
**Infant formula and adult
nutritionals — Determination
of pantothenic acid by ultra high
performance liquid chromatography
and tandem mass spectrometry
method (UHPLC-MS/MS)**

*Formules infantiles et produits nutritionnels pour adultes —
Détermination de la teneur en acide pantothénique par
chromatographie liquide à ultra haute performance et spectrométrie
de masse en tandem (LUHP-SM/SM)*



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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The committee responsible for this document is ISO/TC 34, *Food products* in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this International Standard is equivalent to the AOAC Official Method 2012.16: *Pantothenic acid (vitamin B₅) in infant formula and adult/pediatric nutritional formula ultra high pressure liquid chromatography — tandem mass spectrometry method*.

Infant formula and adult nutritionals — Determination of pantothenic acid by ultra high performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS)

WARNING — The use of this International Standard can involve hazardous materials, operations and equipment. This International Standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies a method for the quantitative determination of pantothenic acid, excluding bound forms, in infant formula and adult nutritionals (i.e. powders) using ultra high performance liquid chromatography and tandem mass spectrometry method (UHPLC-MS/MS).

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1

adult nutritional

nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein

2.2

infant formula

breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

3 Principle

Pantothenic acid is extracted using a 0,4 mol/l 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

4.1.1 **Calcium D-pantothenate**, Sigma¹⁾ or equivalent CAS 137-08-6.

4.1.2 **Calcium pantothenate-[¹³C₆, ¹⁵N₂]**, IsoSciences¹⁾ or equivalent CAS 356786-94-2.

4.2 **α -Amylase**, Sigma A3176¹⁾, from porcine pancreas, about 25 U/mg or equivalent.

4.3 Solvents

4.3.1 **Acetonitrile**, LC grade or equivalent.

4.4 **Ammonium acetate**, ACS grade, > 98 % (Fluka 9690)¹⁾.

4.5 **Acetic acid**, ACS grade.

4.6 **Formic acid**, ACS grade.

4.7 **1 % Formic acid in water**, ACS grade.

4.8 Preparation of standard solutions

4.8.1 **Pantothenic acid (PA) stock solution**, $\rho = 250 \mu\text{g/ml}$. Weigh 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 aliquots at -20 °C.

4.8.2 **Pantothenic acid intermediate solution**, $\rho = 10 \mu\text{g/ml}$. Transfer 1 ml of PA stock solution (4.8.1) into a 25 ml volumetric flask and dilute to volume with water. Store aliquots at -20 °C.

4.8.3 **Calcium pantothenate-[¹³C₆, ¹⁵N₂] solution [IS (Internal Standard)] stock solution**, $\rho = 20 \mu\text{g/ml}$. Weigh 5,0 mg of calcium pantothenate-[¹³C₆, ¹⁵N₂] (4.1.2) into a 250 ml volumetric flask and dilute to volume with water. Store aliquots at -20 °C.

4.8.4 **Solutions for the five-level standard curve**. Transfer appropriate volumes of the PA intermediate solution (10 $\mu\text{g/ml}$) (4.8.2) into 10 ml volumetric flasks to obtain five different concentrations of PA (0,08 $\mu\text{g/ml}$, 0,16 $\mu\text{g/ml}$, 0,32 $\mu\text{g/ml}$, 0,64 $\mu\text{g/ml}$ and 1,2 $\mu\text{g/ml}$). Add 500 μl of the IS stock solution (20 $\mu\text{g/ml}$) (4.8.3) and dilute to volume with water. The concentration of IS in each standard solution is 1 $\mu\text{g/ml}$. Store aliquots of these solutions at -20 °C for no longer than one month before use.

4.8.5 **Ammonium acetate solution**, $c = 400 \text{ mmol/l}$, pH = 3,8 (used for sample extraction). Into a 500 ml beaker, add (30,8 \pm 0,10) g ammonium acetate. Add about 300 ml water and stir to dissolve with a magnetic stirrer. Adjust to pH = 3,8 \pm 0,1, carefully adding glacial acetic acid (about 150 ml is needed). Transfer into a 1 000 ml volumetric flask and make up to volume with water. This solution is stable for one month at 4 °C.

1) 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 ISO 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, in particular, the following.

5.1 Balances, with readability of 0,1 mg, capacity 210 g; with readability of 0,1 g, capacity 4 100 g.

5.2 pH-meter, with readability of 0,01 pH unit.

5.3 Homogenizer²⁾.

5.4 Stir plate with magnetic stirrers.

5.5 Filters. Syringe filters, 0,22 µm pore size, 33 mm internal diameter, Millex-GV, PVDF (Millipore)³⁾. Membrane disc filters, 0,45 µm pore size (Millipore)³⁾ or equivalent.

