.3 *Acetic acid***,** CAS Registry Number® (CAS RN®): 64-19-7; purity ≥99.5%; Glacial Extrapure.

4.3.4 *Formic acid***,** CAS Registry Number® (CAS RN®): 64-18-6, >98% and LCMS/MS Grade.

4.3.5 *Formic acid in water***,** 1 % and LCMSMS Grade.

4.3.6 *Reagent grade water*, Grade 1 as per IS 1070.

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

Usual laboratory glassware and equipment, and particularly the following:

5.1 Balances, with readability of 0.1 mg and capacity 210 g; with readability of 0.1 g and capacity 4.1 kg.

5.2 *p*H meter, with readability of 0.01 pH unit.

5.3 Homogenizer

5.4 Stir plate with magnetic stirrers

5.5 Filters, Syringe filters, 0.22 µm pore size, 33 mm internal diameter, Membrane disc filters, 0.45 µm pore size.

5.6 UHPLC-MS/MS system, UPLC column, e.g. UPLC coupled with triple quadrupole detector equipped with electrospray ionization (ESI) source and C18 column (100 mm × 2.1 mm internal diameter, Particle Size-1.8 µm).

6 PROCEDURE

6.1 Preparation of standard solutions

6.1.1 *Pantothenic acid (PA) stock solution, ρ* = 250 µg/ml

Prepare by weighing 54.5 mg of calcium pantothenate (**4.1.1**) into a 200 ml volumetric flask (take into account the moisture content given in the supplier's certificate or dry to constant mass at 105 °C) and dilute to volume with water. Store the aliquots at −20 °C.

6.1.2 *Pantothenic acid intermediate solution, ρ* = 10 µg/ml

Prepare by transferring 1 ml of PA stock solution (**6.1.1**) into a 25 ml volumetric flask and dilute to volume with water. Store the aliquots at –20 °C.

6.1.3 *Calcium pantothenate-[$^{13}C_6$, $^{15}N_2$] solution [Internal Standard (IS) stock solution], ρ* = 20 *µg/ml*

Prepare by weighing 5 mg of calcium pantothenate-[$^{13}C_6$, $^{15}N_2$] (**4.1.2**) into a 250 ml volumetric flask and dilute to volume with water. Store aliquots at –20 °C.

6.1.4 *Solutions for the five-level standard curve*

Transfer appropriate volumes of the PA intermediate solution (10 µg/ml) (3.8.2) into 10 ml volumetric flasks to obtain five different concentrations of PA (0.08 µg/ml, 0.16 µg/ml, 0.32 µg/ml, 0.64 µg/ml

and 1.2 µg/ml). Add 500 µl of the IS stock solution (20 µg/ml) (**6.1.3)** and dilute to volume with water. The concentration of IS in each standard solution is 1 µg/ml. Store aliquots of these solutions at –20 °C for no longer than one month before use.

6.1.5 *Ammonium acetate solution, c* = 400 mmol/l, pH = 3.8 *(used for sample extraction)*

Into a 500 ml beaker, add (30.8 ± 0.10) g ammonium acetate (**4.3.2**). Add about 300 ml water and stir to dissolve with a magnetic stirrer. Adjust to pH of 3.8 ± 0.1, carefully adding glacial acetic acid (about 150 ml is needed). Transfer into a 1000 ml volumetric flask and make up to volume with water. This solution is stable for one month at 4 °C.

6.2 Sample preparation

6.2.1 *General*

As the product contains starch, add 50mg α-amylase or Taka diastase to the suspensions and incubate for 15 min at 40 °C to decrease viscosity and facilitate handling. Mix liquid samples well to ensure homogeneity and continue directly to extraction. If the powder sample homogeneity is unknown, assume that it is non- homogenous and proceed with **6.2.2**. For matrices containing high starch, amount of α-amylase or Takadiatase can be increased.

6.2.2 *Dry blended powder samples*

For dry blended/non-homogenous powder samples, accurately weigh approximately 25 g (m_1). Add 2 kg (m_2) water at 40 °C before mixing until a homogeneous suspension is obtained. A homogenizer (**5.3**) can be used when necessary. Accurately weigh approximately 15 g (m_3) aliquot of homogenized sample suspension into a 50 ml volumetric flask. Calculate the sample mass (m_S is the powder equivalent) using as follows:

$$m_s = \frac{m_1 \, X \, m_3}{(m_1 + m_2)}$$

Where

m_1 is the mass of sample weighed, in g;

m_2 is the mass of water added before mixing, in g

m_3 is the mass of homogenized sample suspension, in g.

6.2.3 *Wet blended powder samples*

For wet blended homogenous powder samples, accurately weigh approximately 2 g of sample (m_S) into a 50 ml volumetric flask. Add 14 g of water at 40 °C. Mix until a homogeneous suspension is obtained.

6.2.4 *Liquid samples*

For liquid sample samples, accurately weigh approximately 20 g (m_S) into a 50 ml volumetric flask.

6.3 Extraction

Using the prepared sample (**6.2**) and to it add a 25 ml volume of a 0.4 mol/l ammonium acetate solution having pH of 3.8. Dilute the sample extract to volume with water. Add a stir bar and stir for 10 min. Filter a 20 ml portion through folded paper (Grade 597½). Run chromatographic analysis as described in **6.4**.

6.4 Analysis

6.4.1 *Chromatographic analysis*

Transfer a 1 ml aliquot of the filtrate obtained in **6.3** into a 15 ml polypropylene tube containing 500 µl of the IS stock solution (**6.1.3**). It is critical to use the same IS solution as used in the preparation of the standard curve (**6.1.4**). Dilute the solution to 10 ml with water, cap and mix. Filter through a 0.22 µm syringe filter. Inject into the UHPLC-MS/MS system.

6.4.2 *UHPLC conditions*

The UHPLC conditions are as follows:

a) Injection volume, 2 µL.

b) Column temperature, 30 °C.

c) Flow rate, 0.45 ml/min.

d) Mobile phase A, 0.1 % (v/v) formic acid in water.

e) Mobile phase B, Acetonitrile.

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

NOTE — Direct the liquid chromatography flow into the MS detector only between 0 min and 2 min to prevent source fouling as much as possible.

Table 1 Gradient Program for column
(Clause 6.4.2)

Time (Min)	Mobile phase A %	Mobile phase B %
0	92	8
2.2	80	20
2.4	50	50
4.0	50	50
4.1	92	8
7.0	92	8

6.4.3 *MS/MS conditions*

6.4.3.1 The MS/MS conditions are as follows:

a) Positive ESI

b) Capillary voltage, 2.2 kV

c) Cone voltage, 25 V

d) Extractor voltage, 3.0 V

e) Source temperature, 140 °C

f) Desolvation temperature, 350 °C

g) Cone gas flow, 40 l/h

h) Desolvation gas flow, 700 l/h

6.4.3.2 Set the collision energy at 14 V with a dwell time for each monitored transition of 0.1 s. These values are indicative and need to be optimized for each instrument used. Monitor between 0 min and 2.1 min, the transitions m/z 220.2 → 90.1 for PA and m/z 224.2 → 94.1 for the isotope-labelled IS.

6.4.4 *Identification*

MS detection in the single-reaction monitoring mode includes simultaneous detection of molecular ions corresponding to PA and isotopically labelled PA. The selected mass transitions are m/z 220.2 → 90.1 and m/z 224.2 → 94.1 respectively.

7 CALCULATIONS

7.1 Calculate for each standard the peak area ratio between PA and IS. Establish a 5-point calibration curve (ranging from 0.16 ng to 2.4 ng on column) by plotting peak area ratio (y-axis) versus PA concentration (x-axis). Calculate the linear regression. It is recommended to use a weighed regression curve (1/x).

7.2 Calculate the slope (*S*) and the intercept (*I*) of the calibration

curve.

7.3 Calculate the PA mass fraction, *w*, in mg/100 g, using formula given below

$$w = \frac{(A - I) \times V_1 \times V_3 \times 100}{S \times m \times V_2 \times 1000}$$

Where,

A = peak area ratio PA/IS in the test solution;

I = intercept of the calibration curve;

S = slope of the calibration curve;

V_1 = volume of the of sample extract, in ml (= 50);

V_2 = volume of the filtrate pipetted, in ml (= 1);

V_3 = final volume of the of the test solution, in ml (= 10);

M = mass of the test portion, in g;

100 is the conversion to 100 g basis;

1000 is the conversion from µg to mg.