5.6 UHPLC-MS/MS system, UPLC column, e.g. ACQUITY UPLC^{®3)} coupled with triple quadrupole detector equipped with electrospray ionization (ESI) source and T3 column (1,8 µm, 100 mm × 2,1 mm internal diameter; Waters Corp.)³⁾ or equivalent.

6 Procedure

6.1 Sample preparation

6.1.1 General

If the product contains starch, add 50 mg α-amylase 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 [6.1.2](#).

6.1.2 Dry blended powder samples

For dry blended/non-homogenous powder samples, accurately weigh approximately 25,0 g (m_1). Add 200,0 g (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 15,0 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 Formula (1):

$$m_s = \frac{m_1 \times m_3}{m_2} \quad (1)$$

where

m_1 is the mass of sample weighed, in g;

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

2) Polytron PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematics, Lucerne, Switzerland) are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

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

6.1.3 Wet blended powder samples

For wet blended homogenous powder samples, accurately weigh approximately 2,0 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.

6.1.4 Liquid samples

For liquid sample samples, accurately weigh approximately 20,0 g (m_s) into a 50 ml volumetric flask.

6.2 Extraction

Using the prepared sample (6.1), add a 25 ml volume of a 0,4 mol/l ammonium acetate solution, pH = 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.

6.3 Analysis

6.3.1 Chromatographic analysis

Transfer a 1,0 ml aliquot of the filtrate obtained in 6.2 into a 15 ml polypropylene tube containing 500 µl of the IS stock solution (4.8.3). It is critical to use the same IS solution as used in the preparation of the standard curve (4.8.4). Dilute the solution to 10 ml with water, cap and mix. Filter through a 0,22 µm syringe filter (5.5). Inject into the UHPLC-MS/MS system.

Examples of typical chromatograms are given in Annex A.

6.3.2 UHPLC conditions

Injection volume:	2 µl
Column temperature:	30 °C
Flow rate:	0,45 ml/min
Mobile phase A:	0,1 % (v/v) formic acid in water
Mobile phase B:	Acetonitrile

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

Table 1 — Gradient for column

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

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

6.3.3 MS/MS conditions

- Positive ESI
- Capillary voltage, 2,2 kV
- Cone voltage, 25 V
- Extractor voltage, 3,0 V
- Source temperature, 140 °C
- Desolvation temperature, 350 °C
- Cone gas flow, 40 l/h
- Desolvation gas flow, 700 l/h

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 \rightarrow 90,1 for PA and m/z 224,2 \rightarrow 94,1 for the isotope-labelled IS.

6.3.4 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 \rightarrow 90,1 and m/z 224,2 \rightarrow 94,1, respectively.

7 Calculations

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).

Calculate the slope (S) and the intercept (I) of the calibration curve.

Calculate the PA mass fraction, w , in mg/100 g, using Formula (2):

$$w = \frac{(A - I) \times V_1 \times V_3 \times 100}{S \times m \times V_2 \times 1\,000} \quad (2)$$

where

- A is the peak area ratio PA/IS in the test solution;
- I is the intercept of the calibration curve;
- S is the slope of the calibration curve;
- V_1 is the volume of the of sample extract, in ml (= 50);
- V_2 is the volume of the filtrate pipetted, in ml (= 1);
- V_3 is the final volume of the of the test solution, in ml (= 10);
- m is the mass of the test portion, in g;
- 100 is the conversion to 100 g basis;
- 1 000 is the conversion from μg to mg.

Annex A (informative)

Examples of chromatograms

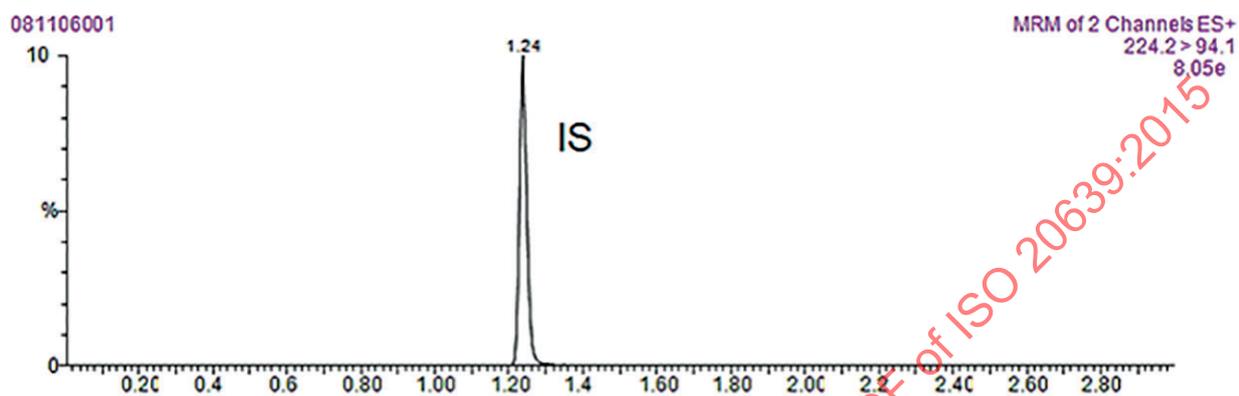


Figure A.1 — Chromatogram of the internal standard (IS) calcium pantothenate-[$^{12}\text{C}_6$, $^{15}\text{N}_2$] by UHPLC-MS/MS