Annex A
(*Foreword*)

PERFORMANCE CHARACTERISTICS OBTAINED DURING SINGLE LAB VALIDATION

Table 2 Performance Characteristics of Vitamin B5 for New Matrices

	Breakfast Cereal	**Beverage spiked at 5 mg/100 g**	**Nuts spiked at 4 mg/100g**	**Fruits spiked at 4 mg/100g**	**Vegetable spiked at 4 mg/100g**
Mean	2.082	12.008	4.252	3.983	4.108
SD_r (Standard deviation of repeatability)	0.071	0.255	0.117	0.067	0.075
C_{vr} %	3.4%	2.1 %	2.7%	1.7%	1.8%
SD_{iR}	0.076	0.292	0.091	0.058	0.088
CV_{iR} %	4%	2.4 %	2%	1%	2 %
$HORRAT_r$	0.25	0.22	0.17	0.11	0.17

Table 3 Performance Characteristics of Vitamin B5 for New Matrices (spiked at 0.8 mg/100g)

	Nuts spiked at 0.8 mg/100g	**Fruits spiked at 0.8 mg/100g**	**Vegetable spiked at 0.8 mg/100g**
Mean	0.872	0.818	0.862
SD_r	0.037	0.046	0.046
C_{vr} %	4.3 %	5.7%	5.4%
SD_{iR}	0.034	0.039	0.034
CV_{iR} %	4%	5%	4%
$HORRAT_r$	0.24	0.29	0.24

Annex B
(I*nformative*)

EXAMPLE CHROMATOGRAMS ON SELECTED PRODUCTS AND STANDARD SOLUTIONS

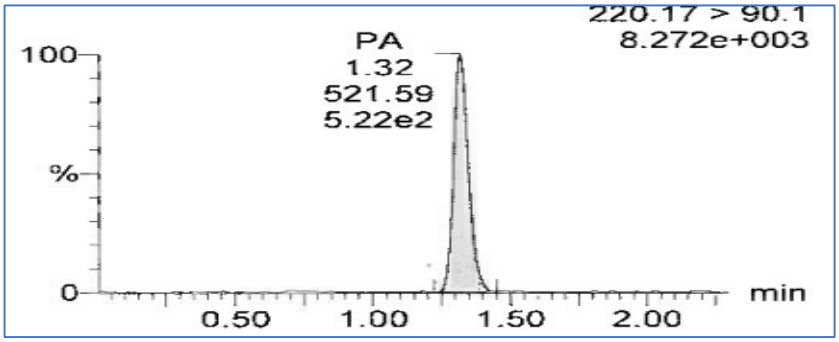

FIG. 1 CHROMATOGRAM OF PANTOTHENIC ACID IN VEGETABLE SAMPLE BY UHPLC-MS/MS

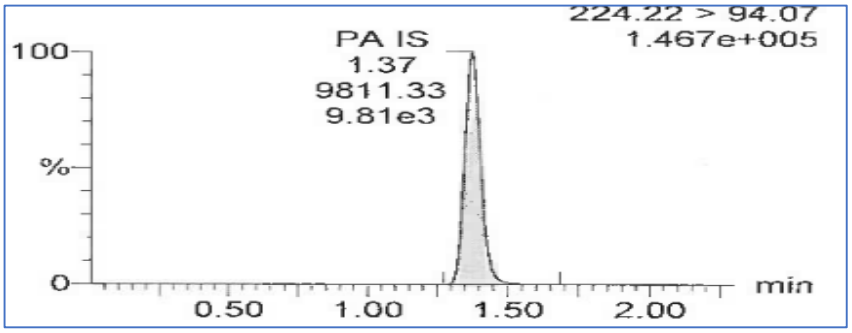

FIG. 2 CHROMATOGRAM OF THE INTERNAL STANDARD (IS) CALCIUM PANTOTHENATE-[12C6, 15N2] BY UHPLC-MS/MS