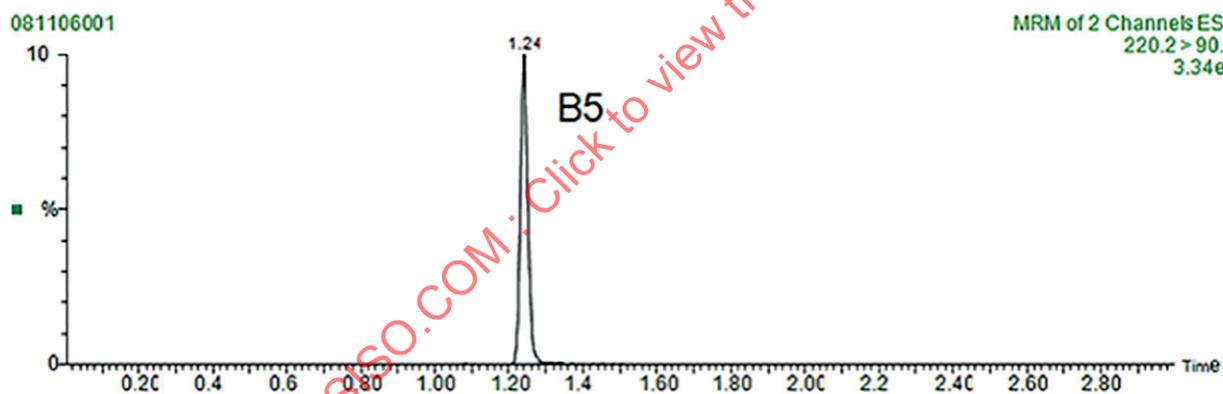


Figure A.2 — Chromatogram of pantothenic acid in an infant formula sample by UHPLC-MS/MS

Annex B (informative)

Precision data

The data given in [Table B.1](#) were obtained in an interlaboratory study and published in 2015^[1], in accordance with ISO 5725-2^[2] and AOAC-IUPAC Harmonized Protocol for collaborative study procedures, to assess precision characteristics of a method of analysis.^[3] The study was performed based on requirements given in Reference [\[4\]](#).

More information on the validation of the method can be found at <http://standards.iso.org/iso/20639>

Table B.1 — Precision data for pantothenic acid

Sample	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h	9 ⁱ	10 ^j
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014	2014	2014
Number of laboratories retained after eliminating outliers	13	14	14	13	13	14	13	14	14	13
Mean value, \bar{x} , mg/100 g	2,59	3,85	6,96	8,07	5,04	5,91	0,549	6,65	2,07	1,57
Repeatability standard deviation s_r , mg/100 g	0,05	0,05	0,14	0,13	0,14	0,17	0,008	0,22	0,06	0,03
Reproducibility standard deviation s_R , mg/100 g	0,13	0,20	0,35	0,33	0,23	0,29	0,022	0,36	0,14	0,09
Coefficient of variation of repeatability, $C_{V,r}$, %	1,9	1,3	2,0	1,6	2,8	2,8	1,5	3,3	2,9	1,7
Coefficient of variation of reproducibility, $C_{V,R}$, %	5,0	5,3	5,1	4,1	4,7	4,9	4,1	5,4	7,0	5,5
Repeatability limit r [$r = 2,8 \times s_r$], mg/100 g	0,14	0,14	0,39	0,36	0,39	0,48	0,022	0,62	0,17	0,08
Reproducibility limit R [$R = 2,8 \times s_R$], mg/100 g	0,36	0,56	0,98	0,92	0,64	0,81	0,061	1,01	0,39	0,25
HorRat value, according to Reference [5]	0,51	0,57	0,60	0,50	0,53	0,57	0,33	0,63	0,69	0,52
^a Adult nutritional powder milk protein based, ^b Infant formula powder partially hydrolysed soy based, ^c SRM 1849a, ^d Adult nutritional powder low fat, ^e Infant formula powder soy based, ^f Child formula powder, ^g Infant formula RTF milk based, ^h Infant elemental powder, ⁱ Adult nutritional RTF high fat, ^j Adult nutritional RTF high protein RTF is ready-to-feed.										